

Quantitation of Maillard Reaction Products in Commercially Available Pet Foods

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S Supporting Information

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 analyzed 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 tissues⁵ 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 etiology 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 hydrolyzed 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

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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 (ISO 6496, 1999) and combustion at 550 °C (ISO 5984, 2002), respectively. Crude protein (CP; N \times 6.25) was determined using the Dumas method (AOAC 990.03), and crude fat (CFat) was gravimetrically determined after hydrolysis with HCl and extraction with light petroleum ether (boiling point 40–60 °C; ISO 6492, 1999). Crude fiber (CFiber) was gravimetrically determined as the remaining insoluble organic fraction after acid and alkaline digestion (ISO 6865, 2000). 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 metabolizable energy (ME, in kJ “as is”) content was calculated using predictive equations for ME (eq 2, 3, 4, and 5).¹⁴

$$\text{NFE (g/kg DM)} = 1000 - \text{CFat} - \text{CP} - \text{crude ash} - \text{CFiber} \quad (1)$$

$$\begin{aligned} \text{Dogs: ME (kJ “as is”)} \\ = \text{digestible energy (DE, kJ)} - (4.35 \times \text{g CP}) \end{aligned}$$

$$\text{Cats: ME (kJ “as is”) = (DE, kJ) - (3.22 \times \text{g CP}) \quad (2)$$

$$\text{DE (kJ)} = \text{gross energy (GE, kJ)} \times \text{energy digestibility (\%)/100} \quad (3)$$

$$\begin{aligned} \text{Dogs: energy digestibility (\%)} \\ = 91.2 - (1.43 \times \% \text{CFiber in DM}) \end{aligned}$$

$$\begin{aligned} \text{Cats: energy digestibility (\%)} \\ = 87.9 - (0.88 \times \% \text{CFiber in DM}) \end{aligned} \quad (4)$$

$$\begin{aligned} \text{GE (kJ)} = (23.85 \times \text{g CP}) + (39.33 \times \text{g CFat}) \\ + (17.15 \times (\text{g NFE} + \text{g CFiber})) \end{aligned} \quad (5)$$

Amino Acid Analyses. Samples were defatted by extraction with light petroleum ether without acid hydrolysis (ISO 6492, 1999) and ground using a mixer mill (Retsch MM2000, Retsch BV). Total lysine (TL) was analyzed 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.¹⁵ Samples (5 mg) were hydrolyzed 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 analyzer (Biochrom, Cambridge, UK) and analyzed 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 Rutherford.¹⁶ 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.

$$\text{RL/TL ratio} = \text{OMIU} - \text{reactive lysine}/\text{total lysine} \quad (6)$$

RP-UPLC-MS. The MRPs furosine (as an indirect measurement for FL, see below), CML, HMF, and the cross-linked amino acid LAL were analyzed. Defatted samples (10 mg) were hydrolyzed according to the amino acid analysis procedure described previously. The samples were redissolved in 2 mL of 5 mM NFPA and filtered (0.2 μ m) 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 Ameer et al.¹⁷ For this, 10 mg of defatted sample was dissolved in 1 mL of Milli-Q and 250 μ L of 40% TCA. The sample was stirred for 5 min and centrifuged for 5 min at 9000g. Then, 800 μ L of the supernatant was separated, dried under vacuum (Savant SpeedVac Plus, SC210A), redissolved in 1 mL of 5 mM NFPA, and filtered (0.2 μ m) into vials for RP-UPLC-MS analysis.

Samples were analyzed using an Acella RP-UHPLC system (Thermo Scientific, San Jose, CA, USA). Samples (5 μ L) were injected on an Acquity UPLC BEH C18 column (2.1 \times 150 mm, 1.7 μ m particle size; Waters, Milford, MA, USA) with an Acquity UPLC BEH C18 Vanguard precolumn (2.1 \times 5 mm, 1.7 μ m 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 μ L/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 analyzing the eluate on a Thermo Scientific LTQ-Velos Pro equipped with an HESI-MS probe coupled to the RP-UHPLC; 300 μ L/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

1. Data-dependent MSⁿ analyses were performed with a normalized collision energy of 35%. Settings were optimized using “LTQ tune

Table 1. Selected Reaction Monitoring Conditions

compound	parent mass (Da)	fragment mass (Da)	range (<i>m/z</i>)
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

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. 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.^{18,19}

$$\text{FL (g/kg DM)} = \text{furosine (g/kg DM)} / (32/100) \quad (7)$$

$$\begin{aligned} &\text{regenerated (unreactive) lysine (g/kg DM)} \\ &= \text{FL (g/kg DM)} \times (56/100) \end{aligned} \quad (8)$$

$$\begin{aligned} &\text{FUR-RL (g/kg DM)} \\ &= \text{total lysine (g/kg DM)} - \text{regenerated lysine (g/kg DM)} \end{aligned} \quad (9)$$

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 Shapiro–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, CFiber, 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 2). With regard to animal species, cat foods contain, on average, higher CP and CFat but lower DM and lower CFiber 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.¹⁴ 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 CFiber 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 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 analyze 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.¹⁹ The regenerated (unreactive) lysine calculated from the furosine procedure originates specifically from the Amadori compound,¹⁸ 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.^{18,20} 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 analyzed is unknown. The analyzed 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 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. Rutherford 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 (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

Table 2. Proximate Composition and Metabolizable Energy Content of 67 Commercial Pet Foods Categorized by Animal Species, Processing Type, and Life Stage (Mean \pm Standard Error of the Mean, g/kg Dry Matter unless Defined Differently)^a

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

^an = 5, except for extruded junior dog foods (n = 16) and extruded junior cat foods (n = 11). ^bCalculated as (g/DM) 1000 – crude fat – crude protein – crude ash – crude fiber. ^cCalculated using predictive equations for ME.¹⁴

Table 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 \pm Standard Error of the Mean, g/kg Dry Matter unless Defined Differently)^a

variable	dog						cat					
	extruded			pelleted			extruded			canned		
	junior	adult	junior	adult	junior	adult	junior	adult	junior	adult	junior	adult
TL	mean	13.7 \pm 0.9	14.5 \pm 3.0	28.4 \pm 5.0	24.2 \pm 3.2	12.7 \pm 0.9	11.6 \pm 0.8	17.4 \pm 1.4	16.4 \pm 1.6	44.3 \pm 3.7	36.5 \pm 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 \pm 0.9	13.2 \pm 2.8	27.1 \pm 4.5	23.9 \pm 3.6	10.9 \pm 0.8	9.9 \pm 0.8	15.0 \pm 1.1	15.9 \pm 1.8	40.7 \pm 4.9	32.1 \pm 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 \pm 1.0	14.1 \pm 3.0	26.2 \pm 4.8	21.7 \pm 2.8	11.9 \pm 1.2	11.5 \pm 0.8	16.9 \pm 1.4	15.9 \pm 1.6	41.3 \pm 4.1	34.1 \pm 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 \pm 0.02	0.90 \pm 0.03	0.96 \pm 0.02	0.98 \pm 0.04	0.86 \pm 0.02	0.84 \pm 0.01	0.88 \pm 0.03	0.97 \pm 0.02	0.91 \pm 0.05	0.88 \pm 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	0.87–0.98	0.91–1.03	0.75–1.03	0.82–0.93	
FL ^e	mean	0.58 \pm 0.04	0.71 \pm 0.19	4.00 \pm 1.26	4.46 \pm 0.94	1.48 \pm 0.71	0.21 \pm 0.05	0.80 \pm 0.08	0.73 \pm 0.18	5.38 \pm 1.67	4.30 \pm 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 \pm 0.66	15.40 \pm 1.31	29.88 \pm 7.39	36.50 \pm 6.00	24.39 \pm 4.63	16.07 \pm 2.15	10.87 \pm 0.82	13.25 \pm 2.81	38.76 \pm 4.11	41.61 \pm 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 \pm 0.06	1.11 \pm 0.27	1.79 \pm 0.59	1.70 \pm 0.33	0.92 \pm 0.21	1.40 \pm 0.26	0.64 \pm 0.08	0.47 \pm 0.07	1.24 \pm 0.37	0.93 \pm 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 \pm 0.63	6.41 \pm 1.19	5.80 \pm 0.64	7.64 \pm 1.06	6.14 \pm 0.89	10.24 \pm 1.82	5.55 \pm 0.78	7.23 \pm 0.82	6.77 \pm 1.41	7.32 \pm 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 ($n = 11$). ^bReactive lysine analyzed using the O-methylisourea procedure. ^cReactive lysine analyzed using the furosine procedure; calculated as total lysine – regenerated lysine. ^dRatio, calculated as reactive lysine/total lysine (TL and RL in g/kg dry matter). ^eCalculated as furosine/(32/100). ^f¹⁸F/¹⁹F In mg/kg dry matter.

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 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).²¹ 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, HMF, 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.^{22–27} 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).²⁸ 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)²² 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),^{29–31} 6.6–241 mg/kg in breakfast cereals,³² 0.2–69 mg/kg in white bread,^{31,33} 0.5–74.6 mg/kg in cookies,¹⁷ and 0.2–34.7 mg/L in infant formulas.^{34–37} 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, Raymond³⁸ 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 analyzed pet foods are within the range reported for human foods (Table 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 ME¹⁴ 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 1). An adult cat (4 kg) with a daily energy requirement of 1.06 MJ ME¹⁴ 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 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

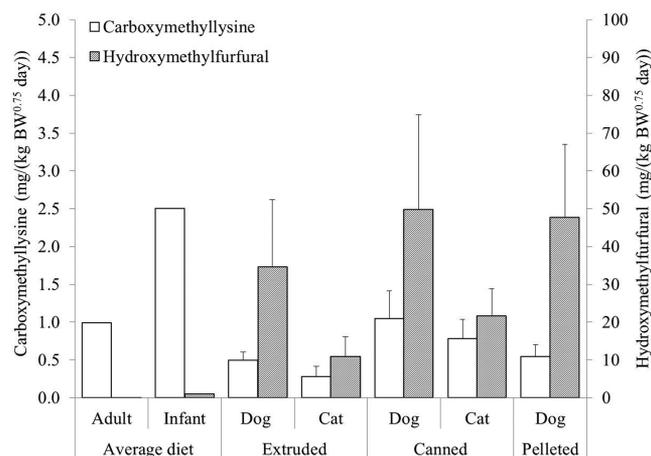


Figure 1. Estimated daily intake (mg/kg body weight (BW)^{0.75}) of carboxymethyllysine and hydroxymethylfurfural of a 70 kg adult human consuming a standard Western diet,^{22,31,32,39} a 6 kg human infant consuming 1 L of an average infant formula,^{23–27,34–37} and a 20 kg adult dog and a 4 kg adult cat fed either an extruded, canned, or pelleted diet for adult dogs and cats at maintenance (using a standard daily energy requirement¹⁴) containing an average \pm standard deviation content of carboxymethyllysine and hydroxymethylfurfural as reported in the current study.

0.99 mg CML/kg BW^{0.75} (70 kg BW) was estimated.³⁹ The CML content in infant formulas was quantified in five studies. Assuming a daily intake of 1 L 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.^{23–27} For HMF, the mean intake for adult humans in three different studies was 5.08, 5.56, and 10 mg per day,^{31,32,39} 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,^{34–37} 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 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, metabolized 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.⁴⁰ No evidence for prolonged accumulation in body tissues was observed. No adverse effects regarding acute and subacute toxicity in rats and mice ingesting HMF concentrations of 80–100 mg/kg body weight was reported.⁴¹ In humans, intake of dietary CML resulted in an increase in circulatory CML concentration.⁴² 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.^{8,43} Around 50% of the ingested CML was not recovered, and it is unknown if it is deposited in organs, degraded by colonic

Table 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 \pm 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 \pm 0.13	7.04 \pm 0.93	59.15 \pm 7.07	7.03 \pm 0.33
animal species	– ^b	–	–	–
dog				
cat				
processing type ^c				–
extruded	1.84 \pm 0.17	5.93 \pm 2.7	–24.91 \pm 3.40	
canned	0	0	0	
pelleted	2.25 \pm 0.22	6.85 \pm 2.64	–22.79 \pm 4.57	
life stage	–	–	–	–
adult				
junior				
dry matter (g/kg)	–	–0.01 \pm 0.004	–	–
crude protein	–	–	–0.05 \pm 0.01	–
crude fat	–	0.006 \pm 0.002	–	–
crude fiber	–	–	0.35 \pm 0.17	–
crude ash	–	–	–	–
NFE ^d	–	–	–	–
RMSE ^e	0.59	0.73	7.29	2.72

^aCalculated as furosine/(32/100).¹⁸ ^b–, no significant correlation. ^cExtruded pet foods $n = 37$, canned pet foods $n = 20$, pelleted pet foods $n = 10$.

^dNFE, nitrogen-free extract calculated as 1000 – crude fat – crude protein – crude ash – crude fiber. ^eRMSE, root mean squared error.

microbiota, or metabolized. 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. 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). The processing conditions between pelleting, extrusion, and canning differ considerably. Extruded kibbles are processed at temperatures of 80–200 °C for 10–270 s under high pressure.⁴⁴ Canned foods are heated until the center of the can reaches temperatures of 121 °C for >10 min to sterilize the product.⁴⁵ Pelleted foods are relatively mildly heat treated using temperatures of 60–90 °C for 30–45 s.⁴⁶ In addition, pet food 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 preprocessed. Carbohydrate sources, for example, are often pretreated as pelleting processing temperatures and residence times are not sufficiently high to fully gelatinize the starch in the raw ingredients during processing.⁴⁷ It could, therefore, be that the MRP contents in pellets mainly originate from the preprocessed 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.⁴⁸ 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.⁴⁹ HMF was associated with a decreased CP content and increased CFiber 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 analyzed 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.

■ ASSOCIATED CONTENT

📄 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>.

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Author Contributions

All authors contributed fundamentally to the present manuscript. C.v.R. contributed to all facets including data collection, calculations, and writing the initial manuscript. Other authors contributed to study design, data interpretation, and manuscript preparation.

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Notes

The authors declare the following competing financial interest(s): Lucille Alexander is an employee of Mars Petcare.

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ABBREVIATIONS USED

ACN, acetonitrile; AGEs, advanced glycation end-products; BW, body weight; CML, ϵ -N-carboxymethyllysine; CFat, crude fat; CFiber, crude fiber; CP, crude protein; DM, dry matter; FL, fructoselysine; HCl, hydrochloric acid; HMF, (5-hydroxymethyl)-2-furfural; LAL, lysinoalanine; ME, metabolizable energy; MR, Maillard reaction; MRPs, Maillard reaction products; MS, mass spectrometric; NFE, nitrogen-free extract; NFPA, non-afuropentanoic acid; OMIU, O-methylisourea; RL, reactive lysine; SEM, standard error of the mean; SRM, selected reaction monitoring; TCA, trichloroacetic acid; TL, total lysine

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