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Identification and Quantification of Cyanide and its Metabolites in Lemur Urine

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Identification and Quantification of Cyanide and its Metabolites in Lemur Urine

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ABSTRACT

Cyanide is a chemical compound that contains the monovalent groups of carbon and nitrogen. This substance is considered a rapidly acting deadly chemical. However, some animals such as lemurs consume food products that are known to contain elevated levels of cyanide without major consequences. The mechanism by which the lemur can handle this high exposure to cyanide is unknown. In this study, we developed and validated two analytical methods for the determination of cyanide and two metabolites, thiocyanate and 2-aminothiazoline-4-carboxylic acid (ATCA) in lemur urine. The method was applied to 47 authentic lemur urine samples collected from 4 different species of lemurs in Madagascar and 20 authentic lemur urine samples from captive lemur. The cyanide and thiocyanate procedure involved a derivatization technique followed by a liquid-liquid extraction, the ATCA procedure involved and solid phase extraction followed by a derivatization step before analysis. Of all the samples tested, the concentration range that was discovered for cyanide was 0- to greater than 200 µg/mL, the range for thiocyanate (major metabolite) was 0- to greater than 200 µg/mL. The method had an accuracy of 89-99% accuracy with a 18% imprecision for cyanide and 81-108 % accuracy and an imprecision of 27% for thiocyanate. ATCA (minor metabolite) had a concentration range from 126.6 ng/mL- to over 4000 ng/mL with a 105-114% accuracy and 4.87% imprecision. These analytical results along with genetic information will be employed in future research to investigate the pathway of cyanide metabolism in lemur.

Keywords: urine; lemur; gas chromatography-mass spectrometry; cyanide; thiocyanate; 2-aminothiazioline-4-carboxyclic acid.

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Introduction

There are far and few known toxins that can rapidly kill an organism since most toxins take a momentous period and or high concentrations to be considered deadly. Cyanide in the form of hydrogen cyanide also known as HCN is not one of those slower acting toxins. Cyanide is a deadly chemical that can be introduced into an organism in several ways including ingestion of edible plants (e.g. spinach), inhalation of smoke from cigarettes or fires, as a chemical warfare agent, or accidental exposure during industrial operations (e.g. pesticide production). Once absorbed into the body, cyanide is introduced to the cells and binds to the iron in the heme groups in cytochrome oxidase, inhibiting the function of the electron transport chain (Bhandari, R. K. et al. 2012). As a result, redox reactions in the cellular respiratory chain halts, cellular hypoxia, cytotoxic anoxia and, with continued exposure, death occurs. There are several analytical methods that have described the detection of cyanide, but these methods are usually hindered by cyanide's high volatility and nucleophilic nature (Bhandari, R. K. et al. 2012). Due to these properties cyanide has a short half-life and is often not detected in biological samples after absorption, (Bhandari, R. K. et al. 2012). Some papers have even stated that depending on the route of exposure and the duration of exposure, cyanide is typically eliminated from the blood within 20 minutes of exposure. (Louge, B. et al. 2005). In addition, from its rapid decreasing elimination from biological fluids, cyanide sometimes forms as an artifact under certain storage conditions in a variety of samples; some sources of endogenous cyanide include normal metabolism of amino acids within the body along with external sources such as smoking, inhalation of smoke from fires, and some types of food. (Logue, B. et al. 2005). Therefore, other markers of cyanide exposure have been proposed. Two such markers are the major and minor metabolite of cyanide. The major metabolite is thiocyanate (SCN^-), about 80% of cyanide its

metabolized to this form through an enzyme catalyzed reaction in the presence of sulfur (Bhandari, R. K. et al 2012). Thiocyanate is not as volatile as CN; therefore, it must be modified to a semi-volatile compound to be analyzed by Gas Chromatography methods. One such manner is via alkylation with pentafluorobenzyl-bromide (PFB-BR) (Toraño, J. S., & Kan, H. J. 2003). It is important to note that some studies have shown the thiocyanate can be formed by other methods other than cyanide metabolism (Logue, B. et al. 2005). The minor metabolite of cyanide, 2-aminothiazoline-4-carboxylic acid (ATCA), is another great indicator that has a higher stability than cyanide. (Logue, B. et al. 2005). ATCA accounts for approximately 20% of cyanide metabolism and increases as exposure increases (Logue, B. et al. 2005). ATCA is formed when cyanide reacts with l-cystine through a proposed intermediate, -thiocyanoalanine, that is transformed to ATCA. ATCA may be present as a tautomer between itself and 2-iminothiazolidine-4-carboxylic acid (ITCA) (Logue, B. et al. 2005). The structure of ATCA is similar to an amino acid. ATCA represents an alternative metabolite of cyanide exposure, and a simple assay for ATCA may circumvent the disadvantages of cyanide and thiocyanate analysis ((Logue, B. et al. 2005). In this study, a method was developed for the simultaneous identification and quantification of cyanide and thiocyanate in urine, as well as a method for the detection and quantification of 2-aminothiazoline-4-carboxylic acid in the same biological matrix. The method was applied to 67 authentic samples from wild and captive lemurs. Lemurs are known to consume food products (such as bamboo) that contain high concentrations of cyanide and aren’t dying because of these high concentrations.

Materials

Sodium cyanide powder, sodium tetraborate decahydrate, 2,5-dibromotouluene (2,5-DBT) and tetra butylammonium sulfate (TBAS) solution were purchased from Sigma-Aldrich (St. Louis, MO). Sodium thiocyanate powder was purchased from Fluka Analytical (St. Louis, MO). Pentafluorobenzyl bromide (PFB-Br) was obtained from Supelco (Bellefonte, PA). Racemic 2-aminothiazoline-4-carboxylic acid (ATCA) and racemic 2-aminothiazoline-4-carboxylic acid- ^{13}C $^{15}N_2$ (ATCA-13C, $15N_2$) were received from Toronto Research Chemicals (Toronto, Canada). OASIS MCX 1cc, 30mg, 60um, extraction cartridges were purchased from Waters (Milford, MA). Hydrochloric acid and LC/MS grade methanol were obtained from Fisher Scientific (Waltham, MA). N-Hexane, ethyl acetate and ammonium hydroxide were all purchased from Pharmco-AAPER (Shelbyville, Kentucky). N-Methyl-N-(trimethylsilyl) trifluoroacetamide was purchased from Thermo Scientific (Waltham, MA). Synthetic urine was acquired from Ricca Chemical Company (Arlington, TX).

Preparation of Standards & Reagents

Cyanide & Thiocyanate Procedure

A stock solution of cyanide and thiocyanate (at 300 µg/mL) was prepared by dissolving 56.55 mg of sodium cyanide and 41.91 mg of sodium thiocyanate in 10 mL of water. Working solutions were created so that the calibration range was 10 µg/mL-200 µg/mL by serial dilutions in water. Derivatizing agent PFB-Br was prepared at a concentration of 20 mM by mixing 30 µL of PFB-Br into 10 mL of ethyl acetate. A stock solution of the internal standard 2,5-DBT at 50 mM was prepared by mixing 20 µL of 2,5-DBT in 2.8 mL of ethyl acetate. Serial dilutions were

made in ethyl acetate until the concentration of 0.05 mM was obtained. The phase-transfer catalyst (TBAS, 10mM) was made by dissolving 15.05 g of sodium tetraborate decahydrate in 300 mL of water, so that a pH of 9.4 was achieved. All solutions were stored at 4°C, except the TBAS and PFB-Br solutions, which was left at room temperature.

ATCA Procedure

A stock solution of ATCA (1000 µg/mL) was prepared by dissolving 10 mg of ATCA in 10 mL of a 0.1 M hydrochloric acid solution. Serial dilutions were preformed to obtain the concentrations of working solution at 0.375 µg/mL – 20 µg/mL. A stock solution of deuterated internal standard ATCA-^{13}C, $^{15}N_2$ was prepared by dissolving 1.6 mg of ATCA-^{13}C,$^{15}N_2$ in 1.6 mL of methanol. Serial dilutions were performed in methanol to obtain a working solution at 5 µg/mL. The 10 mL elution solvent was composed of 5 mL of methanol, 2.5 mL of ammonium hydroxide and 2.5 mL of water. A thirty percent derivatization solution of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was made by adding 1mL of MSTFA to 2.3mL of n-hexane.

Authentic Samples

Elise Lauterbur from the Department of Ecology and Evolution at Stony Brook, worked through Centre ValBio in Ranomafana, Madagascar for sample collection. Forty-seven wild lemur urine samples were obtained from free-living, habituated lemurs (*Prolemur simus,* *Hapelemur aureus,* and *Hapalemur griseus*) in their natural habitat. Urine samples were collected at the height of the bamboo growing season through-out January and February 2016, when dietary cyanide concentrations are the highest, cyanide concentrations are highest in the shoots of bamboo. Individuals were followed from dawn to dusk, urine samples were free-caught

mid-stream in a funnel held below the urinating animal, and collected in a fresh 50 mL Falcon^{tm} tube. Samples were transferred to 1.5 – 2 mL vials using sterile transfer pipettes. To minimize volatilization into headspace, vials were nearly completely filled, only leaving room for expansion during freezing. They were then wrapped in parafilm^{tm}. Any samples contaminated with tree debris were discarded, and samples were not collected when it was raining. Funnels were cleaned and wiped with alcohol swabs between uses. Samples were frozen as soon after collection as possible in a -20° C or -80° C freezer, and the elapsed time between collection and freezing was recorded. Samples were shipped using dry ice to keep them frozen, and upon receiving the samples at John Jay College in New York for analysis, they were stored in a freezer at -20° C.

Elise Lauterbur from the Department of Ecology and Evolution at Stony Brook, worked with the Duke Lemur Center for sample collection of captive samples. Twenty wild lemur urine samples were obtained from captive lemurs (*Varecia variegata, Hapalemur griseus, Eulemur flavifrons*, and *Lemur catta*). Samples were collected in clean tubes; a total of 1 mL was collected from each lemur. Samples were frozen as soon after collection as possible in a -20° C or -80° C freezer, and the elapsed time between collection and freezing was recorded. Samples were shipped using dry ice to keep them frozen, and upon receiving the samples at John Jay College in New York for analysis, they were stored in a freezer at -20° C.

Instrumentation

Gas Chromatography-Mass spectrometry analysis was performed on the GCMS-QP2010 Ultra from Shimadzu (Columbia, MD). The ion source was electron impact (EI). The chromatographic separation was achieved using a DB-5ms Column (length of 30 m, thickness of

0.25 um and internal diameter of 0.25um) from Thermo Scientific, using helium as carrier gas at a flow of 0.99 mL/min in a constant flow rate mode.

For the cyanide and thiocyanate procedure the injection port temperature was 200^0C. The initial oven temperature was 75^0C, then elevated to 165^0C at a rate of 30^0C/min and held for 2 minutes. After that, it ramped to 270^0C at a rate of 50^0C/minute and held for 1 minute. The interface and ion source temperatures were 265^0C and 200^0C, respectively, this was based on a previous study by Paul, B. D., & Smith, M. L. (2006) and Bhandari, R. K. et al. (2012). The GC-MS was programmed to inject on a splitless mode. The MS was programmed to run in selected ion-monitoring (SIM) mode with a solvent cut of time of 2.5 minutes. The ions that were monitored were 207 *m/z*, 188 *m/z* and 157 *m/z* for cyanide with a start time of 2.5 minutes and an end time of 3.5 minutes and 239 *m/z*, 181 *m/z*, 161 *m/z* for thiocyanate and 250 *m/z* and 169 *m/z* for the internal standard 2,5-DBT with a start time of 3.5 minutes and end time of 6.5 minutes.

For the ATCA procedure, the injection port temperature was set to 290^0C. The initial oven temperature was 100^0C, held for 1 minute, then elevated to 230^0C at a rate of 15^0C/minute. After that, the oven was ramped to 300^0C at a rate of 30^0C/min and held for 1 minute. The interface and ion source temperatures were 265^0C and 230^0C, respectively, this was based on a previous study by Logue et al. (2012). The GC-MS was programmed to inject on a splitless mode. The MS was programmed to run in SIM mode with a solvent cut of time of 6 minutes. The ions that were monitored were 362 *m/z*, 347 *m/z*, 245 *m/z*, 316 *m/z* for ATCA and 365 *m/z*, 350 *m/z*, 248 *m/z* and 319 *m/z* for the internal standard ATCA-^{13}C, $^{15}N_2$ with a start time of 2.5 minutes and an end time of 3.5 minutes.

Preparation of Calibrators

For the cyanide and thiocyanate procedure, calibration curve (200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, and 10 µg/mL) was prepared by the addition of 20 µL of the corresponding working solution of CN^- and SCN^- to 200 µL of synthetic urine. For the ATCA procedure, a calibration curve (4000 ng/mL, 2000 ng/mL, 1000 ng/mL, 500 ng/mL, 125 ng/mL, and 75 ng/mL) was prepared by the addition of 40 µL of the corresponding working solution of ATCA to 200 µL of synthetic urine. In the main species of bamboo that *Prolemur simus* and *Hapalemur aureus* eat, it was found that the mean concentrations of cyanide ranged from 139.3 +/- 19.32 in ground shoots to 217.7 +/- 16.80 micromole cyanide [HCN] per gram dry weight in branch shoots." (Ballhorn, D. J., Kautz, S., & Rakotoarivelo, F. P. 2009). It is estimated that the amount of bamboo *Hapalemur aureus* (about 1.5kg in size) are consuming is 500 g of bamboo per day (Glander, K. E., Wright, P. C., Seigler, D. S., Randrianasolo, V., & Randrianasolo, B. 1989). *Prolemur simus* is about 30%-50% larger (2 - 2.5 kg) than *Hapalemur aureus*, thus likely eating more bamboo. One gram of fresh bamboo corresponds to about 0.1 grams dry. Therefore, lemurs could be consuming up to 250 mg of cyanide over the course of a single day, hence the concentration ranges were determined based studies that determined the high-level concentration range of cyanide in the bamboo that lemurs consume (Ballhorn et al 2009) and (Glander et al 1989).

Sample Preparation and Extraction

Cyanide & Thiocyanate Procedure

To 200 µL of urine, 300 µL of the derivatization reagent PFB-Br was added, as well as 200 µL of 2, 5-DBT (internal standard) and 800 µL of the TBAS solution, the catalyzing agent.

The solutions were vortexed for 1 minute and placed in a heating block set to 55^0C for 120 minutes. The samples were centrifuged at 1700 x g using a Thermo Scientific Sorvall Legend X1 Centrifuge (Waltham, MA). One hundred microliters of the clear ethyl acetate (top layer) were transfer to a GC vial and 3 µL were injected into the GC-MS for analysis

ATCA Procedure

Forty microliters of the internal standard and 900 µL of 0.1 M hydrochloric acid were added to 200 µL of urine. OASIS MCX extraction cartridges were conditioned with 1mL of methanol and 1mL of water. Samples were then loaded onto the cartridges. The cartridges were washed with 1mL of 0.1M hydrochloric acid and 1 mL of methanol. The samples were eluted into clean test tube using the elution solvent. After the elution, 200 µL of 0.1M hydrochloric acid was added to reduce the pH of the samples to ensure that the ring structure in ATCA did not open (it opens under high heat and higher basic conditions). The ATCA samples were dried in a Turbovap (Biotage, Charlotte, NC) at 50^0C for 20 minutes. An amount of 150 µL of 30% MSTFA solution in n-hexane was added to each sample as the derivatization step. The samples were placed on a heating block at 50^0C for 60 minutes. The 150 µL were transferred to GC-MS auto sampler vials and 1µL was injected into the GC-MS.

Results and Discussion

Cyanide and Thiocyanate Procedure

The cyanide and thiocyanate determination and quantification method in urine was adapted and modified from Paul, B. D., & Smith, M. L. (2006), Bhandari, R. K. et al. (2012) and Kage, S., Nagata, T., & Kudo, K. (1996). Cyanide and thiocyanate were reacted with PFB-Br to create substances that were volatile (the major metabolite is not as volatile as cyanide) allowing

for analysis via GC-MS. The TBAS solution was used as a phase catalyzing agent allowing for the reaction of cyanide and thiocyanate with the PFB-Br, increasing the areas achieved under the peaks. Identification was made possible by the retention time, *m/z* of the precursor ions and fragments, and the peak area ratios of the monitored ions: 207 *m/z* quantifier ion, 188 *m/z*, 157 *m/z* qualifier ions for cyanide and 239 *m/z* quantifier ion, 181 *m/z*, 161 *m/z* qualifier ions for thiocyanate, Although the 181 ion is in more abundance for PFB-SCN it was observed at high concentrated samples this ion could over saturate the detector, thus 239 *m/z* was used as quantifier. The retention time for PFB-CN was determined to be 3.13 minutes and for PFB-SCN 4.18 minutes. Broader peaks or peaks with slight tailing were observed for PFB-CN (Fig 1), while sharp symmetrical peaks were observed for PFB-SCN (Fig 2) and the internal standard 2,5-DBT.

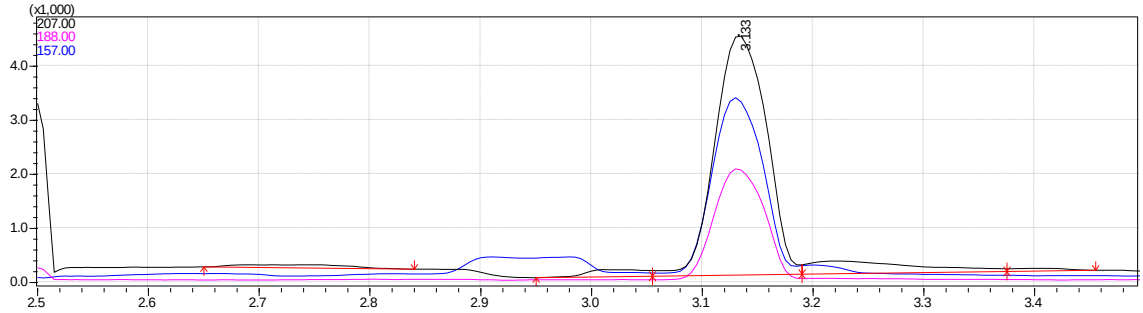

Fig # 1 Single ion monitoring chromatogram of *m/z* 207, 188 and 157 of calibrator 10 µg/mL prepared in synthetic urine analyzed for cyanide

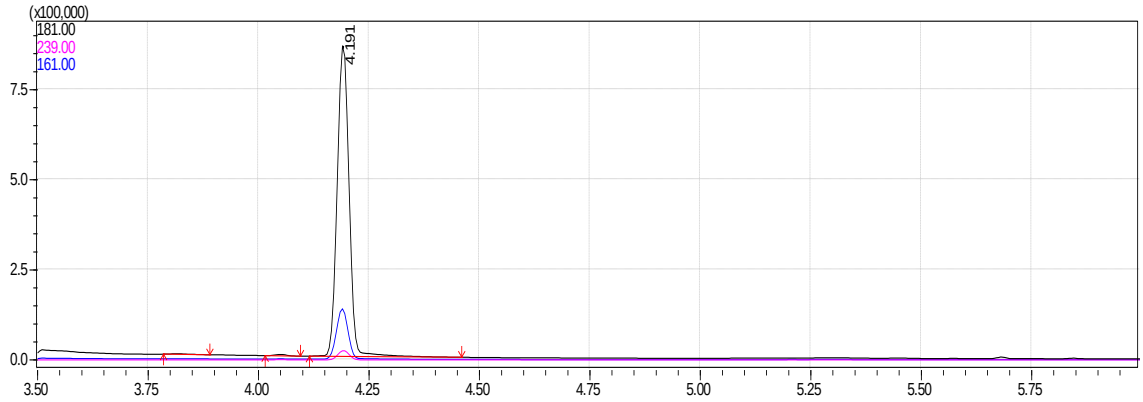

Fig # 2 Single ion monitoring ion chromatogram of *m/z* 239, 181 and 161 of calibrator 50 µg/mL prepared in synthetic urine analyzed for thiocyanate

Method Validation

Calibration curves were constructed in the range of 10 to 200 µg/mL, with five concentrations (10, 25, 50, 100 and 200 µg/mL) in addition to a blank. The calibration curve was assessed for a period of five days. The data was represented by a weighted ($1/C^2$) linear least square line. Calibration curves for CN^- were found to be linear with correlation coefficients greater than 0.90, except one day (0.86), which could have been due to the volatile nature and instability of cyanide. The correlation coefficient for thiocyanate was found to be greater than 0.94 for all five days. All residuals were below the allowed ±20 % range. The linearity data can be seen in the following Table 1.

Table 1. Linearity and Regression of cyanide and thiocyanate in synthetic urine

Analyte	Curve	intercept	slope	r^2		Analyte	Curve	intercept	slope	r^2
CN^-	1	0.133	1.82E-02	0.997		SCN^-	1	2.77	7.42E-01	0.998
	2	4.20E-02	2.10E-02	0.997			2	2.99	6.78E-01	0.964
	3	7.66E-04	2.11E-02	0.984			3	2.90	7.10E-01	0.952
	4	2.43E-02	2.14E-02	0.866			4	1.43	8.23E-01	0.996
	5	4.12E-03	2.89E-02	0.930			5	1.64	1.20E+00	0.995

The limited of detection was determined to be 1µg/mL for CN and SCN and the limit of quantification was determined to be 2.5 µg/mL. Both limits were determined by triplicate analysis over several days, using synthetic urine.

The accuracy and imprecision of the method was determined by triplicate analysis of low- and high-quality controls (30 µg/mL and 150 µg/mL, respectively) over five different days. Table 2 summarizes the accuracy and the inter- day imprecision data that was observed for CN^- and Table 3 summarizes the intra-day imprecision data obtained for CN^-. Table 4 summarizes the accuracy and the inter-day imprecision data that was observed for SCN^- and Table 5 summarizes the intra-day imprecision data obtained for SCN^-.

Table 2. Analytical and imprecision data for cyanide at quality controls 30 and 150 µg/mL in synthetic urine by gas chromatography mass spectrometry

Analyte	Date		Low QC 30 µg/mL	High QC 150 µg/mL
CN^-	9-Mar	#1	37.04	144.34

		40.36	136.18
		30.14	167.35
11-Mar	#2	30.16	131.23
		29.28	133.37
		27.24	129.55
13-Mar	#3	29.79	121.22
		26.53	129.38
		22.73	135.1
16-Mar	#4	30.6	192.77
		38.19	133.17
		33.84	136.14
23-Mar	#5	23.59	111.76
		23.88	106.8
		25.28	99.45
Total	**Mean**	29.91	133.85
	SD	5.43	22.87

	Accuracy	99.70	89.24
	Imprecision	18.16	17.10

Table 3. Intra-day imprecision data for cyanide at quality controls 30 and 150 µg/mL in synthetic urine by gas chromatography mass spectrometry

Analyte	Date		Low QC 30 µg/mL	High QC 150 µg/mL
CN^-	9-Mar			
		Mean	35.845	149.29
		SD	5.21	16.16
		Imprecision	**14.54**	**10.83**
	11-Mar	**Mean**	28.89	131.38
		SD	1.50	1.91
		Imprecision	**5.18**	**1.46**
	13-Mar	**Mean**	26.35	128.57
		SD	3.53	7.00
		Imprecision	**13.41**	**5.43**
	16-Mar	**Mean**	34.21	154.03

		SD	3.81	33.59
		Imprecision	**11.13**	**21.81**
	23-Mar	**Mean**	24.25	106.00
		SD	0.90	6.19
		Imprecision	**3.73**	**5.84**

Table 4. Analytical and imprecision data for thiocyanate at quality controls 30 and 150 µg/mL in synthetic urine by gas chromatography mass spectrometry

Analyte	Date		Low QC 30 µg/mL	High QC 150 µg/mL
SCN^-	9-Mar	#1	33.03	120.19
			33.79	91.52
			32.98	121.94
	11-Mar	#2	34.94	125.01
			33.85	92.82
			33.89	138.03
	13-Mar	#3	31.66	125.41
			32.3	90.52

		25.38	132.12
16-Mar	#4	36.66	167.93
		35.96	90.05
		31.82	142.88
23-Mar	#5	30.64	155.72
		30.58	92.27
		31.59	156.63
Total	**Mean**	32.60	122.87
	SD	2.68	26.63240547
	Accuracy	108.68	81.91
	Imprecision	8.23	21.68

Table 5. Intra-day imprecision data for thiocyanate at quality controls 30 and 150 µg/mL in synthetic urine by gas chromatography mass spectrometry

Analyte	Date		Low QC 30 µg/mL	High QC 150 µg/mL
SCN-	9-Mar			
		Mean	33.27	111.22

		SD	0.45	17.08
		Imprecision	1.36	15.36
	11-Mar	**Mean**	34.23	118.62
		SD	0.62	23.27
		Imprecision	1.81	19.62
	13-Mar	**Mean**	29.78	116.02
		SD	3.82	22.33
		Imprecision	12.84	19.25
	16-Mar	**Mean**	34.81	133.62
		SD	2.62	39.76
		Imprecision	7.51	29.75
	23-Mar	**Mean**	30.94	134.87
		SD	0.57	36.90
		Imprecision	1.83	27.36

According to the lower quality control samples the method is considered highly accurate and has a low level of imprecision for both CN^- and SCN^-. There was a lower than expected accuracy (and higher imprecision) in the higher quality control samples for CN^- and SCN^-. This could

have been due to the high concentration of the compound and the limit of the column's ability to handle higher concentration. An over saturation was not detected by the column, but broader peaks were observed for ion 181 (for the SCN^- analysis), this could account for the lower accuracy observed in addition to the high instability of the CN^- compound to begin with. The intra-day imprecision for CN^- showed the highest observed percentage of the low QC to be 14.54 and 12.80 for CN^- and SCN^- and the high QC to be 21.80 and 29.75 for CN^- and SCN^-, respectively. The higher QC imprecision was above the allowed 20% for both CN^- and SCN^- which could have been due to the instability of the compounds and the volatile of the compounds at high concentrations.

Carry Over

To assess the possibility of carry over blank samples were injected after high calibration points to determine if CN^- or SCN^- were being carried over. Carry over was not detected at these concentration range. Table 6 & 7 summarizes the data that was recovered from the carry over analysis of CN^- and SCN^- respectively.

Table 6. Carry over assessment for CN^- in blank synthetic urine samples

CN^-	Data Filename	after conc (µg/mL)	Ret. Time	Area	Std. Conc.	Conc. (µg/mL)	ISTD Area	Area Ratio
1	BLANK2.qgd	25	3.142	547	-----	0.31505	109,591	0.004991
2	BLANK3.qgd	50	3.149	987	-----	0.47732	101,925	0.009684
3	BLANK4.qgd	100	3.144	1,625	-----	1	99,931	0.016261
4	BLANK5.qgd	200	-----	-----	-----	-----	-----	-----

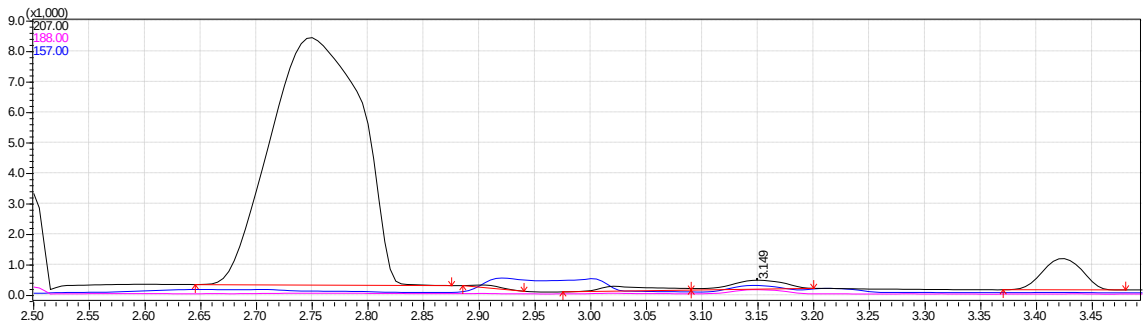

Fig # 3 Single ion monitoring chromatogram of *m/z* 207, 188 and 157 of a blank synthetic urine sample injected after 100 µg/mL of cyanide

Table 7. Carry over assessment for SCN^- in blank synthetic urine samples

SCN-	Data Filename	after conc	Ret. Time	Area	Std. Conc.	Conc. (µg/mL)	ISTD Area	Area Ratio
1	BLANK2.qgd	25	4.187	1,182	-----	0.25133	109,591	0.010786
2	BLANK3.qgd	50	4.191	2,615	-----	0.59784	101,925	0.025656
3	BLANK4.qgd	100	4.188	4,739	-----	1.10506	99,931	0.047423
4	BLANK5.qgd	200	4.207	342	-----	2.14173	3,721	0.091911

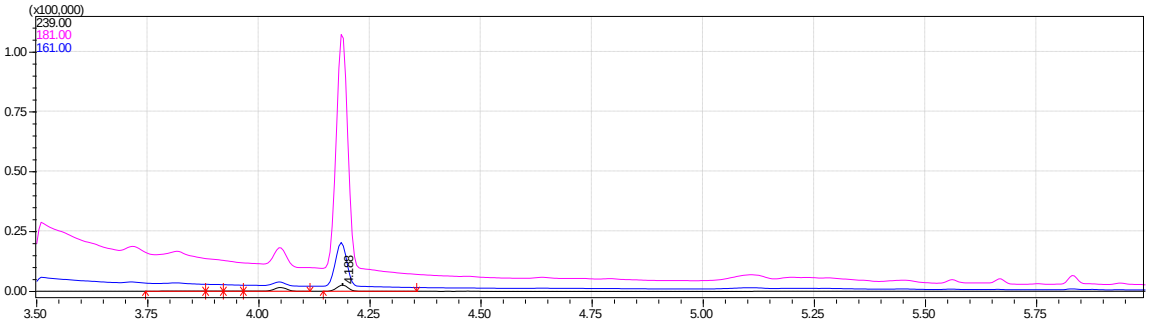

Fig # 4 Single ion monitoring ion chromatogram of *m/z* 239, 181 and 161 of a blank synthetic urine sample injected after 100 µg/mL of thiocyanate

ATCA Procedure

The ATCA determination in urine was adapted and modified from the Logue et al. (2012) method. The sample preparation of ATCA took advantage of the positive charge amine group at a low pH. Samples were acidified and electrostatically bound to cation exchange mix-mode cartridges while allowing negative and neutral compounds to pass through the cartridges. A basic solvent was then used to neutralize the amine group and elute the ATCA samples. After the

extraction, the samples were derivatized with MSTFA, replacing hydrogen groups with trimethylsilyl (TMS) groups, to form a volatile substance for GC-MS analysis. Identification was made possible by the retention time of 9.2 minutes from the total ion chromatogram, *m/z* of the fragments and the peak area ratios of the monitored ions. The 362 *m/z* is the quantifier ion of ATCA and the fragments 347 *m/z*, 245 *m/z*, and 316 *m/z* were used as qualifier ions. Sharp symmetrical peak shapes were observed. Figures 5 & 6 shows a non-spike synthetic urine sample (blank) and a spike synthetic urine sample at 4000 ng/mL, respectively.

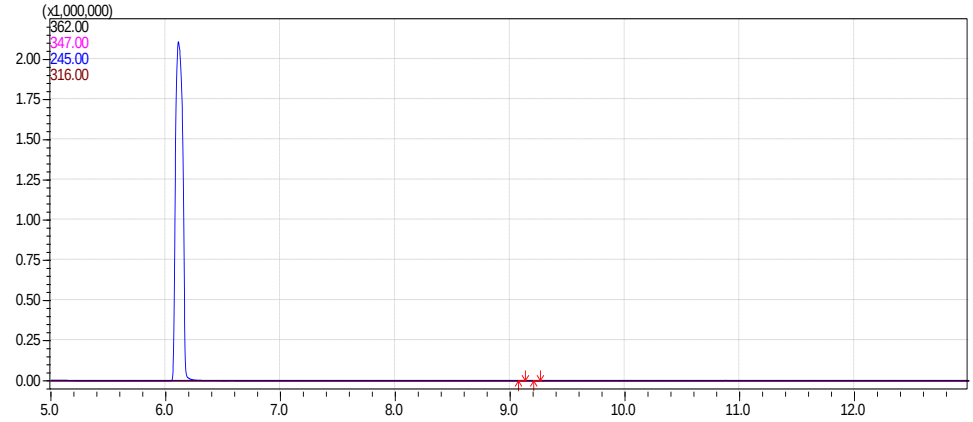

Fig # 5. Single ion monitoring chromatogram of *m/z* 362, 347, 245 and 316 of a blank synthetic urine sample.

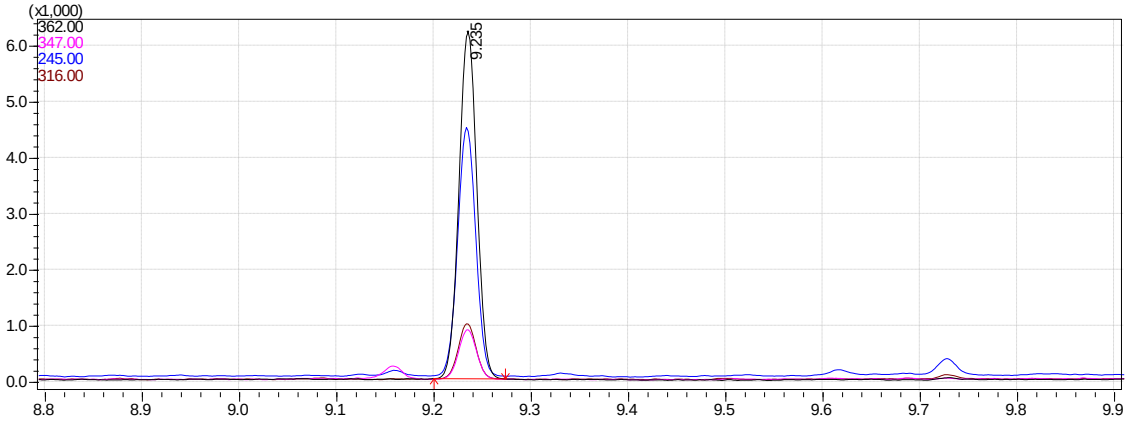

Fig # 6. Single ion monitoring chromatogram of *m/z* 362, 347, 245 and 316 of a spiked synthetic urine sample at 4000ng/mL.

Method Validation

Calibration curves were constructed in the range of 4000ng/mL-75 ng/mL with six concentrations (4000, 2000, 1000, 500, 125, 75 ng/mL) in addition to a blank. The calibration curve was assessed for a period of five days. The data was represented by a weighted ($1/C^2$) linear least square line. Calibration curves for ATCA were found to be linear with correlation coefficients greater than 0.99. The linear range and regression model can be seen in the following Table 8.

Table 8. Linearity and Regression of ATCA in synthetic urine

Analyte	Curve	intercept	slope	R^2
ATCA	1	0.0180	9.22E-04	0.994
	2	0.0140	8.83E-04	0.995
	3	5.16E-04	9.81E-04	0.990
	4	0.00840	8.19E-04	0.996
	5	0.0190	8.10E-04	0.994

The LOD and the LOQ were determined by triplicate analysis over several days using synthetic urine. Sharp clear peaks were able to be seen in low concentrations, such as the LOD 1 ng/mL and the LOQ 5 ng/mL (Fig 7).

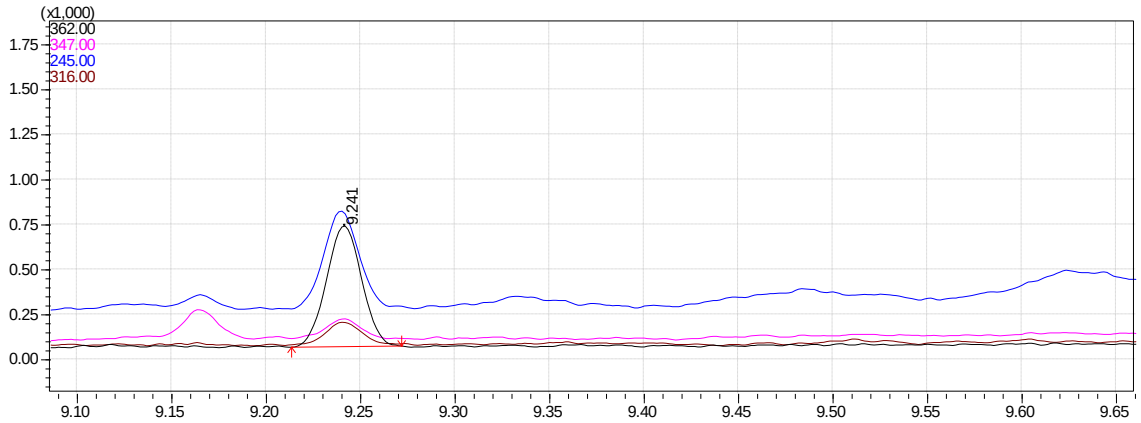

Fig # 7. Single ion monitoring chromatogram of *m/z* 362, 347, 245 and 316 of a spiked synthetic urine sample with a concentration of 5 ng/mL of ATCA.

The accuracy and precision of the method was determined by triplicate analysis of a high and low-quality controls (750 ng/mL and 225 ng/mL respectively) over five different days. Table 6 summarizes the accuracy and inter-day imprecision data that was observed, and Table 9 summarizes the intra-day imprecision and Table 10 summarizes the inter-day imprecision.

Table 9. Accuracy and imprecision data acquired over a five-day period for Low Quality Control (225ng/mL) and High-Quality Control (750ng/mL)

Analyte		Date		Low QC 225	High QC 750
ATCA		18-Apr	#1	261.89	794.58
				278.98	731.29
				295.47	803.51
		21-Apr	#2	272.34	800.84
				264.36	817.26

				261	764.19
		26-Apr	#3	255.08	821.03
				231.17	799.04
				232.11	747.79
		3-May	#4	259.79	870.36
				228.58	787.58
				no data	766.23
		4-May	#5		797.47
				250.83	818.45
				266.58	744.16
		Total	**Mean**	258.32	790.92
			SD	19.377	35.84
			Accuracy	114.81	105.46
			Imprecision	7.50	4.53

Table 10. Inter-day imprecision data acquired over a five-day period for Low Quality Control (225ng/mL) and High-Quality Control (750ng/mL)

Analyte	Date range		Low QC 30	High QC 150
ATCA	18-Apr			
		Mean	278.78	776.46
		SD	16.79	39.37
		Imprecision	6.02	5.07
	21-Apr	**Mean**	265.90	794.10
		SD	5.82	27.17
		Imprecision	2.19	3.42
	26-Apr	**Mean**	241.02	789.29
		SD	13.54	37.58
		Imprecision	5.62	4.76
	3-May	**Mean**	244.19	808.06
		SD	22.07	55.00
		Imprecision	9.04	6.81

	4-May	**Mean**	258.32	786.69
		SD	7.90	38.30
		Imprecision	3.06	4.87

According to the quality control samples the method is considered highly accurate and has a low level of imprecision. The higher accuracy percentage of the lower quality control sample was within the allotted range of 80-120% according to method requirements. The higher quality control sample showed a higher accuracy with a smaller imprecision. The intra-day imprecision showed that the highest percentage of imprecision was 9.04% of the low QC and 6.81% for the high QC, again demonstrating that ATCA is a highly stable compound and an excellent marker to use.

Carry Over

Carry over experimentation was done by injecting blank samples after high calibration points to determine if ATCA was being carried over. Table 11 summarizes the data that was recovered from the carry over analysis.

Table 11. Carry over studies in three non-spike human urine samples and three spiked human samples

	Data Filename	Injection after concentration (ng/mL)	Ret. Time	Area		Conc. (µg/mL)	ISTD Area	Area Ratio
1	blank	0	-----	-----		-----	16,354	-----
2	blank_2	500	-----	-----		-----	14,947	-----
3	blank_3	1000	9.232	91		-2.26495	14,225	0.006397
4	blank_4	2000	9.234	120		-1.51364	16,980	0.007067

It can be seen from the above table and Fig 8 that carry over was not present in the any of the blank samples after injecting concentrations of 500ng/mL, 1000ng/mL, and 2000ng/mL.

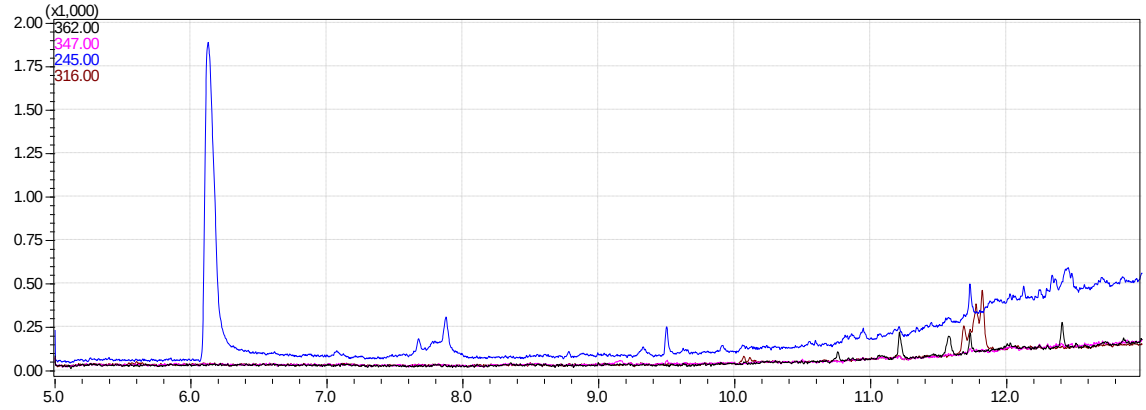

Fig # 8 Single ion monitoring chromatogram of *m/z* 362, 347, 245 and 316 of a blank synthetic urine sample injected after 2000 ng/mL calibrator.

Authentic Lemur Samples

The described method for CN^- and SCN^- and ATCA analysis was applied to 47 wild lemur urine samples from Madagascar to determine the detection and concentrations of CN^-, SCN^- and ATCA. The following Table 12 contains the concentrations of the lemur samples. The CN^- & SCN^- concentrations are in micrograms per milliliter and the ATCA concentrations are in nanograms per milliliter.

Table 12. Concentration data obtained from the authentic lemur samples of CN^- and SCN^- and ATCA

Sample ID	**CN^- concentration µg/mL**	**SCN^- concentration µg/mL**	**ATCA concentration ng/mL**
C0004	78.9	294	2763.5
C005	0.0	0	243.5
C011A	94.2	137.3	1027.1
C012	158.9	>200	1754.5
C015	125.7	109.7	1598.3
C017	99.5	117.8	1974.6
C018	28.0	94.1	1120.2
C020	0.0	0	349.3
C022	0.0	21.9	623.6
C023	51.5	128.1	559.8
C026	51.5	83.7	578.9
C028	0.0	0	126.6

C030	109.8	88.1	1349.4
C032	85.7	>200	3380.7
C034	>200	>200	2348.3
C035	87.2	>200	>4000
C036	177.7	122	2565.2
C038	81.2	135.1	1214.1
CO39	0.0	82.6	1161.6
C041	0.0	0	436.5
C042	0.0	0	493.4
C043	0.0	0	484.2
C064	0.0	57.9	371.5
C071	59.9	143.2	678.5
C072	89.0	102.3	867.6
C073	>200	136.2	1473.7
C074	37.7	146.3	1321.4
C075	79.8	151.6	1526.3
C076	111.5	>200	1307.7
C078	60.4	135.7	1977
C079	99.5	146.2	2933.5
C087	28.2	109.3	527.9
C088	103.6	179.4	1076.2
C089	0.0	73.3	132.5
C091	0.0	97.2	290.4

C093	50.2	186.8	895.1
C094	0.0	120	2467.2
C095	0.0	141.6	1656.6
C096	0.0	78.6	2312.5
C097	60.1	128.5	1396.1
C100	46.5	145.1	905.1
C101	46.8	120	2909.8
C102	0.0	185.2	1162.2
C103	0.0	183.5	1387.4
C104	42.7	>200	2113.9
C107	96.4	>200	1861.8
C108	0.0	45.7	461.5

The CN^- concentration range was from 0 to over 200 µg/mL with an average concentration of 57.8 µg/mL, the SCN^- concentration range was from 0 to over 200 µg/mL with an average concentration of 147.78 µg/mL. Finally, the concentration range of ATCA was from 126 ng/mL to over 4000 ng/mL with an average concentration range of 1308 ng/mL. As it can be seen in Fig 9 the major metabolite (thiocyanate) concentration range was above that of the cyanide concentrations, which suggest that lemurs are metabolizing cyanide and after metabolizing processes are depleted are excreting cyanide itself. The SCN^- samples tended to have the higher concentrations compared to the CN^- data obtained. The authentic samples had a lower concentration of ATCA than the parent compound and the major metabolite (since it only accounts for 20% of metabolization this was expected).

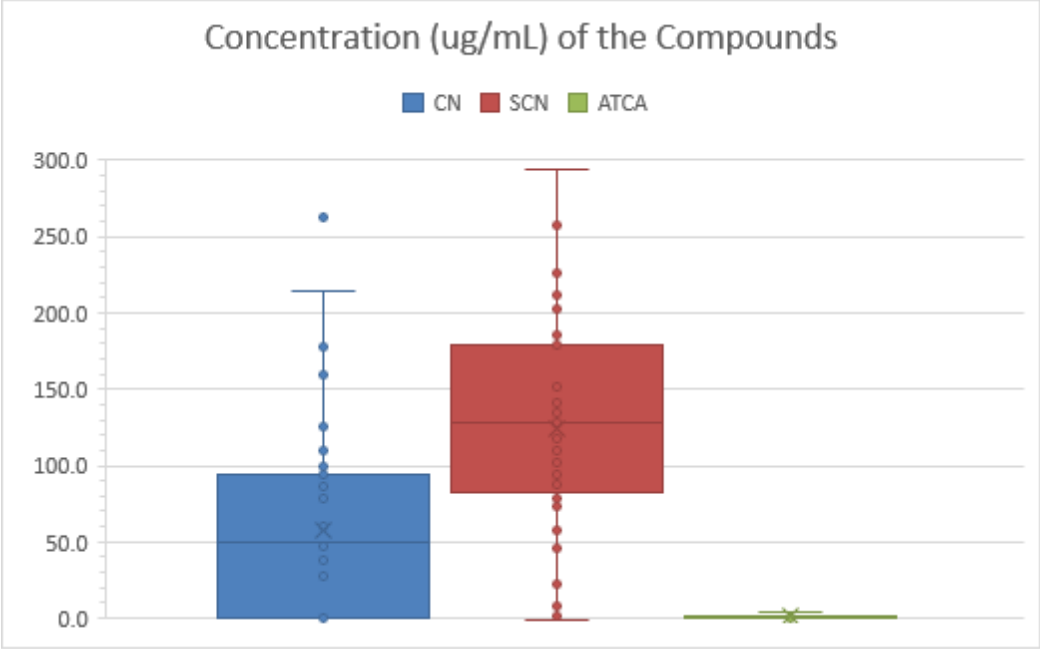

Fig # 9 comparison of concentration data obtained to each compound

Fig 10 shows the total ion chromatogram of the sample labeled CO 108 positive for CN^-. Fig 11 shows the chromatograms for sample CO 88 positive for SCN. The retention time for CN^- was observed to be roughly 3.1 minutes and for SCN^- roughly 4.2 minutes throughout all samples.

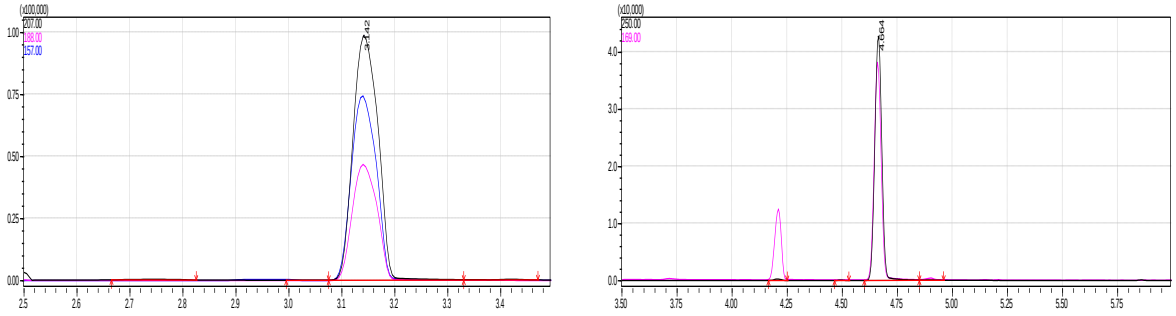

Fig # 10. Single ion monitoring chromatogram of authentic sample CO 108 for cynaide (left) and internal standard (right)

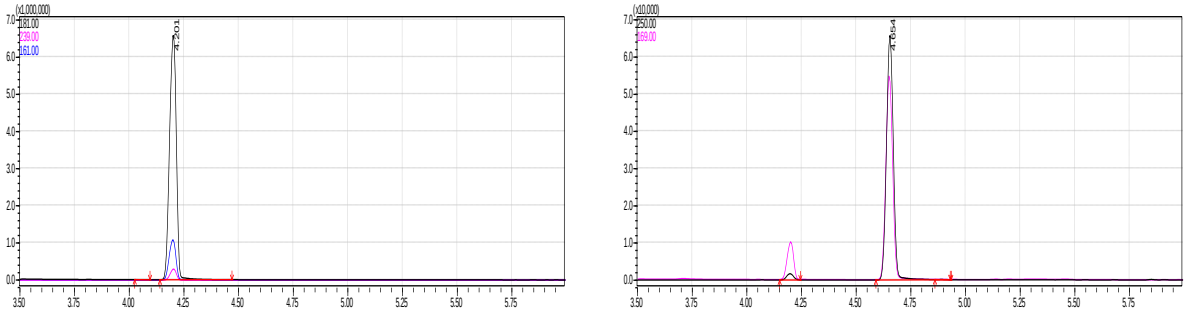

Fig # 11. Single ion monitoring chromatogram of authentic sample CO 88 for thiocyanate (left) and internal standard TIC (right)

Fig 12 shows the GC-MS chromatogram of one of the samples positive for ATCA coded as CO89 with internal standard, related results were seen in all the samples, the retention times was 9.2 minutes throughout the authentic samples.

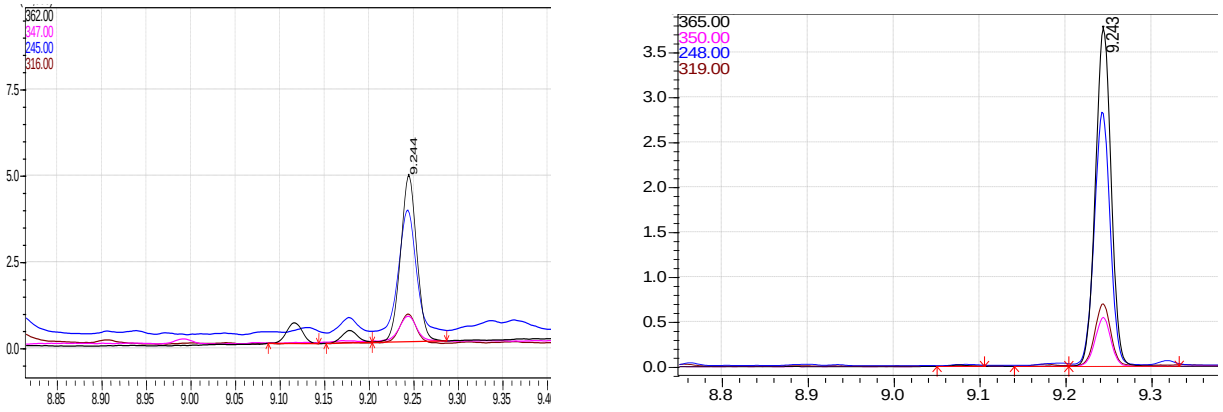

Fig # 12 Single ion monitoring chromatogram of authentic sample CO 89 for ATCA (left) and internal standard TIC (right)

An additional 20 samples were analyzed from captive lemurs from the Duke Lemur Center. All samples tested negative for CN^- and SCN^-. Of the twenty, only three samples showed ATCA concentrations above the LOQ. Fig 13 shows the GC-MS chromatogram of one sample of the captive lemurs positive for ATCA. The retention time was shorter (8.6 minutes) than the calibrators due to a different company's column being used at the time of the analysis, the

chemistry and dimension of the column were similar to the column that was used in the validation studies.

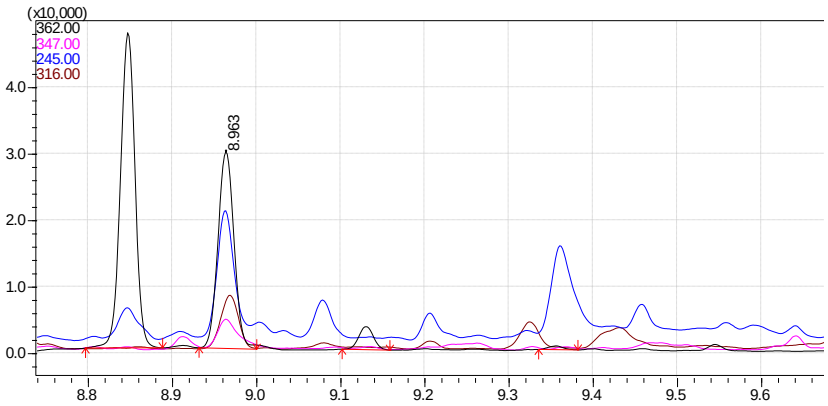

Fig # 13 captive Lemur sample "Babel", authentic urine sample retention time 8.6 minutes and proper ions observed analyzed for ATCA

Correlation data was calculated to determine if concentration data observed was directly related to factors such as gender, age, location of sample collection, species type and urine pH. When comparing gender to concentration of the compounds, there was no significant difference between male or female. The follow Fig 14 demonstrates the data that was observed for ATCA, 22 samples were male samples, 22 samples were female and for 3 samples the gender was not determined.

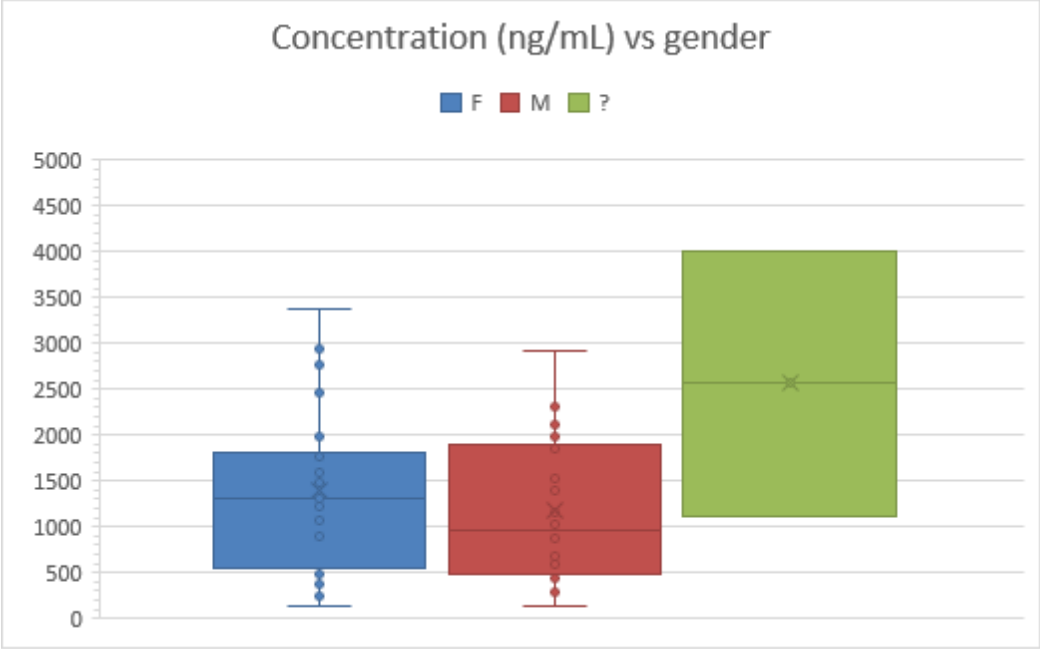

Fig # 14 Correlation data was calculated to determine if concentration data observed was directly related to gender, analyzed for ATCA

Although there are some female samples that are above the concentration 2500ng/mL and below 500ng/mL, majority of the samples were in the range of 500ng/mL-1750ng/mL and the same was observed in general for the male samples. It is important to note that the female concentrations were slightly higher than males and that the three unknown gender samples had the highest concentrations; however, since the gender is unknown no correlation data can be concluded from the data. Similar data was observed for the correlation data between cyanide and thiocyanate and the concentration.

Another correlation study that was computed was the concentration versus the lemur species type. The following Fig 15 demonstrates the data that was calculated.

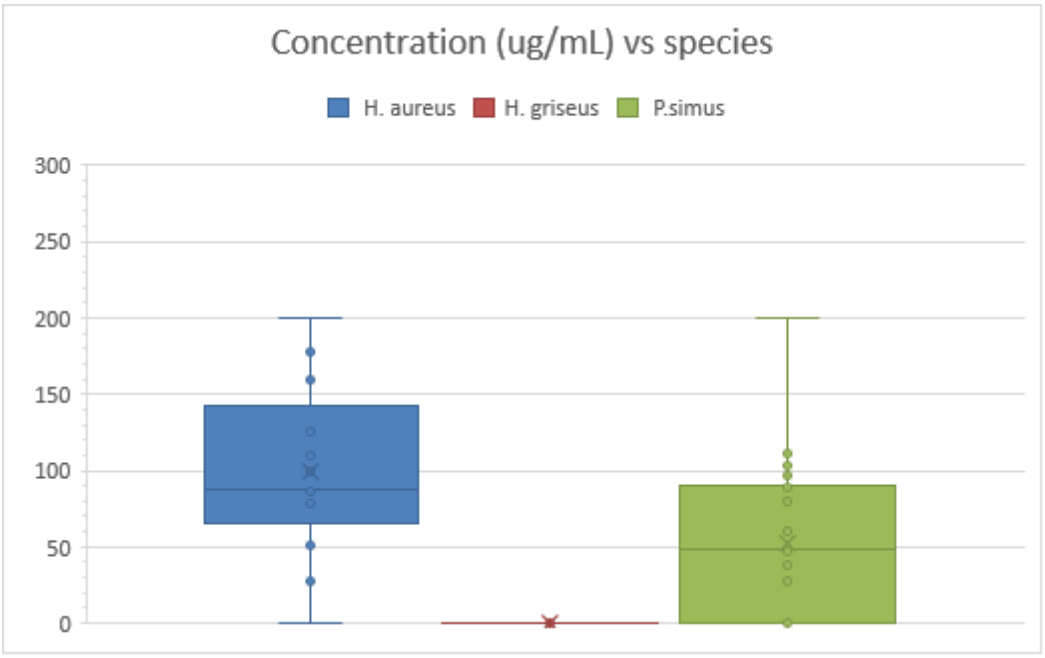

Fig # 15 Correlation data was calculated to determine if concentration data observed was directly related to species, analyzed for cyanide

A significant difference was seen between the cyanide concentration and species type. The H. *aureus* (n=13) species had higher concentrations while the *H. griseus* (n= 8) species tended to have lower concentration. The *P. simus* (n=26) species showed concentrations in between the two-other species. It is important to note that there were not as many *H. aureus* and P. *griseus* samples as *P. simus*, and although the *H. griseus* species had the lower concentrations there were only a few samples of this species. Similar results were seen when comparing thiocyanate and ATCA to the concentration. *H. aureus* species had the highest concentration range and the *H. griseus* had the lowest concentration. *H. aureus* and *P. simus* are believed to take in the most cyanide, because they prefer a cyanide rich species of bamboo, and the bamboo parts they consume have the highest concentration of cyanide. Over 90% of their diets is bamboo. *H. griseus* has a ~70% bamboo diet, but prefers the species with less cyanide and the parts (leaves

and vines) with less cyanide. Glander, K. E., et. Al. (1989) and Ballhorn, D. J., Kautz, S., & Rakotoarivelo, F. P. (2009)

There were no significant differences when correlating between concentration and age and concentration with location. A few juvenile samples were slightly higher in concentration for cyanide and its metabolites than adult samples, but the majority of the juvenile samples were in the same range as most of the adult samples. One factor to note is that most of the authentic samples were adults (n=32), compared to the other major set juveniles (n=12), and the remainder of the samples classified as sub adults.

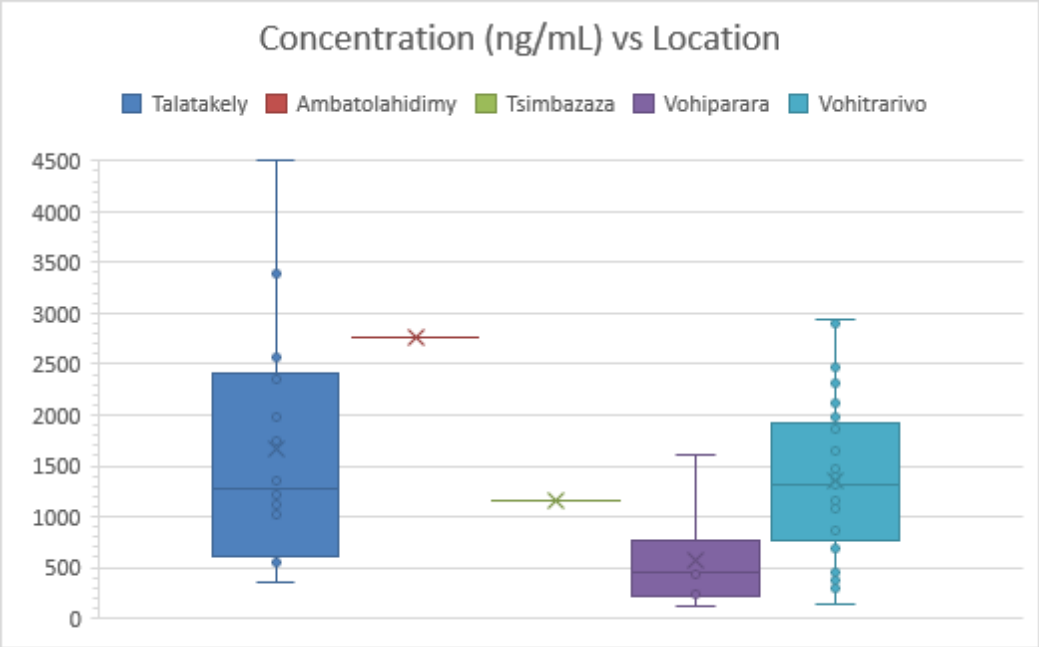

Fig # 16 Correlation data was calculated to determine if concentration data observed was directly related to species, analyzed for ATCA

In terms of sample collection location overall samples collected from Talatakely (n=13) had a higher concentration range in cyanide and its metabolites, these samples were all *H. aureus*. Most of the Talatakely concentrations like the samples from Vohitrarivo (n= 16). In terms of cyanide and thiocyanate, non-zero concentrations were only observed in samples from

Talatakely and Vohitrarivo. Samples from Vohiparara (n=4) tended to have lower concentrations than samples Talatakely (for ATCA only), see Fig # 16. All other locations Ambatolahidmy and Tsimbazaza only had one sample to test so no comparison data was done.

Conclusion

We developed and validated a noninvasive, sensitive and specific method for the determination of cyanide, and its major (thiocyanate) and minor (ATCA) metabolites in urine. The method was applied for the analysis of 67 authentic urine samples from wild and captive lemurs. By developing and validating these methods and applying them, an initial assessment was conducted in order to help understand how lemurs can ingest high amount of cyanide without fatal consequences. Future work would require to study cellular levels to determine how these lemurs are processing and metabolizing cyanide and possibly test other animals that consume bamboo products. Cyanide is a volatile substance, but it seems that in higher concentrations cyanide is able to stay within the body (in lemurs) and with proper storage can be analyzed in a non-invasive manner as was applied in this study.

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