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Amino Acid Analysis of Feedstuffs: Determination of Methionine and Cystine after Oxidation with Performic Acid and Hydrolysis[1]

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Whereas the cystine content of feedstuffs is usually analyzed after oxidation of cystine to cysteic acid and subsequent hydrolysis of the protein, methionine is analyzed in many cases from the unoxidized hydrolysate. With corn, wheat, soybean meal, and feather meal, such values for methionine are 11–15% lower compared to the recovery obtained from oxidized hydrolysates. Only meat and bone meal and poultry byproduct meal showed smaller losses for methionine after direct hydrolysis. For complete oxidation an amount of 5 mL of performic acid/10 mg of nitrogen is sufficient. From multiple analyses of broiler feeds the repeatability (relative standard deviation) was determined to be 2.1% for methionine and 2.7% for cystine.

When analyzing the amino acid content of feedstuffs after protein hydrolysis, methionine and cystine present special problems due to their instability during hydrolysis conditions. Values obtained after direct hydrolysis of feedstuffs would lead to an underestimation of the requirement for methionine and cystine in animal nutrition. The oxidation of cystine to cysteic acid with performic acid before hydrolyzing the protein is widely accepted in order to determine the cystine content of feedstuffs (Moore, 1963; de Belsunce and Pion, 1963; Heese et al., 1971; Davies and Thomas, 1973; Wall and Gehrke, 1976; Mason et al., 1979). Concerning methionine, however, a number of laboratories use the direct hydrolysis of feedstuffs under nitrogen atmosphere (Jamalian and Pellett, 1967; Mondino and Bongiovanni, 1970; Hackler, 1971; Wall and Gehrke, 1976; Moodie et al., 1982). Attempts have been made to reduce losses of methionine during hydrolysis by increasing the temperature and reducing the time of hydrolysis (Kaiser et al., 1974; Wall and Gehrke, 1976; Lucas and Sotelo, 1982; Philipps, 1983). Other reports, however, indicate that for maximum recoveries methionine needs to be oxidized with performic acid to its sulfone (Jennings and Lewis, 1969; Slump, 1969; Heese et al., 1971; Beck et al., 1978; Mason et al., 1979, 1980b; Sarwar et al., 1983). Part of the discrepancies described in the literature may be due to the fact that the analysis of amino acids in food and feed presents problems that are not encountered when analyzing pure proteins (Blackburn, 1978). Various collaborative studies have shown that methionine and cystine usually give the highest variation of the results, compared with other amino acids (Porter et al., 1968; Knipfel et al., 1971; Kwolek and Cavins, 1971; Cavins et al., 1972; Williams et al., 1979; Chavana et al., 1980; Kreienbring, 1981; Sarwar et al., 1983). Considering the economic importance of amino acid levels for feed formulation, a standardization of the method to analyze amino acids in feedstuffs is necessary. Since neither in the United States nor in the European Community (Andersen et al., 1984) such a standard method has been established, further work is necessary.

The objectives of the present investigation were to determine the reliability of the partially contradicting results from literature on the necessity to protect methionine by oxidation before hydrolysis, to investigate oxidation conditions, and to check the accuracy of a standard oxidation procedure for the simultaneous determination of methionine and cystine, as well as other amino acids in feedstuffs and complete feeds.

MATERIALS AND METHODS

Chemicals and sources were as follows: formic acid, 88% (Merck, prepared from 98 to 100%, p.a.); hydrogen peroxide, 30%, p.a. (Merck); phenol, p.a. (Merck); hydrobromic acid, minimum 47%, p.a. (Merck); hydrochloric acid, 6 N, prepared by dilution (1:1) of 37%, p.a. (Merck); trisodium citrate·$2H_2O$, p.a. (Merck); sodium chloride, p.a. (Merck); thiodiglycol, ca. 98% (Serva); Brij 35 solution, prepared from Brij 35, practical quality (Serva), (Brij–water, 1:3); 1-propanol, p.a. (Merck); amino acids for calibration (Serva, Degussa).

Amino acid analysis conditions were as follows: pH Meter CG 803 (Schott); Biotronik Amino Acid Analyzer LC 2000, resin type Durrum DC-6A; resin height 27 cm; column diameter 0.6 cm; flow rate of buffer 35 mL/h; flow rate of ninhydrin 20 mL/h. The buffer composition and chromatographic program were as follows: A, sodium citrate, 0.173 N, pH 3.35, run time 15 min; B, sodium citrate, 0.2 N, pH 4.35, run time 40 min; C, sodium citrate, 0.2 N, sodium chloride, 0.5 N, pH 6.41, run time 33 min; D, sodium citrate, 0.2 N, sodium chloride, 1.4 N, pH 7.16, run time 29 min. Each buffer contained 0.1% of Brij 35–detergent solution and 0.01% of phenol. Buffer A also contained 0.5% of thiodiglycol as antioxidant and 2% of 1-propanol (Atkin and Ferdinand, 1970).

Other conditions were as follows: temperature T_1 = 48.5 °C (48 min) and T_2 = 60 °C (69 min); regeneration with 0.4 N NaOH solution, 10 min at 60 °C; equilibration with

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[1]Dedicated to Dr. Wilhelm A. Schuler in honor of his 70th birthday.

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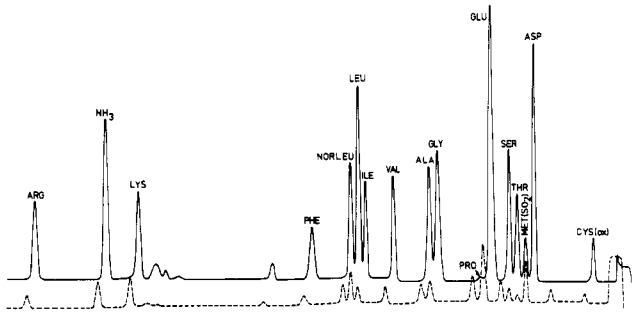

Figure 1. Amino acid chromatogram of a soybean meal hydrolysate after oxidative pretreatment [Cys(ox) = cysteic acid; Met(SO_2) = methionine sulfone].

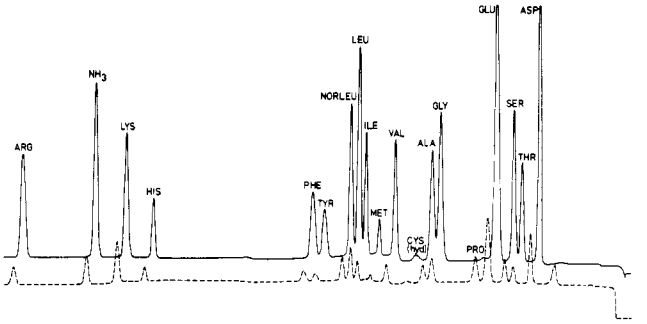

Figure 2. Amino acid chromatogram of a soybean meal hydrolysate without oxidative pretreatment.

buffer A 15 min at 60 °C and 47 min at 48.5 °C; dilution buffer sodium citrate, 0.2 N, pH 2.20, containing 0.1% of Brij 35–detergent solution, 2% of thiodigylcol, and 0.01% of phenol.

Preparation of Norleucine Standard Solution. A total of 200 mg of norleucine was weighed into a 1-L volumetric flask. The norleucine was dissolved by adding 100 mL of 1 N HCl. Then distilled water was added to the mark. Twenty milliliter of this solution contained ca. 30 μmol of norleucine.

Preparation of Performic Acid. A total of 0.5 mL of hydrogen peroxide, 30%, and 4.5 mL of formic acid, 88%, were mixed together with 25 mg of phenol (Mason et al., 1980a) in a glass tube. The mixture was left at room temperature for 30 min, cooled to 0 °C for 15 min in an ice bath, and then used immediately for oxidation of the sample.

Oxidation with Performic Acid. The finely ground sample was weighed into a 100-mL single-necked flask. The sample weight corresponded to 10 mg of nitrogen (62.5 mg of crude protein). The flask was put in an ice bath, and a small magnetic stirring rod and the performic acid were added. After being stirred approximately 15 min, the mixture was left overnight (approximately 16 h), cooled in an ice bath, which is kept in a Dewar flask, and put in the refrigerator.

The excess of performic acid was reduced by adding 0.7 mL of hydrobromic acid, 48% (Moore, 1963). After the mixture was stirred approximately $^1/_2$ h, the rest of bromine and the formic acid were evaporated by using a rotation evaporator with water-jet vacuum (15–20 mmHg) at 50–60 °C.

Acid Hydrolysis Procedure. The finely ground sample corresponding to 10 mg of N was weighed into a 100-mL double-necked flask, the sample weight corresponding to 10 mg of nitrogen, and mixed with 50 mg of phenol and 50 mL of 6 N hydrochloric acid. The solution was mixed for a short period by a magnetic stirring rod, the magnetic stirring rod was removed, a boiling stone was added, a continuous flow of nitrogen was made by bubbling through the solution by a separate inlet tube, and the solution was refluxed for 24 h. The nitrogen was kept bubbling for the complete hydrolysis procedure.

In the case of oxidation pretreatment, the oxidized sample, after evaporation of residual bromine and formic acid, was left in the single-necked flask and treated in the same way, without using a nitrogen atmosphere.

Preparation of the Hydrolysate for Chromatography. After cooling, the hydrolysate was filtered through a glass frit, type G4, into a 1-L single-necked flask. Exactly 20 mL of the norleucine standard solution was added. Then the solution was concentrated almost to dryness with a rotation evaporator, 15–20 mmHg, 50–60 °C. Twenty milliliters of distilled water was added and evaporated off again, this being repeated 5 times. The residue was mixed thoroughly with approximately 50 mL of dilution buffer (pH 2.20).

Table I. Analysis of the Methionine Content of Yellow Corn, from Different Provenances[a]

	provenance	sample size	methionine, % a	methionine, % b	difference, %, (ox value = 100%)
$\bar{x}$	United States	6	0.202	0.173	−14.0
s_x			0.015	0.015	4.6
$\bar{x}$	France	14	0.202	0.166	−18.0
s_x			0.022	0.019	3.6
$\bar{x}$	Austria	20	0.215	0.185	−13.8
s_x			0.012	0.010	3.7

[a] a = after oxidative pretreatment; b = after direct hydrolysis without oxidative pretreatment; mean value and standard deviation are given. The differences are significant at a level of p = 0.001.

For chromatography 4–5 mL of the prepared filtered hydrolysate was diluted with 50 mL of dilution buffer (pH 2.20). The pH was adjusted accurately to 2.20 with HCl or NaOH. A total of 250 μL of this solution was used for the analysis. Typical chromatograms are given in Figures 1 and 2.

RESULTS AND DISCUSSION

Recovery of Methionine. The methionine content of yellow corn, obtained from the United States, France, and Austria (Schmidtborn et al., 1981), and of soybean meal, c.p. 44% and 48%, obtained from the United States was analyzed after oxidative pretreatment with performic acid and hyrolysis with 6 N hydrochloric acid, as well as after direct hydrolysis under a nitrogen atmosphere. Each sample was analyzed by both methods, and the differences in recovery were calculated as [100 − Met(hyd)]/Met(ox) × 100 (%).

The individual results on each sample of corn are given in Table I. After direct hydrolysis, without oxidative pretreatment, the loss in the recovery of methionine for corn was 14.0 ± 4.6% (corn, United States), 18.0 ± 3.6% (corn, France), and 13.8 ± 3.7% (corn, Austria), compared to the recovery after preoxidation of methionine to methionine sulfone. Calculating all data from the analyzes of corn (n = 40 samples), the average loss in methionine recovery was 15.3 ± 3.8% after direct hydrolysis, compared to the recovery after oxidation.

The methionine content of soybean meal has been analyzed in the same way, comparing the relative recoveries by the two procedures on each sample. Eight samples of soybean meal, c.p. 44%, and 18 samples of soybean meal, c.p. 48%, all obtained from the United States, have been evaluated, as shown in Table II.

With soybean meal, 44%, a loss of 13.6 ± 4.0% has been observed after direct hydrolysis. With soybean meal, 48%,

Table II. Analysis of the Methionine Content of Soybean Meal (United States) from Different Provenances[a]

	content of protein	sample size	methionine a	methionine b	difference, % (ox value = 100%)
$\bar{x}$	44	8	0.685	0.592	−13.6
s_x			0.030	0.018	4.0
$\bar{x}$	48	18	0.740	0.644	−13.3
s_x			0.041	0.036	5.9

[a] a = after oxidative pretreatment; b = after direct hydrolysis without oxidative pretreatment; (mean value and standard deviation are given). The differences are significant at a level of p = 0.001.

Table III. Analysis of the Methionine Content of Different Feedstuffs after Oxidation with Different Amounts of Performic Acid and after Direct Hydrolysis (Each Feedstuff with Two Samples)

	methionine, %			
	oxidation with performic acid			
sample	5 mL	15 mL	100 mL	direct hydrolysis
corn				
1	0.195	0.201	0.217	0.186
2	0.210	0.197	0.217	0.187
av	0.203	0.199	0.217	0.187
soybean meal				
1	0.745	0.689	0.740	0.681
2	0.756		0.756	0.672
av	0.751	0.689	0.748	0.677
corn–soybean meal mixture				
1	0.481	0.483	0.488	0.438
2	0.490	0.489	0.487	0.423
av	0.486	0.486	0.488	0.431
wheat				
1	0.226	0.254	0.245	0.207
2	0.249	0.258	0.222	0.211
av	0.238	0.256	0.234	0.209
fish meal				
1	1.834	1.905	1.894	1.711
2	1.841	1.883	1.843	1.754
av	1.838	1.894	1.869	1.733
wheat–fish meal mixture				
1	1.037	1.134	1.029	1.032
2	1.007	1.077	1.063	1.003
av	1.023	1.106	1.046	1.018

the respective loss has been 13.3 ± 5.9%. The average loss in methionine recovery from the parallel evaluation of all soybean meal samples (n = 26) has been 13.4 ± 5.3%, when no oxidative pretreatment has been performed.

The loss in methionine recovery after direct hydrolysis, as observed with corn and soybean meal, is also observed with corn–soybean meal mixtures (−11.3%), as indicated in Table III from a double determination.

The results of other feedstuffs being analyzed for both methionine and cystine after direct hydrolysis as well as after oxidation and hydrolysis are given in Table IV. In this assay with corn (n = 14 samples), a loss of methionine of 11.7 ± 5.6% was observed, which is slightly lower than the above mentioned loss of 15.3 ± 3.8%.

The methionine content of wheat—another feedstuff rich in carbohydrates—was 22.2 ± 4.5% lower when carrying out the hyrolysis directly (n = 12 samples analyzed). The recovery of methionine from soybean meal, c.p. 47.9 (10 samples analyzed), was 11.2 ± 15.4% lower after direct hydrolysis. This result is close to the above-mentioned loss of 13.4%, observed in the previous assay (Table II), however, with a higher variation.

Meat and bone meal, a feedstuff with a c.p. content (49.5%) comparable to that of soybean meal, did not show a significant difference in the methionine content, when comparing recovery after direct hydrolysis to that after oxidation and hydrolysis. The difference was only −1.9 ± 9.9%. This result, based on the analysis of 18 samples, indicates that with meat and bone meal either direct hydrolysis in fact gives maximum recovery, methionine not being degraded, or the oxidation procedure does not give the full value, since the oxidation is not complete. An incomplete oxidation of methionine in meat and bone meal might be explained by the relatively high chloride content, which also is oxidized by performic acid under formation of chlorine (Hirs, 1967). This interpretation already was raised by Slump (1969) but needs further clarification. Also, poultry byproduct meal, c.p. 64.1 ± 8.0%, gave only slight losses in methionine recovery when hydrolyzing without oxidation, the difference being −3.7 ± 6.1% (n = 15 samples). However, with feathermeal, a product rich in crude protein (82.1%), again higher methionine recoveries were observed with the oxidation procedure, the losses after direct hydrolysis being 12.0 ± 10.0% (n = 22 samples analyzed).

The reason for the losses of methionine during hydrolysis might be partial oxidation (Schram et al., 1953) despite the presence of a nitrogen atmosphere, and the presence of carbohydrates (Smith et al., 1965; Hoppe, 1971; Robel, 1973). The results presented in Tables I–IV indicate that for maximum recovery of methionine from feedstuffs an oxidation to the sulfone is necessary, before hydrolyzing the protein. This is essential for carbohydrate-rich feedstuffs, such as corn, wheat, and soybean meal, but also for protein-rich feedstuffs such as feathermeal.

Such a conclusion is supported by the findings of Mason et al. (1979), who also observed that oxidation gave higher

Table IV. Parallel Analysis of the Methionine and Cystine Content of Feedstuffs[a]

	sample	sample size	content of protein, %	methionine a	methionine b	methionine c	cystine a	cystine b	cystine c
$\bar{x}$	corn	14	8.8	0.224	0.198	−11.7	0.213	0.077	−64.1
s_x			0.8	0.016	0.010	5.6	0.015	0.010	4.0
$\bar{x}$	wheat (winter)	12	12.2	0.214	0.170	−22.2	0.292	0.169	−39.6
s_x			1.8	0.030	0.018	4.5	0.025	0.039	8.6
$\bar{x}$	soybean meal	10	47.9	0.716	0.636	−11.2	0.769	0.411	−46.6
s_x			1.8	0.048	0.121	15.4	0.041	0.148	19.4
$\bar{x}$	meat and bone meal	18	49.5	0.656	0.643	−1.9	0.808	0.396	−55.6
s_x			3.7	0.090	0.103	9.9	0.384	0.290	13.8
$\bar{x}$	poultry byproduct	15	64.1	0.813	0.787	−3.7	1.700	1.242	−31.6
s_x			8.0	0.245	0.246	6.1	1.056	0.884	18.2
$\bar{x}$	feather meal	22	82.1	0.659	0.562	−12.0	4.524	3.582	−20.3
s_x			5.7	0.112	0.081	10.0	0.570	0.740	14.7

[a] a = after oxidative pretreatment; b = after direct hydrolysis without oxidative pretreatment; c = percent difference (oxidative pretreatment = 100%). All differences are significant at a level of p = 0.01, except methionine in soybean meal and poultry byproduct (p = 0.05) and meat and bone meal (not significant).

recoveries for methionine, the average loss being 13.4% when comparing direct hydrolysis (reflux with 25 mL of 6 N HCl) of feedstuffs such as barley, maize, grass meal, soybean meal, fish meal, and casein with the cold oxidation procedure. Similarly, an average loss of 14% for methionine, hydrolyzed without oxidation, was observed with different complete feeds for poultry, calf, and pigs (Mason et al., 1980b).

Recovery of Cystine. With regard to cystine an accurate evaluation after direct hydrolysis is difficult due to the rather small and broad peak (see chromatogram, Figure 2), which in some cases can hardly be evaluated. Furthermore, the shape of the cystine peak often is unsymmetrical, due to racemization during acid hydrolysis (Friedman and Noma, 1975). However, since some laboratories still analyze cystine without oxidation, the losses that are observed with such a procedure have been analyzed, the results being given in Table IV.

High losses in cystine recovery are observed with carbohydrate-rich feedstuffs, such as corn (loss of 64.1 ± 4.0%, n = 14 samples), wheat (39.6 ± 8.6%, n = 12), and soybean meal (46.6 ± 19.4%, n = 10). But also feedstuffs that are poor in carbohydrate content gave losses of 55 ± 13.8% (meat and bone meal, n = 18), 31.6 ± 18.2% (poultry byproduct meal, n = 15), or at least 20.3 ± 14.7% (feather meal, n = 22).

In the case of feather meal and also of poultry byproduct meal, special attention should be given to the content of lanthionine, which also is a sulfur-containing amino acid. It is formed from cystine during the steam heating of animal byproducts and is similar to cystine in structure but characterized by a thioether bonding instead of the disulfide bonding. Since cysteic acid is formed by oxidation not only of cystine but also of lanthionine (Lipton et al., 1977), the lanthionine content needs to be analyzed separately after direct hydrolysis (Friedman and Noma, 1975) and the respective amount of cysteic acid needs to be taken into account when calculating the cystine content from the analyzed cysteic acid value (Spindler and Tanner, 1981). The losses observed for cystine during direct hydrolysis are in agreement with previous results (Wall and Gehrke, 1976; Mason et al., 1979, 1980a).

Oxidation Conditions. To assure that oxidation of a feedstuff sample corresponding to 10 mg of nitrogen, i.e., 62.5 mg of crude protein, with 5 mL of performic acid will be sufficient to oxidize methionine and cystine completely in routine feedstuff analysis, a comparison was made between oxidation of 10 mg of N samples with 5 and 15 mL of performic acid, as described above (refluxing with 50 mL of 6 N HCl), and between oxidation of a 32 mg of N sample with 100 mL of performic acid (using 12 mL of hyrobromic acid for reduction and refluxing with 800 mL of 6 N HCl), as described by Heese et al. (1971) and Beck et al. (1978). Samples of corn, soybean meal, wheat, fish meal, and defined mixtures of corn–soybean meal (50:50) and wheat–fish meal (50:50) have been analyzed by double determination including a comparison with direct hydrolysis. The results concerning the methionine content are given in Table III, the results concerning cystine are given in Table V. From the data given in Tables III and V, it can be concluded that a complete oxidation of samples, corresponding to 10 mg of nitrogen, can be achieved with 5 mL of performic acid, since the average recovery of methionine was increased only by 1.7% with 15 mL and only by 1.5% with 100 mL of performic acid. The average recovery of cystine was increased by 3.3% with 15 mL, and by 3.1% with 100 mL of performic acid, compared to the oxidation with 5 mL.

Table V. Analysis of the Cystine Content of Different Feedstuffs after Oxidation with Different Amounts of Performic Acid (Each Feedstuff with Two Samples)

	cystine, %, oxidation with performic acid		
sample	5 mL	15 mL	100 mL
corn			
1	0.187	0.197	0.204
2	0.204	0.209	0.207
av	0.196	0.203	0.206
soybean meal			
1	0.753	0.755	0.738
2	0.739		0.750
av	0.746	0.755	0.744
corn–soybean meal mixture			
1	0.461	0.497	0.498
2	0.501	0.490	0.495
av	0.481	0.494	0.497
wheat			
1	0.315	0.333	0.330
2	0.325	0.334	0.299
av	0.320	0.334	0.315
fish meal			
1	0.480	0.523	0.526
2	0.493	0.477	0.508
av	0.487	0.500	0.517
wheat–fish meal mixture			
1	0.395	0.429	0.417
2	0.395	0.401	0.424
av	0.395	0.415	0.421

This is in agreement with the results from Mason et al. (1980b), who analyzed 10 mg of N samples of different feedstuffs using 5 or 10 mL of performic acid and observed an average increase in recovery of only 0.6% of methionine and 1.2% of cystine, when using more performic acid.

Added methionine, which can easily be analyzed separately by extraction and ion-exchange chromatography (Schmidtborn and Spindler, 1982), cystine, and lysine were shown to be recovered at a rate of 95–102% after oxidation pretreatment, compared to the unsupplemented feed (Mason et al., 1980a).

Reduction of Excess Performic Acid. Excess performic acid should be removed in order to avoid overoxidation of amino acids during the subsequent hydrolysis (Moore, 1963; Hirs, 1967). As an alternative to hydrobromic acid (Moore, 1963) used in the present procedure, one might also use sodium pyrosulfite (Weidner and Eggum, 1966; Mason et al., 1979), which is oxidized by excess performic acid to sodium sulfate. The use of sodium pyrosulfite has the advantage that no bromine is evolved and that no halogenation peaks of tyrosine and histidine are formed (Sanger and Thompson, 1963), which disturb the chromatography of basic amino acids, especially of lysine (Degussa, 1978; see Figure 1). With sodium pyrosulfite, however, the hydrolysate needs to be neutralized instead of being concentrated by evaporation, in order to avoid formation of *O*-sulfates of serine and threonine (Murray and Milstein, 1967; Spindler and Stadler, 1984).

Repeatability. The accuracy of the determination of methionine and cystine after oxidation pretreatment, defined as repeatability of the complete oxidation, hydrolysis, and ion-exchange chromatography procedure on a sample within the same laboratory, was evaluated by double determination of commercial broiler feed samples, obtained from Georgia and Alabama.

The data for the methionine and cystine content are given in Table VI. These data show that the standard deviation of the methionine determination of a broiler feed with the oxidation method, calculated as

$$s = [(x_1 - x_2)^2/(2n)]^{1/2}$$

Table VI. Repeatability of the Analysis of the Methionine and Cystine Content of Commercial Broiler Feeds after Oxidation Pretreatment (n = 23, Each Sample Analyzed by Double Determination)

	methionine, %				cysteine, %		
	x_1	x_2	$\Delta(x_1 - x_2)$		x_1	x_2	$\Delta(x_1 - x_2)$
$\bar{x}$	0.539	0.531	0.011	$\bar{x}$	0.419	0.407	0.011

Table VII. Analysis of the Content (Percent) of Two Amino Acids of Some Feedstuffs and a Defined Mixture (50:50) of Two Feedstuffs (c = Corn; s = Soybean Meal; w = Wheat; f = Fish Meal)

amino acid	starch-rich feedstuff	protein-rich feedstuff	mixture		% diff (found = 100)
			calcd	found	
methionine	0.203 (c)	0.751 (s)	0.477	0.486	1.9
cystine	0.196 (c)	0.746 (s)	0.471	0.481	2.1
methionine	0.238 (w)	1.838 (f)	1.038	1.023	-1.4
cystine	0.320 (w)	0.487 (f)	0.404	0.395	-2.2

is 0.011. In connection with the mean methionine content of 0.535, the coefficient of variation is 0.011/0.535 × 100 = 2.1%. The standard deviation of the cystine determination of a broiler feed is 0.011, as shown in the same table. The coefficient of variation of the cystine determination of a broiler feed is calculated in the same manner to be 2.7%. These results are in good agreement with earlier findings, using a similar method (Heese et al., 1971; Degussa, 1980; Mason et al., 1980b).

Mixture Linearity Response. A different approach to check the accuracy of the oxidation method was made by analyzing one sample of corn, wheat, soybean meal, and fish meal, each by double determination, and then analyzing a defined mixture of corn–soybean meal (50:50), as shown in Tables III and V. The comparison of the value analyzed for the mixture with that calculated from the analyzed values of the individual feedstuffs gives information on the accuracy of the response to the change in feed composition.

The results concerning the methionine and cystine content of corn, soybean meal, and the corn–soybean mixture, as obtained with the routine oxidation procedure (5 mL of performic acid), and the corresponding results of the wheat–fish meal mixture are shown in Table VII. The deviation of the methionine content from the expected values was +1.9% (corn–soybean meal) and −1.4% (wheat–fish meal). The deviation of the cystine content from the expected values was +2.1% (corn–soybean meal) and −2.2% (wheat–fish meal), which is within the variation of analysis. These data are in good agreement with a similar study by Bech-Andersen et al. (1979) and clearly demonstrate the accuracy of the routine oxidation procedure to determine methionine and cystine in feedstuffs and complete feeds.

The same method is also applicable for the simultaneous determination of lysine, if halogenation products of tyrosine and histidine are separated from the lysine peak in chromatography (Degussa, 1978), as well as of threonine and other amino acids (Beck et al., 1978; Mason et al., 1979). This method therefore offers the possibility to determine the main essential amino acids from one single hydrolysate.

Analytical Control and Nutrient Requirement Data. Since complete feeds used in poultry and hog nutrition to a large extent consist of corn and soybean meal, the content of methionine would be found too low if the analysis would be done without oxidation of methionine to its sulfone. With regard to the analytical control of complete feeds used in feeding trials to establish nutrient requirement data, these figures will be at least 10% too low, if direct hydrolysis is performed without oxidative pretreatment. Thus, there is a close relationship between nutrient requirement data, the nutrient content of commercial feeds, and the analytical method of control used to establish them.

If microbiological methods are used to control the methionine and cystine content of feeds (Fry and Stadelman, 1960; Wünsche, 1971; Robel, 1980), discrepancies in analytical results and therefore also in amino acids requirement data might become even greater due to losses of methionine and cystine during hydrolysis and the different method of detection.

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Registry No. Cystine, 56-89-3; methionine, 63-68-3; performic acid, 107-32-4; starch, 9005-25-8.

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Covalent Attachment of Poly(L-methionine) to Food Proteins for Nutritional and Functional Improvement

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Methionine, the first limiting essential amino acid of many plant proteins, has been covalently attached to casein and β-lactoglobulin by using the *N*-carboxyanhydride method. Introduction of as much amino acid as 30% of protein weight led to the formation of polymethionine chains linked to the lysyl residues through isopeptide bonds. Under polymerization conditions at pH 6.5, attachment efficiency was close to 80% and about 40% of the lysyl residues were acylated. In contrast, successive addition of reagent at pH 10.2 reduced the efficiency to 25–30% while all the ε-amino groups were modified. Important changes in solubility properties were observed with polymethionylcasein and to a lesser extent with methionylcasein. "In vitro" digestion studies, performed with bovine α-chymotrypsin and trypsin at pH 8.2 and 38 °C, resulted in a significant decrease of hydrolysis of casein derivatives that was correlated with chain length and distribution of methionine polymers as well as conformational changes as indicated by fluorescence studies. Polymerization of amino acids onto food proteins may be a valuable procedure for improving their nutritional and functional properties.

Supplementation of foods and feeds with free essential amino acids is attractive since it represents a simple and effective means to improve protein quality. It has nevertheless some well-known disadvantages such as deteriorative reactions resulting in the modification of sensory properties and in decreased biological utilization of the supplemented free amino acids as compared to peptides (Puigserver et al., 1982). During the past few years, a number of authors have investigated the feasibility of covalently attaching limiting amino acids to food and feed proteins in order to improve their nutritional and/or functional properties.

Papain-catalyzed incorporation of methionine into soy proteins through a single-step process has been successfully used to increase their content in this limiting amino acid (Yamashita et al., 1979b; Arai et al., 1979). The enzymatic method has the obvious advantage of incorporating exclusively L-methionine with a yield as high as 80% when the racemic amino acid ethyl ester is used (Yamashita et al., 1979a). Chemical methods already available for covalently attaching amino acids to proteins include those used to modify carboxyl and amino groups. The carbodiimide condensation reaction, which requires carboxyl group activation, has recently been used to covalently link methionine, tryptophan, or lysine to soy protein isolates and wheat gluten (Li-Chan et al., 1979; Voutsinas and Nakai, 1979). Although peptide and isopeptide bonds are formed in this case, it has nevertheless been shown that α-carboxyl groups reacted more rapidly than β- or γ-carboxyl groups and that the resulting covalently linked amino acids were readily released by a pepsin–pancreatin mixture. When the ε-amino group of lysyl residues was modified by use of *N*-hydroxysuccinimide esters (Puigserver et al., 1978, 1979b) or *N*-carboxyanhydrides of limiting amino acids (Bjarnason-Baumann et al., 1977), isopeptide bonds only are formed. However, it should be mentioned that no polypeptidyl derivatives were obtained under experimental conditions of the latter study. Amino acids covalently attached to the ε-amino group of lysyl residues were as readily available as the free forms (Puigserver et al., 1979a). Intestinal membrane-bound aminopeptidase appears to be the unique enzymatic activity able to hydrolyze the isopeptide bond effectively.

Since the first methionyl residue attached to each lysine of casein by an isopeptide bond was biologically available in the rat, it may therefore be expected that subsequent attachment of additional residues through a polymerization reaction will also lead to methionine available in vivo. Protein amino groups will act as initiators of the polymerization reaction of *N*-carboxymethionine anhydride yielding polymethionine chains of different length. By use

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