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Research Article

Modification of a Targeted Metabolomics Method Using Hydrophilic Interaction Liquid Chromatography-High Resolution Mass Spectrometry to Measure Nine Purines in Commercial Dog Foods

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ABSTRACT

Urate is the third most common component of canine uroliths. Dogs with a genetic mutation or hepatic dysfunction are prone to forming urate uroliths because they excrete more poorly soluble urate as the end product of purine metabolism. Dietary purine restriction has been reported to reduce urinary urate concentrations in dogs, but the amount and types of purine in commercial diets have not been well documented. A previous targeted metabolomics method was modified to measure eleven purine metabolites in commercial dog foods. The method utilized hydrophilic interaction liquid chromatography-high-resolution tandem mass spectrometry with stable isotope-labeled internal standards. The within-day and between-day coefficient of variation of each purine metabolite concentration in quality control dog food samples averaged 6% (range: 4%-13%) and 15% (range: 11%-19%), respectively. Recoveries were within 100% ± 20% for adenine, guanine, xanthine, adenosine, inosine, guanosine, and adenosine monophosphate at added purine concentrations similar to those in a low and a high purine food chosen to assess quality control. Average recoveries were higher (128%-138%) for adenosine, guanosine, and adenosine monophosphate at added concentrations 4-8 times higher than in either food. Uric acid recovery was within 100% ± 20% at low added concentrations similar to that in the 'high-purine' food, but was high (156%) at the highest concentration, similar to that in the 'low-purine' food. Inosine monophosphate and guanosine monophosphate were below the limit of quantification in both food samples. This methodology achieved effective polar separation with fewer sample preparation steps and enabled comprehensive accurate quantification of purine metabolites in commercial dog foods.

Keywords: Dog food; HILIC (Hydrophilic Interaction Liquid Chromatography); HRMS (High-Resolution Mass Spectrometry); HRMS/MS (High-Resolution Tandem Mass Spectrometry); Purines; Targeted metabolomics

Abbreviations: AMP: Adenosine Monophosphate; GMP: Guanosine Monophosphate; IMP: Inosine Monophosphate; Ade: Adenine; Ado: Adenosine; Gua: Guanine; Guo: Guanosine; Ino: Inosine; Xan: Xanthine; Hxa: Hypoxanthine; UA: Uric Acid; HILIC: Hydrophilic Interaction Liquid Chromatography; HRMS: High-Resolution Mass Spectrometry; HRMS/MS: High-Resolution Tandem Mass Spectrometry; H-ESI: Heated Electrospray Ionization; LOQ: Limit Of Quantitation; QC: Quality Control; CV: Coefficient of Variation; H_2O: Ultra-Pure Water; HCl: Hydrochloric Acid; NaCl: Sodium Chloride; NaOH: Sodium Hydroxide; DAP: 2,6-Diaminopurine Monohydrate.

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INTRODUCTION

Purines in the body, including nucleotides, nucleosides, and nucleobases, are derived from ingested foods or can be synthesized *de-novo*. In dogs, purines are usually metabolized to poorly water-soluble Uric Acid (UA) and further metabolized and excreted in urine as water-soluble allantoin. Urate uroliths are prone to form when excess uric acid is excreted in the urine of dogs with hepatic dysfunction or a genetic defect that impairs purine metabolism [1-4]. Preventative measures include feeding diets that manufacturers report to be low in purines or diets that contain raw materials with low purine content [5-9]. However, the purine content of commercial dog foods has not been well documented in peer-reviewed journals [10,11]. Therefore, a method that measures purines in different types of commercial diets would facilitate the selection of low-purine diets for individual patients to prevent urate urolithiasis.

Conventional methods for measuring purines in food ingredients use acid hydrolysis to break down all purines into four nucleobases [12,13]. Those methods quantify four nucleobases, which are then added together as total purine content, but do not differentiate between nucleotides, nucleosides, and nucleobases. Previous studies in people and rats have indicated that the different purine metabolites vary in their capacity to affect urinary UA concentrations [14-16]. Furthermore, purine metabolite content has been shown to change during storage when measured using thin-layer chromatography [17]. Ardente et al., showed that eight purine metabolite concentrations, quantified using LCMS/MS after aqueous extraction, differed between stored and fresh fish commonly consumed by dolphins [18]. They found differences in total and individual purine concentrations among fish, especially hypoxanthine concentrations, which may explain why dolphins fed by human beings are more likely than wild dolphins to develop urate uroliths.

This report describes how the purine analysis previously developed by Ardente et al., was adapted and validated for the measurement of purines in commercial dog foods [19]. Some adaptation was necessary because dog foods present a complex matrix composed of many ingredients, including protein and fat from animal or vegetable sources, soluble and insoluble fibers, and additives, such as vitamins and minerals. Also, a more sensitive detection method was desired because purine concentrations in dog foods designed for preventing urate uroliths were expected to contain much lower concentrations of purines than were previously measured in fish. Hydrophilic Interaction Liquid Chromatography (HILIC) was utilized because highly selective polar separation of purines has been reported [20,21]. Tandem High-Resolution Mass Spectrometry (HRMS/MS) with wide-isolation quantitative strategies described by Rubio et al., were also utilized to allow faster scan rates and higher selectivity [22]. An internal standard was used instead of more time-consuming standard additions to reduce sample processing time.

MATERIALS AND METHODS

Chemicals and solutions

Stock solutions including the mobile phase, internal standards, and external standards were all prepared freshly at least bi-weekly using high-performance-liquid-chromatography or liquid chromatography-mass spectrometry grade solvents, when available. Ammonium formate, ammonium hydroxide, acetonitrile, acetone, Ultrapure Water (H_2O), Hydrochloric Acid (HCl), Sodium Chloride (NaCl), Sodium Hydroxide (NaOH), methanol, formic acid, and hexane were purchased from Fisher Scientific company, LLC (Suwanee, GA, USA).

Chromatography solutions differed from those used by Ardente, et al., [19]. Buffer stock solution for the mobile phase was 100 mM ammonium formate solution in H_2O, adjusted to a pH 9.2 with 30% ammonium hydroxide in H_2O. The additive stock solution was 5 mM methylene diphosphonic acid (TCI America, Portland, OR, USA) in H_2O to help improve peak shapes. Mobile phase A was 100 mL of buffer stock solution mixed with 900 mL of acetonitrile, while mobile phase B was 100 mL of buffer stock solution mixed with 60 mL of acetonitrile and 840 mL of H_2O. Each 999 mL of mobile phase was spiked with 1 mL of 5 mM methylene diphosphonic acid stock solution. Each mobile phase was shaken well and sonicated for 15 minutes.

Purine external standard solutions were prepared for calibration of the chromatographic method. Adenine (Ade), Adenosine (Ado), Hypoxanthine (Hxa), Xanthine (Xan), Guanine (Gua), Guanosine (Guo), Inosine (Ino), and Uric Acid (UA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Adenosine Monophosphate (AMP), Inosine Monophosphate (IMP), and Guanosine Monophosphate (GMP) were purchased from TCI America. Aqueous 10 mM stock solutions of each purine were prepared. Solubility varied among purines, so Gua, Hxa, Xan, and UA were dissolved in 0.1 N NaOH, while Ade, Ado, Ino, and Guo were dissolved in 0.1 N HCl, and AMP, IMP and GMP were dissolved in 0.9% NaCl. These initial stock solutions were then combined to make three mixtures each of 5 mL final volume: Mixture A contained 1 mL of 0.1 N NaOH and 1 mL of each of the 10 mM stock solutions of Gua, Hxa, Xan, and UA; mixture B contained 1 mL of 0.1 N HCl and 1 mL of each of the 10 mM stock solutions of Ade, Ado, Ino, and Guo; mixture C contained 2 mL of 0.9% NaCl and 1 mL of each of the 10 mM stock solutions of AMP, IMP and GMP. A final stock solution containing 500 µM of each purine standard was prepared by combining 2.5 mL each of mixtures A, B, and C and 2.5 mL of 0.9% NaCl, and stored at -20℃. An aliquot of the final stock solution was thawed and sonicated for 10 minutes and diluted with 0.9% NaCl before each chromatographic run to prepare external standard solutions containing 100 nM, 500 nM, 1, 5, 10, 50, 100, and 250 µM of each individual purine.

This method used an internal standard, 2,6-Diaminopurine Monohydrate (DAP; 97.0+%, Fisher Scientific) to compensate for variations in recoveries and strong matrix effects where standard additions had been used previously [19]. A solution containing 400 µg/mL of DAP in H_2O was added to all food samples before extraction. An internal standard solution of stable isotope-labeled purines was added to each sample solution after extraction in conjunction with the external standards to calibrate the amount of purine in the sample solution. The internal standard solution included Ade-$^{15}N_5$, Xan-^{13}C, $^{15}N_2$, Hxa-$^{13}C_2$, ^{15}N, Ado-$^{13}C_5$, Guo-^{13}C, $^{15}N_2$, UA-$^{13}C_3$, AMP-$^{13}C_5$ (Toronto Research Chemicals, Toronto, ON, Canada) and Ino-$^{15}N_4$, and Gua-$^{15}N_5$ (Cambridge Isotope Laboratories, Cambridge, MA, USA). Labeled IMP and GMP were not readily available at the time of this study. Therefore, labeled AMP-$^{13}C_5$ was used as an alternative because it has the closest chemical structure and retention time to IMP and GMP. Individual solutions for each internal standard were prepared to a concentration of 1 mg/mL: Ade-$^{15}N_5$, Gua-$^{13}C_2$, ^{15}N, Xan-^{13}C, $^{15}N_2$, Hxa-$^{13}C_2$, ^{15}N, and UA-$^{13}C_3$, were prepared in 0.1N NaOH; Ado-$^{13}C_5$, Guo-^{13}C, $^{15}N_2$, Ino-$^{15}N_4$, and AMP-$^{13}C_5$ were prepared in 0.1 N HCl. These internal standard solutions were combined and diluted in methanol before each run to achieve a final concentration of 10 µg/mL of each stable isotope-labeled compound.

Food samples and processing

Two lots of canned dog food were selected, one from each of two different readily available commercial dog foods (Supplementary table). One dog food was an adult maintenance diet presumed to contain increased purines because it contained organ meats and a minimum of 10% as fed crude protein. The other was presumed to be low in purine because it is marketed as a urate-urolith prevention diet. The adult maintenance diet was used for Quality Control (QC) and to evaluate between-day and within-day variability. Both diets were used to assess recovery.

The entire contents of 1 large can (369 grams) of each canned diet was individually ground through a 2 mm sieve (Wiley Mill, Thomas Scientific., Swedesboro, NJ, USA), then homogenized by hand in a polyethylene bag with zip closure, and numbered for identification. Authors who performed the chromatographic analysis of samples were blind as to which sample identification numbers corresponded to which diet or replicate. All samples were lyophilized to stable weight (Free Zone 2.5 Liter -50°C Benchtop Freeze Dryers with 3-shelf drying compartment, Labconco, Kansas City, MO, USA). Dried samples were stored in polyethylene bags with zip closures, in a climate-controlled (25°C) room, then lyophilized again just before preparation for purine analysis to eliminate any moisture accumulated from ambient air. Duplicated QC food samples were prepared whenever purine extraction was performed and then analyzed with the samples extracted on the same day.

Purine extraction

Purine extraction was performed largely according to the method described previously, with the following modifications to accommodate the different nature of the samples and the large number of samples being analyzed [19]. Homogenization, sonication, and heating of samples were performed as described by Clariana et al., [23]. After cooling in an ice water bath for 10 minutes, the solution was centrifuged at 18,100 rpm for 18 min at 20°C (Sorval RC5C, Marshall Scientific, Hampton, NH, USA). The supernatant was filtered (Grade P5, Fisherbrand Qualitative-Grade Filter Paper Circles, Fisher Scientific), and an aliquot was mixed with an equal amount of hexane using a vortex mixer (Fisher Scientific) for 30 seconds and then centrifuged at 6,500 rpm, for 7 min, at 20°C (Sorval RC5C, Marshall Scientific). The aqueous (bottom) layer was frozen at -20°C until final processing and analysis.

A 100 µL aliquot of thawed extract was mixed with 20 µL of 10 µg/mL stable isotope-labeled internal standard solution, and 500 µL of protein precipitation solution (0.015% ammonium hydroxide in 50 mL of acetonitrile: acetone 9:1), using a vortex mixer for 10 seconds. The samples were incubated at 4°C for 10 minutes to allow further precipitation, then centrifuged at 14,000 rpm at 4°C for 10 minutes. An aliquot of the supernatant was transferred to an autosampler vial from which, a 10 µl sample was analyzed using Hydrophilic Interaction Liquid Chromatography (HILIC): High-Resolution Tandem Mass Spectrometry (HRMS/MS). The order of extracted samples, blanks, external standards, and DAP injected was shuffled randomly (RAND function of Microsoft Office Excel 2019).

HILIC separation

Separation of eleven purine metabolites, Ade, Gua, Hxa, Xan, UA, Ado, Guo, Ino, AMP, IMP, and GMP was carried out using HILIC (Dionex Ultimate 3000 UHPLC, Thermo Fisher Scientific). Separation was achieved on an InfinityLab Poroshell 120 HILIC-Z column, 150 mm × 2.1 mm, 1.9 µm with a 2.1 mm × 5 mm, 2.7 µm guard column (Agilent, Santa Clara, CA, USA). The elution conditions for separation began with 100%mobile phase A for 0.5 minutes, then changed to 77% mobile phase A: 23% mobile phase B over 1 minute, then held for 2.5 minutes, before further change to 50%:50% mobile phase A:B over 0.5 minutes, then held for 1 minute, then being increased back to 100% A over 0.5 minute, and then held for 4 minutes.

Detection and quantification

Purine metabolites were identified by mass and retention time using mass spectrometry with a Thermo Q-Exactive hybrid orbitrap Mass Spectrometer (Thermo Fisher Scientific) with Heated Electrospray Ionization (H-ESI) operated in both HRMS and HRMS/MS modes. Purines were detected by the full scan (HRMS) initially, and then ions were selected in the time window specified for tandem Mass Spectrometry (HRMS/MS).

The H-ESI source was operated in negative-ion mode with the following settings: Spray voltage 2500 V, capillary temperature 350°C, 35 arbitrary units of sheath gas pressure, 1.0 arbitrary units of ion sweep gas pressure, 10 arbitrary units of auxiliary gas pressure, probe heater temperature 275°C, and S-lens at 40. Data was processed using computer software (Xcalibur Quan Browser 4.2, Thermo Fisher). The FDA Bioanalytical method validation guidance for industry to standardize analytical methods was followed [24]. The sensitivity was determined by the lowest non-zero external standard (50 nM, 100 nM, 500 nM, 1, 5, 10, 50, 100, and 250 µM of each individual purine) on the calibration curve. The peak area ratio of the external standards relative to labeled internal standards at the different concentrations of the external standards was used to create a calibration curve. The purine concentration of the sample (µM) was determined by substituting the peak area ratio of the sample to the internal standard into the equation obtained from the calibration curve. Recovery of DAP in individual samples was calculated by dividing the measured DAP concentration by the added DAP concentration. Purine concentration (µmol/100 g food DM) in each sample was calculated by adjusting for sample weight and dilution during sample preparation, and dividing the measured concentration in the extract by the recovery of DAP. Spreadsheet software was used for these calculations. (Microsoft Excel for Mac version 16.75.2, Microsoft Corporation, Redmond, WA, USA).

Method validation

The Coefficient of Variation (CV) for within-day (n=6, six replicate sub-samples) and between-days (n=18, two duplicated samples for nine days) was calculated for the content of each purine metabolite (µmol/100 g dry matter) in the high purine QC diet. To evaluate whether the recovery of the internal standard, DAP, accurately represented the recovery of individual purines, increasing amounts of mixed purine standard stock solution (500 µM of each purine in H_2O), were mixed, before extraction and analysis, with decreasing amounts of H_2O, 0.6 g of lyophilized sample of either the 'high purine' or 'low purine' QC diet, and 0.5 mL of 400 µg/mL DAP in H_2O to a final volume of 20 mL containing either 0 (no addition), 2.5 µM, 25 µM, and 100 µM of each added purine. Recovery (%) was calculated as follows: Firstly, the measured amount of each purine standard in the samples was determined as the difference in measured amount of each purine in food samples with and without a known amount of purine standard added; then, the measured amount of each purine standard was divided by the known amount of purine added and multiplied by 100. Recoveries were averaged for the two diets at low, moderate, and high internal standard additions to assess the accuracy of the method at each concentration.

RESULTS AND DISCUSSION

Purine metabolites were separated and quantified by mass and retention time by HILIC-HRMS/MS or HILIC-HRMS (Figure 1).

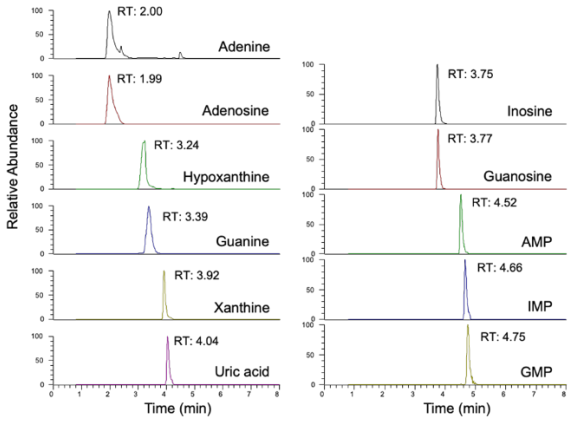

Figure 1: Extracted ion chromatograms of eleven targeted purine metabolites in the high purine dog food. Time (minutes) and relative abundance are represented along the x-axis and y-axis, respectively. The Retention Time (RT) of each purine is noted next to each metabolite peak.

Most purines were quantified from the HRMS/MS data, whereas the full scan data with HRMS were used for only Xan and UA because product ion intensity was low. Instrument settings used for parallel reaction monitoring are listed in Table 1. Additional steps were necessary after extraction to clean samples because dog foods are complex matrices typically containing grains, meat, animal fat, vegetable oil, fiber, and mineral and vitamin supplements. Adding a mixture of acetonitrile and methanol and centrifugation achieved efficient protein precipitation and removal. The mobile phase being basic (pH 9.2) facilitated deprotonation and ionization of the purines and improved separation and response in negative ion mode. Flushing the system and chelating metals are expected to minimize ion suppression because phosphorylated organic compounds can interact with metals from iron-derived columns or solvents, which may affect peak detection [25,26]. Adding methylene diphosphonic acid to a mobile phase for this purpose improved chromatographic performance in HILIC mode.

We used external standards and two internal standards (DAP and stable-isotope-labeled purines) to improve the accuracy of quantification. Comparable information measuring purines in dog foods was not available from previous studies. Our aqueous extraction might not release all the purine in the diets compared to acid hydrolysis. Nevertheless, recoveries have previously been found to be poor in our laboratory when acid hydrolysis was used instead of aqueous extraction at least in part because acid conditions affect the solubility of some purines, such as UA [23]. Each sample was freeze-dried because pet foods can vary widely in moisture content but freeze-drying may have affected purine extraction [27].

Diaminopurine (DAP) was used as an internal standard after caffeine proved unsuitable in negative mode [21]. Recovery of DAP was only about 60% after the filtration step used in sample preparation but DAP proved to be suitable as an internal standard because recovery was consistent and mimicked the recovery of individual purines at concentrations found in the

commercial dog foods. The between-day CV of each purine metabolite concentration (µmol/100g food dry matter) in 18 high purine QC diet samples (2 duplicates, 9 days) averaged 15% (range: 11%-19%). The mean recovery of DAP in between-day QC samples was 59% with a CV of 12%. The within-day CV of each purine metabolite concentration (µmol/100g food dry matter) in six QC samples was 6% on average (range: 4-13%) (Table 2). The mean recovery rate of DAP was 64% with a CV of 3% in within-day QC samples.

The concentration of most purines (µmol/100g DM) were between 4 and 56 in both diets but higher for XA, Hxa and Ino in the high purine diet and higher for UA in the low purine diet (131, 306, 99, and 584, respectively) (Table 3). Both IMP and GMP were below the Limit of Quantitation (LOQ) in both diets. Adding standards to measure recoveries increased the concentration in the diets by 8-333 µmol/100g DM.

Average recoveries for the two diets were within 100% ± 20% for Ade, Gua, XA, Hxa, Ado, Guo, Ino, and AMP for the range of purine concentrations in the food samples (Table 4). Average recoveries for the two diets were higher for Ado, Guo and UA (133%, 138% and 156%, respectively) at the highest added concentration. Average recoveries for the two diets were within 100% ± 20% for IMP and GMP at the highest added concentration and at the intermediate added concentration for GMP but was inconsistent at other added concentrations.

Recoveries were within acceptable limits for most of the purines at all concentrations evaluated but were unacceptable at lower concentrations for IMP and GMP. Recoveries were slightly high at the highest concentrations for Guo, Ado, and UA in both diets. Changing ratio of solvent volume to sample weight before extraction would improve accuracy because recoveries were consistent at low concentrations for all purines except IMP and GMP. Such modifications may not be necessary to measure Guo and Ado, and possibly also UA, however, because the highest concentration added was much higher than that found in both foods for Guo and Ado, and in the high purine diet for UA. Why a 'low purine' diet should contain so much more UA than the 'high purine' diet and whether that is true of all low purine diets needs to be determined. It is unlikely that the analytical errors overestimated UA concentrations, because repeat analyses of additional subsamples of each lot showed reproducible results.

The nucleotides, IMP and GMP were below the limit of quantification in these foods. It is likely that concentrations of these nucleotides were indeed low because IMP is degraded during storage to Ino and then Hxa [17]. Ardente et al. detected lower concentrations of IMP and higher concentrations of Hxa in frozen fish compared to fresh fish using their more laborious method, likely due to degradation during storage [18]. Purine degradation may also occur with cooking [27–29]. This study did not measure XMP (xanthine monophosphate) and xanthosine because internal standards that are stable in basic solutions were unavailable at the time of the study. Nevertheless, XMP and its nucleoside are unlikely to be present in more than negligible quantities because XMP is an intermediate in the metabolism of IMP to GMP [30].

Table 1: Instrument settings for parallel reaction monitoring.

Mass, m/z[1]	Polarity	Start, min	End, min	NCE[2]	Name
134.05	Negative	1.3	2.4	110	Adenine
302.07	Negative	1.4	2.5	30	Adenosine+Cl
135.03	Negative	1.6	2.6	65	Hypoxanthine
151.03	Negative	2	2.7	80	Xanthine
267.07	Negative	3	3.9	40	Inosine
150.04	Negative	3	3.9	55	Guanine
282.08	Negative	3.6	4.3	30	Guanosine
167.02	Negative	4.1	4.8	60	Uric acid
346.06	Negative	5.2	6.5	40	AMP
347.04	Negative	5.2	6.5	35	IMP
362.05	Negative	5.05	6.5	35	GMP
271.06	Negative	2.5	3.6	40	Inosine-$^{15}N_4$
139.03	Negative	1.2	2.5	110	Adenine-$^{15}N_5$
154.1	Negative	2	3	80	Xanthine-^{13}C, $^{15}N_2$

138.03	Negative	1.35	2.7	60	Hypoxanthine-$^{13}C_2$, ^{15}N
155.1	Negative	1.65	4	55	Guanine-$^{15}N_5$
149.06	Negative	1	2.6	65	DAP
351.07	Negative	5.2	6.5	40	AMP-$^{13}C_5$
266.09	Negative	1.4	2.5	30	Adenosine-$^{13}C_5$
285.08	Negative	3.3	4.3	30	Guanosine-^{13}C,$^{15}N_2$
170.03	Negative	4.1	4.8	60	Uric acid-$^{13}C_3$

[1] m/z: Mass to charge ratio. Full scan was 70-1000 m/z.

[2] NCE: Normalized collision energy which was used for fragmentation of the ions.

Table 2: Purine metabolite concentrations (μmol/100 g DM) and assay variability of the high purine diet.

	Within day		**Between day**	
Metabolites	**Mean ± SD***	**CV (%)†**	**Mean ± SD***	**CV (%)†**
Each purine metabolite				
Adenine	39 ± 2	5	44 ± 6	14
Guanine	42 ± 2	4	47 ± 6	13
Xanthine	116 ± 5	4	131 ± 17	13
Hypoxanthine	270 ± 13	5	306 ± 42	14
Uric acid	55 ± 3	6	53 ± 10	18
Adenosine	28 ± 2	7	33 ± 6	19
Guanosine	47 ± 6	13	56 ± 10	18
Inosine	90 ± 5	6	99 ± 11	11
AMP	25 ± 1	5	28 ± 4	14
IMP	0 ± 0	N/A	6 ± 0	N/A
GMP	0 ± 0	N/A	0 ± 0	N/A
All purine metabolites				
Average		6		15
Range		4-13		11-19
Recovery of DAP (%)	64 ± 2	3	58 ± 7	12

†Purine metabolites mean variability was calculated within day (n=6) and between day (n=18); *SD (Standard Deviation); N/A (Not applicable because concentration is zero); AMP (Adenine Monophosphate); IMP (Inosine Monophosphate); GMP (Guanosine Monophosphate)

Table 3: Mean purine concentrations (µmol/100 g DM) in two commercial canned dog foods.

Diet	Ade	Gua	XA	Hxa	UA	Ado	Guo	Ino	AMP	IMP	GMP	Total
Low purine	6	12	56	51	584	14	23	28	4	<LOQ	<LOQ	778
High purine	44	47	131	306	53	33	56	99	28	<LOQ	<LOQ	798

DM: Dry Matter; Ade: Adenine; Gua: Guanine; XA: Xanthine; Hxa: Hypoxanthine; UA: Uric Acid; Ado: Adenosine; Guo: Guanosine; Ino: Inosine; AMP: Adenosine Monophosphate; IMP: Inosine Monophosphate; GMP: Guanosine Monophosphate; <LOQ: Below Limit Of Quantification

Table 4: Recoveries of purine metabolites added to two commercial foods using diaminopurine as an internal standard.

Diet	Purine added (µM)	Change in purine content (µmol/ 100 g DM)	Recoveries of purines, % of added amount										
			Ade	Gua	XA	Hxa	UA	Ado	Guo	Ino	AMP	IMP	GMP
Low purine	2.5	8	94%	86%	95%	97%	92%	96%	108%	95%	119%	0%	0%
	25	83	91%	92%	99%	100%	117%	109%	140%	103%	93%	0%	90%
	100	333	104%	107%	111%	116%	157%	132%	126%	120%	137%	108%	119%
High purine	2.5	8	104%	106%	106%	111%	105%	99%	94%	105%	104%	0%	0%
	25	83	95%	96%	105%	111%	115%	112%	101%	115%	92%	68%	131%
	100	333	110%	96%	115%	123%	156%	134%	150%	121%	117%	86%	89%

Ade: Adenine; Gua: Guanine; XA: Xanthine; Hxa: Hypoxanthine; UA: Uric Acid; Ado: Adenosine; Guo: Guanosine; Ino: Inosine; AMP: Adenosine Monophosphate; IMP: Inosine Monophosphate; GMP: Guanosine Monophosphate; DM: Dry Matter

CONCLUSION

This report describes how a previously reported targeted metabolomics assay was adapted and validated to enable quantification of nine purine metabolites in commercial dog foods. Use of external standards, two internal standards (DAP and stable-isotope-labeled purines), a basic mobile phase with added methylene diphosphonic acid, and HILIC-HRMS/MS or HILIC-HRMS enabled accurate measurement of poorly soluble purines in foods with a complex matrix. These adaptations saved at least 9 hours of extraction time processing one sample by omitting the evaporation, reconstitution, and standard addition steps in the previously reported method. Between-day and within-day variation and recoveries were within acceptable limits for nine purines at all concentrations evaluated but recoveries were unacceptable at lower concentrations for IMP and GMP. Thus, a broader range of purine metabolites in dog foods was successfully recovered in a more reproducible fashion than has been previously reported using conventional approaches. As a result, the optimal dietary composition to prevent canine urate uroliths can be assessed in a more direct and efficient way.

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