PAPER

Monitoring of the declining trend of Polychlorobifenyls concentration in milk of contaminated dairy cows

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Abstract

Six pregnant heifers, coming from a herd with a history of high concentration of Polychlorobifenyls (PCB) into the milk, were fed with a very low-PCB diet starting from the 6th month of pregnancy. After parturition cows were milked for at least 190 d with a maximum of 270 d. Diet was made of: corn silage (31.7% of DMI), dehydrated alfalfa (13.3% of DMI), grass hay (3.7% of DMI) and concentrate (51.3% of DMI). The average DMI was 23.12 kg/d. Milk production was recorded and samples of milk and blood were taken and analyzed for PCB (18, 28, 31, 52, 44, 101, 149, 118, 153, 138, 180 and 194 congeners) content using a GC-MS technique. The average milk yield (kg/d) of the 6 cows was 26.0, 22.5, 23.2, 24.5, 28.9, 29.3. The maximum PCB concentration of 100 ng/g of fat allowed by the Italian law was reached after 144-209 days of lactation. One animal after 204 days had a PCB concentration of 102 ng/g fat. If $\log_{10}$ of PCB concentration (ppb) in milk fat is regressed against days in milking (DIM) the following significant equation was obtained:

$$\log_{10} \text{PCB (ng/g of milk fat)} = 2.796 - 0.00474 \text{ DIM}; r^2\ 0.72; P<0.01$$

The initial level of contamination is not the main factor affecting the time required in order for PCB to return to below the legal threshold of 100 ng/g fat, while daily milk yield significantly affects PCB excretion. Body condition and energy balance could be important factors affecting PCB excretion.

In the presence of high contamination, about 6 months of lactation are required in order to obtain milk with a sufficiently low PCB content.

Among the seven congeners considered by Italian legislation, PCB 101, PCB 118, PCB 138, PCB 153 constitute almost all the PCBs found in tissues and milk, with a much smaller presence of PCB 180, and the numbers of the congeners PCB 28 and PCB 52 are almost insignificant, probably because they accumulate little in the body.

The congeners PCB 138 and PCB 153 are those most frequently found. For PCB 138 the liver is the principal organ of accumulation, while for PCB 153 we found equal accumulations in the liver, kidneys and tail, but a low presence in milk. The PCBs 118 and 138 are those most abundant in milks.

Introduction

Polychlorobifenyls (PCB) are compounds constituted by two enjoined phenylic rings, variously substituted with chlorine atoms (from 1 to 10 per molecule), of which there are 209 isomers. PCBs have been used as lubricants, insulators, heat conductors and fire-retardants; due to their elasticity they have also been widely used in varnishing. Currently their use is in marked decline because of their toxicity, as they are, in fact, endocrine disruptors, which induce alterations in the sexual cycle of mammals (including man), reduce spermatogenesis (Hays and Aylward, 2003) and damage thyroid (Langer *et al.*, 2005). They can also cause dermatitis and are considered by the International Agency for Research on Cancer (IARC) to be human cancerogens (IARC, 1987; European Food Safety Authority, 2005).

Being lipophile molecules, they tend to accumulate in the adipose tissue of animals and then enter the human food chain through the ingestion of milk, eggs and meat, and fish have the highest levels of contamination, due to the accumulation of PCBs in marine sediments (Larsen, 1995; Dùarte-Davidson and Jones, 1995). The contamination of foods of vegetable origin is also possible by root absorption and atmospheric deposition. In literature there are cases of intoxication due to accidental contamination in food production (Bernard *et al*., 1999). Recent cases of contamination by PCBs have occurred in Belgium and Denmark (Bernard *et al*., 2002).

Milk, which contains from 3.5 to 4% fat, can be contaminated by PCBs through ingestion of the molecule by animals, and for this and other foods EU legislation has fixed limits for the quantities of PCBs present in foods (European Commission, 2006).

PCB excretion in milk is a process governed primarily by the lipophilicity of the congener and the lipid content of the carrier (McLachlan, 1994; Moser and McLachlan, 1999). Fox *et al.* (1994) observed that an increase in the degree of chlorination and the planarity of the molecule causes a slow transportation of the congener and consequently a reduction in its excretion through milk.

The aim of this work was to monitor the concentration of PCBs in primiparous cows' milk accidentally exposed to contamination during pregnancy, to determine the time necessary for levels of contamination to return within legal limits and to monitor the excretion of individual congeners.

Materials and methods

Animals and diets

Six pregnant heifers (540±42 kg), coming from a herd with a history of PCB contamination through feeds, were fed witha low-PCB diet from the 6th month of pregnancy. After parturition, cows were milked for at least 190 d with a maximum of 270 d. The diets used for the last month of pregnancy and during lactation are reported in Table 1. At the end of the

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Table 1. Chemical composition and nutritional traits of the diets.

Feeds	Dry period (kg/day)	Lactating period (kg/day)
Corn silage	6.0	22.0
Alfalfa hay	-	3.5
Grass hay	9.0	1.0
Concentrate	1.6*	12.0°
Mix corn (70%) and barley (30%)	-	1.5
Straw	1.0	-
Dry matter intake, kg/d	9.49	19.9
Moisture, %	45.42	46.22
Crude protein, % DM	12.77	16.50
Crude fibre, % DM	28.39	30.82
Fats, % DM	2.21	2.36
Starch, % DM	10.95	16.56

*Mineral-vitamin supplementation (kg as fed): Vit. A U 25,000; Vit. D_3 U 1000; Vit. E mg 40; Cu mg 15; Zn mg 100; Mn mg 23; I mg 1.50; Se mg 0.34; Co mg 0.30. °Mineral-vitamin supplementation (kg as fed): Vit. A U 25,000; Vit D_3 U 1500; Vit. E mg 40; Cu mg 20; Zn mg 110; Mn mg 23; I mg 1.80; Se mg 0.34; Co mg 0.38.

Table 2. PCBs precursor and monitoring ion in MS-MS.

PCBs sorted in order of retention time	Precursor ion	Monitoring ions
PCB 18	258	186, 220
PCB 28, 31	258	186, 220
PCB 52	292	222, 258
PCB 44	292	222, 256
PCB 101	326	256, 291
PCB 149	326	254, 290
PCB 118	360	290, 325
PCB 153	360	290, 325
PCB 138	360	290, 325
PCB 180	394	324, 360
PCB 194	430	360, 395

trial the animals were slaughtered and internal organs (tail fat, body fat, liver, kidney) were sampled for xenobiotic determination.

Milk and blood samples

Milk production was recorded daily and individual milk samples (250 mL) and blood (50 mL) were taken every 15 days. Milk samples were taken from the two milkings and mixed in proportion to the respective production. Milk samples were analysed to determinate the fat and protein content with Milkoscan, (Foss, Denmark). Blood samples were taken by venupuncture in Li-eparin Vacutainer (Becton & Dickinson, Franklin Lakes, NJ, USA) and centrifuged at 3300 g for 10 min. Plasma was recovered and stored in a separate tube at -20°C before the PCB analysis.

All the milk produced was destroyed in accordance with the Italian Law.

Milk and serum analysis

In a separating funnel, an aliquot of 50 mL of milk or 20 of mL serum was diluted with an equal volume of ethanol 95°C, then a liquid-liquid extraction was performed by adding 100 mL hexane. After shaking vigorously for about 1 min, the organic layer was transferred into a 500 mL round-bottomed flask and the extraction was repeated twice. The combined hexane extracts were evaporated to dryness using a rotavapor at 35°C; the residue was redissolved with three portions of 2 mL hexane using ultrasonication and quantitatively transferred to a 10 mL glass vial; then, 2 mL of sulphuric acid (96-98%) was added. After shaking and centrifugation, the organic phase was applied to a Florisil column (5 g Florisil 60-100 mesh pre-treated at 450°C for 2 h and tapped with 2 g anhydrous sodium sulphate) and the PCBs were eluted with 90 mL hexane (recommended for pesticide analysis). The eluate was concentrated and blown to 1 mL for milk and 0.5 mL for serum.

Kidney and liver analysis

A 30 g homogenised kidney or liver was extracted with 100 mL hexane and shaken vigorously for 2 h; then the mixture was filtered through an anhydrous sodium sulphate layer. The solution was treated with sulphuric acid and purified by a Florisil column as described above.

Body fat analysis

An aliquot of 5 g homogenised fat was treated with 100 mL methanolic KOH 2N for 1 h at 80°C in a round-flask with bubble condenser. The PCBs were extracted with 100 mL of hexane three times in a separating funnel. Then the organic phase was treated as described above.

GC-MS-MS analysis

The PCB standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). The PCBs congeners 18, 28, 31, 52, 44, 101, 149, 118, 153, 138, 180 and 194 were determined. The extract was injected (2 L) into a Thermo-Quest Trace GC coupled to a Polaris Q ion trap mass-spectrometer. The instrument was controlled by Thermo-Quest Excalibur 1.2 software. Helium was used as a carrier gas at a flow rate of 1 mL/min and the analysis was carried out with a fused-silica capillary column RTX-5 MS (Restek Comp.) 30 m length, 0.25 mm i.d. The injection was in PTV solvent split mode (from 70° to 250 C at 5°C/sec) and split ratio 1:50. The GC program was: 70°C hold 5 min., increased to 220°C (10°C/min), hold 3 min, increased to 300°C (10°C/min), hold 3 min. Transfer line temperature was 300°C. The analysis was carried out with MS-MS mode selecting a characteristic ion for each PCB (Table 2). The quantification was made on an external standard procedure monitoring the areas of the ions listed in Table 2. Recoveries were determined by adding known volumes of a PCBs standard to blank samples. Results were found to be within 89-102%; the limit of quantification was 0.1 μg/kg.

Data analysis

Statistical analyses were performed using the statistical package SAS (Release 8.0); PROC GLM was utilized for the analysis of congener pattern in milk and tissues. The comparison among regression's equation of PCB congeners was performed using the parallel test as described by Camussi *et al.* (1995).

Table 3. Initial PCB content and time required in order that PCB content become lower than maximum acceptable level.

	Cow 1 214	Cow 2 228	Cow 3 235	Cow 4 242	Cow 5 240	Cow 6 233
Initial PCB content of milk, ng/g fat	1388	1142	709	958	596	490
Days to drop under 100, ng/g fat	167	165	209	144	not reach	189
Average milk yield, kg/d	29.3	28.9	24.5	23.2	22.5	26.0

Table 4. PCB concentration (ng/g wet weight) in blood, milk and several tissues at the end of the experiment.

	Cow 1 214	Cow 2 228	Cow 3 235	Cow 4 242	Cow 5 240	Cow 6 233
Body fat	90.5	194.3	460.7	224.4	241.3	150.4
Tail fat	98.3	134.0	375.0	206.7	177.8	155.8
Liver	0.4	0.9	0.8	1.1	1.2	0.9
Kidney	2.3	5.5	10.2	6.0	8.7	7.9
Blood	0.5	0.6	1.0	0.5	0.7	0.6
Milk	1.1	1.5	1.6	1.3	1.9	1.6

Results and discussion

At the end of the trial the animals were slaughtered and internal organs were taken and analysed to determine levels of xenobiotic concentration. The PCB content of liver ranged from 0.35-1.2 ppb and was negatively correlated with average PCB blood levels (r=-0.84; P<0.05).

Rate of excretion of PCBs in milk

All the animals started lactation with very high levels of PCB in milk, ranging from 490 to 1388 ng/g fat, and the maximum PCB concentration of 100 ng/g of fat allowed by the Italian law was reached after 144-209 d of lactation (Table 3). After 204 days one animal had a PCB concentration of 102 ng/g fat.

In order to avoid painful biopsies for the animals, the initial PCB concentration in body fat was not determined, so it was not possible to correlate the total PCB excreted into the milk with PCB body burden.

The initial level of contamination is not the main factor affecting the time required for PCB levels in milk to return to below the legal threshold of 100 ng/g fat, actually the relationship between initial PCB concentration in milk and the time required to reach the legal threshold of 100 ng/g fat, is not significant: (r^2 0.423; P 0.156). This could means that the amount of PCB mobilized from body fat storage is not proportional to the initial amount of PCB.

Daily milk yield and days in milk (DIM) significantly affect PCB concentration; if the initial PCB concentration in any cow is assumed to be =100, the following equation can be obtained:

PCB in milk=
107.87 - 1.678 daily milk yield - 0.299 DIM
(r^2 = 0.58; P< 0.0001)

This could be explained by the lipophilicity of PCB molecules, that mainly accumulate in body fat, the main route of excretion being through milk. After parturition feed intake does not match energy requirements for milk yield, so a strong fat mobilization occurs to support milk synthesis; this is believed to increase PCB excretion in milk. As lactation continues, feed intake increases, compensating for the energy balance of the animals and reducing the need for fat mobilization, which helps to lower PCB concentration in milk.

If $\log_{10}$ of PCB concentration (ppb) in milk fat is regressed against days in milk (DIM), the following significant equation is obtained:

$\log_{10}$ PCB (ng/g of milk fat)=
2.796 - 0.00474 DIM; r^2 0.72; P<0.01

Assuming the initial concentration of PCBs=100, the rate of excretion in milk was determined for each of PCBs and the following equations (significant at P<0.0001) were calculated:

PCB 101 in milk fat:
59.750 - 0.3037 DIM r^2= 0.52

PCB 118 in milk fat:
61.926 - 0.3259 DIM r^2= 0.52

PCB 138 in milk fat:
71.658 - 0.3281 DIM r^2= 0.48

PCB 153 in milk fat:
62.364 - 0.2949 DIM r^2= 0.39

The slopes of each equation were compared with the parallel test and found not different.

PCBs in tissues and organs

As already mentioned, PCBs are lipophile substances and therefore are deposited in adipose tissue, which is confirmed in Table 4, where PCB levels are compared for the animals slaughtered in our study, and which indicates that it is in adipose tissue that PCBs are principally found. The concentration of PCBs in adipose tissue is around 30 times more than in the kidneys, 130 times more than in milk, 250 times more than in the liver and 300 times more than for haematic contamination. Considering that even with variations due to the nutritional and physiological state, adipose tissue represents around 15% of a cow's weight (Gibb *et al*., 1992), this confirms that for milk cows this is definitely where most PCBs are found (Thomas *et al*., 1999a).

Among the seven congeners considered by

Table 5. Variations of PCB congeners percentage in milk (on sum of PCB) during the trial.

	PCB congeners						
	28	52	101	118	138	153	180
First week	0.61^{Aa}	0.15^{a}	14.55^{Bc}	34.91^{Bc}	26.99^{ABa}	18.75	4.04^{b}
First month	1.35^{Abab}	0.31^{ab}	14.13^{Bbc}	35.64^{Bc}	26.55^{Aa}	18.67	3.34^{ab}
Second month	3.02^{BCbc}	0.75^{abc}	12.79^{ABb}	30.61^{ABb}	32.63^{BCb}	19.63	0.57^{a}
Third month	4.26^{CDcd}	1.22^{bc}	12.01^{Aa}	25.54^{Aab}	34.23^{Cbc}	22.22	0.53^{a}
End trial	5.59^{Dd}	1.32^{c}	11.12^{Aa}	23.07^{Aa}	37.67^{Cc}	21.22	nd*
SE	0.592	0.326	0.477	1.424	1.504	1.648	1.053

*Under the detection level. Means in the same column with different upper letter significantly differ. SE = standard error of the means. ^{a,b,c,d}: P<0.05; ^{A,B,C,D}: P<0.01.

Table 6. Congener profiles of blood, milk and tissue (percentage of PCB's sum).

	PCB congeners							
	28	52	101	118	138	153	180	$SE_{congeners}$
Body fat	^{A}0.99^{Aa}	^{Aa}0.24^{Aa}	^{ABb}8.98^{Bc}	^{Aa}24.08^{Cd}	^{Bb}31.44^{Ef}	^{ABa}27.73^{De}	^{b}6.53^{Bb}	0.648
Tail fat	^{A}1.04^{Aa}	^{Aa}0.25^{Aa}	^{ABb}8.67^{Bb}	^{Aa}22.08^{Cc}	^{BCb}31.80^{Dd}	^{Bb}29.27^{Dd}	^{b}6.88^{Bb}	1.099
Liver	^{A}0.48^{Aa}	^{Aa}0.12^{Aa}	^{ABb}8.91^{Bb}	^{Aa}20.35^{Cc}	^{Dc}38.90^{Ee}	^{Bb}28.72^{Dd}	^{ab}3.17^{ABa}	1.969
Kidney	^{A}0.31^{Aa}	^{Aa}0.07^{Aa}	^{Bb}9.96^{Bb}	^{ABa}24.61^{Cc}	^{BCa}31.93^{Dd}	^{Bb}31.12^{Dd}	^{a}2.00^{Aa}	0.980
Blood	^{A}0.67^{Aa}	^{ABa}0.33^{Aa}	^{Aa}4.44^{Aa}	^{Bb}34.92^{Bc}	^{Aa}24.76^{Bb}	^{Bb}32.54^{Bbc}	^{a}1.23^{Aa}	3.205
Milk	^{B}5.59^{ABa}	^{Bb}1.32^{Aa}	^{Bb}11.12^{Bb}	^{Aa}23.07^{Cc}	^{CDc}37.68^{Dd}	^{Aa}21.22^{Cc}	udl	1.575
SE_{tissue}	0.619	0.252	1.343	2.624	1.544	1.741	1.284	

Means in the same row, with different right upper letter significantly differ. Means in the same column with different left upper letter significantly differ. SE = standard error of the means. ^{a,b,c,d,e,f}: P<0.05; ^{A,B,C,D,E}: P<0.01. udl= Under the detection level.

the Italian legislation, PCB 101, PCB 118, PCB 138, PCB 153 constitute almost all the PCBs found in tissues and milk, with a much smaller presence of PCB 180, and the numbers of the congeners PCB 28 and PCB 52 are almost insignificant (Table 5), probably because they accumulate little in the body (Thomas *et al.*, 1999c).

The congeners PCB 138 and PCB 153 are those most frequently found, confirming, as reported by Thomas *et al.* (1999c) and by Wycklund-Glynn *et al.* (2000), that PCB 153 is the most correlated congener of all PCBs.

For PCB 138 the liver is the principal organ of accumulation, as reported also by Thomas *et al.* (1999a), while for PCB 153 we found equal accumulations in the liver, kidneys and tail, but a low presence in milk. PCB 153 is excreted very slowly due to its non-planarity (Antunes *et al.*, 2007) and shows concentrations in milk lower than in other organs (Table 6). This is coherent with a slower mobilisation of the congener compared to the other PCBs and confirms the results of Thomas *et al.* (1999b,1999c) and McLachlan (1993) that insert PCB 153 in the group of persistent and more slowly metabolised congeners. A greater persistency of PCB 153 was observed both in humans (Linderholm *et al.*, 2007) and in sardines (Antunes *et al.*, 2007).

PCB101 and 118 are found prevalently in milk or blood (Table 6), which is justifiable by the planarity of the molecules which facilitates mobilisation and excretion (Antunes *et al.*, 2007).

PCBs in milk

The PCBs 118, 138, and 153 are those most abundant in milks (Table 5), confirming the results of Thomas *et al.*, (1999a). The concentration of PCB 118 in milk is lower than in blood (Table 6), according to Thomas *et al.* (1999c), which can be explained by the fact that the congeners contained in blood are not necessarily totally amenable to being incorporated in tissues, perhaps because they are associated with haematic components, which could explain the discrepancy (Vrecl *et al.*, 2005).

Further, after the first month of lactation the percentage of PCB 118 drops considerably, which is in itself strange, considering that this congener belongs to the group of those of elevated persistence, but it must not be forgotten that among these PCB 118 is the one with the shortest period of permanence (Antunes *et al.*, 2007).

The percentage of PCB 101 in milk observed in our study (11.12%) is clearly higher than the one reported in the data of Thomas *et al.* (1999c) (0.8 %), but it must be pointed out that in that study the percentage of PCB 101 deposited in bodies was also low compared to milk (0.5%). This behaviour is therefore analogous to the observations in our study, where greater corporeal accumulations of PCB 101 correspond to greater excretions in milk.

PCB138 accumulates predominantly in the liver, but its concentration in milk is also higher than what observed in other tissues and according to Thomas *et al.* (1999b) and McLachlan (1993) this molecule is among the most slowly metabolised ones.

From the first week of lactation the presence of the congeners PCB 28 and 52 is markedly reduced (Table 5), which could seem to derive from the slow initial concentration in corporeal deposits and a low carry over rate (COR) in milk, as reported by Thomas *et al.* (1999a, 1999b, 1999c) and clearly lower than that of

the congeners 138, 153 and 180.

Although remaining low, the percentage of these congeners of the total number of PCBs tends to increase during lactation, probably due to the drop in the number of the more abundant congeners, but which are also excreted more rapidly due to a higher COR.

The low concentration PCB 180, markedly lower than congeners 138 and 153 particularly in milk, contrasts with the studies by Thomas *et al*. (1999a, 1999c) in which PCB 180 showed elevated persistence and constituted almost 10% of all PCBs in milk (Thomas *et al*., 1999c).

A low initial presence of this congener can be hypothesised, seeing that already after a week of lactation it constitutes only 4.04% of PCBs excreted in milk; the high value of COR reported by Thomas *et al.* (1999a, 1999c) indicates a consistent transfer of the congener into milk, exhausting the probably unimportant corporeal reserves.

The percentage of PCB 138 increases during lactation probably for two reasons: on one hand its elevated persistence and on the other one the elevated carry-over rate in milk (Thomas *et al*., 1999a, 1999c).

Conclusions

The initial level of contamination is not the main factor affecting the time required in order for PCB to return to below the legal threshold of 100 ng/g fat and milk yield did not affect the rate of PCB excretion (r^2=0.35; P<0.22). Body condition and energy balance could be the most important factors.

In the presence of high contamination, about 6 months of lactation are required in order to obtain milk with a sufficiently low PCB content.

There are differences in the excretion of different congeners, at the start of lactation PCB 118 is the most abundant, while at the end of the study PCB 138 was most present.

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