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Effect of Exposure to Soil-Bound Polycyclic Aromatic Hydrocarbons on Milk Contaminations of Parent Compounds and Their Monohydroxylated Metabolites

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The aim of this study was to determine the transfer kinetics of soil-bound polycyclic aromatic hydrocarbons to milk in lactating cows. Soil (500 g/day) fortified with fluorene (104 μg/g dry soil), phenanthrene (82 μg/g), pyrene (78 μg/g), and benzo[*a*]pyrene (33 μg/g) was administered to three dairy cows via a rumen cannulas for 28 consecutive days. Parent compounds and their major metabolites in milk were measured using gas chromatography−mass spectrometry. Secretion of parent compounds in milk did not increase significantly ($P > 0.05$) over the control values measured before supply. Target monohydroxylated metabolites were not detected in control samples, but 2-hydroxy fluorene, 3-hydroxy phenanthrene, and 1-hydroxy pyrene were present in milk by the second day of dosing. The highest concentrations of metabolites in milk (31−39 ng/mL) were for 1-hydroxy pyrene at days 7 and 14 of dosing. The observed plateaus for 3-hydroxy phenanthrene and 2-hydroxy fluorene were lower (respectively, 0.69 and 2.79 ng/mL) but significantly increased in comparison to the control samples. Contrarily, 3-hydroxy benzo[*a*]pyrene was not detected in milk at any sampling time. These results suggested a notable metabolism of the parent compounds after their extraction from soil during the digestive transfer. Thus, the metabolization of fluorene and pyrene can lead to higher concentrations of metabolites than of parent compounds in milk. Despite the absence of a significant transfer of parent PAHs to milk, the appearance of metabolites raises the questions of their impact on human health.

KEYWORDS: PAH; metabolites; soil; cow milk; mass spectrometry

INTRODUCTION

Increasing occurrences of polycyclic aromatic hydrocarbon (PAH) pollutions in developed countries have attracted the attention of the scientific community. The carcinogenic risk of some PAHs is well-established, and ingestion of foods is considered as the principal source of human exposure (*1*, *2*). Thus, milk from lactating ruminants grazing near anthropogenic PAHs sources may provide significant human exposure to PAH. Indeed, cows may ingest up to 2 mg daily of these pollutants via roughage (*3*, *4*), especially on pastures close to highly frequented roads, where soil is much more heavily contaminated than grass (*5*−*7*).

The daily intake of soil by grazing dairy cows may reach 1.5 kg depending on the climatic zone, season, grass density, and herd size (*8*−*10*). Contamination levels up to 100 μg PAH/g of soil have been reported for 16 PAHs (*11*, *12*), so that PAH intake by cattle consuming highly contaminated soil can reach 50 mg daily if cattle were consuming highly contaminated soils. Few data are available on the transfer of soil-bound PAHs into milk of exposed dairy cows.

Indeed, the specificity of the digestive tract of ruminants enhances the question if bacterial degradation of feed in the rumen prior to intestinal digestion would modify the PAH release from the soil when compared to monogastric animals. Moreover, modified release, the enzymatic activity of the flora in the rumen, suggests that parent PAH molecules may be metabolized during transfer and prior to excretion in milk as shown for the flora in the soil (*6*).

Thus, the aim of this study was to answer two questions: first, to precisely conclude if soil-bound PAHs can be transferred into the milk of dairy cows. If there is transfer, second, we want to know in which form (parent or metabolized molecule) it will be.

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MATERIAL AND METHODS

Three lactating cows received 500 g of PAH-fortified soil via a ruminal cannula for a period of 28 days. The kinetics of parent PAHs and their major monohydroxylated metabolites in milk were established to assess their transfer.

Soil Preparation. Topsoil (20 kg) of permanent grassland was collected at our experimental farm La Bouzule (Champenoux) near Nancy, France (calcaric cambisoil, FAO classification; 2.8% organic matter; distribution of particle size: 34% clay, 53% silt, and 13% sand). After it was dried for 20 days at room temperature, i.e., 18−20 °C, the soil was crushed and sieved (at 5 mm) prior to fortification with PAHs. The soil contained initially less than 0.1 μg/g dry soil for each studied molecule. Soil samples of 500 g were placed after this preparation in individual jars to be fortified. In this way, target PAHs purchased from Sigma Aldrich (St. Quentin-Fallavier, France) were dissolved in acetonitrile (Carlo Erba, Val de Reuil, France) and homogenized, and then, 35 mL of this solution was added to each soil sample placed previously in the open jars. Thus, each soil sample was fortified with 85 mg of fluorene (Fluo), 72 mg of phenanthrene (Phe), 60 mg of pyrene (Pyr), and 25 mg of benzo[*a*]pyrene (B[*a*]P) in order to reach levels reported for highly contaminated soils (*11*−*13*). Deionized water was then added to bring the soil to 34% moisture (80% from field capacity). The prepared soil was aged for 30 days in the dark and at 20 °C to allow physical and chemical binding (*14*). During aging, the soil samples were weighed regularly to determine moisture content, and deionized water was added to maintain the moisture content at 34%. The aged soil was stored at −20 °C, and each sample was thawed 12 h before use.

Animals. Three Holstein cows used in this trial were permanently housed indoors to avoid any soil during grazing. Each cow was in the second half of the lactation cycle and yielded 15−20 kg of milk daily. The animals were fitted with ruminal cannulas (#3C; Bar Diamond; Parma, ID) at least 1 year before the trial according to the General Guidelines of the Council of the European Communities (1986, no. 86/609/CEE). Cows were fed a maize silage-based total mixed ration (maize silage, wheat straw, cracked wheat, soybean meal, and minerals) and received water ad libitum. The target PAHs in the diet (Fluo 1.8 ng/g DM, Phe 9.3 ng/g DM, Pyr 0.9 ng/g DM, and B[*a*]P not detected) and water (Fluo and B[a]P not detected, Phe 24 ng/L, and Pyr 16 ng/L) have been analyzed (method ISO 15302, *15*), and the found concentrations revealed a total daily supply of less than 1%. Each of three cows received daily 500 g of fortified soil via its rumen cannula every morning after milking and before feeding.

Sampling. A control sample of milk was collected from each cow 3 days before starting the daily administration of contaminated soil. Milk samples were then collected on days 2, 7, 14, 21, and 28 of the trial. All samples were taken at the evening and subsequent morning milking before being pooled within cow (50:50) and stored at −20°C. The milk yield was recorded at each milking (Isalait system 2045, Bou-Matic, France) and subsequently multiplied with PAH concentration to determine the total daily secretion of PAHs.

Analyses. *Soil.* PAH concentrations in soil samples prior and after contamination were checked using the ISO 15302 method (*15*).

Milk. Milk concentrations of parent PAHs (Fluo, Phe, Pyr, and B[*a*]P) and their major monohydroxylated metabolites, 2-hydroxyfluorene (2-OH Fluo), 3-hydroxyphenanthrene (3-OH Phe), 1-hydroxypyrene (1-OH Pyr), and 3-hydroxybenzo[*a*]pyrene (3-OH B[*a*]P), respectively, were determined as follows. Prior to extraction, deconjugation from sulfate was performed with 3750 units of purified *Helix pomatia* juice (Biosepra, Villeneuve la Garenne, France) in 10 mL of milk fortified with 100 μL of glacial acetic acid and an internal standard (d10-Phe, d10-Pyr, and d12-perylene, Interchim, Montluçon, France). Milk samples were incubated for 16 h at 37 °C to enable hydrolysis of glucuronide and sulfate conjugates of hydroxymetabolites. Milk samples were then agitated with 20 mL of cyclohexane/ethyl acetate (50:50, v/v; SDS, Peypin, France) for 30 min. After centrifugation (15 min at 1000*g*), the supernatant was evaporated. The residue was dissolved in 3 mL of cyclohexane and applied onto an Envi-Chrom P SPE (styrene−divinylbenzene copolymer resin, Envi Chrom P: 0.5 g) column previously conditioned with water, methanol (SDS), and cyclohexane. After they were rinsed with 3 mL of cyclohexane, PAHs were eluted with 12 mL of cyclohexane/ethyl acetate (50:50, v/v). After evaporation to dryness, 2 mL of cyclohexane and 2 mL of methanol/water (80:20, v/v) were added and mixed for 30 s, and the cyclohexane phase was separated after centrifugation (1000*g*, 5 min). The methanol/water phase was washed again with 2 mL of cyclohexane and centrifuged (1000*g*, 5 min), and the cyclohexane layer was added to the first cyclohexane phase. At this point, the methanol layer was set aside for later analysis of the hydroxylated metabolites. The supernatant containing the PAHs in cyclohexane was evaporated; saponification was achieved with 5 mL of 10% KOH for 80 min at 90 °C to prevent any fatty matter from being present in the final product. Three milliliters of water and 5 mL of cyclohexane were then added, and the mixture was shaken prior to centrifugation (1000*g*, 5 min). The supernatant was harvested, an external standard (d12-chrysene) was added, and the cyclohexane was evaporated. The residue was dissolved in 20 μL of toluene.

The methanol fraction, described above, was evaporated and extracted with 4 mL of water/ethyl acetate (50:50; v/v). After it was vortexed and centrifuged (1000*g*, 5 min), the supernatant was evaporated and supplemented with an external standard (1-OH chrysene), and hydroxymetabolites were derivatized with 20 μL of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA, Fluka, Buchs, Switzerland).

Chromatography Conditions. A quadrupole MS (HP-5973) monoselective detection was coupled to a Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA). The split/splitless injector, maintained at 250 °C, was set at 1.5 min in the splitless mode, and the injected volume was 2 μL. An OV-1 (Ohio-Valley) column (30 m × 0.25 mm i.d.; film thickness, 0.25 μm) was used to separate parent and PAH metabolites. The temperature gradient was 110 °C (4 min), 10 °C min^{-1} up to 160 °C, 5 °C min^{-1} up to 300 °C, and then 15 °C min^{-1} up to 300 °C (10 min) for the analysis of PAHs and PAH metabolites. All of the analyses were performed in the electron ionization mode (70 eV) and single ion monitoring acquisition mode. The detection limits of all samples were based on a signal-to-noise ratio of 3/1 and are shown in **Table 1**.

Table 1. Limits of Detection of Parent PAH and Their Principal Metabolites in Milk

PAH	concn (ng/mL of milk)	PAH	concn (ng/mL of milk)
Fluo	0.3	2-OH Fluo	0.05
Phe	0.2	3-OH Phe	0.04
Pyr	0.2	1-OH Pyr	0.05
B[*a*]P	0.2	3-OH B[*a*]P	0.39

Statistical Analysis. The concentrations of PAHs and their metabolites in milk, as well as their secreted quantities, were analyzed statistically using the SAS mixed procedure (version 9.1, SAS Institute, Cary, NC) with the repeated time option. The model took into account the sampling day as a fixed factor. The cows were considered as replicates. The covariance structure between the different sampling times was defined as being autoregressive after checking with Akaike and Schwarz-Baysan criteria (*16*). The values at a given exposure time were compared in the form of least-squares means using Tukey's *t*-test, and significance was declared at a threshold of 0.05.

RESULTS

Soil. PAH concentrations in fortified soil with subsequent aging were as follows: Fluo 104 μg/g DM, Phe 82 μg/g DM, Pyr 78 μg/g DM, and B[*a*]P 33 μg/g DM (**Table 2**). The four PAHs added to the soil were, as expected, the most abundant, while the other 12 compounds were present at negligible concentrations.

Milk. Fluo and B[*a*]P were not detected in any of the milk samples (**Table 3**). Concentrations of Phe and Pyr varied but did not increase significantly relative to their predosing level. A small but significant increse was observed in the concentrations of Phe and Pyr compounds during the last 2 weeks of the

Table 2. Final PAH Concentrations in Soil Obtained after Fortification and Aging

compound	concn (μg/g dry soil)	compound	concn (μg/g dry soil)
naphthalene	0.02	benz[*a*]anthracene	0.06
acenaphthylene	0.02	chrysene	0.07
acenaphthene	<0.01	benzo[*b*]fluoranthene	0.09
fluorene	**104**	benzo[*k*]fluoranthene	0.05
phenanthrene	**82**	**benzo[*a*]pyrene**	**33**
anthracene	0.25	indeno[1,2,3-*cd*]pyrene	0.04
fluoranthene	<0.01	dibenz[*a,h*]anthracene	<0.01
pyrene	**78**	benzo[*g,h,i*]perylene	0.03

Table 3. Concentrations of Parent PAH and Their Daily Quantities Secreted in Milk[a]

PAH	control	day 2	day 7	day 14	day 21	day 28	RMSE[b]	effect
				concentration (ng/ mL)				
Fluo	<0.3[c]	<0.3	<0.3	<0.3	<0.3	<0.3		
Phe	4.3 ab	2.8 b	3.0 b	2.8 b	4.0 ab	5.1 a	0.54	$P < 0.01$
Pyr	2.1 ab	1.5 b	1.4 b	2.0 b	2.7 ab	3.1 a	0.43	$P < 0.01$
B[*a*]P	<0.2[c]	<0.2	<0.2	<0.2	<0.2	<0.2		
				secreted quantity (μg/day)				
Phe	80	78	82	69	70	92	17	NS[d]
Pyr	42	39	43	63	47	55	11	NS

[a] Values within a row that do not have common superscripts differ significantly ($P < 0.05$). [b] Root mean square error. [c] Below the detection level. [d] Not significant.

study. No significant differences occurred in the daily quantities of PAH secreted in milk (**Table 3**). However, milk yield seemed to modify the concentrations of PAH by their being more or less diluted and parent PAHs as soil-bound materials were not significantly transferred to milk.

Target PAH metabolites were not detected in control milk (**Table 4**). 3-OH B[*a*]P was not present in control or test milk samples. 2-OH Fluo, 3-OH Phe, and 1-OH Pyr were present in milk by the second day of the soil-dosing regimen (**Figure 1a**,**b**).

Concentrations of 1-OH Pyr in milk showed a significant peak in the second week of the study reaching over 30 ng/mL (**Table 4**). The concentrations of 3-OH Phe and 2-OH Fluo were also highest in the second week. Nevertheless, statistical evidence of a peak was not reached for these metabolites and their concentrations in milk were less elevated. At these moments of the highest concentrations, cows secreted daily in milk 500, 50, and 10 μg, respectively, for 1-OH Pyr, 2-OH Fluo, and 3-OH Phe. The transfer of metabolites into milk, expressed as a proportion of the daily supply of parent PAHs, was only 0.09, 0.03, and 1.6%, respectively, for 2-OH Fluo, 3-OH Phe, and 1-OH Pyr at the days of apparent peak concentrations.

Concentrations of hydroxy metabolites appeared to plateau from about 7 to 14 days (particularly for 1-OH Pyr), which was in the middle of the experimental period. Metabolite concentrations in milk varied considerably among the three cows, particularly on days 2 and 7, as illustrated by the high root mean square error (RMSE).

However, the calculated recovery rate in milk (sum of parent compound and its metabolite) of supplied PAHs at peaks (day 7) differed widely: 0.1% for Fluo (only as metabolite), 0.2% for Phe (mainly as parent compound), and 1.7% for Pyr (mainly as metabolite).

DISCUSSION

PAH burdens of fortified soil declined approximately two-thirds of the fortification level after 30 days of aging. Supposing that biodegradation of the PAH occurred (*17*), fortified soil was still the main source of PAHs, because PAH concentrations in water and feed were negligible. Thus, cows were highly exposed by the daily intake of approximately 150 mg (**Table 2**).

Secretion of Parent Compounds. Although the concentrations of parent PAHs in milk varied slightly during the period of supply, these variations mainly reflected concentration or dilution effects as a function of the cow's milk yield. Indeed, the quantities of parent PAHs excreted in milk on a daily basis did not significantly increase during the period of chronic supply via contaminated soil. During our study, neither parent Fluo nor parent B[*a*]P was measured in milk, consistent with previous studies (*18*), which showed the absence of Fluo and B[*a*]P in milk from "very remote, rural areas" and "highly exposed" farms. Moreover, Phe and Pyr were measured in milk collected in a "very remote, rural area" without significantly higher concentrations in "highly exposed farm". The secretion of Fluo in milk is difficult to study because of its volatility (Henry's law constant 1.01×10^{-2}) limiting its environmental accumulation.

Another study (*19*) confirmed a low transfer of free (not matrix-bound) radiolabeled compounds into goat milk (1.9, 1.6, and 0.2%, respectively, for Pyr, Phe, and B[*a*]P), after a single oral intake in goats. The use of radiolabeled compounds in this study does not allow us to know if metabolites have been formed or only parent PAHs were transferred. Our study demonstrates that PAH concentration in milk is not a sensitive measure of soil-bound PAH exposure.

Secretion of Metabolites. The secretion of PAH metabolites in milk during an exposure to PAH contaminated soil was clearly demonstrated. The rapid appearance of PAH metabolites in milk soon after the beginning of supply was in line with the hypothesis of PAHs extraction from soil followed by its gastrointestinal absorption. Parent PAHs may be transformed in the rumen by bacterial activity and then be absorbed and secreted in milk. Indeed, it was shown in a simulator of the human gastrointestinal tract that PAHs were hydroxylated after

Table 4. Daily Quantities of Metabolites Excreted in Milk during Chronic Supply[a]

PAH	control	day 2	day 7	day 14	day 21	day 28	RMSE[b]	effect
				concentration (ng/mL)				
2-OH Fluo	<0.3[c]	1.73	2.79	2.62	2.30	0.99	0.79	NS[d]
3-OH Phe	<0.2[c]	0.61 a	0.69 a	0.69 a	0.40 ab	0.13 b	0.17	$P < 0.01$
1-OH Pyr	<0.2[c]	18.4 bc	38.6 a	30.6 ab	7.2 c	1.3 c	7.3	$P < 0.01$
3-OH B[*a*]P	<0.2[c]	<0.2	<0.2	<0.2	<0.2	<0.2		
				secreted quantity (μg/day)				
2-OH Fluo	ND[c]	32.5	46.7	43.0	40.3	17.8	15.8	NS
3-OH Phe	ND[c]	10.9 a	11.4 a	10.4 a	6.6 ab	2.2 b	2.3	$P < 0.01$
1-OH Pyr	ND[c]	344 ab	633 a	478 a	126 bc	24 c	10.4	$P < 0.01$
3-OH B[*a*]P	ND[c]	ND	ND	ND	ND	ND		

[a] Values within a row that do not have common superscripts differ significantly ($P < 0.05$). [b] Root mean square error. [c] Not detected. [d] Not significant.

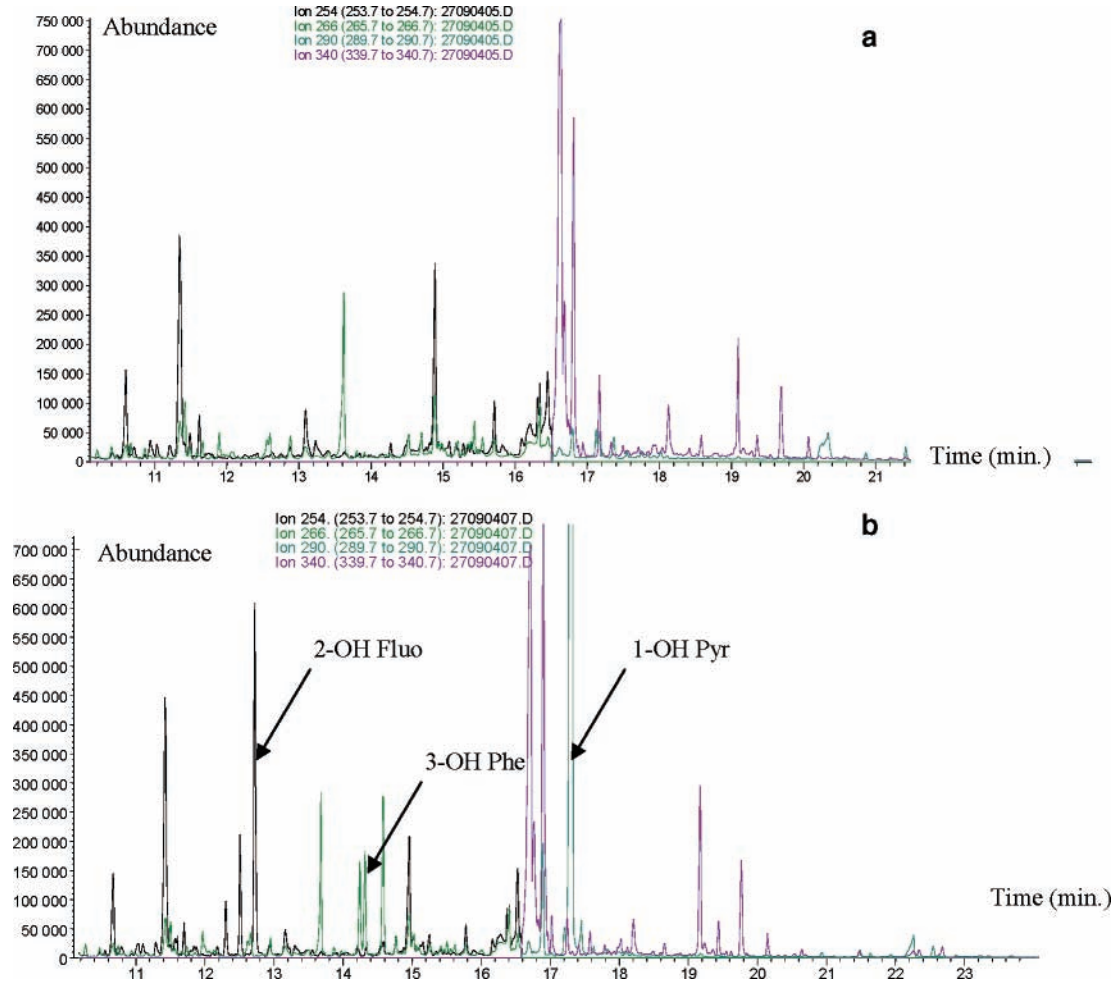

Figure 1. (**a**) GC-MS chromatogram of PAH metabolites in control milk (MW: 2-OH Fluo 254, 3-OH Phe 266, 1-OH Pyr 290, and 3-OH B[*a*]P 340). (**b**) GC-MS chromatogram of PAH metabolites in test milk at day 7 (MW: 2-OH Fluo 254, 3-OH Phe 266, 1-OH Pyr 290, and 3-OH B[*a*]P 340).

incubation for 24 h in a colon suspension enriched with microorganisms (*20*). Moreover, PAH biotransformation has been observed in the Caco-2 enterocyte model (*21*, *22*). Finally, the transformation of parent PAHs has been demonstrated after absorption in the bodies of animals (*23*). Several studies have shown that the concentrations of major PAH metabolites were increased in the blood, urine, and feces of monogastric animals exposed to PAH-contaminated soil (*24*−*29*).

The apparent decrease in metabolite concentration in milk after 7−14 days of exposure suggests that biotransformation of PAHs into metabolites other than the monohydroxylated forms may have occurred. Indeed, Pyr is mainly monohydroxylated into its 1-OH Pyr form, whereas Phe can be monohydroxylated to different forms, which may spread output concentrations over several metabolites (*30*). Fully understanding the decreased concentrations of metabolites needs further studies as other metabolic pathways cannot be excluded.

Transfer Rates. The generally low transfer of PAHs into milk raises the question of whether the fortified PAHs remained sequestered in aged soil and were excreted in the feces. Indeed, increasing polarity of individual PAHs has been reported to favor sequestering by soil aggregates during aging, thus reducing their extractability (*14*, *31*, *32*). Two hypotheses can be proposed to explain their low transfer into milk.

First, PAHs were extracted from the soil and absorbed by the mammalian organism but preferentially excreted in urine. The presence of Phe and Pyr in the blood at day 28 (no PAH being found in the blood prior to treatment, data not shown) suggests absorption. However, their monohydroxylation and subsequent elimination could occur rapidly (*24*) so that the concentrations of these compounds would not rise markedly in the blood. Moreover, Kotin et al. (*33*) showed that B[*a*]P was nearly cleared 10 min after an intravenous injection in rats, which could explain its absence in our trial. PAH metabolites seem to be excreted mainly in urine as demonstrated in rats (*34*).

Second, PAHs may be extracted from the soil but not absorbed by the organism and would therefore be available for metabolism by microorganisms in the gastrointestinal tract. In the feces of soil-treated rats (*24*) were found amounts of 1-OH Pyr and 3-OH B[*a*]P corresponding to, respectively, 5.1 and 8.8% of ingested parent compound, thus demonstrating metabolism. Other studies (*19*, *35*, *36*) reported that excretion of PAHs in feces corresponds mainly to metabolized forms of supplied compounds.

Thus, although large quantities of PAHs were supplied chronically to dairy cows, only a small proportion of the dose was recovered in milk. It is therefore possible that the PAHs and/or their metabolites could have been distributed throughout the body, for instance in organs such as the liver, lungs, intestine, or adipose tissue (*33*, *37*), or have been excreted in the urine.

The results of this study indicate that following a chronic supply of PAH-contaminated soil to dairy cattle, PAH contamination of milk with the parent pollutants was not significantly affected. The marked increase in the concentrations of their principal metabolites in milk suggests the extraction of PAHs

from the soil and their absorption by the animals in the gastrointestinal tract. The quantity of parent PAHs secreted in milk would constitute only a low risk to human consumers. Nevertheless, the relative toxicity of the resulting metabolites is at present unknown in mammals. The determination of the true health risk by metabolites would allow the integration of these compounds in food safety standards for milk.

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