JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Absorption, Distribution, and Milk Secretion of the Perfluoroalkyl Acids PFBS, PFHxS, PFOS, and PFOA by Dairy Cows Fed Naturally Contaminated Feed

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Supporting Information

ABSTRACT: The transfer of the perfluoroalkyl acids (PFAAs) perfluorobutanesulfonate (PFBS), perfluorohexanesulfonate (PFHxS), perfluorooctanesulfonate (PFOS), and perfluorooctanoate (PFOA) from feed into tissue and milk of dairy cows was investigated. Holstein cows (n = 6) were fed a PFAA-contaminated feed for 28 days. After the PFAA-feeding period, three cows were slaughtered while the others were fed PFAA-free feed for another 21 days (depuration period). For PFAA analysis plasma, liver, kidney, and muscle tissue, urine, and milk were sampled and analyzed using high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS). The average daily intake of dairy cows was 3.4 ± 0.7, 4.6 ± 1.0, 7.6 ± 3.7 and 2.0 ± 1.2 μg/kg body weight (bw) for PFBS, PFHxS, PFOS, and PFOA, respectively. Overall, PFBS, PFHxS, PFOS, and PFOA showed different kinetics in dairy cows. In plasma, concentrations of PFBS (mean = 1.2 ± 0.8 μg/L) and PFOA (mean = 8.5 ± 5.7 μg/L) were low, whereas PFHxS and PFOS continuously increased during the PFAA-feeding period up to maximal concentrations of 419 ± 172 and 1903 ± 525 μg/L, respectively. PFOS in plasma remained constantly high during the depuration period. PFOS levels were highest in liver, followed by kidney, without significant differences between feeding periods. The highest PFHxS levels were detected in liver and kidney of cows slaughtered on day 29 (61 ± 24 and 98 ± 31 μg/kg wet weight (ww)). The lowest PFAA levels were detected in muscle tissue. At the end of the feeding study, cumulative secretion in milk was determined for PFOS (14 ± 3.6%) and PFHxS (2.5 ± 0.2%). The other two chemicals were barely secreted into milk: PFBS (0.01 ± 0.02%) and PFOA (0.1 ± 0.06%). Overall, the kinetics of PFOA were similar to those of PFBS and substantially differed from those of PFHxS and PFOS. The very low concentration of PFBS in plasma and milk, the relatively high urinary excretion, and only traces of PFBS in liver (0.3 ± 0.3 μg/kg ww) and kidney (1.0 ± 0.3 μg/kg ww) support the conclusion that PFBS does not accumulate in the body of dairy cows.

KEYWORDS: *perfluoroalkyl acids, PFAA, PFBS, PFHxS, PFOS, PFOA, toxicokinetics, ADMET, dairy cows, milk*

INTRODUCTION

Perfluoroalkyl acids (PFAAs) belong to the overall family of perfluoroalkyl and polyfluoroalkyl substances (PFAS), which consist of substances with unique thermal and chemical stability, as well as water-, oil-, and stain-repellent properties. PFAAs are used for a variety of applications, such as repellents for paper and textiles, technical additives in electroplating, or emulsifiers in the production of fluorpolymers.[1,2] The two most discussed PFAAs are perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA). Perfluorohexanesulfonate (PFHxS) has been the third most frequently detected PFAA in blood and milk in the general population,[3,4] which indicates a potential for accumulation and biomagnifications.[5,6] All three substances are persistent in the environment and have already been detected in a variety of wildlife species (e.g., polar bears, seals, tigers, pandas, caribous) far from populated or industrial regions.[7−11] In vivo studies show a moderate acute toxicological profile for PFAA. However, subchronic and chronic exposure to PFOS and PFOA on rodents showed hepatotoxic, carcinogenic, and teratogenic effects.[12−14] Butenhoff et al.[15] investigated the potential for reproductive and developmental toxicity of PFHxS in rats and demonstrated hepatic hypertrophic effects and reduced total cholesterol and triglycerides in serum of males. Because of the concern for potential adverse effects in humans, animals, and the environment, the industry began to develop new technologies to replace the six- and eight-carbon homologue chemicals. One of the new technologies is based on products made from perfluorobutanesulfonyl fluoride, a short-chain precursor of PFBS.[16] Because of the more rapid elimination of PFBS shown in pharmacokinetic studies in monkeys and rats, it is expected that PFBS should have lower potential for accumulation with less concern for human health.[17,18] Olsen et al.[18] suspected that marked differences in PFBS elimination, compared to longer chained PFAAs, possibly result from its smaller molecular size, higher water solubility, and reduced protein binding affinity.

Received: November 6, 2012
Revised: February 26, 2013
Accepted: February 27, 2013
Published: February 27, 2013

Table 1. Average Concentrations of PFBS, PFHxS, PFOS, and PFOA (μg/kg DM) Analyzed in Grass Silage and Hay (LOD 0.2 μg/kg)

		PFBS	PFHxS	PFOS	PFOA	total PFAA
grass silage[a]	mean ± SD	68.4 ± 23.1	149.0 ± 64.6	79.3 ± 76.2	200.0 ± 168.2	4285 ± 1391
(n = 7)	min−max	29−98	55−243	12−240	29−537	2312−6335
hay[b]	mean ± SD	993.6 ± 224.4	1034.4 ± 355.5	333.3 ± 171.7	1923.6 ± 760.6	497 ± 321
(n = 7)	min−max	843−1419	558−1641	123−597	857−2845	125−1118
	p value[c]	<0.0001	0.0005	0.0037	0.0008	0.0003

[a]41% dry matter (DM). [b]86% DM. [c]Comparison of means between grass silage and hay for the same substance was performed using t test.

PFAAs are distributed in the body by blood circulation after binding to plasma proteins. When incubated with plasma protein fractions, PFOS, PFHxS, and PFOA were found to bind (99%) to serum albumin of rats, bovines, or humans.[19−21] The principal differences between PFBS, PFHxS, PFOS, and PFOA were observed in serum elimination half-life. In rodent species, monkeys, and humans, PFBS consistently had the shortest serum elimination half-life (0.2, 4, and 26 days) compared to PFHxS (30, 100, and 3000 days), PFOS (25, 45, and 1970 days), and PFOA (≤5, 14−42, and 1300 days).[5,21,22] Several studies have shown that renal elimination of perfluoroalkyl carboxylic acids is mediated by differential expression of renal organic anion transporters among species and sex within species.[23−25] At this time, it is not known to what extent the kinetics of the perfluoroalkyl sulfonic acids may be determined by organic anion transporter mediated processes.[5] The excretion of PFAAs via feces is much slower. This lower excretion is hypothesized to be due to enterohepatic circulation.[26] Studies on rodents exhibited a total fecal excretion of PFOA of 4−9% of the total oral single dose over a 5 day depuration period.[27] In consideration of the longer depuration period (9 and 11 days), total fecal excretion of PFOA (5%) and PFOS (6%) in beef cattle is less rapid.[28] Lactation is an additional pathway for females to reduce the PFAA body burden. Transfer of several PFAAs including PFHxS, PFOS, and PFOA to human milk has been confirmed in Swedish women by Kärrman et al.[29] PFHxS and PFOS were detected in all maternal milk samples at mean concentrations of 0.2 and 0.09 μg/L, respectively. However, PFOA was detected less frequently. The concentration of PFOS and PFOA in rat milk was found on average to be 1−10% of the corresponding maternal plasma concentration.[30,31] In sheep, the milk:plasma ratio of PFOS and PFOA (approximately 1:20) was somewhat lower.[32] At present, the specific transfer mechanisms are not clear. Distribution patterns of PFOS and PFOA seem to be similar among species, with the highest levels found in liver followed by blood and kidney.[27,34,35] In ruminants, residues of PFOA in organ and muscle tissues were not detectable after a depuration period, consistent with the finding that the majority of PFOA was eliminated via urine.[28,32]

Studies to quantify the transfer of PFAA from feed into livestock and food of animal origin (meat, milk, and eggs) are limited to PFOS and PFOA.[35−37] In addition to PFOS and PFOA, Guruge et al.[38] analyzed levels of PFHxS in farm animals from several locations in Japan. The levels of PFHxS, PFOS, and PFOA in serum of ruminants ranged from <0.01 to 0.65 μg/L, from 0.53 to 10 μg/L, and from 0.05 to 0.24 μg/L, respectively. In liver, PFOS ranged from 8.8 to 72 μg/kg, and PFHxS and PFOA levels were negligible. No data are available about levels and duration of PFAA exposure of these animals. A Norwegian study reported PFAA levels in certain foods and beverages.[39] Rather low levels of PFBS, PFHxS, PFOS, and PFOA were measured in milk (<0.24, <0.11, 7.0, and 4.7 pg/kg) and beef (<0.6, <0.3, 60.0, and 12.0 pg/kg wet weight (ww)). There is also no information about the exposure dose of livestock in this study.

In 2007, the national food monitoring report in North Rhine-Westphalia in Germany reported extraordinarily high PFOS levels in kidney (1332 μg/kg) and meat (154 μg/kg) in one beef animal.[40] The investigations that followed confirmed that high tissue levels were associated with a 2006 incident in which PFAA-containing industrial waste was illegally mixed into organic fertilizer then sold to farmers and spread on cropland.[40] In general, background levels are low, but levels of PFAA in food of animal origin such as meat, milk, and eggs suggest that livestock are exposed to PFAA.[41] Thus, PFAA intake via feed (and soil by grazing livestock) might be a significant exposure pathway to PFAA for livestock.

In the current study, a feeding experiment was performed using feed obtained from the incident of environmental pollution in Germany in 2006.[40] To determine the transfer of PFAA from feed into the food chain under real-life conditions, grass silage and hay were cultivated on the PFAA-contaminated farmland in Lower Saxony. Until now, no information has been available on how long and to which quantities the PFAA-contaminated fertilizer was applied. However, screening tests of cropping soil indicate a high contamination with PFOA and PFOS ranging from <10 to 240 μg/kg dry matter (DM) and from 31 to 3300 μg/kg DM, respectively.[40] The present study was conducted in dairy cows to determine the kinetics and transfer of PFBS, PFHxS, PFOS, and PFOA from contaminated feed into meat and milk. The generated data will be used to estimate the extent to which these substances are found in the milk and meat of dairy cows being fed PFAA-contaminated feed and, furthermore, constitute the basis to assess consumer exposure.

MATERIALS AND METHODS

Animals, Housing, and Feeding. Six lactating cows (Holstein Friesian) with an average body weight (bw) of 583 ± 31 kg were housed at the experimental farm of the German Federal Institute for Risk Assessment (BfR). After 4 days of adaptation, dairy cows were allocated to two groups, both receiving PFAA-containing feed for 28 days. Animals of group 1 (three cows) were slaughtered directly after the PFAA-feeding period (day 29), whereas animals of group 2 (three cows) were fed PFAA-free feed for another 21 days (depuration period) before slaughter on day 50. During the PFAA-feeding period, the experimental diet contained PFAA-contaminated grass silage and hay, which grew on a PFAA-contaminated farmland in Lower Saxony, Germany. The average concentrations of PFBS, PFHxS, PFOS, and PFOA analyzed in grass silage and hay are shown in Table 1. Because

no information was available on how long and to which quantities PFAA-contaminated fertilizer was spread on farmland, the PFAA dose level for dairy cows was based on analytical data in feed. The composition of the experimental and control diets fulfilled the recommendations of nutrient and energy requirements of dairy cows according to GfE.[42] Feed and water were offered ad libitum. Cows were kept in tie-stalls for individual feeding.

Sampling. The individual intake of grass silage and hay was quantified every day. For determination of PFAA intake, grass silage and hay were separately mixed every 4 days to representative samples of those four consecutive days. Blood samples for groups 1 and 2 were taken on days 0, 1, 4, 7, 10, 15, 20, and 25 (PFAA-feeding period). For group 2, blood samples were taken during the succeeding PFAA-free feeding period (days 29−49) on days 29, 32, 35, 38, 41, 44, and 47. Blood from all cows was also sampled at the time of slaughter (day 29 for group 1 and day 50 for group 2). Blood was collected by puncturing of the *vena jugularis* in a lithium-heparin monovette. To obtain plasma, the blood was centrifuged at 3000*g* for 10 min at room temperature (20 °C), pipetted off, and stored frozen at −20 °C. The individual milk yield was recorded every day. Milk samples were taken twice a day, always at the same time (7 a.m., 4 p.m.) on days 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 17, 20, 23, 25, and 27. Throughout the PFAA-free feeding period, milk was sampled on days 29, 32, 35, 38, 41, 44, 47, and 49. Milk samples were preserved in PFAA-free vessels made from polypropylene (PP) and frozen at −20 °C until analysis. During the first 10 days of the PFAA-feeding period, urine was taken from both groups on days 4, 7, 10, 15, 20, and 25 and throughout the PFAA-free feeding period for group 2 on days 29, 32, 35, 38, 41, 44, and 47. Samples of urine were nonrepresentative because no effort was taken to determine the total daily urinary excretion; thus, collected samples were not taken from the total amount of urine. The nonrepresentative samples of urine were placed in PP vessels and frozen at −20 °C until analysis. Samples of liver, kidney, and muscle tissue from the *musculus longissimus dorsi* were taken immediately after slaughter and stored frozen (−20 °C) until PFAA analysis. Analyses of PFAA in feed, plasma, milk, urine, and liver, kidney, and muscle tissue were performed by the Chemical and Veterinary Analytical Institute Münsterland-Emscher-Lippe (CVUA-MEL).

Analysis. *Reagents.* Native and ^{13}C-labeled PFAA were purchased from Wellington Laboratories, USA. Methanol absolute, acetonitrile, formic acid (99%), and ammonium acetate (all ULC/MS) were purchased from Biosolve, The Netherlands. Sodium acetate anhydrous p.a. was obtained from Merck, Germany. Protease Type XIV (Sigma L 1754-5G) and Lipase Type VII (Sigma P 5147-1G) as well as Pepsine (from porcine gastric mucosa) were purchased from Sigma-Aldrich, German. Water was double distilled by using the distillation unit 2001/2 from GFL.

Sample Preparation. Because of possible interferences between PFAA and proteins, matrix specific sample preparations were performed. For sample storage and sample preparation, only vessels made from PP were used. A blank sample was analyzed in each measurement series. Depending on the matrix, 1−5 g of sample matrix was extracted. Plasma was treated with half-concentrated formic acid.[43] Feed samples were extracted with methanol, and an aliquot of this solution was diluted with water (VDLUFA method). Milk was hydrolyzed using the enzymes lipase and protease according to a method published by Bernsmann and Fuerst.[44] Liver and kidney as well as meat were hydrolyzed using pepsin.[45] All sample solutions were purified and concentrated using solid phase extraction on an OasisWAX (60 mg/3 mL).[46] The final extract was reconstituted in 100−1000 μL of methanol (50%) and water (50%), depending on the expected concentration.

Measurement. The purified solutions were measured using high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) run in negative ion multiple reaction monitoring (MRM) mode. The separation was performed on an Agilent 1200 SL HPLC system. A mixture of 2 mM ammonium acetate (95%) and acetonitrile (5%) (v/v) and a mixture of methanol (40%) and acetonitrile (60%) (v/v) were used as solvents in a gradient elution. MS/MS detection was performed with an Agilent 6460 triple-quadrupole mass spectrometer equipped with an electrospray interface (ESI) operating in the negative ion mode. The MRM settings are published elsewhere.[44]

Quantification. Quantification was performed with isotope-labeled standards and a seven-point calibration curve. ^{18}O-PFHxS and later ^{13}C-PFHxS were used as internal standards for PFBS and PFHxS. ^{13}C-PFOA was used as internal standard for PFOA and ^{13}C-PFOS for PFOS. The internal standards were added at the beginning of the sample preparation. The limit of detection (LOD) was defined as a signal-to-noise ratio of 3:1 of the qualifier ion. The limit of quantification (LOQ) was defined as the concentration on which a substance is identified unequivocally and quantified with a relative standard deviation of 20% or lower.

A small HPLC column was placed as a precolumn between the purge valve and autosampler to separate background perfluoroalkyl carboxylic acids (PFCA) and PFAS from the analytes of the samples. An injector program was used to minimize potential cross-contamination from heavily contaminated samples as far as possible. Interferences of PFOS with taurodeoxycholic acid are excluded, because both substances are separated chromatographically, and furthermore the relationship of the two most intensive transitions of PFOS in comparison to a standard solution was used to check possible interferences. Taurodeoxycholic acid does not show the m/z transition 499 to 99 Da/e, specific for PFOS. The analytical method is described in detail by Ehlers.[45]

Statistics. All statistical analyses were performed using SPSS1201 version 7.0.1.4. A t test was used to assess the significance of differences in DM intake for each day between groups 1 and 2. Differences of PFAA concentrations in plasma and milk between the days were determined by one-way analysis of variance (ANOVA). A Bonferroni test was used to compare mean concentrations of each substance between each sampling day for each feeding period, if homogeneity of variance was given. If not, a Dunett-T3 test was used. Missing values of milk secretion were interpolated for each substance and each cow using linear regression analysis. Predicted milk concentrations (C) were separately calculated for each feeding period using the equation

$$C = A_{yx} + B_{yx}d$$

where d is the day of feeding period and A and B are the intercept and slope of the linear regression line, respectively. Figures describing the PFAA concentration in plasma and milk and percentage of ingested PFAAs recovered in tissues were obtained using Microsoft Office Excel 2003. Comparison of the mean levels in tissue between the groups within the same tissue sample for the same substance was performed using a simple t test. All data are reported as the mean ± standard deviation (SD) of six cows for the PFAA-feeding period and of three cows for the PFAA-free feeding period. The significance level used for all tests was $p = 0.05$.

RESULTS

Daily Intake. The average daily intake of PFAA feed was 10.3 ± 1.3 kg DM (grass silage, 8.9 ± 1.2 kg DM; hay, 1.4 ± 0.7 kg DM) during the PFAA-feeding period. Daily DM intake did not vary significantly among both groups (p value ≥ 0.5). On the basis of the daily intake of PFAA-contaminated grass silage and hay, the total oral intake of PFBS, PFHxS, PFOS, and PFOA were 3.4 ± 0.7 (range = 2.2−5.3 μg/kg bw/day), 4.6 ± 1.0 (range = 3.3−7.4 μg/kg bw/day), 7.6 ± 3.2 μg/kg bw/day (range = 4.6−15.8 μg/kg bw/day), and 2.0 ± 1.2 μg/kg bw/day (range = 0.8−4.6 μg/kg bw/day), respectively. Hay (123−2845 μg/kg DM) was more highly contaminated with PFAA than grass silage was (29−537 μg/kg DM) (Table 1). Concerning the daily DM intake, hay contributed 70% of PFBS, 52% of PFHxS, 60% of PFOS, and 40% of PFOA of the total intake of dairy cows.

Concentration in Plasma. The plasma concentrations of PFHxS and PFOS as well as PFBS and PFOA are presented in Figure 1, panels a and b, respectively. The lowest mean concentration in plasma was observed for PFBS, with 1.8 ± 0.8 μg/L between the 1st and 28th days. PFBS was not detectable in plasma 4 days after the end of the PFAA-feeding period (day 32). PFOS had the highest relative plasma levels of the PFAA measured in the study. PFOS plasma concentration increased to 1903 ± 525 μg/L until the end of the PFAA-feeding period (day 29). PFOS in plasma of dairy cows slaughtered after the PFAA-feeding period increased to 2464 ± 411 μg/L (day 44), which was not significantly different from the concentration on day 29 (p value > 0.05). PFHxS plasma concentration increased to 419 ± 172 μg/L until day 29 and decreased during the following PFAA-free period to a concentration of 108 ± 53 μg/L (day 50). During the PFAA-feeding period, indicated by the slope of the linear regression line, PFHxS plasma concentration ($y = 14.2x + 52.7$, $r^2 = 0.93$) increased to a lower extent than did the plasma concentration of PFOS ($y = 69.3x - 221.2$, $r^2 = 0.98$). The average PFOA plasma concentration was 8.6 ± 4.2 μg/L during the feeding of PFAA-contaminated feed. PFOA rapidly decreased after the end of the PFAA-feeding period and reached the LOD (0.2 μg/L) on day 41 of the PFAA-free feeding period.

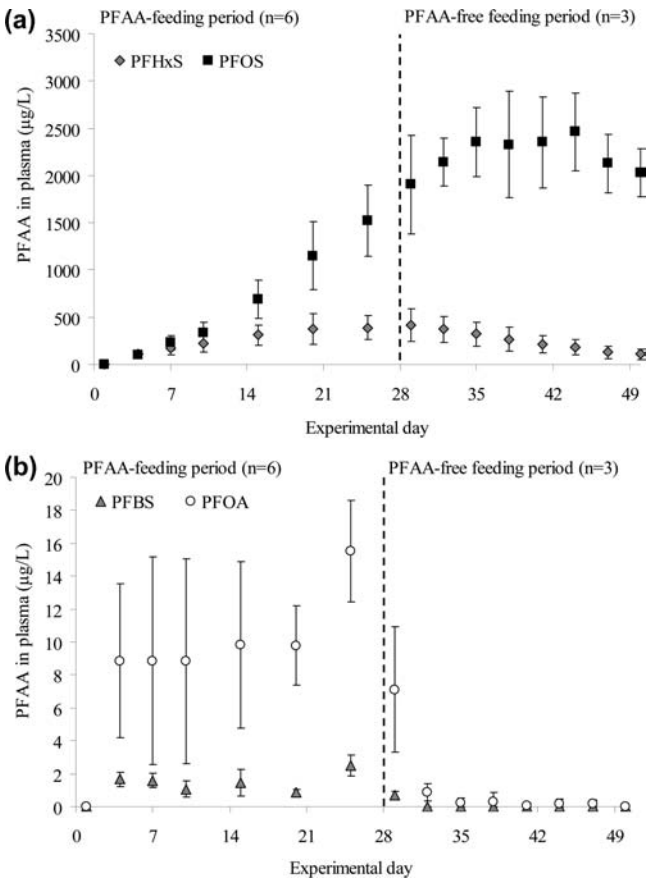

Figure 1. Plasma concentrations (μg/L) of (a) PFHxS and PFOS and (b) PFBS and PFOA during days 1−49 of the feeding study. Data represent mean concentrations ± SD of six cows during the PFAA-feeding period and of three cows during the PFAA-free period, respectively.

Concentration in Urine. The nonrepresentative samples of urine indicated that the PFBS concentration remained relatively constant (60 ± 23 μg/L) as long as PFAA feed was fed. PFBS in urine decreased during the PFAA-free feeding period and was below the detection limit on day 38.

During the PFAA-feeding period, the average concentration of PFHxS in urine was 82 ± 59 μg/L. An increasing PFHxS concentration in urine was observed during the PFAA-feeding period, with the highest observed concentration on day 29 (Figure 2). During the PFAA-free period, the average PFHxS concentration in urine was 88 ± 45 μg/L. PFHxS was detected in urine in every sample up to and including the last day of the PFAA-free feeding period (40 ± 11 μg/L). In contrast, the renal excretion of PFOS was negligible throughout the entire feeding study (days 1−28, 0.8 ± 0.9 μg/L; days 29−49, 2.4 ± 1.1 μg/L). The maximum PFOS concentration in urine was detected in the PFAA-free feeding period on days 32 and 44.

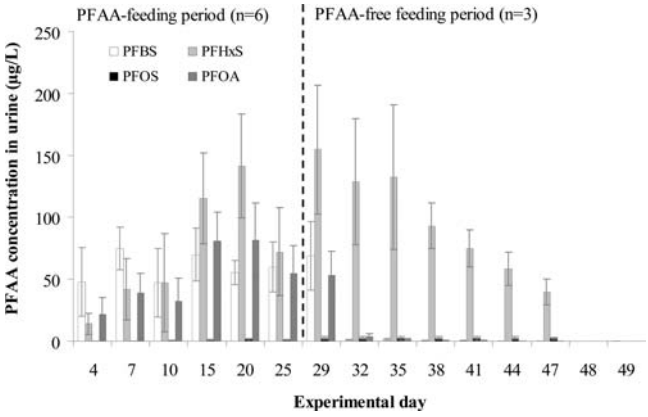

Figure 2. Urine concentrations (μg/L, mean ± SD) of PFBS (white bars), PFHxS (light gray bars), PFOS (black bars), and PFOA (dark gray bars) during the PFAA-feeding period with six cows and the PFAA-free feeding period with three cows.

The nonrepresentative urinary samples indicated an average PFOA concentration of 51 ± 29 μg/L during the PFAA-feeding period. The highest mean concentration of PFOA in urine was observed on days 15−20. PFOA decreased below the detection limit (0.1 μg/L) until day 38 of the PFAA-free feeding period.

Concentration in Milk. When the feeding study started, cows were in their last period of lactation. Thus, the average daily milk yield during the feeding study was low, at 18 ± 4 L. Overall, the four PFAAs showed quite different milk elimination patterns (Figure 3).

Appreciable milk secretion of PFBS did not occur. During the PFAA-feeding period, PFBS concentrations above the detection limit (LOD of 0.1 μg/L) were detected only in milk samples ($n = 11$) of one cow with a mean concentration of 0.12 ± 0.02 μg/L. PFBS was not detected in milk during the PFAA-free feeding period.

PFHxS remained relatively constant (1.8 ± 0.9 μg/L) throughout the feeding study. The concentration of PFHxS in milk decreased slowly during the PFAA-free period and did not reach the LOD (0.1 μg/L) until the day of slaughter.

The highest concentration in milk was observed for PFOS. PFOS steadily increased, as long as PFAA feed was fed, to a concentration of 24.2 ± 9.0 μg/L. The PFOS concentration in milk briefly increased during the subsequent PFAA-free feeding period and reached the highest observed concentration (36.3 ± 9.1 μg/L) on day 35.

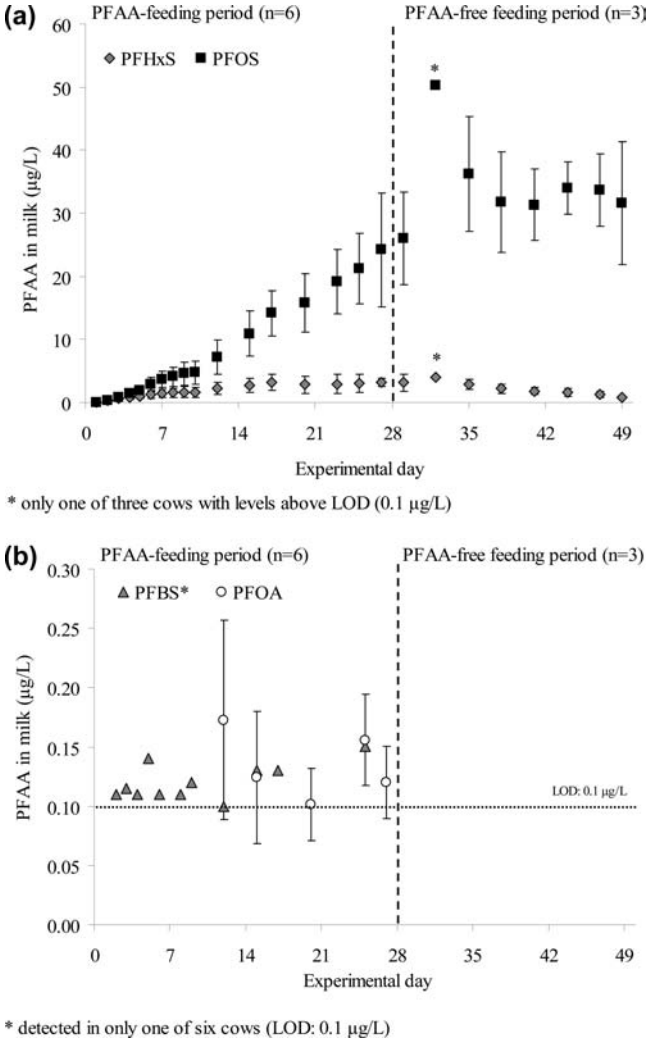

Figure 3. Milk concentrations (μg/L) of (a) PFHxS and PFOS and (b) PFBS and PFOA presented as the mean ± SD during the PFAA-feeding period (six cows) and the PFAA-free feeding period (three cows).

PFOA was detectable in milk only in five occasions during the PFAA-feeding period, with a mean concentration of 0.07 ± 0.07 μg/L, indicating that PFOA secretion via milk was low. PFOA was not detected in milk after PFAA-feeding was stopped (Figure 3b).

Plasma/Milk Ratio. Milk levels were lower compared with the matched plasma levels based on concentration per liter. The mean ratio between milk and plasma (M:P) concentration was 0.007:1 for PFHxS and 0.013:1 for PFOS. The milk:plasma ratio for PFBS and PFOA cannot be calculated as only a few milk samples were found to be above the detection limit. Simple regression analysis and Spearman's correlation test of the matched plasma and milk samples show a significant association between levels of PFHxS (r^2 = 0.91, p = 0.01) and PFOS (r^2 = 0.97, p = 0.01) in plasma and milk (Figure 4).

Total Mass of Accumulation and Secretion. Table 2 shows the estimated fraction of ingested PFAA dose recovered in liver, kidney, and muscle tissue and milk of dairy cows slaughtered after the PFAA-feeding period (day 29) and of dairy cows slaughtered after the PFAA-free feeding period (day 50). For the calculation of the total mass secretion via milk, missing values were calculated by interpolation using linear regression analysis and summed by taking into account the individual daily milk yield. The percentage of PFAAs accumulated in tissues was estimated using the mean concentration of PFAA in liver, kidney, and muscle tissue. It was assumed that the cows' liver weight represents 1.2% of the bw,[47] whereas the weight of the kidneys is assumed to be 0.23% of the bw.[48] The fraction of muscle tissue was estimated to be 59.4% of the bw.[49]

As shown in Table 2, 0.005 and 0.003% of PFBS were quantified in liver and kidney, respectively, of cows slaughtered directly after the PFAA-feeding period. The fraction of the total intake of PFBS secreted via milk was negligible (0.01 ± 0.02%) for dairy cows.

The average fractions of the total intake of PFHxS at time of slaughter were 0.6% in liver and 0.2% in kidney of cows. Highest accumulation of PFHxS (9%) was measured in muscle tissue of cows slaughtered after the PFAA-feeding period. Two percent of the intake of PFHxS was found in muscle tissue of dairy cows slaughtered on day 50. During the entire feeding study, 2.5 ± 0.2% of the total PFHxS intake was recovered in milk. Because PFHxS in milk was detected even on the last day of the PFAA-free period, it can be concluded that PFHxS is secreted over a long period.

The average fractions of total ingested PFOS in liver, kidney, and muscle tissue in cows (group 1) slaughtered after the PFAA-feeding period were 18, 1, and 43%, respectively, and remained at these levels in cows (group 2) slaughtered after the PFAA-free feeding period. The average fraction of the total PFOS dose secreted in milk was calculated to be 5.1 ± 1.1% during the PFAA-feeding period and resulted in a total of 14.1 ± 3.6% PFOS cumulative secretion at the end of the study.

Negligible amounts of PFOA were observed in all tissue samples. Furthermore, an appreciable PFOA milk secretion (0.1 ± 0.06%) did not occur in cows.

DISCUSSION

The present feeding study with dairy cows was conducted to quantify the transfer of PFBS, PFHxS, PFOS, and PFOA from feed into tissues and milk of dairy cows. Especially milk but also edible tissues of dairy cows are considered to be a source of PFAA exposure for consumers. Literature on the fate of PFAA in ruminants is sparse and mainly limited to PFOA and PFOS.[28,32] In contrast to the published toxicokinetic studies, where PFAAs were administered to animals as pure substances or mixtures, dairy cows were fed contaminated feed from a real-world pollution event. Grass for feed, which was ensilaged or dried to produce grass silage or hay, was cultivated on the same contaminated field of farmland. PFAA analyses showed on average a 10-fold higher PFAA level in hay compared to grass silage. The inhomogeneous application of the PFAA-contaminated fertilizer on farmland possibly resulted in varying PFAA levels in soil that consequently caused different levels of PFAA in grass silage and hay. This assumption is confirmed by the analysis of the Landesamt für Natur, Umwelt and Verbraucherschutz (LANUV) in cropping soil with a high distribution variance of PFAA levels.[40] PFAAs are persistent compounds, and biodegradation has not been observed so far. There are no indices of biodegradation by the ensilage process. The heterogeneous PFAA levels in grass silage and hay analyzed in collective samples throughout the PFAA-feeding period could have been caused by the inhomogeneity of the plant material (whole plant), rendering the taking of representative samples more difficult. The presence of contaminated soil in

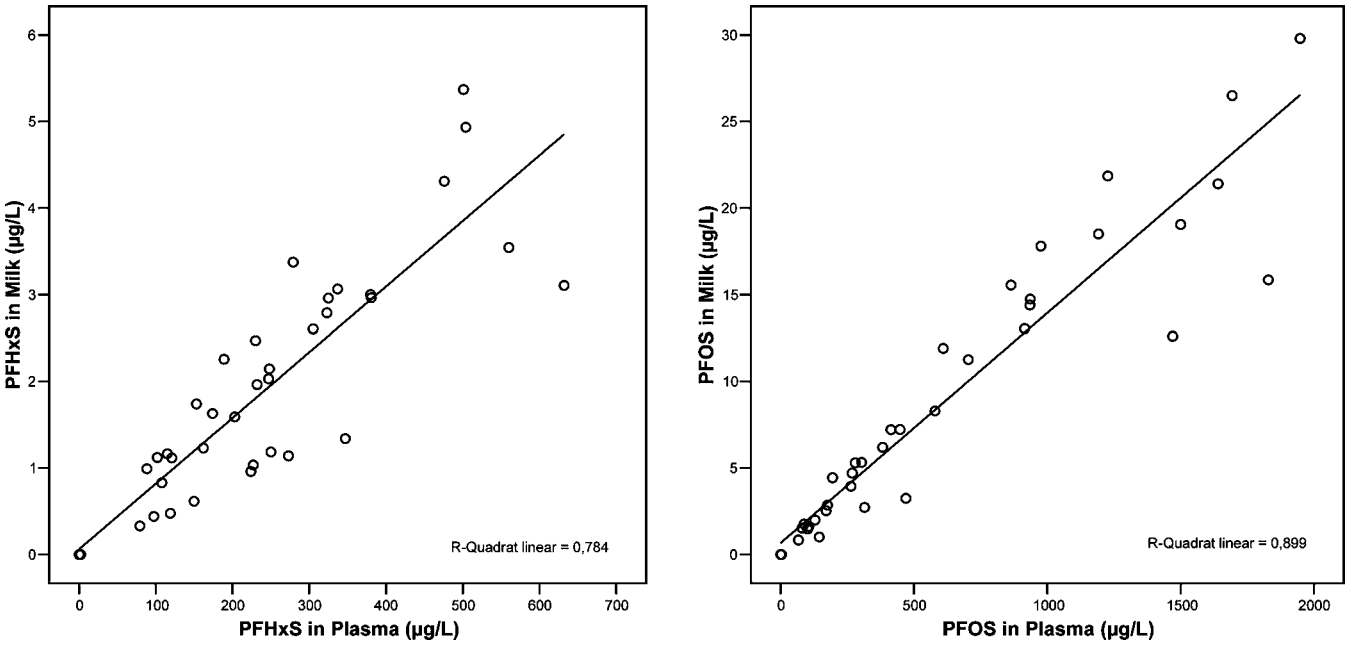

Figure 4. Scatter plot for the correlation of PFHxS and PFOS levels in matched plasma and milk samples from six cows during the PFAA-feeding period: (left) PFHxS (r^2 = 0.784); (right) PFOS (r^2 = 0.899).

Table 2. Percent of Ingested Dose and PFAA Concentrations in Liver, Kidney, and Muscle Tissue and Milk of Dairy Cows (LOD = 0.2 µg/kg; Presented as the Mean ± SD)

	PFBS		PFHxS		PFOS		PFOA	
	concn	% of ingested dose	concn	% of ingested dose	concn	% of ingested dose	concn	% of ingested dose
liver (µg/kg FM)								
group 1[a]	0.3 ± 0.3[c]	0.005 ± 0.004	60.8 ± 23.7	0.58 ± 0.14	2952 ± 718	17.93 ± 2.91	10.1 ± 1.9	0.23 ± 0.02
group 2[b]	<LOD	<0.001	18.6 ± 9.9	0.16 ± 0.05	3964 ± 1035	20.74 ± 1.35	0.8 ± 0.7	0.02 ± 0.01
p value[e]	0.191	0.187	0.046	0.007	0.237	0.204	0.001	<0.001
kidney (µg/kg FM)								
group 1	1.0 ± 0.3	0.003 ± <0.001	98.2 ± 30.8	0.182 ± 0.032	1074 ± 153	1.26 ± 0.13	8.7 ± 3.9	0.036 ± 0.011
group 2	<LOD	<0.001	39.4 ± 22.4	0.062 ± 0.025	1408 ± 303	1.42 ± 0.13	0.4[d]	<0.001
p value[e]	0.033	0.022	0.056	0.007	0.164	0.204	0.019	0.031
muscle tissue (µg/kg FM)								
group 1	<LOD	<0.001	19.1 ± 7.1	9.06 ± 1.99	145 ± 36	43.11 ± 3.09	0.6 ± 0.3	0.69 ± 0.28
group 2	<LOD	<0.001	4.9 ± 2.7	2.02 ± 0.66	178 ± 48	46.47 ± 7.69	<LOD	0.034 ± 0.058
p value[e]			0.032	0.004	0.387	0.522	0.028	0.017
milk (µg/L)								
group 1	0.016 ± 0.041	0.013 ± 0.021	1.86 ± 1.36	1.47 ± 0.54	9.06 ± 9.17	4.68 ± 1.50	0.07 ± 0.07	0.10 ± 0.06
group 2	<LOD	0.004 ± 0.003	1.75 ± 1.56	2.45 ± 0.19	33.09 ± 6.42	14.08 ± 3.62	<LOD	0.09 ± 0.03
p value[f]	0.094	0.517	0.895	0.022	<0.001	0.001	<0.001	0.705

[a]Slaughtered after PFAA-feeding period (n = 3, day 29). [b]Slaughtered after PFAA-free feeding period (n = 3, day 50). [c]Two of three dairy cows with levels above LOD. [d]Two dairy cows with levels below LOD. [e]Comparison between the groups for the same substance in tissue samples was performed using t test. [f]Comparison between the groups for the same substance in milk was performed using Kruskal–Wallis test.

feed could also account for elevated PFAA intake. In the present study, the amounts of crude ash in grass silage and hay analyzed by Weender analysis were 7.48 ± 1.05 and 8.01 ± 2.0% of DM content, indicating that the presence of soil in feeds was not elevated, and dairy cows' exposure to PFAA was primarily caused by the ingestion of contaminated grass silage and hay.

Plasma. Concentrations of PFAA in plasma of dairy cows indicated different kinetic patterns between PFBS, PFHxS, PFOS, and PFOA. As long as PFAA-contaminated feed was fed, PFBS was detected in plasma at a low level. The ratio of PFBS in plasma and feed was 1:1500, showing no tendency of accumulation in dairy cows. Similar ratios were observed in pharmacokinetic studies in male rats.[18] A single oral dose of 30 mg PFBS/kg bw resulted in a 0.4 µg PFBS/L serum concentration 24 h after application.

The mean plasma concentration of PFHxS increased to a maximum of 419 ± 172 µg/L on the last day of the PFAA-feeding period and then slowly declined. In consideration of the daily 1.7-fold lower PFHxS intake compared to PFOS, the PFHxS plasma concentration increased by a factor of 4.5 more slowly compared to PFOS (Figure 1a), indicating a lower tendency for PFHxS to accumulate in plasma. A continuous increase of PFOS and a consistently low concentration of

PFOA in plasma were also found in a previously performed pilot study with dairy sheep.[32] Comparing the PFOS intake of dairy cows (7.6 ± 3.2 μg/kg bw) and dairy sheep (sheep 1 and 2, 1.2 and 1.5 μg/kg bw), the maximum PFOS concentration was higher in the plasma of dairy cows than in the plasma of dairy sheep as long as PFAA was fed, indicating a dose-related increase of PFAA in plasma (dairy cows, 1903 ± 525 μg/L; sheep 1 and 2, 103 and 240 μg/L). The largest differences in plasma concentrations were observed between PFOS and PFOA. In contrast, comparable PFOA plasma concentrations were detected in dairy cows (8.6 ± 4.2 μg/L) and dairy sheep (3.3 ± 2.2 to 15.6 ± 8.3 μg/L), although dairy cows had a 4−5-fold higher PFOA intake.

When the dairy cows' PFOS plasma concentration was compared to that of beef steers that were given a single oral bolus containing PFOS,[28] it could be seen for both animals that the PFOS plasma concentration remained elevated and did not decline during the elimination period. The PFOS plasma concentrations in beef steers following an oral dose of 10 mg/kg bw increased to an extent similar to that observed for dairy cows, although dose and exposure period of these studies were considerably different. However, the maximum PFOA plasma concentration of beef steers was 4.9 ± 0.4 mg/L after a single bolus dose of 1 mg (1-^{14}C)-PFOA/kg.[37] In contrast, the PFOA plasma concentrations in dairy cows that received a PFOA dose of 2.0 ± 1.2 μg/kg bw for 28 days were much lower, possibly indicating a more rapid elimination of PFOA for dairy cows compared to beef steers. Male chickens also showed a steady increase of PFOS in blood as long as the PFOS/PFOA/PFDA mixture (0.1 μg/kg bw) was applied (285 ± 33 μg/L).[35] Similarly to dairy cows, PFOS did not decline in plasma (320 ± 125 μg/L) of chickens after termination of the exposure period. In light of the similar PFOS kinetics in plasma of both species, the specific role of rumen as feed reservoir with a capacity up to 150 L per cow, enabling a sustained release of PFAA from plant cell walls of hay and grass silage by microbial fermentation, seemed to be less important. Moreover, Yeung et al.[35] observed relatively consistent PFOA concentrations of 200 ± 28 μg/L in blood during the PFOS/PFOA/PFDA exposure period and a rapid decrease (17 ± 11 μg/L) in the following PFAA-free feeding period. In contrast to dairy cows, male chickens showed a 500−1000-fold higher tendency to accumulate PFOA and PFOS in blood and a more rapid elimination of PFOA from blood, liver, and kidney. Overall, both studies demonstrate different kinetic patterns for PFOS and PFOA among male chickens and dairy cows.

Urinary Excretion. The urine samples are nonrepresentative, and the daily urinary excretion volume was not quantified. Although the data of PFAA concentration in urine are of limited explanatory power, they were used to make a rough estimate of PFAA elimination rates in dairy cows.

Studies on different animals demonstrate that urine is the major route of elimination for PFBS[18,33] and for PFHxS and PFOA.[5,28,50] For dairy cows, it was therefore assumed that urine is the predominant way of excretion. Figure 2 shows that PFBS and PFOA are rapidly eliminated via urine, resulting in lower systemic exposure. Olsen et al.[18] stated that the urinary excretion of PFBS, PFHxS, and PFOS is a function of chain length for rats, monkeys, and humans. Dairy cows also cleared PFBS more rapidly than PFHxS. In rats, specific organic anion transporters were identified that are involved in the elimination of PFOA by mediated reabsorption in kidney.[23,24] For PFCA, Weaver et al.[25] concluded that the longer the carbon chain, the less the excretion via urine. Whether the expression of organic anion transporter, as demonstrated for PFOA in rats, might influence the differences in PFAA elimination in cows has to be further investigated because not much is known about the identity of such transporters in ruminants.

Sundström et al.[5] investigated the elimination kinetics of PFHxS in rats and reported kinetics similar to those of PFOS. For dairy cows, urinary elimination kinetics of PFOS differed from those of PFHxS. According to Lupton et al.,[28] there is no relevant urinary excretion of PFOS either in beef steers or in dairy cows.

Transfer into Milk. Apart from urine, milk is another possibility for dairy cows to eliminate PFAA. The concentrations of PFBS, PFHxS, PFOS, and PFOA in milk were proportional to plasma. No literature data are available for PFBS concentrations in milk. In recent years, PFBS has caused less concern, because it has been assumed that short-chained PFAAs have much lower potential for accumulation, because their elimination is considered to be more rapid than for longer chained PFAAs.[18,33] In dairy cows, transfer of PFBS from plasma into milk was negligible, caused by low PFBS concentrations in plasma. Only a few milk samples contained PFBS concentrations above the detection limit. A similar picture emerged for PFOA. Because of their low concentrations, no numerically significant mean ratio between milk and plasma could be calculated for PFBS and PFOA. In contrast, PFHxS and PFOS were frequently detected in milk. Only in one human study from Sweden were PFHxS, PFOS, and PFOA analyzed in matched samples of serum and milk.[29] They reported a higher secretion of PFHxS via milk compared to PFOS. A PFOA ratio between milk and serum could not be calculated, because only a few milk samples contained PFOA concentrations above the LOD. In the present study, a significant relationship (p = 0.01) with correlation coefficients of r^2 = 0.784 for PFHxS and 0.899 for PFOS was established between milk and plasma concentrations (Figure 4). The correlation between plasma and milk PFAA concentrations in dairy cows were closer for PFOS than for PFHxS, indicating a higher transfer to milk. In Swedish women, Kärrman et al.[29] determined a correlation of r^2 = 0.7−0.8 (p < 0.05) for both compounds in breast milk and serum. The presented correlation between PFHxS and PFOS concentrations in plasma and milk of dairy cows indicates a rapid transfer mechanism for elimination, which could partly be explained by the highly pronounced vascular system of the udder. Milk yield of cows is dependent on the intensity of the blood flow (300 L/h) at the udder.[51] A bloodstream of 300−500 L of blood flowing through the udder is necessary for the production of 1 L of milk in dairy cows.[52] This intense blood flow in the udder may enable the fast elimination of PFAA from the dairy cow's body.

Because serum albumin is the major binding protein for PFOS and PFOA,[53,54] it was assumed that the binding to protein is the most likely partitioning mechanism for lactation.[55] Only 10% of the milk proteins consist of nonspecific milk proteins, such as immunoglobulin and serum albumin, which can directly pass the blood−milk barrier.[51] Due to the whey protein concentration of 0.6% of the total protein in milk (~3.4 g/100 mL milk), a serum albumin concentration in milk is calculated to be approximately 0.42 g/L, which corresponds to a lactational transfer of 1.2%. In comparison to the serum albumin concentration in plasma of humans (35−50 g/L),[56] an albumin transfer is assumed from blood into the

milk of only 1%. In the present study, transfers of approximately 0.8% for PFHxS and 1.5% for PFOS could be presumed from plasma to milk of dairy cows. The transfer of PFAAs through binding to albumin might be one reason for the relatively low concentration in milk compared to plasma. However, a steady state of PFAA in plasma was not observed in the present study. Consequently, a saturation of albumin binding with PFAAs in plasma was excluded and a transfer of <1% was expected. Besides the binding to albumin, further transport mechanisms might be of significance. As the accumulation kinetics of PFOS, which were estimated by modeling, indicated a behavior comparable to that of fatty acids,[57] a transfer of PFAA through binding on the surface of fat molecules could be possible. In addition, several studies reported high PFAA levels in high-fat food items such as egg yolk, raw milk, or butter.[58−60] Nevertheless, a statistical analysis of milk parameters detected no correlation between the concentrations of PFAAs and the content of milk protein or the content of milk fat.

Accumulation in Tissue Samples. The different excretion rates of the investigated PFAAs caused different concentrations in tissue samples. The estimation of the distribution volume of PFBS, PFHxS, and PFOS indicated that the longer the chain, the higher the accumulation in liver, kidney, and muscle tissue of dairy cows. The very low concentration of PFBS in plasma and milk, the relatively high urinary excretion, and only traces of PFBS in liver (0.3 ± 0.3 μg/kg ww) and kidney (1.0 ± 0.3 μg/kg ww) support the conclusion that PFBS does not accumulate in the body of dairy cows.

PFHxS levels in dairy cows slaughtered on days 29 and 50 of the feeding study were highest in kidney (98.2 ± 30.8 and 39.4 ± 22.4 μg/kg ww) followed by liver (60.8 ± 23.7 and 18.6 ± 9.9 μg/kg ww) and muscle tissue (19.1 ± 7.1 and 4.9 ± 2.7 μg/kg ww). This distribution pattern in dairy cows was similar to PFHxS concentrations in rats 0.5 and 1 h after dosing, as reported by Gannon et al.[22] In the same study, mice with a less rapid urinary elimination showed PFHxS concentrations in the order of liver > kidney > muscle tissue.

In the present study, PFOS primarily accumulated in liver (2952 ± 718 μg/kg ww) and kidney (1074 ± 153 μg/kg ww) and to a lesser extent in muscle tissue (145 ± 36 μg/kg ww). Interestingly, PFOS levels in tissue samples did not decline until the day of slaughter. This was not observed in the feeding study with dairy sheep and in the studies on chicken in which PFOS depuration was investigated.[32,35,36] With regard to the high PFOS plasma concentrations (Figure 1) during the PFAA-free feeding period, it was hypothesized that a release of PFOS from tissues that were not examined in the present study caused the constantly high concentrations of PFOS in liver, kidney, and muscle tissue. Because no decline ($p > 0.05$) of PFOS could be observed in plasma and tissue of dairy cows that were slaughtered after the PFAA-free feeding period, it was assumed that a steady state was reached in these examined tissues, possibly balanced by PFOS release from nonexamined tissues and the PFOS elimination via feces and milk.

PFOA levels in liver, kidney, and muscle tissue were different according to the time of slaughter of dairy cows. The mean PFOA level in muscle tissue of diary cows directly slaughtered after the PFAA-feeding period was 0.6 ± 0.3 μg/kg ww. When cows were fed PFAA-free for another 21 days, PFOA could be detected in the muscle tissue of only one of the three cows. Dairy cows of group 1 (slaughtered directly after the PFAA-feeding period) showed similar levels of PFOA between liver (10.1 ± 1.9 μg/kg ww) and kidney (8.7 ± 3.9 μg/kg ww). In contrast, marked differences in PFOA accumulation were observed in kidney and liver of rodents and chicken, where the highest concentrations occurred in liver.[27,34−36,61] Similar levels of PFOA were also detected among the organs liver (2.6 μg/kg ww) and kidney (4.8 μg/kg ww) of dairy sheep after feeding a PFOA dose of 0.5 μg/kg bw for 21 days.[32] PFOA levels in liver and kidney of dairy cows slaughtered after the PFAA-free feeding period were near (liver) or slightly below (kidney) the detection limit, indicating that PFOA was almost completely removed from animal tissue within 21 days. This is consistent with observations in other livestock species.[32,35,36]

The calculated relative accumulated mass of PFAA in tissue samples of dairy cows showed the highest accumulation potential for PFOS, less for PFHxS as well as negligible potential for PFOA and PFBS accumulation. The observed fraction of total ingested PFOS was similar in liver (18%) and muscle tissue (43%) of dairy cows slaughtered after a 21 day depuration period compared to dairy cows slaughtered directly after the PFAA-feeding period (21 and 47%, respectively) (Table 2). In contrast, the relative accumulated mass of PFHxS declined both in liver (from 0.6 to 0.2%, $p = 0.007$) and in muscle tissue (from 9 to 2%, $p = 0.004$) after 21 days of PFAA-free feeding. In kidney, differences in relative accumulated mass were observed between groups 1 and 2 for PFHxS (0.2 and 0.1%, $p = 0.007$), but not for PFOS (1.3 and 1.4%, $p = 0.204$). The recovered dose of PFOS in muscle, liver, and kidney of beef steers after 28 days of a single oral dosing of 10 mg PFOS/kg have been reported to be 4, 2, and 0.1%, respectively.[28] There seem to be large differences between distribution and accumulation of PFOS from acute and chronic exposure among beef steers and dairy cows. For PFOA, a relative accumulated mass in liver and kidney of dairy cows was estimated at 0.2 and 0.04%, respectively. Assuming weights for liver and kidney of 1.2 and 0.2% per bw in dairy sheep, the relative accumulated mass of PFOA was calculated to be 0.3 and 0.08%, respectively,[47,48] when sheep were directly slaughtered after the PFAA-feeding period, which is comparable to the results in dairy cows. However, chickens seem to eliminate PFOA more rapidly, reflected by high PFOA liver concentrations (87 ± 28 and 950 ± 740 μg/kg ww) that decreased below the LOD after 21 days of depuration.[35] Because the milk production did not affect the clearance of PFOA (0.1 ± 0.06%) from tissue of dairy cows, it was assumed that PFOA elimination kinetics in tissue differ significantly between dairy cows and chickens. Overall, analysis of the relative accumulated mass of PFAA in tissue samples following the PFAA-feeding and PFAA-free feeding periods demonstrated that muscle tissue, due to its large volume, and liver, due to its extraordinarily high concentration, are the largest reservoirs for PFAA in the body of dairy cows.

In conclusion, PFBS, PFHxS, PFOS, and PFOA showed different kinetic patterns in dairy cows. The accumulation of PFAA differed depending on its elimination pattern via urine and milk. The results of PFBS, PFHxS, and PFOS kinetics indicate that the longer the chain, the lower the elimination rate via urine and milk, corresponding to higher accumulation in tissue samples. Interestingly, the kinetics of PFOA were similar to those of PFBS and substantially differed from those of PFHxS and PFOS. Overall, the kinetics of PFAA clearly differed in dairy cows depending on the PFAA carbon chain length and/or functional group. These conclusions are based on limited data, in particular for PFBS and PFHxS in animals. The

study points to species-specific differences in PFAA kinetics among livestock species.

■ ASSOCIATED CONTENT

Supporting Information

Additional tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

We greatly acknowledge financial support from the INTERREG project SafeGuard.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

bw, body weight; DM, dry matter; LOD, limit of detection; PFAA, perfluoralkyl acid; PFAS, polyfluoroalkyl substances; PFBS, perfluorobutanesulfonate; PFCA, perfluoroalkyl carboxylic acids; PFHxS, perfluorohexanesulfonate; PFOA, perfluorooctanoate; PFOS, perfluorooctanesulfonate; PP, polypropylene; ww, wet weight

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