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Urinary excretion of dietary Maillard reaction products in healthy adult female cats[1,2]

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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 etiology 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 2 parallel randomized, 36-d Latin square designs. The 24-h urine was collected quantitatively using modified litter boxes, and fructoselysine (FL), carboxymethyllysine (CML), and lysinoalanine (LAL) were analyzed using ultra high performance liquid chromatography (UHPLC) - mass spectrometer. 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 foods ($P < 0.01$, $P < 0.01$, and $P = 0.08$, respectively) and for CML and LAL in the moist foods ($P < 0.01$). The observed increase in urinary excretion with increasing dietary intake indicates that dietary MRP were absorbed from the gastrointestinal 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% and 23%, 25% and 73%, and 6% and 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: carboxymethyllysine, cat, dietary Maillard reaction products, fructoselysine, metabolic transit, urinary recovery

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INTRODUCTION

Pet cats and dogs are often fed commercially processed foods throughout their lives (Laflamme et al., 2008). 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**; Hodge, 1953; Friedman, 1999). These compounds are also endogenously formed during naturally occurring processes in body tissues (Thorpe and Baynes, 2003). Elevated MRP levels in tissue proteins are associated not only in humans but also in dogs with various age-related diseases, such as diabetes, cataracts, osteoarthritis, vascular dysfunction, and atherosclerosis (DeGroot et al., 2004; Bras et al., 2007; Comazzi et al., 2008; Shapiro et al., 2008; Chiers et al., 2010). Dietary MRP can be absorbed and contribute to the endogenous pool in human subjects (Förster et al., 2005; Delgado-Andrade et al., 2012). The absorptive capacity depends on whether MRP are present in a free or protein-bound form; the latter has to first be released by proteolytic enzymes (Finot and Magnenat, 1981). Once absorbed, dietary MRP can be metabolized or directly excreted in urine. Maillard reaction products have been reported in commercially processed pet foods (Van Rooijen et al., 2014). Whether these MRP are present in a free or protein-bound form is unknown, and information on the absorption of dietary MRP by the gastrointestinal 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 hematology and biochemistry values, and were deemed healthy. All cats were habituated to and trained for the experimental procedures before 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 to 0900 h) and for 1 h (1200 to 1300 h) during daytime 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 aluminum 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 1 side. Urine collection was performed using a modified litter box as described by Hendriks et al. (1999), 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 approximately 200 g polyethylene grains (diameter 2 to 4 mm) to allow cats to express normal behavior of covering feces. The bottom tray of the litter box contained 5 mL of 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 stored at −20°C.

Pilot Study

Ten adult healthy European shorthair cats (intact females, 5 to 6 yr of age) with an average BW of 3.1 ± 0.07 kg (range 2.6 to 3.3 kg) were divided into 2 groups of 5 cats each and were fed 2 commercially available moist foods. The foods were chosen on the basis of their expected contents of MRP based on previously conducted analyses (Van Rooijen et al., 2014) and were analyzed for their actual MRP content (Table 1). The foods were fed for 20 d, with an adaptation period of 10 d to the first food and a collection period of 10 d, starting at the change of the 2 foods. One group changed from food A to food B at d 11; the other group changed from

Table 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											
			Dry food						Moist food					
Component	Moist food A	Moist 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 fiber	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, [2] MJ/kg	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														
Furosine	70.0	338.7												
Fructoselysine[3]			894.9	504.6	1,240.7	1,344.8	1,460.5	1,355.0	258.6	252.2	234.9	1,256.4	249.0	263.9
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]Nitrogen free extract =1000 – (moisture + CP + crude fat + crude ash + crude fiber).

[2]ME = 14.6 × CP + 35.6 × crude fat + 14.6 × nitrogen free extract (NRC, 2006).

[3]Fructoselysine = mol furosine × 3.1 (Krause et al., 2003).

food B to food A. Food intake to maintain a stable body score condition for each cat was determined before the study (score C/D on the WALTHAM Size Health And Physical Evaluation-BCS system), and food allowance during the study was adjusted 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 the base value before diet change, whereas on d 11 to 20, urine produced per cat was collected daily and stored (−20°C). The acidified urine samples were analyzed for specific gravity and the content of furosine, CML, and LAL. During housing in the group rooms during the day, urine produced was also collected if voided.

Main Study

Twelve adult healthy European shorthair cats (intact females, 5 to 6 yr of age) with an average of 3.1 ± 0.09 kg BW (range 2.7 to 3.7 kg BW) were allocated to 1 of 2 parallel balanced Latin square designs. The 2 Latin square designs were completely separate and were analyzed independently; however, they were performed in a similar way and at the same time. One Latin square consisted of 6 cats receiving 6 commercially processed dry foods, whereas the other 6 cats received 6 commercially processed moist foods (Table 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 analyzed for MRP content before 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 for each cat was determined before the study (score C/D on the WALTHAM S.H.A.P.E.-BCS system), and food allowance was adjusted during the study on the basis of the calculated energy content of the food, if required. Individual feed intake was recorded daily, and fresh water was provided ad libitum daily. The urine of each cat was collected from the bottom tray into a single bottle and weighed, pooled over 3 d, and stored at −20°C. The acidified urine samples were analyzed 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 before analyses. The foods were analyzed for DM and crude ash (**CAsh**) by drying to a constant weight at 103°C (International Organization for Standardization, 1999a) and combustion at 550°C (International Organization for Standardization, 2002), respectively. Crude protein (N × 6.25) was determined using the Kjeldahl method (International Organization for Standardization, 2005) and crude fat (**CFat**) was determined gravimetrically after hydrolysis with HCl and extraction with light petroleum (boiling point 40°C to 60°C; International Organization for Standardization, 1999b). Crude fiber (**CFiber**) was determined gravimetrically as the remaining insoluble organic fraction after

acid and alkaline digestion (International Organization for Standardization, 2000). All analyses were performed in duplicate. Metabolizable energy (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 1,000 − moisture − CFat − CP − CAsh − CFiber.

Specific gravity of the acidified urine was determined during sample collection by measuring the weight of 1 mL of 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 analyzed in food and urine samples. Food samples were defatted by extraction with light petroleum ether without acid hydrolysis (International Organization for Standardization, 1999b) and finely ground using a mixer mill (Retsch MM2000, Retsch BV, Retsch Benelux, Aartselaar, Belgium). Food samples (10 mg) and freeze-dried urine samples (0.5 mL) were hydrolyzed using 1.0 and 0.5 mL of 6 *M* HCl, respectively, for 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, Thermo Fisher Scientific Inc., Waltham, MA). The samples were redissolved in 1.0 and 0.5 mL ultra performance liquid chromatography-grade Milli-Q water (Merck KGaA, Darmstadt, Germany), respectively, vortexed, sonicated, and filtered (0.2 µm). Samples were analyzed using an Accela Ultra High Performance Liquid Chromatography System (Thermo Scientific, San Jose, CA) 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% (vol/vol) formic acid, and eluent B was acetonitrile containing 1% (vol/vol) formic acid. The solubilized urine samples were diluted 50 times in 10 m*M* HCl containing 0.5 mg/L $^{13}C_6{}^{15}N_2$-lysine (Sigma-Aldrich, Steinheim, Germany) as the 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 to 2 min isocratic on 90% B, 2 to 10 min linear gradient from 90% to 40% B, 10 to 12 min isocratic 40% B, 12 to 13 min linear gradient 40% to 90% B, and 13 to 23 min isocratic on 90% B. The flow rate was 300 µL/min. Mass spectrometric data were obtained by analyzing samples on a LTQ-VelosPro (Thermo Scientific) equipped with a heated electrospray ionization 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 auxiliary gas flow rate was set at 5 (arbitrary units). The compounds were analyzed using a selected reaction monitoring (**SRM**) method (Table 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 mass spectrometry 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, *E*-*N*-carboxymethyl-L-lysine and lysinoalanine; Sigma-Aldrich). Data were acquired and analyzed using Xcalibur 2.1 (Thermo Scientific).

Table 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 (Krause et al., 2003). As such, the amount of FL (mg/kg) was calculated by multiplying the molar furosine values by 3.1, the known conversion factor for the degradation of fructoselysine during acid hydrolysis.

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 Inst. Inc., Cary, NC). 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 2-phase linear models (NLIN procedure; Koops and Grossman, 1993). The effect of food type (i.e., dry or wet) on urinary excretion and recovery 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 the fixed effect; cat was included as a random effect, with period used as a repeated model statement. The covariance structure was selected on the basis of the Bayesian information criterion and the Akaike information criterion. Significant effects of diet were explored using the Tukey honest significant difference 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 SE are reported with *P*-values. Normal distribution of the residuals was evaluated with the Shapiro-Wilk test. Data were log transformed when the residuals were found to have increasing variance. Overall differences were considered significant if $P < 0.05$. Values are expressed as least squares means ± 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 contents of the foods used in the pilot study were 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 foods A and B, respectively (Table 1). Mean food intakes of the cats fed foods A and B were 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. Daily urinary excretion varied between 84.3 and 130.2 mL, with an average of 105.5 mL, for food A and between 51.6 and 70.0 mL, with an average of 61.6 mL, for food B. The difference in diet MRP content of the urinary MRP excreted is clearly shown for furosine (Fig. 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 (Fig. 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 (Fig. 1B and 1C).

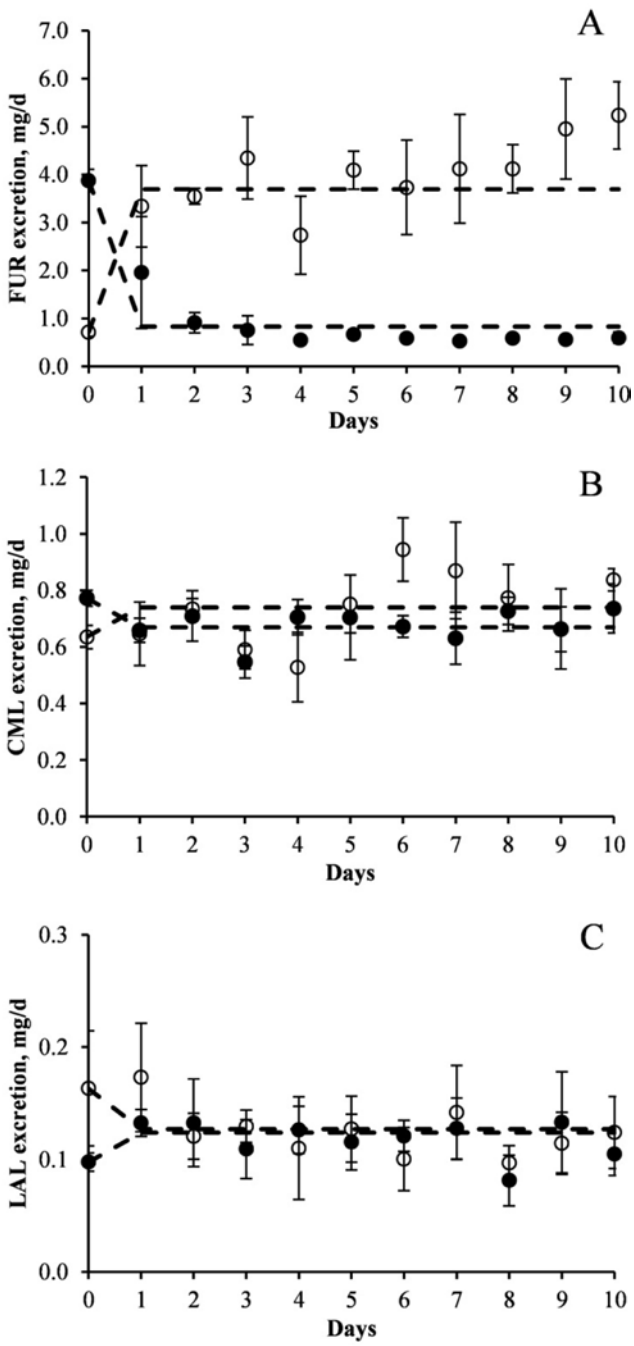

Figure 1. Mean ± SE daily urinary excretion of (A) furosine (FUR), (B) carboxymethyllysine (CML), and (C) lysinoalanine (LAL) for 10 d by adult cats after a change in diet (open circles = food A to B, solid circles = food B to A) and the linear-plateau model fitted through the data.

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% to 47% for furosine, 7% to 44% for CML, and 17% to 63% for LAL. This range is comparable to the variation observed in urinary excretion among rats, which was 22% to 41% for FL, 22% to 35% for CML, and 16% to 20% for LAL (Somoza et al., 2006). The linear-plateau model fitted through the data showed that for furosine an estimated plateau phase was reached within

Table 3. Daily food intake, urinary excretion, and intake, excretion, and recovery of the analyzed Maillard reaction products in the main study

	Dry food							Moist food						
Component	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]	41.02	20.35	49.42	58.79	57.43	57.15	2.50	67.41	40.39	47.25	177.58	55.73	44.03	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	6.39^{a}	4.76^{a}	6.60^{a}	4.36^{a}	11.07^{b}	6.78^{a}	0.88	7.34^{w}	7.93^{w}	4.84^{x}	37.21^{y}	4.65^{x}	4.74^{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^{d}	$18.2^{a,e}$	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

$^{a–e}$Within a row, means for dry food without a common superscript differ ($P < 0.05$).

$^{v–z}$Within a row, means for moist food without a common superscript differ ($P < 0.05$).

[1]Fructoselysine = mol furosine × 3.1(Krause et al., 2003).

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 is high. On the basis of these results, the minimum urine collection period of 3 d that was recommended for accurate total urine collection by Hendriks et al. (1999) 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 1): in the dry cat foods FL ranged from 504.6 to 1,460.5 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 234.9 to 1,256.4 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 contents were chosen intentionally to create contrasts in dietary MRP between the foods. As in the pilot study, FL was the predominant MRP in both dry and moist cat foods. Daily food intake is shown in Table 3 and corresponds to an average daily MRP intake of 20.4 to 58.8 mg FL, 2.0 to 4.8 mg CML, and 3.1 to 6.1 mg LAL in the dry cat foods and of 40.4 to 177.6 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 values 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 mL for the moist cat foods. Daily MRP excretions for FL and LAL were significantly different ($P < 0.05$; Table 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 ($\beta = 1.18$, $P = 0.20$; Fig. 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 ($\beta = 1.03$, $P = 0.45$; Fig. 3). The positive correlations for FL and CML between daily dietary intake and daily urinary excretion indicate 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/($BW^{0.75}$ d) and CML (89.2 vs. 284.0 mg/($BW^{0.75}$/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 than those for the foods used in the present study (Somoza et al., 2006). In human adolescents, a 5.9 mg/d increase in dietary CML did result in a 25%

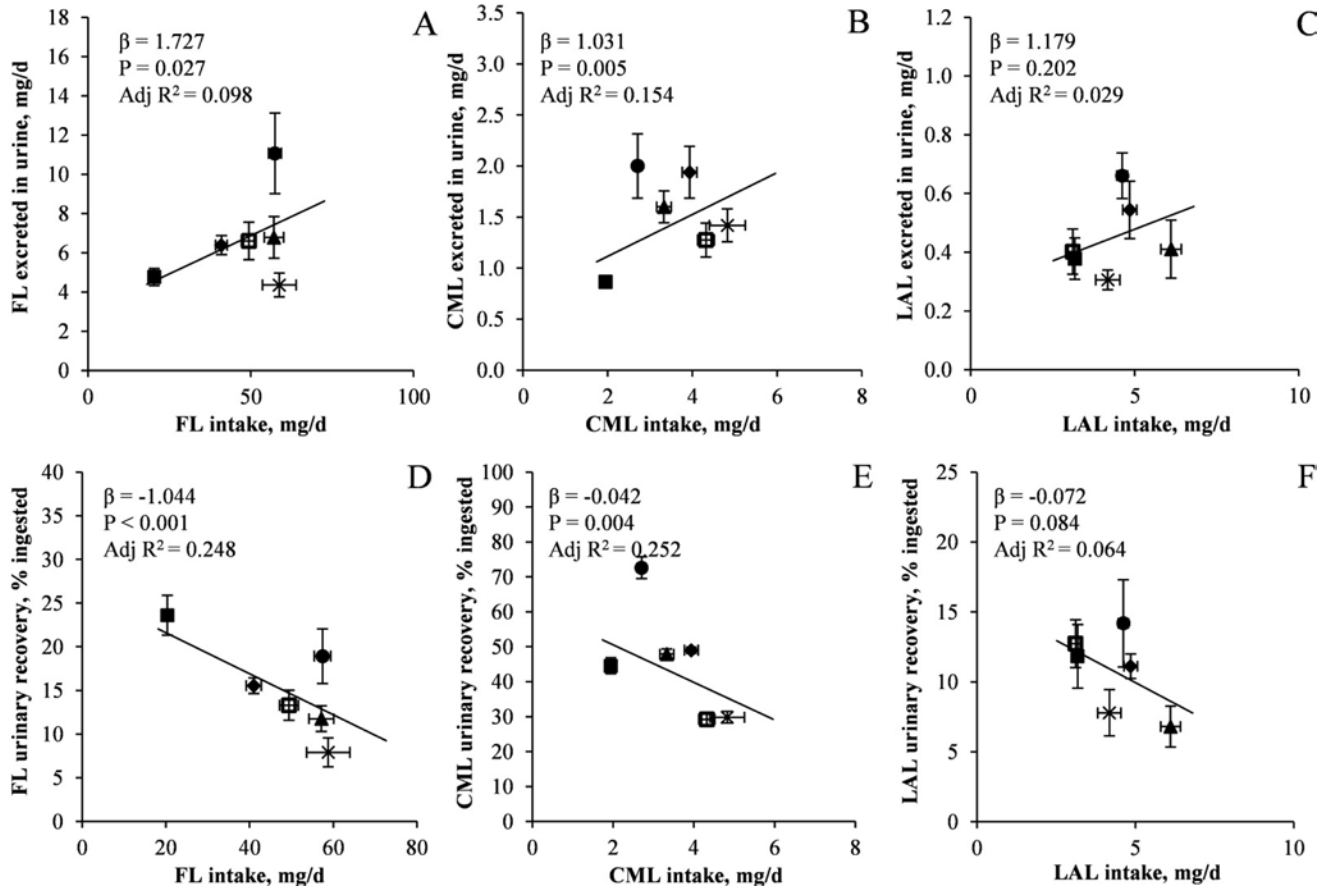

Figure 2. (A)–(C) Relationship of fructoselysine (FL), carboxymethyllysine (CML), and lysinoalanine (LAL) excretion in urine (mg/d) and intake (mg/d) and (D)–(F) relationship of FL, CML, and LAL urinary recovery (% ingested) and intake (mg/d) for 6 commercial dry cat foods (diamonds = food 1, solid squares = food 2, open squares = food 3, crosses = food 4, circles = food 5, triangles = food 6).

increase in daily urinary excretion (Delgado-Andrade et al., 2012). For the moist cat foods in the present study, the result for FL was strongly influenced by food 4, which contained a 3-fold higher FL content compared to the other 5 foods. Additional data points with daily intakes in the range of 60 to 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 to results found in rats, where a 4.9-fold increase in dietary LAL (139.0 vs. 681.6 mg/($BW^{0.75}$/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 among the foods used in the present study, highest and lowest dietary intakes do not always correspond to highest and lowest urinary excretions. Daily MRP urinary recovery (as a percentage of dietary ingested) showed significant differences ($P < 0.05$; Table 3) between the foods within both food types. In addition, urinary recovery of CML was higher in the dry foods than in 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 ($\beta = -0.07$, $P = 0.08$; Fig. 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 ($\beta = -0.20$, $P < 0.01$; Fig. 3). Comparable results were reported in literature for rats; urinary recovery of FL and LAL was reported to decrease from 5.2% to 3.7% and from 5%.6 to 4.9%, respectively, with increasing dietary intake (Somoza et al., 2006). In Somoza et al. (2006), 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 (Delgado-Andrade et al., 2012). 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; Alamir et al., 2013). A

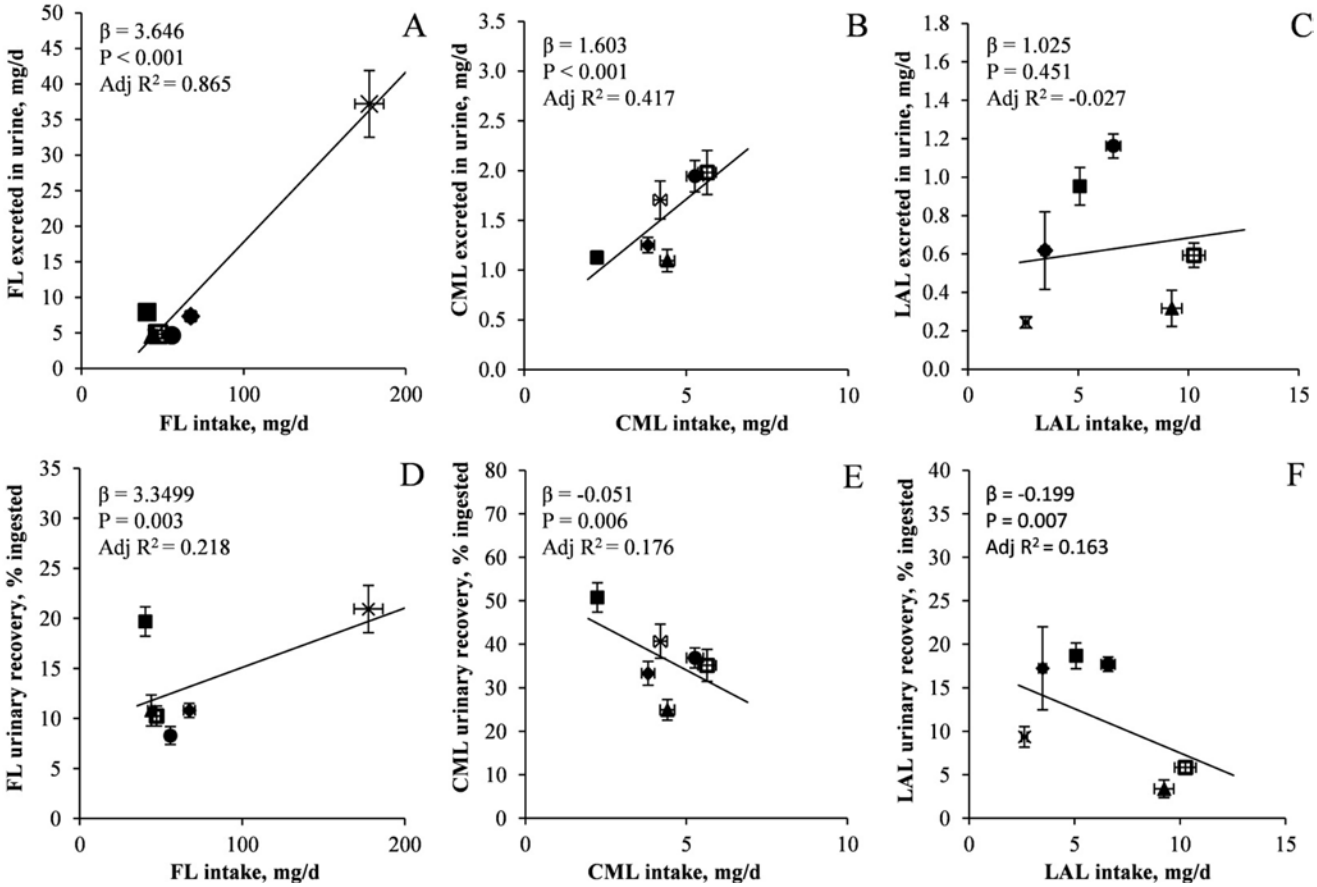

Figure 3. (A)–(C) Relationship of fructoselysine (FL), carboxymethyllysine (CML), and lysinoalanine (LAL) excretion in urine (mg/d) and intake (mg/d) and (D)–(F) relationship of FL, CML, and LAL urinary recovery (% ingested) and intake (mg/d) for 6 commercial moist cat foods (diamonds = food 1, solid squares = food 2, open squares = food 3, crosses = food 4, circles = food 5, triangles = food 6).

decrease in urinary recovery with increasing dietary intake suggests that either 1) digestion and absorption capacity were 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 of whether inhibited digestion and absorption can cause the lower urinary recovery with increasing dietary intake. From the 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 up to 60% in rats compared with 10% for the dietary protein-bound derivative (Finot and Magnenat, 1981). In addition, it was suggested that protein-bound LAL is absorbed in much lower levels compared with free LAL (De Groot et al., 1976; Wenzel et al., 2002). Free FL and CML can be absorbed by passive diffusion (Somoza et al., 2006); 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 (Hellwig et al., 2014). 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 (Hellwig et al., 2011). 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 with CML (between 24.9% and 72.6%) in the current study as well as in the study of Somoza et al. (2006) 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 (Finot, 2005). Dietary MRP that are not absorbed in the small intestine enter the large intestine. It is known that microbiota in the large intestine metabolize and/or degrade FL into different, unknown components that are absorbed in the intestine or excreted in the feces (Somoza, 2005). It is suggested that CML and LAL can be degraded by colonic microbiota as well (Delgado-Andrade et al., 2012; Friedman, 1999), although the microbial degradability may be lower, as suggested by the higher recovery of CML and LAL compared with that of FL in feces of rats (Somoza et al., 2006). Whether the FL, CML, and LAL that were not absorbed and that entered the large intestine in the current study were metabolized by gut microbiota or excreted in feces 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 (Somoza et al., 2006). In addition, a significant correlation was found between dietary intake and serum CML as measured by ELISA using monoclonal antibody against CML (Uribarri et al., 2005). In rats, more than 80% of intravenously injected ^{14}C-labeled FL was excreted in urine after 24 h, indicating that most of the FL that enters the body is excreted in the urine (Erbersdobler and Faist, 2001). Hultsch et al. (2006) reported that 45% of intravenously administered [^{18}F]fluorobenzoylated FL was excreted nearly unchanged in the urine 60 min postinjection in male Wistar rats. However, large accumulation of radioactivity (34%) was observed in the kidneys 60 min postinjection, 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 radiolabeled FL was possibly metabolized. When injected intravenously in the tail of rats, [^{18}F]fluorobenzoylated CML was found to accumulate quickly in the liver and 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 (Xu et al., 2013). 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 by Bergmann et al. (2001) 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 (Struthers et al., 1980).

In line with these reports it can reasonably be assumed that part of the dietary MRP in the present study was 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. (1987) detected CML in urine when rats were fed additional free FL compared with 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. (2006) 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 before 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 of this topic is lacking in the literature.

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 low-MRP diet (Uribarri et al., 2005, 2007). It should be noted, however, that these studies use MRP-sensitive enzyme-linked immunosorbent assays. Only 1 study is known to use gas chromatography mass spectrometry to quantify CML, showing that an increase in dietary CML resulted in an increase in markers associated with an enhanced risk of type 2 diabetes and cardiovascular diseases in

healthy human subjects (Birlouez-Aragon et al., 2010). 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 8.9 to 25.8 mg/kg $BW^{0.75}$ for dry foods and 17.7 to 77.9 mg/kg $BW^{0.75}$ for moist foods; daily intake of CML ranged from 0.88 to 2.11 mg/kg $BW^{0.75}$ for dry foods and from 0.97 to 2.46 mg/kg $BW^{0.75}$ for moist foods, and daily intake of LAL ranged from 1.36 to 2.68 mg/kg $BW^{0.75}$ for dry foods and 1.14 to 4.52 mg/kg $BW^{0.75}$ for moist foods. This daily CML intake was higher than that in a previous study, in which the average intake from extruded dry diets was 0.28 mg CML/kg $BW^{0.75}$ (Van Rooijen et al., 2014). Taking the urinary recovery values into account (Table 3), an average recovery value of 15.2% for FL in dry foods results from a daily absorption and excretion of 1.35 to 3.92 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 to 0.96 mg CML/kg BW0.75. Despite the natural capacity of the body to protect against MRP, over time, endogenous and dietary MRP may accumulate in body tissues (Singh et al., 2001; Basta et al., 2004; DeGroot et al., 2004).

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% and 23% for FL, 25% and 73% for CML, and 6% and 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, and urinary excretion can be limiting factors. Urinary recovery indicated that either absorption or excretion of dietary CML is higher than that of 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.

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