Food Chemistry 150 (2014) 166–173

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Determination of free sulphydryl groups in wheat gluten under the influence of different time and temperature of incubation: Method validation

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ARTICLE INFO

Article history:
Received 7 March 2013
Received in revised form 27 September 2013
Accepted 26 October 2013
Available online 5 November 2013

Keywords:
Free sulphydryl groups
Validation protocol
Incubation time
Incubation temperature

ABSTRACT

The aim of the present study was to determine the characteristics of an analytical method for determination of free sulphydryl (SH) groups of wheat gluten performed with previous gluten incubation for variable times (45, 90 and 135 min) at variable temperatures (30 and 37 °C), in order to determine its fitness-for-purpose. It was observed that the increase in temperature and gluten incubation time caused the increase in the amount of free SH groups, with more dynamic changes at 37 °C. The method characteristics identified as relevant were: linearity, limit of detection, limit of quantification, precision (repeatability and reproducibility) and measurement uncertainty, which were checked within the validation protocol, while the method performance was monitored by X- and R-control charts. Identified method characteristics demonstrated its acceptable fitness-for-purpose, when assay included previous gluten incubation at 30 °C. Although the method repeatability at 37 °C was acceptable, the corresponding reproducibility did not meet the performance criterion on the basis of HORRAT value (HORRAT < 2).

1. Introduction

The importance of free sulphydryl (SH) groups, disulphide (SS) bonds, and their interchange reactions has been emphasized in many studies dealing with wheat and flour quality. It is generally considered that SH groups and SS bonds have significant influence on dough structure formation and dough stability. During dough mixing, the oxidation of sulphydryl groups of cysteine residues within protein (intrachain) and/or between proteins (interchain) occurs. The established SS bonds are responsible for gluten network formation and, therefore, they are key determinants of rheological and baking properties of dough and flour (Delcour et al., 2012; Johansson et al., 2013; Wieser, 2007).

The numerous methods for quantification of free SH content have been developed. The amperometric titration was considered a convenient method for determination of SH groups in purified proteins and amino acids (Carter, 1959). Determination of SH groups of wheat glutenin by direct amino acid analysis was reported by Ewart (1985). Andrews, Caldwell, and Quail (1995) determined free SH content of wheat flour and dough spectrophotometrically using NBD-Cl (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) as a colour developing reagent. For the determination of SH groups of durum wheat semolina and soft wheat flour, covalent bonding of SH groups by 5-iodoacetamide-fluorescein (specific reagent) as a radioactive tracer was employed (Iametti et al., 2013). However, the content of free SH groups has been mostly examined using Ellman's reagent, not only for wheat samples, but for soymilk and rice flour, as well (Gujral & Rosell, 2004; Hayta & Schofield, 2004; Ou, Kwok, Wang, & Bao, 2004; Pérez, Bonet, & Rosell, 2005).

Since its introduction in 1959, 5,5′-dithio*bis*-2-nitrobenzoic acid (DNTB or Ellman's reagent) has been mostly used for the quantification of SH content (Ellman, 1959). The use of Ellman's reagent for this purpose is convenient for several reasons: it is commercially available, water soluble, while its reduction is easily spectrophotometrically monitored (Wilson, Wu, Moth-DeGrood, & Hupe, 1980). However, one of disadvantages of its application is the possibility of occurrence of turbidity when testing milk-based matrices. Due to its sensitivity to daylight, Ellman's reagent and its solutions must be protected in order to prevent formation of degradation products that may yield inaccurate results (Ou et al., 2004). Ellman's reagent reacts rapidly and specifically with free SH groups releasing one equivalent of intensively chromogenic anion – 5-thio-2-nitrobenzoic acid (TNB), which is a yellow coloured substance with maximum absorbance at 412 nm and soluble in aqueous solutions (Andrews et al., 1995; Chan & Wasserman, 1993). Although, the present method has undoubted significance for cereal chemists, no effort has been made to standardise it. Therefore, the aim of the present study was to determine the characteristics of the analytical method for determination of free sulphydryl (SH) groups of wheat gluten performed with previous

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http://dx.doi.org/10.1016/j.foodchem.2013.10.128

gluten incubation for variable times (45, 90 and 135 min) at variable temperatures (30 and 37 °C), in order to determine its capabilities and limitations and demonstrate its fitness-for-purpose.

2. Materials and methods

2.1. Samples, sample preparation and characterisation

Twenty-nine wheat varieties of different technological quality and rheological properties, harvested in 2011 in Serbia, were selected for the study. Wheat samples were cleaned and tempered according to AACC 26-10 (AACC International, 2000) and milled to laboratory flour using a Bühler MLU 202 (Bühler, Uzwil, Switzerland) according to AACC 26-31 (AACC International, 2000). The samples were characterised in terms of Mixolab (Chopin Technologies, Villeneuve-la-Garenne, France) parameters according to ICC standard method 173 (ICC Standards, 2006), wet gluten content and gluten index (GI) according to the ICC standard method 155 (ICC Standards, 1996) and parameters obtained by Kieffer dough extensibility rig for texture analyser TA.XT2 (Stable Micro Systems Ltd., Godalming, UK), which included previous dough incubation at 30 and 37 °C (Kieffer, Garnreiter, & Belitz, 1981). The samples were also characterised in terms of GI values obtained by standard ICC method 155 (ICC Standards, 1996) after incubation of a piece of dough at 37 °C for 90 min (Torbica, Antov, Mastilović, & Knežević, 2007).

2.2. Chemicals

Tris, guanidine-hydrochloride (GuHCl) and 5,5′-dithio*bis*-2-nitrobenzoic acid (DNTB or Ellman's reagent) were purchased from Sigma Chemical Co. (Munich, Germany). Glycine (trihydroxymethyl aminomethane) and L-cysteine hydrochloride monohydrate were purchased from Fisher Scientific (Loughborough, UK), ethylenediaminetetraacetic acid (EDTA) was purchased from Kemika (Zagreb, Croatia) and 1 N NaOH was purchased from LACH-NER (Neratovice, Czech Republic).

2.3. Preparation of solutions

Tris–glycine (Tris–Gly) buffer contained 10.4 g Tris, 6.9 g glycine and 12 g EDTA in 1 L of deionized water, and the pH was adjusted to 8.0 with 1 N NaOH solution. GuHCl/Tris–Gly solution contained 5 M guanidine hydrochloride. Ellman's reagent was prepared by dissolving 40 mg of DTNB (5,5′-dithio*bis*-2-nitrobenzoic acid) in 10 ml of Tris–Gly buffer pH 8.0, and it was freshly prepared before use.

2.4. Calibration curve

Standard stock solution (concentration of 0.83 μmol/ml) was prepared in deionized water and used to prepare following dilutions: 0.018 μmol/ml, 0.036 μmol/ml, 0.050 μmol/ml, 0.080 μmol/ml, 0.100 μmol/ml, 0.120 μmol/ml, 0.150 μmol/ml, 0.200 μmol/ml and 0.250 μmol/ml. 400 μl of each standard solution was added to a test tube with 600 μl of GuHCl/Tris–Gly solution and 250 μl of Ellman's reagent. The mixture was vortexed and developed colour was measured at 412 nm. Calibration curve was established by plotting the absorbance values versus the corresponding concentrations.

2.5. Determination of free sulphydryl (SH) groups

Determination of free sulphydryl (SH) groups was carried out from wet gluten using method of Pérez et al. (2005), which included previous sample incubation at two different temperatures, 30 °C and 37 °C, during three different time intervals 45, 90 and 135 min. The content of SH groups from incubated samples were compared with the SH content of the control sample, determined immediately after gluten washing without previous incubation. Water content of wet gluten samples were 66.21 ± 0.62%, determined at 105 °C until the constant weight was reached.

100 mg of wet gluten was suspended in 1.0 ml of GuHCl/Tris–Gly solution, vortexed for 5 min and centrifuged at 14,500 rpm for 6 min. 600 μl of GuHCl/Tris–Gly solution was added to 400 μl of the supernatant, and the resulting solution was mixed with 250 μl of Ellman's reagent and vortexed. The absorbance was read at 412 nm. Results were calculated against a cysteine standard curve. The scheme of the method applied was presented in Fig. 1.

Sulphydryl content expressed on the crude protein content of flour was calculated according to the following equation (Eq. (1)):

$$\mathrm{SH}\left[\frac{\mu\mathrm{mol}}{\mathrm{g\ protein}}\right] = \left(\frac{A_s - B_0}{B_1}\right) \times \frac{1.25}{1.20} \times \frac{1}{m} \times \frac{G}{P} \times \frac{10^4}{(100 - W)} \quad (1)$$

where A_s is an absorbance (at 412 nm) corresponding to the tested sample, B_0 is an intercept of the calibration curve, B_1 is a slope of the calibration curve, 1.25; 1.20 are volume correction due to dilution, m is a sample mass (g), G is wet gluten content in 1 g of flour (g), P is protein content of flour on dry matter basis (g/100 g), W is moisture content of flour (g/100 g).

Protein and moisture content of flour was determined with scanning monochromator Infratec 1241 Grain Analyzer (Foss Analytical, Hillerød, Denmark) in transmittance mode (850–1050 nm) using flour module.

2.6. Statistical analysis

The data obtained in this study were statistically analysed with the Software XLSTAT, version (2012.2.02) using two-way analysis of variance (ANOVA). Fisher's least significant differences (LSD) test was used to describe the means at 5% significance level.

2.7. Method validation

The method characteristics were assessed by single-laboratory validation procedure according to the Decision 2002/657/EC (European Commission, 2002). Validation protocol comprised the examination of linearity (range), limit of detection (LOD), limit of quantification (LOQ), precision (repeatability and reproducibility), and measurement uncertainty. The method fitness-for-purpose was assessed on the basis of the acceptance criteria for each performance characteristics (Eurachem, 1998).

2.7.1. Linearity and range

Linearity was studied in the range of 0.018–0.250 μmol/ml. The calibration curve was prepared as previously described in Section 2.4 with ten concentrations of the standard solution (0.83 μmol/ml). The linearity was assessed by linear regression analysis, calculated by the least square method.

2.7.2. Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection was the lowest concentration of free SH groups that could be detectable, but not necessarily quantified and confirmed as an exact value. The limit of quantification (LOQ) was the lowest concentration of free SH groups in the test sample that could be quantified with acceptable precision and accuracy. LOD and LOQ were calculated as follows (Eurachem, 1998):

$$\mathrm{LOD} = 3 \times \mathrm{SD_{bl}} \quad (2)$$