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

AUTHORS: Slađana Rakita, Milica Pojić, Jelena Tomić, Aleksandra Torbica

This article is provided by author(s) and FINS Repository in accordance with publisher policies.

The correct citation is available in the FINS Repository record for this article.

NOTICE: This is the author's version of a work that was accepted for publication in *Food Chemistry.* Changes resulting from the publishing process, such as peer review, editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published in *Food Chemistry*, Volume 150, 1 May 2014, Pages 158–165. DOI: 10.1016/j.foodchem.2013.10.128

This item is made available to you under the Creative Commons Attribution-NonCommercial-NoDerivative Works – CC BY-NC-ND 3.0 Serbia

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

Slađana Rakita*, Milica Pojić, Jelena Tomić, Aleksandra Torbica

University of Novi Sad, Institute of Food Technology, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia

*Corresponding author:

Slađana Rakita

E-mail: sladjana.rakita@fins.uns.ac.rs

Tel. +381 21 485 3811

Fax: +381 21 450 730

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).

Key words: Free sulphydryl groups; Incubation time; Incubation temperature; Validation protocol

47 **1. Introduction**

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

56 The numerous methods for quantification of free SH content have been developed. The
57 amperometric titration was considered a convenient method for determination of SH groups in
58 purified proteins and amino acids (Carter, 1959). Determination of SH groups of wheat glutenin
59 by direct amino acid analysis was reported by Ewart (1985). Andrews, Caldwell, and Quail
60 (1995) determined free SH content of wheat flour and dough spectrophotometrically using NBD-
61 Cl (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) as a colour developing reagent. For the
62 determination of SH groups of durum wheat semolina and soft wheat flour, covalent bonding of
63 SH groups by 5-iodoacetamide-fluorescein (specific reagent) as a radioactive tracer was
64 employed (Iametti et al., 2013). However, the content of free SH groups has been mostly
65 examined using Ellman's reagent, not only for wheat samples, but for soymilk and rice flour, as
66 well (Gujral & Rosell, 2004; Hayta & Schofield, 2004; Ou, Kwok, Wang, & Bao, 2004; Pérez,
67 Bonet, & Rosell, 2005).

68 Since its introduction in 1959, 5,50-dithiobis-2-nitrobenzoic acid (DNTB or Ellman's
69 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 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 and sample preparation

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, 2000) and milled to laboratory flour using a Bühler MLU 202 (Bühler, Uzwil, Switzerland) according to AACC 26-31 (AACC, 2000). The samples were characterized in terms of Mixolab (Chopin Technologies, Villeneuve-la-Garenne, France)

93 parameters according to ICC standard method 173 (ICC, 2006), wet gluten content and gluten
94 index (GI) according to the ICC standard method 155 (ICC, 1996) and parameters obtained by
95 Kieffer dough extensibility rig for texture analyser TA.XT2 (Stable Micro Systems Ltd.,
96 Godalming, UK) at 30 and 37 °C (Kieffer, Garnreiter, & Belitz, 1981). The samples were also
97 characterized in terms of GI values obtained by standard ICC method 155 (ICC, 1996) after
98 incubation of a piece of dough at 37 °C for 90 minutes (Torbica, Antov, Mastilović, & Knežević,
99 2007).

100 **2.2 Chemicals**

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

107

108 **2.3 Preparation of solutions**

109 Tris-glycine (Tris-Gly) buffer contained 10.4 g Tris, 6.9 g glycine and 12 g EDTA in one
110 litre of deionized water, and the pH was adjusted to 8.0 with 1N NaOH solution. GuHCl/Tris-
111 Gly solution contained 5M guanidine hydrochloride. Ellman's reagent was prepared by
112 dissolving of 40 mg DTNB (5,5'-dithio*bis*-2-nitrobenzoic acid) in 10 ml of Tris-Gly buffer pH
113 8.0, and it was freshly prepared before use.

114 **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 modified method of Pérez et al. (2005), which involved 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 14500 rpm for 6 min. To 400 µl of the supernatant obtained, 600 µl of GuHCl/Tris-Gly solution was added, 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):

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

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 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/100g); W is moisture content of flour (g/100g).

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 means at the 5% significance level.

2.7 Method validation

The method characteristics were assessed by single-laboratory validation procedure according to Decision 2002/657/EC (European Commission, 2002). Validation protocol comprised 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 obtained results of the assessed 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

160 (0.83 µmol/ml). The linearity was assessed by linear regression analysis, calculated by the least
161 square method.

162 **2.7.2 Limit of detection (LOD) and limit of quantification (LOQ)**

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

168 $$LOD = 3 \times SD_{bl} \qquad \text{(Eq. 2)}$$

169 $$LOQ = 10 \times SD_{bl} \qquad \text{(Eq.3)}$$

170 where:

171 SD_{bl} is standard deviation of eight consecutive measurements of a blank sample.

172 **2.7.3 Precision**

173 Method precision was assessed in terms of repeatability (intra-day precision) and
174 reproducibility (inter-day precision). Repeatability was determined by analyzing 5 subsamples of
175 a single-washed gluten sample under repeatability conditions (same apparatus, identical reagents,
176 short interval of time and same analyst) within a single day. Reproducibility was assessed under
177 reproducibility conditions within 5 days by analyzing 5 samples of gluten being individually
178 washed each time before analysis. All samples were analyzed in duplicates. Repeatability (r) and
179 reproducibility (R) was expressed as relative standard deviations (RSD_r and RSD_R), while the
180 acceptability of method precision was assessed on the basis of HORRAT values ($HORRAT_r$ and
181 $HORRAT_R$) defined as the ratio of the actual relative standard deviation (either repeatability

(RSD$_r$) or reproducibility (RSD$_R$)) and relative standard deviations calculated from the Horwitz and modified Horwitz equation (Horwitz, 2000).

2.7.4 Method performance

The performance of a chosen method was demonstrated by means of X- and R-control charts (Mullins, 2003). The central line (CL) and control limits - warning (WL) and action limits (AL) were calculated as follows:

$$CL=\bar{x}=\frac{\sum x_i}{n} \quad \text{(Eq. 4)}$$

$$WL=\bar{x} \pm 2SD \quad \text{(Eq. 5)}$$

$$AL=\bar{x} \pm 3SD \quad \text{(Eq. 6)}$$

where:

SD is standard deviation from a series of measurement results obtained over 29 days. In the X-control chart the plotted values corresponded to the mean values of two replicates, whilst in the R-control chart the plotted values were the percentage of difference between two replicates.

2.7.5 Measurement uncertainty

The measurement uncertainty was estimated starting from the model equation (Eq. 1) comprising thorough identification of the individual sources of uncertainty that influence the measurement results. The identified uncertainty sources were quantified and combined (‘’bottom-up’’ approach) to obtain combined standard uncertainty (u_c) (Eq.7):

$$\frac{u_c(SH)}{SH}=\sqrt{\left[\frac{u(A_s-B_0)}{A_s-B_0}\right]^2+\left[\frac{u(B_1)}{B_1}\right]^2+\left[\frac{u(G)}{G}\right]^2+\left[\frac{u(m)}{m}\right]^2+\left[\frac{u(P)}{P}\right]^2+\left[\frac{u(W)}{100-W}\right]^2}$$

where: $u(A_s\text{-}B_0)$, $u(B_1)$, $u(G)$, $u(m)$, $u(P)$ and $u(W)$ are individual standard measurement uncertainties.

$u(A_s\text{-}B_0)$ was obtained as follows (Eq. 8):

$$u(A_s - B_0) = \sqrt{u(A_s)^2 + u(B_0)^2}$$

The individual standard measurement uncertainties were obtained by combining experimental standard deviation of the mean (Type A) and data obtained by other means than statistical methods (calibration data provided by the measuring equipment) (Type B) (GUM, Ramachndran & Rasmi, 1999). The expanded uncertainty (U) was calculated using a coverage factor k=2, which gives a level of confidence of approximately 95% (Eq. 9):

$$U = u_c(SH) \times 2$$

The expanded uncertainty was reported as relative (%) and absolute value (μmol/g). Moreover, the budget of measurement uncertainty was reported in order to indentify the most dominant uncertainty contributors.

3. Results and discussion

3.1 Characterization of selected sample set

The sample set was chosen to cover as wide ranges in protein content and gluten strength as possible and it was characterized in terms of parameters commonly used for the characterization of protein complex status of wheat and wheat dough. The status of wheat flour protein complex was estimated on the basis of Mixolab parameters comprising water absorption (WA), torque at development time (C1), dough development time (DevMix), dough stability

(StabMix), torque in a point of minimal torsion (C2) and protein network weakening rate (C2-C1). The characteristics of the chosen sample set in terms of selected parameters are given in Table 1. Water absorption, as an amount of water required for protein hydration and gluten network formation, were in the range of 50.70–59.30%, showing the wide variability in terms of protein water absorption ability. Dough development time and dough stability proved to be highly significantly correlated with protein strength (Cervantes-Espinoza, Dubat, Ortiz-Monasterio, Peña, & Posadas, 2008). Dough development time defined as the time required to reach the optimal dough consistency of 1.1 Nm (in C1), varied between 0.56 and 9.00 min and dough stability, which indicates its resistance to kneading, varied between 8.85 and 11.22 min indicating diversity of selected samples in terms of protein strength. C2 values, being in the range of 0.46-0.58, indicated that the chosen samples were slightly different in terms of protein network weakening due to the mechanical work and temperature increase. The measurement of the gluten index before and after incubation (at 37 °C) has been proposed as a useful and objective way to detect the wheat proteins hydrolysis caused by heteropterous insects (Aja, Pérez, & Rosell, 2004). GI values were in the range of 74.03–99.19%, indicating that chosen samples were of different gluten strength – from strong to very strong gluten (AbuHammad, Elias, Manthey, Alamri, & Mergoum, 2012). However, the corresponding gluten index values obtained after previous incubation of piece of dough at 37 °C ($GI_{37\,°C}$) were lower and within the range of 53.51–93.64% indicating that certain size redistribution of the gluten proteins occurred (Rosell, Aja, Bean, & Lookhart, 2002; Aja et al., 2004). The same trend was noticed for the values of uniaxial extension parameters measured by the method of Kieffer, such as maximum extensibility (D and $D_{37\,°C}$) and maximum force (F and $F_{37\,°C}$).

3.2 The content of free SH groups of gluten

249 The determination of free sulphydryl groups was performed at two different temperatures
250 (30 and 37 °C) that were selected on the basis of standardized conditions prescribed for most
251 rheological measurements (30 °C) as well as favourable conditions for the activity of potentially
252 present proteolytic enzymes (37 °C) (Pérez et al., 2005). Incubation times of 45, 90 and 135
253 minutes corresponded to the operating conditions prescribed by standard Brabender
254 Extensograph method 114 (ICC, 1996) to measure extensional properties of wheat dough.
255 The changes in free sulphydryl content of wet gluten in relation to the incubation temperature
256 and time is presented in Fig. 2. Free SH content of tested samples incubated at 30 °C was in the
257 range of 2.10-2.57 μmol/g protein, being in agreement with the results previously reported by
258 Andrews et al. (1995) and Hayta et al. (2004). The obtained results were also in compliance with
259 the results of Stathopoulos, Tsiami, Schofield, and Dobraszczyk (2008) who reported the
260 variation of SH groups of gluten in the range 2–4 μmol/g protein at incubation temperature of 25
261 °C for 20 min. During the first 90 min of incubation at 30 °C a slight increase in SH content ($p > 0.05$) was observed in relation to the control sample. However, the extension of incubation time
263 to 135 min resulted in significantly higher content of free sulphydryls ($p < 0.05$).
264 Quantification of free SH groups has been extensively studied by many cereal
265 researchers. The direct comparison of the actual results with previously published results is often
266 difficult due to the inconsistency in the material used for testing (flour, dough, gluten) and
267 expression of results, which have been expressed either on the flour, gluten or protein basis.
268 Koehler (2003) indicated that data on free SH groups would be more meaningful if they are
269 expressed on a protein instead of flour basis. In this respect, the standardization of a method for
270 determination of free SH content in wheat-based matrices would be considerably beneficial. As a

contribution to this remark, within the framework of this study, the analytical validation of the method for determination of free SH content of gluten was performed.

The measurement of free SH groups of flour could beproblematic due to low concentrations in which they occur. Antes and Wieser (2000) reported the total SH content of flour in the range of 1–1.5 μmol/g of flour, where approximately 30% of them could be assigned to the glutenins. Koehler (2003), Koehler (2004) reported that free SH groups of dough were preferably located in the glutenin fraction, where their concentration was in the range of 0.22–0.33 μmol/g of flour which corresponded to the 5.6–8.2 μmol/g of protein. Kieffer, Schurer, Köhler, and Wieser (2007) reported the SH content of gluten, previously treated with atmospheric pressure and temperature of 30 °C for 10 min, was 1.61 μmol/g protein. Gómez, Ferrero, Calvelo, Añón, and Puppo (2011) reported the content of free SH groups in the range of 1.42–2.43 μmol/g flour. The apparent discrepancy between the content of free sulphydryls obtained by different authors might be explained either by the differences originating from different varieties, different quality and/or different conditions of the treatment employed (temperature, pressure, chemicals, etc.).

The higher incubation temperature (37 °C) influenced the increase in content of free sulphydryl groups of gluten, being in the range of 2.10-3.45 μmol/g protein and significantly higher ($p<0.05$) then of those samples tempered at 30 °C (Fig. 2). While the SH content of gluten samples incubated at 30 °C for 135 minutes was approximately 20% higher than that of control gluten sample, the SH content of gluten samples tempered at 37 °C for 45, 90 and 135 minutes were about 35, 50 and 65%, respectively, higher than that of control sample. The presented results indicated that higher incubation temperature promoted enzymatic hydrolysis process and gluten structure breakdown, leading the corresponding increase in the amount of free

294 sulphydryls. The temperature of 37 °C corresponds to optimal conditions for the hydrolytic
295 enzyme degradation; therefore it could be assumed that gluten proteins were hydrolysed during
296 incubation either by endogenous or exogenous proteolytic enzymes into smaller polypeptides or
297 amino acids (Pérez et al., 2005). As a result of enzymatic hydrolysis and protein cleavage,
298 molecular weights of peptides decrease while their solubility increase (Wang, Wei, Li, Bian, &
299 Zhao, 2009). The present results were consistent with the results of GI values showing
300 significant decrease from 92.95 to 74.21% ($p<0.05$) as incubation temperature increased. The
301 same trend was observed by Aja et al. (2004) who reported a steady decrease in GI values
302 induced by incubation at 37 °C, suggesting rapid hydrolysis involving a size redistribution of the
303 gluten proteins. By studying the impact of wheat bugs (*Aelia spp* and *Eurygaster spp*) on gluten
304 degradation, Pérez et al. (2005) indicated a rapid increase in free sulphydryls during the first
305 hour of incubation at 37 °C of gluten from damaged wheat. Simultaneously, a slight increase in
306 free sulphydryl groups was registered during 7-hour incubation of gluten of sound wheat. By
307 examining the effects of increased temperature on the level of SH groups of gluten, Stathopoulos
308 et al. (2008) observed that SH content decreased with increasing temperature from 25 to 40 °C,
309 implying that heating always affected the number of SS bonds formed. Wang et al. (2009)
310 reported that the free SH-content of wheat gluten remained constant when incubated at 50 and 60
311 °C, as well as the decrease in sulphydryl groups with further increase in the incubation
312 temperature up to 90 °C which was explained by the lower efficiency of enzymatic hydrolysis
313 due to thermal treatment.

314 **3.3 Analytical validation of method for determination of free SH content of gluten**

315 The suitability of a certain procedure for intended purpose is evaluated during method
316 validation, where depending on the type of analytical procedure, different characteristics of

317 analytical procedure are evaluated. The method characteristics that are commonly evaluated
318 include: accuracy, linearity, precision, specificity, range, detection limit, quantification limit,
319 robustness and measurement uncertainty (Dejaegher, Dumarey, Capron, Bloomfield, & Vander
320 Heyden, 2007). However, each analytical application does not require verification of all
321 characteristics, but only those indicated as relevant for the intended purpose. In this respect,
322 method characteristics indicated as relevant within the frame of this study were: linearity, limit
323 of detection, limit of quantification, precision (repeatability and reproducibility) and
324 measurement uncertainty. The method performance was monitored by X- and R-control charts.
325

326 **3.3.1 Linearity**

327 Linearity of a method is defined as the ability of a method to obtain test results
328 proportional to the concentration of analyte. Consequently, the linear range is the range of
329 analyte concentrations over which the method gives test results proportional to the concentration
330 of the analyte (Eurachem, 1998). The absorbance values at 412 nm increased linearly with the
331 increase in concentration of reactive sulfhydryl groups in the standard solutions. The linear
332 regression analysis resulted in the following calibration equations (Eq. 10):

333 $$y = 3.643x + 0.0109$$

334 where:

335 x is the free sulphydryl concentration in μmol/ml.

336 The obtained linear regression equation indicated a good linearity between the
337 absorbance and concentration in the range of 0.018-0.250 μmol/ml, with a correlation coefficient
338 of r=0.9991, being highly significant for the method.

339 **3.3.2 Limit of detection and limit of quantification**

340 Limit of detection (LOD), as the lowest concentration of the analyte that could be reliably
341 detected by the method, was calculated to be 0.0096 μmol/ml. Limit of quantification (LOQ), as
342 the lowest concentration of free SH groups in the test sample that could be quantified with
343 acceptable precision and accuracy, was calculated to be 0.0320 μmol/ml.

344 **3.3.3 Precision (repeatability and reproducibility)**

345 The acceptability of a method precision was estimated on the basis of HORRAT values
346 calculated as the ratio of relative standard deviations (repeatability - RSDr or reproducibility -
347 RSDR) and respective relative standard deviations calculated from the modified Horwitz
348 equation (Table 2). Repeatability was demonstrated by analysing five samples in duplicate from
349 a single-washed gluten sample under repeatability conditions. Method reproducibility was
350 assessed by quantification of free sulphydryls from 5 gluten samples being individually washed
351 each time before the analysis during five independent days. Since previous measurements
352 showed no significant differences in the content of sulfhydryl groups during 45 and 90 minutes
353 incubation at 30 °C, they were omitted from precision experiment.

354 The HORRAT values calculated from repeatability data ($HORRAT_r$) were less than 2 for
355 all targeted incubation temperatures and times indicating that the performance criterion (<2) was
356 met and demonstrating the validity of the method repeatability (Table 2). Furthermore, the
357 HORRAT values calculated from reproducibility data ($HORRAT_R$) obtained for the incubation
358 temperature of 30 °C were less than 2, demonstrating the validity of the method reproducibility
359 under the above mentioned conditions. However, when the assay included the incubation of
360 gluten samples at 37 °C, HORRAT values calculated from reproducibility data ($HORRAT_R$)
361 were greater than 2 indicating that the performance criterion (HORRAT<2) was not met. Hence,
362 the reproducibility of the method when performed with incubation at 37 °C was questionable.

363 Aja et al. (2004) indicated that between 60 and 90 minutes of incubation intense hydrolysis of
364 gluten occurred followed by the releasing of water soluble compounds. It is likely that during
365 incubation at 37 °C enzymatic hydrolysis occurred, where the progress of enzymatic reactions is
366 more difficult to control in relation to the chemical reactions. Therefore, the adequacy of the
367 classical interpretation of the acceptability of method reproducibility on the basis of HORRAT
368 values (HORRAT<2) has remained questionable.

369 **3.3.4 Method performance**

370 Two types of control charts were selected to monitor the analytical stability of the
371 selected method: X-control chart to monitor the bias on the basis of the measurement of a control
372 sample and R-control chart to monitor the precision on the basis of duplicate measurements of
373 every tenth test sample (Mullins, 2003). Regardless of incubation temperature (30 and 37 °C),
374 the obtained results were located within the warning limits for X-control chart (between 1.0 and
375 3.6 μmol/g protein for incubation temperature of 30 °C and between 1.7 and 3.5 μmol/g protein
376 for incubation temperature of 37 °C (data not shown)) as well as below the upper warning limit
377 for R-control chart (below 4.5 μmol/g protein for incubation temperature of 30 °C and below 6.0
378 μmol/g protein for incubation temperature of 37 °C (data not shown)). X- and R-control chart
379 obtained for incubation temperature of 30 °C are presented in Fig. 3. Therefore, it was indicated
380 that present analytical method is of long-term stability both in terms of changes in measurement
381 level (biases) and variability (precision) (Mullins, 2003).

382 **3.3.5 Measurement uncertainty**

383 In order to ensure a level of confidence of the measurement results and provide reliable
384 and comparable data demanded by scientific community nowadays, measurement uncertainty
385 evaluation included the estimation of all possible sources of uncertainty originating from the

386 model equation (Eq. 1 and Eq. 7). The measurement uncertainty was estimated by measurement
387 of SH groups performed immediately after gluten washing without previous incubation (Table
388 3). The expanded uncertainty was estimated to be 0.4742 μmol/g expressed as absolute value
389 that was 20% expressed as the relative value. Data from uncertainty budget demonstrated that the
390 main contributors to the overall uncertainty were the absorbance measurements (53.14%), the
391 construction of the calibration curve (slope) (13.28%) and the wet gluten content determination
392 (29.38%). Sample mass (4.00%), protein (0.19%) and moisture (0.01%) measurements were
393 components with the smallest contribution to the overall uncertainty. Grande, Falcón, Comesaña,
394 and Gándara (2001) previously revealed that the preparation of standards and the construction of
395 the calibration curve were the dominant source of uncertainty in HPLC measurements. Sooväli,
396 Rõõm, Kütt, Kaljurand, and Leito (2006) demonstrated that those sources of uncertainty
397 originating from the UV-Vis spectrophotometer itself (repeatability of reading, drift, stray light)
398 generally have significantly lower contributions to the combined uncertainty of the result than
399 those sources that originate from the tested sample (interference from the constituents of the
400 matrix, decomposition of the photometric complex).

401 **4. Conclusion**

402 The method applied herein was successfully employed to quantify the content of free
403 sulphydryl groups in wheat gluten samples incubated at two selected temperatures (30 and 37
404 °C) during three incubation times (45, 90, 135 minutes). It was observed that the increase in
405 temperature and gluten incubation time had caused the increase in amount of free sulphydryl
406 groups, with more dynamic change at 37 °C. The method characteristics tested within the
407 validation experiment demonstrated the acceptable quality for method fitness-for-purpose,
408 especially in terms of limit of detection, limit of quantification, linearity, stability, repeatability

409 and reproducibility when assay was performed at 30 °C. Although the method repeatability at 37
410 °C was acceptable, the corresponding reproducibility had not met the performance criterion on
411 the basis of HORRAT value (HORRAT < 2). Therefore, the adequacy of the classical
412 interpretation of the acceptability of method reproducibility on the basis of HORRAT values
413 when there is possibility for enzymatic activity involvement should be reconsidered. The
414 validation protocol presented in the current study represents an important contribution to the
415 standardization of the method for determination of free sulphydryl groups in wheat gluten.
416
417 **Acknowledgment**
418 This paper is a result of the research within the project TR 31007 financed by the Ministry of
419 Education, Science and Technological Development, Republic of Serbia.
420
421
422 **References**
423 AACC International (2000). Approved Methods of Analysis. St. Paul, USA: American
424 Association of Cereal Chemists.
425 AbuHammad, W. A., Elias, E. M., Manthey, F. A., Alamri, M. S., & Mergoum, M. (2012). A
426 comparison of methods for assessing dough and gluten strength of durum wheat and their
427 relationship to pasta cooking quality. *International Journal of Food Science and*
428 *Technology*, 47, 2561-2573.
429 Aja, S., Perez, G., Rosell, C. M. (2004). Wheat damage by *Aelia spp.* and *Erygaster spp.*: effects
430 on gluten and water-soluble compounds released by gluten hydrolysis. *Journal of Cereal*
431 *Science*, 39, 187-193.

Andrews, D. C., Caldwell, R. A., & Quail, K. J. (1995). Sulfhydryl analysis. I. Determination of free sulfhydryls in wheat flour doughs. *Cereal Chemistry*, 72, 326-329.

Antes, S., & Wieser, H. (2000). Quantitative determination and localisation of thiol groups in wheat flour. In P. R. Shewry & A. S. Tatham (Eds.), *Wheat Gluten: Proceedings of the 7th International Workshop* (pp. 211-214). Cambridge, United Kingdom: Royal Society of Chemistry.

Carter, J. R. (1959). Amperometric titration of disulfide and sulfhydryl in proteins in 8 M urea. *The Journal of Biological Chemistry*, 234, 1705-1710.

Cervantes-Espinoza, M. I., Dubat, A., Ortiz-Monasterio, J. I., Peña, R J., & Posadas G. (2008). Gluten composition, gluten quality, and dough mixing properties (National-Mixograph; Chopin-Mixolab) of high yielding wheats derived from crosses between common (*T. aestivum*) and synthetic (Triticum dicoccon x Aegilopstauschii) wheats. In J. L. Molina-Cano, P. Christou, A. Graner, K. Hammer, N. Jouve, B. Keller, J. M. Lasa, W. Powell, C. Royo, P. Shewry, & A. M. Stanca (Eds). *Cereal science and technology for feeding ten billion people: genomics era and beyond* (pp. 357-359). Zaragoza: CIHEAM/IRTA.

Chan, K. Y., & Wasserman, B. P. (1993). Direct colorimetric assay of free thiol groups and disulfide bonds in suspensions of solubilized and particulate cereal proteins. *Cereal Chemistry*, 70, 22-26.

Dejaegher, B., Dumarey, M., Capron , X., Bloomfield, M. S., & Vander Heyden, Y. (2007). Comparison of Plackett–Burman and supersaturated designs in robustness testing. *Analytica Chimica Acta*, 595, 59-71.

453 Delcour, J. A., Joye, I. J., Pareyt, B., Wilderjans, E., Brijs, K., & Lagrain, B. (2012). Wheat
454 gluten functionality as a quality determinant in cereal-based food products. *Annual Review*
455 *of Food Science and Technology*, 3, 469-492.
456 Ellman, G. L. (1959). Tissue sulfhydyl groups. *Archives of Biochemistry and Biophysics*, 82, 70-
457 77.
458 Eurachem (1998). The fitness for purpose of analytical methods: a laboratory guide to method
459 validation and related topics. Uppsala, Sweden: Eurachem.
460 European Commission (2002). Commission decision 2002/657/EC of 12 August 2002
461 implementing Council Directive 96/23/EC concerning the performance of analytical
462 methods and the interpretation of results. *Official Journal of the European Communities,*
463 L221, 8–36.
464 Ewart, J. A. D. (1985). Blocked thiols in glutenin and protein quality. *Journal of the Science of*
465 *Food and Agriculture*. 36, 101-112.
466 Gómez, A., Ferrero, C., Calvelo, A., Añón, M. C., & Puppo, M. C. (2011). Effect of mixing time
467 on structural and rheological properties of wheat flour dough for breadmaking.
468 *International Journal of Food Properties*, 14, 583-598.
469 Grande, B. C., Falcón, M. S. G., Comesaña M. R., & Gándara, J. S. (2001). Determination of
470 sulfamethazine and trimethoprim in liquid feed premixes by HPLC and diode array
471 detection, with an analysis of the uncertainty of the analytical results. *Journal of*
472 *Agriculture and Food Chemistry*, 49, 3145-3150.
473 Gujral, H. S., & Rosell, C. M. (2004). Improvement of the breadmaking quality of rice flour by
474 glucose oxidase. *Food Research International*, 37, 75-81.

475 Hayta, M., & Schofield, J. D. (2004). Heat and additive induced biochemical transitions in gluten
476 from good and poor breadmaking quality wheats. *Journal of Cereal Science*, 40, 245-256.
477 Horwitz, W. (2000). The potential use of quality control data to validate pesticide residue
478 method performance. In A. Fajgelj, & Á. Ambrus (Eds.), *Principles and Practices of*
479 *Method Validation*, (pp. 1-8). Cambridge, UK: The Royal Society of Chemistry.
480 Iametti, S., Marengo, M., Miriani, M., Pagani, M. A., Marti A., & Bonomi, F. (2013). Integrating
481 the information from proteomic approaches: a "thiolomics" approach to assess the role of
482 thiols in protein-based networks. *Food Research International*, in press.
483 ICC Standards (1996). Standard Methods of the International Association for Cereal Science and
484 Technology. Vienna, Austria: International Association for Cereal Science and
485 Technology.
486 ICC Standards (2006). Standard Methods of the International Association for Cereal Science and
487 Technology. Vienna, Austria: International Association for Cereal Science and
488 Technology.
489 ISO GUM - Guide to the expression of uncertainty in measurement (1995). International
490 Organization for Standardization (Geneva, Switzerland).
491 Johansson, E., Malik, A. H., Hussain, A., Rasheed, F., Newson, W. R., Plivelic, T., Hedenqvist,
492 M. S., Gällstedt, M., & Kuktaite, R. (2013). Wheat gluten polymer structures: The impact
493 of genotype, environment, and processing on their functionality in various applications.
494 *Cereal Chemistry*, 90, 367-376.
495 Kieffer, R., Garnreiter, F., & Belitz, H. D., (1981). Beurteilung von teigeigenschaften durch
496 zugversuche im mikromassstab. *Zeitschrift fur Lebensmittel-Untersuchung und -*
497 *Forschung*, 127, 193-194.

498 Kieffer, R., Schurer, F., Köhler, P., & Wieser, H. (2007). Effect of hydrostatic pressure and
499 temperature on the chemical and functional properties of wheat gluten: Studies on gluten,
500 gliadin and glutenin. *Journal of Cereal Science*, 45, 285-292.
501 Koehler, P. (2003). Concentrations of low and high molecular weight thiols in wheat dough as
502 affected by different concentrations of ascorbic acid. *Journal of Agricultural and Food*
503 *Chemistry*, 51, 4948-4953.
504 Koehler, P. (2004). Effect of ascorbic acid in dough: reaction of oxidised glutathione with
505 reactive thiol groups of wheat glutelin. In D. Lafiandra, S. Masci, & R. D'Ovidio (Eds.),
506 *The Gluten Proteins*, Cambridge, UK: The Royal Society of Chemistry.
507 Mullins, E. (2003). *Statistics for the Quality Control Chemistry Laboratory*. Cambridge, UK:
508 The Royal Society of Chemistry.
509 Ou, S., Kwok, K. C., Wang, Y., & Bao, H. (2004). An improved method to determine SH and –
510 S–S– group content in soymilk protein. *Food Chemistry*, 88, 317-320.
511 Perez, G., Bonet, A., & Rosell, C. M. (2005). Relationship between gluten degradation by Aelia
512 spp and Eurygaster spp and protein structure. *Journal of the Science of Food and*
513 *Agriculture*, 85, 1125-1130.
514 Ramachandran, R. & Rashmi (1999). Uncertainty of measurement in spectrophotometric
515 analysis: A case study. *Analyst*, 124, 1099-1103.
516 Rosell, C. M., Aja, S., Bean, S., & Lookhart, G. (2002). Effect of *Aelia* and *Eurygaster* damage
517 on wheat proteins. *Cereal Chemistry*, 79, 801-805.
518 Sooväli, L., Rõõm, E. I, Kütt, A., Kaljurand, I. & Leito, I. (2006). Uncertainty sources in UV-Vis
519 spectrophotometric measurement. *Accreditation and Quality Assurance*, 11, 246-255.

520 Stathopoulos, C. E., Tsiami, A. A., Schofield, J. D., & Dobraszczyk, B. J. (2008). Effect of heat
521 on rheology, surface hydrophobicity and molecular weight distribution of glutens extracted
522 from flours with different bread-making quality. *Journal of Cereal Science*, 47, 134-143.
523 Torbica, A., Antov, M., Mastilović, J., & Knežević, D. (2007). The influence of changes in
524 gluten complex structure on technological quality of wheat (*Triticum aestivum* L.). *Food*
525 *Research International*, 40, 1038-1045.
526 Wang, J. S., Wei, Z. Y., Li, L., Bian, K., & Zhao, M. M. (2009). Characteristics of enzymatic
527 hydrolysis of thermal-treated wheat gluten . *Journal of Cereal Science*, 50, 205-209.
528 Wieser, H. (2007). Chemistry of gluten proteins. *Food Microbiology*, 24, 115–119.
529 Wilson, J. M., Wu, D., Moth-DeGrood, R,. & Hupe, D. J. (1980). A spectrophotometric method
530 for studying the rates of reaction of disulfides with protein thiol groups applied to bovine
531 serum albumin. *Journal of the American Chemical Society*, 102, 359-363.
532

533 **Table 1 – Characteristics of chosen sample set**

Parameters	Mean	Range		SD	CV
		Min	Max		
WA_{Mix}	56.12	50.70	59.30	3.18	5.67
C1	1.11	1.07	1.19	0.03	2.66
Dev_{Mix}	4.64	0.56	9.00	2.77	59.67
$Stab_{Mix}$	10.13	8.85	11.22	0.74	7.33
C2	0.52	0.46	0.58	0.03	5.39
C2-C1	0.60	0.52	0.71	0.04	7.34
GI	92.95	74.03	99.19	6.20	6.67
GI $^{(37\ ºC)}$	74.21	53.51	93.64	10.81	14.57
F $^{(30\ ºC)}$	16.40	11.97	20.55	2.50	15.25
D $^{(30\ ºC)}$	35.86	22.52	57.64	8.66	24.15
F $^{(37\ ºC)}$	11.80	8.24	19.44	2.40	20.35
D $^{(37\ ºC)}$	39.48	16.35	50.71	7.61	19.28
Wet gluten content (%)	31.00	24.00	38.00	0.04	11.83
Protein content (% d.m.)	11.82	10.45	12.83	0.79	6.71

534 *WA_{Mix} - Mixolab water absorption (%); Dev_{Mix} - Mixolab development time (min); $Elast_{Mix}$ Mixolab dough elasticity*
535 *(Nm); $Stab_{Mix}$ - Mixolab stability (min); C1 - Mixolab torque at development time (Nm); C2 - Mixolab torque in*
536 *point of minimal torsion (Nm); α - Protein network weakening rate (Nm/min); GI - Gluten index (%); GI (37 ºC) -*
537 *Gluten index at 37 ºC (%); D (30 ºC) - Kieffer extensibility (mm); F (30 ºC) - Kieffer resistance to extension (g); D*
538 *(37 ºC) - Kieffer extensibility (mm); F (37 ºC) - Kieffer resistance to extension (g); SD - standard deviation; CV -*
539 *coefficient of variation*

Table 2 – Precision parameters of the method for determination of free sulphydryl groups

Time of incubation (min)	Temperature (°C)	Repeatability			Reproducibility		
		RSD_r (%)	$RSD_{r,H}$ (%)	$HORRAT_r$	RSD_R (%)	$RSD_{R,H}$ (%)	$HORRAT_R$
0	-	1.634	2.377	0.688	6.275	3.513	1.786
135	30	1.683	2.475	0.680	6.529	3.424	1.907
45	37	1.598	2.357	0.678	9.112	3.495	2.607
90	37	1.765	2.266	0.779	9.595	3.412	2.812
135	37	2.342	2.347	0.998	15.666	3.427	4.572

RSD_r – repeatability relative standard deviation; RSD_R – reproducibility relative standard deviation; $RSD_{r,H}$ – Horwitz repeatability relative standard deviation; $RSD_{R,H}$ –Horwitz reproducibility relative standard deviation; $HORRAT_r = RSD_r/RSD_{r,H}$; $HORRAT_R = RSD_R/RSD_{R,H}$

545 **Table 3 – The overview of measurement data and corresponding standard uncertainties**

Input quantity		Value	Standard uncertainty
Concentration of calibration solutions	C1, μmol/ml	0.018	0.13×10^{-3}
	C2, μmol/ml	0.036	0.32×10^{-3}
	C3, μmol/ml	0.050	0.32×10^{-3}
	C4, μmol/ml	0.080	1.26×10^{-3}
	C5, μmol/ml	0.100	1.57×10^{-3}
	C6, μmol/ml	0.120	1.88×10^{-3}
	C7, μmol/ml	0.150	1.63×10^{-3}
	C8, μmol/ml	0.200	2.17×10^{-3}
	C9, μmol/ml	0.250	2.72×10^{-3}
Absorbance of the calibration solutions	A1	0.0734	0.0164
	A2	0.1351	0.0111
	A3	0.2003	0.0049
	A4	0.3023	0.0051
	A5	0.3933	0.0218
	A6	0.4481	0.0324
	A7	0.5646	0.0168
	A8	0.7382	0.0246
	A9	0.9107	0.0427
Intercept of the calibration curve	B_0	0.0109	0.0038
Slope of the calibration curve	B_1	3.6430	0.1332
Absorbance of the sample solution	A_s	0.3008	0.0209
Wet gluten content	G, g/100 g	25.16	1.37
Sample mass	m, g	0.1016	0.0020
Protein content	P, g/100 g	11.24	0.05
Moisture content	W, g/100 g	13.26	0.05
Concentration of SH groups in the sample	μmol/g	2.1054	0.1698
Combined standard uncertainty	u_c(SH), μmol/g	**0.2112**	
Expanded uncertainty (k=2) Absolute value	U(SH), μmol/g	**0.4224**	
Expanded uncertainty (k=2) Relative value	%	**20.06**	

546

547

548

549 **Figure captions**

550 Fig. 1 – Scheme of applied method for determination of sulphydyl content in wheat gluten
551 samples

552 Fig. 2 – Effect of incubation temperature (a) and time (b) on the free sulphydryl content of wet
553 gluten

554 Fig. 3 – Calibration curve for standard solutions (L-cistein)

555 Fig. 4 – X-control chart for the determination of free sulphydryls with the incubation temperature
556 of 30 °C (a) and 37 °C (b) (CL - central line; UWL - upper warning limit; LWL - lower warning
557 limit; UAL - upper action limit; LAL - lower action limit)

558 Fig. 5 – R-control chart for the determination of free sulphydryls with the incubation temperature
559 of 30 °C (a) and 37 °C (b) (CL - central line; UWL - upper warning limit; UAL - upper action
560 limit)

561 Fig. 6 – Uncertainty contributions of different uncertainty components for spectrophotometric
562 determination of free sulphydryl groups.

563

564

565

566

567

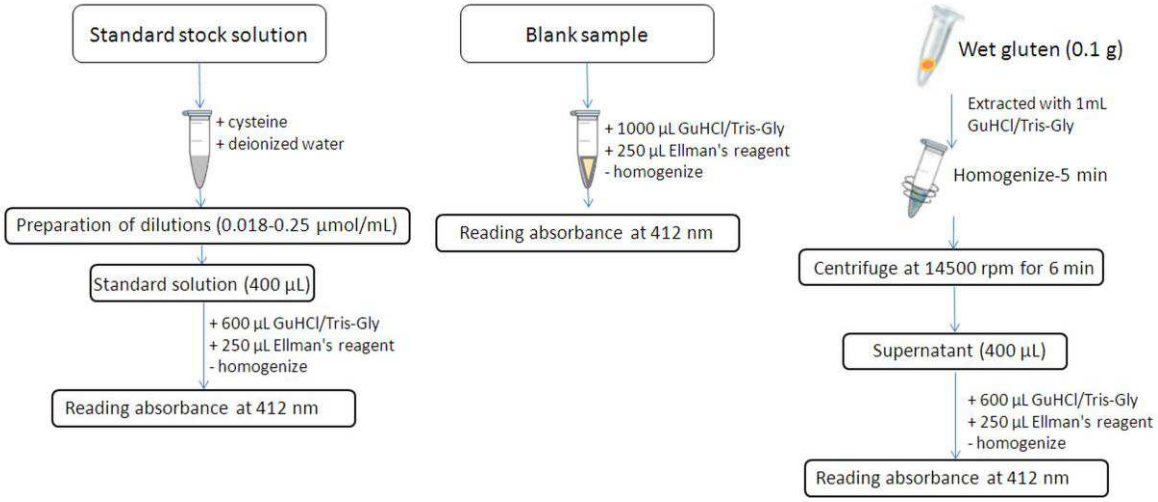
568

569

570

571

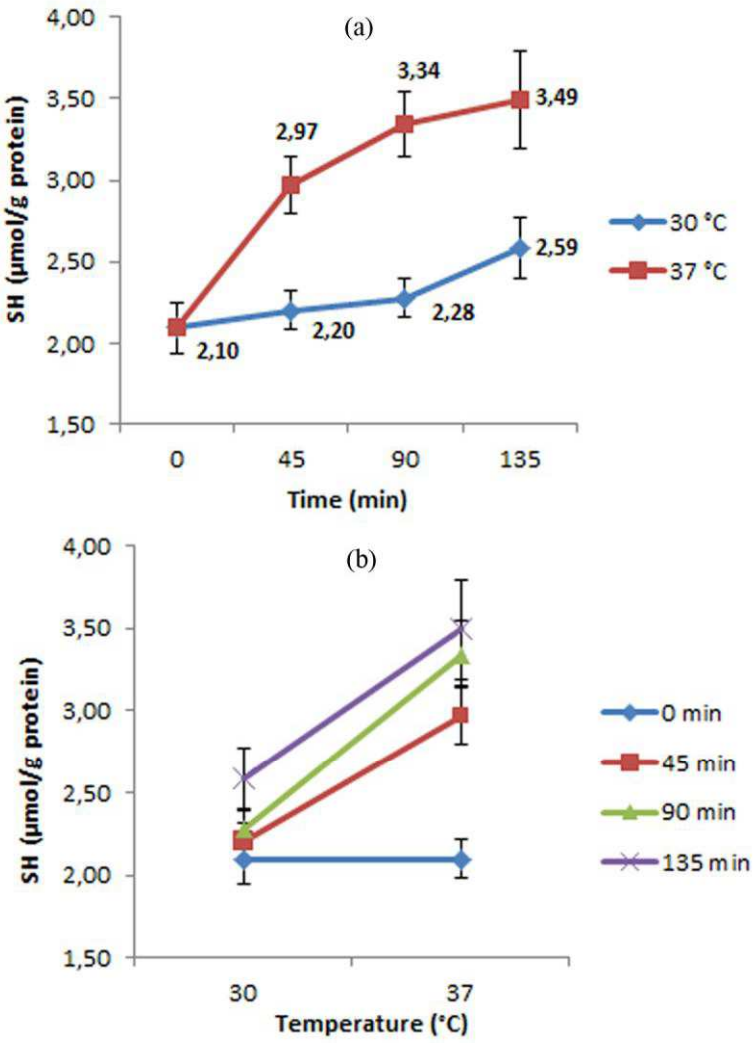
572 Figure 1.



573

574

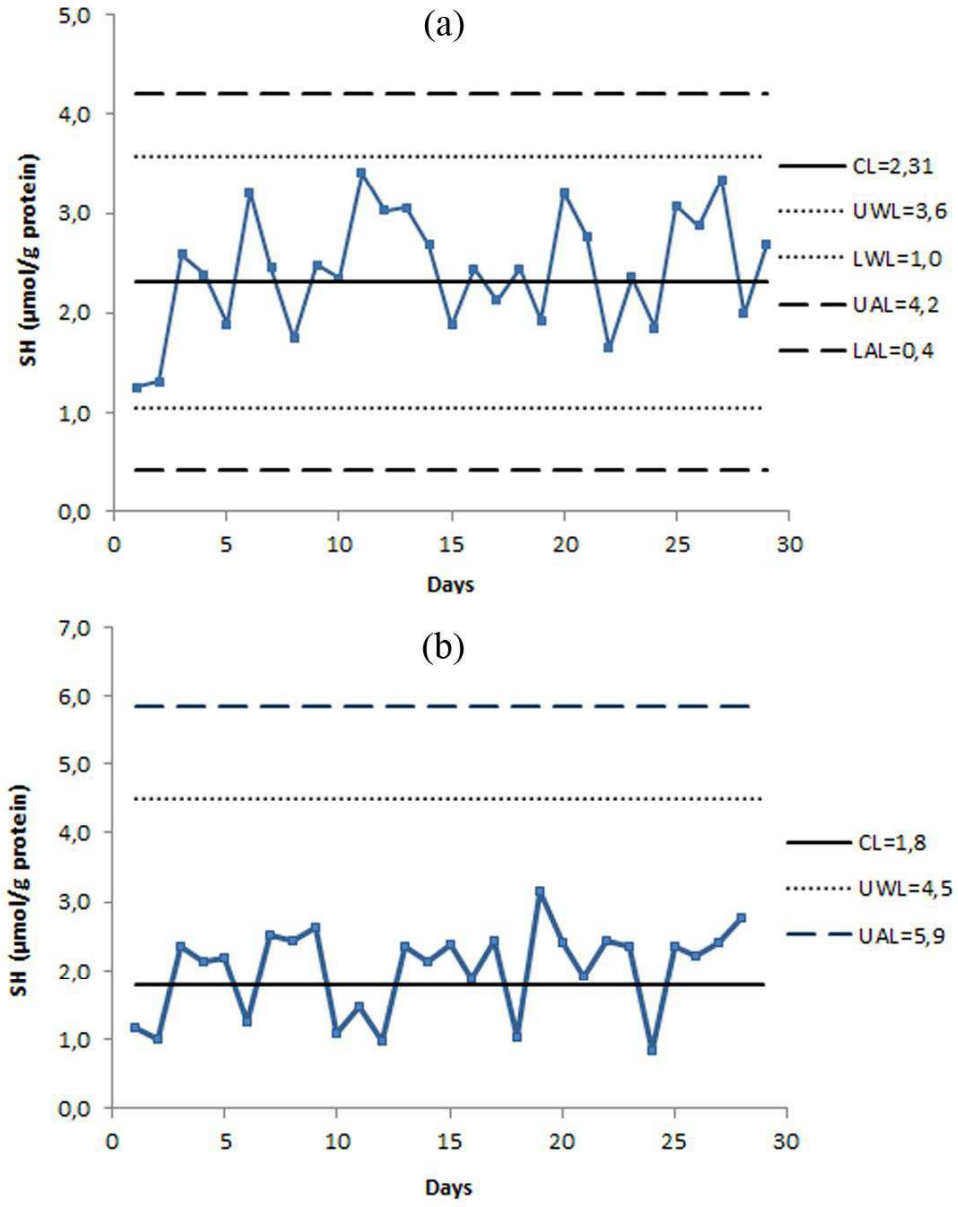
575 Figure 2.



576

577

578 Figure 3.



579