UNIVERSITEIT ANTWERPEN

UNIVERSITAIRE INSTELLING ANTWERPEN

FACULTEIT WETENSCHAPPEN

Departement Scheikunde

ENVIRONMENTAL ANALYSIS AND BIOMONITORING OF PERSISTENT ORGANOHALOGENATED POLLUTANTS

MILIEUANALYSE EN BIOMONITORING VAN PERSISTENTE ORGANOGEHALOGENEERDE POLLUENTEN

Proefschrift voorgelegd tot het behalen van de graad van doctor in de Scheikunde aan de Universiteit Antwerpen te verdedigen door

Adrian COVACI

Promotor : **prof. dr. P. Schepens**

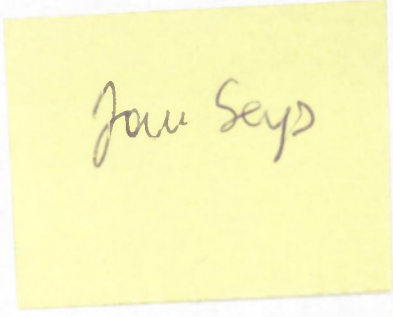
Co-promotor : **prof. dr. F. Adams**

Antwerpen, 2002

To VLIZ

Kindest regards

Adrian



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Thesis Committee

Head
Prof. Dr. Magda Claeys (Laboratory of Mass Spectrometry, UIA, Belgium)

Promotor
Prof. Dr. Paul Schepens (Toxicological Centre, UIA, Belgium)

Co-promotor
Prof. Dr. Freddy Adams (Department of Chemistry, UIA, Belgium)

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'The world was not left to us by our parents,

it was lent to us by our children'.

African proverb

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Part 2. POPs in the environment

Chapter 1

Introduction

1.1. Persistent, bioaccumulative and toxic organic pollutants (POPs)

The presence of persistent man-made chemicals in our environment is not a new problem. However, it was not until the beginning of the 1960s that environmental pollutants aroused debate and concern (Carson, 1962). Since then, a large number of chemicals have been identified in environmental samples, and the time trends of their concentrations have been the subject of continuous interest. Apart from the heavy metals, the group of persistent organic pollutants (POPs) (Jones and de Voogt, 1999) includes many compounds regarded as major environmental problems, e.g. organochlorine pesticides, polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs) and dibenzofurans (PCDFs), the two latter groups generally called dioxins. These compounds and their harmful effects on the environment and humans have been extensively reviewed. Today the effects of these substances are relatively well known, although their mechanism(s) of action remains largely unresolved. The toxicity of the organohalogenated chemicals and their presence in certain food items, mostly of animal origin, have resulted in the introduction of dietary restrictions and recommendations by food administrations in different countries. Continuous monitoring of the environmental levels of these chemicals has shown a decreasing trend in their occurrence over the last 10 years or more in many Western countries.

POPs are persistent, bioaccumulative chemicals, and posses toxic characteristics likely to cause adverse human health or environmental effects. They are prone to long-range atmospheric transport and deposition (Wallack et al., 1998, UN ECE, 1996).

During the past three decades, analytical data have revealed global contamination of aquatic and terrestrial environments (Tanabe et al., 1994; Brydon et al., 1995). In large measure, this is the logical consequence of the physical and chemical properties of POPs:

1. POPs are highly resistant to chemical and biological degradation. Polychlorinated biphenyls (PCBs) and other chlorinated pollutants, particularly the highly chlorinated ones, have been known to persist in soil, water, sediment and biota for long periods of time (Jones and de Voogt, 1999);
2. POPs are non-polar molecules that can accumulate in fatty tissues. This results in their biomagnification in the higher trophic levels of the food chain (Shaw and Connell, 1986);
3. Many POPs were, and still are found in pristine areas where there are no known sources of release to the environment, demonstrating that POPs are subject to long-large transport from their initial source (Muir et al., 1992).

It was suggested that the major mechanism for this mobility is a cyclical evaporation from soil and water surfaces in which winds lift POPs into the air along with water vapour and dust, eventually depositing them with rain, snow, or adsorbed to particles. With repeated evaporation and deposition, the net result is movement of POPs over long distances in the direction of atmospheric air movements. Models of this mobile behaviour correlate well with the measured POP concentrations in the Northern Hemisphere (Muir et al., 1992). The most serious data gap for the prediction of environmental behaviour is the degradation rate and their regional variability based on specific transport conditions. More data are needed in this area. The main topic of further research is the study of deposition/emission processes, transformation processes and bioavailability of POPs in terrestrial and marine ecosystems (Wallack et al., 1998).

1.2. Organochlorine pesticides

Organochlorine pesticides are chlorinated hydrocarbons used predominantly as insecticides which can be divided according to their chemical structure in the several groups. The following pesticides were investigated in this study: DDT and analogues, hexachlorocyclohexane isomers and hexachlorobenzene. Other groups include cyclodienes (aldrin, dieldrin, heptachlor and chlordane) and toxaphene.

a. DDT and metabolites

DDT (or 4,4′-DDT), an acronym for 1,1,1-trichloro-2,2-*bis*(4-chlorophenyl)ethane, is a broad spectrum, persistent insecticide. It has been used for the control of insect pests in gardens, fields, forests, and orchards, especially on cotton, soybean, and peanut crops (Kutz et al., 1991). The worldwide introduction of DDT started in 1945. Its use in the Western World is now severely restricted or banned. In Belgium, the use of DDT has been banned since 1974 (Ministerieel Besluit, 1974). However, it is still extensively used in some developing countries for the control of malaria vector (Roberts et al., 2000). Technical DDT contains mainly the p,p'-isomer (75-80%), while o,p'-DDT constitutes 10-25%. The o,p'-isomers are less stable than the p,p'-isomers, and are therefore only found in low concentrations in nature. p,p'-DDT is metabolized to p,p'-DDE (Figure 1.1), the latter compound being especially important because of its stability and accumulation potential resulting in high residue values in biota.

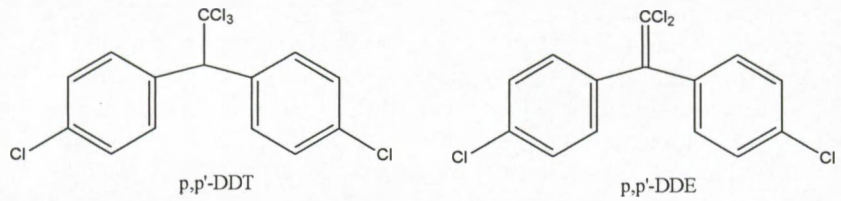


Figure 1.1. Chemical structures for p,p'-DDT and p,p'-DDE.

The ratio between p,p'-DDT and p,p'-DDE gives an indication of the exposure time. Higher ratios indicate a recent exposure to the undegraded technical pesticide, whereas lower ratios usually indicate an earlier exposure or chronic exposure through the food chain. All DDT derivatives are stable under normal environmental conditions and are resistant to complete breakdown by the enzymes present in soil microorganisms and higher organisms. DDT and its derivatives are very soluble in lipids and organic solvents and practically insoluble in water. DDT is stable in strong acids and can withstand acid permanganate oxidation.

Environmental fate

DDT has entered surface water, soil and sediments either by direct spraying of the water/soil during insecticide use or indirectly when rain-washed soil containing DDT entered surface waters. DDT, like all the organochlorine pollutants has a strong tendency to adsorb on surfaces. While in the past, DDT entered the air directly when used as an insecticide, in the present time, DDT may be released into the air due to evaporation from soil and surface waters, soil erosion and manufacture and use in developing countries.

Once in the environment, DDT captured in soil or sediment persists for a very long time (the half-life is estimated to be more than 5 years) (Corona-Cruz et al., 1999). DDT may evaporate from the soil and may be broken down partially by atmospheric oxidation or by

microorganisms. DDT in soil can be absorbed by specific plants and can expose the animals and human eating those crops. DDT in water and sediment can be absorbed by aquatic organisms and then concentrates in fish eating these organisms. The level of DDT in animals and humans are higher than in the environment because lipid-containing cells accumulate DDT and because its metabolism is very slow.

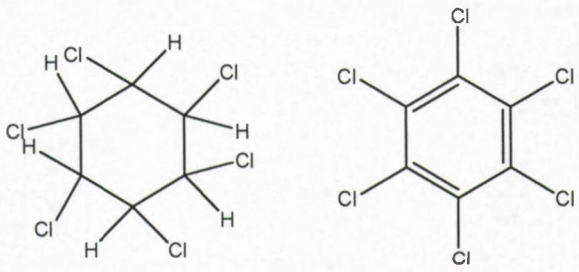
Exposure pathways and metabolism
Human general population is exposed to DDT mainly by food (meat, milk, fish, eggs) containing small amounts of this compound. Infants can be exposed by drinking of breast milk. DDT inhaled with air represents less than 1 % of its total daily intake. DDT does not enter the body through the skin except direct skin exposure during spraying (Geyer et al., 1986). Once inside the body, DDT is slowly metabolised to DDE and DDD, both persistent and lipid soluble. DDE can also be metabolised to a persistent and lipid soluble methyl-sulphonyl compound, which, in some species of experimental animals, influences adrenal cortex activity.

Toxicological effects
In high oral doses, DDT was shown to affect the nervous system (US DHHS, 1994a), but these effects disappear once the exposure stopped. Workers manufacturing DDT exposed for a long time to DDT had some reversible changes in the levels of liver enzymes. However, there was no sign that DDT caused permanent harmful effects. Animal studies have shown that long term exposure to DDT may affect the liver and adversely influences reproduction. In birds, DDT acts by lowering Ca-ATP-ase activity and by a dose depending inhibition of the progesteron binding (Fry, 1995). It also affects fertility by acting as "anti-androgen" (Fry, 1995). There is sufficient evidence for the carcinogenity of DDT in experimental animals (IARC, 1982; Sturgeon et al., 1998). When administered orally, DDT induced hepatomas in mice and rats and lymphomas and lung carcinomas and adenomas in mice. In male rats, oral administrated DDD induced follicular cell carcinomas and adenomas of the thyroid, while DDE induced hepatocellular carcinomas in mice of both sexes. The International Agency for Research of Cancer (IARC) has determined that DDT, DDE, and DDD are possible carcinogenic in humans (Group 2B). The U.S. Environmental Protection Agency (EPA) considers them to be probable human carcinogens.

b. Hexachlorocyclohexane
Hexachlorocychohexane (HCH), one of the oldest organochlorine pesticides, was developed in 1940, and used primarily in agriculture and malaria control (Kutz et al., 1991; Breivik, 1999) (Figure 1.2). Technical grade HCH consists of a mixture of five isomers: roughly 65-70% alfa, 7-10% beta, 14-15% gamma, and approximately 10% of other isomers and compounds (Ahlborg et al., 1995). The γ- isomer (commonly referred to as lindane) has the highest acute mammalian and insecticide toxicity. γ-HCH is biotransformed to conjugated chlorophenols and is excreted very rapidly. Therefore, the detection of lindane in biological matrices is an indication of recent exposure. Although banned in some countries, lindane remains in use in Belgium for restricted applications: desinfection of seeds, insecticide on soils, sugar beet, flowers and ornamental plants, and in shampoos used for head lice (Belgian Ministry of Agriculture, 1997). Contamination occurs through inhalation, dermal exposure, and ingestion (Kutz et al., 1991).

Due to long-lasting systematic application, HCH isomers have slowly spread in the environment. They became target environmental pollutants. The determination of HCH

accumulation levels in soils and consequently in agricultural products has still remained highly actual due to its chemical and biological stability.



HCH HCB

Figure 1.2. Chemical structures of HCH and HCB

β-HCH is essentially non-insecticidal, but it became an environmental contaminant though the use of technical HCH, and is more stable than γ-HCH. Food is the main exposure route for the general population. β-HCH is the most prevalent isomer in fatty tissues, because of its greatest stability, lipophilicity and accumulation potential. It is metabolised very slowly, and is thus eliminated from the human body much more slowly than the other isomers. In most industrial countries, levels of β-HCH are higher than those of lindane (Jensen, 1983).

c. Hexachlorobenzene

Hexachlorobenzene (HCB) is a chlorinated aromatic hydrocarbon with moderate volatility (Figure 1.2). It is practically insoluble in water, but is highly lipid-soluble and bioaccumulative. Technical grade HCB contains up to 2% impurities (1.8% pentachlorobenzene and 0.2% 1,2,4,5-tetrachlorobenzene), including traces of higher chlorinated dibenzo-p-dioxins, dibenzofurans and biphenyls.

Hexachlorobenzene (HCB) was widely used as a pesticide, mainly as a seed dressing to prevent fungal disease on grain and field crops. In industry, HCB has been used directly in the manufacture of pentachlorophenol, as a peptising agent in the production of nitroso and styrene rubber for tyres in the synthetic rubber manufacture, as a fluxing agent in primary aluminium production, as a wood preservative, as a chemical intermediate in dye manufacturing and as a plasticiser for polyvinylchloride. It is generated as a by-product in the manufacture of chlorinated solvents (tri- and tetrachloroethylene, carbon tetrachloride), pesticides (pentachloronitrobenzene, chlorothalonil, or pentachlorophenol), and other chlorinated compounds (vinyl chloride), which are used in metal smelting and electrolyses. Another important source of HCB (Bailey, 2001) is represented by high-temperature processes (incineration of waste, plastics, PCBs and coal, metallurgical processes, including metal recycling, internal-combustion engine operation, fires).

Environmental fate

HCB is distributed throughout the environment because it is mobile and resistant to degradation. Volatilisation from water to air and sedimentation following adsorption to suspended particles are the major removal processes from water. Once in the sediments, HCB will tend to accumulate and becomes trapped by overlying sediments. Although HCB is not readily leached from soils and sediments, some desorption may occur and may be a

continuous source to the environment, even if input to the system cease. Biological degradation is not considered to be important for the removal of HCB from water or sediments.
In the troposphere, HCB is transported over long distances, but undergoes slow photolytic degradation. In soil, volatilisation is the major removal process at the surface, while slow aerobic and anaerobic biodegradation are the major removal processes at lower depths. Organisms generally accumulate HCB from water and food, although benthic organisms may also accumulate HCB directly from sediment.

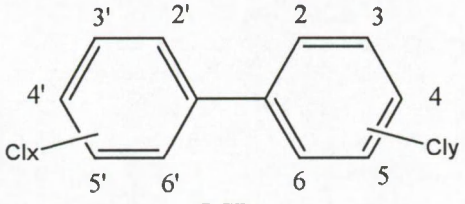
Exposure pathways and metabolism
The general human population is exposed to HCB mainly via food intake (about 92 %), and much less via breathing (7%), drinking (1%) or skin contact. Workers may be exposed to higher concentrations of HCB than the general population, particularly in the manufacture of chlorinated solvents, and in the manufacture and application of pesticides contaminated with HCB. In animals and humans, HCB accumulates in lipid-rich tissues (Schlummer et al., 1998) and can be transferred to offspring both across the placenta and via mother's milk (Sala et al., 2001). HCB undergoes limited metabolism, yielding pentachlorophenol, tetrachlorohydroquinone and pentachlorothiophenol as the major metabolites in urine (To-Figueras et al., 1997). Elimination half-lives for HCB range from approximately one month in rats and rabbits to 2 or 3 years in monkeys.

Toxicological effects
The acute toxicity of HCB to experimental animals is low (1000 to 10000 mg/kg body weight). (EHC, 1997). The available data on the systemic toxicity of HCB indicate that the pathway for the biosynthesis of heme is a major target of HCB toxicity (Van Birgelen, 1998). Elevated levels of porphyrins and/or porphyrin precursors have been found in the liver, other tissues and excreta of several species of laboratory mammals exposed to HCB. Repeated exposure to HCB has also been shown to affect a wide range of organ systems (including the liver, lung, kidney, thyroid, skin, and nervous and immune systems), although these have been reported less than porphyria (EHC, 1997). In studies of reproduction, maternal exposure to HCB was found to be hepatoxic and/or affected the survival or growth of nursing springs, birth weight, neurobehavioural development, and others (Van Birgelen, 1998).
Most data on the effects of HCB on humans originate from accidental poisonings that took place in Turkey in 1955-1959, in which more than 600 cases of porphyria cutanea tarda (PCT) were identified (To-Figueras et al., 1997). In this incident, disturbances in porphyria metabolism, dermatological lesions, hyperpigmetation, hypertrichosis, enlarged liver, enlargement of thyroid gland and lymph nodes, and osteoporosis or arthritis were observed, primarily in children. (EHC, 1997). There is sufficient evidence for the carcinogenity of HCB in experimental animals. IARC and US EPA have classified HCB as possibly carcinogenic to humans (Group 2B) (IARC, 1987; US DHHS, 1994b).

1.3. Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) were commercially produced as complex mixtures for a variety of uses, including dielectric fluids in capacitors and transformers, heat transfer fluids, hydraulic fluids, lubricating and cutting oils, and as additives in pesticides, paints, copying paper, carbonless copy paper, adhesives, sealants and plastics (Erickson, 1997). The major producer (Monsanto, USA), marketed PCBs under the trade name of Aroclor from 1933 to 1977. They consist of 209 isomers and congeners with different numbers and positions of

chlorine atoms on the biphenyl part (Giesy et al., 1999). The chlorine atoms at the biphenyl nucleus are numbered according to Figure 1.3. The position of the chlorine atoms on the PCB molecule can also be indicated by their position relative to the phenyl-phenyl bond (ortho, meta, para).



PCB

Figure 1.3. Chemical structure of PCBs

Ballschmiter and Zell (1980) proposed a numbering system for PCB congeners which was later adopted by the International Union of Pure and Applied Chemistry (IUPAC). Although 209 congeners of PCBs are theoretically possible, only about 130 individual congeners have been identified in commercial mixtures above 0.05% (Giesy et al., 1999). Annex 1 provides the IUPAC numbering and corresponding chlorine substitution pattern of selected PCB congeners. Individual PCB congeners exhibit different physicochemical properties resulting in different profiles for environmental distribution and toxicity.

PCBs have a low solubility in water (Paschke et al., 1998), but high solubility in organic solvents, oils and fats. PCBs have a high fire resistance, thermal stability and electrical properties. Because they are persistent, bioaccumulative and toxic contaminants in the environment, their use in open systems has been banned world wide since the end of 1970s in most industrialised countries. However, they will still be in use in closed systems and other applications until 2010, when their total phasing-out is planned.

Environmental distribution and fate

In contrast to pesticides, which were directly and widely applied to agricultural products and used in human housing, PCBs entered the environment and the food chain as a contaminant (De Voogt et al., 1990). Even after their production was stopped, PCBs still entered the environment, firstly by escaping from 'closed systems' like small condensators, transformers or hydraulic systems, secondly by accidental loss from 'open systems' e.g., during incineration of industrial and municipal waste (Kodavanti et al., 1998), and also due to revolatilization and evaporation from previously contaminated sites, sediments, and soils (Mackay and Fraser, 2000). Furthermore, significant amounts of PCBs may be released in the case of explosions or overheating of transformers and capacitors (Dewailly et al., 1991).

PCBs are known to possess high biomagnification capacities, which means that the concentration increases through a food chain, i.e. from prey to predator. For humans, the major route of exposure to PCBs is via food contamination. It is widely accepted that PCBs will remain present in foods for many years to come (Battershill, 1994). Because of their lipophilic and persisting properties, foodstuffs of animal origin are of special importance. Dairy products, meat and meat products, and fish contribute each for approximately 30% of the daily PCB intake (Liem and Theelen, 1997). However, this share differs to a certain extent due to variations in consumption habits of specific populations and whether or not foodstuffs from contaminated areas are consumed.

Bioaccumulation, tissue distribution and metabolism

Classical pharmacokinetic modelling of PCB disposition in higher animals has been complicated by the existence of PCBs as complex mixtures and by the variable capacity of different animal species to metabolise and clear these compounds (Matthews and Dedrick, 1984, Hansen, 1999). The individual congeners vary considerably in vulnerability to metabolism, and differ in physicochemical properties, with the less chlorinated congeners possessing comparatively greater vapour pressure and water solubility than the higher chlorinated congeners, whilst the higher chlorinated compounds are more lipophilic.

Transport in blood is apparently achieved by nonspecific association of PCBs with both blood cells and plasma proteins (Maliwal and Guthrie, 1982). The partition of PCBs between blood and tissues is determined by lipid content and concentration gradient. Liver and muscle are the primary early depots because liver is highly perfused and has affinity for some of these compounds, and muscle has by far the largest tissue volume. However, since PCBs are highly lipid-soluble compounds, they will accumulate in lipid-rich tissues. A dynamic equilibrium of PCB concentrations is established among all fatty tissues for each PCB homologue (Matthews and Dedrick, 1984). The process of bioaccumulation results in relative enrichment of the higher chlorinated congeners, because they show the greatest lipophilicity (Mackay and Fraser, 2000).

Metabolism of PCBs is achieved primarily by the hepatic mixed-function oxidases. In general, these enzymes are much more efficient in metabolising the lower chlorinated PCBs by generating hydroxylated products that are further metabolised. These observations indicate that different congeners have different half-lives in humans, varying from some days for the lower chlorinated congeners to ten or more years for the more highly chlorinated congeners (Carpenter, 1998).

Toxicological effects

Toxic effects are difficult to predict because of the complex nature of PCBs and the common mixture of other chemicals as impurities. The dioxin-like toxicity of a PCB congener depends on its ability to adapt a planar configuration similar to that of 2,3,7,8-TCDD. Safe (1990) reported that PCB congeners containing no ortho, but two para and at least two meta chlorines (coplanar PCBs: CB-77, -126, and -169) resemble 2,3,7,8-TCDD most in their biochemical and toxic effects, while the addition of ortho chlorines ('mono-ortho' and 'di-ortho') reduces this resemblance significantly (Liem and Theelen, 1997). The coplanar PCBs are minor overall constituents in both commercial mixtures and biological samples. These compounds induce several toxic effects in mammals and birds, such as hepato-toxicity, immunotoxicity, and reproductive toxicity (Eisler and Belisle, 1996). PCBs have a low acute toxicity and adverse effects have been more commonly associated with chronic exposure to PCBs. No documented incidents of human cancer have been associated with PCB exposure although experiments on animals have shown carcinogenic effects (Cogliano, 1998).

Chronic (long-term) exposure to PCBs by inhalation in humans has been reported to result in respiratory tract symptoms, such as cough and tightness of the chest, gastrointestinal effects, including anorexia, weight loss, nausea, vomiting, and abdominal pain, mild liver effects, and effects on the skin and eyes, such as chloracne, skin rashes, and eye irritation. Oral exposure to PCBs in humans has been associated with cardiovascular effects, including hypertension, mild liver effects, and effects on the skin such as pigmentation and acne (US DHHS, 1993).

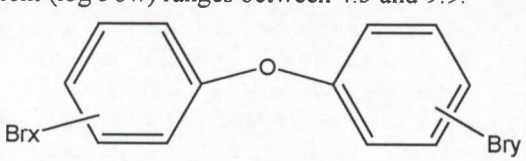
PCBs can reach the developing foetus (across the placenta) or be transferred to a new born (via mother milk) which is a circumstance of great concern. An epidemiological study (Vartiainen et al., 1998) reported that babies born to women occupationally exposed to high levels of PCBs had lower birth weights and shortened gestational age, as compared with

babies born to women exposed to low concentrations of PCBs. Two human studies that investigated exposure to PCBs through the consumption of contaminated fish suggest that exposure to PCBs may cause developmental effects in humans (Winneke et al., 1998; Patandin et al., 1999). Both studies reported neurodevelopmental effects, such as motor deficits at birth, impaired psychomotor index, impaired visual recognition, and deficits in short-term memory in infants of mothers exposed to PCBs.
Reproductive effects, such as decreased fertility, decreased conception, and prolonged menstruation, have also been noted in oral animal studies (US DHHS, 1993), but were not conclusive for human studies.
Oral exposure studies in animals show an increase in liver tumors in rats and mice exposed to several commercial mixtures of PCBs and to several specific congeners. No animal inhalation studies are available on PCBs. EPA has classified all PCBs as Group B2, probable human carcinogens (US DHHS, 1993). Equally, IARC concluded that PCBs are probable carcinogenic to humans (Group 2A) (IARC, 1987).

1.4. Polybrominated diphenyl ethers

There are significant amounts of other chemicals in the environment we know less about, and one such group is the brominated flame retardants. Even today, limited data are available on these compounds, with regard to their presence and levels in various products, environmental levels, transformation products, disposition, and toxic effects. Some overviews have compiled the present knowledge about brominated flame retardants (de Boer et al., 2000; Darnerud et al., 2001; Rahman et al., 2001).
Polybrominated diphenyl ethers (PBDEs) constitute an important group of brominated flame retardants. The compounds are mostly found in ready-made plastic products. PBDEs are used in large quantities world wide and are persistent in the environment. Over the last 10-15 years, there have been indications of increased environmental and human levels of these compounds (Darnerud et al., 2001), although the levels are still lower than those for PCBs and DDT.

The theoretical number of possible congeners is 209 and is divided into 10 congener groups (from mono- to decabromodiphenyl ethers). However, compounds with less than three bromine atoms are generally not found in commercial PBDE products. The number of PBDE congeners used in commercial products, and thus found in environmental samples, is quite small compared to the number of PCB congeners commonly found. PBDE congeners (Figure 1.4) are numbered according to the IUPAC system originally designed for PCBs (Ballschmitter and Zell, 1980). Commercial PBDEs are resistant to physical, chemical and biological degradation. The boiling point is between 301 and 425°C and their vapour pressure is low at room temperature (between $2x10^{-4}$ to $3x10^{-7}$ Pa). PBDEs are lipophilic, and their solubility in water is very low especially for higher brominated compounds. The n-octanol-water partition coefficient (log Pow) ranges between 4.3 and 9.9.



PBDE

Figure 1.4. Chemical structures of PBDEs

Commercial PBDEs are synthesized by bromination of diphenyl ethers under conditions resulting in mixtures of brominated diphenyl ethers (Rahman et al., 2001). The commercial products predominantly consist of penta-, octa-, and decabromobiphenyl ethers. The number of different congeners found in each commercial product is relatively small (Sjödin, 2000).

PBDEs are used only for flame retardant purposes. The rationale for using brominated compounds as flame retardants is based on the ability of halogen atoms, generated from the thermal decomposition of the bromo-organic compound, to chemically reduce and retard the development of fire. Factors favorising the use of of PBDEs are therefore the high bromine content, thermal stability and relatively low cost. They are used as additive flame retardants at concentrations of 5-30% in many polymers, resins and common plastics, including acrylonitrile butadiene styrene (ABS) and high impact polystyrene (Darnerud et al., 2001).

Additive flame retardants (e.g. PBDEs) leach and escape from the finished polymer product more easily than reactive flame retardants (e.g. tetrabromobisphenol A, TBBP-A). Products containing PBDEs include many components of electronic devices (51%), e.g., cabinets for and circuit boards in personal computers and TV sets and various other products (electrical cables, switches and capacitors), building materials (31%) and textiles (10%).

The annual production of flame retardants is roughly 600,000 metric tons, of which about 60,000 are chlorinated and 300,000 are brominated compounds (Table 1.1). Of the brominated products, about one-third contains TBBP-A and derivatives, another third contains PBDEs, and the last third contains various bromine compounds, including hexabromocyclododecane (HBCD).

Table 1.1. Major brominated flame retardants volume estimates (metric tons) - total market demand (1999) (BSEF, 2000).

	Americas	Europe	Asia	Total (tons)
TBBP-A	21,600	13,800	85,900	121,300
HBCD	3,100	8,900	3,900	15,900
Deca-BDE	24,300	7,500	23,000	54,800
Octa-BDE	1,375	450	2,000	3,825
Penta-BDE	8,290	210	-	8,500

Environmental distribution and fate

PBDEs have been detected in environmental samples from aquatic environments and organisms. On a congener basis, levels of PBDEs are similar with those of some PCB congeners, but the levels of total PBDEs are lower because fewer PBDE congeners are present in technical mixtures and in the environment. There are some recent reviews of environmental levels of PBDEs (de Boer et al., 2000; Darnerud et al., 2001).

PBDEs have been measured in air at concentrations varying between 1 and 50 pg/m^3 at different recycling plants from Sweden (Sjödin et al., 2001), Japan and Taiwan (Watanabe et al., 1992). PBDEs with 3-7 bromine atome were identified in German sewage sludge samples (Hagenmeier et al., 1992), while concentrations up to 190 ng/g dry weight (sum of BDE 47 and 99) were measured in recent Swedish samples (Sellström et al., 1999a). It was concluded that the primary PBDE sources to this matrix are household and industrial effluents and not washout from the atmosphere (Sellström, 1999b). Only low brominated congeners were found in water samples (EPA-Japan, 1991), while deca BDE was found in sediment samples at concentrations up to 11,600 ng/g dry weight (Sellström et al., 1998). In sediment, levels of

BDE 47 and 99 were always considerably lower than those of BDE 209 (Sellström et al., 1999a).
In aquatic organisms, PBDEs have been identified and measured in a variety of species. Simultaneous analyses of sediment and fish (Sellström et al., 1998) have shown that tetra- to penta-BDEs and HBCD are much more bioavailable than the full-brominated BDE 209. The highest levels were found in fish from waters with known or suspected local sources of contamination (Jansson et al., 1987). However, fish from other sampling spots also contained measurable levels. Very high concentrations of tetra- to hexa –BDEs, up to 8 µg/g lipid weight, were measured in some marine mammals such as bottlenose dolphins (Kuehl et al., 1995) or harbour porpoises and seals (de Boer et al., 1998). Recent studies from the American continent have shown relatively high levels of PBDEs in certain samples from the aquatic environment. About 3,000 ng PBDE/g lipid weight were measured in steelhead trout from Lake Michigan (Asplund et al., 1999) and on a wet weight basis, it contained almost 6 times more PBDE than the Baltic salmon.
Only few studies have reported PBDEs in terrestrial/avian organisms. The highest concentrations (up to 1,800 ng/g lipid) were measured in osprey, a bird that feeds exclusively on fish (Sellström et al., 1993). In addition, eggs from other fish-eating birds (guillemot) contained relatively high levels of PBDEs. There are several indications that, in contrast with other environmental pollutants such as PBCBs and DDT, the levels of PBDEs have increased exponentially since 1970s (Sellström et al., 1993). However, time trends in recent years are often difficult to follow because of small amount of samples and/or lack of data.

Bioaccumulation, tissue distribution and metabolism
Although limited data are available, existing information strongly suggests that PBDEs are globally transported and distributed in the environment in a manner similar to PCBs. They are probably of minor importance in terrestrial systems, but may reach levels of concern in aquatic environments. In addition, PBDEs are persistent and have very low water solubility, high binding affinity to particles, and tendencies to accumulate in sediments. Available data indicate that the higher brominated compounds (hepta BDE and above) do not bioaccumulate to a significant degree, due to a low uptake in organisms (Kierkegaard et al., 1995). The uptake of the lower brominated BDEs in biota is more significant. It was suggested that PBDEs have a biomagnification potential in the food chain (Burreau et al., 1999), even higher than any PCB congener.
Deca-BDE was rapidly eliminated following a single oral dose (Norris et al., 1973, 1975) with liver, adrenal glands and spleen as target organs for the unchanged parent compound or for some metabolites. Studies with BDE 47 revealed several hydroxylated BDEs metabolites in faeces and tissue, although BDE 47 was the major compound detected (Orn, 1997). Limited metabolism and excretion of another BDE congener, BDE 99 was observed in rats (Hakk et al., 1999). Small amounts of mono-hydroxylated metabolites of penta- and tetra BDE were detected in faeces, which indicates *in vivo* debromination. Mono-and dihydroxy penta BDEs as well as two thiosubstituted penta BDEs were detected in bile tissue.

Toxicological effects
Limited amounts of data are available about the toxicity of PBDEs. Most of the studies have been carried out using technical- or commercial grade PBDEs, the purity of which has been known in several cases, but the isomer composition unknown. However, available data suggest that acute toxicity of PBDEs is low, they show only slight irritating properties, and they are not skin sensitisers. No severe signs of toxicity were observed in subacute and subchronic toxicity studies (NTP, 1986). Target organs were the liver, kidney, and thyroid

gland. Deca-BDE was less potent than the other congeners. Reproduction toxicity studies revealed increased sensitivity of pregnant animals and fetuses to PBDEs (Norris et al., 1975). Except for penta BDEs, toxic effects on fetuses were observed already at dose levels not toxic to mothers. Neurobehavioural effects were seen in adult mice after single relatively low oral doses of tetra- and penta BDEs were given neonatally during the sensitive brain growth period (Eriksson et al., 1998). In spite of certain structural similarity to dioxins, PBDEs are weak agonists of the Ah receptor (Sanderson et al., 1996). It appears likely that PBDEs and hydroxylated metabolites bind effectively to transport proteins for thyroid hormones (Marsh et al., 1998).

1.5. Scope of the thesis

The work presented in this thesis covers three areas: analysis, validation and quality control procedures and monitoring of complex mixtures of persistent halogenated contaminants. Since only a limited set of data were available on the fate of PCBs and other POPs in Belgium and in my native country, Romania, we intended to evaluate the presence of those chemicals in different parts of the environment from soil, fish, animal meat to humans. The main objectives are:

1. To develop and improve the necessary analytical methods for the determination of "classical" persistent halogenated micro-contaminants (PCBs and organochlorine pesticides) in environmental and human samples.
2. To develop analytical methods for the determination of "emerging" priority pollutants, such as PBDEs in environmental and human samples.
3. To improve the quality of data to the necessary level of precision and bias to meet the purposes of the monitoring programs.
4. To measure concentrations of halogenated contaminants in different human tissues (serum, milk, hair and adipose tissue).
5. To assess the level of contamination with POPs in different environmental matrices such as farmed raised animals, fish, marine mammals and soil.
6. To estimate and compare current levels of contamination with POPs in Romanian and Belgian environment and population.

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Chapter 2

General information on analytical methods for POPs

Abstract

Several considerations concerning the analytical procedures used in the determination of different classes of POPs from various matrices are discussed. The principal steps of the chemical analysis of POPs are presented and critically reviewed.

This chapter focuses on some theoretical and practical considerations for the analytical procedures used in the determination of different classes of POPs in various matrices. The most recent methods are presented and compared with methods used throughout this study.

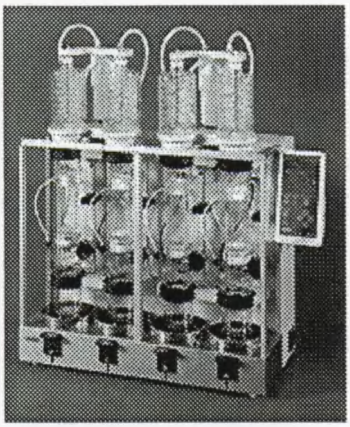
2.1. Extraction methods

Reliable trace organic analysis begins with the quantitative extraction of the analytes from the sample matrix. Different procedures are applied for liquid and solid samples and are summarised below.

For liquid samples, the liquid-liquid extraction is the most applied technique, while more recent applications use alternative techniques, such as solid-phase extraction (SPE). The classical liquid-liquid extraction (LLE) involves simple shaking of the aqueous phase and solvent (hexane or dichloromethane) in a separatory funnel (Erickson, 1997). It is a straight forward extraction, but high volumes of solvents are necessary. The technique is sequential, thus allowing that only the analysis of a limited number of samples per day. With the solid-phase extraction, the liquid sample is passed through a sorbent column, filter or disk where the organic compounds are retained (Erickson, 1997). The adsorbed compounds are then eluted from the column with an organic solvent. A variant, solid-phase microextraction (SPME), involves sorption onto a coated fibre, which is then directly desorbed in the injection port of a gas chromatograph (Rohrig et al., 1998; Yang et al., 1998). One advantage of SPE is the ability to extract large volumes of liquids to enhance the limit of detection. Other advantages include reduction of time, solvent use and, thus, cost of analysis. The most used type of adsorbent is octadecyl-modified silica, but in function of analytes of interest, other adsorbents, such as octyl-, phenyl- or cyanopropyl-modified silica can be used.

For solids, the extraction solvent must come into contact with the entire sample to reliably extract the POPs. With soil, tissue and other solids, physical mixing or maceration is necessary to ensure effective contact. Even aggressive techniques such as ultrasonic disruption or chemical degradation may be required to break up cells, macromolecules or other matrix components. The rigour of the technique must be balanced between efficient extraction on one side and degradation or loss of the analyte on the other. Extraction of organic compounds (polycyclic aromatic hydrocarbons, PCBs, pesticides) from solid environmental samples has frequently been done using organic solvents with or without the addition of heat. This process is typified by the techniques of ultrasonic (Covaci et al., 2001a), shake-flask (Dean and Xiong, 2000) and Soxhlet extraction (Luque de Castro and Garcia-Ayuso, 1998).

Classical Soxhlet extraction requires large volumes of solvents to be refluxed through the solid sample between 6 and 24 h. While several experimental set-ups can be assembled and operated at the same time, it is both labour-intensive (at least at the start and end of the process) and time consuming. The Büchi B-811 Soxhlet extractor system (Figure 2.1) offers four modes of extraction (standard, warm, hot Soxhlet and continuous extraction mode). Each consists of three steps: extraction, rinsing and evaporation and/or drying) with up to six additional evaporation or drying steps. During hot Soxhlet extraction mode, the solvent is distilled into the extraction chamber, while the upper heating element is turned on. The solvent is always kept above a fixed level by means of an optical sensor. This insures equilibrium between the rate of fresh solvent entering the extraction chamber and solvent leaving the chamber. Thus, the sample is permanently in contact with hot solvent.



Process automation reduces labour costs and, by selecting the optimal extraction conditions, also saves time.
The system has an inert gas supply to avoid oxidation during extraction and to accelerate the evaporation and drying process. With the B-811 system, high boiling point solvents (up to 150°C) need no longer to be a problem and several in-built monitoring functions guarantee a high degree of safety.

Figure 2.1. The Büchi B-811 Soxhlet extractor

Alternative strategies, such as supercritical fluid extraction (SFE), microwave-assisted extraction (MAE) and accelerated solvent extraction (ASE), were developed and applied. These newer extraction techniques are largely instrumental. SFE has been used successfully for the determination of PCBs in soil (Bowadt et al., 1995), sediment (Bowadt et al., 1994a) and lyophilised fish tissue (Bowadt et al., 1994b). However, the interest in this environmentally friendly technique (with a possibility to introduce an organic modifier for the more polar analytes) has been neglected in recent years. In MAE, organic solvent and the sample are subjected to radiation from a magnetron in either a sealed vessel (pressurised MAE) or an open vessel (atmospheric MAE). (Dean and Xiong, 2000). This technique was applied with success to the extraction of organohalogenated pollutants from soil (Lopez-Avila et al., 1995; Jayaraman et al., 2001), sediment (Cicero et al., 2000), sewage sludge (Dupont et al., 1999) and biota. (Jayaraman et al., 2001). Unlike in SFE, where samples are extracted sequentially, pressurised MAE allows up to 14 samples to be extracted simultaneously. ASE uses organic solvent to sequentially extract analytes from the sample matrix with the addition of pressure and heat (Richter et al., 1996). Typical extraction times are between 10 and 30 min. Typical applications for ASE are the extraction of organic pollutants from soil (Hubert et al., 2000) and food matrices (Müller et al., 2001).

The development and validation of sample preparation techniques did not appeal to many researchers, and thus received less developmental emphasis. The areas where there is potential for a substantial contribution to improved sample extraction for POPs include:

a. on-line extraction/clean-up/analysis
b. automation
c. micro-scale preparations
d. solvent minimisation and recycling
e. waste minimisation

2.2. Clean-up and fractionation

The clean-up step in an analytical procedure removes other compounds which may interfere with the determination of specific analytes such as POPs. The clean-up takes advantage of the difference in physical or chemical properties of POPs and interferences to remove the unwanted constituents. Several review articles have addressed clean-up of POPs, especially of PCBs (Duinker et al., 1991; Lang, 1992; Creaser et al., 1992; Hess et al., 1995). The extent

of clean-up required is dependent on the specificity of the detection step (see Chapter 2.6). With a highly selective detector such as mass spectrometer, less clean-up to remove other halogenated organics is required than for electron capture detector (ECD). On the other hand, low resolution electron impact MS requires extensive clean-up to remove the oil matrix components prior to the determination of trace levels of PCBs, while ECD is virtually "blind" to the components of a transformer oil matrix, and little clean-up is required.

a. Adsorption chromatography is the most common sample clean-up. In this technique, a solvent elutes a sample extract through a column containing at least one adsorbent. The differences in polarity, solubility, and partition cause PCBs and other POPs to move at different rates. Common adsorbents include Florisil, silica gel, alumina and carbon. In addition, a combination of adsorbent materials in one column, a column clean-up after liquid-liquid partition, or a column clean-up after matrix destruction by sulphuric acid or saponification have all been reported (Pietrogrande et al., 1998; Tan and Liem, 1998, Jang and Li, 2001). The affinity of POPs and other components of the sample for the adsorbent depends on their polarity and the surface activity of the adsorbent. While silica gel, alumina and Florisil exhibit relatively similar abilities for clean-up, the carbon column technique exhibits excellent selectivity, but poor capacity. Thus, it is used as a second clean-up after major interferences have been removed by another technique (Sericano et al., 1991; Concejero et al., 2001).
b. Gel permeation chromatography (GPC) is used extensively for the separation of analytes from interferences on the basis of molecular size. It is used widely for the removal of macromolecular interferences (e.g. humic acids and lipids) from environmental samples. Examples include biological materials containing high levels of lipid materials (Noren and Sjövall, 1987) or oils with molecular weights in the range of 600 to 1500 amu. GPC can be fully automated to accommodate large number of sample extracts (van Rhijn et al., 1992; Pauwels et al., 1999).
c. Chemical degradation is another technique used for the separation of the PCBs and other POPs from interferences. Chemical degradation techniques must be used with caution to ensure that target analytes are not destroyed along with the interferences. Sulphuric acid treatment is used to remove potential chromatographic interferents (fatty acids) and organic macromolecules. Two approaches are used: simple shaking of the sample extract with concentrated sulphuric acid for a short time, followed by washing of the acid phase with hexane and elution of the sample extract through a column of acid sulphuric–treated silica gel (Erickson, 1997). The latter technique has been found to be efficient and is considerably easier to use than the shakeout technique, especially for large-volume samples. PCBs, PBDEs, together with a large number of organochlorine pesticides (e.g. HCHs, DDTs and HCB) were found to be stable in these conditions (Covaci and Schepens, 2001b). However, dieldrin, aldrin, heptachlor and chlordanes were destroyed during the acid clean-up (Pauwels et al., 1998). Treatment of a sample with strong base (e.g. NaOH) can selectively degrade interferences without affecting the target analytes. The most common reaction is the saponification of fats to their corresponding glycerols and carboxylates. However, losses of PCB congeners have been reported (van der Valk and Dao, 1988; Kannan et al., 1993) at high temperature and during long time of contact between the base and the sample.
d. Dialysis performs separations based on differential diffusion of substances through a semi-permeable membrane (Sodergren, 1990; Strandberg et al., 1998). Huckins et al (1990) cites some advantages of the technique over GPC or adsorbent chromatography such as simplicity, large sample capacity and solvent minimisation. However, excessively long times are required (up to 48 h), while the lipid carryover is sometimes considerable.

e. Elemental sulphur frequently occurs in sediment, sewage sludge and some soils. Several chemical treatments with mercury, copper and tetrabutylammonium sulphite can be used for sulphur removal (Smedes and de Boer, 1997).
f. High performance liquid chromatography (HPLC) can be used either as a clean-up technique (following the same chromatographic principles as the open adsorption column technique), or as a separation tool for PCBs and PCDD/Fs according to their planarity (Erickson, 1997). When used as clean-up technique (Grob et al., 1987; van der Hoff et al., 1997), HPLC is faster, has a better resolution, reproducibility, uses less solvent that the open-column technique. However, it has a low capacity for dirty samples and provokes high costs due to sequential operation. HPLC using carbon-based sorbents and specific derivatised silicagel (e.g. 2-(1-pyrenyl) ethyldimethylsilylated silica gel) has been successfully applied for the separation of PCDD/Fs from PCBs (Creaser and Al-Haddad, 1989; Hong et al., 1992; Pyell and Garrigues, 1994; Feltz et al., 1995; Echols et al., 1997) and for the separation of PCBs according to the number of *ortho*-chlorine atoms (Haglund et al., 1990; Creaser et al., 1992; Huang et al., 1997; Kimata et al., 1997).

The analyst is often forced to adapt the clean-up to an actual problem. The criteria for choice of a clean-up include:

1. Reported success at removing interferences
2. Reported success at retaining any of the target analytes in the sample extract
3. Appropriate capacity for the sample size to be used
4. Previous experience in the laboratory
5. Cost of setup and execution relative to other candidate clean-up techniques

The basic principles of the separation chemistry involved both in the general clean-up as well as in specific isolation techniques (e.g. separation of non-*ortho* congeners) are not fully understood and most separations rely on empirical, often trial-and-error, experimental development. Research to understand the chemical principles would improve the efficiency of separations. Matching the scale of separations to the needed analysis is another area for potential improvements. Thus, micro-scale chemical separations can achieve a better match of the preparation with the determination by gas chromatography (GC).

2.3. Injection techniques for the analysis of POPs

The injector is an integral and important part of the chromatographic process. Grob (1994) presented an informative overview of the injection techniques for capillary GC. Application to PCB analysis has also been reviewed (Grob et al., 1988). While often overlooked, the type of injector can affect resolution, discriminations, and sensitivity. Most applications are based on splitless injection technique, with the analytes being swept onto the column after vaporization. For splitless injection, the optimisation parameters include the minimum closing time of the splitter, the initial column temperature and isothermal period, and the injector temperature. On-column injectors provide generally superior chromatography, but in this case, the sample has to be subjected to an extensive clean-up. Dirty matrices, including inorganics, polymeric materials, stay on the front of the column, degrading performance and increasing the background. For on-column injection, the initial column temperature, the secondary cooling time and the initial isothermal period have to be optimised.
Splitless and on-column injections were compared by Alford-Stevens et al., (1986). For PCB congeners, the precision of the response factors was similar for both types. Although on-column injections provided more efficient analyte transfer than splitless injections, especially

for mixtures with a wide range in volatility, the authors concluded that splitless injection provided the best combination of precision, sensitivity and convenience.

Although a 1 to 5-µl injection volume is traditional in PCB analysis, larger injection volumes are possible. Using concurrent solvent evaporation device, injections up to 200 µl were possible onto a GC-ECD (Hogendoorn et al., 1989). Some extraction and clean-up techniques (namely HPLC) are amenable to direct interfacing with the GC system. Thus, Grob et al. (1987) has developed a LC-GC interface in which 100% of the sample was deposited on the GC column, thereby reducing detection limits for trace analysis. The advantages of integration of the various analysis steps into a unified instrument include a reduction in potential for analyte loss, high precision through automation and reduction in labour and in analysis cost.

2.4. Capillary GC columns used for analysis of POPs

*-based on Covaci A, Schepens P, (2001). *Chromatographia*, 53, S 366-S 371.

It was already shown (Frame, 1997) that no capillary column commercially available is able to separate all 209 congeners and some PCBs even co-elute with the 7 ICES marker PCBs. Thus, it was recommended that the chromatographic analysis of PCBs and organochlorine pesticides should be done on two capillary columns of different polarity or different separation mechanism. Cochran and Frame (1999) have recently described the newest developments in the high-resolution gas chromatography of PCBs.
Although a comprehensive paper published by Frame (1997) compares 20 different capillary columns through their ability of separating PCB congeners, no indication on possible co-elution with chlorinated pesticides is provided. We have tested 6 different capillary columns and compared them on their ability to separate PCB congeners and organochlorine pesticides (Table 2.1). It was shown that the HT-8 or DB-1 columns offer less co-elutions of target analytes than the DB-5 or equivalent (the most used column in organochlorine analysis).

Table 2.1. Co-elutions of target PCB congeners and DDT metabolites on different stationary phases. (Individual PCBs are identified by their IUPAC numbers.)

Column	Length	Co-elutions of target analytes
DB – XLB	60 m	90/101, **118**/131, 138/163/**178**, **156**/172, **138**/p,p'-DDT
CP-Sil 5/C18	50 m	90/101, 138/163, 156/157, **141**/p,p'- DDT
DB-5ms	30 m	28/31, 46/52, 90/101, 118/**149/p,p'-DDD**, 138/**p,p'-DDT**, **105**/132, **170**/190, 138/163/164/158
DB-17	30 m	28/31, 90/101, 114/**143**, 179/**138**, 137/138, **105**/141, **156**/172, 157/**180**/197/193, **170**/196
DB-1	30 m	28/31, 90/101, **pp-DDD**/**118**/149, 114/**143**/**op-DDT**, 163/164/138, 138/**pp-DDT**, **156**/171
HT-8	25 m	**28**/53, 90/101, 77/**149**, 131/**118**, 163/**pp-DDT**
DB-XLB + CP-Sil 5/C18	110 m	43/52, 90/101, 138/163

- bold: isomer quantification possible by MS (different degree of chlorination)
- underline: target analytes

The separation of individual PCBs on these types of GC stationary phases have previously been investigated (Ballschmiter et al., 1993; Larsen et al., 1995; Frame, 1997). Although most columns offer better separation and avoid co-elution of target congeners, most of researchers prefer the 5%-phenyl methyl polysiloxane (DB-5ms) or equivalent (SE-54)

stationary phase, due to its robustness and complete characterisation of elution order for PCBs (Mullin et al, 1984). PCB 46 and PCB 143, used as internal standards in our experiments, were checked for possible co-elution with other PCBs on all columns. Except for DB-5ms, DB-1, and DB-17, no co-elution with PCBs present in environmental samples was found.
Table 2.1 presents co-elutions for major PCBs in biota samples. The congeners marked in bold can be identified only by MS, due to different degree of chlorination. It is important to mention that all pesticides investigated (HCHs, HCB and DDTs) can be easily measured by MS, even if they co-elute with some PCB congeners.

For simplicity, only 3 columns (the most used in our laboratory), namely HT-8, DB-XLB and CP-Sil 5/C18 will be compared in their ability to separate key PCB congeners. Thus, the pair PCB 90/101 could not be separated on any type of column. PCB 156 and PCB 157 were not baseline separated on CP-Sil 5/C18, but well separated on HT-8 and DB-XLB. PCB 170 and PCB 190 were reported as co-eluting pair on a 5% phenyl stationary phase (Mullin et al., 1984). All three columns used in this study resolved this pair, but, on DB-XLB, PCB 190 co-eluted with PCB 198 and PCB 199. It can be concluded that the use of CB 198 as internal standard, as indicated by other studies (Grimvall et al., 1995; Poster et al., 1999), is not recommended due to possible overestimation.
On the CP-Sil 5/C18 column, baseline separation of PCB 105 and PCB 153 was not achieved. The separation was improved on HT-8 and DB-XLB, with inversion of the elution order. The elution order of PCB 183 and PCB 128 was different on the columns used, with better separation from possible interferences on the HT-8 and DB-XLB columns. Furthermore, PCB 167 was found to have an interference on CP-Sil 5/C18, which was resolved on HT-8 and DB-XLB. All three columns could separate PCB 138 from PCB 164 (when compared with a 5% phenyl stationary phase), but only HT-8 is able to separate PCB 138 from PCB 163. Values of PCB 138 on CP-Sil 5/C18 are about 15-20% higher than on HT-8. Furthermore, p,p'-DDT was not baseline separated from CB 138 on XLB column, values of CB 138 on this column being up to 10 % higher than the values calculated from CP-Sil 5/C18. An attempt was made to couple two long columns in series. The resulting column (DB-XLB + CP-Sil 5/C18) showed only few co-elutions (Table 2.1), but the retention times were too high (run time of more than 2 h).

2.5. Fast GC with narrow bore capillary columns

* -based on Covaci A and Schepens P, (2001). *J Chromatogr A* 923 (1-2), 287-293.

Since the introduction of capillary GC, there has been a permanent request for faster and more sensitive analytical methods. From a theoretical point of view, the reduction of the internal diameter (I.D.) of the column is an attractive way towards shorter analysis times (Cramers et al., 1988; Blumberg, 1997a,b). Furthermore, the use of narrow-bore capillary columns (I.D. less than 0.10 mm), near the conditions for minimum plate height allows a good chromatographic separation when structurally related compounds are analysed (Blumberg, 1997b; Cramers et al., 1999). Despite of the high number of samples to be analysed for organochlorine pollutants, only limited attention has been paid to the coupling of narrow-bore capillary columns with electron capture detectors or mass spectrometers. A 5 m CP Sil-8 column with 0.05 mm I.D. was used in combination with ECD for the separation of PCBs from different matrices and Aroclor mixtures (Van Ysacker et al., 1995). However, relatively long run times (up to 15 min) were used for complete separation of PCB congeners

from interfering materials. Separation of selected organochlorine pesticides was done in less than 4 min using a 10 m column with 0.10 mm I.D. and pulse-discharge ECD (de Jager and Andrews, 2000), but relatively high detection limits were obtained from water extracts. In another application (Reiner et al., 2000), two 20 m DB-5 and DB-1701 capillary columns with 0.10 mm I.D. in combination with ECD were used for complete characterisation of PCB congeners in sediment extracts. Fast GC using a 3 m column with 0.25 mm I.D. was used for PCB determinations (Alvarado et al., 1997). Beside an important loss of resolution, no improvement of detection limits was observed and restricted applicability to complex mixtures was observed. Although there is a need for improvement of the selectivity for complex mixtures, the use of a selective detector such as a quadrupole mass spectrometer in combination with narrow-bore capillary columns was not fully investigated.

2.5.1. Materials

PCB congeners (IUPAC no. 28, 46, 52, 74, 99, 101, 105, 110, 118, 138, 143, 149, 153, 156, 170, 180, 187 and 194 were available in solution (10 ng/µl in iso-octane) from Dr. Ehrenstorfer Laboratories (Augsburg, Germany). PCB 46 and PCB 143 were used as internal standards.

GC-MS: A Hewlett Packard (Palo Alto, CA, USA) 6890 GC was connected via a direct interface to a HP 5973 quadrupole mass spectrometer. The interface temperature was set at 300°C. Helium was used as carrier gas. Samples were injected into an empty baffled liner (1.5 mm I.D.) of a Gerstel (CIS 4) programmable temperature vaporiser (PTV). Characteristics of columns used in this study together with injection parameters are presented in Table 2.2. The mass spectrometer was operated at 70 eV in selected ion monitoring (SIM) mode. Dwell times were set to 10 ms for the 0.10 mm column and to 20-40 ms for the 0.18 and 0.22 mm columns, respectively. Two ions from the molecular ion cluster of each congener (M^+ and $[M+2]^+$) were monitored for each level of chlorination. Target ions were grouped following a similar procedure used for GC-MS analysis of PCB in 0.25 mm I.D. columns (Erickson, 1997). Retention times relatives to the nearest internal standard, ion chromatograms and ratio between the monitored ions were used as identification criteria. A deviation of ion ratios of less than ±20% from the theoretical value was considered acceptable for identification. A HP Method Translation Software (1997) was used for calculation of optimal GC parameters for the narrow-bore capillary column.

2.5.2. Injection in narrow-bore GC

Hot splitless: Only low injection volumes (typically 0.2 - 0.4 µl) can be injected by hot splitless in narrow bore columns without peak distortion (Van Ysacker et al., 1998). Fast injection is needed to reduce discrimination for low volatile compounds. Because of the low flow rate (typically 0.3 - 0.4 ml/min), long splitless times are required, while liners with small internal diameter should be used (0.8 - 1.2 mm I.D.) to reduce the injector band broadening. Sufficiently strong focusing mechanisms (e.g cold trapping and solvent effect) should occur (Grob, 1993). Hot splitless can be used without any problems for columns of more than 0.15 mm I.D.

Cold splitless: Peak distortion can be observed in hot splitless when volumes of more than 0.4 µl are injected into a 0.10 mm capillary column (Van Ysacker et al., 1998). However, up to 2 µl can be injected in cold splitless, before distortion is observed. There is no solute discrimination and the limiting factor is the volume of liquid which can be handled in the liner. If the sample volume is too high, the liquid may be shoot through an empty liner. The use of a baffled liner, with higher contact surface, allows the handling of volumes of few microliters. No fast injection is mandatory. The injector should be kept at low temperature (to

be optimised for each solvent used) for a longer time. In this way, part of the solvent is already transferred before the analytes enter the column, thus allowing an efficient solvent effect (Grob, 1993). However, at a too low initial oven temperature, excessive solvent recondensation occurs. Initial inlet and column temperatures did not influence greatly the area of the peak, but affected seriously their peak shape, especially for volatile analytes (serious distortion). Using iso-octane as solvent, the best conditions were 90°C and 100°C for the oven and injector initial temperatures, respectively. No solvent focussing effect occurred at oven temperatures of 120°C or higher.

Table 2.2. Column characteristics and injection parameters

Parameter	**Column**		
	AT-5	AT-5	HT-8
Column description	5% phenyl polydimethyl siloxane	5% phenyl polydimethyl siloxane	1,7-dicarba-closo-dodecarborane 8% phenyl methyl siloxane
Manufacturer	Alltech (Lokeren, Belgium)	Alltech (Lokeren, Belgium)	SGE (Zulte, Belgium)
Dimensions	10 m x 0.1 mm x 0.1 µm	20 m x 0.18 mm x 0.25 µm	50 m x 0.22 mm x 0.25 µm
Carrier flow	0.4 ml/min	0.8 ml/min	0.7 ml/min
Oven program	90°C (1 min) at 50°C/min to 200°C, (0.5 min), at 25°C/min to 250°C (0.2 min), at 75°C/min to 280°C (2 min)	90°C (1 min) at 35°Cmin to 200°C, (1 min), at 10°C/min to 250°C (0.5 min), at 50°C/min to 280°C (3 min)	90°C (1 min) at 15°C/min to 170°C (2 min), at 3.5°C/min to 290°C (14 min)
Injection type	Cold splitless	Hot pulsed splitless	Hot pulsed splitless
Injector program	100°C (0.1 min) at 700°C/min to 270°C (stay 7 min)	Pressure pulse 30 psi, pulse time 1 min, 270°C	Pressure pulse 30 psi, pulse time 1.20 min, 270°C
Injection vol.	1 µl	1 µl	1 µl
Splitless time	1.00 min	1.00 min	1.25 min
Theoretical Plates/m	8,600	5,300	3,900
Theoretical Plates (total)	86,000	106,000	195,000

For low splitless times (0.50 - 0.75 min), analytes are incompletely transferred to the column, resulting in low peak areas. For splitless times higher than 1 min, similar areas were obtained for high volatile compounds (hexachlorobenzene and PCB 28), while the increase in area was more evident for low volatile compounds (e.g. PCB 180 and 194) (Figure 2.2).

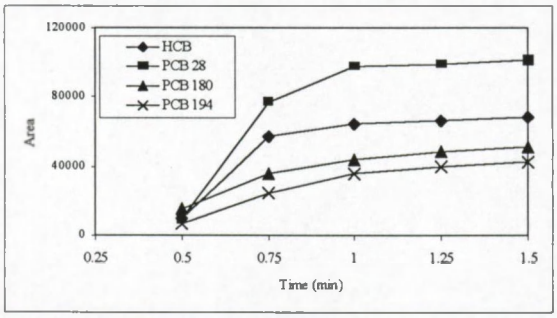


Figure 2.2. Influence of splitless time (for cold splitless injection) on area of HCB, PCB 28, 180 and 194. Oven and injector conditions, as inTable 1, column AT-5, 10 m x 0.10 mm I.D.

However, splitless injection times longer than 1 min lead to more than 25% increase of the peak width, which affected some separations. A splitless time of 1 min was further used in experiments.

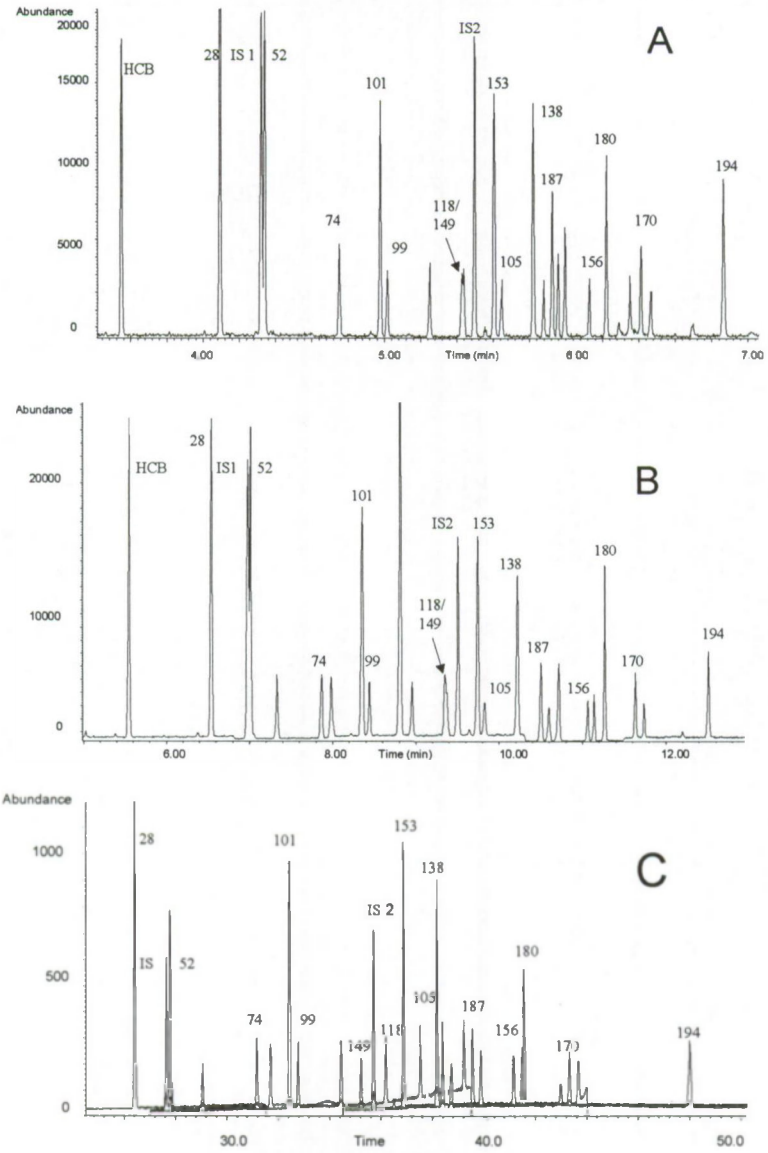


Figure 2.3. Separation of a PCB standard mixture (IUPAC numbering) on 3 different capillary columns: 10m x 0.10 mm I.D. (chromatogram A), 20m x 0.18 mm I.D. (chromatogram B) and 50m x 0.22 mm I.D. (chromatogram C).

A standard mixture of PCBs in iso-octane was injected in cold splitless into a 10 m AT-5 capillary column with 0.10 mm I.D. (Figure 2.3 A). The resulting chromatogram could be compared with similar chromatograms obtained by injecting the same PCB standard solution into a 20 m AT-5 column with 0.18 mm I.D. and 50 m HT-8 column with 0.22 mm I.D.

(Figure 2.3 B and 2.3 C, respectively). No loss of resolution was observed for PCB congeners (see pairs PCB 101/PCB 99 and PCB 153/PCB 105), while run times were reduced from 50 min to 7 min (a reduction of more than 85% of the analysis time).

Large volume injection: On programmable temperature vaporizers, large sample volume introduction is possible. For semi-volatile compounds (e.g. PCBs), optimisation of the method is not as critical as for volatile analytes. To minimise losses of volatile analytes, low solvent amounts should be still present in the injector, before the closure of the vent line (Grob, 1993). A procedure involving the injection of 8 x 5 µl extract was already demonstrated for the determination of various pesticides from water using similar equipment as the one described above (Hada et al., 2000). Thus, it is now possible to increase sample capacity by an order of magnitude, which will allow lower limits of detection. The procedure is currently being tested for the analysis of PCBs in human body fluids.

2.5.3. Quadrupole MS detection for narrow-bore GC

The use of narrow-bore columns (less than 0.10 mm I.D.) requires a fast scanning detector, which should also be sensitive and selective. The use of mass spectrometers based on a magnetic sector (Leclercq et al., 1989) or a time-of-flight analyser (van Bavel et al., 1999) has already been demonstrated to allow short run times (< 3 min). Due to lower scanning rates, the quadrupole mass spectrometer can be used only for reasonable runtimes (> 5 min). Selected ion monitoring and low dwell times (10 ms) are a requirement for a higher number of scans across the chromatographic peak, thus resulting in a better peak shape.

It has already been shown (Grimm et al., 1996) that quadrupole mass spectrometers can be operated for qualitative fast GC analysis in full scan acquisition mode and that excellent quantitative data can be obtained in selected ion monitoring. Thus, for conditions described in Table 2.2, the peak width at the base is maximum 1.2 s for the 10 m column. Assuming that a peak is sampled 8-10 times to obtain a good peak shape, a minimum of 120 ms will be necessary for one chromatographic point. When using dwell times as low as 10 ms, a maximum of 12 ions can be monitored in one acquisition window. For PCB determination, this is not a problem due to specific ions for each level of chlorination. In these conditions, detection limits for individual PCB congeners as low as 0.4 pg/µl for a standard solution and 0.2 ng/g fat for milk extracts could be achieved. The observed gain in sensitivity of capillary GC-MS by decreasing the column diameter might be explained by a reduction of the noise level by faster scanning (less background ions detected). Moreover, the gas flow through narrow-bore columns is much less than for conventional ones (proportional to the square diameter of the column for constant plate numbers) and so is the column bleeding mass flow. The use of low flow rates is compatible with pumping capacity of most systems. A quadrupole mass spectrometer is easy to use and to maintain and can be easily changed for routine operation if needed.

However, some disadvantages of narrow bore capillaries should be mentioned here. The use of these columns requires higher inlet pressure which are incompatible with old GCs (capable to sustain pressures up to 30 psi only). It is prone to leaks through ferrules, septum and sample flashback through leaking syringe plungers. There is little room for trimming the column when column performance begins to deteriorate with use.

2.5.4. Quantitative PCB analysis

Human milk extracts were injected on the 0.10 and 0.18 mm columns using a quadrupole analyser in the selected ion monitoring mode. No loss of resolution for critical PCB pairs was

observed (Figure 2.4). Similar quantitative data (Table 2.3) were obtained on both columns after injection of a certified reference material extract (CRM 450 – PCBs in powdered milk). Milk samples were processed as described in Chapter 4. Very small quantities can be detected on narrow-bore columns, because sharper peaks result in a higher signal and, thus, in a better S/N ratio when a fixed amount of sample is introduced (Cramers et al., 1988).

Table 2.3. PCB concentrations (ng/g) in CRM 450 (PCBs in powder milk) obtained on two AT-5 capillary columns with 0.10 and 0.18 mm I.D.

Compound	Certified values	AT-5 20 m x 0.18 mm	AT-5 10 m x 0.10 mm
PCB 118	3.3 ± 0.4	3.2 ± 0.3	3.1 ± 0.3
PCB 153	19.0 ± 0.7	18.3 ± 1.6	18.6 ± 0.6
PCB 156	1.62 ± 0.20	1.51 ± 0.02	1.56 ± 0.08
PCB 180	11.0 ± 0.7	11.2 ± 0.9	11.4 ± 1.0

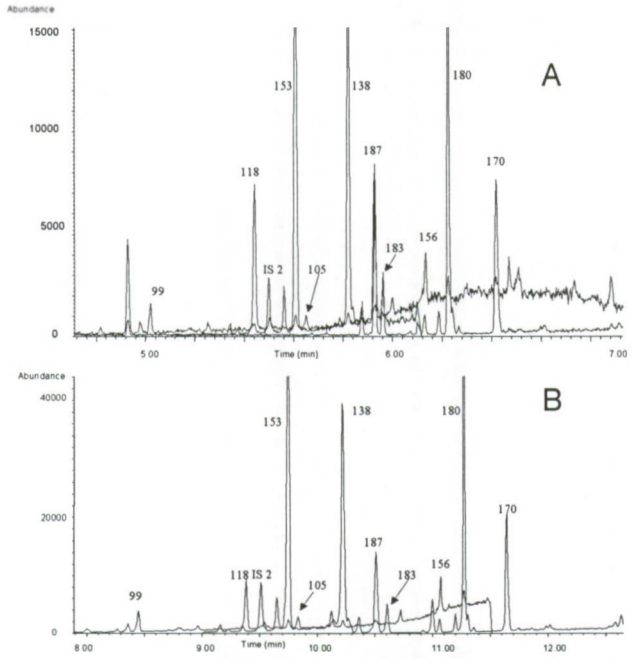


Figure 2.4. GC-MS chromatograms of human milk extract on 10 m x 0.10 mm I.D. (chromatogram A) and 20 m x 0.18 mm I.D. (chromatogram B). IUPAC numbering for PCB congeners.

Thus, narrow-bore (0.10 mm I.D.) capillary columns can be used in combination with quadrupole MS for the selective and sensitive determination of PCBs in human matrices. By using cold splitless injection, relatively high volumes (1 µl) could be injected on narrow-bore capillaries without any peak distortion. The technique can be used with success in trace analysis for environmental matrices.

2.6. Detection systems

All analytical methods are designed to answer "is the analyte present?", "how much analyte is in the sample?" or both questions. The identification and quantitation is generally accomplished in the same step. The analysis of POPs generally requires selectivity and sensitivity. Even after clean-up, PCBs are usually a minor component of the sample, present in a mixture with other organohalogens (e.g. DDE), hydrocarbons and/or lipids. Thus, the detector often must selectively detect PCBs (and other POPs) in the presence of other compounds present in much higher concentrations. Furthermore, the levels typically observed for these compounds in food, biota, soil and other matrices of interest are in the parts-per-billion range (ng/g level). The choice of a detector often depends upon the level of analytes, with low concentrations demanding a highly sensitive detector.

The *electron capture detector (ECD)* is selective toward halogenated compounds. The ECD detects the drop in current caused by the absorption of electrons by the analyte. A radioactive source (^{63}Ni) emits electrons which are swept toward the anode by an applied voltage across the electrodes. This electron migration produces a current of approximately 10^{-9} A. When a molecule or ion capable of absorbing an electron enters the detector, the current is reduced and the current drop is proportional to the concentration of the analyte in the gas stream. Its selectivity, coupled with its extreme sensitivity (down to 1 pg), has made ECD very popular for the analysis of trace levels of organochlorine pesticides and PCBs and has a significant role in regulatory actions on these classes of compounds. However, while it is generally considered as a selective detector, it DOES detect many other chlorinated and nonchlorinated compounds (e.g. polychloronaphthalenes, chloroaromatics, phthalates) which may be differentiated from PCBs and pesticides only on the basis of retention time. A major disadvantage of ECD is the range of response factors (RFs) which different organochlorine compounds exhibit. Thus, for PCBs, the RFs for mono- to decachlorinated congeners varies over 3 orders of magnitude (Luotamo, 1985).

M*ass spectrometers* are becoming increasingly common as GC detectors as sensitivity, cost, performance, reliability and user friendliness improves. Molecules introduced into a mass spectrometer via gas chromatography are generally ionised by a beam of high-energy electrons ("electron impact") or by reaction with ionised molecules such as methane ("chemical ionisation"). The ionised molecule and/or its fragments are then swept to the analyser section (magnetic sector, quadrupole, ion trap or time-of-flight), sorted as a function of their mass/charge ratio and finally focused on an electron multiplier for detection. Important features of a MS method are the type of ionisation and polarity of ions detected (electron impact/positive ion detection, EI; chemical ionisation/negative ion detection, NCI), as well as the resolution, low (able to separate at least unit masses) or high (able to separate masses different with less than 1 mass unit). MS is particularly suited to detect PCBs and other organochlorine compounds because it results in an abundant molecular ion and the characteristic chlorine cluster (Erickson, 1997). MS provides better characterisation of complex mixtures, better discrimination against interferences and thus better quantitative accuracy. Two types of data acquisition modes are available for MS: the scan mode (providing full spectra for each point of the chromatogram) and the selected ion monitoring mode (allowing the measurement only of several analyte-specific ions). The latter mode enhances both instrument sensitivity and selectivity and simplifies data interpretation. The most used techniques in POPs analysis are EI-MS and NCI-MS (especially for PBDEs),

while techniques as high resolution MS (used mostly for dioxins measurements) and MS/MS have a less frequent use (Erickson, 1997).

Other detectors for GC such as an infrared spectrometer (FTIR), a flame ionisation detector (FID), a photoionisation detector (PID) and an atomic emission spectrometer (AED) have found a much more narrow applicability in the determination of POPs from environmental matrices. Recently, several non-chromatographic techniques, such as immunoassays and bioassays (see Chapter 3.2.1) are becoming available for the fast screening of POPs in various matrices.

2.7. Quality assurance

Emphasis on quality assurance (QA) in chemical analysis has increased dramatically in the past few years with the realisation that data of unknown quality are virtually useless. **QA** is generally defined as the system, program, or structure which plans, designs and monitors the quality control (QC) procedures and affirms the data quality in reports. **QC** is the term used to describe the "overall" system of activities whose purpose is to monitor for and identify errors and provide information to define the data quality.
Some QC considerations in PCB analysis are presented below (Erickson, 1997):

a. Method validation
   - analyse blanks, spikes, replicates (precision)
   - assess potential interferences
   - compare to other methods (accuracy)
   - establish instrumental performance criteria (e.g. sensitivity, resolution)
   - determine instrumental limit of detection/quantification
   - determine method limit of detection/quantification
   - determine range of quantification
   - conduct periodically a collaborative study
   - conduct periodic confirmatory analyses
b. Sampling
   - use field controls and blanks
   - use a well-designed sampling scheme
   - evaluate the representativeness of sampling scheme
   - use a validated sample preservation and storage procedure
c. Sample preparation and analysis
   - include blanks, replicates, spikes, check samples with each sample set
   - repeat analyses on a regular basis
   - use surrogates to measure method recovery on each sample
   - use internal standards for quantification
   - verify reagent and standard purity, identity and concentration
   - control and monitor glassware contamination
   - monitor retention times for correct identification
d. Confirmation
   - analyse sample using alternative technique
   - use a confirmatory technique
   - send to another laboratory for a duplicate analysis

A major component of a good QC program is the inclusion of **reference materials** as QC check samples. de Boer and McGovern (2001) have reviewed the existing reference materials for determination of selected POPs in aquatic environment. Another important topic is the choice and use of **internal standards** and **surrogates** in POP analysis, which allows the analyst to quantify accurately compounds present in the sample. While for ECD, it is necessary to include compounds structurally related to the target analytes, but not present in the sample, for MS, ^{13}C-labelled compounds are now available. The later compounds have the advantage of being identical as structure and properties with the target analytes and thus leading in general to a better accuracy during determination. Finally, interlaboratory tests for the determination of selected POPs in various matrices should be regularly conducted (de Boer, 1994, 1996a,b, 1997).

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Part 1

Persistent Organohalogenated Pollutants in Humans

Chapter 3

Determination of POPs in human serum

Abstract

Solid-phase disk extraction (SPDE) was developed and evaluated for the isolation and concentration of trace levels of selected organochlorine pesticides and PCB congeners from human serum. An Empore™ C_{18} bonded silica extraction disk cartridge was used for the initial extraction and enrichment of the analytes. Subsequent clean-up was done by adsorption chromatography on concentrated sulphuric acid: silica gel (1:1, w/w). Analysis was achieved by gas chromatography with mass spectrometric or electron capture detection on two capillary columns of different polarity. Recoveries of target analytes and internal standards were ranging from 62 to 74% and a good reproducibility (RSD < 14%) was demonstrated. By using the SPDE procedure, a high throughput and parallel sample processing could be achieved. The method was validated through successful participation to several interlaboratory tests and by analysis of human serum with different organochlorine loadings.

3.1. Analytical methodology for the determination of POPs in human serum

* - based on: Pauwels A, Wells DA, Covaci A, Schepens PJC, (1998). *J Chromatogr B*, 723 (1-2), 117-125; Covaci A, Pauwels A, Schepens P, (2000). *Intern J Environ Anal Chem*, 76 (3), 167-178; Covaci A, Schepens P, (2001). *Chemosphere*, 43, 439-447.

Due to potential health effects (dermal toxicity, immunotoxicity, reproductive effects and teratogenicity, endocrine disruption and carcinogenicity) of persistent organochlorine pollutants (POPs), such as polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs), their monitoring in humans is of high general concern (WHO, 1998). Monitoring of human exposure to POPs is most conveniently performed by analysis of the blood plasma or blood serum. The requirements for risk assessment in epidemiological studies have created the need for efficient, fast and less-costly analytical methods.

Due to trace levels of POPs in biological fluids and the presence of other extraneous chemicals at higher concentration levels, a highly sensitive and selective multistage analytical procedure is needed. The determination of POPs by high-resolution gas chromatography (HRGC) usually requires preliminary purification of the extracts before instrumental analysis in order to improve the process of quantification.

Conventional methods of separating POPs from human body fluids involve liquid-liquid extraction with non-polar solvents (Grimvall et al., 1995; Gill et al., 1996; Greizerstein et al., 1997; Pauwels and Schepens, 1998; Lino et al., 1998; Najam et al., 1999). They are very complex, labour intensive, time consuming and use excessive amounts of solvents and reagents. Solid-phase extraction (SPE), using commercially available columns pre-packed with various stationary phases has been previously investigated as an alternative method for extraction and clean-up (Seady and Poklis, 1990; Burse et al., 1990; Luotamo et al., 1991; Brock et al., 1996; Guardino et al., 1996).

We were the first to report the use of solid-phase disk extraction (SPDE) technology for the analysis of selected POPs in human serum (Pauwels et al., 1999). The procedure involves denaturation of serum proteins with formic acid, solid-phase extraction using C_{18} Empore™ disk cartridges, followed by elimination of lipids using a sulphuric acid clean-up of the eluate. The use of SPDE improved assay throughput and allowed reduced volumes for elution. However, due to residual interferences, identification and quantification was only possible for PCB 118, 138, 153, 180, and p,p'-DDE (method detection limit ~ 150 pg/ml). The method did not allow the determination of minor PCB congeners, which contribute substantially to the total dioxin-like toxicity. By using an additional clean-up step (e.g. chromatography on polar adsorbents) combined with a more rigorous choice of SPDE conditions, the background noise becomes lower (method limit of detection ~ 10 pg/ml), thus allowing the determination of minor peaks.

MATERIALS AND METHODS

Chemicals

Based on reported abundance and toxicity, the following PCB congeners (IUPAC numbering) were targeted for analysis: 28, 52, 66, 74, 99, 101, 105, 110, 118, 128, 138, 149, 153, 156, 157, 167, 170, 180, 183, 187, 194, 199. Additionally, we included HCB, γ-HCH, p,p'-DDT

and p,p'-DDE, as the major organochlorine pesticides found in human serum. PCB 46 and PCB 143 were used as internal standards and 1,2,3,4-tetrachloronaphthalene (TCN) as recovery standard. The criteria for their selection were the elution characteristics in the GC columns and their absence in human samples. All solvents (methanol, acetonitrile, hexane, dichloromethane, acetone, iso-octane) were pesticide grade (Merck, Darmstadt, Germany). Formic acid (Across, Geel, Belgium) and concentrated sulphuric acid 95-97% (Merck) were analytical grade reagents. Individual PCB congeners (10 ng/µl in iso-octane) were purchased from Dr. Ehrenstorfer Laboratories (Augsburg, Germany). Dilutions were made in order to cover the entire expected range of POPs in human serum from non-exposed subjects. All solutions and dilutions were stored at -20°C. Anhydrous sodium sulphate (Merck) for residue analysis and silica gel 60-200 Mesh (Merck) were used after heating overnight at 120°C. Solvents were tested for interferences by concentration from 15 ml to 50 µl.

Apparatus and Materials

Human serum used for method evaluation was provided by the Blood Transfusion Centre, University Hospital of Antwerp (Belgium). Blood was collected in a vacuum system tube and centrifuged (15 min, 2000 g) within 24 h after collection. The serum was kept frozen at -20°C until analysed. All glassware was washed with detergent, rinsed with water, soaked for 24 h in chromic acid and rinsed with distilled water, acetone, and hexane. Prior to use, the treated glassware was rinsed with the solvent used for extraction. Empore™ extraction disk cartridges (C_{18}, 10 mm/6 ml) from 3M Company (St. Paul, MN, USA) and a Varian Positive Pressure Manifold (part 1223-420X) were used for solid-phase extraction. Empty cartridges (1 ml) were purchased from Supelco (Bellefonte, PA, USA). The modified silica gel was prepared as follows: to 50 g silica gel, 27 ml concentrated sulphuric acid was added dropwise, while the mixture was stirred to ensure good homogeneity. After all the acid was added, the modified silica was stirred for another 30 min.

A Hewlett Packard (Palo Alto, CA, USA) 6890 GC-µECD was equipped with a 50 m x 0.25 mm x 0.10 µm, CP-Sil 5/C18 capillary column, with 10% octadecyl (C18) chains incorporated in the methyl siloxane stationary phase (Chrompack, Middelburg, The Netherlands). Helium was used as carrier gas (0.9 ml/min) and Ar/CH_4 (95:5) as make-up gas (40 ml/min). Two µl were injected in pulsed splitless mode (pulse pressure = 25 psi, pulse time = 1 min) with the split outlet opened after 1.5 min. Injector and detector temperatures were set at 270°C and 300°C, respectively. The temperature program of the CP-Sil 5/C18 column was set to 90°C for 1 min, then with 15°C/min to 180°C, kept for 5 min, then to 250°C by 2°C/min and further by 4°C/min to 270°C, kept for 8 min.

A Hewlett Packard (Palo Alto, CA, USA) 6890 GC was connected via direct interface with a HP 5973 mass spectrometer. A 50 m x 0.22 mm x 0.25 µm, HT-8 (SGE, Zulte, Belgium) 1,7-dicarba-closo-dodecarborane 8% phenyl methyl siloxane capillary column was used with helium as carrier gas at a constant flow of 0.7 ml/min. One µl was injected in the pulsed splitless mode (pulse pressure = 30 psi, pulse time = 1.20 min) with the split outlet opened after 1.25 min. Injector and interface temperatures were set at 270°C and 310°C, respectively. The temperature program of the HT-8 column was starting from 90°C, kept for 1 min, then with 15°C/min to 170°C, stay 2 min and further by 4°C/min to 290°C, kept for 14 min.

The mass spectrometer was operated in the electron impact ionisation mode at 70 eV. A SIM table was constructed for GC-MS quantification. Three ions (two from molecular ion cluster M^+ and $[M+2]^+$ and the $[M-70]^+$ ion) were monitored for each level of chlorination for PCBs. For organochlorine pesticides, the two most abundant ions were monitored (Table 3.1).

Retention time, masses and relative abundance of the confirmation ions to the quantification ion were used as identification criteria. A deviation of ion ratios of less than ±20% from the theoretical value was considered acceptable.
All samples were analysed on both columns, the lowest value for each congener was further considered for calculations. Calibration curves ($r^2 > 0.99$) were created for the quantification using standard solutions in iso-octane, covering the entire range of expected values for each congener. Compounds were quantified against the closest internal standard. Peak area ratios (POP response/internal standard response) were plotted against the concentrations of POPs. Method limits of detection range between 5 and 10 pg/ml serum (whole weight) for ECD and between 10 and 30 pg/ml serum for MS detection.

Table 3.1. Ion fragments monitored in MS analysis for each compound or group of compounds.

Group of compounds	Quantification ion	Qualitative ion 1	Qualitative ion 2
Tri-PCB	256	258	186
Tetra-PCB	290	292	220
Penta-PCB	326	324	256
Hexa-PCB	360	362	290
Hepta-PCB	394	396	326
Octa-PCB	428	430	360
Nona-PCB	464	466	396
Deca-PCB	498	500	428
o,p'- and p,p'-DDE	248	318	-
o,p'- and p,p'-DDD and DDT	235	237	-
α–, β–, γ–HCH	181	219	-
HCB	284	286	-

Sample preparation and clean-up
Ten ml of human serum was spiked with 5 ng internal standards PCB 46 and PCB 143 (to give a concentration of 0.5 ng/ml serum). Before extraction, the spiked sample was equilibrated in an ultrasonic bath for 5 min. The serum was then mixed with 10 ml formic acid and 500 µl acetonitrile and was equilibrated by ultrasonic treatment for 30 min. Prior to the sample application, the Empore™ disk cartridges were washed with two 500 µl portions of dichloromethane and dried thoroughly. Then, each cartridge was activated with 250 µl of methanol followed by two 250 µl portions of deionized water. After conditioning, the cartridges were not allowed to dry.

To avoid overloading and breakthrough of the analytes, two cartridges in parallel, each containing 5 ml of sample, were used. After sample loading at a positive pressure of 2-4 psi, each cartridge was rinsed with two 500 µl portions of deionized water. The sorbent bed was dried thoroughly under a nitrogen stream at 20 psi positive pressure (10 min) and by centrifugation (15 min, 2000 g). Each column was eluted with two 500 µl portions of hexane and 500 µl of dichloromethane:hexane = 1:1 (*v/v*) mixture.

An empty cartridge filled with 0.5 g of acid silica and topped with 100 mg Na_2SO_4 was pre-washed with 2 ml of a mixture of dichloromethane and hexane (1:1, v/v) and 2 ml of hexane. The concentrated sample was applied to the acid silica cartridge. POPs were eluted with 4 ml hexane. After the addition of 50 µl of iso-octane as a keeper, the final eluate was concentrated

under a gentle nitrogen stream at room temperature to approximately 50 µl and transferred to a vial. The tube containing the extract was rinsed with 50 µl iso-octane which was transferred to the vial. Twenty-five µl of recovery standard TCN (500 pg/µl in iso-octane) was added to the final concentrate, prior to GC analysis.

Solid-phase extraction

The SPE method eliminates the elaborate procedures required by traditional methods for total lipid extraction, followed by various treatments to remove lipids. Pre-treatment of the serum by denaturation was found to release the compounds from the protein binding sites. The protein denaturation method without precipitation has been previously evaluated because of the possible loss of analytes by occlusion in the precipitate (Pauwels et al., 1999). Acetonitrile and reduced pH (adjusted by addition of formic acid) inhibit protein binding and increase the extraction efficiency of the analyte by the C_{18} sorbent. Furthermore, as described before (Pauwels et al., 1999), the use of Empore disk technology (90% sorbent, 10% matrix-PTFE) allows reduced elution volumes due to a small bed volume. The C_{18} disk cartridge employed for sample clean-up and analyte enrichment has a non-polar character causing retention of organochlorine and other non-polar compounds. It has also a size exclusion function to eliminate macromolecular interferences (such as serum proteins) in biological extracts. The small bed volume disk approach to SPE reduces elution volume (Plumb et al., 1997), thus making this method more attractive.

The use of slower flow rates (at 2-4 psi) allows maximal residence time of the solvents with the sorbent bed and yields slightly improved recoveries than higher flow rates (at 10-15 psi). The drying step is essential because the non-polar eluent needs to interact with all areas of the sorbent and should not be stopped by residual water trapped in the pores. Centrifugation of the cartridges before elution of POPs, is necessary due to the high compactness of the adsorbent bed, making complete drying difficult. Removal of lipids and other interfering compounds from the SPE eluate could be achieved by using a clean-up on column chromatography on silica gel impregnated with concentrated sulphuric acid. Additional sample clean-up on acidified silica resulted in a lower background, which facilitated peak identification of minor congeners, providing better instrumental performance and longer column lifetime.

Recovery experiments and reproducibility

In order to test the method performance, recoveries of internal standards and of all investigated compounds from 10 ml spiked serum were determined. Five replicates at one spiking level and five non-spiked replicates from the same batch of pooled serum were analysed. Absolute recoveries of POPs were calculated after subtraction of levels found in the non-spiked replicates from the spiked ones. Recoveries for selected POPs ranged from 62 to 74% and a good reproducibility (RSD < 14%) was demonstrated. As in other studies (Tan and Liem, 1998), there were no differences in recoveries between PCB congeners with a low or high degree of chlorination or with respect to the degree of chlorination in *ortho*-position. Results are presented in Table 3.2. Recoveries (n=5) for internal standards were 68 ± 8% for PCB 46 and 65 ± 9% for PCB 143, and they were considered satisfactory for our protocol.

Table 3.2. Relative retention times and recovery for investigated compounds.

Compound	Chlorine substitution pattern	RRT*		Recovery (%)	RSD (%)
		HT-8	CP-Sil5/C18		
PCB 28	2,4,4'	0.88	0.83	65	10
PCB 52	2,2',5,5'	0.93	0.90	71	11
PCB 66	2,3,4,4'	1.06	1.11	73	8
PCB 74	2,4,4',5	1.05	1.10	71	10
PCB 99	2,2',4,4',5	1.10	1.20	68	6
PCB 101	2,2',4,5,5'	1.09	1.18	69	9
PCB 105	2,3,3',4,4'	1.26	1.48	72	4
PCB 110	2,3,3',4',6	1.16	1.28	67	12
PCB 118	2,3',4,4',5	1.21	1.41	73	14
PCB 128	2,2',3,3',4,4'	1.34	1.62	68	11
PCB 138	2,2',3,4,4',5'	1.29	1.55	70	7
PCB 149	2,2',3,4',5',6	1.18	1.35	72	12
PCB 153	2,2',4,4',5,5'	1.24	1.49	68	10
PCB 156	2,3,3',4,4',5	1.39	1.76	70	4
PCB 157	2,3,3',4,4',5'	1.41	1.77	74	9
PCB 167	2,3',4,4',5,5'	1.35	1.70	71	5
PCB 170	2,2',3,3',4,4',5	1.47	1.88	65	7
PCB 180	2,2',3,4,4',5,5'	1.41	1.81	66	6
PCB 183	2,2',3,4,4',5',6	1.31	1.63	68	10
PCB 187	2,2',3,4',5,5',6	1.30	1.61	70	8
PCB 194	2,2',3,3',4,4',5,5'	1.63	2.10	67	5
PCB 199	2,2',3,3',4',5,5',6	1.48	1.90	65	12
HCB		0.76	0.66	62	7
p,p'-DDE		1.13	1.25	70	10
PCB 46**	2,2',3,6'	0.93	0.85	68	8
PCB 143**	2,2',3,4,5,6'	1.20	1.37	65	9

* - retention times relative to TCN (t_R = 25.25 min on CP-Sil5/C18 and t_R = 29.01 min on HT-8)
** - internal standards

ECD chromatogram of a serum sample analysed on the CP-Sil5/C18 column and is shown in Figure 3.1.

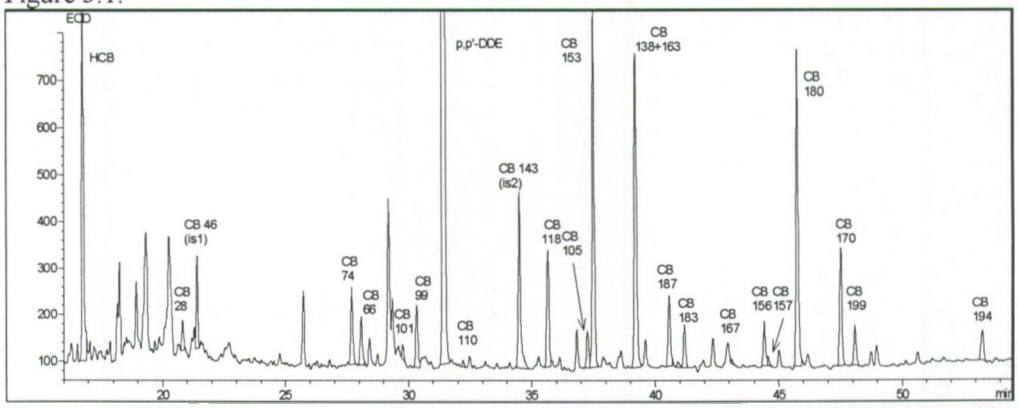


Figure 3.1. ECD chromatogram of human serum analysed on a 50 m CP-Sil 5/C18 capillary column.

Ion mass chromatograms for penta-, hexa- and hepta-PCBs of a serum sample analysed on HT-8 column are presented in Figure 3.2.

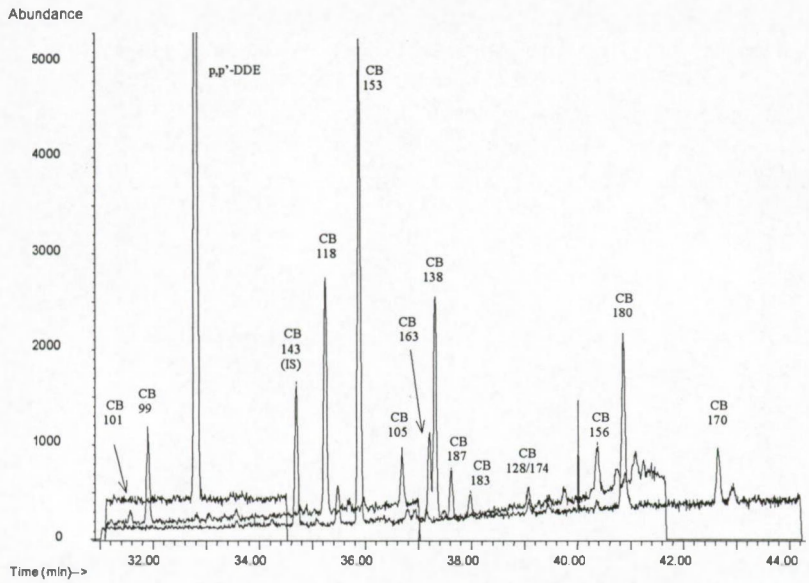


Figure 3.2. Ion chromatograms of human serum analysed on a 50 m HT-8 capillary column.

Considering the arguments presented in Chapter 2.4 concerning the GC columns used in POPs analysis, a good reproducibility for quantification was achieved. The concentration reported for each compound, was the average of the values obtained from the two GC columns used when the difference between the values was less then 20%, otherwise, the lower level was reported.

Quality Control

The following procedures were used to ensure adequate quality. All peaks were manually reviewed for proper integration. For ECD, the identification of POPs was based on their relative retention times (RRT) to the recovery standard (Table 3.1), while for MS, additional identification criteria were the selected ion chromatograms and the ratios of the abundances of the quantification ion and the qualifier ion (Table 3.1). Calibration curves were run with each batch of samples and correlation coefficients were always $r^2 > 0.99$. A standard solution including all congeners and reagent blank were analysed every sixth injection to correct for variations in chromatographic and instrument performance and to check for interferences. Recoveries of internal standards were monitored to ensure their maintenance at acceptable levels.

Internal quality control

Beside these regular procedures, internal quality control was also ensured by regular analysis of an in-house control serum and of standard reference material (SRM 1589a).

a. Human serum (about 50 donations) was obtained from the Blood Transfusion Center, University Hospital of Antwerp (UZA). The serum (approximately 120 ml) was pooled and

homogenised. Aliquots of 7 ml were divided into hexane-washed glass vials and kept at – 20°C until analysis. With each sample batch, a vial containing the in-house control serum was analysed using the described analytical procedure. Major PCB congeners and p,p'-DDE were measured. Confidence and control intervals were established as mean ± 2SD and mean ± 3 SD, respectively. Upper and lower control limits were set as proposed by Luotamo et al., 1997. All measurements from 10 runs fall into the confidence interval (Table 3.3).

Table 3.3. Internal quality control scheme

Parameters	Mean ± SD (n=10)	Min	Max	Confidence interval (mean ± 2 SD)	Control interval (mean ± 3 SD)
PCB 99	0.10 ± 0.03	0.08	0.16	0.04 – 0.16	0.02 – 0.19
PCB 118	0.15 ± 0.02	0.13	0.19	0.11 - 0.19	0.09 - 0.21
PCB 153	1.16 ± 0.12	0.92	1.32	0.92 - 1.41	0.80- 1.53
PCB 138	0.74 ± 0.06	0.65	0.90	0.61 - 0.86	0.55 - 0.92
PCB 187	0.21 ± 0.03	0.18	0.27	0.16 – 0.27	0.13– 0.30
PCB 156	0.11 ± 0.03	0.08	0.15	0.05 – 0.16	0.02 – 0.19
PCB 180	0.81 ± 0.11	0.60	0.94	0.59 - 1.03	0.48 - 1.13
PCB 170	0.32 ± 0.09	0.21	0.45	0.15 - 0.50	0.06 - 0.59
HCB	0.35 ± 0.12	0.22	0.54	0.12 - 0.58	0.02 - 0.69
p,p'-DDE	5.44 ± 0.49	5.00	6.26	4.46 - 6.42	3.97 – 6.91

As exemple, a control chart was drawn for PCB 153. It can be observed that all measured values of PCB 153 fall into the (mean – 2SD, mean + 2SD) interval (Figure 3.3).

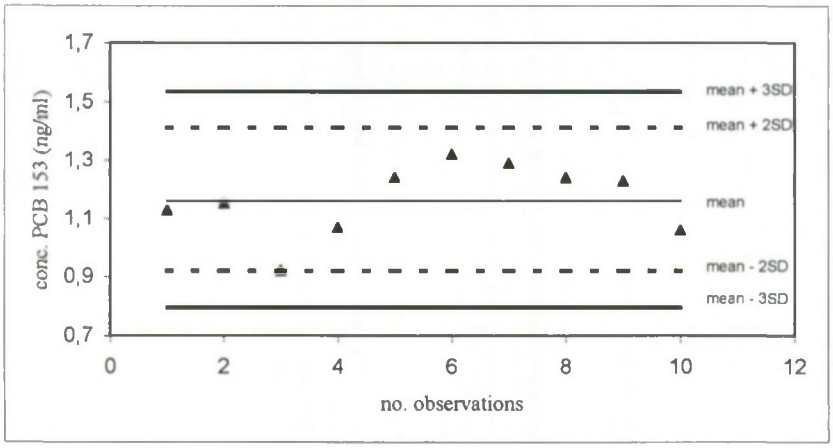


Figure 3.3. Flow chart for PCB 153.

b. The Standard Reference Material (SRM 1589a) is intendend for use in evaluating analytical methods for the determination of selected PCB congeners and chlorinated pesticides in human serum. Reference values are also provided for selected PCDDs and PCDFs. Details of certification procedure can be found at http://www.nist.gov

Sample description: freeze-dried human serum containing natural concentrations of organochlorine pollutants. It was recommended that the content of one vial should be reconstituted with 10 ml distilled or HPLC grade water.

Table 3.4. Concentrations of selected PCB congeners and organochlorine pesticides (ng/g reconstituted serum) obtained with the SPDE method from SRM 1589a (organochlorines in human serum).

Compounds	UIA	NIST	
	Mean ± SD (N=4)	Certified	Uncertified
PCB 99	0.21 ± 0.03	0.12 ± 0.01	
PCB 101	0.08	0.03 ± 0.01	
PCB 105	0.085 ± 0.02	0.03 ± 0.005	
PCB 118	0.19 ± 0.02	0.12 ± 0.01	
PCB 138	0.46 ± 0.05	0.48 ± 0.04	
PCB 153	0.75 ± 0.04	0.67 ± 0.04	
PCB 156	0.07 ± 0.02	0.07 ± 0.005	
PCB 180	0.41 ± 0.1	0.48 ± 0.03	
PCB 183	0.07 ± 0.03	0.07 ± 0.005	
PCB 187	0.22 ± 0.03	0.17 ± 0.03	
p,p'-DDE	10.61 ± 0.56	6.60 ± 1.00	
HCB	0.21 ± 0.05		0.05 ± 0.01
PCB 74	0.17 ± 0.02		0.23 ± 0.005
PCB 170	0.11 ± 0.04		0.19 ± 0.005
PCB 199	0.08		0.10 ± 0.02

Instructions: After careful removal of the stopper, 10 ml water were added at 20°C to the sides of the vial, while continually turning the vial. Mixing by gently swirling and standing were alternating for approximately 1 h. After reconstitution, the vial should be stored between 2°C and 8°C until analysis (preferably within 4 h).

It can be observed that values obtained for most PCB congeners fall inside the certified interval. However, discrepancies were observed for some compounds, such as p,p'-DDE, and PCB 99, 101, 105 and 118, probably due to inadequate calibration (for DDE) or to very low concentrations to be measured (for some PCB congeners).

External quality control

The SPDE method for the determination of organochlorine pollutants in serum was validated by participation in testing programs organised by Health Canada (Dr. J.J. Ryan) and by the Centres for Disease Control and Prevention, Atlanta, USA (Dr. V.W. Burse) as well as to the 24th round robin organised by University of Erlangen (Prof. Dr. J. Angerer).

a. Health Canada

An interlaboratory comparison test organised by Health Canada involved eight different laboratories (6 from North America and 2 from Europe). Two samples of human plasma (HC1 and HC2) had to be analysed for major PCB congeners. Results obtained by the Toxicological Centre were in good agreement with means for target analytes obtained from the participating laboratories (Table 3.5).

b. Centers for Disease Control

Five serum samples were received from Centres for Disease Control (Atlanta, USA). These serum samples were taken from goats that were fed technical Aroclors mixtures (single dose) and allowed to recover for 30 days before blood withdraw. Sample identification was as follows: Alpha – goat fed Aroclor 1254, Beta – control goat (not fed with Aroclors), Gamma

– goat fed Aroclor 1260 (serum was diluted with control serum), Delta – goat fed Aroclor 1016, Epsilon – goat fed Aroclor 1242. The samples were shipped in dry ice to the Toxicological Center.

Table 3.5. Canadian External Quality assessment scheme, results (ng/ml plasma). Two specimens were analysed (HC1 and HC2).

PCB congeners	HC1		HC2	
	UA	Mean*	UA	Mean*
99	0.21	0.16	0.09	0.09
105	0.09	0.07	0.03	0.05
118	0.38	0.30	0.17	0.14
138	0.67	0.59	0.40	0.32
153	0.63	0.52	0.51	0.45
156	0.12	0.10	0.06	0.05
170	0.11	0.09	0.11	0.15
180	0.21	0.17	0.28	0.28
sum PCBs	2.22	2.02	1.65	1.50
RSD (%)		9.90		10.0

* - total of 8 main congener values reported by 8 laboratories

Table 3.6. Centers for Disease Control serum samples

Compounds	Alpha		Gamma		Delta		Epsilon	
	UIA	CDC	UIA	CDC	UIA	CDC	UIA	CDC
PCB 28	nd	nd	nd	nd	17.9	16.8	2.18	1.63
PCB 52	1.31	1.03	nd	nd	1.08	0.83	0.59	0.37
PCB 66	1.28	1.54	nd	nd	2.01	2.68	3.36	3.72
PCB 74	1.57	1.95	nd	nd	2.38	3.44	4.72	5.32
PCB 99	5.51	5.76	nd	nd	nd	nd	1.78	1.93
PCB 101	1.93	1.78	nd	nd	nd	nd	0.56	0.55
PCB 105	8.20	6.18	nd	nd	nd	nd	2.33	2.02
PCB 110	0.27	0.13	nd	0.03	nd	nd	0.09	0.55
PCB 118	15.1	15.8	nd	0.09	nd	nd	3.28	3.73
PCB 138*	5.78	7.19	0.48	0.69	nd	nd	0.26	0.38
PCB 153	9.43	8.24	1.35	1.31	nd	nd	0.32	0.33
PCB 156	2.44	2.24	nd	0.15	nd	nd	nd	nd
PCB 170	1.55	1.89	0.59	0.79	nd	nd	nd	nd
PCB 180	2.05	1.70	1.53	1.34	nd	nd	nd	nd
PCB 183	0.24	0.22	0.25	0.21	nd	nd	nd	nd
PCB 187	0.35	0.29	0.44	0.33	nd	nd	nd	nd
PCB 194	nd	nd	0.35	0.40	nd	nd	nd	nd
PCB 199	nd	nd	0.40	0.36	nd	nd	nd	nd

* UIA: PCB 138; CDC: PCB 138/163

- the Beta sample had all measurements below LOD for both UIA and CDC

As seen in Table 3.6, with exception of PCB 105 and PCB 153, results are in good agreement for sample Alpha. For Beta, no peaks above the detection limit were found in both laboratories. Due to dilution of goat serum (fed with Aroclor 1260) with control serum, sample Gamma contained lower concentrations of high chlorinated PCB congeners than samples Alpha, Delta or Epsilon. All results for sample Gamma are in good agreement

between the two laboratories. Finally, with exception of PCB 74, PCB concentrations found in samples Delta and Epsilon are in good agreement between CDC and UIA (Toxicological Centre).

c. In November 1999, the Toxicological Center has participated in the 24th round robin organised by University of Erlangen. Two samples of human plasma (A and B) containing environmental concentrations of 6 marker CBs (28, 52, 101, 138, 153, 180), HCB, p,p'-DDE and PCP were received for analysis.

As seen in Table 3.7, all measured parameters were inside the tolerance range established by the organisers. Furthermore for most measurements, the difference between the obtained values and the reference value (established by the organisers) was less than 10%

Table 3.7. Results of the 24th Interlaboratory test for organochlorine pollutants in human serum organised by the University of Erlangen.

Compounds	Sample	UIA	Reference value	Tolerance range
PCB 28	A	0.44	0.44	0.24 – 0.65
	B	1.53	1.80	1.08 – 2.52
PCB 52	A	0.81	0.76	0.42 – 0.98
	B	1.80	1.84	1.24 – 2.43
PCB 101	A	0.81	0.76	0.44 – 1.09
	B	2.68	2.46	1.63 – 3.30
PCB 138	A	0.84	1.02	0.65 – 1.39
	B	1.89	2.46	1.63 – 3.29
PCB 153	A	1.39	1.51	0.99 – 2.04
	B	2.35	2.69	1.81 – 3.57
PCB 180	A	1.54	1.83	1.23 – 2.42
	B	3.05	3.27	2.27 – 4.28
HCB	A	0.78	1.08	0.62 – 1.53
	B	4.57	4.82	3.19 – 6.45
p,p'-DDE	A	2.08	1.79	1.10 – 2.48
	B	4.50	3.69	2.38 – 5.00
PCP	A	5.41	5.00	3.46 – 6.64
	B	13.04	11.58	8.45 – 14.71

Conclusion

The solid-phase disk extraction provides an effective method for monitoring selected organochlorine pollutants in a large number of samples. The use of commercially available pre-packed disposable SPDE cartridges reduces significantly the time required for the sample preparation and minimises cross contamination from high-level samples and glassware. Moreover, this clean-up method requires relatively small quantities of expensive and hazardous solvents. With the concentration on a C_{18} cartridge, removal of interference by adsorption chromatography on sulphuric acid: silica gel, and quantification by high resolution GC-ECD or high resolution GC/MSD, this method can be used for exposure assessment.

3.2. Applications

3.2.1. The Flanders Environment and Health study (1999-2000)

*- based on Koppen G, Covaci A, Van Cleuvenbergen R, Schepens P, Winneke G, van Larebeke N, Nelen V, Vlietinck R, Schoeters G, (2001). *Chemosphere*, submitted; Covaci A, Koppen G, Van Cleuvenbergen R, Schepens P, Winneke G, van Larebeke N, Nelen V, Vlietinck R, Schoeters G, (2001). *Chemosphere*, submitted; Koppen G, Covaci A, Van Cleuvenbergen R, Schepens P, Winneke G, Nelen V, Schoeters G, (2001). *Toxicol Lett* 123(1), 59-67.

Enough information on the presence of persistent organochlorine pollutants (POPs) in human tissues from industrialised countries is available to suggest that the concentration of these compounds have decreased during the last ten years (Van Cleuvenbergen et al., 1994; Ewers et al., 1996; EU Dioxin Exposure Data, 1999). Past measurements of polychlorinated dibenzo-*p*-dioxins (PCDD) and furans (PCDF) in human milk showed that Belgium had among the highest values in Europe (Tarkowski et al., 1989).
Few analyses of POPs in the Belgian population have been done until now. It was shown that PCDD/PCDF concentrations in pooled mother milk from Brabant-Walloon (rural), Brussels (urban) and Liège (industrial) collected in 1988 were among the highest in the world (Tarkowski et al., 1989). The mean value of these three pooled human milk samples was 37.6 pg I-TEQ/g fat. In 1993, three pooled samples from the same regions were measured. The mean concentration was now 24.8 pg I-TEQ/g fat (EU Dioxin Exposure Data, 1999).
Measurements of PCDD/Fs in Belgian human serum were done only very recently. In Wallonia, PCDD/F loads were measured in the general population in 1999. A mean PCDD/F concentration of 36.7 pg I-TEQ/g fat was found in a group of 54 men and women (mean age 50 yrs, range 10 to 80 yrs) living near to a municipal waste incinerator. This was 34% higher than the mean concentration (27.2 pg I-TEQ/g fat) measured in 32 persons of similar age range living in a rural area (Bernard, personal communication).
In a detailed study set up by the Flemish Government, concentrations of persistent organochlorine pollutants were evaluated in human populations from two regions in Flanders, Belgium. Primary objectives were: (i) determination of mean concentrations and regional differences of these pollutants based on pooled and individual results, (ii) calculation of correlations between concentrations of organochlorine pollutants measured in human serum and evaluation of potential markers, (iii) comparison of CALUX-TEQ with chemically measured TEQs values. This is the first study with such amplitude done in Belgium.

Study area
The rural area of Peer is situated 15-25 km from the nearest non-ferrous and chemical plants and is remote from motorways. The urban area (Wilrijk and Hoboken, two suburbs of Antwerp city) is located 11-13 km SE from the chemical and petrochemical industry established in the harbour, but close to a non-ferrous smelter, two municipal waste incinerators, a crematory, printing works, several small or medium-sized enterprises and a major motorway. The two waste incinerators have been in operation from 1971 and 1980, respectively. In 1997, they had annual turnovers of 23 000 and 110 000 tons. They were shut down in November 1997, because PCDD/PCDFs emissions were between 2 and 7 ng I-TEQ/Nm^3, exceeding the limit value of 0.1 ng I-TEQ/Nm^3 (Schoeters, 1998).

Study group

The study group consisted of 200 healthy women from Antwerp (n=100) and Peer (n=100) recruited between June and September 1999. The initial selection comprised of 2898 randomly selected women between 50 and 65 years old, which were contacted by letter. About half of the 40.1% and 30.8% responders in Antwerp and Peer respectively, were further selected (N=685) because of compliance to the following four criteria: non- or ex-smoker, minimal residence time of 10 years in the study area, working in the town of residence or at home and exclusion of jobs with specific risks of exposure. From those selected women, 255 were contacted by telephone, and 200 individuals decided to participate in the study. Each participant filled in an informed consent. This age group was selected because, due to bioaccumulation, elderly people have a higher POP body burden which theoretically would facilitate comparison of all different analyses.

Dietary information was obtained by a semi-quantitative food frequency questionnaire on meat, fish, eggs, milk and cheese. In order to estimate the dietary intake of PCBs and dioxins, fat scores and dairy consumption frequencies were calculated. Fat scores were calculated taken into account the average amount per consumption event and the average fat content for each food-group, according to the Dutch food composition table (van Erp-Baart, 1993). Consumption frequencies of milk, cheese and eggs were added and expressed as times of dairy intake per day. In case of outliers in the food categories, the upper and lower categories of intake were reduced until there were at least five observations in the category representing the highest respectively the lowest frequencies. Because the concentrations of investigated compounds in locally produced food may vary by region, information on the consumption of locally produced food was requested. The body mass index (BMI), expressed as weight (kg) divided by the square of the height (m) was calculated for each individual.

Sample collection and pooling procedure

Approximately 40 ml of blood was collected from each individual. Blood samples were collected in polyethylene recipients. Immediately after sampling, serum was separated and divided into one part for individual analysis of indicator PCBs congeners (3 ml) and CALUX-TEQ (2.5 ml) and another part for pooling. Pooling was done by ranking the women in the order of decreasing daily intake of meat and fish, decreasing daily intake of eggs and milk, increasing total number of weeks of lactation and increasing body mass index. The available serum of 3 to 5 subsequently listed individuals was pooled to approximately 50 ml. Each of the 47 pooled samples was divided in three aliquots for the analysis of PCDD/PCDFs (25 ml), PCBs/organochlorine pesticides (13 ml) and CALUX-TEQ (4 ml). All samples were stored in glass vials pre-cleaned with hexane and acetone, and kept at -20°C until analysis.

Target analytes

The analysed compounds are listed in Table 3.8. In 200 individual serum samples, the indicator PCBs and CALUX-TEQ values were determined. In 47 pooled serum samples, the following PCB congeners were measured: mono-ortho PCBs (PCB 105, 118, 156, 157, 167), indicator PCBs (28, 52, 101, 138, 153, 180) and PCB 44, 66, 74, 99, 110, 128, 149, 170, 183, 187, 194, 199. The pooled samples were also analyzed for the non-ortho PCBs (77, 81, 126, 169) and the 17 PCDD/PCDF toxic congeners. The analysis of mono-ortho, non-ortho PCBs and PCDD/PCDF congeners allowed the calculation of the toxicity equivalents (TEQ) for each sample using the toxicity equivalent factors (TEFs) (Table 3.8.) of the WHO (Van den Berg et al., 1998). Hexachlorobenzene (HCB), p,p'-dichlorodiphenyldichloroethylene (p,p'-DDE), p,p'-dichlorodiphenyl-trichloroethane (p,p'-DDT), lindane (γ-HCH) and

pentachlorophenol (PCP) were also measured in the pooled samples as major organochlorine pesticides found in human serum. For all compounds, measurements under the dectection limit were set at half of this detection limit ('medium bound' method). Serum concentrations of triglycerides, cholesterol and phospholipids were determined enzymatically (Pauwels et al., 2000b). All solvents were of pesticide grade purity and were used without any further treatment.

Table 3.8. Persistent polychlorinated compounds analysed in 47 pooled and 200 individual serum samples originating from 200 women (50 to 65 years).

Compound*	TEF-value**	Serum sample (N)	Analysis***
Indicator PCBs		pool (47)	GC-LRMS
PCB 28, 52, 101, 138, 153, 180	/	individual (200)	GC-ECD
Mono-ortho PCBs		pool (47)	GC-LRMS
PCB 105	0.0001		
PCB 118	0.0001		
PCB 156	0.0005		
PCB 157	0.0005		
PCB 167	0.00001		
Non-ortho PCBs		pool (47)	GC-HRMS
PCB 77	0.0001		
PCB 81	0.0001		
PCB 126	0.1		
PCB 169	0.01		
Other PCBs		pool (47)	GC-LRMS
PCB 44, 66, 74, 99, 110, 128, 149, 170, 183, 187, 194, 199	/		
PCDF/PCDDs		pool (47)	GC-HRMS
2,3,7,8-T_4CDF	0.1		
1,2,3,7,8-P_5CDF	0.05		
2,3,4,7,8-P_5CDF	0.5		
1,2,3,4,7,8-H_6CDF	0.1		
1,2,3,6,7,8-H_6CDF	0.1		
2,3,4,6,7,8-H_6CDF	0.1		
1,2,3,7,8,9-H_6CDF	0.1		
1,2,3,4,6,7,8-H_7CDF	0.01		
1,2,3,4,7,8,9-H_7CDF	0.01		
O_8CDF	0.0001		
2,3,7,8-T_4CDD	1		
1,2,3,7,8,P_5CDD	1		
1,2,3,4,7,8-H_6CDD	0.1		
1,2,3,6,7,8-H_6CDD	0.1		
1,2,3,7,8,9-H_6CDD	0.1		
1,2,3,4,6,7,8-H_7CDD	0.01		
O_8CDD	0.0001		
CALUX bioassay		pool (47)	CALUX
TEQ-parameter	/	individual (197)	
Pesticides		pool (47)	GC-LRMS
hexachlorobenzene (HCB), p,p'-DDE, p,p'-DDT, lindane (γ-HCH) pentachlorophenol (PCP)	/		

* IUPAC numbering

** Toxic Equivalency Factors from Van den Berg et al., 1998

*** LRMS - low resolution mass spectrometry, HRMS - high resolution mass spectrometry, ECD - electron capture detection

Analysis

PCB and pesticides analysis

Details of the procedure are previously described (see Chapter 3.1). Detection limits were 0.03 ng/ml (HCB), 0.02 ng/ml (γ-HCH), 0.04 ng/ml (p,p'-DDE), 0.015 ng/ml (p,p'-DDT) and ranged between 0.01 and 0.02 ng/ml for individual PCBs. When expressed based on lipid weight, limits of detection for selected compounds were between 1 and 2 ng/g fat. For PCP analysis, ^{13}C-PCP (internal standard) was added to 0.5 ml serum mixed with formic acid and extracted with hexane. After concentration, the extract was derivatised with diazomethane (to form Me-PCP) and purified on an acid silica micro-column. After concentration, 1 µl was injected on a GC/MS instrument in splitless mode. Recovery of PCP was 72 ± 6%. The detection limit for PCP was 0.07 ng/ml serum or 10 ng/g fat.

PCDD, PCDF and non-ortho PCB analysis (VITO laboratory)

Approximately 20 g human serum was mixed with isopropanol and spiked with internal standard solutions, containing the ^{13}C-labelled compounds (PCDD/PCDFs and non-ortho PCBs). After homogenisation, 45 g of silica and 45 g of anhydrous sodium sulphate were added, grounded to a fine free-flowing powder and extracted for 5 h in a Soxhlet apparatus with n-hexane:acetone (2:1, *v/v*). A first clean-up of the concentrated extract was performed on a multilayer chromatographic column filled with, from bottom to top, 2 g of silica/NaOH 33%, 10 g of silica/H_2SO_4 44% and 10 g of anhydrous sodium sulphate. After elution with hexane and concentration, the extract was purified by HPLC on a Hypercarb column and on alumina column. The analysis was performed on a Micromass Autospec Ultima GC-HRMS, equipped with a splitless injector and a 60 m x 0.25 mm DB5-ms capillary column (J&W Scientific). The HRMS instrument was operated in the electron impact ionisation mode at 34 eV with a resolution of 6000 and the acquisition was based on selected ion recording, using six groups which contained the two most abundant ions of the molecular ion cluster of each compound. Identification of the analytes was based on the m/z values of the selected ions, the relative retention time and the calculated vs. theoretic isotopic ratio for the monitored ions.

For quality control purposes, a procedure blank starting from 20 ml of ultrapure water was included with each series of five serum samples. Blanks averaged at 0.08 and 0.14 pg TEQ/ g serum for PCDD/PCDFs and non-ortho PCBs, respectively. The recovery of the ^{13}C-labelled internal standards was in the range of 70-130%. A milk powder reference material (CRM 534) was analysed together with the samples. The recovery on TEQ basis amounted to 95-102 %. The VITO laboratory has frequently and successfully participated to interlaboratory studies. It has been accredited according to EN 45001 for the analysis of PCDD/PCDFs in emissions, milk and dairy products.

CALUX bioassay (VITO laboratory)

Polychlorinated chemicals in serum were estimated with a Chemical-Activated Luciferase gene eXpression (CALUX) assay variant based on a previously described procedure (Murk et al., 1998). In this assay, dioxin-like compounds are assessed via *in-vitro* activation of the aryl hydrocarbon receptor (AhR) of cultured H4IIE cells. The method involves n-hexane extraction of blood serum (4 mL from the pooled serum and 2 ml from the individual samples) and removal of matrix components by passage through a 33% H_2SO_4 silica column. The extract was evaporated and reconstituted in dimethyl sulphoxide (DMSO, Acros Organics). Cells were grown in 96-well plates in 100 µl minimal essential medium (Gibco) with 10% fetal calf serum (Gibco) at a temperature of 37°C and 5% CO_2. After removal of the medium,

cells were washed with 100 µl phosphate-buffered saline without Ca/Mg (Life Technologies) and 30 µl of cell lysis reagent (Promega) was added. The well plates were then shaked for at least 45 min and stored at -80°C for at least 1 h.
For determination of luciferase activity, the cells were thawed on ice and 100 µl luciferin assay mix (Promega) was added at room temperature. The light production was measured by a Victor 2 Luminometer (EG&G Wallac). The CALUX-based TEQs were calculated by comparing the luciferase activity induced by the sample against a dose-response curve generated from 2,3,7,8-T_4CDD concentration standards analysed simultaneously. The limit of detection varied with cell growth and volume of the blood samples. For 4 ml serum with 700 mg fat/dl the limit of detection was 5.2 ± 3.5 pg TEQ/g fat. Measurements below were set at half of the detection limit. A fetal calf serum sample was run for each series of study samples as internal standard. The inter-experiment variation was less than 30 % and accepted as normal for this low-loaded sample.

Statistical analysis
Database management and statistical analysis were performed with Statistica version'99 (Statsoft Inc.). Analytical data that were not normally distributed were log-transformed. Means and proportions were compared across the two areas by t-test and χ^2-test, respectively. The statistical methods also included analysis of covariance to adjust for confounders in the interregional comparison. Personal attributes such as age, fasting status, body mass index, number of children, lactation history, and food consumption behaviour (intake dairy, fat, local food) were considered in the multivariate models to assess regional differences in the individual measurements. This was not possible for regional comparison of pooled data. The statistical power of the analysis test was calculated being the certainty of not finding false negative results when comparing mean concentrations of two regions. It was calculated for the respective number of samples analysed, based on the formula for comparison of two groups of normally distributed values.

Results and discussion
Study group
The study group had an average age of 58.5 years. There were no differences in age or anthropometric characteristics (BMI=26) between the women living in the rural area of Peer and the urban area of Antwerp (Table 3.9). However, the women from Peer were in average working for almost 30 years, whereas in Antwerp this was only 10 years. The consumption of dairy products was slightly higher in Peer, while the local product consumption was significantly higher in Peer. The number of children and months of breastfeeding were significantly higher in Peer (Table 3.9).

Pooled samples versus individual serum samples
Pooling of serum samples was done because of large volumes of serum needed for all analyses and to reduce the number of samples to be analysed, while keeping the resulting analytical information at acceptable levels. To the best of our knowledge, no other study has addressed the measurement of indicator PCBs and CALUX-TEQs on serum pools as well as on the individuals constituting the pools. The geometric mean of indicator PCBs and CALUX-TEQs in 47 pools and 200 individuals was 365 and 392 ng/g fat, and 36 and 38 pg TEQ/g fat respectively. As it can be seen in Table 3.10, the concentration of PCB 138 was somewhat higher for the analyses done on individual samples. This was due to the use of a Ultra-2 GC column for the individual samples (on which PCB 138 co-elutes with PCB 163 and 164),

whereas for the pooled samples, the separation of PCB 138 from PCB 163 was done using a HT-8 column.

Table 3.9. Description of studied population

Characteristics	Peer n=100		Antwerp n=100		p*
	Mean (SD)	Median	Mean (SD)	Median	
Clinical measurements					
Length(cm)	158 (6)	157	159 (6)	159	NS
Weight (kg)	69 (12)	67	68 (13)	66	NS
Body mass index(kg/m^2)	27.5 (4.4)	26.4	26.6 (5.1)	26.2	NS
Blood fat (mg/dL)	712 (139)	692	714 (165)	705	NS
Questionnaire data					
Age (years)	58.4 (4.1)	59.0	57.8 (4.1)	58.0	NS
Number of years living in the region	42 (14)	41	38 (14)	35	NS
Number of years working	27 (13)	32	10 (10)	8	<0.001
Ex-smokers (%)	7.1		9.7		NS
Number of years stopped smoking	34.0 (9.3)	30.0	26.9 (6.4)	28.0	NS
Passive smoking (hours/day)	0.8 (2.2)	0.0	2.5 (4.6)	0.0	0.04
Dairy consumption (frequency/day)**	0.49 (0.25)	0.45	0.48 (0.26)	0.43	NS
Consumption of animal fat***(score, g/day)	0.55 (0.20)	0.53	0.56 (0.23)	0.53	NS
Consumption of local food (%)	72		28		<0.001
Total number of breastfeeding weeks	22 (34)	10	8 (22)	0	<0.001
Number of children	2.9 (1.6)	3.0	2.0 (1.4)	2.0	<0.001
Age at birth of first child (years)	24.6 (3.8)	23.9	23.8 (3.6)	23.1	NS

NS – not significant for p>0.05

* Significant difference (p<0.05) between the 2 regions determined with the χ^2-test for 2x2 tables.

** Average for milk, cheese and eggs.

*** Fat from fish, shrimps, mussels, meat, cheese, milk, eggs, based on the average daily consumption and average fat content of each nutrition group.

Concentrations of the indicator PCBs were significantly higher in the urban area (423.6 versus 362.8 ng/g fat, p=0.002), when adjusted for age, animal fat and dairy consumption, and fasting status. Assessing regional differences without adjustment for confounding factors resulted in a lower significance of the difference (417.2 versus 369.1 ng/g fat, p=0.02). The individual CALUX-TEQ values were different from the results from the pooled samples (Table 3.10). Whereas there was no regional difference observed for the pooled samples, the individual CALUX-TEQ values were significantly higher in Peer (43.3 pg TEQ/g fat) compared to Antwerp (33.2 pg TEQ/g fat) (p=0.03), when adjusted for number of weeks lactation. This difference was more significant for non-adjusted measurements (44.5 versus 32.2 pg TEQ/g fat, p=0.002). The difference in results between pooled and individual samples could not be explained through confounder adjustment, which was possible only for the individual samples. The nutritional and life style parameters obtained where not powerful enough to be of much influence.

Table 3.10. Arithmetic means of persistent organochlorine pollutants measured in pooled and individual serum samples of 200 women (50-65 years) living in two regions of Belgium.

Compound*	**POOL (N=47)**				**INDIVIDUALS (N=200)**			
	P+A	P	A	p**	P + A	P	A	p**
indicator PCBs (ng/g fat)								
PCB 28	nd	nd	nd	–	nd	nd	nd	
PCB 52	nd	nd	nd	–	nd	nd	nd	
PCB 101	nd	nd	nd	–	nd	nd	nd	
PCB 138	95.2	83.9	105.1	**0.001**	114.9	105.0	125.6	**<0.001**[a]
PCB 153	167.3	152.6	181.4	**0.008**	159.2	148.5	170.6	**<0.001**[a]
PCB 180	104.8	102	107.7	0.42	116.4	113.5	119.4	0.15[a]
sum indicator PCBs	365.4	337.4	392.0	**0.020**	392.5	369.1	417.2	**0.002**[a]
mono-ortho PCBs (ng/g fat)								
PCB 105	7.3	6.2	8.4	**<0.001**		–	–	–
PCB 118	29.7	24.4	35.3	**<0.001**		–	–	–
PCB 156	15.8	14.5	17.1	**0.007**		–	–	–
PCB 157	2.4	2.4	2.4	0.99		–	–	–
PCB 167	4.4	2.8	5.8	**0.042**		–	–	–
mono-ortho PCB TEQ (pg/g fat)	12.9	11.6	14.2	**0.001**				
non-ortho PCBs (pg/g fat)								
PCB 77	nd	nd	nd	–		–	–	–
PCB 126	110.8	92.7	129.6	**0.005**		–	–	–
PCB 169	116.5	116.9	116.1	0.90		–	–	–
non-ortho PCB TEQ	12.6	10.8	14.5	**0.006**		–	–	–
other PCBs (ng/g fat)								
PCB 74	13.2	11.5	15.9	**<0.001**		–	–	–
PCB 99	14.1	11.5	16.7	**0.001**		–	–	–
PCB 170	42.2	40.2	44.0	0.11		–	–	–
PCB 183	8.5	7.6	9.4	**0.008**		–	–	–
PCB 187	20.1	18.3	21.7	0.13		–	–	–
PCB 194	15.3	15.4	15.3	0.94		–	–	–
PCB 199	16.6	16.2	17.0	0.50		–	–	–
sum PCBs (ng/g fat)	550.6	498.6	600.8	**0.005**		–	–	–
PCDFs + PCDDs (pg/g fat)								
2,3,7,8-T_4CDF	nd	nd	nd	–		–	–	–
1,2,3,7,8-P_5CDF	3.7	4.1	3.5	0.44		–	–	–
2,3,4,7,8-P_5CDF	31.8	32.1	31.6)	0.86		–	–	–
1,2,3,4,7,8-H_6CDF	12.7	14.0	11.7	0.18		–	–	–
1,2,3,6,7,8-H_6CDF	11.6	11.9	11.3	0.63		–	–	–
2,3,4,6,7,8-H_6CDF	6.2	6.7	5.8	0.40		–	–	–
1,2,3,7,8,9-H_6CDF	2.8	3.1	2.6	0.44		–	–	–
1,2,3,4,6,7,8-H_7CDF	15.7	13.1	17.9	0.17		–	–	–
1,2,3,4,7,8,9-H_7CDF	5.3	5.9	4.8	0.31		–	–	–
O_8CDF	20.3	26.2	16.3	0.07		–	–	–
2,3,7,8-T_4CDD	4.8	4.6	5.1	0.39		–	–	–
1,2,3,7,8-P_5CDD	13.2	12.7	13.6	0.42		–	–	–
1,2,3,4,7,8-H_6CDD	11.1	11.0	11.3	0.74		–	–	–
1,2,3,6,7,8-H_6CDD	43.4	43.6	43.1	0.83		–	–	–
1,2,3,7,8,9-H_6CDD	8.4	7.9	8.8	0.28		–	–	–
1,2,3,4,6,7,8-H_7CDD	78.4	65.8	91.5	**0.05**		–	–	–
O_8CDD	767.4	769.4	765.7	0.96		–	–	–
PCDFs TEQ	23.2	23.2	23.1	0.95		–	–	–
PCDDs TEQ	25.6	24.8	26.4	0.33		–	–	–
PCDFs+PCDDs TEQ	48.6	47.9	49.2	0.68		–	–	–
Total WHO-TEQ (pg TEQ/g fat)	75.0	70.9	78.9	0.06		–	–	–
CALUX bioassay (pg TEQ/ g fat)	36.0	37.2	35.0	0.61	38.1	44.5	32.2	**0.03**[a]
Pesticides (ng/g fat)								
Hexachlorobenzene	110.3	95.6	125.2	**0.001**		–	–	–
Pentachlorophenol	622.5	714.6	551.3	0.08		–	–	–
p,p'-DDT	2.8	3.6	2.0	**0.003**		–	–	–
p,p'-DDE	950.8	997.6	911.5	0.46		–	–	–
Lindane (γ-HCH)	5.9	7.2	4.8	0.06		–	–	–

–: not measured; nd: not detectable (below detection limit) in all or nearly all samples, *IUPAC numbers, ** p value for regional comparison of the data expressed per g serum fat. [a] Regional comparison of the individual data was done taking into account the covariates: number of lactation weeks (for CALUX-TEQ) or age, fasting status, and fat and dairy consumption frequency (for indicator PCBs). PCB 44, 66, 81, 110, 128 and 149 had all measurement below the detection limit.

Therefore, pooling should be avoided if the purpose of the study is to compare pollutant concentrations between different areas. Besides loss of information (less statistical power, thus more samples needed), there is no possibility to adjust for possibly essential confounders like age, antropometric characteristics and food consumption. On the other hand, if the mean concentration of POPs in human serum for all people in both regions is of interest, the pooling procedure offers a good and cheaper alternative.

Concentrations of POPs and regional differences for pooled serum samples

Concentrations of some organochlorine pesticides (PCP, p,p'-DDE and γ-HCH) were higher (but not significantly) in Peer, while the concentration of p,p'-DDT was significantly higher in that region. Hexachlorobenzene concentration was significantly higher in Antwerp (125.2 versus 95.6 ng/g fat, p=0.001).

Three PCB congeners (IUPAC n° 138, 153 and 180) were present in all pooled samples and contributed with approximately 65% to the total PCB concentration (sum of 27 congeners). Their concentration was significantly higher in the urban region (392.0 ng/g fat versus 337.4 ng/g fat) (Table 3.10). Concentrations of other congeners such as PCB 118 and 156 were also significantly higher in the urban region (35.3 versus 24.4 ng/g fat for PCB 118 and 17.1 versus 14.5 ng/g fat for PCB 156, respectively). Concentrations of PCB 170, 180, 187, 194 and 199 were not significantly different between the two regions. The total PCB concentration was significantly higher in Antwerp (600.8 versus 498.6 ng/g fat, p<0.005).

The mean TEQ concentrations were 25.6 and 23.2 pg TEQ/g fat for PCDDs and PCDFs, respectively. When non- and mono-ortho PCBs were added, the mean total WHO-TEQ value increased to 75.0 pg TEQ/g fat. There was no statistical difference in the PCDD or PCDF concentrations in the two regions, except for 1,2,3,4,6,7,8-H_7CDD, which was slightly significantly higher in Antwerp (p=0.05). Non- and mono-ortho PCB-TEQ values were significantly higher in the urban area (Table 3.10), while the PCDD/PCDF-TEQ and the total WHO-TEQ (sum of PCDDs, PCDFs, non-ortho and mono-ortho PCBs) were not statistically different for both regions. PCDDs and PCDFs contributed almost equally to the PCDD/PCDF-TEQ (average of 53% and 47%, respectively). The mean PCDD/PCDF contribution to the total WHO-TEQ value was 67%. The non- and mono-ortho PCBs contributed to the total WHO-TEQ with 16 and 17 %, respectively. The principal contributors to the total WHO-TEQ value were 2,3,4,7,8-P_5CDF (PCDFs), 1,2,3,7,8-P_5CDD (PCDDs), PCB 126 (non-ortho PCBs) and PCB 156 and 118 (mono-ortho PCBs). Dioxin-like toxicity was also assessed by the CALUX bioassay (mean value of 36.0 pg TEQ/g fat). No significant regional difference was observed in dioxin-like toxicity of the pooled serum samples (37.2 pg TEQ/g fat in Peer versus 35.0 pg TEQ/g fat in Antwerp, p=0.61).

Regional differences in correlations between organochlorines

Comparative to the rural region, higher correlation coefficients were found in the urban area between almost all TEQ values and between TEQ values and indicator PCBs (Table 3.11). The main intake route of those POPs is considered to be the food consumption. There was no difference in reported food consumption in both regions, except for local food, which was more consumed in the rural area (70% of women reported local food consumption vs. 28% in

Antwerp). However, higher PCB concentrations in the urban area Antwerp were observed. Their overall correlation coefficients with the total TEQs were r= 0.70 and 0.57 respectively (Table 3.11). Both increased to 0.77 and 0.73 when considering only the women in the urban region (Table 3.11).

Table 3.11. Pearson correlation coefficients between the different TEQ-measurements and between TEQs and indicator PCBs, for all regions together and separated per region (all log-transformed, except for the CALUX values).

	TEQ	MO-PCB	NO-PCB	MO+NO-PCB	PCDD/F	Total TEQ	IND-PCB
P+A: N=47	CALUX	0.39**	0.53***	0.51***	0.43**	0.57***	0.24
	MO-PCB	-	0.65***	0.86***	0.49***	0.77***	0.84***
	NO-PCB		-	0.95***	0.23	0.65***	0.41**
	MO+NO-PCB			-	0.34*	0.75***	0.62***
	PCDD/F				-	0.88***	0.55***
	Total TEQ					-	0.70***
P: N=22	CALUX	0.24	0.36	0.34	0.39	0.43*	0.07
	MO-PCB	-	0.67***	0.86***	0.29	0.57**	0.78***
	NO-PCB		-	0.95***	0.30	0.62	0.42*
	MO+NO-PCB			-	0.32	0.64**	0.61**
	PCDD/F				-	0.92***	0.34
	Total TEQ					-	0.52*
A: N=25	CALUX	0.61***	0.76***	0.79***	0.46*	0.73***	0.39
	MO-PCB	-	0.53**	0.79***	0.68***	0.88***	0.84***
	NO-PCB		-	0.93***	0.17	0.60**	0.26
	MO+NO-PCB			-	0.39	0.79***	0.53**
	PCDD/F				-	0.87***	0.71***
	Total TEQ					-	0.77***

P= Peer, A= Antwerp, IND-PCB: indicator PCB, NO-PCB: non-ortho PCB, MO-PCB: mono-ortho PCB, Total TEQ = sum TEQ values of mono-ortho PCB, non-ortho PCB, PCDDs and PCDFs.
*p< 0.05, ** p<0.01, ***p<0.001

For the relations: CALUX-TEQ ⇔ total TEQ and indicator PCB ⇔ total TEQ, regression equations were calculated. The respective equations were very similar if calculated for all samples of the women in Peer and Antwerp as well as for both regions seperatly. Using those three different regression lines, the concentrations of total TEQ for all 47 serum samples was estimated from their concentrations of CALUX-TEQ or indicator PCBs (Figure 3.4).

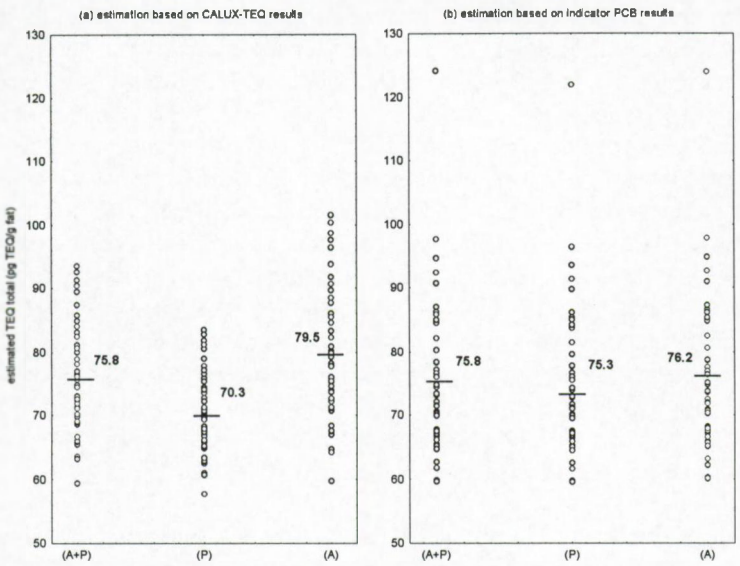


Figure 3.4. Estimation of total TEQ for all 47 pooled serum samples from measured **(a)** CALUX-TEQ results and **(b)** indicator PCB results, using the overall regression equations obtained for individual samples in both regions (A+P), or for the region Peer (P) or Antwerp (A). The experimental TEQ values in the pooled samples were: 75.0, 70.9 and 78.9 pg TEQ/g fat for A+P, P and A, respectively.

This was done to find out if the regional differences in correlation were of large influence on the predictive value for total TEQ of these two examined marker measurements. Estimating the mean total TEQ based on marker PCB results there was no difference whether using the regression found in Peer (75.3 pg TEQ/g fat), Antwerp (76.2 pg TEQ/g fat) or overall (75.8 pg TEQ/g fat). Moreover, the estimated values were statistically identical with the measured value of 75.0 pg TEQ/g fat. However, mean total TEQ values calculated based on CALUX-TEQs using the three regression lines were 70.3, 75.8 and 79.5 pg TEQ/ g fat respectively. The first value was significantly different from the others ($p<0.001$), which could be expected based on the rather low correlation coefficient found between CALUX-TEQs and total TEQ in the rural area ($r=0.43$). All three equations lead to a reasonable equal predicted total TEQ value within certain confidence range. The difference between observed and estimated concentration of total TEQ was < 20% in 77% of the samples. The maximal discrepancy between the measurements of total-TEQ and the estimated total-TEQ was 40%. This is probably due to the high value for the intercept (51.15 pg TEQ/g fat). This means that even for a low value for the CALUX-TEQ measurements, the total TEQ is now estimated to be above 50 pg TEQ/g fat in any case. This is mostly improbable situation because in reality some samples will have values lower than 50 pg TEQ/g fat (Table 3.11).

If another population would be examined with background contamination of POPs, it therefore would be possible to 'predict' TEQ values – within an confidence interval – by measuring indicator compounds. This opens the possibility to estimate the total TEQ values from measurements that are easier and cheaper and need lower amounts of blood.

Serum POP values of Flemish women: comparison with other Belgian and foreign data
Mean concentrations and profiles of PCBs of all individual and pooled serum samples were comparable with individual results obtained from Swedish (Grimvall et al., 1997) and Dutch women (Koopman-Esseboom et al., 1994a,b). In these studies the women were younger, in average 38.8 and 29 years old, respectively (Table 3.12). Furthermore, comparison is possible with two recent Belgian studies (Pauwels et al., 2000b, Nawrot et al., 2001). In the first study, PCBs were measured in young infertile women (mean age 32 years). The second study was part of the FLEHS, and was composed of 17-18 years old adolescents living in the two regions Antwerp and Peer. As expected, due to selection of elderly persons, concentrations of PCB 118, 138, 153 and 180 were higher in this population. A Canadian study (Longnecker et al., 2000) conducted in 1994 on 63 blood donors (33 females, 30 males) with mean age of 45 years showed approximately 3-fold lower levels of indicator, mono- and non-ortho PCBs. The burden of indicator PCBs was even 1.5-2 fold lower than found in young Flemish female populations (Pauwels et al., 2000b; Nawrot et al., 2001). This confirms that PCB concentrations in Belgium and Europe remain a matter of concern.

Table 3.12. Mean (and range) serum PCB concentrations (ng/g fat) in women living in Belgium and other European countries.

Country	**Belgium**				**Sweden**	**The Netherlands**
Sampling Year	1996-1998	1999	1999	1999	1986-1991	1990-1992
Reference	Pauwels, 2000a	Present study		Nawrot, 2001	Grimval, 1997	Koopman, 1994 a
N	106 indiv	47 pools	200 indiv	120 indiv	50 indiv	415 indiv
Age	31.9 (24-42)	58.5 (50-65)		17.4 (17-18)	42 (29-53)	29
PCB 28	nd	3.2	nd	nd	3.0	-
PCB 52	nd	1.7	nd	nd	na	-
PCB 101	nd	1.5	nd	nd	1.0	-
PCB 118	27.7	29.7	-	-	31.0	32.0
PCB 138	69.9	95.2	125.2	69.0	120.0	120.0
PCB 153	94.5	167.3	170.8	92.5	210.0	182.0
PCB 170	-	42.4	-	-	52.0	-
PCB 180	72.0	104.8	122.9	49.7	140.0	108.0

-: not available, nd: not detectable
* geometric mean

The TEQ values in the serum of the Flemish women were higher than values found in Wallonia (Belgium) and other countries (Table 3.13). The data presented in Table 3.13 should be interpreted with caution due to differences in: sampling years, age of individuals and TEF values used (Safe, 1990; Ahlborg et al., 1994; Van den Berg et al., 1998). However, present levels in this Flemish age group can be compared with values obtained in other industrialised countries about 10 years ago. Moreover, a similar German population (43-71 years old) sampled in 1996 showed average values two times lower than PCDD/PCDF-TEQ values measured in the Flemish population (Table 3.10).
When including the non- and mono-ortho PCBs in the TEQ calculations, the total TEQ value increased with 33%. This was also observed in Canadian Red Cross blood donors from Toronto (Longnecker et al., 2000). In the latter study, both mean PCDD/PCDF-TEQs (20 pg TEQ/g fat) and total TEQs (35 pg TEQ/g fat) were lower than in the present study. It was one of the few studies where all dioxin-like compounds were measured in the same population.

Table 3.13. TEQ blood values from the CALUX-bioassay or chemical PCDD/PCDF analysis with GC-HRMS in some European studies.

Country	Year	Age	Sex	N	Mean (range) pg TEQ/g fat	Analysis method	Reference
*Belgium (Flanders)***	*1999*	*58.5*	*F*	*47 p*	*36.1 (4.2-64.9)*	*CALUX*	*Present study*
				200 i	*38.2* (2.1-139.6)*	*CALUX*	*Present study*
Belgium (Flanders)	1999	17-18	M+F	200	30.9* (2.0-243.5)	CALUX	Nawrot,2001
	1996-1998	32	F	106	46.8 (2.0-160.2)	CALUX	Pauwels, 2000a
Netherlands	1990-1992	± 30	F	13	103.7 (-)	CALUX	Brouwer, 1997
Belgium (Flanders)	*1999*	*58.5*	*F*	*47 p*	*48.6[a] (31.2-81.3)*	*GC-HRMS*	*Present study*
Belgium (Wallonia)	1999	10-80	M+F	*54*	36.7[b] (incin.) 27.2[b] (controls)	GC-HRMS	unpublished
Finland	1989-1990	41	M	*14*	49[b] (20-99)	GC-HRMS	EU, 1999
	1993	43	M	*18*	37[b] 26-86)	GC-HRMS	EU, 1999
Finland rural	1999	< 30	M		- (10-15)	GC-HRMS	EU, 1999
	1999	> 30	M		-	GC-HRMS	EU, 1999
Germany	1988	-	M+F	*10*	46.3[b] (-)	GC-HRMS	EU, 1999
	1989	37		*102*	40.8[b] (11.6-93.5)	GC-HRMS	EU, 1999
	1991	44.7		*95*	40.8[b] (11.2-113.6)	GC-HRMS	Ewers, 1996
	1992	37		*44*	26.0[b] (12.0-61.0)	GC-HRMS	EU, 1999
	1993	37		*70*	21.7[b] (10.3-48.8)	GC-HRMS	EU, 1999
	1994	40.4		*134*	19.1[b] (5.2-43.9)	GC-HRMS	EU, 1999
	1996	36.7		180	16.5[b] (7.0-?)	GC-HRMS	EU, 1999
		18-30		*59*	13.0[b] (7.3-?)		
		31-42		*68*	16.9[b] (7.0-?)		
		43-71		*53*	19.9[b] (9.6-?)		
Spain (ind.)	1997	28-62	M+F	*20*	27[b] (14.8-48.9)	GC-HRMS	EU, 1999

* geometric mean, ** both analyses on same persons, [a] WHO-TEQ, [b] I-TEQ

Another approach for assessing the total TEQ burden is the measurement of Ah-receptor activity of POPs in serum using a bioassay (in our case, the CALUX). CALUX-TEQ values reflect the toxicity of all POPs having a synergistic, additive and/or antagonistic interaction with the Ah-receptor. Therefore, the CALUX-TEQ values will differ from the chemically estimated TEQs, which are simply summated. In the present study, the absolute value of the CALUX-TEQ was somewhat lower than the PCDD/PCDF-TEQ, and about half of the total WHO-TEQ. The observed CALUX-TEQ was comparable to the CALUX-TEQ of the young Flemish women (Pauwels et al., 2000a) and considerably lower than the CALUX-TEQ values (mean of 103.7 pg TEQ/g fat) measured in plasma of young Dutch women in 1990-1992 (Brouwer, 1997). This latter discrepancy could be due to different methodologies used. It was clear that this assay might offer new possibilities in monitoring TEQ values in human serum, but further interlaboratory validation of the absolute CALUX-TEQ values is necessary.

Concentrations of most organochlorine pesticides in serum of the women were comparable to other studies. A recent study showed that plasma levels of PCP in men from Sweden and Latvia were between 170 and 1800 ng/g fat (Sjödin et al., 2000). The same range of PCP

levels was found in blood from Canadian men and women participating in 1992 in the Santé Québec Health Survey and from a general population in southern Quebec (Sandau et al., 2000). The levels of p,p'-DDE, HCB and γ-HCH observed in our study were similar or lower than levels from Great Lakes fish consumers (Anderson et al., 1998) and more than 10 times lower than plasma organochlorine levels in 65-74 years old German men and women, recruited in the early eighties (De Voto et al., 1998). Both in Peer and Antwerp, the DDT/DDE ratio was very low (0.0036 and 0.0022 respectively), indicating a past exposure to p,p'-DDT. The use of p,p'-DDT in Belgium has been banned more than 25 years ago. As the women have been living on average for almost 40 years in Peer and Antwerp, they might have been more exposed particularly in the rural area. This could also explain the higher (though not significant) average p,p'-DDE serum concentrations in Peer. Lindane concentrations were non-significantly higher in Peer (p=0.056). Lindane remains in use in Belgium for restricted applications like desinfection of seeds, insecticide on soils, sugar beet, flowers and ornamental plants. Food is the main exposure route for the general population, so this might have smoothed the effect of lindane use in the rural area Peer. Dietary intake of fatty foods is also thought to be the main exposure way to HCB. However, the higher serum concentrations of HCB in Antwerp (p= 0.001) might suggest intake from industrial activities near to the city.

Single markers of exposure

Due to high correlations between organochlorine compounds, less expensive measurements can be used for estimation of e.g. total TEQ (Longnecker et al., 2000). It was shown that the analysis of even single marker substances in serum could provide cost-effective assessment of human exposure to complex mixtures of organochlorines (Glynn et al., 2000).

Table 3.14. Pearson correlation coefficients between single markers and organochlorine compound groups (all log-transformed). For each group and single markers, the best correlation coefficients was highlighted.

Compound	PCBs		MO-PCBs		PCDD/Fs	
	PCB 153	PCB 180	PCB 118	PCB 156	23478-P_5CDF	12378-P_5CDD
IND-PCB	**0.99*****	0.92***	0.63***	0.80***	0.52***	0.43**
PCB total	**0.96*****	0.89***	0.74***	0.82***	0.47**	0.37*
PCB-TEQ	0.60***	0.57***	**0.83*****	0.77***	0.25	0.18
MO-PCB TEQ	0.83***	0.76***	0.82***	**0.94*****	0.40**	0.14
PCDF-TEQ	0.43***	0.47***	0.21	0.35*	**0.97*****	0.35*
PCDD-TEQ	0.53***	0.50***	0.33*	0.55***	0.39**	**0.91*****
PCDD/F-TEQ	0.55***	0.57***	0.30*	0.51***	**0.87*****	0.72***
Total TEQ	0.69***	0.68***	0.64***	0.73***	**0.75*****	0.58***

IND-PCB: indicator PCB, MO-PCB: mono-ortho PCB, Total TEQ = sum TEQ mono-ortho PCB, non-ortho PCB, PCDDs and PCDFs.

*p< 0.05, ** p<0.01, ***p<0.001

Strong relationships were found between the concentrations of single mono-ortho and di-ortho PCBs and groups of PCB congeners in serum (Table 3.14). The preferential analysis of only these compounds (preferably PCBs) is possible by the use of fast analytical methodologies (Covaci et al., 2001b). For correlation coefficients higher than r=0.75, linear regression was performed between the concentration of the potential marker substance and groups of organochlorine compounds. The difference between observed and estimated

concentration for each serum sample of indicator PCBs, mono-ortho PCB TEQs, PCB TEQs, PCDD/F TEQs and total TEQs are given (Figure 3.5).

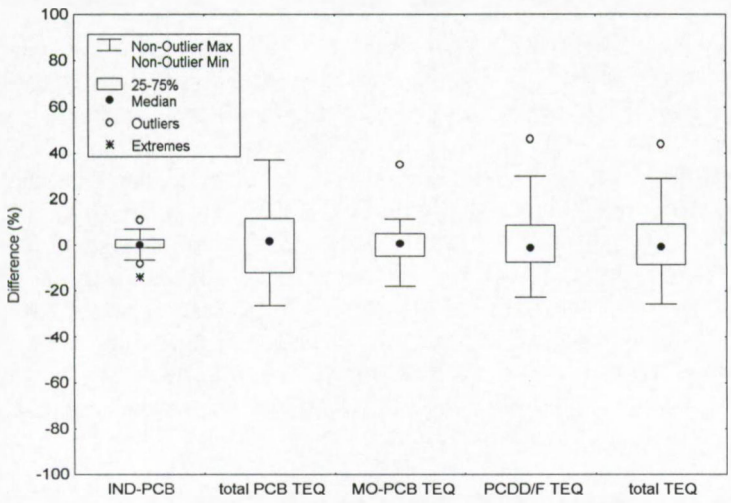


Figure 3.5. Box plots of percentage difference between observed and estimated concentration in each sample of indicator PCBs (IND-PCB), total PCB TEQ, mono-ortho PCB TEQ (MO-PCB TEQ), PCDD/F TEQ and total TEQ. The estimated concentration was calculated using the concentration of single marker substances: PCB 153 (for indicator PCBs), PCB 118 (total PCB TEQ), PCB 156 (mono-ortho PCBs), 2,3,4,7,8-P_5CDF (PCDD/F TEQ and total TEQ). Outliers = if data point value > UBV+1.5*(UBV-LBV), or data point value < LBV–1.5*(UBV-LBV), with UBV and LBV the upper and lower value of the box.

The estimated concentration was calculated using the concentration of single marker substances: PCB 153 (for indicator PCBs), PCB 118 (total PCB TEQ), PCB 156 (mono-ortho PCBs), and 2,3,4,7,8-P_5CDF (PCDD/F-TEQ and total TEQ). All of them were good markers with more than 50% of the pooled serum samples having a difference lower than 15% between observed and estimated concentrations of the respective compound groups' concentrations.

Relationships between TEQs of organochlorines in pooled serum samples

Lognecker et al (2000) reported that for populations with background-exposure (like in our case), it is impossible to sort out the possible contribution of the various organochlorine compounds to health effects because of their strong associations. In Table 3.11, Pearson correlation coefficients between the different TEQ values in the 47 pooled serum samples are given. The total WHO-TEQ value was in good correlation with the values of the individual contributors: mono-ortho PCBs (r=0.77), non-ortho PCBs (r=0.65) and PCDD/Fs (r=0.88) considering all serum pools in both regions (Table 3.11). TEQ values from non-ortho PCBs were poorly correlated (r=0.23) with PCDD/F-TEQs. This could indicate that, beside main intake through the diet, these two groups of POPs might have different exposure sources. A better correlation was found with mono-ortho PCBs (r=0.65). TEQ values from mono-ortho PCBs also showed a higher correlation with PCDD/F (r=0.49). All correlation coefficients calculated from these data were similar with those found for individual measurements in Dutch mothers by Koopman-Esseboom et al. (1994a) and Gladen et al. (1999) or Longnecker

et al. (2000) in individuals of a Michigan and Canadian population respectively. This suggests that correlations among classes of TEQs are not lost in pooled serum data.

TEQ values: chemical analysis versus CALUX-bioassay
Because of historically elevated values in the Flemish population, it was decided that a more rigorous monitoring program should be implemented. For this reason, valid methods (in terms of speed, simplicity and accuracy) had to be proposed. One approach could be the use of the CALUX-bioassay. The total WHO-TEQ values, calculated as the sum of PCDD/Fs, non-ortho and mono-ortho PCBs TEQ values, were two times higher than the TEQ values determined with the CALUX-bioassay (36 pg TEQ/g fat). Similar CALUX-TEQ values were observed in 1996-1998 for young Flemish women (mean age 32 years) (Pauwels et al., 2000a). Considerably higher CALUX-TEQ values (mean of 103.7 pg TEQ/g fat) were measured in 1990-1992 in plasma of Dutch women (mean age 29 years) (Brouwer, 1997). However, differences in results might be (partly) explained by differences in fat extraction and efficiency of clean-up methods used. As the CALUX-TEQ reflects synergistic, additive and/or antagonistic interaction of any compound (including "non-dioxin-like" compounds) with the Ah-receptor, sample cleanup is very important.

Bovee et al. (1998) reported similar values for CALUX-assay and TEQs of PCDD/F and several mono- and di-*ortho*-substituted PCBs determined by GC/MS in 22 cow milk samples. The correlation between the two sets of data was 0.74. The same extraction and cleanup protocol was used for both determinations. In another study, Aarts et al. (1996) have found that average CALUX-TEQ values are almost double than the TEQs obtained by GC/MS measurements of PCDD/F, non- and mono-ortho PCBs in human milk samples. When they applied a fat extraction with hexane and fat destruction through a H_2SO_4 silica column, the CALUX-TEQ values were lower or equal to the GC-MS TEQs. In this case, the correlation coefficient between the two sets of TEQ measurements was 0.71. Similar methodology as the latter was used in our study for human serum samples.

When cleanup was not used after fat extraction, Schecter et al. (1999) observed in human blood 1000-4000 times higher CALUX-TEQ values compared to the sum of TEQs from PCDD/F and coplanar PCBs. These higher values suggest that there were other biologically active compounds in human blood samples which could interact and activate the Ah-receptor pathway in the CALUX-bioassay. Possible compounds include dietary compounds such as indole and tryptophan metabolites, heme breakdown products, as well as polyaromatic hydrocarbons (PAHs), polybrominated biphenyls, polyhalogenated naphthalenes, hexachlorobenzene, azo- and azoxybenzenes. In addition, PCBs have been known to act as Ah-receptor antagonists in a species-specific manner (Schecter et al., 1999). Depending on the extraction method and the cell exposure protocol used, those factors may influence the CALUX-TEQ readings to a higher or lesser extent. Thus, further interlaboratory validation and standardization of the CALUX protocol is necessary in order to obtain a valid and comparable tool for biomonitoring.

Relationships between CALUX-TEQ and total-TEQ
Regardless of the large difference in absolute values, there was no regional difference in human serum from elderly women neither for CALUX-TEQs, PCDD/F or total TEQs. It was clear that these measurements give similar information concerning relative concentrations of "dioxin-like" compounds. In Table 3.11, Pearson correlation coefficients between CALUX-

TEQ and other chemically measured WHO-TEQs are given. CALUX-TEQ values were correlated with all other TEQ groups in the range of r=0.34-0.57. The relatively low correlation coefficients indicate that the AhR mediated response from other compounds (probably including non-organochlorines) together with all interaction effects is also measured in the CALUX-bioassay. The correlation coefficient between CALUX measurements and total TEQ value (r=0.57) increased to 0.73 when considering only the women in the urban region. This important variability in correlation coefficients suggests a difference in the concentration and composition of "dioxin-like" compounds in human serum. While higher concentrations of most PCB congeners were measured in the women living in the urban area (Table 3.10), no measurements were done for other compounds with AhR activity. Therefore it was not possible find a specific reason for this regional difference with respect to correlation. The observed correlations in the urban area were similar with values found by comparing GC/MS determined total WHO-TEQs (including PCBs) and CALUX-TEQ in: human serum, r=0. 71 (Aarts et al., 1996) and cow's milk, r=0.74 (Bovee et al., 1998).

Conclusions

The levels of indicator PCBs and organochlorine pesticides were comparable to those found in other European countries, while TEQ values were clearly higher. Based on pooled serum samples, concentrations of PCBs including indicator, mono-ortho and non-ortho PCBs were higher in the urban region compared to the more rural area. For these pooled samples total WHO-TEQ, PCDD/PCDF TEQ and CALUX-TEQ values were not different between the regions. Measuring TEQs in individual samples by the CALUX bioassay surprisingly revealed slightly higher values in the rural area. It was shown that, especially for comparison of "dioxin-like" activity between groups of people living in different regions or of different age, CALUX-bioassay can be a useful tool for biomonitoring purposes with similar conclusions as deducted from the chemically measured TEQ values. The discrepancy in the absolute values is influenced by still unknown factors and needs to be understood in order to use the CALUX-bioassay as an exposure marker for TEQ values in serum samples. Further interlaboratory validation and standardization of the protocol will further improve the use of CALUX-bioassay for biomonitoring purposes.

Acknowledgments: The Flemish Environment and Health Study (FLEHS) was commissioned and financed by the Flemish Ministry of Health (Brussels, Belgium). The CALUX-bioassay was a gift from Prof. A. Brouwer (Biodetection Systems, The Netherlands).

3.2.2. Distribution of PCBs and organochlorine pesticides in umbilical cord and maternal serum

* based on Covaci A, Jorens P, Jacquemyn Y, Schepens P, *Sci Total Environ,* submitted.

Introduction

Human adults are mainly exposed to PCBs and related compounds by consuming diary products, meat and fish. The foetus and embryo receive small amounts transplacentally and the breast-fed infant is exposed to relatively high doses of PCBs and dioxins via breast feeding. Knowledge of biological levels of these contaminants in new borns and infants is required for the risk assessment of adverse health effects and for the identification of vulnerable groups.

The major developmental endpoints related to *in utero* and lactational exposure to organochlorine pollutants in human infants are reduced birth weight, altered circulating thyroid hormone levels, psychomotor and cognitive functions.

The possibility of transfer of organochlorine chemicals (such as PCBs and organochlorine pesticides) across the human placenta has been well documented (Polishuk et al., 1977; Saxena et al., 1980). Investigators have linked high prenatal exposure to PCBs to neurodevelopmental deficits in children (Fein et al., 1984; Rogan et al., 1986; Jacobson et al., 1990a). The observed PCB-associated neurodevelopmental deficits were associated with intrauterine growth retardation (Jacobson et al., 1990b; Lai et al., 1993; Patandin et al., 1996). However, whether the associations observed were attributable to PCBs remains uncertain.

Dioxins and PCBs can alter thyroid hormone status in laboratory animals (decreased plasma T4 levels accompanied by increased concentrations of TSH). Furthermore, there is evidence that human exposure to these compounds may result in altered thyroid hormone status (reduced serum T4 levels have been observed in transformer repair workers exposed to PCBs (Emmett et al., 1988).

Information regarding prenatal exposure to organochlorines in Belgium is scarce. Pollutant concentrations have been determined in the infant blood collected at delivery to study the significance of the *in utero* exposure. In this study we measured the levels of PCBs, HCB and DDE in maternal and cord blood of 44 Belgian mothers and compared the obtained values with concentrations reported in the literature together with possible adverse effects.

Materials and methods

Study group

The studied population was composed of 44 pregnant women who delivered apparently healthy babies between November and December 1999 at the University Hospital of Antwerp, Belgium. All deliveries were at term and no complications were reported during pregnancy. The mean age of the mothers was 30 years (range 21-37 years). Samples of umbilical cord and maternal blood were taken immediately after the delivery. All blood samples were collected in heparin tubes and centrifuged at 2000 g. The collected serum was kept at –20°C until chemical analysis. Because information concerning the nutritional habits, living and professional characteristics of the subjects was not complete, these data were not included in the final calculations.

Analysis method

The analytical method was previously described (see Chapter 3.1). Sample size of umbilical cord and maternal serum was between 0.5 and 3 ml. Due to relatively low sample volumes, lipid analysis was not performed for this set of samples. The following compounds were analysed: HCB, p,p'-DDE and PCB no. 28, 52, 99, 101, 118, 138, 153, 170 and 180. The limit of quantification (LOQ) for individual congeners was set to 0.02 ng/ml fresh weight).

Statistical analyses

Data analysis was performed by means of the statistical software package Statistica (StatSoft, 1998). Means, medians, ranges and standard deviations are reported in terms of original distributions. Pearson correlation coefficients were measured between concentrations of organochlorine pollutants in umbilical cord and maternal serum. A p value < 0.05 was estimated as being statistically significant.

Results and discussion

All measurements of PCB 28, 52 and 101 were below the LOQ and therefore, were not taken into further calculations. Some measurements for PCB 99, 118 and 170 were below LOQ in the umbilical cord serum, but above LOQ in all maternal serum samples. PCB 138, 153 and 180 had all measurements in umbilical cord and maternal above LOQ. Typical chromatograms for umbilical cord and maternal serum are presented in Figure 3.6. Results including means, medians, standard deviations and ranges of values for all organochlorine pollutants in umbilical cord and maternal serum are presented in Table 3.15.

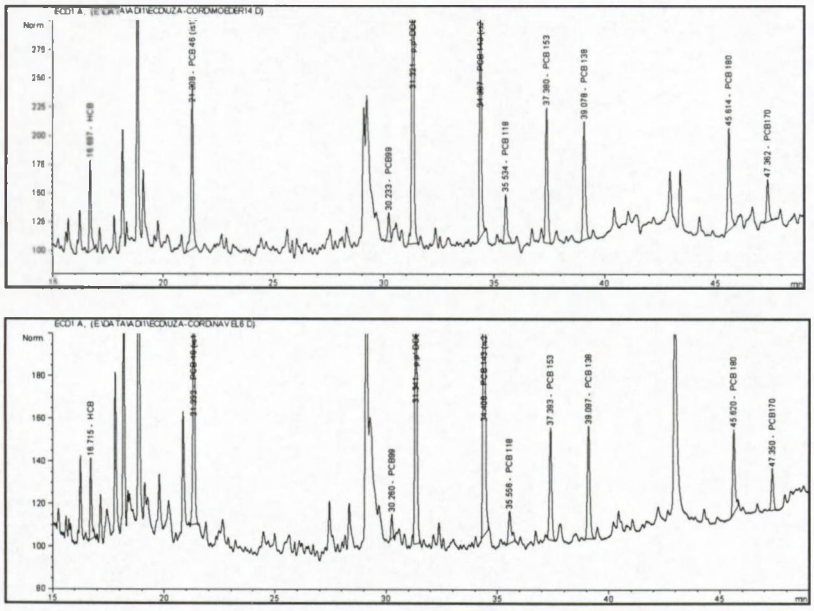


Figure.3.6. Typical chromatograms for umbilical cord and maternal serum.

As expected, concentrations of all organochlorine pollutants in umbilical cord serum are lower than the corresponding concentrations in maternal serum. p,p'-DDE was the major organochlorine contaminant in both matrices, followed by PCBs 153, 138 and 180.

Total lipids measurements were only available for 3 individual pairs of umbilical cord/maternal serum. The lipid concentration in umbilical cord serum is approximately two times lower than in maternal serum (means 2.94 and 6.02 g/l, respectively). Thus, when concentrations of pollutants are calculated on a lipid basis, the concentration of PCBs and p,p'-DDE in umbilical cord serum in the 44 Belgian pairs will be approximately two times lower than in maternal serum. Similar findings were reported in an Inuit population from Greenland (Bjeeregaar et al., 2000), where in 126 pairs umbilical cord - maternal serum, higher concentrations of PCBs and p,p'-DDE (calculated on lipid basis) were found in the maternal than in cord serum. However, other studies have reported that, on a lipid basis, concentrations of PCBs (Koopman-Esseboom et al., 1994a,c) and p,p'-DDE (Walisewski et al., 2000) are similar in umbilical cord and maternal serum.

Table 3.15. Concentrations (pg/ml fresh weight) of HCB, p,p'-DDE and individual PCB congeners in umbilical cord and maternal serum.

Compound	Umbilical cord serum (pg/ml)			Maternal serum (pg/ml)		
	Mean ± SD	Median	Range	Mean ± SD	Median	Range
PCB 99	35 ± 25	30	nd - 100	100 ± 60	100	40 - 275
PCB 118	65 ± 40	55	nd - 155	185 ± 90	180	55 - 365
PCB 138	110 ± 60	95	30 - 355	445 ± 220	420	150 - 1 150
PCB 153	160 ± 90	145	40 - 545	670 ± 350	580	175 - 1 840
PCB 170	30 ± 20	25	nd - 130	150 ± 90	140	40 - 500
PCB 180	80 ± 55	60	15 - 340	345 ± 200	310	85 - 1 130
Sum PCBs	**470 ± 260**	**450**	**120 - 1 580**	**1 900 ± 930**	**1 755**	**535 - 5 140**
PCB153/sum PCB	0.33 ± 0.06	0.33	0.18 – 0.49	0.35 ± 0.04	0.34	0.28 – 0.47
HCB	90 ± 50	70	25 - 240	205 ± 95	180	60 - 515
p,p'-DDE	580 ± 400	490	120 - 2 915	2 160 ± 1 765	1 560	565 - 20 740

It seems that the capacity for pollutant accumulation in specific compartments of the human body depends on the proper physical-chemical properties of each pesticide. This permits the formation of an equilibrium pattern between in umbilical cord and maternal serum, their specific accumulation caused by the inability of rapid metabolic transformation and detoxification processes.

Profile of contaminants in both matrices

Similar profiles of individual PCB congeners, HCB and p,p'-DDE were found in umbilical cord and maternal serum (Figure 3.7).

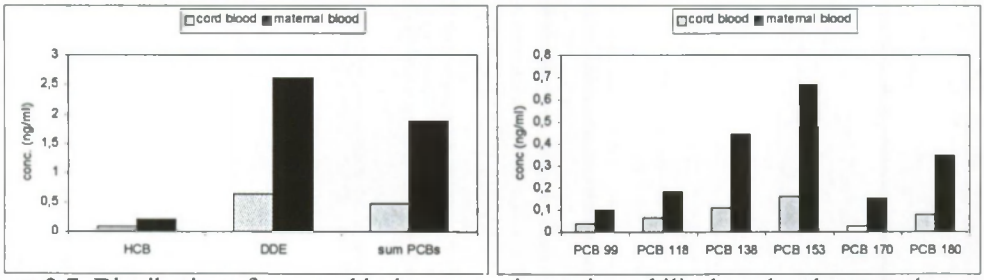


Figure 3.7. Distribution of organochlorine contaminants in umbilical cord and maternal serum.

Levels of HCB, p,p'-DDE and PCB congeners in umbilical cord serum were on average around 40%, 29%, and 26% of maternal levels (Table 3.16). Similar ratios for PCBs (around 20%) were found by Koopman-Esseboom (1994b) in a population of 418 Dutch women.

Table 3.16. Ratios between pollutants concentrations in umbilical cord and maternal serum.

	HCB	p,p'-DDE	PCB 99	PCB 118	PCB 138	PCB 153	PCB 170	PCB 180	Sum PCBs
Mean	0.403	0.2880	0.327	0.360	0.255	0.257	0.201	0.239	0.259
SD	0.141	0.094	0.132	0.199	0.081	0.126	0.106	0.123	0.087

Pearson correlation coefficients between individual contaminants in umbilical cord and maternal serum

Moderate Pearson correlation coefficients for organochlorine pollutants calculated in paired maternal and umbilical cord serum (Table 3.17), indicate an equilibrium pattern between these body compartments during pregnancy. Correlation coefficients are similar with those found in the Dutch cohort (Koopman-Esseboom et al., 1994b).

Table 3.17. Correlation coefficients between organochlorine pollutants in cord blood and maternal serum.

Parameters	Pearson coefficient*
c-HCB - m-HCB	0.49
c-DDE - m-DDE	0.92
c-PCB 99 - m-PCB 99	0.48
c-PCB 118 - m-PCB 118	0.51
c-PCB 138 - m-PCB 138	0.73
c-PCB 153 - m-PCB 153	0.65
c-PCB 170 - m-PCB 170	0.75
c-PCB 180 - m-PCB 180	0.76
c-sumPCB - m-sumPCB	0.74
c-PCB 153 - c-sumPCB	0.95
c-PCB 180 - c-sumPCB	0.93
m-PCB 153 - m-sumPCB	0.98
m-PCB 180 - m-sumPCB	0.95
c-sumPCB - age	0.43
m-sumPCB - age	0.51
c-DDE - age	0.48
m-DDE - age	0.49

c-: concentrations in cord blood serum
m-: concentrations in maternal serum
*- all correlations were statistically significant ($p<0.05$)

Because correlation coefficients between individual PCB congener levels in maternal and umbilical cord serum are not high (r=0.48-0.76), it is difficult to predict the pre- and postnatal exposure levels of an individual infant accurately, on the basis of maternal serum levels. In our present study, maternal age was correlated positively to both PCB and DDE levels in umbilical cord and maternal serum. Dewailly (1996) observed that mean levels of both PCBs and DDE in breast milk were greater in females aged 30y or more than in younger women.

Levels in Belgian population compared with other population

Several studies are available for comparison with our results regarding organochlorine levels in umbilical cord serum (Table 3.18). However, methodological problems (e.g., small samples sizes or differences in analytical methods used for quantification) restrict comparison with these studies. Furthermore, PCB concentrations are reported by some authors as individual congeners or as a total concentration of several congeners, whereas other investigators chose the equivalent concentration of a commercial mixture (Aroclor 1260). Despite these differences in analytical methods, the mean concentration of PCBs in umbilical cord serum appears to be of similar order of magnitude as in other European countries. Similar conclusions can be drawn for p,p'-DDE concentrations.

Table 3.18. Mean concentrations of organochlorine pollutants in maternal and cord blood serum from different countries

Country	No. samples	Maternal serum (ng/ml)		Cord Serum (ng/ml)		Reference
		PCBs*	p,p'-DDE	PCBs*	p,p'-DDE	
Belgium	44	1.64	2.2	0.41	0.58	This study
Mexico	60	-	14.5	-	6.0	Waliszewski et al., 2001
Greenland	126	6.7	5.0	1.5	1.4	Bjerregaard et al., 2000
Canada	656	-	-	0.14	0.41	Rhainds et al., 1999
The Netherlands	395	2.04	-	0.38	-	Patadin et al., 1998
Finland	67	1.35	-	0.46	-	Hagmar et al., 1998
Germany	84	-	-	0.63	-	Brouwer et al., 1995
The Netherlands	206	2.25	-	-	-	Weisglass et al., 1995
The Netherlands	382	-	-	0.38	-	Janousek et al., 1994
Poland	13	-	-	0.23	-	Janousek et al., 1994
Czech Republic	8	-	-	0.77	-	Janousek et al., 1994
Kenya	11	-	6.6	-	1.9	Kanja et al., 1992
The Netherlands	17	2.76	-	0.42	-	van Kaam et al., 1991

* - sum of PCB 118, 138, 153 and 180.

Concentrations of p,p'-DDE in the Mexican blood samples (Walisewski et al., 2001) are the highest, due to a very recent use of DDT (until 1996) for malaria vector control. Due to an extensive marine diet, the population from Greenland contains the highest levels of PCBs in umbilical cord and maternal serum. Concentrations of PCBs and p,p'-DDE in Canadian samples are the lowest reported in the literature (Rhainds et al, 1999), while concentrations from European countries lie between the two extremes.

Effects

It was found (Koopman-Esseboom et al., 1994c) that even under low levels of prenatal exposure, there is a slight but significant effect on thyroid hormone metabolism. The no-observed-adverse-effect level (NOAEL) of organochlorines on impairement of neurological development remains unclear. Tilson (1990) suggested that 1 µg/g PCB concentration in milk fat was the NOAEL for this endpoint. The differences in analytical method and in lipid content were taken into account, and the corresponding umbilical cord serum concentration was calculated as 5 ng/ml (Rhainds et al., 1999). In our population, no new born had a PCB or p,p'-DDE concentrations that exceeded 5 ng/ml. Measured concentrations were 5 to 10 times lower than the NOAEL.

Conclusions
In conclusion, our results show that prenatal exposure to organochlorine compounds in Belgium is similar with that of other industrialised countries. In addition, no children reach the toxic levels associated with alterations of the cognitive development. Finally, results of this study underline the importance for public health authorities to evaluate the magnitude of exposure to environmental contaminants in humans.
A short exposure to high PCB levels, as in the case of the Belgian PCB crisis (see Chapter 7.2), might have different effects on thyroid hormone parameters compared with a long-term background exposure.

3.2.3. Human serum samples from Timisoara and Iassy (Romania)

* - based on Covaci A, Hura C, Schepens P, (2001). *Sci Total Environ*, 280 (1-3), 143-152.

Data on POPs available from industrialised countries are abundant and inventories of sources and possible pollution sites are building up. Although high amounts of POPs were used for industrial and agricultural purposes, less data (Holoubek, 2000) are available from developing countries (including Eastern European countries).

Most of the data available from Romania (Hura et al., 1995, 1997, 1999) are limited to organochlorine pesticides (OCP). OCPs residues in humans from Eastern Romania were measured in 65 adipose tissue and in 180 maternal milk samples (Hura, 1988). A significant reduction in the concentration of ΣHCH and ΣDDT was found in 1985 as compared with 1975. Relatively high concentrations of pesticides were found indicating on-going pollution due to previous contaminated sites or improper disposal. Other studies (Aurigi et al., 2000) have indicated relatively high concentrations of pesticides in wildlife and environment.

This study aims to assess concentrations of POPs in individual human serum from two major towns of Romania.

Samples
Human serum was provided by the Municipal Hospital of Timisoara, Romania (20 samples from general population) and the Gynaecology Unit, University Hospital of Iassy, Romania (20 samples from mothers with at term delivery). After centrifugation, serum was kept frozen at -20°C until analysis.

Results and discussion
Concentrations of organochlorine pollutants in Romanian samples are presented in Table 3.19 Mean concentrations of all compounds in samples from Timisoara were higher than in Iassy samples due to inclusion in the study of individuals of different age (1-68 years). In human serum, β-HCH and p,p'-DDE were the principal organochlorine pesticides, while PCB profile is dominated by the persistent congeners (PCB 153, 138 and 180). In serum samples from Timisoara, correlation coefficients between age and concentrations of DDTs or PCBs were 0.46 and 0.29, respectively.

β–HCH was the principal contributor (80%) to ΣHCHs, except for some samples from Timisoara, where the γ–HCH isomer was present up to 40%. A relatively high ratio p,p'-DDT/ΣDDT (up to 0.66) was found in few samples probably due to a more recent

contamination with technical DDT. The use of DDT was banned in Western Europe in the 1970s, while in Romania, it happened only in 1987. To the best of our knowledge, the data presented in Table 3.19 are the first PCB measurements in human serum from Romanian specimens.

Table 3.19. Levels of selected POPs (ng/ml) in human serum from two Romanian towns.

Compound	Iassy mothers (n=20) (19-32 y)		Timisoara (n=20) (1-68 y)	
	mean ± SD	range	mean ± SD	range
HCB	0.07 ± 0.08	nd – 0.36	0.17 ± 0.15	nd – 0.52
ΣHCH (α–,β–,γ–HCH)	0.69 ± 0.89	nd – 3.8	6.15 ± 6.77	0.84 – 26.5
γ-HCH/Σ HCH	0.26 ± 0.22		0.14 ± 0.17	
ΣDDT	5.33 ± 4.08	1.7 – 12.0	24.84 ± 23.50	1.45 – 85.8
p,p'-DDT /Σ DDT	0.18 ± 0.08	0.06 – 0.32	0.08 ± 0.04	0.03 – 0.18
Σ PCB	0.26 ± 0.22*	nd – 1.04	4.24 ± 4.76**	0.4 – 17.70
PCB 153 / Σ PCB	0.35 ± 0.03	0.30 – 0.42	0.28 ± 0.04	0.22 – 0.35

*-sum of PCB 99, 118, 138, 153, 170 and 180

**-sum of PCB 99, 101, 118, 138, 149, 153, 156, 170, 180 and 187.

The group of mothers from Iassy has similar characteristics with a group of mothers from Antwerp (see Chapter 3.2.2) and thus, results can easily be compared. As expected, p,p'-DDE levels are higher in the Iassy samples, probably due to a more recent restriction on DDT usage. It is possible that levels of other organochlorine pesticides which were not measured in Antwerp samples (HCHs and p,p'-DDT) are also higher in the Romanian group. Concentrations of HCB and PCBs are significantly higher in the Antwerp cohort (Table 3.20).

Table 3.20. Comparison between concentrations of HCB, p,p'-DDE and PCBs in mothers from Romania (n=20) and Belgium (n=44).

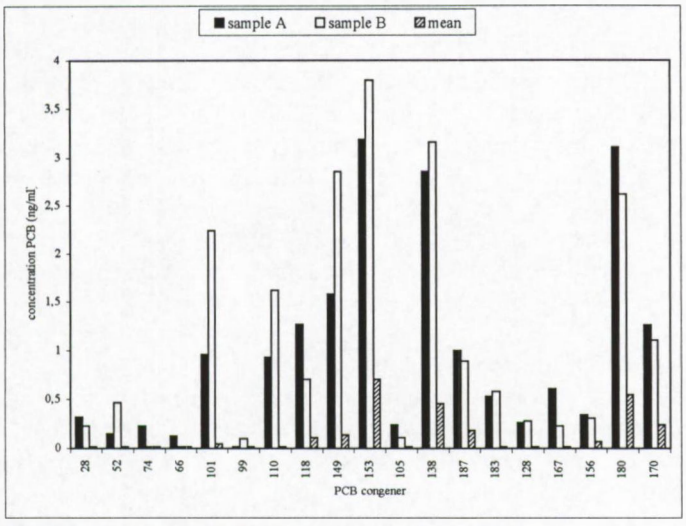
Compound	Iassy mothers (n=20) (19-32 y)		Antwerp mothers (n=44) (21-37 y)		p
	mean ± SD	range	mean ± SD	range	
HCB	0.07 ± 0.08	nd – 0.36	0.20 ± 0.09	0.06 – 0.52	< 0.01
p,p'-DDE	3.98 ± 2.72	1.03 – 11.32	2.16 ± 1.77	0.57 – 20.74	< 0.01
Σ PCB*	0.26 ± 0.22	nd – 1.04	1.90 ± 0.93	0.54 – 5.14	< 0.01

*-sum of PCB 99, 118, 138, 153, 170 and 180

The mean organochlorine pesticide levels in serum (Hura, 1999) collected between 1995 and 1997 from mothers living in the Iassy county (n=50, age range between 20 and 30 years) were 0.92 ng/ml for Σ HCH (mean γ-HCH/Σ HCH ratio of 0.50) and 8.75 ng/ml for Σ DDT (mean p,p'-DDT/Σ DDT of 0.05). A decrease in pesticide levels was observed when comparing samples from 1995-1997 with the present study.

The PCB 153/Σ PCBs ratio in Iassy samples was different from the ratio observed in samples from Timisoara. This is due to the presence of a different PCB profile in two samples from Timisoara (A and B). Figure 3.8 shows that high amounts of non persistent PCB congeners (IUPAC no. 28, 52, 66, 74, 101, 105, 110, 128 and 149) were present in these samples (24 and 27 years, respectively).

Total PCB concentrations measured in these samples (17.7 and 14.7 ng/ml, respectively) are higher than the mean PCB concentration in the remaining 18 samples from Timisoara (mean: 2.51 ng/ml). These findings suggest a local and recent contamination with PCB commercial



mixtures of the specimens at their working or residence place.
Figure 3.8. Profiles of PCBs in samples from Timisoara. Individual PCB congeners are presented in the elution order from the HT-8 column.

No monitoring studies for humans were and are undertaken in Romania and thus, the current degree of contamination with POPs is not known. More effort should be put in the monitoring of persistent pollutants in food and human population from Eastern Europe.

3.2.4. Distribution of persistent organochlorine pollutants in protein derivatives obtained from human plasma fractionation

*- based on Covaci A, Laub R, Di Giambattista M, Branckaert T, Hougardy V, Schepens P, (2001), *Vox Sanguinis*, submitted.

Introduction
Due to the relative easy sampling and to the accumulation of POPs in lipid-rich human tissues (Noren et al., 1999), human serum or plasma are the matrices of choice for the determination of organochlorine contaminants. Human plasma is an important starting material for the production of different derivatives such as therapeutic proteins for managing life-threatening coagulation, immune disorders and for shock treatment. The Cohn process (Cohn, 1946), based on cold ethanol fractionation of human plasma, involves extensive purification by a series of precipitations, virucidal treatment with solvent-detergent, and ion-exchange chromatography, yielding the proteins of interest, such as immunoglobulins, albumin, and clotting Factor VIII.

The distribution and removal of these pollutants were examined in intermediate fractions along the Cohn fractionation process and in the final plasma derivatives. To the best of our

knowledge, this is the first study to discuss the distribution of persistent pollutants in protein derivatives obtained by plasma fractionation.

Methods

Samples

Three starting pooled plasma batches (1500 l plasma/batch) of approximately 5000 donations from healthy individuals were collected in 2000 at the Belgian Red Cross Centers following the PCB food crisis in Belgium (van Larebeke et al., 2001). The plasma batches were tested with serological, viral, and biochemical tests. Sample size for the determination of lipids, proteins and selected contaminants was either 10 ml (for plasma and supernatants) or 50 mg (for the intermediate fractions). Precipitates were re-suspended according to standard production procedures. All fractions were stored at –20°C, except those to be used for pollutant determinations that were kept at room temperature and analysed within 48 h of their re-suspension.

Protein lipid and organochlorine analysis

In each process fraction, proteins, lipoproteins, lipids, and selected organochlorine contaminants were measured. The total protein concentration was determined by Biuret's method using albumin as standard. Lipoproteins (ApoA and ApoB) were measured by nephelometry (BNA100, Behring) using standardized assay protocols and calibrated standards. Lipids (cholesterol and triglycerides) were measured by an enzymatic assay. The plasma and plasma fractions were analyzed for HCB, p,p'-DDE and PCB congeners no. 28, 52, 101, 118, 138, 153, 156, 170 and 180, according to the procedure described in Chapter 3.1. Total PCB concentrations were calculated by summation of all PCB congeners. Method limits detection ranged between 10 and 30 pg/ml liquid. Results below detection limit were set to zero.

Results and discussion

Fractionation procedure

The Cohn process (Figure 3.9) is based on cold ethanol fractionation and is used to manufacture several plasma proteins such as Factor VIII, Immunoglobulins and Albumin. They are of therapeutic use for traumatic, burned or injured patients (Albumin), managing coagulation (Factor VIII) or immunological disorders (Immunoglobulins).

The starting plasma was thawed at 0°C and the cryoprecipitate was centrifuged. The clotting Factor VIII is obtained from cryoprecipitate by a process including a series of precipitations, virucidal treatment with solvent-detergent, and ion-exchange chromatography. Immunoglobulins were purified from Fraction II (FII) and treated with the virucide beta-propiolactone. Filtered Fraction V (FV) (containing about 98% albumin) was obtained before the pasteurisation step.

Concentrations of proteins, lipids, and organochlorines in plasma pools from healthy donors

The mean concentration of proteins, lipoproteins (Apo B), triglycerides (TG) and total cholesterol (CHOL) in the starting plasma pools were 65.8, 0.85, 1.72 and 1.49 g/l, respectively. Total lipids were calculated using the following formula (Phillips et al., 1990): Total lipids (g/l) = 2.27 x CHOL + TG + 0.62. The mean total lipid concentration was 6.01 ± 0.34 g/l.

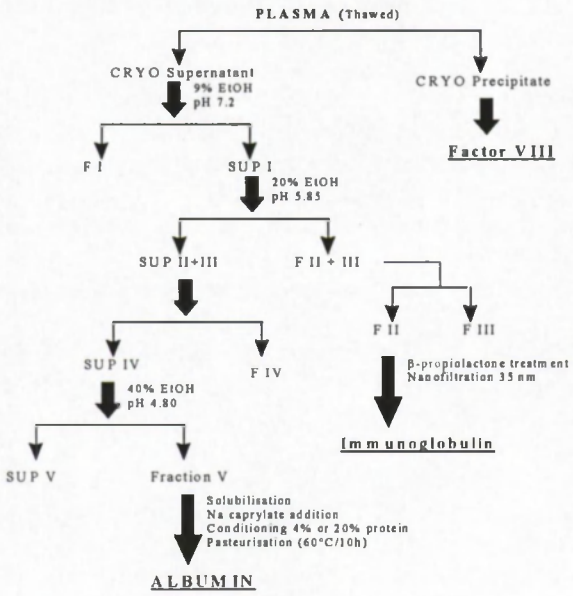


Figure 3.9. Flow chart of the Cohn fractionation procedure.

Concentrations of PCBs 28, 52, and 101 were below the detection limit (0.02 ng/ml for each individual congeners from 10 ml liquid) in the starting plasma pools. The mean concentrations of PCBs (sum of congeners 118, 138, 153, 156, 170, 180), HCB, and p,p'-DDE in the plasma pools were respectively 2.38 ± 0.34, 0.20 ± 0.02 and 1.54 ± 0.38 ng/ml.

When expressed on lipid basis, the mean concentrations of PCBs, HCB and p,p'-DDE in the plasma pools were 394.1 ± 38.2, 32.6 ± 2.5, and 254.1 ± 54.5 ng/g lipid weight, respectively.

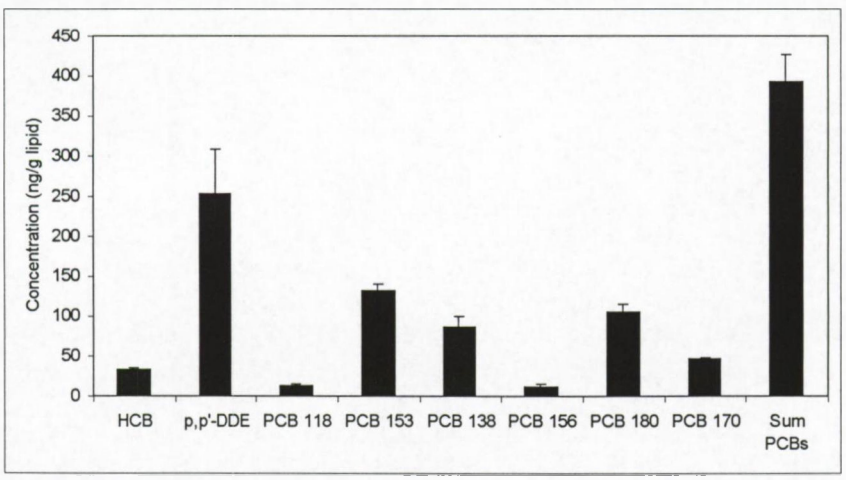


Figure 3.10. Mean concentrations (ng/g fat) and standard deviations of organochlorine pollutants in pooled plasma.

The mean concentration and standard deviation for all organochlorine pollutants (expressed in ng/g fat) are presented in Figure 3.10. Ratios of detected compounds were similar in the

three batches. p,p'-DDE was the major organochlorine pollutant present in the plasma pools, followed by PCB 153 and PCB 180.

The age of the plasma donors varies between 18 and 65 years. Thus, assuming a linear increase with age (Voorspoels et al., 2001), the mean value of 322.8 ng/g fat for the sum of 3 PCB congeners (no. 138, 153 and 180) was in good agreement with values of 245.1 and 390.0 ng/g fat obtained for groups of adolescents (17-18 years, 200 individuals) and elderly women (50-65 years, 200 individuals) (Nawrot et al, 2001; Koppen et al., 2001).

Distribution of PCBs, total protein, and total cholesterol and ApoB in Cohn fractions and cryoprecipitate

Process recoveries for the different products ranged from 71 to 89%. Due to the large scale of the process (involving thousands liters of fluids), it may be assumed that the obtained recoveries are very good. Two fractions (cryoprecipitate and re-suspended Fraction I) displayed no PCBs and very low levels of ApoB and cholesterol (Table 3.21). The other fractions (cryosupernatant, re-suspended FII+III, IV, and V) showed variable contents in organochlorine pesticides and PCBs. The distribution of different classes of compounds is shown in Table 3.21. Due to continuous increase throughout the procedure in the ethanolic content of the fractions, triglycerides could not be quantified in each fraction. Thus, PCB concentrations were calculated in function of the protein content of each fraction. Results are presented in Table 3.21.

Table 3.21. Distribution (in %) of PCBs, proteins, and cholesterol and PCB concentrations expressed in ng/g protein among different Cohn fractions.

Fractions	Proteins	Sum PCBs*	Cholesterol	Apo B	PCBs (ng/g protein)
Plasma	-	-	-	-	36.1
Cryoprecipitate	2.3	0	0.2	0	0
Fraction I	4.9	0	2.1	3.3	0
Fraction II+III	32.5	35.9	67.2	89.2	31.9
Fraction IV	12.3	16.0	9.7	7.5	37.3
Fraction V	47.1	48.1	1.2	0	29.4
Supernatant V	0.8	0	19.6	0	0
Recovery (%)	89	71	73	80	-

*- sum of PCB 118, 138, 153, 156, 170 and 180

Surprisingly, PCBs and proteins were similarly distributed throughout the fractions. Moreover, their distribution differed from that of cholesterol and ApoB. PCBs were found mostly in FV and FII+III. FV was found to contain only low concentrations of cholesterol, while FII+III was enriched in cholesterol and lipoproteins (ApoB). The continuous change in pH and polarity of the fractionation medium affected the differential elimination of selected PCB congeners.

An enrichment of PCB 180 (the most lipophilic congener) was observed in the re-suspended FV. This congener represented 35.3% of the total PCB content in FV, while in the starting plasma pool and re-suspended FII+III, it represented only 26.6 and 20.4%, respectively. The distribution of organochlorine pollutants in Cohn fractions of detected organochlorine concentrations (FII+III, FV and FIV) is presented in Figure 3.11.

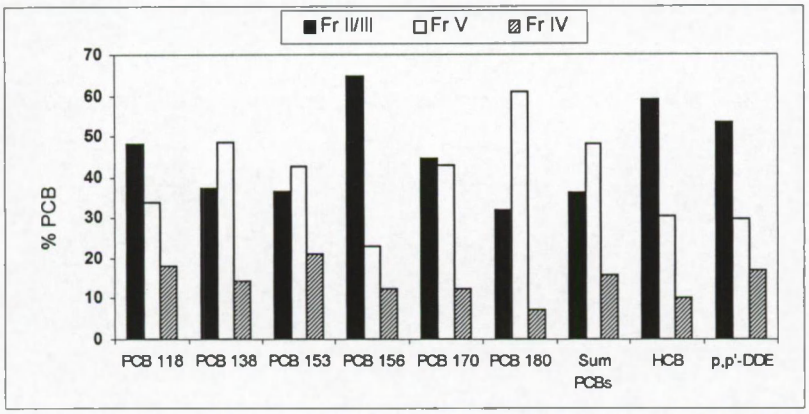


Figure 3.11. Distribution of organochlorine pollutants in Cohn Fraction II+III and Fraction V

Levels of PCBs 118 and 156, HCB, and p,p'-DDE were substantially decreased in FV and increased in FII+III (Figure 3.11), while the PCBs congeners 138, 153 and especially 180 showed an increased accumulation in FV. PCB 170 was found to distribute evenly between FII+III and FV. For all organochlorine pollutants, FIV contained the lowest concentrations. This difference in repartition between fractions was probably due to the lowering of the polarity of the medium during the fractionation procedure.

Concentrations of PCBs and major organochlorine pesticides in therapeutic protein derivatives

Due to selective elimination, the levels of all organochlorine pollutants were below the detection limit in therapeutic proteins prepared from cryoprecipitate (Factor VIII, von Willebrand Factor) and from FI (fibrinogen). PCB congeners found in FII+III (rich in IgG and IgM) were completely eliminated in the subsequent steps of the manufacturing process of Immunoglobulins. However, FV (with 98% albumin) was found to contain measurable levels of organochlorine contaminants. As a result of fractionation and filtration, the PCB concentration (sum of 6 congeners) decreased from 36.1 ng/g protein in the starting plasma pools to 29.4 or to 26.0 ng/g protein in the non-filtered or filtered FV, respectively. Similarly, the p,p'-DDE concentration decreased from 23.4 ng/g protein in the plasma pools to 14.6 and 13.5 in the non-filtered and filtered FV, respectively. It has been reported previously that lipophilic contaminants are transported in the blood by lipoproproteins or proteins such as albumin (Gomez-Catalan and To-Figueras, 1991). The authors of this report, however, used a single ultracentrifugation step to purify albumin, and this purification method does not exclude the presence of another possible carrier.

Estimation of risk associated with medical use of protein derivatives

Because Immunoglobulins and Factor VIII preparates are free of PCBs and organochlorine pesticides, the risk associated with the medical consumption is zero or very low. For albumin, due to residual levels of organochlorines in the final products, the risk was estimated through the calculation of the PCB body burden increase after an albumin treatment. A daily treatment for an adult person consists in 2 x 400 ml of 4% albumin solution. The treatment is lasting only for a couple of weeks, until partial recovery of the patient. Thus, 32 g albumin are used for a daily treatment. The concentration of PCBs (sum of 6 congeners) in the albumin fraction is 26 ng/g protein or 23.1 ng/g protein for the sum of PCBs 138, 153 and 180. The PCB amount (sum of 3 congeners) from an albumin treatment is estimated to 740

ng/day. For an adult person of 70 kg, this means a maximum intake of 14 ng PCBs/kg body weight/day.

Table 3.22. PCB body burden in two groups of Belgian population and PCB increment due to daily albumin treatment.

	Adolescents	**Adults**
Sex	M+F (n=200)	F (n=200)
Age	17-18 years	50-65 years
Average body weight (kg)	70	70
% fat	20.7	41.9
Weight fat (kg)	14.49	29.33
Serum concentration for the sum of 3 PCB (153, 138, 180) (ng/g fat)	245.1	390.0
PCB body burden for the 3 congeners (mg)	3.55	11.44
Daily increase in body burden after albumin treatment	0.021 %	0.006 %

For two Belgian populations (Staessen et al., 2001; Koppen et al., 2001), adolescents (17-18 years) and women (50-65 years) from the same living area, the mean PCB body burden can be estimated from the PCB serum concentrations (see Table 3.22). The percentage of body weight fat with age (Duarte-Davidson and Jones, 1994) is given in Table 3.22. The increment due to albumin treatment is the mass of PCBs in the albumin given for treatment related to the total mass of PCBs in body burden.
Thus, for the two selected populations, it is estimated that a treatment with albumin will increase the PCB body burden with 0.021 and 0.006% for adolescents and elderly women, respectively.

Thus, the increase in POPs body burden for patients administered with a treatment dose of this therapeutic concentrate is negligible. This procedure is affected by errors on the estimation of the mean body weight and body fat content and of the measurement of PCB serum concentration in the population.

Conclusions
Processing of plasma pools leads to complete elimination of PCBs and pesticides from cryoprecipitate, i.e. the starting fraction for producing important clotting factors such as Factor VIII, von Willebrand Factor, and fibrinogen. Processing also yields, from Cohn Fraction FII+III, a PCB- and pesticide-free immunoglobulin concentrate. Our results show residual concentrations of organochlorine pollutants in the albumin fraction. The exposure to these pollutants through the medical use of protein derivatives obtained by human plasma fractionation products is estimated to be lower than the normal dietary intake.

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Chapter 4

Determination of POPs in human milk and other body fluids

Abstract

A simple and rapid procedure based on solid phase disk extraction (SPDE), adsorption chromatography on acidified silicagel and GC-MS analysis was developed for the determination of 8 organochlorine pesticides and 19 PCB congeners in human milk. By using the SPDE procedure, a high throughput and parallel sample processing could be achieved. Method variables were optimised on whole cow milk (3.5% fat) fortified at levels close to concentrations found in human milk. Recoveries of target analytes were acceptable and ranged from 69 to 102% and 86 to 120% for whole and skimmed milk, respectively. With the use of two stage clean-up and narrow bore capillary columns, detection limits as low as 20 pg/ml could be obtained. The method was used for the determination of organochlorine pollutants in human milk of 19 individuals from Romania. Concentrations of PCBs were low, while concentrations of organochlorine pesticides were higher than reported values from other European countries. Due to similarities in the composition, it was speculated that it would be possible to apply the same method for the determination of POPs in other body fluids, such as follicular and seminal fluid. Recoveries of internal standards calculated from each fluid were ranging from 48% (follicular fluid) to 75% (human milk) and a relatively good reproducibility (RSD < 17%) was demonstrated. The purpose of the present work is to provide a reliable, simple, rapid, sensitive and universal methodology for the routine analysis of organochlorine compounds in human body fluids.

4.1 Analytical methodology for the determination of POPs in human milk

* - based on Covaci A, Hura C, Schepens P, (2001). *Chromatographia* 54 (3-4), 247-252.

The examination of human milk can be used to indicate the general level of contamination, and thus of potential health risk. Such investigations can also be used more specifically to examine the possible risks to infants, either from transplacental exposure, which occurs during the sensitive prenatal period, or from consumption of human milk, which may transfer large quantities of some chemicals from mother to child.
A considerable number of methods have been proposed for the determination of persistent organochlorine pollutants (POPs) in human milk. Most of them use the laborious liquid-liquid extraction procedure (Noren and Sjovall, 1987; Johansen et al., 1994; Tuinstra et al., 1994a). In order to reduce the sample preparation time and to simplify the procedure, solid phase extraction in reversed phase mode has been proposed as an alternative method (Manes et al., 1993; Pico et al., 1995). However, due to the existence of only one clean-up stage, the detection limits are still too high for the trace levels of POPs in human and especially in cow milk.
The use of solid-phase disk extraction (SPDE) technology have been reported for the analysis of a wide range of POPs in human serum prior to GC-MS or GC-ECD analysis (Pauwels et al., 1998; Covaci et al., 2001). The procedure involves disruption of protein-binding with formic acid, solid-phase extraction using C_{18} Empore™ disk cartridges, followed by elimination of lipid interferences using adsorption chromatography on silica gel impregnated with concentrated sulphuric acid. This method enables the use of low volumes of solvents (up to 10 ml) and the possibility of parallel sample processing. Because the method has been applied to other body fluids, such as cord blood (Covaci et al., 2001) and follicular fluid (Pauwels et al., 1999), it was speculated that a similar procedure could be used for the determination of POPs in human milk. Furthermore, with the separation on capillary columns with reduced internal diameter (less than 0.25 mm), it was possible to provide a reliable, simple, rapid and more sensitive methodology for the routine analysis of organochlorine compounds from human milk.

Materials
Based on reported abundance and toxicity, the following PCB congeners (IUPAC no. 28, 52, 74, 99, 101, 105, 110, 118, 128, 138, 149, 153, 156, 170, 180, 183, 187, 194 and 199) were targeted for analysis. Additionally, we included hexachlorobenzene (HCB), three hexachlorocyclohexane isomers (α–, β–, γ–HCH), p,p'-DDT and its metabolites (p,p'-DDE, p,p'-DDD and o,p'-DDT) as the major organochlorine pesticides found in human body fluids. ε-HCH, PCB 46 and PCB 143 were used as internal standards and 1,2,3,4-tetrachloronaphthalene (TCN) as recovery standard.

Methods
To 6 ml of milk, 5 ng of each internal standard (100 pg/μl in iso-octane) were added. The fortified sample was equilibrated by ultrasonication for 5 min. The fluid was then diluted with Milli Q water (1:1, *v/v*), mixed with formic acid (6 ml) and acetonitrile (250 μl) and equilibrated by ultrasonic treatment for 30 min. Prior to the sample application, the Empore™ disk cartridges were washed with 2 x 500 μl dichloromethane and dried thoroughly. Each cartridge was activated with 250 μl of methanol followed by 2 x 250 μl deionized water. After conditioning, the cartridges were not allowed to dry. To avoid overloading and

breakthrough of the analytes, a maximum of 3 ml sample was used per cartridge. After sample loading at a pressure of 2-4 psi, each cartridge was rinsed with 2 x 500 µl deionized water. The sorbent bed was dried thoroughly under a nitrogen stream at 20 psi pressure (10 min) and by centrifugation (15 min, 2000 g). Each column was eluted with 2 x 500 µl hexane and 500 µl dichloromethane and hexane (1:1, *v/v*) which were combined.
A cartridge filled with 0.5 g of acid silica and topped with 100 mg Na_2SO_4 was pre-washed with 2 ml of dichloromethane and hexane (1:1, *v/v*) and 2 ml of hexane. The concentrated sample was applied to the acid silica cartridge. Target analytes were eluted with 6 ml hexane. After the addition of 50 µl of iso-octane as a keeper, the final eluate was concentrated under a gentle nitrogen stream at room temperature to approximately 50 µl. Fifty µl of recovery standard TCN (100 pg/µl in iso-octane) was added to the final concentrate, mixed and transferred to a vial.

Instrumentation
Empore™ C_{18} extraction disk cartridges (10 mm/6 ml) from 3M Company (St. Paul, MN, USA) and a Varian Positive Pressure Manifold (part 1223-420X) were used for solid-phase extraction. Empty cartridges (3 ml) were purchased from Supelco (Bellefonte, PA, USA).

GC-MS was performed with a Hewlett Packard (HP; Palo Alto, CA, USA) 6890 GC connected with a HP 5973 mass spectrometer via a direct interface. The chromatograph was equipped with a 20 m x 0.18 mm x 0.25 µm AT-5 (5% phenyl polydimethylsiloxane) capillary column (Alltech, Lokeren, Belgium). Helium was used as carrier gas at a constant flow of 0.8 ml/min. Samples (1 µl) were injected in the pulsed splitless mode (pulse pressure 30 psi, pulse time 1 min) with the split outlet opened after 1 min. Injector and interface temperatures were set at 270°C and 300°C, respectively. The column oven was held at 90°C for 1 min after injection, then programmed at 35°C/min to 200°C, which was held for 1 min, then programmed at 10°C/min to 250°C, held for 0.5 min and further then programmed at 50°C/min to 280°C, which was held for 3 min. The mass spectrometer was operated in the selected ion monitoring (SIM) mode at 70 eV. Dwell times were set at 50 ms. The two most abundant ions were monitored for each level of chlorination for PCBs or for each pesticide (Table 4.1). The retention time, the m/z values and the ion abundance ratio of the qualifier ion to the quantification ion were used as identification criteria. A deviation of ion ratios of less than ±20% from the theoretical value was considered acceptable.

A HP 6890 GC-µ ECD was equipped with a 25 m x 0.25 mm x 0.25 µm HT-8 (SGE, Zulte, Belgium) 1,7-dicarba-closo-dodecarborane 8% phenyl methyl siloxane capillary column. Helium was used as carrier gas at a constant flow of 1 ml/min; Ar-CH_4 (95:5) was used as make-up gas (40 ml/min). Samples (1 µl) were injected in pulsed splitless mode (pulse pressure 25 psig, pulse time 1 min) with the split outlet opened after 1 min. Injector and detector temperatures were set at 270°C and 320°C, respectively. The column oven was held for 1 min at 90°C, then programmed at 15°C/min to 180°C, which was held for 1 min, then programmed at 3°C/min to 250°C, then at 15°C/min to 290°C, which was held for 6 min.

Quality control
Multi-level calibration curves (r^2 >0.999) were created for the quantification using standard solutions in iso-octane, covering the entire range of expected values. Compounds were

quantified against the closest eluting internal standard. Detection limits were 0.2 ng/ml for HCHs and DDTs and ranged between 10 and 30 pg/ml for individual PCBs.

Table 4.1. Acquisition parameters and recoveries for one fortification level from skimmed and whole milk.

Compound	Retention time (min)		Monitored ions[a]	Fortification level (ng/ml)	Recovery ± SD[b] (%)	
	ECD (HT-8)	MS (AT-5)			Whole (3.5% fat)	Skimmed (0.1% fat)
PCB 28	15.30	6.56	**256**, 258	2.0	71 ± 7	100 ± 9
PCB 52	16.60	7.01	**290**, 292	2.0	72 ± 4	105 ± 11
PCB 74	19.88	7.88	**290**, 292	0.5	75 ± 6	92 ± 12
PCB 99	21.62	8.46	**326**, 324	0.5	74 ± 4	101 ± 3
PCB 101	21.29	8.37	**326**, 324	2.0	71 ± 7	105 ± 5
PCB 105	26.98	9.84	**326**, 324	0.4	69 ± 7	107 ± 3
PCB 110	23.42	8.97	**326**, 324	0.5	74 ± 6	116 ± 14
PCB 118	25.38	9.39	**326**, 324	0.4	77 ± 10	112 ± 8
PCB 128	29.57	10.73	**360**, 362	0.5	81 ± 7	109 ± 3
PCB 138	27.94	10.23	**360**, 362	2.0	80 ± 7	105 ± 2
PCB 149	24.34	9.36	**360**, 362	0.5	83 ±5	105 ± 3
PCB 153	26.34	9.76	**360**, 362	2.0	80 ± 6	100 ± 5
PCB 156	31.45	11.08	**360**, 362	0.4	80 ± 12	103 ± 14
PCB 170	33.07	11.65	**394**, 396	1.0	87 ± 11	102 ± 10
PCB 180	31.93	11.28	**394**, 396	2.0	89 ± 12	96 ± 14
PCB 183	28.72	10.61	**394**, 396	0.5	89 ± 9	97 ± 15
PCB 187	28.29	10.52	**394**, 396	1.0	87 ± 7	97 ± 10
PCB 194	35.09	12.53	**428**, 430	2.0	85 ± 15	91 ± 21
PCB 199	32.94	11.75	**428**, 430	0.5	82 ± 13	99 ± 15
PCB 46 (IS1)	16.38	6.98	**290**, 292	0.83	76 ± 5	113 ± 4
PCB 143 (IS2)	24.81	9.52	**360**, 362	0.83	75 ± 6	94 ± 5
ε-HCH (IS3)	15.15	6.35	**183**, 219	0.83	73 ± 12	86 ± 11
HCB	12.14	5.56	**284**, 286	2.0	77 ± 4	86 ± 6
p,p'-DDE	22.74	8.81	**248**, 318	2.0	72 ± 7	103 ± 13
p,p-'DDD	26.02	9.51	**235**, 237	2.0	70 ± 3	114 ± 12
o,p'-DDT	25.12	9.57	**235**, 237	2.0	82 ±9	120 ± 7
p,p'-DDT	27.77	10.14	**235**, 237	2.0	86 ± 5	112 ± 9
α-HCH	11.89	5.45	**183**, 219	2.0	101 ± 11	87 ± 20
β-HCH	13.55	5.78	**183**, 219	2.0	102 ± 18	102 ± 10
γ-HCH	13.33	5.90	**183**, 219	2.0	96 ± 14	110 ± 12

[a]- ions aquired on mass spectrometer - bold: quantifier, normal: qualifier [b]- n=5

The procedure was validated through regular analysis of blanks, fortified samples and certified material CRM 450 (PCBs in powdered cow milk). Recoveries of all investigated compounds

were measured from 6 ml of cow milk fortified at similar concentrations with those normally found in human milk. Five fortification replicates (see Table 4.1 for fortification level of individual compounds) and five non-fortified replicates from skimmed and whole milk were analysed. Absolute recoveries of POPs were calculated after substraction of levels found in the non-fortified replicates from the fortified ones.
Recoveries of internal standards (ε-HCH, PCB 46 and 143) were higher in skimmed milk (86 to 113%) than in whole milk (73 to 76%), due to lower lipid content, thus less competition for binding sites (Table 4.1). Recoveries of target analytes (19 PCB congeners and 8 organochlorine pesticides) followed the same trend and where higher for most analytes in skimmed milk than in whole milk. Low RSD (less than 15%) were obtained for all analytes at a fortification level similar with normal values in human milk, except for HCHs with RSD between 10 and 20%. There were no differences in recoveries between PCB congeners with a low or high degree of chlorination or with respect to the degree of chlorination in *ortho*-position. Results are presented in Table 4.1. Good linearity was achieved for the tested intervals which included the normal range of pollutants in human milk (0.05 – 15 ng/ml for individual PCB congeners, 0.1 – 500 ng/ml for DDTs and 0.1 – 50 ng/ml for HCHs, respectively).

One g of certified powdered milk CRM 450 (PCBs in powder milk) was reconstituted in 9 mL Milli Q water (10 min at 40°C for complete solubilization). After addition of 5 ng internal standards, the mixture was treated as described above (using 2 SPDE cartridges/sample). Recoveries of PCBs from certified milk (Table 4.2), were acceptable (less than 10%), except for PCB 52 (partial co-elution with internal standard PCB 46) and PCB 170 (possible co-elution with PCB 190). The difference between the certified and found values was expressed as percentage relative error (Table 4.2).

Table 4.2. Concentrations of PCBs in a certified standard (CRM 450 natural milk powder).

Compound	Certified values (ng/g)	SPDE + acid silica (n=4)	
		Found (ng/g)	Relative error[c] (%)
PCB 52	1.16 ± 0.17	0.42 ± 0.16	-64
PCB 118	3.3 ± 0.4	3.2 ± 0.3	-3
PCB 153	19.0 ± 0.7	18.3 ± 1.6	-4
PCB 156	1.62 ± 0.20	1.51 ± 0.02	-7
PCB 170	4.8 ± 0.6	5.5 ± 0.7	15
PCB 180	11.0 ± 0.7	11.2 ± 0.9	2
PCB 105	(0.35)[a]	0.30 ± 0.14	-14
PCB 128+167	(1.43)[a]	1.56 ± 0.10	9
PCB 138[b]	(14.4)[a]	14.0 ± 1.4	-3
PCB 138 + PCB 163	(15.4)[a]	15.5 ± 0.9	1
PCB 149	(0.46)[a]	0.39 ± 0.18	-18

[a] - indicative values
[b] - measured on HT-8 column
[c]- calculated as: (found-certified)x100/certified

Solid-phase disk extraction

Sample pretreatment

Denaturation of milk proteins and disruption of fat globules was done by addition of formic acid and sonication. The treatment without precipitation was found to release the analytes

from the binding sites without the possible loss by occlusion in the precipitate (Pauwels et al., 1998) and inapplicability to SPDE. Sodium oxalate (Johansen et al., 1994) or methanol (or short chain alcohols for reduced viscosity) (Manes et al., 1993) can also be used for this purpose. Before application to the SPDE cartridge, it is necessary to dilute the milk with water to reduce sample viscosity.
However, the method is restricted to fluids with relatively low lipid contents. Even after dilution with water and formic acid, due to their viscosity, human or whole cow milk is more difficult to pass through the cartridge than other body fluids.

Advantages and restrictions of SPDE
When compared with serum (Covaci et al., 2001), higher recoveries are obtained from milk due to lower flow rates through the cartridge, thus longer contact time between the analytes and the sorbent. Lower elution volumes than for classical SPE are needed for the SPDE cartridge (up to 1.5 ml per cartridge). Compared with sequential procedure of liquid-liquid extractions, parallel sample processing (up to 12 samples for this specific manifold) becomes now possible.

Due to higher viscosity of milk samples, lower sample volumes can be loaded (3 ml milk instead of 5 ml serum). Sample capacity will be lower due to the presence of higher amounts of lipids with similar polarity as the organochlorines and competition for the binding sites. Higher pressures are needed for the elution of analytes from the SPDE cartridge. The lipid content needs to be measured separately, because the procedure does not allow the collection of the lipidic fraction.

GC separation
Two capillary columns with different stationary phases were chosen because of complementary abilities for PCB separation (Frame, 1997). ECD chromatograms of a standard solution (A) and human milk (B) are presented in Figure 4.1.

Determination of PCB 138 (co-elution with PCB 163 on AT-5) and PCB 170 (co-elution with PCB 190 on AT-5) was done exclusively on HT-8 column. Due to high background level, HCH isomers were quantified exclusively on the ECD. All other analytes were quantified on the mass spectrometer. Ion mass chromatograms for penta-, hexa-, hepta-CBs and DDTs of a human milk sample analysed on AT-5 column are shown in Figure 4.2

The use of narrow bore columns results in shorter run times (Table 4.1), keeping the resolution similar or even better than normal columns (equal or larger than 0.25 mm). However, the use of narrow bore capillaries is restricted to detectors with high scanning rates. Moreover, high inlet pressures are needed and narrow bore columns are not available for all types of stationary phases.

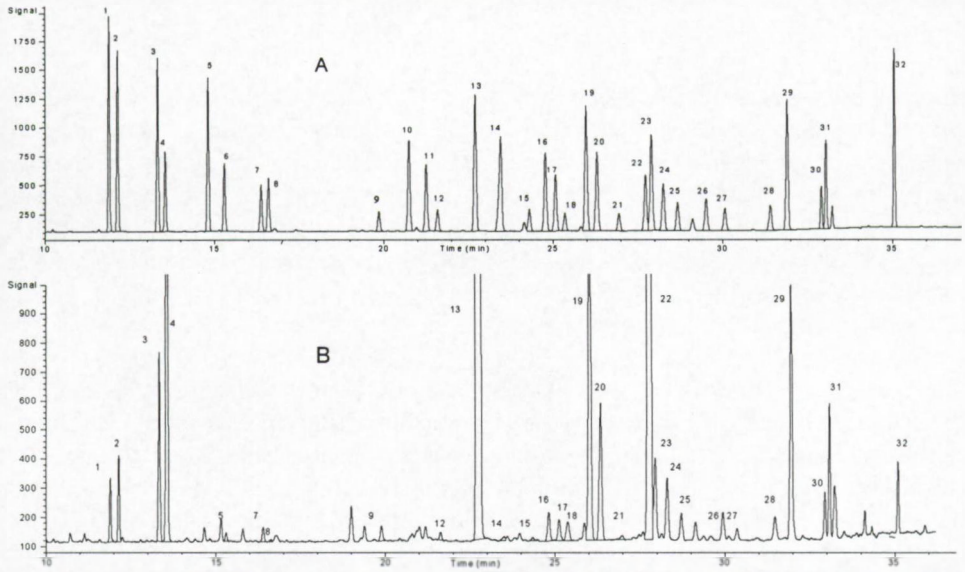

Figure 4.1. Typical ECD chromatograms on HT-8 column of organochlorine mixture (A) and human milk extract (B). Peak identification: 1-α-HCH, 2-HCB, 3-γ-HCH, 4-β-HCH, 5-δ-HCH, 6-PCB 28, 7-PCB 46 (IS), 8-PCB 52, 9-PCB 74, 10-o,p'-DDE, 11-PCB 101, 12-PCB 99, 13-p,p'-DDE, 14-o,p'-DDD, 15-PCB 149, 16-PCB 143 (IS), 17-o,p'-DDT, 18-PCB 118, 19-p,p'-DDD, 20-PCB 153, 21-PCB 105, 22-p,p'-DDT, 23-PCB 138, 24-PCB 187, 25-PCB 183, 26-PCB 128, 27-PCB 167, 28-PCB 156, 29-PCB 180, 30-PCB 199, 31-PCB 170, 32-PCB 194.

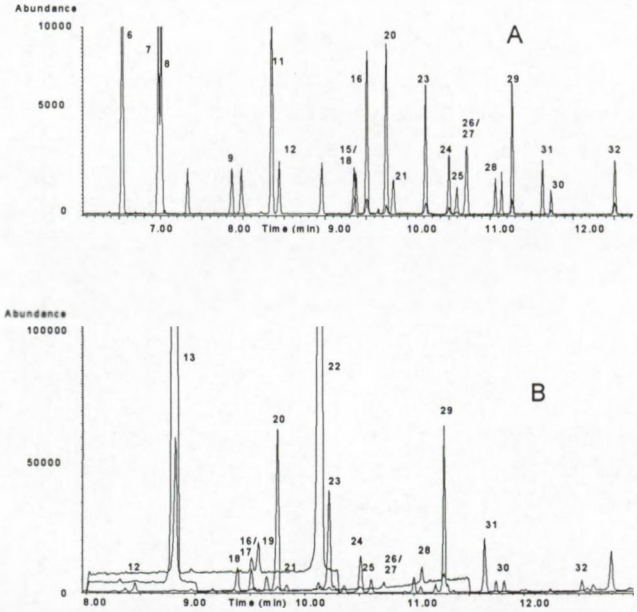

Figure 4.2. Typical mass chromatograms on AT-5 capillary column (0.18 mm) for organochlorine mixture (A) and human milk extract (B). Peak identification as in Figure 4.1.

4.2. Analysis of human milk samples from Romania

Samples
Human milk samples collected between the 2nd and 4th day after delivery were obtained from 19 women attending the Gynaecology Unit of the University Hospital of Iassy, Romania. Individual serum samples and information on the age of the mother, weight of the baby, number of previous children and living area (rural or urban) were also available. Three samples from the same person were collected during the lactation period (2nd, 10th and 18th week). All samples were kept frozen at -20°C. Before analysis, samples were defrosted and homogenised by manual shaking for 5 min.

Results and discussion
To the best of our knowledge, the values presented here are the first reported PCB concentrations in human milk from Romania. Mean and median values together with standard deviations and ranges are presented for 19 individual samples available from Romania (Table 4.3). Concentrations for the sum of PCB congeners were as high as 47 ng/ml with a mean of 9.7 ng/ml (Table 4.3). This value is lower than values reported from the Netherlands (Tuinstra et al., 1994b), Norway (Johansen et al., 1994), Czech Republic (Schoula et al., 1996) or Italy (Larsen et al., 1994).

However, due to two samples with high concentrations of PCBs and DDTs, high SD were obtained while median values are almost half of average values for almost all congeners (Table 4.3). After exclusion of these two values, mean and SD values become 6.9 ± 4.1 and 58.3 ± 34.2 ng/ml for PCBs and DDTs, respectively. The new mean values are similar with median values, thus representing better the levels in the population.

The ratio PCB 153/sum PCBs is an indicator of similar profiles in different samples. A mean value of 0.24 was similar with values found in other European countries (Schoula et al., 1993). A value of 0.15 was found in one sample with high levels of non-persistent congeners (PCB 28, 52, 110, 101) which might come from a local or accidental contamination.

Concentrations of organochlorine pesticides were higher than PCB concentrations and this can be explained by their massive use until their ban in 1987. Organochlorine pesticides were previously measured in Romanian human milk samples and it was found that present concentrations (21 and 81 ng/ml for HCHs and DDTs, respectively) are lower than in 1985 (Hura et al., 1988), but similar with those from 1995 (Hura et al., 1996). p,p'-DDE and β-HCH were the principal contributors to the sum of DDTs and HCHs (80 and 95%, respectively).

For one individual, concentrations of POPs in human milk showed a decrease from the 2nd week of lactation (9.8 and 74.5 ng/ml for PCBs and DDTs, respectively) to the 18th week of lactation (5.8 and 54.9 ng/ml, respectively).

Table 4.3. Mean and median concentrations, standard deviations and ranges of individual organochlorine pollutants detected in 19 individual human milk from Romania.

Compound	Frequency[a] (n=19)	Mean (ng/ml)	SD (ng/ml)	Median (ng/ml)	Range (ng/ml)
PCB 28	17	0.49	0.56	0.30	nd – 2.07
PCB 52	2	nd	-	nd	nd – 0.13
PCB 74	18	0.27	0.25	0.16	nd – 1.11
PCB 99	18	0.16	0.09	0.13	nd – 0.34
PCB 101	3	nd	-	nd	nd – 0.18
PCB 105	11	0.12	0.08	0.10	nd – 0.34
PCB 110	5	0.09	0.06	0.09	nd – 0.18
PCB 118	19	0.26	0.13	0.26	nd – 0.53
PCB 128	6	0.11	0.10	0.06	nd – 0.29
PCB 138	19	0.97	0.92	0.59	0.16 – 4.58
PCB 149	2	nd	-	nd	nd – 0.09
PCB 153	19	2.33	2.41	1.50	0.39 – 10.63
PCB 156	13	0.17	0.13	0.13	nd – 0.49
PCB 170	19	0.65	0.85	0.46	0.09 – 3.55
PCB 180	19	2.48	2.98	1.71	0.40 – 13.12
PCB 183	19	0.28	0.32	0.16	0.04 – 1.37
PCB 187	19	0.79	1.04	0.44	0.09 – 4.58
PCB 194	18	0.41	0.47	0.29	nd – 2.04
PCB 199	15	0.37	0.46	0.18	nd – 1.87
Sum PCBs		**9.73**	**10.00**	**6.49**	**1.60 – 43.85**
PCB 153/sum PCBs		0.24	0.03	0.24	0.15 – 0.29
HCB	19	0.62	0.37	0.56	0.10 – 1.74
pp-DDE	19	66.08	61.22	48.56	9.86 – 256.96
pp-DDD	19	1.79	1.19	1.17	0.26 – 7.21
op-DDT	18	0.77	0.67	0.45	nd – 2.25
pp-DDT	19	12.32	13.73	7.58	1.99 – 39.27
Sum DDTs		**80.92**	**75.83**	**56.62**	**16.73 – 304.54**
pp-DDT/sum DDTs		0.15	0.07	0.13	0.05 – 0.34
α-HCH	12	0.68	0.61	0.59	nd – 2.21
β-HCH	19	19.19	11.77	17.66	4.56 – 42.38
γ-HCH	13	1.40	1.93	0.58	nd – 6.97
Sum HCHs		**20.61**	**12.49**	**17.79**	**6.92 – 46.66**
β-HCH/sum HCHs		0.93	0.10	0.96	0.66 – 0.99

[a]- number of samples with concentrations above LOD; nd – not detected

Concentrations of β-HCH, pp-DDE and pp-DDT are in the same range as values reported from Ukraine (Gladen et al., 1999), but lower than in other European countries (Furst et al., 1994; Albers et al., 1996; Czaja et al., 1997). The mean pp-DDT/sum DDTs ratio was 0.15 and was similar with values from Ukraine (Gladen et al., 1999) and Poland (Czaja et al., 1997), but slightly higher than reported values from Germany (Furst et al., 1994).

No correlation with the number of previous children, birth weight or age of the mother was found. Concentrations of POPs were measured in serum samples collected from the same person (see chapter 3.2.3). Correlation between concentrations in serum and milk of the same individual (Table 4.4) ranged from high for PCBs and DDTs (r=0.89 and 0.80, respectively) to poor for HCHs (r = 0.51).

Table 4.4. Correlation between concentrations of organochlorine pollutants in serum and milk from 19 mothers from Iassy, Romania

Compounds	Correlations (r)	p
HCB	0.01	0.14
HCHs	0.51	< 0.01
DDTs	0.80	< 0.01
PCBs	0.89	< 0.01

Conclusion
Solid-phase disk extraction could be applied to the fast and easy determination of persistent organochlorine pollutants in human milk. With the use of additional clean-up on acidified silica and analysis by GC-MS using capillary columns with reduced internal diameter, detection limits as low as 20 pg/ml could be achieved.

4.3. Analytical methodology for the determination of POPs in other body fluids (folicular and seminal fluid)

* - based on Covaci A, Schepens P, (2001). *Anal Lett* 34 (9), 1449-1460; Pauwels A, Covaci A, Delbeke L, Punjabi U, Schepens P, (1999). *Chemosphere* 39(14), 2433-2441.

Due to similarities in the composition, it was speculated that it would be possible to apply the same method to the determination of POPs in other body fluids. Thus, the SPDE method was thoroughly evaluated for human milk (Chapter 4.1) and serum (Chapter 3.1). To the best of our knowledge, we describe as first the use of SPDE method for the determination of selected POPs in seminal fluid. The purpose of the present work is to provide a reliable, simple, rapid, sensitive and universal methodology for the routine analysis of organochlorine compounds in human body fluids.
Solid-phase disk extraction (SPDE) was developed and evaluated for the isolation and concentration of trace levels of selected persistent organochlorine pollutants (POPs) from human body fluids (serum, cord blood, milk, follicular and seminal fluid). Similar methodology could be used for each matrix, the only restricting factor being the viscosity of the fluid.

Samples
Human follicular and seminal fluid samples were obtained from the Fertility Unit of the University Hospital of Antwerp, Belgium. All samples were kept frozen at -20°C until analysed.

Experimental Procedure
To 2 ml of seminal fluid or 5 ml follicular fluid, 5 ng of internal standards (100 pg/µl in iso-octane) were added. The sample was equilibrated in an ultrasonic bath for 5 min. The fluid

was then mixed with formic acid and acetonitrile (1:1 and 20:1, *v/v*, respectively) and equilibrated by ultrasonic treatment for 30 min.

Prior to the sample application, the Empore™ disk cartridges were washed with 2 x 500 µl dichloromethane and dried thoroughly. Then, each cartridge was activated with 250 µl of methanol followed by 2 x 250 µl deionized water. After conditioning, the cartridges were not allowed to dry. To avoid overloading and breakthrough of the analytes, a maximum of 5 ml sample was used for one cartridge. After sample loading at a positive pressure of 2-4 psi, each cartridge was rinsed with 2 x 500 µl deionized water. The sorbent bed was dried thoroughly under a nitrogen stream at 20 psi positive pressure (10 min) and by centrifugation (15 min, 2000 g). Each column was eluted with 2 x 500 µl hexane and 500 µl dichloromethane and hexane (1:1, v/v) which were combined.

A cartridge filled with 0.5 g of acid silica and topped with 100 mg Na_2SO_4 was pre-washed with 2 ml of dichloromethane and hexane (1:1, v/v) and 2 ml of hexane. The concentrated sample was applied to the acid silica cartridge. Target analytes were eluted with 6 ml hexane. After the addition of 50 µl of iso-octane as a keeper, the final eluate was concentrated under a gentle nitrogen stream at room temperature to approximately 50 µl. Twenty-five µl of recovery standard TCN (200 pg/µl in iso-octane) was added to the final concentrate, mixed and transferred to a vial.

Recovery experiments and reproducibility

In order to test the method performance, recoveries of internal standards were determined from each matrix. Due to small sample volumes of follicular and seminal fluid, only recoveries of internal standards were measured. Recoveries of internal standards calculated from each fluid were ranging from 48% (follicular fluid) to 75% (human milk) and a relatively good reproducibility (RSD < 17%) was demonstrated.

Recoveries from the follicular and seminal fluid were comparable with recoveries obtained from serum and milk (see Chapters 3.1 and 4.1). Recoveries of internal standards (PCB 46 and 143) were lower from follicular fluid (48%), but similar from serum, cord serum and seminal fluid (Table 4.5). They were higher in human milk (up to 76%). Relative standard deviation (RSD) was less than 10% for serum, cord blood and milk and between 12 and 17% for follicular fluid and seminal fluid. The lower recoveries for follicular fluid could be explained by the higher speed through the extraction cartridge (thus a lower contact time), due to lower viscosity.

Analysis of individual samples

The SPDE method was tested on several individual samples. Concentrations of the most persistent compounds (PCB 118, 153, 138 and 180 and p,p'-DDE) were measured in low concentration in follicular fluid and cord blood serum (Table 4.6) and were under the detection limit in seminal fluid.

Table 4.5. Recoveries of internal standards and lipid composition in human body fluids analysed with SPDE fluids.

Matrix	Recoveries (%) (mean ± SD)		Lipids[a] (g/l)			
	PCB 46	PCB 143	Triglycerides	Cholesterol	Phospholipids	Sum lipids
Maternal serum	68 ± 8	65 ± 9	1.85	1.90	2.40	6.15
Cord serum	62 ± 7	61 ± 5	0.75	0.80	1.30	2.85
Human milk	76 ± 5	75 ± 7	34.40	0.30	na[b]	34.70
Follicular fluid	53 ± 17	48 ± 12	0.15	0.30	0.70	1.15
Seminal fluid	67 ± 15	68 ± 14	< 0.10	1.00	0.85	1.85

[a] - mean values (as reported for human milk (Packard, 1982), follicular (Vignon et al., 1991) and seminal fluid (Knobil and Neill, 1988)). Lipids in cord and human serum were determined by enzymatic methods.
[b] - not available

Although very low amounts were found in fluids associated with reproductive system, concentrations of PCBs were highly correlated in serum and follicular fluid (Pauwels et al., 1999) from the same patient (r^2 = 0.56-0.92). Due to higher lipid contents, higher concentrations were measured in serum and milk, compared with follicular and seminal fluid.

Table 4.6. Concentrations of organochlorine pollutants (mean ± SD, expressed in pg/ml whole weight) found in selected individual human samples (n = 5 for each matrix).

Compound	Follicular fluid (n=5)	Seminal fluid (n=5)
PCB 28	< 20	< 20
PCB 99	< 10	< 10
PCB 105	< 20	< 20
PCB 118	25 ± 10	< 15
PCB 138	60 ± 50	< 10
PCB 153	110 ± 140	< 10
PCB 156	< 15	< 15
PCB 170	30 ± 10	< 15
PCB 180	70 ± 80	< 10
PCB 187	< 15	< 15
Sum PCBs	**270 ± 320**	**< 100**
HCB	30 ± 25	< 20
p,p'-DDE	720 ± 230	< 100
p,p'-DDT	< 50	< 50
Sum DDTs	**720 ± 230**	**< 200**
γ-HCH	< 75	< 75
β-HCH	< 75	< 75
Sum HCHs	**< 150**	**< 150**

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Chapter 5

Determination of POPs in human hair

Abstract

Different incubation and extraction methods were evaluated for the determination of PCBs, DDT and HCH isomers from human hair. The best method was found to be overnight incubation with 3N HCl at 40°C and liquid-liquid extraction with hexane:dichloromethane = 4:1 (*v/v*). After clean-up on basic alumina and acid silica, the extract was analysed by GC-ECD or GC-MS. Recoveries of internal standards and analytes under investigation ranged between 87 and 111%. Limits of detection were between 0.5 and 1 ng/g hair for ECD and 1 to 3 ng/g for MS. Good linearity (r^2>0.999) was achieved for the tested intervals (1-30 ng/g for individual PCBs, 1-1000 ng/g for DDT isomers and 1-100 ng/g hair for HCH isomers). No difference between extraction efficiency from powdered and cut hair was observed. The same profiles of PCBs were found in human hair and other body matrices. Similar concentrations of POPs (reported on lipid basis) were found in human milk and hair from the same specimen.

Hair analysis was used for the assessment of exposure to organochlorine pollutants in specimens from Greece, Romania and Belgium. The highest organochlorine load (up to 148 ng/g hair for the sum of PCB, DDT and HCH isomers) was found in samples from a group of Greek women with past occupational exposure to pesticides. DDTs were the main organochlorine pollutants in Greek samples (up to 70%), while in Belgian hair samples their contribution was reduced to 40%. PCB mean concentration was higher in Belgian specimens (up to 14 ng/g hair). Contribution of p,p'-DDT to the sum of DDTs was higher in Greek samples and indicates recent exposure to technical DDT. Similar PCB 153/sum PCBs ratios were found for each of the three countries suggesting similar sources of pollution with PCBs (mainly dietary). Artificially coloured hair samples were found to have lower, but not statistically significant concentrations of organochlorine pollutants than the non-coloured hair. It was concluded that hair can be used for monitoring of any subgroup of population with emphasis on those from which sampling of milk, adipose tissue or blood is not possible or very difficult (e.g. children).

5.1. Analytical methodology for the determination of POPs in human hair

* - based on Covaci A, Schepens P, (2001). *Chromatographia* 53, S366-S371.

Human monitoring of persistent organic pollutants (POPs) has become increasingly important for exposure and risk assessment. Body fluids and tissues are commonly used to evaluate human exposure (Pauwels et al., 2000; Covaci and Schepens, 2001). Adipose tissue analysis gives an estimation of the total exposure, while serum analysis illustrates the present status of exposure to organochlorines. Human milk analysis results in an integrative description of the past exposure, but is restricted to subgroups of population (Lunden and Noren, 1998).

Hair has been identified as a suitable alternative and indicator for short (present) and long-term exposure to organochlorines (Schramm et al., 1992). The average growth speed is 1 cm/month for the capillary hair. Normally, 85% of the hair is in the growth phase, 1% in the transition phase (when metabolism within the matrix ceases in 1-2 weeks) and 14% of hair in the dead phase and stays on the scalp for 1-6 months before it falls out (Bencze, 1990). Hair is composed of 88% proteins (with disulphide bonds between chains), 3.5-4 % lipids (mainly free fatty acids, triglycerides and cholesterol) and water (Spearman, 1977). The relatively high percentage of lipids in the hair (similar with human milk) makes it a suitable matrix for the analysis of persistent organic pollutants. Each hair follicle is surrounded by a system of capillary blood vessels at the root (Spearman, 1977). This ensures a continuous exchange of components between the hair root and the human organism. In this way, the same information, as present in serum, is found in the hair. Internal exposure to pollutants is correlated with compounds incorporated in the hair matrix. The amount of the components deposited depends on its instantaneous concentration in the blood vessels of the hair root.

The external exposure is correlated with gaseous compounds solubilised on the excretions of the sebaceous glands, with which hair is coming in contact. The presence of non-persistent pollutants (e.g. low chlorinated PCBs or α-hexachlorocyclohexane) in higher concentration than in other body matrices can be attributed to external gaseous or particulate exposure (Zupancic-Kralj, 1992). Thus, it might be possible to differentiate between internal and external exposure by looking at the patterns of persistent and non-persistent congeners. There is little knowledge about the elimination kinetics in different body compartments. Available studies suggest a quasi-similar distribution of xenobiotics in different compartments (hair, blood, liver, abdominal fat and muscle), when normalised on lipid content (Klein, 1992).

In comparison with traditional matrices, hair has received little attention for the analysis of organochlorine compounds. Few articles, mostly containing incomplete validated methods, can be found in literature (Schramm, 1997, 1999), but no comprehensive paper on POPs analysis in hair has yet been published. Hair analysis was used for estimation of human exposure to the PCB-containing Yusho oil (Ohgami et al, 1989), for measuring the exposure to γ-HCH and p,p'-DDT from wood preservation products (Neuber et al., 1999) or for air pollution monitoring (Zupancic-Krajl et al., 1992). More attention to POPs (especially PCDD/PCDFs) in hair was given by Schramm (1992, 1997, 1999) concluding that hair can be an important non-invasive biomonitoring tool for assessment of atmospheric exposure. It was concluded that a differentiation between external and internal pathways of exposure becomes now possible.

Materials

The organochlorine pesticides under investigation were α-, β-, γ- isomers of hexachlorocyclohexane (HCHs), o,p'-DDE, o,p'-DDD, o,p'-DDT, p,p'-DDE, p,p'-DDD and p,p'-DDT (expressed here as DDTs) and hexachlorobenzene (HCB). The following PCB congeners (IUPAC numbers) were targeted: 28, 52, 99, 101, 118, 138, 149, 153, 156, 170, 180 and 187. Dilutions were made in iso-octane in order to cover the entire range of POPs expected in human hair. Sodium hydroxide, hydrochloric acid 37%, concentrated sulphuric acid 95-97%, thioglycolic acid (TGA), urea (U), 1,4-dithiothreitol (DTT) and 2-mercaptoethanol (MercEtOH) were analytical grade reagents (Merck, Germany). All reagent dilutions were done with Milli Q water. Anhydrous sodium sulphate (Merck) for residue analysis, basic aluminium oxide 70-230 Mesh and silica gel 60-200 Mesh (Merck) were used after heating overnight at 120°C. Hexane (Hex), acetone (Acet) and dichloromethane (DCM) were available from Merck in pesticide-grade purity

A pooled sample used for method validation was obtained from one individual, by cutting the hair in small pieces (length of ~1 mm). Three hair samples were available in powdered and cut state.

Methods

Two hundred mg from the pooled hair sample were accurately weighted, spiked with 5 ng of internal standards (PCB 46 and PCB 143) and incubated with two ml of each reagent. For samples incubated with acid, solid phase extraction (SPE) efficiency on C_{18} disk cartridges was evaluated as described previously (Covaci and Schepens, 2001). For all incubated samples, liquid-liquid extraction (LLE) with 2 x 3 ml Hex:DCM (4:1, *v/v*) was evaluated as an alternative extraction method. Hot Soxhlet extraction with 50 ml of Hex, Hex:Acet (3:1, *v/v*) or Hex:Acet:DCM (3:1:1, *v/v*) was used for 2 h.

All hexane eluates (from SPE or LLE) were purified on a cartridge filled with 250 mg deactivated alumina (10% water), 500 mg of acid silica and 250 mg anhydrous Na_2SO_4. The cartridge was pre-washed with 2 ml Hex:DCM (1:1, *v/v*) and 2 ml of Hex. The cartridges were eluted with 4 ml Hex, the final eluate was concentrated to approximately 50 µl under a gentle nitrogen stream and spiked with 5 ng recovery standard (1,2,3,4-tetrachloronaphthalene, TCN) and transferred to a vial.

Instrumentation

A Hewlett Packard (Palo Alto, CA, USA) 6890 GC-µ ECD was equipped with a 25 m x 0.25 mm x 0.25 µm, HT-8 (SGE, Zulte, Belgium) 1,7-dicarba-closo-dodecarborane 8% phenyl methyl siloxane capillary column. Helium was used as carrier gas at a constant flow of 1.0 ml/min and Ar/CH_4 (95:5) as make-up gas (40 ml/min). One µl was injected in the pulsed splitless mode (pulse pressure = 20 psi, pulse time = 1 min) with the split outlet opened after 1 min. Injector and detector temperatures were set at 270°C and 320°C, respectively. The temperature program of the HT-8 column was set to 90°C for 1 min, then with 15°C/min to 180°C, kept for 1 min, then to 250°C by 3°C/min and further by 25°C/min to 20°C, kept for 6 min.

A Hewlett Packard (Palo Alto, CA, USA) 6890 GC was connected via direct interface with a HP 5973 mass spectrometer. A 25m x 0.25mm x 0.25µm, DB-1 (J&W Scientific, Folsom, CA, USA) methyl siloxane capillary column was used with helium as carrier gas at a constant flow of 1.0 ml/min. A Gerstel programmable temperature vaporiser with a multi baffled empty liner of 1.5 mm I.D. was kept at 270°C. One µl was injected in the pulsed splitless

mode (pulse pressure=20 psi, pulse time = 1.25 min) with the split outlet opened after 1.25 min. Interface temperature was set at 285°C. The temperature program of the DB-1 column was starting from 90°C, kept for 1 min, then with 15°C/min to 275°C, kept 10 min. MS experimental conditions were: ion source, 200°C; electron impact ionisation energy, 70 eV; and electron multiplier voltage, 2000 V. Two ions from the molecular ion cluster M^+ and $[M+2]^+$ were monitored for each level of chlorination for PCBs or for each pesticide. The retention time, the m/z values of fragment ions and ion abundance ratios of the confirmation ion to the quantification ion were used as identification criteria. A deviation of ion ratios of less than ±20% from the theoretical value was considered acceptable.

Quality assurance

All samples were analyzed on both columns, the lowest value for each congener was further considered for calculations. Multi-level calibration curves were created for the quantification and good linearity (r^2>0.999) was achieved for tested intervals which included the whole concentration range found in samples. The identification of POPs was based on their relative retention times (RRT) to the internal standard used for quantification. Peak area ratios (POP response/internal standard response) were plotted against the concentrations of POPs. Reagent blanks were run to check for interference. Internal standards were monitored to ensure their recovery at acceptable levels.

Incubation Methods

Incubation methods (including reagents, time, temperature) are listed in Table 5.1.

Table 5.1. Comparison of methods used for extraction of selected POPs from human hair

Incubation	Reagent	Conditions	Recoveries (%) PCB 46/PCB 143	p,p'-DDE %[a]	Extraction method
Enzymatic	Protease K	40°C, 12 h	77/94	99	LLE-Hex:DCM
Basic	NaOH 1N, 5N	40°C, 12 h	74/69	121	LLE-Hex:DCM
Acid	H_2SO_4 7M	70°C, 1 h	71/84	61	LLE-Hex:DCM
	TGA+U	40°C, 12 h	77/86	98	LLE-Hex:DCM
	TGA+U	40°C, 12 h	5/8	61	SPE-C_{18}
	HCl 1N, 6N	40°C, 12 h	85/89	87	LLE-Hex:DCM
	HCl 3N	**40°C, 12 h**	**87/90**	**100**	LLE-Hex:DCM
	HCl 3N	40°C, 12 h	8/10	78	SPE-C_{18}
Solvent	MeOH	40°C, 12 h	75/82	103	LLE-Hex:DCM
	DCM	40°C, 12 h	74/74	93	
	Hexane	40°C, 12 h	85/86	88	
	DTT[b]	40°C, 12 h	82/91	97	LLE-Hex:DCM
	MercEtOH[c]	40°C, 12 h	74/95	103	LLE-Hex:DCM
	Hexane	2 h	87/97	98	Hot Soxhlet
	Hex:Acet	2 h	82/100	104	
	Hex:Acet:DCM	2 h	89/101	98	

a- percentage from DDE concentration of a large pooled sample found with HCl 3N and LLE-Hex:DCM
b- 1,4-dithiothreitol (1N) in water
c- 2-mercapto ethanol

Each procedure can be applied to powdered or cut hair after a water wash (recommended for the removal of hair spray preparations or other hair care products). The different incubation methods (for destruction of protein matrix) were compared in terms of recovery of internal

standards and absolute concentration of p,p'-DDE found from the incubated pool sample. It can be seen that almost all incubation methods (except methods involving NaOH which converts DDT in DDE and destroys HCHs and higher chlorinated PCBs) gave comparable recoveries of internal standards (> 75%).

Enzymatic incubation is done with non-specific enzymes (for breaking peptidic bonds). However, optimum conditions for efficient use of the enzyme should be used. Incubation with concentrated sulphuric acid is the fastest method, but emulsions can be easily formed when extracted with hexane. An interesting digestion method using thioglycolic acid was applied with success to the analysis of PCB 153, 138 and 180, p,p'-DDE and HCHs isomers in three human hair samples (Dauberschmidt and Wenning, 1998). Recoveries after incubation with thioglycolic acid and hydrochloric acid are comparable, but HCl extracts result in cleaner chromatograms.

Although widely used in drug analysis, MeOH incubation needs complete drying of the extract before clean-up. Incubation with dithiothreitol and mercaptoethanol, (used for S-S breaking) is found to be acceptable, but difficult to perform due to formation of emulsions and bad odour of the reagents. Hot Soxhlet extraction was also evaluated and found to give high recoveries (>90%). However, the method was restricted to the use of at least 1g of sample. Liquid-liquid extraction with Hex:DCM gave much higher recoveries of internal standards when compared with SPE. After incubation in aqueous reagents, lipophilic compounds are not released in solution and thus not available for interaction with the sorbent.

In all further experiments, HCl 3N (incubation reagent) and LLE with Hex:DCM were used for analysis of real samples. Combined clean-up on deactivated alumina and acid silica was shown to remove effectively lipids and other interferences from the extract.

Detection limits

As expected, detection limits were lower with ECD than with MS detection and were in the range of ng/g hair (Table 5.2). HCHs could not been detected in real samples by MS due to high background for specific ions.

Table 5.2. Limits of detection, linearity ranges and recoveries of spiked hair.

	LOD (ng/g)		Linearity	Spiking level	Powdered hair		Cut hair	
	ECD	MS	(ng/g hair) (r^2>0.999)	(ng/g hair)	mean (%)	RSD	mean (%)	RSD
HCB	1	1.5	1-20	10	na		93	8
PCBs	0.5	1	0.5-30	10	na		91-111	<12
DDTs	1	2	1-1000	25	98-106	<8	91-112	<10
HCHs	1	10	1-100	25	91-103	<10	65-83	<12

-na – not available

Two chromatograms from 2 different samples obtained with ECD and MS detection are presented in Figures 5.1 and 5.2. The presence of sulphur (in high concentration in hair) can be observed in Figure 5.1 at 15.55 min.

This was confirmed by using a desulphurating step (LLE extraction of the cleaned extract with tetrabutylammonium hydrogen sulphate saturated with sodium bisulphite). However,

because the peak does not interfere with any peaks of interest, this additional clean-up step was not used in further experiments.

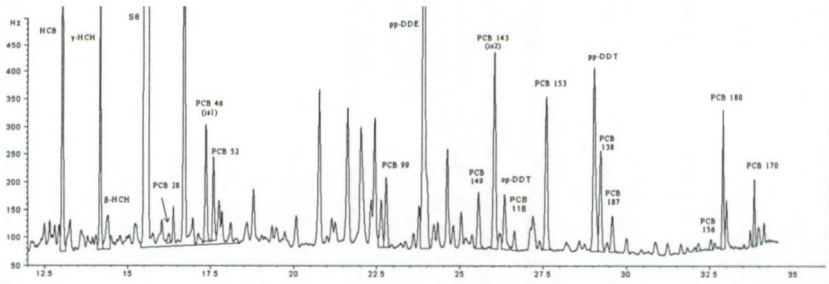


Figure 5.1. GC-ECD chromatogram of human hair sample from Belgium

Chromatograms obtained by electron ionization MS (Figure 5.2) offer more selectivity and correct identification and quantification of co-eluting compounds employing different acquisition ions (e.g. PCB 118–penta PCB from PCB 149-hexa PCB).

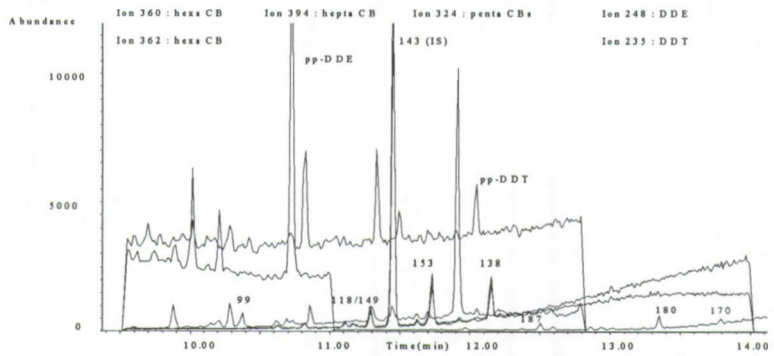


Figure 5.2. GC-MS chromatogram of a human hair sample from Romania

Recoveries

The incubation with 3N HCl was further tested for recovery of spiked products. In all cases, recoveries were calculated after subtraction of values for endogenous compounds. In the pooled hair sample, recoveries for all compounds were found acceptable (>85%) with a relative standard deviation of less than 12% (Table 5.2). The spiking solution was added to powdered or cut hair, hexane was added until the sample was submerged in the solvent and the solution was homogenised. The solvent was then evaporated at ambient temperature.

The extraction efficiency for hair was evaluated through the analysis of three individual non-fortified samples available in powdered and cut states. A good agreement was observed between organochlorine concentrations found from powdered and cut hair (Table 5.3). Thus, cut hair (with less laborious sample preparation) can be used for routine analysis.

Table 5.3. Comparison of extraction efficiency for powdered and cut hair. All results are expressed in ng/g hair.

	Sample A		Sample B		Sample C	
	Powdered	Cut	Powdered	Cut	Powdered	Cut
γ-HCH	15.3	18.4	4.0	3.7	3.4	3.0
o,p'-DDE	5.7	8.0				
p,p'-DDE	34.6	47.7	6.3	6.7	19.6	22.0
o,p'-DDT	16.4	20.4			11.3	11.5
p,p'-DDD	5.4	6.7			10.1	9.5
p,p'-DDT	46.5	58.7	3.6	4.8	88.3	87.1

Profiles of Persistent and Non-persistent PCB Congeners

PCB profiles in hair were compared with profiles in other body matrices (Figure 5.3). With exception of PCB 52 and PCB 149, all other congeners (in higher concentration in hair samples) show similar profiles. The presence of PCB 52 and probably of other low chlorinated PCBs (non-persistent congeners) in higher concentration than in other body matrices may be attributed to external gaseous and/or particulate exposure or is subjected to less metabolisation. Thus, it might be possible to differentiate between internal and external exposure by looking to the patterns of persistent and non-persistent congeners. An external exposure of hair leads to a congener pattern that also contains the non-persistent isomers (Zupancic-Krajl et al., 1992). Hair biomonitoring can thus be used in human residences and working places (Schramm, 1999). A fast adsorption and slow desorption are of importance to rule out exposure before the next hair washing. The main part of accumulation is finished very quickly (<1 h). However, due to much lower concentrations of non-persistent congeners than the persistent congeners, high amounts of sample (500 mg or more) should be used.

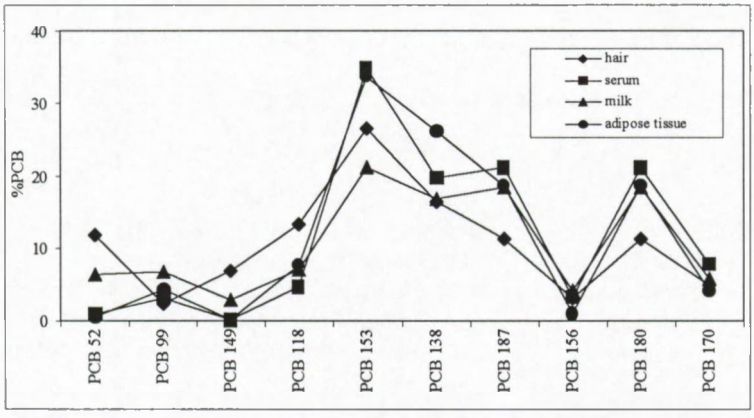


Figure 5.3. PCB profiles in human body matrices (hair, adipose tissue, serum and milk).

When expressed in ng/g fat, concentrations are similar for most of the compounds (Table 5.4). However, differences in concentration between hair and milk can be attributed to the different routes of contamination (ingestion versus air transport), excretion and distribution in body compartments, to the time of exposure and physico-chemical properties (octanol-water partition coefficients). There is little knowledge about the elimination kinetics in different body compartments. Available studies suggest a quasi-similar distribution of xenobiotics in different compartments (hair, blood, liver, abdominal fat and muscle), when normalised on lipid content (Klein et al, 1992).

Table 5.4. Concentrations (ng/g fat) for selected POPs in milk and hair from the same specimen.

Sample	PCB 118	PCB 153	PCB 138	PCB 180	PCB 170	p,p'-DDE	p,p'-DDT	γ-HCH
Human Hair	37	94	57	31	14	1470	314	651
Human Milk	28	83	52	55	18	2250	266	521

Advantages and disadvantages of hair as alternative matrix

Hair is a non-invasive matrix, and this feature becomes interesting for the monitoring of endangered species or children. Beside this, hair sampling is easy and can be done by non-specialised personal. Small amounts of hair (hundreds of mg up to several grams, depending on the compounds under investigation) can be used and no special precautions should be taken. The method developed here is very simple, miniaturised (up to 10 ml of organic solvent is used), cheap and up to 50 samples per analyst can be achieved in one day. Hair analysis can be applied to any group of population (when compared with human milk restricted to women in lactating period or adipose tissue available under surgery).

However, there are some drawbacks of using hair in POP analysis. It is not a homogeneous sample, and as any other solid sample, it is difficult to spike. There are no certified reference materials available (and no signs of future perspectives). Even if human hair is intensively used in drug or heavy metal analysis, existent knowledge cannot be extended to POPs due to their different structure, behaviour and lipophilicity. And finally, there is a great uncertainty for the comparison of concentrations found by different studies in different matrices. Future research on hair analysis should focus on kinetics of distribution of POPs in hair and improvement of the analytical technique for detection of minor PCB congeners and other organohalogen compounds.

5.2. Determination of POPs in human hair from Greece, Belgium and Romania

* - based on: Covaci A, Tutudaki M, Tsatsakis AM, Schepens P, (2002). *Chemosphere* 46(3), 413-418.

Samples

In July 1996, scalp hair strains from the back of the head were obtained from Greek women (n=30) with past occupational (green houses, vineyards or open air plantations of olive) exposure to pesticides (total duration of the exposure between 2 and 35 years). In April 2000, scalp hair strains (back of the head) were obtained from apparently healthy individuals living in Belgium (n=10), Romania (n=2) and Greece (n=5) without known occupational exposure to pesticides

Comparison of profiles and concentrations of selected POPs in different European countries

The assessment of contamination with organochlorine compounds in samples from different European countries (Belgium, Greece and Romania) was done by analysis of scalp hair samples. Complete results are given in Table 5.5.

Table 5.5. Concentrations of organochlorine pollutants (ng/g hair) in hair samples from Greece, Romania and Belgium.

Compound	Greece (n=35)		Romania (n=2)		Belgium (n=10)	
	Mean ± SD	Range	Mean	Range	Mean ± SD	Range
HCB	0.5 ± 0.6	nd-2.6	0.9	nd-1.3	0.9 ± 0.6	nd-3.3
α-HCH	1.4 ± 1.2	nd-5.2	3.5	2.4-4.6	1.5 ± 1.2	nd-2.8
β-HCH	6.1 ± 4.7	nd-16.3	12.0	11.7-12.3	3.5 ± 1.9	1.7-7.7
γ-HCH	33.8 ± 17.0	18-73.7	16.3	9.8-22.8	9.6 ± 10.5	2.7-36.6
Σ HCH	**40.8 ± 14.7**	**21.8-95.2**	**31.8**	**24.5-39.1**	**14.7 ± 10.9**	**6.0-40.8**
o,p'-DDE	20.2 ± 23.1	nd–453.1	nd	nd	nd	nd
p,p'-DDE	37.6 ± 13.4	18-127.4	37.5	23.6-51.4	10.5 ± 7.9	2.1-278.7
p,p'-DDD	4.2 ± 3.7	nd-17.3	nd	nd	2.3 ± 1.6	nd-5.8
o,p'-DDT	5.9 ± 3.6	nd-14.4	3.8	3.4-4.2	2.2 ± 1.0	nd-3.6
p,p'-DDT	22.0 ± 12.2	5-43.6	7.9	4.8-11	5.9 ± 4.3	2.4-13.6
Σ DDTs	**101 ± 41.4**	**12.3-754.2**	**52.2**	**34.8-69.6**	**18.8 ± 12.5**	**8.7-301.8**
Σ PCBs*	**5.2 ± 5.9**	**nd-17.6**	**10.2**	**10.0-10.4**	**13.7 ± 18.1**	**nd - 44.7**

nd – not detected * - sum of PCB 99, 118, 138, 149, 153, 170 and 180.

For PCB 28, 101 and o,p'-DDD all measurements were under the detection limit. For PCB 52, 99, 118, 149, 156 and 187, HCB, α-HCH, β-HCH, o,p'-DDE o,p'-DDT and p,p'-DDD, some measurements were above the detection limit. For calculation of means, concentrations under the detection limit were set to zero.

It can be seen that DDTs (sum of DDT isomers) were the major chlorinated contaminants in human hair (Table 5.5). DDTs and HCHs (sum of α-, β- and γ- HCH) values were higher in Greek specimens, probably due to a more recent restriction on pesticide usage, while PCBs concentrations were higher in Belgian specimens, due to a higher degree of industrialisation (Table 5.5). The organochlorine load of Greek hair specimens was mainly composed of pesticides (> 95%), while Belgian hair specimens contained 29% PCBs (Figure 5.4). Interestingly, the HCHs percentages were almost similar in all three countries (28, 34 and 31% for Greek, Romanian and Belgian hair samples, respectively).

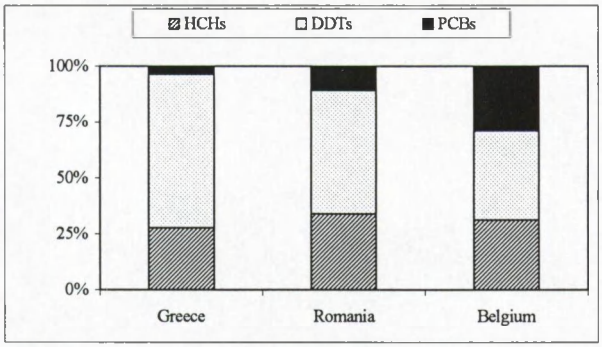
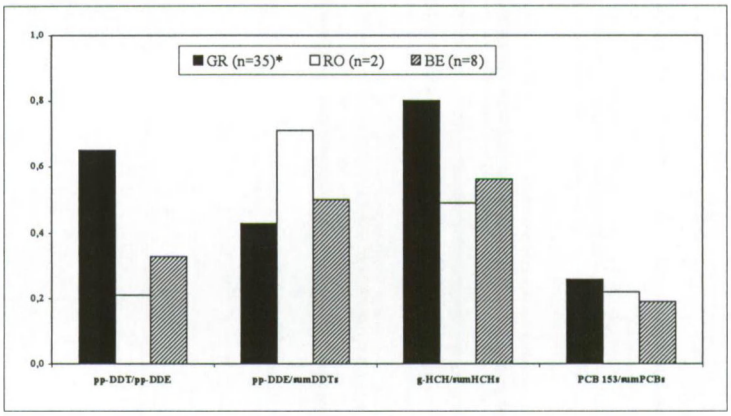


Figure 5.4. Relative contribution of PCBs, HCHs and DDTs to the total organochlorine load in the three countries

Mean p,p'-DDT/p,p'-DDE ratio was higher in Greece than in other countries (Figure 5.5). This can be explained by possibly recent use of technical DDT (containing p,p'- and o,p'-DDT). This hypothesis is supported by the presence in some Greek samples of

concentrations up to 453 ng/g hair of o,p'-DDE, a metabolite of o,p'-DDT, with shorter lifetime than the p,p'-isomer. Higher p,p'-DDE/sum DDTs ratios (up to 0.71 in Romanian hair) suggest past exposure to DDT.



*-n=30 for PCBs from Greece

Figure 5.5. Relative contribution of p,p',DDT, p,p'-DDE, γ-HCH and PCB 153 to the total sum of DDTs, HCHs and PCBs

Lindane (γ-HCH) was the major HCH isomer present in hair samples (up to 82% of total HCHs for the Greek specimens). The γ-HCH/sum HCHs ratio gives an indication of the exposure time and of the HCH mixtures used. Thus, the high ratio in Greek specimens can be explained by a recent usage of pure lindane, while lower ratios (as found in Romanian and Belgian samples) indicate past exposures and the use of technical lindane (with high percentage of the less persistent isomer, α-HCH). Additionally, β-HCH, the most persistent HCH isomer, indicates the exposure time to HCHs. A high β-HCH/sum HCHs ratio (0.4 and 0.3 for Romanian and Belgian specimens, respectively) indicates past exposure to technical lindane.

Ratios PCB 153/sum PCBs in all 3 countries (Figure 5.5) indicated similar pattern and source of pollution with PCBs (mainly dietary exposure).

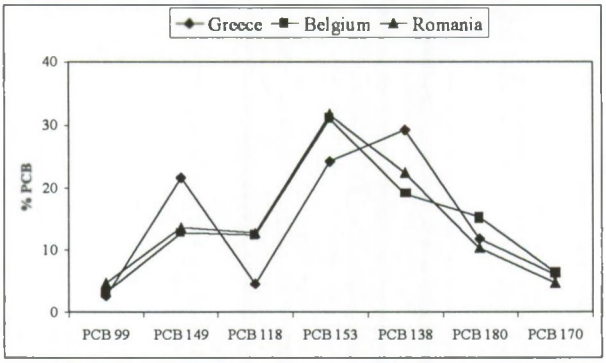


Figure 5.6. Profiles of PCB congeners in hair samples from the three European countries.

However, the lower ratio for Belgian samples (0.19 vs. 0.26 in Greek specimens) was due to the quantification of a higher number of congeners because of higher PCB concentrations in the Belgian samples (Table 5.5). It can be observed (Figure 5.6) that PCB profiles for

Belgian and Romanian specimens are almost similar, but slightly different from the profile in Greek samples. Differences are more pronounced for PCB 118 and 149, possibly because of some measurements under the detection limit in the Greek samples.

Hair treatment and occupational exposure evaluation

Hair treatment was evaluated for Greek samples (obtained from persons occupationally exposed to pesticides) for which information on hair colour was available. Concentrations of investigated compounds in specimens of artificially coloured hair were lower (though not significantly) for all compounds (Table 5.6), suggesting that the structural integrity of hair is altered due to treatment. Adsorptive properties of hair are reduced by the treatment and lipidic components from hair structure are destroyed and lipophilic pollutants can not be adsorbed or, when adsorbed, are easily released from the matrix. This would explain the lower PCB (the most lipophilic pollutants) concentrations in coloured hair. It might be possible that some compounds are degraded during hair colouring process (which in general involves oxidation with hydrogen peroxide in basic medium).

Table 5.6. Mean concentrations of organochlorine pollutants (ng/g hair) and statistical comparison for hair treatment and exposure to pesticides in Greek specimens

	PCBs	HCHs	DDTs
		(ng/g hair)	
colored hair (n=15)	4.9	40.6	101.5
non-coloured hair (n=15)	9.2	52.3	149.1
p-value	0.513	0.053	0.314
exposed (n=30)	7.1	46.6	125.9
non-exposed (n=5)	n.a.	11.6	80.4
p-value	n.a.	0.02	0.212
		r	
age-dependence	0.04	0.55	0.08

-significant at $p < 0.05$ -n.a. – not available

In the same samples, HCHs were found to be significantly higher ($p<0.05$) in persons exposed to pesticides when compared with non-exposed persons. No significant difference between the two groups was found for DDTs. No correlation between occupational exposure time and concentrations of HCHs or DDTs was found for the same group. However, no information was available about the types of pesticides used.

The correlation between age and concentration of contaminants was very weak (Table 5.6). The highest correlation coefficient was found for HCHs (0.33). This is probably due to the fact that, when analysing hair, the sum of internal and external exposure is measured. Thus, correlation cannot be as strong as in the case of, for example, serum analysis, which only gives a measure of internal exposure. For hair analysis, one should take into account the eventually high contribution of external exposure from diffuse sources.

Conclusion

Different incubation, extraction and analysis methods for selected POPs in human hair were compared in terms of recovery of internal standards. A simple method (using 3N HCl as incubation reagent, liquid-liquid extraction with hexane and dichloromethane, alumina/acid silica clean-up and GC-ECD/GC-MS analysis) was validated and used for the analysis of human hair samples.

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Chapter 6

Determination of POPs in human adipose tissue

Abstract

A new analytical method has been developed for the quantification of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) in human adipose tissue samples. After Soxhlet extraction and a clean-up procedure with two successive solid phase extraction cartridges containing acid silica and acid silica : neutral silica : deactivated basic alumina (from top to bottom), detection can been achieved by narrow bore (0.10 mm internal diameter) capillary gas chromatography/electron impact low resolution mass spectrometry using a large volume injection technique. The method allows the determination of five major PBDE congeners (BDE 28, 47, 99, 100 and 153) at concentrations below 1 ng/g lipid weight. Detection limits in the selected ion mode varied between 0.05 and 0.30 ng/g lipid weight, depending on the degree of bromination. Levels of PBDEs (sum of five congeners) in twenty Belgian human adipose tissue samples ranged between 2.18 and 11.70 ng/g lipid weight and were similar to previously reported values from Europe.

The median value for the sum of PCBs (35 congeners) was 841 ng/g lipid weight and ranged from 286 to 1802 ng/g lipid weight. BDE 47 and PCB 153 were highly correlated with the sum PBDEs ($r=0.95$, $p<0.05$) and sum PCBs ($r=0.98$, $p<0.05$), respectively. Sum PCBs showed good correlation with the sum DDTs ($r=0.77$, $p<0.05$), while the correlation with sum PBDEs was weaker ($r=0.56$, $p<0.05$). No age-dependency was found for PBDEs ($r=0.09$), while PCBs and DDTs showed higher correlation coefficients with age ($r=0.59$ and 0.40, respectively).

6.1. Analysis methodology for the determination of POPs in human adipose tissue

* - based on Covaci A, De Boer J, Ryan JJ, Voorspoels S, Schepens P, (2001). *Anal Chem*, in press

Polybrominated diphenyl ethers (PBDEs) have been used extensively over the past two decades as flame retardants in most types of polymers used in electronic circuit boards and computer and TV housing, furniture, building materials, textiles, carpets and vehicles (BSEF, 2000). Due to their persistence and bioaccumulation potential, various PBDEs have been reported in various environmental matrices (Sellström et al., 1993; Alaee et al., 1999; De Wit, 1999) and in humans ((Lindström et al., 1997; Meironyté et al., 1999; Meneses et al., 1999; Sjödin et al., 1999; Strandman et al., 1999).
The monitoring of levels in humans is important to estimate the population exposure to persistent organohalogenated compounds and possible related health risks. To assess possible occupational human exposure to PBDEs, it is essential to establish background values for the general population. PBDE monitoring in humans has started only some years ago, and a limited set of data is available. Data are available from Sweden (Meironyté et al., 1999; Lindström et al., 1997; Meironyté et al., 2001a), Spain (Meneses et al., 1999), Finland (Strandman et al., 1999), and Canada (Ryan and Patry, 2000), but PBDEs have not yet been reported in the human population from Belgium. Despite the fact that PBDE concentrations in humans are significantly lower than those of PCBs and p,p'-dichlorodiphenyldichloroethylene (p,p'-DDE) in human tissues (Sjödin et al., 2000), a dramatic increase in PBDE concentration in Swedish human milk collected between 1972 and 2000 has been reported, corresponding to a doubling every 5 years until 1997, although recent samples showed somewhat lower concentrations (Meironyté and Norén, 2001b).

There is a growing body of research describing toxic aspects of PBDEs (Pijnenburg et al., 1995; de Boer et al., 1999). Although PBDEs seem to have low toxicity in many tests (Darnerud et al., 2001), recent data indicate that PBDE congeners may be more harmful than previously expected. Several PBDE congeners interfere weakly with the aryl hydrocarbon (Ah) receptor (Meerts et al., 1998a). BDE 47 is transformed in rats and mice to hydroxylated metabolites (Örn and Klasson-Wehler, 1998) that compete with thyroxin for the binding site on transthyretin (Meerts et al., 1998b) while some hydroxylated PBDE metabolites also bind to the thyroid receptor (Marsh et al., 1998). These observations indicate that some PBDEs might act as endocrine disruptors. Recently, it was suggested that developmental (neonatal) exposure to some PBDEs (eg BDE 99, BDE 153 and BDE 209) can induce behavioural disorder in mice, a condition that gets worsens with age (Eriksson et al., 2001). However, there is still little information on the effective levels.

Because concentrations of PBDEs in humans are in the order of ng/g lipid weight (approximately 50 to 200 times lower than PCBs), most analytical work has been carried out using highly sensitive systems. Brominated substances are often analysed under electron capture negative ionisation (NCI) conditions (Sellström et al., 1993) using low resolution mass spectrometry (LRMS) by monitoring the negative ions formed by electron capture reactions. The predominant ions formed from organobromine substances under such conditions are the bromine isotopic ions m/z 79 and 81. This technique is more sensitive and less costly than other alternatives such as electron impact-high resolution mass spectrometry (EI-HRMS). However, the latter technique has a higher selectivity than the NCI-LRMS since

the accurate mass of the molecular ion or fragment ion for each level of bromination is recorded (Ryan and Patry, 2000). The higher specificity of the electron ionisation mode is needed to reduce the risk of misinterpretations of interfering substances. Furthermore, it allows the use of ^{13}C-labeled standards (as internal standards) which makes the quantification procedure more accurate. The use of electron impact-low resolution mass spectrometry (EI-LRMS) can be useful due to easy maintenance of the instrumentation and lower costs. Until now, EI-LRMS in combination with a classical injection technique (e.g. 1-2 µL injected in hot splitless) was used for the determination of PBDEs in samples with relatively high concentrations (Lindström et al., 1999), but its use in human monitoring was limited due to a lower detectability (1 to 2 orders of magnitude) for congeners with more than four Br atoms (for lower brominated ones, EI is more sensitive).
In the present study, the use of large volume (up to 20 µL) injection (LVI) combined with narrow-bore capillary column gas chromatography and EI-LRMS for the determination of individual PBDE congeners in human adipose tissue at the low ppb level was investigated.

Materials
Hexane, acetone, dichloromethane, iso-octane, residue analysis grade were obtained from Merck (Darmstadt, Germany). Silica gel 60 (63-230 mesh), alumina 60 (70-230 mesh) and anhydrous sodium sulphate p.a. (Merck) were heated at 150°C for 24 h. The following individual PCB standards: IUPAC no. 28, 31, 46, 52, 74, 95, 99, 101, 105, 110, 118, 128, 138, 143, 146, 149, 153, 156, 163, 167, 170, 171, 172, 177, 178, 180, 183, 187, 189, 190, 194, 195, 196, 199, 203, 206 and 209 were obtained in iso-octane from Dr. Ehrenstorfer Laboratories (Augsburg, Germany). Native PBDE standards in iso-octane (No. 28, 47, 66, 71, 75, 77, 85, 99, 100, 138, 153, 154) were obtained from Promochem (Wessel, Germany), while ^{13}C-labeled PBDEs (^{13}C-BDEs 47, 99, 153) were obtained in nonane from Wellington Laboratories (Guelph, Ontatio, Canada).

Extraction of adipose tissue
One gram of each sample was accurately weighed and mixed with 6 g anhydrous Na_2SO_4 until a fine floating powder was obtained. After addition of internal standards (100 µl from a mixture of ^{13}C-BDE 47, 99 and 153, 13.06 pg/µl in iso-octane and 200 µl from a mixture of PCB 46 and 143, 100 pg/µl in iso-octane), the powder was extracted by hot Soxhlet extraction for 2 h with a mixture of hexane : acetone : dichloromethane (75 ml, 3:1:1, *v/v*). The extract was evaporated to dryness and lipids were determined gravimetrically.

Clean-up
Two successive solid phase extraction (SPE) cartridges containing 6 g acid silica and 2 g acid silica : 1 g neutral silica : 2 g deactivated basic alumina (from top to bottom), respectively, were used for cleanup. After rinsing the cartridges with 20 ml hexane, the sample extract was applied to the acid silica SPE cartridge and PBDEs (together with PCBs and DDTs) were eluted with 40 ml hexane and 20 ml hexane:dichloromethane (1:1, *v/v*) and the two fractions were combined. The eluate was concentrated to near dryness and 100 µl of the recovery standard (polybromobiphenyl (PBB 80), 18 pg/µl in isooctane) was added. The extract was concentrated under a N_2 stream to approximately 60 µl and transferred to an injection vial.

GC/MS system
The measurements were performed with a HP 6890/5973 GC/MS equipped with a 10 m x 0.10 mm x 0.10 µm AT-5 (5% phenyl polydimethyl siloxane) capillary column (Alltech,

Lokeren, Belgium) for the PBDE and PCB determination or with a 50 m x 0.22 mm x 0.25 µm HT-8 (1,7-dicarba-closo-dodecarborane 8% phenyl methyl siloxane) capillary column (SGE, Zulte, Belgium) only for PCBs. Sample introduction was done by a HP 6890 automated liquid sampler. The temperature of the ion source was set at 230°C and the interface at 300°C. Helium was used as carrier gas at constant flow (0.4 and 0.7 ml/min for the 10 m and 50 m columns, respectively). For EI, 70 eV electrons were used and the quadrupole LRMS was operated in the selected ion monitoring (SIM) mode. The ion source and quadrupole temperatures were 230 and 250°C, respectively. The electron multiplier voltage was set at 2300 V.

PBDE determination. Twenty µl (4 x 5 µl) of the extracts were injected into a Gerstel (CIS 4) programmable temperature vaporiser (PTV) in the solvent vent mode (vent flow 100 mL/min for 1.1 min, injector at 70°C for 1.1 min and then heated with 700°C/min to 270°C) with the split outlet opened after 2.1 min (Figure 6.1). The temperature program of the AT-5 column was programmed from 70°C (2.2 min) to 230°C at a rate of 40°C/min, then to 280°C (5 min) at a rate of 25°C/min. The run time was 13.2 min. Dwell times were set at 10 ms. Two most abundant ions (see Table 6.1) were monitored for each level of bromination for native and labeled PBDEs.

Table 6.1. Acquisition parameters, recoveries and detection limits for target BDE congeners.

Target compounds		RT (min)	Ions	Recovery (%)	LOD (ng/g fat)
	PBB 80	7.02	470, 472		
Tri	BDE 28	6.07	406, 408		0.05
Tetra	^{13}C-BDE 47*	7.05	496, 498	81 ± 12	
	BDE 47	7.05	484, 486		0.1
	BDE 66	7.17	484, 486		0.1
	BDE 71	6.94	484, 486		0.1
	BDE 75	6.82	484, 486		0.1
	BDE 77	7.37	484, 486		0.1
Penta	^{13}C-BDE 99*	7.94	576, 578	84 ± 17	
	BDE 85	8.38	564, 566		0.20
	BDE 99	7.93	564, 566		0.15
	BDE 100	7.72	564, 566		0.15
	BDE 119	7.80	564, 566		0.15
Hexa	^{13}C-BDE 153*	9.06	496, 498	103 ± 21	
	BDE 138	9.79	484, 486		0.30
	BDE 153	9.06	484, 486		0.25
	BDE 154	8.64	484, 486		0.25

PCB determination. For the 10 m AT-5 column, 1 µl of the extract was injected in a cold splitless (injector temperature at 100°C (0.1 min), then heated with 700°C/min to 270°C). The splitless time was 1 min. The temperature program of the AT-5 column was programmed from 90°C (1 min) to 200°C (0.5 min) at a rate of 50°C/min, then to 250°C (0.2 min) at a rate of 25°C/min and finally to 280°C (2 min) at a rate of 75°C/min. Run time was 8.3 min and dwell times were set at 10 ms. For the 50 m HT-8 column, 1 µl extract was injected in a hot pulsed splitless (pressure pulse 30 psi, pulse time 1.20 min) at an injector temperature of 270°C. The splitless time was 1.25 min. The temperature was programmed from 90°C (1 min) to 170°C (2 min) at a rate of 15°C/min, and finally to 290°C (14 min) at a rate of 4°C/min. The run time was 52.3 min and dwell times were set at 50 ms.

The two most abundant ions (m/z=256,258 for tri-CBs, 290,292 for tetra-CBs, 324,326 for penta-CBs, 360,362 for hexa-CBs, 394,396 for hepta-CBs, 428,430 for octa-CBs, 464,466 for nona-CBs and 498,500 for deca-CB) were monitored for each level of chlorination.

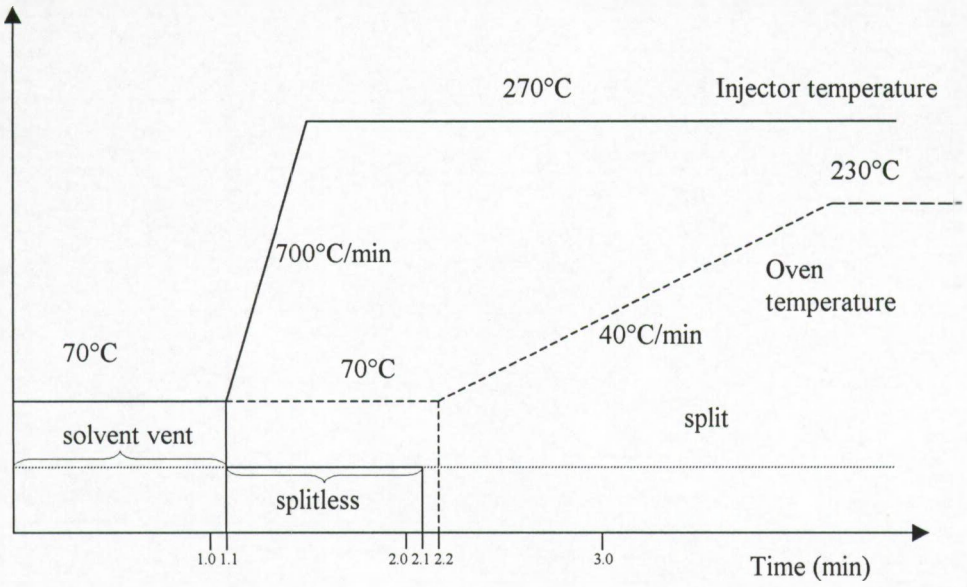

Figure 6.1. Time relationships of oven temperature, PTV temperature and split status in large volume injection mode.

Quality control
Retention times, ion chromatograms and intensity ratios of the monitored ions were used as identification criteria. A deviation of the ion intensity ratios within 20% of the mean values of the calibration standards was considered acceptable.

PBDE. Recoveries of internal standards, ^{13}C-labeled BDEs (calculated based on PBB 80 added prior to injection) were between 81 and 103% with a standard deviation of less than 21% (Table 6.1). Limits of detection (LOD) were calculated for a signal to noise ratio of 3:1 and ranged between 0.05 ng/g fat for tri-BDEs to 0.30 ng/g fat for hexa-BDEs. Multilevel calibration curves (6 levels) were created and a good linearity (r^2>0.995) was achieved for each compound between 2 x LOD and 10 ng/g lipid weight.
External quality control was achieved through interlaboratory comparison (with the Netherlands Institute for Fisheries Research and Health Canada) as shown in Table 6.2. Furthermore, the analysis of two samples of biota (eel and porpoise liver) used for the first world wide interlaboratory test on PBDE (de Boer, 2000), showed a variation of less than 10% from mean values obtained by the participating laboratories (Table 6.2).

Table 6.2. Interlaboratory comparison for PBDEs in different samples (concentrations are expressed in ng/g lipid weight for samples A-D and in ng/g whole weight for E and F).

Sample Description	Lab	Detection	BDE 28	BDE 47	BDE 99	BDE 100	BDE 153	Sum BDE	Diff. (%)
HAT[a]	UA[b]	EI-LRMS[e]	< 0.05	0.87	0.11	0.32	1.43	2.73	- 33
	RIVO[c]	NCI-LRMS[f]	< 0.07	1.47	0.44	0.33	1.82	4.06	
HAT	UA	EI-LRMS	0.14	4.42	1.61	1.04	4.36	11.43	- 20
	RIVO	NCI-LRMS	0.32	5.80	2.32	0.88	5.36	14.36	
HAT	UA	EI-LRMS	0.11	1.34	0.24	0.34	0.87	2.79	- 17
	HC[d]	EI-HRMS[g]	0.10	1.28	0.47	0.33	1.28	3.36	
HAT	UA	EI-LRMS	0.26	2.86	0.29	1.50	1.94	6.59	- 23
	HC	EI-HRMS	0.32	3.33	0.73	1.82	2.71	8.59	
Eel	UA	EI-LRMS	na[h]	12.03	0.78	3.55	0.66	17.02	+ 4
	BSEF[i]		na	11.60	0.92	3.40	0.54	16.46	
Porpoise liver	UA	EI-LRMS	na	121.9	14.3	33.5	8.3	178.0	- 7
	BSEF		na	133.0	20.4	29.9	7.3	190.6	

a- human adipose tissue; b- University of Antwerp; c-Netherlands Institute for Fisheries Research; d-Health Canada; e-electron impact-low resolution mass spectrometry; f-negative chemical ionisation-low resolution mass spectrometry; g-electron impact-high resolution mass spectrometry; h-not available; i- first world wide interlaboratory study organised by Bromine Science and Environmental Forum (mean from 18 participating laboratories)

Results obtained by EI-LRMS in combination with large volume injection and narrow bore capillary GC, correlate well with results obtained with methods based on HRMS or ECNI-LRMS. Results for BDE 28, 47 and 100 were more consistent between the labs, while most of differences were encountered for BDE 99. The difference between results obtained in two different laboratories was always lower than 35%, which is considered acceptable for the low ppb levels measured. The analysis of two samples of biota (eel and porpoise liver) used for the first world wide interlaboratory test on PBDEs (de Boer, 2000) showed an overall variation of less than 10% from the mean values (Table 6.2). Except for the eel sample, the results obtained by EI-LRMS were lower than results obtained by other techniques. Good agreements were obtained for all congeners, with exception of BDE 99. In the BSEF interlaboratory study, the variation for this congener was also high due to unknown factors. Coefficients of variation were lower when the matrix analysed contained higher levels of PBDEs.

PCBs. Mean recoveries of the internal standards were 73 ± 7 and 79 ± 10% for PCB 46 and 143, respectively. LODs ranged between 0.2 and 0.5 ng/g lipid weight. Co-eluting PCB congeners were identified based on their retention times and on their reported elution order (Frame, 1997) for each capillary column. The method performance was assessed through rigorous internal quality control, which included daily check of calibration curves and regular analysis of procedural blanks and certified material CRM 350 (PCBs in mackerel oil). Furthermore, we have participated in an interlaboratory test organised by the Institute for Reference Measurements and Materials (IRMM, Geel, Belgium). The seven marker PCB congeners (no. 28, 52, 101, 118, 138, 153 and 180) were determined in standard solution, medium- and high-level spiked pork fat. The results of the individual PCB congeners deviated less than 6% from the target values (Table 6.3). Excellent agreement can be seen for all congeners.

Table 6.3. IRMM interlaboratory comparison.

Compound	Standard solution (µg/l)		Medium-spiked fat (ng/g lipid)		High-spiked fat (ng/g lipid)	
	UIA	IRMM	UIA	IRMM	UIA	IRMM
	N=6		N=6		N=9	
PCB 28	17.9	17.6	14.3 ± 1.3	14.8 ± 1.3	29.1 ± 1.9	29.6 ± 2.1
PCB 52	31.4	30.8	12.7 ± 1.2	12.9 ± 0.9	26.1 ± 1.8	25.5 ± 1.8
PCB 101	36.6	34.4	12.4 ± 0.9	12.5 ± 1.2	29.4 ± 1.7	30.0 ± 4.0
PCB 118	43.8	41.6	11.1 ± 0.4	12.7 ± 1.3	27.6 ± 0.8	30.2 ± 2.7
PCB 138	37.3	34.4	13.6 ± 0.4	14.6 ± 1.6	29.7 ± 0.4	32.0 ± 4.0
PCB 153	33.2	31.2	12.7 ± 0.8	13.1 ± 1.1	30.4 ± 0.7	30.8 ± 2.4
PCB 180	18.4	17.4	12.9 ± 1.1	12.6 ± 0.9	28.8 ± 2.1	29.8 ± 2.5
Sum PCBs	218.6	207.4	89.5 ± 3.7	93.0 ± 7.0	201.0 ± 4.8	207.0 ± 11.0
Error	+ 5.4%		- 3.9%		- 2.9%	

Results and discussion

Extraction and clean-up efficiency

When compared with classical Soxhlet extraction, the use of hot Soxhlet extraction results in a reduction of the extraction time, together with a comparable efficiency of lipid extraction (Manirakiza et al., 2001). Two hours was a convenient extraction time to obtain high lipid yields, while longer extraction times lead to an insignificant increase of the extracted material. During the hot extraction mode, the solvent is distilled into a heated extraction chamber, while the upper heating is turned on. The sample is permanently in contact with hot, but not boiling solvent. The present procedure has some advantages over classical extraction procedures (such as column percolation (Pauwels and Schepens, 1998) or ultrasonic extraction (Kallenborn et al., 1998) of fatty samples): automation, use of lower volumes of solvents and higher efficiencies and reproducibility. Furthermore, it has similar efficiencies and shorter extraction times compared to conventional Soxhlet extraction, and lower costs when compared with new extraction techniques such as accelerated solvent extraction (Richter et al., 1996) or microwave assisted extraction (Xiong et al., 2000).

A period of 2 h was recommended (de Boer, 1988) between sodium sulphate grinding of the fatty fish samples and the extraction to allow adsorption of the moisture. However, the adipose tissue contains only low amounts of water and the extraction can be performed immediately. Sodium sulphate was used here for good matrix dispersion. The solvent mixture (hexane : dichloromethane : acetone, 3:1:1, *v/v*) was found to be efficient for lipid extraction (Manirakiza et al., 2001), because most of the lipids of adipose tissue are triglycerides, easily extractable in non- or medium- polar solvents. The lipid determination was done gravimetrically.

The dual system of SPE cartridges containing acid silica and alumina allows an efficient clean-up because lipids present in the adipose tissue were readily destroyed by the acidic treatment. Additional clean-up was not necessary for this amount of sample, but it may be required when a higher sample amount is used. Furthermore, compared with the classical clean-up techniques, lower amounts of solvents were used and less manipulation was needed, while the use of disposable cartridges minimised cross contamination. Because of the high selectivity of the electron impact ionisation mode, there was no need for extract fractionation.

Large volume injection for PBDE determination

Because PBDEs are found in low concentrations in human tissues (low ng/g fat), a highly sensitive technique is needed. The response of the analytical system can be increased with the introduction of a larger volume (tens of µl instead of 1-2 µl, as used in a classical splitless injection) of the final extract into the GC/MS instrument. Large volume injection (LVI) using a PTV injector is based on the multiple injection of extracts and selective evaporation of the sample solvent from the liner of the injector, while simultaneously trapping the less volatile components in the cold liner (Mol et al., 1995).

During the injection procedure, the split exit is open and the injector kept at a low temperature to minimise losses of volatile analytes. For iso-octane, the injector temperature was set at 70°C (some 30°C below its boiling point). A lower temperature was not necessary due to the relatively high boiling points of the PBDEs and thus, no significant losses occurred at this temperature. Due to the small volume capacity of the empty baffled liner, a maximum of 5 µl can be injected at once. The multiple injections are performed at regular time intervals and the solvent is vented at a high flow of carrier gas in the period between two injections (solvent elimination time). An optimum of 100 ml/min for the split vent flow was used in order to increase the solvent evaporation rate, but to minimise losses of volatile compounds (Figure 6.2).

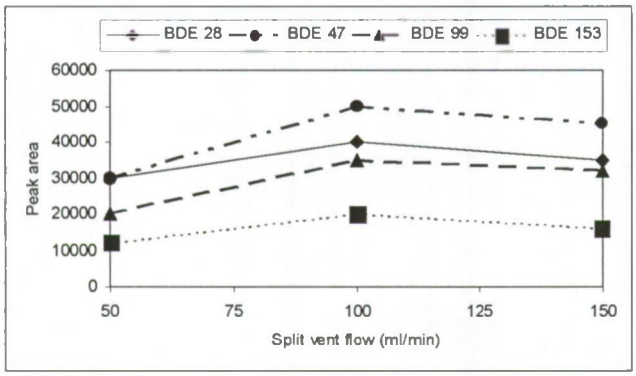

Figure 6.2. Effect of split vent flow on peak areas. Initial PTV temperature: 70°C, delay between injections 10 s, concentration of PBDEs (10 pg/µl).

The solvent elimination time used should be sufficiently long to allow all the solvent to evaporate. Too short interval times might result in losses of components due to flooding of the injector and loss of the liquid sample via the split exit. If, on the other hand, too long interval times are used, excessive losses of the more volatile compounds might occur (Figure 6.3). The interval time depends on the liner temperature, solvent boiling point and sample volume. A solvent elimination time of 10 s was found to be optimal.

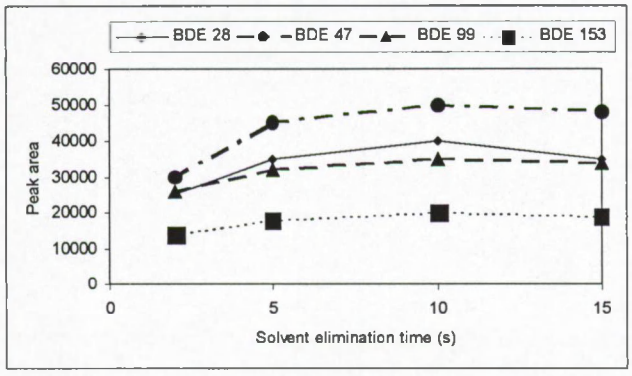

Figure 6.3. Effect of solvent elimination time on peak areas. Initial PTV temperature: 70°C, split vent flow 100 ml/min, concentration of PBDEs (10 pg/µl).

When the solvent elimination is complete, the split exit is closed and the components are transferred to the column in the splitless mode by a rapid temperature-programmed heating of the injector. When automated, the multiple injection method is a very fast and convenient method for the introduction of sample volumes up to approximately 25 µl. However, for the analysis of real samples, the clean-up procedure should be very efficient as interferences may easily disturb the chromatogram.
For semi-volatile compounds (such as PCBs and PBDEs), the method optimisation is not as critical as for volatile analytes. Combining a cool injection step with a controlled vaporisation eliminates a number of important disadvantages associated with the use of conventional hot inlets and especially reduces the discrimination of less volatile compounds. The gain in sensitivity (due to large volume introduction) can be obtained without the need to make any changes to the instrument. Moreover, its small internal volume allows the same empty liner to be used in the injection port liner for any other type of splitless injection.

Narrow bore capillary GC for the determination of PBDEs and PCBs
Narrow-bore columns (equal or less than 0.10 mm internal diameter) emerged from the need for faster and more efficient separations. Extremely narrow peaks (peak width of less than 1 s) are obtained and mass sensitivity is increased. Narrow-bore capillaries can be used in combination with quadrupole mass spectrometers for the rapid determination of organohalogenated pollutants in human matrices (Covaci and Schepens, 2001b). It is possible to analyse complex mixtures in a short time (up to 10 min), saving 50% or more of the analysis time of conventional columns (0.25 mm internal diameter), while maintaining a similar resolution power.

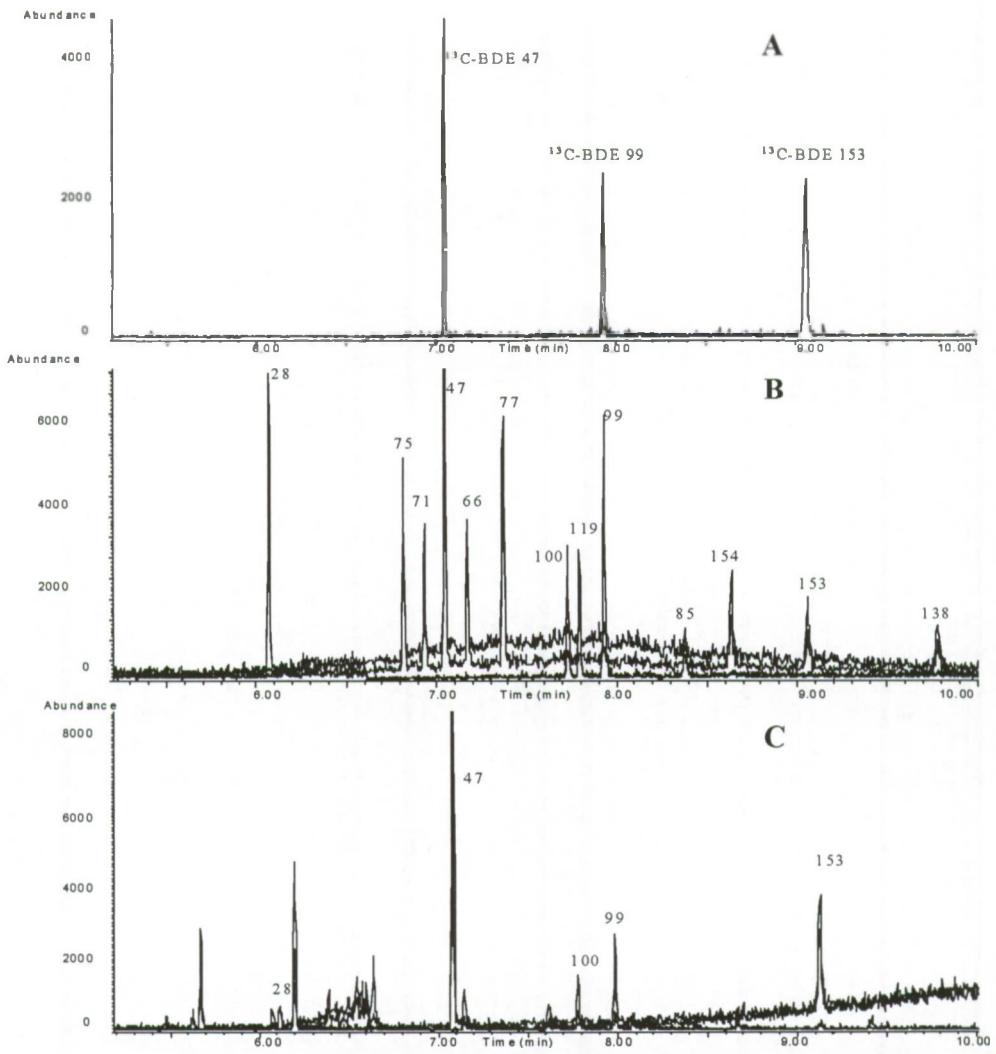

Figure 6.4. Selected ion chromatograms (EI-MS) of a standard mixture (^{13}C-labeled BDEs (A) and target BDEs (B)) and human adipose tissue extract (C) analysed on a 10 m x 0.10 mm I.D. AT-5 capillary column.

On the 10 m x 0.10 mm capillary column, the target PBDEs (tri- to hexa- congeners) eluted from the GC column between 6.0 and 9.8 min (Figure 6.4B), while the PCBs eluted in less than 8 min (Figure 5A). Due to the simplicity of PBDE technical mixtures (Sjödin et al., 1998), a low number of PBDE congeners is expected to be determined in human adipose tissue (Figure 6.4C). Moreover, a lower number of co-elutions for PBDE congeners occur, making their adequate identification possible on a single column. However, for correct a PCB identification and determination, the use of two columns of different polarity was suggested (Frame, 1997).

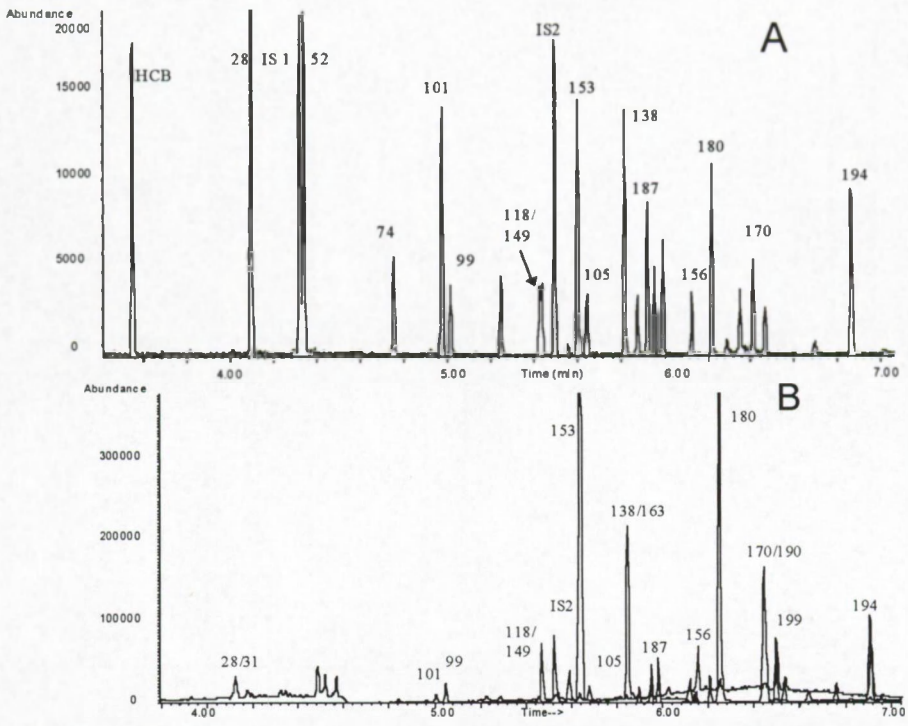

Figure 6.5. Selected ion chromatograms (EI-MS) of a PCB standard mixture (A) and human adipose tissue extract (B) analysed on a 10 m x 0.10 mm i.d. AT-5 capillary column.

Cold splitless injection has been shown to be a viable technique for the determination of PCBs by narrow bore GC/MS (Mol et al., 1995). Relatively high volumes (1 µl) can be injected by cold splitless and there is no solute discrimination or peak distortion. Using iso-octane as solvent, the optimum conditions for PCB determination were splitless times of 1 min and temperatures of 90°C and 100°C for the oven and injector, respectively. No substantial loss of resolution was observed for the elution of PCB congeners on 10 m and 50 m columns (see pairs PCB 101/PCB 99 and PCB 153/PCB 105), while run times were reduced from 50 min to 7 min, corresponding to a reduction of more than 85% of the analysis time (Figures 6.5A and 6.6A).

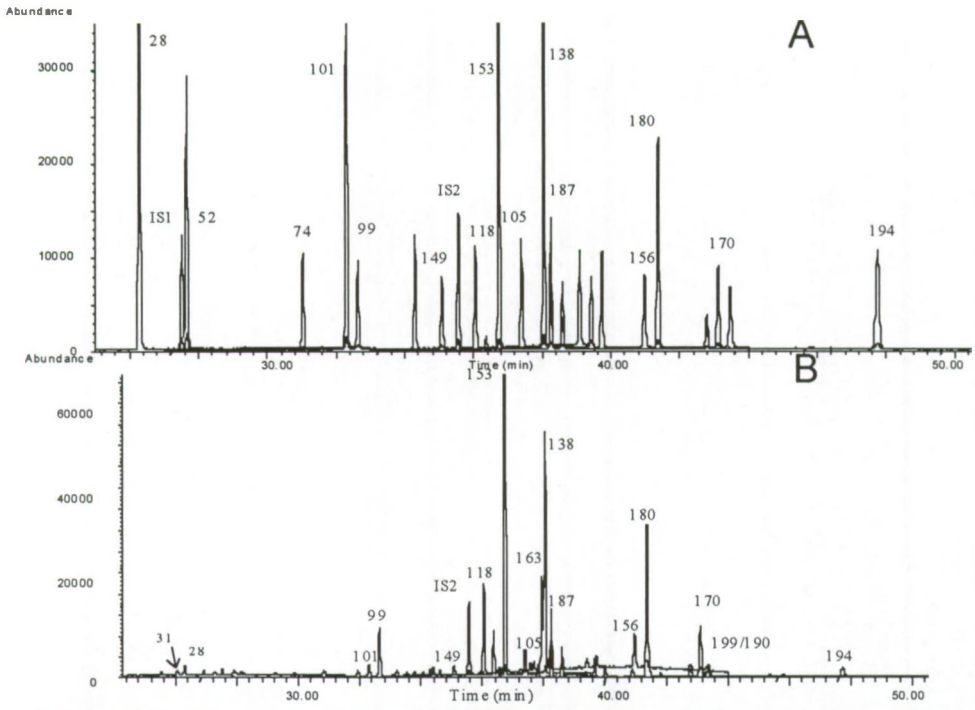

Figure 6.6. Selected ion chromatograms (EI-MS) of a PCB standard mixture (A) and human adipose tissue extract (B) analysed on a 50 m x 0.22 mm i.d. HT-8 capillary column.

Electron impact low resolution mass spectrometry

LRMS is cheaper, easier to maintain and operate, but less sensitive (1 to 2 orders of magnitude) than HRMS. EI allows for the use of ^{13}C-labeled compounds as internal standards, while this procedure is not possible for NCI-LRMS. With the later technique, inadequate internal standards are used due to the existence of a limited number of synthetized PBDE congeners. Moreover, the higher selectivity of EI is important compared to ECNI where often only Br ions (m/z 79 and 81) can be measured. The NCI spectra of organohalogen compounds are strongly dependent on the experimental conditions (such as the type of reagent gas, reagent gas pressure, ion source temperature and the instrument used (Sellström, 1999).

Using classical injection techniques (hot splitless and 1-2 µl injection volume), highly sensitive detectors, such as NCI-LRMS or EI-HRMS, were employed for the determination of PBDEs in environmental and human matrices (de Boer et al., 2000). Moreover, it was also possible to use EI-LRMS for samples with relatively high concentrations (Lindström et al., 1999) of PBDEs (fish, marine mammals). However, for human tissues, the sensitivity offered by this system is not sufficient. As shown above, the use of an alternative injection technique (such as LVI) into narrow bore capillaries could improve drastically the limits of detection (Table 6.1). In this case, it becomes possible to apply EI-LRMS with sufficient accuracy to determine of PBDEs in human samples with relatively high concentrations.

The developed method has the following important advantages: (1) PBDEs (tri to hexa-congeners) present at background levels in human adipose tissue can unequivocally be identified and quantified in the selected ion monitoring mode using the EI mode. (2) An

important increase in sensitivity is achieved by using a large volume injection technique, competing now with the sensitivity achieved by NCI-LRMS. (3) Analysis times are strongly reduced due to the use of a narrow bore capillary column.

However, the present method is limited to the analysis of PBDE congeners with less than 6 bromine atoms. The sensitivity is considerably less for hepta- to deca- congeners, which makes the determination of low concentrations of these congeners very difficult. It is, however, unlikely that higher brominated PBDE congeners will become important with regard to human exposure, as the octa-BDE mixture production is relatively small and deca-BDE does not or hardly bioaccumulates in fish and humans (Strandman et al., 1999). Hepta- to nona-BDEs have not been reported in significant concentrations in environmental and human samples until now.

6.2. Levels of organohalogenated contaminants in Belgian human adipose

* - based on Covaci A, de Boer J, Ryan JJ, Voorspoels S, Schepens P, (2001). *Environ Res,* in press.

Samples
Twenty human adipose tissue samples from abdominal fat region were obtained in 2000 by autopsy at the University Hospital of Antwerp, Belgium from deceased individuals who died from causes unrelated to environmental contaminants as far as could be judged. The mean age of the subjects was 47.2 years, ranging from 19 to 77 years (Table 6.1). They were 9 females and 11 males. No information about possible occupational exposure or diet was available. Samples were collected in hexane-washed polyethylene recipients, frozen immediately and stored at –20°C until analysis. Percentages of extracted lipids in the samples varied between 88.3 and 99.8 % (Table 1).

Concentrations of PBDEs in Belgian human adipose tissue
Due to the simplicity of PBDE technical mixtures (Sjödin et al., 1998), a low number of PBDE congeners is expected to be present in human adipose tissue. Moreover, a lower number of co-elutions for PBDE congeners occur, enabling their adequate identification on a single column. Thus, PBDE measurements were only carried out on the AT-5 capillary column allowing a reduction in analysis time.
Concentrations of PBDE congeners no. 66, 71, 75, 77, 85, 119, 138 and 154 were below the limit of detection in all samples, while BDE 47 and BDE 153 were the predominant PBDE congeners analyzed in the tissues. Concentrations of PBDEs (sum of congeners 28, 47, 99, 100, 153) in Belgian adipose tissue ranged between 2.18 and 11.70 ng/g lipid weight. The ratio of BDE 47 to the sum of PBDEs varied between 0.16 and 0.40, with a mean of 0.29.

Concentrations of PBDEs in 20 samples from Belgian population were at the lower end of PBDE concentrations reported in other countries (Table 6.4). Human adipose tissue samples from Finland (Strandman et al., 1999) contained more than 2 times higher concentrations of PBDEs than the Belgian group. Similar trends were observed in Swedish subjects (Meironyté et al., 2001a). In a Swedish survey, PBDEs (mostly tetra congeners) were measured in more than 400 individuals (Lindström, personal communication). Concentrations of BDE 47 between 0.5 and 4 ng/g lipid weight were measured for most specimens, with few persons

having levels up to 100 ng/g lipid weight, indicating a large variation in the individual values. Adipose tissue samples from the USA (She et al., 2000) contained almost 5 fold higher PBDE concentrations than the Belgian group. The difference in concentrations between countries can be partly explained by the concentrations of PBDEs in the diet and especially in food items with high contribution to the total PBDE intake (fish and meat products).

However, the PBDE concentrations between countries do not vary much, suggesting a widespread pollution.

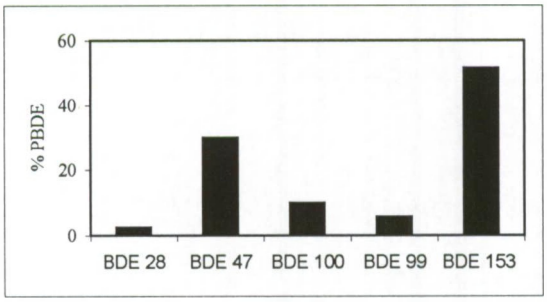

Figure 6.7. Distribution of PBDE congeners in Belgian adipose tissue.

Interestingly, BDE 47 was not always the most abundant congener in the Belgian adipose tissue (Figure 6.7). High values of BDE 153 were obtained in some samples. Blanks were run to check for interferences, but no significant contribution to these high values was observed. High concentrations of BDE 153 were also seen in some samples from Spain (Meneses et al., 1999). However, a different pattern was observed in Finland (Strandman et al., 1999), Sweden (Meironyté et al., 2001a) and USA (She et al., 2000). In those samples, BDE 47 was the major PBDE congener, accounting for 60-70% of the total PBDE content. The difference in profiles of PBDEs observed in human adipose tissue from different countries might be due to a specific diet containing food items with have a preferential accumulation of the higher brominated congeners. Until now, only one study has indicated the presence of higher brominated congeners (hepta- to deca-BDE in adipose tissue (Stanley et al, 1991). Concentrations of higher brominated PBDE congeners ranged between not detected and 6 ng/g lipid weight. However, these data should be interpreted with caution due to the lack of standards of individual PBDE congeners. Deca-BDE has been identified in the blood of occupationally exposed persons, such as electronics dismantlers and computer technicians (Sjödin et al., 1999).

Concentrations of PBDEs in adipose tissue were slightly lower than PBDE concentrations in liver of five Swedish individuals (Meironyté et al., 2001a), indicating a possible specific accumulation of BDE 99 in liver. Furthermore, concentrations and profiles of PBDE congeners in adipose tissue differed from that in human milk (Meironyté et al, 1999; Ryan and Patry, 2000) and in blood from non-occupationally exposed persons (Klasson-Wehler et al., 1997; Sjödin et al., 1999).

Table 6.4. Concentrations (ng/g lipid weight) of PBDE congeners in adipose tissue from female (F) and male (M) subjects.

Sample	Sex	Age (yr)	Weight (kg)	Extractable lipids	BDE 28	BDE 47	BDE 99	BDE 100	BDE 153	Sum PBDE	BDE47/Sum PBDE
1	M	60	72	90.0	nd	1.71	0.16	0.43	2.77	5.07	0.34
2	F	34	100	88.6	nd	0.68	nd	0.27	1.46	2.41	0.28
3	M	19	60	96.3	0.11	1.34	0.24	0.34	1.42	3.45	0.39
4	M	24	70	88.7	nd	0.61	nd	0.26	2.68	3.55	0.17
5	F	30	60	99.6	0.06	1.13	0.28	0.23	1.62	3.32	0.34
6	M	40	68	91.2	0.10	0.95	nd	0.17	2.11	3.33	0.29
7	F	60	65	97.4	nd	0.54	nd	0.18	1.51	2.23	0.24
8	F	40	60	93.5	nd	2.35	0.30	0.99	3.46	7.10	0.33
9	M	54	80	99.1	0.07	1.12	0.15	0.30	4.72	6.36	0.18
10	M	35	70	91.0	nd	1.37	0.27	0.36	1.57	3.57	0.38
11	M	59	70	91.1	nd	1.03	0.17	0.47	2.85	4.52	0.23
12	F	39	55	94.6	0.09	0.95	0.15	0.25	2.48	3.92	0.24
13	F	61	85	91.9	0.26	2.86	0.29	1.50	3.14	8.05	0.36
14	M	28	65	99.6	nd	0.90	0.18	0.60	3.82	5.50	0.16
15	M	77	100	89.9	nd	1.70	0.25	0.68	1.60	4.23	0.40
16	F	65	85	99.8	nd	2.56	1.01	0.76	2.22	6.55	0.39
17	F	61	65	90.4	nd	0.87	0.15	0.32	1.43	2.77	0.31
18	M	75	95	96.6	nd	0.71	nd	0.32	2.02	3.05	0.23
19	M	45	80	97.0	nd	0.82	0.18	0.40	2.47	3.87	0.21
20	F	37	80	88.3	0.14	4.71	1.61	0.88	4.36	11.70	0.40
mean		**47.2**	**74.3**	**93.7**	**0.05**	**1.45**	**0.28**	**0.48**	**2.49**	**4.75**	**0.29**
SD		17.0	13.4	4.1	0.06	1.01	0.38	0.34	1.00	2.30	0.08

nd - not detected

Table 6.5. Mean concentrations of PBDE congeners (in ng/g lipid weight) in human adipose tissue samples form different countries

	Belgium	Spain	Finland	USA	Israel	Sweden	Sweden	Sweden		
Reference	This study	(7)	(9)	(40)	(30)	(31)	(10)	(32)		
Year of sampling	2000	1998	na	1998	1990	1994	1994	1995-1997		
Population	general	general	general	general	exposed	general	general	NHL	Melanoma	No-cancer
No. samples	20	13	10	5	1	1	5	19	23	27
Age range (yrs)	19-77	28-83	36-84	na	21	74	47-83	42-77	28-85	38-79
BDE 28	0.05 ± 0.06	nr	nr	nr	nr	nr	0.08 ± 0.04	nr	nr	nr
BDE 47	1.45 ± 1.01	1.36 ± 1.46	7.28 ± 4.85	17.8 ± 8.6	2.79	8.8	2.48 ± 0.89	13.0	4.8	5.1
BDE 99	0.28 ± 0.38	0.42 ± 0.53	2.07 ± 1.52	4.9 ± 1.9	5.59	1.1	0.32 ± 0.17	nr	nr	nr
BDE 100	0.48 ± 0.34	0.51 ± 0.33	nr	nr	1.40	1.8	1.34 ± 0.37	nr	nr	nr
BDE 153	2.49 ± 1.00	1.83 ± 1.05	2.31 ± 0.92	2.2 ± 0.7	nr	1.7	1.02 ± 0.36	nr	nr	nr
Sum PBDE	4.75 ± 2.30	4.09 ± 2.71	11.68 ± 5.87	24.9 ± 10.8	9.78	13.4	5.16 ± 1.42	-	-	-
BDE 47/sum PBDEs	0.29 ± 0.08	0.30 ± 0.11	0.61 ± 0.12	0.70 ± 0.06	0.29	0.66	0.48 ± 0.06	-	-	-

na-not available, nd-not detected, nr-not recorded

In blood and milk, BDE 47 was the predominant PBDE congener and constituted 70% of the total sum of analysed PBDEs in milk from 1994, whereas in adipose tissue, BDE 47 constituted only 16-40% of PBDE content. Furthermore, the proportion of BDE 153 in adipose tissue and liver (20%) was higher than in human milk (<5%). The ratio BDE 47/sum BDEs in humans is lower than in other species (Law et al., 2001; Zegers et al., 2001), such as fish and marine mammals showing that BDE 47 accumulated stronger in the wildlife species and that pharmacokinetics including half-lives for elimination are not the same for the different species.

Levels of organochlorinated compounds in human adipose

For accurate PCB identification and quantification, the use of two columns different in polarity was recommended (Frame, 1997). The lowest value for each congener measured on the two capillary columns was used for further calculations (Table 3). Several pairs of congeners with the same degree of chlorination (such as 28/31, 138/163, 128/167, 170/190) were found to co-elute on the AT-5 column. All these pairs were separated on the HT-8 column. It was shown that interfering congeners could have a high contribution to the value assigned on the AT-5 column. Thus, PCB 31 represented up to 30% of PCB 28, PCB 163 was up to 33% of PCB 138, while PCB 190 represented up to 25% of PCB 170. Concentrations of PCB congeners with little interferences (such as PCB 118, 153, 180) were identical on both columns (RSD < 10%).

PCB congeners no. 28, 31, 52, 95, 101, 128, 189, 206 and 209 were detected only in some adipose tissue samples. The total PCB concentration was the sum of all PCB congeners measured on each capillary column (Table 6.6). The median value for the sum of PCBs (35 congeners) was 841 ng/g lipid weight and ranged from 286 to 1802 ng/g lipid weight. The sum of 7 ICES marker PCBs (IUPAC no. 28, 52, 101, 118, 138, 153 and 180) was 504 ng/g lipid weight (60 % from the sum of 35 congeners). The median concentration of HCB was 46 ng/g lipid weight and ranged from 13 to 85 ng/g lipid weight. Sum of DDTs was calculated from p,p'-DDE and p,p'-DDT, and was measured only on the AT-5 column. The median value was 290 and ranged from 47 to 2802 ng/g lipid weight (Table 6.6).

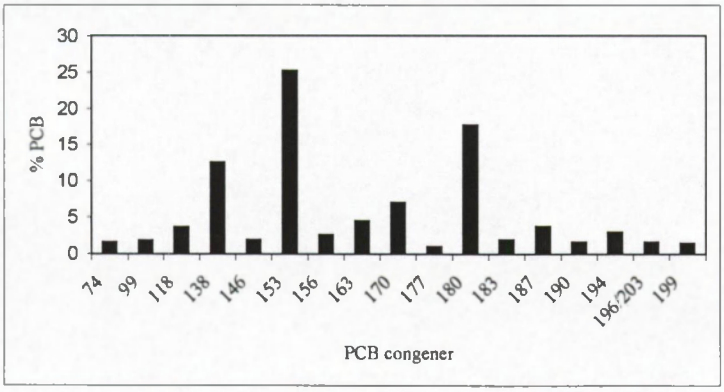

Figure 6.8. Distribution of PCBs in Belgian human adipose tissue (only congeners contributing with more than 1% to the total PCB concentration were represented).

Table 6.6. Concentrations of organochlorine contaminants (ng/g lipid weight) in Belgian human adipose tissue (n=20).

Compound	Median	Range
	ng/g lipid weight	
PCB 28	1.3	nd – 11.8
PCB 31	0.4	nd – 0.9
PCB 52	0.3	nd – 2.2
PCB 74	14.1	4.1 – 42.4
PCB 95	0.6	0.3 – 3.0
PCB 99	15.8	3.8 – 60.5
PCB 101	0.8	nd – 8.8
PCB 105	4.9	1.0 – 19.9
PCB 110	1.0	0.7 – 2.6
PCB 118	31.1	7.9 – 92.6
PCB 128	3.4	nd – 8.3
PCB 138	105.1	29.0 – 227.5
PCB 146	16.7	5.8 – 35.0
PCB 149	1.6	0.8 – 8.0
PCB 153	211.1	67.8 – 399.2
PCB 156	22.4	6.1 – 46.1
PCB 163	38.2	12.0 – 81.6
PCB 167	5.2	0.9 – 14.5
PCB 170	59.2	20.1 – 132.5
PCB 171	5.9	1.6 – 12.6
PCB 172	6.1	2.1 – 14.0
PCB 177	8.9	3.1 – 23.9
PCB 178	7.2	2.6 – 14.0
PCB 180	148.3	50.1 – 342.6
PCB 183	16.6	4.3 – 41.1
PCB 187	31.6	8.5 – 82.4
PCB 189	3.0	nd – 6.4
PCB 190	14.2	5.9 – 29.1
PCB 194	25.7	7.4 – 52.8
PCB 195	4.0	1.4 – 9.9
PCB 196/203	14.3	4.1 – 37.2
PCB 199	13.0	4.2 – 31.8
PCB 206	3.5	nd – 9.7
PCB 209	3.7	nd – 10.7
Sum PCBs	**841**	**286 - 1802**
Sum 7 markers*	**504**	**164 - 1028**
HCB	46	13 – 85
p,p'-DDT	12	7 – 250
p,p'-DDE	280	38 – 2553
Sum DDTs (p,p'-DDE+p,p'-DDT)	**290**	**47 - 2802**

* - IUPAC n° 28, 52, 101, 118, 138, 153, 180; nd - not detected

The median concentration of the 7 ICES indicator PCBs (504 ng/g lipid weight) in the 20 adipose tissue (mean age 47.2 years) was higher than the median concentration (334 ng/g

lipid weight) of 46 samples from young Belgian females with a mean age 31.9 years (Pauwels et al., 2000). This confirms the increase of PCB body burden with age.

PCB 153 and 180 were the major PCB congeners in human adipose tissue. PCB profiles and concentrations in the Belgian samples were similar with profiles observed in other countries such as Italy (Mariottini et al., 2000) and United Kingdom (Duarte-Davidson et al., 1994), showing that the PCB pollution profile in Western European countries is similar. However, PCB concentrations in the Belgian samples were 10 times higher than concentrations found in areas with lower PCB pollution, such as Chile (mean 53 ng/g lipid weight) (Mariottini et al., 2000). Concentrations of p,p'-DDE measured in the Belgian population (290 ng/ g lipid weight) were similar with concentrations found in Swedish subjects (Meironyté et al., 2001a), but much lower than concentrations found in Mexican adipose samples (mean 4 360 ng/g lipid weight) (Waliszewski et al., 2001). Differences in concentrations for PCBs and DDTs between different populations are probably due to the difference in contaminant's load of the food items or to the possible present use in some countries (e.g. DDT in Mexico for malaria control).

Correlations between different groups

High Pearson correlation coefficients were found between BDE 47 and sum PBDEs ($r=0.95$, $p<0.05$) and PCB 153 and sum PCBs ($r=0.98$, $p<0.05$) (Table 6.7). PCBs showed good correlation with the sum DDTs ($r=0.77$, $p<0.05$), while the correlation with PBDEs was weaker ($r=0.56$, $p<0.05$). No age-dependency was found for PBDEs ($r=0.09$), while PCBs and DDTs showed higher correlation coefficients with age ($r=0.59$ and 0.40, respectively).

No significant difference was found between PCB and PBDE concentrations in men and women. Concentrations of PBDEs in human adipose tissue were higher than levels observed in human milk. Similarly to PCBs, it was suggested that the accumulation of PBDEs increases by age, but no clear evidence for this has been reported. In this study, no age-dependency was found for PBDEs ($r=0.09$), while PCBs and DDTs showed higher correlation coefficients with age ($r=0.59$ and 0.40, respectively, $p<0.05$). Meneses (1999) has observed in 13 samples, that the highest as well as the lowest levels of PBDEs correspond to elder men, while for other persistent organohalogenated contaminants (PCBs, DDTs) older persons were found to have higher levels due to bioaccumulation and long half-lives of the compounds. Moreover, in the largest study concerning PBDE levels in human adipose tissue, Hardell (1998) did not find an age-dependency for 77 patients with different categories of cancer. Sjödin (2000) presented data indicating that the concentration of PBDE in blood from Swedish and Latvian men were not related to the age of the subjects. The factors that determine concentrations of PBDEs in humans are most probably different than those for PCBs and the other organochlorines.

Table 6.7. Pearson correlation coefficients between different groups of POPs.

	BDE 47	Sum PBDEs	PCB 153	Sum PCBs	Sum DDTs	age
BDE 47	-	0.95 *	0.44*	0.42*	0.28	0.07
Sum PBDEs		-	0.57*	0.56*	0.31	0.09
PCB 153			-	0.98*	0.81*	0.56*
Sum PCBs				-	0.77*	0.59*
Sum DDTs					-	0.40*
age						-

*- $p<0.05$

Low Pearson correlation coefficients were obtained between PBDEs and PCBs ($r=0.56$, $p<0.05$) or DDTs ($r=0.31$, $p<0.05$) (Table 6.7). Humans are probably exposed to PBDEs through similar routes as many neutral lipophilic organohalogen compounds such as PCBs and DDTs, with food as the major source. However, the intake of contaminants is dependent of the contribution of each food item to the total diet for a specific country. Thus, in Sweden (Darnerud et al., 2001) and Finland (Strandman et al., 2001), it was shown that fish (freshwater and marine) is the principal source of BDEs (up to 70%) in the human diet, while in Canada (Ryan and Patry, 2001), meat products were reported to be the main contributor (up to 70%). Inhalation of particulate-bound PBDEs (especially of higher brominated congeners) in certain occupational settings may also contribute to human exposure. The dietary habits of subjects in the present study are not known, thus no assumptions can be made for the Belgian group.

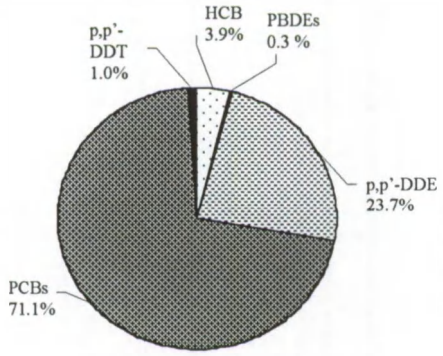

Figure. 6.9. The average distribution percentage of the concentrations of PCBs, p,p'-DDE, p,p'-DDT, HCB, and PBDEs in adipose tissue calculated from twenty subjects.

The distribution percentage of the analysed classes of halogenated compounds in adipose tissue is illustrated in Figure 6.9. PCBs were the main contaminants, followed by p,p'-DDE and HCB. It can be observed that PBDEs constituted only a small fraction (0.3%) of the total contaminant load. Exactly the same proportions for all contaminants were found in a Swedish study (Meironyté et al., 2001a), which included also polychloronaphthalenes (PCNs). Although the increasing concern on PBDEs is justified because of the limited knowledge on effects and possible increasing levels, Figure 6.7 shows that for the time being, the levels are rather low compared with PCBs and DDTs.

Conclusions. The GC/EI-LRMS method in combination with LVI and narrow bore capillary column is suitable for the accurate and rapid identification and quantification of major PBDE congeners in human adipose tissue and may serve as an alternative for GC/NCI-MS and GC/HRMS. As a pre-condition, an efficient clean-up is necessary to allow the introduction of volumes up to 20 µL into the GC system. A remaining problem is the insufficient sensitivity for the determination of higher brominated congeners (hepta- to deca-PBDEs).
The results show that PBDEs are now present in the adipose tissue of the general population in Belgium at the low ppb level. The contamination is lower compared with other Western countries. The correlation between age and concentrations of PBDEs is tenuous and does not follow the trend usually seen for persistent organochlorine compounds. Future work should

further elaborate their age/concentration ratio and focus on the determination of higher brominated congeners for the occupational risk assessment.

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Part 2

Persistent Organohalogenated Pollutants in the Environment

Chapter 7

Determination of POPs in animal feed and meat

Abstract

In January 1999, 500 tons of feed contaminated with approximately 50 kg of polychlorinated biphenyls (PCBs) and 1 g of dioxins was distributed to animal farms in Belgium. This was the start of the PCB/dioxin crisis. More than 20,000 samples of animal feed, cattle, pork, poultry, eggs, milk and various fat containing food items were collected in the database of the Belgian Federal Ministries and analysed for their PCB and/or dioxin content. Dioxin measurements show a clear predominance of polychlorinated dibenzofuran (PCDF) over polychlorinated dibenzodioxin (PCDD) congeners, a dioxin/PCB ratio of approximately 1:50,000 and a PCB fingerprint resembling that of an Aroclor mixture. The contamination was due to transformer oil and not to waste mineral oil or environmental sources, an hypothesis tested by the analysis of mineral oil hydrocarbons content in samples highly contaminated with PCBs. In these conditions, PCBs contribute significantly more to toxic equivalents (TEQ) than dioxins. Since food items differed widely (more than 50-fold) as to the ratio of PCBs to dioxins, other significant sources of contamination and a substantial background contamination are likely to contribute to the exposure of the Belgian population.

The analysis of contaminated samples showed that the patterns for PCB and PCDD/F congeners differed among feed, chicken and pork fat. Lower chlorinated PCBs and PCDFs including those with high TEFs (PCBs 105, 118, 126 and 2,3,4,7,8-PeCDF) were shown to either bioaccumulate more in chicken or to be eliminated more readily in pork. This leads to the possibility that consumption of contaminated chicken would result in a higher TEQ human body burden than consumption of pork. In addition, PCDF congeners with non-2,3,7,8-substitution (e.g. 1,2,4,7,8-PeCDF) were present in chicken fat, but absent in pork fat. Since the residue pattern changes less in avian species, these results reinforce the value of birds rather than mammals as markers of the source of contamination with persistent organochlorine pollutants.

In another study, we found that 12.1% of Belgian export meat and fat samples from chicken or pork, unrelated to the PCB/dioxin crisis, contain more than 50 ng PCBs/g fat and that 6.5% of samples contain more than 20 ng/g fat for the sum of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (p,p'-DDT) and its main metabolite, 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,p'-DDE). Part of this background contamination stems from imported animal feed ingredients (fish flour and grains), sometimes contaminated by recent use of DDT as can be deduced from the ratio between p,p'-DDT and p,p'-DDE. However, after comparison of PCB levels in fish flour and grains with those in meat, it can be suggested that high contamination levels stem from recycled fat.

Lately, animal fat samples from two major towns of Romania (Arad and Iassy) were analysed for their organochlorine load. Animal fat samples from Iassy showed high concentrations of organochlorine pesticides (HCHs concentrations up to 12,370 ng/g fat), but similar PCB concentrations when compared with Arad samples (< 50 ng/g fat). Two samples (out of 24) exceeded the EU norms (1000 ng/g fat) for HCHs and DDTs in animal fat.

7.1. Method for determination of POPs in feed, animal fat and meat

Introduction

There is an enormous amount of literature on PCB and organochlorine pesticides determination in food (Jones, 1988; Sannino et al., 1996; Krokos et al., 1997; Wells and de Boer, 1998). However, for a crisis situation, a suitable method has to meet the following criteria:

a. be simple with a limited number of manipulations (one stage extraction and one stage clean-up)
b. be rapid (clean-up on small, disposable SPE cartridges)
c. allow parallel sample processing
d. minimise sample cross contamination
e. be accurate for samples with high PCB concentrations (see Chapter 7.2), as well as for low PCB concentrations in background contamination assessment (see Chapter 7.4)
f. be reliable (it should maintain its operational parameters in time)
g. be easily applicable to various food items with different fat content.

Materials

All solvents (n-hexane, acetone and dichloromethane) were of pesticide grade purity (Merck, Germany). Chromatographic adsorbents (Merck) were washed with hexane and activated overnight. PCB standard solutions (10 ng/µl in iso-octane) were purchased from Dr. Ehrenstorfer Laboratories (Augsburg, Germany). All glassware was cleaned as required for this type of analysis.

Sample preparation

Animal fat

A homogenised portion (2-3 g) of animal fat was melted at 50°C for chicken fat and 80°C for pork fat, respectively. From this portion, a sample of 0.5 g was weighted accurately and dissolved in 5 ml hexane. Internal standards, ε-HCH, PCB 46 and 143 were added in order to give a concentration of 10 ng/g fat. The mixture was equilibrated in an ultrasonic bath for 5 min and was loaded on top of a polyethylene cartridge (2.5 cm diameter) filled with 5 g acid silica (silica:sulphuric acid=1:1, *w/w*) and 0.5 g sodium sulphate. The eluate was collected and the cartridge was washed with 20 ml hexane and 10 ml dichloromethane:hexane=1:1, *v/v* (for recuperation of polar pesticides). The eluate was concentrated to approximately 200 µl with a rotary evaporator and nitrogen stream. Twenty-five µl of recovery standard, 1,2,3,4-tetrachloronaphthalene (TCN) was added to calculate the recovery of the internal standard. After mixing, the final concentrate was introduced into a vial and one µl was injected into GC-ECD or GC/MS instrument.

Animal feed

To 0.5 g feed, 5 ml n-hexane was added together with 20 ng internal standards (PCB 46, 143 and ε-HCH). After ultrasonication for 20 min and fat content determination, the extract was subjected to the same clean-up procedure as described above.

Animal meat and other food commodities with < 20 % fat

Approximately 10 g of meat or other food commodities were accurately weighted and mixed with 20 g anhydrous sodium sulphate until a fine floating powder was obtained. To the powder, 20 ng of internal standards PCB 46, PCB 143 and 10 ng of internal standard ε-HCH were added and the mixture was extracted with 60 ml hexane:dichloromethane

:acetone=3:1:1 for 30 min in an ultrasonic bath. After concentration and fat content determination, the extract was applied to a hexane pre-washed SPE cartridge filled with 5 g acid silica. Thirty ml of hexane were used for complete elution of the organochlorine contaminants. The final eluate was concentrated under nitrogen and TCN was added prior to GC analysis.

Fat content determination

The extract was concentrated and then transferred to a pre-weighted tube. The extract was completely dried under a gentle nitrogen stream and then kept at 60°C till constant mass (usually 10 min). The difference between the weight of the tube with extract and an empty tube represented the fat content of the sample. No losses of PCBs were observed when CRM 350 (PCBs in mackerel oil) was kept for 15 min at 80°C.

Analysis

A Hewlett Packard HP 6890 GC with μ-ECD was equipped with a HT-8 (SGE, Australia) capillary column (30 m x 0.22 mm x 0.25 μm). Injector and detector temperatures were 270°C and 320°C, respectively. Helium was used as carrier gas at a flow rate of 1 ml/min. Argon:CH_4 was used as make-up gas at a flow rate of 40 ml/min. One μl was injected in the pulsed splitless mode (pressure pulse of 25 psi for 1.5 min) with the split outlet opened after 1.5 min. The temperature program started from 90°C, kept for 1 min and then the temperature increased with 15°C/min to 180°C, kept for 1 min, further by 3°C/min to 250°C and further by 15°C/min to 290°C, kept for 6 min.

A HP 6890 GC was connected to a HP 5793 mass spectrometer (MS) and equipped with a DB-5ms (J&W Scientific, Folsom, USA) capillary column (30 m x 0.25 mm x 0.25 μm). Injector and transfer line temperatures were 265°C and 280°C, respectively. Helium was used as carrier gas at a flow rate of 1 ml/min. One μl was manually injected in pulsed splitless mode (pressure pulse of 20 psi for 1 min) with the split outlet opened after 1.25 min. The temperature program started at 90°C, kept for 1 min and gradually increasing the temperature by 15°C/min up to 275°C, which was maintained for 10 min.

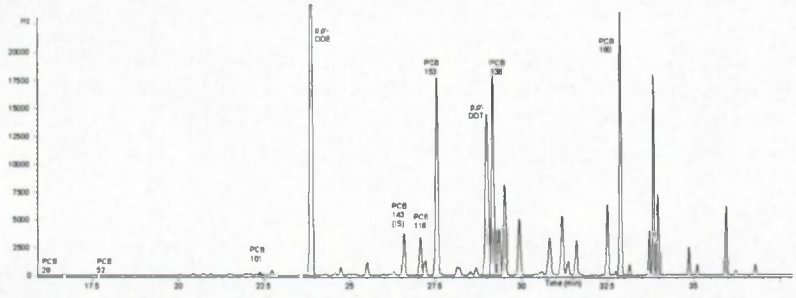

Figure 7.1. Typical GC-ECD chromatogram of contaminated fat sample (PCB concentration > 500 ng/ g fat and DDTs > 1000 ng/g fat).

Quality control

The above mentioned method was used for analysis of 2500 animal fat, meat and feed samples during the PCB crisis. For internal quality control, peak identification was based on relative retention time (to TCN) and for MS on characteristic ions. Because of higher detection limits using MS, HCH isomers were quantified exclusively with ECD. Recoveries of internals standards were 72 ± 8 % for PCB 46, 78 ± 10% for PCB 143 and 82 ± 11% for ε-

HCH, respectively. Multi-level calibration curves ($r^2 > 0.99$) were created for the quantification using the above analytical conditions. Limits of detection were close to 0.5 ng/g fat for the target analytes. The procedure was validated through regular analysis of blanks, fortified samples, certified material CRM 349, 350 (PCBs and organochlorine pesticides in cod liver and mackerel oil) (Table 7.1). Within laboratory Quality control, one blind duplicate was analysed to check repeatability/reproducibility.

Table 7.1. Concentration of PCBs and organochlorine pesticides (all results are expressed in ng/g fat) measured in two CRM fish oils.

Compound	Type[a]	CRM 349		CRM 350	
		UIA	Reference	UIA	Reference
PCB 28	C	49	68 ± 7	6[b]	22.5 ± 4
PCB 52	C	145	149 ± 20	51	62 ± 9
PCB 101	C	351	370 ± 17	148	165 ± 9
PCB 118	C	468	456 ± 31	131	143 ± 20
PCB 153	C	979	938 ± 40	299	318 ± 20
PCB 180	C	288	282 ± 22	59	73 ± 13
PCB 141	I	54	68 ± 5	22	28 ± 4
PCB 138[c]	I	629	765 ± 45	186	274 ± 27
PCB 163[d]		205		93	
PCB 170	I	132	149 ± 15	23	35 ± 2
PCB 187	I	241	277 ± 15	74	95 ± 10
HCB	I	29	40 ± 22	10	20 ± 6
α-HCH	I	26	34 ± 14	25	22 ± 3
γ-HCH	I	65	72 ± 12	52	44 ± 14
p,p'-DDT	I	39	58 ± 11	65	71 ± 29
p,p'-DDE	I	223	237 ± 31	84	89 ± 13

a - C-certified, I-indicative; b - interferences
c - PCB 138+PCB 163 (for the reference), only PCB 138 (for our results)
d - separation of PCB 138 from PCB 163 is possible on HT-8 column

In November 1999, the Toxicological Centre (University of Antwerp) has successfully participated in an intercomparison study of PCBs in feed, animal fat and food organised by the Federal Ministries of Public Health and Agriculture, Belgium. All 22 laboratories involved in the analysis of PCB contaminated feed and meat, participated in this study. Two identical samples (A1 and A2) of highly contaminated feed, a contaminated and non-contaminated egg-yolk (A3 and A4), a contaminated pork fat (A5) and two identical samples of slightly contaminated lyophilised milk (A6 and A7) were prepared and sent to the participating laboratories. Samples A1, A2, A6 and A7 had to be analysed in duplicate. Each laboratory had the opportunity to use its own experienced analytical method.

Results of the participation in the intercomparison study are presented in Table 7.2. A relatively good agreement between results of the Toxicological Centre and the mean value of the participating labs (after exclusion of extreme values) was observed for animal feed, eggs and animal fat. Higher variance was seen for powder milk, due to low concentrations and to relative dispersed results obtained by the participating laboratories. Overall, the results were considered satisfactory as the laboratories were using different sample preparation methodology and most of them were not prepared for high sample throughput.

Table 7.2. Concentration of individual PCB congeners and sum of PCBs in animal feed, lyophilised eggs, pork fat and powder milk used for the intercomparison study of PCBs in feed and food organised by the Belgian Federal Ministries (November 1999).

Compound	A1		A2		A3		A4		A5		A6		A7	
	UIA	Mean	UIA	Mean	UIA	Mean	UIA	Mean	UIA	Mean	UIA	Mean	UIA	Mean
Sample	Animal feed		Animal feed		Lyophilised egg		Lyophilised egg		Pork fat		Powder milk		Powder milk	
N labs		21		21		20		20		22		14		13
Unit	µg/g fat		µg/g fat		ng/g fat		ng/g fat		ng/g fat		ng/g fat		ng/g fat	
PCB 28	0.3	0.3	0.3	0.3	6	7	5	6	2	4	2	2	2	2
PCB 52	7.6	6.5	7.8	6.6	3	7	2	5	2	4	2	5	2	3
PCB 101	16.9	15.5	17.1	15.6	9	11	2	6	3	6	2	13	2	5
PCB 118	9.5	10.1	9.4	10.0	80	125	11	10	12	15	2	7	2	3
PCB 138	24.2	21.6	24.7	21.8	275	298	31	28	287	288	31	26	14	16
PCB 153	22.2	21.0	22.5	21.1	240	260	27	25	243	266	9	16	11	15
PCB 180	14.8	13.2	14.9	13.3	140	155	13	12	129	161	9	8	3	5
Sum PCBs	95.5	88.1	96.7	88.6	793	863	91	92	678	734	57	76	36	49
Difference (%)	+ 8.4		+ 9.1		- 8.1		- 1.1		- 7.6		- 25.0		- 26.5	

7.2. Belgian PCB contamination

*-based on van Larebeke N, Hens L, Schepens P, Covaci A, Baeyens J, Everaert K, Bernheim JL, Vlietinck R, De Poorter G, (2001). *Environ Health Perspect* 109, 265-273.

Brief history of the incident

In Belgium, approximately 20 companies collect animal fat from slaughterhouses and melt it into a homogenous substance, which is sold to animal-food producers. It was a common practice to include household waste fat collected at community waste recycling centers in this product. In January 1999, at the Flemish fat-melting company Verkest, 40 to 50 kg of oil from discarded transformers originating from a waste recycling centre containing polychlorinated biphenyls (PCBs) was admixed to the fat delivered to 10 animal-feed producers. Between 15 and 31 January, the resulting 500 tons of contaminated animal feed, containing approximately 60-80 tons of fat contaminated with 40-50 kg of PCBs and almost 1 g of dioxins, were distributed to poultry and pig farms and to a lesser extent also to rabbit, calf, and cow breeding and raising farms. In Belgium, 445 poultry farms, 393 cattle farms, 746 pig farms (or 25% of this type of farm in Belgium) and 237 dairy farms (representing 1,5 % of the total number of dairy farms in Belgium) used animal feed from the 10 contaminated animal-feed producers. The 500 tons of contaminated feed represent a limited percentage of the total amount of feed produced and used in Belgium, which is estimated to exceed 28,000 tons/week.

Pathologic conditions were first recorded on 4 February in Belgian chicken farms. They included a decrease in egg production and hatching and an epidemic of chicken edema disease. After excluding other causes of the epidemic, samples of animal feed, chicken carcasses and eggs were sent for toxicological analysis (RIKILT – the Netherlands) on 18 March. The authorities were informed of the presence of increased concentrations of dioxins in animal feed, chicken and eggs on 26 April. The first measures to protect public health were taken, but the public was not informed. On 27 May, the Flemish television broke the news; the incident became public and resulted in a political, communication, and economic crisis. The authorities tried to identify the extent of the crisis by identifying the companies involved in the contamination. All available laboratories in Belgium and neighboring countries were mobilized for analytical work. The analyses revealed that the dioxins were part of a PCB contamination, and average PCB/dioxin ratios were determined. During the first days of June, the Ministry of Public Health ordered removal of poultry, derived products (meat, eggs, mayonnaise, custards, cakes, etc), and all meat products with a fat content > 25% from the market. A wide-spread product sampling and analysis was organized, resulting in the data used in the present study. Only products with a PCB concentration of < 200 ng/g fat were released for human consumption. Products with excessive levels were destroyed, including some 2 million chickens. Hence, the duration of exposure of the population can be estimated as four months (February-May).

Dioxin exposure in Belgium before the incident

Some studies (Baeyens, 1993; Mira-T, 1998) showed a gradual reduction in emissions from over 600 g I-TEQ/year in 1994 to approximately 100 g I-TEQ/year in 1999. Whereas municipal solid waste incineration accounted for nearly 60% in 1993, its contribution is now limited to a low percentage (< 10%). The non-ferrous, ferrous and steel industries remain major sources, together with household heating and traffic.

Limited data on the food load of dioxins and PCBs are available. Cox (1999) reviewed the existing data and completed lacking information with Dutch measurements on dioxins in the

food basket. Cox concluded that the average total daily intake was 179 pg I-TEQ/day. These data are a rough estimate because the dioxin concentrations in food are only partially known, and because the official food basket composition as used by the government (Belgisch Staatsblad, 1990) is outdated.
Dioxins and furans in milk of lactating women were studied in 1988 and 1993 by the WHO (WHO, 1996). The dioxin concentration in the Belgian samples was higher (average value of 34 pg I-TEQ/g fat, range 27.3 - 43.2 pg I-TEQ/g fat) than in the samples of all other industrialized countries involved in the study. Levels were stationary between 1988 and 1993. This illustrates that dioxin background values in Belgium often exceed the reference values. The situation is most pronounced for babies. Assuming the 34 pg I-TEQ/g fat in mother's milk as indicative for the country as a whole, babies are fed an average of 43 pg I-TEQ/kg/day. This is substantially more than the 1-4 pg/kg/day that has been proposed by WHO. It is also 20 times higher than the average intake by an adult and indicates that during 3 months of breastfeeding, Belgian babies take in 6% of their lifetime dioxin dose. Assuming that fat amounts to 20% of body weight, 34.4 pg I-TEQ/g fat corresponds to a body burden of 6.88 ng I-TEQ/kg body weight in these young women. The global picture which emerges is that Belgians are exposed to high background concentrations of dioxins and furans.

The data presented here assess the initial sample results (June-August 1999) of analyses performed to respond to the PCB-dioxin contamination episode in Belgium during the first half of 1999. They are used to estimate the overall population exposure and the exposure which might have resulted from selected diets.

Materials and methods
Dioxin and PCB measurements in animals and food products
The data are based on the analyses ordered by the Belgian Ministries of Health and Agriculture. As shown in Table 7.3, a total of 20,491 samples from cattle, pigs, poultry, eggs, milk and various fat containing food items (ranging from mayonnaise to Belgian chocolates) were collected by different control departments of the Ministries of Public Health and Agriculture.

Table 7.3. Number of samples analysed by the Belgian authorities during June-August 1999.

Product	PCBs	Dioxins	Total
Bovine meat or fat	683	19	702
Milk/Butter	864	86	950
Poultry meat or fat	1 890	62	1 952
Eggs	718	55	773
Pig fat or pork meat	7 759	137	7 896
Other food products	3 903	44	3 947
Unspecified animal fat	3 052	31	3 083
Waste	381	2	383
Animal feed	1 040	10	1 050

The sample record form included information on the commercial origin of animal feed for meat or the dairy products, or of dairy ingredients in food. PCB measurements included seven marker congeners - namely PCBs 28, 52, 101, 118, 138, 153 and 180 - and are expressed as the sum of these congeners in ng/g fat. For PCDD/PCDF, the "dirty 17" congeners with chlorine substitution of at least the 2,3,7 and 8 positions were measured and the total dioxin

content of the sample was expressed in pg TEQ/g fat, using the WHO-TEF values (Van den Berg et al., 1998).

Quality control

Dioxins were measured using GC/HRMS, while PCBs were quantified using gas chromatographic techniques combined with electron capture or mass spectrometric detection. All, but 18 of the PCB measurements were performed by a pool of 23 accredited laboratories, while dioxin measurements were performed by 18 accredited laboratories, 5 of which accounted for 398 of all 446 measurements (Beernaert and de Poorter, 1999). Twenty-two of the laboratories which participated in PCB measurements were also included in a quality ring test of PCB measurements, organised by the Dioxin/PCB unit of the Belgian Ministries of Public Health and Agriculture (see Chapter 7.1). The results showed that for contaminated feeds, egg-yolk, and fat of pork, coefficients of variation for reproducibility ranging between 16 and 35% were found. However, for slightly contaminated lyophilised milk unacceptably high coefficients of variations were recorded.

Table 7.3 reviews the number of PCB and PCDD/F sample analyses: 71% of the samples tested for PCBs showed values below the detection threshold, which was specific for each laboratory. For dioxins, only 10% of the samples had concentrations lower than the detection limits of the individual congeners.

Results and discussion

Nature of the contamination and impact on the food chain

The contamination chain is illustrated in Figure 7.2. It shows how 40 to 50 kg of PCBs and less than 1 g of dioxins were distributed over an estimated 500 tons of animal feed and a still undefined amount of animals and derived animal food products. During the initial response to the incident, a limited number of mostly heavily contaminated samples were analysed for both dioxins and PCBs.

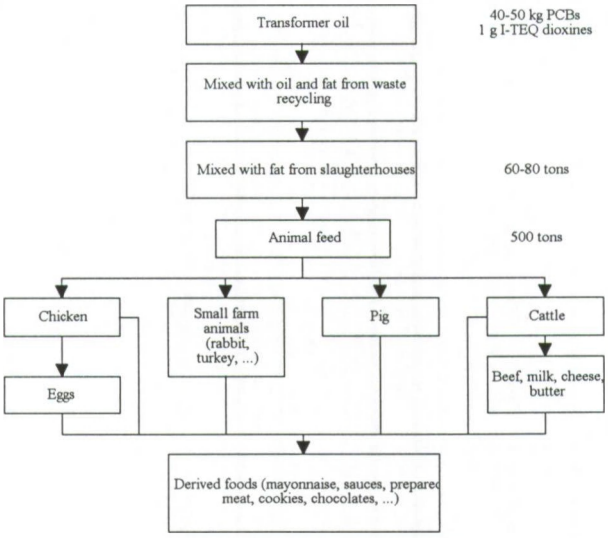

Figure 7.2. Fate of dioxin and PCBs in the food chain during the contamination incident.

Table 7.4 provides concentrations of PCBs and dioxins in the food items sampled between June and August 1999. The data show that the highest dioxin concentrations were found in poultry fat and eggs. Pork, beef and milk were also contaminated, but to a much lower extent. Of note, poultry and eggs without a track record leading to the contamination also contained high concentrations of dioxins and PCBs.

Table 7.4. Dioxins and PCBs in Belgian food and animal feed constituents. Overview of all measurements done in the period May-August 1999.

Sample	PCBs					Dioxins				
	Concentration (ng/g fat)		Max (ng/g fat)	% samples (ng/g fat)		Concentration (pg I-TEQ/g fat)		Max (pg I-TEQ /g fat)	% samples (pg/g fat)	
	AM ± SD	GM		>200	>1000	AM ± SD	GM		≥ 2	≥ 5
Beef	65 ± 65	49	1,024	1.2	0.1	3.9 ± 5.2	2.4	23.0	68.4	21.1
Cattle milk	34 ± 31	26	314	0.1	0	1.9 ± 0.8	1.8	4.3	42.9	0
Butter	38 ± 14	35	50	0	0	1.7 ± 1.3	1.4	4.0	21.7	
Poultry	241 ± 2,037	74	51,059	6.5	1.9	170 ± 488	4.2	2,613.4	41.9	30.6
Eggs	393 ± 2,883	71	46,000	8.1	2.7	32 ± 104	5.1	713.1	63.6	40
Pork	293 ± 956	80	25,472	16.5	7.1	2.6 ± 6.0	1.0	64.0	39.4	8.8
Animal fat (un-specified origin)[1]	67 ± 124	50	4,092	0.8	0.2	2.8 ± 2.9	1.3	7.6	36.4	27.3
Fat of un-specified origin	101 ± 272	53	3,900	5.0	1.1	15.9 ± 19	4.1	61.7	60	55
Waste[2]	265 ± 2,815	67	54,909	2.9	1.8	0.1 ± 0.1	0	0	0	0
Animal feed	1,659±23,585	61	519,000	6.7	1.4	2320±3852	97.7	11,163.0	70	70

AM, arithmetic mean; GM, geometric mean; Max, maximal value

1 - animal fat not specified as being taken from cattle, pigs or poultry.

2 - waste oils that are sometimes incorporated in animal feed

Milk and beef were less contaminated with PCBs and dioxins than pork, chicken and eggs (Table 7.4). Although during the incident, the EU established 200 ng PCB/g fat and 5 pg TEQ/g fat as a guideline for contaminated food, it is reasonable to consider that food for human consumption should contain lower amounts of these highly toxic compounds. Table 7.4 shows, however, that the percentage of samples with dioxin levels above 2 pg I-TEQ/g fat is high. This percentage was higher than the percentage of samples with > 200 ng PCBs/g fat. Dioxins stemming from environmental contamination are probably responsible for background levels up to about 5 pg I-TEQ/g fat. Environmental PCBs probably only rarely lead to contamination levels above 100 ng PCBs/g fat (Schepens et al., 2001). These data provide strong support for the hypothesis that, independent from the incident, the use of recycled fats, oils and animal waste can lead to high levels of organochlorines in human food.

The PCDD/PCDF congener distributions in four samples of animal feed heavily contaminated by the incident are shown in Figure 7.3.

The congeners profile shows a clear predominance of PCDF over PCDD congeners. This is compatible with a PCB contamination by a substance (such as transformer oil) containing PCBs rather than by dioxins originating from thermal processes such as waste incineration. The fingerprint of PCDD/F-emissions by municipal waste incinerators is substantially different from the one found in animal feed (Figure 7.3). The PCDF/PCDD ratio is 1.7 for the municipal waste incinerators (MWI) and 16 for the animal food.

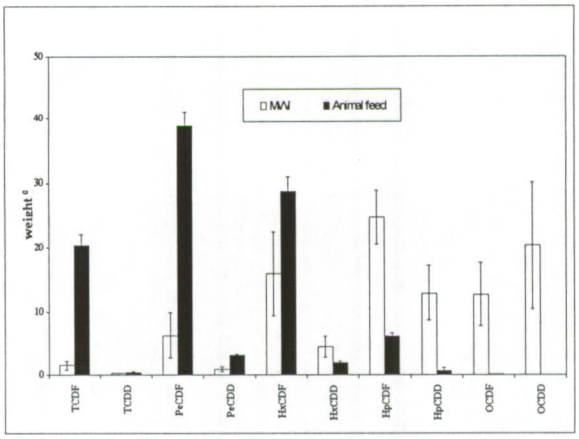

Figure 7.3. PCDD/PCDF congeners distribution (in percentages of weight) measured in four samples of contaminated feed (mean ± SD). The results are compared with the congener distribution as found in emissions of MWI.

To further identify the nature of the PCB mixture, different commercial PCB formulations were analysed under the same conditions as the contaminated feed samples. The results for the 7 marker PCBs in 47 incidental contaminated animal feed samples (containing more than 1,000 ng PCB/g fat) are given in Figure 7.4, which also includes the profiles for a 50/50 mixture of Aroclors 1254 and 1260. There is a good agreement between the contamination pattern found in animal feed and the profile of the mixture of both Aroclors.

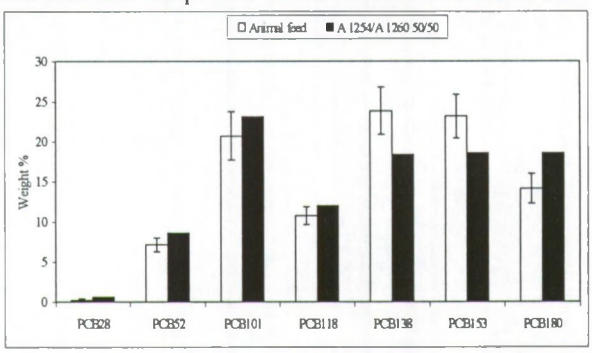

Figure 7.4. Weight percent distribution of 7 PCB marker congeners in 47 contaminated animal feed samples (mean and SD) and a 50/50 mixture of Aroclors 1254 and 1260.

The congener distribution in 11 egg-samples contaminated with > 1,000 ng PCB/g fat is shown in Figure 7.5. The data for eggs, reflecting the result of metabolic conversion and accumulation, are compared with the profiles found in the 47 samples of animal feed. The comparison shows that the higher PCBs (PCB 118, 138, 153, and 180) are the most persistent ones. The lower chlorinated PCBs (PCB 52 and 101) are more easily metabolised or excreted.

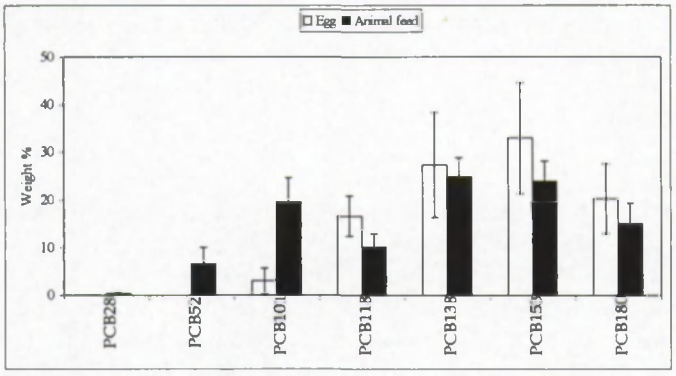

Figure 7.5. PCB congener distribution (weight %) in contaminated eggs, compared to contaminated animal feed (mean and SD).

Figure 7.6 shows the concentration of PCBs plotted against the ratio between the sum of the 7 marker PCBs and dioxins found in the respective samples, for all samples for which both PCB and dioxin data are available. The figure reveals that for most (12 out of 16) samples with concentrations of PCBs > 5,000 ng/g fat, about 50,000 times more PCBs than dioxins are found. This coincides with the ratio found in transformer oil. Numerous less-contaminated samples (between 500 and 1 000 ng PCBs/g fat) show different ratios.

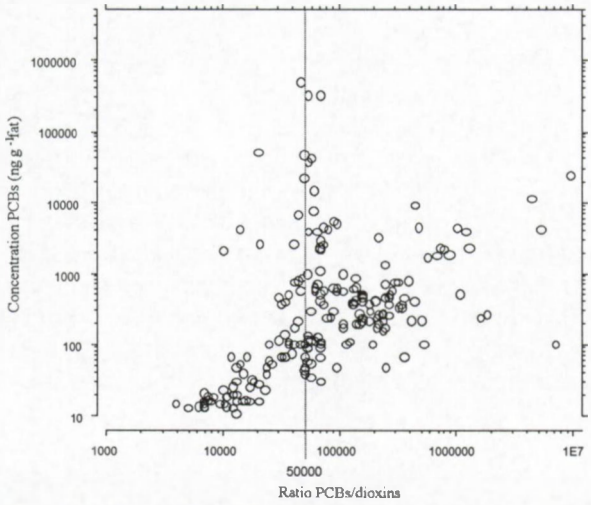

Figure 7.6. PCB/dioxin ratio in 246 samples for which both dioxin and PCB concentrations were measured.

Figure 7.6 shows a peak of measurements with increased PCB concentrations and a PCB:dioxin ratio of approximately 50,000:1 in samples that could be traced back to the feed producers who used ingredients from the incriminated fat-melting company. Figure 7.6 also shows samples with a PCB/dioxin ratio which is clearly different from the 50,000:1 ratio. One reason is that the PCB/dioxin ratio depend on the matrix used for analysis and on the metabolic conversion of some PCB and PCDD/PCDF congeners. Thus, in these samples, for feed, the mean PCB/dioxin ratio was close to 10,000, while for poultry and eggs, the ratio ranged between 35,000 and 65,000. The effect of metabolic conversion is more pronounced for pork, where PCB/dioxin ratios were higher than 150,000 (up to 500,000). The metabolic

conversion of PCB and PCDD/PCDF congeners in chicken and pork is discussed in detail in Chapter 7.4. Another reason for these variable PCB/dioxin ratios is the existence of other contamination sources, different from transformer oil. The wide range of dioxin/PCB ratios in contaminated products suggests that many smaller unidentified contamination events occurred, some of which resulting in high levels of contamination.

Population exposure

Although more than 20,000 PCBs and hundreds of dioxin measurements in animal feed, animal fat and different food items have been performed, there is still an important uncertainty about the extent to which the Belgian population has been exposed to these toxicants. These uncertainties derive essentially from the fact that few measurements of body burdens before the crisis are available and that until now no measurements of body burdens during or after the crisis were performed. There is also uncertainty about the extent to which consumed food was contaminated, as sampling of animal fat and food items were not performed in a systematic way, but evolved during the crisis in response to many different needs and pressures, some from national and European regulatory authorities, others commercial in nature. Some of these demands have biased sampling to the more suspect items, others to less suspect products. Further uncertainty about the extent to which the consumed food was contaminated originates from the period of sampling. During January until the end of May 1999, no systematic sampling of the food chain was performed. Food items may have been contaminated more often and at higher levels than is evident from the data presented above.

Comparison with other contamination episodes

In the Yusho (Japan, 1968) and Yucheng (Taiwan, 1979) incidents, respectively 1,700 and 2,000 victims ate contaminated rice oil and ingested respectively 600 mg PCBs (equivalent to 10 mg/kg body weight) plus 3.5 mg PCDFs, and 1,000 mg PCBs (equivalent to 16.6 mg/kg body weight) plus 3.8 mg PCDFs. In the Belgian crisis, we estimated that a modal Belgian ingested 1.5 mg PCBs (equivalent to 0.025 mg/kg bodyweight). In the Seveso accident (Italy), in which the main toxicant involved was TCDD, individuals studied in the different areas were exposed to between 16 and 78 ng TCDD/kg body weight. The Ranch Hand study on US Air Force veterans concerned individuals with an average exposure of 10 ng TCDD/kg body weight. The mean exposure due to the Belgian incident is estimated at approximately 0.5 ng I-TEQ/kg body weight. In conclusion, exposure during the Belgian incident amounts to only a fraction of that during other episodes, but far more people were involved.

Individual exposure

Significant numbers of Belgians who consumed contaminated products, have temporarily increased their intake of PCDDs/PCDFs up to a factor 100 over the WHO guidelines (1 to 4 pg I-TEQ/person/day). In interpreting the chronic toxicity of persistent toxic chemicals, the body burden is very important (De Vito et al., 1995). Here we estimated that the average incident-related increase in body burden in Belgium was 7% for PCDD/PCDFS and 42% for PCBs. However, because of geographic and dietary reasons, it is likely that the PCB/dioxin burden of the crisis was unevenly distributed among the Belgian population. The U.S. EPA has estimated that dioxin body burdens might be 3 to 4 times above the average in about 10% of the population. It is therefore likely that for some sub-populations the increase in body burdens and the associated risk has been substantial. It is important to identify these sub-populations to study the long-term effects associated with these levels of body burdens.

Effects

Acute clinical health effects have not been reported during the Belgian incident. However, it is most likely that this contamination episode will have delayed effects on the health of exposed individuals. Adverse and other biological effects of dioxin-like substances in animals are observed at low doses. In extrapolating animal study data to human risk, it must be considered that short living species such as rodents require 100 to 200 fold higher doses to reach equivalent body burdens than humans exposed to background contamination.

The Belgian crisis, which probably entailed a higher exposure to TEQ through its PCB content (estimated value equivalent to 0.9 g I-TEQ) than through its PCDDs/PCDFs (estimated value 300 mg I-TEQ), should be considered a potentially important public health event. The incident had a significant impact on the body burden of the modal citizen, and has probably doubled or tripled the body burdens of selected sub-populations who were intensely exposed to contaminated food. Dioxin-like compounds are among the reactive or hormone-disturbing substances whose long-term effects may be insidious and particularly hard to detect because of the high background levels. These high background levels should go down, while the individuals exposed during the incident to high PCB and dioxin amounts should be traced and their health status should be monitored.

7.3. Determination of mineral oil content in contaminated Belgian animal feed and meat samples (in collaboration with Dr. K. Grob).

Introduction

Oils and fats for production of animal feed can become contaminated with mineral oil material originating from gas oils (C_{18}-C_{35}) or synthetic oils (polyolefins, C_{25} to beyond C_{45}). An important cause is assumed to be the discharge of waste oils, such as motor oil and hydraulic oils. Thus, contamination of animal feed is not restricted to PCBs and dioxins: many more non-edible materials are intentionally or unintentionally discharged into feeds.

Waste oils may contain a wide variety of other chemicals, including solvents and diluents from cleaning, diesel-like oils use to clean engine parts, paints, protective coats and others. In Switzerland, a legal limit of 100 µg/g of mineral oil in fats and oils for animal feeds was imposed for 1999) and of 30 µg/g valid after 2000 (Swiss Federal Research Station for Animal Production, 1999).

There is no realistic possibility of analysing fats for animal feeds on all possible waste components and, hence, absence of toxic materials cannot be checked through analysis. Since there is no reason to tolerate any such waste in feeds, it is sufficient to determine their presence through a marker which is as general as possible. As waste oils are likely to contain motor and diesel-type oils, we analysed feeds and foodstuffs of animal origin for mineral oil hydrocarbons. However, the hydrocarbons are probably the least toxic waste components. There is no hint whether they are accompanied by other highly toxic components, nor does a high concentration of mineral oil material necessarily go along with a high concentration of toxic material, nor a low concentration of mineral oil go along with a low concentration of toxicants.

The purpose of this research was to investigate whether the PCB contamination (see Chapter 7.2) was due to the use of waste oils and fats into the animal feed production or to the intentionally/unintentionally discharge of pure transformer oil.

Samples
Samples of eggs, animal feed, chicken and pork fat, all containing concentrations of marker PCBs higher than 200 ng/g fat were collected during the PCB crisis (See Chapter 7.2). Samples of ingredients (oils) for feed production, complete animal feed, eggs, butter and pork fat, with concentrations of marker PCBs < 30 ng/g fat were obtained through the Veterinary Belgian Inspection or were purchased from the supermarket.

Methods
The analyses were carried out at the Official Food Control Authority of the Canton of Zurich, Switzerland, under the supervision of Dr. Koni Grob. Samples were dissolved or extracted into pentane and analysed with on-line normal phase liquid chromatography (NPLC)-GC-FID for the determination of hydrocarbons content (Grob et al, 2001). Four internal standards were added: n-tridecane, n-tetradecane, n-pentadecane and 1-hexadecene.

Oils and animal body fats. After melting at 100°C, 300 mg of oil or fat were dissolved in 1.5 ml pentane and internal standards (to give a concentration in relation to the oil of 100 µg/g) were added.
Animal feed. Three g of homogenised sample was immersed in 15 ml pentane for at least 1 h. Internal standards were added at 10 µg/g feed.
Egg yolk. Two g of egg yolk were mixed with 10 g anhydrous Na_2SO_4 and immersed in 10 ml pentane for at least 1 h with repeated shaking. Internal standards were added at 1 µg/g egg yolk.
On-line LC-GC. LC-GC-FID was performed on two Dualchrom 3000 (Thermoquest, Milan, Italy). For egg yolk, four 25 cm x 2 mm i.d. columns packed with silica gel were used in series. For all other materials, 2 silica gel columns were sufficient. After LC separation, the desired fraction (between 150 and 600 µl) was transferred to the GC instrument via a large volume injection interface (full details in Grob et al., 2001).

Results and discussion
Concentration of mineral oil hydrocarbons, considered as indicators for contamination with waste oils, was determined in Belgian samples with varying concentrations of PCBs (from < 30 up to 100,000 ng/PCBs/g fat. Results are presented in Table 7.5.

The concentrations of mineral oil hydrocarbons in samples highly contaminated with PCBs (due to the PCB contamination of animal food chain, see Chapter 7.2) are identical with those observed in samples with very low concentrations of PCB (Table 7.5). This leads to the conclusion that the introduction of PCBs in the animal food chain was done by the use of pure transformer oil and not from waste oils (used edible, hydraulic or motor oils), collected at the recycling centres. The highest results for hydrocarbons were obtained for animal feed. However, in some countries (including Switzerland), the use of paraffin oil for the preparation of mixtures of minerals is permitted. Unfortunately, the oil cannot be distinguished from the mineral oil of wastes.
All samples contained lower concentrations of mineral oil hydrocarbons than similar samples collected and analysed in Switzerland during the summer of 1999. In June 1999, inspections in Switzerland revealed that separation between edible oil wastes (intended to be used for animal feeds) and other oil wastes was insufficient, both being collected at the same public sites (without supervision!) and that some mixing (even unintentionally) was likely to occur. It was lately decided that edible oil would now be disposed of in the same way as mineral oil

wastes. Analyses done in samples from Switzerland collected in December 1999 revealed an important decrease in mineral oil hydrocarbons content of animal feed and body fat.

Table 7.5. Mineral oil hydrocarbons in the feed ingredients, complete feeds, animal body fats and eggs of Belgium samples non-contaminated and contaminated with PCBs.

Sample Type		Conc. PCBs* (ng/g fat)	Conc. Hydrocarbons (µg/g)	
			Belgium	Switzerland, Summer 1999
Ingredients for feed production	Cereals	< 30	5	-
	Oil 1	< 30	< 3	Mean: 320 Range: 100 – 1,000
	Oil 2	< 30	< 3	
	Oil 3	< 30	< 3	
	Raw fat	<30	36	Range: 100 - 1,000
Animal feed	Feed 1	< 30	35	Mean: 100 (range: <5 – 1,000)
	Feed 2	< 30	5	
	Feed 3	< 30	5	
	Feed 4	100,000	16	
	Feed 5	> 100,000	20	
	Feed 6	> 100,000	25	
Animal body fat	Pork fat 1	< 30	5	Pork Mean: 25 (range: <3 – 100) Chicken Range: <5 – 150
	Pork fat 2	600	< 3	
	Pork fat 3	1,500	< 3	
	Pork fat 4	5,000	< 3	
	Chicken fat 1	200	< 3	
	Chicken fat 2	3,000	< 5	
	Chicken fat 3	4,800	< 3	
Egg Yolk	Egg 1	< 30	12	Mean: 30 Range: < 2 - 80
	Egg 2	500	8	
Butter	Butter 1	< 30	< 3	-

* - sum of IUPAC no. 28, 52, 101, 118, 138, 153, 180.

In Belgium, to the best of our knowledge, the waste oils collected at the recycling centre are not anymore used for animal feed production. However, there are no monitoring programs for determination of mineral oil hydrocarbons in the animal food chain. Apart from risk considerations, it is certain that consumers are not willing to tolerate food from animals fed with waste oils, even if they are not toxic. This should mobilise all forces necessary to stop the dumping of waste into feeds and avoid other types of contamination.

7.4. Profiles of PCBs/dioxins in contaminated samples

* - based on Covaci A, Ryan JJ, Schepens P, (2001). *Chemosphere*, in press.

Introduction

During the PCB contamination episode, the use of 60 tons of contaminated fat from a Belgian fat rendering company for production of approximately 500 tons of animal feed resulted in the introduction of approximately 50 kg PCBs and 1 g of mostly furans in the commercial food chain (see Chapter 7.2). This incident caused widespread concern both within and outside Belgium and forced the Belgian authorities to take drastic measures, including measurements of PCBs on thousands, and of dioxins on hundreds of samples of animal feed,

animal fat and various fat containing food items. Analysis of contaminated foodstuff showed a pattern of PCBs closely matching a mixture of Aroclor 1254 and 1260 and a consistent pattern of dioxin-like compounds, dominated by PCDFs. These patterns were virtually identical to those in the Yusho rice poisoning caused by heat degraded PCBs (Tanabe et al., 1989). The occurrence of the Belgian PCB/dioxin contamination is similar to other past poultry poisonings with PCBs (Drotman et al., 1983; Gilbertson et al., 1991). Pathologic conditions were first recorded in Belgian chicken batteries and hatcheries. They included a decrease in egg production and hatching, and an epidemic of chicken oedema disease.

For poultry, PCBs have been reported to adversely affect hatchability through embryotoxicity (Bernard et al., 1999), along with reduced weight gain. Other pathologic effects reported are generalised oedema and liver necrosis. PCBs have also been shown to induce embryotoxicty (Ax and Hansen, 1975) and hepatic drug metabolizing enzyme activity in birds (Hansen et al., 1981). Circulating thyroid hormones and growth are also decreased by PCB exposure (Gould, 1999). Dioxins have been shown to induce mortality in chicken (Ryan et al., 1985) and young pigs (Ryan, 1983) raised on PCP–treated wooden floors.

Some data are available on the accumulation or changes in the relative distribution of PCB congeners during ingestion studies in chicken (Hansen et al., 1976, 1983), pork (Borchard et al., 1976; Hansen and Welborn, 1977; Hansen et al. 1981, 1983) or sheep (Borchard et al., 1976). PCB residues in animals only vaguely resemble the mixture from which they originate. Changes in Aroclor 1254 component composition in tissues were less apparent than in Aroclor 1242. Subtle differences between congeners are further amplified by species differences in absorption, distribution, biotransformation and excretion. Most of the previous work done in controlled feeding studies used packed GC columns, thus making the correct identification of congeners more difficult. There is no information on specific isomer accumulation when animals are fed a diet containing a mixture of Aroclor 1254 and 1260, as seen in the Belgian episode.

The study was undertaken to examine the accumulation patterns of PCBs and PCDD/PCDFs in chicken and pork resulting from the consumption of commercial feed accidentally adulterated by a mixture of Aroclor 1254 and 1260. Compositional changes were also examined to determine the relative importance of single PCB isomers as residues in human food.

Materials and Methods

Three samples of chicken fat (one low (#10078), medium (#6134) and high (#6133) PCB levels) and two of pork fat (low (#7129) and medium (#7133)) along with a highly adulterated chicken feed sample obtained during the Belgian PCB/dioxin crisis were chosen for detailed analyses. They were collected through the Veterinary Commission of Ministry of Agriculture, sent sealed to the Toxicological Centre (Antwerp) and kept at –20°C until analysis. PCBs were measured according to the procedure described in Chapter 7.1.

Determination of PCDD/Fs and non-ortho PCBs

A mixture of seventeen ^{13}C-2378 substituted PCDD/Fs and three non-ortho PCBs was added to 1 to 5 g of chicken or pork fat or 0.1 g of chicken feed. The samples were homogenised and extracted with acetone-hexane (a small aliquot was used for the lipid determination gravimetrically), defatted with concentrated sulphuric acid, adsorbed on acid/base silica and Florisil, and separated on activated carbon. Identification was by gas chromatography-high resolution mass spectrometry and quantification by the isotope dilution internal standard method (Ryan et al, 1992). Detection limits varied depending on congener and sample size and were as low as 0.2 pg/g for tetra- to hexa-PCDD/Fs. Each batch of 12 samples for

analysis contained a laboratory reagent blank to check for contamination within and a commercial butter sample with low natural background levels of PCDDs/PCDFs. The relative standard deviations (RSD) for repeated analyses were the following: for TEQ <10%; for 2,3,4,7,8-PeCDF <15%; and for 2,3,7,8-TCDD <20%. The Health Canada laboratory has participated in several recent interlaboratory analytical studies for dioxin-like compounds including those organised by WHO.

Results and discussion

The Belgian governmental program required the monitoring of 7 marker PCBs (IUPAC no. 28, 52, 101, 118, 138, 153 and 180). The profiles among the two species and contaminated feed were different (Figure 7.7). In particular, the two animal species contained lower concentrations of tri- to penta-PCBs than the contaminated feed. Moreover, except for PCB 118 in pork fat, the two animal species contained correspondingly more of the higher congeners (penta- to hepta-PCBs). The inclusion of a larger number of congeners in the analysis (Hansen, 1998) will give a greater potential for estimation of congener profile changes and facilitates resolution of the ambiguities in risk assessment.

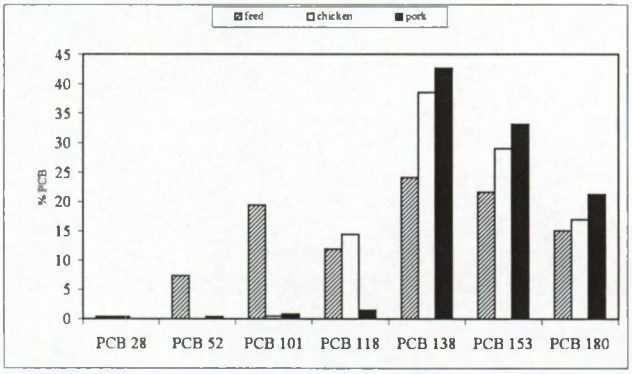

Figure 7.7. Patterns of the 7 marker PCBs in feed, chicken and pork samples.

Total concentrations and individual percentages in three chicken fat, two pork fat and one feed for 56 PCB congeners (ng/g lipid weight) and for 3 non-ortho PCB and 17 2,3,7,8-chlorosubstituted PCDD/F (pg/g lipid weight) are presented in Tables 7.6 and 7.7, respectively. Because the samples were intended for human consumption, TEF values for mammals (Van den Berg et al., 1998) were used for I-TEQ calculation from PCDD/F congeners, and non- and mono-ortho PCBs (Table 7.8).

The patterns found (the individual number and/or relative proportion of each congener within a group) are approximately constant within each species analysed, which in turn are representative of other contaminated samples (same percentage of the 7 markers).

Patterns of PCBs in chicken and pork

Accumulation patterns of PCBs in the two analysed species are presented as homologues in Figure 7.8. Differences in metabolism are suggested by the pattern shown in the region of lower chlorinated PCBs (tri- to penta-PCB congeners). Levels of tri- to penta-CBs are lower in chicken or pork than in the feed, indicating that both species are able to metabolise to some extent these congeners (except PCB 28 in chicken). However, higher levels of lower chlorinated congeners can be found in chicken when compared with pork (Figure 7.8).

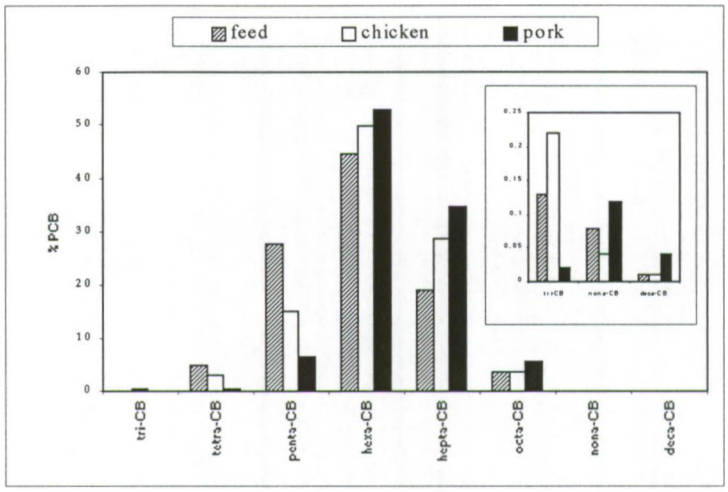

Figure 7.8. Distribution of PCB homologue groups.

Levels of higher chlorinated PCBs (hexa to octa-CB) are higher in animals than in the feed (except octa-PCBs in chicken), suggesting an accumulation for these congeners especially the persistent ones (PCB 138, 153 and 180) in the following sequence: feedstuff < chicken < pork (Figure 7.8).

The PCB congener profiles for chicken, pork and feed are presented in Figure 7.9. Percentages of each congener were normalised to PCB 153, one of the most abundant congeners (only compounds with a contribution to the total PCB content > 0.5% were included).

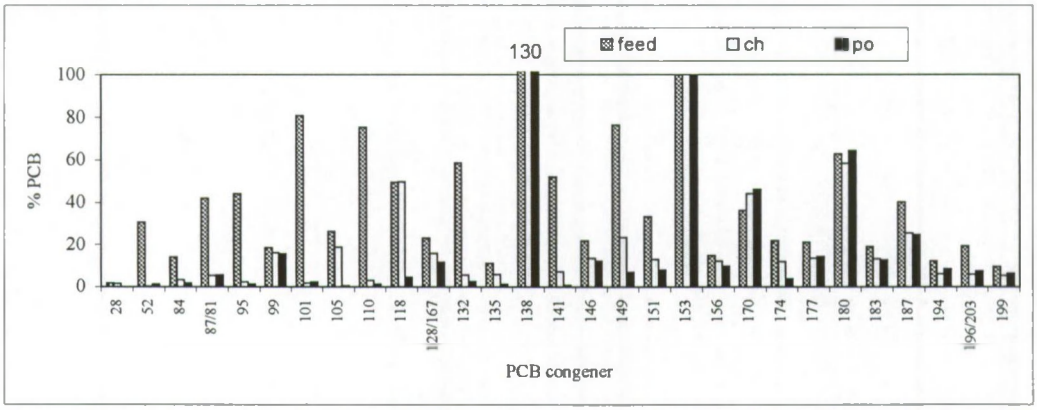

Figure 7.9. Relative distribution of PCB congeners in feed, chicken and pork fat. All congeners were normalised to PCB 153.

The following PCB congeners were found to have a higher accumulation in chicken than in pork: 28, 49, 56/60, 74, 105, 114, 118, 126, 135, 141, 149, 174, 176 and 179. Unless there is a difference in uptake, it seems that there is an ability of mammals to metabolise those PCB congeners with 2 vicinal hydrogen atoms in meta/para or meta/ortho positions. A similar selective metabolism of PCBs has been reported in humans in the Yusho and Yucheng rice

oil poisonings. PCB 118, present in high concentrations in the rice oil, was rapidly metabolised in humans (Masuda et al., 1995).

Table 7.6. Concentrations (ng/g fat) of PCBs in 3 chicken, 2 pork and 1 feedstuff samples.

PCBs	feed-7057	*% feed*	ch-6133	ch-6134	ch-10078	*% ch*	po-7129	po-7133	*% po*
28	441	*0.1*	19	8	1	*0.2*	ND	1	*0.1*
49	2090	*0.7*	15	6	1	*0.2*	ND	2	*0.1*
52	7588	*2.4*	4	2	ND	*0.1*	3	12	*0.2*
56/60	534	*0.2*	26	10	1	*0.3*	ND	2	*0.1*
66	1579	*0.5*	109	43	5	*1.3*	ND	2	*0.1*
74	1197	*0.4*	89	36	4	*1.0*	ND	1	*0.1*
84	3507	*1.1*	44	18	2	*0.5*	3	16	*0.3*
87/81	10373	*3.3*	69	28	3	*0.8*	11	55	*1.1*
95	10897	*3.4*	28	11	1	*0.3*	3	12	*0.2*
99	4623	*1.5*	205	82	9	*2.4*	31	150	*2.9*
101	19973	*6.3*	23	9	1	*0.8*	5	24	*0.5*
105	6521	*2.7*	241	96	11	*2.8*	1	4	*0.1*
110	18637	*5.9*	40	16	1	*0.5*	3	12	*0.2*
114	302	*0.1*	6	2	ND	*0.1*	ND	1	*0.1*
118	12244	*3.9*	634	253	29	*7.4*	9	45	*0.9*
123	298	*0.1*	3	1	ND	*0.1*	ND	ND	*0.1*
128	4120	*1.3*	41	4	ND	*0.5*	1	7	*0.6*
132	14520	*4.6*	74	30	3	*0.9*	5	26	*0.5*
135	2784	*0.9*	76	30	3	*0.9*	3	12	*0.2*
138/163	33933	*10.7*	1691	674	77	*19.8*	254	1232	*23.9*
141	12906	*4.1*	93	37	4	*1.1*	2	8	*0.2*
146	5429	*1.7*	170	68	8	*2.0*	24	118	*2.3*
149	18944	*6.0*	299	119	14	*3.5*	14	67	*1.3*
151	8291	*2.6*	166	66	8	*1.9*	16	77	*1.5*
153	24750	*7.8*	1273	508	58	*14.9*	198	959	*18.6*
156	3676	*1.2*	157	63	7	*1.8*	20	95	*1.9*
157	554	*0.2*	26	10	1	*0.3*	3	16	*0.3*
167	1617	*0.5*	163	12	1	*1.9*	5	21	*2.0*
170	8992	*2.8*	560	223	26	*6.6*	92	444	*8.6*
171	3091	*1.0*	86	34	4	*1.0*	11	51	*1.0*
172	2035	*0.6*	60	24	3	*0.7*	10	49	*0.9*
174	5426	*1.7*	154	61	7	*1.8*	8	39	*0.8*
176	1078	*0.3*	25	10	1	*0.3*	ND	1	*0.1*
177	5207	*1.7*	175	70	8	*2.1*	28	138	*2.7*
178	8400	*0.3*	72	29	3	*0.8*	12	57	*1.1*
179	1020	*0.3*	49	20	2	*0.6*	1	2	*0.1*
180	15533	*4.9*	743	296	34	*8.7*	127	616	*11.9*
183	4738	*1.5*	168	67	8	*2.0*	25	122	*2.4*
187	9929	*3.1*	325	130	15	*3.8*	49	239	*4.6*
189	106	*0.1*	18	7	1	*0.2*	3	16	*0.3*
191	549	*0.2*	21	8	1	*0.3*	3	14	*0.3*
194	3043	*1.0*	80	32	4	*0.9*	17	81	*1.6*
195	562	*0.2*	38	15	2	*0.4*	8	37	*0.7*
196/203	4746	*1.5*	76	30	4	*0.9*	15	73	*1.4*
199	2380	*0.8*	68	27	3	*0.8*	13	63	*1.2*
200	789	*0.3*	14	6	1	*0.2*	2	8	*0.2*
202	nr	*nr*	14	6	1	*0.2*	3	14	*0.3*
206	265	*0.1*	3	1	ND	*0.1*	1	6	*0.1*
209	43	*0.1*	1	0.5	ND	*0.1*	ND	2	*0.1*
sum PCB	316330	*100*	8540	340	390	*100*	1065	5160	*100*
sum 7 markers	114463	*36.2*	4387	1750	200	*51.4*	600	2900	*56.0*

It is worth mentioning that some congeners (PCB 101 and 110) in the contaminated feed and Aroclor mixtures show a relatively high percentage, while their concentration in the two investigated species is very low, probably due to a lack of uptake or rapid metabolism. Congeners 132, 141, 149 and 151 are easily metabolised in both species and as expected, some congeners (PCB 99, 138, 153, 156, 170, 177, 180 and 187) accumulate in both species. Data in the literature are similar with findings presented in this paper. In an earlier study (Hansen et al., 1983), chickens were fed a diet containing Aroclor 1254 (technical diet) or a diet containing fat of swine fed with Aroclor 1254 (residue diet). The percentages of congeners 49, 70, 95, 101, 118, 141 and 149 were higher in the technical diet than in the residue diet, indicating that pork selectively degrades and/or eliminates these PCBs. Likewise their relative concentration is higher in chicken body fat and chicken liver fat. Chickens accumulate mainly PCBs 138, 153 and 118 in their body fat and liver fat when fed diets formulated from swine residues. However, PCBs 118 and 95 accumulate to a higher extent when technical product (Aroclor 1254) is given.

The differential growth rates of tissues important in accumulation of PCBs (such as fat) and those involved in elimination (such as blood and liver) have a significant effect on actual tissue concentrations. Abdominal fat is the best tissue for residue detection because of the 15-22 times higher residue concentrations than in other tissues. Liver was reported (Hansen et al., 1976) to be distinct from other tissues in that it did not concentrate tri-chlorinated congeners, but tetra- and penta-chlorinated ones. Further investigations will be needed to establish if fat is a reliable and good indicator matrix or whether concentrations have to be measured in different tissues.

Patterns of PCDD/PCDFs in chicken and pork

The presence of PCDFs in the feedstuff in a higher proportion than the PCDDs excludes the possibility of contamination from the use of technical pentachlorophenol (PCP) as wood preservative (Ryan et al., 1985) (which is known to contain traces of higher chlorinated PCDD/Fs) or from incineration processes (Domingo et al., 1999), where the PCDD/PCDF ratio is approximately equal with the unity. This is consistent with the presence in Aroclor mixtures of PCDF congeners in much higher proportion than PCDDs, particularly when the PCB has been aged or heat degraded, as observed in the Yusho episode (Tanabe et al., 1989). The Belgian incident is similar to the Yusho one.

Contaminated chicken tissues contain more PCDD/F congeners than pork tissues, including some non-2,3,7,8 substituted ones. Similar results were presented previously (Ryan et al., 1985). This might be related to different metabolic capabilities of mammals and birds. Feed contains the same congeners, but in higher concentrations. Nevertheless, in both animal species, the 2,3,7,8-substituted congeners are accumulating to a higher degree than the non 2,3,7,8-substituted ones (Figure 7.10).

Selective absorption of 2,3,7,8-substituted PCDDs has been noted previously in chickens (Firestone et al., 1971) and dairy cows (Firestone et al., 1979). Higher chlorinated dioxins were implicated in the mortality of young pigs kept on a PCP treated wooden floor (Ryan, 1983). For HxCDDs, the predominant isomers were 1,2,3,6,7,9- and 1,2,3,6,7,8-HxCDD in wood, while in pork liver, only 1,2,3,6,7,8-HxCDD was found. 1,2,6,7,8-HxCDD is most abundant among the toxic 2,3,7,8-substituted HxCDDs in animals and the environment under normal exposure. For hepta congeners, the same pattern of accumulation of 1,2,3,4,6,7,8-HpCDD (more toxic) versus 1,2,3,4,6,7,9-HpCDD was found.

Table 7.7. PCDD/Fs concentrations (pg/g fat) in 3 chicken, 2 pork and 1 feedstuff samples.

	WHO TEFs	feed-7057	ch-6134	ch-6133	po-7129	po-7133	Aroclor 1260
Σ marker PCBs (ng/g fat)			1750	4380	600	2900	E+06
PCDDs (pg/g fat)							
TCDDs							
2,3,7,8-TCDD	1.0	137	0.7	1.6	ND(.1)	ND(.2)	ND(20)
PnCDDs							
1,2,3,7,8-PnCDD	1.0	1319	3.0	7.9	ND(.1)	ND(.2)	ND(20)
1,2,4,7,8-PnCDD	0	10060	2.6	4.3	ND(.1)	ND(.2)	ND(20)
HxCDDs							
1,2,3,4,7,8-HxCDD	0.1	756	3.1	5.8	0.7	2.2	ND(20)
1,2,3,6,7,8-HxCDD	0.1	122	1.9	1.5	0.3	ND(.3)	ND(20)
1,2,3,7,8,9-HxCDD	0.1	196	0.7	1.1	ND(.2)	ND(.3)	ND(20)
1,2,4,6,7(8),9-HxCDD	0	7007	0.8	0.9	ND(.2)	ND(.3)	ND(20)
1,2,3,6,7(8),9-HxCDD	0	2930	2.0	2.1	ND(.2)	ND(.3)	ND(20)
HpCDDs							
1,2,3,4,6,7,8-HpCDD	0.01	390	4.1	3.4	0.9	1.3	ND(30)
1,2,3,4,6,7,9-HpCDD	0	1150	2.0	2.7	ND(.2)	ND(.3)	ND(30)
OCDD	0.0001	ND(1060)	13.3	ND(7.7)	ND(8.8)	ND(10)	ND(50)
PCDFs (pg/g fat)							
TCDFs							
2,3,7,8-TCDF	0.1	17683	46.7	95.4	ND(.1)	ND(.2)	77
1,2,7,8-TCDF	0	4417	6.3	2.6	ND(.1)	ND(.2)	60
PnCDFs							
1,2,3,7,8-PnCDF	0.05	3819	11.6	28.1	ND(.1)	ND(.2)	124
2,3,4,7,8-PnCDF	0.5	18573	59.8	172.9	3.8	6.6	140
1,2,4,7,8-PnCDF	0	54061	31.5	42.1	ND(.1)	ND(.2)	148
HxCDFs							
1,2,3,4,7,8-HxCDF	0.1	16495	39.1	87.3	11.3	19.3	370
1,2,3,6,7,8-HxCDF	0.1	2193	4.5	11.0	1.4	2.1	55
2,3,4,6,7,8-HxCDF	0.1	3075	4.0	9.8	0.6	0.7	65
1,2,3,7,8,9-HxCDF	0.1	574	ND(.2)	ND(.5)	ND(.1)	ND(.2)	ND(20)
1,2,4,6,7,8-HxCDF	0	10880	2.5	3.3	ND(.1)	ND(.2)	265
1,2,4,6,8,9-HxCDF	0	4022	2.3	3.5	ND(.1)	ND(.2)	183
HpCDFs							
1,2,3,4,6,7,8-HpCDF	0.01	2507	1.7	2.2	2.5	3.2	398
1,2,3,4,7,8,9-HpCDF	0.01	1158	1.2	1.4	0.6	ND(.3)	291
1,2,3,4,6,8,9-HpCDF	0	3110	1.5	1.2	ND(.1)	ND(.3)	879
OCDF	0.0001	2969	1.0	ND(1.2)	ND(3)	ND(4)	1490
non ortho PCBs							
TeCB-77	0.0001	317274	303	413	4.4	5.0	54800
PeCB-126	0.1	56832	304	618	7.9	12.1	607
HxCB-169	0.01	3171	18.4	33.0	9.2	25.4	46

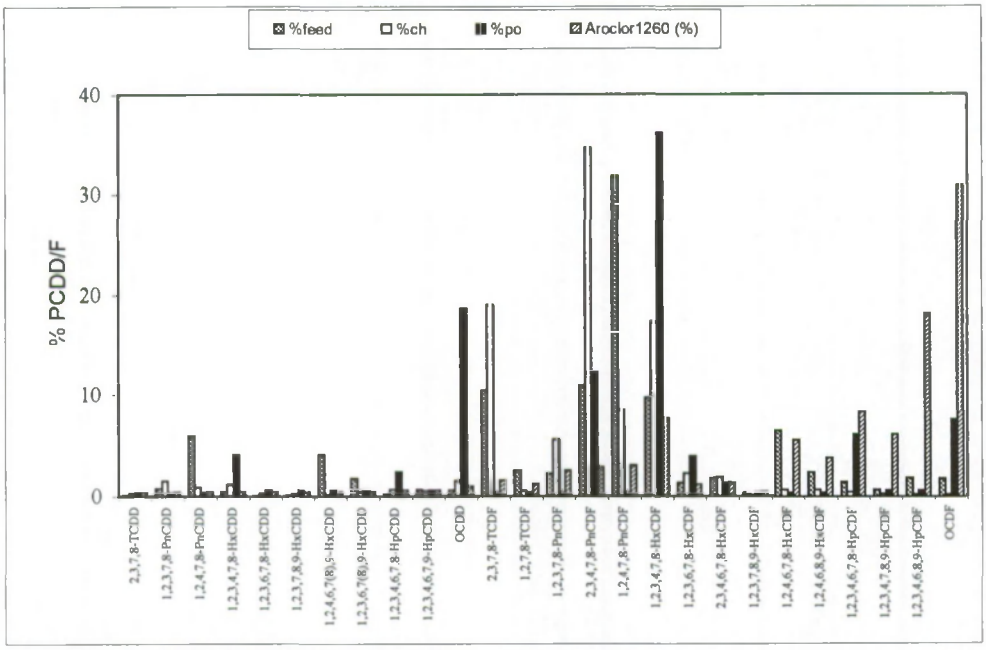

Figure 7.10. Patterns of PCDD/PCDF congeners in feed, chicken, pork and Aroclor 1260.

Figure 7.11 shows different accumulation patterns between PCDD/F homologues with a different degree of chlorination. As for PCBs, tetra and penta-PCDD/Fs are selectively accumulating in chicken rather than in pork whereas the opposite is true for the hexa- and higher homologues.

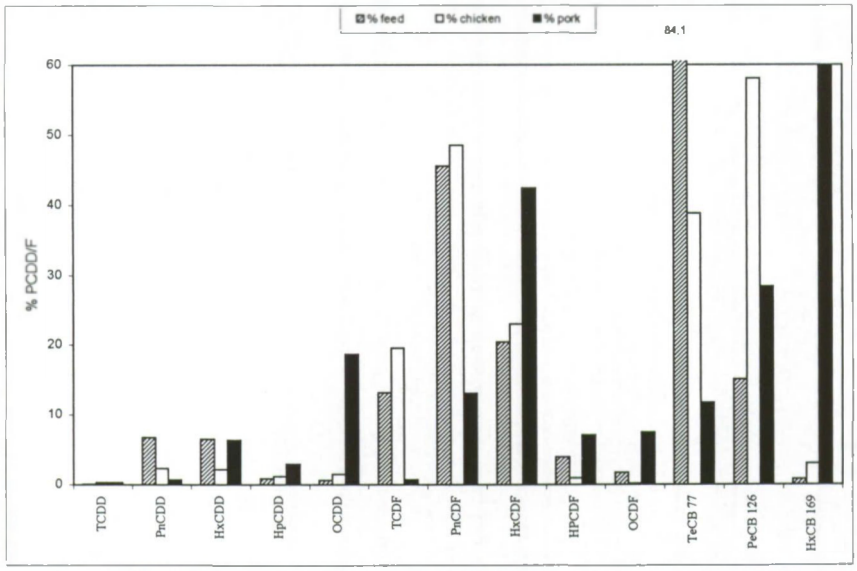

Figure 7.11. Patterns of PCDD/F homologues and non-ortho PCBs at different levels of chlorination in chicken, pork and feed.

TEQ values of PCDD/Fs and PCBs

The higher accumulation rate of some PCB congeners in birds (e.g. PCB 105, 118, 126) is partially responsible for the higher contribution of PCBs to the total TEQ relative to that for feed. These congeners are non or mono-ortho chlorine substituted and have assigned TEF values (Van den Berg, 1998).

Table 7.8. TEQ values (pg/g fat) in 3 chicken, 2 pork and 1 feedstuff samples.

	TEF	feed-7057	ch-6133	ch-6134	ch-10078	po-7129	po-7133
PCB 105	0.0001	652.1	24.1	9.6	1.1	0.1	0.4
PCB 114	0.0005	151	3.0	1.2	0.1	0.1	0.5
PCB 118	0.0001	1224.4	63.4	25.3	2.9	0.9	4.5
PCB 123	0.0005	148.5	1.5	0.6	0.1	0.1	0.2
PCB 156	0.0005	1838	78.5	31.3	3.6	9.8	47.6
PCB 157	0.0005	277	13.0	5.2	0.6	1.7	8.1
PCB 167	0.00001	16.2	1.6	0.1	0.0	0.1	0.2
PCB 189	0.0001	10.6	1.8	0.7	0.1	0.3	1.6
PCB 77	0.0001	31.7	0.04	0.03	0.0	0.0	0.0
PCB 126	0.1	5683.2	61.8	30.4	2.8	0.8	1.2
PCB 169	0.01	31.7	0.3	0.2	0.0	0.1	0.3
PCDF		13517	108.2	40.0	2.3	3.3	5.6
PCDD		1566	10.3	4.3	0.7	0.1	0.2
PCDD/F		15083	118.5	44.3	3.0	3.4	5.8
non-ortho PCB		5747	62.2	30.6	2.8	0.9	1.5
mono-ortho PCB		4317.8	186.9	74.0	8.5	13.1	63.2
total TEQ		25147	367.6	148.9	14.3	17.4	70.5
sum PCB (ng/g fat)		316330	8540	3405	390	1065	5160
sum 7marker PCB (ng/g fat)		114463	4387	1750	200	600	2900
PCB/Dioxin ratio		7,588	37,021	39,503	66,667	176,470	500,000

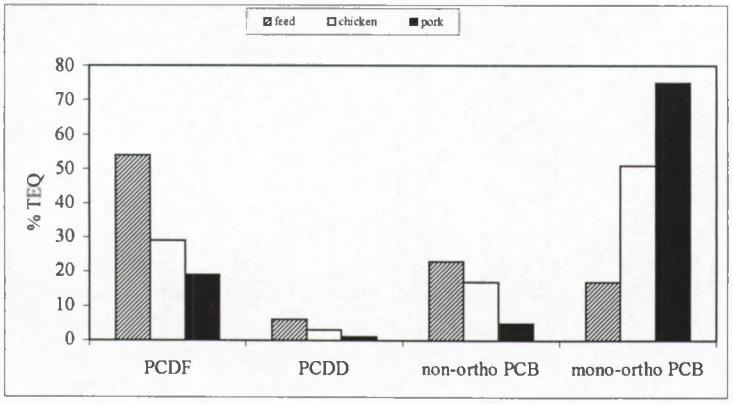

Figure 7.12. Relative contribution of PCBs and PCDD/Fs to the total TEQ values in feed, chicken and pork fat.

Due to PCB 156 and 157, the contribution of mono-ortho PCBs to the total TEQ values is even higher in pork (75%) than in chicken (51%) (Figure 7.12). However, the difference in

the overall concentration values between pork and chicken may also be a factor in these variations in relative contributions to the total TEQ. Because of the ability of pigs to metabolise or excrete tetra and penta-PCBs and PCDD/Fs, the relative contributions to the total TEQ values are different. Uptake rates, exposure times and species-specific metabolism are factors accounting for the observed differences.

The 2,3,4,7,8-PeCDF isomer is the more predominant penta-CDF and accounts for more than 60% of the PCDD/F TEQ value, which together with 1,2,3,4,7,8-HxCDF, accounts for 75%. The PCDFs and PCDDs congeners contributing to total TEQ have lower concentrations in pork than in chicken (Table 7.7).

For chicken, samples with levels of 7 marker PCBs higher than the tolerance level imposed by the European Union (200 ng/g lipid weight) also showed higher TEQ values than the tolerance level for PCDD/Fs (5 pg I-TEQ/g lipid weight). However, in pork, a high value of 600 ng/g lipid weight for the 7 markers PCBs is associated with a value of only 3.4 pg I-TEQ/g lipid weight for PCDD/Fs (Table 7.7). Furthermore, the mean ratios between the concentration of the 7 marker PCBs (ng/g lipid weight) and total TEQ values (pg/g lipid weight) are 12,590 and 37,800 in chicken and pork, respectively. This difference can be explained by the metabolism in pork of congeners with high TEF value (PCB 126 and 2,3,4,7,8-PeCDF).
Normal values for PCDD/Fs in poultry from Europe and US (1991-1994) are between 1.3 and 2.6 pg I-TEQ/g lipid weight (Ferrario and Byrne, 2000). When expressed per lipid weight, the distribution of PCDD/Fs was similar for all analysed organs (fat, liver, skin, gizzard). Contaminated Belgian chicken samples were shown to contain up to 2,000 pg I-TEQ/g lipid weight (see Chapter 7.2).

Remarks on the metabolism of different congeners in other species.
PCB patterns in fish and birds are different than those in mammals including humans, due to different metabolic capabilities of each species.

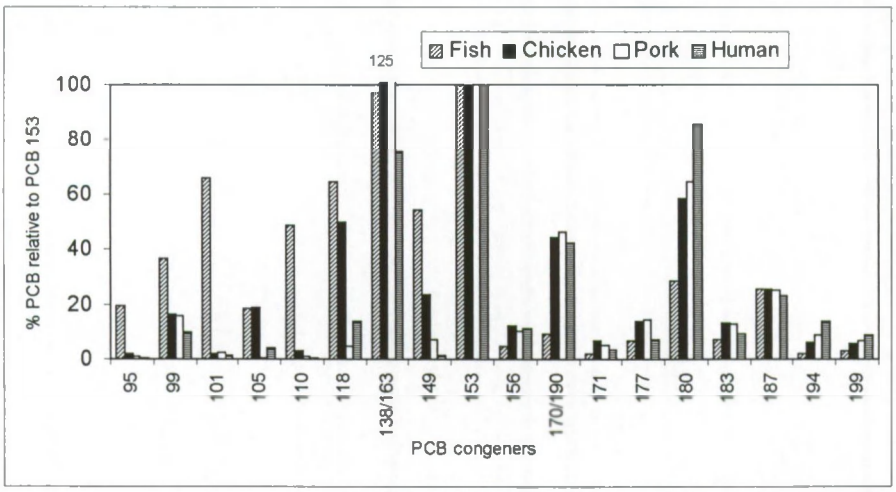

Figure 7.13. Relative percentage (normalised to PCB 153) of selected PCB congeners in fish, chicken, pork and humans.

The higher relative abundance of tri-, tetra- and penta-PCBs, tetra- and penta-PCDD/Fs and even non-2,3,7,8-substituted PCDFs is observed in fish and birds. The appearance of these congeners in fish and birds can in part be explained by metabolism that leads to a selective accumulation (Figure 7.13 and 7.14).

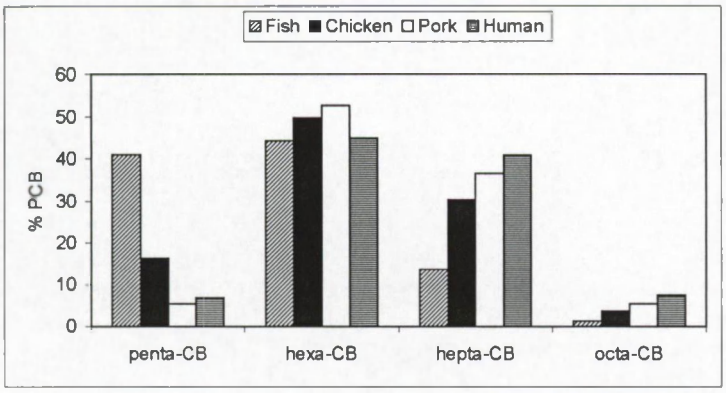

Figure 7.14. Distribution of PCB homologues in fish, chicken, pork and humans

The lower chlorinated PCBs and/or PCDD/Fs usually have higher TEF values. Thus, when exposed to the same source and level of contamination, fish or chicken tissues are liable to have higher TEQ values (lipid based) than mammalian tissues. A human diet based on contaminated fish or chicken can lead to higher TEQ body burdens than diets based on pork (or other mammals). It was shown that consumers of large amounts of fish from polluted areas (e.g. Baltic Sea) have higher blood levels of PCDD/Fs than moderate or non consumers of fish (Svensson et al., 1991). Furthermore, it can be suggested that birds are better markers of the source of contamination with persistent organochlorine pollutants than mammals, since the residue pattern changes less.

7.5. Background levels of organochlorine contaminants in Belgian animal meat used for export

* - based on Schepens P, Covaci A, Jorens P, Hens L, Scharpe S, van Larebeke N, (2001) *Environ Health Perspect* 109, 101-103.

In the period following the PCB crisis (September-November 1999), the Toxicological Centre received and analysed 1,850 samples of Belgian meat for export (chicken and pork) from farms that did not obtain animal feed from producers who might have incorporated ingredients from the incriminated fat melting company. The analysis procedure was described in Chapter 7.1.

Results, summarised in Table 7.9, are expressed as the sum of the 7 marker PCBs (IUPAC no. 28, 52, 101, 118, 138, 153 and 180). For chicken and for pork, 1.8% of the samples tested contained more than 200 ng PCBs/g fat, the tolerated level imposed to Belgium by the European Union (Belgische Staatsblad, 1999).

Table 7.9. PCB concentrations in 1850 samples of Belgian export meat. Percentages of samples within given concentration ranges with the mean and standard deviation for each range are given.

Type of meat	< 30 ng PCBs/g fat		> 30 to < 50 ng PCBs/g fat		>50 to < 200 ng PCBs/g fat		> 200 ng PCBs/g fat	
	(%)	Mean ± SD	(%)	Mean ± SD	(%)	Mean ± SD	(%)	Mean ± SD
Chicken (n=384)	86.2	11.7 ± 6.1	2.9	39.7 ± 6.1	9.1	88.3 ± 33.7	1.8	244 ± 48
Pork (n=1,466)	81.9	12.0 ± 6.3	5.7	39.9 ± 5.0	10.6	91.9 ± 39.9	1.8	356.7 ± 161

- differences between mean PCB concentrations in chicken and pork for the same category were not statistically significant ($p > 0.05$).

Concentrations between 50 and 200 ng PCBs/g fat, considered as moderately elevated, were found in 9.1% of chicken and 10.6% of pork samples tested. Meat from older pork (mean age of 30 months) contained significantly ($p<0.05$) higher levels of PCBs (44 ± 38 ng/g fat, n=578) than did young pork (6-7 months of age; 31 ± 72 ng/g fat, n=888).

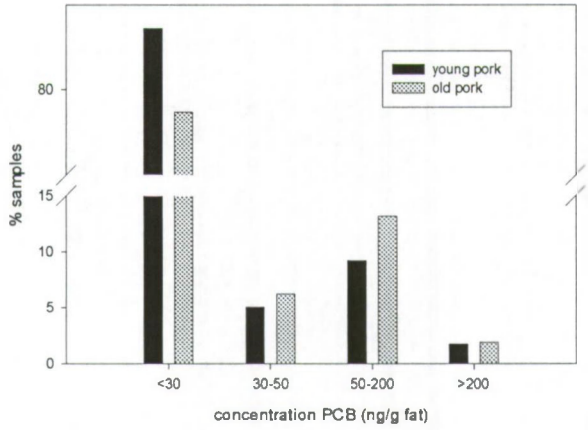

Figure 7.15. Concentrations of PCBs in Belgian export pork meat (n=1,466).

The high variability in the results is due to the fact that samples came from different breeders and food suppliers all over Belgium. Moreover, the percentage of samples with concentrations above 50 ng PCBs/g fat was higher (13.2 %) for old pork than for young pork (9.2 %) (Figure 7.15). These findings support the hypothesis that animals accumulate PCBs with age, due to continuous exposure (Hansen and Welborn, 1977).

Furthermore, the distribution of the 7 marker PCBs in export pork samples containing different concentrations of PCBs is identical (Figure 7.16). This might suggest that the accumulation PCB profile is similar at different doses which lead to low or very high PCB concentrations in the fat or that the profile of PCB contamination in the feed is similar for feeds with low (background) or relatively high concentrations.

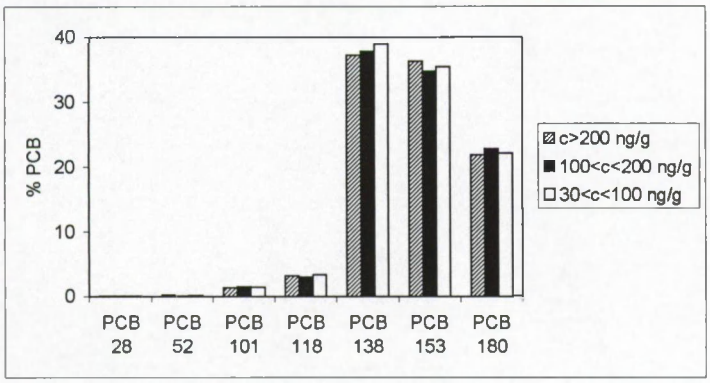

Figure 7.16. Distribution of the 7 marker PCBs in export pork samples with different load of PCBs.

We also measured the sum of p,p'-DDT (here as DDT) and its main metabolite, p,p'-DDE (DDE), expressed here as DDTs, in 750 of the above mentioned export meat samples. Percentage of samples with different concentrations of DDTs, together with mean and standard deviation are shown in Table 7.10.

Table 7.10. DDTs concentrations in export Belgian meat (n=750).

Concentration (ng DDTs/g fat)	Percentage of samples	C_{DDT} (ng/g fat) Mean ± SD	C_{DDE} (ng/g fat) Mean ± SD
<20	93.5	2.4 ± 1.8	12.1 ± 3.9
20-50	4.8	7.3 ± 4.6	24.3 ± 7.0
50-1000	1.4	61.4 ± 80.3	83.6 ± 61.2
> 1000	0.3	1011.5 ± 709.2	5021 ± 2829.8

In some samples, concentrations of DDTs exceeded the maximum limit set by the European Union of 1000 ng/g fat (EEC 93/57), up to 8,535 ng DDTs/g fat. Concentrations of PCBs and DDTs levels were not correlated (r^2=0.04), indicating different sources of contamination. In some cases low PCB levels were associated with high levels of DDTs. The mean DDTs/PCBs ratio was 1.9 ± 2.0.

Animal food chain

The data presented above indicate the existence of a diffuse background contamination of the animal food chain with PCBs and DDTs. To identify the sources of this background contamination, we measured fish flour (n=6) and grains (n=15) used in animal feed production for amounts of PCBs and DDTs (Figures 7.17 and 7.18). Fish flour contained a mean of 126 ± 121 ng PCBs/g fat, 25 ± 17 ng DDT/g fat and 86 ± 71 ng DDE/g fat with a mean DDT/DDE ratio of 0.33 ± 0.09. Fish flour from Peru showed significantly lower concentrations of PCBs and DDTs than the fish flours originating from Northern European Countries (Figure 7.14).

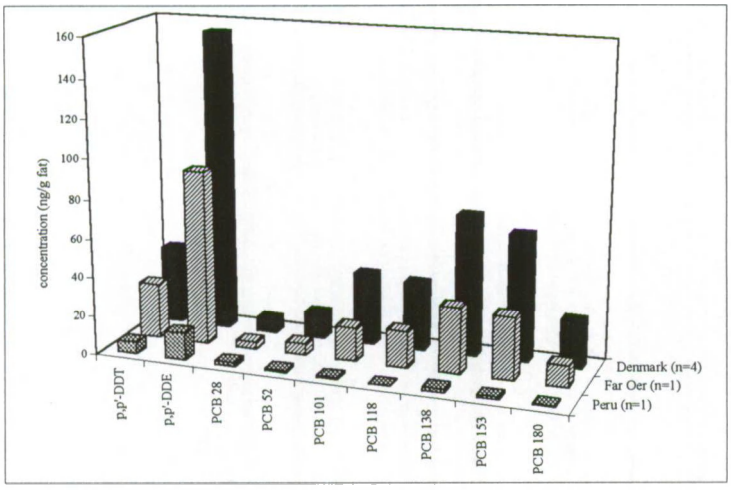

Figure 7.17. Organochlorine distribution in imported fish flour (n=6).

Grains for incorporation into animal feed (mostly imported in Belgium) contained a mean of 0.84 ± 0.21 ng PCBs/g and 0.33 ± 0.55 ng DDTs/g product with widely different DDT/DDE ratio varying from 0.034 to 92 (Figure 7.18).

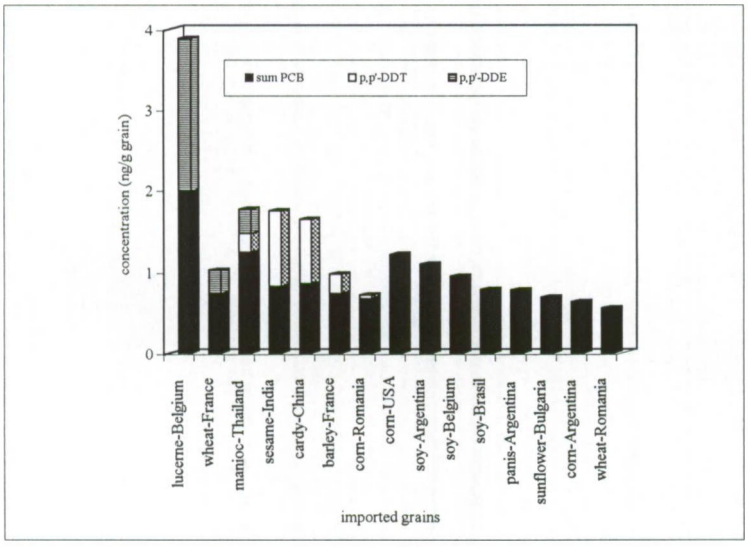

Figure 7.18. Organochlorine distribution in imported grains (n=15).

Taking into account the amount of animal feed that pigs and chicken eat before being slaughtered, their life span, and their body fat content, we could calculate that grains contribute with 15.4 ng/g to PCBs concentration in the pork body fat, while the contribution of fish flour is 1.1 ng/g. In chicken, grains contribute with 14.0 ng/g to the PCBs concentration in the body fat, while fish flour contributes with 6.2 ng/g. Thus, the grains and fish flour in animal feed account for 16.5 ng PCBs/g fat in pork and 20.2 ng PCBs/g fat in

chicken and can explain the concentrations that are found in most of the meat samples (with concentrations of PCBs lower than 30 ng/g fat).

The PCBs content of grains and fish flour does not explain the moderately elevated to high PCB levels (50 ng/g fat and higher) observed in 12.1% of the meat samples analysed. However, in animal feed for both chicken and pork, variable amounts of fat (on average 6.5% for chicken and 3% for pork) are incorporated in addition to grains and fish flour. In Belgium, approximately 20 companies collected residual animal fat from slaughterhouses and melt it into a homogeneous substance that is sold to animal food producers. Moreover, until recently it was a common practice to incorporate into this product household waste fat collected at community waste collection centres. It seems likely that this recycled fat sometimes contains considerable amounts of PCBs and that its use might be responsible for the higher PCB levels observed in 12.1 % of the meat samples included in this study.

The ratio between DDT and DDE diminishes in the course of time due to metabolism and offers some clues concerning the nature of the pollution source. A value close to 0.3 points to fish flour as the primary source of feed contamination with DDTs. A DDT/DDE ratio higher than 0.43, the highest ratio observed for fish flour, was obtained in some of the vegetable ingredients analysed (Figure 7.17). Moreover, such high values were seen in 30.6% of meat samples with a concentration of DDTs higher than 20 ng/g fat. These findings indicate recent contamination of animal feed with DDT due to large-scale incorporation of contaminated grains imported from countries where DDT is still legally in use (e.g., against malaria) or has very local illegal use. This last hypothesis is sustained by extremely high values (7,000 and 1,500 ng/g fat for DDE and DDT, respectively) observed in chicken samples from one farm.

The levels of background contamination with PCBs in Belgium are higher than those measured in Sweden (Glynn et al., 2000). In pork, for 1991-1997, after excluding three samples (out of 490) with abnormally high concentrations, Glynn et al. (2000) found PCB 153 concentrations with annual means between 1.2 and 3.1 ng/g fat, with a mean over 7 years of 1.9 ng/g fat. In Belgium, except for the most contaminated samples (0.6%), pork meat for export contained 25.2 ng PCBs/ g fat. Assuming a mean of 0.30 for the ratio between PCB 153 and sum of 7 marker PCB content (calculated from the 1,466 pork samples), the concentration of PCB 153 in Belgian export pork would be 8.5 ng/g fat. If feed ingredients would have been the sole sources of pork meat contamination with PCBs (previously calculated to account for 16.5 ng PCBs/g fat), the Belgian pork would still contain 2.9 times more PCBs than the Swedish pork. The higher concentrations in Belgian meat might be due at least partly to the common practice of incorporating contaminated fat of slaughtered animals (already containing a certain load of PCBs) into animal feed. Recycling of fats also means a recycling of PCBs.

Although the question of the carcinogenicity (as well as other health effects) of PCBs and DDTs in humans is far from being settled (Cogliano, 1998), there is a high concern for the continuous decrease of their concentrations in the food chain and humans. Instead of focusing only on accidental contamination, public policy should also aim to eliminate as much as feasible the many existing sources of diffuse contamination responsible for almost whole the human body burden. Together with dairy products and fish, animal meat is considered one of the most important sources of organochlorine pollutants in humans. Because concentrations of PCBs and DDTs are far below the existing tolerance limits in almost 90% of the meat samples analysed, it appears feasible and reasonable to lower the tolerance limits for these

hazardous substances without further delay. Our results demonstrate that the use of at least some types of recycled fat should be banned. Rigorous control of organochlorines in the animal feed production is a cost-efficient way to avoid large scale feed contamination incidents. In addition, public health requires a permanent monitoring of organochlorines in animals used for human consumption and in humans because of their top position in the food chain.

7.6. Determination of POPs in Romanian animal fat

* - based on Covaci A, Hura C, Schepens P, (2001). *Sci Total Environ*, 280 (1-3), 143-152.

Introduction
In a pilot study, assessment of pollution with organochlorine contaminants was done in animal fat collected from 2 major towns of Romania (Iassy and Arad).

Samples
Fat from retailed animals was collected from slaughterhouses in Iassy (17 samples of chicken, pork, sheep, and turkey) and from the market or private farms from Arad (6 pork samples). All samples were kept frozen at -20°C until analysed.

Results and discussion
Animal fat samples from Iassy showed higher concentrations of organochlorine pesticides, but similar PCB concentrations when compared with fat samples from Arad (Table 7.11).

Table 7.11. Levels of selected POPs (mean ± SD, ng/g fat) in animal fat from Romania.

Compound	Conc.	Iassy (n=17)			Arad (n=6)		
	(ng/g fat)	mean ± SD	range	Freq.	mean ± SD	range	Freq.
HCB	< 10	2 ± 1	2 – 5	16	1 ± 0.5	1 – 2	6
	> 10	34		1			
ΣHCH	< 200	81 ± 61	29 – 193	10	44 ± 54	ND – 138	6
(α–,β–,γ–HCH)	200-1000	626 ± 243	295 – 652	5			
	> 1000	1 337 and 12 368		2			
γ-HCH/ΣHCH		0.14 ± 0.17	0.02 – 0.60		0.30 ± 0.20	0.07 – 0.65	
	< 200	56 ± 25	10 – 92	11	85 ± 74	16 – 197	6
ΣDDT	200-1000	607 ± 242	376 - 948	4			
	> 1000	1 242 and 1 396		2			
p.p'-DDT/ΣDDT		0.28 ± 0. 12	0.09 – 0.52		0.37 ± 0.14	0.23 – 0.60	
Σ PCBs*	< 30	10 ± 9	2 – 30	14	4 ± 2	2 – 6	5
	> 30	45 ± 10	33 – 52	3	44		1
PCB 153/Σ PCB		0.34 ± 0.03	0.27 - .41		0.28 ± 0.04	0.24 – 0.33	

* - sum of PCB no. : 28, 52, 99, 101, 118, 138, 153, 170, and 180.

For the Iassy samples, the α–HCH isomer was the principal contributor to the sum HCHs (mean 60%), while in Arad samples, its contribution was reduced to 40%. Two chicken samples were exceeding the EU norms for HCHs in animal fat of 1000 ng/g fat (EU Directive, 1993). DDTs concentrations were lower in samples from Arad, while two samples from Iassy exceeded the EU norms of 1000 ng/g fat (EU Directive, 1993).

Higher ratios γ-HCH/Σ HCH and p,p'-DDT/Σ DDT were found in Arad samples. However, some samples from Iassy showed particular ratios correlated with high concentrations of HCHs and DDTs. Thus, a sample containing 1,242 ng/g DDTs and 1,338 ng/g HCHs showed high percentages of β-HCH (94%) and p,p'-DDE (78%), while another sample with 948 ng/g DDTs and 12,370 ng/g HCHs had high percentage of α-HCH (77%) and p,p'-DDT (52%).

A sample from an Iassy farm with a very high value of 12,368 ng HCHs/g fat had a composition matching that of technical lindane (65% α–, 10% β–, 15% γ– and 10% δ–, ε–HCH) suggesting a very recent use of it in that farm.

Due to partial metabolism, a different profile of pollutants is found in animal fat when compared with soil (see Chapter 9.2). Ratios p,p'-DDT/Σ DDT in animal fat are lower than in soil, due to partial metabolism to DDD and DDE. Ratios p,p'-DDT/Σ DDTs are higher in Arad fat samples, but DDTs concentrations are lower than in Iassy samples. This is probably due to a low scale recent contamination. All analysed samples contained PCB concentrations lower than the Belgian limit of 200 ng/g fat (Bernard et al., 1999).

As a general trend, samples included in this study contained lower organochlorine pesticide residues than samples from Eastern Romania collected between 1995 and 1999 (Hura et al., 1999) or between 1980-1995 (Hura 1995). However, p,p'-DDT/Σ DDT and γ-HCH/Σ HCH varied greatly in time due to the use of pesticide formulations in the specified areas.

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Chapter 8

Determination of POPs in aquatic biota

Abstract

Farmed and wild Scottish Atlantic salmon fish, aquaculture feed and fish oils used to supplement the feed were obtained from retail suppliers, producers and a fisheries research centre in Scotland during January 1999 for determination of a wide range of polychlorinated biphenyls (PCBs), organochlorine pesticides and polybrominated diphenyl ethers (PBDEs). Five additional European salmon samples obtained from the Belgian market in 2001 were also analysed. The study confirms previous reports of relatively high concentrations of PCBs, and indicates moderate concentrations of organochlorine pesticides and PBDEs in farmed Scottish and European Salmon. For the salmon samples obtained in Scotland, the PCB WHO-TEQ was twice that of polychlorinated dibenzo-*p*-dioxins and furans (PCDD/PCDFs). The results indicate that high consumption of salmon, particularly by children under 5 years, could lead to intakes above the tolerable daily intake (TDI) and tolerable weekly intake (TWI) for these chemicals, especially PCBs, in combination with mean or high level intakes from the UK diet. These results suggest further specific contaminant investigation of farmed salmon and salmon feed, including feed fortified with fish oil and feed fortified with selected vegetable oils.

Liver samples from 21 harbour porpoises (*Phocoena phocoena*) stranded on the Belgian North Sea coast were analysed for 59 PCB congeners, 10 organochlorine pesticides and 9 PBDEs.

PCBs were the most important organochlorine contaminants in the harbour porpoises. Two samples had more than 100 µg/g lipid for the sum of 59 congeners (359 and 404 µg/g lipid) and they were excluded from further calculations. For the remaining 19 samples, the mean concentration for 59 congeners was similar with those found in other studies. The major contributing PCB congeners were 153 (24%), followed by 138 (13%), 149 (8.1%), 187 (7.4%), 180 (6.8%) and 99 (4.2%). The most abundant organochlorine pesticides were DDT and its metabolites, followed by HCB and HCHs. The mean concentration for the sum of 10 PBDE congeners was 2290 ± 1790 ng/g lipid, ranging from 410 to 5810 ng/g lipid. The principal contributor was BDE 47 with a mean concentration.

Concentrations of PCBs, DDTs and PBDEs were significantly higher ($p < 0.05$) in the adult group (n=8) than in the juveniles (n=13). For HCB and HCHs, no difference was observed between the age groups. Concentrations of PCBs, DDTs, PBDEs and HCB were significantly higher in males (n=15) than in females (n=6), probably due to a loss of PCB load of females through gestation and lactation.

8.1. Analytical methodology for the determination of POPs in aquatic biota

Introduction

Aquatic biota and especially edible fish, are of interest because of their human consumption and their indication of contaminant's movement through aquatic and marine trophic levels. Samples of aquatic biota can often be extracted by techniques similar to those used for adipose tissue (see Chapter 6), since fish or other marine material can contain several percent lipids. Hot Soxhlet extraction was preferred to the classical Soxhlet extraction technique because the extraction time is decreased to 2 hours and because it shows similar extraction efficiencies for the target compounds. Moreover, due to a large spectrum of target analytes, a simple clean-up method has been proposed to allow high recoveries and the determination of the analytes from a single sample aliquot. Because relatively high concentrations of contaminants are expected in marine biota, the method has to be precise and accurate at both low and high contamination level.

Materials and methods

Sample preparation: The samples were thawed, filleted, skinned and the dorsal muscle was homogenised before being subdivided into smaller replicate portions of approximately 100 grams. The portions were weighted, stored in tightly sealed polythene bags and frozen at -20°C. The samples were sent packed in dry ice to the Toxicological Centre (Antwerp), where they were logged-in and stored at -20^{0}C prior to analysis. The samples obtained from Scotland were analysed for 59 PCB congeners (IUPAC no. 16, 17, 18, 20, 28, 31, 33, 44, 47, 48, 49, 52, 66, 70, 74, 84, 85, 87, 91, 95, 97, 99, 101, 105, 110, 114, 118, 123, 128, 130, 132,137, 138, 141, 144, 146, 149, 151, 153, 156, 158, 163, 167, 170, 171, 172, 174, 176, 177, 178, 179, 180, 183, 187, 194, 195, 196, 199 and 203). Seven PCBs were the mono-ortho PCBs 105, 118, 114, 123, 156, 167 and 189 with an assigned toxic equivalency value (TEF) [13]. In addition, the following PCBs (IUPAC no. 22, 56, 60, 135, 157, 191, 193, 200, 201, 202, 205, 206, 207, 208, 209) were tested for but not detected, and so they have been omitted from the results when calculating the mean with ND=1/2 LOD. All samples were also analysed for the following organochlorine pesticides: α-HCH, β-HCH, γ-HCH, HCB and DDT and metabolites (6 *op*- and *pp*-isomers) and for 9 PBDE congeners (IUPAC no. 28, 47, 66, 71, 75, 99, 100, 153 and 154). The procedures used to analyse the salmon samples were similar to those used to analyse organochlorine compounds in chicken and pork fat as described elsewhere (Schepens et al., 2001). For PBDEs, the method was described by Covaci et al., (2001). PCB 46, PCB 143, ε-HCH and ^{13}C-BDE 47, 99 and 153 were used as internal standards, while 1,2,3,4-tetrachloronaphthalene (TCN) and PBB 80 were used as recovery standards. All individual standards of PCBs and pesticides were obtained from Dr. Ehrenstorfer Laboratories (Augsburg, Germany). PBDE native and labelled standards were available from Wellington Laboratories (Guelph, Canada). Acetone, hexane, dichloromethane and iso-octane were of pesticide grade (Merck, Germany). Anhydrous sodium sulphate, basic aluminium oxide and silica gel (Merck) were used after heating overnight at 120°C. An accelerated Soxhlet extractor B-811 (Büchi, Switzerland) was used for the extraction of target compounds from fish tissues and feed.

Fish tissue: The fish fillet was defrosted at room temperature and approximately 10 g precisely weighted, ground with 20 g anhydrous sodium sulphate, and placed into an extraction thimble. 20 ng of the internal standards PCB 46, PCB 143, 10 ng ε-HCH and 1.5 ng of a mixture of ^{13}C-BDE 47, 99 and 153 were added.

Fish oil: 0.5 g fish oil was precisely weighted, and solubilised in 3 ml hexane. Twenty (20) ng of internal standards PCB 46, PCB 143, 10 ng of internal standard ε-HCH and 1.5 ng of

^{13}C-BDE 47, 99 and 153 were added and the mixture equilibrated in an ultrasonic bath for 5 minutes, after which it was ready for application to the SPE cartridges.
Fish feed: Approximately 3 g of fish feed were weighted accurately and placed into an extraction thimble. Twenty (20) ng of internal standards PCB 46, PCB 143, 10 ng ε-HCH and 1.5 ng of ^{13}C-BDE 47, 99 and 153 were added.

Porpoise liver: The liver was defrosted and approximately 5 g were accurately weighted and ground with 20 g anhydrous sodium sulphate till a fine floating powder was obtained. 20ng of internal standards PCB 46, PCB 143, 10 ng ε-HCH and 1.5 ng of a mixture of ^{13}C-BDE 47, 99 and 153 were added.

Extraction
The powder/fish feed was placed into an extraction thimble (Schleicher & Schuell, Dassel, Germany) and the mixture was extracted with 75 ml hexane:dichloromethane:acetone (3:1:1, *v/v*) for 2 h by hot Soxhlet extraction.

Fat content determination: The Soxhlet extract was concentrated and then transferred to a pre-weighted tube. The extract was completely dried under a gentle nitrogen stream and then kept at 60°C until constant mass was obtained. The measured weight represented the fat content of the sample.

Clean-up: The fat extract was solubilised in 2 ml of hexane and applied to a hexane pre-washed SPE cartridge filled (from the bottom to top) with 2 g deactivated alumina and 5 g acid silica. Thirty (30) ml of hexane were used for complete elution of all POPs. The final eluate was concentrated under nitrogen and recovery standards (1,2,3,4 – TCN and PBB 80) were added prior to GC analysis.

Organochlorines determination: All analyses were performed on a Hewlett Packard 6890 GC connected via direct interface with a HP 5973 mass spectrometer. A 50 m x 0.22 mm x 0.25 μm, HT-8 capillary column (SGE, Zulte, Belgium) was used with helium as carrier gas at a constant flow of 0.7 ml/min. One μl was injected in the pulsed splitless mode (splitless time 1 min). Injector and interface temperatures were set at 270°C and 300°C, respectively. The temperature program of oven was starting from 90°C, kept for 1 min, then with 15°C/min to 170°C, hold 3 min, then with 4°C/min to 270°C, hold 1 min and further with 10°C/min to 290°C, hold 15 min. The mass spectrometer was operated in electron impact ionisation mode. The three most abundant ions were monitored for each level of chlorination for PCBs or for each pesticide. Method limits of detection (LOD) for individual PCB congeners ranged between 0.1–0.5 ng/g lipid. For HCHs and DDTs, the detection limit was 0.2 ng/g lipid for each isomer. Recoveries of target compounds ranged between 72 and 80%.

PBDE determination: The analysis, previously described in Covaci et al, 2001, was performed on a Hewlett Packard 6890 GC equipped with a 10 m x 0.10 mm x 0.10 μm AT-5 (5% phenyl polydimethyl siloxane) capillary column (Alltech, Belgium) and connected via direct interface with a HP 5973 mass spectrometer. Helium was used as carrier gas at a constant flow (0.4 ml/min). The low-resolution quadrupole mass spectrometer (EI) was operated at 70 eV in the selected ion monitoring (SIM) mode. The ion source and quadrupole temperatures were 230 and 250°C, respectively. The electron multiplier voltage was set at 2300 V. The program of the AT-5 column was programmed from 70°C, kept for 2.2 min, then with 40°C/min to 230°C, then with 25°C/min to 280°C, hold 5 min. The run time was 13.2 min. Dwell times were set at 10 ms. The two most abundant ions (M^+ and $[M+2]^+$ for the

tri-, tetra-, and penta- congeners and $[M-2Br]^+$ and $[M-2Br+2]^+$ for the hexa-congeners) were monitored for each level of bromination for native and labeled PBDEs. Recoveries of internal standards, ^{13}C-labeled BDEs (calculated based on PBB 80 added prior to injection) ranged between 81 and 103% with a standard deviation of less than 21%. Method limits of detection (LOD) for individual PBDE congeners ranged between 0.1 and 0.4 ng/g lipid.

Quality control/Quality assurance. Retention times, ion chromatograms and intensity ratios of the monitored ions were used as identification criteria. A deviation of the ion intensity ratios within 20% of the mean values of the calibration standards was considered acceptable. The method performance was assessed through rigorous internal quality control, which included a daily check of calibration curves and regular analysis of procedural blanks and certified materials CRM 349 and 350 (PCBs in cod liver and mackerel oil) (Table 8.1). The method was tested through participation in an interlaboratory test organised by the Institute for Reference Measurements and Materials (IRMM, Geel, Belgium). Seven PCB congeners (no. 28, 52, 101, 118, 138, 153 and 180) were determined in non-spiked, medium- and high-level spiked pork fat. The results of the individual PCB congeners deviated less than 10% from the target values (see Chapter 6.1). For PBDEs, the analysis of two samples of biota (eel and porpoise liver) used for the first interlaboratory test on PBDE (de Boer, 2000), showed a variation of less than 10 % from mean values obtained from 18 participating laboratories.

Table 8.1. CRM analysis (all results expressed in ng/g lipid weight)

Compound	Type[a]	CRM 349		CRM 350	
		Our results	Reference	Our results	Reference
PCB 28	C	49	68±7	6[b]	22.5±4
PCB 52	C	145	149±20	51	62±9
PCB 101	C	351	370±17	148	165±9
PCB 118	C	468	456±31	131	143±20
PCB 153	C	979	938±40	299	318±20
PCB 180	C	288	282±22	59	73±13
PCB 44	I	65	75±4	30	43±5
PCB 47	I	69	74±3	n.a.	34
PCB 66	I	208	210±23	n.a.	112±21
PCB 141	I	54	68±5	22	28±4
PCB 138[c]	I	629	765±45	186	274±27
PCB 163[d]		205		93	
PCB 170	I	132	149±15	23	35±2
PCB 187	I	241	277±15	74	95±10
HCB	I	29	40±22	10	20±6
α-HCH	I	26	34±14	25	22±3
γ-HCH	I	65	72±12	52	44±14
pp-DDT	I	39	58±11	65	71±29
pp-DDE	I	223	237±31	84	89±13

-a: C-certified, I-indicative b: interferences
-c: PCB 138+PCB 163 (for the reference), only PCB 138 (for our results)
-d: separation of PCB 138 from PCB 163 possible on HT-8 column

Our laboratory also received a fish meal sample used for an interlaboratory test (PCBs in fish meal) organised in 2000 by the Joint Research Centre (Ispra, Italy). Different mixtures of solvents, amount of sample and extraction time were investigated and compared with the official value assigned by the organisers (Table 8.2).

Table 8.2. Influence of the extraction time, extraction solvent, amount of sample and internal standard on the lipid extraction and concentrations of PCB congeners in fish meal. The reference value was given by the organisers of the interlaboratory test.

Sample	Solvent	Time (h)	Lipid (%)	PCB congeners (ng/g lipid weight)							
				28	52	101	118	138	153	180	Σ PCB
1g, 20 ng IS	H:A 3:1	2h	11.1	7.0	5.9	39.0	25.1	89.8	125.1	36.8	328.7
1g, 20 ng IS	H:D 3:1	2h	10.8	6.7	< 5.0	37.6	25.7	87.9	120.7	35.6	316.7
1g, 20 ng IS	PB	2h	10.1	5.1	11.1	40.2	25.4	90.2	122.2	38.6	332.8
2 g, 15 ng IS	H:A 3:1	3h	11.9	8.2	13.5	34.9	20.7	88.8	128.9	38.9	333.9
2 g, 15 ng IS	H:D 3:1	3h	11.6	6.0	10.5	34.3	21.3	90.4	127.6	36.1	326.2
2 g, 15 ng IS	PE	3h	10.2	6.4	12.6	38.5	24.4	91.2	129.6	37.6	340.3
Mean			**11.0**	**6.6**	**9.4**	**37.4**	**23.8**	**89.7**	**125.7**	**37.3**	**329.8**
Reference			11.0	5.1	11.9	37.0	25.1	86.8	122.7	39.3	327.3

Observations:

1. There was no significant increase in the concentrations of extracted PCBs when extraction time was increased from 2 h to 3 h. Thus, an extraction time of 2 h was further used in the experiments.
2. Concentrations of PCB were higher when fish meal was extracted with petroleum ether, but the lipid content was too low. In contrast, extraction with a mixture of hexane and dichloromethane leads to the lowest concentrations of extracted PCBs. Thus, the hexane: acetone mixture was used for the extraction of POPs.
3. There was no significant effect on the concentrations of extracted PCBs when the amount of sample or internal standard was varied.

8.2. Determination of POPs in salmon, fish feed and fish oil

*- based on Jacobs MN, Covaci A, Schepens P, (2002). *Environ Sci Technol*, in press

Introduction

Although there is an extensive literature on the presence and fate of chlorinated organic compounds in the aquatic environment and biota, most data relate to the natural environment, and little has been published on chlorinated compounds in aquaculture systems or products from aquaculture. Data are available on concentrations of polychlorinated biphenyls (PCBs), and other organochlorine contaminants such as polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzo-*p*-furans (PCDFs) in fish from wild stocks, where exposure is associated with chronic contamination due to leaching of agricultural or industrial chemicals from treated or contaminated soils into surface waters and the global distribution and deposition by atmospheric transport (North Sea Task Force, 1993). With farmed fish, potential hazards associated with the ingredients and additives used in aquaculture feed were not considered significant until very recently (WHO, 1999; EC, 2000a,b).

Fish oil is a by-product of the fish meal manufacturing industry and originatess from many different parts of the world. Chlorinated compounds have low solubility in water and bioaccumulate in the food chain. As they accumulate in the lipid compartment of the animal, oil extracted from fish caught in polluted waters may be contaminated with chlorinated hydrocarbons (MAFF, 1997; Jacobs et al., 1998). Existing data on the levels of PCBs, PCDDs, PCDFs and organochlorine pesticides consumed in the UK have been mainly

derived from total diet surveys and surveillance data for specific food types (MAFF, 1996; FSA, 2000). These data indicate the presence of elevated organochlorine contamination of farmed fish. Of 161 salmon samples tested in 1997, all were found to contain residues of PCBs (ICES 7 set of PCBs) at concentrations of 23-620 ng/g lipid weight. The UK Ministry of Agriculture Fisheries and Food recently reported on a survey of marine fish sampled in 1995/6 (Parsley et al., 1999; MAFF, 1999). The mean TEQ concentrations of PCDDs, PCDFs and PCBs in 12 salmon samples were 25 pg TEQ /g lipid. In both these reports, the World Health Organisation Toxic Equivalent Factors (WHO-TEF) were not the recently revised values (Van den Berg, 1998). DDT and hexachlorobenzene are the predominant organochlorine pesticides detected in farmed salmon (MAFF, 1996).

Polybrominated diphenyl ethers (PBDEs) are widely used flame retardants that are also persistent and ubiquitous organic pollutants (POPs) and that may have endocrine disrupting effects (WHO, 1994; Bergman, 2000; Darnerud et al., 2001). Due to their low solubility in water and bioaccumulation in the food chain they accumulate in the lipid compartment of the animal and within the aquatic food chain in a similar and possibly more efficient way to other lipophilic contaminants such that fatty fish may be contaminated with appreciable amounts of PBDEs due to biomagnification (Burreau et al., 2000; Ohta et al., 2000; Akutsu et al., 2001). Concern about their effect on human health, particularly in infants, is increasing due to observations of increasing PBDE concentrations in human breast milk and human blood (Darnerud et al., 2001; Meironyté et al., 1999; Sjodin et al., 1999), although available data, including that reported here suggest that current levels of PBDE are an order of magnitude lower than those of PCBs. A major source of human exposure to the brominated congeners is through food, but there are other exposure routes. Existing data on the levels of PBDEs consumed in the UK is very limited, although these compounds are in extensive production and use. Data on PBDEs in wild fish from the Northern Hemisphere are available (Schroter-Kermani et al., 2000), and surveys to determine the distribution and fate of PBDEs in marine life in waters around the UK are underway (Allchin et al., 2000). However, there is little or no data in the public domain for aquaculture products and data for farmed fish is very limited (Akutsu et al., 2001).

The determination of the organohalogenated contaminants in fish and identification of the original sources of the contamination is, therefore, important for dietary exposure assessment and the protection of public health, particularly in view of the increasing availability to the consumer of farmed salmon. The farmed salmon industry is rapidly expanding; it has tripled in production between 1990 and 1998. The three major producers are Norway, the United Kingdom and Chile, but other countries, notably the US, are also increasing the growth rate of their salmon farming industries. It is known that fish is a significant source of organochlorine contaminants in the diet (EC, 2000a), and so consequently the use of fish meal and fish oil of European origin are considered to be of critical concern when used in diets for farmed fish and other food producing animals (EC, 2000b). The aquaculture sectors most dependent on the use of fish oil as a source of dietary lipids are the salmon and marine finfish industries.
Currently, the aquaculture industry accounts for around 17% of the global production of fish meal and oil but this has been predicted to double in the next 15 years by the FAO in Rome (Bell, 1998a). This is in line with the manufacturers prediction of UK consumption of fish oil in the next ten years, with a substantial reduction in the use of fish oils in food manufacturing (as hardened animal fats) and doubling of fish oil use in farming (Barlow, 1994). Fish oil is now being strongly promoted by the aquaculture industry as an aquaculture additive in a 'High Energy diet' that encourages fast growth of fish whilst minimising protein utilisation as

an energy source, and improves the nutritional value to humans through raised omega-3 fatty acid levels. Its use has increased significantly since 1995 from less than 30% to 33% and even 36.3% of the total diet for Salmon, in recent years (Bell, et al., 1998b).

To our knowledge, this is the first independent assessment of levels of organochlorine and brominated compounds in the farmed salmon food chain. Farmed European Salmon, feed and fish oil samples were analysed for a wide range of PCBs, organochlorine pesticides and PBDEs. The results of analyses of eight of the salmon samples for PCDDs and PCDFs and selected coplanar PCBs, carried out in 1999 have already been presented (Jacobs et al., 2000). The current paper builds on this initial study, including data on a further five salmon samples (obtained in March 2001), eight aquaculture feed samples and five oils (obtained in January 1999). These values are compared with fish and feed data reported in the literature. The potential contribution to the bioaccumulation of PCBs, organochlorine pesticides in addition to earlier data provided on PCDDs and PCDFs and selected PCBs in farmed salmon is discussed together with the potential contribution of fish oils to the bioaccumulation of PCBs in farmed fish. Suggestions on how to reduce these contaminant levels are indicated, whilst also achieving nutritional and environmental benefits in an economically viable way for both industry and the consumer.

Materials and methods

Sites and sampling: Seven British salmon samples (of which six samples were from individual fish, and one sample was a composite of two fish from the same source and of the same age and size) and one Norwegian salmon (*Salmo salar)* sample that enter the European fish market were analysed. The samples were of variable age, both farm raised and wild, and were obtained from five different Scottish sites, as documented previously (Jacobs et al., 2000). Whole body weights of the fish ranged from 400 g to 5.2 kg. In addition, five salmon samples, two originating from Ireland (one wild, one farmed) and three farmed samples for which no further information was available, purchased from the Belgian market, were analysed. The fresh and frozen samples were wrapped in polyethylene bags and frozen immediately at -20^0C. Eight salmon feeds were analysed (from four different Scottish sources) and five fish oils, one vegetable oil were analysed, (all but one of which were obtained from the same source, on the same date, but originally from widely varying sources). The fish oil and feed were not samples fed directly to the salmon collected as these were not available, but were collected from Scottish sites in January 1999. Table 8.3 gives complete sample details.

Sample preparation: The samples were thawed, filleted, skinned and the dorsal muscle homogenised before being subdivided into smaller replicate portions of approximately 100 grams. The portions were weighted, stored in tightly sealed polythene bags and frozen at -20°C. The samples were stored at -20^0C prior to analysis. The samples obtained from Scotland were analysed for 59 PCB congeners (IUPAC no. 16, 17, 18, 20, 28, 31, 33, 44, 47, 48, 49, 52, 66, 70, 74, 84, 85, 87, 91, 95, 97, 99, 101, 105, 110, 114, 118, 123, 128, 130, 132,137, 138, 141, 144, 146, 149, 151, 153, 156, 158, 163, 167, 170, 171, 172, 174, 176, 177, 178, 179, 180, 183, 187, 194, 195, 196, 199 and 203). Seven PCBs were the mono-ortho PCBs 105, 118, 114, 123, 156, 167 and 189 with an assigned toxic equivalency value (TEF) [13]. In addition, the following 15 PCBs (IUPAC no. 22, 56, 60, 135, 157, 191, 193, 200, 201, 202, 205, 206, 207, 208, 209) were tested for but not detected, and so they have been omitted from the results when calculating the mean with ND=1/2 LOD. All dioxin-like PCB levels compared favourably with those found in duplicate salmon samples earlier by another laboratory (Jacobs et al., 2000). The samples obtained in Belgium were analysed for 23

PCBs. All samples were also analysed for the following organochlorine pesticides: α-HCH, β-HCH, γ-HCH, HCB and DDT and metabolites (6 *op'*- and *pp'*-isomers) and for 9 PBDE congeners (IUPAC no. 28, 47, 66, 71, 75, 99, 100, 153 and 154).

Table 8.3. Sample details for the salmon, feed and fish oil.

Sample codes	Sample type and collection	Information	% lipid	Sample Weight (g)*	Lipid Weight (g)
M11		(f) 3+ yrs fresh	13.5	8.19	1.10
M12		(f) 2+ yrs fresh	14	8.13	1.14
M13		Norway frozen	13.7	8.39	1.15
M14	Salmon: Scotland	(w) 2+yrs frozen	4.9	9.73	0.47
M24	Jan 1999	(f) 3+ yrs fresh	19.9	9.69	1.93
M25		(f) 3+ yrs fresh	19.1	9.04	1.73
M28		(w)	12.3	8.19	1.17
M31		(f) N=2, Smolt, fresh	9.3	8.13	0.85
M18		(f) sm Bio.	3.9	4.60	0.18
M19	Salmon:	(f) Fresh Bio.	16.5	5.15	0.84
M20	Belgian Market	(f) Fresh	11.0	5.48	0.60
M21	March 2001	(w) Ireland, sm.	11.6	4.51	0.52
M22		(f) Ireland, sm.	14.8	7.25	1.07
M01		Fry 1-5g (A)	18.1	3.00	0.54
M02		1000-2200g (B)	30.1	3.00	0.90
M03		350-1000g (B)	31.0	3.03	0.94
M04	S. feed	1000-2200g (C)	35.9	3.07	1.10
M05	Jan 1999	Fry (C)	19.8	2.26	0.45
M15		500- 1300g (D)	28.1	2.00	0.56
M16		1300-2200g (D)	34.3	2.11	0.72
M17		500-1300g (D)	32.7	1.97	0.64
M06		Fish oil (C)	100	0.5	
M07		Fish oil (C)	100	0.5	
M08	Fish oils	Fish oil (C)	100	0.5	
M09	Jan 1999	Fish oil (C)	100	0.5	
M10		Vegetable Oil (C)	100	0.5	
MDEPA		Fish oil (E) March 01	100	0.5	

* - Sample weight taken for analyses; ND = Non Detects (treated as ½ LOD for calculations);
(w)=wild; (f)=farmed; Sources A, B, C, D, E denoted in brackets;
S. Feed=Scotland aquaculture salmon feed; Sm=smoked; Bio=bioculture;

Full details of the extraction, clean-up and analysis procedure for organochlorine and organobromine contaminants are given is Chapter 8.1.

Results

All samples examined contained detectable residues of organochlorine contaminants. Summary results are presented in Table 8.4, with total sum (Σ) values given using a value equal to ½ LOD where compounds were not detected. Detailed results of PCB concentrations together with TEQ values are presented in Table 8.5. All concentrations were adjusted to the lipid content of the sample by dividing the whole weight concentration by the percent lipid in each sample. *o,p'* isomers of DDTs were not found in any sample. PBDEs were detected in all fish samples, all feed samples but only one of the fish oil samples (see Table 8.6). The total concentration of selected PBDEs is the sum of concentrations of the congeners

measured and is an underestimate of the total BDE value as other isomers present were not measured.

Fish samples: PCB concentrations in the farmed samples were between 145.1 and 482.1 ng/g lipid weight. Profiles and concentrations of PCBs were similar and of the same order of magnitude for all the farmed samples, but lower in a Norwegian sample (M13) and a magnitude lower in a wild Irish sample (M21). In the fish samples (M11, M12, M14, M18-M20, M22, M24, M25, M29 and M31), the lower chlorinated congeners (tri-, tetra- and some penta-PCB congeners) had a higher contribution to the ΣPCBs than in the sample M13, where the PCB profile is dominated by the hexa- and hepta-PCB congeners. Together with sample M21, this sample contained the lowest PCB concentration. The mean of the 7 ICES marker PCBs was 129.5 ± 38.1 ng/g lipid and they constituted 41.1 ± 1.8 % of the total ΣPCB congeners for the Scottish salmon samples. In the Norwegian sample (M13), the 7 markers PCB concentration was 69.9 ng/g lipid weight and accounted for 48% of the total PCB concentration.
For the salmon samples obtained in Scotland (n=8), the mean concentration for HCB was 16.7 ± 12.2 ng/g lipid weight, but was much lower in sample M13 (under the detection limit of 0.2 ng/g lipid), the mean for all the salmon samples (n=13) was similar, at 11.6 ± 11.9 ng/g lipid. The mean concentration for HCHs (Σα, β, γ-HCH) was 13.8 ± 7.6 ng/g lipid for the samples obtained in Scotland and 8.5 ± 9.1 ng/g lipid for all the salmon samples (n=13). γ-HCH was the predominant HCH isomer with a mean ratio γ-HCH/ΣHCHs of 0.69 ± 0.01. For M13, the γ isomer accounted for 90% of sum HCHs. The mean concentration of DDTs (Σ*p,p'*-DDE, *p,p'*-DDD and *p,p'*-DDT) was 79.1 ± 74.4 ng/g lipid (n=8) and 69.6 ± 58.4 ng/g lipid for all the samples (n=13). Concentration of DDTs in M13 was much lower (5.1 ng/g lipid) and *p,p'*-DDE was the predominant contributor to the ΣDDTs. The ratio of *p,p'*-DDT/ΣDDTs ranged from 0.1 to 0.31 (mean 0.22). A higher ratio (0.25) was found for sample M13.

The PCB, organochlorine pesticide and PBDE contaminant loading of the bioculture farmed salmon (n=2) appears to be of the same magnitude as that of the farmed salmon, however the sample size is too small to draw any firm conclusions. Bioculture salmon diets are similar to that of farmed salmon with regard to fishmeal content, but the maximum fish oil content is 28% of the total diet (UK, 2000).

For the Scottish and Norwegian salmon samples, non-ortho PCBs and PCDD/PCDFs concentrations were previously measured (Jacobs et al., 2000). PCB-TEQ values were recalculated due to inclusion of other congeners with TEF values: PCB 123 and 114, using the WHO-TEF values for humans (van den Berg et al., 1998). However, their contribution to the PCB-TEQ value is not significant (less than 5%). As previously documented, it was found that the PCB fraction provide a greater contribution to the total TEQ value. The ratio PCB-TEQ/ dioxins-TEQ was 2.39 ± 0.60, while the total TEQ was 25.66 ± 9.92 pg TEQ/g lipid. With most of the fish samples, PCB 126 was the principal contributor (70%) to the PCB-TEQ, followed by PCB 118 (12.5%), PCB 156 (4.7%) and PCB 105 (3.6%). In sample M13, the contribution of PCB 126 was 82%, followed by PCB 118 (6.7%), PCB 156 (5.8%) and PCB 105 (2.0%).

Table 8.5. PCBs and TEQs concentrations in European (Scottish, Irish and Norwegian) Atlantic Salmon, aquaculture feed and fish oil (ng/g lipid weight)

	Sample	Sum PCBs[a]	Sum 7 ICES markers	%7 marker /sum	sum PCBs[b]	MO-PCB-TEQ	NO-PCB-TEQ	PCB-TEQ	diox-TEQ	TEQ	PCB/ Diox-TEQ
		ng/g lipid			ng/g lipid	pg TEQ/g lipid					
Salmon	M11	375.5	162.8	43.4	273.9	5.8	20.7	26.5	11.5	38.1	2.3
	M12	300.5	119.5	39.8	205.7	3.7	6.7	10.4	5.1	15.4	2.1
	M13	145.1	70.1	48.3	115.4	2.9	14.4	17.3	6.2	23.5	2.8
	M14	459.9	183.0	39.8	318.3	6.8	18.7	25.5	18.2	43.7	1.4
	M24	279.1	118.4	42.4	202.0	4.1	11.0	15.1	6.8	21.9	2.2
	M25	277.1	116.6	42.1	199.5	3.9	13.2	17.1	6.1	23.2	2.8
	M28	300.0	125.9	42.0	217.5	4.1	12.8	16.9	5.0	21.9	3.4
	M31	261.4	100.4	38.4	180.8	3.7	8.3	12.0	5.6	17.6	2.1
	M18	NA	140.4	-	226.6	2.9	NA	NA	NA	NA	NA
	M19	NA	167.9	-	265.1	3.0	NA	NA	NA	NA	NA
	M20	NA	141.0	-	219.8	2.6	NA	NA	NA	NA	NA
	M21	NA	60.6	-	94.0	1.1	NA	NA	NA	NA	NA
	M22	NA	176.6	-	284.4	3.4	NA	NA	NA	NA	NA
Feed	M01	124.0	49.4	39.9	81.5	2.3	NA	NA	NA	NA	NA
	M02	1049.5	258.6	24.6	456.8	2.8	NA	NA	NA	NA	NA
	M03	678.2	168.6	24.9	299.7	2.1	NA	NA	NA	NA	NA
	M04	182.0	68.4	37.6	130.3	2.6	NA	NA	NA	NA	NA
	M05	81.2	31.7	39.1	55.8	1.2	NA	NA	NA	NA	NA
	M15	163.6	65.4	40.0	113.8	2.5	NA	NA	NA	NA	NA
	M16	183.5	74.8	40.8	131.9	2.9	NA	NA	NA	NA	NA
	M17	156.4	63.8	40.8	111.6	2.5	NA	NA	NA	NA	NA
Fish oil	M06	72.9	27.7	38.0	48.5	1.1	NA	NA	NA	NA	NA
	M07	8.2	2.4	39.3	4.7	0.2	NA	NA	NA	NA	NA
	M08	251.8	101.7	40.4	185.9	0.3	NA	NA	NA	NA	NA
	M09	17.7	7.9	44.6	12.4	0.3	NA	NA	NA	NA	NA
	M10	5.8	0.7	12.1	2.3	0.2	NA	NA	NA	NA	NA
	MDEPA	450.2	220.6	49.0	353.0	7.7	NA	NA	NA	NA	NA

a- sum of 59 congeners; b- IUPAC no. 28, 52, 101, 118, 138, 153 and 180;
c- sum of 23 PCB congeners as measured in samples M18-M22; NA-not available;
ND-not detected (values below limit of detection were substituted with ½ LOD, upper-bound method)

All fishes contained detectable levels of PBDEs, the sum of the BDEs (ΣBDEs) ranged from 1.2 to 85.2 ng/g lipid for the thirteen salmon samples. The highest levels were found in a wild (or possible farm escapee) salmon sample. The levels of PBDEs found in the salmon analysed in this study are comparable with those found in other cultured fatty fish, particularly gray mullets and yellow tails reported recently in a Japanese study (Akutsu et al., 2001), and in wild fish (Bergman, 2000). As expected, BDE 47 dominated the profiles, with a mean ratio BDE 47/sum BDEs was 0.53 ± 0.04. The highest correlation coefficients in the salmon samples were calculated between PCBs and DDTs. Pearson correlation coefficients between PCBs-DDTs, PCBs-PBDEs and DDTs-PBDEs were 0.57 ($p<0.05$), 0.28 ($p=0.36$) and 0.03 ($p=0.91$), respectively.

Aquaculture feeds: Concentrations of PCBs in the eight feed samples ranged between 76 and 1050 ng/g lipid. Six feed samples presented the same profile of PCBs, with the penta- and hexa-PCB congeners representing approximately 80% of the total PCB content. The 7 marker PCBs constituted approximately 40% of the total ΣPCB congeners. As expected, the concentration of PCBs were high (147 ± 41 ng/g lipid) probably due to the high fish oil content of some samples (up to 35%), indeed the feeds with higher fish oil contents had

Table 8.4. Summary of PCBs and organochlorine pesticides in European (Scottish, Irish and Norwegian) Atlantic Salmon, aquaculture feed and fish oil (ng/g lipid weight)

	Sample codes	PCBs								HCB	HCHs				DDTs				
		Σ tri	Σ tetra	Σ penta	Σ hexa	Σ hepta	Σ octa	Σ PCBs[a]	Σ 7 markers[b]		α-HCH	β-HCH	γ-HCH	Σ HCHs	*pp*-DDE	*pp*-DDT	*pp*-DDD	Σ DDTs	*pp*DDT /ΣDDT
Salmon	M11	13.5	50.3	113.4	151.8	42.5	3.8	375.5	162.8	19.5	6.8	ND	16.1	23.0	71.4	10.5	22.7	104.7	0.10
	M12	33.2	46.2	79.6	110.2	29.4	2.0	300.5	119.5	13.1	4.7	ND	10.3	15.1	50.1	8.9	18.4	77.5	0.11
	M13	1.5	1.5	25.2	75.1	38.3	3.8	145.1	69.9	ND	1.1	ND	9.7	10.9	2.8	1.3	1.0	5.2	0.25
	M14	20.6	76.1	145	167.2	47.3	3.6	459.9	183.0	43.5	4.0	2.0	12.4	18.5	123.0	40.1	46.0	249.5	0.16
	M24	10.2	34.2	80.4	112.6	37.1	4.6	279.1	118.4	15.6	9.7	ND	ND	9.8	29.0	9.1	8.7	46.9	0.21
	M25	10.2	34.5	79.5	111.3	37.0	4.6	277.1	116.6	15.7	10.8	ND	ND	10.9	29.6	10.3	8.5	48.5	0.26
	M28	9.2	29.8	77.6	131.5	47.3	4.8	300.0	125.9	13.8	ND	ND	ND	ND	30.0	12.0	3.3	45.4	0.29
	M31	19.5	33.1	71.4	105.2	29.7	2.5	261.4	100.4	12.6	ND	8.7	14.1	22.9	33.1	14.0	9.3	56.5	0.25
	M18	7.2	8.3	71.5	112.1	26.9	0.6	226.6[c]	140.4	ND	ND	ND	ND	ND	37.1	9.2	ND	46.4	0.20
	M19	7.5	18.6	65.1	130.7	40.3	2.9	265.1[c]	167.9	ND	ND	ND	ND	ND	42.2	19.1	ND	61.4	0.31
	M20	4.4	21.4	63.2	103.1	26.5	1.2	219.8[c]	141.0	ND	ND	ND	ND	ND	46.1	18.5	ND	64.7	0.29
	M21	2.8	8.6	29.0	40.6	12.0	1.0	94.0[c]	60.6	4.1	ND	ND	ND	ND	38.8	11.5	ND	50.5	0.23
	M22	5.5	16.9	79.9	134.6	43.8	3.7	284.4[c]	176.6	12.0	ND	ND	ND	ND	39.2	9.2	ND	48.5	0.19
Feed	M01	14.6	15.8	29.2	53.7	9.8	0.6	123.8	49.3	2.3	4.2	ND	11.2	15.5	22.1	7.6	5.9	35.7	0.21
	M02	688.4	306.3	84.4	43.4	10.1	0.2	1153.2	258.7	4.2	31.0	ND	15.7	46.8	25.6	4.6	7.8	38.1	0.12
	M03	412.6	210.4	65.4	52.8	8.0	0.1	749.4	168.6	4.9	15.0	ND	12.5	27.6	23.0	4.3	7.7	35.1	0.12
	M04	3.3	18.1	63.5	81.0	14.4	0.9	181.3	68.5	5.9	4.6	1.8	36.6	43.1	24.4	4.7	9.6	38.8	0.12
	M05	2.0	5.9	23.0	40.9	3.7	ND	75.6	31.7	0.6	2.3	ND	ND	2.4	21.4	5.4	6.9	33.8	0.16
	M15	1.6	26.8	50.6	72.6	11.0	0.6	163.1	65.5	7.6	5.6	ND	15.7	21.4	33.5	6.0	12.2	51.7	0.12
	M16	3.8	27.8	57.4	79.4	14.7	0.8	181.0	74.7	9.3	5.2	ND	9.5	14.8	35.6	2.8	12.8	51.3	0.05
	M17	3.9	22.9	49.8	68.8	10.6	0.4	157.3	63.7	7.1	5.9	ND	12.7	18.7	30.4	6.0	11.4	47.9	0.13
Fish oil	M06	1.4	11.5	24.3	30.9	3.7	ND	74.0	27.7	3.7	6.7	ND	5.1	11.9	19.3	3.4	7.2	30.0	0.11
	M07	0.3	ND	1.5	1.3	ND	ND	8.8	2.2	1.5	1.3	ND	1.4	2.8	7.8	3.8	3.7	15.4	0.25
	M08	0.2	15.2	51.5	132.6	46.3	4.4	252.5	101.8	0.2	4.7	ND	15.7	20.5	36.1	5.5	12.3	54.0	0.10
	M09	0.5	ND	2.9	9.2	1.6	0.0	18.6	7.9	0.2	2.0	2.0	1.5	5.6	7.4	1.6	1.8	10.9	0.15
	M10	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.1	ND	14.5	15.7	ND	ND	ND	ND	ND
	MDEPA	0.6	31.5	130.4	225.6	57.5	4.7	450.3[c]	220.6	2.3	4.2	0.9	4.4	9.6	152.4	21.4	44.4	218.3	0.10

ND = not detected (1/2 LOD); [a] 59 PCB congeners; [b] ICES 7 marker PCBs: IUPAC no's; 28, 52, 101, 118, 138, 153, 180

[c] 23 PCBs: 28+31, 52,74,101,99,110,118,105,149,153,138+163,128+167,156,187,183,180,199,170+190,194

higher PCB contents also. Two samples from the same manufacturer (M02 and M03) presented a total different profile of PCB congeners when compared with the other six feed samples. Tri and tetra congeners (approximately 55 and 28%, respectively) dominated in both samples, and the profile was close to that of Aroclor 1242. This different profile correlated with the high PCB concentrations found for these two samples (the highest concentrations detected in all the samples) and could point to the existence of local PCB sources or illegal dumping of used Aroclor mixtures in the animal food chain (see the Belgian PCB poisoning episode-summer 1999 (Schepens et al., 2001; Covaci et al., 2001a). The mean for HCB was 9.2 ± 2.9 ng/g lipid (n=8), for HCHs (Σα, β, γ-HCH) the mean was 23.7 ± 14.9 ng/g lipid, and γ-HCH was the predominant HCH isomer with a mean ratio γ-HCH/ΣHCHs of 0.63 ± 0.16. The mean for the ΣDDTs was 42 ± 8 ng/g lipid, with *p,p'*-DDE the predominant contributor to the ΣDDTs (*p,p'*-DDE mean 27 ± 6 ng/g lipid, *p,p'*-DDT mean 5.2 ± 1.4 ng/g lipid). The mean ratio *p,p'*-DDT/ΣDDTs was 0.13 ± 0.04. The levels of PBDEs ranged from 8.1 to 23.8 ng/g lipid for the eight feed samples. The fry feed samples, with a far lower fish oil content, had lower residue levels than the feed designed for smolts and adult salmon with a fish oil content over 20%.

Table 8.6. Selected PBDEs in European Atlantic salmon, aquaculture feed and fish oils (ng/g, lipid weight).

	Sample	BDE 28	BDE 71	BDE 47	BDE 75	BDE 66	BDE 100	BDE 99	BDE 154	BDE 153	sum BDEs	BDE47 /sum
Salmon	M 11	1.3	7.1	29.3	0.3	2.0	6.5	4.9	1.3	1.0	53.7	0.55
	M 12	0.9	4.4	14.7	ND	ND	2.9	2.8	0.7	0.8	27.2	0.55
	M 13	ND	ND	0.4	ND	ND	ND	0.7	ND	ND	1.1	0.37
	M 14	0.8	1.7	12.9	ND	0.9	2.4	5.9	ND	ND	24.6	0.52
	M 24	1.5	7.1	28.9	0.2	2.2	6.4	5.0	1.6	ND	52.9	0.55
	M 25	1.5	7.1	28.0	0.4	1.8	6.1	4.4	1.0	ND	50.3	0.56
	M 28	2.0	7.9	43.0	0.3	3.2	9.9	14.0	3.6	1.3	85.2	0.50
	M 31	1.3	6.7	25.0	0.5	1.6	5.6	4.0	0.9	ND	45.6	0.55
	M 18	ND	ND	3.1	ND	ND	ND	ND	ND	ND	3.1	-
	M 19	0.5	2.8	10.2	ND	0.8	2.8	1.6	0.2	ND	18.9	0.54
	M 20	0.5	2.4	9.4	ND	0.4	1.5	1.5	ND	ND	15.7	0.60
	M 21	0.2	0.7	5.0	ND	0.1	1.1	1.3	ND	ND	8.4	0.59
	M 22	1.0	8.1	25.8	0.7	1.7	7.9	5.6	1.3	ND	52.1	0.49
Feed	M 01	0.3	1.4	7.0	ND	ND	ND	ND	ND	ND	8.7	0.81
	M 02	0.5	3.1	8.2	0.2	0.5	1.5	1.7	ND	ND	15.6	0.53
	M 03	0.5	3.1	8.2	ND	0.7	1.5	1.4	ND	ND	15.4	0.54
	M 04	0.7	4.3	12.3	0.3	0.7	1.8	2.7	ND	ND	22.8	0.54
	M 05	ND	1.4	5.1	ND	ND	0.9	0.7	ND	ND	8.1	0.63
	M 15	0.6	4.6	13.2	ND	ND	2.3	2.3	ND	ND	23.0	0.57
	M 16	0.6	4.4	13.0	0.3	0.9	2.4	2.2	ND	ND	23.8	0.54
	M 17	0.5	3.0	9.5	ND	ND	1.8	1.4	ND	ND	16.2	0.59
Fish oil	M 06	0.3	2.7	7.1	ND	ND	1.4	1.2	ND	ND	12.7	0.56
	M 07, 08, 09, 10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-

ND = not detected

Fish oils: The fish oil samples showed varying PCB contents of 8.8, 18.6, 74.0 and 252.5 ng/g lipid (ND = ½ LOD). Hexa and penta-PCB congeners dominated the PCB profile for all samples. The same profile was found in fish oil used for diet supplement (sample MDEPA) which contained higher PCB amounts (450 ng/g lipid). However, the tri- and tetra-PCB

congeners were higher contributors to the total sum in the fish oil M06 than in the fish oils M08 and MDEPA. Furthermore, the hepta- and octa- congeners were more important for the last two oils. The difference in profiles can be due to different species used for collection of the oil and to different processing procedure. During the oil processing (usually a steam distillation), some of the low chlorinated PCB congeners can be lost through their higher volatility. The ICES 7 marker PCBs constituted approximately 40% of the total sum of PCB congeners (for the samples with high content of PCBs, M06, M08 and MDEPA). Samples M09 and M10 had a high number of measurements below the limit of detection, but the contribution of the ICES 7 PCBs (where the concentrations detected were above the detection limit) was higher, reaching 70%.

Mean HCB concentrations were 2.5 ± 1.5 ng/g lipid, mean HCHs (Σα, β, γ-HCH) were 10 ± 7 ng/g lipid and again γ-HCH was the predominant HCH isomer with a mean ratio γ-HCH/ΣHCHs of 0.50 ± 0.21. Mean ΣDDT concentrations were 66 ± 87 ng/g lipid and pp-DDE was the predominant contributor to the ΣDDTs, the mean ratio *pp*-DDT/ΣDDTs was 0.15 ± 0.07. The levels and ratios of the HCB, HCHs and DDT are reflected in the aquaculture feed, and the DDT levels show a narrower range in the feeds. PBDE concentrations ranged from ND to 12.7 ng/g lipid for the four fish oils and one vegetable oil samples.

Comparison between the three types of samples: The three types of samples were compared using median values for all groups of compounds (Figure 8.1). Except for HCHs, all other groups of contaminants show an increase in concentration in the order fish oil < feed < salmon. Samples with concentrations close to the median value can be considered as representative for each type of sample. The following samples were chosen for this purpose: M06 for fish oil, M04 for feed and M24 for salmon.

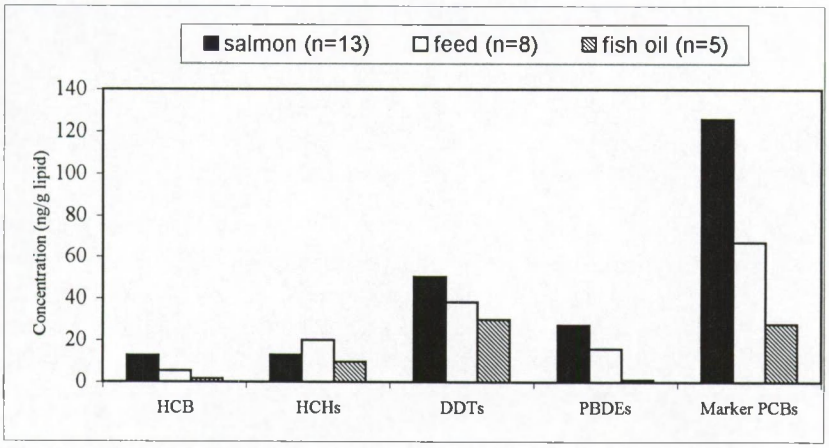

Figure 8.1. Median concentrations of HCB, HCHs, DDTs, PBDEs and marker PCBs in salmon, feed and fish oil samples

When comparing individual congeners (Figure 8.2), a higher contribution of PCB 18, 28, 31 (tri-PCBs), 66 (tetra-PCB), 101, 105 (penta-PCBs), 138 (hexa-PCBs) and all hepta and octa-PCB congeners was found for the salmon, together with a lower contribution of PCB 47/48, 49, 52 (tetra-PCBs), 87, 110 (penta-PCBs), 132, 146 and 149 (hexa-PCBs). The profiles of homologues were similar, with an increase of contribution of hepta- and octa-PCBs in the fish.

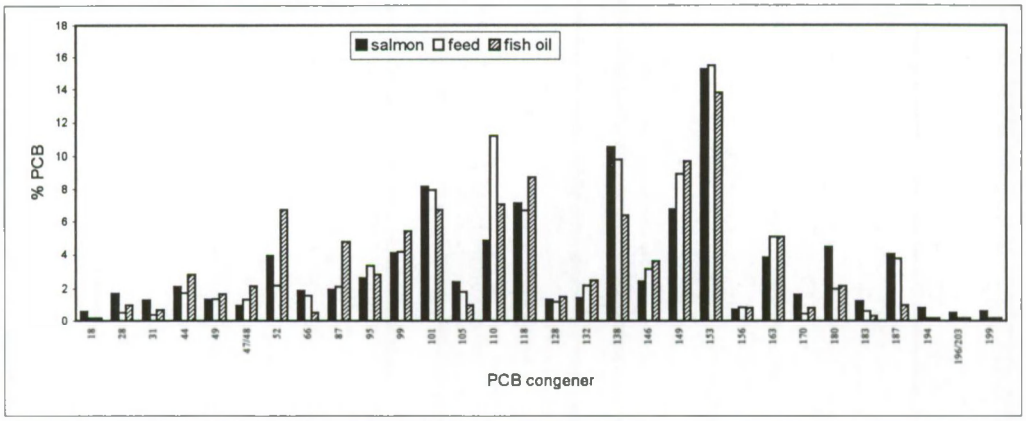

Figure 8.2. Profiles of individual PCB congeners in salmon, feed and fish oil.

Profiles of DDTs were similar in all three types of samples. For HCHs, the α-HCH isomer had a higher contribution in the fish oil, while the γ-HCH isomer had a lower contribution. The β-HCH isomer was in most cases under the detection limit. With a constant contribution to the total PCB content, the ICES 7 PCBs appear to be reliable predictors of the PCB contamination profile through all the samples. For PBDEs, BDE 47 dominated the profiles, as expected, ranging between 50 to 100% of the ΣBDEs included in the analyses. There was no significant difference in the PBDE profiles for the three matrices. Samples with higher PCB contents generally showed higher levels of the pesticide residues, but this was not the case with the PBDEs, indicating the existence of different pollution sources.

Discussion

The PCB results from this study are in good agreement and of a similar order of magnitude to the values for salmon reported in recent studies (Parsley et al., 1999; MAFF, 1999) (see Table 8.7). The comparability of the PCB data suggests that both data sets can be considered fairly representative of PCB concentrations in British farmed salmon. Comparisons with background data from the MAFF study suggest that for wild species such as cod, the PCB concentrations on a lipid adjusted basis are of a similar magnitude, but somewhat higher. This increases on a whole weight basis due to the higher lipid content of the salmon. Species with a higher fat content in the body tissues, such as herring, are reported to have consistently higher organochlorine concentrations both on a lipid adjusted and whole weight basis (Tables 8.7 and 8.8). However, mackerel seems to be an exception, containing relatively low PCB concentrations. Flat fish with a low fat content such as plaice, approach the concentrations seen in herring on a lipid adjusted basis (Parsley et al., 1999), but not on a whole weight basis. Previous reports have detected significant levels of PCBs and organochlorine pesticides in fatty fish such as herring and salmon (Bergqvist et al., 1990; Atuma et al., 1998; Becher et al., 1998).

Table 8.7. Comparison of mean congener specific PCB (ng/g lipid weight) with means of Salmon samples from this study (n=8) and Parsley et al., 1999 (n=12).

PCB congener	Salmon Mean ± SD (ng/g lipid weight)		Herring Mean ± SD (ng/g lipid weight)
	This study (n=8)	MAFF 99 (n=12)	MAFF 99 (n=10)
18	2.8 ± 1.8	1.3 ± 0.6	1.2 ± 0.5
28	7.4 ± 8.7	4.3 ± 1.3	8.0 ± 3.6
31	4.0 ± 1.6	2.7 ± 0.8	3.5 ± 1.5
47	3.0* ± 1.0	2.1 ± 0.6	4.4 ± 2.2
49	4.4 ± 1.9	4.0 ± 1.2	4.4 ± 2.1
52	10.2 ± 3.7	10.2 ± 3.4	17.8 ± 8.7
99	10.2 ± 4.2	9.6 ± 2.9	29.8 ± 14.2
101	19.0 ± 9.0	22.8 ± 6.8	55.7 ± 25.9
105	5.5 ± 1.8	6.3 ± 1.8	20.3 ± 10.5
114	0.8 ± 0.4	0.4 ± 0.1	0.9 ± 0.5
118	18.3 ± 6.5	19.0 ± 5.8	60.3 ± 28.7
123	1.4 ± 0.5	0.9 ± 0.3	2.4 ± 1.2
128	2.7 ± 0.6	4.6 ± 1.4	14.7± 7.2
138	25.8 ± 5.4	34.3 ± 10.0	110.5 ± 46.8
153	37.0 ± 8.7	37.2 ± 11.1	129.7 ± 70.0
156	1.9 ± 0.4	1.8 ± 0.6	5.3 ± 3.1
167	1.0 ± 0.3	1.1 ± 0.3	2.5 ± 1.3
180	10.9 ± 2.7	11.6 ± 4.5	40.7 ± 20.4
Lipid (%)	13.3 ± 4.9	13.6 ± 3.3	9.6 ± 2.9

*PCB 47/48

Table 8.8. Comparison of mean PCB concentrations (pg/g, ppt, unless otherwise indicated) in various fish from the Northern hemisphere (data from Parsley et al., 1999).

Compounds	Herring (n=10)	Mackerel (n=13)	Cod (n=30)	Plaice (n=13)
	Mean ± SD (pg/g, ppt)			
Σ21 MO- PCBs (lwt)*	514.3 ± 237.1	106.7 ± 66.5	117.9 ± 162.8	276.5 ± 142.6
MO- PCB TEQ lwt	12.8 ± 6.3	2.3 ± 1.5	2.9 ± 3.7	6.8 ± 3.4
MO- PCB TEQ wwt	1.3 ± 0.8	0.4 ± 0.3	0.01 or less	0.08 ± 0.04
NO-PCBs TEQ lwt	47 ± 28	11.6 ± 7.3	11.1 ± 14.4	34.8 ± 13.9
NO-PCBs TEQ wwt	4.9 ± 3.3	2.0 ± 1.6	0.11 ± 0.20	0.4 ± 0.2

*ng/g, ppb; lwt = lipid weight; wwt = whole weight.

The 7 marker PCB concentrations in the farmed salmon samples from this study and a Dutch study (RIVO, 2000) were very similar. The Dutch study found PCB concentrations of 110 and 170 ng/g lipid in farmed salmon from Norway, while farmed salmon from Scotland contained 135 and 210 ng/g lipid ranging from 61 to 183 ng/g lipid. The Dutch study also found farmed salmon from Norway or Scotland had relatively low TEQ values (less than 4 pg TEQ/g whole weight). Assuming a mean value of 20% of the lipid content (as presented in the study), the TEQ value becomes approximately 20 pg TEQ/g lipid, similar with or

somehow lower than values found in our study (see Table 8.5). Mean ratio PCB-TEQ/Diox-TEQ in the farm raised salmon from Norway and Scotland was close to 2, similar with the value found by Jacobs et al (2000).
Wild salmon from polluted waters was found to contain higher concentrations of PCBs. Thus, in Baltic salmon, Asplund et al. (1999) reported values up to 3500 ng/g lipid for the sum of 6 PCB congeners. In Lake Michigan Coho and Chinook salmon (*Oncorhynchus kisutch* and *Oncorhynchus tshawytscha*), mean concentrations of PCBs (78 congeners) were 39870 and 53638 ng/ g lipid (Jackson et al., 2001). These concentrations are much higher than in the farmed salmon analysed in this study.

PCB profiles are more species dependent for fish and birds than for mammals due to selective metabolism depending upon the degree of chlorination. Indeed, levels of PCBs are higher in fish compared with other farmed raised animal (such as chicken, beef or pork) intended for human consumption. It was shown that fish species are able to accumulate lower chlorinated PCB congeners (such as tetra- and penta-PCBs). Some of these congeners have the highest TEF values among PCBs. Thus, in comparison with other species (birds or mammals), the ingestion of fish would lead to a higher intake for PCBs in general and in particular for dioxin-like PCBs (see Chapter 7.4).

The concentrations of organochlorine pesticides in salmon compare favourably with those reported for farmed salmon obtained during 1997 in the UK. Sum DDT concentrations ranged from 10 to 40 ng/g whole weight (n=14), γ-HCH concentrations ranged from ND in 11 samples to 2 in 3 samples, and HCB ranged from 2-5 ng/g whole weight (n=14). The UK does not have statutory Maximum Residue Levels (MRLs) for these pesticides in fish, although they exist for other food products (MAFF, 1999).

The average concentration across all salmon samples of the ΣBDE congeners was 33.7 ng/g lipid. Moreover, the results from this study are an order of magnitude lower than the respective PBDE values for salmon reported in recent studies from polluted open waters. Previous reports have detected significant levels of PBDEs in fatty fish such as sprats, herring and salmon from the Atlantic and more polluted waters such as the Baltic (Bergman, 2000; Darnerud et al., 2001) showing age related accumulation and biomagnification. The concentrations of PBDEs in the Atlantic salmon were markedly lower than Lake Michigan Coho and Chinook salmon (Manchester-Neesvig et al., 2001), (mean 2440 ng/g lipid, range 773 - 8120 ng/g lipid). Salmon from Lake Michigan tributaries in the US appear to have the highest PBDE concentrations so far reported world wide, several orders of magnitude greater than the concentrations reported here, but with a similar congener distribution (Manchester-Neesvig et al., 2001).
PCB contamination for the farm-raised salmon comes exclusively via aquaculture feed, while for the wild salmon sources are more diffuse, but clearly biomagnify through trophic levels (Jackson et al., 2001).
One of the routes of food chain contamination may be partly caused by ingestion of plastic resin pellets (used in the manufacture of plastics) by the marine wild fish. PCBs, DDE and nonylphenols (NP) were detected in polypropylene (PP) resin pellets collected from four Japanese coasts (Mato et al., 2001). Concentrations of PCBs (4-117 ng/g), DDE (0.16-3.1 ng/g) and NP (0.13-16 μg/g) varied among the sampling sites and were comparable to those for suspended particles and bottom sediments collected from the same area as the pellets. Comparison of PCBs and DDE concentrations in marine PP resin pellets with those in seawater indicates a likelihood of a high accumulation potential, as observed in wild salmon

feeding in polluted waters, suggesting that plastic resin pellets (unintentionally released) may provide both a transport medium and a potential source of toxic chemicals in the marine environment.

There is very little literature available for PCB and organochlorine pesticide comparison in fish feed. Fish meal, a principal component of fish feed, shows a much higher degree of contamination with organochlorines of North Atlantic stocks than those from South Pacific. Thus, fish meal originating from Denmark and the Faroe Islands contained 250 and 120 ng/g lipid for the 7 marker PCBs, respectively, while fish meal from Peru had a concentration of 10 ng/g lipid (Schepens et al., 2001). TEQ values for PCDDs and PCDFs in fish meal, have been reported to range between 3.28 - 8 pg I-TEQ/g (n=5), and 2.02-2.96 pg I-TEQ/g in 3 fish oil samples originating from UK, while in similar stocks from Chile, the mean values were 0.14 and 0.61 pg I-TEQ/g (CX/FAC, 2001). ICES 7 PCB data in nine compound feed (poultry, cattle, pig) samples marketed in the UK and sampled in 1999 ranged from <0.5 to 6.6 ng/g dry matter (MAFF, 2001) and were similar with concentrations found in samples not contaminated with PCBs collected during the Belgian PCB food contamination from 1999 (Covaci et al., 2001a). The PCB and organochlorine pesticide content in fish oils is highly variable (Jacobs et al., 1998) and of known concern for animal feeds (WHO, 1999).

The PCB concentrations in fish oils from this study are consistent with the study by Parsley et al (1999), reporting surprisingly high levels of PCB congeners. From the literature, one might speculate that the source of contamination was from herring and fish oil based aquaculture feed consumed by the salmon (Bergqvist et al., 1990). A previous study (Jacobs et al., 1998) has found relatively high levels of PCBs 101, 118, 138, 149, 153, 170, 180 in both the herring oil sample from Germany and salmon oil sample. For example, the concentration of PCB 118 was 60 ng/g lipid in the salmon oil and 158 ng/g lipid in the herring oil. The average concentration of the ΣBDE congeners in the analysed feed samples was 16.6 ng/g lipid. At the present time, comparative data are not available in the public domain. The target PBDEs were not detected in all but one of the oil samples, a fish oil from the Northern hemisphere, with a sum PBDE concentration of 12.7 ng/g lipid. The data available in the literature concerning organohalogen contamination of fish oils varies widely, with higher contamination levels observed from more polluted Northern hemisphere waters (Falandysz et al., 1994; Jacobs et al., 1998).

The preliminary data presented here on the analyses of salmon aquaculture feeds and fish oil components of the feeds for organochlorine pesticides and selected PCBs indicate that this is a probable route of contamination, especially when comparing the farmed to the wild salmon samples, although the wild salmon samples were insufficient in this study to make any conclusive comparisons. Wild salmon from Lake Michigan have been shown to have a significant relationship with the congener concentrations in lower trophic levels of invertebrates. PCB congeners biomagnify 20-30 fold, with a more efficient transfer of penta- and hexa-PCBs through the pelagic food web (Jackson et al., 2001).

The risks of exposure to organochlorine contaminants from eating relatively contaminated fish have been known for many years, but how to quantify the risks to human health is frequently disputed. PCBs and *p,p'*-DDE provide the bulk of organochlorine residues in human tissues and there is growing evidence of the insidious effects of background exposure (Longnecker et al., 1997). Regarding the possible exposure of humans through the diet, there is a lot of variability in regulations for PCB and pesticide concentrations in food world wide.

Many EU member states do not have national maximum limits for concentrations of PCBs in food (EC, 2001a). As example, in Holland, the current norm for fish designated for human consumption is 620 ng/g lipid for the sum of the 7 marker PCBs (RIVO, 2000), a value much lower than the concentrations observed in the Baltic or Lake Michigan salmon. Due to comparatively lower toxicity, the risk assessment of non-dioxin-like PCBs have been given a lower priority than dioxin-like PCBs, but with the provision that the PCB risk assessment (IPCS, 1993) should be updated on a congener specific toxicological evaluation to provide an adequate risk assessment in the near future. The risk assessment of PCDD/Fs and the dioxin-like PCBs using the TEF system assesses toxicity through the Ah receptor alone (Brouwer et al, 1999). This does not estimate the risks of organochlorine pesticides and PCBs for health and potential endocrine related health effects for which the literature is extensive. The possible contribution to dietary intakes of organochlorines from farmed fish could be significant for high consumers, and may be congener specific, similar to the species being consumed, as recently observed in a Finnish study (Kiviranta et al., 2001) and observed in high fish eaters generally (Asplund et al., 1994; Humphrey et al., 2000).

In terms of risk assessment, the potential contribution to the human diet of PCBs, organochlorine pesticides and PBDEs from farmed European Salmon will vary according to the diet and age of the fish, the frequency of consumption, portion size, cooking practices and the age of the consumer. The possible contribution to dietary intakes of organohalogen compounds from farmed salmon could clearly be significant for high consumers, particularly pregnant women and breast feeding mothers.

Further investigation of farmed salmon and salmon feed, including feed fortified with fish oil and feed fortified with selected vegetable oils, are warranted, together with consistent regular vigilance by health authorities for PCBs organochlorine pesticides and PBDEs, to monitor and reduce current human dietary exposure. While PCBs and many organochlorine pesticides have been banned in most of the world, they are still being delivered in the diet and European farmed salmon can be a significant source. In addition, in some cases there are suggestions of recent usage, particularly of DDT. Whilst diets based on marine fish oils are currently favoured by the aquaculture industry, it is likely that these oils are contributing greatly to the contamination of farmed salmon by POPs. The degree of variations in levels of contaminants for the feed and fish oil data indicate the significance of changes in feedstock and variations in oil sources and processing procedures that can result in batch–to-batch differences in contaminant levels even in the final product from a single manufacturer. The generation of additional data is needed to further assess the importance of batch variation in animal feeds.

Fish oil originating from seas around the equator and eventually from the less polluted Southern hemisphere should be recommended, because they contain far less organohalogenated contaminants. Furthermore, aquaculture diets utilising vegetable oils (with both n-3 and n-6 fatty acids), and having fatty acid compositions which resemble those of invertebrates that comprise the natural diet of salmon, could be more beneficial in accommodating successful sea water adaptation than diets based on marine fish oils (Bell, 1998a). Vegetable oil based diets could also facilitate the requirement for high-energy aquaculture feed on an economically competitive basis, whilst reducing the problems of organochlorine contamination. This would reduce the reliance on fish meal and oils from non-sustainable natural resources and demonstrate sensitivity to public confidence after the recent PCB and dioxin food crises, producing a nutritious food using environmentally friendly methods, delivering easily utilised essential n-3 fatty acids to the human consumer.

8.3. Determination of organohalogen contaminants in liver of stranded harbour porpoises (*Phocoena phocoena*) on the Belgian North Sea coast.

*- based on Covaci A, De Coen WM, Bouquegneau JM, Blust R, Schepens P, (2001). *Mar Pollut Bull*, submitted

Introduction

The harbour porpoise (*Phocoena phocoena*) is one of the smallest cetaceans in the world. At birth, they are not longer than 65-85 cm, while at adult life, they can reach between 1.4 and 1.9 m. Females are larger than males and they weigh between 55 and 65 kg. Their mean life span is between 3 to 5 years, while occasionally individuals can reach 13 years. They live in small groups of 2 to 5 individuals. Their diet is very varied and consists mainly of herring (*Clupea harengus*), mackerel (*Scomber scombrus*), sprat (*Sprattus sprattus*) and whiting (*Merlangius merlangius*). Daily, they eat an equivalent of 4 to 9.5% of their total body weight. Seasonal migrations can occur and their trajectory follows the availability of food supply. In summer they stay in coastal waters (not deeper than 150 m), while in winter they move to open waters, together with a north-south migration. Thus, they might be considered indicators of coastal pollution and high concentrations of anthropogenic contaminants in the organism are expected from animals living in polluted seas (such as the North or Baltic Seas).

The harbour porpoise is listed as a vulnerable species in the European waters. During recent decades the number of observations of harbour porpoise has drastically decreased, especially in the Baltic and North Seas. One of the main threats to the harbour porpoise is the accidental capture in different fishing gear. High levels of contaminants, declining fish stocks and other anthropogenic stress factors are also considered potential threats to them. Organohalogen contaminants (such as PCBs and DDTs) have been found at levels that may present risk to cetaceans. This is a serious cause of concern for the already threatened harbour porpoises. The relationship between organochlorines and reproductive, endocrine and immunological disorders has also been strongly suspected in marine mammals from highly contaminated areas (De Guise et al., 1995) The capacity to metabolize PCBs and DDTs is known to be low in small cetaceans compared to birds and terrestrial mammals (Tanabe et al., 1988; Boon et al., 1997). Thus the less efficient metabolism of DDTs and *ortho*-substituted PCBs in cetaceans compared to seals and terrestrial mammals could also lead to greater biomagnification and adverse health effects.

Here, concentrations of organohalogenated contaminants, including organochlorine pesticides, PCBs and PBDEs (reported for the first time) were measured in 21 harbour porpoises stranded between 1997 and 2000 on the Belgian North Sea coast. In contrast with other studies reporting concentrations of organohalogenated compounds in blubber of marine mammals, liver was used to assess the contamination in the Belgian porpoises as different biomarker analyses and gene expression assays were conducted on the same samples.

Samples

Liver samples were obtained from 21 harbour porpoises stranded at the Belgian North Sea coast between 1997-2000 and were kept at –20°C until analysis. All samples to be analysed were cut out from the middle of the tissue sample to avoid possible contamination from handling and storage. Life-history data for the porpoises are presented in Table 8.9. No information about the health condition of the animal at the time of stranding was available.

Methods

Complete method details were given in Chapter 8.1.

Table 8.9. Life-history data for the harbour porpoises stranded on the Belgian North Sea Coast (1997-2000).

Liver	Code	Stranding year	Amount (g)	% fat	Age	Sex	Length (cm)	Weight (kg)	Blubber thickness (mm)
1	**A97/759**	1997	24.42	1.63	j	M	108	21	11
2	**A97/850**	1997	2.27	3.28	j	F	125	39	35
3	**A97/944F**	1997	4.57	3.94	j	F	118	28	25
4	**A97/967**	1997	2.22	3.63	j	M	118	22	10
5	**A97/1237F**	1997	4.28	4.06	a	M	151	39	11
6	**A98/356F**	1998	4.7	9.17	a	M	144	33	13
7	**A99/707**	1999	2.92	6.15	a	F	134	30	15
8	**A99/923**	1999	4.55	1.57	j	F	116	17	8
9	**A99/924**	1999	4.56	3.79	j	M	106	17	4
10	**A99/1041F**	1999	3.95	7.12	j	M	103	16	8
11	**A99/1094**	1999	3.96	3.08	j	M	108	15	8
12	**A99/1374F**	1999	3.99	21.74	j	M	80	7	6
13	**00/212**	2000	4.05	10.75	j	M	99	16	8
14	**00/258**	2000	21.45	6.42	j	M	108	18	10
15	**00/307**	2000	4.47	7.48	a	M	142	41	10
16	**00/308F**	2000	4.38	3.92	a	F	151	42	10
17	**00/342F**	2000	4.02	6.74	a	M	144	43	14
18	**00/559**	2000	3.58	3.41	a	M	149	37	8
19	**00/600F**	2000	4.39	11.96	j	M	103	27	8
20	**00/974**	2000	4.44	15.78	j	F	114	22	20
21	**00/1019**	2000	3.87	3.24	a	M	150	39	9

j- juvenile; a- adult

Results

The most abundant organochlorine pesticides were DDT and its metabolites, followed by HCB and HCHs (Table 8.10). Dieldrin, reported to be an important contaminant in cetaceans, was not measured in this study, due to its degradation during the clean-up on acidified silica gel. Mean and standard deviations (SD) concentrations of DDTs were 3380 ± 2310 ng/g lipid (range 250-44 330 ng/g lipid), with a contribution of 69 % from the p,p'-DDE isomer (range 55 to 77%). As to the importance, p,p'-DDD was the second isomer (mean 760 ± 550 ng/g lipid, range 70-14550 ng/g lipid) followed by p,p'-DDT (mean 200 ± 300, range ND-1860 ng/g lipid). Two porpoise samples contained extreme values of DDTs (16820 and 44330 ng/g lipid, repectively) and they were not included in the calculations of means. For HCB, the mean concentration was 650 ± 380 ng/g lipid, range: 130 to 5680 ng/g lipid. HCH concentrations (sum of α–, β–, γ- isomers) were ranging from 40 to 2250 ng/g lipid with a mean of 180 ± 150 ng/g lipid. The γ-HCH isomer was the most abundant isomer with a contribution in average of 96% to the total HCH concentration (range 71 to 100%). The α- and β-HCH isomers were detected only in 5 and 3 samples, respectively, out of 21.

PCBs were the most important organochlorine contaminants in the harbour porpoises. Two samples had more than 100 µg/g lipid for the sum of 59 congeners (359 and 404 µg/g lipid) and they were excluded from further calculations. These abnormal values are probably not due to the analytical procedure since other parameters (concentrations of PBDEs, HCHs, HCB and lipid percentage) were all within the range observed in the other 19 samples. For these remaining 19 samples, the mean concentration was 36.40 ± 26.40 µg/g lipid (Table

8.11).

Table 8.10. Means, standard deviations, medians and ranges of concentrations for the organochlorine pesticides measured in the liver of 21 harbour porpoises stranded at the Belgian North Sea coast.

Compounds	Nr samples	Mean ± SD	Median	Range
			ng/g lipid	
HCB	21	650 ± 380	600	130- 5680
α–HCH	21	10 ± 30	nd	nd – 130
β–HCH	21	10 ± 50	nd	nd - 210
γ-HCH	21	170 ± 130	140	40 - 1910
Σ HCHs	21	200 ± 150	180	50 - 2250
γ-HCH/ Σ HCHs	21	0.96 ± 0.08	1.00	0.71 – 1.00
p,p'-DDE	19	2310 ± 1470	1930	170 – 24350
o,p'-DDD	19	160 ± 150	120	nd – 2680
p,p'-DDD	19	760 ± 550	560	70 – 14550
o,p'-DDT	19	100 ± 200	10	nd – 900
p,p'-DDT	19	200 ± 300	50	nd – 1860
Σ DDTs	19	3380 ± 2310	2620	250 - 44330
p,p'-DDE/ Σ DDTs	19	0.69 ± 0.06	0.70	0.55 – 0.77

Table 8.11. PCB concentrations in harbour porpoises stranded at the Belgian North Sea coast.

Compounds	No. samples	Mean* ± SD	Median	Range
			ng/g fat	
PCB 153	21	8.5 ± 7.1	7.0	0.3 – 98.8
Tri-PCB	21	0.1 ± 0.1	0.1	nd – 0.4
Tetra-PCB	21	1.0 ± 0.6	0.9	0.2 – 16.5
Penta-PCB	21	4.0 ± 2.6	3.8	0.5 – 37.1
Hexa-PCB	21	20.7 ± 15.4	18.4	0.9 – 241.5
Hepta-PCB	21	9.2 ± 7.5	7.4	0.3 – 99.5
Octa-PCB	21	1.2 ± 1.2	1.0	nd – 8.6
Nona-PCB	21	0.1 ± 0.1	0.1	nd – 0.8
Deca-PCB	21	0.1 ± 0.1	0.1	nd – 0.3
Sum PCBs (70 congeners)	21	36.4 ± 26.4	33.8	1.9 – 404.5
Sum 7 congeners**	21	17.8 ± 13.7	15.2	0.8 – 198.4

* - two extremely high values were not included
** - IUPAC no. 28, 52, 101, 118, 153, 138, 180

To enable comparison with other studies, the mean of the 7 ICES PCB congeners (IUPAC no. 28, 52, 101, 118, 138, 153 and 180) was calculated and found to be 17.8 ± 13.7 µg/g lipid, ranging from 0.8 to 198.4 µg/g lipid. The 7 ICES PCBs constituted on average 48% of the total sum of 70 PCB congeners. Similar with what was found in another study (Karlson et al., 2000), the major contributing PCB congeners were 153 (24%), followed by 138 (13%), 149 (8.1%), 187 (7.4%), 180 (6.8%) and 99 (4.2%). The mean concentration of PCB 153 was 8.5 ± 7.1 µg/g lipid. The median concentration was 7.0 µg/g lipid. Furthermore, the hexa-PCB congeners dominated the profile (58%) followed by hepta- (26%) and penta-PCBs (11%) (Figure 8.3).

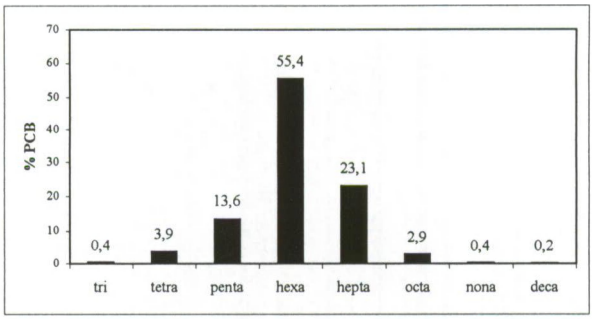

Figure 8.3. Relative distribution of PCB homologues in harbour porpoise liver.

PBDEs were found in relatively high concentrations. The mean concentration for the sum of 10 congeners was 2290 ± 1790 ng/g lipid, ranging from 410 to 5810 ng/g lipid (Table 8.12). The median value was 2180 ng/g lipid.

Table 8.12. Concentrations of PBDEs in harbour porpoise liver.

Compounds	Nr samples	Mean ± SD	Median	Range
			ng/g lipid	
BDE 28	20	10 ± 10	5	nd - 30
BDE 47	20	1060 ± 860	900	190 – 2830
BDE 66	20	10 ± 10	nd	nd – 40
BDE 71	20	50 ± 50	30	nd – 210
BDE 99	20	350 ± 320	270	30 – 990
BDE 100	20	360 ± 320	260	50 – 960
BDE 153	20	200 ± 170	160	10 – 580
BDE 154	20	240 ± 210	170	10 - 610
Sum PBDEs (8 congeners)	20	2290 ± 1790	2180	410 – 5810

In contrast with PCB values, there were no extremely high values for PBDEs, the range was relatively small (one order of magnitude). As found in other studies (Zegers et al., 2001; Law et Allchin, 2001), the principal contributor was BDE 47 with a mean concentration of 1060 ± 860 ng/g lipid and ranging from 190 to 2830 ng/g lipid. Other important PBDE congeners were BDE 99, 100, 154 and 153. These congeners are routinely detected in marine biota.

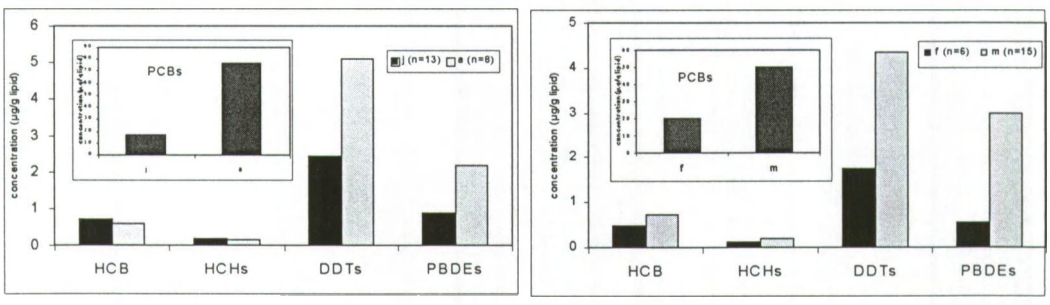

Figure 8.4. Distribution of median concentrations of organohalogens with age (a) and sex (b).

Concentrations of PCBs, DDTs and PBDEs were significantly higher ($p < 0.05$) in the adult group (n=8) than in the juveniles (n=13) (Figure 8.4a). For HCB and HCHs, no difference

was observed between the age groups. Concentrations of PCBs, DDTs, PBDEs and HCB were significantly higher in males (n=15) than in females (n=6), probably due to a loss of PCB load of females through gestation and lactation (Figure 8.4b).

Furthermore, it was shown that the rate of increase in PCB body burden in males is higher than in females (Figure 8.5).

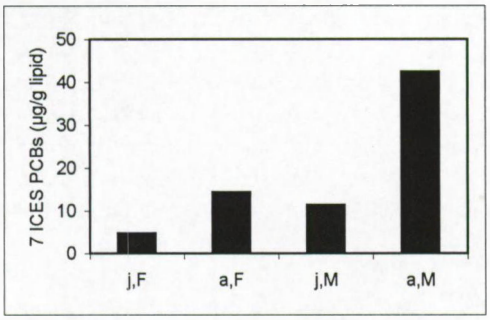

Figure 8.5. Distribution of median for the 7 ICES PCBs in males and females function of age.

It was suggested in other studies (van Scheppingen et al., 1996) that in harbour porpoises, the TEQ toxicity of PCBs is more important than the PCDD/PCDF TEQ toxicity. TEQ values for mono-ortho PCBs (MO-PCBs) were calculated in Table 8.13. These values are similar with values obtained from specimens caught in the Black Sea.

Table 8.13. TEQ values (pg TEQ/g lipid) for mono-ortho PCBs in 21 porpoise livers.

PCB	TEF	j,M (n=9)	a,M (n=6)	j,F (n=4)	a,F (n=2)
105	0.0001	18.7	9.9	13.6	12.6
114	0.0005	14.7	12.3	8.0	2.4
123	0.0005	1.1	1.1	0	0
118	0.0001	100.6	72.8	78.1	70.5
156	0.0005	23.6	14.7	19.6	20.3
157	0.00001	3.9	0.0	0	0
167	0.0005	0.8	0.6	0.7	0.5
189	0.0001	2.6	3.6	2.4	4.0
Total MO-PCBs		166.0	115.0	122.4	110.3

Discussion

The liver of harbour porpoises contained a wide variety of organohalogenated contaminants, as would be expected in a coastal ichthyophagous odontocete. Contemporary contaminant levels in other harbour porpoises populations have been mainly reported for PCBs and DDTs. As previously shown (Berggrena et al., 1999), p,p'-DDE was the main contributor to the total DDT. Concentrations of p,p'-DDT and total DDT were relatively low, lower than those reported from North Sea (Granby and Kinze, 1991) or Baltic Sea porpoises (Bruhn et al., 1999). This is in accordance with the continuous decline in DDT concentrations in biota observed after the ban in the '70s. The p,p'-DDE/sum DDTs (value) is similar with the ratio found by Bruhn (1999) in specimens from North Sea, but higher than the ratio observed in samples from the Arctic. One explanation might be that marine mammals and fish have induced levels of the cytochrome P450-1A and 2B enzymes capable of metabolizing p,p'-DDT (Boon et al., 1998). Higher values of the ratio indicate old input of DDT to the environment. Concentrations of HCHs showed a similar trend with lower values for the most important isomer (γ-HCH) and the sum of HCHs observed in this study is comparable with

previous studies from North Sea (Bruhn et al., 1999) and Baltic Sea (Berggrena et al., 1999) porpoises. The α- and β-HCH isomers were found to have a low contribution to the sum of HCHs for the specimens caught in the North Sea. However, in Arctic animals (Bruhn et al., 1999), higher concentrations of α-HCH were measured. This might be due to atmospheric long-range transport and to the photochemical isomerisation of γ- to α-HCH.

PCB concentrations found in the porpoises stranded on the Belgian North Sea coast are also in agreement with concentrations reported in the literature. The mean value for the sum of the 7 ICES PCB congeners (17.8 μg/g lipid) was similar with mean values found in samples from the Baltic region (mean ranging from 11 to 46 μg/g fat, function of the location and year) (Berggrena et al., 1999). Moreover, these concentration s compare favourably with concentrations measured in harbour porpoises captured on the English and Dutch coast of the North Sea or on the Welsh Atlantic coast (Table 8.14) (Law et al, unpublished data).

Table 8.14. Values of the 7 ICES PCBs (μg/g lipid) in harbour porpoises collected in different locations from Scotland, Wales, England, the Netherlands and Belgium.

Location	Sex	n	ICES 7 PCBs (μg/g lipid)	
			Mean ± SD	Range
Moray Firth	F	10	2.2 ± 1.5	1.0 to 5.3
Cardigan Bay	F	11	9.4 ± 7.1	2.9 to 23.5
E coast of England	F	21	11.0 ± 6.8	0.2 to 22.1
Netherlands	F	12	13.4 ± 8.3	2.6 to 26.5
Belgium (this study)	**F**	**6**	**9.5 ± 9.1**	**0.8 to 18.8**
Moray Firth	M	11	6.3 ± 4.7	1.0 to 16.7
Cardigan Bay	M	10	22.3 ± 18.7	0.3 to 64.8
E coast of England	M	35	14.1 ± 12.4	2.8 to 50.9
Netherlands	M	10	24.7 ± 13.4	9.1 to 43.6
Denmark	M	12	12.7 ± 9.7	5.5 to 37.6
Norway	M	22	10.5 ± 6.3	2.0 to 23.7
Belgium (this study)	**M**	**15**	**19.0 ± 23.1**	**4.0 to 58.4**

Higher concentrations of organochlorine compounds were found in porpoises stranded on the Belgian/Dutch coast of the North Sea in comparison with the English coast (Table 8.14). This is likely to be caused by the discharges from the Rhine, Meuse and Scheldt estuaries or the coastal currents from the French to the Dutch coast. Not too long ago harbour porpoises from the Dutch coast of the North Sea (Smeenk, 1987) or seals (Ries et al., 1999) had high enough PCB levels to compromise their reproductive capacity. A concentration gradient for organochlorine pollutants is expected for the North Sea, with the highest values in the Southern part. This hypothesis is in accordance with the data presented in Table 6, as the porpoises stranded on Belgian coast have higher PCB concentrations than the Danish porpoises.

High concentrations of PCBs were already measured in blubber of porpoises from the North Sea (47-160 μg/g lipid) (Duinker et al., 1989). de Yanés and Buthe (1998) have found relatively high concentrations of PCB (sum of 51 congeners) in young (median: 31.6μg/g lipid, range: 5.7-180.3), female (64.7 μg/g lipid, range: 5.7-192.6) and male (139.6 μg/g lipid, range: 68.7-247.1) seals from the North Sea. Moreover, in comparison with a porpoise population from Iceland, the North Sea group contained much higher concentrations of PCBs and had a much higher variation between individuals (de Yanés and Buthe, 1998).

Lower PCB concentrations than those measured in the North Sea were found in samples from the Gulf of St. Lawrence and Bay of Fundy/Gulf of Maine, NW Atlantic (Westgate et al., 1997). In 62 harbour porpoises from the Gulf of St. Lawrence, PCB blubber concentrations (sum of 68 congeners) in males and females were 10.6 ± 5.4 and 7.2 ± 3.9 μg/g fresh weight, respectively. These concentrations were lower than those measured in 105 samples from the Bay of Fundy/Gulf of Maine where PCB concentrations in blubber were 17.3 ± 11.2 and 11.4 ± 4.8 μg/g fresh weight in males and females, respectively. These values have shown a decrease in concentration of organochlorine contaminants in porpoises during the last 2 decades in the NW Atlantic. Similar trends have been seen in the Baltic or Arctic populations (Bruhn et al., 1999).

However, some individuals from this study showed extremely high PCB values (up to 198.8 μg/g lipid for the sum of 7 ICES markers) which might be due to local pollution or discharges. Resuspension and run-off from old deposits of PCBs and DDTs are possibly higher in locations closer to industrialised regions. It has been shown (Kleivane et al., 1995) that for harbour porpoises, there is no large-scale migration between different locations and that these mammals are a good indicator of local pollution. Another possible explanation might be a particularly bad health condition of the animal (unfortunately no information on the health status at the time of catching is available). It was shown (Jepson et al., 1999) that, due to infectious diseases, animals can loose weight rapidly. This will lead to a thinning of the blubber layer (in parallel with a redistribution of contaminants from the blubber) and thus to an increased concentration of organic pollutants in the blubber. However, these values are at the lower end of the concentrations (up to 3000 μg/g lipid) observed in marine mammals stranded during the 1988 and 1990 epizootic episodes (Aguilar and Borrell, 1994). Seasonal changes, availability of food and ambient water temperature at different locations are also factors that may affect the blubber thickness.

Distribution of PCBs in different tissues: blubber, muscle, liver, brain
It was suggested that the tissue distribution of pollutants might be slightly different when considering tissues with different lipid types and contents (Karlson et al., 2000). For 3 porpoises caught in the Baltic Sea, PCB concentrations (expressed per lipid weight) in the liver were slightly higher (1.25 times) than the concentrations in blubber, but almost double than concentrations found in muscle. Moreover, Zegers et al (2001) has shown that for PBDEs, the concentration of pollutants in the liver is 1.2 times higher than in the blubber. Brain was found to contain the lower concentrations (per lipid weight) due to the presence of phospholipids as major lipids.

Metabolism
Percentage distribution of PCB homologues was consistent between samples (SD < 3% for the mean of percentages). Similar profiles of PCBs were found by Karlson et al. (2000). Importance of single congeners was as follows: 153 > 138 > 149 > 187 > 180 > 99 > 170. These congeners are difficult to metabolise and are accumulating in female and male as well as in juvenile and adult porpoises. Distribution of some specific groups of PCBs (according to Boon et al., 1994) in porpoises was different in juveniles than in adults. Values of the ratio between the concentration of individual PCB congeners and PCB 153 in porpoise liver were calculated for different groups (jm-juvenile/male, am-adult/male, jf-juvenile/female and af-adult/female). The congeners have been divided into five structural groups with respect to the presence of vicinal H atoms and the number of ortho-Cl substituents (Boon et al., 1994). Congeners 101, 110, 105, 118 (from groups III and IV) were found to have a higher

contribution in juvenile individuals to the sum of total PCBs than the adults. This might be due to an increased capacity of metabolism with the PCB body burden increase and thus with the age (Boon et al., 1998). All congeners in the groups I, II and V have been found to have similar ratios for all investigated groups of animals.
Most cetacean species have low hepatic cytochrome CYP450 2B activity, causing them to have little or no capacity for the metabolism of pure [phenobarbital]-type PCBs (those with no vicinal *meta-para* hydrogen atoms) in contrast to seals and terrestrial mammals, which have a higher capacity to metabolise [PB]-type PCBs (Boon et al., 1994). It was shown (Troisi et al., 1998) that, compared with whales or dolphins, the harbour porpoises have a higher capacity for PCB methyl sulphone formation. Porpoises are able to metabolise to hydroxylated or methyl sulphones, non-ortho PCBs (77, 126 and 169) and some mono-ortho-PCBs (114, 123,156), thus the TEQ toxicity is mainly deriving from PCBs 105 and 118 and to a lesser extent from PCB 167 and 189. It was found (Tanabe et al., 1997) that tri- to penta-PCB concentrations are lower in porpoises than in its prey (fish), thus the porpoises have the capacity to degrade lower chlorinated congeners. However, the ability of marine mammals to metabolise PCBs appears to be much lower compared to birds and terrestrial mammals (Tanabe et al., 1994) and even more in harbour porpoises than in many other cetaceans (Duinker et al., 1989). Therefore, the risk posed to porpoises from chronic exposure to PCBs may be greater than in other species.

PBDEs
Very limited data is available on the contamination of harbour porpoises with PBDEs. Zegers et al. (2001) has shown a mean concentration of 864 ng/g lipid (range: 245-1312 ng/g lipid) in 3 harbour porpoises caught on the Dutch coast of the North Sea. Higher levels (mean: 2371, range: 78-7666 ng/g lipid) were measured in 39 harbour porpoises stranded on the English coast of North Sea and on the Welsh coast (Law et al., 2000). BDE 47 was the principal congener measured in all populations. These values are comparable with values measured in harbour porpoises from the Belgian coast (Table 8.11). It was suggested (Zegers et al., 2001) that, at least for invertebrates, concentrations of PBDEs are higher on the English coast when compared with the Dutch coast of the North Sea. This geographical distribution is in sharp contrast with the PCB distribution.

Life-history data versus contaminant load
Different lipid solubilities may to some extent explain the differences between organohalogenated compounds with regard to accumulation, distribution and elimination (Aguilar, 1985). The absence of accumulation of HCH isomers and HCB with age may indicate that these compounds are more easily metabolised and/or more water soluble so that a distribution equilibrium has been reached. Gaskin et al. (1983) revealed sex-related differences associated with organochlorine accumulation in porpoises, with no correlation between organochlorine blubber levels with age in mature females. This indicates an important organochlorine transfer from mother to offspring during the 6-8 month lactation period. Due to possible variation in organochlorine concentrations during development and maturation (Gaskin et al., 1983), it was essential to separate younger animals in the present study. Levels of other organohalogenated compounds (PCBs, DDTs and PBDEs) were significantly higher in the older age-groups, thus revealing an age-dependent accumulation. Differences in food preferences with age may also play a role. These results are comparable with corresponding findings of Kleivane et al. (1995) on harbour porpoises and indicate a relatively low metabolic capacity of small cetaceans with regard to the latter compounds. However, this can not be solely attributed to a relatively low metabolic capacity, since it has

been demonstrated by Reijnders (1994) that harbour porpoises exhibit phenobarbital-type (PB-type) P450-enzyme activity.
Specific bioaccumulation of DDTs, HCHs and HCB, and variation in PCB composition between juvenile and mature harbour porpoises indicate different kinetics of these compounds. The difference found in PCB pattern between juvenile and mature porpoises may indicate the presence of a blood/placenta barrier and/or a selective mammary transport of PCBs with specific structures.

TEQ contribution
From 8 male samples from the Black Sea (Tanabe et al., 1997), TEQ values of non- and mono-ortho PCBs were recalculated using WHO-TEF factors (Van den Berg et al., 1998). TEQ values for non- and mono-ortho PCBs (NO- and MO-PCBs) were 56.6 and 113 pg/g lipid, with PCB 126 and 118 being the major contributors. Total TEQ value was 169.6 pg/g lipid which is similar with the value for MO-PCB TEQ found in male porpoises from this study. PCBs 118 and 126 account for approximately 80% of the PCB-TEQ. In 4 porpoises from the Baltic Sea (Falandysz et al., 1998), NO- and MO-PCB TEQ were 9.8 and 74.3 pg/g lipid, respectively. PCB 126 and 118 constituted approximately 62% of PCB TEQ toxicity.
It was shown (van Scheppingen et al., 1996) that contribution of PCDD/PCDFs is very low compared to the contribution of MO-PCBs (especially PCB 105 and 118) and NO-PCBs (PCB 126). It seems that the harbour porpoises have a high metabolic capacity for PCDD/PCDFs, NO- and some of the MO-PCB congeners (PCB 114, 123 and 156) (van Scheppingen et al., 1996; Tanabe et al., 1997). In harbour porpoises from the North Sea, TEQ values for PCDD/PCDFs were between 1.1 and 3.6 pg/g lipid (Bruhn et al., 1999) or 1.07 and 1.80 pg/g lipid (van Scheppingen et al., 1996). It was recently shown (Jimenez et al., 2001) that also for other marine mammals (such as pilot whale and some dolphin species), the contribution of mono-ortho PCBs to the total TEQs was between 60 and 99%, while the contribution of PCDD/PCDFs was almost negligible. This behaviour differs from that seen in humans, where PCDD/PCDFs account for more than 50% of the total TEQ value, while PCB 126 is the major PCB contributor, followed by PCB 156 and 118 (Koppen et al., 2001). It was also shown (Ishaq et al, 2000) that polychlorinated naphthalenes (PCNs) have an insignificant contribution to total TEQ toxicity, lower than the TEQ toxicity from NO-PCBs. However, comparison between data presented in the literature is rather difficult due to different TEF values used for TEQ calculation.

Conclusion
While concentrations of organochlorine pesticides (HCB, DDTs and HCHs) in harbour porpoises stranded on the Belgian North Sea coast were low, relatively high concentration of PCBs were measured. This indicates that there are still significant sources of PCBs in marine environment. Furthermore, additional compounds must be included in monitoring campaigns (such as PBDEs) as, due to their massive use, concentrations in the aquatic biota increased several fold in the last decades.
As suggested already in the literature, juvenile animals are at risk due to high transfer of contaminants from mother and to differences between PCB congener profiles seen in juvenile and adults animals. PCBs have a much higher contribution to TEQ toxicity than PCDD/PCDFs, as the harbour porpoises seem to have a decrease capacity of metabolizing PCB congeners.

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Chapter 9

Determination of POPs in soil

Abstract

Evaluation of hot Soxhlet extraction of persistent organochlorine pollutants (POPs) from soil was conducted using an industrial soil certified for PCBs. The method was rigorously tested and adequate quality control was ensured. Recoveries of PCBs in the reference material ranged between 70 and 85% from the certified values. Method limits of detection ranged between 0.1 and 0.3 ng/g dry weight. The method was further used for the determination of selected POPs in soils from different European countries.

Analysis of 47 Romanian soil showed that samples from Iassy county showed a lower contamination with organochlorines than samples from other Romanian regions. While DDTs concentrations in soil were significantly higher at rural sites, only few samples (3 out of 47) exceeded the official Romanian norms for DDTs. PCBs concentrations were low in rural sites (less than 8 ng/g soil), but rather high (up to 134 ng/g soil) in urban soils collected mostly from parks (Bucharest, Arad, Baia Mare, Pitesti and Ploiesti).

Accumulation profiles of organochlorines were determined in samples of soil, animal fat and human serum collected in Iassy county. The profiles were different in the three matrices and the accumulation order was soil < animal fat < human serum

For comparison, soil samples from Belgium (16), Italy (6) and Greece (2) were also analysed. These samples were found to contain similar concentrations of HCB and PCBs, but lower concentrations of DDTs and HCHs than Romanian samples. The highest PCB concentrations were found in soils from industrial sites (1 from Belgium and 2 from Romania).

9.1. Analytical methodology for the determination of POPs in soil

* - based on Covaci, Manirakiza P, Schepens P, (2002). *Bull Environ Contam Toxicol* 68(1), 97-103.

Due to their persistence and bioaccumulation potential, organochlorine pollutants (such as polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs)) can be found in the environment even decades after being banned (Leonards et al., 1990; Mackay et Fraser, 2000). They have become a major issue of research in order to investigate their ubiquitous environmental occurrence, biochemical and toxic effects, human exposure and health risk assessment (Duarte-Davidson et Jones, 1994; Hoyer et al., 1998; Glynn et al., 2000).

Soil has been identified as a sink for these toxic chemicals from where they can be released into water or air. However, it was shown that only a part of total organochlorine pollutant concentration in soil is available for uptake by biota or microbial degradation (Reid et al., 2000). The bioavailability decreased with increasing residence time of the pesticide in soil (ageing process). Moreover, the interaction between soil and contaminants depends on the properties of soil and the compounds, which include size, shape, configuration, molecular structure, chemical functions, solubility, polarity and its acid-base nature (Gevao et al., 2000). For organochlorine contaminants, the most important mechanisms of interaction with soil are hydrophobic partitioning (non-active adsorption regarded as partitioning between a non polar compound and a non-specific surface) and sequestration (slow sorption due to prolonged residence in soil). For high extraction efficiencies, the extraction methods should take into account the above mentioned interactions.

Different procedures are reported for persistent organochlorine pollutants (POPs) extraction from the soil. While the Soxhlet extraction represents the classical methodology (Luque de Castro and Garcia-Ayuso, 1998) for the extraction of lipophilic compounds from solid samples, new methodologies using microwave-assisted (Lopez-Avila et al., 1995a,b; Cicero 2000; Xiong et al., 2000), accelerated solvent extraction (Fisher 1997) or supercritical fluid extraction (Bøwadt et al., 1995) have been developed. However, the efficiency of these procedures is often inferior to the Soxhlet procedure or they require costly equipment.

In this study, an evaluation of the hot Soxhlet extraction of POPs from soil was conducted using an industrial soil certified for PCBs. The method was further applied to the determination of selected POPs in soils from different European countries (Romania, Belgium, Italy and Greece).

Materials and methods
Samples
Multiple soil cores (n=2-5, 5 cm deep) were collected from each location (urban, rural, industrial and waste incineration sites). Samples were homogenised by sifting through a steel mesh (~2 mm grid size), dried at room temperature and sealed in air-tight polyethylene containers for storage at room temperature until analysis.

Standards and reagents
The following PCB congeners (IUPAC numbering) were targeted for analysis: 28, 52, 99, 101, 118, 138, 149, 153, 156, 170, 180, and 187. Additionally, we included hexachlorobenzene (HCB), α–, β– and γ– hexachlorocyclohexane isomers (the sum expressed as HCHs), o,p'-DDE, p,p'-DDE, o,p'-DDD, p,p'-DDD, o,p'-DDT and p,p'-DDT

(the sum expressed as DDTs). ε-HCH, PCB 46 and 143 were used as internal standards and 1,2,3,4-tetrachloronaphthalene (TCN) as recovery standard. All individual standards at a concentration of 10 ng/µl in iso-octane were purchased from Dr. Ehrenstorfer Laboratories (Augsburg, Germany).

Dilutions were made in iso-octane to cover the range of POPs expected in the samples. All solutions were stored at -20°C. Certified reference material CRM 481, industrial soil certified for PCBs (IUPAC Nos. 101, 118, 149, 153, 156, 170 and 180) was purchased from BCR (Brussels, Belgium). Acetone, hexane, dichloromethane and iso-octane were of pesticide grade (Merck, Darmstadt, Germany). All solvents were tested for interferences using concentration from 15 ml to 50 µl and analysis by GC-ECD. Analytical grade concentrated sulphuric acid 95-97% was purchased from Merck. Anhydrous sodium sulphate (Merck) for residue analysis, basic aluminium oxide 70-230 Mesh and silica gel 60-200 Mesh (Merck) were used after heating overnight at 120°C. A Soxhlet extractor B-811 (Büchi, Switzerland) was used for the extraction of organic residue from soil (see Chapter 2.1).

Portions of 100 mg for CRM 481 and 2 g of soil samples were spiked with internal standards (PCB 46 and 143) and extracted using hot Soxhlet manifold with 50 ml of solvent (see Table 9.1). The extract was concentrated to 5 ml in the extractor and was further purified on acidified silica: deactivated alumina cartridge. After elution with 25 ml hexane and concentration under a nitrogen stream, TCN (recovery standard) was added prior to GC analysis. Recoveries of target analytes and internal standards ranged between 70 and 85%. Method limits of detection ranged between 0.10-0.25 ng/g soil. The procedure was validated through regular analysis of blanks, fortified soil and certified material, CRM 481.

GC

A Hewlett Packard (Palo Alto, CA, USA) 6890 GC-µ ECD was equipped with a 25 m x 0.22 mm x 0.25 µm, HT-8 capillary column (SGE, Zulte, Belgium). One µl was injected in pulsed splitless mode (pulse pressure = 25 psi, pulse time = 1 min) with the split outlet opened after 1 min. Injector and detector temperatures were set at 275°C and 320°C, respectively. The temperature program of the oven was set to 90°C for 1 min, then with 15°C/min to 180°C, kept for 1 min, then to 250°C by 3°C/min and further by 15°C/min to 290°C, kept for 6 min.

A Hewlett Packard (Palo Alto, CA, USA) 6890 GC was connected via direct interface with a HP 5973 mass spectrometer. A 25 m x 0.25 mm x 0.25 µm, DB-1 (J&W Scientific, Folsom, CA, USA) was used with helium as carrier gas at a constant flow of 1.0 ml/min. One µl was injected in pulsed splitless mode (pulse pressure = 20 psi, pulse time = 1.0 min) with the split outlet opened after 1.0 min. Injector and interface temperatures were set at 270°C and 280°C, respectively. The temperature program of oven was starting from 90°C, kept for 1 min, then with 15°C/min to 275°C, kept for 10 min.

The mass spectrometer was operated in the electron impact ionisation and the selected ion monitoring mode. The two most abundant ions were monitored for each level of chlorination for PCBs or for each pesticide. Retention time, ion chromatograms and relative abundance of the ions were used for identification. All samples were analysed on both columns and the lowest value for each compound was further considered for calculations. A typical chromatogram obtained from soil is presented in Figure 9.1.

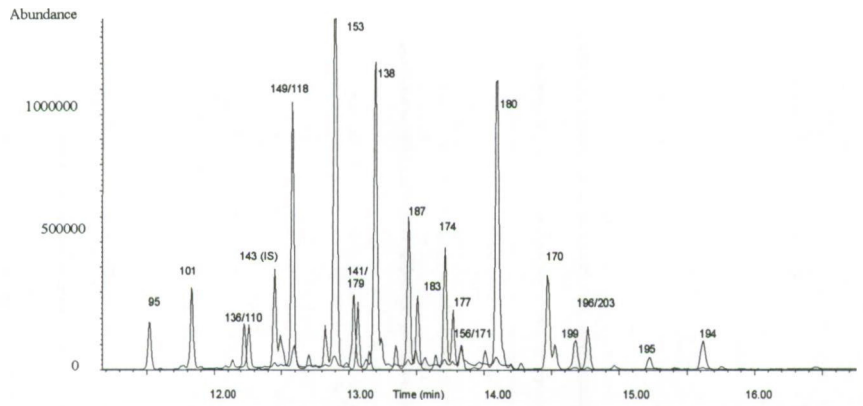

Figure 9.1. Chromatogram of soil sample with a concentration of PCBs higher than 500 ng/g soil.

Results and discussion

Different solvent mixtures were compared for the hot Soxhlet extraction (HSE) by analysing a soil with considerable native PCB concentration. The method efficiency was evaluated by comparing the concentration of PCB 153 and sum of PCBs (sum of 12 congeners) found in the soil (Table 9.1). Hot Soxhlet extraction gave higher concentrations of PCB 153 with lower standard deviations than the sonication extraction.

The yield of Soxhlet extraction is influenced by solvent composition (Table 9.1). The amount of extracted analytes as well as the amount of interfering material extracted increases with the polarity of the mixture used (extraction with hexane:acetone = 1:1, *v/v* showed more interference peaks on ECD chromatogram). Hexane:acetone (3:1, *v/v*) was the best mixture for extraction by HSE (highest concentrations of PCB 153 and sum PCBs). This mixture also lead to an easy evaporation step through azeotropic distillation (Smedes et al., 1997). Pretreatment of soil with hydrochloric acid (as proposed by Smedes et al., 1997) did not increase the concentration of PCB 153 found in the soil.

Table 9.1. Evaluation of sonication and Soxhlet extraction of a native soil sample (n=3).

Method	Solvent mixture	PCB 153		Sum PCBs	
		Mean (ng/g soil)	RSD (%)	Mean (ng/g soil)	RSD (%)
Sonication (30 min)	H:D=4:1	2.4	22	9.4	35
Hot Soxhlet (2hr)	H	4.9	15	22.3	19
	H:D=4:1	5.6	8	24.0	13
	H:D:A=3:1:1	6.7	18	28.2	16
	H:A=1:1	6.6	20	30.1	18
	H:A=3:1	**8.1**	**14**	**34.1**	**16**
HCl + Hot Soxhlet (2 hr)	H:D:A=3:1:1	6.8	16	29.2	15
	H:A=3:1	7.9	13	33.9	18

* - H-hexane, D-dichloromethane, A-acetone

To check method recoveries, a soil sample (previously found to contain very low amounts of POPs) was fortified by addition of 20 ng of each analyte to four 2 g portions of soil. Average recoveries ranged from 70% to 85% after correcting for the amounts in the soil blanks. The

method blank was carried out by extracting a thimble filled with sodium sulphate and treating the extract as a sample. Blank values for all compounds were below quantification limits.
The Soxhlet system has been applied in hot and standard extraction mode to determine the optimum extraction time of PCBs from the certified soil. Two hours was a convenient extraction time to obtain high yields, while longer extraction times failed to increase PCB concentrations (Table 9.2). Recoveries ranged between 73 and 84% with RSD<15%. Compared with standard Soxhlet mode, the use of hot Soxhlet reduced the extraction time from 8 h or more (Smedes et al., 1997) to 2 h. This probably results from the permanent contact with hot solvent.
PCB concentrations in the certified soil (CRM 481) are in the μg/g range and do not reflect concentrations in the environment (in the ng/g range). Because recoveries of analytes at two concentration levels might be different, the validation of the method in the high range of concentrations does not indicate if the method is efficient at low concentrations. This hypothesis was tested by 1:20 dilution of CRM 481 (200 mg soil homogenised with 3800 mg anhydrous Na_2SO_4). Recoveries from the certified values (Table 9.2) were higher in this case and ranged between 83 and 96% probably as a result of increased dispersion of the certified soil. However, higher relative standard deviations were obtained due to adsorption and incomplete homogenisation of the material.

9.2. Determination of POPs in soil from Romania

* - based on Covaci A, Hura C, Schepens P, (2001), *Sci Total Environ*, 280 (1-3), 143-152.

Selected persistent organochlorine pollutants, including PCBs, DDT and its metabolites (DDTs) and hexachlorocyclohexane isomers (HCHs) were determined in soil from Romania.
Soil samples (0-5 cm depth) were collected from 20 rural sites (Iassy county) and from 27 urban, rural, industrial and waste incineration sites (different locations all over Romania). All samples were dried at room temperature and stones and vegetation were removed. Soil samples were sieved and kept in closed vessels until analysis.
Soil samples from Iassy county showed a lower contamination with organochlorines (Table 9.3) than samples from other Romanian regions, which included various sites: rural (near Timisoara, Arad, Ploiesti, Cernavoda), urban (Bucharest, Timisoara, Arad, Baia Mare, Ploiesti, Calimanesti), industrial (Copsa Mica, Ramnicu Valcea) and near municipal waste disposal facilities (Timisoara, Arad) (Figure 9.2).

Figure 9.2. Map of Romania.

Table 9.2. Evaluation of extraction efficiencies of PCBs from certified soil CRM 481 using standard and hot Soxhlet extraction.

Congener n°	Certified value (µg/g soil)	Standard Soxhlet 8 hr (n=3)	Hot Soxhlet mean ± SD, µg/g soil			
			2 hr (n=3)	3 hr (n=3)	4 hr (n=3)	2 hr (diluted CRM)* (n=3)
101	37 ± 3	28 ± 3 (75.7%)**	27.5 ± 1.5 (74.3%)	29.1 ± 3 (78.6%)	29.5 ± 3.2 (79.7%)	31.4 ± 4.2 (84.9%)
118	9.4 ± 0.7	8.9 ± 0.7 (94.5%)	8.7 ± 0.4 (92.5%)	8.7 ± 0.5 (92.5%)	8.9 ± 0.5 (94.5%)	9.0 ± 0.8 (95.7%)
149	97 ± 7	77 ± 6 (79.4%)	71 ± 4 (73.2%)	73 ± 4 (75.3%)	75 ± 3 (77.3%)	82 ± 9 (84.5%)
153	137 ± 7	116 ± 6 (84.7%)	115 ± 3 (83.9%)	117 ± 5 (85.4%)	118 ± 6 (86.1%)	125 ± 10 (91.2%)
156	7.0 ± 0.5	5.6 ± 0.5 (80%)	5.4 ± 0.7 (77.1%)	5.5 ± 0.9 (78.6%)	5.5 ± 0.7 (78.6%)	5.8 ± 0.7 (82.9%)
170	52 ± 4	41 ± 4 (78.8%)	38 ± 5 (73.1%)	40 ± 5 (76.9%)	40 ± 6 (76.9%)	45 ± 5 (86.5%)
180	124 ± 6	100 ± 7 (80.6%)	95 ± 8 (76.6%)	96 ± 10 (77.4%)	95 ± 8 (76.6%)	108 ± 12 (87.1%)

* - 1: 20 dilution of CRM 481 with anhydrous Na_2SO_4

** - percentage of the certified value

Iassy samples showed a significantly lower contamination with organochlorines especially for HCHs and PCBs. Ratios of γ-HCH/Σ HCH were higher in Iassy soil samples (sometimes more than 0.5) with a higher mean value for Iassy soil samples. Concentrations of DDTs were statistically higher at rural sites ($p<0.05$), while ratios p,p'-DDT/Σ DDT were similar (Table 9.4). Concentrations of HCHs and ratios of γ-HCH/Σ HCH were similar at rural and urban sites. Only 3 samples (1 from Iassy, Calimanesti and Bucharest) exceeded the official Romanian norms for DDTs of 500 ng/g soil (Monitorul, 1997). Even more than 10 years after the DDT ban, DDTs concentrations in soil were significantly higher at rural sites, sometimes close to the actual tolerance limits for agricultural purposes (500 ng/g soil). Ratios p,p'-DDT/Σ DDT (often more than 0.5) suggest a probable continuous exposure of the population due to a recent use of DDT in the area correlated with a very slow degradation.

PCB concentrations were low in rural sites (less than 8 ng/g soil), but rather high (up to 134 ng/g soil) in urban soils collected mostly from parks (Bucharest, Arad, Baia Mare, Pitesti and Ploiesti). This might reflect a higher exposure of urban population to PCBs.
A comparison of concentrations of POPs in soils from different sites revealed that highest concentrations could be found near industrial sites. Soil samples near an organochlorine producing factory (OLTCHIM –Rm. Valcea) showed that, except DDTs, all other POPs were significantly higher than in other locations, exceeding the official norms (Monitorul, 1997).

Accumulation profiles
Due to slow degradation, profiles of different compounds in soil were closely related to the technical mixtures used: Aroclors or similar mixtures for PCBs (high percentage of PCBs 101, 110 and 149 found in soil), technical DDT (high percentage of p,p'-DDT and o,p'-DDT) and technical lindane (high percentage of α–HCH). However, a broad range of γ-HCH/Σ HCH and p,p'-DDT/Σ DDT could be explained by exposure at different times and use of different mixtures.

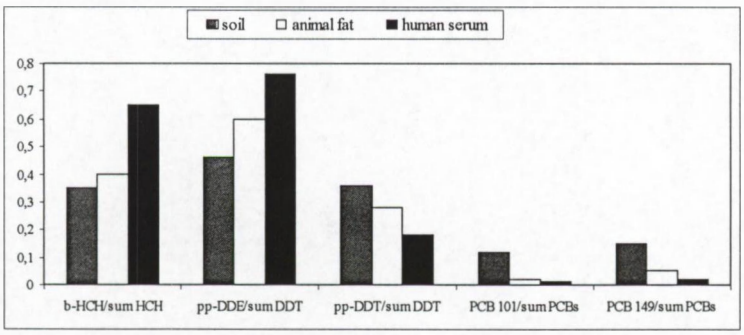

Figure 9.3. Relative contribution of selected pollutants in soil, animal fat and human serum.

Profiles of POPs were determined in samples of soil, animal fat (chapter 7.6) and human serum (chapter 3.2.2) collected in Iassy county. The profiles were different in the three matrices and the accumulation order was soil-animal fat-human serum (Figure 9.3). Thus, high percentages of compounds present in the technical mixtures (eg. γ-HCH, p,p'-DDT and o,p'-DDT, PCB 101, 110 and 149) were found in the soil due to slow degradation. In the animal fat, percentages of β-HCH (the most stable HCH isomer) increased together with the appearance of p,p'-DDD and p,p'-DDE (metabolites of p,p'-DDT).

Table 9.3. Concentrations of selected POPs (mean ± SD, ng/g soil) in soil from Iassy county (n=20) and different Romanian locations (n=26).

	Concentration	Iassy (n=20)			Romanian locations (n=26)		
	(ng/g soil)	mean ± SD	range	frequency	mean ± SD	range	frequency
HCB	< 10	0.1 ± 0.1	nd – 0.2	20	1 ± 2	nd – 5.5	24
	> 10				64 ± 3	61 and 67	2
Σ HCH (α–, β–, γ–HCH)	< 250	2 ± 2	1 – 7	20	26 ± 28	3 - 90	26
γ–HCH/Σ HCH		0.54 ± 0.16	0.21 – 0.76		0.42 ± 0.25	0.16 – 0.94	
ΣDDT	< 200	36 ± 43	4 – 120	18	63 ± 44	9 – 187	18
	200-500	493		1	304 ± 87	207 – 435	6
	> 500	1331		1	1052 ± 694	561 – 1542	2
p,p'-DDT/Σ DDT		0.36 ± 0. 10	0.14 –0.51		0.44 ± 0.14	0.18 – 0.66	
Σ PCBs	< 250	4 ± 2	2 – 9	20	41 ± 40	nd - 134	26
PCB 153/Σ PCB		0.24 ± 0.02	0.17 - 0.26		0.20 ± 0.03	0.13 – 0.24	

nd – not detected

Table 9.4. Concentrations of selected POPs (mean ± SD, ng/g soil) in 27 Romanian soils in function of their provenience.

Compound	Tolerated limits* (ng/g soil)	Rural (n=7)	Urban (n=13)	Industrial (n=2)	Waste sites (n=4)	Industrial** (ng/g soil)
		mean ± SD	mean ± SD	mean ± SD	mean ± SD	
HCB	5000	0.3 ± 0.1	2 ± 2	1 ± 1	1 ± 1	337
Σ HCH (α–,β–,γ–HCH)	250	28 ± 34	30 ± 27	7 ± 2	na	2585
γ-HCH/Σ HCH		0.40 ± 0.35	0.42 ± 0.24	0.46 ± 0.21	na	0.83
ΣDDT	500	227 ± 157	113 ± 152	72 ± 36	101 ±120	80
p,p'-DDT/Σ DDT		0.49 ± 0. 10	0.48 ± 0. 16	0.40 ± 0.26	0.34 ± 0. 10	0.41
Σ PCBs	250	4 ± 3	57 ± 41	23 ± 17	63 ± 35	722
PCB 153/Σ PCB		0.22 ± 0.02	0.19 ± 0.03	0.22 ± 0.01	0.19 ± 0.01	0.20

*-Romanian norms (Monitorul Oficial, 1997) na - not available

**-Rm. Valcea – near OLTCHIM factory

Only persistent PCB congeners could be found in the animal fat, due to rapid metabolism of the non-persistent congeners (PCB 101, 110 and 149). In human serum, β-HCH and p,p'-DDE were the principal organochlorine pesticides, while PCB profile is dominated by the persistent congeners (PCB 153, 138 and 180). The presence of non-persistent congeners (see Chapter 3.2.3) can be attributed to recent exposure of the specimens to PCB commercial mixtures (Covaci et al., 2001).

5.2. Determination of POPs in soil from Belgium, Italy and Greece

* - based on Covaci A, Manirakiza P, Schepens P, (2002). *Bull Environ Contam Toxicol* 68(1), 97-103.

Selected POPs were determined in soils from Belgium (BE), Italy (IT), and Greece (GR). Concentrations of various pollutants vary greatly between countries (Table 9.5). Samples from Romania (see chapter 5.2) were included for comparison and showed similar concentrations of HCB and PCBs, but higher concentrations of DDTs and HCHs than in other countries.

HCB concentrations were lower than 6 ng/g soil and were similar in all countries. With exception of some Romanian rural sites with values up to 134 ng/g soil, low concentrations of HCHs (< 2 ng/g) were found at all sites (Table 9.5). Similar γ-HCH/Σ HCH ratios (close to 0.8) were found in all countries except for Romania (ratio of 0.48) suggesting the use of different mixtures (pure lindane versus technical HCH mixture). Concentrations of HCHs and ratios of γ-HCH/Σ HCH were similar at rural and urban sites.

Table 9.5. Concentrations of selected POPs (ng/g soil) from Belgium (BE), Italy (IT), Greece (GR) and Romania (RO). Concentrations below detection limit were set at half of the detection limit.

Analytes	BE n=16		IT n=6		GR n=2	RO n=46	
	mean (SD)	range	mean (SD)	range	mean	mean (SD)	range
HCB	0.3 (0.3)	nd - 1	1 (2)	nd - 5	nd	0.6 (1.2)	nd - 6
ΣHCH	1 (0.5)	nd - 2	1 (1)	nd - 2	0.9	10 (20)	1 - 90
γ-HCH/Σ HCH	0.8 (0.1)	0.66-0.94	0.8 (0.1)	0.75-0.81	0.80	0.5 (0.2)	0.21- 0.94
ΣDDT	7 (7)	nd - 22	26 (21)	2 - 60	24	96 (126)	3 -561
pp-DDT/Σ DDT	0.4 (0.1)	0.17 -0.58	0.3 (0.1)	0.22 -0.50	0.5	0.4 (0.1)	0.18 - 0.66
Σ PCB	15 (11)	4 - 39	11(8)	4 - 91	4	25 (35)	1 - 134
PCB 153/Σ PCB	0.2 (0.0)	0.17 - 0.22	0.2 (0.0)	0.17-0.22	0.2	0.2 (0.03)	0.13 - 0.26

Concentrations of DDTs from Belgian and Italian soils were low (Table 9.5) and in the same range as other European soils (Harrad et al., 1994; Wilcke et al., 1998), while DDT concentrations in some rural Romanian soils were higher (up to 560 ng/g soil). Three Romanian samples exceeded the official norms for DDTs of 500 ng/g soil (Monitorul, 1997). Concentrations of DDTs were statistically higher at rural sites ($p<0.05$) in all countries except Greece. Similar p,p'-DDT/Σ DDT ratios (up to 0.35) were found in Italian and Belgian soil, suggesting a past DDT exposure. Ratios from Greece and Romania are higher (up to 0.66)

suggesting a more recent use. Similar p,p'-DDT/Σ DDT ratios were calculated form rural and urban sites in each country.

PCB 28, 52, 99 and 156 values were sometimes under the detection limit, while all other congeners were detected at all sites. PCB concentrations (sum of congeners 101, 118, 138, 149, 153, 170, 180 and 187) were similar in all countries, with slightly lower values (3.5 ng/g soil) in Greece and higher ones in Romania (up to 134 ng/g soil). PCBs concentrations at rural sites were significantly lower than in urban soils collected mostly from parks. PCB concentrations were similar in Italian and Romanian urban sites. The highest PCB concentrations were found in soils from industrial sites (1 from Belgium and 2 from Romania). Similar PCB 153/Σ PCB ratios were found in all countries.

Due to slow degradation, profiles of different pollutants in soil are closely related to the technical mixtures used: Aroclors or similar mixtures for PCBs (high percentage of PCBs 101 and 149 found in soil), technical DDT (high percentage of pp-DDT and op-DDT) and pure lindane (high γ-HCH/Σ HCH ratios). However, a broad range of γ-HCH/Σ HCH and pp-DDT/Σ DDT could be explained by different exposure in time and by use of different mixtures.

Generally, with exception of some contaminated sites (rural for DDTs and industrial for PCBs), soils contained relatively low concentrations of organochlorines with an increased load in urban soils collected from parks.

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CONCLUSIONS

Concluding remarks

Persistent organic pollutants (POPs), such as dioxins, polychlorinated biphenyls (PCBs), organochlorine pesticides and polybrominated diphenyl ethers (PBDEs) have considerable endocrine disrupting potency, are linked to the occurrence of endometriosis and breast cancer and have been reported to adversely affect wildlife and humans. More importantly, background levels of PCBs and dioxins, including those occurring in pregnant women and lactating mothers in the Netherlands, were reported to affect the immunological status, neurological and cognitive development of infants and young children. Therefore, there is a permanent need to monitor these compounds in environmental and human matrices.

The work presented in this thesis is the result of interdisciplinary collaboration, but in which the analytical environmental chemistry, including the development and improvements of analytical methods, had an essential part.

The first objectives of this thesis were the development and optimisation of analytical methods for monitoring of POPs (including PCBs, organochlorine pesticides and PBDEs) in human and environmental matrices. Several matrices, such as soil, animal tissues and feed, fish and human tissues and body fluids, were investigated. The emphasis was put on the simplicity and efficiency of the sample preparation and on the speed of the analysis (using fast gas chromatography on narrow bore capillary columns). By using hot Soxhlet extraction for solid samples, an important decrease in the extraction time was observed, in parallel with an efficient extraction. For liquid samples, such as human body fluids (serum, milk, follicular and seminal fluid), a universal method based on solid-phase disk extraction (SPDE) was developed. The SPDE method showed several advantages over the classical techniques (liquid-liquid extraction and solid-phase extraction). Reduced solvent volumes, possibility of combination with a miniaturised clean-up on adsorbents, high flexibility, reliability and easiness to use were some of the most interesting features.

Secondly, an important parameter in trace analysis is the quality assurance protocol. For each of the established methods, validation was necessary in order to improve the quality of the analytical data reported of the investigated compounds and to obtain reliable data that can be easily compared with literature data. In each case, certified reference materials were used for this purpose in parallel with regular participation in interlaboratory tests.

Thirdly, these methods were applied to provide an insight into the distribution of POPs in different European human populations and into the occurrence of pollutants in several environmental compartments.
It was shown in this work, that POPs are widely found in the environment and that they accumulate in species on the top of the food chain in the terrestrial (humans) and aquatic systems (marine mammals). The profiles of specific POPs were different in the various parts of the food chain and the accumulation order was soil-animal fat-human serum. Due to slow degradation, profiles of pollutants in soil were closely related to the technical mixtures used (Aroclor, technical DDT and HCH). In animal fat, percentages of β-HCH (the most stable HCH isomer) increased together with the appearance of p,p'-DDD and p,p'-DDE (metabolites of p,p'-DDT), while, only persistent PCB congeners could be found in animal fat, due to rapid metabolism of non-persistent congeners. Moreover, in human serum, β-HCH and p,p'-DDE were the principal organochlorine pesticides, while PCB profile was

SUMMARY

First objectives of this thesis were the development and optimisation of analytical methods for monitoring of persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), organochlorine pesticides and polybrominated diphenyl ethers (PBDEs) in human and environmental matrices. Secondly, these methods were applied to gain insight into the distribution of POPs in different European human populations and into the occurrence of pollutants in several environmental compartments.

Chapter 1 gives a short introduction of the production, use, human exposure, environmental fate and toxicity for each of the studied class of pollutants. An overview of the most widely used and efficient analytical methods for the determination of POPs is presented in **Chapter 2**. Specific steps, such as extraction, clean-up, gas chromatographic (GC) separation, detection systems and quality control/quality assurance are reviewed and emphasis is put on the techniques used in this thesis. It was also shown that fast GC (using narrow bore capillaries) can be used in connection with quadrupole mass spectrometric (MS) detection for the rapid analysis of PCBs and PBDEs.

Chapter 3 and 4 present a simple, rapid, sensitive and universal procedure based on solid-phase disk extraction (SPDE) which was developed and evaluated for the isolation and concentration of trace levels of selected organochlorine pesticides and PCB congeners from different human body fluids (serum, cord blood, milk, follicular and seminal fluid). Similar methodology could be used for each matrix with the only restricting factor being the viscosity of the fluid. An Empore^{TM} C_{18} bonded silica disk cartridge was used for the initial extraction and enrichment of the analytes. Subsequent clean-up was done by adsorption chromatography on concentrated sulphuric acid: silica gel (1:1, w/w). Analysis was achieved by GC/MS or GC-ECD on two capillary columns of different polarity. By using the SPDE procedure, a high throughput and parallel sample processing could be achieved. The method was validated through successful participation in several interlaboratory tests. To the best of our knowledge, we were the first to describe the use of the SPDE method for the determination of selected POPs in different human body fluids.
The method was successfully used for the analysis of selected organochlorine pesticides and PCBs in human serum from 200 women between 50 and 65 years living in two areas of Flanders. In another study which was aimed to investigate the significance of the *in utero* exposure, concentrations of PCBs, HCB and p,p'-DDE have been determined in the maternal and cord blood collected at delivery from 44 Belgian mothers samples. Then, we have measured at first concentrations of POPs in individual human serum from two major cities of Romania (Timisoara and Iassy). Next, the distribution and removal of PCBs were examined in intermediate fractions along the Cohn fractionation process and in the final plasma derivatives obtained from the blood bank of the Belgian Red Cross. To the best of our knowledge, this is the first study to discuss the distribution of persistent pollutants in protein derivatives obtained by plasma fractionation.
Moreover, POPs were determined in human milk from Romanian individuals. Concentrations of PCBs were low, while concentrations of organochlorine pesticides were higher than reported values from other European countries. Finally, follicular and seminal fluids from several Belgian specimens were investigated for their POP load. Levels of DDE and PCBs (the major organohalogen compounds) were very low, most of the time near detection limit.

Different incubation and extraction methods were evaluated in **Chapter 5** for the determination of POPs in human hair. The best method was found to be overnight incubation with HCl and liquid-liquid extraction with hexane:dichloromethane. After clean-up on basic alumina and acid silica, the extract was analysed by GC-ECD or GC/MS. Recoveries of internal standards and analytes under investigation ranged from 87 to 111%. Similar concentrations of POPs (reported on lipid basis) were found in human milk and hair from the same specimen. Hair analysis was used for the assessment of exposure to organochlorine pollutants in specimens from Greece, Romania and Belgium. The highest organochlorine load (mainly DDTs) was found in samples from a group of Greek women with past occupational exposure to pesticides. It was concluded that hair can be used for monitoring of any subgroup of a population with emphasis on those from which sampling of milk, adipose tissue or blood is not possible or very difficult (e.g. children or endangered animal species).

A new analytical method has been developed for the quantification of PBDEs in human adipose tissue and is presented in **Chapter 6**. After Soxhlet extraction and a clean-up procedure with two successive solid phase extraction cartridges containing acid silica and acid silica : neutral silica : deactivated basic alumina, detection could be achieved by narrow bore (0.10 mm internal diameter) capillary GC/electron impact low resolution MS using a large volume injection technique. Detection limits in the selected ion mode varied between 0.05 and 0.30 ng/g lipid weight, depending on the degree of bromination. The sensitivity of this method can at least compete with low resolution mass spectrometry with electron capture ionization, while a much better selectivity is obtained.
The method allows the determination in Belgian human adipose tissue samples of 5 major PBDE congeners (BDE 28, 47, 99, 100 and 153) at concentrations below 1 ng/g lipid weight and 35 PCB congeners, together with p,p'-DDT and p,p'-DDE. Levels of selected POPs were similar to previously reported values from Europe. Sum PCBs showed good correlation with the sum DDTs ($r=0.77$, $p<0.05$), while the correlation with sum PBDEs was weaker ($r=0.56$, $p<0.05$). No age-dependency was found for PBDEs, while PCBs and DDTs showed good correlation with age.

In January 1999, several Belgian animal farms received feed contaminated with transformer oil containing PCBs and dioxins. The Toxicological Centre (UIA) took part in the national program of food monitoring and, in less than 6 months, analysed almost 2,500 samples of animal feed and meat. Results of these analyses are discussed in **Chapter 7**. Analysis of contaminated samples showed that the patterns for PCB and PCDD/F congeners differed among feed, chicken fat and pork fat. Lower chlorinated PCBs and PCDFs including those with high TEFs (PCBs 105, 118, 126 and 2,3,4,7,8-PeCDF) were shown to either bioaccumulate more in chicken fat or to be eliminated more readily in pork. This leads to the possibility that consumption of chicken would result in a higher TEQ human body burden than consumption of pork. In addition, we found that 12.1% of Belgian export meat and fat samples from chicken or pork, unrelated to the PCB/dioxin crisis, contained more than 50 ng PCBs/g fat and that 6.5% of samples contained more than 20 ng/g fat for the sum p,p'-DDT and p,p'-DDE. We have shown that part of this background contamination stems from imported animal feed ingredients (fish flour and grains), sometimes contaminated by recent use of DDT as can be deduced from the ratio between p,p'-DDT and p,p'-DDE. However, after comparison of PCB levels in fish flour and grains with those in meat, it can be suggested that high contamination levels stem from recycled fat. Animal fat samples from two major towns of Romania (Arad and Iassy) were analysed for their organochlorine load. Some

dominated by the persistent congeners. The presence of non-persistent congeners in some specimens can only be attributed to recent exposure to PCB technical mixtures.

The optimised methods should allow for the measurement of subtle changes in profiles and levels of different pollutants (especially of PCB congeners) in various species (fish, chicken, pork and humans). While fish was found to contain higher concentrations of lower chlorinated PCB congeners (tri- to penta-), in chicken, these congeners were found to occur only in low amounts. Moreover, in mammals, due to a higher metabolism capacity, the predominant congeners were hexa- to octa- PCBs, which also have higher bioaccumulation factors. However, the lower chlorinated PCBs have higher TEF values (e.g PCB 105, 118 and 126). Thus, when exposed to the same source and level of contamination, fish or chicken tissues are liable to have higher TEQ values (lipid based) than mammalian tissues. A human diet based on contaminated fish or chicken can lead to higher TEQ body burdens than diets based on pork (or other mammals: cow, sheep). As a result of the Belgian PCB crisis, it was found that birds are better indicators of the source of contamination than mammals. Furthermore, the continuous monitoring of human food chain was found to be of vital importance in order to reduce the risk of unwanted poisoning episodes and for a continuous decrease in the concentrations of POPs in food.

Finally, it should be mentioned that several studies included in this thesis were presented for the first time. Thus, PCBs and other organochlorine pollutants were investigated in different fractions and final protein derivatives (albumin, immunoglobulins and Factor VIII) from human plasma obtained from the Belgian Red Cross. Moreover, PCBs in cord blood and PBDEs in human adipose tissue were investigated for the first time in Belgian population. Lastly, measurements of PCBs in human serum and milk samples were done for the first time in people from Romania. High concentrations of some pollutants found in the Romanian population, as well as in the environment, point to the necessity to introduce monitoring programs for humans and the human food chain. These monitoring programs should be able to show trends in the general population and to identify groups at risk. In its process of European integration, the increasing interest for controlling environmental pollution should be an absolute requirement.

In conclusion, analytical methodologies used in the determination of POPs have to be adapted to the permanent need of reaching lower levels. More data will be needed for inventories and risk assessment of 'classic' priority POPs. As about half of the PCBs produced are still in use in closed systems and the fasing out is planned to end in 2010, illegal dumping of important amounts of PCBs might be expected. This fact together with the observation that PCBs and other POPs were trapped in the snow and icelayers of glacier from the Arctic, indicates that pollution will affect environmental life for decades to come. In the future, new classes of 'emerging' pollutants (such as PBDEs, organofluorinated compounds, PCB hydroxylated and methyl sulphone metabolites) should be included in monitoring schemes as recent work suggest that these compounds have similar toxic effects as classical POPs.

animal fat samples from Iassy exceeded the EU norms for organochlorine pesticides (HCHs and DDTs), but showed similar PCB concentrations when compared with Arad samples.

Chapter 8 contains two applications of the determination of POPs in aquatic biota. Farmed and wild Scottish Atlantic salmon, aquaculture feeds and fish oils, obtained in January 1999, were investigated for the determination of a wide range of PCBs, organochlorine pesticides and PBDEs. The study confirms previous reports of relatively high concentrations of PCBs, and indicates moderate concentrations of organochlorine pesticides and PBDEs in farmed salmon. For the Scottish salmon samples, the PCB TEQ was twice that of PCDD/Fs. The results indicate that high consumption of salmon, particularly by children under 5 years, could lead to intakes above the tolerable daily intake (TDI) and tolerable weekly intake (TWI) for these chemicals, especially PCBs, in combination with mean or high level intakes from the UK diet.
Liver samples from 21 harbour porpoises (*Phocoena phocoena*) stranded on the Belgian North Sea coast were analysed for a wide range of PCBs, organochlorine pesticides and PBDEs. PCBs were the most important organochlorine contaminants in the harbour porpoises, while the most abundant organochlorine pesticides were DDT and its metabolites, followed by HCB and HCHs. The mean concentrations of groups of pollutants were, with some exceptions, similar with those reported in the literature. Differences between levels and profiles of POPs were observed between adults and juveniles, as well as between males and females.

Hot Soxhlet extraction of POPs from soil was investigated in **Chapter 9** and the optimised method was rigorously tested. Analysis of Romanian soil samples showed that samples from Iassy county showed a lower contamination with organochlorines than samples from other Romanian regions. While DDTs concentrations in soil were significantly higher at rural sites, only few samples (6%) exceeded the official Romanian norms for DDTs. PCBs concentrations were low in rural sites, but rather high in urban soils collected from parks. Accumulation profiles of organochlorines were determined in samples of soil, animal fat and human serum collected in Iassy county. The profiles were different in the three matrices and the accumulation order was soil < animal fat < human serum.
For comparison, soil samples from Belgium (n=16), Italy (n=6) and Greece (n=2) were also analysed. These samples were found to contain similar concentrations of HCB and PCBs, but lower concentrations of DDTs and HCHs than Romanian samples. The highest PCB concentrations were found in soils from industrial sites.

In conclusion, analytical methodologies used in the determination of POPs have to be adapted to the permanent need of reaching lower levels. The methods should be able to measure subtle changes in profiles and levels of different pollutants such as PCB congeners, in various species. Adequate quality control is thus needed for method validation. In this thesis, it was shown that POPs are widely found in the environment and that they accumulate in species on the top of the food chain in terrestrial (humans) and aquatic systems (marine mammals). For this reason, the continuous monitoring of human food chain and of human population is an absolute requirement.
In the future, new classes of 'emerging' pollutants (such as PBDEs, organofluorinated compounds, PCB metabolites) should be included in monitoring schemes, while also more data will be needed for inventories and risk assessment of 'classical' priority POPs.

SAMENVATTING

Een eerste doelstelling van deze thesis was de ontwikkeling en optimalisatie van analytische methoden voor monitoring van persistente organische polluenten (POPs) zoals polychloorbifenylen (PCBs), organochloor pesticiden en polygebromeerde difenyl ethers (PBDEs), in humane- en milieumatrices. Ten tweede werden deze methoden toegepast om meer inzicht te verkrijgen in de distributie van POPs in verschillende Europese humane populaties en in het voorkomen van polluenten in verscheidene milieu compartimenten.

Hoofdstuk 1 geeft een korte inleiding in de produktie, gebruik, menselijke blootstelling, voorkomen in het milieu en toxiciteit voor elk van de bestudeerde groepen van contaminanten. Een overzicht van de meest gebruikte en efficiëntste analytische methoden voor de determinatie van POPs wordt voorgesteld in **Hoofdstuk 2**. Specifieke stappen, zoals extractie, opzuivering, gaschromatografische (GC) scheiding, detectiesystemen en kwaliteitscontrole/kwaliteitsborging worden bekeken en de nadruk wordt gelegd op de technieken gebruikt in deze thesis. Ook wordt aangetoond dat "fast GC" (gebruik makende van "narrow bore" capillaire kolommen) gebruikt kan worden gekoppeld met quadrupool massa spectrometrische (MS) detectie voor de snelle analyse van PCBs en PBDEs.

In **Hoofdstuk 3 en 4** wordt een simpele, snelle, gevoelige en universele procedure, gebaseerd op vaste fase kolom extractie (SPDE) voorgesteld, die was ontwikkeld en geëvalueerd voor de isolatie en concentratie van sporehoeveelheden geselecteerde organochloor pesticiden en PCB congeneren uit verschillende humane lichaamsvloeistoffen (serum, navelstrengbloed, moedermelk, folliculaire en seminale vloeistof). Een vergelijkbare methodologie kon worden gebruikt voor elke matrix, met als enige beperking de viscositeit van de vloeistof. Een Empore™ C_{18} gebonden silica kolom werd gebruikt voor de initiële extractie en aanrijking van de analyten. De verdere opzuivering werd gedaan door adsorptie chromatografie op geconcentreerd zwavelzuur : silica gel (1:1, w/w). Analyse werd gedaan door gas chromatografie – massa spectrometrie (GC-MS) of electron capture detection (ECD) op twee capillaire kolommen van verschillende polariteit. Door gebruik te maken van de SPDE procedure kon een hoge doorvoersnelheid en parallelle staalbehandeling bereikt worden. De methode werd gevalideerd door succesvolle deelname aan verscheidene interlaboratorium tests. We beschrijven als eersten het gebruik van een SPDE methode voor de determinatie van geselecteerde POPs in verschillende menselijke lichaamsvloeidstoffen. De methode werd succesvol gebruikt voor de analyse van geselecteerde organische pesticiden en PCBs in humaan serum van 200 vrouwen tussen 50 en 65 jaar uit 2 streken van Vlaanderen. In een andere studie naar het belang van *in utero blootstelling*, werden PCBs, HCB en p,p'-DDE concentraties bepaald in maternaal en umbilicaal bloed, gecollecteerd bij de bevalling van 44 Belgische moeders. Vervolgens hebben wij als eersten POPs concentraties gemeten in individueel humaan serum van 2 grote steden in Roemenië (Timisoara en Iassy). En tenslotte werd de distributie en verwijdering van PCBs in intermediaire fracties van het Cohn fractioneringsproces en in de uiteindelijke plasma derivaten onderzocht van stalen afkomstig van de bloedbank van het Belgisch Rode Kruis. Volgens ons is dit de eerste studie over de distributie van persistente polluenten in proteïnederivaten verkregen door plasma fractionering. Daarbovenop werden POPs bepaald in moedermelk van Roemeense vrouwen. PCB concentraties waren laag terwijl concentraties van organochloor pesticiden hoger waren dan de geraporteerde waarden van andere Europese landen. Tenslotte werden folliculaire en seminale vloeistoffen van verscheidene Belgische proefpersonen onderzocht voor hun POP

belasting. Concentraties van DDE en PCBs (de vooraamste organohalogeen verbindingen) waren zeer laag, meestal dicht tegen de detectie limiet.

In **Hoofdstuk 5** werden verschillende incubatie en extractie methoden geëvalueerd voor de determinatie van POPs in menselijk haar. De beste methode was een overnacht incubatie met HCl en vervolgens vloeistof – vloeistof extractie met hexaan:dichloromethaan. Na de zuivering op basische alumina en zure silica, werd het extract geanalyseerd met GC-ECD of GC-MS. Recoveries van interne standaaarden en te onderzoeken analyten lagen tussen 87 en 111%. Vergelijkbare concentraties POPs (geraporteerd in verhouding tot de lipiden) werden gevonden in moedermelk en haar van dezelfde persoon. Haaranalyse werd gebruikt voor de blootstellingsschatting aan organochloor polluenten van personen uit Griekenland, Roemenië en België. De hoogste organochloor belasting werd gevonden in stalen van een groep Griekse vrouwen met vroegere occupationele blootstelling aan pesticiden. Er werd besloten dat haar kan worden gebruikt voor monitoring voor elke subgroep van een populatie met de nadruk op diegenen waarvan de staalname van melk, vetweefsel of bloed niet mogelijk of zeer moeilijk is (bijv. kinderen of bedreigde diersoorten).

Een nieuwe analytische methode voor de kwantificatie van PBDEs in humaan vetweefsel werd ontwikkeld en wordt voorgesteld in **Hoofdstuk 6**. Na Soxhlet extractie en een zuiveringsprocedure met twee opeenvolgende vaste fase extraktie kolommen met daarin respectievelijk zure silica en zure silica : neutrale silica : gedeactiveerde basische alumina, kon detectie gebeuren met "narrow bore" capillaire GC/electron impact lage resolutie MS d.m.v. groot volume injectie techniek. Detectielimieten in geselecteerde ion modus varieerde tussen 0.05 en 0.30 ng/g vet, afhankelijk van de bromeringsgraad. De gevoeligheid van deze methode kan minstens wedijveren met lage resolutie massa spectrometrie met "electron capture" ionisatie, terwijl een veel betere resolutie wordt gehaald. De methode laat de determinatie in Belgische vetweefselstalen toe van 5 veel voorkomende PBDE congeneren (BDE 28, 47, 99, 100 en 153) in concentraties lager dan 1 ng/g vet en van 35 PCB congeneren, samen met p,p'-DDT en p,p'DDE. Concentraties aan POPs waren vergelijkbaar met voordien gerapporteerde waarden uit Europa. De som van de PCBs vertoonde een goede correlatie met de som van de DDTs ($r=0.77$, $p<0.05$), terwijl de correlatie met de som van de PBDEs zwakker was ($r=0.56$, $p<0.05$). Geen leeftijdsafhankelijkheid werd gevonden voor PBDEs, terwijl PCBs en DDTs een goede correlatie met de leeftijd vertoonden.

In januari van het jaar 1999 ontvingen verscheidene Belgische dierenkwekerijen voeder dat gecontamineerd was met transformatorolie die PCBs en dioxines bevatte. Het Toxicologische Center (UIA) nam deel aan het nationaal programma voor voedsel monitoring en analyseerde zodoende, in enkele maanden tijd, bijna 2500 stalen diervoeder en vlees. Resultaten van deze analyses worden besproken in **Hoofdstuk 7**. Analyse van de gecontamineerde stalen toonde aan dat de patronen voor PCB en PCDD/F congeneren verschilden tussen voeder, kippenvet en varkensvet. Aangetoond werd dat lager gechloreerde PCBs en PCDFs, die met hoge TEF incluis (PCBs 105, 118, 126 en 2,3,4,7,8-PeCDF), meer accumuleren in kippenvet of sneller worden geëlimineerd in varkens. Dit leidt tot de mogelijkheid dat consumptie van kip zou resulteren in een hogere TEQ humane body burden dan de consumptie van varkensvlees. Daarbovenop vonden we dat 12.1 % van Belgisch exportvlees en vetstalen van kippen of varkens, ongerelateerd aan de PCB/dioxine crisis, meer dan 50 ng PCBs/g vet bevatten en dat 6.5 % van de stalen meer dan 20 ng/g vet bevatten voor de som van p,p'-DDT en p,p'-DDE. Wij hebben aangetoond dat een deel van deze achtergrond contaminatie afkomstig is van geïmporteerde diervoederingrediënten (vismeel en granen), dat soms gecontamineerd is door

recent gebruik van DDT zoals kan worden afgeleid uit de verhouding tussen p,p'-DDT en p,p'-DDE. Na vergelijking van PCB concentraties in vismeel en granen met deze in vlees kan nochtans worden gesuggereerd dat hoge contaminatie afkomstig is van gerecycleerd vet. Dierlijke vetstalen van twee voorname Roemeense dorpen (Arad en Iassy) werden geanalyseerd voor hun organochloor belasting. Sommige dierlijke vetstalen van Iassy overschreden de EU normen voor organochloor pesticiden, maar vertoonden vergelijkbare PCB concentraties met de stalen van Arad.

Hoofdstuk 8 bevat 2 applicaties voor de bepaling van POP in aquatische biota. Gekweekte en wilde Schotse Atlantische zalm, aquacultuur voeder en visoliën, verkregen in januari van het jaar 1999, werden onderzocht voor de bepaling van een brede waaier van PCBs, organochloor pesticiden en PBDEs. De studie bevestigt vorige rapporten van relatief hoge concentraties aan PCBs en toont gemiddelde concentraties aan van organochloor pesticiden en PBDEs in gekweekte zalm. Voor de Schotse zalmstalen was de PCB TEQ twee maal die van PCDD/Fs. De resultaten tonen aan dat hoge zalmconsumptie, vooral door kinderen onder vijf jaar, zou kunnen leiden tot innames boven de aanvaardbare dagelijkse inname (TDI) en tolereerbare wekelijkse inname (TWI) voor deze chemicaliën, vooral voor PCBs, in combinatie met gemiddelde of hoge innames door het dieet in het Verenigd Koninkrijk. Leverstalen van 21 bruinvissen (*Phocoena phocoena*) die gestrand zijn aan de Belgische Noordzeekust werden geanalyseerd voor een brede waaier aan PCBs, organochloor pesticiden en PBDEs. PCBs waren de belangrijkste organochloor contaminanten in de bruinvissen, terwijl het meest voorkomende organochloor pesticide DDT was, gevolgd door HCB en HCHs. De gemiddelde concentraties van groepen polluenten waren, op enkele enkele uitzonderingen na, vergelijkbaar met deze gerapporteerd in de literatuur. Verschillen tussen concentraties en profielen van POPs werden waargenomen tussen volwassen en jonge dieren, evenals tussen mannelijke en vrouwelijke dieren.

Hot Soxhlet extractie van POPs uit bodemstalen werd onderzocht in **Hoofdstuk 9** en de geoptimaliseerde methode werd grondig getest. Analyse van Roemeense grond toonde aan dat stalen van de provincie Iassy een lagere contaminatie met organochloorverbindingen vertoonden dan stalen uit andere Roemeense regio's. Hoewel concentraties aan DDTs in grond aanzienlijk hoger waren in rurale sites, overschreden slechts enkele stalen (6%) de officiële Roemeense normen voor DDTs. PCBs concentraties waren laag in rurale sites, maar redelijk hoog in stedelijke grond, verzameld in parken. Accumulatieprofielen van organochloorverbindingen werden bepaald in bodemstalen, dierlijk vet en humaan serum, gecollecteerd in de provincie Iassy. De profielen waren verschillend in de drie matrices en de accumulatie orde was grond < dierlijk vet < humaan serum. Ter vergelijking werden ook bodemstalen van België (n=16), Italië (n=6) en Griekenland (n=2) geanalyseerd. Deze stalen bevatten vergelijkbare concentraties HCB en PCBs, maar lagere concentraties DDTs en HCHs dan de Roemeense stalen. De hoogste PCB concentraties werden gevonden in grond van industriële sites.

Als conclusie kunnen we zeggen dat analytische methodologies gebruikt voor de determinatie van POPs moeten worden aangepast aan de permanente nood van steeds lagere detectielimieten. De methodes zouden in staat moeten zijn om subtiele veranderingen te meten in profielen en concentraties van verschillende polluenten zoals PCB congeneren, in verschillende species. Voldoende kwaliteitscontrole is aldus vereist voor methode validatie. In deze thesis werd aangetoond dat POPs zeer wijd verspreid in het milieu worden teruggevonden en dat ze accumuleren in species die bovenaan de voedselketen staan, zowel

in land- (mensen) als in aquatische systemen (mariene zoogdieren). Hierdoor is de voortdurende monitoring van de menselijke voedselketen en van de menselijke populatie een vereiste. In de toekomst zouden nieuwe klassen van polluenten (zoals PBDEs, organofluor verbindingen, PCB metabolieten) moeten worden opgenomen in monitoring schema's, terwijl ook meer gegevens nodig zullen zijn voor de inventarisatie en "risk assessment" van 'klassieke' POPs.

ANNEX. Systematic numbering of PCB congeners. Ref : Ballscmiter K, Bacher R, Mennel A, Fischer R, Riehle U, Swerev M, (1992). *J High Resolut Chromatogr* 15, 260-270.

IUPAC Number	IUPAC Name
1	2-Chlorobiphenyl
2	3-Chlorobiphenyl
3	4-Chlorobiphenyl
4	2,2'-Dichlorobiphenyl
5	2,3-Dichlorobiphenyl
6	2,3'-Dichlorobiphenyl
7	2,4-Dichlorobiphenyl
8	2,4'-Dichlorobiphenyl
9	2,5-Dichlorobiphenyl
10	2,6-Dichlorobiphenyl
11	3,3'-Dichlorobiphenyl
12	3,4-Dichlorobiphenyl
13	3,4'-Dichlorobiphenyl
14	3,5-Dichlorobiphenyl
15	4,4'-Dichlorobiphenyl
16	2,2',3-Trichlorobiphenyl
17	2,2',4-Trichlorobiphenyl
18	2,2',5-Trichlorobiphenyl
19	2,2',6-Trichlorobiphenyl
20	2,3,3'-Trichlorobiphenyl
21	2,3,4-Trichlorobiphenyl
22	2,3,4'-Trichlorobiphenyl
23	2,3,5-Trichlorobiphenyl
24	2,3,6-Trichlorobiphenyl
25	2,3',4-Trichlorobiphenyl
26	2,3',5-Trichlorobiphenyl
27	2,3',6-Trichlorobiphenyl
28	2,4,4'-Trichlorobiphenyl
29	2,4,5-Trichlorobiphenyl
30	2,4,6-Trichlorobiphenyl
31	2,4',5-Trichlorobiphenyl
32	2,4',6-Trichlorobiphenyl
33	2,3',4'-Trichlorobiphenyl
34	2,3',5'-Trichlorobiphenyl
35	3,3',4-Trichlorobiphenyl
36	3,3',5-Trichlorobiphenyl
37	3,4,4'-Trichlorobiphenyl
38	3,4,5-Trichlorobiphenyl
39	3,4',5-Trichlorobiphenyl
40	2,2',3,3'-Tetrachlorobiphenyl
41	2,2',3,4-Tetrachlorobiphenyl
42	2,2',3,4'-Tetrachlorobiphenyl
43	2,2',3,5-Tetrachlorobiphenyl
44	2,2',3,5'-Tetrachlorobiphenyl
45	2,2',3,6-Tetrachlorobiphenyl
46	2,2',3,6'-Tetrachlorobiphenyl
47	2,2',4,4'-Tetrachlorobiphenyl
48	2,2',4,5-Tetrachlorobiphenyl
49	2,2',4,5'-Tetrachlorobiphenyl
50	2,2',4,6-Tetrachlorobiphenyl
51	2,2',4,6'-Tetrachlorobiphenyl
52	2,2',5,5'-Tetrachlorobiphenyl
53	2,2',5,6'-Tetrachlorobiphenyl
54	2,2',6,6'-Tetrachlorobiphenyl
55	2,3,3',4-Tetrachlorobiphenyl
56	2,3,3',4'-Tetrachlorobiphenyl
57	2,3,3',5-Tetrachlorobiphenyl
58	2,3,3',5'-Tetrachlorobiphenyl
59	2,3,3',6-Tetrachlorobiphenyl
60	2,3,4,4'-Tetrachlorobiphenyl
61	2,3,4,5-Tetrachlorobiphenyl
62	2,3,4,6-Tetrachlorobiphenyl
63	2,3,4',5-Tetrachlorobiphenyl
64	2,3,4',6-Tetrachlorobiphenyl
65	2,3,5,6-Tetrachlorobiphenyl
66	2,3',4,4'-Tetrachlorobiphenyl
67	2,3',4,5-Tetrachlorobiphenyl
68	2,3',4,5'-Tetrachlorobiphenyl
69	2,3',4,6-Tetrachlorobiphenyl
70	2,3',4',5-Tetrachlorobiphenyl
71	2,3',4',6-Tetrachlorobiphenyl
72	2,3',5,5'-Tetrachlorobiphenyl
73	2,3',5',6-Tetrachlorobiphenyl
74	2,4,4',5-Tetrachlorobiphenyl
75	2,4,4',6-Tetrachlorobiphenyl
76	2,3',4',5'-Tetrachlorobiphenyl

IUPAC Number	IUPAC Name
77	3,3',4,4'-Tetrachlorobiphenyl
78	3,3',4,5-Tetrachlorobiphenyl
79	3,3',4,5'-Tetrachlorobiphenyl
80	3,3',5,5'-Tetrachlorobiphenyl
81	3,4,4',5-Tetrachlorobiphenyl
82	2,2',3,3',4-Pentachlorobiphenyl
83	2,2',3,3',5-Pentachlorobiphenyl
84	2,2',3,3',6-Pentachlorobiphenyl
85	2,2',3,4,4'-Pentachlorobiphenyl
86	2,2',3,4,5-Pentachlorobiphenyl
87	2,2',3,4,5'-Pentachlorobiphenyl
88	2,2',3,4,6-Pentachlorobiphenyl
89	2,2',3,4,6'-Pentachlorobiphenyl
90	2,2',3,4',5-Pentachlorobiphenyl
91	2,2',3,4',6-Pentachlorobiphenyl
92	2,2',3,5,5'-Pentachlorobiphenyl
93	2,2',3,5,6-Pentachlorobiphenyl
94	2,2',3,5,6'-Pentachlorobiphenyl
95	2,2',3,5',6-Pentachlorobiphenyl
96	2,2',3,6,6'-Pentachlorobiphenyl
97	2,2',3,4',5'-Pentachlorobiphenyl
98	2,2',3,4',6'-Pentachlorobiphenyl
99	2,2',4,4',5-Pentachlorobiphenyl
100	2,2',4,4',6-Pentachlorobiphenyl
101	2,2',4,5,5'-Pentachlorobiphenyl
102	2,2',4,5,6'-Pentachlorobiphenyl
103	2,2',4,5',6-Pentachlorobiphenyl
104	2,2',4,6,6'-Pentachlorobiphenyl
105	2,3,3',4,4'-Pentachlorobiphenyl
106	2,3,3',4,5-Pentachlorobiphenyl
107	2,3,3',4',5-Pentachlorobiphenyl
108	2,3,3',4,5'-Pentachlorobiphenyl
109	2,3,3',4,6-Pentachlorobiphenyl
110	2,3,3',4',6-Pentachlorobiphenyl
111	2,3,3',5,5'-Pentachlorobiphenyl
112	2,3,3',5,6-Pentachlorobiphenyl
113	2,3,3',5',6-Pentachlorobiphenyl
114	2,3,4,4',5-Pentachlorobiphenyl
115	2,3,4,4',6-Pentachlorobiphenyl
116	2,3,4,5,6-Pentachlorobiphenyl
117	2,3,4',5,6-Pentachlorobiphenyl
118	2,3',4,4',5-Pentachlorobiphenyl
119	2,3',4,4',6-Pentachlorobiphenyl
120	2,3',4,5,5'-Pentachlorobiphenyl
121	2,3',4,5',6-Pentachlorobiphenyl
122	2,3,3',4',5-Pentachlorobiphenyl
123	2,3',4,4',5'-Pentachlorobiphenyl
124	2,3',4',5,5'-Pentachlorobiphenyl
125	2,3',4',5',6-Pentachlorobiphenyl
126	3,3',4,4',5-Pentachlorobiphenyl
127	3,3',4,5,5'-Pentachlorobiphenyl
128	2,2',3,3',4,4'-Hexachlorobiphenyl
129	2,2',3,3',4,5-Hexachlorobiphenyl
130	2,2',3,3',4,5'-Hexachlorobiphenyl
131	2,2',3,3',4,6-Hexachlorobiphenyl
132	2,2',3,3',4,6'-Hexachlorobiphenyl
133	2,2',3,3',5,5'-Hexachlorobiphenyl
134	2,2',3,3',5,6-Hexachlorobiphenyl
135	2,2',3,3',5,6'-Hexachlorobiphenyl
136	2,2',3,3',6,6'-Hexachlorobiphenyl
137	2,2',3,4,4',5-Hexachlorobiphenyl
138	2,2',3,4,4',5'-Hexachlorobiphenyl
139	2,2',3,4,4',6-Hexachlorobiphenyl
140	2,2',3,4,4',6'-Hexachlorobiphenyl
141	2,2',3,4,5,5'-Hexachlorobiphenyl
142	2,2',3,4,5,6-Hexachlorobiphenyl
143	2,2',3,4,5,6'-Hexachlorobiphenyl
144	2,2',3,4,5',6-Hexachlorobiphenyl
145	2,2',3,4,6,6'-Hexachlorobiphenyl
146	2,2',3,4',5,5'-Hexachlorobiphenyl
147	2,2',3,4',5,6-Hexachlorobiphenyl
148	2,2',3,4',5,6'-Hexachlorobiphenyl
149	2,2',3,4',5',6-Hexachlorobiphenyl
150	2,2',3,4',6,6'-Hexachlorobiphenyl
151	2,2',3,5,5',6-Hexachlorobiphenyl
152	2,2',3,5,6,6'-Hexachlorobiphenyl

IUPAC Number	IUPAC Name
153	2,2',4,4',5,5'-Hexachlorobiphenyl
154	2,2',4,4',5,6'-Hexachlorobiphenyl
155	2,2',4,4',6,6'-Hexachlorobiphenyl
156	2,3,3',4,4',5-Hexachlorobiphenyl
157	2,3,3',4,4',5'-Hexachlorobiphenyl
158	2,3,3',4,4',6-Hexachlorobiphenyl
159	2,3,3',4,5,5'-Hexachlorobiphenyl
160	2,3,3',4,5,6-Hexachlorobiphenyl
161	2,3,3',4,5',6-Hexachlorobiphenyl
162	2,3,3',4',5,5'-Hexachlorobiphenyl
163	2,3,3',4',5,6-Hexachlorobiphenyl
164	2,3,3',4',5',6-Hexachlorobiphenyl
165	2,3,3',5,5',6-Hexachlorobiphenyl
166	2,3,4,4',5,6-Hexachlorobiphenyl
167	2,3',4,4',5,5'-Hexachlorobiphenyl
168	2,3',4,4',5',6-Hexachlorobiphenyl
169	3,3',4,4',5,5'-Hexachlorobiphenyl
170	2,2',3,3',4,4',5-Heptachlorobiphenyl
171	2,2',3,3',4,4',6-Heptachlorobiphenyl
172	2,2',3,3',4,5,5'-Heptachlorobiphenyl
173	2,2',3,3',4,5,6-Heptachlorobiphenyl
174	2,2',3,3',4,5,6'-Heptachlorobiphenyl
175	2,2',3,3',4,5',6-Heptachlorobiphenyl
176	2,2',3,3',4,6,6'-Heptachlorobiphenyl
177	2,2',3,3',4,5',6'-Heptachlorobiphenyl
178	2,2',3,3',5,5',6-Heptachlorobiphenyl
179	2,2',3,3',5,6,6'-Heptachlorobiphenyl
180	2,2',3,4,4',5,5'-Heptachlorobiphenyl
181	2,2',3,4,4',5,6-Heptachlorobiphenyl
182	2,2',3,4,4',5,6'-Heptachlorobiphenyl
183	2,2',3,4,4',5',6-Heptachlorobiphenyl
184	2,2',3,4,4',6,6'-Heptachlorobiphenyl
185	2,2',3,4,5,5',6-Heptachlorobiphenyl
186	2,2',3,4,5,6,6'-Heptachlorobiphenyl
187	2,2',3,4',5,5',6-Heptachlorobiphenyl
188	2,2',3,4',5,6,6'-Heptachlorobiphenyl
189	2,3,3',4,4',5,5'-Heptachlorobiphenyl
190	2,3,3',4,4',5,6-Heptachlorobiphenyl
191	2,3,3',4,4',5',6-Heptachlorobiphenyl
192	2,3,3',4,5,5',6-Heptachlorobiphenyl
193	2,3,3',4',5,5',6-Heptachlorobiphenyl
194	2,2',3,3',4,4',5,5'-Octachlorobiphenyl
195	2,2',3,3',4,4',5,6-Octachlorobiphenyl
196	2,2',3,3',4,4',5,6'-Octachlorobiphenyl
197	2,2',3,3',4,4',6,6'-Octachlorobiphenyl
198	2,2',3,3',4,5,5',6-Octachlorobiphenyl
199	2,2',3,3',4,5,5',6'-Octachlorobiphenyl
200	2,2',3,3',4,5,6,6'-Octachlorobiphenyl
201	2,2',3,3',4,5',6,6'-Octachlorobiphenyl
202	2,2',3,3',5,5',6,6'-Octachlorobiphenyl
203	2,2',3,4,4',5,5',6-Octachlorobiphenyl
204	2,2',3,4,4',5,6,6'-Octachlorobiphenyl
205	2,3,3',4,4',5,5',6-Octachlorobiphenyl
206	2,2',3,3',4,4',5,5',6-Nonachlorobiphen
207	2,2',3,3',4,4',5,6,6'-Nonachlorobiphen
208	2,2',3,3',4,5,5',6,6'-Nonachlorobiphen
209	Decachlorobiphenyl

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List of abbreviations

CRM	certified reference material
DDD	dichlorodiphenyldichloroethane
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
ECD	electron capture detector
EI	electron impact
EU	European Union
GC	gas chromatography
HCB	hexachlorobenzene
HCH	hexachlorocyclohexane
HPLC	high performance liquid chromatography
ICES	International Council for the Exploration of the Sea
ID	internal diameter
IUPAC	Intaernational Union of Pure and Applied Chemistry
LOD	limit of detection
MAFF	Ministry of Agriculture, Fisheries and Food (United Kingdom)
MS	mass spectrometry
MWI	municipal waste incinerator
NCI	negative chemical ionisation
NIST	National Institute for standards and Technology
PBB	polybrominated biphenyl
PBDE	polybrominated diphenyl ether
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-p-dioxin
PCDF	polychlorinated dibenzofuran
PCN	polychlorinated naphthalene
POP	persistent organohalogenated pollutant
PTV	programmable temperature vaporizer
PYE	2-(1-pyrenyl)ethyldimethylsililated silica
QA	quality assurance
QC	quality control
SPDE	solid-phase disk extraction
SPE	solid-phase extraction
TCN	tetrachloronaphthalene
TEF	toxic equivalency factor
TEQ	2,3,7,8-TCDD equivalent
TIC	total ion chromatogram
WHO	World Health Organisation