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INVESTIGATION OF TWO METHODS FOR DETERMINATION OF SULPHYDRYL GROUPS AND THEIR APPLICATION TO FISH MEAL

by Dr G E Trout
15 Lower Hope Road, Rosebank, Cape Town, South Africa

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G E TROUT

EXECUTIVE SUMMARY

It has been established by Miller (IFOMA Research Report 1993-5) that a broadly linear relationship exists between the digestibility of fish meal by mink and the level of free sulphydryl (-SH) residue in the meal. Some exception to the general trend, however, has been observed together with limitations to the analytical method employed for the determination of sulphydryl (thiol) groups and these have prompted a survey into alternative methods of analysis. The method employed in the original study was based on the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to yield the yellow 2-nitro-5-mercaptobenzoate ion (at pH 8,2) in the presence of free thiols. This widely used method has the disadvantage that, at the alkaline pH at which the reaction is carried out, the yellow product of the reaction (2-nitro-5-thiobenzoate) is subject to re-oxidation by atmospheric oxygen and steps to prevent this are required. Furthermore, the DTNB is susceptible to hydrolysis of the disulphide bond in this region of the pH scale, thus increasing the risk of error.

Two alternative methods were therefore investigated to see if they would prove better for free sulphydryls groups analysis in fish meal. These were:

a) reaction with 2,2'-dithiopyridine or 4,4'-dithiopyridine
b) a method based on selective reaction of thiols with nitrous acid due to Saville (1958).

The findings of the study are presented in some detail in subsequent sections of the report. In summary, however, both methods were found to give excellent results when applied to reduced glutathione. This latter thiol is a tripeptide and was the only standard small thiol used in the study.

The first of these methods (Part I) has a mechanism analogous to that of DTNB but, at the lower pH of 7,2 at which it can be carried out, the problems of DTNB are overcome. As will be indicated later, the work in this study was limited predominantly to the 4,4'-isomer which proved very satisfactory with reduced glutathione. Its application to the estimation of accessible thiol residues in bovine albumen was likewise successful. In this protein an estimated 0,42 mole SH groups per mole of protein was low compared with 0,68 reported by Grassetti *et al* (loc. cit.) but was within the range found by others quoted in the above reference. By contrast application to fish meal was, at first, unsuccessful because of large blank readings from both the reagent and chromophores from the meal extracted into the buffer. This resulted in the absorbance readings being too high for Beer's law to be valid and potentially high errors arising from the required absorbance being the small difference of two large values. The difficulty was eventually overcome by methanol precipitation of much of the chromophoric material. However, methanol was shown to markedly diminish the absorbance of 4-mercaptopyridine liberated by free thiols. When this factor was taken into consideration the method appeared less sensitive than originally expected but still viable. In the present study the effect of methanol on the absorbance of the above reaction was determined indirectly. It is recommended that pure 4-mercaptopyridine be obtained and used for a direct determination of the molar absorptivity in buffer/methanol solution. It is further noted that the values for sulphydryl groups in fish meal determined by the 4,4'-dithiopyridine method and quoted elsewhere in this report have not been confirmed by an alternative procedure. Nevertheless, within these provisos the method appears satisfactory.

The second of the above methods (Part II) was quite different in principle and was carried out in strongly acid medium. Additionally, it was thought that the nitrous acid used in the reaction, being smaller than the complex dithiols of the first procedure, may have greater access and hence reactivity with SH groups of an insoluble, heterogeneous material such as fish meal. The Saville procedure resulted in a mauve colour proportional to the thiol. Both the sensitivity of the method and the fact that the absorption maximum was far from that of protein and other extraneous chromophores extracted from fish meal by buffer solutions were features which were desirable. Regrettably, when applied to albumin or fish meal, colour development was not reproducible and was subject to a prolonged drift which prevented a stable end point. Despite several attempts to overcome these factors the method, when applied to fish meal, remained unsatisfactory and cannot be recommended.

PART I

REVIEW OF FINDINGS ON THE APPLICATION OF THE 4,4'-DITHIOPYRIDINE METHOD FOR SULPHYDRYL RESIDUES IN FISH MEAL

INTRODUCTION

Two isomers of dithiopyridine have been proposed for the estimation of sulphydryl groups namely 2,2'-dithiopyridine and the 4,4'-dithiopyridine. Studies by Grassetti *et al.* (1967) indicated that the 4-isomer has a greater molar absorptivity than the 2-isomer and may be expected to be the more sensitive reagent. In addition, both compounds are only slightly soluble in water but the 4-isomer appears somewhat more soluble. Grassetti recommends the use of a 2 mM solution for each compound but considerable difficulty was experienced in preparing the 2,2'-dithiopyridine at this concentration. Other workers (Brocklehurst *et al.* 1973) reported a maximum solubility of the 2-isomer of 1,5 mM. In the initial experiments in this study the 4,4' -dithiopyridine was dissolved directly in the phosphate buffer with warming and prolonged stirring. Later, the solid was dissolved in 0,5 mℓ methanol in which it is readily soluble and sufficient buffer (0,1M phosphate pH 7,2) was added rapidly to produce an effective 0,2 mM aqueous solution. A clear solution with no sign of insoluble matter resulted.

1. Procedure for Glutathione (reduced) and similar thiols

1.1 Solutions

0,1M Phosphate buffer pH 7,2
250 μM glutathione (reduced) i.e. 250 nmoles/mℓ
4,4'-dithiopyridine (11,0 mg in 25 mℓ phosphate buffer).

1.2 Method

Into a series of small vials with sealed screw caps (a glass scintillation vial of 20mℓ volume was used in the present work) 1,5 mℓ of glutathione standard solutions were added (see protocol below) and each was treated with 1,5 mℓ of 4,4' -dithiopyridine solution (4,4' -DTP; 0,2 mM). Blank solution containing buffer but no thiol were included. After a few minutes the absorbance of each sample was measured at 324 nm in a Unicam spectrophotometer against buffer and each sample was corrected by the mean blank reading. A plot of absorbance against concentration gave a good straight line relationship (Fig. 1).

A typical example:

Samples were prepared according to the following protocol:

Vial No.	Blank	1	2	3	4	5
Buffer (mℓ)	1.5	1,4	1,3	1,2	1,1	1,0
Std Thiol (250 μM)	0	0,1	0,2	0,3	0,4	0,5
4,4' -DTP	1,5	1,5	1,5	1,5	1,5	1,5

Mix and read at 324 nm.

1.3 Results

The following results were obtained:

Sample No.	nmole Glutathione	A (324 nm)
1	25	0,159
2	50*	0,321
3	75	0,475
4	100	0,636
5	125	0,787

* small pipette error - approx 53 nmoles

1.4 Test of repeatability of assay on constant amounts of glutathione

Conditions pertaining to Vial 3 above (75 nmole thiol) were employed and 5 replicate analyses were performed. The results were as follows:

Vial	A
1	0,466
2	0,462
3	0,458
4	0,456
5	0,453
Mean	0,459

Note: This value is slightly lower (0,459 *vs* 0,475) than obtained for the standard graph.

2. Extension of method to proteins

2.1 Application to bovin serum albumen

A sample of crystalline bovine albumen (BDH) was used to test the method for application to protein thiol estimation.

Method: 52,5 mg albumen was dissolved in 10,0 mℓ 0,1M phosphate buffer pH 7,2 and the following protocol was followed:

Vial No.	Buffer	Albumen	4,4' -DTP	Mean A
1	1,5	1,5	-	0,056
2	1,5	1,5	-	
3	-	1,5	1,5	
4	-	1,5	1,5	0,678
5	-	1,5	1,5	
6	1,5	-	1,5	
7	1,5	-	1,5	0,256

Absorbance due to thiol residue = 0,678 - (0,256 + 0,056)
= 0,366

Using an millimolar absorptivity of 19,8 $mM^{-1}cm^{-1}$ quoted by Grassetti (loc.cit.) concentration of SH residues in BSA = 3,0/1,5 x 0,366/19,8 mM
= 2 x 18,485 μM
= 36,97 μM

Assuming mol. weight of BSA is 60000

52,5 mg/10 mℓ = 5250000/60000 μM
= 87,5 μM
hence 87,485 μM BSA = 36,97 μM in SH groups
1 M BSA = 36,97/87,485
= 4,22 M in SH residues

lit value = 0,67, 0,68, 0,60, 0,35 (quoted by Grassetti *et al.* 1967)

2.2 The application to fish meal

2.2.1 Introduction

Attempts to apply the above method as described for glutathione or albumen was essentially unsuccessful when applied to fish meal because of the high blank values arising from both non-specific chromophores in the meal leached into the buffer together with the relatively high reagent blank. In addition, the suspension needed to be filtered in order to remove fine sediment which conferred a turbidity on the supernatant often even after centrifugation.

After some trial it was observed that protein and much extraneous chromophoric material could be removed by precipitation with methanol as described in the currently employed DTNB method. Because of the heterogeneous nature of the reaction an attempt was made to continuously shake meal samples by means of a wrist-action shaker. It was eventually concluded that little advantage was gained by this compared to occasional shaking as described in the DTNB method. Attempts were made to compare the methanol precipitation method with the direct approach in the case of BSA. With BSA solutions the strength of methanol as employed for fish meal (70%) was insufficient to cause precipitation. At greater methanol concentration protein was precipitated but the degree of dilution was excessive and the procedure became impractical.

Nevertheless, these trials served an important purpose as they indicated that methanol severely reduced the absorption at 324 nm of the 4-mercaptopyridine end-product of the reaction so that the 19,8 millimolar absorptivity can no longer be applied and the method becomes 2,5-3 times less sensitive. As methanol treatment was essential for the method applied to fish meal, an accurate determination of the absorptivity of 4-mercaptopyridine (4-thiopyridone) in buffer/ methanol solution is needed. As this compound was not available to us at the time of this study the results quoted below depended on an indirect assessment of this value. It is recommended that this deficiency be remedied for future use of the assay.

2.2.2 Procedure

About 30 mg of fish meal was accurately weighed into a small screw-capped vial. Each analysis was conducted in triplicate and 3 vials were prepared for a blank determination. The three blank samples were treated with 3,0 mℓ 0,1 M phosphate buffer pH 7,2 while 3,0 mℓ of 4,4'-dithiopyridine in phosphate buffer (11 mg in 25 mℓ buffer) was added to each of the three test samples. A reagent blank (in duplicate) was also prepared in which 3,0 mℓ of 4,4' -dithiopyridine in buffer was allowed to stand for the same time as the test samples. The samples were shaken in a wrist-action shaker for 2 hours. Seven millilitres of methanol were then added to each vial to precipitate protein and after 5 minutes, the bulk of the supernatant was transferred to 15 mℓ tubes and centrifuged (10 minutes at 2500 rpm). The clear supernatant was decanted from the precipitate and read in a Unicam UV/visible spectrophotometer at 324 nm.

2.2.3 Calculation

The absorbance in the test sample was corrected for the absorbances of both the meal and the reagent blank. Because differing weights of meal were involved in each vial, readings were normalised to the reading of 100 mg meal. (Hence a reading of A due to W mg meal became (A x 100)/W). Using these derived values the sulphydryl content in mmoles/100 g meal was:

$$\frac{A - (\text{meal blank} + \text{reagent blank}) \times 1000}{19{,}8 \times 100}$$

where 19,8 is the mmolar absorptivity of 4-thiopyridone (lit)

$$= \frac{A \text{ corrected}}{1{,}98} \quad \text{mmole SH residues/100 g meal}$$

As will be shown below, later work demonstrated that in methanol/buffer solutions the above value required modification and a value of 6,17 was indirectly established. In the Table below, the SH residues estimated in the fish meals provided by IFOMA are given using both millimolar absorptivity (ϵ) values.

No. of Meal	SH groups in mmoles/100 g meal	
	ϵ = 19,8	ϵ = 6,17
A3481	0,42	1,35 (0,99)
3664	0,34	1,09 (0,91)
163/94	0,36	1,16 (0,76)
11858	0,90	2,90 (2,73)
1156	0,40	1,28 (1,06)

the values in ( ) were determined in a later series of analyses.

3. Influence of methanol on absorbance of 4-mercaptopyridine

Precipitation of fish meal soluble protein with methanol was essential for viable absorbance readings. It should be noted however, that attempts to analyse Bovine Serum Albumin by the same method failed because methanol, even when present in twice the above described amounts, did not effectively remove protein. In the case of the pure BSA assayed in the present study, it was not necessary to remove protein by methanol precipitation and the unmodified method could be used (see section 1.2, page 3). Subsequently, credence was given to the possibility that the absorbance of 4-mercaptopyridine, the reaction product of a thiol and 4,4' -dithiopyridine, might be affected by the presence of methanol. Such a possibility was therefore investigated.

The direct measurement of the absorbance of standard solutions of 4-mercaptopyridine in buffer/methanol mixtures was not possible because the substance was not available to us. A comparison was therefore made in which glutathione standards were prepared and treated with 4,4' -dithiopyridine reagent and the product diluted with either pH 7,2 phosphate buffer or methanol in the proportions described in the method for fish meal. The absorbance of each series of standards were then recorded and graphs prepared. As the data below showed, methanol markedly reduced the absorbance of the reaction product.

3.1 Solutions: 250 μM glutathione in buffer
0,1 M phosphate buffer pH 7,2
2 mM 4,4'-dithiopyridine prepared by dissolving 11 mg solid in 0,5 mℓ methanol and rapidly adding 24,5 mℓ buffer solution and mixing well.

3.2 Procedure

Two groups of vials were prepared according to the following protocol:

Protocol (mℓ)						
Buffer	1,5	1,25	1,0	0,75	0,5	0,25
Std Glutathione	-	0,25	0,5	0,75	1,0	1,25
4,4'-DTP solution	1,5	1,5	1,5	1,5	1,5	1,5

The two sets of solutions were allowed to stand for 5 minutes for reaction to be completed. The first set was then diluted with 7,0 mℓ buffer and the second set with 7,0 mℓ methanol. After mixing, each set was measured at 324 nm.

3.3 Results

The following results were obtained:

Absorbance at 324 nm		
nmoles GSH	in buffer	in methanol
0,0	0,000	0,000
62,5	0,132	-
125	0,270	0,083
187,5	0,408	0,126
250	0,528	0,165
375	-	0,230

A calibration plot of these values gave two straight lines as shown in the accompanying graph (Fig. 2). The slope of the calibration graph in methanol was only about ⅓ of that in buffer. Relating the gradient of each line to the molar absorptivity (ϵ) gave the values:

in buffer ϵ = 21,4 $mM^{-1}.\ell.cm^{-1}$ in methanol ϵ = 6,17 $mM^{-1}.\ell.cm^{-1}$

The lit value of ϵ in aqueous solution is 19,8 $mM^{-1}.\ell.cm^{-1}$ which indicates a possible 92,5% purity for our reduced glutathione. Using a value of ϵ = 6,17 and increased level of sulphydryl residues per 100 g meal were computed and values for the IFOMA meals listed in the table above.

3.4 Comment

In general these values are consistent with values quoted in a previous IFOMA research report but are not supported in an independent assay.

4. Recovery experiment on meal 1156

4.1 Introduction

An attempt to assess the recovery of a known amount of thiol (as glutathione) when added to a fish meal was undertaken using the 4,4'-dithiopyridine method. Because of time constraints, only one such experiment was completed.

4.2 Procedure

Because of complications arising in correcting samples for varying amounts of meal plus a fixed amount of glutathione in calculating the recovery, attempts were made to weigh the same amount of meal into each vial. 30 mg of meal was selected as a suitable amount of meal but in practice the amounts varied between 29,9 and 30,3 mg a variation of 0,4 mg in 30,3 mg or 1,3%. This was regarded as acceptable. The following vials were prepared in triplicate:

Code No.	Treatment
1.	Meal +3,0 mℓ phosphate buffer (pH 7,2).
2.	Meal +1,5 mℓ buffer + 1,5 mℓ reagent in buffer.
3.	Meal + 0,5 mℓ buffer + 1,0 mℓ 250 μM glutathione in buffer + 1,5 mℓ reagent.
4.	0,5 mℓ buffer + 1,0 mℓ 250 μM glutathione + 1,5 mℓ reagent.
5.	1,5 mℓ buffer + 1,5 mℓ reagent.

All samples were allowed to stand for 2 hours with occasional gentle swirling. After the 2 hours reaction time, 7,0 mℓ methanol was added to each vial, which was sealed, shaken well and allowed to stand for 10 minutes to allow precipitation to proceed. The contents of each vial was transferred to a centrifuge tube and centrifuged at 2500 rpm for 10 minutes. The clear supernatant was decanted and the absorbance read at 324 nm.

4.3 Results

The following values were obtained with meal 1156:

Sample No.	Treatment	Weight (mg)	A	A (mean)
1	1 *	30,0	0,211	
2		30,3	0,226	0,220
3		29,9	0,233	
4	2	30,3	0,450	
5		30,3	0,467	0,456
6		30,0	0,450	
7	3	29,9	0,680	
8		30,1	0,700	0,684
9		30,1	0,673	
10	4	-	0,288	
11		-	0,262	0,264
12		-	0,261	
13	5	-	0,077	
14		-	0,078	0,078
15		-	0,079	

* See above for code

Absorbance due to SH in meal = (2) - (1 + 5)

Absorbance due to SH in meal + glutathione = (3) - (1 + 5)

Therefore absorbance due to glutathione in mixture with meal = (3) - (2)

= 0,684 - 0,456

= 0,228

using ϵ of 8,1 (see Part III)

conc. of SH from glutathione = 0,228/8,1 mmolar

= 0,228 x 1000/8,1 μmolar

= 0,228 x 1000/8,1 x 100 μmoles/mℓ

in 10 mℓ solution = 0,228 x 10/8,1 μmoles

= 0,281 μmoles

as 0,250 μmoles added, recovery = 0,281 x 100/0,250%

= 112,6%

Calculating the glutathione from the vlaues for sample 4 and 5.

recovery of glutathione alone = $\frac{0,186 \times 10}{8,1*}$

= 0,2296 μmoles

i.e. = 0,2296 x 100/0,250

= 91,8% recovery

* See Part III

GENERAL CONCLUSION

Even though the methanol precipitation step markedly reduces the sensitivity of the reaction, the absorbance still remains relatively high. The method therefore appears to be useful. An accurate molar absorptivity of 4-mercaptopyridine under the buffer/methanol conditions should be determined for more reliable calculations. The results of assays should also be compared with those from an independent method.

PART II

REVIEW OF FINDINGS ON THE APPLICATION OF THE SAVILLE METHOD FOR SULPHYDRYLGROUP ESTIMATION IN FISH MEAL

INTRODUCTION

The method utilises the facile conversion of SH groups to S-nitrosothiols by treatment with nitrous acid which, after removal of excess nitrous acid, can be selectively decomposed in the presence of mercuric ions. The nitrous acid which results can be colorimetrically measured after conversion to an intense mauve azo dye.

1. Procedure for small soluble thiols such as glutathione (reduced)

1.1 Reagents

Solution A - Mix 1 volume of 0,01M aqueous solution of sodium nitrite with 9 volumes 0,2-1,0N sulphuric acid. Prepare as required.

Solution B - 0,5% solution of ammonium sulphamate in water.

Solution C - Mix 1 volume of a 1,0% solution of mercuric chloride with 4 volumes of a 3,4% solution of sulphanilamide in 0,4N HCl.

Solution D - 0,1% of N-1-naphthylethylenediamine dihydrochloride in 0,4N HCl. (1 mg/mℓ) (freshly prepared each day).

1.2 Method

To 5 mℓ solution A in a 25 mℓ stoppered measuring cylinder 1 mℓ of the thiol (20-500 μM) were added in water. Five minutes were allowed for reaction and then the excess nitrous acid was destroyed with 1 mℓ solution B (stopper and shake well).

After 1-2 minutes , 10 mℓ of solution C was added rapidly and the volume made up to 25 mℓ with solution D. After 10 minutes to allow complete colour development, the mauve colour was read at 540 nm in a Unicam spectrophotometer.

1.3 Results

1.3.1 Test on reproducibility of method

Reduced glutathione as standard (BDH). The stock solution (5 mM) was diluted to 500 μM with 0,001M Hcl.

Run replicate tests on 100 nmole standards:

Run	Sample No.	A
1	1	0,212
	2	0,208
	3	0,205
	Mean	0,210
2	1	0,210
	2	0,208
	3	0,200
	4	0,209
	Mean	0,207

1.3.2 Preparation of a calibration curve

Glutathione (reduced) was used as standard. Freshly prepare stock solution (5 mM) was diluted to a working solution 250 μM:

Solution	0	25	50	100	150	200	250
Sol. A (mℓ)	5	5	5	5	5	5	5
Water (mℓ)	1,0	0,9	0,8	0,6	0,4	0,2	-
Std (250 μM)	-	0,1	0,2	0,4	0,6	0,8	1,0
Stand 5 minutes after mixing							
Sol. C (mℓ)	10	10	10	10	10	10	10
Sol. D (mℓ)	In all cylinders to 25 mℓ. Mix and stand 10 minutes.						
Read at 540 nm against blank.							

Sample No.	nmoles glutathione	A	A
1	25	0,050	
2	25	0,048	0,049
3	50	0,100	
4	50	0,105	0,103
5	100	0,188	
6	100	0,192	0,190
7	150	0,294	
8	150	0,298	0,296
9	200	0,385	
10	200	0,384	0,385
11	250	0,485	
12	250	0,480	0,483

The above results show a good linear correlation (Fig. 3) between thiol concentration and absorbance.

1.4 Application to Bovine serum albumen (BSA)

BSA dissolved freely in 1N sulphuric acid with no sign of precipitation. Analysis by the above protocol but allowing longer time for reaction with nitrous acid was attempted. The absorbance of the mauve end colour showed a rapid increase over the first ten minutes of colour development but, unlike glutathione, continued to increase slowly over an indefinite period making it impossible to determine a satisfactory endpoint. As will be described later, the same phenomenon was observed with fish meal and satisfactory results were not obtained.

1.5 Application to fish meal

1.5.1 Introduction

The insoluble nature of fish meals presents potential difficulties when attempting to estimate SH residues within the proteins. The small nature of the nitrous acid molecule suggests it might pass readily into the protein matrix. In addition, the absorption maximum of the mauve colour developed in the final step of the assay is well removed from chromophores commonly present in fish meal which are particularly troublesome in the violet region of the spectrum. As will be shown in the case of the proteinaceous materials examined here, a serious drift and a lack of

consistency in the colour development was experienced. As a result, it is unlikely that this procedure is adaptable to fish meal despite its otherwise excellent features. The method, as it was used in the present study, is described in detail below.

1.5.2 Procedure

Approximately 30 mg dry finely ground fish meal was accurately weighed in a 25 mℓ stoppered measuring cylinder. This operation was facilitated by a long plastic funnel made from a 5 mℓ "Pipetman" tip from which the last 10-12 mm was cut off. 4,5 mℓ 1,0N sulphuric acid was added and after a short period to allow solution of any material, 0,5 mℓ of 0,01N sodium nitrite was added with shaking. This was equivalent to the addition of 5 mℓ solution A. Because of the proteinaceous environment of the SH residues considerable time (varying between 1 and 4 hours) was allowed for reaction to occur. Because of problems observed later, this time period was not optimised but various times were investigated. Solution B was then added to destroy excess nitrous acid but again an extended time period was used. As the method depends upon reactions of SH residues within the protein, time for ammonium sulphamate to percolate into the protein and react with excess nitrous acid entrained in the protein was possibly longer than for small thiols. Various times were explored in the present study but again, no optimum time was established.

Decomposition of S-nitrosothiol within the protein by mercuric ions, followed by colour development, was investigated under a variety of conditions but in all cases a stable final absorption was not established. The general procedure was to solutions C and D and allow time for the colour to develop. During this time the heavy particles of meal rapidly settled leaving a cloudy mauve supernatant. Filtration through paper invariably left a faintly cloudy solution and it was found best to filter directly through a 0,45 μ nylon membrane filter by means of a syringe. It was noticed that the absorbance of this filtered solution gradually increased as illustrated in the graph (Fig. 4). At the same time, unfiltered final mixture also darkened and when filtered and measured, higher absorbance values were determined compared with the initial sample. It was concluded that entrainment of reactive material within the protein was probably responsible. It could not be determined whether such variation was due to residual nitrous acid entrained in the meal or to slowly reacting S-nitroso groups within the protein. Whatever the cause, it was not possible to determine a definite absorbance and the "drift phase" appeared to be lengthy and significant. Furthermore, it may be noted that the two results in Figure 2, which show an approximately two-fold difference in intensity, are associated with only a small difference in the mass of sample analysed. From these and similar results it was apparent that the presence of protein presents additional difficulties in obtaining reproducibility.

1.6 Some studies on the "drift phase" and its elimination

The following experiments were carried out to investigate this problem:

i) to increase the concentration of mercuric ions (Hg^{++}) of solution C to 2% so as to accelerate decomposition of the S-nitroso group;
ii) to increase the time allowed for colour development;
iii) to heat the final mixture (50°C).

The first of these was without effect; neither did longer times at selected stages eliminate the final drift. Heating greatly increased the colour intensity but this did not appear to correlate with the amount of meal originally present in each sample. It was tentatively concluded that extraneous nitrous acid was still present entrained within the meal. It was at one stage considered that the initial phase of colour development may reflect rapidly reacting thiol residues whereas the slow phase reflects more inaccessible residues. If so, it might be possible to extrapolate the slow phase as shown in Figure 4 and use the intercept as a measure of rapidly reaction SH residues. This was not explored further because of the difficulty of obtaining consistent data.

A few experiments were briefly explored in which the two methods investigated in this study were combined. As treatment of the meal with 4,4'-dithiopyridine yielded 4-mercaptopyridine, a UV-detectable thiol which, in fish meal, was masked by interfering chromophores, consideration was given to estimating this species indirectly via Saville's method. This approach would appear to have the advantage of the favourable absorption maximum of the Saville method and avoid the blank problem of the dithiopyridine method. In a preliminary trial, development of the mauve colour was incomplete, possibly because the mercaptopyridine was predominantly in the pyridine form.

GENERAL CONCLUSION

The intense purple colour developed in the Saville procedure was analytically attractive, both for its high sensitivity and the fact that the wavelength of maximum absorption falls in the visible spectrum. Not only does this latter feature require simpler instrumentation, but it is far from that of a number of possible contaminants. The method proved excellent for compounds such as glutathion and other small thiols. Extension of the method to protein thiols such as in fish meal was not successful by virtue of incomplete colour development and poor reproducibility and cannot be recommended.

PART III

THE DETERMINATION OF THE ABSORPTIVITY (mM ABSORPTION COEFFICIENT) OF 4-MERCAPTOPYRIDINE IN AQUEOUS BUFFER PH 7,2 AND IN A 70% METHANOL/BUFFER MIXTURE

INTRODUCTION

As described in the report (Part I), the absorption at 324 nm resulting from the reaction between sulphydryl groups and 4,4'dithiodipyridine was markedly decreased when the mixture contained 70% methanol as compared with the value in 0.1M phosphate buffer pH 7,2 alone. It was therefore desirable to clarify the effect of methanol on the absorptivity of 4-mercaptopyridine (4-thiopyridone), the product of the reaction of sulphydryl groups and 4.4'dithiodipyridine, by direct estimation. Accordingly, 4-mercaptopyridine was obtained (Aldrich) as a yellow crystalline compound (95% pure) and used without further purification.

1. Method

1.1 Reagents

0,1M phosphate buffer pH 7,2 containing 0,1% EDTA

10mM stock 4-mercaptoyridine. Dissolve 11,12 mg in 10,0 mℓ of the above buffer solution and use within one day.

0,2mM 4-mercaptopyridine: prepared by diluting 100 $\mu\ell$ stock solution to 5,0 mℓ with buffer.

1.2 Determination of the millimolar absorption coefficient in buffer

1,0 mℓ of 0,2mM 4-mercaptopyridine was diluted with 6,0 mℓ buffer and the absorbance of the mixed solution determined at 324 nm against a buffer blank. The absorption reading was stable and a mean value of 21,5 $mM^{-1}.\ell.cm^{-1}$ (triplicate determinations) was obtained.

1.3 Absorption coefficient in buffer/methanol

The condition of 70% methanol/buffer was established by preparing the following mixture:

4,2 mℓ methanol AR
0,8 mℓ phosphate buffer pH 7,2
1,0 mℓ 0,2 mM 4-mercaptopyridine

The absorbance was determined in the spectrophotometer against a 70% methanol/buffer blank. Initial experiments showed that the absorption at 324 nm measured immediately after mixing was, by contrast to that in 100% buffer, subject to a considerable degree of drift. A typical determination showed the following change:

Time (min)	Absorbance
Immediately after mixing	0,228
+ 5	0,252
+ 10	0,258
+ 15	0,262
+ 20	0,268
+ 25	0,270

The latter reading was within the fluctuation of the spectrophotometer at this wavelength and was considered to be essentially stable. Triplicate analyses employing two independent stock solutions gave the following results:

No.	Absorptivity ($mM^{-1}.\ell.cm^{-1}$)	
	in 100% buffer	in 70% methanol/30% buffer
1	21,17	8,07
2	21,72	8,12
3	21,67	8,13
Mean	21,5(2)	8,1(1)

GENERAL CONCLUSION

Experiment showed that not only did 70% methanol in the reaction mixture reduce the absorptivity of 4-mercaptopyridine compared with that observed in buffer alone, but introduced a slow drift in absorbance not found under solely aqueous conditions. However, stable readings can be obtained by attention to the time factor. It is not clear why methanol has this effect, but it may be anticipated that concentrations of methanol other than 70% may modify both the rate at which stable absorption readings are established and the final absorptivity. In the experiments with fish meal, the drift described above was not observed. It appears probable that the time required to prepare and centrifuge the samples was of the same order as that for stable reading to be established. Furthermore, a drift in absorbance was not observed in the standard curve determination (p.8 and Fig. 2) with reduced glutathione in 70% methanol in which readings were taken shortly after mixing. Such a drift would increase the slope of the graph measured at a later time. Thus the millimolar absorptivity calculated indirectly would be higher in keeping with the value determined directly.

FINAL CONCLUSIONS

The two methods investigated give excellent results when applied to small thiols such as glutathione, but only the 4-4[1] dithiodipyridine procedure appears applicable to the protein thiols in fish meal. In the present trials the Saville nitrous acid procedure gave variable and incomplete colour development when applied to fish meal and cannot be recommended. Despite the investigation of a variety of possible factors, the failure to obtain constant results could not be explained. Thus the sensitivity of the method and its potential freedom from interference by impurities could not be realised.

In applying the 4-4[1] dithiodipyridine method to fish meal care must be taken to ensure that stable absorption readings are obtained, as it was subsequently shown that the chromophoric compound 4-mercaptopyridine when dissolved in 70% methanol/phosphate buffer showed a small but significant measure in absorption with time. Within this provision the method appears to be useful.

REFERENCES

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Grassetti, DR & Murray, Jn JF. 1967. *Arch. Biochem. Biophys.*, 119 : 41-49.

Saville, B. 1958. *Analyst,* 83 : 670-672.

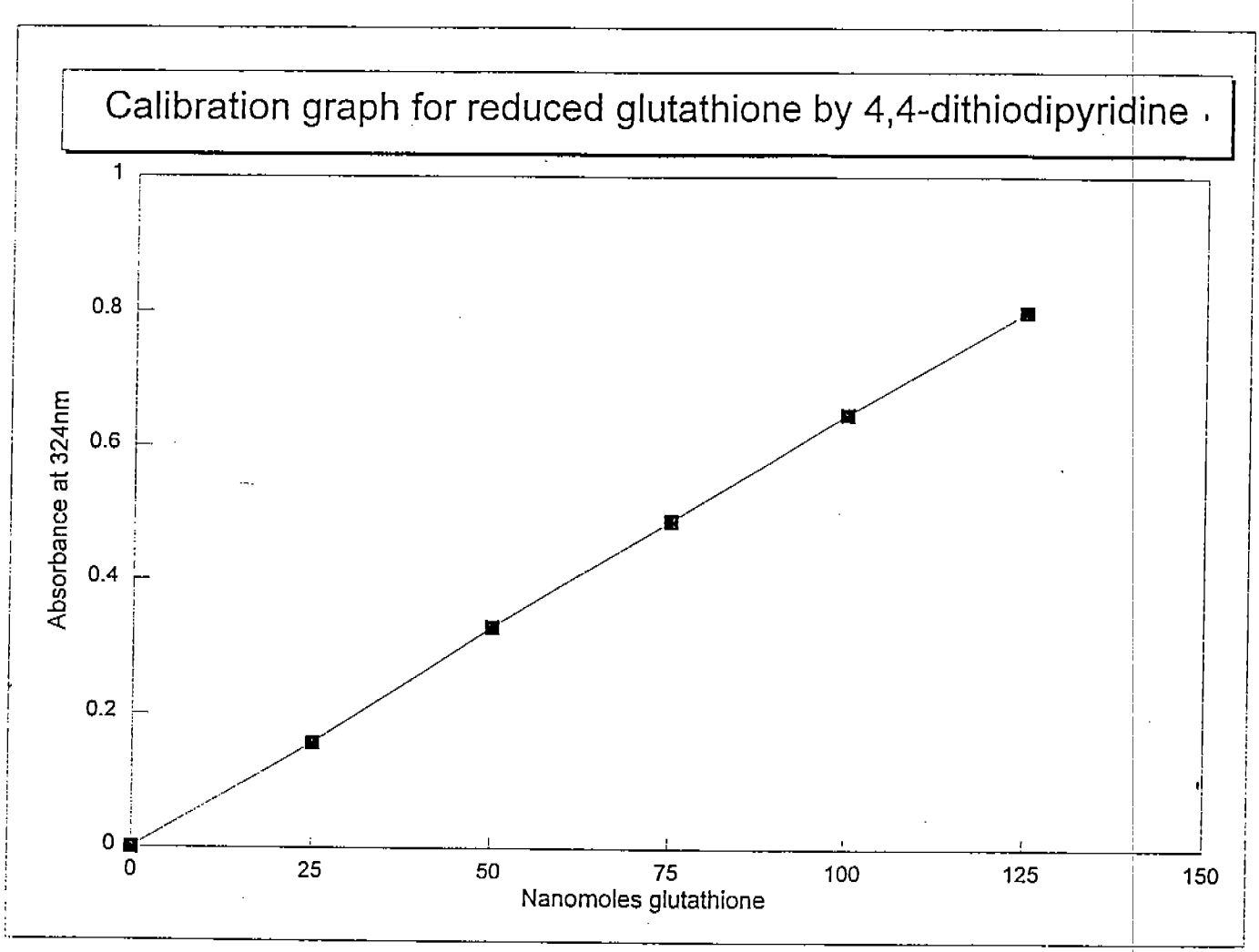
Calibration graph for reduced glutathione by 4,4-dithiodipyridine
Absorbance at 324nm
1
0.8
0.6
0.4
0.2
0
0
25
50
75
100
125
150
Nanomoles glutathione

FIGURE 1

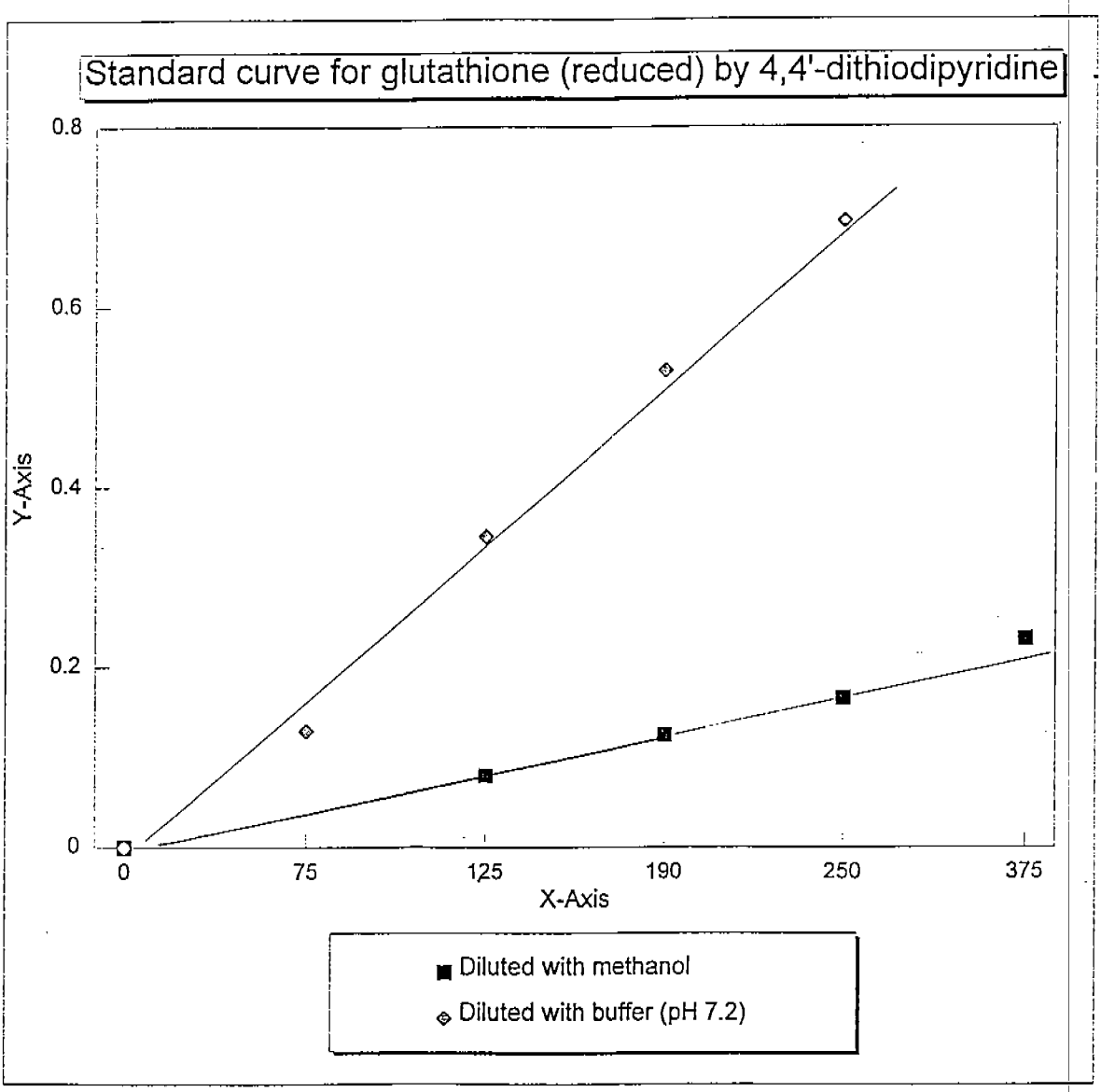
Standard curve for glutathione (reduced) by 4,4'-dithiodipyridine
0.8
0.6
0.4
0.2
0
Y-Axis
0
75
125
190
250
375
X-Axis
Diluted with methanol
Diluted with buffer (pH 7.2)

FIGURE 2

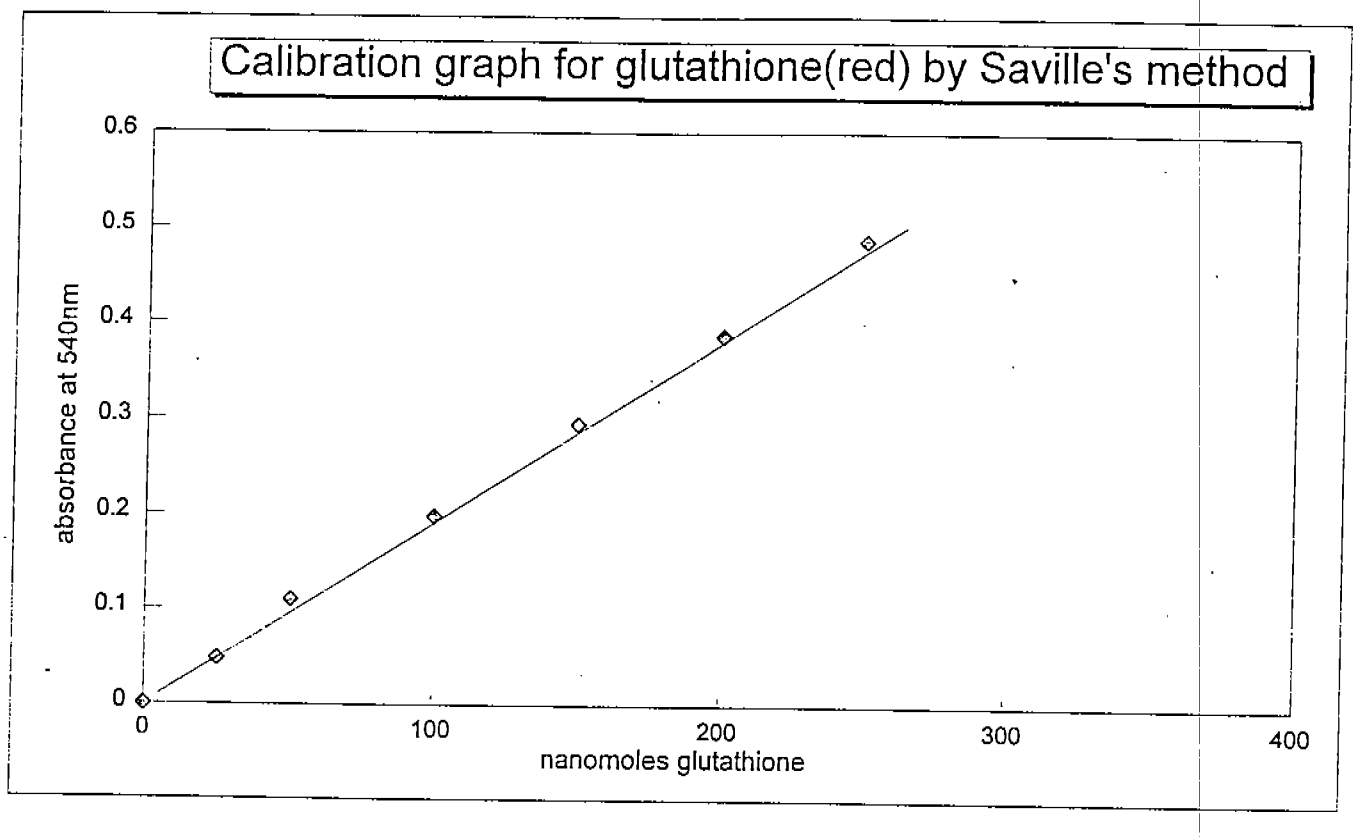
Calibration graph for glutathione(red) by Saville's method
absorbance at 540nm
0.6
0.5
0.4
0.3
0.2
0.1
0
0
100
200
300
400
nanomoles glutathione

FIGURE 3

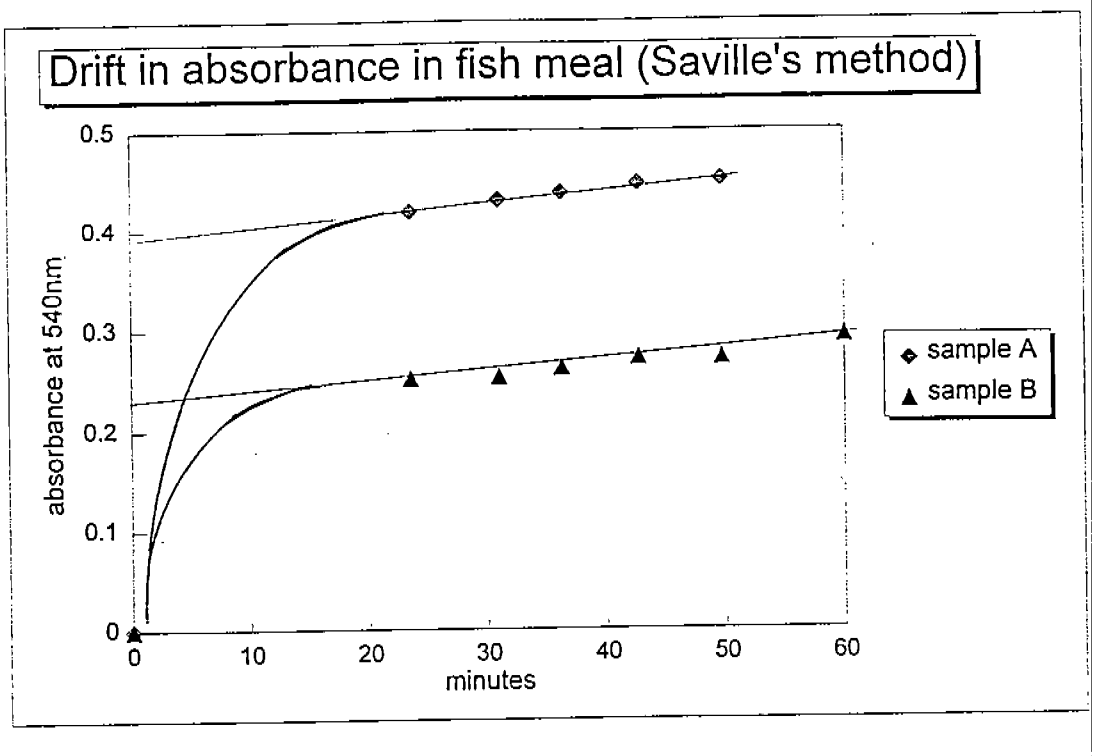
Drift in absorbance in fish meal (Saville's method)
absorbance at 540nm
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
minutes
sample A
sample B

FIGURE 4