Advance Publication

The Journal of Veterinary Medical Science

Accepted Date: 6 Dec 2017
J-STAGE Advance Published Date: 27 Dec 2017

Toxicology [Full Paper]

Sex and site differences in urinary excretion of conjugated pyrene metabolites in the West African Shorthorn cattle

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Running head: PYRENE METABOLITES IN CATTLE URINE

ABSTRACT

Industrialization, economic and population growth rates in Ghana have increased the release of contaminants including polycyclic aromatic hydrocarbons (PAHs) into the environment through which humans and animals are exposed. Cattle is reported to be exposed to high levels of PAHs through feed and inhalation. Once exposed, PAHs are metabolized and excreted in urine, feces or bile. In a previous study, cattle in Ghana was reported to excrete high levels of 1-hydroxypyrene (1-OHPyr) due to high exposure to the parent compound, pyrene. 1-OHPyr is further metabolized to glucuronide and sulfate conjugates. Thus, the aim of this study was to investigate the sex and site differences in urinary excretion of conjugated pyrene metabolites using cattle urine collected from rural and urban sites of the Ashanti region, Ghana. From the results, geometric mean concentration adjusted by specific gravity indicated that 1-OHPyreneGlucuronide (PyG) was the most abundant conjugate followed by PyrenediolSulfate (M3). The sum of conjugated pyrene metabolites and sum of both conjugated and deconjugated pyrene metabolites correlated significantly with PyG, PydiolSulfate (M2) and PydiolSulfate (M3). The study revealed no significant difference in urinary excretion of conjugated pyrene metabolites between rural and urban sites. This indicated that similar to urban sites, cattle in rural sites were exposed to high levels of pyrene. There was no significant difference in urinary concentrations of conjugated pyrene metabolites between sexes.

KEY WORDS: cattle, Kumasi, metabolites, PAHs, urine

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs), the 9th most hazardous substance based on the Agency for Toxic Substance and Disease Registry's (ATSDR) list [1], are formed during incomplete combustion of organic materials. Anthropogenic activities are the major sources of PAHs in the environment. PAHs are ubiquitous and found in vehicle exhaust, wood and cigarette smoke. Human and animal exposure to PAHs are mainly through consumption of contaminated food and water and inhalation [2, 16]. A number of PAHs gain promutagenic and procarcinogenic activities that could contribute to the incidence of cancer in humans and animals [35]. In both humans and animals, PAHs are metabolized by cytochrome P450 enzymes and excreted in urine, feces or bile [10, 26]. Pyrene, a four ring PAH, is also metabolized by cytochrome P450 enzymes to 1-hydroxypyrene (1-OHPyr) which has been suggested as a biomarker of PAHs exposure [10, 12]. Hydroxypyrene is further metabolized by phase II reactions to form conjugates, such as glucuronide and sulfate [37].

In recent years, Ghana's Kumasi region has seen tremendous increase in population, industrialization and economic activities. These activities and many more could lead to deterioration of the environment and pollution likely to reach disturbing levels [8]. Some of these activities and combustion processes have caused an increase in the levels of PAHs and its metabolites in both environmental and biological samples in Kumasi [4-8].

Cattle is reported to excrete large amount of PAH metabolites due to high intake or exposure to the parent compound [33]. In addition to inhalation, feed is one of the dominant sources of cattle's exposure to PAHs [6, 13]. In previous studies, high levels of 1-OHPyr

was detected in cattle urine in rural and urban sites of Ghana. Although not significant, the levels of 1-OHPyr detected in cattle urine from urban sites was higher than rural sites and vehicular traffic was the major contributing factor [6]. Bortey-Sam *et al.* [6] further highlighted that 1-OHPyr was higher (P>0.05) in female compared to male cattle. The high urinary concentrations of 1-OHPyr could be due to high exposure of cattle to PAHs including pyrene [6]. Despite this, there is limited/no study from literature that has determined the levels of conjugated pyrene metabolites in cattle urine. Based on these findings and gaps, the objectives of the current study were to: determine the concentrations of conjugated pyrene metabolites in urine of cattle that has been environmentally exposed to pyrene; and find any sex and site differences in urinary excretion of these conjugated metabolites.

MATERIALS AND METHODS

Sampling

In August 2014, urine of healthy cattle (West African Shorthorn) were randomly collected from 5 communities in Kumasi and Offinso, both in the Ashanti Region of Ghana. Offinso is about 33 km from the city centre of Kumasi (Fig. 1). In Kumasi (urban), samples were collected from Oforikrom and Santasi, which are 5.1 and 3.5 km from the city centre, respectively, where previous studies reported high concentrations of PAHs including pyrene in particulate matter with diameter 10 µm or less (PM10), soils and livers of wild rats [4, 5, 7, 8]. On the other hand, the three sites in Offinso (Twumasen Estate, Saboa and Kokote) selected for cattle urine sampling (Fig. 1) are in rural and agricultural areas.

A total of 95 spot urine (30 males and 65 females) were collected from cattle within these rural and urban sites (Fig. 1). The samples collected were transferred to labeled corning tubes (Corning Incorporated, Corning, NY, U.S.A) and kept frozen at the Department of Chemistry, Kwame Nkrumah University Science and Technology (KNUST), Ghana. Only ages of cattle from two sites (Twumasen Estate and Saboa) were obtained from the herdsmen and the average ages were 2.9 ± 1.0 years (Twumasen Estate) and 4.2 ± 2.9 years (Saboa). Later, the samples collected were transported to the Laboratory of Toxicology, Graduate School of Veterinary Medicine, Hokkaido University, Japan where they were stored at –30°C until analysis (quarantine number for importing is 26 douken 383).

Extraction and analysis of conjugated pyrene metabolites

The extraction process was modified from previous protocols [32, 33]. Briefly, 5 m*l* of urine was acidified (to pH 6.8) with 1 M formic acid (Wako Pure Chemicals, Osaka, Japan) and 6-hydroxychrysene (AccuStandard Incorporation, New Haven, CT, U.S.A) added as an internal standard. The acidified samples were loaded onto an Oasis WAX plus solid-phase extraction cartridge (50 mg; Waters) conditioned with 10 m*l* methanol and MilliQ water (10 m*l*). The loaded samples were washed with 5 m*l* each of 0.1 M sodium hydroxide solution, 0.1 M sodium phosphate buffer (pH 7.4), and Milli Q water. Cartridges were then dried under vacuum. The target analytes were sequentially eluted with methanol/10% formic acid solution (9:1 v/v, 10 m*l*), and then with methanol/ethyl acetate/diethylamine solution (50:50:1 v/v, 2 m*l*). The eluate was reduced to 100 μl under a gentle nitrogen flow,

and re-dissolved to 0.5 m*l* using methanol for LC-MS/MS analysis with an ODS-120 T column (ODS-120T 2.1 mm × 300 mm; Tosoh).

Samples were analyzed for 1-OHPyrene glucuronide (PyG), 1-OHPyrene sulfate (PyS), and two isomers of Pyrenedoil sulfate (M2 and M3) (represented as PydiolS, M2 and M3). Mobile phase A consisted of 10 mM ammonium acetate buffer (pH 5.0), and mobile phase B was a methanol/acetonitrile/water solution (38:57:5, v/v/v). The solvent gradient was as follows: 10% mobile phase B at the first 2 min, followed by a linear gradient to 100% mobile phase B from 2 to 35 min, and then 100% mobile phase B at 35 to 45 min. Solvent flow rate was 0.5 m*l*/min, and column temperature was 45°C. Target compounds were determined by multiple-reaction monitoring (MRM) in the negative ionization mode. A Shimadzu 8030 triple quadrupole mass spectrometer, upgraded to 8040 with UF lens, (ESI MS-MS; Shimadzu Corporations, Kyoto, Japan), equipped with a Prominence UFLC system (Shimadzu Corporations, Kyoto, Japan) was used for analysis.

Specific gravity (SG) of cattle urine

In this study, specific gravity (SG) illustrated by Nermell *et al.* [30] was used to adjust urinary concentrations of conjugated pyrene metabolites. The mean (ranges) SG detected in cattle urine using a refractometer (ATAGO Company Ltd., PAL-095, Tokyo, Japan) were Oforikrom (1.013; [1.004-1.029]), Santasi (1.035; [1.03-1.041]), Twumasen Estate (1.035; [1.028-1.04]), Saboa (1.036; [1.026-1.049]) and Kokote (1.037; [1.029-1.042]). The formula applied [30] to each urine concentration was as follows:

$$\text{SG\_corrected concentration} = \text{urinary OH} - \text{PAH concentration} \times \frac{(\text{SG}_{\text{target}} - 1.0)}{(\text{SG}_{\text{sample}} - 1.0)}$$

128 Where, SG_{target} is the mean specific gravity of cattle urine per community; SG_{sample} is the
129 specific gravity of a particular sample.

130 *Quality control and quality assurance*

131 For measurement of conjugated pyrene metabolites, quantitation was performed using
132 six–point calibration; 1, 5, 10, 25, 50 and 100 $\mu g/l$), and linearity (r^2) were all greater than
133 0.995. Analytical methods were checked for precision and accuracy. Limits of detection
134 (LODs) were calculated based on 3SD/S (SD is the standard deviation of the response of
135 seven replicate standard solution measurements and S is the slope of the calibration curve).
136 LOD ranged from 0.57–1.70 *n*g/m*l* for PyG and PydiolS (M3), respectively and average
137 recovery rate (%) for 6-hydroxy chrysene was 93.3 ± 10.5. For every batch of 10 samples, a
138 solvent blank, a spiked solvent blank (internal standard spiked into solvent), and duplicate
139 sample were analyzed. The average recovery in spiked blanks was 97 ± 9.3%. Blanks were
140 run periodically and contained no detectable amount of target analyte. The coefficients of
141 variation was less than 20%.

142 *Statistical analysis*

143 Data analysis was performed using IBM SPSS v 20 (SPSS Incorporation, Chicago, IL,
144 U.S.A). Kolmogorov–Smirnov (K–S) and Shapiro-Wilks (S-W) tests were used to
145 determine the normality of data and were considered statistically significant if *P* value was
146 less than 0.05. Concentrations of conjugated pyrene metabolites below their respective
147 LODs were replaced with a value of LOD/2. ANOVA and Tukey analyses of log
148 transformed data were used to compare concentrations in cattle urine from the study areas

and differences were considered statistically significant with P value < 0.05. Student's T-Test was also used to compare concentrations between male and female cattle; and, between urban and rural sites. Pearson's correlation of log transformed data was used to determine the relationship between 1-OHPyr and conjugated pyrene metabolites. Statistical significance for the correlation analysis was at a P value < 0.05. Data for 1-OHPyr was obtained from Bortey-Sam *et al.* [6].

RESULTS

The normality tests (K-S and S-W's tests) showed a significant variation (P<0.01) in the distribution of the conjugated pyrene metabolites measured. As shown in Table 1, there was no significant difference (P>0.05) in the levels of PyG, PyS and PydiolS (M3) excreted in cattle urine from the study areas except PydiolS (M3) which was significantly lower in cattle in Oforikrom. Moreover, PydiolS (M2) was significantly higher (P<0.05) in Kokote compared to Twumasen Estate and Oforikrom.

Specific gravity adjusted geometric mean concentrations (GM_{SG}) revealed PyG (4.10 ± 4.44 *n*g/m*l*) as the most dominant conjugate in cattle urine from all study sites followed by PydiolS (M3) (3.14 ± 2.96 *n*g/m*l*) > PydiolS (M2) (1.24 ± 1.68 *n*g/m*l*) and > PyS (0.424 ± 0.435 *n*g/m*l*).

With the exception of PyS, urinary concentrations of conjugated pyrene metabolites were higher (P>0.05) in rural sites compared to urban sites (Table 2). Moreover, no significant gender differences (P> 0.05) were observed for the conjugated pyrene metabolites studied (Table 3).

The study revealed significant correlation ($P< 0.05$) between 1-OHPyr and PydiolS (M2) (Table 4) although, there was no significant association between 1-OHPyr and the other conjugated metabolites (Table 4). The study further showed that the sum of conjugated pyrene metabolites (∑Conj Pyr met) and sum of both conjugated and deconjugated pyrene metabolites (∑Pyrene met) correlated significantly ($P< 0.01$) with PyG, PydiolS (M2) and PydiolS (M3) (Table 4). Similarly, there was a significant correlation ($P< 0.01$) between PyG and PydiolS (M3). As shown in Table 4, there was no significant correlation between other conjugated pyrene metabolites.

DISCUSSION

Excretion of conjugated pyrene metabolites in cattle urine

The significantly higher ($P<0.05$) levels of PydiolS (M2) in Kokote compared to Twumasen Estate and Oforikrom could be due to differences in levels of exposure and/or metabolism. In a previous study, urinary concentrations of 1-OHPyr was significantly higher ($P<0.05$) in cattle in Kokote than levels in cattle in Saboa and Twumasen Estate [6].

In a study by Saengtienchai *et al.* in Japan and Thailand, PyG was the dominant conjugate excreted via urine in the majority of mammals including cattle [33]. Pyrene-1-sulfate, pyrenediol-sulfate and pyrenediol-disulfate were also detected in urine of cattle and other mammals [33]. A wide range of species including mice, rats, dogs, cattle, rabbits, and pigs all have genomes containing a single SULT1A1 gene [9, 18, 27]. However, in ungulates, the UGT activities may be higher than SULT [15, 36].

Unadjusted urinary levels of PyG in cattle from this study were comparable to higher than the study conducted in Japan and Thailand [33]. The higher levels in this study could

be attributed to cattle's exposure to higher levels of pyrene from the study area [6]. Bortey-Sam *et al.* highlighted that catttle's exposure to pyrene in the Ashanti Region of Ghana could mainly be attributed to vehicular activities or traffic [6]. In Kumasi, fuel combustion was the dominant source of PAHs in PM10 and soils, and pyrene was highly abundant [7, 8]. Moreover, some farms in Ghana are located near major road with high vehicular activities or traffic and grazing animals could be exposed to pyrene through this process [40]. According to Laflamme and Hites, during vehicular emission, the high molecular weight PAHs, including pyrene, are dominant [23]. PAHs in the atmosphere are known to settle in soil [31] and this could also increase cattle's exposure, because these free-range cattle pick food and/or water from the ground.

Site differences in urinary excretions of conjugated pyrene metabolites

This study revealed that cattle in rural sites were exposed to higher levels of pyrene. In previous studies there was no significant difference ($P>0.05$) in urinary excretion of 1-OHPyr in cattle in rural and urban sites. In a study by Ferrari *et al.* [17], higher levels of 1-OHPyr were detected in urine of cattle in rural areas compared to urban. Ferrari *et al.* therefore suggested that there could be other sources of pyrene exposure besides traffic [17] such as barn dust, soils, indoor air and/or forage [13].

Sex differences in urinary excretions of conjugated pyrene metabolites

There are only a few studies in literature that have assessed gender differences in urinary excretion of PAH metabolites in cattle [6]. Bortey-Sam *et al.* [6] indicated that there was no significant sex difference in urinary concentrations of 1-OHPyr between male and

213 female cattle. However, differences or similarities in urinary excretion PAH metabolite in
214 humans have been documented [3, 11, 25, 39]. Thai *et al.* [41] revealed no differences
215 between male and female in urinary concentrations of OHPyr in humans, which was similar
216 to other results obtained [3, 11, 25]. On the other hand, urinary 1-OHPyr levels were higher
217 in women than men workers [20]. The reason for these similarities and differences could be
218 due to the fact that intake, accumulation and excretion rates of chemicals differ by sex in
219 cattle, although information on other factors such as ADME (absorption, distribution,
220 metabolism, and excretion) would be needed to support this statement [22].

221 The differences or similarities in urinary excretion of conjugated pyrene metabolites
222 between sexes could also be due to non-pharmacogenetic factors including age, species,
223 disease factors or exposure to environmental pollutants which could contribute to the
224 expression and regulation of hepatic P450 in these domestic animals [19, 29]. In addition,
225 although no gender difference was observed in male and female limousin cattle, male
226 piedmontese cattle showed significantly higher CYP3A-dependent drug metabolizing
227 enzyme activities compared to females [14].

228 *Correlation between 1-OHPyr and conjugated pyrene metabolites*

229 The results obtained from this study was similar to previous studies (mammalian
230 urine) where no significant correlations existed between 1-OHPyr and total pyrene
231 metabolites. Moreover, 1-OHPyr did not show any significant correlation with conjugated
232 metabolites such as PyG and PyS. Furthermore, PyS showed no association ($P > 0.05$) with
233 PyG and total pyrene metabolites in mammalian urine. Nonetheless, a positive association
234 ($r=0.996$, $P<0.05$) was observed between urinary PyG and total pyrene metabolites [33].

The possible reason for these correlations, especially, between PyG and ∑Pyrene met/ ∑Conj Pyr met could be due to the fact that glucuronide have been shown to account for over 80% of total pyrene metabolites in human urine [34]. Moreover, in a study of healthy and non-smoking humans, Saengtienchai *et al.* [33] found that over 75% of total pyrene metabolites existed as glucuronide conjugate. This trend could be due to high activity of UGT enzymes, which have been known to be involved in glucuronidation of 1-OHPyr in human, mice and ungulates [21, 24, 28, 33, 34, 37, 38]. The UGT activity in addition to other substrates have been suggested to be higher than SULT in ungulates [15, 33, 36].

In conclusion, cattle in Kumasi and Offinso (Ghana) have been exposed to high levels of pyrene. Of the urinary concentrations of conjugated pyrene metabolites studied, PyG was the most abundant followed by PydiolS (M3). The study revealed no significant difference in urinary concentrations of conjugated pyrene metabolites between rural and urban sites, and similar to urban areas, cattle in rural sites were exposed to high levels of pyrene. From this study, there was no significant difference in urinary excretion of conjugated pyrene metabolites between sexes.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan awarded to M. Ishizuka (No. 16H0177906), Y. Ikenaka (No. 26304043, 15H0282505, 15K1221305), S. Nakayama (16K16197), and the foundations of Sumitomo and JSPS Core to Core Program (AA Science Platforms) and Bilateral Joint Research Project (PG36150002 and PG36150003). We also acknowledge the financial support by The Mitsui & Co., Ltd. Environment Fund, the Soroptimist Japan Foundation and the Nakajima Foundation. We are grateful to Mr. Takahiro Ichise for technical support.

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FIGURE LEGEND

Fig. 1. Map showing cattle urine sampling locations in the Ashanti Region, Ghana (yellow pins indicate sampled locations and red pin indicates city center in Kumasi) (Obtained from Bortey-Sam *et al.* [6]).

Table 1. Specific gravity adjusted concentrations (*n*g/m*l*) of conjugated pyrene metabolites in cattle urine in the Ashanti Region of Ghana

Sample site		n	Location	PyG	PyS	PydiolS (M2)	PydiolS (M3)
Oforikrom	$GM_{SG} \pm SD$	8	urban	2.88 ± 3.58^{a}	0.684 ± 0.634^{a}	0.260 ± 0.198^{c}	1.19 ± 1.09^{b}
Santasi	$GM_{SG} \pm SD$	9	urban	4.19 ± 2.49^{a}	0.516 ± 0.365^{a}	1.66 ± 1.59^{ab}	3.29 ± 2.72^{a}
Twumasen Estate	$GM_{SG} \pm SD$	31	rural	4.33 ± 4.27^{a}	0.341 ± 0.197^{a}	1.04 ± 0.866^{bc}	2.71 ± 2.06^{a}
Saboa	$GM_{SG} \pm SD$	40	rural	4.64 ± 5.07^{a}	0.417 ± 0.497^{a}	1.51 ± 2.09^{a}	4.10 ± 3.41^{a}
Kokote	$GM_{SG} \pm SD$	7	rural	2.28 ± 1.77^{a}	0.553 ± 0.437^{a}	2.35 ± 1.31^{a}	1.81 ± 0.997^{a}

n: number of samples; different letter (a, b, c and d) within a column indicate significant difference ($P<0.05$) among communities; GM_{SG}: geometric mean concentration adjusted by specific gravity; SD: standard deviation

Table 2. Specific gravity adjusted concentrations (*n*g/m*l*) of conjugated pyrene metabolites in cattle urine from urban and rural sites

Site		n	PyG	PyS	PydiolS (M2)	PydiolS (M3)
Urban	$GM_{SG} \pm SD$	17	3.52 ± 2.99^{a}	0.589 ± 0.514^{a}	0.793 ± 1.52^{a}	2.55 ± 2.63^{a}
Rural	$GM_{SG} \pm SD$	78	4.24 ± 4.67^{a}	0.395 ± 0.409^{a}	1.36 ± 1.71^{a}	3.24 ± 3.01^{a}

n: number of samples; GM_{SG}: geometric mean concentration adjusted by specific gravity; SD: standard deviation; different letters (a and b) within a column indicate significant differences (Student's T-Test; $P<0.05$)

Table 3. Sex differences in urinary excretion (*n*g/m*l*) of conjugated pyrene metabolites

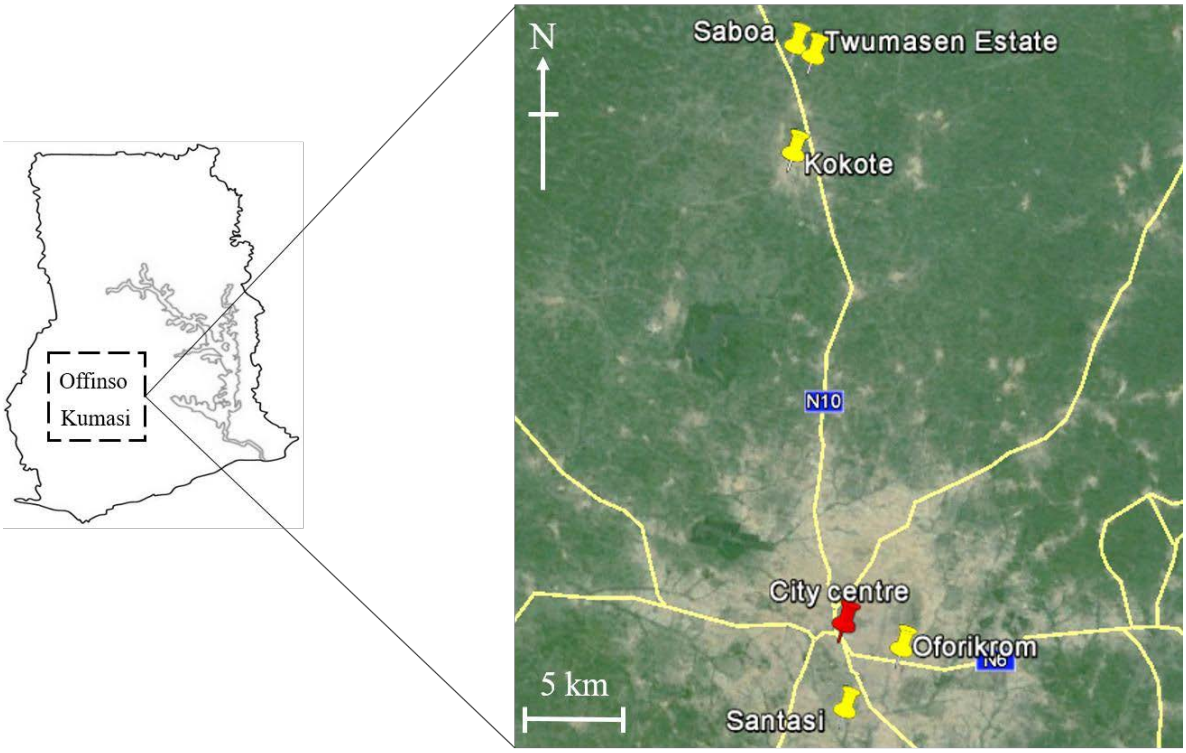
Sex		n	PyG	PyS	PydiolS (M2)	PydiolS (M3)
Male	$GM_{SG} \pm SD$	30	4.16 ± 3.72^{a}	0.545 ± 0.535^{a}	1.00 ± 1.17^{a}	2.71 ± 2.74^{a}
Female	$GM_{SG} \pm SD$	65	4.07 ± 4.76^{a}	0.378 ± 0.363^{a}	1.37 ± 1.86^{a}	3.31 ± 3.02^{a}

n: number of samples; GM_{SG}: geometric mean concentration adjusted by specific gravity; SD: standard deviation; different letters (a and b) within a column indicate significant differences (Student's T-Test; $P<0.05$)

Table 4. Pearson's correlation of 1-OHPyr and conjugated pyrene metabolites in cattle urine

Variables	PyG	PyS	PydiolS (M2)	PydiolS (M3)	∑Conj Pyr met	1-OHPyr	∑Pyrene met
PyG	1						
PyS	-0.0804	1					
PydiolS (M2)	0.0520	-0.169	1				
PydiolS (M3)	0.340**	0.133	0.186	1			
∑Conj Pyr met	0.796**	-0.0073	0.398**	0.695**	1		
1-OHPyr	-0.181	-0.205	0.256*	-0.135	-0.0717	1	
∑Pyrene met	0.768**	-0.0445	0.436**	0.678**	0.986**	0.0747	1

1-OHPyr: 1-hydroxy pyrene; PyG: 1-hydroxy pyrene glucuronide; PyS: 1-hydroxy pyrene sulfate; PydiolS (M2): pyrenediol sulfate (M2); PydiolS (M3): pyrenediol sulfate (M3); ∑Conjugated Pyr: sum of conjugated pyrene metabolites; ∑Pyrene met: sum of conjugated and deconjugated pyrene metabolites; *: $P<0.05$; **: $P<0.01$


Offinso
Kumasi
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Saboa
Twumasen Estate
Kokote
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City centre
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Fig. 1.