

## **The Origin of Sulphur in Wool.—III. An Improved Cuprous Mercaptide Method for the Determination of Cystine in Material low in Cystine.**

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In a previous paper (Rossouw and Wilken-Jorden, 1935), it was shown that cystine can be precipitated as the cuprous mercaptide, thus eliminating many substances which Rossouw and Wilken-Jorden (1934) found to interfere with the determination. In certain cases sulphuric acid instead of hydrochloric acid hydrolysates were used and excessive humin formation was eliminated, also little or no oxidation to cysteic acid took place as found by Lugg (1933). It can be safely said that with the present available methods it will be impossible to detect cystine in a hydrochloric acid hydrolysate of substances in the nature of grasses, which are low in cystine. Andrews (1933) found traces of copper to oxidise cystine in hydrochloric acid solutions but not in sulphuric acid.

In the present modification the amino acid is determined as cysteine, resulting in double sensitivity and also eliminating all interfering colouration in the same process. Where excessive colouring material is present as is the case with hydrolysates of some grasses and blood, this method simplifies the process and leaves a better margin of safety. Many of the interfering substances found by Andrews and Andrews (1936) are thus eliminated from these hydrolysates. In grasses of low cystine content it is, however, advisable to reprecipitate the mercaptide, but as will be shown, this is quick and safe with an excellent regain.

### METHOD.

The method described, applies to grasses which are low in cystine as found by Rossouw (1935).

To 9 grams of grass dried to constant weight in an oven at 105° C. is added 80 ml. of 10 N sulphuric acid. This is warmed on a medium heat hot plate until the material is well soaked. The flask with the material, whilst still hot is covered with a beaker and placed in a gas heated autoclave controlled at 130° C. It is kept at this temperature for 5 hours. The hydrolysate is filtered hot through a G3 or G2 Jena

fritted glass filter funnel, washed with hot water and made up to 200 ml. Depending on the cystine content, 50 to 100 ml. of this solution is measured into a 500 ml. flask and as a buffer 2 ml. glacial acetic acid is added. With 5 N. sodium hydroxide this solution is then brought to pH 4.6. A flocculent precipitate results which is filtered off through a G4 Jena filter and washed with dilute acetic-acid-sodium acetate buffer at pH 4.6. Medes and Padis (1936). To this filtrate is added, with gentle shaking, 0.5 to 1 ml. of freshly prepared solution of cuprous chloride in 25 per cent. potassium chloride. After one hour standing flocculation of the cystine cuprous mercaptide will be complete. For quickest and safest precipitation a temperature of 30° to 35° appears to be best.

The solution is then centrifuged simultaneously in two or more centrifuge tubes of the conical type. The solution containing the precipitate should be disturbed as little as possible, as this precipitate is apparently soluble in cupric chloride.

At this stage, depending on the amount of dark material present, two procedures are possible:—

(1) When excess of dark material is present: Here reprecipitation is resorted to. The precipitate in the tubes is dissolved in 4 ml. of 0.3 N. hydrochloric acid and washed with water into a 50 ml. centrifuge tube. To this solution is added about 200 mg. of pure zinc dust and the contents well stirred or gently shaken until foaming has subsided. This is then filtered and washed into another 50 ml. tube. The reduced solution is now much lighter. At the same time a standard solution containing 0.8 mg. of cystine in a decinormal solution of hydrochloric acid is pipetted into another centrifuge tube and treated similarly to the above. The volumes are made up to about 30 ml. each and the tubes placed in a water bath at about 30°. To the assay and the standard are each added 5 ml. of acetic acid-sodium acetate buffer and 10 per cent. potassium hydroxide dropwise to pH 4.6. To each is now added 0.25 ml. 25 per cent. potassium chloride followed by 6 drops (about 0.2 ml.) of freshly prepared cuprous chloride reagent. This is done dropwise with gentle shaking. Flocculation usually sets in after a few minutes. The tubes are left in the bath for about one hour and then centrifuged.

(2) When less dark material is present: When reprecipitation is not made use of the precipitates should be washed in a 4.6 pH buffer solution consisting of a mixture of acetic acid and sodium acetate. The washings are combined in one tube and centrifuged. A standard is prepared as already described in (1).

Whichever procedure has been followed, the precipitates of the assay and of the standard are separately dissolved in 4 ml. of a 0.3 N. solution of hydrochloric acid. To each solution is added 100 to 200 mg. of zinc dust and it is well stirred or gently shaken. During this process the temperature is kept below 15°. The reaction is allowed to continue for half an hour with frequent stirring or shaking. Each solution is then washed into a 20 ml. measuring cylinder and 2.5 ml. of a 2.5 per cent. acetic acid followed by 1.0 ml. of a 15 per cent. solution of potassium sulpho-cyanide is added.

The contents are mixed and 0.25 ml. pyridine is added dropwise. It is shaken and made up to 20 ml., keeping the temperature constant at 15°. It is then immediately filtered and 5 ml. aliquots used for the cystine determination. To aliquots of both standard and assay is now added 0.5 ml. of 10 per cent. potassium hydroxide solution and the temperature raised to 18° to 20°. As described by Rossouw and Wilken-Jordan (1934) 2 ml. 10 per cent. aqueous solution of sodium cyanide is then immediately added. After an interval of 10 minutes 1 ml. of a 0.5 per cent. aqueous solution, of 1,2-naphthoquinone-4-sodium sulphonate is added, the solution is gently shaken and 5 ml. of 10 per cent. anhydrous sodium sulphite in 0.5 N. sodium hydroxide added after 20 seconds. After mixing the solutions are allowed to stand for 40 to 60 minutes at 18° to 20° when 1 ml. of a 1 per cent. solution of sodium Hydrosulphite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) in 0.5 N. sodium hydroxide is added. The colours are immediately compared colorimetrically.

#### REAGENTS.

1. 10 N. sulphuric acid.
2. 10 Per cent. solution of potassium hydroxide.
3. Merck's pure cuprous chloride. If this is visibly oxidised to cupric chloride it is best to purify a small sample to last for about a month as follows: The oxidised product is dissolved in a minimum quantity of 3 N. hydrochloric acid and a large excess of water is then added which precipitates the pure white cuprous chloride. The solution is quickly filtered through a G 3 filter, the precipitate washed with ethanol and placed in a vacuum at about 40° until dry.  
0.5 Gm. of this powder is transferred to a stoppered cylinder, 13 ml. of 25 per cent. potassium chloride in decinormal hydrochloric acid added and the contents well shaken to dissolve. This solution is covered with a thin layer of petroleum ether and should last for a day.
4. 0.3 N Hydrochloric acid solution.
5. Merck's pure zinc dust.
6. 4.6 pH Buffer solution of Medes and Padis (1936) made up by using equal volumes of 2 M sodium acetate and 2 M acetic acid.
7. 2.5 per cent. solution of acetic acid.
8. 15 Per cent. solution of potassium sulphocyanide.
9. Pyridine, redistilled.
10. 10 Per cent. freshly prepared aqueous solution of sodium cyanide.
11. 1 Per cent. freshly prepared aqueous solution of 1,2-naphthoquinone-4-sodium sulphonate. This material should be tested for purity as described by Sullivan (1926).

12. 10 Per cent. freshly prepared solution of anhydrous sodium sulphite in 0.5 N sodium hydroxide.

13. 1 Per cent. *freshly prepared* solution of sodium hydro sulphite in 0.5 N sodium hydroxide.

## DISCUSSION.

Under the conditions of hydrolysis described above there is a cystine recovery of more than 90 per cent. when 2 mg. cystine is added to a sample of lucerne hay as indicated in the following table:—

Temperature of Autoclave.	Time of Hydrolysis in Hours.	Cystine Content in mg. per cent.	Percentage regain of added Cystine.
122°	7.0	101	95
125°	5.5	117	91
130°	6.0	106	93
136°	2.5	91	57

During hydrolysis the normality of the acid is reduced to about 8.5 N. When hydrolysed for too long a period or at too high a temperature the cystine figures will be too low due for instance to carbonisation, oxidation, etc. A temperature of 125° and hydrolysis for about 6 hours have been found satisfactory. The addition of zinc during hydrolysis is unnecessary in the case of sulphuric acid in contrast to hydrochloric acid but if resorted to the results are not effected.

The cystine cuprous mercaptide is soluble in cupric chloride. It was established that with vigorous shaking of the stoppered centrifuge tube containing the precipitating reagent all the mercaptide would dissolve in the cupric chloride formed. As a result of this oxidation it was decided to resort to centrifuging rather than risking filtration.

Probably due to traces of copper salt in solution the cysteine set free by the zinc dust reduction is apt to be oxidised to cystine but at 15° such cysteine solution will remain stable for 24 hours.

An additional cystine standard (direct standard) can be used as a check to that already described (indirect standard) by omitting the reduction of cystine to cysteine and remembering that in this case the colour intensity will be only half that of the indirect standard.

It was found necessary to control the temperature during the colour development carefully. At 18°-20° C. the colour development is much slower than at the usual 25° and it is for this reason that a time interval of 40-60 minutes should elapse before the hydrosulphite is added, when the colour development-time curve becomes asymptotic.

With the acetic and pyridine in the solution it is evident that their strongly buffering action will prevent the very high hydrogen ion concentration required in this reaction to be obtained by employing the customary concentrations of reagents. As a result alkali is added to the solution prior to the cyanide solution. A similar procedure with good results was followed by Sullivan and Hess (1936).

Very sharp and more reliable colorimetric readings were obtained by using a light filter in the order of 530.

#### SUMMARY.

Cystine is determined as cysteine in hydrolysates of plant or biological material of low cystine content. This method is an improvement and also more accurate than the previous one described.

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