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Routine Techniques for Monitoring the Nutritional Value of Animal Meals

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INDUSTRY SUMMARY

Introduction

To prevent the large variation in nutritional quality of animal meals from becoming a bottleneck for the incorporation in feeds, techniques are needed that allow for the rapid quantification of their actual nutritional value. A method that was previously pioneered relied on using in vivo digestibility data to train Near Infrared Spectroscopic (NIRS) instruments such that the NIRS could predict the in vivo response. Although this method was proven to work, it is practically too costly and cumbersome as it relies on in vivo digestibility assays and a bare minimum 40 to 50 samples are needed to train the NIRS, which would mean that a method for a single feedstuff would cost over k$100. A more practical alternative would be to develop a validated in vitro method on which techniques such as NIRS could be based. Using an in vitro method 50 to 100 samples can be assayed in a short period of time for a fraction of the cost of the in vivo assays, and if the data are biologically relevant, indirect methods can be based on the in vitro data.

Objectives

In brief, an in vivo validated in vitro technique is to be developed, and the in vitro digestible amino acid content is quantified for approximately 70 animal meal samples. These samples are subsequently used to develop NIRS, Fourier Transform Infra Red spectroscopy (FTIR), and Raman spectroscopy[1] calibrations relating spectral data to total amino acids, and in vitro and in vivo digestible amino acids. These calibrations can then be used to predict the nutritional value of an animal meal sample in 2-5 min with a variable cost that is not much more than the labor required for the handling of the sample

[1] A detailed explanation of these spectroscopic techniques has been published in the March issue of the World Poultry Science Journal.

Industry summary

An in vitro digestibility method has been developed that yields data that approximate in vivo digestibility better than existing in vitro techniques. Nevertheless, on average, the in vitro digestibility was a couple percent (2-10%) lower for lysine than the in vivo measurements, making extrapolation from in vitro to in vivo digestibility unreliable. The reasons are possibly 1) solubility problems of the in vitro digested sample; 2) lack of intestinal peptidases in the in vitro system; 3) particle size effects of the meat and bone samples on digestibility, and 4) lack of removal of end products of digestion. The in vitro method thus requires additional work before it can be used as a reference method for the development of spectroscopic prediction equations.

Using spectroscopic methods such as NIRS and FTIR, calibrations could be developed for total, in vitro, and in vivo digestible amino acids. The prediction error for lysine was approximately 0.25 (%) and the r^2 was 0.85 for both methods. Using additional samples, further improvements in this prediction error are very feasible.

Upon careful analysis of in vivo digestibility data it was found that digestibility of the samples is actually a minor component of the variation in digestible amino acids (less than 10% of the total variation). This suggests that a method for predicting total amino acids and using a fixed digestibility coefficient for calculating digestible amino acids is nearly as valuable as a method for measuring digestible amino acids. The estimated prediction error for this method is 0.21 (%) for digestible lysine.

MANUSCRIPT

Scientific abstract

The animal industry is in need of methods that can assess the nutritional value of feed ingredients such as animal meals, which are suitable for routine use. Infrared spectroscopy can be trained to predict digestible amino acids. However, the use of in vivo reference data makes this an expensive and time-consuming proposition. As an alternative the possibility to develop an in vitro digestibility assay that could be used for training infrared spectroscopy was investigated.

The in vitro digestibility assay was developed on the premise that in vitro digestion should be maximized using pepsin and pancreatin as the digestive enzymes but using minimal enzyme levels such that contamination from enzyme catalysis can be minimized. The method developed uses 500 mg of meat and bone meal incubated with 0.7 mg of pepsin for 24 h at pH 2, followed by incubation with 13 mg trypsin-enriched pancreatin for 96 h at pH 8. Although this method maximally digested animal meal samples, the actual digestibility coefficients were still somewhat lower than the in vivo digestibility coefficients (up to 10%), making this method not yet suitable as a reference. The reason for this is not known, but one possibility is inadequate solubilization of the digested material prior to analysis.

Using in vivo poultry digestibility data obtained on 25 animal meal samples and total amino acid data on over 70 animal meal samples, calibrations using both NIRS and infrared calibrations could be developed that explained approximately 85% of the variation in total and digestible lysine. These data also showed that digestibility was responsible for less than 10% of the variation in digestible amino acids; thus, total amino acid data together with fixed digestibility coefficients can be used to predict digestible amino acids with good accuracy.

In conclusion, this research confirmed that infrared spectroscopy could be trained to predict digestible amino acids based on in vivo digestibility data. Digestible amino acids, though, can also be predicted with good accuracy from total amino acids for which infrared calibrations can be developed with great ease.

Key words: Digestible amino acids; FTIR; NIRS; Meat and bone meal; swine; poultry

Introduction

To prevent the large variation in nutritional quality of animal meals from becoming a bottleneck for the incorporation in feeds, techniques are needed that allow for the rapid quantification of their actual nutritional value. A method that was previously pioneered relied on using in vivo digestibility data to train Near Infrared Spectroscopic (NIRS) instruments such that the NIRS could predict the in vivo response. Although this method was proven to work, it is practically too costly and cumbersome as it relies on in vivo digestibility assays; at a bare minimum 40 to 50 samples are needed to train the NIRS, which would mean that a single method would cost over k$100. A more practical alternative would be to develop a validated in vitro method on which techniques such as NIRS or FTIR could be based. Using an in vitro method, 50 to 100 samples can be assayed in a short period of time for a fraction of the cost of the in vivo assays, and if the data are biologically relevant, indirect methods can be based on in vitro data.

Experiment procedures (Materials and Methods)

The objective of this study was to develop methods, thus materials and methods are described under 'results and discussion'.

Results and discussion

In vitro digestibility assay:

The in vitro digestibility assay that serves as a basis for this project had as objective to mimic the digestive process in the animal. Although this seems like a logical assumption, many digestibility assays have been proposed that only attempted to correlate with *in vivo* digestibility. The following assumptions were used for the development of this assay: 1) Digestive capacity in the animal is not limiting the breakdown of feedstuffs, which means that the animal digests feedstuffs to completion (as far as it can be digested with the available enzymes). 2) Enzymes can be hydrolyzed, and contamination of the digested fraction with enzymes should be minimized or quantified. 3) Material that is solubilized (through enzymatic action) is considered digestible (measured after centrifugation of the sample using amino acid analysis).

Based on these assumptions the following design criteria for the in vitro digestibility assay were chosen. 1) Enzymes used will be pepsin and pancreatin (a crude mixture of pancreatic enzymes); the latter is supplemented with trypsin to boost trypsin activity. 2) A combination of incubation time and enzyme levels needs to be used that maximizes the degradation of the feedstuff such that added time and/or enzyme does not result in a further breakdown of the feedstuff. 3) The enzyme levels should be minimized such that autolysis of the enzyme does not yield substantial contamination of the digested fraction. These assumptions were used to design experiments to determine the enzyme levels and incubation times that were needed to maximally digest meat and bone meal samples.

The findings from these experiments are that pepsin plays an insignificant role in total degradation of meat and bone if sufficient levels of pancreatin are used (Fig. 1). Pepsin only degrades a small number of peptide bonds, a number that is easily and quickly surpassed by pancreatin. Although we thus judged pepsin to not be required for the in vitro digestibility assay, it was decided to include a minimal amount of pepsin in the final assay to keep our assay more in line with that of others. The level chosen was 0.25% pepsin-protein relative to the meat and bone

meal protein to be digested, and the selected incubation time was 24 h at pH 2. This level of pepsin corresponds well with the pepsin secretion in vivo.

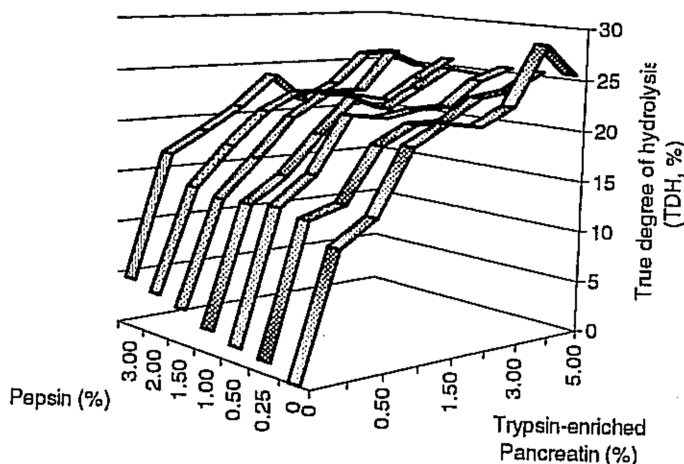

FIGURE 1. Hydrolysis (% total peptide bonds) of meat and bone meal after incubation with pepsin (%, pepsin to substrate protein ratio) for 24 h and trypsin-enriched pancreatin (%, trypsin-enriched pancreatin to substrate ratio) for 96 h.

Pancreatin, under in vitro conditions, breaks down approximately 30% of the peptide bonds in a typical meat and bone meal sample (Fig. 1). The average peptide that is thus produced is three amino acids long. This degree of degradation is achieved by using a pancreatin protein level equivalent to 4.8% of the MBM protein and a 96 h incubation period (Table 1).

Autolysis of enzymes does occur and may interfere with the determination of digestibility. This autolysis is dependent on the time of incubation and the original enzyme concentration. The extent of autolysis, however, was assumed not substantially affected by the presence of meat and bone meal, and thus contamination can be determined by running the in vitro assay in the absence of substrate (thus as an enzyme-blank).

TABLE 1. Maximal digestion of meat and bone meal protein by trypsin-enriched pancreatin in the 2nd stage following the digestion by 0.25% pepsin in the 1st stage, and estimated minimal pancreatin usage required for meat and bone meal samples.

	Parameter			
	c (%)[1]	b_0[1]	k_n (%$^{-1}$)[1]	E_{min} (%)[2]
Mean (n=9)	26.9 ± 1.6	0.12 ± 0.01	0.56 ± 0.05	5.4 ± 0.4

[1]Parameters were expressed as estimate ± SE (standard error). The nonlinear model used to obtain the parameters, the true degree of hydrolysis (TDH) as a function of the pancreatic enzyme usage, was $TDH=c_{(i)}*(1-(1-b_{0(i)}*\exp(-k_{n(i)}*E)))$ where $c_{(i)}$ is the maximal degree of hydrolysis of sample i, $b_{0(i)}$ is the parameter used to estimate the non-zero starting point for sample i ($c_{(i)}*b_{0(i)}$=TDH at pancreatin level of 0), $k_{n(i)}$ is the digestion constant for sample i, and E is the added pancreatin usage (%, enzyme weight to substrate protein weight ratio).

[2]$E_{min(i)}$, the minimal pancreatic enzyme level required to reach 95% of the maximally true degree of hydrolysis ($c_{(i)}$) for sample i, was calculated from $E_{min(i)}=\ln(20-20*b_{0(i)})/k_{n(i)}$.

The final in vitro digestibility assay that was developed is a two-phase digestibility procedure. In the first phase, the sample of meat and bone meal (approximately 500 mg) is incubated with 0.25% pepsin (relative to meat and bone meal protein) for a period of 24 h in a citrate buffer at pH 2. After 24 h, the pH of the mixture is adjusted to 8 with phosphate buffer,

and trypsin-enriched pancreatin is added at 4.8% protein (relative to meat and bone meal protein), and the sample is incubated for an additional 96 h at 38°C with continuous agitation.

In this assay, enzymes add 5% additional protein, and approximately 30% of the enzyme peptide bonds are hydrolyzed (thus the enzyme autolysates are on average 3.3 amino acids long and can be fractionated as digestible feed protein). This autolysis is corrected for by using an enzyme blank.

For measuring amino acid digestibility, the sample is centrifuged at 15,000 g after digestion. The supernatant is subsequently analyzed for amino acids that are considered digestible.

Validation of the in vitro assay:

Typically, in vitro digestibility assays are 'validated' by comparing a large number of feedstuff samples that have been assayed both in vivo and in vitro. Although this procedure is the preferred, it is too expensive. Consequently, a more functional validation approach was selected. A further benefit of this approach was that it enabled us to develop a better understanding of digestive system functioning.

Three meat and bone meal samples were selected that have poor, medium, and high digestibility. The meat and bone meal samples were treated with methyl-iso-urea to convert lysine to homo-arginine, a process called guanidination. The homo-arginine serves as a unique marker for MBM digestion since pigs do not produce it and it reportedly behaves like lysine in the digestive tract.

These modified meat and bone meal samples were subjected to two different treatments:

1. Ileal digestibility was determined in pigs using standard procedures. As the pig does not produce homo-arginine, any homo-arginine excreted through the ileal canula can be considered undigested meat and bone meal. This allows for the determination of the real sample digestibility (which is what is determined in vitro as well).
2. In vitro digestibility was determined using the procedure outlined above.

The in vivo digestibility experiment was carried out in six ilealy-cannulated pigs. Test diets were comprised of meat and bone meal such that it provided 17% CP, sucrose, starch, and corn oil (to improve palatability and reduce dust), premix, and chromium oxide as a marker. Animals were limit-fed twice daily. During the adaptation period of 5 days, animals were fed non-guanidinated diets and on the collection days, animals were fed guanidinated meat and bone meal. Collections were carried out over a 15 h period starting at the first feeding and ending 5 h after the second feeding.

The primary objective of this experiment was to compare the extent of homo-arginine digestion in vivo and in vitro. Our hypothesis was that in vivo, a part of the meat and bone meal was not digestible but present in large molecular weight soluble peptides that could confound the in vitro assay if the entire soluble fraction was considered digestible.

Digesta from both the in vivo and the in vitro assays were centrifuged to remove insoluble material, and the soluble fraction was size-separated using HPLC and homo-arginine was quantified in each size fraction. The relative amounts of homo-arginine in digestible and indigestible fractions could then be compared (Fig. 2).

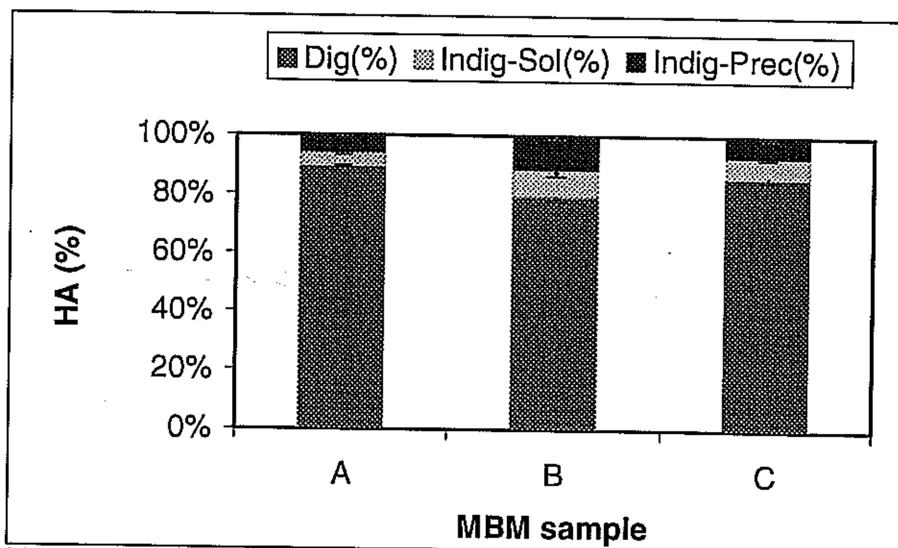

Fig. 2. In vivo homo-arginine digestibility of the three guanidinated meat and bone meal samples. Three fractions were observed: homo-arginine that was absorbed (Dig), homo-arginine that was in insoluble and un-absorbed proteins (Indig-Prec), and homo-arginine that was soluble but not absorbed (Indig-sol).

TABLE 2. In vivo and in vitro digestion of homoarginine in guanidinated meat and bone meal (MBM) samples[1]

MBM Sample	Homoarginine Content (%)			Homoarginine Digestibility Coefficient (%)	
	Total	In Vivo dig. (N=6)	In Vitro dig. (N=3)	In Vivo (N=6)	In Vitro (N=3)
A	1.86	1.66 (0.01)[b]	1.33 (0.07)[a]	89.5 (0.4)[c]	70.9 (3.7)[ab]
B	1.69	1.34 (0.03)[a]	1.36 (0.01)[a]	79.4 (1.6)[b]	79.7 (0.3)[ab]
C	2.50	2.14 (0.04)[d]	1.95 (0.01)[c]	85.7 (1.5)[bc]	77.7 (0.4)[a]

[1]Values are expressed as mean (SEM). Significant difference ($P<0.05$) is denoted by the difference in superscripts.

The in vivo homo-arginine digestibility ranged from 79.4 to 89.5% for the three test samples (Table 2). The indigestible fraction was made up for 55% of precipitated (centrifuged at 1,000*g*) or insoluble proteins; the remainder consisted of small molecular weight peptides (less than 1,000 Dalton or 8 amino acids, see Fig. 3). Although it was not completely unexpected to find such small peptides, the magnitude of this fraction was rather surprising. It suggests that a significant portion of the amino acids in these meat and bone meal samples could be broken down to a size that should be digested, but was in fact excreted.

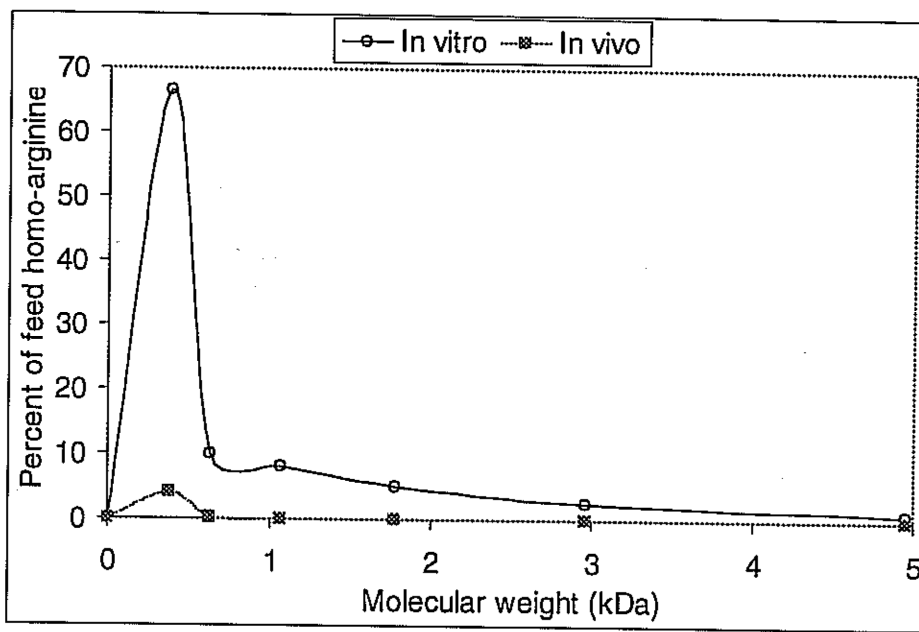

Fig. 3. Size fractionation results for the soluble digesta obtained after in vitro or in vivo digestion of meat and bone meal. In vivo resulted in 6.7% of the homo-arginine being soluble but not digested. The peptides in this fraction were less than 1kDa in average size. In vitro, a wider distribution is seen, with approximately 12% of the homo-arginine being contained in peptides between 1 and 5 kDa in size.

The reason for finding such small peptides is not clear. It is possible that this is a normal physiological phenomenon; however, since feedstuffs such as casein are virtually 100% digestible, it is unlikely that these small peptides simply reflect limits in absorptive capacity. More likely these peptides consist of damaged protein fragments or damaged amino acids, or combinations of amino acids and, e.g., carbohydrates, which inhibit absorption. Such compounds may well originate during regular processing of meat and bone meal when these products are heated to high temperatures. Another possibility is that these compounds arise from the guanidination process needed to convert the lysine in meat and bone meal to homo-arginine. This process is carried out at high pH and at room temperature, and these conditions are known to lead to the production of D-isomers of amino acids, which are poorly absorbed. Homo-arginine itself may also lead to problems with absorption, as has been shown in poultry when administered at high levels (our animals were fed diets containing 17% crude protein (CP) derived solely from guanidinated meat and bone meal.

These problems limit the usefulness of the validation experiment; absolute digestibility data are probably not comparable to in vitro data. However, opposite to what would be expected, the in vitro digestibility assay of these guanidinated meat and bone meals suggested that these samples had a real digestibility that was lower than that observed in vivo. This is proof that the in vitro assay did not yield digestibility coefficients comparable to in vivo.

Notwithstanding the problems with the in vivo assay, these in vivo data provide us with useful information. The size fractionation data showed that the soluble fraction in vivo consisted only of very small peptides/molecules (< 1000 Dalton). In vitro, a large portion of the homo-arginine was in the same fraction, but approximately 12% was in the fraction from 1,000 Dalton to 5,000 Dalton (no peptides were observed between 5,000 and 14,000 Daltons). This fraction was not observed in vivo. These data suggest that in vitro digestion is less complete than in vivo digestion (possibly due to product inhibition of the enzyme activity), but they also suggest that

peptides of this size should be considered digestible, as apparently they have no structural flaws that make them indigestible as shown by the in vivo results.

Other findings that were interesting are based on the distribution of lysine and homo-arginine in these fractions (Fig. 4). The guanidination efficiency for our MBM samples was 74.3%. Thus, the ratio of homo-arginine to lysine was 3. If the MBM samples were broken down indiscriminately (the guanidination had occurred randomly), then each size fraction should contain the same ratio of homo-arginine to lysine from MBM. Degradation of enzymes in vitro and endogenous secretions in vivo can lower this ratio, as only lysine should be present in these enzymes/secretions.

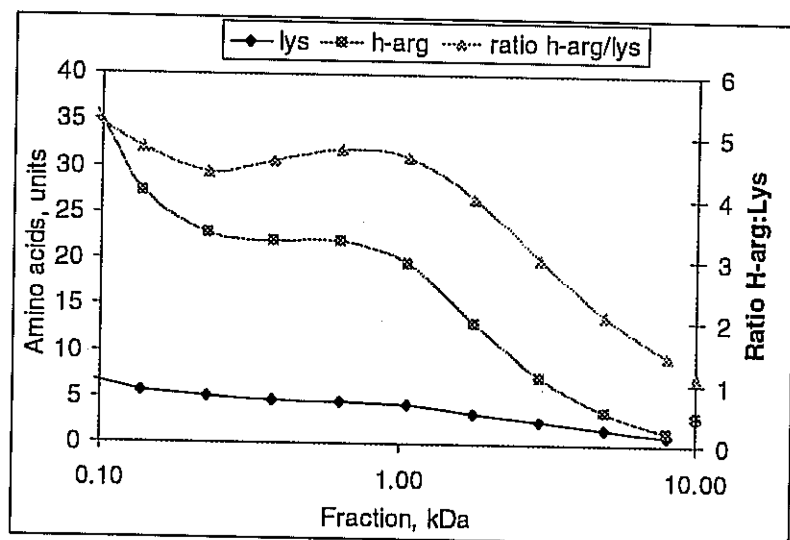

Fig. 4. Lysine and homo-arginine in in vitro digesta as a function of the molecular weight of the peptides, and ratio of homo-arginine to lysine in these molecular weight fractions. Both lysine and homo-arginine were found in all fractions, but lysine was relatively more abundant in the large molecular weight fractions. The fraction listed at 10 kDa is the insoluble fraction, which has an unknown molecular weight.

In vitro, however, a ratio of 5 was observed in the small molecular weight fractions, and ratios of less than 3 (1 to 2) were observed in the large molecular weight fractions. This implies that in the guanidinated meat and bone meal homo-arginine was more digestible than lysine. Although this contradicts the observation of researchers in the field of homo-arginine digestion, it does make sense intuitively that the peptides that were easiest to guanidinate were also the easiest to digest, and that those peptides that were inaccessible by methyl-iso-urea were also the most difficult to digest.

In vivo, differences in distribution of homo-arginine and lysine were even more pronounced (Fig. 5). Homo-arginine was only found in the low molecular weight fractions and in the insoluble fraction, but lysine was present in all fractions. In the small molecular weight fraction, the ratio of homo-arginine to lysine was much less (0.3 to 0.5) than expected based on the conversion efficiency of lysine to homo-arginine and based on the in vitro data. This indicates that 90% of the lysine found was of endogenous origin. In the in vivo insoluble fraction, a ratio of homo-arginine to lysine of 0.8 was observed, indicating a large presence of endogenous lysine as well as indigested meat and bone meal lysine.

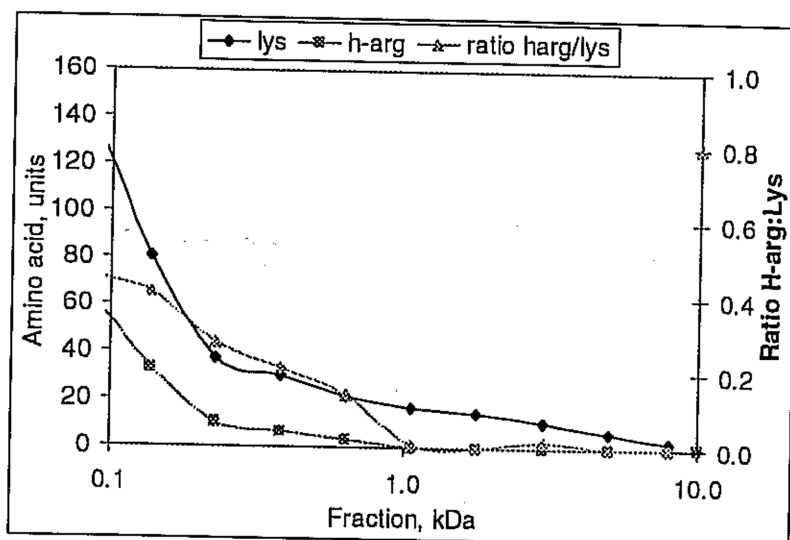

Fig. 5. Lysine and homo-arginine in ileal digesta as a function of the molecular weight of the peptides, and ratio of homo-arginine to lysine in these molecular weight fractions. Homo-arginine was only found in fractions smaller than 1 kDa, as discussed in the text. Lysine, however, was found in all fractions as a result of endogenous proteins being secreted and partially digested. These data indicate that endogenous excretions are present in the small molecular weight fractions. The fraction listed at 10 kDa is the insoluble fraction, which has an unknown molecular weight.

Our interpretation of these data is that in the in vitro digestibility assay, proteins of 1,000 Daltons to 5,000 Daltons should be considered digestible. Proteins smaller than 1,000 Daltons, however, may not be 100% digestible, possibly because of structural flaws in the proteins. Whether this is practically relevant in vivo is unclear.

Because of problems with the planned validation experiment, an additional validation was thus needed, and a unique opportunity was presented through FPRF. FPRF made 25 samples of animal meals available (of which one was considered an outlier) with known in vivo digestible amino acid contents, as determined by Dr. Carl Parsons of the University of Illinois. These samples were assayed using the outlined in vitro digestibility assay, in which all soluble proteins were considered digestible.

Good correlation between in vitro digestible amino acids and in vivo digestible amino acids were found. The results of this comparison are provided in Fig. 6, which looks encouraging as it is based on digestible amino acids rather than digestibility. For digestibility (Table 3), 64% of the Parsons' samples yielded an in vitro lysine digestibility that was within 10% of the in vivo value. However, 36% of the samples were predicted with an error that exceeded 10%. Statistically, no correlation was found between the in vivo and in vitro digestibility coefficients. We thus deemed this distribution unacceptable for practical use of the method.

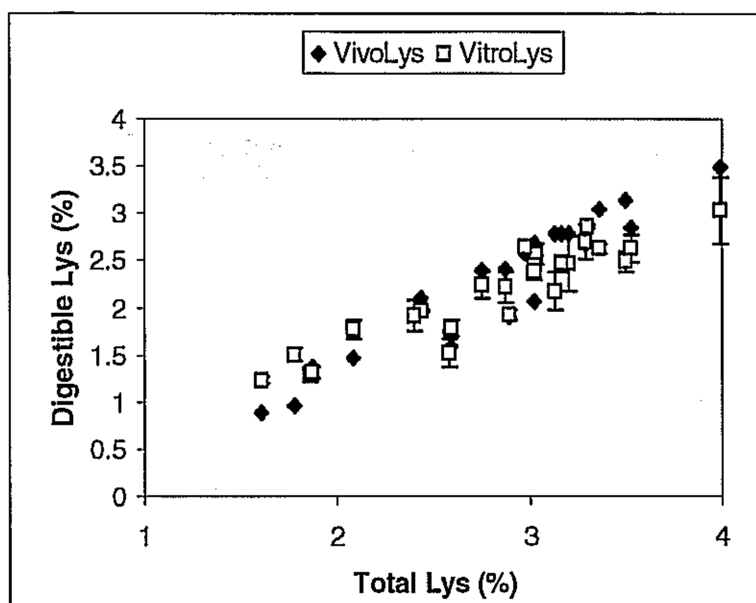

Fig. 6. Digestible lysine measured in vivo (VivoLys) and in vitro (VitroLys), as a function of lysine content in the test samples.

TABLE 3. Variation of in vivo ileal amino acid digestibility and in vitro amino acid digestibility of animal meals

	Digestibility Coefficient (%)				Absolute difference between in vivo and in vitro digestibility coefficients		
	N=25				Range		
	In vivo		In vitro		<10%	10%-20%	>20%
AA	mean	SD	Mean	SD	% of samples		
His	76.2	8.6	68.2	10.5	48	20	32
Ile	84.5	3.9	74.5	8.7	44	40	16
Leu	84.8	5.0	76.2	8.3	48	44	8
Lys	78.7	11.2	77.2	8.4	64	28	8
Met	80.6	9.3	75.2	13.0	48	32	20
Phe	87.4	13.5	77.7	9.4	32	36	32
Thr	79.2	6.8	74.9	14.0	52	32	16
Val	82.7	4.3	79.2	9.8	68	32	0

These validation results also showed an underestimating of digestibility. Digestibility in only two of the Parsons' samples was substantially overestimated; most of the variation comes from underestimation. In vivo, the lysine digestibility averaged 78.7%, and in vitro, it averaged 77.2%.

It is interesting to note that in vivo and in vitro digestible amino acids are correlated but not the in vivo and in vitro digestibility coefficients. The variation in digestibility coefficients is small (approximately 20%) compared with variation in digestible amino acid contents (over 100%). Thus it is more difficult to detect variation in digestibility coefficients than digestible amino acid contents.

Practically speaking, it is the digestible amino acid content of a sample that is of importance, not the digestibility, even though the industry typically talks about digestibility. The latter parameter is also subject to larger errors in our in vitro setup as it accumulates the measurement error in total and in digestible amino acids: in the in vitro assay, digestible amino

acids are measured directly with an error in the amino acid assay of approximately 5% (as the assay is only repeated once). The error in the digestibility coefficient associated with measurement error of amino acid is thus at approximately 7%, leaving no room for other errors. In addition, the in vivo assay for measuring digestibility is subject to error as well (even though it is the gold standard). This error is estimated at 3% for the poultry assay, which implies that an error of 8% between the in vitro and in vivo assay is unavoidable using the current methods for measuring in vivo digestibility and amino acids. Such an error clearly borders on what is acceptable but is in line with what is observed here. Possibly the conclusion that the in vitro method does not work is not because it really doesn't work but because we are caught up comparing the wrong parameter that is subject to too many measurement errors...

Spectroscopic methods for predicting the nutritional value of animal meals:

The original objective of this project was to develop rapid analytical techniques for predicting the nutritional value of meat and bone meals. The above described in vitro digestibility assay was to serve as the reference for this rapid technique, not the actual rapid technique. For rapid techniques, it was originally proposed to work with near infrared spectroscopy (NIRS), mid infrared spectroscopy (FTIR), and nuclear magnetic resonance (NMR). Upon discussing the state of the bench-top NMR technology with experts it was decided to drop this comparison as the instrumentation was considered too expensive and of too low a resolution to be helpful at this point. The NMR was, therefore, replaced with a third spectroscopic technique: Raman. Raman is a variant of FTIR but has as a major advantage of being easier to use, and is expected to become a major quality control method in the near future. All these techniques are indirect measures. In other words, they are not able to directly determine the nutrient content of a sample. Instead, they work by correlating the nutrient content of a series of known samples with spectral information, and based on that, they can predict the composition of an unknown sample.

In order to generate a set of reference samples that were sufficiently diverse to cover the type of material typically found in the field, FPRF members were asked to collect meat and bone meal samples that were both typical and a-typical. From this effort, over 200 samples of meat and bone meal were collected. These samples were subsequently scanned with a NIRS (NIRSystems model 6500) spectrometer, and based on spectral information, 50 samples were selected that covered the range of material most uniformly.

These samples, as well as 25 Parsons' samples (for a total of 75 samples), were evaluated by the in vitro digestibility assay as outlined above but due to the problems with the in vitro digestibility assay these results will not be discussed (the calibrations for in vitro digestible amino acids were actually better than those for in vivo digestible amino acids, suggesting that in vitro digestibility corresponded better with the organic composition of the samples). Instead, calibrations were developed for total amino acids and in vivo digestible amino acids. Results suggest that by using either NIRS or FTIR (Nicolet model Magna 760; samples were ground through a 0.5 mm screen prior to analysis, which was carried out with a multi-bounce horizontal ATR crystal) it is possible to predict the total or in vivo digestible lysine content with an accuracy of approximately 0.25% (Table 4 and 5). Overall, NIRS and FTIR yielded very comparable results and both instruments were very easy to use.

TABLE 4. Prediction of total, in vivo, and in vitro amino acid contents through NIRS spectra obtained from animal meal samples scanned as-is[1, 2]

AA	Total			In vivo digestible			In vitro digestible		
	N	R^2	RMSEP	N	R^2	RMSEP	N	R^2	RMSEP
Cys	50	0.49	0.08	18	0.10	0.08	not analyzed		
Ile	60	0.58	0.20	18	0.06	0.25	59	0.72	0.16
Leu	59	0.69	0.31	17	0.52	0.27	59	0.69	0.26
Lys	64	0.83	0.25	23	0.88	0.23	65	0.85	0.21
Met	66	0.67	0.10	23	0.58	0.11	62	0.58	0.09
Thr	61	0.64	0.18	18	0.21	0.16	58	0.67	0.16
Val	57	0.59	0.21	18	0.46	0.22	58	0.58	0.22

[1]Samples were scanned in the near infrared region (1100-2500 nm). For lysine, methionine, and histidine, the in vivo samples used for regression were composed of meat and bone meals and feather meals (N=6). For other amino acids, the in vivo samples were composed of meat and bone meal only.
[2] RMSEP (in %): root mean square error of prediction (%). N: number of samples.

TABLE 5. Prediction of total, in vivo, and in vitro amino acid contents through FTIR spectra obtained from ground animal meal samples[1, 2]

AA	Total			In vivo digestible			In vitro digestible		
	N	R^2	RMSEP	N	R^2	RMSEP	N	R^2	RMSEP
Cys	47	0.72	0.06	15	0.49	0.05	Not analyzed		
Ile	55	0.56	0.20	15	0.18	0.14	55	0.72	0.16
Leu	52	0.64	0.32	18	0.37	0.38	50	0.69	0.26
Lys	56	0.81	0.25	21	0.85	0.26	54	0.69	0.28
Met	57	0.66	0.10	20	0.90	0.06	54	0.62	0.09
Thr	53	0.58	0.19	No calibration			51	0.44	0.21
Val	51	0.56	0.20	13	0.53	0.15	50	0.48	0.23

[1]For lysine, methionine, and histidine, the in vivo samples used for calibration were composed of meat and bone meals and feather meals (N=6). For other amino acids, the in vivo samples were composed of meat and bone meal only.
[2]RMSEP (in %): root mean square error of prediction (%). N: number of samples.

Somewhat surprising, Raman (obtained with a Nicolet bench) calibrations were not successful (data not shown). The reason for this was obvious: the Raman spectra were very difficult to collect as the meat and bone meal samples emitted too little Raman-shifted light to allow for accurate measurements. The resulting spectra thus had a poor signal to noise ratio, making calibrations unsuccessful.

Upon careful analysis of in vivo digestibility data it was found that digestibility of the samples is actually a minor component of the variation in digestible amino acids (less than 10% of the total variation; variation in the total amino acid content was much more important, Table 6). This suggests that a method for measuring or predicting total amino acids and using a fixed digestibility coefficient for calculating digestible amino acids in meat and bone is nearly as valuable as a method for measuring digestible amino acids. The estimated prediction error for

this method is 0.21 (%) for digestible lysine. An infrared method for predicting total amino acids, however, is much easier to develop.

TABLE 6. Prediction of in vivo digestible amino acid contents from total amino acids acids[1]

AA	R^2	RMSEP
His	0.93	0.08
Ile	0.99	0.10
Leu	0.97	0.20
Lys	0.91	0.21
Met	0.92	0.05
Phe	0.91	0.27
Thr	0.97	0.12
Val	0.99	0.16

[1]RMSEP: root mean square error of prediction (%).

Conclusion

Knowledge of the digestible amino acid content of animal meal samples is an important quality control tool for the animal meal industry and also for the feed industry utilizing animal meals. The tools that were evaluated in this project were intended for the rapid evaluation of the digestible amino acids in animal meals. Major advantages of spectroscopic tools are that they are easy to use for quality control, and that they are easy to update when considering the reference database based on in vitro digestibility.

Findings from this project are:

- An easy to use in vitro digestibility assay has been developed that maximally digests animal meals with minimal use of enzymes (to minimize contamination by enzymes)
    - This method was expected to mimic in vivo digestion, but a direct comparison with in vivo data has shown that digestibility is underestimated with 1.5-10.3% on average, likely because of improper solubilization of the digested fraction prior to analysis.
- Both Near Infrared Reflectance Spectroscopy (NIRS) and Fourier Transform Infrared Spectroscopy (FTIR) were well suited for predicting total and digestible amino acids
    - As a smaller sample size was used for the FTIR analysis it is preferable that samples are ground prior to analysis, making NIRS analysis more convenient.
- Raman spectroscopy was not suited for predicting amino acids in animal meals, as the signal strength was too weak.
- Variation in the digestible amino acid content in animal meals was attributed for at least 91% to variation in total amino acids, and for the rest to variation in digestibility and amino acid analysis.
    - Digestible amino acids can thus be predicted from total amino acids with a very good accuracy

Industry application:

An in vitro digestibility method has been developed that yields data that approximate in vivo digestibility better than existing in vitro techniques. Nevertheless, on average, the in vitro

digestibility was a couple percent (2-10%) lower for lysine than the in vivo measurements with a measurement error that makes extrapolation from in vitro to in vivo digestibility unreliable. The reasons for the lower digestibility are possibly 1) solubility problems of the in vitro digested sample; 2) lack of intestinal peptidases in the in vitro system; 3) non-uniform particle size of the meat and bone samples, and 4) lack of removal of end products of digestion. The in vitro method thus requires additional work before it can be used as a reference method for the development of spectroscopic prediction equations.

Using spectroscopic methods such as near (NIRS) and mid (FTIR) infrared spectroscopy, calibrations could be developed for total and in vivo digestible amino acids. The prediction error for lysine was approximately 0.25 (%) and the r^2 0.85 for both parameters and both methods. Using additional samples, further improvements in this prediction error are very feasible.

Upon careful analysis of in vivo digestibility data it was found that digestibility of the samples is actually a minor component of the variation in digestible amino acids (less than 10% of the total variation; variation in the total amino acid content was much more important). This suggests that a method for measuring or predicting total amino acids and using a fixed digestibility coefficient for calculating digestible amino acids in meat and bone is nearly as valuable as a method for measuring digestible amino acids. The estimated prediction error for this method is 0.21 (%) for digestible lysine. A method for total amino acids, however, is much easier to develop.