# Free Amino Acids and Sugars in Rye Grain: Implications for Acrylamide Formation

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Acrylamide forms from free asparagine and sugars during cooking, and products derived from the grain of cereals, including rye, contribute a large proportion of total dietary intake. In this study, free amino acid and sugar concentrations were 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. Genetic and environmental (location and harvest year) effects on the levels of acrylamide precursors were assessed. The data showed free asparagine concentration to be the main determinant of acrylamide formation in heated rye flour, as it is in wheat. However, in contrast to wheat, sugar, particularly sucrose, concentration also correlated both with asparagine concentration and with acrylamide formed. Free asparagine concentration was shown to be under genetic (G), environmental (E), and integrated (G × E) control. The same was true for glucose, whereas maltose and fructose were affected mainly by environmental factors and sucrose was largely under genetic control. The ratio of variation due to varieties (genotype) to the total variation (a measure of heritability) for free asparagine concentration in the grain was 23%. Free asparagine concentration was closely associated with bran yield, whereas 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 in wheat flour.

KEYWORDS: Acrylamide; amino acids; asparagine; plant breeding; *Secale cereale*; sugars

## INTRODUCTION

The formation of acrylamide during high-temperature cooking and processing of a range of mainly plant-derived raw materials was first reported in 2002 (*1*), and the presence of acrylamide in foods is now recognized as a difficult and important problem for the agricultural and food industries. The International Agency for Research on Cancer has classified acrylamide as probably carcinogenic to humans, on the basis of its carcinogenic action in rodents; acrylamide also has neurological and reproductive effects (*2*). Concern has been heightened by the publication of two epidemiological studies linking high dietary intake of acrylamide with cancer in humans (*3*, *4*), although the results of all the epidemiological studies conducted to date, taken together, remain inconclusive (*5*).

Acrylamide is formed predominantly from the amino group of asparagine and a carbonyl compound derived from reducing sugars (mainly glucose, fructose, and maltose) (*6*, *7*). Sucrose can also participate in the reaction after high-temperature breakdown (*8*).

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Alternative mechanisms for acrylamide formation involving, for example, gluten breakdown at high temperature (*9*) or 3-amino-propionamide as a transient intermediate (*10*), have been proposed, but it is not clear how important these mechanisms are compared with the asparagine/carbonyl compound route.

Foods with the highest levels of acrylamide include those produced from potatoes and cereals by frying, roasting, or baking. For adults, estimates of dietary intake of acrylamide range from approximately 0.3 to 0.6 $\mu$g/kg of body weight per day, and the average intake for children and teenagers is higher (*5*). The contribution of cereal products to this intake varies from country to country according to dietary preferences, but in the United States, for example, cereal products make up 33% of the total (*5*). Coincidentally, some of the first studies on dietary acrylamide intake were undertaken in Sweden, where per capita consumption of rye crispbreads is particularly high (*1*, *11*). Acrylamide levels in some rye crispbreads were found to exceed, by a considerable margin, 1000 $\mu$g/kg (parts per billion), a de facto limit used by regulators in some countries, and rye crispbreads were identified as an important potential source of dietary acrylamide, accounting for 6% of the total intake.

Crispbread manufacturers responded rapidly, and there are published and anecdotal reports that levels have been reduced significantly through changes in baking processes (*12, 13*) (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). However, further reduction is desirable and would be facilitated by reducing the levels of acrylamide precursors in rye grain.

Rye is closely related to wheat, although it is generally more tolerant of stress and is higher yielding in poor soils (*14*). It is therefore often grown where wheat does not perform well. Rye grain has unique properties, however, and consequently products made from it have a distinctive flavor, texture, and color. Rye products are rich in dietary fiber, notably arabinoxylan and $\beta$-glucan, derived from cell wall polysaccharides; these are beneficial to health, although arabinoxylan has been shown to have a negative effect on the baking quality of wheat (*15*). Rye products are also rich in beneficial phytochemicals, including folate, phenolic acids, alkylresorcinols (phenolic lipids), and sterols (*16*). Furthermore, rye seed storage proteins have a relatively high lysine content compared with wheat and are therefore of better nutritional quality (*14*), although storage protein content is very sensitive to environmental factors and agricultural practices. As a result of these properties, rye products have well-established health benefits (*16*), and it is important that these are retained while acrylamide levels are reduced.

The acrylamide potential of rye has hitherto been considered to be higher than that of its close relative, wheat, because of the presence of relatively high levels of free asparagine and sugars in rye grain (*17, 18*). In a previous study, the levels of free asparagine were shown to be affected by genotype, nitrogen fertilization, and preharvest sprouting (*12*), although this study concerned only two rye varieties. In another study, a correlation was observed between free asparagine levels and the concentration of fructose and glucose, and it was suggested that low fructose and glucose content could be used in plant breeding programs to identify lines with low grain asparagine. The advantage of this would be the relative ease of measuring glucose and fructose concentrations (*19*). In our view it would be premature to adopt such a strategy until much more is known about the factors that affect free asparagine accumulation in rye grain.

We have shown previously that sulfur deprivation causes a many-fold increase in free asparagine in wheat grain and that low sulfur and high free asparagine concentrations in wheat grain are closely correlated with increased levels of acrylamide formation in wheat flour on heating (*20−23*). The concentration of free asparagine and other amino acids in wheat grain is also affected by genotype and other environmental factors, most likely including the availability of other minerals in addition to sulfur, as well as temperature and precipitation during grain filling (*23, 24*). The aim of the present study was to establish the relative contributions of genotype and environment to the determination of levels of acrylamide precursors (asparagine, fructose, glucose, maltose, and sucrose) in rye by analyzing grain samples from a range of varieties grown in different countries in 2005−2007. The results indicate significant genetic (G), environmental (E), and G × E interactions in the determination of acrylamide precursor levels and reveal surprising and important differences between wheat and rye.

## MATERIALS AND METHODS

**Rye Grain.** Samples of rye grain were provided by the EU FP6 HEALTHGRAIN diversity program (*25*). Eleven old and modern

varieties/populations had been grown at Martonvasar, Hungary, in 2005 (*16*). Five selected varieties were then grown at Martonvasar in 2006 and 2007 and at sites in the United Kingdom (Nickersons, Suffolk), 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 and was stored at −20 °C until it was analyzed.

**Concentration of Free Amino Acids.** Three random samples were taken from the pool of flour for each combination of site, year, and variety, and processed separately. Twenty-one amino acids were extracted and quantified as described previously (*20*). Briefly, 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 and 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 $\mu$L) of the supernatant was then derivatized using the EZ-Faast amino acid derivatization technique for gas chromatography and mass spectrometry (GC-MS) (Phenomenex, Torrance, CA) as described previously (*17*). GC-MS analysis of the derivatized samples was carried out using an Agilent 6890 GC-5975-MS system (Agilent, Santa Clara, CA) in electron impact mode. An aliquot of the derivatized amino acid solution (1 $\mu$L) was injected at 280 °C in split mode (40:1) onto a Phenomenex capillary column (10 m × 0.25 mm; 0.25 $\mu$m film thickness). The oven temperature was held at 110 °C for 1 min and then increased at 30 °C/min to 310 °C. The transfer line and ion source were maintained at 320 and 230 °C, respectively; carrier gas flow rate was kept constant throughout the run at 1.5 mL/min.

**Analysis of Sugars by Ion Chromatography.** Analyses were performed using methods adapted from Elmore et al. (*26*). Each flour sample (0.200 ± 0.005 g) was weighed into a 14 mL screw-top bottle. Aqueous methanol (10 mL; 50% v/v) containing 100 mg/L trehalose was added to the bottle, and the sample was stirred for 15 min at room temperature. After a further 15 min hold, 1.5 mL of supernatant was removed from the bottle and centrifuged at 7200*g* for 15 min. An aliquot (500 $\mu$L) of the centrifuged supernatant was diluted 10-fold in water, and 2 mL of the diluted extract were then filtered through a 0.2 $\mu$m syringe filter.

The extracts were analyzed using a Dionex ion chromatography system with a 250 × 4 mm Carbopac PA1 column (Dionex Corp., Sunnyvale, CA), operated using Chromeleon software. The ion chromatography system consisted of an AS50 autosampler, an LC25 column oven, GS50 pumps, and an ED50 pulsed amperometric detector, running in internal amperometric mode. Injection volume was 25 $\mu$L. 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; that is, 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.

**Measurements of Total Grain Nitrogen and Sulfur.** Measurements of total grain nitrogen and sulfur were made by the Analytical Unit of the Soil Science Department, Rothamsted Research. Total grain nitrogen was determined according to the Dumas digestion method, using a LECO CNS 2000 combustion analyzer. Total sulfur 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, U.K.) after the samples had been hydrolyzed with a mixture of $HNO_3$ and $HClO_4$.

**Production and Analysis of Acrylamide.** Acrylamide was produced and analyzed essentially as described by Muttucumaru and co-workers (*20*). Flour samples (0.5 g) in unsealed glass ampules (1 mL capacity) were heated for 20 min at 180 °C. Acrylamide was extracted by refluxing for 2 h with 25% aqueous methanol containing an internal standard (1.2 $\mu$g of $^{13}C_3$-acrylamide in 1 mL of methanol). The extract was filtered and the methanol removed from the filtrate on a rotary evaporator at < 40 °C. The residual aqueous solution was passed though a Sep-Pak $C_{18}$ cartridge (Waters Ltd., Elstree, U.K.), and the cartridge was washed with 5 mL of water. The extracted acrylamide was converted to the dibromo derivative prior to analysis by GC-MS, using the method of Castle (*27*). The brominated extracts (2 $\mu$L) were injected onto an Agilent 6890 GC-5975-MS

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system in pulsed splitless mode at 250 °C, the splitter opening after 0.5 min. The helium carrier gas pressure was 21 psi in pulsed mode, falling to 9.6 psi for the rest of the run. A DB-5 MS capillary column was used (30 m × 0.25 m × 1 μm; Agilent). The oven temperature was 85 °C for 1 min, rising at 8 °C/min to 200 °C and then at 30 °C/min to 280 °C, at which temperature it was held for 10 min. The transfer line was held at 280 °C and the ion source at 180 °C. The mass spectrometer was operated in electron impact mode with selected ion monitoring. Two ions were used to monitor brominated $^{13}C_3$-acrylamide (*m/z* 153 and 155), and two closely related ions (*m/z* 150 and 152) were used for brominated acrylamide. The ion *m/z* 155 was used to quantify brominated $^{13}C_3$-acrylamide, and the ion *m/z* 150 was used to quantify brominated acrylamide. Three technical replicates for each sample of flour for each combination of site, year, and variety were used; the means of technical replicates were used in statistical analysis.

**Grain Properties.** One thousand kernel weight, kernel hardness, and protein and starch contents of the grain samples were measured as described by Rakszegi and co-workers (*28*).

**Statistical Analyses.** The GenStat statistical system (GenStat, 2007, 10th ed., Lawes Agricultural Trust (Rothamsted Research), VSN International Ltd., U.K.) was used for residual maximum likelihood (REML) analyses, canonical variate analyses (CVA), and principal component analyses (PCA) of the data with reference to Payne and co-workers (*29*). Prior to any analysis, data were transformed to the natural logarithm ($\log_e$) scale to account for heterogeneity of variance across the treatment (i.e., site, year, and variety) combinations. CVA (*30*) was used to analyze the data from all 21 amino acids together, whereas REML analyses (taking the treatments as fixed effects) were used to analyze the individual (univariate) amino acid and other data sets. Following REML analyses, predicted means for different treatment combinations were compared using the least significant difference (LSD) at the 5% ($p = 0.05$) level of significance. Data from the CVA were visualized on the dimensions formed by the CVs by plotting the CV scores for each sample. The means of CV scores in each dimension, for each treatment combination, were also plotted. Making the assumption of multivariate normality for the data on the natural logarithm ($\log_e$) scale, 95% confidence circles were placed around the means. PCA was used for the variates of grain properties, as there was no replication of site, year, and variety combinations for these data to allow application of CVA or REML.

Pearson's correlation coefficient (*r*) was calculated between the mean acrylamide, asparagine, and sugars (fructose, glucose, sucrose, maltose, and total sugars) data, with the means being taken across the three replicates of country by variety by year combinations in each case ($n = 11$) to give paired 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, again having calculated the means of these variates across replicates. For this, the samples of wheat were chosen from previously analyzed data (*23*) to provide a full range of values across both variates.

For each amino acid, to investigate the contribution of sources of variation to the total variation observed, REML was used to fit a random effects model, with components being (the sum of) variety (*G*), environment (*E*), their interaction (*G* × *E*), and residual (*ε*) effects. For this investigation, the six site-by-year combinations were used as the environments. The model was

$$y_{ijk} = \mu + G_i + E_j + (G \times E)_{ij} + \varepsilon_{ijk}$$

where, for an observation of a particular amino acid, $y_{ijk}$; $\mu$ is the mean, $G_i$ is the effect for variety *i* (*i* = 1, ..., 11); $E_j$ is the effect of environment *j* (*j* = 1, ..., 6); $(G \times E)_{ij}$ is the interaction; and $\varepsilon_{ijk}$ (*k* = 1, ..., 3) is the residual for fitting each replicate of each variety by environment combination. The variance components $\sigma^2_G$, $\sigma^2_E$, and $\sigma^2_{G \times E}$ (corresponding to the *G*, *E*, and *G* × *E* effects) were then extracted, and the ratio $\sigma^2_G/(\sigma^2_G + \sigma^2_E + \sigma^2_{G \times E})$ was calculated. This ratio is a measure of heritability.

## RESULTS

**Free Amino Acid and Sugar Concentrations in the Grain of 11 Rye (*Secale cereale*) Varieties.** Grain samples were provided from 11 varieties of rye that had been grown at Martonvasar, Hungary, in 2005, as part of the HEALTHGRAIN program (*16*). The
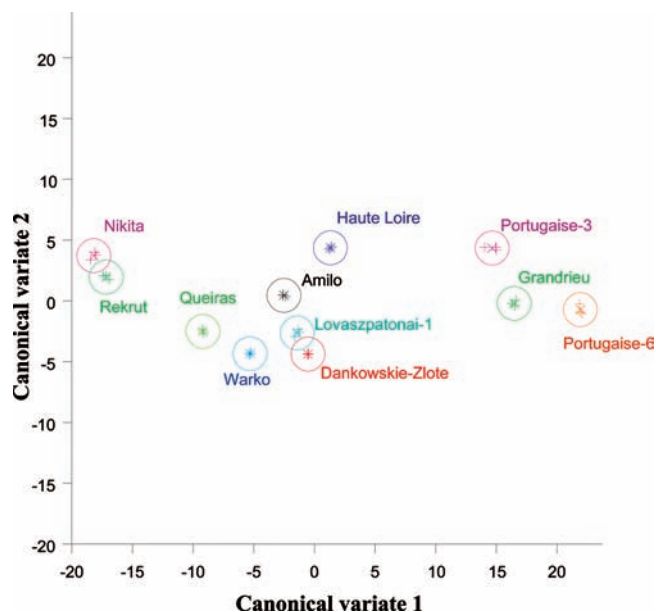


**Figure 1.** Canonical variate analysis plot of data on free amino acid concentrations in grain of 11 rye (*Secale cereale*) varieties harvested in Hungary in 2005 (Table S1, sheet 1, Supporting Information). CV scores (+), means (×), and 95% confidence circles are shown. The major contributors to the discrimination were alanine, asparagine, aspartate, glycine, proline, and threonine for CV1 and asparagine and valine for CV2 (**Table 1**).

varieties chosen included old and modern types. The older varieties 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.

We will continue to use the term variety in this paper, but it should be noted that rye is an open-pollinating species and varieties are genetically heterogeneous, particularly the older ones.

Free amino acid concentrations (for 21 amino acids) in the flour of all the varieties are presented in Table S1 (sheet 1) of the Supporting Information. The data were subjected to REML analyses and CVA (*31*). The REML analysis showed that the varieties accumulated significantly different ($p < 0.001$) concentrations of free asparagine (with means varying from 3.91 to 8.16 mmol/kg) and other amino acids in the grain when grown together, indicating that there is genetic control of this parameter in rye. CVA was used to show the differences between the varieties graphically (**Figure 1**) and to identify which amino acids were responsible for the separation. Most of the variation was accounted for by CV1 (92%; with alanine, asparagine, aspartate, glycine, proline, and threonine being most important to the separation (having the greatest magnitudinal loadings) in this dimension) followed by CV2 (6%; with asparagine and valine being most important). The concentrations of the amino acids that were responsible for the separation of the varieties, for each of these CVs, are given in **Table 1**. The significant differences in concentrations of free asparagine and proline in the grain samples were particularly notable because free asparagine is the principal amino acid precursor of acrylamide, whereas free proline may inhibit acrylamide formation or react with acrylamide and reduce its levels (*32*).

**Table 1.** Means (*n* = 3) (in Bold) and REML Predicted Mean Natural Log (Log$_e$) Values (Normal Print) of Free Alanine, Asparagine, Aspartate, Glycine, Proline, Threonine, and Valine Concentrations (Millimoles per Kilogram of Fresh Weight) in Flour from 11 Varieties of Rye (*Secale cereale*) Harvested in Hungary in 2005[a]

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

[a] The standard error of difference (SED) between log$_e$ data means, least significant difference (LSD), between predicted means (5% level, comparisons made on the log$_e$ scale), and *p* values are also given.
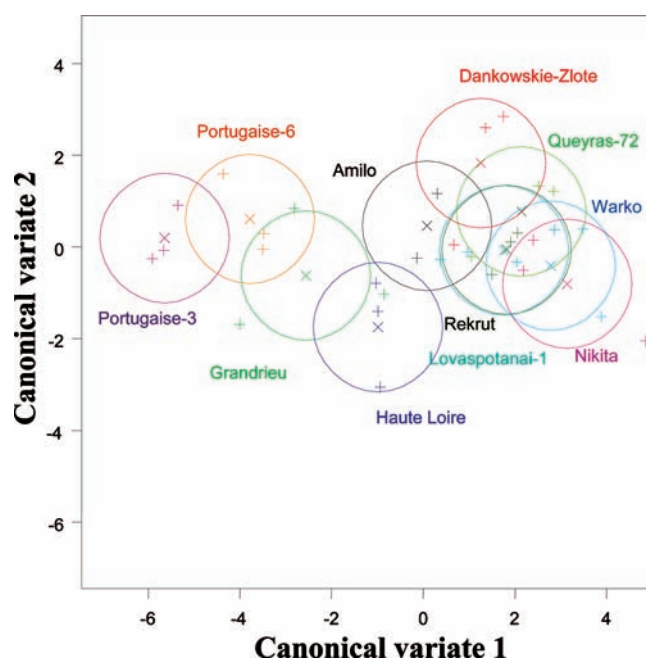
**Table 2.** Means (*n* = 3) of Sucrose, Fructose, Maltose, and Glucose Concentrations (Millimoles per Kilogram of Fresh Weight) in Flour from 11 Rye (*Secale cereale*) Varieties Harvested in Hungary in 2005[a]

| | 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 |
| Portugaise-3 | 1.40 | 1.66 | 38.85 | 3.05 (1.09) | 41.91 |
| Portugaise-6 | 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 | | | | 0.23 | |
| LSD | | | | 0.46 | |
| p value | NS | NS | NS | <0.001 | |

[a] 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).



**Figure 2.** Canonical variate analysis plot of data on sugar concentrations in grain of 11 rye (*Secale cereale*) varieties harvested in Hungary in 2005 (**Table 2**). CV scores (+), means (×), and 95% confidence circles are shown. The major contributors to the discrimination were glucose and sucrose for CV1 and sucrose and maltose for CV2.

The modern German varieties Nikita and Rekrut grouped together in the CVA, whereas Warko was grouped with the old varieties (**Figure 1**). Portugaise-3, Grandrieu, and Haute Loire had the highest concentrations of free asparagine (8.01, 8.16, and 7.39 mmol/kg, respectively), and 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 (3.91 mmol/kg).

Grain sugar concentrations are given in **Table 2**. CVA of the data showed groupings similar to those for the analysis of free amino acids, with CV1 (87.51%; glucose and sucrose) separating the old varieties from the new and CV2 (9.16%; sucrose and maltose) discriminating between some of the varieties within these two groups (**Figure 2**). Rekrut, Nikita, and Warko were similar, with no statistically significant difference (*p* > 0.05) in glucose levels (**Table 2**). Of the older but still commercially available varieties, the 95% confidence circle for Lavanspatonai-1 over-lapped almost entirely with that of Rekrut (the sucrose, glucose, and maltose concentrations in these two varieties were almost

identical), whereas Amilo and Dankowskie-Zlote were also close to the grouping of new varieties. Little difference was observed between the older varieties, with the confidence circles for Portugaise-3 and Portugaise-6, Grandrieu, and Haute Loire consecutively overlapping (**Figure 2**). These older populations had higher levels of sucrose, in particular, than the new varieties, ranging from 30.9 mmol/kg for Grandrieu to 38.9 mmol/kg for Portugaise-3, compared with 26.9 mmol/kg (Rekrut) to 29.5 mmol/kg (Nikita) for the new varieties. Amilo had the lowest concentration of sucrose at 22.8 mmol/kg.

**Effects of Environment on Amino Acid and Sugar Concentrations.** 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

**Table 3.** Means (*n* = 3) (in Bold) and REML Predicted Mean Natural Log (log$_e$) Values (Normal Print) of Free Amino Acid Concentrations (Millimoles per Kilogram of Fresh Weight) in Flour from Rye (*Secale cereale*) Varieties Amilo (A), Dankowskie-Zlote (DZ), Haute Loire (HL), Nikita (N), and Rekrut (R) Grown in Hungary, Poland, France, and the United Kingdom in 2005−2007[a]

| | Hungary, 2005 | | | | | Hungary, 2006 | | | | | Hungary, 2007 | | | | | SED | LSD | *p* value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | DZ | HL | N | R | A | DZ | HL | N | R | A | DZ | HL | N | R | | | |
| alanine | **0.85** | **1.65** | **1.33** | **1.36** | **1.33** | **1.36** | **2.66** | **2.52** | **3.63** | **3.34** | **3.72** | **3.09** | **3.79** | **2.57** | **2.63** | | | |
| | −0.16 | 0.39 | 0.28 | 0.30 | 0.28 | 0.31 | 0.98 | 0.92 | 1.29 | 1.20 | 1.31 | 1.13 | 1.33 | 0.94 | 0.97 | 0.09 | 0.18 | <0.001 |
| asparagine | **3.91** | **6.05** | **7.39** | **6.84** | **5.85** | **3.63** | **9.67** | **10.80** | **9.62** | **8.94** | **9.87** | **8.45** | **15.14** | **9.22** | **9.71** | | | |
| | 1.35 | 1.77 | 2.00 | 1.92 | 1.76 | 1.29 | 2.27 | 2.38 | 2.25 | 2.18 | 2.29 | 2.13 | 2.72 | 2.22 | 2.27 | 0.09 | 0.05 | <0.001 |
| aspartate | **2.75** | **4.19** | **4.37** | **3.97** | **4.12** | **3.38** | **5.34** | **6.32** | **7.13** | **5.82** | **7.70** | **6.99** | **8.98** | **7.27** | **7.72** | | | |
| | 1.01 | 1.42 | 1.46 | 1.38 | 1.41 | 1.22 | 1.68 | 1.84 | 1.96 | 1.75 | 2.03 | 1.94 | 2.19 | 1.98 | 2.04 | 0.12 | 0.06 | 0.003 |
| glutamate | **1.62** | **2.56** | **1.60** | **2.08** | **1.70** | **1.27** | **3.37** | **2.75** | **4.66** | **3.37** | **7.00** | **4.99** | **7.71** | **5.63** | **5.45** | | | |
| | 0.48 | 0.86 | 0.45 | 0.72 | 0.52 | 0.22 | 1.21 | 1.01 | 1.50 | 1.19 | 1.93 | 1.60 | 2.03 | 1.73 | 1.69 | 0.17 | 0.08 | <0.001 |
| glutamine | **0.40** | **0.95** | **0.93** | **0.39** | **0.39** | **0.21** | **1.16** | **1.43** | **1.50** | **1.36** | **1.47** | **0.96** | **2.52** | **1.38** | **0.82** | | | |
| | −0.97 | −0.62 | −0.15 | −0.96 | −0.98 | −0.57 | 0.13 | 0.35 | 0.40 | 0.30 | 0.37 | −0.04 | 0.92 | 0.32 | −0.16 | 0.25 | 0.12 | <0.001 |
| proline | **0.69** | **1.06** | **1.48** | **0.49** | **0.46** | **0.38** | **2.99** | **3.03** | **3.74** | **4.06** | **5.44** | **3.91** | **4.62** | **4.49** | **3.89** | | | |
| | −0.37 | −0.08 | 0.39 | −0.72 | −0.77 | −0.97 | 1.09 | 1.11 | 1.32 | 1.40 | 1.69 | 1.36 | 1.53 | 1.50 | 1.36 | 0.10 | 0.05 | <0.001 |
| threonine | **0.22** | **0.78** | **0.44** | **0.26** | **0.33** | **0.16** | **0.34** | **0.41** | **0.40** | **0.34** | **0.43** | **0.35** | **0.50** | **0.34** | **0.34** | | | |
| | −1.54 | −1.02 | −0.84 | −1.35 | −1.12 | −1.83 | −1.08 | −0.90 | −0.92 | −1.08 | −0.84 | −1.04 | −0.69 | −1.09 | −1.07 | 0.24 | 0.12 | 0.05 |
| total free amino acids | **15.39** | **30.55** | **24.67** | **20.79** | **19.69** | **14.88** | **31.87** | **34.64** | **37.82** | **33.91** | **43.34** | **35.53** | **51.55** | **37.88** | **37.21** | | | |

| | France, 2007 | | | | | Poland, 2007 | | | | | United Kingdom, 2007 | | | | | SED | LSD | *p* value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | DZ | HL | N | R | A | DZ | HL | N | R | A | DZ | HL | N | R | | | |
| alanine | **3.40** | **3.60** | **2.86** | **4.57** | **4.24** | **1.66** | **1.48** | **1.55** | **1.54** | **1.53** | **1.87** | **2.64** | **4.84** | **3.28** | **2.79** | | | |
| | 1.22 | 1.28 | 1.05 | 1.52 | 1.44 | 0.51 | 0.39 | 0.44 | 0.45 | 0.42 | 0.62 | 0.97 | 1.58 | 1.19 | 1.02 | 0.09 | 0.18 | <0.001 |
| asparagine | **9.09** | **11.18** | **13.53** | **9.04** | **9.16** | **8.14** | **9.90** | **11.59** | **8.72** | **7.64** | **5.37** | **6.19** | **7.61** | **7.09** | **5.79** | | | |
| | 2.21 | 2.41 | 2.61 | 2.20 | 2.21 | 2.10 | 2.29 | 2.45 | 2.17 | 2.03 | 1.67 | 1.82 | 2.03 | 1.95 | 1.75 | 0.09 | 0.05 | <0.001 |
| aspartate | **8.03** | **8.93** | **7.10** | **7.65** | **7.47** | **6.59** | **8.25** | **6.00** | **6.36** | **5.36** | **3.45** | **3.71** | **3.70** | **5.57** | **3.84** | | | |
| | 2.08 | 2.18 | 1.96 | 2.03 | 2.01 | 1.89 | 2.11 | 1.78 | 1.85 | 1.66 | 1.23 | 1.31 | 1.30 | 1.70 | 1.34 | 0.12 | 0.06 | 0.003 |
| glutamate | **9.84** | **9.34** | **6.59** | **7.93** | **8.35** | **3.55** | **3.77** | **3.00** | **2.95** | **2.68** | **2.52** | **2.23** | **2.03** | **3.19** | **2.30** | | | |
| | 2.29 | 2.23 | 1.88 | 2.07 | 2.11 | 1.26 | 1.32 | 1.08 | 1.06 | 0.96 | 0.90 | 0.80 | 0.71 | 1.14 | 0.81 | 0.17 | 0.08 | <0.001 |
| glutamine | **5.68** | **4.20** | **2.60** | **4.28** | **4.00** | **1.01** | **1.03** | **1.50** | **1.00** | **0.81** | **1.41** | **3.40** | **6.45** | **5.46** | **3.23** | | | |
| | 1.73 | 1.42 | 0.95 | 1.45 | 1.38 | 0.01 | 0.02 | 0.38 | −0.001 | −0.22 | 0.32 | 1.21 | 1.85 | 1.69 | 1.14 | 0.25 | 0.12 | <0.001 |
| proline | **11.68** | **9.72** | **7.47** | **12.35** | **11.43** | **2.73** | **2.72** | **3.07** | **1.88** | **2.16** | **3.64** | **6.26** | **12.62** | **7.95** | **5.95** | | | |
| | 2.46 | 2.27 | 2.01 | 2.51 | 2.44 | 1.00 | 1.00 | 1.12 | 0.63 | 0.77 | 1.29 | 1.83 | 2.54 | 2.07 | 1.78 | 0.10 | 0.05 | <0.001 |
| threonine | **0.47** | **0.42** | **0.52** | **0.47** | **0.46** | **0.23** | **0.24** | **0.36** | **0.26** | **0.19** | **0.33** | **0.59** | **1.16** | **0.84** | **0.56** | | | |
| | −0.75 | −0.88 | −0.67 | −0.76 | −0.77 | −1.49 | −1.43 | −1.06 | −1.36 | −1.64 | −1.12 | −0.53 | 0.15 | −0.18 | −0.59 | 0.24 | 0.12 | 0.05 |
| total free amino acids | **57.42** | **55.53** | **49.04** | **55.16** | **53.66** | **29.43** | **32.82** | **33.64** | **28.25** | **25.55** | **25.78** | **37.16** | **60.49** | **48.94** | **36.28** | | | |

[a] The standard error of difference (SED) between predicted means, least significant difference (LSD) (5% level, comparisons made on the log$_e$ scale), and *p* values for the country by variety by year interaction are also given.

2007. The same varieties were also grown at locations in the United Kingdom, Poland, and France and harvested in 2007. This enabled an analysis of the effects of variety (G) and of the combination of country and harvest year (collectively E) and of the G × E interaction. REML analyses and CVA were applied to the data for all free amino acids. The mean concentrations are presented in Table S1, sheets 1 and 2, of the Supporting Information and were examined along with the data for these same varieties harvested in Hungary in 2005. REML analysis showed significant effects (*p* < 0.05) of country, harvest year, and variety for all of the amino acids except cysteine and histidine, for which only country had a significant effect, and lysine and tyrosine, for which country and variety but not year of harvest had significant effects. The main contributors to the variation between samples were alanine, asparagine, aspartate, glutamate, glutamine, proline, and threonine (notably *p* < 0.001 for aspar- agine, glutamine, and glutamate and *p* = 0.003 for aspartate). The concentrations of these amino acids are given in **Table 3**.

CVA was applied to the data to show the separation of the samples graphically (**Figure 3**). Most (77%) of the variation was accounted for by CV1 and CV2, which together separated the varieties into five groups. CV1, for which the main contributors to the discrimination were alanine, asparagine, aspartate, glutamate,

glutamine, proline, and threonine, separated the varieties mainly on the basis of harvest year. CV2, for which the main contributors were alanine and asparagine, separated the samples on the basis of country, with those grown in the United Kingdom in particular clearly differentiated from those grown at the other locations.

The levels of total free amino acids in the grain grown at the same location in Hungary in the three harvest years ranged from 14.8 to 26.3 mmol/kg in 2005, from 14.6 to 37.3 mmol/kg in 2006, and from 36.8 to 50.7 mmol/kg in 2007. In the case of Amilo, the concentration of free amino acids in 2005 was 14.8 mmol/kg, whereas in 2007 it was 42.7 mmol/kg, almost 3 times higher. G × E interactions were also evident here in that, for example, Amilo had the lowest concentration in 2005 but the second highest in 2007. It is notable that Hungary had an unusually hot summer in 2007.

Asparagine was responsible for much of the increase in the free amino acid pool in Amilo in 2007 compared with 2005 and 2006, and the same was true for Rekrut. In Dankowskie-Zlote, on the other hand, the asparagine levels were highest in 2006, whereas in Nikita asparagine levels were low in 2005 but greater in 2006 and 2007. Clearly, as with total free amino acids, asparagine concen- tration was affected by harvest year, and there was evidence of G × E interactions with different genotypes reacting in contrasting ways to the different environments.

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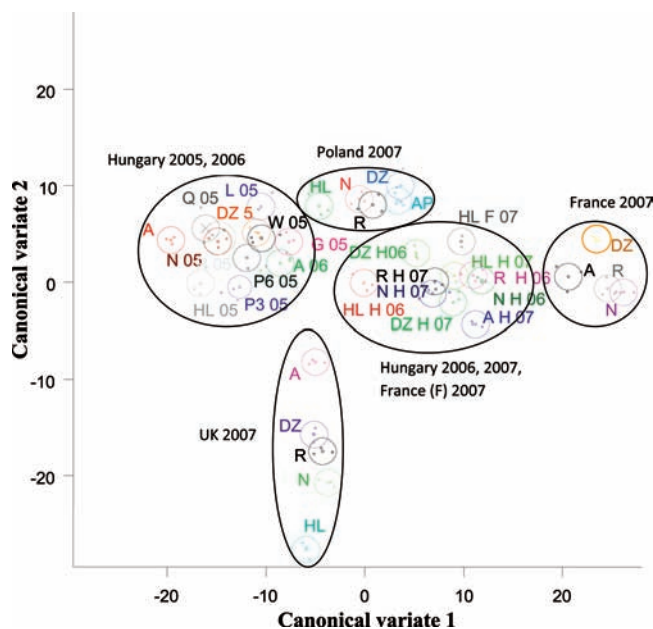
**Figure 3.** Canonical variate analysis plot showing combined effects of variety, location, and year on free amino acid concentrations in grain of rye (*Secale cereale*) varieties Haute Loire (HL), Rekrut (R), Nikita (N), Dankowskie-Zlote (DZ), Amilo (A), Queyras (Q), Warko (W), Lovaszpatonai-1 (L), Grandrieu (G), Portugaise-3 (P3), and Portugaise-6 (P6) grown at sites in Hungary in 2005, 2006, and 2007 and in Poland, France, and the United Kingdom in 2007 (Table S1, sheets 1 and 2, of the Suppoprting Information). Groupings are indicated with solid black lines, with the country and year of harvest indicated for each group. In those groupings that include samples from more than one year, the year is shown for each sample as 05, 06, or 07. In the grouping that includes samples from both Hungary and France, the country is shown by H or F for each sample. As well as individual data points, 95% confidence circles and CV means (at the center of each circle) are shown. The main contributors to the variation were alanine, asparagine, aspartate, glutamate, glutamine, proline, and threonine for CV1 and alanine and asparagine for CV2 (**Table 3**).

The ratio of variation due to varieties (genotype) $(\sigma^2_G)$ to the sum of varietal, environmental $(\sigma^2_E)$, and interaction $(\sigma^2_{G \times E})$ variation, $\sigma^2_G/(\sigma^2_G + \sigma^2_E + \sigma^2_{G \times E})$, is a measure of heritability. This was calculated for the free amino acids that contributed most to the discrimination between samples (Table S1, sheet 3, of the Supporting Information), and the percentage contributions of the different variance components are represented in **Figure 4**. Asparagine and threonine had the greatest genetic effect: for asparagine $\sigma^2_G$ was calculated as 23% of the total variance, with $\sigma^2_E = 42\%$ and $\sigma^2_{G \times E} = 24\%$. For the other amino acids, the greatest effect was due to the environment (alanine, 73%; aspartate, 61%; glycine, 55%; glutamine, 62%; glutamic acid, 80%; proline, 68%; and valine, 58%).

The mean concentrations of glucose, fructose, maltose, and sucrose in the samples are presented in **Table 4**. Of these four sugars, only glucose was affected significantly by all three variables of location, harvest year, and variety ($p < 0.001$, for the interaction of these factors). Hence, $\log_e$ values of the means for glucose are included in **Table 4** for statistical comparison. REML analysis showed that maltose and fructose were affected significantly ($p < 0.001$) by environmental factors (country and year of harvest) (**Table 5**) but not by variety, whereas sucrose concentration, in contrast, was affected significantly ($p < 0.05$) by variety only (**Table 6**).

**Relationships between Free Amino Acids, Sugars, and Grain Properties.** 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 (**Table 7**). PCA was applied because there was only a single determination of each grain property for each of the 36 combinations of country, year, and variety. The first principal component (PC1) accounted for 37.9% of the variation, followed by the second (PC2) with 30.7% (**Figure 5**).

The major contributors to PC1 were flour protein, grain protein, thousand kernel weight, and test weight, all with negative loadings, and bran yield, Hagberg falling number, and flour yield with positive loadings. PC1 discriminated between the old and new varieties. The major contributors to PC2 were bran yield, Hagberg falling number, and test weight with positive loadings and flour yield and flour protein with negative loadings. PC2 discriminated between the varieties within the old and new variety groups.

The PCA separated the varieties in a similar way to the CVA of grain sugar concentrations (**Figure 2**), and it was notable that the separation was due in part to significant differences in the Hagberg falling number. A falling number under 100 is indicative of substantial α-amylase activity, resulting usually from preharvest sprouting. Such grain would contain high concentrations of sugars due to starch breakdown. Portugaise-3, Portugaise-6, Grandrieu, and Haute Loire had the lowest falling numbers, and these varieties also had the highest levels of free proline and asparagine (**Tables 1** and **3**).

Another interesting comparison is made between the bran and flour yield for the grain from the United Kingdom and France. The grain from France had a higher bran and lower flour yield in comparison to the same varieties grown in the United Kingdom. They also had a higher concentration of free asparagine. This is consistent with the bran having a higher concentration of free asparagine than the flour, as has been reported for wheat (*33*).

**Acrylamide Formation.** Acrylamide formation in flour heated for 20 min at 180 °C was determined for a selection of grain samples having a range of free asparagine concentrations (**Table 8A**). Acrylamide was significantly and highly correlated with asparagine ($r = 0.958$, $p < 0.001$), sucrose ($r = 0.911$, $p < 0.001$), and total sugars ($r = 0.838$, $p = 0.001$). A lower and less significant correlation was found with fructose ($r = 0.672$, $p = 0.024$). It should be noted that asparagine was also significantly correlated with sucrose ($r = 0.852$, $p < 0.001$) and total sugars ($r = 0.758$, $p = 0.007$). Acrylamide formation was not affected by moisture content (data not shown).

In wheat flour, acrylamide formation correlates closely with asparagine concentration but not with sugar concentrations (*20−23*). A regression analysis was performed to assess the linear relationship between asparagine concentration and acrylamide formation in rye and wheat (**Figure 6**; **Table 8**), using data from a previous study (*23*) for the latter. This showed that the fitted lines (slopes and intercepts) for the wheat and rye data were separate ($p < 0.001$) and that acrylamide accumulated at a much greater rate for wheat (estimated as $697 \pm 64$ $\mu$g/kg per unit of asparagine concentration (mmol/kg) compared with rye, estimated as $176 \pm 19$ $\mu$g/kg per unit of asparagine (mmol/kg)).

**Grain Nitrogen and Sulfur Contents.** The grain samples were analyzed for total grain nitrogen and sulfur concentrations (Table S1, sheet 4, of the Supporting Information). The level of extractable sulfur in the soil ranged from 4.1 to 25.3 mg/kg (Table S1, sheet 4, of the Supporting Information), but this was not related to the amount of sulfur in the grain, which ranged from 1.30 to 1.86 g/kg. Grain sulfur concentrations did not correlate with levels of free amino acids, sugars, or the amount of acrylamide that formed in heated flour.

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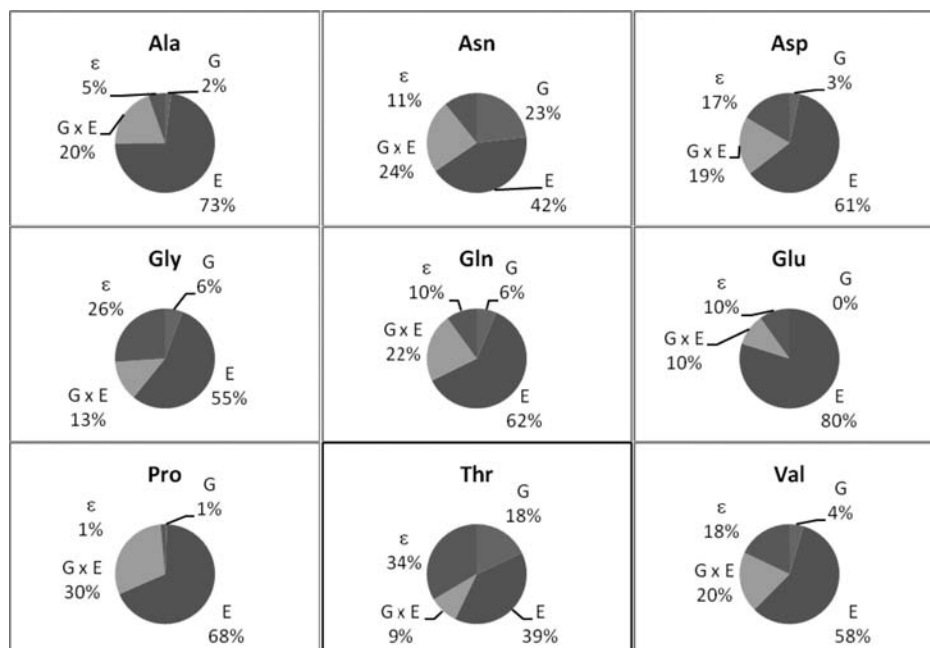


**Figure 4.** Pie charts showing variance components for the concentration of 9 free amino acids in the grain of 11 varieties (G) of rye (*Secale cereale*) grown at locations in France, Hungary, Poland, and the United Kingdom from 2005 to 2007 (six environments, E) (Table S1, sheet 3, of the Supporting Information). The results for these nine amino acids are shown because they were found to be the major contributors to the differences between grain samples.

**Table 4.** Means (*n* = 3) of Sugar Concentrations (Millimoles per Kilogram of Fresh Weight) 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 (U.K.) in 2007[a]

| country | year | variety | fructose | maltose | sucrose | glucose | total |
|---|---|---|---|---|---|---|---|
| Hungary | 2006 | Amilo | 0.61 | 0.74 | 26.81 | 0.64 (−0.46) | 28.16 |
| | | Dankowskie-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 |
| | | Dankowskie-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 |
| | | Dankowskie-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 |
| | | Dankowskie-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 |
| U.K. | 2007 | Amilo | 1.16 | 5.4 | 36.59 | 5.29 (1.66) | 43.15 |
| | | Dankowskie-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 | | ns | ns | ns | 0.46 | |
| | *p* value | | | | | <0.001 | |

[a] Predicted mean natural log (log$_e$) values from REML analyses of glucose concentrations are in parentheses. The standard error of difference (SED) between predicted means, least significant difference (LSD) (5% level, comparisons made on the log$_e$ scale), and the *p* value for the country by variety by year interaction are also given for this sugar. The three-way interaction was not significant (NS) for the other sugars; see **Tables 5** and **6**.

Grain nitrogen concentrations were lower for the new varieties (Nikita, Rekrut, and Warko, 18.26, 17.56, and 17.62 g/kg, respectively) compared with the old varieties (Dankowskie-Zlote, Queiras, Lovanspatonai-1, Haute Loire, Portugaise-3, Portugaise-6, and Queiras, 18.47, 19.34, 21.76, 22.29, 22.92, 23.80, and 24.44 g/kg, respectively), with the exception once again of the

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old variety Amilo, which had the lowest grain nitrogen concentration of 17.09 g/kg (Table S1, sheet 4, of the Supporting Information). High levels of grain nitrogen were associated with high concentrations of free amino acids, including asparagine, and sugars, and, not surprisingly, therefore, with acrylamide formation.

## DISCUSSION

The purpose of this study was to identify the factor or factors determining acrylamide formation in rye flour. It was also important to establish the relationships between varietal and environmental contributors to the control of acrylamide precursor concentrations. A clear conclusion of the study is that the major limiting factor in acrylamide formation in rye flour is free asparagine, just as in wheat. A possible strategy for mitigation of the acrylamide problem is therefore the screening of existing rye

**Table 5.** Predicted Mean Natural Log ($\log_e$) Values from REML 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 (U.K.) in 2007[a]

|  | 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 |
| U.K. |  |  | 1.16 |  |  | 3.68 |  |  | 2.05 |
| SED | 0.28 |  |  | 0.11 |  |  | 0.28 |  |  |
| LSD | 0.55 |  |  | 0.22 |  |  | 0.56 |  |  |

[a] The $p$ values for the country by year interaction were $p < 0.001$ for all three sugars. The standard error of difference (SED) between the predicted means and least significant difference (LSD) (5% level) are also given.

**Table 6.** Predicted Mean Natural Log ($\log_e$) Values from REML Analysis for Sucrose Concentrations in the Grain of 11 Rye Varieties[a]

| 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 |

[a] The $p$ value for main effect of variety was $p = 0.05$. The standard error of difference (SED) between the predicted means and least significant difference (LSD) (5% level) are also given.

**Table 7.** Grain Properties of Different Varieties of Rye (*Secale cereale*) Grown at Locations in Hungary in 2005, 2006, and 2007 and in France, Poland, and the United Kingdom (U.K.) in 2007 ($n = 1$)

| country | year | variety | thousand kernel wt (g) | test wt (g/L) | protein content flour (%) | protein content grain (%) | Hagberg falling no. (s) | flour yield (%) | bran yield (%) |
|---|---|---|---|---|---|---|---|---|---|
| Hungary | 2005 | Haute Loire | 37.6 | 67.1 | 7.72 | 12.26 | 103 | 43.1 | 42.8 |
|  |  | Nikita | 31.0 | 65.2 | 5.92 | 9.92 | 195 | 45.9 | 41.5 |
|  |  | Rekrut | 30.0 | 64.9 | 5.71 | 9.73 | 200 | 46.9 | 40.4 |
|  |  | Dankowskie-Zlote | 37.5 | 73.8 | 6.39 | 10.05 | 228 | 48.1 | 36.4 |
|  |  | Warko | 33.6 | 69.4 | 8.07 | 9.85 | 187 | 46.1 | 37.9 |
|  |  | Lovaspatonai-1 | 33.6 | 70.8 | 7.2 | 12.15 | 226 | 45.7 | 39.7 |
|  |  | Grandrieu | 33.6 | 73.4 | 8.1 | 13.62 | 93 | 41.6 | 42.9 |
|  |  | Queyras-72 | 33.6 | 68.7 | 6.61 | 10.71 | 188 | 45.4 | 41.7 |
|  |  | Portugaise-3 | 38.8 | 73.8 | 5.95 | 13.05 | 71 | 47 | 39.8 |
|  |  | Portugaise-6 | 39.6 | 74.4 | 7.97 | 13.20 | 82 | 45.5 | 38.0 |
|  | 2006 | Haute Loire | 42.6 | 72.3 | 9.35 | 15.71 | 100 | 39.1 | 39.1 |
|  |  | Nikita | 42.6 | 74.1 | 7.39 | 13.16 | 320 | 38.7 | 42.3 |
|  |  | Rekrut | 44.0 | 74.3 | 7.56 | 13.39 | 298 | 40.7 | 39.4 |
|  |  | Dankowskie-Zlote | 44.0 | 75.8 | 7.69 | 13.80 | 295 | 39.8 | 40.0 |
|  |  | Amilo | nd[a] | 76.1 | 5.51 | 9.75 | nd | 44.5 | 35.4 |
|  | 2007 | Haute Loire | 28.0 | 75.8 | 6.46 | 11.27 | 342 | 37.5 | 39.1 |
|  |  | Nikita | 35.4 | 76.9 | 5.4 | 10.17 | 364 | 39.2 | 39.2 |
|  |  | Rekrut | 36.1 | 76.8 | 5.33 | 9.95 | 326 | 39.4 | 37.5 |
|  |  | Dankowskie-Zlote | 35.0 | 76.7 | 5.14 | 9.52 | 345 | 40.7 | 37.0 |
|  |  | Amilo | 34.3 | 76.5 | 5.19 | 10.07 | 326 | 37.5 | 39.4 |
| Poland | 2007 | Haute Loire | 36.6 | 73.2 | 7.65 | 13.81 | 86 | 33.3 | 40.1 |
|  |  | Nikita | 34.6 | 73.5 | 6.11 | 11.31 | 140 | 36.7 | 39.4 |
|  |  | Rekrut | 35.2 | 74.6 | 5.87 | 11.32 | 183 | 37.3 | 38.7 |
|  |  | Dankowskie-Zlote | 35.0 | 76.3 | 6.31 | 11.61 | 198 | 37 | 38.6 |
|  |  | Amilo | 33.9 | 76.1 | 6.03 | 11.81 | 315 | 35.6 | 41.3 |
| U.K. | 2007 | Haute Loire | 34.3 | 66.6 | 8.07 | 12.30 | 62 | 40.9 | 25.9 |
|  |  | Nikita | 40.7 | 70.2 | 6.6 | 10.36 | 62 | 45.6 | 26.3 |
|  |  | Rekrut | 38.4 | 72.2 | 6.93 | 10.69 | 62 | 45.9 | 28.4 |
|  |  | Dankowskie-Zlote | 38.6 | 71.9 | 7.32 | 12.31 | 62 | 42.8 | 27.7 |
|  |  | Amilo | 40.2 | 73.9 | 7.06 | 11.16 | 70 | 41.5 | 31.3 |
| France | 2007 | Haute Loire | 41.4 | 75 | 9.13 | 15.09 | 108 | 34.4 | 32.2 |
|  |  | Nikita | 42.7 | 77.8 | 7.45 | 12.8 | 219 | 33.7 | 35.3 |
|  |  | Rekrut | 42.0 | 77.5 | 7.41 | 13.16 | 248 | 32.5 | 35.0 |
|  |  | Dankowskie-Zlote | 41.3 | 77.9 | 7.09 | 12.54 | 210 | 33.2 | 35.7 |
|  |  | Amilo | 43.3 | 78.3 | 7.39 | 13.20 | 303 | 35.5 | 34.1 |

[a] nd, not determined.
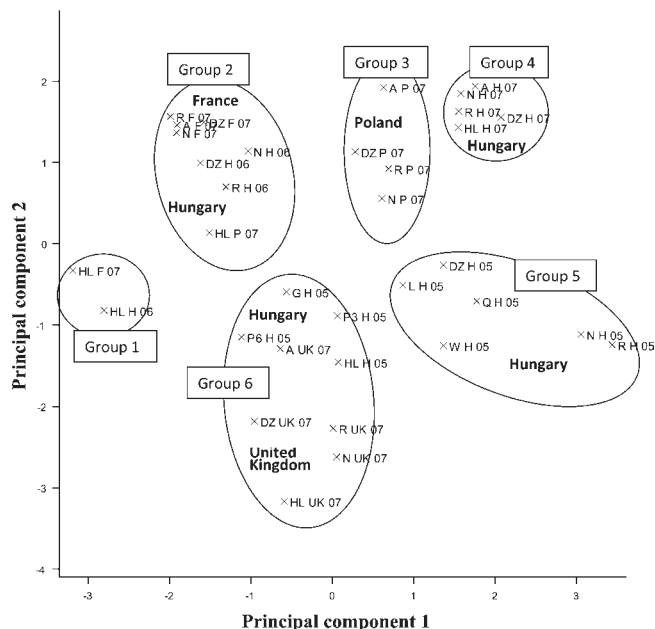
**Figure 5.** Principal component analysis plot showing combined effects of variety, location, and year on grain properties of rye (*Secale cereale*) varieties Haute Loire (HL), Rekrut (R), Nikita (N), Dankowskie-Zlote (DZ), Amilo (A), Queyras (Q), Warko (W), Lovaszpatonai-1 (L), Grandrieu (G), Portugaise-3 (P3), and Portugaise-6 (P6) grown at sites in Hungary (H) in 2005, 2006, and 2007 (Shown as 05, 06, and 07) and in Poland, France, and the United Kingdom in 2007 (**Table 7**). PC scores (×) are shown for each sample, and groupings identified by the analysis are indicated by labeled ovals.

varieties and breeding of new varieties for low free asparagine concentration in the grain.

Variance component analyses showed that the concentration of free asparagine in the grain was under genetic control (G), but was also affected by the environment (E), as has been demonstrated for wheat (*23*), with both country of origin and year of harvest having important effects (notably, rye grown in the United Kingdom had low free asparagine concentrations in the grain compared with rye grown in the other countries). It should be noted that the effects of harvest year and location would each likely be the result of several factors, including temperature, rainfall, and the influences of pests and diseases. There was also a statistically significant three-way interaction between country of origin, year of harvest, and variety (G × E). Nevertheless, a measure of heritability (the ratio of the variance for G to the sum of the variances of G, E, and G × E interaction) was calculated at 0.23, which indicates that the trait may be amenable to improvement by breeding. There was also evidence of G, E, and G × E effects on grain glucose concentration, whereas fructose and maltose were affected principally by environmental factors and sucrose was largely under genetic control.

Total free asparagine in the grain was related to bran yield, the concentration being higher in grain giving 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 the white flour fractions (*33*), and our data indicate that the same is true for rye. Rye crispbreads are generally made from the whole grain and, although this means that acrylamide levels are probably higher than they would be if the bran fraction were removed, it should be noted that there are well-established health benefits associated with eating wholegrain cereal products (*16*).

**Table 8.** Acrylamide (Micrograms per Kilogram) Formed in Rye and Wheat Flour after Heating at 180 °C for 20 min[a]

(**A**) Rye Samples

| variety | harvest year and country of cultivation | acrylamide | asparagine |
|---|---|---|---|
| Amilo | 2006, Hungary | 954 | 3.63 |
| Amilo | 2007, U.K. | 1354 | 5.37 |
| Dankowskie-Zlote | 2005, Hungary | 1266 | 6.05 |
| Dankowskie-Zlote | 2007, France | 2196 | 11.18 |
| Dankowskie-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 |

(**B**) Wheat Samples (Data from Curtis et al. (*23*))

| variety/genotype | harvest year and cultivation site | acrylamide | asparagine |
|---|---|---|---|
| Robigus | 2006, Kent, U.K. | 3115 | 4.46 |
| Rialto | 2007, Rothamsted, U.K. (under glass) | 3059 | 3.27 |
| Claire | 2006, Kent, U.K. | 2224 | 2.50 |
| Spark/Rialto doubled-haploid line SR3 | 2007, Rothamsted, U.K. (under glass) | 1902 | 2.05 |
| Claire | 2006, Lincolnshire, U.K. | 877 | 0.82 |
| Solstice | 2006, Lincolnshire, U.K. | 826 | 0.70 |
| Einstein | 2006, Lincolnshire, U.K. | 690 | 0.67 |

[a] Samples with a range of free asparagine concentrations (mmol/kg) were selected for analysis.
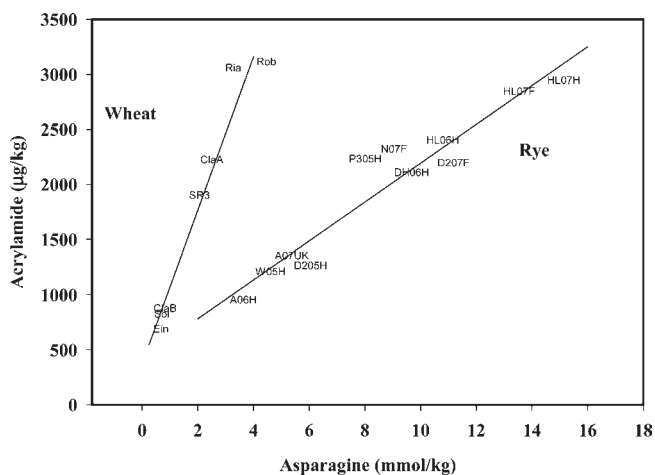


**Figure 6.** Free asparagine concentration (mmol/kg of fresh weight) in wheat (*Triticum aestivum*) (data from Curtis et al. (*23*)) and rye (*Secale cereale*) grain plotted against acrylamide formed in heated flour ($\mu$g/kg). The equations of the fitted lines and standard errors (SE) of estimated intercepts and slopes are $y = 429 + 176x$ (SE 185, 19) for rye and $y = 372 + 697x$ (SE 158, 64) for wheat. The variance explained was 92.2%, and the residual standard deviation was 228 on 14 degrees of freedom.

The study enabled old and new varieties of rye to be compared. In general, the varieties currently being used for commercial production (Nikita, Rekrut, and Warko) had lower levels of free amino acids and sugars than older varieties (Haute Loire, Dankowskie-Zlote, Queyras, Lovaszpatonai-1, Grandrieu, Portugaise-3, and Portugaise-6). Of the free amino acids, the

concentrations of proline and asparagine, in particular, were lower in the new varieties. Free amino acid and sugar levels have not been targets for selection in rye breeding programs up to now, and the fact that modern varieties contain less free asparagine and sugars in the grain than older varieties that are no longer grown is therefore fortuitous. An exception to the rule was Amilo, a relatively old, although still commercially grown, variety, which had consistently low free asparagine and sugar concentrations in the grain.

In contrast to wheat flour, sugar concentrations appeared to be important in determining the amount of acrylamide that formed during heating of rye flour; in addition to free asparagine, sucrose, glucose, and, to a lesser extent, fructose concentrations all correlated with acrylamide formation in the samples studied. However, any conclusion drawn from this must be qualified because the asparagine concentration was itself closely correlated with the concentration of sucrose and total sugars and was shown in a previous study to correlate with glucose and fructose concentrations (*19*). The correlation between sugar concentration and acrylamide formation could therefore be indirect. In the model plant *Arabidopsis*, asparagine and sugar metabolism are linked through the action of the metabolic regulator, sucrose nonfermenting-1 (SNF1)-related protein kinase-1 (SnRK1). This protein kinase is activated, in part, in response to high sucrose concentrations and regulates carbon metabolism through the modulation of enzyme activity and gene expression (*34*). It has also been shown to be required for sugar-repressed and dark-induced expression of an asparagine synthetase gene (*35*). The data from this study suggest asparagine and sugar metabolism to be linked in rye grain, although it would be premature to suggest that SnRK1 could be involved.

Rye grain contains relatively high levels of sugars compared with wheat (*20*, *23*), and in the current study the samples with the highest levels also had the lowest Hagberg falling number. This suggests that the high sugar concentration resulted from high α-amylase activity. The two α-amylase isoenzymes, α-AMY-1 and α-AMY-2, are usually synthesized as a result of preharvest sprouting (*36*). However, certain genotypes of wheat, rye, and *Triticale* also accumulate high levels of α-AMY-1 in the endosperm during late maturity without preharvest sprouting; this is known as late-maturity α-amylase (LMA) or prematurity α-amylase and may be induced by a sudden decrease in temperature during the middle to late stages of grain ripening (*37*, *38*). α-Amylase may also be retained in the pericarp due to synthesis of α-AMY-2. This retained pericarp α-amylase (RPAA) usually vanishes with grain desiccation, but in humid weather conditions may persist to grain maturity and harvest (*39*).

Wheat responds dramatically to sulfur deprivation by accumulating high levels of free asparagine in the grain (*20*, *23*). This increases the risk of acrylamide formation, and ensuring that wheat has an adequate supply of sulfur (current Rothamsted recommendation is 15−20 kg/ha) is a key factor for the mitigation of acrylamide risk in wheat products (*21*). However, in this study, grain sulfur concentration in rye was not related to the availability of sulfur in the soil, even when soil sulfur levels were as low as 4.1 mg/kg, suggesting that the rye plants were able to acquire enough sulfur even when soil levels were relatively low. Consequently, none of the grain samples had a sulfur concentration under 1.0 g/kg, a level that has been associated with very high levels of free asparagine and acrylamide formation in wheat (*23*). Furthermore, grain sulfur concentration did not correlate with the concentrations of free amino acids, including asparagine, or with acrylamide formation. On the other hand, high concentrations of nitrogen were associated with high concentrations of free amino acids, including asparagine, and sugars, and with acrylamide risk, as in wheat grain (*12*).

Another difference between rye and wheat that was revealed in the study was the relatively high concentration of proline present in the rye samples. High proline concentrations may be an intrinsic property of rye, but proline is known to accumulate in plants in response to reactive oxygen species (ROS) produced under drought and high light intensity (*40*); it appears to prevent protein denaturation, preserve enzyme activity, and protect membranes. The samples analyzed in this study did not come from plants that had been deliberately stressed, but it is notable, for example, that the levels of proline were higher in the samples from Hungary in 2007, when Hungary had an unusually hot summer, than in previous years. Whatever the cause of the high proline concentrations, they may explain the wholly unexpected finding that considerably less acrylamide was formed per unit of asparagine in rye flour than has been reported previously for wheat flour. Proline has been shown to reduce acrylamide formation from asparagine in model systems and has been proposed to compete with asparagine for carbonyl compounds with which to react and/or to cause acrylamide to break down after it is formed (*32*). The mitigating effect of free proline on acrylamide formation may be masked when the different rye samples are compared with each other, however, because free asparagine also accumulates under stress conditions. Hence, free asparagine concentrations were high in the samples from Hungary in 2007.

An important conclusion to be drawn from the study is that the reputation of rye as a high acrylamide risk cereal may be undeserved and that it is rye's use almost exclusively in wholegrain products that are baked to give a relatively dark color and crisp texture rather than its intrinsic properties that gives rise to high acrylamide levels. Consideration of the potential but still unknown health risks associated with the acrylamide levels in these products should also take into account the health benefits associated with eating wholegrain rye products (*16*).

**Supporting Information Available:** Table S1 (sheet 1, means of free amino acid concentrations (mmol per kg fresh weight) in flour from 11 varieties of rye (*Secale cereale*) harvested in Hungary in 2005 and 5 varieties harvested in 2006 (GABA, γ-aminobutyric acid; 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); *p* values for country by variety by year (C.V.Y.) interaction are also given); sheet 2, means of free amino acid concentrations (mmol per kg fresh weight) in flour from 5 varieties of rye (*S. cereale*) harvested in Hungary, Poland, France, and the United Kingdom in 2007; sheet 3, REML analysis showing variance components for the concentrations of free amino acids in the grain of 11 varieties of rye (*S. cereale*) grown at locations in France, Hungary, Poland, and the United Kingdom from 2005 to 2007; sheet 4, total sulfur (S), nitrogen (N), and carbon (C) content (g/kg) in grain from 11 varieties of rye (*S. cereale*) harvested in Hungary in 2005 and 5 varieties harvested in Hungary, Poland, France, and the United Kingdom in 2006 and 2007; extractable soil sulfur (mg/kg) is also given for each site (data provided by Fangjie Zhao, Rothamsted Research); the data for the 9 amino acids that were the major contributors to the variance between grain samples are presented). This material is available free of charge via the Internet at http://pubs.acs.org.

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