A METHOD FOR THE POLAROGRAFIC DETERMINATION OF CYSTINE AND CYSTEINE IN WHEAT AND FLOUR HYDROLYSATES

by

WERNER FERDINAND DAUM

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MASTER OF SCIENCE

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INTRODUCTION

Specific methods of analysis for cystine and cysteine involving both colorimetric and polarographic procedures have been the subject of numerous investigations. All of these methods require either laborious laboratory manipulations or give only approximations of the cystine concentrations. It would appear, therefore, that the development of a simplified procedure which would give accurate results for this compound would be highly desirable.

The most commonly employed colorimetric methods are those of Vassel (13) and Sullivan (12). In Vassel's method a blue color is developed by reaction of the reduction products with ferric iron and p-aminodimethylaniline. Vassel's method permits the selective determination of cystine and cysteine. Sullivan's method utilizes the formation of a "red color complex" with sodium $\beta$-naphthoquinone 4-sulfonate.

The polarographic method for the determination of cystine and cysteine was developed by Brdicka (2) in 1933. This method may lend itself to routine analysis because of its simplicity and sensitivity. The basis for the polarographic method of analysis of cystine and cysteine lies in the fact that in the presence of cobaltous ion, ammonia, and ammonium chloride, they exhibit a catalytic hydrogen wave which is proportional to the concentration of cysteine. Brdicka (3) showed that this catalytic wave was due to the mercapto group in the cysteine molecule and further postulated that cystine is reduced to cysteine before the
potential of the catalytic wave is reached.

\[ R-S-S-R + 2H^+ + 2e^- \rightarrow 2RSH \]

The cysteine reacts with cobaltous ion near the electrode forming an internal complex which greatly reduces the overvoltage of hydrogen on mercury. The mercapto group is adsorbed on the surface of the mercury and becomes a strong dipole, thereby enabling the hydrogen to be split off at a lower potential. The negative mercapto group then reacts with the ammonium ion to replace the liberated hydrogen according to the reaction,

\[ R-S^- + NH_4^+ \rightarrow R-SH + NH_3 \]

Brdicka found a linear relationship between wave height and concentrations of cystine between \(5 \times 10^{-6} M\) and \(10^{-4} M\). Kolthoff and Lingane (5) recommended \(10^{-5} M\) to \(2 \times 10^{-5} M\).

The catalytic wave is not specific for cystine and cysteine since the wave is given by other compounds containing any other structure which can be reduced to a mercapto group. Brdicka (3) found that cysteiny1-glycine and thioglycolic acid can produce a catalytic wave with divalent cobalt. Stern and Beach (10) found that homocystine and homocysteine give catalytic waves identical to those of cystine. Smith and Rodden (9) found that methionine, djenkolic acid, and benzylcysteine do not give catalytic waves and that these amino acids did not affect the cystine wave as long as their concentration was no more than twice the cystine concentration. Sládek and Lipschütz (8) found that tryptophane, histidine, arginine, \(\beta\)-phenyl \(\beta\)-alanine, and \(\beta\)-phenyl \(\alpha\)-alanine exert a suppressive effect on the waves. The quantities of cystine and cysteine also influence both the height of the cobalt
maximum and the shape of the catalytic wave minimum (4), however, these variations do not affect the measurement of the cystine concentration.

Brdicka (3) determined cystine in wool, hair, serum, brains, rye flour, and other proteins. Stern, Beach, and Macy (11) prepared a new type of calibration curve by adding small amounts of cystine to a hydrolysate and estimating the actual cystine concentration of the hydrolysate by comparing hydrolysate wave heights with known addition of pure cysteine. These workers established values for the cystine content of casein, edestin, beef globin, and sheep globin which compare favorably with values obtained by colorimetric methods.

It has become apparent that the cystine content of proteins may be an important factor in biological systems. Since the analysis of this amino acid is performed only with difficulty, it has been the object of this work to investigate the polarographic method of analysis with special attention devoted to the preparation of an accurate calibration curve.

EXPERIMENTAL

Materials

The polarographic analyses were performed using a Sargent-Heyrovsky Model XII Polarograph, with the galvanometer sensitivity of 0.0025 microamps/mm. A drop time of approximately 2.5 seconds was used.

All chemicals used in this investigation were of reagent grade. The sample of L-cystine used as a standard was 99+.
percent pure, and it was found to have a moisture content of 0.12 percent.

The wheat and flour samples were obtained from the Department of Milling Industry, Kansas State College, and represented different varieties of wheat grown in Kansas during 1949 and 1950.

**Polarographic Technique**

Wöstmann (14) based the measurement of the cystine wave upon the difference between the cobalt minimum and the minimum of the catalytic wave due to cystine. Below concentrations of $4 \times 10^{-5}$M cystine there was a linear relationship of wave height and concentration of cystine. This method of measurement was verified and used throughout this work.

The electrolytic medium containing $1.6 \times 10^{-3}$M cobaltous chloride, 0.105M ammonium chloride, and 0.105M ammonium hydroxide was prepared in 500 ml portions from stock solutions. One hundred milliliters of $8.0 \times 10^{-3}$M cobaltous chloride plus 50 ml of 1.05N ammonium hydroxide was then added and the solution diluted to 500 ml. In addition an equivalent amount of ammonia was added to neutralize the hydrochloric acid present in the unneutralized hydrolysate. Solutions suitable for assay were prepared by diluting 1.5 ml of protein hydrolysate to 50 ml with the electrolytic medium. The polarogram of this solution was recorded within five minutes after thorough mixing.

**Preparation of Hydrolysates**

There are many and varied conditions for the hydrolysis of proteins reported in the literature. Miller et al. (7) have
reported that optimum conditions for the microbiological assay of cystine were obtained by autoclaving a one gram flour sample with 25 ml of 4N hydrochloric acid for one hour at 15 pounds pressure. Employing the method of Miller, a series of hydrolysates, utilizing different acid concentrations and different heating times, was prepared. The hydrolysis time was recorded after 15 pounds pressure had been reached in a preheated autoclave and ended at the beginning of the "cracking" process. Fifteen minutes "cracking" time was used in all cases. The results are shown in Fig. 1. Optimum conditions included 4N hydrochloric acid and an autoclaving time of thirty minutes. Additional data for flour were obtained under these same conditions and the same optimum conditions established. No values were obtained using hydrolysis times of less than one hour with 2N and 3N hydrochloric acid. The polarograms of these hydrolysates did not exhibit the characteristic maxima and minima of the catalytic wave and could not be resolved.

All hydrolysates were prepared using the optimum conditions established. One gram of wheat or flour on a moisture free basis was hydrolyzed with 25 ml of 4N hydrochloric acid for 30 minutes at 15 pounds pressure in 125 ml Erlenmeyer flasks covered with inverted beakers. After cooling rapidly the hydrolysates were made up to 100 ml volumes and filtered. It was established that the acidity of the resulting hydrolysate did not influence the cystine wave.

Preparation of Standard Curves

Selective destruction of cystine and cysteine in a wheat or flour hydrolysate should allow the development of an absolute
Fig. 1. Effect of hydrolysis time and acid strength on the rate of cystine and cysteine liberation from wheat protein.
calibration curve with the other constituents of the protein exerting their effect as in a normal hydrolysate. The method utilized by Lyman et al. (6) for destruction of cystine, cysteine, tryosine, tryptophane, and methionine in protein hydrolysates for culture media was employed. A series of normal hydrolysates was treated with varying amounts of 1.0 percent hydrogen peroxide and allowed to stand at room temperature for 17 hours. Temperatures of standing ranging from 18° to 40° C. caused no significant variations in the results. The hydrolysates were then heated three hours on a steam bath to destroy the excess of hydrogen peroxide. This treatment resulted in the complete elimination of the catalytic hydrogen wave of cystine. Polarograms recorded for cystine added to the hydrolysate produced a normal cystine wave. The resulting wave heights versus the amount of hydrogen peroxide added, per 100 ml of hydrolysate are listed in Table 1.

Table 1. Effect of hydrogen peroxide concentration on wave height.

<table>
<thead>
<tr>
<th>1.0 percent hydrogen peroxide ml</th>
<th>Wave height of 10x10^{-6}M cystine mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>49.2</td>
</tr>
<tr>
<td>8</td>
<td>55.0</td>
</tr>
<tr>
<td>10</td>
<td>57.2</td>
</tr>
<tr>
<td>12</td>
<td>54.5</td>
</tr>
<tr>
<td>14</td>
<td>53.0</td>
</tr>
</tbody>
</table>

Two methods for the destruction of excess hydrogen peroxide were studied. The use of sodium sulfite gave conflicting results and was abandoned in favor of a heat treatment. The wave heights
corresponding to various times of heating on a steam bath are tabulated in Table 2. The maximum wave height was obtained when the hydrogen peroxide treated hydrolysate was heated for one half hour.

Table 2. The effect of heating on wave height.

<table>
<thead>
<tr>
<th>Heating time (hours)</th>
<th>Wave height 5x10^-6 M cystine (mm)</th>
<th>Wave height 10x10^-6 M cystine (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>33.0</td>
<td>51.6</td>
</tr>
<tr>
<td>0.50</td>
<td>34.3</td>
<td>56.0</td>
</tr>
<tr>
<td>0.75</td>
<td>33.8</td>
<td>55.1</td>
</tr>
<tr>
<td>1.00</td>
<td>32.5</td>
<td>53.5</td>
</tr>
</tbody>
</table>

Three wheat samples containing 10.5, 15.1, and 19.5 percent protein were hydrolyzed, filtered, and treated with 10 ml of hydrogen peroxide using the optimum conditions established. Polarograms were recorded for 1.5 ml of each protein hydrolysate with varying amounts of pure cystine solution. The calibration curve in Fig. 2 is a composite plot of the values obtained using all three hydrolysates and was established by the method of least squares. A correlation coefficient of 0.997 with 16 degrees of freedom was obtained for these data.

Factors Affecting Wave Height

Several components of biological material may influence the catalytic wave of cystine. Wöstmann (14) reported that starch caused considerable destruction of cystine during hydrolysis. In order to determine the loss under the hydrolysis conditions
Fig. 2. A calibration curve for the polarographic determination of cystine and cysteine in wheat and flour hydrolysates.

WAVE HEIGHT (mm)

<table>
<thead>
<tr>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$y = 4.65x + 9.77$

$r = 0.997$, 16 d.f.
established for this work, 8 mg samples of cystine were hydro-
lyzed with amounts of starch ranging from 0.0 to 1.0 gm. Since
the wave heights for all hydrolysates were identical, there would
appear to be no destruction of cystine during hydrolysis.

Accompanying amino acids may also complicate the analysis
of cystine. Since tyrosine, tryptophane, and methionine are
destroyed by treatment with hydrogen peroxide, the influence of
these amino acids on the cystine wave was studied. Methionine
had no effect and corroborated the work of Smith and Rodden (9)
and Sládek and Lipschütz (8). Tryptophane did not suppress the
wave in concentrations up to four times that present in wheat.
Tyrosine was found to suppress the catalytic wave only at three
times its normal concentration in wheat. Amino acids other than
those destroyed by hydrogen peroxide and which are normally present
in wheat were also found to have no significant effect on the
cystine wave. This was shown by the similarity of standard curves
prepared by using hydrogen peroxide treated hydrolysates repre-
senting protein levels ranging from 10 to 20 percent. It would
appear, therefore, that the presence of starch and the various
component amino acids of wheat need not be considered in the
analysis of cystine under the experimental conditions employed.

The Cystine Content of Hard Red Winter Wheat Flour

The cystine content of two sets of flour samples was deter-
mined utilizing the established methods of analysis. One set
consisted of 11 varieties composited by location and the other
set consisted of the same samples composited by variety. A
summary of the assay data is given in Table 3, and the analyses of variance are shown in Table 4.

Table 3. Summary of polarographic assay data of 22 flour samples composited by variety and location.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Protein 1: cystine 2</th>
<th>Location: Protein 1: cystine 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turkey</td>
<td>1.1</td>
<td>1.08</td>
</tr>
<tr>
<td>Pawnee</td>
<td>1.3</td>
<td>2.02</td>
</tr>
<tr>
<td>Comanche</td>
<td>1.9</td>
<td>1.96</td>
</tr>
<tr>
<td>Wichita</td>
<td>1.7</td>
<td>1.67</td>
</tr>
<tr>
<td>Red Chief</td>
<td>1.2</td>
<td>1.91</td>
</tr>
<tr>
<td>Wester</td>
<td>1.3</td>
<td>1.95</td>
</tr>
<tr>
<td>K. M. K. T.</td>
<td>1.7</td>
<td>1.81</td>
</tr>
<tr>
<td>Triumph</td>
<td>1.6</td>
<td>1.78</td>
</tr>
<tr>
<td>COT 12133</td>
<td>1.7</td>
<td>1.87</td>
</tr>
<tr>
<td>Blue Jacket</td>
<td>1.1</td>
<td>1.93</td>
</tr>
<tr>
<td>Blackhull Sel</td>
<td>1.0</td>
<td>1.76</td>
</tr>
</tbody>
</table>

1 Moisture free basis.
2 Mean for two separate analyses.
3 Areas in Kansas where wheat was grown.

Table 4. Analyses of variance of the percent cystine present in 22 flour samples computed by variety and location.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variety</td>
<td>10.0</td>
<td>0.00114**</td>
<td>Location</td>
<td>10.0</td>
<td>0.00286**</td>
</tr>
<tr>
<td>Error</td>
<td>11.0</td>
<td>0.0037</td>
<td>Error</td>
<td>11.0</td>
<td>0.0032</td>
</tr>
</tbody>
</table>

* Significance exceeds the 5 percent level.
** Significance exceeds the 1 percent level.

Differences in the cystine content in both variety and station composites were significant at the 1.0 and 5.0 percent levels.
respectively. Since the two series of composite samples contain the same flours combined in a different manner, the average value of 1.88 percent cystine obtained for each series lends additional confidence in the experimental technique.

**DISCUSSION**

A measurable curve is recorded only when the proper amounts of hydrolysate are employed. Hydrolysates containing little cystine do not exhibit a minimum when too much hydrolysate is used. This difficulty can be corrected either by decreasing the sensitivity of the galvanometer, thereby causing a reduction of the resulting wave height, or by decreasing the amount of hydrolysate. In this work 1.5 ml of hydrolysate was diluted to 50 ml and the polarogram recorded at 1:50 galvanometer sensitivity except when no wave minimum was obtained. On these samples the sensitivity was changed to 1:100.

Certain capillary effects were noted in this study which have not been mentioned by other workers. The maximum of the catalytic wave exhibited large irregular fluctuations which made certain polarograms difficult to measure. This was particularly true of those samples with high protein content. Periodical cleaning of the capillary with concentrated hydrochloric acid virtually eliminated this effect and indicated that such irregularities were caused by adherence of some components of the hydrolysate to the capillary.

The standard deviation for 10 replicate determinations of cystine performed in sequence using a freshly prepared hydrolysate
was calculated to be 1.0 mm difference in wave height. Transformed to percent cystine this value was equivalent to 6.1 percent cystine. Similar determinations made on the same hydrolysate on 10 successive days resulted in a standard deviation of 1.3 mm and was equivalent to 8.8 percent cystine.

Recovery experiments were performed by adding known increments of pure cystine to the flour samples before hydrolysis. Polarograms of the hydrolysates prepared with and without the addition of cystine were recorded and the amount of cystine present was calculated from the calibration curve. The average recovery of cystine was 115 percent for 9 determinations using the average of four individual polarograms for each determination.

Failure to find any inherent shortcomings in the method led to the following explanation for the high recovery based on the lack of further suppression of the cystine wave beyond a certain initial level. Suppression of the cystine wave heights is the result of the combined action of the various components in the hydrolysate. In recovery experiments the cystine content of the flour hydrolysate was determined from the calibration curve and recovery percentage was based upon the difference between this value and that obtained from the wave height of the hydrolysate with added cystine. The error involved in the calculation of recovery lies in the measurement of the cystine originally present in the flour in comparison with that added. Thus the value of cystine originally present in the flour has been measured while suppression due to other components has been exerted on the wave height. When additional cystine is added further suppression does
not appear to take place. An average recovery value of 99.1 percent was obtained when the concentration of cystine was obtained from a calibration curve of pure cystine.

The cystine content of flour obtained by chemical methods has been reported as 1.9 ± 0.1 percent (1). Employing polarographic techniques, Wöstemann (14) reported results giving an average of 3.8 percent cystine. Using the calibration curve prepared according to the procedure of Stern, Beach, and Macy (11) the average cystine content of the flour listed in Table 3 was 2.20 percent. Using the calibration curve developed in this study, the average cystine content of 1.88 percent was calculated. This latter value compares favorably with the values obtained by chemical methods.

Although the method described was applied only to the measurement of cystine in wheat and flour, it would appear reasonable to assume that it could be adapted to the measurement of cystine in other biological systems as well. The optimum conditions for hydrolysis and for preparing the standard curve using other systems may vary, however.

SUMMARY

1. Optimum conditions for hydrolysis of wheat and flour were established using 4N hydrochloric acid for one half hour at 15 pounds pressure.

2. A new type of calibration curve has been established based on the addition of increments of pure cystine to a hydrolysate in which the native cystine had been destroyed by treatment with hydrogen peroxide. Optimum conditions for proper hydrogen
peroxide concentration and for destruction of excess hydrogen peroxide were established.

3. Tyrosine, tryptophane, and methionine were found to have no effect on the catalytic wave height at normal concentrations.

4. Cystine content of numerous flour samples was determined. The average value obtained was compared to those reported in the literature and found to be in close agreement with those obtained by chemical methods.
ACKNOWLEDGMENT

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The author is also indebted to Dr. Byron S. Miller of the Department of Milling Industry for his suggestions and guidance throughout the course of this work. Appreciation is also expressed to all members of the Department of Chemistry and the Department of Milling Industry who aided in this investigation.

The l-cystine used in this investigation was furnished by Merck and Company.
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1951
The analysis of protein for cystine and cysteine is complicated by the complexity of the protein hydrolysate. The polarographic method based on the catalytic hydrogen wave of cystine and cysteine is sensitive and lends itself to routine analysis. The objective of this investigation was the establishment of a convenient analytical procedure with special attention given to the development of an accurate calibration curve.

The calibration curve was established by addition of pure cystine to flour hydrolysates in which the native cystine had been destroyed by treatment with hydrogen peroxide.

Preparatory to the establishment of the calibration curve, optimum conditions for hydrolysis, concentration of hydrogen peroxide for destruction of native cystine and cysteine, and proper heating time for the destruction of excess peroxide were established. Optimum conditions for hydrolysis of wheat and flour included heating with 4N hydrochloric acid for one half hour under 15 pounds pressure. Proper hydrogen peroxide concentration was found to be 10 ml of 1 percent hydrogen peroxide per 100 ml of hydrolysate. Heating time for the destruction of excess hydrogen peroxide was found to be one half hour.

Factors which might influence the cystine wave height were also investigated. Hydrolysis of starch did not cause destruction of cystine. Tyrosine, tryptophane, and methionine were found to have no effect on wave height at their normal concentration in wheat and flour.

Data for the plot of the calibration curve were obtained by recording polarograms of pure cystine added to 1.5 ml of hydrogen
peroxide treated hydrolysate. This plot gave a straight line between concentrations of $2 \times 10^{-6}$M and $10 \times 10^{-6}$M cystine. Wheat and flour of varying protein content (10 to 20 percent) were used in the preparation of the standard curve indicating that the amount of protein had no effect on the wave height beyond a certain initial amount. A correlation coefficient of 0.997 with 16 degrees of freedom was established for this curve.

Average percent cystine based on the total protein content of flour was 1.88 percent. This value compares favorably with the values of 1.9 ± 0.1 percent listed in the literature. An average recovery value of 99.1 percent was obtained.

Although the method described has been applied only to wheat and flour, it would appear reasonable to assume that it could be adapted to other biological systems.