International Fishmeal & Oil Manufacturers Association

INVESTIGATION OF METHODS FOR DETERMINATION OF SULPHYDRYL GROUPS AND THEIR APPLICATION TO FISH MEAL - PART II

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REPORT ON AN INVESTIGATION INTO METHODS OTHER THAN METHANOL TREATMENT FOR THE ELIMINATION OF INTERFERING TURBIDITY AFTER THE REACTION OF FISH MEAL WITH 4,4'-DITHIODIPYRIDINE

by Dr.G.E.Trout, August 1997

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) residues in the meal. However some limitations have been observed on the method employed for determination of sulphydryl groups and the Fishing Industry Research Institute (FIRI) in South Africa undertook a survey into alternative methods of analysis.

FIRI found that one of the methods tested (reaction with 4,4'-dithiodipyridine) proved satisfactory with standard test proteins but fish meal resulted in a loss of sensitivity of the method caused by turbidity experienced during execution of the method (Research Report 1996-3).

FIRI was invited to continue with this work to study methods for reducing the turbidity and therefore increasing the sensitivity of the method. The following conclusions were reached.

The turbidity which seriously interferes with the estimation of available sulphydryl residues in fish meal by 4,4'-dithiodipyridine (4,4'-DTP) can be avoided by markedly reducing agitation of the meal with reagent. It was established that consistent results could be obtained in suspensions subjected only to very occasional gentle swirling. Such suspensions yield a clear supernatant on centrifugation and the sulphydryl content was consistent with that in which continuous vigorous agitation occurred.

A number of potential methods for the clarification of turbid reaction mixtures were explored and the most successful depended upon treatment with perchloric acid. Clarification only occurred, however, after precipitation of the bulk of the perchlorate ion as potassium perchlorate from which it was concluded that the mechanism involved is more complex than precipitation of proteinaceous impurities. While the nature of the interference was not explored in detail, it seemed probable that the turbidity was, at least in part, colloidal in nature.

Comparison between methods avoiding turbidity and removal of turbidity by perchloric acid treatment showed that comparable results could be obtained but the perchlorate acid method gave reduced reproducibility consistent with the more complex manipulations involved.

It was concluded that satisfactory analyses of available sulphydryl residues can be carried on fish meal by the direct treatment of meal with buffer/4,4'-DTP reagent provided the mixture is not subject to vigorous agitation.

1. INTRODUCTION

In an earlier study (Trout 1996) the estimation of thiol residues in fish meal with 4,4'-dithiodipyridine (4,4'-DTP) was accomplished by shaking ground meal (30 mg) in buffer/reagent solution either continuously in a wrist action shaker or with occasional vigorous agitation at intervals over a 2 or 3 hour period. The 4-mercaptopyridine liberated during this incubation period was proportional to the free sulphydryl residues available for reaction. When the assay was applied to soluble proteins or a simple thiol such as glutathione, the 4-mercaptopyridine could be measured directly from the absorption at 324 nm and used to calculate the thiol content of the sample. In the case of fish meal, the assay was complicated by the failure to obtain a clear solution after the period of reaction. Invariably, the samples were turbid and unsuitable for direct measurement in the spectrophotometer. It was observed that treatment of this suspension with methanol resulted in the formation of a white precipitate from which a clear supernatant was obtained after centrifugation. The absorption at 324 nm could then be measured. This procedure was very similar to that described for the estimation of thiols in fish meal by the dithionitrobenzoic acid (DTNB) procedure (Miller 1993).

Two complications were observed, however, in the application of methanol precipitation method. Firstly, by contrast with buffered aqueous solution where the absorption of 4-mercaptopyridine was stable over the period of the analysis, the presence of methanol in the reaction mixture resulted in a "drift phase" during which the absorbance gradually increased. Secondly, the 70% methanol/buffer solution markedly lowered the effective absorption coefficient of 4- mercaptopyridine. The present study was undertaken in an attempt to find conditions which would avoid the above objections and allow the development of a simple and improved assay.

2. NATURE OF THE INTERFERENCE

In the manufacture of fish meal, the cooked fish is pressed and the oil is separated from the press liquor. It may be anticipated that this aqueous fraction would contain soluble proteins as well as pigments such as haem derivatives. During further manufacture this "stickwater" fraction is returned to the meal and contributes to the final product. It was considered probable that during the determination of sulphydryl residue in meal these soluble components would be extracted and might contribute to the background impurities. Methanol, a known protein precipitating agent, when added to the turbid assay mixture, resulted in the formation of a white precipitate and, after centrifugation, a clear supernatant. This observation supported the view that proteins may be the source of the high absorption and led to the suggestion that alternative denaturating or precipitating agents might be successful in clarifying the meal suspension. Thus heat treatment, high salt concentrations, acids such as perchloric acid (PCA) and organic solvents other than methanol, were proposed as worth investigation. At the same time it was accepted that aromatic amino acids absorb maximally around 280 nm and haem derivative around 415 nm and would appear to contribute very little to the spectral absorption around 324 nm used in the present method. Filtration through a membrane filter (0.45 μm) while affecting some improvement in the clarity of the suspension, was not completely successful indicating that the problem was not one of suspended particulate matter. After investigating some of the above proposed methods it was considered probable that the turbidity of the supernatant as described, was, at least in part, colloidal in nature. Thus in experiments with perchloric acid in which (see later) successful clarification of the suspension was achieved, acid treatment alone did not result in a clear solution after centrifugation; in conflict with what would have been expected if proteins were instrumental in the turbidity. Initial experiments with ammonium sulphate precipitation also suggested that any clarification which occurred was not a direct precipitation of protein. Depending on the type of colloid formed, some of the conditions explored in the present

study may be expected to affect colloid destabilisation and may account for the clarification by perchloric acid which only occurs subsequent to precipitation of the perchlorate ion by potassium carbonate.

The formation of turbid suspension of fish meal in the assay was a consequence of the agitation deemed desirable for effective reaction between the soluble reagent and the insoluble meal. The need for such agitation was partially questioned in the earlier study (s.v.) in which continuous shaking in a wrist-action shaker showed no significant change compared with occasional vigorous shaking. In the present study it was established that consistent results for the available thiol residue determination in fish meal could be obtained in reaction mixtures subjected to only occasional GENTLE SHAKING or SWIRLING. A satisfactory recovery of added glutathione from such treatment was also achieved (see Table 6). It is therefore proposed that by limiting the meal/reaction mixture to very mild agitation, 4,4'-DTP can be successfully applied to fish meal without the formation of troublesome turbidity. If turbidity remains a problem, a method for such suspensions was investigated and is described below. In the course of the investigation a number of further conditions were examined for their possible role in obtaining a clear solution for the assay and a number of observations were made which are summarised below.

3. EFFECT OF HEAT TREATMENT

A suspension of fish meal in buffer, when heated to about 90°C for 12 mins showed no obvious change in opalescence and did not leave a clear supernatant on standing. A sample of 4,4'-DTP in the same buffer and treated similarly showed marked increase in absorption at 324 nm (absorbance >2) indicative of conversion to 4-mercaptopyridine. Heat treatment was clearly unsuitable and was not examined further.

4. AMMONIUM SULPHATE AS A REAGENT FOR CLARIFICATION OF FISH MEAL SUSPENSIONS

Ammonium sulphate is commonly used in salt precipitation of proteins partly because of its high solubility in water. For the present purpose a 50% final concentration was explored as this was conveniently produced by treatment of the suspension with an equal volume of a saturated ammonium sulphate stock solution. In view of the effect of methanol on the reduction of the absorption at 324 nm in the assay, the effect of ammonium sulphate on glutathione estimation by 4,4'-DTP was determined.

4.1 EFFECT OF AMMONIUM SULPHATE ON THE ASSAY OF GLUTATHIONE WITH 4,4' DITHIODIPYRIDINE:

Conditions: As outlined in initial report (Trout 1996).

Solutions: 250μM glutathione (dilute 10 mM stock 40x)
4,4' DTP solution 11 mg in 0.5 ml methanol diluted to 25 ml with buffer
0.1M phosphate buffer pH 7.2
Saturated ammonium sulphate solution

TABLE 1

Protocol to determine if ammonium sulphate influences the estimation of sulphydryl groups by 4,4'- dithiodipyridine

Reagent	Tube No.				
	1	2	3	4	5
Buffer ml	1.5	1.25	1.0	0.75	0.5
Std glutathione ml	-	0.25	0.5	0.75	1.0
DTP ml	1.5	1.5	1.5	1.5	1.5

Two sets of solution were prepared and one set was diluted with 7 ml of buffer and the other set with 7 ml saturated ammonium sulphate solution. Each was read at 324 nm against the blank sample.

TABLE 2

Estimation of glutathione in the presence of ammonium sulphate

Tube No.	Absorbance at 324 nm	
	Buffer	Ammonium sulphate
1	0.00	0.00
2	0.217	0.190
3	0.404	0.426
4	0.620	0.627
5	0.814	0.864

A plot of the above figures was linear and little significant difference could be discerned between the two lines It was concluded that ammonium sulphate had no significant effect on colour development and would not change the absorbance of any 4-mercaptopyridine formed during the incubation of meal with 4,4' DTP. A repeat experiments gave very similar results.

4.2 APPLICATION TO FISH MEAL

Studies on fish meal have been problematic because the formation of the stable suspension is to a very large extent dependent upon the vigour of shaking. In most of the experiments in which ammonium sulphate was tested in the present work, shaking of the meal and buffer or reagent was relatively mild and the intensity of the opalescence was low. Under these conditions ammonium sulphate appeared to yield a clear solution and treated samples showed lower absorbance readings than controls. In other samples with a more marked turbidity, ammonium sulphate, at the concentration tested, did not clear the solution. Even filtration through a membrane filter (0.45μm) failed to yield a clear solution. Some soluble protein may have been precipitated by 50% ammonium sulphate as suggested by some of the results but the remaining turbidity was still too high for the method to be useful. As further work suggested a more positive alternative, this approach was terminated.

5. TREATMENT OF THE SUSPENSION WITH ORGANIC SOLVENTS

Methanol treatment of the meal suspension was successful in clarifying the turbidity as described earlier and this effect was confirmed. It was considered possible that other solvents may also result in clarification of the suspension without the disadvantages observed with methanol. Consequently a number of common water-miscible organic solvents were examined. As a first step the effect of these solvents on the absorbance of 4 mercaptopyridine, the absorbing product of the assay, was examined. A stock solution of 4-mercaptopyridine in buffer (10 mM) was prepared and diluted 100x for a working standard. Three millilitres of this solution were transferred to a test tube and diluted with 7 ml of solvent as listed in the table. Phosphate buffer was included for comparison. The absorbance at 324 nm was recorded (Table 3).

TABLE 3

The effect of organic solvents on the absorbance of 4-mercaptopridine at 324 nm

Solvent	A 324 nm
Phosphate buffer pH 7.2	0.626
Methanol	0.265
Ethanol	turbid
Acetonitrile	0.437
Acetone	turbid
Dimethylformamide	turbid

Stability of absorbance in presence of diluent: The samples prepared above were examined at intervals to assess their stability with time.

TABLE 4

Stability of 4-mercaptopyridine in buffer/acetonitrile

Minutes	Absorbance at 324 nm	
	Buffer	Acetonitrile
1'	0.668	-
1 30"	-	0.466
5'	-	0.448
5 16"	0.665	-
10'	0.666	0.433
15'	-	0.425

By contrast with methanol, there was no increase in absorbance over the initial 10 minute period. The presence of acetonitrile, however, resulted in a 8-9% loss in absorbance over 15 mins. This was considered too unstable for reliable use. It was concluded that the solvents explored offered no advantage over methanol.

6. EFFECT OF AGITATION ON THE ASSAY OF THIOL RESIDUES IN FISH MEAL

The estimation of thiol residues in a heterogeneous mixture such as exists in the assay of thiol residues in fish meal would appear, prima facae, to be less efficient than in a mixture in which the thiol residues are soluble. It was for this reason that earlier experiments attempted to facilitate reaction by vigorous agitation. In the present study the effect of agitation was examined by comparing continuous vigorous mechanical shaking with samples subjected to only occasional gentle manual swirling. The first of these actions yielded turbid solution which gave very high reading in the spectrophotometer and could not be used without further treatment to effect clarification. Gentle swirling of the mixture, by contrast resulted in a suspension from which centrifugation yielded a clear solution suitable for direct analysis. Subsequently, perchloric acid was used to clarify the suspension from vigorous agitation (see Method B for details) and although this latter procedure gave a somewhat wider range of replicate values, satisfactory agreement between the two methods suggested that vigorous shaking was unnecessary for complete reaction of the available sulphydryl residues.

6.1 METHODS

Method A: Gentle swirling method

A number of screw capped glass scintillation vials were employed and 30 mg amounts of fish meal, which had been finely ground in a hammer mill, were weighed according to the protocol below. Three millilitres of the 4,4'-DTP solution in phosphate/EDTA buffer (pH 7.2) were dispensed into each tube. The vials were allowed to stand in the dark with occasional gentle swirling for 2-3 hours. Blanks and standards were also prepared and allowed to stand for the same time. After reaction was complete, 7.0 ml of buffer as added to each vial and, after

mixing, the contents were transferred to a tube and centrifuged (2000 rpm for 15 mins, 100 x g). The clear supernatant was carefully decanted into a clean tube and the absorbance at 324 nm against a buffer blank was determined.

Method B: With vigorous shaking

The initial steps followed the above description with the exception that vials were shaken in a wrist-action shaker throughout the 2-3 hour incubation period. At this stage 10µl methyl orange indicator was added to each vial followed by 2.0 ml 2M perchloric acid/4 mM K_2 - EDTA. After mixing, each vial was allowed to stand a few mins and then neutralised dropwise with potassium carbonate solution to the first yellow colour. This step was carried out on a top-pan balance and the weight of potassium carbonate was determined. Phosphate buffer (pH 7.2) was then added to bring the increase in weight to 2.0 g and the assumption was made that this represented the addition of 2.0 ml solution. A further 2.0 ml buffer was then added (total volume 10 ml) and each vial shaken and the contents transferred to a centrifuge tube. Centrifugation at 10°C for 15 mins at 2000 rpm (100 g). The low temperature improved the precipitation of insoluble potassium perchlorate. The clear supernatant was then decanted into clean tubes and the absorption at 324 nm determined. A protocol for both Method A and Method B is included in the Appendix.

6.2 RESULTS

6.2.1 Comparison of method A and method B on a single random meal

A meal was selected at random from samples submitted for routine proximate analysis and had been finely ground in a hammer mill. Replicate simultaneous analyses by Methods A and B of available sulphydryl residues as well as the recovery of 200 nmoles of added glutathione were performed at intervals over a period of several weeks. The results are presented in Table 5.

6.2.2 Application of method A to number of different meal samples

A number of different meals submitted to the Institute for routine analysis were employed for the study. All were recent samples and had been finely ground in a hammer mill prior to analysis. The available sulphydryl residues were determined by Method A only and the result expressed as mmoles SH/100 g meal. The data are recorded in Table 6.

TABLE 5

Available sulphydryl residues in a series of random fish meal samples analysed by Method A

Meal Code	Available SH groups in mmoles/100 g meal
9700603	2.10
9700726	2.42
9700887	0.41
9700724	1.16
9701052	1.52
9701059	2.69

TABLE 6

Repeatability and recovery of available sulphydryl groups in a single meal

EXPERIMENT NUMBER	METHOD A		METHOD B	
	mmole/100 g meal	% * recovery	mmole/100 g	% * recovery
1	1.18	89.9	1.15	75.3
2	1.16	87.7	1.22	87.4
3	1.14	89.1	1.11	70.2
4	1.18	93.5	1.09	105.5
5	1.16	91.5	1.08	98.0
Mean value	1.16	90.3	1.13	87.3
Standard deviation	0.0167	2.2379	0.0570	14.8505
Coef. of variance	1.43%	2.48%	5.04%	17.01%

* 200 nmoles reduced glutathione added to meal.

Mean values for the mmoles SH/100 g meal by the two methods were not significantly different at the 5% level but the SD for both the SH content and % recovery of added glutathione were significantly different at the same 5% level. This suggests that while the two methods gave similar mean results the precision of each method differed. The much wider range of both total thiol and percentage recovery in Method B compared with Method A was confirmed by the larger coefficients of variation.

7. REFERENCES

Miller E.L. Determination of SH content of fish meals. *IFOMA Research Report* 1993(5).

Trout G.E. Investigation of two methods for the determination of sulphydryl groups and their application to fish meal. *IFOMA Research Report* 1996(3).

APPENDIX

Experimental details for the estimation of sulphydryl residues in fish meal by both Method A and Method B.

Reagents: 0.1M Potassium phosphate buffer pH 7.2 containing 0.001M EDTA (as potassium salt).

4,4'-DTP reagent: Dissolve 11 mg DTP in 0.5 ml methanol and add 25 ml buffer with vigorous stirring. Make fresh for each run.

2M Perchloric acid/2mM EDTA reagent (PCA): Add 28.7 ml 70% perchloric acid to approximately 60 ml water. Add 162 mg dipotassium EDTA and adjust the volume to 100 ml with water.

Potassium carbonate solution: A hot saturated solution was prepared and allowed to stand overnight for crystals to appear. The clear solution was used. To assist in neutralising the sample, some of this concentrated solution was diluted and used when it was apparent that the indicator was close to its change point.

METHOD A

Finely ground fish meal (30 mg) was weighed into glass scintillation vials according to the protocol below and 3 ml of either 4,4'-DTP reagent or, in the case of the blank sample, with buffer. A second blank sample without fish meal but with 4,4'-DTP was similarly prepared to assess the decomposition of the reagent over the time of reaction. All analyses were carried out in duplicate. In Method A the samples were allowed to stand for 2-3 hours with occasional gently swirling before being diluted to 10 ml final volume with buffer. Centrifuge and read clear supernatant at 324 nm. Dilute sample if absorbance too great.

Tube	Mass of meal (mg)	Buffer/DTP (ml)	Buffer (ml)	Reaction	Buffer (ml)
1 + 2	30	3.0	-	Stand 2-3 hour with occasional gentle swirling	7.0
3 + 4	30	-	3.0		7.0
5 + 6	-	3.0	-		7.0

METHOD B (for turbid solutions):

The initial procedure was identical to that described above except that the vials were vigorously shaken with a wrist-action shaker. After reaction was complete methyl orange (10µl) and perchloric acid reagent (2 ml) were added and each vial was transferred to a top-pan balance and tared. Potassium carbonate solution was added dropwise allowing time for reaction to take place until the solution reached the first permanent yellow. Phosphate buffer was then added until the increase in weight was 2.0 g. The volume was then made up to 10 ml as outlined above. All vials were then well mixed, transferred to centrifuge tubes and centrifuges for 15 mins (100 x g) in a centrifuge at 10°C. The low temperature facilitates the precipitation of potassium perchlorate. The supernatants were then decanted into clean tubes, diluted when necessary, and warmed to room temperature before the absorbance was measured at 324 nm.

Tube	Mass of meal (mg)	Buffer/ DTP (ml)	Buffer (ml)	Reaction	MO (ul)	PCA (ml)	Buffer (ml)
7 + 8	30	3.0	-	As above	10	2.0	2.0
9 + 10	30	-	3.0	but shake	10	2.0	2.0
11 + 12	-	3.0	-	vigorously	10	2.0	2.0