

# Development of a novel bioassay for determining the available lysine contents of foods and feedstuffs

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Lysine is an important indispensable amino acid, and describing the lysine content of a food or feedstuff provides useful information about nutritional value. However, when a food or feedstuff is subjected to heating the lysine present can be altered to nutritionally unavailable derivatives. These derivatives can revert back to lysine during the acid hydrolysis step used in amino acid analysis causing an overestimate of the lysine content. There have been many chemical methods developed to determine the reactive (unmodified) lysine content of foods and feedstuffs, but these do not take into account the incomplete absorption of lysine from the small intestine. There are also a number of animal-based assays for determining available lysine (the lysine that can be absorbed in a form that can be used for protein synthesis). The true ileal amino acid digestibility assay is commonly used to determine amino acid availability and is accurate for application to unprocessed foods and feedstuffs but is not accurate for lysine and possibly other amino acids when applied to heat-processed foods or feedstuffs. For such protein sources, assays such as the slope-ratio assay, indicator amino acid oxidation assay and the BIOLYSINE™ assay (true ileal digestible reactive lysine assay) have been developed to determine available lysine. The present paper discusses the efficacy of the BIOLYSINE™ assay as well as other assays for determining available lysine in processed foods and feedstuffs.

**Digestible reactive lysine: Processed foods: Available lysine: Protein**

## Introduction

Lysine (2,6-diaminohexanoic acid) is one of the twenty-two amino acids found in plant and animal proteins. Lysine is found in relatively low amounts in many cereals, such as wheat and rice, but tends to be more abundant in legumes, milk and meat-based foods. From a nutritional standpoint, lysine is an indispensable dietary essential amino acid that can only be derived from the diet and is often the first limiting amino acid for production animals (pigs and poultry). Lysine can also be limiting in diets for man, especially diets that are high in cereals and low in animal proteins.

During the processing of foods and feedstuffs, lysine can be chemically modified to form acid-labile derivatives. As a result of the presence of these derivatives, conventional methods of assessing lysine availability which use traditional amino acid analysis (for example, ileal amino acid digestibility assays) are inaccurate. Considerable effort has gone into developing alternative methods that accurately determine lysine and digestible lysine content. In the present

review, we outline and discuss a bioassay which has been developed in our laboratory that overcomes the analytical problems associated with lysine to accurately predict the available lysine content of processed foods and feedstuffs.

## Lysine and processing

Lysine is a basic amino acid and possesses a reactive amino group on its side chain. This  $\epsilon$ -amino group can undergo reactions with a wide variety of compounds including reducing sugars, fats and their oxidation products, polyphenols, vitamins, food additives and other amino acids<sup>1</sup>. Perhaps the most important lysine modification is that which occurs with reducing sugars (Maillard reaction). Several excellent reviews have been published describing this reaction<sup>2,3</sup> and it is not the intention to repeat that discussion here. However, a brief synopsis of the reaction is presented. The reducing sugar–lysine Maillard reaction initially involves a reversible condensation reaction which

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**Abbreviations:** EHC, enzymically hydrolysed casein; FDNB, fluorodinitrobenzene.

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results in the formation of a Schiff's base. The Schiff's base then undergoes irreversible rearrangement to produce  $\epsilon$ -*N*-deoxyketosyllysine (Amadori product), also known as the early Maillard product<sup>3</sup>. The Amadori product can then further react to produce brown pigments or melanoidins (late Maillard products) but these reactions are not well defined<sup>1</sup>. Other amino acids are also believed to undergo Maillard-type reactions, for example, proline<sup>4,5</sup>, tryptophan<sup>6,7</sup> and arginine<sup>8</sup> but these reactions are less well studied.

The nutritional availability of the Maillard reaction intermediates has been studied. Using a rat assay where the growth of rats fed graded levels of lysine was compared with those fed Schiff's bases similar to those formed during the Maillard reaction, Finot *et al.*<sup>3</sup> reported near-complete utilisation for the Schiff's base. It has been postulated that utilisation is via reversion to the aldiosylamine derivative of lysine which can then be easily hydrolysed to lysine<sup>3</sup>. In contrast, biological availability of  $\epsilon$ -*N*-deoxyketosyllysine (Amadori product, early Maillard product) has been found to be low (5–15%) by some workers<sup>9</sup> and nil by others<sup>3,10</sup>.

Maillard damage of lysine is generally slow, sometimes taking many months before significant amounts of lysine in a food or feedstuff are modified even at ambient temperatures<sup>11</sup>. However, there are a number of factors that control the rate of Maillard product formation, including temperature, pH, water activity and reactant concentration<sup>12</sup>. Many foods or feedstuffs undergo processing during their manufacture to either improve palatability, functionality or food safety<sup>13</sup>, during which, foods and feedstuffs are subjected to heat, pH extremes and pressure, all of which can greatly accelerate Maillard product formation.

In the intensive livestock industry accurate diet formulation is critical for maximising financial return and since lysine is the first limiting amino acid for growth in most pig and poultry diets, protecting lysine from damage during processing is important. Accurate data on the lysine content of diets and protein sources is also critical for efficient diet formulation. In human nutrition, many of the protein sources consumed by infants, children and adults are processed (for example, milk products, breakfast cereals and breads) and food is often cooked for safety or to enhance flavour and aroma. These practices accelerate the formation of advanced Maillard products. Indeed it is some of these advanced Maillard products that are responsible for flavour and aroma enhancement. Many Western diets tend to be high in meat and milk products. Since these foodstuffs contain high amounts of lysine, lysine damage may not be a critical problem nutritionally, in practice. However, for diets high in cereals and low in meat and milk, lysine can be limiting and this can have health implications, particularly for growing children.

### The fate of lysine during chemical analysis

The lysine content of foods is usually determined using amino acid analysis. Proteins consist of a chain of amino acids held together with peptide bonds. During analysis the peptide bonds are hydrolysed by heating the protein in concentrated acid (6 M-HCl) at 110°C for 24h<sup>14</sup>. The

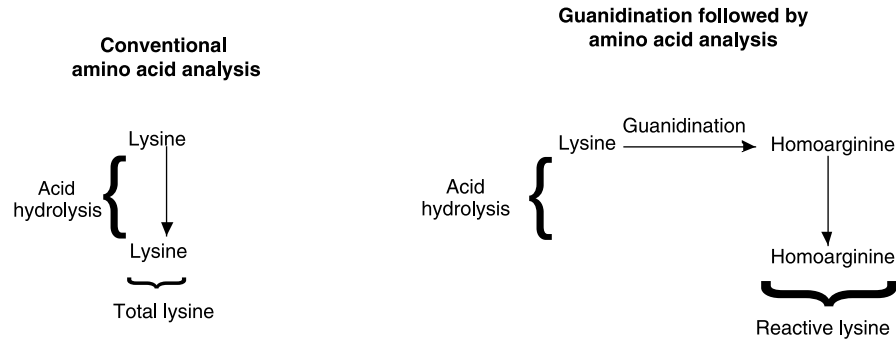
resulting free amino acids are then quantified using HPLC. This hydrolysis procedure was developed by Moore & Stein in the 1950s and has changed little over the decades. However, when heat-processed protein sources which may contain early Maillard products undergo acid hydrolysis these products are further modified to a number of other compounds. For example, hydrolysis of processed malt products can lead to the formation of carboxymethyllysine<sup>15</sup>. Acid hydrolysis of heated milk converts fructosyl lysine to a mixture of lysine<sup>16</sup>, furosine<sup>17,18</sup> and pyridosine<sup>19</sup>. Since some of the early Maillard products revert back to lysine during acid hydrolysis, the traditional amino acid analysis procedure is not suitable for quantifying lysine in processed protein sources (Fig. 1). Inaccurate and misleading terminology has evolved, for example, the term 'total lysine' is deemed to be the lysine determined by traditional amino acid analysis of a food in which early Maillard compounds are present. Total lysine constitutes the unmodified lysine present in the food plus the lysine that has reverted back from the early Maillard products during acid hydrolysis. The term total lysine is therefore as inaccurate as calling two different compounds one compound, simply because they co-elute on the HPLC. Lysine that has reverted back from early Maillard compounds during acid hydrolysis should not be referred to as lysine in any way and does not represent the lysine in the food in any way. It is simply an artifact of the amino acid analysis procedure. The early Maillard products only revert to lysine under conditions of acid hydrolysis and do not revert to lysine during the milder hydrolysis conditions encountered in the mammalian or avian digestive tract thus rendering them nutritionally unavailable.

### Lysine terminology

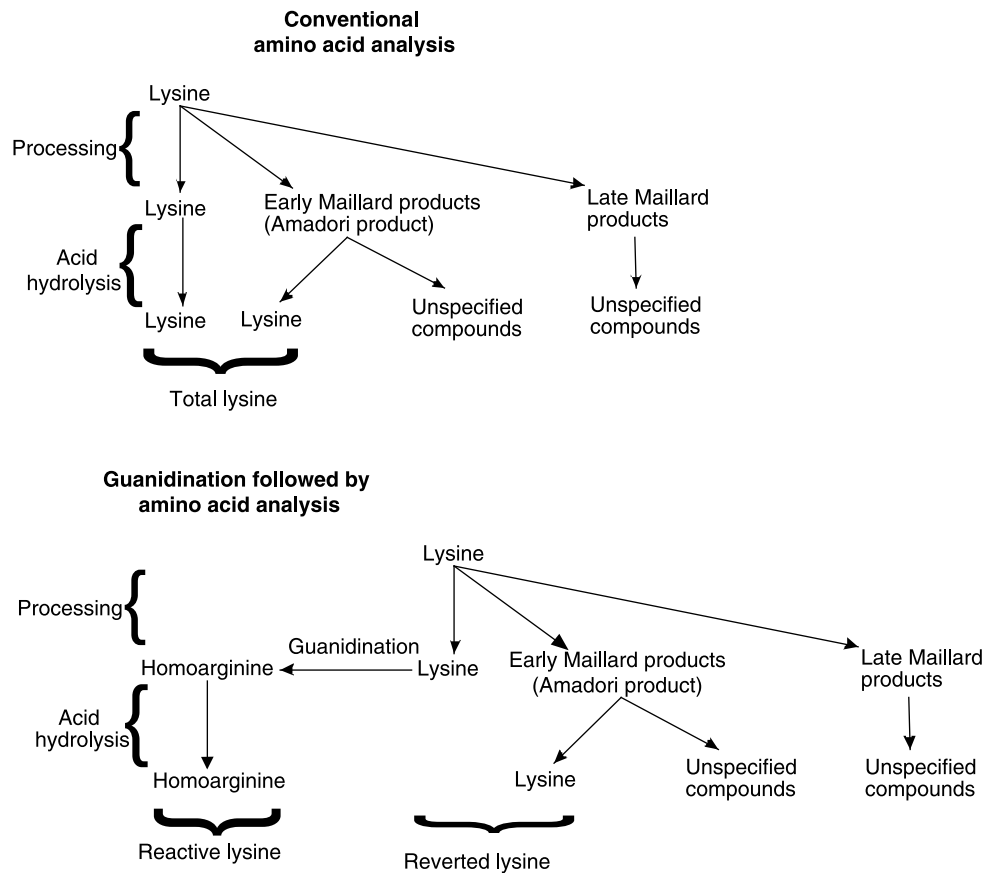
Total lysine, reactive lysine, chemically available lysine, available lysine and biologically available lysine are all terms used to describe the lysine content of foods and there appears to be considerable confusion as to the appropriate terminology to use. Many workers have determined chemically reactive lysine using chemical tests and described it as available lysine<sup>20–27</sup>, reactive lysine<sup>11,28–40</sup>, chemically available lysine<sup>41,42</sup>, chemically reactive lysine<sup>43</sup> and total available lysine<sup>44</sup>. Furthermore, some workers have determined lysine using conventional amino acid analysis and refer to this as available lysine<sup>45</sup> and furosine levels have also been determined and related to undamaged lysine which has then been termed bioavailable lysine<sup>46,47</sup>. Moreover, the terms available lysine and bioavailable lysine<sup>11,28–30,32,33</sup> have been used to describe the unmodified lysine units that are absorbed in a form that can be potentially utilised for protein synthesis, catabolism or conversion. The terms available lysine<sup>48–50</sup> and bioavailable lysine<sup>51–57</sup> have also been used to describe the unmodified lysine units that are utilised for protein synthesis only.

Clearly, there is considerable discrepancy and perhaps misunderstanding surrounding the appropriate terminology for describing 'available lysine' depending on the method used to determine it. In our laboratory and following Hurrell & Carpenter<sup>38</sup>, we refer to the undamaged lysine residues

(A)



(B)



**Fig. 1.** The fate of lysine during processing and analysis. (A) Unprocessed protein source; (B) processed protein source.

(lysine that has not undergone Maillard reactions or similar and possesses a side-chain amino group that is free to react) determined using any chemical method that targets the unreacted  $\epsilon$ -amino group of lysine (for example, fluorodinitrobenzene (FDNB), trinitrobenzenesulfonic acid, sodium borohydride, guanidination, dye-binding method, or any chemical method that can be related back to undamaged lysine, such as the furosine method), as reactive lysine or

chemically reactive lysine. Furthermore, we refer to the undamaged lysine residues that are digested and absorbed (i.e. absorbed reactive lysine) by an animal or human subject consuming the feed or food as available or bioavailable lysine (i.e. potentially available for body protein synthesis). We use the term total lysine to indicate the reactive lysine plus the lysine that has reverted back from Maillard products during acid hydrolysis (reactive + reverted lysine). The

latter terminology will be used for the remainder of the present review. We would view any undamaged lysine residues that are digested, absorbed and potentially available for utilisation (either protein synthesis, catabolism or conversion) by an animal or human subject as utilisable lysine. Finot & Hurrell<sup>58</sup> concur that availability and utilisability are two separate parameters and should not be confused. Batterham<sup>59</sup> is also in agreement in that an appropriate definition for amino acid availability would be 'the proportion of the total amino acid that is digested and absorbed in a form suitable for protein synthesis'.

### Determining reactive lysine

There have been many methods developed to determine reactive lysine, including chemical and biological assays. For the chemical assays, most are based on specific reactions with the  $\epsilon$ -amino group of lysine. The most well-known method is the FDNB method<sup>60</sup> which uses the Sanger reaction to convert lysine to dinitrophenyl-lysine which is extracted and measured spectrophotometrically or by HPLC. This method has been applied to animal feedstuffs<sup>34,57</sup>, breakfast cereals<sup>24,25,31,61</sup>, meat<sup>61,62</sup> and milk<sup>61</sup>. However, the method underestimates the reactive lysine content of foods since some of the dinitrophenyl-lysine may be destroyed during the acid-hydrolysis step used to liberate the dinitrophenyl-lysine from the protein<sup>63</sup>, necessitating the use of correction factors. Rao *et al.*<sup>40</sup> developed the FDNB-difference method where the lysine content was determined both before and after reaction with FDNB and the difference represented the reactive lysine<sup>32,40,64</sup>. Another shortcoming with this assay is that FDNB will react with  $\alpha$ -amino groups as well as the  $\epsilon$ -amino group of lysine, so significant levels of free amino acids or peptides in the food will cause an overestimation of reactive lysine content<sup>65</sup>. Recently, HPLC has been used to separate and quantify the dinitrophenyl-lysine, but this still does not overcome the problem of doubly labelled free lysine, a particular problem whereby synthetic lysine has been added to the diet or food. Other methods for determining reactive lysine include the trinitrobenzenesulfonic acid method<sup>66</sup>, sodium borohydride method<sup>38</sup>, furosine method<sup>41</sup>, dye-binding method<sup>34</sup>, ninhydrin-reactive lysine method<sup>36</sup>, *o*-phthalaldehyde-reactive lysine method<sup>35</sup> and guanidination method<sup>26,31,33,67,68</sup>.

The guanidination method involves the reaction of the  $\epsilon$ -amino group of lysine with *o*-methylisourea to produce homoarginine. Homoarginine is acid stable, so after guanidination proteins can undergo amino acid analysis in the traditional manner and the determined homoarginine represents the reactive lysine present. This reaction has been used to modify protein for functional studies<sup>69–73</sup>, to produce low-lysine or lysine-free diets for determining endogenous ileal lysine loss in pigs and poultry<sup>74–80</sup> as well as for determining the reactive lysine content of foods and feedstuffs<sup>26,30–33,81</sup>.

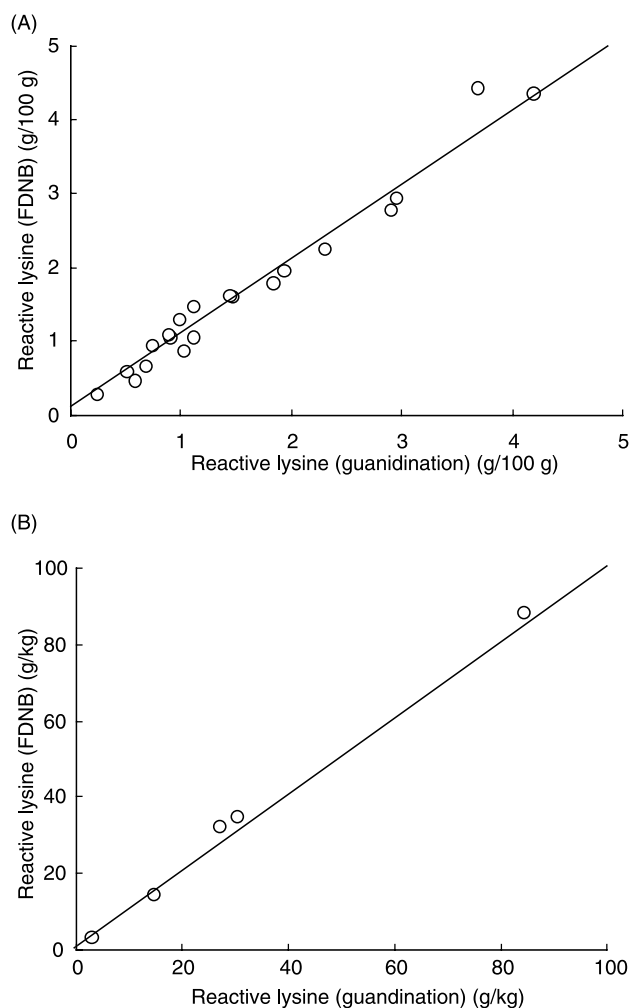
An important prerequisite for the successful application of the guanidination reaction for determining the reactive lysine content of foods or feedstuffs is that the conversion of lysine to homoarginine is complete. Consequently, considerable work has been conducted to this end<sup>68,82,83</sup>. In our

laboratory we routinely use unheated purified proteins (for example, lysozyme) as standards which are guanidinated along with test samples to ensure that the guanidination reagent is adequately prepared and that the incubation conditions are optimal.

The guanidination method works equally well with both unprocessed and processed proteins. In an unprocessed food or feed, there are no early Maillard products and, consequently, total lysine is equivalent to reactive lysine (Fig. 1). However, in a processed product where early Maillard products, or any other acid-labile lysine derivative which reverts to lysine in the presence of hot acid, are present, then total lysine overestimates reactive lysine by including reverted lysine in its estimation. When guanidination is used, the reaction takes place before acid hydrolysis so all the reactive lysine is converted to acid-stable homoarginine before being exposed to acid. During acid hydrolysis, some of the early Maillard products revert to lysine but this reverted lysine does not get included in the reactive lysine measurement since reactive lysine is represented by the homoarginine content only. The presence of a lysine peak in the chromatogram of a guanidinated processed feed or food has created certain confusion around the use of the guanidination reaction to determine reactive lysine. It has been thought that the presence of a lysine peak suggests that the guanidination reaction has not been complete. This is not the case.

Rigorous quality control using unprocessed protein standards and the development of appropriate reaction conditions for the reaction are required. If this is ensured, then the analyst can be confident that all lysine observed after hydrolysis of a guanidinated feed or food is not unguanidated reactive lysine but rather reverted lysine. In carefully controlled studies, we have shown an excellent correlation between the reactive lysine content determined using the guanidination reaction with that determined using the FDNB method for a range of animal feedstuffs and breakfast cereals (Fig. 2)<sup>31,33</sup>.

There has also been confusion around the presence of reverted lysine in that some workers who have used the guanidination reaction to determine the reactive lysine content of feedstuffs or foods believe that the sum of the reactive lysine and the reverted lysine determined after guanidination should equal the total lysine determined without guanidination. While this may occur sometimes, it is our experience that this is generally not the case and should not necessarily be expected to be so. Guanidination of a feedstuff or food is conducted in the presence of concentrated *o*-methylisourea at a high pH and may take place over several days. During this time it is possible (indeed likely) that early Maillard products are converted to other acid-stable products, such as late Maillard products. The resulting lower concentration of early Maillard products will result in a lower amount of reverted lysine than is observed in the unguanidinated corresponding food or feedstuff. Furthermore, during acid hydrolysis it is possible that the kinetics of the reversion reaction from early Maillard product to lysine are altered in the presence of high concentrations of *o*-methylisourea and barium sulfate, leading to different proportions of early Maillard product reverting to lysine than would occur in the feedstuff or food alone during conventional amino acid analysis without prior guanidination.



**Fig. 2.** Comparison of the reactive lysine content of (A) selected breakfast cereals ( $r$  0.985 ( $P < 0.001$ );  $y = 1.05x + 0.01$ ) and (B) selected animal feedstuffs (blood meal, meat and bone meal, wheat meal, soyabean meal and cottonseed meal ( $r$  0.996 ( $P < 0.001$ );  $y = 1.04x + 1.21$ )) determined using either the guanidination reaction or the fluorodinitrobenzene (FDNB) method. (—), Complete agreement between the two methods. (Reproduced with permission from Torbatinejad *et al.*<sup>31</sup> and Rutherford *et al.*<sup>33</sup>; copyright 2005, 1997 American Chemical Society.)

Another advantage of the guanidination method over all the other reactive lysine assays is that *o*-methylisourea will only react with the  $\epsilon$ -amino group of lysine and does not react with the  $\alpha$ -amino group of lysine or any other amino acid<sup>70–72,81,84–87</sup> with the exception of glycine<sup>88</sup>. Consequently, the guanidination reaction can be used to determine free lysine or peptide-bound lysine, and permits the accurate determination of reactive lysine content of diets that have been supplemented with synthetic lysine. The same cannot be said for the FDNB assay or any other reactive lysine assays.

### Determining available lysine

There are a number of assays that determine protein-bound reactive lysine in foods and feedstuffs with reasonable

accuracy, some of which have been described earlier. However, the failing of these assays is that they do not take into account the possibility that the digestion and absorption of unmodified lysine in a food protein may be less than 100%. This is an important consideration since the amino acid (including reactive lysine) digestibility of processed foods or feedstuffs is often far from complete<sup>29,32,48,64,89</sup>. Moughan *et al.*<sup>46</sup> using a heated glucose–casein mixture clearly demonstrated that considerable amounts of dietary reactive lysine were not absorbed from the small intestine of the growing pig (Table 1).

The most accurate means of determining amino acid digestibility is the true ileal amino acid digestibility assay. This methodology is discussed in detail by Moughan<sup>90</sup>. A test diet containing the feedstuff or food that is being tested is fed to an animal or human subject and digesta are collected from the terminal ileum just anterior to the ileo-caecum junction (ileal digesta). The amino acid content of the diet and digesta are determined using amino acid analysis and related to dietary intake by use of an indigestible marker. Ileal amino acid digestibility is calculated from the difference in amino acid content of the diet and digesta. There are a number of methods used to collect ileal digesta including nasogastric tube intubation, the cooperation of ileostomates, cannulating the terminal ileum of animals (ileal cannulation), removing the large intestine (ileo-rectal anastomosis) and collection under anaesthesia and direct sampling. Several reviews discussing these and other methods of ileal digesta collection have been published<sup>91–93</sup>.

For protein sources that have not been damaged during processing or storage, the ileal amino acid digestibility assay seems to accurately determine the amount of amino acid that is delivered to the animal's body in a form that can be utilised (available amino acid content). However, since amino acid analysis is used to determine the amino acid content of both the diet and digesta, then for processed protein sources, ileal digestibility may not be accurate for all amino acids, especially lysine, threonine, methionine and tryptophan<sup>59</sup>. The analytical problems associated with determining lysine in processed feedstuffs resurface when trying to measure the lysine content of ileal digesta leading to erroneous digestibility coefficients.

### Slope-ratio assay

One alternative to using ileal digestibility to determine lysine availability in processed protein sources is the use of

**Table 1.** The amount of total lysine, fluorodinitrobenzene (FDNB)-reactive lysine, reactive lysine and absorbed reactive (available) lysine in a heated glucose–casein mixture (reproduced with permission from Moughan *et al.*<sup>46</sup>; copyright 1996 American Chemical Society)

	Total lysine*	FDNB-reactive lysine	Reactive lysine†	Absorbed reactive lysine‡
Lysine (mmol/100 g)	31.6	22.7	25.0	14.7

\* Determined using conventional amino acid analysis.

† Determined using the furosine method.

‡ Determined from the furosine levels in the diet and digesta of pigs fed a heated glucose–casein diet.

animal growth assays. These assays include the protein efficiency ratio, net protein utilisation, biological value<sup>94</sup> and, arguably the most important, the slope-ratio assay. These methods are all based on the ability of an animal to deposit amino acids or protein from a test diet into the animal's body. Protein deposition in the animals fed the test protein source is compared with that in animals fed a series of standard diets with known and often graded levels of a limiting amino acid supplied in its synthetic form. While these assays overcome the analytical difficulties in determining lysine in processed protein sources and the resulting inaccuracy of the ileal digestibility assay when applied to lysine in processed foods or feedstuffs, they may also be flawed in that the efficiency with which protein-bound lysine, which would be present in the test protein, is utilised may be different from that of the synthetic form. Batterham *et al.*<sup>95</sup> developed and used the slope-ratio assay to determine the biologically available lysine content of processed meals for pigs. The assay was based on feeding test animals diets containing graded levels of synthetic lysine and a curve relating body growth to lysine addition was plotted. The same was repeated for a test protein with graded amounts of protein added to a series of test diets. By comparing the slopes for the synthetic lysine diets (standard diet) (where all the lysine is assumed to be utilised) with those for the test-protein diets, the available lysine content of the test protein can be estimated.

The slope-ratio assay has been applied to cottonseed meal, meat meals and sunflowerseed meal, rapeseed meal, skimmed milk powder and soyabean meal<sup>55,95</sup>, meat and bone meal<sup>50,53</sup>, blood meal<sup>50</sup>, distillers dried grains<sup>51</sup>, maize<sup>54</sup> and heated field peas<sup>49</sup>. Similar methods have been developed<sup>56,96</sup> that use rat growth assays to determine the available lysine content in heated protein sources. These methods are similar to the slope-ratio assay but instead of using standard diets containing graded levels of synthetic lysine to plot against animal growth, Faldet *et al.*<sup>96</sup> used wheat gluten-based standard diets with graded levels of available lysine supplied from the wheat gluten where the reactive lysine had previously been determined using the FDNB-difference method. Adeola<sup>97</sup> used the slope-ratio assay to determine the available tryptophan in soyabean meal using 10 kg pigs. For severely processed protein sources, the slope-ratio assay gives superior information to the traditional ileal digestibility assay and considerable work has been conducted to compare these two assays. Batterham *et al.*<sup>48</sup> showed that ileal digestible lysine determined using traditional amino acid analysis overestimated the available lysine content of cottonseed meal but not high-quality soyabean meal when fed to growing pigs. Furthermore, ileal digestible lysine also overestimated bioavailable lysine determined using the slope-ratio assay for heated field peas when fed to pigs<sup>49</sup>. Similar results were found for maize and high-oil maize<sup>54</sup> and autoclaved and non-autoclaved soyabean meal<sup>98</sup> when fed to poultry. Mavromichalis & Baker<sup>52</sup> reported good agreement between lysine bioavailability, determined using a standard-curve-based method where chick growth was related to lysine intake, and true ileal lysine digestibility for a high-quality complex nursery pig diet. Furthermore, Wang & Parsons<sup>53</sup> fed chicks maize-soya diets containing a

high-quality meat and bone meal formulated based on either the ileal digestible lysine content determined using caecectomised roosters or the bioavailable lysine content determined using a slope-ratio assay. They reported similar feed intake, weight gain and feed conversion ratio for the birds fed the two diets formulated using the two methods.

The slope-ratio assay may accurately predict available lysine content in processed protein sources. However, lysine availability determined using the slope-ratio assay is calculated using the total lysine content of the test diet. When applied to a diet containing a processed protein source where early Maillard products are present, total lysine is an inaccurate measure of lysine content, and lysine availability data generated using the total lysine content of the test diet will also be inaccurate. Furthermore, in the above studies where ileal lysine digestibility was compared with the slope-ratio assay estimates, total lysine, not reactive lysine, digestibility was determined. Consequently, this comparison is fundamentally flawed when applied to processed feedstuffs that contain early Maillard products. In cases where good agreement was obtained between slope-ratio assay data and ileal lysine digestibility data, it may simply highlight the inadequacy of the slope-ratio assay. Data generated using growth-based assays such as the slope-ratio assay tend to be highly variable, often making interpretation difficult<sup>1</sup>.

Another problem with the slope-ratio assay is that it does not distinguish between the inevitable catabolic lysine losses of the animal and the lysine used for growth. This can lead to an underestimate of lysine availability in processed feedstuffs. In other words, the slope-ratio assay is a predictor of lysine utilisation rather than lysine availability. Furthermore, utilisation is highly dependent on factors unrelated to the feed protein itself; for example, altering the dietary non-protein energy fraction, the dietary vitamin content or using animals of differing genotype can significantly change the utilisation of dietary amino acids<sup>99</sup>. We would argue that inevitable catabolic lysine losses are a function of the animal and not the food or feedstuff and occur not because the lysine has been damaged during processing but rather as a result of normal metabolic function. These losses form part of the animal's natural requirement for lysine and must be accounted for when formulating diets.

### Indicator amino acid oxidation technique

Another assay has recently been developed for determining amino acid availability based on the indicator amino acid oxidation technique<sup>100</sup>. This method can be applied to any amino acid and Moehn *et al.*<sup>100</sup> have described a study investigating the availability of lysine. This involved either feeding pigs radioactive phenylalanine or infusing it directly into the bloodstream at the same time as the pig received a test diet formulated to contain lysine at levels which render it the first limiting amino acid. The oxidation of phenylalanine was determined in the pigs fed a test diet and compared with pigs fed control diets for which the lysine content was known, and was also first limiting. Lysine availability was then calculated based on the proportion of phenylalanine oxidation in the pigs fed the test diet

compared with that on the control diet. Moehn *et al.*<sup>100</sup> tested heated field peas similar to those used by van Barneveld *et al.*<sup>49</sup> and found good agreement between lysine availability data generated using the isotope amino acid oxidation technique and that determined using the slope-ratio assay. However, given that van Barneveld *et al.*<sup>49</sup> calculated lysine availability based on the total lysine content of the heated peas then the accuracy of their estimates may be in question. Consequently, Moehn *et al.*<sup>100</sup> may be comparing the accuracy of their method against inaccurate estimates of lysine availability. Once again, strictly, the method is based on a measure of utilisation rather than uptake from the digestive tract. In spite of the latter reservations, this method is soundly conceived and appears to have practical application.

### The Biolysine™ (digestible reactive lysine) assay

True ileal lysine digestibility is an accurate measure of lysine availability when applied to unheated or minimally processed protein sources<sup>48</sup>. However, it is not accurate when applied to processed protein sources that have sustained lysine damage, since total lysine digestibility is being determined rather than reactive lysine digestibility. Total lysine digestibility is not an accurate measure of lysine availability in processed protein sources since total lysine is not an accurate measure of undamaged lysine. In contrast, reactive lysine is an accurate measure of undamaged lysine in a processed protein source. Therefore, by definition, true ileal reactive lysine digestibility is equivalent to lysine availability.

Most chemical methods used for determining reactive lysine are not specific for the side-chain amino group of lysine and will react with the N-terminal amino group of free amino acids or peptides. Consequently, determining reactive lysine in digesta which contains significant amounts of peptides and amino acids has been problematic. In contrast, the guanidination reaction is specific for the  $\epsilon$ -amino group of lysine<sup>70–72,81,84–87</sup>. In our laboratory, we have developed the BIOLYSINE™ assay which accurately determines true ileal reactive lysine digestibility (lysine availability) in processed feedstuffs and foods by coupling the guanidination reaction with the true ileal amino acid digestibility assay<sup>81</sup>.

Briefly, the assay involves feeding a test diet to an animal or human subject, digesta are collected from the terminal ileum and the reactive lysine content of both diet and digesta are determined using the guanidination reaction. Whenever digesta have been sampled as opposed to total collection, the reactive lysine content at the terminal ileum is related to dietary intake using an indigestible marker. Apparent ileal reactive lysine digestibility is calculated as the difference between diet amino acid intake and ileal digesta output. Apparent digestibility is adjusted to true digestibility by correcting for endogenous lysine (assumed to be reactive lysine) flow at the terminal ileum.

### Demonstrating the accuracy of the Biolysine™ assay

The BIOLYSINE™ assay has undergone rigorous validation. A well-controlled study based on body lysine retention was used to investigate the accuracy of the assay<sup>101</sup>. Three diets were formulated to be identical except for the protein source. These included two control diets containing enzymically hydrolysed casein (EHC) as the sole protein source and a test diet for which a heated skimmed milk powder was the sole protein source. The EHC was assumed to be completely digested and absorbed and all diets were limiting in lysine and were isoenergetic. One of the EHC diets contained lysine at the same level as the true ileal digestible total lysine content of the heated skimmed milk powder diet determined based on traditional amino acid analysis, while the other EHC diet contained lysine at the same level as the true ileal digestible reactive lysine content of the heated skimmed milk powder diet determined using the new true ileal digestible reactive lysine assay (guanidination). The diets were then fed to growing pigs and body lysine deposition determined<sup>101</sup> (Table 2). There was no significant difference in lysine deposition for the pigs fed the heated skimmed milk powder diet compared with those fed the EHC control diet for which the lysine content was formulated to match the lysine content of the heated skimmed milk powder diet determined using the true ileal digestible reactive lysine content. In contrast, the lysine deposition of the pigs fed the heated skimmed milk powder diet was significantly higher than for the pigs fed the EHC control diet formulated based on the true ileal digestible total lysine content (traditional assay). The experiment

**Table 2.** Whole-body lysine deposition (g/d) for pigs fed a heated skimmed milk powder-based diet, and two enzymically hydrolysed casein (EHC) control diets (reproduced with permission from Rutherford *et al.*<sup>101</sup>; copyright 1997 American Chemical Society)  
(Least-squares means with their standard errors)

	Heated skimmed milk powder		EHC diet A†		EHC diet B‡		P
	Mean	SEM	Mean	SEM	Mean	SEM	
Lysine deposition	9.1*	0.62	5.4	0.63	9.1*	0.58	<0.001

\* Mean value was significantly different from that for EHC diet A ( $P < 0.05$ ).

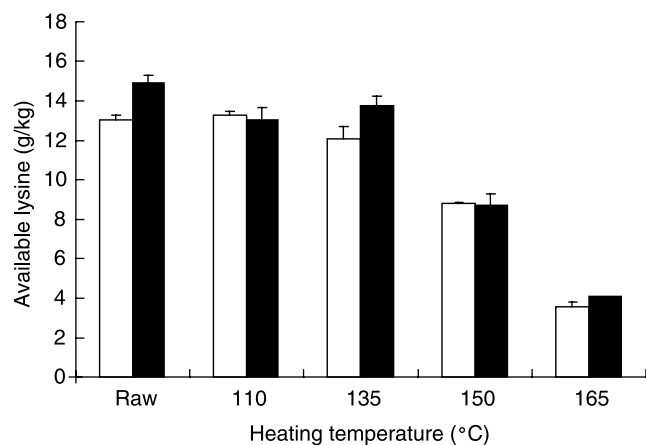
† EHC diet A was formulated to contain lysine equal to the digestible lysine content of the heated skimmed milk powder determined using the conventional ileal digestibility assay (reactive lysine in heated skimmed milk powder  $\times$  true digestibility of total lysine (determined using conventional methods) for the heated skimmed milk powder).

‡ EHC diet B was formulated to contain lysine equal to the digestible lysine content of the heated skimmed milk powder determined using the new ileal reactive lysine digestibility assay (reactive lysine in heated skimmed milk powder  $\times$  true digestibility of reactive lysine (determined using the new method) for the heated skimmed milk powder).

elegantly demonstrated the accuracy of the BIOLYSINE™ (true ileal reactive lysine digestibility) assay in predicting lysine availability and available lysine content in processed feedstuffs and the inaccuracy of the traditional ileal digestibility assay that uses traditional amino acid analysis to determine total lysine.

The BIOLYSINE™ assay was further validated by comparing the available lysine content of heated field peas determined using growth assays (from the late Dr Batterham's laboratory in Wollongbar, NSW, Australia) with that determined using the BIOLYSINE™ (true ileal reactive lysine digestibility) assay<sup>32,49</sup> (Fig. 3). There was good agreement between the available lysine content determined using growth assays<sup>49</sup> and that obtained using the BIOLYSINE™ assay<sup>32</sup>, while ileal total lysine digestibility significantly overestimated the available lysine content particularly for the more severely heated peas. This study further demonstrates the accuracy of the BIOLYSINE™ (digestible reactive lysine) assay and the inaccuracy of the ileal total lysine digestibility (conventional assay) for determining available lysine in processed feedstuffs.

Rutherford & Moughan<sup>32</sup> have also demonstrated the level of disparity between true ileal total lysine and reactive lysine digestibility estimates that can occur in foods as they are progressively heat treated. In this study, we took a skimmed milk powder and autoclaved it for 1–10 min then determined the true ileal digestible reactive and total lysine contents of the heated powders<sup>32</sup> (Table 3). There was little difference between true ileal digestible reactive and total lysine contents in the unheated milk powder, demonstrating that the traditional ileal digestibility assay using amino acid analysis is suitable for application with unprocessed protein sources. However, for the skimmed milk powder autoclaved for 10 min, the digestible total lysine overestimated digestible reactive lysine (available lysine) by 100% and even after only 1 min autoclaving, this overestimation was 12%.



**Fig. 3.** Comparison of available lysine (g/kg) in field peas determined by the digestible reactive lysine assay (□) or growth assay<sup>49</sup> (■). Values are means, with their standard errors indicated by vertical bars. (Reproduced with permission from Rutherford *et al.*<sup>33</sup>, copyright 1997 American Chemical Society).

**Table 3.** Digestible total lysine and digestible reactive (available) lysine contents (g/kg air-dry weight) for variably heated skimmed milk powder (reproduced with permission from Rutherford & Moughan<sup>32</sup>; copyright 1997 American Chemical Society)

Heat treatment	Digestible lysine			Significance
	Total*	Reactive†	Overall SEM	
Unheated	36.8	38.1	0.09	$P < 0.001$
121°C for 1 min	31.6	28.0	0.53	$P < 0.001$
121°C for 3 min	19.8	16.6	0.25	$P < 0.001$
121°C for 5 min	13.7	11.0	0.62	$P < 0.05$
121°C for 10 min	11.2	5.7	0.73	$P < 0.001$

\* Digestible total lysine was calculated from total lysine digestibility determined using a true ileal amino acid digestibility assay (rat) where conventional amino acid analysis was used to quantify lysine and from total lysine content in the protein source determined using conventional amino acid analysis.

† Digestible reactive lysine was calculated from reactive lysine digestibility determined using a true ileal amino acid digestibility assay (rat) where the guanidination reaction was used to detect reactive lysine in both diet and digesta and the reactive lysine content in the protein source was determined using the guanidination reaction and amino acid analysis.

#### Application of the Biolysine™ assay

The BIOLYSINE™ assay has been applied to a wide range of processed foods and feedstuffs including animal feedstuffs<sup>33</sup>, milk-based products<sup>11,30</sup>, 'ready-to-eat' breakfast cereals<sup>29</sup> and pet foods<sup>28</sup> (Table 4). For most of these foods and feedstuffs the assay was sufficiently sensitive to detect small differences between the digestible total lysine content and the available lysine content. Indeed, statistically significant differences of as little as 1.2% (ultra heat-treated milk) are easily detected. However, for many of the processed protein foods and feedstuffs the overestimation of digestible total lysine was far in excess of that observed for ultra heat-treated milk. For all of the samples shown in Table 4, the overestimation ranged from 0 to 143%, with the average overestimation being 31%.

The BIOLYSINE™ assay has also been used to highlight the high quality of milk-based foods<sup>30</sup>. Twelve milk-based foods were tested with only one product showing a greater than 10% difference between digestible total and reactive lysine content. In contrast, the severe effect of processing on lysine that occurs in some foods and feedstuffs has also been demonstrated. Breakfast cereals are perceived to be a healthy balanced high-quality food product. However, when the BIOLYSINE™ assay was applied to twenty 'ready-to-eat' breakfast cereals, high levels of lysine damage were detected in most of the twenty cereals tested<sup>29</sup> (Table 4). On average, digestible total lysine was 42% higher than the digestible reactive (available) lysine content, suggesting the very large amounts of early Maillard product present in the cereals. Similarly, when applied to twenty processed cat foods digestible total lysine overestimated digestible reactive lysine by between 18 and 143% with an average overestimation of 72%<sup>28</sup>.

The assay has not only been used to assess the available lysine content of the final processed product, but it has also been used to monitor the effect of the manufacturing processing itself on lysine availability. SM Rutherford and PJ Moughan (personal communication), using the BIOLYSINE™ assay, found that the lactose hydrolysis process, used to produce low-lactose milk powders, may



**Table 4.** Digestible total and reactive lysine contents (available lysine) (g/kg) for a range of protein sources\*

	Total†	Reactive‡	Difference (%)		Total†	Reactive‡	Difference (%)
Breakfast cereal 1	1.8	1.6	16.1	Split peas	16.1	15.4	4.6
Breakfast cereal 2	1.7	1.3	31.4	Flaked wheat cereal	0.59	0.63	5.8
Breakfast cereal 3	1.3	0.8	53.1	Extruded maize cereal	0.15	0.08	88.9
Breakfast cereal 4	2.0	1.4	41.8	Broll	4.9	4.6	6.5
Breakfast cereal 5	1.4	1.1	35.1	Evaporated milk	18.8	19.9	5.2
Breakfast cereal 6	0.66	0.56	17.0	Maize	1.9	1.8	5.0
Breakfast cereal 7	0.38	0.21	78.7	Wholegrain bread	2.8	2.4	17.5
Breakfast cereal 8	1.1	0.69	52.8	Soyabean meal	26.6	27.5	3.1
Breakfast cereal 9	0.88	0.67	31.3	Soya and linseed	6.8	5.7	19.4
Breakfast cereal 10	1.1	0.74	51.1	Wheat	3.3	2.4	37.9
Breakfast cereal 11	1.1	0.61	76.8	Whole-milk powder	18.6	18.4	1.2
Breakfast cereal 12	0.84	0.69	22.4	Whole-milk protein	26.2	24.0	9.2
Breakfast cereal 13	0.44	0.27	65.9	Infant formula A	8.3	8.6	3.5
Breakfast cereal 14	1.4	1.0	43.2	Infant formula B	9.1	9.2	1.1
Breakfast cereal 15	0.83	0.54	52.8	Infant formula C	11.1	11.7	5.1
Breakfast cereal 16	3.6	2.5	43.5	Whey protein concentrate	79.9	77.5	3.1
Breakfast cereal 17	3.8	3.2	17.0	Ultra heat-treated milk	31.7	31.4	1.0
Breakfast cereal 18	3.7	3.5	4.0	Evaporated milk	23.4	20.5	14.1
Breakfast cereal 19	3.7	2.8	35.5	Weight-gain formula	24.4	24.1	1.2
Breakfast cereal 20	3.2	1.9	68.3	Sports formula	20.4	19.1	6.8
Dried maize	2.6	1.9	36.8	Elderly formula	11.7	11.8	0.8
Lucerne-based diet	14.4	10.8	33.3	Protein supplement	14.3	14.3	0.0
Dried maize	2.8	2.2	27.3	Moist cat food 1	26.4	11.9	121.8
Cottonseed meal	12.9	10.3	25.2	Moist cat food 2	21.5	11.6	85.3
Heated skimmed milk powder	19.8	16.6	19.3	Moist cat food 3	17.1	9.6	78.1
Mixed diet	22.2	18.9	17.5	Moist cat food 4	27.4	15.8	73.4
Evaporated milk	23.4	20.5	14.1	Moist cat food 5	25.0	12.7	96.9
Moist cat food	29.6	26.8	10.4	Moist cat food 6	27.0	11.1	143.2
Wheat meal	3.2	2.9	10.3	Moist cat food 7	22.3	14.0	59.3
Whole-milk powder	26.2	24.0	9.2	Moist cat food 8	10.3	7.0	47.1
Low-lactose milk powder	27.2	25.1	8.4	Moist cat food 9	16.3	11.6	40.5
Heated peas	9.5	8.8	8.0	Moist cat food 10	28.9	18.2	58.8
Milk-based sports drink	20.4	19.1	6.8	Dry cat food 1	15.7	8.8	78.4
Dried peas	12.5	13.1	4.6	Dry cat food 2	8.9	5.3	67.9
Dry cat food	16.3	16.8	3.0	Dry cat food 3	13.4	8.8	52.3
Infant formula	9.1	9.2	1.1	Dry cat food 4	13.4	8.5	57.6
Soyabean meal	30.6	31.2	1.9	Dry cat food 5	12.7	7.2	76.4
Ultra heat-treated milk	31.7	31.4	1.0	Dry cat food 6	16.6	10.0	66.0
Milk-based meal replacer	14.3	14.3	0.0	Dry cat food 7	17.5	9.2	90.2
Blood meal	85.9	85.1	0.9	Dry cat food 8	18.1	12.4	46.0
Meat and bone meal	32.5	31.6	2.8	Dry cat food 9	9.1	5.1	78.4
Indian soyabean meal	23.4	22.7	2.7	Dry cat food 10	18.5	15.7	17.8
Split lentils	13.3	12.3	7.7				

\* Reproduced with permission from Rutherford *et al.*<sup>28,29,33</sup>; copyright 2007, 2006, 1997 American Chemical Society. Reproduced with permission from Rutherford & Moughan<sup>102</sup>. Reproduced with permission from Rutherford & Moughan<sup>30</sup>; copyright 2005.

† Digestible total lysine was calculated from the true ileal total lysine digestibility determined using the true ileal amino acid digestibility assay (rat) using traditional amino acid analysis to determine the total lysine content of the diets and digesta and from the total lysine content of the protein source also determined using traditional amino acid analysis.

‡ Digestible reactive lysine was calculated from the true ileal reactive lysine digestibility determined using the BIOLYSINE™ assay (rat) using guanidination and amino acid analysis to determine the reactive lysine content of the diets and digesta and from the reactive lysine content of the cereal also determined using guanidination and amino acid analysis.

have a negative impact on available lysine content (Table 5), while the processing method required to manufacture skimmed milk powder had little effect on the available lysine content.

The BIOLYSINE™ assay has also been used to assess the shelf life of milk powders by examining the effect of long-term storage on available lysine content of a skimmed milk powder and a lactose hydrolysed skimmed milk powder<sup>11</sup> (Table 6). The BIOLYSINE™ assay showed that as much as 40 % of the original lysine in the skimmed milk powder was damaged after 9 months storage at 40°C, while for the lactose hydrolysed skimmed milk powder 70 % of the lysine was modified after 6 months storage at 40°C. However, at lower storage temperatures, much less lysine modification was observed.

### Lysine determined using acid hydrolysis overestimates reactive lysine but total lysine digestibility underestimates reactive lysine digestibility – an explanation

It is well known that lysine determined using acid hydrolysis overestimates reactive lysine in processed protein sources and it may be expected that ileal total lysine digestibility would overestimate ileal reactive lysine digestibility. However, this is not the case and total lysine digestibility, determined using acid hydrolysis without guanidination, actually underestimates reactive lysine digestibility in processed protein sources (Fig. 4). In a processed feed or food there may be early and late Maillard products; the late Maillard products do not revert back to lysine during acid hydrolysis. However, a proportion of the early Maillard products do revert back to lysine during acid hydrolysis. Fig. 4 shows a hypothetical processed feedstuff that contains 16 units reactive lysine and 5 units reverted lysine, the sum of which is determined as total lysine (although it should be noted that in practice, reactive lysine plus reverted lysine does not always add up to lysine determined using acid hydrolysis. The reason for this has been discussed earlier). The reactive lysine reflects the undamaged lysine in the feedstuff and the reverted lysine is the lysine that reverts from the early Maillard products during acid hydrolysis. When the feed is eaten the proteins are digested by gut proteases, but because of the modified lysine residues in the protein, and possible other factors, digestion will not be complete. The resulting undigested peptides are called limit peptides. These limit peptides will contain proportionally more damaged lysine than undamaged lysine. Moreover, the limit peptides are absorbed to a lesser degree than the normal products of protein digestion, amino acids and small

**Table 5.** Digestible reactive (available) lysine contents (g/kg air-dry weight) of a skimmed milk powder and a hydrolysed lactose skimmed milk powder and the raw milks used to produce the products (reproduced with permission from SM Rutherfurd and PJ Moughan, unpublished results)

	Skimmed milk powder	Hydrolysed lactose skimmed milk powder
Raw milk	32.7	32.6
Processed product	33.0	28.0

**Table 6.** Digestible reactive lysine (available) contents (g/kg air-dry weight) for a skimmed milk powder and a hydrolysed lactose skimmed milk powder stored at 30, 35 and 40°C for varying lengths of time determined using the BIOLYSINE™ assay (reproduced with permission from SM Rutherfurd and PJ Moughan, unpublished results)

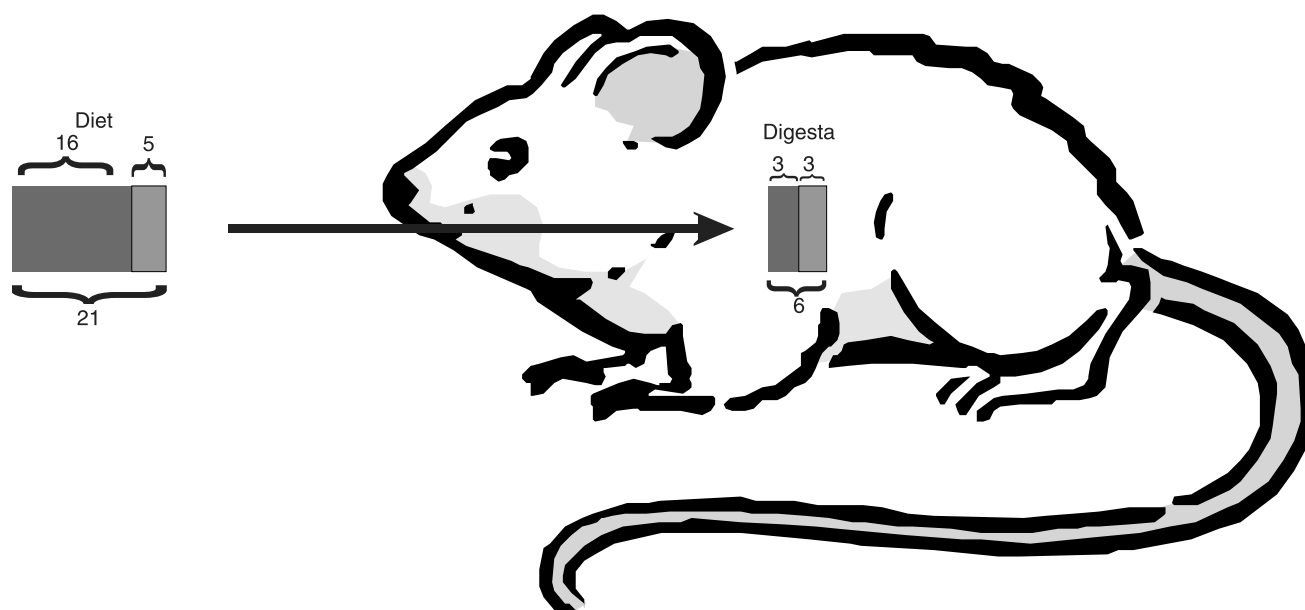
Storage time (months)	Storage temperature (°C)					
	Skimmed milk powder			Hydrolysed- lactose skimmed milk powder		
	30	35	40	30	35	40
0	32.9	32.9	32.9	28.1	28.1	28.1
3	31.2	31.3	27.9	26.6	22.3	15.5
6	29.8	30.3	25.9	23.9	18.5	9.7
9	29.9	28.9	21.5	22.0	17.3	–
12	26.5	25.7	19.8	19.8	14.5	–
18	26.3	25.2	–	16.7	9.9	–

peptides. This leads to a greater proportion of damaged lysine residues compared with the undamaged residues in the digesta at the terminal ileum compared with that in the food or feedstuff being tested. In the example given in Fig. 4, when the ileal digesta are analysed for reactive and total lysine, we find 3 units reactive lysine and 3 units reverted lysine giving 6 units total lysine. This is quite a different proportion to that found in the original feedstuff (16 units reactive lysine and 5 units reverted lysine). When digestibility is calculated, total lysine (reactive + reverted) digestibility is 71 % while reactive lysine digestibility is 81 %. Overall, total lysine (21 units) overestimates reactive lysine (16 units), while total lysine digestibility (71 %) underestimates reactive lysine digestibility (81 %).

It might be imagined that since total lysine overestimates reactive lysine in the diet, and total lysine digestibility underestimates reactive lysine digestibility and that digestible lysine is calculated by multiplying the lysine in the diet by ileal lysine digestibility, then there will be no net difference between digestible reactive lysine and digestible total lysine when determined in processed protein sources. However, this is generally not the case and if digestible reactive lysine and digestible total lysine estimates are similar in a processed protein source it is only by coincidence rather than some methodological artifact or real phenomenon. Using Fig. 4 as an example we find that the digestible total lysine, which is 15 units, is clearly not the same as the digestible reactive lysine, which is 13 units.

### Conclusion

Accurate determination of the available lysine content of processed foods and feedstuffs is particularly important since lysine is often the first limiting amino acid in diets for intensive livestock and human diets high in cereals and low in meat and milk products. Furthermore, the unique chemistry of lysine makes it difficult to determine lysine accurately in processed foods or feedstuffs. While there are many chemical methods available that allow the assessment of reactive lysine levels in such foods and feedstuffs, there are only a few that permit the determination of bioavailable lysine. These include the slope-ratio assay and other growth-based assays and the indicator amino acid oxidation method.



**Fig. 4.** Calculation of the total lysine and reactive lysine (■) digestibility in a processed protein source. Total lysine determined using amino acid analysis of processed diets and digesta for an animal fed the processed diet is made up of reactive lysine (the undamaged lysine) and reverted lysine (■), where reverted lysine is the lysine that reverts from early Maillard products (Amadori compound) to lysine during the acid hydrolysis step of amino acid analysis. Reverted lysine = total lysine – reactive lysine. Reactive lysine digestibility =  $(16 - 3)/16 \times 100 = 81\%$ . Total lysine digestibility =  $(21 - 6)/21 \times 100 = 71\%$ .

The slope-ratio and other growth-based assays tend to be labour intensive, expensive and highly variable. The BIOLYSINE™ assay offers an accurate and sensitive means to determining available lysine content of processed foods and feedstuffs.

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