Of proteins and processing

Mechanisms of protein damage upon rapeseed processing and their effects on nutritional value

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Mechanisms of protein damage upon rapeseed processing and their effects on nutritional value

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CHAPTER 1

General introduction

Amino acids are essential nutrients for the performance of livestock animals. The greatest part of the amino acids in the rations are supplied through the protein fraction of vegetable ingredients. Many of these ingredients are protein-rich co-products originating from the vegetable oil industry, which undergo hydrothermal processing during their manufacture. These protein-rich co-products (e.g. defatted rapeseed meal and soybean meal) are mixed with other (non-processed) ingredients and are processed further in order to manufacture complete feeds.

Processing can cause changes to the structure of proteins and chemical changes to the amino acid residues, changes that can decrease the nutritional value of the proteins [(1)]. Reduced protein digestibility has been frequently reported for ingredients or diets after hydrothermal processing [(2-6)]. Most of the undigested or unabsorbed proteins/amino acids will be fermented at the large intestine. Fermentation results in growth of the bacterial biomass and the production of short chain- and branched chain-fatty acids after deamination of amino acids [(7)]. The bacterial biomass is excreted in the faeces; approximately 60 to 90% of the nitrogen in the faeces originates from bacterial origin. The fraction of chemically modified amino acids that can be absorbed but are not utilized for protein deposition will be excreted in the urine after deamination. The excreted nitrogen can leach into water reservoirs and increase their nitrate contents [(8)]. Furthermore, some of the fermentation breakdown products (e.g. ammonia and amines) can affect the development of intestinal mucosa and villus height, which in turn reduce protein digestion and absorption [(7)]. High levels of nitrogen reaching the large intestine can also predispose the host for the growth of harmful bacteria, leading to diarrhoea. Therefore, it would be beneficial for the health of the gut to reduce the amount of nitrogen that reaches the large intestine. In addition, all the nitrogen that is included in the feeds but is not converted into animal protein represents an important economic loss.

PROCESSING OF INGREDIENTS: THE CASE OF OILSEED MEALS

Soybean meal and rapeseed meal are two common protein-rich ingredients included in monogastric animal diets. These ingredients undergo similar processing-steps in order to extract the oil present in the seeds, to remove the remnant organic solvents after oil extraction and to inactivate the antinutritional constituents present. Processing of the seeds (Fig. 1.1) involves several thermal processing steps.

It has been shown previously [(6, 9, 10)] that most of the reduction in the crude protein and amino acid digestibility occurs after the desolventization/toasting step. The decrease in digestibility has been linked to the formation of Maillard reaction products, which decrease the content of lysine and reactive lysine [(5, 11)]. However, hydrothermal treatments also result

in changes to the secondary [12] and tertiary structure of proteins. These modifications cause formation of protein aggregates that may hinder enzymatic access for protein hydrolysis. In addition, large variation between processing plants on the conditions used during toasting has been reported [11, 13], which reflects the large variation in protein quality of the processed ingredients (e.g. protein solubility, contents of lysine and reactive lysine).

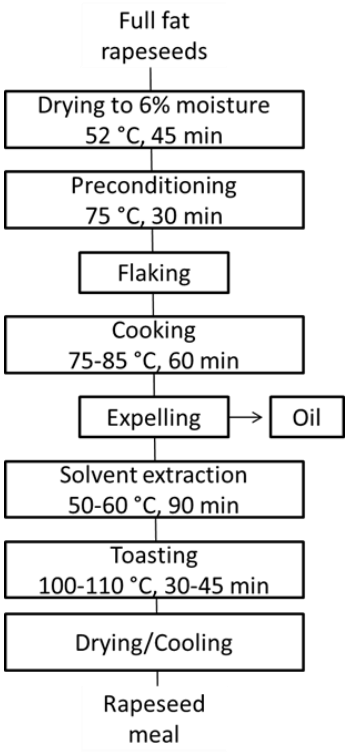

Fig. 1.1. Diagram of the production process of rapeseed meal. Adapted from Classen *et al.* [6].

PROCESSING OF FEEDS

Animal feeds consist of a mixture of previously processed ingredients (e.g. oilseed meals) and ingredients that have not been subject to thermal-processing (e.g. cereals). In order to control the physical properties of the ingredients and to reduce the microbiological burden of the feeds, these ingredient mixtures are usually thermally processed. The most common thermal-processing techniques used for feed manufacturing (e.g. pelleting, expander processing, extrusion) involve heat, moisture and mechanical energy. The amounts of heat, moisture and mechanical energy input depend on the (feed) agglomeration technology employed, as it can be seen from Table 1.1.

During commercial feed manufacturing, the physical quality of the agglomerated feeds is usually considered more important than the effects on their nutritional value [14]. Previous studies have shown that damage to the proteins of protein-rich ingredients by a previous thermal-processing step can occur, resulting in a reduced nutritional value [2, 3]. Therefore, harsh processing conditions employed during the production of feeds might also reduce the nutritional quality of previously processed ingredients (e.g. soybean meal, rapeseed meal, DDGS).

In contrast, positive effects of feed processing technologies on nitrogen and essential amino acid digestibility have been identified as well. These effects have been attributed to increased protein denaturation [14, 20] that may not always occur if the ingredients have undergone a previous hydrothermal processing step and their proteins are already denatured. Further effects are the increased digesta retention time due to higher

starch gelatinization that leads to increased viscosity [20], or the reduction of particle size due to the processing treatments [14]. Other studies have shown limited effects of secondary processing on protein digestion and N-retention [21]. These studies tested the effects of expander processing, pelleting and expander processing with subsequent pelleting of diets containing barley, wheat, soybean meal and cassava on ileal and total tract digestibility nitrogen digestibility of starter, grower and finisher pigs. Thus, results of processing of feeds on protein and amino acid digestibility are not always consistent and this might depend on the extent of damage of the proteins during production of the ingredients.

Table 1.1. Processing parameters employed in common feed manufacturing technologies [a].

Technology	Temperature (°C)	Moisture (%)	Residence time (s)	Mechanical energy (kJ/kg)
Pelleting	60 – 100	12 – 18	-	27 – 99
Expander processing	90 – 130	16 – 18	15 – 25	54 – 83
Universal Pellet Cooker®	115 – 170	14 – 18	3 – 4	88 – 95
Extrusion	60 – 160	20 – 35	20 – 30	839 – 1277

[a] References: Abdollahi *et al.* [14], Thomas *et al.* [15], Chae and Han [16], Fahrenholz *et al.* [17], Fang *et al.* [18], Bokelman *et al.* [19].

PROTEIN EVALUATION

Physico-chemical analysis of proteins allows to determine the properties that could impact its nutritional value. Furthermore, an estimation of the digestibility of an ingredient can also be measured through several *in vitro* or *in vivo* methods. Monitoring the changes of the physico-chemical properties of the proteins and of their digestibility allows to determine the mechanisms by which processing influences protein digestibility. The toolbox for protein analysis is very diverse and the present description does not intend to be complete, but only the methods included in this thesis will be discussed.

Protein analysis involves an inherent difficulty, as many of the techniques available only allow to analyse the soluble protein fraction. Processing reduces protein solubility [22, 23]. Depending on the severity of processing, protein solubility can be as low as 5 and 7% for rapeseed meal and soybean meal [24], respectively, meaning that conclusions obtained from the analysis of the soluble fraction only could be misleading.

Physico-chemical properties

Analysis of physical properties provides information of the structural conformation of proteins. The secondary (and tertiary) structure of native proteins, which is determined by the amino acid composition of the protein, can change due to hydrothermal processing [12, 25-27]. It was previously reported that the secondary structure of the native and processed proteins influences the *in vitro* protein digestibility [12]. Changes in the structure of proteins

result after protein denaturation and refolding, which usually lead to the formation of bonds or interactions (e.g. hydrogen bonds, ionic interactions, disulfide bonds) within and between proteins, resulting in protein aggregation [28, 29]. Overall changes in the structure of proteins due to processing can be determined by measuring their denaturation enthalpy, which is a measure of their degree of denaturation. The denaturation enthalpy of proteins usually decreases, along with protein solubility, as the severity of the process increases. Therefore, protein solubility can be considered as an indication for protein aggregation. Protein solubility tests with different solvents, i.e. cleaving of non-covalent (e.g. sodium dodecyl sulfate [SDS]) or disulfide bonds (e.g. dithiothreitol [DTT]), have previously been used to demonstrate the type of bonds formed during protein aggregation [30-32]. The type of bonds formed could facilitate or limit enzymatic accessibility for proteolysis [30].

Chemical changes consist of the reaction between two or more compounds resulting in the formation of a new compound. Hydrothermal processing of feed ingredients has been reported to facilitate the formation of disulfide bonds [32], Maillard reaction products [33] and amino acid crosslinking [34], which can be considered as chemical changes. Maillard reactions occur due to the reaction of a free reactive group of an amino acid and a reducing sugar. The occurrence of these type of reactions has been linked to the reduction in ileal protein and amino acid digestibility in pigs [2, 3, 5] and poultry [10]. Formation of amino acid crosslinks (e.g. lysinoalanine [LAL], lanthionine [LAN]) could be less common during processing of feed ingredients, as it is favoured at alkaline pH [35]. The determination of Maillard reaction products in processed feeds allows to get insight into the severity of the processing conditions. Other methods, such as the determination of the content of reactive lysine, allow to quantify the amount of lysine which has not been blocked by a reducing sugar.

Crude protein and amino acid digestibility

Protein digestibility is a complex phenomenon that comprises the enzymatic cleavage of peptide bonds and absorption of the free amino acids and the di- and tri-peptides formed. During protein digestion, large complex proteins (e.g. globulins of 60 kDa) are degraded to small peptides or free amino acids that can be absorbed. Enzymes secreted in the gastrointestinal tract (e.g. trypsin and chymotrypsin) are highly specific and the access for cleavage of peptide bonds depends on the structural conformation of the proteins and the chemical availability of the target amino acids. Changes in the structural conformation of proteins and chemical modifications of the amino acids occur simultaneously during hydrothermal processing. However, most literature points to Maillard reactions as the main cause for reduced protein digestibility [3, 36, 37], without considering the influence of reduced accessibility for protein digestion due to structural changes to proteins.

Important reductions in the digestibility of the other essential and non-essential amino acids have also been noticed [4], indicating that changes to the structure of proteins might also contribute to the reduction in protein digestibility.

Methods used to measure protein digestibility intend to mimic the digestion process (i.e. *in vitro* digestibility) or they measure the end result of the digestion process (i.e. *in vivo* digestibility). *In vitro* protein digestibility provides an indication of the hydrolysis of proteins, but most of the methods do not take into account the subsequent absorption process. In this *in vitro* closed systems, the peptides released during protein hydrolysis can inhibit the enzymatic activity. Multistep enzymatic digestibility methods, e.g. the method described by Boisen and Fernández (1995) are based on the quantification of soluble nitrogen. Therefore, soluble proteins might be considered as digested by this method, even when no protein hydrolysis occurred. Furthermore, most of these methods use varying substrate:enzyme ratios, which depends on the protein content of the ingredient or diet tested [38, 39]. The influence of the enzyme:protein ratio on the end-point degree of hydrolysis and hydrolysis kinetics has been shown before [40]. Other *in vitro* digestibility methods rely on the quantification of the release of hydrogen ions after enzymatic cleavage of peptide bonds, either by the measurement of the drop in pH or the addition of alkali to maintain a constant pH (e.g. pH-STAT method). In this way, the pH-based methods provide a quantifiable indication of the enzymatic hydrolysis. In addition, fixed enzyme:substrate ratios are used in this method for every ingredient or diet tested. Several feed ingredients containing vegetable or animal proteins were tested by the pH-STAT method and good correlations were obtained with faecal digestibility in rats [41].

Most *in vitro* methods show good correlations with the *in vivo* digestibility of feed ingredients and diets [38, 41, 42]. However, the predictive accuracy of these methods is far from the *in vivo* values. Moreover, scarce research has tested *in vitro* methods that can predict the *in vivo* digestibility of processed ingredients or feeds.

The goal of the *in vivo* determination of protein digestibility is to accurately quantify the absorption of nutrients from the gastrointestinal tract. The determination of ileal digestibility values of nitrogen and amino acids is considered the 'golden standard' for ingredient evaluation. The apparent digestibility values can be corrected for the secretion of basal (standardised digestibility) and basal plus specific (true digestibility) endogenous losses [43]. Decreased secretion of specific endogenous losses in pigs have been reported after pelleting and high-flow extrusion of wheat based diets, leading to improvements of the standardized ileal digestibility [44-46].

As it has been mentioned before, hydrothermal processing can decrease the *in vivo* protein and amino acids digestibility. The absorption of compounds from the gut does not necessarily mean that these compounds are bioavailable and can be utilized by the

animal for its metabolism or protein deposition [47]. In addition, the physico-chemical changes of the processed proteins could affect the rate of protein digestion. Techniques that allow the collection of samples from different sections of the gut, such as the slaughter method or cannulas at multiple sections (e.g. duodenal and ileal cannulas) can be used to reflect kinetics of protein digestion.

IDENTIFICATION OF THE KNOWLEDGE GAPS

The effects of thermal processing on protein digestibility have been studied in multiple ingredients and diets. The results of processing on protein and amino acid digestibility are highly variable, as they depend on the combination of processing conditions and equipment used. This is noticed in the large variation in the outcome of primary (Table 1.2.1) and secondary (Table 1.2.2) processing in terms of protein or amino acid digestibility in previous studies.

Negative effects on the contents of lysine or reactive lysine usually lead to negative effects on crude protein or amino acid digestibility (Table 1.2.1 and Table 1.2.2). Most of the experiments performed in the desolventization/toasting process reported negative effects on lysine/reactive lysine contents and protein digestibility. In contrast, the effects of hydrothermal processing techniques used for feed manufacturing (pelleting, expander processing or extrusion) on protein digestibility do not seem to be constant. When primary or secondary processing did not reduce the content of lysine or reactive lysine, no effects or positive effects were reported on protein or amino acid digestibility. This could lead to the consideration that chemical changes to proteins are the sole origin of the negative effects on protein digestibility, neglecting the effects of the physical changes that occur to the conformation of proteins during processing. Characterization of the changes to the physical conformation of proteins after processing of feed ingredients and diets is lacking, especially in combination with the simultaneous description of the chemical changes. This could provide a more complete overview of the mechanisms by which processing affects protein and amino acid digestibility.

Table 1.2.1. Overview of the effects of primary processing of ingredients on physical and chemical changes of their proteins and crude protein and amino acid digestibility.

		Physical changes		Chemical changes		CP/AA digestibility				
Ingredient	Treatment	Sec. Struc.	Solubility	Lys	React. Lys	*In vitro*	*In vivo*	Specie	Determination	Reference
Canola	Desolventization/toasting		-				=	broilers	SID	9
Canola	Desolventization/toasting			-			-	broilers	AID	10
Canola	Desolventization/toasting						-	broilers	AID	6
Canola	Desolventization/toasting 91 - 95°C			=			=	pigs	SID	48
Corn	Extrusion (no temp)			=			=	pigs	AID / TT	49
Corn	Extrusion 130 - 135°C						-	pigs	AID	50
Corn	Extrusion 130°C			-			-	pigs	TT	51
Corn	Pelleting						+	pigs	TID	45
Corn	Extrusion 140°C			=			=	pigs	SID	52
DDGS	5 different sources, thermal damage			-			-	pigs	SID	36
DDGS	12 different sources, thermal damage			-	-		-	pigs	SID	53
Flaxseed-field pea	Extrusion 110 - 135°C			=	=		+	pigs	AID / TT	54
Peas	Heated 110 - 165°C			-				pigs		55
Peas	Extrusion (no temp)			=			+	pigs	SID	56
Rapeseed	Desolventization/toasting		-	-			-	rats	TT	11
Rapeseed	Desolventization/toasting			-	-	-	-	pigs	SID	5
Soybeans	Autoclaving at 120°C	-				+				12
Soybean	Toasting			-	-		+/-	pigs	SID	57
Wheat	Extrusion 130°C			-			-	pigs	TT	51
Wheat	Pelleting						+	pigs	TID	45
Wheat	Pelleting			=			+	pigs	TID	46

* Abbreviations: Sec. Struc., secondary structure; Lys, lysine; CP, crude protein; AA, amino acids; AID, apparent ileal digestibility; SID, standardised ileal digestibility; TID, true ileal digestibility; TT, total tract digestibility; -, negative effect; +, positive effect; =, no effect.

Table 1.2.2. Overview of the effects of secondary processing of ingredients or diets on physical and chemical changes of their proteins and crude protein and amino acid digestibility.

		Physical changes		Chemical changes		CP/AA digestibility				
Processed ingredients	Secondary processing	Sec. Struc.	Solubility	Lys	React. Lys	*In vitro*	*In vivo*	Specie	Determination	Reference
Canola meal	Autoclaving 121°C		-	-			-	broilers	SID	9
Canola meal	Autoclaving 130°C			-	-		-	pigs	SID	2
DDGS	Autoclaving 130°C			-	-		-	pigs	SID	58
DDGS-Wheat	Pelleting, extrusion			=	=		=	pigs	TT	19
Fishmeal-soybean meal-wheat	Pelleting, expander, extrusion			=			+	pigs	AID	20
Rapeseed meal	Toasting with reducing sugars			-	-		-	pigs	SID	4
Rapeseed meal	Toasting with reducing sugars		-	-	-		-	pigs	SID	59
Rapeseed meal	Extrusion		-			+				24
Soybean meal	Extrusion (no temp)			-			=	pigs	AID / TT	60
Soybean meal	Extrusion 130 - 135°C						+	pigs	AID	50
Soybean meal	Autoclaving 125°C			-	-		-	pigs	SID	3
Soybean meal	Autoclaving 135°C			-	-		-	pigs	TID	61
Soybean meal	Toasting with reducing sugars			-	-		-	pigs	SID	4
Soybean meal	Toasting with reducing sugars		-	-	-		-	pigs	SID	59
Soybean meal	Extrusion		-			+				24
Soybean meal-barley-wheat-cassava	Expander 100°C		=	=	+		=	pigs	AID / TT	21
Soybean meal-barley-wheat-cassava	Pelleting 80°C		+	=	+		+	pigs	AID / TT	21
Sunflower meal-Wheat	Pelleting 80°C						=	pigs	SID	44
Sunflower meal-Wheat	Extrusion 145°C (1-2 s)						+	pigs	SID	44
Wheat-barley-soybean meal-rapeseed meal	Expander 105°C (5 s)						=	pigs	TT	62

* Abbreviations: Sec. Struc., secondary structure; Lys, lysine; CP, crude protein; AA, amino acids; AID, apparent ileal digestibility; SID, standardised ileal digestibility; TID, true ileal digestibility; TT, total tract digestibility; -, negative effect; +, positive effect; =, no effect.

PROJECT OUTLINE

This PhD thesis is the outcome of a project entitled “Effects of ingredient processing and ingredient interactions on protein nutritional value”. This project was part of IPOP Customised Nutrition, Research Line 1, which dealt with the impact of processing on the nutritional value of (novel) protein-containing products. This research line was financially supported by Wageningen University & Research, the Dutch Ministry of Economic Affairs, WIAS, Agrifirm Innovation Center BV, ORFFA Additives BV, Ajinomoto Eurolysine s.a.s and Stichting VICTAM BV.

In this thesis, rapeseed meal is used as a model ingredient, i.e. it is the example of ingredients that are processed during their production and that are re-processed for the manufacture of complete feeds. The effects of both types of processing on the nutritional value of proteins are analysed using diverse *in vitro* and *in vivo* techniques.

AIMS AND OUTLINE OF THE THESIS

The main aim of this thesis was to provide further insight into the mechanisms of protein damage that occur during ingredient and feed processing, and their effects on protein hydrolysis and digestibility. Chapter 2 consists of a literature study that describes the structural properties of proteins from vegetable ingredients used in swine diets, the changes that the structure of proteins undergo after processing, and the link between these changes and the protein digestibility. Chapter 3 describes the physico-chemical changes of rapeseed proteins during toasting and the effects on *in vitro* crude protein digestibility. In Chapter 4, the role of protein solubility on enzymatic hydrolysis of toasted rapeseed meal proteins is evaluated. The relation between the *in vitro* digestibility methods discussed in Chapters 3 and 4 with the *in vivo* standardized ileal crude protein digestibility of heat-processed ingredients is described in Chapter 5. In Chapter 6, the interaction between processing of ingredients (i.e. toasting time of rapeseed meal) and processing of diets (i.e. pelleting and extrusion) on protein and amino acid digestibility is analyzed. A method to quantify the ileal digestibility of Maillard reaction products in growing pigs is described in Chapter 7. Finally, Chapter 8 (General Discussion) provides an overview of the mechanisms by which protein damage after ingredient/feed processing, according to the results described in this thesis, affects protein digestibility.

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CHAPTER 2

Protein structural changes during processing of vegetable feed ingredients used in swine diets: Implications for nutritional value

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ABSTRACT

Protein structure influences the accessibility of enzymes for digestion. The proportion of intramolecular β-sheets in the secondary structure of native proteins has been related to a decrease in protein digestibility. Changes to proteins that can be considered positive (e.g. denaturation and random coil formation) or negative (e.g. aggregation and Maillard reactions) for protein digestibility can occur simultaneously during processing. The final result of these changes on digestibility seems to be a counterbalance of the occurrence of each phenomenon. Occurrence of each phenomenon depends on the conditions applied, but also on the source and type of the protein that is processed. The correlation between denaturation enthalpy after processing and protein digestibility seems to be dependent on the protein source. Heat seems to be the processing parameter with the largest influence on changes in the structure of proteins. The effect of moisture is usually limited to the simultaneous application of heat, but increasing level of moisture during processing usually increases structural changes in proteins. The effect of shear on protein structure is commonly studied using extrusion, although the multifactorial essence of this technology does not allow disentanglement of the separate effects of each processing parameter (e.g. heat, shear, moisture). Although most of the available literature on the processing of feed ingredients reports effects on protein digestibility, the mechanisms that explain these effects are usually lacking. Clarifying these mechanisms could aid in the prediction of the nutritional consequences of processing conditions.

Keywords: Processing, protein structure, protein digestibility, secondary structure

INTRODUCTION

The increasing world population, along with a higher income, especially in developing countries, is predicted to increase the demand for animal protein. This increased demand and the limits to arable land increase the demand for feed ingredients and their price. As feed represents approximately 50% of the total production costs in animal farming activities [1], a rise in ingredient price requires a higher efficiency in nutrient utilisation in order to be able to produce animal protein without increasing production costs.

Protein deposition in single-stomach production animals is directly linked to the digestibility of protein in the feed and the adequacy of the amino acid profile to match the animal's requirements [2]. This is evident from the vast amount of research conducted over the last decades to define the nutritional requirements of production animals (to maximise protein deposition and to minimise protein oxidation) and to evaluate the nutritional quality of the ingredients or feeds that are fed to meet these requirements [2-7].

Large variation exists in the nutritional quality of protein in feed ingredients, especially in those that received a thermal processing step, such as the most commonly used oilseed co-products (e.g. soyabean (*Glycine max*) meal or rapeseed (*Brassica spp.*) meal) [8, 9]. Heat used during production of ingredients facilitates the separation of the oil fraction from the full fat oilseed, inactivates antinutritional factors present, and removes the residual organic solvents used for oil extraction [10, 11]. However, depending on the severity of the conditions employed, thermal processing may negatively affect the digestibility and nutritional value of proteins [12]. As many of the ingredients used for compound feed production have already undergone a processing step, compound feed production is described here as secondary processing. Secondary processing can alter the protein quality of native as well as previously heat-processed protein sources [3, 13]. Compound feeds are processed in order to control the physical properties of ingredients and improve nutrient availability, which leads to improvements in performance, and to the reduction of the pathogenic burden in feed [14]. It is usual yet unintentional, however, that during processing of ingredients and feed compounding, the factory throughput of material and the physical quality of the feed are of prime importance over the nutritional consequences of the processing parameters employed [15]. These processes could potentially decrease protein digestibility and amino acid bioavailability.

Most nutritional studies conducted on feed or feed ingredient processing focus on digestibility of nutrients as an end parameter, and fail to identify the underlying mechanisms for the differences found [13, 16-18]. For example, extrusion of field peas (*Pisum sativum*) at 75, 115 and 155°C increased the standardised ileal digestibility of crude protein from 81% in untreated field peas to 89, 94 and 92% for the extrusion treatments, respectively [13]. These

authors suggested that denaturation of either storage proteins or antinutritional factors explain the observed effects, but did not measure this. Furthermore, processing conditions in many studies are poorly described and when complex equipment (e.g. pelleting or extrusion) was used, simultaneously involving several processing parameters, such as heat, pressure and shear, it is impossible to disentangle their effects [19-21]. It is important to understand the effects of each processing parameter individually, as well as interdependency of parameters, on the structural and chemical changes of the protein fraction, which could influence its nutritional value.

Processing, as described in the present review, is defined as any action that results in physical or chemical changes or disrupts the conformation of a native or previously processed ingredient or mixture of ingredients for compound feed production. Here, we address the effects of processing on the physico-chemical changes of proteins and the consequences of these changes for the nutritional quality of protein, linked to the end point of protein digestion. Selection of literature was limited to vegetable ingredients commonly used in swine diets, which reported effects of processing on both protein structure and digestibility. The review starts with a brief summary of the biochemistry of native proteins and its relation with crude protein digestibility. Although subject to discussion, a protein with a structural conformation equal to its presence in the original source can be considered as native. We continue with an overview of the available literature on the effects of processing at various conditions on protein structure of ingredients used in swine feeds, and the consequences of these changes for protein digestibility in single-stomach production animals. When possible, correlations between studies are evaluated with the CORR procedure of Statistical Analysis System software version 9.3 (SAS Institute, Inc.) [22] using the information also included in the tables and online Supplementary tables. The correlations were used to identify trends on the mechanisms by which protein structure and structural modifications due to processing influence digestibility.

There are shortcomings to the different digestibility techniques reported in literature which might confound the correlations between structural properties of proteins and digestibility. For example, the effects of gut microbiota, antinutritional factors and dietary fibre on digestion are difficult to simulate using *in vitro* methods[23]. In addition, the standardised ileal digestibility technique does not take into account the specific endogenous losses originating from antinutritional factors and dietary fibre. Finally, the measurement of faecal protein digestibility includes the fermentation of proteins in the large intestine, which might overestimate protein digestibility at the level of the ileum. For this reason, correlations between structural properties and digestibility were performed for studies in which digestibility was analysed using similar techniques (e.g. *in vitro* or faecal).

BIOCHEMISTRY AND BIOAVAILABILITY OF PROTEIN IN VEGETABLE FEED INGREDIENTS

Structure of native proteins

Proteins are polymers made up of different amino acids linked by peptide bonds. This linear chain of amino acids forms the primary structure of the protein and their sequence, specific for every protein, also determines the secondary, tertiary and quaternary structures of the native form of the protein [24]. The main configurations of the secondary structure of protein are the α-helices and β-sheets (Fig. 2.1), although others exist, mostly resulting from variations of these configurations. The α-helices and β-sheets provide strength and rigidity to proteins [27]. Regions in the primary structure of a protein that lack structural elements are considered as random coils [28], which by definition are less stable [27]. The secondary structure of proteins can be determined by several techniques, such as Fourier transform IR (FTIR) spectroscopy and circular dichroism, the latter one used solely for soluble proteins. Circular dichroism uses the polarisation angle of polarised light in the far-UV region for the determination of the secondary structure of proteins and the near-UV region for the determination of changes in the tertiary structure. With FTIR spectroscopy, the amide I region (1600 – 1700/cm) is used to measure the secondary structure of proteins by detecting the vibrations of the carboxyl groups of amino acids [29, 30]. This region of the spectra includes bands for the main secondary structures: α-helices (1650 – 1660/cm) and β-sheets (1630 – 1638/cm). Within the amide I region, also bands that indicate modifications to the native structural conformation can be distinguished, such as the intermolecular β-sheets (1620 – 1630/cm) and A2 bands (1690 – 1695/cm), indicative for protein aggregation [30]. Intermolecular β-sheet structures originate from intermolecular hydrogen bonds [31] and A2 regions from intermolecular aggregation of proteins or the vibration of the carboxyl groups of the amino acid side chains [30].

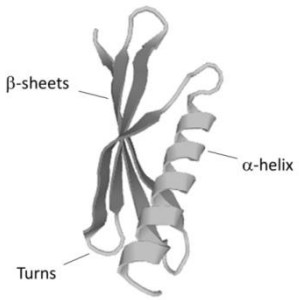

Fig. 2.1. Cartoon view of the main secondary structures of proteins. Protein in the cartoon is PDB ID: 4R80 [25, 26].

Intramolecular formation of non-covalent and disulfide bonds between side chains of amino acids holds the three-dimensional tertiary structure of protein together. The hydrophobic amino acids are hidden in the interior of the structure. Non-covalent bonds between amino acids can be due to Van der Waals interactions between hydrophobic amino acids (e.g. proline, tryptophan), to hydrogen bonding, or to electrostatic interactions between amino acids with opposite charges (e.g. lysine and glutamate) [27]. Moreover, proteins in their tertiary structure can interact with each other and form non-

covalent and covalent (disulfide) intermolecular bonds, which give rise to the quaternary structure of proteins. Most of the storage proteins in vegetable sources are present in a quaternary conformation, which can also be considered their native conformation. Examples of such proteins are napin (albumin) and cruciferin (globulin) in rapeseed, and the globulins β-conglycinin and glycinin in soyabean. Rupture of intermolecular bonds releases the individual proteins that compose the quaternary structure. For example, under reducing conditions, the acidic and basic polypeptides of soyabean glycinin and rapeseed cruciferin are separated as seen by electrophoresis (SDS-Page) [32, 33]. The energy required to break disulfide covalent bonds is higher than the energy required to break non-covalent bonds (Table 2.1). Proteins with large numbers of disulfide bonds are regarded to have a higher resistance to enzymic activity during digestion and also a lower sensitivity to structural changes during processing than proteins with small numbers of disulfide bonds [34].

Proteins from cereals and legumes are usually stored as protein bodies. In general, approximately 80% of the protein content of legumes can be classified as storage proteins and is stored in protein bodies [35]. It has been defined that any protein present at least in 5% of the total protein content can be considered as storage protein [36]. The remainder of the proteins, which comprise the proteins with biological activity (e.g. enzymes and inhibitors), are not contained within protein bodies.

An overview of the structural properties of the major proteins that are present in important vegetable ingredients used for commercial swine compound feed production is shown in Table 2.2. The proteins are described based on the most abundant protein types for each ingredient. For example, although classified under the same name (i.e. zein), the prolamins from maize consist of four types of proteins with different secondary structures (i.e. α-, β-, γ- and δ- zein). The secondary structure of α-zein is mostly α-helical, whilst that of β-zein (which is also related to γ-zein) consists mostly of β-sheets and turns [37]. The difference in the secondary structure of these proteins originates from their different amino acid compositions [38].

Table 2.1. Types, strength and amino acids involved in bonds found in proteins and solvents that can dissolve these bonds*.

Type of bond	Description	Strength (kJ/mol)	Distance (nm)	Amino acids involved	Broken by:
Non-covalent					
- van der Waals	Induced or permanent interactions between dipoles	0.4 - 4	0.30 - 0.60	Tyrosine, tryptophan, phenylalanine, proline, methionine, leucine, isoleucine, valine, alanine, glycine	Ionic and non-ionic detergents (e.g. SDS, thiourea, triton, CHAPS, sodium salts of long chain fatty acids)
- Hydrophobic interactions	Interactions between non-polar molecules following localised water structuring	< 40	1.00		
- Hydrogen bonds	Between hydrogen and strong electronegative atom	NH→O: 8 NH→N: 13 OH→O: 21 OH→N: 29	0.30 0.31 0.27 0.28	Asparagine, glutamine, threonine, serine, cysteine	Molecules with strong polarity (e.g. urea, thiourea, SDS)
- Ionic/electrostatic	Between opposite charged groups	20	0.25	Aspartic acid, glutamic acid, lysine, arginine, histidine	Acids, alkali or salt solutions
Covalent					
- Disulfide bonds	Two sulfur atoms linked together	215 - 251	0.21	Cysteine	Reducing reagents (e.g. DTT, Na_2SO_3, 2-mercaptoethanol)
- Peptide bonds	Amide bonds between amino acids	C-N: 308	0.13 - 0.15	All amino acids	Very strong forces (e.g. combustion, hot acid hydrolysis)
- Isopeptide bonds	Amide bonds between side chains of amino acids	305 - 308	0.13 - 0.15	Lysine, glutamic acid, aspartic acid, glycine	Very strong forces (e.g. combustion, hot acid hydrolysis)

CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol.
* Modified after Liu & Hsieh [81].

Table 2.2. Biochemical characteristics reported for major proteins of vegetable ingredients frequently used in pig feed.

Ingredient	Protein	Osborne class (protein type)	Content (% of total protein)	MM (kDa)	pI (pH)	Secondary structure				Protein digestibility coefficient *	Reference
						α-helices (%)	β-sheets (%)	α : β ratio	Random coils (%)		
Rapeseed	Cruciferin	Globulin	60	300	7.2	11	31	0.35	58	0.89 ± 0.003	35, 42, 115
	Napin	Albumin	15 - 45	12 - 14	9.0 - 12.0	25	38	0.66		0.87 ± 0.002	35, 42, 116
Soyabean	Glycinin	Globulin (legumin)	31	300 - 380	4.6 - 5.0	15	37	0.41	23	0.79 ± 0.008	33, 35, 42, 117, 118
	β-Conglycinin	Globulin (vicilin)	30 - 50	150 - 200	4.9 - 6.0	13	38	0.34	23	0.86 ± 0.011	35, 42, 43, 119
Pea	Legumin	Globulin	65 - 80	398	4.5 - 8.8	9	46	0.20		0.63 ± 0.02*	41, 120, 121
	Vicilin	Globulin		180	5.0 - 6.0	35 - 36	40 - 49	0.80	7 - 15	0.88 ± 0.02*	41, 120, 122
		Albumin	20 - 35	26		3	49	0.06	31	0.41 ± 0.12*	41, 123
Kidney beans	Phaseolin	Globulin (vicilin)	36 - 46	163 - 600	4.2	16 ± 0.2	39 ± 0.4	0.41	26 ± 0.2	0.11 ± 0.02 - 0.27 ± 0.02*	35, 46, 124
Sunflower	Helianthinin	Globulin (legumin)	55 - 60	300 - 350	5.4 - 6.8	2	28	0.07	70		125, 126
Wheat	Gliadin	Prolamin	34	28 - 55	6.5 - 7.8	22 - 36	25 – 31	1.04	11 - 16	0.90 ± 0.007	42, 127, 128, 129, 130
	Glutenin	Glutelin	47	100 - 1000	7.0			1.29 - 3.63		0.91 ± 0.005	42, 127, 130
Maize	Zein	Prolamin	40 - 52	22 - 25	6.8	44 - 65	0 – 30		41 - 51	0.53 - 0.99 ± 0.001*	131, 132, 133, 134, 135
Barley	Hordein	Prolamin	80	36 - 44	6.0	18 ± 2.3 - 34 ± 1.6	14 ± 1.3 - 25 ± 2.4	1.40 - 2.00		0.54 - 0.70*	38, 136, 137, 138
Sorghum	Kafirin	Prolamin	68 - 73	13 - 27	6.0	40 - 60	36 - 43	1.27		0.76 ± 0.03*	135, 139

MM, molecular mass; pI, isoelectric point.

* Values were determined from *in vitro* measurements.

Overall, globulins are the predominant protein types in oil (e.g. rapeseed) and legume seeds (e.g. soyabean), whilst prolamins and glutelins are the most abundant cereal proteins. In addition, proteins with very different structural characteristics can be found within the same ingredient. For example, cruciferin in rapeseed has a higher molecular weight and α-helix:β-sheet ratio than napin. According to the 'Osborne' [39] classification of proteins, albumins are soluble in water, globulins in diluted salt solutions, prolamins in ethanol-water solutions and glutelins in diluted alkali solutions. Although many proteins are classified as being from the same 'Osborne' type [39], proteins from different ingredients usually differ in their amino acid composition. This is reflected in different conformations, thereby making it impossible to generalize on the types of bonds present and their secondary, tertiary or quaternary structures. Globulins and albumins seem to have a higher content of intramolecular β-sheet structures compared with prolamins. This is also reflected in a lower α-helix:β-sheet ratio of the globulins and albumins compared with the prolamins.

Carbonaro *et al.* [30] suggested that the secondary structure of the protein in a (processed) ingredient is a good predictor of digestibility. These authors reported that the proportion of intramolecular β-sheet structures in food sources from vegetable and animal origin is negatively correlated with *in vitro* crude protein digestibility ($r = -0.98$) using porcine trypsin and peptidase, bovine chymotrypsin and a bacterial protease (the latter added in a subsequent incubation). A higher α-helix:β-sheet ratio has been described to negatively influence *in vitro* intestinal digestibility of rumen undegraded protein in ruminants [40], although the mechanism of digestion in ruminants differs from that in single-stomached animals. The relationship between the α-helix:β-sheet ratio and digestibility was also studied *in vitro* [41]. It was found that the *in vitro* digestibility of the albumin, globulin and vicilin fractions of peas (41, 63 and 88%, respectively), using porcine pepsin and pancreatin, corresponded with the α-helix:β-sheet ratio of 0.06, 0.20 and 0.80, respectively. Variation in the secondary structure of proteins, however, does not seem to explain the variation in digestibility completely. Napin and cruciferin, which have very different secondary structures (e.g. ratio α-helix:β-sheet is 0.66 in napin and 0.35 in cruciferin), do not differ much in their apparent faecal digestibility in rats [42]. Also, proteins with similar secondary structures, such as glycinin and β-conglycinin from soyabeans, differ in their apparent faecal digestion coefficients [42]. The larger number of disulfide bonds in glycinin compared with β-conglycinin has been postulated to provide a further explanation for the differences in the digestibility values between these proteins [33, 43].

There was no correlation ($P > 0.05$) between the secondary structure of isolated native proteins and *in vitro* or faecal digestibility (Table 2.2). However, literature on food allergens [44, 45] suggests that the native structure of some proteins (e.g. Ara h 1 from groundnuts or β-conglycinin from soyabeans) influences the accessibility of gastric

proteases, which might also limit the access of intestinal peptidases for proteolysis. For example the main globulin from kidney beans (*Phaseolus vulgaris*), phaseolin, has high resistance to pepsin and pancreatin hydrolysis (19% *in vitro* digestibility), which was attributed to its closed tertiary or quaternary structure that restricts enzyme accessibility [46].

Structural changes due to protein denaturation

Exposure of plant storage proteins to harsh conditions, like extreme pH values, heat or pressure causes protein denaturation. For example, a pH higher than 11.5 or lower than 3.0 or pressure at 400 MPa for 10 min cause complete denaturation of soya glycinin [47, 48]. Denaturation is the unfolding of proteins from their tertiary or secondary structures and will occur when the denaturing influence is sufficiently large to break the non-covalent or covalent bonds that hold the structure together. Conditions for denaturation depend on the type and structure of each native protein [49]. Refolded proteins with a structural conformation different from their native state can also be considered as denatured. Proteins refold in an attempt to minimize their free energy state, with simultaneous formation of new bonds or interactions [50]. It has been suggested [51] that the new bonds formed are not representative of the original conformation of the protein, as this is statistically unlikely to occur. These new bonds result from intramolecular electrostatic and hydrophobic interactions between amino acids, or from intermolecular non-covalent and/or disulfide bonds between two unfolded proteins. The formation of these bonds or interactions between amino acids may lead to protein aggregates, which can be soluble or insoluble depending on the molecular weight of the aggregate. As defined by Wang *et al.* [52], protein aggregates consist of proteins that have lost their native state and are at least twice the size of the native protein. The mechanisms that explain the aggregation of proteins were extensively reviewed by these authors. Protein aggregation can also occur due to crosslinking reactions between proteins, resulting from protein oxidation and the formation of covalent bonds between amino acids, which is favoured at alkaline pH [53]. An example of the latter is the formation of lysinoalanine (LAL), which is a crosslink between lysine and alanine and is mediated through the formation of dehydroalanine (DHA) from β-substituted amino acids (e.g. phosphoserine) and the subsequent nucleophilic addition of the ε-amino group of lysine [53].

During digestion, proteins are partially denatured by the acidic pH conditions in the stomach, which facilitates the accessibility of proteases to the peptide bonds. Pepsin is secreted in the stomach and cleaves peptide bonds between hydrophobic or neutral amino acids, except for proline. Proteins that are resistant to the acidic and proteolytic conditions in the stomach can reach the intestinal mucosa and cause allergic reactions [44]. Trypsin and chymotrypsin are secreted in the pancreatic juices in the duodenum. Trypsin is highly

specific to lysine and arginine, whilst chymotrypsin has a preference for large hydrophobic amino acids, such as tryptophan, tyrosine and phenylalanine. This specificity becomes important when the cleavage sites for these enzymes are physically or chemically blocked by structural constrains, such as protein aggregation due to non-covalent or covalent bonds formation or by modification of the amino acid residues due to protein oxidation (e.g. dityrosine bonds) or Maillard-type reactions.

Changes in the structure of proteins due to denaturing treatments, such as heat, pH and pressure, can lead to proteins that become either more susceptible or more resistant to proteolysis. Protein denaturation can result in the formation of random coils, which exposes groups that are not usually accessible in the native form, thus becoming more susceptible to enzymic hydrolysis [30, 54]. The formation of random coils due to processing result from the breakdown of the tertiary structure and that of the secondary structure, such as α-helices and intramolecular β-sheets. Denaturation, especially at extreme conditions involving high temperatures or pH extremes, promotes protein aggregation [30, 55-59] and crosslinking between amino acids [60]. Aggregation and crosslinking reactions could decrease the accessibility of digestive enzymes, thereby reducing protein digestibility [61, 62].

The extent of structural changes in proteins depends on the conditions employed during processing, but also on the types of proteins present. Upon thermal processing of rapeseed protein isolates, the globulin fraction (cruciferin) was more affected and formed a larger amount of aggregates than the albumin fraction (napin) [63]. Similar results have been reported for soyabean proteins, in which β-conglycinin is more heat-sensitive than glycinin due to the disulfide bonds present in the latter protein type [51]. Disulfide bonds can be broken during processing, but depending on the severity of the conditions, the free thiol residues can also react to form new bonds via sulfydryl-disulfide interchange reactions and increase aggregation [59]. Differences in amino acid composition and structure between different types of the same protein (e.g. α-, β- and γ-kafirins) might also influence the response of the proteins to denaturing conditions and digestion [64].

Furthermore, it has been suggested that the net charge of the protein influences the structural response to thermal processing [59]. Increasing NaCl concentrations from 0 to 1.5 M at pH 8 increases the denaturation temperature of soya glycinin from 79.1 to 98.5°C [47]. This is an indication of a protective effect of the salt on the native structure of glycinin from protein denaturation at increasing temperatures. When similar processing conditions were used, a higher content of hydrophobic and uncharged polar amino acids and higher acidic:basic amino acid ratio were linked to a decrease in aggregation [59]. It has been proposed [55] that heat-induced aggregation promoted by basic amino acids (lysine, arginine or histidine) could have detrimental effects on protein digestibility.

In addition, proteins are highly susceptible to react with reducing sugars upon heating to produce Maillard reaction products. Some essential amino acid residues, such as lysine and arginine, are more susceptible than other amino acids to these type of reactions. Lysine susceptibility is due to the presence of the additional amino group, whilst in arginine it is due to the additional guanidinium group [65]. The nature of these reactions and the wide range of compounds formed have been reviewed elsewhere [65]. It must be considered that structural changes *per se* (e.g. protein aggregation) and chemical changes due to Maillard reactions may occur simultaneously [66] and that the effects of both on proteolysis might be confounded.

Protein digestion is a multifactorial process that is influenced not only by the physico-chemical properties of the proteins, but also by the matrix in which the protein is embedded. Microstructural components of the feed are not often described in literature and this aspect should be taken into account in future research that links modifications of proteins due to processing to protein digestibility.

PROCESSING, PROTEIN STRUCTURE AND PROTEIN DIGESTIBILITY

Extrusion and pelleting, which are frequently used agglomeration processes in feed production, involve the simultaneous utilisation of heat, moisture, shear and pressure. These agglomerating technologies can be considered as complex processes, in which process parameters (e.g. temperature, moisture, screw configuration) and system parameters (e.g. mechanical energy, throughput, residence time) are interrelated and can influence each other [67-69]. Both process and system parameters influence the final structural conformation of proteins and enzyme accessibility for proteolysis, and consequently affect protein digestion. Hence, it would be important for further research efforts to disentangle the effects of individual processing factors on protein, which will allow maximisation of dietary protein utilisation in animal production.

Heat induced changes

Thermal treatments are involved in most common processing technologies to achieve the desired physical state of the ingredients and ingredient mixtures. Other reasons are to improve the digestibility of starch and proteins, to eliminate residual organic solvents after oil extraction and antinutritional factors, and to maintain hygienic levels of the processed feeds [15, 70-72].

The maximum temperature achieved during commercial oil extraction of soyabeans and rapeseeds ranges between 100 and 110°C [10, 11, 73, 74], a temperature which is held constant for 60-90 min [12]. It must be emphasised that these temperatures are achieved by

toasting, which comprises the use of steam, which is a source of heat that involves large amounts of moisture. Large differences with respect to formation of Maillard reaction products in soyabean meal proteins have been detected between dry (e.g. oven-drying) and wet (e.g. autoclaving) sources of heat [75], making it difficult to disentangle the individual effects of heat and moisture. The temperature applied during compound feed production (secondary processing) depends on the technology employed. Common pelleting temperatures for swine feed production range between 60 and 100°C, whilst higher temperatures are achieved with expander processing (90-130°C) and extrusion (60-160°C) [76].

Changes in the secondary structural conformation of proteins due to thermal processing have been reported (Table 2.3), although only a limited number of studies have linked these changes to protein digestibility. After autoclaving at 120°C for 20 min, an increase in the relative amount of random coil conformation in different whole ingredients was reported [30]. Heating at 100°C for 15 min also increased the proportion of random coils in rapeseed protein isolate from 23% in the unheated material to 28% after heating, whilst increasing simultaneously the proportion of β-sheets from 10 to 33% [63]. Formation of random coils is an indication of successful protein denaturation, also linked to a positive correlation with *in vitro* digestibility (r = 0.91) [30]. The increase of the *in vitro* digestibility reported in this study after processing ranges from 2 to 6%, which can be considered as low. In addition, inactivation of protease inhibitors was not considered in this study. The effect of the formation of random coils on the increase in digestibility after the thermal treatment might be confounded with the inactivation of protease inhibitors. These authors also reported that autoclaving induced the disappearance of the intramolecular β-sheet structures and the appearance of intermolecular β-sheet structures and A2 bands in FTIR spectra. These two bands in the spectra (intermolecular β-sheets and A2 bands) have been related to protein aggregation and the proportion of intermolecular β-sheets was highly predictive (r = -0.99) for *in vitro* protein digestibility. As formation of aggregates and random coils occur simultaneously during processing, the net effect on digestibility is related to the relative frequency of each phenomenon. When taken together (Fig. 2.2), the total content of intramolecular β-sheet structures from native proteins and the intermolecular β-sheet structures from proteins after thermal treatment has a high negative correlation (r = -0.95, $P < 0.001$, n = 8) with *in vitro* crude protein digestibility. The total content of intramolecular and intermolecular β-sheets can be used as a fast predictor for protein digestibility, at least for legume protein sources. As both β-sheet structures within the protein and between proteins correlate well with *in vitro* crude protein digestibility, it appears that the structure itself (i.e. β-sheets), but not its location, limits proteolysis. The correlation between the α-helix:β-sheet ratio and *in vitro* crude protein digestibility was not significant, but exhibits a trend (r = 0.65,

P = 0.08). Nevertheless, native legume ingredients tend to have a lower ratio and digestibility compared with thermally treated ingredients. It should be noted, however, that these correlations originate from a single study [30] and that the ingredients used probably contained protease inhibitors. It is likely that these protease inhibitors were inactivated during thermal treatment and that the increase in digestibility can be partly attributed to the inactivation of protease inhibitors and partly to the modifications of the secondary structure.

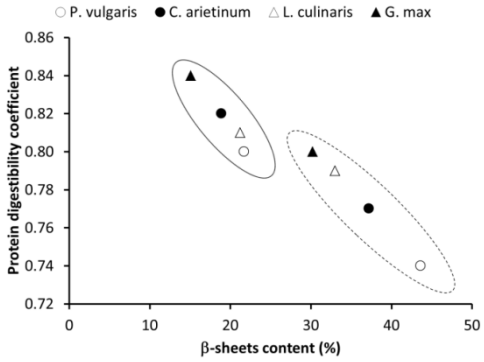

Fig. 2.2. Correlation between the β-sheets content and *in vitro* crude protein digestibility coefficient of native (dashed line) and thermally treated (solid line) whole legume seeds. Adapted from Carbonaro *et al.* [30].

Although the *in vitro* digestibility of the most frequent protein fraction present in kidney beans (phaseolin) was improved after thermal processing (Table 2.4), the digestibility of other protein fractions from the same source (total albumins, protease inhibitor and lectins) was largely reduced [77]. This could explain why minor or no changes in digestibility were reported after thermal treatment of whole kidney beans or protein isolates thereof [30, 55, 59]. The overall effect on total protein in the feedstuff seems to be a combination of the positive and negative effects on each individual protein fraction. This means that for a complete understanding of the underlying mechanisms that explain the effects of processing on protein digestibility in feed ingredients, the effects on each individual protein type needs to be studied. Positive effects of the thermal treatment of kidney beans on apparent ileal crude protein digestibility in pigs have been reported [78]. It was discussed that these effects might be due to inactivation of antinutritional factors and conformational changes of proteins during 'high temperature short time' treatments (136°C; 1.5 min), which is in contrast to the lower temperature and longer times (ranging from 95 to 121°C; 15 to 30 min) applied in other studies [30, 55, 59, 77].

Table 2.3. Relative spectral weights of the secondary structure of proteins and *in vitro* crude protein digestibility coefficient after processing under different conditions.

Feedstuff/material	Treatment	Secondary structure (relative spectral weights)				Protein digestibility coefficient		Reference
		α-helices (%)	β-sheets (%)	α-helix:β-sheet ratio	Random coils (%)	Reported average	SE	
Kidney bean	Control	11 - 18	32 - 44	0.34 - 0.41	0	0.74	0.003	30, 95
	Dry heated 120°C, 30 min	0	13		18			
	Autoclaved 120°C, 20 min	0 - 13	0 - 22	0.58	18 - 33	0.80	0.005	
Lentil	Control	8 - 18	33 - 47	0.17 - 0.54	0	0.79	0.005	30, 95
	Dry heated 120°C, 30 min	8	46	0.17	0			
	Autoclaved 120°C, 20 min	0 - 22	7 - 21	1.02	9 - 23	0.81	0.004	
Chickpea	Control	20	37	0.53	0	0.77	0.004	30
	Autoclaved 120°C, 20 min	19	19	1.02	15	0.82	0.005	
Soyabean	Control	12	30	0.39	0	0.80	0.003	30
	Autoclaved 120°C, 20 min	12	15	0.80	17	0.84	0.005	

Table 2.4. Change in *in vitro* and *in vivo* protein digestibility compared with a control of feed ingredients as affected by various processing treatments.

Feedstuff/material	Treatment	Δ Digestibility (%) *	Reference
In vitro digestibility			
Red bean isolate	Cooked 95°C, 30 min	-9	59
Mung bean isolate	Cooked 95°C, 30 min	5	59
Kidney bean isolate	Cooked 95°C, 30 min	2	59
Kidney bean	Autoclaved 120°C, 20 min	7	30, 55
Faba bean	Autoclaved 120°C, 20 min	-4	55
Lentil	Autoclaved 120°C, 20 min	-1 to 2	30, 55
Chickpea	Autoclaved 120°C, 20 min	5	30, 55
Kidney bean isolated proteins			
Phaseolin	Cooked 99°C, 30 min	>100	77
Total albumins	Cooked 99°C, 30 min	-33	
	Autoclaved 121°C, 15 min	-47	
Protease inhibitor - lectins	Cooked 99°C, 30 min	-11	
	Autoclaved 121°C, 15 min	-44	
Glutelins	Cooked 99°C, 30 min	3	
	Autoclaved 121°C, 15 min	-9	
Soyabean meal †	Extruded 115°C, 26% moisture, 80 rpm	27	104
	Extruded 115°C, 35% moisture, 80 rpm	22	
	Extruded 115°C, 26% moisture, 140 rpm	32	
	Extruded 115°C, 35% moisture, 140 rpm	22	
In vivo digestibility			
Faba bean ‡	Autoclaved 120°C, 20 min	-30	140
Soyabean meal §	Extruded 116°C, 23 kWh/t	1	141
Soyabean white flakes §	Extruded 116°C, 23 kWh/t	18	141

* Change (%) within studies with respect to control.
† Data extrapolated from graphs.
‡ Standardised ileal digestibility, rats.
§ Fecal digestibility, mink

Changes in the solubility of proteins, due to the heat-induced formation of insoluble aggregates, have been reported for a wide range of experimental conditions and were linked to changes in the types of bonds within and between proteins (see Supplementary Table S1). There is a parallel increase in the amount of insoluble protein aggregates with the severity of the thermal treatments applied. Already at 80°C, 31% of the protein content in rapeseed protein isolate formed insoluble aggregates, compared with 0% in the unheated material [63]. In the isolate, cruciferin (globulin) was more susceptible to aggregate formation than napin (albumin) [63]. Most studies agree on a decreased solubility of a protein after a thermal treatment, compared with that of the native protein [57, 79-81]. The reduction in protein solubility is an indication of the formation of insoluble protein aggregates. Many native proteins are soluble as their hydrophobic groups are located on the inside of the molecule. After denaturation, involving reorientation of the hydrophobic groups and refolding, the initial

protein aggregates formed are soluble, but they become insoluble when their size exceeds the solubility limit [31].

Protein solubility studies with agents capable of cleaving different types of bonds (e.g. urea, SDS and dithiothreitol (DTT)) have been used in order to study the main causes for protein insolubility (see Supplementary Table S1). Urea and SDS are good agents to cleave non-covalent bonds, whilst DTT is adequate for the reduction of disulfide bonds into thiols. The solubility of unheated proteins in buffer solutions is similar to the solubility of aggregated proteins with solutions that contain both non-covalent and covalent cleaving agents. Combining these two types of cleaving agents has a synergistic effect on the amount of solubilised protein, compared with the amount of protein solubilised by using them separately [57, 79-81]. This indicates that non-covalent and covalent bonds are mutually important in maintaining the structure of aggregated proteins.

After thermal processing, there is an increase in the disulfide bond content with a simultaneous reduction in the content of thiols (Table 2.5). For example, thiol content was reduced from 6.6 μmol/g protein in the albumin and the protein inhibitors plus lectins fractions isolated from native kidney beans to 0.2 and 0.6 μmol/g protein, respectively, after heating at 121°C for 15 min [77]. At the same time, the disulfide bond content was increased from 24.8 to 28 μmol/g protein in the albumin fraction and from 22.5 to 25.5 μmol/g protein in the protein inhibitors plus lectins fraction. These changes were more evident with autoclaving at 121°C for 15 min than with cooking at 99°C for 30 min. This could be an indication that the effect of temperature on protein denaturation is more relevant than the effect of time, as reported elsewhere [78] for the storage protein phaseolin. There was a positive correlation between the *in vitro* crude protein digestibility and the content of thiol groups in thermally treated isolated albumins from kidney beans ($r = 0.80$, $P < 0.003$, $n = 11$) (Fig. 2.3). Correlations for the rest of the protein sources analysed were not significant. Some of these sources (*Phaseolus* beans and soyabeans) probably contained protease inhibitors and lectins. An increase in digestibility that resulted from heat inactivation of protease inhibitors and lectins could mask the decrease in digestibility resulting from heat-induced disulfide bonding.

Table 2.5. Free thiol and disulfide bonds content, and protein digestibility coefficient after processing under different conditions†.

Feedstuff/material	Treatment	Disulfide bonds	Free thiol groups	Protein digestibility coefficient	Reference
		(μmol/g protein)			
Kidney bean isolate	Control		4.5 ± 0.20	0.64 ± 0.02	59
	Cooked 95°C, 30 min		1.3 ± 0.02	0.65 ± 0.02	
Mung bean isolate	Control		8.7 ± 0.07	0.56 ± 0.01	59
	Cooked 95°C, 30 min		2.2 ± 0.04	0.61 ± 0.01	
Red bean isolate	Control		10.3 ± 0.21	0.53 ± 0.01	59
	Cooked 95°C, 30 min		1.8 ± 0.01	0.48 ± 0.01	
Rapeseed protein isolate	Control		25		63
	Cooked 60°C, 15 min		24		
	Cooked 80°C, 15 min		17		
	Cooked 100°C, 15 min		13		
Soyabean meal	Control	29.5 ± 0.5	9.5 ± 0.4	0.85 ± 0.006*	141
	Extruded 122°C, 27 kWh/t	31.4 ± 0.3	7.8 ± 0.2	0.86 ± 0.002*	
Soyabean white flakes	Control	23.3 ± 0.8	10.6 ± 0.4	0.71 ± 0.020*	141
	Extruded 119°C, 24 kWh/t	32.7 ± 0.4	7.2 ± 0.1	0.84 ± 0.003*	
Kidney beans isolated proteins					
Albumins	Control	24.8 ± 0.3	6.6 ± 0.1 - 19.2 ± 0.2	0.29 ± 0.009 - 0.32 ± 0.004	77, 87
	Heated 60°C, 30 min		14	0.28 ± 0.002	
	Heated 80°C, 30 min		13	0.25 ± 0.003	
	Heated 100°C, 30 min		4.8	0.25 ± 0.006	
	Heated 121°C, 30 min		2	0.21 ± 0.010	
	Autoclaved 121°C, 15 min	28 ± 0.3	0.2 ± 0.0	0.15 ± 0.008	
	Heated 135°C, 30 min		0.1	0.18 ± 0.008	
Protease inhibitor lectin	Control	22.5 ± 0.1	6.6 ± 0.1	0.28 ± 0.001	77
	Cooked 99°C, 30 min	24.9 ± 0.1	1.9 ± 0.0	0.25 ± 0.015	
	Autoclaved 121°C, 15 min	25.5 ± 0.1	0.6 ± 0.1	0.16 ± 0.010	
Glutelins	Native	ND	ND		77
Red sorghum	Control	30.6 - 34.0 ± 0.16	0.9 - 1.5 ± 0.03	0.72 ± 0.001 - 0.79 ± 0.001	82, 83
	Pelleted 65°C	28.5	0.8	0.72 ± 0.001	
	Pelleted 80°C	29.7	0.9	0.72 ± 0.001	

Table 2.5. Continued

Feedstuff/material	Treatment	Disulfide bonds	Free thiol groups	Protein digestibility coefficient	Reference
		(μmol/g protein)			
	Pelleted 90°C	35.9 ± 0.16	1.3 ± 0.03	0.74 ± 0.001	
	Pelleted 95°C	29.0	0.8	0.76 ± 0.001	
White sorghum	Control	33.9 ± 0.16	1.2 ± 0.03	0.75 ± 0.001	82
	Pelleted 90°C	34.4 ± 0.16	1.1 ± 0.03	0.75 ± 0.001	
Yellow sorghum	Control	33.8 ± 0.16	1.5 ± 0.03	0.71 ± 0.001	
	Pelleted 90°C	34.7 ± 0.16	1.4 ± 0.03	0.73 ± 0.001	

ND, not detected. † Reported average ± standard error. * Values correspond to faecal crude protein digestibility in minks.

It has been suggested [82, 83] that the formation of disulfide bonds after heat treatment of sorghum is responsible for the decreased digestibility of kafirin. However, correlations between disulfide bond content of sorghum after thermal treatments and standardised ileal digestibility were not significant (P > 0.05). Oddly, the disulfide bond content in the study of Selle *et al.* [83] did not increase along with the decrease in the thiol content. With increasing temperatures, there is also an increase in the surface hydrophobicity of the proteins in rapeseed protein isolate, which is a reflection of the exposure to the surface of the hydrophobic groups buried inside the molecules [63]. Surface hydrophobicity (S_O) increased from 600 in the unheated rapeseed protein isolate to 650, 1500 and 1100 after thermal treatments at 60, 80 and 100°C for 15 min, respectively [63].

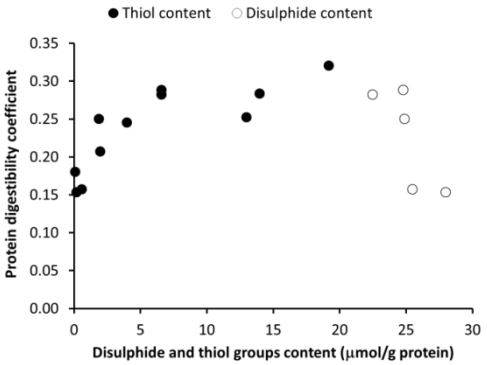

Fig. 2.3. Correlation between the content of disulfide or thiol groups and *in vitro* protein digestibility coefficient for thermally treated albumins isolated from kidney beans.

Overall changes in the structure of protein can be estimated by comparing the denaturation enthalpy of native protein with that of the protein after processing (see Supplementary Table S2). Denaturation enthalpy is a measure of the amount of energy required to denature a protein. Hence, denatured proteins exhibit a lower or a lack of denaturation enthalpy compared with the native ones. Protein denaturation usually increases with the intensity of the heat treatment and has been suggested as the main reason for improved protein digestibility in thermally heat-treated ingredients [70, 84]. According to these authors, unfolding of the native secondary/tertiary structure of the protein could facilitate enzymic attack and their suggestion was based on several proteins of vegetable origin (e.g. wheat and soya). The conditions applied during processing probably determine whether proteins become more susceptible (e.g. unfolding and random coil formation) or more resistant (e.g. protein aggregation and chemical changes to amino acids) to enzymic attack. However, enthalpy of denaturation can also increase after long thermal treatments as reported [85] for soyabean protein isolates prepared from heat-treated soyabeans at 40 and 80°C for 4, 8, 12 and 16 h. The denaturation enthalpy of this material decreased after 4 and 8 h of thermal treatment, but increased again with longer heating times. It is possible that protein refolding occurred at longer heating times causing the denaturation enthalpy to increase. Most of the denaturation enthalpy observed, ranging from 36 to 70% of the enthalpy in the original material (2.85 J/g), originated from glycinin, which was less denatured than β-conglycinin under these conditions.

Only a limited number of studies has been performed on the link between denaturation enthalpy and digestibility of processed vegetable protein sources [85-88]. The positive correlation between the relative degree of denaturation (i.e. decrease of enthalpy after thermal treatment with respect to the enthalpy of the native material) and *in vitro* crude protein digestibility for protein-containing ingredients (i.e. sweet potato protein, cowpeas and soyabeans) was significant ($r = 0.49$, $P = 0.003$, $n = 34$) (Fig. 2.4). However, these ingredients probably contained protease inhibitors. In that case, the relation between the degree of protein denaturation and the *in vitro* crude protein digestibility would be somewhat confounded with increased apparent digestibility due to the heat inactivation of protease inhibitors. The amino acid sequence of sporamin (sweet potato protein) resembles that of Kunitz-type trypsin inhibitors, possibly explaining the increase in digestibility after denaturation [88]. Proteins from cowpeas and soyabeans can be classified under the same 'Osborne' type. However, their amino acid compositions are different, which possibly lead to different structural conformations (e.g. secondary and tertiary) and explain the differences in the correlations between the relative degree of denaturation and *in vitro* digestibility (Fig. 2.4). In addition, whilst the *in vitro* digestibility of sweet potato protein and cowpeas was determined using multiple enzymes (e.g. porcine pepsin and pancreatin), the digestibility of soyabean proteins was determined after incubation with only pepsin. Incubation with a single enzyme might lead to an underestimation of protein digestibility. For isolated albumins from *P. vulgaris* there is a trend for a negative correlation between the relative degree of denaturation and the *in vitro* digestibility ($r = -0.53$, $P = 0.09$, $n = 11$) (Fig. 2.4). The degree of purity of the isolated albumin fraction is not described in that study [87], thus this fraction might still contain trypsin inhibitors. The limited number of studies available, the inclusion of sources that contain protease inhibitors and the inconsistency in the results do not allow us to draw firm conclusions on the effects of protein denaturation on protein digestibility. The effect of protein denaturation on protein digestibility could be linked to the nature of the protein.

Effects of moisture

Water or steam are usually added as co-factors during processing in order to manage the moisture content and the physical properties of the processed material. The moisture content ranges between 12 and 18% (w/w) for pelleting [15, 76], whereas extrusion processing tolerates higher moisture inclusion ranging from 20 to 35% (w/w) [76].

Proteins in dry conditions have high glass transition temperatures (T_g), which can be close to their degradation temperature [89, 90]. The T_g can be reduced by the addition of water, which act as a protein plasticiser, and decreases T_g approximately 10°C for every 1% increase in moisture content [90]. Due to the small size of its molecules, water can move

freely through the small openings of the structure of proteins. Water decreases the T_g by disrupting hydrogen bonds, van der Waals and ionic interactions that hold the structure of protein together, thereby increasing its flexibility [89]. Increased flexibility and rupture of the bonds also decrease the denaturation temperature of proteins. However, the flexibility of proteins is limited below water contents of 5-9%. Exposure of hydrophobic amino acid residues due to changes in the conformation of proteins after the addition of water can lead to irreversible protein aggregation [91]. The denaturation temperature of sunflower (*Helianthus annuus*) oil cake globulins decreased from 189.5°C to 154.4, 133.4 and 119.9°C with increasing moisture contents of 0, 10, 20 and 30%, respectively [92]. The T_g of these proteins showed a similar pattern, decreasing from 180.8°C with no water to 5.3°C at 26% moisture content. Similar results were also reported for β-conglycinin [93] and glycinin [93, 94] from soyabean meal. The denaturation temperature of β-conglycinin and glycinin were 76.5°C and 93.3°C, respectively, with a moisture content of 94%. However, when the moisture content was decreased to 29%, the denaturation temperature of β-conglycinin was increased to 180°C and no denaturation temperature could be found for glycinin. The addition of water decreases the T_g of both native and denatured glycinin, with a faster rate for the native proteins [94].

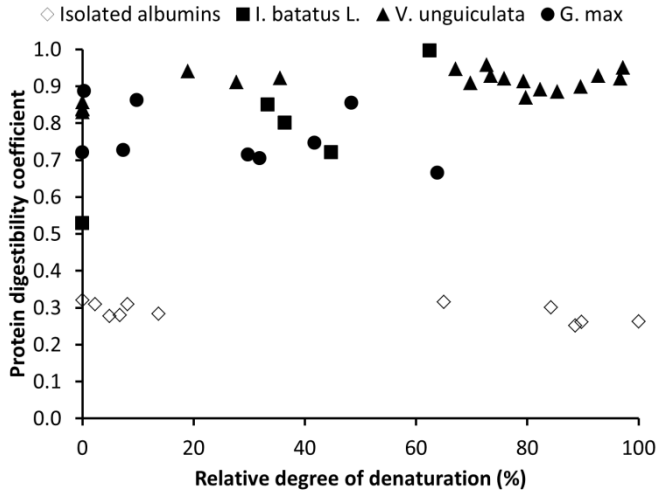

Fig. 2.4. Correlation between the relative degree of denaturation and *in vitro* crude protein digestibility coefficient of thermally treated isolated albumins from kidney beans (open) and other protein sources (closed).

Thus, water addition increases the flexibility of protein structure and in combination with heat, shear or pressure, changes the effects of these factors on the secondary protein structure (Table 2.3), *in vitro* crude protein digestibility (Table 2.4) and protein solubility (see online Supplementary Table S1). Increasing the moisture content during extrusion

processing, whilst keeping a constant temperature, increases protein denaturation and refolding, with the formation of new bonds. For example, the percentage of protein solubilised by a solution of 50 mM DTT in a phosphate buffer after extrusion at 170°C of a soya protein, wheat gluten and wheat starch mix (weight ratio 60:40:5) increased from 10 to 30 when moisture content (w/w) was increased from 60 to 72% [81]. This indicates that during processing the extents of both covalent and non-covalent interactions increase with increasing moisture contents, possibly related to unfolding, which is the rate-limiting step. The increased interaction between proteins could explain the reduction of the *in vitro* digestibility of soyabean meal with increasing moisture contents at constant temperature and extrusion screw speed (Table 2.4). Alternatively, inactivation of trypsin inhibitors could have been higher at lower moisture contents, leading to higher *in vitro* crude protein digestibility.

Changes in the secondary structure of proteins have been reported when moisture-including sources of heat were used (e.g. using autoclaving, desolventization/toasting) in contrast with dry sources of heat (e.g. roasting, IR radiation) at similar temperatures (Table 2.3). This was the case for lentils (*Lens culinaris*), in which no effects were reported for dry heating at 120°C for 30 min, whilst autoclaving at similar temperatures (120°C for 20 min) increases the α-helices and random coil contributions. The latter treatment also reduces the contribution of the intramolecular β-sheets conformation in exchange for a larger amount of intermolecular β-sheets [30]. However, the response of the secondary structure of proteins to changes in moisture could be source-dependent, as not all the ingredients analysed follow the same pattern. Whilst a dry source of heat at 120°C for 30 min induces the appearance of random coil structures in kidney beans, the same thermal treatment does not induce the formation of these structures in lentils [30, 95]. These differences could be related to the proportion of the different types of proteins in each ingredient (e.g. globulins *vs.* albumins), or to the inherent structural characteristics of the proteins of each source. In these studies [30, 95] the seeds were autoclaved, along with the water that was used for soaking them (1:4, w/v) during 2 h.

Shear effects

Multiple definitions have been provided for shear. As defined for extrusion processing [96], shear is the dissipation of the mechanical energy input by friction of the particles inside the extruder barrel. The estimation of shear is based on the torque produced by the engine and on the calculation of specific mechanical energy (SME) input. Different levels of shear are involved in the equipment, which is commonly used for compound feed production. Pelleting consumes a low level of SME, ranging between 23 and 45 kJ/kg, and the levels are highly dependent on the fat level inclusion in the diet [97, 98] and the size of the die. The technology that is considered to cause the highest levels of shear is extrusion, with

SME ranging from 839 to 1277 kJ/kg [68]. As a system parameter, torque is the result of the combination of several process parameters, such as temperature, moisture content, feeder-screw speed, extruder-screw speed and screw configuration. Thus, any change in the process parameters during extrusion can lead to an alteration of the SME input. Separating the effects of SME input from other process parameters used during processing in complex systems such as extrusion is difficult, and literature describing changes in protein structure due to shear only is scarce.

The effects of simple shear flow on protein structure and function have been reviewed before [99]. However, most of these research has been performed on model systems of proteins in solution [100, 101], which do not resemble the conditions used during feed production. Simple shear of proteins can induce conformational changes to the structure of proteins by disrupting the secondary/tertiary structure, even at relatively low temperature, as illustrated by the work of Dunstan and colleagues [100, 101].

Protein aggregation in soya isolates has been reported as a consequence of extrusion [68]. Vital factors during extrusion, such as a high temperature and pressure, were suggested to be responsible for protein aggregation [68]. However, with increasing SME levels, the molecular weight of the protein aggregates was reduced. The molecular weight of the aggregates was reduced by 5, 9 and 17% at SME inputs of 1050, 1093 and 1277 kJ/kg, respectively, compared with the molecular weight at the 839 kJ/kg extruder setting [68]. It was suggested by Meade *et al.* [54] that the mechanical forces during extrusion could break the peptide bonds in the amino acid chain (effects on primary protein structure), thus producing a similar effect as proteolysis.

Increased levels of non-covalent and covalent bonds, which lead to larger amounts of aggregation, were reported [102] in soya proteins with increasing SME. In this experiment, shear contrasts were produced by adaptations to the screw configuration of the extruder. With the screw configurations that produced increased levels of shear, there was also a decrease in the solubility after the extraction of proteins with urea or DTT and the successive extractions with solutions of urea/DTT or DTT/urea. This could indicate that after extrusion at high mechanical energy inputs, proteins may interact by mechanisms other than the formation of disulfide or non-covalent bonds. These mechanisms could be covalent bonds related to crosslinking between amino acids [102].

The formation of Maillard reaction products (and possibly also protein structural modifications) is affected by the interaction between temperature and mechanical energy [103]. High SME inputs produced large amounts of Maillard reaction products when extrusion of a rice (*Oryza spp.*)-glucose-lysine model mixture was performed at barrel temperatures of 100 and 130°C, but not at 70°C [103]. Increasing the screw speed from 80 to 140 rpm had

limited effect on the *in vitro* crude protein digestibility of commercial soyabean meal after extrusion [104].

Although there is little evidence that crude protein digestibility might be largely affected after shear-processing, the formation of crosslinked or Maillard-modified amino acids has a large impact on the nutritional value of the feed. The crosslinked and modified amino acids cannot be utilised after absorption [105], and therefore animal performance is decreased.

Particle size reduction processing

The amount of mechanical energy involved in particle size reduction is related to the type of equipment employed. SME ranges from negligible in roller milling to 104.4-126 kJ/kg for hammer milling [106] and 6.5-10.1 kJ/kg for the multicracker system [107]. Hammer and roller milling are the most frequently used technologies for particle size reduction of compound feed ingredients, although new technologies, such as the multicracker, have been developed [107].

The effects of particle size reduction on digestibility has been analysed before [21, 108-110]. During particle size reduction, rupture of protein bodies from grains and legume seeds that contain storage proteins increases protein solubility [111], which in turn could facilitate enzyme accessibility. However, only few studies report improved enzyme accessibility due to a larger area of exposure of the substrate [112]. Few studies have been performed on the effects of particle size reduction on protein structure. This is probably related to the fact that particle size reduction is regarded as only a physical change and as such, it is assumed not to have major effects on the structural characteristics of proteins.

Some of the technologies that are used to reduce particle size of ingredients involve the use of moisture, whilst others are performed under dry conditions. Wet-milling of maize dried distillers grains with solubles increased *in vitro* crude protein digestibility [113]. It is possible, as suggested by these authors, that the disruption of the cell wall structure leads to increased enzyme accessibility and that the improved *in vitro* digestion is not linked directly to changes in protein structure, although the latter point was not measured by the authors. Supporting this suggestion, is the observation that there were no changes in the secondary protein structure after wet-milling of silk from *Philosomia cynthia ricini* and *Bombyx mori* silkworms [114]. In contrast, when dry milling was used in the same study, the intramolecular β-sheet structures in these proteins completely disappeared, probably due to the processes involved in dry milling, including shear. The silk in that study was wet (i.e. addition of water) or dry milled using a ball mill followed by air jet milling. In the case of wet milling, water could act as a lubricant, thereby decreasing the amount of shear. It is possible that the disappearance of the intramolecular β-sheets after dry milling could increase protein

digestibility as previously reported by Carbonaro *et al.* [30]. Furthermore, dry milling could have the additional benefit of the disruption of cell wall structures (as mentioned before for wet-milling), which can increase the access of enzymes for protein hydrolysis.

CONCLUSIONS

The structure of proteins influences enzyme accessibility for protein digestion. Some commonly used feed ingredients in swine diets exhibit structural constraints for digestion in their native proteins. Both primary processing of ingredients and compound feed production as a secondary treatment can affect the structure of native and partially denatured proteins. However, the link between the conformational changes and protein digestibility is not clear and further research should help to elucidate these underlying mechanisms. Most literature available that links protein structure to digestibility describes protein sources or ingredients which still contain protease inhibitors. It is necessary for future research to take this aspect into account and study the relationship between the structure of proteins (native or processed) and digestibility in isolated proteins, excluding the effect of protease inhibitors on digestibility. Correlations between structural properties of proteins (e.g. free thiol content or degree of denaturation) with protein digestibility after processing seem to be dependent on the protein nature. Heat-induced modifications in the structure of proteins during primary processing render bonds (intra- and intermolecular) and structures, which become irresponsive to secondary processing and allow limited access to proteolytic enzymes. When proteins did not undergo complete denaturation during primary processing, secondary processing might change protein structure, with positive or negative effects on digestibility. Heat seems to be the parameter during secondary processing with the largest influence on structural changes of proteins. Nevertheless, other factors involved during processing (e.g. moisture and shear) could also have a decisive role. However, the multifactorial essence of the complex processing technologies used in compound feed production does not allow yet to disentangle and explain the effects of each separate processing factor. In this sense, model systems could aid in separating the processing factors and explaining their effects on the structural conformation and nutritional value of proteins.

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Supplementary Table S1. Protein solubility in phosphate buffer and solutions of non-covalent and covalent cleaving agents after processing under different conditions [a].

Feedstuff/material	Treatment	Protein solubility (% of total protein)				Reference
		PB	PB + non-covalent cleaving agent	PB + covalent cleaving agent	PB + both	
Wheat flour[b]	Control	17	67	21	99	57
	Extrusion 160°C	9	16	12	88	
	Extrusion 170°C	7	16	14	79	
	Extrusion 185°C	9	15	13	79	
Wheat flour	Control		87			142
	Extrusion 140°C, 20% moist., 41.1 Wh/kg		28			
	Extrusion, 160°C, 20% moist., 34.9 Wh/kg		22			
	Extrusion 180°C, 20% moist., 32.1 Wh/kg		29			
	Extrusion 140°C, 24% moist., 35.5 Wh/kg		38			
	Extrusion 160°C, 24% moist., 30.8 Wh/kg		25			
	Extrusion 180°C, 24% moist., 28.6 Wh/kg		21			
Rapeseed meal	Control	60	29			143
	Dried at 37°C	51	27			
	Dried at 60°C	34	30			
Soybean meal	Control / Untoasted SBM	47				102
	Toasted SBM	9				
	Extrusion 0 flights, 120°C, 100 rpm	4				
	Extrusion 4 flights, 120°C, 100 rpm	3				
	Extrusion 8 flights, 120°C, 100 rpm	4				
Soybean meal	Control	20			32	144
	Heat treatment low humidity	25			39	
	Heat treatment high humidity	17			21	
SPI	Control	20	75	43	80	80
	Cold induced gel 25°C	10	71	50	71	
	Heat induced gel 85°C, 60 min	8	68	45	77	
	Heat induced gel 95°C, 60 min	9	70	42	70	

Supplementary Table S1. continued

Feedstuff/material	Treatment	Protein solubility (% of total protein				Reference
		PB	PB + non-covalent cleaving agent	PB + covalent cleaving agent	PB + both	
SPI:wheat starch (90:10)[c]	Control	7.5	33.1	30.2	83.9	79
	Extrusion 129°C, 70% moist, 7.1% torque	1.4	27.4	17.2	71.1	
	Extrusion 129°C, 65% moist, 9% torque	2	21.5	10.8	62.9	
	Extrusion 130°C, 60% moist, 12% torque	2.2	18.7	12.6	63.3	
SPI:wheat gluten:wheat starch (60:40:5)[c]	Control	20	85	37	86	80, 81
	Extrusion 170°C, 60% moist	4	22	10	80	
	Extrusion 170°C, 67% moist	3	58	15	83	
	Extrusion 170°C, 72% moist	4	70	30	81	

[a] PB: phosphate buffer; non-covalent cleaving agents: SDS, urea; covalent cleaving agents: DTT, 2-mercaptoethanol; SPI, soy protein isolate.
[b] Analysis determined in the soluble phase or filtrate of processed proteins.
[c] Reported mixing ratio.

Supplementary Table S2. Denaturation enthalpy (J/g) and *in vitro* crude protein digestibility coefficient after thermal treatments under different conditions.

Feedstuff/material	Treatment	Denaturation enthalpy (J/g)	Protein digestibility coefficient	Reference
Cowpea				
variety Cuarenton	Control	7.76	0.86	86
	Cooked 100°C, 20 min	5.00	0.92	
	Cooked 100°C, 60 min	2.55	0.95	
	Autoclaved 121°C, 10 min	2.12	0.96	
	Autoclaved 121°C, 30 min	0.22	0.95	
variety Colorado	Control	7.22	0.84	86
	Cooked 100°C, 20 min	5.22	0.91	
	Cooked 100°C, 60 min	2.18	0.91	
	Autoclaved 121°C, 10 min	5.85	0.94	
	Autoclaved 121°C, 30 min	1.92	0.93	
variety San Francisco	Control	9.01	0.84	86
	Cooked 100°C, 20 min	1.83	0.87	
	Cooked 100°C, 60 min	1.59	0.89	
	Autoclaved 121°C, 10 min	2.18	0.92	
	Autoclaved 121°C, 30 min	1.86	0.91	
variety Z_1	Control	8.57	0.83	86
	Cooked 100°C, 20 min	1.25	0.89	
	Cooked 100°C, 60 min	0.89	0.90	
	Autoclaved 121°C, 10 min	0.62	0.93	
	Autoclaved 121°C, 30 min	0.28	0.92	
Soybean	Control	2.85	0.71	85
	40°C, 4 h	2.00	0.86	
	40°C, 8 h	1.47	0.86	
	40°C, 12 h	2.57	0.89	
	40°C, 16 h	2.84	0.67	
	80°C, 4 h	1.03	0.71	
	80°C, 8 h	1.94	0.73	
	80°C, 12 h	2.64	0.73	
	80°C, 16 h	1.66	0.75	
Kidney beans isolated proteins				
Albumins	Control	7.74	0.32	87
	60°C, 1 min	7.11	0.31	
	60°C, 5 min	7.56	0.31	
	60°C, 10 min	7.22	0.28	
	60°C, 30 min	6.68	0.28	
	60°C, 60 min	6.12		
	80°C, 1 min	7.36	0.28	
	80°C, 5 min	2.71	0.32	
	80 °C, 10 min	1.22	0.30	
	80°C, 30 min	0.88	0.25	
	100°C, 1 min	0.79	0.26	
	100°C, 5 min	0	0.26	
Sweet potato	Control	9.6	0.56	88
	60°C, 20 min	5.3	0.72	
	80°C, 20 min	6.1	0.80	
	100°C, 20 min	6.4	0.85	
	127°C, 20 min	3.6	1.00	

CHAPTER 3

Physical and chemical changes of rapeseed meal proteins during toasting and their effects on *in vitro* digestibility

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ABSTRACT

Toasting during the production of rapeseed meal (RSM) decreases ileal crude protein (CP) and amino acid (AA) digestibility. The mechanisms that determine the decrease in digestibility have not been fully elucidated. A high protein quality, low-denatured, RSM was produced and toasted up to 120 min, with samples taken every 20 min. The aim of this study was to characterize secondary structure and chemical changes of proteins and glucosinolates occurring during toasting of RSM and the effects on its in vitro CP digestibility. The decrease in protein solubility and the increase of intermolecular β-sheets with increasing toasting time were indications of protein aggregation. The contents of NDF and ADIN increased with increasing toasting time. Content of arginine, lysine and *O*-methylisourea reactive lysine (OMIU-RL) linearly decreased with increasing toasting time, with a larger decrease of OMIU-RL than lysine. First-order reactions calculated from the measured parameters show that glucosinolates were degraded faster than lysine, OMIU-RL and arginine and that physical changes to proteins seem to occur before chemical changes during toasting. Despite the drastic physical and chemical changes noticed on the proteins, the coefficient of *in vitro* CP digestibility ranged from 0.776 to 0.750 and there were no effects on the extent of protein hydrolysis after 120 min. In contrast, the rate of protein hydrolysis linearly decreased with increasing toasting time, which was largely correlated to the decrease in protein solubility, lysine and OMIU-RL observed. Rate of protein hydrolysis was more than 2-fold higher for the untoasted RSM compared to the 120 min toasted material. Increasing the toasting time for the production of RSM causes physical and chemical changes to the proteins that decrease the rate of protein hydrolysis. The observed decrease in the rate of protein hydrolysis could impact protein digestion and utilization.

Keywords: hydrolysis rate, *in vitro* protein digestibility, rapeseed meal, reactive lysine, secondary structure.

INTRODUCTION

Rapeseed meal (RSM) is the most important protein source utilized in commercial swine and poultry diets after soybean meal [1-3]. The production process of RSM involves toasting to remove the organic solvent remaining after solvent extraction of the oil and to inactivate antinutritional factors present such as glucosinolates [4]. Direct application of live steam is used during toasting to complete the solvent removal, which also increases the moisture content. The toasting process time usually ranges from 60 to 90 min at 100 to 110°C, which can increase the variation in the lysine content and ileal digestibility of most AA in RSM [4, 5]. The coefficient of variation of the apparent ileal digestibility of lysine in poultry increased from 1.4% in the solvent extracted meal to 5.4% after toasting [5].

Both physical and chemical changes of proteins due to thermal processing can influence protein digestibility [6]. Autoclaving at 120°C for 20 min increased the proportion of random coil in the secondary protein structure of legume seeds, which is related to protein denaturation and increased CP *in vitro* digestibility [7]. At the same time, appearance of intermolecular β-sheets, linked to decreased protein digestibility, was reported in the same study. The net effect on the *in vitro* crude protein (CP) digestibility seems to be related to the ratio between both types of physical changes. Chemical changes can be the result of Maillard reactions or covalent crosslinking between amino acids (AA). Increasing the toasting time decreased the lysine and reactive lysine contents [8]. In addition, the standardized ileal digestibility of CP was reduced from 66 to 60% and that of lysine from 64 to 54% when the toasting time was increased from 48 to 93 min [8]. Chemical changes due to Maillard reactions were suggested to be responsible for the decrease in protein and amino acid digestibility. However, chemical changes of proteins due to Maillard reactions do not completely explain the reduction in the digestibility of all AA, as observed in that study. This suggests that also changes in the structure of proteins (e.g. secondary and tertiary) affect the digestion process.

The aim of the present experiment was to characterize the physical and chemical changes that occur to rapeseed proteins during toasting and the influence of these changes on *in vitro* CP digestibility. We hypothesize that increasing toasting times causes physical and chemical changes to rapeseed proteins, resulting in decreased *in vitro* CP digestibility.

MATERIALS AND METHODS

Materials

A batch of commercially available winter 00-rapeseed (*Brassica napus*), harvested in the southwest of France in 2013, was used. All chemicals used were of analytical grade. Pepsin (2,000 FIP U/g) was obtained from Merck (Darmstadt, Germany), whilst pancreatin (grade IV from porcine pancreas), trypsin (type IX-S, 13,000 – 20,000 BAEE units/mg protein), chymotrypsin (type II, >40 units/mg protein), and peptidase from porcine intestinal mucosa (50-100 units/g solid) were obtained from Sigma-Aldrich (St Louis, MO, USA).

Rapeseed meals preparation

All processing of the rapeseed was conducted at the pilot plant of CREOL (Pessac, France). The batch of rapeseed was dried in a warm-air dryer at 70°C to a moisture level of 5% (w/w) (Fig. 3.1). After drying, the rapeseed was cold pressed (La Mecanique Moderne MBU 75 type, Arras, France) at 250 kg/h. Temperatures during pressing did not exceed 80°C. Continuous extraction of the cake by hexane was performed on a belt extractor (B-1930, Desmet-Ballestra, Zaventem, Belgium) at 160 kg/h flow of the cake and 230 L/h flow of solvent. Temperature of the rapeseed cake during solvent extraction did not exceed 55°C. The solvent remaining after solvent extraction of the oil was removed using indirect heat (i.e. without use of direct live steam) in a desolventizer-toaster (Schumacher type, Desmet-Ballestra, Zaventem, Belgium; 6 trays with a rotating arm and 1 m internal diameter) for 60 min. Temperatures during desolventization were 90 ± 3°C. Spot samples (5 kg) were obtained after drying, cold-pressing, hexane extraction, and desolventization with indirect heat.

A batch of 150 kg of the desolventized-untoasted RSM was toasted in the lower tray of the desolventizer-toaster with injection of live steam (30 kg/h), whilst indirect steam pressure was set at 3 bars and arm rotations at 20 rpm. These conditions were maintained for 120 min, with spot samples (5 kg) taken every 20 min through a door in the desolventizer-toaster. Monitoring of time was initiated when the temperature inside the desolventizer-toaster reached 100°C. A second batch of 150 kg of the desolventized-untoasted RSM was used for duplication of the toasting experiment on the next day (Fig. 3.1). The samples obtained from both toasted batches (in total 12 toasted RSM plus the untoasted RSM) were analyzed separately. Temperatures during toasting ranged from 107 to 112°C on the first day and from 109 to 112°C on the second day.

Analytical methods

The desolventized RSM and the toasted RSMs were ground to pass a 1 mm sieve using a centrifugal mill (ZM200, Retsch, Haan, Germany) at 8,000 rpm prior to chemical analysis. Samples (5 g) were re-ground using a ball (Ø 12 mm) mill (MM2000, Retsch) at a frequency of 80 during 3 min and used for secondary structure, degree of denaturation, amino acid and reactive lysine analysis. All chemical and secondary structure analyses were performed in duplicate, except for reactive lysine, which was performed in triplicate.

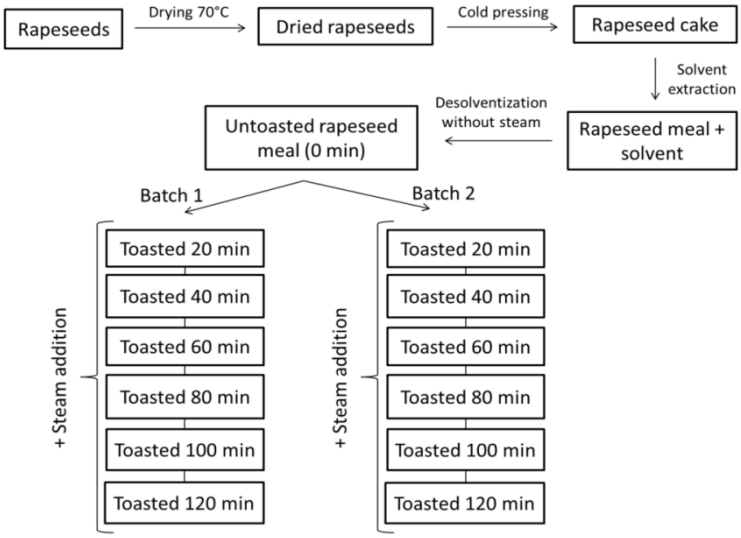

Fig. 3.1. Schematic view of the experimental rapeseed treatment.

Nutrient contents

Dry matter (DM) content was determined by oven-drying at 103°C to constant weight according to ISO 6496 [(9)]. Nitrogen content was analyzed by combustion according to AOAC 968.06 (Thermo Quest NA 2100 Nitrogen and Protein Analyzer; Breda, The Netherlands) [(10)]. Nitrogen content in the nitrogen solubility index (NSI) and nitrogen linked to the acid detergent fiber (ADIN) determinations were measured using the Kjeldahl method according to ISO 5983 [(11)]. A conversion factor of 6.25 was used for the calculation of CP content from nitrogen. Crude fat content was determined according to ISO 734-2 [(12)]. The neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were determined using a fiber analyzer equipment (Fiber Analyzer, Ankom Technology, Macedon, NY) according to a modification of the procedure of Van Soest *et al*. [(13)]. The NDF determination involved enzymatic incubation with α-amylase and alcalase, but without addition of sodium

sulfite. The ADIN content was determined in the residues after hydrolysis with acid detergent reagents.

For the determination of amino acid content, samples were hydrolyzed with 6 M HCl at 110°C for 23 h and the hydrolysates were adjusted to pH 2.2 using NaOH. Amino acids were determined by post column reaction with ninhydrin, after separation by ion exchange chromatography. Photometric detection was performed at 570 nm and at 440 nm for proline according to ISO 13903 [14]. Norleucine was used as an internal standard.

Reactive lysine was determined using a method described by Moughan and Rutherfurd [15]. In short, reactive lysine was converted into homoarginine by incubation with *O*-methylisourea (OMIU) for 7 d. Samples were subsequently hydrolyzed with 6 M HCl at 110°C for 23 h and the hydrolysates were adjusted using NaOH to pH 2.2. After separation by ion exchange chromatography, the homoarginine content was determined by post column reaction with ninhydrin using photometric detection at 570 nm. The amount of OMIU-reactive lysine (OMIU-RL) was calculated from the molar amount of homoarginine and the molecular weight of lysine.

Glucosinolates were quantified according to ISO 9167-1 [16]. Glucosinolates were extracted by a 70% (v/v) methanol in water mixture at 70°C, using sinigrin as internal standard. The glucosinolates were then linked on an anion-exchange column, purified and on-column desulphated by overnight action of sulphatase enzyme. Desulphoderivatives were eluted with water and analyzed using reverse phase liquid chromatography with gradient elution and UV detection at 229 nm.

Differential scanning calorimetry

Degree of denaturation of the samples was studied using differential scanning calorimetry (DSC12E, Mettler-Toledo, Greifensee, Switzerland). Samples (15 – 20 mg) were weighed into medium pressure crucibles (ME-29990, Mettler-Toledo, Greifensee, Switzerland) and approximately 60 mg of demineralized water was added. Samples were left overnight to equilibrate at 4°C. The heating program ranged from 15 to 120°C at a rate of 5°C/min, with an initial isothermal step of 5 min at 15°C. A crucible filled with demineralized water was used in the reference cell. Enthalpy (J/g CP) of denaturation was determined using the TA89E software (Version 3, Mettler-Toledo) for analysis of thermo-analytical data.

Protein solubility

Protein dispersibility index (PDI) in water was measured using a modification of the method of AOCS [17]. Approximately 75 mg of sample was weighed and mixed for 30 s with 1.5 mL of water in a vortex. The sample was then mixed for 20 min in a rotator SB2 (Stuart-

Barloworld Scientific Staffordshire, UK) with an angle of 90° and a speed of 20 rpm. Centrifugation was performed at 13,000 × g for 10 min at room temperature and the supernatant analyzed for nitrogen content. Soluble protein in 0.2% (w/v) KOH, equivalent to nitrogen solubility index (NSI), was measured according to ISO 14244 [18].

Protein secondary structure

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) (Tensor 27, Bruker, Billerica, MA, USA) was used to measure the spectra ranging from 600 to 4,000/cm. The spectra were measured as absorbance with a resolution of 4/cm and using 16 scans per spectra. Spectral measurements were performed in duplicate and corrected for background. The OPUS software Version 7.2 (Bruker) was used for all spectral transformations and calculations according to the procedure described by Hu *et al.* [19] with minor modifications. Briefly, Fourier self-deconvolution was applied to the Amide I region (1,595 – 1,705/cm) of the original spectra, using a Lorentzian correction with a bandwidth of 25/cm and a noise reduction factor of 0.3. The second derivative was applied to the original spectra and used for peak selection. Curve fitting of the selected peaks was performed using a Gaussian approximation with the Levenberg-Marquardt algorithm as the fitting method and an iteration time of 10 s. Selected peaks were identified using existing literature [7, 20].

***In vitro* digestibility**

Two-step enzymatic digestibility

In vitro dry matter and CP digestibility were determined using a modification of the method from Boisen and Fernández [21]. Briefly, 5 g of material was mixed with 125 mL of a pH 6.0 disodium phosphate buffer (0.1 M) and 50 mL of 0.2 M HCl. This mixture was incubated with 5 mL of a freshly prepared pepsin solution (0.025 g/mL) for 2 h at 39°C and pH 2.0. After this incubation, 50 mL of pH 6.8 sodium phosphate buffer (0.2 M) and 25 mL of 0.6 M NaOH were added. The pH was adjusted to 6.8, 5 mL of a freshly prepared pancreatin solution (0.10 g/mL) were added and the mixture incubated for 4 h. All buffers and solutions were preheated at 39°C before addition, with the exception of the enzyme solutions. After the latter incubation, 5 mL of a 20% (w/v) sulfosalicylic acid solution was added and the mixture centrifuged at 4,500 × g for 10 min at room temperature. The insoluble residue was collected, freeze-dried and analyzed for dry matter and nitrogen content.

pH-STAT enzymatic hydrolysis

Enzymatic hydrolysis was performed with the addition of porcine trypsin, bovine chymotrypsin and porcine intestinal peptidase using a modification of the pH-STAT method from Pedersen and Eggum [22]. In contrast to the original method, the hydrolysis was extended for 120 min after the addition of the enzymes. The volume of 0.1 M NaOH added was used for the calculation of the degree of hydrolysis (DH) according to Eq. 1:

(1) $\mathrm{DH} = (Vb \times Nb)/(\alpha \times mp \times \mathrm{htot})$

in which *Vb* is the volume of NaOH solution added, *Nb* is the normality of the titration solution, α is the degree of dissociation of the α-NH_2 group (i.e. 0.794 at 37°C and pH 8), *mp* is the mass of protein in grams and *htot* the total number of peptide bonds per gram of substrate (7.8 eq/g). The DH was used to calculate the rate of protein hydrolysis (*k*) based on the model described by Butré *et al.* [23] shown in Eq. 2:

(2) $\mathrm{DH} = 1/b \times ln(k \times ht + 1)$

In this model *b* is a parameter that defines the shape of the curve, *k* is the constant for the rate of protein hydrolysis (s^{-1}) and *ht* is the hydrolysis time (s). The model was fitted using the MODEL procedure of SAS.[24]

Calculations and statistical analysis

Degradation rate constants and half-life for the parameters were calculated according to first-order reactions which were selected after fitting zero and second order reactions. Regression equations for the effect of toasting time were generated using the GLM procedure of the statistical software SAS [24]. Correlations between parameters related to protein changes (e.g. NSI, PDI, lysine and OMIU-RL content, secondary structure) and *in vitro* digestibility (e.g. CP digestibility, DH after 120 min, *k*) were determined using the CORR procedure of SAS. Linear or quadratic effects were considered as significant when the *P*-values were lower than 0.05 and as trends when *P*-values were between 0.05 to 0.10. The experimental unit was the RSM at each toasting time point.

RESULTS

During the oil extraction process of the rapeseed seeds, the crude fat content was reduced from 493 g/kg DM in the seeds to 16 g/kg DM in the untoasted meal (Table 3.1). At the same time, the NSI was decreased from 86.9% in the seed to 79.9% in the untoasted meal.

There was no effect of toasting time on the CP content of RSM, whilst there was a linear increase (*P* = 0.02) of the DM content with increasing toasting time (Table 3.1). There

was a 33% linear increase ($P < 0.001$) in the NDF content with increasing toasting time from the untoasted to the 120 min toasted RSM. In contrast, the ADF content was not affected ($P > 0.05$) by toasting time. Linear ($P < 0.001$) and quadratic ($P = 0.02$) effects of toasting time were found on the content of ADIN. The increase in the content of ADIN was more evident after 60 min toasting. Toasting time had a linear effect ($P = 0.02$) and a tendency for a quadratic effect ($P = 0.07$) on the denaturation enthalpy, which decreased with increasing toasting time. There were linear ($P < 0.001$) and quadratic ($P < 0.001$) effects of toasting time on NSI and PDI, with more apparent effects at low toasting times.

Glucosinolates content

There were linear and quadratic effects of toasting time on the content of total ($P = 0.001$), alkenyl ($P < 0.01$) and indolyl plus aralkyl ($P < 0.001$) glucosinolates (Table 3.2). The largest decrease seems to occur after 60 to 80 min of toasting. Not all the glucosinolate types, however, responded to toasting in the same manner. Whilst the contents of epi-progoitrin, sinalbin and neoglucobrassicin were linearly reduced ($P < 0.001$) with increased toasting times, the effect of toasting time was both linear and quadratic ($P < 0.05$) for the other glucosinolates. The most abundant alkenyl glucosinolates was progoitrin (Table 3.2) which, even after toasting for 120 min, remained present at 9% of its content in the untoasted RSM. Gluconapoleiferin was the most resilient alkenyl glucosinolate after toasting, as 16% of the content of the untoasted RSM can still be found after 120 min toasting.

Amino acids content

The amino acid content is reported in Table 3.3. There was a linear decrease ($P < 0.05$) in the content of alanine, aspartic acid, glutamic acid and glycine with increasing toasting time. Increasing toasting time also caused a linear decrease ($P < 0.001$) of the lysine and arginine content. Arginine and lysine contents were reduced by 7 and 23%, respectively, after toasting for 120 min in comparison with the untoasted RSM. The content of OMIU-RL was also reduced ($P < 0.001$) linearly with increasing toasting time. After 120 min of toasting, the OMIU-RL content was 38% lower than that in the untoasted sample. The reduction of the OMIU-RL content after toasting was more pronounced than the reduction of the lysine content. This is reflected in the reduction of the OMIU-RL to lysine ratio from 0.98 in the untoasted RSM sample to 0.80 in the RSM toasted for 120 min.

Table 3.1. Characterization of rapeseed samples before and after toasting [1].

Material	DM (g/kg)	CP (g/kg DM)	Crude fat (g/kg DM)	NDF (g/kg DM)	ADF (g/kg DM)	ADIN (g/kg DM)	Denaturation enthalpy (J/g CP)	NSI (g/kg CP)	PDI (g/kg CP)
Rapeseed	937	-	493	-	-	-	2.23	869	-
Dried rapeseed	947	-	489	-	-	-	-	861	-
Rapeseed cake	923	-	135	-	-	-	-	861	-
RSM + solvent	907	-	13	-	-	-	-	825	-
Toasting time RSM									
0 min	913.0	360.0	16	274.4	217.4	3.0	2.34	799.0	260.7
20 min	917.7	362.0	-	278.2	211.7	3.0	1.29	695.5	161.8
40 min	918.9	366.3	-	291.4	215.5	3.1	1.23	597.5	129.3
60 min	922.8	372.9	-	319.1	213.3	3.1	1.13	537.0	105.3
80 min	924.6	368.4	-	338.9	216.0	3.3	1.07	513.0	84.7
100 min	917.8	363.7	-	354.6	217.1	3.4	1.03	475.5	72.6
120 min	930.8	369.1	-	365.3	218.2	3.8	0.74	431.0	63.3
SEM	1.7	1.4		9.8	1.1	0.3	0.11	31	15
P-value									
Linear	0.02	0.19	-	<0.001	0.21	<0.001	0.02	< 0.001	< 0.001
Quadratic	0.90	0.15	-	0.91	0.40	0.02	0.07	< 0.001	< 0.001

[1] DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADIN, nitrogen linked to the acid detergent fiber; NSI, nitrogen solubility index; PDI, protein dispersibility index; RSM, rapeseed meal; SEM, standard error of the mean.

Table 3.2. Content (μmol/g DM) of glucosinolates in rapeseed meal samples toasted for different times [1].

Toasting time	PRO	EPRO	GNL	GNA	GBN	SNB	GST	4-OHGBS	GBS	NGBS	Alk	Ara+Ind	Total
0 min	12.62	0.36	0.81	4.83	2.66	0.23	0.63	5.50	0.23	0.11	21.28	6.70	27.98
20 min	10.18	0.28	0.68	4.01	2.08	0.25	nd	2.69	0.18	0.09	17.21	3.20	20.41
40 min	8.15	0.25	0.56	3.22	1.67	0.18	nd	1.39	0.13	0.08	13.83	1.77	15.60
60 min	5.94	0.16	0.41	2.47	1.11	0.16	nd	0.58	0.09	0.06	10.09	0.89	10.97
80 min	3.84	0.06	0.28	1.65	0.71	nd	nd	0.20	0.06	nd	6.52	0.26	6.78
100 min	2.27	nd	0.19	0.99	0.36	0.03	nd	0.06	nd	nd	3.81	0.08	3.89
120 min	1.13	0.02	0.13	0.46	0.20	nd	nd	nd	nd	nd	1.94	0.00	1.94
SEM	1.05	0.04	0.06	0.40	0.23	0.03	-	0.45	0.02	0.01	1.78	0.54	2.28
P-value													
Linear	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	-	< 0.001	< 0.001	< 0.001	<0.001	<0.001	<0.001
Quadratic	0.001	0.19	0.006	0.04	0.003	0.73	-	< 0.001	0.01	0.55	0.002	<0.001	<0.001

[1] PRO, progoitrin; EPRO, epi-progoitrin; GNL, gluconapoleiferin; GNA, gluconapin; GBN, glucobrassicanapin; SNB, sinalbin; GST, gluconasturtin; 4-OHGBS, 4-hydroxyglucobrassicin; GBS, glucobrassicin; NGBS, neoglucobrassicin; Alk, alkenyl glucosinolates; Ara+Ind, aralkyl plus indolyl glucosinolates; nd, not detected; SEM, standard error of the means. Alkenyl glucosinolates: PRO, EPRO, GNL, GNA, GBN; aralkyl glucosinolates: SNB, GST; indolyl glucosinolates: 4-OHGBS, GBS, NGBS.

Table 3.3. Amino acid contents (g/16 g N) and ratio between OMIU-RL and lysine in rapeseed meal samples toasted for different times [1].

Toasting time	Indispensable amino acids										Dispensable amino acids					
	Arg	His	Ile	Leu	Lys	OMIU-RL	Phe	Thr	Val	Ratio	Ala	Asp	Glu	Gly	Ser	Tyr
0 min	5.47	2.93	4.11	7.04	6.31	6.20	4.07	4.67	5.38	0.98	4.63	7.38	17.34	5.33	4.48	3.30
20 min	5.50	2.95	4.17	7.13	6.08	5.66	4.12	4.70	5.43	0.93	4.68	7.39	17.45	5.38	4.56	3.34
40 min	5.42	2.93	4.13	7.06	5.83	5.32	4.06	4.67	5.38	0.91	4.64	7.35	17.30	5.33	4.51	3.26
60 min	5.18	2.85	4.03	6.90	5.46	4.85	3.97	4.55	5.28	0.89	4.53	7.15	16.83	5.19	4.39	3.17
80 min	5.23	2.91	4.08	6.97	5.39	4.61	4.03	4.59	5.34	0.86	4.58	7.21	17.05	5.25	4.45	3.24
100 min	5.25	3.01	4.18	7.15	5.23	4.22	4.12	4.68	5.47	0.81	4.69	7.36	17.40	5.38	4.55	3.27
120 min	5.08	2.94	4.09	6.99	4.85	3.86	4.02	4.61	5.38	0.80	4.60	7.18	17.06	5.27	4.47	3.23
SEM	0.04	0.01	0.02	0.03	0.13	0.21	0.02	0.02	0.02	0.02	0.02	0.03	0.07	0.02	0.02	0.02
P-value																
Linear	<0.001	0.09	0.09	0.15	<0.001	<0.001	0.37	0.15	0.07	<0.001	0.05	0.02	0.03	0.04	0.06	0.35
Quadratic	0.72	0.24	0.23	0.24	0.74	0.32	0.29	0.17	0.17	0.92	0.14	0.30	0.16	0.16	0.12	0.16

[1] Arg, arginine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; OMIU-RL, O-methylisourea reactive lysine; Phe, phenylalanine; Thr, threonine; Val, valine; Ratio, ratio OMIU-RL to lysine; Ala, alanine; Asp, aspartic acid; Glu, glutamic acid; Gly, glycine; Ser, serine; Tyr, tyrosine; SEM, standard error of the mean.

Secondary protein structure

The proportion of intermolecular β-sheets tended (*P* = 0.06) to be affected by the quadratic effect of toasting time (Table 3.4). This proportion markedly increased after the initial 20 min of toasting and thereafter gradually decreased with longer toasting times. In contrast, the proportion of α-helix decreased by half after the initial 20 min of toasting and increased thereafter with toasting time from 16.2% after 20 min to 19.7% after 120 min toasting. Toasting time had a quadratic effect (*P* = 0.04) on the α-helix proportion. Linear (*P* = 0.04) and quadratic (*P* = 0.01) effects of toasting time were noticed on the T2 proportion. This element increased after the first 20 min toasting, but stabilize thereafter. Linear (*P* < 0.001) and quadratic (*P* = 0.004) effects of toasting time were also found for the proportion of A2 elements (Table 3.4). The increase in these elements was more apparent after the first 20 min of toasting than thereafter.

Table 3.4. Proportion (%) of the secondary structures of rapeseed meals toasted for different times [1].

Toasting time	Intermolecular β-sheets (1,627 – 1,630/cm) [2]	Intramolecular β-sheets (1,634 – 1,635/cm)	α-helix (1,655 – 1,656/cm)	T2 (1,674/cm)	A2 (1,692/cm)	α-helix : β-sheet
0 min	49.0	5.5	30.5	8.9	6.1	0.62
20 min	63.1	nd	16.2	13.7	6.9	0.26
40 min	60.8	nd	17.5	14.4	7.2	0.29
60 min	60.0	nd	18.0	14.6	7.4	0.30
80 min	59.4	nd	18.0	15.0	7.5	0.30
100 min	57.5	nd	20.4	14.6	7.5	0.36
120 min	57.9	nd	19.7	14.7	7.7	0.34
SEM	1.0	-	1.1	0.5	0.1	0.03
P-value						
Linear	0.94	-	0.63	0.04	< 0.001	0.56
Quadratic	0.06	-	0.04	0.01	0.004	0.03

[1] T2, turns; A2, intermolecular hydrogen-bonded β-sheets; nd, not detected; SEM, standard error of the mean.
[2] Regions of the Fourier transform infrared spectra.

Degradation rate constants

Indolyl, alkenyl and total glucosinolates had the highest degradation rate constants and the shortest half-life compared to the other parameters (Table 3.5). The degradation rate constant of indolyl glucosinolates was 2-fold higher than that of alkenyl glucosinolates. The half-life of total glucosinolates was approximately 5.5-fold lower than that of OMIU-RL and 10-fold lower than that of lysine. The degradation rate constant of OMIU-RL was almost twice that of lysine. The NSI had a degradation rate constant 2-fold as low as that of PDI, which is also reflected in a longer half-life.

In vitro CP digestibility

With the two-step enzymatic digestibility method, there was a tendency (P = 0.08) for a linear increase of the *in vitro* dry matter digestibility along with toasting time (Table 3.6). In addition, there was a quadratic effect of toasting time on the *in vitro* CP digestibility (P = 0.005), increasing before 60 min of toasting and decreasing thereafter. With the pH-STAT enzymatic hydrolysis method, there were no effects of toasting time on the DH after 120 min hydrolysis. However, the rate of protein hydrolysis was linearly (P < 0.001) reduced with increasing toasting time.

Table 3.5. Degradation rate constants and half-life (first order reactions) of parameters measured after toasting of rapeseed meal.

Parameter [1]	Degradation rate constant, $\times 10^{-3}$/min	Half-life, min
Enthalpy of denaturation	6.1	114
NSI	4.8	144
PDI	10.6	65
Alkenyl glucosinolates	20.4	34
Indolyl glucosinolates	44.3	16
Total glucosinolates	22.3	31
Arginine	0.7	990
Lysine	2.1	330
OMIU-reactive lysine	3.8	182

[1] NSI, nitrogen solubility index; PDI, protein dispersibility index.

The *in vitro* CP digestibility with the two-step enzymatic method did not correlate with any of the parameters of protein changes measured. In contrast, significant correlations were found between k and NSI (r = 0.88, P < 0.001), PDI (r = 0.79, P = 0.001), lysine (r = 0.92, P < 0.001), OMIU-RL content (r = 0.91, P < 0.001), and the proportion of A2 in the secondary structure (r = -0.74, P = 0.004). Significant correlations were also found between the DH after 120 min hydrolysis and the proportion of intermolecular β-sheets (r = -0.66, P = 0.01), α-helices (r = 0.60, P = 0.03) and the ratio of α-helices to β-sheets (r = 0.58, P = 0.04) in the secondary structure.

DISCUSSION

The small reduction in the enthalpy of denaturation and the NSI, along with a high ratio of OMIU-RL to total lysine (0.98) in the untoasted RSM can be considered indicators of a RSM with low protein denaturation and high protein nutritional quality. A decrease in protein solubility in heat-treated materials is an indication of the aggregation of proteins after denaturation [25, 26]. As more proteins become denatured and unfolded with increasing toasting time, intra and intermolecular interactions within and between proteins promote aggregation.

Both NSI and PDI have been used before as indicators of the extent of thermal damage in processed protein-rich ingredients (e.g. soybean meal and RSM) [27-29]. Protein solubility and the standardized ileal digestibility of AA in cecectomized broilers were reduced with increasing autoclaving time of a commercial RSM [29]. Pastuszewska *et al*. [30] suggested that rapeseed meals with a NSI in 0.5% KOH between 55 to 60% can be considered of a high nutritional value. These values were achieved in our experiment between 40 to 60 min toasting, which correspond to toasting times used during commercial RSM production [30].

The increasing NDF and ADIN contents with increasing toasting time in the present experiment was previously described after hydrothermal treatments of canola and RSM [8, 30, 31]. These authors, however, also reported an increase in the ADF content, which was not found in the present study. The difference in the results could be due to milder conditions used in the present experiment compared to those reported previously. The increase of the ADIN content was linked to a decrease of the standardized ileal protein digestibility and was proposed as a good indicator for protein damage [31]. Although it has been suggested that heat treatment increases the linkage between proteins and fiber [30], it is possible that the increase in the content of NDF, ADF and ADIN results from the inability of the solvents used to solubilize the aggregated and chemically modified proteins (e.g. melanoidins) [31].

Table 3.6. Coefficients of *in vitro* digestibility and degree of hydrolysis of rapeseed meal samples toasted for different times [1].

Toasting time	Two-step enzymatic digestibility		pH-STAT enzymatic hydrolysis	
	CDMD	CCPD	DH 120 min (%)	k (s^{-1})
0 min	0.382	0.752	18.6	0.029
20 min	0.393	0.758	17.3	0.032
40 min	0.406	0.773	17.9	0.027
60 min	0.418	0.776	17.5	0.024
80 min	0.415	0.764	18.3	0.018
100 min	0.411	0.753	17.9	0.017
120 min	0.415	0.750	18.5	0.013
SEM	0.005	0.003	0.2	0.002
P-value				
Linear	0.08	0.32	0.23	<0.001
Quadratic	0.19	0.005	0.18	0.47

[1] CDMD, coefficient of dry matter digestibility; CCPD, coefficient of crude protein digestibility; DH, degree of hydrolysis; SEM, standard error of the mean.

The changes observed in protein denaturation and solubility with increasing toasting time do not parallel the changes observed in the secondary structure of proteins. Contrary to what we expected, there was an increase in the proportion of α-helix and a decrease of intermolecular β-sheets with increasing toasting time after the initial 20 min of

toasting. Previous research [7, 20] described a decrease in the proportion of α-helix and an increase in that of intermolecular β-sheet structures after thermal treatment, which was also expected in the present experiment with increasing toasting time. The increase in the proportion of intermolecular β-sheets was linked to a decrease in the in vitro CP digestibility [7]. It is possible that with increasing denaturation, which is the rate limiting step, there is partial unfolding of the proteins with a simultaneous increase of aggregation and (partial) refolding of the secondary structure. Most of the literature on thermal-induced changes to the secondary structure of proteins reports the effects after a certain period of time (e.g. autoclaving at 120°C for 20 min) [7, 20, 32], but do not include the changes occurring during that time period. When considering all time points analyzed in the present study, the net results for secondary structure are still comparable to the results described in literature after autoclaving [7, 20, 32]. The presence of A2 bands has been related to aggregation of proteins due to intermolecular hydrogen-bonded anti-parallel β-sheets [33] or to absorption of infrared light from the amino acid side chains [7].

The formation of Maillard reaction products (MRP) results from chemical changes to AA, for which the most susceptible ones are lysine and arginine [34]. With increasing toasting time, chemical changes of AA continue to occur resulting in the formation of more early MRP and the conversion of the early into advanced MRP and melanoidins [34]. In contrast to early MRP, advanced MRP cannot be reverted into lysine under conditions of 6 M acid hydrolysis [15]. This was noticed by a decrease of lysine content with increasing toasting time. The decrease of the OMIU-RL to lysine ratio is probably the result of higher rate of formation of early MRP compared to advanced ones. Previous research only showed a reduction in the ratio between lysine and reactive lysine after 64 min of toasting [8]. This might be due to the low reactive lysine to lysine ratio determined already in the rapeseed cake (i.e. 0.81). In a recent experiment [3], values of lysine for commercial RSM of German oil mills ranged from 5.5 to 5.3 g/100 g CP, which correspond in our experiment to toasting times of approximately 71 and 91 min, respectively. However, in that same experiment, the OMIU-RL content ranged from 4.4 to 4.0 g/16 g N for the same RSM. This makes the ratio of OMIU-RL to lysine much lower compared to those reported here. The variation in the results could be due to the shorter incubation times for the reaction with OMIU used in the those studies [3, 8] compared to the longer incubation times used in the present study (2 – 2.5 vs. 7 days). It is possible that proteins with a large extent of thermal damage and high aggregation (low solubility) might need longer incubation times for the OMIU reactive to penetrate within the aggregate structure and bind with the free lysine. Alternatively, free lysine may have been formed during toasting, which cannot be analyzed by the OMIU-RL procedure.

The decrease in the ratio lysine to CP with thermal treatments has been reported before [8, 31, 35-37]. According to these authors, lower ratios, as compared to higher ones, indicate that protein damage occurred due to the formation of MRP. A decrease of this ratio led to a decrease of the ileal digestibility of CP and AA [8, 31, 35]. Therefore, the decrease in the lysine to CP ratio reported in our experiment with increasing toasting time is indicative of protein damage and could lead to a decrease of the in vivo protein digestibility. The ratio lysine to CP of the 0 min toasted RSM in the present study (6.3 g lysine/100 g CP) corresponds well to values previously reported for non-toasted canola meal [37]. The apparent ileal digestibility of lysine of the non-toasted canola meal in broilers ranged from 87 to 92% [37]. A lower ratio of lysine to CP (5.55 g lysine/100 g CP) was reported in that study for solvent-extracted canola meals from 7 different production plants. The apparent ileal digestibility of lysine for the solvent-extracted canola meals was lower and more variable (ranging from 65.5 to 85.7%) than the values reported for non-toasted canola meal [37]. Other authors [8, 31] have reported lysine to CP ratios of 5.2 g/100 g CP in commercial canola meals and 5.1 g/100 g CP in RSM toasted for 48 min, indicating damage of the proteins. These values corresponded to standardized ileal digestibilities of lysine in growing pigs of 68.2 and 64%, respectively. In the present experiment, values of lysine to CP ratio that resemble the ones reported by these authors were obtained after 100 min toasting, indicating that the thermal treatments applied by these authors were likely more severe than the ones used herein.

First order reactions have been used previously to model the decrease in glucosinolate content of red cabbage [38] and reactive (available) lysine in model systems [39]. One of the aims of toasting during the production of RSM is to inactivate the glucosinolates without affecting the nutritional quality of proteins (e.g. lysine content). Glucosinolates were degraded at a faster rate than the degradation of OMIU-RL and lysine. Furthermore, the rate constant of decrease of the solubility parameters (i.e. NSI and PDI) is higher than that of OMIU-RL and lysine. This could be an indication that changes in the structure of proteins occur earlier during toasting than chemical (i.e. Maillard) changes. The higher rate constant of decrease of PDI could make it a better indicator of the changes in solubility after toasting of RSM than NSI. Previous research in soybeans indicated that PDI reflects protein quality better than NSI, especially after processing at mild conditions [40].

The range of values obtained with the two-step *in vitro* CP digestibility can be considered as narrow (75.0 – 77.6%). A linear decrease of the *in vitro* CP digestibility from 71% in the 48 min toasted RSM to 62% in the 93 min toasted meal was reported in a recent study [8]. This also matched the reported decrease in standardized ileal CP digestibility in that study. Toasting time did not affect the DH after 120 min indicating that the observed protein changes are not a restriction for protein hydrolysis when the enzymes are available

sufficiently long to hydrolyze. However, the rate at which the enzymes access the substrate during hydrolysis was linearly reduced by toasting time. A reduction in the rate of hydrolysis with increasing heating time has been reported before [41] for glycinin from soybeans. The high correlation of the rate of hydrolysis with protein solubility (in alkali and water), and lysine or OMIU-RL contents could explain the decrease in the rate of hydrolysis with increasing toasting time. It is not possible, however, to distinguish if the formation of aggregates (i.e. lower solubility) or the chemical modification of the Maillard-sensitive AA is the major factor controlling the rate of hydrolysis, as both occur simultaneously during toasting. The decrease in the rate of protein hydrolysis with increasing toasting time could explain the reduction of the ileal protein digestibility reported in other studies after toasting [5, 8].

Extensive reviews have suggested inclusion levels of total glucosinolates ranging from 2 to 2.5 μmol/g diet for pigs, whilst for poultry, the inclusion level ranges from 2 to 10 μmol/g diet [42, 43]. To maintain these total glucosinolates level, at the maximum rate of protein hydrolysis (i.e. 20 min of toasting) in this study, the inclusion level of RSM in the diets for pigs and poultry can be 9.8 and 49%, respectively. This would also involve a loss of 4% lysine and 9% OMIU-RL with respect to the untoasted RSM. At the maximum *in vitro* CP digestibility (i.e. 60 min of toasting) in this study, the inclusion level in the diets for pigs can increase to 18.2%, whilst there would be no limit to the inclusion level for poultry diets. This would involve a loss of 13% lysine and 22% OMIU-RL with respect to the untoasted RSM. However, the inclusion level of rapeseed or canola meal in the diets for pigs might depend not only on the content of total glucosinolates, but also on the type of glucosinolates included [44]. Whilst the feed intake of weanling pigs did not decrease after the inclusion of 2.2 μmol/g diet of total glucosinolates from *Brassica napus* [45], the inclusion of 2.2 μmol/g diet of total glucosinolates from Brassica juncea in diets for growing-finishing pigs resulted in a decrease of feed intake and weight gain [44]. The major glucosinolate in *B. juncea* is gluconapin, whilst *B. napus* contains higher levels of progoitrin than gluconapin [46].

CONCLUSIONS

Toasting of RSM for increasing time induces physical and chemical changes to the proteins and affects its nutritional value. These changes are correlated to the rate of protein hydrolysis but not the *in vitro* CP digestibility or the extent of hydrolysis. Degradation of glucosinolates occurs earlier during toasting and at higher rates than that of OMIU-RL and lysine.

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Authors' contributions

SSV, EMAMB, PC, AQ and AFBvdP conceived and designed the experiment. PC and AQ performed the production of the experimental samples. HG, EMAMB and AFBvdP supervised the experimental work. SSV performed all chemical analyses and wrote the manuscript. EMAMB, AFBvdP, HG and WHH checked the results and revised the manuscript.

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CHAPTER 4

Effects of toasting time on hydrolysis of soluble and insoluble rapeseed meal proteins

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ABSTRACT

Thermal damage to proteins can reduce their nutritional value. The effects of toasting time on the hydrolytic profile and the resulting molecular weight distribution of rapeseed meal (RSM) and the soluble and insoluble protein fractions separated from these RSM were studied. Increasing the toasting time of RSM linearly decreased the rate of protein hydrolysis of RSM and the insoluble protein fractions. The extent of hydrolysis was higher for the insoluble compared to the soluble protein fraction. In contrast, the rate of protein hydrolysis of the soluble protein fraction was 3-9 fold higher than that of the insoluble protein fraction. Increasing the toasting time elicited the formation of Maillard reaction products (furosine, N^{ε}-carboxymethyl-lysine and N^{ε}-carboxyethyl-lysine) and disulfide bonds in the insoluble protein fraction, which is proposed to explain the reduction in the hydrolysis rate of this fraction. Overall, longer toasting times increased the size of the peptides resulting after hydrolysis of the RSM and the insoluble protein fraction. The hydrolysis kinetics of the soluble and insoluble protein fractions and the proportion soluble:insoluble proteins in the RSM explain the reduction in the rate of protein hydrolysis observed in the RSM with increasing toasting time.

Keywords: hydrolysis rate, Maillard, protein solubility, rapeseed meal, toasting.

INTRODUCTION

The production of defatted rapeseed meal (RSM) involves toasting for the removal of remnant solvent after oil extraction and the degradation of glucosinolates [1, 2], the main antinutritional factor of RSM for monogastric animals. Increasing the toasting time decreases protein solubility [1] and the contents of lysine (Lys) [1] and reactive Lys [2] of the resulting RSM. Nutritionally, these changes have been noticed as reduction of *in vitro* crude protein digestibility [2], apparent ileal protein digestibility in broilers [3], standardized ileal protein digestibility in pigs [2] and faecal crude protein digestibility in rats [1].

Chemical and physical modifications can impair the accessibility of proteins for enzymatic hydrolysis [4]. The proteases in the pancreatic secretions at the small intestine are highly specific. Trypsin cleaves peptide bonds involving the carboxyl groups of either Lys or arginine, which are also the most susceptible amino acids to heat damage. Lysine and arginine residues that are modified via Maillard reactions could, therefore, reduce enzyme accessibility for proteolysis, finally reducing their standardized ileal digestibility [5]. Also physical modifications, such as protein aggregation, might have a similar effect. Protein aggregates can be formed after hydrothermal processing and are noticed as a reduction in protein solubility [6]. The formation of aggregates can reduce protein accessibility for enzymatic hydrolysis [7].

In proteins that are accessible for enzymatic hydrolysis, the gastric and intestinal enzymes cleave proteins into peptides, followed by cleavage into free small peptides and free amino acids by the brush border enzymes in the gut's epithelium. The size of the peptides produced during hydrolysis depends on the accessibility of the protein for the enzymes. Even at similar degrees of protein hydrolysis, the molecular size distribution of the resulting peptides can be different [8]. This might be the result of the selectivity of the enzymes or the accessibility of the proteins for cleavage [8].

The aim of the present study was to determine the effects of toasting time during the production process of RSM on the kinetics of hydrolysis of proteins present in the complete material, and in its soluble and insoluble fractions, and on the resulting molecular size distribution of the peptides after hydrolysis. Previous (unpublished) results from our research group indicate that there is a highly significant positive correlation between nitrogen solubility and the rate of protein hydrolysis. Therefore, we hypothesize that the rate of hydrolysis will be higher for the soluble protein fraction compared to the insoluble protein fraction, but that the rates at different toasting times would not vary within the soluble and the insoluble protein fractions. We also hypothesize that damage to the proteins due to prolonged toasting times would change the molecular size distribution of the peptides obtained after hydrolysis towards a larger size.

MATERIALS AND METHODS

Materials

Rapeseed meals were prepared from 00-rapeseed (*Brassica napus*) at the pilot plant of CREOL/OLEAD (Pessac, France). Trypsin (type IX-S, 13000 – 20000 BAEE units/mg protein, EC 232-650-8), chymotrypsin (type II, ≥40 units/mg protein, EC 232-671-2) and peptidase from porcine intestinal mucosa (50-100 units/g solid, EC 232-875-1) were obtained from Sigma-Aldrich (St Louis, MO, USA).

Rapeseed meals preparation

An untoasted RSM was prepared by cold-pressing of the rapeseeds, solvent-extraction and desolventization using indirect heat. Rapeseeds were cold-pressed (La Mecanique Moderne MBU 75 type, Arras, France) at 250 kg/h with temperatures not exceeding 80°C. Solvent extraction was performed at temperatures not higher than 55°C on a belt-extractor (B-1930, Desmet-Ballestra, Zaventem, Belgium) at 230 L/h flow of hexane and 160 kg/h flow of the rapeseed cake. Desolventization with indirect heat (without direct steam) was performed in a desolventizer-toaster (Schumacher type, Desmet-Ballestra) for 60 min at temperatures of 90 ± 3°C.

A batch of 150 kg of the untoasted RSM was toasted with the use of direct steam (30 kg/h) for 120 min, with spot samples of 5 kg taken every 20 min (Fig. 4.1). Toasting of a separate batch of 150 kg of RSM was performed during the next day under the same conditions for replication. Temperatures during toasting on the first day ranged from 107 to 112°C and between 109 to 112°C on the second day. In total, 13 samples of RSM (1 untoasted RSM and 12 toasted RSM) were obtained. Untoasted and toasted RSM were ground with a centrifugal mill (ZM200, Retsch, Haan, Germany) at 8000 rpm to pass a 1 mm sieve.

Fractionation of proteins into soluble and insoluble fractions

The water soluble and insoluble fractions of the meals were separated by suspending 25 g of the RSM in 250 mL water. The pH of the suspension was adjusted to 8.0 with NaOH and magnetic stirring was applied for 20 min at room temperature. The soluble fraction was separated by centrifugation (11900 × g, 20 min, room temperature). Transparent solutions were obtained, which were dialyzed extensively against a 0.01M NaCl solution. After dialysis, the pH was re-adjusted to 8.0 with NaOH. The soluble fractions were kept at 4°C and hydrolyzed within 24 h. The insoluble fraction was filtered through a nylon

cloth and washed three times with 250 mL water in order to remove soluble proteins and was freeze-dried prior to hydrolysis.

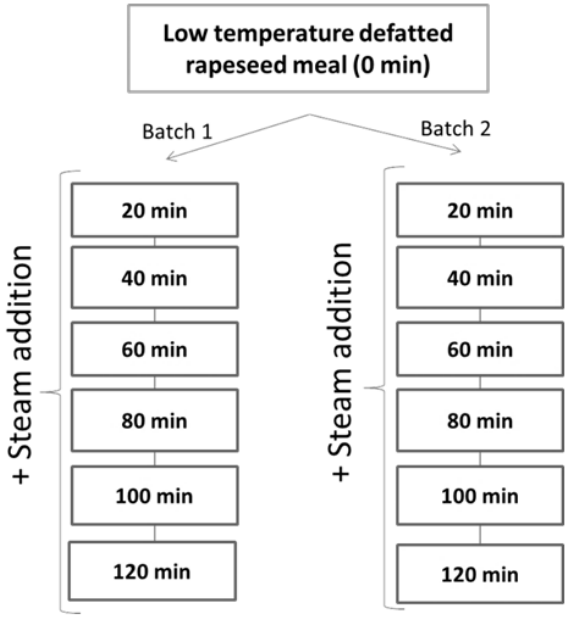

Fig. 4.1. Design of the experiment.

Analytical methods

Nitrogen contents of the complete meals and the insoluble fractions were determined by combustion (AOAC 968.06, Thermo Quest NA 2100 Nitrogen and Protein Analyzer, Breda, The Netherlands). The N concentration of the soluble protein fractions was measured after an aliquot of 0.3 mL was oven-dried at 60°C overnight.

Protein hydrolysis

In vitro protein hydrolysis was performed for 120 min using a modification of the method described previously [(9)]. Briefly, 10 mL aqueous suspension containing 1 mg N/mL was adjusted to pH 8.0 in a titration unit (719 S Titrino, Metrohm, Herisau, Switzerland) at 39°C using 0.1 M NaOH. At this point, 1 mL enzyme solution containing 1.61 mg trypsin, 3.96 mg chymotrypsin and 1.18 mg porcine intestinal peptidase was added and the 120 min titration was initiated. This procedure was used for the complete RSM and the insoluble fractions.

Concerning the soluble fraction, different concentrations of N in the soluble fractions were obtained upon solubilizing the RSM in water. Demineralized water was used for diluting these solutions to the concentration of the lowest one of the solutions (0.30 mg N/mL), to a volume of 10 mL. The solutions were adjusted to pH 8.0 in the titration unit using 0.05 M NaOH. At this point, 1 mL of an enzyme solution containing 0.48 mg trypsin, 1.19 mg chymotrypsin and 0.35 mg porcine intestinal peptidase was added and the 120 min titration initiated. The same substrate to enzyme ratio was used for hydrolysis of RSM, soluble and insoluble fractions. All hydrolyses were performed in duplicate.

The volume of alkali added was used for the calculation of the degree of hydrolysis (DH) according to Eq. 1,

(1) $DH\ (\%) = \frac{Vb \times Nb}{\alpha \times mp \times htot} \times 100$

in which *Vb* is the volume added (mL), *Nb* is the normality of the titration solution, α is the degree of dissociation of the α-NH_2 group (in this case 0.794 at 37°C and pH 8.0), *mp* is the mass of protein (g) and *htot* is the total number of peptide bonds per gram of protein (7.8 meq/g) [10].

Modelling of the DH curves was based on second order reaction kinetics, which is described in Eq. 2,

(2) $DH\ (\%) = \ DHmax - \frac{DHmax}{1 + k \times t \times DHmax}$

in which DH_{max} is the maximum DH (%), *k* is the hydrolysis rate constant ($M^{-1} \times s^{-1}$) and *t* is the hydrolysis time. Fitting of this model was performed using the MODEL procedure in SAS [11].

Nitrogen solubility

Five hundred mg RSM were suspended in 10 mL water and the suspension was adjusted to pH 8.0 using 2 M NaOH. The suspension was stirred for 20 min at room temperature and centrifuged (16100 × g, 15 min, room temperature). An aliquot (0.3 mL) was oven-dried overnight at 60 °C and analyzed for N content. Nitrogen solubility of the insoluble protein fractions were determined in water, 100 mM sodium phosphate buffer pH 7.5, and the same buffer containing either 2% (w/v) SDS, 10 mM DTT or both 2% (w/v) SDS and 10 mM DTT. Briefly, 1.5 mL of these solutions were added to 75 mg of the insoluble protein fractions. The suspensions were vortexed for 20 s and mixed in a head-over-tail rotator for 20 min at 20 rpm. Following centrifugation (16100 × g, 15 min, room temperature), 0.3 mL of the supernatant was oven-dried overnight at 60°C and analyzed for N content.

Size exclusion chromatography (SEC)

After hydrolysis, a sample of 1.5 mL was taken from the supernatant of the hydrolysate, heated at 99°C for 15 min and centrifuged (16100 × g, 10 min, room temperature). In addition, samples from the water soluble fraction of RSM were analyzed after centrifugation (16100 × g, 15 min, room temperature). The samples were analyzed in the ÄKTA micro system (GE Healthcare, Uppsala, Sweden) using a Superdex 75 column (GE Healthcare) at a flow rate of 100 µL/min with UV detection at 220 nm. The eluent used was 10 mM sodium phosphate buffer pH 7.0 containing 150 mM NaCl and 2% (w/v) SDS. The volume of injection was 50 µL. A calibration curve of the elution volumes in the column was obtained using threonine (119 Da), proline-glycine-glycine (229 Da), vitamin B12 (1355 Da), lysozyme (14307 Da), β-lactoglobulin (18400 Da), and ovalbumin (42700 Da). Areas under the curve were integrated manually and the proportion of peptides based on AU response in each region (>10 kDa, 10 – 1.5 kDa and <1.5 kDa) were calculated relative to the total area under the curve.

Maillard reaction products, crosslinked compounds and lysine

The contents of furosine, N^{ε}-[carboxymethyl]-lysine (CML), lysinoalanine (LAL), lanthionine (LAN), N^{ε}-[carboxyethyl]-lysine (CEL) and Lys in the RSM and the insoluble protein fractions were quantified by UHPLC-MS. The samples (10 mg) were hydrolyzed with 1 mL 6 M HCl during 24 h at 110°C. The tubes were dried under N_2 flow and the dried material was re-suspended in 1 mL UPLC-grade Milli-Q water, sonicated and centrifuged (16100 × g, 3.5 min, room temperature). The supernatant was diluted 50 times in eluent A that contained 1 mg/L (w/v) $^{13}C_6$ $^{15}N_2$-lysine (Sigma-Aldrich, Steinheim, Germany) as internal standard. Eluent A was UPLC-grade Millipore water containing 0.1% (v/v) formic acid and eluent B was acetonitrile containing 0.1% (v/v) formic acid. The samples were analyzed using an Accela RP-UHPLC system (Thermo Scientific, San Jose, CA, USA) with an Acquity BEH Amide Vanguard precolumn (2.1 × 50 mm, 1.7 µm particle size) and an Acquity UPLC BEH 300 Amide column (2.1 × 150 mm, 1.7 µm particle size). The column was maintained at 35°C and the injection volume was 1 µL. The elution profile was as follows: 0-2 min isocratic on 80% B, 2-3 min linear gradient from 80% B to 65% B, 3-5 min isocratic on 65% B, 5-7 min linear gradient from 65% B to 40% B, 7-10 min isocratic on 40% B, 10-12 min linear gradient from 40% B to 80% B and 12-28 min isocratic on 80% B. The flow rate was 350 µL/min. Mass spectrometric data were obtained using a LTQ-VelosPro (Thermo Scientific) equipped with a heated ESI probe, coupled to the UHPLC system. The capillary voltage was set to 3 kV. The sheath gas flow rate was set at 20 and the auxiliary gas flow rate at 5 (arbitrary units). A selected reaction monitoring (SRM) method (Table 4.1) was used for fragments analysis in negative ion mode for lysinoalanine and in positive ion mode

for the other compounds. The normalized collision energy was set at 30 for furosine, Lys and LAL and at 35 for the other compounds and the m/z width on the fragment was set to 1. An external standard calibration curve with concentrations of 0.01, 0.1, 1, 2.5, 5 and 10 mg/L was used to calculate the content of each compound. Compounds were quantified using the external standard calibration curve by plotting MS peak area divided by the MS peak area of the labelled Lys, used as internal standard. Data were acquired and analyzed using XCalibur 2.2 software (Thermo Scientific).

Table 4.1. Selected reaction monitoring conditions.

Compound	Parent mass (Da)	Fragment (m/z)
Lysine	146	130
$^{13}C_6$ $^{15}N_2$-lysine	154	137
Carboxymethyl-lysine	204	84, 130
Lanthionine	208	120
Carboxyethyl-lysine	218	84, 130
Lysinoalanine	233	128, 145
Furosine	255	84, 130

Statistical analysis

Linear and quadratic regressions were fitted using toasting time as fixed effect in the model. Linear and quadratic effects were considered to be significant if the *P*-value was lower than 0.05 and as trends when the *P*-value was between 0.05 – 0.10. Correlations between hydrolysis parameters and molecular size distribution after hydrolysis were performed using the CORR procedure of SAS [(11)].

RESULTS AND DISCUSSION

There were linear ($P < 0.001$) and quadratic ($P = 0.001$) effects of toasting time on N solubility of the RSM in water at pH 8.0 (Table 4.2). Solubility seems to decrease faster at shorter toasting times compared to longer ones. The decrease in N solubility can be caused by physical aggregation of proteins following protein unfolding [(6)]. In addition, chemical modifications to proteins (e.g. formation of intermolecular disulfide bonds, Maillard reactions) might also be involved in the solubility decrease [(6)]. Both of these phenomena (physical aggregation and chemical modifications) might decrease the accessibility of proteins for enzymatic hydrolysis.

Hydrolysis kinetics

Increasing the toasting time affected the hydrolysis profile of RSM (Fig. 4.2a). The hydrolysis rate constant (*k*) of the RSM decreased linearly ($P < 0.001$) with increasing toasting time (Table 4.2). The *k* after toasting for 120 min was approximately 2-fold lower

compared to the *k* of the untoasted RSM (6.8E-05 *vs*. 3.4E-05, respectively). The decrease in *k* was probably related to the restricted enzyme accessibility for proteolysis due to protein aggregation or chemical protein modifications [4]. Increasing the toasting time caused a linear (P = 0.006) increase in the DH_{max} of RSM (Table 4.2). The DH_{max} decreased 9% after the initial 20 min toasting and subsequently gradually increased with increasing toasting time, up to a level in the 120 min toasted RSM similar to the untoasted RSM (Fig. 4.2a).

Table 4.2. N solubility at pH 8.0 of the rapeseed meals (RSM) and kinetic parameters for the hydrolysis curves of RSM toasted for different times and the soluble and insoluble fractions separated from these RSM.

Toasting time	N solubility RSM	RSM		Soluble protein fraction		Insoluble protein fraction	
	(% of total N)	DH_{max} (%)	*k* ($M^{-1}\times s^{-1}$)	DH_{max} (%)	*k* ($M^{-1}\times s^{-1}$)	DH_{max} (%)	*k* ($M^{-1}\times s^{-1}$)
0 min	31.3	20.0	6.8E-05	14.7	1.2E-04	19.5	4.0E-05
20 min	21.1	18.3	8.0E-05	14.8	1.4E-04	22.8	4.0E-05
40 min	17.7	19.2	7.0E-05	15.3	1.3E-04	21.1	4.3E-05
60 min	15.3	19.0	6.7E-05	14.3	1.5E-04	21.4	2.4E-05
80 min	12.3	20.4	5.0E-05	15.8	1.6E-04	21.9	2.2E-05
100 min	11.4	20.2	4.8E-05	14.5	1.7E-04	21.7	2.4E-05
120 min	9.9	21.7	3.4E-05	15.3	1.4E-04	22.3	1.5E-05
SEM	0.2	0.3	4.5E-06	0.2	6.7E-06	0.3	3.0E-06
P-value							
Linear	<0.001	0.006[b]	<0.001[c]	0.47	0.18	0.34	<0.001[d]
Quadratic	0.001[a]	0.06	0.07	0.96	0.34	0.86	0.87

[a] N solubility = 28.84 – 0.33 × time + 0.0015 × time^2 (R^2 = 0.95).
[b] RSM DH_{max} = 18.33 + 0.023 × time (R^2 = 0.52).
[c] RSM *k* = 8.30E-05 – 3.71E-07 × time (R^2 = 0.80).
[d] Insolubles *k* = 4.46E-05 – 2.40E-07 × time (R^2 = 0.78).

Toasting time had no effect on the *k* or the DH_{max} of the soluble protein fraction (Table 4.2), as can be seen from the similar shapes of the DH curves (Fig. 4.2b). The soluble proteins could either be native or aggregated proteins with a molecular size that allows them to stay in solution [6, 12]. Aggregation of proteins in solution into spherical particles occurs when heating is performed at a pH close to the isoelectric point of the protein [13]. With longer heating times, there is secondary aggregation of these spherical particles [13] into protein condensates [12], which results in the reduction of protein solubility. Protein aggregation mechanisms for proteins in solution have been clearly identified before [12, 13]. We assume that proteins thermally treated under semi-dry conditions (e.g. toasting or autoclaving) follow similar aggregation mechanisms as reported for proteins in solution.

The *k* of the soluble protein fraction was 3-9 fold higher compared to that of the insoluble protein fraction (Table 4.2), which matches our hypothesis. Higher rates of hydrolysis were reported previously [14] for soluble sodium caseinate compared to the

insoluble form of the same ingredient (casein), although these protein sources were not heat-processed before hydrolysis. Both native and denatured proteins can remain in solution and this depends on their concentration and on their extent of aggregation [15, 16]. Due to their high flexibility, native proteins in solution are in dynamic equilibrium with their distorted forms and these distorted forms could be considered as denatured [15], which could make them more accessible for enzymatic cleavage. Furthermore, the relatively small size of the protein aggregates present in the soluble fraction allows them to stay in solution [6]. Therefore, the lower degree of aggregation of the proteins in the soluble fraction as compared to the proteins in the insoluble fraction probably facilitated enzymatic hydrolysis, thus increasing *k*. This would mean that a decrease in the proportion of soluble to insoluble proteins after thermal processing leads to a decrease in the *k* for the complete material, as observed for the RSM in this study. Another possibility for the differences in *k* between the soluble and insoluble fractions is that the insoluble fiber matrix structures present in the latter, and not in the soluble fraction, might limit and decrease the rate of protein hydrolysis.

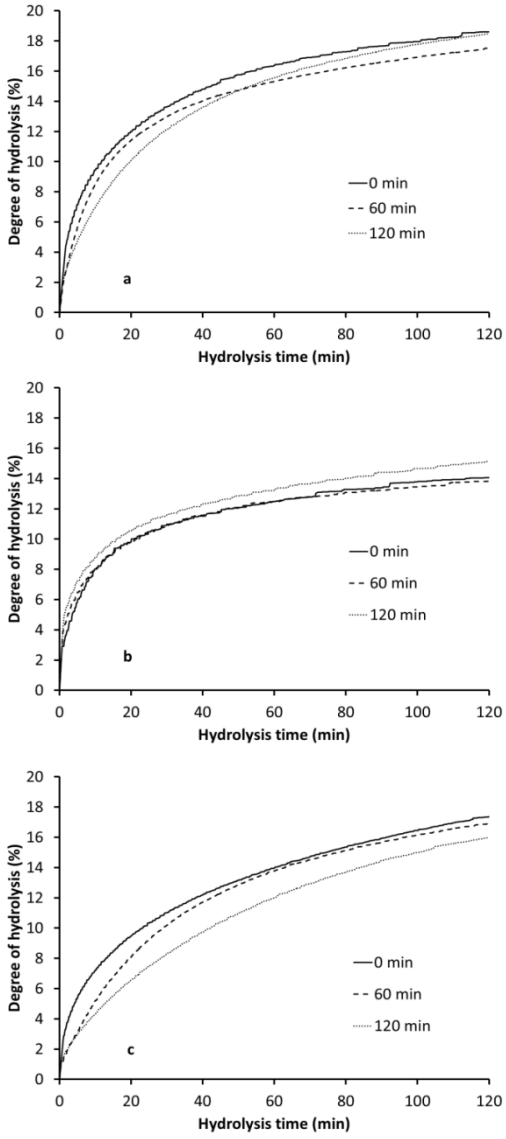

Fig. 4.2. Degree of hydrolysis during 120 min hydrolysis of (a) rapeseed meals toasted for different times and (b) soluble and (c) insoluble protein fractions separated from these rapeseed meals.

The *k* of the insoluble protein fraction decreased linearly ($P < 0.001$) by 62% when comparing the untoasted RSM to the 120 min toasted RSM (Table 4.2), whilst no effects were noticed on the DH_{max} of these fractions. As 70 – 90% of the proteins in RSM correspond to insoluble proteins, the pattern of the hydrolysis curves of the insoluble protein fractions (Fig. 4.2c) were, as expected, similar to those from the RSM. It is possible that there is an increase in the

size of protein aggregates formed at increasing toasting times, which can hamper the penetration of the hydrolytic enzymes. Insolubility of the proteins in water does not seem to limit the extent of enzymatic hydrolysis. The DH_{max} of the insoluble protein fractions was numerically higher compared to that of the soluble protein fractions (Table 4.2).

In vivo, enzymatic protein digestion starts at the stomach with pepsin and is followed by secretion of trypsin and chymotrypsin at the small intestine, where most of the digestion occurs. The length of the small intestine and the transit time, however, are limited and the extent of protein digestion probably also depends on their rate of digestion. These factors might be even more important for poultry than for pigs, due to the short digestive tract of the former [17].

Nitrogen solubility of the insoluble fractions

The N solubilities of the insoluble protein fraction in the sodium phosphate buffer pH 7.5, buffer with 2% (w/v) SDS, buffer with 10 mM DTT and buffer with 2% (w/v) SDS and 10 mM DTT decreased with increasing toasting time (Fig. 4.3a). All solvents show a reduction in their solubilizing power with increasing toasting time, which might be related to the increase in chemical modifications of the residues (e.g. Maillard reactions or crosslinking). Non-covalent electrostatic interactions can be cleaved by salt solutions [18], such as the phosphate buffer used in this experiment. Furthermore, SDS can cleave hydrogen bonds and hydrophobic interactions, whilst DTT can cleave disulfide bonds [18]. Non-covalent bonds seem to be important for the stability of the insoluble aggregates. The SDS solution solubilizes twice the amount of N than the phosphate buffer alone, while the increase of N solubility with additional DTT was minimal (Fig. 4.3a). The increase in N solubility relative to the solubility by the phosphate buffer is reported in Fig. 4.3b. The relative importance of non-covalent bonds (solubilized by SDS solution) for the stability of the aggregates does not seem to change with the increasing toasting time (Fig. 4.3b). With increasing toasting time, there is an increase in the relative amount of protein solubilized by the DTT containing buffer (Fig. 4.3b). This indicates that there is formation of disulfide bonds in the insoluble aggregates with increasing toasting time. The SDS – DTT solution solubilizes more protein than SDS or DTT containing solutions separately. Synergy of SDS and DTT for N solubility has been reported before [19] for extruded soy proteins. In addition, the relative amount of protein solubilized by this solution increases with increasing toasting time (Fig. 4.3b). We suggest that cleavage of non-covalent bonds by SDS exposes extra disulfide bonds that can subsequently be cleaved by the DTT.

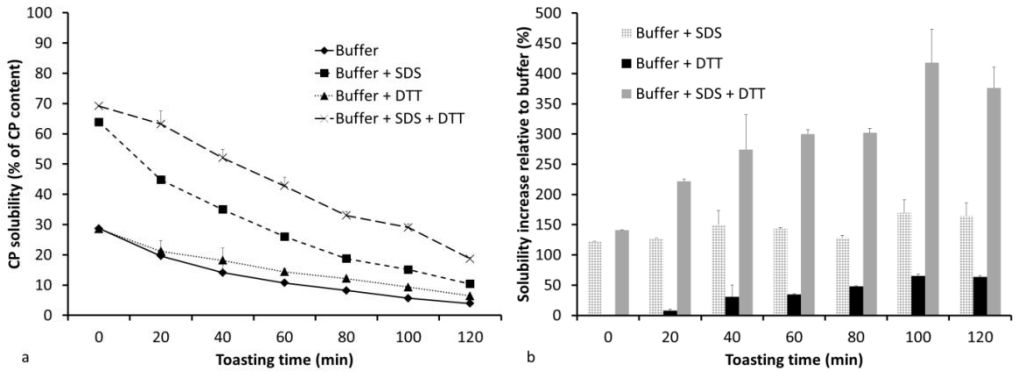

Fig. 4.3. (a) Nitrogen solubility of the insoluble protein fractions in 100 mM phosphate buffer and the buffer containing either 2% SDS, 10 mM DTT or 2% SDS and 10 mM DTT. (b) Increase in N solubility of the insoluble protein fractions relative to the phosphate buffer of solutions containing either 2% SDS, 10 mM DTT, or 2% SDS and 10 mM DTT.

Maillard reaction products

Formation of early (fructosyl-lysine) and advanced Maillard reaction products (CML and CEL) in the RSM was noticed with increasing toasting time, along with a decrease in the Lys content (Fig. 4.4). Conversion of fructosyl-lysine during 6M HCl hydrolysis yields furosine (32%), pyridosine (16%) and regenerated Lys (56%) [(20)]. The CML (Fig. 4.4a), furosine (Fig. 4.4c) and CEL (Fig. 4.4d) contents in the RSM linearly ($P < 0.001$) increased with increasing toasting time. The formation of these compounds with increasing toasting time does not completely account for the reduction in the Lys content, which also decreases linearly ($P < 0.001$) (Fig. 4.4e). Whilst the Lys content decreases 15.7 µg/mg CP from the 0 min to the 120 min toasted RSM, the sum of fructosyl-lysine (calculated from furosine), CML and CEL only increases 11.1 µg/mg CP in the same toasting time range. Other Maillard derived compounds (e.g. [5-hydroxymethyl]-2-furfural) that were not determined in this experiment were probably also formed during toasting, which could account for this difference. The content of LAL (Fig. 4.4b) decreased during the initial 20 min of toasting, but remained constant with increasing toasting times. Formation of LAL is favored at alkaline pH [(21)], which is unlikely to have been applied during toasting of the RSM. No LAN could be detected in any of the samples.

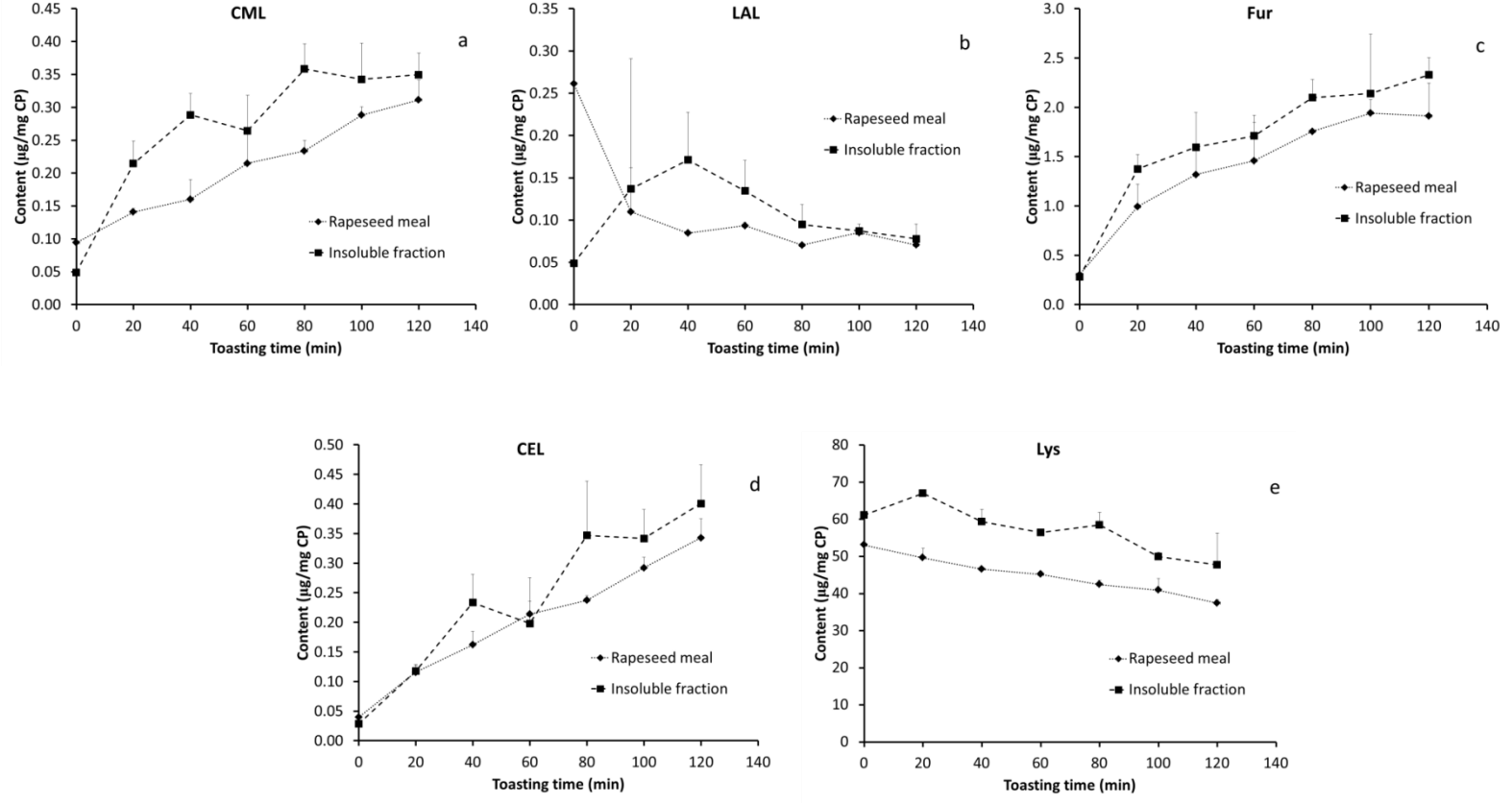

Fig. 4.4. (a) N^{ε}-[carboxymethyl]-lysine (CML), (b) lysinoalanine (LAL), (c) furosine (Fur), (d) N^{ε}-[carboxyethyl]-lysine (CEL) and (e) lysine (Lys) contents (µg/mg crude protein) in the rapeseed meals toasted for different times and the insoluble protein fraction separated from these rapeseed meals.

Most of the chemically modified compounds formed were present in the insoluble protein fraction. The contents of furosine (Fig. 4.4c) and CEL (Fig. 4.4d) increased linearly ($P < 0.001$) with increasing toasting times, whilst there were linear ($P = 0.006$) and quadratic ($P = 0.009$) effects of toasting time on the CML (Fig. 4.4a) content. The content of Lys (Fig. 4.4e) decreased linearly ($P < 0.001$) with increasing toasting time. Contents (µg/mg CP) of furosine, CML, CEL and Lys were higher in the insoluble protein fractions compared to the complete RSM. The removal of the soluble protein fraction probably concentrates the aggregated and chemically modified insoluble fraction. The presence of these Maillard-derived compounds in the insoluble fraction could delay the enzymatic cleavage of the available peptide bonds due to steric hindrance [22]. In addition to protein aggregation, as described above, the presence of these chemically modified compounds in the insoluble fraction could also explain the observed reduction of the *k* of the insoluble protein fractions. There were no indications of other chemical crosslinks occurring: LAL (Fig. 4.4b) contents in the insoluble fraction do not change with increasing toasting time, whilst no LAN could be detected in any of the samples.

Molecular size distribution

Toasting time influenced the molecular weight distribution of the proteins in solution before hydrolysis (Fig. 4.5a) and the peptides obtained after hydrolysis of the RSM (Fig. 4.5b), the soluble (Fig. 4.5c) and the insoluble (Fig. 4.5d) protein fractions. The fractions in the chromatograms that elute after 2 mL consist of protein/peptides that a-specific bind to the column.

The largest fraction of soluble proteins from the intact RSM elute at a molecular mass >10 kDa (Fig. 4.5a). Intact soluble proteins and soluble protein aggregates can be located in this region [23]. The proportion of material >10 kDa decreased (linear $P < 0.001$, quadratic $P = 0.04$) with increasing toasting time (Table 4.3). This was also reflected by the linear increase ($P < 0.001$) of the proportion of proteins/peptides <10 kDa with increasing toasting time. This increase was probably the result of a higher relative representation of this highly soluble fraction compared to the decreasing contents of soluble intact proteins at longer toasting times.

Hydrolysis of the RSM and their soluble and insoluble fractions changed their elution profile compared to the soluble intact RSM, as higher proportions of material can be found at lower molecular masses (<10 kDa) (Table 4.3). In the hydrolysates of RSM, the proportion of material <10 kDa corresponded to 80 – 86% of the quantified area (i.e. excluding the a-specific binding, retention volume higher than 2 mL). The proportion of peptides >10 kDa in the hydrolysates of the RSM decreased after the initial 20 min of toasting and was not largely affected by a further increase in toasting time. Increasing the

toasting time increased the overall molecular weight of these hydrolysates, as an increasing (linear, $P < 0.001$) proportion of peptides 1.5 – 10 kDa and a decreasing (linear, $P < 0.001$) proportion of peptides <1.5 kDa were found at higher toasting times compared to lower ones. There was a negative correlation ($r = -0.73$, $P < 0.01$) between the proportion of peptides 1.5 – 10 kDa and the *k* of hydrolysis of the RSM, whilst a positive correlation ($r = 0.84$, $P < 0.001$) was found for the proportion of peptides <1.5 kDa (Table 4.4). The proportion of peptides 1.5 – 10 kDa in the RSM hydrolysates was also positively correlated ($r = 0.56$, $P < 0.05$) to the DH_{max}, whilst a negative correlation ($r = -0.74$, $P < 0.01$) was found between the proportion of peptides <1.5 kDa and the DH_{max}.

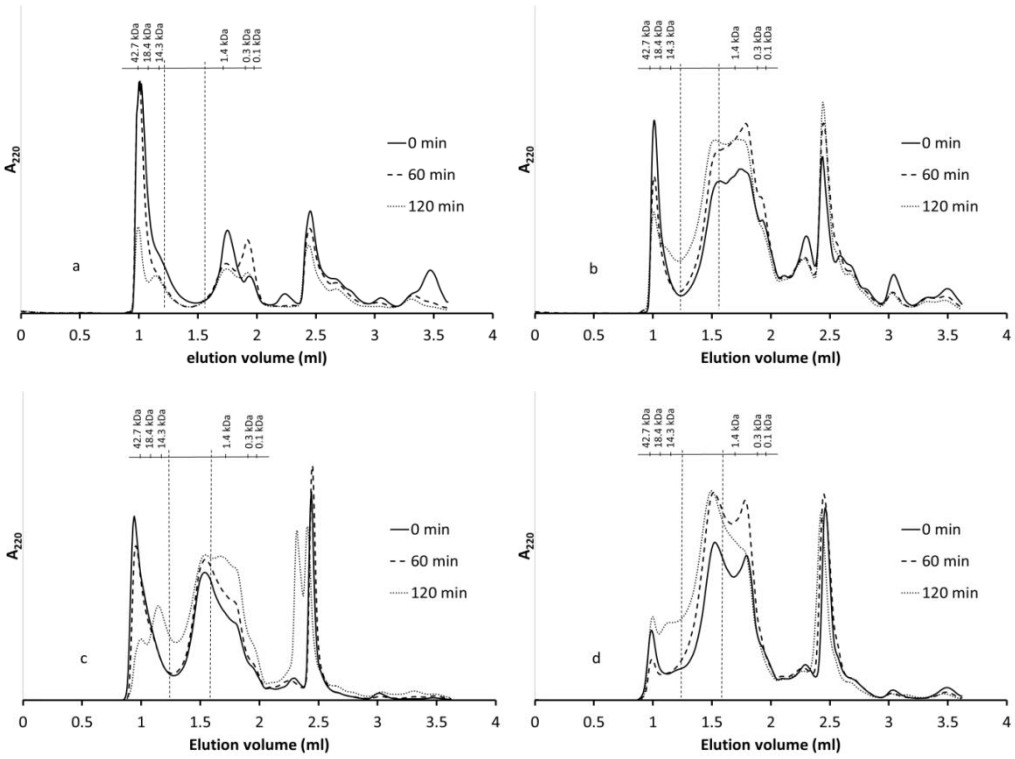

Fig. 4.5. Size exclusion chromatograms of the (a) soluble fractions of rapeseed meals toasted for different times before hydrolysis and the hydrolysates of (b) rapeseed meals, (c) soluble and (d) insoluble protein fractions of the rapeseed meals toasted for different times. Vertical dashed lines represent the cut points of 10 and 1.5 kDa.

Table 4.3. Relative molecular weight distribution (%) of intact rapeseed meals (RSM) toasted for different times and the hydrolysates of these RSM, soluble and insoluble protein fractions separated from these RSM.

Toasting time	Intact RSM			Hydrolysates								
				RSM			Soluble protein fraction			Insoluble protein fraction		
	>10 kDa	10 – 1.5 kDa	<1.5 kDa	>10 kDa	10 – 1.5 kDa	<1.5 kDa	>10 kDa	10 – 1.5 kDa	<1.5 kDa	>10 kDa	10 – 1.5 kDa	<1.5 kDa
0 min	52.3	12.8	34.9	19.2	21.4	59.5	33.6	26.4	40.0	11.7	32.0	56.3
20 min	50.9	9.0	40.1	13.1	24.3	62.6	27.8	27.8	44.4	7.0	35.8	57.2
40 min	49.5	10.1	40.5	12.7	25.7	61.6	24.9	28.1	47.0	7.5	36.6	56.0
60 min	48.6	9.8	41.6	13.3	28.6	58.1	22.8	30.4	46.8	9.1	37.3	53.6
80 min	45.2	10.3	44.5	12.6	28.7	58.7	20.3	30.6	49.1	11.2	38.3	50.5
100 min	42.4	11.3	46.3	13.7	30.7	55.6	19.2	31.3	49.5	13.1	39.3	47.6
120 min	38.3	12.4	49.3	14.0	30.5	55.5	18.8	30.7	50.5	15.6	39.6	44.7
SEM	1.4	0.4	1.2	0.5	0.9	0.8	1.3	0.5	0.9	0.9	0.6	1.3
P-value												
Linear	<0.001	0.15	<0.001[c]	0.34	<0.001[e]	<0.001[f]	<0.001	0.002[h]	<0.001[i]	0.003	<0.001	<0.001
Quadratic	0.04[a]	0.006[b]	0.76	0.006[d]	0.13	0.32	0.005[g]	0.18	0.07	<0.001[j]	0.02[k]	0.002[l]

[a] intact >10 kDa = 51.84 – 0.017× time – 0.00079 × time^2 (R^2 = 0.91).
[b] intact 10 – 1.5 kDa = 11.45 – 0.07× time + 0.00066 × time^2 (R^2 = 0.63).
[c] intact <1.5 kDa = 36.38 + 0.10 × time (R^2 = 0.85).
[d] RSM > 10 kDa = 16.97 – 0.13× time + 0.00093 × time^2 (R^2 = 0.58).
[e] RSM 10 – 1.5 kDa = 22.83 + 0.073 × time (R^2 = 0.76).
[f] RSM < 1 kDa = 62.63 – 0.060 × time (R^2 = 0.67).
[g] soluble > 10 kDa = 32.81 – 0.23 × time + 0.00099 × time^2 (R^2 = 0.93).
[h] soluble 10 – 1.5 kDa = 27.09 + 0.038 × time (R^2 = 0.60).
[i] soluble < 1 kDa = 42.68 + 0.071 × time (R^2 = 0.77).
[j] insoluble > 10 kDa = 10.10 – 0.093 × time + 0.0012 × time^2 (R^2 = 0.91).
[k] insoluble 10 – 1.5 kDa = 32.74 + 0.11 × time – 0.00041× time^2 (R^2 = 0.92).
[l] insoluble < 1 kDa = 57.16 – 0.014 × time – 0.00078 × time^2 (R^2 = 0.97).

The hydrolysates of the soluble protein fraction contained a larger proportion of material >10 kDa compared to the RSM hydrolysates (Table 4.3). This can be explained by the presence of intact soluble proteins, which were detected by SDS-PAGE in the hydrolysates of the RSM and the soluble protein fraction (results not shown). However, increasing the toasting time decreased (linear $P < 0.001$, quadratic $P = 0.005$) the proportion of material >10 kDa (Table 4.3). The nitrogen concentration before hydrolysis was similar for all the hydrolyzed soluble fractions. Therefore, the decrease in the proportion of material >10 kDa is not expected to be due to the decrease in solubility with increasing toasting times (as explained previously for the soluble intact RSM), but to the facilitated hydrolysis of soluble intact proteins. This was also reflected by the overall decrease in the molecular weight of the hydrolysates, as the proportion of peptides 1.5 – 10 kDa ($P = 0.002$) and <1.5 kDa ($P < 0.001$) linearly increased with increasing toasting times. The increase in the proportion of peptides <1.5 kDa with increasing toasting time obtained after hydrolysis of the soluble protein fraction can be explained by an increased denaturation of the soluble proteins, exposing cleavage sites that were initially buried [24]. A positive correlation ($r = 0.63$, $P < 0.05$) was found between the proportion of peptides 1.5 – 10 kDa and the *k* of hydrolysis of the soluble protein fraction (Table 4.4).

Table 4.4. Pearson correlation coefficients between the proportion of peptides after hydrolysis and the maximum degree of hydrolysis (DH_{max}) and rate of hydrolysis (*k*) of the rapeseed meals (RSM) and the soluble and insoluble protein fractions separated from these RSM.

Proportion of peptides after hydrolysis	RSM		Soluble protein fraction		Insoluble protein fraction	
	DH_{max}	*k*	DH_{max}	*k*	DH_{max}	*k*
> 10 kDa	0.16	-0.01	-0.25	-0.44	0.02	-0.77**
1.5 – 10 kDa	0.56*	-0.73**	0.08	0.63*	0.44	-0.74**
< 1.5 kDa	-0.74**	0.84***	0.32	0.25	-0.23	0.87***

Significance level: * p<0.05, ** p<0.01, *** p<0.001.

In contrast to what was observed in the hydrolysates of the soluble protein fraction, increasing the toasting time increased the overall molecular weight of the peptides present in the hydrolysates of the insoluble protein fraction (Fig. 4.5d). The proportion of material >10 kDa decreased after the initial 20 min of toasting, but steadily increased with increasing toasting time (Table 4.3). This is not expected to be caused by the presence of intact proteins, as no clear bands were detected by SDS-PAGE in these hydrolysates (results not shown). The proportion of material >10 kDa was negatively correlated to the *k* of hydrolysis ($r = -0.77$, $P < 0.01$) of the insoluble protein fraction (Table 4.4). Furthermore, increasing the toasting time increased (linear $P < 0.001$, quadratic $P = 0.02$) the proportion of peptides 1.5 – 10 kDa and decreased (linear $P < 0.001$, quadratic $P = 0.002$) the proportion of peptides

<1.5 kDa. There was a negative correlation (r = -0.74, $P < 0.01$) between the proportion of peptides 1.5 – 10 kDa and the *k* of hydrolysis of the insoluble protein fraction, whilst a positive correlation (r = 0.87, $P < 0.001$) was found with the proportion of peptides <1.5 kDa (Table 4.4).

The correlations between *k* and the size distribution of the peptides in the hydrolysates of RSM and the insoluble protein fraction could be explained by a shift of the hydrolytic mechanism from a more one-by-one-type to a more zipper-type dominated system with increasing toasting time [15, 25]. Hydrolysis of most proteins shows an intermediate behavior between these two types of hydrolytic mechanisms [15]. In the one-by-one type of hydrolysis, the cleavage of peptide bonds in one protein is followed by a fast cleavage into smaller peptides. Overall, a higher proportion of intermediate peptides (Table 4.3) was found in this study at short toasting times compared to long toasting times. In contrast, in the zipper-type hydrolysis, several peptide bonds are cleaved simultaneously, which is followed by a slow conversion of large peptides into smaller peptides. The shift towards a zipper-type enzymatic cleavage renders peptides of larger sizes, compared to the more one-by-one type of hydrolysis. Hydrolysates that contain peptides with larger sizes, in addition to intact proteins, can probably be considered to be less digestible compared to those with smaller sizes.

In conclusion, the rate of protein hydrolysis of the soluble protein fraction was 3-9 fold higher than that of the insoluble protein fraction. The decrease in the rate of hydrolysis of the RSM observed with increasing toasting time results from a combination of 1) the reduction in the proportion of fast hydrolysable soluble proteins to slowly digestible insoluble proteins and 2) the decrease in the rate of hydrolysis of the insoluble proteins with increasing toasting time due to the formation of disulfide bonds and/or chemically-modified amino acid residues. In addition, increasing the toasting time results in an overall increase of the size of the peptides after hydrolysis. Positive correlations were obtained between the rates of protein hydrolysis of the RSM and the insoluble protein fraction with the proportion of small peptides (<1.5 kDa) after hydrolysis.

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CHAPTER 5

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

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ABSTRACT

A study was conducted to compare protein digestibility of processed ingredients using two *in vitro* methods with known standardized ileal digestibility of CP (SIDCP) measured in growing pigs. The SIDCP 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 8.0. Size exclusion profiles of the resulting peptides after hydrolysis were also determined. Crude protein digestibility was determined using a two-step enzymatic method, with pepsin at pH 2 and pancreatin at pH 6.8. The SIDCP 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 SIDCP ($r = 0.99$, $P < 0.01$). The DH10 using the pH-STAT method was 10.8, 7.3, 8.7, and 7.0% and was positively correlated to SIDCP ($r = 0.95$, $P = 0.046$). There was no correlation between DH120 and SIDCP. Similarly to the SIDCP, 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 two-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 SIDCP ($r = 0.91$, $P = 0.092$). In conclusion, both *in vitro* methods gave similar correlations to SIDCP indicating that both might be used as indication for the SIDCP of thermally processed SBM and RSM in growing pigs.

Keywords: growing pigs, *in vitro* digestibility, pH-STAT, processing, standardized ileal digestibility, two-step enzymatic method

INTRODUCTION

The determination of standardized ileal digestibility of CP (SIDCP) and AA is necessary for ingredient evaluation and formulation of swine diets. This involves the use of *in vivo* experiments, which is expensive and laborious [1]. 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 (e.g., protein solubility, AA content, and protein digestibility) [2]. *In vitro* methods have been used to predict protein digestibility for many ingredients [1, 3]. However, studies on *in vitro* methods that can accurately predict SIDCP for thermally processed ingredients are scarce (e.g., Eklund *et al.* [4]).

The objective of the present study was to evaluate characteristics of protein digestibility from the two-step *in vitro* enzymatic method and the pH-STAT method with the SIDCP 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.

MATERIALS AND METHODS

Ingredients, processing conditions and standardized ileal digestibility

The experiment consisted of 10 growing barrows (initial BW of 30.8 ± 1.0 kg) fed 1 of 4 experimental diets in each of 3 periods in an incomplete cross-over design [5]. 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 was performed to induce protein damage. The SIDCP was calculated by correcting the apparent ileal digestibility of the ingredients for basal endogenous losses as described by Jansman *et al.* [6].

Two-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 [3]. The method was upscaled 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®.

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 [7]. 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 SIDCP. The DH curve was used to calculate the rate of protein hydrolysis (*k*) based on the model described by Butré *et al.* [8]. The model was fitted using the MODEL procedure of SAS (Version 9.3, SAS Inst. Inc., Cary, NC, USA).

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 mM phosphate buffer at pH 7 containing 150 mM NaCl and 2% (w/v) SDS 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.

Statistical analysis

Results of the *in vitro* analysis were analyzed using the PROC GLM procedure of SAS. The model included the fixed effects of ingredient, processing, and their interaction. Correlations between SIDCP and the *in vitro* results (two-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.

RESULTS AND DISCUSSION

Significant effects of type of ingredient ($P < 0.001$) and processing ($P < 0.001$) were found for SIDCP (Table 5.1). Soybean meal had a higher SIDCP compared to RSM, in

accordance with Eklund *et al.* [9]. Processing reduced SIDCP of SBM and RSM in a similar manner (i.e., without interaction), largely caused by Maillard reactions occurring during toasting with lignosulfonate [5]. Such reactions occurred as shown by the lowered Lys:CP ratio and the formation of furosine and carboxymethyl-lysine [5]. These reactions reduce accessibility of enzymes to specific cleavage sites of proteins [10]. Nevertheless, processing in that study also reduced the standardized ileal digestibility 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.* [11].

Table 5.1. Standardized ileal digestibility of CP (SIDCP) 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 ratio[1]	0.063	0.046	0.056	0.043				
SIDCP[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. [5].
[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.

The two-step enzymatic method showed similar results to SIDCP, as significant ingredient and processing effects were found ($P < 0.05$) without interactions (Table 5.1). The two-step enzymatic method and SIDCP 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 SIDCP. The DH10 was a criterion selected by Pedersen and Eggum [7], 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 [12, 13]. Protein aggregation can hamper enzyme accessibility required for protein

hydrolysis [14, 15], 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 SIDCP (r = 0.91, *P* = 0.094).

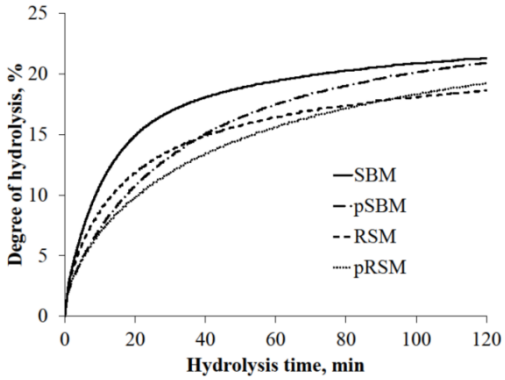

Fig. 5.1. Degree of hydrolysis of the soybean meal (SBM), rapeseed meal (RSM) and both ingredients toasted in the presence of lignosulfonate resulting in processed SBM (pSBM) and processed RSM (pRSM).

The DH120 of SBM and pSBM was higher than that of RSM and pRSM (*P* = 0.02) (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. The content of AA that are specific for trypsin (i.e., Lys and Arg) and chymotrypsin (e.g., Trp, Tyr, Phe), the enzymes used during the pH-STAT hydrolysis, is lower in rapeseed compared to soybean proteins [16]. 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.

Initial pH of the pH-STAT method was highly correlated (r = 0.99, *P* < 0.01) with SIDCP. 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 [17]. Thus, initial pH may be used as a rapid indicator for protein damage because of its high correlation with SIDCP. 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.

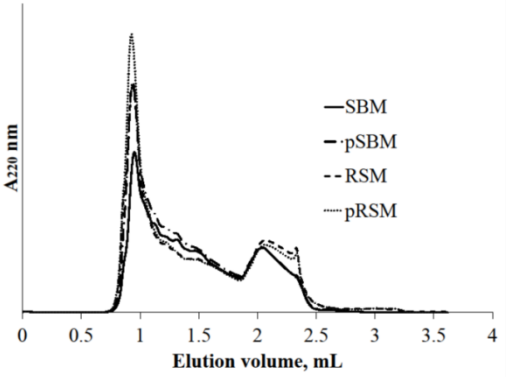

Fig. 5.2. Size exclusion chromatograms of hydrolysates after 2 hours of hydrolysis of soybean meal (SBM), rapeseed meal (RSM) and both ingredients toasted in the presence of lignosulfonate resulting in processed SBM (pSBM) and processed RSM (pRSM).

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 [18]. 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 SIDCP 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 SIDCP for a wider range of feed ingredients and processing conditions.

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CHAPTER 6

Pelleting and extrusion can ameliorate negative effects of toasting of rapeseed meal on protein digestibility in growing pigs

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ABSTRACT

Toasting time (TT) of rapeseed meal (RSM), the secondary diet processing (DP) method and the interaction between both on the apparent CP digestion along the gastrointestinal tract and the apparent and standardized ileal digestibility of AA (AID and SID, respectively) of growing pigs were investigated. The experiment consisted of a 3×3 factorial design of TT of RSM (0, 60 and 120 min) and DP method (mash, pellet and extrusion). Seventy-five boars with a starting body weight of 20 kg were fed incremental amounts of the test diet mixed with a commercial diet for 9 days, followed by 4.5 days of 100% test diet. The pigs were euthanized 4 hours after their last feeding. The gastrointestinal tract was dissected and the small intestine divided in 3 sections of similar length. Samples were collected from the stomach, 1.5 m from the ends of each of the 3 sections of the small intestine and the rectum. The apparent digestibility (AD) of CP for each of the gastrointestinal sections was used to calculate the rate of CP digestion based on a second order equation. Increasing the TT of RSM resulted in lower protein solubility, lower Lys/reactive Lys contents and higher protein denaturation, indicative of the occurrence of protein aggregation and Maillard reactions. There were significant effects ($P \leq 0.01$) of TT on the AD of CP in the different sections of the gastrointestinal tract. The rate of CP digestion of the 0 min toasted RSM diets was 23 and 35% higher than that of the 60 and 120 min toasted RSM diets, respectively. There was a significant interaction ($P = 0.04$) between TT and DP for the AID of CP. Pelleting of the 60 and 120 min toasted RSM diets increased the standardized ileal digestible CP content by 6 and 15%, respectively, compared to the 60 and 120 min toasted mash diets. Extrusion of the 0, 60 and 120 min toasted RSM diets increased the standardized ileal digestible CP content by 5, 9 and 12%, respectively, compared to the 0, 60 and 120 min toasted mash diets. Similar positive effects of pelleting and extrusion were obtained for the apparent ileal digestible contents of Lys and reactive Lys, especially in the diets with higher TT. Processing (pelleting and extrusion) of RSM containing diets can ameliorate the negative effects of RSM toasting on protein and amino acid digestibility.

Keywords: digestibility, extrusion, growing pigs, pelleting, rapeseed meal, rate of protein digestion.

INTRODUCTION

The production process of defatted oilseed meals, such as soybean meal and rapeseed meal (RSM), can cause protein damage, with negative effects on the nutritional value of proteins [1, 2]. According to these authors, most of the protein damage results from the desolventization/toasting process. Toasting is important for the reduction of antinutritional factors, for example glucosinolates in RSM [3] and trypsin inhibitors in soybean meal. Increasing the toasting time (TT) of RSM from 48 to 93 min linearly decreased the standardized ileal digestibility (SID) of most AA in growing pigs [4]. In addition, post-absorptive utilization of the AA may also be reduced due to the Maillard reaction products (MRP) formation [5].

During compound feed manufacturing, the processed oilseed meals are mixed with other ingredients and re-processed (secondary processing) using agglomeration technologies (e.g. pelleting or extrusion) that involve hydrothermal treatments. Contrasting results of secondary processing of ingredients or diets on protein digestibility have been reported [6-8]. The lack of consistency in reported effects of secondary processing on protein damage and protein digestibility is probably caused by variation in the process conditions employed (temperature, time) as well as by variation of the initial extent of protein damage of the ingredients (primary processing). Previous research has not involved secondary processing of diets that contained ingredients with different degrees of protein damage.

Hence, the aim of this study was to test the effects of TT of RSM, the secondary diet processing (DP) method and the interaction between both on the apparent CP digestion along the gastrointestinal tract and the AID and SID of AA. We hypothesize that there will be additive negative effects of TT and DP on CP and AA digestibility.

MATERIALS AND METHODS

Ethical approval

The experiment was approved by the Central Committee of Animal Experiments (The Netherlands) under the authorization number AVD260002015139.

Experimental setup

The experiment involved a 3×3 factorial arrangement of treatments including TT of RSM (0, 60 and 120 min) and secondary processing of diets that contained those RSM (mash, pelleting, and extrusion) as factors. This design resulted in 9 experimental diets: 0, 60 and 120 min mash (M-0, M-60 and M-120, respectively), 0, 60 and 120 min pelleted (P-0,

P-60 and P-120, respectively) and 0, 60 and 120 min extruded (E-0, E-60 and E-120, respectively).

Processing of the RSM

Commercial 00-rapeseeds (*Brassica napus*) harvested in 2015 were purchased from the Agricultural Cooperative Arterris (Toulouse, France). The RSM was processed at the pilot plant of CREOL/OLEAD (Pessac, France). Production of the untoasted (0 min) RSM was achieved following the procedure described previously [(9)].The 0 min toasted RSM was divided in three batches: one untoasted and the other two undergoing toasting for 60 min and 120 min. Approximately 450 kg of the untoasted RSM was toasted in 3 batches for 60 or 120 min each in the lower tray of a desolventizer-toaster (Schumacher type, Desmet-Ballestra, Zaventem, Belgium) with injection of live steam (30 kg/h). During toasting, the arm rotation in the tray was set at 20 rpm and indirect steam pressure at 3 bar. Temperatures during toasting ranged between 100 – 110°C.

Processing of the diets

Mash diets including the 0, 60 and 120 min toasted RSM were prepared according to the formulation described in Table 6.1. Rapeseed meal was the only protein source included in the diets and was ground by hammer milling to pass a 3 mm sieve. Each of these mash diets was divided into 3 batches in order to prepare the mash, pelleted or extruded pig feeds. The mash diets were mixed without any further treatment.

Table 6.1. Ingredient composition of the experimental diets.

Ingredient	Inclusion level (g/kg as is)
Rapeseed meal	410.0
Potato starch [1]	503.9
Soy oil	60.0
Monocalcium phosphate	8.1
Calcium carbonate	5.5
Premix vitamins and minerals [2]	5.0
Titanium dioxide	3.0
Salt	3.0
Sodium bicarbonate	1.5

[1] Paselli™ (Avebe, Veendam, The Netherlands).

[2] Composition premix per kg of feed: 100 mg Fe (as $FeSO_4 \cdot H_2O$), 70 mg Zn (as $ZnSO_4 \cdot H_2O$), 20 mg Cu (as $CuSO_4 \cdot 5H_2O$), 30 mg Mn (as MnO), 1.2 mg I (as KI), 0.25 mg Se (as Na_2SeO_3), 125 mg antioxidant (Oxytrap PXN), 10000 IU Vit. A, 2000 IU Vit. D_3, 150 mg choline chloride, 40 mg Vit. E, 30 mg niacin, 15 mg D-pantothenic acid, 4 mg Vit. B_2, 1.5 mg Vit. K_3, 1.5 mg Vit. B_6, 1.0 mg Vit. B_1, 0.4 mg folic acid, 0.05 mg biotin, 20 µg Vit. B_{12}. Carrier was potato starch (Paselli).

The RSM containing diets were pelleted (Research Diet Services, Wijk bij Duurstede, The Netherlands) at a mash conditioning temperature of 80°C to pass through a 4×40 mm die. Pellet temperatures after the die reached 86, 87 and 89°C for the P-0, P-60

and P-120 diets, respectively. The corresponding energy consumptions of the pellet press were 7.50, 7.55, and 7.61 kWh/t, respectively.

Extrusion was performed in a co-rotating twin-screw extruder (MPF50, Baker Perkins, Peterborough, UK) after the diets were mixed with water to 850 g DM/kg in a paddle mixer (type F60, Halvor Forberg, Bygland, Norway). The screw configuration in the extruder has been described elsewhere [10]. The extruder consisted of 9 heating zones and a die with 2 orifices (Ø 1.9 mm). Temperatures in the 9 heating zones were set at 30, 40, 50, 60, 70, 80, 90, 105 and 115°C, respectively. During extrusion of the 3 diets the speed of the extruder screw was fixed at 200 rpm and the measured internal temperatures at the die ranged from 111 – 118, 110 – 118 and 114 – 120°C for the E-0, E-60 and E-120 diets, respectively. Water was added directly in the extruder with a water pump at 4.3, 3.2 and 4.5 L/h for the E-0, E-60 and E-120 diets, respectively. Corresponding measured throughputs were 41.1, 32.7 and 42.2 kg/h, respectively. The extruded diets were subsequently air-dried at 60°C in air-forced ovens until a dry matter content of ~900 g/kg (IR 200 Moisture Analyzer, Denver Instrument Company, Arvada, CO, USA) was obtained. The analyzed chemical composition of the diets is reported in Table 6.2.

Animals and housing

Eighty-one crossbred boars (Pietrain × Topigs 20) weighing on average 20.4 ± 0.9 kg were used. The experiment was performed in 3 trials of 24 pigs each, plus a reposition trial with 9 animals. Pigs were housed in groups of 4 animals per pen, except during the reposition trial, when each pen housed 3 animals. To enable individual feeding, animals were separated twice per day (0800 and 1600 h) using physical barriers. The animals remained individually housed for the duration of feeding (approximately 1 h), where after they were again group housed. All pigs had free access to water both during group and individual housing. The pens (0.71 m^2/animal) had a 3:1 ratio of solid to slatted floor. Temperature in the barn was 28 ± 1°C and the lights were turned off from 1900 h till 0700 h of the next day.

Animals were fed at a rate of 2.8 × maintenance energy requirement (293 kJ NE/kg $BW^{0.75}$; CVB, 2011). Sucrose (Van Gilse, Oud Gastel, The Netherlands) was added (10% w/w in final diet) on top of the diets before feeding to the M-0, P-0 and E-0 diets in order to mask the bitter taste of the glucosinolates and promote feed intake. To stimulate feed intake and to prevent sucrose separation, all the 0 min toasted RSM and the mash diets were mixed with water (1:1.5 w/w feed to water) just before feeding. The experiment consisted of 10 adaptation days, during which the animals were fed meals consisting of 30% (w/w) of the test diet and 70% (w/w) of commercial grower diet (Agrifirm Feed, Apeldoorn, The Netherlands) for the first 3 days. Incremental amounts of the test diet (10% extra per

day) were exchanged for the commercial diet, until 100% test diet was achieved on the 10th day. Hence, the test diet (100%) was fed for 4.5 consecutive days. On the morning of the 14th day, the animals were fed 4 hours before being euthanized. Pigs were weighed on days 8 and 13 in order to calculate the amount of feed provided.

Sample collection and chemical analysis

For euthanasia, animals were injected with pentobarbital in the ear vein and exsanguinated. Immediately after exsanguination, the stomach, small intestine and rectum were removed. The contents of the stomach were vigorously mixed in the stomach to obtain a homogeneous suspension before sampling. The small intestine was carefully spread on a table and divided with clamps in 3 segments of equal length. Sections of 1.5 m from the ends of each segment (SI1, SI2 and SI3) were dissected and their contents flushed out with 50 mL demineralized water. Section SI3 was considered to represent the ileum. A section of 0.2 m of the rectum was removed and its contents collected by gentle stripping. Digesta samples were immediately frozen on dry-ice and kept at -20°C until freeze drying. After freeze drying, samples were ground to pass a 1 mm sieve using a centrifugal mill at 12000 rpm (ZM200, Retsch, Haan, Germany).

Nitrogen content was analyzed by combustion (AOAC 968.06; Thermo Quest NA 2100 Nitrogen and Protein Analyzer; Breda, The Netherlands) and the CP content calculated using a 6.25 conversion factor. Dry matter (DM) content was determined according to ISO 6496 [(12)]. Ash was determined according to ISO 5984 [(13)]. Crude fat was determined according to ISO 6492 [(14)] after hydrolysis with HCl. Crude fiber was determined according to ISO 6865 [(15)]. The NDIN was determined as the nitrogen (determined by the Kjeldahl method, ISO 5983, ISO 2005b) remaining after 60 min hydrolysis with neutral detergent reagents (Boom, Meppel, The Netherlands), containing Termamyl (Novozymes, Bagsvaerd, Denmark) and Alcalase (Novozymes) at pH 7.0. Protein solubility in water and the protein denaturation enthalpy were determined according to Salazar-Villanea *et al.* [(9)]. For particle size determination, 250 – 300 mg of the diets were suspended in 3 mL demineralized water and mixed in a rotator until the pellets or extrudates completely disintegrated.

Table 6.2. Chemically analyzed nutrient composition of the RSM and the experimental diets [1].

	Toasting time of the RSM (min)			Experimental diets								
				Mash			Pelleted			Extruded		
Content (g/kg DM)	0	60	120	M-0	M-60	M-120	P-0	P-60	P-120	E-0	E-60	E-120
DM (g/kg as is)	924.4	925.1	924.3	915.7	916.6	917.7	895.2	900.7	905.4	906.0	911.2	909.7
CP	381.9	396.1	395.3	158.9	155.8	157.4	159.3	161.1	157.0	155.6	161.0	157.7
A-EE	38.9	36.8	34.6	81.9	76.4	77.4	78.2	77.7	77.3	77.3	74.6	76.9
Crude fiber	169.1	167.8	162.0	72.6	71.5	71.0	74.1	72.1	71.7	65.8	67.9	68.6
Ash	74.9	75.6	75.9	51.7	52.0	51.7	51.5	51.5	51.4	50.4	50.7	50.8
Titanium dioxide	-	-	-	2.4	2.2	2.4	2.3	2.3	2.3	2.3	2.3	2.4
NDIN	4.7	8.4	14.6	1.9	3.7	6.7	<0.4	3.1	5.5	2.0	3.4	4.9
Soluble CP	92.0	51.9	37.6	29.7	14.0	10.4	30.6	13.9	9.3	8.2	7.4	6.9
Indispensable AA												
Arg	22.8	22.8	22.1	9.2	9.3	9.0	9.5	9.3	9.3	9.5	8.9	8.8
His	11.3	10.9	10.6	4.4	4.3	4.3	4.6	4.3	4.3	4.8	4.6	4.5
Ile	14.8	15.0	14.4	6.1	6.2	6.2	6.3	6.2	6.2	6.2	6.0	6.2
Leu	26.8	27.1	26.6	10.9	11.0	11.0	11.1	11.1	11.2	11.0	11.0	11.0
Lys	24.9	23.2	21.3	10.2	9.4	8.7	10.4	9.4	8.8	10.2	9.3	8.6
OMIU-RL	23.6	21.2	18.7	9.6	8.5	7.5	9.8	7.9	7.6	9.7	8.3	7.5
Met	8.0	8.2	8.0	3.4	3.3	3.3	3.4	3.4	3.4	2.9	2.9	3.1
Phe	15.1	15.5	15.3	6.2	6.3	6.4	6.6	6.4	6.4	6.4	6.5	6.3
Thr	17.4	17.6	17.5	7.2	7.3	7.2	7.3	7.3	7.4	7.2	7.2	7.3
Trp	4.6	4.9	4.7	2.0	1.7	2.1	2.0	2.0	1.9	2.0	2.0	1.9
Val	21.3	21.1	20.8	8.7	8.5	8.6	8.8	8.9	8.7	8.4	8.9	9.0
Dispensable AA												
Ala	17.1	17.2	17.1	7.0	7.0	7.0	7.0	7.0	7.1	7.1	7.0	7.0
Asp	27.3	27.5	26.9	11.0	10.7	10.7	11.0	10.9	10.9	10.9	11.0	10.9
Cys	9.4	9.3	9.3	3.9	3.9	3.8	4.1	3.9	3.9	3.4	3.3	3.6
Glu	68.3	69.2	67.9	27.7	27.9	27.7	28.2	27.8	28.3	28.4	27.2	27.0
Gly	20.0	20.2	20.0	8.2	8.2	8.2	8.3	8.3	8.4	8.3	8.2	8.2
Hyp	5.5	5.6	5.4	2.6	1.8	2.0	2.2	2.4	2.5	1.9	2.4	2.5
Pro	24.0	24.1	23.9	9.5	9.2	9.4	9.4	9.4	9.5	9.7	9.4	9.6
Ser	17.2	17.2	17.3	6.9	7.0	6.9	7.0	7.0	7.1	7.0	7.0	6.9
Tyr	10.8	11.0	10.9	4.3	4.5	4.4	4.5	4.3	4.4	4.4	4.5	4.3

[1] Abbreviations: RSM, rapeseed meal; A-EE, acid hydrolyzed ether extract; OMIU-RL, O-methylisourea reactive lysine.

Particle size was determined by laser diffraction (Mastersizer 3000, Malvern, Worcestershire, UK) using demineralized water as dispersant. Measurements were performed in the range 0.01 – 5000 µm. The reference material was wood flour (refraction index 1.53, absorption index 0.1) and 5 measurements per sample were obtained. The particle size distribution (D_{10}, D_{50} and D_{90}) and the mean particle size ($D_{4,3}$) were obtained using the Mastersizer V3.50 software. Titanium dioxide, added as marker, was determined according to the spectroscopic method described elsewhere [17]. Amino acids in the diets and ileal-digesta were analyzed according to ISO 13903 [18]. Oxidation using a performic acid/phenol mixture was performed prior to hydrolysis for the analysis of Met and Cys. Tryptophan was analyzed according to ISO 13904 [19]. Reactive Lys was determined after reaction of the diet and ileal-digesta samples with O-methylisourea (OMIU) for 3 days and subsequent hydrolysis with 6M HCl for 23 hours [20]. The OMIU-reactive Lys content was calculated from the molar amount of homoarginine and the molecular weight of Lys after separation by ion exchange chromatography, post-column reaction with ninhydrin and detection at 570 nm.

Calculations and statistical analysis

Apparent digestibility (AD) of CP for each section of the gastrointestinal tract (i.e. stomach, SI1, SI2, SI3 and rectum) and of AID of AA was calculated according to Eq. 1

(1) AD or AID of $x = 100 - [(x_{chyme} / x_{diet}) \times (TiO_{2\ diet} / TiO_{2\ chyme}) \times 100]$

The SID was calculated according to Eq. 2, using the composition of basal endogenous losses previously described [21].

(2) SID of x = AID of x + [(basal endogenous x / x_{diet}) × 100]

The rate of protein digestion (k) was estimated for each individual pig using the AD of CP of each intestine section. Retention times for each of the sections of the small intestine (SI1, 27 min; SI2, 74 min; SI3, 101 min) were obtained from the values reported by Asche *et al.* [22] for weanling pigs. A cumulative retention time (CRT) of 27, 101 and 202 min was used for sections SI1, SI2 and SI3, respectively. Equal retention times were assumed for all diets based on their identical ingredient composition. The MODEL procedure of SAS (version 9.3) was used to fit k according to a second order kinetic model, in which the maximum possible digestibility (D_{max}) was set to 100. The model is described in Eq. 3

(3) AD of CP = $D_{max} - [D_{max} / (1 + k \times CRT \times D_{max})]$

Effects of experimental treatments were tested by restricted maximum likelihood using the MIXED procedure of SAS (version 9.3) by means of an F-test according to a model with trial, TT of RSM, DP and their interaction as fixed factors. Feed intake of each of the animals on the day of euthanasia corrected for the average intake per treatment was

used as covariate in the model. Feed intake was not included as a covariate in the model for the analysis of SID, as these had already been corrected for the basal endogenous losses, which are proportional to the feed intake level [21]. Significance was assumed at *P*-values lower than 0.05, whilst trends were considered at *P*-values between 0.05 and 0.1. Post-hoc comparison between treatment means was performed using the Bonferroni adjustment.

RESULTS

Chemical composition and particle size distribution of the RSM and the diets

Increasing TT increased the contents of NDIN, whilst these were decreased in the P-120 and E-120 diets (Table 6.2). Increasing TT of the RSM resulted in a reduction of the Lys and OMIU-reactive Lys contents and the ratio between them, which was also reflected in their contents in the diets (Table 6.2). The DP method did not seem to influence the content of any of the AA in the diets. Increasing the TT reduced the denaturation enthalpy and the content of soluble CP in the RSM (Table 6.3). In the diets, whilst pelleting did not change the denaturation enthalpy or content of soluble CP compared to the mash diets, these appeared to be reduced after extrusion, mainly in the E-0 diet.

Overall, there was a decrease in particle size after pelleting and, even further, after extrusion compared to the particle size in the mash diets (Table 6.3). The reduction in particle size after extrusion and pelleting was notorious across the whole distribution (D_{10}, D_{50} and D_{90}). The mean particle size of the P-0, P-60 and P-120 diets was 42, 33 and 31% lower compared to that of the M-0, M-60 and M-120 diets, respectively (Table 6.3). Extrusion had a bigger impact on the mean particle size compared to pelleting, as this was decreased by 60, 60 and 47% when comparing the E-0, E-60 and E-120 diets to the M-0, M-60 and M-120 diets, respectively.

Crude protein digestion along the gastrointestinal tract

Due to low feed intake four animals (3 from diet M-0 and 1 from diet M-120) were excluded before the end of the experiment. Another two animals (both from diet P-0) were excluded from the experiment because low amounts of digesta collected after euthanasia. There was a significant interaction ($P = 0.002$) between TT and DP for the feed intake on the day of euthanasia. Feed intakes on this day were 0.279, 0.427, 0.397, 0.288, 0.417, 0.371, 0.449, 0.380 and 0.424 kg for the M-0, M-60, M-120, P-0, P-60, P-120, E-0, E-60 and E-120 diets, respectively.

Table 6.3. Denaturation enthalpy and particle size distribution of the RSM and the experimental diets [1].

Physical parameter	Toasting time of the RSM (min)			Experimental diets								
				Mash			Pelleted			Extruded		
	0	60	120	M-0	M-60	M-120	P-0	P-60	P-120	E-0	E-60	E-120
Denaturation enthalpy (J/g CP)	2.57	0.56	0.51	2.52	0.51	0.44	2.45	0.43	0.38	0.51	0.56	0.38
Particle size (µm)												
$D_{4,3}$	-	-	-	494	489	454	286	329	313	196	198	240
D_{10}	-	-	-	33	53	49	18	28	31	17	17	27
D_{50}	-	-	-	368	393	339	146	197	192	107	111	154
D_{90}	-	-	-	1154	1093	1042	785	828	782	534	529	592

[1] Abbreviations: RSM, rapeseed meal.

In general, the variation in the AD of CP decreased from the proximal to the distal sections of the small intestine (Table 6.4). There were no interactions ($P > 0.05$) between TT and DP for the AD of CP in the stomach, SI1, SI2 and rectum. The AD of CP in the stomach was higher ($P = 0.002$) in the 0 and 60 min toasted RSM diets compared to the 120 min toasted diets. Mash and pelleted diets tended ($P = 0.08$) to have higher AD of CP in the stomach compared to the extruded diets. The AD of CP of the 0 min toasted RSM diets in SI1 was higher ($P = 0.01$) compared to that of the 60 and 120 min diets. The TT also affected the AD of CP in SI2 ($P = 0.004$). The AD of CP in SI2 of pigs fed the 0 min toasted RSM diets was higher than that of pigs fed the 120 min toasted diets whereas, the AD of CP in SI2 of pigs fed the 60 min toasted diets was intermediate. There was an interaction ($P < 0.05$) between TT and DP with regard to the AD of CP in the ileum (SI3). Subclass means are presented in Table 6.5. The ileal AD of CP in pigs fed the M-120 diet was lower than in pigs fed the other diets, apart from the intermediate value in pigs fed the E-120 diet (Table 6.5). The AD of CP in the rectum of pigs fed the 0 min toasted RSM was higher ($P < 0.001$) than for pigs fed the 120 min diets, whilst the digestibility of the 60 min diets was not different between either of them (Table 6.4).

There were significant effects of TT ($P < 0.001$) and DP ($P = 0.002$) on the SID of CP, whilst there was a tendency ($P = 0.07$) for the interaction between TT and DP to be significant (Table 6.6). The content of standardized ileal digestible CP was similar in the P-0 compared to the M-0 diet, whereas it increased by 6 and 15% when comparing the P-60 to the M-60 diet and the P-120 to the M-120 diet, respectively. Extrusion increased the standardized ileal digestible CP content of the by 5, 9 and 12% comparing the E-0, E-60 and E-120 to the M-0, M-60 and M-120 diets, respectively.

Kinetics of CP digestion (Table 6.4) were derived from the AD of CP at the different sections of the small intestine. Significant effects of TT ($P < 0.001$) were observed on *k*. The *k* for the 0 min toasted RSM diets was 23 and 35% higher than that of the 60 min and 120 min toasted RSM diets, respectively. The DP method tended ($P = 0.08$) to affect the *k*, with higher values for the extruded > pelleted > mash diets.

Table 6.4. Apparent crude protein digestibility in the different sections of the gastrointestinal tract and the derived kinetic digestion parameters as affected by toasting time of RSM and diet processing method [1, 2].

Gastrointestinal section	Toasting time (min)			Pooled SE	*P*-value	Diet processing method			Pooled SE	*P*-value
	0	60	120			Mash	Pellet	Extruded		
n	24	26	25			25	25	25		
Stomach	2.7[a]	1.0[a]	-3.4[b]	0.44	0.002	2.2	-0.1	-1.8	0.44	0.08
Small intestine 1	14.9[a]	-3.0[b]	-1.1[b]	1.64	0.01	2.5	-1.2	9.5	1.64	0.24
Small intestine 2	51.7[a]	46.9[ab]	38.5[b]	0.99	0.004	41.4	49.7	46.0	0.99	0.11
Small intestine 3 [3]	68.1[a]	67.4[a]	61.7[b]	0.35	<0.001	62.9[b]	66.5[a]	67.8[a]	0.35	0.002
Rectum	71.2[a]	69.1[ab]	66.8[b]	0.25	<0.001	68.2	70.4	68.4	0.25	0.05
Digestion kinetics										
k ($M^{-1} \times s^{-1}$)	1.04E-04[a]	8.0E-05[b]	6.7E-05[b]	1.7E-06	<0.001	7.6E-05	8.5E-05	9.0E-05	1.7E-06	0.08

[1] Means with different superscript letters ([a, b]) within a row and treatment are significantly ($P < 0.05$) different.

[2] Abbreviations: *n*, number of pigs; SE, standard error; *k*, rate of digestion.

[3] Significant interaction Toasting time × Diet Processing ($P = 0.04$). See Table 6.5.

Table 6.5. Toasting time × diet processing method subclass means for the apparent ileal CP and AA digestibility (%) [1, 2].

Nutrient	Experimental diets									Pooled SE	*P*-values		
	Mash			Pelleted			Extruded						
	M-0	M-60	M-120	P-0	P-60	P-120	E-0	E-60	E-120		TT	DP	TT×DP
n	8	9	8	8	8	9	8	9	8				
CP	67.3^{a}	65.2^{a}	56.3^{b}	66.2^{a}	67.5^{a}	65.6^{a}	70.7^{a}	69.5^{a}	63.2^{ab}	1.77	<0.001	0.002	0.04
Indispensable AA													
Arg	80.2^{a}	80.2^{a}	73.9^{b}	81.0^{a}	82.2^{a}	82.6^{a}	86.0^{a}	84.6^{a}	81.9^{a}	1.31	0.01	<0.001	0.02
His	78.3	76.2	70.1	81.7	79.1	77.2	84.9	81.6	78.4	1.08	<0.001	<0.001	0.26
Ile	68.7^{bc}	71.4^{abc}	66.7^{c}	72.4^{abc}	76.2^{a}	74.3^{ab}	74.3^{ab}	71.6^{abc}	69.7^{bc}	1.35	0.03	<0.001	0.04
Leu	71.9^{c}	75.4^{abc}	70.7^{c}	75.9^{abc}	80.0^{a}	78.7^{ab}	77.9^{ab}	75.5^{abc}	74.0^{bc}	1.24	0.03	<0.001	0.01
Lys	75.2	68.8	58.1	76.9	73.2	67.0	78.9	73.4	66.1	1.59	<0.001	<0.001	0.21
OMIU-RL	76.5^{ab}	70.2^{bc}	59.5^{d}	77.1^{ab}	72.4^{bc}	68.6^{c}	80.4^{a}	75.1^{abc}	68.6^{c}	1.58	<0.001	<0.001	0.06
Met	82.7^{bc}	82.5^{bc}	79.3^{c}	84.7^{ab}	87.5^{a}	85.7^{ab}	85.4^{ab}	83.2^{bc}	83.3^{bc}	0.88	0.03	<0.001	0.02
Phe	74.0	75.7	71.4	78.4	81.1	79.4	80.2	78.4	76.1	1.18	0.01	<0.001	0.11
Thr	63.6^{ab}	64.3^{a}	56.2^{b}	63.7^{ab}	68.6^{a}	66.0^{a}	67.6^{a}	65.3^{a}	61.6^{ab}	1.75	0.002	0.003	0.02
Trp	65.7	64.1	63.3	68.7	72.5	70.8	71.8	70.7	68.3	1.77	0.44	<0.001	0.36
Val	68.4	68.3	63.2	70.4	74.5	71.6	72.4	72.0	69.5	1.54	0.01	<0.001	0.21
Dispensable AA													
Ala	69.8^{ab}	72.4^{a}	65.3^{b}	72.0^{a}	75.0^{a}	73.5^{a}	75.7^{a}	73.2^{a}	70.2^{ab}	1.46	0.003	<0.001	0.03
Asp	69.9	66.0	57.3	70.6	71.1	66.9	74.0	69.8	64.9	1.72	<0.001	<0.001	0.19
Cys	69.2	68.0	57.3	74.0	73.2	68.1	70.3	68.8	65.0	2.22	<0.001	<0.001	0.39
Glu	81.3	80.5	74.0	84.6	85.1	81.9	86.2	85.0	82.2	1.32	<0.001	<0.001	0.37
Gly	48.8^{ab}	54.2^{ab}	34.6^{b}	46.3^{ab}	44.6^{ab}	54.5^{ab}	58.0^{ab}	60.4^{a}	48.3^{ab}	5.24	0.18	0.06	0.04
Hyp	45.0^{a}	-14.1^{bc}	-20.0^{c}	10.0^{bc}	19.6^{ab}	19.6^{ab}	-6.2^{bc}	17.6^{ab}	10.4^{abc}	8.05	0.12	0.10	<0.001
Pro	67.2^{a}	56.1^{ab}	37.5^{ab}	25.3^{b}	29.6^{ab}	46.1^{ab}	49.4^{ab}	54.6^{ab}	34.8^{ab}	8.78	0.43	0.01	0.03
Ser	64.2^{ab}	66.7^{a}	58.9^{b}	65.6^{ab}	69.6^{a}	67.9^{a}	70.1^{a}	67.9^{a}	63.7^{ab}	1.57	0.001	0.001	0.02
Tyr	65.1	67.4	61.1	68.0	71.1	70.1	72.9	72.3	67.8	1.78	0.02	<0.001	0.19

[1] Means with different superscript letters ($^{a, b, c, d}$) within a row represent significant differences ($P < 0.05$) or trends ($P \geq 0.05$ and < 0.10).

[2] Abbreviations: AID, apparent ileal digestibility; *n*, number of pigs; SE, standard error; M, mash; P, pelleted; E, extruded; TT, toasting time; DP, diet processing; OMIU-RL, O-methylisourea reactive lysine.

Table 6.6. Toasting time × diet processing method subclass means for the standardised ileal CP and AA digestibility (%) [1, 2].

Nutrient	Experimental diets									Pooled SE	*P*-values		
	Mash			Pelleted			Extruded						
	M-0	M-60	M-120	P-0	P-60	P-120	E-0	E-60	E-120		TT	DP	TT×DP
CP	69.3^{a}	68.5^{a}	59.4^{b}	68.9^{a}	70.5^{a}	68.3^{a}	74.2^{a}	72.3^{a}	66.5^{ab}	1.8	<0.001	0.002	0.07
Indispensable AA													
Arg	81.4^{bc}	82.0^{ab}	75.7^{c}	82.4^{ab}	83.9^{ab}	84.1^{ab}	87.9^{a}	86.3^{ab}	83.8^{ab}	1.3	0.01	<0.001	0.02
His	79.5	78.1	71.9	83.1	80.9	78.8	86.7	83.2	80.3	1.1	<0.001	<0.001	0.26
Ile	70.4^{c}	74.1^{abc}	69.1^{c}	74.6^{abc}	78.8^{a}	76.6^{ab}	77.2^{ab}	74.0^{abc}	72.4^{bc}	1.4	0.03	<0.001	0.02
Leu	73.1^{c}	77.3^{abc}	72.5^{c}	77.5^{abc}	81.8^{a}	80.3^{ab}	79.9^{ab}	77.1^{abc}	75.9^{bc}	1.3	0.03	<0.001	0.01
Lys	76.2	70.6	60.0	78.3	75.0	68.6	80.7	75.0	68.1	1.6	<0.001	<0.001	0.28
Met	85.5^{cd}	87.0^{bcd}	83.5^{d}	88.1^{abc}	91.6^{a}	89.3^{abc}	90.8^{ab}	87.8^{abcd}	88.0^{abc}	1.0	0.05	<0.001	0.01
Phe	79.1^{b}	83.5^{ab}	78.5^{b}	84.0^{ab}	88.5^{a}	86.0^{a}	88.3^{a}	85.1^{a}	83.9^{ab}	1.3	0.02	<0.001	0.01
Thr	64.1^{ab}	65.2^{a}	57.0^{b}	64.8^{ab}	69.3^{a}	66.6^{a}	68.6^{a}	66.0^{a}	62.4^{ab}	1.8	0.002	0.003	0.04
Trp	69.9	71.6	69.2	73.9	78.7	76.5	78.7	76.5	75.0	1.9	0.36	<0.001	0.34
Val	68.4	68.3	63.3	70.6	74.5	71.5	72.4	71.9	69.5	1.5	0.02	<0.001	0.27
Dispensable AA													
Ala	71.8^{bc}	75.5^{ab}	68.2^{c}	74.6^{abc}	77.9^{ab}	76.1^{ab}	79.0^{a}	75.8^{ab}	73.3^{abc}	1.5	0.005	<0.001	0.02
Asp	69.9	69.3	60.4	73.2	74.3	69.6	77.4	72.6	68.1	1.7	<0.001	<0.001	0.16
Cys	70.7	70.4	59.6	76.0	75.3	70.1	73.1	71.1	67.6	2.3	<0.001	<0.001	0.37
Glu	82.6	82.5	75.9	86.1	87.1	83.5	88.2	86.8	84.2	1.3	<0.001	<0.001	0.37
Gly	51.9^{ab}	59.1^{ab}	39.2^{b}	50.3^{ab}	49.1^{ab}	58.5^{ab}	63.1^{ab}	64.6^{a}	53.1^{ab}	5.2	0.19	0.04	0.04
Pro	69.2^{a}	59.3^{ab}	40.4^{ab}	27.8^{b}	32.6^{ab}	48.7^{ab}	52.6^{ab}	57.3^{ab}	37.9^{ab}	8.7	0.44	0.01	0.03
Ser	66.6^{ab}	70.5^{a}	62.5^{b}	68.7^{ab}	73.2^{a}	71.1^{a}	74.1^{a}	71.1^{a}	67.5^{ab}	1.7	0.002	0.001	0.02
Tyr	68.6	72.7	66.2	72.1	76.3	74.6	78.4	76.8	73.3	1.9	0.03	<0.001	0.15

[1] Means with different superscript letters ($^{a, b, c, d}$) within a row represent significant differences ($P < 0.05$).

[2] Abbreviations: SID, standardized ileal digestibility; M, mash; P, pelleted; E, extruded; TT, toasting time; DP, diet processing.

Apparent and standardised ileal digestibility of AA

Significant interactions ($P < 0.05$) between the TT and DP were noticed on the AID of Arg, Ile, Leu, Met, Thr, Ala, Gly, Pro and Ser (Table 6.5). The interaction between TT and DP for the AID of OMIU-reactive Lys tended to be significant ($P = 0.06$). Significant effects ($P < 0.05$) of TT and DP were obtained for the AID of His, Lys, Phe, Val, Asp, Cys, Glu and Tyr. The AID of Trp was only affected ($P < 0.001$) by DP. The effect of feed intake, which was used as a covariate in the statistical model, was not significant for the AID of any of the AA. The apparent ileal digestible Lys content increased by 4, 6 and 17% comparing the P-0, P-60 and P-120 to the M-0, M-60 and M-120 diets, respectively. Similarly, the apparent ileal digestible OMIU-reactive Lys content increased by 3 and 17% comparing the P-0 and P-120 to the M-0 and M-120 diets, whilst it decreased by 4% comparing the P-60 to the M-60 diet. Extrusion increased the apparent ileal digestible Lys content by 5, 6 and 12% comparing the E-0, E-60 and E-120 to the M-0, M-60 and M-120 diets, respectively. The apparent ileal digestible OMIU-reactive Lys content was increased by 6, 4 and 15% comparing the E-0, E-60 and E-120 to the M-0, M-60 and M-120 diets, respectively.

A significant ($P < 0.05$) interaction between the effects of TT and DP was found on the SID of Arg, Ile, Leu, Met, Phe, Thr, Ala, Gly, Pro and Ser (Table 6.6). The effects of TT and DP were significant ($P < 0.05$) for the SID of His, Lys, Val, Asp, Cys, Glu and Tyr. The SID of Trp was only affected by the effect of DP ($P < 0.001$).

DISCUSSION

Chemical composition of RSM and diets

Protein solubility has been identified as an important predictor for CP digestibility [23]. The reduction in protein solubility with increasing intensity (e.g. temperature, time) of hydrothermal processing has been linked to protein denaturation and refolding of the proteins into protein aggregates [24]. Thus, the 60 and 120 min toasted RSM diets can be considered to contain larger amounts of aggregated proteins than the 0 min toasted RSM diets. However, not all protein denaturation resulted in a reduction of protein solubility. Whilst protein solubility of the M-120 diet was reduced by 65% compared to the M-0 diet, the denaturation enthalpy was reduced by 83%. Pelleting of the diets including the 60 and 120 min toasted RSM did not result in a further decrease of protein solubility compared to the mash diets, indicating that the conditions applied during pelleting do not induce further protein denaturation and aggregation. In contrast, extrusion of the diets decreased their protein solubility, mainly for the diet including the 0 min toasted RSM (decreased 72%), resulting from an increase in protein denaturation (increased 80%) and aggregation. The

decrease in protein solubility from the M-60 to the P-60 and E-60 and from the M-120 to the P-120 and E-120 diets was lower than those in the 0 min toasted RSM because their proteins were already denatured and aggregated after toasting.

Similar to our findings, decreasing contents of Lys and OMIU-reactive Lys in the RSM with increasing TT have been reported before [3, 4, 25]. Lysine content of the 120 min toasted RSM decreased by 14% compared to the 0 min toasted RSM, whilst the decrease in the content of OMIU-reactive Lys was 21%. The decreasing contents of these compounds were likely caused by chemical changes of proteins due to MRP formation. Pelleting or extrusion of the diets did not further affect the contents of Lys or OMIU-reactive Lys suggesting that no chemical changes to the proteins occurred during processing of the diets.

Crude protein digestion along the gastrointestinal tract

Several actions (e.g. restriction at 2.8× maintenance, sugar and water addition) were taken to maximize the feed intake in the present experiment, especially for those diets including the 0 min toasted RSM. However, the feed intake of the M-0 and P-0 diets were still lower than those of the rest of the diets. The lower feed intake of the M-0 and P-0 diets on the day of euthanasia might be explained by a higher content of glucosinolates and the activity of myrosinase (thioglucoside glucohydrolase) in the 0 min toasted RSM. According to Tripathi and Mishra [26], glucosinolates (e.g. progoitrin) are non-bitter compounds, but hydrolysis by myrosinase produces bitter compounds (e.g. goitrin, thiocyanates, isothiocyanates). Extrusion probably denatured the myrosinase [27] from the E-0 diet, which increased the intake of this diet, without decreasing the content and AID of Lys and OMIU-reactive Lys. Extrusion has also been reported to decrease the content of glucosinolates of rapeseed meal [26].

Increasing thermal protein damage with increasing TT, as determined by the decreasing denaturation enthalpy and contents of soluble protein, Lys and OMIU-reactive Lys, decreased CP digestibility at all intestinal sections. Similar results were recently reported [28] after toasting of soybean meal and RSM in presence of reducing sugars. This severe thermal process was reported to induce the formation of early (e.g. fructosyl-lysine measured as furosine) and advanced (e.g. carboxymethyl-lysine) MRP [29]. The AD of CP of a commercial RSM reported by Hulshof *et al.* [28] at the end of the first and second sections of the small intestine (-28.7 and 23.3%, respectively) were lower than the ones reported here for all TT (ranging from -3.0 to 14.9% in the first section and from 38.5 to 51.7% in the second section). These are large differences considering that the contents of Lys and OMIU-reactive Lys reported by Hulshof *et al.* [29] in the commercial RSM (5.6 and 5.0 g/100 g CP,

respectively) were comparable to the contents of the 60 min toasted RSM reported in the present study (5.8 and 5.4 g/100 g CP, respectively).

The AID and SID of CP did not differ between the diets containing 0 and 60 min toasted RSM for any of the DP methods (i.e. mash, pelleting, extrusion), even though the ratio of Lys to CP and the contents of soluble protein and OMIU-reactive Lys were lower for the 60 min toasted RSM diets. Previous research has identified a link between the reduction in the Lys to CP ratio and the apparent or standardized ileal digestibility [4, 30-32]. Although damage to the proteins after toasting for 60 min reduced the rate of protein digestion compared to the 0 min toasted diet, the extent of digestion (i.e. ileal digestibility) remained unaffected.

The reduction of the Lys to CP ratio when comparing the 0 min to the 120 min toasted RSM was 14, 14 and 17% for the mash, pelleted and extruded diets, respectively. However, no effects of toasting for 120 min were observed on the AID or SID of CP after pelleting or extrusion of the diets. Although Maillard reactions occurred due to toasting of RSM, protein digestibility was improved by pelleting or extrusion of the diets. Positive effects of diet processing on CP and AA digestibility have been associated with an increase in protein denaturation [8, 33, 34]. However, proteins in the 60 and 120 min toasted RSM were already denatured during the toasting treatment and no further denaturation occurred during pelleting or extrusion of the diets, as demonstrated by the absence of changes in denaturation enthalpy of their proteins. Therefore, protein denaturation during pelleting or extrusion does not explain the observed increase in protein digestibility.

Factors during pelleting and extrusion of the diets, such as shear and pressure, can reduce the particle size of the feeds as observed in the present experiment. The reduction in particle size likely explains the overall increase in the AID and SID of CP and AA after pelleting and extrusion of the diets. However, the reduction in particle size after pelleting and extrusion was constant amongst the different TT. This does not explain that the increase in AID and SID of CP and most AA after pelleting and extrusion was higher for the diets that contained 60 and 120 min toasted RSM compared to 0 min toasted RSM. Reducing the particle size of wheat or maize from 1000 to 500 µm did not result in improved SID of CP in growing pigs [35, 36]. In addition, reducing the particle size of soybean meal in the range from 900 to 150 µm resulted in only a small increase (1.3%) of the essential AA standardized ileal digestibility in grower-finisher pigs [37]. Therefore, it seems unlikely that the decrease in particle size after pelleting or extrusion caused the increase in digestibility for the different toasting times.

The increase in the AID and SID of CP of the 60 and 120 min toasted RSM after pelleting and extrusion matches the reduction in the NDIN content after pelleting and

extrusion. It is possible that the disruption of cell walls by shear increased the release of protein. However, the reduction in the NDIN content of the diets only partly accounts for the increase in standardized ileal digestible CP content of the diets. In addition, shear can decrease the size of the protein aggregates formed after thermal treatments. The mechanical energy or shear input during extrusion is usually higher compared to pelleting [38]. The size of the protein aggregates formed during extrusion of a soy protein isolate decreased with increasing mechanical energy input [39]. Smaller protein aggregates might be more accessible for protein hydrolysis and facilitate digestion.

Alternatively, processing could reduce specific endogenous losses as previously reported [7]. These authors suggested that by increasing the digestion rate, high-flow extrusion processing might decrease the proliferation of epithelial cells and secretion of mucus due to production of short chain fatty acids following fermentation. However, lower CP digestion rates were obtained in the present experiment after pelleting or extrusion processing compared to the mash diets. The decrease in the rate of CP digestion of the 60 and 120 min toasted RSM compared to the 0 min toasted RSM could either be caused by the formation of MRP or the formation of protein aggregates after toasting. Both factors have been identified to limit access of enzymes for protein hydrolysis [40, 41], the first step of protein digestion.

Apparent and standardized ileal digestibility of AA

The AIDs and SIDs of most indispensable and dispensable AA followed the same trend as the AID and SID of CP. The AIDs and SIDs of AA were comparable to the values previously reported for rapeseed or canola meal [4, 32]. In contrast, thermal protein damage due to autoclaving of canola meal at 130°C for 20, 30 or 45 min decreased the AID and SID of AA in the study of Almeida *et al.* [32] to values much lower than reported herein. Due to the use of steam for the thermal treatment, effects of autoclaving can be comparable to those of toasting; however, the temperature used by these authors was higher than those used in our experiment.

Previous studies indicated that thermal processing reduces the digestibility of the chemically reactive Lys [42]. The decrease in reactive Lys digestibility with increasing TT might be due to a restricted access of the enzymes for protein hydrolysis due to the formation of protein aggregates or the formation of MRP. Maillard reaction products decrease enzyme access to the proteins due to steric hindrance [43]. Alternatively, proteins that are more difficult to digest could increase the secretion of enzymes [44, 45], which could be considered as specific endogenous losses, resulting in lower apparent and standardized digestibility. Pelleting and extrusion of the diets reduced the negative effects of toasting of

RSM on reactive Lys digestibility. The effects of pelleting and extrusion could be due to the factors mentioned before (i.e. decrease of protein aggregate size, decrease of NDIN content or reduction of specific endogenous losses). Although the negative effects of higher TT of RSM on CP digestibility seem to be compensated by thermal processing of the diets, chemically modified AA that are absorbed (mainly lysine derivatives) may not be utilized for protein deposition [5].

Digestible reactive Lys content was previously suggested as a better predictor of the bioavailable Lys to the pig [46]. However, recent results from our laboratory (Hulshof et al. 2016 Submitted) show that OMIU binds to both the ε- and the α-amino groups of free Lys. Double-bound Lys is not detected as homoarginine after acid hydrolysis. Pelleting or extrusion could potentially increase the content of free Lys in the ileal digesta, which would underestimate the content of homoarginine, thereby overestimating OMIU-reactive Lys digestibility.

The low AID values of Pro and Gly reported here might be associated with the high proportion of these AA in the endogenous secretions [21], for example saliva. Similar results have been reported before [47]. Hydroxyproline is a major AA of the cell wall protein extensin, which can crosslink cell wall polysaccharides [48]. The crosslink between Hyp and the cell wall polysaccharides likely justify the overall low AID of this amino acid.

In conclusion, toasting of RSM decreased the rate of CP digestion along the gastrointestinal tract. The negative effects of toasting of rapeseed meal on the AID and SID of CP and AA, however, can be ameliorated by pelleting or extrusion of the diets. Future research should be directed towards elucidating the mechanisms by which processing of the diets increases protein digestibility of protein-damaged ingredients and to investigate whether the extra protein and AA absorbed can be utilized for protein deposition.

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CHAPTER 7

Apparent ileal digestibility of Maillard reaction products in growing pigs

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ABSTRACT

The aim of the present study was to determine the apparent ileal digestibility (AID) of lysine, fructosyl-lysine (FL), carboxymethyl-lysine (CML), carboxyethyl-lysine (CEL), lysinoalanine and lanthionine in growing pigs fed rapeseed meal (RSM) based diets with different levels of toasting and different diet processing methods. The study consisted of a 2×3 factorial design with toasting time of RSM (60, 120 min) and diet processing method (mash, pelleted, extruded) as factors. Fifty growing pigs were individually fed one of the experimental diets for 4.5 consecutive days. Following euthanasia, samples of digesta were collected from the terminal 1.5 m of the small intestine. Increasing the toasting time of RSM increased the contents of FL, CML and CEL, whereas the additional effects of the diet processing methods were relatively small. Lysinoalanine and lanthionine were below the limit of quantification in the diets; therefore, digestibility of these compounds could not be determined. The contents of FL, CML and CEL in the diets were positively correlated to their contents in the ileal chyme. The AID of the Maillard reaction products from thermally-treated RSM was overall low and did not seem to be related to the content of their contents in the diets. The AID of FL ranged between -8.5 and 19.1%, whilst AID of CML and CEL ranged from -0.2 to 18.3 and 3.6 to 30%, respectively. In conclusion, although clear effects of thermal treatments were observed on the contents of Maillard reaction products in the diets, these compounds have a relatively low digestibility in growing pigs.

Keywords: carboxymethyl-lysine, carboxyethyl-lysine, digestibility, growing pigs, Maillard, processing

INTRODUCTION

Maillard reaction products (MRP) are formed during thermal processing of feed ingredients and diets. This complex group of compounds results from covalent bonding between a free reactive group of an amino acid and the carbonyl group of a reducing sugar or lipid oxidation product [1]. Most commonly, formation of MRP occurs due to bonding of reducing sugars to the ε-amino group of protein-bound lysine, although arginine has also been reported to be susceptible [2]. Crosslinks between amino acids (e.g. lysinoalanine [LAL] or lanthionine [LAN]), mediated through formation of dehydroalanine, also occur during processing without reducing sugars involved.

The absorption of free MRP is higher than that of protein-bound MRP [3]. Protein-bound MRP have to be degraded by digestive proteases into low molecular weight peptides before they can be absorbed [2]. The formation of MRP or amino acid crosslinks can reduce protein digestibility [4, 5] due to restricted enzymatic access for proteolysis. Free carboxymethyl-lysine (CML) can be absorbed by simple diffusion, whereas absorption of CML in dipeptides occurs through peptide transporters (e.g. PEPT1) [2]. This is also the case for other MRP, such as CEL and pyrraline [2]. Absorption of MRP and crosslinked amino acids has been estimated previously using several indirect methods, such as measurements in blood, urine and faeces [6-10]. The estimation of absorption by these methods could be confounded by endogenous formation of MRP [11] and deposition in tissues [8, 12], unless isotope tracer techniques are used [5]. However, in the tracer isotope studies it was not possible to distinguish between differences in absorption of the different MRP compounds. Hence, accurate measurements of absorption of the individual MRP, such as the determination of their ileal digestibility, are lacking. The absorbed MRP can promote oxidative stress and initiate inflammatory responses linked to atherosclerosis [13], although there are also reports on their positive antioxidant effects [14].

The dietary intake of MRP increases their concentration in serum [11]. There was a significant positive correlation ($r = 0.44$, $P = 0.04$) between the dietary intake of CML and its concentration in serum in humans [15]. However, quantification of total absorption requires measurement of blood flow, which is a highly invasive technique [16], and does not account for deposition in target tissues or endogenous formation. There is evidence that the urinary excretion of MRP depends on the rate of renal clearance, which has been suggested to reach saturation [7, 10] and could lead to an underestimation of the absorption of MRP. Urinary excretion also does not consider retention of MRP in the organs, although evidence supporting lack of retention has been reported [17]. Faecal determination of MRP excretion is biased by microbiota fermentation in the large intestine [18]. Using a thermal-damaged

mixture of casein-glucose in growing pigs, Moughan *et al.* [19] reported 46% apparent ileal digestibility (AID) of fructosyl-lysine (FL). Reports on the intestinal absorption of dietary advanced MRP, such as CML and carboxyethyl-lysine (CEL), and crosslinking products, such as LAL and LAN, are lacking.

In addition to the above, many of the studies dealing with absorption and metabolism of MRP have used rats [6, 9, 20] as model animals for humans. However, rats might be able to digest protein sources that are poorly digestible by humans [21]. For example, the true ileal nitrogen digestibility of rapeseed proteins was 84% in humans [22], whilst 95% in rats [23]. Feeding pigs using the same rapeseed protein isolate resulted in values closer to the ones measured in humans (91%) [24]. Pigs have been suggested as a better model animal than rats for digestion in humans due to their digestive anatomical and physiological similarity [16, 21].

The aim of the present study was to determine the AID of lysine, FL, CML, CEL, LAN and LAL in growing pigs fed rapeseed meal-based diets with two levels of toasting and different diet processing methods. We hypothesize that higher contents of the MRP due to longer toasting times and harsher diet processing methods would lead to lower AID of MRP, linked to a lower AID of lysine.

MATERIALS AND METHODS

The Central Committee of Animal Experiments (The Netherlands) approved the use of experimental animals under the authorization number AVD260002015139. The furosine and CML standards were obtained from Polypeptides (Strasbourg, France), whilst the rest of the standards ($^{13}C_6{}^{15}N_2$-lysine, lysine, LAL, LAN) were obtained from Sigma-Aldrich (Steinheim, Germany).

Production of the rapeseed meals and diets

The experiment consisted of a 2×3 factorial arrangement, in which the factors were toasting time of the rapeseed meal (60, 120 min) and diet processing method (mash, pelleting, extrusion). This design resulted in 6 experimental diets: 60 min mash/pelleted/extruded (M-60, P-60 and E-60, respectively) and 120 min mash/pelleted/extruded (M-120, P-120 and E-120, respectively).

An untoasted rapeseed meal (RSM) was produced according to a procedure previously described [25]. The untoasted RSM was toasted for 60 and 120 min. Toasting was performed in batches of 150 kg, which were pooled afterwards, for a total of 450 kg of 60 and 120 min toasted RSM each. The rapeseed meals were toasted by injection of direct

steam (30 kg/h) in the lower tray of a desolventizer-toaster (Schumacher type, Desmet-Ballestra, Zaventem, Belgium). Temperatures during toasting ranged from 100 – 110°C.

The 60 and 120 min toasted RSM were mixed into diets, in which RSM was the only protein-containing ingredient (Table 7.1). Each of the diets including 60 min and 120 min toasted RSM was divided into 3 batches, which either remained as mash or were pelleted or extruded. Pelleting (Research Diet Services, Wijk bij Duurstede, The Netherlands) was performed at a mash conditioning temperature of 80°C to pass through a 4×40 mm die. At the die, the P-60 and P-120 diets reached temperatures of 87 and 89°C, respectively. Extrusion was performed using a co-rotating twin-screw extruder (M.P.F.50, Baker Perkins, Peterborough, UK), after the diets were mixed with water to 850 g DM/kg in a paddle mixer (type F60, Halvor Forberg, Bygland, Norway). The measured internal temperatures at the die during extrusion ranged from 110 to 118 and from 114 to 120°C for the E-60 and E-120 diets, respectively. The extruded products were air-dried at 60°C in air-forced ovens until a dry matter content (IR 200 Moisture Analyzer, Denver Instrument Company, Arvada, Colorado) of ~900 g/kg was obtained. Titanium dioxide (3 g/kg) was added to all diets for the calculation of digestibility.

Animals, feeding and housing

The experiment was performed with 81 crossbred boars (Pietrain × Topigs 20) divided in 3 trials of 24 pigs each, plus a reposition trial with 9 pigs. Out of the 81 pigs, 31 were fed with diets that correspond to a different experiment. Pigs had a starting body weight of 20.4 ± 0.9 kg and were housed in groups of 4 animals per pen, except for the reposition trial, in which 3 animals per pen were used. Pigs were separated and individually fed twice per day (0800 and 1600 h) and remained separated for 1 h. After feeding, the pigs were re-grouped into their original groups. Pigs had free access to water during group and individual housing. Temperature in the experimental barn was 28 ± 1°C and the lights were turned off 12 h per day. The pens (0.71 m^2/animal) had a 3:1 ratio of solid to slatted floor.

Each trial period consisted of 10 adaptation days, during which the animals were fed 30% test diet mixed with a commercial growing pig diet for the first 3 days and incremental amounts of the test diet (10% extra per day) until 100% was achieved on the 10^{th} day. In total, the animals were fed 100% test diet during 4.5 consecutive days. Pigs were fed 4 h before euthanasia on the morning of the 14^{th} day. The animals were fed at 2.8 × maintenance energy requirement (239 kJ NE/kg $BW^{0.75}$; CVB, 2011), based on the measured body weight on days 8 and 13. Water (1:1.5 feed to water) was added before feeding to the mash diets to stimulate feed intake and to reduce dustiness of the diet. One

animal from the M-120 diet was excluded before the end of the experiment due to low feed intake.

Table 7.1. Ingredient composition of the experimental diets.

Ingredient	Inclusion level (g/kg as is)
Rapeseed meal	410.0
Potato starch [1]	503.9
Soy oil	60.0
Monocalcium phosphate	8.1
Calcium carbonate	5.5
Premix vitamins and minerals [2]	5.0
Titanium dioxide	3.0
Salt	3.0
Sodium bicarbonate	1.5

[1] Paselli™ (Avebe, Veendam, The Netherlands).

[2] Composition premix per kg of feed: 100 mg Fe (as $FeSO_4 \cdot H_2O$), 70 mg Zn (as $ZnSO_4 \cdot H_2O$), 20 mg Cu (as $CuSO_4 \cdot 5H_2O$), 30 mg Mn (as MnO), 1.2 mg I (as KI), 0.25 mg Se (as Na_2SeO_3), 125 mg antioxidant (Oxytrap PXN), 10000 IU Vit. A, 2000 IU Vit. D_3, 150 mg choline chloride, 40 mg Vit. E, 30 mg niacine, 15 mg D-pantothenic acid, 4 mg Vit. B_2, 1.5 mg Vit. K_3, 1.5 mg Vit. B_6, 1.0 mg Vit. B_1, 0.4 mg folic acid, 0.05 mg biotine, 20 µg Vit. B_{12}. Carrier was potato starch (Paselli).

Euthanasia was performed by injection of pentobarbital in the ear vein and exsanguination. Following exsanguination, the small intestine was removed and 1.5 m anterior to the ileo-cecal valve were dissected. The contents from this section were flushed out with 50 mL demineralized water, immediately frozen in dry-ice and kept at -20°C until freeze-drying. After freeze-drying, the samples were ground through a 1 mm sieve using a centrifugal mill at 12000 rpm (ZM200, Retsch, Haan, Germany).

Chemical analyses

Dry matter (DM) content was determined according to ISO 6496 [(26)]. Nitrogen content was analyzed by combustion [(27)] (Thermo Quest NA 2100 Nitrogen and Protein Analyzer; Breda, The Netherlands) and the CP content calculated using a 6.25 conversion factor. Titanium dioxide was determined by spectroscopy as described before [(28)].

The contents of furosine, CML, LAL, LAN, CEL and Lys in the RSM, diets and ileal samples were quantified by UHPLC-MS. The samples (10 mg) were hydrolyzed with 1 ml 6 M HCl during 24 h at 110°C. The tubes were dried under N_2 flow and the dried material was re-suspended in 1 mL UPLC-grade Milli-Q water, sonicated and centrifuged (16100 × g, 3.5 min, room temperature). The samples were kept at -20°C until analysis.

To test for ionisation efficiency, the recovery of a spike was measured by adding a known concentration of standard to the supernatant of 18 randomly selected samples (after diluting the samples 10 or 50 times), using $^{13}C_6{}^{15}N_2$-lysine as internal standard. Ionisation efficiency was calculated as the difference between the spiked and non-spiked samples,

divided by the standard injection alone, after correction of all values by the internal standard. Average efficiencies for the samples diluted 50 times were 103, 103, 103, 103, 117 and 128% for furosine, CML, CEL, LAL, lysine and LAN. Corresponding average efficiencies for the samples diluted 10 times were 112, 81, 93, 85, 113 and 53%. As the effect of the sample matrix seems stronger in the 10 times diluted samples, it was decided to use the samples diluted 50 times for quantification.

The supernatant of all samples was diluted 50 times in eluent A that contained 1 mg/L (w/v) $^{13}C_6{}^{15}N_2$-lysine as internal standard. Eluent A was UPLC-grade Millipore water containing 0.1% (v/v) formic acid and eluent B was acetonitrile containing 0.1% (v/v) formic acid. The samples were analyzed using an Acella RP-UHPLC system (Thermo Scientific, San Jose, CA, USA) with an Acquity BEH Amide Vanguard precolumn (2.1 × 50 mm, 1.7 µm particle size) and an Acquity UPLC BEH 300 Amide column (2.1 × 150 mm, 1.7 µm particle size). The column was maintained at 35°C and the injection volume was 1 µL. The elution profile was as follows: 0-2 min isocratic on 80% B, 2-3 min linear gradient from 80% B to 65% B, 3-5 min isocratic on 65% B, 5-7 min linear gradient from 65% B to 40% B, 7-10 min isocratic on 40% B, 10-12 min linear gradient from 40% B to 80% B and 12-28 min isocratic on 80% B. The flow rate was 350 µL/min. Mass spectrometric data were obtained using a LTQ-VelosPro (Thermo Scientific) equipped with a heated ESI probe, coupled to the UHPLC system. The capillary voltage was set to 3 kV. The sheath gas flow rate was set at 20 and the auxiliary gas flow rate at 5 (arbitrary units). A selected reaction monitoring (SRM) method (Table 7.2) was used in negative ion mode for LAL and LAN and in positive ion mode for the other compounds. The normalized collision energy was set at 30 for furosine, Lys and LAL and at 35 for the other compounds and the m/z width on the parent fragment was set to 1. An external standard calibration curve with concentrations of 10, 5, 2.5, 1, 0.1 and 0.01 mg/L of each standard was used to calculate the content of each compound. Compounds were quantified using the external standard calibration curve by plotting MS peak area divided by the MS peak area of the labelled Lys, used as internal standard. The coefficient of variation in the intensity of the internal standard was 15.2%. Data were acquired and analyzed using XCalibur 2.2 software (Thermo Scientific). The quantification limits were 0.05 g/kg for LAN, 0.005 g/kg for LAL, 0.0005 g/kg for CML and CEL and 0.0004 g/kg for furosine and lysine.

Calculations and statistical analysis

Furosine was analysed in the present experiment as an indirect measurement for FL [(10)]. Furosine is produced after hydrolysis with 6M HCl at 110°C for 23 hours, where FL is degraded into 30.0 ± 1.2% furosine [(29)]. It is assumed in this study that the same reaction

occurs with protein-bound FL originating from RSM. The content of FL in the diets and the ileal chyme was calculated by multiplying the determined furosine content by 3.3, based on the conversion of FL into furosine during acid hydrolysis [29]. These contents were used for the calculation of the AID of FL.

The AID was calculated according to Eq. 1

(1) AID of $x = 100 - [(x_{chyme} / x_{diet}) \times (TiO_{2\ diet} / TiO_{2\ chyme}) \times 100]$

Effects of experimental treatments were tested by restricted maximum likelihood using the MIXED procedure of SAS (version 9.3) by means of an *F*-test according to a model with trial, toasting time of RSM (TT), type of diet processing (DP) and their interaction as fixed factors. Feed intake of each of the animals on the day of euthanasia corrected for the average intake per treatment was used as covariate in the model. The CORR procedure of SAS was used to estimate correlations between MRP contents in the diets, the ileal chyme and their AID. Significance was assumed at *P*-values lower than 0.05, whilst trends were considered at *P*-values between 0.05 and 0.1. Post-hoc comparison between treatment means was performed using the Bonferroni adjustment.

Table 7.2. Selected reaction monitoring conditions.

Compound	Parent mass (Da)	Fragment (m/z)
Lysine	146	130
$^{13}C_6\ ^{15}N_2$-Lysine	154	137
Carboxymethyl-lysine	204	84, 130
Lanthionine	208	120
Carboxyethyl-lysine	218	84, 130
Lysinoalanine	233	128, 145
Furosine	255	84, 130

RESULTS AND DISCUSSION

MRP content of the diets and the ileal chyme

The analysed MRP compositions of the rapeseed meals and the diets are reported in Table 7.3. LAL and LAN concentrations in the diets were below the limit of quantification. Therefore, these were excluded from the digestibility calculations. Increasing the toasting time of RSM from 60 to 120 min, decreased the content of lysine by 9% and increased the contents of FL, CML and CEL by 20, 60 and 45%, respectively. Autoclaving of canola meal at 130°C for 20, 30 or 45 min has been previously reported to decrease the lysine content, whilst increasing the content of FL (determined as furosine) [30]. Comparing the 60 to the 120 min toasted RSM in the present study, the sum of the amounts of FL, CML and CEL formed, only accounts for 25% of the decrease in lysine content. Similar results have been reported previously [31], as the sum of the Amadori products (calculated from measured furosine),

CML and CEL after heating sodium caseinate suspensions with glucose or lactose (molar ratio 10:1) at 120 and 130°C for 30 min, only represented approximately 21% of the lysine loss. The remainder of the lysine could probably have reacted to other MRP products that were not analysed in the present study, for example 5-hydroxymethyl-2-furaldehyde (HMF) or pyrraline (amongst others) [32, 33].

Table 7.3. Chemically analyzed composition of the RSM and the experimental diets [1].

Ingredient/diet	Content (g/kg DM)							
	DM (g/kg as is)	CP	Titanium dioxide	Lysine	Furosine	FL [2]	CML	CEL
Toasting time RSM								
60 min	925.1	396.1	-	17.1	0.490	1.6	0.074	0.064
120 min	924.3	395.3	-	15.5	0.588	2.0	0.118	0.093
Experimental diets								
60 min toasted RSM								
Mash	916.6	155.8	2.2	7.1	0.179	0.6	0.038	0.028
Pelleted	900.7	161.1	2.3	6.4	0.127	0.4	0.038	0.026
Extruded	911.2	161.0	2.3	7.0	0.115	0.4	0.048	0.034
120 min toasted RSM								
Mash	917.7	157.4	2.4	6.6	0.206	0.7	0.062	0.044
Pelleted	905.4	157.0	2.3	5.8	0.212	0.7	0.059	0.042
Extruded	909.7	157.7	2.4	6.6	0.141	0.5	0.060	0.058

[1] Abbreviations: CP, crude protein; DM, dry matter; RSM, rapeseed meal; FL, fructosyl-lysine; CML, carboxymethyl-lysine; CEL, carboxyethyl-lysine.

[2] FL = Furosine (g/kg DM) × 3.3. Based on Krause *et al.* [29].

The additional effect of the diet processing methods on the content of MRP was relatively small compared to the effects of toasting time (Table 7.3). A decrease in the content of lysine of approximately 10% was noticed after pelleting of the diets, but not after extrusion (Table 7.3). The decrease in the content of lysine after pelleting was not reflected in higher contents of FL, CML or CEL of the pelleted diets. The content of FL decreased from (on average) 0.65 g/kg DM in the mash diets to 0.55 and 0.45 g/kg DM in the pelleted and extruded diets, respectively. The average content of CML increased after extrusion of the 60 min toasted RSM (0.048 g/kg DM) compared to the mash and pelleted diets (0.038 g/kg DM). No effects of pelleting or extrusion compared to the mash diets were detected on the diets containing 120 min toasted RSM. Pelleting did not affect the average content of CEL compared to the mash diet (0.036 *vs* 0.034 g/kg DM, respectively). There was an increase in the average content after extrusion compared to the mash diet (0.036 *vs* 0.046 g/kg DM, respectively). The conditions employed during extrusion (temperature, shear) could facilitate the conversion of early MRP (e.g. FL) into advanced MRP (e.g. CML, CEL), as reported before for extrusion of casein-based biscuits [6].

The contents of CML in the diets used in the present study are within the range of values reported for foods consumed by humans as part of a western style diet (0 - 0.424 g/kg) [34]. The lowest CML-containing diets in the present study (M-60 and P-60) had higher contents than cereals (0.025 g/kg food), the highest CML food category analysed in the western style diets [34]. The highest content of CML in the present study (M-120) was more than 2-fold higher compared to that of cereals.

There was no correlation ($P > 0.05$) between the content of lysine in the diets and that in the ileal chyme. The content of lysine in the ileal chyme was affected ($P = 0.04$) by the interaction between toasting time and the diet processing method (Fig. 7.1a). Whilst the lysine content in the ileal chyme increased after extrusion of the 60 min toasted RSM compared to the mash and pelleted diets, in the 120 min toasted RSM diets there was a reduction after pelleting and an even further reduction after extrusion compared to the mash diets. The content of FL in the ileal chyme increased ($P < 0.001$) with a higher toasting time, whereas it was reduced ($P < 0.001$) after extrusion compared to the mash and pelleted treatments (Fig 7.1b). There was a positive correlation ($r = 0.83$, $P = 0.04$) between the content of FL in the diets and that in the ileal chyme. Significant effects ($P < 0.001$) of toasting time and diet processing method were also obtained on the content of CML in the ileal chyme (Fig 7.1c). The average CML content in the ileal chyme was higher for the pigs fed the 120 min toasted RSM diets (0.147 g/kg DM) than for the pigs fed the 60 min toasted RSM diets (0.110 g/kg DM). It was also higher for those fed the extruded diets (0.143 g/kg DM) compared to the mash (0.116 g/kg DM) and pelleted (0.127 g/kg DM) diets. The CML content in the diets correlated positively ($r = 0.93$, $P = 0.007$) with that of the ileal chyme. Faecal excretion of CML has been reported to be proportional to the level of CML in the diets [6, 8]. The ileal content of CEL (Fig 7.1d) was also affected by toasting time ($P < 0.001$) and diet processing method ($P = 0.009$) and it was positively correlated ($r = 0.98$, $P < 0.001$) to the CEL content of the diets. The significant positive correlations between the contents of MRP in the diets and the ileal chyme were likely due to the low and rather similar digestibility of the MRP analysed.

Apparent ileal digestibility of MRP

The AID of lysine decreased ($P < 0.001$) from 73.3% in the 60 min toasted RSM diets to 65.3% in the 120 min toasted RSM diets (Table 7.4). The AID of lysine was negatively correlated to the content of FL in the diets ($r = -0.82$, $P < 0.05$) and tended to be negatively correlated to the CML content in the diets ($r = -0.79$, $P = 0.06$). Formation of MRP due to hydrothermal processing has been reported to have negative effects on lysine digestibility [30, 35, 36]. The AID of lysine in the mash diets (67.4%) was lower ($P < 0.001$) than

that of the pelleted (68.9%) and extruded (72.0%) diets (Table 7.4). Pelleting and extrusion can drastically reduce particle size of the diets [37], which likely explains the increase in AID of lysine. In addition, it was shown previously that increasing the mechanical energy input during extrusion, resulting in higher shear, can decrease the size of the protein aggregates [38], which might facilitate enzymatic accessibility for protein hydrolysis.

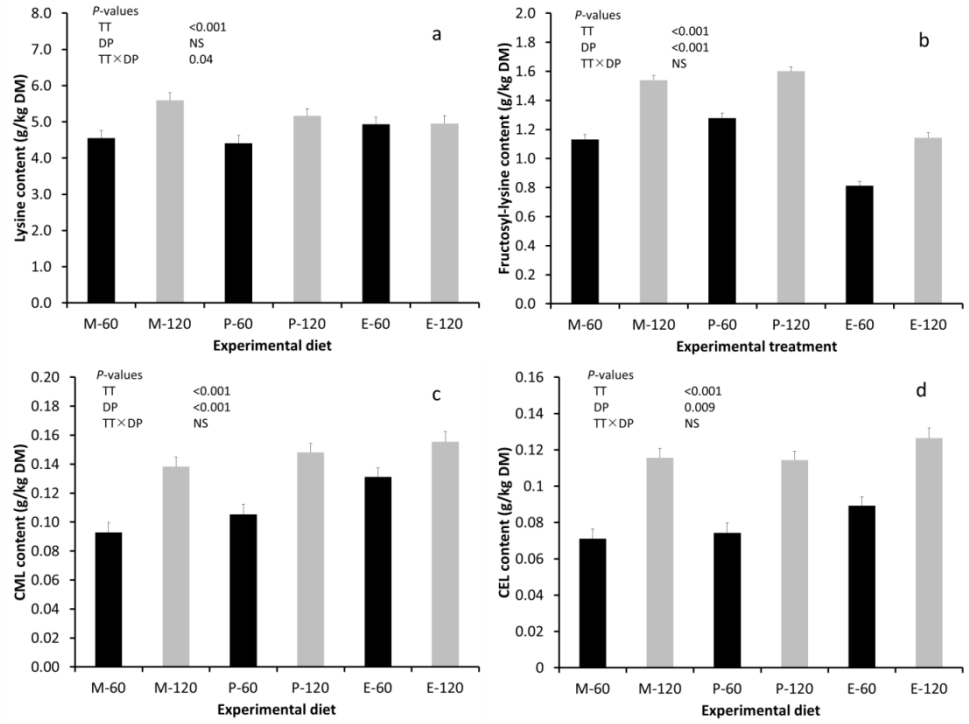

Fig. 7.1. Contents (g/kg DM) of (a) lysine, (b) fructosyl-lysine, (c) carboxymethyl-lysine (CML) and (d) carboxyethyl-lysine (CEL) in the ileal chyme of RSM diets toasted for 60 or 120 min and either fed as mash, pelleted or extruded. Means plus standard errors. Abbreviations: TT, toasting time; DP, diet processing; NS, not significant. Fructosyl-lysine (g/kg DM) = furosine (g/kg DM) × 3.3; based on Krause *et al.* [29].

An interaction (P = 0.01) between the toasting time and the diet processing method was observed for the AID of FL (Table 7.4). Although the effect of the interaction was significant for the AID of FL, there were no statistical pairwise differences between the AID of FL for the different diets. Previous studies [9] have suggested, based on urinary excretion, that only 3 – 5% FL is absorbed from the gastrointestinal tract of rats, in diets based on high levels of casein-bound FL (15.8 g/kg). Other studies [39] reported that only 16% of the FL intake in children fed a glucose-containing milk formula was excreted in urine. The values suggested by these studies [9, 39] match the overall AID of FL values reported in the present study. In contrast, another study [19] reported an AID of FL of 46% (± 5.9%) for a heated

casein-glucose mixture in growing pigs. The furosine content in that study (approximately 5.0 g/100 g crude protein), which was used to calculate FL, was more than 50-fold higher than the average furosine content of the diets in the present study (0.1 g/100 g crude protein). This could indicate that FL digestibility is related to its content in the diet. The negative AID of FL values for the P-60 and M-120 treatments (Table 7.4) could originate from endogenous FL production. Faecal excretion of FL has been reported in rats from control groups fed diets devoid of FL [9]. Alternatively, it has been suggested that the acidic environment in the stomach can revert up to 50% of the FL into lysine [11]. This could also contribute to the large variation in AID of FL reported in the present study.

Table 7.4. Apparent ileal digestibility of lysine, furosine, CML and CEL [1, 2].

Experimental diet	*n*	Apparent ileal digestibility (%)			
		Lysine	FL	CML	CEL
60 min toasted RSM					
Mash	8	73.0	19.1	10.6	17.2^{ab}
Pelleted	8	72.8	-8.5	-0.2	6.0^{b}
Extruded	9	74.0	18.6	10.9	21.3^{ab}
120 min toasted RSM					
Mash	8	61.8	-1.2	10.8	3.6^{b}
Pelleted	9	65.1	12.9	18.3	14.3^{ab}
Extruded	8	70.0	0.2	13.9	30.0^{a}
SEM		1.4	6.5	4.0	3.7
P-values					
TT		<0.001	0.33	0.05	0.74
DP		0.02	0.54	0.75	<0.001
TT×DP		0.10	0.01	0.10	0.01

[1] Abbreviations: n, number of animals; FL, fructosyl-lysine; CML, carboxymethyl-lysine; CEL, carboxyethyl-lysine; TT, toasting time; DP, diet processing method.

[2] "Different superscripts within a column per main effect represent significant differences between means (P<0.05).

Toasting time tended (*P* = 0.05) to affect the AID of CML, which ranged from -0.2 to 18.3% (Table 7.4). Diet processing method or the interaction between toasting time and diet processing method did not affect the AID of CML. The urinary excretion of CML in rats fed various ingredients ranged between 4 and 19% [40], which matches the values reported in the present study. In other studies [6], urinary excretion of CML in rats was reported to reach 38.2% (± 12.2%) and 22.7% (± 6.5%) of the dietary intake of unextruded and extruded casein-based diets, respectively. Rats fed diets with high or low casein-bound CML contents were reported to excrete between 26 to 29% of their dietary intake in urine [9]. As CML was not excreted in the urine of rats fed CML-free diets, all CML was assumed to be from dietary origin [9]. It could be possible that the AID of the several early and advanced MRP are related to the overall digestibility of the protein source. As protein from casein-based diets

are more digestible than protein from the diets used in the present study (80-90% *vs* 60-75%, respectively), this might explain that the excretion of MRP (as a measurement of absorption) in those studies was higher than the absorption reported in our study.

The average AID of CEL ranged from 6.0 to 30.0% (Table 7.4) and was affected ($P < 0.01$) by the interaction between toasting time and diet processing method. The AID of CEL decreased after pelleting of the 60 min toasted RSM diets, whilst it increased after extrusion. In contrast, the AID of CEL in the 120 min toasted RSM diets increased after pelleting and even further after extrusion. The increase in AID of CEL after extrusion of the 120 min toasted RSM diets could be related to the increase in CEL concentration in the diet. There are no previous reports on urinary/faecal excretion of CEL. The plasma level of CEL in rats fed bread crust was 25% higher compared to that of rats fed non-heated wheat starch diets [(20)].

In conclusion, AID of MRP from thermally-treated RSM was overall low and did not seem to be related to the content of MRP in the diets. Fructosyl-lysine had a very limited digestibility (-8.5 to 19.1%), whilst AID of CML and CEL ranged from -0.2 to 18.3 and 3.6 to 30%, respectively.

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CHAPTER 8

General discussion

AIM AND MAIN CONCLUSIONS OF THE THESIS

The main aim of this thesis was to provide further insight into the mechanisms of protein damage that occur during ingredient and feed processing, and their effects on protein hydrolysis and digestibility.

The main conclusions of this thesis are:

- Thermal processing increases the contents of intra- or intermolecular β-sheets and disulfide bonds, which negatively impact protein digestibility. The impact of the changes to the physical conformation of proteins, however, depend on the processing conditions and the amino acid composition of the protein.
- In rapeseed meal (RSM) proteins, these physical/chemical changes occurred simultaneously during toasting. The changes to the physical conformation of proteins occurred approximately 2.5 times faster than chemical changes.
- Increasing the toasting time of rapeseed meal from 0 to 120 min decreases protein solubility of rapeseed meal by 3.2-fold and the rate of protein hydrolysis by 2-fold.
- The rate of hydrolysis of the soluble rapeseed meal protein fraction is 3-9 fold higher than the rate of hydrolysis of the insoluble fraction. Toasting time does not influence the rate of hydrolysis of the soluble protein fraction. In contrast, the measured formation of disulfide bonds and Maillard reaction products likely caused the linear decrease in the rate of hydrolysis of the insoluble protein fraction with increasing toasting time.
- Increasing the toasting time of rapeseed meal results in an overall increase of the peptide size after hydrolysis.
- The 10 min degree of hydrolysis using the pH-STAT *in vitro* protein digestibility method is positively correlated ($r = 0.95$, $P < 0.05$) with the standardized ileal digestibility of severely processed soybean and rapeseed meals in growing pigs.
- The rate of protein digestion of the untoasted RSM in growing pigs is 1.3 and 1.6-fold higher than that of the 60 and 120 min toasted RSM, respectively.
- The negative effects of increasing toasting times of rapeseed meal on protein digestibility can be ameliorated by pelleting and extrusion of the diets. Pelleting increased the standardised ileal digestible crude protein content of RSM diets by 6 – 15% compared to the mash diets. Extrusion increased the standardised ileal digestible crude protein content up to 12% compared to the mash diets.
- Ileal digestion of Maillard reaction products (fructosyl-lysine, carboxymethyl-lysine and carboxyethyl-lysine) in growing pigs is overall low and does not seem to be related to the concentration of these products in the diets.

WHY IS PROCESSING OF INGREDIENTS/FEEDS REQUIRED?

Hydrothermally processed ingredients and diets are used for livestock production. The extraction of oil from oilseeds requires hydrothermal processing for the recovery of the organic solvents (commonly hexane) used during oil extraction and for the inactivation of antinutritional factors [1-5]. Hydrothermal processing during the production of animal feeds is also used in order to control the physical properties of the feed (e.g. agglomeration of particles, preventing selection of ingredients) and to decrease its pathogenic burden [6]. Agglomeration techniques for feed production (e.g. pelleting, expander processing or extrusion) are commonly used due to their positive effects on feed intake [7-10], which have a large impact on animal performance. Additionally, hydrothermal processing beyond the extent of inactivation of antinutritional factors has been reported to exert positive effects on the digestibility of some nutrients, for example starch [11], whilst negative effects have been reported for amino acid (AA) digestibility [12-16].

As mentioned before in this thesis, a reduction in AA digestibility not only decreases the performance of animals and the revenues of the farmers, but could also have negative environmental impacts. Protein damage due to hydrothermal processing can result from physical changes to the structural conformation of the proteins or chemical changes to the AA residues.

CHANGES IN RAPESEED PROTEINS DURING TOASTING AND THEIR EFFECT ON HYDROLYSIS

Physical and chemical changes that occur to proteins after thermal and thermo-mechanical processing impact on their nutritional value. The mechanisms that describe these changes for rapeseed meal proteins are summarized in Fig. 8.1 and 8.2, with data reported in the previous chapters of this thesis included.

Protein denaturation leads to changes in the structural conformation of proteins. As indicated by changes in the denaturation enthalpy, protein denaturation increased 66% after toasting for 120 min compared to the untoasted RSM (Chapter 3). Denaturation results in the exposure of hydrophobic AA that were initially buried inside the molecule and could result in the formation of new bonds within and between proteins.

Random coils are formed during processing following protein denaturation and represent the absence of a defined secondary structure. Although, there are some proteins, such as casein, which in their native state contain large amounts of random coils [17]. The random coils formed after thermal processing result from the stable configurations of the secondary structure of proteins: α-helices and β-sheets. Higher contents of random coils in native animal proteins compared to native vegetable proteins were suggested to explain the higher *in vitro* digestibility of the former [17]. In the current study, formation of random coils was not observed after toasting of RSM using attenuated total reflectance Fourier transform IR spectroscopy (Chapter 3). In contrast to the results reported in this thesis, autoclaving of

legume seeds at 120°C for 20 min has been reported to result in increasing proportions (8.5 – 33% of the total secondary structure) of random coils [18].

It was suggested in Chapter 2, based on previous reports [17], that the proportion of secondary structures resulting after thermal processing might explain the decrease in protein and subsequent AA digestibility. The proportion of intermolecular β-sheets increased during the initial 20 min of RSM toasting, with a concomitant decrease in the proportion of α-helices (Chapter 3). However, increasing the toasting time further than 20 min decreased the proportion of intermolecular β-sheets, whilst increasing the proportion of α-helices. The changes in the secondary structure of proteins with increasing toasting time did not parallel the decrease in the rate of protein hydrolysis (Chapter 3). Therefore, the influence of the formation of insoluble protein aggregates or chemical modifications in the proteins might be more important than the changes in the secondary structure for the accessibility of enzymes for protein hydrolysis.

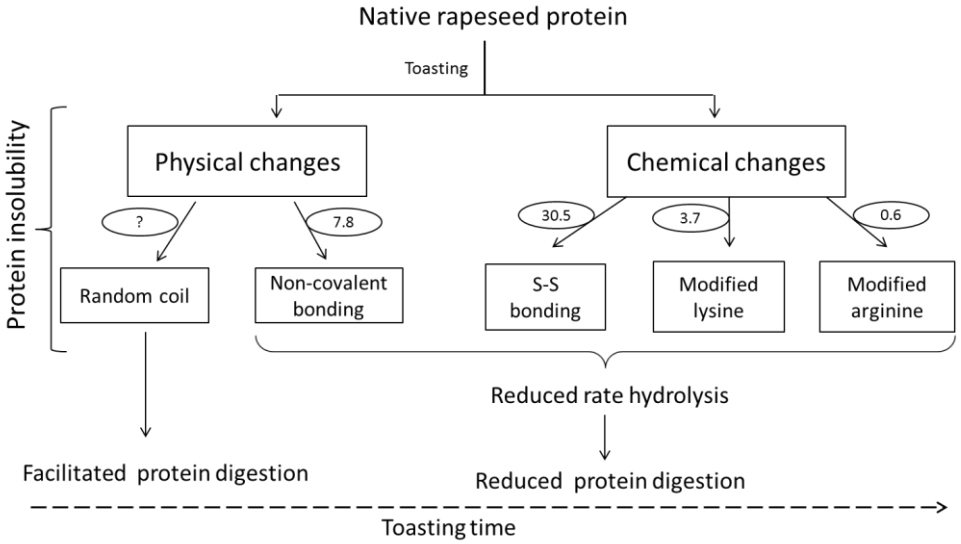

Fig. 8.1. Schematic representation of physical and chemical changes measured in this thesis that occur after toasting of untoasted rapeseed meal and their effects on protein hydrolysis/digestion. Numbers in ovals represent rates of change ($\times 10^{-3}$ /min). Abbreviations: S-S, disulfide.

Protein aggregation due to formation of non-covalent and disulfide bonds within and between (partially) denatured proteins results in protein condensation [19], which reduces protein solubility [20]. Protein solubility is defined in this thesis as the proportion of nitrogen that remained in solution after high speed centrifugation (higher than 13000 × g). Solubility of the rapeseed protein decreased 75% when comparing the 120 min with the 0 min toasted RSM (Chapter 3). Processing can disrupt the disulfide bonds present in the protein, which results in disulfide interchange reactions [21] with the subsequent formation of new disulfide bonds and changes to the original protein structure. In that sense, disulfide bonds can be considered as chemical changes with effects on the physical structure of proteins. The energy required to disrupt disulfide bonds (215 – 251 kJ/mol) is lower

compared to other types of covalent bonds (e.g. peptide bonds, 308 kJ/mol). The rate of formation of disulfide bonds during toasting of RSM was almost 4-fold higher compared to that of non-covalent bonds (Fig. 8.1). The lower rate of formation of non-covalent bonds compared to that of disulfide bonds indicates that the increase in protein aggregation, thus the decrease in protein solubility, was not primarily caused by non-covalent bond formation. Non-covalent bonds are very important in maintaining the structure of the insoluble proteins, also in untoasted rapeseed proteins (Chapter 4), but their abundance does not seem to change drastically due to hydro-thermal processing. Similar results were reported previously [22] upon extrusion of a mixture of soy proteins, wheat gluten and wheat starch. It was reported that as the mixture moved forward through the extruder and faced harsher processing conditions, formation of non-covalent bonds was limited, whilst there was increased formation of disulfide bonds. Formation of disulfide bonds can decrease the accessibility of trypsin for protein hydrolysis, as reported [23] for chickpea albumins. Hence, protein digestibility could be lowered by formation of disulfide bonds, especially in proteins with relatively high cysteine contents. Therefore, formation of disulfide bonds due to hydrothermal processing could be a larger problem for rapeseed proteins compared to soybean proteins, as the former has a higher content of cysteine (2.25 *vs* 1.48 g/100 g CP) [24].

Chemical modifications of AA can occur due to covalent bonding of the carbonyl group of a reducing sugar to a reactive group of an AA or formation of crosslinks between AA. Lysine, as noticed by the decrease in O-methylisourea reactive lysine (Chapter 3), and arginine modifications occur at lower rates compared to formation of non-covalent and disulfide bonds (Fig. 8.1). In other words, harsher conditions are needed in order to obtain chemical modifications to proteins than modifications to their physical structure. Arginine residues are less reactive compared to lysine [25], which explains the lower rates of chemical modifications observed for arginine compared to lysine.

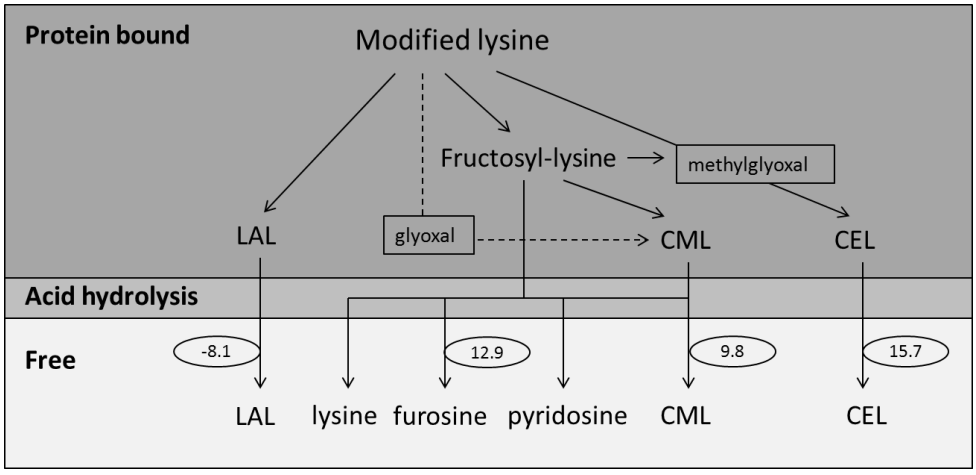

Fig. 8.2. Tentative scheme for chemical modifications to protein-bound lysine and the analysed compounds resulting after acid hydrolysis. Numbers in ovals represent rates of change. Abbreviations: LAL, lysinoalanine; CML, carboxymethyl-lysine; CEL, carboxyethyl-lysine.

Condensation between the reducing sugars and the reactive groups of AA causes the formation of reversible Schiff's bases, which undergo rearrangement into Amadori (aldoses as starting materials) or Heyns (ketoses as starting materials) products [6, 26]. Amadori products, such as fructosyl-lysine (Fig. 8.2), are considered as early Maillard reaction products (MRP). In order to analyse the amount of protein-bound fructosyl-lysine (and overall MRP) formation, peptide bonds have to cleaved by acid hydrolysis. However, fructosyl-lysine is not stable during acid hydrolysis. As reported previously [27], hydrolysis at 110°C with 6 M HCl converts fructosyl-lysine into furosine (30.0%), pyridosine (14.9%) and carboxymethyl-lysine (CML, 1.5%) and also reverts fructosyl-lysine into lysine (56.1%). It must be noted that the sum of these compounds is higher than 100%. Carboxymethyl-lysine can be formed following oxidation of fructosyl-lysine [28]. Other pathways for the formation of CML includes the reaction of glyoxal directly with the ε-amino group of lysine, whilst the corresponding reaction with methylglyoxal yields carboxyethyl-lysine (CEL) [29]. Glyoxal and methylglyoxal can originate from the retro-aldol cleavage of the carbon chain of reducing sugars, also called the Namiki pathway [29-31]. Methlyglyoxal, involved in the subsequent formation of CEL, can also be formed through the degradation of Amadori products [32]. The rate of formation of the early MRP fructosyl-lysine (determined from furosine) with increasing toasting time of RSM was higher compared to that of CML, which is considered an advanced MRP. As the rate of formation of fructosyl-lysine is possibly the limiting step for the formation of CML, this explains the higher rate of the former. In contrast to what was described [32] for thermally-treated casein-saccharide mixtures, the rate of formation of CEL in the present study was higher than the rate of formation of CML. This could suggest that there was also formation of methylglyoxal from other sources, for example the peroxidation of polyunsaturated fatty acids. However, glyoxal and methylglyoxal concentrations were not determined in this thesis. Therefore, it is difficult to distinguish between the different pathways of formation of MRP.

Crosslinking of AA, such as the formation of lysinoalanine (LAL), are favoured during thermal treatments under alkaline conditions (pH > 8.0) [33]. In a previous study, formation of LAL could not be detected after thermal treatment (25 to 120°C) of 7.7% (w/v) solutions of soy protein isolate (pH 6.7), lactalbumin and casein (pH 5.1) [33]. The pH of the RSM decreased with increasing toasting time (results not shown) and ranged between 5.6 to 6.3. The decrease in the pH is likely related to the formation of organic acids, following degradation of saccharides [32, 34]. Hence, the conditions during toasting cannot be considered to favour formation of LAL. Although the content of LAL in the RSM decreased during the initial 20 min of toasting compared to the untoasted RSM (Chapter 4), a further increase of the toasting time did not affect the content of LAL.

As demonstrated *in vitro* (Chapters 3 and 4) and *in vivo* (Chapter 6), increasing the toasting time of RSM caused a decrease in the rate of protein hydrolysis and digestion. This decrease could be caused by aggregation of proteins due to formation of non-covalent and disulfide bonds or the formation of MRP. However, as these changes occur simultaneously during thermal processing of ingredients, it is not possible to disentangle their effects on

protein hydrolysis/digestion. The use of model systems in experiments could aid to discriminate between these effects.

DISENTANGLING THE EFFECTS OF PHYSICAL AND CHEMICAL CHANGES ON PROTEIN HYDROLYSIS

Text Box 8.1 describes a preliminary experiment using a model system, which consisted of a commercial soy protein isolate (SPI) mixed with reducing sugars (glucose and

Text box 8.1 – Temperature×sugar type interactions on protein hydrolysis

Aim. To discriminate between the effects of physical or chemical modifications to proteins during thermal treatments on protein hydrolysis.

Methods. A 3×3 factorial design was used to test the effects of autoclaving temperature (not autoclaved, 100°C, 120°C) and type of sugar (not added, glucose, xylose). Sugars were added at a 2:1 molar proportion of sugars to lysine residues in the SPI. Sugars and SPI were mixed as suspensions and freeze-dried. Autoclaving was performed for 30 min in sealed tubes after mixing with 25% (w/w) water and was followed by freeze-drying. Hydrolysis was performed for 120 min at pH 8.0 by the addition of trypsin, chymotrypsin and an intestinal peptidase (see Chapters 3 and 4). Hydrolysis kinetics were calculated using a second order equation (see Chapter 4). Protein solubility in water was measured as described in Chapters 3 and 4. Content of lysine, furosine, CML, CEL, LAL and LAN (Table 8.1) were measured by UHPLC-MS after diluting the samples 20 times (see Chapters 4 and 7).

Table 8.1. Content of lysine and Maillard reaction products in soy protein isolate thermally treated without sugar (No) or with glucose (G) or xylose (X).

Treatment	Content (g/kg CP)					
(temperature – sugar type)	Lysine	Furosine	CML	CEL	LAL	LAN
Room-No	44.24	0.40	0.08	0.03	0.06	nd
Room-G	36.55	15.61	0.45	0.04	0.05	nd
Room-X	26.71	0.41	1.95	0.24	0.06	nd
100 °C-No	49.86	0.20	0.11	0.07	0.16	0.09
100 °C -G	33.08	16.28	2.05	0.28	0.07	nd
100 °C -X	20.77	0.24	2.48	0.38	0.05	nd
120 °C -No	42.64	0.03	0.30	0.19	0.85	0.20
120 °C -G	24.58	0.45	1.87	0.67	0.10	0.16
120 °C -X	19.08	0.10	2.48	0.53	0.05	0.13

Results. Limited formation of Maillard reaction products occurred in the absence of sugars. Crosslinks between amino acids (LAL, LAN) occurred with increasing temperatures, with sugar addition being inhibitoring. The decrease in N solubility after autoclaving was higher at 100°C compared to 120°C (Table 8.2). At 100°C in the absence of sugars, N solubility decreased 84% with respect to the un-autoclaved no sugar treatment (Room-No), whilst glucose and xylose addition further decreased N solubility by 6 and 11%, respectively. At 120°C in the absence of sugars, N solubility decreased 63% compared to the Room-No treatment, whilst the decrease after glucose and xylose addition was 20 and 21% extra compared to the 120°C-No treatment.

Text box 8.1 (Continued) – Temperature×sugar type interactions on protein hydrolysis

Samples autoclaved at 100°C and the 120°C-glucose treatment had higher maximum degrees of hydrolysis (DH_{max}) than those of the other treatments (Table 8.2). At 100°C in the absence of sugars, *k* was reduced by 35% compared to the Room-No treatment, whilst chemical changes due to glucose and xylose addition further decreased *k* by 22 and 31%.

In contrast, at 120°C the effect of chemical changes on *k* was higher compared to thermal treatments in the absence of sugars. Whilst the 120°C-No treatment reduced *k* by 32%, the addition of glucose and xylose further decreased *k* by 48 and 44%.

Table 8.2. Maximum degree of hydrolysis (DH_{max}), rate of hydrolysis (*k*) and N solubility of a soy protein isolate thermally treated without sugar (No) or with glucose (G) or xylose (X) hydrolysed for 120 min.

Treatment (temperature – sugar type)	DH_{max} (%)	*k* ($M^{-1}s^{-1}$)	N solubility (%)
Room-No	18.06^{bc}	0.0036^{a}	21.1^{a}
Room-G	17.60^{c}	0.0038^{a}	22.4^{a}
Room-X	18.77^{abc}	0.0030^{ab}	2.7^{cd}
100 °C -No	18.99^{abc}	0.0023^{bc}	3.2^{cd}
100 °C -G	20.06^{a}	0.0015^{cd}	2.1^{de}
100 °C -X	19.77^{a}	0.0012^{d}	1.1^{e}
120 °C -No	17.83^{c}	0.0025^{b}	7.8^{b}
120 °C -G	19.44^{ab}	0.0007^{d}	3.6^{c}
120 °C -X	17.71^{c}	0.0009^{d}	3.4^{cd}
SEM	0.29	0.0001	0.2
P-values			
Temperature	<0.001	<0.001	<0.001
Sugar	0.02	<0.001	<0.001
Temperature×Sugar	0.002	<0.001	<0.001

Conclusions. At 100°C, the effects observed on the rate of protein hydrolysis (*k*) by processing in the absence of sugars (physical changes to protein conformation) can be as harsh as the chemical changes. In contrast, after autoclaving at 120°C, chemical changes reduced *k* to a further extent (46% more in average) compared to processing in the absence of sugars (physical changes to protein conformation).

xylose). The SPI (Unisol, Barentz, Hoofddorp, The Netherlands) was selected due to the absence of sugars, as reported by the supplier. The aim of the experiment was to discriminate between the effects of physical and chemical changes, due to hydrothermal processing, on protein hydrolysis.

Xylose is more reactive than glucose concerning the formation of MRP [35]. Therefore, its effect on protein hydrolysis was higher (Table 8.1), especially at low processing temperatures. Despite the formation of MRP in the unheated samples due to the addition of glucose and xylose, no effects on the maximum degree of hydrolysis (DH_{max}) or on the rate of hydrolysis (*k*) were noticed. Formation of MRP due to autoclaving in the presence of sugars reduced *k* further than the thermal treatment without sugar addition,

especially at the elevated autoclaving temperatures. Furthermore, the content of MRP or crosslinked AA after autoclaving at 120°C was similar to that after autoclaving at 100°C. This did not match the decrease in *k* observed after autoclaving at 120°C with the addition of sugars. It is possible that formation of other MRP (e.g. hydroxymethylfurfural), different from the ones reported in Text Box 8.1, might also contribute to the large decrease in *k* observed after autoclaving at 120°C. In addition, the effects of autoclaving on the DH_{max} did not seem to be related to the increase in formation of MRP or crosslinked AA.

Based on the results reported in Text Box 8.1, it can be concluded that the effects of chemical changes on the rate of protein hydrolysis seem dominant at high processing temperatures, whilst physical and chemical changes have similar impact on hydrolysis kinetics when processing is performed at lower temperatures (≤ 100°C). Both physical and chemical changes seem to negatively influence protein solubility. In Chapter 4 it was suggested that the proportion of soluble to insoluble proteins determines the rate of protein hydrolysis. In the following section further evidence for this is provided.

PROTEIN SOLUBILITY AS A DETERMINANT FOR RATE OF PROTEIN HYDROLYSIS

In Chapter 4, soluble and insoluble proteins were separated from RSM that was toasted for different times, in order to determine the effects of toasting on the hydrolysis kinetics of both protein fractions. The degree of hydrolysis of the soluble and insoluble protein fractions was calculated under the assumption that these fractions contain the same type of proteins, but in different concentrations. The same molecular weight bands, although with different intensity, were observed in the SDS-PAGE analysis of the rapeseed meals toasted for different times (results not shown). This suggests that protein aggregation (and consequently precipitation) was not different for the different protein types and it was mostly dependent on the toasting time. The maximum degree of hydrolysis (DH_{max}) and the rate of hydrolysis (*k*) were determined for both fractions and for the whole RSM and modelled according to second order reaction kinetics (Chapter 4). The DH_{max} and the *k* of both fractions (Chapter 4) and the contents of soluble and insoluble proteins of each fraction (Chapter 4) were used to calculate the reconstituted DH_{max} (Fig. 3a) and *k* (Fig. 3b) for the whole material, according to Eq 1 and Eq 2, respectively.

(1) Calculated $DH_{max} = DH_{max\ sol} \times S + DH_{max\ insol} \times (1 - S)$
(2) Calculated $k = k_{sol} \times S + k_{insol} \times (1 - S)$

where $DH_{max\ sol}$ is the DH_{max} for the soluble protein fraction, $DH_{max\ insol}$ is the DH_{max} for the insoluble protein fraction, k_{sol} is the *k* for the soluble protein fraction, k_{insol} is the *k* for insoluble protein fraction and *S* is the proportion of soluble proteins in the rapeseed meals.

The average difference between the measured and the calculated DH_{max} for the complete RSM was 4% (Fig. 8.3a), whilst that for *k* was 20% (Fig. 8.3b). Although the errors were higher for *k*, similar trends were observed between the measured and calculated values. Whilst the measured *k* of the 0 min toasted RSM was 1.8-fold higher compared to

that of the 120 min toasted RSM, the calculated *k* of the 0 min toasted RSM was 2.2-fold higher compared to that of the 120 min toasted RSM. This shows good agreement between the measured values and those calculated based on protein solubility. Hence, the protein fractions obtained are representative for the protein fraction present in the complete RSM.

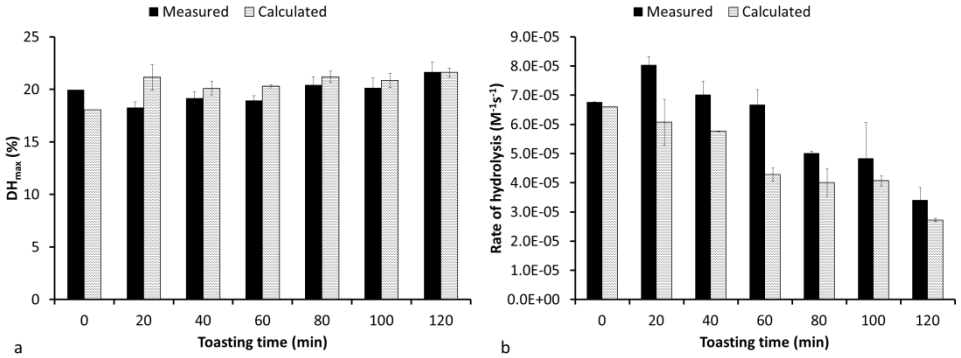

Fig. 8.3. (a) Maximum degree of protein hydrolysis (DH_{max}) and (b) rate of protein hydrolysis measured in rapeseed meal toasted for increasing times or calculated using the DH_{max} and rate of the soluble and insoluble protein fractions and the proportion of soluble proteins for each toasting time.

The measured DH_{max} increased with toasting time. This was due to the higher extent of hydrolysis of the insoluble protein fraction compared to that of the soluble protein fraction and higher proportion of insoluble proteins with increasing toasting time (Chapter 4). Physical and chemical changes of proteins due to hydrothermal processing, as demonstrated in the different chapters of this thesis, does not seem to limit the extent, but only the rate of protein hydrolysis. Even when thermal damage to the proteins occurred (protein aggregation and Maillard reactions), enzymes could still access the proteins for hydrolysis if provided sufficient time. In contrast, protein hydrolysis by trypsin can be inhibited by protein glycation as reported previously [36]. In this respect, enzyme complementarity seems to be important, as incubation with trypsin, chymotrypsin and intestinal peptidase seems to overcome the reported reduction in tryptic hydrolysis. Nevertheless, the rate of protein hydrolysis of the RSM gradually decreased with increasing toasting time. This probably means that for proteins with low rates of hydrolysis, a short hydrolysis time would limit their extent of hydrolysis.

In vivo, protein hydrolysis starts at the stomach, although the major part occurs in the small intestine, with absorption being limited only to the small intestine. Proteins with low *k* values might not have the time required for their complete hydrolysis in the total length of the small intestine. This might be more relevant for animals with shorter gastrointestinal tracts, such as poultry, compared to animals with longer ones, such as pigs [37, 38]. It could also be more relevant for younger animals compared to older ones. The length of the small intestine of broilers increases with age and the increase is more extensive for the jejunum and ileum, which are the main absorptive regions, than for the duodenum [39]. Furthermore,

the morphology of the small intestine also changes with age, as villus height along the whole small intestine increased with age, whilst the crypt depth only increases in the duodenum and jejunum sections [39]. The increased height of the villi and increased depth of the crypts provide an increased surface area. This is not only important for absorption, but also for digestion. Peptidases located in the brush border hydrolyse larger peptides into tri- and dipeptides and free AA that can be absorbed. This could mean that the effects of damage of proteins due to thermal processing could be more severe in younger animals compared to older ones, as the latter have a higher intestinal area available for protein hydrolysis. In addition, synthesis and secretion of pancreatic enzymes increases with age in pigs, mainly due to the increase in their feed intake [40]. This could cause a further negative impact on the rate of protein hydrolysis and digestion of young animals compared to older ones.

It would be of interest to determine if an increase of the retention time of thermally damaged proteins in the small intestine could lead to an increase in protein digestibility. Increasing the content of soluble arabinoxylans in a red meat based diet fed to pigs led to a decreased retention time of the digesta and to a decrease in protein digestibility [41]. These authors suggested that at least part of the effects on protein digestion was due to shorter access time of digestive proteases to the substrate. Other studies also attempted to alter the retention time in the gut through the use of soluble (guar gum) and insoluble (cellulose) non-starch polysaccharides [42]. The retention time was increased from 4.48 h in the control diet to 4.70 and 4.62 h after the inclusion of the guar gum and cellulose in the diets, respectively. Nevertheless, both (purified) polysaccharide sources also decreased the apparent ileal CP digestibility, feed intake and daily weight gain of growing pigs. The reduction in protein digestibility was likely linked to the increase in the viscosity of the digesta, which could restrict enzymatic access for protein hydrolysis. Future challenges are finding methods to increase the retention time in the gastrointestinal tract without increasing the viscosity of the digesta or reducing the feed intake of the animals. Alternatively, to overcome the restriction in the rate of protein hydrolysis, the inclusion of exogenous proteases in the diets could function as an aid for protein hydrolysis in the gastrointestinal tract [37, 38]. This strategy could be more valuable for proteins with a high extent of thermal damage, thus proteins with lower rates of hydrolysis/digestion.

Methods for secondary processing of protein damaged ingredients, for example pelleting and extrusion, could also aid to improve CP and AA digestibility (Chapter 6). The observed increase in CP and AA digestibility after pelleting and extrusion compared to the mash diets was possibly related (at least partly) to the decrease in particle size and the amount of nitrogen linked to the neutral detergent fibre fraction. Nevertheless, the reported increase in CP and AA digested might not be used by the animal for growth if part of the lysine absorbed has been chemically modified. As chemically modified lysine cannot be metabolised [43], the imbalanced surplus AA will be catabolised and excreted in urine [16]. There are energy costs associated to amino acid catabolism and effects on carcass composition [16]. Each gram of total tract digestible CP that is not utilized and is subsequently deaminated increases the urinary energy loss in pigs by 3 kJ [44]. As an example, a pig of 20 kg BW fed a diet containing 10,012 kJ/kg with 16% CP at 3×

maintenance energy requirement (293 kJ NE / kg $BW^{0.75}$), has a daily intake of 132.8 g CP. Using the standardised ileal digestibility described in Chapter 6, the net increase in digested CP was 11.8 and 9.4 g CP for the pelleted and extruded diets, respectively, containing 120 min toasted RSM. Deamination of the extra digested CP (if it cannot be utilized for growth) would involve 0.4 and 0.3% of the daily energy intake of the pig. Therefore, the contribution of AA catabolism to the energetic balance of the pig, relative to the daily energy intake, seems negligible. Future research should elucidate whether the increase in digested protein after secondary processing of protein damaged ingredients (e.g. rapeseed meal) has an impact on protein retention.

IMPORTANCE OF HYDROLYSIS KINETICS: COMPARING *IN VITRO* AND *IN VIVO* RESULTS

The *in vitro* hydrolysis profile of the insoluble protein fraction of RSM toasted for 0, 60 and 120 min as determined using the pH-STAT method (Fig. 8.4a) was similar to the apparent CP digestion along the gastrointestinal tract of mash diets that contained the 0, 60 and 120 min toasted RSM as the sole protein source (Fig. 8.4b). The insoluble protein fraction of the RSM represented the major part (70 – 90%) of the total protein content of the RSM.

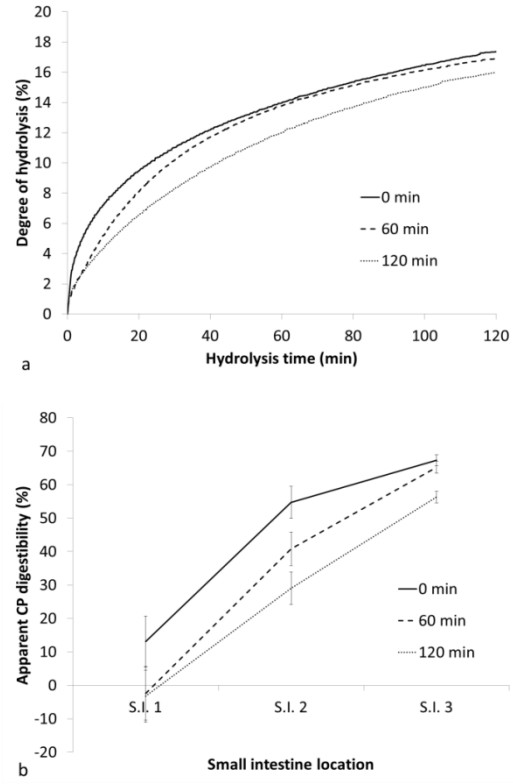

Fig. 8.4. (a) Degree of hydrolysis of the insoluble fraction of 0, 60 and 120 min toasted rapeseed meals (Chapter 4) and (b) apparent crude protein digestibility in different sections of the small intestine of mash pig diets with 0, 60 and 120 min toasted rapeseed meals as sole protein source (Chapter 6).

The *in vitro k* value of the 0 min toasted RSM was 1.4 and 2.0-fold greater than that for the 60 and 120 min toasted RSM, respectively. Similar ratios were found for the *in vivo k* of the 0 min toasted RSM and of the 60 (1.5-fold) or 120 min (2.3-fold) toasted RSM. The highly significant positive correlation ($r = 0.99$, $P < 0.05$) between the *in vitro* and *in vivo k* indicates that the *in vitro k* could be used as a good predictor for the rate of protein digestion *in vivo*.

The rate of protein digestion is not only important for the extent of protein digested at the end of the ileum, but also for the rate at which AA are absorbed and appear in the blood stream. It has been shown [45] that

asynchrony between AA and glucose supply reduces protein retention in growing pigs fed 2 meals per day. It is possible that the slower rate of hydrolysis and absorption of the toasted RSM might also result in catabolism of AA that are asynchronous with the energy supply. In addition, the rate of release of AA from the protein during digestion might differ due to the specificity of the digestive enzymes. This might be more relevant for meal-fed animals, in contrast to animals fed *ad libitum*. In the latter ones, the continuous supply of feed is reflected on a relatively constant absorption of AA, which might reduce the asynchrony due to differences in the rate of digestion. Furthermore, caution is warranted for the application of the results of the study described before [45], as the asynchrony between energy and AA supply was quite extreme.

EFFECTS OF SECONDARY PROCESSING ON INGREDIENT EVALUATION SYSTEMS

The importance of the rate of protein hydrolysis has been highlighted in view of the limitation in the length of the gastrointestinal tract. Interestingly, as demonstrated in Chapter 6, secondary processing (pelleting and extrusion) can ameliorate some of the negative effects of thermally damaged ingredients on protein digestibility.

The evaluation of the effects of thermal processing of ingredients on protein digestibility has been performed before either without an adequate description of the physical form in which the feed was fed [12-14, 46-52] or with the diets fed as mash [53-55]. This means that the compensatory effects of secondary processing (pelleting or extrusion of the diets) on protein digestibility have not been accounted for in those studies. Only few studies have taken into account effects of secondary processing of diets in their design [15, 16]. Furthermore, as demonstrated in Chapter 6, the partial compensatory effect of secondary processing on protein and AA digestibility depends on the extent of damage of the ingredients. A higher increase in protein and AA digestibility was measured after pelleting and extrusion for the diets that incorporated RSM that were toasted for longer times compared to the untoasted RSM or short toasted RSM. Future research should evaluate if the effects of secondary processing on protein digestibility are also observed for other ingredients that undergo hydrothermal processing during their manufacture, for example soybean meal and dried distilled grains with solubles.

PRACTICAL IMPLICATIONS

The physical and chemical changes of the proteins in RSM occurring after hydrothermal processing have an effect on protein solubility. Protein solubility is an important determinant of the rate of protein hydrolysis/digestion, although also chemical changes (formation of disulfide bonds and MRP) of the insoluble protein fraction of RSM have a major influence. Therefore, controlling the extent of the damage to the proteins can also allow control of the rate of protein hydrolysis. The rate of protein hydrolysis not only impacts on the extent of protein absorption at the terminal ileum, but could also affect protein deposition in pigs and nitrogen excretion to the environment.

In vitro methods, such as the pH-STAT method, allow to simulate *in vivo* protein digestion of hydrothermally treated RSM. *In vitro* methods have been routinely used to compare protein digestibility of different ingredients, but not so often to compare hydrothermally processed ingredients. One of the advantages of the pH-STAT method (over other methods that only provide endpoint measurements) is that it allows to determine the rate of protein hydrolysis without extra sampling during the procedure. Future studies should analyse the optimum hydrolysis time during the pH-STAT incubation to also reflect *in vivo* protein digestibility. In that way, the analytical method would provide information on the rate of protein digestion, but also predict protein digestibility.

The effects of secondary processing (pelleting or extrusion) of diets seem more beneficial for protein digestibility of ingredients with a higher degree of protein damage due to thermal processing than for less damaged ingredients. This could have a large impact in the currently used feed evaluation systems, as the separate effects of secondary processing on protein and AA digestibility of ingredients with different extents of protein damage are currently not taken into account. Correcting for the increase in protein digestibility due to secondary processing allows animal nutritionists and farmers to get closer to the concept of precision feeding and reduce nitrogen excretion to the environment.

RECOMMENDATIONS FOR THE FEED INDUSTRY

The negative effects of hydrothermal processing on protein digestibility have been well-established. The animal feed industry would, therefore, benefit from any strategy that can effectively minimize protein damage. Within these strategies, omitting the desolventization/toasting step during the defatting process of oilseeds seems to be a very attractive alternative. Desolventization/toasting are considered necessary steps during the production of rapeseed meal for the recovery of the organic solvent (usually hexane) remaining after solvent extraction of the oil and the degradation of glucosinolates. Plant breeding programmes have decreased the content of glucosinolates in 00-rapeseeds to levels below 30 µmol/g [(56)], which might allow excluding the toasting step from the production process. Nevertheless, the long-term impact of feeding diets with glucosinolates contents higher than the current commercial RSM on animal performance needs to be evaluated. The hexane-loaded oilseed cake would still need to be desolventized. The rapeseed defatting industry should, therefore, devise strategies or develop equipment that allows recovery of the hexane without the use of excessive amounts of heat. This would yield rapeseed meals with a minimum amount of protein damage, with a high ratio of soluble:insoluble proteins and high lysine and reactive lysine contents. In addition, omitting the desolventization/toasting step would decrease the variation in the nutritional value of the rapeseed meals. This could also be achieved by the use of rapeseed expeller in feeds, in which the oil is removed by mechanical pressure. Rapeseed expeller, thus, do not undergo the solvent extraction and the desolventization/toasting steps. Therefore, the oil content of rapeseed expeller is relatively high (99 g/kg crude fat) compared to commercial RSM (32

g/kg) [24]. This would involve the loss of a major part of the oil for the oilseed crushers, which is finally, their primary objective.

In RSM, myrosinase catalyses the degradation of glucosinolates into isothiocyanates, nitriles and thiocyanates. These are bitter compounds and reduce feed intake and consequently animal performance [57]. The activity of myrosinase is lost by its denaturation due to thermal treatment. Extrusion or similar agglomerating techniques during compound feed production could be appropriate methods to denature myrosinase. It was shown in this thesis that the lysine and reactive lysine contents of untoasted or toasted (60 and 120 min) rapeseed meals were not decreased after extrusion. However, the intake of the extruded-untoasted rapeseed meal diet was improved compared to the mash and pelleted diets, which is likely related to the denaturation of myrosinase. Extrusion of the untoasted rapeseed meal also increased protein and AA digestibility compared to the mash and pelleted diets, which is likely related to a reduction in particle size (higher than the one determined after pelleting of the diets). In summary, extrusion of diets including untoasted rapeseed meal yields a diet that can be readily consumed by the animals and provides high lysine contents, with relatively high digestibility coefficients.

As suggested before, future research should evaluate whether the positive effects of secondary processing (pelleting and extrusion) on protein and AA digestibility are also observed in other thermally-treated and protein-damaged ingredients, as observed for RSM here. If this is the case, secondary processing methods should be considered to be included as an important factor in linear programming tools for diet formulation.

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SUMMARY

Hydrothermal processing is a common practice during the manufacture of protein-rich feed ingredients, such as rapeseed meal (RSM), and feeds. This processing step can induce physical and chemical changes to the proteins, thereby reducing the digestibility and utilization of crude protein (CP) and amino acids (AA). Whilst most literature has linked the chemical changes to the proteins with negative effects on protein digestibility, the effects of the physical changes of the protein conformation have not been considered simultaneously. Hence, the aim of this thesis was to provide further insight into the mechanisms of protein damage during ingredient/feed processing and their effects on protein hydrolysis/digestibility. In Chapter 2, the available literature on the physical changes that occur to vegetable proteins used in swine diets after processing was reviewed. Overall, hydrothermal processing increases the contents of intermolecular/intramolecular β-sheets and disulfide bonds, which were negatively correlated to protein digestibility. The correlations, however, were dependent on the type of protein analysed. When the physico-chemical changes in the proteins occur during processing of the ingredients, proteins usually become less responsive to further processing treatments. Rapeseed proteins were used as model, as this oilseed is hydrothermally processed during the oil extraction process and is further processed when incorporated in animal diets.

Protein damage during production of rapeseed meal

There is high variability in the nutritional value of commercial RSM. The variation is mainly due to the conditions used during the desolventization/toasting step. Therefore, the aim of the experiment in Chapter 3 was to characterize the secondary structure and chemical changes that occur during toasting of RSM and their effects on *in vitro* protein digestibility. A cold defatted RSM was toasted for 120 min with samples obtained every 20 min. Increasing the toasting time from 0 to 120 min increased protein denaturation by 3-fold and decreased protein solubility by 4-fold, lysine content by 23% and the reactive lysine content by 37%. The proportion of intermolecular β-sheets increased after the initial 20 min of toasting, but steadily decreased thereafter. The contrary was observed for the proportion of α-helices. The changes in the secondary structure of proteins were not correlated to the rest of the physical and chemical changes. Therefore, changes in the secondary structure of proteins cannot be considered good indicators of damage to proteins due to hydrothermal processing. The rate of protein hydrolysis decreased by 2-fold when toasting time was increased from 0 min to 120 min. The changes in protein solubility and lysine/reactive lysine contents were positively correlated to the rate of protein hydrolysis. Changes to the physical

conformation of rapeseed proteins occur at faster rates during toasting compared to chemical changes.

In Chapter 4, it was hypothesized that the decrease in the rate of protein hydrolysis with increasing toasting time was due to the reduction in protein solubility. In order to test this hypothesis, the soluble and insoluble protein fractions from each of the RSM studied in Chapter 3 were separated and hydrolysed. Hydrolysis kinetics and the molecular size distribution of the peptides resulting after hydrolysis were analysed. The extent of hydrolysis of the insoluble protein fraction was 44% higher than that of the soluble protein fraction. The rate of hydrolysis of the soluble protein fraction separated from the hydrothermally treated RSM was 3-9 fold higher than that of the insoluble protein fraction. In the insoluble fraction, formation of both disulfide bonds and Maillard reaction products (MRP) (fructosyl-lysine [FL], carboxymethyl-lysine [CML] and carboxyethyl-lysine [CEL]) was noticed, which explains the decrease in the rate of protein hydrolysis with longer toasting times. Overall, increasing the toasting time of the whole RSM and the insoluble protein fraction increased the size of the peptides resulting after enzymatic hydrolysis. A shift in the mechanism of protein hydrolysis from a more one-by-one type to a more zipper-type likely explained the correlations between the rate of hydrolysis and the molecular size distribution after hydrolysis. Protein solubility seems to be a key parameter for understanding the decrease in the rate of protein hydrolysis with increasing toasting time.

The correlations between two *in vitro* protein digestibility methods and the standardised ileal digestibility in growing pigs of severe thermally-treated soybean and rapeseed meals were studied in Chapter 5. Soybean meal and RSM were toasted in the presence of lignosulfonate in order to induce severe thermal damage to the proteins. *In vitro* protein digestibility was analysed using the two-step enzymatic method (pepsin at pH 2.0 and pancreatin at pH 6.8) and the pH-STAT method. The standardised ileal digestibility values were obtained from a previous experiment, in which ileal-cannulated growing pigs were used. The degree of hydrolysis after 10 min was positively correlated ($r = 0.95$, $P = 0.046$) to the standardised ileal CP digestibility. The *in vitro* rate of protein hydrolysis using the pH-STAT method and CP digestibility using the two-step enzymatic method tended to be positively correlated to the standardised ileal digestibility of CP ($r = 0.91$, $P = 0.09$, for both *in vitro* methods). In conclusion, both *in vitro* methods might be used for the *in vivo* digestibility of severe thermally-treated ingredients.

Effects of diet processing on protein digestibility of RSM with different extents of damage

The processed ingredients (e.g. rapeseed meal) are mixed with other ingredients and processed further during the compound feed manufacturing process. The effects of the

diet processing methods (e.g. pelleting and extrusion) on protein digestibility could depend on the extent of the damage of the ingredients used. The aim of Chapter 6 was to test the effects of toasting time of rapeseed meal, diet processing method and the interaction between both on protein digestion along the gastrointestinal tract and apparent/standardised ileal digestibility of CP and AA. Mash, pelleted and extruded diets were manufactured using either 0, 60 or 120 min toasted RSM as the only protein source, for a total of 9 different experimental diets. Whilst increasing the toasting time decreased the contents of lysine and reactive lysine in the diets, no effects were noticed after pelleting or extrusion of the diets compared to the mash. The mean particle size of the diets was reduced from 479 µm in the mash diets to 309 and 211 µm after pelleting and extrusion, respectively. A total of 81 growing boars were individually fed with one of the experimental diets. Following euthanasia, the small intestine was divided in 3 sections of equal length and the contents of the final 1.5 m of each small intestine section were sampled. The apparent CP digestibility for each section of the gastrointestinal tract was used to calculate the rate of CP digestion based on a second order equation. The rate of digestion was higher in the diets containing 0 min toasted RSM compared to the diets that contained 60 or 120 min toasted RSM. The diet processing method tended to affect the rate of protein digestion, with higher rates for the extruded > pelleted > mash diets. Significant effects of the interaction between toasting time and diet processing method were found on the apparent ileal CP digestibility. Whilst a lower apparent ileal CP digestibility was found in the 120 min toasted RSM mash diet compared to the 0 and 60 min toasted RSM mash diets, no differences were observed between the different toasting times in the pelleted and extruded diets. Similar significant interactions were noticed for the apparent ileal digestibility of some dispensable and indispensable AA (e.g. arginine, isoleucine, leucine, methionine, threonine, alanine, glycine, proline, serine). Pelleting of the 60 and 120 min toasted RSM diets increased the standardized ileal digestible CP content by 6 and 15%, respectively, compared to the 60 and 120 min toasted mash diets. Extrusion of the 0, 60 and 120 min toasted RSM diets increased the standardized ileal digestible CP content by 5, 9 and 12%, respectively, compared to the 0, 60 and 120 min toasted mash diets. Similar positive effects of pelleting and extrusion were obtained for the apparent ileal digestible contents of lysine and reactive lysine, especially for the diets that contained RSM toasted for longer times. In conclusion, the severe effects of protein damage during the production of RSM on protein digestibility can be (partially) ameliorated by processing of the diets.

Processing of ingredients and diets can lead to the formation of early (e.g. FL) and advanced (e.g. CML and CEL) MRP. These MRP have been associated with common metabolic disorders, for example atherosclerosis. Absorption of dietary MRP has been previously estimated based on indirect measurements, such as concentrations in blood,

urine and faeces, which could be biased by endogenous formation of MRP and deposition in tissues. Hence, the aim of Chapter 7 was to measure the apparent ileal digestibility of early (FL, determined as furosine after acid hydrolysis) and advanced (CML and CEL) MRP. The same diets and ileal digesta samples as in Chapter 6 were used in this study. The 0 min toasted RSM diets (mash, pelleted and extruded) were excluded from this study. Sucrose was added to these diets immediately before feeding in order to increase their feed intake, which could confound the determination of the apparent ileal digestibility of the MRP for these treatments. The content of FL was higher in the 120 min compared to the 60 min toasted RSM diets, whereas it was lower in the extruded diets compared to the pelleted and mash diets. The decrease in FL content after extrusion of the diets can probably be related to conversion of the early into advanced MRP, as the content of CML was higher in the extruded diets compared to the pelleted and mash diets. The contents of FL, CML and CEL were positively correlated to the contents of these compounds in the ileal chyme. The apparent ileal digestibility of FL, CML and CEL for the different diets ranged from -8.5 to 19.1%, -0.2 to 18.3% and 3.6 to 30%, respectively. In conclusion, the apparent ileal digestibility of the early and advanced MRP from thermally-treated RSM diets in growing pigs were overall low and did not seem to be related to the contents of these compounds in the diets.

The results of this thesis indicate that the changes to the physical conformation of proteins during toasting of RSM occur at a faster rate than chemical changes. Both types of changes affect protein solubility, which is important in determining the rate of protein hydrolysis. It is suggested in this thesis that the rate of protein hydrolysis of hydrothermally-processed ingredients is probably the main contributing factor for the *in vivo* protein digestibility. This was demonstrated, as increasing the toasting time of RSM decreased the rate of CP digestion in growing pigs. The negative effects of longer toasting times on CP and AA digestibility, however, could be ameliorated by pelleting and extrusion of the diets. The positive effects of diet processing methods (pelleting and extrusion) on the digestibility of damaged proteins from thermally-treated ingredients should be taken into account in feed evaluation studies and formulation of practical diets.

RESUMEN

El procesamiento hidro-térmico es una práctica común durante la producción de ingredientes altos en proteína, por ejemplo la harina de colza (HC), y de alimentos para animales. Este procesamiento puede inducir cambios físicos y químicos en las proteínas, reduciendo la digestibilidad y utilización de la proteína cruda (PC) y los amino ácidos (AA). Mientras gran parte de la literatura científica ha vinculado los cambios químicos en las proteínas con efectos negativos en su digestibilidad, los efectos de los cambios físicos en la conformación de las proteínas no han sido considerados de forma simultánea. Por ende, el objetivo principal de esta tesis era describir los mecanismos de daño a las proteínas como resultado del procesamiento de ingredientes y de alimentos animales y sus efectos en hidrólisis y digestibilidad de las proteínas. El Capítulo 2 consiste de una revisión de la literatura científica disponible acerca de los cambios físicos que ocurren debido al procesamiento en las proteínas de origen vegetal utilizadas en dietas para cerdos. En general, el procesamiento hidro-térmico incrementa los contenidos inter- e intra-moleculares de hojas plegadas beta y de enlaces disulfídicos, los cuales están negativamente correlacionados con la digestibilidad de las proteínas. Sin embargo, las correlaciones dependen del tipo de proteína analizado. Los cambios físico-químicos que ocurren en las proteínas durante el procesamiento de los ingredientes reducen la sensibilidad de las proteínas al procesamiento adicional. Las proteínas de colza fueron utilizadas como modelo, debido a que este ingrediente oleaginoso conlleva procesamiento hidro-térmico durante la extracción del aceite y procesamiento adicional cuando se incorpora en las dietas para animales.

Daño a las proteínas durante la producción de harina de colza

Existe gran variabilidad en la calidad nutricional de la HC disponible comercialmente. Esta variabilidad tiene su origen en las condiciones que se utilizan durante la etapa de desolventización/tostado. Por consiguiente, el objetivo del experimento descrito en el Capítulo 3 era caracterizar los cambios en la estructura secundaria y cambios químicos que ocurren durante el tostado de la HC y sus efectos en la digestibilidad *in vitro* de las proteínas. Una HC desgrasada en frío fue tostada durante 120 minutos, con muestras obtenidas cada 20 minutos. Incrementar el tiempo de tostado de 0 a 120 minutos resultó en un incremento de 3-veces en la desnaturalización de las proteínas, una disminución de 4-veces en la solubilidad de las proteínas y una reducción de 23% y 37% en el contenido de lisina y lisina reactiva, respectivamente. La proporción de hojas plegadas beta entre proteínas incrementó durante los 20 minutos iniciales de tostado, pero disminuyó de forma constante con tiempos mayores de tostado. Lo contrario ocurrió con la proporción

de hélices alfa. No se encontró correlación entre los cambios en la estructura secundaria de las proteínas y el resto de cambios físicos y químicos. Por lo tanto, los cambios en la estructura secundaria de las proteínas no pueden considerarse buenos indicadores del daño a las proteínas producto del procesamiento hidro-térmico. La velocidad de hidrólisis de las proteínas se redujo a la mitad cuando el tiempo de tostado se incrementó de 0 a 120 minutos. Los cambios en la solubilidad de las proteínas y el contenido de lisina y lisina reactiva se correlacionaron positivamente con la velocidad de hidrólisis de las proteínas. Comparados a los cambios químicos, los cambios físicos a la conformación de las proteínas de colza ocurrieron a velocidades mayores durante el tostado.

En el Capítulo 4, se probó la hipótesis de que la disminución en la velocidad de hidrólisis, que ocurre con el incremento en el tiempo de tostado, se debía a la reducción en la solubilidad de las proteínas. Para probar esta hipótesis, se separó e hidrolizó la fracción proteica soluble e insoluble de cada una de las HC descritas en el Capítulo 3. La cinética de hidrólisis y la distribución del tamaño molecular de los péptidos después de la hidrólisis fueron analizados. El grado de hidrólisis de la fracción proteica insoluble fue 44% mayor que el grado de hidrólisis de la fracción proteica soluble. La velocidad de hidrólisis de las fracciones solubles separadas de las HC fueron entre 3 y 9 veces mayores que la velocidad de las fracciones insolubles. La formación de enlaces disulfídicos y productos de la reacción de Maillard (fructosil-lisina [FL], carboximetil-lisina [CML] y carboxietil-lisina [CEL]) en las fracciones insolubles puede explicar la reducción en la velocidad de hidrólisis de estas fracciones con mayores tiempos de tostado. En general, el incremento en el tiempo de tostado de las HC y de las fracciones insolubles aumentó el tamaño de los péptidos resultantes después de la hidrólisis enzimática. Un cambio en el mecanismo de hidrólisis de las proteínas de uno-por-uno a tipo-zipper pueda probablemente explicar las correlaciones entre la velocidad de hidrólisis y la distribución del tamaño molecular de los péptidos después de la hidrólisis. La solubilidad de las proteínas es un parámetro esencial para entender la disminución en la velocidad de hidrólisis producto del aumento del tiempo de tostado.

En el Capítulo 5 se describe la correlación entre dos métodos *in vitro* de digestibilidad de proteína y la digestibilidad ileal estandarizada en cerdos en crecimiento para harinas de soya y colza con tratamientos térmicos severos. Las harinas de soya y colza fueron tostadas en presencia de lignosulfonato con el fin de inducir daño térmico severo a las proteínas. La digestibilidad *in vitro* de las proteínas se analizó utilizando el método enzimático de dos pasos (pepsina a pH 2.0 y pancreatina a pH 6.8) y el método pH-STAT. La digestibilidad ileal estandarizada de estos ingredientes se obtuvo de un experimento previo, en el cual se utilizaron cerdos en crecimiento con cánulas en el íleo. El grado de hidrólisis después de 10 minutos de hidrólisis se correlacionó positivamente (r =

0.95, *P* = 0.046) con la digestibilidad ileal estandarizada de la PC. La velocidad de hidrólisis *in vitro* de la proteína utilizando el método pH-STAT y la digestibilidad de la PC utilizando el método enzimático de dos pasos tendieron a estar positivamente correlacionados con la digestibilidad ileal estandarizada de la PC (r = 0.91, *P* = 0.09, para ambos métodos *in vitro*). En conclusión, ambos métodos *in vitro* pueden ser utilizados para predecir la digestibilidad *in vivo* de ingredientes con severos tratamientos térmicos.

Efectos del procesamiento de dietas en la digestibilidad de las proteínas de harina de colza con diferentes grados de daño térmico

Los ingredientes procesados (por ejemplo, HC) son mezclados con otros ingredientes y reprocesados durante la producción de alimentos para animales. Los efectos de los métodos de procesamiento (por ejemplo, peletizado y extrusión) en la digestibilidad de las proteínas pueden depender del grado de daño de las proteínas en los ingredientes utilizados. El objetivo del Capítulo 6 era examinar los efectos del tiempo de tostado de la HC, el método de procesamiento de las dietas y la interacción entre ambos factores en la digestión de las proteínas a lo largo del tracto gastrointestinal y la digestibilidad ileal aparente y estandarizada de la PC y los AA. Dietas en harina, peletizadas y extrusadas fueron producidas utilizando HC tostadas por 0, 60 o 120 minutos como única fuente de proteína, para un total de 9 dietas. Los contenidos de lisina y lisina reactiva disminuyeron al incrementar el tiempo de tostado, mientras no hubo efectos del peletizado o la extrusión de las dietas en comparación con las harinas. El tamaño de partícula promedio de las dietas se redujo de 479 µm en las dietas en harina a 309 y 211 µm en las dietas peletizadas y extrusadas, respectivamente. Un total de 81 machos enteros en crecimiento fueron alimentados individualmente con una de las dietas experimentales. Después de la eutanasia, el intestino delgado se dividió en 3 segmentos iguales y se colectaron los contenidos de los últimos 1.5 m de cada sección del intestino. La digestibilidad aparente de la PC en cada sección del intestino delgado se utilizó para calcular la velocidad de digestión basada en una ecuación de segundo orden. La velocidad de digestión de la PC fue mayor en las dietas que contenían HC sin tostar, comparada a las dietas que contenían HC tostadas por 60 y 120 minutos. Hubo una tendencia del método de procesamiento de las dietas de afectar la velocidad de digestión de las proteínas, con velocidades mayores para las dietas extrusadas > peletizadas > harina. Se obtuvieron efectos significativos de la interacción entre el tiempo de tostado y el método de procesamiento de las dietas en la digestibilidad ileal aparente de la PC. Mientras la digestibilidad ileal aparente de la dieta en harina tostada por 120 minutos fue menor que las dietas en harina sin tostar y tostada por 60 minutos, no se observaron diferencias entre los diferentes tiempos de tostado en las dietas peletizadas o extrusadas. Interacciones similares fueron observadas en la

digestibilidad ileal aparente de algunos AA esenciales y no esenciales (por ejemplo, arginina, isoleucina, leucina, metionina, treonina, alanina, glicina, prolina y serina). El contenido de PC digestible estandarizada a nivel del íleo aumentó en 6 y 15% después de peletizar las dietas que incluían HC tostadas por 60 y 120 minutos, respectivamente, comparado con las dietas en harina. El contenido de PC digestible estandarizada a nivel del íleo aumentó en 5, 9 y 12% después de extrusar las dietas que incluían HC tostadas por 0, 60 y 120 minutos, respectivamente, comparado con las dietas en harina. Efectos positivos similares del peletizado y del extrusado fueron obtenidos en los contenidos de lisina y lisina reactiva digestibles aparentes a nivel ileal, especialmente para las dietas que contenían HC tostadas por tiempos más largos. En conclusión, los severos efectos del daño a las proteínas durante la producción de la HC en la digestibilidad de las proteínas pueden ser (parcialmente) mejorados por el procesamiento de las dietas.

El procesamiento de los ingredientes y las dietas puede llevar a la formación de productos de reacción de Maillard (PRM) tempranos (por ejemplo, FL) y avanzados (por ejemplo, CML y CEL). Estos PRM han sido asociados con desórdenes metabólicos comunes, por ejemplo aterosclerosis. La absorción de PRM ha sido estimada anteriormente con base en métodos indirectos, por ejemplo la concentración en sangre, orina y heces, los cuales pueden resultar erróneos debido a la formación endógena de PRM y al depósito de estos en los tejidos. Por lo tanto, el objetivo del Capítulo 7 era determinar la digestibilidad ileal aparente de los PRM tempranos (FL, calculado a partir de furosina, después de hidrólisis con ácidos) y avanzados (CML y CEL). Las mismas dietas y contenidos digestivos ileales que en el Capítulo 6 fueron utilizadas en este experimento. Las dietas basadas en la HC sin tostar (harina, peletizada y extrusada) fueron excluidas de este experimento. A estas dietas se añadió sucrosa justo antes de la alimentación con el fin de aumentar el consumo de alimento, lo cual puede confundir la determinación de la digestibilidad ileal aparente de los PRM. El contenido de FL fue mayor en las dietas que incluían la HC tostada por 120 minutos comparado a aquellas tostadas por 60 minutos, mientras fue menor en las dietas extrusadas comparado a las dietas peletizadas y en harina. La disminución en el contenido de FL después de la extrusión de las dietas puede estar relacionado a la conversión de PRM tempranos en productos avanzados, ya que el contenido de CML fue mayor en las dietas extrusadas comparado a las dietas peletizadas y en harina. Hubo una correlación positiva entre los contenidos de FL, CML y CEL en las dietas y en los contenidos del íleo. La digestibilidad ileal aparente de FL, CML y CEL para las distintas dietas estuvo en un rango entre -8.5 a 19.1%, -0.2 a 18.3% y 3.6 a 30%, respectivamente. En conclusión, las digestibilidades ileales aparentes de PRM tempranos y avanzados de HC tratadas térmicamente en cerdos en crecimiento fueron en general bajas y no parecen estar relacionadas con los contenidos de estos compuestos en las dietas.

Los resultados de esta tesis indican que los cambios físicos a la conformación de las proteínas ocurren a velocidades mayores que los cambios químicos durante el tostado de la HC. Ambos tipos de cambios afectan la solubilidad de las proteínas, la cual es un factor determinante de la velocidad de hidrólisis. Es sugerido en esta tesis que la velocidad de hidrólisis proteica de ingredientes tratados hidro-térmicamente es probablemente el principal factor que determina la digestibilidad proteica *in vivo*. Esto fue demostrado ya que al incrementar el tiempo de tostado de la HC se disminuyó la velocidad de digestión en cerdos en crecimiento. Sin embargo, los efectos negativos al incrementar el tiempo de tostado en la digestibilidad de las proteínas y los AA pueden ser atenuados por la peletización y extrusión de las dietas. Los efectos positivos de los métodos de procesamiento de dietas (peletizado y extrusión) en la digestibilidad de proteínas dañadas provenientes de ingredientes tratados térmicamente deben ser considerados en los sistemas de evaluación de alimentos para animales y en la formulación de dietas comerciales.

Curriculum vitae

Sergio Salazar Villanea was born in San José, Costa Rica on May 14th, 1982. He obtained his bachelor's degree in Zootechnics in the University of Costa Rica in 2007. In 2008, he started working as a researcher and lecturer in the Zootechnics department of the University of Costa Rica, position which he still holds up to this date. In 2009 he was awarded a Nuffic scholarship to perform M.Sc. studies at the Animal Sciences department of Wageningen University. During his M.Sc. studies, he did his minor at the Adaptation Physiology group on glucose tolerance in sows during the peripartum and the effects on piglet vitality. His major thesis was performed at the Animal Nutrition group on processing technologies to improve the in vitro digestibility of non-starch polysaccharides from corn dried distiller grain with solubles. In 2011, he obtained his M.Sc. degree. From 2011 to 2012, he resumed his position at the Zootechnics department of the University of Costa Rica. In November 2012, he was selected to start a Ph.D. at Wageningen University, within the IPOP Customised Nutrition project. During his Ph.D., Sergio focused on the effects of processing of ingredients and feeds on the nutritional value of proteins, using rapeseed meal as model ingredient. The results of this work are presented in this thesis. After concluding his Ph.D., Sergio will resume his position at the Zootechnics department of the University of Costa Rica, San José, Costa Rica.

List of publications

Peer-reviewed scientific publications

De Vries, S., A. M. Pustjens, M. A. Kabel, S. Salazar-Villanea, W. H. Hendriks and W. J. J. Gerrits. 2013. Processing technologies and cell wall degrading enzymes to improve nutritional value of dried distillers grain with solubles for animal feed: an *in vitro* digestion study. Journal of Agricultural and Food Chemistry 61(37): 8821-8828.

Salazar-Villanea, S., W. H. Hendriks, E. M. A. M. Bruininx, H. Gruppen and A. F. B. van der Poel. 2016. Protein structural changes during processing of vegetable feed ingredients used in swine diets: implications for nutritional value. Nutrition Research Reviews 29: 126-141.

Salazar-Villanea, S., E. M. A. M. Bruininx, H. Gruppen, W. H. Hendriks, P. Carré, A. Quinsac and A. F. B. van der Poel. 2016. Physical and chemical changes of rapeseed meal proteins during toasting and their effects on *in vitro* digestibility. Journal of Animal Science and Biotechnology 7:62.

Salazar-Villanea, S., T. G. Hulshof, A. F. B. van der Poel, E. M. A. M. Bruininx and P. Bikker. 2016. Predicting the standardized ileal digestibility of processed soybean meal and rapeseed meal in growing pigs using two *in vitro* methods. Journal of Animal Science 94: 202-206.

Salazar-Villanea, S., E. M. A. M. Bruininx, H. Gruppen, P. Carré, A. Quinsac and A. F. B. van der Poel. Effects of toasting time on hydrolysis of soluble and insoluble rapeseed meal proteins. Submitted to Journal of the American Oil Chemists' Society.

Salazar-Villanea, S., E. M. A. M. Bruininx, H. Gruppen, W. H. Hendriks, P. Carré, A. Quinsac and A. F. B. van der Poel. Pelleting and extrusion can ameliorate negative effects of toasting of rapeseed meal on protein digestibility in growing pigs. Submitted to Journal of Animal Science.

Salazar-Villanea, S., C. I. Butré, P. A. Wierenga, E. M. A. M. Bruininx, H. Gruppen, W. H. Hendriks and A. F. B. van der Poel. Apparent ileal digestibility of Maillard reaction products. To be submitted to Amino Acids.

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. 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.

Salazar-Villanea, S., A. F. B. van der Poel and E. M. A. M. Bruininx. 2014. Shear processing of rapeseed meal: effects on protein solubility. Proceeding of the 39th Animal Nutrition Research Forum, 3 April, 2014, Utrecht, the Netherlands.

Salazar-Villanea, S., E. M. A. M. Bruininx, A. Quinsac, P. Carré and A. F. B. van der Poel. 2015. Effects of toasting during the production of rapeseed meal on protein hydrolysis. Proceedings of the WIAS Science Day, 5 February, 2015, Wageningen, the Netherlands.

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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. Proceeding of the 13th Digestive Physiology of Pigs, 19-21 May, 2015, Kliczków, Poland.

Salazar-Villanea, S., E. M. A. M. Bruininx and A. F. B. van der Poel. 2015. Effects of structural and chemical changes of soy proteins during thermal processing on proteolysis. Proceedings of the 66th Annual Meeting of the European Federation of Animal Science. 31 August - 4 September, 2015, Warsaw, Poland.

Salazar-Villanea, S., E. M. A. M. Bruininx and A. F. B. van der Poel. 2016. Thermal and shear processing of pea protein concentrate: effects on secondary structure and protein hydrolysis. Proceedings of the 41st Animal Nutrition Research Forum, 15 April, 2016, Wageningen, the Netherlands.

Salazar-Villanea, S., E. M. A. M. Bruininx, P. Carré, A. Quinsac and A. F. B. van der Poel. 2016. Effect of toasting time on proteolysis of soluble and insoluble protein fractions of rapeseed meal. Proceedings of the 5th International Symposium on Energy and Protein Metabolism and Nutrition, 12-15 September, 2016, Krakow, Poland.

Salazar-Villanea, S., E. M. A. M. Bruininx, W. H. Hendriks, H. Gruppen and A. F. B. van der Poel. 2016. Processing of rapeseed meal: effects on protein hydrolysis and digestibility. Proceeding 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
Ethics and Phylosophy in Life Sciences	2013
Scientific exposure (13 ECTS)	
International conferences	
66th Annual Meeting of the European Federation of Animal Science, Warsaw, Poland	2015
13th International Symposium - 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
Animal Nutrition Research Forum, Utrecht, the Netherlands	2014
Animal Nutrition Research Forum, Wageningen, the Netherlands	2016
Presentations	
'Shear processing of rapeseed meal: effects on protein solubility', Animal Nutrition Research Forum, Utrecht, the Netherlands, oral presentation	2014
'Protein modifications during the production of rapeseed meal and the effects on the rate of protein hydrolysis', Digestive Physiology of Pigs, Kliczków, Poland, poster presentation	2015
'Predicting the *in vivo* standardized ileal digestibility of over-processed ingredients using two *in vitro* methods', Digestive Physiology of Pigs, Kliczków, Poland, poster presentation	2015
'Effects of toasting during the production of rapeseed meal on protein hydrolysis', WIAS Science Day, Wageningen, the Netherlands, oral presentation	2015
'Using protein chemistry to explain differences in digestibility', WBS Course Quality of Protein in Animal Diets, Wageningen, the Netherlands, oral presentation	2015
'Effects of structural and chemical changes of soy proteins during thermal processing on proteolysis', 66th Annual Meeting of the European Federation of Animal Science, Warsaw, Poland, oral presentation	2015
'Thermal and shear processing of pea protein concentrate: effects on secondary structure and protein hydrolysis', Animal Nutrition Research Forum, Wageningen, the Netherlands, oral presentation	2016
'Effect of toasting time on proteolysis of soluble and insoluble protein fractions of rapeseed meal', 5th International Symposium on Energy and Protein Metabolism and Nutrition, Krakow, Poland, oral presentation	2016
In-depth studies (7 ECTS)	
WBS Course Advances in Feed Evaluation Science, Wageningen, the Netherlands	2013
VLAG Course Industrial Food Proteins 1st Edition, Wageningen, the Netherlands	2013
WIAS Advanced Statistics Course: Design of Experiments, Wageningen, the Netherlands	2013
WIAS Statistics for Life Sciences, Wageningen, the Netherlands	2014
WBS Course Quality of Protein in Animal Diets, Wageningen, the Netherlands	2015
Course Indirect Calorimetry, Krakow, Poland	2016
Statutory courses (3 ECTS)	
Use of Laboratory Animals	2014

Professional skills support courses (3 ECTS)	
Supervising MSc thesis work	2013
Techniques for scientific writing	2015
Mobilising your -scientific- network	2016
Scientific publishing	2016
Research skills training (3 ECTS)	
Preparing own PhD research proposal	2013
Didactic skills training (6 ECTS)	
Lecturing	
Principles of Animal Nutrition, BSc.	2013 - 2015
Feed Technology, MSc.	2013 - 2015
Supervising thesis	
Supervising MSc. thesis (3×)	2014 - 2016
Management skills training (2 ECTS)	
Organisation WIAS Science Day	2014
Education and training total	**40 ECTS**

[1] Completed in fulfilment of the requirement for the education certificate of the Graduate School WIAS (Wageningen Institute of Animal Science)
[2] One ECTS equals a study load of 28 hours

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