JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

1-Hydroxypyrene in Milk and Urine as a Bioindicator of Polycyclic Aromatic Hydrocarbon Exposure of Ruminants

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Urinary 1-hydroxypyrene (1-OH-pyrene) is now largely considered to be a valuable biomarker of exposure of man and animals to pyrene and other polycyclic aromatic hydrocarbons (PAHs). However, from a practical and agronomic standpoint, the question remains whether such biomarking capability still holds when 1-OH-pyrene is analyzed in milk produced by ruminants. To assess this hypothesis, four goats were daily submitted to three different amounts of pyrene oral ingestion, together with phenanthrene and benzo(*a*)pyrene (1, 7, and 49 mg/day during 1 week each). An HPLC−fluorometric analysis of 1-OH-pyrene in milk revealed a perfect correlation between pyrene doses and 1-OH-pyrene detected in milk, thus fully confirming the biomarking capability of 1-OH-pyrene and providing information on its transfer coefficient toward milk. Transfer equations such as the ones found in the present study could be used as a valuable and practical risk assessment tool in (i) the accurate monitoring of exposure of ruminants to pyrene and (ii) the evaluation of occupational and environmental exposure of ruminants to PAH mixtures.

KEYWORDS: Polycyclic aromatic hydrocarbons; 1-hydroxypyrene; biomarker of exposure; ruminant; milk

INTRODUCTION

Among environmental and health issues, polycyclic aromatic hydrocarbons (PAHs) and their metabolites are the subject of numerous studies in terms of production (*1*), spatial deposition (*2–4*), and impact on health (*5, 6*), as well as transfers in native or metabolized forms (*7, 8*). A significant environmental persistence of PAHs in crop soils amended with sewage sludges was also observed over a 25-year record, which also clearly demonstrated the environmental persistence of PAHs (*9*). PAHs belong to the group of persistent organic pollutants (POPs), which, according to the Protocol on Persistent Organic Pollutants signed on June 24, 1998, in Denmark (*10*), fulfill the five following points: (i) they possess toxic characteristics; (ii) they are persistent; (iii) they can bioaccumulate; (iv) they are prone to long-range transboundary atmospheric transport and deposition; and (v) they are likely to cause significant adverse human health or environmental effects near to and distant from their source. PAHs are a group of over 100 different chemicals that are formed during the incomplete burning of coal, oil, and gas, garbage, or other organic substances such as tobacco or charbroiled meat. PAHs are usually found as a mixture containing two or more of these compounds, such as soot. Considering their toxicity and presence in our environment, the U.S. Environmental Protection Agency (EPA) has identified 16 unsubstituted PAHs as priority pollutants, these being approximately 80% of the PAHs found in the United States. Besides quantitative considerations, the physical−chemical properties and danger of PAHs is highly related to their number of cycles (2−6 cycles for the 16 priority pollutants). Considered as hydrophobic molecules, their log K_{ow} ranges from 3.4 with naphthalene to 6.5 with benzo(*g*,*h*,*i*)perylene (*11*), which means that benzo(*g*,*h*,*i*)perylene is a 1000-fold more hydrophobic molecule than naphthalene. With regard to health aspects, PAHs such as benzo[*a*]anthracene and benzo(*a*)pyrene (respectively, 4 and 5 cycles) have, for instance, been classified as highly toxic, confirmed to be carcinogenic, and observed mutagenic components, whereas phenanthrene and anthracene have been identified as "only" moderately toxic and observed as mutagenic compounds (*12*).

Next to the intensive studies of native PAHs focusing on their production, atmospheric transport, deposition, transfer, stability, or toxicity, PAH metabolites have also been considered with an increased interest over the past decade. Indeed, PAHs are absorbed inside the body through the skin but can also be absorbed through the lung and the gastrointestinal tract before being metabolized inside the liver. There, they are converted into monohydroxylated PAHs and may be subsequently conjugated with glucuronides and sulfates before being almost fully

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10.1021/jf072757g CCC: $40.75
Published on Web 02/14/2008

eliminated in urine or bile. Most studies attempt to answer the following questions: What is the rate of PAH conversion into metabolites? What is the fraction of PAHs converted into metabolites and in which form? Are they simply hydroxylated or also glucuronoconjugated or sulfoconjugated and, if so, in which proportions depending of the metabolites? What is their toxicity at the cellular, organic, and body scale? Can they reflect the level of global exposure to PAHs of human beings and animals (food, respiration, absorption through the skin). That is, could they be used as relevant exposure bioindicators of a global exposure to PAHs? The latter question has recently been investigated by several authors who analyzed metabolite concentrations in animal or human urine and fish bile mainly. With regard to human exposure, Huang et al. (*13*) analyzed 1-hydroxypyrene (1-OH-pyrene) in 2312 urine samples and used results to establish the first U.S. reference range of OHPAH as biomarkers to assess human exposure. But 1-OH-pyrene was also demonstrated to be a potential biomarker of exposure to PAHs of workers in an artificial shooting target factory (*14*), in the petrochemical industry (*15*), in a carbon anode plant (*16*), in a coke oven (*17*), and also in an aluminum production plan (*18*). Multimetabolite monitoring in the urine of smokers and nonsmokers was also performed by Chetiyanukornkul et al. (*19*), who recently developed an excellent analytical method for the analysis of up to 10 different metabolites in a single HPLC−fluorometric run. In this last valuable study, 10 metabolites from 2 cycles up to 4 cycles were accurately quantified, thus allowing the multimetabolite-based evaluation of global exposure to PAHs, instead of a single-metabolite (1-OH-pyrene) based evaluation.

With regard to animal exposure, the urinary excretion kinetics of 1-hydroxypyrene following intravenous or oral administration of PAHs in rat was seriously investigated (*20–24*). 1-OH-pyrene was also, for instance, successfully analyzed in fish bile as a bioindicator of PAH exposure of *Solea solea* fish after the Erika oil spill (*25*). Ferrari et al. (*26*) analyzed 1-OH-pyrene in bovine urine samples and observed a significant increase of concentrations when bovines were living close to a highway.

At the moment, there is a real lack of information on the possibility of using the analysis of PAH metabolites inside ruminant milk to evaluate their global level of exposure to PAHs. Milk samples would, however, be much more convenient to collect than urine samples for subsequent PAH metabolites analysis. Grova et al. (*27*) were the first to detect PAH metabolites in the milk of lactating goats, thus demonstrating that the detection of native molecules in milk is not representative of the real contamination of this biological matrix. As mentioned by the latter authors, in terms of food safety, knowledge of PAH metabolite levels in food is of real interest since these metabolites appear to be more reactive than their parent compounds in terms of mutagenesis and carcinogenesis (*28, 29*) or endocrine disruption (*30, 31*).

The purpose of the present study was therefore to evaluate the potential of 1-OH-pyrene in milk and urine to be used as a relevant bioindicator of PAH exposure of lactating goats submitted to three different levels of PAH chronic oral uptake of phenanthrene, the most often found PAH under metabolite form in milk (*27*), pyrene, the usual indicator of environmental PAH contamination according to ref *32*, and benzo(*a*)pyrene, the reference PAH in terms of toxicity according to the EPA.

MATERIALS AND METHODS

Animals. Four alpine goats (50 ± 5 kg; second and third lactations, second month postpartum) from the herd of the "Domaine Experimental de la Bouzule" (Champenoux, France) were used for this experiment as a model of the lactating ruminant. They received a 7 day adaptation period within individual boxes at an average day temperature of 22 °C and under natural light conditions. The animals were mechanically milked twice a day, providing an average milk production of 2000 ± 200 mL during the 7 days before the experiment was run. The goats were fed with meadow hay, water, and mineral salt ad libitum and received 850 g of granulated mix consisting of dehydrated maize, alfalfa, wheat, sunflower grains, sugar beet, colza and soybean cattle cakes mixed with palm oil, sugar beet molasses, and other organic additives and specific salts. This diet met the nutritional requirements of goats and has already been proven to be sufficient to maintain milk production at about 3 L/day over three milkings (*33*). Measurements of the milk production were performed each day as well as renewal of the litters.

Experimental Design. The experimental design involved the oral and daily intake of three different amounts of PAHs (1, 7, and 49 mg), each level being applied during a 7 day period. The animal protocol was in accordance with the general directive 86/609/EEC on animal care used in the European Community (*34*). After 1 week of adaptation period, and just after the morning milking, each animal received 2 mL of cremophor oil (Fluka) containing 1 mg of pyrene, 1 mg of phenanthrene, and 1 mg of benzo(*a*)pyrene (0.02 mg/kg of body weight) for 7 consecutive days. The 7 mg dose was applied for 7 successive days and the 49 mg dose for a further 7 successive days. PAHs were directly administrated into the mouth of the animal with a syringe. The latter was then flushed once with another 2 mL of cremophor oil, and the contaminated oil was given to the animals. It was previously demonstrated that this amount of ingested oil did not affect the rumen activity (*35*). Urine samples and milk samples (the last two milkings) from the 24 h before each increase of dose (every 7 days) were collected and stored at −20 °C.

1-OH-pyrene Extraction in Milk. Due to the complex composition of milk, which contains approximately 49, 39, and 33 g/L of lactose, fat, and proteins, respectively, the extraction of 1-OH-pyrene required both liquid/liquid and liquid/solid separation processes. Extractions were performed in triplicate for pyrene, phenanthrene, and benzo(*a*)pyrene metabolites but, for technical and priority reasons, only 1-OH-pyrene was dosed as described below. The procedure used was the one designed in our laboratory by Grova et al. (*27*). Ten milliliter milk samples were collected in 50 mL Falcon tubes (Greiner bio-one, Frickenhausen, Germany), adjusted at pH 5.2 with glacial acetic acid (Eastman, Kingsport, TN), and complemented with 50 μL of β-glucuronidase and sulfatase solutions, respectively. β-Glucuronidase type H-2 from *Helix pomatia* was provided in crude solution by Sigma-Aldrich (Poole, U.K.) with a volume activity of 99000 units/mL, where 1 unit is the amount of glucuronidase that can liberate 1 μg of phenolphthalein from phenolphthalein glucuronide at 37 °C and pH 5 in 30 min. Sulfatase type H-2 from *H. pomatia* was provided in crude solution by Sigma-Aldrich (Poole, U.K.) with a volume activity of 2000 units/mL, where 1 unit is the amount of sulfatase that can hydrolyze 1 μmol of *p*-nitrocatechol sulfate per hour at pH 5 and at 37 °C. Milk samples were then incubated at 37 °C during 16 h to convert glucuronide and sulfate conjugates of 1-OH-pyrene into 1-OH-pyrene. Internal standard 6-OH-chrysene was then added to the samples, which were subsequently mixed in 20 mL of cyclohexane (Sigma-Aldrich, Schnelldorf, Germany) and 20 mL of ethyl acetate (Fluka, Saint-Quentin Fallavier, France) before being thoroughly shaken with a horizontal Agitest shaker (CAT, Staufen, Germany) at a maximum speed of 700 shakings per minute during 30 min at ambient temperature. After centrifugation (15 min at 1000*g*) in a Jouan CR4I centrifuge (Chateau Gontier, France), the supernatant was evaporated with a Buchi RE121 rotovapor (Flawil, Switzerland) under automated vacuum control. Residual volume was recovered with 6 mL of cyclohexane and applied onto an Envi-Chrom P SPE columm (Supelco, Bellefonte, PA) previously conditioned with 5 mL of ultrapure water, 5 mL of methanol (Prolabo, Leuven, Belgium), and 5 mL of cyclohexane. After elution of interfering compounds with 2 × 3 mL of cyclohexane, pyrene and 1-OH -pyrene were coeluted with 12 mL of cyclohexane/ethyl acetate (50:50, v/v). The samples were totally evaporated to dryness at 40 °C using a nitrogen flow and a multichannel evaporator (Liebisch, Bielefeld, Germany). The dry

residue was then dissolved in 2 mL of cyclohexane and 2 mL of methanol–water (80/20; v/v), vortexed for at least 10 s, and centrifuged at 1500*g* and ambient temperature during 5 min; the two phases were then separated with a Pasteur pipet. This step, which was repeated once, allowed the separation of pyrene and fatty acids (cyclohexane fraction) from 1-OH-pyrene and 6-OH-chrysene metabolites (methanol/water fraction). The methanol/water fraction was collected and totally evaporated to dryness at 40 °C under nitrogen flow before being redissolved in 4 mL of ethyl acetate/water solution (50:50, v/v) and then centrifuged at 1500*g* and ambient temperature during 5 min. Forty microliters of 1000 ng/mL chrysene-d_{12} as an external standard (Cluzeau Synthe Labo, Paris, France) was then added to the supernatant. Extracts were finally evaporated to dryness and redissolved into 250 μL of acetonitrile before HPLC−fluorometry analysis.

1-OH-pyrene Extraction in Urine. Samples were treated using the method described by Wang et al. (*36*). A 5 mL urine sample was transferred to a 50 mL flask (Greiner bio-one). The pH of the solution was adjusted to 5.0 with 1 M HCl, and then 5 mL of 0.5 M acetate buffer (pH 5.0) was added and complemented with 50 μL of β-glucuronidase and sulfatase solutions, respectively, before incubation at 37 °C during 16 h. Internal standard 6-OH-chrysene was then added to the samples. After hydrolysis, samples were centrifuged at 1500*g* for 3 min in a Jouan CR41 centrifuge, and supernatant was used for solid phase extraction. C_{18} cartridges were pretreated with 5 mL of methanol and 5 mL of ultrapure water. The hydrolyzed urine sample was then loaded onto the cartridge at a flow rate lower than 1 mL/min. The column was washed with 10 mL of water and 10 mL of methanol/water (30:70, v/v) to remove the matrix interferences. The purity of methanol was 99.8%. 1-OH-pyrene and internal standard were eluted with 4 mL of methanol. The eluate was totally evaporated to dryness at 40 °C using a gentle flow of nitrogen and multichannel evaporator (Liebisch, Bielefeld, Germany). The dry residue was then dissolved in 1 mL of methanol and vortexed for at least 10 s. The solution was filtered through a 0.2 μm filter, and 40 μL of external standard chrysen-d_{12} (Cluzeau Synthe Labo, Paris, France) was finally added before storage at −20 °C and subsequent HPLC−fluorometry analysis. Before injection, the extracts must be diluted at 50:50 (v/v) with a 10 mM acetate buffer solution at pH 4.

HPLC Analysis in Milk. The HPLC system consisted of an autosampler 717+ programmed for 100 μL of volume injection, a gradient controller 600 with column temperature control, and a 2475 multiwavelength fluorescence detector (all from Waters, Milford, MA). 1-OH-pyrene, internal standard 6-OH-chrysene, and external standard chrysene-d_{12} were separated on a reverse phase C_{18} column (250 × 4.6 mm i.d., 5 μm, Grace-Vydac, Hesperia, CA) with a guard column (C_{18}, 20 × 4 mm i.d., 5 μm, Grace-Vydac). Based on a method recently designed in our laboratory, HPLC conditions were as follows: eluent A, 4 mg/L ascorbic acid solution (daily prepared and stored in brown glass bottle); eluent B, methanol; gradient program at constant temperature (35 °C) and flow rate (0.8 mL/min), 0–21 min (eluent B composition, 65%), 21–22 min (B, 65–100%), 22–37 min (B, 100%), 37–38 min (B, 100–65%); detection wavelength program (excitation/emission in nm), 0–21 min (346/400) for 1-OH-pyrene and 6-OH-chrysene, 22–53 min (270–378) for chrysene-d_{12}. After the end of the washing step (38 min), the initial mobile phase conditions were kept from 15 to 53 min before the next sample was injected.

HPLC Analysis in Urine. The analysis was carried out using a method different from the one used with milk. On the basis of the recent study of Chetiyanoukornkul et al. (*10*), we recently developed a method for the simultaneous analysis of 1-, 2-, 3-, and 4-OH-phenanthrene, 1-OH-pyrene, and 3-OH-benzo(*a*)pyrene. In the present paper only quantitative results about 1-OH-pyrene will be introduced for comparison with results found in milk. The autosampler, gradient controller, and fluorescence detector were similar to the ones used for analysis in milk. 1-OH-pyrene (and other metabolites), internal standard 6-OH-chrysene, and external standard chrysene-d_{12} were separated on a Discovery reverse phase amide C_{16} column (250 × 4.6 mm i.d., 5 μm, Supelco) with a guard column (C_{16}, 20 × 4 mm i.d., 5 μm, Supelco). HPLC conditions were as follows: eluent A, acetonitrile (purity of 99.8%); eluent B, 10 mM acetate buffer at pH 4 (daily prepared and stored in brown glass bottle); gradient program at constant temperature (40 °C) and flow rate (1 mL/min), 0–25 min (eluent A composition, 45%), 25–50 min (A, 45–65%), 50–60 min (A, 65%), 60–63 min (A, 65–90%), 63–68 min (A, 90%), 68–71 min (A, 90–45%). 71–86 min (A, 45%); detection wavelength program (excitation/emission in nm), 0–35 min (256–370) for 1-, 2-, 3-, and 4-OH-phenanthrene, 35–46.5 min (240–387) for 1-OH-pyrene, 46.5–56 min (270–378) for internal standard 6-OH-chrysene and external standard chrysene-d_{12}, over 56 min (375–435) for 3-OH-benzo(*a*)pyrene. Injection volume was 100 μL. After the end of the washing step (71 min), the initial mobile phase conditions were kept from 15 to 86 min before the next sample was injected.

Quantification Limits. Briefly, the calculation of the QL value for 1-OH-pyrene was based on the former calculation of a response factor *R* by taking the slope of the standard curve obtained with five different standard solutions of 1-OH-pyrene at, respectively, 10, 20, 30, 40, and 50 ng/mL. *R* was taken as the inverse of the slope of the standard curve peak height versus amount of 1-OH -pyrene injected. For instance, in the HPLC method used with urine, these amounts were, respectively, 1, 2, 3, 4, and 5 ng because the injection volume was 100 μL. After definition of the h_{av} and h_{max} on both sides of the 1-OH-pyrene peak, we could calculate two different QL values for 1-OH-pyrene: $QL_1 = 10R \times h_{av} = 0.017$ ng and $QL_2 = 10R \times h_{max} = 0.043$ ng. Choosing the method based on h_{max} logically provided the highest QL value. Taking the average of these two QL values, we obtained an estimated QL of 0.03 ng for 1-OH-pyrene, which was largely sufficient given the amounts of pyrene used in our experimental design.

RESULTS AND DISCUSSION

HPLC Analysis, Calibration Curve, Extraction Yield, and Quantification Limits. Panels **a** and **b** of **Figure 1**, respectively, show typical milk and urine extract chromatograms. Our six-point standard curve in milk showed an excellent linearity ($R^2 = 0.99$) in the range of 0–400 ng of 1-OH-pyrene/g of milk, which was investigated. Our five-point standard curve in urine also showed a good linearity ($R^2 = 0.99$ in the range of 0–1000 ng of 1-OH-pyrene/g of urine. In addition, on the basis of the use of our external and internal standards, we obtained an average extraction yield of 35.1 ± 10.2% within the range 23.9−53.9% for 1-OH-pyrene in milk, which is acceptable given the complexity of the milk composition (caseins, fat compounds, lactose, etc.). On the basis of a specific low-concentration standard curve with five points in the range of 0–0.1 ng/g and a R^2 of 0.99, we found a noise concentration of 0.12 ng of 1-OH-pyrene/g of milk.

1-OH-pyrene in Milk. Figure 2 presents the correlation between the doses of pyrene ingested and the pyrene concentration in the milk volume produced 24 h after the last ingestion of each dose (two milking volumes). To take into account the variations in milk volumes produced for each dose (1, 7, and 49 mg/day), **Figure 3** presents the correlation between the doses of pyrene ingested and the 1-OH-pyrene mass amount found in the milk volume produced 24 h after the last ingestion of a pyrene dose (two milking volumes). **Table 1** provides details on percent transfer of pyrene under 1-OH -pyrene form in the milk for each of the three doses tested as well as goat to goat variations in 1-OH-pyrene concentration in milk and milk volumes. From these data several interesting points deserve to be discussed. Of course, not surprisingly, we observe an increase of 1-OH-pyrene concentration in milk when ingested doses increase. However, when considering the average transfer coefficients, we can see that the latter are not significantly different whatever the pyrene daily doses used (1.10 ± 0.55, 1.26 ± 0.57, and 1.23 ± 0.57% for 1, 7, and 49 mg/day, respectively). Therefore, it is obvious that transfer coefficients, even with large differences between ingested doses of pyrene, are not at all affected by these differences. However, the key

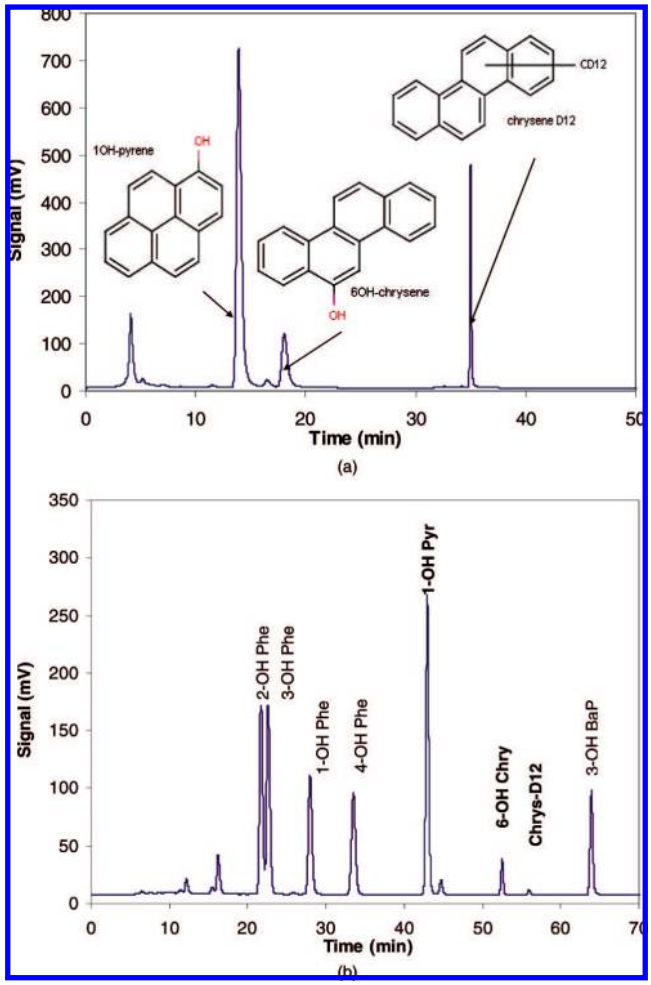

Figure 1. HPLC−fluorometry chromatograms of milk extracted from milk at 50 ng of 1-OH-pyrene/g of milk (**a**) and urine extracted from urine at 1000 ng of 1-OH-pyrene/g of urine (**b**).

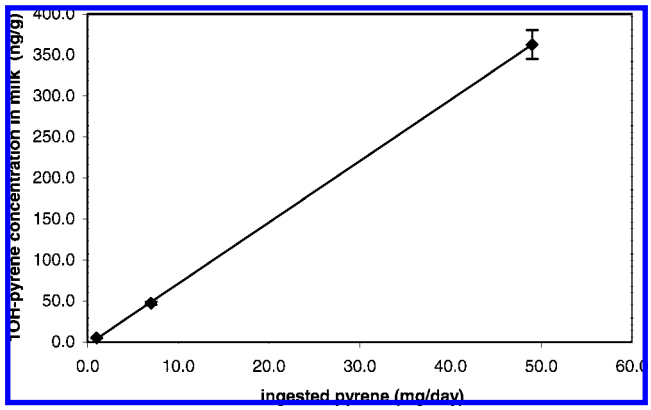

Figure 2. Average 1-OH-pyrene concentrations (ng/g) and their associated standard deviation in milk produced 24 h after the last ingestion of a 7 day period at 1 mg of pyrene/day followed by a 7 day period at 7 mg of pyrene/day and finally a 7 day period at 49 mg of pyrene/day (concentration = 7.47 × ingested dose − 3.59, $R^2 = 0.99$). Averages are based on the milking of four goats.

point in the present study is that the correlations between doses and concentration as well as mass amounts of 1-OH-pyrene in the milk are excellent as can be seen from R^2 values. Logically, the correlation is better when the mass amount of 1-OH-pyrene is considered instead of the concentration of 1-OH-pyrene. However, the latter difference in correlation is extremely low because we did not observe significant variations in goat to goat

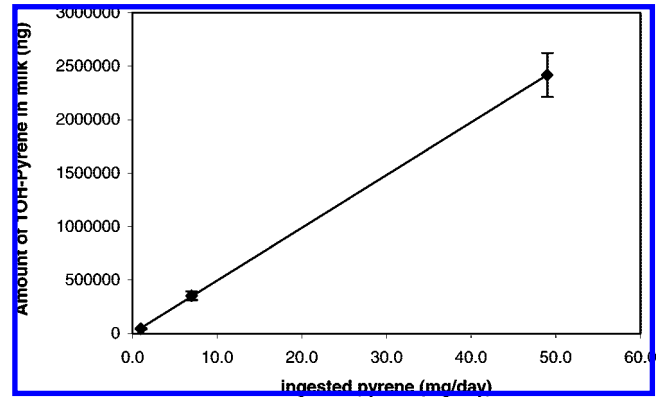

Figure 3. Average 1-OH-pyrene amounts (ng) and their associated standard deviation found in milk produced 24 h after the last ingestion of a 7 day period at 1 mg og pyrene/day followed by a 7 day period at 7 mg of pyrene/day and finally a 7 day period at 49 mg of pyrene/day (amount = 49345 × ingested dose + 1170.7, $R^2 = 1$). Averages are based on the milking of four goats.

and dose to dose milking volumes (**Table 1**). Consequently, in a herd of goats (and probably other ruminants), it can be concluded that the monitoring of 1-OH-pyrene in milk, when used together with one or another of the two transfer equations that we experimentally generated (in urine or milk), should properly reflect the level of chronic exposure of goats to pyrene and other PAHs. However, it is obvious, here, that the transfer equation of pyrene under the 1-OH-pyrene form into milk or urine should be designed with and for much lower daily intakes of pyrene in order to increase the accuracy in the evaluation of exposure to pyrene and other daily ingested PAHs. For instance, if we enter the "natural" 1-OH-pyrene concentration in milk (0.12 ng/g) into our transfer equation from **Figure 2**, we will find a negative value that is, of course, not providing any information on the "natural" and, however truly existing, daily dietary intake of pyrene by the goat. Along with the good correlations that were observed, it should also be stressed here that, in terms of sampling, the analysis of 1-OH-pyrene in milk is much easier to perform than in urine. Naturally, the question of whether metabolism of pyrene into 1-OH-pyrene can be affected when pyrene is ingested together with other PAHs, such as phenanthrene and benzo(*a*)pyrene, should be discussed. Indeed, according to Bouchard et al. (*20*) various individual PAHs are likely to be biotransformed via the same cytochrome P450 oxidase system, thus generating potential competition for accessing to the metabolism pathway (hydroxylation, glucuronide, and sulfate conjugates synthesis). On the basis of binary and ternary mixtures of PAHs intravenously administered to Sprague–Dawley rats, they demonstrated that sufficiently high doses of benzo(*a*)pyrene could generate a significant increase of 1-OH-pyrene metabolism resulting in higher urinary concentrations. Such an observation, which precluded the use of 1-OH-pyrene as a relevant bioindicator of exposure to PAHs, was, however, true for only benzo(*a*)pyrene doses that were not in accordance with realistic levels of exposure. In the present study, it is clear that even the high amounts of ingested phenanthrene and benzo(*a*)pyrene did not affect at all the metabolism of pyrene into 1-OH -pyrene because we observed a perfect linearity between the amounts of ingested pyrene and 1-OH-pyrene concentration in the milk. This observation confirms that analysis of 1-OH-pyrene in milk is a performing, practical, and robust way to evaluate the level of exposure of goats (and likely other ruminants) to PAHs.

Table 1. 1-Hydroxypyrene Concentrations in Milk and Percent Recovery of Ingested Pyrene under the 1-Hydroxypyrene Form with Their Associated Standard Deviations

sample								
dose/day pyrene (mg)	goat	av 1-OH-pyrene[a] [(ng/g)/goat]	variation (%)	av/dose[b]	variation (%)	milk[c] (g)	% recovery in milk[d]	av transfer (%)
1	1	5.5 ± 0.4	6.8	5.2 ± 0.1	2.9	1696	0.93	1.10 ± 0.55
	2	2.7 ± 0.4	15.2			1703	0.46	
	3	7.6 ± 0.2	2.7			2364	1.79	
	4	4.9 ± 0.2	3.4			2484	1.22	
7	1	65.0 ± 4.5	6.9	47.2 ± 1.9	4.0	1523	1.41	1.26 ± 0.57
	2	36.1 ± 2.1	5.8			1873	0.97	
	3	68.2 ± 5.0	7.3			2042	1.99	
	4	19.6 ± 2.9	14.9			2460	0.69	
49	1	568.0 ± 59.4	10.5	362.7 ± 17.8	4.9	1200	1.39	1.23 ± 0.42
	2	228.2 ± 3.8	1.7			1521	0.71	
	3	418.5 ± 32.4	7.7			1988	1.70	
	4	235.9 ± 43.5	18.5			2363	1.14	

[a] Average based on three extractions on the same milk sample. [b] Standard deviation derived from rules in error propagation in arithmetic calculations. [c] Milk produced during the 24 h (three last milkings) before the increase of dose. [d] Percent recovery (or transfer coefficient) into milk of ingested pyrene under the 1-OH-pyrene form 24 h after the last ingestion of a dose calculated as [milk (in g) × average 1-OH-pyrene concentration in milk (in ng/g)] × 100/daily intake (converted in ng).

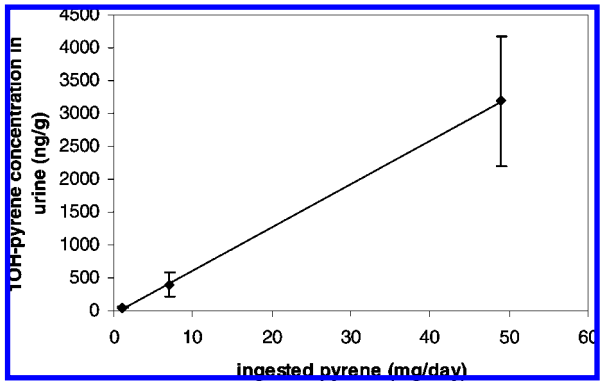

Figure 4. Average 1-OH-pyrene concentrations (ng/g) and their associated standard deviation in urine produced 24 h after the last ingestion of a 7 day period at 1 mg of pyrene/day followed by a 7 day period at 7 mg of pyrene/day and finally a 7 day period at 49 mg of pyrene/day (concentration = 65.684 × ingested dose − 36.395, R^2 = 0.99). Averages are based on the milking of four goats.

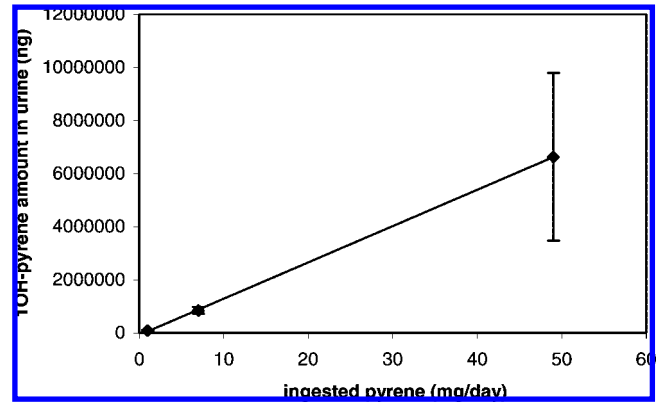

Figure 5. Average 1-OH-pyrene amounts (ng) and their associated standard deviation found in urine produced 24 h after the last ingestion of a 7 day period at 1 mg of pyrene/day followed by a 7 day period at 7 mg of pyrene/day and finally a 7 day period at 49 mg of pyrene/day (amount= 136752 × ingested dose − 76264, R^2 = 1). Averages are based on the milking of four goats.

1-OH-pyrene in Urine. Figure 4 presents the correlation between the doses of pyrene ingested and the pyrene concentration in the urine volume produced 24 h after the last ingestion of a pyrene dose. To take into account the variations in urine volumes produced for each dose (1, 7, and 49 mg/day), **Figure 5** presents the correlation between the doses of pyrene ingested and the 1-OH-pyrene mass amount found in the urine volume produced 24 h after the last ingestion of a pyrene dose. **Table 2** provides details on percent transfer of pyrene under 1-OH-pyrene form in the urine for each of the three doses tested as well as goat to goat variations in 1-OH-pyrene concentration in urine and urine volumes. First of all, the excellent linearity observed in **Figures 4** and **5** clearly demonstrates the potential of 1-OH-pyrene to be used as a good bioindicator of exposure of goat, and probably other ruminants, to pyrene. Another important observation is that the percent transfer of ingested pyrene toward urine under the 1-OH-pyrene form was found to be about 10-fold higher than the one observed in milk, which is in accordance with the fact that urine is a major elimination pathway for xenobiotics. However, when the percent transfers of pyrene under the 1-OH-form into milk and urine are summed, not more than 15% transfer is found into these two compartments. Given the fact that it was recently shown that, even 24 h after a 100 mg pyrene ingestion by goats, the transfer of pyrene under native form was <0.01% into milk and <0.05% into urine (*7*), it can reasonably be assumed that feces is by far the main elimination pathway for pyrene (nonabsorbed or absorbed and eliminated via the bile) and its metabolites, such as 1-OH-pyrene. It can also be reasonably assumed that other metabolites of pyrene, different from 1-OH-pyrene, represent a major source of pyrene metabolites as compared to 1-OH-pyrene. Whereas the first assumption is actually generally recognized in the literature, the second assumption can be supported by the recent studies of Ruzgyte et al. (*37, 38*). These authors recently showed that about 27% of pyrene intravenously injected into Sprague–Dawley rats was metabolized under the pyrene-1,6-dione and pyrene-1,8-dione forms against about 1% only under the 1-OH-pyrene form (analysis in urine over a 48 h sampling period). Finally, as observed with milk, no significant differences in percent transfer of pyrene as 1-OH-pyrene into urine were observed when ingested doses increased from 1 to 7 to 49 mg/day. We do not observe any plateau curve (which would reflect a

Table 2. 1-Hydroxypyrene Concentrations in Urine and Percent Recovery of Ingested Pyrene under the 1-Hydroxypyrene Form with Their Associated Standard Deviations

sample							
dose/day pyrene (mg)	goat	av 1-OH-pyrene [(ng/g)/goat]	av/dose	variation (%)	urine[a] (g)	% recovery in urine[b]	av transfer (%)
1	1	32.2	47.9 ± 11.4	23.8	3407	10.9	8.9 ± 2.6
	2	51.8			986	5.1	
	3	59.1			1715	10.1	
	4	48.7			1929	9.4	
7	1	245.8	402.7 ± 185.48	46.1	2910	10.2	12.1 ± 1.8
	2	572.6			1499	12.3	
	3	552.5			1759	13.9	
	4	237.5			871	3.0	
49	1	2365.0	3184.8 ± 982.9	30.9	4690	22.6	13.5 ± 6.4
	2	3776.8			1020	7.9	
	3	4260.4			1520	13.2	
	4	2336.9			2180	10.4	

[a] Urine produced during the 24 h before the increase of dose. [b] Percent recovery (or transfer coefficient) into urine of ingested pyrene under the 1-OH-pyrene form 24 h after the last ingestion of a dose calculated as [urine (in g) × average 1-OH-pyrene concentration in urine (in ng/g)] × 100/daily intake (converted in ng).

saturation in metabolism capabilities of goats) or exponential type curves (which would reflect a progressive increase of adaptation in metabolism capabilities of goats). As seen with milk, we simply observe a linear relationship between ingested dose and 1-OH-pyrene amounts found in urine. From a metabolic standpoint, it remains difficult to know whether this linear relationship (observed in milk and urine) reflects or not a preexisting sufficient metabolic machinery or an induced metabolic machinery. Further investigations on cytochrome P450 oxidase induction might provide a definitive answer to such a question.

From a general standpoint, it is now more than 20 years that urinary 1-OH-pyrene has been suggested to be a good indicator of exposure to pyrene and other PAHs because it is always present in PAH mixtures and is a ubiquitous PAH (*39–41*). Jongeneelen notes that the conclusion of the first international workshop on 1-OH-pyrene in 1993 was that urinary 1-OH-pyrene is a solid biological exposure indicator of PAH (*41*). Actually, whereas 700 papers reporting on the urinary concentrations of 1-OH-pyrene in workers' urines have been published (*42*), fewer than 50 papers related to PAHs in milk can be found in the literature (personal search on SCOPUS database, 2007), of which only 4 are aimed toward PAH metabolites in milk (*7, 27, 33, 43*). The evidence that PAH metabolites can be transferred to milk has only been recently demonstrated (*27*). From an agronomic and food safety standpoint, this lack of information on metabolites in milk and more precisely in ruminant milk is somewhat surprising because milk is a highly consumed animal product, in a natural or processed form (concentrate, cheese, yogurt, powder, etc.) and may, in our opinion, represent an issue in terms of "mother to child" transfer of lipophilic persistent organic pollutants such as PAH and their metabolites.

In this context, the present work clearly demonstrates that 1-OH -pyrene in milk could be used as a reliable and convenient agronomic bioindicator of exposure to pyrene and should therefore be considered with a clear interest as a bioindicator of global exposure of ruminants to PAHs. It is therefore our expectation that transfer equations such as the one identified in this study will be used as a valuable and practical risk assessment tool for (i) the accurate monitoring of global exposure of ruminants to pyrene and (ii) the evaluation of occupational and environmental exposure of ruminants to PAH mixtures. A similar approach based on more realistic daily ingested PAHs doses will be necessary to achieve a more reliable level of bioindication accuracy at low exposure levels.

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Received for review September 16, 2007. Revised manuscript received January 7, 2008. Accepted January 9, 2008.

JF072757G