Nonconventional enzymatic method to determine free asparagine level in whole-grain wheat

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Abstract

A new enzymatic methodology is herein proposed to measure free asparagine content in wheat grains and to predict their potential for Maillard reaction products. Our model estimates the acrylamide levels generated during the industrial heat treatment of whole-grain wheat based on free asparagine and glucose measurements. We selected fifteen wheat varieties currently grown in Belgium as benchmark for the present study. While conventional chromatographic methods require a long and tedious multi-step sample preparation, the proposed method takes advantage of being simple and quick. Statistical analysis of free asparagine content indicates that selected wheat varieties can be classified into seven content levels from 0.0149% to 0.0216% of the dry matter. Based on our analysis, the varieties KWS Ozon, Benchmark and Pionier appears to be the most suitable for thermal processing (i.e. cooking applications).

Keywords: asparagine, acrylamide, wheat grain, enzymatic method

1. Introduction

Acrylamide, an odorless and water-soluble vinyl molecule, is detectable in many foods, including wheat and potato derivatives, after high temperature processing (Douny, Widart, Maghuin-Rogister, De Pauw, & Scippo, 2012; Tareke, Rydberg, Karlsson, Eriksson, & Törnqvist, 2002). This decomposition product is generated during Maillard cascade reactions, involving free reducing sugars and a specific amino acid, i.e. asparagine, when temperatures typically exceed 120°C (Stadler et al., 2002). Acrylamide is suspected to be reprotoxic, genotoxic and carcinogenic with effects reported on animals (Dearfield, Abernathy, Ottley, Brantner, & Hayes, 1988; Erkekoglu & Baydar, 2014). Despite the lack of conclusive studies demonstrating its carcinogenic effect on humans (Lipworth, Sonderman, Tarone, & McLaughlin, 2012; Wilson et al., 2009), its discovery in processed food has raised public health concerns. Based on the animal studies, World Health Organization and International Agency for Research on Cancer classified acrylamide as A2 (probable carcinogen) and committees of experts encouraged the food industry to reduce the acrylamide level in their products to obtain a dietary exposure as low as reasonably achievable (Loaëc et al., 2014).

Many parameters of the food processing can have an impact on the acrylamide content of the final product (Claus, Carle, & Schieber, 2008). Numerous efforts have thus been undertaken to minimize the acrylamide formation in thermal processing conditions by accurately controlling operating factors including moisture, temperature and time. The potential for acrylamide formation during the thermal processing depends on both the quantities of free reducing sugars (mostly glucose) and free asparagine in the starting amylaceous materials (Loaëc et al., 2014; Stadler et al., 2002). The quantity of free asparagine in wheat grains has been identified as a key factor to reduce acrylamide formation (Surdyk et al., 2004). Because free asparagine content can vary from simple to double in wheat grains as a function of the

variety (Martinek et al., 2009), selection of wheat varieties with low levels of free asparagine has been targeted by the agro-food industry. The measurement of acrylamide in cereal products has been reported previously and correlated with the free asparagine level using a multi-step protocol involving high performance liquid chromatography system (HPLC)-based determination of asparagine content. Moreover, precise quantification of amino acids by HPLC-based methods can be limited by the overlap or co-elution of several amino acids. The pioneering study on acrylamide production in wheat reported quantification of free asparagine in wheat endosperm (Surdyk et al., 2004). Because both wheat germs and brans are more concentrated in free asparagine than the endosperm (Hamlet, Sadd, & Liang, 2008), the implementation of methods to precisely and rapidly assess the potential for acrylamide production in whole-grain wheat are required.

In the present study, we describe the development and optimization of a fast enzymatic method to measure free asparagine content in wheat. Our methodology has been developed on the whole-grain rather than on the endosperm fraction in order to appreciate the global free asparagine content as whole-grain flours are increasingly used in bakery, biscuits and breakfast wheats to increase the fiber content of the product. Fifteen wheat varieties cropped in Belgium were selected as benchmark for this study. We also attempt to establish relationships between free asparagine, free reducing sugars and proteins in the whole-grain of those fifteen wheat varieties.

2. Materials and methods

All chemicals were purchased from commercial suppliers and used as received. The K-ASNAM L-Asparagine/L-Glutamine/Ammonia kit was purchased from Megazyme (Illinois, USA) and was used for the free asparagine quantification. This kit contains three specific enzymes and their appropriated buffers, namely glutaminase, glutamate dehydrogenase (GIDH) and asparaginase, as well as a colored reagent (nicotinamide-adenine dinucleotide phosphate, NADPH) required for spectrophotometric quantification at λ 340 nm.

2.1. Varieties and processing of wheat samples

This study was conducted on the following wheat varieties: Albert, Anapolis, Benchmark, Cellule, Edgar, Gedser, Homeros, KWS Ozon, Mentor, Pionier, Reflection, RGT Reform, Sherlock, Sy Epson, Toback. Field trials were conducted in Lonzée (Belgium) in 2014. Sowing was performed in October and harvest occurred in August 2015 for all varieties. Three nitrogen inputs (50 kg/ha at BBCH 21 (tillering), 60 kg/ha at BBCH 30 (elongation) and 70 kg/ha at BBCH 37) and two fungicide treatments, one in early May and the second in early June 2015 were performed. All the whole-grain samples were ground through a 0.5mm grid diameter mesh using a Cyclotec 1093 to obtain flour-like powder. Grinded samples were stored in the dark at room temperature before analysis.

2.2. Analytical protocols

2.2.1. Free glucose and protein measurement

The water-extractable monosaccharide composition which we considered as free monosaccharides, was determined, after derivatization as alditol acetates, by gas chromatography (Blakeney, Harris, Henry, & Stone, 1983). Analyses were carried out with a Hewlett-Packard (HP 7890) gas chromatograph equipped with a flame ionization detector.

The components were separated using a high performance capillary column, HP1-methylsiloxane (30 m×320 µm, 0.25 µm, Scientific Glass Engineering, S.G.E. Pty. Ltd, Melbourne, Australia). Kjeldahl procedure with a conversion factor of 6.25 was used for the determination of protein content (EN ISO 20483:2006).

2.2.2. Free asparagine measurement

Quantification of free asparagine levels in the whole-grain wheat samples was conducted using a spectrophotometric analysis after total deproteination and extraction of free asparagine. Determination of asparagine takes place in a three step reaction. In the first step glutamine from the sample is converted to glutamate and ammonium ions (reaction 1). Then both ammonium ions from the sample and from the reaction 1 react with 2-oxoglutarate and NADPH, which absorb light at 340 nm, to form glutamate and $NADP^+$, which is colourless at 340 nm (reaction 2). Finally asparagine is hydrolyzed to aspartate and ammonium ions (reaction 3). The liberated ammonium ions enter into the reaction 2 which lead to a fall in absorbance that is stoichiometric with the amount of asparagine.

$$\text{reaction 1}: \ \text{L-glutamine} + H_2O \xrightarrow{(\text{glutaminase})} \text{L-glutamate} + NH_4^+$$

$$\text{reaction 2}: \ NH_4^+ + \text{2-oxoglutarate} + \text{NADPH} \xrightarrow{(\text{GIDH})} \text{L-glutamate} + NADP^+ + H_2O$$

$$\text{reaction 3}: \ \text{L-asparagine} + H_2O \xrightarrow{(\text{asparaginase})} \text{L-aspartate} + NH_4^+$$

Practically, 10 g of wheat sample were added to 30 mL of perchloric acid (1 mole/L) and vigorously stirred for 30 minutes. The solution was then brought to pH 8 by adding 2M KOH and adjusted to 100 mL in gauged flask. The sample was then refrigerated in an iced bath for 20 minutes to enhance precipitation. Samples were subsequently centrifuged at 5251 RCF for 15 minutes at 0°C. The supernatant was then filtered through a 0.45 µm syringe filter. 100 µL of this deproteinised sample was collected in a 1.5 mL spectrophotometric cuvette and mixed with 100 µL of the pH 4.9 buffer (offered in the K-ASNAM L-Asparagine/L-Glutamine/Ammonia kit) and 10 µL of glutaminase. This operation is expected to prevent

glutamine interference. After a 5 minutes time lapse at room temperature, 150 µL of the second buffer at pH 8.0 was added together with 100 µL of NADPH and 700 µL of distilled water. After a second delay of 5 minutes at room temperature, 10 µL of glutamate dehydrogenase was added. The initial and maximal absorbance at 340 nm for this sample was then measured using a UV 1800 Shimadzu spectrophotometer. After measurement, 10 µL of asparaginase was added and the decrease of the absorbance at 340 nm was estimated.

The concentration of asparagine in the sample is calculated as follows:

$$\text{Free asparagine (\%DM)} = 100 \times \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A / w$$

where:

V = final volume [mL] = 1.18

MW = molecular weight of asparagine [g/mol] = 132.1

ε = extinction coefficient of NADPH at 340 nm [l x mol^{-1} x cm^{-1}] = 6300

d = light path [cm] = 1

v = sample volume [mL] = 0.1

ΔA = difference of measured absorbance at 340 nm between the product from reaction 2 and reaction 3

w = amount of sample weighted [g/L]

2.3. Statistical analyses

All statistical analysis were performed using R software version 3.2.4 (2016-03-10). Homoscedasticity was validated using the Shapiro-Wilk test for mean normality, while Levene (median-center) test was used to determine variance homogeneity. When homoscedasticity conditions were met, ANOVA coupled with Fisher-LSD test was performed to assess significant differences. Significance level was set to $p < 0.05$ for Homoscedasticity

150 and $p < 0.01$ for Fisher-LSD test. Correlation relationships were tested using the Pearson
151 correlation coefficient.

3. Results and discussion

The fifteen varieties selected for this study are amongst the most common cropped in Belgium and they are used for several industrial food applications. Results presented in **Figure 1** underline the free asparagine contents (A), the free glucose contents (B) and the total proteins (C) found in the fifteen whole-grain samples after determination with the enzymatic experimental protocol. The selected enzymatic method was found reliable for each sample and can therefore constitute a promising tool for the routine quantification of free asparagine in wheat grains batches. The free asparagine level was calculated based on the arithmetic mean of 9 replicates ± standard deviation for each variety (excepted for Edgar, Sherlock, Gedser and Reflection with respectively 7, 8, 12 and 13 replicates). The homogeneity of variance was confirmed with the Bartlett test ($p = 0.067$) and the Levene median-center test ($p = 0.3232$).

As depicted in **Figure 1A**, the free asparagine content varies significantly between the different wheat varieties and the ANOVA indicates that the selected wheat varieties can be classified according to their free asparagine levels ($p < 0.01$). The class with the lowest level of free asparagine (mean value 0.0149 ± 0.0012 % of the dry matter) is constituted of KWS Ozon, Benchmark and Pionier varieties. The class with the highest level of free asparagine (mean value 0.0216 ± 0.0010 % of the dry matter) includes Sy Epson and RGT Reform varieties. Previous studies using a multi-step liquid chromatography method to quantify free asparagine in wheat endosperm, reported values for free asparagine content ranging from 0.0137 to 0.0471% of the dry matter (Emebiri, 2014).

Profiling of the free reducing sugars reveals that glucose is the most abundant sugar in all samples with an average content of 0.5% of the dry matter (**Figure 1B**). The free glucose amount differs significantly from one variety to another one. As for free asparagine, the

ANOVA indicates that varieties can be classified in different groups according to their free glucose content ($p < 0.01$). The lowest level in free glucose (0.31 ± 0.02 % of the dry matter) was measured in Homeros, whilst Toback displayed the highest level (0.53 ± 0.07 %) of free glucose content. For proteins, results demonstrated that the average content was constant as a function of the variety (about 10% of the dry matter) with a minimum of 9.09 ± 0.16 % for Reflection and a maximum of 11.55 ± 0.36 % for RGT Reform (**Figure 1C**).

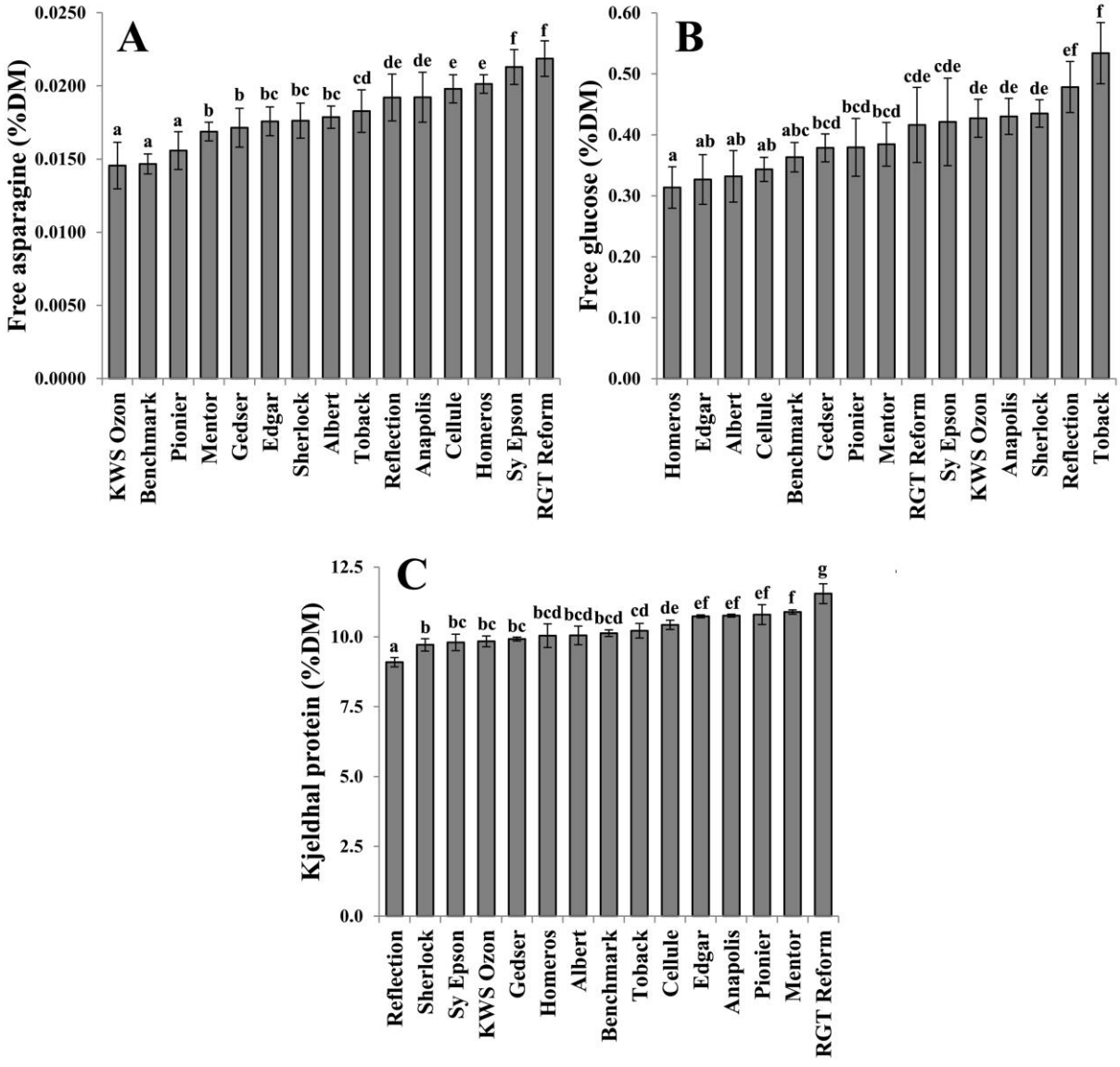

Figure 1. Compositional analyses of whole-grain samples from 15 wheat varieties: mean free asparagine content (Fig. 1A), free glucose (Fig. 1B) and protein contents (Fig. 1C)

referred as % in dry matter. The vertical bars show the standard deviation. Letters show the groups obtained by a Fisher-LSD test after an analysis of variance.

Establishment of relationships between the individual chemical components of interest in the production of acrylamide are presented in **Figure 2**, namely the correlation between free asparagine and protein (**Figure 2A**), free glucose and protein (**Figure 2B**) and free asparagine and glucose (**Figure 2C**). The figure is completed with a three dimensional visualization of the relationships between the individual components (**Figure 2D**).

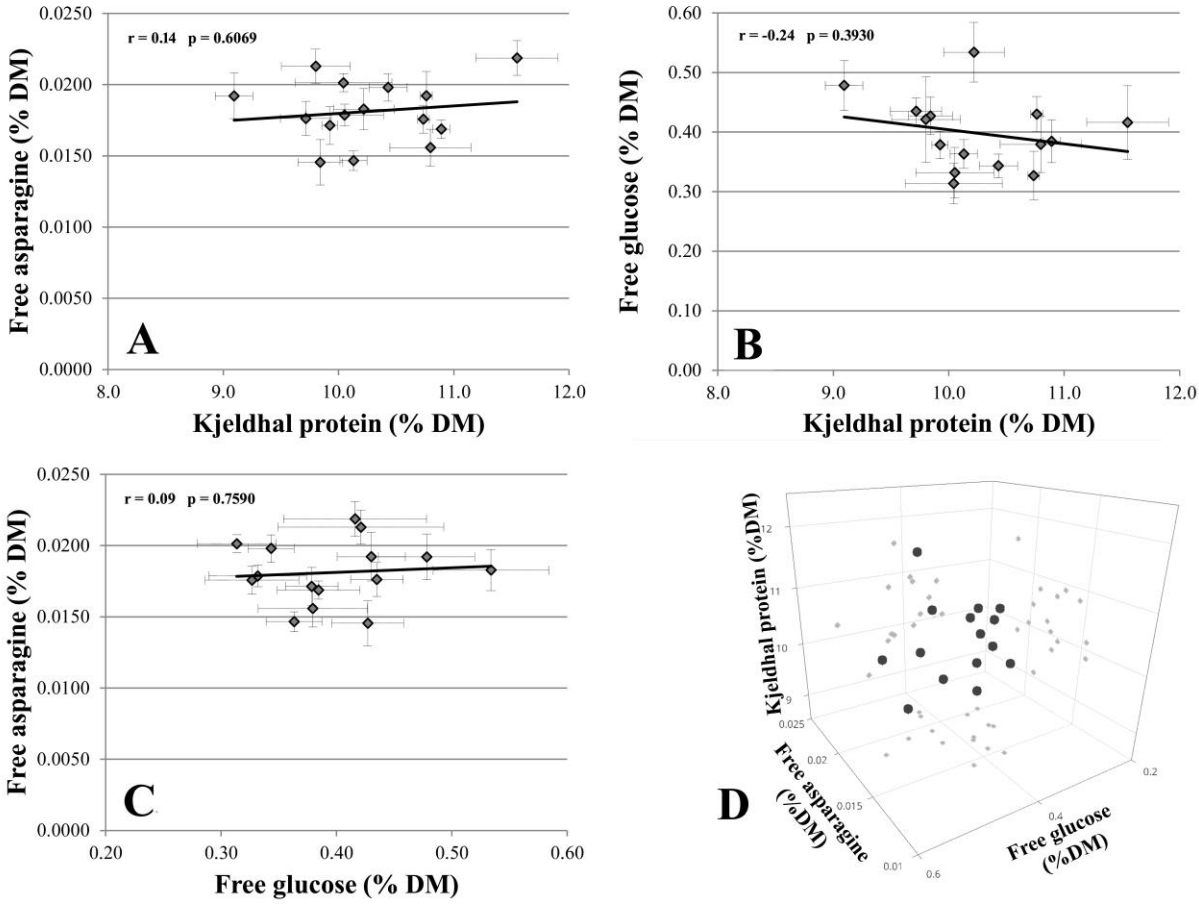

Figure 2. Correlation diagrams between free asparagine and protein contents (Fig. 2A), free glucose and protein (Fig. 2B), free asparagine and glucose (Fig. 2C) and 3-D plot between the three components (Fig. 2D) for whole-grain samples from 15 wheat varieties. All contents are referred as % in dry matter. The plots A, B and C show

Pearson's correlation with r and p-value. Vertical and horizontal bars show the standard deviation. In the plot D black dots represent the projection of the varieties on the three axis and gray dots on two axis.

In some plant samples, protein and free asparagine contents are strongly correlated and the protein level can thus be used to predict the content of free asparagine (Loaëc et al., 2014; Weber et al., 2008). In the present study, no such a rigorous correlation between proteins and free asparagine ($p = 0.6069$) (**Figure 2A&D**) and between proteins and glucose ($p = 0.3930$) (**Figure 2B&D**) could be observed in the whole-grain wheat samples. In a previous study, working for two years on one cultivar grown with different level of nitrogen, authors reported the correlation between protein and free asparagine level to be nonlinear (Weber et al., 2008). Their data revealed that wheat flour with protein content ranging from 8% to 12% had low free asparagine levels and that free asparagine levels increased significantly in wheat samples with protein content above 13%. As the protein contents in our whole-grain samples ranged between 9 to 11.5% of protein level, correlation is too low to be noticeable.

As free glucose and free asparagine are the two main identified acrylamide precursors in wheat we investigate on whether their content evolves in the same trend in each variety sample. As shown in **Figure 2C and Figure 2D**, no significant correlation was found between free glucose and free asparagine ($R^2 = 0.0866$ and $p = 0.7590$). However, varieties Benchmark and Pionier were found to have significantly inferior level of both free glucose and free asparagine while varieties Reflection, RGT Reform and Sy Epson were found to have significantly higher level in at least one of these precursors. Considering that free asparagine and free reducing sugars are documented to have an important impact on acrylamide production from Maillard reaction (Hamlet et al., 2008; Wicklund et al., 2006) those varieties are expected to produce significantly different amounts of acrylamide after

thermal processing. However, regarding that expected stoichiometric ratio of acrylamide formation from free asparagine in presence of reducing sugar is 1:1:1 (Blank et al., 2005), it appears that glucose, which accumulates to levels that are about 20 times higher than free asparagine levels in wheat whole grains, is largely in excess. Therefore it is possible that two varieties with a significant difference in glucose content, for similar free asparagine level, have the same acrylamide potential.

Additionally, the correlation between free asparagine and acrylamide production is well described in literature (Hamlet et al., 2008; Loaëc et al., 2014; Weber et al., 2008) but the impact of protein degradation during thermal processing has so far not been studied. Proteins subjected to thermal treatment tend to degrade and liberate amino acids, such as asparagine, that could enhance the acrylamide production. The impact of the protein degradation could be observed by measuring the change in acrylamide production from flour spiked with increasing amount of asparagine-rich proteins.

4. Conclusion

Our results have demonstrated that a new enzymatic approach was a reliable methodology to quantify free asparagine in wheat sample. This spectrophotometric methodology is rapid, simple and applicable on whole-grain sample, in contrary to conventional liquid chromatographic approaches that require tedious sample preparation. Our statistical results suggest the existence of different classes of wheat with different levels of free asparagine. The lowest quantities of free asparagine was found in KWS Ozon, Benchmark and Pionier varieties with a mean of about 0,015 % of the dry matter.

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Author declaration

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We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He/she is responsible for communicating with the other authors about progress, submissions of

revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from Food Chemistry.

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Figure caption

Figure 1. Compositional analyses of whole-grain samples from 15 wheat varieties: mean free asparagine content (Fig. 1A), free glucose (Fig. 1B) and protein contents (Fig. 1C) referred as % in dry matter. The vertical bars show the standard deviation. Letters show the groups obtained by a Fisher-LSD test after an analysis of variance.

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