ACCUMULATION OF PERFLUOROALKYLACIDS IN PLASMA, ORGANS AND MUSCLE TISSUE OF FOOD PRODUCING ANIMALS

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Introduction

Perfluoroalkylacids (PFAA) are used in a wide range of applications, because of their strong stability and their inert and non adhering surface properties. But it was observed, that perfluoroalkylcarboxylic acids (PFCA) and perfluoroalkanesulfonic acids (PFSA), in particular perfluorooctanoic acid (PFOA) and perfluorooctanesulfonate (PFOS) are persistent, bioaccumulative and toxic in animal studies. There is a lack of data for the longer and shorter chain PFAA and thus there is a need for data on the occurrence and the behaviour of these substances in food producing animals. In a survey[1], a concentration dependent carry-over of PFAA from soil to plant was shown. If plants are used as feed for food producing animals, PFAA can enter the food chain. In order to investigate the carry-over from naturally contaminated feed (grown on contaminated fields) into food producing animals, feeding studies with lactating cows and fattening pigs were performed at the Federal Institute for Risk Assessment (BfR) in Berlin, Germany[2,3]. The analyses of all samples from these feeding studies were performed at the Chemical and Veterinary Analytical Institute Muensterland-Emscher-Lippe (CVUA-MEL) in Muenster, Germany[4]. On the one hand, the contamination of food, such as meat, organs and milk were analysed in these studies and on the other hand, concentrations in plasma were also analysed in order to learn more about the behaviour of these substances in animal bodies. In this paper, the carry-over of PFCA and PFSA with special emphasis on compounds other than PFOS and PFOA from contaminated feed into pigs and cows is presented.

Materials and methods

Animal studies

The feeding experiments were performed at the BfR in Berlin. Six lactating cows were fed with naturally contaminated feed for 29 days. After 29 days, three cows were slaughtered. The other three cows were fed with PFAA-free feed for another period of 21 days. They were slaughtered after 50 days.

Eight sows, eight barrows and eight boars were fed with PFAA-contaminated feed for 21 days. All pigs were slaughtered after these 21 days.

Samples from plasma were taken during and after the feeding with PFAA contaminated feed. After slaughter, samples from muscle tissue, liver and kidney were taken. Feed samples were also analyzed.

Sample 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. Pepsine (from porcine gastric mucosa) was purchased from Sigma-Aldrich, Germany. Water was double distilled by using the distillation unit 2001/2 from GFL.

Apparatus: HPLC-MS/MS: Agilent 1290 SL LC/ Agilent 6460 Triple Quadrupole LC/MS.

Sample preparation: In plasma, PFCA and PFAS were determined based on a method published by Kärrman et al. (2005)[5]. Internal standard solution and 4 ml of a mixture of formic acid 99 % / water (1:1) were added to 1 ml sample. The solution was sonicated for 15 minutes. After sonication, 3 ml water was added and after centrifugation the samples were purified and concentrated using solid phase extraction (SPE) on an OasisWAX (60 mg/3 ml) column. The conditioning-, washing- and eluting steps were described previously[6]. The SPE-columns were each preconditioned with 2 ml 0.1 % NH_4OH in methanol, methanol and water. After the transfer of the samples onto the weak anion exchanger, the SPE-columns were each washed with 2 ml 0.025 mol/l

sodium acetate (pH 4) and methanol and subsequently put under vacuum suction until dryness. Elution was performed with 2 ml 0.1 % NH_4OH in methanol.
Muscle tissue and organs were hydrolysed using pepsine. Internal standard solution and 20 ml of a pepsine solution (1 g/100 ml; pH 2 – 2.5) were added to 1 - 3 g of the grinded sample. The samples were incubated at 37 °C for 12 hours. After incubation, the samples were heated up to > 90 °C for 10 minutes. After addition of 20 ml methanol the samples were sonicated for 15 minutes. After a centrifugation step, the samples were purified and concentrated using SPE on an OasisWAX (150 mg/6 ml) column. The conditioning-, washing- and eluting steps were performed in the same way as described for plasma with the exception that 4 ml instead of 2 ml were used for conditioning, washing and eluting. After the elution step, all sample solutions were evaporated in a gentle stream of nitrogen after addition of 30 µl glycerol as a keeper. After reconstitution with a mixture of methanol (40 %) and water (60 %), the samples were measured using HPLC-MS/MS. Quantification was done by using isotope dilution analysis.
HPLC-MS/MS Analysis: A small column (Eclipse Plus, C18, 3.5 µm, 4.6 x 30 mm Agilent Technologies) was placed as a pre column between purge valve and autosampler to separate background PFCA and PFAS from the analytes of the samples. Also an in-line filter (Replacement frits 4.6 mm, 0.2 µm Agilent Technologies) between autosampler and column was used to filter remaining particles from the samples. An injector program was used to minimize a potential cross-contamination from heavily contaminated samples as far as possible.
HPLC-Parameters: The separation was performed on an Agilent 1200 SL HPLC-System. A Gemini column (3 µm, C18, 110 A, 150 x 2 mm) from Phenomenex was the appropriate column to separate the individual analytes. The column temperature was held at 50 °C. A mixture of 2 mM ammoniumacetate (95 %) and acetonitrile (5 %) (v/v) was used as solvent A and a mixture of methanol (40 %) and acetonitrile (60 %) (v/v) was used as solvent B. The flow rate was 0.3 ml/min.; Gradient: 10 % B (2 min. hold), 10 % B to 85 % B (7 min.), 85 % B to 98 % B (3 min.), 98 % B (hold 7 min.), equilibration 10 min. 5 µl of the extract were injected into the HPLC-MS/MS system. The total run time was 30 minutes.
*Source parameters:*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.
Gas temperature: 300 °C; gas flow: 4 l/min; sheath gas temperature: 350 °C; sheath gas flow: 10 l/min; nebulizer: 43 psi; capillary: 3000 V (negative); nozzle voltage: 0 V. MRM settings for linear PFC are published elsewhere[4].

Results and discussion

Analysis

In validation experiments, good recoveries for PFCA with six to twelve carbon atoms and PFSA with four, six, seven, eight and ten carbon atoms could be achieved in all matrices by using the described methods. Because of the surface activity of the PFCA and PFSA, it was important to minimize a potential cross-contamination from Teflon® containing parts of the analytical instrument and/or from highly contaminated samples as far as possible. This is successfully performed by using a trap column between pump and autosampler and an injector-program. By using a mixture of methanol and acetonitrile as one solvent component, it was possible to separate all PFAA of interest chromatographically from each other. The limits of quantification were low, despite of the strict criteria of a signal to noise ratio of 3:1 of the least intensive transition at the limit of detection according to SANCO 10684/2009.

Calculation

A carry-over from naturally PFAA-contaminated feed into food of animal origin could be observed. For example, high concentrations of PFAA were found in liver of the animals from the feeding study. Cows and pigs were fed with different kinds of PFAA-contaminated feed and the amount of feed consumed was also different. To be able to compare the results of both feeding studies, the fractions of the individual PFAA in tissue in relation to the cumulative intake were calculated. In this way, it was also possible to identify correlations between the chain length of the PFAA and the absorption in the various tissues.

Results for Plasma, Muscle Tissue and Organs

In the pig experiment, results of two control animals for each of the three groups were available. Neither in plasma nor in muscle tissue and organs of the control animals relevant PFAA concentrations could be observed.

In plasma samples from the cows taken before the beginning of the feeding study, PFAA could not be detected in relevant concentrations. In feed used for the feeding experiment with the cows, PFCA from six to nine carbon atoms and PFSA with four, six, seven and eight carbon atoms could be quantified. In feed used for the feeding experiment with the pigs, PFCA from six to eight carbon atoms and PFSA with four, six, seven and eight carbon atoms could be quantified.
In the cow experiment, less than 10 % of the ingested PFOA[2] was found in plasma during and after the PFAA-feeding-period (Figure 1). The fractions of PFHxA and PFHpA in plasma were even smaller (not shown). However, in contrast to these compounds, a considerably higher fraction of PFNA could be detected in cow plasma (Figure 1).

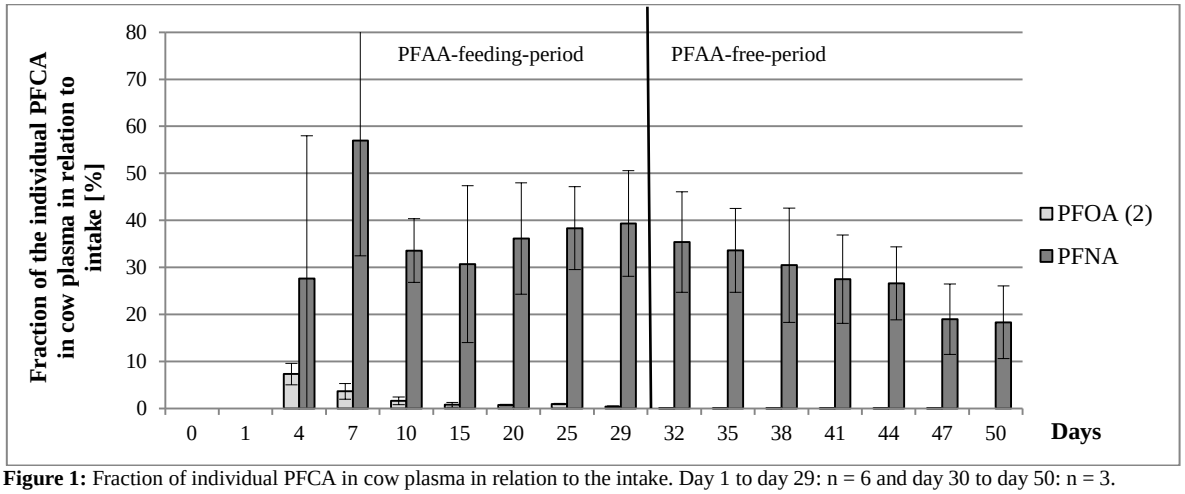

Figure 1: Fraction of individual PFCA in cow plasma in relation to the intake. Day 1 to day 29: n = 6 and day 30 to day 50: n = 3.

After the end of the PFAA-feeding period, the PFNA-concentration in cow plasma decreased slowly and thus, the PFNA fraction in relation to the intake also decreased slowly (Figure 1).

The situation in pig plasma was very different. The fractions of PFHpA and PFOA[3] in pig plasma contributed to around 30 % to 35 % to the cumulative intake during the 21 days long feeding period (Figure 2). The fraction of PFHxA decreased constantly from about 25 % to about 10 % during the PFAA feeding period (Figure 2). Because of the fact that no relevant differences between sows, barrows and boars could be observed, the mean of all 24 pigs were taken.

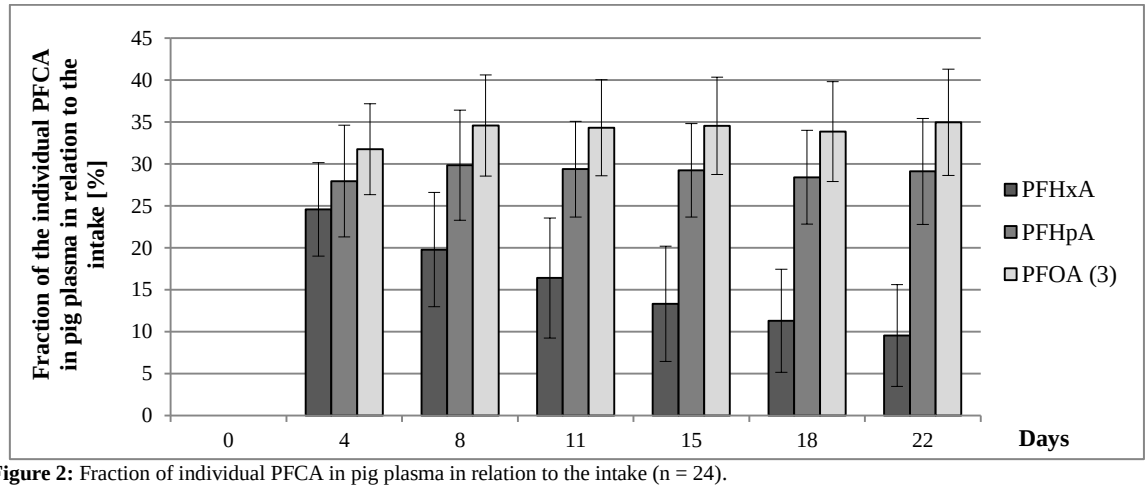

Figure 2: Fraction of individual PFCA in pig plasma in relation to the intake (n = 24).

PFNA could not be detected in the PFAA contaminated feed used in the pig experiment. Differences could also be observed for PFSA in plasma of cows and pigs. The fractions of PFBS, PFHxS and PFHpS in pig plasma were about 40 % in relation to the intake during the 21 days long feeding period. The fraction of $PFOS^3$ in pig plasma was about 15 % to 20 %. In cow experiment, nearly no ingested PFBS could be observed in the plasma. The fractions of PFHxS and PFHpS were lower in the cow than in the pig experiment. Only the fractions of PFOS were similar in the pig and the cow experiment[2,3].
About 5% of the ingested PFHpS and about 20 % of the ingested $PFOS^2$ was found in cow livers. The concentrations as well as the fractions of the other PFAA, with the exception of PFNA, were much lower. In cow kidney, fractions were ten times lower than in cow liver. About 40 % of the ingested $PFOS^2$, about 25 % of the ingested PFHpS and about 10 % of the ingested PFHxS was accumulated in muscle tissue of cows slaughtered directly after the PFAA-feeding-period and in cows slaughtered three weeks after the end of the PFAA-feeding period. The results of the pig experiment are shown in Table 1.

Table 1: Fraction of PFAA in relation to the intake [%] in muscle tissue and liver of pigs (n = 24).

	PFBS	**PFHxA**	**PFHpA**	**PFHxS**	**$PFOA^3$**	**PFHpS**	**$PFOS^3$**
Muscle tissue	29.3 ± 7.2	7.0 ± 4.6	21.3 ± 6.2	31.8 ± 8.7	29.8 ± 7.9	34.3 ± 9.2	28.2 ± 5.6
Liver	5.6 ± 1.4	0.9 ± 0.6	1.8 ± 0.5	2.7 ± 0.7	4.1 ± 1.1	8.2 ± 2.8	29.4 ± 8.7

The larger fractions of PFBS, PFHxS, PFHxA, PFHpA and $PFOA^3$ in liver and muscle tissue of pigs compared to cows could be explained with the higher concentration of these substances in pig plasma in contrast to cow plasma.

Comparison with other studies
In studies with cows performed by Lupton *et al.* (2011)[7], one group of animals got a single dose of radioactive PFOA and another group received a single dose of natural PFOS. The obtained results for plasma are comparable with the results of the feeding study with cows presented in this paper. Lupton *et al.* (2011)[7] reported a smaller fraction of PFOS in liver and muscle tissue compared to the present study. From other studies, data on PFAA with other chain length as well as data for pigs were not identified.

Summary
The feeding studies have shown that even under realistic conditions a carry-over of PFCA and PFSA from naturally contaminated feed into food of animal origin is possible. An accumulation could not only be observed for PFOS and PFOA but also for PFCA and PFSA with other chain length. Furthermore, considerable differences between their accumulation in cows and pigs could be observed.

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