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Concentrations of Total Glutathione and Cysteine in Wheat Flour as Affected by Sulfur Deficiency and Correlation to Quality Parameters

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A method for the simultaneous quantitation of total glutathione and total cysteine in wheat flour by a stable isotope dilution assay using high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) was developed. As internal standards, L-[$^{13}C_3$, ^{15}N]cysteine and L-γ-glutamyl-L-[$^{13}C_3$, ^{15}N]cysteinyl-glycine were used. The method consisted of the extraction and reduction of flour with tris(2-carboxyethyl) phosphine after the addition of internal standards, protection of free thiol groups with iodoacetic acid, derivatization of free amino groups with dansyl chloride, and HPLC-MS/MS. The limits of detection and quantitation for glutathione were 0.75 nmol/g and 2.23 nmol/ g flour, respectively. For cysteine, the limits of detection and quantitation were 0.72 nmol/g and 2.12 nmol/g flour, respectively. The developed method was found to be sensitive enough for quantitation of total glutathione and cysteine levels in wheat flour. This method was then utilized to investigate the effect of sulfur (S) deficiency on the amount of total glutathione and cysteine in flour. In S-deficient wheat, the concentrations of total glutathione and cysteine were proportional to the amount of S supplied during growth. The calculation of correlations revealed that GSH and Cys concentrations influenced the rheological dough properties and the baking performance at least as much as protein parameters. Thus, the low concentration of GSH and Cys in flour from S-deficient wheat had a similar effect on the technological properties as the altered composition of gluten proteins.

INTRODUCTION

Since the fundamental discoveries by Justus von Liebig in the first half of the 19th century, sulfur (S) has been known as one of the major plant nutrients along with nitrogen (N), phosphorus, and potassium. However, S fertilization was not common for wheat crops until the 1980s with air pollution from traffic and industry providing enough S in the soil for wheat growth. After the 1979 Geneva Convention on Long-Range Transboundary Air Pollution (*1*), sulfur dioxide (SO_2) emissions were dramatically reduced, and as a consequence, S deficiency in soil was observed (*2*). Since then, S fertilization and the effect of S deficiency on growing plants, crops, and the properties of dough prepared from wheat flour with S deficiency have attracted considerable interest.

Several studies (*3–7*) indicated that S deficiency affects the amount and the proportions of different gluten protein types in flour, while the content of albumins, globulins, and total gluten proteins were hardly influenced by S fertilization. S deficiency caused a significant increase in the amounts of S-free ω-gliadins and moderately increased the amount of S-poor high molecular weight (HMW) glutenin subunits. Conversely, S rich γ-gliadins as well as low molecular weight (LMW) glutenin subunits decreased dramatically in the case of S deficiency, while the amount of α-gliadins declined only moderately. Additionally, it has been shown that S deficiency causes low yield and poor technological properties of wheat, the latter of which results in doughs that are less extensible and more resistant to extension and in loaves of smaller volume and poorer texture (*3, 5, 6, 8–13*). Changes in the proportions of gluten protein fractions and types have been proposed to be responsible for these impairments (*3, 5, 7, 14*). However, another possible effect of S deficiency on dough properties and baking quality has been discussed by Zhao et al. (*14*) through the involvement of low molecular weight (LMW) thiols present in flour such as cysteine (Cys) and glutathione (GSH). Numerous studies have demonstrated the large weakening effect of these thiols on dough by SH/SS interchange reactions with gluten proteins (*15*). However, a certain amount of thiols in the developing dough is thought to be necessary owing to an optimal arrangement of gluten proteins (*16*). Thus, Zhao et al. (*14*) speculated that low S supply to wheat may decrease the concentration of endogenous thiols in flour resulting

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in a stronger dough. Because this hypothesis has not been tested directly until now, the quantitation of total Cys and GSH in flours from wheat supplied with different amounts of S fertilizer and the determination of correlations of their concentrations to technological flour properties were the aims of the present study.

Several methods have been reported for the quantitation of GSH and Cys in wheat flour and dough (*17–21*). But, until today, no consensus emerged about the exact levels of these thiols. The values documented in literature vary by orders of magnitude. This could be due to varietal differences, milling extraction grade, or storage time of wheat flours but could be due to the analytical methods used for the quantitation. During the past 15 years, different HPLC methods after the derivatization of thiol groups have been developed for the quantitation of GSH and Cys. The first developed method involved the reaction of GSH with iodoacetic acid (IAA) and fluorodinitrobenzene (*18*). More recently, Niskijima et al. (*22*) developed a method to simultaneously measure the amounts of GSH and Cys in biological samples. This involved the alkylation of GSH and Cys with IAA and the derivatization of free amino groups with 1-dimethylaminonaphathlene 5-sulfonyl chloride (dansyl chloride), with samples being measured using HPLC with fluorescence detection. Because of its higher specificity, LC-MS/MS was also applied for thiol determination. For example, Hammermeister et al. (*23*) reported LC-MS detection of dansylated thiols but did not quantify them. To compensate for matrix interferences, an assay based on radioactive labeled internal standards was devised by Sarwin et al. (*21*). However, the procedure was rather tedious as it required preparative HPLC with subsequent HPLC-radiodetection. A simpler isotope dilution assay (SIDA) for GSH quantitation based on stable isotope-labeled internal standards with LC-MS/MS detection was introduced by Rellan-Alvarez et al. (*24*). However, the latter assay was not able to determine Cys simultaneously with GSH. Therefore, a stable isotope dilution assay was developed for the rapid and simultaneous determination of GSH and Cys levels in flour to examine the effect of S fertilization on the amount of total GSH and Cys.

MATERIALS AND METHODS

Reagents. Acetonitrile Lichrosolv, formic acid (purity of 98−100%), methanol Lichrosolv, dichlormethane (distilled; DCM), dimethyl formamide, diisopropylamine, Fmoc-Glu-*tert*-butyl ester, Fmoc-glycine, trityl-resin, sodium phosphate, trisodium citrate, hydrochloric acid (25%, w/w; HCl), potassium hydroxide (KOH), and lithium hydroxide were obtained from Merck, Darmstadt, Germany. Chloroform stabilized with ethanol was obtained from Biosolve, Valkenswaard, Netherlands. [$^{13}C_3$, ^{15}N,]cysteine and *N*-Fmoc, *S*-trityl-L-[$^{13}C_3$, ^{15}N]cysteine were purchased from Cambridge Isotope Laboratories Inc., Andover, Massachusetts. Boric acid was purchased from Serva, Heidelberg, Germany. L-Cysteine, tris(2-carboxyethyl) phosphine (TCEP) hydrochloride, *N*-ethylmaleimide (NEMI), iodoacetic acid (IAA), and trifluoroacetic acid (TFA) were obtained from Fluka, Steinheim, Germany, and L-glutathione, 4-vinylpyridine (distilled; 4-VP), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Ellman′s reagent), and 1-naphthalenesulfonyl chloride (dansyl chloride) were purchased from Sigma Aldrich, Steinheim, Germany. All reagents were of p.a. or higher grade. All standard solutions and aqueous solvents were prepared with water purified by a Milli-Q system (Millipore GmbH, Schwalbach, Germany).

S-Fertilization of Wheat and Analytical Characterization of Flour. The German spring wheat cultivar 'Star' was grown in six pots (5 L, each) supplied with either 0 mg (flour no. 0), 30 mg (no. 1), 60 mg (no. 2), or 90 mg (no. 3) of S (applied as potassium sulfate) as well as with a combination of 60 mg (no. 4) or 90 mg (no. 5) of S before sowings and an additional 60 mg during growth, as described previously (*7, 13*). The supply of nitrogen, phosphorus, potassium, and magnesium was optimal for normal development. The mature grains were milled by means of a Quadrumat Junior mill (Brabender, Duisburg, Germany) into white flours, sieved (0.2 mm), and stored at room temperature prior to analysis. After microwave digestion, the S content of flours was determined by inductively coupled plasma atomic emission spectrometry (ICP-AES). The water content was determined gravimetrically according to ICC method 110/1 (*25*). The ash content was determined according to ICC method 104/1 (*25*). The nitrogen content of flours was determined according to Dumas (ICC method 167 (*25*)) using a FP-328 nitrogen analyzer (Leco, Kirchheim, Germany). For the quantitation of flour protein fractions and gluten protein types, a previously developed (*26*) combined extraction/HPLC procedure was used. At least two determinations were performed for each analytical assay.

Rheological Methods and Baking Tests. Dough preparation and extension tests on doughs were performed on a microscale as described previously (*27*) (3−7 strands per experiment). Creep-recovery tests on doughs were made on a dynamic stress rheometer (SR500, TA Instruments, Rheometric Scientific, New Castle, DE) using the conditions described previously (*28*), and baking tests were also performed on a microscale (*27*) using 10 g of flour (single determinations due to limited amounts of flours).

Synthesis of L-γ-Glutamyl-L-[$^{13}C_3$, ^{15}N]cysteinyl-glycine. Labeled GSH was synthesized by Fmoc-chemistry by means of an ABI 433A peptide synthesizer (Applied Biosystems, Darmstadt, Germany) using trityl-resin (96.4 mg), Fmoc-glycine (36.25 mg; 0.12 mmol), *S*-trityl-, Fmoc-L-[$^{13}C_3$, ^{15}N]cysteine (91.9 mg; 0.16 mmol), and Fmoc-Glu-*tert*-butyl ester (426.2 mg;1 mmol). The resulting peptide−resin adduct was dried over KOH in a vacuum desiccator, and the peptide was liberated from the resin and protecting groups by addition of TFA (10 mL; 95%) and 1,2-ethandithiol (0.25 mL) according to the manufacturer's recommendation. The resulting crude solution of the tripeptide in 20% methanol was purified by HPLC with UV detection (HPLC-UV) using system 1 and concentrated in a stream of nitrogen. The reaction product was checked by direct infusion into the LC-MS system. LC-MS data (ESI^+): *m/z* (%), 312 (100), 623 (15).

To prepare a standard solution, the synthetic L-γ-glutamyl-L-[$^{13}C_3$, ^{15}N]cysteinyl-glycine was concentrated under a stream of nitrogen. The solution had a pH of 2, which minimized any potential oxidation. The concentration of this solution was determined by HPLC-UV (system 1) after recording a linear calibration curve with different dilutions of the unlabeled reference compound in 0.1% aqueous formic acid. A standard solution of the commercially available L-[$^{13}C_2$,^{15}N]cysteine was prepared by dissolving the amino acid in 0.1% aqueous formic acid. Both standard solutions were stored at −20 °C and diluted prior to analysis.

Derivatization with Different Reagents. Thiol derivatization with different reagents such as IAA, NEMI, and 4-VP was performed to elucidate the best method for final LC-MS/MS detection.

Reaction of GSH with NEMI was carried out as described by Walther and Grosch (*29*) with slight modifications. In brief, 0.5 mL of a GSH solution (6 mmol/L in 0.02 mol/L sodium citrate buffer, pH 4.5) was mixed with NEMI solution (0.5 mL; 7 mmol/L in ethanol/water, 3/2, v/v) and stirred for 30 min at room temperature. The reaction was checked by using HPLC-UV system 2. For derivatization with IAA, a solution of GSH (200 μL; 3.3 mmol/L) was stirred for 30 min with a solution of IAA (200 μL; 0.02 mol/L) in boric acid/lithium hydroxide buffer (0.5 mol/L, pH 8.5) according to Niskijima and Yoshida (*22*). Reaction of GSH with IAA was examined with HPLC-UV system 3. 4-VP derivatization was performed according to Schliekelmann (*30*) with slight modifications. A solution of GSH (200 μL; 3.3 mmol/L in phosphate/HCl buffer, 0.05 mol/L at pH 7.0) was stirred with a 4-VP solution in acetonitrile (100 μL; 28 mmol/L) for 2 h at room temperature. This reaction was monitored with HPLC-UV system 4. All products were pooled from several HPLC runs and checked by direct infusion into the LC-MS system.

HPLC-UV. To purify labeled GSH and to check the reaction of GSH with different thiol reagents, a HPLC-UV system (Jasco, Groβ-Umstadt, Germany) was used. This consisted of a pump PU-1580, a solvent mixer and degasser type 2080-04, and a UV detector UV-2075. Four different gradient systems were used for the purification of labeled GSH and to monitor the different derivatization reactions. The stationary

Table 1. Most Abundant Mass Transitions and Collision Energies for the SRM Detection of Dansylated Carboxymethyl-glutathione, Dansylated Carboxymethyl-cysteine, and Their Dansylated Isotopologues

dansylated carboxymethyl-thiol	ion transition	collision energy (V)
glutathione	*m/z* 599 → *m/z* 496	20
	m/z 599 → *m/z* 524	15
[$^{13}C_2$, ^{15}N]glutathione	*m/z* 603 → *m/z* 499	20
	m/z 603 → *m/z* 528	15
cysteine	*m/z* 413 → *m/z* 170	24
	m/z 413 → *m/z* 252	11
[$^{13}C_3$, ^{15}N]cysteine	*m/z* 417 → *m/z* 170	24
	m/z 417 → *m/z* 253	11

phase of system 1 (for purification of labeled GSH) was a Synergi Aqua-RP18 column (250 mm × 4.6 mm, 5 μm particle size, 12.5 nm pores, Phenomenex, Aschaffenburg, Germany) equipped with a C_{18} guard column (Phenomenex). The mobile phase consisted of 0.1% aqueous TFA (solvent A) and 0.1% TFA in acetonitrile (solvent B). Gradient 1 started with 0% B and increased within 10 min to 3.6% B. A washing phase of 5 min with 95% B followed before returning the mobile phase to initial conditions, which were held for 17 min before the next injection. All other gradient systems consisted of a Synergy Hydro-RP column (250 mm × 3.0 mm, 4 μm particle size, 8 nm pores, Phenomenex, Aschaffenburg, Germany) equipped with a C_{18} guard column (Phenomenex, Aschaffenburg, Germany) as stationary phase. Mobile phases consisted of variable mixtures of formic acid (0.1%, solvent A) and acetonitrile (solvent B).

System 2 (for monitoring reaction of GSH with NEMI) started with 0% B held for 2 min. Then, the content of B was increased to 60% within 11 min and maintained for 2 min before being raised to 90%.

System 3 (for pursuing the reaction of GSH with IAA) started with 0% B, which was held for 8 min. Then, the content of B was raised to 1% and held for further 12 min before being raised to 90%.

System 4 (for monitoring the reaction of GSH with 4-VP) began with 0% B, which was held for 2 min. Then, the percentage of B was increased to 40% in 10 min and held for 3 min before being brought to 90%. For all gradients, a 5 min washing phase at 90% B followed before returning to initial conditions held for 10 min before the next injection.

Sample Preparation. To flour (20 mg), a solution (20 μL) containing labeled GSH (c = 24.6 μg/mL) and labeled Cys (c = 15.5 μg/mL), as well as a solution containing tris (2-carboxyethyl) phosphine in 0.1% (v/v) formic acid (200 μL; c = 5 mg/mL) was added, with the mixture being stirred for 15 min at room temperature. Subsequently, IAA (600 μL; 0.02 mol/L) in boric acid/lithium hydroxide buffer (0.5 mol/L, pH 8.5) was added, and the resulting mixture was stirred for 30 min in the dark at room temperature. Thereafter, a solution of dansyl chloride (400 μL; 1 mg/mL in acetonitrile) was added and stirred for 1 h under the same conditions. To stop the reaction, the mixture was shaken with chloroform (400 μL) and centrifuged (16 000*g*, 10 min, 20 °C). The supernatant was filtered (0.45 μm, Schleicher & Schuell, Dassel, Germany) and analyzed by LC-MS/MS.

LC-MS. A LCQ Classic mass spectrometer (Thermo Finnigan, Dreieich, Germany) was used for analysis of isotopologic GSH and derivatization products by direct infusion. The ion source was operated in the positive electrospray ionization (ESI^+) mode. Spray voltage was set to 4.5 kV, and sheath gas pressure and auxiliary gas pressure were set to 60 and 5 arbitrary units, respectively. Capillary temperature was 150 °C, and capillary voltage was 3 V.

LC-MS/MS. A Finnigan TSQ Quantum Discovery quadrupole mass spectrometer coupled with a Finnigan Surveyor Plus HPLC system (Thermo Electron Corporation, Waltham, MA) was used for LC-MS/MS analysis. The stationary phase was a Synergi HydroRP C_{18} column (2.0 mm × 150 mm, 4 μm particle size, 8 nm pores; Phenomenex), which was equipped with a C_{18} guard column (Phenomenex). For separation of GSH and Cys derivatives, gradient elution (flow rate: 0.2 mL/min) was employed with aqueous 0.1% formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Initial conditions were 100% A and were raised to 100% B within 26 min. The gradient

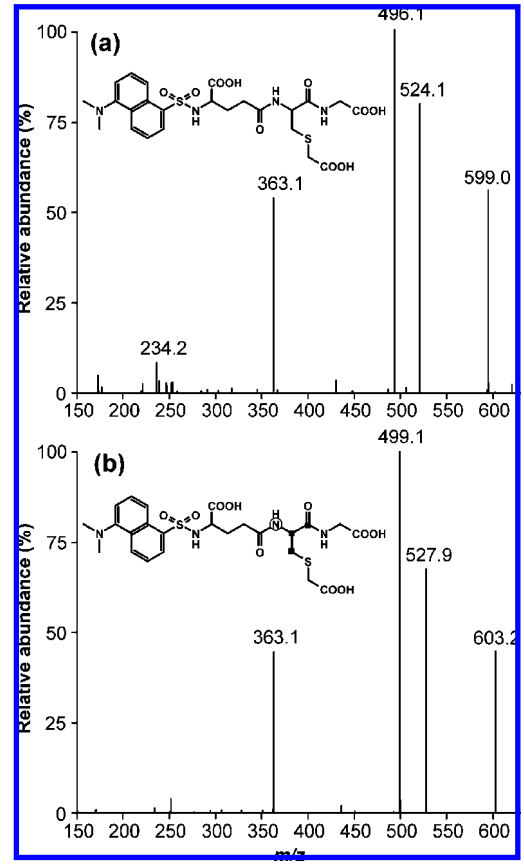

Figure 1. LC-ESI^+-MS/MS spectrum (collision energy: 20 V) of (**a**) dansylated carboxymethyl-glutathione and (**b**) dansylated carboxymethyl-[$^{13}C_3$, ^{15}N]glutathione. ■: [^{13}C]-label; ○: [^{15}N]-label.

was left at this composition for 2 min and then returned back to initial conditions within 1 min. Before each injection, the column was equilibrated for 15 min. Injection was carried out at full loop mode, and injection volume was 10 μL. The LC eluate from 0 to 14 min and from 23 min to the end of gradient was directed into waste.

The effluent between 14 and 23 min was introduced into the mass spectrometer, which was operated in the ESI^+ mode with a spray needle voltage of 3.7 kV. The temperature of the capillary was 300 °C, and the capillary offset was set to 35 V. The sheath and auxiliary gas were adjusted to 35 and 10 arbitrary units, respectively. The collision cell was operated at a collision gas (argon) pressure of 6.7×10^{-2} Pa, and source CID (collision-induced dissociation) was used with the collision energy set at 12 V. On both mass filter quadrupoles, the peak width was adjusted to 0.7 full width at half-maximum, the scan time for each transition was 0.2 s, and the scan width was 0.7 amu. During method development, the unlabeled derivatized compounds were subjected to MS/MS using a full scan in the product mode to find the two most intense and specific product ions for selected reaction monitoring (SRM). To optimize the intensity of these product ions, different collision energies in quadrupole 2 were tested using SRM mode. The results of the optimization are summarized in **Table 1**.

Calibration and Calculation. For determination of response factors, solutions of unlabeled and labeled Cys and GSH in 0.1% formic acid were mixed in seven mass ratios between 0.1 and 9. The derivatization procedure was performed as described above. After LC-MS/MS analysis, a calibration curve of area ratios in relation to mass ratios was obtained. From the mass ratios, the added amounts of labeled standards and the weighted samples, the mass, and molar concentrations of unlabeled thiols in the samples were calculated.

Further Validation. To check the precision, three flours with different Cys and GSH contents were analyzed in triplicate three times within 2 weeks by SIDA as previously described in this study. The recovery was determined by adding four different amounts of analytes to two different flour samples, which had different total thiol contents. To determine the detection and quantification limits, the method

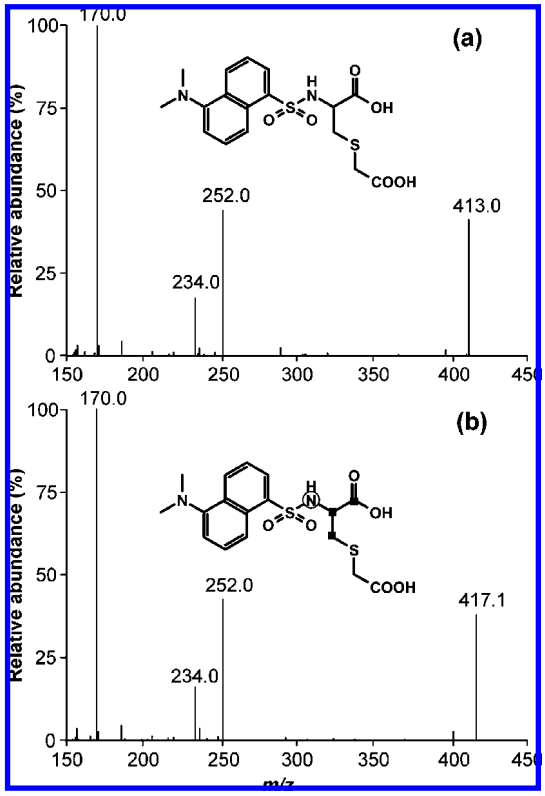

Figure 2. LC-ESI$^+$-MS/MS spectrum (collision energy: 24 V) of (**a**) dansylated carboxymethyl-cysteine and (**b**) dansylated carboxymethyl-[$^{13}C_3$, ^{15}N]cysteine. ■: [^{13}C]-label; ○: [^{15}N]-label.

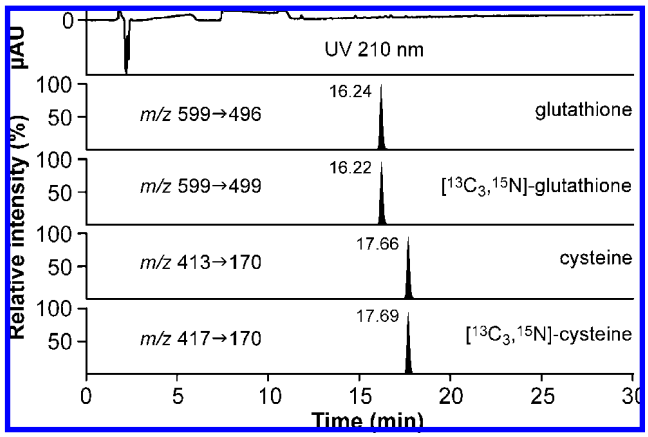

Figure 3. LC-MS/MS chromatogram of a derivatized flour extract showing the UV and mass traces of the derivatives of glutathione and cysteine along with their labeled internal standards.

suggested by Hädrich and Vogelgesang (*31*) was utilized. The analytes Cys and GSH were added in four different amounts to a surrogate flour matrix free of analyte consisting of 90% rice starch and 10% cellulose. As required by the method, each of the four addition assays was performed in triplicate and analyzed as described above.

Statistical Calculations. For the calculation of correlation coefficients (R) the computer program Slide Write Plus (Advanced Graphics Software, Inc., Carlsbad, CA) was used.

RESULTS AND DISCUSSION

Stable Isotope Dilution Assay for Glutathione and Cysteine.

Synthesis of Isotopologic Glutathione. In order to obtain labeled GSH, [$^{13}C_3$, ^{15}N]cysteine was chosen for introducing the labels into the tripeptide. In this way, L-γ-glutamyl-L-[$^{13}C_3$, ^{15}N]cysteinyl-glycine was synthesized by automated Merrifield synthesis. The MS spectra of purified standard solution clearly showed a base peak at $m/z = 312$ ($[M + H]^+$), which confirmed the introduction of the four labels in comparison with the base peak of unlabeled GSH at $m/z = 308$. Further signals in labeled and unlabeled GSH, respectively, can be explained by elimination of glutamic acid ($m/z = 183$ and 179) and by elimination of H_2O ($m/z = 294$ and 290).

Table 2. Calibration Functions Relating Mass Ratio of Labeled (IS) and Unlabeled (A) Compounds with Their Peak Area Ratio

substance	product ion[a]	equation[c]	(R^2)[b]
glutathione	496/499	$\left(\frac{A(\mathrm{IS})}{A(\mathrm{A})} = 1.00 \times \frac{m(\mathrm{IS})}{m(\mathrm{A})}\right)$	1.0000
cysteine	170/170	$\left(\frac{A(\mathrm{IS})}{A(\mathrm{A})} = 0.94 \times \frac{m(\mathrm{IS})}{m(\mathrm{A})}\right)$	0.9975

[a] Labeled/unlabeled compound. [b] Coefficient of correlation. [c] A(IS), area of internal standard peak; m(IS), mass of internals standard; A(A): area of analyte peak, m(A), mass of analyte.

Derivatization. In preliminary studies, 4-VP, NEMI, and IAA were used for derivatization of free thiol groups. 4-VP showed poor chromatographic properties with the resulting derivative not being separated from the reagent. The best reagents to protect the thiol groups from reoxidation were NEMI and IAA. However, Smyth (*32*) reported that NEMI is able to react with thiol groups as well as with amino groups. This can lead to duplicate derivatization of LMW thiols. Therefore, NEMI was deemed unsuitable as a protective agent for thiol groups against reoxidation, and IAA was selected for derivatization of the free thiol groups.

LC-MS/MS. Because of increased specificity of detection, MS/MS was applied for identification and quantitation of GSH and Cys derivatives. ESI$^+$ mode was used to produce protonated molecule ions $[M + H]^+$. Adduct ions such as $[M + Na]^+$ or $[M + K]^+$ were not observed. After fragmentation of the protonated molecule ions, MS/MS spectra of dansylated carboxymethyl-GSH (**Figure 1**) and dansylated carboxymethyl-Cys (**Figure 2**) were obtained. The most intense and characteristic ion transitions and collision energies for SRM detection of derivatized analytes and internal standards are summarized in **Table 1**. For dansylated carboxymethyl-GSH ($[M + H]^+$ at $m/z = 599$), ion transitions m/z 599 → m/z 496 and m/z 599 → m/z 524 resulting from the loss of formylglycine and glycine, respectively, were the most abundant and characteristic ones. For the internal standard (dansylated carboxymethyl-L-γ-glutamyl-L-cysteinyl-[$^{13}C_3$, ^{15}N]glycine), the analogous ion transitions m/z 603 → m/z 499 and m/z 603 → m/z 528 were recorded for quantification. For dansylated carboxymethyl-Cys ($[M + H]^+$ at $m/z = 413$) ion transitions m/z 413 → m/z 170 and m/z 413 → m/z 252 were obtained, which corresponded to the formation of the *N*,*N*-dimethylaminonapthylium ion and the dansyl ammonium adduct, respectively. The analogous fragments were recorded for dansylated carboxymethyl-[$^{13}C_3$, ^{15}N]Cys ($[M + H]^+$ at $m/z = 417$): m/z 417 → m/z 170 and m/z 417 → m/z 253. A chromatogram of a derivatized wheat flour extract with all mass transition traces is shown in **Figure 3**. For quantitation purposes, only the most abundant transitions (m/z 599 → m/z 496 and m/z 603 → m/z 499 for GSH quantitation and m/z 413 → m/z 170 and m/z 417 → m/z 170 for Cys quantitation) were used.

Calibration. Solutions of GSH and L-γ-glutamyl-L-cysteinyl-[$^{13}C_3$, ^{15}N]glycine as well as Cys and [$^{13}C_3$, ^{15}N]Cys were mixed

Table 3. Levels of Sulfur (S) Fertilization, Crop Yield, S Content, N Content, N/S Ratio, Ash Content, Concentration of Total Glutathione (GSH), Total Cysteine (Cys), and Sum of Total GSH and Total Cys in Six Wheat Flours from Plants Cultivated in Pots

flour no.	S/pot[a] (mg)	yield[a] (g/pot)	S[a] (% dm[c])	N[b] (% dm[c])	N/S[b]	ash[a] (% dm[c])	GSH[b] (nmol/g flour)	Cys[b] (nmol/g flour)	GSH + Cys[b] (nmol/g flour)
0	0	15.1	0.087	2.54	25	0.57	84 ± 4	82 ± 4	166 ± 5
1	30	30.3	0.066	2.09	32	0.40	24 ± 1	43 ± 2	66 ± 2
2	60	41.3	0.084	2.44	29	0.46	28 ± 2	60 ± 2	88 ± 2
3	90	42.0	0.128	2.75	21	0.43	67 ± 2	68 ± 4	135 ± 5
4	120	38.3	0.143	2.79	20	0.41	114 ± 5	108 ± 4	222 ± 4
5	150	44.3	0.158	2.74	17	0.43	135 ± 6	125 ± 3	261 ± 9

[a] Data from ref (*17*). [b] Mean values of at least triplicate determinations ± standard deviation. [c] Dry mass.

Table 4. Technological Properties of Flours[a]

flour no.	DDT[b] (min)	R_{max}[c] (mN)	ext[c] (mm)	EA[c] (N·mm)	η^*[b] (Pa·s)	γ (t)[b] (%)	V/E[b]	BV[b] (mL)
0	9.0	218	48	5.6	3261	67	0.93	30.7
1	2.0	138	29	1.5	4337	29	0.91	19.8
2	2.0	169	38	2.1	3585	32	0.83	24.8
3	16.5	331	61	11.3	2607	79	0.74	46.7
4	15.5	293	84	14.2	1950	138	1.06	47.5
5	14.5	316	92	17.1	1505	281	1.86	55.3

[a] DDT, dough development time; R_{max}, maximum resistance to extension; Ext, extensibility; EA, extension area; η^*, dynamic viscosity; γ (t), deformation; V/E, viscous/elastic ratio; BV, bread volume. [b] Single determination. [c] Mean value (measurement of 3−7 dough strands).

in seven mass ratios between 0.1 and 9 for GSH and between 0.1 and 8 for Cys. After derivatization, calibration functions were determined from the obtained data. The resulting curves showed good response linearity (**Table 2**). The difference of 4 amu between internal standards and analytes was high enough to avoid interfering signals caused by the naturally occurring isotopes in the analyte.

Further Validation. To calculate the limit of detection (LOD) and the limit of quantitation (LOQ), the method according to Hädrich and Vogelgesang (*31*) was utilized. This method considers losses during sample preparation, matrix effects, and variation in results at low concentration ranges. With the calibration curve obtained by analyzing the data of the performed addition experiments, a prediction was calculated. The LOD results from the intersection of upper limit of the one sided prediction with the ordinate and the following projection to the *x*-axis. After further statistical calculations, the LOQ was obtained. The LOD and LOQ were 0.75 and 2.23 nmol/g for GSH and 0.72 and 2.12 nmol/g for Cys, respectively. When regarding the LOQs and LODs obtained, the developed method appeared sensitive enough to quantify the expected amounts of total GSH and total Cys in wheat flour. Recovery was determined by adding different amounts of analyte to two flours with different thiol contents. Recovery levels were found to range between 84 and 105% for GSH and between 88 and 109% for Cys. Interassay precision was checked by analyzing four flours differing in the total thiol content (nos. 1−4) three times within two weeks as previously described. The relative standard deviation was below ± 7% for GSH and below ± 6% for Cys.

Total Glutathione and Cysteine in Wheat Flour as Affected by Sulfur Fertilization. The developed method was used to determine the concentration of total GSH and total Cys in six flours (nos. 0−5) from wheat grown in pots, with each having been fertilized differently with S (0−150 mg/pot). The results are summarized in **Table 3**. With the exception of flour no. 0, a clear correlation between sulfur fertilization, flour S content, and total thiol content was observed. The increase in total GSH and total Cys went in parallel to the increasing amount of S that had been supplied during growth. This correlation is an interesting extension of the findings of Granvogl et al. (*33*), which showed an inverse proportionality between the level of S fertilization and the concentration of free asparagine and some other non-S containing amino acids. The variation between flour no. 0 and the other samples was likely due to the significant lower kernel yield due to extreme S deficiency (see below).

Correlation of Glutathione and Cysteine Concentrations with Quality Parameters of Flours.

Chemical Analyses of Flours. As expected, the different levels of S fertilization (0−150 mg per pot) caused substantial differences in the S content (0.066−0.158%) of flours (**Table 3**). An exception with respect to the relation between S supply and S content was flour no. 0 (S supply = 0 mg); its grain yield (15.1 g/pot) was so low in comparison with the other samples (30.3−44.3 g) that the S content of the soil was obviously sufficient to produce flour with an S content of 0.087%. The N content of flours nos. 3−5 (2.74−2.79%) was similar and much higher than that of flours nos. 0−2 (2.09−2.54%). On the basis of an S content of <0.120%, sample nos. 0−2 were judged as S-deficient; on the basis of the ratio of N/S > 17, only sample no. 5 was sufficiently supplied with S (*9*). The quantitation of flour protein fractions and gluten protein types (data not shown) confirmed previous results (*7*): the proportions of S-poor proteins such as ω-gliadins were increased and those of S-rich proteins such as γ-gliadins and LMW subunits of glutenin were reduced by S deficiency.

Technological Properties of Flours. Flour and dough properties were characterized by means of a farinograph (water absorption, dough development time), extensograph (maximum resistance to extension, extensibility, extension area), and stress rheometer (viscosity, deformation, ratio of viscosity/elasticity). Flour samples nos. 0−2 showed generally poor properties such as short dough development time, low resistance, and low extensibility (**Table 4**). These doughs had a very high dynamic viscosity, and correspondingly, the viscous proportion was low. Flour nos. 3−5 had much better dough properties; nevertheless, differences were obvious in accordance with the S content though the N contents were almost identical. Dough from flour no. 5 showed the highest extensibility and extension area, the lowest dynamic viscosity, and the highest viscous proportion. In agreement with the literature (*5, 6, 8*), it can be concluded that S deficiency provokes tough, less extensible doughs, even if the protein content is similar. These different dough properties affected the bread volume drastically (**Table 4**). Sample no. 5

Table 5. Correlation Coefficients (R)[a] for the Relationship of Total Glutathione (GSH) and Total Cysteine (Cys) Concentrations and Quantities of Gluten Proteins with Dough Properties[b]

	DDT	R_{max}	ext	EA	η^*	γ (t)	V/E	BV
GSH	0.81	0.78	0.95	0.92	−0.93	0.89	0.74	0.88
Cys	0.72	0.72	0.95	0.89	−0.93	0.91	0.79	0.85
GSH + Cys	0.78	0.76	0.95	0.91	−0.94	0.91	0.77	0.87
CP	0.90	0.92	0.83	0.84	−0.89	0.60	0.32	0.88
gliadins	0.88	0.93	0.90	0.90	−0.95	0.70	0.43	0.94
glutenins	0.93	0.96	0.88	0.91	−0.93	0.66	0.37	0.95
GLI/GLU	−0.97	−0.96	−0.74	−0.82	0.78	−0.49	−0.16	−0.87
HMW/LMW	−0.78	−0.72	−0.86	−0.85	0.83	−0.82	−0.69	−0.80

[a] Level of significance: $R = 0.54-0.66$, $p = 0.05$; $R = 0.67-0.78$, $p = 0.01$; $R > 0.78$, $p = 0.001$. [b] CP, crude protein; GLI/GLU, ratio gliadins/glutenins; HMW/LMW, ratio high molecular weight/low molecular weight glutenin subunits; for other abbreviations see **Table 4**.

had by far the highest volume (55.3 mL) followed by no. 4 (47.5 mL) and no. 3 (46.7 mL). Bread volumes of sample nos. 0−2 were much lower (19.8−30.7 mL). Thus, flour S status strongly influenced breadmaking quality as already suggested by Zhao et al. (*11, 12*).

Correlations with Total Glutathione and Cysteine. The correlation coefficients (R) calculated for the relations between GSH and Cys concentrations and technological flour properties are given in **Table 5**. Coefficients for protein parameters important for flour properties such as crude protein, gliadin and glutenin contents, ratio of gliadins to glutenins, and HMW to LMW glutenin subunits are included for comparison. Significant differences between single GSH and Cys concentrations and the sum of both were not detected. Dough development time and maximum resistance to extension were correlated with GSH and Cys concentrations in a range of $R = 0.72-0.81$. This was somewhat lower than for crude protein, gliadin, and glutenin contents. Coefficients for the correlations of GSH and Cys concentrations to dough extensibility and extension area were extremely high ($R = 0.89-0.95$) and exceeded those of all other protein parameters showing that GSH and Cys directly affect the dough properties. Also dynamic viscosity (negatively) and deformation (positively) were closely related to GSH and Cys concentrations. In contrast, the correlations with viscous/elastic ratios were somewhat lower ($R = 0.74-0.79$) but still higher than those of the proteins. Remarkably, the frequently described positive effect of the gliadin/glutenin ratio on dough extensibility and viscous properties was not present in the case of S deficiency, and the ratio of HMW/LMW glutenin subunits proposed to be important for dough rheology (*14*) showed lower correlation coefficients than GSH and Cys (**Table 5**). In conclusion, the concentrations of GSH and Cys in S-deficient flours influenced the rheological dough properties at least as much as protein parameters. In accordance to dough properties, bread volume was also highly correlated to GSH and Cys concentrations ($R = 0.85-0.88$). This was within the same range as the crude protein content, which is an important factor for baking quality. In agreement with the hypothesis of Zhao et al. (*14*), the low concentrations of GSH and Cys in flour from S-deficient wheat had a similar effect on the technological properties as the altered composition of gluten proteins.

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