Propositions belonging to the thesis

Bioavailability of lysine in heat-treated foods and feedstuffs

Shane M. Rutherfurd

28th September 2010, Wageningen

1. Currently, a "growth gap" exists between the actual growth of an animal fed a particular diet and the predicted growth for that diet based on growth models. The use of digestible reactive lysine rather than digestible total lysine as a predictor of available lysine will overcome this growth gap.

   This Thesis

2. The chemical composition of a food will determine the nature of the different lysine derivatives that are generated during processing. Not all these derivatives will result in a difference between digestible total lysine and digestible reactive lysine. Not nearly enough work has been done to determine which lysine derivatives are problematic.

   This Thesis

3. The results obtained from similar studies derived from different laboratories often vary, sometimes to the point where they lead to completely different conclusions. The main reason for this is that different methods of experimental conduct and analysis are often used in different laboratories. Consequently, there needs to be a standardisation of methodologies across laboratories to reduce this inter-laboratory variation.

4. There is no need to supplement endurance athletes with amino acids as long as they are receiving a mixed diet (containing 10% - 15% protein) and their intake meets their caloric needs. This is because energy will have a sparing effect on the dietary amino acids leading to a greater and sufficient amino acid pool to maintain muscle repair and growth.
   Tarnopolsky, M. 2004. Protein requirements for endurance athletes. Nutrition. 20, 662-668.

5. Men are destroyers while women are creators. Consequently while men, as opposed to women, are in power there will be no world peace.

6. Sailing is important in New Zealand since Kiwis are flightless and it is a long way to swim to our nearest neighbour.

7. Abel Tasman discovered New Zealand 125 years before Captain James Cook, but Captain Cook claimed New Zealand as part of the British Commonwealth. If Abel Tasman had claimed New Zealand as a territory of The Netherlands, then this thesis would probably have more spelling mistakes.

BIOAVAILABILITY OF LYSINE IN HEAT-TREATED FOODS AND FEEDSTUFFS

Shane M^{c}Artney Rutherfurd

Thesis committee

Thesis supervisors

Prof.dr.ir. W. H. Hendriks
Professor of Animal Nutrition
Department of Animal Science
Wageningen University

Prof.dr.ir. M. W. A. Verstegen
Emeritus Professor of Animal Nutrition
Department of Animal Science
Wageningen University

Thesis co-supervisor

Prof.dr. P. J. Moughan
Distinguished Professor
Riddet Institute, Massey University
Palmerston North, New Zealand.

Other members

Prof.dr.ir. H. Gruppen, Wageningen University
Prof.dr. D. A. Bender, University College, London, UK
Prof.dr. G. Janssens, University of Ghent, Belgium
Dr.ir. P. Bikker, Wageningen University

This research was conducted under the auspices of the Graduate School of Wageningen Institute of Animal Sciences

BIOAVAILABILITY OF LYSINE IN HEAT-TREATED FOODS AND FEEDSTUFFS

Shane M^{c}Artney Rutherfurd

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M. J. Kropff
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 28 September 2010
at 1.30 pm in the Aula

S. M. Rutherfurd, 2010
Bioavailability of lysine in heat-treated foods and feedstuffs

Ph.D Thesis, Wageningen University, Wageningen, The Netherlands
Summary in English and Dutch
ISBN: 978-90-8585-682-5

Abstract

During the processing of foodstuffs, lysine can react with other compounds present to form nutritionally unavailable derivatives, the most common example of which are Maillard products. Maillard products can cause serious problems when determining the available lysine content of processed foods or feedstuffs as they can revert to lysine during amino acid analysis. Several methods have been developed to determine the dietary lysine available for the metabolic processes of animals including animal growth-based assays, reactive lysine chemical methods and digestibility assays. However, growth-based assays are laborious, highly variable and tend to determine utilisation rather than availability. Chemically reactive lysine assays do accurately determine the unmodified lysine in a food or feedstuff, but do not determine available lysine as they incorrectly assume that reactive lysine digestion and absorption is 100%. Ileal digestibility assays measure digestible total lysine rather than digestible reactive lysine (available lysine) and so are inaccurate, especially when applied to processed protein sources. This thesis describes the development of a true ileal digestible reactive lysine assay for determining dietary (bio)available lysine. This assay couples the guanidination reaction, for determining reactive lysine, with a true ileal digestibility assay. The resulting apparent digestibility estimate is corrected to a true digestibility value by accounting for the endogenous ileal lysine flow.

Selected reaction conditions for the guanidination of lysine in a heated lactose/casein mixture and digesta of rats fed unheated casein and heated lactose/casein was examined. Overall, suitable reaction conditions were 0.6 M O-methylisourea for 7 d in a shaking waterbath at 21 ± 2 ^{o}C with an O-methylisourea to lysine ratio of 1000 and a reaction mixture pH of 10.6 for casein and heated lactose/casein and 11.0 for digesta. The accuracy of the guanidination method for determining reactive lysine in a range of "ready-to-eat" cereal-based breakfast foods and selected feedstuffs was tested by comparison with the reactive lysine content of the same protein sources when determined using the fluorodinitrobenzene method. Overall, there was excellent agreement between the two methods. The accuracy of the newly developed bioassay for determining digestible reactive (available) lysine for predicting lysine deposition was also tested using a heated skim milk powder. The true ileal total and reactive lysine digestibilities were determined for the heated skim milk powder which was then fed to pigs, along with two control diets which were formulated based on either total lysine digestibility or reactive lysine digestibility. All diets were limiting in lysine. The pigs fed the heated skim milk powder deposited the same ($P > 0.05$) amount of lysine (9.1 g d^{-1}) as the pigs fed the control diet that was formulated based on reactive lysine digestibility (9.1 g d^{-1}) but deposited significantly ($P < 0.05$) more than the pigs fed the control diet that was formulated based on total lysine digestibility (5.4 g d^{-1}). Consequently for the heated skim milk powder at least, the true ileal digestible reactive lysine assay accurately determined the available lysine content.

The new assay demonstrated that for a range of milk protein-based foods, there was little difference between digestible total lysine and digestible reactive lysine for most of the milk products tested. In contrast, for a range of "ready-to-eat" cereal-based breakfast foods, available lysine was 5 - 50% lower than that determined using the traditional assay, which is of concern given that breakfast cereals are perceived to be "healthy" foods. Similarly, the available lysine content of a range of moist and dry commercial cat foods was significantly

($P < 0.05$) lower (15 - 55% lower) than previously estimated using the traditional true ileal digestible total lysine assay. The assay was also used to examine the effect of storage for extended periods at elevated temperatures on a hydrolysed-lactose skim milk powder and overall, there was a significant decrease in the available lysine content over time, as much as 60% over 6 mth when the powder was stored at 40 °C. In addition, the decrease in available lysine content of the hydrolysed-lactose skim milk powder was 2 - 5.5 times greater than observed for a normal skim milk powder depending on the storage time and temperature. Overall, foods and feedstuffs that have undergone processing often contain lower amounts of available lysine than thought previously. This new assay not only highlights the inaccuracy of the traditional true ileal digestible total lysine assay as a method for determining available lysine in processed protein sources, but permits the accurate assessment of the available lysine content of processed foods and feedstuffs.

Contents

Chapter 1

General introduction

Parts of this chapter have been published in Rutherfurd, S.M., Moughan, P.J. 2007. Development of a novel bioassay for determining the available lysine contents of foods and feedstuffs. Nutr. Res. Rev. 20, 3-16 and are reproduced with permission.

Introduction

Lysine (2,6-diaminohexanoic acid) is one of the 22 amino acids found in plant and animal proteins. Compared to amounts normally found in the body protein of animals and humans, 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 a dietary indispensable 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 humans, especially diets that are high in cereals and low in animal proteins.

Lysine and processing

Lysine is a basic amino acid that possesses a reactive amino group on its side chain. This ε-amino group can undergo reactions with a wide variety of compounds that are present in foods and feedstuffs including reducing sugars, fats and their oxidation products, polyphenols, vitamins, food additives and other amino acids to produce modified lysine derivatives (Hurrell and Carpenter, 1981). When a food or feedstuff undergoes processing, particularly heat processing, the rate of these reactions are greatly accelerated and since many protein sources undergo extensive heat treatment during manufacture, significant amounts of these derivatives can be present in the protein source. Perhaps the most important lysine modification is that which occurs when it reacts with reducing sugars (Maillard reaction). Several excellent reviews have been published (Friedman, 1996; Finot et al., 1977) describing this reaction 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 results in the formation of a Schiff base (Fig. 1). The Schiff base then undergoes irreversible rearrangement to produce ε-N-deoxyketosyllysine (Amadori product) also known as the early Maillard product (Finot et al., 1977). The Amadori product can then further react to produce brown pigments or melanoidins (advanced Maillard products) but these reactions are less well defined (Hurrell and Carpenter, 1981). Other amino acids are also thought to undergo Maillard type reactions, for example, proline (Tressl et al., 1994; Mills and Hodge, 1976), tryptophan (Friedman and Cuq, 1988; Saito et al., 1986) and arginine (Konishi et al., 1994) but these reactions are less well studied.

The nutritional availability of Maillard reaction intermediates has been studied. Using a rat assay where the growth of rats fed graded levels of lysine was compared to those fed Schiff bases similar to those formed during the Maillard reaction, Finot et al. (1977) reported near complete utilisation of the Schiff base. It has been postulated that utilisation is via reversion to the aldosylamine derivative of lysine which can then be easily hydrolysed to lysine (Finot et al., 1997). In contrast, biological availability of ε-N-deoxyketosyllysine (Amadori product, early Maillard product) has been found to be low (5 - 15%) by some workers (Finot, 1990) and nil by others (Hurrell and Carpenter, 1977; Finot et al., 1977).

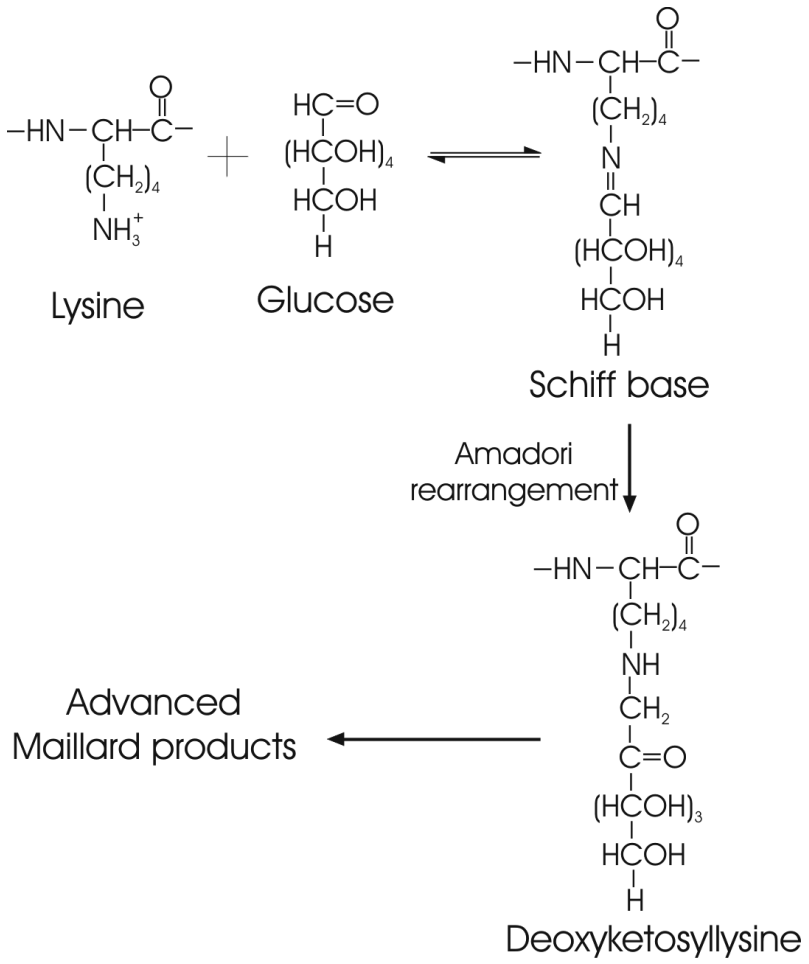


Figure 1. The reaction of protein bound lysine with glucose (Maillard reaction).

In human nutrition, many of the protein sources consumed by infants, children and adults are processed (for example, infant formulas, milk products, breakfast cereals and breads) and food is often cooked for safety and/or to enhance flavour and aroma. These practices accelerate the formation of advanced Maillard products which indeed are responsible for flavour and aroma enhancement. Advanced Maillard products that form *in vivo* are referred to as advanced glycation end products (AGE's) and have been observed to increase during the normal ageing process (Schleicher et al., 1997). In addition, AGE's have also been linked to a number of diseases in humans such as complications in diabetes (Cai et al., 2004), atherosclerosis (Kume et al., 1995) and renal failure (Šebeková et al., 2001). The significance of the contribution of dietary (exogenous) AGE to these diseases is largely unresolved (Nass et al., 2007). While the range of AGE compounds present in foods that are absorbed in the intestine is not fully known, there is evidence that at least some are absorbed into the blood stream and subsequently excreted in the urine (Koschinsky et al., 1997). Moughan et al. (1996) showed that early Maillard products (deoxyketosyllysine)

were also absorbed into the bloodstream of pigs fed diets containing a heated glucose/casein mixture. Dietary AGE's may also be affected by digestive enzymes and gut microbes and it is generally thought that it is mainly the smaller compounds that are absorbed into the bloodstream. Approximately two thirds of the absorbed AGE's are incorporated into tissues and components in the blood (Koschinsky et al., 1997) and dietary restriction of AGE's has been reported to reduce atherosclerosis (Lin et al., 2003).

Lysine in foods or feedstuffs can undergo Maillard reactions at any temperature but the rate is greatly accelerated at elevated temperatures. For example, the reactive lysine content of skim milk powder stored for 3 mth decreased by 5% when stored at 30 °C but by 14% when stored at 45 °C (Chapter 6). However, there are a number of other factors that influence the rate of Maillard product formation, including pH, water activity and reactant concentration (Franzen et al., 1990). Many foods or feedstuffs undergo processing during their manufacture to either improve palatability, functionality or food safety (Hendriks et al., 1999), 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 returns 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. Furthermore, for human diets, accurate information on the lysine content is required for diets that are likely to be low in lysine (cereal-based diets) and also those diets for humans with a particular requirement for protein such as growing children, athletes or the elderly. Consequently, it would be useful to accurately determine the lysine content in extensively heat-treated foods and feedstuffs such as pet foods, breakfast cereals and protein supplements. In this study, such an assay was developed and used to determine the available lysine content in commonly consumed foods and feedstuffs. In addition, it is important to obtain knowledge about the extent to which AGE's that may be present in processed foods and feedstuffs can contribute to human and animal health.

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 24 h (Hirs et al., 1954). The resulting free amino acids are then quantified using HPLC. This hydrolysis procedure was developed by Moore and Stein in the 1950's and has changed little over the decades. When heat-processed protein sources which 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 (Hartkopf et al., 1994). Acid hydrolysis of heated milk converts fructosyllysine to a mixture of lysine (Mauron et al., 1955), furosine (Finot et al., 1968; Heyns et al., 1968) and pyridosine (Finot et al., 1969). 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. 2).

There is a need therefore to be able to distinguish between the reactive lysine and reverted lysine in foods and feedstuffs and there are a number of assays that will do this for intact proteins (e.g. fluorodinitrobenzene (FDNB) method). However, for samples that contain free amino acids or peptides such as protein hydrolysates, lysine supplemented feeds or intestinal digesta, many of these assays are inaccurate and the optimisation of an assay that is robust enough for such samples is required. The guanidination method offers the potential for such an assay and it's optimisation for processed protein sources and digesta from animals fed such protein sources is described in Chapter 2.

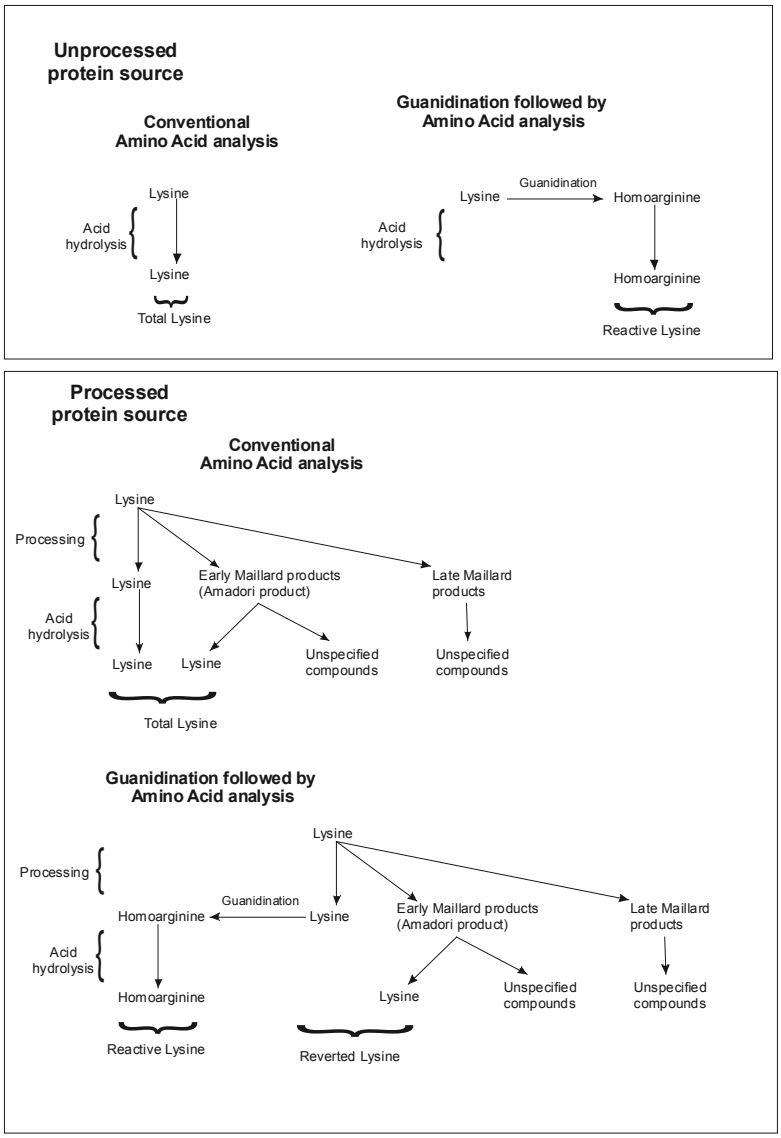


Figure 2. The fate of lysine during processing and analysis.

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 (Milán-Carillo et al., 2006; Rufián-Henares, et al., 2006; Malec et al., 2005; Siskos et al., 2005; Fernandez-Artigas et al., 1999; Horvatić and Guterman, 1997; Mao et al., 1993; Couch and Thomas, 1976), reactive lysine (Torbatinejad et al., 2005; Hendriks et al., 1994; Vigo et al., 1992; Friedman et al., 1984; Taverner and Farrell, 1981; Hurrell and Carpenter, 1974, Roach et al., 1967; Rao et al., 1963), chemically available lysine (Desrosiers et al., 1989; Moughan et al., 1989), chemically reactive lysine (Henle et al., 1991) and total available lysine (Rehman, 2006). Furthermore, some workers have determined lysine using amino acid analysis and have referred to this as available lysine (Undi et al., 1996) and furosine levels have also been determined and related to undamaged lysine which has then been termed bio-available lysine (Moughan et al., 1996; Erbersdobler and Hupe, 1991). Moreover, the terms available lysine and bioavailable lysine, as is the case in this thesis, 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 (van Barneveld et al., 1994a; Batterham et al., 1990; Batterham et al., 1986) and bioavailable lysine (Lumpkins and Batal, 2005; Mavromichalis and Baker, 2000; Parsons et al., 1998; Wang and Parsons, 1998; Fernandez and Parsons, 1996; McDonough et al., 1989; Noll et al., 1984) have also been used to describe the unmodified lysine units that have been absorbed into the body and deposited in body protein.

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 this thesis and following Hurrell and Carpenter (1974), undamaged lysine residues (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 ε-amino group of lysine (e.g. FDNB, trinitrobenzenesulphonic acid (TNBS), sodium borohydride, guanidination, dye binding methods, or any chemical method that can be related back to undamaged lysine, such as the furosine method), are referred to as reactive lysine or chemically reactive lysine. Furthermore, the undamaged lysine residues that are digested and absorbed (i.e. absorbed reactive lysine) by a human or animal consuming the food or feedstuff are referred to as available or bioavailable lysine (i.e. potentially available for body protein synthesis) (Fig. 3). In this thesis the term total lysine is used to indicate the reactive lysine plus the lysine that has reverted back during standard acid hydrolysis (reactive + reverted lysine). In practice, total lysine should not be used for describing lysine in feedstuffs that contain appreciable amounts of Maillard products as it will overestimate the actual lysine (reactive lysine) present. Any undamaged lysine residues that are digested, absorbed and utilised for protein synthesis should be viewed as utilisable lysine. Finot and Hurrell (1985) concur that availability and utilisability are two separate parameters and should not be confused. Batterham (1992) is also in agreement in that an appropriate definition for amino acid availability would be "the proportion of the amino acid that is digested and absorbed in a form suitable for protein synthesis".

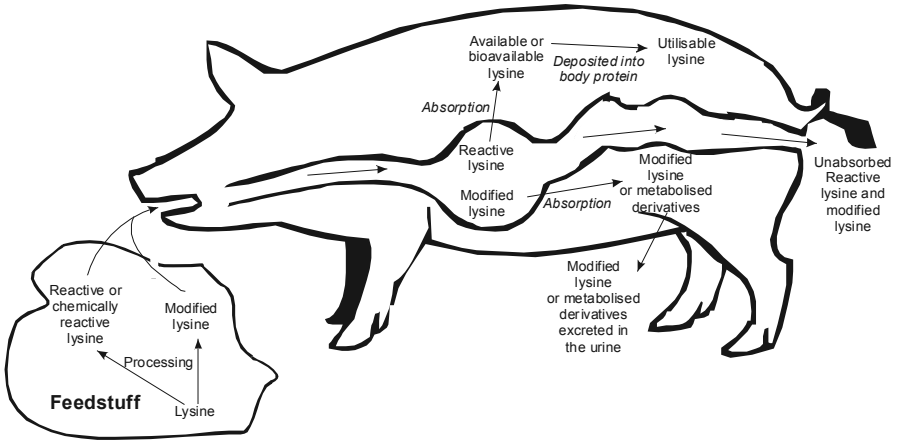


Figure 3. Definition of the terminology used to describe lysine in processed feedstuffs.

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 ε-amino group of lysine. The most well known method is the FDNB method (Carpenter, 1960; modified by Booth, 1971) which uses the Sanger reaction to convert lysine to dinitrophenyllysine (DNP-lysine) which is extracted and measured spectrophotometrically or by HPLC. This method has been applied to animal feedstuffs (Hendriks et al., 1994; Noll et al., 1984), breakfast cereals (Torbatinejad et al., 2005; Fernandez-Artigas et al., 1999; Horvatić and Guterman, 1997; Carpenter et al., 1989), meat (Carpenter et al., 1989; Rayner and Fox, 1978) and milk (Carpenter et al., 1989). However, the FDNB method underestimates the reactive lysine content of foods since some of the DNP-lysine may be destroyed during the acid hydrolysis step used to liberate the DNP-lysine from the protein (Booth, 1971), necessitating the use of correction factors. Rao et al. (1963) 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 (van Barneveld et al., 1994b; Taverner and Farrell, 1981; Rao et al., 1963). Another shortcoming with this assay is that FDNB will react with the α-amino group of amino acids as well as the ε-amino group of lysine, so significant levels of free amino acids or peptides present in the food or feedstuff will cause an overestimation of the reactive lysine content (Carpenter and Bjarnason, 1968). HPLC has also been used to separate and quantify the DNP-lysine (Marquié et al., 1997), but this still does not overcome the problem of doubly labelled free lysine, a particular problem when synthetic lysine has been added to the diet or feedstuff. Other methods for determining reactive lysine include the TNBS method (Kakade and Leiner, 1969), sodium borohydride method (Hurrell and Carpenter, 1974), furosine method (Desrosiers et al., 1989), dye binding method (Hendriks et al., 1994), ninhydrin-reactive lysine method (Friedman et al., 1984), *o*-phthaldialdehyde reactive lysine method (Vigo et al., 1992) and guanidination method (Torbatinejad et al., 2005; Maga, 1981; Mauron and Bujard, 1964; Mao et al., 1993). Of these methods, only the guanidination method shows promise for foods and feedstuffs that contain free amino acids or peptides.

The guanidination method involves the reaction of the ε-amino group of lysine with O-methylisourea (OMIU) 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 proteins for functional studies (Kassell and Chow, 1966; Hettinger and Harbury, 1965; Sheilds et al., 1959; Klee and Richards, 1957; Hughes et al., 1949), to produce low lysine or lysine free diets for determining endogenous ileal lysine loss in rats, pigs and poultry (Rutherfurd et al., 2007; Ravindran et al., 2004; Nyachoti et al., 1997; Siriwan et al., 1994; Moughan and Rutherfurd, 1991; Moughan and Rutherfurd, 1990; Hagemeister and Erbersdobler, 1985) as well as for determining the reactive lysine content of foods and feedstuffs (Torbatinejad et al., 2005; Mao et al., 1993).

The main advantage of the guanidination method over all the other reactive lysine assays is that OMIU will only react with the ε-amino group of lysine and does not react with the α-amino group of lysine or any other amino acid (Zhang et al., 2006; Catrein et al., 2005; Yamaguchi et al., 2005; Kassel and Chow, 1966; Shields et al., 1959; Klee and Richards, 1957; Chervenka and Wilcox, 1956) with the exception of glycine (Wang et al., 2005). 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.

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 (Rutherfurd and Moughan, 1990; Maga 1981; Möller et al., 1977). It is recommended to use unheated purified proteins (e.g. lysozyme) as external standards which can be guanidinated along with test samples to ensure that the guanidination reagent is adequately prepared and that the incubation conditions are optimal.

From a theoretical standpoint, the guanidination method works equally well with both unprocessed and processed proteins. In an unprocessed food or feedstuff, there are no early Maillard products and consequently, total lysine is equivalent to reactive lysine (Fig. 2). However, in a processed product where early Maillard products, or any other acid-labile lysine derivatives which revert 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 back to lysine but this reverted lysine is not included in the reactive lysine measurement since reactive lysine is represented by the homoarginine content only.

While the guanidination reaction has been used to determine the reactive lysine content of processed protein sources it does not take into account the incomplete digestion of proteins from the digestive tract and as such cannot be used to predict the biologically available lysine content of processed protein sources. In Chapter 2, the development of an assay that determines the true ileal digestibility of reactive lysine, and so does account for the incomplete digestion of reactive lysine, is described.

Determining available lysine

Accounting for the incomplete digestion of processed protein sources is an important consideration since the amino acid (including reactive lysine) digestibility of processed foods or feedstuffs is often far from complete (van Barneveld et al., 1994b; Wiseman et al., 1991; Batterham et al., 1990). Moughan et al. (1996) 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).

Table 1.
The amount of total lysine, FDNB reactive lysine, reactive lysine and absorbed reactive (available) lysine in a heated glucose/casein mixture (From Moughan et al., 1996).

	Total lysine[1]	FDNB reactive lysine	Reactive lysine[2]	Absorbed reactive lysine[3]
Lysine (mmol / 100 g)	31.6	22.7	25.0	14.7

[1]*Determined using conventional amino acid analysis.*
[2]*Determined using the furosine method.*
[3]*Determined from the furosine levels in the diet and digesta of pigs fed a heated glucose/casein-based diet.*

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 (2003). A test diet containing the food or feedstuff that is being tested is fed to a human subject or animal and digesta are collected from the terminal ileum just anterior to the ileo-caecal 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 naso-gastric 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 (Hodgkinson and Moughan, 2000; Batterham, 1994; Moughan, 1991).

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 lysine 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 (Batterham, 1992). 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. However, since many ingredients, foods and feedstuffs undergo extensive processing, there is a need to be able to accurately determine the available lysine content to permit the accurate formulation of diets for animals as well as assessment of the nutritional

quality of diets for animals and humans. In Chapter 2, the development of a new assay for determining the available lysine content based around the true ileal amino acid digestibility assay and the guanidination reaction is described. In Chapters 3-5, the new assay is used to determine the available lysine content of processed milk protein-based foods, "ready-to-eat" cereal-based breakfast foods and "complete and balanced" pet foods. In Chapter 6, the assay is used to evaluate the extent of processing and long-term storage on the available lysine content of skim milk powder and hydrolysed-lactose skim milk powder.

Slope-ratio assay

One alternative to using ileal digestibility to determine lysine availability in processed protein sources is the use of animal growth assays. These assays include the protein efficiency ratio, net protein utilisation, biological value (Bodwell, 1977) and arguably the most important, the slope-ratio assay. These methods are all based on the ability of an animal to deposit lysine from a test diet that is limiting in lysine, into the animal's body protein. Protein deposition in animals fed the test protein source is compared to 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 to that of the synthetic form. Batterham et al. (1979) 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, sunflower meal, rapeseed meal, skim milk powder and soyabean meal (Fernandez and Parsons, 1996; Batterham et al., 1979), meat and bone meal (Wang and Parsons, 1998; Batterham et al., 1986), blood meal (Batterham et al., 1986), distillers dried grains (Lumpkins and Batal, 2005), corn (Parsons et al., 1998) and heated field peas (van Barneveld et al., 1994a). Similar methods have been developed that use rat growth assays to determine the available lysine content in heated protein sources (Faldet et al., 1992; McDonough et al., 1989). 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., (1992) 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 (1996) 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. (1990) 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 (van Barneveld et al., 1994a). Similar results were found for corn and high oil corn (Parsons et al., 1998) and autoclaved and non autoclaved soyabean meal (Parsons et al., 1992) when fed to poultry. Mavromichalis and Baker (2000) 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 and Parsons (1998) fed chicks corn-soyabean meal diets containing a high quality meat and bone meal formulated based on either the ileal digestible lysine content determined using cecectomised roosters or the bioavailable lysine content determined using a slope-ratio assay. They reported similar feed intake, weight gain and FCR 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 to 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 (Hurrell and Carpenter, 1981). In addition, the slope-ratio assay is a predictor of lysine utilisation rather than lysine availability and utilisation is highly dependent on factors unrelated to the feed protein itself.

Indicator amino acid oxidation technique

Another assay has recently been developed for determining amino acid availability based on the indicator amino acid oxidation technique (Moehn et al., 2005). This method can be applied to any amino acid and Moehn et al. (2005) 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 to 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 to that on the control diet. Moehn et al., (2005) tested heated field peas similar to those used by van Barneveld et al. (1994a) and found good agreement between lysine availability data generated using the indicator amino acid oxidation technique and that determined using the slope-ratio assay. However, given that van Barneveld et al. (1994a) 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. (2005) 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 true ileal digestible reactive (available) lysine assay

True ileal lysine digestibility is an accurate measure of lysine availability when applied to unheated or minimally processed protein sources (Batterham et al., 1990). 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 and available lysine is then calculated as the reactive lysine content in the protein source multiplied by the true ileal reactive lysine digestibility.

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 ε-amino group of lysine (Zhang et al., 2006; Catrein et al., 2005; Yamaguchi et al., 2005; Kassel and Chow, 1966; Shields et al., 1959; Klee and Richards, 1957; Chervenka and Wilcox, 1956). In this thesis, we aimed to accurately determine the available lysine contents of a range of processed foods and feedstuffs and explore the effects of long-term storage on the available lysine content of selected milk products. In order to fulfil this aim, an assay was developed to accurately determine true ileal reactive lysine digestibility (lysine availability) in processed foods and feedstuffs by coupling the guanidination reaction with the true ileal amino acid digestibility assay.

In summary, available lysine is the unmodified lysine in foods and feedstuffs that is absorbed by the animal or human after consuming the food or feedstuff. It is important to be able to accurately determine available lysine for the optimal formulation of diets for intensive livestock, such as pigs or poultry, and for the assessment of the nutritional adequacy of diets for humans. Currently, assays are available to determine unmodified lysine (reactive lysine) in foods and feedstuffs but these do not take into account the incomplete absorption of amino acids from the small intestine. Furthermore, the true ileal amino acid digestibility assay does accurately determine available lysine in unprocessed and some processed protein sources, but since it uses traditional amino acid analysis it does not accurately determine the available lysine content of processed protein sources for which significant amounts of early Maillard products (or other acid-labile modified lysine derivatives) are present. Clearly an assay is required that determines the true ileal digestibility of reactive lysine. In this thesis, the development and validation of such an assay is described in Chapter 2. In addition, there is little information about the available lysine content of processed milk protein-based foods and almost no data on the available lysine content of other processed foods and feedstuffs such as pet foods and breakfast cereals. In this thesis, this gap in information is addressed with the assessment of the available lysine content of milk protein-based foods, “ready-to-eat”

cereal-based breakfast foods and "complete and balanced" cat foods described in Chapters 3, 4 and 5, respectively. In addition, there are no reports describing the effect of long-term storage at elevated temperatures on the digestible reactive lysine (available lysine) content of milk powders and in Chapter 6 the newly developed assay is used to address this gap in knowledge. The development of the available lysine assay described in this thesis will lead to better information about the amounts of available lysine present in processed foods and feedstuffs. This can give a more accurate prediction of protein deposition in animals which will enable feed companies to optimise the lysine contents in diets and minimise feed costs for intensive livestock production. In addition, the assay will provide a better assessment of the nutritional adequacy of human and companion animal diets in terms of dietary lysine leading to healthier foods for humans and companion animals and contribute to the knowledge of the presence of AGE's in foods and feedstuffs.

Literature cited

Adeola, O. 1996. Bioavailability of tryptophan in soybean meal for 10-kg pigs using slope-ratio assay. J. Anim. Sci. 74, 2411-2419.

Batterham, E.S. 1992. Availability and utilization of amino acids for growing pigs. Nutr. Res. Rev. 5, 1-18.

Batterham, E.S. 1994. Ileal digestibility of amino acids in feedstuffs for pigs. In Amino Acids in Farm Animal Nutrition. Pp. 113-131 [JPF D'Mello, editors]. Wallingford, U.K.: CAB International.

Batterham, E.S., Andersen, L.M., Baigent, D.R., Darnell, R.E., Taverner, M.R. 1990. A comparison of the availability and ileal digestibility of lysine in cottonseed and soya-bean meals for grower/finisher pigs. Brit. J. Nutr. 64, 663-677.

Batterham, E.S., Lowe, R.F., Darnell, R.E., Major, E.J. 1986. Availability of lysine in meat meal, meat and bone meal and blood meal as determined by the slope-ratio assay with growing pigs, rats and chicks and by chemical techniques. Brit. J. Nutr. 55, 427-440.

Batterham, E.S., Murison, R.D., Lewis, C.E. 1979. Availability of lysine in protein concentrates as determined by the slope-ratio assay with growing pigs and rats and by chemical techniques. Brit. J. Nutr. 41, 383-391.

Bodwell, C.E. 1977. Application of animal data to human protein nutrition: A review. Cereal Chem. 54, 958-983.

Booth, V.H. 1971. Problems in the determination of FDNB-available lysine. J. Sci. Food Agric. 22, 658-666.

Cai, W., He, J.C., Zhu, L., Peppa, M., Lu, C., Uribarri, J., Vlassara, H. 2004. High levels of dietary advanced glycation end products transform low-density lipoprotein into a potent redox-sensitive mitogen-activated protein kinase stimulant in diabetic patients. Circulation. 110, 285-291.

Carpenter, K.J. 1960. The estimation of available lysine in animal-protein foods. Biochem. J. 77, 604-610.

Carpenter, K.J., Bjarnason, J. 1968. Nutritional evaluation of proteins by chemical methods. In Evaluation of Novel Protein Products. Pp. 161 [AE Bender, R Kihlberg, B Löfqvist, and L Munck, editors]. Oxford: Pergamon Press.

Carpenter, K.J., Steinke, F.H., Catignani, G.L., Swaisgood, H.E., Allred, M.C., MacDonald, J.L., Schelstraete, M. 1989. The estimation of "available lysine" in human foods by

three chemical procedures. Plant Foods Hum. Nutr. 39, 129-135.
Catrein, I., Herrmann, R., Bosserhoff, A., Ruppert, T. 2005. Experimental proof for a signal peptidase 1 like activity in *Mycoplasma pneumoniae*, but absence of a gene encoding a conserved bacterial type 1 SPase. FEBS J. 272, 2892-2900.
Chervenka, C.H., Wilcox, P.E. 1956. Chemical derivatives of chymotrypsinogen. II. Reaction with O-methylisourea. J. Biol. Chem. 222, 635-647.
Couch, J.R., Thomas, M.C. 1976. A comparison of chemical methods for the determination of available lysine in various proteins. J. Agric. Food Chem. 24, 943-946.
Desrosiers, T., Savoie, L., Bergeron, G., Parent, G. 1989. Estimation of lysine damage in heated whey proteins by furosine determinations in conjunction with the digestion cell technique. J. Agric. Food Chem. 37, 1385-1391.
Erbersdobler, H.F., Hupe, A. 1991. Determination of lysine damage and calculation of lysine bio-availability in several processed foods. Z. Ernährungswiss. 30, 46-49.
Faldet, M.A., Satter, L.D., Broderick, G.A. 1992. Determining optimal heat treatment of soybeans by measuring available lysine chemically and biologically with rats to maximize protein utilization by ruminants. J. Nutr. 122, 151-160.
Fernandez-Artigas, P., Garcia-Villanova, B., Guerra-Hernandez, E. 1999. Blockage of available lysine at different stages of infant cereal production. J. Sci. Food Agric. 79, 851-854.
Fernandez, S.R., Parsons, C.M. 1996. Bioavailability of the digestible lysine and valine in cottonseed meal and soybean meal for chicks. Poult. Sci. 75, 216-223.
Finot, P.A. 1990. Metabolism and physiological effects of Maillard reaction products. In The Maillard Reaction in Food Processing, Human Nutrition, and Physiology. Pp. 259-271 [PA Finot, HU Aeschbacher, RF Hurrell and R Liardon, editors]. Basal, Switzerland: Birkhauser.
Finot, P.A., Bricout, J., Viani, R., Mauron, J. 1968. Identification of a new lysine derivative obtained upon hydrolysis of heated milk. Experientia. 24, 1097-1099.
Finot, P.A., Bujard, E., Mottu, F., Mauron, J. 1977. Availability of the true Schiff's bases of lysine. Chemical evaluation of the Schiff's base between lysine and lactose in milk. In Protein Crosslinking-B: Nutritional and Medical Consequences. Pp. 343-366 [M Friedman, editor]. New York: Plenum.
Finot, P.A., Hurrell, R.F. 1985. *In vitro* methods to predict lysine availability. In Digestibility and Amino Acid Availability in Cereals and Oilseeds. Pp. 247-258 [JW Finley and DT Hopkins, editors]. St. Paul Minnesota: American Association of Cereal Chemists Inc.
Finot, P.A., Viani, R., Bricout, J., Mauron, J. 1969. Detection and identification of pyridosine, a second lysine derivative obtained upon acid hydrolysis of heated milk. Experientia. 25, 134-135.
Franzen, K., Singh, R.F., Okos, M.R. 1990. Kinetics of nonenzymatic browning in dried skim milk. J. Food Eng. 11, 225-239.
Friedman, M. 1996. Food browning and its prevention: An overview. J. Agric. Food Chem. 44, 631-653.
Friedman, M., Cuq, J-L. 1988. Chemistry, analysis, nutritional value, and toxicology of tryptophan. A review. J. Agric. Food Chem. 36, 1079-1093.
Friedman, M., Pang, J., Smith, G.A. 1984. Ninhydrin-reactive lysine in food proteins. J. Food Sci. 49, 10-13.
Hagemeister, H., Erbersdobler, H. 1985. Chemical labelling of dietary protein by

transformation of lysine to homoarginine: A new technique to follow intestinal digestion and absorption. Proc. Nutr. Soc. 44, 133A.

Hartkopf, J., Pahlke, C., Lüdeman, G., Erbersdobler, H.F. 1994. Determination of N^{ε}-carboxymethyllysine by a reversed-phase high-performance liquid chromatography method. J. Chromatogr. A. 672, 242-246.

Hendriks, W.H., Emmens, M.M.A., Trass, B., Pluske, J.R. 1999. Heat processing changes the protein quality of canned cat foods as measured with a rat bioassay. J. Anim. Sci. 77, 669-676.

Hendriks, W.H., Moughan, P.J., Boer, H., van der Poel, A.F.B. 1994. Effects of extrusion on the dye-binding, fluorodinitrobenzene-reactive and total lysine content of soybean meal and peas. Anim. Feed Sci. Technol. 48, 99-109.

Henle, T., Walter, H., Klostermeyer, H. 1991. Evaluation of the extent of the early Maillard-reaction in milk products by direct measurement of the Amadori-product lactuloselysine. Zeitschrift für Lebensmitteluntersuchung und -Forschung A 193, 119-122.

Hettinger, T.P., Harbury, H.A. 1965. Guanidinated cytochrome *c*. II. Biochem. 4, 2585-2589.

Heyns, K., Heukeshoven, J., Brose, K.H. 1968. Der Abbau von Fructose-aminosäuren zu N-(2-Furosylmethyl) Aminosäuren: Zwishenprodukte von Bräunungsreaktionen. Agnew. Chem. 80, 627.

Hirs, C.H.W., Stein, W.H., Moore, S. 1954. The amino acid composition of ribonuclease. J. Biol. Chem. 211, 941-950.

Hodgkinson, S.M., Moughan, P.J. 2000. Amino acids – The collection of ileal digesta and characterisation of the endogenous component. In Developments in the Determination of Protein and Amino Acids. Feed Evaluation: Principles and Practice. Pp. 105-124 [PJ Moughan, MWA Verstegen and MI Visser-Reyneveld, editors]. Wageningen, Netherlands: Wageningen Pers.

Horvatić, M., Guterman, M. 1997. Available lysine content during cereal flake production. J. Sci. Food Agric. 74, 354-358.

Hughes, W.L. Jr., Saroff, H.A., Carney, A.L. 1949. Preparation and properties of serum and plasma proteins. XXII. A crystallizable guanidinated derivative of human serum albumin. J. Am. Chem. Soc. 71, 2476-2480.

Hurrell, R.F., Carpenter, K.J. 1981. The estimation of available lysine in foodstuffs after Maillard reactions. Prog. Food Nutr. Sci. 5, 159-176.

Hurrell, R.F., Carpenter, K.J. 1977. Nutritional significance of cross-linking formation during food processing. In Protein Crosslinking: Nutritional and Medical Consequences, Pp. 225-238. [M Friedman, editor]. New York: Plenum.

Hurrell, R.F., Carpenter, K.J. 1974. Mechanisms of heat damage in proteins 4. The reactive lysine content of heat-damaged material as measured in different ways. Brit. J. Nutr. 32, 589-604.

Kakade, M.L., Leiner, I.E. 1969. Determination of available lysine in proteins. Anal. Biochem. 27, 273-280.

Kassell, B., Chow, R.B. 1966. Modification of the basic trypsin inhibitor of bovine pancreas. The ε-amino groups of lysine and the amino terminal sequence. Biochem. 5, 3449-3453.

Klee, W.A., Richards, F.M. 1957. The reaction of *O*-methylisourea with bovine pancreatic ribonuclease. J. Biol. Chem. 229, 489-504.

Konishi, Y., Hayase, F., Kato, H. 1994. Novel imidazolone compound formed by the advanced Maillard reaction of 3-deoxyglucosone and arginine residues in proteins. Biosci. Biotechnol. Biochem. 58, 1953-1955.

Koschinsky, T., He, C.J., Mitsuhashi, T., Bucala, R., Liu, C., Buenting, C., Heitmann, K., Vlassara, H. 1997. Orally absorbed reactive glycation products (glycotoxins): An environmental risk factor in diabetic nephropathy. Proc. Natl. Acad. Sci. USA. 94, 6474–6479.

Kume, S., Takeya, M., Mori, T., Araki, N., Suzuki, H., Horiuchi, S., Kodama, T., Miyauchi, Y., Takahashi, K. 1995. Immunohistochemical and ultrastructural detection of advanced glycation end products in atherosclerotic lesions of human aorta with a novel specific monoclonal antibody. Am. J. Pathol. 147, 654-667.

Lin, R.Y., Choudhury, R., Cai, W., Lu, M., Fallon, J.T., Fisher, E.A., Vlassara, H. 2003. Suppression of atherosclerotic lesions by dietary restriction of advanced glycation end products (AGEs) in diabetic apolipoprotein E-deficient C57BL/6J mice. Atherosclerosis. 168, 213–220

Lumpkins, B.S., Batal, A.B. 2005. The bioavailability of lysine and phosphorus in distillers dried grains with solubles. Poult. Sci. 84, 581-586.

Maga, J.A. 1981. Measurement of available lysine using the guanidination reaction. J. Food Sci. 46, 132-134.

Malec, L.S., Llosa, R.A., Naranjo, G.B., Vigo, M.S. 2005. Loss of available lysine during processing of different *dulche de leche* formations. Int. J. Dairy Technol. 58, 164-168.

Mao, L-C., Lee, K-H., Erbersbobler, H.F. 1993. Effects of heat treatment on lysine in soya protein. J. Sci. Food Agric. 62, 307-309.

Marquié, C., Tessier, A-M., Aymard, C., Guilbert, S. 1997. HPLC determination of the reactive lysine content of cottonseed protein films to monitor the extent of cross-linking by formaldehyde, glutaraldehyde, and glyoxal. J. Agric. Food Chem. 45, 922–926

Mavromichalis, I., Baker, D.H. 2000. Effects of pelleting and storage of a complex nursery pig diet on lysine bioavailability. J. Anim. Sci. 78, 341-347.

Mauron, J., Bujard, E. 1964. Guanidination, an alternative approach to the determination of available lysine in foods. Proc. 6th Int. Nutr. Congr. 489-490.

Mauron, J., Mottu, F., Bujard, E., Egli, R.H. 1955. The availability of lysine, methionine and tryptophan in condensed milk and milk powder. *In vitro* digestion studies. Arch. Biochem. Biophys. 59, 433-451.

McDonough, F.E., Hitchins, A.D., Bodwell, CE., Staples, R.S. 1989. Bioavailability of lysine in selected foods by rat growth assay. Plant Foods Hum. Nutr. 69, 67-75.

Milán-Carillo, J., Gutiérrez-Dorado, R., Perales-Sánchez, J.X.K., Cuevas-Rodríguez, E.O., Ramírez-Wong, B., Reyes-Moreno, C. 2006. The optimization of the extrusion process when using maize flour with a modified amino acid profile for making tortillas. Int. J. Food Sci. Technol. 41, 727-736.

Mills, F.D., Hodge, J.E. 1976. Amadori compounds: vacuum thermolysis of 1-deoxy-1-L-prolino-D-fructose. Carbohyr. Res. 51, 9-12.

Moehn, S., Bertolo, R.F.P., Pencharz, P.B., Ball, R.O. 2005. Development of the indicator amino acid oxidation technique to determine the availability of amino acids from dietary protein in pigs. J. Nutr. 135, 2866-2870.

Möller, A.B., Andrews, A.T., Cheeseman, G.C. 1977. Chemical changes in ultra-heat-treated

milk during storage. III. Methods for the estimation of lysine and sugar-lysine derivatives formed by the Maillard reaction. J. Dairy Res. 44, 277-281.

Moughan, P.J. 2003. Amino acid availability: Aspects of chemical and bioassay methodology. Nutr. Res. Rev. 16, 127-141.

Moughan, P.J. 1991. Towards an improved utilization of dietary amino acids by the growing pig. In Recent Advances in Animal Nutrition. Pp. 45-64 [W Haresign and DJA Cole, editors]. Oxford, UK: Butterworth-Heinemann.

Moughan, P.J., Gall, M.P.J., Rutherfurd, S.M. 1996. Absorption of lysine and deoxyketosyllysine in an early-Maillard browned casein by the growing pig. J. Agric. Food Chem. 44, 1520-1525.

Moughan, P.J., Rutherfurd, S.M. 1991. Endogenous lysine flow at the distal ileum of the protein-fed rat: Investigation of the effect of protein source using radioactively labelled acetylated lysine or lysine transformed to homoarginine. J. Sci. Food Agric. 55, 163-174.

Moughan, P.J., Rutherfurd, S.M. 1990. Endogenous flow of total lysine and other amino acids at the distal ileum of the protein- or peptide-fed rat: The chemical labelling of gelatin protein by transformation of lysine to homoarginine. J. Sci. Food Agric. 52, 179-192.

Moughan, P.J., Schrama, J., Skilton, G.A., Smith, W.C. 1989. *In-vitro* determination of nitrogen digestibility and lysine availability in meat and bone meals and comparison with *in-vivo* ileal digestibility estimates. J. Sci. Food Agric. 47, 281-292.

Nass, N.B., Bartling, B., Navarrete Santos, A., Scheubel, R.J., Börgermann, J., Silber, R.E., Simm, A. 2007. Advanced glycation end products, diabetes and ageing. Z. Gerontol. Geriat. 40, 349–356.

Noll, S.L., Waibel, P.E., Behrends, B.R. 1984. Available lysine content of blood meals determined by turkey bioassay and fluorodinitrobenzene assay. Poult. Sci. 63, 144-152.

Nyachoti, C.M., de Lange, C.F.M., Schulze, H. 1997. Estimating endogenous amino acid flows at the terminal ileum and true ileal amino acid digestibilities in feedstuffs for growing pigs using the homoarginine method. J. Anim. Sci. 75, 3206-3213.

Parsons, C.M., Hashimoto, K., Wedekind, K.J., Han, Y., Baker, D.H. 1992. Effect of over processing on availability of amino acids and energy in soybean meal. Poult. Sci. 71, 133-140.

Parsons, C.M., Zhang, Y., Araba, M. 1998. Availability of amino acids in high-oil corn. Poult. Sci. 77, 1016-1019.

Rao, S.R., Carter, F.L., Frampton, V.L. 1963. Determination of available lysine in oilseed meal proteins. Anal. Chem. 35, 1927-1930.

Ravindran, V., Hew, L.I., Ravindran, G., Bryden, W.L. 2004. Endogenous amino acid flow in the avian ileum: quantification using three techniques. Brit. J. Nutr. 92, 217-223.

Rayner, C.J., Fox, M. 1978. Measurement of available lysine in processed beef muscle by various laboratory techniques. J. Agric. Food Chem. 26, 494-497.

Rehman, Z-U. 2006. Storage effects on nutritional quality of commonly consumed cereals. Food Chem. 92, 53-57.

Roach, A.G., Sanderson, P., Williams, D.R. 1967. Comparison of methods for the determination of available lysine value in animal and vegetable protein sources. J. Sci. Food Agric. 18, 274-278.

Rufián-Henares, J.A., Guerra-Hernández, E., García-Villanova, B. 2006. Available lysine

and fluorescence in heated milk proteins/dextrinomaltose or lactose solutions. Food Chem. 98, 685-692.

Rutherfurd, S.M., Moughan, P.J. 1990. Guanidination of lysine in selected dietary proteins. J. Agric. Food Chem. 38, 209-211.

Rutherfurd, S.M., Chung, T.K., Moughan, P.J. 2007. The effect of a commercial enzyme preparation on apparent metabolizable energy, the true ileal amino acid digestibility, and endogenous ileal lysine losses in broiler chickens. Poult. Sci. 86, 665-672.

Saito, G., Okitani, A., Hayase, F., Kato, H. 1986. Characterization of tryptophan derivatives from the reaction of N-α-acetyl-tryptophan with carbonyl compounds. Agric. Biol. Chem. 50, 2315-2323.

Schleicher, E.D., Wagner, E., Nerlich, A.G. 1997. Increased accumulation of the glycoxidation product N(epsilon)-(carboxymethyl)lysine in human tissues in diabetes and aging. J. Clin. Invest. 99, 457–468.

Šebeková, K., Blažíček, P., Syrová, D., Šíková, Z.K., Spustová, V., Heidland, A., Schinzel, R. 2001. Circulating advanced glycation end product levels in rats rapidly increase with acute renal failure. Kidney Int. 59, S58–S62.

Shields, G.S., Hill, R.L., Smith, E.L. 1959. Preparation and properties of guanidinated mercuripapain. J. Biol. Chem. 234, 1747-1753.

Siriwan, P., Bryden, W.L., Annison, E.F. 1994. Use of guanidinated dietary protein to measure losses of endogenous amino acids in poultry. Brit. J. Nutr. 71, 515-529.

Siskos, I., Zotos, A., Taylor, K.D.A. 2005. The effect of drying, pressure and processing time on the quality of liquid-smoked trout (*Salmo gairdnerii*) fillets. J. Sci. Food Agric. 85, 2054-2060.

Taverner, M.R., Farrell, D.J. 1981. Availability to pigs of amino acids in cereal grains. 3. A comparison of ileal availability values with faecal, chemical and enzymic estimates. Brit. J. Nutr. 46, 173-180.

Torbatinejad, N.M., Rutherfurd, S.M., Moughan, P.J. 2005. Total and reactive lysine contents in selected cereal-based food products. J. Agric. Food Chem. 53, 4454-4458.

Tressl, R., Wondrak, G., Kersten, E., Rewicki, D. 1994. Structure and potential cross-linking reactivity of a new pentose-specific Maillard product. J. Agric. Food Chem. 42, 2692-2697.

Undi, M., Moshtaghi, S.S., Wittenberg, K.M., Ingalls, J.R. 1996. A comparative study on amino acid availability of moist heated canola meal for poultry vs. ruminants. Anim. Feed Sci. Technol. 63, 179-186.

van Barneveld, R.J., Batterham, E.S., Norton, B.W. 1994a. The effect of heat on amino acids for growing pigs. 3. The availability of lysine from heat-treated field peas (*Pisum sativum* cultivar Dundale) determined using the slope-ratio assay. Brit. J. Nutr. 72, 257-275.

van Barneveld, R.J., Batterham, E.S., Norton, B.W. 1994b The effect of heat on amino acids for growing pigs. 1. A comparison of ileal and faecal digestibilities of amino acids in raw and heat-treated field peas (*Pisum sativum* cultivar Dundale). Brit. J. Nutr. 72, 221-241.

Vigo, M.S., Malec, L.S., Gomez, R.G., Llosa. R.A. 1992. Spectrophotometric assay using *o*-phthaldialdehyde for determination of reactive lysine in dairy products. Food Chem. 44, 363-365.

Wang, X., Parsons, C.M. 1998. Dietary formulation with meat and bone meal on a total versus a digestible or bioavailable amino acid basis. Poult. Sci. 77, 1010-1015.

Wang, D., Xu, W., McGrath, S.C., Patterson, C., Neckers, L., Cotter, R.J. 2005. Direct identification of ubiquitination sites on ubiquitin-conjugated CHIP using MALDI mass spectrometry. J. Proteome. Res. 4, 1554-1560.

Wiseman, J., Jagger, S., Cole, D.J.A., Haresign, W. 1991. The digestion and utilization of amino acids of heat-treated fish meal by growing/finishing pigs. Anim. Prod. 53, 215-225.

Yamaguchi, M., Nakazawa, T., Kuyama, H., Obama, T., Ando, E., Okamura, T-A., Ueyama, N., Norioka, S. 2005. High-throughput method for N-terminal sequencing of proteins by MALDI mass spectrometry. Anal. Chem. 77, 645-651.

Zhang, H.L., Li, D.F., Qiao, S.Y., Wang, F.L., Chen, X.J., Thacker, P.A. 2006. The effect of dietary homoarginine derived from guanidination of synthetic lysine on endogenous amino acid loss and apparent and true ileal amino acid digestibility in the pig. Anim. Sci. 82, 23-30.

Chapter 2

Development and validation of an assay for determining true ileal digestible reactive (available) lysine in processed foods and feedstuffs

Shane Rutherfurd was part of the original research team that first discovered that not all the reactive lysine in a processed food or feedstuff is absorbed from the small intestine of an animal or human. Based on this finding the same team recognised the need to have an assay that could determine the absorbed reactive lysine content of a processed food or feedstuff and went on to develop an assay that could determine the digestible reactive lysine. This work is described in two pivotal papers (Moughan et al., 1996; Moughan and Rutherfurd, 1996). In publishing these papers, our team shifted the focus, which had previously been on the fate of the modified lysine derivatives in the gastrointestinal tract, to the fate of the unmodified lysine in processed foodstuffs when consumed by an animal or human. The original assay required further development and testing, and this thesis describes the development, validation and application of the bioavailability assay.

Reproduced in part with permission from the publishers from: Moughan, P.J., Rutherfurd, S.M. 1996. A new method for determining digestible reactive lysine in foods. J. Agric. Food Chem. 44, 2202-2209. Rutherfurd, S.M., Moughan, P.J. 1997. Application of a new method for determining digestible reactive lysine to variably heated protein sources. J. Agric. Food Chem. 45, 1582-1586. Rutherfurd, S.M., Moughan, P.J., Morel, P.C.H. 1997. Assessment of the true ileal digestibility of reactive lysine as a predictor of lysine uptake from the small intestine of the growing pig. J. Agric. Food Chem. 45, 4378-4383. Rutherfurd, S.M., Moughan, P.J., van Osch, L. 1997. Digestible reactive lysine in processed feedstuffs: Application of a new bioassay. J. Agric. Food Chem. 45, 1189-1194. Torbatinejad, N.M., Rutherfurd, S.M., Moughan, P.J. 2005. Total and reactive lysine contents in selected cereal-based food products. J. Agric. Food Chem. 53, 4454-4458.

Introduction

Available lysine is defined as the lysine present in a food or feedstuff that is absorbed from the small intestine in a form capable of being used for protein synthesis. A number of methods have been developed to determine available lysine in both unprocessed and processed foods and feedstuffs, including growth-based assays, chemical methods, indicator amino acid oxidation technique and ileal digestibility assays. Briefly, growth assays determine the lysine deposition or growth rate of an animal fed control diets containing increasing amounts of dietary lysine but for which lysine is the first limiting indispensible amino acid. The lysine deposition or growth rate of the control animals is then compared to animals fed a test food or feedstuff (that is also limiting in lysine) and based on this comparison the available lysine content of the food or feedstuff can be estimated. These assays are laborious and time consuming, are highly variable and the results are difficult to interpret. Furthermore, growth-based assays tend to determine lysine utilisation rather than lysine availability. The indicator amino acid oxidation technique uses phenylalanine oxidation as the measure of lysine utilisation and as such does not specifically determine lysine availability. Reactive lysine assays, most of which are chemical-based, accurately determine the unmodified lysine content of a food or feedstuff but assume that digestion and absorption of lysine from the small intestine is complete, an assumption that is likely to be flawed when applied to many processed protein sources. Ileal digestibility assays determine the lysine content in the food or feedstuff and also the undigested lysine at the end of the small intestine (ileal digesta) of an animal or ileostomised human fed the test food or feedstuff. From the difference between the dietary lysine and undigested lysine in the digesta, the proportion of lysine that has been digested and absorbed can be calculated. The ileal digestibility assay does accurately determine lysine availability for unprocessed foods and feedstuffs as well as the availability of most amino acids in processed foods and feedstuffs assuming that microbial amino acid breakdown or production in the small intestine is negligible. However, since this method uses conventional amino acid analysis to determine the lysine content in diets and digesta, it does not accurately determine lysine digestibility when applied to processed foods or feedstuffs that may contain early Maillard products or other acid-labile modified lysine derivatives. Consequently, there is a need for an assay that can accurately determine the available lysine in processed protein sources which can be used to formulate animal feeds or human diets that meet the lysine requirement of animals or humans.

The aim of this study was to develop such an assay by combining the guanidination reaction and a true ileal digestibility assay. Essentially, a test foodstuff would be fed to a group of test animals and the digesta collected from the terminal ileum. The reactive lysine content of both the diet and digesta would be determined using the guanidination reaction (using OMIU to convert reactive lysine to the acid-stable derivative, homoarginine) and the digestibility of reactive lysine calculated. This apparent digestibility estimate would then be corrected to a true digestibility value by correcting for the endogenous lysine that is secreted into the small intestine in mucus, digestive enzymes and sloughed gut cells. Correction for endogenous ileal lysine loss is made using the enzymatically hydrolysed casein (EHC) technique (Butts et al., 1991; Moughan et al., 1990). The reactive lysine content of the original food or feedstuff can also be determined and multiplied by the true ileal reactive lysine digestibility providing an estimate of the true ileal digestible reactive lysine content of the food or feedstuff. The digestible reactive lysine content is by definition the bioavailable

or available lysine. In this method it is assumed that there is no net microbial lysine synthesis in the gut.

Developing the true ileal digestible reactive (available) lysine assay

Optimisation of the guanidination reaction

In this study, the effect of both reaction time and reaction mixture pH on the guanidination of lysine in food protein models and in terminal ileal digesta was investigated. The food protein models consisted of an unprocessed (or minimally processed) protein source and a processed protein source. Unheated casein was chosen as the unprocessed protein source while a heated lactose/casein mixture was chosen as the processed protein source. The lactose/casein mixture, which was derived from the same batch of casein as the unheated casein, was autoclaved at 121 °C for 3.5 min to ensure the presence of early Maillard products (Amadori compound). To obtain digesta material for the optimisation of the guanidination reaction for digesta, terminal ileal digesta were collected from rats fed wheat starch-based diets containing either the unheated casein (unheated casein digesta) or the heated lactose/casein mixture (heated lactose/casein digesta) as the sole protein source.

The optimal reaction mixture pH and reaction time was determined for the guanidination of both protein sources and digesta. The most suitable OMIU concentration (0.6 M) and reaction mixture pH (10.6) for guanidinating unheated casein had been previously determined (Rutherfurd and Moughan, 1990). Consequently, these conditions were used for the first experiment in this study aimed at determining the optimal reaction time for maximal guanidination of the casein and the heated lactose/casein mixture. When guanidination was carried out with 0.6 M OMIU at pH 10.6 in a shaking waterbath at 21 ± 2 °C using a OMIU to lysine molar ratio of 1000, the lysine in the unheated casein was almost completely guanidinated (> 98%) after incubation for 1 d. For the heated lactose/casein mixture, as with any processed food or feedstuff, the extent of conversion of lysine to homoarginine cannot be determined directly. This is because the lysine that is detected during the amino acid analysis of a guanidinated processed protein source or digesta may result from the reversion of early Maillard products during acid hydrolysis rather than from incomplete guanidination. Consequently, the reaction time that resulted in the maximal guanidination of the heated lactose/casein mixture was deemed to be the optimal reaction time. For the heated lactose/casein mixture, the optimal incubation time was 7 d (Fig. 1).

For digesta, the optimal incubation time for the guanidination of digesta was determined. Both digesta (unheated casein derived and heated lactose/casein derived digesta) were incubated with 0.6 M OMIU, at pH 11.0 in a shaking waterbath at 21 ± 2 °C and an OMIU to lysine ratio of 1000 for 1 - 14 d (Fig. 2A). For the digesta collected from rats fed the unheated casein-based diet, the reaction time that resulted in the greatest amount of guanidination ranged from 1 - 7 d. For the digesta collected from rats fed the heated lactose/casein-based diet, the reaction time that resulted in maximal guanidination was 7 d. Based on these results, it was concluded that 7 d was a suitable incubation time for the maximal guanidination of lysine in digesta containing either no Maillard products or early Maillard products.

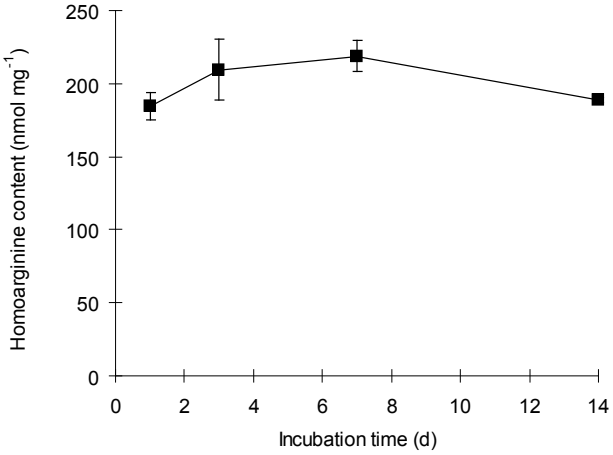


Figure 1. Homoarginine (reactive lysine) content (± SD) of a heated lactose/casein incubated with 0.6 M OMIU pH 10.6 in a shaking waterbath at 21 ± 2 °C for 1 to 14 d, with an OMIU to lysine ratio greater than 1000.

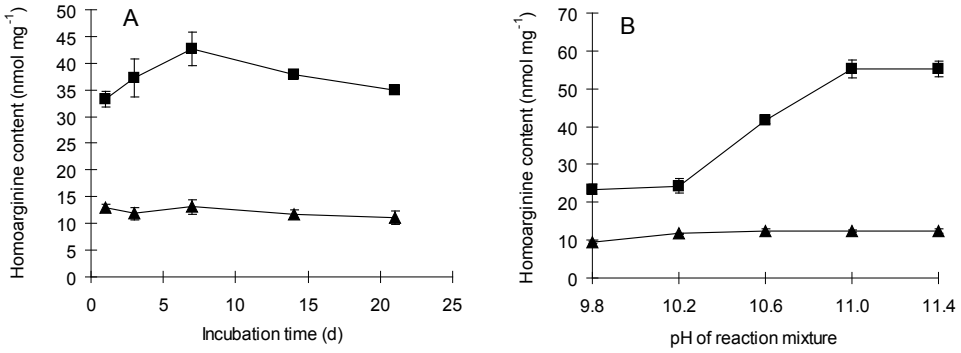


Figure 2. Amount of homoarginine (reactive lysine) (nmol mg^{-1} sample) present in the digesta of rats fed unheated casein (▲) and heated lactose/casein (■) determined using the guanidination reaction varying reaction time (A) and reaction mixture pH (B).

(A) Guanidination conditions were incubation in 0.6 M OMIU (pH 10.6) at 21 ± 2 °C for 1 - 21 d, with the OMIU to lysine ratio greater than 1000, followed by conventional amino acid analysis. (B) Guanidination conditions for the digesta of rats fed unheated casein were incubation for 1 d at 21 ± 2 °C in 0.6 M OMIU (pH 9.8 - 11.4), with the reagent to lysine ratio greater than 1000, followed by conventional amino acid analysis. The guanidination conditions for the digesta of rats fed heated lactose/casein were incubation for 7 d at 21 ± 2 °C in 0.6 M OMIU (pH 9.8 - 11.4), with the reagent to lysine ratio greater than 1000, followed by conventional amino acid analysis. For (A) n=13 for unheated casein digesta, 1 d incubation; n=8 for heated lactose/casein digesta, 1 d incubation; n=7 for unheated casein digesta, 3 and 7 d incubations and heated lactose/casein digesta, 7 d incubation; n=5 for heated lactose/casein digesta, 7 d incubation; n=3 for both digesta at 14 and 21 d incubations. For (B) n=3 for all analyses. Values are means ± SE.

To determine the optimum pH for the guanidination of lysine, incubation with 0.6 M OMIU was carried out for either 1 d (unheated casein derived digesta) or 7 d (heated lactose/casein derived digesta) in a shaking waterbath at 21 ± 2 °C with an OMIU to lysine ratio of 1000. The optimal pH of the reaction mixture for the maximal guanidination of lysine in the digesta of rats fed the unheated casein-based diets and the heated lactose/casein-based diets ranged from 10.6 - 11.4 and 11.0 - 11.4 respectively (Fig. 2B). Consequently, it was concluded that a pH of 11.0 was suitable for the maximal guanidination of lysine in both digesta that did contained early Maillard products and digesta that did not contained early Maillard products.

Investigating the specificity of the guanidination reaction for the ε-amino group of lysine in digesta

One advantage of OMIU as a derivitising reagent for reactive lysine is that it has been reported to react only with the ε-amino group of lysine and not with the α-amino group of lysine (Zhang et al., 2006; Catrein et al., 2005; Yamaguchi et al., 2005; Kassel and Chow, 1966; Shields et al., 1959; Klee and Richards, 1957; Chervenka and Wilcox, 1956). This characteristic is not relevant when lysine is present in intact proteins, such as those present in foods and feedstuffs, since the α-amino group of lysine will already be bound through peptide bonds to other amino acids. However, in digesta, free amino acids and small peptides are present and the likelihood of unbound lysine α-amino groups being present is much greater. Consequently, it was deemed necessary to test the specificity of OMIU for the ε-amino group of lysine for application to the guanidination of reactive lysine in digesta.

The specificity of OMIU for the ε-amino group of lysine in digesta cannot be determined directly since the doubly derivatised amino acid (OMIU bound to both ε- and α-amino groups) and the α-N-guanidinolysine (OMIU bound to the α-amino group) cannot be easily detected. With this limitation in mind, two approaches were adopted. Firstly, the recovery of lysine as homoarginine after guanidination of pure synthetic lysine was determined. Approximately 95% of the synthetic lysine was converted to homoarginine during guanidination suggesting a high specificity of OMIU for the ε-amino group of lysine. The second approach was conducted with digesta directly and was based on the hypothesis that if the α-amino group of amino acids other than lysine that are present in digesta did not react with the OMIU then it was likely that the α-amino group of lysine would similarly not react. In this study, digesta was collected from rats fed one of five different processed protein sources (blood meal, soyabean meal, wheat meal, meat and bone meal and cottonseed meal). The amino acid content (of amino acids other than lysine) in the rat digesta was determined using either traditional amino acid analysis or guanidination followed by traditional amino acid analysis and then compared. The results are presented in (Table 1).

Table 1.
The concentration (µg g^{-1} dry matter intake (DMI)) of amino acids (other than lysine) determined using either conventional amino acid analysis or guanidination followed by conventional amino acid analysis.

	Blood meal				Soyabean meal				Wheat meal				Meat and bone meal				Cottonseed meal			
	U[1]	G[2]	SE[3]		U	G	SE		U	G	SE		U	G	SE		U	G	SE	
Aspartic acid	1.30	1.54	0.10	***	1.98	2.36	0.08	**	1.59	1.64	0.21	NS	2.96	3.07	0.20	NS	2.90	3.42	0.15	***
Threonine	0.64	0.82	0.05	***	0.84	1.12	0.03	***	0.84	0.91	0.10	NS	1.27	1.63	0.06	**	1.52	1.92	0.08	***
Serine	0.57	0.74	0.05	***	0.80	1.05	0.03	***	0.79	0.97	0.11	NS	1.53	1.84	0.08	*	1.45	1.85	0.08	***
Glutamic acid	1.50	1.43	0.11	*	2.03	2.02	0.09	NS	1.78	1.50	0.22	NS	3.67	3.35	0.23	NS	3.96	3.91	0.18	NS
Proline	0.54	0.71	0.05	*	0.93	0.99	0.08	NS	1.15	0.93	0.18	NS	2.83	2.51	0.22	NS	1.67	1.97	0.14	*
Glycine	0.90	0.81	0.06	**	1.39	1.38	0.10	NS	1.56	1.31	0.21	NS	5.05	3.89	0.45	NS	2.27	2.21	0.17	NS
Alanine	0.91	0.86	0.07	NS	0.65	0.72	0.03	*	0.80	0.84	0.13	NS	1.90	1.75	0.11	NS	1.57	1.62	0.06	NS
Valine	0.70	0.66	0.06	NS	0.71	0.75	0.03	NS	0.71	0.63	0.08	NS	1.21	1.14	0.07	NS	1.47	1.51	0.07	NS
Isoleucine	0.38	0.38	0.03	NS	0.57	0.55	0.03	NS	0.45	0.40	0.05	NS	0.69	0.69	0.03	NS	1.12	1.11	0.06	NS
Leucine	1.01	0.93	0.09	NS	1.09	1.10	0.05	NS	0.82	0.74	0.10	NS	1.61	1.56	0.06	NS	2.07	2.07	0.09	NS
Tyrosine	0.45	0.41	0.03	NS	0.47	0.45	0.03	NS	0.52	0.44	0.06	NS	0.74	0.67	0.07	NS	0.83	0.87	0.05	NS
Phenylalanine	0.60	0.64	0.05	NS	0.79	0.86	0.05	NS	0.64	0.64	0.10	NS	0.86	1.06	0.07	NS	1.34	1.43	0.09	NS

[1]Unguanidinated digesta.
[2]Guanidinated digesta.
[3]Overall SE
*NS not significant, P > 0.05; *, 0.05 > P > 0.01; **, 0.01 > P > 0.001; ***, P < 0.001.*

Across all of the five feedstuffs and the 12 amino acids tested in the present study (histidine and arginine were not determined since these amino acids coeluted with a large ammonia peak present during the ion-exchange HPLC analysis of the guanidinated samples), there was no significant ($P > 0.05$) difference between the amino acid content determined either with or without guanidination prior to amino acid analysis for 73% of the amino acid determinations. It should be noted that for serine and threonine there were some relatively large differences between the values determined with guanidination followed by amino acid analysis or amino acid analysis alone. However, since the determined content of these amino acids was higher when guanidination was used, the differences could not have been a result of guanidination of the α-amino group. Furthermore, for the amino acids where significant differences were observed, for only 2 amino acids (glutamic acid and glycine) in the blood meal were the values lower when guanidination was used. It would be expected that if the α-amino group of amino acids was being guanidinated in the presence of OMIU, then the amino acid content would be lower when guanidination and amino acid analysis was used compared to amino acid analysis alone. Since in this experiment that was not the case there is strong indirect evidence to suggest that the α-amino group of lysine does not undergo guanidination in digesta.

Comparison of the guanidination method with the FDNB method for determining reactive lysine in foods

The reactive lysine content of a selection of processed protein sources were determined using either the newly optimised guanidination reaction conditions or the FDNB method (Carpenter 1960) with modifications as described by Booth (1971). The latter method is generally accepted as being suitable for determining the reactive lysine content of processed foods and feedstuffs (Hurrell and Carpenter, 1981). The results of this experiment are shown in Fig. 3A. For the five processed feedstuffs tested, there was excellent agreement between the reactive lysine content determined using the guanidination reaction followed by amino acid analysis and that determined using the FDNB method. Torbatinejad et al. (2005) made a similar comparison for 20 "ready-to-eat" cereal-based breakfast foods (Fig. 3B) and also showed an excellent correlation between the reactive lysine content determined using the guanidination reaction with that determined using the FDNB method. Overall, the guanidination method using the reaction conditions established in the present study appeared to accurately determine the reactive lysine content of processed foods and feedstuffs based on comparison with the FDNB method. In addition, since the guanidination method generally did not underestimate reactive lysine it would appear that guanidination condition used in this study resulted in the complete, or at least near complete, conversion of reactive lysine to homoarginine. Unfortunately, the same comparison cannot be made with digesta since FDNB reacts with free amino acids and peptides in the digesta.

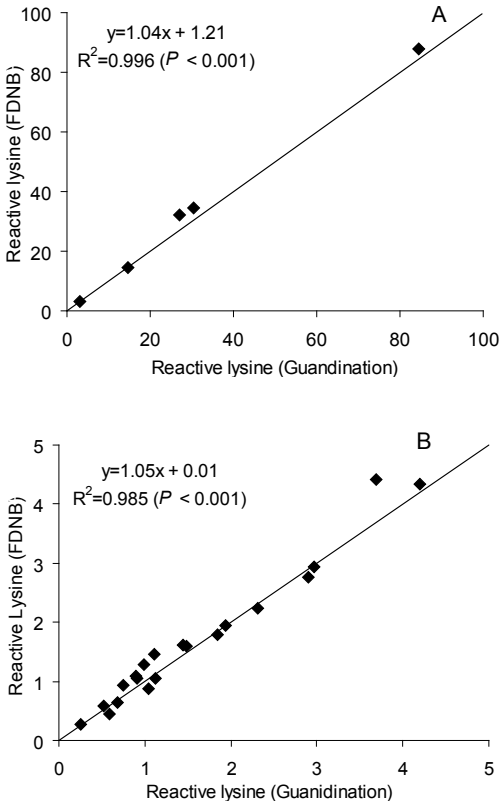


Figure 3. Comparison of the reactive lysine content (g kg^{-1}) of selected animal feedstuffs (A) and selected "ready-to-eat" cereal-based breakfast foods (B) determined using either the guanidination reaction or the FDNB method.
The solid lines denote agreement between the two methods. The animal feeds included blood meal, meat and bone meal, wheat meal, soyabean meal and cottonseed meal.

Comparison of the true ileal total lysine digestibility with the true ileal reactive lysine digestibility in a heated lactose/casein

Having determined the optimal guanidination reaction conditions for lysine in food proteins and digesta, the aim of this study was to compare the true ileal total lysine digestibility of the heated lactose/casein mixture (determined using the traditional true ileal digestibility assay with conventional amino acid analysis of diets and digesta) with the true ileal reactive lysine digestibility (determined using the new true ileal reactive lysine digestibility assay where the guanidination reaction was used to determine the reactive lysine content of both diets and digesta).

An unheated casein-based diet and a heated lactose/casein-based diet were fed to growing rats and digesta were collected from the terminal ileum. The total lysine and reactive lysine contents in the diets and digesta were determined using either amino acid analysis or

guanidination followed by amino acid analysis, respectively. Digestibility was calculated as the absorbed total or reactive lysine at the terminal ileum, corrected for endogenous ileal lysine flows, as a proportion of the dietary lysine intake (total or reactive) (Table 2). The true ileal total lysine digestibility of the heated lactose/casein was significantly ($P < 0.001$) lower than the true ileal reactive lysine digestibility and the actual difference was large (15% units) demonstrating that the total lysine digestibility is not a good predictor of reactive lysine digestibility, at least for the heated lactose/casein mixture. In addition, the true ileal reactive lysine digestibility of the heated lactose/casein mixture was significantly lower ($P < 0.001$) than the true ileal lysine digestibility of the unheated casein, suggesting that processing, and in turn lysine damage, reduces the lysine digestibility of food proteins and that the lysine digestibility of an unheated protein cannot be assumed to be the same once the protein has undergone heat processing.

Table 2.
Mean (n=6) true ileal lysine digestibility (%) for an unheated casein determined using the rat ileal digestibility assay and based on conventional amino acid analysis and for a heated lactose/casein mixture (n=6) determined using the rat ileal digestibility assay coupled with either conventional amino acid analysis (AAA) or the guanidination method (reactive lysine digestibility coefficient).

	Unheated casein	Heated lactose/casein		Overall SE	Significance
		Conventional AAA	Guanidination method		
Lysine digestibility	98.8[a]	70.5[b]	85.9[c]	2.01	***

Means with difference superscripts were significantly (P < 0.05) different.

Accuracy of the true ileal digestible reactive lysine (available lysine) assay for determining the available lysine content

While the true ileal digestible reactive lysine assay appeared to produce sensible lysine digestibility data for the heated lactose/casein mixture, it was important that the accuracy of the assay be rigorously tested. To that end, a well controlled study based on body lysine retention was conducted to investigate the accuracy of the assay to predict the available lysine content of a heated skim milk powder. Three diets were formulated to be identical except for the nitrogen source. These included two control diets containing EHC and free amino acids as the sole nitrogen source and a test diet for which a heated skim milk powder was the sole nitrogen source. The lysine in the EHC-based diet was assumed to be completely digested and absorbed and all diets were isocaloric and shown experimentally to be limiting in lysine (data not shown). One of the EHC diets was formulated to contain lysine at the same level as the heated skim milk powder diet based on determination with the true ileal total lysine digestibility assay using traditional amino acid analysis, while the other EHC diet contained lysine at the same level as the heated skim milk powder diet based on determination with the true ileal reactive lysine digestibility assay using guanidination. The diets were then fed to growing pigs and body lysine deposition determined (Table 2.3).

Table 3.
Least squares means (n=8; ± SE) of the whole body lysine deposition (g d^{-1}) in pigs fed a heated skim milk powder based diet and one of two EHC[1] control diets.

	Heated skim milk powder	EHC Diet A[2]	EHC Diet B[3]	Significance
Lysine deposition	9.1[a](0.62)	5.4(0.63)	9.1[a](0.58)	$P < 0.001$

[1]Enzymatically hydrolysed casein.
[2]EHC Diet A was formulated to contain lysine equal to the digestible lysine content of the heated skim milk powder determined using the conventional ileal digestibility assay (reactive lysine in heated skim milk powder x true digestibility of total lysine (determined using conventional methods) for the heated skim milk powder).
[3]EHC Diet B was formulated to contain lysine equal to the digestible lysine content of the heated skim milk powder determined using the new ileal reactive lysine digestibility assay (reactive lysine in heated skim milk powder x true digestibility of reactive lysine (determined using the new method) for the heated skim milk powder).
[a]Means with difference superscripts were significantly ($P < 0.05$) different.

There was no significant ($P > 0.05$) difference between the lysine deposition in the pigs fed the heated skim milk powder diet compared to those fed the EHC control diet for which the lysine content was formulated to match the lysine content of the heated skim milk powder diet determined using the true ileal reactive lysine digestibility assay. In contrast, the lysine deposition of the pigs fed the heated skim milk powder diet was significantly ($P < 0.05$) higher than for the pigs fed the EHC control diet formulated based on the true ileal total lysine digestibility assay (traditional assay). The experiment demonstrated the accuracy of the true ileal reactive lysine digestibility assay in predicting lysine availability and available lysine content in processed foods and feedstuffs and in doing so provides confidence that the guanidination reaction conditions used lead to the complete, or at least near complete, conversion of reactive lysine to homoarginine in both diets and digesta. Finally, the inaccuracy of the traditional ileal digestibility assay that uses traditional amino acid analysis to determine total lysine was clearly demonstrated.

While the experiment described above clearly demonstrated the accuracy of the new true ileal reactive lysine digestibility assay for determining available lysine in a processed protein source (skim milk powder), a further validation study was undertaken. For this study, the digestible reactive lysine (available lysine) content of heated field peas (from the late Dr Batterham's laboratory in Wollongbar, Australia), determined using the true ileal digestible reactive lysine assay was compared with the available lysine content of the same peas determined using the slope-ratio assay (van Barneveld et al., 1994) (Fig. 4).

The available lysine content determined using the growth-based assays (van Barneveld et al., 1994) was similar to that obtained using the true ileal digestible reactive lysine (available lysine) assay for most of the heated pea samples. In addition, true ileal total lysine digestibility significantly ($P < 0.05$) underestimated (3.4 - 37% underestimation) the true ileal reactive lysine digestibility particularly for the more severely heated peas (Table 4). While it must be remembered that strictly, the growth-based assays determine utilisation rather than availability it is encouraging that the available lysine content determined using the two approaches was very similar. Overall, this study further demonstrates the

suitability of the true ileal digestible reactive lysine assay and the inaccuracy of the true ileal total lysine digestibility (conventional assay) for determining the available lysine content of processed foods and feedstuffs.

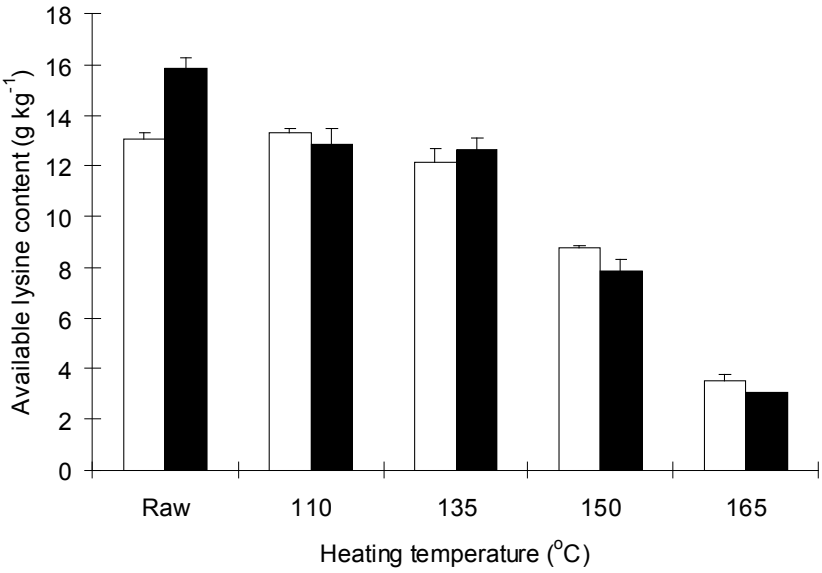


Figure 4. Comparison of available lysine in field peas determined using the true ileal digestible reactive lysine assay (□) or using growth-based assays (van Barneveld et al., 1994) (■).

Table 4.
The true ileal total and reactive lysine digestibility for variably heated field peas[1].

Heating temp (°C)	Lysine digestibility (%)		Overall SE	Significance[4]	Difference (%)
	Total[2]	Reactive[3]			
Raw	83	88	1.7	***	6.0
110	86	90	1.6	NS	4.9
135	90	93	4.1	**	3.4
150	78	83	0.8	*	5.5
165	43	67	4.9	*	36.5

[1]The peas were heated for 15 min.
[2]True ileal total lysine digestibility was determined using conventional amino acid analysis.
[3]True ileal reactive lysine digestibility was determined using the guanidination reaction followed by amino acid analysis.
*[4]NS not significant, P > 0.05; *, 0.05 > P > 0.01; **, 0.01 > P > 0.001; ***, P < 0.001.*

Highlighting the inadequacy of the true ileal total lysine digestibility assay.

The relationship between true ileal digestible total lysine and true ileal digestible reactive (available) lysine in a skim milk powder that had been progressively heat-treated was investigated. In this study, skim milk powder was autoclaved for 1 - 10 min and the true ileal digestible reactive and total lysine contents of the heated powders were determined (Table 5). For the unheated skim milk powder, although there was a significant ($P < 0.001$) difference between the true ileal digestible reactive and total lysine contents, the actual difference was small (< 4%). However, as the heat treatment became more severe, the observed significant ($P < 0.001$) difference between the digestible total and reactive lysine contents also increased such that, for the skim milk powder that received the maximum heat treatment (121 ^{o}C for 10 min), the digestible total lysine content overestimated digestible reactive lysine content by 96%. This study demonstrated that the traditional ileal total lysine digestibility assay, using traditional amino acid analysis, may be suitable for determining available lysine in unprocessed protein sources. However, using heated skim milk powder as an example of a processed protein source, this study demonstrated that the overestimation of the available lysine content when determined using the true ileal digestible total lysine assay increases as protein sources are increasingly heat damaged.

Table 5.
Digestible total lysine and digestible reactive (available) lysine contents (g kg^{-1} air dry weight) for a variably heated skim milk powder.

Heat treatment	Digestible lysine		Overall SE	Significance[3]
	Total[1]	Reactive[2]		
Unheated	36.8	38.1	0.09	***
121^{o}C for 1 min	31.6	28.0	0.53	***
121^{o}C for 3 min	19.8	16.6	0.25	***
121^{o}C for 5 min	13.7	11.0	0.62	*
121^{o}C for 10 min	11.2	5.7	0.73	***

[1]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 quantitate lysine and from the total lysine content of the protein source determined using conventional amino acid analysis.
[2]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 of the protein source was determined using the guanidination reaction followed by amino acid analysis.
[3], 0.05 > P > 0.01; ***, P < 0.001.*

Conclusion

There are a number of techniques currently published for determining available lysine in processed foods and feedstuffs for example, protein efficiency ratio, biological value, slope-ratio and indicator amino acid oxidation techniques. However, these assays tend to determine lysine utilisation rather than lysine availability. For this reason, the development of an assay that determines available lysine in processed foods and feedstuffs is important. This chapter described the development and validation of a true ileal digestible reactive lysine (available lysine) assay. This assay couples a true ileal digestibility assay with the guanidination reaction which is used to determine the reactive lysine content of diets and digesta. The guanidination reaction conditions were optimised for an unheated casein and a heated lactose/casein and the digesta of rats fed either a unheated casein or heated lactose/casein mixture. The guanidination reaction, using the newly established reaction conditions, compared favourably with the FDNB method when applied to a range of foods and feedstuffs. The accuracy of the new assay was demonstrated using a carefully controlled lysine deposition study. Furthermore, the available lysine content of heated peas determined using the true ileal digestible reactive lysine assay was similar to that determined using growth-based assays. Overall, true ileal digestible reactive lysine accurately determines available lysine in processed foods and feedstuffs, while true ileal digestible total lysine overestimates available lysine in severely processed foods and feedstuffs.

Literature cited

Booth, V.H. 1971. Problems in the determination of FDNB-available lysine. J. Sci. Food Agric. 22, 658-666.

Butts, C.A., Moughan, P.J., Smith, W.C. 1991. Endogenous amino acid flow at the terminal ileum of the rat determined under conditions of peptide alimentation. J. Sci. Food Agric. 55, 175-187.

Carpenter, K.J. 1960. The estimation of available lysine in animal-protein foods. Biochem. J. 77, 604-610.

Catrein, I., Herrmann, R., Bosserhoff, A., Ruppert, T. 2005. Experimental proof for a signal peptidase 1 like activity in *Mycoplasma pneumoniae*, but absence of a gene encoding a conserved bacterial type 1 SPase. FEBS J. 272, 2892-2900.

Chervenka, C.H., Wilcox, P.E. 1956. Chemical derivatives of chymotrypsinogen. II. Reaction with O-methylisourea. J. Biol. Chem. 222, 635-647.

Hurrell, R.F., Carpenter, K.J. 1981. The estimation of available lysine in foodstuffs after Maillard reactions. Prog. Food Nutr. Sci. 5, 159-176.

Kassell, B., Chow, R.B. 1966. Modification of the basic trypsin inhibitor of bovine pancreas. The ε-amino groups of lysine and the amino terminal sequence. Biochem. 5, 3449-3453.

Klee, W.A., Richards, F.M. 1957. The reaction of *O*-methylisourea with bovine pancreatic ribonuclease. J. Biol. Chem. 229, 489-504.

Moughan, P.J., Darragh, A.J., Smith, W.C., Butts, C.A. 1990. Perchloric and trichloroacetic acids as precipitants of protein in endogenous ileal digesta from the rat. J. Sci. Food Agric. 52, 13-21.

Moughan, P. J., Gall, M. P. J. and Rutherfurd, S. M. 1996. Absorption of lysine and deoxyketosyllysine in an early Maillard browned casein by the growing pig. J.

Agric. Food Chem. 44, 1520-1525.
Rutherfurd, S.M., Moughan, P.J. 1990. Guanidination of lysine in selected dietary proteins. J. Agric. Food Chem. 38, 209-211.
Shields, G.S., Hill, R.L., Smith, E.L. 1959. Preparation and properties of guanidinated mercuripapain. J. Biol. Chem. 234, 1747-1753.
Torbatinejad, N.M., Rutherfurd, S.M., Moughan, P.J. 2005. Total and reactive lysine contents in selected cereal-based food products. J. Agric. Food Chem. 53, 4454-4458.
van Barneveld, R.J., Batterham, E.S., Norton, B.W. 1994. The effect of heat on amino acids for growing pigs. 3. The availability of lysine from heat-treated field peas (*Pisum sativum* cultivar Dundale) determined using the slope-ratio assay. Brit. J. Nutr. 72, 257-275.
Yamaguchi, M., Nakazawa, T., Kuyama, H., Obama, T., Ando, E., Okamura, T-A., Ueyama, N., Norioka, S. 2005. High-throughput method for N-terminal sequencing of proteins by MALDI mass spectrometry. Anal. Chem. 77, 645-651.
Zhang, H.L., Li, D.F., Qiao, S.Y., Wang, F.L., Chen, X.J., Thacker, P.A. 2006. The effect of dietary homoarginine derived from guanidination of synthetic lysine on endogenous amino acid loss and apparent and true ileal amino acid digestibility in the pig. Anim. Sci. 82, 23-30.

Chapter 3

Digestible reactive lysine in selected milk-based products

Milk products are high quality protein sources but also undergo processing as part of their manufacture. In this thesis an assay was developed that can determine the digestible reactive lysine (available lysine) of processed protein sources. This chapter describes the determination of the digestible reactive lysine content of a range of milk protein-based foods using the newly developed assay and the digestible total lysine content using the traditional ileal digestibility assay.

Digestible reactive lysine in selected milk-based products

Shane M. Rutherfurd and Paul J. Moughan

Riddet Institute, Massey University, Palmerston North, New Zealand.

Reproduced with permission from the publishers from: Rutherfurd, S.M., Moughan, P.J. 2005. Digestible reactive lysine in selected milk-based products. J. Dairy Sci. 88, 40-48.

Abstract

Reactive lysine content, true ileal reactive lysine digestibility, and true ileal digestible reactive lysine content were determined in a wide range of processed milk products. A previously validated assay based on determining reactive lysine in both foods and ileal digesta, after reaction of these materials with OMIU, was applied. Semisynthetic diets containing milk products as the sole sources of protein and including chromic oxide as an indigestible marker were fed to growing rats. Digesta from the terminal ileum were collected posteuthanasia and, with samples of the diets, were analysed for reactive lysine (homoarginine) content. True ileal reactive lysine digestibility was determined after correcting for endogenous lysine loss at the terminal ileum of rats fed an EHC-based diet, followed by ultrafiltration (5000 Da MW cutoff) of the digesta. Digestible total lysine (determined using conventional methods) was also determined. The true ileal reactive lysine digestibility was high (> 91%) for all the milk products tested, but was highest for the ultra-heat-treated (UHT) milk (100%) and lowest in the infant formulas (91 - 93%). Total lysine digestibility (conventional assay) significantly ($P < 0.05$) underestimated reactive lysine digestibility for all the products tested. The mean underestimation ranged from 1.3 to 7.1% units. The mean digestible total lysine content was significantly different from the available lysine content for most of the products examined. In some cases this difference was small (< 3%), but for a number of the products (evaporated milk, whole milk protein, lactose-hydrolysed milk powder and a sports formula) the difference was greater (6.5 - 14%). This would suggest firstly that total lysine and total lysine digestibility determined using conventional methods were inaccurate when applied to some milk-based foods, and secondly that some of the milk products had undergone lysine modification. In general, milk proteins are a highly digestible source of amino acids and lysine.

Introduction

Lysine is a dietary essential amino acid that is sometimes first limiting in diets for humans, particularly diets high in cereals. Generally, milk proteins contain relatively high amounts of lysine, especially in comparison with cereal protein sources; therefore, milk protein products are a valued source of lysine. Lysine, however, is susceptible to modification during processing or prolonged storage, whereupon the side chain amino group of lysine can react with lactose or other compounds present in the food to produce nutritionally unavailable (unreactive) derivatives (Moughan, 2003; Hurrell and Carpenter, 1981). Some of these derivatives are acid-labile and will revert back to lysine under the acid hydrolysis conditions used to determine lysine contents in food samples. This reversion is not quantitative and leads to inaccurate estimates of amino acid digestibility, and generally an overestimation of the reactive lysine content and the digestible reactive (available) lysine content of some processed foods. Numerous methods have been developed to determine chemically reactive lysine, often incorrectly referred to as available lysine, in milk products (Ramirez-Jimenez et al., 2004; Ferrer et al., 2003; Pereyra-Gonzáles, 2003), but few researchers have determined biologically available lysine in any protein sources (Batterham et al., 1990), including milk products. A method has been developed (Moughan and Rutherfurd, 1996; Rutherfurd et al., 1997) that allows the accurate measurement of the available lysine content of processed foods. The assay (true ileal reactive lysine digestibility) involves feeding a test diet to an

animal. Ileal digesta are collected and reactive lysine is measured in both diet and digesta. The difference between the two determinations reflects the reactive lysine that has been digested and absorbed. Ileal rather than faecal measurements are used since firstly, faecal measurements are confounded by microorganisms in the hindgut that metabolise amino acids, and secondly, it does not appear that amino acids are absorbed from the hindgut in quantitatively significant amounts (Moughan, 2003). The aim of this study was to determine reactive lysine content, true ileal reactive lysine digestibility and true ileal digestible reactive lysine content in a range of high-quality milk protein sources, using the new assay and to compare these values with total lysine content, true ileal total lysine digestibility and true ileal digestible total lysine content determined using the traditional approach. Information on the available lysine content of a range of milk-based products was obtained.

Materials and methods

Materials

OMIU was obtained from Sigma Chemicals (St Louis, MO) and barium hydroxide octahydrate was obtained from BDH Laboratory Supplies (Poole, England). A total of 12 milk-based protein products were obtained locally. These included a whole milk powder, three infant formulas, whey protein concentrate, UHT milk, evaporated milk, weight gain formula, milk powder-based sports drink, milk powder-based formula for the elderly, lactose-hydrolysed milk powder and a milk powder-based high protein supplement. The crude protein, total fat, and total carbohydrate contents of the 12 products (as given in the product specifications) are shown in Table 1. EHC was obtained from New Zealand Pharmaceuticals Ltd. (Palmerston North, New Zealand) and contained free amino acids and peptides ≤ 2000 Da. Centriprep YM-3 disposable ultrafiltration devices (with a 3000 Da MW-cutoff) were obtained from Amicon Inc (Beverly, MA). Laboratory rats were obtained from the Small Animal Production Unit, Massey University (Palmerston North, New Zealand).

Preparation of protein sources

Most products were purchased as finely ground powders. Any products purchased as liquids were freeze dried then ground through a 1 mm mesh.

Digestibility study

A total of 60 male Sprague-Dawley rats, approximately 150 g body weight, were housed individually in stainless steel wire-bottomed cages in a room that was maintained at 22 ± 2 ^{o}C, with a 12 h light/dark cycle. Twelve semisynthetic test diets were formulated (Table 2a,b) to each contain 100 g kg^{-1} of crude protein. An EHC-based diet was also formulated (Table 2b), in order to allow the determination of endogenous ileal lysine flows (Butts et al., 1991; Moughan et al., 1990). Chromic oxide was also included (0.5%) in each diet as an indigestible marker. All diets met the nutritional requirements for the growing rat with the exception of protein (National Research Council, 1995). The animals were randomly allocated to the dietary treatments and were fed the diets for a 14 d experimental period. On each day, each rat received its respective diet as nine meals given hourly (0830 h to 1630 h),

each meal time consisted of a 10 min period during which the rats had unrestricted access to their diet. Water was available at all times. On the final day of the study, from 5.5 to 7 h after the start of feeding, the rats were asphyxiated in carbon dioxide gas and then decapitated. The 20 cm of ileum immediately anterior to the ileo-caecal junction was dissected out. The dissected ileum was washed with distilled deionised water to remove any blood and hair and, carefully dried on absorbent paper. The digesta were gently flushed from the ileum section and freeze dried in preparation for chemical analysis. The digesta of rats fed the EHC-based diet were adjusted to approximately pH 3 with 6 M HCl in order to minimise protease activity. The EHC digesta were centrifuged and ultrafiltered (3000 Da MW-cutoff) and then freeze dried in preparation for analysis (Butts et al., 1991).

Ethics approval for the animal trial was obtained from the Animal Ethics Committee, Massey University.

Table 1.
Crude protein, total fat and total carbohydrate contents[1] (g kg^{-1} air dry weight) for the 12 protein sources.

	Crude protein	Total fat	Total carbohydrate
Whole milk powder	322	288	362
Infant formula A	114	293	570
Infant formula B	120	280	510
Infant formula C	154	210	560
Whey protein concentrate	785	49	86
UHT milk[2]	35	5	48
Evaporated milk[2]	84	86	382
Weight gain formula[3]	280	18	620
Sports formula[4]	245	6	661
Elderly formula[5]	140	159	607
Lactose-hydrolysed milk powder[6]	340	120	442
High protein supplement	199	57	303

[1]Data taken from the statutory label information appearing on the product.
[2]These products are liquids and as such the protein, carbohydrate contents are g L^{-1}.
[3]Milk based formula designed for bodybuilders and people needing to gain body weight.
[4]Milk based formula designed for athletes and sports people.
[5]Milk based formula for the elderly.
[6]Hydrolysed-lactose milk based food for lactose intolerance.

Table 2a.
Ingredient composition[1] (g kg^{-1} air dry weight) of the experimental diets.

	WMP[2]	Infant formula A	Infant formula B	Infant formula C	Sports formula	UHT milk[3]	Evaporated milk
Wheat starch	466.0	89.8	133.7	317.6	358.8	493.9	462.7
Soyabean oil	50.0	-	-	-	50.0	50.0	50.0
Cellulose	50.0	-	-	-	50.0	50.0	50.0
Sucrose	100.0	-	-	-	100.0	100.0	100.0
Vitamin premix[4]	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Mineral premix[4]	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Dicalcium Phosphate	24.0	24.0	24.0	24.0	24.0	24.0	24.0
WMP	301.0	-	-	-	-	-	-
Infant formula A	-	877.2	-	-	-	-	-
Infant formula B	-	-	833.3	-	-	-	-
Infant formula C	-	-	-	649.4	-	-	-
Sports formula	-	-	-	-	408.2	-	-
UHT milk	-	-	-	-	-	273.1	-
Evaporated milk	-	-	-	-	-	-	304.3
Chromic oxide	5.0	5.0	5.0	5.0	5.0	5.0	5.0

[1]All diets were formulated to contain equal crude protein contents.
[2]Whole milk powder.
[3]Ultra heat-treated milk
[4]Vitamin/mineral mix was formulated to meet the requirement of the growing rat for vitamins and minerals in the final diets (National Research Council, 1995).

Table 2b.
Ingredient composition[1] (g kg^{-1} air dry weight) of the experimental diets.

	WPC[2]	Weight gain formula	Elderly formula	Lactose-hydrolysed milk powder	High-protein supplement	EHC[3]
Wheat starch	639.6	409.9	52.7	472.9	264.5	637.0
Soyabean oil	50.0	50.0	50.0	50.0	50.0	50.0
Cellulose	50.0	50.0	50.0	50.0	50.0	50.0
Sucrose	100.0	100.0	100.0	100.0	100.0	100.0
Vitamin premix[4]	0.5	0.5	0.5	0.5	0.5	0.5
Mineral premix[4]	3.5	3.5	3.5	3.5	3.5	3.5
Dicalcium Phosphate	24.0	24.0	24.0	24.0	24.0	24.0
WPC[4]	127.4	-	-	-	-	-
Weight gain formula	-	357.1	-	-	-	-
Elderly formula	-	-	714.3	-	-	-
Lactose-hydrolysed milk powder	-	-	-	294.1	-	-
High-protein supplement		-	-	-	502.5	-
EHC	-	-	-	-	-	130.0
Chromic oxide	-	5.0	5.0	5.0	5.0	5.0

[1]All diets were formulated to contain equal crude protein contents.
[2]Whey protein concentrate.
[3]Enzymatically hydrolysed casein (EHC) diet used for determining endogenous amino acid losses at the terminal ileum. The EHC contained free amino acids and small peptides (< 2000 Da).
[4]Vitamin/mineral mix was formulated to meet the requirement of the growing rat for vitamins and minerals in the final diets (National Research Council, 1995).

Chemical analysis

Amino acid contents were determined in duplicate 5 mg protein source and digesta samples and quadruplicate 5 mg semisynthetic diet samples using a Waters ion-exchange HPLC system, utilising postcolumn ninhydrin derivatisation and detection using absorbance at 570 nm and 440 nm, following hydrolysis in 6 M glass-distilled HCl containing 0.1% phenol for 24 h at 110 ± 2 °C in evacuated sealed tubes. Cysteine, methionine and tryptophan were not determined as they are destroyed during acid hydrolysis. The weight of each amino acid was calculated using free amino acid molecular weights.

Reactive lysine contents were determined in duplicate 5 mg protein source and digesta samples and quadruplicate 5 mg diet samples after incubation for 1, 7 and 7 d respectively in 0.6 M OMIU, pH 10.6 (pH 11.0 for the digesta samples), at 21 ± 2 °C in a shaking waterbath, with the reagent to lysine ratio being greater than 1000, according to the procedure of Moughan and Rutherfurd (1996). The 0.6 M O-methylisourea solution was prepared as described by Moughan and Rutherfurd (1996). After incubation, the samples were dried using a Speedvac concentrator (Savant Instruments, Inc. (Farmingdale, NY)) and then hydrolysed and analysed for amino acid content as described previously.

The chromium content of the diets and ileal digesta were determined in duplicate using a GBC 902 AA absorption/emission spectrophotometer (GBC Scientific NZ Ltd, Auckland, New Zealand) following the method described by Costigan and Ellis (1987).

Data analysis

Ileal total and ileal endogenous (EHC diet) amino acid (AA) flows at the terminal ileum were calculated as follows (units are μg g^{-1} dry matter (DM)):

$$\text{Ileal AA flow (μg g}^{-1}\text{ dry matter intake (DMI))} = \text{Ileal AA content} \times \frac{\text{Diet chromium}}{\text{Ileal chromium}}$$

True ileal amino acid (AA) digestibility was calculated using the following equation (units are μg g^{-1} DMI):

$$\text{True AA digestibility (\%)} = \frac{(\text{Dietary AA intake} - (\text{Ileal AA flow} - \text{Endogenous AA flow}))}{\text{Dietary AA intake}} \times 100$$

True ileal reactive lysine (RL) digestibility was calculated using the following equation (units are μg g^{-1} DMI):

$$\text{True RL digestibility (\%)} = \frac{(\text{Dietary RL intake} - (\text{Ileal RL flow} - \text{Endogenous lysine flow}))}{\text{Dietary RL intake}} \times 100$$

The true ileal amino acid digestibility data were subjected to a one-way ANOVA for each amino acid singly (GLM Procedure, SAS 1999).

Results

Comparison of total and reactive lysine contents for the milk protein sources

Total and reactive lysine contents were determined for the 12 milk protein products (Table 3). For the three infant formulas, UHT milk, elderly formula and high-protein supplement the difference between total and reactive lysine contents was ≤ 2.2%. For the other products, total lysine overestimated reactive lysine from 3.8% for the weight gain formula to as much as 23% for evaporated milk.

Table 3.
Total and reactive lysine contents (g kg^{-1} air dry weight) of 12 dairy protein sources.

	Total[1]	Reactive[1]	Overestimation[2] (%)
Whole milk powder	27.5	24.4	12.8
Infant formula A	9.6	9.5	0.9
Infant formula B	10.2	10.0	2.2
Infant formula C	12.5	12.5	-0.5
Sports formula	21.4	19.5	9.7
UHT milk	32.0	31.4	1.8
Evaporated milk	26.1	21.2	23.0
Whey protein concentrate	83.0	78.6	5.6
Weight gain formula	25.2	24.3	3.8
Elderly formula	12.4	12.2	1.7
Lactose- hydrolysed milk powder	28.3	25.5	11.1
High-protein supplement	14.6	14.4	1.9

[1]Mean values based on duplicates.
[2]Overestimation (%) = $\frac{\text{Total lysine – Reactive lysine}}{\text{Reactive lysine}} \times 100$

Comparison of true ileal lysine digestibility based on either reactive lysine or total lysine

True ileal lysine digestibility based on total lysine was determined for the 12 milk-based protein sources using conventional amino acid analysis and compared with true ileal reactive lysine digestibility, determined after guanidination of the underivatised lysine in the

diet and digesta, to form homoarginine and subsequent analysis of homoarginine by HPLC (Table 4). The reactive lysine digestibility (lysine availability) was in excess of 90% for all of the protein products tested, indicating that most of the reactive lysine was absorbed.

Table 4.
Mean (n=5) true ileal total lysine digestibility (%) (conventional amino acid analysis) and true ileal reactive lysine digestibility (%) based on reactive lysine determined using guanidination prior to amino acid analysis.

	Lysine digestibility		Overall	Significance[4]
	Total[1]	Reactive[2]	SE[3]	
Whole milk powder	95.2	98.3	0.87	***
Infant formula A	86.9	91.0	1.71	**
Infant formula B	88.6	92.3	1.03	***
Infant formula C	89.0	93.1	1.20	**
Sports formula	95.2	98.0	0.56	**
UHT milk	99.2	100.2	0.31	*
Evaporated milk	89.6	96.7	2.02	**
Whey protein concentrate	96.3	98.5	0.71	***
Weight gain formula	96.7	99.0	0.61	***
Elderly formula	94.3	97.1	0.54	**
Lactose-hydrolysed milk powder	96.2	98.6	0.62	***
High-protein supplement	97.4	99.9	1.11	**

[1]Lysine digestibility was determined using a true ileal amino acid digestibility assay (rat) and conventional amino acid analysis was used to quantitate total lysine in the diets and digesta.
[2]Lysine digestibility was determined using a true ileal amino acid digestibility assay (rat) and the guanidination reaction was used to quantitate reactive lysine in the diets and digesta.
[3]Overall SE is the standard error for total and reactive lysine digestibility combined.
[4], 0.05 > P > 0.01; **, 0.01 > P > 0.001; ***, P < 0.001.*

For all of the products tested, true ileal reactive lysine digestibility (lysine availability), determined using the new assay, was statistically significantly higher than true ileal total lysine digestibility, determined using the conventional assay. On average, total lysine digestibility underestimated lysine availability by 3.3% and this underestimation ranged from 1.2% for UHT milk to 7.4% for evaporated milk.

Digestible total lysine (conventional analysis) and digestible reactive lysine (available lysine) contents are shown in Table 5. True ileal digestible total lysine was statistically significantly different from true ileal digestible reactive lysine (available lysine)

for all of the milk protein products tested with the exception of the high-protein supplement. For the whole milk protein, whey protein concentrate, UHT milk, evaporated milk, weight gain formula, sports formula and lactose-hydrolysed milk powder, conventional analysis (digestible total lysine) overestimated digestible reactive lysine; for the infant formulas and elderly formula, conventional analysis underestimated digestible reactive lysine. For infant formula B, UHT milk, weight gain formula and the elderly formula, the difference between digestible total lysine and digestible reactive lysine was less than 3%. For the other milk protein products, where statistically significant differences were observed, these differences ranged from 3.2% to 13.9% but were on average approximately 4.7%.

Table 5.
Mean[1] digestible total and reactive lysine contents (g kg^{-1} air dry weight) for 12 dairy protein sources.

	Digestible lysine		Overall	Significance[4]
	Total[1]	Reactive[2]	SE[3]	
Whole milk powder	26.2	24.0	0.42	***
Infant formula A	8.3	8.6	0.12	**
Infant formula B	9.1	9.2	0.08	**
Infant formula C	11.1	11.7	0.14	**
Sports formula	20.4	19.1	0.22	***
UHT milk	31.7	31.4	0.06	*
Evaporated milk	23.4	20.5	0.60	***
Whey protein concentrate	79.9	77.5	0.58	***
Weight gain formula	24.4	24.1	0.12	**
Elderly formula	11.7	11.8	0.05	*
Lactose-hydrolysed milk powder	27.2	25.1	0.38	***
High-protein supplement	14.3	14.3	0.11	NS

[1]Digestible total lysine was calculated from true ileal lysine digestibility (rat) with lysine determined by conventional amino acid analysis and from the total lysine content in the protein source, also determined using conventional amino acid analysis.
[2]Digestible reactive lysine was calculated from true ileal reactive lysine digestibility (rat, guanidination analysis) and the reactive lysine content of the protein source, also determined using guanidination.
[3]Overall SE is the standard error for total and reactive lysine digestibility combined.
*[4]NS not significant, $P > 0.05$; *, $0.05 > P > 0.01$; **, $0.01 > P > 0.001$; ***, $P < 0.001$.*

Comparison of the ileal digestibility of acid-stable amino acids in protein sources with and without guanidination treatment

True ileal amino acid digestibility values for the acid-stable amino acids except lysine, proline and arginine, were also determined and are shown in Table 6a,b. Overall, amino acid digestibility was high with a mean true digestibility for all amino acids over all protein products of 91%. Generally, digestibility was lowest for glycine (69%) and highest for tyrosine (98%). The mean digestibility for all amino acids was lowest for the infant formula A (81%) and highest for UHT milk (97%).

A paired *t*-test was used to compare the mean true ileal digestibilities (for each amino acid individually) for the acid-stable amino acids determined using the new and conventional methods for the 12 protein products. For most (61%) of the amino acids, there was no significant difference between digestibility determined either with or without guanidination prior to amino acid analysis. For a further 19% of the amino acids, there was a statistically significant difference between methods, but the actual difference between the mean digestibility for the two methods was < 3%, which in practical terms may not be meaningful. For the remaining 20% of the amino acids determined, there was a statistically significant difference between the two methods and the difference was > 3%.

Discussion

It is important to have accurate information on the amount of lysine present in foods and the digestibility and availability of lysine, as lysine is an important dietary essential amino acid that is often found in relatively low amounts in cereal-based diets. Lysine possesses a reactive amino group on its side chain which is capable of reacting with other compounds present in a food to form nutritionally unavailable derivatives (e.g. Maillard products) when a food is processed or stored (Hurrell and Carpenter, 1981). Some of these products, although being structurally different from lysine, can revert back to lysine when the food is subjected to the acid hydrolysis step of amino acid analysis, resulting in an overestimate of the lysine content. Moreover, the presence of Maillard compounds in a food results in inaccurate digestibility coefficients being generated using traditional amino acid digestibility assays. Recently, a new assay methodology for accurately determining ileal digestible reactive lysine (available lysine) has been developed (Moughan and Rutherfurd, 1996; Rutherfurd et al., 1997). The aim of the present study was to investigate the application of this new assay to a range of high quality dairy protein-based products and compare results from the new assay with those found with the traditional true ileal amino acid digestibility assay.

Table 6a.
Mean (n=5) true ileal amino acid digestibility (± SE) for 12 dairy protein sources[1].

	Whole milk powder		Infant formula A		Infant formula B	
	AAA[2]	G[3]	AAA	G	AAA	G
ASP	96.9(1.05)*	93.0(1.94)	81.3(3.02)*	70.8(5.89)	85.3(1.66)*	69.5(4.66)
THR	97.9(1.30)*	96.4(1.71)	83.1(2.95)*	75.5(4.99)	86.6(1.36)*	84.1(1.99)
SER	89.9(1.82)**	94.2(2.22)	81.3(3.96)	79.0(4.87)	81.6(2.14)	83.6(1.49)
GLU	94.1(0.86)	94.2(1.02)	86.7(2.27)	86.7(2.93	87.9(1.03)**	91.7(0.65)
GLY	76.3(6.83)	76.0(7.03)	42.8(17.26)*	54.4(13.99)	56.0(3.95)	56.5(4.12)
ALA	100.6(0.88)***	98.0(1.09)	72.1(3.07)	73.4(4.21)	79.6(3.30)	80.0(2.68)
VAL	95.8(0.83)	96.3(0.99)	85.9(2.36)	85.9(2.96)	87.6(1.23)	89.1(1.04)
ILE	93.5(0.97)	93.7(1.28)	84.1(2.73)	83.0(3.71)	87.4(1.15)	88.6(0.94)
LEU	99.3(0.43)	99.5(0.48)	91.7(1.25)	91.7(1.61)	92.5(0.92)	93.6(0.80)
TYR	101.4(0.32)	101.2(0.44)	92.9(0.96)	92.5(1.44)	91.8(1.37)	92.6(1.44)
PHE	100.8(0.35)*	101.1(0.40)	91.8(1.03)	92.1(1.34)	91.2(1.32)*	92.6(1.29)
HIS	99.1(0.78)***	89.7(1.14)	88.6(1.85)	84.2(4.43)	88.6(1.42)	87.2(1.80)

	Infant formula C		Sports formula		UHT Milk	
	AAA	G	AAA	G	AAA	G
ASP	81.9(2.05)	74.6(4.08)	90.4(1.09)	91.4(1.12)	98.2(0.46)*	93.2(1.92)
THR	83.6(1.65)**	79.4(1.62)	91.6(1.35)	91.7(1.08)	98.7(0.65)	97.4(1.04)
SER	75.7(3.25)*	80.1(1.92)	82.1(2.38)**	86.6(2.86)	92.7(0.73)*	95.7(1.39)
GLU	85.6(1.56)*	88.5(1.13)	89.4(1.09)	91.2(0.87)	95.5(0.39)	95.7(0.52)
GLY	32.7(12.25)*	43.9(11.69)	65.3(7.47)	65.0(7.03)	84.3(3.78)	81.7(4.78)
ALA	76.8(4.07)	80.3(2.43)	94.7(0.91)*	93.0(0.93)	101.1(0.39)**	99.9(0.52)
VAL	85.6(1.53)*	88.5(0.92)	91.1(1.01)	91.9(1.24)	97.1(0.38)	97.3(0.48)
ILE	82.5(2.01)*	85.6(1.31)	88.9(1.19)	89.7(1.59)	95.6(0.44)	95.6(0.50)
LEU	92.1(0.95)*	93.6(0.62)	96.5(0.51)	96.6(0.64)	99.9(0.17)	99.9(0.25)
TYR	93.2(1.00)	94.0(0.69)	98.6(0.38)	98.4(0.43)	101.6(0.11)	101.5(0.22)
PHE	93.1(1.10)*	94.3(0.80)	97.5(0.38)	97.6(0.49)	101.2(0.12)	101.3(0.20)
HIS	90.2(0.91)	86.7(2.60)	96.0(1.36)***	87.2(1.40)	100.3(0.19)**	92.9(1.38)

[1]Values were corrected for endogenous amino acid flow using the EHC method (Butts et al., 1991; Moughan et al., 1990).
[2]True ileal amino acid digestibility determined using conventional amino acid analysis.
[3]True ileal amino acid digestibility determined using guanidination followed by amino acid analysis.
Superscripts denote statistically significant differences (, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) between the mean total and reactive lysine digestibilities.*

Table 6b.
Mean (n=5) true ileal amino acid digestibility (± SE) for 12 dairy protein sources[1].

	Evaporated milk		Whey protein concentrate		Weight gain formula	
	AAA[2]	G[3]	AAA	G	AAA	G
ASP	91.0(2.48)	91.2(1.98)	95.0(1.03)	92.7(1.87)	92.9(1.15)*	88.8(1.70)
THR	95.9(1.96)	95.6(1.65)	92.4(1.45)	92.5(2.09)	94.8(1.22)	93.4(1.45)
SER	88.5(2.74)*	93.3(1.85)	92.8(1.58)	94.5(2.01)	87.6(1.79)*	89.6(2.07)
GLU	92.6(1.36)**	93.2(1.26)	95.6(0.86)	95.4(0.92)	92.3(0.79)	92.6(0.84)
GLY	76.4(6.99)	77.4(6.36)	86.2(3.59)	86.4(3.90)	82.2(3.96)	82.1(3.96)
ALA	97.6(1.85)**	96.4(1.87)	97.2(0.93)	96.8(0.94)	96.2(1.11)**	95.3(1.17)
VAL	94.6(1.42)*	95.4(1.29)	96.0(0.96)	96.2(1.02)	93.3(0.88)	93.9(0.95)
ILE	92.3(1.68)**	93.2(1.46)	97.1(0.64)	97.2(0.72)	91.5(0.95)	92.1(1.06)
LEU	98.3(0.80)*	98.7(0.74)	98.4(0.49)	98.5(0.52)	97.3(0.51)*	97.7(0.55)
TYR	100.2(0.70)	100.2(0.66)	100.2(0.83)	100.1(0.78	99.1(0.46)	98.9(0.66)
PHE	99.9(0.70)**	100.2(0.68)	98.5(0.80)	98.7(0.83)	98.0(0.50)*	98.3(0.58)
HIS	95.2(1.75)*	91.2(1.73)	95.1(1.50)*	87.0(2.29)	96.7(0.82)*	90.4(1.41)

	Elderly formula		Lactose-hydrolysed milk powder		High-protein supplement	
	AAA	G	AAA	G	AAA	G
ASP	88.3(1.18)	83.0(1.13)	96.4(1.00)*	92.2(1.81)	94.9(2.75)	92.4(2.38)
THR	88.3(1.63)***	85.8(1.46)	97.8(1.09)	96.6(1.89)	96.5(3.32)*	94.6(2.56)
SER	86.3(1.79)	86.8(1.37)	88.9(1.83)***	94.9(1.75)	91.6(4.24)	92.7(3.86)
GLU	91.5(0.94)	91.1(0.85)	93.7(0.76)	94.0(1.47)	93.8(2.17)	93.6(2.13)
GLY	63.8(7.61)	64.0(7.30)	87.4(3.70)	86.3(2.70)	70.0(11.65)	72.4(10.80)
ALA	93.7(1.09)	92.4(0.76)	100.2(0.77)**	97.1(3.28)	98.7(2.34)**	96.6(2.55)
VAL	93.0(0.93)	92.9(0.75)	95.6(0.78)**	96.0(0.93)	94.9(2.19)	95.1(2.13)
ILE	91.9(1.22)	91.8(0.99)	93.1(1.00)	93.3(1.05)	93.8(2.48)	93.7(2.50)
LEU	96.7(0.51)	96.8(0.41)	99.3(0.36)*	99.6(1.63)	98.2(1.19)	98.4(1.18)
TYR	98.2(0.31)	98.2(0.40)	101.3(0.26)	101.1(0.50)	100.1(0.84)	99.5(1.18)
PHE	98.1(0.35)*	98.3(0.39)	100.7(0.29)*	100.9(0.31)	98.8(1.06)	99.0(1.12)
HIS	94.6(0.69)***	86.5(0.88)	98.9(0.74)**	90.4(2.73)	97.6(1.89)***	91.2(2.54)

1Values were corrected for endogenous amino acid flow using the EHC method (Butts et al., 1991; Moughan et al., 1990).
2True ileal amino acid digestibility determined using conventional amino acid analysis.
3True ileal amino acid digestibility determined using guanidination followed by amino acid analysis.
Superscripts denote statistically significant differences (, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) between the mean total and reactive lysine digestibilities.*

Comparison of total lysine and reactive lysine contents for 12 high quality protein sources

There was a high degree of variation among protein sources for agreement between the total lysine and reactive lysine contents. For some protein sources (the infant formulas A and C, elderly formula and high-protein supplement), there was close agreement. In contrast, for sources such as whole milk powder, evaporated milk and lactose-hydrolysed milk powder, total lysine overestimated reactive lysine by ≥ 10%, suggesting the presence of labile lysine derivatives (Maillard products).

It is possible that during the guanidination reaction there is incomplete conversion of lysine to homoarginine and that the determined reactive lysine values underestimate lysine content. This is unlikely, however, given that conversion of lysine to homoarginine in lysozyme (for which the lysine content is known) which was included in the present study as a quality control was in excess of 98%. At high pH, the formation of lysinoalanine may also compete with the guanidination reaction for reactive lysine. However, based on studies investigating the formation of lysinoalanine in casein at high pH (Karayiannis et al., 1979) only a maximum of about 2.5% of the reactive lysine will convert to lysinoalanine after a 6 h incubation under the guanidination conditions used here. Furthermore, given that as much as 95% of guanidination is complete in a soluble protein in the first 8 h of incubation (Rutherfurd and Moughan, unpublished data), the actual amounts of lysinoalanine formed during guanidination are likely to be much lower than 2.5% and only a negligible error.

It is apparent that some purified high-protein products, marketed on the basis of high protein quality, have structural lysine damage during manufacture and storage, and for these products, the traditional total lysine determination overestimates the available lysine.

Comparison of true ileal reactive lysine digestibility and conventional total lysine digestibility

For all the protein sources, true ileal total lysine digestibility was significantly ($P < 0.05$) lower than true ileal reactive lysine digestibility. However, for many of the protein sources tested, including whole milk protein, whey protein concentrate, UHT milk, weight gain formula, elderly formula, sports formula, high-protein supplement and lactose-hydrolysed milk powder, the numerical difference between true ileal total and reactive digestibility was small (< 3%) and of little practical relevance. This suggests the presence of minimal amounts of acid-labile lysine derivatives, and for these particular samples, both the conventional true ileal amino acid digestibility assay and the new true ileal reactive lysine digestibility assay were suitable methods for determining lysine digestibility. For the infant formulas and evaporated milk, however, the difference between true ileal total and reactive lysine digestibility was > 3% and as high as 7%. For these protein sources, the new assay provides more meaningful estimates of lysine availability.

It has been appreciated for some time that the conventional true ileal lysine digestibility assay overestimates lysine availability in processed feedstuffs (Batterham et al., 1990). In the present study, the digestible total lysine content was statistically significantly

different from the digestible reactive lysine content for all protein sources except the high-protein supplement. Ultimately, and in relation to nutrition, it is the digestible lysine content that is of importance. For several of the protein sources (infant formula B, UHT milk, weight gain formula and elderly formula) the actual difference between the digestible total lysine and available lysine (digestible reactive lysine) contents was small (< 3%). For whole milk protein, whey protein concentrate, evaporated milk, sports formula and hydrolysed-lactose milk powder, the overestimation of available lysine by digestible total lysine ranged from 3.2% (whey protein concentrate) to 14% (evaporated milk).

It would appear that even for purified dairy-based protein sources, such as those tested in the present study, the traditional true ileal total lysine assay does not always accurately predict available lysine content. The true ileal reactive lysine digestibility assay described here measures the uptake from the small intestine of structurally unaltered lysine molecules and provides more accurate estimates of available lysine.

The true ileal digestibility of acid-stable amino acids in protein sources determined following traditional amino acid analysis or after guanidination of the protein

Although the newly developed digestible reactive lysine assay can be used to determine available lysine with accuracy, it would be useful if the other acid-stable amino acids could also be determined with the same procedure. To this end, a statistical comparison was conducted of the mean true ileal amino acid digestibility determined using either conventional amino acid analysis or amino acid analysis following guanidination. Arginine was not examined, as it coeluted with a very large ammonia peak present. For most (61%) of the acid-stable amino acids, there were no significant ($P > 0.05$) differences between the two methods tested, and for a further 19% of the amino acids studied, the difference between means, although significant statistically, was < 3% and arguably not meaningful in practical terms. For the remaining 20% of amino acids tested, the new method did not predict ileal digestibility accurately compared to the conventional method. Histidine was the amino acid that showed the greatest absolute differences between the two methods for most protein products; while tyrosine showed the least difference.

It appears that the digestible reactive lysine assay can be used to determine available lysine without affecting the estimation of the majority of the acid-stable amino acids.

Amino acid quality of the milk based products

All the milk-based products tested were highly digestible. This is consistent with other milk products that have been tested in our laboratory, such as sodium and calcium caseinate, milk protein isolate, α-lactalbumin, lactic casein and whey protein concentrates (Rutherfurd and Moughan 1998). The most poorly digested amino acid was glycine, with a true ileal digestibility for the 12 milk products ranging from 33 to 87% and a mean digestibility of 70%. The most highly digestible amino acid was tyrosine, with a digestibility ranging from 92 to 102% and a mean digestibility of 98%. The digestibility of a few of the amino acids was slightly greater than 100%, reflecting complete digestion and absorption

of these amino acids. Experimental error explains the overestimate in digestibility. The infant formulas tended to have the lowest amino acid digestibility, with mean true amino acid digestibility for the formulas ranging from 81 to 84% for the acid-stable amino acids (apart from lysine) tested. The cause of the relatively low digestibility is unknown but should be investigated further.

The gross reactive lysine content of the milk products ranged from 9.5 to 78.6 mg g^{-1}. This variation is largely due to the inclusion level of milk proteins into the products. The digestibility of reactive lysine was similar to the digestibility values obtained for most of the other amino acids and ranged from 91 to 100%. As was the case with the acid-stable amino acids, the digestibility of reactive lysine tended to be lowest in the infant formulas. The UHT milk was the most digestible protein source in terms of reactive lysine.

Conclusions

The traditional true ileal amino acid digestibility assay is inaccurate for measuring available lysine in some processed protein sources. This is particularly true for protein sources that have been heat-processed and that contain reducing sugars. The true ileal digestible reactive lysine assay, used in this study, to measure available lysine does accurately determine available lysine. Although for a number of the protein sources tested there was little difference between the total lysine digestibility and the reactive lysine digestibility, there were statistically significant and sizable differences in digestible reactive lysine content and digestible total lysine content for some of the milk products. Consequently, it would appear that the conventional ileal total lysine digestibility assay may not be accurate for all processed milk products. Finally, it would appear in general that milk provides a highly digestible protein source for inclusion into other foods.

Literature cited

Batterham, E.S., Andersen L.M., Baigent D.R., Darnell, R.E., Taverner, M.R. 1990. A comparison of the availability and ileal digestibility of lysine in cottonseed and soya-bean meals for grower/finisher pigs. Brit. J. Nut. 64, 663-677.

Butts, C.A., Moughan, P.J., Smith, W.C. 1991. Endogenous amino acid flow at the terminal ileum of the rat determined under conditions of peptide alimentation. J. Sci. Food Agric. 55, 175-187.

Chervenka, C.H., Wilcox, P.E. 1956. Chemical derivatives of chymotrypsinogen. II. Reaction with O methylisourea. J. Biol. Chem. 222, 635-647.

Costigan, P., Ellis, K.J. 1987. Analysis of faecal chromium derived from controlled release marker devices. N.Z. J. Tech. 3, 89-92.

Ferrer, E., Alegría, A., Farré, R., Abellán, P., Romero, F. 2003. Fluorometric determination of chemically available lysine: Adaptation, validation and application to different milk products. Nahrung. 47, 403-407.

Hurrell, R.F., Carpenter, K.J. 1981. The estimation of available lysine in foodstuffs after Maillard reactions. Prog. Food Nutr. Sci. 5, 159-176.

Karayiannis, N.I., MacGregor, J.T., Bjeldanes, L.F. 1979. Lysinoalanine formation in alkali-treated proteins and model peptides. Food Cosmet. Toxicol. 17, 585-590.

Kassell, B., Chow, R.B. 1966. Modification of the basic trypsin inhibitor of bovine pancreas. The ε-amino groups of lysine and the amino terminal sequence. Biochem. 5, 3449-3453.

Mauron, J., Bujard, E. 1964. Guanidination, an alternative approach to the determination of available lysine in foods. Proc. 6th Int. Nutr. Congr. 489-490.

Moughan, P.J. 2003. Amino acid availability: Aspects of chemical and bioassay methodology. Nutr. Res. Rev. 16, 127-141.

Moughan, P.J., Darragh, A.J., Smith, W.C., Butts, C.A. 1990. Perchloric and trichloroacetic acids as precipitants of protein in endogenous ileal digesta from the rat. J. Sci. Food Agric. 52, 13-21.

Moughan, P.J., Rutherfurd, S.M. 1996. A new method for determining digestible reactive lysine in foods. J. Agric. Food Chem. 44, 2202-2209.

National Research Council. 1995. Nutrient requirement of the laboratory rat. In Nutrient Requirements of Laboratory Animals. 4th ed. Washington, DC: Natl. Acad. Press.

Pereyra-Gonzáles, A.S., Naranjo, G.B., Malec, L.S., Vigo, M.S. 2003. Available lysine, protein digestibility and lactulose in commercial infant formulas. Int. Dairy J. 13, 95-99.

Ramirez-Jiménez, A., García-Villanova, B., Guerra-Hernández, E. 2004. Effect of storage conditions and inclusion of milk on available lysine in infant cereals. Food Chem. 85, 239-244.

Rutherfurd, S.M., Moughan. P.J. 1998. The digestible amino acid composition of several milk proteins: Application of a new bioassay. J. Dairy Sci. 81, 909-917.

Rutherfurd, S.M., Moughan, P.J., Morel, P.C.H. 1997. Assessment of the true ileal digestibility of reactive lysine as a predictor of lysine uptake from the small intestine of the growing pig. J. Agric. Food Chem. 45, 4378-4383.

SAS Institute Inc. SAS User's Guide: Statistics. Version 8.02. SAS Institute Inc, Gary, NC. 1999.

Shields, G.S., Hill, R.L., Smith, E.L. 1959. Preparation and properties of guanidinated mercuripapain. J. Biol. Chem. 234, 1747-1753.

Chapter 4

Available (ileal digestible reactive) lysine in selected cereal-based food products

Cereal-based breakfast foods are perceived to be high quality human foods. However, breakfast cereals often contain significant amounts of sugar and can undergo quite severe processing during their manufacture. Consequently, this chapter describes the determination of the digestible reactive lysine (available lysine) and digestible total lysine content of a range of processed "ready-to-eat" cereal-based breakfast foods using the newly developed assay.

Available (ileal digestible reactive) lysine in selected cereal-based food products

Shane M. Rutherfurd*, Nour Mohammad Torbatinejad† and Paul J. Moughan*

**Riddet Institute, Massey University, Palmerston North, New Zealand; †Department of Animal Science, Gorgan University of Agricultural Sciences & Natural Resources, Iran.*

Reproduced with permission from the publishers from: Rutherfurd, S.M., Torbatinejad, N.M., Moughan, P.J. 2006. Available (ileal digestible reactive) lysine in selected cereal-based food products. J. Agric. Food Chem. 54, 9453-9457.

Abstract

True ileal total lysine digestibility was determined and compared with the true ileal reactive lysine digestibility for 20 cereal-based breakfast foods. Semisynthetic diets each containing a breakfast cereal as the sole protein source were formulated and fed to growing rats. Titanium dioxide was included as an indigestible marker. Digesta were collected from the rats and total (conventional amino acid analysis) and reactive (guanidination) lysine were determined in both diets and digesta. The true ileal reactive lysine digestibility ranged from 53 to 108% and was significantly higher than the true ileal total lysine digestibility for most of the breakfast cereals. Available lysine content (digestible reactive lysine content) ranged from 0.21 - 3.5 g kg^{-1} across the breakfast cereals. The conventional measure of digestible total lysine content significantly overestimated (on average by 37%) available lysine for the majority of the cereals. Breakfast cereals undergo a significant degree of lysine modification probably as a result of processing during manufacture.

Introduction

Although cereals are an important source of dietary protein for humans, they tend to contain lower amounts of the amino acid lysine compared to other protein sources such as milk or meat. Consequently, lysine is often the first limiting amino acid in diets that are high in cereals.

A unique property of the amino acid lysine is that it possesses a reactive side chain amino group which can react with a variety of chemical entities, particularly reducing sugars, to produce biologically unavailable lysine derivatives. These reactions occur when protein sources are manufactured or stored for prolonged periods of time, with the rate of reaction being greatly accelerated during processing, particularly heat processing (Moughan, 2003; Hurrell and Carpenter, 1981). Simple sugars are often added to cereal-based breakfast foods during processing as sweetening agents, which is expected to exacerbate the extent of lysine side chain reactions. Erbersdobler and Hupe (1991) reported that some 20% of lysine was inactivated and 10% was destroyed in a single processed breakfast cereal. Moreover, a recent study from our laboratory (Torbatinejad et al., 2005) found large differences between the total lysine and reactive lysine contents of 20 commercial breakfast cereal products indicating that considerable lysine damage had occurred. Given that breakfast cereals are an important staple food, such deterioration in nutrient quality is a matter of concern.

When lysine reacts with other compounds it is expected, in addition to the formation of biologically unavailable lysine derivatives, that overall protein digestibility is also adversely affected (Moughan, 2003) meaning that otherwise reactive lysine (available) units may not be released and absorbed from the parent protein. Thus, the digestible reactive lysine content of a processed food may be considerably lower than the reactive lysine content, which is lower in turn than total lysine. It has been reported that breakfast cereals based on maize, wheat, rice or oats tend to have low protein digestibility (Hopkins, 1981) and it is possible that Maillard product formation is at least partly responsible for such a reduction in amino acid digestibility. It is important, therefore, to determine digestible reactive lysine in addition to the chemically reactive lysine content. The latter can be accomplished by applying an in vivo ileal digestibility assay which determines the disappearance of reactive lysine from the upper

digestive tract of the laboratory rat (Moughan and Rutherfurd, 1996). The new assay has been validated (Rutherfurd et al., 1997a) and applied to a range of processed food products including milk-based foods (Rutherfurd and Moughan, 2005) and animal feeds (Rutherfurd et al., 1997b).

The presently reported study follows that of Torbatinejad et al. (2005) and aimed to determine the *in vivo* digestible reactive (available) lysine content of 20 processed cereal–based breakfast foods using the true ileal reactive lysine digestibility assay.

Materials and methods

Cereal samples

Twenty commercially available packaged breakfast cereal products were selected as described fully by Torbatinejad et al. (2005). In brief, six different batches each of 20 cereal products (120 samples in total) were purchased from supermarkets in Palmerston North, New Zealand. Equal weights of each batch were pooled for each cereal product and the resulting 20 composite samples were ground through a 1 mm mesh and stored at -20 °C prior to being tested for true ileal digestible reactive lysine content using an *in vivo* digestibility assay.

The major ingredients of the cereal products as reported on the statutory labels given on the packages consisted of either wheat, corn, oatmeal or rice (Torbatinejad et al., 2005). The proximate composition of each of the breakfast cereals was reported by Torbatinejad et al. (2005). In brief, the crude protein content of the breakfast cereals ranged from 52 to 253 g kg^{-1} DM, crude fibre from 4 to 38 g kg^{-1} DM, total fat from 14 to 144 g kg^{-1} DM and ash from 7 to 32 g kg^{-1} DM. The nitrogen free extractive (NFE) ranged from 678 to 908 g kg^{-1} DM.

Preparation of 0.6 M O-Methylisourea Solution.

A 0.6 M O-methylisourea solution was prepared as described by Moughan and Rutherfurd (1996), based on the procedures of Chervenka and Wilcox (1956), Shields et al. (1959), Mauron and Bujard (1964), and Kassell and Chow (1966).

Digestibility study.

Ethics approval for the animal trial was obtained from the Animal Ethics Committee, Massey University, Palmerston North, New Zealand. One hundred entire male Sprague-Dawley rats of approximately 150 g body weight, were obtained from the Small Animal Production Unit (Massey University, Palmerston North, New Zealand). The rats were housed individually in stainless steel wire-bottomed cages in a room maintained at 22 ± 2 °C, with a 12 h light/dark cycle. Twenty semisynthetic test diets were formulated. The protein content of the cereal products ranged from 5.2% to 25.3%, and in the main, the experimental diets consisted of the cereal product alone with the addition of titanium dioxide (0.3%). For cereal products that had a crude protein content greater that 10%, dilution with soyabean oil and cornstarch was used to reduce the crude protein content to 100 g kg^{-1}. A basal diet containing 100 g kg^{-1} protein was also formulated using skim milk powder as the protein source and

this diet met the nutritional requirements for the growing rat for all nutrients except protein (National Research Council, 1995). The latter diet contained 27.2% skim milk powder, 5% proprietary vitamin premix, 5% proprietary mineral premix, 5% soyabean meal, 10% sucrose, 5% purified cellulose and 42.5% wheat starch. Titanium dioxide (0.3%) was added to all diets as an indigestible marker. For the first 10 d of the experimental period, all the rats were fed the basal skim milk powder-based diet. The test diets were then randomly allocated to the rats such that there were five rats per diet. The animals were then fed their respective diets for the final 4 d. The test diets were not fed for the entire experimental period as they may not have met the rat's requirement for all vitamins and minerals. On each day, each rat had unrestricted access to its respective diet from 0830 h to 1130 h. Water was available at all times. On the final day of the study, between 3 and 4 h after the start of feeding, the rats were asphyxiated using carbon dioxide gas and then decapitated. The 20 cm of ileum immediately anterior to the ileo-caecal junction was dissected out. The dissected ileum was washed with distilled deionised water to remove any blood and hair and was carefully dried on an absorbent paper towel. The digesta were gently flushed from the ileum section with distilled deionised water from a syringe. The digesta were then freeze dried ready for chemical analysis.

Chemical analysis.

Amino acid contents were determined in duplicate 5 mg diet and digesta samples using a Waters ion-exchange HPLC system, utilising postcolumn ninhydrin derivatisation and detection using absorbance at 570 nm and 440 nm, following hydrolysis in 6 M glass-distilled HCl containing 0.1% phenol for 24 h at 110 ± 2 °C in evacuated sealed tubes. Cysteine, methionine and tryptophan were not determined as they are destroyed, at least in part, during acid hydrolysis. The weight of each amino acid was calculated using free amino acid molecular weights.

Reactive lysine contents were determined in duplicate 5 mg digesta and diet samples by incubation for 7 d in 0.6 M OMIU, pH 10.6 (pH 11.0 for the digesta samples), at 21 ± 2 °C in a shaking waterbath, with the reagent to lysine ratio being greater than 1000 according to the procedure of Moughan and Rutherfurd (1996). After incubation, the samples were dried using a Speedvac concentrator (Savant Instruments, Inc, Farmingdale, NY) and were analysed for homoarginine content in a similar manner as for the amino acid content described above. The reactive lysine content of the breakfast cereals themselves was those determined by Torbatinejad et al. (2005).

The titanium contents of both the diet and ileal digesta samples were determined in duplicate. Titanium was determined on the basis of the method of Short et al. (1996). Samples were ashed before being digested in 60% (v/v) sulphuric acid and then incubated with 30% hydrogen peroxide, and the absorbance was read at 405 nm.

Data analysis.

Ileal and endogenous ileal amino acid flows were calculated as follows (units are $\mu g\ g^{-1}$ DM):

$$\text{Ileal amino acid flow } (\mu g\ g^{-1}\ DMI) = \text{Ileal amino acid content} \times \frac{\text{Diet titanium}}{\text{Ileal titanium}}$$

True ileal amino acid (AA) digestibility was calculated using the following equation (units are $\mu g\ g^{-1}$ DMI):

$$\text{True AA digestibility } (\%) = \frac{(\text{Dietary AA intake} - (\text{Ileal AA flow} - \text{Endogenous AA flow}))}{\text{Dietary AA intake}} \times 100$$

Endogenous amino acid flow is based on the endogenous amino acid flows of the growing rat reported by Rutherfurd and Moughan (1998).

True ileal reactive lysine (RL) digestibility was calculated as follows (units are $\mu g\ g^{-1}$ DMI):

$$\text{True RL digestibility } (\%) = \frac{(\text{Dietary RL intake} - (\text{Ileal RL flow} - \text{Endogenous lysine flow}))}{\text{Dietary RL intake}} \times 100$$

Reactive lysine was determined using the guanidination method and endogenous lysine flow was based on the endogenous lysine flow reported by Rutherfurd and Moughan (1998).

True ileal digestible reactive lysine content of the cereals was calculated as follows (units are $g\ kg^{-1}$):

True ileal digestible RL content = RL content x True ileal RL digestibility (%)

True ileal digestible amino acid content of the cereals was calculated as follows (units are $g\ kg^{-1}$):

True ileal digestible AA content = AA content x True ileal AA digestibility (%)

The amino acid digestibility data were subjected to a one-way analysis of variance for each amino acid singly (GLM Procedure) (SAS, 1999).

Results

The amino acid composition of the 20 cereal-based breakfast foods examined in this study has been presented in a previously published paper (Torbatinejad et al., 2005).

True ileal total and reactive lysine digestibility for 20 cereal-based foods

True ileal total lysine digestibility, which is based on the conventional amino acid analysis of both diets and digesta, was determined in 20 breakfast cereals and was compared to true ileal reactive lysine digestibility, which is based on using the guanidination reaction to

determine the reactive lysine contents in both diets and digesta. These results are presented in Table 1. The mean reactive lysine digestibility (lysine availability) across all the cereals was 80% but ranged from 53% for cereal 13 (a puffed wheat product) to 108% for cereal 6 (a flaked corn product). For half of the cereals tested, the true ileal reactive lysine digestibility was significantly ($P < 0.05$) higher than true ileal total lysine digestibility determined using conventional amino acid analysis. For these cereals, total lysine digestibility underestimated lysine digestibility by between 12% and 65% with the average underestimation being 31%. For three breakfast cereals (8, 10 and 15) the difference between total lysine digestibility and reactive lysine digestibility was large (17%, 14% and 14% respectively) although these differences were not statistically ($P > 0.05$) significant.

True ileal digestible total and reactive lysine content for 20 cereal-based foods

The true ileal digestible total lysine content was determined and compared with the true ileal digestible reactive lysine (available lysine) content for the 20 selected breakfast cereals (Table 2). For 15 of the 20 cereal products, the digestible total lysine content significantly ($P < 0.05$) overestimated digestible reactive lysine content (available lysine). This overestimation ranged from 16% for cereal product 1 (a shredded wheat product) to 77% for product 11 (a puffed rice product). The mean overestimation was 40%. For the other five cereals, there was no significant ($P > 0.05$) difference between digestible total lysine content and digestible reactive lysine content. However, for these five cereal products, there were some sizable numerical differences (4 – 79%, mean 47%) between digestible total and reactive lysine contents.

True ileal amino acid digestibility for the 20 cereal-based foods

True ileal amino acid digestibility values for the amino acids other than lysine for the 20 breakfast cereals are given in Table 3. Glycine digestibility was not determined since, in protein sources that contain low levels of protein, endogenous glycine may be underestimated using the EHC method since bile acids may be included in the endogenous amino acid fraction. The overall true ileal amino acid digestibility across amino acids for each cereal ranged from 61% for cereal 12 (a puffed rice product) to 89% for cereal 8 (an extruded wheat product) with a mean overall digestibility of 79%. The least digestible amino acid across all cereals was histidine (63%) and the most digestible amino acid was phenylalanine (88%).

True ileal digestible amino acid content for the 20 cereal-based foods

True ileal digestible amino acid content of the 20 breakfast cereals is presented in Table 4. There was considerable variation in digestible amino acid content between the 20 cereal products, with a greater than 10-fold range in digestible amino acid content across cereal products for glutamic acid, proline, histidine and arginine. For threonine, serine, valine, isoleucine, leucine, tyrosine and phenylalanine there was a relatively lower (5-fold) difference in digestible amino acid content across products.

Table 1.
Mean (n=5) true ileal total and reactive lysine digestibility (%) for 20 selected cereal-based foods.

Breakfast cereal	Lysine Digestibility		Overall SE	Statistical significance[3]
	Total[1]	Reactive[2]		
1	67.7	84.3	4.0	**
2	71.5	86.3	4.8	*
3	67.2	80.5	3.6	*
4	74.3	75.7	7.0	NS
5	64.4	68.3	5.7	NS
6	71.1	108.3	3.3	***
7	76.8	74.2	14.9	NS
8	53.7	62.8	5.5	NS
9	40.2	66.3	7.2	***
10	57.7	66.0	7.0	NS
11	84.1	86.8	5.6	NS
12	63.0	90.2	4.2	***
13	55.4	53.5	18.4	NS
14	66.9	91.1	1.2	***
15	52.2	59.7	8.7	NS
16	81.8	84.0	5.7	NS
17	76.8	89.8	3.8	**
18	72.2	86.1	3.4	**
19	81.2	90.8	1.5	**
20	78.6	79.3	3.6	NS

[1]Total lysine digestibility was determined using the true ileal amino acid digestibility assay in the rat using traditional amino acid analysis to determine the total lysine content of the diets and digesta.
[2]Reactive lysine digestibility was determined using the true ileal amino acid digestibility assay in the rat using guanidination and homoarginine analysis to determine the reactive lysine content of the diets and digesta.
*[3]NS not significant, $P > 0.05$; *, $0.05 > P > 0.01$; **, $0.01 > P > 0.001$; ***, $P < 0.001$.*

Table 2.
Mean (n=5) digestible total and reactive lysine contents (available lysine) (g kg^{-1} freeze dried matter) for 20 selected cereal-based foods.

Breakfast cereal	Digestible lysine		Overall SE	Statistical Significance[3]
	Total[1]	Reactive[2]		
1	1.8	1.6	0.08	*
2	1.7	1.3	0.08	**
3	1.3	0.8	0.05	**
4	2.0	1.4	0.13	*
5	1.4	1.1	0.09	*
6	0.7	0.6	0.02	*
7	0.4	0.2	0.05	NS
8	1.1	0.7	0.07	*
9	0.9	0.7	0.11	NS
10	1.1	0.7	0.10	**
11	1.1	0.6	0.05	**
12	0.8	0.7	0.04	*
13	0.4	0.3	0.10	NS
14	1.4	1.0	0.02	***
15	0.8	0.5	0.09	NS
16	3.6	2.5	0.19	*
17	3.8	3.2	0.15	*
18	3.7	3.5	0.15	NS
19	3.7	2.8	0.06	***
20	3.2	1.9	0.08	***

[1]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 cereal also determined using traditional amino acid analysis.
[2]Digestible reactive lysine was calculated from the true ileal reactive lysine digestibility determined using the true ileal amino acid digestibility 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.
*[3]NS not significant, $P > 0.05$; *, $0.05 > P > 0.01$; **, $0.01 > P > 0.001$; ***, $P < 0.001$.*

Table 3.
Mean (n=5) true ileal amino acid digestibility (%) for the 20 cereal-based breakfast products[1].

Amino acid	Cereal																				Overall
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	SE
Aspartic acid	63	54	59	64	48	74	70	71	49	62	63	49	46	67	46	82	76	73	87	77	3.9
Threonine	76	76	77	72	70	75	80	84	62	74	76	57	60	74	71	81	77	73	84	75	3.8
Serine	85	86	84	83	82	84	90	90	69	83	80	61	78	78	83	85	80	75	88	81	3.0
Glutamic acid	92	93	91	91	90	83	85	96	81	92	68	58	83	86	91	91	88	86	92	89	1.6
Proline	90	83	89	88	89	68	77	95	69	90	60	56	78	78	89	85	83	79	86	82	2.7
Alanine	78	80	77	75	75	87	85	85	63	77	67	59	71	76	75	82	79	72	86	82	2.8
Valine	83	86	82	80	80	79	81	90	68	82	74	65	75	80	84	86	82	78	89	85	2.5
Isoleucine	85	89	85	84	84	87	85	93	74	85	75	66	81	83	87	88	85	81	91	87	2.2
Leucine	87	90	87	84	86	90	90	94	76	88	71	62	83	84	90	88	85	81	91	87	1.8
Tyrosine	88	88	87	85	86	87	89	93	75	87	70	60	83	82	89	88	82	77	90	85	2.0
Phenylalanine	91	94	91	87	90	90	91	97	80	92	75	65	88	86	93	91	86	82	93	90	1.7
Histidine	51	52	76	43	48	71	71	78	49	79	71	54	49	68	52	74	71	67	74	51	4.7
Arginine	85	86	85	80	84	59	50	89	70	83	71	78	80	79	87	89	85	80	90	84	3.0

[1]Values were corrected for endogenous amino acid flow using the EHC method (Butts et al., 1991; Moughan et al., 1990) reported by (Rutherfurd and Moughan, 1998).

Table 4.
Mean (n=5) true ileal digestible amino acid content (g kg^{-1}) for the 20 cereal-based breakfast products.

	Breakfast cereal product										Overall
Amino acid	1	2	3	4	5	6	7	8	9	10	SE[1]
Aspartic acid	4.2	3.5	3.1	3.6	3.2	2.9	2.4	7.0	2.9	3.8	
Threonine	2.5	2.5	1.9	1.9	2.3	1.4	1.3	4.5	1.8	2.3	
Serine	4.0	4.3	3.1	3.4	4.0	2.2	2.1	8.1	2.6	4.0	
Glutamic acid	28.2	34.4	24.9	24.4	32.4	10.0	8.8	77.7	17.9	32.9	
Proline	9.3	9.9	8.3	8.2	10.4	3.5	3.2	25.8	5.5	10.4	
Alanine	3.8	3.7	2.9	2.8	3.4	3.5	3.1	6.1	2.8	3.3	
Valine	4.1	4.3	3.3	3.4	4.0	2.2	2.0	7.9	2.8	4.0	
Isoleucine	3.1	3.5	2.6	2.7	3.2	1.7	1.6	7.1	2.2	3.3	
Leucine	6.4	7.2	5.8	5.3	6.8	5.9	5.5	14.9	4.8	6.8	
Tyrosine	3.0	3.2	2.6	2.4	3.0	2.0	2.0	6.8	2.1	3.1	
Phenylalanine	4.3	5.0	3.7	3.5	4.6	2.5	2.3	10.4	3.0	4.6	
Histidine	1.0	1.2	1.7	1.0	1.3	1.4	1.2	4.3	1.5	2.5	
Arginine	4.4	4.0	2.9	3.8	3.6	1.4	0.8	6.1	3.5	3.7	
Amino acid	11	12	13	14	15	16	17	18	19	20	
Aspartic acid	3.4	2.7	1.5	4.1	3.1	7.3	8.0	7.9	9.4	6.5	0.23
Threonine	1.5	1.1	0.9	1.9	2.4	2.7	3.1	3.1	3.4	2.4	0.10
Serine	2.3	1.8	1.8	3.0	4.1	3.9	4.4	4.5	4.8	3.7	0.11
Glutamic acid	8.0	7.2	10.8	19.5	29.8	21.3	25.6	24.3	23.8	20.6	0.31
Proline	1.9	1.8	3.4	5.5	9.3	5.3	6.3	5.7	5.6	4.8	0.18
Alanine	2.2	2.1	2.1	2.8	3.6	4.2	4.7	4.8	5.6	4.1	0.12
Valine	2.4	2.3	1.9	3.2	4.4	4.6	5.3	5.4	5.8	4.6	0.10
Isoleucine	1.7	1.6	1.5	2.5	3.6	3.4	4.0	3.9	4.2	3.3	0.07
Leucine	3.4	3.3	4.0	5.2	7.3	6.9	8.2	8.3	8.7	6.7	0.12
Tyrosine	1.9	1.9	1.7	2.5	3.5	3.3	3.9	3.9	4.1	3.2	0.06
Phenylalanine	2.2	2.1	2.2	3.4	4.8	4.6	5.5	5.5	5.7	4.6	0.07
Histidine	1.0	0.8	0.3	1.2	1.2	1.9	2.2	1.8	2.3	1.3	0.10
Arginine	3.0	3.3	1.8	3.5	4.6	6.9	6.9	7.5	8.5	6.0	0.12

[1]*Overall SE was calculated across all breakfast cereal products.*

Discussion

Lysine is prone to undergo chemical modification when foodstuffs are processed (Maillard reaction). This modified lysine is generally nutritionally unavailable and its presence leads to an overestimate of determined available lysine in foodstuffs when traditional techniques such as the true ileal amino acid digestibility assay are used. In this study, the new and accurate method (Moughan and Rutherfurd, 1996) for determining available lysine (true ileal digestible reactive lysine assay) was applied to 20 commercially available cereal-based breakfast foods.

True ileal total and reactive lysine digestibility for the 20 cereal-based foods

In processed foods, such as breakfast cereals, a proportion of the lysine will have inevitably been chemically modified to form Maillard products (Moughan, 2003; Hurrell and Carpenter, 1981). Torbatinejad et al. (2005) reported sizable differences between the amounts of total lysine and reactive lysine in cereal foods, suggesting the presence of acid-labile lysine derivatives. Erbersdobler and Hupe (1991) also reported lysine damage in breakfast cereals. The presence of Maillard products (modified lysine residues) in dietary proteins is believed to reduce the effectiveness of digestive enzymes, resulting in the presence of undigested peptides (limit peptides) at the terminal ileum (Moughan et al., 1996) and a proportionally higher concentration of modified lysine in the digesta compared to the diet. Consequently, total lysine digestibility is expected to underestimate the actual lysine digestibility. Reactive lysine digestibility focuses only on the lysine that has remained intact during processing and as such is an accurate measure of lysine digestibility.

For the majority of the cereal products, total lysine digestibility underestimated lysine digestibility in comparison to reactive lysine digestibility. This underestimation was quantitatively large, being on average 33%. Reactive lysine digestibility also ranged widely across breakfast cereals, likely reflecting variation in the processing methods used and in the ingredient composition.

For cereal 6 (a flaked corn-based product), the mean true ileal digestibility of reactive lysine was 108% which from a theoretical standpoint is not possible. In this study, endogenous amino acid losses were determined using an EHC-based diet containing 100 g kg^{-1} peptides while the cereal 6 diet contained only 60 g kg^{-1} protein. Since the protein/peptide concentration of a diet influences the amount of endogenous amino acids present at the terminal ileum (Hodgkinson et al., 2000), it is likely that the endogenous lysine loss in the rats fed the cereal 6 diet was lower than that determined using the EHC-based diet, which would lead to an overestimate of the true reactive lysine digestibility.

True ileal digestible total and reactive lysine contents for the 20 cereal-based foods

There have been few reports in the literature describing available lysine contents of breakfast cereals. Clarke and Kennedy (1962) reported available lysine data for two breakfast cereals, but their estimates were based on the faecal digestibility of total lysine and are likely to be misleading (McNeil, 1988). While several workers have examined total lysine levels and *in vitro* protein digestibility in processed wheat-based cereals (Abdel-Aal and Hucl, 2002; McAuley et al., 1987), biologically available lysine in breakfast cereals has not been investigated. In the present work, digestible total lysine content overestimated digestible reactive lysine content (available lysine) in 15 of the cereal foods tested. The degree of overestimation was considerable, being greater than 20% for at least 60% of the cereal products tested. For many of the breakfast cereals tested, there was a considerable amount of lysine damage, and for such foods, digestible total lysine is an inaccurate measure of available lysine. Given the public perception that breakfast cereals are high-quality healthy foods, it is somewhat surprising that the protein quality of many of the breakfast cereals was so poor. Lysine is naturally low in cereals and as such any further losses from processing should be viewed with some concern.

True ileal amino acid digestibility for the 20 cereal-based foods

The true ileal digestibility of the acid-stable amino acids (apart from lysine) was determined. Overall, amino acid digestibility was low (79%), which concurs with the low lysine availability observed. Khan and Eggum (1979) also reported a reduction in overall protein digestibility in breakfast cereals after processing. There appeared to be some reasonably highly digestible cereal products with products 1, 2, 3, 7, 8, 10, 16, 17, 19, and 20 having mean true ileal amino acid digestibilities above 80%. These cereals were predominantly wheat and rolled oat based cereal foods. In contrast, there were several cereal-based products that were poorly digested, and these included cereals 9, 11, 12 and 13 all of which had mean amino acid digestibilities ranging from 61% to 74%. These cereal products generally contained significant amounts of puffed rice. It is likely that the type of processing method used and differences in the cereal bases used resulted in the differences in lysine damage and the varying amino acid digestibility among the cereal products. Without more detailed information, however, it is difficult to draw definitive conclusions.

True ileal digestible amino acid content for the 20 cereal-based foods

Overall, there was considerable variation (more than a 3-fold difference) in the true ileal digestible amino acid content for each amino acid across the breakfast cereal products. This variation was predominantly a result of the different cereal bases used to formulate each of the breakfast cereal products. For example, wheat and the wheat-based cereals (1-5, 8-10, 13-15) both have high amounts of glutamic acid and proline (Torbatinejad et al., 2005). Furthermore, amino acid digestibility will impact the digestible amino acid contents across the cereal products.

Conclusion

True ileal total lysine digestibility, based on conventional amino acid analysis of diets and digesta is an inaccurate measure of lysine availability in processed protein sources (Batterham, 1990). The reactive lysine digestibility assay used in this study and based on the guanidination of lysine in both diets and digesta is an alternative accurate measure of lysine availability (Rutherfurd et al., 1997a). For most of the breakfast cereals tested in this study, the traditional measure of true ileal digestible total lysine considerably overestimated available lysine.

Literature cited

Abdel-Aal, E-S. M., Hucl, P. 2002. Amino acid composition and in vitro protein digestibility of selected ancient wheats and their end products. J. Food Sci. 15, 737-747.

Batterham, E.S., Andersen, L.M., Baigent, R.D., Darnell, R.E., Taverner, M.R. 1990. A comparison of the availability and ileal digestibility of lysine in cottonseed and soya-bean meals for grower/finisher pigs. Brit. J. Nut. 64, 663-677.

Butts, C.A., Moughan, P.J., Smith, W.C. 1991. Endogenous amino acid flow at the terminal ileum of the rat determined under conditions of peptide alimentation. J. Sci. Food Agric. 55, 175-187.

Chervenka, C.H., Wilcox, P.E. 1956. Chemical derivatives of chymotrypsinogen. II. Reaction with O methylisourea. J. Biol. Chem. 222, 635-647.

Clarke, J-A.K., Kennedy, B.M. 1962. Availability of lysine in wholewheat bread and in selected breakfast cereals. J. Food Sci. 27, 609-616.

Erbersdobler, H.F., Hupe, A. 1991. Determination of lysine damage and calculation of lysine bio-availability in several processed foods. Z. Ernährungswiss. 30, 46-49.

Hodgkinson, S.M., Moughan, P.J., Reynolds, G.W., James, K.A.C. 2000. The effect of dietary peptide concentration on endogenous ileal amino acid loss in the growing pig. Brit. J. Nutr. 83, 421-430.

Hopkins, D.T. 1981. Effects of variation in protein digestibility. In Protein Quality in Humans: Assessment and In Vitro Estimation. Pp. 169-193 [CE Bodwell, JS Adkins and DT Hopkins, editors]. Westport, Connecticut: AVI Publishing.

Hurrell, R.F., Carpenter, K.J. 1981. The estimation of available lysine in foodstuffs after Maillard reactions. Prog. Food Nutr. Sci. 5, 159-176.

Kassell, B., Chow, R.B. 1966. Modification of the basic trypsin inhibitor of bovine pancreas. The ε-amino groups of lysine and the amino terminal sequence. Biochem. 5, 3449-3453.

Khan M.A., Eggum B.O. 1979. Effect of home and industrial processing on protein quality of baby foods and breakfast cereals. J Sci. Food Agric. 30, 369-376.

Mauron, J., Bujard, E. 1964. Guanidination, an alternative approach to the determination of available lysine in foods. Proc. 6th Int. Nutr. Congr. 489-490.

McAuley, J.A., Kunkel, M.E., Acton, J.C. 1987. Relationships of available lysine to lignin, color and protein digestibility of selected wheat-based breakfast cereals. J. Food Sci. 52, 1580-1583.

McNeil, N.I. 1988. Nutritional implications of human and mammalian large intestinal function. World Rev. Nutr. Diet. 56, 1-42.

Moughan, P.J. 2003. Amino acid availability: Aspects of chemical and bioassay methodology.

Nutr. Res. Rev. 16, 127-141.
Moughan, P.J., Darragh, A.J., Smith, W.C., Butts, C.A. 1990. Perchloric and trichloroacetic acids as precipitants of protein in endogenous ileal digesta from the rat. J. Sci. Food Agric. 52, 13-21.
Moughan, P.J., Gall, M.P.J., Rutherfurd, S.M. 1996. Absorption of lysine and deoxyketosyllysine in an early-Maillard browned casein by the growing pig. J. Agric. Food Chem. 44, 1520-1525.
Moughan, P.J., Rutherfurd, S.M. 1996. A new method for determining digestible reactive lysine in foods. J. Agric. Food Chem. 44, 2202-2209.
National Research Council. 1995. Nutrient requirement of the laboratory rat. In Nutrient Requirements of Laboratory Animals. 4th ed. Washington, DC: Natl. Acad. Press.
Rutherfurd, S.M., Moughan, P.J. 2005. Digestible reactive lysine in selected milk-based products. J. Dairy Sci. 88, 40-48.
Rutherfurd, S.M., Moughan. P.J. 1998. The digestible amino acid composition of several milk proteins: Application of a new bioassay. J. Dairy Sci. 81, 909-917.
Rutherfurd, S.M., Moughan, P.J., Morel, P.C.H. 1997a. Assessment of the true ileal digestibility of reactive lysine as a predictor of lysine uptake from the small intestine of the growing pig. J. Agric. Food Chem. 45, 4378-4383.
Rutherfurd, S.M., Moughan, P.J., van Osch, L. 1997b. Digestible reactive lysine in processed feedstuffs: Application of a new bioassay. J. Agric. Food Chem. 45, 1189-1194.
SAS Institute Inc. SAS User's Guide: Statistics. Version 8.02. SAS Institute Inc, Gary, NC. 1999.
Shields, G.S., Hill, R.L., Smith, E.L. 1959. Preparation and properties of guanidinated mercuripapain. J. Biol. Chem. 234, 1747-1753.
Short, F.J., Gorton, P., Wiseman, J., Boorman, K.N. 1996. Determination of titanium dioxide added as an inert marker in chicken digestibility studies. Anim. Feed Sci. Technol. 59, 215-221.
Torbatinejad, N.M., Rutherfurd, S.M., Moughan, P.J. 2005. Total and reactive lysine contents in selected cereal-based food products. J. Agric. Food Chem. 53, 4454-4458.

Chapter 5

Available (ileal digestible reactive) lysine in selected pet foods

This chapter describes the determination of the digestible reactive lysine (available lysine) content of a range of moist and dry cat foods using the newly develop available lysine assay. Digestible total lysine was also determined and compared with digestible reactive lysine which highlighted the inadequacy of digestible total lysine values for pet foods.

Available (ileal digestible reactive) lysine in selected pet foods

Shane M. Rutherfurd*, Kay J. Rutherfurd-Markwick† and Paul J. Moughan*

**Riddet Institute and*
†Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand.

Reproduced with permission from the publishers from: Rutherfurd, S.M., Markwick-Rutherfurd, K.J., Moughan, P.J. 2007. Available (ileal digestible reactive) lysine in selected pet foods. J. Agric. Food Chem. 55, 3517-3522.

Abstract

A recently developed assay for determining available lysine (true ileal digestible reactive lysine) in foods and feedstuffs was applied to 20 commercially available cat foods. Semisynthetic diets, containing cat food as the sole source of protein, were prepared. Titanium dioxide was included in the diets as an indigestible marker. The diets were fed to growing rats and digesta were collected from the terminal ileum. The digesta were then analysed, along with the diets, for reactive lysine using the guanidination method. True ileal reactive lysine digestibility was determined after correction for endogenous lysine loss at the terminal ileum of rats fed an EHC-based diet. The amounts of digestible total lysine (conventional method) were also determined. Ileal total lysine digestibility significantly ($P < 0.05$) underestimated (3.6 - 10.2%) lysine availability (ileal reactive lysine digestibility) for most of the cat foods tested in the study. Ileal digestible total lysine significantly ($P < 0.05$) overestimated the amount of dietary available lysine for all of the cat foods tested by between 38 and 143%. Total lysine digestibility determined using the conventional method of lysine analysis was inaccurate when applied to commercially available cat foods. Consequently, acid-labile modified lysine derivatives are present in cat foods and the available lysine content of cat foods would appear to be much less than previously thought.

Introduction

Both moist and dry cat foods are subjected to high temperatures for considerable periods of time during their processing in order to sterilise the food, achieve an acceptable form and enhance the palatability (Hendriks et al., 1999). When a food is heated, the ε-amino group (side chain) of lysine can react with other compounds present to produce derivatives that are nutritionally unavailable to the animal that consumes the food (Hurrell and Carpenter 1981). Furthermore, traditional digestibility and chemical analysis techniques (true ileal digestible total lysine) overestimate the available lysine content of such foods, because some of the lysine derivatives revert to lysine during the hydrolysis step of amino acid analysis, a key step in the amino acid digestibility procedure (Moughan, 2003).

A new method that can accurately determine the available lysine content in processed foods and feedstuffs has been developed and described (Moughan and Rutherfurd, 1996). With this assay, chemically reactive (underivatised) lysine in both the diet and ileal digesta collected from a test animal that has consumed the food or feedstuff is determined. After correction for ileal endogenous lysine the digestible reactive lysine (available lysine) is then calculated. The assay has been applied to a small selection of feedstuffs used in the pig and poultry industry (Rutherfurd et al., 1997a), a range of milk products (Rutherfurd and Moughan, 2005), breakfast cereals (Rutherfurd et al., 2006), but has not been applied to pet foods.

Ileal digestibility measurements are superior to faecal digestibility because faecal digestibility is confounded by the activity of hindgut microflora (Mason et al., 1976). From an ethical standpoint, however, determining ileal digestibility in cats is undesirable because sacrifice of the animal is usually required. An alternative is to use a laboratory animal model. The laboratory rat was used as an animal model for the cat in the present study for determining true ileal amino acid digestibility of foods consumed by cats. While there are some obvious

differences between the two species, the main one being that the cat is an obligate carnivore whereas the rat is an omnivore, there are also a number of similarities. The gut mucosal areas are similar between the two species when related to body weight (Woods, 1944). The laboratory rat has also been used previously as an animal model to investigate the effect of heat treatment on processed canned cat foods (Hendriks et al., 1999).

Cat foods, being predominantly meat based, are unlikely to contain high levels of reducing sugars but do contain elevated amounts of fatty acids and their oxidation products. During the processing that cat diets undergo, these compounds may react with lysine leading to a reduction in the available lysine content. Meat offal, which is commonly used as an ingredient in cat foods, also contains significant amounts of collagen (Davey and Winger, 1979) which is a natural source of ε-amino bound lysine (Singh, 1991; Asghar and Henricksen, 1982).

Hendriks et al. (1999) reported true ileal amino acid digestibility values (based on conventional total lysine analysis) for a canned cat food heated for different times, but to date no one has examined the available lysine content of commercially available cat foods. The aim of this study was to compare the digestible total lysine (traditional digestibility assay) with the digestible reactive lysine content (new assay) for a range of commercial cat foods, to highlight the potential inaccuracy of the traditional assay with respect to this type of food product.

Materials and methods

Cat food samples

Twenty commercially available cat foods were purchased from a local supermarket in Palmerston North. These included 10 moist canned cat foods and 10 dry biscuit cat foods. The moist cat foods were freeze dried and the dried material, along with the samples of dry biscuits, was ground using a standard kitchen food processor. The finely ground material was then stored at -20 ^{o}C prior to analysis and incorporation into the experimental diets.

Digestibility study

Ethics approval for the animal trial was obtained from the Animal Ethics Committee, Massey University, Palmerston North, New Zealand. One hundred entire male Sprague-Dawley rats (150 - 200 g body weight), were obtained from the Small Animal Production Unit (Massey University, Palmerston North, New Zealand). The rats were housed individually in wire-bottomed cages designed to prevent the rats from practising coprophagy. The rats were kept in a room maintained at 22 ± 2^{o}C, with a 12 h light/dark cycle. Twenty experimental diets were formulated to each contain 100 g kg^{-1} protein, such that each cat food was the sole source of protein in the respective diet. The respective inclusion rates for the 20 cat foods (diets 1 - 20) were 236, 221, 235, 172, 217, 215, 224, 295, 224, 216, 287, 411, 350, 293, 342, 260, 285, 308, 340 and 243 g kg^{-1}. Other dietary ingredients were 50 g kg^{-1} purified cellulose, 100 g kg^{-1} sucrose, 50 g kg^{-1} soyabean oil, 50 g kg^{-1} proprietary vitamin mix and 50 g kg^{-1} proprietary mineral mix. The vitamin and mineral mixes were formulated to meet

the requirement of the growing rat for vitamins and minerals, respectively, in the final diets (National Research Council, 1995). Purified corn starch was added as necessary to make the diets up to 1 kg. Titanium dioxide was included (0.3%) in each diet as an indigestible marker. The rats were randomly allocated to the diets such that there were five rats per diet. The experimental period lasted 14 d, during which time each rat had unrestricted access to its respective diet from 0830 h to 1130 h. Water was available at all times. On the final day of the study, between 3 and 4 h after the start of feeding, the rats were asphyxiated using carbon dioxide gas and then decapitated (Butts et al., 2002). The 20 cm of ileum immediately anterior to the ileo-caecal junction was dissected out. The dissected ileum was washed with distilled deionised water to remove any blood and hair and carefully dried on an absorbent paper towel. The digesta were gently flushed from the ileum section with distilled deionised water from a syringe. The digesta were then freeze dried for chemical analysis.

Chemical analysis

The nitrogen content of the 20 cat food products was determined using a LECO analyser based on the Dumas method (AOAC, 1995).

Amino acid contents were determined using a method based on that reported by the AOAC (1995). Duplicate 5 mg diet and digesta samples were analysed using a Waters ion-exchange HPLC system, utilising postcolumn ninhydrin derivatisation and detection using absorbance at 570 nm and 440 nm (for proline), following hydrolysis in 6 M glass-distilled HCl containing 0.1% phenol for 24 h at 110 ± 2 ^{o}C in evacuated sealed tubes. Cysteine and tryptophan were not determined as they are destroyed during acid hydrolysis. The weight of each amino acid was calculated using free amino acid molecular weights.

Reactive lysine contents were determined in duplicate 5 mg cat food, test diet and digesta samples after incubation for 7 d in 0.6 M OMIU, pH 10.6 (pH 11.0 for the digesta samples), at 21 ± 2 ^{o}C in a shaking waterbath, with the reagent to lysine ratio being greater than 1000. The 0.6 M OMIU solution was prepared as described by Moughan at Rutherfurd, (1996). After incubation, the samples were dried using a Speedvac concentrator (Savant Instruments, Inc. Farmingdale, NY) and analysed for amino acids as described above.

Titanium was determined according to the method of Short et al. (1996). Essentially, samples were ashed before being digested in 60% (v/v) sulphuric acid. The mixture was then incubated with 30% hydrogen peroxide and absorbance read at 405 nm.

Data analysis

Ileal amino acid flows were calculated as follows (units are μg g^{-1} DM):

$$\text{Ileal amino acid flow } (\mu g\ g^{-1}\ DMI) = \text{Ileal amino acid content} \times \frac{\text{Diet titanium}}{\text{Ileal titanium}}$$

True ileal amino acid (AA) digestibility was calculated using the following equation (units are μg g^{-1} DMI):

True AA digestibility (%) =
(Dietary AA intake - (Ileal AA flow - Endogenous AA flow)) x 100
Dietary AA intake

Endogenous AA flows are based on endogenous amino acid flows determined in the growing rat as reported by Rutherfurd and Moughan (1998).

True ileal reactive lysine (RL) digestibility was calculated as follows (units are μg g^{-1} DMI):

True RL digestibility (%) =
(Dietary RL intake - (Ileal RL flow - Endogenous lysine flow)) x 100
Dietary RL intake

Reactive lysine was determined using the guanidination method. Endogenous lysine flow is the endogenous lysine flow as reported by Rutherfurd and Moughan (1998).

True ileal digestible reactive lysine content of the cat foods was calculated as follows (units are g kg^{-1}):

True ileal digestible RL content = RL content x True ileal RL digestibility (%)

True ileal digestible amino acid content of the cat foods was calculated as follows (units are g kg^{-1}):

True ileal digestible AA content = AA content x True ileal AA digestibility (%)

The amino acid digestibility data were subjected to a one-way analysis of variance for each cat food singly (GLM Procedure, SAS 1999).

Results

Crude protein and total and reactive lysine contents

The crude protein content of the 20 cat foods was determined and these data are presented in Table 1. For the moist cat foods, the crude protein content ranged from 34 to 58% with a mean value of 45%, whereas for the dry diets, the crude protein content ranged from 24 to 41% with a mean value of 33%. Overall the mean crude protein content was 39%.

The total and reactive lysine contents of the 20 cat foods were determined and are also shown in Table 1. Total lysine content ranged from 10 - 33 g kg^{-1}, whereas the range for the moist and dry cat foods was 12 - 33 g kg^{-1} and 10 - 20 g kg^{-1} respectively. On average, total lysine overestimated reactive lysine content by 86% across all diets, 99% across the moist diets and 73% across the dry diets.

Table 1.
Crude protein and total and reactive lysine contents (g kg^{-1} freeze dried matter) for 20 selected commercial cat foods.

Cat food	Crude protein [1]	Lysine		Overestimation[4]
	(%)	Total[2]	Reactive[3]	(%)
Moist				
1	42	30.9	13.6	127
2	45	27.4	13.5	103
3	43	20.8	12.0	73
4	58	33.3	18.3	82
5	46	31.3	14.3	119
6	47	33.0	12.6	162
7	45	28.3	16.2	75
8	34	12.3	7.5	64
9	45	28.6	12.9	122
10	46	31.0	18.8	65
Dry				
11	35	16.9	9.0	88
12	24	10.5	5.7	84
13	29	15.0	9.5	58
14	34	15.4	9.0	71
15	29	13.8	7.5	84
16	38	18.1	10.3	76
17	35	19.9	10.2	95
18	32	20.2	12.9	57
19	29	10.2	5.3	92
20	41	20.4	16.3	25

[1]Crude protein was calculated as total nitrogen multiplied by 6.25.
[2]Total lysine was determined using traditional amino acid analysis.
[3]Reactive lysine was determined using guanidination and subsequent homoarginine analysis.
[4]Overestimation (%) = $\frac{\text{Total lysine} - \text{Reactive lysine}}{\text{Reactive lysine}} \times 100$

True ileal total and reactive lysine digestibility

True ileal total lysine digestibility, based on conventional amino acid analysis of both diets and digesta, was determined for the 20 commercially available cat foods and compared to the true ileal reactive lysine digestibility, based on using the guanidination reaction followed by amino acid analysis to determine the reactive lysine contents in both diets and digesta and these results are shown in Table 2. The mean reactive lysine digestibility (lysine availability) across all cat foods was 91% but ranged from 80% for cat food 3 (moist cat food) to 98% for cat food 11 (dry cat food). When the moist and dry diets were examined separately, the lysine availability ranged from 80% to 97% for the moist cat foods, with a mean of 88%, and 90% to 98% for the dry foods, with a mean of 95%. For 16 of the 20 cat foods, true ileal reactive lysine digestibility was significantly ($P < 0.05$) higher than the true ileal total lysine digestibility determined using conventional amino acid analysis. For these cat foods, total lysine digestibility underestimated lysine digestibility by between 3.6% and 10% with the average degree of underestimation being 6.6%. For the moist and dry cat foods, total lysine digestibility underestimated lysine availability by 8% and 6% respectively.

True ileal digestible total and reactive lysine content

The true ileal digestible total lysine content was determined and compared with the true ileal digestible reactive lysine (available lysine) content of the 20 selected cat foods and these data are presented in Table 3. For all the cat foods tested, digestible total lysine content significantly ($P < 0.05$) overestimated digestible reactive lysine content (available lysine). This overestimation ranged from 41 to 143% (mean = 79%) for the moist food and 51 to 90% (mean = 66%) for the dry foods.

True ileal amino acid digestibility

True ileal amino acid digestibility values for the amino acids other than lysine for the 20 cat foods are given in Table 4. The overall true ileal amino acid digestibility across amino acids ranged from 71% for cat food 7 (moist food) to 93% for cat food 11 (dry food) with a mean overall digestibility of 84%. When the moist and dry cat foods were examined separately, the mean digestibility across amino acids ranged from 71% to 89% (mean = 79%) for the moist foods and 84% to 93% (mean = 89%) for the dry foods. The least digestible amino acids across all cat foods were aspartic acid and glycine (72%), and the most digestible amino acid was arginine (91%). For the moist diets, the least digestible amino acid was aspartic acid (64%) and the most digestible arginine (90%). For the dry diets, the least digestible amino acid was glycine (79%), and the most digestible were arginine and phenylalanine (93%).

True ileal digestible amino acid contents

The true ileal digestible amino acid contents of the 20 commercially available cat foods are presented in Table 5. There was variation in digestible amino acid content among the 20 cat foods, with a 1.8 - 3.1-fold range in digestible amino acid content across cat foods.

Table 2.
Mean (n=5) true ileal total and reactive lysine digestibility (%) for 20 selected commercial cat foods.

Cat food	Lysine Digestibility		Overall SE	Significance[3]
	Total[1]	Reactive[2]		
Moist				
1	85.3	87.8	1.6	NS
2	78.3	85.4	1.5	*
3	82.3	79.9	1.0	NS
4	82.2	86.3	1.3	*
5	79.9	88.7	0.7	**
6	81.9	88.5	0.9	**
7	78.7	86.4	1.7	***
8	83.9	93.5	1.5	***
9	87.3	87.7	1.0	NS
10	93.1	97.1	0.9	***
Dry				
11	92.9	97.7	0.9	**
12	85.0	93.1	1.0	***
13	88.9	92.2	0.8	*
14	87.1	94.7	0.8	***
15	91.9	95.9	1.2	**
16	92.0	96.8	0.9	***
17	87.8	89.9	1.3	NS
18	89.6	95.7	0.8	***
19	89.9	95.4	0.5	***
20	90.8	96.3	0.5	***

[1]Total lysine digestibility was determined using the true ileal amino acid digestibility assay in the rat using traditional amino acid analysis to determine the total lysine content of the diets and digesta.
[2]Reactive lysine digestibility was determined using the true ileal amino acid digestibility assay in the rat using guanidination and homoarginine analysis to determine the reactive lysine content of the diets and digesta.
*[3]NS not significant, P > 0.05; *, 0.05 > P > 0.01; **, 0.01 > P > 0.001; ***, P < 0.001.*

Table 3.
Mean (n=5) digestible total and reactive lysine (available lysine) contents (g kg^{-1} freeze dried matter) for 20 selected commercial cat foods.

Cat food	Digestible lysine		Overall SE	Significance[3]
	Total[1]	Reactive[2]		
Moist				
1	26.4	11.9	0.31	***
2	21.5	11.6	0.28	***
3	17.1	9.6	0.12	***
4	27.4	15.8	0.31	***
5	25.0	12.7	0.18	***
6	27.0	11.1	0.17	***
7	22.3	14.0	0.32	***
8	10.3	7.0	0.14	***
9	24.9	11.6	0.13	***
10	28.9	18.2	0.22	***
Dry				
11	15.7	8.8	0.11	***
12	8.9	5.3	0.07	***
13	13.4	8.8	0.09	***
14	13.4	8.5	0.09	***
15	12.7	7.2	0.12	***
16	16.6	10.0	0.11	***
17	17.5	9.2	0.18	***
18	18.1	12.4	0.12	***
19	9.1	5.1	0.04	***
20	18.5	15.7	0.08	***

[1]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 cat food also determined using traditional amino acid analysis.
[2]Digestible reactive lysine was calculated from the true ileal reactive lysine digestibility determined using the true ileal amino acid digestibility 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 cat food also determined using guanidination and amino acid analysis.
*[3] ***, $P < 0.001$.*

Table 4.
Mean (n=5) true ileal amino acid digestibility (%) for 20 selected commercial cat foods[1].

Amino acid	Moist cat food										
	1	2	3	4	5	6	7	8	9	10	
Aspartic acid	72	58	60	58	55	61	55	73	68	84	
Threonine	80	71	78	77	73	76	68	84	84	92	
Serine	80	71	80	76	75	76	67	85	83	91	
Glutamic acid	84	77	87	78	77	80	75	87	84	92	
Proline	82	76	85	72	74	76	70	82	83	90	
Glycine	70	60	68	59	59	61	56	72	75	83	
Alanine	85	76	85	79	80	81	77	82	86	92	
Valine	82	76	83	78	78	80	73	87	85	93	
Isoleucine	84	75	83	80	79	82	74	90	87	92	
Leucine	85	78	88	82	81	83	76	88	87	92	
Tyrosine	84	78	85	82	80	83	73	87	86	92	
Phenylalanine	86	78	88	83	82	84	76	91	88	92	
Histidine	76	65	77	72	72	75	66	83	80	69	
Arginine	93	88	91	91	89	89	84	88	92	95	
Amino acid	Dry cat food										Overall
	11	12	13	14	15	16	17	18	19	20	SE[2]
Aspartic acid	87	77	77	79	78	83	75	79	81	84	1.9
Threonine	94	85	86	88	90	90	81	89	90	90	1.4
Serine	94	86	85	89	88	92	81	89	91	91	1.5
Glutamic acid	94	91	90	90	93	94	84	89	95	92	1
Proline	93	87	86	86	89	92	86	87	92	89	1.3
Glycine	84	76	77	76	81	78	78	78	82	77	2.2
Alanine	96	88	88	88	90	92	88	89	91	91	1.1
Valine	95	88	88	89	91	92	87	90	93	92	1.2
Isoleucine	95	90	90	91	92	94	85	92	95	93	1.1
Leucine	91	90	90	92	93	95	84	92	94	94	1
Tyrosine	95	91	91	92	93	94	87	91	94	94	1.1
Phenylalanine	95	92	91	93	93	95	86	93	96	94	0.9
Histidine	90	84	84	85	89	88	79	86	90	88	1.5
Arginine	94	91	94	93	92	95	91	92	93	94	0.9

[1]Values were corrected for endogenous amino acid flow using the EHC method (Butts et al., 1991; Moughan et al., 1990) reported by Rutherfurd and Moughan (1998).
[2]Overall SE for both wet and dry cat foods.

Table 5.
Mean (n=5) true ileal digestible amino acid contents (g / 100 g of freeze-dried matter) for 20 selected commercial cat foods.

Amino acid	Moist cat food										
	1	2	3	4	5	6	7	8	9	10	
Aspartic acid	2.9	2.2	1.8	2.4	2.3	2.9	1.9	1.6	2.5	3.1	
Threonine	1.6	1.2	1.2	1.6	1.4	1.6	1.2	0.9	1.5	1.6	
Serine	1.6	1.3	1.5	1.7	1.6	1.8	1.2	1.5	1.5	1.6	
Glutamic acid	4.9	4.3	5.9	4.6	5.0	5.8	3.7	4.4	4.3	4.7	
Proline	2.4	2.1	2.7	2.7	2.2	2.6	1.7	2.5	1.8	2.0	
Glycine	2.5	2.0	2.2	2.7	1.9	2.5	1.7	2.1	2.5	2.8	
Alanine	2.6	2.0	1.9	2.8	2.4	2.8	2.1	1.5	2.3	2.5	
Valine	2.1	1.7	1.6	2.2	2.1	2.5	1.6	1.3	0.7	2.1	
Isoleucine	1.5	1.2	1.3	1.3	1.6	1.8	1.3	1.0	2.8	1.6	
Leucine	3.3	2.6	2.6	3.1	3.1	3.5	2.4	1.8	1.2	2.9	
Tyrosine	1.4	1.1	1.2	1.3	1.3	1.6	1.0	0.8	1.6	1.3	
Phenylalanine	1.9	1.5	1.6	1.9	1.9	2.1	1.3	1.2	0.1	1.7	
Histidine	1.2	0.9	0.8	1.1	1.1	1.2	1.0	0.5	2.5	0.1	
Arginine	3.1	2.7	2.4	3.3	3.1	3.5	2.4	2.1	2.9	2.8	
Amino acid	Dry cat food										Overall
	11	12	13	14	15	16	17	18	19	20	SE[1]
Aspartic acid	2.7	1.4	1.7	2.2	1.6	2.2	2.0	2.1	1.5	2.8	0.05
Threonine	1.2	0.7	0.8	1.0	0.9	1.2	1.1	1.1	0.9	1.3	0.02
Serine	1.5	0.8	0.9	1.3	1.2	1.6	1.1	1.2	1.4	1.7	0.02
Glutamic acid	5.8	4.0	4.1	4.8	4.9	7.2	3.3	3.9	6.5	6.1	0.05
Proline	2.5	1.7	2.0	2.2	2.2	3.1	1.8	1.8	2.8	2.7	0.03
Glycine	1.7	1.3	1.8	1.8	2.0	1.6	2.1	1.8	1.4	1.9	0.06
Alanine	2.4	1.3	1.5	1.8	1.5	2.5	1.8	2.0	1.4	2.5	0.03
Valine	1.6	1.0	1.1	1.3	1.2	1.7	1.6	1.4	1.3	1.7	0.02
Isoleucine	1.3	0.8	0.9	1.1	1.0	1.4	1.0	1.1	1.1	1.4	0.02
Leucine	3.3	1.8	1.7	2.5	2.0	3.8	2.1	2.3	2.1	3.6	0.03
Tyrosine	1.4	0.7	0.7	1.0	0.9	1.5	0.9	0.9	0.9	1.4	0.01
Phenylalanine	1.8	1.0	1.0	1.4	1.2	1.9	1.2	1.3	1.4	1.8	0.01
Histidine	0.8	0.5	0.6	0.7	0.6	0.8	0.8	0.8	0.6	0.9	0.01
Arginine	2.2	1.4	1.9	2.1	1.8	2.1	1.9	2.1	1.7	2.6	0.02

[1]*Overall SE for both wet and dry cat foods.*

Discussion

When foodstuffs are processed, lysine is susceptible to chemical modification through reaction with reducing sugars, fats and their oxidation products, polyphenols and some food additives (Hurrell and Carpenter, 1981). Modified lysine is generally nutritionally unavailable, and its presence leads to an overestimation of determined available lysine in foodstuffs when traditional techniques such as the true ileal amino acid digestibility assay are used (Batterham, 1990). Cat foods, both moist and dry, are processed during manufacture, to sterilise the diet, modify functionality, or increase palatability (Hendriks et al. 1999) and as such the lysine present is susceptible to modification. In this thesis, a new assay (true ileal digestible reactive lysine (available lysine) assay) was developed that accurately determines the available lysine (true ileal digestible reactive lysine) content of processed foods or feedstuffs (Moughan and Rutherfurd, 1996; Rutherfurd et al., 1997b). This assay has been applied to selected milk protein-based foods (Rutherfurd and Moughan, 2005) and breakfast cereals (Rutherfurd et al., 2006). In this study, the assay was applied to 20 commercially available cat foods.

True total and reactive lysine contents

In processed foods such as cat foods, a proportion of the lysine will have inevitably been chemically modified to form Maillard products (Moughan 2003; Hurrell and Carpenter, 1981) or cross linked in the form of collagen. Hendriks et al. (1999) found differences (13 - 21%) between total lysine and reactive lysine contents of a canned cat food that had been autoclaved at 121 °C for 80 - 120 min suggesting the presence of acid-labile lysine derivatives. In the present study, total lysine considerably overestimated (by 25 to 162%) the actual lysine content (reactive lysine) of all the cat foods tested. This would suggest considerable processing damage to lysine has occurred and/or the presence of high levels of collagen in the cat foods (Singh, 1991; Asghar and Henricksen, 1982).

True ileal total and reactive lysine digestibility

Overall, reactive lysine digestibility was high, but less than complete. It is interesting to observe that the overall reactive lysine digestibility was 7% units lower in the moist canned diets compared to the dry diets. This difference may result from variation in ingredient composition or differences in the processing methods for moist and dry cat foods. However, further conclusions cannot be drawn because we do not know the details of the manufacturers' processing conditions used to produce the cat foods tested in this study.

Conventional true ileal total lysine digestibility significantly underestimated lysine availability (true ileal reactive lysine digestibility) for most of the cat foods tested, but the mean underestimation (6.6%) was not large. For the dry cat foods, there were more diets for which total lysine digestibility underestimated lysine availability than for the moist foods, but the absolute degrees of underestimation were similar between moist and dry cat foods.

There have been only a few reports published describing the lysine bioavailability of cat foods. Hendriks et al. (1999) investigated the effects of processing on amino acid

digestibility using the rat as a model for the cat, but did not examine available lysine. Larsen et al. (2002) investigated the effect of processing on the lysine bioavailability of casein-based kitten diets. Using a kitten growth assay, they found that heat treatment of the diets resulted in a 41% decrease in the lysine bioavailability when compared to an unheated diet. Rutherfurd et al. (1997a) determined the true ileal total and reactive lysine digestibility of a meat and bone meal using an assay similar to that employed in this study. They found that the total and reactive lysine digestibilities of the meat and bone meal was 88% and 92%, respectively. These values were similar to those observed for several of the cat foods tested in the present study. This is not unexpected because both meat and bone meal and cat foods are largely derived from by-products of the meat industry.

True ileal digestible total and reactive lysine contents

True ileal digestible total lysine overestimated available lysine (true ileal digestible reactive lysine) content for all of the cat foods tested. The degree of overestimation was considerable, being greater than 38% and as much as 143%. There appears to be considerable lysine damage in the cat foods tested in the present study. Furthermore, the traditional assay for determining available lysine is inaccurate for processed cat foods. This is of concern given that commercial cat foods are often the only source of nutrition for many cats. For some of those cat foods tested in this study, lysine is likely to be limiting, leading to a surplus of the nonlimiting amino acids in the bodies of cats consuming these diets. Excess amino acids are excreted in the urine, placing increased load on the kidneys, and it is known that renal failure is a common cause of death for domestic cats.

True ileal amino acid digestibility

The true ileal digestibility of the acid-stable amino acids (apart from lysine) was also determined. Overall, the average amino acid digestibility across all amino acids tested (except lysine) was moderate (84%) but was similar to values reported for some processed meat and bone meals (Hendriks et al., 2002; Donkoh et al., 1994). Although meat and bone meal and cat foods are not strictly the same, they are both derived from meat industry by-products.

When the moist and dry diets were examined separately, the dry diets appeared to be considerably more digestible than the moist cat foods. This difference most likely reflects the different processing methods used to produce the two product types. The mean amino acid digestibility for the moist cat foods was similar to that of an unheated moist canned cat food tested by Hendriks et al. (1999) for which the mean amino acid digestibility was 78%. However, when the latter workers heated the diet for 120 min at 121 °C the digestibility decreased to 67%, which was similar to the least digestible (71%) cat food tested in this study. For the moist cat foods, aspartic acid was the least digestible amino acid. Hendriks et al. (1999) observed that for an unheated moist canned diet aspartic acid was not the least digestible amino acid but that once the can had been heat-treated, even for a short time, aspartic acid digestibility decreased markedly and became least digestible. Why heat treatment would reduce aspartic acid digestibility to a much greater extent that other amino acids is unknown, but it may be related to the amino acid sequence of the meat by-product proteins and the potential for aspartic acid placement in limit peptides.

Overall, it is likely that the type of processing method used and differences in the ingredient composition resulted in the differences in lysine damage and the varying amino acid digestibility observed here for the cat foods.

True ileal digestible amino acid content

Overall, there was a high degree of variation (2 - 3-fold difference) in the true ileal digestible amino acid content for each amino acid across the cat foods. This variation was most likely a result of the types of meat by-products used and inclusion rate of these into the cat foods. Overall, the amino acid pattern across cat foods was similar, with glutamic acid, leucine, and arginine being the amino acids in greatest amounts across all diets and histidine being the least abundant amino acid. A similar amino acid pattern across all cat foods is expected because a similar protein base (meat by-products) was most likely used for all the cat foods tested.

Conclusion

True ileal total lysine digestibility, based on conventional amino acid analysis of diets and digesta, is an inaccurate measure of lysine availability in processed protein sources (Batterham et al., 1990). The reactive lysine digestibility assay used in this study and based on the guanidination of lysine in both diets and digesta is an alternative accurate measure of lysine availability (Moughan and Rutherfurd, 1996). For most of the cat foods tested in this study, the traditional measure of true ileal digestible total lysine considerably overestimated available lysine. Consequently, the amino acid pattern relative to lysine in processed cat foods may be quite different from that assumed. This has nutritional and health implications for the domestic cat, and closer examination of the amino acid pattern in processed cat foods in relation to the amino acid requirement of the cat is warranted.

Literature cited

AOAC 1995. Official Methods of Analysis of AOAC International. 16th ed. AOAC, Gaithersburg, MD.

Asghar, A., Henrickson, R.L. 1982. Chemical, biochemical, functional, and nutritional characteristics of collagen in food systems. Adv. Food Res. 28, 231-272.

Batterham, E.S., Andersen, L.M., Baigent, R.D., Darnell, R.E., Taverner, M.R. 1990. A comparison of the availability and ileal digestibility of lysine in cottonseed and soya-bean meals for grower/finisher pigs. Brit. J. Nut. 64, 663-677.

Butts, C.A., James, K.A.C., Koolaard, J.P., Booth, C.L., Donaldson, H.E., Scott, M.F., Moughan, P.J. 2002. The effect of digesta sampling time and dietary protein source on ileal nitrogen digestibility for the growing rat. J. Sci. Food Agric. 82, 343-350.

Butts, C.A., Moughan, P.J., Smith, W.C. 1991. Endogenous amino acid flow at the terminal ileum of the rat determined under conditions of peptide alimentation. J. Sci. Food Agric. 55, 175-187.

Davey, C.L., Winger, R.J. 1979. The structure of skeletal muscle and meat toughness. In Fibrous Proteins: Scientific, Industrial and Medical Aspects. Pp. 97-133 [DAD Parry, and LK Creamer, editors]. London: Academic Press.

Donkoh, A., Moughan, P.J., Smith, W.C. 1994. The laboratory rat as a model animal for determining ileal amino acid digestibility in meat and bone meal for the growing pig. Anim. Feed Sci. Technol. 49, 57-71.

Hendriks, W.H., Butts, C.A., Thomas, D.V., James, K.A.C., Morel, P.C.H., Verstegen, M.W.A. 2002. Nutritional quality and variation of meat and bone meal. Asian-Aust. J. Anim. Sci. 15, 1507-1516.

Hendriks, W.H., Emmens, M.M.A., Trass, B., Pluske, J.R. 1999. Heat processing changes the protein quality of canned cat foods as measured with a rat bioassay. J. Anim. Sci. 77, 669-676.

Hurrell, R.F., Carpenter, K.J. 1981. The estimation of available lysine in foodstuffs after Maillard reactions. Prog. Food Nutr. Sci. 5, 159-176.

Larsen, J.A., Calvert, C., Rogers, Q.R. 2002. Processing of dietary casein decreases bioavailability of lysine in growing kittens. J. Nutr. 132, 1748S-1750S.

Mason, V.C., Just, A., Bech-Anderson, S. 1976. Bacterial activity in the hindgut of piglets. 2. It's influence on apparent digestibility of nitrogen and amino acids. Z. Tierphysiol. Tierernaehr. Futtermittelkd. 36, 310-324.

Moughan, P.J. 2003. Amino acid availability: Aspects of chemical and bioassay methodology. Nutr. Res. Rev. 16, 127-141.

Moughan, P.J., Darragh, A.J., Smith, W.C., Butts, C.A. 1990. Perchloric and trichloroacetic acids as precipitants of protein in endogenous ileal digesta from the rat. J. Sci. Food Agric. 52, 13-21.

Moughan, P.J., Rutherfurd, S.M. 1996. A new method for determining digestible reactive lysine in foods. J. Agric. Food Chem. 44, 2202-2209.

National Research Council. 1995. Nutrient requirement of the laboratory rat. In Nutrient Requirements of Laboratory Animals. 4th ed. Washington, DC: Natl. Acad. Press.

Rutherfurd, S.M., Moughan, P.J. 2005. Digestible reactive lysine in selected milk-based products. J. Dairy Sci. 88, 40-48.

Rutherfurd, S.M., Moughan. P.J. 1998. The digestible amino acid composition of several milk proteins: Application of a new bioassay. J. Dairy Sci. 81, 909-917.

Rutherfurd, S.M., Moughan, P.J., Morel, P.C.H. 1997b. Assessment of the true ileal digestibility of reactive lysine as a predictor of lysine uptake from the small intestine of the growing pig. J. Agric. Food Chem. 45, 4378-4383.

Rutherfurd, S.M., Moughan, P.J., van Osch, L. 1997a. Digestible reactive lysine in processed feedstuffs: Application of a new bioassay. J. Agric. Food Chem. 45, 1189-1194.

Rutherfurd, S.M., Torbatinejad, N.M., Moughan, P.J. 2006. Available (ileal digestible reactive) lysine in selected cereal-based food products. J. Agric. Food Chem. 54, 9453-9457.

SAS Institute Inc. SAS User's Guide: Statistics. Version 8.02. SAS Institute Inc, Gary, NC. 1999.

Short, F.J., Gorton, P., Wiseman, J., Boorman, K.N. 1996. Determination of titanium dioxide added as an inert marker in chicken digestibility studies. Anim. Feed Sci. Technol. 59, 215-221.

Singh, H. 1991. Modification of food proteins by covalent crosslinking. Trends Food Sci. Technol. 8, 196-200.

Woods, H.O. 1944. The surface area of the intestinal mucosa in the rat and in the cat. J. Anat. 78, 103-105.

Chapter 6

Effect of elevated temperature storage on the digestible reactive lysine content of unhydrolysed- and hydrolysed-lactose milk-based products

This chapter uses the true ileal digestible reactive lysine assay developed as part of this thesis to examine the effect of prolonged storage at elevated temperatures on the digestible reactive lysine (available lysine) content in a skim milk powder and a hydrolysed-lactose skim milk powder.

Effect of elevated temperature storage on the digestible reactive lysine content of unhydrolysed- and hydrolysed-lactose milk-based products

Shane M. Rutherfurd and Paul J. Moughan

Riddet Institute, Massey University, Palmerston North, New Zealand.

Reproduced with permission from the publishers from: Rutherfurd, S.M., Moughan, P.J. 2008. Effect of elevated temperature storage on the digestible reactive lysine content of unhydrolysed- and hydrolysed-lactose milk-based products. J. Dairy Sci. 91, 477-482.

Abstract

The study aimed to evaluate the effects of storage at elevated temperatures on reactive lysine content and true ileal reactive lysine digestibility in a skim milk powder and a hydrolysed-lactose skim milk powder. A validated bioassay based on guanidination of food and digesta samples was applied. Semisynthetic diets containing the milk powders as the sole sources of protein were formulated and fed to growing rats. Chromic oxide was included in each diet as an indigestible marker. Digesta were collected posteuthanasia and analysed along with the diets for reactive lysine (homoarginine), and true ileal reactive lysine digestibility was calculated after correction for endogenous lysine loss. For the skim milk powder, there was no decrease in reactive lysine digestibility (lysine availability) when the powder was stored at 30 °C and 35 °C for 18 mth. In contrast, when stored at 40 °C for 12 mth, a small but statistically significant ($P < 0.05$) decrease (6%) was observed. For the hydrolysed-lactose product, a 22% decrease in lysine availability was observed after storage at 35 °C for 18 mth and a 17% decrease was observed when stored at 40 °C for only 6 mth. Digestible reactive (available) lysine content decreased by more than 20% for the skim milk powder stored at 30 °C and 35 °C for 18 mth and 40% when stored at 40 °C for 12 mth. Furthermore, available lysine decreased in the hydrolysed-lactose skim milk powder by 41% when stored at 30 °C for 18 mth and 34% and 65% when stored at 35 °C and 40 °C respectively, for only 6 mth. Elevated temperatures and prolonged storage periods negatively impacted the available lysine contents of both milk powders. The decrease in available lysine content and lysine availability was greater for the hydrolysed-lactose skim milk powder compared with the normal skim milk powder, after prolonged storage at elevated temperatures.

Introduction

Lysine is often the first-limiting indispensable amino acid in diets for humans, underlining the nutritional importance of milk proteins, which are significant dietary sources of lysine. In milk products that undergo heat processing or during storage, however, the ε amino group of lysine can react with other compounds in milk, particularly milk sugars, to produce nutritionally unavailable derivatives (Moughan, 2003; Hurrell and Carpenter, 1981). Some of these derivatives are acid-labile and revert back to lysine during the acid hydrolysis step of conventional amino acid analysis, leading to inaccuracy in the determination of the lysine and digestible lysine contents of processed milk products (Rutherfurd and Moughan, 2005). There are a number of methods that can be used to accurately determine the chemically reactive lysine content of processed protein sources, including the FDNB, TNBS and guanidination methods (Hurrell and Carpenter, 1974). However, these techniques do not take into account the incomplete absorption from the small intestine that can occur with a damaged protein. A method has been developed (Moughan and Rutherfurd, 1996) that accurately determines digestible reactive (available) lysine in processed foods (Rutherfurd et al., 1997). The assay utilises the guanidination reaction, which is specific to the ε-amino group of lysine (Zhang et al., 2006; Catrein et al., 2005; Yamaguchi et al., 2005; Kassell and Chow, 1966; Klee and Richards, 1957), to determine the amounts of reactive lysine in both diet and digesta of an animal fed that test diet. Reactive lysine digestibility is then calculated as the difference between dietary reactive lysine intake and ileal reactive lysine output. The assay has been applied to selected milk-based products (Rutherfurd and Moughan, 2005) to a variably heated skim milk powder (Rutherfurd and Moughan, 1997), to a range of cereal-

based breakfast foods (Rutherfurd et al., 2006). The aim of the present study was to apply the assay to determine the effect of long-term storage at elevated temperatures (30 to 40 oC) on digestible reactive lysine content in an unhydrolysed and hydrolysed-lactose skim milk powder. The study sought to determine the degree of protein damage and indicate shelf life for products stored under temperatures encountered in warmer world climates.

Materials and methods

Milk protein-based products

Two milk-based products were obtained locally; these included a commercial skim milk powder and a commercial hydrolysed-lactose skim milk powder. The protein content of the milk powders was determined on a LECO analyser using the Dumas method (IDF, 1993), and crude protein was calculated as the total nitrogen content multiplied by 6.38. The skim milk powder and the hydrolysed-lactose skim milk powder contained 37.5% and 38.2% protein, respectively.

Storage conditions

The skim milk powder and the corresponding hydrolysed-lactose skim milk powder were stored in a temperature-controlled room at 30 °C, 35 °C and 40 °C in 500 g lots in vacuum sealed sample bags for up to 18 mth. The storage regimen is shown in Table 1.

Table 1.
Conditions used to test the effect of storage duration and air temperature on digestible reactive lysine contents in a skim milk powder and a hydrolysed-lactose skim milk powder.

Storage time (mth)	Storage temperature (°C)					
	Skim milk powder			Hydrolysed-lactose skim milk powder		
	30	35	40	30	35	40
0	X[1]	X	X	X	X	X
1.5			X			X
3	X	X	X	X	X	X
4.5						X
6	X	X	X	X	X	X
9	X	X	X	X	X	
12	X	X	X	X	X	
15					X	
18	X	X		X	X	

[1]*An X indicates that a sample of either skim milk powder or hydrolysed-lactose skim milk powder underwent storage at the corresponding time and temperature*

Digestibility study

Ethics approval for the animal trial was obtained from the Animal Ethics Committee, Massey University, Palmerston North, New Zealand. One hundred and sixty entire male Sprague-Dawley rats, of approximately 150 g body weight, were obtained from the Small Animal Production Unit (Massey University, Palmerston North, New Zealand). The rats were housed individually in stainless steel wire-bottomed cages in a room maintained at 22 ± 2 °C, with a 12 h light/dark cycle. Thirty two semisynthetic wheat starch-based test diets were formulated to each contain 100 g kg^{-1} crude protein. The ingredient composition of the experimental diets is shown in Table 2. Chromic oxide was included (0.5%) in each diet as an indigestible marker. All diets met the nutritional requirements of the growing rat with the exception of protein (National Research Council, 1995). The animals were randomly allocated to the dietary treatments such that there were 5 animals per treatment and they were fed their respective diets for a 14 d experimental period. On each day, each rat received its diet as nine meals given hourly (0830 h to 1630 h); each meal time consisted of a 10 min period in which the rats had unrestricted access to their diet. Water was available at all times. On the final day of the study, from 5.5 to 7 h after the start of feeding, the rats were asphyxiated using carbon dioxide gas and then decapitated. The 20 cm of ileum immediately anterior to the ileo-caecal junction was dissected out, carefully washed with distilled deionised water and dried on absorbent paper. The digesta were gently flushed from the ileum section with distilled deionised water and freeze dried ready for chemical analysis.

Table 2.
Ingredient compositions[1] (g kg^{-1} air dry weight) of the experimental diets.

	Skim milk powder[2]	Hydrolysed-lactose skim milk powder[3]
Wheat starch	433	428
Soyabean oil	50	50
Cellulose	50	50
Sucrose	100	100
Vitamin premix[4]	50	50
Mineral premix[4]	50	50
Skim milk powder	262	-
Hydrolysed-lactose milk powder	-	267
Chromic oxide	5	5

[1]All diets were formulated to contain equal crude protein contents.
[2]All the skim milk powder-based diets for samples stored at the varying times and temperatures were prepared using this formulation.
[3]All the hydrolysed-lactose skim milk powder-based diets for samples stored at the varying times and temperatures were prepared using this formulation.
[4]Vitamin-mineral mix was formulated to meet the requirements of the growing rat for vitamins and minerals in the final diets (National Research Council, 1995).

Chemical analysis

Reactive lysine contents were determined in duplicate 5 mg milk powder and digesta samples and quadruplicate 5 mg diet samples after incubation for 1, 7 and 7 d, respectively in 0.6 M OMIU, pH 10.6 (pH 11.0 for the digesta samples), at 21 ± 2 °C in a shaking waterbath, with the OMIU to lysine ratio being greater than 1000 according to the procedure of Moughan and Rutherfurd (1996). The 0.6 M OMIU solution was prepared as described by Moughan and Rutherfurd (1996). After incubation, the samples were dehydrated using a Speedvac concentrator (Savant Instruments, Inc. Farmingdale, NY) and analysed for homoarginine using a Waters ion-exchange HPLC system, utilising postcolumn ninhydrin derivatisation and detection using absorbance at 570 nm, following hydrolysis in 6 M glass distilled HCl containing 0.1% phenol for 24 h at 110 ± 2 °C in evacuated sealed tubes. The weight of reactive lysine was calculated from the determined molar quantity of homoarginine and the molecular weight for free lysine.

The chromium contents of the diet and ileal digesta samples were determined in duplicate on a GBC 902 AA absorption-emission spectrophotometer (GBC Scientific NZ Ltd. Auckland, New Zealand) following the method of Costigan and Ellis (1987).

Data analysis

Ileal reactive lysine (RL) flows at the terminal ileum were calculated using the following equation (units are μg g^{-1} DM):

$$\text{Ileal RL flow } (\mu g\ g^{-1}\ \text{DMI}) = \text{Ileal RL content} \times \frac{\text{Diet chromium}}{\text{Ileal chromium}}$$

True ileal reactive lysine (RL) digestibility was calculated as described below (units are μg g^{-1} DMI):

$$\text{True RL digestibility } (\%) = \frac{(\text{Dietary RL intake} - (\text{Ileal RL flow} - \text{Endogenous lysine flow}))}{\text{Dietary RL intake}} \times 100$$

The endogenous lysine flow was based on the endogenous amino acid flows reported by Rutherfurd and Moughan (1998).

The reactive lysine digestibility and digestible reactive lysine data were analysed, using an analysis of covariance. The covariate was fitted as an exponential form. Temperature and milk powder type were fixed factors and time was the covariate. Fixed factor effects were incorporated into the model by means of the dummy variable technique (Zar, 1984). SigmaPlot (Jandel Scientific, San Raphael, CA) software was used for model fitting.

Results

The effect of storage on the reactive lysine content of a skim milk powder and a hydrolysed-lactose skim milk powder

The two milk powders (skim milk powder and hydrolysed-lactose skim milk powder) were stored at 30 °C, 35 °C and 40 °C from 0 and 18 mth. The reactive lysine content of the milk powders was determined periodically throughout this storage period, and these results are shown in Table 3. For the skim milk powder that was stored at 30 °C and 35 °C, the reactive lysine content decreased linearly (R^2 = 0.93 and 0.94 for the skim milk powder stored at 30 °C and 35 °C respectively) from 33 g kg^{-1} initially to approximately 26 g kg^{-1} after 18 mth of storage. The reactive lysine content of the skim milk powder stored at 40 °C decreased linearly (R^2 = 0.94) from 33 g kg^{-1} to 21 g kg^{-1} after a 12 mth storage.

Table 3.
Mean (± SE) reactive lysine content[1] (g kg^{-1} air dry weight) for a skim milk powder and a hydrolysed-lactose skim milk powder stored at 30 °C, 35 °C and 40 °C for varying time periods.

Storage period (mth)	Storage temperature (°C)					
	Skim milk powder			Hydrolysed-lactose skim milk powder		
	30	35	40	30	35	40
0	33.0 (0.06)	33.0 (0.06)	33.0 (0.06)	28.0 (1.8)	28.0 (1.8)	28.0 (1.8)
1.5	-	-	28.3 (1.18)	-	-	19.6 (0.30)
3	31.3 (1.38)	31.3 (2.14)	28.1 (0.70)	27.0 (0.10)	22.7 (0.82)	16.8 (1.05)
4.5	-	-	-	-	-	13.3 (1.06)
6	29.8 (0.01)	30.1 (0.13)	26.1 (0.07)	24.0 (0.04)	18.9 (0.65)	11.7 (0.40)
9	29.7 (0.19)	28.8 (0.14)	22.1 (5.03)	22.4 (0.10)	18.2 (0.08)	-
12	26.4 (1.99)	25.7 (0.21)	21.0 (0.10)	21.2 (0.43)	17.0 (0.34)	-
15	-	-	-	-	15.9 (0.10)	-
18	26.1 (1.47)	25.6 (0.15)	-	17.9 (0.64)	12.6 (0.00)	-

[1]*Mean values based on duplicate determinations.*

For the hydrolysed-lactose skim milk powder that was stored at 30 °C, the reactive lysine content decreased linearly (R^2 = 0.99) from 28 g kg^{-1} initially to 18 g kg^{-1} after 18 mth of storage, whereas over the same storage period the reactive lysine content in the hydrolysed-lactose milk powder stored at 35 °C decreased linearly (R^2 = 0.90) from 28 g kg^{-1} to 13 g kg^{-1}. When this hydrolysed-lactose milk powder was stored at 40 °C, the reactive lysine content decreased linearly (R^2 = 0.91) from 28 g kg^{-1} to 12 g kg^{-1} after a storage period of only 6 mth.

True ileal reactive lysine digestibility of a skim milk powder and a hydrolysed-lactose skim milk powder before and after storage

The determined mean true ileal reactive lysine digestibilities of the two products determined before and after storage are shown in Table 4. The reactive lysine digestibility of both the skim milk powder and hydrolysed-lactose skim milk powder before storage was 100%. There was a statistically significant effect of time and temperature on the true ileal reactive lysine digestibility ($P < 0.001$ and 0.05 respectively), in which digestibility decreased with increasing storage temperature and storage time. There was a significant ($P < 0.001$) interaction between milk powder type and time ($P < 0.001$) and temperature ($P < 0.001$) in which digestibility decreased to a greater degree in the hydrolysed-lactose skim milk powder than the normal skim milk powder as storage temperature and time increased. There was also a significant ($P < 0.001$) interaction between time and temperature.

Table 4.
Mean (n=5) true ileal reactive lysine digestibility[1] (%) for a skim milk powder and a hydrolysed-lactose skim milk powder stored at 30 °C, 35 °C and 40 °C for varying times.

Storage Period	Storage temperature (°C)					
	Skim milk powder			Hydrolysed-lactose skim milk powder		
(mth)	30	35	40	30	35	40
0	100	100	100	100	100	100
1.5	-	-	99	-	-	94
3	100	100	99	98	98	93
4.5	-	-	-	-	-	96
6	100	100	99	99	98	83
9	101	100	98	98	95	-
12	101	100	94	94	85	-
15	-	-	-	-	85	-
18	101	98	-	94	78	-

[1]Reactive lysine digestibility was determined using a true ileal amino acid digestibility assay (rat). The guanidination reaction was used to quantitate reactive lysine in the diets and digesta.

True ileal digestible reactive (available) lysine content of a skim milk powder and a hydrolysed-lactose skim milk powder before and after storage

True ileal digestible reactive lysine contents for the skim milk powder and the hydrolysed-lactose skim milk powder stored at elevated temperatures for up to 18 mth were

determined (Table 5). There was a 20% and 23% ($P < 0.001$) decrease in digestible reactive lysine content for the skim milk powder stored for 18 mth at 30 °C and 35 °C, respectively, whereas for the skim milk powder stored at 40 °C for 12 mth, there was a 40% decrease ($P < 0.001$) in digestible reactive lysine content. For the hydrolysed-lactose skim milk powder stored for 18 mth at 30 °C and 35 °C there was a 41% and 65% ($P < 0.001$) reduction in the digestible reactive lysine content, respectively. After only 6 mth of storage at 40 °C, the digestible reactive lysine content of the hydrolysed-lactose skim milk powder was also decreased by 65%. The hydrolysed-lactose skim milk powder suffered greater digestible reactive lysine loss with increasing storage time and temperature compared with the normal skim milk powder.

Table 5.
Mean (± SE, n=5) digestible reactive lysine contents[1] (g kg^{-1} air dry weight) for a skim milk powder and a hydrolysed-lactose skim milk powder stored at 30 °C, 35 °C and 40 °C for varying times.

Storage Period (mth)	Storage temperature (°C)					
	Skim milk powder			Hydrolysed-lactose skim milk powder		
	30	35	40	30	35	40
0	32.9 (0.05)	32.9 (0.05)	32.9 (0.05)	28.1 (0.03)	28.1 (0.03)	28.1 (0.03)
1.5	-	-	28.0 (0.08)	-	-	18.4 (0.13)
3	31.2 (0.06)	31.3 (0.07)	27.9 (0.12)	26.6 (0.11)	22.3 (0.10)	15.5 (0.12)
4.5	-	-		-	-	12.7 (0.23)
6	29.8 (0.12)	30.3 (0.07)	25.9 (0.39)	23.9 (0.27)	18.5 (0.21)	9.7 (0.58)
9	29.9 (0.01)	28.9 (0.17)	21.5 (0.12)	22.0 (0.32)	17.3 (0.23)	-
12	26.5 (0.10)	25.7 (0.21)	19.8 (0.45)	19.8 (0.19)	14.5 (0.37)	-
15	-	-	-	-	13.5 (0.36)	-
18	26.3 (0.15)	25.2 (0.26)	-	16.7 (0.23)	9.9 (0.35)	-

[1]Digestible reactive lysine was calculated from true ileal reactive lysine digestibility (rat, guanidination analysis) and the reactive lysine content of the respective milk powder which was also determined using guanidination.

Discussion

Lysine is a nutritionally important amino acid, and because of this, the accurate assessment of its content and availability in foods is important. Lysine can be chemically modified during the processing or storage of foods to form nutritionally unavailable derivatives (e.g. Maillard products). Because some of these products can interfere with the chemical analysis of lysine, traditional methods for determining available lysine may not always be accurate. Recently, a new assay methodology for accurately determining ileal digestible reactive lysine (available lysine) has been developed (Moughan and Rutherfurd, 1996). This assay was used here to determine the available lysine content of a skim milk powder and a hydrolysed-lactose skim milk powder after long-term storage at elevated temperatures.

The effect of storage on the reactive lysine content of a skim milk powder and a hydrolysed-lactose skim milk powder

For the skim milk powder, the reactive lysine content decreased by approximately 22% when stored at 30 °C and 35 °C for 18 mth, whereas during storage at 40 °C, the reactive lysine content decreased by 36% in only 12 mth. For the hydrolysed-lactose skim milk powder, the reduction in reactive lysine content during storage at elevated temperatures was greater, with decreases of 36% after 18 mth storage at 30 °C, 55% after 18 mth storage at 35 °C, and 58% after 6 mth storage at 40 °C. In all cases, there was a considerable reduction in reactive lysine content after storage, suggesting extensive lysine damage had occurred to both milk powders after storage at elevated temperatures. Furthermore, and as expected, the reduction in lysine was greater in the milk powders stored at the higher temperatures. It is also interesting that the loss of lysine over time was very nearly linear for both products over all the temperatures tested.

It is of note that the lysine content decreased to a much greater extent and more rapidly in the hydrolysed-lactose skim milk powder than in the normal skim milk powder. The reducing sugar content of the hydrolysed-lactose skim milk powder is twice that of the skim milk powder, because lactose (a reducing sugar) is hydrolysed to glucose and galactose which are in turn both reducing sugars. It is likely that the higher concentration of reducing sugars in the hydrolysed-lactose product compared with its unhydrolysed counterpart explains the difference in lysine damage observed for the two milk powders.

True ileal reactive lysine digestibility of a skim milk powder and a hydrolysed-lactose skim milk powder before and after storage

The reactive lysine in the fresh skim milk powder was completely digested. In Chapter 2 the reactive lysine in skim milk powder was also highly digestible (98.8%). For the skim milk powder stored at 30 °C and 35 °C, there was no reduction in reactive lysine digestibility over the 18-mth storage period. When stored at 40 °C, the reactive lysine digestibility did decrease after 12 mth of storage but the decrease was not large (6%). In contrast, a similar small decrease (6%) in reactive lysine digestibility was observed for the hydrolysed-lactose skim milk powder when stored for 12 mth at only 30 °C. When stored at 35 °C, a similar (5%) decrease was observed after only 9 mth, and after 18 mth of storage, reactive lysine digestibility had decreased by 22%. When stored at 40 °C, digestibility had dropped markedly (a 17% decrease) after only 6 mth.

Clearly, when skim milk powder underwent prolonged storage at elevated temperatures, lysine availability decreased, but this reduction was not large. In contrast, for a skim milk powder that had undergone lactose hydrolysis before storage, the decrease in lysine availability was large. The lactose hydrolysis process clearly had a major effect on the digestibility of reactive lysine when the products were stored for long periods of time at elevated temperatures, most likely an effect of greater lysine damage due to the higher concentration of reducing sugars in the hydrolysed products. The decrease in reactive lysine digestibility may be brought about by a reduction in the effectiveness of trypsin in hydrolysing the peptide bonds in proximity to the modified lysine residues. This would then

further hinder digestion by other intestinal proteases such as chymotrypsin, resulting in the presence of indigestible limit peptides (Moughan et al., 1996). These limit peptides may contain any of the amino acids present in proteins and will therefore result in a reduction in the digestibility of all amino acids, not just lysine (Moughan and Rutherfurd, 1996).

True ileal digestible reactive (available) lysine content of a skim milk powder and a hydrolysed-lactose skim milk powder before and after storage

For the skim milk powder stored at 30 °C and 35 °C, the reduction in the true ileal digestible reactive lysine content after storage for 18 mth was large and was similar to the reduction observed for the gross reactive lysine content of the skim milk powder, because digestibility was essentially complete for these samples. When the skim milk powder was stored at 40 °C, the reduction in digestible reactive lysine was almost double that observed after storage at the lower temperatures after only two-thirds the storage time. For the hydrolysed-lactose skim milk powder, the reduction in digestible reactive lysine content was very large even at the lower storage temperatures (30 °C and 35 °C), whereas for the samples stored at 40 °C well over half of the digestible reactive lysine was destroyed after only 6 mth of storage.

Clearly, temperature plays a significant role in the stability of lysine in milk powders. Digestible reactive lysine content was 34% higher when the powders were stored at 30 °C compared to storage at 40 °C for the same length of time (12 mth). For the hydrolysed-lactose product, this difference was even more dramatic with digestible reactive lysine content, being as much as 146% higher in the product stored for 6 mth at 30 °C compared with the same time at 40 °C. Other studies have shown similar effects, van Barneveld et al. (1994) found a 73% decrease in the available lysine content of field peas heated from 110 °C to 165 °C when determined using an animal growth assay.

Overall, skim milk powder is a highly digestible, high quality protein source. However, there was a sizable loss of reactive lysine and digestible reactive lysine in the skim milk powder when stored at elevated temperatures for prolonged periods. Again, the effect was much more dramatic for the hydrolysed-lactose product. The study demonstrates that at elevated temperatures, such as those experienced in warmer world climates, considerable lysine damage can occur in relatively short periods of time (6 mth) and that these may be high enough to warrant concern from a nutritional viewpoint. The study also demonstrates the utility of the digestible reactive lysine (available lysine) assay (Moughan and Rutherfurd, 1996) in predicting the effects of storage on protein quality and evaluating product shelf-life.

Literature cited

Catrein, I., Herrmann, R., Bosserhoff, A., Ruppert, T. 2005. Experimental proof for a signal peptidase 1 like activity in Mycoplasma pneumoniae, but absence of a gene encoding a conserved bacterial type 1 SPase. FEBS J. 272, 2892-2900.

Costigan, P., Ellis, K.J. 1987. Analysis of faecal chromium derived from controlled release marker devices. N.Z. J. Tech. 3, 89-92.

Hurrell, R.F., Carpenter, K.J. 1981. The estimation of available lysine in foodstuffs after Maillard reactions. Prog. Food Nutr. Sci. 5, 159-176.

Hurrell, R.F., Carpenter, K.J. 1974. Mechanisms of heat damage in proteins 4. The reactive lysine content of heat-damaged material as measured in different ways. Brit. J. Nutr. 32, 589-604.

International Dairy Federation. 1993. Milk determination of nitrogen content. FIL-IDF Standard no. 20B. IDF, Brussels, Belgium.

Kassell, B., Chow, R.B. 1966. Modification of the basic trypsin inhibitor of bovine pancreas. The ε-amino groups of lysine and the amino terminal sequence. Biochem. 5, 3449-3453.

Klee, W.A., Richards, F.M. 1957. The reaction of O-methylisourea with bovine pancreatic ribonuclease. J. Biol. Chem. 229, 489-504.

Moughan, P.J. 2003. Amino acid availability: Aspects of chemical and bioassay methodology. Nutr. Res. Rev. 16, 127-141.

Moughan, P.J., Gall, M.P.J., Rutherfurd, S.M. 1996. Absorption of lysine and deoxyketosyllysine in an early-Maillard browned casein by the growing pig. J. Agric. Food Chem. 44, 1520-1525.

Moughan, P.J., Rutherfurd, S.M. 1996. A new method for determining digestible reactive lysine in foods. J. Agric. Food Chem. 44, 2202-2209.

National Research Council. 1995. Nutrient requirement of the laboratory rat. In Nutrient Requirements of Laboratory Animals. 4th ed. Washington, DC: Natl. Acad. Press.

Rutherfurd, S.M., Moughan, P.J. 2005. Digestible reactive lysine in selected milk-based products. J. Dairy Sci. 88, 40-48.

Rutherfurd, S.M., Moughan, P.J. 1998. The digestible amino acid composition of several milk proteins: Application of a new assay. J. Dairy Sci. 81, 909-917.

Rutherfurd, S. M. and Moughan, P. J. 1997. Application of a new method for determining digestible reactive lysine to variably heated protein sources. J. Agric. Food Chem. 45, 1582-1586.

Rutherfurd, S.M., Moughan, P.J., van Osch, L. 1997. Digestible reactive lysine in processed feedstuffs: Application of a new bioassay. J. Agric. Food Chem. 45, 1189-1194.

Rutherfurd, S.M., Torbatinejad, N.M., Moughan, P.J. 2006. Available (ileal digestible reactive) lysine in selected cereal-based food products. J. Agric. Food Chem. 54, 9453-9457.

van Barneveld, R.J., Batterham, E.S., Norton, B.W. 1994. The effect of heat on amino acids for growing pigs. 3. The availability of lysine from heat-treated field peas (Pisum sativum cultivar Dundale) determined using the slope-ratio assay. Brit. J. Nutr. 72, 257-275.

Yamaguchi, M., Nakazawa, T., Kuyama, H., Obama, T., Ando, E., Okamura, T-A., Ueyama, N., Norioka, S. 2005. High-throughput method for N-terminal sequencing of proteins by MALDI mass spectrometry. Anal. Chem. 77, 645-651.

Zar, J.H. 1984. Biostatistical Analysis (2nd Ed). Pp 346-347. Englewood Cliffs, NJ: Prentice-Hall.

Zhang, H.L., Li, D.F., Qiao, S.Y., Wang, F.L., Chen, X.J., Thacker, P.A. 2006. The effect of dietary homoarginine derived from guanidination of synthetic lysine on endogenous amino acid loss and apparent and true ileal amino acid digestibility in the pig. Anim. Sci. 82, 23-30.

Chapter 7

General discussion

Parts of this chapter have been published in Rutherfurd, S.M., Moughan, P.J. 2007. Development of a novel bioassay for determining the available lysine contents of foods and feedstuffs. Nutr. Res. Rev. 20, 3-16 and are reproduced with permission.

Introduction

Lysine is an important amino acid in the nutrition of monogastric animals and humans and is often the first limiting dietary indispensible amino acid in pig and poultry diets. The inefficient formulation of diets for pigs and poultry has a direct effect on production costs and in turn feed formulation is dependent on the accurate assessment of the amino acid content of the respective ingredients. Furthermore, the prediction of body protein gain for intensive livestock such as pigs and poultry, which is based on the determined available nutrient content of formulated diets, is far from perfect and while there may be a number of reasons for this, the inaccuracy of traditional methods for determining available lysine is a contributing factor. For humans receiving western type diets which tend to be high in meat and milk products, lysine may be adequate, but lysine may be limiting for those people receiving cereal-based diets. In addition, many of our foods are processed, during which lysine is chemically modified leading to lower, and possibly inadequate, dietary intakes of unmodified lysine. Having accurate information about the lysine content in foods for humans is important to ensure diets are balanced and meet the nutritional requirements. For companion animals, which generally consume manufactured diets for their entire lives, the protein quality of those diets is particularly critical. Diets for which lysine has been modified during processing may not only contain AGE's, which if absorbed may impact on the health of the animal, but can also have an inappropriate dietary amino acid balance, especially for growth.

A number of assays have been reported to be suitable for determining available lysine in processed foods and feedstuffs including the indicator amino acid oxidation technique (Moehn et al., 2005) and a range of growth-based assays including the protein efficiency ratio, net protein utilisation, biological value (Bodwell, 1977) and slope-ratio assay (Batterham et al., 1979). However, these assays determine protein or amino acid utilisation rather than availability. Moreover, utilisation is a function of the animal rather than just the food protein, and so does not adequately describe the available lysine (the lysine that is digested and absorbed by an animal or human in a form capable of being used for protein synthesis) content of a food or feedstuff. Finally, assays that determine utilisation are laborious and expensive to perform and only one amino acid can be examined at a time.

While determining the reactive lysine content of foods and feedstuffs is important, Moughan et al. (1996) demonstrated that not all the reactive lysine present in a processed food or feedstuff is absorbed from the small intestine of the growing pig. They suggested that the presence of modified lysine derivatives in heat processed food proteins reduced the effectiveness of digestive enzymes, leading to the formation of unabsorbed limit peptides. Other workers have also shown that the amino acid digestibility and absorption, in general, is often far from complete in processed proteins (van Barneveld et al., 1994a; Wiseman et al., 1991; Batterham et al., 1990). Consequently, chemically reactive lysine is not an accurate predictor of bioavailable lysine and the digestibility of reactive lysine is required in order to derive digestible reactive lysine (available lysine).

The true ileal amino acid digestibility assay is suitable for determining the bioavailable amino acid content of unprocessed foods and feedstuffs (Moughan, 2003). However, Batterham et al. (1990) reported that true ileal amino acid digestibility overestimated the availability of several amino acids, including lysine, in a number of processed feedstuffs when compared

to the available amino acid content determined using the slope-ratio assay. The inaccuracy of the ileal digestibility assay for processed foods and feedstuffs is a consequence of using conventional amino acid analysis to determine the total lysine content of diets and digesta when determining true ileal amino acid digestibility. In contrast, digestible reactive lysine, at least from a theoretical standpoint, would accurately determine the available lysine content in processed foods and feedstuffs since the unmodified (reactive) lysine content, rather than the total lysine content, of the diets and digesta would be determined. Chapter 2 describes the validation and application of the available lysine assay developed by Moughan and Rutherfurd (1996) which greatly improved the accuracy of the available lysine determination for processed protein sources. This assay has been applied to a range of processed foods and feedstuffs including milk protein-based products (Rutherfurd and Moughan, 2005) presented in Chapter 3, "ready-to-eat" cereal-based breakfast foods (Rutherfurd et al., 2006) presented in Chapter 4 and "complete and balanced" cat foods (Rutherfurd et al., 2007) presented in Chapter 5. In addition, the assay was used to evaluate the effect of long-term storage of two milk protein powders at elevated temperatures (Rutherfurd and Moughan, 2007) presented in Chapter 6.

Development and validation of the true ileal digestible reactive (available) lysine assay

The development of a new assay for determining available lysine. The assay is based around the guanidination reaction (the reaction of OMIU with lysine to form homoarginine) and the true ileal amino acid digestibility assay. The reactive lysine contents of the diets and digesta were then determined using the guanidination reaction. The determined dietary reactive lysine content and the content of undigested reactive lysine at the terminal ileum were used to calculate apparent ileal reactive lysine digestibility. This apparent digestibility coefficient was corrected to a true value by correcting for endogenous ileal lysine flow determined using the EHC technique (Butts et al., 1991; Moughan et al., 1990). The true ileal digestible reactive lysine content of the protein source was then derived by multiplying the true ileal reactive lysine digestibility by the reactive lysine content of the protein source. Digestible reactive lysine is by definition the available or bioavailable lysine and describes the amount of unmodified lysine in a food or feedstuff that is digested and absorbed from the small intestine of the human or animal consuming the food or feedstuff.

The optimal reaction time required to obtain either complete guanidination of unheated casein or maximal guanidination of a heated lactose/casein mixture (due to the presence of reverted lysine after acid hydrolysis of processed protein sources complete guanidination cannot be tested directly) was determined. The optimal pH of the reaction mixture and optimal reaction time required to achieve maximal guanidination of digesta from rats fed either the unheated casein or the heated lactose/casein was also determined. The optimised guanidination reaction was then coupled with the true ileal digestibility assay to determine the true ileal reactive lysine digestibility of the heated lactose/casein. It was found that true ileal total lysine digestibility significantly underestimated lysine availability compared to the true ileal reactive lysine digestibility assay for the heated lactose/casein.

The accuracy of the new assay is dependent on the complete conversion of lysine to homoarginine in diets and digesta during the guanidination reaction and it is possible that

lysinoalanine formation, which also occurs at high pH, may compete with the guanidination reaction for reactive lysine. However, Karayiannis et al. (1979) reported that for casein only 2.5% of the lysine present underwent lysinoalanine formation after a 6 h incubation at high pH, yet as much as 95% of lysine is guanidinated to homoarginine in soluble proteins in the first 8 h of incubation (Rutherfurd and Moughan, unpublished data). Consequently, the amount of lysinoalanine formation during guanidination is likely to be negligible, particularly for soluble proteins. Despite this, it is good practice to guanidinate a pure unheated protein as an external standard along side the guanidination of samples to give confidence that the conversion of lysine to homoarginine is complete.

Racemisation is another effect of processing, particularly at high pH, leading to the formation of D-amino acids. Liardon and Hurrell (1983) have reviewed the nutritional availability of D-amino acids and reported that many D-amino acids, including D-lysine, are not nutritionally available. As the assay currently stands, it is likely that D-lysine will react with OMIU to the same degree and at a similar rate as L-lysine yet the chromatographic separation described in this thesis cannot distinguish between D- and L-homoarginine. Consequently, the assay may overestimate available lysine if significant amounts of D-lysine are present. For most processing conditions however, racemisation is negligible (Liardon and Hurrell, 1983) and for those few processed protein sources that may contain D-lysine, such as alkali-treated proteins, there are a number of methods available that can distinguish between D- and L-amino acids (Rutherfurd and Sarwar-Gilani, 2009).

It is also possible that racemisation can occur during the guanidination process itself given that guanidination is carried out at high pH over an extended period. However, de Vrese et al. (1994) reported that for guanidination carried out at pH 10.5 - 11 at 4 ^{o}C or pH 10 at 22 ^{o}C racemisation was negligible. The guanidination conditions used for the digestible reactive lysine assay developed as part of this thesis are pH 10.6 (diets) and 11.0 (digesta) at 21 ^{o}C and may result in the formation of minor amounts of D-homoarginine. However, since D- and L-amino acids cannot be separated using the chromatographic separation used in this thesis then the sum of D- and L-homoarginine will still accurately equate to the reactive lysine content.

Ultimately, the definitive demonstration of the accuracy of the digestible reactive lysine assay is to compare the actual lysine deposition in animals fed a processed protein source with the lysine deposition predicted based on the digestible reactive lysine content of the protein source. To that end, a controlled study based on lysine retention in the bodies of growing pigs was conducted. In this study, a heat-treated skim milk powder, for which both the true ileal total and reactive lysine digestibilities had been experimentally determined, was prepared and incorporated into a test diet. Two EHC-based control diets were formulated to contain a digestible lysine content based on either the total lysine digestibility or reactive lysine digestibility of the heated skim milk powder. The amino acids in the control diets were assumed to be completely digested and absorbed and all diets were isocaloric and limiting in lysine. It was found that the pigs fed the heated skim milk powder diet deposited the same amount of lysine as the pigs fed the control diet that had been formulated based on reactive lysine digestibility and deposited more lysine than the pigs fed the control diet formulated based on total lysine digestibility. This study clearly demonstrated the accuracy of the new true ileal reactive lysine digestibility assay and the inaccuracy of the traditional ileal total

lysine digestibility assay, which uses conventional amino acid analysis, for predicting the lysine availability of a heated protein source.

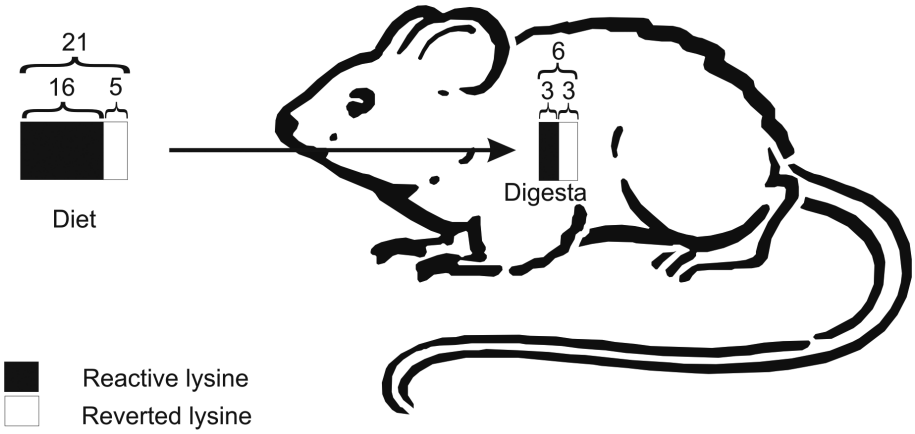
In addition, the accuracy of the new assay was further evaluated by comparing the true ileal digestible reactive lysine content of heat-treated field peas with the available lysine content of the same peas when determined using growth-based assays (van Barneveld et al., 1994b). Overall, there was good agreement between the digestible reactive lysine and available lysine (growth-based assays) contents of the heated peas further demonstrating the accuracy of the digestible reactive lysine (available lysine) assay when applied to processed protein sources. Although it must be remembered that the growth-based assays tends to measure utilisation rather than availability.

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 was also investigated in this study. A skim milk powder was autoclaved for 1 - 10 min and the true ileal digestible reactive and total lysine contents of the heated powders determined. There was little difference between true ileal digestible reactive and total lysine contents in the unheated milk powder demonstrating that the traditional true ileal digestibility assay is suitable for determining available lysine in unprocessed skim milk powder. However, for the skim 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 the overestimation was 12%.

While from a theoretical viewpoint the assay is straightforward there are several apparent contradictions that have created confusion. The first is that it has been observed that for some processed foods or feedstuffs or for the digesta of animals fed processed foods or feedstuffs, the sum of the reactive plus reverted lysine sample does not always equate to the total lysine content (Fontaine et al., 2007). This apparent discrepancy can be explained by the fact that during the guanidination reaction, which can take up to several days to complete for some protein sources, it is likely that early Maillard products are converted to other acid-stable products, such as late Maillard products which will no longer revert back to lysine during the acid hydrolysis step of amino acid analysis (Fontaine et al., 2007).

Another apparent discrepancy is that lysine determined using acid hydrolysis overestimates reactive lysine in processed protein sources but, and perhaps unexpectedly, ileal total lysine digestibility underestimates ileal reactive lysine digestibility. This apparent inconsistency is explained in Fig. 1 which shows a hypothetical processed feedstuff that contains 16 units of reactive lysine and five units of 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 equal total lysine). 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 eaten, the proteins are digested by gut proteases, but because of the modified lysine residues in the proteins, and possibly other factors, digestion is not complete. The resulting undigested peptides are called limit peptides and these will contain proportionally more damaged lysine than undamaged lysine. Moreover, limit peptides are absorbed to a lesser degree than the normal products of protein digestion, amino acids and small peptides. This leads to a greater proportion of damaged lysine residues compared to

the undamaged residues in terminal ileal digesta compared to the feedstuff being tested. In the example in Fig. 1, when the ileal digesta are analysed for reactive and total lysine, three units of reactive lysine and three units of reverted lysine are present giving six units of total lysine. This is quite a different proportion to that found in the original feedstuff (16 units of reactive lysine and five units of 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%).



Reverted lysine = Total lysine - Reactive lysine

$$\text{Reactive lysine digestibility} = \frac{(16 - 3)}{16} \times 100 = 81\%$$

$$\text{Total lysine digestibility} = \frac{(21 - 6)}{21} \times 100 = 71\%$$

Figure 1. 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 of 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 acid hydrolysis.

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 content is calculated by multiplying the lysine in the diet by ileal lysine digestibility, there will be no net difference between the digestible reactive lysine and the digestible total lysine when determined in processed foods or feedstuffs. However, this is generally not the case and if digestible reactive lysine and digestible total lysine estimates are similar in a processed food or feedstuff it is only by coincidence rather than some methodological artefact or real phenomenon. Again using Fig. 1 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.

Determining the available lysine content of heat-processed foods and feedstuffs using the true ileal digestible reactive lysine assay

The true ileal digestible reactive lysine (available lysine) assay was used to determine the available lysine content of a range of processed foods and feedstuffs including milk protein based foods (Rutherfurd and Moughan, 2005) presented in Chapter 3, "ready-to-eat" cereal-based breakfast foods (Rutherfurd et al., 2006) presented in Chapter 4 and "complete and balanced" cat foods (Rutherfurd et al., 2007) presented in Chapter 5. For the cat foods, the digestible total lysine significantly overestimated (mean overestimation of 86%) the digestible reactive lysine (available lysine) content of the 20 commercially available cat foods tested. This large difference between digestible total and reactive lysine suggested a high concentration of acid labile modified lysine derivatives and consequently a large amount of lysine damage in the processed cat foods. Overall, the moist cat foods appeared to have more lysine damage than the dry foods and this may be attributable to the higher water content in the moist cat foods (Franzen et al., 1990) and more severe processing conditions. Hendriks et al. (1999) also found significant amounts of reverted lysine, and consequently lysine damage, in cat foods that had undergone autoclaving. The high degree of lysine damage will inevitably alter the amino acid balance of a cat food and possibly result in an excess of unutilised amino acids that must be excreted in the urine. It is known that renal failure is a common cause of death for domestic cats and it is possible that an elevated urinary amino acid output due to the high degree of lysine damage is in part responsible.

The true ileal digestible reactive lysine and total lysine content of 20 "ready-to-eat" cereal-based breakfast foods was also determined. The digestible total lysine content was considerably higher (mean overestimation was 40%) than the digestible reactive (available) lysine content for most of the breakfast cereals tested. Torbatinejad et al. (2005) also determined the gross total and reactive lysine content of the same breakfast cereals. They reported that gross total lysine considerably overestimated (25 - 120% overestimation) the gross reactive lysine content, suggesting very large amounts of early Maillard product present in the cereals. Breakfast cereals are perceived to be a healthy balanced high-quality food product. However, when the bioavailable lysine content is compared to the gross total lysine content (the currently accepted method for presenting the amino acid composition of foods), the bioavailable lysine content is only on average half (31 - 69%) of the gross total lysine content. Given that lysine tends to be naturally low in cereals, the significant loss of the lysine present as a result of processing may be of concern. There are few reports in the literature describing the available lysine content of breakfast cereals. The comparison of the data presented in this study with those few published data is also not possible since the published data is based on flawed methodologies such as total lysine estimates and faecal digestibility.

The digestible reactive lysine assay is a sensitive tool for detecting small differences between true ileal digestible total lysine and digestible reactive lysine (available lysine) estimates. Indeed, when the assay was applied to a range of milk protein products, statistically significant ($P < 0.05$) differences of as little as 1.2% (for a UHT milk) could be detected. The difference between digestible total lysine content and digestible reactive lysine (available lysine) content was much smaller than that found for the cat foods and the "ready-to-eat" cereal-based breakfast foods tested in this study and perhaps reflects milder processing

treatment for milk-based foods compared to cat foods and breakfast cereals. Indeed, of the 12 milk-based foods examined, only one product showed a difference of greater than 10% between the digestible total and reactive lysine content. For eight of the 12 products tested there was also little difference (< 6%) between gross total lysine content and gross reactive lysine content. UHT milk is processed at up to 161 °C but only for a few seconds, while milk powders undergo evaporation under reduced pressure at low temperatures and then spray drying which exposes the atomised partially evaporated milk to high temperatures (200 °C) although the milk never reaches the air temperature as it undergoes evaporative cooling during the drying process. In contrast, pet foods undergo considerable processing as part of the manufacturing process. Meat by-products used for pet foods are often rendered prior to inclusion into pet foods. Wet pet foods are autoclaved at 121 °C for up to 80 min to sterilize the product and dry pet foods undergo drying and extrusion. Breakfast cereals also undergo considerable processing, including drying, flaking, toasting and extrusion which can expose proteins to high temperatures (> 100 °C) for considerable periods (> 60 min) (Fast, 2000).

The hydrolysis of milk protein-based foods during amino acid analysis has been well characterised and it has been estimated that approximately only 40% of the ε-N-lactulosyllysine present in processed milk proteins reverts to lysine during acid hydrolysis (Bujard and Finot, 1978). Consequently, the difference between gross total and reactive lysine content of the milk protein-based foods tested in this study will significantly underestimate the actual amount of ε-N-deoxyketosyllysine present. Overall however, given that the true ileal reactive lysine digestibility for the 12 milk-based foods tested in this study was greater than 91%, it is reasonable to conclude that milk-based foods are high quality protein sources.

Other workers have used the digestible reactive lysine assay developed in this thesis to determine the available lysine in pea-based pigs diets (Friesen et al., 2006) and distiller dried grains with solubles (Pahm et al., 2009). Friesen et al. (2006) reported that apparent ileal total lysine digestibility overestimated the lysine availability of 4 cultivars of field peas compared to apparent ileal reactive lysine digestibility. While Pahm et al. (2009) reported that for 12 distiller dried grains, true ileal digestible total lysine significantly ($P < 0.05$) overestimated available lysine compared to true ileal digestible reactive lysine. In contrast, true ileal total lysine digestibility was significantly ($P < 0.05$) lower than true ileal reactive lysine digestibility for only 2 of the 12 distiller dried grain samples. The latter workers agreed with the findings in this study that the overestimation of available lysine can be avoided if diets containing distiller dried grains are formulated based on digestible reactive lysine rather than digestible total lysine.

The digestible reactive lysine assay has also been used to assess the shelf-life of milk powders by examining the effect of long-term storage at elevated temperatures on the available lysine content of skim milk powder and hydrolysed-lactose skim milk powder (Rutherfurd and Moughan, 2007) presented in Chapter 6. The true ileal digestible reactive lysine assay showed that as much as 40% of the original lysine in the skim milk powder was damaged after 9 mth storage at 40 °C, while for the lactose-hydrolysed skim milk powder, 70% of the lysine was modified after 6 mth storage at 40 °C. However, at lower storage temperatures (30 and 35 °C), much less lysine modification was observed for both the milk powders tested. The greater decrease of available lysine in the hydrolysed-lactose skim milk powder compared to its unhydrolysed counterpart was most likely due to a greater

concentration of reducing sugars (glucose + galactose) in the hydrolysed-lactose product. These results suggest that the manner in which milk powders, particularly hydrolysed-lactose milk powder, are stored in countries that have hotter climates will impact available lysine content. This may be particularly important given that many third world countries, where nutrition is likely to be poor, have such climates.

Predicting protein deposition and nutritional adequacy based on dietary available lysine estimates

In order to maximise the growth of intensive livestock, such as pigs, diets are often formulated to contain energy and lysine at a level 3 to 4-times that required for maintenance. In contrast for humans, particularly adults, and companion animals, energy and protein intakes are similar to the maintenance requirement (Table 1). Production animal diets that are high in cereals are often limiting in protein and this is exacerbated since for many of these proteins sources the lysine content is also low (Table 2). Often synthetic lysine is added to diets for pigs and poultry in order to provide a dietary amino acid balance more suitable for achieving optimum lean deposition. Deficiencies in dietary available lysine may have implications for the production efficiency as well as the health of the animals. For growing children, a diet very high in cereals may result in an insufficient lysine intake to meet their nutritional requirement and this may be aggravated in third world countries where food intake is often marginal. Since high cereal diets, which tend to be low in lysine, are commonly consumed in third world countries it would be valuable to determine the digestible reactive lysine content of a range of food ingredients and diets that are commonly consumed in third world countries in order to evaluate the lysine intake status of both children and adults in those countries, particularly given that current available lysine content data for foods are most likely to be based on total lysine rather than reactive lysine estimates.

For pig and poultry production, diets are formulated to maximise protein deposition while minimising both fat deposition and urinary nitrogen excretion. To achieve this goal, careful control of the ratio of energy and individual essential amino acids in the diet is required. Since lysine is often the first limiting amino acid in pig and poultry diets, determining the available lysine content of those diets accurately is important. If the predicted available lysine content of a diet is greater than that actually present then protein deposition will be overestimated. As an example, in Fig. 2 the protein deposition of a 40-kg male pig was calculated based on the available lysine content of a corn-soyabean meal-based diet when determined using one of six different methods. For this example, it was assumed that the digestible reactive lysine assay, slope-ratio assay and indicator amino acid oxidation method would generate similar available lysine content values. Based on data presented in Fig. 2, it can be seen that protein deposition would have been overestimated by 17, 5 and 9% when determined using gross total lysine, gross reactive lysine or true ileal digestible total lysine respectively. These overestimations would result in the under performance of the pigs and an increased nitrogen output from the excess unutilised amino acids.

Table 1.
Daily lysine and digestible energy (DE) requirement and intake of the adult human and growing pig and the daily lysine and metabolizable energy (ME) requirement and intake of the domestic cat.

	Maintenance Requirement		Daily Intake	
	Lysine (g d^{-1})	DE/ME (MJ d^{-1})	Lysine (g d^{-1})	DE/ME (MJ d^{-1})
Pig (40 kg)	0.57[1]	7.3[2]	2.1[3]	27.9[4]
Human (70 kg)	2.1[5]	10.2[6]	2.8-12.6[7]	11[8]
Cat (4 kg)	0.17[9]	1.18[10]	0.21[11]	1.13[12]

[1]NRC (1998). Calculated as 0.036 x $kg^{0.75}$ body weight.
[2]NRC (1998). DE calculated as the 110 kcal of DE x $kg^{0.75}$ body weight.
[3]Calculated assuming a corn-soyabean meal-based diet containing 1.04% lysine and a dry matter intake of 2 kg d^{-1}.
[4]ARC (1981). DE calculated using the ad libitum energy intake for swine (DE (MJ) = 50(1-$e^{(-0.0204w)}$).
[5]Young and el-Khoury (1995) and assuming a lysine requirement for maintenance of 30 mg kg^{-1} body weight d^{-1}.
[6]FAO (2001). Calculated assuming a 30 year old 70 kg male who has a lifestyle based around light-moderate activity.
[7]Tomé and Bos (2007). Calculated as 40-180 kg^{-1} d^{-1} for a 70 kg adult male.
[8]Calculated assuming a diet for weight maintenance for a 30 year old 70 kg male who has a lifestyle of light-moderate activity.
[9]NRC (2006). Calculated as 0.067 x $kg^{0.67}$ body weight and assuming a 4 kg adult cat.
[10]NRC (2006). ME was calculated as 70 kcal x kg^{-1} body weight and assuming a 4 kg adult cat.
[11]NRC (2006). Calculated as 0.084 x $kg^{0.67}$ body weight and assuming a 4 kg adult cat.
[12]ME was calculated based on the recommended daily ration of Eukanuba Adult Weight Control Formula (dry diet) prescribed by Eukanuba for weight maintenance.

For humans, and companion animals such as cats and dogs, where the daily nutrient intake is much more closely aligned with the maintenance requirement, sufficient dietary available lysine is even more important since a shortfall in dietary lysine may impact directly on health due to an imbalance of the absorbed amino acids resulting in an increased renal load and an undersupply of lysine for protein turnover. For growing children and companion animals the supply of adequate lysine is even more important. As an example, the estimated daily lysine requirement for a 1 kg kitten is 0.44 g d^{-1} (NRC, 2006). The estimated daily lysine intake for a 1 kg kitten and based on the digestible total lysine content of the dry diets tested in this study would range from 0.50 - 1.04 g d^{-1}, while when based on digestible reactive lysine the corresponding daily lysine intake ranged from 0.29 – 0.88 g d^{-1}. While it is recognised that the adult cat diets analysed in this study contain slightly less protein that their counterparts for kittens, it is still of note that based on digestible total lysine it would have been predicted that all the dry diets would be adequate for lysine while when based on digestible reactive lysine it is clear that 3 diets would not have met the kittens requirement for growth.

Table 2.
Protein and lysine content of common diet ingredients (Degussa, 1996).

Protein source	Protein content (g /100 g)	Lysine content[1] (g/100 g)	Lysine content[1] (g/100 g protein)
Cereals			
Millet	11.62	0.23	2.0
Corn	8.53	0.25	2.9
Rice	7.29	0.26	3.6
Wheat	13.28	0.38	2.9
Barley	10.55	0.38	3.6
Corn gluten	18.43	0.51	2.8
Oats	12.63	0.53	4.2
Rice bran	16.72	0.76	4.5
Dried brewer's grains	22.80	0.84	3.7
Legumes			
Peas	20.92	1.50	7.2
Beans	25.41	1.63	6.4
Soyabean meal	45.64	2.86	6.3
Animal products			
Skim milk powder	35.79	2.76	7.7
Meat and bone meal	53.04	2.82	5.3
Fish meal	62.94	4.81	7.6
Blood meal	88.80	7.96	9.0

[1]*Determined using conventional amino acid analysis*

Conclusions and implications

There has been a need for an assay that can determine available lysine (the unmodified lysine absorbed from the small intestine in a form capable of being used for protein synthesis) in processed foods and feedstuffs for some time. Chemically reactive lysine assays do not take into account the incomplete intestinal absorption of lysine from heat damaged proteins and the true ileal digestibility assay determines the digestibility of total lysine, a fundamentally flawed measurement, when determined in processed foods that

contain early Maillard products or other acid-labile lysine derivatives. Growth-based assays which determine utilisation rather than availability may also not be suitable. In this study, a new assay is described that couples the guanidination reaction with a true ileal amino acid digestibility assay to determine true ileal digestible reactive lysine which is, by definition, available lysine. The assay has been validated and used to highlight the effect of processing on the available lysine content for several different processed foods and feedstuffs including milk protein-based foods, "ready-to-eat" breakfast cereals and "complete and balanced" cat foods.

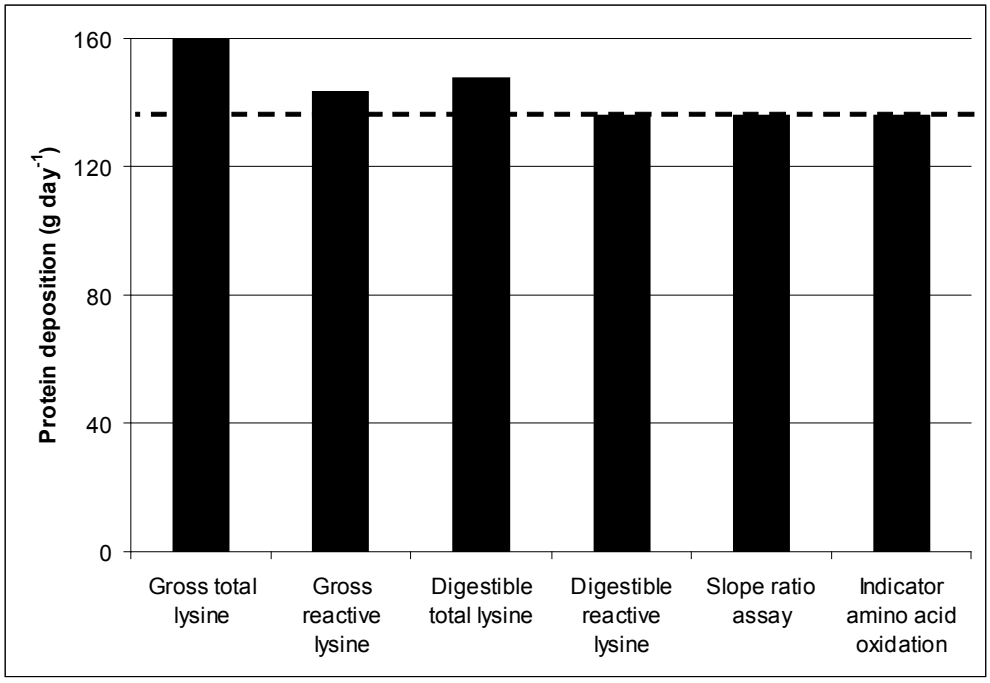


Figure 2. The predicted protein deposition in a 40-kg male pig fed a corn-soyabean meal (74.1%; 23.4%; NRC 1998) diet based on the available lysine content estimated using six different methods.
The protein deposition was calculated using the following equation and assuming that lysine was first limiting in the diet:

Protein deposition (g d^{-1}) = (Daily DMI (kg d^{-1}) x Dietary available lys (g kg^{-1}) – Maint lys req (g d^{-1})) (Lys required to deposit protein (g g^{-1}) x Efficiency of lys deposition (%))

Where daily DMI is the dry matter intake and was assumed to be 2 kg d^{-1}. Dietary available lys is the available lysine content of the diet and was based on either gross total lysine, gross reactive lysine, true ileal digestible total lysine, true ileal digestible reactive lysine, the slope ratio assay or the indicator amino acid oxidation method. Maint lys req was the lysine requirement for maintenance and was calculated as 0.036 x $kg^{0.75}$ body weight (NRC 1998). The amount of lysine required to deposit protein was assumed to be 0.07 g g^{-1} and the efficiency of lysine deposition was assumed to be 83%.

For cat foods and breakfast cereals, processing appeared to have a major impact on available lysine and for such foods the presence of dietary AGE's may be an issue for

animal and human health. Furthermore, digestible total lysine significantly overestimated the available lysine content of these products and, as highlighted in the example for dry cat foods shown above, this may impact on the prediction of lysine adequacy for such foods and feedstuffs. For milk protein-based foods the amount of lysine modification during processing and storage appeared to be much less, but given that milk products are perceived to be high quality from a nutritional standpoint, the reduction in digestible reactive lysine content observed for some milk-based products may be noteworthy.

Literature cited

Agricultural Research Council, 1981. Requirements for vitamins and minerals. In: The nutrient requirement of pigs. Pp. 125–299. Farnham Royal, England: Commonwealth Agricultural Bureaux.

Batterham, E.S., Andersen, L.M., Baigent, D.R., Darnell, R.E., Taverner, M.R. 1990. A comparison of the availability and ileal digestibility of lysine in cottonseed and soya-bean meals for grower/finisher pigs. Brit. J. Nutr. 64, 663-677.

Batterham, E.S., Murison, R.D., Lewis, C.E. 1979. Availability of lysine in protein concentrates as determined by the slope-ratio assay with growing pigs and rats and by chemical techniques. Brit. J. Nutr. 41, 383-391.

Bodwell, C.E. 1977. Application of animal data to human protein nutrition: A review. Cereal Chem. 54, 958-983.

Bujard. E., Finot, P.A. 1978. Mesure de la disponibilité et du blocage de la lysine dans les laits industriels. Ann. Nutr. Alim. 32, 291-305.

Butts, C.A., Moughan, P.J., Smith, W.C. 1991. Endogenous amino acid flow at the terminal ileum of the rat determined under conditions of peptide alimentation. J. Sci. Food Agric. 55, 175-187.

Degussa. 1996. The amino acid composition of feedstuffs. 4th revised edition. Degussa Corporation Additives.

de Vrese, M., Middendorf, K., Hagemeister, H. 1994. Prevention of amino acid racemization during guanidination – a prerequisite for measurement of protein digestibility by homoarginine labeling. Z. Ernährungswiss. 33, 310-312.

FAO/WHO/UNU. 2001. Human energy requirements. In: Food and Nutrition Technical Report Series. Report of a joint FAO/WHO/UNU expert consultation. Rome.

Fast, R.B. 2000. Manufacturing technology of ready-to-eat cereals. In Breakfast Cereals and How They are Made; 2nd ed. Pp. 15-42 [RB Fast and EF Caldwell, editors]. St. Paul, Minnesota: American Association of Cereal Chemist, Inc.

Fontaine, J., Zimmer, U., Moughan, P.J., Rutherfurd, S.M. 2007. Effect of heat damage in an autoclave on the reactive lysine contents of soy products and corn distillers dried grains with solubles. Use of the results to check on lysine damage in common qualities of these ingredients. J. Agric. Food Chem. 55, 10737-10743.

Franzen, K., Singh, R.F., Okos, M.R. 1990. Kinetics of nonenzymatic browning in dried skim milk. J. Food Eng. 11, 225-239.

Friesen, M.J., Kiarie, E., Nyachoti, C.M. 2006. Ileal amino acid digestibility and reactive lysine content in peas (Pisum sativum) fed to growing pigs. Anim. Feed Sci. Technol. 129, 210-223.

Hendriks, W.H., Emmens, M.M.A., Trass, B., Pluske, J.R. 1999. Heat processing changes the protein quality of canned cat foods as measured with a rat bioassay. J. Anim. Sci.

77, 669-676.
Karayiannis, N.I., MacGregor, J.T., Bjeldanes, L.F. 1979. Lysinoalanine formation in alkali-treated proteins and model peptides. Food Cosmet. Toxicol. 17, 585-590.
Liardon, R., Hurrell, R.F. 1983. Amino acid racemization in heated and alkali-treated proteins. J. Agric. Food Chem. 31, 432-437.
Moehn, S., Bertolo, R.F.P., Pencharz, P.B., Ball, R.O. 2005. Development of the indicator amino acid oxidation technique to determine the availability of amino acids from dietary protein in pigs. J. Nutr. 135, 2866-2870.
Moughan, P.J. 2003. Amino acid availability: Aspects of chemical and bioassay methodology. Nutr. Res. Rev. 16, 127-141.
Moughan, P.J., Darragh, A.J., Smith, W.C., Butts, C.A. 1990. Perchloric and trichloroacetic acids as precipitants of protein in endogenous ileal digesta from the rat. J. Sci. Food Agric. 52, 13-21.
Moughan, P.J., Gall, M.P.J., Rutherfurd, S.M. 1996. Absorption of lysine and deoxyketosyllysine in an early-Maillard browned casein by the growing pig. J. Agric. Food Chem. 44, 1520-1525.
Moughan, P.J., Rutherfurd, S.M. 1996. A new method for determining digestible reactive lysine in foods. J. Agric. Food Chem. 44, 2202-2209.
National Research Council. 2006. Nutrient Requirements of Dogs and Cats. Washington, DC: National Academies Press.
National Research Council. 1998. Nutrient requirements of swine. In: Nutrient Requirements of Domestic Animals. 10th ed. Washington, DC: Natl. Acad. Sci.
Pahm, A.A., Pedersen, C., Stein, H.H. 2009. Standardized ileal digestibility of reactive lysine in distillers dried grains with solubles fed to growing pigs. J. Agric. Food Chem. 57, 535-539.
Rutherfurd, S.M., Markwick-Rutherfurd, K.J., Moughan, P.J. 2007. Available (ileal digestible reactive) lysine in selected pet foods. J. Agric. Food Chem. 55, 3517-3522.
Rutherfurd, S.M., Moughan, P.J. 2008. Effect of elevated temperature storage on the digestible reactive lysine content of unhydrolysed- and hydrolysed-lactose milk-based products. J. Dairy Sci. 91, 477-482.
Rutherfurd, S.M., Moughan, P.J. 2005. Digestible reactive lysine in selected milk-based products. J. Dairy Sci. 88, 40-48.
Rutherfurd, S.M., Sarwar-Gilani, G. 2009. Amino Acid Analysis. Curr. Protoc. Protein. Sci. 58, 11.9.1-11.9.37.
Rutherfurd, S.M., Torbatinejad, N.M., Moughan, P.J. 2006. Available (ileal digestible reactive) lysine in selected cereal-based food products. J. Agric. Food Chem. 54, 9453-9457.
Torbatinejad, N.M., Rutherfurd, S.M., Moughan, P.J. 2005. Total and reactive lysine contents in selected cereal-based food products. J. Agric. Food Chem. 53, 4454-4458.
Tomé, D., Bos, C. 2007. Lysine requirement through the human life cycle. J. Nutr. 137, 1642S-1645S.
van Barneveld, R.J., Batterham, E.S., Norton, B.W. 1994a The effect of heat on amino acids for growing pigs. 1. A comparison of ileal and faecal digestibilities of amino acids in raw and heat-treated field peas (Pisum sativum cultivar Dundale). Brit. J. Nutr. 72, 221-241.
van Barneveld, R.J., Batterham, E.S., Norton, B.W. 1994b. The effect of heat on amino acids for growing pigs. 3. The availability of lysine from heat-treated field peas (Pisum

sativum cultivar Dundale) determined using the slope-ratio assay. Brit. J. Nutr. 72, 257-275.

Wiseman, J., Jagger, S., Cole, D.J.A., Haresign, W. 1991. The digestion and utilization of amino acids of heat-treated fish meal by growing/finishing pigs. Anim. Prod. 53, 215-225.

Young, V.R., el-Khoury, A.K. 1995. Can amino acid requirements for nutritional maintenance in adult humans be approximated from the amino acid composition of body mixed proteins? Proc. Natl. Acad. Sci. USA. 92, 300-304.

Summary

Many foods produced for human consumption, and feedstuffs produced for farm and companion animals, undergo some form of processing. During processing, proteins in these foods or feedstuffs can be exposed to heat, both wet and dry, pressure and alkali. When foods or feedstuffs are subjected to these kinds of conditions, certain amino acids, such as lysine, can react with other compounds, especially reducing sugars, in a food or feedstuff to form Maillard compounds. Some Maillard compounds are not acid-stable and during amino acid analysis these compounds revert back to lysine leading to an overestimation of the amount of lysine (total lysine) present in the food or feedstuff.

This study has developed an assay that accurately measures available lysine in processed foods and feedstuffs. The assay combines the guanidination method with the true ileal digestibility assay and can be applied to humans or animal models such as the rat or pig. The guanidination method (a method for determining reactive lysine) involves the conversion of reactive lysine to the acid-stable compound homoarginine which can then be determined by conventional amino acid analysis. Essentially, the test foodstuff is fed to a group of test animals and the digesta are collected from the terminal ileum of those animals. The reactive lysine contents of both the diet and digesta are determined using the guanidination method and the ileal digestibility of reactive lysine is then calculated. The digestible reactive lysine content is, by definition, the available lysine content. The newly developed assay was then used to determine the available lysine content of processed milk protein-based products, cereal-based breakfast foods and cat foods. It was also used to investigate the effects of long-term storage at elevated temperatures on selected milk powders.

This dissertation consists of seven chapters. **Chapter 1** is a literature review that discusses:

1 The fate of lysine during processing, including the chemical modification that lysine undergoes during processing and the implications on determining the lysine content of processed foods and feedstuffs.
2 A discussion about the terminology used to describe reactive lysine and available lysine.
3 A review of current methods available for determining reactive lysine including a detailed treatment of the most suitable methods (FDNB and guanidination methods).
4 A review of several methods available for determining bioavailable lysine including the shortcomings of these methods. Several key methods are discussed in detail including the slope-ratio assay and the indicator amino acid oxidation technique.
5 The concept of the true ileal digestible reactive lysine assay as a method for determining available lysine. How the combination of the guanidination reaction and a true ileal digestibility assay could be used as a method for determining true ileal digestible reactive lysine. The aims of the thesis are also outlined.

Chapter 2 describes the development of the true ileal digestible reactive lysine (available lysine) assay. This includes the optimisation of the guanidination reaction for heated protein sources, using heated lactose/casein as a model heated protein source, as well as for the terminal ileal digesta of animals fed unheated casein and heated lactose/casein. Experiments based on lysine deposition in growing pigs were also conducted to evaluate the accuracy of the new assay and these experiments are described. Overall, the newly developed assay was shown to be an accurate predictor of available lysine for a heated skim milk powder, while true ileal total lysine digestibility did not accurately predict lysine availability in the heated skim milk powder.

Chapter 3 describes a study which determined the available lysine content of a range of milk protein-based foods. The true ileal reactive lysine digestibility and total lysine digestibility were determined. The true ileal reactive lysine digestibility was high (> 91%) for all the milk products tested. Total lysine digestibility (conventional measurement) underestimated reactive lysine digestibility for all the products tested by between 1.3 to 7.1% units. The mean digestible total lysine content was significantly different from the available lysine content for most of the products examined but for some products the difference was small (< 3%). However for several products the difference was greater (6.5 - 14%). Overall, total lysine and total lysine digestibility determined using conventional methods were inaccurate when applied to some milk-based foods. In general, milk proteins are a highly digestible source of amino acids and lysine. Furthermore, the extent of lysine damage in these foods, as reflected in the difference between digestible total lysine and digestible reactive lysine estimates, was considerably lower than observed for the cat foods or cereal-based breakfast foods also tested as part of this thesis.

Chapter 4 describes the determination of available lysine (true ileal digestible reactive lysine) and true ileal digestible total lysine in 20 "ready-to-eat" cereal-based breakfast foods using the new assay. True ileal reactive lysine digestibility ranged from 53 to 108% across the 20 breakfast cereals and was significantly ($P < 0.05$) higher than the true ileal total lysine digestibility for most of the products. Available lysine content (true ileal digestible reactive lysine content) ranged from 0.21 – 3.5 g kg^{-1} across the breakfast cereals. The conventional measure of true ileal digestible total lysine content significantly overestimated (on average 37%) available lysine for the majority of the cereals tested. Breakfast cereals undergo a significant degree of lysine modification and this may be of concern given the perception that breakfast cereals are high quality foods.

Chapter 5 describes the determination of the available lysine content of a range of moist and dry commercial cat foods. Again both true ileal digestible reactive lysine and digestible total lysine was determined. A comparison of the true ileal digestible reactive lysine content with the true ileal digestible total lysine (traditional method for determining available lysine) content was also made. True ileal total lysine digestibility underestimated (3.6 - 10.2% underestimation) lysine availability (true ileal reactive lysine digestibility) for most of the cat foods tested. True ileal digestible total lysine overestimated the amount of dietary available lysine for all the cat foods tested by between 18 to 143%. Given that the latter overestimation reflects lysine that has reverted from damaged lysine during amino acid analysis, there appeared to be a significant amount of lysine damage in the cat foods tested.

Chapter 6 describes a study where the true ileal digestible reactive lysine assay was used to investigate the effect of storage at elevated temperatures on the available lysine content of skim milk powder and a hydrolysed-lactose skim milk powder. For the skim milk powder, there was essentially no decrease in reactive lysine digestibility (lysine availability) when the powder was stored at 30 °C and 35 °C for 18 mth, but a small decrease was observed when the powder was stored at 40 °C. For the hydrolysed-lactose product, lysine availability decreased by 22% after storage at 35 °C for 18 mth and 17% when stored at 40 °C for 6 mth. Digestible reactive lysine (available lysine) content decreased by more than 20% and 41% for the skim milk powder and hydrolysed-lactose skim milk powder respectively when stored for 18 mth at 30 °C. At higher storage temperatures even greater decreases in available lysine

content over shorter storage periods were observed for both powders. Overall, prolonged storage, particularly at elevated temperatures, resulted in a lower available lysine content for both milk powders. The decrease in available lysine content and lysine availability after storage was greater for the hydrolysed-lactose skim milk powder compared to the normal skim milk powder, most likely due to the higher concentration of reducing sugars in the hydrolysed-lactose skim milk powder.

Chapter 7 is a general discussion that sums up the overall study. The findings of Chapters 2-6 are then briefly discussed and an overall conclusion is given. This chapter also explains apparent contradictions with the assay such as why total lysine overestimates reactive lysine contents but that total lysine digestibility underestimates reactive lysine digestibility. The impact of different methods for determining the lysine content of processed feedstuffs on the prediction of lysine deposition in pigs is also illustrated.

Overall the general conclusions of the study were:

1 An assay was developed that accurately determined the available lysine content of processed protein sources.
2 True ileal total lysine digestibility underestimates lysine availability when applied to processed foods.
3 True ileal digestible total lysine overestimates available lysine when applied to processed foods.
4 Processed foods such as cat foods and “ready-to-eat” cereal-based breakfast foods have a high degree of lysine damage.
5 Milk protein-based foods are generally high quality protein foods. These products had less lysine damage than the cat foods or breakfast cereals tested.
6 Storage of skim milk powder and hydrolysed-lactose skim milk powder led to lower available lysine contents particularly at elevated temperatures and particularly for the hydrolysed-lactose product.

Samenvatting

Een groot deel van de huidige voedingsmiddelen voor humane consumptie en ook van de grondstoffen die gebruikt worden in de voeding van gezelschaps- en landbouwhuisdieren hebben één of andere technologische behandeling ondergaan. Tijdens deze technologische behandelingen kunnen de eiwitten in grondstoffen blootgesteld worden aan hitte (bij verschillende vochtgehalten), druk, wrijving of aan behandeling met alkalische stoffen. Gedurende deze behandelingen kunnen aminozuren zoals lysine reageren met andere componenten aanwezig in de grondstof, in het bijzonder met reduceerde suikers waarna Maillard producten kunnen ontstaan. Sommige van deze lysine houdende Maillard producten zijn gevoelig voor zuur en tijdens standaard aminozuuranalyse kunnen deze teruggevormd worden tot lysine. Dit leidt dan tot een overschatting van het gehalte aan lysine in deze grondstoffen of in behandelde voeders.

Tijdens het onderzoek beschreven in dit proefschrift is een nieuw bio-assay ontwikkeld die nauwkeurig het biologische beschikbare lysine in technologisch behandelde voedings- en voedermiddelen kan meten. Deze methodiek combineert de guanidinisatie-reactie met een ware ileale verteerbaarheidbepaling uitgevoerd in ratten of varkens. In de guanidinisatie-reactie wordt lysine met een vrije ε-amino group (reactief lysine) omgezet in het zuurstabiele aminozuur homoarginine door middel van *O*-methylisourea. Homoarginine is zuurstabiel en kan via de conventionele aminozuuranalyse worden gekwantificeerd. In deze nieuwe bio-assay wordt het testvoeder (of de test grondstof) aan een groep dieren gevoerd en wordt de chymus (digesta) aan het einde van het ileum verzameld. Het gehalte aan reactief lysine in voer en de digesta wordt vervolgens bepaald via de guanidinisatie-methode en de verteerbaarheid van het reactief lysine berekend conform de standaard methode voor het bepalen van de verteerbaarheid van aminozuren. De hoeveelheid verteerbaar reactief lysine is per definitie de hoeveelheid beschikbare lysine voor metabolische processen voor mens en dier. Deze nieuwe bio-assay is in dit onderzoek gebruikt om de hoeveelheid beschikbaar lysine aanwezig in hittebehandelde melkproducten ontbijtgranen en kattenvoeders te bepalen. Bovendien werd deze techniek gebruikt om te bepalen wat het effect is van langdurige opslag bij verhoogde omgevingstemperatuur op reactief lysine in een aantal melkpoeders. Het proefschrift omvat 7 hoofdstukken.

Hoofdstuk 1 is een overzicht van de literatuur waarin verschillende aspecten bediscussieerd worden:
*Het lot van lysine gedurende technologische behandeling met onder andere hitteprocessen zoals vermeld in de literatuur is weergegeven. Ook de chemische verandering die lysine ondergaat gedurende processing is beschreven zoals gemeten in de literatuur. Er wordt nagegaan wat de uitkomsten betekenen voor de lysine bepaling in hittebehandelde voedingsmiddelen en in grondstoffen in de diervoeding.
*Er wordt ingegaan op de terminologie die gebruikt wordt om reactief en beschikbaar lysine te definiëren.
*Een overzicht wordt gegeven van de huidige methoden die gebruikt worden om reactief lysine te meten inclusief een gedetailleerde beschrijving van de meest geschikte methoden (FDNB en guanidinisatie methode)
*Een overzicht van verschillende methoden is gegeven om de biologische beschikbaarheid te bepalen met een discussie van de beperkingen van deze methoden. Sommige belangrijke methoden worden in detail bediscussieerd inclusief de slope-ratio methode en indicator methode voor aminozuuroxidatie.

*Er wordt ingegaan op het concept van de ware ileale lysine beschikbaarheid. In het bijzonder wordt bediscussieerd hoe een combinatie van guanidinisatie en ware ileale verteerbaarheid gebruikt kan worden om de ware ileale hoeveelheid beschikbare lysine te bepalen. Verder wordt in dit hoofdstuk de doelstelling van het gehele onderzoek in dit proefschrift nader aangegeven.

Hoofdstuk 2 beschrijft de ontwikkeling van een assay voor het meting van de ware ileale verteerbare hoeveelheid lysine in voedingsgrondstoffen welke door mensen en dieren worden geconsumeerd inclusief de optimalisatie van de guanidinisatie-reactie voor verhitte eiwitbronnen. Daarvoor werd een verhit mengsel van lactose\caseïne als modelstof gebruikt in dieren die of onverhitte of verhitte caseïne/lactose kregen. Van beide proefbehandelingen werd per dier de ileale digesta opgevangen. Bij dieren gevoerd met deze voeders werd de lysine in de eiwitaanzet van het lichaam gemeten om zo de nauwkeurigheid van de ontwikkelde beschikbare lysine bepaling te meten. Immers alleen beschikbare lysine kan door een dier voor eiwitsynthese worden gebruikt. Het bleek dat de nieuwe methode voor het meten van beschikbare lysine een nauwkeurige methode is om de verandering in beschikbaar lysine door hittebehandeling van bijvoorbeeld magere melkpoeder te voorspellen. De ware totale ileale lysine bepaling zelf kan de beschikbare lysine in hitte behandelde magere melkpoeder niet goed voorspellen.

Hoofdstuk 3 beschrijft een studie waarin de beschikbare lysine gehalten in een aantal verschillende voedingsmiddelen (vooral melkproducten) wordt bepaald. In deze studie werden de ware verteerbaarheden van het reactief lysine en van totaal lysine gemeten. De ware verteerbaarheid van reactief lysine was erg hoog (>91 %) in alle geteste melkproducten. De totale lysine verteerbaarheid (bepaald met de conventionele bepalingsmethode) als maat voor de reactieve lysine verteerbaarheid, onderschatte de verteerbaarheid van lysine in alle gevallen. De onderschatting was tussen de 1,3 en 7,1 % eenheden. Het gemiddelde gehalte aan totaal lysine verschilde significant van het reactief lysine gehalte voor bijna alle producten. Voor sommige was het verschil klein, minder dan 3 % terwijl voor andere producten het verschil veel groter was, tussen de 6,5 en 14 %. Er werd geconcludeerd, dat de verteerbaarheid van totale lysine met de conventionele methode onnauwkeurig is voor een aantal, op melk gebaseerde voedingsmiddelen. De melkproducten zijn een goede bron van goed verteerbare aminozuren en ook van lysine. Bovendien is de mate van lysine beschadiging in deze voedingsmiddelen zoals deze tot uiting komt in de verschillen tussen verteerbare totaal lysine en verteerbaar reactief lysine niet gelijk. In voedingsmiddelen voor de humane consumptie en diervoeders is het verschil tussen totaal en beschikbare lysine veel groter dan het verschil bij melkproducten.

Hoofdstuk 4 beschrijft de bepaling van beschikbare lysine (ware ileaal verteerbaar reactief lysine) en de ware ileaal verteerbare totale lysine in 20 kant-en-klaar, op graan gebaseerde ontbijtproducten zoals gemeten met de nieuwe assay. De ware ileaal verteerbare reactief lysine coëfficiënten varieerden tussen de 53 en 108 % in de 20 producten en deze waarden zijn significant hoger dan de ware ileaal verteerbare totale lysine gehalten voor de meeste producten. De hoeveelheid beschikbare lysine (de hoeveelheid waar verteerbare reactief lysine) varieerde tussen 0,21 en 3,5 g per kg in de ontbijtgraanproducten. Wanneer men via de conventionele methode de totaal verteerbare lysine meet, overschat men het beschikbare lysinegehalte voor de meerderheid van de geteste granen wel met gemiddeld 37 %. Lysine

in ontbijtgranen ondergaat een aanzienlijke modificatie en dit is een zorgelijk aspect van de perceptie dat ontbijtgranenproducten kwalitief hoogwaardige voedingsmiddelen zijn voor de mens.

Hoofdstuk 5 handelt over de bepaling van beschikbare lysine in een reeks van droge en natte kattenvoeders. Zoals in de vorige hoofdstukken werd vermeld is zowel het ware ileale totale lysine (conventionele methode) alsook het ware ileale reactief (beschikbare lysine) gemeten. De waarden voor de ware ileale totale lysine verteerbaarheid meting onderschatte de ware ileale reactieve lysine verteerbaarheid met 3,6 tot 10,2 % eenheden voor bijna alle geteste kattenvoeders. Wanneer deze omgerekend worden naar hoeveelheden, dan blijkt dat met de conventionele ware ileaal verteerbare lysine methode er een overschatting is van de ware reactieve hoeveelheid lysine in kattenvoeders met 18 to 143 %. Deze overschatting wijst erop dat lysine, dat bijvoorbeeld door een technologische behandeling eerst gebonden was, tijdens de aminozuurmeting weer terug wordt gevormd tot lysine. Dit wil ook zeggen dat een aanzienlijk deel van de lysine in de kattenvoeders daar aanwezig is als gebonden lysine en dus niet beschikbaar is voor de dieren.

Hoofdstuk 6 beschrijft een vervolgstudie naar de invloed van opslag bij verhoogde temperaturen op de ware ileaal verteerbare hoeveelheid reactief lysine in magere melkpoeder en in gehydrolyseerde, lactose houdende magere melkpoeder. In magere melkpoeder was er geen afname in reactief (verteerbare) lysine wanneer de poeder bewaard werd bij 30° C of 35° C gedurende 18 maanden. Na bewaring bij 40° C werd echter wel een kleine afname gevonden. In het gehydrolyseerde, lactose houdende product nam de lysine beschikbaarheid af met 22 % na bewaren bij 35° C gedurende 18 maanden en met 17 % na bewaring bij 40° C gedurende 6 maanden. Het verteerbare reactief lysine gehalte nam met meer dan 20 % af in magere melkpoeder en met meer dan 41 % in gehydrolyseerde caseïne magere melkpoeder na bewaring bij 30° C gedurende 18 maanden. Wanneer de bewaartemperatuur steeg nam de beschikbare lysine nog meer af en gedurende een veel kortere bewaarduur. Een lange bewaarduur speciaal bij hoge temperaturen bleek in beide melkproducten de beschikbare lysine nog meer te doen afnemen De afname in lysine en in beschikbare lysine was het grootst in het gehydrolyseerde lactose houdende magere melkpoederproduct vergeleken met normale magere melkpoeder. Dit is hoogstwaarschijnlijk veroorzaakt door de aanwezigheid van de reducerende suikers in het hydrolyse product.

Hoofdstuk 7 bevat de algemene discussie waarin de resultaten van de verschillende studies met elkaar worden vergeleken en met de literatuur. De vergelijkingen worden verder bediscussieerd en een aantal algemene conclusies worden getrokken. De discussie verheldert ook de schijnbare tegenstelling tussen toename in ware ileale reactief lysine verteerbaarheid en de afname in het ware reactief lysine gehalte na warmtebehandeling. Dit hoofdstuk illustreert tevens wat de betekenis is van de verschillende methoden voor de bepaling van het lysine gehalte in technologisch behandelde producten voor de voorspelling van de lysineaanzet (en dus ook potentiële eiwitaanzet) in het dier.

De algemene conclusies zijn als volgt weer te geven:

1. Er is een assay ontwikkeld die nauwkeurig het gehalte aan beschikbare lysine van technologisch behandelde voedingsmiddelen en veevoedergrondstoffen kan meten.
2. Ware ileale totale lysine verteerbaarheid onderschat de lysine beschikbaarheid in

technologisch behandelde voedingsmiddelen.

3. Gehaltes aan ware ileale totaal lysine in technologisch behandelde voedingsmiddelen overschatten de beschikbare lysine gehaltes.
4. Technologisch behandelde voeders zoals kattenvoeders en klant-en-klare ontbijtengranen voor mensen hebben een hoge graad van lysine beschadiging.
5. Melkeiwitten zijn van hoge kwaliteit en ondergaan bij hittebehandeling minder lysine beschadiging dan kattenvoeders of ontbijtproducten gebaseerd op granen.
6. Opslag bij hoge bewaartemperaturen van magere melkpoeders en gehydrolyseerde, lactose houdende magere melkpoeders leidt tot een verlaging van het beschikbaar lysine gehalte. In gehydrolyseerde, lactose houdende magere melkpoeders daalt dit gehalte sterker dan in magere melkpoeder zelf.

Publications

Original papers

1. Rutherfurd, S.M., Moughan, P.J. 1990. Guanidination of lysine in selected dietary proteins. J. Agric. Food Chem. 38, 209-211.
2. Moughan, P.J., Rutherfurd, S.M. 1990. Endogenous flow of total lysine and other amino acids at the distal ileum of the protein- and peptide-fed rat: The chemical labelling of gelatin protein by transformation of lysine to homoarginine. J. Sci. Food Agric. 52, 179-192.
3. Sauter, S.L., Rutherfurd, S.M., Wagener, C., Shively, J.E., Hefta, S.A. 1991. Binding of nonspecific cross-reacting antigen, a granulocyte membrane glycoprotein, to *Escherichia coli* expressing type 1 fimbriae. Infection and Immunity. 59, 2485-2493.
4. Moughan, P.J., Rutherfurd, S.M. 1991. Endogenous lysine flow at the distal ileum of the protein-fed rat: Investigation of the effect of protein source using radioactively labelled acetylated lysine or lysine transformed to homoarginine. J. Sci. Food Agric. 55 163-174.
5. Enghild, J.J., Salvesen, G., Hefta, S.A., Thørgersen, I.B., Rutherfurd, S.M., Pizzo, S.V. 1991. Chondriotin 4-sulphate covalently cross-links the chains of the human blood protein pre-α-inhibitor. J. Biol. Chem. 266, 747-751.
6. Sauter, S.L., Rutherfurd, S.M., Wagener, C., Shively, J.E., Hefta, S.A. 1993. Identification of the specific oligosaccharide sites recognised by type 1 fimbriae from *Escherichia coli* on nonspecific cross-reacting antigen, a CD66 cluster granulocyte glycoprotein. J. Biol. Chem., 268, 15510-15516.
7. Moughan, P.J., Gall, M.P.J., Rutherfurd, S.M. 1996. Absorption of lysine and deoxyketosyllysine in an early-Maillard browned casein by the growing pig. J. Agric. Food Chem. 44, 1520-1525.
8. Moughan, P.J., Rutherfurd, S.M. 1996. A new method for determining digestible reactive lysine in foods. J. Agric. Food Chem. 44, 2202-2209.
9. Rutherfurd S.M., Moughan, P.J., van Osch, L. 1997. Digestible reactive lysine in processed feedstuffs: Application of a new bioassay. J. Agric. Food Chem. 45, 1189-1194.
10. Rutherfurd, S.M. and Moughan, P.J. 1997. Application of a new method for determining digestible reactive lysine to variably heated protein sources. J. Agric. Food Chem. 45, 1582-1586.
11. Rutherfurd, S.M., Moughan, P.J., Morel, P.C.H. 1997. Assessment of the true ileal digestibility of reactive lysine as a predictor of lysine uptake from the small intestine of the growing pig. J. Agric. Food Chem. 45, 4378-4383.
12. Rutherfurd, S.M., Moughan, P.J. 1998. The digestible amino acid composition of several milk proteins: Application of a new bioassay. J. Dairy Sci. 81, 909-917.
13. Hendriks, W.H., O'Conner, S., Thomas, D.V., Rutherfurd, S.M., Taylor, G.A., Guilford, W.G. 2000. Nutrient composition of the crop contents of growing and adult grey-faced petrels (*Pterodroma macroptera*): a preliminary investigation. J. Royal Soc. N. Z. 30, 105-111.
14. Hendriks W.H., Rutherfurd S.M., Rutherfurd K.J. 2001. Importance of sulphate, cysteine and methionine as precursors to felinine synthesis by domestic cats (*Felis catus*). Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 129, 211-216.
15. Rutherfurd, K.J., Rutherfurd, S.M., Moughan, P.J., Hendriks, W.H. 2002. Isolation

and characterization of a felinine-containing peptide from the blood of the domestic cat (*Felis catus*). J. Biol. Chem. 277, 114-119.

16. Rutherfurd, S.M., Chung, T.K., Moughan, P.J. 2002. The effect of microbial phytase on ileal phosphorus and amino acid digestibility in the broiler chicken. Brit. Poult. Sci. 44, 598-606.
17. Rutherfurd, S.M., Moughan, P.J. 2003. The rat as a model animal for the growing pig in determining ileal amino acid digestibility in soya and milk proteins. J. Anim. Physiol. Anim. Nutr. 87, 292-300.
18. Purchas, R.W., Rutherfurd, S.M., Pearce, P.D., Vather, R., Wilkinson, B.H.P. 2004. Concentrations in beef and lamb of taurine, carnosine, coenzyme Q_{10}, and creatine. Meat Sci. 66, 629-637.
19. Rutherfurd, S.M., Chung, T.K., Morel, P.C.H., Moughan, P.J. 2004. Effect of microbial phytase on the ileal digestibility of phytate phosphorus, total phosphorus, and amino acids in a low-phosphorus diet for broilers. Poult. Sci. 83, 61-68.
20. Purchas, R.W., Rutherfurd, S.M., Pearce, P.D., Vather, R., Wilkinson, B.H.P. 2004. Cooking temperature effects on the forms of iron and levels of several other compounds in beef semitendinosus muscle. Meat Sci. 68, 201-207.
21. Rutherfurd, S.M., Zhang, F., Harding, D.R.K., Woolhouse, A.D., Hendriks W.H. 2004. Use of capillary (zone) electrophoresis for determining felinine and it's application to investigate the stability of felinine. Amino Acids. 27, 49-55.
22. Hendriks, W.H., Vather, R., Rutherfurd, S.M., Weidgraaf, K., Rutherfurd-Markwick, K.J. 2004. Urinary isovalthine excretion in adult cats is not gender dependent or increased by oral leucine supplementation. J. Nutr. 134, 2114S-2116S.
23. Rutherfurd, S.M., Chung, T.K., Moughan, P.J. 2004. The effect of a commercial microbial phytase preparation on the *in vitro* release of phosphorus and amino acids from selected plant feedstuffs supplemented with free amino acids. J. Anim. Feed Sci. 13, 677-690.
24. Simcock, S.E., Rutherfurd, S.M., Wester, T.J., Hendriks, W.H. 2004. Total selenium concentrations in canine and feline foods commercially available in New Zealand. N.Z. Vet. J. 53, 1-5.
25. Rutherfurd, S.M., Moughan, P.J. 2005. Digestible reactive lysine in selected milk-based products. J. Dairy Sci. 88, 40-48.
26. Torbatinejad, N.M., Rutherfurd, S.M., Moughan, P.J. 2005. Total and reactive lysine contents in selected cereal-based food products. J. Agric. Food Chem. 53, 4454-4458.
27. Bell, K.M., Rutherfurd, S.M., Hendriks, W.H. 2006. The isoflavone content of commercially-available feline diets in New Zealand. N.Z. Vet. J. 54, 103-108.
28. Williams, P.A., Hodgkinson, S.M., Rutherfurd, S.M., Hendriks, W.H. 2006. Lysine content in canine diets can be severely heat damaged. J. Nutr. 136, 1998S-2000S.
29. Rutherfurd, S.M., Darragh, A.J., Hendriks, W.H., Prosser, C.G., Lowry, D. 2006. True ileal amino acid digestibility of goat and cow milk infant formulas. J. Dairy Sci. 89, 2408-2413.
30. Rutherfurd, S.M., Darragh, A.J., Hendriks, W.H., Prosser, C.G., Lowry, D. 2006. Mineral retention in three-week-old piglets fed goat and cow milk infant formulas. J. Dairy Sci. 89, 4520-4526.
31. Rutherfurd, S.M., Torbatinejad, N.M., Moughan, P.J. 2006. Available (ileal digestible reactive) lysine in selected cereal-based food products. J. Agric. Food Chem. 54, 9453-9457.

32. Rutherfurd, S.M., Kitson, T.M., Woolhouse, A.D., McGrath, M. C., Hendriks, W.H. 2007. Felinine stability in the presence of selected urine compounds. Amino Acids. 32, 235-242.
33. Rutherfurd, S.M., Chung, T.K., Moughan, P.J. 2007. The effect of a commercial enzyme preparation on apparent metabolizable energy, the true ileal amino acid digestibility, and endogenous ileal lysine losses in broiler chickens. Poult. Sci. 86, 665-672.
34. Rutherfurd, S.M., Markwick-Rutherfurd, K.J., Moughan, P.J. 2007. Available (ileal digestible reactive) lysine in selected pet foods. J. Agric. Food Chem. 55, 3517-3522.
35. Rutherfurd, S.M., Schneuwly, A., Moughan P.J. 2007. Analyzing sulfur amino acids in selected feedstuffs using least-squares nonlinear regression. J. Agric. Food Chem. 55, 8019-8024.
36. Deglaire, A., Moughan, P.J., Rutherfurd, S.M., Bos, C., Tome, D. 2007. Feeding dietary peptides to growing rats enhances gut endogenous protein flows compared with feeding protein-free or free amino acid-based diets. J. Nutr. 137, 2431-2436.
37. Fontaine, J., Zimmer, U., Moughan, P.J., Rutherfurd, S.M. 2007. Effect of heat damage in an autoclave on the reactive lysine contents of soy products and corn distillers dried grains with solubles. Use of the results to check on lysine damage in common qualities of these ingredients. J. Agric. Food Chem. 55, 10737-10743.
38. Deglaire, A., Moughan, P.J., Bos, C., Petzke, K., Rutherfurd, S.M., Tomé D. 2008. A casein hydrolysate does not enhance gut endogenous protein flows compared with intact casein when fed to growing rats. J. Nutr. 138, 556-561.
39. Rutherfurd, S.M., Moughan, P.J. 2008. Effect of elevated temperature storage on the digestible reactive lysine content of unhydrolysed- and hydrolysed-lactose milk-based products. J. Dairy Sci. 91, 477-482.
40. Rutherfurd, S.M., Moughan, P.J., Lowry, D., Prosser, C.G. 2008. Amino acid composition determined using multiple hydrolysis times for three goat milk formulations. Int. J. Food Sci. Nutr. 59, 679-690.
41. Balan, P., Han, K-S, Rutherfurd, S.M., Singh, H., Moughan, P.J. 2009. Orally administered ovine serum immunoglobulins influence growth performance, organ weights, and gut morphology in growing rats. J. Nutr. 139, 244-249.
42. Ravindran, V., Morel, P.C.H., Rutherfurd, S.M., Thomas, D.V. 2009. Endogenous flow of amino acids in the avian ileum as influenced by increasing dietary peptide concentrations. Brit. J. Nutr. 101, 822-828.
43. Awati, A., Rutherfurd, S.M., Plugge, W., Reynolds, G.W., Marrant, H., Kies, A.K., Moughan, P.J. 2009. Ussing chamber results for amino acid absorption of protein hydrolysates in porcine jejunum must be corrected for endogenous protein. J. Sci. Food Agric. 89, 1857-1861.
44. Awati, A., Rutherfurd, S.M., Kies, A.K., Veyry, A., Moughan, P.J. 2009. Endogenous lysine in ileal digesta in the growing rat determined using different methods. J. Sci. Food Agric. 89, 2200-2206.
45. Rutherfurd, S.M. 2009. Accurate determination of the amino acid content of selected feedstuffs. Int. J. Food Sci. Nutr. 60 Suppl. 7, 53-62.
46. Kaur, L., Rutherfurd, S.M., Moughan, P.J., Drummond, L. and Boland, M.J. 2010. Actinidin enhances protein digestion in the small intestine as assessed using an *in vitro* digestion model. J. Agric. Food Chem. 58, 5074-5080.

47. Kaur, L., Rutherfurd, S.M., Moughan, P.J., Drummond, L. and Boland, M.J. 2010. Actinidin enhances gastric protein digestion as assessed using an *in vitro* gastric digestion model. J. Agric. Food Chem. 58, 5068-5073.
48. Bell, K.M., Rutherfurd, S.M., Hendriks, W.H. 2010. Exposure of growing and adult captive cheetahs (*Acinonyx jubatus*) to dietary isoflavones: twenty years later. J. Anim. Phys. Nutr. In press.
49. Bell, K. M., Rutherfurd, S. M., Cottam, Y. H., Hendriks, W. H. 2010. Evaluation of two milk replacers fed to hand-reared cheetah cubs (Acinonyx jubatus): nutrient composition, apparent total tract digestibility, and comparison to maternal cheetah milk. Zoo. Biol. In press.

Review articles

1. Darragh A.J., Moughan P.J., Rutherfurd S.M., Boisen S. 1995. Amino acids availability in feedstuffs for the growing pig. In Recent Advances in Animal Nutrition in Australia. Pp 23-29 [JB Rowe and JV Nolan, editors]. Armidale, University of New England.
2. Rutherfurd, S.M., Moughan, P.J. 1997. Determining reactive lysine and the digestibility of reactive lysine in heat processed foods. Proc. Nutr. Soc. N.Z. 22, 222-234.
3. Moughan, P.J. Annison, G. Rutherfurd, S.M., Wiseman, J. 1999. The chemical and physical description of feedstuffs. In A quantitative biology of the pig. Pp. 39-69 [I Kyriazakis editor]. Wallingford, UK: CAB International.
4. Rutherfurd, S.M., Moughan, P.J. 2000. Principles of chemical analysis. In Feed Evaluation: Principles and Practice. Pp. 33-43 [PJ Moughan, MWA Verstegen and MI Visser Reyneveld, editors]. Wageningen, Netherlands: Wageningen Pers.
5. Rutherfurd, S.M., Moughan, P.J. 2000. Developments in the determination of protein and amino acids. In Feed Evaluation: Principles and Practice. Pp. 45-56 [PJ Moughan, MWA Verstegen and MI Visser Reyneveld, editors]. Wageningen, Netherlands: Wageningen Pers.
6. Rutherfurd, S.M., Rutherfurd-Markwick, K.J. and Hendriks, W.H. 2002. Model animals for human nutritional studies. Proc. Nutr. Soc. N.Z. 27, 25-35.
7. Simcock, S.E., Rutherfurd, S.M., Hendriks, W.H. 2002. The role of selenium in companion animal health and nutrition. In Nutritional Biotechnology in the Feed and Food Industries Proceedings of Alltech's 18th Annual Symposium. Pp. 511-520 [TP Lyons and KA Jacques, editors] Nottingham, England: Nottingham University Press.
8. Rutherfurd, S.M., Moughan, P.J. 2007. Development of a novel bioassay for determining the available lysine contents of foods and feedstuffs. Nutr. Res. Rev. 20, 3-16.
9. Rutherfurd, S.M., Moughan, P.J. 2008. The determination of sulfur amino acids in foods as related to bioavailability. J. AOAC Int. 91, 907-913.
10. Moughan, P.J., Rutherfurd, S.M. 2008. Available lysine in foods: A brief historical overview. J. AOAC Int. 91, 901-906.
11. Rutherfurd, S.M., Sarwar-Gilani, G. 2009. Amino acid analysis. Curr. Protoc. Protein. Sci. 58, 11.9.1-11.9.37.

12. Rutherfurd, S.M. and Dunn, B. 2010. Quantitative amino acid analysis. Curr. Protoc. Protein. Sci. In press.
13. Rutherfurd, S.M. 2010. Methodology for the determination of degree of hydrolysis of proteins in hydrolysates: A review. J. AOAC Int. In press.
14. Prosser, C.G., Rutherfurd, S.M. 2010. Glutamine and Taurine. In Handbook of Analysis of Active Compounds in Functional Foods. [LML Nollet and F Toldrá, editors] CRC Press, a Taylor & Francis Company. In press.

Abstracts

1. Sauter, S.L., Rutherfurd, S.M., Wagener, C., Shively, J.E., Hefta, S.A. 1990. Role of non-specific cross-reacting antigen (NCA), a granulocyte membrane glycoprotein, in bacterial binding: Demonstration of preferential binding of a unique glycoform to Escherichia Coli expressing type 1 fimbriae. 1st International CEA-Workshop, Montreal, Canada.
2. Rutherfurd, S.M., Moughan, P.J. 1993. Use of free or bound amino acid molecular weights in the determination of amino acid compositions. In Manipulating Pig Production IV. Pp 229 [ES Batterham, editor]. Attwood, Victoria: Australasian Pig Science Association.
3. Rutherfurd, S.M., Hodgkinson, S.M., Moughan, P.J. 1995. The rat as a model for the pig for determining true ileal reactive lysine digestibility. In Manipulating Pig Production V. Pp 35 [ES Batterham, editor]. Attwood, Victoria: Australasian Pig Science Association.
4. Rutherfurd, S.M., Moughan, P.J. 1995. A new method for determining available lysine in feedstuffs. In Manipulating Pig Production V. Pp 25 [ES Batterham, editor]. Attwood, Victoria: Australasian Pig Science Association.
5. Rutherfurd, S. M., Moughan, P. J. 1997. Evaluation of a new available lysine assay. In Manipulating Pig Production VI. Pp 232 [ES Batterham, editor]. Attwood, Victoria: Australasian Pig Science Association.
6. Rutherfurd, S.M., Edwards, A.C. Selle, P.H. 1997. Effect of phytase on lysine-rice pollard complexes. In Manipulating Pig Production VI. Pp248 [ES Batterham, editor]. Attwood, Victoria: Australasian Pig Science Association.
7. Zou, M.L., Rutherfurd, S.M., Rutherfurd, K.J., Moughan, P.J. 2002. Assessment of the accuracy of Atwater's general factors calculating metabolisable energy in New Zealand foodstuffs. Proc. Nutr. Soc. N.Z. 27, 117.
8. Simcock, S.E., Rutherfurd, S.M., Hendriks, W. H. 2002. Selenium content of commercial pet foods. Proc. Nutr. Soc. N.Z. 27, 115.
9. Prosser, C.G., McLaren, R., Rutherfurd, S.M., Darragh A.J., Hendriks, W.H., Lowry, D. 2003. Digestion of milk proteins from cow or goat milk infant formula. The 11th Asian Congress of paediatrics & The 1st Asian Congress on paediatric Nursing. Pp 87.
10. Rutherfurd-Markwick, K.J., Rutherfurd, S.M., Gill, H.S., Moughan, P.J. 2003. Bioactive peptides and human health. Worldnutra's 4th International Conference and Exhibition on Nutraceuticals and Functional Foods. September 29-October 1 Las Vegas, Nevada, USA
11. Ravindran, V., Rutherfurd, S.M., Thomas, D.V. 2005. Influence of dietary peptide levels on endogenous nitrogen flow in broiler chickens. Proc. Aust. Poult. Sci.

Symp. 17, 296-297.
12. Darragh A.J., Prosser C., Rutherfurd S.M., Hendriks W.H., Lowry D. 2005. Protein quality of dairy goat milk for inclusion in infant formulas. Proc. Perinat. Soc. Aust. N.Z. Pp 37.
13. Williams, P.A., Hodgkinson, S.M., Rutherfurd, S.M., Hendriks, W. H. 2005. Lysine content in commercial canine growth and maintenance diets can be severely heat damaged. In: Innovations in Companion Animal Nutrition. The Waltham International Sciences Symposium. Waltham Centre for Pet Nutrition. Pp 43.
14. Rutherfurd, S.M., Moughan, P.J., Kruger M., Lowry D., Prosser C.G. 2007. An accurate determination of amino acids in goat milk. 5th International Symposium on The Challenge to Sheep and Goats Milk Sectors. In Press
15. Awati, A., Moughan, P.J., Rutherfurd, S.M., Reynolds, G.W., Kies, A. 2007. Hiccups in the application of Ussing chamber techniques to amino acid absorption kinetics. Annals of Nutr. Metabol. 51, 207 Suppl. 1.
16. Rutherfurd, S.M., Moughan, P.J. 2007. The analysis of sulphur amino acids in foods as related to bioavailability. AOAC International symposium on "Accurate Analysis of Amino Acids and Bioactive Peptides in Foods and Dietary Supplements" at the 121th AOAC International Annual Meeting to be held in Anaheim, California. Pp 51.
17. Moughan P.J., Rutherfurd S.M. 2007. An overview of lysine bioavailability in processed foods – The need for new analytical approaches. AOAC International symposium on "Accurate Analysis of Amino Acids and Bioactive Peptides in Foods and Dietary Supplements" at the 121th AOAC International Annual Meeting to be held in Anaheim, California. Pp 50.
18. Weidgraaf, K., Rutherfurd, S.M., O'Flaherty, K.A., Thomas, D.G., Rutherfurd-Markwick, K.J. 2007. Effect of dietary form on nutrient digestibility in the cat and dog. Nestle Purina Nutrition Forum: Focus on Felines.
19. Rutherfurd, S.M., Moughan, P.J. 2009. Determining available lysine in processed feedstuffs. Proceedings of the Massey University Advancing Pork Production Seminar. Palmerston North, New Zealand. Pp 7-10.
20. Kaur, L., Rutherfurd, S.M. Boland, M.J., Moughan, P J. 2009. Eating kiwifruit: A key to healthy digestion. Proceedings of the NZIFST conference, Christchurch, New Zealand. Pp 34.
21. Moughan, P.J., Coles, L. Rutherfurd, S.M., Zou, M.L. 2010. Describing dietary energy – do we need a new approach. Proceedings of the Nutrition Society of Australia. 33, 76.
22. Zhu, X., Moughan, P.J., Rutherfurd, S.M. 2010. Eating kiwifruit: A comparison of selected methods for determining eicosapentanoic acid and docosahexaenoic acid in cereal-based foods. Proceedings of the NZIFST conference, Auckland, New Zealand. Pp 40.

About The Author

Shane M^{c}Artney Rutherfurd was born in Elsdon, New Zealand, a small town just north of the capital city, Wellington. He attended Massey University in Palmerston North, New Zealand from 1983 to 1988, where he obtained a Bachelors of Science (majoring in biochemistry) and a Masters of Science also majoring in biochemistry. From 1989-1992, he worked at the Beckman Research Institute of the City of Hope National Medical Center, Los Angeles, California, where he worked in the cancer research field on a project aimed at determining the oligosaccharide structures of the glycoprotein, non-specific cross-reacting antigen. In 1992, he returned to Massey University and took up a position as a research officer at the Monogastric Research Centre where he worked with Professor Paul Moughan conducting research in a number of topics within the nutrition and feed evaluation science fields. In 2002, he was promoted to Senior Research Officer within the newly formed Institute of Food, Nutrition and Human Health, Massey University. In 2007, he started with the prestigious Riddet Institute, Massey University again working with Professor Paul Moughan and is now responsible for the delivery of the nutrition research platform within the Institute.

Correspondence address:
Mr Shane M. Rutherfurd
Riddet Institute, Massey University
Tennent Drive, Palmerston North
New Zealand.
Phone 64 6 350 5894, Fax 64 6 350 5655.
Email: S.M.Rutherfurd@massey.ac.nz

Acknowledgements

Firstly I sincerely would like to thank my supervisors Professor Wouter Hendriks, Professor Martin Verstegen and Professor Paul Moughan for their contribution to this thesis and many useful discussions. In particular though I would like to pay special mention to Professor Paul Moughan with whom I have worked with for almost 20 years. Professor Moughan has mentored me and guided my career over that period and a large amount of the success I have attained is due to the opportunities Professor Moughan has provided me.

I would also like to thank Dr Patrick Morel for his statistical advice, Dr Kay Rutherfurd-Markwick and Dr Nour Mohammad Torbatinejad for their useful discussions, Miss Leonie van Osch, Dr Katherine Whitehouse-Tedd (Bell), Dr Suzanne Hodgkinson, Ms Margret Hekman and Mr Darren Hartley for assistance with the animal trials and Professor Ravi Ravindran for his encouragement.

I would also like to thank my wife Kelly-Ann Rutherfurd, for proof reading this thesis and providing encouragement and support.

The research described in this thesis was financially supported by the Australian Pig Research and Development Corporation, New Zealand Dairy Board and Monogastric Research Centre, Massey University.

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.
Financial support from the Riddet Institute, Massey University for the production and defence of this thesis is also gratefully acknowledged.