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REVIEW ARTICLE

Use of the guanidination reaction for determining reactive lysine, bioavailable lysine and gut endogenous lysine

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Abstract Determining the bioavailability of lysine in foods and feedstuffs is important since lysine is often the first limiting indispensable amino acid in diets for intensively farmed livestock (pigs and poultry) and also in many cereal-based diets consumed by humans. When foods or feedstuffs are heat processed, lysine can undergo Maillard reactions to produce nutritionally unavailable products. The guanidination reaction, the reaction of O-methylisourea with the side chain amino group of lysine that produces homoarginine, has been used to determine the unmodified lysine (reactive lysine) in processed foods and feedstuffs and also true ileal digestible reactive lysine (bioavailable lysine). The advantages of the guanidination method in comparison with other reactive lysine methods such as the fluorodinitrobenzene, trinitrobenzenesulphonic acid and dye-binding methods are that it is very specific for reactive lysine and also that the method is relatively straightforward to conduct. The specificity of the guanidination reaction for the lysine side chain amino group is particularly important, since ileal digesta will contain N-terminal groups in the form of free amino acids and peptides. The main disadvantage is that complete conversion of lysine to homoarginine is required, yet it is not straightforward to test for complete guanidination in processed foods and feedstuffs. Another disadvantage is that the guanidination reaction conditions may vary for different food types and sometimes within the same food type. Consequently, food-specific guanidination reaction conditions may be required and more work is needed to optimise the reaction conditions across different foods and feedstuffs.

Keywords Homoarginine · Lysine · Guanidination · Reactive · Bioavailable

Introduction

From a nutritional perspective, lysine is a dietary indispensable amino acid since it cannot be synthesised in the bodies of animals and humans. Moreover, lysine is the most limiting (first limiting) amino acid in many cereal-based foods (e.g. rice, wheat, barley, oats, corn, millet), the latter of which are staple foods for much of the world's population. For production animals, lysine is often the first limiting essential amino acid in diets for pigs and the second limiting essential amino acid in diets for poultry. Consequently, information about the amount of lysine present in foods and feedstuffs and its bioavailability is important.

Many food protein sources undergo processing during their manufacture, including heat processing, or may be cooked prior to being consumed. During heating or long-term storage, the amino side chain of lysine can react with other compounds present in the foods and feedstuffs to produce nutritionally unavailable derivatives (e.g. Maillard products) (Hurrell and Carpenter 1981). Traditional amino acid analysis can be inaccurate for measuring lysine in processed foods and feedstuffs that contain some of these modified lysine derivatives (e.g. Amadori compounds) and alternative methods for determining the unmodified lysine must be used. One such method employs the guanidination reaction [the reaction of O-methylisourea (OMIU) and lysine to produce homoarginine] and it is the guanidination reaction within the latter context that will form the

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basis of this review. Specifically, the fate of lysine during processing and the use of guanidination to determine reactive lysine and bioavailable lysine will be discussed. In addition, the use of guanidination to determine endogenous lysine present in the gastrointestinal tract will also be reviewed.

The fate of lysine during heat processing

As mentioned above, during heat processing, cooking or long-term storage at elevated ambient temperatures, the amino side chain of lysine can react with other compounds present in foods (Hurrell and Carpenter 1981). These "other" compounds include reducing sugars, fats and their oxidation products, polyphenols, vitamins, food additives and other amino acids to produce modified lysine derivatives (Hurrell and Carpenter 1981). The most important reaction is that with reducing sugars (Maillard reaction). Essentially, the reducing sugar–lysine Maillard reaction initially involves a reversible condensation reaction which forms a Schiff base (Fig. 1). The Schiff base then undergoes irreversible rearrangement to produce ε-*N*-deoxyketosyllysine (Amadori product) (Finot et al. 1977). The Amadori product, also referred to as the early Maillard product, can then further react to produce melanoidins (advanced Maillard products or advanced glycation products) which are characterised by their brown pigments (Hurrell and Carpenter 1981). While the early Maillard reaction is well understood, the advanced Maillard reactions are less well defined. Both early and late Maillard products are not nutritionally available since they are chemically distinct from lysine and cannot be used in place of lysine for metabolism in the body.

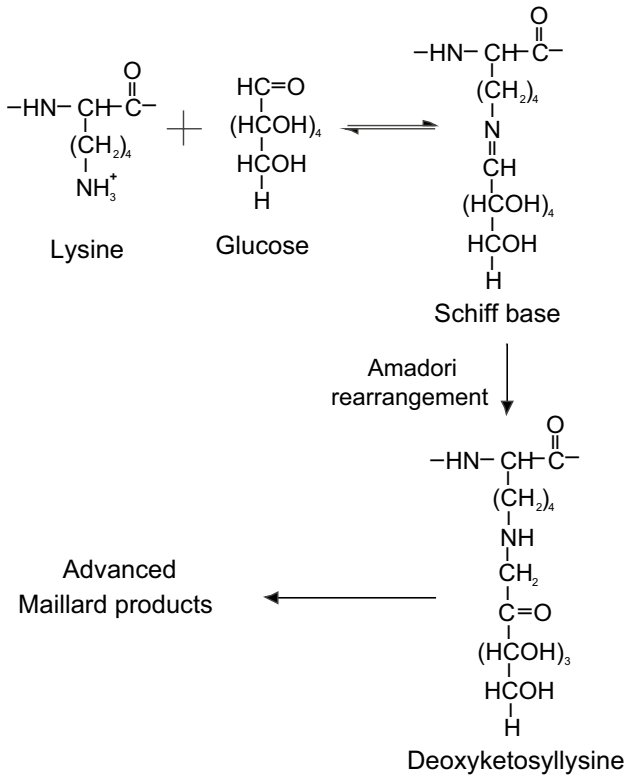

Fig. 1 The reaction of protein-bound lysine with glucose (Maillard reaction)

Clearly, the presence of early and late Maillard products in foods is of consequence to the consumer, since their presence equates to a lower amount of lysine present in a food. However, the presence of early Maillard products also has consequences for analysts attempting to determine the amount of lysine in processed foods and feedstuffs, since the typical amino acid analysis procedure employs an HCl hydrolysis step (6 M HCl at 110 °C for 24 h in an oxygen-free environment) to liberate the amino acids from the protein (Rutherfurd and Gilani 2009). While lysine is stable during HCl hydrolysis, the early Maillard products are not stable. The fate of early Maillard products during HCl hydrolysis has been studied in milk powders where lactulosyl-lysine (the early Maillard product of the reaction between lysine and lactose) has been shown to revert to lysine (Mauron et al. 1955), furosine and to pyridosine (Finot et al. 1969) (Hurrell and Carpenter 1974). From an analytical point of view, the reversion to lysine is problematic since reverted lysine will be co-analysed with the unmodified lysine (i.e. the lysine that has not undergone Maillard reactions) leading to an overestimate of the amount of unmodified lysine present. In contrast to Amadori compounds, the structure of melanoidins is so different

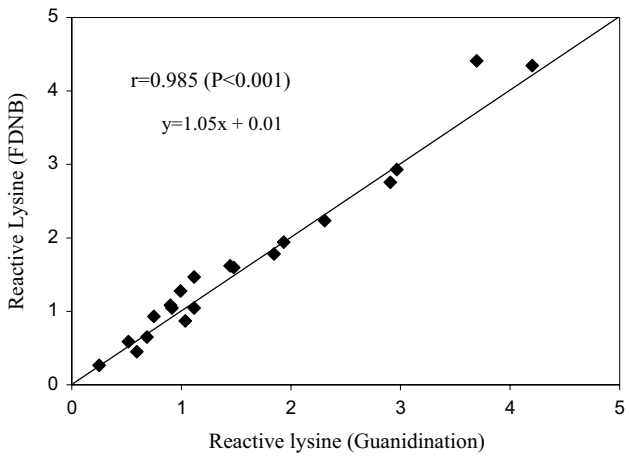

Fig. 2 Reactive lysine content of 20 ready-to-eat breakfast cereals determined using guanidination and the FDNB method. The *solid line* denotes complete agreement between the two methods. Reprinted with permission from Torbatinejad et al. (2005). Total and reactive lysine contents in selected cereal-based food products. J Agric Food Chem 53:4454–4458. Copyright (2005) American Chemical Society

from lysine that it would be virtually impossible for them to revert back to lysine under any circumstances. For other modified lysine derivatives such as isopeptides formed by the cross-linking of lysine with aspartic acid or glutamic acid, reversion of the isopeptide-lysine to lysine during HCl hydrolysis may be complete.

While determining the unmodified lysine present in processed foods and feedstuffs (termed reactive lysine) is important, determining the amount of reactive lysine that is digested and absorbed in the gastrointestinal tract is of greater importance since it reflects the lysine that is available for metabolism in the body. This is because for many food proteins, the digestibility (disappearance from the gastrointestinal tract) can be far less than complete. For example, true ileal reactive lysine digestibility of 81 % has been reported for rajmah (Rutherfurd et al. 2012), 84 % for cooked rolled oats (Rutherfurd et al. 2015) and digestibilities ranging from 53 to 91 % for 19 different ready-to-eat breakfast cereals (Rutherfurd et al. 2006). For the latter foods, the reactive lysine content will markedly overestimate the amount of unmodified lysine that is absorbed into the body and therefore "available" for metabolism in the body.

Describing lysine in foods

Historically, the lysine present in processed foods and feedstuffs has been referred to using a range of descriptors often with little consensus or consistency. For example, lysine has been determined using conventional amino acid analysis and referred to as available lysine (Undi et al. 1996), while reactive lysine has been determined using chemical tests and described as reactive lysine (Hurrell and Carpenter 1974), chemically reactive lysine (Henle et al. 1991), chemically available lysine (Desrosiers et al. 1989), available lysine (Mao et al. 1993) and total available lysine (Rehman 2006). Furthermore, unmodified lysine has been estimated based on the presence of furosine after HCl hydrolysis and referred to as bioavailable lysine (Erbersdobler and Hupe 1991). Moreover, the terms available lysine and bioavailable lysine have been used to describe the unmodified lysine residues that are absorbed from the gastrointestinal tract. Overall, there is clearly a lack of consistency in describing lysine and perhaps there is also misunderstanding around the appropriate terminology for describing lysine as a function of the method used to determine it. A summary of the recommended lysine terminology is presented in Table 1.

Hurrell and Carpenter (1974) have published a clear and logical description for lysine nomenclature. They defined reactive lysine or chemically reactive lysine as the undamaged lysine residues (lysine that has not undergone Maillard reactions or similar and that possesses an unmodified side chain amino group) that has been determined using any chemical method that targets the unreacted ε-amino group of lysine (e.g. guanidination, fluorodinitrobenzene (FDNB), trinitrobenzenesulphonic acid (TNBS), sodium borohydride and dye-binding methods, or the use of furosine as a predictor of undamaged lysine). In addition, Hurrell and Carpenter (1974) defined available or bioavailable lysine as the undamaged lysine residues that are digested and absorbed from the gastrointestinal tract and therefore

Table 1 Lysine terminology and definitions

Recommended term	Definition	Terms that have been used	Method of determination
Reactive lysine	Unmodified lysine, possesses a free side chain amino group and can be either free or protein bound	Reactive lysine Chemically reactive lysine Chemically available lysine Available lysine Total available lysine Bioavailable lysine	Fluorodinitrobenzene Guanidination Dye-binding Trinitrobenzenesulphonic acid Sodium borohydride Furosine
Modified lysine	Lysine for which the side chain amino group has reacted with another compound, e.g. Maillard products	Bound lysine	Cannot be determined directly
Reverted lysine	Lysine that has reverted from modified lysine during the acid hydrolysis step of conventional amino acid analysis	Reverted lysine	Cannot be determined directly
Total lysine	Lysine determined using conventional amino acid analysis. For protein sources that contain modified lysine, total lysine comprises reactive lysine and reverted lysine	Total lysine	Conventional amino acid analysis
Bioavailable lysine	Dietary lysine that is absorbed in a form that can be used for metabolism in the body	Available lysine Bioavailable lysine	True ileal reactive lysine digestibility assay

potentially "available" for body protein synthesis. An additional term, total lysine, has been used to describe the lysine determined using HCl hydrolysis and represents the sum of the reactive and reverted lysine. In this author's view, the term total lysine is misleading, since the reverted lysine does not represent lysine in any nutritional sense but rather is an interfering artefact of the method used to determine lysine in foods and feedstuffs.

Determining reactive lysine

A number of methods have been reported for determining reactive lysine in processed foods. Most are based on chemical reactions with the ε-amino group of lysine but some, such as the furosine method, are based on the presence of reversion products of early Maillard products during HCl hydrolysis. Arguably, the three most commonly reported methods are the FDNB, guanidination and furosine methods.

FDNB method

The FDNB method (Booth 1971; Carpenter 1960) is based on the reaction with FDNB resulting in the formation of dinitrophenyllysine (DNP-lysine). The sample is then hydrolysed in HCl to release the protein-bound DNP-lysine which is then solvent extracted (with DNP-lysine in the aqueous layer) to remove unreacted FDNB and other interfering compounds. The DNP-lysine is then measured photometrically either with or without separation by HPLC. One of the problems with the FDNB method is that DNP-lysine can be degraded during acid hydrolysis leading to an underestimation of the reactive lysine content (Booth 1971).

Furosine method

The principle of the furosine method is based on the fact that when processed foods or feedstuffs that contain early Maillard products undergo HCl hydrolysis, Amadori compounds degrade to a number of derivatives including lysine and furosine. The amount of furosine present can then be used to predict the amount of reverted lysine which can in turn be subtracted from the determined total lysine content to give the reactive lysine content (Almeida et al. 2014). While in principle the latter method is robust, it is an indirect method and therefore likely to be less accurate than direct methods such as the FDNB or guanidination methods. Moreover, the relative proportion of lysine, furosine and pyridosine present after HCl hydrolysis differs for different Amadori compounds. For example, the relative proportion of lysine and furosine present after HCl hydrolysis is 56 and 30 %, respectively, for fructosyl-lysine (derived from the reaction of glucose with lysine), 58 and 34 % respectively for lactulosyl-lysine (derived from the reaction of lactose with lysine) and 29 and 42 % for tagatosyl-lysine (derived from the reaction of galactose with lysine) (Krause et al. 2003). Consequently, the conversion factor used to predict the reverted lysine content from the furosine content in hydrochloric acid hydrolysates is likely to vary depending on the processed foods and feedstuffs being analysed. Food-specific furosine to reverted lysine conversion factors may help to improve the accuracy of the furosine method, but more work needs to be conducted in this area.

Guanidination method

Within the context of determining reactive lysine, the guanidination reaction is the reaction of OMIU with the ε-amino group of lysine to produce homoarginine. For the latter purpose, reactive lysine is converted to homoarginine in the food or feedstuff and the amount of homoarginine is then determined following HCl hydrolysis. Homoarginine is stable under HCl hydrolysis conditions (Chervenka and Wilcox 1956; Klee and Richards 1957). As long as the conversion of reactive lysine to homoarginine prior to HCl hydrolysis is complete, the moles of homoarginine present will be equivalent to the moles of reactive lysine. If Amadori compounds are present in the food or feedstuff, then some reversion of the Amadori compounds back to lysine during HCl hydrolysis may occur, but that is inconsequential since reactive lysine has been converted to homoarginine prior to HCl hydrolysis and it is then that the homoarginine, and not lysine, is quantified.

The guanidination reaction itself is carried out at high pH, since the ε-amino group of lysine, which has a p*K*a of 10.5, must be deprotonated for guanidination to occur. Interestingly, OMIU does not appear to react with the N-terminal amino group of lysine or any other amino acids to any significant extent (Catrein et al. 2005; Maga 1981; Yamaguchi et al. 2005; Zhang et al. 2006) with perhaps the exception of glycine (Allen and Viswanatha 1970; Beardsley and Reilly 2002). The latter characteristic makes guanidination a suitable approach for determining the reactive lysine content of protein hydrolysates and compound animal feeds where synthetic lysine has been supplemented. Why OMIU readily reacts with the ε-amino group of lysine, but not the N-terminal amino group is not clear. The lower p*K*a of the N-terminal amino group as compared with the ε-amino group may be one explanation. However, Allen and Viswanatha (1970) reported very different reactivity towards OMIU for glycine and glutamic acid, yet the p*K*a of each of their respective N-terminal amino groups are very similar (9.60 and 9.67 respectively). The latter workers concluded that it was difficult to explain the selectivity of OMIU based on differences in either the basicity or the

electronic charge of the amino group (Allen and Viswanatha 1970).

As mentioned above, the efficacy of the guanidination method for determining reactive lysine hinges on the fact that the guanidination reaction goes to completion. Consequently, considerable work has been reported focussing on the reaction conditions that convert all of the reactive lysine to homoarginine in processed foods and feedstuffs. The most important factors affecting the extent of guanidination are the pH of the reaction mixture, the time and temperature of incubation and the relative amount of OMIU and lysine in the reaction mixture. Maga (1981) reported that complete guanidination of synthetic lysine took 96 h when conducted at pH 10.5, 20 °C with 0.5 M OMIU. In contrast, Moughan and Rutherfurd (1996) reported 95 % guanidination of synthetic lysine after only 24 h incubation with 0.6 M OMIU, pH 10.6 at 20 °C. After 24 h incubation, Maga (1981) reported only 63 % guanidination of synthetic lysine. However, the latter workers used an OMIU to lysine ratio of 1.4, while Moughan and Rutherfurd (1996) used an OMIU to lysine ratio of 1000 which likely explains the marked difference in the extent of guanidination after 24 h incubation observed across the two studies. Maga (1981) also noted that the pH of the incubation mixture would drop during incubation at a rate of 0.1–0.2 pH units per 24 h and recommended pH adjustment on a daily basis. OMIU can be used as the buffer for maintaining the pH of the guanidination reaction mixture (Imbeah et al. 1996). However, the low OMIU to lysine ratio used by Maga (1981) meant that as OMIU was consumed by the guanidination reaction, the amount of unreacted OMIU (the buffer) decreased and the pH dropped. Moughan and Rutherfurd (1996) did not adjust pH during the reaction and assumed that the high OMIU to lysine ratio (>1000) would lead to a negligible decrease in the unreacted OMIU concentration even after guanidination was complete.

The optimum pH for guanidinating food proteins has also been investigated. Maga (1981) reported that the optimum pH varied across several foods, ranging from 9.4 for whole egg to 10.8 for whole milk, while an optimum pH of 12.0 was reported for distiller dried grains with solubles (DDGS) (Fontaine et al. 2007). The optimum pH for guanidination may also differ within food categories. For example, an optimum pH of 10.3 has been reported to be the optimum pH for soy (Maga 1981), 10.8 for soy protein isolate (Rutherfurd and Moughan 1990) and 11.5 for soybean meals and soybeans (Fontaine et al. 2007). For DDGS, however, Fontaine et al. (2007) and Pahm (2008) reported similar optimum pH values for guanidination (11.4 and 12.0 respectively).

For guanidinating the proteins in foods and feedstuffs, OMIU has been used as either the free base (Eklund et al. 2013; Friesen et al. 2006; Imbeah et al. 1996; Mao et al. 1993; Moughan and Rutherfurd 1996; Pahm et al. 2010; Ravindran et al. 2004; Rutherfurd and Moughan 1990; Torbatinejad et al. 2005) or as the hydrogen sulphate salt (Fontaine et al. 2007; Imbeah et al. 1996). While the use of the free base is more prevalent, both forms of OMIU have been reported to be equally effective at guanidinating proteins (Imbeah et al. 1996). The free base form of OMIU is commonly prepared from the hydrogen sulphate form after removal of the sulphate by precipitation with barium hydroxide.

Overall, while much work has been conducted to optimise guanidination reaction conditions, there remains considerable diversity in the optimal conditions reported. The optimal guanidination conditions appear to vary depending largely on the protein source and it may be necessary to develop food-specific guanidination conditions in the future. More work is desperately required in this area.

Assessing the extent of guanidination for foods or feedstuffs that do not contain early Maillard products is straightforward, since any lysine present after HCl hydrolysis of a guanidinated food or feedstuff can be assumed to be a result of incomplete guanidination. However for processed foods and feedstuffs, the lysine present after HCl hydrolysis may be derived from either incomplete guanidination or from the reversion of the Amadori compounds to lysine (reverted lysine) and discerning the two sources of lysine is difficult. In the latter case, it may be possible to use the furosine method to estimate the amount of reverted lysine to distinguish the reverted lysine from the unguanidinated lysine. However, even using the furosine method may not be accurate for all foods and feedstuffs, as the extent of reversion from Amadori compounds to lysine during acid hydrolysis may differ across different protein sources. Overall, there is currently no straightforward method to assess the extent of guanidination in foods and feedstuffs that contain early Maillard products.

Several studies have compared the guanidination method with other methods for determining reactive lysine. Torbatinejad et al. (2005) reported excellent agreement between the guanidination method and the FDNB method when applied to 20 ready-to-eat breakfast cereals (Fig. 2). Rutherfurd et al. (1997b) also compared the guanidination and FDNB methods and reported excellent inter-method agreement when applied to animal feedstuffs such as blood meal, wheat meal and cottonseed meal (<4 % difference), but poorer agreement (12–16 % difference) for meat and bone meal and soybean meal. Pahm et al. (2008) used both the guanidination and furosine methods to determine reactive lysine in 33 DDGS samples (an animal feedstuff derived from the bioethanol industry). The main aim of the latter study was to attempt to correlate reactive lysine content with standardised ileal amino acid digestibility (amino acid digestibility determined to the end of the small

intestine) in pigs. As a consequence, the complete reactive lysine dataset for the DDGS samples was not presented. However, summary information was presented that suggested that reactive lysine determined using the guanidination method was approximately 12 % lower than equivalent values determined using the furosine method. It should be noted, however, that the furosine to reverted lysine conversion factors used were those derived for milk and may not be accurate for determining reactive lysine in DDGS.

The difference between the total lysine and reactive lysine contents has been used to describe the amount of bound lysine (the lysine bound as Maillard products) as an indicator of lysine damage to processed foods and feedstuffs (Tran et al. 2007). However, the latter strategy is likely to be too simplistic as it assumes that all the modified lysine reverts back to lysine during HCl hydrolysis. The latter assumption is almost certainly flawed given the chemical diversity of the modified lysine derivatives that can be present in processed foods.

Overall, a number of methods have been developed for determining reactive lysine in processed foods and feedstuffs. Each method possesses its own inherent strengths and weakness and in this author's opinion there is no method that clearly sets itself apart as being the method of choice, although the FDNB and guanidination methods are arguably the most commonly used. It is important that the method users fully understand the limitations of each method to obtain the best reactive lysine compositional data.

Determining lysine bioavailability

Bioavailable lysine, as opposed to reactive lysine, takes into account the fact that not all the unmodified lysine present in the food or feedstuff will be absorbed into the body during digestion in the gastrointestinal tract. A number of methods have been developed to determine bioavailable lysine. These include the slope ratio assay (Batterham et al. 1979), the indicator amino acid oxidation technique (Moehn et al. 2005) and the true ileal digestible reactive lysine assay (Moughan and Rutherfurd 1996). These assays are discussed in detail by Rutherfurd and Moughan (2007), but briefly the slope ratio method is based on the comparison of the growth of animals fed either (1) control diets containing known, and first limiting, amounts of bioavailable lysine and (2) the test diet. The indicator amino acid oxidation technique is based on similar principles to the slope ratio assay, but uses amino acid oxidation as the end point rather than animal growth. It is important to note that both the slope ratio and indicator amino acid oxidation assays measure lysine utilisation rather than lysine bioavailability and that, while bioavailability is a function of the food, utilisation is a function not only of the food, but also the metabolic status of the animal or human consuming the food and may also be influenced by dietary nutrient balance.

Bioavailable lysine can also be measured directly as the true ileal digestible reactive lysine which is based on the reactive lysine content of the food or feedstuff and true ileal reactive lysine digestibility (Moughan and Rutherfurd 1996) and this method is particularly applicable to processed foods and feedstuff. For this method, a test food or feedstuff is fed to either test animals or human subjects. Digesta is then collected from the end of the small intestine (terminal ileum) and the reactive lysine content of the diets and digesta determined. The difference between the reactive lysine intake and the ileal reactive lysine outflow represents the reactive lysine that has been digested and absorbed. True ileal reactive lysine digestibility is then calculated by correcting for the endogenous (non-dietary) lysine present in the ileal digesta, which in turn is derived from proteins secreted into the gastrointestinal tract such as mucins, digestive enzymes, serum albumin as well as sloughed epithelial cells and microbes.

Conceptually, any method that determines reactive lysine could be used to determine the reactive lysine in the digesta. However, in practice only the guanidination method and possibly the furosine method are suitable, since other reagents for determining reactive lysine (e.g. FDNB, TNBS, dye-binding methods) can react with N-terminal amino groups in addition to the side chain amino group of lysine. While this is not problematic for determining reactive lysine in foods and feedstuffs, since almost all the N-terminal amino groups are bound within peptide bonds, it can be a problem for ileal digesta as significant amounts of peptides and free amino acids are present. For example, ileal digesta from growing pigs (Moughan and Schuttert 1991) and growing rats (Butts et al. 1992) has been reported to contain 69 and 21 % respectively of the total nitrogen as peptides and amino acids. Since OMIU does not react with the N-terminal groups of lysine (or any other amino acid), it is an ideal reagent for determining reactive lysine in digesta. Moreover, homoarginine is not found naturally in body proteins and is therefore not naturally present in ileal digesta (Angkanaporn et al. 1997).

The composition of ileal digesta is quite different from that of foods and feedstuffs and contains endogenous proteins as well as undigested dietary proteins and peptides. Consequently, the guanidination conditions for ileal digesta may not be the same as for foods and feedstuffs in general or for the specific food or feedstuff that has been consumed. Moughan and Rutherfurd (1996) reported that a pH of 11.0 to 11.4 and an incubation time of 7 days was optimal for ileal digesta collected from rats fed either unheated casein or a heated lactose–casein mixture. Moreover, these

workers reported that the reaction mixture pH had a much greater effect on the extent of guanidination for the digesta of rats fed the heated lactose/casein mixture as compared to digesta collected from rats fed unheated casein. Specifically, the extent of guanidination doubled when the reaction mixture pH was increased from 10.2 to 11.0 for the heated lactose/casein digesta, as compared to the unheated casein digesta where the same pH change caused no significant change to the extent of guanidination. For ileal digesta collected from pigs fed DDGS, Pahm (2008) reported a wide pH range of 10.0–12.0 and a reaction time of between 1 and 6 days was optimal. Overall, it would be prudent to establish the optimal guanidination conditions for ileal digesta for a wide range of foods and feedstuffs, but to the author's knowledge this has not yet been reported.

True ileal amino acid digestibility uses conventional amino acid analysis to determine amino acids in diets and digesta. Consequently with respect to lysine, it is true ileal digestibility of total lysine, rather than reactive lysine, which is determined. Since total lysine represents the sum of the reactive lysine and reverted lysine, if early Maillard products are present in the diets and digesta, estimates of true ileal total lysine digestibility will not accurately reflect the true ileal reactive lysine digestibility (lysine bioavailability). Generally, the greater the amount of early Maillard products present, the greater is the inaccuracy of true ileal total lysine digestibility estimates. This is exemplified in Table 2 which shows the total and reactive lysine content, true ileal total and reactive lysine digestibility and true ileal digestible total and reactive lysine content of skim milk powder autoclaved at 121 °C for 1 to 10 min. True ileal total and reactive lysine digestibilities were similar in the unheated skim milk powder. For the heated powders, true ileal total and reactive lysine digestibility both decreased with increasing heating time, but total lysine digestibility decreased to a much greater extent. The greater decrease in total lysine digestibility as compared to that for reactive lysine may appear to be contradictory, since the total lysine content in the heated skim milk powders decreased to a lesser degree when compared to reactive lysine. The apparent contradiction can be explained by the formation of limit peptides (Rutherfurd and Moughan 2007). Limit peptides are relatively large indigestible peptides that cannot be absorbed and most likely result from the inability of trypsin to cleave at lysine residues that have undergone Maillard reactions (Moughan et al. 1996).

To the author's knowledge, only one published study exists that has aimed to determine the accuracy for estimating lysine bioavailability. Using a growth study approach in pigs and using a heated skim milk powder as a model protein source, Rutherfurd et al. (1997a) were able to report that true ileal reactive lysine digestibility, but not true ileal total lysine digestibility, was an accurate predictor of lysine bioavailability. This type of validation is desperately needed for other types of foods and feedstuffs.

Using guanidination to determine endogenous ileal lysine losses

While guanidination has been used to determine both reactive lysine and bioavailable lysine, it has also been used in the determination of endogenous ileal lysine losses. Determining endogenous ileal amino acid (including lysine)

Table 2 Lysine content (g/kg), true ileal lysine digestibility (%) and true ileal digestible lysine content (g/kg) of unheated and heated skim milk powder determined in the growing rat. Adapted from Rutherfurd and Moughan (1997)

Heating	Lysine content		True ileal lysine digestibility		Digestible lysine content	
Time[a] (min)	Total[b]	Reactive[c]	Total[d]	Reactive[e]	Total[f]	Reactive[g]
Unheated	38.1	38.1	96.6	100.0	36.8	38.1
1	35.7	28.1	88.5	99.4	31.6	28.0
3	28.6	17.7	69.1	94.0	19.8	16.6
5	26.5	11.9	51.6	92.3	13.7	11.0
10	25.6	6.7	43.7	84.7	11.2	5.7

[a] Skim milk powder was autoclaved at 121 °C for 1, 3, 5 and 10 min

[b] Determined using conventional amino acid analysis

[c] Determined using guanidination (conversion of lysine to homoarginine)

[d] Determined using conventional amino acid analysis of diets and digesta

[e] Determined using the guanidination (conversion of lysine to homoarginine) of diets and digesta

[f] Calculated as the total lysine content of the skim milk powder sample multiplied by the true ileal total lysine digestibility

[g] Calculated as the reactive lysine content of the skim milk powder sample multiplied by the true ileal reactive lysine digestibility

losses is important as these values are required for correcting apparent estimates of ileal amino acid digestibility to true estimates of ileal amino acid digestibility.

A number of techniques have been published for determining endogenous ileal amino acid losses. The traditional technique involves feeding a protein-free diet ensuring that any amino acids present in ileal digesta must be of endogenous origin (de Lange et al. 1989). However, the protein-free technique is flawed for a number of reasons (de Lange et al. 1989), including the fact that the amount of endogenous protein present in the gastrointestinal tract is much greater when protein or peptides are present in the diet (Hodgkinson et al. 2000; Hodgkinson and Moughan 2007). Consequently, while determining endogenous ileal amino acid losses in animals or humans given diets that contain protein is preferable, it raises the problem in that the protein/amino acids of endogenous origin must be distinguished from the undigested dietary protein/peptides and amino acids. Making this distinction is not straightforward and a wide range of methods have been developed in an attempt to differentiate endogenous from undigested dietary amino acids in ileal digesta. Many of these methods have been reviewed thoroughly (Moughan 2003; Stein et al. 2007), so this review will focus only on the use of the guanidination reaction for this purpose. In contrast to many of the methods for determining endogenous ileal amino acid loss, the guanidination method determines the losses of lysine only. This drawback, however, has been overcome to some degree by determining the endogenous ileal lysine loss using guanidination and then estimating the losses of the remaining amino acids based on the published ratio of endogenous amino acids to endogenous lysine (Marty et al. 1994; Nyachoti et al. 1997).

When determining the endogenous ileal lysine loss, the diet is first guanidinated and then fed to test animals or human subjects, from which ileal digesta are collected and the lysine content analysed. In contrast to using guanidination for determining bioavailable lysine, the amount of homoarginine in the digesta need not be determined as long as guanidination is complete. This is because homoarginine is not being used as a proxy for reactive lysine (as is the case for determining reactive and bioavailable lysine), but rather guanidination is used to make a lysine-free protein. Consequently, any lysine present in the ileal digesta of an animal or human fed the lysine-free protein must be of endogenous origin and therefore it is lysine and not homoarginine that needs to be analysed. Furthermore, there is no need to make any assumptions about how guanidination might affect the digestibility of the dietary protein source, since there will be no undigested dietary lysine present in the digesta that would interfere with the determination of endogenous lysine. What may be an issue, however, is if during the guanidination process, changes are made to the guanidinated material that impact endogenous ileal amino acid losses directly. One example may be the removal of anti-nutritional factors during the washing step (used to remove unreacted OMIU) which may in turn lead to a reduction in the amount of endogenous losses (Eklund et al. 2013). If guanidination of the diet is not complete, then any residual undigested dietary lysine present in ileal digesta can be corrected based on the digestibility of homoarginine present in the diet (Marty et al. 1994; Moughan and Rutherfurd 1990; Nyachoti et al. 2002), where it is assumed that the true ileal homoarginine digestibility is equivalent to the true ileal lysine digestibility. However, in this case homoarginine does need to be determined in the digesta.

Very few studies reporting the use of guanidination for determining endogenous ileal lysine loss have achieved complete guanidination of test protein sources or complex diets even when the guanidination reaction conditions have been optimised. There may be a number of reasons for this, but the most likely is that most dietary protein sources are not soluble in the guanidination mixture and therefore the physical penetration of the OMIU reagent into the particles and further penetration into the internal structure of the proteins make it difficult to obtain complete guanidination of many food protein sources. This may be particularly important for plant protein sources which contain fibrous material (e.g. bran layer and hulls seeds) where grinding to a very small particle size may not always be possible. Consequently, the assumption that true ileal homoarginine digestibility is equivalent to true ileal lysine digestibility is pivotal, particularly if the extent of guanidination is low, or for studies where guanidinated protein sources are diluted with their undiluted counterparts prior to testing (Nyachoti et al. 2002). Several studies have been conducted, however, that have compared the in vitro digestion and apparent and true ileal amino acid digestibility of unguanidinated and guanidinated dietary protein sources (Caine et al. 2008; Eklund et al. 2013; Nyachoti et al. 2002; Schmitz et al. 1991). However in this author's view, these study designs do not demonstrate whether lysine and homoarginine digestibility within the guanidinated protein is the same, as that would require the determination of the true ileal digestibility of homoarginine and unguanidinated lysine with the same partially guanidinated protein source.

Another problem with using homoarginine digestibility as a proxy for lysine digestibility is that it is assumed that the guanidination process itself does not affect amino acid digestibility per se. An example of one potential issue is amino acid racemisation which can occur under alkali guanidination conditions and can in turn affect digestibility, at least in vitro (Bunjapamai et al. 1982). To combat any possible racemisation during guanidination, an incubation temperature of 4 °C, rather than room temperature, has been used and largely eliminates the problem of racemisation (de Vrese et al. 1994). Certainly, Nyachoti et al.

(2002) have carried out guanidination at 4 °C and reported that for unguanidinated and guanidinated barley and canola meal-based diets the apparent ileal digestibilities of amino acids including lysine (unguanidinated diet) and lysine plus homoarginine (guanidinated diet) were similar. In contrast, however, Ekland et al. (2013) also carried out guanidination at 4 °C and reported that for some feedstuffs (rapeseed meal, soybean meal and peas), the standardised ileal digestibility of most amino acids was quite different between unguanidinated and guanidinated feedstuffs. More work is therefore required to develop guanidination conditions that do not interfere with protein digestion in the gastrointestinal tract if the apparent digestibility of homoarginine is to be used as a measure of the true ileal digestibility of lysine. Alternatively, the greater the extent of guanidination of the test food or feedstuff, the lesser is the importance of using homoarginine digestibility as a proxy for lysine digestibility. Consequently, more work on developing reaction conditions that yield the complete guanidination of complex food and feed materials, without influencing true digestibility or endogenous gut amino acid losses, may be a better alternative.

Another assumption for the guanidination method is that homoarginine is not recycled back into the gut lumen. This assumption has been validated in pigs in a study where homoarginine was infused into the jugular vein and little homoarginine (0.12 %) was found in terminal ileal digesta (Schmitz et al. 1991). Moreover, Angkanaporn et al. (1997) reported a similar negligible re-secretion of absorbed homoarginine back into the gastrointestinal tract lumen of chickens.

The accuracy of the guanidination method has been tested by comparison with other methods for determining endogenous ileal amino acid losses, in particular, the enzyme-hydrolysed casein/ultrafiltration method (Butts et al. 1991; Moughan et al. 1990). The latter method is arguably the gold standard for this purpose, since it determines endogenous losses during protein/peptide alimentation. Overall good agreement between the enzyme-hydrolysed casein/ultrafiltration approach and the use of guanidinated casein has been reported for the growing rat (Awati et al. 2009; Hodgkinson et al. 2003), broiler chicken (Ravindran et al. 2004) and growing pig (Hodgkinson et al. 2003). Moreover, good agreement has also been reported between the enzyme-hydrolysed casein/ultrafiltration approach and the use of guanidinated gelatin (Rutherfurd and Moughan 1990).

Overall, many methods have been developed to determine endogenous ileal amino acid losses and that fact in itself may suggest that there is no one ideal method. While the guanidination method has its disadvantages, the main one being that it only applies to lysine, the single most important advantage is that it can be applied to any food or feedstuff directly, thereby permitting the determination of specific endogenous ileal lysine losses. This is particularly important for foods or feedstuffs that contain anti-nutritional factors or dietary fibre that can elicit a greater endogenous ileal amino acid loss as compared to foods without anti-nutritional factors or fibre (Fuller 2004).

Conclusions

The guanidination reaction is an important reaction for food evaluation science. It is arguably the most suitable method for determining reactive lysine, the only direct method for determining bioavailable lysine (true ileal digestible reactive lysine) and one of the few methods that allows the determination of specific endogenous ileal lysine losses. The main advantage of the guanidination reaction is its specificity for the lysine side chain amino group which is particularly important when determining bioavailable lysine. Its main disadvantage is that complete conversion of lysine to homoarginine is required when determining reactive lysine and bioavailable lysine and is also preferable when determining endogenous lysine losses. However, given the chemical and physical diversity of materials (foods, feedstuffs and digesta) to which the guanidination reaction is applied, it is unlikely that a single set of guanidination reaction conditions is applicable for all cases. Consequently, it is the view of this author that if more work is to be done is this area, the focus should be on developing food-specific reactions conditions for complete guanidination and developing methods that can prove that guanidination is complete for processed foods and feedstuffs.

Conflict of interest The author declares that there is no conflict of interest.

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