DRAFT

LIMITATIONS OF ELLMAN'S REAGENT - 5,5' DITHIOBIS (2-NITROBENZOIC ACID) - FOR THE ASSAY OF SH GROUPS AND S-S BONDS IN FEED AND DIGESTA PROTEIN.

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Running title: Assay of SH groups

SUMMARY

Ellman's reagent, 5,5'dithiobis(2-nitrobenzoic acid), was used to determine native SH groups and total SH groups after reduction with sodium borohydride of S-S in feedstuffs, diets and digesta. S-S bonds were calculated by difference. The molar extinction coefficient of the 2-nitro-thiobenzoic acid (NTB) anion formed using reduced glutathione was 14323 under carefully defined conditions, but decreased with increasing time of incubation. The method gave satisfactory results with feedstuffs but problems were encountered with diets and digesta. Coefficients of variation (CV), based on a single analysis carried out on different days in one laboratory, within feedstuffs and diets were 15.8 and 22.8% for native SH, and 9.9 and 17.1% for total SH, respectively. In a collaborative study of analysis of fish meals, taking a unit determination as the mean of triplicate analysis, the within and between laboratory CV were 7.6 and 19.5% for native SH and 6.9 and 7.8% for total SH repectively. Recovery rates of added SH (as reduced glutathione) were 89.9% (range 75.4 - 96.5%) in feedstuffs, 33.3% (range 18.4 - 48.5%) in diets and -1.2% (range -6.4 to +4.2%) in digesta. A further limitation of the method was that absorbance from coloured secretions in digesta overlaps the 412 nm absorption maximum of the NTB anion.

Key words sulphydryl, thiol, disulphide, Ellman's reagent, pig feeds, fish meal, soya bean meal, pig diets, pig digesta

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INTRODUCTION

Sulphydryl (SH) and disulphide (S-S) bonds make a major contribution to the functional properties and stability of the native conformation of proteins (see Wolf, 1970; Kinsella, 1982; Shimada and Cheftel, 1988a). Mild heat treatment (< 50-70 °C) of muscle protein may increase SH but slightly more severe treatment, yet still within the normal range of heat processing (70-115 °C), decreases SH and increases S-S content without loss of cyst(e)ine (Hamm and Hoffmann, 1965; Opstvedt *et al* 1984; Synowiecki and Sikorski, 1988; Synowiecki and Shahadi, 1991). Severe heating at temperatures above 115 °C results in loss of cyst(e)ine, reduced protein digestibility in rats, reduced availability of several amino acids for growth of *Streptococcus zymogenes* (Miller *et al* 1965a and b) and reduced ileal digestibility of cyst(e)ine in pigs (Wiseman *et al* 1991). Loss of SH and increase in S-S is accompanied by loss of solubility of the protein and reduced digestibility in rainbow trout (Opstvedt *et al* 1984). Consequently, disulphides may influence the nutritive value of food proteins by conferring increased resistance to enzymatic hydrolysis. Although this resistance has been demonstrated with purified protein *in vitro* (Reddy et al 1988), it has not been well evaluated for conventional protein sources or practical diets. The question arises whether alteration in the SH and S-S bond content of normally processed feedstuffs can have detrimental effects on the quality of feed proteins and whether measurement of SH or S-S content of processed proteins can be an indicator of mild processing damage.

Ellman's reagent 5,5'dithiobis(2-nitrobenzoic acid) (DTNB) has received the widest application in the determination of SH groups in native and denatured proteins. The procedure is based on the reaction of thiol groups of a protein with DTNB to give a mixed disulphide and the intense yellow coloured dianion 2-nitro-5-thiobenzoic acid (NTB) with maximum absorbance at 412 nm according to the equation:

$$RS^- + NBA^-\text{-S-S-}NBA^- \rightarrow R\text{-S-S-}NBA + {}^-\text{S-}NBA^- \qquad (I)$$

(DTNB) (NTB)

where NBA^- = 2-nitrobenzoic acid

The method has generally been used only with purified protein sources (e.g. Buttkus, 1971; Shimada and Cheftel 1988ab; Reddy *et al*, 1988) and high protein feeds (e.g. Opstvedt *et al* 1984) but not in low protein feeds or digesta samples. The reaction is normally carried out on native or denatured proteins in solution. In applying the reaction to feedstuffs a period of 2 to 3 hours in Tris buffer with sodium dodecyl sulphate (SDS) at pH 8.2 has been used to solubilise the protein (Opstvedt *et al* 1984). Oxidation of SH may take place under such conditions. A further major disadvantage of the method is that oxidation of NTB can occur during reaction with DTNB resulting in loss of colour (Torchinsky 1981) and consequently strict precautions are necessary to remove oxygen while EDTA is added to chelate prooxidant minerals. In contrast there is also a slow increase in colour due to hydrolysis of DTNB (Jocelyn 1987a). The aim of the present study was to evaluate DTNB as a reagent for the determination of SH and S-S groups in pig feeds (cereal and protein concentrates), pig diets and digesta samples using the method of Opstvedt *et al* (1984).

EXPERIMENTAL

Materials

Apparatus Reactions were performed in 70 ml Beckman polycarbonate centrifuge tubes with rubber seal assembly caps and centrifugation was carried out on a Beckman J2-21ME centrifuge (Beckman Instruments Inc California, USA). Absorbance was measured on a SP6-500 uv spectrophotometer fitted with a 1 cm quartz flow cell (Pye Unicam Limited, Cambridge, England).

Reagents Reduced glutathione (GSH) and ethylenediaminetetra-acetic acid disodium salt (EDTA) were obtained from Sigma Chemical Company, St. Louis, USA. Ellman's reagent (DTNB) (Biochemical) and absolute methanol (Analar), hydrochloric acid (Analar), Tris base [(2-amino-2(hydroxymethyl)-1,3 propanediol], sodium dodecyl sulphate (SDS), n-octanol, sodium borohydride (GPR), sodium hydroxide (Analar) and urea (GP) were obtained from Merck Limited, Poole, England.

DTNB solutions (0.016 M and 0.005 M) DTNB was dissolved in 20 ml absolute methanol on the day of analysis.

0.2 M Tris buffer (pH 8.2) - 0.02 M EDTA - 2% SDS Tris base, 24.22 g and 20 g SDS were dissolved in 800 ml of distilled water deoxygenated by passing nitrogen, followed by the addition of 90 ml of 1 N HCl and 100 ml of 0.2 M EDTA. The pH was adjusted to 8.2 by the addition of 1 N HCl and the solution was then transferred into a 1 litre volumetric flask and made to volume with deoxygenated distilled water, bubbled with nitrogen for a further 5 minutes and capped under nitrogen.

Samples Barley meal (D1243): obtained locally.

Casein: lactic acid casein (30 mesh) New Zealand Dairy Board.

Dried Skim Milk (D1241) supplied by Volac Ltd, Royston, Herts, UK..

Herring fish meal (D1240).

Low temperature processed fish meal (LTFM) (D1278): prepared from fresh herrings using a low temperature process. Supplied by International Fishmeal & Oil Manufacturers Association (IFOMA), St Albans, Herts, UK.

Normal temperature processed fish meal (NTFM) (D 1279): prepared from the same batch of herring as used in D 1278 by normal temperature processing. Supplied by IFOMA, St Albans, UK.

Soyabean meal (D1242). Hipro soya supplied by Pauls Agriculture Ltd, Ipswich, Suffolk, UK.

Soy Protein Concentrate (SPC). Soycomil™ Loders Croklaan, Wormerveer, The Netherlands.

Four semi-purified pig diets containing (g/kg) casein, 130; either alone or in conjunction with LTFM, 150; or NTFM, 150; or SPC, 160; together with lactose, 200; sucrose, 100; soya oil, 50; trace elements and vitamins, 10; chromium mordanted α-cellulose, 20; Ca, P, Na, K, and Cl supplements to balance diets, 30 to 54.5; corn starch to 1kg, 282.7-435.5.

Digesta, containing chromium and Co-EDTA as flow markers, were obtained post-mortem from the stomach, proximal and distal ileum and rectum of early-weaned 6 week old pigs that had been fed the casein-LTFM diet. The digesta were immediately frozen in liquid nitrogen and then stored at -20°C prior to freeze-drying

Methods

Determination of the molar extinction coefficient of DTNB and recovery rate of SH groups in reduced glutathione solutions Different concentrations of GSH in 0.02 M EDTA were prepared in triplicate ranging from 5.0×10^{-5} to 1.0×10^{-3} M. The absorbance was determined by adding 1 ml samples of GSH solution to 8.0 ml of 0.2 M Tris buffer (pH 8.2) - 0.02 M EDTA - 2% SDS solution, followed by 0.5 ml of 0.016 M DTNB and 31.5 ml of absolute methanol. The tubes were flushed with N, capped, mixed and allowed to stand for 5, 25, 30 or 45 minutes before reading the absorbance at 412 nm against a reagent blank. The total volume of the reaction mixture was 41 ml, which would give an equivalent SH group concentration range of 1.22 to 48.78 mmoles/L.

Determination of native sulphydryl (SH) groups in protein Triplicate samples containing about 30 mg of protein were dissolved in 8.0 ml 0.2 M Tris buffer (pH 8.2) - 0.02 M EDTA - 2% SDS solution. Nitrogen was flushed over the solution for a few seconds. The tubes were capped and left to stand for 3 hours with intermittent shaking. Two centrifuge tubes, each containing 8 ml buffer, were similarly set up for reagent blanks.

After 3 h, 0.5 ml of 0.016 M DTNB and 31.5 ml of absolute methanol were added with mixing. The solution was flushed with nitrogen and allowed to stand in capped tubes at room temperature for 15 minutes, centrifuged at 3000 g for 15 minutes, filtered through a prepared Whatman No 4 filter paper and the absorbance determined immediately at 412 nm. Reagents blank absorbance values were determined and subtracted from the sample absorbance. The SH content was calculated using the molar extinction coefficient of NTB of 14323 determined in this study.

Determination of total SH and disulphide bonds in protein Total SH was determined after $NaBH_4$ reduction. The previously determined native SH groups were then subtracted to give the number of S-S bonds. To triplicate samples containing about 35 mg of protein and triplicate reagent blank tubes were added 4.0 ml of 0.6 M $NaBH_4$ in 8 M urea and 0.1 ml of octyl alcohol (to avoid foaming) and stood for 3 h at room temperature. The remaining $NaBH_4$ was destroyed by the addition of 1.1 ml of 2 N HCl and the pH of the solution brought to 8.2 by the addition of 0.9 ml of 2 N NaOH.

To an aliquot of 0.5 ml, 1.5 ml of 0.2 M Tris buffer (pH 8.2) - 0.02 M EDTA - 2% SDS solution was added, followed by 0.2 ml of 0.005 M DTNB and 7.8 ml of absolute methanol. The solution was mixed, flushed with nitrogen and stood in capped tubes at room temperature for 15 minutes, centrifuged at 3000 g for 15 minutes, filtered and absorbance determined as above.

Determination of recovery rates of GSH from feed and diet samples Nine samples (30 mg of protein) of each test material were dissolved in nitrogen-saturated 0.2 M Tris buffer (pH 8.2) - 0.02 M EDTA - 2% SDS by standing with occasional shaking for 3 hours, with 2.0 μmols of GSH (i.e. 1 ml of 0.002 M GSH) added to three of these samples. Samples were analysed as for native SH. The total time from addition of GSH to determination of absorbance was 3.5 h. To three other samples GSH was added after three hours and immediately prior to adding DTNB giving a total of 0.5 h from time of addition of GSH to determining absorbance. The remaining three samples were analysed without GSH. Samples containing GSH only were incubated simultaneously as controls. Absorbance values from reagent blanks were determined in duplicate and deducted from sample readings.

Collaborative study on the determination of sulphydryl and disulphide bonds in fish meals Twelve samples representing 10 fish meals selected to represent a range of low temperature and normal temperature processings were sent to 9 participating laboratories for analysis of native and total SH contents. The analyses was carried out according to the protocols described above except that the dissolution time in Tris buffer

for native SH was 2 h and use of reagent blanks and flushing the reaction tubes with nitrogen after addition of DTNB were not specified. Two of the samples were hidden duplicates unknown to participating laboratories. Laboratories reported analyses on a meal basis. A common N content of each meal was used to express results on protein basis so that variation reflects only that due to the estimation of thiol.

Determination of dry matter and crude protein Samples were dried *in vaccuo* at 70°C for 5 h according to AOAC (1984). N was determined by the Kjeldahl method using a Kjeltec 1015 System 20 digester and auto 1030 analyzer (Tecator AB, Hoganas, Sweden).

Experimental Design and Statistical Analysis In the main all samples were analyzed either in duplicate or triplicate. This allowed for the determination of variation within analysis. When the repeatability of the methods needed testing the analyses were repeated on at least two days, usually 3 to 6 days, to obtain estimates of between day coefficients of variation. Analysis of variance and regression analysis were carried out using the GENSTAT 5 Statistical Package, Lawes Agricultural Trust (1988). A hierarchical experimental design was used to partition the sources of variation according to diet or feedstuff, within day and between day effects in the determination of SH as shown in Table 1.

[Table 1 about here]

RESULTS

Determination of the Molar Extinction Coefficient of NTB.

Absorbance at 412 nm increased linearly (r^2 0.99 - 1.00) with increasing concentration of GSH (Table 2). The molar extinction coefficient (ε_{412}) of NTB was calculated from the regression coefficient. This decreased with increase in reaction time suggesting oxidation of the NTB anion at pH 8.2. With the 30 minutes reaction time adopted for the analysis of SH in feedstuffs, the ε_{412} was determined as 14323. This value was used to calculate the SH content in subsequent analyses.

[Table 2 about here]

Repeatability of determining SH groups in feeds and diets.

Native SH and total SH in feedstuffs, together with estimates of between day and within day variability for each feedstuff, are presented in Table 3. The standard deviation (SD) for native SH was greater between days than within days (pooled values 0.147 and 0.065 respectively). For total SH, the SDs were greater than for native SH and was greater between than within days (pooled values 0.512 and 0.343 respectively). The CV for native SH of casein is high reflecting the very low native SH content. The results in Table 4 give the corresponding values for analyses on two separate batches of the four pig diets together with the expected mean value based on analysis of the component feedstuffs. The determined mean values of the diets were substantially less than the expected values, the SDs were lower than for the concentrates and the CVs were greater, especially the between day CV for both native and total SH.

[Tables 3 and 4 about here]

Collaborative study of determination of native SH and total SH of fishmeals.

For each laboratory the differences between the two sets of hidden duplicates were tested against the pooled variation of the 'open' triplicate determinations. In a high proportion of tests the differences between hidden duplicates were judged significant ($P<0.05$). Consequently, the variation between 'open' triplicates does not provide an unbiased estimate of 'repeatability' or 'within laboratory' variation. The means of the triplicate determinations were used in the further calculations. 'Repeatability' was calculated from the difference between hidden duplicates. Data from 4 of the 9 participating laboratories were omitted as they differed significantly from the others by analysis of variance and were also classified as outliers in a principal component analysis. The pooled mean values and estimates of 'reproducibility' or 'between laboratory' variation and 'repeatability' or 'within laboratory' variation are given in Table 5. The interpretation of these statistics is, that if a randomly selected laboratory were asked to analyse a sample in triplicate, and to present the resulting mean value as an estimate, then these figures give an indication of the errors to be expected on such estimates for

comparison with other determinations made in the same (within laboratory) or a different (between laboratory) centre. Between and within laboratory variation were similar for total SH but between laboratory variation was about twice as great for native SH and consequently also for native SH expressed as a proportion of the total.

[Table 5 about here]

Recovery of added glutathione

Glutathione carried through the whole native SH procedure, involving 3 h standing in Tris buffer, followed by 30 minutes reaction with DTNB was recovered close to 100% (Table 6). GSH added to protein feedstuffs immediately prior to adding DTNB was also well recovered but when added to the feedstuff at the beginning of the 3 h period in Tris buffer recovery averaged 89.9%. Recovery of GSH from the diets averaged only 33.3% although when GSH was added immediately prior to DTNB recovery was still high at 88-95% (Table 7). With digesta (Table 8) recovery of GSH was zero when carried through the whole procedure. Even when GSH was added immediately prior to DTNB, recovery was reduced and decreased from 82% to zero with progression down the intestinal tract from stomach to faeces.

[Tables 6,7 and 8 about here]

Addition of starch to each of Casein, LTFM, NTFM and SPC to represent the major non-protein component of the diets had no effect on the determination of native SH. Increasing the concentration of EDTA in the Tris buffer from 0.02M to 0.05M, 0.10M or 0.20M had no effect on the determination of native SH of LTFM or the recovery of GSH added to LTFM (data not shown).

DISCUSSION

Ellman's reagent is usually dissolved in phosphate or Tris buffer pH 8 at a concentration of 10 mM and is added into a solution of protein containing 1 mM EDTA in a five to ten fold excess with respect to the number of reactive SH groups in the protein (Torchinsky 1981). The final pH of the complete reaction mixture should be above 8.0 as the absorbance of the NTB anion has been found to depend on the pH of the solution. Its

absorbance decreases at pH below 8.0 as a consequence of protonation of the sulphur atom (Paula and Daban 1974), but there is little variation in the pH range 8.0 - 9.0 (Sedlak and Lindsay 1968; Torchinsky 1981). In contrast, Riddles et al (1979) report no difference in ε_{412} at pH 7.27, 7.6 or 8.6.

In most studies the molar extinction coefficient (ε_{412}) for the liberated NTB is routinely taken to be 13600 M^{-1} cm^{-1} at 412 nm, after Ellman (1959). However, several other sources indicate large variation in the extinction coefficient values determined. Riddles *et al* (1979) cites values ranging from 11400 to 14140 and, using stringent analytical conditions, they found an ε_{412} value of 14150. Sedlak and Lindsay (1968) found an ε_{412} value of 13000 while Thannhauser *et al* (1984) found a value of 13900. The present study indicates the colour yield is not stable but declines with time from addition of reagent to reading the absorbance. Consequently this time must be carefully standardised. In the collaborative trial, laboratories reported ε_{412} values which ranged from 12096 to 14771 and this variation must contribute to the between laboratory variation in the determination of native SH and total SH after reduction but would be self-cancelling in the determination of the native SH proportion.

The within laboratory CV for the determination of native SH in fish meals was about 7% both in this laboratory and in the collaborative assay. In both situations the variability between open triplicates carried out side by side on the same day underestimated the real within laboratory variation, indicated by the between day CV or between hidden duplicate samples which were not necessarily analysed on the same day. The high CV for casein reflects the very low native SH content. However, dried skim milk also gave an unacceptably high CV despite a high native SH content. In contrast, soyabean meal was assayed with good repeatability. The variability of determination of total SH after reduction was considerably greater but as the mean values were also much greater the CV was smaller than that for native SH and within an acceptable range. Even after removing 4 outlier laboratories in the collaborative study significant differences ($P<0.001$) in mean values reported by the laboratories remained for both native and total

SH and the CV between laboratories was unacceptably high. Consequently, the current method cannot be recommended for estimation of protein quality for the purposes of commercial trade although it may have utility within one laboratory for relative ranking of materials and processing variables.

Recovery of SH added as reduced glutathione to protein concentrates averaged 89.9% when carried through the whole procedure for native SH but was quite unsatisfactory when applied to semi-purified diets containing the same protein concentrates. Since the recovery of SH added to the diets immediately before adding DTNB was still high, loss of SH must have occurred during the three hour period for dissolution of the protein in Tris buffer rather than loss of NTB anion. The lower than expected values for the analysis of native SH in the diets reflects a similar loss of SH due to oxidation despite the precautions taken. Since adding starch to the protein concentrates had no adverse effect it seems possible that the addition of minerals in the diet catalysed the oxidation but this was not tested. Recovery of SH was effectively zero when applied to freeze-dried digesta samples and in this case recovery was also low and variable when the SH was added immediately before the DTNB suggesting either very rapid oxidation of SH or loss of NTB anion. Mineral ions are most likely involved as the recovery decreased with progression down the gastro-intestinal tract while mineral ions would be expected to increase in concentration. Consequently, the partial or total loss of added SH groups invalidated the use of the DTNB method for the determination of thiols in samples and digesta containing high levels of minerals.

Another problem observed was a high and variable sample blank (methanol replacing DTNB reagent) absorbance at 412 nm, especially for ileal digesta (absorbance readings of 0.6) and less so for faeces (absorbance readings of 0.3) compared with readings of 0.05 for protein concentrates and 0.02 for the reagent blank. The major source of the endogenous colours was probably bile pigments which are concentrated in the ileal digesta but subjected to microbial degradation prior to reaching the rectum. The problems of coloured pigments affecting the DTNB method have been reported before

for haemoglobin (Torchinsky 1981) and for animal tissues (Sedlak and Lindsay 1968). The method of Opstvedt et al (1984) did not specify correction for either reagent or sample blanks and consequently the need for the latter was not initially appreciated. Values given in this paper for feeds and diets have not been corrected for the sample blank but values (Table 7) for digesta and faeces have been so corrected.

The assay of native SH seems to be more prone to oxidative losses than total SH after reduction as the method requires the sample be incubated for at least 2 hours in Tris-buffer to denature and solubilise the protein. EDTA is included in the reaction mixture not only to protect SH-groups from oxidation but also because metal ions can affect the development of the colour. Catalyzed reoxidation of thiols to disulphides was probably the major problem encountered during the assay of SH groups in diets and digesta. The use of EDTA, as a chelating agent (see Buttkus 1971; Jocelyn 1972; Friedman 1973; Torchinsky 1981) in the current studies was not sufficient to eliminate thiol oxidation completely. Increasing the concentration of EDTA ten fold, to suppress the effect of transition metal ions, gave no significant improvement in the recovery of SH groups from LTFM but the more critical test using diets or digesta was not carried out.

The greater variability in the determination of total SH after reduction may reflect failure to completely destroy all the excess $NaBH_4$ by acidification with 1.1 ml 2N HCl. This is essential. To achieve this it was necessary to leave the tubes standing for about 5 minutes, with occasional shaking. Other methods recommend removing the last traces of borohydride by adding a little acetone (Jocelyn 1987b). When sodium borohydride was destroyed completely, the absorbency of reagent blank was in the range 0.016 - 0.037. Cavillini *et al* (1966) reported blank absorbance values of 0.030 - 0.050 after complete removal of sodium borohydride. Another problem was in controlling pH prior to adding DTNB. The pH was brought to around 8.2 with 0.9 ml of 2N NaOH and it was necessary to shake the tubes and leave them standing for another 5 min. This provides opportunity for reoxidation of thiols. Actual pH varied and it was both difficult to further adjust to 8.2 with 2N NaOH, without significant change in volume, and the adjustment

seemed to be affected by the type of protein, indicating probably the influence of proteins of different buffering capacities. The pH in the system after adding Tris buffer was normally around 7.5-8.0. According to Riddles *et al* (1979) pH of 7-8 is normally chosen for absorbance measurements to provide maximum stability of DTNB while assuming that at least 99.8% of NTB would be in the form of the intensely coloured anion. In contrast, absorbance decreases below pH 8.0 according to Sedlak and Lindsay (1968) Palau and Daban (1974) and Torchinsky (1981). On the other hand too high a pH increases hydrolysis of excess DTNB to NTB with consequent increase in absorbance (Riddles et al 1979).

No precautions were taken in these studies to exclude light though for half the reaction period the samples were in a centrifuge in the dark. Damodaran (1985) re-evaluating the method by Thannhauser *et al* (1984) involving the use of a similar reagent 2-nitro-5-thiosulfobenzoate (NTSB) reported that the NTB produced is readily converted to a nonchromophoric derivative in the presence of excess sulphite, used to reduce disulphide bonds, and room light (Riddles *et al* 1979). It was therefore recommended that the NTSB protein reaction be carried out in the dark before measuring absorbance at 412 nm. In the present studies it is not clear whether an analogous reaction to the one described by Damodaran (1985) takes place when SH groups react with DTNB in the presence of light.

The data presented in this study illustrates the inherent problems that exist in using DTNB for the assay of native and total SH after reduction in feedstuffs, diets and digesta protein. However, variations in native SH group and S-S bond determination in feedstuffs or high protein sources could be reduced when the method was familiarised. It appears that the DTNB method gives reasonable results of SH and total SH after reduction in high protein feedstuffs, in particular fish meals, and the latter are comparable to values for cysteic acids determined by amino acid analysis. However, it is concluded that the DTNB method, in its present form, cannot be relied upon for the quantitative

assay of thiol groups in complex feedstuffs, mixed diets or digesta obtained in nutritional studies.

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Table 1

Model of an analysis of variance of a hierarchical experimental design showing the partitioning of degrees of freedom ascribed to variation according to FEEDS or DIETS (F), between day (D) and within day analyses.

SOURCE	d.f.	MODEL MS
FEEDS	(f-1)	$\sigma^2 + a\sigma_D^2 + adF^2$
Between days within feeds	f(d-1)	$\sigma^2 + a\sigma_D^2$
Analysis within days	df(a-1)	σ^2

SD within days = σ

CV (%) within days = (σ/ mean) 100

SD between days = $\sqrt{(\sigma^2 + \sigma_D^2)}$

CV (%) between days = ($\sqrt{(\sigma^2 + \sigma_D^2)}$/ mean) 100

TABLE 2

Determination of the molar extinction coefficient (ε_{412}) of from the regression of reduce glutathione (GSH) concentration on the absorption of the anion NTB at 412 nm. The effect of duration of incubation on the (ε_{412}) value.

SH concentration (μmols/L)	DURATION OF INCUBATION Absorbance at 412 nm after incubating GSH + DTNB for:			
	~ 5 min	~ 25 min	~ 30 min	~ 45 min
48.780	-	-	0.743	-
36.585	-	-	0.536	-
24.390	0.371	0.363	0.367	0.355
19.512	0.292	0.281	0.283	0.269
12.195	0.181	0.176	0.177	0.171
8.049	0.121	0.115	-	0.109
4.878	0.074	0.069	0.069	0.064
2.439	0.040	0.036	-	0.031
1.610	0.028	0.024	-	0.019
1.220	0.021	0.019	-	0.016
ε_{412}	15092	14632	14323	14153
se	80.0	95.9	23.7	163.0
R^2	1.000	0.999	0.999	0.998

TABLE 3

Estimates of variance for the determination of native SH and total SH after reduction in protein sources used in pig experiments (mMol/16g N).

SAMPLE	Mean SH content mMol/16g N	n	Within day SD	Within day CV(%)	Between days SD	Between days CV(%)
Barley meal						
Total SH	7.64	6	0.851	11.14	1.108	14.5
Native SH	0.75	12	0.100	13.34	0.117	15.6
Casein						
Total SH	2.73	9	0.057	2.1	0.177	6.6
Native SH	0.02	9	0.004	20.2	0.019	95.7
Dried skim milk						
Total SH	6.59	12	0.115	1.8	0.294	4.5
Native SH	2.26	12	0.146	6.5	0.374	16.5
Herring fish meal						
Total SH	6.80	12	0.218	3.2	0.251	3.7
Native SH	0.92	12	0.040	4.4	0.066	7.2
LTFM[1]						
Total SH	5.76	9	0.334	5.8	0.338	5.9
Native SH	1.67	9	0.015	0.9	0.097	5.8
NTFM[2]						
Total SH	5.62	9	0.124	2.2	0.353	6.3
Native SH	0.63	9	0.006	0.9	0.059	9.4
Soyabean meal						
Total SH	10.01	12	0.567	1.8	1.303	13.0
Native SH	0.75	15	0.025	3.3	0.036	4.8
SPC[3]						
Total SH	9.30	9	0.130	1.4	0.551	5.9
Native SH	0.44	9	0.014	3.2	0.034	7.7
Pooled Total SH	6.81		0.396	5.8	0.675	9.9
Pooled Native SH	0.93		0.065	7.0	0.147	15.8

1 LTFM low temperature processed fish meal. 2 NTFM normal temperature processed fish meal. 3. SPC soy protein concentrate

TABLE 4

Estimates of variance for the determination of native SH and total SH after reduction in pig diets (mMol/16g N). Two separate mixes of each diet were analysed.

SAMPLE	Expected SH content	Mean SH content	n	Within day SD	Within day CV(%)	Between days SD	Between days CV(%)
Casein-1							
Total SH	2.74	0.94	9	0.077	8.2	0.457	48.7
Native SH	0.02	0.12	9	0.023	19.4	0.032	26.4
Casein-2							
Total SH	2.74	1.31	9	0.148	11.3	0.320	24.4
Native SH	0.02	0.14	9	0.004	3.1	0.052	37.0
Casein-LTFM-1							
Total SH	4.25	1.48	9	0.112	7.6	0.429	29.0
Native SH	0.84	0.36	9	0.019	5.2	0.079	21.9
Casein-LTFM-2							
Total SH	4.25	1.87	9	0.114	6.1	0.162	8.7
Native SH	0.84	0.27	9	0.009	3.3	0.039	14.5
Casein-NTFM-1							
Total SH	4.18	2.46	9	0.156	6.4	0.235	9.6
Native SH	0.32	0.21	9	0.009	4.2	0.030	14.1
Casein-NTFM-2							
Total SH	4.18	2.12	9	0.212	10.0	0.217	10.2
Native SH	0.32	0.24	9	0.013	5.4	0.043	17.9
Casein-SPC-1							
Total SH	6.02	4.36	9	0.349	8.0	0.687	15.8
Native SH	0.21	0.10	9	0.010	9.7	0.023	23.4
Casein-SPC-2							
Total SH	6.02	3.84	9	0.273	7.1	0.364	9.5
Native SH	0.21	0.08	9	0.012	14.9	0.015	18.1
Pooled Total SH	4.30	2.30		0.200	8.7	0.392	17.1
Pooled Native SH	0.35	0.19		0.014	7.2	0.043	22.8

TABLE 5

Estimates of between and within laboratory variation for determination of native SH and total SH in 10 fish meals.

Analysis	Units	Overall mean	Range	Between Laboratory Variation *		Within Laboratory Variation *†	
				SD	CV	SD	CV
Native SH	mmol/16g N	1.62	0.67-3.14	0.315	19.5	0.123	7.6
Total SH	mmol/16 g N	6.13	5.23-7.00	0.479	7.8	0.422	6.9
SH proprtion	mol/mol	0.26	0.10-0.46	0.047	18.0	0.024	9.1

* A unit determination is taken as the mean of triplicate 'open' analyses.

† Within laboratory variation determined from differences between hidden duplicates.

TABLE 6

Recovery of glutathione (GSH) added to casein, low temperature fish meal (LTFM), normal temperature fish meal (NTFM) and soy protein concentrate (SPC).

PROTEIN SOURCE	TREATMENT	SH groups in sample (mmol/ 16gN)	SH groups in sample (mols)	SH groups added as GSH (mols)	Recovery of added SH (%)
CASEIN	Native SH	0.06	0.04	-	-
	Native SH + GSH 0.5h	3.82	2.07	2.0	101.5
	Native SH + GSH 3.5h	3.61	1.97	2.0	96.5
LTFM	Native SH	2.01	0.71	-	-
	Native SH + GSH 0.5h	7.77	2.71	2.0	100.6
	Native SH + GSH 3.5h	7.35	2.58	2.0	93.7
NTFM	Native SH	0.59	0.20	-	-
	Native SH + GSH 0.5h	6.17	2.14	2.0	96.9
	Native SH + GSH 3.5h	6.05	2.08	2.0	94.0
SPC	Native SH	0.60	0.21	-	-
	Native SH + GSH 0.5h	6.02	2.14	2.0	96.1
	Native SH + GSH 3.5h	4.82	1.72	2.0	75.4
GSH	GSH 0.5h	-	2.00	2.0	100.2
	GSH 3.5h	-	2.05	2.0	102.5

TABLE 7

Recovery of glutathione (GSH) added to pig diets[1].

PROTEIN SOURCE	TREATMENT	SH groups in sample (mmol/ 16gN)	SH groups in sample (mols)	SH groups added as GSH (mols)	Recovery of added SH (%)
CASEIN	Native SH	0.89	0.03	-	-
	Native SH + GSH 0.5h	6.08	1.77	2.0	88.5
	Native SH + GSH 3.5h	1.63	0.45	2.0	22.6
CASEIN-LTFM	Native SH	0.40	0.14	-	-
	Native SH + GSH 0.5h	5.58	1.90	2.0	94.8
	Native SH + GSH 3.5h	3.09	0.97	2.0	48.5
CASEIN-NTFM	Native SH	0.29	0.10	-	-
	Native SH + GSH 0.5h	5.51	1.88	2.0	93.9
	Native SH + GSH 3.5h	2.72	0.88	2.0	43.8
CASEIN-SPC	Native SH	0.08	0.03	-	-
	Native SH + GSH 0.5h	5.03	1.77	2.0	88.5
	Native SH + GSH 3.5h	1.13	0.37	2.0	18.4

[1]Diet formulations see text.

TABLE 8

Recovery of glutathione (GSH) added to digesta samples from pigs fed a diet with protein supplied solely by casein + low temperature fish meal.

SITE	TREATMENT	SH groups (mMol/ 16g N)	SH groups in sample (mols)	Added SH groups (mols)	Recovery of added SH (%)
Duodenum	Native SH	0.20	0.08	-	-
	Native + GSH 0.5h	3.92	1.65	2.0	82.4
	Native + GSH 3.5h	0.39	0.16	2.0	4.2
Proximal ileum	Native SH	1.20	0.35	-	-
	Native + GSH 0.5h	5.23	1.53	2.0	59.1
	Native + GSH 3.5h	0.91	0.26	2.0	-4.3
Distal ileum	Native SH	0.29	0.07	-	-
	Native + GSH 0.5h	4.65	1.17	2.0	55.0
	Native + GSH 3.5h	0.43	0.11	2.0	1.8
Faeces	Native SH	1.92	0.35	-	-
	Native + GSH 0.5h	1.92	0.35	2.0	0.2
	Native + GSH 3.5h	1.21	0.22	2.0	-6.4