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Amino Acid Contents in Raw Materials Can Be Precisely Analyzed in a Global Network of Near-Infrared Spectrometers: Collaborative Trials Prove the Positive Effects of Instrument Standardization and Repeatability Files

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Four international ring trials for NIRS amino acid analysis of feed raw materials have demonstrated for the first time that the calibrations developed by Degussa allow reliable and precise predictions of essential amino acids in up to 44 NIR spectrometers. Different standardization techniques were compared, and the effectiveness of using spectra of the host instruments as repeatability files was studied. The ultimately achieved reproducibility of 2−3% CV for almost all analyses in the network is considerably better than ring trial results for wet chemical amino acid analysis.

KEYWORDS: Collaborative trial; ring test; standardization of NIR spectrometer; repeatability file; NIRS calibration; check cell; WinISI; master NIRS; host NIRS; precision; reproducibility; amino acids; crude protein; methionine; cystine; lysine; threonine; tryptophan

INTRODUCTION

The formulation of compound feeds based on amino acid requirements is practiced worldwide today because deficiency of methionine, lysine, threonine, and other essential amino acids will adversely affect the growth of farm animals. Wet chemical amino acid analysis is quite complicated, labor intensive, and requires a minimum of 3 days processing time. This is why feed calculation is still based on mean amino acid concentrations in raw materials, for example, by referring to raw materials tables created and regularly updated by Degussa (*1*). However, this approach does not allow for either the variation or the quality of the specific raw material source of an individual feed manufacturer and therefore carries a substantial risk of under- or oversupplementation with synthetic amino acids and hence adverse effects on animal growth and feed costs.

We have developed accurate, global NIR calibrations for all major feed raw materials, whether protein carriers or cereal products, for accurate estimation of essential amino acids (*2*, *3*). As the determination of crude protein, water, crude fat, and other feed components by NIRS has become increasingly accepted in laboratories and feed mills and such instruments are now widely available, we aim to transfer our amino acid calibrations to NIRS instruments in the laboratories of feed manufacturers.

It is well-known that minute changes in the spectra produce different readings, and the process of exchanging calibrations, even between instruments of the same model, is far from straightforward. Light source, ray path, monochromator, reference ceramic, and detectors are always slightly different, leading to variations in the measured wavelength, absorption, and optical density. Fearn (*4*) and Bouveresse and Massart (*5*) published reviews of the different attempts at standardizing NIR spectrometers. This was done either by adapting the spectra of the host instrument to those of the master NIR spectrometer, the instrument on which the calibration was established, or by evening out the differences in the results by means of bias and slope corrections (adaptation of the calibration). For adaptation of spectra, it is important that the samples measured for this purpose in the different NIR spectrometers are truly identical, which is best achieved by sealing them in an airtight check cell, which prevents any alteration of the reflecting sample surface and the moisture content of the material. Shenk and Westerhaus (*6*, *7*), who developed the ISI calibration software that we use, have created algorithms for this purpose, which permit instrument standardization. Westerhaus (*8*) suggested additionally that any remaining, and ultimately unavoidable, small differences in the measurements, which may be due to the effect of room temperature, atmospheric humidity, and filling of the samples, should be evened out by a rep-file, which is factored into the calibration and consists of spectra of the same sample, measured on as many standardized NIR instruments as possible. The improvement is achieved by reducing the effect of the wavelengths with a particularly high analytical variation for the MPLS calibration algorithms (*9*, *10*). Calibrations with and without rep-file, despite identical spectra and reference data, thus differ in the use and weighting of spectral information. This approach is designed to produce calibrations, which, despite slightly poorer calibration statistics (SEC slightly higher, RSQ slightly poorer), have a distinctly improved, reduced variation in the network (SEP improved).

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As there is currently no published information on the transfer of NIR calibrations of amino acids, the purpose of our work was to develop an optimal strategy in several international ring trials for standardization and calibration to allow precise amino acid analysis in a global NIRS network.

MATERIALS AND METHODS

Samples. All raw materials for which amino acid calibrations are available were tested; these are the protein-rich feedingstuffs soybean meal, soya beans, rapeseed meal, sunflower meal, fish meal, meat and bone meal, poultry byproduct meal, feather meal, and peas and the cereal products wheat, barley, corn, triticale, wheat bran/middlings, rice bran, and sorghum. All samples were of feed grade quality. The samples used in the ring trials were ground with a Retsch ZM 100, using a 0.5 mm sieve, and scanned by NIRS to test whether they match the respective calibration. Representative samples were produced with a Retsch sample divider PT 100 with dosing unit DR 100. In the first and fourth collaborative trial representatively divided, unground samples were also sent out, which had to be ground prior to measurement by the participating laboratory. Samples of about 50 g, in tightly sealed containers, were sent out for the ring test.

Check Cells. Foss NIRSystems supply with each machine a tightly sealed check cell containing a soya product, which is used to check the day-to-day performance of the NIR spectrometer comparing the actual results with the last 64 measurements. Additonally, further check cells were produced for the calibrations by evenly filling ring cup cells to a level of about 8 mm with sample material, which, on spectral analysis by the principal component method, should preferably be in the middle of the calibration population (see refs *7*, *9*, and *10*), i.e., have a GH of less than 1. The cell was enclosed up to the edge in a matching piece of about 2 mm thick foam and tightly sealed by gluing on an aluminum lid (leave clamp on for some time) or with a screw top. It is important to ensure a slight pressure in the cell, which reliably prevents any change in the surface below the quartz window, and that the seal is airtight. Before being used for instrument standardization, a new check cell had to be challenged by shaking and the spectrum had to remain stable during multiple measurements over several weeks. A root mean square (RMS) test of below 200 for the first derivative of the spectrum was used as the stability criterion (see below). Storage in a freezer should be avoided as this can cause the feed raw materials to dry out if there is even the slightest leakage, which would result in major changes to the spectrum.

NIR Spectroscopy: Instrumentation. Up to 44 instruments took part in the collaborative studies; only NIRSystems Monochromator 5000 or 6500 instruments with spinning sample module or transport module employing a reflectance detector were involved. For measurements mainly the WinISI II routine and calibration software for PC, in some cases the corresponding DOS program NIRS 2, version 3 or 4, was available in the feedmill (Foss NIR Systems Inc., Silver Spring, MD). We have not tried to transfer the NIRS amino acid calibrations to other brands of NIR spectrometers or even filter instruments.

NIRS: Standardization Procedure. Before applying amino acid calibrations on other NIR instruments, a standardization was performed using the simplified version of the standardization procedure by Shenk, which is contained in the WinISI equipment software (*6*). This involves calculating a correction file from the spectral difference between the master and the host NIR spectrometer, which is then used to adapt all spectra measured by the host instrument. The ideal situation, where both NIR instruments are located in the same room for standardization, thus avoiding effects of temperature and atmospheric humidity on the spectrum of the check cell used, was impossible to achieve in most cases as the locations were a very long distance apart. The instrument had to be warmed up prior to measuring the check cell for standardization by switching on the lamp at least 1 h in advance or better still overnight. As suggested by Shenk, the host NIR spectrometer was first examined by the instrument response test to check lamp and detector, the wavelength accuracy test to check the need for adjusting the monochromator with the aid of the spectrum of the built-in polystyrene film (wavelength error < 0.3 nm), and the NIR repeatability test, adhering to the limit values specified in the Foss NIR System or making corrections where necessary. The check cell supplied by Foss was also measured, and its results had to be within the tolerance range.

At the beginning of our work, we performed standardizations only with the Foss check cell that came with the Degussa master instrument and used the correction file for all calibrations. Later, we produced check cells for the various raw materials ourselves, using a different correction file for each calibration (ingredient.STD). If the host NIR spectrometer featured a transport module for sample measurement, our check cells had to be measured in the small ring cup with an adaptor. The missing rotation in the measurement was simulated by averaging at least three spectra, having rotated the respective ring cups in the adaptor the appropriate number of times. The agreement of the host NIR spectrometer with the master instrument was always validated by measuring a set of samples taken along (three per raw material and calibration, about 50 in total), i.e., the analytical results of the two instruments were compared. The standardization was deemed successful if no conspicuous deviations occurred in the analytical results for all calibrations and if the following requirements were met across all results: (i) crude protein: $>80\%$ of validation samples with relative difference of $<3\%$; $<20\%$ of validation samples with relative difference of $>3\%$; and $<5\%$ of validation samples with relative difference of $>5\%$. (ii) Amino acids: $>80\%$ of validation samples with relative difference of $<5\%$; $<20\%$ of validation samples with relative difference of $>5\%$; and $<5\%$ of validation samples with relative difference of $>10\%$. (iii) Dry matter: $>60\%$ of validation samples with relative difference of $<0.5\%$; $<40\%$ of validation samples with relative difference of $>0.5\%$; and $<10\%$ of validation samples with relative difference of $>1\%$.

These criteria are derived from the accuracy of the corresponding wet chemical reference analysis, which also has a profound effect on the SECV of the NIR calibration (see refs *2* and *3*). These requirements were consistently met and in many cases substantially exceeded.

NIRS: Sample Measurement. The participants of ring tests were asked to proceed in the following way: Two ring or quarter cups were filled with the finely ground material and scanned in standardized mode between 1100 and 2500 nm in 2 nm steps. In the case of the NIRSystems 6500 with an enlarged range from 400 to 2500 nm, only the above-mentioned range was used for the prediction. The reflectance at each wavelength was expressed as $\log(1/R)$ using a ceramic plate as reference (see ref *7*). As for the calibration development, the spectra for prediction were also first treated with the ISI scatter correction program "standard normal variate (SNV) and detrending transformation" as recommended for samples with less than 15% moisture to reduce artifacts in the spectra caused by particle size effects only and not by changes in the constituents. The spectra were then smoothed over four data points (8 nm), and the first or the second derivatives of the calibration spectra were calculated using a gap of four data points, depending on the applied calibration equation. The RMS error test of the ISI software was used to check for important spectral differences caused by imperfect sample cup filling. It calculates for the compared spectra the square root of the mean of the squares of spectral differences at each wavelength. A RMS value of below 200 for the first derivatives of the two scans was the accepted limit; otherwise, measurements had to be repeated.

Applied Calibration Equations and Evaluation of Ring Test Results. The NIRS calibration equations tested in the collaborative studies were the actual versions of amino acid calibrations described in refs *2* and *3*. In these articles, the prediction accuracy of these calibrations (SEC, RSQ, SECV, and 1-VR) and validation data were described. In the first two ring tests, participants were asked to enter their analytical results in a table and to send this to us. Later, only the files of the NIR spectra of the tested samples were sent to us. The predictions were carried out by us, and the spectra were additionally available for the construction of rep-files. In this way, we were also able to check the effect of updated calibrations on the accuracy in the network.

The results of master and host instruments for dry matter, crude protein, and the individual amino acids of each sample were summarized, and the mean and SD were calculated. To enable a better

Table 1. Variation of Results in the First Collaborative Trial for NIRS Amino Acid Analysis in Feed Ingredients

		reproducibility CV (%) of NIRS prediction											
sample	*N*	DM	CP	MET	CYS	M + C	LYS	THR	TRP	ARG	ILE	LEU	VAL
soybean meal A, unground[a]	5	0.32	0.50	2.46	0.63	0.36	1.00	0.99	0.67	1.35	2.81	1.04	1.63
soybean meal B	5	0.66	0.87	3.18	0.96	0.81	1.16	1.40	0.66	1.32	2.65	0.67	1.46
corn A, unground[a]	5	0.81	3.83	7.45	5.52	4.88	21.04	4.38	12.62	14.18	6.21	3.81	4.82
corn B	5	1.15	3.88	7.75	2.43	3.69	15.95	4.18	8.07	10.75	4.86	2.72	4.57
barley A, unground[a]	5	2.44	6.89	8.36	9.28	8.14	8.59	9.52	15.82	4.36	9.98	8.99	10.04
wheat A, unground[a]	5	1.72	6.45	5.04	6.06	5.15	3.63	5.69	n.d.	6.68	7.45	6.33	6.54
fish meal A, unground[a]	5	0.43	2.73	6.13	1.86	4.23	4.15	2.91	4.93	3.89	4.59	3.57	4.97
fish meal B	5	0.22	2.46	4.95	1.47	4.21	3.83	2.97	5.55	3.61	3.97	3.40	4.52
rapeseed meal A	5	0.31	1.45	3.34	2.37	2.30	3.54	1.27	1.85	1.49	1.76	2.14	1.24
meat and bone meal A	5	0.36	2.08	10.13	n.d.	12.37	7.42	4.86	28.65	4.91	6.54	2.85	6.47

[a] These samples were ground in the host laboratories applying a Retsch ultracentrifugal mill with 0.5 mm ring sieve.

comparison of the obtained network precision of individual contents per sample, the CVs (reproducibility CV) were calculated because the sizes of the SDs are very different.

RESULTS AND DISCUSSION

First Collaborative Study. Ten samples, five of which unground, were sent to four external customer laboratories. Soybean meal A and B, corn A and B, and fish meal A and B were sent, sample A unground and B ground; the other samples were wheat A and barley A as grains, ground rapeseed meal A, and meat and bone meal A. All participants were asked to grind the coarse samples with a Retsch ultracentrifugal mill using a 0.5 mm ring sieve and then to analyze the samples in the NIR spectrometer. All four host instruments had been standardized with the Foss check cell to our master instrument; at that time, the calibrations did not yet contain a rep-file. **Table 1** shows the reproducibility CV (%) obtained for the predictions of dry matter, crude protein, and the different amino acids. The estimation of Met + Cys was performed with a separate calibration equation. All results were included in the statistics because there was no evidence of outliers.

Conclusions were drawn as follows: (i) The variation among the products that were sent unground and ground is similar. This indicates that the sample preparation is sufficiently standardized. (ii) Soybean meals A/B and rapeseed meal A had a good precision in the network, with a CV of the amino acids of 0.36−3.54%; fish meal A is slightly poorer, but the results for corn samples A/B, barley A, wheat A, and meat and bone meal A are fairly unsatisfactory with variations of up to 28.65%.

Our explanation for these findings is that the Foss check cell contains finely ground soybean meal and that the spectrum is therefore similar to that of soybean meal and rapeseed meal samples. Bouveresse et al. (*11*) arrived at the following conclusion in extensive comparisons of NIR instrument standardization with different sample sets: ''It appears that one needs standardization samples which cover exactly the same optical density range as the prediction samples." This is not the case for corn, barley, and wheat, and the instrument differences for these calibrations cannot be adequately compensated by using the Foss check cell. In our subsequent work, we therefore produced check cells for all raw materials for calibration and used these to standardize the host NIR spectrometers to our master (standardization per product).

Second Collaborative Study. Fifteen samples were selected for testing, namely, two fish meals B/C, two meat meals A/B, barley B, corn C, rapeseed meal B, rice bran A, two soybean meals C/D, soya full fat A, sunflower meal A, sorghum A, triticale A, and wheat B. These samples were distributed ground, as the chief objective was to check the effect of instrument standardization. In addition to the master instrument, nine customer NIR spectrometers were involved, three of which were still standardized to the master with the Foss check cell as in the first ring test, five by the new procedure with the aid of 11 cells specifically made by us for the respective calibrations, and one at their own request with the Universal Set for Standardization supplied by Foss, which contains 30 different feedingstuffs and pure substances in sealed and waterproof ring cups (*6*). One laboratory reported major problems with its Foss 5000 NIR spectrometer, and almost all its measurements produced GHs that were distinctly higher than average, so that the results of this laboratory were not included in the evaluation. No further checking and elimination of outliers took place.

Table 2 contains the reproducibility CV (%) of the remaining nine laboratories. Reproducibility is markedly improved, excellent for soybean meals C and D with a CV between 0.7 and 2.9% for all amino acids and crude protein, followed by fish meal B and C, barley B, rapeseed meal B, rice bran A, and full fat soya A with a similarly low variation and individual CV of up to 4.5% for these nutrients. Sunflower meal A also has good agreement (CV 2−3%), except for lysine with a CV of 5.4%. A slightly poorer reproducibility was obtained mainly for the cereals due to far lower CP and amino acid concentrations (corn C, sorghum A, triticale A, and wheat B) and for meat meals A and B, with the latter and corn being clearly in need of improvement. Higher variations among individual amino acids may also be due to a greater sensitivity of this calibration equation to spectral noise, such as changes in reflectance, which are not caused by the calibrated contents but by differences in temperature, humidity, cell filling, etc. Such variations were also observed after multiple measurements of the same sample over several weeks in the master instrument. **Figure 1** shows that those effects consistently lead to a variation of 1−2% CV, even in the master instrument, and that distinctly higher variations (in some cases from 3 to 6.5% CV) were obtained in corn and meat meal for some amino acid concentrations. Precise measurements in the network are thus achieved not only by optimal instrument standardization but also by making the calibration equations insensitive to unavoidable changes in the NIR spectra due to the aforementioned factors. Analytical differences between the customer instruments and the master in relation to the method of standardization and applied sample modules (spinning sample or transport module) were also studied, but because of the paucity of data, no really conclusive results could be obtained. However, it seemed that both sample modules give similarly good agreement with the master NIR spectrometer and that results for fish meals B/C, sunflower meal A, rice bran A, sorghum A, barley B, and wheat B were clearly better when

Table 2. Variation of Results in the Second Collaborative Study for NIRS Amino Acid Analysis in Feed Ingredients

		reproducibility CV (%) of NIRS prediction											
sample	*N*	DM	CP	MET	CYS	M + C	LYS	THR	TRP	ARG	ILE	LEU	VAL
fish meal B	9	0.84	2.10	4.50	1.75	3.20	2.74	3.13	3.61	2.00	3.23	2.87	3.20
fish meal C	9	0.73	2.50	3.70	1.91	2.93	2.89	2.69	3.79	2.35	3.10	2.76	3.63
meat meal A	9	0.42	3.62	8.25	n.d.	14.67	5.39	7.89	8.40	5.58	6.43	6.01	7.16
meat meal B	9	0.56	4.07	10.7	n.d.	11.6	5.68	7.60	5.07	5.97	6.55	6.78	7.93
barley B	9	1.16	3.17	4.54	4.07	3.28	2.89	3.38	3.78	3.74	3.74	3.09	2.95
corn C	9	0.65	5.40	8.11	4.90	5.70	8.43	4.51	9.76	9.00	7.72	7.29	6.37
rapeseed meal B	9	0.82	2.17	2.82	1.86	2.45	4.23	1.37	2.43	3.08	2.66	3.25	2.54
rice bran A	9	0.65	2.41	1.62	0.55	0.89	2.59	2.52	3.21	1.47	1.45	1.77	1.24
soybean meal C	9	0.58	1.24	1.24	0.97	0.73	1.92	1.03	1.55	2.49	1.43	1.21	1.37
soybean meal D	9	0.64	1.88	0.93	1.09	1.05	1.96	1.50	2.29	2.92	2.14	1.77	1.87
soya full fat A	9	0.39	1.13	1.71	4.28	2.88	2.49	1.15	2.11	3.10	1.16	1.12	1.13
sunflower meal A	9	0.37	2.28	2.83	2.75	2.48	5.36	2.96	3.16	2.89	2.54	2.36	2.57
sorghum A	9	0.36	3.62	4.14	2.42	2.20	7.81	3.82	2.75	5.20	4.06	5.78	2.91
triticale A	9	1.10	2.63	4.60	9.06	7.28	5.59	5.13	5.55	8.30	4.25	2.61	4.83
wheat B	9	0.42	3.63	4.92	3.10	3.46	6.00	3.14	4.23	5.67	4.39	3.48	5.56

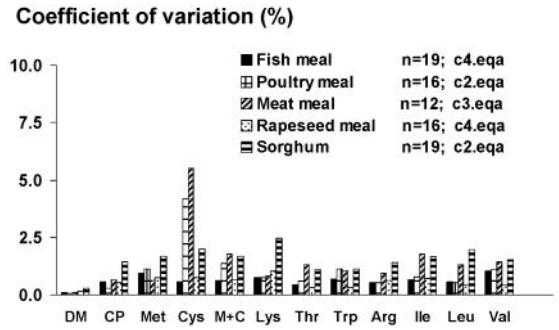

Figure 1. Variation at the master NIR instrument. Feed ingredients repeatedly scanned and predicted on the master instrument over many weeks.

measured with instruments that had been standardized per product using the customized check cell produced by us.

Effect of Host Spectra in Calibration Equations on the Accuracy in a Network. Tillmann et al. (*12*) reported that they obtained the best precision in a network with a combined approach of standardizing all NIR instruments and integrating rep-files obtained from spectra of the host instruments into the calibration equations. As some participants in the second ring trial had sent all of their spectra to us at our request, we too were able to perform tests with rep-files as additional factor in calibration algorithm as programmed in the WinISI software (*8*).

An alternative to the use of a rep-file in the calibration is to integrate spectra of the same sample from different NIR spectrometers into the CAL-file (spectra and reference data) for calculating the calibration. A ring trial was set up to objectively study the efficacy of the various techniques as compared with the "normal calibration". We selected products for which we had distributed 2 or 3 samples per calibration in the second ring trial because this provided us with the most host spectra. In one case, the current calibration equations of the three products fish meal, meat and bone meal, and soya were extended by the existing host spectra, without any further changes in the number of samples or the calibrated reference data, in the CAL-file; that is, they were assigned the identical reference data in the calibration set. In the second case, the spectra of the host instruments were combined into a NIR spectra file, which was used in the calibration model as additional factor, called a rep-file. In this way, two new versions of the three calibration equations (soybean meal + full fat soybean, fish meal, and meat meal products) were calculated simply, i.e., without further changes to reference data or sample selection, and distributed for testing. Two meat meal samples C and D, two fish meals D and E, and a soya full fat sample B were selected for this comparison, and the ground samples were sent to seven participants whose analytical instruments had all been standardized per product to our NIR spectrometer. **Table 3** shows the results for the reproducibilty CV (%) obtained in the network. Meat meal C is clearly analyzed with greater agreement when host spectra were integrated into the calibration. This is evident mainly from the low CV obtained for crude protein, methionine, and lysine; in the case of meat meal D, the improvement was not as marked but still apparent. The analysis of the two fish meals D and E with the calibrations containing host spectra is still a slight improvement on the excellent level of the normal calibration, especially when the calibration contained a rep-file. This variant is also distinctly more precise for soya full fat B. A comparison of the mean of all CV for dry matter, crude protein, and amino acids shows that the calibration with rep-file always performed best, albeit not by a wide margin. Looking at the central question of this ring trial, the application of the rep-file obviously minimizes the differences in NIR prediction between the host and the master NIR spectrometer. This is most obvious for the meat meal C and the soybean full fat B samples. These results and the consideration that integration of numerous host spectra into the CAL-file also falsely increases the number of samples and affects the statistical parameters led to the decision to use host spectra as rep-files in the updates of calibration models together with spectra of the master instrument from samples measured at different temperatures.

Third Collaborative Study. Nineteen samples were selected, which covered 13 different calibration equations, namely, fish meals F and G, meat meal E, poultry meal A, rapeseed meal C, soybean meals E and F, soya full fat C, sunflower meal B, rice bran B, sorghum B, triticale B, wheat bran A, and two of barley (C/D), corn (D/E), and wheat (C/D). These samples were again distributed ground and analyzed with up to 29 different NIR instruments, having previously greatly extended the network; two of these were standardized to the master with the individual Foss check cell as in the first ring trial, 10 with the Universal Set previously supplied by Foss, which contained 30 different feedingstuffs and pure substances (see above), and 17 NIR spectrometers with the customized check cells produced by us specifically for each calibration. Five of the tested calibrations, those of barley, fish meal, rapeseed meal, rice bran, and sorghum, already contained a rep-file. In this ring trial, the prediction was performed almost exclusively on the basis of

Table 3. Collaborative Study on the Effect of Host Spectra Built in Calibration Equations, Variation of Results

		reproducibility CV (%) of NIRS prediction												
sample	*N*	DM	CP	MET	CYS	M + C	LYS	THR	TRP	ARG	ILE	LEU	VAL	mean CV (%)
						meat meal C								
without host spectra	8	0.44	1.78	4.28	11.03	3.59	3.17	4.04	3.62	2.52	2.63	2.23	2.73	3.51
with host spectra as cal-file	8	0.42	1.27	2.99	9.99	3.89	1.90	3.42	2.95	1.89	2.78	1.52	2.81	2.99
with host spectra as rep-file	8	0.43	1.12	2.20	12.01	4.45	1.24	2.46	2.67	1.59	2.76	1.57	2.45	2.91
						meat meal D								
without host spectra	8	0.27	1.22	3.18	14.01	3.64	1.71	2.69	5.26	1.53	3.06	2.24	1.86	3.39
with host spectra as cal-file	8	0.28	0.83	3.29	16.54	3.90	1.24	2.23	4.44	1.58	2.13	1.58	1.81	3.32
with host spectra as rep-file	8	0.31	0.86	3.21	15.05	4.51	0.94	2.49	4.56	1.12	2.50	2.06	1.67	3.27
						fish meal D								
without host spectra	8	0.33	1.44	1.27	2.16	1.01	2.16	0.97	2.51	1.04	2.33	1.99	2.00	1.60
with host spectra as cal-file	8	0.31	1.36	1.26	2.07	1.03	2.09	1.07	2.18	0.83	2.22	1.93	1.95	1.53
with host spectra as rep-file	8	0.29	1.26	1.23	2.04	1.06	1.71	0.91	1.65	1.10	1.77	1.64	1.80	1.37
						fish meal E								
without host spectra	8	0.26	1.61	1.59	1.91	1.18	1.87	1.26	2.82	1.25	2.88	2.47	2.50	1.80
with host spectra as cal-file	8	0.25	1.63	1.50	1.90	1.20	1.84	1.36	2.60	0.89	2.73	2.39	2.50	1.73
with host spectra as rep-file	8	0.28	1.48	1.60	1.97	1.26	1.73	1.26	2.10	1.32	2.28	2.14	2.34	1.65
						soya full fat B								
without host spectra	8	0.29	1.57	1.45	3.27	2.56	2.25	1.58	2.08	2.41	1.67	1.60	1.58	1.86
with host spectra as cal-file	8	0.55	0.60	2.22	4.32	3.60	1.87	1.41	1.61	1.09	1.24	1.20	1.46	1.76
with host spectra as rep-file	8	0.59	0.93	2.30	1.44	1.37	1.06	1.07	1.13	2.48	0.96	1.21	0.95	1.29

Table 4. Variation of Results in the Third Collaborative Study for NIRS Amino Acid Analysis in Feed Ingredients

		reproducibility CV (%) of NIRS prediction											
sample	*N*	DM	CP	MET	CYS	M + C	LYS	THR	TRP	ARG	ILE	LEU	VAL
fish meal F	29	0.31	1.2	1.27	1.53	0.97	1.42	0.83	1.46	1.05	1.5	1.32	1.82
fish meal G	29	0.41	1.58	1.79	2.3	1.36	1.78	1.29	2.12	1.4	2.02	1.83	2.76
meat meal E	28	0.68	2.1	2.94	9.46	3.81	2.01	2.77	3.03	2.34	3.19	2.59	2.57
poultry meal A	27	0.23	0.85	2.92	6.95	1.62	1.13	1.34	2.17	1.55	2.34	1.23	2.78
barley C	28	0.51	2.61	3.09	6.72	4.34	3.32	2.76	4.06	2.38	2.74	2.35	2.57
barley D	28	0.45	2.94	3.3	6.63	4.23	3.04	2.63	3.35	3.25	3.12	2.56	3.03
corn D	29	0.33	5.5	7.53	5.04	5.99	9.71	6.23	7.25	7.7	7.65	6.21	5.73
corn E	29	0.22	4.93	6.26	4.26	5.45	8.78	4.89	8.74	6.92	6.03	5.15	4.86
rapeseed meal C	28	0.52	1.79	2.5	2.48	2.44	3.16	1.56	1.77	2.45	1.79	1.93	1.56
rice bran B	27	0.54	2.94	2.69	3.8	2.76	5.72	3.31	3.21	2.87	3.22	2.78	2.62
soybean meal E	29	0.4	1.4	1.46	1.6	1.36	2.22	1.46	1.67	2.85	1.61	1.43	1.47
soybean meal F	28	0.26	1.99	1.66	2.24	2.01	2.45	2.15	2.46	2.93	2.24	2.07	2.03
soya full fat C	27	0.28	1.33	1.54	3.23	2.31	2.46	1.48	1.81	3.43	1.44	1.38	1.46
sunflower meal B	28	0.3	3.74	3.55	3.81	3.62	4.62	3.37	3.97	4.85	4.15	3.92	3.33
sorghum B	27	0.4	4.83	4.89	3.58	3.52	6.66	4.47	6.67	6.52	5.42	5.88	4.64
triticale B	26	0.25	3.22	4.61	7.72	2.93	6.36	5.08	5.5	5.4	4.26	3.52	4.9
wheat bran A	25	0.24	2.51	2.39	2.39	1.9	3.1	3.5	5.69	2.58	3.15	3.53	2.02
wheat C	28	0.42	2.6	3.74	4.42	3.85	5.78	3.36	3.51	4.02	3.32	2.4	3.66
wheat D	28	0.43	2.79	3.89	3.77	3.69	5.26	3.73	2.52	4.05	2.69	2.53	3.52

spectral files sent in, again with a view to obtaining a large number of host spectra for integration at the next update. As not all instruments had already been standardized for each raw material (applies to rice bran, sorghum, triticale, and wheat bran) and as also not all laboratories wanted to analyze each raw material, the number of participants per samples varied. The results for fish meal from one participating instrument were excluded from the statistical analysis because its spectra showed in the first derivative a RMS of >3000 relative to the master NIR spectrometer, while all other results were <500. We interpreted this result as a fault or possibly inadequate instrument standardization. **Table 4** shows the reproducibility CV (%) obtained for the samples measured in 25−29 NIR spectrometers. For the majority of the 19 measured feed samples, the determined CV values were far below 5%. All results demonstrate a high level of agreement with the master instrument in the network. Optimal agreement is found for the fish meal samples, the three soya products, and rapeseed meal; the new calibration for poultry meal had excellent precision in the network except for cystine, and that of meat meal was also greatly improved. The relatively high variation for cystine in these products is associated with the reference data (see comments in ref *2*) and cannot be improved by standardization or rep-files.

While the rep-file calibration gave a markedly improved agreement over the second ring test for wheat C and D, the improvement for barley samples C and D is only minimal, and rice bran B and sorghum B even showed slight deteriorations. Other raw materials with calibrations without rep-file show some good CV (%) (sunflower meal B and wheat bran A), acceptable for triticale B, and an unsatisfactorily high variation for both corn samples D and E. Shortly after termination of this ring trial, an update of the corn calibration was completed, which contained a rep-file. As shown in **Figure 2**, all existing spectra of the ring test participants ($N = 24$) for the sample corn D were also predicted with this equation, resulting in far better agreement and a corresponding improvement also for corn E. Calculations with other calibrations, used with and without rep-

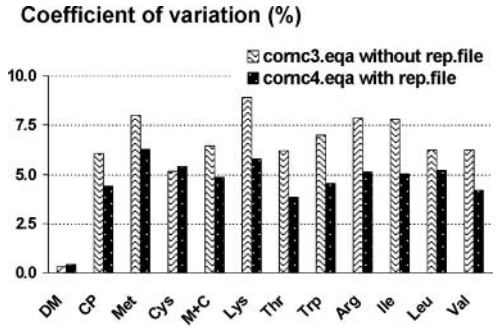

Figure 2. Calibration update of corn—the new equation with a rep-file reduces variation. NIR scans of sample corn D from 24 standardized instruments were predicted.

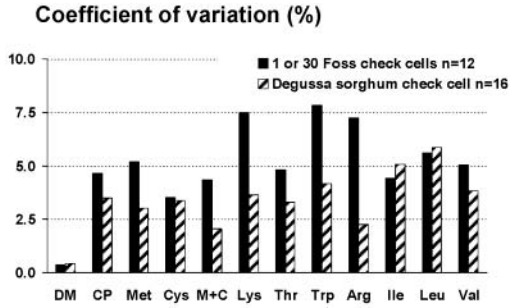

Figure 3. Effect of standardization technique. Instrument adaption with a specific check cell reduced the CV for the sorghum sample B.

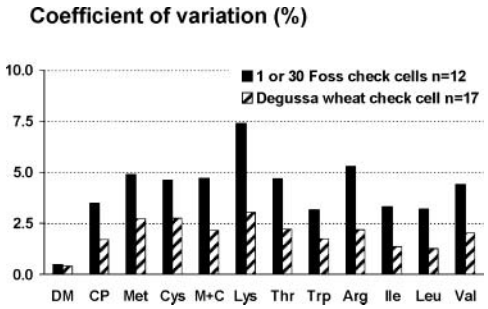

Figure 4. Effect of standardization technique. Instrument adaption with a specific check cell reduces the CV for the wheat sample D.

file for the spectra from the ring test, consistently gave a similar improvement for cereal products in the reproducibility CV for crude protein and amino acids but a slight deterioration in the prediction of dry matter.

We have also checked the influence of applying a rep-file on the accuracy of the NIRS prediction by comparing SECV obtained with and without it for the same calibration samples. In most cases, there is an increase of SECV when using a rep-file, but with only a few exceptions, it is below 5% relatively, thus neglectable. A real damage of the accuracy, that is an increase of 20% and more of the standard error, was not observed for amino acid calibrations.

As the number of results for NIR spectrometers standardized per product with Degussa check cells and those standardized with cells supplied by Foss were very similar, it was possible to compare both variants with the regard to the variation reached in the network. It was evident for all raw materials with low protein and hence low amino acid concentrations that product specific standardization with material having a spectrum close to the center of the calibration population (low GH) clearly helps to reduce the variation in the network. **Figures 3** and **4** show the respective improvement in the CV of crude protein and

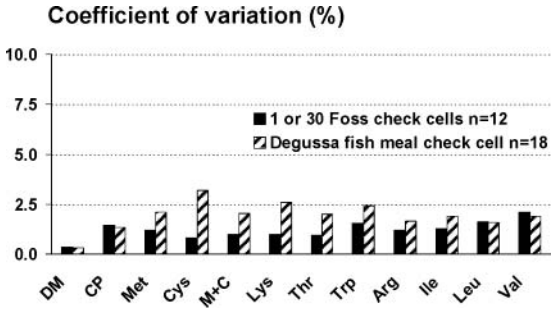

Figure 5. Effect of standardization technique. Instrument adaption with a specific check cell doesn't further improve variation for the protein rich fish meal sample F.

amino acids for the samples sorghum B and wheat D. The other cereal samples also clearly demonstrated this improvement through our standardization technique. The same comparison with the protein-rich raw materials fish meal, meat meal, and soybean meal gave a different picture, however, with CV values for both variants distinctly below 5% throughout, with the exception of cystine in fish meal. This was most marked in the sample fish meal F, as illustrated in **Figure 5**; although agreement for all nutrient concentrations is excellent (CV < 3%) in the network, instruments standardized with the Foss check cell set performed best. However, as the positive effect for cereals is much more marked and more important, we persisted with standardization using Degussa check cells.

Fourth Collaborative Study. One year later, the most extensive international study to date on the use of amino acid calibrations took place among feed manufacturers worldwide. Seventeen feed raw materials, namely, barley E and F, corn F, feather meal A, fish meal H, meat and bone meal B, meat meal F, peas A, poultry meal B, rapeseed meal D, rice bran C, sorghum C, soya full fat D, soybean meal G, sunflower meal C, wheat E, and wheat bran B were sent to the laboratories of 25 feed producers and three Degussa satellite laboratories. All 14 amino acid calibrations available at this time were tested. As some companies have small networks themselves, our samples were analyzed in up to 44 NIR spectrometers. Almost all samples were again sent finely ground, but the sample barley F as grain was included additionally to test the effect of sample preparation by the participant. It had to be ground on site prior to the NIR analysis, and the use of a 0.5 mm sieve in the sample mill was stipulated. All laboratories receiving our calibrations are in any event checked for the existence of a suitable mill as sample grinding has a profound effect on the spectra and must be standardized.

Results with a GH greater than 3 were not included in the final assessment as they indicate errors in sample grinding, measurement, or instrument standardization. In addition, as is customary in ring trials, a test for outliers according to Grubbs (*13*) was performed. If the test was positive for one parameter, all results for the sample concerned were excluded because inadequate instrument standardization or errors in sample analysis were assumed. Laboratories with outlying results were contacted in a follow-up operation, the calibrations in question were tested, and improvements were made where necessary. In the case of the new feather meal calibration, many NIR machines could not be standardized prior to the ring trial and were therefore not be able for participation. **Table 5** shows the reproducibility CV (%) obtained in this way from 31 to 43 valid results per sample and 11 for feather meal. The number of participating NIR spectrometers N_{instr} and the number of evaluated results N are separated. In the majority of results, the

Table 5. Variation of Results in the Fourth Collaborative Study for NIRS Amino Acid Analysis in Feed Ingredients[a]

			reproducibility CV (%) of NIRS prediction											
sample	N_{instr}	N	DM	CP	MET	CYS	M + C	LYS	THR	TRP	ARG	ILE	LEU	VAL
barley E	44	42	0.40	1.84	2.58	5.23	3.71	4.32	2.61	3.65	2.16	2.51	2.41	2.44
barley F, ground by client	42	30	0.81	2.54	3.02	6.29	4.48	4.30	3.33	4.31	2.52	3.40	2.94	3.05
corn F	44	38	0.41	1.80	4.88	2.84	2.99	4.15	1.96	3.40	3.90	2.68	2.42	2.35
feather meal A	13	11	0.31	1.03	11.6	2.52	2.38	6.52	1.25	1.69	2.71	1.01	1.25	1.10
fishmeal H	36	34	0.45	1.68	1.46	1.98	1.45	2.06	0.99	1.45	1.43	1.72	1.36	2.54
meat and bone meal B	36	34	0.33	1.68	2.39	13.2	3.84	2.77	1.85	2.68	2.04	2.53	2.28	3.47
meat meal F	36	33	0.31	2.14	2.85	18.2	4.87	3.97	2.65	3.24	3.32	2.79	3.03	3.36
peas A	37	31	0.28	3.43	2.08	1.1	1.41	3.61	2.53	2.04	5.09	3.60	2.70	3.12
poultry meal B	35	33	0.50	1.24	2.18	2.99	2.06	1.71	1.69	2.20	1.62	1.82	1.61	1.93
rapeseed meal D	43	40	0.31	1.06	1.97	2.43	2.17	2.34	1.11	1.31	1.93	1.37	1.54	1.05
rice bran C	36	34	0.49	2.28	2.26	2.36	2.12	4.01	2.26	2.12	2.24	2.34	2.04	1.93
sorghum C	37	32	0.43	2.25	2.24	2.84	2.30	3.81	2.05	2.26	2.89	2.86	3.19	2.29
soya full fat D	44	41	0.38	1.05	1.54	1.87	1.46	1.32	1.21	1.73	1.54	1.24	1.26	1.07
soybean meal G	44	40	0.25	0.69	1.23	1.22	1.18	0.82	0.84	1.12	1.13	0.80	0.79	0.78
sunflower meal C	44	43	0.30	5.44	5.64	5.06	5.44	6.92	5.08	6.44	6.48	5.08	5.74	5.39
wheat E	43	39	0.31	2.01	2.20	3.23	2.75	3.22	2.55	1.74	2.00	2.04	1.96	2.19
wheat bran E	41	36	0.30	1.34	2.01	2.35	1.77	2.87	1.81	2.48	1.86	2.35	1.98	1.58

[a] N_{instr} = number of participating NIR spectrometer. N = number of evaluated results; predictions of spectra having a GH above 3 were removed. The outlier test by Grubbs was applied; if an individual prediction value was detected as outlier, all results of this instrument for the respective sample were excluded from the evaluation.

Table 6. Obtained SDs for Amino Acids Predictions in the Network as Compared to SECV

		SD relative to the respective SECV									
sample	N	MET	CYS	M + C	LYS	THR	TRP	ARG	ILE	LEU	VAL
barley E	42	0.48	1.02	0.88	1.17	0.81	0.90	0.55	0.82	0.97	0.98
corn F	38	0.68	0.63	0.63	0.80	0.81	0.68	0.96	0.95	0.78	0.84
feather meal A	11	0.75	0.50	0.48	0.74	0.45	0.31	0.96	0.42	0.49	0.33
fish meal H	34	0.41	0.25	0.43	0.63	0.33	0.40	0.43	0.57	0.47	0.74
meat and bone meal B	34	0.48	0.92	0.67	0.64	0.50	0.49	0.58	0.59	0.66	0.82
meat meal F	33	0.46	0.85	0.66	0.82	0.61	0.58	0.75	0.61	0.77	0.69
peas A	31	0.40	0.17	0.26	1.22	0.77	0.51	1.02	1.19	0.89	1.04
poultry meal B	33	0.55	0.34	0.59	0.50	0.67	0.51	0.67	0.73	0.74	0.59
rapeseed meal D	40	0.65	0.67	0.75	0.78	0.56	0.63	0.78	0.63	1.05	0.44
rice bran C	34	0.56	0.65	0.63	0.82	0.81	0.46	0.64	0.99	1.14	0.82
sorghum C	32	0.42	0.62	0.55	0.73	0.87	0.79	0.61	0.95	0.93	0.85
soya full fat D	41	0.32	0.31	0.34	0.48	0.47	0.66	0.49	0.46	0.62	0.39
soybean meal G	40	0.31	0.25	0.34	0.37	0.40	0.51	0.45	0.37	0.48	0.36
wheat E	39	0.45	0.72	0.67	0.71	0.87	0.32	0.40	0.66	0.83	0.56
wheat bran B	36	0.64	0.68	0.73	0.94	0.82	0.59	0.72	0.96	1.28	0.64

determined CV values were about 0.4% for dry matter, about 2% for crude protein, and less than 3% for the analyzed amino acids. Excellent results in ring tests of the wet chemical amino acid analysis however show CV values of about 2% for crude protein and 5−8% for the amino acids (*14*). Thus, when accurately adapted, NIR spectrometers and robust equations are applied, and amino acid analysis by NIRS produces more consistent values than amino acid analysis by the chromatographic method. CVs exceeding 5% were only found in some cystine results, for methionine in feather meal A and for sunflower meal C. As regards sunflower meal C, we believe the problem was due to the packing of the measuring cell. It was hard to fill the material homogeneously into the sample cup because of electrostatic effects. As we had much better results for the other sunflower meals A and B in previous ring tests, we concluded that the problem was specific to that sample.

The barley sample F, distributed unground to check the grinding process in the customer lab, did indeed reveal numerous problems. Out of the 42 NIR spectra received, we had to exclude 10 from the final evaluation due to GHs > 3. However, the results of the unground barley sample showed clearly that a GH value > 3 is a good criterion for pinpointing problems with grinding or instrument standardization because in most cases the relative differences between host and master were significantly higher for samples with GH values > 3 than for samples with GH values < 3. Before outlier elimination, the CV results of barley F were 6.9% for crude protein and 5.9−13.7% for the amino acids. After eliminating samples that were ground in a very different way (10 GH outliers and two Grubbs outliers), the CV values of the barley F turned out to be relatively low, just like those of the barley E ground in the Degussa laboratory. In the next ring trial, we will focus on the problem of sample preparation in the customer lab.

Precise and accurate measurements in a NIR spectrometer network are only possible if the error due to the individual instrument is smaller than the SECV of the calibration applied. Under these circumstances, the instrument error in a validation of the NIRS prediction vs wet chemical analysis has a negligible impact. Bouveresse, Massart, and Dardenne (*11*) measured three sample sets at the master and host instruments and predicted results using different standardization methods. They compared the standard error of the differences between master and host predictions, here called SEP, to the SEC of the respective constituent in the applied calibration equation. They recommended that for successful standardizations SEP/SEC should be smaller than 0.5. If larger than 1, the standardization method was not useful. Dardenne (*15*) applies today the relation of the SD of NIR predictions from ring tests in a Belgian network relative to the SECV of the applied calibration model with the same criteria to judge the precision and accuracy obtained. Thus,

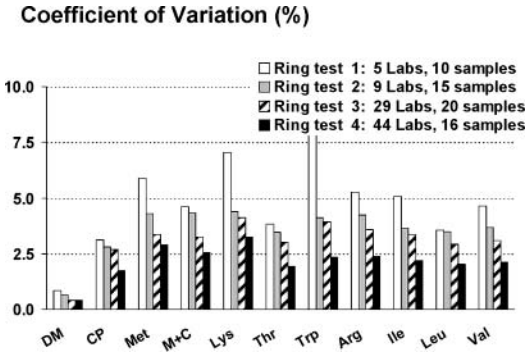

Figure 6. Average variation in the worldwide network was continuously reduced by described measures.

we also divided the SDs per amino acid obtained in our fourth ring test in the different raw materials by the respective SECV of the calibration tested (see our calibration statistics in refs *2* and *3*). **Table 6** shows this evaluation. In 44 of the 15 × 10 amino acid readings, the SD obtained in the ring test was less than half (0.5) of the corresponding SECV. This was frequently the case for the methionine prediction in particular, where the highest relative SD/SECV was 0.75. Both soya products showed this high precision for almost all amino acids, followed by feather meal and fish meal. In 101 of the 150 estimates, the SD was less than 0.75 relative to the corresponding SECV, and only in five cases, it was greater than the SECV. This analysis is further evidence that a high level of agreement was reached in the network.

Conclusion. Continuous updating of calibrations by using the rep-file and product specific standardization of customer instruments has led over the years to a marked reduction in the variation of estimated NIR concentrations for amino acids, crude protein, and dry matter in a worldwide network.

The CV for the major amino acids, determined from the results of the fourth ring trial, is in the order of 2−3%. **Figure 6** illustrates the continuous improvement of the precision in the network documented with four worldwide collaborative studies. Following the transfer of calibration equations, amino acids in raw materials can be estimated in many labs worldwide in good agreement with our master instrument and the reference method. This brings substantial benefits for the feed producer by enabling a fast, cost effective, and comprehensive analysis of incoming raw materials for optimal use in feed formulations.

ABBREVIATIONS USED

NIRS, near-infrared reflectance spectroscopy; SEC, standard error of calibration; SECV, standard error of cross-validation; CV, coefficient of variation (relative standard deviation); RSQ, fraction of explained variance for the calibration samples (square of correlation coefficient *r*); 1-VR, fraction of explained variance for cross-validation (square of correlation coefficient *r*); SD, standard deviation of the variable; GH, global *H* value (Mahalanobis distance); rep-file, repeatability file.

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