Protein quality of pig diets

Processing effects on amino acid digestibility and post-absorptive utilization

Tetske G. Hulshof

Thesis committee

Promotor
Prof. Dr W. H. Hendriks
Professor of Animal Nutrition
Wageningen University

Co-promotors
Dr P. Bikker
Wageningen Livestock Research
Wageningen University & Research

Dr A. F. B. van der Poel
Associate Professor, Animal Nutrition Group
Wageningen University

Other members
Prof. Dr M. A. J. S. van Boekel, Wageningen University
Prof. Dr J. E. Lindberg, Swedish University of Agricultural Sciences, Uppsala, Sweden
Dr J. van Milgen, INRA, Rennes, France
Dr T. A. T. G. van Kempen, Trouw Nutrition, Boxmeer, the Netherlands

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

Protein quality of pig diets

Processing effects on amino acid digestibility and post-absorptive utilization

Tetske G. Hulshof

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A. P. J. Mol,
in the presence of the
Thesis Committee appointed by the Academic board
to be defended in public
on Wednesday 2 November 2016
at 4 p.m. in the Aula.

Tetske G. Hulshof
Protein quality of pig diets
Processing effects on amino acid digestibility and post-absorptive utilization,
176 pages.

PhD thesis, Wageningen University, Wageningen, NL (2016)
With references, with summary in English

ISBN 978-94-6257-902-6
DOI http://dx.doi.org/10.18174/388361

Table of contents

Chapter 1

General introduction

1.1 Increased need for an efficient use of ingredients

The world population is expected to increase from 7.3 billion in 2015 to 9.7 billion in 2050 and 11.2 billion in 2100 which can mainly be attributed to growth in the developing world (UN, 2015). To feed this growing world population, food production should increase by 70% in 2050 and, thus, the demand for plant and animal-based products increases the coming years. Moreover, the expected increase in per capita income will further increase the demand for food products that are responsive to higher incomes, such as livestock and dairy products, and vegetable oils. These livestock and dairy products are important protein sources for humans (FAO, 2009). Plant-based ingredients are needed to produce such products. The subsequent increasing demand for protein coming from plant- or animal-based ingredients, for both human food and animal feed, could result in protein scarcity. There is, thus, a need to use current ingredients more efficiently and to search for alternative ingredients for both humans and animals. The protein quality of ingredients should be accurately assessed before ingredients can be used more efficiently.

1.2 Protein quality

Protein quality of feed ingredients is defined in this thesis as the capacity of a dietary protein to meet an animal's requirements for N and amino acids (AA; Boye et al., 2012; FAO, 2013). Animals require AA to meet metabolic demands for protein synthesis (FAO, 2013) and for specific metabolites (Millward et al., 2008), for example hormones or immunoglobulins. In protein research, a distinction is made between indispensable (or essential) and dispensable (or non-essential) AA which are listed in Table 1.1. Animals have a dietary requirement for indispensable AA which cannot be produced by the body and for essential precursor AA because these can only be produced from indispensable AA. Indispensable AA can only be efficiently used when adequate levels of dispensable AA are provided (Boye et al., 2012) and, hence, animals have a dietary requirement for dispensable AA as well. Moreover, a mismatch between the body AA composition and the dietary AA pattern will result in an inefficient utilization of AA (Boisen, 1997). Protein quality depends on AA bioavailability which includes AA digestibility, absorption, and subsequent utilization for protein synthesis and the associated inevitable losses from AA catabolism (Kies, 1981; Mauron, 1990; Friedman, 1996b; Boye et al., 2012). The AA bioavailability and, thus, protein quality depends on the origin of the ingredient (for example, Chinese vs. Argentine soybeans or soybean meal (SBM) from USA, Brazil or Argentina; Qin et al., 1998; Frikha et al., 2012), cultivar (for example, canola meal from *Brassica napus* or *Brassica campestris*; Bell and Keith, 1991), growing conditions (for example, fertilization of sulfur-deficient soil; Finlayson et al., 1970), storage conditions

(Hurrell et al., 1983; Mavromichalis and Baker, 2000), analytical variation in the determination of the AA content (for example, different optimum hydrolysis intervals for each AA; Moughan, 2003), and processing conditions (for example, Fernandez and Parsons, 1996). Processing can induce protein denaturation which will inactivate certain anti-nutritional factors and, thereby, enhances protein quality (Messerschmidt et al., 2012; Boye et al., 2012). However, severe processing can induce reactions among AA and between AA and other nutrients. These reactions render the AA unavailable (Hurrell and Carpenter, 1981) and, thus, reduce protein quality.

Table 1.1. Overview of indispensable and dispensable amino acid (AA) for growing pigs (Boisen, 1997)

Indispensable AA	Essential precursor AA[1]	Dispensable AA
Arginine (Arg)		Alanine (Ala)
Histidine (His)		Asparagine (Asn)
Isoleucine (Ile)		Aspartic acid (Asp)
Leucine (Leu)		Glutamine (Gln)
Lysine (Lys)		Glutamic acid (Glu)
Threonine (Thr)		Glycine (Gly)
Tryptophan (Trp)		Proline (Pro)
Valine (Val)		Serine (Ser)
Methionine (Met)	Cysteine (Cys)	
Phenylalanine (Phe)	Tyrosine (Tyr)	

[1]Essential precursor AA have to be consumed with the diet to prevent the use of their respective indispensable AA for their synthesis.

Ingredients are often processed to obtain products with a desired composition mainly for the human food industry. This processing results in the formation of co-products which have a certain composition and are a valuable source of nutrients for several industries such as animal feed. For example, oil is extracted from soybeans by a solvent where after the remaining product is toasted and desolventized resulting in the co-product SBM. Such co-products have an increased CP content compared with the original ingredient but are often not used as ingredient for human food. These co-products, therefore, can be a valuable source of protein for animal feed. The protein quality of these co-products is, however, highly variable due to the factors listed above. For example, the CP and Lys content of 10 wheat distillers dried grains with solubles samples varied from 326-389 g/kg DM and 0.83-3.01 g/100 g CP, respectively. The accompanying standardized ileal digestibility (SID) of N and Lys were also highly variable and ranged between 62 to 88% and 9 to 83%, respectively (Cozannet et al., 2010). Thus, the effects of processing on protein quality can be substantial and are further described in the next section.

1.3 Effect of processing on protein quality

The ultimate effect of processing on protein quality depends on the duration of heating (Kwak and Lim, 2004), temperature (Mauron, 1981; Friedman, 1992; Hendriks et al., 1994), shear (Marsman et al., 1995), pressure (Batterham et al., 1986b), particle size (Messerschmidt et al., 2012), water content and activity (Eichner and Karel, 1972; Hendriks et al., 1994), pH of the reaction environment (Annan and Manson, 1981; Mauron, 1990; Friedman, 1992), and the type and amount of reducing sugars and AA present (O'Brien and Morrissey, 1989; Kwak and Lim, 2004). Reactions that affect protein quality are racemization (that is, conversion of L-AA to their D-form), formation of isopeptides, protein-oxidized lipid interactions, protein-polyphenol interactions, cross-link reactions, and the Maillard reaction (Mauron, 1990). These reactions involve the amino groups of several AA but Lys is the most susceptible AA to react because of its free ε-amino group in a protein structure. Since Lys is often the first limiting AA in pig diets, reactions with Lys may reduce its content and, thereby, potentially the protein quality of the ingredient.

1.3.1 Cross-link reactions

Cross-link reactions occur between or within protein structures at high temperatures in combination with or without alkaline pH (Annan and Manson, 1981). Alkaline conditions are, for example, used to extract proteins in the production of protein concentrates and isolates (Friedman, 1999). At alkaline conditions, dehydroalanine is formed from Cys or Ser by β-elimination (removal of an SH- or OH-group, respectively). The dehydroalanine can bind to the ε-amino group of Lys which results in the formation of lysinoalanine (LAL). Dehydroalanine can also bind to Cys, ornithine (a degradation product of Arg; Friedman, 1999), and ammonia resulting in lanthionine, ornithinoalanine, and β-aminoalanine, respectively (Annan and Manson, 1981). Moreover, β-elimination of Thr yields 3-methyldehydroalanine which can react to the ε-amino group of Lys subsequently resulting in lysinomethylalanine (Mauron, 1990). Several other cross-link products are known and their chemical structures are described in Friedman (1999). The formation of LAL depends on the amount of free ε-amino groups of Lys and on the location of the Lys and dehydroalanine molecules in the protein structure (Annan and Manson, 1981; Friedman, 1999). Moreover, the formation of dehydroalanine depends on the amount of Ser and Cys molecules and on their susceptibility to β-elimination. Cross-link reactions are, thus, complex and the final concentration of cross-link products can be highly variable between ingredients. Lysinoalanine was found to be present in varying amounts in several food products such as baby food (10-70 μg/g), cereal products (200-390 μg/g), and (alkali treated) eggs (160-1820 μg/g). Lysinoalanine is partly absorbed in rats, mice, and hamster

and subsequently excreted in urine as free LAL (Friedman, 1999). The amount of LAL in plasma, liver, kidneys, and feces of rats was found to be dependent on dietary intake and this dose-dependent effect was most pronounced in the kidneys (Somoza et al., 2006). Thus, the kidneys can be regarded as a site for storage of LAL. Lysinoalanine can be toxic (Mauron, 1990) and is associated with the occurrence of nephrocytomegaly which is an increase in the size of the nucleus and cytoplasm and disturbed DNA synthesis and mitosis of kidney cells resulting in renal defect (Friedman, 1999; Finot, 2005b). The absorption and metabolism of cross-link products in growing pigs has not been studied.

1.3.2 Maillard reaction

The Maillard reaction is a non-enzymatic browning reaction between reducing sugars, aldehydes, or ketones and amines, AA, peptides, and proteins. In ingredients, the reaction mainly occurs between reducing sugars and free amino groups with the free ε-amino group of Lys being most susceptible. The Maillard reaction is a sequence of chemical reactions that can be divided in three phases: early, advanced, and final. The formation of each Maillard reaction product (MRP) depends on the severity of processing. A schematic representation of the Maillard reaction can be found in Fig. 1.1.

The first step of the Maillard reaction is a reversible condensation reaction between reducing sugars and AA resulting in Schiff's bases. The rather unstable Schiff's bases are in equilibrium with reducing sugars and AA. Lysine released from Schiff's bases in the acidic environment of the stomach was 100% bioavailable in rats (Finot, 2005b). The Schiff's bases react further through cyclization and the irreversible Amadori rearrangement to 1-amino-1-deoxy-2-ketoses which are known as the Amadori compounds (Hodge, 1953; Friedman, 1996a). The pathway via the irreversible Heyn's rearrangement occurs when the reducing sugar is a ketose resulting in 2-amino-2-deoxyaldoses (O'Brien and Morrissey, 1989). The formation of Schiff's bases, Amadori compounds, and Heyn's rearrangement products belong to the early Maillard reaction. Furosine is formed from the Amadori product fructoselysine (FL) upon acid hydrolysis and is, therefore, regarded as a marker for the early Maillard reaction (Mauron, 1981; Erbersdobler and Somoza, 2007). During the advanced Maillard reaction, several products are formed from Amadori compounds and Heyn's rearrangement products. Due to the complexity of chemical reactions in this phase, not all products that are formed have been identified. There are at least three main reactions identified which are 1,2-enolization at pH < 7 resulting in 3-deoxyoson, 2,3-enolization at pH > 7 resulting in 1-deoxyoson (O'Brien and Morrissey, 1989), and dehydration resulting in reductones and carbonyls (Hodge, 1953). Further reactions of 3-deoxyoson results in the formation of hydroxymethylfurfural (HMF), pyrraline, pentosidine, and imidazolone. Further reactions of 1-deoxyoson first result in

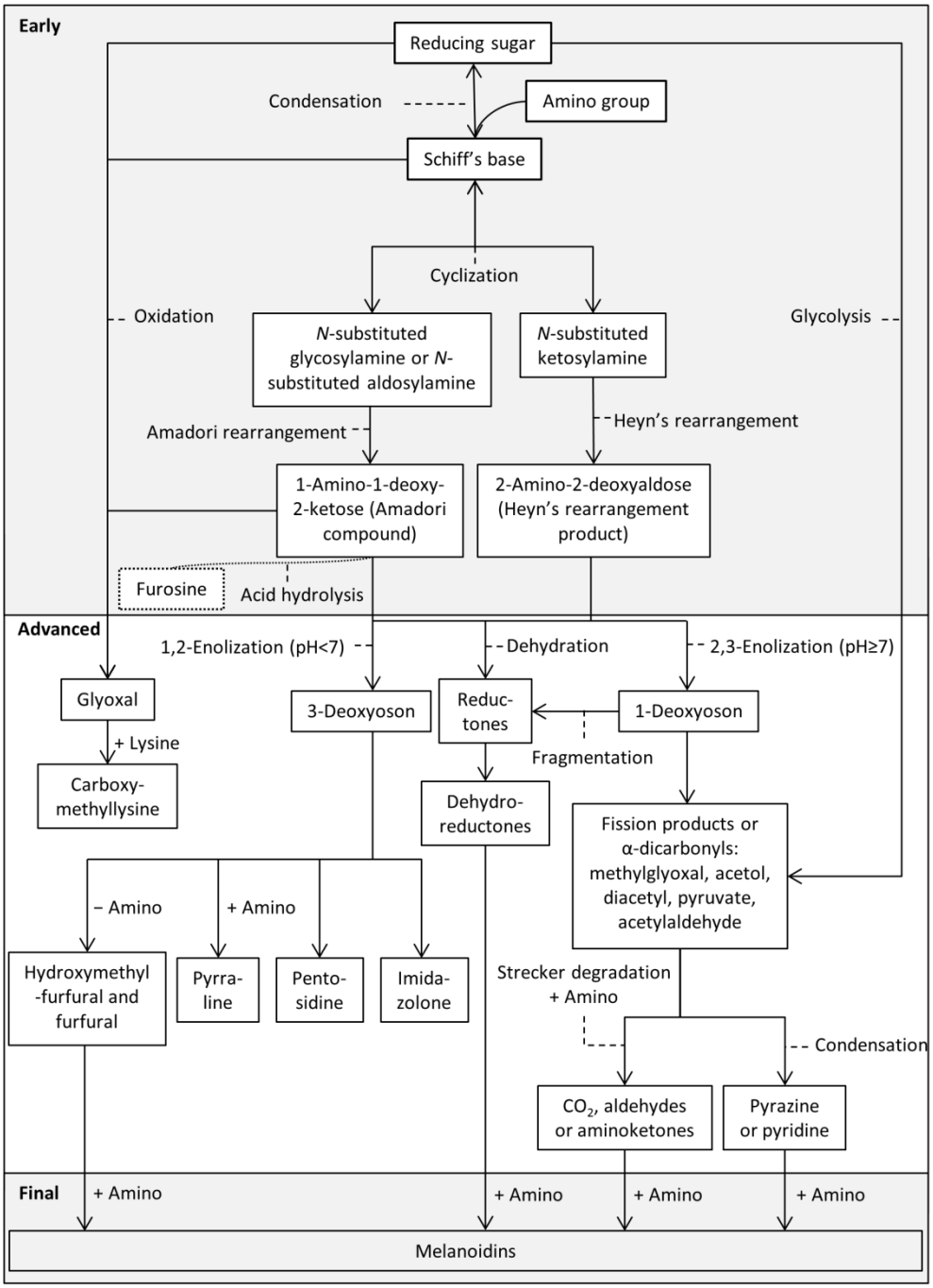


Figure 1.1. Schematic representation of the Maillard reaction (adapted from Hodge, 1953; O'Brien and Morrissey, 1989; Friedman, 1996a; Van Boekel, 1998; Van Rooijen et al., 2013). Dashed lines indicate the reaction that occurs and the dotted line indicates the formation of furosine from 1-amino-1-deoxy-2-ketose during acid hydrolysis.

Fission products and carbonyls which react further to aldehydes or aminoketones by the Strecker degradation and to pyrazine or pyridine by condensation. Pyrazine and pyridine are heterocyclic amines and give flavor to ingredients (Mauron, 1981). A fourth pathway results in the formation of carboxymethyllysine (CML) from the reaction between Lys and glyoxal, which is formed by oxidation of reducing sugars, Schiff's bases, or Amadori compounds (Van Boekel, 1998; Finot, 2005a). In the final Maillard reaction, melanoidins are formed by aldol condensation, aldehyde-amine polymerization, and subsequent formation of heterocyclic compounds. Moreover, reactions between advanced MRP such as pyruvate and furfural lead to the formation of melanoidins (Hodge, 1953). The melanoidins cause the brown coloring of processed ingredients. The chemical structures of most final MRP are unfortunately still unknown because of the large diversity in melanoidins that can be formed from multiple pathways.

The Maillard reaction can have both positive and negative nutritional consequences. Positive consequences are the formation of flavors and aromas such as nutty, roasted or caramel which are appreciated in human foods. However, also off-flavors can be produced such as an aged or stale flavor during the storage of dairy products. Other negative consequences are the loss of AA, loss of AA digestibility and bioavailability, and the formation of toxic and mutagenic compounds (Mauron, 1981; O'Brien and Morrissey, 1989; Friedman, 1996a; Van Boekel, 1998). Losses of AA, especially of Lys, Met, Cys, and Trp, are also found for protein-oxidized lipid interactions and protein-polyphenol interactions. Racemization and the formation of isopeptides do not result in AA loss (Mauron, 1990). Moreover, Lys bioavailability, as measured by a slope-ratio assay with pigs, was not affected by the D-Lys content in 15 protein-containing meals (that is, rapeseed meal (RSM), meat and bone meal, meal of field peas, fish meal, meal of skimmed milk, SBM, peanut meal, sunflower meal, and cottonseed meal; Davies, 1993). The amount of AA, in particular Lys, Cys, and Ser, involved in the formation of LAL are considered negligible when normal processing treatments, that is, not involving excessive heat and alkali, are used (Mauron, 1990). For example, Davies (1993) found that LAL was < 0.3% of the Lys content in 15 protein-containing meals and that LAL was not related to Lys bioavailability as determined with pigs and rats. Thus, the ultimate loss in AA can be highly variable and depends on the reactions that occur during processing.

1.3.3 Effect of processing on digestibility

Processing influences digestibility of AA by (1) inactivating anti-nutritional factors, (2) modifying the tertiary and secondary structure (that is, denaturation), (3) changing AA side-chains, thereby, hindering peptide bonds from enzymatic hydrolysis, (4) forming cross-links within or between molecules, and (5) the presence of D-AA resulting from

racemization. The first two increase digestibility while the latter three decrease digestibility (Mauron, 1990). Anti-nutritional factors in field peas were found to be inactivated by extrusion which subsequently resulted in an increased SID of AA (Stein and Bohlke, 2007).

Several enzymes are involved in the breakdown of peptide bonds which are either produced by the pancreas or by the brush border of the small intestine. The pancreatic enzymes can be divided in the endopeptidases trypsin and chymotrypsin which cleave the carboxyl end of basic (Lys and Arg) or aromatic (Tyr, Phe, and Trp) AA, respectively, and the exopeptidases carboxypeptidase-A and -B which release single AA from the carboxyl terminal ends of peptides. Carboxypeptidase-A is specific for aromatic AA while carboxypeptidase-B is specific for basic AA. Polypeptides are further hydrolyzed to AA, di- and tri-peptides by several peptidases, such as aminopeptidases, located on the brush border membrane (Erickson and Kim, 1990). The inhibition of enzymes by MRP have been studied using model systems. Hansen and Millington (1979) heated a mixture of glucose and a polymer of Lys at temperatures ranging from 101 to 170°C up to 60 min. The heated solutions were incubated for 24 h at 37°C with carboxypeptidase-B. It was found that carboxypeptidase-B was unable to release Lys when as little as 12% of the Lys-glucose reaction had occurred. Thus, even small amounts of early Maillard damage could inhibit enzymatic hydrolysis of peptides. Öste et al. (1986) added a low molecular weight (MW) fraction produced from the reaction between glucose and Lys to different substrates to study the *in vitro* activity of different enzymes. Carboxypeptidase-A and aminopeptidase-N were inhibited by the addition of 0.5 and 0.25 mg/mL, respectively, of the low MW fraction to the substrate. Thus, the brush border enzyme aminopeptidase-N was inhibited to a stronger extent than carboxypeptidase-A. A follow-up study showed that carboxypeptidase-A was inhibited by heterocyclic compounds that are formed during the advanced Maillard reaction. Two of these compounds also inhibited aminopeptidase-N. These compounds were shown to reduce protein digestion in rats. Moreover, one of them (formyl-(hydroxymethyl)pyrrole-norleucine) might also interfere with the absorption of Lys by binding to the same carrier on the epithelial cells as Lys (Öste et al., 1987). Thus, MRP from model systems can inhibit several enzymes *in vitro* and *in vivo*. Moreover, peptides were found to aggregate in the intestinal tract of growing pigs (Fischer et al., 2007) which hinders absorption. The largest aggregates were found for SBM heat-treated at high humidity (addition of 1.8 L of water to 200 g SBM followed by 1 h stirring and autoclaving for 15 min at 125°C; Fischer et al., 2002). Thus, processing reduced digestibility of protein by the joint action of enzyme inhibition, interference with carriers, and formation of peptide aggregates.

Several studies have been performed using different processing conditions for several feed ingredients. Most of these studies reported a reduction in *in vivo* digestibility of protein and AA due to processing. For example, De Vrese et al. (2000) fed diets containing ^{15}N labelled casein, β-lactoglobulin, or wheat subjected to heat (65°C for 6 or 24 h) and alkali (pH 10.5-11.5) to miniature pigs. The true ileal digestibility (TID) of protein was reduced by this processing treatment which was mainly caused by the presence of D-AA while the presence of LAL was of minor importance. The presence of MRP, from heating egg albumen in the presence of glucose, increased the amount of N present in digesta in the small intestine of rats indicating a reduced protein digestion (Valle-Riestra and Barnes, 1970). Feeding a heat-treated mixture of glucose and casein to growing pigs reduced the apparent ileal digestibility (AID) of Lys by 37 percentage points and the AID of other AA by 4 percentage points. Moreover, a greater amount of peptides with a MW of 220-1500 Da was present in digesta of growing pigs fed a heated glucose/casein mixture compared with an unheated glucose/casein mixture. The reduced digestibility and increased amount of peptides were hypothesized to be caused by a decreased enzymatic hydrolysis and competition or blockage of carriers by FL present in the heated glucose/casein mixture (Moughan et al., 1996). Table 1.2 summarizes the scientific literature that reported effects of processing on the AID or SID of CP and Lys for feed ingredients used in pig diets. Autoclaving canola meal at 130°C for 20 min or SBM at 125°C for 15 min reduced the SID of Lys with 12 and 3.7 percentage points, respectively. The SID of Lys was further reduced by extending autoclaving time to 45 min for canola meal (Almeida et al., 2014b) or 30 min for SBM. Oven-drying of SBM at 125°C for 30 min did not affect SID of Lys (González-Vega et al., 2011) indicating that the effects on digestibility are dependent on the method used to process a feed ingredient and not solely on the temperature and duration of heating. With regard to field peas, heating at 150°C for 15 min reduced the Lys content but the AID of Lys was only affected with a higher temperature (165°C). Messerschmidt et al. (2012) used different toasting conditions during the desolventizing/toasting step in which SBM is obtained from soybeans. Increasing steam pressure from 150 to 500 kPa, steam contact from 34 to 45 min, and temperature from 105 to 115°C resulted in an increased SID of Lys. The increase in SID of Lys was accompanied by a reduction in trypsin inhibitors with no Lys loss. Another batch of soybeans was toasted with a steam pressure of 850 kPa, temperature of 139°C, and processing time of 30 min but with 7 min steam contact to obtain SBM. These conditions were not optimal to decrease trypsin inhibitors and resulted in a loss of Lys and a decreased SID of Lys. Thus, processing can have substantial negative effects on the digestibility of AA which subsequently affects pig growth performance.

Table 1.2. Overview of processing conditions in relation to CP (g/kg DM) and Lys (g/100 g CP) content and the standardized or apparent ileal digestibility (SID/AID)[1] of CP and Lys (%) for different feed ingredients. The studies found a significant effect of processing on ID of Lys

Ingredient	Processing conditions[2]	CP content	SID/AID of CP	Lys content	SID/AID of Lys	Reference
Canola meal	None	405	71.7	5.2	68.2	Almeida et al., 2014b
	Autoclaving at 130°C for 20 min	408	62.0	4.4	56.2	
	Autoclaving at 130°C for 30 min	411	64.8	4.1	57.4	
	Autoclaving at 130°C for 45 min	417	34.5	3.8	20.8	
Soybean meal	None	551	93.1	6.3	93.0	González-Vega et al., 2011
	Autoclaving at 125°C for 15 min	562	88.8	5.8	89.3	
	Autoclaving at 125°C for 30 min	560	84.0	5.6	84.2	
	Oven drying at 125°C for 30 min	553	91.4	6.3	91.3	
Soybean meal[3]	Toasting at 150 kPa steam pressure, 34 min steam contact, at 105°C for 20 min	567	38.7	5.8	44.9	Messerschmidt et al., 2012
	Toasting at 500 kPa steam pressure, 45 min steam contact, at 115°C for 20 min	572	71.9	5.6	68.2	
	Toasting at 850 kPa steam pressure, 7 min steam contact, at 139°C for 30 min	569	48.9	5.3	47.5	
Field peas	None	210	60*	7.0	75*	Van Barneveld et al., 1994a
	Heating at 110°C for 15 min	224	61*	6.8	79*	
	Heating at 135°C for 15 min	222	59*	6.8	74*	
	Heating at 150°C for 15 min	227	64*	5.6	74*	
	Heating at 165°C for 15 min	216	53*	4.0	56*	

[1]Values for SID are provided unless indicated by an asterisk which indicates AID.

[2]None = no additional heat processing was applied to the feed ingredient.

[3]The processing treatment was applied during the desolventizing/toasting step to produce soybean meal from soybeans.

1.3.4 Effect of processing on amino acid utilization

Processing could affect the utilization of AA after absorption if the products formed during processing are absorbed. Lysinoalanine, FL, and CML were found to be bound in peptides with a MW of 4.5-20 kDa (30-40 AA), 1-4.5 kDa, and 250-1,000 Da, respectively, after *in vitro* enzymatic digestion simulating the stomach with pepsin and the small intestine with pancreatic juice (mucin, pancreatin, trypsin, and bile extract). The peptides containing LAL are too large to be absorbed, for which sizes of smaller than 1,000 Da are required. Fructoselysine and CML are present in smaller peptides than LAL and are, therefore, considered to be able to cross the small intestinal wall (Hellwig et al., 2014). The absorption of MRP was confirmed by several authors. Around half of the FL present in a heated glucose/casein mixture was absorbed in the small intestine of growing pigs. The FL concentration in portal serum was about twice as high as in jugular serum (24 vs. 11 nmol/mL of serum) indicating that most was metabolized in the liver (Moughan et al., 1996). The urinary excretion of FL, pyrraline, and pentosidine was found to be dependent on diet in humans (Förster et al., 2005). Carboxymethyllysine was found to be excreted in urine in studies with rats and humans (Delgado-Andrade et al., 2012; Alamir et al., 2013). Moreover, protein-bound CML in plasma increased after administration of a single dose of free CML (Alamir et al., 2013). Up to 65 and 82% of the ingested HMF was excreted in urine of mice and rats, respectively, within 48 h after administration. Hydroxymethylfurfural was also deposited in several organs, such as the liver and kidneys, but the compound was cleared from these organs within 48 h after administration (Godfrey et al., 1999). A study in rats showed that the urinary excretion of ^{14}C was higher for rats fed heated (60 min at 120°C in the presence of glucose) egg albumen labelled with ^{14}C-Lys compared with non-heated egg albumen. Moreover, the utilization of heated egg albumen was reduced as measured by the amount of $^{14}CO_2$ expired air. This indicates that Lys or a derivative of Lys was absorbed but could not be utilized and was, therefore, excreted in urine (Valle-Riestra and Barnes, 1970). Thus, several products of the Maillard reaction can be (partially) digested and absorbed but seem to be unavailable post-absorption.

Amino acid bioavailability can be determined using the slope-ratio assay in which the response to incremental amounts of the test AA is compared with the response to a standard free AA. The most appropriate response criterion of an animal in slope-ratio assays is the retention of the AA in the empty body. This is, however, the most expensive criterion and, therefore, alternative criteria, such as feed conversion efficiency on a carcass weight basis, are often used. The slope-ratio assay requires the test AA to be limiting in the diet. The major disadvantages of the slope-ratio assay are that the bioavailability of only one AA at a time can be determined, making such an assay time

consuming, and that the variation in the results is high (standard deviation of 10%) resulting in inaccurate estimates (Batterham, 1992). Therefore, the ileal digestibility of AA would be a good alternative to assess AA bioavailability. The ileal digestibility of Lys is thought to accurately reflect Lys bioavailability for unprocessed ingredients. However, ileal digestibility of Lys might overestimate Lys bioavailability for processed ingredients because Lys might be absorbed in an unavailable form. This was found for cottonseed meal, meat-and-bone meal (Batterham et al., 1990a), and additionally heat-treated fish meal (Wiseman et al., 1991) and field peas (Van Barneveld et al., 1994b). Almeida et al. (2014a) formulated diets for weanling pigs based on SID of Lys in SBM or autoclaved SBM. The average daily gain (ADG), gain to feed ratio, and plasma urea N were (numerically) lower for the autoclaved SBM diets compared with the SBM diet. Lysine bioavailability was not determined but the results indicated that the absorbed Lys might have been (partly) in an unavailable form. Formulating a cottonseed meal diet based on Lys content resulted in a significantly lower ADG and feed conversion efficiency compared with a SBM diet containing a similar Lys content. Supplementing crystalline L-Lys HCl to the cottonseed meal diet to equalize the bioavailable Lys content, increased the ADG and feed conversion efficiency. Moreover, ileal digestibility of Lys overestimated Lys bioavailability in the cottonseed meal. Therefore, diets containing feed ingredients with a low bioavailability should be formulated based on bioavailable Lys content rather than Lys or ileal digestible Lys content (Batterham et al., 1990b). Lysine bioavailability was highest for SBM and field peas (> 80%) followed by lupin seed meal and groundnut meal (44-65%), and meat-and-bone meal and cottonseed meal (< 42%) as determined with a slope-ratio assay with pigs using feed conversion efficiency on a carcass weight basis as response criterion (Batterham et al., 1984, 1986a, 1990b). Lysine bioavailability, as determined with a slope-ratio assay with pigs using feed conversion efficiency on an empty body gain basis as response criterion, was 96% for raw field peas but decreased to 71, 77, 56, and 18% for heat treatments at 110, 135, 150, and 165°C, respectively (Van Barneveld et al., 1994c). Processing not only affected the bioavailability of Lys but also the bioavailability of Thr, Met, and Trp. The branched-chain AA (Ile, Leu, and Val) were not affected by processing. Thus, also the ileal digestibility of Thr, Met, and Trp might not accurately reflect their bioavailability (Batterham, 1992). Amino acid bioavailability is, therefore, preferred above ileal AA digestibility for processed ingredients as a measure for protein quality. However, the determination of AA bioavailability has several disadvantages (listed above) resulting in the frequent use of ileal AA digestibility as a measure for protein quality in practice.

1.4 Aim and outline of this thesis

Currently, pig diets are formulated on SID AA content. The AA are analyzed after acid hydrolysis which breaks down all peptides bonds. Amadori compounds partially revert

back to Lys during acid hydrolysis (Hurrell and Carpenter, 1981). The Lys determined after acid hydrolysis is, therefore, referred to as total Lys. The reversion of Amadori compounds does not occur in the animal (Rérat et al., 2002) and, thus, the SID total Lys content may provide an inaccurate estimate of Lys bioavailability. The SID reactive Lys (having a free ε-amino group) content is thought to be a better measure for Lys bioavailability than total Lys. The study of Rutherfurd et al. (1997) reported that the TID reactive Lys content predicted Lys deposition (that is, Lys bioavailability) more accurately than the TID total Lys content. This was determined by formulating a diet containing enzymatically hydrolyzed casein to the TID reactive or total Lys content of a diet containing heated skim milk powder. The AA in enzymatically hydrolyzed casein were considered to be 100% digestible. The diets were fed to growing pigs and the daily Lys deposition was determined. The Lys deposition did not differ between the heated skim milk diet and enzymatically hydrolyzed casein diet formulated on the TID reactive Lys content. The Lys deposition for these diets was higher than for the enzymatically hydrolyzed casein diet formulated on the TID total Lys content. These results indicated that the TID reactive Lys content is a better measure for Lys bioavailability than the TID total Lys content. This is, unfortunately, the only study that examined this. Moreover, most studies report the effects of processing on ileal AA digestibility while post-absorptive utilization of AA is rarely measured.

The initial aim of this thesis was to evaluate the ileal digestible reactive Lys assay as a more accurate measure for protein quality of processed protein sources than the ileal digestible total Lys assay. Moreover, two alternative *in vitro* methods for determining protein digestibility for processed protein sources were evaluated. Soybean meal and RSM were used as sole protein sources throughout this thesis. Soybean meal is a major protein source in pig diets while RSM is considered as an alternative for SBM. Processing of SBM and RSM by toasting at 95°C for 30 min in the presence of a sugar-rich lignosulfonate was used as model for over-processed protein sources. This model was validated in a preliminary trial in which the SID of AA and reactive Lys were determined (Chapter 2). In this study, reactive Lys was analyzed by guanidination using the chemical reagent O-methylisourea (OMIU) which results in the formation of homoarginine, an acid stable AA that can be accurately analyzed after acid hydrolysis. O-methylisourea is used in this assay (Moughan and Rutherfurd, 1996) as it is reported to react specifically to the ε-amino groups of Lys (Greenstein, 1935; Chervenka and Wilcox, 1956; Klee and Richards, 1957; Shields et al., 1959; Kassell and Chow, 1966; Keough et al., 2000). The results of Chapter 2, however, cast doubt on the specificity of OMIU to react only with the ε-amino group of Lys. The serendipitous finding reported in Chapter 2 resulted in a redirection of the aim of

this thesis to include further research into the specificity of OMIU for the ε-amino group of Lys (Chapter 6).

The SID AA contents of the protein sources from Chapter 2 were used to formulate the diets of the main *in vivo* experiment. Its results are presented in Chapters 3 and 4. Chapter 3 describes the effects of processing on digestibility and solubilization of N along the small intestine, metabolic load as assessed by organ weight, and nutrient composition of the empty body. Chapter 4 describes the effects of processing on whole body AA composition, nutrient retention, and post-absorptive utilization of AA for retention. The *in vitro* digestibility of the protein sources was determined using two methods as rapid and less expensive alternative methods for assessing protein digestibility for processed protein sources (Chapter 5). Chapter 6 describes the series of experiments that were conducted to study the specificity of OMIU to react with the ε-amino group of Lys. Finally, Chapter 7 summarizes and discusses the results of the previous chapters.

1.5 Literature cited

Alamir, I., C. Niquet-Leridon, P. Jacolot, C. Rodriguez, M. Orosco, P. M. Anton, and F. J. Tessier. 2013. Digestibility of extruded proteins and metabolic transit of N^{ε}-carboxymethyllysine in rats. Amino Acids 44:1441-1449.

Almeida, F. N., J. K. Htoo, J. Thomson, and H. H. Stein. 2014a. Effects of balancing crystalline amino acids in diets containing heat-damaged soybean meal or distillers dried grains with solubles fed to weanling pigs. Animal 8:1594-1602.

Almeida, F. N., J. K. Htoo, J. Thomson, and H. H. Stein. 2014b. Effects of heat treatment on the apparent and standardized ileal digestibility of amino acids in canola meal fed to growing pigs. Anim. Feed Sci. Technol. 187:44-52.

Annan, W. D., and W. Manson. 1981. The production of lysinoalanine and related substances during processing of proteins. Food Chem. 6:255-261.

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

Batterham, E. S., L. M. Andersen, D. R. Baigent, S. A. Beech, and R. Elliott. 1990a. Utilization of ileal digestible amino acids by pigs: Lysine. Brit. J. Nutr. 64: 679-690.

Batterham, E. S., L. M. Andersen, D. R. Baigent, R. E. Darnell, and M. R. Taverner. 1990b. 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., L. M. Andersen, R. F. Lowe, and R. E. Darnell. 1986a. Nutritional value of lupin (*Lupinus albus*)-seed meal for growing pigs: Availability of lysine, effect of autoclaving and net energy content. Brit. J. Nutr. 56:645-659.

Batterham, E. S., R. E. Darnell, L. S. Herbert, and E. J. Major. 1986b. Effect of pressure and temperature on the availability of lysine in meat and bone meal as determined by slope-

ratio assays with growing pigs, rats and chicks and by chemical techniques. Brit. J. Nutr. 55:441-453.

Batterham, E. S., R. D. Murison, and L. M. Andersen. 1984. Availability of lysine in vegetable protein concentrates as determined by the slope-ratio assay with growing pigs and rats and by chemical techniques. Brit. J. Nutr. 51:85-99.

Bell, J. M., and M. O. Keith. 1991. A survey of variation in the chemical composition of commercial canola meal produced in Western Canadian crushing plants. Can. J. Anim. Sci. 71:469-480.

Boisen, S. 1997. Ideal protein – and its suitability to characterize protein quality in pig feeds. A review. Acta Agric. Scand., Sect. A 47:31-38.

Boye, J., R. Wijesinha-Bettoni, and B. Burlingame. 2012. Protein quality evaluation twenty years after the introduction of the protein digestibility corrected amino acid score method. Brit. J. Nutr. 108:S183-S211.

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

Cozannet, P., Y. Primot, C. Gady, J. P. Métayer, P. Callu, M. Lessire, F. Skiba, and J. Noblet. 2010. Ileal digestibility of amino acids in wheat distillers dried grains with solubles for pigs. Anim. Feed Sci. Technol. 158:177-186.

Davies, R. L. 1993. D-Lysine, alloisoleucine and lysinoalanine in supplementary proteins with different lysine availabilities. J. Sci. Food Agric. 61:151-154.

Delgado-Andrade, C., F. J. Tessier, C. Niquet-Leridon, I. Seiquer, and M. P. Navarro. 2012. Study of the urinary and faecal excretion of N^{ε}-carboxymethyllysine in young human volunteers. Amino Acids 43:595-602.

De Vrese, M., R. Frik, N. Roos, and H. Hagemeister. 2000. Protein-bound D-amino acids, and to a lesser extent lysinoalanine, decrease true ileal protein digestibility in minipigs as determined with ^{15}N-labeling. J. Nutr. 130:2026-2031.

Eichner, K., and M. Karel. 1972. The influence of water content and water activity on the sugar-amino browning reaction in model systems under various conditions. J. Agric. Food Chem. 20:218-223.

Erbersdobler, H. F., and V. Somoza. 2007. Forty years of furosine – forty years of using Maillard reaction products as indicators of the nutritional quality of foods. Mol. Nutr. Food Res. 51:423-430.

Erickson, R. H., and Y. S. Kim. 1990. Digestion and absorption of dietary protein. Annu. Rev. Med. 41:133-139.

Fernandez, S. R., and C. M. Parsons. 1996. Bioavailability of digestible lysine in heat-damaged soybean meal for chick growth. Poult. Sci. 75:224-231.

Finlayson, A. J., C. M. Christ, and R. K. Downey. 1970. Changes in the nitrogenous components of rapeseed (*Brassica napus*) grown on a nitrogen and sulfur deficient soil. Can. J. Plant Sci. 50:705-709.

Finot, P. A. 2005a. Historical perspective of the Maillard reaction in food science. Ann. N. Y. Acad. Sci. 1043:1-8.

Finot, P. A. 2005b. The absorption and metabolism of modified amino acids in processed foods. J. AOAC Int. 88:894-903.

Fischer, M., H. Gruppen, S. R. Piersma, L. V. Kofod, H. A. Schols, A. G. J. Voragen. 2002. Aggregation of peptides during hydrolysis as a cause of reduced enzymatic extractability of soybean meal proteins. J. Agric. Food Chem. 50:4512-4519.

Fischer, M., A. G. J. Voragen, S. R. Piersma, L. V. Kofod, C. I. Jörgensen, P. Guggenbuhl, C. S. Nunes, and H. Gruppen. 2007. Presence of indigestible peptide aggregates of soybean meal in pig ileal digesta residue. J. Sci. Food Agric. 87:2229-2238.

Food and Agricultural Organization of the United Nations (FAO). 2009. Global Agriculture Towards 2050. Proceedings of 'High Level Expert Forum – How to feed the world in 2050'. Rome, Italy, October 12-13, 2009.

Food and Agricultural Organization of the United Nations (FAO). 2013. Dietary Protein Quality Evaluation in Human Nutrition. Report of an FAO Expert Consultation, FAO Food and Nutrition paper 92, Auckland, New Zealand from March 31 to April 2, 2011.

Förster, A., Y. Kühne, and T. Henle. 2005. Studies on absorption and elimination of dietary Maillard reaction products. Ann. N. Y. Acad. Sci. 1043:474-481.

Friedman, M. 1992. Dietary impact of food processing. Annu. Rev. Nutr. 12:119-137.

Friedman, M. 1996a. Food browning and its prevention: An overview. J. Agric. Food Chem. 44:631-653.

Friedman, M. 1996b. Nutritional value of proteins from different food sources. A review. J. Agric. Food Chem. 44:6-29.

Friedman, M. 1999. Chemistry, biochemistry, nutrition, and microbiology of lysinoalanine, lanthionine, and histidinoalanine in food and other proteins. J. Agric. Food Chem. 47:1295-1319.

Frikha, M., M. P. Serrano, D. G. Valencia, P. G. Rebollar, J. Fickler, and G. G. Mateos. 2012. Correlation between ileal digestibility of amino acids and chemical composition of soybean meals in broilers at 21 days of age. Anim. Feed Sci. Technol. 178:103-114.

Godfrey, V. B., L-.J. Chen, R. J. Griffin, E. H. Lebetkin, and L. T. Burka. 1999. Distribution and metabolism of (5-hydroxymethyl)furfural in male F344 rats and B6C3F1 mice after oral administration. J. Toxicol. Environ. Health 57:199-210.

González-Vega, J. C., B. G. Kim, J. K. Htoo, A. Lemme, and H. H. Stein. 2011. Amino acid digestibility in heated soybean meal fed to growing pigs. J. Anim. Sci. 89:3617-3625.

Greenstein, J. P. 1935. Studies on multivalent amino acids and peptides. II. The synthesis of certain derivatives of lysyl-glutamic acid. J. Biol. Chem. 109:541-544.

Hansen, L. P., and R. J. Millington. 1979. Blockage of protein enzymatic digestion (carboxypeptidase-B) by heat-induced sugar-lysine reactions. J. Food Sci. 44:1173-1177.

Hellwig, M., R. Matthes, A. Peto, J. Löbner, and T. Henle. 2014. *N*-ε-fructosyllysine and *N*-ε-carboxymethyllysine, but not lysinoalanine, are available for absorption after simulated gastrointestinal digestion. Amino Acids 46:289-299.

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

Hodge, J. E. 1953. Dehydrated foods – Chemistry of browning reactions in model systems. J. Agric. Food Chem. 1:928-943.

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

Hurrell, R. F., P. A. Finot, and J. E. Ford. 1983. Storage of milk powders under adverse conditions. I. Losses of lysine and of other essential amino acids as determined by chemical and microbiological methods. Brit. J. Nutr. 49:343-354.

Kassell, B., and R. B. Chow. 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.

Keough, T., M. P. Lacey, and R. S. Youngquist. 2000. Derivatization procedures to facilitate *de novo* sequencing of lysine-terminated tryptic peptides using postsource decay matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun. Mass Spectrom. 14:2348-2356.

Kies, C. 1981. Bioavailability: A factor in protein quality. J. Agric. Food Chem. 29:435-440.

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

Kwak, E.-J., and S.-I. Lim. 2004. The effect of sugar, amino acid, metal ion, and NaCl on model Maillard reaction under pH control. Amino Acids 27:85-90.

Marsman, G. J. P., H. Gruppen, D. J van Zuilichem, J. W. Resink, and A. G. J. Voragen. 1995. The influence of screw configuration on the *in vitro* digestibility and protein solubility of soybean and rapeseed meals. J. Food Eng. 26:13-28.

Mauron, J. 1981. The Maillard reaction in food; A critical review from the nutritional standpoint. Prog. Food Nutr. Sci. 5:5-35.

Mauron, J. 1990. Influence of processing on protein quality. J. Nutr. Sci. Vitaminol. 36:S57-S69.

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

Messerschmidt, U., M. Eklund, V. T. S. Rist, P. Rosenfelder, H. K. Spindler, J. K. Htoo, and R. Mosenthin. 2012. Effect of particle size and heat treatment of soybean meal on standardized ileal digestibility of amino acids in growing pigs. J. Anim. Sci. 90(Suppl. 4):119-121.

Millward, D. J., D. K. Layman, D. Tomé, and G. Schaafsma. 2008. Protein quality assessment: Impact of expanding understanding of protein and amino acid needs for optimal health. Am. J. Clin. Nutr. 87:1576S-1581S.

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

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

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

O'Brien, J., and P. A. Morrissey. 1989. Nutritional and toxicological aspects of the Maillard browning reaction in foods. Crit. Rev. Food Sci. Nutr. 28:211-248.

Öste, R. E., A. Dahlqvist, H. Sjöström, O. Norén, and R. Miller. 1986. Effect of Maillard reaction products on protein digestion. *In vitro* studies. J. Agric. Food Chem. 34:355-358.

Öste, R. E., R. Miller, H. Sjöström, and O. Norén. 1987. Effect of Maillard reaction products on protein digestion. Studies on pure compounds. J. Agric. Food Chem. 35:938-942.

Qin, G. X., M. W. A. Verstegen, and A. F. B. van der Poel. 1998. Effect of temperature and time during steam treatment on the protein quality of full-fat soybeans from different origins. J. Sci. Food Agric. 77:393-398.

Rérat, A., R. Calmes, P. Vaissade, and P. A. Finot. 2002. Nutritional and metabolic consequences of the early Maillard reaction of heat treated milk in the pig. Significance for man. Eur. J. Nutr. 41:1-11.

Rutherfurd, S. M., P. J. Moughan, and P. C. H. Morel. 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.

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

Somoza, V., E. Wenzel, C. Weiβ, I. Clawin-Rädecker, N. Grübel, and H. F. Erbersdobler. 2006. Dose-dependent utilisation of casein-linked lysinoalanine, *N*(epsilon)-fructoselysine and *N*(epsilon)-carboxymethyllysine in rats. Mol. Nutr. Food Res. 50:833-841.

Stein, H. H., and R. A. Bohlke. 2007. The effects of thermal treatment of field peas (*Pisum sativum* L.) on nutrient and energy digestibility by growing pigs. J. Anim. Sci. 85:1424-1431.

United Nations (UN). 2015. World Population Prospects: The 2015 Revision, Key Findings and Advance Tables. United Nations, Department of Economic and Social Affairs, Population Division. Working Paper No. ESA/P/WP.241.

Valle-Riestra, J., and R. H. Barnes. 1970. Digestion of heat-damaged egg albumen by the rat. J. Nutr. 100:873-882.

Van Barneveld, R. J., E. S. Batterham, and B. W. Norton. 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., E. S. Batterham, and B. W. Norton. 1994b. The effect of heat on amino acids for growing pigs. 2. Utilization of ileal-digestible lysine from heat-treated field peas (*Pisum sativum* cultivar Dundale). Brit. J. Nutr. 72:243-256.

Van Barneveld, R. J., E. S. Batterham, and B. W. Norton. 1994c. 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 Boekel, M. A. J. S. 1998. Effect of heating on Maillard reactions in milk. Food Chem. 62:403-414.

Van Rooijen, C., G. Bosch, A. F. B. van der Poel, P. A. Wierenga, L. Alexander, and W. H. Hendriks. 2013. The Maillard reaction and pet food processing: Effects on nutritive value and pet health. Nutr. Res. Rev. 26:130-148.

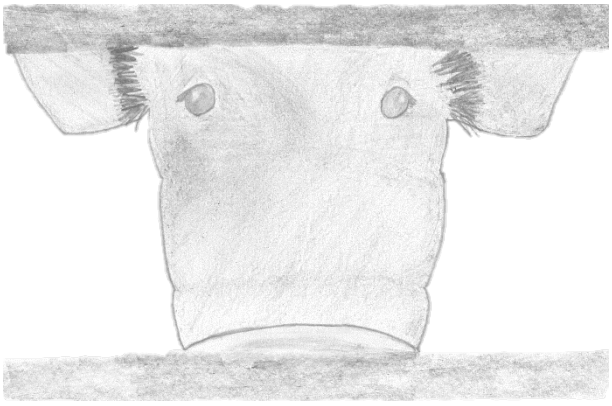
Wiseman, J., S. Jagger, D. J. A. Cole, and W. Haresign. 1991. The digestion and utilization of amino acids of heat- treated fish meal by growing/finishing pigs. Anim. Prod. 53:215-225.

Assessment of protein quality of soybean meal and 00-rapeseed meal toasted in the presence of lignosulfonate by amino acid digestibility in growing pigs and Maillard reaction products

T. G. Hulshof,[*†] P. Bikker,[*] A. F. B. van der Poel,[†] and W. H. Hendriks[†]

[*]Wageningen UR Livestock Research, Wageningen, the Netherlands, and [†]Animal Nutrition Group, Wageningen University, Wageningen, the Netherlands

Journal of Animal Science 2016, 94 (3):1020-1030



Abstract

An experiment was conducted to determine protein quality in processed protein sources using the content of amino acids (AA), O-methylisourea (OMIU)-reactive Lys, Maillard reaction products (MRP), and cross-link products; the standardized ileal digestibility (SID) of CP and AA; and growth performance in growing pigs as criteria. Differences in protein quality were created by secondary toasting (at 95°C for 30 min) of soybean meal (SBM) and rapeseed meal (RSM) in the presence of lignosulfonate resulting in processed SBM (pSBM) and processed RSM (pRSM). The processing treatment was used as a model for over-processed protein sources. Ten growing pigs were each fed 1 of the 4 diets containing SBM, pSBM, RSM, or pRSM in each of 3 periods. Ileal digesta was collected at the end of each period and analyzed for CP, AA, and OMIU-reactive Lys. Diets were analyzed for furosine and carboxymethyllysine (CML) as an indicator for MRP and lysinoalanine (LAL) which is a cross-link product. The SBM and RSM diets contained furosine, CML, and LAL indicating that the Maillard reaction and cross-linking had taken place in SBM and RSM, presumably during the oil extraction/desolventizing process. The amounts of furosine, CML, and LAL were elevated in pSBM and pRSM due to further processing. Processing resulted in a reduction in total and OMIU-reactive Lys contents, a decrease in gain to feed ratio from 0.52 to 0.42 for SBM and 0.46 to 0.39 for RSM (P = 0.006), SID of CP from 83.9 to 71.6% for SBM and 74.9 to 64.6% for RSM (P < 0.001) and SID of AA (P < 0.001), with the largest effects for total and OMIU-reactive Lys. The effects of processing could be substantial and should be taken into account when using processed protein sources in diets for growing pigs. The extent of protein damage may be assessed by additional analyses of MRP and cross-link products.

Key words: feed efficiency, growing pigs, O-methylisourea-reactive Lys, processing, protein quality, standardized ileal digestibility

2.1 Introduction

Processing of feed ingredients such as soybean meal (SBM) and rapeseed meal (RSM) may have negative effects on the content and ileal digestibility of amino acids (AA) in growing pigs (Van Barneveld et al., 1994; González-Vega et al., 2011; Kim et al., 2012). These negative effects are caused by Maillard-type reactions, for example, the reaction of Lys (or other AA) with reducing sugars (Hurrell and Carpenter, 1981; Rérat et al., 2002; Van Boekel, 2006; Moughan and Rutherfurd, 2008), or cross-link reactions between AA resulting in, for example, lysinoalanine (LAL; Friedman, 1999; De Vrese et al., 2000). Maillard reaction products (MRP), for example, furosine and carboxymethyllysine (CML), or LAL may indicate protein damage in feed ingredients (Van Rooijen et al., 2013). Diets for growing pigs are commonly formulated on standardized ileal digestible (SID) Lys content (CVB, 2011). Total Lys, determined using conventional analysis, may consist of Lys with a free ε-amino group (reactive Lys) plus Lys reacted to early MRP but reverted back under the strong acidic conditions of the test (Moughan and Rutherfurd, 2008). Only ileal digestible reactive Lys is considered bioavailable for protein synthesis (Moughan and Rutherfurd, 2008). Using the guanidination method with O-methylisourea (OMIU) to determine SID of reactive Lys may better estimate Lys bioavailability than SID of total Lys (Rutherfurd et al., 1997a).

The aim of the experiment was to determine protein quality in processed protein sources using 1) the AA, OMIU-reactive Lys, furosine, CML, and LAL contents; 2) SID of CP, AA, and OMIU-reactive Lys; 3) apparent total tract digestibility (ATTD) of nutrients; and 4) growth performance in growing pigs as criteria. Processing by secondary toasting of SBM and RSM in the presence of lignosulfonate was used as a model to achieve differences in protein quality. It was hypothesized that ileal digestible Lys may overestimate Lys bioavailability compared with OMIU-reactive Lys.

2.2 Materials and methods

2.2.1 Animals and housing

This study was approved by the Animal Care and Use Committee of Wageningen University and Research Centre Livestock Research (Lelystad, the Netherlands). Ten growing barrows (Dutch Landrace × Yorkshire from VOF van Beek, Lelystad, the Netherlands) with a high health status and an average initial BW of 30.8 ± 1.0 kg were surgically fitted with a steered ileocecal valve cannula (Mroz et al., 1996). Pigs were individually housed in metabolism cages (1.3 by 1.3 m) for collecting ileal digesta and feces and to enable individual supply of the experimental diets. The metabolism cages had one transparent wall, to enable visual contact between two pigs, three opaque walls, and a

plastic coated tenderfoot slatted floor. A feeding trough and a nipple drinker were present in each cage. The ambient temperature in the barn was kept constant at ±24°C during the experimental period, and the lights were turned on from 0500 until 2000 h and dimmed at night.

2.2.2 Diets and feeding

The study consisted of an incomplete crossover design with a 2 × 2 factorial arrangement of treatments. The study consisted of three periods of 11 d in which three of four experimental diets were fed to each pig to obtain a sufficient number of replicates in a short period of time. Pigs were gradually adapted from a commercial diet to the experimental diets during 3 d before the start of period 1. At the end of period 1 and 2, pigs were gradually adapted to the following experimental diet during 3 d.

All four experimental diets (Research Diet Services, Wijk bij Duurstede, the Netherlands) consisted of a basal CP-free diet based on gelatinized potato starch. The diets were supplemented with either SBM (*Glycine max*) or 00-RSM (*Brassica napus*) and both were supplied by Feed Valid B.V. (Poederoijen, the Netherlands) as the sole source of protein. The 00-RSM variety contains low levels of erucic acid and glucosinolates. Soybean meal and RSM were used either as such, referred to as the SBM and RSM diet, respectively, or processed by secondary toasting in the presence of lignosulfonate (Xylig from Lenzing Aktiengesellschaft, Lenzing, Austria) to obtain processed SBM (pSBM) and processed RSM (pRSM). Lignosulfonate, a commercially available source of xylose and glucose, was used to induce protein damage, in particular to Lys by the Maillard reaction. The latter was confirmed by analysis of OMIU-reactive Lys levels before the start of the study. The process is normally used to produce bypass protein for application in ruminant diets (Prestløkken and Rise, 2003). The lignosulfonate was added at a constant ratio to CP, that is, at 7% (*wt*/*wt*) of the SBM content in the SBM and pSBM diets and at 5% (*wt*/*wt*) of the RSM content to the RSM and pRSM diets. The SBM and RSM diets were produced by mixing the ingredients with air-heated (± 50°C) lignosulfonate syrup to make it fluid and subsequent pelleting with steam (± 75°C) through a 4-mm die. For the pSBM and pRSM diet, lignosulfonate was added to the SBM and RSM and the mixture was subsequently toasted in a seven-floor cascade toaster for 30 min at 95 ± 2°C, then dried with hot air (140°C for 10 min), and, thereafter, cooled on a conveyor belt to produce the pSBM and pRSM ingredients. The pSBM and pRSM ingredients were then mixed with the other feed ingredients and pelleted using the same procedure as for the SBM and RSM diets. Chromic oxide was added to all diets as an indigestible marker at an amount of 0.25 g/kg (as-fed basis). The SBM diets were supplemented with DL-Met and the RSM diets with L-Lys HCl, DL-Met, L-Thr, and L-Trp to meet 80% of the requirements for SID Lys, Met, Thr, and Trp

content as required for digestibility studies (CVB, 2011). The analyzed nutrient composition of the four protein sources and the ingredient and nutrient composition of the four experimental diets are provided in Tables 2.1, 2.2, and 2.3, respectively.

Table 2.1. Analyzed nutrient composition of the four protein sources: soybean meal (SBM), processed SBM (pSBM)[1], rapeseed meal (RSM), or processed RSM (pRSM)[1]

	Protein source			
Analyzed nutrient composition, g/kg DM[2]	SBM	pSBM[3]	RSM	pRSM[3]
DM, g/kg as-is	900	895	895	897
Ash	73	72	75	77
CP	531	511	383	371
Acid-hydrolyzed ether extract	28	28	39	42
Starch	10	10	7	7
Total sugars	134	122	102	96
Indispensable amino acid, g/100 g CP				
Arg	7.3	6.4	5.8	5.0
His	2.8	2.7	2.7	2.7
Ile	4.6	4.6	3.9	3.9
Leu	7.8	7.8	6.9	7.0
Lys	6.3	4.6	5.6	4.3
OMIU[4]-reactive Lys	6.0	3.7	5.0	3.1
Met	1.3	1.3	1.9	1.9
Phe	5.2	5.1	4.0	4.0
Thr	4.0	3.9	4.4	4.4
Trp	1.3	1.3	1.3	1.3
Val	4.9	4.9	5.2	5.2
Dispensable amino acid, g/100 g CP				
Ala	4.5	4.4	4.4	4.5
Asp	11.5	11.4	7.3	7.2
Cys	1.4	1.3	2.2	2.1
Glu	18.2	18.0	16.2	16.1
Gly	4.3	4.3	5.1	5.2
Pro	4.8	4.8	5.8	5.7
Ser	4.9	4.8	4.1	4.1
Tyr	3.6	3.6	3.1	3.1

[1]Processing consisted of addition of lignosulfonate to SBM and RSM and secondary toasting at 95 ± 2°C for 30 min.

[2]Unless stated otherwise.

[3]Processed SBM and pRSM include lignosulfonate at 7 and 5% (*wt*/*wt*), respectively.

[4]OMIU = O-methylisourea.

Table 2.2. Ingredient composition of four experimental diets containing soybean meal (SBM), processed SBM (pSBM)[1], rapeseed meal (RSM), or processed RSM (pRSM)[1] fed to growing pigs

	Diet			
Ingredient composition, g/kg, as-fed basis	SBM	pSBM	RSM	pRSM
Gelatinized potato starch	472.0	472.0	481.2	481.2
SBM	350.0	350.0	-	-
RSM	-	-	350.0	350.0
Lignosulfonate[2]	26.75	26.75	18.81	18.81
Dextrose	100.0	100.0	100.0	100.0
Soybean oil	20.0	20.0	20.0	20.0
Monocalcium phosphate	9.47	9.47	7.61	7.61
Limestone	11.63	11.63	6.90	6.90
Vitamin-mineral premix[3]	5.0	5.0	5.0	5.0
Salt	3.65	3.65	2.46	2.46
Sodium-bicarbonate	-	-	1.51	1.51
Cr_2O_3 – maize starch mix[4]	1.0	1.0	1.0	1.0
L-Lys HCl (78% Lys)	-	-	3.45	3.45
DL-Met (99% Met)	0.50	0.50	0.48	0.48
L-Thr (98% Thr)	-	-	0.86	0.86
L-Trp (98% Trp)	-	-	0.72	0.72

[1]Processing consisted of addition of lignosulfonate to SBM or RSM and secondary toasting at 95 ± 2°C for 30 min.

[2]Lignosulfonate (Feed Valid B.V., Poederoijen, the Netherlands) typically contains 35 to 42% Mg-lignosulfonate, 15 to 25% xylose, 3 to 7% glucose, 3 to 7% mannose, 3 to 7% rhamnose, 12 to 41% water (added up to 100%). The CP level of lignosulfonate was analyzed to be 3.2 g/kg (as-is).

[3]The vitamin-mineral premix provided, per kilogram of complete diet: Vitamin A, 6,000 IU; vitamin D_3, 1,200 IU; vitamin E, 15 mg; vitamin K_3, 1.5 mg; thiamin, 1.0 mg; riboflavin, 3.0 mg; pyridoxine, 1.0 mg; vitamin B_{12}, 15 µg; niacin, 20 mg; D-pantothenic acid, 10 mg; choline Cl, 150 mg; folic acid, 0.1 mg; Fe, 75 mg Fe as $FeSO_4$; Cu, 10 mg as $CuSO_4$; Mn, 30 mg as MnO; Zn, 50 mg as $ZnSO_4$; Co, 0.15 mg as $CoSO_4$; I, 0.50 mg as KIO_3; Se, 0.18 mg as Na selenite; antioxidant, 125 mg.

[4]Contains 25% Cr_2O_3.

Pigs were fed twice a day, equal portions at 0730 and 1530 h on d 1 to 7 in each period. Pigs were fed at 0600 and 1800 h on d 8 to 11 in each period to allow 12 h of ileal digesta collection. Feeding level was 2.8 times maintenance energy requirement (293 kJ net energy/kg $BW^{0.75}$; CVB, 2011). At the beginning of each period, the BW of pigs was determined and individual feed allowance was calculated. Average daily feed intake (ADFI) was measured daily by weighing the feed refusals before the morning feeding. Diets were fed as liquid feed by adding 2.5 L of water per kilogram of diet to ensure a similar flow of ileal digesta after morning and evening feeding.

Table 2.3. Analyzed nutrient composition of four experimental diets containing soybean meal (SBM), processed SBM (pSBM)[1], rapeseed meal (RSM), or processed RSM (pRSM)[1] fed to growing pigs

	Diet			
Nutrient composition, g/kg DM[2]	SBM	pSBM	RSM	pRSM
DM, g/kg as-is	889	896	890	895
Ash	51	51	44	45
CP	185	189	136	138
Acid-hydrolyzed ether extract	34	30	36	39
Crude fiber	14	12	48	53
Starch	476	473	488	488
Total sugars	160	152	153	145
Net energy, MJ/kg feed[3]	10.7	10.5	10.2	10.3
Indispensable amino acid, g/100 g CP				
Arg	6.9	5.9	5.4	4.6
His	2.8	2.7	2.8	2.7
Ile	4.5	4.4	3.8	3.7
Leu	7.7	7.5	6.8	6.6
Lys	6.0	4.3	7.7	6.3
OMIU[4]-reactive Lys	5.7	3.5	5.1	3.6
Reactive Lys from furosine	5.4	3.6	6.9	4.8
Met	1.6	1.4	2.1	2.0
Phe	5.1	5.0	3.9	3.8
Thr	3.9	3.9	5.1	5.0
Trp	1.3	1.3	1.7	1.8
Val	4.8	4.7	5.2	5.0
Dispensable amino acid, g/100 g CP				
Ala	4.4	4.3	4.4	4.3
Asp	11.3	11.1	7.2	6.9
Cys	1.4	1.3	2.0	1.9
Glu	18.2	17.9	16.2	15.7
Gly	4.3	4.2	5.1	5.0
Pro	4.8	4.9	5.5	5.6
Ser	4.8	4.9	4.1	4.0
Tyr	3.4	3.3	2.6	2.7
Maillard reaction products, mg/100 g CP				
Furosine	444	571	627	1,194
Carboxymethyllysine	42	67	57	130
Cross-link product, mg/100 g CP				
Lysinoalanine	28	37	27	199

[1]Processing consisted of addition of lignosulfonate to SBM and RSM and secondary toasting at 95 ± 2°C for 30 min.

[2]Unless stated otherwise.

[3]Net energy = 10.8 × digestible CP + 36.1 × digestible acid-hydrolyzed ether extract + 13.7 × starch + 12.4 × sugars + 9.6 × digestible non-starch polysaccharides (CVB, 2011). The calculated net energy contents for SBM and RSM were 10.8 and 9.8 MJ/kg feed.

[4]OMIU = O-methylisourea.

2.2.3 Sample collection and chemical analysis

Samples of the protein sources and the four experimental diets were collected at the beginning of the study, ground to pass a 1-mm screen using a Retsch ZM 100 mill (Retsch GmbH, Haan, Germany), and stored at –20°C until analysis. Feces were quantitatively collected on d 6 to 8. Ileal digesta was collected for 12 h between 0600 and 1800 h on d 9 and 11 of each period by attaching a plastic bag to the cannula. Bags were replaced once per hour. Ileal digesta and feces were pooled per pig per collection period, subsampled, freeze-dried, ground to pass a 1-mm screen using a Retsch ZM 100 mill, and stored at –20°C until analysis.

Diet, ileal digesta, and fecal samples were analyzed for DM (method ISO 6496; ISO, 1999b) by drying to a constant weight at 103°C; N was analyzed by the Kjeldahl method (method ISO 5983-1; ISO, 2005a); CP was calculated as N × 6.25; and Cr_2O_3 was analyzed by ionization and mass spectrometry using a pure grade Cr standard (PerkinElmer, Groningen, the Netherlands; Williams et al., 1962). Diet and fecal samples were analyzed for ash (method ISO 5984; ISO, 2002) after 3 h ashing at 550°C and acid-hydrolyzed ether extract (AEE; method ISO 6492; ISO, 1999a) was analyzed by gravimetric determination after hydrolysis with HCl followed by extraction with petroleum ether. Diet samples were analyzed for crude fiber (method ISO 6865; ISO, 2000) by boiling in sulfuric acid and subsequent ashing, starch (method ISO 15914; ISO, 2004) was analyzed by enzymatic determination of the amount of hexokinase/glucose-6-phosphate-dehydrogenase, total sugars were analyzed as reducing sugars and easily hydrolysable disaccharides (method NEN 3571; NEN, 1974) by hydrolysis in a diluted strong acidic solution with Luff-Schoorl reagent, furosine and CML as indicators for the Maillard reaction and LAL as indicator for cross-linking using reverse-phase ultra-high-performance liquid chromatography and mass spectrometry with $^{13}C_6$ $^{15}N_2$-Lys (Sigma-Aldrich, Steinheim, Germany) as an internal standard (Van Rooijen et al., 2014). Protein source, diet, and ileal digesta samples were analyzed for total AA profile (method ISO 13903; ISO, 2005b) by acid hydrolysis at 110°C for 23 h and ion-exchange chromatography with post-column derivatization with ninhydrin, including sulfur-containing AA measured as cysteic acid and methionine sulfone after oxidation with performic acid; Trp was measured by alkaline hydrolysis at 110°C for 20 h and ion-exchange chromatography with fluorescence detection (method ISO 13904; ISO, 2005c); and OMIU-reactive Lys was measured according to Moughan and Rutherfurd (1996).

For the determination of OMIU-reactive Lys, a 0.6 *M* OMIU solution was prepared as described by Moughan and Rutherfurd (1996) which converts Lys with a free ε-amino group to homoarginine. Briefly, 4 g of barium hydroxide octahydrate (Sigma-Aldrich,

Zwijndrecht, the Netherlands) was added to approximately 16 mL of boiling distilled deionized water that had been boiled for at least 10 min to remove CO_2, after which 2 g of OMIU sulfate salt was added. The solution was transferred to a centrifuge tube and cooled for 30 min at room temperature before being centrifuged at 6,400 × *g* for 10 min at 20°C. The supernatant was retained and the precipitate was washed with approximately 2 mL of boiled distilled deionized water and centrifuged again at 6,400 × *g* for 10 min at 20°C. Both supernatants were combined and the pH was checked to verify that it was above 12 to ensure complete conversion of the sulfate salt to the free base. Thereafter, the pH was adjusted to 11.5, necessary for guanidination, by adding 6 *M* HCl and made up to 20 mL with boiled distilled deionized water. The homoarginine content was analyzed in duplicate in 5-mg ball milled diet and ileal digesta samples. The samples were gently mixed in 1 mL 0.6 *M* OMIU solution for 7 d in a shaker before being dried under vacuum (Savant SpeedVac Concentrator SC210A; Savant Instruments Inc., Farmingdale, NY) and subjected to acid hydrolysis following the procedure for total AA profile described above using L-norleucine (Sigma-Aldrich, Zwijndrecht, the Netherlands) as an internal standard. The OMIU-reactive Lys content was calculated from the homoarginine content. Results showed that crystalline L-Lys HCl was not properly analyzed as OMIU-reactive Lys. The OMIU-reactive Lys contents of the experimental diets were, therefore, unreliable and data on OMIU-reactive Lys contents from the protein sources were used for further calculations.

2.2.4 Calculations and statistical analysis

The ADFI, average daily gain (ADG), and gain to feed ratio (G:F) were calculated per pig per period. Apparent ileal digestibility (AID) and, subsequently, SID of nutrients were calculated using Eq. [1] (Stein et al., 2007; CVB, 2011) and [2] (Stein et al., 2007):

$$\text{AID of } x = 100 - [(x_{\text{digesta}} / x_{\text{diet}}) \times (Cr_2O_{3\text{diet}} / Cr_2O_{3\text{digesta}}) \times 100] \quad [1]$$

and

$$\text{SID of } x = \text{AID of } x + [(\text{basal endogenous } x / x_{\text{diet}}) \times 100], \quad [2]$$

in which x (%) is CP, individual AA, or OMIU-reactive Lys; x_{digesta} and x_{diet} are the CP, individual AA, or OMIU-reactive Lys content (g/kg DM) in the ileal digesta and diet, respectively; $Cr_2O_{3\text{diet}}$ and $Cr_2O_{3\text{digesta}}$ are the analyzed Cr_2O_3 content (mg/kg DM) in the diet and ileal digesta, respectively; and basal endogenous x is the basal endogenous loss (in g/kg DMI) of CP or individual AA as described by Jansman et al. (2002). For the calculation of SID of OMIU-reactive Lys, the basal endogenous Lys loss was used. To determine the SID of CP and AA from the protein sources in diets supplemented with crystalline AA, the crystalline AA were considered to be 100% digested.

The reactive Lys content was also calculated from the furosine content based on the assumption that acid hydrolysis of Amadori products yields 32% furosine and 40% of reverted Lys using Eq. [3] (Desrosiers et al., 1989):

reactive Lys = total Lys – 1.24 × furosine. [3]

The ATTD of DM, OM, ash, CP, AEE, and non-starch polysaccharides (NSP; defined in the current study as DM – ash – CP – AEE – starch – sugar) were calculated using Eq. [4] (CVB, 2011):

ATTD of x = 100 – [(x_{feces} /x_{diet}) × (Cr_2O_{3diet} /Cr_2O_{3feces}) × 100], [4]

in which x (%) is DM (g/kg, as-is), OM, ash, CP, AEE, or NSP (g/kg DM) and Cr_2O_{3diet} and Cr_2O_{3feces} are the analyzed Cr_2O_3 content (mg/kg, as-is for ATTD of DM and mg/kg DM for the other nutrients) in the diet and feces, respectively. For calculating the NSP fraction in feces, starch and sugars were considered to be 100% digested.

The SID of CP, individual AA and OMIU-reactive Lys, ATTD of proximate components, ADG, ADFI, and G:F were statistically analyzed using a 2-tailed mixed model (PROC MIXED procedure) in SAS 9.2 (SAS Inst. Inc., Cary, NC) with pig as the experimental unit. The model included the fixed effects of protein source, processing, and their interaction to test the effect of SBM or RSM and processing on response variables. The random effect included the interaction between period and pig to account for multiple measurements per pig. Least squares means were calculated per experimental diet. The *P*-values < 0.05 were considered significant and *P*-values between 0.05 and 0.10 were considered indicative of a trend.

2.3 Results and discussion

Protein quality in processed protein sources was assessed using the content of AA, OMIU-reactive Lys, MRP, and LAL; SID of CP, AA, and OMIU-reactive Lys; ATTD of nutrients; and growth performance in growing pigs as response criteria. It was hypothesized that SID total Lys content would overestimate Lys bioavailability compared with SID OMIU-reactive Lys content.

2.3.1 Effect of processing on diet composition

The amount of sugar, Arg, and total Lys in the pSBM and pRSM diets were lower compared with the amounts in the SBM and RSM diets, indicating that processing reduced the quantity of these nutrients in the diets (Table 2.3). A decrease in Arg and total Lys in SBM was also found in the study of Harstad and Prestløkken (2000), in which a similar processing treatment was used, and in a study using autoclaved SBM (Kim et al., 2012).

The most reactive reducing sugar in the experimental diets was xylose coming from the lignosulfonate. Xylose has the highest non-enzymatic browning (Maillard reaction) activity (Lievonen et al., 2002). The highly reactive free ε-amino group of Lys is mostly involved in the Maillard reaction followed by Arg (Kwak and Lim, 2004). Both the current study and the study of Harstad and Prestløkken (2000) showed that total Lys decreased more than Arg by applying this type of processing to SBM. Hence, intense processing may reduce the Arg and total and OMIU-reactive Lys contents of diets containing SBM or RSM. Results showed that the SBM and RSM diets already contained furosine, CML, and LAL (Table 2.3), indicating that Maillard and cross-link reactions had taken place in SBM and RSM, presumably during the oil extraction/desolventizing process. Processing increased the furosine, CML, and LAL contents (Table 2.3). Therefore, the amount of early MRP, with furosine as marker, increased due to processing. Moreover, the amount of advanced MRP, with CML as marker, increased as well, and this was also reflected by the loss of total Lys. The loss of total Lys due to processing was larger than the amount of CML formed indicating that other advanced MRP were also formed during processing. The formation of final MRP, that is, melanoidins, was clearly indicated by a darker color of the pSBM and pRSM protein sources compared with the SBM and RSM protein sources, respectively (data not shown). Therefore, the total Lys content in the SBM and RSM protein sources already overestimated the amount of bioavailable Lys as indicated by the lower OMIU-reactive Lys content. Additional determinations of furosine, CML, and LAL could provide more insight in the type of reactions that occurred.

2.3.2 Crystalline L-Lys HCl and the guanidination reaction

The OMIU used during the guanidination reaction of Lys was considered to specifically bind to the free ε-amino group of Lys (Mauron, 1981). However, unlike the SBM and pSBM diets and protein sources, the difference between total and OMIU-reactive Lys in the RSM and pRSM diets (Table 2.3) was much larger than in the RSM and pRSM protein sources (Table 2.1). There was no difference in OMIU-reactive Lys and total Lys contents in samples taken before and after pelleting, indicating that pelleting did not result in additional heat damage to Lys and a reduction in OMIU-reactive Lys (data not shown). It is more likely that crystalline L-Lys HCl, having a free α- and ε-amino group and being present only in the RSM and pRSM diets, was not adequately measured as reactive Lys because OMIU may not be specific for the ε-amino group of Lys. Therefore, we analyzed crystalline L-Lys HCl using the protocol of Moughan and Rutherfurd (1996) with a calculated OMIU to Lys ratio of 20:1, resulting in an OMIU-reactive Lys content of 246 g/kg while 780 g/kg was expected (data not shown). This indicated that OMIU may have been bound to both the α- and ε-amino group of Lys, under the conditions of the analysis, resulting in double derivatized Lys instead of homoarginine. The guanidination method, therefore, may be

unsuitable to determine reactive Lys in diets containing crystalline L-Lys HCl or hydrolyzed protein sources.

In contrast to the OMIU-reactive Lys content in diets with a relatively high content of crystalline L-Lys HCl, the OMIU-reactive Lys content in ileal digesta was considered to be correctly analyzed. The free Lys concentration as percentage of the total amount of Lys present was approximately 10% in the ileal digesta samples and 27% in the RSM diets (T. G. Hulshof, unpublished data). Additionally, there was no difference in free Lys content between the four protein sources (T. G. Hulshof, unpublished data), indicating that the free Lys might come from endogenous sources and not from dietary crystalline L-Lys HCl. The assumption that crystalline L-Lys HCl is 100% digestible, therefore, seems valid. Moughan and Schuttert (1991) found that the concentration of free Lys in endogenous losses was 3.1% of the total amount of Lys in the ileal digesta. The greater value in the current study perhaps can be explained by auto-digestion of endogenous and dietary peptides because enzyme activity was not immediately stopped after digesta collection. Moreover, a reaction time of 7 d and a pH > 11 were found to be optimal for converting Lys to homoarginine in rat digesta (Moughan and Rutherfurd, 1996) and these conditions were used in the current study. Further research on the specificity of OMIU to the α- and ε-amino group of Lys is necessary to fully explain the results found for the RSM and pRSM diets.

2.3.3 Effect of processing on standardized ileal digestibility of CP and amino acids

Processing decreased ($P < 0.001$) the SID of CP and all AA except Pro ($P = 0.140$; Table 2.4). The SID of CP and AA for the SBM and RSM diets were comparable with that found in other studies (Stein et al., 2001; Eklund et al., 2012). The processing of SBM used in this study caused a larger reduction in SID of CP and total and OMIU-reactive Lys in growing pigs than autoclaving of SBM at 125°C for 30 min (González-Vega et al., 2011) and at 135°C for 28 min (Kim et al., 2012). The processing of RSM used in this study had an effect on SID of CP in growing pigs similar to autoclaving of canola meal at 130°C for 30 min (Almeida et al., 2014b). Hence, the processing in combination with lignosulfonate applied in this study to SBM and RSM was suitable to induce a contrast in SID of CP and AA and can be compared with other conditions for processing.

The decrease in SID of CP and AA due to processing might be explained by a less effective denaturation and pepsin activity in the stomach due to conformational changes in protein structure resulting from Maillard and cross-link reactions. Research has shown that pancreatic enzymes, such as carboxypeptidases and the brush border enzyme aminopeptidase N (Hansen and Millington, 1979; Öste et al., 1986), are less effective in

Table 2.4. The effect of protein source, processing,[1] and their interaction on standardized ileal digestibility (%) of CP and individual AA for the four experimental diets containing either soybean meal (SBM), processed SBM (pSBM), rapeseed meal (RSM), or processed RSM (pRSM) fed to growing pigs[2]

	Diet					*P*-value		
Item	SBM	pSBM	RSM	pRSM	SEM	Protein source	Processing	Protein source × processing
CP	83.9	71.6	74.9	64.6	1.3	< 0.001	< 0.001	0.420
Indispensable amino acid								
Arg	94.6	87.4	89.5	84.1	0.7	< 0.001	< 0.001	0.231
His	89.1	77.0	84.0	75.9	1.3	0.022	< 0.001	0.141
Ile	87.6	79.4	78.5	71.6	0.9	< 0.001	< 0.001	0.457
Leu	85.4	78.6	78.9	72.6	0.9	< 0.001	< 0.001	0.797
Lys[3]	85.7	66.8	76.0	57.6	1.2	< 0.001	< 0.001	0.833
Met[3]	89.5	82.0	87.3	82.5	1.0	0.369	< 0.001	0.144
Phe	87.1	80.4	80.8	75.1	1.0	< 0.001	< 0.001	0.603
Thr[3]	83.4	73.6	74.8	65.9	1.3	< 0.001	< 0.001	0.713
Trp[3]	84.2	74.5	78.2	72.2	1.7	0.025	0.001	0.297
Val	85.8	76.8	77.7	69.3	1.0	< 0.001	< 0.001	0.729
Dispensable amino acid								
Ala	81.0	71.7	77.6	68.3	1.3	0.008	< 0.001	0.978
Asp	85.2	72.9	76.0	66.5	1.3	< 0.001	< 0.001	0.277
Cys	83.5	67.4	80.5	68.6	2.2	0.698	< 0.001	0.342
Glu	88.7	78.6	87.4	81.3	1.1	0.492	< 0.001	0.063
Gly	83.3	70.8	79.3	68.7	2.3	0.182	< 0.001	0.664
Pro[4]	83.7	73.5	71.9	58.5	8.0	0.093	0.140	0.838
Ser	88.8	80.0	79.8	71.7	1.2	< 0.001	< 0.001	0.722
Tyr	85.7	79.5	74.0	67.8	1.6	< 0.001	< 0.001	0.994

[1]Processing consisted of addition of lignosulfonate to SBM and RSM and secondary toasting at 95 ± 2°C for 30 min.

[2]Least squares means are reported for seven pigs for SBM diet and RSM diet and for eight pigs for pSBM diet and pRSM diet.

[3]Standardized ileal digestibility was calculated by considering 100% digestibility of L-Lys HCl, DL-Met, L-Thr, and L-Trp.

[4]One pig had an aberrant standardized ileal digestibility for Pro irrespective of diet with values of 38.9% in period 1 (SBM diet), 1.2% in period 2 (pRSM diet), and 15.0% in period 3 (RSM diet). Standardized ileal digestibility of Pro per diet after excluding this pig was: 93.2, 76.0, 83.1, and 69.2% for the SBM, pSBM, RSM, and pRSM diet, respectively with a SEM of 2.9. *P*-values for protein source, processing, and the interaction term were 0.008, < 0.001, and 0.557, respectively.

hydrolyzing peptide bonds located near modified Lys residues, which are highly present in processed protein sources (Moughan et al., 1996). Moreover, aggregation of peptides can occur by cross-linking of unabsorbed AA and smaller peptides resulting in peptides that are unable to cross the small intestinal wall (Mauron, 1981). This lower effectiveness of

hydrolysis and the formation of aggregations may explain the overall decrease in SID of CP and AA seen for the processed protein sources.

2.3.4 Effect of processing on standardized ileal digestibility of total Lys and O-methylisourea-reactive Lys

Processing decreased ($P < 0.001$) the SID of total Lys when considering crystalline L-Lys HCl to be 100% digestible for the SBM and RSM protein sources (Table 2.5). Moreover, the SID of OMIU-reactive Lys when considering crystalline L-Lys HCl to be 100% digestible was also decreased ($P < 0.001$) for the SBM and RSM protein sources. The SID of OMIU-reactive Lys was greater than SID of total Lys for the four experimental diets. Ileal digestibility of total Lys also includes, next to reactive Lys, the digestibility of early MRP, which are found to be less absorbed than reactive Lys (Moughan et al., 1996). The SID total and OMIU-reactive Lys contents for each protein source were similar and were reduced by processing (Table 2.5). The total Lys content overestimated the OMIU-reactive Lys content and the SID of total Lys underestimated the SID of OMIU-reactive Lys. These over- and underestimations were proportional to each other and, in this case, resulted in similar SID total and OMIU-reactive Lys contents. Because SID total and OMIU-reactive Lys are similar, it seems that the early MRP, represented by the difference between total and OMIU-reactive Lys content, were not digested and absorbed in the small intestine of the pigs. Rutherfurd and Moughan (1997) found that the true ileal digestible (TID) total and OMIU-reactive Lys contents were similar for field peas heated at 110, 135, and 165°C for 15 min. Rutherfurd et al. (1997b) found that the TID OMIU-reactive Lys content was greater than the TID total Lys content (31.2 vs. 30.6 g/kg, respectively) in SBM. Differences between SID total and OMIU-reactive Lys contents were also found for distillers dried grains with solubles (Pahm et al., 2009) and wheat, dried maize, heated skim milk powder, cottonseed meal, and an alfalfa-based mix (Rutherfurd et al., 1997b). Therefore, the SID OMIU-reactive Lys content might be a better measure for bioavailable Lys than the SID total Lys content, but this was not found for the SBM and RSM in this study. Despite the fact that the SID contents of total and OMIU-reactive Lys were similar in this study, the SID OMIU-reactive Lys content may still be preferred because the total Lys content and SID of total Lys were significantly different from the values for OMIU-reactive Lys, which is in agreement with Rutherfurd et al. (1997b). Diets are commonly formulated on ileal digestible total Lys, which does not reflect available Lys for processed feed ingredients. Ileal digestible reactive Lys, being a better measure for available Lys, is not often measured and data are, therefore, not available for diet formulation. Therefore, reactive Lys content of the processed feed ingredients may be analyzed and used together with the table values for ileal digestibility of total Lys of the non-processed ingredient to get an indication of the actual available Lys content.

Table 2.5. Standardized ileal digestibility (SID) of total and O-methylisourea (OMIU)-reactive Lys and standardized ileal digestible total and OMIU-reactive Lys content of the four protein sources fed to growing pigs: soybean meal (SBM), processed SBM (pSBM)[1], rapeseed meal (RSM), or processed RSM (pRSM)[1,2]

	Diet					*P*-value		
Item	SBM	pSBM	RSM	pRSM	SEM	Protein source	Processing	Protein source × processing
SID based on analyzed content in the protein source, %								
Total Lys	86.7	66.6	81.8	70.1	1.0	0.525	< 0.001	< 0.001
Total Lys, 100% digestibility of L-Lys HCl[3]	86.7	66.6	74.5	54.0	1.2	< 0.001	< 0.001	0.826
OMIU-reactive Lys	93.1	81.2	88.8	81.7	0.9	0.047	< 0.001	0.012
OMIU-reactive Lys, 100% digestibility of L-Lys HCl[3]	92.8	80.7	83.5	67.7	1.3	< 0.001	< 0.001	0.142
SID based on analyzed content in the diet, %								
Total Lys	85.7	66.8	81.7	70.3	1.1	0.840	< 0.001	0.001
Total Lys, 100% digestibility of L-Lys HCl[3]	85.7	66.8	76.0	57.6	1.2	< 0.001	< 0.001	0.833
OMIU-reactive Lys	92.6	80.9	84.2	74.1	1.1	< 0.001	< 0.001	0.488
SID content of the protein source, g/100 g CP[4]								
Total Lys	5.5	3.1	4.2	2.3				
OMIU-reactive Lys	5.6	3.0	4.2	2.1				

[1]Processing consisted of addition of lignosulfonate to SBM and RSM and secondary toasting at 95 ± 2°C for 30 min.

[2]Least squares means are reported for seven pigs for the SBM diet and the RSM diet and for eight pigs for the pSBM diet and the pRSM diet.

[3]Calculated to derive the SID of total and OMIU-reactive Lys of the RSM and pRSM protein sources.

[4]Calculated using total and OMIU-reactive Lys content in the protein source and SID based on content in the protein source of total and OMIU-reactive Lys when considering 100% digestibility of L-Lys HCl.

2.3.5 Effect of processing on apparent total tract digestibility of CP

Processing decreased ($P < 0.001$) ATTD of CP for SBM and RSM (Table 2.6), which was lower than the decrease observed for SID of CP. Ileal digestibility of CP includes the undigested N from advanced and final MRP because these products are resistant to enzymatic degradation. Some advanced MRP can be metabolized by gut microbiota whereas final MRP (that is, melanoidins) are resistant to this (Van Rooijen et al., 2013). Part of the N from advanced MRP is, therefore, lost in the large intestine, resulting in lower amounts of N in the feces, which may explain the observed smaller difference in ATTD of CP compared with SID of CP between the experimental diets.

Table 2.6. The effect of protein source, processing,[1] and their interaction on apparent total tract digestibility (%) of proximate components in four experimental diets containing soybean meal (SBM), processed SBM (pSBM), rapeseed meal (RSM), or processed RSM (pRSM) fed to growing pigs[2]

	Diet					*P*-value		
Item	SBM	pSBM	RSM	pRSM	SEM	Protein source	Processing	Protein source × processing
DM	94.2	92.3	87.2	86.5	0.3	< 0.001	< 0.001	0.080
OM[3]	95.8	94.0	89.2	88.5	0.3	< 0.001	< 0.001	0.066
Ash	63.6	60.8	44.0	43.7	1.1	< 0.001	0.146	0.269
CP	88.8	81.3	76.5	72.1	1.1	< 0.001	< 0.001	0.167
Acid-hydrolyzed ether extract	82.3	81.7	81.5	82.3	0.8	0.926	0.852	0.350
Non-starch polysaccha-rides[4]	86.1	85.0	55.0	55.4	0.9	< 0.001	0.688	0.427

[1]Processing consisted of addition of lignosulfonate to SBM and RSM and secondary toasting at 95 ± 2°C for 30 min.

[2]Two pigs were not fully adapted in the first fecal collection period; hence, least squares means for six pigs for the SBM diet, seven pigs for the pSBM diet and the RSM diet, and eight pigs for the pRSM diet.

[3]Organic matter calculated as DM – ash.

[4]Non-starch polysaccharides were defined in the current study as DM – ash – CP – acid-hydrolyzed ether extract – starch – sugar in the diet and DM – ash – CP – acid-hydrolyzed ether extract in feces considering complete digestion of starch and sugars.

2.3.6 Effect of processing on growth performance

The ADFI was approximately 200 g/d greater ($P = 0.007$; Table 2.7) for the RSM diets compared with the SBM diets because of the greater feed allowance to account for the lower calculated net energy content of the RSM diet (9.8 MJ/kg feed) compared with that

of the SBM diet (10.8 MJ/kg feed). Processing decreased ADG (P = 0.002) and G:F (P = 0.006; Table 2.7). The decrease in growth performance was presumably caused by the lower digestibility of nutrients, in particular, the lower Lys bioavailability for pSBM and pRSM compared with SBM and RSM. The lower amount of bioavailable AA would limit protein deposition. The surplus available energy that could not be used for protein deposition would be deposited as fat resulting in a lower weight gain because fat deposition requires more energy and is deposited without water. The conditions of the study, that is, using pigs with a high health status, surgery to insert the cannula, individual housing, and collection of ileal digesta and feces, influenced G:F and ADG but these effects were considered to be equal between experimental diets. The study design allowed for measurements of ADG over a short period of time (that is, 11 d) but the negative effects of processing on ADG are also expected to occur for longer growth periods. Lignosulfonate treatment of SBM in a study with broiler chicks also decreased Lys, reactive Lys (measured with 1-fluoro-2,4-dinitrobenzene), and ADG and increased feed conversion ratio (ADFI/ADG; Awawdeh et al., 2007). Feeding autoclaved SBM (125°C for 60 min) to weanling pigs also resulted in a decreased ADG and G:F (Almeida et al., 2014a). Feeding processed protein sources, therefore, may negatively affect growth performance in different monogastric species in different stages of growth.

Table 2.7. The effect of protein source, processing[1], and their interaction on growth performance of growing pigs fed one of four experimental diets containing soybean meal (SBM), processed SBM (pSBM), rapeseed meal (RSM), or processed RSM (pRSM)[2]

	Diet					*P*-value		
Item	SBM	pSBM	RSM	pRSM	SEM	Protein source	Proces-sing	Protein source × processing
Average daily feed intake, kg/d	1.12	1.13	1.31	1.33	0.07	0.007	0.815	0.976
Average daily gain, kg/d	0.58	0.45	0.59	0.51	0.03	0.248	0.002	0.494
Gain to feed ratio	0.52	0.42	0.46	0.39	0.03	0.133	0.006	0.525

[1]Processing consisted of addition of lignosulfonate to SBM and RSM and secondary toasting at 95 ± 2°C for 30 min.

[2]Least squares means are reported for seven pigs for the SBM diet and the RSM diet and for eight pigs for the pSBM diet and the pRSM diet.

In conclusion, the intense processing used in the current study is not commonly applied in pig feed industry but could be used as model to evaluate protein quality. There was no difference in SID total and OMIU-reactive Lys content for each protein source. Therefore, the protein quality of these four protein sources was equally well predicted by SID OMIU-

reactive Lys and SID total Lys. This result was caused by a proportional overestimation of OMIU-reactive Lys content by total Lys and underestimation of OMIU-reactive Lys digestibility by the SID of total Lys. Crystalline L-Lys HCl was not properly analyzed as reactive Lys when using the protocol of Moughan and Rutherfurd (1996). Further research to the specificity of OMIU to the ε-amino group of Lys is necessary to explain the results found for the RSM and pRSM diets in more detail. Analysis of MRP and LAL in SBM and RSM has received little attention and the results showed that the original, commercial SBM and RSM already contained these products. Processing resulted in greater amounts of MRP and LAL and it negatively affected nutrient digestibility and pig growth performance. The effect of processing on post-absorptive nutrient utilization to elucidate the effects of processing on pig growth performance warrants further study.

2.4 Acknowledgement

The authors acknowledge the financial support from the Wageningen UR "IPOP Customized Nutrition" program financed by Wageningen UR (Wageningen, the Netherlands), the Dutch Ministry of Economic Affairs (The Hague, the Netherlands), WIAS (Wageningen, the Netherlands), Agrifirm Innovation Center (Apeldoorn, the Netherlands), ORFFA Additives BV (Werkendam, the Netherlands), Ajinomoto Eurolysine s.a.s. (Paris, France), and Stichting VICTAM BV (Nijkerk, the Netherlands). The authors thank J. Th. M. van Diepen, R. A. Dekker, and the laboratories of Food Chemistry (T. F. M. Kuijpers) and Animal Nutrition (M. J. H. Breuer, H. Beukers-Van Laar, C. van Rooijen, and R. van Erp) of Wageningen UR for their contributions. The authors thank Feed Valid B.V., Poederoijen, the Netherlands, for providing the lignosulfonate and the two soybean meals and two rapeseed meals.

2.5 Literature cited

Almeida, F. N., J. K. Htoo, J. Thomson, and H. H. Stein. 2014a. Effects of balancing crystalline amino acids in diets containing heat-damaged soybean meal or distillers dried grains with solubles fed to weanling pigs. Animal 8:1594-1602.

Almeida, F. N., J. K. Htoo, J. Thomson, and H. H. Stein. 2014b. Effects of heat treatment on the apparent and standardized ileal digestibility of amino acids in canola meal fed to growing pigs. Anim. Feed Sci. Technol. 187:44-52.

Awawdeh, M. S., E. C. Titgemeyer, J. S. Drouillard, R. S. Beyer, and J. E. Shirley. 2007. Ruminal degradability and lysine bioavailability of soybean meals and effects on performance of dairy cows. J. Dairy Sci. 90:4740-4753.

Centraal Veevoeder Bureau (CVB). 2011. Chemical compositions and nutritional values of feed ingredients. CVB, Lelystad, the Netherlands.

Desrosiers, T., L. Savoie, G. Bergeron, and G. Parent. 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.

De Vrese, M., R. Frik, N. Roos, and H. Hagemeister. 2000. Protein-bound D-amino acids, and to a lesser extent lysinoalanine, decrease true ileal protein digestibility in minipigs as determined with ^{15}N-labelling. J. Nutr. 130:2026-2031.

Eklund, M., W. R. Caine, W. C. Sauer, G. S. Huang, G. Diebold, M. Schollenberger, and R. Mosenthin. 2012. True and standardized ileal digestibilities and specific ileal endogenous recoveries of crude protein and amino acid in soybean meal, rapeseed meal and peas fed to growing pigs. Livest. Sci. 145:174-182.

Friedman, M. 1999. Chemistry, biochemistry, nutrition, and microbiology of lysinoalanine, lanthionine, and histidinoalanine in food and other proteins. J. Agric. Food Chem. 47:1295-1319.

González-Vega, J. C., B. G. Kim, J. K. Htoo, A. Lemme, and H. H. Stein. 2011. Amino acid digestibility in heated soybean meal fed to growing pigs. J. Anim. Sci. 89:3617-3625.

Hansen, L. P., and R. J. Millington. 1979. Blockage of protein enzymatic digestion (carboxypeptidase-B) by heat-induced sugar-lysine reactions. J. Food Sci. 44:1173-1177.

Harstad, O. M., and E. Prestløkken. 2000. Effective rumen degradability and intestinal indigestibility of individual amino acids in solvent-extracted soybean meal (SBM) and xylose-treated SBM (SoyPass®) determined *in situ*. Anim. Feed Sci. Technol. 83:31-47.

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

International Organization for Standardization (ISO). 1999a. ISO 6492:1999. Animal feeding stuffs - Determination of fat content. ISO, Geneva, Switzerland.

International Organization for Standardization (ISO). 1999b. ISO 6496:1999. Animal feeding stuffs - Determination of moisture and other volatile matter content. ISO, Geneva, Switzerland.

International Organization for Standardization (ISO). 2000. ISO 6865:2000. Animal feeding stuffs - Determination of crude fibre content - Method with intermediate filtration. ISO, Geneva, Switzerland.

International Organization for Standardization (ISO). 2002. ISO 5984:2002. Animal feeding stuffs - Determination of crude ash content. ISO, Geneva, Switzerland.

International Organization for Standardization (ISO). 2004. ISO 15914:2004. Animal feeding stuffs - Enzymatic determination of total starch content. ISO, Geneva, Switzerland.

International Organization for Standardization (ISO). 2005a. ISO 5983-1:2005. Animal feeding stuffs - Determination of nitrogen content and calculation of crude protein content - Part 1: Kjeldahl method. ISO, Geneva, Switzerland.

International Organization for Standardization (ISO). 2005b. ISO 13903:2005. Animal feeding stuffs - Determination of amino acids content. ISO, Geneva, Switzerland.

International Organization for Standardization (ISO). 2005c. ISO 13904:2005. Animal feeding stuffs - Determination of tryptophan content. ISO, Geneva, Switzerland.

Jansman, A. J. M., W. Smink, P. van Leeuwen, and M. Rademacher. 2002. Evaluation through literature data of the amount and amino acid composition of basal endogenous crude protein at the terminal ileum of pigs. Anim. Feed Sci. Technol. 98:49-60.

Kim, J. C., B. P. Mullan, and J. R. Pluske. 2012. Prediction of apparent, standardized, and true ileal digestible total and reactive lysine contents in heat-damaged soybean meal samples. J. Anim. Sci. 90(Suppl. 4):137-139.

Kwak, E.-J., and S.-I. Lim. 2004. The effect of sugar, amino acid, metal ion, and NaCl on model Maillard reaction under pH control. Amino Acids 27:85-90.

Lievonen, S. M., T. J. Laaksonen, and Y. H. Roos. 2002. Nonenzymatic browning in food models in the vicinity of the glass transition: Effects of fructose, glucose, and xylose as reducing sugar. J. Agric. Food Chem. 50:7034-7041.

Mauron, J. 1981. The Maillard reaction in food; A critical review from the nutritional standpoint. Prog. Food Nutr. Sci. 5:5-35.

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

Moughan, P. J., and S. M. Rutherfurd. 2008. Available lysine in foods: A brief historical overview. J. AOAC Int. 91:901-906.

Moughan, P. J., and G. Schuttert. 1991. Composition of nitrogen-containing fractions in digesta from the distal ileum of pigs fed a protein-free diet. J. Nutr. 121:1570-1574.

Mroz, Z., G. C. Bakker, A. W. Jongbloed, R. A. Dekker, R. Jongbloed, and A. van Beers. 1996. Apparent digestibility of nutrients in diets with different energy density, as estimated by direct and marker methods for pigs with or without ileo-cecal cannulas. J. Anim. Sci. 74:403-412.

Netherlands Standardization Institute (NEN). 1974. NEN 3571:1974 nl. Test method for feeding stuffs - Determination of reducing sugar, crude total sugar, saccharose and lactose. Nederlands Normalisatie Instituut, Delft, the Netherlands.

Öste, R. E., A. Dahlqvist, H. Sjöström, O. Norén, and R. Miller. 1986. Effect of Maillard reaction products on protein digestion. *In vitro* studies. J. Agric. Food Chem. 34:355-358.

Pahm, A. A., C. Pedersen, and H. H. Stein. 2009. Standardized ileal digestibility of reactive lysine in distillers dried grains with solubles fed to growing pigs. J. Agric. Food Chem. 57:535-539.

Prestløkken, E., and O. Rise. 2003. Protein and amino acid digestibility in dairy cows measured with mobile nylon bags recovered in ileum or in faeces. Acta Agric. Scand., Sect. A 53:11-20.

Rérat, A., R. Calmes, P. Vaissade, and P. A. Finot. 2002. Nutritional and metabolic consequences of the early Maillard reaction of heat treated milk in the pig. Significance for man. Eur. J. Nutr. 41:1-11.

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

Stein, H. H., M. F. Fuller, P. J. Moughan, B. Sève, R. Mosenthin, A. J. M. Jansman, J. A. Fernández, and C. F. M. de Lange. 2007. Definition of apparent, true, and standardized ileal digestibility of amino acids in pigs. Livest. Sci. 109:282-285.

Stein, H. H., S. W. Kim, T. T. Nielsen, and R. A. Easter. 2001. Standardized ileal protein and amino acid digestibility by growing pigs and sows. J. Anim. Sci. 79:2113-2122.

Van Barneveld, R. J., E. S. Batterham, and B. W. Norton. 1994. 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 Boekel, M. A. J. S. 2006. Formation of flavour compounds in the Maillard reaction. Biotechnol. Adv. 24:230-233.

Van Rooijen, C., G. Bosch, A. F. B. van der Poel, P. A. Wierenga, L. Alexander, and W. H. Hendriks. 2013. The Maillard reaction and pet food processing: Effects on nutritive value and pet health. Nutr. Res. Rev. 26:130-148.

Van Rooijen, C., G. Bosch, A. F. B. van der Poel, P. A. Wierenga, L. Alexander, and W. H. Hendriks. 2014. Quantitation of Maillard reaction products in commercially available pet foods. J. Agric. Food Chem. 62:8883-8891.

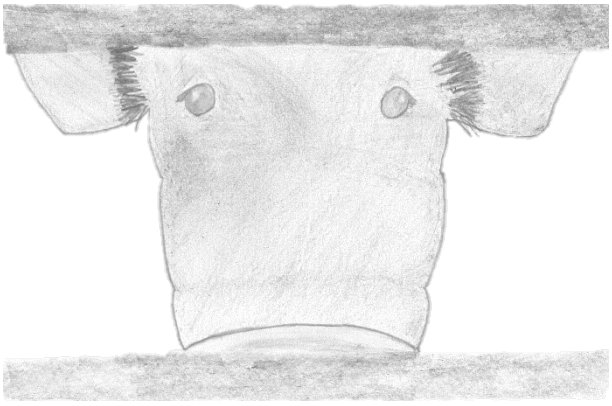
Williams, C. H., D. J. David, and O. Iismaa, 1962. The determination of chromic oxide in faeces samples by atomic absorption spectrophotometry. J. Agric. Sci. 59:381-385.

Processing of soybean meal and 00-rapeseed meal reduces protein digestibility and pig growth performance but does not affect nitrogen solubilization along the small intestine

T. G. Hulshof,[*†] A. F. B. van der Poel,[*] W. H. Hendriks,[*] and P. Bikker[†]

*Animal Nutrition Group, Wageningen University, Wageningen, the Netherlands, and [†]Wageningen UR Livestock Research, Wageningen, the Netherlands

Journal of Animal Science 2016, 94 (6):2403-2414



Abstract

An experiment was conducted to determine the effects of processing of soybean meal (SBM) and 00-rapeseed meal (RSM) on N solubilization in digesta, CP digestibility along the small intestine, metabolic load as determined by organ weight, body composition, and growth performance in growing pigs. The SBM and RSM were processed by secondary toasting (at 95°C for 30 min) in the presence of lignosulfonate resulting in processed SBM (pSBM) and processed RSM (pRSM) as a model for over-processed protein sources. Fifty-four growing pigs were each fed one of the six experimental diets. Four of the diets contained SBM, pSBM, RSM, or pRSM as sole protein source. The remaining two experimental diets contained pSBM or pRSM and were supplemented with crystalline AA to the same standardized ileal digestible (SID) AA levels as the SBM or RSM diet. Pigs were slaughtered at 40 kg, and organ weights were recorded. The organs plus blood and empty carcass were analyzed for CP content. The small intestine was divided into 3 segments, and digesta samples were taken from the last meter of each segment. Digesta of the SBM, pSBM, RSM, and pRSM diets was centrifuged to separate the soluble and insoluble fractions, and N content was determined in the latter. The amount of insoluble N as a fraction of N in digesta at each small intestinal segment was not affected by processing. Diet type, comprising effects of processing and supplementing crystalline AA, affected (P < 0.05) the gain to feed ratio (G:F) and SID of CP. Processing reduced G:F from 0.56 to 0.38 for SBM and 0.49 to 0.40 for RSM whereas supplementing crystalline AA increased G:F to the level of the SBM and RSM diets. Processing reduced the SID of CP from 87.2% to 69.2% for SBM and 71.0% to 52.2% for RSM. Diet type affected (P < 0.05) the CP content in the empty body with processing reducing this content from 170 to 144 g/kg empty BW for SBM and 157 to 149 g/kg empty BW for RSM and supplementing crystalline AA restoring this content. Processing reduced (P < 0.05) the weight of several organs, and supplementing crystalline AA restored organ weight. In conclusion, processing increased the amount of N in the digesta, reduced organ weight, body CP content, and G:F. These effects were caused by a reduction in available AA as supplementing crystalline AA restored organ weight, body CP content, and G:F.

Key words: amino acid availability, amino acid supplementation, growing pigs, nitrogen solubility, organ weight, secondary toasting

3.1 Introduction

Processing of feed ingredients such as soybean meal (SBM) and rapeseed meal (RSM) is commonly applied before inclusion as protein source in pig and poultry feed. Processing of protein-rich ingredients may result in modified Lys residues because of Maillard-type reactions, for example, reactions between reducing sugars and free amino groups, especially the ε-amino group of Lys (Mauron, 1981), or cross-link reactions (Friedman, 1999), leading to a decrease in protein quality (Hurrell and Carpenter, 1981). *In vitro* studies have shown that pancreatic and brush border enzymes were less effective in hydrolyzing peptide bonds near these modified Lys residues (Hansen and Millington, 1979; Öste et al., 1986), therefore lowering CP digestibility. Previous research in our laboratory showed that toasting of SBM and RSM in the presence of lignosulfonate reduced nutrient availability, that is, standardized ileal digestible (SID) amino acid (AA) content, and growth performance of growing pigs and resulted in the formation of Maillard reaction products (MRP; Hulshof et al., 2016). However, the mode of action of these effects and the metabolic consequences are hitherto not clear. Processing decreases N solubility in feed ingredients (Araba and Dale, 1990). The current study determined the effect of processing on N solubilization and rate of degradation in the digestive tract to explain the observed decrease in CP digestibility. Processing has been shown to reduce growth performance, which may be mediated by a reduced AA digestibility and availability, imbalance between essential and nonessential AA and net energy due to a greater loss of SID Lys compared with other AA, or presence of MRP. Supplementation with crystalline AA was used to determine the contribution of reduced AA availability to the effects of processing. Growth performance was assessed by carcass composition and gain to feed ratio (G:F), and effects of dietary treatments on metabolic load were assessed by measuring organ weights.

3.2 Materials and methods

3.2.1 Animals and housing

This study was approved by the Animal Care and Use Committee of Wageningen University and Research Centre (Wageningen, the Netherlands). The experiment consisted of 54 growing gilts (Tempo × Topigs 40 from Van Haaren, Horssen, the Netherlands) with an average initial BW of 15.6 ± 0.7 kg on the day of arrival (d 1). The pigs were housed in four identical rooms in individual pens (0.9 by 3.8 m). Pigs were fed a commercial diet and were gradually adapted during the first week to a mixture of the SBM and RSM diets (50:50) and to a feeding level of 3.0 times net energy requirements for maintenance (321 kJ net energy/kg $BW^{0.75}$; ARC, 1981). After the first week, pigs were allocated to an experimental diet and subsequently to a pen. The pens consisted of a half-slatted and half-concrete floor with no bedding material and a feeding trough and nipple drinker. The

pigs had free access to water during the whole experimental period. The ambient temperature in each room was kept constant at 24°C during the whole experimental period, and the lights were turned on from 0700 until 1900 h and dimmed at night.

3.2.2 Experimental design and diets

The study consisted of a randomized complete block design with BW on d 8 as the blocking factor and 9 blocks each consisting of 6 pigs. Pigs within a block were randomly allocated to one of six experimental diets. The blocks were allocated to four rooms with two blocks per room. Because of a limited number of pens per room, one block was divided over two rooms.

All six isoenergetic (net energy 9.96 MJ/kg) experimental diets (Research Diet Services, Wijk bij Duurstede, the Netherlands) consisted of a basal CP-free diet based on gelatinized potato starch. Commercially toasted/desolventized SBM (*Glycine max*) and 00-RSM (*Brassica napus* containing low levels of erucic acid and glucosinolates; both supplied by Feed Valid B.V., Poederoijen, the Netherlands) were each included as the sole protein source in three experimental diets (2 × 3 factorial treatment arrangement). Soybean meal and RSM were used either as such, referred to as SBM and RSM diets, respectively, or processed by secondary toasting (steam treatment) in the presence of lignosulfonate at 95°C for 30 min in a seven-floor cascade toaster to obtain processed SBM (pSBM) and processed RSM (pRSM). The process is normally used in ruminant nutrition to produce bypass protein (Prestløkken and Rise, 2003) but was used in the current study to induce protein damage through the Maillard reaction. The SBM and RSM diets were supplemented with crystalline AA to meet 90% of the requirements for SID Lys (SID Lys/net energy ratio of 0.91) and the other indispensable AA as a percentage of Lys according to nutrient recommendations (CVB, 2011). The remaining two experimental diets contained pSBM and pRSM and were supplemented with crystalline AA to meet the SID AA content in the SBM and RSM diets, respectively, on the basis of the SID AA content in the pSBM and pRSM protein sources (Hulshof et al., 2016). These diets were formulated to compensate for the loss in SID AA by processing and are referred to as the pSBM+AA and pRSM+AA diets. L-Glutamine was added to these diets to account for the loss in SID dispensable AA. Titanium dioxide was added to all diets as an indigestible marker at an amount of 2.5 g/kg (as-fed basis). The protein sources and diets were produced as described in Hulshof et al. (2016) and pelleted with steam through a 3-mm die. The ingredient and analyzed nutrient composition of the six experimental diets are provided in Tables 3.1 and 3.2, respectively.

Table 3.1. Ingredient composition of six experimental diets containing soybean meal (SBM), processed SBM (pSBM), pSBM supplemented with crystalline AA (pSBM+AA), rapeseed meal (RSM), processed RSM (pRSM), or pRSM supplemented with crystalline AA (pRSM+AA) fed to growing pigs[1]

Ingredient composition, g/kg as fed	Diet					
	SBM	pSBM	pSBM+AA	RSM	pRSM	pRSM+AA
Gelatinized potato starch	420.1	420.1	385.0	447.4	447.4	430.8
SBM	350.0	350.0	350.0	-	-	-
RSM	-	-	-	350.0	350.0	350.0
Lignosulfonate[2]	26.75	26.75	26.75	18.81	18.81	18.81
Dextrose	100.0	100.0	100.0	100.0	100.0	100.0
Arbocel[3]	49.9	49.9	49.9	-	-	-
Soybean oil	20.0	20.0	23.5	35.0	35.0	36.0
Limestone	11.37	11.37	11.37	7.40	7.40	7.38
Monocalcium phosphate	8.68	8.68	8.79	7.97	7.97	7.99
Vitamin-mineral premix[4]	5.0	5.0	5.0	5.0	5.0	5.0
Salt	3.68	3.68	3.68	3.55	3.55	3.55
Potassium carbonate[5]	-	-	3.41	7.79	7.79	9.30
Titanium dioxide	2.5	2.5	2.5	2.5	2.5	2.5
L-Arg HCl (83% Arg)	-	-	3.20	-	-	1.47
L-His (99% His)	-	-	0.84	-	-	0.35
L-Ile (99% Ile)	-	-	0.91	1.66	1.66	2.04
L-Leu (99% Leu)	-	-	1.40	3.12	3.12	3.82
L-Lys HCl (78% Lys)	0.10	0.10	5.45	4.52	4.52	7.01
DL-Met (99% Met)	1.58	1.58	1.86	1.40	1.40	1.54
L-Cys (99% Cys)	-	-	0.49	-	-	0.45
L-Phe (98% Phe)	-	-	0.91	-	-	0.35
L-Thr (98% Thr)	0.34	0.34	1.21	1.68	1.68	2.20
L-Trp (98% Trp)	-	-	0.35	0.52	0.52	0.62
L-Tyr (98% Tyr)	-	-	0.56	-	-	0.31
L-Val (98% Val)	-	-	1.01	1.68	1.68	2.31
L-Gln (99% Gln)	-	-	11.91	-	-	6.20

[1]Processing consisted of the addition of lignosulfonate to SBM and RSM and secondary toasting at 95 ± 2°C for 30 min. The pSBM+AA and pRSM+AA diets were formulated on standardized ileal digestible amino acid levels in pSBM and pRSM and supplemented with crystalline amino acid to meet standardized ileal digestible amino acid levels in the SBM and RSM diets, respectively.

[2]Lignosulfonate typically contains 35% to 42% Mg-lignosulfonate, 15% to 25% xylose, 3% to 7% glucose, 3% to 7% mannose, 3% to 7% rhamnose, 12% to 41% water (add up to 100%). The CP level was analyzed as 3.2 g/kg as is.

[3]Rettenmaier & Söhne Group, Rosenberg, Germany.

[4]The vitamin-mineral premix provided per kilogram complete diet: Vitamin A, 10,000 IU; vitamin D_3, 2,000 IU; vitamin E, 40 mg; vitamin K_3, 1.5 mg; thiamin, 1.0 mg; riboflavin, 4.0 mg; pyridoxine, 1.5 mg; vitamin B_{12}, 20 µg; niacin, 30 mg; D-pantothenic acid, 15 mg; choline Cl, 150 mg; folic acid, 0.4 mg; biotin, 0.05 mg; Fe, 100 mg as $FeSO_4$; Cu, 160 mg as $CuSO_4$; Mn, 30 mg as MnO; Zn, 70 mg as $ZnSO_4$; Co, 0.21 mg as $CoSO_4$; I, 0.70 mg as KIO_3; Se, 0.25 mg as Na selenite; antioxidant, 125 mg.

[5]Added to maintain a constant diet cation-anion balance.

Table 3.2. Analyzed nutrient composition of six experimental diets fed to growing pigs

	Diet[1]					
Composition	SBM	pSBM	pSBM+AA	RSM	pRSM	pRSM+AA
DM, g/kg	894	901	898	887	894	896
Ash, g/kg DM	53	54	58	58	59	60
CP, g/kg DM	184	186	215	147	148	161
Acid-hydrolyzed ether extract, g/kg DM	29	26	33	51	52	53
Crude fiber, g/kg DM	44	50	49	52	55	58
Starch, g/kg DM	395	401	379	432	428	411
Total sugars, g/kg DM	156	150	144	139	133	129
Net energy,[2] MJ/kg feed	9.9	9.5	9.7	10.0	9.9	10.0
Carboxymethyllysine,[3] mg/100 g CP	42	67	-	53	121	-
Lysinoalanine,[3] mg/100 g CP	28	38	-	26	186	-
Furosine,[3] mg/100 g CP	444	577	-	581	1,114	-
Indispensable amino acid, g/100 g CP						
Arg	6.9	6.2	6.7	5.1	4.5	4.9
His	2.5	2.3	2.5	2.3	2.2	2.3
Ile	4.4	4.5	4.4	4.7	4.6	4.6
Leu	7.5	7.6	7.3	8.4	8.5	8.2
Total Lys	6.0	4.6	6.1	7.3	6.1	6.8
OMIU-reactive Lys[4]	4.8	3.2	3.3	4.0	2.9	2.9
Met	2.1	2.0	1.7	2.5	2.5	2.3
Phe	5.0	5.0	4.8	3.6	3.6	3.5
Thr	4.1	4.1	4.0	5.1	5.1	5.0
Trp	1.3	1.3	1.3	1.6	1.6	1.5
Val	4.7	4.8	4.7	5.8	5.7	5.8
Dispensable amino acid, g/100 g CP						
Ala	4.3	4.3	3.9	4.0	4.0	3.7
Asp	11.0	11.1	9.7	6.6	6.5	6.0

Cys	1.4	1.3	1.3	1.9	1.9	1.9
Glu	17.6	17.8	21.3	14.7	14.7	17.4
Gly	4.1	4.2	3.7	4.6	4.6	4.3
Pro	5.1	5.1	4.4	5.5	5.5	5.1
Ser	4.9	5.0	4.4	3.9	3.9	3.5
Tyr	3.3	3.3	3.3	2.5	2.5	2.6

[1]SBM = soybean meal and RSM = rapeseed meal. Processing consisted of the addition of lignosulfonate to SBM and RSM and secondary toasting at 95 ± 2°C for 30 min yielding the pSBM and pRSM diets. Processing plus supplementing with crystalline AA to standardized ileal digestible amino acid levels in the SBM and RSM diets is indicated as pSBM+AA and pRSM+AA, respectively.

[2]Net energy = 10.8 × digestible CP + 36.1 × digestible acid-hydrolyzed ether extract + 13.7 × starch + 12.4 × sugars + 9.6 × digestible non-starch polysaccharides (CVB, 2011).

[3]Calculated from data reported in Hulshof et al. (2016).

[4]OMIU = O-methylisourea.

The experimental diets were fed from d 9 until a BW of 40 ± 2 kg was reached. Pigs were fed equal portions at 0800 and 1600 h daily. Body weight was measured twice a week, and feed allowance was calculated as 3.0 times net energy requirements for maintenance (ARC, 1981) on the basis of BW and expected growth. Feed refusals were refed with the next morning feeding, and total feed intake was determined at the end of the experiment by weighing feed refusals that were not ingested.

3.2.3 Sample collection and slaughter procedure

Samples of the six experimental diets were collected during feed production, ground to pass a 1-mm screen using a Retsch ZM 200 mill (Retsch GmbH, Haan, Germany), and stored at 4°C until analysis. Fecal samples of all pigs were taken twice a day on d 31 to 33 by rectal stimulation. Feces were pooled per pig, subsampled, oven-dried (70°C), ground to pass a 1-mm screen using a Retsch ZM 200 mill, and stored at 4°C until analysis.

Pigs were fed half of their daily feed allowance 4 h before slaughter. Pigs were killed at a BW of 40 ± 2 kg by electrical stunning, followed by hanging the pig by the ankles and exsanguination by cutting the carotid artery. Blood was collected in a plastic bag and weighed. Immediately after opening the abdominal cavity, four clamps were placed distributed over the length of the small intestine to prevent digesta relocation. The gastrointestinal tract (GIT) from stomach to anus was removed and carefully weighed to prevent mixing of digesta; the full GIT was weighed together with the other thoracic and abdominal organs to obtain the full organ weight. The small intestine was placed horizontally after weighing and ligated at the pylorus and ileocecal junction. The small intestine was thereafter carefully separated from the stomach and large intestine. The small intestine was kept horizontally during the removal of the mesentery, spread out on a table, and divided in three segments of equal length. Care was taken during small intestinal handling to minimize mixing of digesta between segments before the segments were ligated. Clamps were placed at the end and approximately 100 cm before the end of each segment. Digesta was collected by flushing the contents of these 100 cm parts with water acclimatized to room temperature into a container while avoiding contact between the outside of the intestine and the container. The collected digesta was subsequently frozen in liquid nitrogen to avoid degradation of proteins and stored at –80°C.

The remainder of the small intestine, the stomach, and large intestine, were emptied by squeezing, followed by rinsing with water. The urine and gall bladders were emptied by squeezing, and afterward, all emptied thoracic and abdominal organs were weighed individually, subsequently combined, and weighed to obtain a measure of empty organ weight. The organs were collected together with the blood in a bag and weighed to obtain

the weight of the blood and organs which is referred to as the organ fraction. The empty carcass, including the head, teeth, feet, hair, and skin, was collected in a bag and weighed to obtain the empty carcass weight. The organ fraction and empty carcass were frozen at –20°C and thereafter cut separately in small blocks using a band saw (Butcher Boy B 14-9, Montebello, CA) and homogenized using a commercial butcher's mincer (Alexanderwerk, Remscheid, Germany). The organ fraction and empty carcass were subsampled after mincing and stored at –20°C pending analysis.

Digesta from all three small intestinal segments of pigs fed the SBM, pSBM, RSM, and pRSM diets (n = 108) was thawed and subsampled for the determination of apparent digestibility of CP along the small intestine and for separation into soluble and insoluble phases. For the separation in phases, approximately 40 g of digesta were subsampled into a 50-mL tube. The digesta sample was quantitatively transferred into a 100-mL centrifuge tube and centrifuged at 20,000 × g for 15 min at 4°C. The weight of the emptied 50-mL tube and the amount of Milli-Q water to balance the tube were noted. After centrifugation, the supernatant was pipetted back into the 50-mL tube. The residue was centrifuged again after the addition of 5 mL Milli-Q water. Both supernatants were combined, and the weight of the supernatant, that is, soluble phase, including the 50-mL tube was noted. The residue, that is, the insoluble phase, was transferred to a 40-mL beaker using a spatula and oven-dried at 70°C, ground using a mortar and pestle, and stored at 4°C pending analysis. The soluble phase was stored at –20°C for future analysis.

For the pSBM+AA and pRSM+AA diets, only ileal digesta (that is, the most posterior sample of the three segments; n = 18) was thawed and subsampled for the determination of SID of CP. The digesta subsampled for the determination of digestibility of CP was oven-dried (70°C), ground to pass a 1-mm screen using a Culatti mill (Culatti AG, Zürich, Switzerland), and stored at 4°C until analysis.

3.2.4 Chemical analysis

The organ fraction and empty carcass were separately analyzed for moisture by freeze-drying; ash (method ISO 5984; ISO, 2002) was analyzed after 3 h of heating at 550°C, ether extract (method ISO 6492; ISO, 1999a) was analyzed by extraction with petroleum ether, and N (method ISO 5983-1; ISO, 2005a) was analyzed by the Kjeldahl method with CP calculated as N × 6.25. Diet, complete digesta, the insoluble phase of the digesta samples, and fecal samples were analyzed for N (method ISO 16634-1; ISO, 2008) by combustion according to the Dumas principle and calculation of CP as N × 6.25. Diet, complete digesta, and fecal samples were analyzed for DM (method ISO 6496; ISO, 1999b) by drying at 103°C to a constant weight and for Ti (Short et al., 1996; Myers et al., 2004). Diet and fecal

samples were analyzed for ash and acid-hydrolyzed ether extract (AEE; method ISO 6492; ISO 1999a) by gravimetric determination after hydrolysis with HCl followed by extraction with petroleum ether. Diet samples were analyzed for crude fiber (method ISO 6865; ISO, 2000) by boiling in sulfuric acid and subsequent ashing. Starch (method ISO 15914; ISO, 2004) was analyzed by enzymatic determination of the amount of hexokinase/glucose-6-phosphate-dehydrogenase. Total sugars as reducing sugars and easily hydrolyzable disaccharides (method NEN 3571; NEN, 1974) were analyzed by hydrolysis in a diluted strong acidic solution with Luff-Schoorl reagent. The total AA profile (method ISO 13903; ISO, 2005b) was analyzed by acid hydrolysis at 110°C for 23 h and ion-exchange chromatography with postcolumn derivatization with ninhydrin, including sulfur-containing AA measured as cysteic acid and methionine sulfone after oxidation with performic acid. Tryptophan (method ISO 13904; ISO, 2005c) was measured by alkaline hydrolysis at 110°C for 20 h and ion-exchange chromatography with fluorescence detection, and O-methylisourea (OMIU)-reactive Lys was analyzed according to the method described in Moughan and Rutherfurd (1996). The four protein sources were analyzed for N solubility index (NSI; AOCS, 2009) by dissolving samples in 0.2% KOH, centrifugation at 13,000 × *g* for 10 min, and analyzing the supernatant for N according to the Dumas principle.

3.2.5 Calculations and statistical analysis

Apparent digestibility of CP at the end of the three small intestinal segments and SID of CP were calculated using Eq. [1] (Stein et al., 2007; CVB, 2011) and [2] (Stein et al., 2007):

Apparent digestibility of N (%) = $100 - [(N_{digesta}/N_{diet}) \times (Ti_{diet}/Ti_{digesta}) \times 100]$, [1]

SID of CP (%) = AID of CP + [(basal endogenous CP/CP_{diet}) × 100], [2]

in which $N_{digesta}$, N_{diet}, Ti_{diet}, $Ti_{digesta}$, and CP_{diet} are the N, Ti, and CP contents (g/kg DM) in the digesta or diet, AID of CP is the apparent ileal digestibility of CP calculated using Eq. [1], and basal endogenous CP is the basal endogenous loss (g/kg DMI) of CP as reported by Jansman et al. (2002). The apparent digestibility and SID of CP of the protein sources were calculated on the basis of the CP contents in the diets excluding the CP coming from the crystalline AA.

The apparent total tract digestibility (ATTD) of DM, OM, ash, CP, AEE, and non-starch polysaccharides (NSP determined as DM – ash – CP – AEE – starch – sugar) was calculated using Eq. [3] (CVB, 2011):

ATTD of *x* (%) = $100 - [(x_{feces}/x_{diet}) \times (Ti_{diet}/Ti_{feces}) \times 100]$, [3]

in which *x* is DM (g/kg as is), OM, ash, CP, AEE, or NSP (g/kg DM) and Ti_{diet} and Ti_{feces} are the Ti contents (g/kg as is for ATTD DM and g/kg DM for the other nutrients) in the diet

and feces, respectively. To calculate the NSP fraction in feces, starch and sugars were considered to be 100% digestible.

The amount of insoluble N was expressed as a fraction of the total amount of N in the corresponding digesta sample. The latter was calculated using the N content of the digesta and the weight of the sample taken for separation into a soluble and insoluble phase.

The average daily feed intake (ADFI in g/d), average daily gain (ADG in g/d), and gain to feed ratio (G:F) were calculated per pig. The empty BW (EBW) per pig was calculated by adding the weights of the empty carcass, blood, and empty individual organs. The weights of the carcass, blood, and individual organs were expressed as grams per kilogram EBW to account for variation in EBW. Nutrient composition of the empty body (in g/kg EBW) was based on the composition of the organ fraction and the carcass fraction of a particular nutrient. The SID of CP, ATTD of proximate components, growth performance, nutrient composition of the empty body, carcass characteristics, and weights of individual organs were statistically analyzed using a 2-tailed general linear model (Proc GLM procedure) in SAS 9.3 (SAS Inst. Inc., Cary, NC) with pig as the experimental unit. The model included the fixed effects of protein source, diet type, and their interaction to test the effect of SBM/RSM and the effect of processing and supplementing crystalline AA on response variables. The insoluble N as a fraction of N in digesta and apparent digestibility of CP in each segment of the small intestine were analyzed using the Proc GLM procedure with protein source, processing, and their interaction as fixed effects. Body weight block was included in the random statement of the GLM procedure for all statistical analyses. For each diet, the differences between small intestinal segments in insoluble N as a fraction of N in the digesta and apparent digestibility of CP were statistically analyzed using the Proc Mixed procedure with BW block and pig as random effects. Least squares means were calculated per experimental diet, and statistical differences between least squares means were calculated using a post hoc Tukey test. The response variables were tested for normality using Studentized residuals. Non-normal data were log transformed, and these *P*-values are included in the tables. The presented least squares means are of non-log-transformed data. *P*-values < 0.05 were considered significant, and *P*-values between 0.05 and 0.10 were considered indicative of a trend.

3.3 Results

All pigs remained healthy throughout the whole experimental period.

3.3.1 Dietary nutrient composition

The pSBM and pRSM diets contained a lower content of sugar, Arg, total Lys, and OMIU-reactive Lys than the SBM and RSM diets, respectively (Table 3.2).

3.3.2 Solubilization of N and digestibility of CP along the small intestine

Toasting in the presence of lignosulfonate reduced the NSI of the protein sources (Table 3.3). The amount of insoluble N as a fraction of N in the digesta was not affected ($P > 0.05$) by the processing treatment but was higher ($P < 0.05$) at the end of the third small intestinal segment compared to the other two segments except for the pSBM diet (Table 3.3). The apparent digestibility of CP increased ($P < 0.05$) along the small intestine for each diet. Toasting in the presence of lignosulfonate reduced ($P < 0.05$) the apparent digestibility of CP at the end of the third small intestinal segment (that is, AID of CP) for both protein sources (Table 3.3). Diet type affected ($P < 0.05$) the SID of CP, with the SID being significantly higher for the SBM and RSM diets compared with the diets containing pSBM and pRSM (with or without supplementation of crystalline AA), respectively. The apparent digestibility of CP at the ends of the first and third small intestinal segments (Table 3.3) and the SID of CP (Table 3.4) were greater ($P < 0.05$) for the SBM diets than for the RSM diets.

3.3.3 Apparent total tract nutrient digestibility

The ATTD of all proximate components, except AEE, were greater ($P < 0.05$; Table 3.4) for the SBM diets than for the RSM diets. Diet type affected ($P < 0.05$) the ATTD of all proximate components except NSP. Within diet type, toasting in the presence of lignosulfonate decreased ($P < 0.05$) the ATTD of DM, OM, CP, and AEE, with the biggest decrease in the ATTD of CP. Supplementing crystalline AA to pSBM and pRSM resulted in an ATTD of DM, OM, and CP intermediate between the SBM and pSBM diets and RSM and pRSM diets, respectively.

3.3.4 Growth performance and organ weights

The interaction between protein source and diet type was significant ($P < 0.05$) for ADG and G:F (Table 3.5). Comparison of the least squares means showed that toasting in the presence of lignosulfonate reduced ADG and G:F more for SBM than for RSM and that supplementing crystalline AA improved ADG and G:F, with the latter increasing to the level of the SBM and RSM diets. There were no effects of protein source and diet type on the proportion of carcass and organs in the empty body. Pigs fed the SBM diets had, on average, 3.8 g more ($P < 0.05$) blood per kilogram EBW than pigs fed the RSM diets. The

Table 3.3. The N solubility index of the protein sources and the effect of protein source, processing, and their interaction on the insoluble N as a fraction of total N in the digesta sample and apparent digestibility of CP determined at the end of three segments of the small intestine of growing pigs fed one of four experimental diets[1]

	Diet					*P*-value		
Item	SBM	pSBM	RSM	pRSM	SEM	Protein source	Processing	Protein source × processing
N solubility index,[2] %	0.672	0.186	0.317	0.184				
End of first segment								
Insoluble N/total N	0.283	0.334	0.345	0.333	0.046	0.531	0.685	0.500
Apparent CP digestibility,[3] %	-5.6[a] *	-11.5[a]	-28.7[a]	-31.6[a] *	6.7	0.004	0.519	0.827
End of second segment								
Insoluble N/total N	0.295	0.301	0.298	0.391	0.035	0.197	0.174	0.231
Apparent CP digestibility,[3] %	33.9	24.9	23.3	4.4	8.9	0.093	0.129	0.584
End of third segment								
Insoluble N/total N	0.478	0.422	0.546	0.540	0.048	0.061	0.521	0.600
Apparent CP digestibility,[3] %	80.8[a] *	63.0[b] *	62.2[b]	43.2[c]	2.3	< 0.001	< 0.001	0.811
P-value segment								
Insoluble N/total N	0.002	0.212	0.003	0.001				
Apparent CP digestibility	< 0.001	< 0.001	< 0.001	< 0.001				

[a-c]Least squares means within a row lacking a common superscript letter differ significantly ($P < 0.05$).

[1]SBM = soybean meal and RSM = rapeseed meal. Processing consisted of addition of lignosulfonate to SBM and RSM and secondary toasting at 95 ± 2°C for 30 min yielding the pSBM and pRSM diets. Least squares means for nine pigs per experimental diet except for the least squares means denoted with an asterisk (*), where $n = 8$ pigs.

[2]N solubility index in 0.2% KOH of the protein sources.

[3]Apparent digestibility of the protein sources excluding crystalline amino acids.

Table 3.4. The effect of protein source, diet type, and their interaction on standardized ileal digestibility (SID) of CP and apparent total tract digestibility (ATTD) of proximate components in six experimental diets fed to growing pigs[1]

	Diet							*P*-value		
Item	SBM	pSBM	pSBM+AA	RSM	pRSM	pRSM+AA	SEM	Protein source	Diet type	Protein source × diet type
SID of CP,[2] %	87.2[a]	69.2[b]	69.5[b]	71.0[b]	52.2[c]	59.3[c]	2.8	< 0.001	< 0.001	0.416
ATTD of, %										
DM	88.8[a]	84.6[bcd]	86.0[b]	84.8[bc]	83.2[d]	83.7[cd]	0.4	< 0.001	< 0.001	0.005
OM	90.4[a]	86.2[bcd]	87.5[b]	86.8[bc]	85.2[d]	85.7[cd]	0.4	< 0.001	< 0.001	0.003
Ash	61.3[a]	55.6[b]	62.9[a]	52.6[bc]	50.4[c]	52.6[bc]	0.9	< 0.001	< 0.001	0.030
CP	82.6[a]	67.9[c]	75.3[b]	71.7[bc]	61.7[d]	66.1[cd]	1.3	< 0.001	< 0.001	0.215
Acid-hydrolyzed ether extract	79.1[bc]	74.2[c]	82.0[ab]	85.1[ab]	83.0[ab]	86.0[a]	1.5	< 0.001	0.003	0.274
Non-starch polysaccharides[3]	71.6[a]	65.2[b]	65.2[b]	57.0[d]	59.3[cd]	61.4[bc]	1.0	< 0.001	0.144	< 0.001

[a-d]Least squares means within a row lacking a common superscript letter differ significantly ($P < 0.05$).

[1]SBM = soybean meal and RSM = rapeseed meal. Diet type included the factor processing (pSBM and pRSM) and processing plus supplementing with crystalline amino acids to standardized ileal digestible amino acid levels in the SBM (pSBM+AA) and RSM (pRSM+AA) diets. Least squares means for nine pigs per experimental diet except for SID of CP for SBM and pSBM diets, where $n = 8$ pigs.

[2]The SID of CP of the protein sources excluding crystalline amino acids, assuming their complete digestibility.

[3]Non-starch polysaccharides were defined in the current study as DM – ash – CP – acid-hydrolyzed ether extract – starch – sugar in the diet and DM – ash – CP – acid-hydrolyzed ether extract in feces considering complete digestion of starch and sugars.

Table 3.5. The effect of protein source, diet type, and their interaction on performance and carcass characteristics of growing pigs fed one of six experimental diets[1]

	Diet							*P*-value		
Item	SBM	pSBM	pSBM+AA	RSM	pRSM	pRSM+AA	SEM	Protein source	Diet type	Protein source × diet type
Average daily feed intake, kg/d	1.14[a]	1.13[ab]	1.12[b]	1.12[ab]	1.12[ab]	1.14[a]	0.05	0.753	0.982	< 0.001
Average daily gain, kg/d	0.64[a]	0.43[c]	0.61[a]	0.54[b]	0.45[c]	0.56[b]	0.01	< 0.001	< 0.001	< 0.001
Gain to feed ratio	0.56[a]	0.38[c]	0.55[a]	0.49[b]	0.40[c]	0.49[b]	0.01	< 0.001	< 0.001	< 0.001
Days to slaughter	39[d]	57[a]	39[d]	44[c]	52[b]	45[c]	1	0.001	< 0.001	< 0.001
BW at slaughter, kg	42.1[a]	41.5[abc]	40.7[bc]	41.0[abc]	40.6[c]	41.8[ab]	0.3	0.184	0.175	< 0.001
EBW,[2] g/kg BW at slaughter	931[a]	923[ab]	926[ab]	910[b]	924[ab]	914[ab]	5	0.006	0.696	0.055
Carcass, g/kg EBW[2]	827[a,b]	825[a,b]	816[b]	819[ab]	829[a]	827[ab]	3	0.333	0.163	0.010
Blood, g/kg EBW[2]	50.0[a]	49.3[a]	50.4[a]	47.6[a]	45.6[a]	44.7[a]	1.5	0.003	0.590	0.542
Organ fraction,[3] g/kg EBW[2]	173.3[ab]	175.9[ab]	183.6[a]	181.3[ab]	170.9[b]	173.1[ab]	2.9	0.296	0.217	0.009

[a-d]Least squares means within a row lacking a common superscript letter differ significantly ($P < 0.05$).

[1]SBM = soybean meal and RSM = rapeseed meal. Diet type included the factor processing (pSBM and pRSM) and processing plus supplementing with crystalline amino acids to standardized ileal digestible amino acid levels in the SBM (pSBM+AA) and RSM (pRSM+AA) diets. Least squares means for nine pigs per experimental diet.

[2]EBW = empty BW = sum of empty carcass and weights of emptied individual thoracic and abdominal organs and blood.

[3]Organ fraction is calculated as the sum of the blood and individual thoracic and abdominal organs.

water, crude fat (that is, ether extract), and CP content in the organ fraction, empty carcass, and empty body were affected ($P < 0.05$ for all; Table 3.6) by diet type. Within diet type, toasting in the presence of lignosulfonate increased the crude fat and decreased the water and CP contents in the organ fraction, empty carcass, and empty body, and this was compensated by supplementing crystalline AA to similar contents as for the SBM and RSM diets. Toasting of SBM in the presence of lignosulfonate and supplementing crystalline AA had a larger ($P < 0.05$) effect on empty body composition, except ash, than applying the same treatment to RSM.

The weight of the organs excluding the GIT was affected ($P < 0.05$; Table 3.7) by the interaction between protein source and diet type. Within diet type, the weight of the organs was higher for pSBM compared with SBM, whereas it was lower for pRSM compared with RSM. This effect was largely caused by effects on the weight of the heart, reproductive tract, and liver. The weight of the organs for the pSBM+AA and pRSM+AA diets was not significantly different from the other experimental diets. Diet type affected ($P < 0.05$) the weight of the kidneys, pancreas, and spleen. Within diet type, toasting in the presence of lignosulfonate lowered the weights of the kidneys, pancreas, and spleen, and supplementing crystalline AA resulted in organ weights similar to those for the SBM and RSM diets. The weight of the liver was affected ($P < 0.05$) by the interaction between protein source and diet type. Comparison of the least squares means showed that processing increased the liver weight of pigs fed the pSBM diet but not those fed the pRSM diet. Supplementing crystalline AA to pRSM decreased liver weight, whereas there was no effect of supplementing crystalline AA to pSBM on liver weight.

The weight of the GIT was affected ($P < 0.05$) by the interaction between protein source and diet type (Table 3.7). Diet type affected ($P < 0.05$) the weights of the small and large intestines. For the small intestine, toasting in the presence of lignosulfonate decreased its weight, and supplementing crystalline AA largely restored this effect. Toasting in the presence of lignosulfonate increased the weight of the large intestine, whereas supplementing crystalline AA resulted in an intermediate weight (least squares means for the main effect of diet type were 283.5, 301.8, and 296.6 g/kg GIT for SBM/RSM, pSBM/pRSM, and pSBM+AA/pRSM+AA, respectively, with SEM = 4.4).

3.4 Discussion

3.4.1 Solubilization of N and digestibility of CP

The apparent digestibility of CP increased for all four diets as digesta passed through the small intestine in accordance with Low (1980) using 60-kg pigs fed different diets and

Table 3.6. The effect of protein source, diet type, and their interaction on nutrient composition in the organ fraction[1], empty carcass, and empty body of growing pigs fed one of six experimental diets[2]

	Diet							*P*-value		
Item	SBM	pSBM	pSBM+AA	RSM	pRSM	pRSM+AA	SEM	Protein source	Diet type	Protein source × diet type
Organ fraction,[1] g/kg										
Water	779.7[a]	758.9[b]	783.0[a]	776.2[a]	758.1[b]	772.6[a]	2.7	0.030	< 0.001	0.191
Ash	11.3[a]	10.3[b]	10.3[b]	10.2[b]	10.1[b]	10.2[b]	0.2	0.013	0.026	0.072
Ether extract	41.7[bc]	63.8[a]	36.3[c]	46.6[b]	62.3[a]	50.3[b]	2.1	0.002	< 0.001	0.003
CP	146.3[a]	138.1[c]	144.3[ab]	141.5[abc]	139.5[bc]	142.3[abc]	1.2	0.080	< 0.001	0.044
Empty carcass, g/kg										
Water	634.6[a]	570.8[c]	636.1[a]	605.3[b]	568.1[c]	608.3[b]	3.9	< 0.001	< 0.001	0.002
Ash	33.9	35.4	34.6	33.1	34.6	32.6	0.8	0.077	0.126	0.697
Ether extract	153.5[c]	244.1[a]	151.2[c]	196.7[b]	242.7[a]	192.2[b]	4.7	< 0.001	< 0.001	< 0.001
CP	174.5[a]	145.0[c]	173.6[a]	160.9[b]	151.2[c]	160.1[b]	1.7	< 0.001	< 0.001	< 0.001
Empty body, g/kg EBW[3]										
Water	659.8[a]	603.8[c]	663.1[a]	636.2[b]	600.5[c]	636.8[b]	3.7	< 0.001	< 0.001	0.007
Ash	30.0	31.0	30.1	29.0	30.4	28.7	0.6	0.067	0.097	0.806
Ether extract	134.1[c]	212.4[a]	130.1[c]	169.5[b]	212.0[a]	167.7[b]	4.3	< 0.001	< 0.001	< 0.001
CP	169.6[a]	143.8[c]	168.1[a]	157.4[b]	149.2[c]	157.0[b]	1.5	< 0.001	< 0.001	< 0.001

[a-c]Least squares means within a row lacking a common superscript letter differ significantly ($P < 0.05$).

[1]Organ fraction is calculated as the sum of the blood and individual thoracic and abdominal organs.

[2]SBM = soybean meal and RSM = rapeseed meal. Diet type included the factor processing (pSBM and pRSM) and processing plus supplementing with crystalline amino acids to standardized ileal digestible amino acid levels in the SBM (pSBM+AA) and RSM (pRSM+AA) diets. Least squares means for nine pigs per experimental diet.

[3]EBW = empty BW = sum of empty carcass and weights of emptied individual thoracic and abdominal organs and blood.

Table 3.7. The effect of protein source, diet type, and their interaction on weight of individual organs of growing pigs fed one of six experimental diets[1]

	Diet							*P*-value		
Item	SBM	pSBM	pSBM+AA	RSM	pRSM	pRSM+AA	SEM	Protein source	Diet type	Protein source × diet type
Organs excluding GIT,[2] g/kg EBW[3]	58.8^{b}	62.5^{ab}	62.1^{ab}	64.7^{a}	60.9^{ab}	61.1^{ab}	1.3	0.311	0.995	0.009
Heart	5.0^{ab}	5.4^{a}	4.8^{b}	4.9^{ab}	4.7^{b}	4.8^{b}	0.1	0.016	0.120	0.017
Lungs	11.3^{ab}	10.2^{b}	12.6^{ab}	15.1^{a}	12.4^{ab}	14.8^{a}	1.0	0.003	0.074	0.568
Kidneys	4.58^{ab}	4.43^{ab}	4.71^{a}	4.65^{ab}	4.16^{b}	4.48^{ab}	0.12	0.139	0.013	0.302
Reproductive tract	1.6^{bc}	2.2^{a}	2.0^{ab}	1.2^{c}	1.2^{c}	1.1^{c}	0.1	< 0.001	0.054	0.045
Liver	23.1^{bc}	25.8^{a}	24.3^{abc}	25.3^{ab}	24.8^{abc}	22.9^{c}	0.5	0.783	0.013	0.004
Pancreas	1.9^{a}	1.5^{bc}	1.8^{ab}	1.5^{bc}	1.4^{c}	1.6^{abc}	0.1	0.002	0.003	0.311
Spleen	1.77^{abc}	1.56^{c}	1.86^{ab}	1.82^{abc}	1.60^{bc}	1.95^{a}	0.07	0.301	< 0.001	0.909
GIT,[2] g/kg EBW[3]	65.3^{ab}	64.7^{b}	71.2^{a}	69.6^{ab}	64.9^{b}	67.6^{ab}	1.5	0.794	0.013	0.036
Stomach, g/kg GIT[2]	102.5	107.3	105.5	104.5	103.7	106.5	3.2	0.942	0.720	0.647
Small intestine, g/kg GIT[2]	516.8^{ab}	483.8^{c}	508.4^{abc}	527.4^{a}	497.3^{bc}	510.1^{abc}	7.0	0.141	< 0.001	0.684
Large intestine, g/kg GIT[2]	286.1^{a}	302.9^{a}	306.9^{a}	280.8^{a}	300.7^{a}	286.2^{a}	6.3	0.074	0.017	0.301

[a-c]Least squares means within a row lacking a common superscript letter differ significantly ($P < 0.05$).

[1]SBM = soybean meal and RSM = rapeseed meal. Diet type included the factor processing (pSBM and pRSM) and processing plus supplementing with crystalline amino acids to standardized ileal digestible amino acid levels in the SBM (pSBM+AA) and RSM (pRSM+AA) diets. Least squares means for nine pigs per experimental diet.

[2]GIT = gastrointestinal tract.

[3]EBW = empty BW = sum of empty carcass and weights of emptied individual thoracic and abdominal organs and blood.

Asche et al. (1989) using weanling pigs fed a corn-SBM diet. In the first segment, the apparent digestibility of CP was negative for all four experimental diets, indicating that the addition of protein in endogenous secretions exceeded the absorption of dietary protein. The SID of CP was greater for the SBM diets than for the RSM diets, in accordance with other studies (Stein et al., 2001; Eklund et al., 2012; Hulshof et al., 2016). Jondreville et al. (2000) found that endogenous AA losses at the end of the ileum were greater for RSM diets than for SBM diets. In addition, the true ileal digestibility (TID) of N and AA was found to be higher for SBM than for RSM (Jondreville et al., 2000; Eklund et al., 2012). Therefore, the difference in apparent digestibility and SID of CP between protein sources in the current study can be explained by the combination of a difference in endogenous losses and in TID.

Mild heat treatment generally denatures the protein in feed ingredients, which increases its solubility and digestibility through an enhanced enzyme accessibility to peptide bonds (Adler-Nissen, 1976). Severe processing may lead to protein aggregation, which lowers the solubility (Carbonaro et al., 2012). The severe processing in the current study indeed reduced the NSI of the protein sources. This led to the hypothesis that N solubilization in the digesta of the processed protein sources would be lower as well. However, the data showed that toasting in the presence of lignosulfonate had no effect on the amount of insoluble N as a fraction of total N in the digesta as determined by centrifugation. The reduction in digestibility due to toasting in the presence of lignosulfonate, therefore, was not caused by a change in N solubilization but by an increased absolute amount of N present in the digesta. Processing numerically decreased the apparent CP digestibility at the end of the first and second small intestinal segments and significantly decreased it at the end of the third small intestinal segment (reflecting AID of CP). This might result from a decrease in spontaneous, non-enzymatic breakdown of peptides (Butré et al., 2015), a reduction in trypsin and chymotrypsin activity as seen for processed protein sources, and the formation of non-digestible peptide aggregates (Fischer et al., 2002, 2007). The N solubility of endogenous losses in the small intestine as determined using a protein-free diet was found to be 86.6% (Asche et al., 1989). A greater secretion of endogenous losses, especially in the first small intestinal segment, would contribute to the increased amount of N in the digesta, thereby contributing to the (numerical) differences in apparent digestibility of CP. Toasting in the presence of lignosulfonate decreased the SID of CP and the ATTD of CP, giving results similar to those found in the previous study (Hulshof et al., 2016). The data indicate that the decrease in CP digestibility at the end of the ileum due to processing was not solely mediated by an increased amount of insoluble N but also by an increased amount of soluble N, thereby increasing the total amount of N in the digesta.

3.4.2 Performance and organ weights

Toasting of SBM in the presence of lignosulfonate had a larger effect on ADG and G:F than applying this processing treatment to RSM. This result can be explained by the greater inclusion of crystalline AA in the RSM diets, which were not subjected to the processing treatment and were added to realize a SID AA/net energy ratio of 90% of requirements in the RSM diet. This result is in accordance with the previous study (Hulshof et al., 2016). Pigs fed the pSBM+AA and pRSM+AA diets performed better than pigs fed the pSBM and pRSM diets and performed as well as pigs fed the SBM and RSM diets, respectively. Similar results were found in a broiler feeding trial in which a diet was formulated on SID AA in heat-damaged SBM (115°C for 120 min) and supplemented with crystalline AA (Helmbrecht et al., 2010) and in a trial with weanling pigs in which autoclaved distillers dried grains with solubles (125°C for 60 min) were used (Almeida et al., 2014).

Toasting in the presence of lignosulfonate reduced the CP content in the organ fraction, carcass, and empty body, which was restored by supplementing crystalline AA. The pSBM and pRSM diets contained a lower amount of SID AA than the SBM and RSM diets, respectively. The SBM and RSM diets were formulated to be limiting in AA, and the processing treatment mainly affected the SID Lys (Hulshof et al., 2016), resulting in a reduced AA availability and imbalance between AA and between AA and net energy. This imbalance is reflected by the increased fat content for the pSBM and pRSM diets. Protein is deposited in combination with water, in contrast to fat, which explains the reduced water content in the empty body for the pSBM and pRSM diets compared with the SBM and RSM diets. The body water:CP in the current study was approximately 4 gram of water per gram of CP, which is in accordance with Bikker et al. (1994, 1995). The AA availability and imbalance were restored by supplementing crystalline AA in the pSBM and pRSM diets. This result is reflected by the similar nutrient compositions in the empty body between the SBM and pSBM+AA diets and RSM and pRSM+AA diets. The whole-body nutrient composition of pigs fed the SBM and RSM diets was comparable to values found in other studies (Kyriazakis et al., 1993; Bikker et al., 1994, 1995). Therefore, toasting in the presence of lignosulfonate changed whole-body nutrient composition as result of a lower nutrient availability and imbalance between AA and between AA and net energy.

In general, toasting in the presence of lignosulfonate decreased the weight of several organs in response to total and post-absorptive nutrient supply (metabolic load). Organ development and weight are dependent on metabolic load (Roe, 1970). Supplementing crystalline AA restored organ weight, indicating that the metabolic load of nutrients post-absorption was similar for these diets compared with the SBM and RSM diets. The lower weight of the pancreas for the pSBM diet was unexpected because this diet would require

a greater amount of enzymes to be digested. However, Brannon (1990) showed that greater amounts of enzymes are released for a high protein diet, a high-quality protein, and a low-quality protein supplemented with indispensable AA. The smaller amount of enzymes released for low-quality protein without supplementing crystalline AA could be explained by a limited availability of indispensable AA and the adaptive response of the pancreas to spare AA for other tissues (Brannon, 1990). In the RSM, pRSM, and pRSM+AA diets an effect on pancreas weight may have been negated by the large amounts of crystalline AA supplemented to these diets. Toasting in the presence of lignosulfonate also decreased the weight of the small intestine, which may be a direct effect of the lower availability of all AA due to the processing treatment and the presence of modified Lys residues and MRP (Hulshof et al., 2016). Processing may affect the weight of the liver and kidneys because of the metabolic load resulting from AA availability, imbalance between absorbed AA, and MRP. An imbalance in AA would increase deamination in the liver and excretion of urea by the kidneys (Blachier et al., 2013). Indeed, pSBM showed an increased liver but not kidney weight in the current study. The lower AA absorption presumably reduced the urea excretion in urine, resulting in a lower weight of the kidneys, as was also seen in studies of Kerr et al. (1995, 2003) and Chen et al. (1999). The weight of the liver of pigs fed the pRSM diet was not greater than that of pigs fed the RSM diet. The imbalance between absorbed AA may have been lower for the pRSM diet because of supplementation of a greater amount of crystalline AA. The kidneys and liver are a site for metabolism and deposition of absorbed MRP (Ames, 2007). The presence of MRP post-absorption might therefore affect the metabolic load and weight of these organs. In literature, excessive intakes of MRP resulted in increased organ weights (Šebeková and Somoza, 2007). The intake of MRP in the current study may have been too low to alter organ weight because of the absence of a consistent increase in kidney and liver weight due to processing. The data indicate that the weights of several organs were affected by toasting in the presence of lignosulfonate because of a direct effect on digestibility and post-absorptive availability of AA and an imbalance in available AA in relation to net energy but organ weights were presumably not affected by MRP.

In conclusion, toasting in the presence of lignosulfonate reduced the CP digestibility along the small intestine, but this effect could not be explained by an effect of processing on N solubilization in the GIT. Toasting in the presence of lignosulfonate affected the weight of several organs and reduced body protein content and pig growth performance. Supplementing crystalline AA in diets containing pSBM and pRSM to the SID AA level of the SBM and RSM diets restored these characteristics to the level of the SBM and RSM diets. Therefore, *in vivo* effects of feeding processed protein sources were mediated by a reduction in AA availability and not by the presence of MRP. Further research on how

processing affects digestibility by studying peptide aggregation, spontaneous non-enzymatic breakdown, enzyme activity, and the involvement of endogenous N in the (in)soluble phase should be undertaken.

3.5 Acknowledgement

The authors acknowledge the financial support from the Wageningen UR "IPOP Customized Nutrition" program financed by Wageningen UR (Wageningen, the Netherlands), the Dutch Ministry of Economic Affairs (The Hague, the Netherlands), WIAS (Wageningen, the Netherlands), Agrifirm Innovation Center (Apeldoorn, the Netherlands), ORFFA Additives BV (Werkendam, the Netherlands), Ajinomoto Eurolysine s.a.s. (Paris, France), and Stichting VICTAM BV (Nijkerk, the Netherlands). The authors thank T. Zandstra, S. Salazar-Villanea, H. Gruppen, M. W. A. Verstegen, the staff of the experimental facility of Wageningen UR, and the laboratories of Food Chemistry and Animal Nutrition of Wageningen UR for their contributions. The authors thank Feed Valid B.V., Poederoijen, the Netherlands, for providing the lignosulfonate and the two soybean meals and two rapeseed meals.

3.6 Literature cited

Adler-Nissen, J. 1976. Enzymatic hydrolysis of proteins for increased solubility. J. Agric. Food Chem. 24:1090-1093.

Agricultural Research Council (ARC). 1981. The nutrient requirements of pigs. Commonwealth Agricultural Bureaux., Slough, UK. p. 16.

Almeida, F. N., J. K. Htoo, J. Thomson, and H. H. Stein. 2014. Effects of balancing crystalline amino acids in diets containing heat-damaged soybean meal or distillers dried grains with solubles fed to weanling pigs. Animal 8:1594-1602.

American Oil Chemists Society (AOCS). 2009. Nitrogen Solubility Index Official Method Ba 11-65. AOCS, Champaign, Illinois.

Ames, J. M. 2007. Evidence against dietary advanced glycation endproducts being a risk to human health. Mol. Nutr. Food Res. 51:1085-1090.

Araba, M., and N. M. Dale. 1990. Evaluation of protein solubility as an indicator of overprocessing soybean meal. Poult. Sci. 69:76-83.

Asche, G. L., A. J. Lewis, and E. R. Peo Jr. 1989. Protein digestion in weanling pigs: Effect of feeding regimen and endogenous protein secretion. J. Nutr. 119:1083-1092.

Bikker, P., V. Karabinas, M. W. A. Verstegen, and R. G. Campbell. 1995. Protein and lipid accretion in body components of growing gilts (20 to 45 kilograms) as affected by energy intake. J. Anim. Sci. 73:2355-2363.

Bikker, P., M. W. A. Verstegen, R. G. Campbell, and B. Kemp. 1994. Digestible lysine requirement of gilts with high genetic potential for lean gain, in relation to the level of energy intake. J. Anim. Sci. 72:1744-1753.

Blachier, F., G. Wu, and Y. Yin. 2013. Nutritional and physiological functions of amino acids in pigs. Springer, Vienna, Austria.

Brannon, P. M. 1990. Adaptation of the exocrine pancreas to diet. Annu. Rev. Nutr. 10:85-105.

Butré, C. I., S. Buhler, S. Sforza, H. Gruppen, and P. A. Wierenga. 2015. Spontaneous, non-enzymatic breakdown of peptides during enzymatic protein hydrolysis. Biochim. Biophys. Acta 1854:987-994.

Carbonaro, M., P. Maselli, and A. Nucara. 2012. Relationship between digestibility and secondary structure of raw and thermally treated legume proteins: A Fourier transform infrared (FT-IR) spectroscopic study. Amino Acids 43:911-921.

Centraal Veevoeder Bureau (CVB). 2011. Chemical compositions and nutritional values of feed ingredients. CVB, Lelystad, the Netherlands.

Chen, H. Y., A. J. Lewis, P. S. Miller, and J. T. Yen. 1999. The effect of excess protein on growth performance and protein metabolism of finishing barrows and gilts. J. Anim. Sci. 77:3238-3247.

Eklund, M., W. R. Caine, W. C. Sauer, G. S. Huang, G. Diebold, M. Schollenberger, and R. Mosenthin. 2012. True and standardized ileal digestibilities and specific ileal endogenous recoveries of crude protein and amino acid in soybean meal, rapeseed meal and peas fed to growing pigs. Livest. Sci. 145:174-182.

Fischer, M., H. Gruppen, S. R. Piersma, L. V. Kofod, H. A. Schols, and A. G. J. Voragen. 2002. Aggregation of peptides during hydrolysis as a cause of reduced enzymatic extractability of soybean meal proteins. J. Agric. Food Chem. 50:4512-4519.

Fischer, M., A. G. J. Voragen, S. R. Piersma, L. V. Kofod, C. I. Jörgensen, P. Guggenbuhl, C. S. Nunes, and H. Gruppen. 2007. Presence of indigestible peptide aggregates of soybean meal in pig ileal digesta residue. J. Sci. Food Agric. 87:2229-2238.

Friedman, M. 1999. Chemistry, biochemistry, nutrition, and microbiology of lysinoalanine, lanthionine, and histidinoalanine in food and other proteins. J. Agric. Food Chem. 47:1295-1319.

Hansen, L. P., and R. J. Millington. 1979. Blockage of protein enzymatic digestion (carboxypeptidase-B) by heat-induced sugar-lysine reactions. J. Food Sci. 44:1173-1177.

Helmbrecht, A., M. S. Redshaw, C. Elwert, T. Veldkamp, and A. Lemme. 2010. Detection of heat damage in soybean meal by rapid method and consideration in feed formulation - Validation of a concept by broiler feeding trials. In: Proc. XIIIth Eur. Poultry Conf., Tours, France. p. 146-150.

Hulshof, T. G., P. Bikker, A. F. B. van der Poel, and W. H. Hendriks. 2016. Assessment of protein quality of soybean meal and 00-rapeseed meal toasted in the presence of lignosulfonate by amino acid digestibility in growing pigs and Maillard reaction products. J. Anim. Sci. 94:1020-1030.

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

International Organization for Standardization (ISO). 1999a. ISO 6492:1999. Animal feeding stuffs - Determination of fat content. ISO, Geneva, Switzerland.

International Organization for Standardization (ISO). 1999b. ISO 6496:1999. Animal feeding stuffs - Determination of moisture and other volatile matter content. ISO, Geneva, Switzerland.

International Organization for Standardization (ISO). 2000. ISO 6865:2000. Animal feeding stuffs - Determination of crude fibre content - Method with intermediate filtration. ISO, Geneva, Switzerland.

International Organization for Standardization (ISO). 2002. ISO 5984:2002. Animal feeding stuffs - Determination of crude ash content. ISO, Geneva, Switzerland.

International Organization for Standardization (ISO). 2004. ISO 15914:2004. Animal feeding stuffs - Enzymatic determination of total starch content. ISO, Geneva, Switzerland.

International Organization for Standardization (ISO). 2005a. ISO 5983-1:2005. Animal feeding stuffs - Determination of nitrogen content and calculation of crude protein content - Part 1: Kjeldahl method. ISO, Geneva, Switzerland.

International Organization for Standardization (ISO). 2005b. ISO 13903:2005. Animal feeding stuffs - Determination of amino acids content. ISO, Geneva, Switzerland.

International Organization for Standardization (ISO). 2005c. ISO 13904:2005. Animal feeding stuffs - Determination of tryptophan content. ISO, Geneva, Switzerland.

International Organization for Standardization (ISO). 2008. ISO 16634-1:2008. Food products - Determination of the total nitrogen content by combustion according to the Dumas principle and calculation of the crude protein content - Part 1: Oilseeds and animal feeding stuffs. ISO, Geneva, Switzerland.

Jansman, A. J. M., W. Smink, P. van Leeuwen, and M. Rademacher. 2002. Evaluation through literature data of the amount and amino acid composition of basal endogenous crude protein at the terminal ileum of pigs. Anim. Feed Sci. Technol. 98:49-60.

Jondreville, C., J. van den Broecke, F. Gâtel, F. Grosjean, S. van Cauwenberghe, and B. Sève. 2000. Ileal amino acid digestibility and estimates of endogenous amino acid losses in pigs fed rapeseed meal, sunflower meal and soybean meal. Can. J. Anim. Sci. 80:495-506.

Kerr, B. J., F. K. McKeith, and R. A. Easter. 1995. Effect on performance and carcass characteristics of nursery to finisher pigs fed reduced crude protein, amino acid-supplemented diets. J. Anim. Sci. 73:433-440.

Kerr, B. J., J. T. Yen, J. A. Nienaber, and R. A. Easter. 2003. Influences of dietary protein level, amino acid supplementation and environmental temperature on performance, body composition, organ weights and total heat production of growing pigs. J. Anim. Sci. 81:1998-2007.

Kyriazakis, I., G. C. Emmans, and R. McDaniel. 1993. Whole body amino acid composition of the growing pig. J. Sci. Food Agric. 62:29-33.

Low, A. G. 1980. Nutrient absorption in pigs. J. Sci. Food Agric. 31:1087-1130.

Mauron, J. 1981. The Maillard reaction in food; A critical review from the nutritional standpoint. Prog. Food Nutr. Sci. 5:5-35.

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

Myers, W. D., P. A. Ludden, V. Nayigihugu, and B. W. Hess. 2004. Technical note: A procedure for the preparation and quantitative analysis of samples for titanium dioxide. J. Anim. Sci. 82:179-183.

Netherlands Standardization Institute (NEN). 1974. NEN 3571:1974 nl. Test method for feeding stuffs - Determination of reducing sugar, crude total sugar, saccharose and lactose. Nederlands Normalisatie Instituut, Delft, the Netherlands.

Öste, R. E., A. Dahlqvist, H. Sjöström, O. Norén, and R. Miller. 1986. Effect of Maillard reaction products on protein digestion. *In vitro* studies. J. Agric. Food Chem. 34:355-358.

Prestløkken, E., and O. Rise. 2003. Protein and amino acid digestibility in dairy cows measured with mobile nylon bags recovered in ileum or in faeces. Acta Agric. Scand., Sect. A 53:11-20.

Roe, F. J. C. 1970. Metabolic aspects of food safety. Blackwell, Oxford, UK.

Šebeková, K., and V. Somoza. 2007. Dietary advanced glycation endproducts (AGEs) and their health effects–PRO. Mol. Nutr. Food Res. 51:1079-1084.

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

Stein, H. H., M. F. Fuller, P. J. Moughan, B. Sève, R. Mosenthin, A. J. M. Jansman, J. A. Fernández, and C. F. M. de Lange. 2007. Definition of apparent, true, and standardized ileal digestibility of amino acids in pigs. Livest. Sci. 109:282-285.

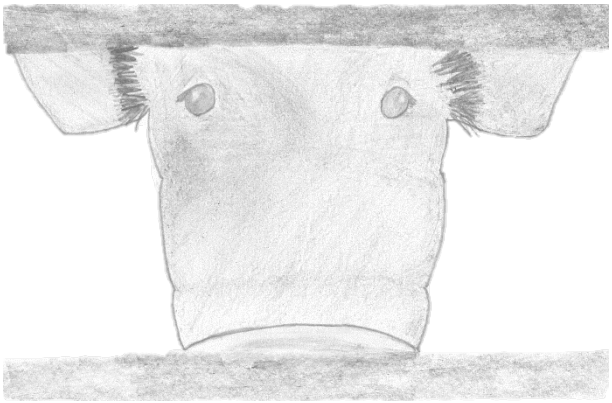
Stein, H. H., S. W. Kim, T. T. Nielsen, and R. A. Easter. 2001. Standardized ileal protein and amino acid digestibility by growing pigs and sows. J. Anim. Sci. 79:2113-2122.

Amino acid utilization and body composition of growing pigs fed processed soybean meal or 00-rapeseed meal with or without amino acid supplementation

T. G. Hulshof,[*†] A. F. B. van der Poel,[*] W. H. Hendriks,[*] and P. Bikker[†]

*Animal Nutrition Group, Wageningen University, Wageningen, the Netherlands, and [†]Wageningen UR Livestock Research, Wageningen, the Netherlands

Submitted to Animal



Abstract

This study aimed to determine effects of processing of soybean meal (SBM) and rapeseed meal (RSM) on post-absorptive utilization of ileal digestible amino acids (AA) for retention and on body AA composition of growing pigs. Processing was expected to reduce AA retention due to a reduced AA ileal digestibility but not by a reduction in post-absorptive AA utilization. Soybean meal and RSM were processed by secondary toasting (at 95°C for 30 min) in the presence of lignosulfonate to obtain processed SBM (pSBM) and processed RSM (pRSM). Four diets contained SBM, pSBM, RSM, or pRSM as sole protein source. Two other diets contained pSBM or pRSM but were supplemented with crystalline AA to the same standardized ileal digestible (SID) AA level as the SBM or RSM diet. These two diets were used to verify that processing affected AA retention by affecting AA digestibility rather than post-absorptive AA utilization. At the start of the experiment, initial body composition of five pigs was determined and 54 pigs were fed one of the six experimental diets. At a BW of 40 kg, pigs were slaughtered and the empty organs plus blood (referred to as organ fraction) and empty carcass were analyzed separately for N and AA content. Post-absorptive AA utilization was calculated from AA retention and SID AA intake. Diet type, comprising effects of processing and supplementing crystalline AA, affected the AA composition of protein in the organ fraction, carcass, and empty body. Processing mainly reduced (P < 0.10) the Lys concentration with greater effects for SBM than for RSM. Supplementing crystalline AA restored the Lys concentration for both protein sources. Processing reduced AA retention more for SBM than for RSM (P < 0.001 for interaction effect) while supplementing crystalline AA restored AA retention for both protein sources indicating that processing affected retention by reducing digestibility. Thus, correcting AA retention for SID AA intake would result in similar post-absorptive AA utilization which was found for most AA for the RSM diets. However, the post-absorptive AA utilization was lower for the pSBM diet than for the SBM diet which might be related to an imbalanced supply of AA after absorption in the first diet. In conclusion, processing negatively affected post-absorptive utilization of ileal digestible AA for retention for SBM but not for RSM and altered the Lys concentration of body protein in growing pigs.

Key words: amino acid, body composition, nutrient retention, post-absorptive utilization, toasting with lignosulfonate

4.1 Introduction

Research into effects of (over-)processing of protein sources has focused on (ileal) amino acid (AA) digestibility (Van Barneveld et al., 1994a; González-Vega et al., 2011; Almeida et al., 2014; Hulshof et al., 2016a). For protein sources with a high AA availability such as soybean meal (SBM), there is close agreement between ileal digestibility and availability measured using the slope-ratio assay and based on feed conversion efficiency on a carcass basis. For protein sources with a low AA availability such as cottonseed meal, however, ileal digestibility overestimated AA availability (Batterham et al., 1990b) because the absorbed AA could not be fully utilized by the pig. The latter was also found for heat-treated fish meal (Wiseman et al., 1991) and heat-treated field peas (Van Barneveld et al., 1994b). The negative effects of processing on digestibility and availability are, at least partly, caused by the formation of Maillard reaction products (MRP) which are formed by the reaction between amino groups and reducing sugars (Hurrell and Carpenter, 1981). Lysine is often the first limiting AA in diets for growing pigs and the most susceptible AA to react with sugars because of its free ε-amino group. Reactive Lys is thought to provide a better estimation of Lys availability than total Lys because the latter includes the Lys reverted back from early MRP during acid hydrolysis (Moughan and Rutherfurd, 2008). Reactions occurring during processing, could lead to an imbalance among available AA and between AA and net energy. This was shown to affect organ weight, carcass composition, and the gain to feed ratio (Hulshof et al., 2016b). Conde-Aguilera et al. (2010) found that pigs were able to alter the AA composition of retained protein when fed a diet deficient in sulfur AA. However, the effects of processing of SBM and rapeseed meal (RSM) on the utilization of ileal digestible AA for retention and the metabolic flexibility of an animal to cope with changes in post-absorptive AA availability have received little attention.

The current study aimed to determine the effects of processing of SBM and RSM on (post-absorptive) utilization of ileal digestible AA for retention and on the body AA composition of growing pigs. It was hypothesized that processing would reduce AA retention because of an effect on ileal digestibility of AA but not via an effect on the post-absorptive utilization of AA. The latter was determined by supplementing diets containing processed SBM (pSBM) or processed RSM (pRSM) with crystalline AA based on standardized ileal digestible (SID) AA concentration. It was further hypothesized that processing and subsequent AA availability would not affect body AA composition.

4.2 Materials and methods

4.2.1 Animals and housing

This study was approved by the Animal Care and Use Committee of Wageningen University and Research Centre (Wageningen, the Netherlands). The experiment consisted of 59 growing gilts (Tempo × Topigs 40 from Van Haaren, Horssen, the Netherlands) with an average initial BW of 15.6 ± 0.7 kg at the day of arrival (d 1). Pigs were fed a commercial diet and were gradually adapted during the first week to a mixture of the SBM and RSM diets (50:50) and to a feeding level of 3.0 times net energy requirements for maintenance (321 kJ net energy/kg $BW^{0.75}$; ARC, 1981). After this week, pigs were allocated either to the initial slaughter group (0-pig) or to an experimental diet and subsequently to a pen. The housing conditions of the pigs are described elsewhere (Hulshof et al., 2016b).

4.2.2 Experimental design and diets

The study consisted of a randomized complete block design with BW at d 8 as blocking factor and nine blocks consisting alternately of seven (five blocks) and six (four blocks) pigs. From each BW block consisting of seven pigs, one pig was randomly chosen and allocated to the initial slaughter group. The other 54 pigs were randomly allocated to one of six experimental diets within a block and allocated to a pen in each of four rooms as described previously (Hulshof et al., 2016b). The five 0-pigs were divided over two rooms and housed in groups of two and three pigs, respectively.

All six isoenergetic (9.96 MJ of net energy per kg) experimental diets (Research Diet Services, Wijk bij Duurstede, the Netherlands) consisted of a basal CP-free component based on gelatinized potato starch. Three experimental diets contained commercially toasted/desolventized SBM (*Glycine max*) while the other three experimental diets contained 00-RSM (*Brassica napus* containing low levels of erucic acid and glucosinolates) as sole protein source (2 × 3 factorial treatment arrangement). Soybean meal and RSM (supplied by Feed Valid B.V., Poederoijen, the Netherlands) were used either as such, referred to as SBM and RSM diet, respectively, or processed by secondary toasting in the presence of lignosulfonate as described in Hulshof et al. (2016a) to obtain pSBM and pRSM. This processing treatment, normally applied in ruminant nutrition to produce rumen by-pass protein, was used to induce additional protein damage through the Maillard reaction. The SBM and RSM diets were supplemented with crystalline L-Ile, L-Leu, L-Lys, DL-Met, L-Thr, L-Trp, and L-Val to meet 90% of the requirements for SID Lys (SID Lys to net energy ratio of 0.98 g/MJ) for growing pigs (CVB, 2008) and the other indispensable AA as percentage of Lys according to INRA recommendations (Gloaguen et al., 2013). The

remaining two experimental diets contained pSBM and pRSM and were supplemented with crystalline AA to meet the SID AA concentration in the SBM and RSM diets, respectively, based on the SID AA concentration in the pSBM and pRSM protein sources (Hulshof et al., 2016a). These diets were formulated to compensate for the losses in SID AA by processing and are referred to as pSBM+AA and pRSM+AA diet. L-Glutamine was added to these diets to account for the loss in SID of dispensable AA. The manufacturing and the ingredient and chemical compositions of the experimental diets have been described previously (Hulshof et al., 2016b). The calculated SID AA concentration and SID AA to SID Lys ratio of the six experimental diets are provided in Table 4.1. Pigs were fed the experimental diets twice a day in equal portions at 0800 and 1600 h from d 9 until a BW of 40 ± 2 kg was reached.

4.2.3 Slaughter procedure, sample preparation, and chemical analysis

The five 0-pigs were killed at d 9 (average BW before slaughter 18.2 ± 0.6 kg) and the 54 pigs receiving the experimental diets were killed at a BW of 40 ± 2 kg by electrical stunning followed by hanging the pig by the ankles and exsanguination by cutting the carotid artery. Blood and the empty visceral organs, referred to as organ fraction, were collected together in a bag as described previously (Hulshof et al., 2016b) and frozen at –20°C. The carcass, including head, teeth, feet, hair, and skin, was collected in plastic bags, and frozen at –20°C. The frozen organ fraction and carcass were cut separately in small blocks using a band saw (Butcher Boy B 14-9, Montebello, CA) and homogenized using a commercial butcher mincer (Alexanderwerk, Remscheid, Germany). The organ fraction and carcass were subsampled after mincing and stored at –20°C pending analysis. The organ fraction and carcass of all pigs (n = 59) were analyzed separately for moisture by freeze-drying, ash (method ISO 5984; ISO, 2002) after 3 h heating at 550°C, ether extract (method ISO 6492; ISO, 1999) by extraction with petroleum ether and N (method ISO 5983-1; ISO, 2005a) by the Kjeldahl method with CP calculated as N × 6.25. Defatted samples of the organ fraction and carcass of the five 0-pigs and six pigs per experimental diet representative for the BW blocks (n = 41) were analyzed separately for total AA profile (method ISO 13903; ISO, 2005b) by acid hydrolysis at 110°C for 23 h and ion-exchange chromatography with post-column derivatization with ninhydrin, including sulfur containing AA measured as cysteic acid and methionine sulfone after oxidation with performic acid; Trp (method ISO 13904; ISO, 2005c) was measured by alkaline hydrolysis at 110°C for 20 h and ion-exchange chromatography with fluorescence detection.

Table 4.1. Calculated standardized ileal digestible (SID) amino acid concentration (g/kg as-is)[1] of six experimental diets containing soybean meal (SBM), processed SBM[2] (pSBM), pSBM supplemented with crystalline amino acids (pSBM+AA[3]), rapeseed meal (RSM), processed RSM[2] (pRSM), or pRSM supplemented with crystalline amino acids (pRSM+AA[3]) fed to growing pigs

	Diet					
Amino acid[4]	SBM	pSBM	pSBM+AA	RSM	pRSM	pRSM+AA
His	3.65 (43)	3.00 (58)	3.89 (42)	2.52 (31)	2.20 (36)	2.59 (32)
Ile	6.39 (75)	5.96 (115)	6.86 (74)	5.14 (64)	4.83 (78)	5.30 (66)
Leu	10.50 (124)	9.98 (193)	11.30 (122)	9.33 (116)	8.98 (146)	9.60 (120)
Lys	8.50 (100)	5.17 (100)	9.23 (100)	8.07 (100)	6.17 (100)	7.97 (100)
OMIU-reactive Lys	8.97 (106)	5.02 (97)	9.09 (98)	7.95 (98)	5.69 (92)	7.53 (94)
Met	3.26 (38)	3.04 (59)	3.07 (33)	3.07 (38)	2.96 (48)	3.01 (38)
Met + Cys	5.14 (60)	4.47 (86)	4.95 (54)	5.07 (63)	4.66 (76)	5.07 (64)
Phe	7.14 (84)	6.75 (131)	7.57 (82)	3.80 (47)	3.53 (57)	3.92 (49)
Phe + Tyr	11.77 (139)	11.21 (217)	12.69 (137)	6.24 (77)	5.77 (94)	6.52 (82)
Thr	5.64 (66)	5.17 (100)	5.98 (65)	5.43 (67)	5.05 (82)	5.48 (69)
Trp	1.84 (22)	1.60 (31)	1.97 (21)	1.75 (22)	1.65 (27)	1.74 (22)
Val	6.61 (78)	6.14 (119)	7.22 (78)	6.20 (77)	5.77 (94)	6.45 (81)

[1]Based on data published in Hulshof et al. (2016a).

[2]Processing consisted of the addition of lignosulfonate to SBM and RSM and secondary toasting at 95 ± 2°C for 30 min.

[3]The pSBM+AA and pRSM+AA diets were formulated on SID amino acid levels in pSBM and pRSM, respectively, and supplemented with crystalline amino acids to meet SID amino acid levels in the SBM and RSM diets, respectively.

[4]The ratio between SID individual amino acids and SID Lys is provided in parentheses. The requirements for SID amino acids to SID Lys were: 31 for His, 52 for Ile, 101 for Leu, 30 for Met, 60 for Met + Cys, 54 for Phe, 94 for Phe + Tyr, 65 for Thr, 22 for Trp, and 70 for Val (Gloaguen et al., 2013).

4.2.4 Calculations and statistical analysis

The nutrient retention in the organ fraction and carcass of pigs fed the experimental diets was calculated from the composition at 40 ± 2 kg and the initial composition at d 9 based on the composition of the 0-pigs (Supplementary Table S4.1). The AA composition of protein in the empty body of the pigs was calculated from the AA composition in the organ fraction and carcass. The nutrient and AA retentions in the empty body were calculated from the retention in the organ fraction (Supplementary Table S4.2) and carcass (Supplementary Table S4.3). The post-absorptive N and AA utilizations were calculated as the ratio between the amount of N and individual AA retained in the empty body and the daily intake of SID N and individual AA. The daily intake of SID N and individual AA was based on the AA concentration in the diet excluding crystalline AA, the SID of AA from the protein sources as reported in Hulshof et al. (2016a) and considering 100% digestibility of crystalline AA. Reactive Lys was analyzed using the guanidination reaction with O-methylisourea (OMIU) and its concentration in the experimental diets are reported elsewhere (Hulshof et al., 2016b). Lysine in the empty body was considered to be reactive. The post-absorptive OMIU-reactive Lys utilization was calculated from the Lys retained in the empty body and the daily intake of SID OMIU-reactive Lys.

The AA composition, nutrient retention, and post-absorptive N and AA utilization were statistically analyzed using a 2-tailed general linear model (PROC GLM) in SAS 9.3 (SAS Inst. Inc., Cary, NC) with pig as experimental unit. The model included the fixed effects of protein source, diet type, and their interaction to test the effect of SBM/RSM and the effect of processing and supplementing crystalline AA on response variables. Body weight block was included in the random statement of the GLM. Least squares means were calculated per experimental diet and statistical differences between least squares means were determined using a post-hoc Tukey test. The response variables were tested for normality using studentized residuals and non-normal data were log transformed and these *P*-values are included in the tables. The presented least squares means are of non-log transformed data. The *P*-values < 0.05 were considered significant and *P*-values between 0.05 and 0.10 were considered indicative of a trend.

4.3 Results

4.3.1 Body composition

All pigs remained healthy during the whole experimental period.

The interaction between diet type, comprising effects of processing and supplementing crystalline AA, and protein source affected the CP concentration in the organ fraction (*P* =

0.044; Table 4.2), carcass ($P < 0.001$; Table 4.3), and empty body ($P < 0.001$; Table 4.4). Processing decreased the CP concentration with greater effects for SBM than for RSM and supplementing pSBM and pRSM with crystalline AA restored the CP concentration. The Arg, Met, Thr, and Cys concentration in the organ fraction was lower ($P < 0.05$) and the His concentration in the carcass was greater ($P < 0.05$) for the SBM diets than for the RSM diets. Diet type affected the Lys ($P < 0.001$), Cys ($P = 0.046$), Glu ($P = 0.003$), and Ser ($P = 0.003$) concentration in the organ fraction and the Trp concentration ($P = 0.027$) in the carcass. Processing lowered the concentrations of these AA and supplementing crystalline AA restored the concentrations. The interaction between diet type and protein source affected the His concentrations in the empty body ($P = 0.023$; Table 4.4). Processing lowered the His concentration in the empty body for SBM while this was the opposite for RSM. Supplementing crystalline AA resulted in an intermediate His concentration for both protein sources. Diet type affected the Lys ($P = 0.015$) and Trp ($P = 0.043$) concentration in the empty body with processing decreasing the concentration and supplementing crystalline AA increasing the concentration to levels in the SBM and RSM diets.

4.3.2 Nutrient retention

The interaction between diet type and protein source affected nutrient retention ($P < 0.05$; $P = 0.079$ for ether extract; Table 4.5). Processing decreased water, ash, N, and AA retention but the differences were smaller for RSM compared with SBM. Supplementing crystalline AA restored nutrient retention for both protein sources. Processing increased fat retention in the empty body and supplementing crystalline AA resulted in a fat retention similar to the SBM and RSM diets ($P < 0.001$ for diet type).

4.3.3 Post-absorptive utilization of N and amino acids

The interaction between diet type and protein source affected the post-absorptive N and AA utilization ($P < 0.05$; Table 4.6). Processing of SBM decreased the post-absorptive utilization of N and all AA while processing of RSM only decreased the post-absorptive utilization of N, Leu, Phe, Glu, and Ser. Supplementing pSBM with crystalline AA increased the post-absorptive utilization but there was a (numerical) difference between the SBM and pSBM+AA diets ranging between 8% (OMIU-reactive Lys) and 31% (Glu). Supplementing pRSM with crystalline AA increased the post-absorptive utilization of N and AA with most AA being (numerically) higher for the pRSM+AA diet than for the RSM diet.

Table 4.2. Effect of protein source, diet type, and their interaction on the amino acid composition of protein in the organ fraction of growing pigs fed one of six experimental diets: SBM, pSBM, pSBM+AA, RSM, pRSM, or pRSM+AA[1,2]

	Diet							*P*-value		
Item	SBM	pSBM	pSBM + AA	RSM	pRSM	pRSM + AA	SEM	Protein source	Diet type	Protein source × diet type
CP, g/kg as-is	146.3[a]	138.1[c]	144.3[ab]	141.5[abc]	139.5[bc]	142.3[abc]	1.2	0.080	< 0.001	0.044
Amino acid, g/100 g CP										
Arg	4.66	4.78	4.65	4.89	4.77	4.82	0.06	0.013	0.743	0.118
His	3.15	3.09	3.11	3.16	3.10	3.11	0.03	0.813	0.218	0.975
Ile	2.95	2.98	2.99	2.97	2.96	2.99	0.02	0.815	0.383	0.621
Leu	9.07	8.95	9.08	9.07	9.02	9.04	0.06	0.850	0.292	0.651
Lys	6.48[a]	5.88[b]	6.46[a]	6.41[a]	6.03[b]	6.52[a]	0.09	0.495	< 0.001	0.442
Met	1.39	1.40	1.39	1.42	1.43	1.43	0.02	0.024	0.884	0.924
Phe	4.76	4.73	4.77	4.79	4.73	4.75	0.03	0.802	0.170	0.665
Thr	3.74	3.75	3.76	3.81	3.77	3.79	0.02	0.038	0.762	0.506
Trp	1.19	1.19	1.16	1.19	1.17	1.16	0.01	0.385	0.083	0.451
Val	6.05	6.13	6.11	6.08	6.07	6.09	0.04	0.632	0.630	0.441
Ala	6.40	6.48	6.49	6.44	6.47	6.46	0.05	0.979	0.476	0.792
Asp	9.10	9.00	9.07	9.08	8.89	9.00	0.07	0.245	0.137	0.838
Cys	1.12[ab]	1.09[b]	1.14[ab]	1.13[ab]	1.14[ab]	1.15[a]	0.01	0.014	0.046	0.275
Glu	11.17[ab]	11.04[ab]	11.19[ab]	11.30[a]	10.98[b]	11.32[a]	0.07	0.266	0.003	0.297
Gly	6.13	6.32	6.15	6.21	6.24	6.22	0.07	0.696	0.237	0.411
Pro	4.57	4.73	4.73	4.67	4.70	4.66	0.05	1.000	0.167	0.225
Ser	4.15[ab]	4.06[ab]	4.14[ab]	4.19[a]	4.05[b]	4.17[ab]	0.03	0.458	0.003	0.662
Tyr	2.72	2.78	2.81	2.81	2.77	2.76	0.06	0.833	0.951	0.484

[a-c]Least squares means within a row lacking a common superscript letter differ significantly ($P < 0.05$).

[1]SBM = soybean meal and RSM = rapeseed meal. Diet type included the factor processing (pSBM and pRSM) and processing plus supplementing with crystalline amino acids to standardized ileal digestible amino acid levels in the SBM (pSBM+AA) and RSM (pRSM+AA) diets.

[2]Least squares means are reported for nine pigs for CP and six pigs for individual amino acids.

Table 4.3. Effect of protein source, diet type, and their interaction on the amino acid composition of protein in the carcass of growing pigs fed one of six experimental diets: SBM, pSBM, pSBM+AA, RSM, pRSM, or pRSM+AA[1,2]

	Diet							*P*-value		
Item	SBM	pSBM	pSBM + AA	RSM	pRSM	pRSM + AA	SEM	Protein source	Diet type	Protein source × diet type
CP, g/kg as-is	174.5[a]	145.0[c]	173.6[a]	160.9[b]	151.2[c]	160.1[b]	1.7	< 0.001	< 0.001	< 0.001
Amino acid, g/100 g CP										
Arg	6.42	6.25	6.37	6.39	6.34	6.32	0.09	0.982	0.487	0.698
His	2.87[a]	2.71[ab]	2.81[a]	2.51[b]	2.77[ab]	2.66[ab]	0.07	0.011	0.703	0.016
Ile	3.80	3.58	3.71	3.68	3.70	3.82	0.10	0.664	0.409	0.390
Leu	6.87	6.47	6.73	6.64	6.67	6.87	0.15	0.780	0.290	0.327
Lys	7.03	6.50	7.01	6.80	6.84	7.12	0.16	0.609	0.067	0.242
Met	2.08	1.97	2.05	2.00	2.00	2.11	0.05	0.919	0.157	0.355
Phe	3.85	3.64	3.81	3.68	3.78	3.79	0.08	0.795	0.473	0.138
Thr	3.80	3.61	3.80	3.71	3.75	3.86	0.08	0.614	0.230	0.383
Trp	0.92[ab]	0.85[b]	0.97[a]	0.89[ab]	0.90[ab]	0.91[ab]	0.02	0.375	0.027	0.073
Val	4.51	4.25	4.49	4.41	4.41	4.51	0.10	0.751	0.204	0.442
Ala	6.19	5.99	6.19	6.14	6.15	6.03	0.09	0.829	0.578	0.247
Asp	8.15	7.72	8.16	7.97	7.99	8.28	0.16	0.609	0.093	0.362
Cys	0.98	0.94	0.95	0.99	0.94	1.00	0.04	0.474	0.412	0.698
Glu	13.48	12.90	13.46	13.35	13.33	13.55	0.25	0.535	0.290	0.554
Gly	8.26	8.33	8.30	8.55	8.22	7.64	0.27	0.458	0.268	0.219
Pro	5.78	5.87	5.98	5.92	5.86	5.54	0.15	0.413	0.776	0.154
Ser	3.78	3.63	3.82	3.73	3.72	3.79	0.07	0.962	0.195	0.559
Tyr	2.88	2.72	2.83	2.78	2.81	2.91	0.07	0.642	0.341	0.339

[a-c]Least squares means within a row lacking a common superscript letter differ significantly ($P < 0.05$).

[1]SBM = soybean meal and RSM = rapeseed meal. Diet type included the factor processing (pSBM and pRSM) and processing plus supplementing with crystalline amino acids to standardized ileal digestible amino acid levels in the SBM (pSBM+AA) and RSM (pRSM+AA) diets.

[2]Least squares means are reported for nine pigs for CP and six pigs for individual amino acids.

Table 4.4. Effect of protein source, diet type, and their interaction on the amino acid composition of protein in the empty body of growing pigs fed one of six experimental diets: SBM, pSBM, pSBM+AA, RSM, pRSM, or pRSM+AA[1,2]

	Diet							*P*-value		
Item	SBM	pSBM	pSBM + AA	RSM	pRSM	pRSM + AA	SEM	Protein source	Diet type	Protein source × diet type
CP, g/kg as-is	169.6[a]	143.8[c]	168.1[a]	157.4[b]	149.2[c]	157.0[b]	1.5	< 0.001	< 0.001	< 0.001
Amino acid, g/100 g CP										
Arg	6.16	6.00	6.11	6.14	6.09	6.08	0.08	0.774	0.390	0.709
His	2.92[a]	2.78[ab]	2.87[ab]	2.61[b]	2.82[ab]	2.74[ab]	0.06	0.013	0.789	0.023
Ile	3.67	3.48	3.60	3.56	3.58	3.68	0.08	0.728	0.375	0.357
Leu	7.20	6.89	7.10	7.04	7.04	7.21	0.13	0.751	0.312	0.430
Lys	6.95[ab]	6.40[b]	6.93[ab]	6.74[ab]	6.71[ab]	7.02[a]	0.14	0.564	0.015	0.180
Met	1.97	1.88	1.94	1.91	1.91	2.00	0.04	0.835	0.158	0.297
Phe	3.98	3.82	3.96	3.86	3.93	3.94	0.06	0.818	0.480	0.207
Thr	3.80	3.63	3.80	3.73	3.76	3.85	0.07	0.555	0.223	0.390
Trp	0.96[ab]	0.91[b]	1.00[a]	0.94[ab]	0.94[ab]	0.95[ab]	0.02	0.346	0.043	0.105
Val	4.74	4.57	4.74	4.69	4.67	4.76	0.08	0.759	0.297	0.625
Ala	6.22	6.08	6.23	6.19	6.20	6.10	0.08	0.825	0.678	0.252
Asp	8.29	7.94	8.31	8.15	8.14	8.39	0.14	0.692	0.092	0.446
Cys	1.00	0.97	0.98	1.01	0.98	1.02	0.03	0.389	0.399	0.805
Glu	13.14	12.58	13.10	13.01	12.95	13.20	0.21	0.519	0.190	0.521
Gly	7.94	7.99	7.96	8.16	7.91	7.41	0.22	0.451	0.253	0.231
Pro	5.60	5.68	5.79	5.72	5.67	5.41	0.13	0.397	0.800	0.137
Ser	3.83	3.70	3.87	3.80	3.77	3.85	0.06	0.900	0.129	0.647
Tyr	2.85	2.73	2.83	2.79	2.81	2.89	0.06	0.640	0.320	0.428

[a-c]Least squares means within a row lacking a common superscript letter differ significantly ($P < 0.05$).

[1]SBM = soybean meal and RSM = rapeseed meal. Diet type included the factor processing (pSBM and pRSM) and processing plus supplementing with crystalline amino acids to standardized ileal digestible amino acid levels in the SBM (pSBM+AA) and RSM (pRSM+AA) diets.

[2]Least squares means are reported for nine pigs for CP and six pigs for individual amino acids.

Table 4.5. Effect of protein source, diet type, and their interaction on nutrient retention (g/d) in the empty body of growing pigs fed one of six experimental diets: SBM, pSBM, pSBM+AA, RSM, pRSM, or pRSM+AA[1,2]

	Diet							*P*-value		
Item	SBM	pSBM	pSBM + AA	RSM	pRSM	pRSM + AA	SEM	Protein source	Diet type	Protein source × diet type
Water	390.5[a]	223.2[c]	374.5[a]	299.1[b]	229.3[c]	310.3[b]	6.3	< 0.001	< 0.001	< 0.001
Ash	17.8[a]	12.6[b]	17.1[a]	13.6[b]	12.8[b]	13.9[b]	0.6	< 0.001	< 0.001	0.001
Ether extract	96.4[c]	118.0[ab]	89.2[c]	110.2[b]	124.1[a]	110.2[b]	3.2	< 0.001	< 0.001	0.079
N	16.6[a]	8.3[c]	15.6[a]	12.0[b]	9.3[c]	12.3[b]	0.2	< 0.001	< 0.001	< 0.001
Amino acid										
Arg	6.53[a]	3.20[c]	5.88[a]	4.51[b]	3.54[c]	4.61[b]	0.15	< 0.001	< 0.001	< 0.001
His	3.32[a]	1.61[c]	2.98[a]	1.97[bc]	1.80[bc]	2.20[b]	0.09	< 0.001	< 0.001	< 0.001
Ile	3.94[a]	1.84[d]	3.49[a]	2.59[bc]	2.10[cd]	2.87[b]	0.14	< 0.001	< 0.001	< 0.001
Leu	7.64[a]	3.61[c]	6.83[a]	5.09[b]	4.07[c]	5.54[b]	0.22	< 0.001	< 0.001	< 0.001
Lys	7.48[a]	3.31[c]	6.81[a]	4.93[b]	3.91[c]	5.52[b]	0.23	< 0.001	< 0.001	< 0.001
Met	2.15[a]	1.02[d]	1.92[a]	1.42[bc]	1.13[cd]	1.60[b]	0.07	< 0.001	< 0.001	< 0.001
Phe	4.24[a]	2.02[d]	3.84[a]	2.78[bc]	2.30[cd]	3.00[b]	0.12	< 0.001	< 0.001	< 0.001
Thr	4.02[a]	1.90[d]	3.68[a]	2.70[bc]	2.19[cd]	2.97[b]	0.12	< 0.001	< 0.001	< 0.001
Trp	1.01[a]	0.46[d]	0.98[a]	0.67[bc]	0.53[cd]	0.71[b]	0.03	< 0.001	< 0.001	< 0.001
Val	4.99[a]	2.38[c]	4.56[a]	3.38[b]	2.69[c]	3.62[b]	0.15	< 0.001	< 0.001	< 0.001
Ala	6.55[a]	3.21[c]	6.02[a]	4.50[b]	3.60[c]	4.56[b]	0.15	< 0.001	< 0.001	< 0.001
Asp	8.81[a]	4.18[c]	8.08[a]	5.92[b]	4.72[c]	6.49[b]	0.23	< 0.001	< 0.001	< 0.001
Cys	1.08[a]	0.52[d]	0.95[ab]	0.76[bc]	0.57[cd]	0.80[b]	0.04	0.001	< 0.001	0.002
Glu	13.95[a]	6.62[c]	12.70[a]	9.52[b]	7.54[c]	10.17[b]	0.39	< 0.001	< 0.001	< 0.001
Gly	8.29[a]	4.30[c]	7.65[a]	6.02[b]	4.53[c]	5.27[bc]	0.30	< 0.001	< 0.001	< 0.001
Pro	5.92[a]	3.12[d]	5.73[a]	4.26[b]	3.35[cd]	4.01[bc]	0.17	< 0.001	< 0.001	< 0.001
Ser	4.04[a]	1.94[c]	3.76[a]	2.76[b]	2.17[c]	2.94[b]	0.10	< 0.001	< 0.001	< 0.001
Tyr	3.01[a]	1.41[d]	2.71[a]	1.99[bc]	1.61[cd]	2.21[b]	0.10	< 0.001	< 0.001	< 0.001

[a-d]Least squares means within a row lacking a common superscript letter differ significantly ($P < 0.05$).

[1]SBM = soybean meal and RSM = rapeseed meal. Diet type included the factor processing (pSBM and pRSM) and processing plus supplementing with crystalline amino acids to standardized ileal digestible amino acid levels in the SBM (pSBM+AA) and RSM (pRSM+AA) diets.

[2]Least squares means are reported for nine pigs for water, ash, ether extract, and N and six pigs for individual amino acids.

Table 4.6. Effect of protein source, diet type, and their interaction on the utilization (%) of standardized ileal digestible N and individual amino acids for retention[1] in the empty body of growing pigs fed one of six experimental diets: SBM, pSBM, pSBM+AA, RSM, pRSM, or pRSM+AA[2,3]

	Diet							*P*-value		
Item	SBM	pSBM	pSBM + AA	RSM	pRSM	pRSM + AA	SEM	Protein source	Diet type	Protein source × diet type
N	65.9^{a}	38.2^{c}	60.7^{b}	67.0^{a}	57.9^{b}	67.6^{a}	1.2	< 0.001	< 0.001	< 0.001
Amino acid										
Arg	53.7^{b}	31.2^{c}	45.2^{b}	68.4^{a}	63.1^{a}	66.5^{a}	2.1	< 0.001	< 0.001	0.002
His	80.3^{a}	47.6^{b}	68.8^{a}	69.9^{a}	72.2^{a}	73.7^{a}	2.9	0.013	< 0.001	< 0.001
Ile	54.1^{a}	27.3^{d}	45.9^{abc}	44.9^{bc}	38.1^{c}	47.4^{ab}	2.1	0.555	< 0.001	< 0.001
Leu	64.0^{a}	32.1^{c}	54.5^{b}	49.0^{b}	40.1^{c}	50.7^{b}	2.0	0.034	< 0.001	< 0.001
Lys	77.6^{a}	56.8^{bc}	66.7^{ab}	54.6^{c}	55.8^{bc}	60.8^{bc}	2.7	< 0.001	0.003	< 0.001
OMIU-reactive Lys[4]	73.3^{a}	58.4^{b}	67.6^{ab}	55.6^{b}	60.7^{b}	64.4^{ab}	2.8	0.011	0.072	0.004
Met	58.0^{a}	29.6^{d}	56.5^{a}	41.4^{bc}	33.8^{cd}	46.6^{b}	2.0	< 0.001	< 0.001	< 0.001
Cys	50.6^{a}	32.3^{b}	45.4^{a}	34.1^{b}	29.5^{b}	34.3^{b}	2.1	< 0.001	< 0.001	0.013
Met + Cys	55.3^{a}	30.4^{c}	52.2^{a}	38.5^{bc}	32.2^{c}	41.6^{b}	2.0	< 0.001	< 0.001	< 0.001
Phe	52.3^{bc}	26.5^{d}	45.7^{c}	65.6^{a}	57.5^{b}	66.8^{a}	2.2	< 0.001	< 0.001	0.002
Thr	62.3^{a}	32.4^{e}	55.5^{ab}	44.8^{cd}	38.5^{de}	47.8^{bc}	1.9	< 0.001	< 0.001	< 0.001
Trp	48.0^{a}	25.6^{d}	44.8^{a}	34.4^{bc}	28.4^{cd}	35.6^{b}	1.6	< 0.001	< 0.001	< 0.001
Val	66.4^{a}	34.3^{d}	57.0^{b}	49.2^{bc}	41.3^{cd}	49.4^{bc}	2.0	0.001	< 0.001	< 0.001
Ala	101.6^{a}	55.1^{c}	100.9^{ab}	100.0^{ab}	87.9^{b}	110.7^{a}	3.1	< 0.001	< 0.001	< 0.001
Asp	50.3^{c}	27.1^{d}	53.2^{c}	81.3^{b}	73.0^{b}	99.7^{a}	2.8	< 0.001	< 0.001	< 0.001
Glu	47.7^{a}	25.0^{d}	32.9^{c}	50.9^{a}	42.3^{b}	41.5^{b}	1.6	< 0.001	< 0.001	< 0.001
Gly	128.7^{ab}	76.9^{d}	137.0^{a}	113.5^{abc}	95.6^{cd}	108.6^{bc}	5.7	0.085	< 0.001	0.001
Pro	73.9^{ab}	43.7^{c}	82.7^{a}	73.7^{ab}	69.3^{b}	82.4^{ab}	3.0	0.002	< 0.001	< 0.001
Ser	49.4^{b}	25.6^{c}	50.5^{b}	60.7^{a}	51.5^{b}	70.7^{a}	2.0	< 0.001	< 0.001	< 0.001
Tyr	57.2^{bc}	28.1^{d}	48.0^{c}	73.3^{a}	63.9^{ab}	74.5^{a}	2.9	< 0.001	< 0.001	< 0.001

[a-e]Least squares means within a row lacking a common superscript letter differ significantly ($P < 0.05$).

[1]Utilization was calculated as the ratio between the amount of nutrient retained (g/d) divided by the daily intake of standardized ileal digestible nutrient

(g/d).

[2]SBM = soybean meal and RSM = rapeseed meal. Diet type included the factor processing (pSBM and pRSM) and processing plus supplementing with crystalline amino acids to standardized ileal digestible amino acid levels in the SBM (pSBM+AA) and RSM (pRSM+AA) diets.

[3]Least squares means are reported for nine pigs for N and six pigs for individual amino acids.

[4]OMIU = O-methylisourea.

4.4 Discussion

In the current study, it was hypothesized that processing would reduce AA retention by a reduced ileal digestibility of AA but not by reduced post-absorptive AA utilization. This was verified by supplementing diets containing pSBM and pRSM with crystalline AA based on SID AA levels in the SBM and RSM diet. According to our hypothesis, the AA supplementation should restore N and AA retentions.

4.4.1 Body amino acid composition

The body AA composition of pigs fed the SBM and RSM diets was comparable to values found in other studies (Kyriazakis et al., 1993, Bikker et al., 1994). Processing reduced the SID AA concentration in the diets which, subsequently, resulted in a decreased body protein (determined as N) concentration and altered AA composition of body protein. The AA composition per unit body protein was found to be affected by dietary Lys level at a constant AA to Lys ratio (Batterham et al., 1990a). However, in the current study the AA profile of the diets changed because processing reduced the SID Lys level more than of other AA. Changing the AA profile by adding crystalline Met at the expense of corn starch was shown to affect the AA composition of body protein (Chung and Baker, 1992, Conde-Aguilera et al., 2010). Moreover, an increase in dietary Met reduced the amount of collagen, that is, Gly and Pro, in the empty body (Chung and Baker, 1992). In the current study, processing might have resulted in the deposition of different types of proteins, for example, less muscle protein in the carcass compared with protein in bone and adipose tissue with the latter being relatively low in Lys (Wünsche et al., 1983), which thereby changed the AA composition of body protein. Since there was a greater difference in SID Lys concentration between the SBM and pSBM diets than between the RSM and pRSM diets, our results could provide insight in the flexibility of pigs to deposit AA either in the organ fraction or in the carcass. With a small reduction in SID Lys level (that is, RSM and pRSM diets), pigs reduce the Lys concentration in the organ fraction. With a greater reduction in SID Lys level (that is, SBM and pSBM diets), also the Lys concentration in the carcass was reduced. Restoring the SID AA levels by supplementing crystalline AA to pSBM and pRSM resulted in similar AA compositions as for the SBM and RSM diets, respectively. Thus, processing affected the body AA composition because of a reduction in absorbed AA.

4.4.2 Limiting dietary amino acids

The experimental diets were formulated to be equally limiting in SID Ile, Leu, Lys, Met, Met + Cys, Thr, and Val. The SBM diet complied well with these requirements while Lys

and Met + Cys were first limiting in the pSBM and pSBM+AA diet, respectively (Table 4.1). The three RSM diets may have been first limiting in aromatic AA (Phe and Tyr) which were not supplemented to the RSM diet. The SID AA concentration was slightly different between the SBM/RSM and pSBM+AA/pRSM+AA diets because of small differences between AA concentration used for diet formulation and for calculating the actual SID AA concentration.

4.4.3 Nutrient retention and post-absorptive utilization of standardized ileal digestible amino acids

Processing decreased nutrient retention for both protein sources. Supplementation with crystalline AA was applied to determine if the decrease in retention was caused by a reduction in AA digestibility or post-absorptive availability. The latter would be the case when, for example, absorbed AA could not fully be utilized by the pig as was shown for cottonseed meal (Batterham et al., 1990b), heat-treated fish meal (Wiseman et al., 1991), and heat-treated field peas (Van Barneveld et al., 1994b). For example, early MRP, resulting from the reaction between sugars and the free ε-amino group of Lys, were found to be absorbed in the small intestine (Moughan et al., 1996) but were unavailable for protein synthesis in growing pigs (Rérat et al., 2002). The results of the current study, however, showed that supplementing the pSBM and pRSM diets with crystalline AA increased nutrient retention to similar levels as for the SBM and RSM diets. Moreover, the SID Lys and SID OMIU-reactive Lys concentrations of the used protein sources were similar (Hulshof et al., 2016a) and the post-absorptive utilization of Lys and OMIU-reactive Lys were comparable within each experimental diet (Table 4.6) indicating that the absorption of early MRP may have played a minor role. Thus, the decrease in nutrient retention was caused by a reduction in AA digestibility. Correcting N and AA retention for SID N and AA intake would, therefore, result in similar post-absorptive utilizations. Processing of SBM, however, reduced the post-absorptive utilization of all AA. This was not because energy or other AA were limiting in the pSBM diet. The utilization of AA has been shown to depend on the indispensable to dispensable AA ratio with higher utilizations at a lower indispensable to dispensable AA ratio (Lenis et al., 1999). The SID indispensable AA to SID dispensable AA ratio of the SBM and pSBM diets were, however, similar for the SBM and pSBM diets (47:53 and 46:54, respectively). The reduced post-absorptive AA utilization might result from a relatively higher amount of SID AA being used for maintenance for the pSBM diet compared with the SBM diet. The post-absorptive AA utilization above maintenance were calculated using maintenance requirements given by Fuller (1991) and Van Milgen et al. (2008). This calculation, however, gave similar results for the SBM and pSBM diets as in Table 4.6 (data not shown) indicating that AA of the pSBM diet were less efficiently used for growth than of the SBM diet. Since Lys was first limiting in the pSBM

diet, other AA could not be fully utilized resulting in an increased oxidation of these AA. The involvement of Lys in metabolic pathways used to remove excess AA, such as deamination via aminotransferase and in the citric acid cycle via acetyl CoA (Berg et al., 2002), might result in an increased catabolism of Lys which subsequently reduces the post-absorptive utilization for growth. An increased catabolism of AA could result in an increased liver weight which was found for pigs fed the pSBM diet (Hulshof et al., 2016b). Another possibility for the reduced post-absorptive utilization of AA for the pSBM diet is an overestimation of SID Lys of the pSBM protein source. The SID Lys was calculated based on values of a previous study (Hulshof et al., 2016a). However, the same batches of protein sources were used for both studies and the quality of the protein sources, as measured by analyzing the OMIU-reactive and total Lys concentrations, had not decreased in time. It is, therefore, unlikely that the digestibility of Lys was decreased by the additional formation of Lys-complexes, such as MRP, in the time between both studies. Moreover, the diets of both studies contained the same feed ingredients in similar proportions and, therefore, digestibility of the diet was expected to be similar as well. Since correcting AA retention for SID AA intake for the RSM and pRSM diets resulted in (largely) similar post-absorptive utilizations, it is likely that SID AA levels were not overestimated. The similar post-absorptive AA utilizations for the RSM and pRSM diets indicate that there was no additional effect of processing of RSM beyond effects on digestibility.

Supplementing crystalline AA to the pSBM diet increased the post-absorptive utilization of N and (most) AA compared with processing. The utilization of most AA for the pSBM+AA diet was, however, numerically lower compared with the SBM diet. Methionine + Cys became limiting for nutrient retention in the pSBM+AA diet which may explain why other AA could not be used with the same efficiency as for the SBM diet. In general, the utilization of Met + Cys is lower than of Lys because Met is used for several metabolic processes and, thus, has higher requirements for maintenance than Lys which is mainly used for protein synthesis (Chung and Baker, 1992; Heger et al., 2002). The post-absorptive utilization of most AA was numerically higher for the pRSM+AA diet compared with the RSM diet. Phenylalanine + Tyr were less limiting in the pRSM+AA diet compared with the RSM diet (Table 4.1) which presumably resulted in a higher utilization of all AA. Moreover, catabolism of AA might have been lower as well as the liver weight was lower for the pRSM+AA diet than for the RSM diet (Hulshof et al., 2016b). The post-absorptive Glu utilization was lower for both supplemented diets because L-Glu was supplemented to compensate for the decrease in SID dispensable AA due to processing. Thus, supplementing crystalline AA restored the post-absorptive utilization of AA to a large extent.

In conclusion, processing of SBM and RSM reduced nutrient retention by reducing AA digestibility. Processing of SBM negatively affected the utilization of ileal digestible AA for retention which might be related to an imbalanced supply of AA after absorption. Supplementing crystalline AA based on SID AA concentration in pSBM largely restored utilization but effects may have been influenced by a limiting Met + Cys supply. Processing of RSM did not affect the post-absorptive AA utilization while supplementing crystalline AA increased utilization presumably because Phe was less limiting. Processing resulted in small differences in AA composition of protein in the organ fraction, carcass, and empty body, especially for Lys, and supplementing crystalline AA restored AA composition for both protein sources.

4.5 Acknowledgements

The authors acknowledge the financial support from the Wageningen UR "IPOP Customized Nutrition" program financed by Wageningen UR (Wageningen, the Netherlands), the Dutch Ministry of Economic Affairs (The Hague, the Netherlands), WIAS (Wageningen, the Netherlands), Agrifirm Innovation Center (Apeldoorn, the Netherlands), ORFFA Additives BV (Werkendam, the Netherlands), Ajinomoto Eurolysine s.a.s. (Paris, France), and Stichting VICTAM BV (Nijkerk, the Netherlands). The authors thank T. Zandstra, M. W. A. Verstegen, the staff of the experimental facility, and the laboratory of Animal Nutrition of Wageningen UR for their contributions. The authors thank Feed Valid B.V., Poederoijen, the Netherlands, for providing the lignosulfonate and the two soybean meals and two rapeseed meals.

4.6 Literature cited

Agricultural Research Council (ARC). 1981. The nutrient requirements of pigs. Commonwealth Agricultural Bureaux, Slough, UK. p. 16.

Almeida, F. N., J. K. Htoo, J. Thomson, and H. H. Stein. 2014. Effects of heat treatment on the apparent and standardized ileal digestibility of amino acids in canola meal fed to growing pigs. Anim. Feed Sci. Technol. 187:44-52.

Batterham, E. S., L. M. Andersen, D. R. Baigent, and E. White. 1990a. Utilization of ileal digestible amino acids by growing pigs: Effect of dietary lysine concentration on efficiency of lysine retention. Brit. J. Nutr. 64:81-94.

Batterham, E. S., L. M. Andersen, D. R. Baigent, R. E. Darnell, and M. R. Taverner. 1990b. 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.

Berg, J. M., J. L. Tymoczko, and L. Stryer. 2002. Biochemistry, 5th edition. W.H. Freeman and Company, New York, NY, USA.

Bikker, P., M. W. A. Verstegen, and M. W. Bosch. 1994. Amino acid composition of growing pigs is affected by protein and energy intake. J. Nutr. 124:1961-1969.

Centraal Veevoeder Bureau (CVB). 2011. Feeding of pigs: Feeding standard, feeding advices and nutritional values of feeding ingredients. CVB series no. 43. CVB, Lelystad, the Netherlands. p. 25.

Chung, T. K., and D. H. Baker. 1992. Efficiency of dietary methionine utilization by young pigs. J. Nutr. 122 :1862-1869.

Conde-Aguilera, J. A., R. Barea, N. Le Floc'h, L. Lefaucheur, and J. van Milgen. 2010. A sulfur amino acid deficiency changes the amino acid composition of body protein in piglets. Animal 4:1349-1358.

Fuller, M. F. 1991. Present knowledge of amino acid requirements for maintenance and production: Non-ruminants. In Proceedings of the 6th International Symposium on Protein Metabolism and Nutrition, 9-14 June 1991, Herning, Denmark, p. 116-126.

Gloaguen, M., N. Le Floc'h, and J. van Milgen. 2013. Couverture des besoins en acides aminés chez le porcelet alimenté avec des régimens à basse teneur en protéines. INRA Prod. Anim. 26:277-288.

González-Vega, J. C., B. G. Kim, J. K. Htoo, A. Lemme, and H. H. Stein. 2011. Amino acid digestibility in heated soybean meal fed to growing pigs. J. Anim. Sci. 89:3617-3625.

Heger, J., T. van Phung, and L. Křížová. 2002. Efficiency of amino acid utilization in the growing pig at suboptimal levels of intake: Lysine, threonine, sulphur amino acids and tryptophan. J. Anim. Physiol. Anim. Nutr. 86:153-165.

Hulshof, T. G., P. Bikker, A. F. B. van der Poel, and W. H. Hendriks. 2016a. Assessment of protein quality of soybean meal and 00-rapeseed meal toasted in the presence of lignosulfonate by amino acid digestibility in growing pigs and Maillard reaction products. J. Anim. Sci. 94:1020-1030.

Hulshof, T. G., A. F. B. van der Poel, W. H. Hendriks, and P. Bikker. 2016b. Processing of soybean meal and 00-rapeseed meal reduces protein digestibility and pig growth performance but does not affect nitrogen solubilization along the small intestine. J. Anim. Sci. 94:2403-2414.

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

International Organization for Standardization (ISO). 1999. ISO 6492:1999. Animal feeding stuffs - Determination of fat content. ISO, Geneva, Switzerland.

International Organization for Standardization (ISO). 2002. ISO 5984:2002. Animal feeding stuffs - Determination of crude ash content. ISO, Geneva, Switzerland.

International Organization for Standardization (ISO). 2005a. ISO 5983-1:2005. Animal feeding stuffs - Determination of nitrogen content and calculation of crude protein content - Part 1: Kjeldahl method. ISO, Geneva, Switzerland.

International Organization for Standardization (ISO). 2005b. ISO 13903:2005. Animal feeding stuffs - Determination of amino acids content. ISO, Geneva, Switzerland.

International Organization for Standardization (ISO). 2005c. ISO 13904:2005. Animal feeding stuffs - Determination of tryptophan content. ISO, Geneva, Switzerland.

Kyriazakis, I., G. C. Emmans, and R. McDaniel. 1993. Whole body amino acid composition of the growing pig. J. Sci. Food Agric. 62:29-33.

Lenis, N. P., H. T. M. van Diepen, P. Bikker, A. W. Jongbloed, and J. van der Meulen. 1999. Effect of the ratio between essential and nonessential amino acids in the diet on utilization of nitrogen and amino acids by growing pigs. J. Anim. Sci. 77:1777-1787.

Moughan, P. J., and S. M. Rutherfurd. 2008. Available lysine in foods: A brief historical overview. J. AOAC Int. 91:901-906.

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

Rérat, A., R. Calmes, P. Vaissade, and P. A. Finot. 2002. Nutritional and metabolic consequences of the early Maillard reaction of heat treated milk in the pig. Significance for man. Eur. J. Nutr. 41:1-11.

Van Barneveld, R. J., E. S. Batterham, and B. W. Norton. 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., E. S. Batterham, and B. W. Norton. 1994b. The effect of heat on amino acids for growing pigs: 2. Utilization of ileal-digestible lysine from heat-treated field peas (*Pisum sativum* cultivar Dundale). Brit. J. Nutr. 72:243-256.

Van Milgen, J., A. Valancogne, S. Dubois, J.-Y. Dourmad, B. Sève, and J. Noblet. 2008. InraPorc: A model and decision support tool for the nutrition of growing pigs. Anim. Feed Sci. Technol. 143:387-405.

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

Wünsche, J., E. Borgmann, U. Hennig, F. Kreienbring, and H.-D. Bock. 1983. Einfluβ einer abgestuften Proteinversorgung bei hohem Energieniveau auf die Mastleitung sowie den Ansatz und die Verwertung von Futterenergie, Protein und Aminosäuren durch weibliche Mastschweine. 4. Mitteilung. Stickstoff- und Aminosäurengehalte in den Schlachtkörpern und Schlachtkörperteilen. Arch. Tierernähr. 33:389-413.

4.7 Supplementary material

Supplementary Table S4.1. Composition of the organ and carcass fraction of pigs (*n* = 5) slaughtered at BW of 17.0 ± 1.2 kg to determine initial body composition

Item	Organ fraction	SEM	Carcass fraction	SEM
Proximate components, g/kg as-is basis				
Water	796.8	3.1	659.8	3.5
Ash	11.2	0.2	37.2	0.6
Ether extract	26.4	1.8	117.7	3.4
N	23.0	0.4	28.1	0.4
Amino acids, g/100 g CP				
Arg	4.81	0.07	6.45	0.08
His	2.96	0.04	2.42	0.11
Ile	3.10	0.03	3.71	0.14
Leu	8.72	0.03	6.76	0.20
Lys	6.33	0.16	6.84	0.27
Met	1.46	0.03	1.98	0.05
Phe	4.63	0.03	3.77	0.10
Thr	3.78	0.02	3.78	0.12
Trp	1.19	0.01	0.92	0.04
Val	5.92	0.04	4.50	0.14
Ala	6.34	0.07	6.22	0.08
Asp	8.73	0.20	8.10	0.20
Cys	1.08	0.01	0.95	0.02
Glu	11.11	0.29	13.49	0.34
Gly	6.21	0.06	8.53	0.36
Pro	4.60	0.04	5.83	0.17
Ser	4.03	0.09	3.79	0.08
Tyr	2.93	0.06	2.85	0.10

Supplementary Table S4.2. Effect of protein source, diet type, and their interaction on nutrient retention (g/d) in the organ fraction of growing pigs fed 1 of 6 experimental diets: SBM, pSBM, pSBM+AA, RSM, pRSM, or pRSM+AA[1,2]

	Diet							*P*-value		
Item	SBM	pSBM	pSBM + AA	RSM	pRSM	pRSM + AA	SEM	Protein source	Diet type	Protein source × diet type
Water	63.7[ab]	41.2[d]	68.1[a]	55.9[bc]	39.6[d]	52.3[c]	2.3	< 0.001	< 0.001	0.013
Ash	1.0[a]	0.5[cd]	0.8[ab]	0.7[bc]	0.5[d]	0.6[cd]	0.0	< 0.001	< 0.001	0.026
Ether extract	4.9[ab]	5.9[a]	4.1[b]	5.1[ab]	5.9[a]	5.4[a]	0.3	0.050	< 0.001	0.055
N	2.0[a]	1.2[c]	2.0[a]	1.6[b]	1.2[c]	1.6[b]	0.1	< 0.001	< 0.001	< 0.001
Amino acid										
Arg	0.57[a]	0.38[c]	0.57[a]	0.50[ab]	0.36[c]	0.47[b]	0.02	0.001	< 0.001	0.157
His	0.42[a]	0.26[c]	0.41[a]	0.34[b]	0.25[c]	0.32[b]	0.01	< 0.001	< 0.001	0.009
Ile	0.35[a]	0.23[c]	0.37[a]	0.28[b]	0.21[c]	0.28[b]	0.01	< 0.001	< 0.001	0.017
Leu	1.19[a]	0.73[c]	1.20[a]	0.95[b]	0.70[c]	0.92[b]	0.03	< 0.001	< 0.001	0.002
Lys	0.84[a]	0.43[c]	0.84[a]	0.65[b]	0.42[c]	0.66[b]	0.03	< 0.001	< 0.001	0.005
Met	0.17[a]	0.11[c]	0.17[a]	0.14[b]	0.10[c]	0.14[b]	0.01	0.001	< 0.001	0.112
Phe	0.62[a]	0.38[c]	0.62[a]	0.50[b]	0.36[c]	0.48[b]	0.02	< 0.001	< 0.001	0.003
Thr	0.47[a]	0.29[c]	0.48[a]	0.39[b]	0.28[c]	0.37[b]	0.01	< 0.001	< 0.001	0.012
Trp	0.15[a]	0.10[c]	0.14[a]	0.12[b]	0.09[c]	0.11[b]	0.00	< 0.001	< 0.001	0.007
Val	0.78[a]	0.51[c]	0.80[a]	0.63[b]	0.47[c]	0.62[b]	0.02	< 0.001	< 0.001	0.008
Ala	0.82[a]	0.53[c]	0.84[a]	0.65[b]	0.50[c]	0.65[b]	0.03	< 0.001	< 0.001	0.008
Asp	1.20[a]	0.74[c]	1.20[a]	0.95[b]	0.68[c]	0.91[b]	0.03	< 0.001	< 0.001	0.005
Cys	0.15[a]	0.09[c]	0.15[a]	0.12[b]	0.09[c]	0.12[b]	0.00	< 0.001	< 0.001	0.001
Glu	1.43[a]	0.87[c]	1.43[a]	1.15[b]	0.81[c]	1.14[b]	0.04	< 0.001	< 0.001	0.020
Gly	0.77[a]	0.51[bc]	0.77[a]	0.62[b]	0.47[c]	0.62[b]	0.03	< 0.001	< 0.001	0.095
Pro	0.58[a]	0.39[bc]	0.62[a]	0.48[b]	0.36[c]	0.47[b]	0.02	< 0.001	< 0.001	0.025
Ser	0.54[a]	0.33[c]	0.54[a]	0.44[b]	0.31[c]	0.43[b]	0.02	< 0.001	< 0.001	0.019
Tyr	0.32[ab]	0.21[cd]	0.34[a]	0.27[bc]	0.19[d]	0.25[c]	0.01	< 0.001	< 0.001	0.049

[a-d]Least squares means within a row lacking a common superscript letter differ significantly ($P < 0.05$).

[1]SBM = soybean meal and RSM = rapeseed meal. Diet type included the factor processing (pSBM and pRSM) and processing plus supplementing with crystalline amino acids to standardized ileal digestible amino acid levels in the SBM (pSBM+AA) and RSM (pRSM+AA) diets.

[2]Least squares means for nine pigs for water, ash, ether extract, and N and six pigs for individual amino acids.

Supplementary Table S4.3. Effect of protein source, diet type, and their interaction on nutrient retention (g/d) in the carcass of growing pigs fed 1 of 6 experimental diets: SBM, pSBM, pSBM+AA, RSM, pRSM, or pRSM+AA[1,2]

	Diet							*P*-value		
Item	SBM	pSBM	pSBM + AA	RSM	pRSM	pRSM + AA	SEM	Protein source	Diet type	Protein source × diet type
Water	326.8[a]	182.0[c]	306.4[b]	243.2[b]	189.6[c]	258.1[b]	5.2	< 0.001	< 0.001	< 0.001
Ash	16.8[a]	12.1[b]	16.2[a]	13.0[b]	12.3[b]	13.3[b]	0.6	< 0.001	< 0.001	0.002
Ether extract	91.5[c]	112.0[ab]	85.1[c]	105.1[ab]	118.2[a]	104.9[b]	3.1	< 0.001	< 0.001	0.107
N	14.7[a]	7.1[d]	13.6[b]	10.3[c]	8.1[d]	10.8[c]	0.2	< 0.001	< 0.001	< 0.001
Amino acid										
Arg	5.96[a]	2.82[c]	5.32[a]	4.01[b]	3.19[c]	4.14[b]	0.15	< 0.001	< 0.001	< 0.001
His	2.91[a]	1.36[c]	2.57[a]	1.63[bc]	1.55[bc]	1.88[b]	0.10	< 0.001	< 0.001	< 0.001
Ile	3.58[a]	1.61[e]	3.12[ab]	2.31[cd]	1.89[de]	2.59[bc]	0.14	< 0.001	< 0.001	< 0.001
Leu	6.45[a]	2.88[d]	5.63[a]	4.14[bc]	3.63[cd]	4.62[b]	0.22	< 0.001	< 0.001	< 0.001
Lys	6.64[a]	2.88[d]	5.98[a]	4.28[bc]	3.49[cd]	4.85[b]	0.23	< 0.001	< 0.001	< 0.001
Met	1.98[a]	0.91[e]	1.76[ab]	1.28[cd]	1.03[de]	1.46[bc]	0.07	< 0.001	< 0.001	< 0.001
Phe	3.63[a]	1.63[d]	3.22[a]	2.28[bc]	1.93[cd]	2.53[b]	0.12	< 0.001	< 0.001	< 0.001
Thr	3.55[a]	1.60[d]	3.21[a]	2.31[bc]	1.90[cd]	2.60[b]	0.12	< 0.001	< 0.001	< 0.001
Trp	0.86[a]	0.37[d]	0.84[a]	0.55[bc]	0.45[cd]	0.59[b]	0.03	< 0.001	< 0.001	< 0.001
Val	4.21[a]	1.88[d]	3.76[a]	2.76[bc]	2.21[cd]	3.01[b]	0.15	< 0.001	< 0.001	< 0.001
Ala	5.73[a]	2.69[c]	5.18[a]	3.84[b]	3.10[c]	3.92[b]	0.14	< 0.001	< 0.001	< 0.001
Asp	7.62[a]	3.44[d]	6.88[a]	4.98[bc]	4.04[cd]	5.57[b]	0.23	< 0.001	< 0.001	< 0.001
Cys	0.93[a]	0.43[d]	0.79[ab]	0.64[bc]	0.48[cd]	0.68[b]	0.04	0.004	< 0.001	0.003
Glu	12.53[a]	5.75[d]	11.27[a]	8.37[bc]	6.73[cd]	9.03[b]	0.39	< 0.001	< 0.001	< 0.001
Gly	7.53[a]	3.79[c]	6.87[a]	5.40[b]	4.06[c]	4.66[bc]	0.29	< 0.001	< 0.001	< 0.001
Pro	5.34[a]	2.73[d]	5.12[a]	3.78[b]	2.99[cd]	3.54[bc]	0.17	< 0.001	< 0.001	< 0.001
Ser	3.50[a]	1.62[c]	3.22[a]	2.32[b]	1.87[c]	2.52[b]	0.10	< 0.001	< 0.001	< 0.001
Tyr	2.70[a]	1.21[e]	2.37[ab]	1.73[cd]	1.43[de]	1.96[bc]	0.10	< 0.001	< 0.001	< 0.001

[a-e]Least squares means within a row lacking a common superscript letter differ significantly ($P < 0.05$).

[1]SBM = soybean meal and RSM = rapeseed meal. Diet type included the factor processing (pSBM and pRSM) and processing plus supplementing with crystalline amino acids to standardized ileal digestible amino acid levels in the SBM (pSBM+AA) and RSM (pRSM+AA) diets.

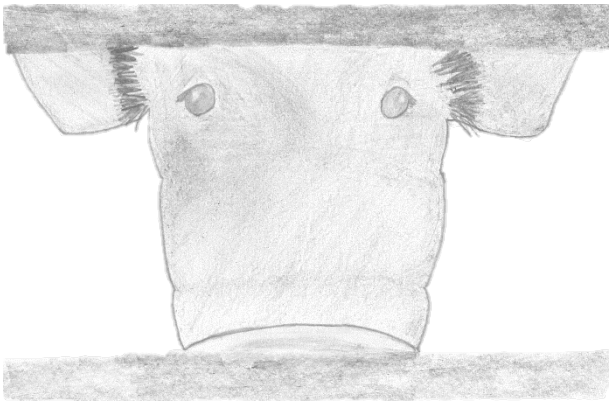
[2]Least squares means for nine pigs for water, ash, ether extract, and N and six pigs for individual amino acids.

Predicting the standardized ileal protein digestibility of processed soybean meal and rapeseed meal in growing pigs using two *in vitro* methods

S. Salazar-Villanea,[*†] T. G. Hulshof,[*†] A. F. B. van der Poel,[*] E. M. A. M. Bruininx,[*‡] and P. Bikker[†]

[*]Animal Nutrition Group, Wageningen University, Wageningen, the Netherlands, [†]Wageningen UR Livestock Research, Wageningen, the Netherlands, and [‡]Agrifirm Innovation Center, Royal Agrifirm Group BV, Apeldoorn, the Netherlands

Accepted in Journal of Animal Science



Abstract

A study was conducted to compare protein digestibility of processed ingredients using two *in vitro* methods with known standardized ileal digestibility (SID) of CP measured in growing pigs. The SID of CP in soybean meal (SBM), rapeseed meal (RSM), and both ingredients re-toasted in the presence of lignosulfonate resulting in processed SBM (pSBM) and processed RSM (pRSM) was determined in a trial with growing pigs surgically fitted with a steered ileo-cecal valve cannula. Toasting in the presence of lignosulfonate was performed to induce protein damage. Initial pH and degree of hydrolysis after 10 (DH10) and 120 (DH120) min were determined using the pH-STAT method. Hydrolysis was performed using trypsin, chymotrypsin, and peptidase at pH 8. Size exclusion profiles of the resulting peptides after hydrolysis were also determined. Crude protein digestibility was determined using a 2-step enzymatic method, with pepsin at pH 2 and pancreatin at pH 6.8. The SID of CP in SBM, pSBM, RSM, and pRSM were 83.9, 71.6, 74.9, and 64.6 %, respectively. Initial pH of ingredient solutions measured at constant N concentration was 6.9, 5.9, 6.1, and 5.5 and was highly positively correlated to SID of CP (r = 0.99, $P < 0.01$). The DH10 using the pH-STAT method was 10.8, 7.3, 8.7, and 7.0 % and was positively correlated to SID of CP (r = 0.95, $P = 0.046$). There was no correlation between DH120 and SID of CP. Similarly to the SID of CP, the size distribution of peptides in the 120 min hydrolysates were affected ($P < 0.001$) by the type of ingredient and processing. Digestibility of CP with the 2-step enzymatic method was 89.6, 83.4, 78.9, and 68.8 % for SBM, pSBM, RSM, and pRSM, respectively, and tended to be correlated to SID of CP (r = 0.91, $P = 0.092$). In conclusion, both *in vitro* methods gave similar correlations to SID of CP indicating that both might be used as indication for the SID of CP of thermally processed SBM and RSM in growing pigs.

Key words: growing pigs, *in vitro* digestibility, pH-STAT, processing, standardized ileal digestibility, 2-step enzymatic method

5.1 Introduction

The determination of standardized ileal digestibility (SID) of CP and amino acids (AA) is necessary for ingredient evaluation and formulation of swine diets. This involves the use of *in vivo* experiments, which is expensive and laborious (Jezierny et al., 2010). Thus, faster and cheaper methods are required for routine ingredient evaluation. This is especially true for processed ingredients and diets, as the wide range in processing conditions increases the variation and influences the protein quality (such as protein solubility, AA content, and protein digestibility; Moughan, 1999). *In vitro* methods have been used to predict protein digestibility for many ingredients (Boisen and Fernández, 1995; Jezierny et al., 2010). However, studies on *in vitro* methods that can accurately predict SID of CP for thermally processed ingredients are scarce (for example, Eklund et al., 2015).

The objective of the present study was to evaluate characteristics of protein digestibility from the 2-step *in vitro* enzymatic method and the pH-STAT method with the SID of CP measured in growing pigs using processed ingredients. Intense processing was used as a way to create a large contrast between commercial and over-processed ingredients.

5.2 Materials and methods

5.2.1 Ingredients, processing conditions and standardized ileal digestibility

The experiment consisted of 10 growing barrows (initial BW of 30.8 ± 1.0 kg) fed one of four experimental diets in each of 3 periods in an incomplete cross-over design (Hulshof et al., 2016). Four experimental diets containing 35% (as-fed) commercial soybean meal (SBM), rapeseed meal (RSM), or both ingredients re-toasted in the presence of lignosulfonate resulting in processed SBM (pSBM) and processed RSM (pRSM) were used to determine *in vivo* apparent ileal digestibility of CP. Toasting in the presence of lignosulfonate, a sugar-rich polymer, was performed to induce protein damage resulting from the Maillard reaction (that is, the reaction between sugars and the ε-amino group of Lys). The SID of CP was calculated by correcting the apparent ileal digestibility of the ingredients for basal endogenous losses as described by Jansman et al. (2002).

5.2.2 2-Step enzymatic in vitro *protein digestibility*

The *in vitro* CP digestibility was determined in duplicate using a modification of the method described by Boisen and Fernández (1995). The method was up-scaled to 5 g of material. Incubation with pepsin was performed at pH 2 for 2 h, followed by incubation with pancreatin at pH 6.8 for 4 h and omitted the incubation with Viscozyme®.

5.2.3 Protein hydrolysis using the pH-STAT method

The degree of hydrolysis (DH) was determined in duplicate using a modification of the method described by Pedersen and Eggum (1983). Enzymatic incubation was extended to 120 min using 1.61 mg porcine trypsin (Sigma, 13,000 to 20,000 BAEE units/mg protein), 3.96 mg bovine chymotrypsin (Sigma, > 40 units/mg protein), and 1.18 mg porcine intestinal peptidase (Sigma, 50 to 100 units/g solid) per milliliter of water. The volume of NaOH added during the titration was used to calculate DH. Initial pH of ingredient solutions and DH after 10 (DH10) and 120 min (DH120) hydrolysis were selected for correlation with SID of CP. The DH curve was used to calculate the rate of protein hydrolysis (*k*) based on the model described by Butré et al. (2012). The model was fitted using the MODEL procedure of SAS 9.3 (SAS Inst. Inc., Cary, NC, USA).

5.2.4 Size exclusion chromatography of hydrolysates

Hydrolysates after pH-STAT incubation were heated to 99°C for 20 min to inactivate the enzymes and centrifuged at 13,000 × *g* for 20 min at room temperature. The supernatant was filtered through 0.45 µm filter and analyzed by size exclusion chromatography using the Superdex peptides column (GE Healthcare, Uppsala, Sweden) in the ÄKTA micro system (GE Healthcare, Uppsala, Sweden). Injection volume was 50 µL, flow rate was 100 µL/min and UV detection was performed at 220 nm. Samples were run with 10 m*M* phosphate buffer at pH 7 containing 150 m*M* NaCl and 2% (*wt*/*vol*) sodium dodecyl sulfate as eluent. Areas under the curve were integrated using the Unicorn software. Relative areas under the curve were calculated using the total area under the curve for each hydrolyzed ingredient.

5.2.5 Statistical analysis

Results of the *in vitro* analysis were analyzed using a general linear model (PROC GLM procedure) of SAS 9.3. The model included the fixed effects of ingredient, processing, and their interaction. Correlations between SID of CP and the *in vitro* results (2-step enzymatic method, initial pH, DH10, DH120, and *k*) were estimated using the PROC CORR procedure of SAS. *P*-values were considered significant when lower than 0.05 and indicative for a trend when between 0.05 and 0.10.

5.3 Results and discussion

Significant effects of type of ingredient ($P < 0.001$) and processing ($P < 0.001$) were found for SID of CP (Table 5.1). Soybean meal had a higher SID of CP compared to RSM, in accordance with Eklund et al. (2012). Processing reduced SID of CP of SBM and RSM in a

Table 5.1. Standardized ileal digestibility (SID) of CP and characterization of *in vitro* CP digestibility of soybean meal (SBM), rapeseed meal (RSM), and both ingredients toasted with lignosulfonate resulting in processed SBM (pSBM) and processed RSM (pRSM)

	Ingredient					*P*-value		
Item	SBM	pSBM	RSM	pRSM	SEM	Ingredient	Processing	Ingredient × processing
Lys:CP[1]	0.063	0.046	0.056	0.043				
SID of CP[1], %	83.9	71.6	74.9	64.6	1.3	< 0.001	< 0.001	NS[2]
Two-step enzymatic method, %	89.6	83.4	78.9	68.8	2.9	< 0.001	< 0.01	NS
pH-STAT								
Initial pH	6.9	5.9	6.1	5.5	0.2	< 0.001	< 0.001	NS
DH10[3], %	10.8	7.3	8.7	7.0	0.6	0.01	< 0.001	0.03
DH120[4], %	21.3	20.9	18.6	19.2	0.5	0.02	NS	NS
k[5], s^{-1}	0.029	0.011	0.022	0.011	0.003	0.03	< 0.001	0.01
SEC relative AUC[6], %								
> 20 – 0.4 kDa	72.5	75.8	69.1	71.7	0.9	< 0.001	0.001	NS
0.4 – 0.1 kDa	27.1	23.9	29.6	27.0	0.8	0.001	< 0.001	NS
< 0.1 kDa	0.5	0.3	1.3	1.4	0.2	< 0.001	NS	NS

[1]Results from Hulshof et al. (2016).

[2]NS = not significant.

[3]DH10 = degree of hydrolysis after 10 min.

[4]DH120 = degree of hydrolysis after 120 min.

[5]Rate of protein hydrolysis.

[6]SEC = size exclusion chromatography and AUC = area under the curve.

similar manner (that is, without interaction), largely caused by Maillard reactions occurring during toasting with lignosulfonate (Hulshof et al., 2016). Such reactions occurred as shown by the lowered Lys:CP (Table 5.1) and the formation of Maillard reaction products as furosine and carboxymethyllysine (Hulshof et al., 2016). These reactions reduce accessibility of enzymes to specific cleavage sites of proteins (Moughan et al., 1996). Nevertheless, processing in that study also reduced the SID of AA that are not susceptible to Maillard reactions (data not shown), which could point at undesired structural modifications of proteins due to processing as suggested by Gerrard et al. (2012).

The 2-step enzymatic method showed similar results to SID of CP, as significant ingredient and processing effects were found ($P < 0.05$) without interactions (Table 5.1). The 2-step enzymatic method and SID of CP tended to correlate (r = 0.91, $P = 0.092$). The DH10 was affected by the interaction between type of ingredient and processing ($P = 0.03$; Table 5.1) and was positively correlated (r = 0.95, $P = 0.046$) with SID of CP. The DH10 was a criterion selected by Pedersen and Eggum (1983), amongst other hydrolysis times tested, as it exhibited the highest correlation with fecal CP digestibility in rats. In the current study, DH10 largely determined the estimate of *k* and was highly correlated to protein solubility (data not shown). Protein solubility is usually reduced after processing due to increased protein aggregation (Liu and Hsieh, 2007, 2008). Protein aggregation can hamper enzyme accessibility required for protein hydrolysis (Carbonaro et al., 2012; Pinto et al., 2014), which may explain the lower *k* values after processing. The *k* values were affected by the interaction between type of ingredient and processing ($P = 0.01$; Table 5.1) and tended to correlate with SID of CP (r = 0.91, $P = 0.094$).

The DH120 of SBM and pSBM was higher than that of RSM and pRSM ($P = 0.02$; Table 5.1 and Fig. 5.1). This could mean that at the same protein concentration soybean proteins can inherently be hydrolyzed to a larger extent than rapeseed proteins. Rapeseed proteins contain a lower amount of AA of which the peptide bonds are specifically cleaved by trypsin (that is, Lys and Arg) and chymotrypsin (for example, Trp, Tyr, Phe), the enzymes used during the pH-STAT hydrolysis, compared with soybean proteins (Grala et al., 1998). Hence, the number of potential cleavage sites for trypsin and chymotrypsin is higher for the soybean proteins. The accessibility of enzymes to the substrate is restricted by structural modifications such as protein aggregation and by Maillard reaction modifications of AA. However, when given sufficient time the enzymes used in the pH-STAT method can access the cleavage sites, as the extent of hydrolysis after 120 min was not affected by processing.

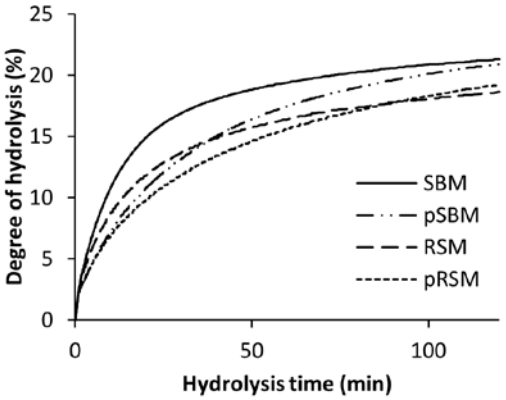


Figure 5.1. Degree of hydrolysis of the soybean meal (SBM), rapeseed meal (RSM) and both ingredients toasted in the presence of lignosulfonate resulting in processed SBM (pSBM) and processed RSM (pRSM).

Initial pH of the pH-STAT method was highly correlated (r = 0.99, $P < 0.01$) with SID of CP. The decrease in pH can be caused by degradation of sugars into organic acids during Maillard reactions as reported earlier for a glucose-Gly model system (Chung et al., 2012). Thus, initial pH may be used as a rapid indicator for protein damage because of its high correlation with SID of CP. Initial pH of the solutions in the present study was measured using the same N concentration (1 mg N/mL water), removing the effect of N concentration on pH.

Size exclusion chromatography allows to separate molecules according to their size, with larger molecules eluting before smaller molecules (Fig. 5.2). The elution of peptides with a size ranging from > 20 to 0.4 kDa and from 0.4 to 0.1 kDa was affected ($P < 0.001$) by ingredient type and processing (Table 5.1). Peptides from hydrolyzed ingredients that include Maillard reaction products have been suggested to be larger than peptides from hydrolyzed sources with lower thermal damage (Wada and Lönnerdal, 2014). Peptides smaller than 0.1 kDa were only affected by type of ingredient ($P < 0.001$).

In conclusion, characterization of protein digestibility by both *in vitro* methods correlated to SID of CP of these four ingredients. Moreover, measuring pH of protein solutions with a constant N content could provide a rapid indication for protein damage of thermally treated ingredients. It is necessary for future research to validate the correlation between these *in vitro* methods and SID of CP for a wider range of feed ingredients and processing conditions.

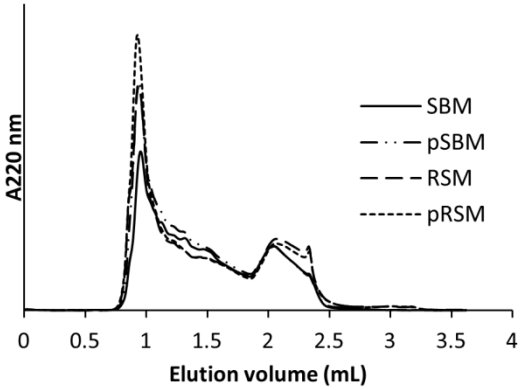


Figure 5.2. Size exclusion chromatograms of hydrolysates after 2 hours of hydrolysis of soybean meal (SBM), rapeseed meal (RSM) and both ingredients toasted in the presence of lignosulfonate resulting in processed SBM (pSBM) and processed RSM (pRSM).

5.4 Acknowledgement

The authors gratefully acknowledge the financial support from the Wageningen UR "IPOP Customized Nutrition" program financed by Wageningen UR (Wageningen, the Netherlands), the Dutch Ministry of Economic Affairs (The Hague, the Netherlands), WIAS (Wageningen, the Netherlands), Agrifirm Innovation Center (Apeldoorn, the Netherlands), ORFFA Additives BV (Werkendam, the Netherlands), Ajinomoto Eurolysine s.a.s. (Paris, France) and Stichting VICTAM BV (Nijkerk, the Netherlands).

5.5 Literature cited

Boisen, S., and J. A. Fernández. 1995. Prediction of the apparent ileal digestibility of protein and amino acids in feedstuffs and feed mixtures for pigs by *in vitro* analyses. Anim. Feed Sci. Technol. 51:29-43.

Butré, C. I., P. A. Wierenga, and H. Gruppen. 2012. Effects of ionic strength on the enzymatic hydrolysis of diluted and concentrated whey protein isolate. J. Agric. Food Chem. 60:5644-5651.

Carbonaro, M., P. Maselli, and A. Nucara. 2012. Relationship between digestibility and secondary structure of raw and thermally treated legume proteins: A Fourier transform infrared (FT-IR) spectroscopic study. Amino Acids 43:911-921.

Chung, S. Y., S. H. Han, S. W. Lee, and C. Rhee. 2012. Effect of Maillard reaction products prepared from glucose-glycine model systems on starch digestibility. Starch/Stärke 64:657-664.

Eklund, M., W. R. Caine, W. C. Sauer, G. S. Huang, G. Diebold, M. Schollenberger, and R. Mosenthin. 2012. True and standardized ileal digestibilities and specific ileal endogenous recoveries of crude protein and amino acid in soybean meal, rapeseed meal and peas fed to growing pigs. Livest. Sci. 145:174-182.

Eklund, M., N. Sauer, F. Schöne, U. Messerschmidt, P. Rosenfelder, J. K. Htoo, and R. Mosenthin. 2015. Effect of processing of rapeseed under defined conditions in a pilot plant on chemical composition and standardized ileal amino acid digestibility in rapeseed meal for pigs. J. Anim. Sci. 93:2813-2825.

Grala, W., M. W. A. Verstegen, A. J. M. Jansman, J. Huisman, and P. van Leeusen. 1998. Ileal apparent protein and amino acid digestibilities and endogenous nitrogen losses in pigs fed soybean and rapeseed products. J. Anim. Sci. 76:557-568.

Hulshof, T. G., P. Bikker, A. F. B. van der Poel, and W. H. Hendriks. 2016. Assessment of protein quality of soybean meal and 00-rapeseed meal toasted in the presence of lignosulfonate by amino acid digestibility in growing pigs and Maillard reaction products. J. Anim. Sci. 94:1020-1030.

Jansman, A. J. M., W. Smink, P. van Leeuwen, and M. Rademacher. 2002. Evaluation through literature data of the amount and amino acid composition of basal endogenous crude protein at the terminal ileum of pigs. Anim. Feed Sci. Technol. 98:49-60.

Jezierny, D., R. Mosenthin, N. Sauer, and M. Eklund. 2010. *In vitro* prediction of standardised ileal crude protein and amino acid digestibilities in grain legumes for growing pigs. Animal 4:1987-1996.

Liu, K. S., and F. H. Hsieh. 2007. Protein-protein interactions in high moisture-extruded meat analogues and heat-induced soy protein gels. J. Amer. Oil Chem. Soc. 84:741-748.

Liu, K. S., and F. H. Hsieh. 2008. Protein-protein interactions during high-moisture extrusion for fibrous meat analogues and comparison of protein solubility methods using different solvent systems. J. Agric. Food Chem. 56:2681-2687.

Moughan, P. J. 1999. *In vitro* techniques for the assessment of the nutritive value of feed grains for pigs: A review. Aust. J. Agric. Res. 50:871-879.

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

Pedersen, B., and B. O. Eggum. 1983. Prediction of protein digestibility by an *in vitro* enzymatic pH-stat procedure. Z. Tierphysiol. Tierernähr. Futtermittelkd. 49:265-277.

Pinto, M. S., J. Léonil, G. Henry, C. Cauty, A. F. Carvalho, and S. Bouhallab. 2014. Heating and glycation of β-lactoglobulin and β-casein: Aggregation and *in vitro* digestion. Food Res. Int. 55:70-76.

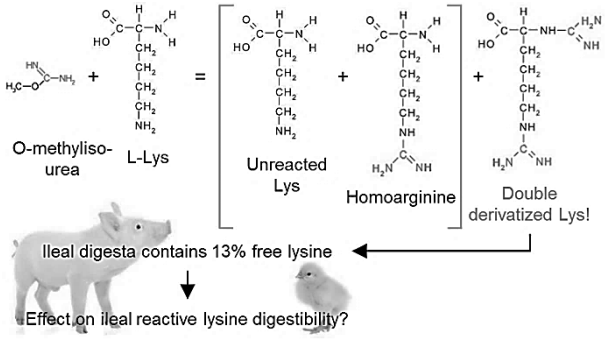
Wada, Y., and B. Lönnerdal. 2014. Effects of different industrial heating processes of milk on site-specific protein modifications and their relationship to *in vitro* and *in vivo* digestibility. J. Agric. Food Chem. 62:4175-4185.

O-methylisourea can react with the α-amino group of lysine: implications for the analysis of reactive lysine

T. G. Hulshof,[*†] S. M. Rutherfurd,[‡] S. Sforza,[§#] P. Bikker,[*] A. F. B. van der Poel,[†] and W. H. Hendriks[†]

[*]Wageningen UR Livestock Research, Wageningen, the Netherlands, [†]Animal Nutrition Group, Wageningen University, Wageningen, the Netherlands, [‡]Riddet Institute, Massey University, Palmerston North, New Zealand, [§]Laboratory of Food Chemistry, Wageningen University, Wageningen, the Netherlands, and [#]Department of Food Science, University of Parma, Parma, Italy

Submitted to Journal of Agricultural and Food Chemistry



Abstract

The specificity of O-methylisourea (OMIU) to bind to the ε-amino group of Lys, an important supposition for the OMIU-reactive Lys analysis of foods, feeds, ingredients and digesta, was investigated. Crystalline L-Lys incubated under standard conditions with OMIU resulted in low homoarginine recoveries. The reaction of OMIU with the α-amino group of Lys was confirmed by mass spectrometry analysis with double derivatized Lys being identified. None of the changes in reaction conditions (OMIU pH, OMIU to Lys ratio, and reaction time) with crystalline L-Lys resulted in 100% recovery of homoarginine. The average free Lys content in ileal digesta of growing pigs and broilers, was found to be 13% of total Lys which could result in a significant underestimation of the reactive Lys content. The reaction of OMIU with α-amino groups may necessitate analysis of free Lys to accurately quantify reactive lysine in samples containing a large proportion of Lys with a free α-amino group.

Key words: guanidination reaction, reaction conditions, specificity, reactive lysine

6.1 Introduction

Protein-bound Lys with its free ε-amino group is considered the amino acid (AA) that is most susceptible to react with other compounds present in ingredients, foods, and feeds during thermal processing (Finot, 1983; Friedman, 1992). One example is the reaction between amino groups and reducing sugars (Maillard reaction) resulting in the formation of Maillard reaction products (MRP). This reaction renders Lys unavailable for protein synthesis and concomitantly reduces the level of bioavailable Lys in foods and feeds (Hurrell and Carpenter, 1981; Finot, 2005; Moughan and Rutherfurd, 2008). Analyzing Lys using conventional AA analysis provides an inaccurate estimate of bioavailable Lys as early MRP can revert back to Lys under the strong acidic conditions used to hydrolyze protein during AA analysis (Hurrell and Carpenter, 1981). A number of methods have been developed that can determine Lys possessing a free ε-amino group, that is, reactive Lys, by reacting the latter group with a chemical reagent. Greenstein (1935) reported that the chemical reagent O-methylisourea (OMIU) was specific for the ε-amino group of Lys in a guanidination reaction which was corroborated in a number of subsequent studies (Chervenka and Wilcox, 1956; Klee and Richards, 1957; Shields et al., 1959; Kassell and Chow, 1966; Keough et al., 2000). The guanidination reaction with OMIU results in the conversion of Lys to homoarginine, an acid stable AA which can be quantified using conventional AA analysis (Moughan and Rutherfurd, 1996), thereby, allowing the OMIU-reactive Lys content to be determined. The guanidination method for determining reactive Lys has been shown to accurately predict Lys availability in feed ingredients for growing pigs (Rutherfurd et al., 1997a) and has been extensively used to determine standardized ileal digestibility (SID) of reactive Lys for different foods and feeds such as wheat, soybean meal, heated skim milk powder (Rutherfurd et al., 1997b), breakfast cereals (Torbatinejad et al., 2005), and cat foods (Rutherfurd et al., 2007). However, Kimmel (1967) stated that the reaction of OMIU is specific for the ε-amino group if the α-amino group is blocked, suggesting that OMIU might be able to bind to the α-amino group of AA under certain conditions. Evidence for the non-specificity of the guanidination reaction has been observed in the binding of OMIU to the free α-amino group of Gly (Beardsley et al., 2000) and to a lesser extent of Met, Ser, Val, Leu, Phe, Glu, and Ala (Beardsley and Reilly, 2002) when OMIU is used to enhance matrix-assisted laser desorption/ionization mass spectra of peptides. In addition, the OMIU-reactive Lys content in diets containing crystalline L-Lys HCl was recently reported to be underestimated when analyzed using the guanidination reaction (Hulshof et al., 2016). The authors hypothesized that OMIU had reacted with the free α-amino group of crystalline L-Lys HCl. Non-specificity of OMIU for the ε-amino group of Lys may have implications when determining reactive Lys if foods, feeds, ingredients, and ileal digesta contain appreciable quantities of free and N-terminal Lys.

Since it has been hypothesized that OMIU also binds to the α-amino groups of AA in addition to the ε-amino group of Lys, the current study investigated the specificity of OMIU for the ε-amino group of crystalline L-Lys and the binding of OMIU to α-amino groups of selected crystalline AA. Reaction conditions (OMIU to Lys ratio, reaction time and pH of the OMIU solution) for the specificity of OMIU to react with the ε-amino group of crystalline L-Lys were investigated. Practical implications of the results are assessed by examining the free Lys content of several food/feed ingredients and ileal digesta. The current study focused on ingredients used in feeds but implications also account for food ingredients.

6.2 Materials and methods

6.2.1 Materials and terminology

Barium hydroxide octahydrate, crystalline L-Lys, L-Arg, L-Phe, L-Val, L-Ile, L-Thr, and Gly were obtained from Sigma-Aldrich (Castle Hill, Australia) and crystalline L-Lys HCl (78% L-Lys) from BDH Laboratory Supplies (Poole, England). The OMIU sulfate salt was obtained from Sigma-Aldrich (St. Louis, MO). All crystalline AA were reagent grade with a purity greater than 98%.

Free Lys was determined after extraction with 0.1 *M* HCl and precipitation of co-extracted nitrogenous macromolecules by sulfosalicylic acid followed by centrifugation, separation, and detection using ion-exchange chromatography employing post-column ninhydrin or *o*-phthalaldehyde derivatization (method ISO 13903; ISO, 2005). Total Lys was determined after acid hydrolysis in 6 *M* HCl for 24 h at 110°C followed by separation and detection using ion-exchange chromatography employing post-column ninhydrin or *o*-phthalaldehyde derivatization (method ISO 13903; ISO, 2005). Reactive Lys was determined as being equivalent to the molar amount of homoarginine quantified after incubation of the sample with OMIU followed by acid hydrolysis with 6 *M* HCl for 24 h at 110°C.

6.2.2 Preparation of 0.6 M *O-methylisourea solution*

A 0.6 *M* OMIU solution was prepared according to the procedure described by Moughan and Rutherfurd (1996) except that 6 g of barium hydroxide octahydrate, instead of 4 g, was added to approximately 16 mL of boiled distilled deionized water, which had been boiled for at least 10 min to remove CO_2, in a centrifuge tube. Thereafter, 2 g of OMIU sulfate salt was added. The solution was cooled for 30 min at room temperature before being centrifuged at 6,400 × *g* for 10 min. The supernatant was retained and the

precipitate was washed with approximately 2 mL of boiled distilled deionized water and centrifuged again. Both supernatants were combined and the pH was determined to ensure it was above 12. Thereafter, the pH was adjusted to 10.6 by adding 6 *M* HCl and made up to 20 mL with boiled distilled deionized water.

6.2.3 Investigating the binding of O-methylisourea to amino groups present in crystalline amino acids

The binding of OMIU to amino groups of seven crystalline AA was investigated. Lysine, Arg, and Phe were selected because these AA have been reported to have the highest browning activity, that is, most likely to react with sugars during processing (Kwak and Lim, 2004). Valine, Ile, and Thr were selected because these AA are acid stable and frequently added in crystalline form to pig diets. Glycine was selected because it was previously reported to react with OMIU via its α-amino group (Beardsley et al., 2000; Beardsley and Reilly, 2002). The following guanidination procedure was used in the present study since it was reported to be optimal for diets and ileal digesta (Moughan and Rutherfurd, 1996): each AA (0.0006 moles) was separately incubated in 0.6 *M* OMIU (OMIU to AA ratio of 1000:1) at 25 ± 2°C in a shaking water bath for 7 d. The samples were reduced to dryness under vacuum (Savant SpeedVac Concentrator SC250EXP, Savant Instruments Inc. Farmingdale, NY), subsequently dissolved in citric acid buffer (pH 2.2), and analyzed in duplicate using a cation-exchange high-performance liquid chromatography system (Shimadzu Corp., Kyoto, Japan) employing post-column *o*-phthalaldehyde derivatization. Each unreacted AA (0.0006 moles) was also analyzed in duplicate to determine the AA content in non-OMIU incubated samples.

6.2.4 Liquid chromatography/mass spectrometry analysis of guanidinated crystalline amino acids

A 0.6 *M* OMIU solution was prepared as described above. Separate solutions of crystalline L-Lys and crystalline L-Tyr (0.0006 M) were incubated with OMIU for 3 d at room temperature in a shaker using an OMIU to AA ratio of either 10:1, 100:1, or 1000:1. Tyrosine was chosen as a model AA, in addition to Lys, because of its relatively high molecular weight (181.19 g/mol) and low polarity, which both favor reverse phase-liquid chromatography detectability. Samples were analyzed by an Acquity ultrahigh-performance liquid chromatography system (Waters, Milford, MA) using an Acquity ultrahigh-performance liquid chromatography BEH C18 column (2.1 × 150 mm, 1.7 µm particle size) with an Acquity BEH C18 Vanguard precolumn (2.1 × 50 mm, 1.7 µm particle size). Eluent A was 1% (*v*/*v*) acetonitrile containing 0.1% (*v*/*v*) trifluoroacetic acid in Millipore water and eluent B was 100% acetonitrile containing 0.1% (*v*/*v*) trifluoroacetic

acid. Samples (1 µL) were injected into the column maintained at 40°C. The analysis was conducted using the following elution profile: for OMIU incubated crystalline L-Lys, isocratic elution with 99.9% eluent A and 0.1% eluent B; for OMIU incubated crystalline L-Tyr, 0 to 2 min isocratic 99.9% eluent A, from 2 to 15 min linear gradient from 99.9% to 50% eluent A, from 15 to 20 min linear gradient from 50% eluent A to 99.9% eluent B, from 20 to 25 min isocratic at 99.9% eluent B, then re-equilibration to the initial conditions. The flow rate was set at 0.35 mL/min. The photodiode array detector was operated at a sampling rate of 40 points/s in the range 200-400 nm, resolution 1.2 nm. The SYNAPT G2Si mass spectrometer was operated in positive ion mode, capillary voltage 3 kV, sampling cone 30 V, source temperature 150°C, desolvation temperature 500°C, cone gas flow (N_2) 200 L/h, desolvation gas flow (N_2) 800 L/h, acquisition in the Full Scan mode, scan time 0.3 s, acquisition range 150-2000 m/z. The mass spectrometer was calibrated using NaI (m/z range: 100-2000). Mass spectrometry data were processed using the software MassLynx v 4.1 (Waters, Milford, MA).

6.2.5 Influence of reaction time and O-methylisourea to Lys ratio on guanidination of crystalline L-Lys

The influence of OMIU to Lys ratio and reaction time on the guanidination of crystalline L-Lys was assessed using a 4 × 3 factorial arrangement with four OMIU to Lys ratios and three reaction times. The OMIU to Lys ratios were 1.5:1 (optimal to convert crystalline L-Lys to homoarginine; Zhang et al., 2006), 10:1 (found to be optimal for casein; Imbeah et al., 1996), 100:1, and 1000:1 (Moughan and Rutherfurd, 1996). The reaction times were 1, 3, and 7 d with the remaining reaction conditions as described above.

6.2.6 Influence of O-methylisourea to Lys ratio and O-methylisourea pH on guanidination of crystalline L-Lys

The influence of pH of the OMIU solution and OMIU to Lys ratio on guanidination of crystalline L-Lys was assessed using a 7 × 2 factorial arrangement with seven pH levels and two OMIU to Lys ratios. The pH values ranged from 8.6 to 11.0 with 0.4 increments, with pH 9.0 and 10.6 being the pKa values for the α- and ε-amino groups of Lys, respectively. The OMIU to Lys ratios were 10:1 and 1000:1. A reaction time of 3 d was used with the remaining reaction conditions as described above.

6.2.7 Analysis of crystalline L-Lys HCl and a mixture of crystalline amino acids using two O-methylisourea to amino acid ratios during guanidination

L-lysine HCl (78% L-Lys), that is, a form of crystalline L-Lys that is supplemented to diets, and an equimolar mixture of the other six selected crystalline AA (that is, Arg, Phe, Val, Ile, Thr, and Gly) were analyzed using an OMIU to AA ratio of 10:1 and 1000:1, an OMIU pH of 10.6 and a reaction time of 3 d. Unreacted and OMIU-incubated solutions of crystalline L-Lys HCl and the mixture of six crystalline AA were analyzed in duplicate using the high-performance liquid chromatography system as described above.

6.2.8 Examining the free Lys content in selected protein sources

Data on the free Lys as percentage of total Lys for 44 different food/feed ingredients were obtained from Ajinomoto Eurolysine s.a.s. (2014). The free and total Lys contents were determined by Ajinomoto Eurolysine s.a.s. using the procedures described above.

6.2.9 Free Lys content in ileal digesta collected from pigs and broilers fed protein-free or selected protein-containing diets

Samples of ileal digesta were selected based on the protein source present in the experimental diets and the method used to collect the digesta during animal trials with growing pigs or broilers previously conducted at the Riddet Institute (Palmerston North, New Zealand) and Animal Nutrition group of Wageningen University (Wageningen, The Netherlands).

With regard to the growing pig trials, samples were obtained from five experiments. In the first experiment (Hulshof et al., 2016), diets contained soybean meal (SBM) or rapeseed meal (RSM) as the sole protein source and were each fed to seven (steered ileo-cecal valve) cannulated growing pigs (n = 14). Crystalline L-Lys HCl was added to the RSM diet. In the second experiment (H. Chen, Wageningen UR Livestock Research, unpublished data), a protein-free diet was fed consisting of corn starch, dextrose, arbocel (fiber source from J. Rettenmaier & Söhne Group, Rosenberg, Germany), soy oil and vitamins/minerals/marker. In the same study, SBM or RSM was added as the sole protein source to the experimental diets at the expense of corn starch. Each diet was fed to three growing pigs and ileal digesta was collected at slaughter (n = 9). In the third experiment (S. M. Rutherfurd, Riddet Institute, unpublished data), a protein-free diet (corn starch, sugar, cellulose, soybean oil, vitamins/minerals/marker) was fed to growing pigs and ileal digesta collected at slaughter. The ileal digesta of four pigs was pooled based on freeze DM content (n = 1). In the fourth experiment (S. M. Rutherfurd, Riddet Institute, unpublished data), a protein-

free diet (wheat starch, sucrose, cellulose, soybean oil and vitamins/minerals/marker) or a 15% gelatin-based diet was fed to growing pigs and ileal digesta was collected at slaughter. One pooled ileal digesta sample was obtained for the protein-free diet by combining samples of two pigs based on the freeze DM content (*n* = 1). Two pooled ileal digesta samples were obtained for the gelatin diet by combining samples of two and four pigs based on the freeze DM content (*n* = 2). In the fifth experiment (S. M. Rutherfurd, Riddet Institute, unpublished data), diets contained one of two whey protein concentrates or a whey protein isolate as the sole protein source and were fed to growing pigs. Ileal digesta was collected at slaughter. The ileal digesta of three, five and four pigs for the two whey protein concentrate diets and the whey protein isolate diet, respectively, were pooled based on the freeze DM content (*n* = 2 for whey protein concentrate and *n* = 1 for whey protein isolate).

Samples from broilers were obtained from two experiments. In the first experiment (De Vries et al., 2014), maize (30%) and RSM (35%) were the main protein-containing ingredients in the experimental diet and ileal digesta samples were collected at slaughter. The experimental diet contained crystalline L-Lys HCl. The ileal digesta from six cages containing 11 broilers were pooled based on freeze DM content (*n* = 2 by pooling samples per three cages). In the second experiment (Abdollahi et al., 2015), wheat (65%) and SBM (28%) were the main protein-containing ingredients in two experimental diets and ileal digesta samples were collected at slaughter. The experimental diets contained crystalline L-Lys HCl. The ileal digesta of six cages per experimental diet containing eight broilers were pooled based on the freeze DM content (*n* = 4 by pooling samples per four and two cages per experimental diet).

The samples were analyzed for free Lys and total Lys content using the methods described above. The contribution of endogenous or dietary free Lys to the total free Lys content in ileal digesta was determined by comparing ileal digesta from growing pigs fed protein-free or protein-containing diets. The free Lys content in ileal digesta collected at slaughter from growing pigs or broilers fed protein-containing diets was also compared.

6.2.10 Calculations

The recovery of AA after OMIU incubation was calculated using Eq. [1]:

$$\text{AA recovery (\%)} = \frac{\text{AA in OMIU incubated sample (nmol/mg)}}{\text{AA in non-OMIU incubated sample (nmol/mg)}} \times 100 \quad [1]$$

For crystalline L-Lys, the difference between Lys in the non-OMIU incubated sample (100% recovery) and the sum of the recovery of unreacted Lys (that is, Lys having a free α- and ε-amino group), and homoarginine (that is, Lys with OMIU bound to the ε-amino group), was attributed to Lys with a CN_2H_3 (that is, OMIU) bound to the α- and ε-amino group (that is, Lys that could not be recovered by high-performance liquid chromatography analysis). For the other crystalline AA, the difference between the recovery of the AA in the non-OMIU incubated sample (100% recovery) and the recovery of the AA in the OMIU incubated sample was attributed to the AA with a CN_2H_3 (that is, OMIU) bound to the α-amino group.

The free Lys contents in ileal digesta collected via a cannula or at slaughter of growing pigs fed SBM or RSM were plotted against the apparent ileal digestible (AID) CP content of the diets. Correlations between the free and total Lys content in ileal digesta of growing pigs fed protein-containing diets and between the AID CP content and the free Lys as percentage of total Lys were statistically analyzed using the PROC CORR procedure in SAS 9.3 (SAS Inst. Inc., Cary, NC).

6.3 Results and discussion

6.3.1 Binding of O-methylisourea to α- and ε-amino groups

As expected, the recovery of unreacted Lys (that is, having free α- and ε-amino groups) when crystalline L-Lys was incubated with OMIU was low. However, the recovery of homoarginine was low as well resulting in a significant amount of Lys (that is, 96%) being unaccounted for after guanidination (Fig. 6.1). The latter was also observed for the other six AA (Fig. 6.1). The unrecovered AA after incubation with OMIU were likely to have reacted with OMIU via their α-amino groups and the subsequent inability of the compound to be retained on the ion-exchange column or to be derivatized by *o*-phthalaldehyde after chromatographic separation. The difference between AA in terms of recovery of the unreacted AA suggests that there may be a different reaction equilibrium for each AA, possibly related to differences between side-chains (that is, charged vs. uncharged and polar vs. non-polar) and the pKa of α-amino groups of the different AA. Binding of OMIU to both the α- and ε-amino groups of crystalline L-Lys and to the α-amino group of crystalline L-Tyr was confirmed by mass spectrometry. After incubation of crystalline L-Lys with OMIU, protonated Lys (1.38 min, m/z 147.11), protonated monoderivatized Lys/homoarginine (~1.8 min, m/z 189.13), and protonated double derivatized Lys (~2.9 min, m/z 231.16) were identified (Fig. 6.2A). Furthermore, the ratio of these three compounds was dependent on the OMIU to Lys ratio used for incubation. The

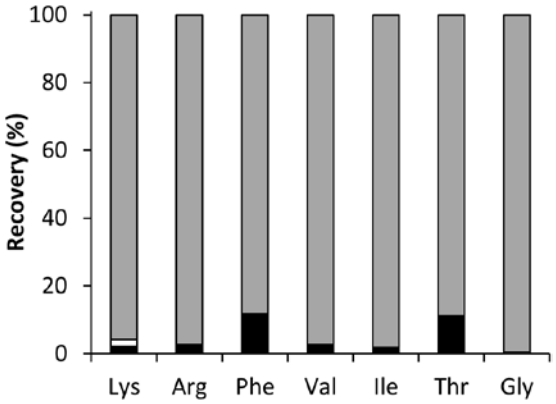


Figure 6.1. Recovery of crystalline amino acids (AA) after the guanidination reaction using an O-methylisourea (OMIU) to crystalline AA ratio of 1000:1, pH of the OMIU solution of 10.6, and a reaction time of 7 d. Black bars indicate unreacted AA, white bars indicate homoarginine, and grey bars indicate non-recovered AA.

m/z for the peak at 1.79 min (Fig. 6.2B) and 2.94 min (Fig. 6.2C) are consistent with those of non-protonated homoarginine (188.23 g/mol) and non-protonated double derivatized Lys (230.27 g/mol). In addition to the m/z for the intact molecules, several m/z corresponding to fragment ions were also visible, such as m/z 172.11 (protonated homoarginine without NH_3) and m/z 213.14 (protonated double derivatized Lys without 2 × H and 1 × O). After incubation of crystalline L-Tyr with OMIU, protonated Tyr (~5.5 min, m/z 182.08) and protonated monoderivatized Tyr (~6.7 min, m/z 224.10) were identified (Fig. 6.2D). As was the case with Lys, the ratio of these two compounds was dependent on the OMIU to Tyr ratio. The m/z for the peak at 6.70 min (Fig. 6.2E) is consistent with those of non-protonated monoderivatized Tyr (223.22 g/mol). In this case, OMIU reacted only with the α-amino group of Tyr because there is no binding site on the aromatic ring. In peptides, OMIU has been reported to bind to the α-amino group of Gly (Beardsley et al., 2000) and partially to the α-amino group of Met, Ser, Val, Leu, Phe, Glu, and Ala when reaction time was extended to several hours (Beardsley and Reilly, 2002). However, since matrix-assisted laser desorption/ionization mass spectrometry was used, only qualitative results were provided and the extent of the binding of OMIU to α-amino groups could not be determined. The latter along with the fact that different reactions times used between the study of Beardsley and Reilly (2002) and the study reported here (5 or 10 min and 3 or 7 d, respectively) makes comparison of results difficult. Nonetheless, under the reaction conditions that were employed in the present study, OMIU was found to bind extensively to the α-amino group of several crystalline AA. Moreover, the OMIU to AA ratio used during incubation appeared to have a major influence on the specificity of OMIU for the ε-

amino group of Lys. The effect of OMIU to AA ratio, pH of the OMIU solution and reaction time were subsequently studied to investigate the specificity of OMIU to react with the ε-amino group of crystalline L-Lys.

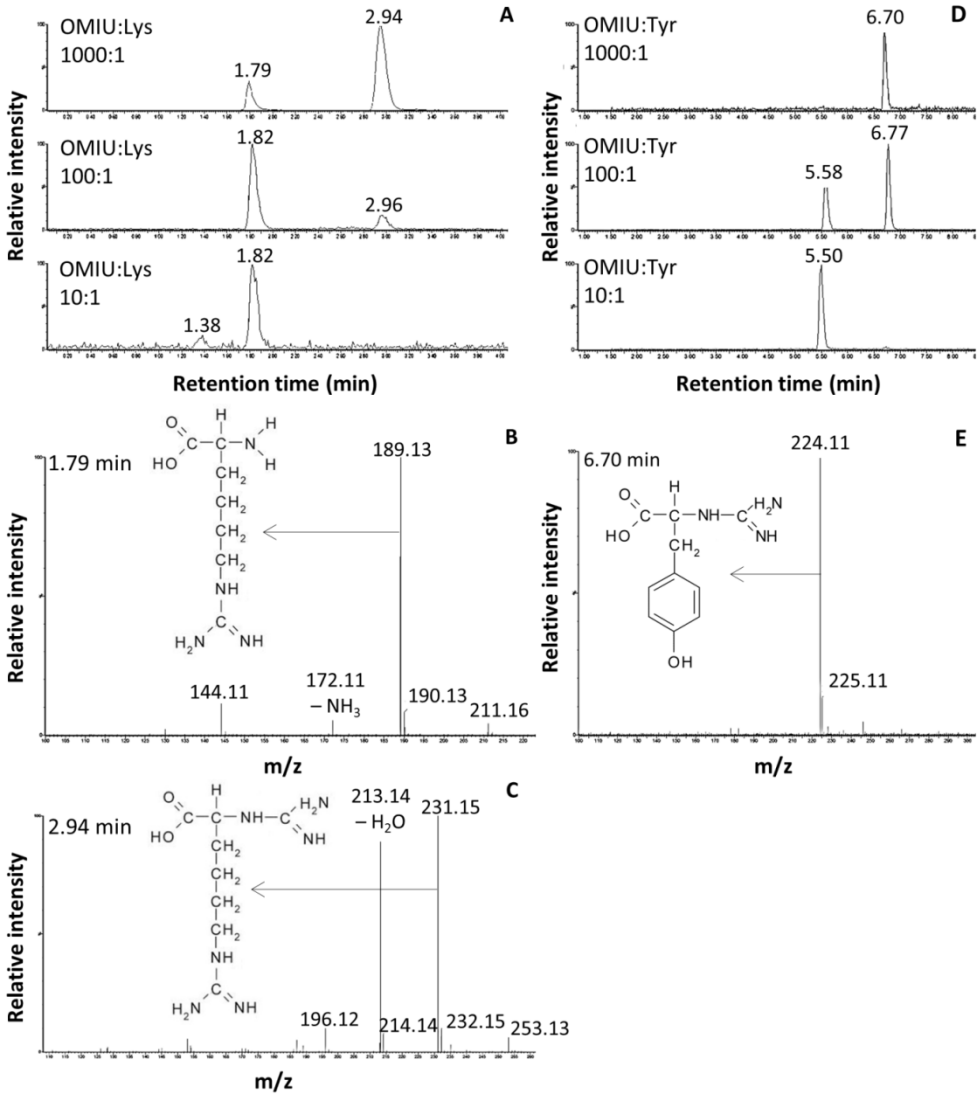


Figure 6.2. Liquid chromatography/mass spectrometry results of O-methylisourea (OMIU) incubated samples: crystalline L-Lys (A) and crystalline L-Tyr (D) incubated at an OMIU to crystalline L-Lys or crystalline L-Tyr ratio of 10:1, 100:1, and 1000:1, and mass spectra of the liquid chromatography peaks at 1.79 min (B) and 2.94 min for crystalline L-Lys (C) and at 6.70 min for crystalline L-Tyr (E).

6.3.2 Optimization of the guanidination reaction for crystalline L-Lys

Regardless of the OMIU to Lys ratio, reaction time had little effect on the recovery of unreacted Lys and homoarginine (Fig. 6.3) and on the quantity of non-recovered Lys (considered to be double derivatized Lys). The recovery of unreacted Lys and homoarginine decreased from 9 to 1% and 51 to 1%, respectively, when the OMIU to Lys

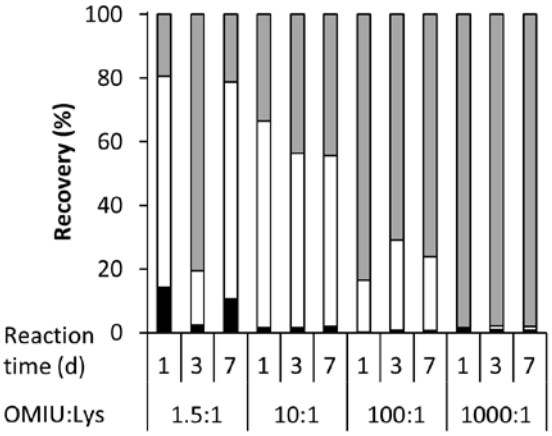


Figure 6.3. Recovery of crystalline L-Lys after the guanidination reaction using four O-methylisourea (OMIU) to free Lys ratios (1.5:1, 10:1, 100:1, or 1000:1), three reaction times (1, 3, or 7 d), and a pH of the OMIU solution of 10.6. Black bars indicate unreacted Lys, white bars indicate homoarginine, and grey bars indicate non-recovered Lys.

ratio increased from 1.5:1 to 1000:1. These results were consistent with the findings obtained using mass spectrometry analysis (Fig. 6.2A). The impact of the reaction mixture pH on the binding of OMIU to the α- and ε-amino group of crystalline L-Lys was also examined (Fig. 6.4). When the OMIU to Lys ratio was 10:1, the recovery of homoarginine increased from 13 to 75% and the recovery of unreacted Lys decreased from 79 to 9% as pH increased from 8.6 to 11.0. When the OMIU to Lys ratio was 1000:1, the recovery of unreacted Lys was highest at pH 8.6 and close to 0% for the other pH values while the recovery of homoarginine was highest for pH values between 8.6 and 9.4 and low for pH values between 9.8 and 11.0. Overall, none of the tested combinations of OMIU to Lys ratio, reaction time, and OMIU pH resulted in the complete recovery of crystalline L-Lys as homoarginine. Moreover, in all cases, between 4 and 99% of the crystalline L-Lys was not recovered either as unreacted Lys or as homoarginine after incubation with OMIU suggesting that OMIU had bound to the α-amino group of Lys to differing extents. Increasing the amount of OMIU appeared to drive the equilibrium of the chemical reaction towards double derivatization of the crystalline L-Lys. Typically, a higher OMIU to Lys ratio

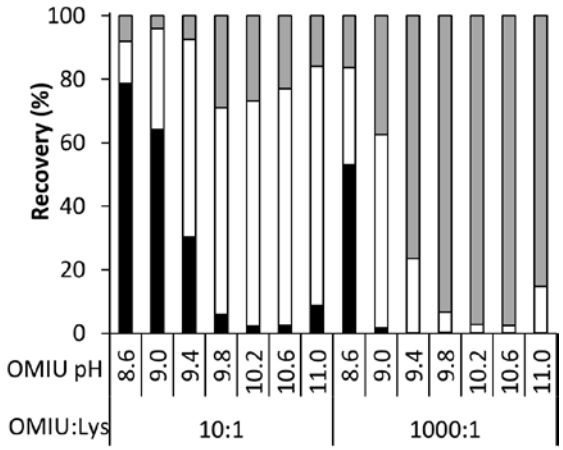


Figure 6.4. Recovery of crystalline L-Lys after the guanidination reaction using two O-methylisourea (OMIU) to free Lys ratios (10:1 or 1000:1), seven pH values (8.6-11.0 with 0.4 increments) of the OMIU solution, and a reaction time of 3 d. Black bars indicate unreacted Lys, white bars indicate homoarginine, and grey bars indicate non-recovered Lys.

is preferred for the guanidination of protein-bound Lys present in food/feed ingredients and diets, having only a free ε-amino group, in order to completely convert protein-bound Lys to homoarginine. However, if Lys with a free α-amino group, that is, crystalline L-Lys, free Lys or N-terminal Lys, is present in those protein sources or diets, then as the OMIU to Lys ratio is increased, the double derivatization of this Lys also appears to increase. Lowering the OMIU to Lys ratio to 1.5:1, however, resulted in a 51% recovery of homoarginine indicating that even at low OMIU to Lys ratios, it is still possible for OMIU to bind to the α-amino group of Lys. These results appear to be in contrast to those of Zhang et al. (2006) who reported a conversion of Lys to homoarginine of 99.5% for an OMIU to Lys ratio of 1.5:1. Conversion of Lys to homoarginine in the latter study, however, was calculated as the molar amount of homoarginine divided by the sum of the molar amounts of homoarginine and unreacted Lys. This manner of expressing conversions is often used (Maga, 1981; Rutherfurd and Moughan, 1990; Imbeah et al., 1996; Moughan and Rutherfurd, 1996; Ravindran et al., 1996; Zhang et al., 2006; Pahm et al., 2008) but does not take into account the conversion of Lys to double derivatized Lys or other Lys derivatives. When applying this equation to the data of the current study, conversions of > 90% were found (data not shown). This is in contrast with the low recovery of homoarginine that was actually observed in the present study. Thus, the conversion of Lys to homoarginine can appear to be high while actually a large proportion of Lys is in the double derivatized form. The low recovery of homoarginine could result in an underestimation of the reactive Lys content and subsequently an overestimation of Lys damage. When considering protein-bound Lys to be fully converted to homoarginine, the

underestimation of the reactive Lys content in food/feed ingredients and diets depends on the amount of Lys with a free α-amino group (free + N-terminal Lys).

The pH of the OMIU solution clearly affected the guanidination reaction. This reaction depends on the amino group being deprotonated (that is, pH > pKa) for the reaction with OMIU to occur (Beardsley and Reilly, 2002). The pKa of the ε-amino group of Lys is 10.6 and the recovery of homoarginine should be highest when the pH of the OMIU solution is greater than 10.6. The latter was also found in the current study (homoarginine recovery of 75%; Fig. 6.4). The pKa of the α-amino group of Lys is 9.0 and the recovery of unreacted Lys (that is, no binding of OMIU to either amino group) should be highest when the pH of the OMIU solution is smaller than 9.0. Again this was found in the current study (average unreacted Lys recovery of 70%; Fig. 6.4). The results are, however, not conclusive with regard to pH of the OMIU solution since homoarginine is also recovered at pH values smaller than 10.6. The effect of pH of the OMIU solution was clearly seen for an OMIU to Lys ratio of 10:1. For an OMIU to Lys ratio of 1000:1, the OMIU pH of 8.6 resulted in a high recovery of unreacted Lys (53%) and an OMIU pH of 9.0 in a high recovery of homoarginine (61%). The excess of OMIU for an OMIU pH greater than 9.0 apparently drove the reaction towards both amino groups, irrespective of protonation/ deprotonation. Several authors have reported different optimal pH values of the OMIU solution for different protein sources (Maga, 1981; Rutherfurd and Moughan, 1990; Moughan and Rutherfurd, 1996; Ravindran et al., 1996). The optimal pH for free Lys is approximately 10.6 (Fig. 6.4) but none of the pH values resulted in a 100% recovery of homoarginine.

To confirm the specificity of OMIU for the ε-amino group of crystalline L-Lys, the homoarginine content after incubation with OMIU should be equal to the level of Lys added to the reaction mixture (that is, complete recovery of Lys as homoarginine). Unfortunately, none of the combinations of reaction time with OMIU to Lys ratio and pH of the OMIU solution with OMIU to Lys ratio used in the present study resulted in specific binding of OMIU to the ε-amino group of crystalline L-Lys. The best reaction conditions (that is, maximal conversion of crystalline L-Lys to homoarginine and minimal conversion of crystalline L-Lys to double derivatized Lys) were reaction at pH 10.6 for 3 d with an OMIU to Lys ratio of 10:1 which resulted in a homoarginine recovery of 75%. It seems unlikely that the guanidination reaction for free + N-terminal Lys can be optimized to obtain complete conversion of Lys to homoarginine. Moreover, it is also unlikely that both protein-bound and free + N-terminal Lys can be measured using one set of reaction conditions.

6.3.3 Analysis of crystalline L-Lys HCl and a mixture of crystalline amino acids using optimized guanidination conditions

The optimized guanidination conditions (pH 10.6, OMIU to AA ratio of 10:1, and reaction time of 3 d) were applied to crystalline L-Lys HCl and a mixture of six AA (Arg, Phe, Val, Ile, Thr, and Gly) in order to test the reactivity of the α-amino groups of crystalline L-Lys HCl (a commercially available form of crystalline L-Lys often used as a supplement for pig and poultry diets) and six other AA under these guanidination conditions. An OMIU to AA ratio of 1000:1 was used since these conditions have been used previously to determine reactive Lys in food/feed ingredients. Incubating crystalline L-Lys HCl with OMIU resulted in a homoarginine recovery of 19.5 and 1.1% whereas the non-recoverable Lys was 79 and 98%, respectively, when the OMIU to Lys ratio was either 10:1 or 1000:1, respectively. The recovery of the other six other AA (Arg, Phe, Val, Ile, Thr and Gly) was also low (< 26 and < 38% for an OMIU to AA ratio of 10:1 and 1000:1, respectively) when incubated with OMIU as described above. Again, these results suggest that OMIU can bind to the free α-amino groups of not only crystalline L-Lys but also of crystalline L-Lys HCl and the free α-amino groups of crystalline AA other than Lys, irrespective of reaction conditions used.

6.3.4 Specificity of O-methylisourea

The results described above clearly demonstrate that OMIU can react with α-amino groups of AA in addition to the ε-amino group of Lys. Furthermore, none of the reaction conditions used in the present study resulted in the complete guanidination of the ε-amino group of Lys without guanidination of the α-amino group. Thus, it is unlikely that guanidination conditions can be optimized in the future to achieve specificity for Lys with a free α-amino group (free + N-terminal Lys). Previously, authors have reported the recovery of all AA after guanidination to approximate 100% for lysozyme, soy protein isolate, skim milk powder, lactic casein, whey protein concentrate, soy protein concentrate, blood meal, and cottonseed meal (Moughan and Rutherfurd, 1996). This suggests that the level of free + N-terminal Lys in these ingredients is low. The free Lys content in 44 different food/feed ingredients was compiled and found to range from 0 to 5.8% of total Lys, with an average of 1.3% (Table 6.1). Consequently, the underestimation of the OMIU-reactive Lys content for these food/feed ingredients is expected to be low. The estimates of the OMIU-reactive Lys content are expected to be inaccurate only in those cases where the test material contains a large proportion of free + N-terminal Lys. Materials for which OMIU-reactive Lys estimates could be inaccurate are materials that contain crystalline L-Lys (for example, practical pig and poultry diets, enteral nutrition formula, and specific pet foods), hydrolyzed products (for example, hydrolyzed feather

Table 6.1. Free Lys and total Lys[1] content (g/kg as-fed basis) and free Lys as percentage of total Lys in 44 different food/feed ingredients (adapted from Ajinomoto Eurolysine s.a.s., 2014)

Class	Food/feed ingredient	*n*	Free Lys content	Total Lys content	Free Lys as % of Lys
Cereals	Wheat	114	0.04	3.16	1.3
	Barley	64	0.04	3.80	1.1
	Corn	89	0.08	2.33	3.4
	Triticale	29	0.02	3.63	0.6
	Oats	4	0.03	4.91	0.6
	Rice	4	0.04	3.03	1.3
	Rye	4	0.03	3.26	0.9
	Sorghum	2	0.02	2.16	0.9
Cereal by-products	Wheat middlings & bran	23	0.09	5.94	1.5
	Wheat gluten	15	0.05	12.51	0.4
	Wheat gluten feed	4	0.07	5.48	1.3
	Wheat DDGS[2]	44	0.05	6.40	0.8
	Corn feed flour	2	0.13	4.05	3.2
	Corn gluten meal 60% CP	16	0.21	10.03	2.1
	Corn germ	2	0.48	8.26	5.8
	Corn DDGS[2]	6	0.06	7.47	0.8
	Rice protein	3	0.02	18.94	0.1
Vegetable protein sources	Soybean meal	132	0.14	28.41	0.5
	Full fat soybean	37	0.16	22.43	0.7
	Soy protein concentrate 52-56% CP	21	1.28	32.55	3.9
	Soy protein concentrate 65% CP	13	0.12	40.96	0.3
	Rapeseed meal	43	0.06	18.60	0.3
	Full fat rapeseed	2	0.07	12.16	0.6
	Sunflower meal 28% CP	14	0.18	10.13	1.8
	Sunflower meal 33% CP	9	0.12	11.54	1.0
	Sunflower meal 37% CP	7	0.26	13.82	1.9
	Palm kernel meal	3	0.00	3.75	0.0

	Faba bean	2	0.10	17.39	0.6
	Lupin seed	10	0.22	16.27	1.4
	Pea	22	0.12	14.78	0.8
	Potato protein concentrate	24	0.16	61.86	0.3
Dairy products	Milk	23	0.11	18.94	0.6
	Whey powder	71	0.09	9.89	0.9
	Whey protein concentrate	10	0.05	30.27	0.2
Miscellaneous	Fish meal	51	0.89	54.01	1.6
	Blood meal	2	0.02	79.02	0.0
	Feather meal	5	0.17	19.69	0.9
	Poultry protein	3	0.49	33.28	1.5
	Plasma	4	0.08	65.02	0.1
	Egg	5	0.92	45.64	2.0
	Cassava	2	0.03	0.95	3.2
	Brewers' yeast	7	1.11	27.36	4.1
	Bakery by-products	5	0.03	2.63	1.1

[1]Determined after acid hydrolysis in 6 *M* HCl at 110°C for 24 h.

[2]DDGS = distillers dried grains with solubles.

meal, hydrolyzed vegetable protein, infant formula, hypoallergenic diets) and potentially digesta obtained from the small intestine.

In order to determine the potential error involved in the measurement of reactive Lys in ileal digesta samples, 23 non-pooled and seven pooled ileal digesta samples from growing pigs and six pooled ileal digesta samples from broilers were analyzed for their free and total Lys content. The free Lys as a percentage of total Lys for two samples from growing pigs (one from a protein-free diet and the other from a SBM diet) were considered outliers (< mean – 2 × SD) and were, therefore, excluded from the data analysis. The mean (± SD) free and total Lys contents across the remaining 34 ileal digesta samples from growing pigs and broilers fed protein-free and protein-containing diets was 0.74 (± 0.39) and 5.74 (± 2.49) g/kg as-is, respectively. The free Lys, therefore, was on average 12.8% of the total Lys present in the ileal digesta. This amount was unexpectedly high considering that trypsin cleaves at the carboxyl terminal of Lys (Erickson and Kim, 1990). Multiple carrier transport systems are involved in the absorption of different AA and absorption rates differ between AA. For example, Thr and branched-chain AA (Leu, Ile, and Val) are rapidly absorbed while Lys and Arg are more slowly absorbed (Webb, 1990). Moreover, peptides use a different carrier transport system from that used by AA (Webb, 1990) and are absorbed more rapidly than free AA (Rerat, 1985). A slow absorption rate of Lys and a preference for the absorption of peptides might explain the relatively large amount of free Lys present in the ileal digesta. Of the Lys in ileal digesta collected from growing pigs fed a protein-free diet or a protein-containing diet, 13.0 or 12.7% was free Lys (Fig. 6.5A). Asche et al. (1989) reported that approximately 20% of proteinaceous material in the soluble fraction of ileal digesta of growing pigs fed a protein-free diet had a molecular weight less than 1,000 Da (considered to consist of free AA and small peptides) while for a corn-SBM diet the equivalent value was approximately 13%. Unfortunately, the individual free AA were not determined. Zebrowska et al. (1976) reported that endogenous proteins are absorbed at a slower rate compared to dietary proteins resulting in an increased concentration of endogenous proteins at the end of the ileum. The data of the current study, however, indicate that the presence of free Lys in ileal digesta is not related to the presence of protein-containing ingredients in the diet. Moreover, the free Lys as percentage of total Lys in ileal digesta of growing pigs fed SBM or RSM diets was independent (R^2 = 0.01, P = 0.71) of the AID CP content in the diet and of collection method (Fig. 6.5B). There appeared to be no difference in the free Lys as a percentage of total Lys between ileal digesta samples collected from growing pigs or broilers (12.7 and 14.4%, respectively; Fig. 6.5A) suggesting that the free Lys content in ileal digesta is not species specific. The amount of free Lys at the terminal ileum of growing pigs and broilers fed protein-containing diets, in the current study, was much higher than the 3.1%

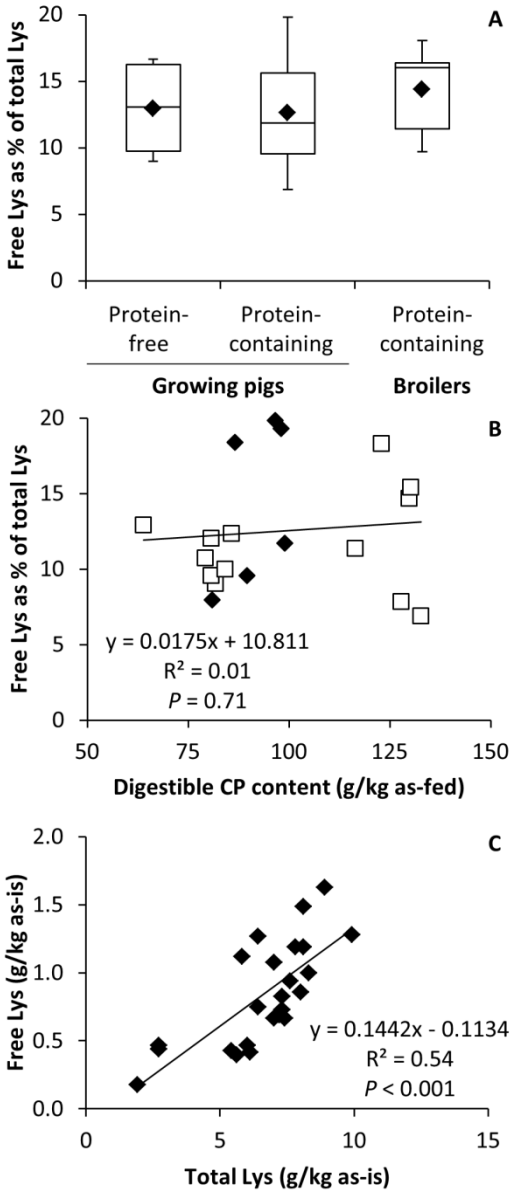


Figure 6.5. Free Lys as percentage of total Lys in ileal digesta samples from growing pigs and broilers fed protein-free or protein-containing diets (A; means are indicated by diamonds), the apparent ileal digestible CP content of the diet in relation to the free Lys as percentage of total Lys in ileal digesta samples collected using an ileal cannula (*n* = 13; open squares) or at slaughter (*n* = 6; closed diamonds) from growing pigs (B), and the free Lys content in relation to the total Lys content in ileal digesta of growing pigs fed protein-containing diets (C).

reported by Moughan and Schuttert (1991). This may be due to the relatively slower absorption of free Lys compared with peptides (Rerat, 1985) or spontaneous non-enzymatic breakdown of peptides due to their instability after hydrolysis (Butré et al., 2015). The thawing of fresh samples for subsampling might also have affected the level of free Lys in ileal digesta but this effect is expected to be low. Separating pig ileal digesta by centrifugation (14,500 relative centrifugal force for 30 min at 4°C) resulted in the separation of porcine and microbial cells (precipitate) from soluble proteins, peptides, free AA, and mucins (supernatant). Approximately half of the protein present in the supernatant was of microbial origin. While the microbial cells are most likely to be present in the precipitate, the supernatant might contain free Lys originating from lysed microbial cells (Miner-Williams et al., 2009). This source of free Lys might also have added to the free Lys content in pig ileal digesta analyzed in the current study. There was a linear relation between the total and free Lys contents in ileal digesta of growing pigs fed protein-containing diets (R^2 = 0.54, P < 0.001; Fig. 6.5C). Therefore, the methodology of determining free AA (method ISO 13903; ISO, 2005) which involves the use of 0.1 *M* HCl and co-extraction of nitrogenous macromolecules by sulfosalicylic acid may have hydrolyzed Lys from peptides or proteins, thereby, overestimating the free Lys content relative to that present in digesta at the terminal ileum. The latter may explain the lower value reported by Moughan and Schuttert (1991) as these authors used a different methodology to determine free AA in ileal digesta of pigs fed protein-free diets.

The impact of the non-specificity of OMIU and the free Lys content in ileal digesta on the SID of OMIU-reactive Lys was assessed using samples from a previous study (Hulshof et al., 2016). The SID was calculated considering supplemented dietary crystalline L-Lys HCl to be completely absorbed from the small intestine before the terminal ileum in growing pigs (Buraczewska et al., 1980). Moreover, it was assumed that all free Lys in ileal digesta was double derivatized and, therefore, not determined as OMIU-reactive Lys. For the SBM and RSM ingredients examined, the determined SID of OMIU-reactive Lys were 92.8 and 83.5%, respectively, and the SID OMIU-reactive Lys content was 5.6 and 4.2 g/100 g CP, respectively (Hulshof et al., 2016). The equivalent recalculated values for SBM and RSM considering all free Lys to be reactive but not analyzed by the guanidination reaction were 91.5 and 80.1%, respectively, and 5.5 and 4.0 g/100 g CP, respectively. Overall, the difference in SID of reactive Lys where the non-specific guanidination of free Lys was taken into account was small. The overestimation will be greater if ileal digesta contains a significant amount of peptides containing an N-terminal Lys residue.

In conclusion, OMIU was found to be not specific for the ε-amino group of crystalline L-Lys (HCl) and able to bind to the α-amino groups of crystalline AA. The various guanidination

conditions of the OMIU-reactive lysine assay investigated did not result in absolute specificity for the ε-amino group of Lys. It is recommended to analyze the reactive Lys content of food/feed ingredients, diets, and ileal digesta using an OMIU pH of 10.6, OMIU to Lys ratio of 1000:1 and a reaction time of at least 3 d to fully convert protein-bound Lys to homoarginine. These samples should subsequently be analyzed for their free Lys content to calculate the reactive Lys content of the samples (that is, assuming free Lys to be 100% reactive). The accurate quantification of free and N-terminal AA in ileal digesta warrants further investigation.

6.4 Acknowledgement

The authors gratefully acknowledge the financial support from the Wageningen UR "IPOP Customized Nutrition" program financed by Wageningen UR (Wageningen, the Netherlands), the Dutch Ministry of Economic Affairs (The Hague, the Netherlands), WIAS (Wageningen, the Netherlands), Agrifirm Innovation Center (Apeldoorn, the Netherlands), ORFFA Additives BV (Werkendam, the Netherlands), Ajinomoto Eurolysine s.a.s. (Paris, France), and Stichting VICTAM BV (Nijkerk, the Netherlands). The authors thank the laboratories of the Riddet Institute and the Institute of Food Nutrition and Human Health of Massey University, Palmerston North, New Zealand and the laboratory of Ajinomoto Eurolysine s.a.s. for their contributions to the analyses.

6.5 Literature cited

Abdollahi, M. R., A. B. J. van Dalen, W. H. Hendriks, and V. Ravindran. 2015. Influence of separate feeding of calcium on nutrient digestibility, energy utilisation and performance of young broilers fed pelleted wheat-based diets. Anim. Feed Sci. Technol. 205:122-130.

Ajinomoto Eurolysine s.a.s. 2014. Technical Bulletin 32: Formulator's handbook – Measuring and predicting amino acid contents in feedingstuffs. Paris, France.

Asche, G. L., A. J. Lewis, E. R. Peo. 1989. Protein digestion in weanling pigs: Effect of feeding regimen and endogenous protein secretion. J. Nutr. 119:1083-1092.

Beardsley, R. L., J. A. Karty, and J. P. Reilly. 2000. Enhancing the intensities of lysine-terminated tryptic peptide ions in matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun. Mass Spectrom. 14:2147-2153.

Beardsley, R. L., and J. P. Reilly. 2002. Optimization of guanidination procedures for MALDI mass mapping. Anal. Chem. 74:1884-1890.

Buraczewska, L., J. Lachowicz, and S. Buraczewski. 1980. The rate of absorption of synthetic lysine and dietary protein in the upper half of the small intestine of pigs. Arch. Tierernähr. 30:751-758.

Butré, C. I., S. Buhler, S. Sforza, H. Gruppen, and P. A. Wierenga. 2015. Spontaneous, non-enzymatic breakdown of peptides during enzymatic protein hydrolysis. Biochim. Biophys. Acta 1854:987-994.

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

De Vries, S., A. M. Pustjens, M. A. Kabel, R. P. Kwakkel, and W. J. J. Gerrits. 2014. Effects of processing technologies and pectolytic enzymes on degradability of nonstarch polysaccharides from rapeseed meal in broilers. Poult. Sci. 93:589-598.

Erickson, R. H., and Y. S. Kim. 1990. Digestion and absorption of dietary protein. Annu. Rev. Med. 41:133-139.

Finot, P. A. 1983. Influence of processing on the nutritional value of proteins. Qual. Plant. Plant Food Hum. Nutr. 32:439-453.

Finot, P. A. 2005. The absorption and metabolism of modified amino acids in processed foods. J. AOAC Int. 88:894-903.

Friedman, M. 1992. Dietary impact of food processing. Annu. Rev. Nutr. 12:119-137.

Greenstein, J. P. 1935. Studies on multivalent amino acids and peptides. II. The synthesis of certain derivatives of lysyl-glutamic acid. J. Biol. Chem. 109:541-544.

Hulshof, T. G., P. Bikker, A. F. B. van der Poel, and W. H. Hendriks. 2016. Assessment of protein quality of soybean meal and 00-rapeseed meal toasted in the presence of lignosulfonate by amino acid digestibility in growing pigs and Maillard reaction products. J. Anim. Sci. 94:1020-1030.

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

Imbeah, M., K. Angkanaporn, V. Ravindran, and W. L. Bryden. 1996. Investigations on the guanidination of lysine in proteins. J. Sci. Food Agric. 72:213-218.

International Organization for Standardization (ISO). 2005. ISO 13903:2005. Animal feeding stuffs - Determination of amino acids content. ISO, Geneva, Switzerland.

Kassell, B., and R. B. Chow. 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.

Keough, T., M. P. Lacey, and R. S. Youngquist. 2000. Derivatization procedures to facilitate *de novo* sequencing of lysine-terminated tryptic peptides using postsource decay matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun. Mass Spectrom. 14:2348-2356.

Kimmel, J. R. 1967. Guanidination of proteins. Methods Enzymol. 11:584-589.

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

Kwak, E.-J., and S.-I. Lim. 2004. The effect of sugar, amino acid, metal ion, and NaCl on model Maillard reaction under pH control. Amino Acids 27:85-90.

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

Miner-Williams, W., P. J. Moughan, and M. F. Fuller. 2009. Endogenous components of digesta protein from the terminal ileum of pigs fed a casein-based diet. J. Agric. Food Chem. 57:2072-2078.

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

Moughan, P. J., and S. M. Rutherfurd. 2008. Available lysine in foods: A brief historical overview. J. AOAC Int. 91:901-906.

Moughan, P. J., and G. Schuttert. 1991. Composition of nitrogen-containing fractions in digesta from the distal ileum of pigs fed a protein-free diet. J. Nutr. 121:1570-1574.

Pahm, A. A., C. Pedersen, and H. H. Stein. 2008. Application of the reactive lysine procedure to estimate lysine digestibility in distillers dried grains with solubles fed to growing pigs. J. Agric. Food Chem. 56:9441-9446.

Ravindran, V., M. Imbeah, K. Angkanaporn, and W. L. Bryden. 1996. Guanidination of lysine in cottonseed protein. J. Agric. Food Chem. 44:1812-1815.

Rerat, A. A. 1985. Intestinal absorption of end products from digestion of carbohydrates and proteins in the pig. Arch. Tierernähr. 35, 461-480.

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

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

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

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

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

Webb, K. E. 1990. Intestinal absorption of protein hydrolysis products: A review. J. Anim. Sci. 68, 3011-3022.

Zebrowska, T., O. Simon, R. Münchmeyer, and H. Bergner. 1976. Untersuchungen zur Sezernierung endogener Aminosäuren in den Verdauungstrakt und zur Aminosäurenresorption beim Schwein. Arch. Tierernähr. 26:69-82.

Zhang, H. L., D. F. Li, S. Y. Qiao, F. L. Wang, X. J. Chen, and P. A. Thacker. 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

7.1 Background and outline

The world population is expected to increase from 7.3 billion in 2015 to 11.2 billion in 2100 (UN, 2015). The expected increase in per capita income will result in an increased demand for livestock and dairy products (FAO, 2009). Protein-containing ingredients are needed to produce such products resulting in an increased demand for protein. However, arable land is limited (FAO, 2009) and there is competition between protein used for human food and for animal feed which ultimately could result in protein scarcity. To avoid the latter, it is necessary to use current ingredients more efficiently which includes the accurate assessment of the quality of the protein before inclusion in animal diets. Protein quality is defined in this thesis as the capacity of a dietary protein to meet an animal's requirements for N and AA. In the case of pigs and poultry, the requirement is for meeting a particular production target. Protein quality is influenced by processing applied to feed ingredients. Processing may lead to the formation of Maillard reaction products (MRP) or cross-link products which are known to hinder enzymatic hydrolysis in the gastrointestinal tract (Moughan et al., 1996) resulting in a decreased digestibility.

The results of the research chapters are integrated in this final chapter. The main aim was to evaluate the ileal digestible reactive Lys assay as a more accurate measure for protein quality of processed protein sources than the ileal digestible total Lys assay. Soybean meal (SBM) and rapeseed meal (RSM) were used as sole protein sources and a model processing treatment (toasting in the presence of lignosulfonate) was used to induce protein damage through the Maillard reaction. This chapter first compares the CP and amino acid (AA) digestibility of SBM and RSM in growing pigs (Chapters 2 and 3). Second, the suitability of the model processing treatment to study effects of processing on protein quality is discussed (Chapters 2, 3, 4, and 5). Third, problems encountered with the analysis of reactive Lys (being a criterion for protein quality) and the implications for the determination of reactive Lys digestibility are discussed (Chapter 2 and 6) using supporting data provided in textboxes. Fourth, the effects of processing on *in vivo* digestibility and post-absorptive utilization of AA for retention are reviewed (Chapter 2, 3, and 4) using data provided in Chapter 5. General conclusions and recommendations are provided at the end of this chapter.

7.2 Protein and amino acid digestibility of soybean meal and rapeseed meal

Soybean meal and RSM were used as sole protein sources throughout this thesis. In general, the CP content in SBM is higher than in RSM (46 vs. 34%, respectively) but the crude fiber, NDF, ADF, and ADL contents are higher in RSM (12, 25, 18 and 7%, respectively) than in SBM (6, 12, 7 and 5%, respectively; CVB, 2016). With regard to

indispensable AA, SBM has a relatively higher Arg and Lys content than RSM (7.5 and 6.2 g/100 g CP for SBM vs. 6.1 and 5.5 g/100 g CP for RSM, respectively) while RSM has a relatively higher sulfur-AA content than SBM (4.5 g/100 g CP for RSM vs. 2.9 g/100 g CP for SBM; CVB, 2016). These values are in accordance with the analyzed AA composition of the SBM and RSM used in this thesis (Chapter 2).

The standardized ileal digestibility (SID) of CP reported in Chapters 2 (Table 7.1) and 3 (87.2 and 71.0% for SBM and RSM, respectively) compare well with values reported in other studies (Table 7.1). The SID of indispensable AA reported in Chapter 2 also compare well with values reported in other studies (Table 7.1).

Table 7.1. Comparison of the standardized ileal digestibility (%) of CP and indispensable amino acids between studies with growing pigs fed soybean meal (SBM) and rapeseed meal (RSM) or canola meal (CM)

	Chapter 2		Jondreville et al., 2000		Stein et al., 2001		Eklund et al., 2012		CVB, 2016[1]	
Item	SBM	RSM	SBM	RSM	SBM	CM	SBM	RSM	SBM	RSM
CP	83.9	74.9	88.4	73.6	84.8	76.1	87	71	87	72
Arg	94.6	89.5	94.6	83.9	92.4	86.9	93	81	93	84
His	89.1	84.0	91.2	82.5	91.1	87.3	91	82	90	80
Ile	87.6	78.5	90.6	76.3	88.8	81.3	91	74	88	75
Leu	85.4	78.9	89.8	79.2	87.4	82.3	88	75	87	77
Lys	85.7	76.0	90.5	70.7	91.4	83.6	88	77	89	74
Met	89.5	87.3	93.0	86.0	89.7	86.7	ND	ND	90	81
Phe	87.1	80.8	90.9	81.4	88.5	82.6	90	76	89	77
Thr	83.4	74.8	86.8	73.2	85.4	78.5	85	70	85	71
Trp	84.2	78.2	89.4	74.8	90.1	88.7	ND	ND	88	71
Val	85.8	77.7	88.6	74.6	86.0	79.4	88	73	87	72

ND = not determined.

[1]Values are given for SBM containing crude fiber 45-70 g/kg as-is and CP > 450 g/kg as-is; RSM containing CP 290 to 370 g/kg as-is.

In general, the SID of CP and AA is lower for RSM than for SBM. This can largely be attributed to the higher fiber content in RSM compared with SBM. An increase in NDF (that is, hemicellulose, cellulose and lignin) content in the diet (Schulze et al., 1994) or ADF (that is, cellulose and lignin) content in SBM (Jondreville et al., 2000) resulted in an increase in endogenous AA losses. Moreover, pigs fed a RSM diet had a greater (total and specific) endogenous AA loss compared with pigs fed a SBM diet. However, the increased endogenous AA loss cannot fully explain the lower SID of CP and AA observed for RSM since the true ileal digestibility of CP and AA, that is, SID corrected for specific endogenous

AA losses, was also lower for RSM than for SBM (Jondreville et al., 2000). Fiber might also enclose N which, thereby, hinders the access of enzymes to peptide bonds resulting in a lower digestibility (Jondreville et al., 2000; Eklund et al., 2012). Moreover, the degree of hydrolysis after 120 min hydrolysis with trypsin, chymotrypsin and peptidase (pH-STAT method reported in Chapter 5) was higher for SBM than for RSM. This was attributed to the AA composition of both protein sources with soybean proteins having a higher amount of AA of which the peptide bonds are specifically cleaved by trypsin (that is, Lys and Arg) and chymotrypsin (that is, Trp, Tyr, and Phe) than rapeseed proteins (Chapter 5). Thus, differences in fiber content, amount of N enclosed by fiber, and AA composition between SBM and RSM result in differences in SID of CP and AA.

7.3 Model processing treatment

In this thesis, toasting in the presence of lignosulfonate was used as model processing treatment to study the effects of processing on protein quality. Toasting in the presence of lignosulfonate of SBM and RSM reduced the O-methylisourea (OMIU)-reactive and total Lys content, SID of all AA and CP, the apparent total tract digestibility (ATTD) of CP, pig growth performance as measured by the gain to feed ratio (Chapters 2 and 3), Lys content of protein in the empty body, and AA retention in the empty body (Chapter 4). Thus, the processing treatment was an effective model to reduce protein quality and to assess the *in vivo* effects of protein damage.

Soybean meal and RSM were processed as follows: (1) lignosulfonate was added to SBM or RSM, (2) the mixture was toasted in a seven-floor cascade toaster for 30 min at 95 ± 2°C, (3) the mixture was subsequently dried with hot air at 140°C for 10 min, and (4) cooled on a conveyor belt. This processing treatment is normally applied in ruminant nutrition to produce rumen by-pass protein. Addition of lignosulfonate to SBM (Windschitl and Stern, 1988; Harstad and Prestløkken, 2000), canola meal (Wright et al., 2005), or canola screenings (Von Keyserlingk et al., 2000) and subsequent heating reduced degradation of dietary protein and AA but also microbial protein synthesis in the rumen of dairy cows. Therefore, the amount of N entering the duodenum was similar between SBM and lignosulfonate-treated SBM. As a consequence of a reduced AA degradation, the amount of Lys entering the small intestine was higher for lignosulfonate treated SBM than for SBM but the intestinal absorption of Lys expressed as percentage of Lys entering the small intestine did not differ between treatments (Windschitl and Stern, 1988). The intestinal digestibility of rumen undegraded dietary N and AA (calculated from rumen undegraded dietary N and AA minus truly indigestible dietary N and AA) was approximately 97-98% for SBM and lignosulfonate-treated SBM. A 16-h rumen incubation numerically increased this intestinal digestibility to approximately 98-99% and was similar

between treatments (Harstad and Prestløkken, 2000; Prestløkken and Rise, 2003). The intestinal disappearance of CP for rumen incubated canola screenings (8-, 12-, or 16-h) was higher for lignosulfonate-treated samples compared with untreated samples while total tract disappearance of CP was not affected. The lack of effect of lignosulfonate-treatment on total tract disappearance was attributed to the absence of indigestible MRP which were apparently not formed during the lignosulfonate treatment (Von Keyserlingk et al., 2000). A slight reduction in intestinal disappearance was found for canola meal heated in the presence of lignosulfonate (Wright et al., 2005). Dairy cows, thus, can shift from protein degradation in the rumen to degradation in the small intestine when the amount of by-pass protein is high as a result of heating in the presence of lignosulfonate (Von Keyserlingk et al., 2000). The studies reported in this thesis are the first that applied this processing treatment to growing pigs. Applying this processing treatment to SBM and RSM significantly reduced the SID and ATTD of CP in growing pigs (Chapters 2 and 3). With regard to starch, calves had a lower level of digestive enzymes compared with pigs and starch mainly disappeared from the small intestine through bacterial fermentation rather than enzymatic digestion (Gilbert et al., 2015). Moreover, ruminal fermentation in dairy cows seemed to affect the lignosulfonate and heat-treated ingredient in such a way that it became accessible for bacterial fermentation in the small intestine. Therefore, differences in fermentation capacity in the small intestine might explain the observed species differences in digestibility of CP and AA from lignosulfonate-treated ingredients.

The main AA that were damaged by toasting in the presence of lignosulfonate were Arg, total Lys, and reactive Lys (Chapter 2). The loss of these AA was comparable to losses found after autoclaving of SBM or canola meal while extrusion and altered toasting conditions during desolventizing/toasting resulted in lower losses (Table 7.2). The greatest losses were found for reactive Lys irrespective of processing method.

The reactive- to total Lys ratio can be considered as measure for protein damage. The variation in this ratio within ingredients can be substantial, especially for ingredients that underwent processing such as distillers dried grains with solubles from corn or wheat (Fig. 7.1). The reactive- to total Lys ratio of SBM, processed SBM (pSBM), RSM, and processed RSM (pRSM) were 0.95, 0.80, 0.89, and 0.72, respectively (Chapter 2). The reactive- to total ratio of the SBM and RSM fell within the range observed in scientific literature (0.94-1.06 for SBM and 0.78-1.01 for RSM). The pSBM and pRSM, however, had a slightly lower reactive- to total Lys ratio than the minimum observed in scientific literature. However, the minimum reactive- to total Lys ratio of canola meal, being a cultivar of rapeseed, was 0.63 which was lower than the ratio for pRSM. There is a substantial risk for protein damage with current bio-refinery practices and it is, therefore, necessary to understand

the effects of such protein damage on the digestibility and post-absorptive utilization of AA in growing pigs. Protein damage is often quantified by analyzing the reactive Lys content using the guanidination reaction with OMIU.

Table 7.2. Loss of Arg, total Lys, and reactive Lys after processing for soybean meal (SBM), rapeseed meal (RSM), and canola meal (CM)

		Amino acid loss (%)			
Ingre-dient	Processing method	Arg	Total Lys	Reactive Lys	Reference
SBM	Toasting at 95°C for 30 min in the presence of lignosulfonate	12	27	38	Chapter 2[1]
	Autoclaving at 125°C for 15 min	3	7	8	González-Vega et al., 2011[2]
	Autoclaving at 125°C for 30 min	7	12	12	
	Autoclaving at 125°C for 60 min	10	14	ND	Almeida et al., 2014a
	Autoclaving at 135°C for 28 min	20	30	50	Kim et al., 2012b[1]
	Toasting at 500 kPa steam pressure, 45 min steam contact, at 115°C for 20 min	NA	2	7	Messerschmidt et al., 2012[3]
	Toasting at 850 kPa steam pressure, 7 min steam contact, at 139°C for 30 min	3	10	18	
	Extrusion at 113°C and 25% moisture	ND	NA	2	Hendriks et al., 1994[4]
	Extrusion at 93°C and 27% moisture	ND	NA	2	
	Extrusion at 143°C and 27% moisture	ND	4	11	
	Extrusion at 114°C and 30% moisture	ND	NA	4	
	Extrusion at 139°C and 30% moisture	ND	2	10	
	Extrusion at 93°C and 40% moisture	ND	NA	4	
	Extrusion at 135°C and 40% moisture	ND	3	14	
RSM	Toasting in the presence of lignosulfonate	14	23	38	Chapter 2[1]
CM	Autoclaving at 130°C for 20 min	9	16	21	Almeida et al., 2014b[2]
	Autoclaving at 130°C for 30 min	14	21	21	
	Autoclaving at 130°C for 45 min	23	26	32	

NA = not affected. ND = not determined.

[1]Reactive Lys by guanidination.

[2]Reactive Lys calculated from furosine as reactive Lys = Lys – 1.24 × furosine (Desrosiers et al., 1989).

[3]Compared with mild treatment conditions, that is, toasting at 150 kPa steam pressure, 34 min steam contact, at 105°C for 20 min. Reactive Lys method not specified.

[4]Reactive Lys by fluorodinitrobenzene method.

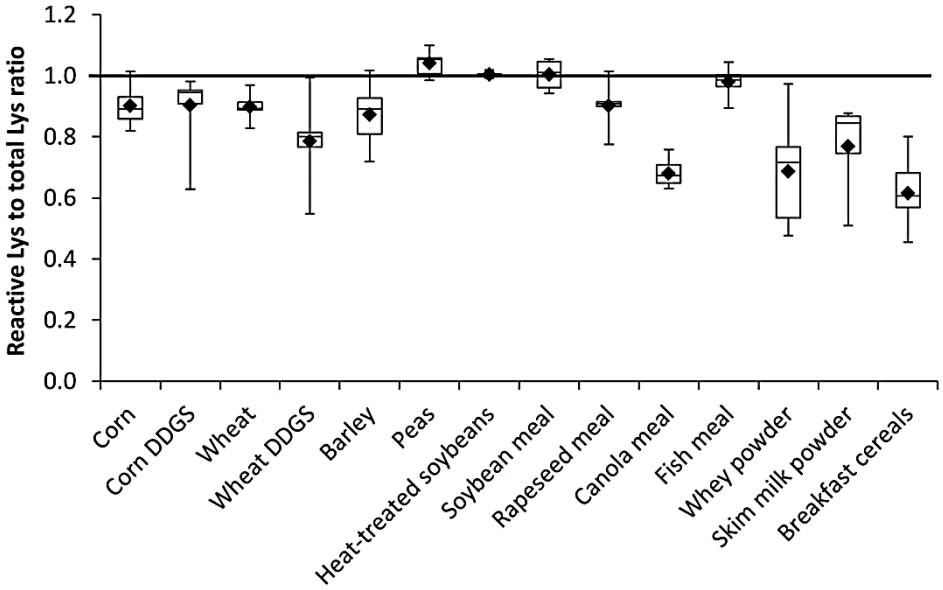


Figure 7.1. Boxplots of the reactive- to total Lys ratio for several food/feed ingredients (Van Barneveld, 2001; Torbatinejad et al., 2005; Cozannet et al., 2010; Kim et al., 2012a; Van der Poel and Bikker, unpublished data). DDGS = distillers dried grains with solubles. Means are indicated by diamonds.

7.4 Consequences of using the guanidination reaction to determine reactive Lys

In this thesis, the guanidination reaction with OMIU was used to analyze the reactive Lys content in the ingredients and experimental diets (Chapters 2 and 3). The serendipitous findings of Chapter 2 were further studied in Chapter 6. An overview of the guanidination reaction and its advantages and disadvantages are provided next.

The guanidination reaction with OMIU is frequently used to determine the reactive Lys content of ingredients, diets, and ileal digesta. This method is preferred over other methods, such as the fluorodinitrobenzene, trinitrobenzenesulfonic acid, and dye-binding methods, because OMIU was considered the only reagent being specific for the ε-amino group of Lys (Rutherfurd, 2015). This specificity was already reported in 1935 for lysylglutamic acid (Greenstein, 1935) and later confirmed in studies where the guanidination reaction with OMIU was used to study enzyme activity (Chervenka and Wilcox, 1956; Klee and Richards, 1957; Shields et al., 1959; Kassell and Chow, 1966). Moreover, OMIU is useful for peptide sequencing because it increases the mass of Lys making it better detectable in mass spectrometry spectra. While using OMIU for the latter

purpose, Keough et al. (2000) found that OMIU bound specifically to the ε-amino group of Lys.

In Chapter 2, the difference between total and OMIU-reactive Lys in the RSM and pRSM diets was much greater than in the corresponding protein sources. This was also observed for all experimental diets in Chapter 3. This finding might have been caused by (1) the presence of sugar-containing compounds in the diets which reacted with protein-bound or crystalline L-Lys HCl during pelleting (that is, heat treatment) or by (2) the presence of crystalline L-Lys HCl which was not measured as reactive Lys because of non-specificity of OMIU. The crystalline L-Lys HCl was not subjected to the processing treatment of heating in the presence of lignosulfonate. These two possible explanations were first investigated in a pilot study (Textbox 7.1). The results showed that for treatments containing crystalline L-Lys HCl, the OMIU-reactive- to total Lys ratio was lower than for treatments without crystalline L-Lys HCl. Moreover, the reactive Lys content in the diets was not affected by the pelleting process (data not shown) indicating that no additional reactions involving Lys had occurred during pelleting. As such, the lowered reactive- to total Lys ratio in treatments with crystalline L-Lys HCl had to be attributed to the binding of OMIU to the free α-amino group of crystalline L-Lys HCl. A free α-amino group is required for binding of the AA to the column during ion-exchange chromatography and for the AA to be derivatized by *o*-phthalaldehyde or ninhydrin after chromatographic separation. Beardsley et al. (2000) and Beardsley and Reilly (2002) reported that OMIU binds to free α-amino groups of Gly, Met, Ser, Val, Leu, Phe, Glu, and Ala. The binding of OMIU to α-amino groups of AA and, thereby, the non-specificity of OMIU to bind to the ε-amino group of Lys was investigated and confirmed in this thesis (Chapter 6).

One of the problems encountered in Chapter 6 was the calculation of AA recoveries using the recovery of the internal standard norleucine (NLE). In standard AA analysis, the recovery of NLE is used to correct for reagent stability. The recovery of NLE is calculated as the area under its peak, multiplied by its concentration in the sample divided by the area under its peak multiplied, by its concentration in the standard. The NLE recovery is usually around 100%. Textbox 7.2 presents the background of the problem encountered when calculating AA recoveries using the recovery of NLE and the experimental design and results of an experiment that was conducted to investigate this problem. The AA recoveries when calculated using the recovery of NLE gave erroneous results for samples containing only crystalline AA either with or without guanidination with OMIU. The low recovery of NLE for guanidinated samples can be explained by the binding of OMIU to the α-amino group of NLE which is a crystalline AA. Thus, all results presented in Chapter 6 were calculated without correction for the recovery of NLE. The concomitant inaccuracy

coming from reagent instability was considered smaller than the inaccuracy when results were calculated using the recovery of NLE and the inaccuracy was considered to be similar between samples. During standard hydrolysis used to analyze the AA content in ingredients, diets, and, ileal digesta, NLE is added after acid hydrolysis followed by drying down the acid under vacuum. The acidic environment might prevent the binding of OMIU (still present in the samples as a salt) to the free α-amino group of NLE. Problems with the recovery of NLE will, therefore, only occur when guanidination is not followed by acid hydrolysis.

Textbox 7.1. Effect of sugar compounds and crystalline L-Lys HCl on the reactive and total Lys content of soybean meal and rapeseed meal

Aims

To determine the effect of the presence of sugar compounds (that is, lignosulfonate, potato starch and dextrose) and crystalline L-Lys HCl in the diet on the reactive and total Lys content using soybean meal (SBM) and rapeseed meal (RSM).

Materials and methods

Six combinations of treatments were made for both SBM and RSM and three combinations were made for both pSBM and pRSM resulting in 18 samples in total (Table 7.3).

Table 7.3. Ingredients added to soybean meal (SBM), processed SBM (pSBM)[1], rapeseed meal (RSM), and processed RSM (pRSM)[1]

	Ingredient(s) added to the treatment		
Treatment	Crystalline L-Lys HCl	Lignosulfonate	Potato starch + dextrose
SBM/RSM1	X	X	
SBM/RSM2		X	
SBM/RSM3	X	X	X
SBM/RSM4	X		
SBM/RSM5			
SBM/RSM6	X		X
pSBM/pRSM1	X		
pSBM/pRSM2			
pSBM/pRSM3	X		X

[1]The pSBM and pRSM contain 7 and 5% (*wt*/*wt*) lignosulfonate and were toasted at 95°C for 30 min after addition of lignosulfonate.

The ingredients were combined, subsequently mixed, and ground to pass a 1-mm screen using a Retsch ZM 100 mill (Retsch GmbH, Haan, Germany). Lignosulfonate was air-heated

to 50°C to make it fluid before combining with the other ingredients. No further heat treatment was applied. Subsamples were analyzed for reactive Lys by guanidination with O-methylisourea (Moughan and Rutherfurd, 1996) and total Lys by acid hydrolysis at 110°C for 23 h followed by ion-exchange chromatography (method ISO 13903; ISO, 2005).

Results

The results of the reactive- to total Lys ratio per treatment are presented in Fig. 7.2. SBM/RSM5 only contained SBM or RSM and was, therefore, regarded as control treatment. The presence of lignosulfonate did not influence the reactive- to total Lys ratio (SBM/RSM2 vs. SBM/RSM5). The treatments containing crystalline L-Lys HCl (SBM/RSM1, 3, 4, and 6 and pSBM/pRSM1 and 3) had a lower reactive- to total Lys ratio than treatments without crystalline L-Lys HCl (SBM/RSM5 and pSBM/pRSM2). The presence of potato starch + dextrose did not affect the reactive- to total Lys ratio (SBM/RSM1, 4, and pSBM/pRSM1 vs. SBM/RSM3, 6, and pSBM/pRSM3, respectively).

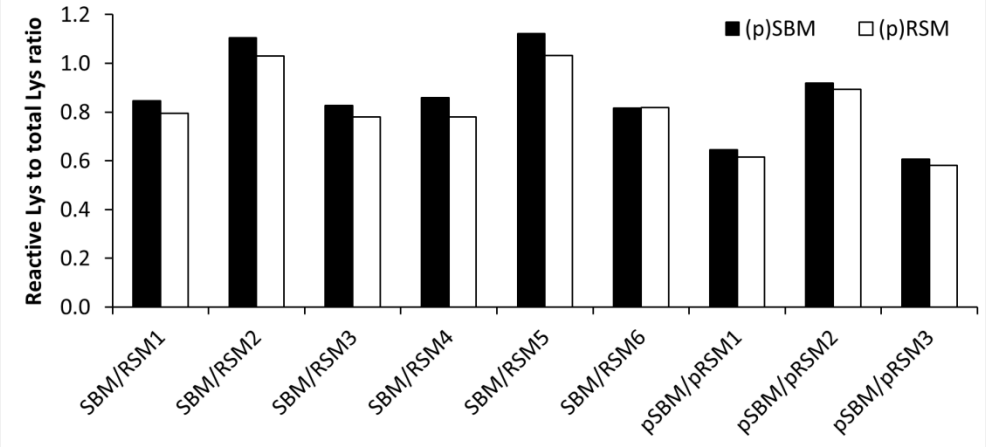


Figure 7.2. Reactive- to total Lys ratio for samples containing soybean meal (SBM), processed SBM (pSBM), rapeseed meal (RSM), or processed RSM (pRSM). The description of the different samples is given in Table 7.3.

Conclusions

The presence of crystalline L-Lys HCl reduced the reactive- to total Lys ratio while the presence of sugar compounds had no effect. The reactive Lys content determined by guanidination with O-methylisourea is underestimated when crystalline L-Lys is present.

Textbox 7.2. Norleucine recovery after incubation with O-methylisourea

Background

The norleucine (NLE) recovery in samples containing free AA was found to be highly variable (minimum of 7% and maximum of 267% with a mean ± SD of 105% ± 81) compared with the NLE recovery (84% ± 10) in lysozyme control samples. A NLE recovery of around 80% was within the normal range for the high-performance liquid chromatography analyses used (L. Turnbull, personal communication). It is unclear what the cause of the high (> 100%) norleucine recoveries is in samples containing only crystalline AA.

Norleucine is a crystalline AA and, therefore, the low NLE recovery might have been caused by the binding of O-methylisourea (OMIU) to the α-amino group of NLE. Norleucine was added before the drying of the OMIU solution. As such, OMIU might have reacted with the α-amino group of NLE in the time that the samples were dried down under vacuum.

Aim

To determine the recovery of NLE after incubation with OMIU when NLE is added before or after drying of the OMIU solution and to determine the effect of NLE recovery on the recovery of crystalline L-Lys and crystalline L-Ile.

Materials and methods

Eight tubes containing crystalline L-Lys (n = 4) or crystalline L-Ile (n = 4) were incubated in a 0.6 *M* OMIU solution for 3 d in a shaker at room temperature and an OMIU to crystalline AA ratio of 1000:1. To two tubes per AA, 240 µL of NLE (2.5 m*M*) was added directly after OMIU incubation and the solution was subsequently dried under vacuum (Savant SpeedVac Concentrator SPD131DDA, Thermo Scientific, Asheville, NC, US). The other two tubes per AA were first dried under vacuum followed by addition of 240 µL of NLE and subsequently a second drying under vacuum. Two additional tubes per AA that were not incubated with OMIU were included. These were used to calculate the expected molar amount of homoarginine and Ile and subsequently their recovery after OMIU incubation. Control samples (rapeseed, peas, and field peas) were included to check if the OMIU solution was properly made and to verify that NLE recovery was not affected by the high-performance liquid chromatography system. Control samples were first incubated with OMIU followed by drying the solution under vacuum. The samples were then hydrolyzed in 6 *M* HCl at 110°C for 23 h followed by the addition of NLE and drying the acid under vacuum. All samples were analyzed using ion-exchange chromatography with post-column derivatization with ninhydrin.

Results

The NLE recovery and the conversion of Lys to homoarginine in the control samples were on average 97 and 93%, respectively. This indicated that the OMIU solution was properly prepared and that the NLE recovery was not affected by the high-performance liquid chromatography system. Thus, a NLE recovery around 100% could be expected for other samples as well. The NLE recovery of samples containing crystalline L-Lys and crystalline L-Ile without OMIU incubation was 101% for both crystalline L-Lys and crystalline L-Ile. The Lys and Ile recoveries in the samples not incubated with OMIU were calculated based on weight of the sample and molecular mass. These recoveries were approximately 100% irrespective of correcting for NLE recovery since the latter was 101%.

The NLE recovery was slightly higher for samples to which norleucine was added after drying of the OMIU solution but this recovery was still well below 100% (Table 7.4). Correcting the data for NLE recovery resulted in a too high homoarginine recovery (1919 and 712% for adding NLE before and after drying). The Ile recovery calculated with corrected data (29 and 13% for adding NLE before and after drying) is, therefore, considered false as well.

Table 7.4. Norleucine (NLE) recovery and recovery of homoarginine and Ile[1] in samples incubated with O-methylisourea

Free AA	Time of adding norleucine[2]	Norleucine recovery (%)	Homoarginine/Ile recovery, uncorrected (%)[3]	Homoarginine/Ile recovery, corrected (%)[4]
Lys	Before	2.6	51	1919
Lys	After	8.2	58	712
Ile	Before	3.1	1	29
Ile	After	5.7	1	13

[1]Average values of two tubes.

[2]Norleucine was added before or after drying of the O-methylisourea solution under vacuum.

[3]Calculated using expected contents calculated from non-incubated samples not corrected for NLE recovery.

[4]Calculated using expected contents calculated from non-incubated samples corrected for NLE recovery.

Conclusions

The recovery of NLE was affected by the time at which it was added to the sample (that is, before or after drying of the OMIU solution). Correcting homoarginine and Ile recoveries for NLE recovery resulted in erroneously high values. The NLE recovery was close to 100% in control samples to which NLE was added under acidic conditions and for crystalline L-Lys and crystalline L-Ile not incubated with OMIU. It seems that the binding of OMIU to the α-amino group of NLE is influenced by the pH of the solution to which the NLE is added.

There is a need to accurately determine reactive Lys since it is a better measure for bioavailable Lys than total Lys. During acid hydrolysis using 6 *M* HCl at 110°C for 23 h, Lys, furosine, and pyridosine are recovered from Amadori compounds which are produced during the early Maillard reaction (Erbersdobler and Somoza, 2007). This reversion of Amadori compounds, however, does not occur in the animal and subsequently Amadori products are considered not bioavailable as Lys in the pig (Rérat et al., 2002). The analysis of reactive Lys is, therefore, preferred above total Lys. Reactive Lys can be analyzed by several methods such as the fluorodinitrobenzene method, dye-binding method, or furosine method. Fluorodinitrobenzene reacts with free ε-amino groups of Lys which yields the yellow compound dinitrophenyl Lys upon acid hydrolysis. Dinitrophenyl Lys can subsequently be measured colorimetrically. When samples contain 20-30% starch, there is a loss of dinitrophenyl Lys during acid hydrolysis which necessitates the use of correction factors to accurately determine reactive Lys (Hurrell and Carpenter, 1981). Dye-binding methods depend upon the uptake of dye (for example, Acid Orange 12) from a solution because of binding of the dye to basic amino groups (that is, Lys, Arg, and His). The reactive Lys content is calculated from the difference between samples to which the dye was added with or without prior propionylation (blocks the basic amino group of Lys). However, inconsistent results were found with the dye-binding method (Hendriks et al., 1994). Furosine is produced during acid hydrolysis from deoxyketosyllysine or fructoselysine (early MRP). Reactive Lys is subsequently calculated from the total Lys and furosine content using fixed conversion factors (32% furosine and 40% reverted Lys formed after acid hydrolysis). The recovery of furosine and reverted Lys after acid hydrolysis is, however, only quantified for milk protein and were reported to be inconsistent (Erbersdobler and Somoza, 2007). Thus, using fixed conversion factors for the calculation of reactive Lys could give inaccurate results. Despite the inaccuracy of the guanidination reaction for samples containing Lys with a free α-amino group (that is, crystalline L-Lys, free Lys, or N-terminal Lys), it is still preferred for the analysis of reactive Lys in ingredients because the method is relatively straightforward, the homoarginine formed by guanidination is acid stable, it does not rely upon conversion factors (Rutherfurd, 2015), and it does not involve vesicant chemicals. The major drawback is the long reaction time needed (at least 3 d) for full conversion of protein-bound Lys to homoarginine.

The reactive Lys content in diets containing free Lys either from crystalline L-Lys HCl or from feed ingredients can be estimated as follows. The reactive Lys content coming from protein-bound Lys can be obtained by guanidination of a diet sample using an OMIU to Lys ratio of 1000:1, a reaction time of at least 3 d, and an OMIU pH of 10.6. By using these conditions, all Lys with a free α-amino group can be considered to be double derivatized

and not to be analyzed as reactive Lys. Full conversion of reactive Lys to homoarginine can be verified by integrating the peaks for (unreacted) Lys and homoarginine. The free Lys content in the diet sample should be analyzed separately and added to the reactive Lys content coming from protein-bound Lys to calculate the total amount of reactive Lys in the diet sample. The free Lys content is generally determined after extraction with 0.1 *M* HCl and precipitation of co-extracted nitrogenous molecules. This is an international standard method (method ISO 13903; ISO, 2005) and it is highly unlikely that the 0.1 *M* HCl results in hydrolysis of Lys from protein. However, the linear relation between the free and total Lys content in ileal digesta reported in Chapter 6 suggests that some hydrolysis of Lys from proteins might occur during free Lys analysis. The proposed method of calculating reactive Lys in diets by guanidinating protein-bound Lys and analyzing free Lys cannot be applied to diets that contain ingredients with a (potentially) high amount of N-terminal Lys from other sources than free Lys, such as hydrolyzed products. Moreover, reactive Lys is now separately analyzed from total AA. When both are analyzed in the same guanidinated sample, the presence of free α-amino groups coming from other AA would result in underestimations of those AA as well.

Approximately 13% of the Lys in ileal digesta was present as free Lys irrespective of protein source in the diet, collection method, or monogastric species (Chapter 6). When considering this Lys not to be measured as OMIU-reactive Lys, the SID of OMIU-reactive Lys reported in Chapter 2 is overestimated by 1 to 6 percentage points (Textbox 7.3). Especially the overestimation of 6 percentage points can have substantial effects on pig growth performance when diets are formulated on SID reactive Lys content. Reactive Lys in ileal digesta can best be determined by analyzing the reactive Lys content by guanidination using pH 10.6, an excess of OMIU (such as 1000:1 but this requires the Lys content in ileal digesta to be known beforehand), and a reaction time of 7 d. The free Lys content can be approximated to be 13%. The analyzed OMIU-reactive Lys content in ileal digesta should subsequently be corrected for the percentage of free Lys.

Despite the drawbacks of the guanidination reaction, the model processing method enabled me to determine the effects of processing on ileal digestibility and post-absorptive utilization of AA in growing pigs.

Textbox 7.3. Influence of free Lys content in ileal digesta on standardized ileal digestibility of O-methylisourea reactive Lys

Aim

To determine the influence of free Lys content in ileal digesta on the standardized ileal digestibility (SID) of reactive Lys analyzed by guanidination with O-methylisourea (OMIU).

Materials and methods

Ileal digesta samples of Chapter 2 were analyzed for free Lys content according to ISO 13903 (ISO, 2005). Ileal digesta samples were extracted using 0.1 *M* HCl followed by precipitation of co-extracted nitrogenous macromolecules by sulfosalicylic acid and subsequent centrifugation. The supernatant was filtered and its pH adjusted to 2.2. The AA were separated using ion-exchange chromatography employing post-column derivatization with ninhydrin. The apparent ileal digestibility (AID) and SID of OMIU-reactive Lys were calculated as (Stein et al., 2007):

AID of OMIU-reactive Lys = 100 – ([(OMIU-reactive $Lys_{digesta}$ + free $Lys_{digesta}$) / OMIU-reactive Lys_{diet}] × [Cr_{diet} / $Cr_{digesta}$] × 100)

and

SID of OMIU-reactive Lys = AID of OMIU-reactive Lys + [(basal endogenous Lys / OMIU-reactive Lys_{diet}) × 100],

in which OMIU-reactive $Lys_{digesta}$, OMIU-reactive Lys_{diet}, and free $Lys_{digesta}$ are the OMIU-reactive Lys content (g/kg DM) in the ileal digesta and diet, and the free Lys content (g/kg DM) in ileal digesta, respectively; Cr_{diet} and $Cr_{digesta}$ are the analyzed Cr contents (mg/kg DM) in the diet and ileal digesta, respectively; and basal endogenous Lys is the basal endogenous loss (in g/kg DM intake) of Lys (Jansman et al., 2002). Basal endogenous Lys is determined using acid hydrolysis and was considered to include free and reactive Lys. The SID can only be calculated when assuming free and reactive Lys to be in similar proportions in basal endogenous losses and in the rest of the ileal digesta. Since approximately 13% of Lys was free Lys both for pigs fed a protein-free diet (basal endogenous Lys) and for protein-containing diets (Chapter 6), this assumption seems valid. The OMIU-reactive Lys content in the diet was considered to exclude crystalline L-Lys HCl. To determine the SID of OMIU-reactive Lys from the protein sources in diets supplemented with crystalline L-Lys HCl, the crystalline L-Lys HCl was considered to be 100% digested.

Results

The SID of OMIU-reactive Lys was overestimated by 1.3 to 6.0 percentage points because free Lys is not analyzed as reactive Lys (Table 7.5). The SID OMIU-reactive Lys content was overestimated by 1.8 to 9.5%.

Table 7.5. Standardized ileal digestibility (SID) of O-methylisourea (OMIU)-reactive Lys (%) and SID OMIU-reactive Lys content (g/100 g CP) of soybean meal (SBM), processed SBM (pSBM), rapeseed meal (RSM), and processed RSM (pRSM) ingredients[1]

Item	SBM	pSBM	RSM	pRSM
OMIU-reactive Lys content in ingredient, g/100 g CP[2]	6.0	3.7	5.0	3.1
SID excluding free Lys in digesta[3], %	92.8	80.7	83.5	67.7
SID including free Lys in digesta[4], %	91.5	77.7	80.1	61.7
SID content, excluding free Lys in digesta[2], g/100 g CP	5.6	3.0	4.2	2.1
SID content, including free Lys in digesta, g/100 g CP	5.5	2.9	4.0	1.9

[1]Processing consisted of addition of lignosulfonate to SBM and RSM and secondary toasting at 95°C for 30 min.
[2]Chapter 2.
[3]Considering crystalline L-Lys HCl in the RSM and pRSM diets to be 100% digestible (Chapter 2).
[4]Free Lys content in digesta added to OMIU-reactive Lys content in digesta for calculating SID.

Conclusions

The SID of reactive Lys and SID reactive Lys content for all four protein sources were overestimated when reactive Lys was determined by the guanidination reaction with OMIU because of the presence of Lys with a free α-amino group.

7.5 Digestibility and post-absorptive utilization of protein and amino acids

In this thesis, the *in vivo* digestibility and post-absorptive utilization of AA for retention in growing pigs were determined (Chapters 2, 3, and 4). The *in vivo* digestibility of CP for the SBM, pSBM, RSM, and pRSM presented in Chapter 2 was correlated to *in vitro* protein digestibility which was measured using two different assays (Chapter 5).

7.5.1 Processing effects on in vivo *digestibility and post-absorptive utilization*

Processing reduced the digestibility of CP and AA (Chapters 2 and 3) which might be mediated through effects on solubility. The N solubility in the feed ingredients was reduced by the processing treatment (Chapter 3) which might be explained by conformational changes in protein structure occurring upon heating such as hydrophobic aggregation (Zheng et al., 1998). Nitrogen solubility can be measured by the protein dispersibility index or by the N solubility index. These two indices were developed for the determination of over-processing of soy products. Distilled water and 0.2% KOH are used for the extraction of N in the protein dispersibility index (AOCS, 2009b) and the N solubility index (AOCS, 2009a), respectively. The indices are calculated as the percentage of CP (N × 6.25) or N in the original sample being present in the supernatant after centrifugation.

Nitrogen solubility of SBM (Araba and Dale, 1990; Parsons et al., 1991) and soy flakes (Batal et al., 2000) reflected the duration of autoclaving and the growth response of chicks and pigs fed SBM or soy flakes autoclaved for different durations. Since an animal's growth response depends on the digestibility of the diet, it was hypothesized that the reduced digestibility reported in Chapters 2 and 3 for pSBM and pRSM could have been caused by a reduced N solubility in the ileal digesta. In Chapter 3, the insoluble N as a fraction of total N in digesta collected along the small intestine was determined after centrifugation of fresh digesta samples and removal of the supernatant. It was considered that the soluble and insoluble N could be separated by this method. If processing would reduce N solubility in digesta, a greater proportion of N would be present in the insoluble fraction. However, processing was found to have no effect on the insoluble N as fraction of total N along the small intestine. This fraction increased irrespective of diet as digesta passed towards the end of the ileum indicating that absorption of soluble N mainly occurred between the end of the second and third small intestinal segment (Chapter 3). A relatively greater amount of soluble peptides was present in the 0.4 to > 20 kDa fraction for pSBM/pRSM compared with SBM/RSM after 120 min hydrolysis with trypsin, chymotrypsin, and peptidase (Chapter 5). Therefore, the greater size of the peptides in the pSBM and pRSM might have hindered the absorption of soluble N and, thereby, decreased digestibility. The peptides are larger after processing because of a reduced action of pancreatic and brush border enzymes (Hansen and Millington, 1979; Öste et al., 1986) and because of the formation of peptide aggregates (Fischer et al., 2007). Moreover, the weight of the pancreas was lower for the pSBM/pRSM suggesting a lower enzyme secretion compared with the SBM/RSM (Chapter 3). Thus, the reduced hydrolysis of dietary protein seems to be the rate limiting step for digestion and absorption of processed feed ingredients.

Processing mainly affected pig growth performance by affecting digestibility since supplementing the pSBM and pRSM diets with crystalline AA resulted in similar gain to feed ratios (Chapter 3) and N and AA retentions (Chapter 4) as for the SBM and RSM diets. Different results were found for cottonseed meal, meat-and-bone meal (Batterham et al., 1990), and additionally heat-treated fish meal and field peas (Wiseman et al., 1991; Van Barneveld et al., 1994). For these feed ingredients, Lys digestibility overestimated Lys bioavailability resulting in reduced growth performance. For processed feed ingredients, reactive Lys is considered to be bioavailable for the pig while total Lys is not fully bioavailable as discussed above. Standardized ileal digestible reactive Lys is, therefore, a better measure for Lys bioavailability than SID total Lys. The SID of total Lys overestimated SID of reactive Lys for a range of feed ingredients including wheat, dried maize, heated skim milk powder, and cottonseed meal (Rutherfurd and Moughan, 2007). However, in

this thesis, there was no difference between the SID total and reactive Lys contents within the SBM, pSBM, RSM, and pRSM (Chapter 2). Thus, digestibility of total Lys did not overestimate Lys bioavailability for these protein sources. This explains why formulating a diet containing pSBM or pRSM and supplementing with crystalline L-Lys HCl to similar SID total Lys content as in the SBM or RSM diet resulted in similar growth performance, and N and AA retentions (Chapters 3 and 4). There is no indication that MRP from these protein sources were digested and absorbed and subsequently were hindering the post-absorptive utilization of N and AA for retention.

The SID reactive Lys content changed when taking into account that free Lys in ileal digesta was not analyzed as reactive Lys by the guanidination method (Textbox 7.3). For pRSM, the SID total Lys content overestimated the SID reactive Lys content while this was not the case for the other three protein sources. The recalculated SID of reactive Lys reported in Textbox 7.3 were used to recalculate the post-absorptive utilization of reactive Lys. The values were 74.3, 60.6, 69.0, 56.9, 62.8, and 66.0% for the SBM, pSBM, pSBM+AA, RSM, pRSM, and pRSM+AA diets, respectively, which are 1.0 to 2.2 percentage points higher than the values reported in Chapter 4. The overestimation of the SID of reactive Lys is greater when peptides with a free N-terminal are present in ileal digesta next to the presence of free Lys. This overestimation of the SID of reactive Lys will result in a greater underestimation of the post-absorptive utilization of reactive Lys. However, the proportion of N-terminal Lys is considered to be small because trypsin cleaves at the carboxyl terminal of Lys (Erickson and Kim, 1990). The latter should be verified by assessing the level of N-terminal Lys in ileal digesta.

7.5.2 In vitro *digestibility*

Two *in vitro* digestibility methods were assessed as alternatives for *in vivo* digestibility of protein using SBM, pSBM, RSM, and pRSM as protein source. The first method was the 2-step enzymatic method according to Boisen and Fernández (1995) in which the *in vivo* digestion is mimicked by incubating a sample with pepsin at pH 2 for 2 h (stomach phase) followed by incubation with pancreatin at pH 6.8 for 4 h (small intestinal phase). Pancreatin includes the enzymes trypsin, amylase, and lipase produced by the porcine pancreas. Boisen and Fernándes (1995) reported a high correlation (r = 0.96) between the 2-step enzymatic method and *in vivo* apparent ileal digestibility (AID) of CP as determined using 15 feed ingredients (wheat, rye, oats, SBM, RSM, sunflower meal, grass meal, peas, skim milk powder, barley grits, barley meal and 4 batches of barley). The resulting regression equation was applied to 48 feed mixtures but the relationship between predicted and observed values for the AID of CP was relatively low (r = 0.75). This was attributed to the presence of endogenous protein in the measurement of AID. The *in vitro*

digestibility of CP was closely related to the *in vitro* digestibility of most individual AA except for Cys, Asp, Glu, and Pro. There was, however, variation in the *in vivo* digestibility of individual AA between feed ingredients (Boisen and Fernández, 1995). Cone and Van der Poel (1993) reported a linear relationship between the *in vitro* (using the 2-step enzymatic method) and *in vivo* AID of CP for wheat but not for peas, rapeseeds and soybeans. Peas and soybeans contain anti-nutritional factors that increase endogenous secretions *in vivo* which does not occur in an *in vitro* system. In general, the 2-step enzymatic method overestimated AID because the *in vitro* measurements exclude endogenous losses and microbial protein. Moreover, the *in vitro* method assumes that all soluble N is absorbed while this is not the case *in vivo* (Cone and Van der Poel, 1993). The 2-step enzymatic method correlated to the SID of CP reported in Chapters 2 (Chapter 5) and 3 (r = 0.96, *P* = 0.042) indicating that the 2-step enzymatic method might predict the effects of processing on the SID of CP.

The second method was the pH-STAT method of Pedersen and Eggum (1983) in which the amount of cleaved peptide bonds (that is, degree of hydrolysis) is calculated from the amount of NaOH added to maintain pH. The degree of hydrolysis after 10 min (DH10) was found to have the highest correlation (r = 0.94) with true fecal CP digestibility in rats and was, therefore, used as criterion for *in vitro* digestibility. Applying the pH-STAT method for 10 min resulted in high correlations (Pedersen and Eggum, 1983) between *in vitro* and *in vivo* digestibility of CP for animal proteins (r = 0.98), plant proteins (r = 0.95), and all tested protein sources (r = 0.96). The DH10 was positively correlated to SID of CP reported in Chapters 2 (Chapter 5) and 3 (r = 0.90, *P* = 0.098). The degree of hydrolysis after 120 min (DH120) was used to determine the maximum degree of hydrolysis of the protein sources. The maximum degree of hydrolysis was higher for SBM/pSBM than for RSM/pRSM irrespective of processing and was not correlated to SID of CP reported in Chapter 2 (Chapter 5) and 3 (*P* = 0.381). The rate of protein hydrolysis (*k*) was calculated from the DH curve and is a measure for the speed of hydrolysis. The rate of protein hydrolysis tended to correlate with SID of CP reported in Chapter 2 (Chapter 5) but did not correlate with SID of CP reported in Chapter 3 (*P* = 0.148). Thus, several *in vitro* digestibility measurements correlated or tended to correlate with *in vivo* digestibility of CP for SBM and RSM and for both processed protein sources. The DH10 and rate of protein hydrolysis also correlated to N retention reported in Chapter 4 (r = 0.99, *P* = 0.015 and r = 0.97, *P* = 0.027, respectively). None of the *in vitro* parameters (*in vitro* digestibility of protein by the 2-step enzymatic method, DH10, and *k*) correlated with post-absorptive utilization of N for retention reported in Chapter 4. It was, however, expected that processing would affect protein digestibility but would have no effect on the post-absorptive utilization of N (Chapter 4). Therefore, *in vitro* digestibility was not expected to correlate with *in vivo* N

utilization. Since, DH10 and rate of protein hydrolysis correlated with SID of CP and N retention, the pH-STAT method might be a suitable alternative for the *in vivo* analysis of CP digestibility and N retention of processed feed ingredients. However, care should be taken to interpret these correlations because only four feed ingredients were examined.

7.6 General conclusions and recommendations

The processing method used in this thesis, being toasting of SBM and RSM in the presence of the sugar-rich lignosulfonate, proved to be effective to study the effects of processing on digestibility and post-absorptive utilization of AA for retention. However, additional processing methods would be valuable to further study the initial aim: to evaluate the ileal digestible reactive Lys assay as a more accurate measure for protein quality of processed protein sources than the ileal digestible total Lys assay. These processing methods should result in a difference between the SID total and reactive Lys contents in order to verify that the ileal digestible reactive Lys assay is a more accurate measure for protein quality than the ileal digestible total Lys assay. Moreover, a difference between the SID total and reactive Lys contents is needed to study the effect of the absorption of MRP on post-absorptive AA utilization.

The presence of Lys with a free α-amino group, either as crystalline L-Lys HCl, free Lys or N-terminal Lys in diets and ileal digesta, respectively, resulted in inaccurate estimates of their reactive Lys content when using the guanidination reaction with OMIU. This subsequently resulted in inaccurate estimations of the ileal reactive Lys digestibility and post-absorptive utilization of reactive Lys for retention. For diets containing free Lys (either from crystalline L-Lys HCl or from feed ingredients), the reactive Lys content can be determined by the guanidination reaction (OMIU to Lys ratio of at least 1000:1, > 3 d incubation time, at pH 10.6) and the analysis of free Lys. This method cannot be applied for diets containing Lys with a free α-amino group coming from other sources than from free Lys. For ileal digesta, the level of free Lys appeared to be stable between protein sources, collection methods, and monogastric species. The level of N-terminal Lys present in peptides is currently unknown but might have an influence on the reactive Lys content in ileal digesta when determined by the guanidination method.

A reduction in N solubility could not explain the reduction in ileal CP and AA digestibility seen for the processed protein sources. Future studies should focus on the size of the peptides present in the soluble and insoluble fraction of ileal digesta to understand better the mechanism behind the reduced digestibility as a consequence of thermal processing.

The 2-step enzymatic method and pH-STAT method were found to be promising alternatives for the assessment of protein digestibility of processed protein sources. However, these methods should be validated for multiple protein sources with varying protein damage before they can be applied in practice. A possible alternative for the assessment of reactive Lys might be the furosine method. This method currently relies upon conversion factors determined in milk samples which might not apply to plant-based protein sources. Therefore, conversion factors for plant-based protein sources should first be determined before the furosine method can be applied in practice as alternative for the guanidination method.

7.7 Literature cited

Almeida, F. N., J. K. Htoo, J. Thomson, and H. H. Stein. 2014a. Effects of balancing crystalline amino acids in diets containing heat-damaged soybean meal or distillers dried grains with solubles fed to weanling pigs. Animal 8:1594-1602.

Almeida, F. N., J. K. Htoo, J. Thomson, and H. H. Stein. 2014b. Effects of heat treatment on the apparent and standardized ileal digestibility of amino acids in canola meal fed to growing pigs. Anim. Feed Sci. Technol. 187:44-52.

American Oil Chemists Society (AOCS). 2009a. Nitrogen Solubility Index Official Method Ba 11-65. AOCS, Champaign, Illinois.

American Oil Chemists Society (AOCS). 2009b. Protein Dispersibility Index Official Method Ba 10a-05. AOCS, Champaign, Illinois.

Araba, M., and N. M. Dale. 1990. Evaluation of protein solubility as an indicator of overprocessing soybean meal. Poult. Sci. 69:76-83.

Batal, A. B., M. W. Douglas, A. E. Engram, and C. M. Parsons. 2000. Protein dispersibility index as an indicator of adequately processed soybean meal. Poult. Sci. 79:1592-1596.

Batterham, E. S., L. M. Andersen, D. R. Baigent, S. A. Beech, and R. Elliot. 1990. Utilization of ileal digestible amino acids by pigs: Lysine. Brit. J. Nutr. 64: 679-690.

Beardsley, R. L., J. A. Karty, and J. P. Reilly. 2000. Enhancing the intensities of lysine-terminated tryptic peptide ions in matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun. Mass Spectrom. 14:2147-2153.

Beardsley, R. L., and J. P. Reilly. 2002. Optimization of guanidination procedures for MALDI mass mapping. Anal. Chem. 74:1884-1890.

Boisen, S., and J. A. Fernández. 1995. Prediction of the apparent ileal digestibility of protein and amino acids in feedstuffs and feed mixtures for pigs by *in vitro* analyses. Anim. Feed Sci. Technol. 51:29-43.

Centraal Veevoeder Bureau (CVB). 2016. Chemical compositions and nutritional values of feed ingredients. CVB, Wageningen, the Netherlands.

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

Cone, J. W., and A. F. B. van der Poel. 1993. Prediction of apparent ileal protein digestibility in pigs with a two-step *in-vitro* method. J. Sci. Food Agric. 62:393-400.

Cozannet, P., Y. Primot, C. Gady, J. P. Métayer, P. Callu, M. Lessire, F. Skiba, and J. Noblet. 2010. Ileal digestibility of amino acids in wheat distillers dried grains with solubles for pigs. Anim. Feed Sci. Technol. 158:177-186.

Desrosiers, T., L. Savoie, G. Bergeron, and G. Parent. 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.

Eklund, M., W. R. Caine, W. C. Sauer, G. S. Huang, G. Diebold, M. Schollenberger, and R. Mosenthin. 2012. True and standardized ileal digestibilities and specific ileal endogenous recoveries of crude protein and amino acid in soybean meal, rapeseed meal and peas fed to growing pigs. Livest. Sci. 145:174-182.

Erbersdobler, H. F., and V. Somoza. 2007. Forty years of furosine – forty years of using Maillard reaction products as indicators of the nutritional quality of foods. Mol. Nutr. Food Res. 51:423-430.

Erickson, R. H., and Y. S. Kim. 1990. Digestion and absorption of dietary protein. Annu. Rev. Med. 41:133-139.

Fischer, M., A. G. J. Voragen, S. R. Piersma, L. V. Kofod, C. I. Jörgensen, P. Guggenbuhl, C. S. Nunes, and H. Gruppen. 2007. Presence of indigestible peptide aggregates of soybean meal in pig ileal digesta residue. J. Sci. Food Agric. 87:2229-2238.

Food and Agricultural Organization of the United Nations (FAO). 2009. Global Agriculture Towards 2050. Proceedings of 'High Level Expert Forum – How to feed the world in 2050'. Rome, Italy, October 12-13, 2009.

Gilbert, M. S., A. J. Pantophlet, H. Berends, A. M. Pluschke, J. J. G. C. van den Borne, W. H. Hendriks, H. A. Schols, and W. J. J. Gerrits. 2015. Fermentation in the small intestine contributes substantially to intestinal starch disappearance in calves. J. Nutr. 145:1147-1155.

González-Vega, J. C., B. G. Kim, J. K. Htoo, A. Lemme, and H. H. Stein. 2011. Amino acid digestibility in heated soybean meal fed to growing pigs. J. Anim. Sci. 89:3617-3625.

Greenstein, J. P. 1935. Studies on multivalent amino acids and peptides. II. The synthesis of certain derivatives of lysyl-glutamic acid. J. Biol. Chem. 109:541-544.

Hansen, L. P., and R. J. Millington. 1979. Blockage of protein enzymatic digestion (carboxypeptidase-B) by heat-induced sugar-lysine reactions. J. Food Sci. 44:1173-1177.

Harstad, O. M., and E. Prestløkken. 2000. Effective rumen degradability and intestinal indigestibility of individual amino acids in solvent-extracted soybean meal (SBM) and xylose-treated SBM (SoyPass®) determined in situ. Anim. Feed Sci. Technol. 83:31-47.

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

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

International Organization for Standardization (ISO). 2005. ISO 13903:2005. Animal feeding stuffs – Determination of amino acids content. ISO, Geneva, Switzerland.

Jansman, A. J. M., W. Smink, P. van Leeuwen, and M. Rademacher. 2002. Evaluation through literature data of the amount and amino acid composition of basal endogenous crude protein at the terminal ileum of pigs. Anim. Feed Sci. Technol. 98:49-60.

Jondreville, C., J. van den Broecke, F. Gâtel, F. Grosjean, S. van Cauwenberghe, and B. Sève. 2000. Ileal amino acid digestibility and estimates of endogenous amino acid losses in pigs fed rapeseed meal, sunflower meal and soybean meal. Can. J. Anim. Sci. 80:495-506.

Kassell, B., and R. B. Chow. 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.

Keough, T., M. P. Lacey, and R. S. Youngquist. 2000. Derivatization procedures to facilitate *de novo* sequencing of lysine-terminated tryptic peptides using postsource decay matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun. Mass Spectrom. 14:2348-2356.

Kim, B. G., D. Y. Kil, Y. Zhang, and H. H. Stein. 2012a. Concentrations of analyzed or reactive lysine, but not crude protein, may predict the concentration of digestible lysine in distillers dried grains with solubles fed to pigs. J. Anim. Sci. 90:3798-3808.

Kim, J. C., B. P. Mullan, and J. R. Pluske. 2012b. Prediction of apparent, standardized, and true ileal digestible total and reactive lysine contents in heat-damaged soybean meal samples. J. Anim. Sci. 90:137-139.

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

Messerschmidt, U., M. Eklund, V. T. S. Rist, P. Rosenfelder, H. K. Spindler, J. K. Htoo, and R. Mosenthin. 2012. Effect of particle size and heat treatment of soybean meal on standardized ileal digestibility of amino acids in growing pigs. J. Anim. Sci. 90(Suppl. 4):119-121.

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

Öste, R. E., A. Dahlqvist, H. Sjöström, O. Norén, and R. Miller. 1986. Effect of Maillard reaction products on protein digestion. *In vitro* studies. J. Agric. Food Chem. 34:355-358.

Parsons, C. M., K. Hashimoto, K. J. Wedekind, and D. H. Baker. 1991. Soybean protein solubility in potassium hydroxide: An *in vitro* test of *in vivo* protein quality. J. Anim. Sci. 69:2918-2924.

Pedersen, B., and B. O. Eggum. 1983. Prediction of protein digestibility by an *in vitro* enzymatic pH-stat procedure. Z. Tierphysiol. Tierernähr. Futtermittelkd. 49:265-277.

Prestløkken, E., and O. Rise. 2003. Protein and amino acid digestibility in dairy cows measured with mobile nylon bags recovered in ileum or in faeces. Acta Agric. Scand., Sect. A 53:11-20.

Rérat, A., R. Calmes, P. Vaissade, and P. A. Finot. 2002. Nutritional and metabolic consequences of the early Maillard reaction of heat treated milk in the pig. Significance for man. Eur. J. Nutr. 41:1-11.

Rutherfurd, S. M. 2015. Use of the guanidination reaction for determining reactive lysine, bioavailable lysine and gut endogenous lysine. Amino Acids 47:1805-1815.

Rutherfurd, S. M., and P. J. Moughan. 2007. Development of a novel bioassay for determining the available lysine contents of foods and feedstuffs. Nutr. Res. Rev. 20:3-16.

Schulze, H., P. van Leeuwen, M. W. A. Verstegen, J. Huisman, W. B. Souffrant, and F. Ahrens. 1994. Effect of level of dietary neutral detergent fiber on ileal apparent digestibility and ileal nitrogen losses in pigs. J. Anim. Sci. 72:2362-2368.

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

Stein, H. H., M. F. Fuller, P. J. Moughan, B. Sève, R. Mosenthin, A. J. M. Jansman, J. A. Fernández, and C. F. M. de Lange. 2007. Definition of apparent, true, and standardized ileal digestibility of amino acids in pigs. Livest. Sci. 109:282-285.

Stein, H. H., S. W. Kim, T. T. Nielsen, and R. A. Easter. 2001. Standardized ileal protein and amino acid digestibility by growing pigs and sows. J. Anim. Sci. 79:2113-2122.

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

United Nations (UN). 2015. World Population Prospects: The 2015 Revision, Key Findings and Advance Tables. United Nations, Department of Economic and Social Affairs, Population Division. Working Paper No. ESA/P/WP.241.

Van Barneveld, R. J. 2001. Pig Research Report – Prediction of reactive lysine in heat-processed feed ingredients used in pig diets. South Australian Research and Development Institute, Adelaide, Australia.

Van Barneveld, R. J., E. S. Batterham, and B. W. Norton. 1994. The effect of heat on amino acids for growing pigs. 2. Utilization of ileal-digestible lysine from heat-treated field peas (*Pisum sativum* cultivar Dundale). Brit. J. Nutr. 72:243-256.

Von Keyserlingk, M. A. G., E. Weurding, M. L. Swift, C. F. Wright, J. A. Shelford, and L. J. Fisher. 2000. Effect of adding lignosulfonate and heat to canola screenings on ruminal and intestinal disappearance of dry matter and crude protein. Can. J. Anim. Sci. 80:215-219.

Windschitl, P. M., and M. D. Stern. 1988. Evaluation of calcium lignosulfonate-treated soybean meal as a source of rumen protected protein for dairy cattle. J. Dairy Sci. 71:3310-3322.

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

Wright, C. F., M. A. G. Von Keyserlingk, M. L. Swift, L. J. Fisher, J. A. Shelford, and N. E. Dinn. 2005. Heat- and lignosulfonate-treated canola meal as a source of ruminal undegradable protein for lactating dairy cows. J. Dairy Sci. 88:238-243.

Zheng, G. H., O. Fasina, F. W. Sosulski, and R. T. Tyler. 1998. Nitrogen solubility of cereals and legumes subjected to micronization. J. Agric. Food Chem. 46:4150-4157.

List of abbreviations

0-pigs	Pigs belonging to the initial slaughter group
AA	Amino acid(s)
ADFI	Average daily feed intake
ADG	Average daily gain
AEE	Acid-hydrolyzed ether extract
AID	Apparent ileal digestibility / Apparent ileal digestible
Ala	Alanine
AOCS	American Oil Chemists Society
ARC	Agricultural Research Council
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATTD	Apparent total tract digestibility
AUC	Area under the curve
BW	Body weight
$BW^{0.75}$	Metabolic body weight
CML	Carboxymethyllysine
CP	Crude protein
Cr_2O_3	Chromic oxide
CVB	Centraal Veevoeder Bureau
Cys	Cysteine
Da	Dalton
DDGS	Distillers dried grains with solubles
DH10	Degree of hydrolysis after 10 min (pH-STAT method)
DH120	Degree of hydrolysis after 120 min (pH-STAT method)
DM	Dry matter
DMI	Dry matter intake
EBW	Empty body weight
Eq.	Equation
FAO	Food and Agricultural Organization of the United Nations
FL	Fructoselysine
G:F	Gain to feed ratio
Gln	Glutamine
GIT	Gastrointestinal tract
GLM	General linear model
Glu	Glutamic acid
Gly	Glycine
His	Histidine

HMF	Hydroxymethylfurfural
Ile	Isoleucine
INRA	Institut National de la Recherche Agronomique (France)
ISO	Internation Organization for Standardization
k	Rate of protein hydrolysis (pH-STAT method)
LAL	Lysinoalanine
Leu	Leucine
Lys	Lysine
Met	Methionine
MRP	Maillard reaction product(s)
MW	Molecular weight
N	Nitrogen
NA	Not affected
ND	Not determined
NLE	Norleucine
NS	Not significant
NSI	Nitrogen solubility index
NSP	Non-starch polysaccharides
OM	Organic matter
OMIU	O-methylisourea
Phe	Phenylalanine
Pro	Proline
pRSM	Processed rapeseed meal
pSBM	Processed soybean meal
RSM	Rapeseed meal
SBM	Soybean meal
SD	Standard deviation
SEC	Size exclusion chromatography
SEM	Standard error of the mean
Ser	Serine
SID	Standardized ileal digestibility / Standardized ileal digestible
Thr	Threonine
TID	True ileal digestibility / True ileal digestible
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

Summary

The increasing world population and per capita income imposes a risk for protein scarcity. It is, therefore, necessary to use current ingredients more efficiently which includes the accurate assessment of protein quality before inclusion in animal diets. Protein quality is defined in this thesis as the capacity of a dietary protein to meet a pig's requirement for nitrogen (N) and amino acids (AA) to meet a particular production target. Protein quality is influenced by processing applied to feed ingredients which may lead to the formation of Maillard reaction products (MRP) or cross-link products. The Maillard and cross-link reactions mainly involve lysine (Lys) and their products may decrease ileal crude protein (CP) digestibility. During the acid hydrolysis step used to analyze AA, part of the early MRP revert back to Lys. This reverted Lys is not bioavailable for animals. Therefore, methods that specifically analyze Lys with a free ε-amino group (that is, not bound to other nutrients) have been developed. The guanidination reaction with O-methylisourea (OMIU) is one such method. The initial aim of this thesis was to evaluate the ileal digestible reactive Lys assay as a more accurate measure for protein quality of processed protein sources than the ileal digestible total Lys assay. Soybean meal (SBM) and rapeseed meal (RSM) were used as sole protein sources throughout this thesis. Processing of SBM and RSM by toasting at 95°C for 30 min in the presence of a sugar-rich lignosulfonate was used as model for over-processed protein sources.

Digestibility, post-absorptive utilization, and pig growth performance

In Chapter 2, protein quality in processed protein sources was determined using the content of AA, OMIU-reactive Lys, MRP, and lysinoalanine (LAL; as cross-link product), the standardized ileal digestibility (SID) of AA and OMIU-reactive Lys, and pig growth performance. The SBM and RSM diets contained furosine and carboxymethyllysine (CML) as MRP, and LAL indicating that Maillard and cross-link reactions had taken place in SBM and RSM, presumably during the oil extraction/desolventizing process. The amounts of furosine, CML, and LAL were elevated in the pSBM and pRSM diets due to further processing. Processing resulted in a reduction in total and OMIU-reactive Lys contents, a decreased pig growth performance as determined by the gain to feed ratio (G:F), and the SID of CP, AA, and OMIU-reactive Lys. The SID AA contents of the protein sources from Chapter 2 were used to formulate the diets of the main *in vivo* experiment (Chapters 3 and 4). In this experiment, six experimental diets were used of which four contained either SBM, pSBM, RSM, or pRSM as sole protein source. The remaining two experimental diets contained pSBM or pRSM and were supplemented with crystalline AA to the same SID AA levels as the SBM or RSM diet. These supplemented diets were used to verify that processing affected AA digestibility rather than post-absorptive AA utilization. The effects of processing on CP digestibility and N solubilization along the small intestine, metabolic load as assessed by organ weight, and nutrient composition of the empty body of growing

pigs are described in Chapter 3. The small intestine was divided in three segments of similar length and digesta was collected from the last 100 cm of each segment. The amount of insoluble N as a fraction of N in digesta at each small intestinal segment was not affected by processing. Thus, the reduced SID of CP and AA reported in Chapter 2 was not caused by a reduced N solubility but by a general increase of N in digesta. Processing reduced the SID of CP, CP content in the empty body, and G:F. Supplementing crystalline AA to diets containing pSBM or pRSM increased the CP content and G:F to the level of the SBM and RSM diets. Processing also reduced the weight of several organs and supplementing crystalline AA restored organ weight. The effects of processing on whole body AA composition, nutrient retention, and post-absorptive utilization of AA in growing pigs are described in Chapter 4. Post-absorptive AA utilization was calculated as percentage of SID AA intake used for AA retention. Processing affected the AA composition of protein in the organ fraction (that is, empty organs and blood), carcass, and empty body. The Lys concentration in body protein was mainly reduced by processing. Supplementing crystalline AA restored the AA composition of body protein for SBM and RSM. Processing reduced AA retention and again supplementing crystalline AA restored AA retention for both SBM and RSM. Since crystalline AA were supplemented on an SID AA basis, the results indicated that processing affected AA digestibility but not post-absorptive AA utilization. Thus, correcting AA retention for SID AA intake would result in a similar post-absorptive AA utilization which was found for most AA for the RSM diets. However, the post-absorptive AA utilization was lower for the pSBM diet than for the SBM diet which might be related to an imbalanced AA supply after absorption in the first diet.

The assessment of ileal digestibility and utilization is expensive and laborious. Therefore, two alternative *in vitro* methods for determining protein digestibility for processed protein sources were evaluated (Chapter 5). The protein digestibility determined using the pH-STAT method and a 2-step enzymatic method was compared with the *in vivo* SID of CP reported in Chapter 2. Initial pH and the degree of hydrolysis assessed in the pH-STAT method were positively correlated the SID of CP. Protein digestibility determined with the 2-step enzymatic method, simulating digestion in the stomach and small intestine, tended to correlate to SID of CP. Both the 2-step enzymatic method and pH-STAT method were suitable alternatives for the assessment of SID of CP. However, only four ingredients were tested. The suitability of the methods should be further studied using multiple (processed) feed ingredients before they can be used as alternatives for *in vivo* assays.

Reactive Lys analysis

O-methylisourea was reported to bind specifically to the ε-amino group of Lys. The results of Chapter 2, however, cast doubt on the specificity of OMIU to react only with the ε-

amino group of Lys. A series of experiments was conducted to study this specificity (Chapter 6). Incubating crystalline L-Lys with OMIU under standard conditions (OMIU pH of 10.6, OMIU to AA ratio of 1000:1, and reaction time of 7 d) resulted in a low homoarginine (that is, Lys with OMIU bound to its ε-amino group) recovery. The reaction of OMIU with the α-amino group of Lys was confirmed by mass spectrometry analysis with double derivatized Lys being identified. Several reaction conditions (OMIU pH, OMIU to Lys ratio, and reaction time) were studied but none of these resulted in 100% recovery of homoarginine. Binding of OMIU to the α-amino group of Lys could result in an underestimation of the reactive Lys content when significant levels of Lys with a free α-amino group (that is, crystalline L-Lys (HCl), free and N-terminal Lys) are present in food/feed ingredients, diets, and ileal digesta. The free Lys content in food/feed ingredients was on average 1.3% of total Lys. The free Lys content can be substantial in certain diets and was reported to be 13% of total Lys in ileal digesta. The latter might result in an overestimation of OMIU-reactive Lys digestibility. The reaction of OMIU with α-amino groups may necessitate analysis of free Lys to accurately quantify reactive lysine in samples containing a large proportion of Lys with a free α-amino group.

The results presented in this thesis indicate that the effects of processing on SID of CP and AA, body composition, nutrient retention, post-absorptive AA utilization, and growth performance could be substantial. These effects should, therefore, be taken into account when using processed feed ingredients in diets for growing pigs. The extent of protein damage in feed ingredients can be assessed by the analysis of OMIU-reactive and total Lys, MRP, and cross-link products. However, OMIU-reactive Lys only provides accurate results when samples contain small levels of Lys with a free α-amino group (that is, crystalline L-Lys (HCl), free and N-terminal Lys). When samples contain significant levels of Lys with a free α-amino group, it is recommended to use standard guanidination conditions (OMIU pH of 10.6, OMIU to AA ratio of 1000:1, and reaction time of 7 d) to convert protein-bound Lys to homoarginine and to separately analyze such samples for free Lys.

Acknowledgements

Na (bijna) 4 jaar is het zover: ik mag mijn dankwoord schrijven. Heel cliché, maar 4 jaar PhD zijn voorbij gevlogen en niet geheel zonder hulp van anderen. Er zijn veel mensen die mij de afgelopen jaren op één of ander moment hebben geholpen met het één en ander.
Allereerst wil ik mijn promotor en co-promotoren bedanken. Wouter, dank je voor je kennis, altijd kritische blik op artikelen en enthousiasme over de serendipitous finding. Het eerste artikel was een lastige met name door een aantal aanpassingen van de scope net wanneer ik dacht dat het klaar was. Uiteindelijk is het een mooi en innovatief artikel geworden mede dankzij jou. Ik wil je ook bedanken voor de kans om een deel van mijn PhD in Nieuw-Zeeland uit te voeren. De ervaringen in Nieuw-Zeeland zowel werk gerelateerd als op sociaal gebied hebben mij geholpen om mezelf verder te ontwikkelen.
Paul, jouw kennis over vertering en benutting in het varken heeft mij geholpen om de data uit de varkensproef beter te begrijpen. We hebben een heel aantal keer bij elkaar gezeten om de resultaten en daaruit volgende berekeningen uit te pluizen met als conclusie dat we niet alles dicht kunnen rekenen. Dat hoort uiteindelijk ook bij onderzoek en geeft handvaten om verder te gaan. Tijdens mijn tweede dissectiedag kwam ik handen tekort maar gelukkig sprong jij bij om te helpen, dank daarvoor.
Thomas, waarvoor kon ik eigenlijk niet bij je terecht? Dank je voor je feedback op artikelen en steun bij het begeleiden van studenten. Ook dank je voor je oplettendheid toen ik in Nieuw-Zeeland zat.
Shane, thank you for being my supervisor at the Riddet Institute. I learned a lot from your experience with the guanidination reaction. I would like to thank Maggie Zou, Janiene Gilliland and Leiza Turnbull for their help at the lab at the Riddet Institute and NutLab. Sorry, that my tubies occupied a waterbath for a considerable amount of time.
Paul, Thomas en Ruud Dekker, hartelijk dank voor het opzetten en uitvoeren van de eerste proef. Dankzij deze proef kon ik beginnen met een vliegende start.
Een aantal mensen hebben mij geholpen met het opzetten en uitvoeren van mijn varkensproef. Martin Verstegen, dank je voor proofreading van het proefplan en later het brainstormen over de orgaangewicht resultaten. Hans van Diepen, dank je voor je hulp bij het formuleren van de voeders. De proef had niet plaats kunnen vinden zonder de hulp van het personeel van de dierfaciliteit Carus. Ries Verkerk, Ronald Wormgoor en met name Rinie Ernste, hartelijk dank voor jullie hulp. Ik wil ook graag Pieter Roskam bedanken voor zijn hulp tijdens de eerste dissectiedag en zijn uitleg over het nemen van monsters.
The experiment ended with quite some dissection days and I would like to thank Bayissa, Geronda, Henk, Hsuan, Myrthe, Sandra, Sanne, and Sergio for their help during these dissection days. Tamme, dank je voor je hulp tijdens de dissectiedagen en het verwerken van de karkassen. De cutter dagen waren lang, maar op een gegeven moment hadden we een goed ritme te pakken. Sorry nog voor de vlek op het plafond in de cutterkamer.

Betty en Yvon, jullie zijn de belangrijke stille krachten achter ANU. Dank jullie voor het regelen van de importvergunning, vliegtickets, declaraties, congresregistraties, printen van de thesis en nog veel meer maar ook voor jullie luisterend oor.
Deze thesis had niet geschreven kunnen worden zonder de inzet van het ANU lab. Leon, dank je voor je hulp met berekeningen met name van de oplosbare/onoplosbare fase en het doorspreken van analyses. Saskia, dank je voor al het werk dat je hebt gedaan om analyses mogelijk te maken en voor je vertrouwen dat ik aminozuur analyses zelfstandig kon uitvoeren. Michel, dank je voor al je hulp bij de aminozuur analyses en het fungeren als vraagbaak terwijl ik aan de andere kant van de wereld zat. Dank aan Jane-Martine, Xuan Huong en Erika voor jullie hulp bij het analyseren van mijn monsters.
Tijdens mijn PhD heb ik samengewerkt met de Levensmiddelenchemie groep van Wageningen Universiteit. Harry Gruppen, bedankt voor je input bij de opzet van de varkensproef en de interesse die je toonde door aanwezig te zijn tijdens een dissectiedag. Peter Wierenga and Stefano Sforza, thank you for your help with the MS analysis of the guanidinated lysine. We knew the double derivatized lysine was there, but thanks to you we could prove it.
I would like to thank Maarten Boerrigter, Jan Wiebrand Dijkstra (BSc students), Catalin Nicolaiciuc, Yihan Wang, Yufei Wang, and Hangtian Jia (MSc students) for their contribution to this PhD thesis and for letting me gain experience with supervising students.
Naast al het werk, was er voldoende tijd voor plezier! De koffiepauzes, playbackshows, borrels, teambuilding activiteiten en kerstbrunches zorgen voor een fijne, informele sfeer binnen de groep wat ik als zeer prettig heb ervaren. Thanks to all (former) PhD students at the Animal Nutrition Group for their interest. I wish you all the best in your (future) career!
Sergio, our projects both belonged to the IPOP Customized Nutrition Research line 1. You have helped me so many times during my PhD: grinding, N analysis with Dumas, collecting samples on dissection days, and performing pH-STAT, a 2-step *in vitro* incubation, and PDI and NSI analysis on my protein sources. You are always willing to help even when you are really busy yourself. Thank you for the nice chats and it was great working with you! Hsuan and Soumya, you also worked in the IPOP Customized Nutrition project but in a different research line. Thank you for the nice discussions about proteins, solubility, digestion kinetics, and –omics. Sergio, Hsuan, and Soumya, we might even going to miss the stamppot buffet after the IPOP meetings ;-)
Geronda, Marijke, Myrthe, Sandra, Sanne, Sonja en Yvonne, dank jullie voor alle momenten, van lunchwandelingen tot borrels en etentjes, die we samen hebben meegemaakt. Sandra en Yvonne, het was leuk om met jullie te winkelen en te squashen. Sandra, we hebben samen vaak borrels en de ANU kerstbrunch georganiseerd. Ik kijk

ernaar uit om weer je collega te worden. Thank you Kasper, Pierre, and Marijke for making me enthusiastic about indoor- and race biking. Sanne, dank je voor het maken van foto's tijdens de dissectie- en cutter dagen, voor alle gezelligheid en voor het samen luisteren van de Foute Top 500. Myrthe en Sonja, dank jullie voor jullie steun en interesse; ik kon altijd bij jullie terecht met vragen over proefopzetten, statistiek en het afronden van de PhD. Marijke, dank je voor alle fijne gesprekken en voor het oppassen op mijn huisje terwijl ik in Nieuw-Zeeland zat. Het was fijn om terug te keren in een opgewarmd huisje. En wat hebben we onwijs gelachen in de kelder van het kasteel tijdens het DPP (met dank aan wodka en wijn). Dank je dat je mijn paranimf wil zijn!

Lieve HANTT-vrienden (Carmen, Demi, Henri, Iwan, Jasmijn, Laura, Maaike, Martijn, Sabine, Yorick), dank jullie voor jullie steun en interesse. Sabine, we doen/deden allebei een PhD bij Wageningen Universiteit. Het was fijn om met jou alle ups en downs te kunnen delen. Dank je dat je mijn paranimf wil zijn!

Een PhD is niet mogelijk zonder steun van familie. Pap en mam, jullie hebben mij altijd mijn eigen keuzes laten maken en me nergens in geremd, ook al betekende dat dat jullie een half jaar zonder jongste dochter zaten. Skype maakte veel goed maar was toch anders. Ik weet dat jullie ontzettend trots op mij zijn. De eerste doctor in de familie is toch heel wat, maar ik blijf gewoon Tetske hoor. Jitske, dank je voor je interesse in een onderwerp wat compleet anders is dan jouw vakgebied (alhoewel jij toch ook een beetje bèta bent). Je wist vrij snel dat mijn PhD over eiwit en OMIU ging en ik vind het heel fijn dat je dat al die tijd onthouden hebt.

Finishing of my booklet gives me mixed feelings: it is nice to finish my PhD but it means that I have to say goodbye to my ANU colleagues. Luckily, the field of animal nutrition is not that big and we will definitely see each other again. Ik ben dankbaar voor alles wat ik tijdens mijn PhD heb geleerd en meegemaakt!

Tetske

Curriculum vitae

List of publications

Training and supervision plan

Curriculum vitae

Tetske Hulshof is born in Varsselder, the Netherlands, on the 2nd of November 1988. She graduated from secondary education (gymnasium) in 2007 at Almende College location Isala, Silvolde, the Netherlands. Thereafter, she studied Animal Sciences at Wageningen University where she obtained her BSc degree in 2010 and, thereafter, her MSc degree in 2012. Tetske did a minor thesis in Animal Nutrition studying the excretion of felinine by male cats of different breeds. She did her major thesis in Adaptation Physiology studying the influence of early-life microbiota on the behavior of laying hens.

In 2012, Tetske started her PhD at the Animal Nutrition group of Wageningen University and Wageningen Livestock Research. In her PhD, she studied protein quality of pig diets by examining the effects of processing on amino acid digestibility and post-absorptive utilization. She also studied the guanidination reaction as analysis method for reactive lysine. This research was part of the IPOP Customized Nutrition project of Wageningen University & Research. In 2014, Tetske received a WIAS fellowship which allowed her to spend 6 months at the Riddet Institute, Massey University, Palmerston North, New Zealand. The results of her PhD project are presented in this thesis.

List of publications

Peer reviewed scientific publications

Hulshof, T. G., P. Bikker, A. F. B. van der Poel, and W. H. Hendriks. 2016. Assessment of protein quality of soybean meal and 00-rapeseed meal toasted in the presence of lignosulfonate by amino acid digestibility in growing pigs and Maillard reaction products. Journal of Animal Science 94:1020-1030.

Hulshof, T. G., A. F. B. van der Poel, W. H. Hendriks, and P. Bikker. 2016. Processing of soybean meal and 00-rapeseed meal reduces protein digestibility and pig growth performance but does not affect nitrogen solubilization along the small intestine. Journal of Animal Science 94:2403-2414.

Salazar-Villanea, S., T. G. Hulshof, A. F. B. van der Poel, E. M. A. M. Bruininx, and P. Bikker. Predicting the standardized ileal protein digestibility of processed soybean meal and rapeseed meal in growing pigs using two *in vitro* methods. Accepted in Journal of Animal Science.

Hulshof, T. G., A. F. B. van der Poel, W. H. Hendriks, and P. Bikker. Amino acid utilization and body composition of growing pigs fed processed soybean meal or 00-rapeseed meal with or without amino acid supplementation. Submitted to Animal.

Hulshof, T. G., S. M. Rutherfurd, S. Sforza, P. Bikker, A. F. B. van der Poel, and W. H. Hendriks. O-methylisourea can react with the α-amino group of lysine: implications for the analysis of reactive lysine. Submitted to Journal of Agricultural and Food Chemistry.

Conference and symposia proceedings

Bikker, P., A. F. B. van der Poel, T. G. Hulshof, S. Salazar-Villanea, E. M. A. M. Bruininx, and G. van Duinkerken. 2013. Impact of technological treatment of feed ingredients on feed efficiency in farm animals. Proceedings of the 64th Annual Meeting of the European Federation of Animal Science, 26-30 August, 2013, Nantes, France.

Hulshof, T. G., P. Bikker, and A. F. B. van der Poel. 2013. Effect of processing of oilseed meals on the apparent ileal digestibility and performance in pigs. Proceedings of the 4th International Symposium on Energy and Protein Metabolism and Nutrition, 9-12 September, 2013, Sacramento, California, USA.

Hulshof, T. G., P. Bikker, A. F. B. van der Poel. 2014. Effect of processing of oilseed meals on ileal digestibility, performance and protein deposition in growing pigs. Proceedings of the 39th Animal Nutrition Research Forum, 3 April, 2014, Utrecht, the Netherlands.

Hulshof, T. G., P. Bikker, and A. F. B. van der Poel. 2015. Over-processing negatively affects digestibility and post-absorptive utilization of protein in growing pigs. Proceedings of the 13th Digestive Physiology of Pigs, 19-21 May, 2015, Kliczków, Poland.

Salazar-Villanea, S., T. G. Hulshof, A. F. B. van der Poel, E. M. A. M. Bruininx, and P. Bikker. 2015. Predicting the *in vivo* standardized ileal digestibility of over-processed ingredients using two *in vitro* methods. Proceedings of the 13th Digestive Physiology of Pigs, 19-21 May, 2015, Kliczków, Poland.

Hulshof, T. G., S. M. Rutherfurd, P. Bikker, A. F. B. van der Poel, and W. H. Hendriks. 2016. Guanidination of amino acids: what is going on? Proceedings of the WIAS Science Day, 4 February, 2016, Wageningen, the Netherlands.

Hulshof, T. G., A. F. B. van der Poel, and P. Bikker. 2016. Oilseed meal processing affects whole body amino acid retention and composition in growing pigs. Proceedings of the 5th International Symposium on Energy and Protein Metabolism and Nutrition, 12-15 September, 2016, Krakow, Poland.

Hulshof, T. G., A. F. B. van der Poel, and P. Bikker. 2016. Oilseed meal processing affects protein digestion kinetics and metabolic organ load of growing pigs. Proceedings of the 5th International Symposium on Energy and Protein Metabolism and Nutrition, 12-15 September, 2016, Krakow, Poland.

Hulshof, T. G., A. F. B. van der Poel, W. H. Hendriks, and P. Bikker. 2016. Processing influence on protein digestion and post-absorptive amino acid utilisation in growing pigs. Proceedings of the Protein for Life conference, 23-26 October, 2016, Ede, the Netherlands.

Hulshof, T. G., S. M. Rutherfurd, S. Sforza, P. Bikker, A. F. B. van der Poel, and W. H. Hendriks. 2016. Impact of free lysine on reactive lysine measurement by guanidination. Proceedings of the Protein for Life conference, 23-26 October, 2016, Ede, the Netherlands.

Training and supervision plan[1]

Basic package (3 ECTS[2])

WIAS Introduction Course	2013
Course on philosophy of science and/or ethics	2013

Scientific exposure (15 ECTS)

International conferences

4th International Symposium on Energy and Protein Metabolism and Nutrition, Sacramento, USA	2013
13th Digestive Physiology of Pigs, Kliczków, Poland	2015
5th International Symposium on Energy and Protein Metabolism and Nutrition, Krakow, Poland	2016

Seminars and workshops

WIAS Science Day, Wageningen, the Netherlands	2013-2016
Aspects of sow and piglet performance seminar, Wageningen, the Netherlands	2013
1st Wageningen PhD Council Symposium, Wageningen, the Netherlands	2013
Animal Nutrition Research Forum, Utrecht, the Netherlands	2014
WIAS Fiber seminar, Wageningen, the Netherlands	2014
WIAS Nutrition, health and welfare of calves seminar, Wageningen, the Netherlands	2014
Schothorst Feed Research symposium: Future dynamics in sustainable animal nutrition, Nijkerk, the Netherlands	2014
Nutrient requirements in relation to animal health symposium, Wageningen, the Netherlands	2015
Animal Nutrition Research Forum, Wageningen, the Netherlands	2016

Presentations

'Ingredient processing affects reactive lysine levels and feed conversion efficiency in pigs', WIAS Science Day, Wageningen, the Netherlands, poster presentation	2013

[1]Completed in the fulfilment of the requirements for the education certificate of the Graduate School Wageningen Institute of Animal Sciences (WIAS).

[2]One ECTS equals a study load of 28 hours.

'Effect of processing of oilseed meals on the apparent ileal protein digestibility and performance in pigs', 4th International Symposium on Energy and Protein Metabolism and Nutrition, Sacramento, USA, oral presentation	2013
'Protein quality in human food and animal feed', 1st Wageningen PhD Council Symposium, Wageningen, the Netherlands, oral presentation	2013
'Effect of processing of oilseed meals on ileal digestibility, performance and protein deposition in growing pigs', Animal Nutrition Research Forum, Utrecht, the Netherlands, oral presentation	2014
'Over-processing negatively affects digestibility and post-absorptive utilization of protein in growing pigs', 13th Digestive Physiology of Pigs, Kliczków, Poland, oral presentation	2015
'How to account for protein damage in diet formulation', Wageningen Academy Course Quality of protein in animal diets, Wageningen, the Netherlands, oral presentation	2015
'Guanidination of amino acids: What is going on?' WIAS Science Day, Wageningen, the Netherlands, oral presentation	2016
'Oilseed meal processing affects whole body amino acid retention and composition in growing pigs', 5th International Symposium on Energy and Protein Metabolism and Nutrition, Krakow, Poland, oral presentation	2016
'Oilseed meal processing affects protein digestion kinetics and metabolic organ loaf of growing pigs', 5th International Symposium on Energy and Protein Metabolism and Nutrition, Krakow, Poland, oral presentation	2016

In-depth studies (8 ECTS)

Rundveevoeding, Wageningen Academy, Wageningen, the Netherlands	2012
Varkensvoeding in de praktijk, Wageningen Academy, Sterksel/Wageningen, the Netherlands	2012
Advances in feed evaluation science, Wageningen Academy, Wageningen, the Netherlands	2013
Advanced statistics course: design of experiments	2013
Indirect Calorimetry and selected applications, University of California, Davis, USA	2013
Industrial food proteins, Graduate School VLAG, Wageningen, the Netherlands	2013
Statistics for the life sciences	2014
Quality of protein in animal diets, Wageningen Academy, Wageningen, the Netherlands	2015

Professional skills support courses (4 ECTS)

Techniques for writing and presenting a scientific paper	2013
Supervising MSc thesis work	2013
Data management	2014
Scientific publishing	2014
Project and time management	2014
Last stretch of the PhD program	2016

Research skills training (2 ECTS)

External training period, Riddet Institute, Massey University, Palmerston North, New Zealand	2015

Didactic skills training (13 ECTS)

Lecturing

Guest lecture, Principles of Animal Nutrition	2014-2015

Supervising practicals and excursions

Practical Feed Formulation Science	2013
Supervising practicals Principles of Animal Nutrition	2014
Supervising practicals Biology of Animal Production	2015

Supervising theses

Supervising BSc thesis (2x)	2013-2014
Supervising MSc thesis (4x)	2013-2015

Tutorship

Integrated Course on Pigs and Poultry	2013
Inleiding Dierwetenschappen	2014

Management skills training (7 ECTS)

WIAS Science Day 2014, chair	2013-2014
Wageningen Associated PhD students (WAPS) council, activity committee	2013-2014
Wageningen Academy Course, Quality of protein in animal diets	2015

Education and training total	**52 ECTS**

Colophon

The research described in this thesis is part of the IPOP Customized Nutrition project of Wageningen University & Research and was financially supported by Wageningen University & Research, the Dutch Ministry of Economic Affairs, Agrifirm Innovation Center, ORFFA Additives BV, Ajinomoto Eurolysine s.a.s., and Stichting VICTAM BV.

Cover design and lay-out: Tetske Hulshof

Printed by Digiforce