

Methods for Determination of Amino Acids Bioavailability in Pigs* - Review -

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ABSTRACT : Methods developed for measuring digestibility and availability of amino acids in feedstuffs used in pig nutrition are reviewed. Digestibility is a proportion of an amino acid in a feed that is absorbed from the digestive tract and should be determined from the difference between the amount of amino acid consumed and passing the distal ileum. Techniques for ileal digesta sampling including various types of cannulas: a re-entrant, T-piece, IPV, IPVC and ileorectal anastomosis are described and comparisons amongst these methods are presented. Other methodologies like mobile bag technique, *in vitro* assays and mathematical prediction method are also described. Significance and methodologies for measurement of endogenous nitrogen and amino acids losses

at the distal ileum and their effect on the apparent and true nitrogen and amino acid digestibilities in feeds are discussed. Factors influencing the apparent and true amino acid digestibilities such as dry matter intake, protein, fibre and antinutritive compounds content in the diet are discussed. Amino acid bioavailability - the proportion of the total amino acid digested and absorbed in a form utilized in metabolism - measured by the growth assay may differ from its ileal digestibility. Chemical methods for determination of available lysine content in heat treated feeds are evaluated.

(Key Words: Amino Acids, Bioavailability, Factors, Methods, Pigs)

INTRODUCTION

The amino acid content of feedstuffs determined by standard chemical analyses does not adequately indicate the extent to which feedstuffs protein can meet the animal's amino acid requirements. In practical pig feeding information on the availability of amino acids has great economic value, permitting the formulation of diets that meet amino acid requirements without providing unnecessary excess.

To formulate diets properly the correct amounts and balance of essential amino acids are needed to cover the pig requirement and to support a specified level or type of production. To do this effectively the amounts of biologically available amino acids in feedstuffs should be measured.

It is important in nutrition to distinguish between amino acid digestibility and amino acid availability. The

terms digestibility and availability have often been used interchangeably to describe the proportion of an amino acid in a feedstuff that is utilized by the pig. Digestibility is a proportion of an amino acid in a feedstuff that disappears from the digestive tract. Availability has been defined as the proportion of an amino acid in a feedstuff that can be utilized by the pig for growth, development, and maintenance, and as such, measures the net result of digestion, absorption, and metabolism.

Numerous papers have been published on the subject of bioavailability of amino acids in feedstuffs; they have been reviewed by Sibbald (1987) and recently by Batterham (1992) and Lewis and Bayley (1995), who described "bioavailability" as the degree to which an ingested nutrient in a particular source is absorbed in a form utilized in metabolism by the animal. Thus for amino acids "availability" is the proportion of the total amino acid that is digested and absorbed in a form suitable for protein synthesis (Batterham, 1992)

An amino acid absorbed from the digestive lumen may be unavailable for protein synthesis. This is particularly important for lysine, often the first amino acid limiting protein value in feedstuffs. It was demonstrated that at least for some feeds, apparent ileal digestibility

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coefficients for lysine overestimate lysine availability. This is mainly due to the reaction between lysine and reducing sugars (the Maillard reaction) which occurs during heat processing.

The digestive process

Feed protein entering the stomach is immediately subjected to the action of pepsin, which begins hydrolysis of proteins to peptides. After passing into the duodenum the chyme is mixed with pancreatic juice containing trypsin, chymotrypsin, elastase and carboxypeptidases and during its transit along the small intestine the contents are subjected to a sequence of pancreatic and intestinal peptidase enzymes which cleave the proteins into amino acids and small peptides. Amino acids and small peptides are absorbed by the enterocytes. The peptides are hydrolyzed within the enterocyte, but some may pass into the portal blood.

In addition to the digestive enzymes, mucins and other nitrogenous compounds enter the gut lumen and are mixed with the feed protein entering the digestive tract. Some of endogenous protein can be digested and reabsorbed in the small intestine, but a large proportion of it passes into the large intestine.

In the large intestine unabsorbed feed and endogenous proteins residues are subjected to the action of a large population of bacteria. The microbes ferment undigested protein to ammonia, which they then utilize for protein synthesis, or the ammonia is absorbed and excreted in urine as urea (Low and Żebrowska, 1989; Low, 1990).

Although the value of ileal amino acid digestibility data is generally recognized, there are several factors modifying digestibility values. It was found that microorganisms present in the small intestine may affect the amount of amino acids passing the terminal ileum (Bergner et al., 1986). Amino acids may be catabolized or synthesized and incorporated into microbial protein and then absorbed in the small intestine (Torrallardona et al., 1996). Studies by Dierick et al. (1986) show that there may be small but measurable catabolism of amino acids by the bacteria in the upper part of the digestive tract. Nevertheless, a number of studies have demonstrated that ileal digestibilities are reasonably accurate in describing the extent of uptake of amino acids from the gut, at least for most feedstuffs.

There are comprehensive reviews on the metabolism of nitrogenous compounds in the large intestine by microflora (Just, 1983; Mason, 1984; Low and Żebrowska, 1989). They showed that there is a net loss of most amino acids between the terminal ileum and the rectum. Early studies (Żebrowska, 1973) showed that protein infused in

the terminal ileum of pigs fed protein free diet was almost completely digested and absorbed from the large intestine, but absorbed nitrogen was not retained and was excreted in the urine. These and other studies (Żebrowska, 1975; Just et al., 1981) have led to the conclusion that the large intestine is not a site of amino acid absorption to a degree having practical importance.

The extent of protein degradation in the large intestine is not constant for all feedstuffs nor for all amino acids within a feedstuff. In general, feeds with lower digestibility in the small intestine undergo greater fermentation in the hind gut than feeds with high digestibility as measured at terminal ileum.

Also the action of bacteria on individual amino acids within the same feed is not constant. Of the essential amino acids, threonine and tryptophan are generally degraded to a greater extent than the other amino acids. There may be a net synthesis of methionine and lysine (Tanksley and Knabe, 1984).

There is evidence (Low and Żebrowska, 1989) that up to 80% of faecal nitrogen is of microbial origin and that only small proportion of the faecal amino acids originate from undigested dietary amino acids entering the large intestine.

Methods for measuring ideal protein and amino acid digestibilities

Reliable sampling of ileal digesta is one of the most important factors affecting correct measuring of amino acid digestibilities. In most experiments with pigs ileal digesta has been collected from cannulas implanted at the end of ileum.

Re-entrant cannula

The early studies were made using pigs with a re-entrant cannula where the distal cannula was placed either in the terminal ileum or in the caecum. In this method digesta passing the proximal cannula is collected quantitatively, sampled, and the remaining digesta is returned through the distal cannula. This method of cannulation requires transection of the small intestine which may alter normal gut motility and function. Re-entrant cannula allow total collection of digesta but blockage of digesta flow through cannula is a practical problem. Blockage occurs more frequently when fibrous or coarsely ground feeds are fed.

Simple T-cannula

The most frequently used method is a simple T-cannula installed in the terminal ileum. Total collection of digesta is not possible using this method and digestibility

must be calculated from the change in concentrations of an indigestible marker. Chromic oxide is the most commonly used marker. The success of this method is wholly dependent on the behavior of the marker in relation to amino acids. The use of a simple T-cannula avoids the transection of the small intestine, the disruption of the myoelectric complex, and maintains normal gut motility.

Post-valvular ileo-colic (or IPVC) method

To avoid the problems associated with complete transection of the small intestine a technique of post-valvular ileo-colic (IPV) cannulation has been developed (Darcy and Laplace, 1980). In this method a cannula is installed past the ileocaecal valve and all digesta flows out of the cannula and is returned via another cannula placed in the large intestine.

Post valvular T-caecum (PVTC) method

The post valvular T-caecum cannula (PVTC) method was recently developed for the total collection of ileal digesta (Van Leeuwen et al., 1988). This technique involves a simple surgical procedure, exerts minor negative physiological effects and causes less discomfort for the animal. The PVTC technique using a simple cannula in the caecum which can be opposed to the ileocaecal valve allows digesta to be collected as it leaves the ileum. The advantage of the PVTC technique includes the possibility of measuring ileal digestibilities of protein and amino acids of coarsely ground and fibre-rich feeds.

Ileo-rectal anastomosis (IRA) method

The technique of ileo-rectal anastomosis (IRA) has been proposed as a method of avoiding the problems of both the simple and the re-entrant cannula (Fuller, 1991). It has been modified and used in several studies (Hennig et al., 1989; Sauer and de Lange, 1992; Laplace et al., 1994). In this method the terminal ileum is transected anterior to the ileocaecal sphincter and anastomosed to the colon (end to side), or to the rectum (end to end) leaving the large intestine isolated.

This procedure has been modified by inserting a T-cannula into the isolated colon to allow for the escape of gases that results from microbial fermentation in the colon. Such modified animals remain in good health and can be used for many months.

The results of collaborative studies, in which four modifications of IRA were compared, showed that end-to-end IRA pigs can be used to determine ileal digestibility of protein and amino acids. In end-to-side pigs residual bacterial activity in the bypassed colon may affect the

amino acid digestibility value (Laplace et al., 1994).

Certain precautions, however, should be taken in studies with IRA pigs. The diets used should contain more minerals, those which are absorbed predominantly in the large intestine. Water intake is also 2-3 times greater than in intact pigs. According to Henning et al. (1988) pigs prepared by the IRA method and intact animals had similar growth rates, plasma indices, stomach mucosa, small and large intestine histology. This was, however, not confirmed by Fuller et al. (1994), who examined the gut of animals 26 weeks after surgery and found histological changes in the ileum with hypertrophy of smooth muscle. The concentration of volatile fatty acids excreted by these animals was higher than in pigs with T-cannulas. Because of these changes and of ethical considerations they not suggest using ileo-rectal anastomosis as a method for ileal digesta collection.

Mobile bag technique

With the methods described above only one feed can be measured at a time. These limitations might be overcome if feed samples placed in small bags could be introduced into the digestive tract and the undigested residue recovered quantitatively in faeces. For this purpose the mobile bag technique for measuring nutrient digestibility, similar to that used in ruminants, was developed (Sauer et al., 1983). In this method, samples of ground feed put into nylon bags, predigested in pepsin solution to simulate gastric digestion are inserted into the duodenum through the T-piece cannula and are recovered in feces to estimate total digestibility. To measure ileal digestibility the bags should be collected through a cannula at the end of ileum which may cause blockages and modification of digesta and bags passage rates. This technique may not be reasonable also for measuring nutrient digestibilities in feeds that contain high levels of antinutritional factors. Bornholdt et al. (1994) concluded that use of this technique for determination or prediction of amino acid digestibility is limited.

Comparison of methodologies

Because several methods have been used to determine ileal amino acid digestibility it is important to know whether results obtained by the different methods are comparable. The earlier studies by Żebrowska et al. (1978) and Taverner et al. (1983) showed no significant differences in digestibility of dry matter, nitrogen and amino acids as measured in pigs with re-entrant or T-cannulas at the terminal ileum. In recent studies on dietary amino acids digestibility in pigs (Fuller et al., 1994) three diets were used in five experiments on

growing pigs equipped with T-cannulas (TC), or with ileocolic postvalve cannulas (IPVC) and with ileorectal anastomosis (IRA). There were considerable differences between results with different techniques but the

differences between results obtained in different experiments using the same technique were equally large (table 1).

Table 1. Apparent and true digestibilities, averaged over nitrogen and all amino acids in the five experiments with three diets, based on barley (B), dried skimmed milk (M) and an isonitrogenous mixture of the two (MB).

Exp.	Digestibility						
	Apparent			True			
	Diets						
	B	MB	M	B	MB	M	
IRA 1	0.70	0.71	0.79	0.81	0.82	0.91	
IRA 2	0.65	0.66	0.82	0.76	0.78	0.96	
IRA 3	0.66	0.75	0.82	0.76	0.84	0.94	
TC	0.59	0.81	0.76	0.70	0.92	0.89	
IPVC	0.76	0.87	0.85	0.84	0.95	0.96	

IRA - ileorectal anastomosis.

(Fuller et al., 1994)

TC - T-cannula.

IPVC - ileocolic postvalve cannula.

Differences between methods found in this study were greater than in other comparisons. Köhler et al. (1990) who compared PVTC with reentrant and T-cannulas found only small differences in amino acid digestibilities between methods and concluded that PVTC technique is a good alternative to the other methods.

Darcy-Vrillon and Laplace (1990) compared nitrogen and amino acid digestibility of a standard cereal-based diet and two semi-synthetic diets containing either wheat

bran or beet pulp with IRA and IPV pigs. They found similar values for standard and wheat diets in IRA and IPV pigs but not in the beet pulp diet. IPV collection resulted in significantly higher apparent digestibility of nitrogen and amino acids than did IRA collection (table 2).

Therefore digestibility values of nitrogen and amino acids based on IRA collection may differ from those measured by IPV collection depending on diet composition.

Table 2. Apparent ileal digestibilities of protein and the essential amino acids in pigs prepared with the ileorectal (IRA) and with the ileocolic postvalve (IPV) method and fed with standard or wheat bran and beet pulp enriched diets.

Nutrient	Diet					
	Standard		Wheat bran		Beet pulp	
	IRA	IPV	IRA	IPV	IRA	IPV
Crude protein	70.0	66.5	78.1	81.9	74.16 ^b	87.20 ^a
Amino acids						
Arginine	84.4	83.4	86.6	91.2	85.0 ^b	92.4 ^a
Histidine	79.1	77.9	88.8	92.2	86.7 ^b	92.5 ^a
Isoleucine	74.4	72.9	82.6	87.2	81.2 ^b	88.6 ^a
Leucine	76.9	75.8	85.5	89.1	88.4 ^b	93.1 ^a
Lysine	74.8	72.4	86.3	89.9	87.5 ^b	93.1 ^a
Methionine	83.3	84.4	87.7	90.9	92.6 ^b	95.0 ^a
Phenylalanine	77.5	76.1	86.8	89.8	89.6	93.5
Threonine	68.8	66.1	77.9	82.5	76.9 ^b	85.8 ^a
Valine	72.7	70.5	82.8	87.6	81.8 ^b	89.0 ^a

^{a,b} Means in the same row (within diet) with different superscripts differ ($p < .05$).

(Darcy-Vrillon and Laplace, 1990)

Apparent and true ileal protein and amino acid digestibilities

Most data on ileal amino acids digestibility of a wide variety of feedstuffs and diets represent apparent values. Apparently undigested protein in the ileal digesta comprises both feed protein and non-absorbed endogenous secretions. In order to measure the true nitrogen and amino acids digestibility the apparent values have to be corrected for the amount of unabsorbed endogenous nitrogen or amino acids. The amount (proportion) of endogenous protein in ileal digesta seems not to be a constant value and may be influenced by such factors as dry matter intake, the content of fibre and antinutritive factors in feedstuffs, the level of protein in the diet (Boisen and Mougham, 1996).

There is evidence that dry matter intake significantly influences endogenous protein passing the terminal ileum. Feeding pigs increasing daily amounts of the same feed clearly showed a linear relationship between endogenous protein losses and dry matter intake (Butts et al., 1993).

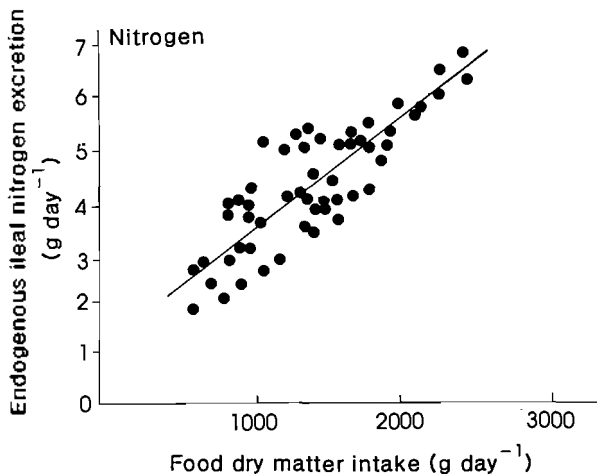


Figure 1. The effect of food dry matter intake on endogenous ileal nitrogen excretion in 50 kg liveweight pig (Butts et al., 1993).

No effect of dry matter intake on endogenous flow through the terminal ileum after feeding with protein-free diet was found by Furuya and Kaji (1992). However, readily digestible nutrients such as starch, have small influence on ileal N-flow (Buraczewska and Horaczyński, 1983). Therefore the content of endogenous protein in the ileal digesta could perhaps be better predicted from undigested dry matter assuming that the undigested part of the diet has the main influence on the addition of endogenous protein into the ileal digesta (Boisen, 1991; Fernandez and Boisen, 1991). At the same dietary DM

intake but different CP intakes a similar amount of undigested endogenous compounds in the terminal ileum will have a disproportionate effect on the determination of apparent digestibility. This is because the apparent digestibility is influenced by the protein level in the test diet (Furuya and Kaji, 1989) at the same DM but different CP intakes.

The amount of endogenous nitrogen and amino acids measured in pigs with ileo-rectal anastomosis, fed diets with graded protein content from 0 to 165 g/kg, calculated from the protein free diet or using regression methods were similar. Apparent protein digestibility increased with the level of protein in the diet while true digestibilities calculated with correction for endogenous losses or from regression slope were very close (Mariscal-Lendin et al., 1995).

Anti-nutritive factors (ANFs) such as trypsin inhibitors, lectins and tannins present in feedstuffs can increase endogenous nitrogen flow through the terminal ileum and impair apparent protein digestibility. ANFs elevate the amount of endogenous N in ileal digesta either by increasing endogenous secretion and/or by decreasing degradation and reabsorption of endogenous nitrogen (Huisman et al., 1992; Schulze 1994). Results of several studies indicate that the effects of ANFs on the amount of endogenous nitrogen and digestibility of nitrogen and amino acids can be substantial, particularly at low dietary protein level (Tamminga et al., 1995).

Fibre may affect the content of endogenous N in ileal digesta (table 3). There are several reasons for the increase in endogenous nitrogen excretion in response to

Table 3. The amount of endogenous protein and amino acids (g/kg DM intake) in the distal ileum digesta of pigs fed protein-free diets alone or supplemented with pectin, cellulose or fat.

	Control	Pectina	Cellulose	Fat
Protein	19.8 ^c	24.0 ^b	22.5 ^{b,c}	20.0 ^c
Arginine	0.73	0.89	0.86	0.67
Histidine	0.22	0.26	0.23	0.24
Isoleucine	0.36	0.39	0.37	0.40
Leucine	0.60	0.62	0.62	0.67
Lysine	0.53	0.58	0.56	0.61
Methionine	0.16	0.13	0.17	0.19
Phenylalanine	0.60	0.63	0.66	0.64
Threonine	0.65	0.69	0.72	0.75
Valine	0.48	0.51	0.50	0.54

a - Pectin 4%, cellulose 10%, fat 10% - included at the expense of corn starch.

^{b,c} - $p \leq 0.05$.

(Sauer and de Lange, 1992)

fibre. Fibre may directly stimulate digestive secretion (Langlois et al., 1987; Żebrowska and Low, 1985). Because the adsorptive properties of fiber some amino acids may not be available for absorption. Amino acids may also be left undigested in ileal digesta because of the accelerated rate of passage of the digesta from fibrous diets. Recent studies on the significance of fibre viscosity in digestion indicate that the amount of nitrogen and amino acids in the terminal ileum is higher when feeding fibers of high compared with low viscosity (Larsen et al. 1993).

Methods for estimating endogenous losses at the terminal ileum

Several methods have been used to measure endogenous nitrogen secretion and flow at the terminal ileum of pigs. The conventional but still used method, is to feed the pig a protein-free diet and to measure the amount of nitrogen in the ileal digesta. The main criticism of this method pertains to its non-physiological nature (Low, 1980) which may affect protein metabolism, and reduce protein secretion and re-absorption thus influencing (reducing) the amount of endogenous N content in ileal digesta as compared with protein-containing diets.

Later studies by de Lange et al. (1989) and Butts et al. (1993) showed only a minor effect of protein-free feeding on endogenous essential amino acids flow at the terminal ileum. This shows that protein-free feeding leading to negative nitrogen balance may not influence endogenous protein loss at the distal ileum.

In the regression method the animals are fed diets containing graded levels of protein, and then endogenous losses of nitrogen or amino acids at the terminal ileum are calculated by mathematical extrapolation. This method may provide better measurements of endogenous nitrogen as compared to feeding a protein-free diet (Fan et al., 1995 Figure 2).

The regression method allows for the evaluation of the effects of dietary proteins of different quality on endogenous nitrogen loss at the distal ileum. Some other studies have shown, however, that the data obtained by both the regression and the protein-free diet feeding methods are not very different and that both methods result in an underestimation of ileal endogenous protein flow (Furuya and Kaji, 1989; Donkoh et al., 1995). The obtained values can be used as the minimum endogenous nitrogen and amino acid losses and for diet formulation they should be used with caution.

Specific feed-induced endogenous protein loss can be determined by isotope dilution techniques. They allow to

distinguish endogenous protein from undigested feed protein after labelling either the feed or animal body protein (Krawielitzki et al., 1990; de Lange et al., 1992; Żebrowska et al., 1992; Huisman et al., 1992; Schulze et al., 1995).

Labelling the animal's nitrogen pool using ^{15}N is commonly used by continuous infusion of labelled amino acids. To measure the amount of labelled amino acids present in the ileal digesta relative to the precursor pool for endogenous gut protein synthesis usually the TCA-soluble fraction of blood plasma is taken.

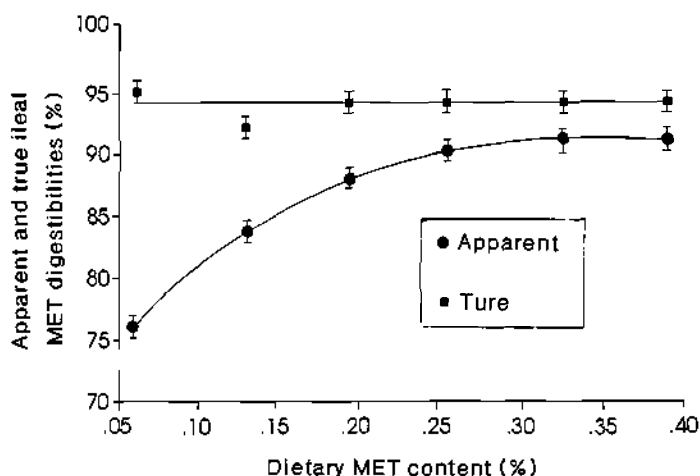


Figure 2. Relationship between dietary methionine (MET) content (percentage, DM basis) and apparent and true ileal digestibilities of MET (percentage) in SBM

(Fan et al., 1995)

The isotope dilution technique is a promising approach because it allows a direct estimate of the feed dependent endogenous ileal protein in distal ileal digesta (table 4). The method does not allow, however, direct estimation of all individual amino acids losses and can only be used to determine total endogenous nitrogen but not the content of individual amino acids. There is also no agreement as to what constitutes are appropriate as precursor pool (Moughan et al., 1992). ^{15}N -labelled compounds are very expensive and this may limit the usefulness of this method.

Growth assays

In a growth assay the response to increasing amounts increments of the test amino acid in a protein is compared to the response to the standard free amino acid. The experiments are arranged in a statistical design named a slope-ratio or regression assays using deficient diets (Sato et al., 1987; Batterham, 1992, Figure 3).

To measure amino acid availability by the growth

Table 4. Apparent and true ileal protein digestibilities measured with the ^{15}N -isotope dilution technique and endogenous protein in the distal ileum (g/kg DM intake) of pigs given diets with soybean meal, canola meal, wheat and barley as the protein source.

	Diets				SE
	Soybean meal	Canola meal	Wheat	Barley	
Digestibility					
apparent	83.8 ^a	66.0 ^b	80.0 ^a	69.5 ^b	1.6
true	97.5 ^a	84.1 ^c	99.0 ^a	94.2 ^b	1.0
Endog. protein (g/kg DM intake)	25.5	30.5	27.4	27.7	2.4

^{a,b,c} - $p < 0.05$.

(de Lange et al. 1990)

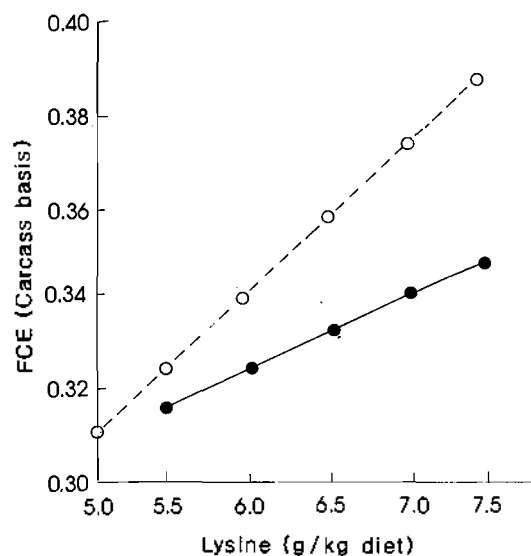


Figure 3. Design of a slope-ratio assay for determining lysine availability. The response to increments of lysine in the test protein (●—●) is determined and expressed as a proportion of the response to standard free lysine (○—○). FCE, feed conversion efficiency.

assay it is important to know that the pig's response is due to the amino acid of interest and is not influenced by other nutrients contributed by the test protein. In the growth assay developed and used for measuring the availability of lysine in protein concentrates for pigs (Batterham et al., 1979; 1992) basal diets based on wheat, wheat gluten and wheat starch with only a small addition of free amino acids to ensure that lysine is limiting are used. Test proteins are incorporated into the basal diet at the expense of wheat starch. The diets are fed on a controlled feeding scale based on live weight. The diets are offered at three-hourly intervals to ensure full utilization of the free amino acids. A multi-assay is used, with up to 5 test proteins assessed per assay and 4 or 5 levels per test protein. The response is measured using feed conversion efficiency on a carcass basis regressed

against lysine dose level in the diets. Availability of lysine in major protein concentrates for growing pigs measured according this procedure (Batterham, 1992) showed that availability (proportion of total) varied from 0.27 in cottonseed meal to 1.13 in blood meal (table 5). Growth response and lysine retention in pigs fed diets formulated on available lysine contents gave similar results (Batterham et al., 1990).

Leibholz (1986) found that the three regressed responses (weight gain, feed : gain, nitrogen retention) of piglets to various test materials did not lead to a single intercept value, though the differences were relatively small, and each response was related to the response of the piglets to free lysine. A recent study by Leibholz (1992) indicate that to measure the availability of lysine in feeds by regression assays it is important to use a wide range of lysine intakes as availability may not be constant and may vary with the lysine level and other factors in the feed.

The ileal digestibility of amino acids is commonly used to estimate the availability of amino acids for growing pigs. The comparative study of ileal digestibility

Table 5. Apparent and true ileal digestibility and availability of lysine in protein concentrates for pigs

Feed	Digestibility ²⁾		Availability ¹⁾
	Apparent	True	
Blood meal	91	4	108
Cottonseed meal	59	64	34
Meat and bone meal	79	93	68
Groundnut meal	80	87	57
Rapeseed meal	75	78	87
Skim milk powder	94	96	85
Soya bean meal	83	88	88
Sunflower meal	74	75	60

¹⁾ Batterham, 1992.

²⁾ RHODIMET NUTRITION GUIDE, 2 nd edition, 1993.

and availability of amino acids of protein concentrates showed that ileal digestible lysine, threonine, methionine and tryptophan are not always a reliable indicators for their availability. It appears that from heat-processed meals, a considerable proportion on lysine, threonine, methionine and tryptophan is absorbed in a form that is inefficiently utilized (Batterham, 1992). In the same experimental procedure it was shown that the branched chain amino acids, isoleucine, leucine and valine, seem to be less sensitive to heat damage and the ileal digestibility of these amino acids reflects availability.

Growth assays, slope-ratio and regression assays, are expensive, time-consuming and limited in that only one amino acid can be assessed at a time. They are only suitable for measuring relatively large differences in amino acid availability. More sensitive, faster and less costly methods are needed for predicting amino acid availability.

***In vitro* methods**

Several attempts were made to find less elaborate and fast methods for amino acid bioavailability determination in feedstuffs and pig diets. These methods do not require animals except as donors of enzymes (digesta) and mostly are based on *in vitro* enzymatic or other procedures. The expected main advantage of such methods was that they may give necessary information on the ileal digestible amino acid content of feeds in a short time in order to use the obtained data for feed mixture preparations in agreement with animal and ecological requirements.

In his review of methods on determination of bioavailable amino acids Sibbald (1987) stressed that although *in vitro* methods are attractive, their usefulness may be satisfactory for limited ranges of feeds. Later Boisen and Eggum (1991) in their critical evaluation of *in vitro* methods stated that it is not possible to simulate *in vitro* digestive tract conditions for digestibility processes, as there are many factors influencing digestion *in vivo*. Therefore it is necessary to standardize procedures and feeds to get acceptable correlation between *in vitro* and *in vivo* results of apparent ileal or true ileal amino acid digestibility.

The *in vitro* enzymatic methods simulating the process of digestion in the stomach and small intestine, are not influenced by endogenous protein and microorganisms, probably the main factors modifying the apparent ileal digestible amino acid in the feedstuffs. Therefore, enzymatic *in vitro* methods would rather indicate the content of the true ileal digestible amino acids.

Enzymatic methods

There are many enzymatic procedures of determining protein and amino acid ileal and total digestibility (Sibbald, 1987; Boisen and Eggum, 1991). It is obvious, that the choice of enzymes and conditions of their action should simulate processes in the digestive tract. Therefore, some methods utilize digesta collected from the small intestine (distal to the pancreatic duct), others use commercially available enzymes (pepsin, trypsin, rennin, chymotrypsin, papain, peptidases, amylase) and enzymatic preparation of composed nature (e.g. pancreatin). Digesta and/or enzymes may be used alone or in various combinations. (Dierick et al., 1985; Graham et al., 1989; Furuya, 1991).

Boisen and Eggum (1991) and Eggum and Boisen (1991) differentiated enzymatic methods into the dialysis cell, pH-drop and pH-stat and filtration methods.

Dialysis cell method

Simulates the stomach compartment by pepsin digestion of the substrate at pH 1.9 followed by pancreatin digestion at pH 8 in a dialysis tube (molecular weight cut-off 1000) simulating the small intestine compartment (Gauthier et al., 1982). Dialyzed digested products are removed by circulation of sodium phosphate buffer and analyzed for nitrogen and amino acids content. The procedure prevents inhibition of digestion by end products.

This method seems to give good prediction of available amino acid content in feedstuffs. The results depend mainly on the specificity of enzymes used. Obtained digestibility values are usually higher than that *in vivo* ileal apparent digestibility values. The method also permits studying the kinetics of amino acid liberation (Savoie, 1991). Galibois et al. (1989) using this method found a close relationship between essential amino acid profiles estimated at successive times of digestion from casein and rapeseed protein *in vitro* and those found postprandially in the portal blood of pigs fed the same proteins.

pH-drop and pH-stat methods

Used to predict amino acid digestibility are based on the measure of pH change of a suspension in which the product is digested by a mixture of trypsin, chymotrypsin and peptidase enzymes (Boisen and Eggum, 1991). Splitting a peptide bond releases protons and decreases the pH of the suspension. A correlation between the rate of peptide bond cleavage and protein digestibility at the initial stages of digestion is assumed. The pH decrease is recorded (pH drop method) or amount of NaOH needed to keep the pH constant during incubation period is noted.

The methods are used mainly for food protein digestibility determination and the pH-stat method gives better results as compared with the true protein digestibility in rats. Foods with a low protein digestibility have not been tested yet.

Filtration method

Seems to be the most adequate *in vitro* method to predict protein and amino acid availability or/and ileal or total digestibility.

The method is based on enzymatic digestion of the sample in one or more steps and subsequent separation of soluble and insoluble parts by filtration and analysis of insoluble part for protein and amino acid content.

The typical a two step procedure ends with collection of the insoluble residue for nitrogen (Cone and van der Poel, 1993) or nitrogen and amino acid determination (Boisen and Fernandez, 1995). The last procedure is presented schematically below:

A two step procedure of *in vitro* protein and amino acid digestibility determination as described by Boisen and Fernandez (1995)

Sample, 1 g, < 1 mm size, of known crude protein and amino acid contents

- + phosphate buffer 0.1 M, pH 6,
 - mix
- + HCl, 0.2 M,
 - adjust to pH 2 (HCl or NaOH)
- + pepsin, porcine, and chloramphenicol
 - gentle stirring at 39°C for 6 h
- + phosphate buffer 0.2 M pH 6.8 and 0.6 M NaOH
 - adjust to pH 6.8
- + pancreatin, porcine
 - gentle stirring at 39°C for 18 h
- + sulphosalicylic acid, 20%,
 - incubation 30 min at room temperature
 - transfer using 1% sulphosalicylic acid to
- + Celite in glass filter crucible
 - wash with ethanol and acetone, dry
 - analysis of undigested material

Boisen and Fernandez (1995) used this method to compare protein and amino acid digestibility *in vitro* in nine feedstuffs of plant origin with the respective value of apparent ileal digestibility measured earlier in pigs. In individual samples low *in vitro* variation was found between digestibility values of amino acids and these values were generally closely related to those of crude protein *in vitro* digestibility.

In their work they found that comparing 15 various

feedstuffs samples, determined *in vitro* protein digestibility values were higher than those obtained *in vivo*, and that there was a close relationship ($r^2 = 0.92$). Authors supposed that *in vitro* values represented real digestibility, and that the difference may reflect endogenous protein losses. The amino acid composition of endogenous protein was then calculated as the difference between individual amino acid values of *in vitro* and apparent ileal *in vivo* digestibility. Obtained sets of amino acids was relatively constant for all feeds and resembled the composition of endogenous protein determined *in vivo* (Wünsche et al., 1987).

The relationship between the digestibility *in vitro* and apparent ileal digestibility *in vivo* of individual amino acids was predicted by the equation (Boisen and Fernandez, 1995):

$$pdAA_i = (a + b \times dN_v) - 100 \times (13.2 + 0.066 \times UDM) \times cAA/AA$$

where: pdAA_i = predicted (p)ileal (i) apparent digestibility (d) of amino acid (AA)

(a+b x dN_v) = regression equation for *in vitro* (v) individual digestibility of amino acids and total nitrogen (N), given in Table

cAA = conversion factor from N to the individual amino acid in the endogenous protein, given in the table

AA = amino acid content in the feed (g/kg DM)

The relationships obtained by Boisen and Fernandez (1995) between measured and predicted values of apparent ileal digestibility of protein and amino acids were then checked by comparing with earlier *in vivo* results on 48 feed mixtures. The correlation r^2 value for protein was 0.57. The r^2 values for essential amino acids were generally higher (lysine 0.65) or similar (methionine 0.55) to that of the protein. For non-essential amino acids these values were generally lower than for protein.

Jaguelin et al. (1994) used enzymatic method to study *in vitro* nitrogen digestibility of four groups of feedstuffs. Results were compared with that obtained in pigs with ileo-rectal anastomosis. They concluded that a single equation predicting ileal N digestibility for all feedstuffs is inadequate because of the different endogenous N losses between groups of feeds.

Mathematical prediction method

Van Leeuwen et al. (1993) collected literature data on *in vivo* determined apparent ileal digestibility of protein and amino acids. The data comprised 290 batches of 40 different feedstuffs and were taken from experiments, in which various techniques of ileal chyme collection were used. From that data the apparent ileal digestibility of each amino acid was described in an equation based on two variables: the apparent ileal digestibility of nitrogen and the reciprocal of the relative contribution of amino acids to the feedstuff protein. The variance (R^2) for essential amino acids ranged from 0.6 to 0.8.

The mathematical model was checked on 18 diets *in vivo* in an experiment on pigs (Van Leeuwen et al., 1996). The obtained results explained 60 to 94% of the variation in apparent ileal amino acid digestibility of 16 diets, except of the phaseolus and meat and bone meal diets. The highest values of R^2 (> 0.8) were obtained for grains and casein diets.

Yin (1994) found also that for single feedstuffs (28 feeds studied) ileal apparent digestibility of essential amino acids could be predicted from ileal apparent digestibility of nitrogen.

Wünsche and Rudolph (1993) collected data of 294 ileal and total apparent digestibilities of protein and lysine, divided into 22 feedstuff categories. The calculated prediction of protein and lysine apparent ileal digestibility was based on their content in feeds and amount of ileal undigested protein and lysine. There were feedstuff groups like some proteinous-feeds with better than 70% predictions at differences not higher than 5 units of ileal lysine digestibility, and groups with a low predictability like rye, barley, meat and bone meal, showing only 20-30% predictions within differences lower than ± 5 units. Five unit difference was accepted as border of digestibility value differentiations for *in vivo* experiments.

Chemical methods

Are mainly used to measure the content of available lysine, that is lysine with a free epsilon amino group in contrast to the unavailable lysine with that group blocked in reaction with reducing sugars and/or other molecules. The unavailability of lysine is not detected using the protein hydrolysis procedure for amino acid determination (Lin-chun Mao et al., 1993; Hendriks et al., 1994).

Unavailable lysine seems to be undetected by ileal digestibility determination *in vivo*, as it may be digested and probably absorbed (Buraczewska et al., 1973; Van Barneveld et al., 1995). In practical nutrition this is a significant problem in respect to heat treated feeds like rape seed meal (Grala et al., 1994; Nyström et al., 1996)

or grain dried at high temperatures (Szamrej and Buraczewska, 1989). The method of free lysine reaction with dinitrofluorobenzene is mainly used for available lysine evaluation (Carpenter and Booth, 1973), as dye binding methods may give inconsistent results (Hendriks et al., 1994).

Chemical methods may give diverse results with various groups of feeds (Sibbald, 1987), although the DFNB lysine method may be helpful in lysine bioavailability evaluation together with the *in vivo* digestibility method.

Physical methods

The application of Near Infrared Reflectance (NIR) for prediction of lysine digestibility, as was tested on blood meal samples (Harrison et al., 1991), indicated the necessity of a large sample set for calibration. Van Leeuwen et al. (1991) evaluated NIR to predict crude protein ileal digestibility. There were 45 feedstuffs and obtained correlation coefficient was 0.90 from accumulated data at 7 different wavelengths, but there were large deviations for some samples. The NIR method has the potential to be used in the future (McNab, 1991).

CONCLUSION

Reliable and fast methods are required to measure the content of amino acids available to the pig in a variety of feeds used for practical diet formulation. There are two main problems to cope with: one is how to assure the additivity of available amino acid contents measured in a single feedstuffs, another is how to evaluate availability of amino acids in feed mixtures taking into account numerous external and internal factors in the organism modifying availability.

There are many approaches to solve these problems. The ileal digesta instead of faecal analysis methods for amino acid digestibilities in feedstuffs are used because it eliminates the modifying effect of bacterial activity in the large intestine. A variety of methodologies have been developed to measure ileal digestibility, and each described method for the collection of digesta has its advantages and disadvantages. Recently the post-valve T-caecum (PVTc) technique appears to be current method of choice. Regardless of the method used the validity of digestibility measurement depends mainly on the sampling of ileal digesta and on validity of the marker used.

The apparent and true ileal digestibility of amino acids in a number of different feedstuffs have been determined. The apparent digestibility values are not corrected for endogenous amino acids losses. A number

of different factors like dry matter intake, amount and type of fibre, protein level, antinutritional factors and development of microflora can influence the loss of both endogenous and feed proteins.

To determine the true ileal digestibility of dietary amino acids the correction for endogenous losses has to be made. A simple and exact method to measure the endogenous nitrogen and amino acid losses is not yet available. The protein-free diet method underestimates the losses of endogenous nitrogen at the distal ileum. The ^{15}N dilution techniques have limitations for routine application. Therefore further research is required to develop methodologies for accurate determination of endogenous nitrogen.

Ileal digestibility may overestimate availability of lysine, threonine, methionine and tryptophan in heat-damaged feeds as measured by growth assays. The branched chain amino acids are less sensitive to heat damage and their ileal digestibility and availability appear to be similar.

Measuring availability of amino acids with growth assays are however expensive, time consuming techniques and estimate relatively large differences in amino acids availability.

To determine true digestibility of amino acids in feeds using the *in vitro* methods could be an alternative to the conventional *in vivo* methods. From chemical methods determination of available lysine in heat treated feeds is very helpful, although the diverse results with various groups of feeds may be observed.

Further studies are required to relate the sensitivity of the ileal *in vivo* and *in vitro* methods to feed efficiency, increased growth rate or protein deposition. Further development of *in vitro* methods are needed for measuring availability of amino acids in variety of feedstuffs.

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