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Near-Infrared Reflectance Spectroscopy for Predicting Amino Acids Content in Intact Processed Animal Proteins

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Near-infrared calibrations were developed for the instantaneous prediction of amino acids composition of processed animal proteins (PAPs). Two sample presentation modes were compared (ground vs intact) for demonstrating the viability of the analysis in the intact form, avoiding the need for milling. Modified partial least-squares (MPLS) equations for the prediction of amino acids in PAPs were developed using the same set of samples ($N = 92$ PAPs) analyzed in ground and intact form and in three cups differing in the optical window size. The standard error for cross validation (SECV) and the coefficient of determination (1-VR) values yielded with the calibrations developed using the samples analyzed in the intact form showed similar or even better accuracy than those obtained with finely ground samples. The excellent predictive ability (1-VR > 0.90; CV < 3.0%) obtained for the prediction of amino acids in intact processed animal proteins opens an enormous expectative for the on-line implementation of NIRS technology in the processing and marketing of these important protein feed ingredients, alleviating the costs and time associated with the routine quality controls.

KEYWORDS: Processed animal proteins; NIRS; amino acids composition; intact NIRS analysis

INTRODUCTION

Processed animal proteins (PAPs) have traditionally made a great contribution to the value of animals by finding their way into a wide variety of applications. For instance, meat and bone meal (MBM) has been included in livestock feeds, pet foods, and fertilizers. Animal protein meals are also excellent sources of calcium, phosphorus, protein, and essential amino acids. Nutritionists traditionally included fat and protein ingredients derived from animal sources for supplying amino acids, energy, minerals, and other trace nutrients when formulating swine and poultry diets (*1*, *2*).

In the European Union (EU), as a consequence of the Bovine Spongiform Encephalopathy (BSE) crises and its association with Creutzfeldt Jakob Disease (CJD) in humans, government and customer restrictions have been established on the use of PAPs. The ban of the use of meat and bone meals (MBM) in compound feeds is one of the measures carried out in the EU to stop the spread of BSE and to prevent its re-occurrence. Another important measure affecting the use of PAPs has been the prohibition of intraspecies recycling, in order to avoid a potential infectivity due to the absence of barrier within species (*3*−*5*).

One of the main consequences of the legislation relating to the use of PAPs has been the loss of commercial and social value of the rendering products (i.e., meat and bone meal) and the increasing customers' inquiries concerning nutrient availability and variability, animal species identification, and quality and safety of the PAPs. Therefore, in the rendering industry, there is a great concern for the implementation of new analytical methods that ensure traceability, safety, and customers demands, allowing a more informative label.

Information about amino acid content in PAPs is an increasing demand of food animal and pet food nutritionists, being a key element for the feed manufacturers and integrated livestock operations to produce precise and cost-effective feeds. The amino acid content of feed ingredients is affected by several factors such as plant breeding or fertilization. In animal and plant byproducts, there are additional factors influencing the amino acid profile, such as the origin of raw material, the processing method, and the storage conditions (*6*).

Chromatographic amino acid analysis requires oxidation and hydrolysis of the protein, followed by ion-exchange chromatography (IEC). This procedure is quite complicated and labor-intensive, needing a minimum of 3 days of processing time (*6*).

Near-infrared spectroscopy (NIRS) is already well-established for routine quality control in feed mills and nutritional feed analytical services (*7*). At present, NIRS is the only technique that allows the analysis of large-scale samples and, consistently, taking decisions in real time (*8*).

Several recent review papers have demonstrated the ability of NIRS technology to predict traditional feed chemical values and other parameters of nutritional interest (*7*, *8*). Although other authors have demonstrated the ability of NIRS to predict the

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amino acid profile in cereals (*9*, *10*) or compound feeds (*11*, *12*), there are a limited number of papers that address the use of NIRS to predict amino acids profile in PAPs. In this way, NIRS calibration equations for predicting amino acids content in PAPs have been reported by Fontaine et al. (*6*) and Qiao and Van Kempen (*13*).

Most of the NIRS works for amino acids prediction have been developed after a fine milling of the product. Pazdernik et al. (*14*), using the same set of soybean samples analyzed ground and intact, concluded that milling improves the accuracy of the amino acid predictions.

In order to fully incorporate NIRS technology into the rendering industry, inspection, and feed industry laboratories, it is desirable to avoid the tedious milling task. Nevertheless, to ensure that, beforehand it is necessary to demonstrate that the results obtained with the analysis of intact samples by NIRS, i.e., in the original marketing presentation, are comparable to those obtained analyzing the samples finely milled.

Brimmer and Hall (*15*) suggested the need to enlarge the scanning surface for the analysis of intact material. In fact, great changes have been made in NIRS instruments within the 1990s. Grating monochromator instruments enjoy the most widespread usage throughout the feed industry. Versatile NIRS analyzers with different sample presentation attachments and large analysis windows, allowing a better representation of the sample and also the analysis of intact material, have appeared in the market (*16*). Pérez-Marín et al. (*17*) using a cup with a large window size (94 cm^2) showed that NIRS calibrations developed for the prediction of the chemical composition of intact feeds had a similar or better accuracy than the equations developed with the same samples that were analyzed ground.

The main aim of the present paper is to evaluate the predictive ability of NIRS equations developed for the prediction of amino acids content in PAPs analyzed in intact form versus ground form, using the same calibration set.

MATERIALS AND METHODS

PAPs Samples. A total of 359 commercial PAPs, provided by the most important rendering plant of Andalusia (Spain), have been collected in the framework of two different projects (STRATFEED G6RD-2000-CT-00414 and MCYT-INIA CAL02-028-C2-2). The samples belonged to different species of terrestrial PAPs: poultry byproducts meal, pig meal, cattle meal, and meat and bone meal (mixture of different species). They were stored in the University of Córdoba Sample Bank.

Reference Analysis. The nitrogen content of the samples was determined by combustion using a LECO FP-428 (LECO Corp., St. Joseph, MI) nitrogen analyzer (Dumas method) according to the AOAC method 990.03 (*18*).

The samples were analyzed for 18 amino acids which included all the essential ones: methionine (Met), cystine (Cys), methionine + cystine (M + C), lysine (Lys), threonine (Thr), tryptophan (Trp), arginine (Arg), isoleucine (Ile), leucine (Leu), valine (Val), histidine (His), phenylalanine (Phe), tyrosine (Tyr), glycine (Gly), serine (Ser), proline (Pro), alanine (Ala), aspartic acid (Asp), and glutamic acid (Glu).

All amino acids, except tryptophan, were analyzed by the method proposed by Llames and Fontaine (*19*) and adopted by the AOAC as Official Method 994.12. This procedure agrees with the Official European Method of amino acid analysis in feed (*20*). The tryptophan content of the samples was analyzed following the Official European Method (*21*).

NIRS Analysis. *Intact Samples.* Samples were scanned as received, i.e., without previous milling and in its original form with a particle size of 6 mm, approximately. A FOSS NIRSystems model 6500 SY-II scanning monochromator (Silver Spring, MD) equipped with a transport module was used to measure reflectance spectra from 400 to 2500 nm, every 2 nm. Two replicates were measured for each sample, using the average of spectra for calibrating.

The analysis of the samples was carried out using the natural product transport cell. This is a rectangular cell with internal dimensions of 4.7 cm wide, 20 cm long, and 4.3 cm deep. The quartz viewing window of 4.7 cm × 20 cm allows 94 cm^2 of the sample surface area to be irradiated.

Ground Samples. Prior to NIRS analysis, a subsample of each animal protein meal was ground to a diameter particle size of 1 mm, using a cyclonic mill. Ground samples were presented in two types of cups and NIR analysis modules:

(1) A FOSS NIRSystems model 6500 SY-I scanning monochromator (Silver Spring, MD) equipped with spinning module was used to measure reflectance spectra from 400 to 2500 nm (every 2 nm), using the standard ring cup with 3.75 cm diameter.

(2) A FOSS NIRSystems instrument was described previously for the analysis of the intact samples and equipped with transport module, using the $^1/_4$ rectangular cup that was 4.6 cm wide and 5.7 cm long.

Spectra were recorded with the WINISI II software version 1.50 (Infrasoft International, Port Matilda, PA).

The instruments, as well as the type of cups used in this study, are the analysis modes more traditionally implemented in the laboratories placed at the feed industry and in feed analytical services.

Sample Selection. The selection of the training samples is one of the critical steps when developing a calibration equation. In the present paper, the algorithm SELECT based on the neighbor concept (*22*) was used to select the calibration samples.

When the SELECT algorithm is working, the user can set the number of samples that will be selected, instead of setting a limit value. In the present work it was decided to select a total of 92 samples from the original set of 359 samples, due to time and money restrictions for the reference chemical analysis.

Development of NIR Calibrations. Calibrations were developed using WINISI software version 1.50. The modified partial least squares (MPLS) regression method was used to obtain NIR equations for all the studied parameters.

Modified partial least squares is similar to partial least squares (PLS), but in this case the NIR residuals at each wavelength are standardized (divided by the standard deviation of the residuals at a wavelength) before calculating the next factor (*23*). Cross-validation separates the samples into groups for prediction. Each prediction group is predicted once, based on calibration from the remaining groups. Predicted results are summarized as the standard error of cross validation (SECV). In this study, cross-validation was performed by splitting the population into six groups.

In all calibrations, the standard normal variate (SNV) and detrending (D) methods were used for scatter correction (*24*). SNV and D are two separate algorithms that are usually applied together. SNV is applied first for correcting the effects of multiplicative interferences of scatter and particle size. Detrending removes the additional variations in baseline shift and curvilinearity, typically present in diffuse reflectance spectra (*8*).

Moreover, four derivative mathematical treatments were tested in the development of NIRS equations. The derivative treatment is one of the best for removing baseline effects (*25*). The first derivative (a simple measure of the slope of the spectral curve at every point) and the second derivative (a measure of the change in the slope of the curve) (*25*) have been tested: 1,5,5,1; 2,5,5,1; 1,10,5,1; 2,10,5,1. The first number denotes the derivative order, while the second denotes the number of nanometers in the segment used to calculate the derivative. The third and fourth numbers denote the number of data points over which running average smoothing is conducted (*26*).

The statistics used to select the best equations were the coefficient of determination (1-VR) and the standard error of cross-validation (SECV). Another statistic used was the coefficient of variation (CV) for the cross-validation, i.e., the ratio of the SECV to the mean of the reference data multiplied by 100 (*16*).

In addition to these statistics, the importance of calibration outliers must be considered. H outliers are samples with spectra very different from the average spectrum (*23*) and a critical *H* value of 10.0 is recommended when calibrating (*26*). *T* outliers are samples with large

Table 1. Amino Acid Composition (%) of the Calibration Set Grouped by the Different Types of PAPs

	poultry byproducts meal ($N = 18$)				pork meal ($N = 20$)				MBM ($n = 53$)				cattle meal ($N = 1$)
	min	mean	max	CV	min	mean	max	CV	min	mean	max	CV	
Met	1.03	1.16	1.29	5.71	0.70	0.93	1.08	12.30	0.72	0.85	1.26	13.12	0.73
Cys	0.48	0.54	0.68	8.29	0.27	0.47	0.59	17.49	0.39	0.55	0.90	24.81	0.71
M+C	1.51	1.70	1.87	5.21	1.11	1.40	1.67	12.40	1.13	1.40	1.88	13.07	1.43
Lys	3.32	3.66	3.93	4.64	2.68	3.67	4.26	12.21	2.68	3.10	4.05	9.15	2.6
Thr	1.98	2.18	2.36	4.54	1.54	2.00	2.29	10.24	1.67	1.90	2.36	7.73	1.88
Trp	0.41	0.47	0.55	7.62	0.33	0.37	0.43	8.16	0.34	0.42	0.56	11.11	0.39
Arg	3.92	4.22	4.45	3.37	3.44	4.90	5.79	13.01	3.07	3.56	4.56	8.81	3.09
Ile	1.85	2.10	2.31	5.72	1.35	1.79	2.08	10.75	1.43	1.71	2.30	11.17	1.67
Leu	3.48	3.86	4.16	4.37	2.99	3.74	4.31	10.27	3.02	3.55	4.22	7.91	3.42
Val	2.36	2.60	2.84	4.35	2.11	2.64	3.02	10.30	2.12	2.48	2.99	7.95	2.55
His	1.10	1.22	1.35	5.67	0.91	1.27	1.49	12.84	0.94	1.15	1.40	10.55	0.94
Phe	2.02	2.21	2.36	3.80	1.70	2.15	2.45	10.23	1.74	2.00	2.39	7.39	1.9
Tyr	1.42	1.66	1.86	6.48	1.17	1.44	1.67	9.45	1.20	1.41	1.89	9.68	1.31
Gly	5.78	7.00	7.86	6.99	6.60	10.35	12.47	15.85	4.94	6.16	8.16	10.00	5.43
Ser	2.14	2.35	2.55	4.43	1.86	2.48	2.84	11.40	1.85	2.21	2.74	10.91	2.46
Pro	3.58	4.42	5.38	10.42	4.97	7.15	9.13	16.88	3.25	4.13	6.10	13.78	4.13
Ala	3.99	4.25	4.40	3.04	3.62	5.15	5.96	13.01	3.31	3.81	4.56	6.28	3.55
Asp	4.42	4.81	5.09	3.46	3.68	4.89	5.59	11.15	3.81	4.25	5.09	6.71	3.86
Glu	7.14	7.77	8.23	3.65	5.81	7.90	9.03	11.39	5.70	6.55	8.28	7.97	5.88

differences between their reference and predicted values (*23*). This statistic allows evaluation criteria for assessing the variation between a predicted value and its primary chemical value. To avoid going to the tables, a rule of thumb is that t values greater than 2.5 are considered significant, and therefore values may possibly be outliers (*27*).

Finally, the results obtained with the different modes of analysis were compared using a Fisher test (*28*), calculating the F value as

$$F = \mathrm{SECV}_2^2/\mathrm{SECV}_1^2, \text{ where } \mathrm{SECV}_1 < \mathrm{SECV}_2$$

The calculated F value was compared with the confidence limit $F_{\mathrm{limit}(1-\alpha,\ n1-1,\ n2-1),}$ obtained from the distribution F table, where α is the test significance level, n_1 the times that the measure is repeated with the first method tested, and n_2 the times that the measure is repeated with the second method tested. The differences between the SECVs are significant when $F > F_{\mathrm{limit}}$.

RESULTS AND DISCUSSION

During the period 2002−2004, a Sample Bank and a NIRS Spectral Data Bank containing PAP samples representative of those produced in different Spanish rendering plants have been created. These samples were provided by the producers with accompanying documents revealing the gross composition (in percentage) in different species (i.e., % cow, % poultry, etc.) used for their production (*29*).

As indicated earlier, the SELECT algorithm (*22*) was used to choose the calibration set ($N = 92$). The application of that algorithm to the original Spectral Data Base of 359 samples computed and suggested a number of 19 principal components to represent all the relevant information existing in the spectrum. The high number of principal components selected suggests that the spectral variation and the diversity of the product are high (*22*).

Table 1 shows the mean, the coefficient of variation, and the range for the AA content of the 92 spectrally selected samples grouped by PAPs categories. In general, the poultry byproduct samples show the lowest CV values for most of the AA. However, the MBM group presents high CV values for many AA. This fact can be explained by the heterogeneity of the samples belonging to this group. While poultry byproduct meals and pork byproduct meals are made of one single animal species, meat and bone meal can include a variety of raw materials derived from different parts of the carcasses of beef, poultry, swine, sheep, goat, and others.

It is also important to highlight that the highest values for glycine and proline and the lowest value for cystine correspond to samples belonging to pork meal. The variations observed for the three groups, measured by the CV values for all the AA, agreed with those reported in animal nutrition tables and databases (*30, 31*).

Regarding the poultry byproducts meal, the mean values for the essential amino acids are very similar to those reported by Fontaine et al. (*6*).

The data reported in **Table 1** confirm that the SELECT spectral algorithm allows the creation of a calibration set that represents the usual variability encountered by each AA and PAP type and the choosing of samples containing variability inherent to intra- and interanimal species. That is important for calibration work, where the time to obtain the chemical data and/or the analytical costs associated with the constituent of interest (i.e., AA) are the main constraints for using a large calibration set.

The main difficulties encountered for the interpretation of NIRS works developed by different authors for the same product and constituent arise from the existing differences in the calibration sets used, in the repeatability quality of the reference data used, in the calibration strategies performed, in the instrumentation and type of cup used, etc. (*15*). Therefore, in the present work, to demonstrate the viability of NIRS for the analysis of intact PAPs, without previous milling, calibrations have been developed with the same set analyzed both in intact and in ground form. No outliers removal was done during calibration development, to ensure that the differences existing among the three calibration sets are due to the sample presentation mode.

The calibration equations obtained for the three analysis modes assayed (**Table 2**) present a high predictive ability. According to their 1-VR values (>0.90), the equations for these three analyses modes should be regarded as usable for quality assurance purposes (*16*). Generally speaking, the calibration statistics obtained for the equations developed with intact samples are better than those obtained with the ground samples analyzed using the standard ring cup, and similar to those obtained with the ground samples using the $^1/_4$ rectangular cup.

The statistical F-test was used to calculate the one-sided probability of the likelihood that the SECV values were different at the 95% confidence level. The comparison of the three

Table 2. Calibration Statistics for Predicting Amino Acid Composition of Ground and Intact PAPs ($N = 92$)

			standard ring cup: ground samples			rectangular $^1/_4$: ground samples			rectangular natural: intact samples		
	mean	S.D.	SECV	1-VR	CV	SECV	1-VR	CV	SECV	1-VR	CV
Met	0.93	0.16	0.042	0.93	4.52	0.037	0.95	3.98	0.035	0.95	3.75
Cys	0.53	0.12	0.048	0.84	9.06	0.041	0.88	7.74	0.040	0.89	7.43
M + C	1.46	0.20	0.067	0.90	4.59	0.058	0.92	3.97	0.053	0.93	3.63
Lys	3.33	0.42	0.110	0.93	3.30	0.095	0.95	2.85	0.086	0.96	2.57
Thr	1.98	0.19	0.064	0.88	3.23	0.054	0.91	2.73	0.051	0.92	2.60
Trp	0.42	0.05	0.019	0.87	4.52	0.015	0.92	3.57	0.015	0.92	3.66
Arg	3.98	0.67	0.155	0.95	3.89	0.113	0.97	2.84	0.132	0.96	3.32
Ile	1.80	0.23	0.064	0.92	3.56	0.055	0.94	3.06	0.047	0.96	2.62
Leu	3.65	0.31	0.096	0.91	2.63	0.083	0.93	2.27	0.087	0.92	2.38
Val	2.54	0.21	0.083	0.85	3.27	0.069	0.89	2.72	0.073	0.88	2.86
His	1.19	0.14	0.045	0.89	3.78	0.038	0.92	3.19	0.035	0.93	2.98
Phe	2.07	0.18	0.055	0.91	2.66	0.048	0.93	2.32	0.048	0.93	2.32
Tyr	1.47	0.16	0.069	0.83	4.69	0.069	0.82	4.69	0.068	0.83	4.66
Gly	7.23	1.92	0.441	0.95	6.10	0.287	0.98	3.97	0.353	0.97	4.89
Ser	2.30	0.25	0.106	0.82	4.61	0.085	0.89	3.70	0.097	0.85	4.20
Pro	4.84	1.43	0.426	0.91	8.80	0.320	0.95	6.61	0.299	0.96	6.18
Ala	4.18	0.65	0.168	0.93	4.02	0.116	0.97	2.78	0.136	0.96	3.24
Asp	4.50	0.45	0.127	0.92	2.82	0.102	0.95	2.27	0.102	0.95	2.28
Glu	7.07	0.87	0.214	0.94	3.03	0.158	0.97	2.23	0.163	0.96	2.31

Table 3. Statistical Comparison between the SECV Values Obtained for the Three Modes of Analysis Used ($F_{limit} = 1.41$; Significance Level = 95%)

	standard ring cup (ground samples)	rectangular natural cup (intact samples)	F	rectangular $^1/_4$ cup (ground samples)	rectangular natural cup (intact samples)	F
Met	0.042	0.035	1.44[a]	0.037	0.035	1.12
Cys	0.048	0.04	1.44[a]	0.041	0.04	1.05
M + C	0.067	0.053	1.60[a]	0.058	0.053	1.20
Lys	0.11	0.086	1.64[a]	0.095	0.086	1.22
Thr	0.064	0.051	1.57[a]	0.054	0.051	1.12
Trp	0.019	0.015	1.60[a]	0.015	0.015	1.00
Arg	0.155	0.132	1.38	0.113	0.132	1.36
Ile	0.064	0.047	1.85[a]	0.055	0.047	1.37
Leu	0.096	0.087	1.22	0.083	0.087	1.10
Val	0.083	0.073	1.29	0.069	0.073	1.12
His	0.045	0.035	1.65[a]	0.038	0.035	1.18
Phe	0.055	0.048	1.31	0.048	0.048	1.00
Tyr	0.069	0.068	1.03	0.069	0.068	1.03
Gly	0.441	0.353	1.56[a]	0.287	0.353	1.51[a]
Ser	0.106	0.097	1.19	0.085	0.097	1.30
Pro	0.426	0.299	2.03[a]	0.32	0.299	1.15
Ala	0.168	0.136	1.53[a]	0.116	0.136	1.37
Asp	0.127	0.102	1.55[a]	0.102	0.102	1.00
Glu	0.214	0.163	1.72[a]	0.158	0.163	1.06

[a] Significantly different ($p < 0.05$), the SECV values versus the values for the natural cup.

analysis modes (compared each two, intact versus ground) was carried out with the applications of the Fisher test. **Table 3** shows the SECV values for each amino acid and analysis mode and the F values obtained in each case, with the F_{limit} value 1.41 for a level of significance of 95%.

For the comparison between the ground (standard ring cup) and intact (rectangular natural cup) modes of analysis, the SECV values obtained for the equations developed using the samples analyzed in the standard ring cup were largest of those obtained with the samples analyzed intact. Moreover, for 13 of the 19 AA the differences between the SECV values were significant according to the F test.

The SECV values corresponding to the equations developed using the samples analyzed in the $^1/_4$ rectangular cup were also larger than those obtained with the samples analyzed intact, for 11 of the 19 AA predicted. However, when the F test is applied to the comparison of these two analysis modes, only the SECV value corresponding to the amino acid glycine result was statistically different for the natural cup. Nevertheless, the 1-VR value for the prediction of glycine in intact samples should be regarded as excellent (0.99) and CV as very good (3.01%). These results clearly confirm that the NIRS analysis of intact PAP samples is feasible and that it can replace the analysis in ground form.

Due to the high price of analyzing AA using HPLC, many nutritionists around the world use published regression equations based on the crude protein content to estimate the amino acid content in feedingstuff, as those reported in Degussa tables (*31*) and other nutritional databases. In the present work, the relationship between protein and amino acid content for the 92 samples studied was measured by the RSQ statistic, reaching values ≤ 0.50 for 11 of the 19 parameters studied, between 0.51 and 0.80 for lysine, phenilalanine, and proline and over 0.80 for arginine, glycine, alanine, aspartic acid, and glutamic acid. The RSQ values for each amino acid were always lower than the 1-VR values corresponding to the NIRS calibrations developed both with intact and ground samples (**Table 2**), offering further confirmation of the significant contribution that the practical implementation of NIRS equations could have from the nutritional point of view. These results allow one to conclude that NIRS prediction of AA in intact meat and bone meals are much better than predictions based on crude protein regressions.

Once the comparison between the three analysis modes, with the same number of samples, was made, the T outliers could then be eliminated as an essential step in the optimization procedure of the equations developed.

During calibration development, spectral outliers were not detected. However, chemical outliers were detected for the three sample presentation modes and for each of the amino acids. **Table 4** shows the statistics of the NIR equations for predicting AA in ground and intact PAPs, after the T outliers samples elimination.

With a few exceptions, the chemical outliers were consistent for the equations developed for the three sample presentation modes and, in most cases, the outlier samples are unique samples, reflecting an extreme chemical value for a given amino acid. For example, there was a sample outlier for glycine and proline that had the highest contents for these amino acids, i.e., 12.47% and 9.13%, respectively (**Table 1**). Moreover, another outlier sample presents the highest values for arginine (5.79%) and glycine (12.47%).

Table 4. NIRS Calibration Statistics for Equations Developed without *T* Outliers for the Two Presentation Modes (Ground vs Intact)

	standard ring cup: ground samples					rectangular 1/4 cup: ground samples					rectangular natural cup: intact samples				
constituent	mean	S.D.	SECV	1-VR	CV	mean	S.D.	SECV	1-VR	CV	mean	S.D.	SECV	1-VR	CV
Met	0.93	0.16	0.042	0.93	4.53	0.93	0.16	0.042	0.93	4.48	0.93	0.16	0.035	0.95	3.75
Cys	0.53	0.12	0.042	0.87	8.22	0.53	0.12	0.048	0.83	9.07	0.53	0.12	0.036	0.91	6.78
M + C	1.46	0.20	0.067	0.90	4.25	1.46	0.20	0.057	0.92	3.91	1.46	0.20	0.053	0.93	3.63
Lys	3.33	0.42	0.102	0.94	3.05	3.33	0.42	0.100	0.94	3.00	3.32	0.41	0.075	0.97	2.26
Thr	1.98	0.19	0.055	0.91	2.78	1.97	0.18	0.053	0.92	2.67	1.98	0.19	0.051	0.92	2.60
Trp	0.42	0.05	0.014	0.92	3.45	0.42	0.05	0.015	0.92	3.61	0.42	0.05	0.015	0.92	3.51
Arg	3.93	0.66	0.052	0.99	1.84	3.93	0.64	0.064	0.99	1.62	3.94	0.65	0.083	0.98	2.12
Ile	1.81	0.23	0.061	0.93	2.74	1.79	0.23	0.047	0.96	2.64	1.80	0.23	0.047	0.96	2.62
Leu	3.65	0.31	0.081	0.94	2.22	3.64	0.32	0.074	0.95	2.02	3.65	0.31	0.087	0.92	2.38
Val	2.55	0.21	0.066	0.90	2.36	2.53	0.21	0.066	0.90	2.62	2.53	0.21	0.067	0.90	2.63
His	1.19	0.13	0.036	0.93	3.00	1.19	0.14	0.038	0.92	3.22	1.19	0.13	0.030	0.95	2.53
Phe	2.07	0.18	0.052	0.92	2.49	2.07	0.18	0.047	0.93	2.27	2.07	0.18	0.048	0.93	2.32
Tyr	1.46	0.15	0.043	0.92	2.96	1.46	0.15	0.038	0.94	2.62	1.47	0.16	0.043	0.93	2.92
Gly	6.96	1.72	0.236	0.98	3.40	7.03	1.73	0.174	0.99	2.47	7.02	1.73	0.211	0.99	3.01
Ser	2.30	0.25	0.102	0.84	4.08	2.28	0.25	0.071	0.92	3.12	2.30	0.25	0.080	0.90	3.49
Pro	4.75	1.37	0.325	0.94	6.83	4.82	1.38	0.307	0.95	6.36	4.80	1.37	0.305	0.95	6.35
Ala	4.13	0.61	0.111	0.97	2.98	4.18	0.65	0.113	0.97	2.70	4.14	0.63	0.088	0.98	2.13
Asp	4.49	0.46	0.139	0.90	3.09	4.49	0.46	0.090	0.96	2.01	4.50	0.46	0.089	0.96	1.98
Glu	7.06	0.86	0.171	0.96	2.73	7.01	0.86	0.127	0.98	1.80	7.07	0.89	0.148	0.97	2.09

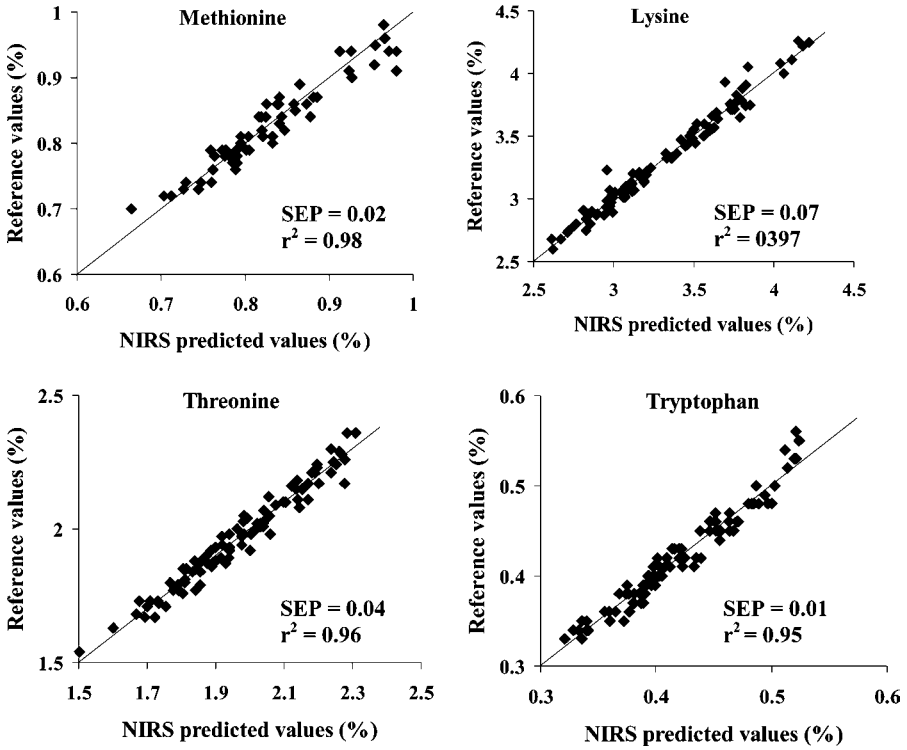

Figure 1. Reference values versus predicted NIRS values for the amino acids methionine, lysine, treonine, and tryptophan.

The outliers removal produced the reduction of the SECV values in all the cases. To better understand the significance of the SECV values obtained for the three analysis modes and in order to facilitate the comparison with the results obtained by other authors, the coefficient of variability (CV) for the NIR data was also calculated (**Table 4**).

The coefficient of variability (CV) relates the SD to the mean and provides a more realistic evaluation of the importance of the SECV. The size and interpretation of the CV depends partly on the source of the data. In the interpretation of NIR data, a CV value between 1.1 and 2.0% is excellent, between 2.1 and 3.0% is very good, between 3.1 and 4.0% is good, from 4.1 to 5.0% is fair, and CV higher than 5% is poor.

From the results shown in **Table 4**, it can be concluded that the CV values obtained for equations developed with ground samples analyzed in the standard ring cup were in most cases excellent, very good, or good, although in five cases (Met, Cys, M + C, Ser, and Pro) presented CV values higher than 4. For the ground samples analyzed in the 1/4 cup, only the amino acids Met, Cys, and Pro obtained CV values higher than 4, whereas the rest of them reached CV values of excellent, very good, or good. Similar conclusions can be obtained from the CV values reached in intact samples; however. in this case only two amino acids (Cys and Pro) offered CV values higher than 4.

These results (**Table 4**) confirm again the viability of analyzing PAPs samples in their natural form, avoiding the tedious and time-consuming milling task, since 1-VR, SECV, and CV values for all the essential AAs were similar to or better than those obtained in the ground samples.

Figure 1 shows the correlation of the values obtained in the laboratory with respect to those predicted by NIR by using the natural cup for four of the most critical amino acids (methionine,

Table 5. Comparison of the Results Obtained by Other Authors (*6* and *13*) and the Results of the Present Paper Regarding the CV Values

	Fontaine et al. (2001) (*6*)	Qiao and Van Kempen (2004) (*11*)	present study (**Table 4**)
Met	6.0	12.82	3.75
Cys	13.33	9.80	7.43
M + C	6.12		3.63
Lys	5.43	7.38	2.57
Thr	4.06	7.10	2.60
Trp	5.59		3.66
Arg	3.69		3.32
Ile	4.32	9.67	2.62
Leu	3.55	6.53	2.38
Val	4.46	9.28	2.86
His		11.01	2.98
Phe		9.04	2.32

lysine, threonine, and tryptophan) for ration formulation. From the SEP and the r^2 values, it may be inferred that the calibration models of all of the amino acids showed in **Figure 1** allow the determination of these amino acids with excellent accuracy.

The interpretation of the PLS spectral loadings for the NIR calibrations models developed showed patterns very similar for all of the amino acids. The main features were found in the regions 1650−1794, 1840−1940, and 2224−2394 nm. The regions 1650−1794 and 2224−2394 nm are characteristic of amino acids absorption, while the region 1840−1940 nm could be indicative of water absorption (*16*).

Further confirmation of the accuracy of the equations developed with intact samples could also be obtained for comparison of the results obtained in the present paper for intact PAPs and those obtained by other authors using ground samples.

Fontaine et al. (*6*) developed NIRS calibrations for prediction of essential amino acids (methionine, cystine, methionine + cystine, lysine, threonine, tryptophan, arginine, isoleucine, leucine, and valine) in ground meat meal ($n = 333$). Qiao and Van Kempen (*13*) also developed NIRS calibration equations for routine evaluation of amino acids in animal meals, scanned as received, without previous milling. In order to make comparable the results obtained by these authors with those obtained in the present work (**Table 4**), the coefficient of variation (CV) based on the SECV and the mean values of the Fontaine et al. (*6*) and Qiao and Van Kempen (*13*) studies have been calculated (**Table 5**).

It should be highlighted that the equations obtained by Fontaine et al. (*6*) and those reported in the present paper have in common that the reference AA data were provided by the same reference laboratory. Thus, the differences in the CV obtained for each AA should be explained by the better repeatability of the spectral data used in the present paper. That may be partially due to the use of the largest scanning surface (94 cm^2) compared to those used in the Fontaine et al. (*7*) study (11 cm^2).

However, the CVs calculated from the data of Qiao and Van Kempen (*13*) for all amino acids were much higher than those found in the present study, as can be seen in **Table 5**. In this case the huge differences existing in the CV values should be attributed to an insufficient number of samples used by Qiao and Van Kempen (*13*) for calibration development ($N = 54$) and, probably, also to the poorest repeatability of the AA reference method (HPLC).

In conclusion, the results obtained demonstrate that NIRS technology is suitable for analyzing processed animal proteins in their marketing presentation, avoiding the need for fine milling and reducing labor and time costs. The accuracy of the equations obtained in the present paper for the prediction of the most important amino acids in intact PAP samples lay within the usual range of values reported by other authors for ground samples. To maintain the desired quality and to fulfill the requirements of the consumers of PAPs, a quality control unit in each individual rendering plant is needed. This work reveals great expectations for the on-line implementation of NIRS technology in individual plants, feed mills, and customs inspection points during transportation.

ABBREVIATIONS USED

NIRS, near-infrared reflectance spectroscopy; SECV, standard error of cross-validation; 1-VR, fraction of explained variance for cross validation (square of the correlation coefficient, *r*); CV, coefficient of variation; RSQ, fraction of explained variance for linear crude protein regression (square of correlation coefficient, *r*); *N*, number of samples of the equation; SD, standard deviation of the variable in the sample population; RPD, ratio of the standard deviation divided by the SECV; PAPs, processed animal proteins; AA, amino acids; SEP, standard error of prediction for independent validation samples; *r*, coefficient of correlation.

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