Maillard reaction products in pet foods

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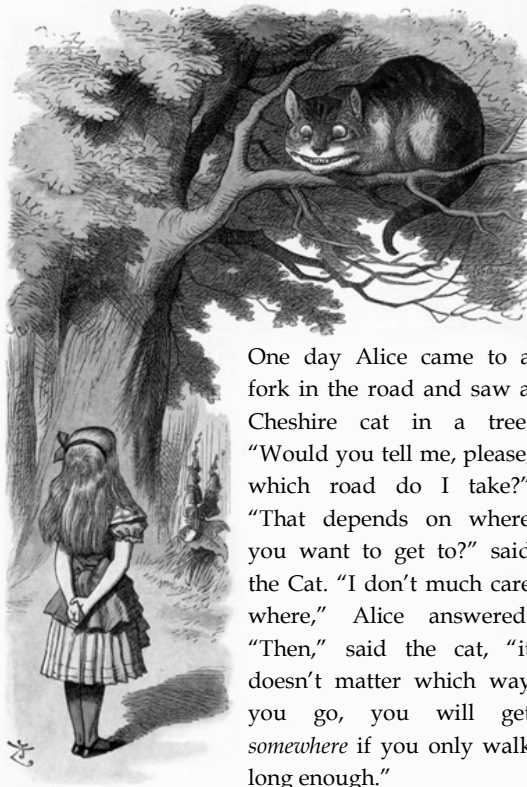to be defended in public
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One day Alice came to a fork in the road and saw a Cheshire cat in a tree. "Would you tell me, please, which road do I take?" "That depends on where you want to get to?" said the Cat. "I don't much care where," Alice answered. "Then," said the cat, "it doesn't matter which way you go, you will get *somewhere* if you only walk long enough."

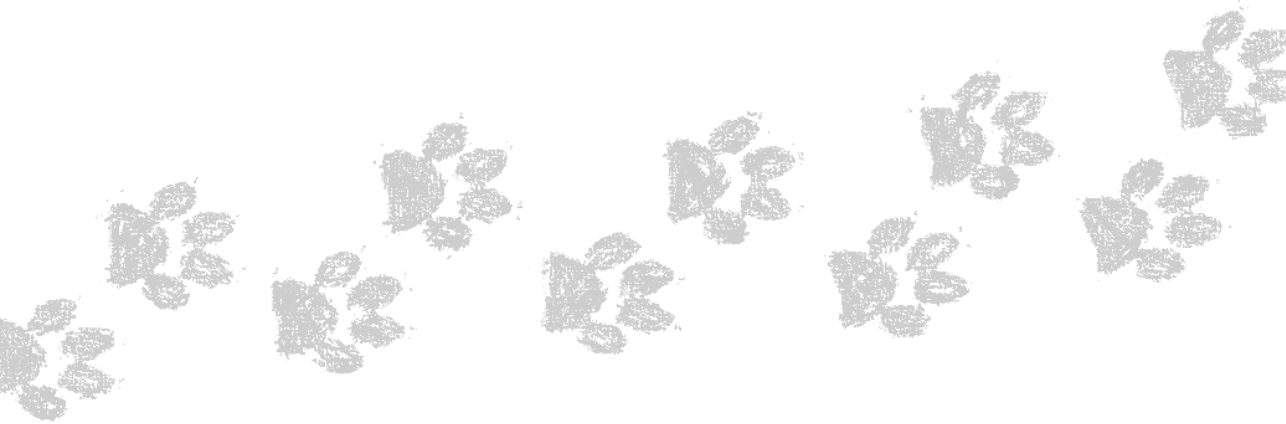*From: Alice's Adventures in Wonderland*

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Chapter 1

General Introduction

In Europe, the estimated percentage of households owning at least one cat is 24%. For dogs this value is also 24%.[1] Together, the number of pet dogs and cats in Europe and US add up to 300 million. Ninety percent of the pet owners regard their pet as a valued family member and, therefore, their health and well-being is becoming more and more important.[2] Most pet dogs and cats in Europe, the US and other developed countries are fed commercially manufactured pet foods. To be able to supply all these animals with food, pet food producing companies own a total of 650 plants, producing 8.5 million tons of pet food products per year, with the market still growing every year.[1] The primary role of these commercial pet foods is to provide safe and nutritionally balanced foods to be able to meet the animal's nutritional needs. As the majority of cats and dogs tends to be fed commercial diets throughout their lives, their health depends to a large extent on the nutritional quality of these foods (**Textbox I**).

Textbox I - Should there be a reason to doubt the quality of commercial pet foods?

By European law (Reg. (EC) 767/2009 art. 3 ad 2(i) and (EC) 1831/2003 art. 2 ad 1(f)), complete feed is defined as feed which, by reason of its composition, is sufficient for a daily ration (i.e. the average quantity required daily to satisfy all needs). Hence, most pet food manufacturers produce their pet foods according to recommended allowances based on scientific principles as presented by either the National Research Council, the European Pet Food Industry Federation, or the Association of American Feed Control Officials.[3-5] Therefore, foods formulated according to these recommended allowances meet all nutritional requirements as far as they are known in literature. These foods support growth, reproduction and maintenance using standard diets, and support animals suffering from several diseases using veterinary diets.[6] However, many essential nutrients for cats and dogs lack precise information on digestibility and bioavailability. The recommendations of pet foods can change over time if scientific knowledge provides new insights. Regarding the safety of the foods, all pet food manufacturers should apply to officially stated safety rules, ensuring safe products to feed pet animals.

Protein quality of pet foods

Dietary proteins provide both essential and non-essential amino acids that are used to build body tissues. Essential means that the animal cannot synthesize the amino acid in the body and, therefore, has to be provided in the diet. There are 10 essential amino acids recognized for dogs and cats. In addition, cats also require the sulphonic amino acid taurine, which is present as a free amino acid in animal tissues.[7] However, also non-essential amino acids can become conditionally essential, for example under special pathophysiological conditions. Whether protein intake is sufficient to meet the animal's

requirement, depends on the protein quality, i.e. the efficiency by which essential and non-essential amino acids from food are converted into body tissue.[8] That efficiency depends on the concentration of amino acids and their bioavailability, which is determined as the amount of amino acids that can be absorbed from the intestinal tract into the body, and can be used for growth, maintenance and lactation. The term protein requirement is used to refer to the quantity of protein (or its single amino acids) needed to be absorbed by the animals body to maintain a healthy physiological state.[6] Protein quality decreases when the amino acids in the food are imbalanced. If one of the essential amino acids is not present in sufficient amounts, one of the building blocks of protein synthesis is lacking and protein synthesis will slow down. The amino acid that is the first to limit protein synthesis is named the limiting amino acid. Undersupply of limiting amino acids can result in a reduced growth in young animals, as well as a decreased muscle mass, decreased immune response and poor skin and hair appearance in young and adult animals.[3] Lysine is often the first or second limiting essential amino acid in commercial foods for cats and dogs,[3] and requires, therefore, particular focus to be available in sufficient amounts in their food.[9]

The essential amino acid lysine

The amino acid lysine has a rather simple structure (Figure 1.1). Free lysine contains an α-amino group ($-NH_2$), an α-carboxyl group (-COOH) and an ε-amino group ($-NH_3^+$). The NH_2 and COOH groups are involved in the peptide bonds formed when the amino acid is present in a peptide or protein sequence. As a result, in peptides and proteins, the ε-amino group of lysine is still reactive and can participate in chemical reactions.

$$-HN-CH-\overset{\overset{\displaystyle O}{\|}}{C}-$$
$$|$$
$$(CH_2)_4$$
$$|$$
$$NH_3^+$$
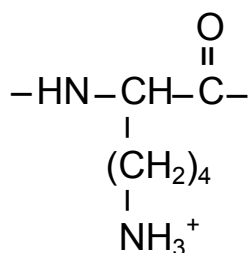
Figure 1.1. Chemical structure of lysine in a peptide or protein.

The lysine content of foods and ingredients is usually determined using traditional amino acid analysis. Proteins are heated in concentrated acid, hydrolysing the peptide bonds and resulting in free amino acids that can be quantified using chromatographic separation. This is referred to as total lysine. However, a distinction should be made between lysine that has a free ε-amino group and lysine of which that ε-amino group is

bound to another component. During the hydrolysis step of amino acid analysis, these modified lysine structures can revert back to lysine. To be able to determine the amount of lysine that contains a reactive free ε-amino group, *O*-methylisourea is used as a reagent. This is a component that binds only to the reactive free ε-amino groups of lysine and therefore this structure refers to as reactive lysine. The difference between total and reactive lysine represents the amount of modified lysine that reverts back to lysine during acid hydrolysis. It is known that the bioavailability of lysine in the body depends on whether the ε-amino group is reactive or modified. Kittens fed an experimental diet including a casein mixture containing a difference between total and reactive lysine of 32.9% resulted in a lower growth rate (mean daily gain of 2.7 g) compared with kittens fed a control diet including casein containing a difference between total and reactive lysine of 0.4% (mean daily gain of 14.9 g).[10,11] Adding 4.0, 5.5 and 7.0 g of synthetic lysine to the first diet increased mean daily gain of the kittens to 5.4, 11.2 and 20.1 g. Hence, bioavailability of lysine is lower than expected with increasing differences in total and reactive lysine; subsequently protein quality of the food is reduced. There have been a few studies that have analysed the reactive lysine content using *O*-methylisourea in processed pet foods.[12-17] Those studies have found that the difference between total and reactive lysine was up to 62% in commercially available dry canine and feline foods. Based on these data, differences in total and reactive lysine may result in lower bioavailable lysine than expected using traditional amino acid analysis, and subsequently a lower nutritive value of the food. The potential consequence of a decreased lysine bioavailability of foods is a supply of lysine below the lysine requirements of animals, especially in growing animals that have higher requirements compared to adult animals. This can result in suboptimal development and growth.

What is the cause of a difference in total and reactive lysine in pet foods?

Proteins and amino acids in foods can undergo several chemical reactions, including crosslinking and glycation reactions. An example of an amino acid crosslink is lysinoalanine. The formation of lysinoalanine includes a dehydroalanine originating from cysteine and serine, that reacts with the ε-amino group of lysine to form a crosslink, thereby modifying the reactive side chain of lysine to form an altered amino acid.[18] An example of a glycation reaction is the Maillard reaction. This reaction is also referred to as a non-enzymatic browning and flavouring reaction.[19] The Maillard reaction can be divided into three phases: an early, advanced and final stage.[*] In the early stage, the α-carbonyl group of a reducing sugar reacts with a free amino group of an amino acid either in a free form or bound in

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[*] A more extensive explanation is provided in Chapter 2

protein or peptides. In many foods, the highly reactive ε-amino group of the amino acid lysine is the most important source of reactive amino groups to produce modified lysine derivatives.[9,20] A Schiff's base is formed,[21] and this reversible but unstable compound usually undergoes the Amadori rearrangement to form the Amadori compound, ε-*N*-deoxyketosyllysine, which is a modified lysine structure. Once this Amadori product has been formed, the reaction becomes irreversible under normal conditions. However, this structure can revert back to lysine during acid hydrolysis, and is therefore the main cause of the difference between total and reactive lysine. In the advanced stage, several other reactions occur which lead to the formation of several advanced Maillard reaction products (**Textbox II**). All these products can react further with amino acids during the final stage of the Maillard reaction to form melanoidins, which are responsible for the brown colouring of foods.[20] Because amino groups are involved in several steps of the Maillard reaction, a strong decrease of the availability of amino acids can occur. In these advanced stages, the structure of the lysine component is modified in such a way that it does not revert back to lysine during acid hydrolysis. As such, the difference between total and reactive lysine is not equal to the lysine that has undergone the Maillard reaction.

Textbox II - Advanced Maillard reaction products and their possible effect on health

The Maillard reaction that takes place during the processing of foods also takes place endogenously as a naturally occurring process in body tissues.[22,23] Dietary advanced Maillard reaction products have, therefore, endogenously formed analogues referred to as advanced glycation end-products. During their formation, advanced glycation end-products can covalently crosslink tissue proteins and, thereby, modify structural and functional properties of the proteins. As these crosslinks are resistant to degradation, turnover rate is delayed and tissue repair hindered, resulting in an accumulation of advanced glycation end-products in body tissues.[24] In addition, receptors for advanced glycation end-products are expressed on a wide range of cells, and binding to these receptors can lead to oxidative stress, vasoconstriction and inflammatory responses.[25-27] Advanced glycation end-products have been associated with the aetiology of age-related diseases in humans, such as atherosclerosis, nephropathy, retinopathy, osteoarthritis, neurodegenerative diseases and diabetes mellitus.[†] These age-related diseases are also seen in dogs, showing many similarities to these diseases in humans. Elevated levels of advanced glycation end-products in tissue proteins were observed in aging dogs with e.g. diabetes mellitus,[28] carteracts,[29] osteoarthritis,[30] neurodegenerative diseases like canine cognitive dysfunction syndrome,[31] vascular dysfunction[32] and atherosclerosis.[33] Recent studies indicate that dietary Maillard reaction products can contribute to the body's

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[†] A more extensive overview is provided in Chapter 2

advanced glycation end-product pool as has been reported in humans,[34-37] and possibly relate to the aetiology of age-related diseases.

Pet food processing can induce the Maillard reaction

Until the mid-1800s, the food of domestic dogs and cats consisted of prey animals scavenged by the animal itself, supplemented with table scraps. From 1860 onwards, the commercial pet food market started to grow. At first, the foods were produced using a baking or pelleting process, later followed by canned pet foods. In those days, canned pet foods represented a major part of the commercial market. During the Second World War, however, metal was rationed and the focus switched to dry foods again. In 1957, the first extruded pet foods were produced. From that time onwards, commercially extruded, canned and pelleted pet foods were marketed as "complete foods" and resulted in a growing market in which the choices are almost unlimited.[38] All these processing technologies have in common that they use heat, moisture, shear and pressure to produce a shaped and ready to feed end-product out of an ingredient mixture.[‡] Thermal treatment improves the nutritive properties of the food by improving the digestibility of raw ingredients and inactivating anti-nutritional components. In addition, safety and shelf life are improved by destruction of viable spores and bacteria.[39-41] Besides these beneficial effects, thermal treatments can also induce the Maillard reaction and the formation of lysinoalanine.[42] The reaction rate of the Maillard reaction is influenced by temperature and heating time, moisture level, pressure, pH and mechanical shear.[43] The Maillard reaction is deliberately induced in pet foods to contribute to desired flavour and colour, as well as in palatability enhancers often used in pet foods. However, as mentioned above, processing of ingredients and foods can induce unfavourable consequences of the Maillard reaction such as a decreased protein quality due to the loss of bioavailable amino acids like lysine and the formation of advanced Maillard reaction products.[20,44] Until now, the occurrence of the Maillard reaction and the presence of Maillard reaction products in pet foods was suggested,[45,46] but only limited quantitative data are available. A furosine level of 0.91 mg/g was reported in a dry dog food of unknown origin, and acrylamide contents ranging from 106 to 358 µg/kg in dry dog foods and from 66 to 269 µg/kg were reported in dry cat foods commercially available on the Czech Republic market.[47,48]

Problem definition

Knowledge to increase the nutritive value of pet foods and minimisation of potential components identified as risk factors for disease would benefit overall pet health. At

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[‡] A detailed overview of the processing technologies is provided in Chapter 2

present there is a paucity of information on the availability of lysine in pet foods and factors affecting this availability. It is, for example, unknown whether reactive lysine levels in commercially processed pet foods meet minimal requirements of dogs and cats. It is known, however, that the reactive lysine content of many pet foods is lower than the total lysine content, suggesting the occurrence of the Maillard reaction in pet foods and, therefore, the presence of Maillard reaction products. The quantity of Maillard reaction products in commercially processed pet foods is only limited reported in literature. To be able to evaluate the formation of Maillard reaction products during processing, it is important to know where in the production process of pet foods and under which conditions Maillard reaction products are formed. This could lead to optimisation of processing conditions in a way that the desired beneficial effects are promoted and the undesired effects are minimized.

Objective and outline of this thesis

The overall aim of the project was to determine the occurrence and progression of the Maillard reaction during the manufacturing of pet foods, and characterize the subsequent impact on nutritive value of the food, and the bioavailability of Maillard reaction products in pet animals. First, literature was reviewed, describing the Maillard reaction and how this reaction affects the nutritive value of pet foods in relation to the lysine requirements of dogs and cats (**Chapter 2**). Occurrence of the Maillard reaction in commercially available pet foods was characterized using total and reactive lysine to evaluate whether available lysine levels in these foods meet minimal requirements of growing and adult dogs and cats (**Chapter 3**). In addition, the Maillard reaction products fructoselysine, carboxymethyllysine, hydroxymethylfurfural and the amino acid lysinoalanine were quantified in commercial pet foods and daily intake was estimated (**Chapter 4**). The results from these studies raised the question whether and how processing, i.e. steam pelleting and extrusion processing, influences the occurrence and progression of the Maillard reaction in pet foods (**Chapter 5 and 6**). In the final stage of the project, explorative research was conducted on whether and to what extent specific Maillard reaction products present in pet foods are absorbed and excreted by adult cats (**Chapter 7**). Finally, the results of this thesis are summarized and discussed in **Chapter 8**.

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Chapter 2

The Maillard reaction and pet food processing: effects on nutritive value and pet health

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Abstract

The Maillard reaction, which can occur during heat processing of pet foods or ingredients, is known to reduce the bioavailability of essential amino acids such as lysine due to the formation of early and advanced Maillard reaction products (MRP) that are unavailable for utilisation by the body. Determination of the difference between total and reactive lysine by chemical methods provides an indication of the amount of early MRP present in foods, feeds and ingredients. Previous research reported that the difference between total and reactive lysine in pet foods can be up to 61.8%, and foods for growing dogs may be at risk of supplying less lysine than the animal may require. The endogenous analogues of advanced MRP, advanced glycation end-products, have been associated with age-related diseases in humans, such as diabetes and impaired renal function. It is unknown to what extent advanced MRP are present in pet foods, and if dietary MRP can be associated with the development of diseases such as diabetes and impaired renal function in pet animals. Avoidance of ingredients with high levels of MRP and processing conditions known to favour the Maillard reaction may be useful strategies to prevent the formation of MRP in manufactured pet food. Future work should further focus on understanding the effects of ingredient choice and processing conditions on the formation of early and advanced MRP, and possible effects on animal health.

Key words: Extrusion, Advanced glycation end-products, Reactive lysine, Dogs, Cats

Introduction

The majority of pet dogs and cats in the developed world are fed processed commercial pet foods throughout their lives.[1] These foods are formulated and manufactured to support the pets' nutritional needs, health and vitality. During the manufacturing of most commercial pet foods, thermal treatments are used to improve the safety and nutritive properties of the foods.[2] These thermal treatments, including pre-conditioning, extrusion cooking, retorting and pelleting, can improve the digestibility of protein through denaturation and starch by gelatinisation. Moreover, vegetable ingredients such as legumes and cereals may contain anti-nutritional components (for example, trypsin inhibitors, lectins) that are inactivated by thermal treatments.[3] Safety and shelf-life are improved by thermal destruction of viable spores and any bacterial contamination.

Besides the above-mentioned beneficial effects, thermal treatments can also negatively influence protein quality due to crosslinking, racemisation, oxidation of sulphur-containing amino acids, and the involvement of amino acids in the Maillard reaction.[4,5] The latter reaction is an important chemical reaction for food manufacturers as it contributes to desired flavour, colour and anti-oxidative properties in many foods.[6-8] However, the Maillard reaction also has unfavourable consequences such as the loss of bioavailable essential amino acids.[9,10] During the Maillard reaction, a reducing sugar binds to a free reactive amino group of an amino acid. In food proteins, the reactive ε-amino group of lysine is the most important source of reactive amino groups.[10,11] Previous research has indicated that up to 61.8% of the lysine in pet foods contains a bound ε-amino group, probably due to its involvement in the Maillard reaction.[12,13] This complex, also referred to as early Maillard reaction products (MRP), may be absorbed from the gastrointestinal tract but cannot be utilised by the animal.[10,14,15] As lysine is the first or second limiting essential amino acid in commercial foods for cats and dogs,[16] a reduced utilisation results in a reduced nutritive value of the food. In addition to the loss of essential amino acids, advanced MRP may have an influence on health. Some of these MRP are also endogenously formed, i.e. formed in the body, and have been associated with age-related diseases in humans and dogs.[17]

As commercially prepared pet foods are often routinely fed throughout the entire life of domestic cats and dogs, it is important to understand the factors that influence the availability of essential amino acids such as lysine and the formation of potentially bioactive MRP. The purpose of this review is to present an overview of the effect of the Maillard reaction on the nutritive value of pet foods in relation to the requirements of the animal. In addition, the potential health implications of MRP for dogs and cats are discussed. Factors influencing the formation of MRP in pet foods, including recipe ingredients and processing techniques used during pet food manufacture, will be

presented in the context to minimising the formation of early and advanced MRP in complex matrices such as pet foods.

The Maillard reaction

The Maillard reaction is a non-enzymatic browning and flavouring reaction that can occur during the processing and storage of foods.[18] Often free amino acids or amino acids in peptides and proteins are involved in the Maillard reaction. Free amino acids contain an α-amino (-NH_2) group as well as a functional side chain that varies between amino acids and can react during the Maillard reaction.[10] In peptides and proteins, amino acids form (poly)peptide chains through a polymerisation reaction, whereby the α-amino group links to an a-carboxyl group and becomes unavailable for the Maillard reaction. As a result, in peptides and proteins only the side chains of amino acids are reactive in the Maillard reaction. In a free as well as a protein-bound form, the ε-amino side chain of lysine is the most susceptible group to the Maillard reaction.[7,10,11,19] Side chains of other amino acids, for example the guanidine side group of arginine, and side chains of histidine and tryptophan, are also known to be involved in the Maillard reaction but are less well studied.[11,20] Next to free amino acids and proteins, amino lipids and nucleic acids can also be involved in the Maillard reaction.

The Maillard reaction can be divided into early, advanced and final stages (Figure 2.1). In the early stage, the carbonyl group of a reducing sugar reacts through a condensation reaction with the ε-amino group of lysine, resulting in the formation of a reversible Schiff's base. The Schiff's base can undergo an Amadori rearrangement resulting in the formation of the Amadori compound ε-*N*-deoxyketosyllysine.[21] It seems that the Amadori rearrangement can be reversible under certain conditions; however, the mechanism is not fully understood and it is unknown whether it is of quantitative relevance in the Maillard reaction.[22] In the advanced stage of the Maillard reaction, the Amadori compound can react further through several pathways, including rearrangement, condensation, oxidation and dehydration (and hydration) reactions, which lead to the formation of advanced MRP. Several α-oxoaldehydes including glyoxal, 1-,3-deoxyglucosones and fission products such as methylglyoxal are formed, either by non-oxidative rearrangements or by oxidation and glycolysation. These compounds are high in oxidative potential and, therefore, tend to be pro-oxidative.[23-26] α-Oxoaldehydes and ε-*N*-deoxyketosyllysine react with proteins (or lipids) to generate oxidants such as N^{ε}-(carboxymethyl)lysine (CML) or crosslink-forming end-products such as pentosidine. Other advanced MRP derived from these precursors include pyrraline and hydroxymethylfurfural (HMF). These advanced MRP are the most common compounds
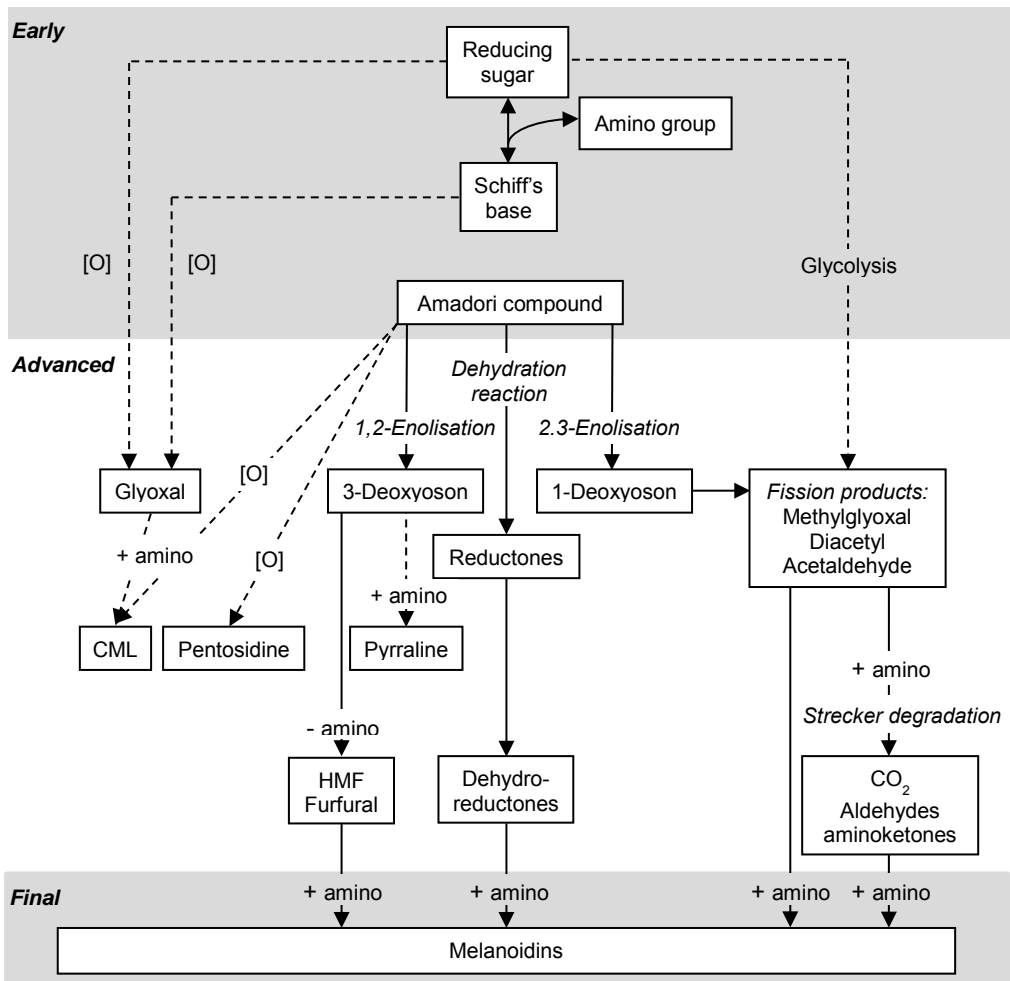
Figure 2.1 Scheme of the early, advanced and final Maillard reaction pathways and formation of Maillard reaction products and melanoidins (modified after Hodge[5]). [O], oxidation; CML, carboxymethyllysine; HMF, hydroxymethylfurfural.

and are used as markers to indicate the extent of the Maillard reaction in foods.[27] Amadori compounds that undergo a dehydration reaction result in reductones and dehydroreductones. In addition, the Strecker degradation converts fission products with an amino group into aldehydes, aminoketones and CO_2. In the final stage, MRP further react with free amino groups to produce melanoidins which are responsible for the characteristic brown colour of heated foods.[10,28] Increased antioxidant activity is observed during the formation of brown colouring,[24,28] which can be mainly attributed to the reductones and dehydroreductones that can act as antioxidants in their reduced state.[29]

Determining Maillard reaction products in processed foods and ingredients

Several methods have been developed to quantify MRP in processed foods and ingredients. Total lysine content of foods and ingredients is usually determined using traditional amino acid analysis. Proteins are heated in concentrated acid (for example, 6 M-HCl) at 110°C for 24 h, hydrolysing the peptide bonds and resulting in free amino acids that can be quantified using chromatographic separation. However, total lysine may not be suitable for quantifying nutritional available lysine in processed foods, feeds and ingredients as the early and some advanced MRP can revert back to lysine during the acid hydrolysis step.[11,30-32] Bioavailable lysine is defined as the reactive lysine that is digested, absorbed and potentially can be utilised for metabolism. Bioavailable lysine can be determined by either an animal growth assay where the ability of an animal to deposit protein or amino acids from a test diet is measured, or the true ileal amino acid digestibility assay.[14,31] These methods are the most accurate but costly and time consuming. Alternative methods based on a reaction of chemical compounds with the free ε-amino group of lysine have been developed to measure reactive lysine in foods, feeds and ingredients. The most well-known method, and considered to be the reference method for determination of reactive lysine, was developed by Carpenter[33] and revised by Booth.[34] Free amino groups of lysine in protein react with fluorodinitrobenzene (FDNB) to form the acid-resistant yellow compound dinitrophenol (DNP)-lysine. After hydrolysis of the protein, the α-DNP-amino acids are removed by ether extraction. The remaining ether-insoluble ε-DNP-lysine is detected spectrophotometrically. Variations on the FDNB-reactive lysine method have been developed. In the 'Silcock' method, reactive lysine in samples is determined as the difference between total lysine before the reaction with FDNB and the residual lysine present after the reaction.[35] Determination of ε-DNP-lysine by HPLC results in higher values compared with the original method of Carpenter,[33] possibly due to compounds formed during hydrolysis that interfere with the spectrophotometric determination of ε-DNP-lysine.[36] Trinitrobenzenesulfonic acid and sodium borohydride have also been used to form acid-stable complexes with the free ε-amino group of lysine; the formed complexes are measured after hydrolysis using a spectrophotometer or amino acid analyser.[37,38] The 'dye binding lysine' method of Hurrell et al.[39] is based on the difference between measurement of unmodified amino acids with Acid Orange 12 and after the ε-amino group of lysine has been blocked through a reaction with propionic anhydride; reactive lysine content is then calculated from the difference.[39] Another binding agent is *ortho*-phthaldialdehyde (OPA), which forms a fluorescent reaction product with the free amino groups in proteins.[40] The fluorescent intensity measured is corrected for the contribution of N-terminal amino groups to determine the reactive lysine content. In the above-mentioned methods, the chemical

compounds bind to both the α- and ε-amino group of lysine and are, therefore, not suitable for accurate determination when free or synthetic lysine is present.[31] The guanidination method uses O-methylisourea (OMIU) as a reagent which is able to bind to only free ε-amino groups, converting lysine into homoarginine.[41] Homoarginine is acid stable[42] and, after acid hydrolysis via traditional amino acid analysis, allows for the accurate determination of the reactive lysine contents of foods, feeds and ingredients. A good correlation has been demonstrated between the FNDB- and OMIU-reactive lysine methods for a range of animal feedstuffs (r = 0.996) and breakfast cereals (r = 0.985), indicating that results obtained by the two methods are comparable.[31] According to Rutherfurd & Moughan,[31] the guanidination method is the most preferred method to analyse reactive lysine in processed foods and ingredients.

In terms of terminology, the present review will refer to total lysine as lysine molecules with a free ε-amino group and lysine that reverts back to lysine after standard acid hydrolysis. Reactive lysine is undamaged lysine that has a reactive ε-amino side chain. The difference between total and reactive lysine, therefore, is a measure of the lysine that reverts back to lysine after acid hydrolysis and includes lysine involved in the early Maillard reaction. Most lysine that has reacted to yield advanced Maillard products does not revert back to lysine during acid hydrolysis and as such the difference between total and reactive lysine is, therefore, not equal to the lysine that has undergone the Maillard reaction. It is, however, often used as an indication of heat damage of processed foods, feeds and ingredients.[4,11]

To be able to measure MRP in foods, feeds and ingredients, other chemical markers can be analysed. Furosine (ε-N-(furoyl-methyl)-L-lysine) is an amino acid formed during acid hydrolysis of the Amadori compound fructoselysine and is produced by reaction of the ε-amino groups of lysine with glucose. As such, furosine is a specific chemical marker of the Amadori compound generated during the early Maillard reaction.[27,43] In the advanced stage, the extent of the Maillard reaction can be measured in several ways. The colour formation during this stage can be measured by absorbance at 420 nm. In addition, dehydration and fission reactions form fluorescent compounds that can be measured by fluorescence spectrophotometry at 347 nm excitation and 415 nm emission.[44] Specific markers of the advanced stage such as HMF and CML are often analysed by ultra-performance liquid chromatography or HPLC, sometimes combined with MS.[45]

From a practical point of view, procedures that can rapidly provide an indication of the occurrence of the Maillard reaction due to heat processing of foods, feeds or ingredients are necessary. Besides the furosine, colour and fluorescence methods, the total or reactive lysine:crude protein ratio seems to be a relatively rapid method to estimate heat damage as the concentration of lysine, but not the concentration of crude protein,

reduces if samples are extensively heat processed.[46,47] This method is, however, mainly tested in distillers dried grains with solubles (DDGS) and soyabean meal. An increase of dark colour in DDGS was related to an increase in acid-detergent fibre (r = 0.62; P = 0.10) and acid-detergent insoluble N (r = 0.79; P = 0.01) during heat processing, indicating that these components can be indicators of the Maillard reaction especially in fibrous feeds or ingredients.[48,49] Near-IR reflectance spectroscopy might be a future method to determine reactive lysine in foods, feeds and ingredients; however, data are limited.

The Maillard reaction in model systems

The Maillard reaction has been extensively studied using pure compounds as well as heat-treated food systems.[10,11,50] Studies of pure compounds in model systems indicate that the type of reactions and the extent to which they occur depend on several reaction conditions. In terms of amino acid type, lysine had the highest reactivity among twelve investigated amino acids (aspartic acid, glutamic acid, alanine, leucine, isoleucine, valine, proline, serine, cysteine, phenylalanine, arginine and lysine) when heated with reducing sugars at 100°C for 3 h as measured by the formation of MRP with absorbance at 420 nm.[19] As the heating time was increased to 12 h, the colour intensity of the MRP from lysine became two to three times higher than that of the other amino acids.[19] In terms of reducing sugar type, glucose has been reported to be the most reactive reducing sugar when heated at 60°C in the presence of casein; OPA-reactive lysine content decreased by about 60% within 10 h, followed by maltose (15 h), lactose (20 h) and fructose (35 h).[51] Brands et al.,[52] however, observed contradictory results when casein was heated at 120°C for 90 min in the presence of glucose or fructose, with fructose being more reactive than glucose. After 60 min of heating, both reducing sugars induced an OPA-reactive lysine reduction of about 60%. In a model system consisting of soyabean protein concentrate, glucose and microcrystalline cellulose, heated at 95°C for 75 min FDNB-reactive lysine content was reduced by 31.9%.[53] OPA-reactive lysine reduction appeared to be faster with increasing temperatures from 37 to 60°C.[51] Increasing the pH from 4 to 12 at a temperature of 100°C decreased total lysine content by up to 50% after 2 h in the presence of fructose.[54] In addition, lowering the water content (w/w) of a sugar-amino acid model system from 100 to 20% increased colour formation.[55] Based on these data, it appears that in model systems the type of amino acid and the type of reducing sugar influence the extent of the Maillard reaction. In addition, increasing heating temperature and time, pH level and decreasing water content increase the reaction of lysine.

Effects of the Maillard reaction on the nutritive value of pet foods

As described above, the early MRP of lysine may be partly absorbed but has no nutritional value.[14,32,56,57] This impaired utilisation of lysine that has undergone the Maillard reaction was recently confirmed in a kitten growth study.[58,59] Kittens fed an experimental diet including a heated casein-dextrose mixture containing a difference between total and reactive lysine of 32.9% resulted in a lower growth rate (mean daily gain of 2.7 g) compared with kittens fed a control diet including unheated casein containing a difference between total and reactive lysine of 0.4% (mean daily gain of 14.9 g). Adding 4.0, 5.5 and 7.0 g of synthetic lysine to the heated casein diet increased mean daily gain of the kittens to 5.4, 11.2 and 20.1 g, respectively. This study clearly indicates that the bioavailability of lysine can be significantly impaired by heating. Quantification of reactive lysine is therefore important for the evaluation of the nutritive value of pet foods. Bioavailability of nutrients, including lysine, is taken into account in the nutritional recommendations for the formulation of complete pet foods. The recommended allowances of lysine for dogs at maintenance based on scientific principles presented by the National Research Council (NRC)[16] and the European Pet Food Industry Federation (FEDIAF)[60] include a bioavailability factor of 0.80 and 0.67, respectively. The Association of American Feed Control Officials (AAFCO)[61] is a regulatory organisation that sets standards for the quality and safety of animal feed and pet food in the USA. AAFCO provides higher recommended lysine allowances compared with NRC, i.e. 6.3 vs. 3.5 g/kg DM, including a bioavailability factor of 0.44 when calculated using the NRC minimal requirement of 2.8 g/kg DM.[16] The fraction of the lysine in foods that is actually bioavailable depends on the extent of the fraction of lysine that has undergone the Maillard reaction and the ileal digestibility of the reactive lysine. The difference between total and reactive lysine of commercially produced canned and dry dog and cat foods can be considerable, with values reported of up to 61.8%.[12,13,62] Furthermore, apparent ileal crude protein digestibility has been shown to be highly variable among 141 dog foods, with values ranging from 51.1% up to 90.5% and a mean digestibility of 73.5%.[64] As variability in amino acid digestibility can be expected to be similar to crude protein digestibility, these results indicate that lysine digestibility is likely to be highly variable as well. Data on ileal reactive and total lysine digestibility in dogs are scarce. However, recently Hendriks et al.[63] reported standardised ileal OMIU-reactive lysine digestibility values in dogs fed five commercial dry dog foods containing varying protein contents (24.4 to 32.7% DM) of 79.5 to 93.7% with a mean of 88.2%. Corresponding standardised ileal total lysine digestibility values of these foods were 64.2 to 87.2%, with a mean of 80.0%. In another study involving dogs,[65] the apparent ileal total lysine digestibility of a
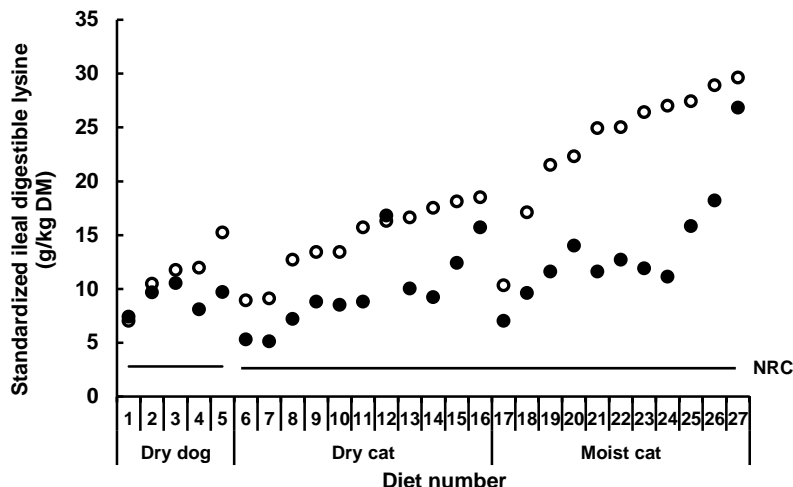
Figure 2.2 Standardised ileal digestible total lysine (○) and standardised ileal digestible *O*-methylisourea (OMIU)-reactive lysine (●) contents for five commercial dry maintenance foods for dogs using ileally cannulated dogs[63] and twenty-two commercial maintenance (SM Rutherfurd, personal communication) foods for cats using the rat as the model animal.[13,31] Horizontal solid lines indicate the minimal lysine requirement for maintenance for dogs and cats presented by the National Research Council (NRC),[16] being, respectively, 2.8 and 2.7 g/kg DM, assuming a dietary energy density of 16.7 MJ (4000 kcal) metabolisable energy/kg.

Figure 2.3 Total lysine (○) and *O*-methylisourea (OMIU)-reactive lysine (●) contents for twenty-seven commercial dry maintenance foods for dogs (reanalysis of original data of Williams et al.[12]).[12,62] The horizontal solid line indicates the minimal lysine requirement for maintenance for dogs presented by the National Research Council (NRC),[16] being 2.8 g/kg DM, assuming a dietary energy density of 16.7 MJ (4000 kcal) metabolisable energy/kg. Dotted lines indicate bioavailability thresholds of 75 and 50% for meeting minimal lysine requirements. The recommended lysine allowance for dog maintenance foods of the Association of American Feed Control Officials (AAFCO)[61] equals a dietary lysine bioavailability of 44%.

commercial dry dog food was reported to be 83.6%. The bioavailable reactive lysine, i.e. standardised ileal digestible OMIU-reactive lysine, contents of the five commercial foods[63] met the minimal lysine requirement of 2.8 g/kg DM for dogs at maintenance[16] when a dietary energy density of 16.7 MJ (4000 kcal) metabolisable energy (ME)/kg is assumed (Figure 2.2). The OMIU-reactive and total lysine contents of twenty-seven commercial maintenance foods for dogs are reported in Figure 2.3.[12,62] These foods varied considerably in terms of differences between total and reactive lysine, with values up to 56.0% and an overall mean of 15.4%. Assuming a dietary energy density of 16.7 MJ (4000 kcal) ME/kg, these foods were well above the minimal lysine requirement. The two foods with the lowest OMIU-reactive lysine contents would have been deficient in lysine if their ileal reactive lysine digestibilities were 51.7 and 46.9%, which may be considered unlikely. The OMIU-reactive and total lysine contents of fourteen commercial growth foods for dogs[12] are reported in Figure 2.4. For growing dogs dietary total lysine contents were all above the recommended allowance as set by the NRC[16] (Figure 2.4). The OMIU-reactive lysine contents of two foods, however, were below the minimal total lysine requirements of 7 g/kg DM for growing dogs between 4 and 14 weeks of age,[16] assuming a dietary energy density of 16.7 MJ (4000 kcal) ME/kg. If these foods for growing dogs were used as weaning diets, the minimal requirement for growing dogs between 4 and 14 weeks would not be met. No ileal reactive lysine digestibility data are available for growing dogs. Assuming that the ileal reactive lysine digestibility was within the range presented by Hendriks et al.[63] (79.5 to 93.7%), three commercial foods would have had reactive lysine contents below the minimal total lysine requirements for 4- to 14-week-old growing dogs. It should be noted that growing dogs (and cats) have lower gastric pepsin secretion,[66] so digestibility values would probably be lower than those observed in adult dogs. As long as the reactive lysine digestibility for the other eleven foods were above 70.0%, these foods would have met the minimum lysine requirements for 4- to 14-week-old growing dogs. Interpretation of these results should take into account that most studies use commercially available single batch pet foods. Batch variation in ingredients as well as processing conditions (see further in the present review) can result in variation in the difference between total and reactive lysine content between batches of pet foods. The difference between total and reactive lysine appears to be greater in commercial cat foods than in dog foods. Rutherfurd et al.[13] reported average differences between total and reactive lysine values in canned cat foods (n = 10) of 48.6% (range 39.0 − 61.8%) and in dry cat foods (n = 10) of 41.2% of total lysine (range 20.1 − 48.7%) values (Figure 2.5). As long as the reactive lysine digestibilities are above 50.0%, these foods will meet the minimum lysine requirements for adult cats.[16] Using the rat as model animal,

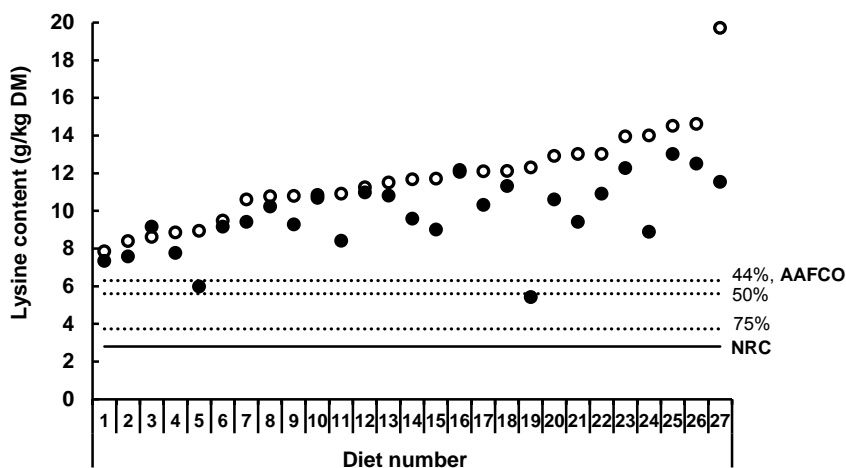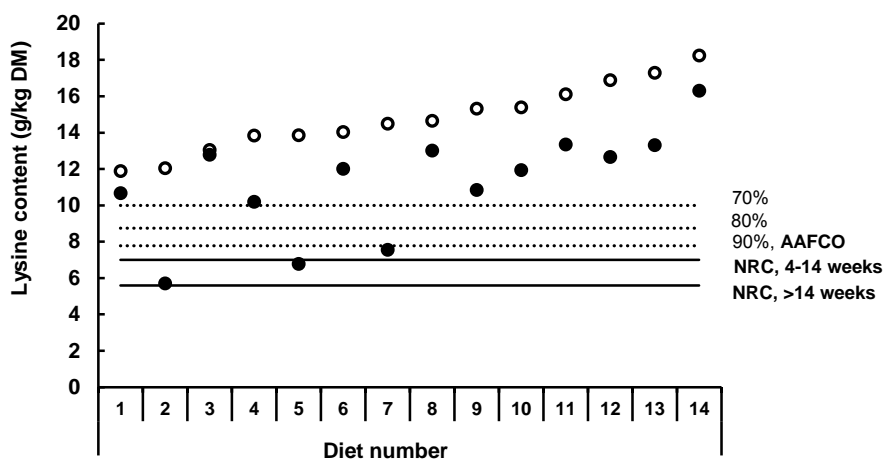**Figure 2.4** Total lysine (○) and *O*-methylisourea (OMIU)-reactive lysine (●) contents for fourteen commercial growth foods for dogs (reanalysis of original data of Williams et al.[12]). Horizontal solid lines indicate the minimal lysine requirement for growing dogs between 4 and 14 weeks old and older than 14 weeks presented by the National Research Council (NRC),[16] being, respectively, 7.0 and 5.6 g/kg DM, assuming a dietary energy density of 16.7 MJ (4000 kcal) metabolisable energy/kg. Dotted lines indicate bioavailability thresholds of 90, 80 and 70% for meeting minimal lysine requirements. The recommended lysine allowance for dog maintenance foods of the Association of American Feed Control Officials (AAFCO)[61] equals a dietary lysine bioavailability of 90%.
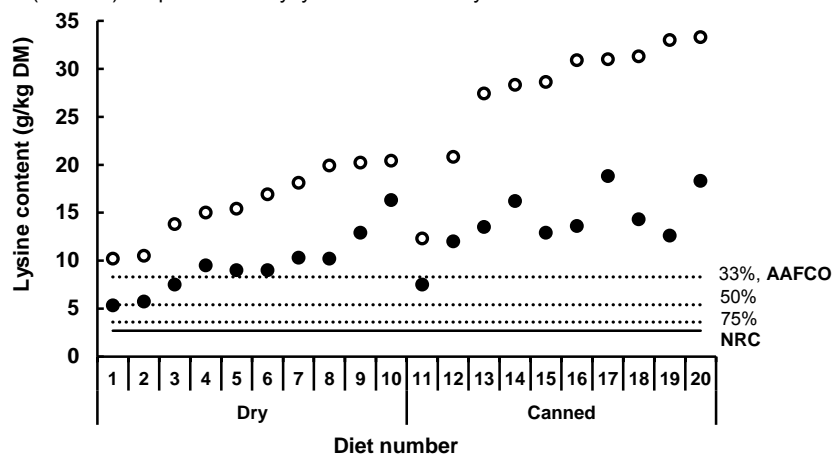
Figure 2.5 Total (○) and *O*-methylisourea (OMIU)-reactive lysine (●) contents of twenty commercial maintenance (SM Rutherfurd, personal communication) foods for cats.[13] The horizontal solid line indicates the minimal lysine requirement for maintenance of cats presented by the National Research Council (NRC),[16] being 2.7 g/kg DM, assuming a dietary energy density of 16.7 MJ (4000 kcal) metabolisable energy/kg. Dotted lines indicate bioavailability thresholds of 75 and 50% for meeting minimal lysine requirements. The recommended lysine allowance for dog maintenance foods of the Association of American Feed Control Officials (AAFCO)[61] equals a dietary lysine bioavailability of 33%.

standardised ileal OMIU-reactive lysine digestibility of the canned cat foods ranged from 79.9 to 97.1% with a mean of 88.1% while for the dry cat foods values ranging from 89.9 to 97.7% with a mean of 94.8% were reported.[13] These values are higher than a previously reported value of 71.5% for the apparent ileal total lysine digestibility of a canned cat food using the rat as the model animal.[67] The latter is probably due to the difference in method (standardised vs. apparent) and the presence of early MRP (OMIU-reactive only vs. OMIU-reactive plus Amadori compounds). Assuming a dietary energy density of 16.7 MJ (4000 kcal) ME/kg,[16] the approximated ileal digestible OMIU-reactive lysine content of these cat foods was higher than the minimal lysine requirements for maintenance in cats (Figure 2.2).

Overall, these data indicate that the difference between total and reactive lysine in commercial dog and cat foods can be considerable, in particular in canned foods. Factors such as ingredients used and processing conditions applied which contribute to the difference between total and reactive lysine in pet foods are described below. The standardised ileal digestibility of reactive lysine in analysed pet foods appears to be 80.0% or higher. However, the number of pet foods evaluated is limited. In particular, the lysine supply of certain commercial foods consumed by growing dogs, and possibly also growing cats, can be compromised due to differences between total and reactive lysine. Further prospective studies are required to identify the effects of early MRP during growth.

Effects of glycated Maillard reaction products on health

The Maillard reaction does not only occur during heat treatment of pure compounds or food systems but is also a naturally occurring process in body tissues. Biological interest in the endogenously occurring Maillard reaction was initiated during the late 1960s with the identification of non-enzymatically glycated Hb in the blood of diabetic patients.[68,69] During normal metabolism at homeostatic concentrations of glucose, endogenous proteins can be glycated resulting in advanced glycation end-products (AGE).[70,71] A large number of AGE have been identified *in vivo* including CML, HMF, pentosidine and pyrraline. These AGE are the most widely studied and used as biomarkers for *in vivo* formation of AGE.[70,72]

AGE have a variety of predominantly adverse biological effects. AGE can be pro-oxidants that can modify physicochemical properties of proteins by covalently crosslinking to proteins and thereby modifying structural and functional properties of the proteins. As the crosslinks of AGE and proteins are resistant to degradation, turnover rate is delayed and tissue repair hindered.[73-75] This results in accumulation of AGE in tissues with amounts depending on the turnover rate of the protein. As a result, proteins with a slow

turnover such as collagens in connective tissue, eye lenses and nerve myelin are sensitive to AGE accumulation.[76,77] Due to the modified physico-chemical properties of tissue proteins, AGE can become pathogenic. This could, for example, lead to stiffening of collagen fibres in the arterial wall leading to vascular complications.[78] Next to physico-chemical protein modifications, AGE can elicit cell-mediated responses through interaction with several cellular AGE receptors and induce cellular signalling, activation of transcription factors and subsequent gene expression. These receptors are expressed on a wide range of cells including smooth muscle cells, monocytes, macrophages, endothelial cells, podocytes, astrocytes and microglia.[79] The best-characterised receptor is RAGE ('receptor for AGE'), which belongs to the Ig superfamily and is capable of interacting with a broad spectrum of ligands, including AGE. Binding of an AGE to the receptor leads to activation of transcription factor NF-kB which in turn can lead to oxidative stress, vasoconstriction and inflammatory responses.[80,81] Under certain pathological conditions, such as hyperglycaemia or oxidative stress in diabetes mellitus, AGE formation can be accelerated. AGE have been, therefore, proposed to contribute to the development of long-term complications of diabetes.[79] In addition, AGE may contribute to the pathogenesis of age-related diseases such as atherosclerosis, nephropathy, retinopathy, osteoarthritis and neurodegenerative diseases such as Alzheimer's disease in humans.[17,79,82] Age-related diseases such as diabetes and renal, cardiovascular and neurodegenerative diseases are also seen in dogs, showing many similarities to these diseases in humans.[83-85] RAGE are reported in dogs,[86] and several studies have been conducted indicating that AGE also accumulate by binding to tissue proteins of ageing and diseased dogs. Comazzi et al.[87] reported higher AGE in plasma from dogs suffering from canine diabetes mellitus compared with control animals. In addition, elevated levels of AGE in tissue proteins in dogs were seen during cataract in the canine eye lens,[88] osteoarthritis,[17] neurodegenerative diseases such as canine cognitive dysfunction syndrome,[89] vascular dysfunction,[90] atherosclerosis,[91] and in skin collagen[92] with increasing age.

As indicated above, AGE formation occurs naturally in the body and defence mechanisms have evolved to protect against the adverse effects of (endogenously) formed AGE. Effects of AGE are avoided or regulated by renal AGE elimination and detoxification, antioxidant systems and suppression of signalling via the AGE receptor AGER1.[75] As in humans, the dietary intake of AGE, such as CML, HMF, pentosidine and pyrraline, formed during the processing of foods potentially provides an additional AGE load to dogs and cats. These dietary components appear to be, at least partially, digestible and transported through the general circulation. A significant correlation (r = 0.8) between ingested MRP and serum concentrations were observed when comparing the ingestion of

a high-MRP diet vs. a low-MRP diet in human diabetes mellitus patients with or without kidney disease.[93] The latter authors reported that about 10% of the dietary AGE were observed in serum. This supports the idea that dietary MRP contribute to the body's AGE pool.[94] As in tissues where the crosslinks of AGE and proteins are resistant to degradation, the majority (about 70%) of dietary AGE is not available either because the crosslinks are resistant to enzymatic hydrolysis, or trypsin digestion is impaired due to the absence of a positive charge on lysine in the intestinal tract.[93] To the authors' knowledge, no data are available in the scientific literature on the absorption and excretion of dietary MRP in dogs and cats.

There are large differences in absorption, metabolism and excretion between the different MRP. Advanced MRP such as CML, pyrraline and pentosidine seem to be absorbed by the gut. Ingested dietary CML in rats appeared to be approximately 26.0 to 29.0% excreted in the urine, and 15.0 to 22.0% excreted in faeces.[95] Approximately 1.7% of dietary CML accumulated in the circulation, kidney and liver and approximately 50.0% of the ingested CML was not recovered. This was confirmed in a later study where 31.2% of ingested dietary CML was excreted in the faeces, 14.4% in the urine, and 54.4% left unrecovered in human subjects.[96] Whether the unrecovered CML is deposited in organs, degraded by colonic microbiota or metabolised is unknown. Approximately 80% of dietary pyrraline is absorbed and excreted via the kidneys in humans within 48 h.[97] Urinary pyrraline is almost exclusively of dietary origin, and pyrraline is most probably not metabolised post-absorption. Approximately 2.0% of dietary pentosidine is recovered in urine in the peptide-bound form; however, approximately 60.0% is recovered in the free form.[98] The remainder may have been metabolised into unknown compounds and also excreted in the urine. Oral administration of HMF in rats resulted in rapid absorption. Excretion was primarily via urine, with between 66.3 and 80.0% of the radioactivity excreted in urine in the first 24 h.[99] In faeces, 8.5 − 12.2% was excreted within 48 h. The highest concentration of HMF in organs was observed in the liver and kidneys, the major organs for metabolism and excretion.[99] There was no evidence of prolonged accumulation in any other tissues. The MRP that are not absorbed can be metabolised by intestinal microbes,[100,101] giving MRP including Amadori products and melanoidins biotic properties.[102-104] The majority of the ingested MRP are not bioavailable; most of the absorbed MRP are excreted via the urine, however, some of the dietary MRP may accumulate in body tissues. In addition, it is seen that dietary MRP increase markers associated with an increased risk of type 2 diabetes and cardiovascular disease in healthy individuals, and dietary MRP promote inflammatory mediators in diabetics, which may lead to tissue injury;[75,105,106] restriction of dietary MRP suppressed these effects. In contrast to human foods, there is at present only one study reporting concentrations of

early MRP, and no data on the concentrations of advanced MRP in pet foods have been reported. The difference between total and reactive lysine reported in pet foods, and a furosine level of 0.91 mg/g reported in a dry dog food[107] indicate that at least the early phase of the Maillard reaction has occurred. In addition, there is no information available on the absorption of MRP in the gastrointestinal tract of dogs and cats and the contribution of dietary MRP to the body's AGE pool. At this stage, it can only be hypothesised that the daily intake of thermally processed foods could provide an additional peak load of AGE that may exceed the natural capacity to protect against AGE. In addition, it is unknown whether endogenous AGE formation is stimulated due to a postprandial increase in blood glucose levels. Dry foods for cats and dogs can contain up to 60% carbohydrates (as is), compared with a voluntary selection of a diet with a protein-fat-carbohydrate energy balance of 52:36:12 for cats[108] and of 30:63:7 for dogs.[109] A test diet with a carbohydrate content at 25% ME resulted in a lower peak and postprandial glucose concentration compared with diets with a carbohydrate content at 45 and 55% ME, although all measured glucose levels were within normal reference ranges.[110] Whether dogs and cats have the capacity to protect against increased blood glucose levels when fed commercial diets compared with their natural diet remains to be determined. It was recently demonstrated that the proportion of dry food intake may not be a risk factor for the development of type 2 diabetes mellitus in cats,[111] however, the study did not take into account the composition of the food or specific absorption of dietary AGE. Whether increased AGE exposure contributes to the pathogenesis of the aforementioned diseases in dogs[17,87-91] and possibly cats warrants further study. Given the key role of the kidney in the elimination and detoxification of AGE, cats and dogs with conditions such as chronic kidney disease will probably have an impaired capacity to eliminate AGE, which causes a build-up of AGE, formation of new AGE in the body and contributes to the pathogenesis of various health conditions. Whether or not dietary AGE intake also plays a pivotal role in the development of diabetes mellitus in dogs and cats, as suggested by Vlassara & Striker[75] in humans, is unknown, as is the potential of AGE restriction as a cost-effective strategy in the prevention and treatment of diabetes mellitus in dogs and cats.

Early Maillard reaction products in ingredients and during processing of pet foods

The amount of early (and advanced) MRP in pet foods may originate from three sources: first, the pet food ingredients may already contain early MRP due to processing; second, the processing conditions applied to produce the actual pet food; and third, lysine may react during coating and storage of the pet food.

Early Maillard reaction products in pet food ingredients

Most pet food manufacturers use co-products from other industries that may have been processed to varying degrees as primary ingredients in pet foods.[12] Proteins in pet foods originate from both animal and vegetable ingredients.[112] Animal protein sources in pet food include meat and bone meals of poultry, beef, pig, lamb and/or fish and other animal co-products, many of which are manufactured by a rendering-process.[113] Rendering separates fat, removes water and eradicates bacteria at temperatures as high as 130°C for several hours. These high temperatures and the duration of processing can influence protein quality. Using a chicken growth assay, total lysine availability in raw animal meals ranged from 86.9 to 107.7% whereas in the rendered animal meals lysine availability ranged from 70.1 to 99.9%.[114] Differences between total and reactive lysine content in several ingredients of animal origin, mostly rendered meat meals, range from 1.0% to up to 36.0% (Table 2.1). The difference between total and reactive lysine content in fish meals is, on average, less (4.0%) compared with meat and bone meal (16.0%), poultry meal (17.0%) and meat meal (20.0%), although variation is also high within these ingredients of animal origin.

Proteins of vegetable origin in pet foods originate from cereal grains and soyabean meal. These proteins are often considered to be of lower nutritional quality compared with animal proteins, because of a lower content of some essential amino acids and the presence of anti-nutritional factors.[122] In particular, cereal proteins are relatively low in lysine (Table 2.1). The difference between total and reactive lysine content in several ingredients of vegetable origin ranges from 0 to 44.0% (Table 2.1). The difference between total and reactive lysine content in peas and soyabean meal is, on average, less (0 and 6.0%, respectively) compared with wheat (15.0%), barley (15.0%), maize (27.0%) and rice (17.0%). In cereals, differences between total and reactive lysine content are higher compared with non-cereal ingredients of vegetable origin, with an average difference of 17.0 vs. 7.0%, respectively. Vegetable ingredients are often dried and ground before being included in the pet food recipe. Both drying and grinding involve heat during the process.

The data in Table 2.1 indicate that part of the lysine in animal and vegetable protein sources has already gone through the early Maillard reaction before inclusion in pet food recipes. Given the use of meat meals as main protein sources in pet foods, it would, therefore, be of interest to evaluate where and how early MRP are formed in these ingredients, which may ultimately contribute to the reduction of the difference between total and reactive lysine content in pet foods.

Table 2.1 Total and reactive lysine contents of common pet food ingredients.*

Ingredient	Lysine (g/kg as is) Total†	Reactive‡	Method	RL/TL	CP (g/kg)	DM (g/kg)	Reference
Animal origin							
Fish meal	46.9	46.5	FDNB	0.99	625§	912§	Hurrell et al.[39]
	51.0	45.9	FDNB	0.90	649	867	Batterham et al.[115]
	51.7	51.0	OMIU	0.99	703	917	Tran[116]
	47.0	45.9	OMIU	0.98	625§	925	vd Poel & Bikker|
	51.1	46.9	OMIU	0.92	635	983	Morel et al.[117]
Meat and bone meal	24.8	23.7	FDNB	0.96	509§	940§	Hurrell et al.[39]
	24.0	18.7	FDNB	0.78	494	967	Batterham et al.[118]
	37.0	32.6	FDNB	0.88	513	952	
	34.0	29.6	FDNB	0.87	430	926	
	22.0	18.7	FDNB	0.85	431	928	
	28.0	22.1	FDNB	0.79	527	935	Batterham et al.[115]
	36.5	34.6	OMIU	0.95	509§	940§	Rutherfurd et al.[41]
	27.4	17.4	OMIU	0.64	491	932	Morel et al.[117]
Meat meal	27.0	21.3	FDNB	0.79	547	946	Batterham et al.[118]
	26.0	21.8	FDNB	0.84	507	936	
	29.0	22.3	FDNB	0.77	559	946	
	32.0	26.2	FDNB	0.82	532	945	
Chicken meat	11.8	9.1	OMIU	0.78	213	442	Tran[116]
Poultry meal	35.4	29.3	OMIU	0.83	652	960	
Blood meal	89.1	88.0	OMIU	0.99	927¶	937¶	Rutherfurd et al.[41]
	85.2	61.5	OMIU	0.72	920	914	Morel et al.[117]
Vegetable origin							
Wheat	3.1	2.4	OMIU	0.78	94	871	Tran[116]
	3.5	3.1	OMIU	0.89	137§	897§	Rutherfurd et al.[41]
	3.1	2.8	OMIU	0.90	137§	873	vd Poel & Bikker|
	3.4	2.9	OMIU	0.85	120	867	Morel et al.[117]
Barley	5.9	5.4	FDNB	0.92	123§	902§	Hurrell et al.[39]
	3.7	3.1	OMIU	0.84	110	883	Tran[116]
	3.6	3.1	OMIU	0.86	123§	875	vd Poel & Bikker|
	4.6	3.6	OMIU	0.78	97	877	Morel et al.[117]
Corn	2.4	1.8	OMIU	0.75	81	876	Tran[116]
	2.5	2.2	OMIU	0.88	84§	869	vd Poel & Bikker|
	2.7	1.5	OMIU	0.56	83	876	Morel et al.[117]
Corn gluten	8.2	8.3	FDNB	1.01	563§	865§	Hurrell et al.[39]
Rice	3.0	2.5	OMIU	0.83	80	870	Tran[116]
Soybean (full fat)	22.4	22.5	OMIU	1.00	381§	907	vd Poel & Bikker|
	24.3	22.1	OMIU	0.91	421	968	Morel et al.[117]
Soybean meal	30.9	30.8	FDNB	1.00	515§	928§	Hendriks et al.[30]
	33.2	31.1	FDNB	0.94	515§	928§	Hurrell et al.[39]
	30.0	23.1	FDNB	0.77	475	890	Batterham et al.[115]
	32.3	32.3	OMIU	1.00	515§	928§	Rutherfurd et al.[41]
	29.3	29.1	OMIU	0.99	515§	886	vd Poel & Bikker|
	31.0	29.0	OMIU	0.94	416	885	Morel et al.[117]
Pea	15.1	14.7	FDNB	0.97	211¶	867¶	Hendriks et al.[30]
	14.8	15.4	OMIU	1.04	211¶	868	vd Poel & Bikker|
	14.9	14.9	FDNB	1.00	210	914	v Barneveld & Batterham[119]
	17.5	13.8	OMIU	0.79	192	875	Morel et al.[117]
Pea flour	15.3	14.5	FDNB	0.95	211¶	867¶	Hurrell et al.[39]
DDGS (wheat)	7.0	5.5	OMIU	0.79	268§	912	vd Poel & Bikker|
	6.4	5.1	FDNB	0.79	335	928	Cozannet et al.[120]

RL, reactive lysine; TL, total lysine; CP, crude protein; FDNB, fluorodinitrobenzene; OMIU, *O*-methylisourea; DDGS, distillers dried grains with solubles.
* Pet food ingredients used in dog and cat foods according to National Research Council.[16]
† Total lysine was determined by conventional amino acid analysis.
‡ Reactive lysine was determined according to the given method.
§ Missing values for DM and CP were completed with the help of data from National Research Council.[16] Values were necessary to calculate results in g/kg from the original publications.
| AFB van der Poel and P Bikker, unpublished results.
¶Missing values for DM and CP were completed with the help of data from the CVB.[121]

Effect of processing of pet foods

Pet foods are produced utilising various process technologies. Dry pet foods are the most popular form of dog and cat food[1] and most often produced by extrusion cooking. Next to extrusion cooking, dry foods can also be manufactured by pelleting the ingredient mixture. Moist foods are manufactured using heat sterilisation using retorting processing. Every processing technology has its own characteristic process conditions, thereby having a greater or lesser impact on the rate and extent of the Maillard reaction. The effect of processing on the Maillard reaction is expressed in terms of a change in total and reactive lysine content. As the Maillard reaction can decrease total lysine content due to its conversion into advanced MRP, the calculated difference between total and reactive lysine content can give an underestimation of the effect of processing on the Maillard reaction. In addition, small changes in total and reactive lysine contents should be interpreted with care, as the CV in amino acid analysis can be as high as 3% for total lysine.[123] For reactive lysine, extra steps in the analysis can cause a higher CV compared with total lysine.

Extrusion. Extrusion cooking is a high-temperature, short-time treatment to improve the digestibility of raw ingredients and allows expansion, dehydration and shaping of the kibbles. The extruder consists of a feeder, pre-conditioner, extruder barrel, die and a knife assembly.[124] The feeder controls the feed rate or throughput of the raw ingredients into the pre-conditioner. The main function of the pre-conditioner is to mix the ingredients with water and steam and to pre-cook the ingredients. The extruder barrel is a fixed metal barrel that contains one or two screws to transport the (pre-cooked) ingredient mix from the inlet zone to the die. The temperature inside the barrel is increased, resulting in cooking of the mix. Pet foods are generally extruded using temperatures between 80 and 200°C for 10 to 270 s, with moisture levels of 10 to 25%.[125] At the end of the extruder barrel, a die is installed rapidly when the extrudate exits the die and encounters ambient pressure and temperature, which causes expansion and creates the characteristic texture of dry extruded pet food.[124] In addition, the die and a knife assembly behind the die are responsible for the shape of the product. The kibbles are then dried to reduce the moisture content to less than 6 – 9%. Dryers usually consist of a heating zone, where the kibbles are heated to about 80 – 100°C. The product is then moved into the drying zone with temperatures of about 120 – 150°C. Finally, the product is cooled to 80 – 100°C. This type of drying takes about 15 min for drying and 7 min for cooling.[125] Finally the pet food is coated with fat and/or a palatability enhancer and subsequently packaged. The extrusion process is controlled by several process parameters. Raw materials and screw configuration as well as die size are set before extrusion. Process parameters such as moisture content, screw speed and barrel temperature can be adjusted and continuously

monitored during extrusion. Variables such as retention time, product temperature, pressure and mechanical energy change as a result of changing one or more of the process parameters.[132] The effects of the process parameters, i.e. temperature, moisture and mechanical shear, on pet foods and pet food ingredients in relation to total and reactive lysine content are summarised in Table 2.2. It is commonly accepted that temperature is the most important process parameter for the Maillard reaction during extrusion.[4,133-135] In general, the rate of Maillard reaction increases with temperature and time, resulting in a decrease in reactive lysine content and an increase in the formation of MRP as indicated in model systems.[50] Several studies have examined the effect of extrusion temperatures on total and reactive lysine content in food recipes and in various single ingredients. Tran[116] reported that extrusion at 120°C of an experimental dog food did not affect the difference between total and OMIU-reactive lysine content, which remained at 25.0%, but resulted in a 12.1 and 12.2% reduction, respectively, of total and OMIU-reactive lysine content compared with the unextruded food mixture. Furthermore, this study reported that extrusion can have contradictory effects on total and OMIU-reactive lysine between ingredients used in this food mixture. Extrusion at 120°C of chicken meat and fish meal resulted in an OMIU-reactive lysine loss of 17.9 and 6.3% whereas extrusion had little effect on OMIU-reactive lysine content of poultry meal. Furthermore, total lysine contents in chicken meat decreased by 18.8% after extrusion, 3.0% for poultry meal and slightly increased for fish meal (0.9%). For ingredients of vegetable origin, total lysine contents were low overall and decreased after extrusion for wheat (2.8%) and dehulled rice (8.6%), whereas total lysine content increased for barley (4.8%) and maize (9.5%). OMIU-reactive lysine contents were decreased after extrusion for barley (28.6%) and wheat (3.6%), whereas increased contents were reported for dehulled rice (20.7%) and maize (9.5%). As the vegetable ingredients studied were low in lysine content, the contribution of a possible error of the assay to a relative change in lysine content would have been larger than that for the animal-derived ingredients that contained considerably more lysine. The data of Tran,[116] however, do not show the effect of lower or higher extrusion temperature on total and reactive lysine content in foods or its ingredients. Extrusion of soyabean meal or peas had no effect on total lysine content and little effect on FDNB-reactive lysine content when extruded at 90 or 115°C and 105 or 125°C, respectively.[30] Extrusion at 140°C, however, decreased FDNB-reactive lysine content by 11.4% for soyabean meal and 10.2% for peas. Extrusion of two rice flour varieties at 120, 130 or 150°C had little effect on total lysine contents.[127] Extrusion of a glutinous rice-based snack with 20% protein at 150°C had no influence on the OMIU-reactive lysine content; however, it reduced OMIU-reactive lysine content by 9.2% in a

Table 2.2 Overview of extrusion parameters in relation to total and reactive lysine content of several foods and ingredients.

Food/ingredient		Lysine (g/kg as is)		RL/TL	Method	Additional settings	Reference
		Total*	Reactive†				
Temperature (°C)							
Dog food	UP	8.3	5.9	0.71	OMIU	Co-rotating twin screw extruder;	Lankhorst
	110	8.4	7.1	0.85		length/diameter ratio 25; Screw speed	*et al.*[126]
	130	**8.5**	8.5	1.01		200 rpm; feed rate 14.1 kg/h; 2 die	
	150	**8.1**	8.0	0.98		orifices 0.8 cm.	
Dog food	UP	9.9	7.4	0.75	OMIU	Co-rotating twin screw extruder;	Tran[116]
	120	8.7	6.5	0.75		length/diameter ratio 25; screw speed	
Fish meal	UP	56.3	55.6	0.99		150 rpm; 2 die orifice diameters 0.8;	
	120	56.8	52.1	0.92		moisture 300 g/kg.	
Poultry meal	UP	36.9	30.6	0.83			
	120	35.8	31.9	0.89			
Chicken meat	UP	26.6	20.7	0.78			
	120	21.6	17.0	0.77			
Barley	UP	4.2	3.5	0.83			
	120	4.4	2.5	0.57			
Wheat	UP	3.6	2.8	0.78			
	120	3.5	2.7	0.77			
Dehulled rice	UP	3.5	2.9	0.83			
	120	3.2	3.5	1.09			
Maize	UP	2.8	2.1	0.75			
	120	2.9	2.3	0.79			
Soybean meal	UP	30.9	30.8	1.00	FDNB	Experimental single screw extruder;	Hendriks
	90	31.0	29.8	0.96		die orifice 0.8 cm; screw speed 80	*et al.*[30]
	115	31.7	29.8	0.94		rev/min; feed rate 200 g/min.	
	140	30.0	27.3	0.91			
Pea	UP	15.1	14.7	0.97	FDNB	Experimental twin screw extruder; die	
	105	15.1	14.7	0.97		surface 1.245 cm^2; screw speed 48	
	125	15.4	14.6	0.95		rev/min; feed rate 500 g/min.	
	140	15.0	13.2	0.88			
Rice flour	UP	2.6	-	-	-	Twin screw extruder; die orifice 0.4	Eggum
variety 1	120	2.7	-	-		cm; barrel length 46 cm; barrel	*et al.*[127]
	135	2.7	-	-		diameter 3.77 cm; screw diameter	
	150	2.4	-	-		3.74 cm; moisture content 15%;	
						screw speed 140 rpm; barrel pressure	
						45 bars.	
Rice flour	UP	3.2	-	-			
variety 2	120	3.0	-	-			
	135	2.7	-	-			
	150	2.9	-	-			
Glutinous rice-	UP	-	8.2	-	OMIU	Co-rotating twin screw extruder;	Chaiyakul
based snack	150	-	8.3	-		screw length 70 cm; feed rate 12	*et al.*[128]
protein 20%	180	-	7.6	-		kg/h; die orifice 0.3 cm; screw speed	
	UP	-	12.0	-		400 rpm.	
Glutinous rice-	150	-	10.9	-			
based snack	180	-	10.0	-			
protein 30%							
Cowpea	UP	-		-	FDNB	Pilot plant extruder; die orifice 0.635	Pham &
	93	-		-		cm; barrel length 115 cm; internal	Delrosario[129]
	112	-	↓‡	-		diameter 5.715 cm; compression type	
	132	-		-		screw.	
Mung bean	UP	-		-			
	93	-	↓	-			

Product	Value	RL†	TL*	Ratio	Method	Extruder type and conditions	Reference
	112	-		-			
	132	-		-			
Moisture content (%)							
Dog food	20	8.2	8.0	0.97	OMIU	Co-rotating twin screw extruder; throughput 14.1 kg/h; die diameter 8 mm; temperature 110°C.	Lankhorst et al.[126]
	30	8.4	7.7	0.92			
Glutinous rice-based snack protein 20%	20	-	7.7	-	OMIU	Co-rotating twin screw extruder; screw length 70 cm; feed rate 12 kg/h; die orifice 0.3 cm; screw speed 400 rpm.	Chaiyakul et al.[128]
	25	-	8.0	-			
	30	-	7.8	-			
Glutinous rice-based snack protein 30%	20	-	9.8	-			
	25	-	10.3	-			
	30	-	11.1	-			
Soybean meal	25	32.3	30.3	0.94	FDNB	Experimental single screw extruder; die orifice 0.8 cm; screw speed 80 rev/min; feed rate 200 g/min.	Hendriks et al.[30]
	27	30.3	28.9	0.95			
	30	30.7	28.6	0.93			
	40	30.5	28.0	0.92			
Pea	15	16.1	14.2	0.88		Experimental twin screw extruder; die surface 1.245 cm^2; screw speed 48 rev/min; feed rate 500 g/min.	
	30	16.3	15.6	0.96			
Cowpea	30	-		-	FDNB	Pilot plant extruder; die orifice 0.635 cm; barrel length 115 cm; internal diameter 5.715 cm; compression type screw; measured at a given temperature.	Pham & Delrosario[129]
	37.5	-		-			
	45	-	↓	-			
Mung bean	30	-		-			
	37.5	-	↓	-			
	45	-		-			
Screw speed (rpm)							
Cowpea	100	-		-	FDNB	Pilot plant extruder; die orifice 0.635 cm; barrel length 115 cm; internal diameter 5.715 cm; compression type screw.	Pham & Delrosario[129]
	140	-	↑	-			
	200	-		-			
Mung bean	100	-		-			
	140	-	↑	-			
	200	-		-			
Soy-sweet potato mixture	80	-		-	OPA	Experimental single screw extruder; length/diameter ratio 16:1; compression ratio 1:1.15; moisture 18%; temperature 100°C.	Iwe et al.[130]
	92	-		-			
	110	-	↑	-			
	127	-		-			
	140	-		-			
Die size (mm)							
Dog food	4	7.9	7.2	0.90	OMIU	Single screw extruder; length/diameter ratio 8; temperature 130°C; moisture 300 g/kg.	Tran et al.[131]
	8	8.3	7.2	0.86			
Soy-sweet potato mixture	6	-			OPA	Experimental single screw extruder; length/diameter ratio 16:1; compression ratio 1:1.15; moisture 18%; temperature 100°C.	Iwe et al.[130]
	7	-					
	8	-	↓				
	9	-					
	10	-					

RL, reactive lysine; TL, total lysine; UP, unprocessed; OMIU, *O*-methylisourea; FDNB, fluorodinitrobenzene; OPA, *ortho*-phthaldialdehyde.

* Total lysine was determined with conventional amino acid analysis.

† Reactive lysine was determined according to the given method.

‡ Data of reactive lysine content were not presented by authors. Arrows indicate a decrease (↓) and an increase (↑) in reactive lysine content with increasing parameter.

snack with 30% protein.[128] Increasing the extrusion temperature to 180°C reduced OMIU reactive lysine content by 8.4% for the 20% protein snack, and by 16.7% for the 30% protein snack. Although absolute values were not reported, extrusion of cowpeas and mung beans at temperatures from 93 up to 132°C decreased FDNB-reactive lysine content by 25.4 and 21.3%, respectively.[129] Lankhorst et al.[126] reported that extrusion of an experimental dog food had no effect on total lysine content, but OMIU-reactive lysine content was increased by 20.3% after extrusion at 110°C and 35.6% after extrusion at 150°C. At these temperatures, the difference between total and OMIU-reactive lysine content decreased from 29.0% (unprocessed) to 15.0 and 2.0%, respectively. The authors hypothesised that the bound ε-amino group of lysine regained its reactivity as a result of the extrusion process. The underlying mechanism is still unknown; however, based on these data, increasing extrusion temperatures generally reduced reactive lysine contents of single ingredients with little or no effect on total lysine content. The number of studies evaluating the change in total and reactive lysine in pet foods at varying extrusion temperatures is limited, and contradictory effects of temperature on reactive lysine were seen. Additional studies are, therefore, required to assess the importance of extrusion temperature to the formation of MRP in pet foods.

The moisture content of the ingredient or mixture can also be adjusted during extrusion. The moisture content is either the original moisture level of the ingredient or mix itself, or from added water or steam during extrusion. Moisture is necessary for the Maillard reaction to take place,[50,136] but moisture also seems to inhibit the browning reaction if present in high concentrations in model systems. Increasing moisture content from 20 to 30% during extrusion of an experimental dog food only slightly affected total lysine content (+2.4%) and OMIU-reactive lysine content (-3.9%) (Table 2.2).[126] Moisture content had little effect on OMIU-reactive lysine content for a 20% protein glutinous rice-based snack but for the 30% protein snack the OMIU-reactive lysine content was increased by 5.1 and 13.3% when moisture content was increased from 20 to 25 and 30%, respectively.[128] Increasing moisture content from 25 to 40% during the extrusion of soyabean meal decreased total and FDNB-reactive lysine content by 5.0 and 6.5%, respectively.[30] Also for cowpeas and mung beans, FDNB-reactive lysine content has been reported to be decreased with increasing moisture content from 30 to 45% during extrusion.[129] For peas, however, increasing moisture content from 15 to 30% during extrusion slightly increased total lysine content (1.2%) and increased FDNB-reactive lysine content by 9.9%.[30] A high moisture content may induce a protective effect by increased or prolonged water evaporation, which keeps product temperature relatively low. In addition, water could reduce friction and, therefore, protects the ingredients from shear during processing.

Mechanical shear force may change during extrusion.[132] The amount of shear force developed during extrusion cooking depends on several process parameters such as screw configuration, compression ratio, screw speed and die size. Literature on the effect of these process parameters in relation to the Maillard reaction is scarce. There are only a few studies that have evaluated the effect of screw speed and die size in relation to reactive lysine content in ingredients or foods, and for most studies no quantitative data are reported (Table 2.2). There is no information available on the effect of screw speed on total and reactive lysine contents during the extrusion of pet foods. Increasing screw speed during the extrusion of cowpeas and mung beans from 100 to 200 rpm resulted in reduced loss of FDNB-reactive lysine content.[129] Similarly, OPA-reactive lysine in a soya-sweet potato mixture resulted in less loss with increasing screw speed from 80 to 140 rpm during extrusion.[130] At constant extrusion temperatures, a higher screw speed increases shear but reduces residence time and hence limits the exposure to heat treatment.[137] Based on these data, shorter residence time and thus less exposure to heat treatment seem to be more important than the higher shear forces that occur when screw speed is increased. Shear and pressure are also affected by the die at the end of the extruder, which restricts the product flow. Increasing die size from 4 to 8 mm in a control sample dried at 40°C increased total lysine content in an experimental dog food by 5.1% with no effect on OMIU-reactive lysine.[131] Increasing die size from 4 to 9 mm in the same experimental dog food at four different drying temperatures (80, 120, 160 and 200°C) on average increased OMIU-reactive lysine content with no effect on total lysine. For extrusion of a soya-sweet potato mixture, an increase in die size from 6 to 10 mm at a temperature of 100°C reduced OPA-reactive lysine content.[130]

Drying and storage. The heat applied during the drying process can also affect the difference between total and reactive lysine. Drying of a dog food at increasing temperatures of 80, 120, 160 and 200°C to an end moisture content of 6 – 9% reduced total lysine content by 15.7% as well as OMIU-reactive lysine content by 15.5%.[131]

Besides drying, addition of palatability enhancers can influence differences between total and reactive lysine in the final product. Flavour and texture are important properties that contribute to the overall palatability of the food, which determines how likely animals are to eat the food. Texture is created during processing; however, dry pet foods often have only moderate inherent flavour.[138] Therefore, palatability enhancers are regularly used during the production of dry pet foods. As well as palatability enhancers such as acidified yeast, hydrochloric acid, phosphoric acid, sugars and spices, most dry pet foods are coated with digests.[125] Digests are hydrolysed proteins from meats, offals and yeasts, produced using heat and enzymes. The digestion process releases amino acids such as lysine and dipeptides that enhance palatability. However, during this process, the

hydrolysed proteins are available to participate in the Maillard reaction. The resulting MRP increase the palatability of the digest,[138] but simultaneously result in the formation of early and advanced MRP. The digest is applied as liquid sprayed on the dry food or applied as a powder. It is possible that the production process of digest adds to the differences in total and reactive lysine and to the amount of MRP in dry pet foods.

Little information has been reported on storage conditions and duration in relation to the Maillard reaction in pet foods. Chiang[107] recorded an increase in furosine from 0.91 mg/g in a control dry dog food to 1.52 mg/g after storage of 12 weeks at 22.2°C. Storing the same control dry dog food for 12 weeks at 37.8°C increased furosine levels to 3.19 mg/g. These data are an indication that early Maillard products can be present in pet foods and that additional fructoselysine is generated during storage. No studies have been conducted to determine the stability of reactive lysine in pet foods during varying storage conditions. The data by Chiang[107] indicate that as the furosine concentration increases during storage, the concentration of reactive lysine decreases.

Retorting. Retorting is a processing method in which food contents are sealed in airtight cans, containers, or flexible pouches and are subsequently heat-treated. Process temperatures sterilise the product, resulting in preservation of these high-moisture foods for extended periods of time.[125] Moist pet foods contain fresh or frozen meat and other animal tissues, which are ground and homogenised. Additional ingredients such as mash grains or other ground starch sources, vitamin and mineral premixes, and water are added and mixed to create a complete food recipe. The mixture is heated up to 85°C to start starch gelatinisation and protein denaturation.[125] The hot mixture is transported to machines that fill and seal the packages. A vacuum is created by either the hot product itself, or by injecting steam over the product just before sealing. The steam replaces air in the container, creating under-pressure as the steam condenses during cooling. After sealing, the packages are sterilised in a retort in a continuous or batch system. Retorting is a temperature/time-dependent process, using an F_o-value as a unit to summarise the lethality of the heat employed on the product. The F_o-value represents the time equivalent in minutes of a heating process to destroy micro-organisms at the reference temperature of 121.1°C.[139] In general, the process time should be at least 3 min at 121.1°C (F_o-value of 3) to kill pathogenic bacteria.[125] However, higher values (F_o-value >10) are employed in practical pet food production in order to destroy spores.[139]

As described above, the difference between total and reactive lysine can be considerable in moist canned foods, with values between 39.0 and 61.8% (Figure 2.5). This indicates that processing conditions applied during retorting of cans favour the Maillard reaction, but effects of processing conditions on total and reactive lysine content have been the subject of only a few experiments. Retorting a standard moist cat food recipe containing

a low content of carbohydrates (maximum 6.7%) at lethality values (F-values) of 5.3, 8.6, 17.2 and 24.3 (temperature set at 121°C, time periods between 80 to 120 min) had no effect on the total or OMIU-reactive lysine content.[139] In the unprocessed food, the difference between total and reactive lysine was 12.0%. The difference between total and reactive lysine content in the pet food ingredients used in this particular food does not explain the high differences between total and reactive lysine reported in some moist canned foods. True ileal total lysine digestibility as measured using growing rats consistently decreased with increasing F-values from 84.2% for the untreated food to 77.4% for the food with the highest lethality value (24.3),[139] indicating that part of the lysine was rendered indigestible. However, total as well as OMIU- and FDNB-reactive lysine did not change due to processing. It is therefore unclear whether the Maillard reaction was the cause of the reduced ileal total lysine digestibility. Results of other experiments indicate a decrease in reactive lysine during retorting. Retorting of tuna at 115°C for 55 and 90 min decreased FDNB-reactive lysine contents by 5.0 and 15.7%, respectively.[140] Autoclaving a diet used for salmonid fish, including fish meal, barley protein concentrate and wheat flour as the main ingredients, at temperatures of 100, 110, 120 and 130°C reduced total lysine by 5.8% and OMIU-reactive lysine by 18.3%.[141] As FDNB- and OMIU-reactive lysine demonstrate a good correlation,[31] results of the effect of retorting on reactive lysine are inconsistent. It is unclear what the contribution is of the ingredient used or the retorting process on the high differences between total and reactive lysine contents reported in moist canned cat foods (Figure 2.5). The effects of retorting temperature and its possible interaction with time on total and reactive lysine content have not been studied to date.

Pelleting. Pelleting dry pet foods is a processing technique that is less severe in terms of temperatures applied compared with extrusion (60 to 90°C vs. 80 to 200°C). The pelleting process includes mash conditioning, pelleting and subsequent drying or cooling. For mash conditioning, heat, water and pressure with temperatures between 60 and 90°C[142,143] are used to induce a wide range of physical and chemical changes including softening of the food, denaturation of proteins and gelatinisation of starch.[142] After conditioning, the mash is pressed through a die in a pellet mill. The die design is either a ring, which is revolving around fixed rollers, or flat, which is static and horizontal rollers rotate around a vertical axis. The rollers continuously press layers of mash inside the die hole, by which the pellet is built up. The extent of compression is dependent on the height of the layer and the gap distance between the die and the roller. Changing these conditions changes pellet quality. Die holes differ in their length:diameter ratio, influencing the amount of shear that the feed mash receives. After leaving the die, pellets are usually cooled by air flow.[142]

Despite the lower processing temperatures, the Maillard reaction occurs during pre-conditioning and/or pelleting, resulting in loss of reactive lysine and formation of MRP. Tran et al.[62] reported a difference of 20.5% between total and reactive lysine in pelleted commercial dog pellets. There are no reports from experiments in which preconditioning or pelleting conditions were evaluated in terms of influencing total and reactive lysine levels in pelleted pet foods. For pig feed, few studies are available but these only compared unprocessed feed mash with pellets under one fixed pelleting condition. Pelleting pig feed at 80°C did not affect total lysine content but slightly increased FDNB-reactive lysine content by up to 1.2%.[144] Pelleting a complex nursery pig diet (steam-conditioned at 60°C for 45 s through a 5 × 38 mm die) had no effect on lysine bioavailability as measured using standard-curve bioassay with 8-d-old chicks.[143] Pelleting a conventional pre-starter diet for suckling piglets (steam-conditioned at 40°C for 30 s; pelleted at 60 to 65°C through a 1.7 × 40 mm die) resulted in a 200% increase of furosine, a marker for the presence of the Amadori compound of lysine (Figure 2.1), from 11.71 to 35.18 mg/kg.[145] Other MRP also increased; HMF increased by 32.6% from 12.56 to 16.66 mg/kg, and furfural increased from non-detectable to 0.034 mg/kg.

Conclusions

Data indicate that significant proportions of the lysine in pet foods can be modified and may be unavailable for metabolism by dogs and cats. OMIU-reactive lysine content was below minimal requirement for growing dogs from 4- to 14-weeks in two out of fourteen analysed dry foods. As such, foods for growing dogs require more careful considerations in terms of lysine supply, as the requirement for growing animals is higher than that of adult animals. As batch-to-batch variation is unknown, the results may be different between batches of the same food due to variation in ingredients or processing. More knowledge of the bioavailability of lysine and other amino acids involved in the Maillard reaction in dog and cat foods is required. Advanced MRP have varying biological activities, and in dogs data indicate higher AGE in plasma from dogs suffering from canine diabetes mellitus compared with control animals. In addition, elevated levels of AGE in tissue proteins in dogs were observed for a number of diseases. It is unknown to what extent advanced MRP are present in pet foods. Most of the dietary ingested MRP are rapidly excreted via the kidneys although no data are available for cats and dogs. Whether or not the presence of dietary MRP in pet foods may result in the development of diseases such as diabetes and impaired renal function in pet animals requires further study. In the regulation of the levels of total and reactive lysine and possible advanced MRP in pet foods, control of ingredient processing and choice of ingredients may be a useful strategy as differences between total and reactive lysine are observed in several

ingredients commonly used in pet foods. Effects of processing conditions on the difference in total and reactive lysine contents in pet foods are inconsistent and do not always correspond to model systems. Processing temperature is the most important factor followed by moisture level. Moist pet foods appear to have a higher difference between total and reactive lysine content compared with dry foods. Further study into choice of ingredients, extrusion, pelleting and retorting of pet food on the progression of the Maillard reaction is recommended, as are studies on the bioavailability of dietary MRP and their possible relationship with pet health.

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Chapter 3

Reactive lysine content in commercially available pet foods

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Abstract

The Maillard reaction can occur during processing of pet foods. During this reaction, the ε-amino group of lysine reacts with reducing sugars to become unavailable for metabolism. The aim of the present study was to determine the reactive lysine (RL; the remaining available lysine) to total lysine (TL) ratio of commercial pet foods and to evaluate whether RL levels meet minimal lysine requirements (MLR). Sixty-seven extruded, canned and pelleted commercially available dog and cat foods for growth and maintenance were analysed for proximate nutrient composition, TL and RL. RL was expressed on a metabolisable energy basis and compared with the MLR for maintenance and growth. In dog foods, average RL/TL ratios were 0.87 (SE 0.02) for extruded, 0.97 (SE 0·02) for canned and 0.85 (SE 0.01) for pelleted foods, with the lowest ratio of 0·77 in an extruded diet for growing dogs. In extruded and canned cat foods, the average ratio was 0·91 (SE 0.02) and 0·90 (SE 0.03), respectively, with the lowest ratio being 0.67 in an extruded diet for growing cats. Variation in the RL/TL ratio between and within processing type indicate that ingredients rather than processing might be the key factor influencing RL content in pet foods. Eight dry foods for growing dogs had RL contents between 96 and 138% of MLR, indicating that RL has to be between 62 and 104% digestible to meet the MLR. Considering the variability in RL digestibility, these foods could be at risk of not meeting the MLR for growing dogs. Ingredients and pet foods should be characterised with respect to the RL content and digestibility, to avoid limitations in the lysine supply to growing dogs.

Key words: Maillard reaction, Minimal lysine requirement, Dogs, Cats, Nutritive value

Introduction

Lysine is an essential amino acid for cats and dogs and sufficient amounts of bioavailable lysine need to be included in pet foods to meet the minimal lysine requirements (MLR) of these animals throughout their lives. Commercial pet foods vary greatly in format, ingredient and nutrient composition and type of processing. Thermal treatment is widely used in pet food production to improve digestibility of nutrients and to increase shelf life and food safety. Extruded kibbles are processed at temperatures of 80 – 200°C for 10 – 270 sec under high pressure, while canned foods are heated until temperatures of 121°C for > 10 min are reached at the centre of the can to sterilise the product[1] and pelleted pet foods are relatively mildly heat-treated using temperatures of 60 – 90°C for 30 – 45 s.[2] Increased temperatures and residence times during processing are known to induce the Maillard reaction where a reducing sugar binds to a free reactive amino group of an amino acid, blocking the reactive site of the amino acid. When the reactive ε-amino group of lysine is the substrate of the reaction, modified lysine derivatives are formed, that may be partially absorbed but cannot be fully utilised by the animal, thereby, reducing the nutritive value of the food.[3-5] These derivatives can revert back to lysine under standard amino acid analyses conditions and, therefore, are assumed to be lysine molecules with a reactive ε-amino group. Direct determination of lysine with a free ε-amino group (reactive lysine; RL) can be performed using the guanidination method, which uses *O*-methylisourea as a reagent to bind specifically. Recent studies using the guanidination method have reported that the difference between total lysine (TL) and RL of commercially produced canned and dry dog and cat foods can be considerable, with RL/TL ratio values reported as low as 0.38.[6-8] The residual RL appears not to be 100% digestible either: standardised ileal *O*-methylisourea-RL digestibility values in dogs fed five commercial dry extruded dog foods were 79.5 to 93.7% with a mean of 88.2%.[9] Using the rat as a model animal, standardised ileal *O*-methylisourea-RL digestibility of canned cat foods ranged from 79.9 to 97.1% with a mean of 88.1%, whereas for the dry cat foods values ranged from 89.9 to 97.7% with a mean of 94.8%[6]. These results indicate that RL content in commercial pet foods can be reduced, possibly due to the Maillard reaction, and that RL content does not equal RL bioavailability.

Previous studies (e.g. Rutherfurd et al.[6] and Williams et al.[7]) focused mainly on the difference between RL and TL contents of processed commercial pet foods. As these foods were not evaluated for metabolisable energy (ME) content, RL contents were not related to MLR in dog and cat foods. The objective of the present study was to determine the RL-to-TL ratio of commercial extruded, canned and pelleted pet foods to evaluate whether RL levels meet MLR for growing and adult dogs and cats as set out by the NRC.[10] Additional extruded junior foods were included to provide insight into the variability of RL

content in the same brand/manufacturer junior foods with similar recipes, but produced in different countries over the world.

Experimental methods

Diets and sample preparation

A dataset was created containing 153 extruded, canned and pelleted pet foods commercially available in The Netherlands. Pet food types were categorised according to species (dogs or cats) and life stage (junior or adult). From each category in the dataset, five pet foods (single batch) were randomly selected. In addition, eleven extruded dry foods for growing dogs and six extruded dry foods for growing cats similar in recipe from a single manufacturer were obtained that were manufactured in Australia, Brazil, China, Germany, Mexico, Thailand, UK and the USA. This resulted in five commercial pellets, sixteen extruded and five canned foods for growing dogs; five commercial pellets, five extruded and five canned foods for adult dogs; eleven commercial extruded and five canned foods for growing cats and five commercial extruded and five canned foods for adult cats. According to the information on the labels of the junior foods, the food could be fed from the age of 4 weeks onwards. The moist canned foods were freeze-dried, and all the foods were ground to pass a 1 mm sieve in a Retch Mill (ZM100, Retch BV). All samples were stored in air-tight plastic containers at 4°C prior to analyses.

Chemical analyses

DM and crude ash were determined by drying to a constant weight at 103°C[11] and combustion at 550°C,[12] respectively. Crude protein (N × 6.25) was determined using the DUMAS method and crude fat was determined gravimetrically after hydrolysis with HCl and extraction with light petroleum (boiling point 40 – 60°C).[13] Crude fibre was determined gravimetrically as the remaining insoluble organic fraction after acid and alkaline digestion.[14] All analyses were performed in duplicate. ME content of the foods was calculated using predictive equations for ME[10]: digestible energy (DE, kJ) – (4.35 × g crude protein) for dogs and DE (kJ) – (3.22 × g crude protein) for cats; DE (kJ) = gross energy (GE, kJ) × energy digestibility (%) / 100; energy digestibility (%) = 91.2 – (1.43 × % crude fibre in DM) for dogs and 87.9 – (0.88 × % crude fibre in DM) for cats; GE (kJ) = (23.85 × g crude protein) + (39.33 × g crude fat) + (17.15 × (g nitrogen-free extract + g crude fibre)); nitrogen-free extract in g/kg DM was calculated as 1000 – crude fat – crude protein – crude ash – crude fibre. Amino acids including TL were determined according to Hendriks et al.[15] with *O*-methylisourea-RL determined according to Moughan & Rutherfurd[16]. RL was expressed on an ME basis and compared with the nutritional requirements for growth and maintenance of dogs and cats.[10]

Statistical analyses

The effect of food type (i.e. extruded, pelleted and canned for dog foods, extruded and canned for cat foods) on RL/TL was tested for significance using ANOVA by Proc GLM of SAS 9.2 for Windows (SAS Institute, Cary, NC, USA). In case $P \leq 0.05$ for significant effects, pairwise comparisons were made using post hoc analysis and corresponding P values were reported. Results are presented as the means with their standard errors.

Results

Proximate analysis is shown in Table 3.1 and was on average for DM, crude protein, crude fat, crude fibre, crude ash, nitrogen free extract (g/kg DM) and ME (MJ/kg), respectively: in extruded dog foods 921.97 (1.77), 300.40 (10.92), 153.27 (7.81), 19.67 (0.82), 74.05 (3.17), 452.61 (17.40) and 16.15 (0.16); in canned dog foods 217.77 (12.34), 497.61 (38.01), 22.96 (17.01), 13.01 (1.38), 102.84 (8.98), 172.22 (49.35) and 4.17 (0.26); in pelleted dog foods 907.40 (4.01), 260.79 (6.48), 123.45 (13.09), 23.55 (1.79), 78.92 (2.22), 513.29 (16.84) and 15.14 (0.28); in extruded cat foods 939.23 (3.38), 374.85 (11.33), 170.61 (10.08), 16.68 (1.38), 80.31 (3.86), 357.55 (18.78) and 16.79 (0.26); and in canned cat foods 210.98 (13.36), 660.09 (26.96), 243.00 (11.61), 9.71 (2.45), 102.12 (8.94), 34.70 (20.82) and 4.34 (0.30). In dog foods, average RL/TL ratios (Table 3.1) were 0.87 (0.02) (range 0.77 − 0.99) for extruded, 0.97 (0.02) (range 0.83 − 1.10) for canned and 0.85 (0.01) (range 0.78 − 0.94) for pelleted foods, with the lowest ratio of 0.77 in an extruded diet for growing dogs. Canned foods differed significantly from extruded ($P = 0.004$) and pelleted ($P = 0.0008$) foods. In extruded and canned cat foods, the average ratio was 0·91 (0.02) (range 0.67 − 1.03) and 0.90 (0.03) (range 0.75 − 1.03), respectively ($P = 0.652$), with the lowest ratio being 0.67 in an extruded diet for growing cats. RL/TL ratio in similar foods from countries around the world produced by the same manufacturer ranged from 0.77 to 0.94 in junior dog foods and 0.82 to 0.90 in junior cat foods. RL contents (g/kg DM) were: 14.5 (3.0), 24.2 (3.2) and 11.6 (0.8) for extruded, canned and pelleted adult dog foods, respectively; 13.7 (0.9), 28.4 (5.0) and 12.7 (0.9) for extruded, canned and pelleted junior dog foods, respectively; 16.4 (1.6) and 36.5 (2.5) for extruded and canned adult cat foods, respectively, and 17.4 (1.4) and 44.3 (3.7) for extruded and canned junior cat foods, respectively. The RL contents of all dog foods but one and all cat foods were higher than MLR for growing and adult cats and dogs as stated by the NRC,[10] ranging from 96 up to 581% of the MLR (Figure 3.1).

Discussion

The variation in RL/TL ratio observed in the present study (Table 3.1) between, as well as within the diet types (i.e. extruded, canned, pelleted) may be due to: 1) the use of different processing conditions, 2) different durations of drying and storage and 3) the use

Table 3.1 Proximate composition, metabolisable energy (ME), total lysine (TL) and *O*-methylisourea-reactive lysine (RL) content, and RL/TL ratio of sixty-seven commercial pet foods categorized in animal species, processing type and life stage (mean ± SEM, g/kg dry matter unless defined differently).[a]

Component		Dog Extruded Junior Mean	SEM	Adult Mean	SEM	Canned Junior Mean	SEM	Adult Mean	SEM	Pelleted Junior Mean	SEM	Adult Mean	SEM	Cat Extruded Junior Mean	SEM	Adult Mean	SEM	Canned Junior Mean	SEM	Adult Mean	SEM
Dry matter[b]		921.9	2.0	922.3	4.2	231.3	18.0	204.2	16.4	909.3	5.6	905.5	6.3	939.5	4.2	938.6	6.4	240.7	18.4	181.2	4.8
	Range	907.9-937.9		910.1-932.4		186.9-296.2		169.0-259.1		899.7-931.1		884.8-922.4		919.4-972.2		914.9-949.0		197.7-296.3		167.6-195.7	
Crude protein		301.5	8.7	297.0	39.8	512.6	57.4	482.6	55.6	260.3	9.6	261.3	9.9	393.6	12.3	333.6	9.2	700.8	30.2	619.4	39.1
	Range	204.9-354.1		210.8-444.9		364.0-710.3		294.0-591.2		229.2-280.0		246.2-300.3		351.5-494.4		310.5-365.9		590.1-757.5		489.3-711.6	
Crude fat		160.0	8.6	131.6	15.7	224.0	26.7	221.9	24.2	154.7	12.1	92.2	11.6	166.8	11.8	178.9	20.8	246.6	15.5	239.4	19.0
	Range	106.7-209.6		98.1-189.5		157.7-308.6		164.9-275.9		116.3-188.2		63.4-128.1		118.9-240.3		115.4-233.1		216.6-300.0		198.3-302.7	
Crude ash		73.8	3.8	74.8	6.2	104.6	9.0	101.0	16.7	78.1	3.0	79.8	3.6	82.6	4.5	75.4	7.6	90.3	14.8	113.9	8.4
	Range	52.4-118.1		58.4-96.4		73.6-126.6		43.3-133.0		69.3-88.3		72.6-92.5		59.3-109.8		55.3-101.3		43.9-124.0		90.8-139.1	
Crude fibre		18.1	0.6	24.6	1.3	11.1	0.9	14.9	2.5	21.6	2.7	25.5	2.3	15.7	1.2	18.9	3.7	9.9	3.9	9.5	3.4
	Range	15.3-23.5		22.2-29.4		8.2-13.5		11.3-24.5		12.2-27.7		21.1-32.9		7.6-21.6		11.7-28.7		1.5-24.1		3.9-21.9	
Nitrogen-free extract[c]		446.6	14.8	472.0	60.0	163.1	55.9	181.3	88.3	485.3	21.0	541.3	21.0	341.3	22.5	393.2	23.4	19.2	19.2	50.2	38.3
	Range	341.5-595.3		239.9-576.8		0.0-341.0		0.0-481.5		443.3-557.5		467.2-588.8		167.9-426.1		320.2-466.1		0.0-95.8		0.0-199.0	
ME[d]		16.3	0.2	15.6	0.3	4.5	0.4	3.9	0.3	15.8	0.3	14.5	0.2	16.8	0.3	16.9	0.6	5.1	0.4	3.6	0.1
	Range	15.0-17.3		14.9-16.4		3.3-5.6		2.9-4.8		15.0-16.5		13.9-15.3		15.5-18.6		15.1-18.3		4.4-6.4		3.3-4.0	
TL		13.7	0.9	14.5	3.0	28.4	5.0	24.2	3.2	12.7	0.9	11.6	0.8	17.4	1.4	16.4	1.6	44.3	3.7	36.5	2.5
	Range	8.1-16.6		9.1-26.0		19.4-44.9		16.3-32.8		10.6-15.7		10.2-14.4		15.5-28		12.5-20.2		36.2-55.1		36.2-55.1	
RL		11.9	0.9	13.2	2.8	27.1	4.5	23.9	3.6	10.9	0.8	9.9	0.8	15.0	1.1	15.9	1.8	40.7	4.9	32.1	2.2
	Range	6.6-12.4		7.1-23.4		18.7-41.5		13.5-32.6		9.1-13.6		8.6-12.9		11.9-23.2		11.7-20.1		30.6-52.9		25.6-36.8	
RL/TL		0.87	0.02	0.90	0.03	0.96	0.02	0.98	0.04	0.86	0.02	0.84	0.01	0.88	0.03	0.97	0.02	0.91	0.05	0.88	0.02
	Range	0.77-0.99		0.78-0.97		0.92-1.03		0.83-1.10		0.78-0.94		0.81-0.89		0.67-0.98		0.91-1.03		0.75-1.03		0.82-0.93	

[a] n = 5, except for extruded junior dog foods (n = 16) and extruded junior cat foods (n = 11).
[b] g/kg
[c] Calculated as: 1000 – crude fat – crude protein – crude ash – crude fibre.
[d] MJ/kg; calculated using predictive equations for ME.[10]

of different ingredients.[17] The low RL/TL ratios in pelleted foods compared with extruded or canned foods is in line with the result of Tran et al.,[8] in which pelleted diets, on average, contained a lower RL/TL ratio (0.80; range 0.72 − 0.93) compared with extruded diets (0.87; range 0.83 − 0.93). These results are unexpected as pelleting is generally carried out at lower temperatures compared with extrusion. Considering extruded and canned pet foods in the present study, the average and minimal RL/TL ratio of extruded dog foods (0.87; range 0.77 − 0.99) was lower compared with canned dog foods (0.97; range 0.83 − 1.10); however, for the cat foods the results were comparable (0.90, range 0.67 − 1.03 for extruded vs. 0.90, range 0.75 − 1.03 for canned cat foods). The results of the present study were higher compared with previous studies. Rutherfurd et al.[6] reported average RL/TL ratios of 0.51 (range 0.38 − 0.61) for canned cat foods and of 0.59 (range 0.51 − 0.80) for dry cat foods. Williams et al.[7] reported average RL/TL ratios of 0.85 (range 0.44 − 1.06) for adult dog foods, and of 0.75 (range 0.47 − 0.98) for foods for growing dogs. Although other factors such as drying and storage can reduce the RL content of extruded diets,[18,19] the unexpected results for the pelleted foods, as well as the discrepancy between results for extruded and canned dog and cat foods, indicate that ingredient choice, and not processing type, can have a major influence on RL/TL ratio.

Ingredients used in recipes for pelleting often include pre-treated ingredients. Carbohydrate sources for example, are often pre-treated as pelleting processing temperatures and residence times are not high enough to fully gelatinise the starch in the raw ingredients during pelleting.[20] The production of meat meal, used in pelleted and extruded foods, includes rendering under high temperatures. Rendered meals generally show a lower protein quality compared with raw animal meals, measured using lysine availability in a chicken growth assay.[21] RL/TL ratio in animal protein sources ranged from 0.64 to 0.99 and in vegetable proteinsources from 0.56 to 1.00.[17] Therefore, it is likely that the loss of RL already starts at the selection of ingredients included in the pet food recipe; however, future studies measuring RL/TL ratio in ingredients and final product testing would need to be carried out to further investigate this hypothesis. MLR reported by NRC[10] relate to highly (95 − 100%) bioavailable lysine. Although most of the dog and cat foods contained RL contents higher than MLR for growing and adult dogs and cats as stated by the NRC (Figure 3.1),[10] actual digestibility and subsequent availability of the RL in practical diets could be lower than the bioavailability assumed by the NRC.[10] Apparent ileal crude protein digestibility has been shown to be highly variable among 141 experimental dog foods, with values ranging from 51.1 up to 90.5% with a mean digestibility of 73.5%.[22] Variability in amino acid digestibility is not fully comparable, but it can be expected to be similar to crude protein digestibility. Therefore, these results
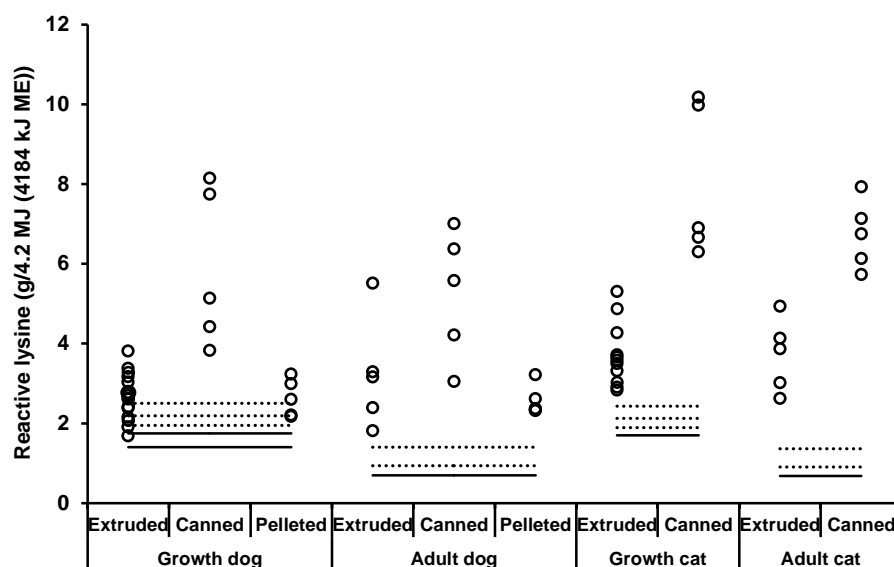
Figure 3.1 Reactive lysine contents of five commercial pellets, sixteen extruded and five canned foods for growing dogs; five commercial pellets, five extruded and five canned foods for adult dogs; eleven commercial extruded and five canned foods for growing cats and five commercial extruded and five canned foods for adult cats. Horizontal solid lines indicate minimum lysine requirements (MLR) for growing dogs between 4 and 14 weeks of age presented by NRC[10] being 1.75 g/4.2 MJ (4184 kJ) ME (lower solid line), for growing dogs from 14 weeks onwards (1.40 g/4.2 MJ (4184 kJ) ME; upper solid line), for dogs at maintenance (0.70 g/4.2 MJ (4184 kJ) ME), for growing cats being 1.70 g/4.2 MJ (4184 kJ) ME and for cats at maintenance being 0.68 g/4.2 MJ (4184 kJ) ME. Dashed lines indicate bioavailability thresholds of 90, 80 and 70% for meeting MLR of growing dogs between 4 and 14 weeks of age and growing cats, and of 75 and 50% for meeting minimal lysine requirements for dogs and cats at maintenance. digestibility in pet foods (dry and moist) ranged between 79.5 and 97.7%,6,9 showing that lysine available to the animal is lower than the RL content.

indicate that lysine digestibility is likely to also vary. As indicated above, standardised ileal RL The reduced lysine availability compared with the RL content might be of particular significance during growth. Growing dogs have a lower gastric pepsin secretion compared with adult dogs, which may result in digestibility values lower than those observed in adult dogs.[23] In the present dataset, eight dry (pelleted and extruded) foods for growing dogs between 4 and 14 weeks of age had RL contents between 96 and 138% of MLR, indicating that RL has to be between 62 and 104% digestible to meet the MLR. Considering the variability in RL digestibility, these foods could be at risk of not meeting the MLR for included two out of fourteen commercial foods for dogs between 4 and 14 weeks of age growing dogs. These results are comparable with the dataset used by Williams et al.,[7] that included two out of fourteen commercial foods for dogs between 4 and 14 weeks of age which had an RL content below MLR, and one food needed an RL

digestibility of more than 90% to meet MLR.[17] Dry foods for growing cats and adult dogs and cats, as well as canned foods contain RL contents that meet MLR if the RL digestibility of these foods is above 55%.

In conclusion, variation in RL/TL ratio is high between and within processing types, and ingredients rather than processing or storage may be a key factor influencing the RL/TL ratio in processed pet foods. Foods for growing dogs that are used as weaning diets could be at risk of not meeting MLR, depending on the digestibility of RL. Ingredients and pet foods should be characterised with respect to the RL content and digestibility, to avoid limitations in the lysine supply to growing dogs.

Acknowledgements

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Chapter 4

Quantitation of Maillard reaction products in commercially available pet foods

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Abstract

During processing of pet food, the Maillard reaction occurs, which reduces the bioavailability of essential amino acids such as lysine and results in the formation of advanced Maillard reaction products (MRPs). The aim of this study was to quantitate MRPs (fructoselysine (FL), carboxymethyllysine (CML), hydroxymethylfurfural (HMF)) and the cross-link lysinoalanine (LAL) in commercial pet foods. Sixty-seven extruded, canned, and pelleted dog and cat foods for growth and maintenance were analysed using UPLC-MS. Canned pet foods contained on average the most FL, CML, and HMF (4534, 37, and 1417 mg/kg dry matter, respectively) followed by pelleted and extruded foods. Average daily intake (mg/kg body weight$^{0.75}$) of HMF is 122 times higher for dogs and 38 times higher for cats than average intake for adult humans. As commercial pet foods are most often the only source of food for dogs and cats, future research focus should be on the bioavailability and long-term health implications of MRP consumption by dogs and cats.

Keywords: Advanced glycation end-products (AGEs), Carboxymethyllysine (CML), Hydroxymethylfurfural (HMF), Pet food processing, Dogs and cats

Introduction

Pet dogs and cats around the world are commonly fed processed commercial foods throughout their lives. Often heat treatments are used during the processing of these foods to improve nutrient digestibility, shelf life, and food safety. In addition, many of the ingredients used to formulate pet foods have already been heat treated. Temperature and residence time during processing of foods or feeds are known to influence Maillard reactions (MR) in which a reducing sugar binds to a free reactive amino group of an amino acid, thereby blocking its reactive site.[1] In intact proteins, the ε-amino group of lysine is the most abundant free amino group. When a reducing sugar, for example, glucose, binds to the reactive ε-amino group of lysine, the Amadori compound fructoselysine (FL) is formed. This structure cannot be utilized by the body, so its formation effectively reduces the bioavailability of lysine.[2] Bioavailability of lysine can also be reduced by the formation of lysinoalanine (LAL),[3] a cross-linked amino acid that forms during heat treatment in an alkali environment when a dehydroalanine reacts with the ε-amino group of lysine. As the MR progresses, the Amadori compound can react further through several pathways, leading to the formation of a range of reaction products typically referred to as advanced Maillard reaction products (MRPs). The MRPs that are most commonly used as markers to indicate the extent of the advanced MR in foods are ε-*N*-carboxymethyllysine (CML) and (5-hydroxymethyl)-2-furfural (HMF).[4]

Advanced Maillard compounds are also endogenously formed during naturally occurring processes in body tissues[5] and referred to as advanced glycation end-products (AGEs). During their formation, AGEs can covalently cross-link tissue proteins and, thereby, modify structural and functional properties of the proteins. Elevated levels of AGEs in tissue proteins are associated with various age-related diseases in humans, rats, and dogs, such as diabetes, cataract, osteoarthritis, vascular dysfunction, atherosclerosis, and pro-inflammatory responses.[6] Dietary MRPs can be absorbed and, those that are, can contribute to the body's AGE pool[7,8] and possibly relate to the aetiology of age-related diseases in humans and animals.

Previous publications reported reactive to total lysine ratios as low as 0.38 in commercial pet foods, possibly due to lysine being involved in the MR.[9-12] These estimates provide an indication that much of the lysine in pet foods is involved in Maillard or other reactions and that this lysine can revert back when the protein is hydrolysed with strong acid. Considering the processing conditions used during pet food manufacturing, it is likely that advanced MRPs are formed during pet food processing. To the authors' knowledge, there is only one scientific publication reporting the presence and contents of furosine levels (0.91 mg/kg) in a dry dog food, providing evidence that at least FL can be found in pet

foods.[13] As pet animals usually consume processed foods throughout their lives, chronic ingestion of advanced MRPs could induce long-term effects on pet health.

The aim of this study was to quantitate key MRPs in commercial pet foods to calculate the daily intake of MRPs in pet animals. The MRP data were subjected to multivariate analysis to identify diet formulation factors such as processing type, animal species, life stage, and nutrient composition that could affect the variation in MRP concentrations in these foods.

Materials and methods

Materials

L-Lysine monohydrochloride (Fluka, Buchs, Switzerland), furosine dihydrochloride, ε-N-carboxymethyl-L-lysine, lysinoalanine (PolyPeptide Group, Strasbourg, France), and 5-hydroxymethyl-2-furaldehyde (Sigma-Aldrich, Steinheim, Germany) were used as external chemical standards. External standards of all other amino acids were purchased from Serva Electrophoresis (Heidelberg, Germany). Other chemicals of analytical grade were nonafluoropentanoic acid (NFPA) (Fluka), barium hydroxide octahydrate and trichloroacetic acid (TCA) (Merck, Darmstadt, Germany), hydrochloric acid 37% (HCl) and *O*-methylisourea hemi sulfate (OMIU) (Sigma-Aldrich), water, acetonitrile (ACN), and methanol (UPLC-MS, Biosolve BV, Valkenswaard, The Netherlands), and buffers for amino acid analyses (Frank Gutjar Chromatographie, Balingen, Germany).

Diets and sample preparation

A sample set was created containing 153 extruded, canned, and pelleted pet foods commercially available on the Dutch market. Pet food types were categorized according to species (dog or cat) and life stage (junior and adult). From each category in the sample set, five pet foods (single batch) were randomly selected, resulting in a selection of 50 foods. In addition, 11 extruded dry foods for growing dogs and 6 extruded dry foods for growing cats were obtained from Australia, Brazil, China, Germany, Mexico, Thailand, the United Kingdom, and the United States. This results in a total of 67 foods that were included in the study. The moist canned foods were freeze-dried, and all of the foods were ground to pass through a 1 mm sieve in a centrifugal mill (Retsch ZM100, Retsch BV, Ochten, The Netherlands). All samples were stored in airtight plastic containers at 4°C prior to analyses.

Proximate analyses

Dry matter (DM) and crude ash contents were determined by drying to a constant weight at 103°C[14] and combustion at 550°C,[15] respectively. Crude protein (CP; N × 6.25) was determined using the Dumas method,[16] and crude fat (CFat) was gravimetrically

determined after hydrolysis with HCl and extraction with light petroleum ether (boiling point 40 – 60°C.[17] Crude fibre (CFibre) was gravimetrically determined as the remaining insoluble organic fraction after acid and alkaline digestion.[18] All analyses were performed in duplicate. The content of nitrogen-free extract (NFE, g/kg DM) of foods was calculated as described by eq 1, and metabolisable energy (ME, in kJ "as is") content was calculated using predictive equations for ME (eq 2, 3, 4, and 5).[19]

NFE (g/kg DM) = 1000 – CFat – CP – crude ash – CFibre   (1)

Dogs: ME (kJ "as is") = digestible energy (DE, kJ) – (4.35 × g CP)

Cats: ME (kJ "as is") = (DE, kJ) – (3.22 × g CP)   (2)

DE (kJ) = gross energy (GE, kJ) × energy digestibility (%)/100   (3)

Dogs: energy digestibility (%) = 91.2 – (1.43 × % CFibre in DM)

Cats: energy digestibility (%) = 87.9 – (0.88 × CFibre in DM)   (4)

GE (kJ) = (23.85 × g CP) + (39.33 × g CFat) + (17.15 × (g NFE + g CFibre))   (5)

Amino acid analyses

Samples were defatted by extraction with light petroleum ether without acid hydrolysis[17] and ground using a mixer mill (Retsch MM2000, Retsch BV). Total lysine (TL) was analysed using traditional amino acid analysis and represents both reactive as well as unreactive lysine that reverts back to reactive lysine during acid hydrolysis. Amino acids including TL were determined according to the method of Hendriks et al.[20] Samples (5 mg) were hydrolysed using 1 ml of 6 M HCl during 23 h at 110°C in glass tubes that were sealed under vacuum. The tubes were opened, norleucine was added as an internal standard, and the tubes were dried under vacuum (Savant SpeedVac Plus, SC210A). The dried pellet was redissolved in 2 ml of loading buffer (0.1 M sodium acetate; pH 2.2). Amino acids were separated by ion exchange chromatography using a Biochrom 20 AA analyser (Biochrom, Cambridge, UK) and analysed by postcolumn derivatization with ninhydrin, using photometric detection at 570 nm. Reactive lysine (RL) was measured using the OMIU procedure that transforms RL into homoarginine through a guanidination reaction. OMIU-RL was determined according to the method of Moughan and Rutherfurd.[21] Samples (5 mg) were incubated during 7 days with 1 ml of OMIU to convert all lysine with a free ε-amino group into homoarginine. Homoarginine was measured in the dried sample according to the amino acid analysis procedure described previously. The amount of OMIU-RL was calculated from the molar amount of homoarginine and the molecular weight of lysine. All analyses were performed in duplicate. The ratio between TL and OMIU-RL was calculated using eq 6.

RL/TL ratio = OMIU – reactive lysine/total lysine   (6)

RP-UPLC-MS

The MRPs furosine (as an indirect measurement for FL, see below), CML, HMF, and the cross-linked amino acid LAL were analysed. Defatted samples (10 mg) were hydrolysed according to the amino acid analysis procedure described previously. The samples were redissolved in 2 ml of 5 mM NFPA and filtered (0.2 μm) into vials for RP-UPLC-MS analysis. Because HMF is not stable during acid hydrolysis, the sample preparation was performed according to the method of Ameur et al.[22] For this, 10 mg of defatted sample was dissolved in 1 ml of Milli-Q and 250 μl of 40% TCA. The sample was stirred for 5 min and centrifuged for 5 min at 9000 × *g*. Then, 800 μl of the supernatant was separated, dried under vacuum (Savant SpeedVac Plus, SC210A), redissolved in 1 ml of 5 mM NFPA, and filtered (0.2 μm) into vials for RP-UPLC-MS analysis.

Samples were analysed using an Acella RP-UHPLC system (Thermo Scientific, San Jose, CA, USA). Samples (5 μL) were injected on an Acquity UPLC BEH C18 column (2.1 × 150 mm, 1.7 μm particle size; Waters, Milford, MA, USA) with an Acquity UPLC BEH C18 Vanguard precolumn (2.1 × 5 mm, 1.7 μm particle size; Waters) housed in a column oven at 40°C. The mobile phases were eluent A, 5 mM NFPA in Milli-Q water, and eluent B, 100% ACN. The flow rate was 300 μl/min. The following elution profile was used: 0 – 11 min, eluent B linear gradient from 20 to 24%; 11 – 12 min, eluent B linear gradient from 24 to 100%; 12 – 17 min, eluent B isocratic on 100%; 17 – 18 min, eluent B linear gradient from 100 to 20%; 18 – 25 min, eluent B isocratic on 20%.

Mass spectrometric (MS) data were obtained by analysing the eluate on a Thermo Scientific LTQ-Velos Pro equipped with an HESI-MS probe coupled to the RP-UHPLC; 300 μl/min of the flow from the RP-UHPLC was directed to the MS. Nitrogen was used as sheath gas and as auxiliary gas. Data were collected in positive ionization mode with selected reaction monitoring (SRM); for details, see Table 4.1. Data dependent MS^n analyses were performed with a normalized collision energy of 35%. Settings ere optimized using "LTQ tune plus" (Velos Pro 2.7, Thermo Scientific) via automatic tuning using standard stock solutions. The capillary temperature was 300°C, the source heater 230°C, and the source voltage 3.0 kV.

Table 4.1 Selected reaction monitoring conditions

Compound	Parent mass (Da)	Fragment mass (Da)	Range (m/z)
Furosine	255	130	127.50 – 132.50
Carboxymethyllysine	205	130	127.50 – 132.50
Hydroxymethylfurfural	127	108	106.50 – 111.50
Lysinoalanine	234	198	195.50 – 200.50

Compounds were quantified by reference to an external standard calibration curve by plotting MS area ratio in base peak SRM against amount ratio using external standard concentrations of 0.1, 0.05, 0.01, 0.005, 0.001, 0.005, and 0.0001 mg/ml. Data acquisition and reprocessing was done with Xcalibur 2.1 software (Thermo Scientific).

Furosine is an indirect measurement of FL; during acid hydrolysis in 6 M HCl, peptide-bound FL is transformed into approximately 32% furosine, 56% regenerated (unreactive) lysine, and 16% pyridosine.[18] RL can be calculated using this furosine procedure. FL, regenerated lysine, and FUR-RL were calculated using eqs 7, 8, and 9, respectively.[23,24]

FL (g/kg DM) = furosine (g/kg DM)/(32/100)   (7)
regenerated (unreactive) lysine (g/kg DM) = FL (g/kg DM) × (56/100)   (8)
FUR- RL (g/kg DM) = total lysine (g/kg DM) – regenerated lysine (g/kg DM)   (9)

Statistical analyses

Statistical analyses were performed using SAS 9.2 for Windows (SAS Institute, Cary, NC, USA). Data analysis to identify factors associated with variation in dietary MRPs was performed by ANOVA using the GLMSELECT procedure. CML and HMF were square root transformed, and FL was logarithmically transformed (Ln) to attain normal distribution of the residuals evaluated with the Sharpiro-Wilk test. Using stepwise regression and the Schwarz Bayesian information criteria, the best model explaining the variance of the dependent variables FL, CML, HMF, and LAL was selected. The following independent variables were used: animal species (dog, cat), processing type (extruded, canned, pelleted), life stage (adult, junior), DM (in g/kg), CP, CFat, CFibre, crude ash, NFE (g/kg DM). Correlations were assessed by use of the Pearson correlation method. Regressions were assumed significant when $P < 0.05$.

Results and discussion

Proximate composition of commercially available pet foods

The selected commercial pet foods differed considerably in proximate composition and ME content (Table 4.2). With regard to animal species, cat foods contain, on average, higher CP and CFat but lower DM and lower CFibre and NFE contents on a DM basis compared to corresponding dog foods. This result could be expected as cats require more protein and fat for growth and maintenance compared to dogs.[19] Crude ash content was comparable among foods in the category animal species. For processing type, canned foods contain on average more crude ash, CP, and CFat on a DM basis compared to extruded and pelleted foods. The lower CFibre and NFE contents of the canned foods are due to little or no inclusion of starch sources in the latter foods. In the category life stage,

foods formulated for animals at different life stages did not differ in proximate composition. However, nutrient composition can vary within this category; CP contents, for example, varied considerably in extruded adult dog foods (range 211 – 445 g/kg DM), canned junior dog foods (range 364 – 710 g/kg DM), canned adult dog foods (range 294 – 591 g/kg DM), and canned adult cat foods (range 489 – 712 g/kg DM). The mean NFE content was higher in pelleted dog foods than in extruded dog and cat foods, with the latter foods showing larger ranges in NFE contents.

Reactive lysine content of commercially available pet foods
The mean values for TL and RL of the commercial pet foods are shown in Table 4.3. RL contents met the minimal lysine requirements for dogs and cats; however, levels could be at risk of not meeting lysine requirements in weaning diets for growing dogs as described in Van Rooijen et al.[12] To estimate the extent to which the MR affected the RL content of pet foods, two procedures (OMIU or FUR method) were applied to analyse the RL contents. The two methods showed a strong correlation (r = 0.98; P < 0.0001). The mean RL content of the pet foods was lower compared to the mean TL content regardless of the procedure that was used to measure RL, with some samples having equal OMIU-RL and TL values. Except for the canned dog diets, mean OMIU-RL values were lower than FUR-RL values. A difference between OMIU-RL and FUR-RL was also reported using corn distillers' dried grains with solubles.[24] The regenerated (unreactive) lysine calculated from the furosine procedure originates specifically from the Amadori compound,[23] and the FUR-RL calculation assumes that the remaining lysine is reactive. The conversion factors used in the furosine calculation are uncertain as these depend on the origin of the Amadori compound and the hydrolysis conditions.[23,25] The OMIU-RL calculated using the OMIU procedure measures the actual amount of free ε-amino groups when complete conversion occurs of lysine to homoarginine. Why the difference between OMIU-RL and FUR-RL is reversed when canned pet foods are analysed is unknown. The analysed concentration of RL can be affected by the measuring procedure, and it is important to take into account using either of the two procedures to measure RL.

The RL/TL ratio is often used as an indication of lysine damage during processing. Pelleted dog foods had the lowest mean RL/TL ratio (0.85), compared to extruded (0.89) and canned (0.97) dog foods and extruded (0.93) and canned (0.90) cat foods, respectively (Table 4.3).[12] Using OMIU-RL, the lowest RL/TL ratio was 0.67 and was found in an extruded junior cat food. The reported mean and minimal RL/TL ratios of the foods were higher compared to previous studies. Rutherfurd et al.[10] reported mean RL/TL ratios of 0.51 (minimum 0.38, maximum 0.61) for canned cat foods and of 0.59 (minimum 0.51, maximum 0.80) for dry cat foods. Williams et al.[9] reported mean RL/TL ratios of 0.85

Table 4.2 Proximate composition and metabolisable energy content of 67 commercial pet foods categorized by animal species, processing type and life stage (mean ± standard error of the mean, g/kg dry matter unless defined differently). [a]

Component		Dog						Cat			
		Extruded		Canned		Pelleted		Extruded		Canned	
		Junior	Adult	Junior	Adult	Junior	Adult	Junior	Adult	Junior	Adult
Dry matter	Mean	922±2.0	922±4.2	231±18.0	204±16.4	909±5.6	906±6.3	940±4.2	939±6.4	241±18.4	181±4.8
(g/kg 'as is')	Range	908-938	910-932	187-296	169-259	900-931	885-922	919-972	915-949	198-296	168-196
Crude protein	Mean	302±8.7	297±39.8	513±57.4	483±56.6	260±9.6	261±9.9	394±12.3	334±9.2	701±30.2	619±39.1
	Range	205-354	211-445	364-710	294-591	229-280	246-300	352-494	311-366	590-758	489-712
Crude fat	Mean	160±8.6	132±15.7	224±26.7	222±24.2	155±12.1	92±12	167±11.8	179±20.8	247±15.5	239±19.0
	Range	107-210	98-190	158-309	165-276	116-188	63-128	119-240	115-233	217-300	198-303
Crude ash	Mean	74±3.8	75±6.2	105±9.0	101±16.7	78±3.0	80±3.6	83±4.5	75±7.6	90±14.8	114±8.4
	Range	52-118	58-96	74-127	43-133	69-88	73-93	59-110	55-101	44-124	91-139
Crude fibre	Mean	18±0.6	25±1.3	11±0.9	15±2.5	22±2.7	26±2.3	16±1.2	19±3.7	10±3.9	10±3.4
	Range	15-24	22-29	8-14	11-25	12-28	21-33	8-22	12-29	2-24	4-22
Nitrogen-free extract[b]	Mean	447±14.8	472±60.0	163±55.9	181±88.3	485±21.0	541±21.0	341±22.5	393±23.4	19±19.2	50±38.3
	Range	342-595	240-577	0-341	0-482	443-558	467-589	168-426	320-466	0-96	0-199
Metabolisable energy	Mean	16±0.2	16±0.3	5±0.4	4±0.3	16±0.3	15±0.2	17±0.3	17±0.6	5±0.4	4±0.1
(MJ/kg 'as is')[c]	Range	15-17	15-16	3-6	3-5	15-17	14-15	13-19	15-18	4-6	3-4

[a] n = 5, except for extruded junior dog foods (n = 16) and extruded junior cat foods (n = 11).
[b] Calculated as (g/DM): 1000 – crude fat – crude protein – crude ash – crude fibre.
[c] Calculated using predictive equations for ME.[19]

Table 4.3 Total lysine (TL), *O*-methylisourea reactive lysine (OMIU-RL), reactive lysine calculated using the furosine method (FUR-RL), RL/TL ratio and Maillard reaction products fructoselysine (FL), carboxymethyllysine (CML), hydroxymethylfurfural (HMF) and the cross-linked amino acid lysinoalanine (LAL) of 67 commercial pet foods categorized by animal species, processing type and life stage (mean ± standard error of the mean, g/kg dry matter unless defined differently). [a]

Variable		Dog						Cat			
		Extruded		Canned		Pelleted		Extruded		Canned	
		Junior	Adult	Junior	Adult	Junior	Adult	Junior	Adult	Junior	Adult
TL	Mean	13.7±0.9	14.5±3.0	28.4±5.0	24.2±3.2	12.7±0.9	11.6±0.8	17.4±1.4	16.4±1.6	44.3±3.7	36.5±2.5
	Range	8.08-24.12	9.08-26.04	19.42-44.88	16.27-32.83	10.62-15.70	10.17-14.44	12.51-28.00	12.50-20.24	36.19-55.09	27.42-40.80
OMIU-RL [b]	Mean	11.9±0.9	13.2±2.8	27.1±4.5	23.9±3.6	10.9±0.8	9.9±0.8	15.0±1.1	15.9±1.8	40.7±4.9	32.1±2.2
	Range	6.63-20.43	7.09-23.38	18.74-41.54	13.45-32.64	9.13-13.58	8.59-12.88	11.86-23.20	11.73-20.08	30.60-52.87	25.63-36.82
FUR-RL [c]	Mean	13.3±1.0	14.1±3.0	26.2±4.8	21.7±2.8	11.9±1.2	11.5±0.8	16.9±1.4	15.9±1.6	41.3±4.1	34.1±2.4
	Range	7.73-23.95	8.83-25.75	16.06-40.84	14.77-28.32	8.35-15.53	10.08-14.26	11.90-27.33	12.28-19.60	34.14-53.88	25.50-38.51
RL/TL [d]	Mean	0.87±0.02	0.90±0.03	0.96±0.02	0.98±0.04	0.86±0.02	0.84±0.01	0.88±0.03	0.97±0.02	0.91±0.05	0.88±0.02
	Range	0.77-0.99	0.78-0.97	0.92-1.03	0.83-1.10	0.78-0.94	0.81-0.89	0.67-0.98	0.91-1.03	0.75-1.03	0.82-0.93
FL [e]	Mean	0.58±0.04	0.71±0.19	4.00±1.26	4.46±0.94	1.48±0.71	0.21±0.05	0.80±0.08	0.73±0.18	5.38±1.67	4.30±0.35
	Range	0.30-0.82	0.38-1.44	1.57-7.21	2.67-8.06	0.31-4.05	0.11-0.33	0.42-1.19	0.38-1.32	2.15-11.45	3.43-5.15
CML [f]	Mean	11.23±0.66	15.40±1.31	29.88±7.39	36.50±6.00	24.39±4.63	16.07±2.15	10.87±0.82	13.25±2.81	38.76±4.11	41.61±5.65
	Range	7.75-16.67	13.24-20.22	12.27-50.61	20.42-54.69	16.00-40.30	11.99-21.58	7.72-17.26	6.01-20.72	27.40-51.65	26.74-60.54
HMF	Mean	0.65±0.06	1.11±0.27	1.79±0.59	1.70±0.33	0.92±0.21	1.40±0.26	0.64±0.08	0.47±0.07	1.24±0.37	0.93±0.12
	Range	0.33-1.25	0.27-1.92	0.34-2.82	1.33-3.02	0.52-1.70	0.91-2.38	0.27-1.02	0.24-0.64	0.07-2.29	0.59-1.24
LAL [f]	Mean	7.76±0.63	6.41±1.19	5.80±0.64	7.64±1.06	6.14±0.89	10.24±1.82	5.55±0.78	7.23±0.82	6.77±1.41	7.32±1.29
	Range	4.25-12.95	3.15-9.33	4.05-7.45	3.49-9.49	4.12-8.54	6.31-16.11	1.39-9.49	5.09-10.02	1.59-9.69	4.22-11.63

[a] n = 5, except for extruded junior dog foods (n = 16) and extruded junior cat foods (n = 11).
[b] Reactive lysine analysed using the *O*-methylisourea procedure.
[c] Reactive lysine analysed using the furosine procedure; calculated as: total lysine – regenerated lysine.[19]
[d] Ratio, calculated as reactive lysine / total lysine (TL and RL in g/kg dry matter).
[e] Calculated as: furosine / (32/100).[18]
[f] in mg/kg dry matter.

(minimum 0.44, maximum 1.06) for adult dog foods and of 0.75 (minimum 0.47, maximum 0.98) for foods for growing dogs. It is, however, known that depending on the extent of the MR, some lysine may be converted to compounds that do not revert back to lysine during acid hydrolysis, thus reducing the total lysine content of the food. The RL/TL ratio, therefore, is valid when the RL/TL ratio of the unprocessed ingredient mixture is known, but is not suitable as an indicator of advanced MRPs that could have formed in processed pet foods.

Presence of common markers of the Maillard reaction

The current study is the first to quantitate levels of various MRPs in commercial pet foods. High variation in MRP content was seen between as well as within processing type. Canned pet foods contained on average the most FL, CML, and HMF followed by pelleted and extruded foods (FL, 4534, 844, 706; CML, 37, 20, 12; HMF, 1417, 1161, 715 mg/ kg DM, respectively; Table 4.3). However, when expressed on an "as is" basis, canned pet foods contain the lowest CML and HMF contents. In dry dog foods, the mean contents of FL, CML, and HMF were higher in pellets than in extrudates (FL, 844 vs. 646 mg/kg DM; CML, 20 vs. 13 mg/kg DM; HMF, 1161 vs. 880 mg/kg DM, respectively). The HMF content in pelleted dog foods is much higher than previously reported in a pelleted piglet starter diet (16.66 mg/kg).[26] For LAL, the means are comparable between processing types. The only study to date focusing on MRP content in pet foods reported a FL content of 2840 mg/kg "as is" in one dry dog food.[13] This level is twice as high as the highest level for the dry extruded dog foods in the present study (1440 mg/kg DM), but not as high as the highest level for pelleted junior dog foods (4050 mg/kg DM). No data are available for comparison of the CML, MF, and LAL in pet foods. However, several studies are available that evaluated these advanced MRPs in human food items. CML concentrations in human foods were found to range between 0.1 and 68.7 mg/kg in meat and fish products, between 1.0 and 423.9 mg/kg in meat dishes, between 7.6 and 54.2 mg/kg in cereals, and between 0.5 and 39.4 mg/l in infant formulas.[27-32] The manner of processing has an influence on the level of CML; raw minced beef contained less CML (0.7 mg/kg) than boiled and fried minced beef (5.0 and 11.2 mg/kg CML, respectively).[33] Increasing CML levels with increasing severity of processing was also found in roasted beef joint (1.5, 2.5, and 4.2 mg/kg for rare, medium-rare, and well-done, respectively)[27] and chicken breast (3.8, 4.6, 5.1, and 23.9 mg/ kg, for boiled, roasted, fried, and casseroled breast, respectively). HMF levels in human foods were found in the ranges of 91.3 – 3060 mg/kg in instant coffee (values for ground roasted coffee beans were found within this range),[34-36] 6.6 – 241 mg/kg in breakfast cereals,[37] 0.2 – 69 mg/kg in white bread,[36,38] 0.5 – 74.6 mg/kg in cookies,[22] and 0.2 – 34.7 mg/l in infant formulas.[39-42] LAL levels of 10 – 70 mg/kg for baby food, 200 – 300 mg/kg for cereal products, 150 – 920 mg/kg for dry infant

formulas, and 140 – 540 mg/kg for meat products were reported in human foods.[3] However, Raymond[43] reported lower levels for LAL, 0 – 70 mg/kg for ready-to-eat cereals, 0 mg/kg for pasta, 0 – 50 mg/kg for infant formula, 0 mg/kg for prepared meat, and 0 – 120 mg/kg for canned fish. The CML, HMF, and LAL levels of the analysed pet foods are within the range reported for human foods (Table 4.3).

Daily intake of common markers of the Maillard reaction
On the basis of the MRPs and the calculated ME contents of the pet foods, daily MRP intake can be estimated. A 20 kg adult dog having a daily energy requirement of 5.15 MJ ME[14] would ingest 0.50 mg CML and 34.58 mg HMF/kg body weight (BW)$^{0.75}$/day when eating an extruded diet with an average content of 3.83 and 266.00 mg CML and HMF/4.18 MJ ME (Figure 4.1). An adult cat (4 kg) with a daily energy requirement of 1.06 MJ ME[14] would ingest 0.28 mg CML and 10.90 mg HMF/kg BW$^{0.75}$/day when consuming an extruded diet with an average of 3.12 and 121.80 mg CML and HMF/ 4.18 MJ ME. For canned and pelleted diets, these values were even higher (Figure 4.1). Limited estimates are available for humans, and these may be influenced by country, region, social class, and age. For Spanish adolescents, a mean daily intake of 0.99 mg CML/kg BW$^{0.75}$ (70 kg BW) was estimated.[44] The CML content in infant formulas was quantified in five studies. Assuming a daily intake of 1 litre for a 6 kg infant, daily CML intake ranges from 0.49 to 39.38 mg corresponding to mean values of 1.23, 3.69, 4.60, 4.75, and 33.77 mg.[28-32] For HMF, the mean intake for adult humans in three different studies was 5.08, 5.56, and 10 mg per day,[36,37,44] resulting in an average daily intake of 0.28 mg HMF/kg BW$^{0.75}$. HMF levels in infant formulas range from 0.158 to 34.7 mg/L,[39-42] resulting in average daily values of 0.16, 0.32, 0.38, and 13.9 mg/L. This results in an average daily intake of 0.96 mg HMF/kg BW$^{0.75}$. Compared to the calculated average for human adults, dogs may ingest up to 122 times more HMF when consuming an average extruded dog food (Figure 4.1). In addition, dogs may ingest up to 36 times more HMF compared to human infants fed infant formula. Cats consuming an average extruded cat food may ingest up to 38 times more HMF compared to adult humans and 11 times more HMF compared to human infants. Dogs and cats fed canned and pelleted diets are more likely to ingest higher amounts of HMF and CML compared to animals that are fed an extruded diet. No data are available regarding the average daily intake of LAL in humans. Both CML and HMF from consumed foods can cross the intestinal wall and enter the circulation. In rats, dietary HMF has been shown to be rapidly absorbed, metabolised into 5-hydroxymethyl-2-furoic acid and N-(5-hydroxymethyl-2-furoyl)glycine as main metabolites, and 70 – 82% of the ingested HMF is excreted via the urine within 48 h.[45] No evidence for prolonged accumulation in body tissues was observed. No adverse effects regarding acute and
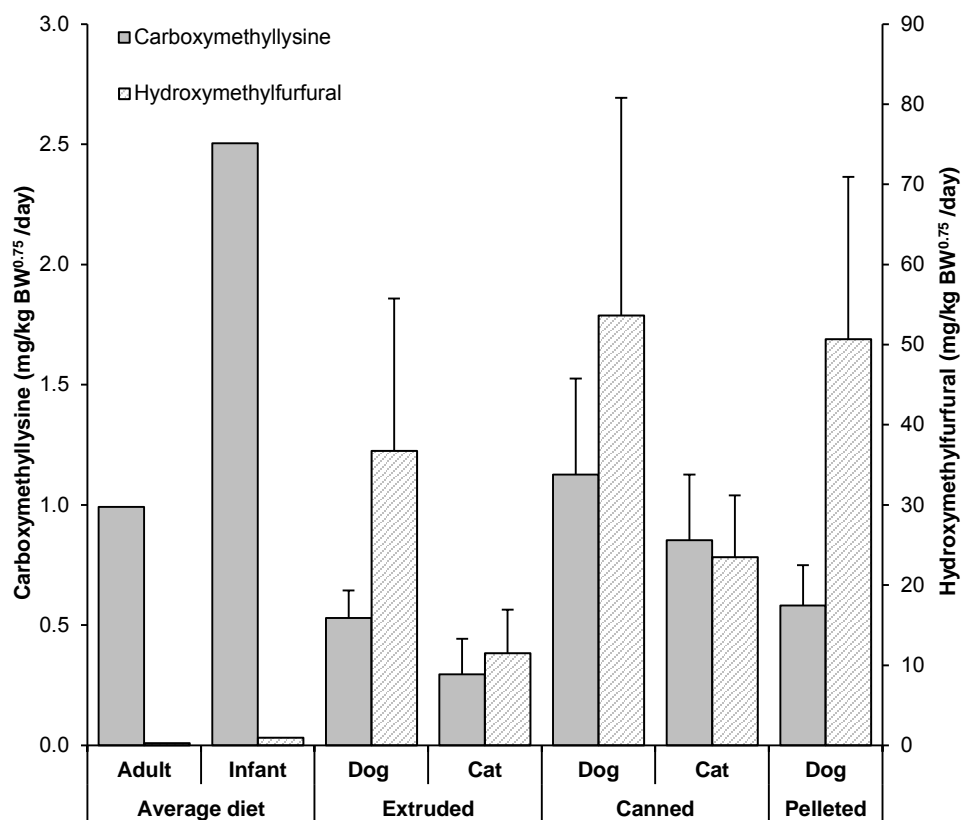
Figure 4.1. Estimated daily intake (mg/kg body weight (BW)$^{0.75}$) of carboxymethyllysine and hydroxymethylfurfural of a 70 kg adult human consuming a standard Western diet,[27,36,37,44] a 6 kg human infant consuming 1 l of an average infant formula,[28-32,39-42] and a 20 kg adult dog and a 4 kg adult cat fed either an extruded, canned, or pelleted diet for adult dogs and cats at maintenance (using a standard daily energy requirement[19]) containing an average ± standard deviation content of carboxymethyllysine and hydroxymethylfurfural as reported in the current study.

subacute toxicity in rats and mice ingesting HMF concentrations of 80 – 100 mg/kg body weight was reported.[46] In humans, intake of dietary CML resulted in an increase in circulatory CML concentration.[47] In rats, approximately 26 – 29% of the ingested CML was excreted in the urine, whereas in humans 14% of ingested dietary CML was excreted in urine.[8,48] Around 50% of the ingested CML was not recovered, and it is unknown if it is deposited in organs, degraded by colonic microbiota, or metabolised. Whether the MRPs in pet foods are absorbed and excreted when consumed, and subsequently add to the endogenous AGE load in dogs and cats, remains to be investigated.

Dietary factors associated with the occurrence of the Maillard reaction

Dietary factors associated with variation in advanced MRP content of pet foods are shown in Table 4.4. The levels of MRPs in pet foods were not related to animal species or life stage; however, type of pet food shows a correlation for FL, CML, and HMF (Table 4.4). The processing conditions between pelleting, extrusion, and canning differ considerably. Extruded kibbles are processed at temperatures of 80 – 200°C for 10 – 270 sec under high pressure.[49] Canned foods are heated until the centre of the can reaches temperatures of 121°C for > 10 min to sterilize the product.[50] Pelleted foods are relatively mildly heat treated using temperatures of 60 – 90°C for 30 – 45 s.[51] In addition, pet food

Table 4.4 Linear regression coefficients animal species, life stage, processing type, proximate composition (g/kg dry matter unless defined differently) on fructoselysine (FL)[a], carboxymethyllysine (CML), hydroxymethylfurfural (HMF) and lysinoalanine (LAL) in 67 commercial pet foods (g/kg dry matter; estimate±standard error).

Variable	Ln(FL) (R^2=0.70)	Sqrt(CML) (R^2=0.73)	Sqrt(HMF) (R^2=0.48)	LAL (R^2=0.00)
Intercept	8.29±0.13	7.04±0.93	59.15±7.07	7.03±0.33
Animal species	-[b]	-	-	-
Dog				
Cat				
Processing type[c]				-
Extruded	1.84±0.17	5.93±2.7	-24.91±3.40	
Canned	0	0	0	
Pelleted	2.25±0.22	6.85±2.64	-22.79±4.57	
Life stage	-	-	-	-
Adult				
Junior				
Dry matter (g/kg)	-	-0.01±0.004	-	-
Crude protein	-	-	-0.05±0.01	-
Crude fat	-	0.006±0.002	-	-
Crude fibre	-	-	0.35±0.17	-
Crude ash	-	-	-	-
NFE[d]	-	-	-	-
RMSE[e]	0.59	0.73	7.29	2.72

[a] Calculated as: furosine / (32/100).[23]
[b] -, no significant correlation.
[c] Extruded pet foods n = 37, canned pet foods n = 20, pelleted pet foods n = 10.
[d] NFE, nitrogen-free extract calculated as: 1000 – crude fat – crude protein – crude ash – crude fibre.
[e] RMSE, root mean squared error.

ingredients can be extensively processed (i.e., rendered, cooked, dried, and/or ground) before inclusion. Depending on the severity of the processing, the MR progresses through several stages of the reaction. Overall, pelleted foods contain more CML and HMF compared to extruded foods (and canned foods when expressed on an "as is" basis). This result is unexpected considering the rather mild heat treatment used during pelleting compared to extrusion conditions. Because of this mild heat treatment, ingredients used during pelleting are often pre-processed. Carbohydrate sources, for example, are often pre-treated as pelleting processing temperatures and residence times are not sufficiently high to fully gelatinize the starch in the raw ingredients during processing.[52] It could, therefore, be that the MRP contents in pellets mainly originate from the pre-processed ingredients rather than de novo formation due to the processing itself. Analyses of ingredients and food mixtures prior to processing are required to confirm this hypothesis. LAL content in pet foods is comparable between processing types and is not associated with type of processing. With regard to the chemical composition, CML was associated with CFat content. Besides the MR, lipid peroxidation during processing results in the intermediate glyoxal, which can react with lysine to form CML.[53] Next to the association with CFat, CML content showed a strong correlation with FL content (r = 0.78; P < 0.0001), which reflects the formation of CML from FL via an oxidation pathway.[54] HMF was associated with a decreased CP content and increased CFibre content. CML is moderately correlated to HMF (r = 0.45; P = 0.0001). The MR pathways that result in the formation of CML and HMF are different but apparently occur in a certain ratio during the processing of pet foods. No factor was associated with the content of LAL in the analysed pet foods.

In conclusion, this study demonstrates that pet foods do contain Maillard reaction products. Type of processing seems to be a key factor for the concentration of FL, CML, and HMF, with on average higher amounts in wet canned pet foods than in dry extruded or pelleted pet foods (DM basis). LAL contents were similar for wet canned and dry pet foods. Further research is required to determine the contribution of pet food processing and the use of processed ingredients in the observed concentrations of Maillard reaction products in pet foods. Average daily intake of MRPs in pet animals can be higher compared to limited adult human and infant data. As commercial pet foods are most often the only source of food for dogs and cats, future research focus should be on the bioavailability and long-term health implications of MRP consumption by dogs and cats.

*S Supporting information

Table S1. Amino acid composition of 67 commercial pet foods categorized by animal species, processing type, and life stage. This material is available free of charge via the Internet at http://pubs.acs.org.

Acknowledgements

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Chapter 5

The effect of steam pelleting of a dry dog food on the Maillard reaction

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Abstract

During processing of pet foods, the Maillard reaction (MR) can occur, which reduces the bioavailability of essential amino acids like lysine and results in the formation of advanced Maillard reaction products (MRPs). This study examined the effect of conditioning temperature (65 and 90°C) and die hole length (ø 5 × 45, 65, and 80 mm) during pelleting processing of a standard dry dog food on selected indicators of the MR (total lysine, reactive lysine, fructoselysine, ε-N-carboxymethyllysine, (5-hydroxymethyl)-2-furfural, lysinoalanine), browning development and CIE-Lab colour. Steam pelleting variables did not cause a significant loss of lysine or change in colour and absorbance values. Analysing the unprocessed ingredient mix suggests that the choice of the ingredients used in the ingredient mix, rather than the pelleting process applied, is responsible for the RL/TL ratio observed in the dry standard dog food used in this study. MRP content increased during steam pelleting (fructoselysine: 366.2 to 538.8 mg/kg DM; ε-N-carboxymethyllysine: 12.6 to 14.8 mg/kg DM; lysinoalanine: 5.7 to 7.7 mg/kg DM; $P <$ 0.05). Increasing conditioning temperature from 65 to 90°C increased fructoselysine (475.9 to 601.6 mg/kg DM; $P <$ 0.01) and ε-N-carboxymethyllysine (14.3 to 15.1 mg/kg DM; $P =$ 0.003). An increased die hole length of 80 mm decreased fructoselysine content compared to 45 and 65 mm (461.3 vs. 573.3 and 581.6 mg/kg DM; $P <$ 0.01) but increased lysinoalanine content (8.8 vs. 7.4 and 6.8 mg/kg DM; $P =$ 0.002). Analysing total and reactive lysine and absorbance values are not accurate enough to predict the MR and formation of MRPs during processing.

Keywords: Conditioning, Pelleting, Reactive lysine, Maillard reaction products, Carboxymethyllysine, Dogs

Introduction

Pelleted dog foods are sold by manufacturers as an alternative to dry extruded dog foods. Compared to the extrusion process, the pelleting process is considered to be less severe with lower temperatures (60 to 90°C vs. 80 to 200°C) and shorter residence times (2 to 20 sec vs. 10 to 120 s).[1] Processing is known to induce the Maillard reaction. (MR) where a reducing sugar reacts with a free reactive amino group of an amino acid. Lysine is the most reactive amino acid due to its ε-amino group although other amino acids are also knows to be affected.[2] As the MR progresses, advanced Maillard reaction products (MRPs) are formed. As the rate of MR depends on temperature, time and pressure during processing, it can be expected that the amount of blocked lysine and formed MRPs would be lower in pellets compared to extrudates. However, it has previously been reported that commercially pelleted dog foods contain, on average, a lower ratio of reactive to total lysine (RL/TL; lysine that contains a reactive ε-amino group/total amount of lysine) compared to commercially extruded dog foods. Reported RL/TL ratios ranged from 0.72 to 0.94 (n = 14) in commercial pelleted dog foods, compared to 0.77 to 0.99 (n = 14) in commercial extruded dog foods.[3,4] The concentration of MRPs was higher in pellets; commercially available pelleted dog foods contained 1.4, 1.7 and 1.5 times more fructoselysine, carboxymethyllysine, and hydroxymethylfurfural, respectively compared to commercially available extruded dog foods.[5] The MR can affect the nutritional quality of pet foods in different ways. Firstly, a loss of essential amino acids, especially lysine, can occur when the reactive amino group of an amino acid is the substrate of the reaction. Modified lysine derivatives like fructoselysine (FL, also referred to as Amadori compound) are formed that may be partially absorbed but cannot be utilized by the body, thereby, reducing the bioavailable lysine content.[6-8] Secondly, during the MR crosslinking of peptides can occur, for example the formation of lysinoalanine (LAL). These compounds will decrease the digestibility of the protein and, therefore, utilization and uptake of all amino acids. Finally, several pathways lead to the formation of advanced MRPs as the MR progresses, such as ε-*N*-carboxymethyllysine (CML) and (5-hydroxymethyl)-2-furfural (HMF). Their endogenous analogues, advanced glycation end-products (AGE), as well as dietary MRPs have been associated with the aetiology of age-related diseases in humans, such as diabetes mellitus and impaired renal function.[9] In dogs, elevated levels of AGEs in tissue proteins were observed in a number of diseases with increasing age[10-13] and it is possible that bioavailable dietary MRPs contribute to the endogenous AGE levels in dogs as has been reported for humans.[14,15] During pelleting, processing parameters like steam conditioning, residence time and pressure can influence the MR, resulting in a decrease in RL and an increase in the formation of MRPs. Pelleting is a processing technique in which mash conditioning, agglomeration (pelleting) and subsequent cooling take place. Mash

conditioning includes heat, water and pressure, often combined in the form of steam, to induce softening of the food, denaturation of proteins and gelatinization of starch before the product enters the pellet press.[16] After conditioning, the mash is compacted and pressed through a die using rollers in the pellet press. The rollers continuously press layers of mash inside the die hole, by which the pellet is actually built up. The application of mechanical energy by rollers on a die results in pressure and friction in the die. The extent of compression depends on the height of the layer and the gap distance between the die and the roller. Die holes can differ in their length to diameter ratio, influencing the amount of shear which the feed mash receives. After leaving the die, pellets are usually cooled by air flow or using a conveyer belt.[17] Studies that report the effect of the pelleting process (unprocessed meal vs. pellets) on RL contain contrasting results. Fluorodinitrobenzene-reactive lysine significantly increased with 0.8, 0.9 and 1.1% by pelleting an unprocessed starter, grower, and finisher pig diet meal (80°C using a 4 mm die), respectively.[18] Pelleting a complex nursery pig diet (steam conditioned at 60°C for 45 sec through a 5 × 38 mm die) had no effect on lysine bioavailability as measured using a standard-curve bioassay with 8-d-old chicks.[19] However, pelleting a pre-starter diet for suckling piglets (steam-conditioned at 40°C for 30 s; pelleted at 60 to 65°C through a 1.7 × 40 mm die) resulted in an increase of furosine (a hydrolysis product and indicator of FL) from 11.7 to 35.2 mg/kg.[20] The same study reported an increase in HMF from 12.6 to 16.7 mg/kg, while Shipe et al.[21] found no effect of change in furfural concentration due to the pelleting process (steam conditioned at 82°C, using 38.1 or 44.5 mm die hole length). All these studies aimed to elucidate the effect of the total pelleting process rather than focusing on the difference in conditioning temperature and shear forces/pressure induced by the die dimensions during pelleting. In addition, studies investigating the effect of the pelleting process for pet food manufacture are lacking. The objective of this study was to examine the effect of steam conditioning temperature and die hole length applied during the pelleting process of a standard dry dog food on the progression of the MR as measured using selected indicators as well as browning index and colour development.

Materials and methods

Experimental food
The ingredient mixture of a standard dry dog food, hammer milled over a 1.5 mm sieve and thoroughly mixed, was provided by a pet food manufacturer. Ingredient composition and analysed chemical composition of the food is shown in Table 5.1.

Experimental design
A 3 × 2 factorial study design was used with pelleting die specifications of 5 × 45, 5 × 65, and 5 × 80 mm (diameter × length), combined with steam temperatures of 65 and 90°C

Table 5.1 Ingredients and analysed chemical composition of the experimental adult dog food.

Ingredients	g/kg	Chemical composition	g/kg
Wheat	273.1	Dry matter	914.6
Corn	253.0	Starch	326.8
Chicken meal	120.0	Crude protein	253.2
Meat meal	65.0	Crude fat	111.3
Liquid fat (bovine, pig)	64.0	Crude ash	65.5
Maize gluten feed	50.0	Neutral detergent fibre	85.9
Chicory	50.0	Reducing sugars	32.6
Poultry meal, hydrolysed	30.0		
Blood meal	25.0		
Maltodextrin	25.0		
Vitamin-mineral Premix[a]	25.0		
Yeast[b]	5.0		
Salmon oil	5.0		
Pellet binder[c]	5.0		
Organic acids[d]	2.0		
Sunflower oil	2.0		
Emulgator (liquid)[e]	0.5		
Fructo-oligosaccharides[f]	0.4		

[a] Premix, provided per kg of diet: vitamin A (retinol), 15000 IU; Vitamin D3 (cholecalciferol), 1500 IU; Vitamin E (all-rac-alpha-tocoferyl acetate), 100 IU; Vitamin B1 (thiamine mononitrate), 12 mg; Vitamin B2 (riboflavin), 12 mg; Vitamin B6 (pyridoxine hydrochloride), 10 mg; Vitamin B12 (cyanocobalamin) , 0.03 mg; Vitamin C (6-palmitoyl-L-ascorbic acid), 17.5 mg; Vitamin C, 100 mg; pantothenic acid (D-calcium pantothenate), 40 mg; choline chloride, 650 mg; niacin, 75 mg; folic acid, 2.5 mg; biotin, 0.4 mg; betaine, 650 mg; copper, 10 mg; iron, 50 mg; iodine, 1 mg; manganese, 35 mg; zinc, 100 mg; selenium, 0.3 mg.
[b] Brocacel.
[c] LignoBond DD.
[d] Nutri-C.
[e] Solumul 484.
[f] Fructomax.

Table 5.2 Measured pelleting process parameters during sampling of the pet food.

	Die size (ø × l) and temperature					
	5 × 45 mm		5 × 65 mm		5 × 80 mm	
Parameters	65°C	90°C	65°C	90°C	65°C	90°C
Product temperature (°C)						
feeder screw	19.6	20.1	18.6	18.6	20.4	20.4
conditioner	65.0	90.2	65.2	90.0	65.3	90.2
die exit	74.5	97.9	75.7	99.7	74.9	99.8
after cooling	21.4	21.5	18.9	20.4	22.5	26.3
Electric consumption (A)	2.20	2.57	2.30	2.62	2.20	2.58
Capacity (kg/h)	604	618	605	620	602	628

set at the conditioning stage (Table 5.2). Die diameter was chosen according to the average diameter of 10 commercial pelleted dog foods, and die length to obtain the highest variation possible using available dies. Temperatures were chosen in order to obtain a large difference and feasibility of production. All settings of the pellet press were kept constant and pellet processing parameters like product temperature, electrical consumption and capacity were monitored during sampling. Each treatment combination was replicated three times.

Pelleting procedures

The experiment was performed at Research Diet Services (Wijk bij Duurstede, The Netherlands) under approval of the Netherlands Food and Consumer Safety Authority (NVWA) using a pilot scale Robinson-Heesen pellet press (seriesV3-30, Heesen, Boxtel, The Netherlands) and a cooler conveyer belt. The ingredient mixture was processed through the conditioner (residence time estimated at ~ 20 s). The pellet press was set at a constant feed rate of 600 kg/h and a steam pressure between 2.7 and 2.8 bar was used throughout the experiment. Electric consumption (A) was recorded during de pelleting process, and capacity (kg/h) was calculated by weighing pellets that were collected at the outflow of the die for 30 s. During the pelleting process, product temperature was measured at the feeder screw site, post steam conditioning, at the die exit and after cooling for 12 min by collecting the product in a thermos bottle using a digital industrial thermometer with sensor. The unprocessed ingredient mix was sampled in triplicate for analysis. In addition, for each treatment, representative samples were collected at the cooling conveyer belt after pelleting conditions reached steady state, i.e. a constant product temperature for at least 5 min. Between sampling the pellets of replicate treatments, the conditioner was cooled down for 5 min by reducing the amount of steam to disturb the steady state of the conditioner. Then, temperature was elevated again to reach target temperature for at least 10 min to create repeated assessments of each treatment. Pellet samples were ground to pass a 1-mm screen in a centrifugal mill (Retsch ZM100, Retsch BV, Ochten, the Netherlands) and all samples were stored in wide neck bottles at 4°C prior to analysis.

Chemical analyses

Samples of the unprocessed ingredient mix were analysed for dry matter (DM) and crude ash by drying to a constant weight at 103°C[22] and combusting at 550°C,[23] respectively. Crude protein (N × 6.25) was determined using the Kjeldahl-method[24] and crude fat was determined gravimetrically after hydrolysis with hydrochloric acid and extraction with light petroleum (boiling point 40 – 60°C.[25] Neutral detergent fibre (aNDFom) was analysed with the addition of α-amylase and without sodium sulphite.[26] Starch was determined through enzymatic hydrolysis using amyloglucosidase.[27] Reducing sugars

were analysed according to Van Vuuren et al.[28] In the unprocessed ingredient mix and processed pellets, TL, *O*-methylisourea-reactive lysine (OMIU-RL), FL, CML, HMF and LAL were determined as described previously.[5] For analysis of absorbance, samples were dissolved in a 2% sodium dodecylsulfate and 10 mmol 2-hydroxy-1-ethanethiol solution at a concentration of 5 mg sample per ml. Samples were incubated overnight at room temperature. Samples were centrifuged and 1 ml of supernatant was used for further analysis. For the 280 nm measurement, 4 ml quartz cuvettes, and for 420 nm measurement, 1.5 ml semi-micro UV-cuvettes (Plastibrand, Sigma Aldrich Nederland, Zwijndrecht, the Netherlands) were used. Absorbance was measured with a UVmini 1240 UV-VIS spectrophotometer (Shimadzu, Columbia, Maryland).With the CIE-Lab system spectro-color spectrophotometer (Dr. Lange, Berlin, Germany) *L**, *a**, and *b** values were determined using D65 artificial daylight. *L** expresses the brightness of the sample (100 = white, 0 = black). The *a** and *b** values are colour coordinates, with the *a** values ranging from red (+) to green (–) and the *b** values ranging from yellow (+) to blue (–). $C* = (a*^2 + b*^2)^{1/2}$ further determines the intensity or the degree of colour saturation, and $E = (L*^2 + a*^2 + b*^2)^{1/2}$ gives an indication about the lightness of the sample.[29] All analyses were performed induplicate.

Physical analysis
Pellet durability was determined using a Holmen Pellet Tester (NHP 100, Holmen Chemical Ltd., Borregaard group, Norfolk,UK). A sample (100 g) was placed in the Holmen pellet tester. Pneumatic transport was simulated for 60 sec and screen size was 80% of the pellet diameter. The remaining intact pellets were sieved and weight was recorded. Durability (%) = weight of material retained after test/weight of material before test × 100. Pellet hardness was determined by force using an automatic Kahl device (Amandus Kahl Nachf., Reinbek, Germany). A single pellet was placed into the device and its breaking force was measured as described by Thomas and van der Poel.[30] Hardness (kg) is reported as an average value of 12 pellets in which the minimum and maximum values were deleted. Pellet bulk density was determined using a device similar to a Pfeuffer bulk density measuring system (Wageningen University, Mechanische Werkplaats, Wageningen, NL). The volume of the cylinder was 1 L and the measurement included three replicates. Bulk density (g/L) = weight of full cylinder (g) – weight of empty cylinder (g).

Statistical analyses
Statistical analyses were conducted using SAS 9.2 for Windows (SAS Institute Inc., Cary, NC, USA). Data analysis to identify pelleting effects was performed by ANOVA using the Proc GLM procedure. Normal distribution of the residuals was evaluated with the

Sharpiro-Wilk test. Effect of steam pelleting (unprocessed ingredient mix vs. pelleted food) was tested using the model $Y_i = \mu + TR_i + \varepsilon_i$, where Y_i = parameter to be tested, μ = mean, TR_i = treatment effect i, and ε_i = error term. Within treatments the effects of conditioning temperature and die hole length was tested using the model $Y_{ijk} = \mu + T_i + D_j + (T \times D)_{ij} + \varepsilon_{ijk}$, where Y = parameter to be tested, μ = mean, T_i = effect of conditioning temperature i, D_j = effect of die hole length j, $T \times D$ = interaction term and ε_{ij} = error term. Correlations between OMIU-RL and RL calculated using FL were assessed by use of the Pearson correlation method. The data were adjusted for multiple comparisons using the Tukey-Kramer adjustment. Overall differences were considered significant if $P < 0.05$. Values are expressed as LSmeans ± SEM.

Results

Pelleting process parameters
Conditioner temperatures reached the target temperatures of 65 and 90°C (Table 5.2), while product temperatures at the die exit were approximately 10°C higher. Electric consumption as well as the capacity of the pellet press increased when temperature was raised from 60 to 90°C.

Unprocessed ingredient mix
The unprocessed ingredient mix consisted of 253.2 g/kg protein, of which approximately 30 g/kg originates from hydrolysed poultry meal (Table 5.1). In addition, the unprocessed ingredient mix contained 326.8 g/kg starch, of which 32.6 g/kg was from the reducing sugars.

Effect of steam pelleting on chemical parameters
The effect of the steam pelleting processes (unprocessed ingredient mix vs. pelleted food) on chemical parameters is shown in Table 5.3. Neither absorbance at 280 nor at 420 nm was affected by steam pelleting, with values for 420 nm close to 1. For the colour values, lightness $L*$ and $a*$ were not affected by steam pelleting. The $b*$ value significantly decreased after steam pelleting from 17.6 to 16.4, as well as the values for $C*$ from 172.4 to 151.6 and E from 1555.4 to 1488.3. The steam pelleting processes applied to the ingredient mix had no effect on TL and OMIU-RL contents and RL/TL ratio. Contrary to the lysine results, FL content significantly increased from 366.2 to 538.8 mg/kg DM, CML content from 12.6 to 14.8 mg/kg DM and LAL content from 5.7 to 7.7 mg/kg DM after the steam pelleting was applied to the ingredient mix. HMF content was not affected by steam pelleting.

Table 5.3 Total and reactive lysine, Maillard reaction products, absorbance and colour values, and physical parameters of a dry dog food as affected by steam conditioning temperature and die hole length during the pelleting process (LSmean ± SEM).

Parameters	Ingredient mix (n = 3)	Pelleted food (n = 18)	P-value	Die size (ø × l) and conditioning temperature						Pooled SEM	P-values		
				5 × 45 mm		5 × 65 mm		5 × 80 mm					
				65°C	90°C	65°C	90°C	65°C	90°C		Temp	Die	Temp×Die
Absorbance (nm)													
280	0.48±0.04	0.42±0.02	0.162	0.45	0.46	0.48	0.39	0.37	0.37	0.04	0.387	0.100	0.281
420	0.99±0.07	1.02±0.03	0.673	0.95	1.01	1.12	1.04	0.93	1.04	0.06	0.559	0.286	0.348
Colour[a]													
L*	52.6±0.7	51.7±0.3	0.227	51.6	52.3	51.5	52.1	51.2	51.6	0.61	0.266	0.690	0.966
a*	5.7±0.3	5.7±0.1	0.959	6.1	5.2	6.3	5.3	6.1	5.3	0.12	**<0.001**	0.601	0.467
b*	17.6±0.4	16.4±0.2	**0.013**	16.5	16.6	16.3	16.0	16.5	16.8	0.20	0.870	0.052	0.407
C*	172.4±7.4	151.6±3.0	**0.022**	154.4	151.2	152.3	141.7	155.1	154.6	3.71	0.142	0.133	0.400
E	1555.4±28.5	1488.3±11.6	**0.042**	1483.5	1517.0	1478.6	1498.7	1465.9	1485.9	29.34	0.326	0.715	0.966
Lysine (g/kg DM)													
Total	10.8±0.2	10.7±0.1	0.528	10.6	10.6	10.8	10.6	10.8	10.9	0.15	0.979	0.300	0.502
OMIU-reactive	9.6±0.2	9.7±0.1	0.577	9.6	9.4	9.7	9.6	10.0	10.0	0.21	0.514	0.095	0.805
RL/TL-ratio	0.88±0.01	0.91±0.01	0.309	0.90	0.88	0.90	0.91	0.92	0.92	0.02	0.534	0.404	0.770
Maillard reaction products (mg/kg DM)													
Fructoselysine	366.2±51.1	538.8±20.9	**0.006**	498.0	648.7	515.2	648.1	414.5	508.1	23.37	**<0.001**	**<0.001**	0.478
ε-N-carboxymethyllysine	12.6±0.4	14.8±0.2	**0.002**	14.3	15.7	14.4	15.5	14.1	14.8	0.35	**0.003**	0.248	0.544
(5-hydroxymethyl)-2-furfural	400.5±22.3	390.5±9.1	0.682	398.9	386.3	378.0	391.3	361.1	425.2	23.35	0.294	0.939	0.282
Lysinoalanine	5.7±0.6	7.7±0.3	**<0.001**	7.3	7.5	6.4	7.3	9.4	8.1	0.43	0.788	**0.002**	0.066
Physical parameters													
Pellet hardness (kg)	-	-	-	4.9	8.5	4.3	7.0	4.9	9.0	0.19	**<0.001**	**<0.001**	**0.011**
Pellet durability (%)	-	-	-	92.5	86.6	90.8	80.7	90.3	89.6	0.54	**<0.001**	**<0.001**	**<0.001**
Bulk density (g/l)	-	-	-	631.4	540.7	613.1	513.8	634.1	569.0	3.52	**<0.001**	**<0.001**	**0.001**

OMIU, O-methylisourea; RL, reactive lysine; TL, total lysine.

[a] L* expresses brightness (100 = white, 0 = black). a* expresses a colour range from red (+) to green (-), b* expresses a colour range from yellow (+) to blue (-). $C* = (a^{*2} + b^{*2})^{1/2}$, and $E = (L^{*2} + a^{*2} + b^{*2})^{1/2}$.

Effect of conditioning temperature and die hole length on chemical parameters

Results for conditioning temperature and die hole length are described as mean value per treatment effect. Absorbance at 280 and 420 nm was not affected by either conditioning temperature or die hole length (Table 5.3). For the colour values, only $a*$ showed a significant decrease from 6.2 to 5.3 with increasing conditioning temperature from 65 to 90°C. Other colour values did not show any significant effects for conditioning temperature and die hole length. For TL, OMIU-RL as well as the RL/TL ratio, no significant effect was seen of conditioning temperature and die hole length applied. However, increasing the conditioning temperature from 65 to 90°C significantly increased the FL content from 475.9 to 601.6 mg/kg DM and the CML content from 14.3 to 15.1 mg/kg DM. Conditioning temperature did not affect HMF and LAL contents. Die hole length had a significant effect on FL content, with a die hole length of 80 mm resulting in lower FL contents compared to die hole lengths 65 and 45 mm (461.3, 581.6 and 573.3 mg/kg DM, respectively). In contrast, a die hole length of 80 mm resulted in increased LAL contents compared to die hole length 65 and 45 mm (8.8, 6.8 and 7.4 mg/kg DM, respectively). Die hole length did not affect CML and HMF contents. In addition, no significant interaction between conditioning temperature and die hole length was observed for all chemical parameters.

Effect of steam pelleting on physical parameters

Results for the physical parameters are described as mean value per treatment effect. Pellet hardness significantly increased with increasing temperature from 65 to 90°C from 4.7 to 8.2 kg (Table 5.3), and was lower for die hole length 65 mm compared to 45 and 80 mm (5.6, 6.7 and 7.0 kg, respectively). Pellet durability significantly decreased from 91.2 to 85.7% with increasing temperature from 65 to 90°C, and was lower for die hole length 65 mm compared to 45 and 80 mm (85.7, 89.6 and 90.0%, respectively). Bulk density of the pellets significantly decreased from 626.2 to 541.2 g/l with increasing temperature from 65 to 90°C, and was lowest for die hole length 65 mm, followed by die hole length 45 and 80 mm (563.5, 586.0 and 601.5 g/l, respectively). For all physical parameters, significant interactions were found between conditioning temperature and die hole length.

Discussion

Pressing the conditioned ingredient mix through the pellet dies used in this study caused a 10°C increase in product temperature compared to the set conditioning temperature. An increase in product temperature of 6 – 10°C using a conditioning temperature of 65 and 80°C and die dimensions of 4.8 × 50.8 mm was reported previously by Skoch et al.[31]

This increase in temperature can be attributed to mechanical friction between the ingredient mix and the die hole surface. Increasing length-to-diameter ratio of the die hole may increase the amount of shear which the ingredient mix receives.[17]

Absorbance and colour

The absorbance and colour of foods are used as indicators of the progression of the MR. Absorbance at 280 nm is related with early-stage, low molecular weight MRPs, while absorbance at 420 nm is used as an indicator of browning development in the advanced and final stage of the MR.[32] The absence of change in absorbance values at 280 nm due to steam pelleting, conditioning temperature and die hole length suggests that no change in early stage MRP content could be observed; either no early stage MRPs were formed during the pelleting process, or the same amount of early stage MRPs reacted further into the advanced stages of the MR. Furthermore, no change in absorbance value at 420 nm implies that no advanced and final stage MRPs were formed during the pelleting process. However, the absorbance values of the unprocessed ingredient mix was already close to 1, which indicates that a high intensity of brown colour is already present in the unprocessed ingredient mix. Absence in change of colour was supported by lightness $L*$ values, which indicate that the pellets were not considerably darker compared to the unprocessed ingredient mix. Opposite of the results in the present study, Delgado-Andrade et al.[20] reported a significant increase during pelleting for absorbance at 280 nm (from 0.527 to 0.631) and at 420 nm (from 0.032 to 0.082), and a significant decrease of $L*$ after pelleting treatment. However, it should be noted that the colour of foods is a combination of the natural colour of the ingredients and the coloured compounds that are formed during processing.[33] It may be possible that the meal of a piglet starter diet is lighter in colour due to the use of wheat flour, extruded wheat and milk whey powder as main ingredients, compared to the unprocessed ingredient mix for the dog food used in the present study containing animal proteins such as blood meal. While $a*$ did not change during steam pelleting, conditioning temperature affected the $a*$ value with increasing conditioning temperature from 65 to 90°C resulting in a decreased $a*$ value equal to a change of colour in the direction of green. Decreasing $b*$ values indicate a change of colour in the direction of blue. A decrease in E index has been previously related to the progression of the MR.[29] The decrease of the E index in the pellets in the present study indicates, in contrast to the L* value, loss of lightness during pelleting due to dark compounds that can be formed during the advanced stage of the MR. In addition to E, $C*$ values decreased significantly after pelleting. Overall, absorbance and colour values do not provide strong evidence that the MR occurs or progresses during pelleting.

Total and reactive lysine

The absence of change in absorbance value due to steam pelleting of the dry dog food was supported by the results of TL and OMIU-RL content; no changes were found between the unprocessed ingredient mix and the steam pelleted food. In addition, no effect was seen of the increased conditioning temperature and increased die hole length applied in this study. A previous study reported no differences in apparent faecal N digestibility in dogs fed either an unprocessed ingredient mixture of a dog food (77.1%) or a pelleted diet (78.4%; 52°C without steam addition, 6 × 60 mm die), while extrusion of the same ingredient mixture significantly decreased apparent faecal N digestibility (72.4%).[34] The results of the latter study indicate that the bioavailability of lysine might not be negatively affected by the MR during the pelleting process of dog foods. The RL/TL ratio observed in the present study is within the range of the reported values (0.72 − 0.94) for commercially pelleted dog foods.[3,4] The RL/TL ratio of the unprocessed ingredient mix, being 0.88, suggests that mainly the processing of the ingredients used in the recipe rather than the subsequent pelleting process underlie the RL/TL ratios for pelleted dog foods. Ingredients used in pet foods show a high variation in RL/TL ratios with RL/TL ratios of ingredients of animal origin ranging from 0.64 to 0.99, and ingredients of vegetable origin from 0.56 to 0.90.[35] Proteinaceous ingredients for pet foods are often heat-processed, as well as carbohydrate sources that are often pre-treated as the conditions used during the pelleting process are not optimal for complete starch gelatinization.[36] It seems that the choice of ingredients used in the ingredient mix, rather than the steam pelleting process applied, is responsible for RL/TL ratio observed in the dry standard dog food used in this study.

Maillard reaction products

In contrast to absorbance, colour, TL, and OMIU-RL contents, most of the analysed MRP contents display an increase during steam pelleting of the ingredient mix into pellets. Fructoselysine can be determined using furosine as an alternative to OMIU-RL.[37,38] The FL content of the current study (Table 5.3) is in the lower range found in commercially available pelleted dog foods (range 110 − 4050 mg/kg DM.[5] The FL content increased after steam pelleting, indicating the net formation of this Amadori compound during the pelleting process. This result is in line with previous results found in a pre-starter diet for suckling piglets.[20] However, FL contents in the present unprocessed dog food ingredient mix is 10 times higher compared to values reported for the pre-starter diet. Besides OMIU-RL, RL can be calculated using the FL contents.[5] Despite the increase in FL content due to the pelleting process, RL calculated using FL was not significantly different between the unprocessed ingredient mix and pelleted food (10.6 and 10.4 g/kg DM, respectively) which is in line with the OMIU-RL results. RL calculated using FL showed a

moderate but significant correlation with RL determined using the OMIU-procedure (r = 0.46; P = 0.035) and resulted in higher RL contents as was reported previously.[5] The FL content in the pelleted food is 18 times lower compared to OMIU-RL content, and changes in FL content would not be reflected equally well in the OMIU-RL values.

Steam pelleting of the ingredient mix into pellets increased CML and LAL. However, HMF was not affected. Several pathways result in the formation of CML namely directly by oxidation of FL, via the α-oxoaldehyde glyoxal that is formed by oxidation of FL or via lipid peroxidation.[39] HMF is formed by dehydration and cyclization of 3-deoxyglucosone.[40] It seems that in the present study, formation of HMF did not occur, which is in contrast to previous studies that report a significant increase in HMF content from 12.6 to 16.7 mg/kg after pelleting of a pig starter diet.[20] However, Shipe et al.[21] reported no changes in furfural concentration after pelleting a practical finisher broiler diet (steam conditioned at 82°C using a 38.1 or 44.5 mm die hole length). As formation of furfural follows the same reaction pathway as HMF, the latter results are in line with the HMF data in the present study. The CML contents of the dog food pelleted in the present study (Table 5.3) were in the lower ranges of CML contents reported in commercially available pelleted dog foods (12.0 – 40.3 mg/kg DM), while HMF contents were below the range reported (520 – 2380 mg/kg DM) in the same foods.[5] The formation of LAL includes a dehydroalanine that reacts with the ε-amino group of lysine to form a LAL-crosslink and is seen in alkali environments.[41] The LAL contents of the pelleted dog food (Table 5.3) is within the range (4.1 – 16.11 mg/kg DM) reported in commercially available pelleted dog foods.[5] Despite the fact that the pH of the product was 5.6 for both unprocessed ingredient mix and pelleted food, LAL content of the dog food increased during steam pelleting. As the amino groups of lysine are used during the formation of LAL, it would be expected that the RL content decreased. However, LAL content of the food is rather low compared to the total lysine content, so the possible small decrease in total or reactive lysine reactivity might not be measurable. The low MRP contents in the current pelleted food implies either less extreme processed ingredients or milder pelleting conditions compared to some of the commercially available pelleted foods.

The increase in MRPs during steam pelleting can be attributed to both conditioning temperature and die hole length. The increase in FL and CML content with increasing conditioning temperature from 65 to 90°C indicates that a reduced conditioning temperature during the pelleting process can reduce the progression of the MR and, therefore, possibly retain nutritive value of the food. This is in line with the general idea that increasing temperatures promote the MR.[35] The absence of an increase in HMF content due to conditioning temperature is in line with the results of HMF for the unprocessed ingredient mix vs. pelleted dog food, although literature indicates that the

formation of HMF is also a temperature/time dependent process.[42] Die hole length had an effect on FL and LAL; however, a decrease in FL content for the 80 mm die hole length is rather unexpected, as a longer die hole is thought to increase the amount of friction and shear forces on the product.[17] Opposite of the results for FL content, LAL content increased using a die hole length of 80 mm compared to die hole length 65 and 45 mm. The increase in MRPs during steam pelleting can be attributed to conditioning temperature and die hole length, however, the effect of the parameters is not consistent between the different components.

Physical pellet quality
Besides nutritional quality and safety, physical characteristics of pellets are important for food manufacturers. Transportation and handling of the product require high quality pellets without fines,[30] as fines can have a negative influence on food consumption of the animal. Pellet hardness represents the forces necessary to crush a pellet, while pellet durability is the amount of fines after mechanical or pneumatic agitation. Bulk density is used to express the weight of the product per volume, and determines the space required to store the feed materials during transport or storage. An increase in conditioning temperature and, therefore, increased amount of steam added to the ingredient mix generally improves pellet hardness and durability. Steam is superior to water in producing good quality pellets; heat induced by steam injection changes the properties of the ingredient mix which leads to harder and more durable pellets.[17,43] The latter corresponds to the pellet hardness measured in the present study, however, the results for pellet durability are opposite with increased conditioning temperature resulting in a decreased pellet durability. Skoch et al.[31] reported an increase in durability between dry-conditioned pellets and steam conditioned pellets. However, no differences in durability between the 65 and 80°C were noted (93.5 and 90.6% for 65°C and 96.5 and 93.8 for 80 and 78°C, respectively). Fat is known for its adverse effect on pellet hardness and durability due to a decreased pressure in the die which is a result of low friction.[44] The ingredient mix used in the present study contained a considerable amount of fat compared to standard pelleted livestock feeds. However, it also contained a lignosulphonate as a pellet binder (Lignobond DD; Table 5.1) to increase the physical quality of the pellets.[44] The latter could be the reason that pellet hardness and durability of the pellets in the present study are within the acceptable range. Decreasing bulk density of the pellets in the present study with increasing conditioning temperatures from 65 to 90°C indicates that transportation or storage of pellets produced using conditioning temperatures of 90°C requires more space compared to pellets produced using 65°C. Pellet hardness and durability of pellets in the present study were lower for die hole length 65 compared to 45 and 80 mm. This result is in contrast to the idea that a longer die hole length increases friction and,

therefore, improves pellet quality.[16] In line with these results, bulk density was lowest for die hole length 65 mm. Interactions found for all physical parameters between conditioning temperature and die hole length support the suggestion that several factors and combinations of factors can affect physical quality of pellets during the pelleting process.[17]

Conclusion

Steam pelleting of a dry dog food does not cause a significant loss of reactive lysine and change of absorbance values, indicating that the effect of steam pelleting on the nutritive value of pelleted dog foods is low. Analysing the unprocessed ingredient mix suggests that the choice of the ingredients used in the ingredient mix, rather than the pelleting process has an influence on the latter parameters. However, steam pelleting does increase early and advanced MRP content. The formation of MRPs can be addressed to an increase in temperature and die hole length during the steam pelleting process. The discrepancy in the results between the analyses in the processed samples indicate that analysing reactive lysine and absorbance values are not sufficiently accurate to predict a change in MRPs during processing. Whether the formed MRP contents during steam pelleting are physiologically relevant in dogs depends on the bioavailability of these components, which warrants further study.

Acknowledgements

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Chapter 6

Effect of extrusion conditions on the Maillard reaction and *in vitro* digestibility in two dry dog foods

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Abstract

During extrusion, the Maillard reaction (MR) occurs, which reduces the bioavailability of essential amino acids such as lysine and results in the formation of Maillard reaction products (MRPs). The aim of this study was to determine the effect of extrusion processing and extrusion parameters of two dry dog foods formulated using either intact or hydrolysed protein, on the progression of the MR and *in vitro* digestibility. Decreasing temperature and moisture content lead to higher total and reactive lysine contents, and lower MRPs in the dog foods. Increasing screw speed has a positive influence on total and reactive lysine, but a negative influence on MRPs. It seems that the unprocessed ingredient mix has more influence on the difference between reactive and total lysine and the presence of MRPs than extrusion processing of this ingredient mix.

Keywords: Pet food, Reactive lysine, Fructoselysine, Carboxymethyllysine, Hydroxymethylfurfural, Lysinoalanine, *in vitro* Digestibility

Introduction

Extrusion cooking is the foremost technique to produce commercial pet foods around the world. Extrusion cooking is a high temperature (80 – 200°C), short time (10 – 270 s) treatment used during pet food manufacturing to improve the nutritive properties and safety of the food, and to be able to expand, dehydrate and shape the kibbles.[1] Heat treatment can, however, also have negative effects such as a decrease in protein quality due to cross-linking and the involvement of amino acids in the Maillard reaction (MR).[2,3] During this reaction, the free reactive α-amino group of free or terminal amino acids in peptides is blocked.[3] Lysine is the most reactive amino acid as it contains a reactive ε-amino group.[4] For example, the ε-amino group of lysine can react with a dehydroalanine resulting in the cross-link lysinoalanine (LAL) or bind to fructose to form fructoselysine (FL) in the early stage of the MR. If the MR continues, advanced Maillard reaction products (MRPs) are formed such as carboxymethyllysine (CML) and hydroxymethylfurfural (HMF).[5]

It was suggested that foods for growing dogs could be at risk of not meeting minimal lysine requirement.[6] This is due to two factors. First, the reaction products of lysine (e.g. FL and LAL) cannot be metabolically utilized by the animal, thereby potentially reducing the bioavailability of lysine in pet foods.[7-9] Second, the remaining reactive lysine (RL; lysine with a free ε-amino group) is not 100% bioavailable; in a study using 5 commercial dry dog foods, apparent ileal crude protein (CP) digestibility ranged from 66 up to 83% with a mean of 76%, whereas apparent ileal total lysine (TL) digestibility ranged from 60 to 84% and RL ranged from 48 to 98%.[7,10] Therefore, CP digestibility can be decreased due to the above mentioned reactions.[11,12]

The extent to which MR or heat induced crosslinking occurred in the pet foods can be considerable, with RL to TL ratio values being as low as 0.67.[6] In addition, the content of MRPs in extruded pet foods can be as high as 1.44 g FL /kg dry matter (DM), 20.7 mg CML /kg DM, 1.92 g HMF /kg DM and 13.0 mg LAL /kg DM.[13] The MRP in these diets can originate from the extensive processing of pet foods, but may also originate from the ingredients used. Commonly used pet food ingredients are often thermally processed and contain appreciable quantities of MRPs.[10] In meat meal, for example, RL/TL ratio was found to range between 0.77 to 0.84.[14] Early MRPs already present in the ingredient mixture, could progress to become advanced MRPs during further processing. In addition, some pet foods that are specifically developed for animals with food allergies contain hydrolysed proteins rather than intact proteins. Although no commercial pet foods containing hydrolysed proteins have been evaluated for RL/TL ratios or MRP contents, it can be speculated that these foods are more vulnerable to the MR as these contain more reactive α-amino groups than intact proteins.

Several authors have indicated that endogenous as well as dietary MRP are associated with the aetiology of age-related diseases in humans, such as diabetes mellitus and impaired renal function.[15-17] Elevated MRP levels in tissue proteins were found in dogs with various age-related diseases, such as diabetes, cataract, osteoarthritis, vascular dysfunction, and atherosclerosis.[18-22] The extent to which MRPs also impact pet health, is largely unknown. However, MRPs can also exert positive effects, such as promoting flavour and improving antioxidant status. If there is a potential impact of the MR on nutritive quality and pet health, it is important to understand what extrusion conditions promote the MR.

Only a limited number of studies have focused on the effect of extrusion on the MR in pet foods, using only TL and RL as parameters.[23] Knowledge on the effect of extrusion on the formation of MRPs in pet foods is lacking. The aim of the present study was to determine the effect of extrusion processing, and the extrusion parameters temperature, moisture content and screw speed on the progression of the MR in a standard dog formulation including intact protein (IPD) and hydrolysed protein (HPD). The effect of extrusion processing on nutritive quality was assessed by RL and TL determination and by using an *in vitro* method simulating the gastric and small intestinal digestive processes in dogs. Furthermore, the progression of the MR was evaluated by quantification of LAL, FL, CML and HMF.

Materials and methods

Materials

L-lysine monohydrochloride (Fluka, Buchs, Switzerland), furosine dihydrochloride, *ε-N*-carboxymethyl-L-lysine, lysinoalanine (PolyPeptide Group, Strasbourg, France) and 5-hydroxymethyl-2-furaldehyde (Sigma-Aldrich, Steinheim, Germany) were used as external chemical standards. External standards of all other amino acids were purchased from Serva Electrophoresis (Heidelberg, Germany). Other chemicals of analytical grade were nonafluoropentanoic acid (NFPA) (Fluka), barium hydroxide octahydrate and trichloroacetic acid (TCA) (Merck, Darmstadt, Germany), hydrochloric acid 37% (HCl) and *O*-methylisourea hemi sulphate (OMIU) (Sigma-Aldrich), water, acetonitrile (ACN) and methanol (UPLC-MS, Biosolve BV, Valkenswaard, The Netherlands), and buffers for amino acid analyses (Frank Gutjar Chromatographie, Balingen, Germany). For *in vitro* digestibility measurement, the following chemicals were used: disodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$) (art. no. 658, Merck), sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$) (art. no. 6345, Merck), sodium hydroxyde (NaOH) (art. no. 6498, Merck), HCl 37% (art. no. 31, Merck), pepsin (Porcine pepsin 2000 FIP U/g, art. no. 7190, Merck), pancreatin (Porcine pancrease grade VI, P1750, Sigma-Aldrich), sulphosalicylic

acid (Sigma-Aldrich), ethanol 95% (Technisolv, VWR International), acetone 99.5% (Brenntag, The Netherlands). For the proximate analyses, the following chemicals were used: α-amylase (Sigma-Aldrich), amyloglucosidase (Megazyme, Bray, Ireland), petroleum (Avantor, BOOM, The Netherlands).

Experimental diets

Two experimental dry canine diets were formulated by pet food manufacturers. The IPD was formulated to specifications of a standard dry diet while the HPD was formulated to specification of a hypoallergenic diet, although only 62% of the animal derived proteins were replaced by hydrolysed protein. Ingredient composition and analysed nutrient composition of both experimental diets are shown in Table 6.1. Prior to extrusion, an appropriate amount of water was weighed and mixed with the ingredients in a Günther-Papenmeier KG pilot mixer, type GHK 20 (Machinen- und Apparatebau, West Germany) to obtain either 200 or 300 g/kg moisture; mixing time was 120 sec for the 200 g/kg and 180 sec for the 300 g/kg.

Experimental design

Per diet, a 2×2×2 factorial study design was used with treatment factors being the extruder temperature (set at 140 and 165°C), feed moisture content (200 and 300 g/kg), and screw speed (100 and 135 rpm). Extruder settings were kept constant and all extruder parameters were monitored during sampling (Table 6.2). Each treatment combination was performed in triplicate.

Extrusion process

The experiments were performed at the facilities of Wageningen University (Wageningen, the Netherlands) on a pilot-scale Battenfeld single-screw extruder (Battenfeld-cincinnati, Bad Oeynhausen, Germany) using a screw with a length to diameter ratio of 26:1, a compression ratio of 1 to 1.1 and a constant pitch of 17 mm. A die with one orifice (4 mm ø, 16 mm length) was used. Temperatures in the eight different sections of the extruder were measured using thermocouples. By the aid of the thermocouples and heaters in the barrel wall, the temperature of the product at the die was adjusted to the desired temperature, however, in practice the product temperatures were lower than the set temperature (Table 6.2). Pressure was recorded in the die during the extrusion process, and throughput was calculated by weighing the ingredient mix that was collected at the feeder screw for 30 s. The unprocessed ingredient mix was sampled for analyses. In addition, representative processed samples were collected after extrusion conditions reached a steady state for at least 5 min. Temperature of the final product

Table 6.1. Ingredient and chemical composition of the intact (IPD) and hydrolysed protein diet (HPD).

IPD		HPD	
Ingredient composition (g/kg)			
Poultry meal	345.0	Wheat	453.9
Corn	287.5	Corn	300.0
Rice	161.0	Hydrolysed fish meal	112.5
Beet pulp	63.3	Poultry meal	70.0
Greaves meal	57.5	Beet pulp	30.0
Maize gluten	46.0	Vitamin premix	10.0
Sodium chloride	10.4	Yeast	10.0
Vitamin premix	8.6	Chalk	7.3
Potassium chloride	8.1	Calcium phosphate	6.2
Mineral premix	6.9		
Choline chloride	5.8		
Chemical composition (g/kg DM[a])			
Crude protein (N × 6.25)	357.5	Crude protein (N × 6.25)	243.4
Starch	335.6	Starch	508.3
Neutral detergent fibre	90.7	Neutral detergent fibre	87.4
Crude fat	79.3	Crude fat	61.1
Crude ash	99.9	Crude ash	58.4

[a] DM content of the IPD and HPD was 916.8 g/kg and 908.9 g/kg.

Table 6.2 Measured extruder parameters of the intact (IPD) and hydrolysed protein diet (HPD) monitored during sampling

	IPD			HPD		
Extruder settings[b]	Temperature[a] (°C)	Pressure (kPa)	Throughput (g/min)	Temperature[a] (°C)	Pressure (kPa)	Throughput (g/min)
140/200/100	107	4500	254	111	2707	182
140/200/135	108	4650	254	110	2817	196
140/300/100	101	2100	190	102	2370	207
140/300/135	99	2100	160	101	2270	196
165/200/100	113	3630	160	121	2689	212
165/200/135	117	4000	160	120	2530	248
165/300/100	112	1800	176	115	1363	236
165/300/135	110	1800	176	116	1467	210

[a] Product temperature measured with thermocouple at the die opening.
[b] Temperature (°C)/ moisture (g/kg)/ screw speed (rpm).

after the die was measured using a Raytek Raynger ST infrared meter. The screw speed was changed between sampling of the replicate treatments to disturb the steady state of the extruder. After resetting the screw speed, the next replicate was sampled, 5 min after reaching the new steady state. After extrusion, all extruded strips were dried at 50°C for 4 h. The dried samples were ground to pass a 1 mm screen in a centrifugal mill (Retsch ZM100, Retsch BV, Ochten, The Netherlands) and all samples were stored at 4°C prior to analyses.

In vitro digestibility

A two-step multi-enzymatic incubation method of Boisen and Fernandez[24] adapted to the canine gastro-intestinal tract as described by Hervera et al.[25] simulating the canine gastric and small intestinal digestive processes was used to determine *in vitro* digestibility. Only samples processed at 135 rpm screw speed were analysed for *in vitro* digestibility. For each sample, 10 g was incubated in beakers with a phosphate buffer solution (250 ml, 0.1 M, pH 6.0) and a HCl solution (30 ml, 0.2 M). The pH was then adjusted to 2.0 with 1 M HCl or 10 M NaOH. Fresh pepsin solution (10 ml, 25 g/l) was added, each beaker was covered and placed in a heating chamber at 39°C for 2 h under constant magnetic stirring. Then, 100 ml phosphate buffer (0.2 M, pH 6.8) and 40 ml of a 0.6 M NaOH were added into the solution. The pH was adjusted to 6.8 with 1 M HCl or 10 M NaOH. Fresh pancreatin solution (10 ml, 100 g/l) was added and incubation was continued for 4 h under the same conditions. After incubation the samples were cooled to room temperature and 5 ml 20% sulphosalicylic acid was added to precipitate solubilized but undigested proteins. The undigested residue of the slurry was collected by filtration on a nylon gauze (37 μm) folded in a Büchner porcelain funnel. The residue was washed twice with acetone (99.5%) followed by ethanol (96%). Then the cloth with the residue was placed on a clean piece of paper to evaporate the remaining ethanol/acetone overnight. The residue was collected from the nylon cloth, freeze-dried and prepared for chemical characterization.

Chemical analyses

The chemical analyses of the unprocessed ingredient mixtures, extruded samples and *in vitro* residues were performed in duplicate. DM was determined by drying to a constant weight at 103°C;[26] crude ash (CAsh) by combustion at 550°C.[27] Organic matter (OM, %) was calculated as 100 % – CAsh %. Nitrogen content was determined in 5 mg of sample by DUMAS[28] in a FlashEA® 1112 N/Protein Nitrogen and Protein Analyser (ThermoFisherScientific, 2007). CP results were calculated using N × 6.25. Crude fat (CF) was determined gravimetrically after hydrolysis with HCl and extraction with light petroleum ether (boiling point 40 – 60°C).[29] Neutral detergent fibre (NDF) was analysed

with the addition of α-amylase and without sodium sulphite according to the method of van Soest et al.[30] Starch was determined through enzymatic hydrolysis using amyloglucosidase.[31] Total lysine, RL, FL, CML, HMF and LAL were determined as described previously.[13] Digestibility values for DM, OM, CP, TL and RL were calculated according to eq. 1 (DM as example):

$$\text{Digestibility (\%)} = (g\,DM_{diet} - g\,DM_{residue})/g\,DM_{diet} \times 100 \hspace{2cm} (1)$$

Statistical analyses

Statistical analyses were conducted using SAS 9.2 for Windows (SAS Institute Inc., Cary, NC, USA). Data analysis to identify extrusion effects was performed by ANOVA using the Proc GLM procedure. Normal distribution of the residuals was evaluated with and confirmed by the Sharpiro-Wilk test. Effect of extrusion (unprocessed ingredient mix vs. extruded diet) was tested using the model $Y_i = \mu + TR_i + \varepsilon_i$, where Y_i = parameter to be tested, μ = mean, TR_i = treatment effect i, and ε_i = error term. Within extrusion treatments the effects of extrusion temperature, moisture content and screw speed was tested using the model $Y_{ijk} = \mu + T_i + M_j + S_k + (T \times M)_{ij} + (T \times S)_{ik} + (M \times S)_{jk} + (T \times M \times S)_{ijk} + \varepsilon_{ijk}$, where Y= parameter to be tested, μ = mean, T_i = effect of temperature i, M_j = effect of moisture content j, S_k = effect of screw speed k, and ε_{ijk} = error term. For the effects of extrusion on *in vitro* digestibility, S_k and interaction effects with S_k were omitted the model. For comparisons of least square means, P-values were adjusted for multiple comparisons using the Tukey-Kramer adjustment. Overall differences were considered significant if $P \leq 0.05$.

Results and discussion

Diet formulation and ingredient composition

The unprocessed IPD contained less TL and RL than the unprocessed HPD (Table 6.3), which is a direct result of diet formulation and ingredient composition. For both unprocessed diets the RL content was lower than the TL content, resulting in a RL/TL ratio of 0.90. The latter indicates that 10% of the ε-amino groups of lysine present in both unprocessed ingredient mixtures were bound to other compounds which were released upon acid hydrolysis. It is known that some of the ε-amino groups of lysine in common pet food ingredients has reacted prior to processing.[10] Most pet food manufacturers use co-products of other industries as primary ingredients which may have been processed to varying degrees before being used as ingredients in pet foods.[32] Many animal protein sources are manufactured by a rendering process, where temperatures as high as 130°C are used for several hours.[33] In these ingredients of animal origin, the difference between RL and TL can be up to 36.0%. Vegetable ingredients are often dried and ground before

Table 6.3 Effect of extrusion on total lysine (TL), *O*-methylisourea reactive lysine (RL), RL/TL ratio, fructoselysine (FL), carboxymethyllysine (CML), hydroxymethylfurfural (HMF) and lysinoalanine (LAL) of an intact (IPD)[a] and hydrolysed protein diet (HPD)[a] (LSmeans; mg/16gN unless defined differently).

Diet	Parameter	Unprocessed	Processed	P-value	Temperature 140	Temperature 165	Moisture 200	Moisture 300	Screw speed 100	Screw speed 135	Pooled SEM	T	M	S	T×M	T×S	M×S	T×M×S
IPD	TL[b]	4.93±0.08	4.79±0.03	0.127	4.86	4.72	4.85	4.73	4.74	4.84	0.03	**0.004**	**0.011**	**0.036**	0.583	0.882	0.420	0.125
	RL[b]	4.43±0.07	4.38±0.02	0.555	4.45	4.32	4.40	4.36	4.35	4.42	0.03	**0.007**	0.324	0.074	0.365	0.432	0.867	**0.013**
	RL/TL	0.90±0.02	0.91±0.01	0.491	0.91	0.91	0.91	0.92	0.91	0.91	0.01	1.000	0.138	1.000	1.000	0.489	0.273	**0.003**
	FL[c]	101.1±75.9	339.9±26.8	**0.008**	307	373	251	428	269	410	13	**0.003**	**<0.001**	**<0.001**	0.528	**0.003**	**0.001**	0.143
	CML	2.4±0.3	3.8±0.1	**<0.001**	3.5	4.1	3.7	4.0	3.5	4.1	0.05	**<.001**	**<0.001**	**<0.001**	0.124	**0.024**	**0.001**	1.000
	HMF	125.5±7.0	140.9±2.5	**0.047**	146	136	137	145	138	144	2.47	**0.010**	0.054	0.155	**0.003**	0.486	0.665	0.148
	LAL	1.7±0.1	1.6±0.1	0.684	1.7	1.6	1.60	1.6	1.5	1.7	0.04	0.191	0.765	**0.004**	0.416	0.990	0.825	0.631
HPD	TL[b]	5.74±0.08	5.41±0.03	**<0.001**	5.48	5.35	5.44	5.38	5.36	5.46	0.03	**0.008**	0.197	**0.041**	0.590	0.540	0.642	0.164
	RL[b]	5.13±0.11	4.85±0.04	**0.024**	4.98	4.72	4.92	4.78	4.83	4.86	0.04	**<0.001**	**0.017**	0.556	0.597	0.303	0.171	0.189
	RL/TL	0.90±0.02	0.90±0.01	0.984	0.91	0.88	0.91	0.89	0.90	0.89	0.01	0.064	0.224	0.445	0.953	0.321	0.517	0.859
	FL[c]	74.1±164.2	562.4±58.0	**0.010**	523	602	279	846	533	592	7.29	**<0.001**	**<0.001**	**<0.001**	**0.001**	**0.002**	**0.004**	0.882
	CML	3.4±1.0	7.0±0.4	**0.002**	6.3	7.7	5.4	8.5	6.8	7.2	0.08	**<0.001**	**<0.001**	**0.001**	**0.001**	0.890	**0.035**	**0.004**
	HMF	203.9±19.7	277.5±7.0	**0.002**	284	271	257	298	280	275	6.47	0.190	**<0.001**	0.588	**0.010**	0.157	0.496	0.528
	LAL	1.5±0.1	1.9±0.1	**<0.001**	1. 9	2.0	2.0	1.9	1.9	1.9	0.03	0.058	0.417	0.973	**0.006**	0.077	0.766	**0.035**

[a] Unprocessed n = 3; Processed n = 24.
[b] g/16gN.
[c] Calculated as furosine / (32/100).[43]

Table 6.4 Effect of extrusion on *in vitro* digestibility of crude protein (CP), total lysine (TL), *O*-methylisourea reactive lysine (RL), dry matter (DM) and organic matter (OM) of the intact protein diet (IPD)[a] and hydrolysed protein diet (HPD)[a] (LSmeans ± SE, %).

Diet	Parameter	Unprocessed	Processed	P-value	Temperature		Moisture		Pooled	P-value		
					140	165	200	300	SEM	T	M	T×M
IPD	CP	86.8±0.62	88.0±0.31	0.103	88.0	88.0	87.1	88.9	0.28	0.962	**0.002**	0.341
	TL	87.9±0.43	88.3±0.22	0.364	87.8	88.9	88.4	88.2	0.22	**0.005**	0.588	0.232
	RL	91.8±0.33	91.8±0.17	0.927	91.5	92.2	91.7	91.9	0.20	**0.022**	0.317	0.101
	DM	79.5±0.51	80.4±0.26	0.123	80.2	80.6	80.3	80.5	0.39	0.545	0.808	0.596
	OM	84.0±0.53	85.4±0.26	**0.040**	85.4	85.4	85.3	85.5	0.43	0.944	0.729	0.886
HPD	CP	94.1±0.32	93.1±0.16	**0.017**	93.2	93.1	93.2	93.0	0.26	0.866	0.611	0.156
	TL	94.1±0.21	93.5±0.10	**0.027**	93.6	93.4	93.4	93.6	0.13	0.242	0.313	0.215
	RL	95.9±0.15	94.8±0.08	**<.001**	94.9	94.6	94.7	94.9	0.09	**0.039**	0.152	0.150
	DM	83.2±0.33	85.8±0.17	**<.001**	85.7	85.8	85.7	85.8	0.23	0.773	0.809	**0.047**
	OM	85.9±0.27	88.0±0.13	**<.001**	88.0	88.1	88.0	88.1	0.21	0.938	0.936	0.231

[a] Unprocessed n = 3; Processed n = 24.

inclusion in the pet food recipe. The difference between RL and TL in ingredients of vegetable origin ranged from 0 to 44.0%, where cereals have a larger difference compared to non-cereal ingredients of vegetable origin.[10] Besides a difference between RL and TL, the unprocessed ingredient mixtures of both experimental diets contained MRPs (Table 6.3), indicating progression of the MR. Although the presence of MRPs has been reported in several foodstuffs,[34-36] there is limited information on the presence of MRPs (mainly furosine and LAL) in feedstuffs.

The unprocessed HPD had a higher digestibility compared to the unprocessed IPD for all parameters analysed (Table 6.4). A plausible cause of this difference is ingredient composition. Wheat is the major ingredient in the HPD followed by corn, whereas the IPD contained corn and rice (Table 6.1). Diets including wheat resulted in higher ileal digestibility values of DM, OM and CP, compared to diets including rice and corn when fed to dogs.[37] Therefore, the 45% wheat included in the HPD may have a major effect on the overall *in vitro* digestibility. Animal proteins are more easily digested than vegetable proteins;[38] but although approximately 40% of the IPD consist of animal proteins compared to approximately 18% of the HPD, the CP in the unprocessed HPD ingredient mix was more digestible (Table 6.4). Hydrolysis of proteins into smaller peptides and amino acids causes a more easy hydrolysis by peptidases, increasing solubility which results in improved digestibility compared to intact proteins.[39,40] The hydrolysed protein in the HPD, therefore, could be the cause of the higher *in vitro* CP digestibility of the HPD compared to the IPD. In addition, inclusion of beet pulp in an extruded dog food decreased ileal digestibility of DM, OM and CP.[41,42] Even though the amount of beet pulp was low in both diets (< 7%), the amount of beet pulp in the IPD was two times higher

compared to the HPD, possibly reducing the *in vitro* DM, OM and CP digestibility of this diet.

Effect of extrusion processing on nutritive value

Extrusion processing did not affect TL and RL contents in the IPD, but lowered these contents in the HPD (5.74 to 5.41 and 5.13 to 4.85 g/16gN, respectively). This result might be due to the hydrolysed fish meal that was included in the HPD recipe; hydrolysed proteins contain peptides < 10 kDa, resulting in more reaction sites for the MR to occur. It was, therefore, expected that this recipe is more susceptible for the MR during extrusion. A decrease in TL and RL after extrusion was also reported by Tran,[44] who extruded an experimental dry canine diet at 120°C and 300 g/kg moisture resulting in a reduction of TL (10.43 to 9.37 mg/g DM) and RL (7.84 to 6.99 mg/g DM). The molecular size of the dietary proteins used in the latter study were not reported. Despite the lower TL and RL contents after extrusion in the present study, RL/TL ratio did not change during extrusion. The RL/TL ratio is in the higher range compared to previous studies; reported means of extruded adult dog foods were 0.90 and 0.85, and of extruded junior dog foods 0.87 and 0.75.[6,32] These results are consistent with the general consensus in literature that thermal processing decreases lysine content.[2,45,46] The reduced protein quality was, besides the reduced TL and RL contents and the formation of MRPs, also noted by the reduced *in vitro* digestibility; CP, TL and RL digestibility decreased significantly in the HPD (Table 6.4). Protein digestibility in the IPD, however, did not change as a result of extrusion. Varying results are also found in literature; extrusion of an experimental dry dog food did not have an effect on *in vitro* small intestinal digestibility of protein being 48% for the unprocessed diet and 49% for the extruded diet, although the overall digestibility was rather low.[23] However, extrusion (140°C) of a standard dog diet containing animal meal as the main protein source decreased apparent faecal CP digestibility in dogs from 77.1 to 72.4%.[47] Hindgut fermentation that is included in the apparent faecal digestibility methodology could possibly cause differences in results,[7] as well as different protein sources. The difference between *in vitro* digestibility results of CP, TL and RL could be either caused by a slightly higher product temperature during extrusion of the HPD despite the similar extruder settings (Table 6.2), or by the fact that hydrolysed proteins are more susceptible to the MR, thereby, reducing the digestibility of the protein. *In vitro* digestibility values of OM increased in the IPD and of DM and OM in the HPD during extrusion processing (Table 6.4). As protein digestibility is decreased, it seems feasible that the increase in DM and OM digestibility is due to starch gelatinization that takes place during the extrusion process and results in higher starch digestibility.

Effect of extrusion on the progression of the Maillard reaction
In both unprocessed diets, MRPs were found. Despite the lack in change of TL and RL in the IPD during extrusion, FL, CML and HMF increased significantly (Table 6.3). It should be noted that an increase in MRP does not have to coincide with changes in total and reactive lysine. For the HPD, all FL, CML, HMF and LAL significantly increased during extrusion processing. An increase in FL and HMF, both intermediate components of the MR, indicates that these components are formed faster than they disappear due to progression into further stages of the MR. The increase in MRP during extrusion was reported previously in pellets for rats that were used in a digestibility study. In the latter study, the extruded protein diet contained significantly less furosine (137.9 mg/16gN vs. 561.3 mg/16gN), but more CML (151.3 vs. 16.7 µg/16gN) and LAL (6294.9 vs. 777.3 µg/16gN) compared to the un-extruded protein diet.[48] Unfortunately, extrusion conditions were not provided by the authors. As FL, measured by furosine, is an intermediate compound, it might be already converted into advanced MRPs.

Effect of extrusion conditions on the Maillard reaction
Temperature Increasing extrusion temperature from 140 to 165°C resulted in significant lower TL and RL for both diets (Table 6.3), thereby decreasing the protein quality of the food by the reduction of this essential amino acid. It should be noted that the product temperature was, on average, 35 to 50°C lower than the set extruder temperature (Table 6.2). In general, it is known that the rate of the MR increases with temperature and time as indicated in model systems. Effect of extrusion temperature on the MR in pet food was also found by Lankhorst et al.;[23] TL decreased from 8.4 to 8.1 g/kg with increasing the extrusion temperature of an experimental dog food from 110 to 150°C. However, in the latter study, RL increased from 7.1 to 8.0 g/kg for which the underlying mechanism causing this increase was left unexplained. In a study using pelleting to process an experimental dog food, no effect was seen of increased conditioning temperature from 65 to 90°C.[49] These results indicate that an increased extrusion temperature does not always result in a decrease in TL and RL in dog foods and might depend on the temperature differences. FL and CML increased with increasing extrusion temperature for both diets in the present study, an effect that was also reported when pelleting an experimental dog food.[49] However, LAL only increased in the HPD and HMF decreased in the IPD. Besides the effect of temperature on TL, RL and MRPs, increased temperature settings resulted in a significant decrease in *in vitro* digestibility of RL in the HPD, further reducing the protein quality although the present reduction of 0.3% will be not biologically relevant. In contrast to the HPD, a significant increase in *in vitro* digestibility with increasing temperature was observed for TL and RL in the IPD (Table 6.4); an increase in digestibility partly corrects for the loss of nutritive value due to loss of lysine.

As mentioned previously, it might be possible that the ingredients used in the recipe can affect digestibility of TL and RL. The *in vitro* digestibility results found here are in contrast to the results of Ljøkjel et al.,[50] where increasing temperature from 100 to 150°C decreases total lysine true total tract digestibility from 95.7 to 94.7% in a fish-meal-based diet fed to mink. No effect of increasing extrusion temperature on *in vitro* ileal digestibility of DM, CP and OM was observed for both diets in the present study (Table 6.4). This is in agreement with Øverland et al.,[51] who did not find effects of extrusion of a dog diet using mild (104°C) vs. moderate (126°C) extrusion temperatures on apparent digestibility of DM, CP, CF, CAsh and starch in mink. In addition, *in vitro* small intestinal protein digestibility of an experimental dry dog food was not affected by extrusion temperatures of 110°C with a digestibility of 50% vs. 150°C with a digestibility of 49%.[23]

Moisture Moisture is necessary for the Maillard reaction to take place,[45] but moisture also inhibits the browning reaction at high concentrations in model systems. In the present study, increasing the moisture level from 200 to 300 g/kg significantly decreased RL in the HPD, while TL decreased in the IPD. Despite the fact that some literature reports less lysine damage with increasing food moisture levels,[45,46,52] our results are in agreement with the data of Lankhorst et al.,[23] Hendriks et al.[53] and Pham and Delrosario[54] where increasing moisture level negatively affects lysine content. Increase in moisture content only resulted in a significant increase of CP digestibility for the IPD. FL and CML increased with increasing moisture content for both diets, while HMF only significantly increased in the HPD.

Screw speed Increasing screw speed from 100 to 135 rpm significantly increases TL content in both diets, indicating a slower reduction of TL with a higher screw speed. Increasing screw speed increases the shear force in the barrel, but decreases the residence time of the product in the extruder which may limit the exposure to adverse conditions. Pham & Delrosario[54] and Iwe et al.[55] report a positive effect of increasing screw speed on RL for cowpeas, mung beans and a mixture of soya and sweet potato, however, no literature is available that reports an effect of screw speed on TL. FL and CML also show an increase with increasing screw speed, which is rather unexpected regarding the higher TL.

In conclusion, to be able to reduce the amount of MRPs in dog foods, one should already start at the choice of ingredients, as there was already lysine damage and were already MRPs found in the unprocessed ingredient mix. Extrusion processing of the ingredient mix in general results in a further progression of the MR as indicated by a decrease in TL and RL content and an increase in FL, CML, HMF and LAL, resulting in a decrease in protein quality of the food. However, this effect was more pronounced in the HPD compared to the IPD, indicating that the result of extrusion processing depends for a

large part on the type of ingredients included in the recipe. The progression of the MR can be reduced during the extrusion of dog foods by a reduction in temperature and moisture content.

Acknowledgements

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Chapter 7

Urinary excretion of dietary Maillard reaction products in healthy adult female cats

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Abstract

During processing of foods, the Maillard reaction occurs, resulting in the formation of advanced Maillard reaction products (MRP). Varying amounts of MRP have been found in commercially processed pet foods. Dietary MRP can be absorbed and contribute to the endogenous pool of MRP, and possibly the aetiology of age-related diseases. The aim of the present study was to determine urinary excretion of dietary MRP in cats fed commercial moist and dry foods. A pilot study with 10 cats, conducted to determine the adaptation time required for stable urinary excretion of MRP when changing to a diet with contrasting MRP content, showed an adaptation time of 1 d for all components. In the main study, 6 commercially processed dry and 6 moist diets were fed to 12 adult female cats in two parallel randomized, 36-day, Latin square designs. Urine was collected quantitatively using modified litter boxes, and fructoselysine (FL), carboxymethyllysine (CML) and lysinoalanine (LAL) were analysed using UHPLC-MS. Daily urinary excretion of FL and CML showed a positive relationship with daily intake in the dry (P = 0.03 and P < 0.01, respectively) and moist (P < 0.01) foods. For LAL, no significant relationship was observed. Urinary recovery (% ingested) showed a negative relationship with daily intake for FL, CML and LAL in the dry (P < 0.01, P < 0.01 and P = 0.08, respectively) and for CML and LAL in the moist (P < 0.01) foods. The observed increase in urinary excretion with increasing dietary intake indicates that dietary MRP were absorbed from the gastro-intestinal tract of cats and excreted in the urine. The adaptation time with change in diet indicates a likely effective excretion of MRP. Minimum apparent absorption of FL, CML and LAL was found to range between 8 to 23%, 25 to 73% and 6 to 19%, respectively. The observed decrease in urinary recovery suggests a limiting factor in digestion, absorption, metabolism or urinary excretion. This study shows that dietary MRP in commercial diets are absorbed and excreted via the kidneys in cats.

Key words: Dietary Maillard reaction products, Cat, Carboxymethyllysine, Fructoselysine, Metabolic transit, Urinary recovery

Introduction

Pet cats and dogs are often fed commercially processed foods throughout their lives.[1] Processing induces the Maillard reaction where reducing sugars bind to amino acids like lysine, yielding various Maillard reaction products (MRP) including fructoselysine (FL) and carboxymethyllysine (CML). Lysine can also react with dehydroalanine residues originating from degradation of serine and cysteine to form lysinoalanine (LAL).[2,3] These compounds are also endogenously formed during naturally occurring processes in body tissues.[4] Elevated MRP levels in tissue proteins are associated not only in humans, but also in dogs with various age-related diseases, such as diabetes, cataract, osteoarthritis, vascular dysfunction, and atherosclerosis.[5-9] Dietary MRP can be absorbed and contribute to the endogenous pool in human subjects.[10,11] The absorptive capacity depends on whether MRP are present in a free or protein-bound form, the latter have to first be released by proteolytic enzymes.[12] Once absorbed, dietary MRP can be metabolized or directly excreted in urine. Maillard reaction products have been reported in commercially processed pet foods.[13] Whether these MRP are present in a free or protein-bound form is unknown, and information on the absorption of dietary MRP by the gastro-intestinal tract of cats and dogs is lacking. The aim of the present study was to determine urinary excretion of dietary MRP in cats fed commercial moist and dry foods. A pilot study determined the adaptation period required for stable urinary excretion when changing to a diet with contrasting MRP content. The main study involved 6 commercial dry and moist cat foods varying in MRP content to determine urinary excretion of dietary FL, CML and LAL.

Materials and methods

Animals and housing

The studies were conducted at the feline unit of Wageningen University, Wageningen, The Netherlands. Animal housing, care and experimental procedures were approved by and conformed to the requirements of the Ethical Committee for Animal Experiments of Wageningen University (authorization number 2013/45 for the pilot study and 2013/68 for the main study) and of the WALTHAM Centre for Pet Nutrition Ethical Review Committee (July 2013). Before initiation of the study, all cats received a veterinary examination including blood analyses for standard haematology and biochemistry values and were deemed healthy. All cats were habituated to, and trained for, the experimental procedures prior to the study.

During the adaptation periods, the cats were housed in groups in rooms (10.4 m^2) with access to an outdoor area of 5.4 m^2. Usable space was increased with multiple shelves varying in height containing rest areas. Surfaces were provided for the deposition of

olfactory and visual signals and for claw abrasion (e.g. scratch posts, rush matting, pieces of carpet, wood). Toys were provided and exchanged on a regular basis. During the collection periods, the cats were housed individually in a metabolism cage overnight (1700 – 0900 h) and for one hour (1200 – 1300 h) during day time feeding. During the remaining time, cats were housed in the group rooms under constant supervision to ensure identification of urine voided. The metabolism cages were constructed of Trespa panels and an aluminium front frame and were 0.80 × 1.00 × 0.75 m. The front contained a feeding and water bowl and in the back corner of the cage a removable litter tray (29 × 29 × 12 cm) was securely positioned sloping to one side. Urine collection was performed using a modified litter box as described by Hendriks et al.,[14] in which the stainless steel wire mesh was replaced by a solid plastic bottom containing a row of 1.5 mm holes at the lowest point. The top tray contained ± 200 g polyethylene grains (diameter 2 to 4 mm) to allow cats to express normal behaviour of covering faeces. The bottom tray of the litter box contained 5 mL boric acid (50 g/L) to immediately conserve the urine. Identical litter trays were also provided to the cats in the group rooms.

Dry and moist foods were obtained from a single-batch. For each food, the content was collected, pooled and homogenously mixed before a representative sample was taken from each food for chemical analyses. Homogenized foods were stored in air tight plastic bags with dry foods stored at room temperature and moist foods at -20°C.

Pilot study

Ten adult healthy European shorthair cats (intact females, 5 to 6 years of age) with an average BW of 3.1±0.07 kg (range, 2.6 – 3.3 kg) were divided into 2 groups of 5 cats each and were fed two commercially available moist foods. The foods were chosen based on their expected contents of MRP based on previously conducted analyses,[13] and analysed for their actual MRP content (Table 7.1). The foods were fed for 20 d, consisting of an adaptation period of 10 d to the first food, and a collection period of 10 d, starting at the change of the two foods. One group changed from food A to food B at d 11, the other group from food B to food A. Food intake to maintain a stable body score condition of each cat was determined before the study (score C/D on the WALTHAM[TM] S.H.A.P.E.-BCS system), and food allowance during the study was adjusted during the study if required. Individual food intake was recorded daily and fresh water was provided *ad libitum.* Urine was collected from the bottom tray of the litter box, weighed and stored (-20°C). On d 8 to 10, collected urine was pooled per cat to determine base value before diet change whereas on d11 to 20, urine produced per cat was collected daily and stored (-20° C). The acidified urine samples were analysed for specific gravity and the content of FL, CML, and LAL. During housing in the group rooms during the day, urine produced was also collected if voided.

Table 7.1 Chemical composition, ME and Maillard reaction product content of the commercial cat foods used in the pilot and main study (g/kg as-fed, unless defined differently).

| | Pilot study | | Main study | | | | | | | | | | | | |
| | Moist food | | Dry food | | | | | | Moist food | | | | | |
Component	Food A	Food B	1	2	3	4	5	6	1	2	3	4	5	6
DM	211	275	944	958	927	918	934	953	151	208	192	270	168	211
CP	110	135	329	333	339	307	356	491	68	89	76	130	92	110
Crude fat	65	62	131	184	213	106	208	201	51	68	43	60	47	65
Crude ash	20	20	87	53	75	80	65	120	18	9	19	19	22	20
Crude fibre	5	8	20	13	14	30	11	15	5	0	3	7	6	5
Nitrogen free extract[1]	10	51	377	376	287	395	293	126	8	41	51	53	1	10
ME, MJ/kg[2]	4.1	4.9	15.0	16.9	16.7	14.1	16.9	16.2	2.9	4.3	3.4	4.8	3.0	4.1
Maillard reaction product, mg/kg as-fed														
Fructoselysine[3]	70.0	338.7	741.6	418.1	1,028.1	1,114.4	1,210.2	1,122.8	236.7	208.9	194.6	1,041.1	206.3	218.7
Carboxymethyllysine	26.4	24.0	85.9	48.3	108.5	110.4	68.9	79.0	16.2	14.0	28.1	29. 7	23.5	26.4
Lysinoalanine	55.4	11.1	105.8	78.7	78.1	95.6	117.5	144.8	14.8	31.7	51.0	18.6	29.5	55.5

[1]1000 – (moisture + CP + crude fat + crude ash + crude fibre).
[2]14.6 × CP + 35.6 × crude fat + 14.6 × nitrogen free extract.[15]
[3]Furosine / (32/100).[16]

Main study

Twelve adult healthy European shorthair cats (intact females, 5 to 6 years of age) with an average of 3.1±0.09 kg BW (range, 2.7 – 3.7 kg BW) were allocated to one of two parallel balanced Latin square designs. One Latin square consisted of 6 cats receiving 6 commercially processed dry foods whereas the other 6 cats receiving 6 commercially processed moist foods (Table 7.1). The foods were selected for contrasting MRP content from 20 different pet foods, all obtained from single batches and different manufacturers. All 20 foods were analysed for MRP content prior to the selection of the 12 diets used. Each food was fed for a 6 d period, consisting of a 3 d adaptation and 3 d urine collection period. Food intake to maintain a stable body score condition of each cat was determined before the study (score C/D on the WALTHAM[TM] S.H.A.P.E.-BCS system), and food allowance was adjusted during the study based on the calculated energy content of the food, if required. Individual feed intake was recorded daily and fresh water was provided *ad libitum* daily. Urine of each cat was collected from the bottom tray into a single bottle and weighed, pooled over three days, and stored at -20°C. The acidified urine samples were analysed for specific gravity, FL, CML and LAL. During the day, urine produced was also collected if voided.

Chemical analyses

Moist foods were freeze dried, and all foods were ground to pass a 1 mm sieve in a Retch Mill (ZM100, Retch BV). All samples were stored in air-tight plastic containers at 4°C prior to analyses. The foods were analysed for DM and crude ash (CAsh) by drying to a constant weight at 103°C[17] and combustion at 550°C,[18] respectively. CP (N × 6.25) was determined using the Kjeldahl method[19] and crude fat (CFat) was determined gravimetrically after hydrolysis with HCl and extraction with light petroleum (boiling point 40 to 60°C).[20] Crude fibre (CFibre) was determined gravimetrically as the remaining insoluble organic fraction after acid and alkaline digestion.[21] All analyses were performed in duplicate. ME (MJ/kg) content of the foods was calculated using modified Atwater factors: 14.6 × CP + 35.6 × CFat + 14.6 × NFE; nitrogen-free extract (NFE) in g/kg was calculated as 1000 – Moisture – CFat – CP – CAsh – CFibre.

Specific gravity of the acidified urine was determined during sample collection by measuring the weight of 1 mL urine. The measurement was repeated 5 times and the average was calculated.

The MRP furosine (as an indirect measurement for FL, see below), CML, and the cross-linked amino acid LAL were analysed in food and urine samples. Food samples were defatted by extraction with light petroleum ether without acid hydrolysis[20] and finely ground using a mixer mill (Retsch MM2000, Retsch BV). Food samples (10 mg) and freeze

dried urine samples (0.5 mL) were hydrolysed using 1.0 and 0.5 mL of 6 M HCl, respectively, during 23 h at 110°C in glass tubes that were sealed under vacuum. The tubes were opened and dried under vacuum (Savant SpeedVac Plus, SC210A). The samples were redissolved in respectively, 1.0 and 0.5 mL UPLC-grade Milli-Q water, vortexed, sonicated and filtered (0.2 µm). Samples were analysed using an Accela UHPLC System (Thermo Scientific, San Jose, CA, USA) using an Acquity UPLC BEH 300 Amide column (2.1 × 150 mm, 1.7 µm particle size) with an Acquity BEH Amide Vanguard precolumn (2.1 × 50 mm, 1.7 µm particle size). Eluent A was Millipore water, containing 1% (v/v) formic acid and eluent B was acetonitrile containing 1% (v/v) formic acid. The solubilized urine samples were diluted 50 times in 10 mM HCl containing 0.5 mg/L $^{13}C_6$$^{15}N_2$-lysine (Sigma-Aldrich, Steinheim, Germany) as internal standard, and centrifuged (5 min, 19,000 × g, 20°C). Supernatants (1 µL) were injected onto the column, which was maintained at 35°C. The elution profile used was as follows: 0 – 2 min isocratic on 90% B; 2 – 10 min linear gradient from 90% – 40% B; 10 – 12 min isocratic 40% B; 12 – 13 min linear gradient 40 – 90% B; 13 – 23 min isocratic on 90% B. The flow rate was 300 µL/min. Mass spectrometric data were obtained by analysing samples on a LTQ-VelosPro (Thermo Scientific) equipped with a heated ESI probe coupled to the UHPLC system. The capillary voltage was set to 3 kV with the source operation in positive ion mode. The heater temperature was set at 225°C and the capillary temperature was set at 300°C. The sheath gas flow rate was set at 20 and the auxillary gas flow rate at 5 (arbitrary units). The compounds were analysed using a selected reaction monitoring (SRM) method (Table 7.2). The normalized collision energy was set at 35 for all compounds and the *m/z* width on the fragment was set to 5. Compounds were quantified by reference to an external standard calibration curve by plotting MS area ratio in base peak SRM against amount ratio using external standard concentrations of 5, 3, 2, 1, 0.5, 0.1, 0.05, and 0.01 µg/mL (furosine dihydrochloride, *ε-N*-carboxymethyl-L-lysine and lysinoalanine; Sigma-Aldrich, Steinheim, Germany). Data were acquired and analysed using Xcalibur 2.1 (Thermo Scientific).

Table 7.2 Selected reaction monitoring conditions.

Compound	Parent mass, Da	Fragment mass, Da
Furosine	254.0	130.0
Carboxymethyllysine	204.2	130.0
Lysinoalanine	233.3	198.0
$^{13}C_6$$^{15}N_2$-Lysine	155.1	137.0

Furosine is an indirect measurement of FL. During acid hydrolysis in 6 M HCl, peptide-bound FL is transformed into approximately 32% furosine, 56% regenerated (unreactive)

lysine, and 16% pyridosine.[16] As such, the amount of FL (mg/kg) was calculated as the amount of furosine (mg/kg)/(32/100).

Calculations and statistical analyses

Urine volume (mL/d) was calculated by first converting the acidified urine collected from a weight to a volume basis using the specific gravity measured, and subsequently subtracting the added amount of boric acid to the bottom tray. Dietary MRP intake, urinary MRP excretion, and MRP urinary recovery were calculated as:

MRP intake (mg) = daily food intake (g) × food MRP content (mg/g)
MRP excretion (mg) = daily urine excreted (mL) × urine MRP concentration (mg/mL)
MRP recovery (%) = MRP excretion (mg) / MRP intake (mg) × 100%

Statistical analyses were performed using SAS 9.2 for Windows (SAS Institute Inc., Cary, NC, USA). The time (d) required for urinary FL, CML and LAL to plateau after the diet change in the pilot study was determined by averaging regression analyses of each individual cat using two-phase linear models (NLIN procedure).[22] The effect of food type (i.e. dry or wet) on urinary excretion and recovery in the main study was tested for significance using mixed model analysis by Proc Mixed. To test the influence of diet (for each food separately) on urinary excretion and recovery, mixed model analysis was performed using Proc Mixed of SAS. Diet was included as fixed effect, cat as random effect with period used as a repeated model statement. The covariance structure was selected based on the BIC and AIC. Significant effects of diet were explored using the Tukey HSD test. Correlations between intake and excretion or recovery were assessed by mixed model analysis, with intake as a fixed covariate effect and cat as a random effect. The regression slopes and SEs are reported with P-values. Normal distribution of the residuals was evaluated with the Sharpiro-Wilk test. Data was log transformed when the residuals were found to have increasing variance. Overall differences were considered significant if $P < 0.05$. Values are expressed as LSmeans ± SEM unless defined differently.

Results and discussion

Adaptation of urinary MRP excretion to dietary changes

The pilot study was conducted to determine the time required for urinary MRP excretion to adapt to dietary changes. Maillard reaction product content of the foods used in the pilot study was 70.0 and 338.7 mg furosine /kg, 26.4 and 24.0 mg CML /kg and 55.4 and 11.1 mg LAL /kg for food A and B, respectively (Table 7.1). Mean food intake of the cats fed food A and B was 179 ± 7.3 and 146 ± 6.0 g, respectively, with all the cats consuming all the food provided. The BW of the cats was, on average, 2.9±0.06 kg at the end of the

study, and the cats remained healthy throughout the study. Uncontaminated urine was quantitatively collected from all cats, on all days. The difference in diet MRP content on the urinary MRP excreted is clearly shown for furosine (Figure 7.1A). The daily urinary excretion of furosine increased from 721 ± 95 to 3,694 ± 745 µg after the change from food A to food B, and decreased from 3,878 ± 233 to 833 ± 213 µg after the change from food B to food A (Figure 7.1A). Urinary furosine excretions stabilized after the dietary change to the same value as the pooled reference value for both groups of cats. For CML and LAL, the effects were less clear (Figure 7.1B and C). In addition, variation in daily excretion was observed throughout the collection period. Moreover, variation between individual cats was considerable, with a range in CV of 15 – 47% for furosine, 7 – 44% for CML and 17 – 63% for LAL. This range is comparable to the variation observed in urinary excretion between rats which was 22 – 41% for FL, 22 – 35% for CML and 16 – 20% for LAL.[23] The linear-plateau model fitted through the data showed that for furosine an estimated plateau phase was reached within 1 d, indicating a likely effective excretion of MRP. For CML and LAL, a plateau level was also estimated within 1 d, however, the contrast in excretion is very small and variation high. Based on these results, the minimum urine collection period of 3 days that was recommended for accurate total urine collection by Hendriks et al.[14] was used in the main study.

Relation between dietary intake and urinary excretion of MRP
The MRP content varied between the foods used in the main study (Table 7.1): in the dry cat foods FL ranged from 418.1 to 1,210.2 mg/kg, CML ranged from 48.3 to 110.4 mg/kg, and LAL ranged from 78.1 to 144.8 mg/kg; in the moist cat foods FL ranged from 194.6 to 1,041.1 mg/kg, CML ranged from 14.0 to 29.7 mg/kg, and LAL ranged from 14.8 to 55.5 mg/kg (as-fed basis). Foods with various MRP content were chosen intentionally to create contrasts in dietary MRP between the foods. Similar to the pilot study, FL was the predominant MRP in both dry and moist cat foods. Daily food intake is shown in Table 7.3 and corresponds to an average daily MRP intake of 16.9 to 48.7 mg FL, 2.0 to 4.8 mg CML and 3.1 to 6.1 mg LAL in the dry cat foods, and of 33.5 to 147.2 mg FL, 2.2 to 5.6 mg CML and 2.6 to 10.3 mg LAL in the moist cat foods. The difference in daily intake between the lowest and the highest intake value when consuming the different foods was 2.9-fold for FL, 2.5-fold for CML and 2-fold for LAL in the dry cat foods, and 4.4-fold for FL, 2.5-fold for CML and 3.9-fold for LAL in the moist cat foods. The BW of the cats was, on average, 3.0±0.09 at the end of the main study, and the cats remained healthy throughout the study.
Uncontaminated urine was collected from all the cats. Daily urinary excretion varied between 35.5 and 74.2 mL for the dry cat foods and between 63.7 and 148.5 for the moist
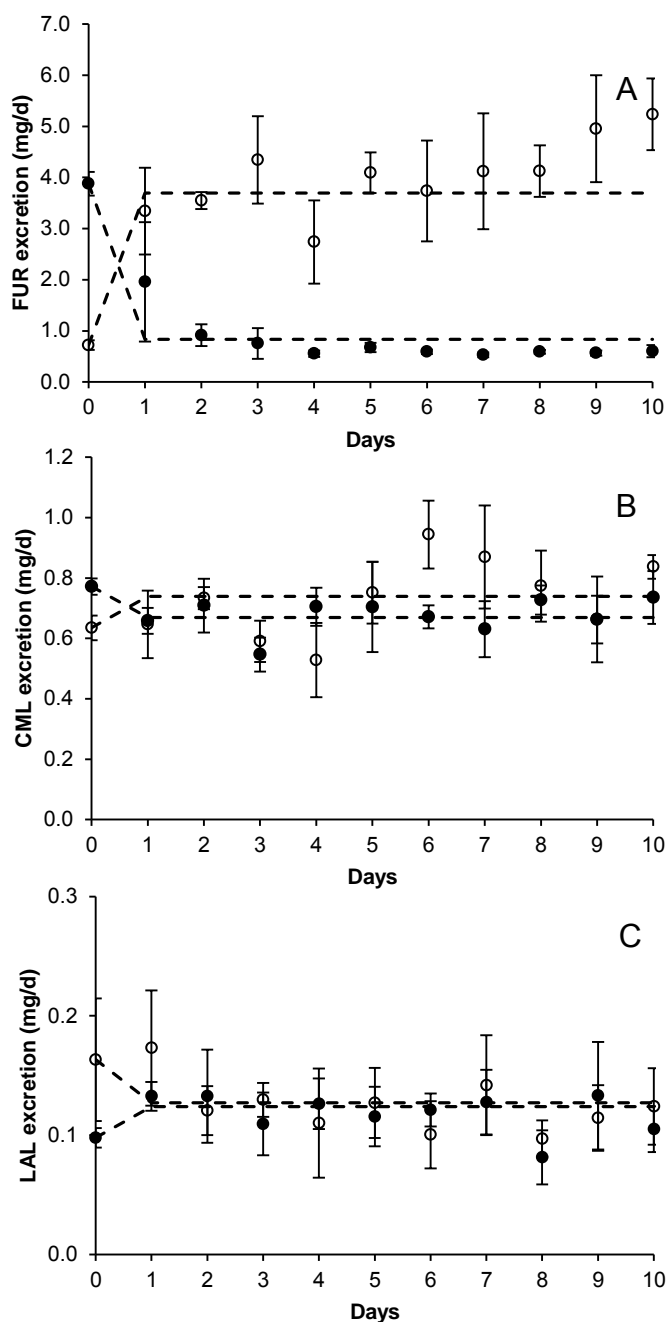
Figure 7.1 Mean ± SE daily urinary excretion of furosine (FUR; A), carboxymethyllysine (CML; B) and lysinoalanine (LAL; C) for 10 d by adult cats after a change in diet (○ = food A to B, ● = food B to A) and the linear-plateau model fitted through the data.

cat foods. Daily MRP excretions for FL and LAL were significantly different ($P < 0.05$; Table 7.3) between the dry and moist foods. Daily excretion by the cats fed the dry cat foods showed a positive regression coefficient with daily intake for FL ($\beta = 1.73$, $P = 0.03$) and CML ($\beta = 1.03$, $P < 0.01$), but no significant regression coefficient for LAL ($\beta = 1.18$, $P = 0.20$) (Figure 7.2). In addition, daily excretion by the moist cat foods showed a positive regression coefficient with daily intake for FL ($\beta = 3.65$, $P < 0.01$) and CML ($\beta = 1.60$, $P < 0.01$), but no significant regression coefficient for LAL ($\beta = 1.03$, $P = 0.45$) (Figure 7.3). The positive correlations for FL and CML between daily dietary intake and daily urinary excretion indicates that increased intake results in increased urinary excretion. However, as these models have very low R^2 values, more data would be needed to confirm these results. Similar correlations were observed in rats, an increase in dietary FL (159.3 vs. 1,057.6 mg/kg $BW^{0.75}$/d) and CML (89.2 vs. 284.0 mg/kg $BW^{0.75}$/d) resulted in an increase in urinary excretion from 38 to 174 mg for FL and from 94 to 283 mg for CML, although the content and difference of FL and CML in the low and high diets was larger compared to the foods used in the present study.[23] In human adolescents, a 5.9 mg/d increase in dietary CML did result in a 25% increase in daily urinary excretion.[11] For the moist cat foods in the present study, the result for FL was strongly influenced by food 4 that contained a ± 3-fold higher FL content compared to the other 5 foods. Additional data points with daily intakes in the range of 60 – 150 mg/d are necessary to confirm the results of the present study. For LAL, no significant increase was observed between daily urinary excretion and daily dietary intake for both dry and moist cat foods. This is in contrast with results found in rats where a 4.9-fold increase in dietary LAL (139.0 vs. 681.6 mg/kg $BW^{0.75}$/d) resulted in a 4.2-fold increase in urinary excretion. Overall, it can be concluded that FL and CML urinary excretion increased with an increase in dietary intake.

Urinary recovery of dietary MRP
Despite the differences in urinary excretion of the MRP between the foods used in the present study, highest and lowest dietary intake does not always correspond to highest and lowest urinary excretion. Daily MRP urinary recovery (as % of dietary ingested) showed significant differences ($P < 0.05$; Table 7.3) between the foods within both food types. In addition, urinary recovery of CML was higher in the dry foods compared with the moist foods ($P < 0.05$). Urinary recovery in the dry cat foods had a negative regression coefficient with daily dietary intake for FL ($\beta = -1.04$, $P < 0.01$), CML ($\beta = -0.04$, $P < 0.01$), and LAL ($\beta = -0.07$, $P = 0.08$) (Figure 7.2). Recovery of FL in the moist cat foods showed a positive regression coefficient with daily dietary intake for FL ($\beta = 3.35$, $P < 0.01$), however, urinary recovery showed a negative regression coefficient with daily intake for CML ($\beta = -0.05$, $P < 0.01$) and LAL ($\beta = -0.20$, $P < 0.01$) (Figure 7.3). Comparable results

Table 7.3 Daily food intake, urinary excretion, and intake, excretion and recovery of the analysed Maillard reaction products in the main study.

Component	Dry food							Moist food						
	1	2	3	4	5	6	SEM	1	2	3	4	5	6	SEM
Food intake, g/d	45.8	40.3	39.8	43.7	39.3	42.2	2.4	236.0	160.2	201.2	141.3	223.8	166.8	10.5
Urine produced, mL/d	61.2	35.6	37.4	44.0	43.9	74.2	7.8	145.6	93.1	130.7	63.7	148.5	88.1	8.7
Intake, mg/d														
Fructoselysine[1]	33.99	16.87	40.95	48.72	47.59	47.36	2.50	55.86	33.47	39.15	147.15	46.18	36.49	3.77
Carboxymethyllysine	3.94	1.95	4.32	4.83	2.71	3.33	0.23	3.81	2.24	5.64	4.19	5.27	4.41	0.24
Lysinoalanine	4.85	3.17	3.11	4.18	4.62	6.11	0.26	3.49	5.08	10.25	2.63	6.60	9.24	0.37
Urinary excretion, mg/d														
Fructoselysine	5.29^a	3.95^a	5.48^a	3.61^a	9.17^b	5.62^a	0.88	6.08^w	6.58^w	4.01^x	30.83^y	3.85^x	3.93^x	1.66
Carboxymethyllysine	$1.94^{c,d}$	0.87^b	1.27^a	1.42^a	$1.62^{a,c}$	$1.60^{a,d}$	0.17	1.25^w	1.13^w	1.98^x	1.71^x	1.95^x	1.10^w	0.15
Lysinoalanine	$0.54^{b,c}$	$0.38^{a,c}$	$0.40^{a,c}$	0.30^a	0.66^b	$0.41^{a,c}$	0.08	$0.40^{w,y}$	0.95^x	0.59^w	0.25^y	1.17^x	0.32^y	0.07
Recovery, % ingested														
Fructoselysine	$14.8^{a,b}$	22.9^e	$15.0^{b,c}$	8.1^a	$18.2^{a,e}$	12.0^c	1.8	10.8^w	19.7^x	10.2^w	21.0^x	8.3^w	10.8^w	1.4
Carboxymethyllysine	49.0^b	44.5^b	29.2^a	29.8^a	72.6^c	47.9^b	5.0	33.3^w	50.7^y	35.1^w	$40.7^{w,y}$	36.9^w	24.9^x	3.2
Lysinoalanine	$11.1^{a,b}$	$11.8^{a,b}$	12.7^b	$7.8^{a,c}$	14.2^b	6.8^c	1.7	$12.8^{v,w}$	18.7^x	5.8^z	$9.4^{w,z}$	$17.7^{v,x}$	3.4^y	1.4

Within a row and diet type ([a,b,c,d,e] dry food, [v,w,x,y,z] moist food), means without a common superscript differ ($P < 0.05$).
[1] Furosine / (32/100).[16]
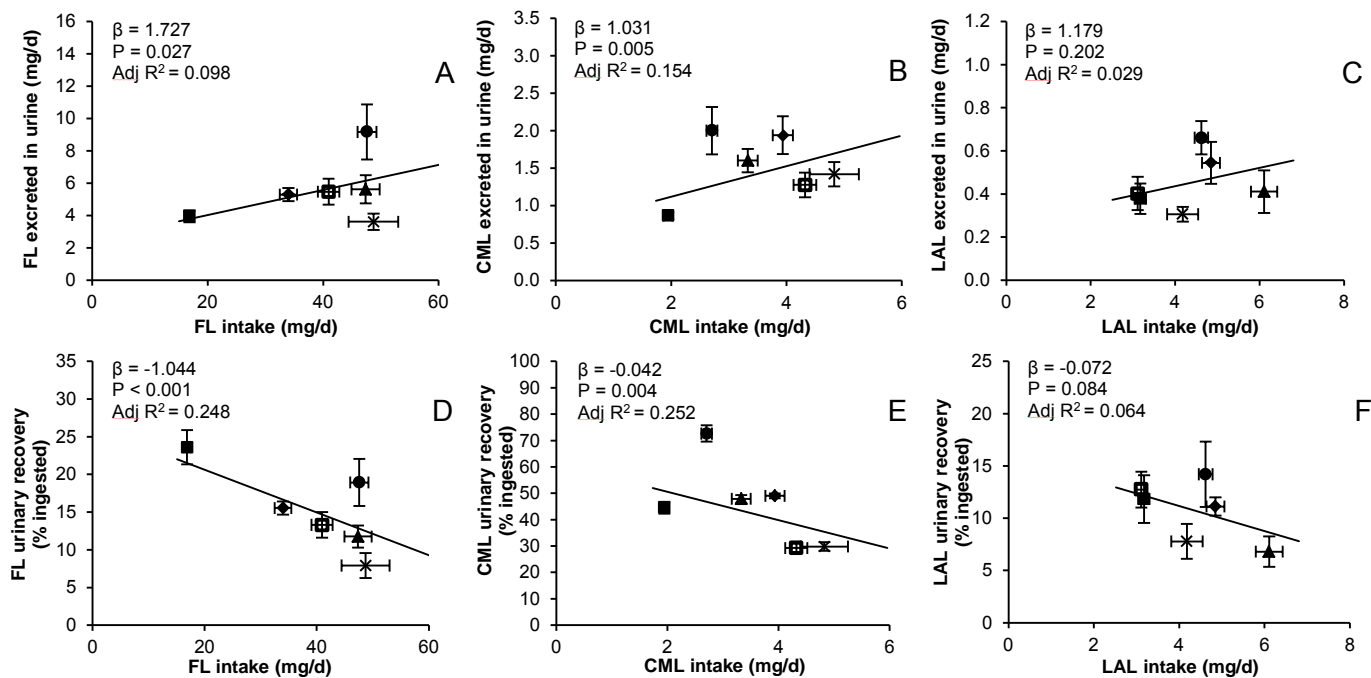
Figure 7.2 Relationship of fructoselysine (FL), carboxymethyllysine (CML) and lysinoalanine (LAL) excretion in urine (mg/d) and intake (mg/d) (A, B and C) and relationship of FL, CML and LAL urinary recovery (% ingested) and intake (mg/d) (D, E, and F) for 6 commercial dry cat foods (♦ = Food 1, ■ = Food 2, □ = Food 3, × = Food 4, ● = Food 5, ▲ = Food 6).
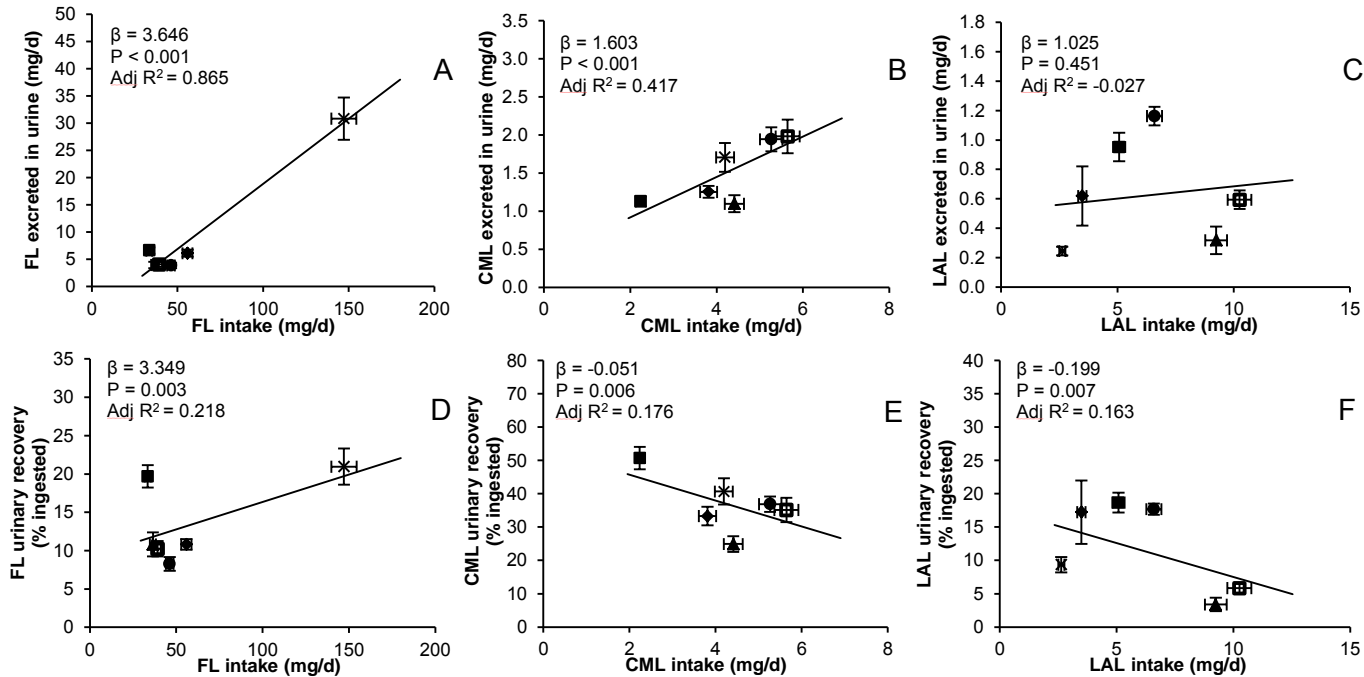
Figure 7.3 Relationship of fructoselysine (FL), carboxymethyllysine (CML) and lysinoalanine (LAL) excretion in urine (mg/d) and intake (mg/d) (A, B and C) and relationship of FL, CML and LAL urinary recovery (% ingested) and intake (mg/d) (D, E, and F) for 6 commercial moist cat foods (♦ = Food 1, ■ = Food 2, □ = Food 3, × = Food 4, ● = Food 5, ▲ = Food 6).

were reported in literature for rats, urinary recovery of FL and LAL were reported to decrease from 5.2 to 3.7% and from 5.6 to 4.9%, respectively, with increasing dietary intake.[23] In the latter study, urinary recovery of CML increased with increased dietary intake from 26 to 29%, which is in contrast to the results in the present study. However, urinary recovery of CML in human adolescents decreased significantly with increasing dietary CML intake.[11] In addition, urinary recovery of dietary CML decreased from 38 to 23% in rats fed an unextruded (low CML) and extruded protein diet (high CML).[24] A decrease in urinary recovery with increasing dietary intake suggests that either: 1) digestion and absorption capacity was exceeded, 2) the rate of *in vivo* metabolism increased as intake increased, or 3) the capacity for renal excretion of the components was exceeded.

Digestion and absorption of dietary MRP
The present results raise the question whether an inhibited digestion and absorption can cause the lower urinary recovery with increasing dietary intake. From literature it is known that dietary MRP can be present in the food in either a free or protein-bound form, and that both forms behave differently in the gastrointestinal tract. Orally administered free FL was excreted in urine for up to 60% in rats, compared with 10% for the dietary protein-bound derivative.[12] In addition, it was suggested that protein-bound LAL is absorbed in much lower levels compared to free LAL.[25,26] Free FL and CML can be absorbed by passive diffusion,[23] however, in processed foods, most FL and CML are present in a protein-bound form. Using simulated gastrointestinal digestion, it was reported that protein-bound FL and CML are released in peptides smaller than 1,000 Da in the small intestine, which are considered available for absorption. The cross-linked LAL seems to be absent in peptides smaller than 1,000 Da.[27] Therefore, LAL is suggested to be poorly available for absorption and most LAL will be transported into the large intestine. Protein-bound FL, although digested into small dipeptides, is not fully transported by the intestinal peptide transporter PEPT1 and might also flow into the large intestine.[28] These mechanisms could explain part of the rather low urinary recovery of both dietary FL (between 8.1 and 22.9%) and LAL (between 3.4 and 18.7%) compared to CML (between 24.9 and 72.6%) in the current study as well as in the study of Somoza et al.[23], and suggest that the foods in the present study contained protein-bound MRP rather than free MRP. In addition, varying protein sources in the different foods could add to the variation in absorption capacity of MRP. Feeding rats FL originating from different protein sources resulted in different urinary recoveries, whereas gastrointestinal simulation showed that more LAL from fish proteins was degraded and available for absorption than LAL from whey proteins.[29] Whether the FL, CML and LAL that were not absorbed and which

entered the large intestine in the current study were metabolized by gut microbiota or excreted in faeces remains unknown.

In vivo metabolism and urinary excretion of dietary MRP

In vivo metabolism was not determined in the present study. Studies in other animals have studied plasma levels and urinary excretion of dietary or intravenously administered MRP. Once absorbed, FL, CML and LAL are transported in the plasma, and can be found in increased concentrations in the liver and kidneys of rats when dietary intake increases.[23] In addition, intake of dietary CML resulted in an increase in serum CML in humans.[30] In rats, intravenously injected ^{14}C labelled FL was excreted in urine for more than 80% after 24 h , indicating that most of the FL that enters the body is excreted in the urine.[31] Hultsch et al.[32] reported that 45% of intravenously administered [^{18}F]fluorobenzoylated FL was excreted nearly unchanged in the urine 60 min post injection in male Wistar rats. However, large accumulation of radioactivity (34%) was observed in the kidneys 60 min post injection, whereas minor accumulation (with a total of less than 10%) was reported in the stomach, lungs, liver and intestine. Approximately one third of the intravenously applied radiolabelled FL was possibly metabolized. When injected intravenously in the tail of rats, [^{18}F]fluorobenzoylated CML was found to accumulate quickly in the liver, kidneys, and in lesser amounts in the muscles and heart. After 120 min, however, 72.3% of the radioactivity was found in the urine bladder, whereas 18.1% had accumulated in the kidneys and hardly any accumulation was reported in other organs.[33] When administered via a stomach tube, most [^{18}F]fluorobenzoylated CML is still located in the stomach and intestines (17.9 and 48.3%) after 120 min, and 29.6% is found in the urine bladder. This result is supported by an earlier study of Bergmann et al.[34] using intravenous distribution of [^{18}F]fluorobenzoylated CML. When ^{14}C-radiolabeled LAL was dosed to rats by stomach tube, 61.7% was excreted within 72 h of which 53.9% was found in urine suggesting the kidney as the primary excretion route of elimination.[35]

In line with these reports it can reasonably be assumed that part of the dietary MRP in the present study were absorbed into the bloodstream and mainly excreted via the kidneys into the urine, a hypothesis that is supported by the increasing urinary excretion of MRP with increasing dietary intake. Although the literature supports the retention of part of the absorbed dietary MRP in body tissues, absorption capacity is likely to be the main factor influencing urinary excretion in healthy subjects.

The urinary excretion of MRP can be influenced by endogenous formation of these compounds. Liardon et al.[36] detected CML in urine when rats were fed additional free FL compared to control diets, in which only trace amounts of CML were reported. It was suggested that there might be a possible transformation from FL to CML endogenously.

The study of Somoza et al.[23] reported a urinary excretion of 0.65 mg FL, no CML and 13.8 mg LAL in rats fed a FL, CML and LAL free diet for 10 d, respectively. Urinary excretion values of MRP can, therefore, be affected by the urinary excretion of endogenous MRP. Whether this endogenous load is formed by *in vivo* glycation, or is accumulated in body tissues from dietary MRP prior to the 10 d study, remains unknown. It is possible that foods can influence endogenous production of MRP by providing sugars that can be absorbed; carbohydrate-rich foods could, therefore, provide more reducing sugars for the endogenously occurring Maillard reaction. However, knowledge on this item from literature is lacking.

Possible consequences of dietary MRP on animal health

Although it seems that not all dietary MRP is absorbed and part of the absorbed concentration is excreted quickly, it is still unknown what happens with the part of the MRP that is metabolized and not accounted for. The quantity of absorbed MRP is unlikely to cause damage when administered in an acute dose. However, effects of long term daily exposure in healthy subjects is suspected to induce oxidative stress and inflammation, and aging subjects might benefit from an MRP-low diet.[30,37] Cats ingest more dietary MRP than humans when compared by metabolic bodyweight ($BW^{0.75}$). In the present study, daily intake of FL ranged from 7.4 – 21.4 mg/kg $BW^{0.75}$ for dry and 14.7 – 64.6 mg/kg $BW^{0.75}$ for moist foods; daily intake of CML ranged from 0.88 – 2.11 mg/kg $BW^{0.75}$ for dry and from 0.97 – 2.46 mg/kg $BW^{0.75}$ for moist foods; daily intake of LAL ranged from 1.36 – 2.68 mg/kg $BW^{0.75}$ for dry and 1.14 – 4.52 mg/kg $BW^{0.75}$ for moist foods. This daily CML intake was higher compared with that in a previous study, in which the average intake from extruded dry diets was 0.28 mg CML/kg $BW^{0.75}$.[13] Taking the urinary recovery values into account (Table 7.3), an average recovery value of 15.2% for FL in dry foods results from a daily absorption and excretion of 1.13 – 3.25 mg FL/kg $BW^{0.75}$, assuming that 100% of the FL excreted in the urine originates from the absorbed dietary FL. In addition, an average recovery value of 45.5% for CML in dry foods results from a daily absorption and excretion of 0.40 – 0.96 mg CML/kg $BW^{0.75}$. Despite the natural capacity of the body to protect against MRP, over time, endogenous and dietary MRP may accumulate in body tissues.[6,38,39]

Conclusions

The minimum apparent absorption of FL, CML and LAL from commercial feline dry and moist foods as measured by urinary excretion was found to range between 8 to 23% for FL, 25 to 73% for CML and 6 to 19% for LAL. Urinary excretion of dietary MRP was rapid and increased with an increase in dietary intake. However, urinary recovery decreased

with increasing dietary intake, suggesting that digestion, absorption, metabolism or urinary excretion can be limiting factors. Urinary recovery indicated that either absorption or excretion of dietary CML is higher than FL and LAL. As dietary MRP is proven to be absorbed and subsequently excreted via the kidneys, the potential contribution of dietary MRP absorption to the pathogenesis of various health conditions requires further study. Similarly further study would be required to determine if dietary MRP restriction has a potential role in the prevention and treatment of such long-term health conditions that may be associated with MRP.

Acknowledgements

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Chapter 8

General Discussion
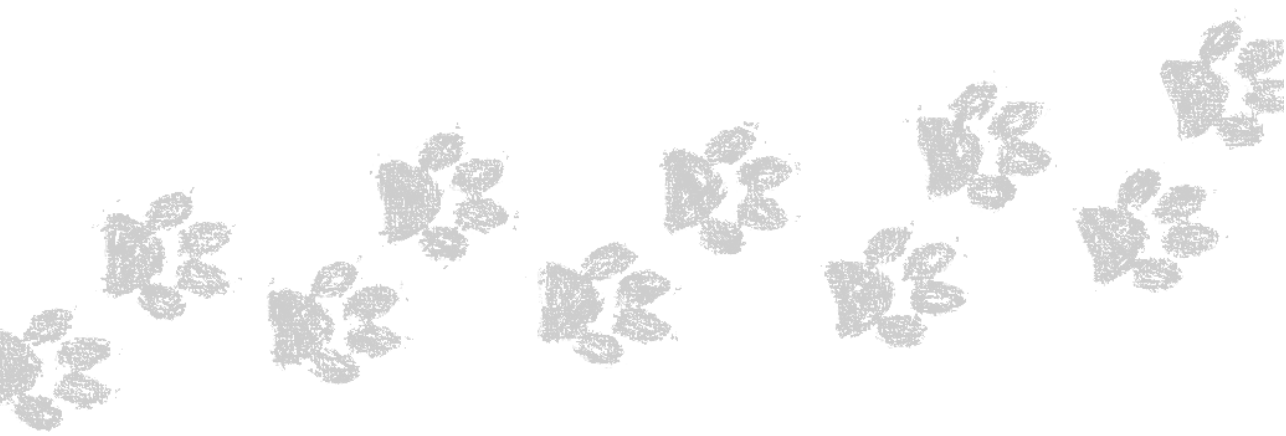
The aims of this thesis were to characterize the occurrence and progression of the Maillard reaction during the manufacturing of pet foods and determine the subsequent impact on nutritive value of the food and the bioavailability of selected Maillard reaction products in pet animals. The results of the work described in this thesis may be summarised as follows:

- The Maillard reaction products carboxymethyllysine and hydroxymethylfurfural are present in high concentrations in commercial pet foods, and their contents are equivalent to the contents in processed human foods such as bread, cereals, meat and fish, dairy products and prunes

- Ingredients, rather than extrusion processing or pelleting are the major contributors to Maillard reaction products in dry pet foods

- Analysing total and reactive lysine is not a good indicator for the quantity of Maillard reaction products present in processed pet foods

- Increasing temperature or moisture content leads to lower total and reactive lysine contents and higher contents of Maillard reaction products during the extrusion of commercial dog foods

- Foods for growing dogs that are used as weaning diets could be at risk of not meeting minimal lysine requirement, depending on the digestibility of reactive lysine

- The dietary Maillard reaction products fructoselysine, carboxymethyllysine, and the amino acid lysinoalanine in commercial pet foods are absorbed and excreted to a variable extent by cats

- Dogs and cats consuming commercial pet foods ingest, on average, comparable carboxymethyllysine levels, but more hydroxymethylfurfural compared to humans

In the following paragraphs, the work described in this thesis is discussed, and conclusions and implications for future scientific research and pet food processing are provided.

Total and reactive lysine contents in pet foods

Nearly all foods in the experimental studies of this thesis (Chapters 3, 5, and 6), regardless of the type of processing technology used, contained a lower reactive than total lysine content (Figure 8.1). This result is in agreement with previous studies determining reactive lysine in commercial pet foods.[1-3] The lowest reactive to total lysine ratio reported in this thesis (0.67) is not as low as reported in one of the previous studies (0.38), however, this discrepancy is mainly caused by the data of the cat foods of Rutherfurd et al.[1] As the studies originate from different countries and continents, ingredients, processing conditions and environmental conditions could influence the results. In addition, although the analytical method using *O*-methylisourea is one of the most suitable methods to determine reactive lysine in processed foods, the large diversity of materials to which the method is applied may have influenced complete guanidination of reactive ε-amino groups and, therefore, the final analytical result.[4]
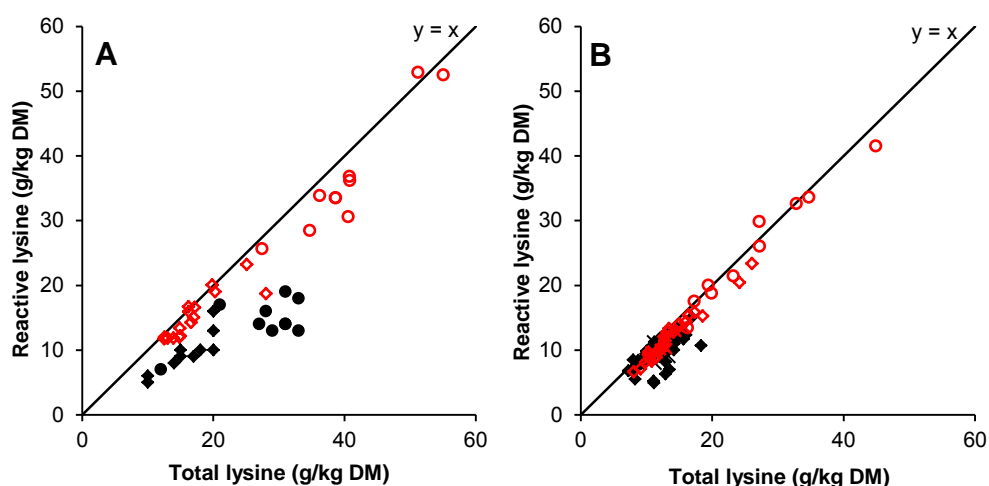
Figure 8.1 Reactive to total lysine ratio for A) cat foods and B) dog foods. Red (◊) = extruded, red (×) = pelleted and red (○) = canned (data from Chapter 3, 5 and 6). Equal closed black symbols represent data from literature.[1-3]

For commercially available dog foods, foods that are manufactured using extrusion processing and pelleting technologies contain lower reactive to total lysine ratios (0.89, $P = 0.004$ and 0.85, $P = 0.0008$, respectively) compared to canned dog foods (0.97), while there was no difference between commercially available dry and canned cat foods (Chapter 3). The higher ratio in canned foods is caused by either the canning process, or the ingredients used in canned foods. Those ingredients are often unprocessed or raw, in contrast to the ingredients used in dry foods, and therefore, most likely do not contain

lysine that is modified by the Maillard reaction before inclusion in the pet food formulation. Pelleted dog foods tend to contain, on average, a lower reactive to total lysine ratio compared to extruded dog foods, which is supported by the data of Tran et al.[3] who report an average ratio of 0.80 for pelleted and 0.88 for extruded dog foods. These results are rather unexpected considering the less severe production conditions during the process of pelleting, with lower temperatures and shorter residence times compared to extrusion (Chapter 2). However, pellets require more pre-processed ingredients e.g. with pre-gelatinized starch, due to the routine pelleting conditions that are not favouring starch gelatinization. To define to which extent pet food processing affects total and reactive lysine contents in pelleted and extruded dog foods, pelleting and extrusion experiments were performed (Chapter 5 and 6).[§]

The presence of Maillard reaction products in pet foods

In all foods analysed as a result from the experimental studies included in this thesis, the Maillard reaction products fructoselysine (FL), carboxymethyllysine (CML) and hydroxymethylfurfural (HMF), and the amino acid lysinoalanine (LAL) were found (Table 8.1; Chapter 4, 5, 6 and 7). A plethora of studies have reported the presence of a number of Maillard reaction products in processed human food. However, in the animal feed

Table 8.1 Overview of selected Maillard reaction products (mg/kg DM) analysed in samples from experiments included in this thesis.

	Chapter 4 (n = 59)	Chapter 5 (n = 1)	Chapter 6 (n = 2)	Chapter 7 (n = 12)
Fructoselysine	1838±272[1]	539	1277±86	1297±244
range	106 – 11453		1228 – 1326	436 – 3861
Carboxymethyllysine	20.5±1.6	14.8	15.1±0.5	101.7±8.6
range	6.0 – 60.5		13.8 – 16.5	50.4 – 146.3
Hydroxymethylfurfural	983.6±79.9	390.5	582.5±14.0	NA[2]
range	65.9 – 3021.5		510.0 – 655.0	
Lysinoalanine	7.0±0.3	7.7	5.2±0.1	139.4±19.3
range	1.4 – 16.1		4.6 – 5.8	69.0 – 265.6

[2] Mean ± SEM.
[3] not analysed.

industry the determination of Maillard reaction products is uncommon, while virtually lacking for pet foods.[**] It should be noted that, although FL, CML and HMF are commonly analysed Maillard reaction products, the Maillard reaction produces a large variation in type of Maillard reaction products in processed foods, both known and unknown. FL (analysed as furosine) represents the Amadori compound formed during the early

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[§] For a more extensive discussion on the effects of processing, see general discussion page 137
[**] A more extensive overview of these studies is provided in Chapter 4

Maillard reaction. However, as FL is an intermediate product of the Maillard reaction, it can react further into other compounds.[5] One study reported furosine levels in pet foods being 910 mg/kg in a dry dog food.[6] HMF is also an intermediate product, formed during the advanced Maillard reaction, and widely used as a marker for quality in processed human foods.[5,7] CML is a stable product representing the advanced and late Maillard reaction, which provides additional information of the Maillard reaction when FL and/or HMF contents have already decreased.[5] As there are no data available in pet foods for CML and HMF, the data reported in this thesis have to be compared to CML and HMF contents in processed human food items. The contents of CML and HMF found in commercial pet foods are on average within the range reported in processed human food products (Figure 8.2). Coffee represents one of the most important contributors to dietary HMF intake in humans,[8] and maximum HMF content in the pet foods analysed in this thesis is close to maximum HMF content of ground coffee.
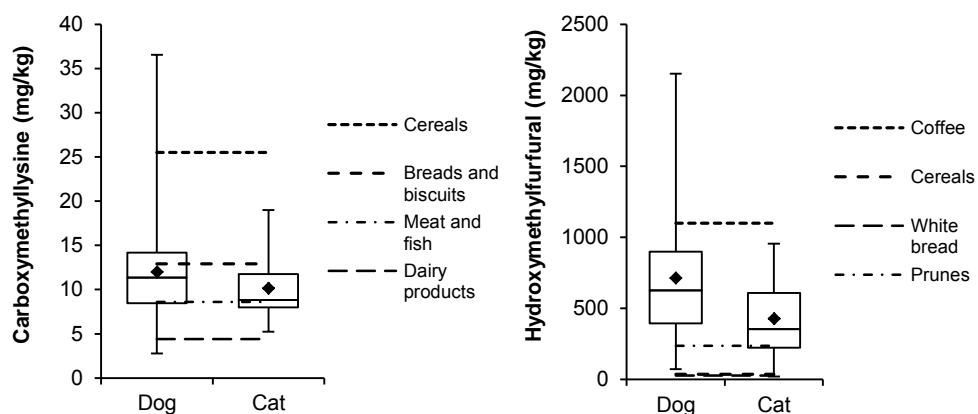
Figure 8.2 Comparison of the Maillard reaction products CML and HMF in dry and canned pet foods analysed in this thesis compared to average CML and HMF content found in several processed human foods (data from Chapter 4).

Commercially available canned pet foods contained, on average, the most FL, CML and HMF, followed by commercially available pelleted and extruded foods (FL: 4534, 844, 706; CML: 37, 20, 12; HMF: 1417, 1161, 715 mg/ kg dry matter, respectively; Chapter 4). For LAL, the means were comparable between processing types. The high contents of FL, CML and HMF in canned pet foods are in contrast to the earlier discussed high reactive to total lysine ratios, which in the given definition of total and reactive lysine indicates the least occurrence of the Maillard reaction compared to extrusion processing and pelleting. However, total lysine content can decrease during heat treatment (Chapter 2). As such, it is possible that the initial total lysine contents of the ingredient mixture of canned pet

foods was higher, and that part of the total lysine was converted into Maillard reaction products that are not analysed as lysine anymore after thermal treatment. De degradation of lysine into Maillard reaction products might be accelerated in high moisture conditions. In addition, possible addition of palatability enhancers might increase the content of FL, CML and HMF in certain foods independent from the processing type used. Similar to the total and reactive lysine results, pelleted foods contain more Maillard reaction products than extruded foods.

The effect of extrusion processing or pelleting on the Maillard reaction in pet foods

Maintaining the lysine content in feed and food during processing preserves the nutritional value of the initial unprocessed ingredient mix. From the data in this thesis, however, it can be concluded that the ingredient mix rather than extrusion or pellet processing might be the key factor to control the presence of Maillard reaction products in pet foods. Both processing technologies had little effect on total and reactive lysine content in the dog food formulas used in the experiments (Figure 8.3, Chapter 5 and 6). The only significant effect was measured after extrusion of the ingredient mixture including hydrolysed proteins (Figure 8.3 C). Total and reactive lysine in this formula decreased by 5.9 and 5.5%, respectively.
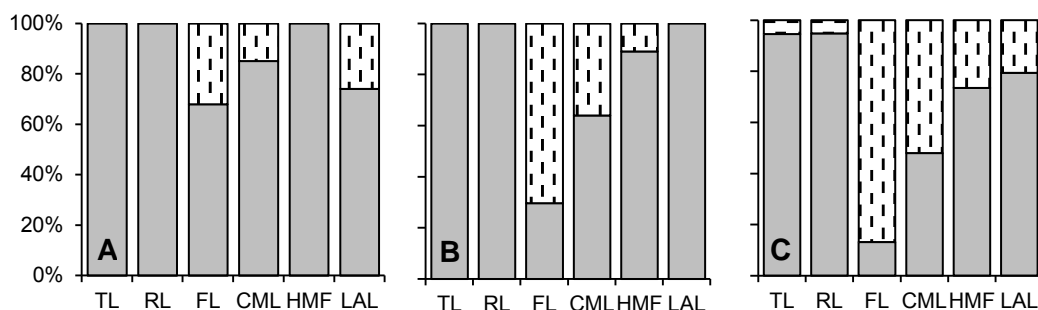
Figure 8.3 Effect of processing technology on total (TL) and reactive lysine (RL), the Maillard reaction products fructoselysine (FL), carboxymethyllysine (CML), hydroxymethylfurfural (HMF) and the amino acid lysinoalanine (LAL). Bar with horizontal stripes represents the proportion that significantly decreased relative to the initial unprocessed ingredient mixture; bar with vertical stripes is the proportion that significantly increased relative to the initial unprocessed ingredient mixture. A) steam pelleting, B) extrusion including intact proteins, C) extrusion including hydrolysed proteins (data from Chapter 5 and 6).

However, because both total and reactive lysine content decreased in similar percentages, the end product contains a similar reactive to total lysine ratio compared to the ingredient mixture. This result supports the idea that the total and/or reactive lysine content of the processed the end-product, such as the commercial pet foods described in

Chapter 3, underestimate the amount of lysine of the initial ingredient mixture and the loss of lysine due to processing. To obtain more insight in the effect of extrusion on total and reactive lysine content, a so called "dead-stop" experiment was performed (Textbox). The samples along the extruder barrel were suggested to have received increasing shear, pressure and temperature treatment, which is confirmed with the decline in total and reactive lysine content in the extruder barrel when passing from feed input to the extruder die (Figure 8.4). For all diets, total and reactive lysine content is lower in the final product compared to the ingredient mixture, hence, the effect of extrusion on total and reactive lysine content varies and is dependent on extruder parameters. Samples of the 5 barrel locations were lower than the final product, due to the longer residence time in the hot extruder barrel during opening. Despite the latter effect, it might be interesting to use this type of experimental setup to determine the progression of chemical reactions like the Maillard reaction and the variety of compounds that can be produced during the extrusion process. Although processing tends to have a minor negative impact on total and reactive lysine, the ingredient mixture seems to have more influence. The unprocessed ingredient mixtures of the commercial pet foods used in Chapter 3 could not be analysed, however, the unprocessed ingredient mixtures used in experiments described in Chapter 5, 6 and the Textbox (which were representable for commercial foods) indicate that the reactive to total lysine ratio is already between 0.85 and 0.90 before processing. Indeed, the overview in Chapter 2 (Table 2.1) shows that frequently used pet food ingredients already contain differences in total and reactive lysine. Differences between total and reactive lysine content in several ingredients of animal origin, mostly rendered meat meals, range from 1 to up to 36% (Chapter 2). Unexpectedly, proteins of vegetable origin, that are most often only ground, can include an even higher difference between total and reactive lysine, up to 44%.

Textbox – lysine reactivity inside the extruder

During extrusion experiments, it is unknown to what extent the Maillard reaction progresses in the extruder barrel; being a black box it is only possible to analyse the input and output. Therefore, a so-called "dead stop" experiment was performed using an extruder which can be opened over the full length, to determine the progression of the Maillard reaction as indicated by changes in lysine reactivity along the extruder screw from feed input up to the extruder die.

Based on the ingredients used in the high protein diet described in Chapter 6, three experimental diets were formulated: diet 1, containing fish meal (as a source of intact proteins), diet 2, containing hydrolysed fish meal (as a source of free amino groups), and diet 3, containing fish meal and supplementary L-lysine HCl (0.2%). On average, the diets

contained 244±0.71 g/kg dry matter (DM) crude protein, 56±4.50 g/kg DM crude fat, 92±4.37 g/kg DM neutral detergent fibre, 64±4.94 g/kg DM crude ash, 508±1.23 g/kg DM starch, and 907±1.50 g/kg DM. Extrusion was performed using a co-rotating double screw extruder (M.P.F.50, Baker Perkins, Peterborough, United Kingdom) with a feed rate of 84 kg/h, a screw speed of 200 rpm and a die size of 5 mm; no die face cutter was used. Extruder barrel temperatures were set at 50, 70, 80, 90, 100, 120, 140, 160, 170, and 170°C from the 1st to the 10th zone, respectively. Ingredient mix and final product were sampled in simplo after reaching a steady state for at least 5 min. After sampling the final product, the extrusion operation was shut down and the barrel was opened. Within 8 min after shutting down the extruder, product temperature could be measured at five locations equally distributed along the extruder barrel and samples were taken from these locations. On average, product temperature of the 5 locations was 41, 65, 80, 97, and 110°C; product temperature of the final product was on average 140°C. All collected samples were dried at 50°C for 4 h, ground to pass a 1 mm screen in a centrifugal mill (Retsch ZM100, Retsch BV, Ochten, the Netherlands) and stored at 4°C. Samples were analysed for total and reactive lysine according to the method discussed in Chapter 3; N was determined by DUMAS.
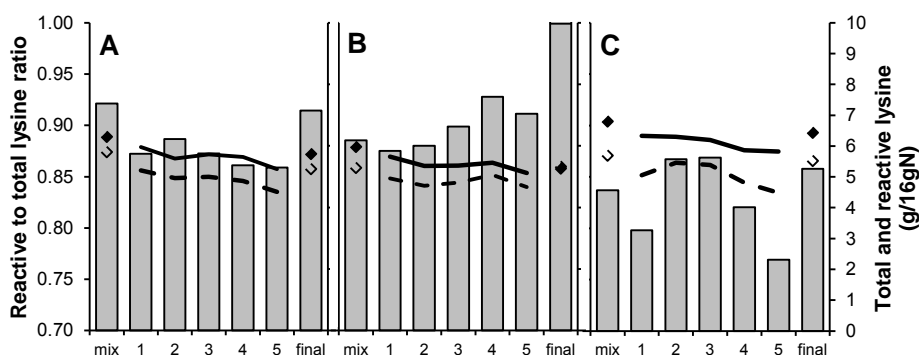
Figure 8.4 Reactive to total lysine ratio (bars), total lysine (♦ and solid line), and reactive lysine (◊ and dashed line) in the ingredient mixture, the 5 locations along the extruder barrel, and the final product of diet 1 (A), diet 2 (B) and diet 3 (C)

Not only the total and reactive lysine contents in the food, but also their digestibility is important to estimate its nutritional value. The data in Chapter 6 indicate that *in vitro* digestibility values change when the ingredient mixture is extruded (Figure 8.5). Dry matter and organic matter digestibility increased after extrusion while crude protein, total and reactive lysine decreased after extrusion processing. However, this result was

only seen in the diet that included hydrolysed proteins. In the diet including intact proteins, no change in digestibility of crude protein, total or reactive lysine was reported. The difference between *in vitro* digestibility results of crude protein, total lysine and reactive lysine could be either caused by a slightly higher product temperature during extrusion of the diet including hydrolysed proteins, despite the similar extrusion conditions (Chapter 6), or by the fact that hydrolysed proteins are more susceptible to the Maillard reaction, thereby, reducing the digestibility of the protein.
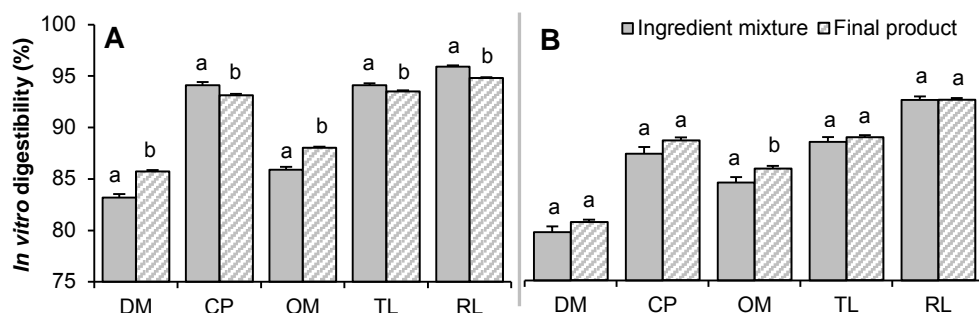
Figure 8.5 *In vitro* digestibility values of dry matter (DM), crude protein (CP), organic matter (OM), total lysine (TL) and reactive lysine (RL) of the ingredient mixture and the final product of A: a diet containing hydrolysed proteins and B: a diet containing intact proteins (data from Chapter 6). Bars without a common superscript differ ($P < 0.05$).

The *in vitro* digestibility results indicate that ingredients have the largest influence on *in vitro* digestibility, as only minor change in digestibility is shown after extrusion. Extrusion reduces the digestibility of total and reactive lysine, but is influenced by extrusion conditions and ingredients included in the ingredient mixture. In addition, Chapter 6 shows that it is possible to estimate total and reactive digestibility values using *in vitro* techniques. This can be of importance especially in research using pet animals, as invasive experimental settings like cannulas or euthanizing the animal for its intestinal digesta are generally not accepted in pet animals.

With respect to the formation of Maillard reaction products during processing, both extrusion and pelleting technologies increased the concentrations of Maillard reaction products (Figure 8.3). FL content shows the highest increase, followed by CML and HMF. As FL and HMF are intermediate products, an increase in FL and HMF content indicates that these components are formed faster than they are used to form other Maillard reaction products. From Figure 8.3 it can also be concluded that although total and reactive lysine do not decrease, FL, CML, HMF and LAL can increase significantly. As the concentrations of the analysed Maillard reaction products are below 0.6 g/kg, it is not surprising that their formation does not immediately affect lysine content. Although the

difference between total and reactive lysine is significantly correlated to FL and CML content (r = 0.36, P = 0.004 and r = 0.32, P = 0.01, respectively), these results do indicate that analysis of total and reactive lysine may not be a good indicator for the level of Maillard reaction products in processed foods. This should be taken into account when total and reactive lysine analyses are used to identify lysine damage due to processing, which is more common in the animal feed industry. The Maillard reaction contents of all analysed components of the pelleted end-product in Chapter 5 (Table 8.1) are within the lower ranges of the commercially available pelleted foods of Chapter 4 (FL, range 310 – 4050 mg/kg DM; CML, 12.0 – 40.3 mg/kg DM; HMF, 520 – 2380 mg/kg DM; LAL, 4.12 – 16.11 mg/kg DM). The Maillard reaction contents of the extruded end-products in Chapter 6 (Table 8.1) vary. FL and CML contents are in the higher ranges of the commercially available foods while HMF and LAL contents at the lower ranges (FL, range 300 – 1440 mg/kg DM; CML, 7.75 – 20.22 mg/kg DM; HMF, 270 – 1920 mg/kg DM; LAL, 3.15 – 12.95 mg/kg DM). Differences between a pilot-scale process-setting and commercial manufacturing of pet foods can cause these differences, like the use of different ingredients and fat coating of the products. Opposite to the commercially available foods, the results of Chapter 5 and 6 show that the pelleted food contains comparable or lower FL, CML and HMF contents compared to the extruded foods. In addition, steam pelleting (Figure 8.3 A) displays less formation of these components compared to extrusion (Figure 8.3 B and C). In all 3 unprocessed ingredient mixtures, Maillard reaction products were found. The content of CML, HMF and LAL in the unprocessed ingredient mixture was higher than the formation of the compounds during processing. This indicates that for both pelleting and extrusion processing, the choice of the ingredient mixture has a greater influence on the amount of Maillard reaction products in the final product compared to the processing itself. As such, pet food manufacturers should carefully consider the quality of the ingredients used in pet food formulation in order to maintain the nutritional value of the food and to reduce the amount of Maillard reaction products.

Although it seems that the analysed Maillard reaction products originate to a large extent from ingredient processing rather than the pet food production processes (i.e. pelleting and extrusion), it can be important to reduce the loss of lysine and the formation of additional Maillard reaction products during processing. To be able to do so, knowledge of the parameters that affect the formation of these components is necessary. During extrusion of the two experimental diets (Chapter 6), temperature had the largest effect on total and reactive lysine content, followed by moisture content and screw speed (Figure 8.6). It should be noted that the measured product temperature was lower than the extruder temperature settings. In the diet containing hydrolysed protein, reactive
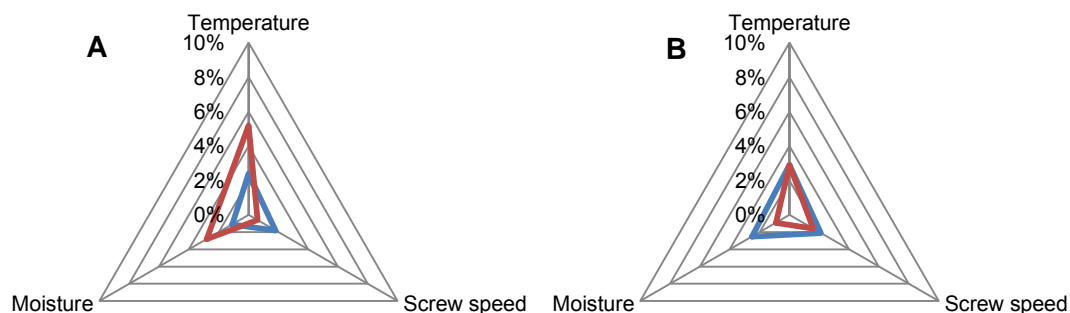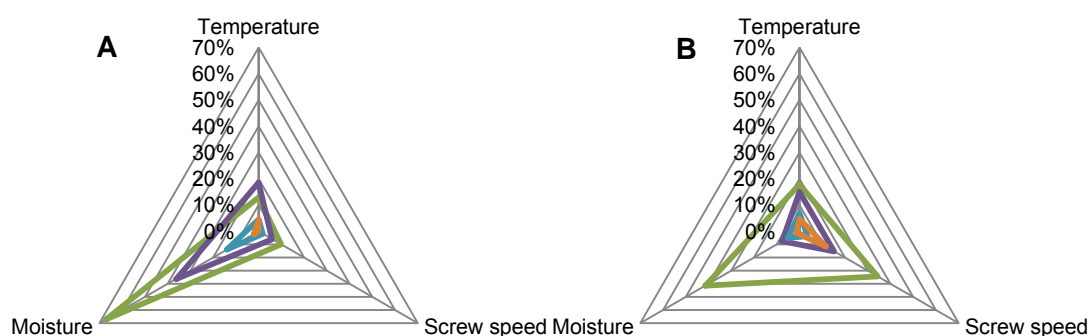
Figure 8.6 Effect of extrusion parameters temperature (140 and 165°C), moisture content (20 and 30%) and screw speed (100 and 200 rpm) of A: a diet containing hydrolysed proteins and B: a diet containing intact proteins on reactive and total lysine. Values are the absolute differences in percent between the two parameter settings. Blue line = total lysine; red line = reactive lysine.

lysine content was most affected by extrusion parameters (Figure 8.6 A), whereas total lysine content was most affected by extrusion parameters in the diet containing intact proteins (Figure 8.6 B). For both diets, increasing extrusion temperature and moisture content had a negative influence on total and reactive lysine content, whereas increasing screw speed had a positive influence on total and reactive lysine content. These effects were more pronounced in the diet containing hydrolysed protein compared to the diet containing intact protein. During pelleting of an experimental diet (Chapter 5), the processing parameters temperature (65 and 90°C) and die hole length (45, 65 and 85 mm) did not have a significant effect on total and reactive lysine. Increasing die hole length tended to have a positive effect on total as well as reactive lysine content; temperature tended to have a negative effect.

Figure 8.7 Effect of extrusion parameters temperature (140 and 165°C), moisture content (20 and 30%) and screw speed (100 and 200 rpm) of A: a diet containing hydrolysed proteins and B: a diet containing intact proteins on fructoselysine, carboxymethyllysine hydroxymethylfurfural and lysinoalanine. Values are the absolute differences in percent between the two parameters. Green line = fructoselysine; purple line = carboxymethyllysine; light blue line = hydroxymethylfurfural; orange line = lysinoalanine.

The effect of extrusion parameters on Maillard reaction products is different to that of total and reactive lysine. In the diet containing hydrolysed proteins, moisture content had the largest effect on FL, CML and HMF contents, followed by temperature and screw speed (Figure 8.7 A). In the diet containing intact proteins, screw speed also influenced Maillard reaction products (Figure 8.7 B). FL and CML content increased with increasing temperature, moisture content and screw speed. HMF content was lower with increasing temperature, however, was higher with increasing moisture content. As HMF is not an end-product, a reasonable explanation of the lower HMF contents with increasing temperature is the fact that HMF disappears due to progression into additional components of the Maillard reaction. Screw speed did not have a significant effect on HMF. LAL contents were not significantly different in both diets with increasing temperature, moisture content and screw speed. During the pelleting process, CML and FL content were higher when temperature increased. Overall, increasing temperature and moisture content lead to lower total and reactive lysine contents, and higher levels of Maillard reaction products in the processed dog foods used in our experiments. Increasing screw speed has a positive influence on total and reactive lysine content, but a negative influence on Maillard reaction products.

Nutritional impact of the Maillard reaction in pet foods

From a nutritional point of view, a difference between total and reactive lysine will result in a reduced nutritional value of the food. Besides the fact that the Amadori compound that is formed cannot be fully utilized, the reactive lysine present is not 100% bioavailable as standardised ileal reactive lysine digestibility of dry extruded and canned pet foods ranges between 80 and 98%.[1,9] These results can raise the question whether lysine levels in pet foods meet the animal's requirements. As stated in the general introduction (Chapter 1), pet food manufacturers produce their pet foods according to recommended allowances as presented by the NRC, FEDIAF or AAFCO.[10-12] Food for specific disease conditions are often formulated to contain low concentrations of certain nutrients, for example low protein content in the case of diets that aim to aid animals with a compromised renal function. In such cases, foods are often formulated to contain the minimum requirements of these nutrients. The guidelines as developed by NRC, FEDIAF or AAFCO, refer to lysine that is analysed using standard amino acid analysis, and therefore to total lysine. Minimal requirements of lysine as provided by the NRC relate to lysine with a high dietary bioavailability.[10] Recommended allowances for practical diets are based on this minimum requirement estimate by using a standard protein digestibility factor of approximately 80% (NRC), 67% (FEDIAF) or 44% (AAFCO). With the data from this thesis and information available in literature, a hypothetical scenario can be

calculated to determine whether these digestibility values are sufficient. In the commercially available pet foods analysed in Chapter 3, reactive lysine content was as low as 77% from the total lysine content in foods for dogs. Considering the lowest reported reactive lysine digestibility of 80% by Hendriks et al.[9], the bioavailability of the analysed total lysine decreases from 77% to 62%. This result indicates that the digestibility safety value of 80% for total lysine has a risk to overestimate the actual available reactive lysine in certain processed pet foods. The digestibility factors of 67 and 44% for the foods analysed in our experiments are in the safe zone. However, data reported in literature suggest that there might be a risk that foods contain even lower reactive to total lysine ratios and as such lower availability of total lysine.[1] In addition, even though pet food manufacturers more often aim for lysine contents higher than the recommended allowance, one food for growing dogs analysed in this thesis contained less reactive lysine than the advised minimal lysine requirement, and an additional 7 foods for growing dogs that had to have had reactive lysine digestibility values between 62 and 100% to meet minimal lysine requirements (Chapter 3). These foods are at risk of not meeting the minimal lysine requirements of growing dogs as stated by the NRC (i.e. 1.75 g/1000 kcal ME), especially when used as weaning diets.[10] On the other hand, all foods for adult and growing cats analysed in Chapter 3 contained reactive lysine levels above the recommended allowance of total lysine as reported by the NRC. Foods for cats contain in general more protein compared to foods for dogs, which explains the higher lysine levels in cat foods. Dietetic foods were not included in the dataset used in this thesis, however, for low protein dietetic foods it is also important that the bioavailability of lysine is not overestimated. It would be recommended to include reactive lysine levels and reactive lysine digestibility as a parameter in pet food formulation instead of using only total lysine levels. In addition, the prevention of a reduction in reactive lysine during food formulation and manufacturing is important, especially in diets for growing dogs and in low protein foods.

Bioavailability of Maillard reaction products in pet animals

To the author's knowledge, Chapter 7 of this thesis reports the first explorative study on the absorption and excretion of dietary Maillard reaction products in cats. A positive correlation was found between dietary intake and urinary excretion: when dietary intake of FL and CML is increased, urinary excretion of these components increased as well (Chapter 7). Hence, the results indicate that these dietary Maillard reaction products are, at least partially, absorbed from the gastrointestinal tract of cats and excreted in the urine. For LAL, no positive correlation was found. It is unknown whether and to which extent endogenously formed FL and CML are also excreted in urine of cats. An estimate

of endogenous excretion can be predicted by extrapolating the regression line through zero intake of dietary Maillard reaction products, however, fasting studies in cats should confirm this prediction. A negative correlation was reported between dietary intake and urinary recovery (as % of intake) for FL and CML in the dry cat food, and for CML and LAL in the wet cat food. Absorption and/or excretion of dietary CML is higher compared to dietary FL and LAL. If the excretion of endogenous formed FL, CML and LAL is negligible, the results in Chapter 7 indicate that 6 to 73% of the dietary FL, CML and LAL is absorbed from the gastrointestinal tract of cats fed commercially available dry and wet foods, and subsequently excreted in the urine.

A decrease in urinary recovery with increasing dietary intake suggests that either 1) digestion and absorption is inhibited due to a higher dietary intake, 2) *in vivo* metabolism of the components is increased with increasing intake, or 3) excretion of the components in the urine is limiting with increasing intake (Figure 8.8). From the data in Chapter 7, however, the fate of the dietary Maillard reaction products not excreted by the kidney is unknown. These may have remained in body tissues or blood plasma, or may have not been absorbed from the gastrointestinal tract. In the latter case, metabolism by the large intestinal microbiota could have occurred or they were unmodified excreted in the faeces. During the experiment, blood plasma and faeces were also collected. Increasing levels of FL, CML and LAL in the blood plasma with an increase in dietary intake would indicate that FL, CML and LAL are absorbed from the small intestine into the bloodstream (Figure 8.8). In addition, clearance rates by the kidney into the urine can be calculated. Dietary Maillard reaction products that are not absorbed in the small intestine enter the large intestine. It is known that microbiota in the large intestine metabolise and/or degrade FL into different, unknown components that are absorbed in the intestine or excreted in the faeces.[16] It is suggested that CML and LAL can be degraded by colonic microbiota as well,[14,17] however, a higher recovery of CML and LAL compared to that of FL in faeces of rats indicates that if CML and LAL are metabolised by microbiota, the amounts will be lower than FL.[13] Therefore, analysis of FL, CML and LAL in faeces might not be an accurate measure of the concentration of these components that are not absorbed in the small intestine. Alternative analysis could include the use of inflammation markers and S100A12 in faeces as biomarkers of gastrointestinal health. Unfortunately, due to a delay in analytical method development, analysis of faeces and blood plasma was not realised within the timeframe of the project. It would, however, be of significant interest to determine the clearance rate of the various Maillard reaction products from plasma. This provides further information about the metabolism and excretion of Maillard reaction products by cats.
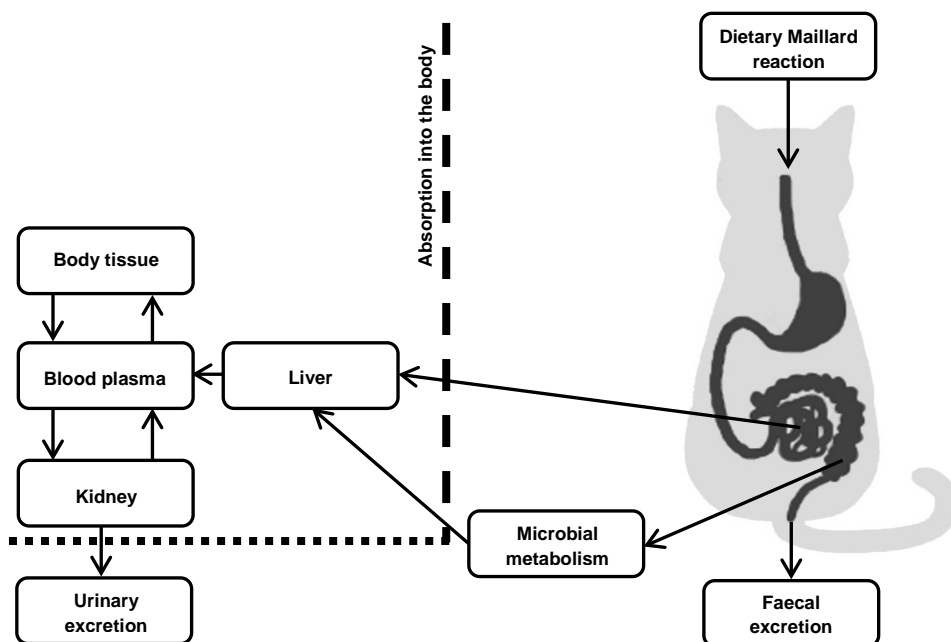
Figure 8.8 Dietary Maillard reaction products that enter the animal's gastrointestinal tract are absorbed, distributed, metabolised, and excreted in either faeces or urine, like has been reported in humans, rats and mice.[13-15]

To keep the experiment close to practice, commercially available dry and wet cat foods were analysed for their content of FL, CML and LAL, and foods contrasting in dietary Maillard reaction product content were chosen to be included in the study. HMF was excluded from the analysis, as HMF is metabolised in the body. The major pathway is oxidation of HMF to 5-hydroxymethyl-2-furanoic acid (HMFA) and subsequent conjugation with glycine to form N-(5-hydroxymethyl-2-furoyl) glycine (HMFG) as main metabolites. These components can be determined in urine of humans and rats,[7] however, in this thesis these components were not taken into account due to lack of commercial standards and analytical methodology.

To be able to cover all the pathways of dietary Maillard reaction products in the animal's body, it is of importance that future research includes analysis of blood plasma and faecal samples. In addition, to be able to survey the complete metabolic pathways of absorption, metabolism and excretion, labelled synthetic compounds are needed to allow quantitative determination of Maillard reaction products in body tissues and excreta.

Consumption of Maillard reaction products from commercial pet foods, a health risk?

Average daily intake (mg/kg body weight$^{0.75}$) of CML of dogs and cats consuming an average extruded food is comparable to the average daily intake of human adults (Chapter 4). HMF average daily intake, however, is 122 times higher for adult dogs and 38 times higher for adult cats compared to the average intake of adult humans. Although the presence of Maillard reaction products in pet foods does not imply a direct risk for the animal, the fact that dietary FL and CML are to a certain extent absorbed in and excreted by cats could indicate that there might be certain health implications for pet animals.

Advanced glycation end-products accumulate in body tissues over time. Elevated levels of advanced glycation end-products in body tissues have been associated with various age-related diseases in humans and rats, as well as in dogs,[18-23] although no direct causality was proven. In addition, plasma of dogs suffering from canine diabetes mellitus contained higher concentrations of advanced glycation end-products compared to control dogs.[24] Cell-associated AGE-specific receptors, like "receptor for advanced glycation end-products" (RAGE) and "advanced glycation end-product receptor 1" (AGER1) are reported in humans and dogs.[18] These receptors are thought to be part of the natural defence system of the body to regulate or avoid adverse effects of these compounds.[25] Up-regulation of RAGE leads to an increase in inflammation and oxidative stress, whereas up-regulation of AGER1 leads to removal and degradation of advanced glycation end-products.[25] The results of these studies indicate that Maillard reaction products might in a certain way be related to various diseases, and it should be questioned whether dietary Maillard reaction products can affect health. As this has never been studied in dogs or cats, results from studies in mice and humans have to provide information.

Lifelong restriction of dietary CML in healthy mice showed higher AGER1 expression and reduced plasma 8-isoprostanes (a lipid peroxidation product), tissue RAGE and p66 levels (oxidative stress regulatory components) compared to a control diet, resulting in an decreased insulin resistance, tissue damage and longer lifespan.[26] It was concluded that the increased AGER1 expression with reduced dietary CML indicate that AGER1 levels remain responsive to increases in advanced glycation end products when the external CML concentrations remain below a certain threshold, but fail to respond in case of chronically excessive exogenous CML levels.[26] Adding synthetic methylglyoxal to the restricted CML diet increased previously mentioned markers for oxidative stress again.[27] Dietary Maillard reaction products (unknown compounds) were related to increased proteinuria in both healthy control and remnant-kidney model rats.[28] In addition, restriction of dietary CML led to improved insulin sensitivity in *db/db* mice.[29] It should be noted, however, that the choice of diets can be a limiting factor in these kind of studies. In

the first study, the difference in CML level was induced by heating of the diets.[26] Other heat-sensitive nutrients could either increase or decrease during the extreme processing necessary to induce high concentrations of Maillard reaction products in the experimental diets. The second study replaces an ingredient to induce dietary Maillard reaction products,[28] while the last study uses complete different diets.[29] Contrasting diets have to be similar in energy and nutrient content to be able to differentiate between effects of Maillard reaction products. Replacing part of the experimental diet with components of ingredients containing high amounts of Maillard reaction products can result in deviant nutritional values. All these diets low and high in Maillard reaction products have a high chance to differ in more parameters than just the Maillard reaction products. It could be questioned whether the effect that is reported in such studies is due to dietary Maillard reaction products, or are biased by other dietary parameters. This phenomenon could be prevented by using, for example, chemically synthesized and well-characterised Maillard reaction products in otherwise exact comparable experimental foods to be able to identify individual effects. In diabetic as well as healthy human subjects, restriction in dietary Maillard reaction products for up to 4 months decreased inflammatory markers of diabetes, while insulin sensitivity increased.[22,23] Compared to the diabetic patients, fewer markers were affected in the healthy individuals. In human subjects with kidney disease, dietary restriction of Maillard reaction products lowered oxidative stress and RAGE levels, and restored biomarkers associated with renal failure.[30] In all the human trials, dietary Maillard reaction products were induced by different cooking methods by either the researchers or the humans themselves. Dietary Maillard reaction products were estimated using food databases present in literature. Also in these diets, it is not clear whether the reported effects are due to dietary Maillard reaction products or other dietary parameters. In addition, the length of the human studies is rather short. In animals like mice and rats it is easier to determine the effects of dietary Maillard reaction products during the entire lifespan of the animal. Unfortunately, the data of the foods in all these studies is analysed using CML-sensitive enzyme-linked immunosorbent assays, expressing the results in arbitrary units/mg. Therefore, the dietary intake of CML that is suggested to induce or suppress the mentioned health risks cannot be related to the intake calculated in this thesis for cats and dogs (Chapter 4). Only one study reports data of dietary CML that can be compared to the data in this thesis. In healthy human subjects, dietary consumption of 5.4±2.3 vs. 2.2±0.9 mg CML per day resulted in increased markers associated with an enhanced risk of type 2 diabetes and cardiovascular diseases.[31] The high-CML diet also contained 40-fold higher HMF and 5-fold higher acrylamide contents compared to the low-CML diet (quantitative data not provided by the authors). When expressed per metabolic body weight, the humans in this study consumed 0.22 vs. 0.09

mg CML/kg $BW^{0.75}$ (assuming an average BW of 70 kg). The results of this study suggests that reducing the intake of dietary Maillard reaction products may help to positively modulate biomarkers associated with an increased risk of diabetes mellitus and cardiovascular diseases. The calculated average daily CML intake of 0.50 mg/kg $BW^{0.75}$ for dogs and 0.28 mg/kg $BW^{0.75}$ for cats is higher compared to the humans in the latter study. Although the general consensus of these studies suggests that diets high in Maillard reaction products can stimulate markers associated with various diseases in healthy and diseased humans and animals, these results should be considered with care. Besides the limitations of the use of experimental diets, most studies only define CML as marker for dietary Maillard reactions in the experimental foods. The use of just one single marker limits knowledge on the amount and impact of other Maillard reaction products. It is unknown whether other dietary Maillard reaction products have the same effects. Furthermore, the analytical methods used are important when comparing results between studies. Standardized validated methods that accurately quantify Maillard reaction products in foods, biological tissues and fluids are necessary to be able to build a reliable database. These analytical methods currently exist, however, should be further developed. The analytical method used in this thesis, UPLC-MS/MS after acid hydrolysis, is one of the most promising methods to determine Maillard reaction products, however should also be further validated and standardized.

Overall, intake of dietary CML in pet animals is comparable to human intake, dietary CML in literature seem to induce biomarkers for several health risks in healthy and diseased human and mice, and dietary CML showed the highest recovery values in urine of cats compared to FL and LAL. Therefore, it might be possible that dietary CML also influences certain biomarkers in cats. In high risk groups, such as pet animals with diabetes or renal failure, the positive effect of a restriction of dietary Maillard reaction products might be more beneficial compared to healthy animals. Therefore, certain veterinary diets like renal, cardio, and diabetic diets could benefit from a reduced content of Maillard reaction products. Cats and dogs ingest much more HMF compared to humans, however, excretion and recovery of dietary HMF was not analysed in this thesis. In addition, it is not clear whether exposure to dietary HMF represents a potential health risk.[7] Some LD_{50} values are reported for HMF,[7,32] however, with limited data on dietary exposure to both CML and HMF it is not possible to establish maximum intake and safe upper limits for humans and pet animals. To be able to determine the health risks of dietary Maillard reaction products, further long term studies in healthy and diseased cats and dogs should be performed, using standardized diets and possibly including synthetic Maillard reaction products.

General conclusions and recommendations

Maillard reaction Based on the lower reactive than total lysine contents found in previous studies, it was suggested that the Maillard reaction would take place during pet food processing. The experiments in this thesis report the presence of the Maillard reaction products FL, CML and HMF and the amino acid LAL in processed commercial pet foods. A large variation in the contents of specific Maillard reaction products was observed in commercially available canine and feline foods. Analysis of additional compounds will provide a better insight into the progression of the Maillard reaction during the manufacturing of pet foods. Using data reported for commercial pet foods in this thesis, the calculated dietary intake of Maillard reaction products by dogs and cats is equal or higher compared to humans.

Processing Extrusion decreases total and reactive lysine content and increases Maillard reaction products. However, the extent of the effect depends on the ingredients used during pet food extrusion and the severity of the processing conditions. Hydrolysed proteins result in a more extensive Maillard reaction during processing than intact proteins. To maintain total and reactive lysine contents and prevent the formation of Maillard reaction products, a reduced temperature and moisture content are recommended. Screw speed shows opposite results, with a higher screw speed resulting in higher total and reactive lysine levels, while increasing Maillard reaction products. Steam pelleting has a minor effect on the Maillard reaction, only FL and CML increase with increasing conditioning temperature. Despite the reported effects of processing, the unprocessed ingredient mix already contained low reactive to total lysine ratios, and more Maillard reaction products than were formed during processing. Therefore, choice of ingredient rather than processing might be the key factor influencing the effect of the Maillard reaction in dog foods. Pet food manufacturers that aim to control bioavailable lysine and the presence of Maillard reaction products should evaluate the main ingredients rather than change pet food processing conditions.

Lysine requirements Analysis of 67 commercially available pet foods showed that most pet foods contain reactive lysine levels lower than total lysine levels, resulting in reactive lysine contents of 90% of total lysine, on average (range 67 – 100%). In comparison to the minimal lysine requirements as set by the NRC, foods for growing dogs, especially the ones used as weaning diets, could be at risk of not meeting minimal requirements due to a low bioavailable lysine content. Nutritional recommendations used for pet food formulation should include reactive lysine content and a measure of its digestibility to avoid limitations in the lysine supply to growing dogs. The results highlight the

importance of the prevention of lysine damage due to the Maillard reaction during pet food manufacturing.

Bioavailability Dietary Maillard reaction products are, at least partially, absorbed from the gastrointestinal tract of cats, where they can contribute to the endogenous pool, and are excreted via the urine. However, urinary recovery of dietary Maillard reaction products decreases with increasing intake, suggesting a limiting factor in digestion, absorption, metabolism or excretion through the kidneys. Future research should focus on both validation and standardization of analytical methods, as well as include blood plasma and faeces analysis to provide a more complete picture of mechanisms responsible for digestion, absorption and excretion of dietary Maillard reaction products.

Health risks Dogs and cats fed commercially available pet foods ingest equal CML, but more HMF compared to humans expressed on a metabolic body weight basis. From the experiments performed in this thesis, it cannot be determined whether the Maillard reaction in pet foods induces a certain health risk for dogs or cats. Although several scientific publications suggest that dietary Maillard reaction products promote biomarkers for age-related diseases like diabetes and renal failure, these studies do not provide evidence for a causal relationship. Unambiguous evidence that dietary Maillard reaction products are responsible for the biological effects is lacking. Future studies should focus on long-term experiments to indicate the risks of dietary Maillard reactions to the pathology of diseases.

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Summary

Summary

Pet dogs and cats around the world are commonly fed processed commercial foods throughout their lives. Often heat treatments are used during the processing of these foods to improve nutrient digestibility, shelf life, and food safety. Processing is known to induce the Maillard reaction, in which a reducing sugar binds to a free reactive amino group of an amino acid. In intact proteins, the ε-amino group of lysine is the most abundant free amino group. The reaction reduces the bioavailability of lysine and results in the formation of advanced Maillard reaction products. The aim of this thesis was to determine the occurrence and progression of the Maillard reaction during the manufacturing of pet foods, the subsequent impact on nutritive value of the food, and the bioavailability of Maillard reaction products in cats.

In Chapter 2, the scientific literature was reviewed to investigate the current state of knowledge on the Maillard reaction and its potential effect on the nutritive value of pet foods and on pet health. Determination of the difference between total and reactive lysine by chemical methods provides an indication of the Maillard reaction in pet foods. Studies reported that the proportion of reactive lysine is on average 73% (range 39 – 100%) of total lysine, and that foods for growing dogs may be at risk of supplying less lysine than the animals require. The endogenous analogues of Maillard reaction products, advanced glycation end-products, have been associated with age-related diseases in humans, such as diabetes and impaired renal function. In dogs, data indicate higher advanced glycation end-product contents in plasma from dogs suffering from canine diabetes mellitus compared with healthy control animals. In addition, elevated levels of advanced glycation end-products in tissue proteins in dogs were observed for a number of diseases. To date it was unknown to what extent Maillard reaction products were present in pet foods, and whether dietary Maillard reaction products can be associated with the development of diseases such as diabetes and impaired renal function in pet animals. As the Maillard reaction is induced by processing, changing processing conditions should have an influence on the severity of the reaction. However, effects of processing conditions on the difference in total and reactive lysine contents in pet foods were inconsistent and did not always correspond to model systems. Processing temperature was reported to be the most important factor followed by moisture level. In addition, differences between total and reactive lysine were observed in several ingredients commonly used in pet foods. Reviewing the literature indicates that it is unknown to which extent the Maillard reaction occurs and whether Maillard reaction products are present in pet foods. There might be a risk for certain foods not meeting

minimal lysine requirements. It is also unknown what the exact effect of processing on the Maillard reaction is in pet foods.

The experiment described in Chapter 3 was designed to evaluate whether commercial pet foods meet minimal lysine requirements. Sixty-seven extruded, canned and pelleted commercially available dog and cat foods formulated for growth and maintenance were analysed using conventional amino acid analysis and *O*-methylisourea as reagent for reactive lysine. Sixty out of the 67 foods in this study, regardless of the type of processing technology used, contained a lower reactive lysine than total lysine content. On average, pelleted and extruded foods contain lower reactive to total lysine ratios compared to canned foods (0.85, 0.89, and 0.93, respectively). All cat foods and foods for adult dogs met minimal lysine requirements. However, eight dry foods for growing dogs contained reactive lysine contents between 96 and 138% of the minimal lysine requirement, indicating that reactive lysine has to be between 62 and 104% digestible to meet minimal requirement. Considering the variability in reactive lysine digestibility, these foods could be at risk of not meeting minimal lysine requirements for growing dogs.

In Chapter 4, the foods from Chapter 3 were used to quantitate the Maillard reaction products fructoselysine (FL), carboxymethyllysine (CML), hydroxymethylfurfural (HMF), and the cross-linked amino acid lysinoalanine (LAL) using UPLC-MS. In all foods, Maillard reaction products and LAL were found but in highly variable amounts. Type of processing seems to be a key factor for the concentration of FL, CML and HMF, with on average higher amounts in canned foods than pelleted and extruded foods (on a dry matter basis). The contents of CML and HMF found in commercial pet foods are, on average, within the range reported in processed human food products. Average daily intake (mg/kg body weight$^{0.75}$) of HMF was 122 times higher for dogs and 38 times higher for cats than the calculated average intake for adult humans. Average daily intake of CML was comparable to the intake of adult humans.

As Chapters 3 and 4 indicated that pelleted foods contain more Maillard reaction products than extruded foods, despite the less severe production process, an experiment was designed to gain insight in the effect of steam pelleting on the Maillard reaction in a dog food (Chapter 5). The aim was to examine the effect of conditioning temperature (65 and 90°C) and die hole length (ø 5 × 45, 65, and 80 mm) during pelleting processing of a standard dry dog food on selected indicators of the Maillard reaction (total lysine, reactive lysine, FL, CML, HMF, LAL), browning development and CIE-Lab colour. Steam pelleting did not cause a significant loss of reactive lysine and change of absorbance values. This indicates that the effect of steam pelleting on the nutritive value of the foods is low. However, steam pelleting did increase the content of Maillard reaction products. The formation of the Maillard reaction products was associated with an increase in

temperature and die hole length during the steam pelleting process. The unprocessed ingredient mix already contained a larger difference between reactive and total lysine, and contents of Maillard reaction products than was induced during steam pelleting. Therefore, the choice of the ingredients used in this study mainly determines reactive lysine content and Maillard reaction products in the pet food formulation.

As it is unknown to which extent extrusion processing influences the Maillard reaction in pet foods, the effect of extrusion processing on selected indicators of the Maillard reaction was determined (Chapter 6). The extrusion parameters temperature (140 and 165°C), moisture content (200 and 300 g/kg) and screw speed (100 and 200 rpm) were applied to two dry dog foods formulated using either intact or hydrolysed proteins. Extrusion processing in general results in a decrease in total and reactive lysine and an increase in FL, CML, HMF and LAL content. However, this effect appeared more pronounced in the diet containing hydrolysed protein. Decreasing temperature and moisture content led to higher total and reactive lysine contents, and less Maillard reaction products in the dog foods. Increasing screw speed had a positive influence on total and reactive lysine, but a negative influence on Maillard reaction products. As was found in Chapter 5, the unprocessed ingredient mixtures in this experiment contained already more Maillard reaction products than was induced during extrusion processing.

Whether the Maillard reaction products reported in pet foods are physiologically relevant in pet animals depends on the bioavailability of these components. Therefore, urinary excretion was studied in adult cats fed commercial moist and dry foods containing varying amounts of FL, CML and the amino acid LAL (Chapter 7). A pilot study was first conducted to determine the adaptation time required for stable urinary excretion of the Maillard reaction products when changing diets with contrasting contents of Maillard reaction products. An adaptation time of 1 d was deemed sufficient in adult cats. The short adaptation time indicates an effective urinary excretion of Maillard reaction products. In the main study, six commercially processed dry and six moist diets were fed to 12 adult female cats in two parallel randomized, 36-day, balanced Latin square designs. Urine was collected quantitatively and FL, CML and LAL were analysed in foods and collected urine using HPLC-MS. Daily urinary excretion of FL and CML showed a positive relationship with daily intake in the dry and moist foods. For LAL, no significant relationship was observed. The observed increase in urinary excretion with increasing dietary intake indicates that dietary Maillard reaction products are absorbed from the gastro-intestinal tract of cats and excreted in the urine. Minimum apparent absorption based on urinary excretion (assuming 100% of the excreted component originates from the diet) of FL, CML and LAL was found to range between 8 to 23%, 25 to 73% and 6 to 19%, respectively. Urinary recovery (% ingested) showed a negative relationship with daily intake for FL, CML and LAL in the dry foods and for CML and LAL in the moist foods.

The observed decrease in urinary recovery with increasing intake suggests a limiting factor in digestion, absorption, metabolism or urinary excretion.

The studies reported in this thesis are one of the first to determine Maillard reaction products in pet foods and the bioavailability of FL, CML and LAL in cats. In addition, the results highlight the importance of reactive lysine measurement in foods for growing dogs used as weaning diets. Contribution of the absorption of dietary Maillard reaction products to the pathogenesis of various health conditions requires further study, as well as the potential role of restriction of dietary Maillard reaction products in prevention and treatment of long-term health implications. Extrusion and pelleting processing do increase the Maillard reaction, however, choice of ingredients appears to have a larger effect on the content of Maillard reaction products and can, therefore, be a useful strategy for pet food manufacturers that want to decrease the content of Maillard reaction products in their pet foods.

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Dan nu het moment dat ik ben aanbeland bij het allerlaatste stuk dat ik zal schrijven voor dit boekwerk, wat op één van de laatste pagina's staat, maar door vrijwel iedereen als eerste gelezen wordt! Voor de meeste mensen blijft het daar ook bij, al wil ik iedereen natuurlijk uitdagen om ook de rest te lezen.

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Logica brengt je van A naar B, stijl en klasse brengen je overal. Myrthe, Sonja en Harma, echte s***jes met stijl en klasse. Net als je denkt dat het niet eigenwijzer kan, dan heb je jullie nog. We wonnen het zelfs van Ponpon de ezel! Samen besteden we de tijd aan de zin en onzin van het leven, euroweddenschappen (ik ben niet zo van de euro's...), verhitte en luidruchtige discussies, karaoke, en tijgerprints shoppen die 'ook wel geschikt zijn voor een congres'. Jullie zijn er, altijd en overal. Bedankt!

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reden dat ik heel erg blij ben dat jij mijn paranymf wilt zijn op de belangrijkste dag van het hele promotie-traject.

Naast het kantoor en lab is er veel werk verricht op Carus. Tamme, bedankt voor al je praktische hulp met extruderen, maar nog meer voor het samen roddelen tijdens dissectiedagen! Jij staat altijd klaar voor iedereen die je nodig heeft. Kasper, bedankt voor je tijd en hulp bij de kattenproef, ik vond het gezellig om op die manier met je samen te werken. De gekke kattenvrouwtjes Debbie en Sabine, bedankt voor jullie betrouwbare hulp en inzet, maar zeker ook voor jullie eindeloze geklets en geleuter! Maar, de belangrijkste medewerkers op Carus waren toch wel 'mijn' dames, vlnr: Diesel, Daisy & Abby, Kaatje, Lady, Miep, Muis, Puck, Queen, Sokje, Trix en Tamzin.

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Naast uni-collega's waren er het afgelopen jaar nog meer collega's: Bedankt Gert-Jan voor alles wat ik van je geleerd heb, Lonneke voor je gezelligheid en
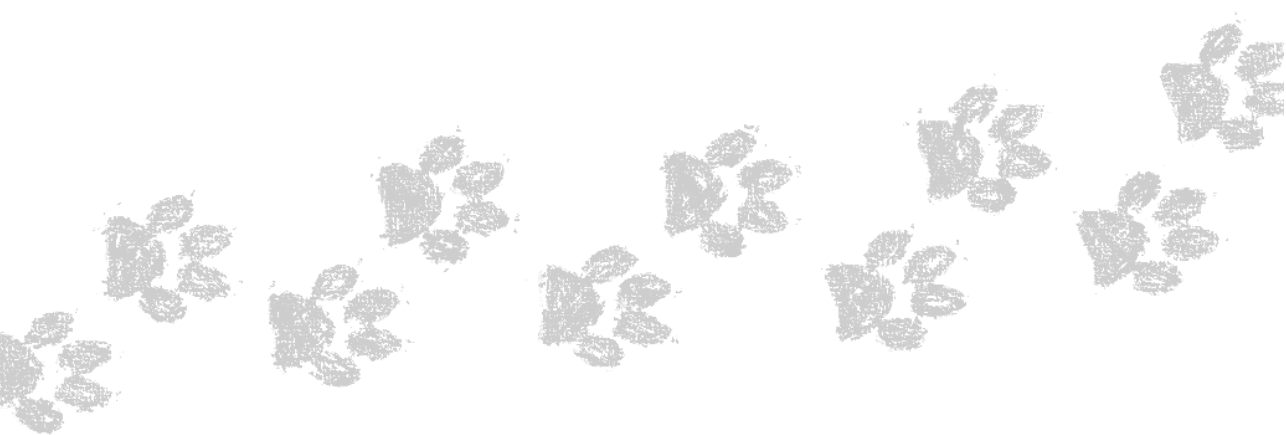
Lotte

About the author

Curriculum Vitae
List of Publications
Training and Supervision Plan

Curriculum Vitae

Charlotte (Lotte) van Rooijen was born on 19 October 1983 in Slochteren (the Netherlands) and grew up in Berlin (Germany), Haren and Maastricht (the Netherlands). She finished secondary school at the Montessori College (Maastricht, the Netherlands) in 2002, whereafter she studied at Wageningen University and obtained her BSc degree in Animal Sciences. During her MSc in Animal Sciences, Lotte followed the specialization Adaptation Physiology and Animal Nutrition. For the specialization Adaptation Physiology she determined embryo survival and development in sows with lactational ovulation induced by intermittent suckling. For the specialization Animal Nutrition she set up a protocol for a digestibility study in dogs under in-house living conditions. During her MSc internship, Lotte participated in several PhD projects at the Wildlife Sciences department of the University of Queensland, Australia. In 2008, she graduated for her MSc degree in Animal Sciences at Wageningen University. In 2010, she started her PhD at the Animal Nutrition group of Wageningen University in cooperation with WALTHAM Centre for Pet Nutrition (Waltham-on-the-Wolds, United Kingdom), within the framework of the Carbohydrate Competence Centre, resulting in this thesis. Meanwhile, she gained experience at pet food manufacturers Prins Petfoods (Veenendaal, the Netherlands; 2008-2009) and Vobra Special Petfoods (Veghel, the Netherlands; 2014-2015).

List of Publications

Peer reviewed scientific publications
Rooijen, C. van, Bosch, G., Poel, A.F.B. van der, Wierenga, P.A., Alexander, L. & Hendriks, W.H. (2013). The Maillard reaction and pet food processing: effects on nutritive value and pet health. Nutrition Research Reviews, 26, pp. 130-148.

Rooijen, C. van, Bosch, G., Poel, A.F.B. van der, Wierenga, P.A., Alexander, L. & Hendriks, W.H. (2014). Reactive lysine content in commercially available pet foods. Journal of Nutritional Science 3, e35.

Rooijen, C. van, Bosch, G., Poel, A.F.B. van der, Wierenga, P.A., Alexander, L. & Hendriks, W.H. (2014). Quantitation of Maillard reaction products in commercially available pet foods. Journal of Agricultural and Food Chemistry, 62, pp. 8883-8891.

Rooijen, C. van, Bosch, G., Wierenga, P.A., Hendriks, W.H., Poel, A.F.B. van der (2014). The effect of steam pelleting of a dry dog food on the Maillard reaction. Animal Feed Science and Technology, 198, pp. 238-247.

Rooijen, C. van, Bosch, G., Poel, A.F.B. van der, Wierenga, P.A., Alexander, L. & Hendriks, W.H. Effect of extrusion conditions on the Maillard reaction and *in vitro* digestibility in two dry dog foods. *To be submitted 2015*

Rooijen, C. van, Bosch, G., Poel, A.F.B. van der, Wierenga, P.A., Alexander, L. & Hendriks, W.H. Urinary excretion of dietary Maillard reaction products in healthy adult female cats. *Submitted to Journal of Animal Science 2015*

Vries, S. de, Pustjens, A.M., Rooijen, C. van, Kabel, M.A., Hendriks, W.H., Gerrits, W.J.J. (2014). Effects of acid extrusion on the degradability of maize distillers dried grain with solubles in pigs. Journal of Animal Science, 92, pp. 5496-5506.

Conference and symposia proceedings
Rooijen, C. van, Bosch, G., Poel, A.F.B. van der, Wierenga, P.A., Alexander, L., Hendriks, W.H. (2013). Reactive lysine content in commercially available pet foods. In: The WALTHAM International Nutritional Sciences Symposium 2013: From pet food to pet care – bridging the gap, 1-4 October 2013, Portland, Oregon, USA (pp. 42).

Rooijen, C. van, Bosch, G., Poel, A.F.B. van der, Wierenga, P.A., Alexander, L., Hendriks, W.H. (2013). Effect of extrusion conditions on protein quality in two dry dog foods. In: Proceedings of the 17th European Society of Veterinary and Comparative Nutrition Congress, 19-21 September 2013, Ghent, Belgium. Zelzate, Belgium: University Press, 2013 – ISBN 9789058643537 (pp. 58).

Rooijen, C. van, Bosch, G., Poel, A.F.B. van der, Alexander, L., Wierenga, P.A., Hendriks, W.H. (2013). Maillard reaction products in commercially available pet foods. Advances in Feed Evaluation Science course, Wageningen, The Netherlands, 23 May 2013.

Rooijen, C. van, Bosch, G., Poel, A.F.B. van der, Alexander, L., Wierenga, P.A., Hendriks, W.H. (2013). Maillard reaction products in commercially available pet foods. In: Proceedings of the WIAS Science Day 2013, 28 February 2013, Wageningen, The Netherlands. – Wageningen: WIAS Science Day, 2013 (pp.7).

Rooijen, C. van, Bosch, G., Alexander, L. & Poel, A.F.B. van der (2012). Evaluation of lysine damage in commercial dog and cat foods in the Netherlands. In John Cone (Ed.), Proceedings of the 37th Animal Nutrition Research Forum, 18 April 2012, Wageningen, The Netherlands. Wageningen: Wageningen University (pp. 23-24).

Poster presentations
Rooijen, C. van, Bosch, G., Alexander, L. & Poel, A.F.B. van der (2012). Lysine damage in commercial pet foods. WIAS Science day, Wageningen University, Wageningen, The Netherlands.

Rooijen, C. van, Poel, A.F.B. van der, Bosch, G., Alexander, L., Wierenga, P.A. & Hendriks, W.H. (2011). Monitoring Maillard products in pet foods – Quantification & nutritional effects. WIAS Science day, Wageningen University, Wageningen, The Netherlands.

Training and Supervision Plan

Basic package	Year	Credits[2]
WIAS Introduction Course	2010	1.5
WIAS Course on philosophy of science and ethics	2010	1.5

Scientific Exposure		
International conferences		
Waltham International Nutritional Sciences Symposium "Pet Nutrition - Art or Science?" Cambridge, UK, 16th – 18th September 2010	2010	0.9
Waltham International Nutritional Sciences Symposium "Petfood to Petcare…Bridging the Gap." Portland, Oregon, USA, October 1-4 2013	2013	0.9
17th European Society of Veterinary & Comparative Nutrition Congress, 19-21 September, Gent, Belgium	2013	0.9
Seminars and workshops		
WIAS Science Day (3x)	2011-2013	0.9
Animal Nutrition Research Forum (2x)	2010/2012	0.6
Seminar "Dietary lysine and the importance of processing of food- and feedstuffs."	2010	0.15
Symposium "100 Doctors of Philosophy in Animal Nutrition, which question can possibly remain?"	2012	0.15
Carbohydrate Competence Centre-symposium (3x)	2011-2013	1.5
Presentations		
WIAS Science Day: Poster Presentation (2x)	2011-2012	2.0
WIAS Science Day: Oral Presentation	2013	1.0
Carbohydrate Competence Centre: Oral presentation (3x)	2011-2013	3.0
Animal Nutrition Research Forum: Oral presentation	2012	1.0
Advances in Feed Evaluation Science: Oral presentation	2013	1.0
17th European Society of Veterinary and Comparative Nutrition Congress: Oral Presentation	2013	1.0
Waltham International Nutritional Sciences Symposium: Oral Presentation	2013	1.0

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

In-Depth Studies		
Disciplinary and interdisciplinary courses		
WBS Course: Advances in Feed Evaluation Science	2013	1.5
Advanced statistics courses		
WIAS Advanced Statistics Course: Design of Experiments	2010	1.0
WIAS Statistics for the Life Sciences	2010	2.0
MSc level courses		
Feed Technology	2013	2.0
Professional Skills Support Courses		
WIAS Course: Techniques for Scientific Writing and Presenting a Scientific Paper	2010	1.2
DO Course: Supervising MSc thesis work	2010	1.0
Scientific publishing	2013	0.3
Career assessment	2013	0.3
Research Skills Training		
Preparing own PhD research proposal	2011	6.0
HPLC course for amino acid analysis	2010	0.3
Didactic Skills Training		
Lecturing		
Principles of Animal Nutrition - Presentation	2011	0.3
Supervising practicals and excursions		
Principles of Animal Nutrition – Chicken growth practical	2012	0.9
Supervising theses		
BSc Thesis (1x)	2012	1.0
MSc Thesis (7x)	2011-2014	14.0
Tutorship		
Introduction to Animal Science	2010	1.0
Management Skills Training		
Membership of boards and committees		
WIAS Science Day	2011	2.0
Education and Training Total		54

Colophon