Near-Infrared Reflectance Spectroscopy Enables the Fast and Accurate Prediction of the Essential Amino Acid Contents in Soy, Rapeseed Meal, Sunflower Meal, Peas, Fishmeal, Meat Meal Products, and Poultry Meal

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Near-infrared reflectance spectroscopy (NIRS) calibrations were developed to enable the accurate and fast prediction of the total contents of methionine, cystine, lysine, threonine, tryptophan, and other essential amino acids, protein, and moisture in the most important protein-rich feed ingredients. More than 1000 samples of global origin collected over four years were analyzed on amino acids following the official methods of the United States and the European Union. Detailed data and graphics are given to characterize the obtained calibration equations. NIRS was validated with independent samples for soy and meat meal products and compared to the amino acid predictions using linear crude protein regressions. With a few exceptions, validation showed that 85−98% of the amino acid variance in the samples could be explained using NIRS. NIRS predictions compared to reference results agree excellently, with relative mean deviations below 5%. Especially for meat and poultry meals, NIRS can predict amino acids much better than crude protein regressions. By enabling the amino acid analysis of many samples to be completed in a short time, NIRS can improve the accuracy of feed formulation and thus the quality and production costs of mixed feeds.

Keywords: *NIRS calibration; NIRS validation; amino acids; crude protein; linear crude protein regression; feed ingredient evaluation; methionine; cystine; lysine; threonine; tryptophan*

INTRODUCTION

Accurate knowledge of the amino acid contents of feedstuffs is crucial for a successful feed compounding because a lack of methionine, lysine, threonine, and other essential amino acids can limit the nutritional efficiency of the feed. Chromatographic amino acid analysis requires oxidation and hydrolysis of the protein followed by ion exchange chromatography. This wet chemical procedure is quite complicated and labor intensive and needs a minimum of 3 days of processing time. Near-infrared reflectance spectroscopy (NIRS) combined with chemometric algorithms for calibration has been used for more than 30 years for the analysis of feed. It is mainly applied to determine moisture, crude protein, and other crude nutrients and combines easy and safe measurements with only a few minutes of analysis time. In 1978 Rubenthaler and Bruinsma (*1*) first reported a sucessful NIRS calibration of an amino acid, actually the lysine content in cereals. In the following years, some further publications of NIRS prediction of amino acids in feedstuffs followed. The results of Bodin et al. (*2*), van Kempen et al. (*3*−*5*), Pazdernik et al. (*6*), Shenk (*7*), Michalski and Mroczyk (*8*), NIRSystems (*9*), and Williams et al. (*10*) dealing with amino acid calibrations for the feedstuffs reported herein will be compared with our data later. Dyer and Feng (*11*) stated in 1997 that NIRS has become a major tool for feedstuff evaluation. They concluded that besides proximate analysis also energy contents and amino acids can be predicted accurately and that this technique will improve feed formulation and quality management in the feed industry tremendously.

For many years, our laboratory has been doing a worldwide analytical service for the feed industry. Thus, many samples of the feed raw materials arrive continuously from our customers and are analyzed chromatographically for amino acids. Therefore, we concluded that we are in an optimum position to develop NIRS calibrations for amino acids. The purpose of this study is to evaluate whether NIRS calibrations of good accuracy can be obtained for protein-rich feedstuffs. It is also our intent to validate NIRS predictions with independent samples against the reference results and amino acid predictions based on linear correlations to the crude protein as described in the amino acid composition tables of Degussa (*12*) and older versions of this data collection.

MATERIALS AND METHODS

Samples. Samples were ground with a Retsch ultracentrifugal mill, using a 0.5 mm sieve, analyzed by chemical and chromatographic methods and scanned by NIRS. Since 1996 ∼50 g portions of the ground samples were filled in tight 50 mL polyethylene bottles with screw caps and stored in a freezer to enable the repetition of chemical analysis and NIRS measurement for subsequent NIRS calibration work. All samples of plant origin were of feed grade quality: soybeans (*Soya hispida*) were either fat extracted or full fat, rapeseed meals (*Brassica napus oleifera*, *B. campestris*), and sunflower meals (*Helianthus anuus*) were fat extracted, and peas (*Pisum sativum*) as harvested. The "meat meal products" used were

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either byproducts of the meat production without or with bones (meat meal or meat and bone meal, respectively) or obtained by processing of complete animal carcasses (meat meal, tankage). The three types of meat meal products were difficult to differentiate and therefore grouped together. The poultry byproduct meals are side products of the chicken meat production and are an important ingredient for integrated broiler producers. Sometimes a large amount of feathers containing high cystine levels is added to these products. To exclude such untypical and less valuable poultry meals, a maximum cystine content of 1.3% was used for the sample selection. During production, meat meal products and poultry byproduct meals are heated, coarse-ground, and dried to >90% dry matter to obtain a safe and stable feed ingredient. The fishmeals were byproducts of the fish meat processing or obtained from complete fish. All were dried and coarse-ground.

Chemical and Chromatographic Methods. The nitrogen content of the samples was determined by combustion using a LECO FP-428 nitrogen analyzer (Dumas method). Two hundred milligrams of the sample was weighed accurately in tin foil and processed according to AOAC Method 990.03 (*13*). Pure EDTA obtained by LECO Corp., St. Joseph, MI, with a guaranteed nitrogen content of 9.57 ± 0.04% was used for the calibration. In a double determination, the two assays must not differ by >1% relatively; otherwise, the average of four determinations was used. Crude protein was obtained using the conversion factor of 6.25.

Dry matter (moisture) was determined by accurately weighing 2 g of the sample in a tared glass, drying it in a ventilated oven for 4 h at 103 °C [VDLUFA Method 3.1 (*14*)], cooling it afterward in a desiccator, and weighing it back.

All amino acids except tryptophan were analyzed by weighing in a sample amount containing ~10 mg of nitrogen and adding 5 mL of performic acid for oxidation of methionine to methionine sulfone and of cystine to cysteic acid during 16 h in an ice bath. After the performic acid had been destroyed by the addition of sodium metabisulfite, 25 mL of 6 mol/L hydrochloric acid was added and the protein was hydrolyzed for 24 h at 110 °C in a closed 50 mL glass bottle with a screw cap. Norleucine was added as internal standard, and the hydrolysate was diluted with sodium citrate buffer and adjusted to a pH of 2.20 by the addition of sodium hydroxide. The amino acids were separated in a cation exchanger resin (polystyrene sulfonate, Na^+ form) and were postcolumn reacted with ninhydrin following a photometric detection at 570 nm. The amino acid analyzer model Biochrom 20 of Biochrom Ltd., Cambridge, U.K., was used, their ninhydrin reagent was purchased, and elution buffers and program were used as recommended by the manufacturer. Llames and Fontaine (*15*) have proposed this analysis procedure to the AOAC, and it was adopted as Official Method 994.12. Our procedure for amino acid analysis (see also ref *16*) also agrees with the Official European Method of amino acid analysis in feed (*17*).

The tryptophan content of the samples was analyzed after alkaline hydrolysis with barium hydroxide for 20 h at 110 °C in an autoclave with a steam atmosphere to exclude oxygen. α-Methyltryptophan was added as internal standard, and the hydrolysate was adjusted to a pH of 3.0 by the addition of phosporic acid and hydrochloric acid, filtered, diluted with 30% methanol, and injected to a C18 reversed phase HPLC column for separation. A very specific fluorescence detection was applied using an excitation wavelength of 280 nm and an emission wavelength of 356 nm. The procedure agrees with the Official European Method (*18*), the development and performance of which were reported by Fontaine et al. (*19*). Details of the analytical procedure were also published by Degussa AG (*16*).

NIR Spectroscopy: Instrumentation. A NIRSystems Composite Monochromator 5000 with spinning sample module and reflectance detector with autogain function was used. WinISI II routine and calibration software for PC (Foss Tecator AB, Höganäs, Sweden) was employed.

NIRS: Sample Measurement. All samples that were used for chemical analysis were also scanned by NIRS. Two ring cups were filled with the finely ground material (<0.5 mm sieve) and scanned between 1100 and 2500 nm in 2 nm steps. The reflectance at each wavelength was expressed as log(1/*R*) using a ceramic plate as reference (see ref *20*). The root-mean-square test (RMS) of the WinISI software was used to check for eventual differences caused by errors in sample cup filling or sample inhomogeneity. The two spectra obtained for each sample were compared with RMS based on their first derivatives. If the difference obtained by the RMS option of the program was <200, the mean of the two NIR spectra was calculated and stored in a NIRS file for further calibration work; otherwise, scans were repeated.

NIRS: Calibration Development. A minimum of 30 samples was collected before a first version of the calibration equation was developed. Updates were prepared regularly with approximately one year intervals. A table containing the laboratory codes and the results of reference analyses for dry matter (DM), crude protein (CP), methionine (Met), cystine (Cys), the sum of methionine and cystine (Met + Cys), lysine (Lys), threonine (Thr), tryptophan (Trp), arginine (Arg), isoleucine (Ile), leucine (Leu), and valine (Val) was prepared and imported in WinISI to establish the calibration CAL file. Other (nonessential) amino acid contents were not calibrated, although available, because they are not commonly used for feed formulation. Typically, all available reference data were single analysis with the exception of crude protein, which is obtained from double nitrogen determination. All data in the calibration set and all spectra were checked carefully to detect and eliminate outlier samples, which were caused by poor information about the incoming feedstuffs. No sample reduction was necessary to minimize the calibration data set and the costs for the amino acid analysis because the amino acid contents of all samples were available.

Different calibration algorithms on spectra or derivatives such as multiple linear regression (MLR) with single wavelengths, full spectra principal component regression (PCR), partial least squares regression (PLS), and modified partial least squares regression (MPLS) were tried (see refs *20* and *21*). The following procedure gave the best results:

Spectra were first treated with the scatter correction "SNV and detrend" as recommended by WinISI to reduce particle size effects. The spectra were smoothed over four or five data points (8 or 10 nm), and the first or second derivatives of the calibration spectra were calculated using a gap of four or five data points. The MPLS algorithm that was used reduces the data points in the spectra population to terms not only based on differences in the spectra but also taking into consideration the reference data. Typically, a limit of eight terms was set to avoid regressions on "spectral noise". In the case of 200−300 samples, up to 16 terms were allowed, although the software usually stopped much below this limit using the cross-validation results as criterion. For cross-validation the setting was five or six groups.

The results of the calibration calculation were checked by observing the *t*-ouliers with $t > 2.5$. In the case of *t*-outliers, the samples were taken from the freezer and analyzed again. The new analytical results were used in the following way: if the deviation to the first analytical result was reasonable compared to the precision of the related reference method, that is a relative difference below 1% for DM, below 3% for CP, below 10% for methionine or cystine, and below 6% for all other amino acids, the average result was used for the second calibration run. If the deviation was higher and the second laboratory value was closer to the NIRS prediction, the first laboratory result was removed as an outlier. Sometimes even a third analysis was made to obtain an accurate laboratory result. In any case, laboratory results were not simply removed using the respective option of the WinISI software. Except for tryptophan, which was not analyzed in all samples, the contents of amino acids, crude protein, and dry matter in all samples were used to obtain the calibration equation. In some cases, the sample spectra were also repeated, which sometimes improved the calibaration statistics, especially if the moisture content of the sample had changed.

In case of calibration updates, care was taken to add deviating samples such as *H*-outliers or samples containing

Table 1. NIRS Calibration Statistics of Soybean Meal and Full-Fat Soybeans[a]

	content (%) of variables in the sample population				NIRS performance data				linear regression of amino acids to CP		
					calibration		cross-validation				
variable	mean	CV	min	max	SEC	RSQ	SECV	1-VR	intercept	slope	RSQ_{CP}
dry matter	91.5	1.96	86.9	95.8	0.328	0.97	0.342	0.96			
crude protein	44.4	12.0	31.5	53.1	0.510	0.99	0.545	0.99			
methionine	0.59	12.2	0.44	0.75	0.029	0.84	0.029	0.83	0.048	0.0122	0.81
cystine	0.67	12.0	0.46	0.87	0.033	0.84	0.036	0.81	0.072	0.0135	0.79
Met + Cys	1.26	11.7	0.90	1.59	0.049	0.89	0.053	0.87	0.121	0.0257	0.85
lysine	2.70	11.6	1.99	3.42	0.070	0.95	0.083	0.93	0.190	0.0565	0.92
threonine	1.72	11.6	1.28	2.08	0.039	0.96	0.040	0.96	0.086	0.0367	0.96
tryptophan	0.60	11.3	0.46	0.71	0.014	0.96	0.015	0.95	0.019	0.0129	0.90
arginine	3.29	12.7	2.20	4.09	0.077	0.97	0.092	0.95	−0.121	0.0768	0.95
isoleucine	2.02	12.5	1.41	2.48	0.044	0.97	0.045	0.97	−0.064	0.0469	0.97
leucine	3.39	11.9	2.43	4.08	0.053	0.98	0.059	0.98	0.046	0.0752	0.98
valine	2.11	12.1	1.46	2.50	0.047	0.97	0.048	0.96	0.018	0.0470	0.97

[a] Number of samples, $n = 209$, Trp, $n = 160$. Linear regression of amino acid contents relative to crude protein for the same sample population.

Table 2. NIRS Calibration Statistics of Rapeseed Meal[a]

	content (%) of variables in the sample population				NIRS performance data				linear regression of amino acids to CP		
					calibration		cross-validation				
variable	mean	CV	min	max	SEC	RSQ	SECV	1-VR	intercept	slope	RSQ_{CP}
dry matter	91.1	1.43	88.1	94.3	0.226	0.97	0.297	0.95			
crude protein	36.1	5.79	31.7	40.1	0.327	0.98	0.561	0.93			
methionine	0.70	6.79	0.59	0.82	0.015	0.90	0.020	0.83	0.119	0.0160	0.50
cystine	0.85	11.5	0.70	1.08	0.034	0.88	0.038	0.85	−0.648	0.0415	0.78
Met + Cys	1.55	8.24	1.29	1.82	0.044	0.88	0.050	0.84	−0.524	0.0574	0.88
lysine	1.88	8.23	1.54	2.25	0.028	0.97	0.063	0.84	0.995	0.0245	0.11
threonine	1.53	4.54	1.37	1.71	0.036	0.73	0.042	0.65	0.889	0.0178	0.29
tryptophan	0.51	7.17	0.44	0.57	0.010	0.92	0.018	0.76	−0.098	0.0164	0.85
arginine	2.20	9.43	1.83	2.67	0.047	0.95	0.075	0.87	−0.960	0.0875	0.78
isoleucine	1.41	5.81	1.21	1.60	0.020	0.94	0.028	0.88	0.090	0.0367	0.87
leucine	2.49	5.78	2.13	2.84	0.027	0.96	0.045	0.90	0.286	0.0611	0.79
valine	1.82	5.56	1.59	2.02	0.030	0.91	0.041	0.84	0.271	0.0429	0.79

[a] Number of samples, $n = 64$; Trp, $n = 25$. Linear regression of amino acid contents relative to crude protein for the same sample population.

low or high crude protein levels compared to the calibration population and not only typical samples laying in its center. The three-dimensional score plots of the principal component analysis (PCA) and the WinISI algorithms CENTER and SELECT were used to avoid too many similar samples in the calibration.

RESULTS AND DISCUSSION

Statistics for NIRS Calibration. Tables 1−7 summarize the performance parameters obtained for the calibration equations. Additionally, a linear regression between amino acid contents and crude protein for the same sample populations was also calculated. Slope, intercept, and RSQ_{CP} between amino acids and crude protein are also given in the tables.

The calibration statistics for *soy*, obtained from the reference results of 209 samples, of which 160 were also analyzed for tryptophan content, are shown in Table 1. With the exception of the sulfur-containing amino acids methionine and cystine, the RSQ of calibration and 1-VR of cross-validation are between 0.93 and 0.98 for all amino acids. For crude protein, a level of 0.99 was reached, proof of a perfect correlation. Obviously, the amino acid composition of the protein is very stable for soy because the CV values of the protein and of all amino acid contents are similar between 11.6 and 12.7% and high crude protein contents correlate with high amino acid levels. As a consequence, also the RSQ_{CP} of the linear regression of amino acids to crude protein is high for the sample population and equal to or only slightly below the results of the NIRS calibration. NIRSystems (*9*) have first published NIRS calibration results for soybean meal. The obtained performance data SECV, SEP, and RSQ were clearly inferior to our data. Shenk (*7*) claims that amino acids can be measured by NIRS and compared with the CP regressions as an alternative. He reported that the latter had clearly an inferior RSQ to NIRS and had generally lower correlations than we have obtained. Pazdernik et al. (*6*) have selected 116 soybeans samples of 408 using the CENTER algorithm of ISI. They calibrated with 90 samples using "grams of amino acid per kilogram of crude protein" as parameter and validated with 26 independent samples. The performance of their calibration can thus not be directly compared to ours, but with RSQ and 1-VR values of 0.65−0.89 for the amino acids, investigated herein, they could show that NIRS can even predict the relatively small amino acid variation in the plant protein. They have also compared calibrations obtained by the same set of ground and unground soybeans and clearly concluded that grinding improves the prediction accuracy.

The calibration statistics for *rapeseed meal*, obtained from the reference results of 64 samples, are shown in Table 2. In contrast to soy results the CV for some amino acids in the sample population differed a lot from that of crude protein (5.79%). Threonine with a CV of only 4.54% was clearly below, whereas for arginine (9.43%) and cystine (11.5%) higher variations were found. The small variation of threonine contents leads to a low RSQ

Table 3. NIRS Calibration Statistics of Sunflower Meal[a]

	content (%) of variables in the sample population				NIRS performance data: calibration		NIRS performance data: cross-validation		linear regression of amino acids to CP		
variable	mean	CV	min	max	SEC	RSQ	SECV	1-VR	intercept	slope	RSQ_{CP}
dry matter	92.0	1.54	88.8	96.1	0.161	0.99	0.261	0.97			
crude protein	33.5	13.4	25.0	49.0	0.741	0.97	0.948	0.96			
methionine	0.73	13.9	0.56	0.97	0.029	0.92	0.034	0.89	−0.001	0.0219	0.92
cystine	0.57	14.5	0.44	0.85	0.028	0.89	0.029	0.88	0.169	0.0069	0.32
Met + Cys	1.30	13.9	1.00	1.82	0.045	0.94	0.053	0.92	0.245	0.0126	0.43
lysine	1.16	13.8	0.84	1.54	0.045	0.92	0.058	0.87	0.436	0.0516	0.87
threonine	1.20	12.8	0.92	1.65	0.041	0.93	0.042	0.93	0.249	0.0252	0.78
tryptophan	0.45	13.3	0.34	0.59	0.009	0.98	0.013	0.96	0.058	0.0064	0.73
arginine	2.69	16.0	1.96	4.25	0.084	0.96	0.108	0.94	−1.522	0.1587	0.91
isoleucine	1.34	14.5	1.02	2.03	0.043	0.95	0.049	0.94	0.211	0.0314	0.88
leucine	2.09	13.6	1.56	2.94	0.053	0.97	0.068	0.94	0.275	0.0586	0.92
valine	1.64	13.4	1.25	2.35	0.048	0.95	0.061	0.92	0.171	0.0387	0.92

[a] Number of samples, $n = 83$; Trp, $n = 45$. Linear regression of amino acid contents relative to crude protein for the same sample population.

Table 4. NIRS Calibration Statistics of Field Peas[a]

	content (%) of variables in the sample population				NIRS performance data: calibration		NIRS performance data: cross-validation		linear regression of amino acids to CP		
variable	mean	CV	min	max	SEC	RSQ	SECV	1-VR	intercept	slope	RSQ_{CP}
dry matter	89.6	2.09	86.0	94.9	0.289	0.98	0.309	0.97			
crude protein	21.0	9.12	16.9	26.4	0.362	0.96	0.460	0.94			
methionine	0.20	8.15	0.16	0.23	0.009	0.68	0.010	0.61	0.073	0.0058	0.49
cystine	0.31	7.54	0.25	0.37	0.018	0.43	0.020	0.31	0.169	0.0069	0.32
Met + Cys	0.51	7.23	0.40	0.59	0.025	0.55	0.027	0.48	0.245	0.0126	0.43
lysine	1.52	6.97	1.25	1.74	0.029	0.93	0.043	0.84	0.436	0.0516	0.87
threonine	0.78	7.04	0.68	0.92	0.020	0.87	0.024	0.80	0.249	0.0252	0.78
tryptophan	0.20	6.99	0.16	0.23	0.006	0.79	0.007	0.72	0.058	0.0064	0.73
arginine	1.82	17.6	1.25	2.98	0.041	0.98	0.082	0.93	−1.522	0.1587	0.91
isoleucine	0.87	7.39	0.73	1.01	0.019	0.91	0.026	0.84	0.211	0.0314	0.88
leucine	1.51	7.78	1.23	1.78	0.036	0.91	0.044	0.86	0.275	0.0586	0.92
valine	0.99	7.85	0.82	1.18	0.021	0.92	0.029	0.86	0.171	0.0387	0.92

[a] Number of samples, $n = 68$; Trp, $n = 48$. Linear regression of amino acid contents relative to crude protein for the same sample population.

of 0.73, a situation typical for NIRS calibrations. All other RSQ values for amino acids are between 0.88 and 0.97. For crude protein a level of 0.98 was reached. The fractions of explained variance of cross-validation, 1-VR, agreed well with RSQ of calibration for all variables. The standard errors SEC and SECV are also equally low and prove the good precision of this calibration equation. For rapeseed meal as well, NIRS is clearly superior to the linear CP regression, which shows especially for the important amino acids methionine, lysine, and threonine very poor RSQ_{CP} values. Michalski and Mroczyk (*8*) reported a NIRS amino acid calibration of unground rapeseed using near-infrared transmittance (NIT) and NIR techniques. They have calibrated using "grams of Lys/16 g of nitrogen" and "grams of Met + Cys/16 g of nitrogen" values. With about the same sample number, they have obtained RSQ values of 0.45 for lysine and 0.83 for sulfur amino acids. The calibration was useful for the evaluation of 1100 field samples. There are no other data reported about amino acid calibrations of rapeseed.

The calibration statistics for *sunflower meal*, obtained from the reference results of 83 samples, are shown in Table 3. Like soy, the CV of most amino acid contents is between 12.8 and 14.5, very similar to that of crude protein; arginine varies somewhat more. With 0.89−0.98 and 0.87−0.96 the obtained RSQ and 1-VR values, respectively, for amino acids were very good. The standard errors SEC and SECV correspond very well and are low compared to the mean amino acid contents. On the basis of the obtained RSQ and RSQ_{CP} values, the linear crude protein regressions of the amino acids cystine, Met + Cys, threonine, and tryptophan were clearly inferior to NIRS. There seemed to be more amino acid variation in sunflower than in soy, caused either by genetics or by processing. Obviously, NIRS can correlate the amino acid contents of the sample population not only to the protein bands of the spectrum but also to other available spectral information. No further amino acid calibration results for sunflower are published.

The calibration statistics for *field peas*, obtained from the reference results of 68 samples, are shown in Table 4. With 7−8% the CV of the amino acid contents in the sample population is somewhat below that of crude protein, except for arginine. The obtained RSQ and 1-VR values for amino acids vary a lot, indicating good correlations for lysine, threonine, arginine, isoleucine, leucine, and valine, less for methionine and tryptophan, and poor correlations for cystine and Met + Cys. However, the standard errors SEC and SECV correspond very well to each other and are small. The ratio of the standard deviation of the amino acid in the samples to the corresponding standard error SECV is between 2.3 and 3.9 for the amino acids with good RSQ, but only 1.9 for tryptophan, 1.6 for methionine, 1.4 for Met + Cys, and 1.2 for cystine. Thus, for cystine with the given samples, no usable NIRS calibration could be obtained. It is interesting to observe that the fractions of explained variance RSQ_{CP} for amino acids obtained by the linear CP regressions are very similar to the 1-VR values of the NIRS cross-validations, with the exception

Table 5. NIRS Calibration Statistics of Fish Meal[a]

	content (%) of variables in the sample population				NIRS performance data				linear regression of amino acids to CP		
					calibration		cross-validation				
variable	mean	CV	min	max	SEC	RSQ	SECV	1-VR	intercept	slope	RSQ_{CP}
dry matter	92.2	1.82	87.7	97.0	0.556	0.89	0.627	0.86			
crude protein	64.3	8.84	45.5	78.0	1.545	0.93	1.989	0.88			
methionine	1.73	15.3	1.08	2.25	0.066	0.94	0.076	0.92	−0.842	0.0400	0.73
cystine	0.58	14.0	0.33	0.81	0.048	0.66	0.052	0.60	0.013	0.0089	0.38
Met + Cys	2.32	13.9	1.46	3.06	0.079	0.94	0.091	0.92	−0.828	0.0489	0.74
lysine	4.67	16.3	2.71	6.17	0.155	0.96	0.203	0.93	−2.876	0.1172	0.77
threonine	2.58	13.8	1.54	3.35	0.088	0.94	0.100	0.92	−0.969	0.0551	0.78
tryptophan	0.70	18.5	0.44	0.97	0.026	0.96	0.034	0.93	−0.509	0.0185	0.63
arginine	3.66	13.0	2.31	4.81	0.134	0.92	0.154	0.90	−0.902	0.0709	0.71
isoleucine	2.59	14.1	1.65	3.47	0.083	0.95	0.102	0.92	−0.956	0.0551	0.74
leucine	4.52	13.5	2.81	5.89	0.138	0.95	0.173	0.92	−1.724	0.0971	0.81
valine	3.09	12.7	2.04	3.98	0.108	0.92	0.130	0.89	−0.784	0.0603	0.77

[a] Number of samples, $n = 204$; Trp, $n = 115$. Linear regression of amino acid contents relative to crude protein for the same sample population.

Table 6. NIRS Calibration Statistics of Meat Meal Products[a]

	content (%) of variables in the sample population				NIRS performance data				linear regression of amino acids to CP		
					calibration		cross-validation				
variable	mean	CV	min	max	SEC	RSQ	SECV	1-VR	intercept	slope	RSQ_{CP}
dry matter	95.1	1.36	91.0	98.0	0.292	0.95	0.316	0.94			
crude protein	53.1	11.6	35.3	66.5	0.944	0.98	1.215	0.96			
methionine	0.70	19.4	0.37	1.08	0.034	0.94	0.042	0.91	−0.316	0.019	0.75
cystine	0.51	38.7	0.14	1.22	0.058	0.92	0.068	0.88	−0.533	0.020	0.38
Met + Cys	1.21	23.7	0.51	2.05	0.064	0.95	0.074	0.93	−0.851	0.039	0.69
lysine	2.67	19.3	1.46	3.79	0.102	0.96	0.121	0.95	−1.285	0.075	0.79
threonine	1.70	21.0	0.87	2.37	0.052	0.98	0.069	0.96	−1.172	0.054	0.88
tryptophan	0.34	34.5	0.11	0.57	0.017	0.98	0.019	0.97	−0.449	0.014	0.24
arginine	3.52	9.25	2.59	4.46	0.105	0.90	0.130	0.84	1.190	0.044	0.69
isoleucine	1.46	22.6	0.69	2.21	0.049	0.98	0.063	0.96	−1.026	0.047	0.77
leucine	3.26	23.5	1.59	4.73	0.085	0.99	0.116	0.98	−2.752	0.113	0.84
valine	2.33	23.1	1.12	3.60	0.084	0.98	0.104	0.96	−1.850	0.079	0.82

[a] Number of samples, $n = 333$; Trp, $n = 270$. Linear regression of amino acid contents relative to crude protein for the same sample population.

of methionine, for which NIRS performed better. Williams et al. (*10*) have developed a NIRS calibration for methionine and crude protein based on 60 pea samples. After several tests concerning wavelength selection and spectra treatment, they reported as optimum RSQ values of 0.88 for methionine and 0.94 for protein and standard errors SEP of 0.011 and 0.76, respectively, based on 20 validation samples. Other amino acids were not investigated.

The calibration statistics for *fish meal*, obtained from the reference results of 204 samples, are shown in Table 5. Like all feed ingredients based on the processing of animals, the CVs of the amino acid contents exceed the CV of the protein in the sample population by a factor of 1.5−2.1. The obtained RSQ and 1-VR values for amino acids, 0.92−0.96 and 0.89−0.93, respectively, were very good, with the exception of cystine again. For fish meal, the CP regression performance was clearly inferior to NIRS. The standard errors SEC and SECV agreed well and were low, compared to the means of the variables. Thus, NIRS is able to give accurate amino acid predictions in fish meals and can use much more spectral information than protein absorbances only.

The calibration statistics for *meat meal products*, obtained from the reference results of 333 samples, are shown in Table 6. In this huge sample population, the CVs of the amino acid contents were very different. Arginine varied less than protein, most amino acids had 2 times higher CVs than protein, and cystine and tryptophan even had a 3-fold CV. The very high variation of amino acid compositions of the global sample population leads obviously to an improvement of the NIRS accuracy. With 0.90−0.99 and 0.84−0.98 the RSQ and 1-VR values, respectively, obtained for amino acids were excellent. The SEC and SECV figures corresponded well with each other and were both small compared to the mean of the variable. Thus, highly informative and accurate predictions were obtained by NIRS. For the meat meal products as well, the CP regressions were clearly inferior to the NIRS performance.

The calibration statistics for *poultry byproduct meal*, obtained from the reference results of 59 samples, are shown in Table 7. The CVs of the amino acid contents in this sample population were similar to those of the meat meal products. However, there were also important differences in the mean amino acid contents and several trials led to the decision to separate both types of animal byproducts in two calibrations. Again, the obtained RSQ and 1-VR values for amino acids, 0.89−0.98 and 0.71−0.97, respectively, were very good. There was a big gap for cystine between the RSQ of calibration and 1-VR of cross-validation, and the standard error SECV of cystine was nearly double that of the SEC, whereas for all other amino acids the SEC and SECV figures corresponded well and were small compared to the mean of the variable. Consequently, here as well highly informative and accurate predictions can be obtained by NIRS. The CP regressions for poultry meal were clearly inferior to the NIRS performance, especially for methionine, cystine, lysine, and tryptophan.

Bodin et al. (*2*) and van Kempen et al. (*3*−*5*) have

Table 7. NIRS Calibration Statistics of Poultry Byproduct Meal[a]

	content (%) of variables in the sample population				NIRS performance data				linear regression of amino acids to CP		
					calibration		cross-validation				
variable	mean	CV	min	max	SEC	RSQ	SECV	1-VR	intercept	slope	RSQ_{CP}
dry matter	95.6	1.04	93.5	97.2	0.321	0.90	0.369	0.87			
crude protein	63.2	9.31	49.5	70.3	1.445	0.94	1.663	0.92			
methionine	1.12	18.1	0.65	1.38	0.040	0.96	0.056	0.92	−0.707	0.029	0.71
cystine	0.75	29.0	0.32	1.25	0.071	0.89	0.116	0.71	−0.977	0.027	0.55
Met + Cys	1.87	19.7	1.02	2.46	0.095	0.93	0.118	0.90	−1.683	0.056	0.80
lysine	3.46	14.4	2.37	4.30	0.119	0.94	0.152	0.91	−0.980	0.070	0.69
threonine	2.29	15.0	1.50	2.72	0.070	0.96	0.094	0.92	−1.165	0.055	0.87
tryptophan	0.52	27.1	0.29	0.73	0.021	0.98	0.029	0.97	−0.619	0.019	0.76
arginine	4.22	8.76	3.37	4.73	0.096	0.93	0.126	0.89	0.516	0.059	0.87
isoleucine	2.24	17.3	1.33	2.75	0.053	0.98	0.087	0.95	−1.629	0.061	0.86
leucine	4.14	14.1	2.86	4.92	0.109	0.96	0.142	0.94	−1.765	0.093	0.89
valine	2.86	14.1	2.00	3.59	0.091	0.95	0.131	0.90	−1.185	0.064	0.87

[a] Number of samples, $n = 59$; Trp, $n = 35$. Linear regression of amino acid contents relative to crude protein for the same sample population.

Table 8. NIRS Validation Statistics for Independent Samples of Soybean Meal and Full-Fat Soybeans[a]

	content (%) as analyzed with the reference method			content (%) as analyzed with the NIRS calibration			NIRS performance data of independent validation			
variable	mean	min	max	mean	min	max	SEP	RSQ_{Val}	slope	SEP_{rel}
dry matter	90.1	87.2	94.4	90.2	87.2	94.3	0.379	0.95	0.97	0.42
crude protein	45.1	31.9	52.7	44.9	32.1	52.0	0.663	0.99	0.99	1.47
methionine	0.59	0.44	0.71	0.59	0.43	0.67	0.026	0.84	0.89	4.38
cystine	0.68	0.50	0.83	0.68	0.51	0.80	0.037	0.78	0.89	5.41
Met + Cys	1.28	0.95	1.49	1.27	0.97	1.47	0.051	0.87	0.96	3.99
lysine	2.73	2.02	3.17	2.71	1.92	3.10	0.078	0.94	0.97	2.86
threonine	1.75	1.27	2.00	1.73	1.27	1.98	0.039	0.96	1.00	2.23
tryptophan	0.59	0.47	0.67	0.58	0.45	0.67	0.014	0.97	1.01	2.39
arginine	3.33	2.27	4.01	3.32	2.29	3.99	0.103	0.94	0.96	3.09
isoleucine	2.03	1.47	2.36	2.03	1.42	2.36	0.046	0.97	1.02	2.27
leucine	3.42	2.41	3.93	3.42	2.44	3.95	0.060	0.98	1.01	1.75
valine	2.14	1.51	2.46	2.13	1.53	2.44	0.065	0.94	0.99	3.03

[a] Number of samples, $n = 100$; Trp, $n = 23$.

Table 9. NIRS Validation Statistics for Independent Samples of Meat Meal Products[a]

	content (%) as analyzed with the reference method			content (%) as analyzed with the NIRS calibration			NIRS performance data of independent validation			
variable	mean	min	max	mean	min	max	SEP	RSQ_{Val}	slope	SEP_{rel}
dry matter	95.1	90.9	98.0	95.1	91.7	98.2	0.357	0.94	0.91	0.38
crude protein	54.4	39.7	69.9	54.0	38.3	68.2	1.550	0.95	0.98	2.85
methionine	0.71	0.43	1.15	0.71	0.39	0.99	0.048	0.87	0.88	6.73
cystine	0.54	0.20	1.23	0.53	0.19	1.04	0.078	0.90	0.83	14.4
Met + Cys	1.26	0.69	2.02	1.24	0.69	1.87	0.089	0.93	0.87	7.08
lysine	2.72	1.78	3.65	2.70	1.62	3.79	0.126	0.93	0.99	4.64
threonine	1.77	1.04	2.49	1.75	1.02	2.43	0.066	0.97	0.97	3.73
tryptophan	0.37	0.16	0.58	0.35	0.16	0.52	0.025	0.96	0.91	6.82
arginine	3.59	2.92	4.57	3.55	2.61	4.42	0.125	0.89	0.94	3.48
isoleucine	1.52	0.86	2.41	1.52	0.85	2.28	0.078	0.95	0.96	5.12
leucine	3.38	1.85	4.84	3.34	1.79	4.78	0.127	0.98	0.98	3.76
valine	2.41	1.38	3.40	2.39	1.19	3.34	0.126	0.95	0.98	5.23

[a] Number of samples, $n = 87$; Trp, $n = 29$.

developed NIRS calibrations for digestible and total amino acid contents. Because of the high costs of the determination of digestible amino acids in animal trials, only a limited number of samples could be used. Therefore, a global calibration for animal meals was developed, first based on 66 samples composed of fish, meat and bone, and poultry byproduct meals and more recently on 150 samples, which were grouped together. Even though the work was focused on the digestibility of amino acids, total contents were also calibrated and some performance data were reported. The best obtained accuracies were for lysine (SEP = 0.22, RSQ = 0.95), for methionine (SEP = 0.11, RSQ = 0.92), and for threonine (SEP = 0.15, RSQ = 0.94). Comparing the standard errors SECV (Tables 5−7) and SEP (Table 9) of these amino acids in our global calibrations for the individual ingredients, the standard errors in this study were higher and NIRS predictions less accurate, which was probably caused by the grouping and the smaller sample numbers. The authors also have checked the performance of NIRS calibrations in comparison with the crude protein regressions and also came to the conclusion that NIRS performs much better in predicting the amino acid contents in animal meals.

Validation with Independent Samples. One hundred samples of soybean meal and full-fat soya and 87 samples of meat meal products were selected to check the NIRS calibration equations independently. A tool of the WinISI software, the global *H*-value was used to eliminate samples that were not reflected in the calibra-

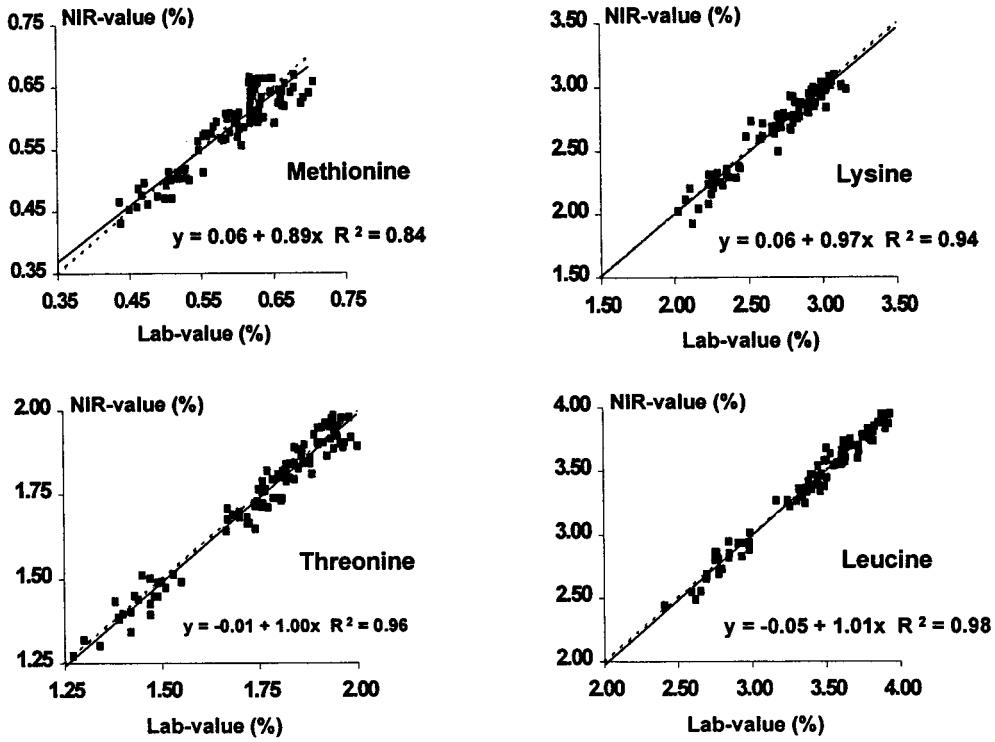


Figure 1. Validation of the NIRS amino acid predictions for soybean meals and full-fat soy: methionine, lysine, threonine, and leucine contents compared to reference analysis (100 samples).

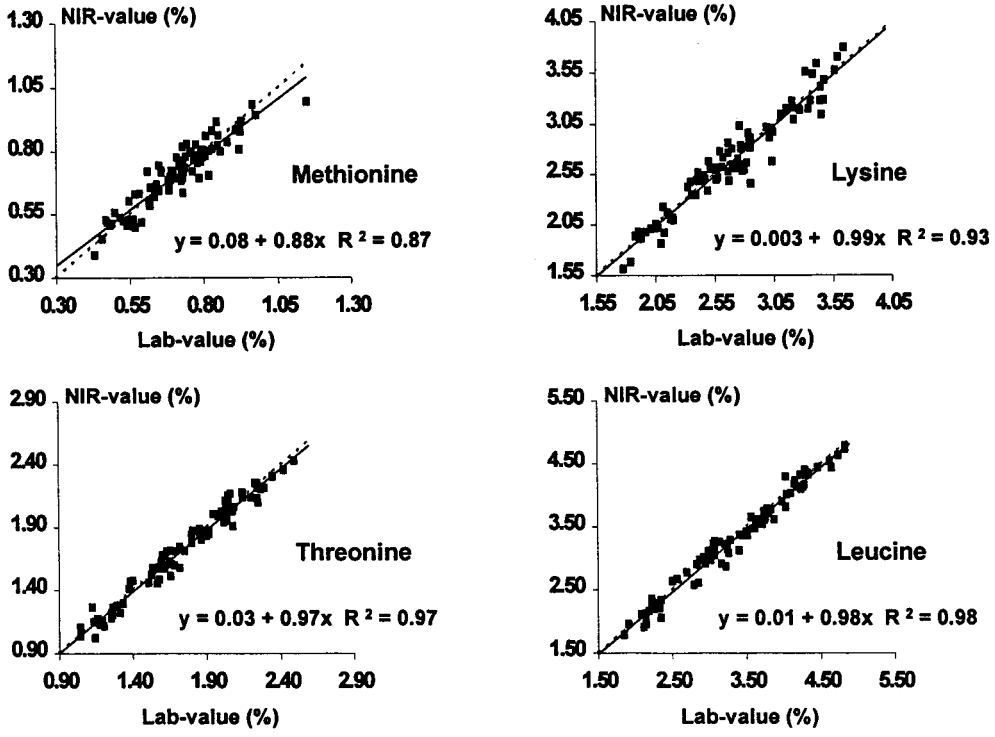
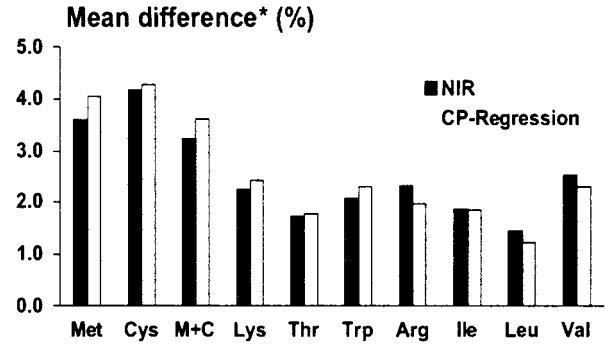


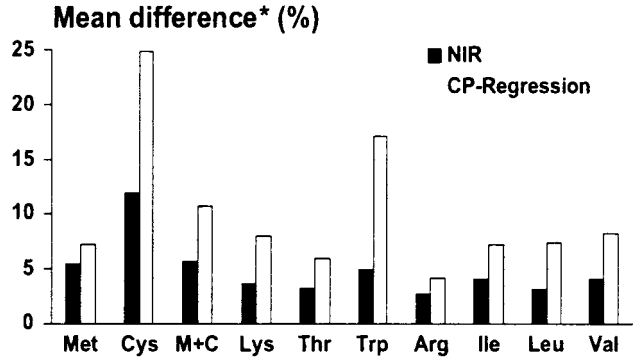
Figure 2. Validation of the NIRS amino acid predictions for meat meal products: methionine, lysine, threonine, and leucine contents compared to reference analysis (87 samples).

tion population, by using 3.0 for maximum limit. This is also necessary when using NIRS equations in daily work. The statistics based on WinISI are summarized in Tables 8 and 9. The mean, maximum, and minimum contents of the variables agreed very well as analyzed with the reference method and with NIRS. The variation of the samples was well depicted by the NIRS predictions. The standard error of prediction SEP and the fraction of explained variation RSQ_{val} were also in good agreement with the related parameters SECV and 1-VR of the cross-validation statistics. It is a normal finding that the standard errors obtained by validation were slightly higher and the RSQ values slightly lower than those parameters of the cross-validation, and therefore the results show that the NIRS equations give robust predictions under practical conditions. Additionally, the slope between laboratory values (x-axis) and NIRS predictions (y-axis) was given. For soybeans, it was very



* Mean of [(Lab$_n$-NIR$_n$)/Lab$_n$*100] and Mean of [(Lab$_n$-CP-Reg$_n$)/Lab$_n$*100]

Figure 3. Mean difference of amino acid contents predicted by NIRS or by the linear CP regression as compared to the reference analysis for 100 independent samples of soybean meal and full-fat soy.



* Mean of [|Lab$_n$-NIR$_n$|/Lab$_n$*100] and Mean of [|Lab$_n$-CP-Reg$_n$|/Lab$_n$*100]

Figure 4. Mean difference of amino acid contents predicted by NIRS or by the linear CP regression as compared to the reference analysis for 87 independent samples of meat meal products.

close to the ideal value of 1 for all variables, with the exception of methionine and cystine. For meat meal products, the slope was between 0.91 and 0.99 for all variables with the exception of the sulfur-containing amino acids. As an indication of the typical relative deviations between laboratory and NIRS, the parameter SEP_{rel} was calculated using the mean of the respective variable. For soybeans, these relative deviations SEP_{rel} were only between 1.75 and 5.41% for the amino acids. For the less homogeneous feed ingredient meat meal products, SEP_{rel} was between 3.48 and 7.08%, except cystine. Results of collaborative trials in chromatographic amino acids analysis (see refs *15* and *22*) show that such deviations were also observed for results of one sample in different laboratories.

In Figure 1 the individual data for the soy validation samples were plotted for methionine, lysine, threonine, and leucine. The illustrations also contain the linear regression equations with slope, intercept, and square of correlation coefficient RSQ_{val}. The gap in the data points was caused by the fact that only fat-extracted soybean meals or full-fat soybeans are used for feed production but not partly defatted materials. Figure 2 shows the results of the individual meat meal validation samples for the same amino acids. It is obvious that the scattering of data points around the ideal curve with the slope = 1 (dotted line) was larger for methionine than for lysine, threonine, and especially leucine, and this agreed with the accuracy parameters in the validation data in Tables 8 and 9.

As a validation of the CP regression equations, we predicted the amino acids based on the analyzed crude protein content and calculated the individual differences to the laboratory value. In Figures 3 and 4, the mean of these differences, averaging the absolute values, is compared with the respective mean of the differences for the NIRS amino acid prediction. For soy the obtained accuracy was very similar. This had to be expected taking the excellent RSQ_{CP} figures of Table 1 into account. A completely different picture was obtained for the meat meal products (Figure 4). The accuracy of the NIRS amino acid predictions was highly superior to the CP regressions. Often the mean difference (prediction error) was twice as high, and this again clearly showed that NIRS calibration derives much more information from the spectra than only the protein bands. If indeed crude protein and amino acids correlate highly as in soy, it is impossible that the NIRS calibrations will perform better than the linear CP regression equations.

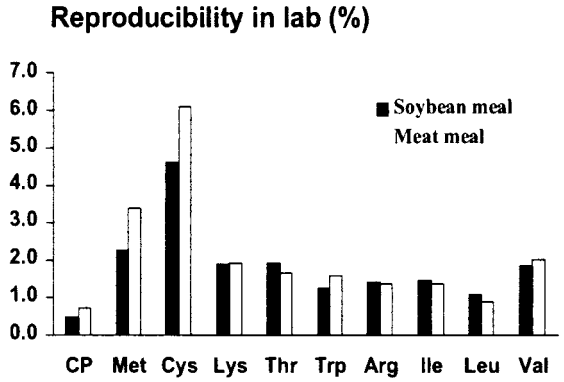


Figure 5. Reproducibility of chromatographic amino acid analysis and crude protein determination in the laboratory. Each sample was analyzed 12 times in 1 month intervals.

Reproducibility of Reference Analysis. It is obvious that the performance data of calibrations and validations for methionine and cystine were inferior to that of other amino acids. The reason for this is shown in Figure 5. A sample of a soybean meal and of a meat meal were analyzed monthly for crude protein and amino acids over a one year period. The samples were stored in a freezer to avoid deterioration. The reproducibility of the analysis (CV) for the different variables was calculated on the basis of the 12 results per sample. For crude protein, it was below 1% and for most amino acids 1−2% with the exceptions of methionine (∼3%) and especially cystine (5−6%). Most of the calibration samples were analyzed only once, and thus the higher error of the reference values for methionine and cystine affected the accuracy of NIRS calibrations. Reasons for this are as follows: (a) the prior oxidation of the sulfur amino acids enlarges the sample preparation error; and (b) due to their low contents and to baseline interferences at the peak position of cysteic acid in the chromatogram, the peak integration is more difficult than for other amino acids. There is no alternative method for the analysis, and therefore the precision of cystine and methionine results could only be improved by repeated analyses. Following the statistical rules the analytical error of each amino acid content could be halved by using the average of four analyses for NIRS calibration. However, due to some laboratory constraints, replicate assays for the large amounts of calibration samples could not be performed.

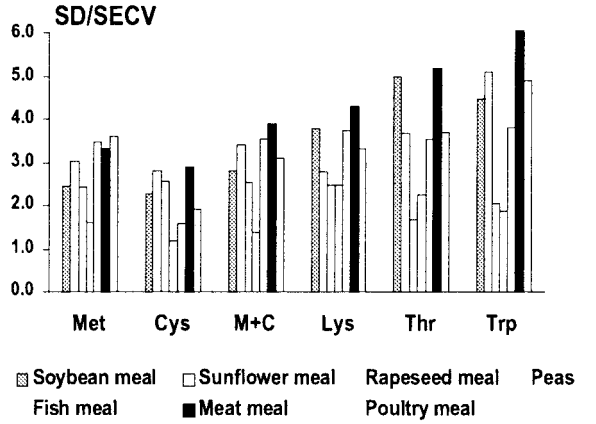


Figure 6. Ratio of the standard deviation (SD) of the amino acid contents in the calibration samples to the standard error SECV of NIRS.

SD/SECV, a Measure for Meaningfulness of NIRS Predictions. A small size of the standard error SECV alone does not clearly reflect the usefulness of a NIRS calibration for feedstuff evaluation. If the ratio of the standard deviation SD of the amino acid in the sample population to the SECV is calculated, a clearer picture appears. If the SD/SECV ratio is high, NIRS predictions enable one to significantly divide a given amount of samples in some subgroups of low, medium, and high contents of the amino acid. Especially for the essential amino acids, this can improve the supplementation rate with crystalline amino acids and enable cost savings. In Figure 6, the ratio SD/SECV is shown for the most important amino acids in animal nutrition for all calibrations. If this ratio exceeds a value of 3, the calibration equation is very meaningful to predict the amino acid, whereas in the case of values below 2, the applicability is limited. We found the latter situation to be true for the sulfur-containing amino acids in peas and for cystine in fishmeal and poultry meal. For these amino acids in soy and rapeseed, SD/SECV values of ~2.5 were obtained. In all other cases, SD/SECV is between 3 and 7 for amino acids in all calibrations. The best results were obtained for leucine, the amino acid with the best reproducibility in the chromatographic assay. The results show that our NIRS calibration equations are mostly able to give very meaningful predictions of the amino acid contents in feedstuff samples.

Applications. For the past two years, our laboratory has used NIRS as a tool for our customer service for amino acid analysis of feedstuff, and currently we have analyzed >3000 samples. The advantage for our customers is not only the short processing time but also the huge series of ingredients that can be analyzed and evaluated in summary tables. This enables a screening of the quality and variation of different sources of feedstuff suppliers for quality improvement and optimum feed formulation. Additionally, we are able to transfer our calibrations to other Foss NIR spectrometers of customers or internal laboratories (hosts). This is accomplished after an accurate standardization of these client instruments by use of sealed check cells, where spectra are compared and corrected to our laboratory instrument (master). The WinISI software offers practical solutions for this purpose. Presently our calibrations can be used in 15 laboratories, and two international collaborative studies have shown that the transferred equations predict amino acids with good accuracy. This network is growing quickly because the amino acid calibrations directly used in the quality laboratory of the feedmill give the highest advantage. NIRS calibrations for grains and brans were also developed. It is intended to publish these data at a later date.

In summary, we conclude that our NIRS calibrations enable meaningful, fast, and accurate predictions of essential amino acids in feedstuff and are recognized as useful by our customers. The calibrations contain high numbers of samples of global origins; consequently, they are very robust and applicable for samples from all continents. It is these calibrations and the continuous updating and enlarging of our equations that make our NIRS work with amino acids unique worldwide.

ABBREVIATIONS USED

NIRS, near-infrared reflectance spectroscopy; SEC, standard error of calibration; RSQ, fraction of explained variance for the calibration samples (square of correlation coefficient *r*); SECV, standard error of cross-validation; 1-VR, fraction of explained variance for cross-validation (square of correlation coefficient *r*); CV, coefficient of variation (relative standard deviation); RSQ_{CP}, fraction of explained variance for linear crude protein regression (square of correlation coefficient *r*); SEP, standard error of prediction for independent validation samples; SEP_{rel}, SEP/(mean of lab values) × 100 (%); slope, slope of regression line between lab values (*x*-axis) and NIRS values (*y*-axis); RSQ_{val}, fraction of explained variance for independat validation samples (square of correlation coefficient *r*); SD, standard deviation of the variable in the sample population.

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