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Analyzing Sulfur Amino Acids in Selected Feedstuffs Using Least-Squares Nonlinear Regression

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Five feedstuffs were oxidized using performic acid, and these, along with their unoxidized counterparts, were acid hydrolyzed for multiple times (0–144 h) in degassed and vacuum-sealed glass tubes. The methionine sulfone, cysteic acid, methionine, and cysteine contents were determined for each hydrolysis time. Least-squares nonlinear regression of the sulfur amino acid contents and hydrolysis time was used to predict the actual sulfur amino acid content as well as the hydrolysis and loss rates. Least-squares nonlinear regression estimates for methionine content compared well with those of methionine sulfone for most of the feedstuffs tested. In contrast, the estimates for cysteine agreed poorly with cysteic acid. The loss rates during acid hydrolysis for methionine, methionine sulfone, and cysteic acid were low. Overall, acid hydrolysis in an evacuated sealed tube for 24 h without prior oxidation is suitable for methionine, but not cysteine, quantitation in some complex feedstuffs.

KEYWORDS: Methionine; cysteine; performic acid; amino acids; oxidation

INTRODUCTION

Methionine and cysteine are collectively indispensable sulfur amino acids present in foods and feedstuffs. The accurate determination of these amino acids is important for the optimal formulation of diets for intensive livestock and for supplying nutritional information about foods for humans. Most amino acids are routinely analyzed using acid hydrolysis in 6 M HCl (*1*) and subsequent separation and quantitation of the liberated amino acids using HPLC. However, methionine and, particularly, cysteine are susceptible to oxidation during acid hydrolysis. Although reasonable recoveries of methionine can be obtained from samples that have been well degassed, considerable losses in the presence of carbohydrates have been reported (*2*). Furthermore, recoveries of cysteine are also poor. Consequently, methionine and cysteine are usually quantitatively oxidized to methionine sulfone and cysteic acid, respectively, using performic acid oxidation, prior to acid hydrolysis (*3*).

For amino acids that are susceptible to degradation during acid hydrolysis, least-squares nonlinear regression of the amino acid content determined using multiple hydrolysis times has been used to accurately predict the actual amino acid content of several protein sources (*4–9*). Robel and Crane (*9*) used least-squares nonlinear regression to determine the hydrolysis and loss rates of amino acids in lysozyme during acid hydrolysis including methionine, when analyzed without performic acid oxidation to methionine sulfone. Moreover, Rutherfurd et al. (*4*) showed that for two goat milk based infant formulas and a whole goat milk powder, the stability of methionine determined using acid hydrolysis without oxidation with performic acid was equal to that of methionine sulfone after oxidation and hydrolysis. However, with the exception of Rutherfurd et al. (*4*), no one has used least-squares nonlinear regression to compare the stability of methionine or cysteine during acid hydrolysis with that of their oxidized counterparts methionine sulfone and cysteic acid.

In this study, we aimed to use least-squares nonlinear regression to investigate the stability of methionine and cysteine during hydrolysis using our standard laboratory method for hydrolyzing protein (sealed tube method) with that of methionine sulfone and cysteic acid after performic acid oxidation and acid hydrolysis for five complex feedstuffs.

MATERIALS AND METHODS

Five feedstuffs including canola meal, skim milk powder, corn meal, soybean meal, and meat and bone meal were used in this study. They were all obtained from local suppliers. The products were ground through a 1 mm mesh and stored at –20 °C prior to analysis

Proximate Analysis. The total nitrogen content of the five selected feedstuffs was determined on a LECO analyzer using the Dumas method (*10*), and crude protein was calculated as the total nitrogen content multiplied by 6.25. Dry matter, crude fiber, ash, and total fat were determined according to the methods described by AOAC (*11*). Nitrogen-free extractive (NFE) (an estimate of the noncellulose carbohydrate) was determined as the difference between the total sample weight and the sum of the moisture, ash, crude protein, crude fiber, and ether extract.

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Analysis of Methionine and Cysteine Using Acid Hydrolysis without Prior Oxidation. Approximately 5 mg of each feedstuff was weighed into 10 sets of duplicate hydrolysis tubes (130 mm × 12 mm Schott Duran glass tubes). One milliliter of 6 M glass-distilled HCl containing 0.1% phenol was added to each tube. The necks of the tubes were stretched to produce a narrowed section. This was carried out by melting the glass approximately 15 mm from the top of the tube with an oxygen/methane gas torch. When the glass was molten, the top of the tube was pulled about 3–4 cm away from the rest of the tube using forceps, producing a thin neck of glass that could be easily melted to seal the tube. The tubes were then attached to a vacuum pump, and a vacuum was very carefully applied, such that the acid would gently bubble but that the acid or sample would not bubble up into the narrowed neck of the tube. Once all of the bubbling had stopped, a Bunsen burner was used to melt the glass in the narrow section of the neck and the tube was allowed to seal. The tubes were then incubated, in duplicate, at 110 °C for 0, 3, 6, 10, 16, 24, 52, 92, 120, and 144 h. After hydrolysis, the tubes were cracked open and norleucine was added as an internal standard before being dried down. Once dry, the amino acids were dissolved by the addition of an HPLC loading buffer of 67 mM sodium citrate (pH 2.2) containing 0.1% (w/v) phenol before being analyzed using a Waters ion-exchange HPLC system, utilizing post-column ninhydrin derivatization and detection using absorbance at 570 nm. A calibration standard containing methionine, cysteine, and norleucine (or methionine sulfone, cysteic acid, and norleucine for the oxidized samples) was used to identify and quantify these amino acids in the samples. When appropriate, the weight of each amino acid was calculated using free amino acid molecular weights.

Analysis of Methionine as Methionine Sulfone and Cysteine as Cysteic Acid after Oxidation and Acid Hydrolysis. Approximately 5 mg of each feedstuff was weighed into 10 sets of duplicate hydrolysis tubes. The tubes were treated with performic acid to quantitatively oxidize methionine and cysteine to methionine sulfone and cysteic acid, respectively, prior to hydrolysis as described below. These tubes were cooled in ice before 1 mL of freshly prepared ice-cold performic acid (9:1 88% formic acid/30% hydrogen peroxide) was added (*11*). The tubes were then incubated on ice in a cold room (5 ± 2 °C) for 16 h. After incubation, 0.15 mL of hydrogen bromide was added to the tubes, which were then dried down. Once dry, the samples underwent acid hydrolysis as described above. Norleucine was used as an internal standard and was added immediately after acid hydrolysis.

The amino acid concentrations were then plotted against hydrolysis time, and the following best-fit equation was applied:

$$B(t) = \frac{A_0 h(e^{-lt} - e^{-ht})}{h - l} + B_0(e^{-lt})$$

$B(t)$ is the amino acid concentration at time t, B_0 is the free amino acid concentration prior to hydrolysis, h is the hydrolysis rate, l is the loss rate, and A_0 is the actual protein-bound amino acid content of the samples. A_0 for each product was derived for each amino acid using least-squares nonlinear regression with the constraints that $A_0 > 0$, $h > 0$, and $B_0 \geq 0$ (*6*).

RESULTS

The overall coefficients of variation between duplicate determinations for methionine and cysteine analyzed using acid hydrolysis without prior performic acid oxidation were 6 and 11%, respectively, whereas those for methionine sulfone and cysteic acid determined after performic acid oxidation and acid hydrolysis were 9 and 11%, respectively.

The proximate composition of the selected feedstuffs was determined and is shown in **Table 1**. Overall, the nutrient composition varied across feedstuffs with the noncellulose carbohydrate (nitrogen-free extract) ranging from 1.4 to 77%, protein ranging from 7.2 to 49%, crude fiber ranging from 0.2 to 11.7%, and total fat ranging from 0.9 to 5.9%.

Hydrolysis yield curves for methionine and methionine sulfone plotted against hydrolysis time for the five feedstuffs are shown in **Figure 1**. The mean R^2 value for methionine sulfone across all feedstuffs was 0.97 (ranging from 0.95 for meat and bone meal to 0.99 for canola meal). For methionine (no oxidation step prior to acid hydrolysis) the R^2 values ranged from 0.94 for soybean meal to 0.99 for canola meal and skim milk powder with a mean R^2 value of 0.97. For both methionine and methionine sulfone, least-squares nonlinear regression provided a close fit to the amino acid yields determined for various hydrolysis intervals.

Table 1. Crude Protein,[a] Ash, Nitrogen-free Extractive[b] (NFE), Crude Fiber, and Total Fat of the Five Feedstuffs Used in This Study

	crude protein	ash	NFE	crude fiber	total fat
canola	37.4	6.6	32.3	11.7	3.8
skim milk powder	31.9	7.7	50.2	1.9	0.9
corn meal	7.2	1.1	76.5	1.8	3.5
SBM	48.3	6.2	30.6	3.8	1.5
meat and bone meal	48.7	36.9	1.4	0.2	5.9

[a] Crude protein was calculated as determined nitrogen multiplied by 6.25. [b] Nitrogen-free extractive (NFE) (an estimate of the noncellulose carbohydrate) was determined as the difference between the total sample weight and the sum of the moisture, ash, crude protein, crude fiber, and ether extract.

Hydrolysis curves for cysteine and cysteic acid for the five feedstuffs are shown in **Figure 2**. The mean R^2 values for cysteine and cysteic acid across all feedstuffs were 0.94 and 0.98, respectively, ranging from 0.84 for corn meal to 0.97 for soybean meal and meat and bone meal for cysteine and from 0.95 for skim milk powder to 0.99 for canola meal and meat and bone meal for cysteic acid. For both cysteine and cysteic acid, least-squares nonlinear regression provided a close fit to the amino acid yields determined for various hydrolysis intervals.

The hydrolysis (h) and loss (l) rates for methionine, methionine sulfone, cysteine, and cysteic acid were determined for each of the five feedstuffs, and these data are shown in **Table 2**. The hydrolysis rate for methionine ranged from 0.353 for skim milk powder to 0.566 for meat and bone meal (mean = 0.462). For methionine sulfone, the hydrolysis rate was lowest for canola meal (0.372) and highest for skim milk powder (0.458) with a mean of 0.411. The hydrolysis rate for cysteine ranged from 0.198 to 0.607 and was generally much lower than that observed for cysteic acid, which ranged from 0.344 for meat and bone meal to 0.769 for skim milk powder.

The loss rate was less than zero for methionine in skim milk powder and corn meal. For the other feedstuffs, the loss rate for methionine ranged from 0.000028 for meat and bone meal to 0.000264 for canola meal. For methionine sulfone, the loss rate was less than zero for all of the feedstuffs except soybean meal ($l = 0.000419$). For cysteine, the loss rate ranged from 0.003584 for meat and bone meal to 0.009327 to skim milk powder. In contrast, the loss rate for cysteic acid was much lower, ranging from less than zero for canola meal and meat and bone meal to 0.000694 for soybean meal.

Comparison of the 24 h Hydrolysis Values with Least-Squares Nonlinear Regression Estimates. The estimated methionine content determined using either least-squares nonlinear regression ($A_0 \pm B_0$) as either methionine or methionine sulfone for the selected feedstuffs is shown in **Table 3**., along with the methionine content determined as either methionine or methionine sulfone using a single 24 h hydrolysis time. The estimated methionine ($A_0 \pm B_0$) content was within 2.5% of the 24 h hydrolysis value for all of the feedstuffs tested, except soybean meal, for which the 24 h hydrolysis values overestimated the methionine content by 7.8%. In contrast, when

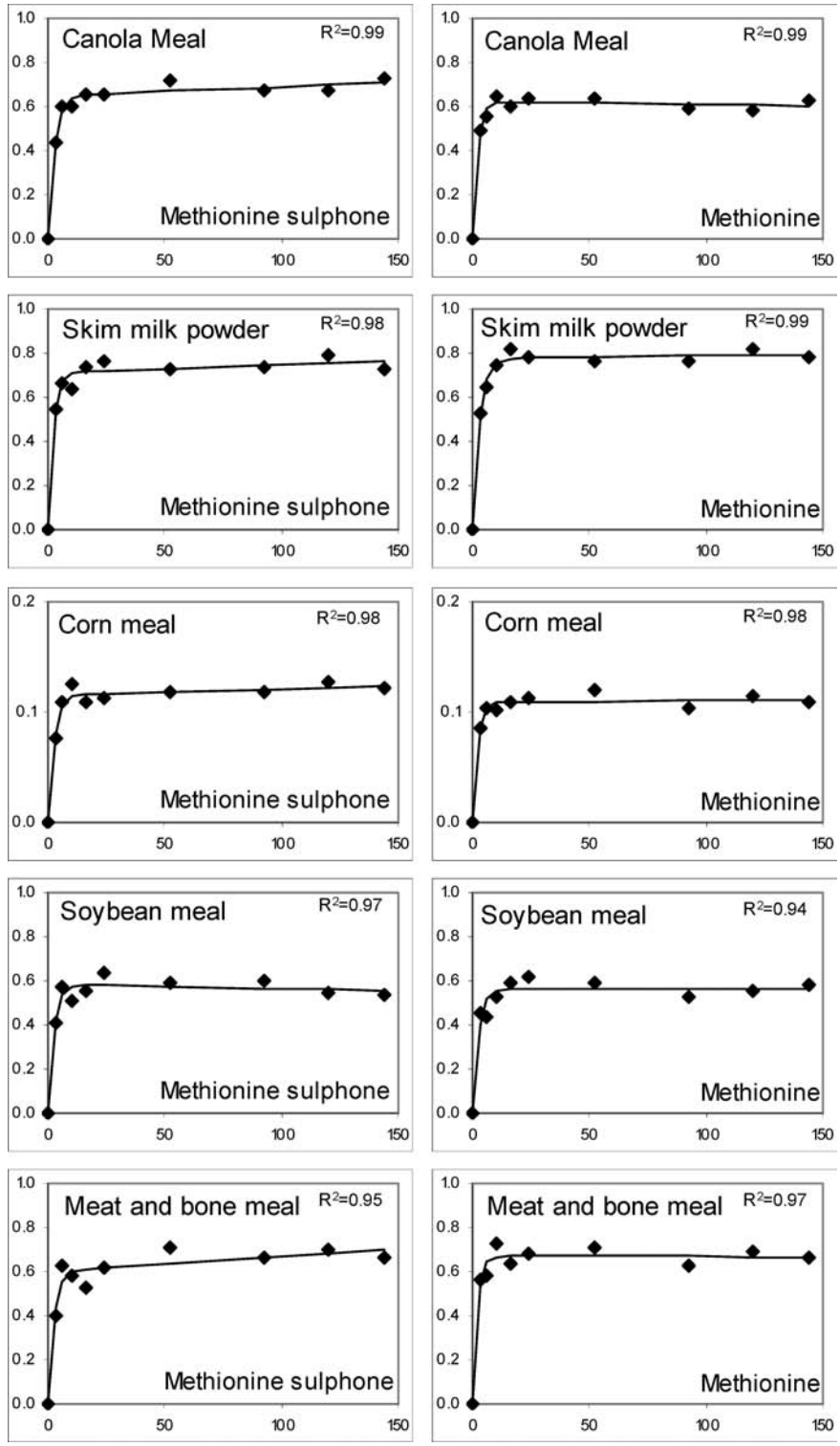

Figure 1. Effect of hydrolysis time (*X*-axis, h) on the yield of methionine and methionine sulfone (*Y*-axis, g/100 g) in five feedstuffs. The mean amino acid yield (duplicate) (♦) is plotted along with the line of best fit predicted from A_0, B_0, h, and I for each data set.

methionine sulfone was examined, for canola meal, corn meal, and meat and bone meal there was a <3% difference between the estimated value ($A_0 \pm B_0$) and the 24 h hydrolysis value. For skim milk powder and soybean meal, the 24 h hydrolysis value overestimated the methionine sulfone (after oxidation) content by 6.9 and 7.0%, respectively, when compared to the methionine sulfone content estimated using least-squares nonlinear regression.

The estimated cysteine content determined using either least-squares nonlinear regression ($A_0 \pm B_0$) as either cysteine or cysteic acid for the selected feedstuffs is also shown in **Table 3**, along with the cysteine content determined as either cysteine or cysteic acid using a single 24 h hydrolysis time. For cysteine, the 24 h hydrolysis value was between 5% (meat and bone meal) and 33% (skim milk powder) lower than the content estimated using least-squares nonlinear regression ($A_0 \pm B_0$). In contrast, the cysteic acid content across all feedstuffs, determined using 24 h hydrolysis, was within 6% of the content estimated using least-squares nonlinear regression ($A_0 \pm B_0$).

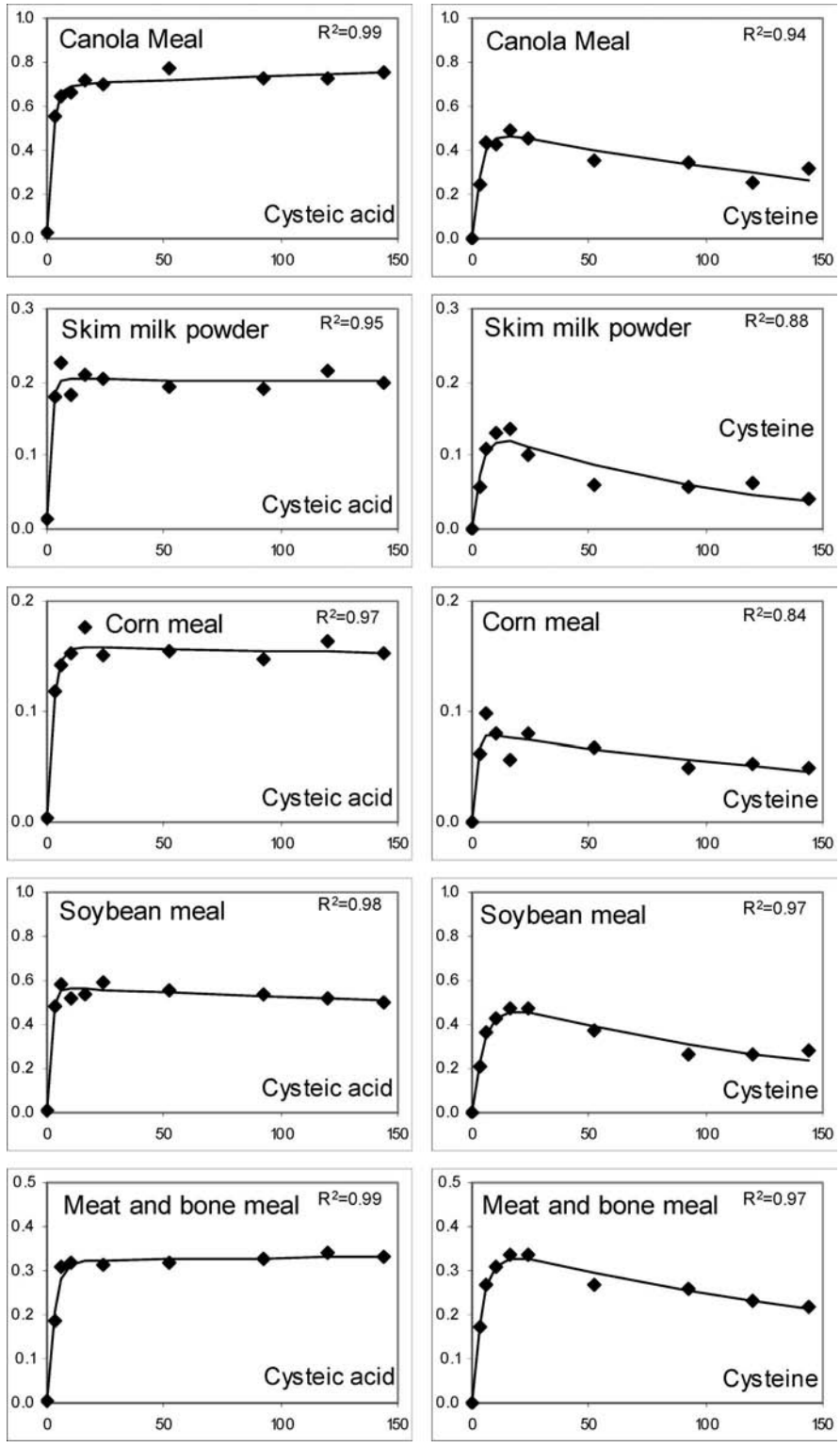

Figure 2. Effect of hydrolysis time (*X*-axis, h) on the yield of cysteine and cysteic acid (*Y*-axis, g/100 g) in five feedstuffs. The mean amino acid yield (duplicate) (♦) is plotted along with the line of best fit predicted from A_0, B_0, h, and I for that data set.

Comparison of the Sulfur Amino Acids Determined either with or without Oxidation prior to Hydrolysis. When the least-squares nonlinear regression estimates of methionine and methionine sulfone were compared, the amount of methionine was within 5% of that determined as methionine sulfone after oxidation with performic acid across the feedstuffs tested. Moreover, the methionine content was slightly lower than the methionine sulfone content for canola meal, corn meal, and soybean meal but was higher for skim milk powder and meat and bone meal. When the 24 h hydrolysis values for methionine and methionine sulfone were compared, both values were within 5% of each other for all of the feedstuffs tested with the exception of meat and bone meal (9.1%).

For meat and bone meal, the least-squares nonlinear regression estimate of cysteine determined using acid hydrolysis without prior oxidation was 9% higher than the estimated cysteic acid content (least-squares nonlinear regression) determined using acid hydrolysis with prior oxidation. In contrast, for the

Table 2. Estimated Hydrolysis Rate[a] (*h*) (Proportion of Bound Amino Acid Hydrolyzed per Hour) and Loss Rate[a] (*l*) (Proportion of Free Amino Acid Hydrolyzed per Hour) for Methionine, Methionine Sulfone, Cysteine, and Cysteic Acid in Five Feedstuffs during Acid Hydrolysis

	hydrolysis rate		loss rate		hydrolysis rate		loss rate	
	methionine[b]	methionine sulfone[c]	methionine[b]	methionine sulfone[c]	cysteine[d]	cysteic acid[e]	cysteine[d]	cysteic acid[e]
canola meal	0.490	0.372	0.000264	–0.000611	0.280	0.482	0.004375	–0.000542
skim milk powder	0.353	0.458	–0.000153	–0.000465	0.259	0.769	0.009327	0.000120
corn meal	0.497	0.392	–0.000141	–0.000454	0.607	0.417	0.004047	0.000270
soybean meal	0.405	0.414	0.000068	0.000419	0.198	0.657	0.005657	0.000694
meat and bone meal	0.566	0.419	0.000028	–0.001055	0.241	0.344	0.003584	–0.000256
mean	0.462	0.411	0.000013	–0.000433	0.317	0.534	0.005398	0.000057

[a] Determined using least-squares nonlinear regression of amino acid concentration plotted against multiple hydrolysis times. [b] Determined as methionine after acid hydrolysis. [c] Determined as methionine sulfone after performic acid oxidation followed by acid hydrolysis. [d] Determined as cysteine after acid hydrolysis. [e] Determined as cysteic acid after performic acid oxidation followed by acid hydrolysis.

Table 3. Estimated Sulfur Amino Acid Composition (Grams per 100 g of Air-Dry Powder) Determined Using Nonlinear Least-Squares Regression after Multiple Interval Hydrolyses[a] Compared with 24 h Hydrolysis Values[b] for Five Selected Feedstuffs

	methionine[c]		methionine sulfone[d]		cysteine[e]		cysteic acid[f]	
	$A_0 \pm B_0$	24 h hydrolysis	$A_0 \pm B_0$	24 h hydrolysis	$A_0 \pm B_0$	24 h hydrolysis	$A_0 \pm B_0$	24 h hydrolysis
canola meal	0.625	0.634	0.649	0.655	0.525	0.458	0.698	0.696
skim milk powder	0.776	0.778	0.713	0.766	0.149	0.100	0.204	0.203
corn meal	0.109	0.112	0.116	0.113	0.086	0.079	0.159	0.150
soybean meal	0.568	0.616	0.588	0.632	0.518	0.471	0.565	0.595
meat and bone meal	0.670	0.678	0.603	0.621	0.354	0.336	0.321	0.312

[a] The total (protein bound, $A_0 \pm$ free, B_0) amino acids determined using least-squares nonlinear regression of amino acid concentration after hydrolysis for a range of hydrolysis times. [b] Values are the mean of duplicate determinations. [c] Determined as methionine after acid hydrolysis. [d] Determined as methionine sulfone after performic acid oxidation followed by acid hydrolysis. [e] Determined as cysteine after acid hydrolysis. [f] Determined as cysteic acid after performic acid oxidation followed by acid hydrolysis.

other feedstuffs, the cysteine content was between 8% (soybean meal) and 46% (corn meal) lower than the cysteine content determined as cysteic acid.

DISCUSSION

The hydrolysis rates estimated using nonlinear least-squares regression for methionine (acid hydrolysis without prior oxidation) across all five feedstuffs found in this study were similar to those reported for two goat milk formulas, a whole goat milk powder (*4*), and lysozyme (*9*). Furthermore, the hydrolysis rates estimated for methionine sulfone (oxidation and acid hydrolysis) across all feedstuffs determined in this study were also similar to those reported for lysozyme (*8*), human milk (*6*), two goat milk infant formulas, and a goat milk powder (*4*). It is interesting to note that the hydrolysis rates for methionine found in this study were generally similar to or higher than those observed for methionine sulfone. Rutherfurd et al. (*4*) also found the hydrolysis rates for methionine to be slightly higher than those observed for methionine sulfone.

For cysteine, the hydrolysis rates were low and the loss rates were very high across all five feedstuffs when determined without prior oxidation. Cysteine appeared to be quite unstable during acid hydrolysis even when the samples had been degassed thoroughly. This was not unexpected for complex feed materials. In contrast, the loss rate for cysteic acid was very low and was $^1/_{50}$ of the loss rate observed for cysteine. Loss rates for cysteic acid of 0.0088 for human milk (*6*), 0.0044 for lysozyme (*8*), 0.0006 and 0.001 for two goat milk infant formulas (*4*), and 0.001 for a whole goat milk powder (*4*) have also been reported. These rates were between 1 and 35 times higher when compared to the loss rates for cysteic acid observed for the five feedstuffs examined in this study.

It is interesting to note that there was a strong positive correlation ($R^2 = 0.97$) between fat content and hydrolysis rate for methionine (no oxidation) and a strong negative correlation ($R^2 = 0.90$) between fat content and the hydrolysis rate for cysteic acid. The reasons for this are not obvious, and further investigation into this finding may be warranted. There was no correlation between the loss rate for either methionine, methionine sulfone, cysteine, or cysteic acid and the composition of any of the nutrients tested using proximate analysis. Clearly, for the samples tested in this study the presence of carbohydrate or fat had no effect on the stability of methionine, cysteine, or their oxidized derivatives during acid hydrolysis.

The loss rate for methionine was low across the five feedstuffs (<0.00027). In contrast, Robel and Crane (*9*) reported a loss rate of 0.0008 for methionine in lysozyme, whereas Rutherfurd et al. (*4*) reported a loss rate of 0.0007 for goat milk infant formula. These loss rates were 3 times greater than found in this study. For two other goat milk based foods, lower loss rates (0.0003) for methionine were reported (*4*). The loss rate for methionine sulfone for the five feedstuffs examined in this study was generally lower than or similar to that reported for human milk (*6*) and three goat milk based foods (*4*). In contrast, the loss rate for methionine sulfone in lysozyme was >10-fold higher than that observed for the five feedstuffs examined in this study.

The loss rate for methionine, determined with acid hydrolysis alone, was similar to that for methionine sulfone, determined using performic acid oxidation followed by acid hydrolysis, for all of the feedstuffs examined. This would suggest that both methionine and methionine sulfone are stable under the hydrolysis conditions used in this study, where care was taken to remove oxygen from the samples and the 6 M HCl solution. Furthermore, because methionine may be accurately determined using acid hydrolysis without prior oxidation, this may negate the necessity to determine methionine using a separate method (performic acid oxidation followed by hydrolysis) from that used to determine the "acid stable" amino acids (acid hydrolysis).

Comparison of the 24 h Hydrolysis Values with Least-Squares Nonlinear Regression Estimates. For most of the feedstuffs tested, the methionine contents determined using least-squares nonlinear regression were similar (<2.1%) to those obtained using 24 h hydrolysis. The exception was for soybean meal, for which the estimated value ($A_0 \pm B_0$) was 8% lower than the 24 h hydrolysis value. In this case, the estimate based on a least-squares nonlinear regression model would be more accurate than the 24 h hydrolysis value because the model estimate is based on the regression of 10 separate data points, whereas the 24 h hydrolysis value is based on only one data point. The mean difference between the least-squares nonlinear regression estimate and the 24 h hydrolysis value across all feedstuffs was 2.9%. For methionine sulfone, the mean difference across all feedstuffs between the two methods was 3.6%, and again the greatest difference between the model estimate and the 24 h hydrolysis value was observed for soybean meal. Overall, it would appear that for both methionine determined with acid hydrolysis alone and methionine sulfone determined using performic acid oxidation followed by acid hydrolysis, 24 h hydrolysis is reasonably accurate for determining the methionine content of a range of feedstuffs when compared to the least-squares nonlinear regression estimate. Given that the loss rates of methionine and methionine sulfone were very low, this result is not surprising.

For cysteic acid, the 24 h hydrolysis values were similar (<6%) to the least-squares nonlinear regression estimates for all of the feedstuffs, whereas, for cysteine, the agreement between the least-squares nonlinear regression estimates and the 24 h hydrolysis values was poor with an average difference of 17% across all feedstuffs.

Comparison of the Sulfur Amino Acids Determined either with or without Oxidation prior to Hydrolysis. The methionine content estimated using least-squares nonlinear regression was within 5% of the estimated methionine sulfone content across all of the feedstuffs tested. For canola meal, corn meal, and soybean meal the methionine content was slightly lower than the methionine sulfone content, whereas for skim milk powder and meat and bone meal, the methionine content was slightly higher than the methionine sulfone content. Again, acid hydrolysis in an evacuated sealed tube appears to be accurate for determining the methionine content of most of the feedstuffs used in this study.

The cysteine content determined after acid hydrolysis and using least-squares nonlinear regression was higher than that determined as cysteic acid after oxidation and hydrolysis for meat and bone meal. Furthermore, for soybean meal, the cysteine and cysteic acid values were similar. However, for the remaining protein sources, the cysteine content when determined using acid hydrolysis without prior oxidation considerably underestimated the cysteine content when determined as cysteic acid.

Cysteine is generally not stable during acid hydrolysis. The least-squares nonlinear regression method can be used to predict the content of both acid stable and acid labile amino acids. Therefore, this method was used in this study to estimate cysteine content after acid hydrolysis without prior oxidation. However, given the large discrepancy in the estimate of cysteine content determined as either cysteine or cysteic acid, it would appear that the least-squares nonlinear regression method may not be suitable for the determination of cysteine using acid hydrolysis without prior oxidation. In contrast and on the basis of this study, cysteic acid does appear to be stable during acid hydrolysis even in feedstuffs with high fat and carbohydrate contents. Therefore, it would appear that the 24 h hydrolysis method used in this study (sealed tube method) can accurately be applied to feedstuffs for determining cysteine content when cysteine is oxidized to cysteic acid. However, it would appear that least-squares nonlinear regression may not be suitable for quantifying cysteine in unoxidized samples.

Methionine can be determined as either methionine without oxidation to methionine sulfone prior to acid hydrolysis or as methionine sulfone. Furthermore, the 24 h hydrolysis values for either methionine or methionine sulfone would appear to be accurate for most of the feedstuffs used in this study. Methionine would appear to be quite stable during acid hydrolysis with the appropriate degassing, at least for the five chemically complex feed materials examined in this study, permitting the analysis of methionine along with the "acid stable" amino acids using a single acid hydrolysis method without the need to oxidize methionine first.

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