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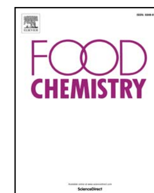

Analytical Methods

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).

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.

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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 0.5 mm grid diameter mesh using a Cyclotec 1093 to obtain flour-like powder. Grinded samples were stored in the dark at room temperature before analysis.

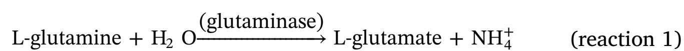
2.2. Analytical protocols

2.2.1. Free glucose and protein measurement

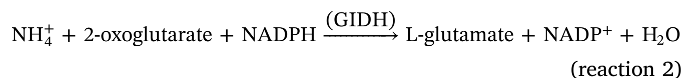
The water-extractable monosaccharide composition which we considered as free monosaccharides, was determined, after derivatization as alditol acetates, by gas chromatography (Blakeney, Harris, Henry, & Stone, 1983). Analyses were carried out with a Hewlett-Packard (HP 7890) gas chromatograph equipped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

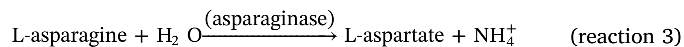
Quantification of free asparagine levels in the whole-grain wheat samples was conducted using a spectrophotometric analysis after total deproteination and extraction of free asparagine. Determination of asparagine takes place in a three step reaction. In the first step glutamine from the sample is converted 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 colorless 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.



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



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



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

Practically, 10 g of wheat sample were added to 30 mL of perchloric acid (1 mol/L) and vigorously stirred for 30 min. The solution was then brought to pH 8 by adding 2 M KOH and adjusted to 100 mL in gauged flask. The sample was then refrigerated in an iced bath for 20 min to enhance precipitation. Samples were subsequently centrifuged at 5251 RCF for 15 min 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 min 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 min 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 $[l \times mol^{-1} \times cm^{-1}]$ = 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 $p < .05$ for Homoscedasticity and $p < .01$ for Fisher-LSD test. Correlation relationships were tested using the Pearson 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 Fig. 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

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