Fluorinated, Chlorinated and Brominated Contaminants in Fish for Human Consumption

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Methods and measurements

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Summary

Fish is an important part of our food. In addition to its general nutritional value fish contains essential nutrients like omega-3 and omega-6 fatty acids, which are believed to be beneficial for our health. Fish is also an important dietary source of selenium. Unfortunately, several contaminants are present in fish, such as heavy metals and organohalogen contaminants. Well-known examples of the latter are polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins and –furans (dioxins) and DDT. Production of PCBs and DDT has started before World War II and were used in industrial and consumer applications as well as in agriculture. PCBs and DDT have entered the environment during production, product formulation, application and disposal or (for dioxins) as a result of combustion processes or as a by-product of chemical synthesis *(chapter 1)*. Environmental residues of these xenobiotic compounds have been found since the late 1960s. Extensive research is carried out to these contaminants since then. This showed that these contaminants are persistent, bioaccumulate, have adverse effects and are transported globally over large distances. They are therefore qualified as persistent organic pollutants (POPs) in the framework of the Stockholm Convention. More recently, new contaminants were found in fish, such as polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD) and perfluorinated compounds (PFCs). These compounds are still being produced for application in consumer products and industrial processes. There is toxicological concern on these contaminants. The toxicity of dioxins and dioxin-like (dl)-PCBs received much attention, and these are among the most toxic substances known. Non-dioxin-like (ndl) PCBs are less toxic, although they can cause neurotoxic effects. The toxicity of PFCs, PBDEs and HBCD is under investigation, and their complete toxic profile is not yet elucidated, although several adverse effects of these compounds were reported. Through the consumption of fish, these contaminants are ingested and may potentially harm consumer's health. Several studies showed that fish is an important contributor to the dietary exposure to e.g. dioxins, PCBs, organochlorine pesticides (OCPs) and PBDEs. Information on the dietary exposure to HBCD and PFCs is urgently needed to allow adequate exposure

assessments. Unfortunately, validated methods for the analysis of HBCD and PFCs were not readily available. To overcome this problem, methods needed to be developed and validated. Apart from that, for dioxins and dioxin-like (dl-)PCBs less expensive and complicated methods were needed to enlarge food safety monitoring capacity.

This study was undertaken with the following aims:

- Development of reliable methods of analysis, including in-house and between laboratory validations;
- Determination of the contaminant concentrations in fish, shellfish and shrimp species relevant for human exposure;
- Estimation of the dietary exposure to a broad suite of contaminants from wild and farmed fish.

This allows determining the possible health risks and the relative importance of specific contaminants and fish species, and may help policy makes and scientists to determine where to put their focus on.

Analytical method development

Current methods for halogenated lipophilic contaminants in fish often start with extraction based on Soxhlet, although accelerated solvent extraction (ASE) becomes more popular (*chapter 2.1*). Clean-up of the sample consists of lipid removal (e.g. by gel permeation chromatography, sulphuric acid treatment of alumina oxide column chromatography). Fractionation of the extract is often achieved by silica column chromatography after which the extract is ready for analysis by gas chromatographic (GC) techniques. Electron capture detection (ECD) is still used as an inexpensive and simple detector, but mass spectrometric (MS) techniques are increasingly being used because of unambiguous identification and for their sensitivity. In recent years, comprehensive multidimensional GC (GCxGC) gained popularity as it enables the separation of very complex mixtures in a single run. Because of the low concentrations in fish samples, dioxins and dl-PCBs require additional clean-up over e.g. graphitised carbon columns. GC-high resolution (HR)MS is being used in order to reduce interferences in the detection. Unfortunately, GC-HRMS equipment requires large investments and, therefore, it is only applied in a limited number of specialized laboratories. In addition, the sample extraction and clean-up is time consuming, further adding to the costs. Within the framework of the EU DIFFERENCE project, integrated sample extraction and clean-up methods were developed based on ASE extraction and combined with within-cell clean-up and fractionation of the extract. For the final determination, alternative detection techniques were developed and optimised (i.e. GC-ion trap MS/MS, GCxGC with ECD and the DR-CALUX bioassay). These techniques were validated in an international framework,

including sensitivity, accuracy and precision tests *(chapter 3.1)*. GC-ion trap MS/MS was most promising in terms of performance and costs. GCxGC-ECD performed also well, but a drawback (for the moment) is the time-consuming manual integration of peaks, especially at low concentrations. DR-CALUX is sensitive but suffered from a bias and limited precision. Nevertheless, DR-CALUX can serve as an excellent screening technique during crises, as well as for finding unknown compounds that exhibit an Ah receptor response. It should be noted that GC-LRMS/MS, GCxGC-ECD and DR-CALUX all three met the EU requirements of screening methods for dioxins and dl-PCBs in food. Extraction by ASE and sample clean-up within the extraction cell is promising, but further method optimisation is needed.

For HBCD, both GC- and liquid chromatography (LC-)MS/MS techniques were available. However, discrepancies were observed between results from both techniques, with the GC results being 4.4-fold higher (on average) than the LC based results *(chapter 3.2)*. Although this difference could not be explained completely, the LC method is preferred for a number of reasons: (i) the specific detection of the three major HBCD diastereomers; (ii) the use of ^{13}C-labelled standards allowing a more accurate analysis; (iii) no thermal degradation of HBCD or interconversion of individual diastereomers.

For PFCs, the methods initially developed internationally were based on ion pair extraction (IPE) and no further clean-up. In recent years, the number of PFCs included in methods increased and more diverse methods were developed for fish, such as extraction by methanol or acetonitrile and clean-up by suspended graphitised carbon, or saponification of the sample followed by solid phase extraction (SPE) for concentration and clean-up (*chapter 2.2*). Chromatographic separation is typically achieved over reversed phase (C18) columns and the best detection method is MS/MS or time-of-flight (TOF) MS, which both provide selectivity and allow unambiguous identification. The lack of high quality standards, mass labelled internal standards, suitable clean-up methods and the presence of interferences has put a pressure on the accuracy of the data produced world-wide. This was reflected in the first worldwide PFC interlaboratory study (ILS), organised within the framework of the EU Perforce project (*chapter 3.3*). The comparability of results for water and fish was very poor, showing the need for improvement of methods and availability of high quality standards (native and mass labelled). Large developments took place on both aspects in recent years. A broad suite of high quality standards and mass labelled standards became commercially available. Furthermore, a variety of analytical approaches were developed delivering accurate data *(chapter 2.2)*. In a follow-up study the between laboratory comparability improved considerably (*chapter 3.4)* as a result of the use of high quality native and internal standards by all participants. Furthermore, the routinely applied method of using solvent based calibration curves (i.e. a calibration curve in solvent) delivers very precise and reproducible (between labs) results in case it is combined with a

mass labelled analogue for each single target compound. Results were more precise and reproducible than those based on standard addition quantification (i.e. a calibration curve in the matrix), although this technique is highly suitable for quantification of those compounds that have no mass labelled analogue in samples with substantial matrix effects.

Contaminant concentrations in fish

A broad selection of contaminants (dioxins, dl-PCBs, indicator-PCBs, OCPs, PBDEs, HBCD and PFCs) was determined in a wide range of fish, shellfish and shrimp species. Emphasis was put on species, which are regularly consumed by Dutch citizens or which – from previous research – were known to contain high PCB concentrations (eel). This study included salmon (farmed), eel (wild and farmed), trout (farmed), pikeperch, herring, mackerel, cod, coalfish, haddock, flounder, sole, shrimp (wild shrimp from the Dutch coast and farmed shrimp, mainly from Asia) and mussels. In addition, recently introduced farmed species like tilapia and pangasius were investigated. Most fish originated from Dutch freshwaters (eel, pikeperch), the North Sea and the Atlantic Ocean. Farmed species like salmon, eel and trout originated mainly from Europe while tilapia, pangasius and farmed shrimp originated mainly from Asia.

Dioxins and dl-PCBs were highest in wild eel samples from the river Meuse and Rhine deltas *(chapter 4.1)*. Total-TEQ concentrations (i.e. the sum of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxicity equivalents) were up to 4.5 times above the EU maximum level (ML) for eel (12 pg TEQ/g ww). The concentrations in fish from other Dutch freshwater locations were (much) lower. Concentrations in marine fish such as herring, flounder, mackerel, salmon etc. were also lower and below the ML of 8 pg TEQ/g ww (for other fish). The farmed species pangasius and tilapia stand out because of their extremely low total-TEQ concentrations *(chapter 4.4)*. In most pangasius and tilapia samples nearly all dioxin congeners and PCB congeners were below the limit of quantification (LOQ). A 250-fold difference was observed between the concentrations in the pangasius and tilapia samples and the highest contaminated eel samples. This difference would be much larger (2,400,000 fold) when the concentrations would be expressed on a lower bound basis. The revised World Health Organisation (WHO) toxic equivalency factors (TEFs) of 2005 result in 10-20% lower TEQ values compared to the 1998 TEFs because mono-ortho PCBs were assigned lower TEFs in the 2005 TEF revision *(chapter 4.1)*. This effect is most pronounced in eel (40% lower TEQ values) as these have relative high mono-ortho PCB concentrations.

The indicator PCBs follow a similar pattern as discussed above, with eel being the highest contaminated fish species (sum of indicator PCBs 1,740 ng/g ww for eel from the river New Merwede, *chapter 4.1*) and tilapia and pangasius being the lowest contaminated species *(chapter 4.4)*. The lowest ΣPCB concentration was found in pangasius from Vietnam with as little as 0.034

ng/g ww. This is 50,000 times lower than in the New Merwede eel sample. Some of the eel samples (New Merwede, Hollands Diep, Meuse at Keizersveer) exceeded the Dutch MLs for indicator PCBs, but no other species exceeded these MLs. CB 153 was in all cases the predominant congener.
The analysis of PBDEs was limited to the congeners proposed by the European Food Safety Agency (EFSA) (BDE 28, 47, 99, 100, 153, 154, 183 and 209). BDE 49 was added to the selection as it was in most samples detected in concentrations comparable to BDE 99. In nearly all samples, these BDEs were detected. BDE 183, also recommended by EFSA, was not detected in most samples. The sum of BDEs 28, 47, 49, 99, 100, 153, 154, 183 and 209 was 0.01-0.15 ng/g ww in farmed shrimps, pangasius and tilapia, 0.34-3.9 ng/g ww in farmed trout, salmon and eel, 0.1-9.3 ng/g ww in mussels and marine fish and 0.2-220 ng/g ww in wild eel (*chapter 4.2 and 4.4*). BDE 209 was not detected in most of the wild fish species. For long it was believed that BDE 209 would not accumulate in fish, or that reported values were debatable because of analytical problems (e.g. blank contributions). Due to a very well controlled low blank contamination, BDE 209 could be detected in most farmed shrimp samples (8-17 pg/g ww) and pangasius samples (7-70 pg/g ww) *(chapter 4.4)*. It was not detected in any of the tilapia samples, and only in two salmon samples (45 and 59 pg/g ww) and in one trout sample (3600 pg/g ww). The cause of this high concentration in trout is unknown. Re-analysis confirmed the concentration. The predominant congeners were BDE 47>49≈99≈100, except for pangasius and farmed shrimps in which BDE 209 contributed approx. 50% to the sum. It is not clear what explains the presence of BDE 209 in several samples. Possibly, it is ingested through the diet. On the other hand, it may be a contamination that occurred during processing, packaging, storage and transportation of these farmed fish samples.
HBCD was detected in approx. 50% of the samples (*chapter 4.2 and 4.4*). In nearly all of these samples, α-HBCD was the only diastereomer present at concentrations from 0.01 ng/g ww (pangasius, farmed shrimps) to 41 ng/g ww (eel), spanning four orders of magnitude. β- and γ-HBCD were present in eel, but at lower concentrations than α-HBCD (e.g. γ-HBCD was approx. 21% of α-HBCD). In other species, these differences were much larger and β- and γ-HBCD were only detected in a few trout and shrimp samples (0.01-0.05 ng/g ww).
Within the group of perfluorinated compounds, PFOS was the dominating contaminant (*chapter 4.3*). Short chain PFCs (C4 to C7) hardly accumulate in fish and also perfluoroctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorododecanoic acid (PFDoA) were only found in a 1-2 samples of the 70 samples analysed. Perfluordecanoic acid (PFDcA) and the odd chain length perfluorundecanoic acid (PFUnA) and perfluortridecanoic acid (PFTrA) were detected more frequently (in 10-20% of the samples). There was no clear relationship with species or origin of the sample. It is not known why these two stand out, as no widespread use was reported. Possibly, these are stable end

products of (bio)degraded precursors. In wild fish (marine and freshwater), 2-150 ng/g ww PFOS was found. Concentrations in Western Scheldt flounder samples were higher due to (historic) contamination caused by PFC manufacturing plant and PFC users. PFOS concentrations in livers were on average 5.3-fold higher as in the fillets from the corresponding fish. PFOS was only detected in 4 out of the 37 farmed fish species (*chapter 4.3 and 4.4*). Surprisingly, PFOS was not detected in any of the salmon samples, whereas the other (earlier mentioned) contaminants often are found in salmon. This may be partly due to the higher LODs for PFOS (approx. 0.5 ng/g ww) as compared to e.g. PCBs (LOD of approx. 0.005 to 0.1 ng/g ww). On the other hand, the distinct accumulation behaviour of PFCs may play a role here as well.
When comparing contaminant concentrations in the investigated samples, they (generally) decrease in the following order ∑indicator PCB > PFOS ≈ ∑3 DDTs (i.e. p,p'-DDT, p,p'-DDD and p,p'-DDE) > ∑8 PBDEs > HCB ≈ α-HBCD >> total-TEQ. This is only a general indication, as the order may vary between species and per contaminant.

Human exposure estimation from fish consumption

The human exposure from fish consumption was estimated for dioxins and dl-PCBs, ∑indicator-PCBs, ∑3 DDTs, HCB, ∑8PBDEs, α-HBCD and PFOS. The exposure was calculated from fish consumption figures from the Dutch National Food Consumption Survey (DNFCS), and multiplied with mean contaminant data. The DNFCS database of 1997/1998 contains mean fish consumption data for a variety of fish including herring, salmon, cod, eel, mackerel, mussels and wild shrimp. New farmed fish species like pangasius and tilapia are not represented in the DNFCS. Because consumption of these species increases rapidly, recent estimates of sales to consumers (2006) were taken and the consumption was derived from these figures. The same holds for farmed shrimp and trout. Contaminant data that consisted mainly of <LOD values was not taken into account. This holds e.g. for BDE 183 and PFOA. On the other hand, BDE 49 was included as it was present in nearly every fish sample.
The exposure estimates are based on fish only, meaning that other sources of dietary exposure (e.g. dairy, cereals and pork) were not taken into account. The absolute exposure amounts decrease in the following order *(chapter 4.5)*: ∑indicator-PCBs (1.1 ng/kg bw per day) > PFOS (1.0 ng/kg bw per day) > ∑3 DDTs (0.45 ng/kg bw per day) > ∑8PBDEs (0.27 ng/kg bw per day) > HCB (0.09 ng/kg bw per day) ≈ α-HBCD (0.06 ng/kg bw per day) >> Dioxins and dl-PCBs (0.26 pg total-TEQ/kg bw per day). The exposure of dioxins and dioxin-like PCBs (as compared to other contaminants) is closest is to the WHO tolerable daily intake (TDI) of 2 pg/kg bw per day, leaving only a small margin of exposure. This margin will even be smaller when exposure to other food items

will be included in the estimation. The human toxicity data of non-dioxin-like PCBs is currently revised, and therefore no comparison is made with reference values. The PFOS exposure is 150-fold lower than the estimate made by the EFSA (2008). There are two underlying reasons for this difference (*chapter 4.3*). First, EFSA used 5-fold higher fish consumption estimates, based on consumer-only data, meaning that it was assumed that every citizen consumes fish every day (which is a conservative approach). Secondly, EFSA used a high estimate for the PFOS concentration in fish in general (68 ng/g ww), which is much higher than was shown in this study. In this study, species-specific PFOS contamination data was available, allowing a more accurate estimation of the exposure of the Dutch population. When looking at the exposure from all contaminants (summarised), herring predominates (41%), followed by salmon (21%) and Gadidae (14%) (total 76%). The other fish species together contribute 24%. The contribution of pangasius, tilapia and farmed shrimp is less than 1%.

Conclusions and future perspectives

The results of this study show that promising alternative techniques (GC-ITMS/MS) to GC-HRMS are available for the detection of dioxins and dl-PCBs in food and feed. DR-CALUX and GCxGC-ECD are valuable screening tools, but they would benefit from further development in terms of accuracy (DR-CALUX) and reduction of labour involved in peak integration (GCxGC-ECD). For the analysis of HBCD, LC-ESI-MS/MS is preferred over GC-ECNI-MS for its better accuracy and because separation of individual diastereomers is feasible. The quality of the analysis of PFCs in fish and water benefited to a large extent from the improved knowledge on the behaviour of PFCs, a broader range of analytical approaches and the availability of a high quality native and mass labelled standards.

Contaminant concentrations were highest in wild eel from polluted areas (e.g. Haringvliet) and lowest in farmed fish samples. From a contaminant point of view, concentrations decreased as follows: ∑7indicatorPCB > PFOS ≈ ∑3 DDTs > ∑8PBDEs > HCB ≈ a-HBCD >> total-TEQ. Although contaminant concentrations in wild eel were the highest, herring and salmon dominate the human exposure from fish consumption. The contribution from newly farmed species (pangasius, tilapia and shrimp) is <1%. The exposure to PFOS was different as compared to that of the more lipophilic contaminants.

Future analytical developments will focus on (i) reduction of time and labour needed for sample pre-treatment (sample extraction and clean-up), (ii) further lowering of detection limits of MS instruments (iii) miniaturisation of analytical methods (iv), improving speed and throughput of analytical methods. This will enable the analysis of hundreds of compounds in a single run. On the other hand, bio-analytical approaches are complementary to chemo-analytical methods. These will develop further allowing rapid analysis

of contaminants with a comparable biological activity and the identification of unknown contaminants.
New contaminants are discovered continuously. Recent examples of new contaminants that drew attention of scientists and policy makers are PFCs, nano particles and siloxanes. Whereas exposure to traditional lipophilic contaminants is dominated by the diet (i.e. foods form animal origin), these contaminants have different properties that require the development of new analytical approaches. In addition, the different properties require a broader exposure assessment including diet, drinking water, beverages, dust and air. Characterisation of these different routes is laborious. The analysis human body fluids (e.g. blood, milk) can provide an integrated picture of all exposure routes and is a valuable complementary approach.