Hydrolysis conditions for the analysis for sulphur amino acids in feedstuffs

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A study of methods of analysis for sulphur amino acids in feeds was undertaken with a view to finding methods suitable for the routine analysis of feedstuffs.

In the case of cyst(e)ine, it was found that the results obtained from simple oxidation and hydrolysis with a mixture of dimethylsulphoxide (DMSO) and hydrochloric acid correlated well with those obtained from performic acid oxidation (PAO) and subsequent hydrolysis (r = 0,94). It was also found that evacuation was not necessary in the DMSO-HCl oxidation-hydrolysis procedure.

Added cyst(e)ine could not be recovered quantitatively; 82% being recovered from a blank hydrolysis, 73% when starch and tryptophan were also present and only 50% when added to a mixed feed.

Methionine, measured after a normal evacuated hydrolysis, did not correlate well with methionine recovered as methionine sulphone after PAO (r = 0,79). However, over 90% of methionine added to a complete feed could be recovered after the normal evacuated hydrolysis.

S. Afr. J. Anim. Sci. 1984, 14: 64 – 69

'n Ondersoek van metodes vir die ontleding van swawelbevattende aminosure in voere is onderneem met die doel om geskikte metodes te vind vir die roetine-ontleding van voergewasse.

In die geval van sist(e)ien is getoon dat die resultate verkry deur eenvoudige oksidasie en hidrolise met 'n mengsel van dimetielsulfoksied (DMSO) en soutsuur goed korreleer met die resultate verkry deur permieresuuroksidasie (PAO) en daaropvolgende hidrolise (r = 0,94). Daar is ook gevind dat evakuering onnodig is in die DMSO-HCl oksidasie-hidrolise-metode.

Bygevoegde sist(e)ien kan nie kwantitatief herwin word nie; 82% is herwin van 'n blanko hidrolise, 73% as stysel en triptofaan ook teenwoordig is en slegs 50% as dit by 'n gemengde voer gevoeg is. Metionien, bepaal na 'n normale geëvakueerde hidrolise, het nie goed gekorreleer met metionien, herwin as metioniensulfoon na PAO nie (r = 0,79). Meer as 90% van die metionien wat by 'n volledige voer gevoeg is kan egter herwin word na die normale geëvakueerde hidrolise.

S.-Afr. Tydskr. Veek. 1984, 14: 64 – 69

Keywords: Sulphur amino acids, analysis, feedstuffs

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Received 25 August 1983

Introduction

In a previous communication (Dennison & Gous, 1980) it was noted that small changes in the levels of limiting amino acids in feed ingredients can have substantial economic repercussions in least cost mixed feeds. Accurate methods of analysis are consequently of paramount importance.

The sulphur-containing amino acids cystine, cysteine and methionine, which are of particular nutritional interest, are to varying degrees unstable during conventional hydrolysis with 6 mol dm^{-3} hydrochloric acid and special procedures are therefore necessary for their analysis. The most common of these methods consists of preliminary oxidation with performic acid, which converts cystine and cysteine to cysteic acid and methionine to methionine sulphone, both derivatives being stable to acid hydrolysis (Moore, 1963). For the routine analysis of feedstuffs however, the performic acid oxidation method is not ideal as it is a multi-step procedure requiring special apparatus and incorporating freeze-drying and other time consuming procedures. As Friedman & Noma (1975) have noted, the accuracy and reproducibility reported for this method when applied to purified proteins, is seldom achieved with feedstuffs.

A simple method of analysis for total cystine plus cysteine has been proposed by Spencer & Wold (1969), consisting simply of the inclusion of 0,2 to 0,35 mol dm^{-3} dimethylsulphoxide (DMSO) in the 6 mol dm^{-3} hydrochloric acid used to effect hydrolysis. The DMSO oxidizes cystine and cysteine to cysteic acid concurrently with the hydrolysis but as other amino acids are destroyed, a separate analysis is necessary to determine the remaining amino acids, including methionine.

In this study the DMSO oxidation method was evaluated in its application to feedstuffs, as opposed to purified proteins, and in comparison with the performic acid oxidation procedure. The effect of not evacuating the vial prior to hydrolysis in the presence of DMSO was investigated as it was reasoned that removal of oxygen should not be necessary for an oxidative reaction. Also, since cysteic acid is eluted from the sulphonic acid resin with virtually no retardation, it was anticipated that high salt concentrations within the sample would not affect its elution time, and the applicability of the neutralization technique of Spitz (1973) was consequently also investigated. These techniques have previously been applied to the specific analysis for lysine (Dennison, 1978).

As a consequence of the rapid elution of cysteic acid, only a very short analytical cycle is needed, but this imposes a need for an internal standard with a similar short elution time. Three candidate internal standard compounds, namely taurine,

aminomethanesulphonic acid and ethanolaminephosphoric acid were evaluated, the criteria for selection being a molecular structure containing an amino group, for reaction with ninhydrin, and a strongly acidic group to ensure a low affinity for the sulphonic acid resin. Ethanolaminesulphonic acid was eventually selected as most suitable, since taurine occurs naturally in feedstuffs such as fishmeal, carcassmeal and poultry by-product meal and aminomethanesulphonic acid could not be recovered quantitatively in blank control experiments.

The DMSO-oxidation method, applied with the modifications outlined above, appeared to be a rapid and feasable alternative to performic acid oxidation, but as methionine is not determined by this procedure, alternative methods of methionine analysis were investigated. Ideally, for a rapid routine method, methionine should be determined concurrently with the remaining amino acids, if a separate analysis is already undertaken for cysteine. The performic acid oxidation method for methionine analysis was consequently compared with conventional hydrolysis, with evacuation (Davies & Thomas, 1973). On the suggestion of J. Tannoch (personal communication) the effect of not evacuating the vial prior to hydrolysis upon the methionine values was investigated and, arising from the report of Keutmann & Potts (1969), the possibility of using mercaptoethanol in the acid, in lieu of evacuation, was studied.

Materials and Methods

1. 'Conventional' hydrolysis for the analysis for methionine.

Reagents

Hydrochloric acid (6 mol dm^{-3}): conc. HCl (54 cm^3 made up to 100 cm^3 with distilled water).

Buffer pH 2,2: Sodium citrate. $2H_2O$ (19,6 g), conc. HCl (16,5 cm^3), thiodiglycol (20 cm^3) and caprylic acid (0,1 cm^3) are dissolved in approximately 900 cm^3 of distilled water, the pH adjusted to 2,2 with HCl, and the solution made up to 1 dm^3.

Norleucine internal standard: A solution containing 6,25 μmol cm^3 is prepared by dissolving norleucine (81,9 mg) in pH 2,2 buffer and making up to 100 cm^3.

Procedure

A finely ground sample for analysis (0,025 g) is measured into a rimless pyrex test tube (12 × 150 mm) and 6 mol dm^{-3} hydrochloric acid (3 cm^3) is added. The mixture is frozen in an acetone/dry ice mixture, evacuated to less than 0,1 mm Hg, thawed under vacuum, refrozen, and the tube sealed in a flame while still under vacuum. Hydrolysis of the sample is effected in an oven at 110°C for 24 hours and, after cooling, the tube is opened and norleucine standard solution (0,4 cm^3) is added and mixed in. The hydrolysate is filtered through glass fibre filter paper (Whatman GF/C) and evaporated twice to the point of dryness at less than 40°C under reduced pressure in a rotary evaporator, before being made up to 5 cm^3 with pH 2,2 buffer. A Beckman 119 analyser was used in our studies and a sample of 0,25 cm^3 was applied for each analysis.

2. Dimethylsulphoxide oxidation method for the analysis for cystine plus cysteine (Spencer and Wold, 1969).

Reagents

Dimethylsulphoxide (0,35 mol dm^{-3}) in 6 mol dm^{-3} HCl: Conc. HCl (54 cm^3) is made up to approximately 90 cm^3 with distilled water, 2,5 cm^3 dimethylsulphoxide is added and the solution is made up to 100 cm^3.

Buffered neutralizing solution: Sodium hydroxide (52,5 g) is made up to 500 cm^3 with pH 2,2 sample buffer.

Ethanolaminephosphoric acid (EPA) internal standard: A solution containing 12,5 μmol cm^3 is prepared by dissolving EPA (176,4 mg) in pH 2,2 sample buffer and making up to 100 cm^3.

Procedure

A finely ground sample for analysis (0,025 g) is measured into a rimless pyrex test tube (12 × 150 mm). The tube is constricted and sealed without evacuation and hydrolysis of the sample is effected in an oven at 110°C for 24 hours. After cooling, the tube is opened carefully, EPA internal standard solution (0,4 cm^3) is added, well mixed in, and the solution is filtered through glass fibre filter paper. To a sample of the filtrate (1 cm^3), buffered neutralizing solution (2 cm^3) is added and the pH is adjusted, if necessary, to pH 2,2 by the dropwise addition of conc. HCl or NaOH. Cysteine is measured as cysteic acid on the long column of the analyser, using the first buffer only on an abbreviated analytical cycle, the column being regenerated immediately after elution of the internal standard, which elutes before aspartic acid.

3. Performic acid oxidation method for analysis for cystine, cysteine and methionine.

Reagents

Performic Acid: Perhydrol (2 cm^3 of a 30% solution) and formic acid (18 cm^3 of an 88% solution) are mixed together and allowed to stand for 1 hour at room temperature. A freshly prepared solution is used for each set of analyses.

Apparatus

Screw cap test tubes (Schott No. 26135215) (16 × 160 mm) were modified into pear-shaped flasks of approximately 15 cm^3 capacity in which all the reactions and evaporations could be effected without transfer. For evaporation, a six-limb radially symmetrical manifold was constructed to which the pear-shaped flasks could be attached by means of screw caps, with aperture (Schott No. 2922706) and sealed with silicone/PTFE washers. A thermostatically controlled sandbath, into which the flasks were inserted with the caps protruding, was used to effect heating for hydrolysis as it was found that heating of the caps in an oven caused them to bind irremovably to the flask neck. The threads of the plastic cap were greased with silicone grease before use.

Procedure

The procedure followed is essentially that of Moore (1963). A finely ground sample for analysis (0,025 g) is measured into a pear-shaped flask. The flasks and the performic acid solution are cooled to 0°C and 2 cm^3 of performic acid is added to each flask, which is shaken to mix the contents and subsequently maintained at 0°C for 20 hours. HBr (0,3 cm^3 of a 40% solution) is added in the cold and the resulting bromine is removed by evaporating twice in a rotary evaporator under reduced pressure at 45°C, care being taken in the initial stages to avoid frothing. The rotary evaporator was found to be incapable of reducing the sample completely to dryness in a reasonable time however, and final drying is therefore effected by diluting the sample with distilled water, freezing and freeze-drying (This procedure cannot be strongly recommended however, as it was discovered that bromine is extremely cor-

rosive to stainless steel, leading to the development of pinholes in the freeze-dryer vacuum drum if this is not thoroughly cleaned immediately after use).

HCl (3,0 cm^3 of 6 mol dm^{-3}) is added to the freeze-dried sample, the flask sealed with a PTFE-lined screw cap and hydrolysis is effected in a sandbath at 110°C for 24 hours. After cooling, EPA internal standard is added and thoroughly mixed in. The solution is filtered through glass fibre filter paper (Whatman GF/C) and the filtrate is evaporated twice to the point of dryness on a rotary evaporator at 45°C and dissolved in 5 cm^3 of pH 2,2 buffer. Samples (0,25 cm^3) of the resulting solution are taken for analysis, cystine plus cysteine being measured as cysteic acid and methionine as methionine sulphone on an abbreviated elution cycle, the column being regenerated after the elution of glutamic acid.

4. Experiment to test reproducibility of cysteine analysis and recovery of added cysteine.

Reagents

Stock cysteine solutions (SCS): Twenty identical cysteine solutions were made up by dissolving, in each case, cysteine-HCl (16 mg) and dimethylsulphoxide (5 cm^3) in distilled water and making up to 100 cm^3.

5% DMSO: Dimethylsulphoxide (5 cm^3) dissolved and made up to 100 cm^3 with distilled water.

Diluted cysteine solutions: Three groups of diluted cysteine solutions were prepared, consisting of five replicates each and using a different stock solution for the preparation of each individual solution. In this way weighing errors were randomized. The three groups were prepared as follows:-

Solution (i) SCS (2,5 cm^3) plus 5% DMSO (7,5 cm^3)
Solution (ii) SCS (5,0 cm^3) plus 5% DMSO (5,0 cm^3)
Solution (iii) SCS (7,5 cm^3) plus 5% DMSO (2,5 cm^3)

Procedure

Twenty five samples (0,025 g) are measured out from a finely ground mixed feed sample into hydrolysis tubes. The tubes are divided into five groups and treated as described below, each preparation being prepared from a separate diluted or stock cysteine solution.

Group 1. Add 1,5 cm^3 5% DMSO and 1,5 cm^3 conc. HCl.
Group 2. Add 1,5 cm^3 solution (i) and 1,5 cm^3 conc. HCl.
Group 3. Add 1,5 cm^3 solution (ii) and 1,5 cm^3 conc. HCl.
Group 4. Add 1,5 cm^3 solution (iii) and 1,5 cm^3 conc. HCl.
Group 5. Add 1,5 cm^3 stock cysteine solution and 1,5 cm^3 conc. HCl.

The hydrolysis tubes are sealed and the samples analysed for cysteine as described above.

5. Experiment to test reproducibility of conventional methionine analysis and recovery of added methionine.

The rationale of this experiment was essentially the same as that of the cysteine experiment described above. However, as it was found from the results of the cysteine experiment that weighing errors are negligible, only one stock solution was made up containing 13,0 mg methionine made up to 100 cm^3 with distilled water.

From the stock methionine solution, three dilute solutions were prepared containing a stock methionine solution and water in the following proportions, 2,5 cm^3: 7,5 cm^3; 5,0 cm^3: 5,0 cm^3 and 7,5 cm^3: 2,5 cm^3.

Procedure

Twenty five samples (0,025 g) are measured out from a single finely ground mixed feed into hydrolysis tubes. The tubes are divided into five groups and treated as follows:-

1. Add 1,5 cm^3 water and 1,5 cm^3 conc. HCl.
2. Add 1,5 cm^3 dilute methionine solution (i) and 1,5 cm^3 conc. HCl.
3. Add 1,5 cm^3 dilute methionine solution (ii) and 1,5 cm^3 conc. HCl.
4. Add 1,5 cm^3 dilute methionine solution (iii) and 1,5 cm^3 conc. HCl.
5. Add 1,5 cm^3 stock methionine solution and 1,5 cm^3 conc. HCl.

The tubes are evacuated, sealed and the samples analysed for methionine by the 'conventional' method described above.

Results and Discussion

The first question addressed concerned a change to the DMSO-oxidation procedure of Spencer & Wold (1969). It was reasoned that since an oxidative reaction is involved and since DMSO itself is a strong oxidizing agent there would appear to be no need to evacuate the vials prior to hydrolysis. This was consequently tested and, as Figure 1 shows, there is a close agreement between the values obtained with and without evacuation.

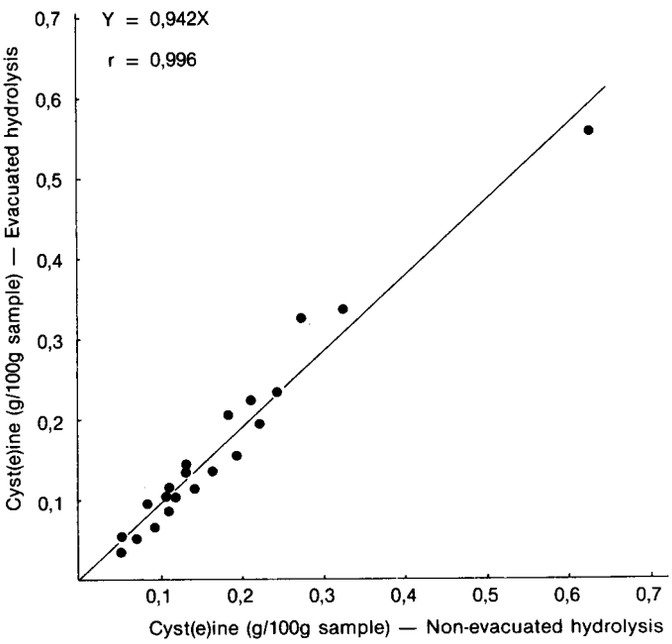

Figure 1 Cyst(e)ine analysis: correlation between evacuated hydrolysis and non-evacuated hydrolysis.

It was also established that the neutralization technique of Spitz (1973) could be applied to the analysis of DMSO-oxidized samples as the resulting salt does not interfere with the elution of either cysteic acid or ethanolaminephosphoric acid, the internal standard. With the modification outlined above, the DMSO-oxidation procedure is particularly straightforward and, as is shown in Figure 2, there is a good correlation between the values obtained for cyst(e)ine by this method and by the performic acid oxidation procedure of Moore (1963). This would seem to indicate that the DMSO-oxidation method is an acceptable alternative to the more laborious performic acid method when applied to the analysis of cyst(e)ine in feedstuffs.

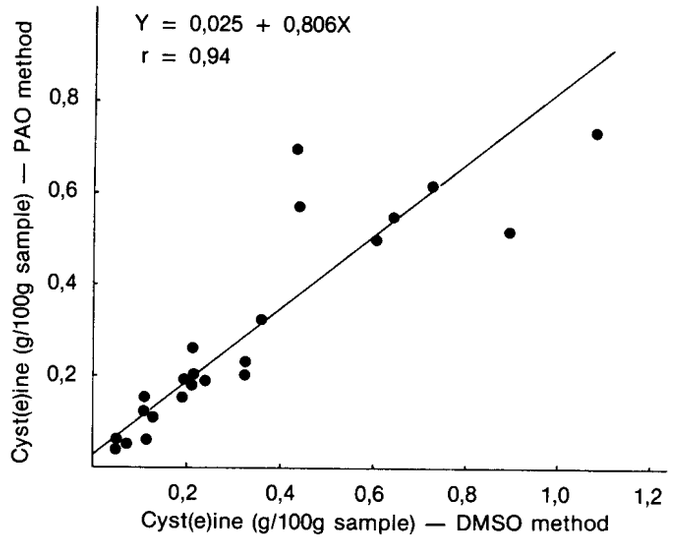

Figure 2 Cyst(e)ine analysis: correlation between performic acid oxidation and DMSO oxidation.

Experiments on the recovery of added cyst(e)ine from DMSO-oxidized hydrolysates revealed that the recovery of cyst(e)ine as cysteic acid is apparently influenced by other compounds present in the hydrolysis mixture (Figure 3). Thus cysteine (or cystine) itself, with no other compounds present, was recovered to the extent of approximately 82%. In the presence of tryptophan and starch the recovery decreased to 73% and in the presence of a complete mixed diet there was a further decrease in recovery to approximately 50% of the added cyst(e)ine.

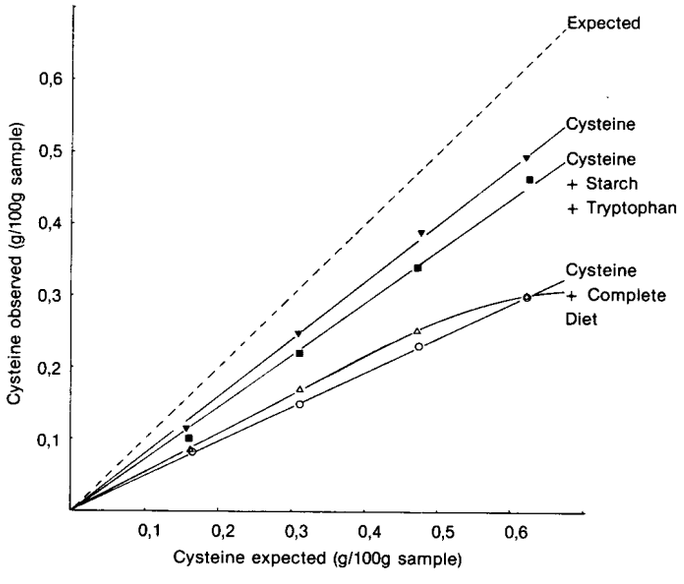

Figure 3 Cysteine analysis: recovery of added cysteine. ---- = expected line for 100% recovery; ▲ – ▲ = observed recovery of cysteine alone; +--+ = observed recovery of cysteine in the presence of starch and tryptophan; ■ – ■ and 0 – 0 = repeat experiments on the recovery of cysteine added to a complete mixed feed. Similar results were obtained for cystine.

The destruction of cyst(e)ine would therefore appear to be effected by reactions involving a number of different compounds and this could be the origin of the variability reported in the analysis for cyst(e)ine in feedstuffs (Friedman & Noma, 1975; Dennison & Gous, 1980; Chavana, Haque, Njaa & Petterson, 1980).

In a collaborative study reported by Williams, Hewitt & Cockburn (1979) most laboratories reported a higher recovery of cyst(e)ine from a blank hydrolysis after DMSO-oxidation than the 82% which we report here. It was not stated that the hydrolysis was carried out in sealed vials although this is most likely. Unfortunately also the participants in this collaborative trial apparently did not test the recovery of cyst(e)ine added to a complete mixed feed. The exceptionally low recovery (50%) which we have noted under such conditions throws some doubt on the validity of the results obtained for cyst(e)ine analysis in feedstuffs. On the other hand Mason, Rudemo & Bech-Anderson (1980) have obtained almost quantitative recoveries of cystine added to barley, maize or wheat using a performic acid oxidation followed by reflux hydrolysis in 6 mol dm^{-3} HCl under argon gas. Their method is particularly interesting in that they have adopted the novel approach of analysing for all of the amino acids in a simple neutralized hydrolysate of a pre-oxidized sample, destruction of the non-sulphur amino acids during hydrolysis being minimised by the use of phenol as a halogen scavenger. In our analytical system the salt arising from the neutralization of the 6 mol dm^{-3} HCl has resulted in a poor resolution of certain amino acids. Another method using a simple hydrolysis has been described by Friedman, Krull & Cavins (1970) and involves conversion of cyst(e)ine to S-β-(4-pyridylethyl) cysteine, but recovery of added cyst(e)ine was apparently not tested.

Recently, Inglis (1983) has reported a novel hydrolysis procedure for the analysis of all amino acids, including cysteine and tryptophan, but again the applicability of this method to the analysis of feedstuffs has apparently not been tested. The status of cyst(e)ine analysis is thus undecided. Williams *et al.* (1979) have decided, from the results of their collaborative study, that both the performic acid oxidation and DMSO-oxidation methods are satisfactory for the analysis of cyst(e)ine in feedstuffs. Chavana *et al.* (1980) conclude however, after a study of three methods, including the performic acid oxidation method but not hydrolysis in the presence of DMSO, that the best that can be hoped for in a cyst(e)ine method is to obtain results which may be used for comparisons within a laboratory.

Our own results suggest that greater attention should be given to the question of the recovery of cyst(e)ine added to complex complete diets, since poor recovery can cast a doubt upon results which otherwise look satisfactory. Clearly to achieve a greater degree of agreement between the results from different laboratories a greater understanding is needed of the chemistry of cyst(e)ine under the conditions used for oxidation and hydrolysis, especially in respect of possible side reactions which might occur with the components of practical feeds.

The results that we have obtained for methionine analysis are in a sense opposite to those obtained for cyst(e)ine. A relatively poor correlation was observed between the performic acid oxidation method and the 'conventional' method of hydrolysis (Figure 4) but confidence in the latter method was supported by the good recoveries of added methionine which were obtained (Figure 5). An investigation of the necessity for the evacuation of the vials, revealed that generally lower values were obtained when the evacuation step was omitted and mercaptoethanol was present instead during hydrolysis (Figure 6). In a previous report (Dennison & Gous, 1980) it was noted that the coefficient of variation of the methionine values obtained by repetitive analysis of a standard sample of maize,

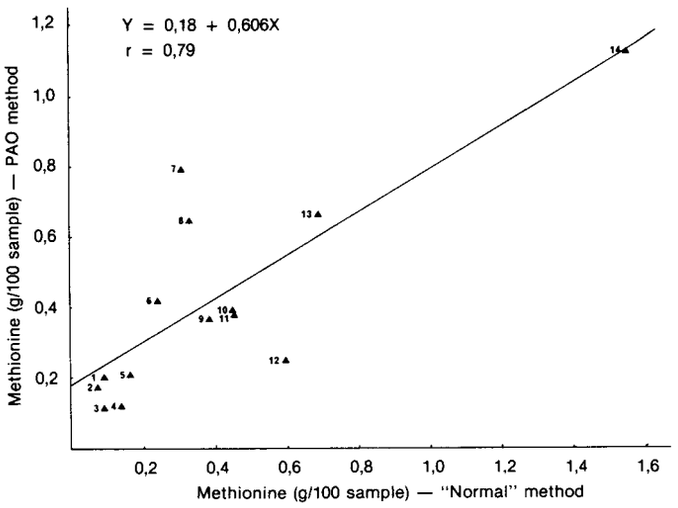

Figure 4 Methionine analysis: correlation between performic acid oxidation method and non-oxidative hydrolysis for a range of sample types. 1,2, lucerne; 3, sorghum, 4, wheat; 5, rice; 6, groundnut oilcake; 7, 13, sunflower oilcake; 8, poultry by-product meal; 9, 10, mixed diets; 11, brewers grain; 12, carcass meal; 14, fishmeal.

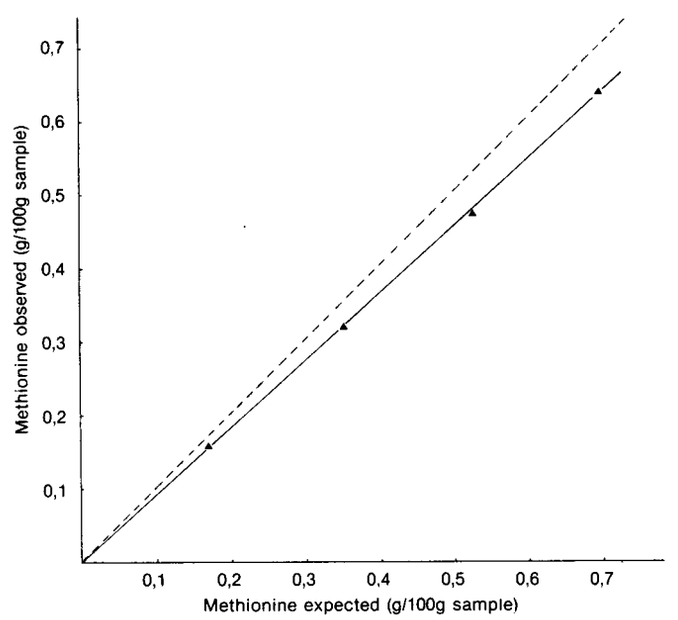

Figure 5 Methionine analysis: recovery of added methionine. ---- = expected line for 100% recovery; ▲ – ▲ = observed recovery.

by the conventional method involving evacuated hydrolysis, was of the same order as that of many of the other amino acids. These results suggest, therefore, that conventional hydrolysis provides methionine values which are sufficiently accurate, provided that adequate precautions are taken to exclude air during hydrolysis.

It should be pointed out that Jennings & Lewis (1969) have found markedly lower values for methionine analyses of leaf material by the 'conventional' method compared to the performic acid oxidation method. The only material which we have tested which might be termed 'leaf material' is lucerne and on the two samples tested our results agree with those of Jennings and Lewis, in that the PAO method gave results nearly twice as high as the 'conventional' method (Figure 4). However, completely different results were obtained for non-leaf material. This could indicate that the other chemical constituents present in samples analysed for methionine might have a specific influence on the results obtained, depending on the

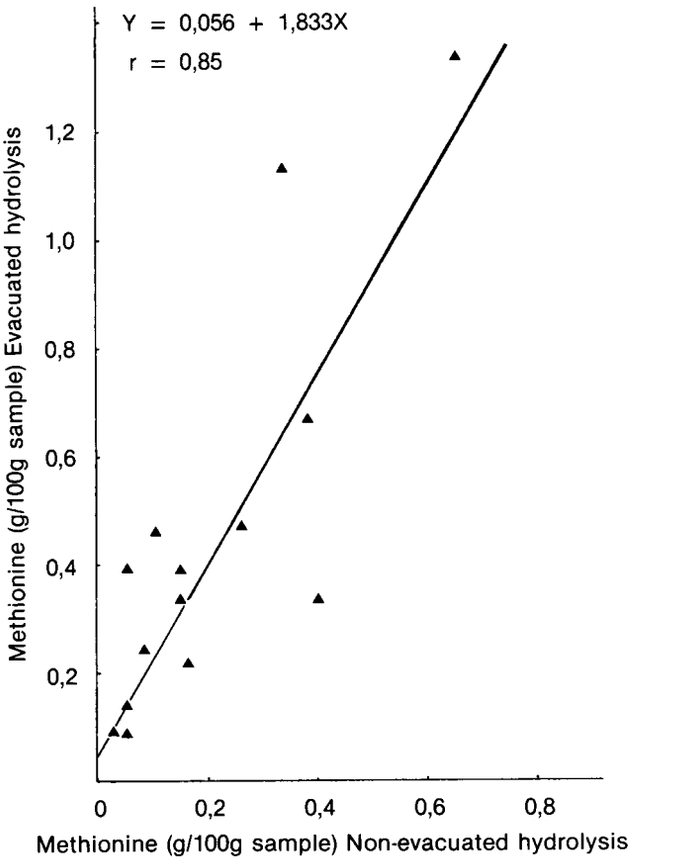

Figure 6 Methionine analysis: correlation between evacuated and non-evacuated hydrolysis.

method of analysis. However, further data are needed before this can be stated with certainty.

Acknowledgements

The authors wish to acknowledge the contributions of Anne M. Phillips and J.P. Marais to this paper and of Mrs E.M. Stephenson, who performed the amino acid analyses.

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