

DETERMINATION OF SULFHYDRYL AND DISULFIDE GROUPS IN FLOUR AND THEIR RELATION TO WHEAT QUALITY¹

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ABSTRACT

A study has been made on the conditions for determining sulfhydryl (-SH) and disulfide (S-S) groups in flour using a rapid-dropping mercury electrode as indicator electrode and ethyl mercuric chloride as titrant. A flour sample of 0.2 g., or less, is dispersed in 8M urea by means of continuous magnetic stirring in nitrogen. The sulfitolysis is carried out at 37°C. and pH 9.0 (NH₄Cl-NH₄OH) for 5 min. The amperometric titration is done at -0.75 v vs. Ag/AgCl reference electrode. The method gives accurate and reproducible results. For a sample of Thatcher flour, the mean value of S-S groups, based on nine determinations, is 16.12 ± 0.06 $\mu\text{eq./g.}$ -SH and S-S contents of representative varieties for three types of wheat flour have been determined by this method. The hard wheat flour contains the highest number of S-S bonds; the soft wheat flour, the least S-S bonds and -SH groups. On the protein basis, the soft wheat flour contains the highest and the hard wheat flour the lowest number of S-S bonds. The durum wheat flour, on the other hand, contains more -SH groups than the others. These findings provide an interesting clue to the "quality" differences among proteins in the three types of wheat flour.

The importance of sulfhydryl and disulfide groups in relation to the rheological properties of dough and the baking qualities of flour has been recognized by cereal chemists for a good many years. For the determination of sulfhydryl groups in flour, the amperometric, argentimetric titration of Benesch *et al.* (2) was successfully adopted and modified by Sokol, Mecham, and Pence (10), and by Matsumota and Hlynka (8), and so was the amperometric, mercurimetric method of Kolthoff *et al.* (6) by Sullivan, Dahle, and Larson (13). For the determination of disulfide groups in flour, Hird and Yates (5) have recently employed Leach's method (7) using methyl mercuric iodide as the titrating reagent, while Axford, Campbell, and Elton still used the argentimetric titration (1). Both groups appeared to have trouble in dispersing flour samples in the titrating medium for the determination of S-S bonds. Hird *et al.* did not detail their method; in one report they mentioned that before estimation of S-S bonds, the protein was dispersed in a homogenizer (5). Axford *et al.* first made a flour paste and then transferred the paste to a titration vessel (1). Following their

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procedures, we found it time-consuming to transfer completely the dispersed medium or paste to a titration vessel. It thus appeared that the problem of obtaining adequate dispersion of the flour for titration remained to be solved. Moreover, the amount of S-S bonds titratable depends on how complete the sulfitolysis reaction is. The reaction, in turn, is affected by reaction time, temperature, pH, titrating reagent, and protein sample. For flour samples, no information appeared to be available on the most suitable conditions for the disulfide determination. This study was therefore undertaken in an attempt to establish the optimal condition for the determination of disulfide groups in flour and then to measure sulfhydryl and disulfide contents of representative varieties of hard, soft, and durum wheat flours. It also appeared that some indication might be obtained of relations between the sulfhydryl and disulfide contents and wheat quality. The results of the study, together with discussion, are presented in this paper.

Materials and Apparatus

Flours. Winter (soft) wheat samples were kindly supplied by the Cereal Division, Central Experimental Farm, Ottawa, and durum wheat samples by the Canadian Agriculture Research Station, Winnipeg. The other wheat samples were from this Laboratory. The wheat samples were milled in this Laboratory with the same conditioning treatment and milling process. The extraction of the flours was 70 to 75%. Unless otherwise stated, the Thatcher flour was used for special experiments throughout this study.

Chemicals. All chemicals used in this study were reagent grade. Methyl mercuric iodide (MMI) was prepared and recrystallized according to Leach's procedure (7). Ethyl mercuric chloride (EMC, Technical grade) was purchased from the Aldrich Chemical Co., Milwaukee, Wisconsin, and purified by the method of Stricks and Chakravarti (11). Crystallized bovine albumin was purchased from Pentex, Inc., and recrystallized insulin from California Corporation for Biochemical Research. Distilled water was passed through a "Deeminizer" before use. Nitrogen used in this study was of commercial grade (at least 99.7% nitrogen) and was passed through two gas-washing bottles containing vanadous sulfate solution to remove traces of oxygen from nitrogen (9).

Reagents. Monofunctional organic mercurials, including MMI (7), EMC (11), and phenylmercuric hydroxide (4), have been recommended as the titrating reagents very recently. Of the three, MMI is the most reactive, but is poisonous (7). Some persons including one of us (C.C.T.),

are quite sensitive to this compound, which causes them to flush and to feel dizzy even when the operations are conducted under a fume hood. EMC is less poisonous and more reactive than phenylmercuric hydroxide (11). Accordingly, EMC was chosen as the titrating reagent. $2 \times 10^{-3}M$ EMC was prepared by dissolving 0.1326 g. of EMC in 25 ml. of dimethyl formamide and diluting with water to 250 ml. The solution was stored in a refrigerator.

Supporting Urea Solution. The solution consists of 0.2M in respect to potassium chloride, 0.1M to ammonium hydroxide and to ammonium chloride, 0.001M to EDTA (disodium salt of ethylenediamine-tetra-acetic acid), and 8M to urea. The pH of the supporting urea solution is 9.2.

Reducing Reagent. Sodium sulfite solution is unstable. It was prepared by dissolving 3.1513 g. of sodium sulfite (anhydrous) in 0.01M EDTA and diluting to 25 ml. The solution was prepared fresh daily and kept in an ice-water bath to minimize oxidation.

Since 1 ml. of the sulfite solution was added to 10 ml. of the supporting urea solution for titration, the final concentration of sulfite in the titration mixture was 0.091M. The concentration was selected in view of the following evidence: Stricks and Kolthoff have found from their kinetic studies on the sulfitolysis that the sulfite concentration should not be lower than 0.05M; otherwise the reaction is slow (12). Very recently, in a procedure recommended by Cecil and Wake to determine S-S bonds in proteins, the sulfite concentration was arranged to give an excess of 200-300 moles per S-S bond (4).

Apparatus. Metrohm Polarecord E 261 and polarographic stand E 354 were used. The polarographic stand is specially designed for rapid polarography. By means of a pulse generator, the capillary tube receives brief and vigorous knocks via a relay to increase the dropping rate of mercury. The chief advantage of rapid polarography is the substantial time reduction. Because of the rapid tap-off operation, the dropping mercury electrode almost appears to be a stationary electrode; the recorded current variations are so small that in most cases there is no need for any additional damping. Ease of evaluation and increased accuracy are thus obtained. For the present work, the normal drop time was 3.5 sec. and the rapid drop time 0.2 sec. The normal rate of flow was 1.85 mg. and the rapid rate of flow 1.77 mg. per sec. The polarograph vessel was equipped with a magnetic stirrer and water jacket in which water was circulated to maintain the solution in the vessel at 37.0°C. Silver-silver chloride electrode was used as reference electrode.

Methods

Separate methods were used for the determination of $-\text{SH}$ content, and of the combined $-\text{SH}$ and S-S contents in flour. The S-S content was determined by difference.

A. The $-\text{SH}$ content was determined according to the method of Sokol, Mecham, and Pence (10) as described previously (14).

B. The determination of the combined $-\text{SH}$ and S-S contents involved the reduction (sulfitolysis) of S-S bonds to $-\text{SH}$ groups by sodium sulfite and the amperometric titration of the total $-\text{SH}$ content. The procedure, recommended for the determination of the combined $-\text{SH}$ and S-S contents, is as follows:

The supporting urea solution (5 ml.) and one drop of *n*-octyl alcohol were pipetted into a polarograph vessel. About 200 mg. of flour sample were introduced into the vessel with continuous magnetic stirring to disperse the sample. Another 5 ml. of the supporting urea solution were used to rinse down any flour particles adhering to the vessel wall. Immediately after 1 ml. of sodium sulfite solution was delivered into the vessel under nitrogen, the vessel was fastened to its top part. The flour dispersion was bubbled with nitrogen to remove any residual oxygen and was constantly stirred by the magnetic stirrer for 5 min.

After 5 min. of bubbling and stirring, the flour sample was dissolved and dispersed. No flour particles or lumps could be observed; this is essential for obtaining accurate and reproducible results with this method. The current reading was stabilized and taken after the rapid tap-off switch was turned on to increase the dropping rate of mercury for 45 sec.; while, during that period, nitrogen was flushing into the vessel instead of the bubbling and the magnetic stirrer was turning off. Then, each time 0.1 ml. of EMC was added from a microburet, the solution was bubbled with nitrogen and stirred for 1 minute. The current reading was taken as described before.

The intercept of the excess EMC line with the base line (current in the absence of EMC) was determined graphically to measure the volume of EMC used.

For an accurate determination of total $-\text{SH}$ and S-S groups, in addition to performing the titration in nitrogen, an excess of titrating reagent should be presented before the titration in order to prevent oxidation of $-\text{SH}$ groups originally present or formed through the reduction. The titration, in the presence of excess titrating reagent, however, required the establishment of a base line which is obtained from a separated titration with no titrating reagent added before

sulfitolysis. When the titrations were done at the sensitivity of 5×10^{-10} amperes per mm. and at -0.75 volts vs. silver-silver chloride reference electrode with various flour samples, we found that the base lines fluctuated from one titration to another. Therefore, the procedure was modified as follows:

Three separate titrations were required for determining total -SH and S-S groups in one flour sample. The first one was done according to the titration procedure with no EMC added before sulfitolysis. The amount of EMC consumed could then be determined. For the next two titrations, 70-75% of the determined amount of EMC was added to the polarograph vessel containing the supporting urea solution and octyl alcohol before introduction of the sample. As the titration was carried on, there were enough current readings to establish a short base line before the current turned upward sharply to form the excess EMC line. With this procedure, the differences in titer, obtained from the titrations with EMC added before sulfitolysis for 16 different flour samples, varied from + 3.3 to + 10.8% with an average of + 6.0%, as compared to the titrations without EMC added before sulfitolysis. A

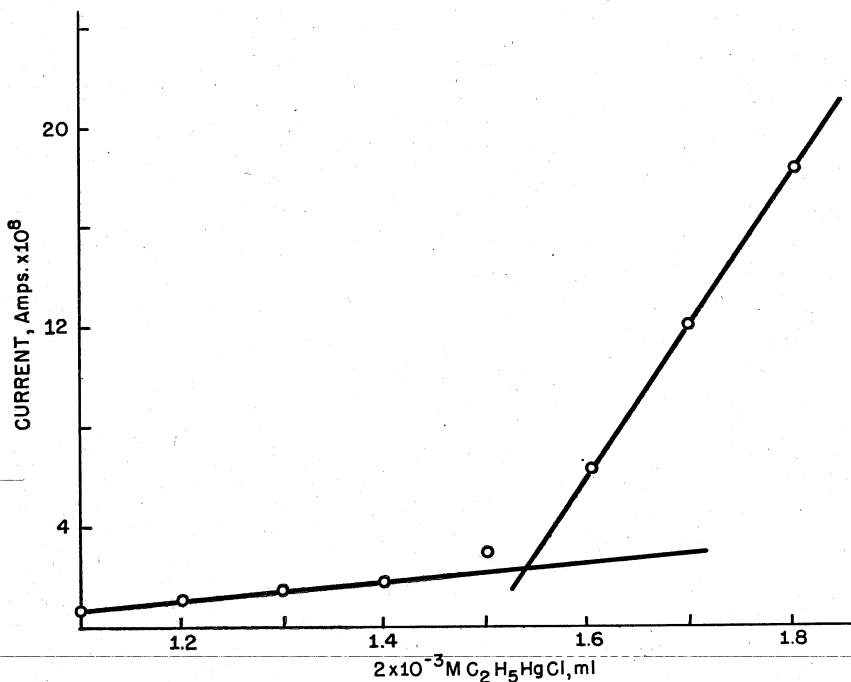


Fig. 1. A typical titration curve of flour (0.2006 g.) by ethyl mercuric chloride.

typical titration curve is illustrated in Fig. 1. All the total -SH and S-S contents in flour samples were determined according to this procedure, and the average of the last two of the three titrations was reported in this study.

Results and Discussion

Current-Voltage Curve. The current-voltage curve of EMC in the presence of cysteine has been reported by Stricks and Chakravarti with their rotated dropping mercuric electrode (11). Figure 2 presents the curves obtained in a titration of flour with EMC in the presence of urea. The polarograms were taken from -0.25 to -1.50 volts vs. Ag/AgCl reference electrode and after the addition of 2, 4, and 7 ml. of $2 \times 10^{-3}M$ EMC. As shown in the figure, the limiting diffusion current plateau covers from -0.65 to -1.05 volts; within this voltage range, any potential can be suitably used for titration.

Reaction Temperature and Time. The extent of reduction of disulfide bonds in proteins depends on the reaction temperature and time. Carter found that the sulfitolysis reaction went to completion almost immediately at $37^{\circ}C$. However, the complete reaction was not achieved at $27^{\circ}C$, with some proteins such as ribonuclease, even at optimal reaction time. He also reported that if the titrations were not carried out immediately, there was a rapid decay in silver-titratable groups for the proteins that he studied (3).

Figure 3A shows that reduced disulfide groups of flour titratable at 7° , 17° , 27° , and $37^{\circ}C$. increase with increasing temperatures, especially rapidly from 7° to $17^{\circ}C$. Figure 3B shows that EMC-titratable

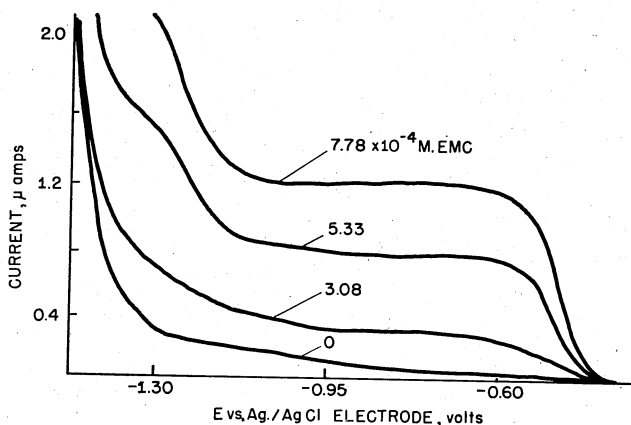


Fig. 2. Current voltage curves obtained in a titration of flour (0.2018 g.) with ethyl mercuric chloride in the presence of $8M$ urea.

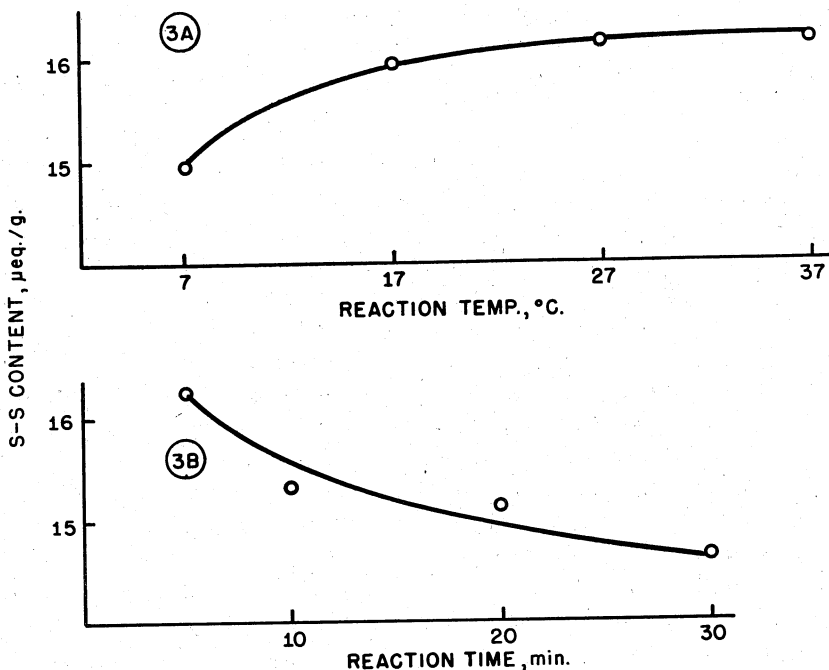


Fig. 3. The effect of reaction temperature (3A) and of reaction time (3B) on the sulfitolysis.

groups decrease with prolonged reaction time even in a nitrogen atmosphere at 37°C. At 27°C., an almost identical decrease in the titrable groups was observed. Since the sulfitolysis and the titration of the reduced disulfide bonds of flour were done in a nitrogen atmosphere, the reason for the decrease in titratable groups with prolonged reaction time still cannot be explained. In general our findings agree well with those of Carter (3). In the present study the sulfitolysis of disulfide groups of flour was therefore carried out at 37°C. for 5 min.; this is the minimum time required for removing any residual oxygen in the dispersion for the titration.

pH and Buffer. The effect of pH on the sulfitolysis reaction and the titration of S-S groups in flour are summarized in the table below.

<i>pH</i>	<i>Titratable S-S groups</i> µeq./g. flour
5.6	16.06
8.6	16.38
9.6	16.39
10.6	15.61

The data indicate that the titratable groups increase with increasing pH of ammonia-ammonium chloride buffer. The pH of the buffer was adjusted by adding hydrochloric acid or sodium hydroxide. However, at pH 10.60 the titratable value decreases.

Three buffer systems (all adjusted to pH 9.6), including tris (hydroxymethyl) aminomethane, borate, and ammonia-ammonium chloride, were used for the determination. Tris gave 15.79 μ eq. of total S-S groups per g., while borate and ammonia-ammonium chloride gave 16.29 to 16.59 μ eq. of S-S groups per g. of flour.

Precision of the Method. The specificity of EMC for the determination of sulfhydryl and disulfide groups was already reported by Stricks and Chakravarti (11). By this method, when bovine plasma albumin and insulin were used as the reference samples, we found the total S-S groups were 17.0 moles and 3.1 moles per mole of protein, respectively. For a sample of Thatcher flour, the mean value of S-S groups based on nine determinations, was 16.12 ± 0.06 μ eq. per g.; the standard error of a single determination was 0.19 μ eq. per g.

Sulfhydryl and Disulfide Content of Various Flours. Sulfhydryl and disulfide contents of representative varieties for three types of wheat flour were measured by the method and are presented in Table I. Results show that, on the weight basis, the hard wheat flour contains the highest number of S-S bonds; while the soft wheat flour contains

TABLE I
SULFHYDRYL AND DISULFIDE CONTENTS OF REPRESENTATIVE VARIETIES FOR
THREE TYPES OF WHEAT FLOUR

TYPE AND VARIETIES	PROTEIN	FLOUR		PROTEIN	
		-SH	S-S	-SH	S-S
	%	μ eq./g.	μ eq./g.	μ eq./g.	μ eq./g.
Hard					
2 Northern	13.63	1.25	16.17	7.92	102.7
Marquis	14.70	1.43	16.35	8.44	96.3
Selkirk	14.63	1.26	15.82	7.45	93.8
Thatcher	15.55	1.48	16.20	8.23	90.3
Pembina	14.57	1.22	16.11	7.28	96.1
Soft					
2 C.E. White Winter	7.51	0.78	10.79	8.92	123.6
U.S. Red Winter	8.06	0.70	11.60	7.44	124.2
Dawson's Golden					
Chaff	9.84	0.89	13.15	7.86	115.8
Genesee	8.54	1.00	11.17	8.89	114.5
Kent	9.32	0.88	12.53	8.49	117.1
Durum					
2 Av. Amber Durum	14.05	1.49	16.93	9.18	104.6
Mindum	11.79	1.27	13.61	9.50	102.2
Sentry	10.65	1.42	15.03	9.90	106.0
Langdon	12.00	1.27	14.17	9.42	104.7
Pelissier	12.62	1.32	15.29	9.27	107.4

the least S-S bonds and -SH groups. The number of S-S and -SH groups agrees well with the protein content and dough properties of the three types of wheat flour. On the protein basis ($N \times 5.7$), in general our results show that there is an inverse relation between the S-S value and the protein content for the three types of wheat flour, as found by Axford *et al.* (1) for their various flour samples. However, this inverse relation apparently does not hold for the varieties within each type of flour. This situation could be caused by a number of factors such as varietal difference and varied agronomic conditions. Nevertheless, our results clearly demonstrate that there is a distinctive difference in the S-S contents among the three types of wheat flour.

The soft wheat flour contains the highest and the hard wheat flour the lowest number of S-S bonds. The durum wheat flour, on the other hand, contains more -SH groups than the others. These findings provide an interesting clue as to the "quality" differences, among wheat proteins in the three types of wheat flour. It is known that the protein consists of one peptide, or more than one peptide, linked together with S-S or other bonds. The difference in the number of S-S bonds indicates that there are likely some variations in the structure of wheat proteins, leading to the qualitative difference in the hard, soft, and durum wheat flours. Further, the fraction or fractions of wheat proteins responsible for the difference in -SH and S-S groups and how the inter- and intra- S-S bonds distribute in these fractions, are problems that remain to be worked out. A study along these lines is presently in progress and will be reported later.

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