CHAPTER - II

SPECTROPHOTOMETRIC DETERMINATION OF CYSTEINE AND CYSTINE WITH AMMONIUM MOLYBDATE IN THE PRESENCE OF PHOSPHORIC ACID
INTRODUCTION

A conventional reagent for the colorimetric estimation of cysteine is phospho-18-tungstic acid\(^1\) with in some cases cystine converted to a reduced form and determined at the same time\(^2\). A spectrophotometric method based on the oxidation of cysteine by nitrilotriacetogerrate(III) in the presence of 1,10-phenanthroline has been described by Bydalek and Padolski\(^3\). The other reagents used for its spectrophotometric determination are: selenium dioxide\(^4\), ninhydrin\(^5\), o-phthalaldehyde\(^6\), 1-methyl-4-nitro-5-chloroimidazole\(^7\), copper sulphate\(^8\), methylglyoxal\(^9\), dichlone\(^10\), ferrozine\(^11\) and 3-\(\text{N}_4\)-(4-sulphophenyl)\(\text{N}_2\)-pyridyl\(\text{N}_2\)-(4-sulphophenyl)\(\text{N}_4\)-1,2,4-triazine or PPTS\(^12\). In addition, the spectrophotometric methods for the determination of cysteine based on the reactions of sulphhydryl group have been reported using Ullman's reagent\(^13,14\), sodium nitroprusside\(^15,16\), 2-vinylquinoline\(^17\), 3,3'-dithio-bis-(6-nitrobenzoic acid)\(^18\), \(Z-\alpha,\beta\)-dinitrostilbene\(^19,20\) and Pd(II) containing reagent\(^21\). Some other methods for its spectrophotometric determination are also available in the literature\(^22-24\).

Cysteine is an aminoacid which has a sulphhydryl group. Due to the presence of this group cysteine has been differentiated from the other aminoacids and sometimes
determined specifically. As the reactions of organic compounds with the metals are considered to be the most sensitive, it was decided to study the colour reactions of cysteine with different metallic reagents. Ammonium molybdate has proved to be an important reagent in this regard. It gave a blue coloration with cysteine on heating in presence of phosphoric acid. The sensitivity of the reaction has been enhanced by setting the experimental conditions. The reduced form of cystine also gave the same blue colour.
EXPERIMENTAL

Apparatus

A Bausch & Lomb Spectronic 20 (USA) was used for the absorbance measurements.

Chemicals and reagents

All the reagents used were of analytical grade.

A 0.1% cysteine and cystine solutions were prepared in conductivity water adding 10 ml of 1.0M hydrochloric acid. These solutions were diluted according to the requirement.

A 0.1M ammonium molybdate solution was prepared by dissolving 12.36 g in 100 ml water by heating.

A 1.5M o-phosphoric acid, 1.0M and 0.1M hydrochloric acid solutions were also prepared.

Determination of Cysteine

Procedure

One millilitre of sample solution containing 10–200 μg cysteine was treated with 2.0 ml of 0.1M hydrochloric acid and 2.5 ml of 0.1M ammonium molybdate in a boiling tube. To the mixture, added 1.0 ml of 1.5M phosphoric acid and it was diluted
by approximately 8 ml conductivity water. The contents were heated on a boiling water bath for 30 minutes. After that it was cooled and transferred to a 10 ml standard flask followed by 1.0 ml of 15M phosphoric acid. The final volume was made up to the mark by adding conductivity water. Absorbance was measured at 780 nm against a blank.

**Determination of Cystine**

**Procedure**

One ml of sample solution containing 10–200 μg cystine was first reduced by heating with zinc fillings containing 2.0 ml of 0.1M hydrochloric acid. It was filtered, the filtrate which contained cysteine, now determined by the procedure mentioned above.
RESULTS

Absorbance of the coloured product obtained by the interaction of cysteine and ammonium molybdate was recorded at stepwise increasing wavelengths. The maximum absorbance was obtained in the wavelength range 750-800 nm (Fig 1). For the determination, 780 nm was selected as the most suitable wavelength. In order to set the conditions for the quantitative determination of cysteine and cystine, the effects of possible variables were studied. The results are summarized as follows.

Effect of reagent concentration

Different volumes of 0.1M ammonium molybdate solution were added to cysteine solution containing 0.2 mg followed by 2.0 ml of 0.1M hydrochloric acid, 1 ml of 1.5M phosphoric acid and approximately 8 ml of water. The contents were heated on a boiling water bath for 30 minutes. The mixture was cooled and transferred to a 10 ml standard flask followed by 1.0 ml of 15M phosphoric acid. It was made up to the mark adding conductivity water. Absorbance was made at 780 nm against a blank reagent. It was observed that the absorbance increased with the increasing volume of ammonium molybdate and reached to maximum at a volume of 1.5 ml (Fig 2). Using a high volume of reagent, it made no appreciable effect on
FIG. 1. ABSORPTION SPECTRUM OF THE PRODUCT OBTAINED WITH AMMONIUM MOLYBDATE AND CYSTEINE.
FIG. 2. EFFECT OF REAGENT CONCENTRATION
absorbance. However, the stability of the coloured complex increased very much and, therefore, a 2.5 ml of the reagent volume was selected for such studies.

**Effect of volume of phosphoric acid**

Different volumes of 1.5M phosphoric acid were added to the mixtures containing 0.2 mg of cysteine, 2 ml of 0.1M hydrochloric acid and 2.5 ml of 0.1M ammonium molybdate solution and the colour was developed using the requisite conditions and absorbance was recorded of each of the coloured complex. It was found that the range 0.8-1.0 ml of 1.5M phosphoric acid gave the optimum results (Fig 3).

**Confirmatory with Beer's law**

Beer's law holds good in the range of 10-200 µg of cysteine and cystine for monochromatic radiation. Experimental results showing a linear relation are presented in Fig 4.

**Study of precision**

The reproducibility of method was checked by ten replicate determinations of 50 µg of cysteine and the relative standard deviation was calculated. The results are given in Table I.
FIG. 3  EFFECT OF VOLUME OF PHOSPHORIC ACID
FIG. 4.  CALIBRATION CURVE OF CYSTEINE
Table I  Magnitude of distribution of random errors in the determination of cysteine

<table>
<thead>
<tr>
<th>Solution No.</th>
<th>Amount taken, µg</th>
<th>Amount found, µg</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
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<td>50</td>
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<td>3</td>
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<td>8</td>
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<td>9</td>
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<td>48</td>
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<tr>
<td>10</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

Standard deviation = 1.47 µg

Relative standard deviation = 2.94%
Study of interferences

A study of interference of some of amino acids was made in the determination of 50 μg of cysteine. It was found that the presence of glycine, lysin, leucin, dl-isoleucin, methionine, proline, aspartic acid, glutamic acid, threonine, dl-alanine, arginine, tryptophan, asperginine, dl-phenylalanine and serine could be tolerated with a maximum amount of 1.0 mg of each. Oxalic acid, tartaric acid and glucose did not interfere in its determination up to an amount of 0.5 mg. Hydrazine and ascorbic acid were found to interfere.
DISCUSSION

The results of this study reveal that this test can be successfully used for the determination of cysteine and cystine. The oxidation of cysteine with a few oxidizing agents has been reported resulting the formation of cysteic acid. On the basis of these studies, a tentative reaction mechanism between cysteine and ammonium heptamolybdate in the presence of phosphoric acid has been proposed. Ammonium heptamolybdate under the given conditions oxidises cysteine into cysteic acid and itself reduces to molybdenum blue.

\[
\text{(NH}_4\text{)}_6\text{Mo}_7\text{O}_{24}\cdot4\text{H}_2\text{O} = 7\text{MoO}_3 + 6\text{NH}_3 + 7\text{H}_2\text{O}
\]

\[
\begin{align*}
\text{SH} & \quad \text{NH}_2 & \quad \text{SO}_3\text{H} & \quad \text{NH}_2 \\
\text{CH}_2-\text{CH} & \quad \text{CO}_2\text{H} + 6\text{MoO}_3 & \quad 9\text{H}_2\text{O} & \quad \text{CH}_2-\text{CH} & \quad \text{CO}_2\text{H} + 6\text{MoO(OH)}_3 \\
\end{align*}
\]

Cysteine Cysteic acid

The formation of molybdenum blue has recently been used in the determination of ascorbic acid and some biologically important compounds. Molybdenum blues are mixed valence oxide-hydroxides containing Mo(V) and Mo(VI). Apparently a series of compounds exists with MoO(OH)_3 and MoO_3 as the limits, the blue compounds have composition between these limits.
The calibration curve for systine is prepared after its reduction with zinc fillings and hydrochloric acid. Cystine has two cysteine units joined together by disulphide bond. On reduction the disulphide bond is split and the free –SH groups are formed.

\[
\text{NH}_2 \quad \text{NH}_2 \\
\text{HCO}_2 - \text{CH}_2 - \text{CH}_2 - S - S - \text{CH}_2 - \text{CH} - \text{CO}_2\text{H} \quad \text{Zn fillings} \quad \text{HCl} \\
\text{Cystine} \\
\text{SH} \quad \text{NH}_2 \\
2 \quad \text{CH}_2 - \text{CH} - \text{CO}_2\text{H} \\
\text{Cysteine}
\]

The method is found to be reproducible with a relative standard deviation of 2.94%.
REFERENCES


27. Y. Sakagishi, Kensa To Giyutsu, 6(8), 609 (1978).