

*Microsphere-based binding assays for
organic pollutants*

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*Microsphere-based binding
assays for organic pollutants*

Anastasia Meimaridou

Thesis

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*Στους γονείς μου Αθανασία Μειμαρίδου-Τζιτζιού , Μειμαρίδη Χαράλαμφο
και στον αδερφό μου Γιάννη.
Ότι είμαι το οφείλω σε εσάς. Σας υπεραγαπώ!!!!*

*To my parents Athanasia Meimaridou-Tzitziou , Charalampos Meimaridis and
my brother Giannis. Whatever I am I own to you. LOVE YOU!!!!*

*As you set out for Ithaka
hope the voyage is a long one,
full of adventure, full of discovery.
Laistrygonians and Cyclops,
angry Poseidon don't be afraid of them:
you'll never find things like that on your way
as long as you keep your thoughts raised high,
as long as a rare excitement
stirs your spirit and your body.
Laistrygonians and Cyclops,
wild Poseidon you won't encounter them
unless you bring them along inside your soul,
unless your soul sets them up in front of you.
Hope the voyage is a long one.
May there be many a summer morning when,
with what pleasure, what joy,
you come into harbours seen for the first time;
may you stop at Phoenician trading stations
to buy fine things, mother of pearl and coral, amber and ebony,
sensual perfume of every kind
as many sensual perfumes as you can;
and may you visit many Egyptian cities
to gather stores of knowledge from their scholars.
Keep Ithaka always in your mind.
Arriving there is what you are destined for.
But do not hurry the journey at all.
Better if it lasts for years,
so you are old by the time you reach the island,
wealthy with all you have gained on the way,
not expecting Ithaka to make you rich.
Ithaka gave you the marvelous journey.
Without her you would not have set out.
She has nothing left to give you now.
And if you find her poor, Ithaka won't have fooled you.
Wise as you will have become, so full of experience,
you will have understood by then what these Ithakas mean.*

C.P. Cavafy

*So here I am reaching one of my Ithakas and hopefully understanding what my Ithaka means in order to be able to explore my next Ithaka. These trips to Ithaka filled in my luggages with new experiences, adventures, knowledge and help me to develop as a human being as long as a scientist. But to this trip I was not alone many people were aboard and help me tremendously to keep on walking. **I would love to thank you all of YOU for being my co-travellers to Ithaka. I would not make it without you!!!!!!!!!!***

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Sabina, Maryam, Christina, Elsakj, Rinoudi, Tonia my great paranymphs. We went through a lot of things and that help us to create strong bonds... My other paranymphs

Gabi, Barbara, Charlotte, Maraki, Aldana, Cata, David, Minoos and Ishay I wish I could have all on the podium but then I think it would have been a theatre performanceIt was so tough to make a choice.

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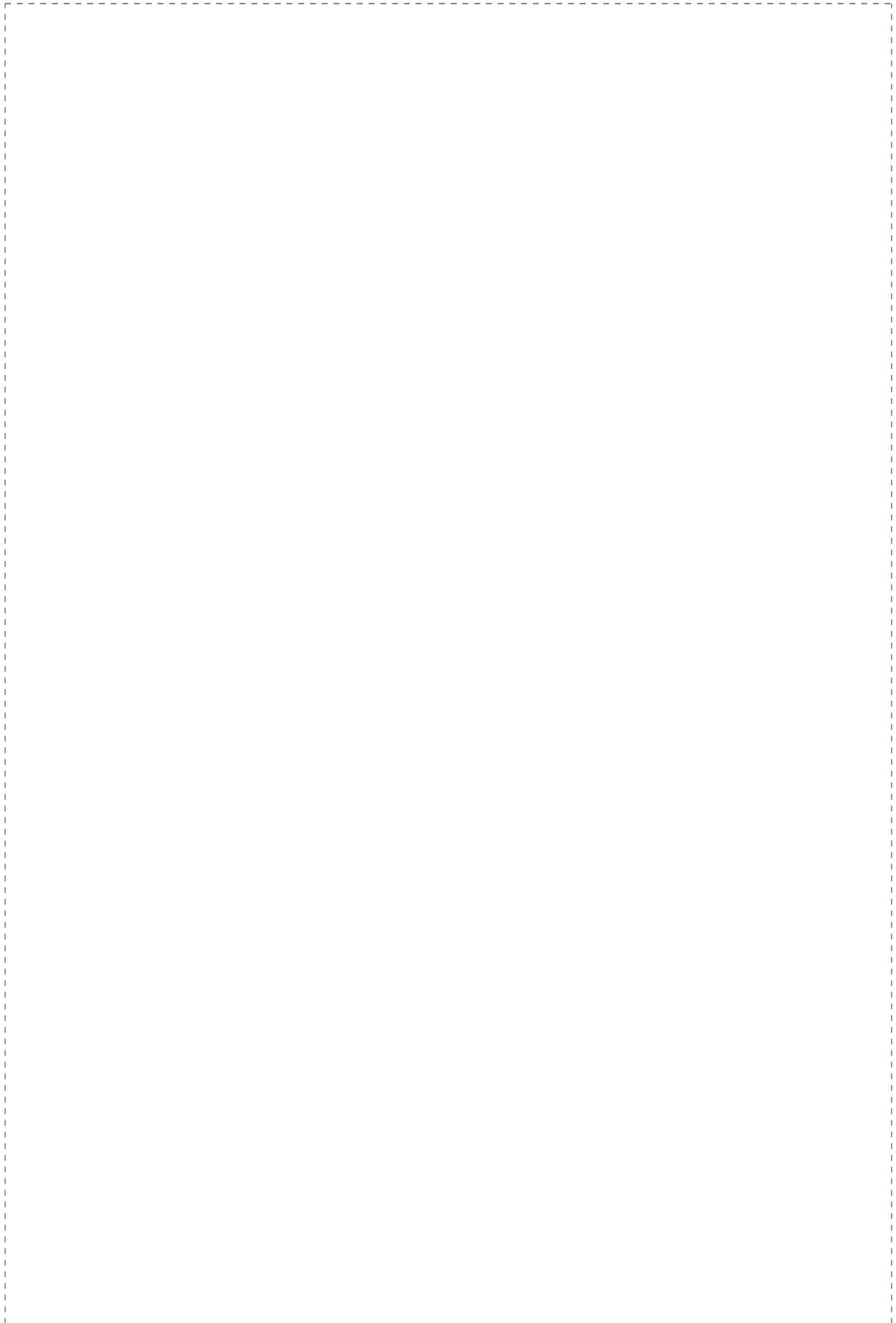
Thanks Helen, Alexandra, Julia, Cozmina, Giannis, Lambros, Rik, Ronald, Karin, Morteza, Iliana, Kristzian, Vicky, Bas, Charlotte, Silvia, Felipe, Alisah, Wietse, and Doenan for the nice dinners, lunches, birthdays parties and and.....Offradio I got addicted to you. You are keeping me a great company all these years with your great unique music. I learned so many great bands due to you. I felt that I was travelling to Thessaloniki. Rafaela, Komninos, Dimitris...Even my non-Greek friends are listening to you. If I could add music "carpet" for each chapter of this thesis would have been Gotan project, Bonobo, Davis Miles, Nina Simone, Frank Sinatra, Marieta Fafouti, Messie Minimal, Ρογκσορ, my angry challenging days in the lab Archive and my really gloomy days Eleftheria Arvanitaki "Tziabaeri mou" and "Oi glaroi". Christinaki we still need to dance nisiotika hehehe...hint??

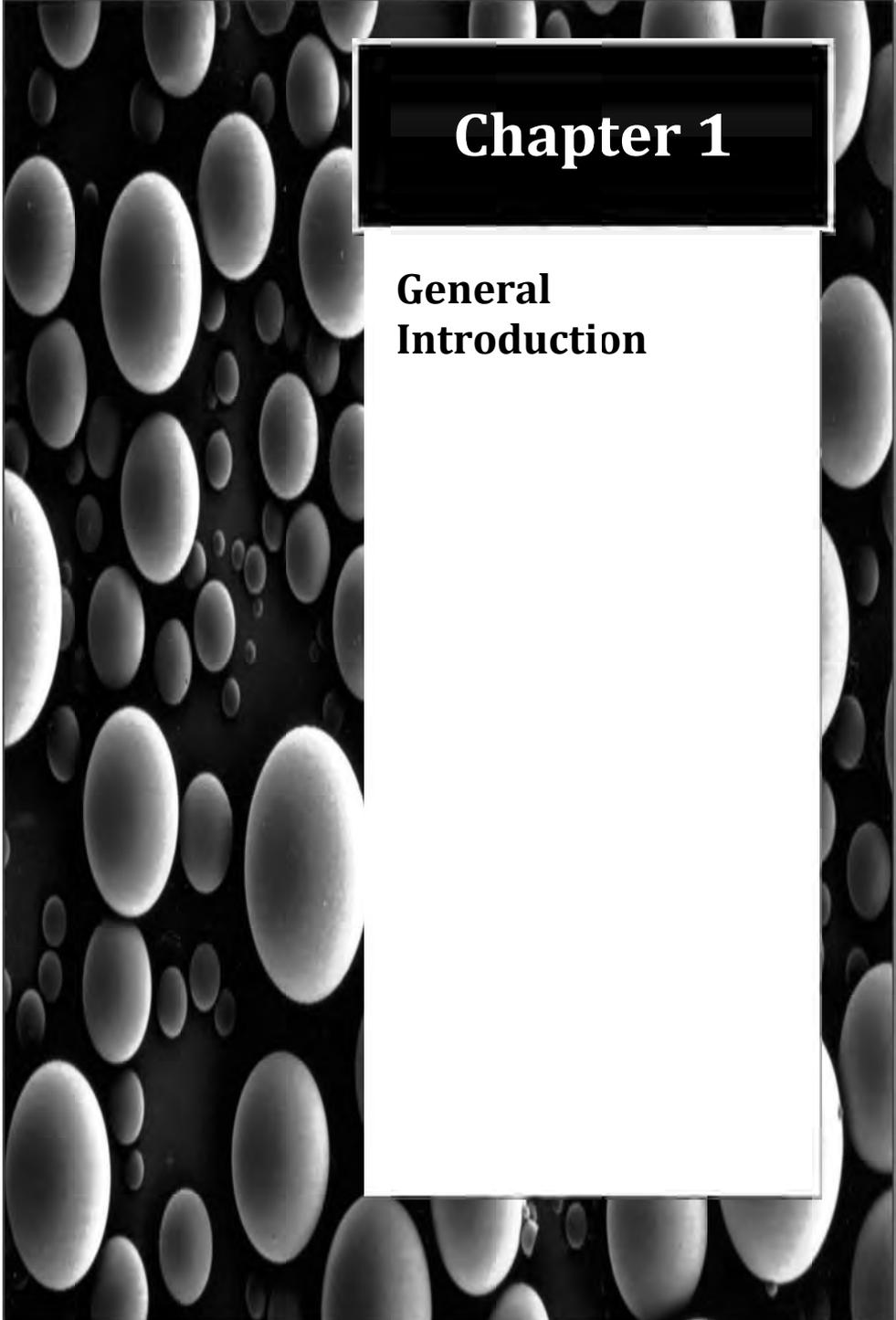
Τόση ώρα μιλούσα για την οικογένεια και τους φίλους που απόκτησα εδώ στο Βαχενίνγκεν αλλά η καρδιά και σκέψη μου ήταν και είναι πίσω στην Ελλάδα στην Θεσσαλονίκη, Βέροια και στο Μυρτόφυτο Καβάλας. Στις θείες μου Βαγγελιώ και Βέτα και τον θείο μου Μιχάλη, Μάχη και τα ξαδέρφια μου Βάγια, Χρήστο, Χαράλαμπο, Παναγιώτη, Σάρρα και την θεία μου Ευδοκία πάντα με θυμάστε και μου στέλνετε την αγάπη σας. Τόσα χρόνια εδώ ήμουν απτούσα από πολλές χαρές και λύπες. Αντωνία μου δεν πρόφτασα να σου πω αντίο πάει ένας χρόνοςδεν μπορώ να το χωνέψω ότι είσαι μακριά μας. Πάππου γίναμε σκορποχώρι.....εάν είχα διαβατήριο θα ερχόμουν μαζί σου στην Ολλανδία δεν θα το ξεχάσω ποτέ αυτό.....Γιαγιά Βαίτσα φορά το κόκκινο σάλι που μου έπλεξες στην Ολλανδία είσαι μαζί μου έστω και έτσι. Γιαγιά Ελευθερία δεν θα σταματήσω να ελπίζω. Θείε Μιχάλη ποτέ δεν με ξεχνάς.....πάντα με νοιάζεσαι.....

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Chapter 1

General Introduction

1.1 Environmental contaminants in food

Environmental contaminants are chemicals that accidentally or deliberately enter the environment, often, but not always, as a result of human activities such as agriculture, mining, industrial operations, or energy production. They are either a) synthetic or natural organic chemicals, b) metals and their organic and inorganic derivatives, or c) natural or synthetic radioactive substances [1]. Numerous recent incidents dramatically illustrate the potential health hazards and economic harm that can be caused by environmental contaminants [2–7]. The severity of food polluted with environmental contaminants in human health is defined by several aspects: the toxicity of the contaminant, the concentrations of the compounds in the food, the amount of the contaminated food consumed and the physiological vulnerability of the individual(s) consuming the food [8]. Apart from the obvious health effects, the economic impacts of a contamination incident have traditionally been stated in terms of the estimated amount of money of the resulting food loss. But additional costs are also involved like health and “other” costs. The health costs include medical expenses and lost workdays from illness resulting from food contamination incidents. The “other” costs of environmental contamination comprise the expenses or losses incurred by affected businesses, individuals, and government bodies. The best action against environmental contamination is to assure that these toxic compounds are not released in the environment. However, regulating mechanisms are not so effective against that. In addition to that, some of the chemical contaminants persist in the environment and bioaccumulate through the food web. Monitoring of food contamination is an essential component of assuring the safety of food supplies and managing health risks at global level.

1.2 Persistent organic pollutants (POPs)

Persistent organic pollutants (POPs) encompass a range of man-made toxic contaminants which are long-lasting in the environment and can travel long distances from their production/contamination source [9–11]. POPs are persistent to degradation so they remain intact and can bioaccumulate in the food chain with exposure of animals, possibly impacting biodiversity, and ultimately humans, causing adverse health impacts for current and future generations [12, 13]. Most of the POPs are lipophilic and they tend to accumulate in the fatty tissues of aquatic and terrestrial organisms and in humans [14–19]. Twelve POPs were initially globally reported by the Stockholm Convention [12] (aldrin, chlordane, dichlorodiphenyltrichloroethane (DDT), dieldrin, endrin, heptachlor, hexachlorobenzene (HCB), mirex, toxaphene, polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). But new emerging POPs are included, such as several pesticides: chlordecone, alpha hexachlorocyclohexane, beta hexachlorocyclohexane, lindane, pentachlorobenzene;

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industrial chemicals: hexabromobiphenyl, hexabromodiphenyl ether and heptabromodiphenyl ether, pentachlorobenzene, perfluorooctane sulfonic acid, its salts and perfluorooctane sulfonyl fluoride, tetrabromodiphenyl ether and pentabromodiphenyl ether; and by-products: alpha hexachlorocyclohexane, beta hexachlorocyclohexane and pentachlorobenzene. Moreover, several chemicals are under review by the Stockholm Convention such as short-chain chlorinated paraffins (SCCPs), hexachlorocyclododecane, chlorinated naphthalenes, hexachlorobutadiene, and pentachlorophenol. In addition to these POPs, polycyclic aromatic hydrocarbons (PAHs) are considered as indicators of environmental pollution and are included in several monitoring programs. However, PAHs do not comply with the POPs definition due to their low bioaccumulation potentials since they metabolize rapidly in biota. Due to their typical physicochemical properties, analytical strategies applicable for their determination are similar to those employed for POPs. In this work, we focused on the development of bioanalytical tools for the screening of two important groups of POPs: PCBs and polybrominated diphenyl ethers (PBDEs) and PAHs belonging to the group of “POPs indicators” [12].

1.2.1 Polychlorinated biphenyls (PCBs)

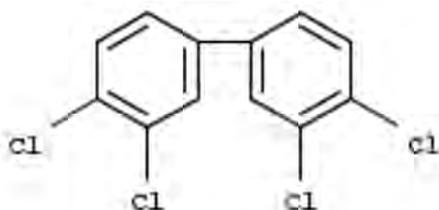


Figure 1: Molecular structure of 3,3',4,4'-tetrachlorobiphenyl (PCB77).

Polychlorinated biphenyls (PCBs) were widely used in various applications due to their low flammability, chemical stability, high boiling point, and electrical insulating properties. The majority of PCBs manufacturing was in the United States of America (USA) from 1929 until 1979 when their production was banned [20]. PCBs were used primarily and mainly as electrical insulating fluids in capacitors and transformers and also as hydraulic, heat transfer, and lubricating fluids. They were blended also with other chemicals such as plasticizers and fire retardants and used in a range of products including caulks, adhesives, plastics, and carbonless copy paper and many other industrial uses [15, 21]. PCBs consist of 209 known congeners. A PCB congener is a unique well-defined chemical compound and the congener's name specifies the total number of chlorine substituents and the position of each chlorine. In most of the cases, PCBs were manufactured as a mixture of various PCB congeners, through progressive

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chlorination of batches of biphenyl until a certain target percentage of chlorine by weight was achieved. The most well know trade names of PCBs mixtures are Aroclor, Clophen and Delor [1]. 3,3',4,4'- Tetrachlorobiphenyl (PCB77; Figure 1)) was chosen as the model PCB congener for the development of our bioanalytical screening assay since it is prevalent in the environment and highly toxic.

1.2.2 Polybrominated diphenyl ethers (PBDEs)

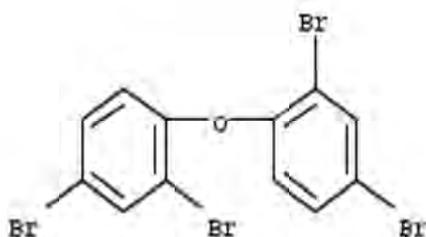


Figure 2: Molecular structure of 2,2',4,4'-tetra-bromodiphenyl ether (PBDE47)

Polybrominated diphenyl ethers (PBDEs) were used since the 1970s in consumer electronics and textiles as flame retardants. PBDEs are either incorporated into the polymeric materials or they are dissolved in the polymer. In the last case, PBDEs can leach, so their flame retardancy may be gradually lost and they can enter the environment more easily. There are 209 PBDE congeners, numbered using the same system as the PCBs. PBDEs were on the market in three mixtures, branded as pentabrominated PBDE (pentaBDE), octabrominated PBDE (octaBDE), and decabrominated PBDE (decaBDE). DecaBDE is the most widely used PBDE globally and is still produced in the USA and in Europe, while pentaBDE and octaBDE have been banned in the European Union since 2004 and also in several states in the USA. PBDEs are still present in several products used in houses or workplace and the exposure risk to them is quite high [1, 22–24]. One of the PBDE congeners with the highest dietary exposure is 2,2',4,4'-tetra-bromodiphenyl ether (PBDE47)[25] (Figure 2), which was chosen as the PBDE congener for the development of the screening assay.

1.2.3 Polycyclic aromatic hydrocarbons (PAHs)

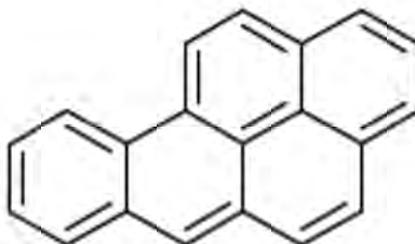


Figure 3: Molecular structure of benzo[a]pyrene (BaP).

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Polycyclic aromatic hydrocarbons (PAHs) belong to a group of semi-volatile organic compounds containing two or more fused aromatic rings. PAHs are mostly produced during the incomplete combustion of organic materials including coal, oil, gasoline and garbage [26, 27]. PAHs are also found in crude oil, coal tar, creosote and asphalt. PAHs are associated with human activities (the combustion of fossil fuels) and natural occurrences (such as forest fires), and they are considered to be ubiquitous in the environment at some level [27]. PAHs are often discussed as a group because they are commonly found as mixtures of two or more compounds in the environment. There are over 100 chemicals in this family of compounds, although a smaller number is routinely reported at disposal sites [11, 26, 28–33]. Benzo[a]pyrene (BaP; Figure 3) was the target PAH for the development of the PAH immunoassay since, so far, it is considered as the indicator of PAHs toxicity and occurrence in food.

1.3 Human exposure routes to PAHs, PCBs and PBDEs and health effects

POPs have been found globally at all major climate zones and geographic sector sites such as oceans, deserts, the Arctic and the Antarctic. In these remote regions, no significant local sources exist and the only reasonable explanation for their presence is long-range transport from other parts of the globe [13,24,28,33-42]. In industrialized areas, concentrations may be several orders of magnitude higher. Human exposure to POPs is either through their diet or inhalation (Figure 4) and can be connected to various adverse health effects, including illness and death [4, 24, 43–47].

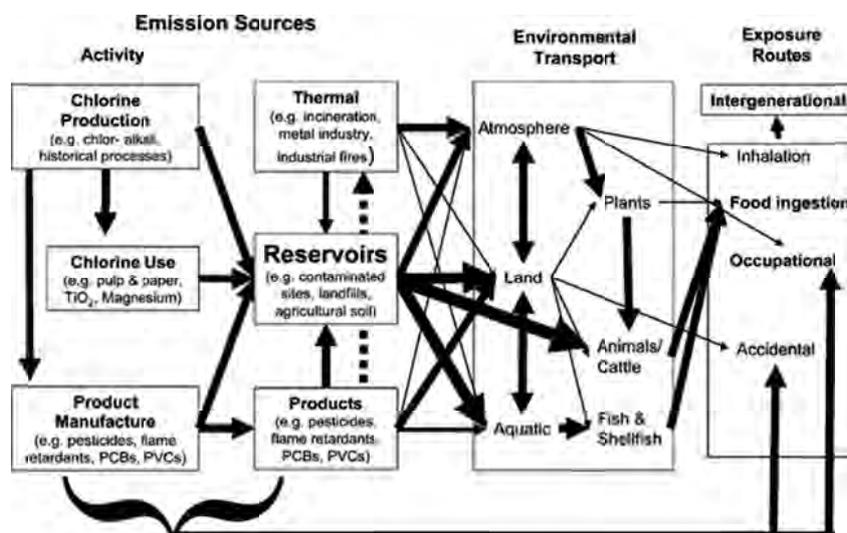


Figure 4: Sources and reservoirs, environmental transport and major human exposure pathways of POPs (reproduced with permission [33]).

Consumption of contaminated food with POPs is the major exposure route for humans compared to other ways, such as inhalation and dermal contact [4, 11, 30, 48]. During the past years, various contamination incidents with organic pollutants in food have been reported [2, 3, 10, 49]. PCBs, PBDEs and PAHs represent major groups of organic toxicants worldwide distributed not only in the environment but also in high fat content food such as fish [15, 18, 36, 49–51].

1.4 Regulations for PCBs, PBDEs and PAHs in fish

PAHs. The United States Environmental Protection Agency (US-EPA) identified the most frequent PAHs in environmental samples (“the 16 EPA PAHs”) [53]. In Europe, maximum levels (MLs) have been established for BaP only, which are $5 \mu\text{g kg}^{-1}$ wet weight for muscle meat of smoked fish and smoked fishery products, excluding bivalve mollusks, and $2 \mu\text{g kg}^{-1}$ for fresh fish [54]. However, the European Food Safety Authority (EFSA) concluded in 2008, in an opinion based on data relating to occurrence and toxicity, that the sum of four PAHs (PAH4: includes BaP, chrysene (CHR), benz[a]anthracene (BaA) and benzo[b]fluoranthene (BbF)) and the sum of eight PAHs (PAH8: includes PAH4 plus benzo[k]fluoranthene (BkF), benzo[ghi]perylene (BghiP), dibenzo[a,h]anthracene (DBaH) and indeno[1,2,3-cd]pyrene (IP)) are the most suitable indicators of PAHs in food, with PAH8 not providing much added value compared to PAH4, but EFSA set no MLs [55].

PCBs. To prevent health risks from exposure to dioxin-like PCBs, both the European Commission (EC) [54] and the US-EPA established monitoring programs according to the Stockholm Convention [12] on POPs. The EU MLs set for dioxins and dioxin-like PCBs are based on the World Health Organization (WHO) toxic equivalent quotients (TEQs) [56, 57], which are used to express the toxicological concentrations of these chemicals and enable risk assessments to be carried out. Toxic equivalence factors (TEFs) are consensus estimates of compound-specific toxicity/potency relative to the toxicity/potency of an index chemical. TEFs are the result of expert scientific judgment using all of the available data and taking into account uncertainties in the available data. TEQ is the product of the concentration of an individual dioxin-like compound in an environmental mixture and the corresponding tetrachlorinated dibenzo-p-dioxin (TCDD)-related TEF for that compound [56, 57]. The ML applied to muscle meat of fish and fishery products, excluding eel and crustaceans, is 8 pg g^{-1} wet weight for the sum of the dioxin-like PCBs (WHO-PCDD/F-PCB-TEQ). The ML for muscle meat of eel (*Anguilla anguilla*) and its products is 16 pg g^{-1} wet weight for the sum of the dioxin-like PCBs (WHO-PCDD/F-PCB-TEQ). In June 2008, the EU established an ML also for the sum of dioxins and the dioxin-like PCBs (WHO-PCDD/F-PCB-TEQ) of 25 pg g^{-1} wet weight in fish liver and derived products since very high levels of dioxins have been found in canned fish liver and were reported through the Rapid Alert System for Feed and Food (RASFF) since 2006.

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PBDEs. So far, no regulatory limits have been established for the PBDEs in food, however, under the European regulatory framework, the authorization procedure foresees that the utilization of PBDEs can be subject to an authorization requirement [58]. In June 2008, the US-EPA set a safe daily exposure level ranging from 0.1 to 7 µg per kg body weight per day for the 4 most common PBDEs [53]. The EU decided to ban the use of two classes of flame retardants, in particular PBDEs and polybrominated biphenyls (PBBs) in electric and electronic devices. In August 2011 the EFSA panel on Contaminants in the Food Chain concluded that for PBDE47, -153 and -209 current dietary exposure in the EU does not raise a health concern, but for PBDE99 there is a potential health concern with respect to current dietary exposure [25].

1.5 Methods used for the detection of organic pollutants in fish

Last years, prominent developments in instrumental- and bioanalytical techniques occurred offering various valuable tools also for the detection of PAHs, PBDEs and PCBs in several food matrices including fish. These methods are mainly either instrumental-based (chromatographic and mass spectrometry instrumentation) or they are based on various biorecognition elements (antibodies, receptors, binding proteins, cells) combined with different platforms for the determination of the biointeractions and subsequently the detection of the analytes of interest. All analytical steps for the determination of POPs such as the collection of the sample, transportation, storage, sample preparation and analysis should be carefully considered prior to the sample analysis (Figure 5). In this study, the focus was mainly on simplifying the extraction procedure and the development of a multiplex bioanalytical screening technique for the organic pollutants.

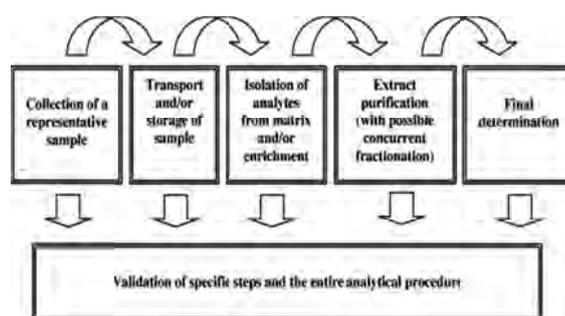


Figure 5: Main steps of analytical procedures used for determining POPs in food samples (reproduced with permission [59]).

1.5.1 Extraction procedure of organic pollutants from fish

Prior to the analysis, sample preparation is a detrimental step to achieve optimum results, sample throughput and analysis costs. The sample preparation for POPs involves the isolation of the lipophilic analytes and preconcentration of the low levels prior to final analysis. Many extraction techniques are described in literature [14, 16, 19, 48, 51, 52, 60–77]. The traditional *Soxhlet extraction* is widely used in analytical labs for the isolation of POPs from solid sample matrices, however, it is rather tedious and both time- and solvent-consuming resulting in being unfriendly to both analysts and the environment [76, 77]. Due to that, several new procedures have been developed in the past such as *ultrasonic-assisted extraction* (UAE) [63, 66], *microwave-assisted extraction* (MAE) [62, 66], *pressurized liquid extraction* (PLE) or *accelerated solvent extraction* (ASE) [76,78,79], *supercritical fluid extraction* (SFE) [62, 66-67] and the *QuEChERS* (Quick, Easy, Cheap, Effective, Rugged, and Safe) method which was initially developed for pesticides analysis [71, 80], but was applied also for POPs analysis [72-74]. Table 1 summarizes and compares the characteristics of some of the extraction techniques for POPs in solid samples. In principle, there are several parameters that influence the extraction efficiency such as type and volume of solvent, extraction time and temperature. Moreover, other criteria that influence the choice of the extraction procedure are the volume of the sample, the toxicity of the organic solvent, the simplicity, the costs and many others.

1.5.2 Cleanup procedures

In most cases, the extraction procedures are not selective enough for the specific isolation of POPs from complex matrices such as fish, demanding an extra thorough cleanup step to eliminate co-extracted substances such as lipids, fatty acids and elemental sulfur, that are present in most cases at much higher concentrations than the extremely low POPs concentration in the environment and food. The impact of matrix effects can be detrimental and might cause erroneous quantification, low method ruggedness, poor analyte detectability, and even reporting of false positive or false negative results. Consequently, the cleanup step is important for the POPs analysis and several procedures have been used (Table 2) such as gel chromatography (GPC), sometimes referred to as size exclusion chromatography (SEC) [79]. Another non-destructive cleanup procedure is adsorption column chromatography. Various sorbents such as alumina, silica, and florisil, available in different mesh sizes, levels of activity and column size, either separately or in combination, were successfully evaluated for this purpose to reduce sample handling and analysis time [66, 79]. Recently, dispersive solid-phase extraction (d-SPE) [71-72, 74, 80] was introduced as a very simple cleanup procedure where a suitable sorbent (primary-secondary amine (PSA), C18, silica, or activated charcoal) is added to an extract aliquot. After mixing and centrifugation, the

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extract is used for subsequent analysis employing either gas chromatography (GC) or liquid chromatography (LC). Destructive lipid removal mainly comprises either alkaline treatment (saponification) or oxidative dehydration by sulphuric acid treatment. It has been shown that PCBs and PBDEs are stable under strong acid conditions [76, 81]. Basic conditions of saponification are critical as too high temperatures and long process time may cause degradation of highly brominated PBDEs and PCBs. Also, silica gel saturated with alcoholic KOH or a multilayer column with neutral silica, acidified silica, and basic silica can be utilized.

Table 1: Main characteristics of several procedures used for the extraction of POPs from (semi)- solid samples (adapted from different sources).

UAE: ultrasonic-assisted extraction; MAE: microwave-assisted extraction; PLE: pressurized liquid extraction; SFE: supercritical fluid extraction; QUECHERS: Quick, Easy, Cheap, Effective, Rugged, and Safe.

UAE	MAE	PLE	SFE	QUECHERS
Organic solvent	Organic solvent	Organic solvent	Organic solvent	Carbon dioxide
1-30	1-30	7-5	Up to 30	1-10
3-48h	30-60 min	20-30 min	10-60 min	30-60 min
60-500 ml	30-200 ml	10-40 ml	10-60 ml	10-40 ml
Boiling point of solvent	30-35°C	100-150°C	100-150°C	70-150°C
AP	AP	AP for open vessel; pressurized MAE for closed vessel	7-15 MPa	15-50 MPa
Low no filtration; not matrix dependent; easy to operate	Low Fast method; large amount of sample; not matrix dependent; easy to operate	Moderate Fast extraction; small amount of solvent and full control of extraction parameters	High Fast technique; small solvent usage; no filtration needed;	High Fast extraction non-toxic environmental friendly; non-flammable extractant selectivity can be tuned by varying pressure, temperature, and modifier; small amount of solvent filtration not required; no solvent exposure;
Long extraction time; large consumption of solvent; exhaustive extraction; pre-concentration of sample required after extraction; clean-up	Large amount of organic solvent; labor intensive; filtration; risk of exposure to solvent vapour relatively	Extracts must be filtered; polar solvent needed; exhaustive extraction	Extraction efficiency is more matrix dependent	Unlimited sample size; extraction efficiency depends on matrix and analyte
				Required optimization of operating conditions for high fat content fishes

Table 2: Main characteristics of several cleanup procedures for POPs extracts (compiled from different sources [71, 80, 74, 72])

	Two steps necessary None observed	Few lipids removed or large amount of adsorbent used None observed	Fair None observed	Fair None observed	Good Organochlorine pesticides	Good Mainly congeners with fully chlorinated aryl-ring	Fair None observed
Good	Good	Good	Good	Good	Good	Fair	Fair
High	Fair	Fair	High	High	Low	Low	Low
High	Fair	Fair	Fair	High	Fair	Fair	Low

1.5.3 POPs determination by instrumental techniques

A wide choice of instrumental techniques combined with different sample preparations is available for the analysis of trace levels of POPs (PCBs, PBDEs and PAHs) present in complex matrices such as food and fish. High-resolution gas chromatography (HR-GC) combined with mass spectrometry (MS) utilizing a suitable single or tandem mass analyzer represents the main separation/detection technique for PCBs, PBDEs and PAHs. Apart from traditional electron ionization (EI) and chemical ionization (CI), some novel ambient desorption ionization techniques are available, such as desorption electrospray ionisation (DESI), atmospheric-pressure solids analysis probe (ASAP) [91] and direct analysis in real time (DART) [92]. The direct option of sample examination in an open atmosphere with minimal or no sample preparation and higher sample throughput are the main advantages compared to conventional techniques but selectivity and sensitivity might be decreased significantly.

PCBs. GC coupled to detectors, either the classical electron-capture detector (ECD) or the currently favored MS, is routinely used in PCBs analysis. For the routine analysis of PCBs, either simultaneous separation on two serial columns differing in polarity or comprehensive two-dimensional GC (GC × GC) separation and detection with electron-capture detector (ECD) or MS are used since even single HR capillary GC columns do not succeed to separate all of the PCB congeners. Typically, non-polar columns such as 100%-methylpolysiloxane or (5%-phenyl)-methylpolysiloxane are employed for their separation. However, because of coelution of a number of congeners (critical pairs), alternative phases such as (50%-phenyl)-methylpolysiloxane, (8%-phenyl)-polycarborane-siloxane, or (14%-cyanopropyl-phenyl)-methylpolysiloxane have been used [14, 49, 59-60,62,67, 82-83].

PBDEs. A single capillary GC column offers sufficient resolution for a congener-specific PBDE determination since not all of the theoretical 209 PBDE congeners can be found in the earlier produced technical PBDE mixtures (e.g. PBDE 28, 47, 99,100, 153, 154, 183, 209) [34]. A non-polar or medium-polar column, e.g. 100%-methylpolysiloxane, (5%-phenyl)-dimethyl polysiloxane, 14%-cyanopropylphenyl–86%-dimethylpolysiloxane, with a length of 25–60 m and small inner diameters (<0.25 mm) are most often used. The use of sufficiently long columns is important for achieving enough separation between PBDE congeners and possible interferences supposing that the selectivity of the detection is not sufficient. The analysis of PBDE 209 is sometimes performed separately from the rest of the PBDEs due to its thermodegradation vulnerability and its very long retention time [79,81,84-86].

PAHs. For the determination of PAHs in food and environmental samples, the most commonly used techniques are either HPLC with fluorescence detection or GC coupled to MS. Some advantages of LC versus GC are the ability to separate some PAH isomers which are difficult to be separated by GC, and a better performance for high molecular weight compounds, since discrimination during injection at high temperatures and during

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analysis does not occur. However, the higher chromatographic resolution, lower detection limits, high selectivity and the use of deuterated or ^{13}C -labelled internal standards result in making GC-MS the preferred technique. Despite the high selectivity and sensitivity provided by MS operated in the selective ion monitoring (SIM) mode, preferably isomers or structurally related compounds should be resolved by GC prior to detection. At this stage, the selection of a capillary column with a suitable stationary phase and dimensions becomes of highest importance. Separation of some isomeric PAHs might be a problem even on long (non-polar) capillaries. If comprehensive analysis of all 16 carcinogenic PAHs is required, then "heavy," low volatile representatives, such as dibenzopyrenes, can be a factor limiting the throughput in GC due to long retention times; moreover, band broadening might contribute to higher detection limits. Under these conditions, low-bleed columns allowing programming up to high temperature limits are needed. If the analysis of these late eluting PAHs is not required, medium-polar stationary phases under a 50% liquid crystalline-methylpolysiloxane stationary phase may provide a greater selectivity for some critical pairs as compared to non-polar low bleed stationary phases. In HPLC analysis of PAHs, LC columns (100–250 mm \times 2–4 mm (ID)) with 3.5–5 μm C18 sorbent (or some special sorbent developed for PAHs separation, e.g. LC-PAH, Chromspher 5PAH) can be used with gradient elution employing most often acetonitril:water mixtures [29-31,72,74, 87–90].

1.5.4 State-of-the-art in bioanalytical techniques for the target POPs

More than two decades ago, instrumental techniques were the only possibility and became the "golden standard" to detect the targeted POPs. Recent progresses in biotechnology offered the development of various in-vitro bioassays and ligand binding assays. The application of "bi detectors", such as bioassays, enzyme-linked immunosorbent assays (ELISAs) or assays based on other biorecognition elements to recognize the structural properties of organic pollutants, or the ability of cells or organisms to have a specific effect-directed response to these compounds is becoming popular. The highlights of bioanalytical techniques are the low cost, high sample throughput and, in some methods, the effect-directed analysis. The state-of-the-art for the bioanalytical detection methods for measuring dioxin-like PCBs, PAHs and PBDEs include several approaches such as, the 7-ethoxyresorufin-O-deethylase (EROD)-bioassay [91], the aryl hydrocarbon hydroxylase (AHH) bioassay [92], the enzyme immunoassay (EIA) using different platforms [93–102], the reporter gene assay [e.g., chemical-activated luciferase gene expression (CALUX) [101, 103–106]], the gel retardation of AhR DNA binding (GRAB) assay [106], the recAhr assay kit [97], the Ah receptor (AhR) (or filtration) assay with radiolabeled dioxins [107], the Ah-immunoassay (AhIA) [108, 99, 109] and the transthyretin-based assays using either radiolabelled thyroxine or no label for the

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detection of thyroid hormone disrupting compounds [110–117]. These methods are based on the ability of key biological molecules (e.g., antibodies, receptors, transport proteins, enzymes) to bind to target analytes. Most of these bioanalytical techniques are requiring cell lab facilities or utilize radiolabels. Enzyme-linked immunosorbent assays (ELISAs) for each POPs target group are developed but not for all three of them simultaneously [94-95, 98, 100,102, 118]. In this work, the focus was the development of antibody and binding protein (thyroid hormone transport proteins) -based screening assays for the simultaneous detection of the non-hydroxylated and hydroxylated POPs within the target groups (PAHs, PCBs and PBDEs).

1.5.4.1 Biorecognition elements used in this study

For the development of the spectrally encoded microspheres (SEMs)- and the surface plasmon resonance (SPR)-based screening assays for the detection of the POPs target groups either monoclonal or polyclonals antibodies and thyroid hormone transport proteins such as transthyretin (TTR) and thyroxine-binding globulin (TBG) were used.

1.5.4.1.1 Antibodies

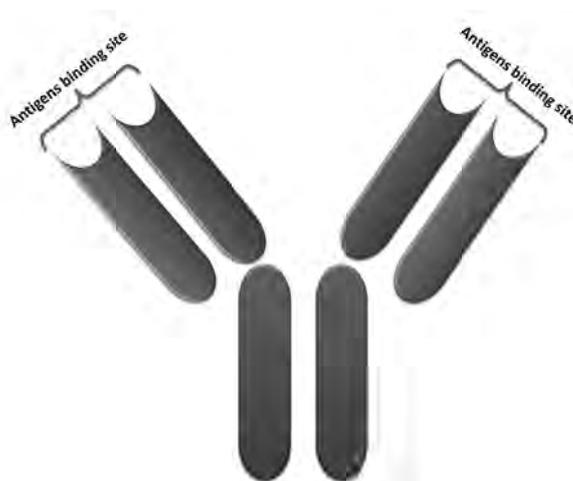


Figure 6: Schematic illustration of a typical antibody molecule.

The fundamental part of an immunoassay is the antibody. Antibodies are immune system-related macromolecules called immunoglobulins (Igs), and they are present at 12-15 mg mL⁻¹ in blood serum, approaching nearly one fifth of its total protein content [119]. Structurally, antibodies are composed of one or more copies of a characteristic unit that can typically be visualized as forming a Y shape (Figure 6). Each Y contains four polypeptides – two identical copies of a polypeptide known as the heavy chain and two

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identical copies of a polypeptide called the light chain. There are two forms of light chains and a single antibody will have light chain subunits of either lambda (λ) or kappa (κ) variety, but not both types are in the same molecule. Igs appear as a very diverse group of proteins that share key structural and functional features. Functionally, they can be characterized by their ability to bind both to antigens and to specialized cells or proteins of the immune system. In mammals, five Igs classes (IgG, IgM, IgA, IgD and IgE) are distinguished by the type of heavy chains found in the molecule. The differences in the heavy-chain polypeptides, primarily in the Fc fragment (for the fragment that crystallizes), allow these proteins to function in different types of immune responses and at particular stages of the maturation of the immune response. Different classes of antibodies may also vary in the number of Y-like units that join to form the complete protein. In mammals, IgM constitutes about 10% of the antibodies. IgG represents 70% of the serum immunoglobulins and constitutes the majority of the secondary immune response to most antigens. IgG molecules have three domains (two Fab domains and one Fc domain). The Fab domains (fragment that carries the antigen binding site) - forming the arms of the Y shape - are identical, which makes IgG molecules bivalent. To their advantage, polyclonal antibodies (Pabs) recognize the antigen from different orientations: this may be important in certain assays. Furthermore, Pabs are rather simple, fast and low-cost to produce compared to Mabs. Besides, the use of larger animals (such as horses, goats, and rabbits) enables the recovery of a large volume of antibody-rich serum. However, the production of Pabs might generally lack reproducibility. Special antibodies termed monoclonal antibodies (Mabs) can be obtained from cells grown in the laboratory and are very specific for their intended targets or they can be group specific. In contrast, the continuous culture of B cell hybridomas offers a reproducible and potentially inexhaustible supply of antibody with exquisite specificity. The hybridoma technology to create Mabs was first described by Kohler and Milstein in 1975. Hybridomas are hybrid cell lines derived by fusing of immortal myeloma cells with B-lymphocytes taken from the spleen of animals immunized with the target antigen. After limiting dilution, cloning hybridomas represent a pure and indefinite source of Mabs with the desired specificity to a target. The antibodies produced by the hybridoma are all of a single specificity and are therefore Mabs. Consequently, Mabs enable the development of standardized and secure immunoassay systems [119–124].

1.5.4.1.2 Thyroid hormone transport proteins

Thyroid hormones are involved in several physiological processes as regulators of metabolism, bone remodeling, cardiac function and mental status. Thyroid hormones are especially crucial in fetal development. Absence of thyroid hormones reduces neuronal growth and differentiation in the cerebral cortex, hippocampus, and cerebellum. During the first part of pregnancy, the fetus relies entirely on transplacental transfer of maternal thyroid hormones and thus on a normal maternal thyroid function, but maternal thyroid

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homeostasis is also contributing substantially to fetal development during the remaining part of pregnancy. Exposure to thyroid-disrupting chemicals may potentially result in subtle reductions of serum hormone levels, which in turn may have significant consequences for public health. Thyroid hormones in humans are mainly bound to thyroxine binding globulin (TBG), whereas transthyretin (TTR), being the most important carrier protein in rodents, binds only a minor proportion of the hormones. However, TTR has been proposed to be of importance by transferring thyroid hormones over the blood–brain barrier as well as over placenta to the fetal compartment. Competitive binding of environmental chemicals such as hydroxylated PCBs and/or PBDEs to transport proteins may result in increased bioavailability of endogenous thyroid hormones [110-111, 113, 115-116, 125–129].

1.5.4.1.2.1 Transthyretin (TTR)

Transthyretin (TTR) is a 127-residue monomer and in humans it functions in the form of a 55-kDa tetramer composed of four identical subunits. The resolution of the crystal structure of human, chicken, and rat TTR has been determined at 1.8 Å [130] (Figure 7). TTR differs from the other THTPs in that it is also synthesized by the brain. Specifically, TTR is synthesized by the epithelial cells of the choroid plexus which is the site of the blood–cerebrospinal fluid barrier, part of the blood–brain barrier [131]. The tetramer has a central channel with two thyroid hormone binding sites. However, only one binding site of TTR is occupied by thyroid hormone under physiological conditions due to negative cooperativity [123-124, 132-133]. For the purposes of this thesis we used a recombinant TTR (rTTR) produced by the group of Matsubara et al., 2003 [134]. A high-expression plasmid of human TTR was constructed in order to facilitate the study of amyloid fibril formation of this protein.

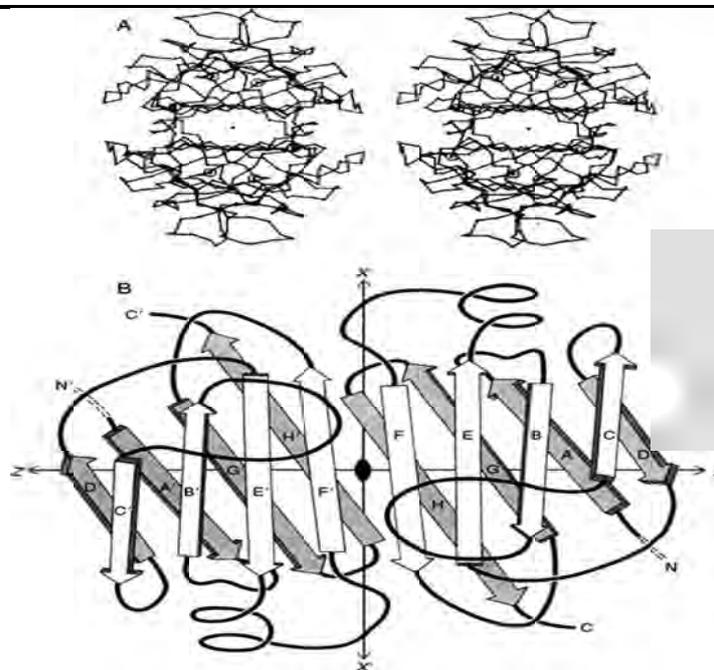


Figure 7: (A) X-ray crystal structure of human TTR. TTR is a homotetramer with a central channel that contains two potential TH-binding sites. Stereo view down the central channel containing the TH-binding sites. (B) Dimer of human TTR showing eight antiparallel β sheets (labeled A–H or A'–H') and one section of α helix in each subunit (reproduced with permission [130]).

The TTR gene was constructed by an assembly of eight chemically synthesized oligonucleotides and amplified by polymerase chain reaction, and the amplified gene was inserted into an *Escherichia coli* expression vector. The expression plasmid was transformed into M15 cells and the gene product was expressed as a polyhistidine-tagged fusion protein. Purified rTTR was obtained by one-step nickel chelation affinity chromatography and the production level of the protein was 130 mg per 1 culture.

1.5.4.1.2.2 Thyroxine binding globulin (TBG)

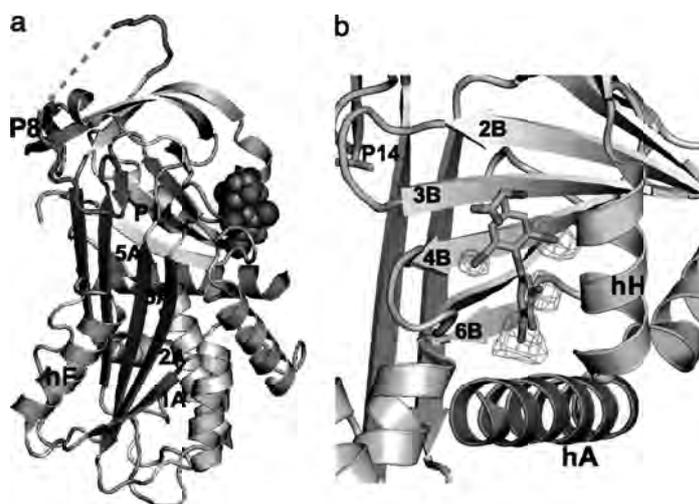


Figure 8: TBG and the thyroxine binding site. (a) Structure of TBG with thyroxine (space-filled). The upper half of the Aβ-sheet (blue) is opened, with initial insertion of the reactive loop (red) to P14 threonine, 14 residues before the reactive center P1. (b) Binding pocket showing thyroxine in stick form enclosed between strands 3–5 of the B-sheet and helices H and A and with iodine atoms, contoured at 5 times rms density in a log-likelihood gradient map for anomalous scattering (reproduced with permission [135]).

Thyroxine binding globulin (TBG)(Figure 8) has the highest affinity for thyroxine (T4), but, of the three THTPs in blood, it is present in the lowest concentration. Due to the very low serum concentration of T4, TBG is rarely more than 25% saturated with ligand. Unlike TTR and albumin, TBG has a single binding site for T4. TBG's upper half of its main β-sheet is fully opened, so its reactive centre peptide loop can readily move in and out of the sheet to give an equilibrated binding and release of T4. The entry of the loop triggers a conformational change, with a linked contraction of the binding pocket and release of the bound T4. Despite its low concentration, TBG carries the majority of T4 in serum. Genomically, TBG is a serpin, although it has no inhibitory function like many other members of this class of proteins. TBG is synthesized primarily in the liver [125-127].

1.6 Platforms with multiplex potential used in this study

1.6.1 Surface plasmon resonance (SPR)-based biosensor

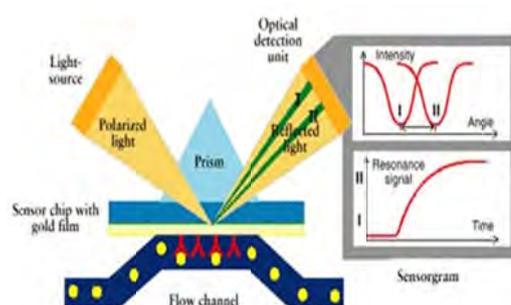


Figure 9: Principle of the applied SPR-based sensor (reproduced with permission from Biacore corporation (GE)).

The use of the surface plasmon resonance (SPR) phenomenon in a biosensor results in a real-time, label-free, optical method that enables the monitoring of a variety of biospecific interactions [136, 137]. SPR biosensors are optical sensors exploiting special electromagnetic waves due to fluctuations in the electron density at the boundary of two materials. In these systems, a light beam coming from the side of the higher refractive index will be partly reflected and partly refracted, but above a certain critical angle of incidence no light will be refracted and total internal reflection is observed. However, a component of the light, the evanescent wave, will propagate towards the medium with the lower refractive index and if the interface between the media is covered with a metal film, e.g. gold, the evanescent wave will interact with free electrons in the metal. Light energy will, thereby, be lost to the metal and the intensity of the reflected light will decrease. This phenomenon is referred to as SPR and only takes place at a sharply defined angle of incidence, the SPR angle. The response from the angular change is expressed in resonance units (RU) and 1 RU corresponds to a shift in the angle of 0.0001° . By plotting the measured angular shift against time, a sensorgram is obtained illustrating the progress of the interaction at the sensor surface in real time (Figure 9)[136–140]). This optical technique detects and quantifies changes in refractive index in the vicinity of the surface of sensor chips to which in most cases ligands/antibodies/binding proteins, are covalently immobilized. Because the changes in the refractive index are proportional to the changes in the adsorbed mass, the SPR technology allows detection of the interaction between biomolecules immobilized on the sensor chip and the ones binding to them. A

few SPR-based assays have been developed for the analysis of the POPs target groups [115-116,139-140].

1.6.2 Flow cytometry

Fluorescence-based flow cytometry dates back to the 1960s, and refers to the measurement of the optical properties of single particles in a flowing sample stream. Originally, the flow cytometer was utilized for the study of individual cells from where also the name derives. Flow cytometers initially were used for a range of medical and clinical purposes such as hematology and oncology; however, their application expanded to other areas, such as bioprocess monitoring, pharmacology, toxicology, environmental sciences, bacteriology and virology. The conventional flow cytometer (Figure 10) performs the cell analysis by passing thousands of cells per second in a directed fluid stream through a laser beam and capturing the light that emerges from each cell as it passes through.

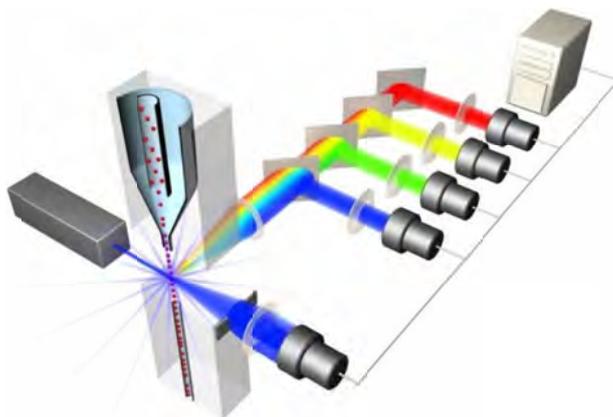


Figure 10: The fluidics, optics and electronics: three basic components of a flow cytometer (reproduced with permission [141])

The data gathered can be analyzed statistically by flow cytometry software to report cellular characteristics such as size, complexity, phenotype, and health. A traditional flow cytometer consists of a light source, fluidics, collection optics, detection hardware, and computing power to convert the signals into data. In most flow cytometers, the light source is a laser that emits coherent light at a specified wavelength focused on the sensing point. The fluidics system is designed to deliver single particles to the sensing point with an accuracy of $\pm 1 \mu\text{m}$. This accuracy is achieved by injecting the sample into the center of an enclosed channel through which a column of liquid (the sheath fluid) is flowing. Particles in the sample are hydrodynamically focused into the center of the column. For a given excitation source, the scattered light and/or fluorescence are collected by two lenses (one opposite the light source and one at right angles) and

resolved by a series of beam splitters and filters. This set up allows photoluminescence at multiple wavelengths, and physical characteristics, such as particle size and shape, to be determined. The laser is focused on a very small volume of solution surrounding the particle. This approach reduces the background signal and even allows assays with no washing steps in between to be carried out. Detectors are usually photomultiplier tubes (PMTs) that can detect as few as 100 reporter molecules per particle. Commercially available instruments that are smaller and have fewer requirements along with new software for data acquisition and interpretation form the basis for the success of the technique [141–145].

1.6.3 Microsphere (bead)-based suspension arrays in food and environmental analysis

Bead-based assays offer a powerful, flexible and high throughput approach for analysing the interactions of a large variety of molecules with biological targets in a single small sample volume (multiplexed assays). The technique has quite a few advantages over alternative methods for studies of protein and DNA interactions, clinical diagnostics and drug discovery. Applications in food and environmental analysis are quite limited [146–149]. On the market there are several multiplex bead-based kits coupled to different detectors for the testing of allergens, autoimmune diseases, cardiac markers, cytokine detection, endocrine markers, infectious disease markers, isotyping, genotyping, kinase and phosphorylated protein activity, metabolic markers, and tissue typing [150–155]. Microsphere-based suspension arrays that are compatible with standard flow cytometers are commercially available from different companies. FlowCytomix™ of eBioscience, Inc. provides bead-based immunoassays kits for the simultaneous detection of up to 20 analytes (a combination of two sets of polystyrene beads with different sizes (4 and 5 µm) and each size consists of bead populations which are differentiated by varying intensities of an internally fluorescent dye). QuantumPlex™ kits (Bangs Laboratories, Inc) provide a platform for the design of multiplexed suspension arrays that may be run on standard flow cytometers (488nm or 633nm excitation). Microsphere populations in five-bead set kits are encoded with different intensities of Starfire Red™, and microspheres in ten-bead set kits are distinguished by both fluorescence intensity and size (4.4 and 5.5 µm). They also provide five populations of superparamagnetic microspheres of 6 µm (QuantumPlex™M). Thermo Scientific Cyto-Plex carboxylated particles provide beads with different sizes (4, 5 and 7 µm) and levels of fluorescent intensities up to twelve levels of red fluorescent intensities for analysis of maximum twelve analytes per diameter, which enables the simultaneous quantification of more than 30 analytes within a single sample. A selection of Dynabeads™ (Invitrogen) similar to the Cyto-plexi is also applied in the Sal Plex™ (RnAassays, Utrecht, the Netherlands) for the measurement of Salmonella antibodies in plasma, serum, meat-drip, eggs and milk. Sal Plex™ beads enable flow

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cytometric detection of any infection caused by any Salmonella serovar belonging to serogroups B, C1, C2, D and E, providing at the same time serogroup information as well. Based on the same beads, SoftFlow Ltd. (Pécs,HU) supplies a fiveplex immunoassay (Fungi-PLEX5) for the simultaneous detection of several mycotoxins in food and feed. These are a few examples of available commercially beads. In this study we focused on the spectrally encoded microspheres (SEMs) MultiAnalyte Profiling (xMAP®) technology from Luminex.

1.6.3.1 Spectrally encoded microsphere (SEM)-based flow cytometry

An emerging technology for multiplex analysis in food is the spectrally encoded microspheres (SEM)-based suspension array in which each SEM set represents a unique binding assay. In recent years, the need for rapid and multiplexed detection turned research toward the development of screening technologies such as the MultiAnalyte Profiling (xMAP®) Luminex technology that can offer great flexibility and cost-effectiveness. The core of this sensing system are the SEMs, such as the 100 differently colored MicroPlex and SeroMap™ SEMs (5.6 µm), the 100 superparamagnetic MagPlex™ SEMs (6.5 µm) and 500 SEMs in the 3D system. Each microsphere set is internally stained with different ratios of red and infrared fluorophores to create 100 unique spectral addresses, excited by the system's red laser (Figure 11). The system utilizes a green laser that quantifies the reporter fluorophore or fluorescent target molecule bound to the bead surface during the assay. Since each bead set can be coated with a different capture antibody, multiplexed assays are simplified. The bead sets are mixed together during the assay, and then the flow cytometer detects each bead individually to identify it and quantify the intensities of tracer fluorescence (Figure 12). SEMs with carboxyl, amine/hydrazide, and maleimide groups for covalent coupling of proteins, peptides, oligonucleotides or other biorecognition elements are available. High- and low-density streptavidin-coated SEMs with the capacity to bind biotinylated analytes are available. Similarly, other affinity tags such as glutathione-GST, Ni+6x-histidine, and protein A and G can be utilized to link capture proteins to microspheres. Several procedures for covalent and non-covalent protein coupling to microspheres have been reviewed previously and some of them are mentioned in the following chapters of this thesis. The Luminex has been shown to be a highly effective platform for sandwich or competitive immunoassays for several applications [146-148, 156-158].

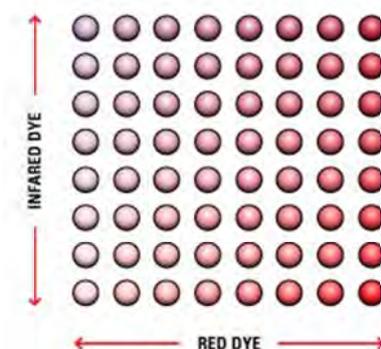


Figure 11: Spectrally encoded polystyrene microspheres (SEMs). Each microsphere set is internally stained with different ratios of a red and infrared fluorophore to create 100 unique spectral addresses, both excited by the system's red laser (reproduced with permission from Luminex corporation).

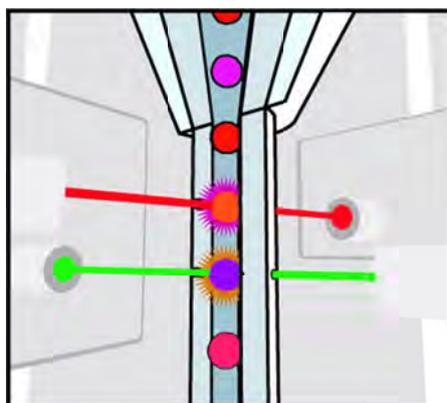


Figure 12: Spectrally encoded microsphere (SEM)-based flow cytometry. the red laser recognizes the bead set and the green one excites any reporter dye captured during the assay (reproduced with permission from Luminex corporation).

1.6.3.2 Spectrally encoded microspheres (SEM)-based Imaging Planar Array

An emerging technology for multiplex analysis in food is in the MAGPIX instrument which was launched in 2010 by the Luminex corporation. This detection platform employs the same superparamagnetic MagPlex™ SEMs suspension array as the flow cytometric set-up, however, it uses light emitting diodes (LEDs) and a charge-coupled device (CCD) imager

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instead of lasers and photo multiplying tubes (PMTs). This SEM- based imaging system is cheaper, more robust, compact sized, and transportable, but has a reduced capacity for multiplexing (up to 50) and a longer read time. The main differences between the flow cytometric- and the imaging-based platforms described in this work are illustrated in Figure 13. In the imaging-based set-up, MagPlex™ SEMs are forming a monolayer and images for the bead classification are taken after excitation by a red LED and for reporter quantification by a green LED and signals are superimposed in order to sort the microspheres making a flow cytometer superfluous [159].

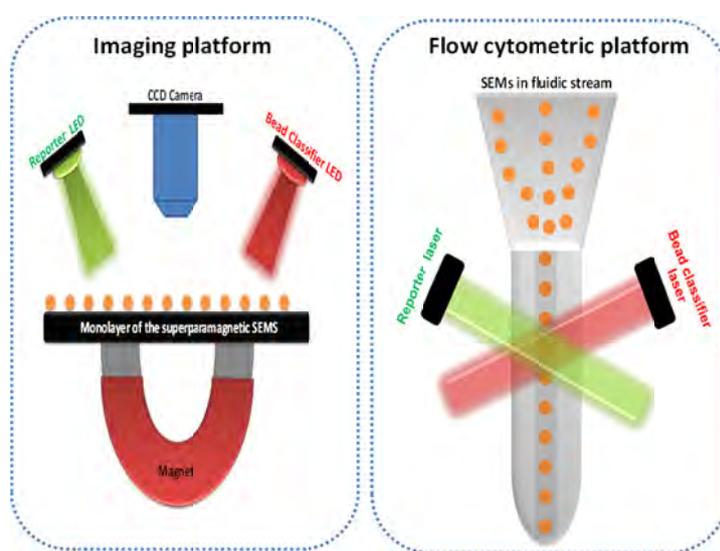


Figure 13: Illustration of the main features of the imaging and the flow cytometric spectrally encoded microspheres (SEM)-based technologies.

1.7 Aims and scope

The objectives within this study in general were the development and application of multiplex assays for the simultaneous screening of several (groups of) organic pollutants in food using mainly the novel SEM-based platforms. So far, this type of technology is known and proven in clinical diagnostics but not yet well explored for use in food analysis. The analytes of interest were bioactive target organic pollutants (PCBs, PBDEs and PAHs) and/or some of their OH-metabolites, all of them recognised for their adverse health effects and their occurrence in environmental and food samples. Several issues have been addressed during this study such as:

- The utilization of different biorecognition elements such as Mabs and Pabs and thyroid hormone transport proteins for the development of the SEM-based screening assays.

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- The investigation of various combinations of coated SEMs (with different analyte-protein conjugates) and antibodies for the optimum performance of the assays in mono- and multiplex format.
- The high hydrophobicity of organic pollutants in the aqueous environment of the screening assays.
- The antibodies specificity in mono- and multiplex immunoassays and solving their inter-assay interactions in the multiplex format.
- The development of a generic and simple sample preparation procedure for the three groups of POPs in combination with the multiplex SEM-based immunoassays.
- Application and comparison of platforms with different multiplex capacities (SEM- and biosensor-based) for the screening of POPs in buffer and fish extracts and for the determination of their thyroid hormone disruption potency.
- In-house preliminary validation of a 3-plex immunoassay for the screening of PCBs, PAHs and PBDEs in fish extracts.

1.8 Outline of this thesis

Chapter 1 “General Introduction” provides a theoretical background about the physicochemical properties of the target POPs, current regulations and state-of-the-art of analysis. Moreover, it describes the multiplex platforms and biorecognition elements used in this study.

Chapter 2 “Spectrally encoded microbead (SEM)-based flow cytometric immunoassay for polycyclic aromatic hydrocarbons in food.” Describes in detail the development of a flow cytometric SEM- based immunoassay for the screening of the PAHs in buffer and fish.

Chapter 3 “Multiplex Screening of Persistent Organic Pollutants in fish using spectrally encoded microspheres.” describes the development and applicability of a 3-plex flow cytometric SEM-based immunoassay for the simultaneous detection of PCBs, PBDEs and PAHs in different types and lipid content fish extracts contaminated with different concentrations of the three target POPs. Moreover, a generic, simplified and modified approach of the QuEChERS-like extraction procedure for PCBs, PAHs and PBDEs is described in combination with the 3-plex FCIA.

Chapter 4 “Multiplex immunoassay for Persistent Organic Pollutants in Tilapia: Comparison of Imaging- and Flow Cytometry-based platforms using spectrally encoded paramagnetic microspheres.” describes the transformation of the 3-plex flow cytometric SEM-based immunoassay for the simultaneous detection of the three target POPs to a new imaging SEM-based platform (IMIA). Moreover, a critical comparison of the performance between the two systems is given in buffer and tilapia extracts. In this chapter also an in-house preliminary validation with 40 tilapia fillets, analyzed as blanks and each spiked with a mixture of relevant concentrations of the model compounds BaP,

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PCB77 and PBDE47, or a cocktail of several POPs belonging to the three target groups (PCBs, PBDEs and PAHs), is described.

Chapter 5 “Comparison of multiplex flow cytometric and biosensor platforms to determine thyroid hormone disruption potency.” deals with the experimental comparison of an SPR-based multichannel biosensor and a flow cytometry-based suspension array, using SEMs, for the screening of thyroid hormone disrupting compounds (THDCs). Moreover, both assays were theoretically compared with other thyroid hormone transport proteins-based screening tools for THDCs.

Chapter 6 “General discussion” provides concluding points on the application of the multiplex platforms and extraction procedures used in this thesis for the screening of the target POPs in fish and gives some suggestions for future research for the screening of POPs.

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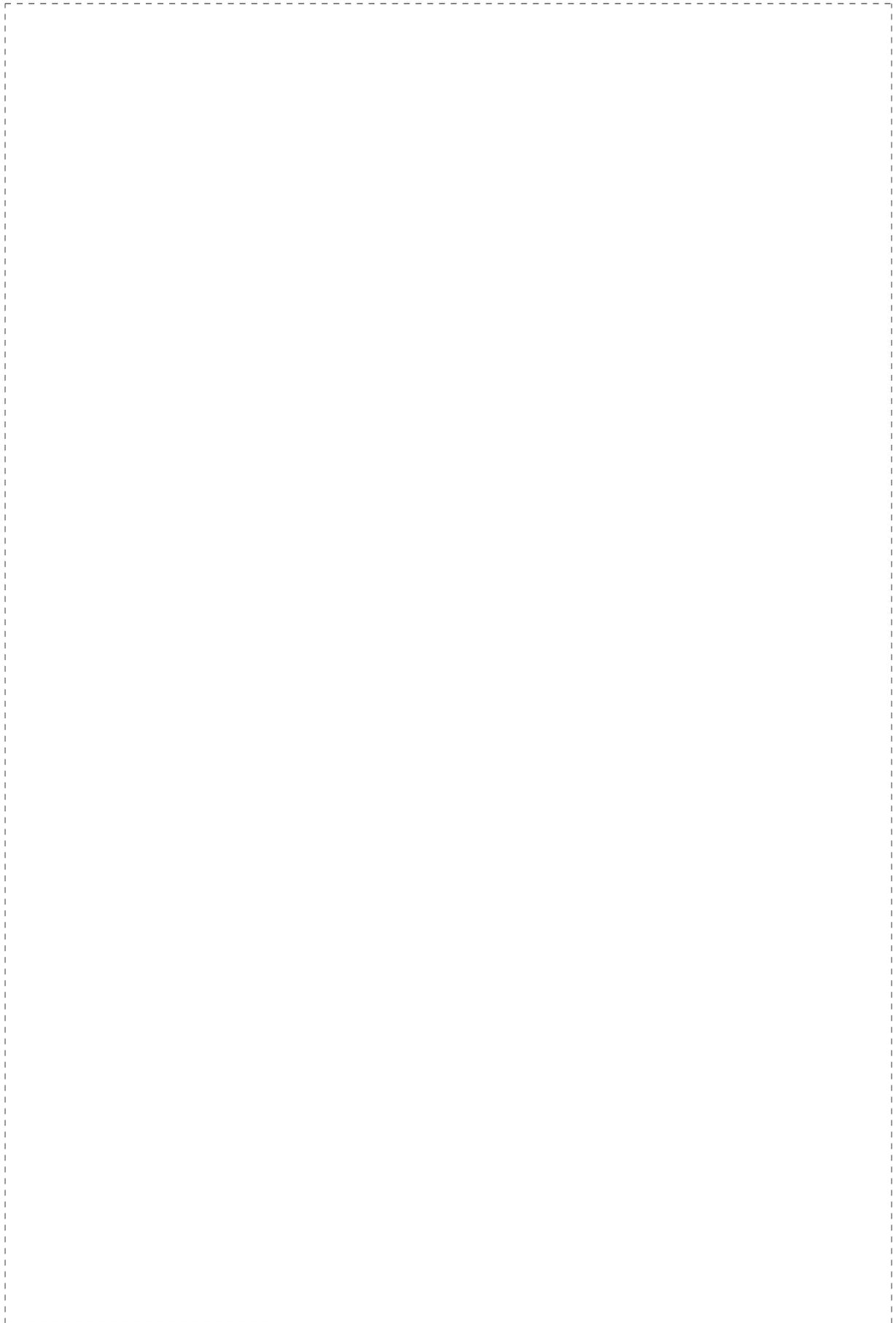
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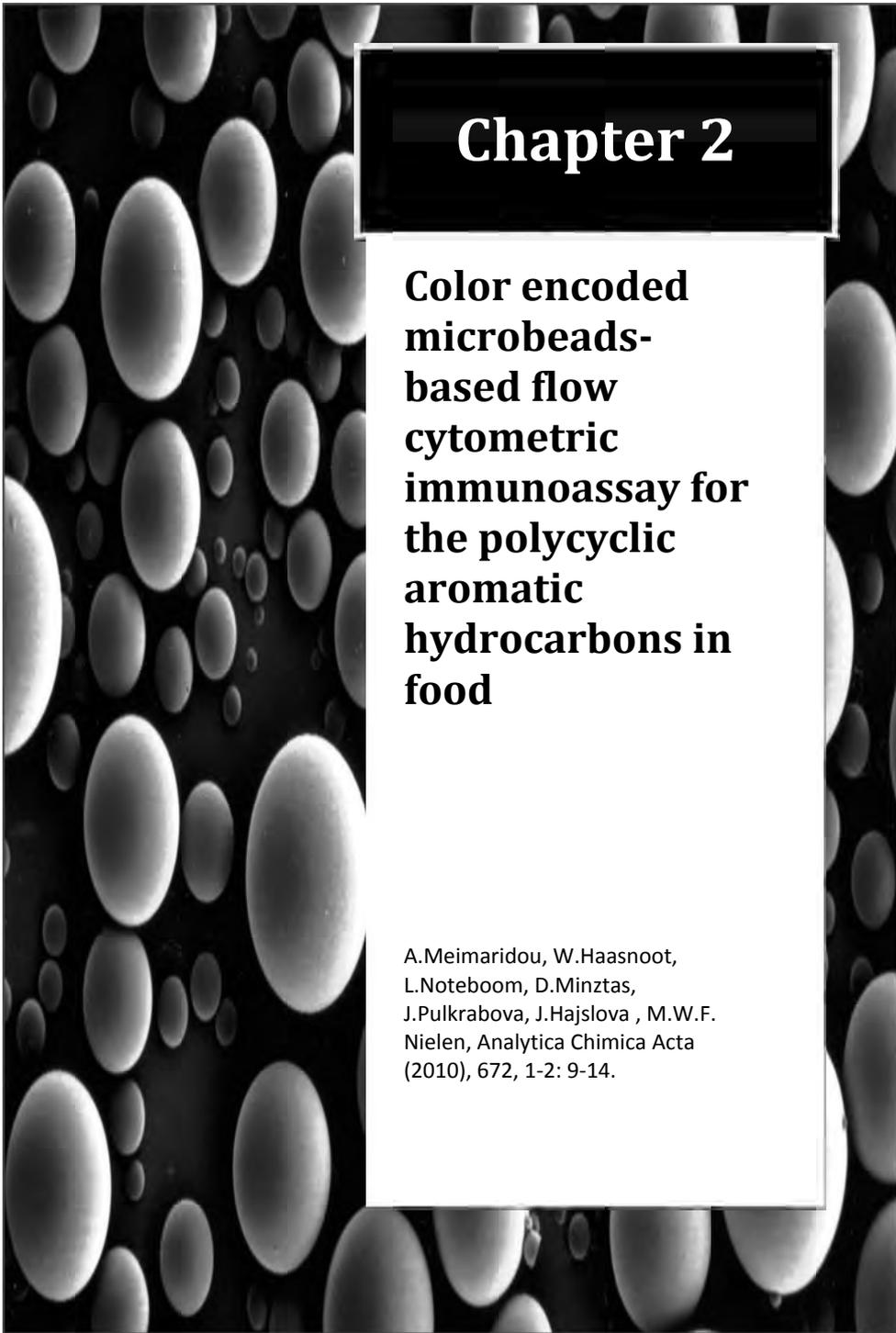
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Chapter 1: General Introduction

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The background of the slide is a black and white photograph of numerous microbeads of various sizes, appearing as bright, oval shapes against a dark background. The beads are scattered across the entire frame, creating a textured, cellular appearance.

Chapter 2

Color encoded microbeads- based flow cytometric immunoassay for the polycyclic aromatic hydrocarbons in food

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Abstract

Food contamination caused by chemical hazards such as persistent organic pollutants (POPs) is a worldwide public health concern and requires continuous monitoring. The chromatography-based analysis methods for POPs are accurate and quite sensitive but they are time-consuming, laborious and expensive. Thus, there is a need for validated simplified screening tools, which are inexpensive, rapid, have automation potential and can detect multiple POPs simultaneously. In this study we developed a flow cytometry-based immunoassay (FCIA) using a color-encoded microbeads technology to detect benzo[a]pyrene (BaP) and other polycyclic aromatic hydrocarbons (PAHs) in buffer and food extracts as a starting point for the future development of rapid multiplex assays including other POPs in food, such as polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs). A highly sensitive assay for BaP was obtained with an IC_{50} of $0.3 \mu\text{g L}^{-1}$ using a monoclonal antibody (Mab22F12) against BaP, similar to the IC_{50} of a previously described enzyme-linked immunosorbent assay (ELISA) using the same Mab. Moreover, the FCIA was 8 times more sensitive for BaP compared to a surface plasmon resonance (SPR)-based biosensor immunoassay (BIA) using the same reagents. The selectivity of the FCIA was tested, with two Mabs against BaP for 25 other PAHs, including two hydroxyl PAH metabolites. Apart from BaP, the FCIA can detect PAHs such as indeno[1,2,3-cd]pyrene (IP), benz[a]anthracene (BaA), and chrysene (CHR) which are also appointed by the European Food Safety Authority (EFSA) as suitable indicators of PAH contamination in food. The FCIA results were in agreement with those obtained with gas chromatography-mass spectrometry (GC-MS) for the detection of PAHs in real food samples of smoked carp and wheat flour and have great potential for the future routine application of this assay in a simplex or multiplex format in combination with simplified extraction procedure which are under development.

Introduction

The presence of chemical contaminants in food, such as polycyclic aromatic hydrocarbons (PAHs), is a fact of life. PAHs belong to a group of organic compounds containing two or more condensed aromatic rings (Figure 1), and their metabolites are well known to be carcinogenic and/or mutagenic because they bind to DNA and can induce mutation and cancers [1-5]. PAHs are mainly formed as pyrolysis by-products, especially during the incomplete combustion of organic materials during industrial and other human activities [6]. For the general population, the major routes of exposure are through food and inhaled air. In smokers, the contributions from smoking and food may be of a similar exposure magnitude [7-11]. Food contamination sources for PAHs can be the environment, industrial food processing and certain home cooking practices [10-13]. The U.S. Environmental Protection Agency (EPA) identified the most frequent PAHs in environmental samples ("the 16 EPA PAHs")[14]. In Europe, maximum levels (MLs) are established for benzo[a]pyrene (BaP) only, which are $1 \mu\text{g kg}^{-1}$ wet weight for processed

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cereal-based food products for infants and young children, and $5 \mu\text{g kg}^{-1}$ wet weight for muscle meat of smoked fish and smoked fishery products, excluding bivalve mollusks.

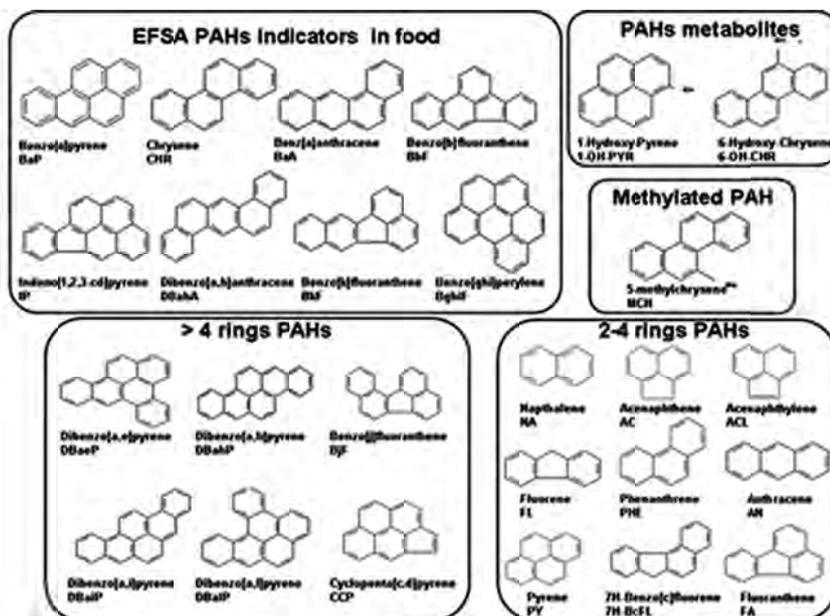


Figure 1: Molecular structures of the 26 food-related PAHs used during the evaluation of the previously developed ELISA and/or the presently described flow cytometry-based immunoassay (FCIA).

However, the European Food Safety Authority (EFSA) concluded in 2008, in an opinion based on data relating to occurrence and toxicity, that the sum of four PAHs (PAH4: includes BaP, chrysene (CHR), benzo[a]anthracene (BaA) and benzo[b]fluoranthene (BbF)) and the sum of eight PAHs (Figure 1) (PAH8: includes PAH4 plus benzo[k]fluoranthene (BbF), benzo[ghi]perylene (BghiP), dibenzo[a,h]anthracene (DBaA) and indeno[1,2,3-cd]pyrene (IP)) are the most suitable indicators of PAHs in food, with PAH8 not providing much added value compared to PAH4 [15].

Several methods for detecting PAHs in food, such as high pressure liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS) are described as accurate and quite sensitive but they are time-consuming, laborious and expensive to operate since most analyses for POPs in food require around 30 min per injection (also for each point of the standard curve) plus the time needed for extensive sample cleanups, which limits the sample throughput and increases the labor costs [10, 16-20]. For the rapid detection of chemical contaminants, many biosensors as well as immunosensors have been developed and utilized to detect BaP, however, mainly in buffer rather than in foods [21, 22]. The regulatory demand for surveilling of numerous chemical contaminants in food creates the

need for analytical screening tools, which are simple, inexpensive, rapid, sensitive, and have the possibility of detecting multiple analytes simultaneously in high throughput automated manner [23].

An interesting multiplex screening alternative can be the use of the color-encoded microbeads technology (MultiAnalyte Profiling (xMAP[®]) technology) combined with flow cytometry (Luminex[®]), so far hardly explored for food applications [24-26]. This technology is frequently applied in clinical diagnostics, for instance for the multiplex detection of several cytokines [27, 28]. In food and related products, applications are described for the detection of plant proteins in milk powder and for drug residues in milk and blood serum [24, 25].

Luminex's xMAP[®] technology uses color-encoded tiny microspheres (beads), such as the 100 differently colored MicroPlex and SeroMap™ beads (5.6 μm) and the 54 superparamagnetic MagPlex™ beads (6.5 μm). In the present flow cytometry-based immunoassay (FCIA), the latter beads were coated with a BaP protein conjugate to capture the mouse monoclonal antibodies (Mabs) against BaP of which the binding was inhibited by the presence of the PAHs in the sample. Detection and quantification of the immunocomplex was obtained via a secondary antibody against mouse IgG conjugated to the fluorescent reporter molecule phycoerythrin (PE).

A dual laser instrumentation system allowed both, the identification of the color-coded bead set by its characteristic color (red laser) and the quantification of the anti-mouse PE bound to the beads (green laser). With this combination it is theoretically possible to simultaneously measure up to 54 different biomolecular reactions in a single well, which is a major advantage compared to analyte specific (simplex) immunoassays such as enzyme-linked immunosorbent assays (ELISAs).

In the developed model assay, one color-encoded microbead-set was used for the detection of one group of persistent organic pollutants (POPs), the PAHs. However, in the future, multiplex assays with multiple bead-sets can be developed for the simultaneous detection of other persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) with the same sample extract. The sensitivity of the FCIA for BaP was compared with those obtained with an ELISA and a Surface Plasmon Resonance (SPR)-biosensor immunoassay (BIA), two well established immunoassay formats in food analysis, by using the same immunochemicals [29]. The selectivity of the FCIA was tested for a wide range of PAHs (Figure 1). Besides that, the assay's performance was tested in real life applications using extracts of smoked carp and white flour, previously analyzed for PAHs with GC-MS.

Materials and methods

Chemicals, materials and instruments

The mouse Mabs against BaP (Mab22F12 and Mab2H3) and the BaP conjugated to bovine serum albumin (BaP-BSA) and to keyhole limpet hemocyanin (KLH) (BaP-KLH) were

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purchased from Dietmar Knopp (Technical University of Munich, Munich, Germany). Another mouse Mab against BaP (MabBaP (MA1-19274)) was supplied by Affinity BioReagents (Colden, CO, USA). The anti-mouse R-Phycoerythrin (PE) conjugate was from Prozyme® (San Leandro, CO, USA). Most of the different PAHs stock solutions (n=22) were supplied by AccuStandard (New Heaven, CT, USA). Dr. Ehrenstorfer Laboratory (Ausburg, Germany) supplied us with dibenzo[a,e]pyrene (DBaP), cyclopenta[c,d]pyrene (CCP), 5-methylchrysene (MCH) and 7H-benzo(c)fluorine (7H-BcFL). The magnetic separator DynaMag™-2 was obtained from Invitrogen Dynal (Oslo, Norway). The test tube rotator was from Snijders (Tilburg, the Netherlands). Protein LoBind Tubes (1.5 mL) were supplied by Eppendorf (Hamburg, Germany) and the LoBind 96-wells microplates were from Greiner Bio-One B.V. (Alphen a/d Rijn, the Netherlands). The 10 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20 (HBS-EP) buffer, the amine coupling kit, CM5 biosensor chips and the Biacore 3000 SPR biosensor system were supplied by GE Healthcare (Uppsala, Sweden). The N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) was provided by Fluka Analytical (Steinheim, Switzerland). All other reagents not specified above were from Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands).

Applied Cytometry Systems (ACS, Dinnington, Sheffield, South Yorkshire, UK) delivered the Luminex 100 IS 2.2 system consisting of a Luminex 100 analyzer and a Luminex XY Platform, which is programmed to analyze a 96-well plate. The system operates with StarStation System control software. The magnetic carboxylated microspheres (MagPlex™ no. 021) and the sheath fluid were supplied by Luminex Corporation (Austin, TX, USA). The automated microplate wash station Bio-Plex™ Pro II, with a magnetic carrier, was provided by Bio-Rad Laboratories B.V. (Veenendaal, the Netherlands). The Agilent 6890 gas chromatograph coupled to, a model 5975 XLD Inert mass spectrometer was bought by Agilent Technologies (Santa Clara, CA, USA).

For the calibration and dose-response curves, a 4 parameter curve fitting was used from GraphPad Prism software, GraphPad Software Inc. (La Jolla, CA, USA). The blank and contaminated wheat flour was purchased from the Crop Research Institute (Prague, Czech Republic). The carp was bought at the Trebon Fishery (Třeboňsko, Czech Republic) and was smoked at the University of South Bohemia, Faculty of Fisheries and Protection of Waters (Budějovice, Czech Republic).

Preparation of BaP-BSA and BaP-KLH coated paramagnetic beads

Different batches of the same microspheres were coated with BaP-BSA and BaP-KLH conjugates using a modification of the protocol provided by Luminex® for a two-step carbodiimide coupling of protein to MagPlex™-C paramagnetic carboxylated microspheres. Briefly, the stock microsphere suspension (1.25×10^7 microspheres mL^{-1}) was resuspended by vortexing for 5 min and sonication for 1 min. Then, 200 μL of the suspension (2.5×10^6 microspheres) was transferred to a Protein LoBind Tube in which the

microspheres were pelleted by a magnetic separator in 20 s. After gently removing of the supernatant, the pellet was resuspended in 100 μL of demineralized water (dH_2O). The suspension was pelleted and after removal of the supernatant, the pellet was resuspended in 80 μL of activation buffer (0.1 M NaH_2PO_4 , pH 6.2). Solutions of sulfo-NHS and EDC, both at 50 mg mL^{-1} in dH_2O , were prepared just before adding 10 μL of each to the 80 μL microsphere suspension. The microspheres were incubated in the dark at room temperature for 20 min. The activated microspheres were pelleted and washed by adding 250 μL of 50 mM MES (2-N-morpholino ethanesulfonic acid) buffer (pH 5.0). To the activated and pelleted microspheres, 50 μg of the BaP-BSA or BaP-KLH conjugates, in a final volume of 500 μL of 50 mM MES buffer (pH 5.0), were added. The suspension was vortexed and incubated for 1 hour under mixing by rotation in the test tube rotator at room temperature in the dark. After incubation, the unbound BaP-protein conjugates were removed by washing twice with 500 μL of blocking buffer consisting of PBS (5.4 mM sodium phosphate, 1.3 mM potassium phosphate, 150 mM sodium chloride pH 7.4) to which 0.1% BSA, 0.02% Tween-20 and 0.05% NaN_3 were added. The microspheres were suspended in 200 μL of the blocking buffer and stored at 4 $^\circ\text{C}$ until used.

Preparation of BaP-BSA and BaP-KLH coated SPR biosensor chip

The immobilization of the two different BaP protein conjugates (BaP-BSA and BaP-KLH) was conducted in the Biacore 3000 SPR instrument. The sensor chip (CM5) consists of a gold surface coated with a carboxymethylated dextran matrix. In the Integrated μ -Fluidic Cartridge (IFC) channels of the biosensor (Biacore 3000), the different BaP protein conjugates were immobilized on the sensor surface using the amine coupling kit and the immobilization wizard of the Biacore 3000 software aiming for immobilization levels of 10000 RU. Solutions were injected directly into the flow channels with the auto sampler needle. At first, the sensor chip surface was activated by injecting a mixture of 0.4 M N-hydroxysuccinimide (EDC) and 0.1 M NHS (1:1; v/v) at a flow rate of 10 $\mu\text{L min}^{-1}$ and with a contact time of 7 min. After washing with 0.1 M sodium hydroxide (NaOH), the BaP protein conjugates, with a concentration of 50 $\mu\text{g mL}^{-1}$ in acetate buffer pH 4, were injected in the different IFCs for 7 min at a flow rate of 5 $\mu\text{L min}^{-1}$. To deactivate the remaining active sites, 1 M ethanolamine was injected for 3 min at a flow rate of 10 $\mu\text{L min}^{-1}$.

SPR biosensor immunoassay (BIA) protocol

The assay was designed as an indirect inhibition assay. The BaP-coated sensor chip, with the two different BaP protein conjugates, was used to bind mouse Mabs against BaP. A known concentration of each antibody was mixed automatically by the instrument (1:5; v/v) in a microtiter plate with the sample (different BaP concentrations in HBS-EP and

10% methanol (MeOH)). Of this mixture, 50 μL was injected over the sensor surface at a flow rate of 25 $\mu\text{L min}^{-1}$ and the response was measured 5 sec before the regeneration started. The surface was regenerated by injecting 10 μL of 5 mM NaOH containing 2% of acetonitrile (MeCN) at 25 $\mu\text{L}\cdot\text{min}^{-1}$ and the sensor chip was ready for the next analysis cycle. The total run time under these conditions was 8 min.

Flow cytometry immunoassay (FCIA) protocol

First, 20 μL of the mouse Mab against BaP (final dilution of 1/4000 for Mab22F12 and 1/2000 for Mab2H3) was pre-incubated for 15 min with 80 μL of sample or standards in a well of a low-binding 96-wells microplate. Then, 10 μL of BaP-BSA or BaP-KLH coated beads suspension were added to the well providing at least 1000 microspheres per test. The mixture was incubated for 45 min at room temperature, in the dark on a plate shaker. After the incubation, three washing steps with PBS, using the magnetic washing plate carrier of the automated wash station removed the excess of the unbound bioreagents. After washing, 25 μL of anti-mouse PE was added followed by 100 μL of HBS-EP and the mixture was incubated for 30 min in the dark, followed by one washing step in the wash station. The microspheres were resuspended in 100 μL of PBS. Finally the measurement in the Luminex[®] was done in 50 sec using 50 μL per well.

To prepare a calibration curve in buffer or in blank carp/cereal extract, a dilution series of BaP and other PAHs (0.01-100 $\mu\text{g L}^{-1}$) was spiked either in HBS-EP with 10% MeOH and 0.1% BSA (working buffer) or in the blank carp or wheat flour samples, diluted in the same working buffer. As negative controls, we used dilution of buffer or blank carp/cereal extract. The contaminated carp and cereal extracts were diluted in the same way as the spiked blank samples.

PAHs extraction from non-contaminated and contaminated carp and wheat flour

Carp

15 g of homogenous fish fillet was mixed with 50 g of anhydrous sodium sulphate to form a flowing powder which was extracted for 8 hours in a Soxhlet apparatus with 170 mL of a solvent mixture of hexane:dichloromethane (1:1, v/v). The crude extract was carefully evaporated and dissolved in 10 mL of chloroform. A cleanup of the crude extracts was carried out by an automatic gel permeation chromatographic (GPC) system employing Bio Microspheres S-X3. As a mobile phase, chloroform was used at a flow rate of 0.6 mL min^{-1} , the injection volume was 1 mL of the crude extract and the fraction corresponding to the elution volume of 16–32 mL was collected. The eluate was evaporated by a rotary vacuum evaporator at 40°C and the residual solvent was carefully removed by a gentle stream of nitrogen. The residue was then redissolved in 0.25 mL of toluene and

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transferred into a glass vial for GC-MS analysis. For the FCIA, the residue (corresponding to 1.5 g of sample) was dissolved in 0.25 mL of MeCN (6 g mL⁻¹).

Wheat flour

In the case of the cereals extraction, the protocol was slightly different from the fish. The 15 g of wheat flour was directly extracted for 8 hours in a Soxhlet apparatus with 170 mL of dichloromethane before being evaporated and the residue was dissolved in 10 mL of chloroform. In the GPC purification, 2 mL of the crude wheat flour extract was injected instead of the 1 mL of the fish extract.

GC-MS measurement

The GC-MS measurement conditions were the same for both samples materials. The concentrated extract was injected onto a DB-17HT (30 m x 250 µm i.d. x 0.15 µm film thickness) and a BPX – 50 (30 m x 250 µm i.d. x 0.25 µm film thickness) capillary column. The following GC conditions were used: capillary column carrier gas: helium, initial flow 2 mL min⁻¹, and constant pressure (145 kPa) mode. PTV injection: 16 µL (4 x 4 µL); vent time: 3.4 min; vent flow: 50 mL min⁻¹; vent pressure 34.4 kPa; splitless period 2 min; initial PTV temperature: 50°C; inlet rating velocity: 400°C; final inlet temperature: 350°C. GC-MS oven temperature program: 110°C (5.4 min), 50°C min⁻¹ to 350°C (5.8 min); temperature of the GC-MS interface: 320°C; temperature of quadrupole: 150°C; temperature of ion source: 230°C. Quantification was based on the isotope dilution technique compensating matrix effects and analytes losses. The following ¹³C PAH analogs were used as internal standards (B[a]A-¹³C6, CHR-¹³C6, B[b]F-¹³C6, B[k]F-¹³C6, B[a]P-¹³C4, DB[ah]A-¹³C6, B[ghi]P-¹³C12, I[1,2,3-cd]P-¹³C6, DB[ai]P-¹³C12; DB[ae]P-¹³C6).

Results and discussion

General considerations

The objective of the present study was to develop a FCIA for the detection of PAHs in food using the color-encoded paramagnetic microbeads technology with multiplex capabilities. So far, this described technology is hardly being explored in food analysis. Our ultimate goal is the extension of this assay format to the simultaneous detection of several classes of POPs in foods. For that reason, we developed and evaluated the performance of an indirect inhibition immunoassay for the detection of BaP and other PAHs in buffer and food extracts using one color-coded paramagnetic microbeads set. During the development, two BaP-protein conjugates (BaP-BSA and BaP-KLH) were available together with three different mouse Mabs raised against BaP (Mab22F12, Mab2H3 and MabBaP). From the ELISA data it was known that Mab22F12 was more sensitive for BaP than Mab2H3, but the last one had a broader cross-reactivity for other PAHs [29]. Cross-reactivity data for the commercially obtained MabBaP were not

available prior to this study. In general, immunoassays are performed in an aqueous and physiological salt-containing environment. However, for hydrophobic analytes such as PAHs, the addition of a suitable organic co-solvent in the buffer is essential for their solubility and the elimination of their adsorption to container walls. In earlier studies, MeOH (methanol) or MeCN-containing solvents were found to be immunoassay compatible without an influence on the assay sensitivity [30-32]. In our assays, the addition of 10% MeOH in the HBS-EP was found to be optimal for all the tested antibodies (results not shown), as was found earlier in the ELISA using the same antibodies [29].

SPR-BIA development for BaP

At first, to investigate the biointeractions between the different available immunoreagents, a label-free Surface Plasmon Resonance (SPR)-based biosensor system (Biacore 3000) with four serially connectable flow channels was used. Both, the FCIA and the SPR biosensor technology use a carboxylated surface (microbead or biosensor chip) for the common EDC/NHS immobilization chemistry of reactants. The major advantage of the SPR biosensor is its label-free detection to investigate the functional nature of binding events. Therefore, this system was used at first, in which the two BaP-protein conjugates were covalently immobilized on the biosensor chip surfaces in two different flow channels (FCs) on a CM5 chip which resulted in immobilization levels of 8500 and 11492 resonance units (RU) for BaP-BSA and BaP-KLH, respectively. The three Mabs were injected in different dilutions over the two immobilized BaP-protein conjugates. With the BaP-BSA conjugate, relative responses of 320 and 125 RU were obtained with 10 times diluted Mab22F12 and Mab2H3, respectively. The MabBaP could be used in a higher dilution (1/500) resulting in a response of 332 RU in combination with the BaP-BSA coated sensor chip. The relative responses of all the antibodies in combination with the BaP-KLH conjugate, using the same antibody dilutions, were much lower and varied from 50-100 RU. The only difference between the two BaP conjugates was the carrier protein. The coupling site of BaP and the length of the linker used were the same [29], though it is possible that the affinity between the antibody and the BaP protein conjugates was affected by the density of the BaP molecules on the carrier proteins. In the article of Matschulat *et al.*, 2005, it is mentioned that 26 BaP moles are attached per mole of BSA however the number of BaP moles attached per molecule of KLH was not mentioned [29]. Under the optimum conditions, using the best performing Mab22F12 in combination with the BaP-BSA conjugate, the dose-response curve for BaP showed an IC_{50} , -the concentration of the analyte at 50% inhibition of the maximum response-, of $2.4 \mu\text{g L}^{-1}$ (Figure 2). This IC_{50} was comparable with the value reported by Shimomura *et al* [21] in an SPR-based immunoassay for BaP. However, the IC_{50} for BaP in the BIA was much higher than in the ELISA ($0.2 \mu\text{g L}^{-1}$) using the same Mab [29].

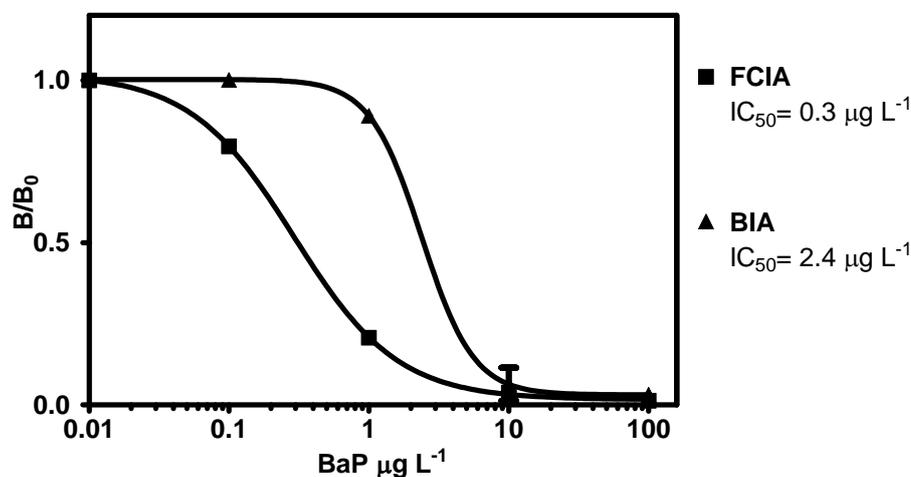


Figure 2: BaP dose-response curves obtained with Mab22F12 in the SPR-biosensor immunoassay (BIA) and the FCIA in which the B_0 is the maximum relative response units (RUs) in the BIA or the maximum median fluorescence intensity (MFI) in the FCIA obtained with a blank measurement and B is the MFI or RU obtained with the different BaP concentrations. Each point represents the mean of three replicates \pm SD.

FCIA development for BaP

To facilitate the development of the FCIA's, the two BaP-protein conjugates were coupled to the color-coded paramagnetic microbeads. For the measurement of the amounts of bound anti-BaP Mabs, a goat anti-mouse Mab labeled with PE was used. In this FCIA format, these essential immunoreagents were tested for their optimum dilutions in combination with different incubation conditions.

The criteria used to evaluate the optimization were the maximum median fluorescence intensity (MFIs), aiming for around 2000 MFI, of the blank measurement and the lowest IC_{50} values. To achieve the maximum performance of the assay, it was essential to remove the unbound biochemicals that interfered with the sensitivity of the assay. For that reason, we implemented washing steps between the incubations with the anti-BaP Mab and the anti-mouse Mab conjugated to PE. In earlier flow cytometry-based assays [24], in which non-paramagnetic beads were used, the washing procedure was performed by centrifugation in a filter plate. In this study, we used the automatic magnetic washer without any influence on the assay sensitivity for BaP (results are not shown). The addition of 0.1% bovine serum albumin (BSA) in our working buffer (HBS-EP+10% MeOH) minimized the adsorption of the polystyrene beads on the surface of the well. The use of the automatic washer resulted in faster, simpler and a more economical assay, since the use of the expensive filter plates could be avoided.

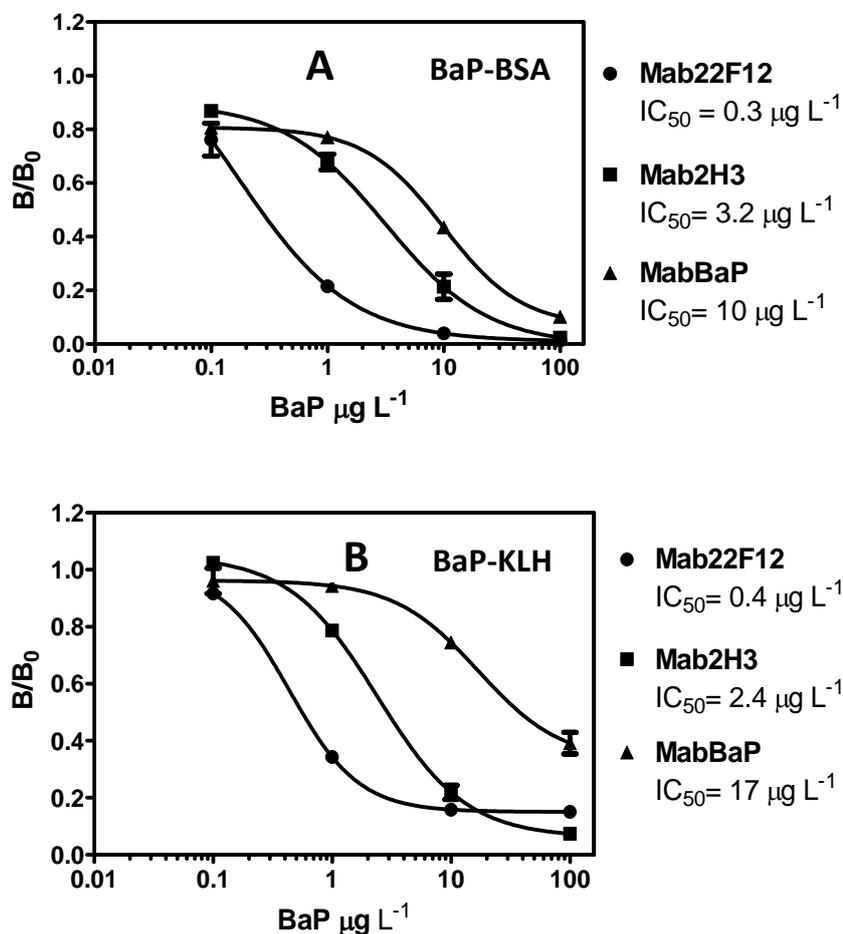


Figure 3: BaP dose–response curves obtained in the FCIA with Mab22F12, Mab2H3 and MabBaP in combination with two different BaP protein conjugates coated beads (BaP-BSA (A) and BaP-KLH (B)) in which B_0 is the maximum MFI of the blank measurement and B the MFI obtained with the different BaP concentrations. Each point represents the mean of three replicates \pm SD.

The normalized BaP dose-response curves obtained in the FCIA's, after the optimization steps with all the different combinations of the available immunoreagents, are shown in Figure 3, where the B_0 is the maximum MFI of the blank measurement and B the MFIs obtained with the different BaP standard solutions. The FCIA using Mab22F12 was the most sensitive for BaP with IC_{50} values of 0.3 and 0.4 $\mu\text{g L}^{-1}$ for the beads coated with BaP-BSA and BaP-KLH, respectively, followed by Mab2H3 with IC_{50} values of 2.5 and 3 $\mu\text{g L}^{-1}$ for the same sets of coated beads (Figure 3). The sensitivities of the ELISA for BaP using

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Mab22F12 and Mab2H3 were 0.2 and 1.5 $\mu\text{g L}^{-1}$, respectively. Compared to the reported ELISA data [29] there is a slight deviation in the IC_{50} for Mab2H3. It seems that the different position of the BaP molecule to which the proteins were attached plays an important role on the sensitivity of the Mab2H3 for BaP. For the FCIA, the protein coupling position was at C6 of BaP and for the ELISA the C7 position was used [29]. Due to the low sensitivity of the MabBaP antibody, with IC_{50} values varying from 10-17 $\mu\text{g L}^{-1}$ (Figure 3), it was not used anymore.

Furthermore, we selected the beads coated with the BaP-BSA conjugate, since it resulted in more stable maximum responses (MFIs) in combination with both antibodies, the antibody dilutions were higher, and the assay's sensitivity for BaP, especially with Mab22F12, was slightly better compared to the BaP-KLH coated beads.

In general, the sensitivity of the FCIA for BaP in buffer was in good agreement with those obtained with the ELISA using the same antibodies [29]. Additionally, using Mab22F12 in combination with the BaP-BSA conjugate on the surface of the biosensor chip resulted in an 8 times less sensitivity for BaP in the SPR-BIA compared to the FCIA (Figure 2). It seems that the use of a label, a longer incubation time and especially a more diluted antibody solution (1/4000 dilution for Mab22F12 in the FCIA and 1/10 dilution in the BIA) contribute to a more sensitive FCIA for BaP.

Multi-PAHs FCIA

Antibodies often recognize not only the hapten used for the immunization but also molecules of similar structure. This is an advantage especially when the target is the development of a chemical group-oriented immunoassay for the simultaneous detection of suitable indicators of PAH contamination in food. For the PAHs cross-reactivity (CR) measurements, the IC_{50} value of BaP was assigned as 100%, and CR for the other tested compounds were reported as the percentage relative to that value. The specificities of Mab22F12 and Mab2H3 were tested using structurally similar PAHs as target analytes (Figure 1). The selection of the PAHs was done according to the EPA 16 priority PAHs and the 2008 EFSA opinion. CR data for the two antibodies found in our assay and also measured previously in the ELISA [29] are compared in Table 1. In most cases, the CRs obtained with the FCIA were comparable to the ELISA data. Mab2H3 exhibited a broader CR for the majority of PAHs than Mab22F12, as expected from the previous ELISA results [29]. No or low CR was obtained in both assays for the two- and three-rings containing aromatic compounds such as naphthalene (NA), fluorene (FL), phenanthrene (PHE), and anthracene (AN), apart from acenaphthylene (ACL) and acenaphthene (AC) which showed a moderate CR in the FCIA (Table 1). Increasing numbers of aromatic rings resulted in higher CR with the exception of dibenzo[a,h]anthracene (DBaHA), dibenzo[a,e]pyrene (DBaeP), dibenzo[a,h]-pyrene (DBahP), dibenzo[a,i]pyrene (DBaiP), dibenzo[a,l]pyrene (DBalP), which showed no or low CR with both antibodies (Table 1). Apparently, these PAHs do not fit properly into the binding pocket of the antibodies. For some PAHs, especially with Mab2H3, lower CRs were observed in the FCIA compared to

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the ELISA (Table 1). However, in the ELISA, as it has already been described, the coating antigen was different compared to the one used to coat the microspheres in the FCIA and this seems to play an important role on the sensitivity and selectivity of the assays for the different PAHs.

Table 1: Percentage of cross-reactivity (CR%) obtained with Mab22F12 and Mab2H3 for the 26 food-related PAHs in the formerly developed ELISA and/or in the present described FCIA. The PAHs marked with the * belong to the group of PAH8 according to EFSA's opinion.

PAHS	CR (%)			
	Mab22F12		Mab2H3	
	FCIA	ELISA	FCIA	ELISA
BaP*	100	100	100	100
CHR*	53	77	24	63
BaA*	7	13	30	149
BbF*	8	24	23	101
BkF*	4	5	6	50
IP*	25	45	81	76
BghiP*	0	1	0	2
DBahA*	0	0	6	38
DBaeP	0	-	0	-
DBahP	1	-	0	-
DBaiP	1	-	10	-
DBalP	0	-	0	-
NA	0	0	0	0
ACL	10	0	27	0
AC	25	0	27	0
FL	0	0	5	1
PHE	0	1	0	9
AN	3	1	0.3	4
FA	0	0	0	0
PY	1	18	0	30
MCH	11	-	30	-
6-OH-CHR	2	-	0	-
1-OH-PYR	2	-	0.1	-
BjF	146	-	127	-
CCP	1	-	16	-
7H-BcFL	9	-	37	-

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Apart from the PAHs tested both in the FCIA and the ELISA, some other additional food-related PAHs were tested in the FCIA only. Both Mab22F12 and Mab2H3 were extremely sensitive for benzo[j]fluoranthene (BjF) with CRs of 146% and 127%, respectively. For cyclopenta[c,d]pyrene (CCP), 5-methylchrysene (MCH) and 7H-benzo[c]fluorine (7H-BcFL), the CR varied from low (1%) to moderate (16-37%) (Table 1). In fresh fish, significant PAHs bioaccumulation has not been reported since these organisms have a high biotransformation potential for these compounds [33]. For that reason we evaluated the selectivity of the Mabs for two commercial available hydroxylated metabolites of PAHs. However, the presence of the hydroxyl (OH) group in the molecule of 6-OH-chrysene and 1-OH-pyrene resulted in no or low CR of both antibodies (Table 1). For the methylated PAHs however such as 5-methylchrysene (MCH) a low CR of Mab22F12 (11%) and a moderate CR of Mab2H3 (30%) were found.

Overall it can be concluded that the developed FCIA in buffer has potential as a rapid screening assay, not only for BaP but also for the other PAHs from the PAH4 and PAH8 groups of EFSA. Although the CRs of Mab2H3 for two of the PAHs from the PAH4 group were higher, the sensitivity of the assay based on Mab22F12 was better for all four PAHs. This was also the case for the PAHs from the PAH8 group of which DBaH and BghiP were not detected with both antibodies. On the other hand, the detection of some additional PAHs (i.e. ACL, AC) from the EPA16 seems to be feasible.

Applicability of the FCIA to real food samples

With the aim to investigate the applicability of the FCIA in a real food matrix, we measured the levels of PAHs in extracts of non-contaminated (blank) and contaminated carp and wheat flour, previously analyzed by GC-MS. In the blank carp and wheat flour extract, the PAHs detected by GC-MS were less than 0.18 and 0.06 $\mu\text{g L}^{-1}$ respectively. The maximum response (B_0) obtained with the blank carp extract (100 times diluted in buffer) for Mab22F12 (1770 MFIs) and Mab2H3 (1315 MFIs) were similar to those obtained in buffer (1700 and 1250 MFIs for both Mabs). That was not the case for the maximum response obtained in the extract of the blank wheat flour with Mab2H3, which increased from 1170 to 1720 MFIs with a 20 times diluted extract. Further dilution of the extract eliminated the matrix effect, however, we could not measure any inhibition caused by the presence of PAHs in the extract of the contaminated wheat flour. Calibration curves of different concentrations of BaP in buffer, blank carp extract (diluted in buffer 100 times for both Mabs) and blank wheat flour extract (diluted 50 times in buffer for Mab22F12 and 20 times for Mab2H3) were measured in the FCIA and compared (Figure 4). The BaP assay's sensitivity for both antibodies, was not affected by the carp extract however in the presence of the wheat flour extract, the sensitivity for BaP became less (Figure 4).

For the extracts from both food samples (carp and wheat flour) differences between the non-contaminated and contaminated samples were distinguished (Figure 5). The BaP assay's sensitivity for both antibodies, was not affected by the carp extract however in the presence of the wheat flour extract, the sensitivity for BaP became less (Figure 4). The

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comparison of the data obtained with an immunoassay and with GC-MS can be problematic because of the fundamental differences in the analytical methods. Chromatography-based assays depend on chemical or physicochemical properties of the molecule of interest, while the critical element of an immunoassay is the affinity between the analyte (antigen) and antibody, coupled with a suitable endpoint detection system. The FCIA reacts with several PAHs with different responses to each of them, but all the responses are added and reported as BaP equivalents because of the use of this PAH for the calibration curve. Moreover, the GC-MS technique used isotope dilution to compensate for any analytes losses, something we could not use for the FCIA.

In the smoked carp extract, the FCIA's based on either Mab22F12 or Mab2H3 measured 800 and 1700 $\mu\text{g L}^{-1}$ of BaP equivalents, respectively. This is higher than the 438 $\mu\text{g L}^{-1}$ of BaP measured by GC-MS. In the contaminated wheat flour extract 96 and >100 $\mu\text{g L}^{-1}$ of BaP equivalents were found with the FCIA's based on Mab22F12 and Mab2H3, respectively, which was also higher than found with GC-MS (49.5 $\mu\text{g L}^{-1}$ of BaP). Indeed the GC-MS results confirmed that apart from BaP several other PAHs were present which contributed to the FCIA result in accordance with the cross-reactivity of the individual PAH.

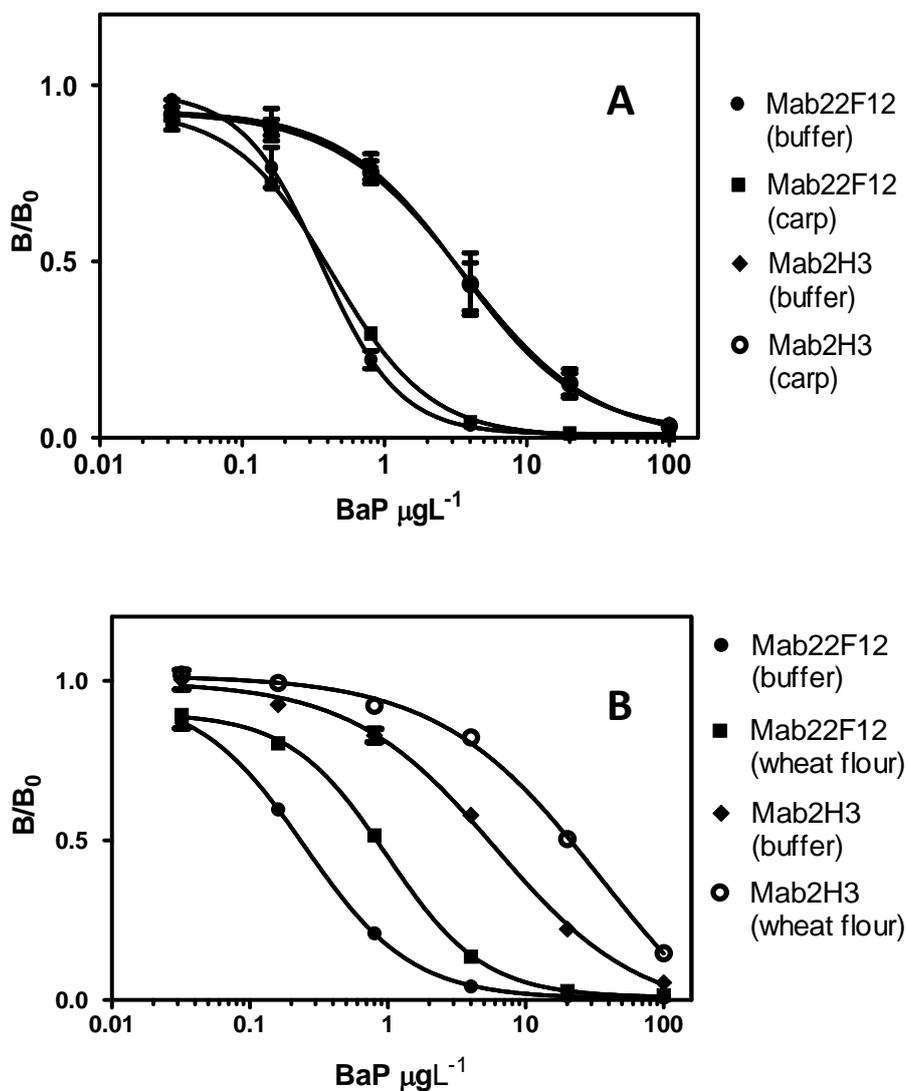


Figure 4: BaP calibration curves in the FCIA using Mab22F12 and Mab2H3, in combination with BaP-BSA coated beads, in buffer and in carp extract (A) (1/100 diluted in assay buffer for both Mabs) and in wheat flour extracts (B) (1/50 diluted for Mab22F12 and 1/20 for Mab2H3 in assay buffer). The B_0 is the MFIs of the blank measurement in buffer or blank food extract and B the MFIs obtained with the different BaP concentrations in buffer or blank food extract. Each point represents the mean of three replicates \pm SD.

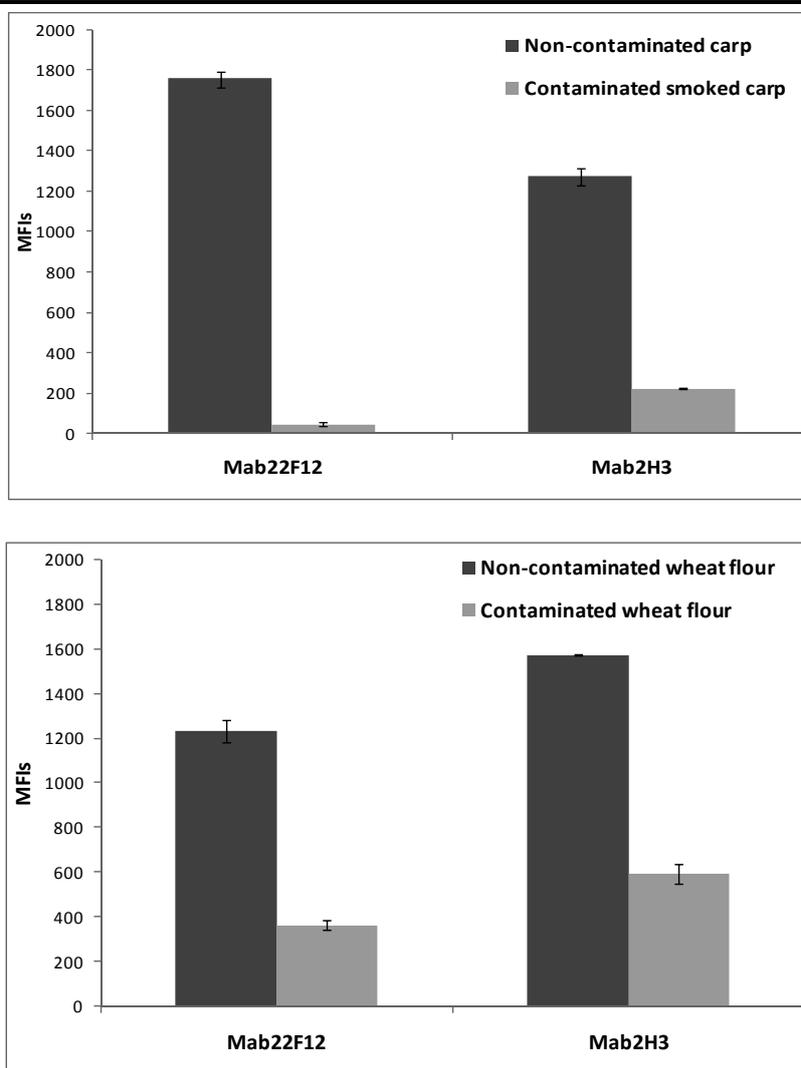


Figure 5: Responses (MFIs) obtained with non-contaminated (black columns) and with PAHs contaminated extracts (grey columns) of (A) carp and (B) wheat flour measured in the FCIA with both Mabs (Mab22F12 and Mab2H3).

Conclusions

For the first time a color-coded paramagnetic microbead immunoassay with flow cytometry detection has been developed and applied to the analysis of PAHs in real food samples. The analytically high sensitivity for BaP with an IC_{50} of $0.3 \mu\text{g L}^{-1}$ using the Mab22F12 against BaP is similar to that obtained in a previously developed ELISA in buffer [29]. An alternative analytical method SPR-based biosensor immunoassay proved to be 8

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times less sensitive than FCIA using the same immunoreagents. Next to BaP, the FCIA detects CHR and IP, with cross-reactivities (CRs) of 81 and 53%, respectively, which are also appointed by the European Food Safety Authority (EFSA) as suitable indicators of PAH contamination in food. The developed FCIA was shown to be applicable to food analysis in real food extracts such as smoked carp and wheat flour extract previously analyzed for PAHs by GC-MS. Due to its multiplexing potential, the FCIA described here offers a better alternative to existing bioanalytical screening methods and it is expected to be suitable for multi-analyte food profiling like the simultaneous detection of PAHs and other POPs such as PCBs and BDEs combined with easy extraction procedures which are under development.

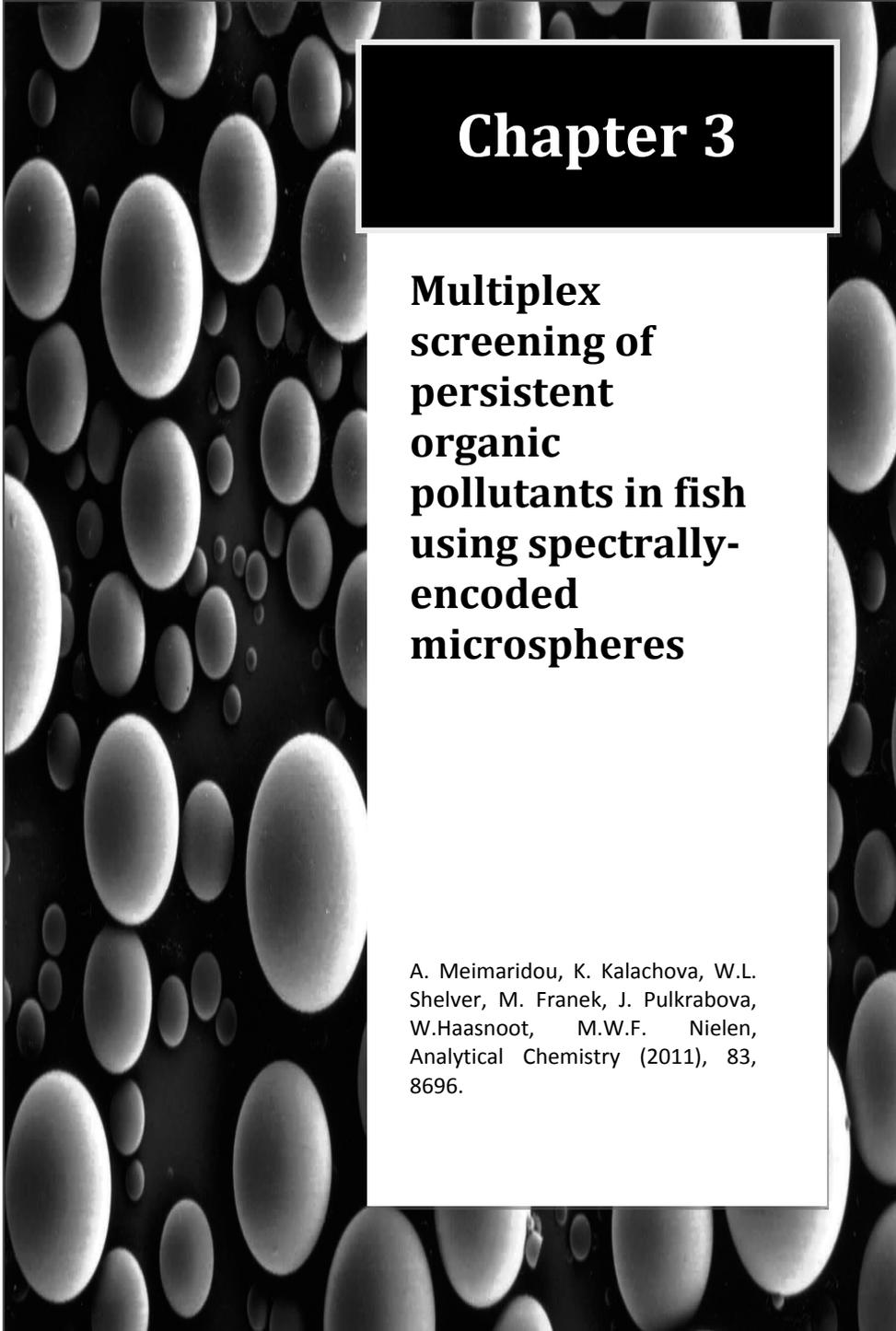
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Chapter 3

Multiplex screening of persistent organic pollutants in fish using spectrally- encoded microspheres

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Abstract

Persistent organic pollutants (POPs) are environmental and food-related contaminants of global public health concern and known to be carcinogenic and endocrine disruptors. Their monitoring is essential, and an easy-to-use, rapid, and affordable multi-analyte screening method with simplified sample preparation can be a valuable tool prior to instrumental analysis. For this purpose, a flow cytometric immunoassay (FCIA), based on a spectrally encoded microbeads technology, was developed for the multiplex detection of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (BDEs) in buffer and fish extracts. The sensitivities of the assays in the 3-plex FCIA format were similar to the individual FCIA for the marker compounds benzo[a]pyrene (BaP), 3,3',4,4' tetrachlorobiphenyl (PCB77), and 2,2',4,4'-tetrabromodiphenyl ether (BDE47) in buffer with IC_{50} values of 0.4, 20, and $2 \mu\text{g L}^{-1}$, respectively. Apart from the three markers, we could detect at least 14 other POPs. Extracts of fish with different fat content, prepared with a simplified extraction and cleanup procedure, had an insignificant influence on the overall 3-plex FCIA performance, with the exception of some impact on the PAHs detection. The performance of the 3-plex FCIA, in combination with the simple extraction procedure, is adequate for regulatory control in accordance with the required limits.

Introduction

These days, it is known that the consumption of food contaminated with persistent organic pollutants (POPs) can cause acute intoxication incidents after high levels of exposure. Furthermore, diseases can appear after low-level chronic exposure of these chemicals [1,2]. During the past years, various contamination incidents with POPs in food have been reported [2-6]. Polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (BDEs), and polycyclic aromatic hydrocarbons (PAHs) represent the major groups of persistent toxicants not only in the environment but also in high fat content foods such as fish, due to their lipophilicity [4, 7-9]. Consumption of food contaminated with POPs is the major exposure route for humans compared to other ways, such as inhalation and dermal contact [10, 11]. To prevent health risks from the exposure to POPs, both the European Commission (EC) [12] and the United States Environmental Protection Agency (US-EPA) [13] established monitoring programs according to the Stockholm Convention [14] on POPs. The EC set maximum levels (MLs) for the sum of dioxins and dioxin-like PCBs and benzo[a]pyrene (BaP) [15] in various foods and animal feed. Apart from BaP, 7 other carcinogenic PAHs [16] are under evaluation to be included as indicators of PAHs occurrence and toxicity in food. So far, no regulatory limits have been established for the BDEs, however, under the European regulatory framework [17], the authorization

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procedure foresees that the utilization of BDEs can be subject to an authorization requirement [18].

Instrumental analytical techniques that have routinely been used to detect POPs [7, 19-22] are quite sensitive, specific and irreplaceable in terms of identification power. However, they require costly equipment, skilled personnel, and they are time- and labor-intensive. In vitro bioanalytical assays, such as the aryl hydrocarbon hydroxylase (Ahh)/ethoxyresorufin-*O*-deethylase bioassay and the chemical-activated luciferase gene expression (CALUX) bioassay [23-26], were developed to analyze Ah receptor agonists, such as several dioxin-like PCBs, as well as some BDEs and PAHs. These assays are cheaper compared to instrumental analytical techniques; however, recombinant cell culture lab facilities are necessary. Several enzyme-linked immunosorbent assays (ELISAs) were developed for the detection of PCBs, PAHs, and BDEs separately [27-34] but, so far, no attempts to simultaneous multiplex analysis were made. Rapid screening methods which are simple, inexpensive, fast, sensitive have high throughput, and the possibility of detecting multiple POPs simultaneously are greatly needed. A new open platform in food analysis that enables the rapid analysis of a large number of samples for multiple analytes is the superparamagnetic (MagPlex) spectrally encoded microbead (xMAP) technology combined with flow cytometry (Luminex), which has been described for the analysis of several contaminants in food [35-36].

In the present work, we utilized this technology for the development of a 3-plex flow cytometric immunoassay (FCIA) for the detection of three major POPs using BaP, 3,3',4,4'-tetrachlorobiphenyl (PCB77), and 2,2',4,4'-tetrabromo- diphenyl ether (BDE47) as marker compounds. After the characterization of the 3-plex FCIA, the performance was tested in fish extracts prepared with a simplified and fast sample extraction and cleanup based on the quick, easy, cheap, effective, rugged, and safe (QuEChERS) [37] approach (Figure 1).

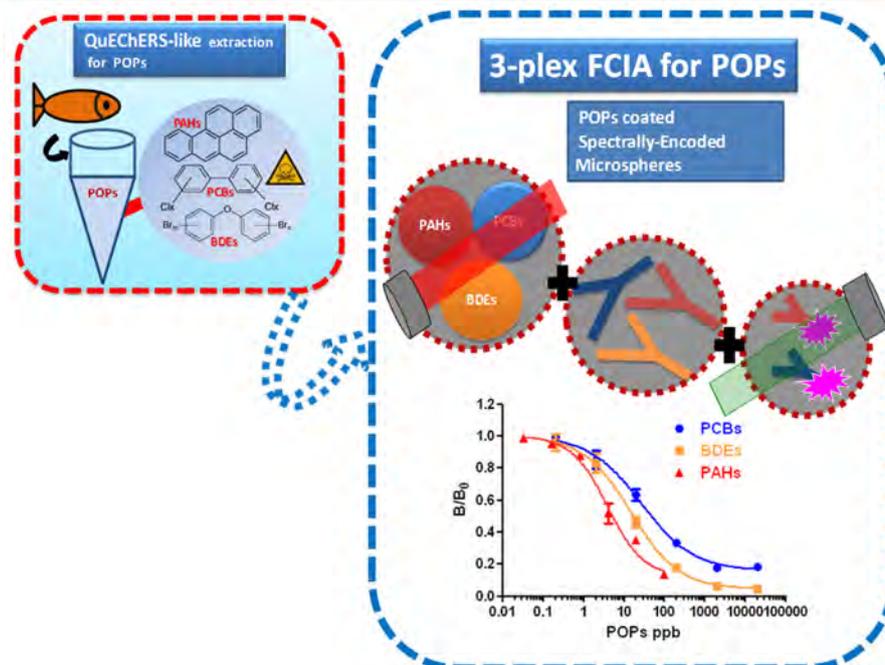


Figure 1. Illustration of the developed 3-plex flow cytometric immunoassay (FCIA) for the simultaneous detection of PCBs, BDEs and PAHs in fish extracts combined with a simple QuEChERS-like extraction.

Experimental Section

Reagents and materials.

The mouse monoclonal antibody (Mab) against BaP (two batches of Mabs of different purities) and the BaP conjugated to bovine serum albumin (BaPBSA) were purchased from the Technical University of Munich (Munich, Germany). Four rabbit polyclonal antibodies (Pabs) against BDE47 (PabBDE47 nos.122,123,124, and 125) and the BDE47 conjugated to BSA (BDE47BSA) were kindly offered by Dr. Weilin L. Shelver of the USDA (Fargo ND, U.S.A.). The two rabbit Pabs against PCB77 (PabPCB77-3TG and PabPCB77-5TG) and the PCB77 conjugated to ovalbumin (PCB77OVA) were gifts from Dr. Milan Franek of the Veterinary Research Institute (Brno, Czech Republic). The goat antimouse and goat antirabbit R-phycoerythrin (PE) conjugates were from Prozyme (San Leandro, CA, U.S.A.). Most of the stock standard solutions of PAHs, PCBs, and BDEs (n=51) (Table 1) were supplied in the water-miscible organic solvents dimethyl sulfoxide (DMSO), methanol (MeOH), or acetonitrile (ACN) by AccuStandard (New Haven, CT, U.S.A.). Dr. Ehrenstorfer Laboratory (Ausborg, Germany) supplied the dibenzo[a,e]pyrene, cyclo- penta[c,d]pyrene,

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5-methylchrysene, benzo(c)fluorine), and BDE47. Aroclor mixtures 1232, 1242, 1248 were provided by Supelco (Bellefonte, PA, U.S.A.). Protein LoBind tubes (1.5 mL) were supplied by Eppendorf (Hamburg, Germany), and the LoBind 96-well microplates were from GreinerBio-One B.V. (Alphen/dRijn, The Netherlands). The N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) was provided by Fluka Analytical (Steinheim, Switzerland). n-Hexane-dichloromethane and silica (0.0630.200 mm) were supplied by Merck (Darmstadt, Germany), and ethyl acetate was from Sigma-Aldrich (Steinheim, Germany). Magnesium sulfate and sodium chloride for the QuEChERS-like extraction were delivered from Sigma-Aldrich and Lach-Ner (Neratovice, Czech Republic), respectively. All other reagents not specified above were from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). The blank and the different POPs-contaminated fish extracts were caught at the locality Vranany on Vltava (Moldau) river located downstream from Prague industrial region and analyzed with gas chromatography/mass spectrometry (GC/MS) by the Institute of Chemical Technology Prague (Prague, Czech Republic).

Equipment

Applied Cytometry Systems (South Yorkshire, UK) delivered the Luminex 100 IS 2.2 system consisting of a Luminex 100 analyzer and a Luminex XY Platform, which was programmed to analyze a 96-well plate. The magnetic carboxylated microbeads (MagPlex™ no. 21, 42 and 70) and the sheath fluid were supplied by Luminex Corporation (Austin, TX, USA). The automated microplate wash station Bio-Plex™ Pro II, with a magnetic carrier, was provided by Bio-Rad Laboratories B.V. (Veenendaal, the Netherlands). The magnetic separator DynaMag™-2 was obtained from Invitrogen Dynal (Oslo, Norway). The test tube rotator was from Snijders (Tilburg, the Netherlands). All GC-MS experiments were performed using a gas chromatograph Agilent 6890N (Agilent Technologies, Palo Alto, CA, USA) coupled to a high-speed time-of-flight mass spectrometer (TOF-MS) Pegasus III (LECO Corp, St Joseph, MI, USA) operated in an electron ionization mode (EI). The GC system was equipped with an electronic pressure control (EPC), a split/splitless injector and an MPS 2 autosampler (Gerstel, Germany). The capillary column BPX-50 (30 m × 0.25 mm i. d. × 0.25 μm film thickness) was obtained from SGE (Austin, TX, USA). The ChromaTOF 4.24 software (LECO Corp.) was used for data processing. A Pasteur pipette (D812, 230 mm length) and glass wool were received from Poulten & Graf GmbH (Wertheim, Germany) and Merck, respectively. The tissue grinder Waring blender (model 38BL40) was supplied by Waring (Torrington, CT, USA). The rotary vacuum evaporators, Buchi Rotavapor R-114 and R-200 with a heating bath, were obtained from Buchi Rotavapor (Flawil, Switzerland). The centrifugal machine Rotina 35R was supplied by Hettich Zentrifugen (Tuttlingen, Germany). For the calibration and dose-response curves of the FCiAs, a 4 parameter curve fitting was used from GraphPad Prism software (GraphPad Software Inc. (La Jolla, CA, USA)).

Preparation of the different POP-coated microbeads

The different protein conjugates (BaP-BSA, PCB77-OVA and BDE47-BSA) were covalently coupled to unique superparamagnetic carboxylated microbead sets (21, 70 and 42, respectively) according to the procedure described before [36]. Briefly, after the activation of each microbead set with 50 mg mL⁻¹ sulfo-NHS and EDC in 0.1 M NaH₂PO₄ (pH 6.2) and twice washing with 200 µL of 50 mM MES (2-N-morpholino ethanesulfonic acid) buffer (pH 5.0), to remove any by-product, 50 µg of the BaP-BSA, or BDE47-BSA or PCB77-OVA conjugate was added to the different activated microbead sets in a final volume of 500 µL of 50 mM MES. The suspension was incubated for 2 hours under mixing by rotation in the test tube rotator at room temperature in the dark. The different coated paramagnetic color-encoded microbeads were washed three times, to remove any unbound protein conjugates, with 500 µL of PBS buffer, resuspended in 200 µL of PBS buffer and stored at 4 °C until use.

GC-MS analysis of the fish extracts

All experiments were performed using an Agilent 6890N GC system coupled to a Pegasus III high-speed time-of-flight mass spectrometer (GC-TOFMS) operated in an electron ionization mode (EI). Target analytes were separated on a BPX-50 capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness). A volume of 8 µL was injected using PTV injection in a solvent vent mode with initial temperature 50°C (2.3 min); inlet heating velocity: 400°C s⁻¹ and final inlet temperature: 300°C. Helium was used as a carrier gas and for the first 19 min the ramped flow was 1,3 ml min⁻¹ and it was increased up to 2 mlmin⁻¹ using the speed of 50 ml min⁻¹. The GC oven temperature program was as follows: 80°C (4.3 min), 30°C min⁻¹ to 220°C, 2°C min⁻¹ to 240°C, and 10°C min⁻¹ to 340°C (15 min). The MS detector was operated under the following conditions: mass range: m/z 45–750; ion source temperature: 250°C; transfer line temperature: 280°C; detector voltage: –1950 V.

Procedures

Purification of the PabBDE47

PabPCB77 anti- serum was affinity purified, in order to remove a BDE47 cross-reacting fraction, by incubating the antiserum (final dilution 1/ 1000) for 1 h with BDE47BSA-coated superparamagnetic microbeads (final number of beads used ~500000). After the incubation, the BDE47 cross-reacting fraction was removed with the help of a magnetic separator. The affinity-purified PabPCB77 was always prepared fresh prior to the analysis.

3-plex FCIA

The protocol for the 3-plex FCIA was similar to the single-plex FCIA protocol with the exception of the application of mixtures of reagents (antibodies, beads, and labels), instead of individual reagents. For the 3-plex FCIA analysis, the POPs standard dilution series was prepared in the working buffer (5.4 mM sodium phosphate, 1.3 mM potassium phosphate, 150 mM sodium chloride pH 7.4 (PBS) with 2% DMSO and 0.7% BSA) of which 40 μL was combined with 40 μL of 50% DMSO in the well of a low-binding 96-well microplate. To obtain a similar DMSO concentration, the sample extract in DMSO was diluted with working buffer (1:1; v/v) and 40 μL of this diluted sample extract was combined with 40 μL of working buffer. After that, 20 μL of the mixture of the three different antibodies in PBS, the Mab against BaP (in a final dilution (fd) of 1/1000) and the two Pabs against PCB77 (affinity purified and with a fd of 1/1000) and BDE47 (fd 1/250), was added. The mixture was incubated for 15 min, and then 10 μL of the mixture of the three different POP-coated microbeads was added to the well providing at least 1000 microbeads per set per test. The mixture was incubated for 45 min at room temperature in the dark on a plate shaker. After the incubation, the unbound bioreagents were removed by three washing steps with PBS, using the magnetic plate carrier of the automated wash station. Next, 25 μL of the mixture of the anti-mousePE and anti-rabbitPE in PBS (both in a fd of 1/500) was added, followed by 100 μL of PBS, and then the mixture was incubated for 20 min in the dark, followed by one washing step with PBS. The microbeads were resuspended in 100 μL of PBS, and finally, the measurement in the Luminex was read for 20 s using 50 μL per well. To prepare dose response curves in buffer or in blank fish extract, a dilution series of the three POPs (0.01-1000 $\mu\text{g L}^{-1}$) was prepared either in the working buffer or in the diluted blank fish extracts. As negative controls, we used buffer or dilutions of blank fish extract (1:1; v/v). The contaminated fish extracts were diluted in the same way as the spiked blank samples.

POPs extraction from the fish samples and GC-MS analysis of the fish extracts

The extraction protocol for fish samples followed a previous described procedure [38]. Briefly, 10 g of homogenized fresh fish (5 g of homogenized smoked fish) muscle tissue was mixed in a polypropylene tube with 5 mL of distilled water and 10 mL of ethyl acetate and shaken vigorously for 1 min. Subsequently, 4 g of magnesium sulfate and 2 g of sodium chloride were added. After 1 min of shaking, the tube was centrifuged for 5 min (11000 rpm), and finally an aliquot of 5 mL in the case of fresh fish (for the PCBs and BDEs extraction) and 4 mL in the case of smoked fish (for the PAHs extraction) from the ethyl acetate layer was removed and evaporated under a gentle flow of nitrogen. If no extra cleanup was needed, the residue was dissolved in 1 mL of DMSO. For the non-

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contaminated fish spiked with different Aroclor solutions, 10 g of fish muscle tissue was spiked 15 min prior to the extraction with 50 μL of original standards (concentration 200000 ng mL^{-1}) of Aroclor 1232, 1242, and 1248, respectively, resulting in 1000 μg of the different Aroclors per kg of fish tissue. For the cleanup, the residue was redissolved in 1 mL of hexane and this solution was introduced into a laboratory-made silica solid-phase extraction (SPE) column (Pasteur pipet filled with glass wool, 1 g of silica, and ca. 0.2 g of sodium sulfate) which was preconditioned with 6 mL of hexane/dichloromethane (3:1, v/v) followed by 4 mL of hexane. After the sample load, the analytes were eluted with 10 mL of hexane/dichloromethane (3:1, v/v). The eluate was evaporated by a rotary vacuum evaporator, and the residual solvents were removed by a gentle stream of nitrogen gas. The residue was dissolved in 1 mL of DMSO. The analysis of the fish extracts by GC/MS was carried out as described above.

Results and Discussion

Development of the singleplex FCIA prior to the 3-plex FCIA

The research started with the development of the individual (single-plex) FCIA for each of the three target model analytes (BaP, PCB77, and BDE47) using the different available immunoreagents (antibodies and protein conjugates of the three POPs). The competitive inhibition format of the single-plex FCIA was based on previously described work with PAHs. [36]. Briefly, we coated PCB77-OVA, BDE47-BSA, and BaP-BSA protein conjugates to three different sets of spectrally encoded microbeads. A Mab against BaP and Pabs against PCB77 and BDE47 were tested during the development of the single-plex FCIA. For the measurement of the bound antibodies to the coated beads, goat antimouse or antirabbit Pabs labeled with PE were used. All these available immunoreagents were tested for their optimum dilutions in combination with different sequential incubation conditions. The water solubility of PCBs, PAHs, and BDEs is very low, so they are usually extracted from food matrixes using organic solvents [39-40]. In order to increase the solubility of POPs and to avoid adsorption to the well plate, the final concentration of DMSO in the well was around 20%, resulting in no influence on the sensitivities of the three single-plex FCIA. Also in earlier described immunoassays for various POPs, several organic solvents such as DMSO, ACN, and MeOH were used up to 50% without a significant influence on the assay sensitivities [29],[30],[32],[34]. The criteria used to evaluate the optimization process were the maximum median fluorescence intensities (MFIs), aiming for around 2000 MFIs for the blank measurements (the maximum responses) in a competitive inhibition format, the dynamic ranges, and the lowest IC_{50} values for each of our target analytes. The optimum combinations for each individual FCIA were PabPCB77-3TG (fd 1/4000) with PCB77 OVA-coated beads for the detection of PCBs, MabBaP (fd of 1/ 1000) with BaPBSA-coated beads for the detection of PAHs, and

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PabBDE47122 (fd 1/1000) with BDE47BSA-coated beads for the detection of BDEs. The normalized PCB77, BaP, and BDE47 dose response curves obtained in the single-plex FCIA are given in Figure 2, where the B_0 is the maximum MFI of the blank measurement and B is the MFI obtained with the different analyte concentrations. The IC_{50} values (the concentrations of the analytes at 50% inhibition of the maximum responses) for each analyte were 20 ± 2 , 2 ± 0.2 and $0.3 \pm 0.1 \mu\text{g L}^{-1}$ for PCB77, BDE47 and BaP, respectively. The IC_{50} values of the single-plex for PCB77 and BaP in buffer were in good agreement with those obtained with the ELISA using the same antibodies, [32,34] only, the sensitivity for BDE47 was higher in the ELISA [30] ($IC_{50}=0.135 \mu\text{g L}^{-1}$).

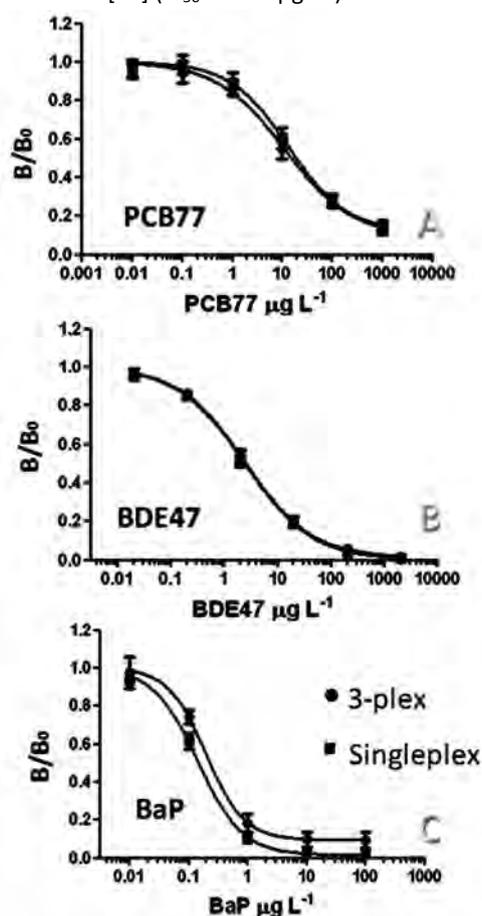


Figure 2. Dose-response curves obtained with the FCIA in 3-plex (●) and single-plex (■) formats in buffer for the three main POPs representatives analyzed in this current study: (A) PCB77, (B) (BDE47), and (C) (BaP). The relative binding (B/B_0) was calculated by dividing the response (B) of each concentration by the maximum response obtained in a solution without the analyte (B_0). Solid lines show curves fitted with the four-parameters (4P) model. Each point represents the average of six replicates SD).

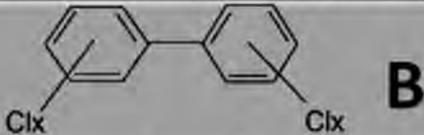
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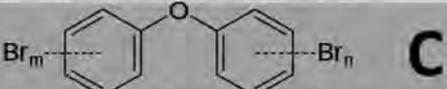
This difference can be due to different BDE47 protein conjugates that were used in both formats. Another BDEs immunoassay [29] described a similar sensitivity as obtained in this study. Note that, for the BDEs no limits are set yet within the European Union (EU) and by EPA. Other immunoassays were developed for other indicators for PCBs, such as 2,3',4,4',5-pentachlorobiphenyl (PCB118) [31],[41], but their sensitivities for dioxin-like PCBs were rather low. The specificities of the three single-plex FCIA were determined by the assessment of the cross-reactivity (CR) pattern with the different target POPs selected on the basis of EU and EPA regulations and their structural similarities to PCB77, BDE47, and BaP. The percentage of CR was determined using the 50% displacement method [42], and the data thus obtained are included in Table 1.

Table 1: Names, abbreviations (Abbr.), CAS-numbers and percentages of cross-reactivity (CR%) as determined in the three different immunoassay formats of formerly developed ELISAs, singleplex and 3-plex FCIA for the 51 tested POPs: (A1 and A2) for PAHs, (B) for PCBs and (C) PBDEs.

Polycyclic Aromatic Hydrocarbons (PAHs)					
					A1
Compound	Abbr.	Cas-number	Cross-reactivity %		
			3plex FCIA	Singleplex FCIA	ELISA*
Benzo[a]pyrene	BaP	50-32-8	100	100	100
Chrysene	CHR	218-01-9	88±13	53±0.2	77
Benz[a]anthracene	BaA	56-66-3	25±5	7±0.1	13
Benzo[b]fluoranthene	BbF	206-99-2	3±0.5	8±0.9	24
Benzo[k]fluoranthene	BkF	207-08-9	3±0.7	4±0.8	5
Indeno[1,2,3-cd]pyrene	IP	193-39-5	8±1	25±0.5	45
Benzo[ghi]perylene	BghiP	191-24-2	4±1	0	1
Dibenzo[a,h]anthracene	DBahA	53-70-3	0	0±0.3	nm
Dibenzo[a,e]pyrene	DBaeP	192-65-4	0	0	nm
Dibenzo[a,h]-pyrene	DBahP	189-64-0	1±0.2	0±0.1	nm
Dibenzo[a,l]pyrene	DBalP	191-30-0	0	0±0.1	nm
Dibenzo[a,i]pyrene	DBaiP	189-55-9	5±0.5	1±0.1	nm
Benzo[j]fluoranthene	BjF	205-82-3	57±10	146±0.03	nm

Polycyclic Aromatic Hydrocarbons (PAHs)					
					A2
Compound	Abbr.	Cas-number	Cross-reactivity %		
			3plex FCIA	Singleplex FCIA	ELISA*
Cyclopenta[c,d]pyrene	CCP	27208-37-3	40±0.5	1±0.5	0
Anthracene	An	120-12-7	0	3±0.5	1
Acenaphthylene	ACL	208-96-8	0	10±1	1
Acenaphthene	AC	83-32-9	0	25±3	0
Pyrene	PY	1718-52-1	0	0.1	18
Fluorene	FL	86-73-7	0	0	0
Phenanthrene	PHE	85-01-8	0	0	1
Benzo[c]fluorene	BcFL	205-12-9	20±1	9±2	nm
Naphthalene	nA	91-20-3	0	0	0
Fluoranthene	FA	206-44-0	0	0	0
5-methylchrysene	MCH	3697-24-3	24±5	11±1	n.m.
OH-chrysene	6-OH-CHR	37515-51-8	7±2	2±0.2	n.m.
1-OHpyrene	1-OH-PYR	5315-79-7	0	2±0.1	n.m.

Polychlorinated Biphenyls (PCBs)					
					
Compounds	Abbr.	Cas-number	Cross-reactivities %		
			3plex FCIA	Singleplex FCIA	ELISA*
1,1'-Biphenyl, 3,3',4,4'-tetrachloro-	PCB 77	32598-13-3	100	100	100
1,1'-Biphenyl, 3,4,4',5-tetrachloro-	PCB 81	70362-50-4	2±0.35	6±3	0
1,1'-Biphenyl, 2,3,3',4,4'-pentachloro-	PCB 105	32598-14-4	12±7	4±2	0
1,1'-Biphenyl, 2,3,4,4',5-pentachloro-	PCB 114	74472-37-0	0	1±0.2	n.m.
1,1'-Biphenyl, 3,3',4,4',5-pentachloro-	PCB 126	57465-28-8	23±7	7±3	7
1,1'-Biphenyl, 3,3',4,4',5,5'-hexachloro-	PCB 169	32774-16-6	9±2	1±0.5	0
1,1'-Biphenyl, 2,3',4,4',5-pentachloro-	PCB 118	31508-00-6	0	1±0.1	n.m.
1,1'-Biphenyl, 2,3',4,4',5'-pentachloro-	PCB 123	65510-44-3	1±0.1	1±0.2	n.m.
1,1'-Biphenyl, 2,3,3',4,4',5-hexachloro-	PCB 156	38380-08-4	10±3	2±0.3	n.m.
1,1'-Biphenyl, 2,3,3',4,4',5'-hexachloro-	PCB 157	69782-90-7	0	1±0.4	0
1,1'-Biphenyl, 2,3',4,4',5,5'-hexachloro-	PCB 167	52663-72-6	0	1±0.1	n.m.
1,1'-Biphenyl, 2,3,3',4,4',5,5'-heptachloro-	PCB 189	39635-31-9	0	1±0.2	0

Polybrominated Diphenyls (PBDEs)					
					
Compounds	Abbr.	Cas-number	Cross-reactivity %		
			3plex FCIA	Singleplex FCIA	ELISA
Benzene, 2,4-dibromo-1-(2,4-dibromophenoxy)-	BDE 47	5436-43-1	100	100	100
Benzene, 2,4-dibromo-1-(4-bromophenoxy)-	BDE 28	41318-75-6	31±5	52±0.5	16
Benzene, 1,2,4-tribromo-5-(2,4-dibromophenoxy)-	BDE 99	60348-60-9	48±11	31±0.5	90
Benzene, 1,3,5-tribromo-2-(2,4-dibromophenoxy)-	BDE 100	189084-64-8	8±0.5	1±0.1	3
1,1'-Biphenyl, 2,2',4,4',5,5'-hexabromo-	BDE 153	59080-40-9	1±0.2	1±0.1	2
Benzene, 1,3,5-tribromo-2-(2,4,5-tribromophenoxy)-	BDE 154	207122-16-4	<0.1	0	0
Benzene, 1,2,3,5-tetrabromo-4-(2,4,5-tribromophenoxy)-	BDE 183	207122-16-6	<0.1	0	0
Benzene, 1,1'-oxybis[2,3,4,5,6-pentabromo	BDE 209	1163-19-6	<0.1	0	0
Phenol, 2,6-dibromo-4-(2,4-dibromophenoxy)-	4'-OH-BDE 49	602326-23-8	1±0.2	2±0.3	4
Phenol, 2,4-dibromo-5-(2,4-dibromophenoxy)-	5'-OH-BDE 47	602326-30-7	3±0.7	18±1	9
Phenol, 3,5-dibromo-2-(2,4-dibromophenoxy)-	6'-OH-BDE 47	79765-43-4	<1	0	1
5-Hydroxy-2,2',4,4'-tetrabromodiphenyl ether	5'-MeO-BDE-047	602326-30-7	180±14	95±2	168
5'-Methoxy-2,2',4,4',5-pentabromodiphenyl ether	5'-MeO-BDE-099	n/A	2±0.7	0	1

Development of the 3-plex FCIA

In the 3-plex FCIA, the three single-plex assays were combined. However, this initially resulted in the PCB77 related cross-interaction of the PabPCB77 antiserum to the BDE47BSA-coated microbeads. Using the purification procedure described in the Experimental Section, the binding of the purified PabPCB77 antiserum to the BDE47BSA-coated microbeads could be strongly reduced from 3000 to 50 MFIs and was no longer PCB77-, BDE47-, or BaP-related. The normalized PCB77, BaP, and BDE47 dose response curves obtained in the 3-plex and single-plex FCIA are shown and compared in Figure 2. The low standard deviations (SDs; n=6) indicate that the curves are highly reproducible. The sensitivities of the single-plex FCIA and the 3-plex FCIA for the analytes in buffer are similar.

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The curve fitting was done with four-parameter fitting, and R^2 varied from 0.98 to 0.99 (Table 2). The accuracy and precision of the dynamic ranges in the three-plex FCIA were determined by replicate analyses of PCB77, BDE47, and BaP curves. Known concentrations of each analyte (0-1000 $\mu\text{g L}^{-1}$) were assayed in different sets of wells in the same plate (intra-assay measurements; $n = 2$) and in different plates on different days (inter assay measurements; $n=6$). Results showed that the sensitivity of the individual assays in the 3-plex FCIA format after the affinity purification of the PabPCB77 antiserum remained the same and is in compliance with the present established EU and U.S. limits.

Table 2: 3-plex FCIA characteristics in buffer and fish extracts and comparison to previously reported ELISAs for the detection of the three target POPs in buffer.

Target POPs	Matrix	Goodness of the 4P R^2 ^a	Curve steepness (mlng^{-1}) ^b	IC_{50} ppb in the 3plex FCIA ^c	IC_{50} ppb in ELISAs
PCB77 (A)	Buffer	0.9968	-0.8	20 ± 2	2-15 ³⁴
	Fish extract	0.9927	-0.6	55 ± 5	Not measured
BDE47 (B)	Buffer	0.9992	-0.7	2 ± 0.1	0.135 ³⁰
	Fish extract	0.9902	-0.7	2 ± 0.4	Not measured
BaP (C)	Buffer	0.9857	-1.3	0.4 ± 0.1	0.3 ³²
	Fish extract	0.9435	-1	4 ± 0.5	Not measured

^a Goodness of the four-parameter model fit to the calibration curve. ^b Calculated from the four-parameter fitted calibration curve. ^c The average half-maximal inhibitory concentration (IC_{50}) for each analyte extrapolated from six standard curves as the concentration of the analyte that provokes 50% inhibition of the maximum response.

Specificity of the 3-plex FCIA

In addition to the sensitivity assessment of the newly developed 3-plex FCIA for the marker POPs, CRs of the three different antibodies toward a panel of 51 POPs were determined and compared with previous results in Table 1. After the optimization process, no cross-interactions were displayed between the assays. The individual calibration curves were also tested in the final 3-plex format, and the individual assays were specific for their own target analytes. The CR patterns obtained with the three different assay formats (classic ELISAs and single-plex and 3-plex FCIA) were similar, with a few exceptions. The comparison between the single-plex FCIA and the ELISA [32] for the PAHs has been described previously [36]. No or low CR was obtained in all assays for the two- and three-rings containing aromatic compounds (Table 1: A1 and A2). For the

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other tested food-related PAHs, the 3-plex FCIA is less sensitive for B_jF compared to the singleplex. These differences between the assays might be explained by the fact that the MabBaP used in the 3-plex FCIA was an extra affinity-purified batch. The cross-reactivities of all assays for non-*ortho* and mono-*ortho* PCBs congeners were relatively low with only slight detection of one mono-*ortho* congener PCB105 and one non-*ortho* PCB126 in the 3-plex FCIA (Table 1: B). In the case of the BDEs (Table 1: C), a higher CR was shown for BDE99 in the ELISA than in the singleplex and 3-plex FCIA; however, in FCIA we used a different protein conjugate of BDE47. To our experience from previous work [36], that can have a big influence on the sensitivity and specificity of the assay. 5'-MeO-BDE47 exhibits high sensitivity in all the assay formats. It seems that the methoxy derivative is fitting more closely to the hapten used to generate the antibody [30]. BDE28 exhibits slightly higher CR in both FCIA. The low or no CR for the rest of the BDEs tested was comparable for all the immunoassays.

The developed 3-plex FCIA in buffer has potential as a rapid screening assay, since it can detect several POPs simultaneously, such as the most abundant flame retardant BDE47 in fish, along with PCB77 belonging to the group of the most toxic dioxin-like PCBs and BaP, CHR and many other PAHs belonging to the group of the 8 European Food Safety Authority (EFSA) designated PAHs.

Applicability of the 3-plex FCIA to fish samples and Aroclors

The direct measurement of POPs in high fat content foods is challenging for most analytical techniques, including immunoassays. POPs tend to accumulate in the fat tissues; therefore, simplified extraction and transportation into an immunoassay compatible solvent (such as DMSO) is essential. To demonstrate the 3-plex FCIA's applicability combined with a simplified sample preparation in a relevant food material, extracts of different fat content fish and different levels of PAHs, PCBs, and BDEs were analyzed (Table 2). The applied QuEChERS-like extraction technique relies on a favorable partition of POPs from the fatty sample material into the extraction materials (organic solvent and a mini silica column) and finally into DMSO. To study the influence of the silica minicolumn cleanup, we used aliquots of the same extracts before and after this cleanup in triplicate. The fish extracts after the cleanup step were also analyzed using capillary gas chromatography/time-of-flight mass spectrometry (GC/TOF-MS) and the levels of contamination for BaP, BDE47 and PCB77 as well as the fat contents are given in Table 3. However, in most fish extracts, more POPs were detected with the GC-TOF-MS than just the marker compounds, for example BaA, CHR, CCP, BDE100, PCB153, etc. Dose-response curves of BaP, PCB77, and BDE47 in buffer and blank fish extracts with no cleanup for the PCBs and PAHs and with silica cleanup for the BDEs were measured and compared in the 3-plex FCIA (Figure 3). In general, the 3-plex assay's sensitivity was not

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affected by the fish extract except for the BaP assay with a 10 times lower sensitivity and an IC_{50} of 4 ppb in matrix compared to the 0.4 ppb in buffer (Table 2). However, the PAH's FCIA is still adequate for screening smoked fish at the EU limit of 5 ng BaP per g wet weight of smoked fish and smoked fishery products; moreover, it can also detect more PAHs than just BaP.

Table 3. Contaminated fish samples with their fat contents, levels of the target POPs (BaP, PCB77, and BDE47) as measured with GC/MS, and the percentages of inhibition of the maximum responses as were measured in the 3-plex FCIA with (+) or without (-) the cleanup.

Fishes	Target POPs measured	Fat content %	μgkg^{-1} as measured in GC-MS	Clean-up	% of inhibition of maximum response in 3plexFCIA
Smoked trout	BaP	10	0.06	-	0
Smoked trout	BaP	11	1	-	80±2
Smoked trout	BaP	14	5	-	80±5
Smoked trout	BaP	13	14.7	-	80±3
Trout	PCBs/BDEs	2	n.d.	+	0±0.1
Chub	BDE47	1.5	0.43	+	45±2
Chub	BDE47	2	4.93	+	56±5
Chub	BDE47	2	9	+	50±4
Chub	PCB77	1.5	1.95	-	22±2

The maximum limit (ML) for the sum of dioxin and dioxin-like PCBs for fish and fishery products, excluding eel, is 8 pg of WHO PCDD/F-PCB- TEQ/g wet weight fish. The PCB77 toxic equivalency factor (TEF) value as stated for humans [43] is 0.0001, which results in a TEQ of 80 μg per kg fish. The IC_{50} value for PCB77 obtained in the 3-plex FCIA was $55\pm 5 \mu\text{g kg}^{-1}$ fish and thus well below the ML. For the emerging BDEs contaminants, no limits are set by both EU and US-EPA; we obtained an IC_{50} for BDE47 screening of $2 \mu\text{g kg}^{-1}$ of fish with a silica cleanup. The maximum responses (B_0) obtained in the 3-plex FCIA for the blank fish extracts with or without cleanup were similar to the B_0 of the assays performed in buffer, with the exception of the PCB assay which showed an increase of 2500 MFIs without cleanup that was decreased after the cleanup. In the presence of PAH contaminants in the fish extracts, the decrease of the response was high (80±3%), and even for the low contaminated samples (Table 3), which indicates the presence of cross-reacting PAHs especially in the case of no extra cleanup. An average similar decrease was measured after the cleanup, but a high SD for the replicates of the three positive PAHs extracts indicates that the cleanup procedure was not yet that reproducible for PAHs (data not shown). Moderate decrease (45±5%) of the B_0 was measured in the BDEs positive fish extracts after the cleanup (Table 3).

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For the positive fish extracts without cleanup, BDEs could not be detected in the samples. Most likely the residual fat in these extracts did not allow the BDEs to interact with the corresponding antibody. With the PCBs contaminated fish we could measure a slight decrease of $22 \pm 2\%$ of the B_0 , even without the cleanup step.

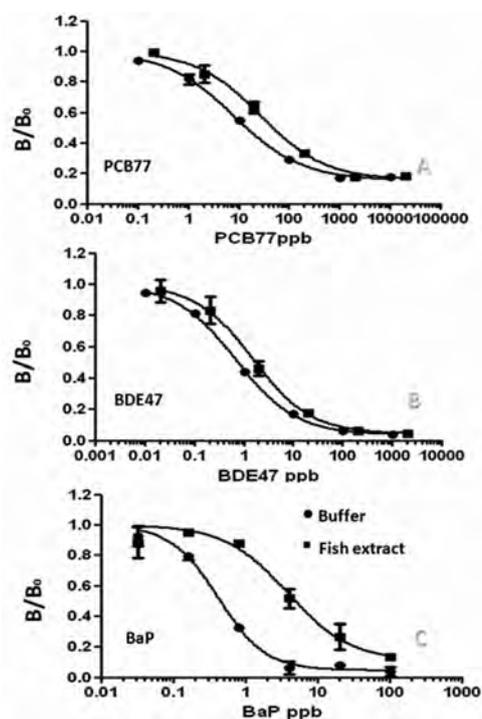


Figure 3: Dose-response curves obtained with the 3-plex FCIA in buffer (●) and fish extract (■) for the three main POPs representatives analyzed in this current study: (A) PCB77 (fish extract (-) cleanup), (B) BDE47 (fish extract (+) cleanup), and (C) BaP (fish extract (-) cleanup). The relative binding (B/B_0) was calculated by dividing the MFI response (B) of each concentration by the MFI obtained in a solution without the analyte (B_0). Solid lines show curves fitted with the four-parameters (4P) model. Each point represents the average of six replicates \pm SD.

However, PCB77 is not the only dioxin-like congener present in food. PCBs were produced in the United States as standard mixtures known as Aroclors. Each Aroclor mixture has a unique dioxin and non-dioxin like PCBs content [44],[45] and they are still abundant in the environment and subsequently in food. In this study, we investigated the possible detection of the Aroclors 1232, 1242 and 1248 in buffer and spiked blank fish (Figure 4). Figure 4A shows 50 to 60% inhibition after the addition of $1000 \mu\text{g L}^{-1}$ of the Aroclors to buffer. This inhibition corresponds to around 20 to $50 \mu\text{g L}^{-1}$ of PCB77 equivalents (Figure

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2). According to Van den Berg et al [43], the Aroclors contain 0.2 to 0.4 % of PCB77, which corresponds to 2 to 4 $\mu\text{g L}^{-1}$ of PCB77 in the Aroclors. This means that other congeners from the Aroclors cross-react with the antiserum. This can partially be explained by the contribution of the known cross-reacting congeners (PCB105, 126, 156 and 169 (Table 1)).

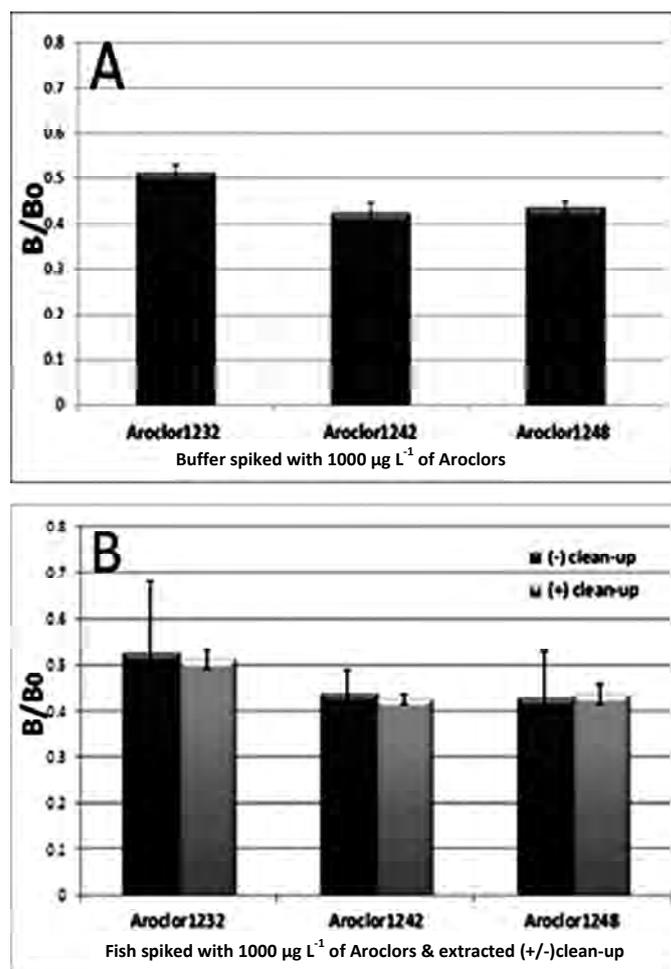


Figure 4: Relative inhibition (B/B_0) of the maximum MFIs (B_0) caused by the addition of 1000 ppb of Aroclor 1232, 1242 and 1248 respectively, to buffer ($\mu\text{g L}^{-1}$) (A) and fish ($\mu\text{g kg}^{-1}$) (B), applying a concentration step of 2.5 (2.5 g of fish mL^{-1} of extract), as measured in the 3-plex FCIA. The different Aroclors-spiked fish samples were extracted with (+) or without (-) cleanup using the simplified extraction procedure described in this paper.

Therefore, other structurally related congeners, such as PCB33 and PCB37 with chlorine positions attached to the 3, 4 position of the benzene ring and present at much higher

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concentrations in the Aroclors [45], may have contributed to this inhibition. The IC_{50} value for PCB77 in our assay is $20 \mu\text{g L}^{-1}$ and the limit of detection (LOD) around $2 \mu\text{g L}^{-1}$ (Figure 2) so we can easily detect $100 \mu\text{g L}^{-1}$ Aroclor in buffer. The total dioxin-like PCB fractions in the Aroclors 1232, 1242 and 1248 are 1, 1.6 and 4.9%, respectively, and the detectable concentrations of the dioxin-like fractions vary from 1 to $5 \mu\text{g L}^{-1}$. With the 3-plex FCIA, we could detect differences in responses between the extracts of the non-spiked blank fish and the spiked fishes with 1000 ppb of the three Aroclors with and without cleanup in which the concentration factor was 2.5 (2.5 g of fish per mL), however, without the cleanup the SDs of repeated analysis of the fish extracts were high. In the case of 100% recovery, the final concentrations of the Aroclors should be 2.5 times higher ($2500 \mu\text{g L}^{-1}$) compared to the buffer. However, for all fish extracts, the inhibited responses are similar (37 to 61%) to those in buffer, indicating losses during the extraction. The addition of $1000 \mu\text{g kg}^{-1}$ of the Aroclors caused significant inhibition. This amount of Aroclor contains 10 to $50 \mu\text{g kg}^{-1}$ dioxin-like PCBs, or 3 to 16 pg TEQ/g (with an average weighted TEF of 0.00032 [44]), which is around the maximum limit for dioxins and dioxin-like PCBs of 8 pg TEQ/g wet weight fish. A previous group [34] also tested the feasibility of an immunoassay to detect Aroclors in buffer but not in a sample material. The 3-plex FCIA seems to be applicable for the simultaneous detection of several POPs in fishes with various fat content and contamination levels.

Conclusions

This work describes the development and performance characteristics of a multiplex FCIA for the screening of POPs in buffer and fatty food material such as fish. For the first time, representatives of three main POP groups -BDEs, PCBs and PAHs- can be detected simultaneously in fish by combining three different immunoassays in one format. Certainly, instrumental analysis offers the identification and quantification of individual POPs, but at high cost and time of the analysis. The 3-plex FCIA can rapidly screen for POPs contamination in food by analyzing about 40 samples in two and half hours (including the sample preparation) and, after further validation, can be a valuable prescreening tool for POPs in fish and other food and environmental samples prior to GC-MS. Furthermore, the developed 3-plex FCIA meets the regulatory requirements of the EU and US food safety authorities for PCBs and PAHs.

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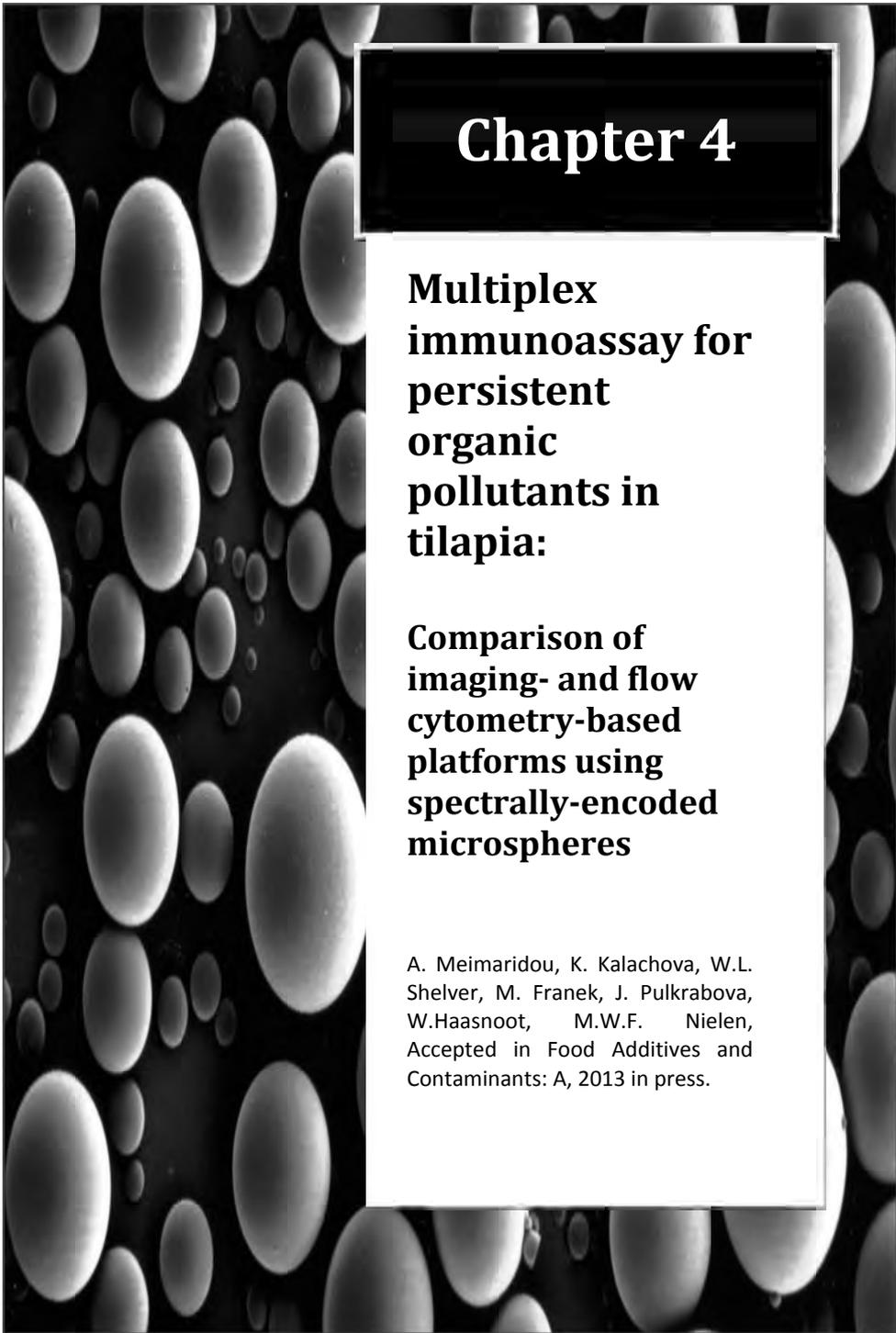
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Chapter 4

Multiplex immunoassay for persistent organic pollutants in tilapia:

Comparison of imaging- and flow cytometry-based platforms using spectrally-encoded microspheres

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Abstract

Recent developments in spectrally encoded microspheres (SEMs)-based technologies provide high multiplexing possibilities. Most SEMs-based assays require a flow cytometer with sophisticated fluidics and optics. A new imaging superparamagnetic SEMs-based alternative platform transports SEMs with considerably less fluid volume into a measuring chamber. There, SEMs are held in a monolayer by a magnet, LEDs instead of lasers are focused on the chamber to illuminate the SEMs, imaged by a CCD detector, thus offering a more compact-sized, transportable and affordable system. The feasibility of utilizing this system to develop a 3-plex SEMs-based imaging immunoassay (IMIA) for the screening of persistent organic pollutants (POPs) was studied. Moreover the performance characteristics of the 3-plex IMIA were critically compared with the conventional 3-plex flow cytometric immunoassay (FCIA). Both SEMs technologies have potential for the multiplex analysis of polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and polycyclic aromatic hydrocarbons (PAHs) in buffer and fish extract with insignificant differences in assay sensitivities. Furthermore, we developed a faster and simpler, modified QuEChERS (quick, easy, cheap, effective, rugged, and safe)-like generic POPs extraction from tilapia fillet using sodium hydrogen carbonate as one of the salt additives and dispersive solid phase extraction (dSPE) as a clean-up. Finally, a preliminary in-house validation using 40 different blank and spiked tilapia fillet samples was performed in both systems and results obtained were critically compared. The lower-cost imaging SEMs-based system performed similar to the original flow cytometer and, in combination with the new quicker QuEChERS-like extraction, it has high potential for future rapid screening of POPs in several other sample matrices such as other fish species, vegetable refined oils and environmental samples.

Introduction

Several food contamination incidents with persistent organic pollutants (POPs) have occurred in the past, emphasizing the demand for a more systematic monitoring of these health-hazardous chemicals in order to exclude them from the consumer's shopping basket [1,2]. Important POPs are polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and polycyclic aromatic hydrocarbons (PAHs). Especially these POPs attract worldwide attention since they are recognized to be persistent, highly toxic, causing a range of adverse health effects and found to bioaccumulate/biomagnify into higher trophic levels in the food chain [3-6]. One of the main routes of human exposure to POPs is via food [7-9]. It has been proven that these lipophilic compounds accumulate in fatty food such as fish [10-12]. The European Food Safety Authority [13] established maximum levels (MLs) for benzo(a)pyrene (BaP) (5 ng g^{-1} smoked fish) and the sum of dioxins and dioxin-like PCBs ($8 \text{ pg of WHO PCDD/F-PCB-TEQ g}^{-1}$ wet weight fish). In the present study, PCB77 was one of the marker compounds and, with a toxic equivalency

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factor (TEF) value of 0.0001 for humans [14], the toxic equivalency quotient (TEQ) is 80 μg PCB77 kg^{-1} fish. For the emerging PBDEs contaminants however, still no regulations are set, despite the strong evidences of posing health risks.

Tilapia is a newly farmed fish species of which its production and consumption are growing rapidly in Europe and in the US [15]. Tilapia's ability to grow well in a variety of environments, its flexible diet, and the increasing global demand for fish make it a practical and economical choice for aquaculture. However, little is known about its POPs contamination profile [10].

The current methods of analysis for POPs in fish involve mainly gas chromatography mass spectrometry (GC-MS) [16-18], but its application is still rather expensive, time-consuming and requires skilled personnel. A rather cheap and simple screening method is the CALUX assay that detects the specific binding of certain POPs to the Aryl hydrocarbon (Ah)-receptor [19] but that assay requires special lab facilities and handling for cell culturing. Several enzyme-linked immunosorbent assays (ELISAs) were developed for certain groups of POPs such as PCBs [20, 21], PAHs [22] and PBDEs [23], but not, until recently ([24], Chapter 3), for the simultaneous detection of different groups. An emerging technology for multiplex analysis in food is the spectrally encoded microspheres (SEMs)-based suspension array in which each encoded microsphere set represents a unique binding assay. The SEMs set-up offers a number of advantages over the existing POPs screening techniques with high multiplexing capacity as the most prominent feature. In previous studies, we utilized SEMs and a flow cytometer to develop either a single immunoassay for the detection of PAHs [25] or a multiplex competitive inhibition immunoassay for the screening of three groups of POPs in fish [24], Chapter 3.

In the present work, we developed for the first time a 3-plex immunoassay for these POPs using an emerging detection platform for a superparamagnetic SEMs suspension array using light emitting diodes (LEDs) and a charge-coupled device (CCD) imager instead of lasers and photo multiplying tubes (PMTs), which offers a more robust, compact sized, transportable and affordable system. The main differences between the flow cytometric- and the imaging-based platforms described in this work are illustrated in Figure 1.

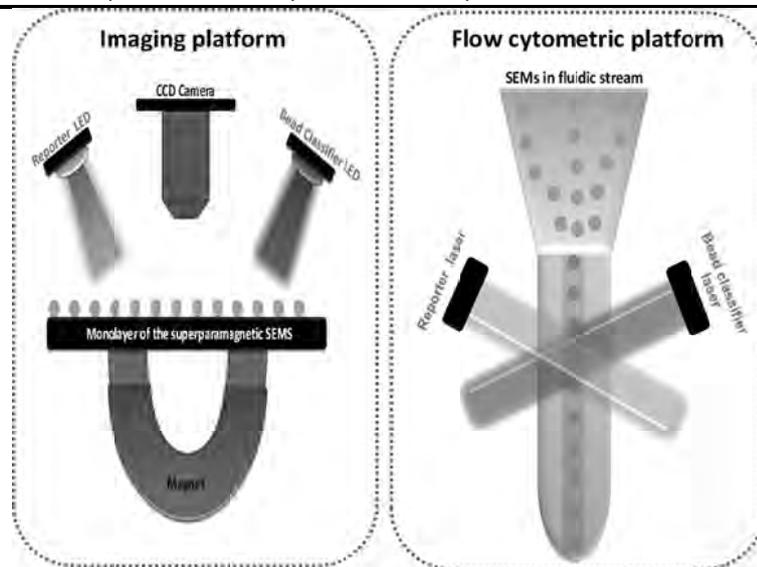


Figure 1: Illustration of the main features of the imaging and the flow cytometric spectrally encoded microspheres (SEMs)-based technologies.

Both technologies require the same assay process prior to multiplex analyte determination by the reader. In the imaging-based set-up, images for the bead classification are taken after excitation by a red LED and for reporter quantification by a green LED and signals are superimposed in order to sort the microspheres making a flow cytometer superfluous.

Apart from transferring the POPs 3-plex flow cytometric immunoassay (FCIA; Chapter 3 [24]) to the new 3-plex imaging immunoassay platform (IMIA), we critically compared the performances of the two platforms for the detection of POPs, both in buffer and in tilapia fish extracts. Moreover, we developed a simpler and faster, modified QuEChERS (quick, easy, cheap, effective, rugged, and safe)-like extraction procedure based on a previously developed protocol [18]. The modified sample preparation differs in the extraction salts composition and the clean-up step (dispersive solid phase extraction procedure (dSPE) instead of silica SPE). Finally, a preliminary in-house validation with 40 tilapia fillets, analysed as blanks and each spiked with a mixture of relevant concentrations of the model compounds BaP, 3,3',4,4'-tetrachlorobiphenyl (PCB77) and 2,2',4,4'-tetrabromodiphenyl ether (PBDE47), or a cocktail of several POPs belonging to the three target groups (PCBs, PBDEs and PAHs), was performed and results obtained with both platforms were compared.

Materials and methods

Reagents & materials

The mouse monoclonal antibody (Mab) against BaP and the BaP conjugated to bovine serum albumin (BaP-BSA) were purchased from the Technical University of Munich (Munich, Germany). The polyclonal antiserum (Pab) against PBDE47 and the PBDE47 conjugated to BSA (PBDE47-BSA) were kindly offered by Dr. Weilin L. Shelver of the USDA (Fargo, USA). The rabbit Pab against PCB77 (PabPCB77-3TG) and the PCB77 conjugated to ovalbumin (PCB77-OVA) were gifts from Dr. Milan Franek of the Veterinary Research Institute (Brno, Czech Republic). The goat anti-mouse and goat anti-rabbit R-phycoerythrin (PE) conjugates were from Prozyme (San Leandro, CO, USA). The standard solutions of POPs were supplied by AccuStandard (New Heaven, CT, USA). Dr. Ehrenstorfer Laboratory (Ausburg, Germany) supplied us with cyclopenta[c,d]pyrene (CCP), chrysene (CHR) and benzo(a)anthracene (BaA). The stock standard solutions were supplied in the water miscible organic solvents dimethyl sulfoxide (DMSO), methanol (MeOH) or acetonitrile (ACN). Protein LoBind Tubes (1.5 mL) were supplied by Eppendorf (Hamburg, Germany) and the LoBind 96-wells microplates were from Greiner Bio-One B.V. (Alphen a/d Rijn, the Netherlands). The N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were provided by Fluka Analytical (Steinheim, Switzerland). Hexane, dichloromethane, magnesium sulphate, sodium chloride and sodium hydrogen carbonate for the QuEChERS-like extraction and sodium phosphate, potassium phosphate and sodium chloride for the preparation of PBS buffer and all other non-specified reagents were from Sigma Aldrich (Zwijndrecht, the Netherlands). All solvents were of analytical grade. The dispersive solid phase extraction (dSPE) tubes were purchased from Agilent (Eindhoven, the Netherlands). We obtained different frozen and fresh tilapia fillets from the local market. The fresh tilapia fillets (*Oreochromis spp* and *Oreochromis mossambica*) were farmed in China and Ecuador, respectively. The frozen tilapia fillets (*Oreochromis niloticus* and *tilapia spp*) were farmed in Indonesia and Malaysia. The fat contents of the tilapia fillets indicated on the packages varied from 2- 3%.

Equipment

The Luminex 100 IS 2.2 flow cytometric system, consisting of a Luminex 100 analyzer and a Luminex XY Platform, programmed to analyze a 96-well plate, was supplied by Applied Cytometry Systems (Dinnington, Sheffield, South Yorkshire, UK). The MagPix[®] imaging analyzer was purchased from Luminex B.V. (Oosterhout, the Netherlands). The superparamagnetic carboxylated microspheres (MagPlex[™] no. 21, 42 and 70) and the sheath fluid were supplied by Luminex Corporation (Austin, TX, USA). The Bio-Plex[™] Pro II automated microplate wash station, with a magnetic carrier, was provided by Bio-Rad

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Laboratories B.V. (Veenendaal, the Netherlands). The DynaMag^{TM-2} magnetic separator was obtained from Invitrogen Dynal (Oslo, Norway). The test tube rotator was from Snijders (Tilburg, the Netherlands). The tissue grinder Waring blender (model 38BL40) was supplied by Waring (Torrington, CT, USA). The rotary vacuum evaporators Buchi Rotavapor R-114 and R-200 with a heating bath were obtained from Buchi Rotavapor (Flawil, Switzerland). The Rotina 35R centrifuge was supplied by Hettich Zentrifugen (Tuttlingen, Germany). For the calibration and dose-response curves of the 3-plex flow cytometric immunoassay (FCIA) and imaging immunoassay (IMIA), a 4 parameter curve fitting was used from GraphPad Prism software GraphPad Software Inc. (La Jolla, CA, USA).

Procedures

POPs-coated superparamagnetic SEMs protocol

The different POPs-protein conjugates (BaP-BSA, PCB77-OVA and BDE47-BSA) were covalently coupled to unique superparamagnetic carboxylated microspheres sets (spectral codes 21, 70 and 42, respectively) according to the procedure described before [24, 25] and the SEMs were compatible with both platforms.

Purification of the PabPCB77

The PabPCB77 antiserum was affinity purified, in order to remove a PBDE47 cross-reacting fraction, by incubating the antiserum (final dilution 1/1000) for one hour with PBDE47-BSA-coated SEMs (final number of used beads was 500,000). After the incubation, the PBDE47 cross-reacting fraction was removed with the help of a magnetic separator. The affinity purified PabPCB77 antiserum was always prepared fresh prior to the analysis.

3-Plex FCIA & IMIA protocols

The initial protocol for the 3-plex FCIA was described previously (see Chapter 3 [24]) in which the applied standard dilution series of each POP were prepared individually in working buffer (5.4 mM sodium phosphate, 1.3 mM potassium phosphate, 150 mM sodium chloride pH 7.4 (PBS) with 2% DMSO and 0.7% BSA) of which 40 μ L were combined with 40 μ L of 50% DMSO in the well of a low-binding 96-wells microplate. In the new optimized protocol for both 3-plex FCIA and IMIA, cocktails of the three model analytes (BaP, PCB77 and PBDE47) were applied in the same final concentrations as in the previous protocol. The rest of the protocol, such as the SEMs numbers, incubation times, antibodies and secondary antibodies dilutions and wash steps remained the same. After the last washing, to remove the excess of the secondary antibodies labelled with PE, the different suspensions in the 96-well plate were measured first in the flow cytometer.

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After that, the recovered microspheres were washed once and resuspended in 100 μL PBS by gently shaking for 5 minutes prior to the final measurement in the MagPix analyser. Dilution series of the cocktail of the three POPs (0.01-1000 ng mL^{-1}) were prepared either in the working buffer or in diluted blank tilapia extracts. As negative controls, we used buffer or dilutions of blank tilapia extract.

QuEChERS-like extraction for POPs

The QuEChERS-like extraction protocol for fish samples followed a previous described procedure [18, 24] which was modified by changing the salt composition (using sodium hydrogen carbonate (NaHCO_3) and magnesium sulfate (MgSO_4) instead of sodium chloride (NaCl) and MgSO_4) during the extraction, and by introducing a dSPE clean-up. Spiked samples were prepared by adding different mixed standard solutions in acetone to homogenized tilapia fillet 30 minutes prior to extraction. One spiking solution contained 2.5 ng BaP , 2 ng PBDE47 and 40 ng PCB77 per g of homogenized tilapia fillet. The second spiking solution was a mixture of 4 PAHs (10 ng g^{-1} fish of BaP, CHR, BaA and CCP), 4 PCBs (40 ng g^{-1} PCB77, 0.04 ng g^{-1} 3,3',4,4',5-pentachlorobiphenyl (PCB126), 0.13 ng g^{-1} 3,3',4,4',5,5'-hexachlorobiphenyl (PCB169) and 133 ng g^{-1} 2,3,3',4,4'-pentachlorobiphenyl (PCB105)) and 2 PBDEs (2 ng g^{-1} PBDE47 and 2,2',4,4',5-penta-bromodiphenyl ether (PBDE99)). For the POPs extraction, 5 g of homogenized tilapia fillet was mixed in a polypropylene tube with 5 mL of distilled water and 5 mL of ethyl acetate and shaken vigorously for 1 min. Subsequently, 4 g of MgSO_4 and 2 g of NaHCO_3 or NaCl were added. After 1 min of shaking, the tube was centrifuged for 8 min (7800 g) and finally an aliquot of 3.5 mL of ethyl acetate layer was removed and evaporated under a gentle flow of nitrogen gas. For the clean-up, the residue was redissolved in 5 mL of acetonitrile and this solution was introduced into the dSPE tube. After 1 min of shaking, the dSPE tubes was centrifuged for 5 min (3400 g) and finally an aliquot of 3 mL from the acetonitrile layer was removed and evaporated under a gentle flow of nitrogen gas. The residue was redissolved in 1 mL of DMSO of which a portion was diluted 1:1 (v/v) with working buffer prior to the analysis.

Results and discussion

General considerations

In contrast to our previous 3-plex FCIA work (Chapter 3, [24]), we investigated the use of calibration curves with cocktails of the three calibration standards instead of individual standards at the same final concentrations (0–1000 ng mL^{-1}). The normalized dose-response curves for PCB77, BaP and PBDE47 obtained in both 3-plex FCIA experimental set-ups showed no differences in the sensitivities and shapes of the curves with individual standards or cocktails. The use of cocktails resulted in a faster and more efficient assay,

since a lower number of wells are needed for the calibration standards and a higher number of samples can be analyzed per 96-well plate.

3-plex IMIA

In 2010, a superparamagnetic SEMs-based imaging analyzer with planar readout (MagPix) was introduced [26]. This system uses the same SEMs principle as the flow cytometric platform but only operates with superparamagnetic microspheres which are imaged in a flow cell after the magnetic formation of a SEMs monolayer (Figure 1). The imaging SEMs-based system main features are its lower costs, compact size and easy transportability. For the preparation of typical normalized dose-response curves in the 3-plex IMIA and FCIA (Figure 2), known concentrations of the three POPs mixture (final concentrations 0-1000 ng mL⁻¹) were assayed as duplicates in the same plate (intra-assay measurements; n = 2) and in six plates on different days (inter-assay measurements; n = 6).

The sensitivities of both 3-plex formats for all three analytes in buffer are identical with IC₅₀ values (the concentrations of the analytes at fifty percentage inhibition of the maximum responses) in the new developed 3-plex IMIA of 20 ± 2, 1.5 ± 0.5, and 0.5 ± 0.1 ng mL⁻¹ and in the FCIA of 20 ± 1, 2 ± 0.5, and 0.4 ± 0.1 ng mL⁻¹ for PCB77, PBDE47, and BaP, respectively. Compared to their performances in buffer, the sensitivities of the assays within the 3-plex IMIA were higher in fish extract. These sensitivities were almost similar to those obtained with ELISA using the same antibodies [20, 22], only the sensitivity for PBDE47 was higher in a magnetic particle ELISA [23]. The reason for this difference between the ELISA and 3-plex IMIA is probably the use of a different PBDE47 conjugate that apparently plays an important role on the sensitivity of the immunoassay. The 3-plex IMIA platform results in a more affordable assay in comparison to three individual ELISAs or the 3-plex FCIA.

Modified QuEChERS-like POPs generic extraction

Sample preparation is the most crucial and time-consuming step for the accurate analysis of POPs in foodstuff. For the simultaneous extraction of PCBs, PBDEs and PAHs from food samples, a one-step, simple and fast procedure is highly desirable especially prior to a rapid multiplex screening assay. QuEChERS-like extraction is a procedure that was initially developed for the detection of pesticides in foods and agricultural products [27]. The technique uses simple glassware, a minimal amount of organic solvent and various salt/buffer additives to partition analytes into an organic phase prior to clean-up with dSPE. Recently, a QuEChERS-like approach was presented for the extraction of PAHs [28] and for PAHs, PCBs and PBDEs [18] prior to the analysis with instrumental analytical techniques. In our previous work (Chapter 3, [24]) we demonstrated the applicability of a simplified extraction procedure in combination with the 3-plex FCIA for different extracts of naturally contaminated fishes having different fat content (varying from 2-15%).

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Nevertheless, that clean-up step, including the handmade mini-silica column and several conditioning and eluting steps, was still rather laborious and time-consuming for the demands of our simplified and fast multiplex screening.

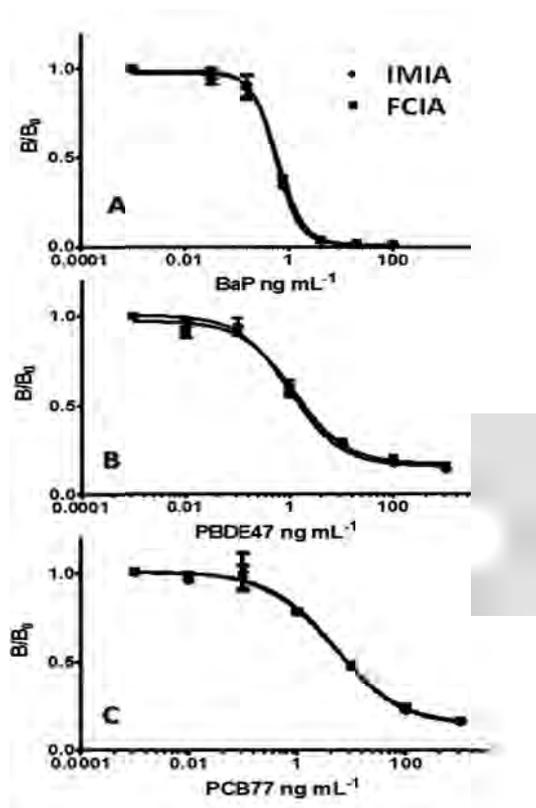


Figure 2: Dose-response curves in the 3-plex IMIA (imaging immunoassay) (●) and FCIA (flow cytometric immunoassay) (■) format in buffer for the three main POP representatives: (A) BaP, (B) PBDE47 and (C) PCB77 using cocktails of standard solutions. Each point represents the average mean of six duplicates \pm SD.

The extraction procedure was improved by the addition of NaHCO_3 , instead of NaCl , in combination with MgSO_4 (Figure 3). NaHCO_3 , when heated or dissolved in water, breaks down to produce carbon dioxide (CO_2) gas. The effervescent effect of the CO_2 produced in situ was acting as dispersant and supported sample homogenization and separation of the water and organic layers. MgSO_4 facilitates solvent partitioning and improves recovery of polar analytes. A base sorbent with primary and secondary amine exchange material (PSA) was used for the dSPE clean-

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up of QuEChERS tilapia extracts to remove any residuals lipids that might act as interferences.

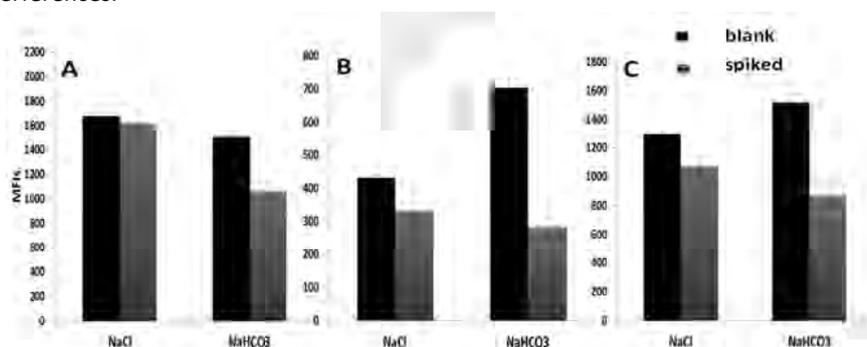


Figure 3: Average responses (MFIs, n=6) obtained in the 3-plex IMIA for (A) BaP, (B) PBDE47 and (C) PCB77 with non-spiked blank fish extracts and with extracts of spiked fishes (2.5, 2 and 40 (ng g⁻¹) BaP, PBDE47 and PCB77, respectively) applying NaCl or NaHCO₃ during the extraction procedure and dSPE clean-up.

A more homogenous mixture of fish, salts and ethyl acetate was obtained during the extraction procedure with NaHCO₃ and one minute shaking. Moreover, after centrifugation, we observed a clearer ethyl acetate aliquot that implied a better clean-up. Both observations can explain that the extraction procedure with NaHCO₃ resulted in more inhibition of the MFIs with the extracts of the spiked tilapia fillets and less matrix effect for all three analytes compared to the extraction with NaCl after the clean-up with dSPE (Figure 3).

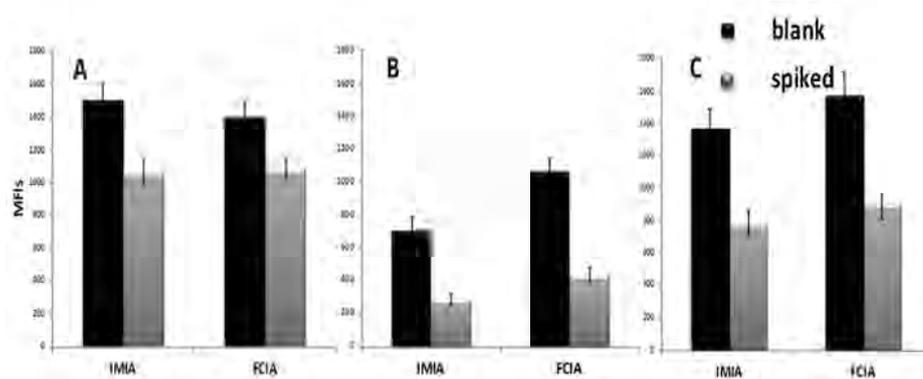


Figure 4: Average responses (MFIs, n=6) obtained in the 3-plex IMIA and FCIA for (A) BaP, (B) PBDE47 and (C) PCB77 with non-spiked blank fish extracts and with extracts of spiked fishes (2.5, 2 and 40 (ng g⁻¹) BaP, PBDE47 and PCB77, respectively), applying NaHCO₃ during the extraction procedure and dSPE clean-up.

The responses (MFIs) obtained with both assays with the new modified extraction step and clean-up for the non-spiked and spiked fishes are shown in Figure 4. The MFIs obtained for PBDE47 and PCB77 with the 3-plex FCIA are slightly higher compared to the 3-plex IMIA, while the other differences are insignificant. Note that in the present study we focused on the analysis of specific POPs (PCBs, PBDEs and PAHs) in lean fish; the application to other more fatty fishes should be demonstrated in future work. An easy, fast, simple and generic POPs extraction from fatty fish is a common challenge for both instrumental and bioanalytical techniques. In the case of fatty fishes, it might be necessary to adjust the extraction procedure by varying the organic solvents and the extraction temperature in order to eliminate the lipids co-extraction. If the clean-up procedure of the dSPE is not effective on the removal of the lipids, then maybe an additional dSPE step or a different dSPE sorbent could be considered.

Applicability of the 3-plex IMIA with tilapia extracts

Dose-response curves of BaP, PCB77 and PBDE47 in blank tilapia fillet extract (2 times diluted in buffer) were measured in the 3-plex IMIA (Figure 5) in order to investigate matrix influences. The tilapia extracts were prepared with the described modified QuEChERS-like extraction. However, in the case of the BaP assay (data not shown) we obtained a significant decrease of the maximum response from 4600 ± 100 MFIs in buffer to 2300 ± 500 in tilapia extract, clearly indicating a matrix influence of the assay. This matrix effect of the BaP assay was also demonstrated in previous works [24,25]. The maximum responses for the PCBs and PBDEs assays were less affected. For the PBDE47 assay, we obtained 1130 ± 60 MFIs in buffer versus 1600 ± 200 MFIs in tilapia extract and for the PCB77 assay 1390 ± 80 MFIs in buffer versus 2000 ± 300 in tilapia extract. Due to this matrix effect, matrix matched calibrators are required for the qualitative or (semi-) quantitative detection of the target POPs in tilapia fillet.

3-plex IMIA and FCIA in-house pre-validation study

The robustness and performance of the 3-plex IMIA and FCIA were tested in combination with the new modified QuEChERS-like extraction procedure. The performance of the 3-plex FCIA for various fish extracts contaminated with different POPs levels was compared previously with the results obtained using capillary GC-MS after a clean-up and they were in good agreement (Chapter 3 and Meimaridou et al. 2011 [24]). Forty different homogenized tilapia fillets (fresh, frozen, from various species, origins and slightly different fat content) were analyzed as blanks and spiked with two different mixtures of POPs standard solutions. The variations of the responses for the 40 different samples as measured with the 3-plex FCIA (results are not shown) were similar to the 3-plex IMIA (Figure 6). The responses obtained with the blank and the spiked tilapia fillets with the

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standard solutions containing only the three targets analytes (2.5, 40 and 2 ng of BaP, PCB77 and PBDE47, respectively per g of tilapia fillet) measured in the 3-plex IMIA are presented in Figure 6 (A-C). Apart from the BaP assay (Figure 6A) the discrimination capabilities between the blank and spiked tilapia fillets were evident in the rest of the assays. We were able to distinguish the blank and spiked tilapia fillets at half of the ML for PCB77 and at a relevant level for PBDE47. However, there is strong evidence that BaP [29], PCB77 [3, 30] and PBDE47 [4, 10, 11, 31-34] are never the only POPs present in food, especially in fish. So, apart from the target compounds, several others contaminants belonging to these PAHs, PCBs and PBDEs groups will be present in contaminated samples.

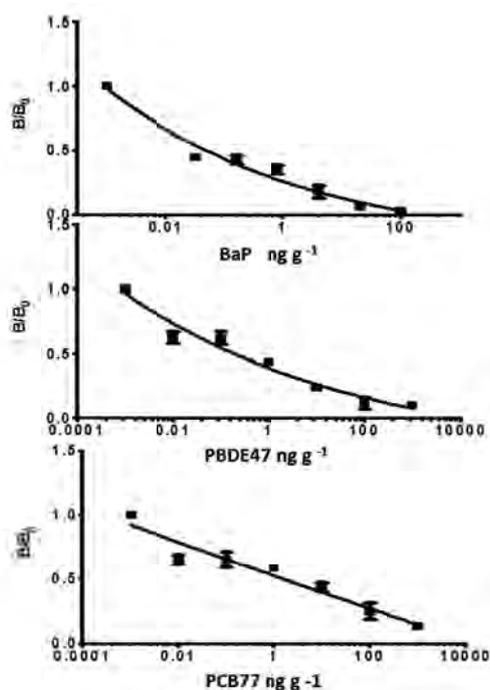


Figure 5: Dose-response curves in tilapia extract (■) obtained in the 3-plex IMIA for the three main POP representatives: (A) BaP, (B) PBDE47 and (C) PCB77. Each point represents the average of six replicates \pm SD.

BaP alone is not an adequate indicator of PAHs presence and toxicity, and the EFSA intends to include other PAHs such as CHR, BaA and five other PAHs. Moreover, apart from that, several other PCBs (PCB126, PCB169, etc.) and other PBDEs (PBDE49, PBDE49, etc.) are found in several food items in practice. For that reason, in a subsequent experiment, we spiked another set of 20 different tilapia fillets with POPs mixtures of

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standard solutions containing four different PAHs (10 ng g⁻¹ tilapia fillet of each BaP, CHR, BaA and CCP); four food-related dioxin-like PCBs (40 ng g⁻¹ of PCB77, 0.04 ng g⁻¹ of PCB126, 0.13 ng g⁻¹ of PCB169 and 133 ng g⁻¹ of PCB105) and two emerging PBDEs (2 ng g⁻¹ of each PBDE47 and PBDE99).

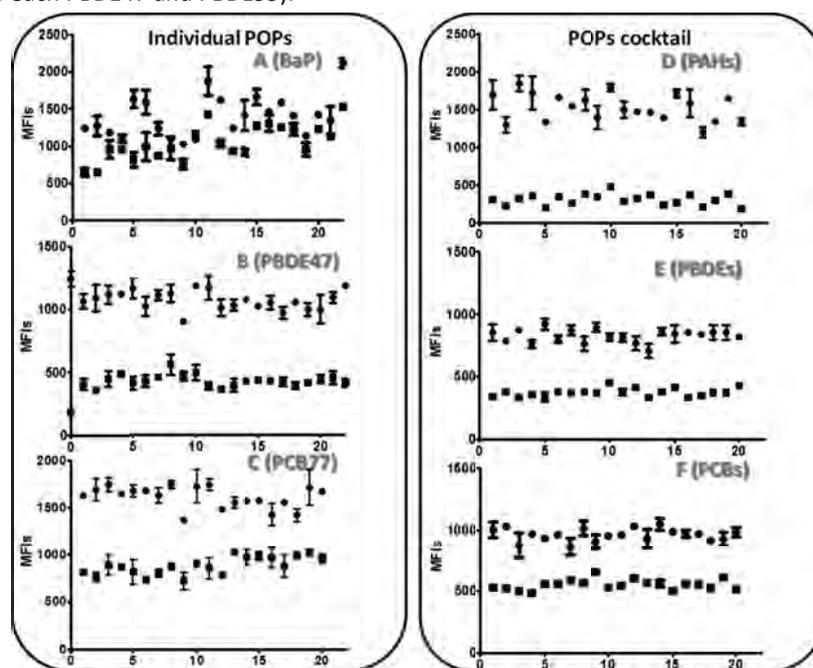


Figure 6: Responses (MFIs) obtained in the 3-plex IMIA with extracts of 2 sets of 20 blank (●) and spiked (■) tilapia fillets for the three main POP representatives. The first set of 20 spiked samples contained BaP (A), PCB77 (B) and PBDE47 (C) in final concentrations of 2.5, 40 and 2 ng per g of fish fillet, respectively. The other set of 20 spiked samples (D,E,F) contained a mixture of 10 ng of BaP, CHR, CCP and BaA (D), 40 ng of PCB77, 105, 126, and 169 (E) and 2 ng PBDE47, 99 (F). The different tilapia fillets (n=40) were extracted with the QuEChERS approach using NaHCO₃.

The POPs spiking concentrations were in most cases selected based on the half values of the existing threshold levels, TEF toxicity values. Moreover the POPs selection was based also on the cross-reactivity data towards a panel of 51 POPs with structural similarities to PCB77, BDE47 and BaP determined in previous work (Chapter 3 [24]). In addition to the previous samples, 20 different blank fillets were extracted and analyzed as above. The variations in MFIs of the blank and spiked samples in the 3plex-IMIA for the three targets POPs are demonstrated in Figure 6 (D, E, F). In this case, for the three individual assays, the separations of the responses of the spiked and blank samples are evident, thanks to the cross-reactivity of the antibodies used towards the other relevant POPs. Some minor

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MFI variations between the different tilapia extracts (spiked only with the target analytes or the mixture of the POPs belonging to PCBs, PBDEs and PAHs) are noticed. The second set of the spiking experiments with more POPs standards was conducted after few months utilizing other tilapia fillets. Unfortunately, in the case of samples spiked with only BaP, it was impossible to discriminate between blank and spiked samples at half of the EU limit set for smoked fish and smoked fishery products but when higher concentration of several PAHs was added the difference between the MFIs of blanks and spiked samples was clear. In previous work [18], the analytes were measured with internal standards to compensate for any analytes losses, something we cannot use for the 3-plex IMIA. Moreover, when higher fat content is present in the fish (> 10%), at least two simultaneous clean-up steps were necessary in order to remove matrix effects. In general PAHs, PCBs and PBDEs are contaminants with similar physicochemical properties but still not identical. POPs high lipophilicity is an extraction bottleneck since lipids present in the sample can be co-extracted which might have a great influence on the sensitivity of both instrumental and bioanalytical assays. Moreover the extraction of several POPs with a generic procedure is not the ideal since there is high risk of low recoveries.

Conclusions

For the first time, a multiplex immunoassay for POPs was developed using superparamagnetic SEMs and a new imaging platform with a planar readout. The performance of the 3-plex IMIA in buffer and tilapia extracts was critically compared to the previously developed 3-plex FCIA (Chapter 3 and [24]) and found to be similar. Moreover, we developed and evaluated the performance of a new quicker and simpler, modified QuEChERS-like simultaneous extraction for all three groups of analytes in both platforms indicating that the combination of NaHCO₃ and dSPE was optimal. A preliminary in-house validation with 40 different tilapia fillet samples, blank and spiked with two mixtures of different POPs standards at relevant levels, was performed in both assay platforms. The outcome of the pre-validation study demonstrated the high potential of both 3-plex immunoassays to screen for POPs in tilapia fillet at half the ML for PCBs and at relevant levels for PAHs and the emerging PBDEs. The 3-plex IMIA has the clear advantage of a lower-cost and easy transportable system, and, after further application and validation in a range of different matrices, can be a useful pre-screening tool for POPs in fish and possibly in other environmental samples, thus eliminating the number of samples to be screened by the laborious and time-consuming GC-MS methods.

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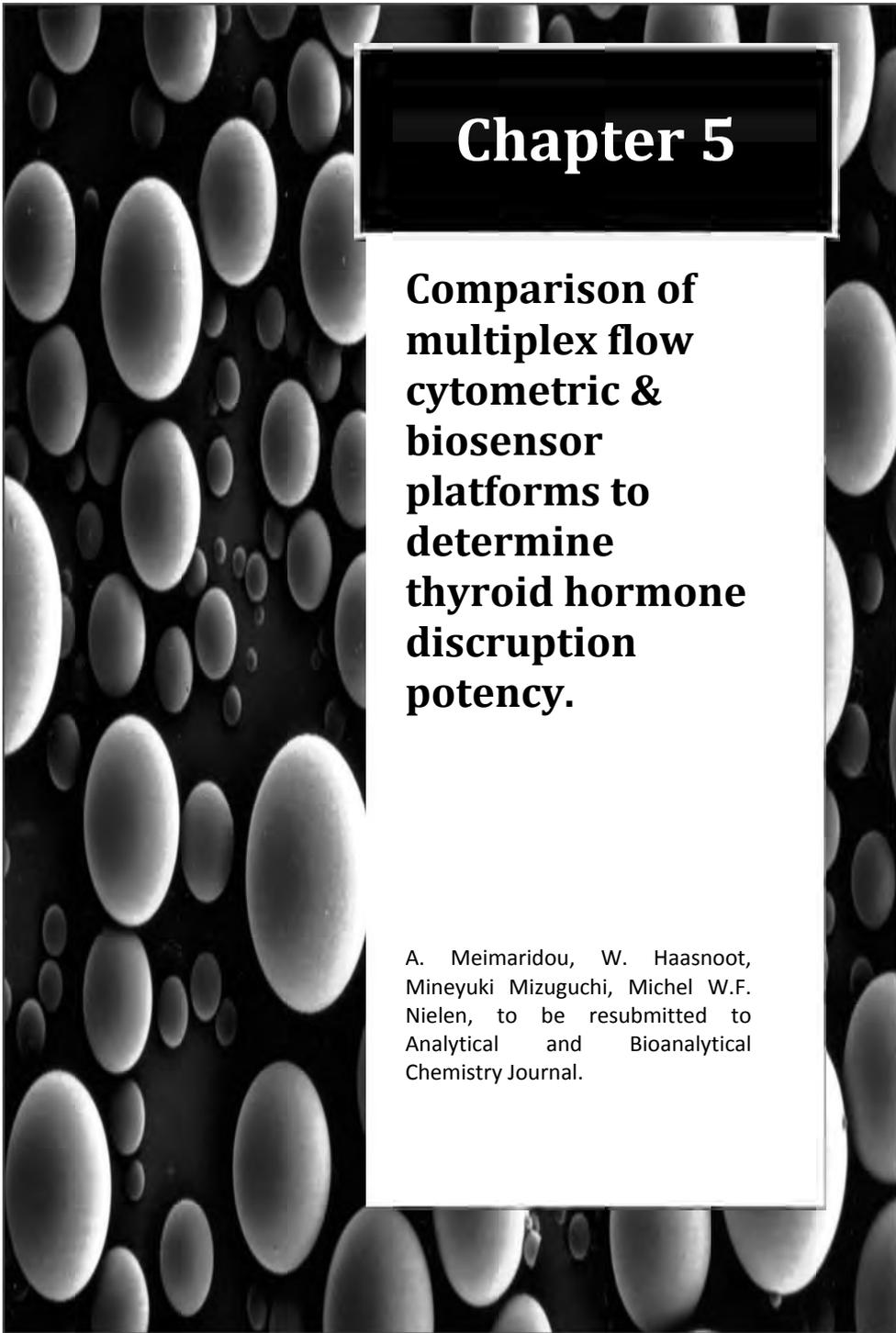
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Chapter 5

Comparison of multiplex flow cytometric & biosensor platforms to determine thyroid hormone disruption potency.

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Mineyuki Mizuguchi, Michel W.F.
Nielen, to be resubmitted to
Analytical and Bioanalytical
Chemistry Journal.

Abstract

Chemical contaminants, such as persistent organic pollutants (POPs), have been associated with numerous toxic effects and lesions in animals and humans. In order to characterize several bioeffect-related properties of chemicals, fast, radiolabel-free, simple and multiplex platforms combined with different biorecognition elements are essential. In this study, a surface plasmon resonance (SPR)-based multichannel biosensor and a spectrally encoded microspheres (SEMs)-based flow cytometer, were critical compared focusing on the screening of thyroid hormone disrupting compounds (THDCs). Several substances with similar molecular structures to thyroxine (T4) can influence T4 transportation by binding to thyroid hormone transport proteins (THTPs) such as transthyretin (TTR) and thyroxine binding globulin (TBG). In both platforms, T4 was covalently coupled to the surfaces of the biosensor chip and SEMs via a linker and the inhibition of the interaction with the THTPs was measured directly in the biosensor, or via antibodies in the case of the flow cytometer. Although the label-free biosensor assay was more sensitive for the natural ligand T4, the thyroid disrupting potency of 4'-OH-PBDE49 was higher in the flow cytometer and in better agreement with data described in the literature. The benefits of the flow cytometer, such as simplified and less expensive instrumentation, disposable SEMs, less system contamination, higher sample throughput and greater multiplexing capability, were evident.

Introduction

Much of the current perceptive of endocrine disruption in wildlife and humans stems from research that has focused on sex hormone disruption in general and oestrogens in particular. However, there is growing evidence that chemicals in our environment, food, and consumer products may affect other functions such as the thyroid gland function in humans [1]. Disturbances in thyroid hormones (TH) function and metabolism can lead to abnormal development, altered growth patterns and a variety of physiological perturbations in mammals as well as in fish, birds and amphibians [2, 3]. Thyroid hormone disrupting chemicals (THDCs) may target any of the multiple pathways in a chemical-dependent manner, including thyroid hormone (TH) production, receptor binding, metabolism, and interaction with thyroid hormone transport proteins (THTPs) such as transthyretin (TTR) and thyroxine-binding globulin (TBG) [1, 4]. TTR is produced in the liver, brain, pancreas, retina and placenta and is responsible for the transportation of L-thyroxine (T4) over the blood-brain barrier and the maternal-fetal transfer. Thyroidogenic activity via interaction with human TTR and TBG has been reported for hydroxylated persistent organic pollutants (POPs), such as hydroxy (OH)-polychlorinated biphenyls (PCBs), and/or OH-polybrominated diphenyl ethers (PBDEs) [5-9]. These congeners, due to their chemical resemblance to the endogenous ligand T4 (Figure 1), have shown competitive displacement of T4 from human TTR and TBG [7, 9-11]. Ample evidences

demonstrated that POPs are environmental contaminants and bioaccumulate through the highest levels of the food chain [3, 12, 13]. POPs are highly liposoluble and minimally degradable. Thus, they accumulate into the body via the food chain, particularly in fish, birds or marine mammals being at the upper level in the food chain [3, 14, 15].

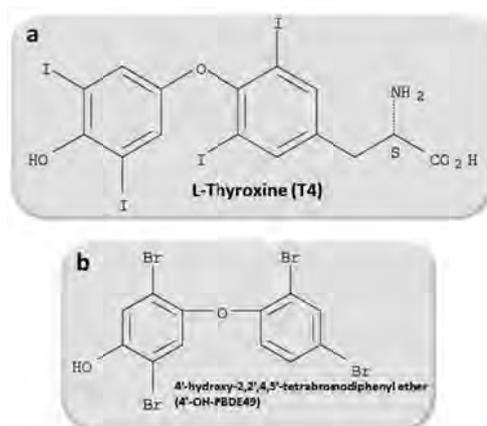


Figure 1: Molecular structures of (a) L-thyroxine (T4) and (b) a T4-like thyroid hormone disrupting compound (THDC) used in this study.

Most of the existing methods used for measuring biointeractions, such as the TTR/TBG binding potency of chemical compounds, are specific, time-consuming, expensive, usually demand large quantities of sample, isotopic labelled internal standards or radioactive tracers [9, 10, 16, 17]. Recently, a surface plasmon resonance (SPR)-based biosensor assay for the determination of chemicals for their thyroid hormone disrupting activity was developed [6, 18]. However, the applied biosensor is expensive and the multiplex capability of that biosensor is limited to three flow channels (FC) plus one reference FC [6]. Moreover, the high risk of system's contamination due to the high hydrophobicity of POPs might result in a less robust assay [6, 18]. An open platform with high multiplexing capability of theoretically up to 500 simultaneous assays, is the multi-analyte profiling (xMAP) technology (Luminex) using spectrally encoded microspheres (SEMs) combined with flow cytometry, which has been described for the analysis of several food contaminants [19-24]. The aim of this work was to investigate the possible transfer of the existing biosensor assay to the new flow cytometric SEMs-based multiplex platform for the determination of chemicals causing disruption in T4 transportation by THDCs. Finally, we critically compared the two in-house developed assays with each other and within literature described screening assays for THDCs.

Materials and methods

Chemicals, materials and instruments

The recombinant transthyretin (rTTR) was kindly offered by the Toyama Medical and Pharmaceutical University (Toyama, Japan). Thyroid binding globulin (TBG) was provided by Scipac (Kent, UK). The EZ-Link Sulfo-NHS-LC biotinylation kit and Sephadex G were provided by Thermo Fisher Scientific (Etten-Leur, the Netherlands). 4'-Hydroxy-2,2',4,5'-tetrabromodiphenyl ether (4'-OH-PBDE49) was purchased from Accu Standard (New Haven, USA). The Alexa Fluor[®] 532 Protein Labeling Kit (A10236) was obtained from Invitrogen (Bleiswijk, the Netherlands). Phycoerythrin (PE) and the Phycolink[®] R-PE Conjugation Kit were obtained from a Prozyme B.V. (Duiven, the Netherlands). The L-thyroxine (T4), 5-aminovaleric acid (AVA), 8-amino octanoic acid (OCT), 12-amino dodecanoic acid (DOD), ovalbumin (OVA), mouse monoclonal antibody against T4 (MabT4) and all other reagents were obtained from Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands). Protein LoBind Tubes (1.5 mL) were supplied by Eppendorf (Hamburg, Germany) and the LoBind 96-wells microplates were from Greiner Bio-One B.V. (Alphen a/d Rijn, the Netherlands). The magnetic separator DynaMag^{TM-2} was obtained from Invitrogen Dynal (Oslo, Norway). The test tube rotator was from Snijders (Tilburg, the Netherlands). Sensor chips (CM5), the amine coupling kit (containing 0.1M N-hydroxysuccinimide (NHS), 0.4M 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC), and 1M ethanolamine hydrochloride (pH 8.5), and the Biacore 3000 SPR system were supplied by GE Healthcare (Uppsala, Sweden). Applied Cytometry Systems (South Yorkshire, UK) delivered the Luminex 100 IS 2.2 system consisting of a Luminex 100 analyzer and a Luminex XY Platform, programmed to analyze a 96-well plate. The MicroPlexTM, MagPlexTM and SeroMapTM SEMs and the sheath fluid were supplied by Luminex Corporation (Austin, TX, USA). The automated microplate wash station BioPlexTM Pro II, with a magnetic carrier, was provided by Bio-Rad Laboratories B.V. (Veenendaal, the Netherlands). The NanoDrop 2000, micro-volume UV-Vis spectrophotometer for nucleic acid and protein quantification was provided by Fisher Scientific, the Netherlands. The calculation of the binding parameters in the SPR-based assay was performed with the BIAevaluation software (GE Healthcare, Sweden). All data was obtained from at least three different experiments performed in duplicate. Data are given as average \pm standard deviation (SD). For the calibration curves, a 4-parameter curve fitting was used from GraphPad Prism software GraphPad Software Inc. (La Jolla, CA, USA).

Procedures

THTPs labeling

Biotin, Alexa Fluor[®] 532 and PE are commonly used labels that can be detected in the applied flow cytometer as such, or using streptavidin- or avidin-PE probes in the case of biotin. Biotinylation of the THTPs was performed with the EZ-Link Sulfo-NHS-LC biotinylation kit according to the manufacturer's protocol. The starting concentration of the THTPs prior to biotinylation was 2 mg mL⁻¹ and the excess of the unbound biotin and the rest of the reagents were removed with a Zeba™ Desalt Spin Column. Alexa Fluor[®] 532 was attached to the THTPs according to the protocol of the protein labeling kit (A10236) and PE via the PhycoLink[®] R-Phycoerythrin (R-PE) conjugation kit. The coupling chemistries used were different in each conjugation procedure: both biotin and Alexa Fluor[®] 532, have a succinimidyl ester moiety that reacts efficiently with the primary amines of THTPs to form stable label-THTPs conjugates. The R-PE was bought already activated with succinimidyl 4-[N-maleimidomethyl]-cyclohexane-1-carboxylate which reacted with the reduced cysteines and other sulfhydryl residues of the THTPs.

For the conjugation of Alexa Fluor[®] 532 to THTPs, the starting concentration was 1 mg mL⁻¹ and the excess of the reagent used for the conjugation was removed with Sephadex G according to the manufacturer protocol. For the THTPs conjugation to PE, the initial concentration of both THTPs was 500 µg mL⁻¹ and the desalting column procedure was applied. The protein yield of the labels was estimated by the NanoDrop 2000 spectrophotometer. All the labeled THTPs were stored at 4 °C in the dark until used.

Covalent coupling of T4 on the surface of a CM5 biosensor chip

Based on the work described by Marchesini et al. (2008) [18], T4 was immobilized on the surface of a CM5 chip using the surface sample preparation device or on the bench. The T4 immobilization was performed via the amino group of the alanyl side chain using different linkers (AVA, OCT and DOD). Briefly, the CM5 sensor chip surface was activated by injecting 140 µL of a mixture of 0.4M EDC and 0.1M NHS (1:1; v/v) at a flow rate of 5 µL min⁻¹. The activation was followed by an 140 µL injection of the different linkers (2 mM of each in 10 mM carbonate buffer pH 9.6) at a flow rate of 5 µL min⁻¹. The immobilized spacer was further activated by a second 140 µL injection of an EDC/NHS mixture as described above. Finally, 140 µL of T4 (2 mM in 10 mM carbonate buffer pH 9.6) was injected at a flow rate of 5 µL min⁻¹. The T4 stock solution (10 mM) was prepared by dissolving T4 in a solution containing DMSO (94%), chloroform (5%) and 1 M NaOH (1%) [24]. When the conjugation was performed on the bench, the incubation time for the activation processes and attachment of the linkers was 20 min and 1 hour for the T4 coupling.

Biosensor-based screening assay for T4 and THDCs.

Standards solutions of the THDCs were measured using the rTTR inhibition assay format as described previously (Marchesini et al., 2008). Briefly, 20 μL of the rTTR solution ($1 \mu\text{g mL}^{-1}$ in phosphate buffer) was mixed in the biosensor with 80 μL of the analyte solution (in 0.1 M phosphate buffer containing 2% of DMSO and 0.5 mg/mL vitamin C, pH 8.5) and immediately after mixing, 50 μL were injected over the sensor chip surface at a flow rate of $25 \mu\text{L min}^{-1}$. The response was measured 5 seconds after the injection ended. The surface was regenerated in one step by the injection of 10 μL of a mixture of NaOH (0.1M) and acetonitril (10 % v/v). The running buffer was phosphate buffer 100 mM, pH 8.5, filtered and degassed. The total run time between samples, including the time for mixing and washing steps was 10 min.

Flow cytometric immunoassay for the evaluation of various T4-coated SEMs.

In order to evaluate the successful immobilization of T4 on the surface of the SEMs via the different linkers, we performed an immunoassay by using a monoclonal antibody against T4 (MabT4).

First, 20 μL of the MabT4 (dilution 1/1000) was incubated for 15 min with 80 μL of standard solution in a well of a low-binding 96-wells microplate. Then, 10 μL of the different T4-coated SEMs suspensions (1000 times diluted) were added to the well providing at least 1000 SEMs per test. The mixture was incubated for 45 min at room temperature in the dark on a plate shaker. After the incubation, three wash steps with PBS, using the washing plate carrier of the automated wash station, removed the excess of the unbound bioreagents. After washing, the microspheres were added to mouse monoclonal antibody conjugated with R-PE, resuspended in 100 μL of PBS and incubated for 20 minutes. Thereafter, one wash step in the wash station was needed prior to the measurement in the flow cytometer performed in 50 sec using 50 μL per well. The schematic presentation of the immunoassay format is shown in Figure 3.

Preparation of the different T4-coated SEMs

Different batches of the three types of SEMs (MicroPlex™, MagPlex™ and SeroMap™) were coated with T4 via several linkers using modifications of the protocols for a two-step carbodiimide coupling of protein to carboxylated microspheres and the T4 coupling on the carboxylated dextrane surface of a CM5 chip (Electronic Supplementary material (SM)). Briefly, 0.2 mL of the microsphere suspension stocks (1.25×10^7 microspheres mL^{-1} in activation buffer) were activated with the addition of 50 mg mL^{-1} sulfo-NHS and EDC in activation buffer (0.1M NaH_2PO_4 , pH 6.2). After 20 min incubation in the dark at room temperature, the activated microspheres were washed by first pelleting with the help of a centrifuge (8000g for 1 min) for the MicroPlex™ and SeroMap™ SEMs or by a magnetic

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separator for MagPlex™ ones and then by adding 250 μL of 10 mM carbonate buffer (pH 9.6). To the activated microspheres, the different linkers (2 mM of aminovaleric acid (AVA), 8-amino octanoic acid (OCT) and 12-amino dodecanoic acid (DOD) (Figure S1 in the SM) in carbonate buffer) were added in final volumes of 500 μL of carbonate buffer. In the case of the SEMs without a linker, a solution of T4 was added following the procedure described below. The microsphere suspensions were vortexed and incubated for 1 hour by rotating them in the test tube rotator at room temperature in the dark. After incubation, the unbound linkers were removed by washing twice with 500 μL of activation buffer and the microspheres were activated again with EDC/NHS as described before. The microspheres with the activated linkers were washed by adding 250 μL of 10 mM carbonate buffer (pH 9.6) which was followed by the addition of 250 μL of 2mM T4, in carbonate buffer. The suspension was incubated for 2 hours under mixing by rotation in the test tube rotator at room temperature in the dark.

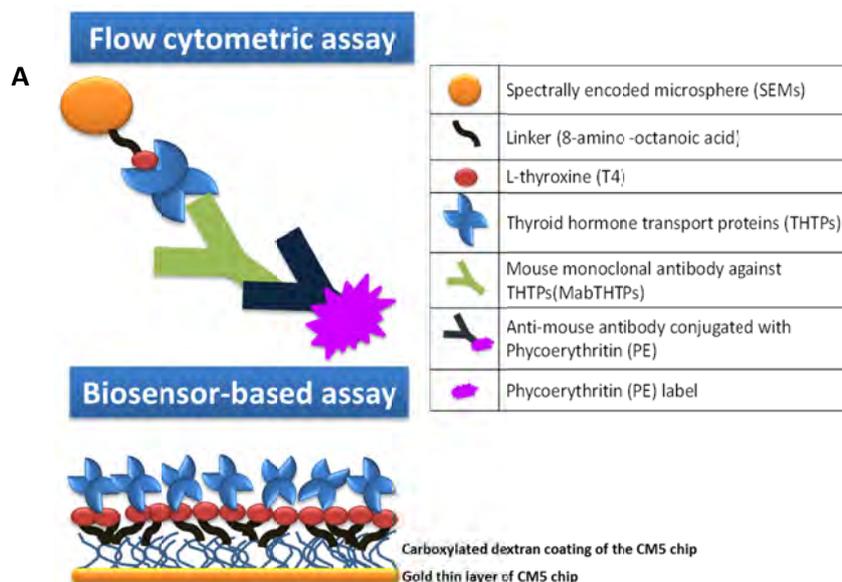


Figure 2: (A) Flow cytometric assay format for the detection of thyroid hormone disrupting compounds (THDCs). SEMs were covalently coupled using octanoic acid (OCT) linker to L-thyroxine (T4) via the amino group of the alanyl side chain to capture the THTPs. The binding was inhibited by the presence of free T4 or THDCs in the sample. Detection and quantification of the complex was obtained via mouse monoclonal antibodies against THTPs, followed by an anti-mouse antibody conjugated to the fluorescent reporter molecule R-phycoerythrin (PE). (B) Surface Plasmon Resonance (SPR) biosensor based assay. On the carboxylated dextran surface of the CM5 chip, T4 is immobilized via OCT linker to capture the THTPs. Also in this format, the binding of THTPs was inhibited by the presence of T4 and THDCs in the sample.

After incubation, the unbound T4 was removed by washing twice with 500 μL of blocking buffer consisting of PBS (5.4 mM sodium phosphate, 1.3 mM potassium phosphate, 150 mM sodium chloride pH 7.4) to which 0.1% BSA, 0.02% Tween-20 and 0.05% NaN_3 were added. The microspheres were suspended in 200 μL of the blocking buffer and stored at 4 $^\circ\text{C}$ until used.

Flow cytometric assay formats

Using labeled THTPs. First, 20 μL of the different labeled THTPs (PE, Alexa and biotin) were pre-incubated in final concentrations of 1, 10 and 100 $\mu\text{g mL}^{-1}$ for 15 min with 80 μL of T4 or OH-PBDE49 (0.01-10000 $\mu\text{g L}^{-1}$) in HBS-EP plus 10% MeOH in a well of a low-binding 96-wells microplate. Then, 10 μL of the different T4-coated SEMs suspensions were added to the well providing at least 1000 microspheres per test. The mixture was incubated for 45 min at room temperature, in the dark on a plate shaker. After the incubation, three wash steps with PBS, using the washing plate carrier of the automated wash station, removed the excess of the unbound bioreagents. After washing, the microspheres plus the PE- or Alexa-labeled THTPs were resuspended in 100 μL of PBS and the measurement in the flow cytometer was performed in 50 sec using 50 μL per well. For the biotinylated THTPs, an additional 30 minutes incubation step in the dark with streptavidin-PE (diluted 1:1000), followed by one wash step in the wash station, was needed prior to the measurement. The formats of the different assays are shown in Figure 2.

Using labeled antibodies. First, 20 μL of the THTPs with different final concentrations (0.1, 1 and 10 $\mu\text{g mL}^{-1}$) was pre-incubated for 15 min with 80 μL of T4/OH-PBDE49 standard solution in a well of a low-binding 96-wells microplate. Then, 10 μL of the different T4-coated SEMs were added to the well providing at least 1000 microspheres per test. The mixture was incubated for 45 min at room temperature in the dark on a plate shaker. After the incubation, three wash steps with PBS, using the washing plate carrier of the automated wash station, removed the excess of the unbound bioreagents. After washing, 100 μL of the two different Mabs against the THTPs (final dilution of 1/10000 (MabTBG) and 1/1000 (MabTTR)) were added to the respective assay and the mixture was incubated for 60 min in the dark, followed by three wash step in the wash station. After washing, 25 μL of anti-mouse PE was added followed by 100 μL of PBS and the mixture was incubated for 30 min in the dark, followed by one wash step in the wash station. The microspheres were resuspended in 100 μL of PBS and the measurement in the flow cytometer was done in 50 sec using 50 μL per well. In Figure 2A the format of the final assay is illustrated. To prepare calibration curves in buffer, dilution series of T4 or OH-PBDE (0.01-10000 $\mu\text{g L}^{-1}$) were prepared in phosphate buffer [100mM NaH_2PO_4 (0.6 g), $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ (1.9 g), NaCl (0.59 g) per liter adjusted to pH 8.5] with 2% DMSO and 0.5 % vitamine C.

Results and discussion

General considerations

Among the critical success factors for robust flow cytometric or biosensor inhibition assays are: (i) the successful immobilization of T4 on the surface of either the biosensor chip or the SEMs, (ii) the optimum solubilisation of the hydrophobic analytes of interest in the aqueous assay buffers and (iii) absence of system contamination and carry-over to the next measurement. Previously, we developed a biosensor-based screening method for chemicals with T4 transport protein disrupting activity [6, 18]. In that work, T4 was immobilized on the carboxylated dextrane of a CM5 biosensor chip, either directly or via a linker. THTPs were injected via a micro fluidic system and the inhibition of the binding to the immobilized T4 on the biosensor chip was detected using the SPR phenomenon (Figure 2B). However, the repeatability of that assay during routine analysis was affected by a combination of several parameters. The biosensor platform suffered from contamination with the hydrophobic T4 and T4-like compounds, which can adsorb to the plastic microfluidics and tubing parts of the biosensor. This also occurred when the immobilization of the T4 on the surface of the CM5 biosensor chip was performed outside the biosensor platform. When the biosensor system was not used for a long period for our T4-THTPs assay, the binding of the THTPs to the T4-coated biosensor chip started with high responses (2000-3000 RU) and reduced responses (150 –1100 RU) were observed in time with the same biosensor chip. Probably, the T4 and/or T4-like compounds still adsorbed in the fluidic system from previous experiments partly retained the newly injected THTPs. Occasionally, a thoroughly and multi-step cleaning procedure of the system was effective, which supported this contamination/carry-over hypothesis. A second explanation of the biosensor-based THTPs assay's inconsistencies could be that the applied AVA linker used for the T4 covalent immobilization on the surface of the CM5 sensor chip was not long enough for the undisturbed binding of the immobilized T4 to the binding sites of the THTPs. Therefore, in this work, T4 was immobilized on four different flow channels of a sensor chip surface in four different ways: either without or via three linkers of different length (AVA, OCT and DOD). The performance of the biosensor-based THTPs assay was optimum with OCT as linker, having the highest relative response units (RU) of 1200 (900, 300 and 150 RU when DOD, AVA and no linker were used, respectively) in combination with rTTR. Also TBG yielded slightly higher values with similar conclusions. Following dose-responses curves, the T4 sensitivity of the biosensor assays was slightly higher when OCT was used as a linker instead of AVA (IC_{50} of 12 ± 1 nM and 18 ± 2 nM, respectively). Unfortunately, our biosensor platform does not allow the cleaning by organic solvents and also the T4-coated chip cannot tolerate harsh regeneration solutions. Both are still major obstacles for a robust biosensor-based THDC screening assay.

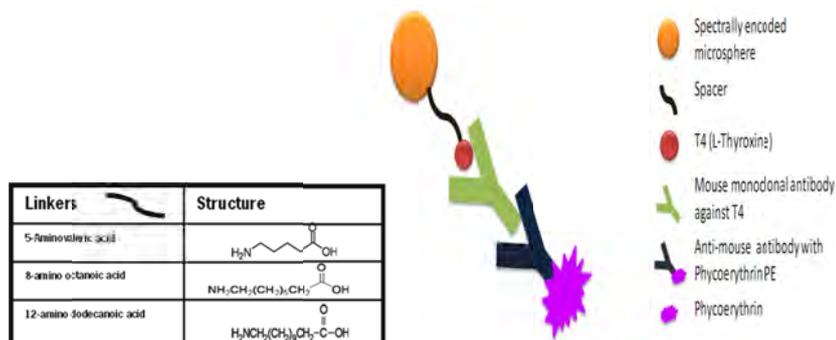


Figure 3: Flow cytometric immunoassay format used for the confirmation of covalent immobilizations of L- thyroxine (T4) on the surface of SEMs via three different linkers.

By using the flow cytometer, we were eliminating the problem of the biosensor system’s contamination with T4 and T4-like compounds since the T4-coated SEMs were prepared and washed outside the flow cytometer and in every analysis a new portion of T4-coated SEMs was used. Based on our previous experiences with the biosensor assay, we covalently coupled T4 to the SEMs via three different linkers (AVA, DOD, and OCT). The successful immobilization of T4 on the surface of the microspheres was confirmed by the binding of a mouse Mab against T4 (MabT4) which was detected by a goat anti-mouse antibody labeled with PE Figure 3. The assay performance was checked by T4 inhibition and the T4 dose-response curves obtained in the flow cytometric-based immunoassay with MabT4 and the different linkers are shown in Figure 4.

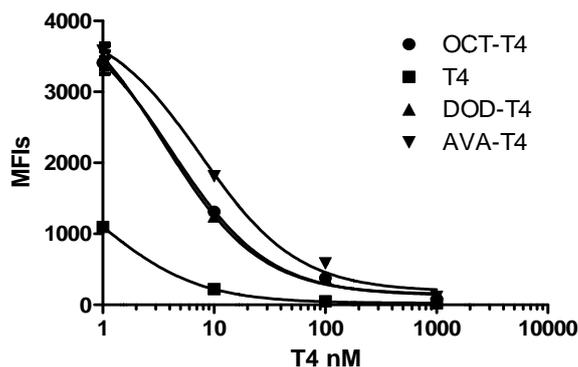


Figure 4: T4 dose-response curves using the format of FigS1. The median fluorescence intensities (MFIs) are the average of three replicates ± SD.

The observed inhibitions in these dose-response curves confirmed that the binding of MabT4 to the T4-coated SEMs was T4-related, that the immobilization of T4 to all microspheres was successful and that the use of linkers resulted in higher responses.

Impact of THTPs labeling

During the development of the flow cytometric THTP-based assay for T4 and THDCs, we labeled rTTR and TBG with biotin and different fluorophores (Alexa Fluor[®] 532 (Alexa) and PE) (Figure 5).

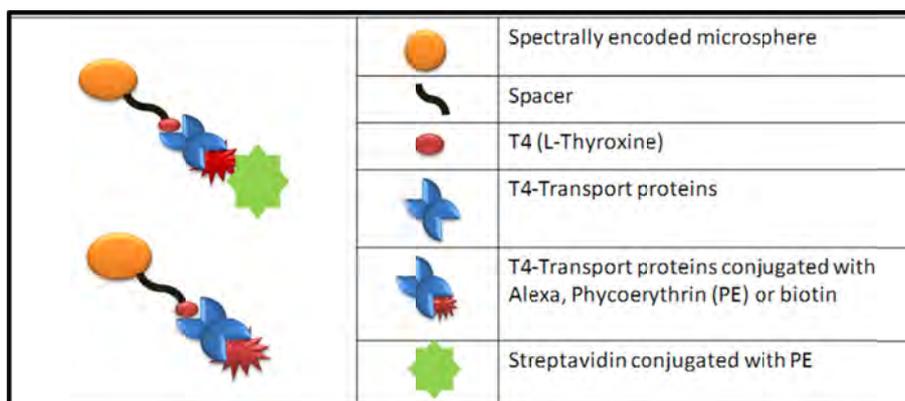


Figure 5: The different inhibition assay formats for the detection of thyroid disrupting compounds (THDCs) were built. The spectrally encoded microspheres were coated with T4 via the amino group of the alanyl side chain using a spacer to capture the thyroid THTPs (rTTR or thyroid-binding globulin (TBG)) labeled with biotin, Alexa or PE. Detection and quantification of the complexes was obtained directly via the different labels. In the case of the biotinylated THTPs conjugate, streptavidin-PE was added for the further detection and quantification.

The binding of the THTPs labeled with Alexa and PE to the various T4-coated SEMs was detected by the green laser of the flow cytometer. For the biotinylated TPs, the binding was detected via streptavidin-PE. In the case of the biotinylated THTPs, we obtained a binding to the T4-coated beads but, unfortunately, that binding was not T4-related, since it could not be inhibited by the presence of a high concentration of free T4 (1000 μ M). When Alexa or PE was attached to THTPs even no binding to the T4-coated microspheres was detected at all. The conjugation chemistries may result in occupying or changing the T4 binding sites for both THTPs.

Final flow cytometric format

From the results presented above and also after literature review [7, 9, 10, 17, 25], we concluded that rTTR or TBG should not be covalently coupled with labels and therefore unmodified THTPs were applied in the final assay format with T4 coupled SEMs and the OCT linker. The T4-coated microspheres indeed captured the unmodified rTTR/TBG and

these bindings could be detected by the mouse monoclonals MabTTR or MabTBG, respectively, followed by a PE-labeled secondary anti-mouse antibody as illustrated in Figure 2A.

Several optimization steps such as THTP and Mabs dilutions were performed. The optimum conditions for the final flow cytometric assay were: $1 \mu\text{g ml}^{-1}$ of rTTR or TBG, MabTTR diluted 1:10000 or MabTBG diluted 1:1000, Mab anti-mouse-PE diluted 1:1000 and SeroMap™ T4-OCT-coated beads. The binding of the Mabs was specific for the THTPs because no binding to the T4-coated beads was observed without the THTPs. For TBG, we could not measure any inhibition of the signal when different concentrations of free T4 were introduced. It seems that TBG has a higher affinity for T4-coated microspheres than free T4.

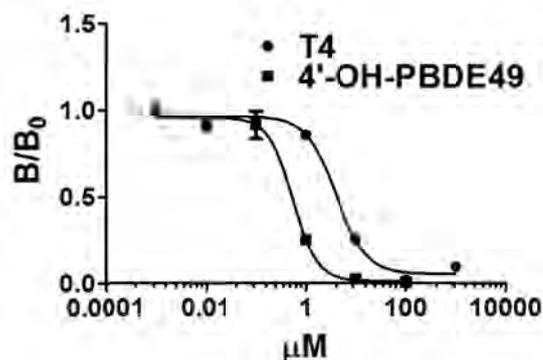


Figure 6: Dose-response curves of L-thyroxine (T4) and the THDC (4'-OH-PBDE49) measured with the final flow cytometric screening assay using rTTR and MabTTR, in combination with T4-coated SeroMap™ beads. B_0 is the maximum MFI of the blank measurement in buffer and B the MFI obtained with different analyte concentrations in buffer. Each point represents the average of three replicates \pm SD.

With other types of microspheres (MicroPlex™ and MagPlex™) we could not detect any inhibition of the binding of rTTR to the T4-beads when high concentrations of free T4 were added. SeroMap™ SEMs are specially formulated to reduce non-specific binding in serum but unfortunately, the exact surface chemistry of the three microspheres types used in this study was not disclosed by the manufacturer. A normalized T4 dose-response curve obtained by the final flow cytometric assay is shown in Figure 6.

The T4 sensitivity (IC_{50}) of the flow cytometric screening assay using rTTR and MabTTR, in combination with T4-coated SeroMap™ beads in buffer is $5.5 \pm 1 \mu\text{M}$ and that is much higher than the IC_{50} of 18 nM previously obtained with the SPR-based assay [18]. The difference can be attributed to dissimilar surfaces, formats and detection systems of the two inhibition assays. In the biosensor-based assay, binding of THTPs to the T4-coated biosensor chip is directly detected using the SPR phenomenon. In the case of the flow cytometer, the binding of the THTPs to the T4-coated SEMs is detected by sequences of

bio-interactions, first the antibody against THTPs that is binding to the bound THTPs to the T4-coated beads and then the anti-mouse antibody conjugated with PE that binds to mouse MabTHTPs. Apart from the natural ligand T4, we also tested a chemical belonging to the group of PBDEs (4'-OH-PBDE49) (Figure 1) known to be a potent THDC. The IC_{50} value obtained with 4'-OH-PBDE49 in the flow cytometer-based assay was $0.7 \pm 0.2 \mu M$ comparable to the one obtained in the biosensor ($0.1 \mu M$) [6]. However, in the biosensor, the relative potency (RP) of 4'-OH-PBDE49, defined as the ratio between the IC_{50} of T4 and the IC_{50} of the compound [IC_{50} (T4) / IC_{50} (THDC)], was much higher compared to the flow cytometric and what it is reported to literature in which 4'-OH-PBDE49 is characterized as stronger binder to TTR than T4 [26, 27].

Comparison with other THTPs-based screening assays

Next to our in-house developed assays, several other in-vitro THTPs inhibition screening assays were described for the determination of the potency of THDCs [7, 16, 17, 25, 28] (Table 1). All of these assays, apart from the biosensor-based one, are using labels including (radioactive) isotopes and different detection platforms such as mass spectrometry (MS) [16] etc. The THTPs were either natural or recombinant TTR. The assay sensitivities for T4 in buffer vary from 18-5500 nM, with the final flow cytometry-based assay having the lowest sensitivity. The MS binding assay uses an ultrahigh-performance-liquid-chromatography electrospray-ionization-triple-quadrupole MS (UPLC-QqQ-MS) as a readout system that results in quite an expensive analysis [16]. The fluorescence displacement method measures the shift in fluorescence peak of the 8-anilino-1-naphthalenesulfonic acid (ANSA) fluorescence probe, after binding to TTR. The ANSA is a promising screening method but not so rugged since the incubation procedure occurs solely at 4°C. Nevertheless, application ability towards a more complicated matrix such as microsomal metabolites of PCB77 and PBDE47 was demonstrated [17]. There are many options available when deciding upon a detection platform for use in THDCs methods and our work demonstrates that flow cytometric SEM-based one, has the highest multiplexing potential for the estimation of the thyroid hormone disruption potency and in the future after further optimization can be combined with other assays detecting other bio-effect related properties of chemicals.

Conclusions

Our ultimate goal was the evaluation of a relatively new platform with high multiplexing potential, and the use of non-radioactive labels, to determine chemicals with thyroid hormone transport protein disrupting potency. In the flow cytometric-based assay, the THDC 4'-OH-PBDE49 exhibited higher thyroid hormone disrupting potency compared to the natural ligand of T4 for which the sensitivity obtained was lower than the ones of the existing methods. Compared to present THDCs screening assays, flow cytometric is proven to be superior mainly due to high multiplexing capacity and sample throughput. In

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addition to that, it is simple, cheap and attributes to less contamination risks. The final flow cytometric assay format, using THTPs as biorecognition elements, turned out to be more complex because direct labeling of the THTPs was impossible. The multiplex potential with other bioreagents for the determination of other chemical's bioeffect related properties still needs to be proven.

Table 1: Characteristics of different THTP (thyroid hormone transport protein)-based assays using different platforms and formats.

*FC-SEMs: Flow cytometric SEMs-based assay; **SPR: surface plasmon resonance (SPR)-based assay; ***ANSA: 8-anilino-1-naphthalenesulfonic acid fluorescence probe; **** LC-MS: Liquid chromatography-mass spectrometry.

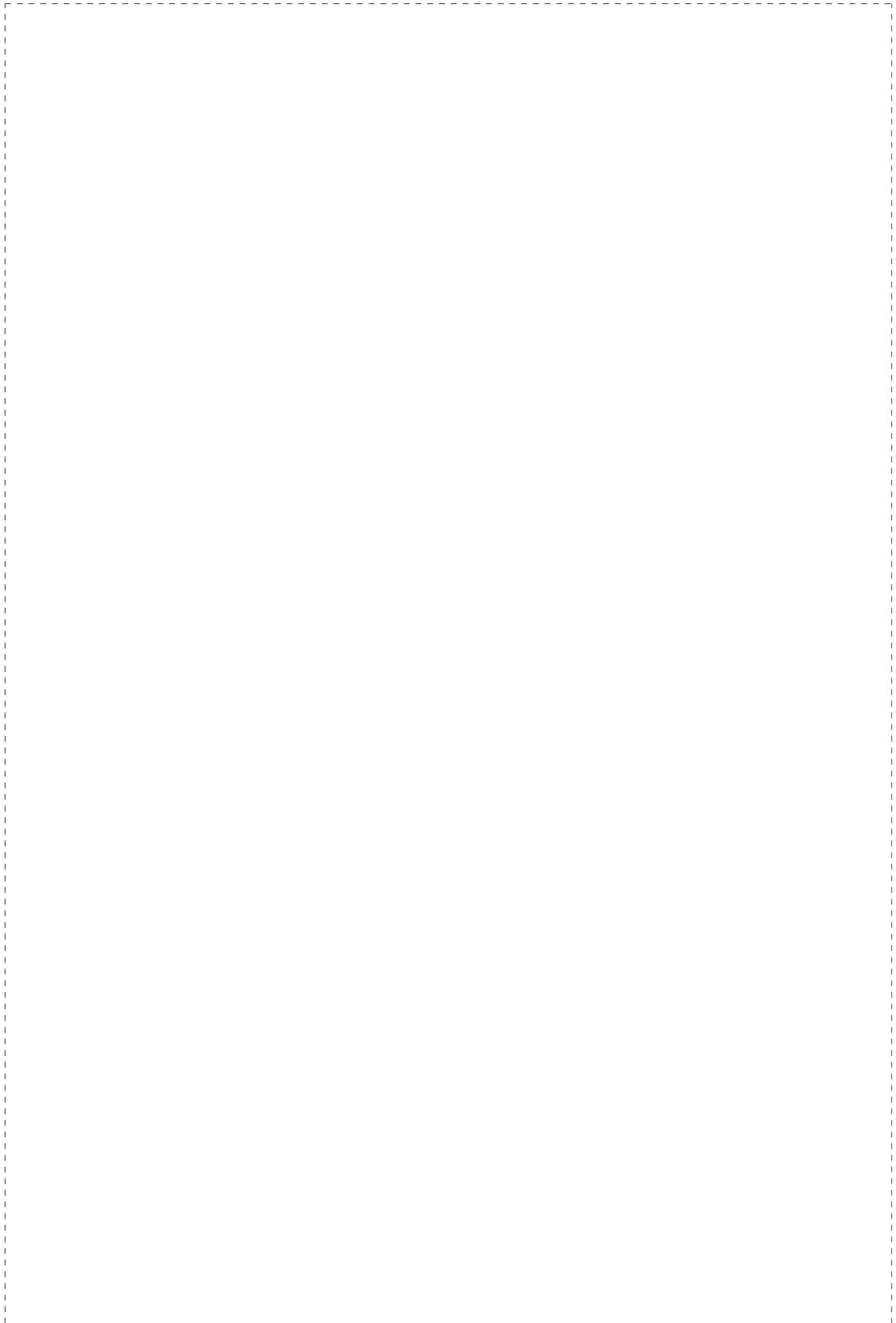
	Different transthyretin (TTR) based assays				
Characteristics	FC-SEMs*	SPR** [5,18]	***ANSA [17]	****LC-MS [16]	Radiolabeled [1,3,5,7,9,11]
Format	Inhibition	Inhibition	Displacement	Displacement	Displacement
Sensitivity for T4	5pM	18nM	265nM	40pM	38nM
THTP type	rTTR	rTTR	pTTR	rTTR	pTTR
Label	Phycoerythrin	No	8-anilino-1-naphthalenesulfonic acid	¹²⁵ C-T4	[¹²⁵ I]T ₄
Analysis time	30secs/sample	8min/sample	Few secs/96well plate	3min/sample	10-60min/sample
Multiplexing potentials	100-500 different assays	Up to 16	No	Yes	No
Simplicity	Simple	Complicated	Work at 4°C	Trained personnel	Trained personnel/radioactivity
Costs (instrument, consumables, maintenance)	Affordable	Expensive	Affordable	Expensive	Expensive
System's contamination	No	High	No	High	Low
Applicability	Buffer	Buffer	Microsomal POPs metabolites	Water	Several applications
System's physical characteristics (size, weight, footprint)	Medium	Medium	Medium	Big	Big

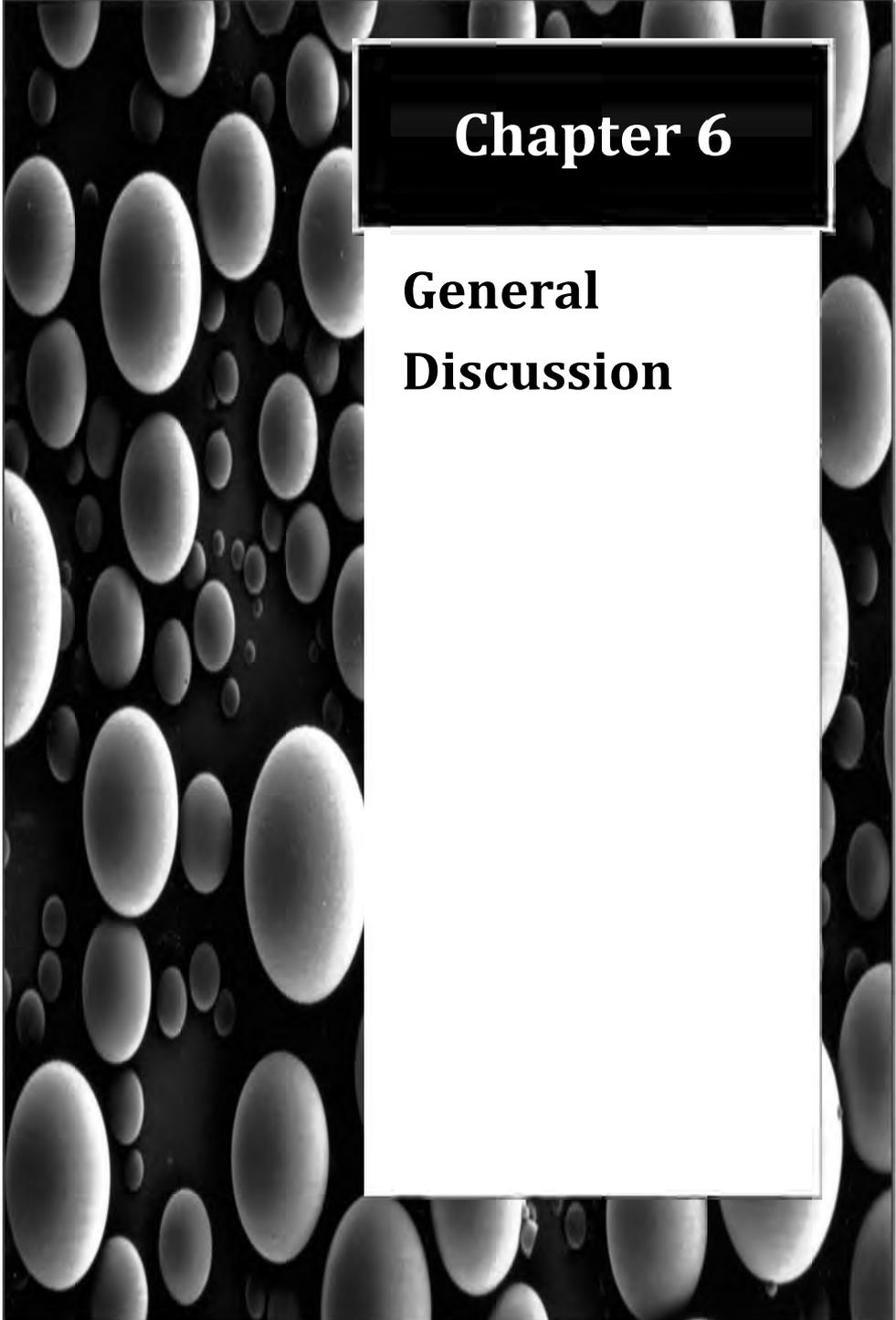
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Chapter 6

General Discussion

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Over the last years, several safety issues in food received wide public attention, including the presence of persistent organic pollutants (POPs). One well-known example of a food safety accident was the Belgian “dioxin” crisis in 1999, which rather than dioxins involved polychlorinated biphenyls (PCBs) [1–3]. This event made it evident that it is still possible for environmental pollutants to be present in our food at health-threatening levels. In addition to that, the incident revealed how easily toxicants can enter the food supply chains, accidentally or by means of illegal practices. This accident led the European authorities to establish more systematic monitoring tools for environmental contaminants, to enforce a maximum action target level strategy, as well as to make the Rapid Alert System for Food and Feed (RASFF) available. All these measurements allow actions to be taken faster and in an organized way in order to reduce the potential human exposure when a contamination incident is reported. Food safety problems have become a universal issue due to the globalization of the production and a more intensive transportation of foods. For instance, new environmental contaminants were discovered in the last 10-15 years such as brominated flame retardants (BFRs), including polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD) [4]. Nevertheless, the chance of another contamination accident in the food chain is still high. Similarly, perfluorinated compounds were discovered as environmental contaminants in the early 2000’s [5,6]. Information on human exposure to these “emerging” contaminants through food consumption was limited. In addition to that, the EU REACH program (Registration, Evaluation, Authorization and restriction of Chemicals) initiated the registration and evaluation (and ultimately authorization) of chemicals in the EU (<http://echa.europa.eu/>). One of the REACH aims is to prevent the emission of chemicals that can do harm to humans, animals and the environment. Under the REACH program, detailed testing programs should be undertaken and completed with satisfactory results before allowing a chemical to be used in products on the European market.

For all these reasons, continuous developments of methods are necessary for (i) identification of unknown chemicals in food, (ii) development of methods that offer inexpensive, simple, fast and high-throughput analysis of food, feed and environmental samples and (iii) to estimate and characterize the human exposure to these chemicals. Development of versatile methods is an ongoing procedure. Mass spectrometry (MS) is still the “golden standard” and nowadays it is routinely applied for identification and quantification of several organic pollutants at low levels (e.g. quadrupole, and ion trap instruments and to a certain degree also time-of-flight instruments) [7–14]. However, MS requires expensive and sophisticated facilities and instruments and well-trained operators. The Chemically-Activated Luciferase eXpression (CALUX) cell assay and other Aryl hydrocarbon receptor (AhR)-related bioassays have been developed and some are used routinely for the screening of bioactive chemicals prior to the confirmation with MS-based methods [15–19]. These bioeffect-based bioassays have the great potential to discover novel unknown contaminants with potentially adverse health effects, however,

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in general, the applied cleanup procedures are laborious, they require special facilities for cell growth, and their performances not always correlate with the legislative maximum limits. Several faster and easier to perform enzyme-linked immunosorbent assays (ELISAs) were developed for each group of organic pollutants [20–27]. However, ELISAs and bioassays lack the high multiplexing possibilities.

Flow cytometry- or imaging-based spectrally encoded microsphere (SEM)-based platforms have many advantages comprising: a) high multiplexing and throughput analysis, b) less sample volume needed, c) efficiency in terms of time and costs. These SEM-based platforms are widely applied to clinical analysis and drug diagnostics but not to food analysis. Within RIKILT- Institute of Food Safety (Wageningen, the Netherlands), a few SEM-based immunoassays for the detection of various contaminants (sulfonamides, coccidiostats, mycotoxins) [28–31] in foods utilizing the flow cytometer system were developed.

The development of a bioaffinity (antibody or receptor/protein binding)-based assay can be divided mainly into four phases: the preliminary investigation, the actual development of the assay, the comparison with existing methods and the final validation. The preliminary investigation includes mainly the inquiry of the analyte's physicochemical properties in terms of solubility and stability, the exploration of suitable bioreagents (antibodies, conjugates, labeled antibodies, transport proteins) and the format of the assay (inhibition or direct assay). Several parameters should be taken into account prior to the choice of the bioreagents according to the assay's desired degree of specificity and sensitivity. Some of those criteria are the nature of the bioreagents, for instance if the antibodies are monoclonal or polyclonal, the purity of the utilized antibodies or binding proteins and the susceptibility of the bioreagents in certain assay's conditions are important. The way that a biochemical reaction is performed should also be defined, by identifying the part of the molecule which is being recognized by the antibody or the binding protein and which is the active part of the binding protein. The latter will help to decide later on the type of conjugation chemistry to be used for the SEMs coating with the specific bioreagents. All this "desk" investigation is extremely important for the smooth development of a bioaffinity assay and to avoid as much as possible potential failures and delays during the development process. During the actual assay development, several parameters need to be optimized in order to achieve the optimal sensitivity of the assay, such as the coating of the SEMs or any other surface, the antibody/transport protein dilution, the labeled antibody dilutions, the incubation time and washing steps, other bioreagents (labels), storing conditions and elimination of any background noise signal. As soon as the monoplex SEM-based assays are ready then it is wise to combine and compare them for the performances in both formats (monoplex and multiplex). In the case that cross-interaction between the assays is observed, it might be necessary to eliminate it by purifying the antibody. The performance of the final developed assay should be compared with existing related assays in buffer and relevant

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matrix. Finally, the assay's robustness, reproducibility and many other parameters should be tested with an in-house validation and small-scale interlaboratory comparison.

Another crucial step in the development of a screening assay is the sample preparation, a key factor of success for most analytical methods. In this thesis, the potential of developing a simple sample preparation for the generic extraction of all target organic pollutants was investigated and tested with the different developed SEM-based immunoassays. Current trends in sample preparation are the reduction of laboratory solvent use and hazardous waste production, saving labor and time, and reduction of the cost per sample, while improving the efficiency of the analyte isolation. Taking these requirements into consideration, QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) methodology [32, 33] for determining pesticide residues in food matrices was an attractive candidate extraction method for the organic pollutants from several food items [9, 34]. Throughout the chapters of this thesis several issues of the assay's development procedure are described. The target of this study was to develop assays for the screening of POPs (polycyclic aromatic hydrocarbons (PAHs), PCBs and PBDEs) in fish using the SEM-based technology and several biorecognition elements (antibodies and thyroid hormone transport proteins). Our ultimate goal was the simultaneous screening of these POPs and their thyroid hormone disrupting hydroxyl (OH)-metabolites in fish and other environmental material.

At first, a SEMs-based flow cytometric immunoassay was developed for the screening of benzo(a)pyrene (BaP) and several other PAHs in naturally contaminated fish samples. The sensitivity of the flow cytometric immunoassay (FCIA) for BaP proved to be comparable to the respective ELISA [22], and 8 times more sensitive than a surface plasmon resonance (SPR)-based biosensor immunoassay developed using the same immunoreagents and assay format. The utilization of the label might be an explanation for this difference, together with the fact that lipophilic compounds such as PAHs, PCBs and PBDEs tend to be adsorbed by the plastic tubing and microfluidics of the SPR instrument. Unfortunately, the SPR-system's chemical resistance is rather low and high concentrations of organic solvents, as we used in the FCIA (up to 25%), cannot be used. The latter was also the reason for the failure of developing PCBs and PBDEs immunoassays using the SPR platform. The developed FCIA was applicable to smoked carp and wheat flour extracts previously analyzed for PAHs by gas chromatography (GC)-MS. This work was the "stepping stone" for the development of a 3-plex FCIA for the simultaneous screening of PAHs, PCBs and PBDEs in several fishes with different fat content by combining the three different immunoassays in one format. For the extraction of the three analytes, a generic QuEChERS-like procedure [9, 34] combined with a solid phase extraction (SPE) cleanup step was used. In another attempt to further simplify this extraction procedure, a modified QuEChERS-like simultaneous extraction for all target POPs from tilapia fillet was applied by using NaHCO₃ as one of the additive salts together with dispersive (d)SPE. However, the generic extraction of three different chemical groups of POPs that have

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some chemical similarities is a cumbersome task, especially when high fat content fish was analyzed. To overcome this problem in the future, it might be useful to adjust the extraction procedure by varying the organic solvents and the extraction temperature in order to eliminate the lipids co-extraction. If the cleanup procedure of the dSPE is not effective in the removal of the lipids present in fish, then an additional dSPE step or different dSPE sorbents could be considered.

The newly developed 3-plex FCIA was successfully transferred to a new, lower-cost and easy transportable imaging platform with a planar readout and using superparamagnetic SEMs. The performance of the 3-plex immunoassays in the two platforms was identical in buffer and tilapia extracts prepared with the modified QuEChERS-like extraction using NaHCO_3 and dSPE as a cleanup step. Moreover, a cell and radiolabelled-free, flow cytometric SEMs-based assay utilizing thyroid hormone transport proteins (THTPs) (transthyretin (rTTR) and thyroxine binding globulin (TBG)) was developed to investigate organic pollutants with thyroid hormone disruption potential. The flow cytometric bead-based assay certainly is superior in terms of multiplexing and sample throughput compared to existing methods [33–38], however, the attachment of the label to the THTPs hampered the development of a highly sensitive assay. The final aim of this assay's development was also its combination with the already 3-plex FCIA for the detection of both parent and hydroxyl-POPs.

In this thesis, the “proof of principle” of a preliminary “prototype kit” for the screening of dioxin-like PCBs, PBDEs and PAHs in fish combined with two different simplified generic cleanup procedures, according to the fish fat content, is presented. Toxicants such as the PCBs, PBDEs and PAHs and POPs mixtures (such as the technical mixtures of PCBs (Aroclors)) can be detected at relative low levels (with IC_{50} values 55 ± 15 , 2 ± 0.4 and 4 ± 0.5 ppb for the PCB77, PBDE47 and BaP, respectively) in fish samples in a multiplex, simple and inexpensive manner compared to existing techniques. The 3-plex immunoassay can be performed in two different detection platforms: the traditional flow cytometer- and the new imaging-based bead analyzers. The latter system offers a cheaper analysis and it is an easier transportable platform than the flow cytometer.

Further work should also be dedicated to the development of cell-based biosensors. Gavlasova et al. [35] developed a whole cell optical biosensor for semi-quantitative detection of PCBs with three chlorine substitutions in soil extracts. In this work they used *Pseudomonas* sp. P2 as biorecognition element based on optical detection. *Pseudomonas* sp. P2 can oxidize PCB molecules, which results in the production of yellow meta ring-fission metabolites that can be measured through the absorption spectra by an optical transducer. However, limits of detection of these whole cell-based biosensors are above those required for feed and food samples and therefore they need further development.

Biorecognition elements that are used in bioanalysis may denature when exposed to chemical reagents that are used during extraction and cleanup procedures, or lose their bioactivity during the attachment to beads or labels, resulting in several cases in low

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assay sensitivity. A solution to this limitation might be the synthesis and design of sensing elements as those based on polymer networks to mimic the natural bioreceptors: either these are antibodies, enzymes, binding proteins or receptors. These biosensing elements can be used for the development of several bioanalytical screening assays using various platforms. A competitive assay to detect dioxins using peptides synthesized on beads was developed by Inuyama et al. [36]. The fluorescence intensity on the bead decreases with increasing dioxin concentration. The concentration of dioxin was determined by measuring the fluorescence intensity using an automatic analyzer of a fluorescence microscope equipped with a CCD camera. However, this still required extraction and cleanup procedures prior to evaluation [36]. Another group, Mascini et al., synthesized oligopeptides to mimic the AhR binding sites and used a biomimetic approach combined with a quartz crystal microbalance piezoelectric transducer to determine dioxin and PCB contamination [37].

Recently, the successful use of single wall carbon nanotube as a detection element for determining non-dioxin-like PCBs has been also reported [38]. In this study, they used an electrochemical impedance sensor for determination of 3,3',4,4'-tetrachlorobiphenyl (PCB77), based on a single-walled carbon nanotube/pyrenecyclodextrin hybrid. With these approaches PCB trace detection has been reported to be successfully established. However, so far, no physical sensor instrument for detection of POPs in food and feed has been commercially developed.

Lately, the group of Aydogan Ozcan has built a prototype cell phone camera sensor that can detect a slide's contents at a cellular level-reading, for example, an increase in white blood cell count that might indicate a new infection or injury. That information can then be forwarded wirelessly to a lab or hospital. The advantage of this devices is that magnification is done electronically, requiring no lens. LEDs are simply added to the phone, and those diodes direct light over the sample, which is analyzed in front of the camera sensor. The resulting hologram is recorded by the camera as a collection of pixels, and can be reconstructed through specialized software into highly detailed images. The applications for this kind of affordable and mobile device abound. Screening for malaria is a big one, or monitoring someone's white blood cell count throughout chemotherapy or applied to monitor several urgent food safety issues [39–42].

The development of assays for the screening of chemical and microbiological hazards in food is an ongoing procedure. Several issues need to be investigated such as all the other potential dangerous molecules present in our food but that we do not search for and/or potential synergic effects of mixture of untargeted toxicants. Apart from the constant discussions about alternative screening tools for chemical contaminant monitoring an idea might be to focus on the development of analytical methods that will increase the target compounds list to more “exotic” (un)suspected persistent chemicals which might threaten the public health. A high level of food safety requires a more proactive and comprehensive approach.

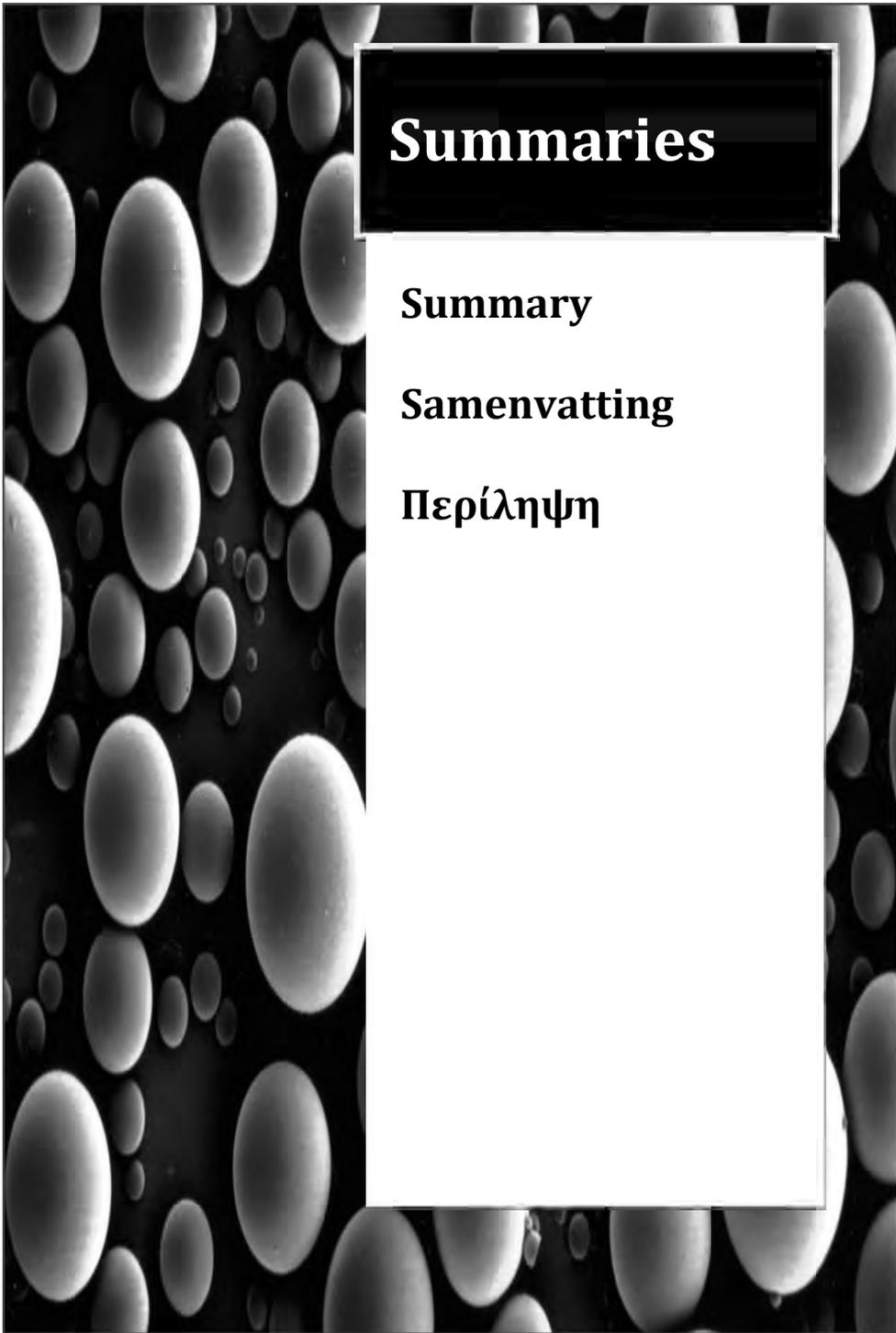
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Summaries

Summary

Samenvatting

Περίληψη

Summary

Within the scope of this research, various bioanalytical platforms with multiplex capabilities, sample preparations and biorecognition elements for the combined detection of three groups of persistent organic pollutants (POPs) in fish and for their endocrine disruption potential to thyroid hormone transport proteins were explored.

For the development of the bioanalytical (monoplex or multiplex) screening assays, the relevant target analytes, the available biorecognition elements (antibodies and transport proteins), other reagents (such as POPs-protein conjugates), suitable buffers and, last but not least, different multiplex detection platforms were selected. For the identification of the thyroid hormone disrupting compounds (THDCs), thyroid hormone transport proteins (THTPs), the recombinant transthyretin (rTTR) and thyroxine binding globulin (TBG), were used. For the detection of POPs, various monoclonal (Mab) or polyclonal (Pab) antibodies raised against the three target analytes were selected. Some background information about all these mentioned elements, which are essential ingredients for the development of multiplex bioanalytical screening assays, together with a short overview of the various existing sample preparation procedures and instrumental/bioanalytical methods used for the specific POPs analysis are given in the general introduction (**Chapter 1**).

The use of the spectrally encoded microsphere (SEM)-based flow cytometric platform was explored during the development of a monoplex immunoassay for the analysis of several polycyclic aromatic hydrocarbons (PAHs) in fish extracts (**Chapter 2**). A Mab raised against benzo(a)pyrene (BaP) was used in this flow cytometric immunoassay (FCIA) but, next to BaP, it detected other PAHs as well which are also assigned as suitable indicators of PAHs contamination in food by the European Food Safety Authority (EFSA). PAHs were detected in divers fish extracts (varying in species, fat content and contamination profile) prepared with the same extended procedure as used for the gas chromatograph mass spectrometry (GC-MS) analysis. The results of the PAHs FCIA proved to be in good agreement with GC-MS analysis when smoked carp and wheat flour extracts contaminated with several PAHs were analyzed.

Following the success of the monoplex FCIA for screening of the lipophilic PAHs, the utilization of the same platform was extended to a triplex for the screening of polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs), next to the PAHs, in fish with various fat content and organic pollutant contamination profiles (**Chapter 3**). Fish extracts were prepared with a simpler procedure based on a modification of the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe)-like extraction approach which was initially developed for pesticides extraction. All assays performed similar in monoplex or triplex format in buffer (with 25% DMSO final concentration), after an elimination step of cross-reacting antibodies from one of the Pabs. The performance of the mono- and multiplex FCIA were critically compared with already developed ELISAs using similar bioreagents and found to be similar. These fish

extracts were also analyzed with GC-MS and the results of both methods were comparable. However, the QuEChERS-like extraction procedure still was rather complicated, especially when fishes with high fat content needed to be extracted.

For that reason, in **Chapter 4**, the use of a dispersive solid phase extraction (dSPE) cleanup was combined with a slightly altered protocol of the QuEChERS-like extraction, by using sodium hydrogen carbonate as one of the salt additives which was optimal for the extraction of the three target contaminants from lean fish. In this study, the triplex FCIA was transferred to a cheaper and more transportable imaging SEM-based platform. A preliminary in-house pre-validation, using 40 different blank and spiked tilapia fillet samples, was performed in the flow cytometer and the imaging SEM-based systems and results obtained were similar.

To explore the performance of the SEMs-based platforms for other biorecognition elements, such as THTPs, a flow cytometric THTP-based assay for the determination of the thyroid hormone disrupting potency was developed (**Chapter 5**) and compared with a previously developed surface plasmon resonance (SPR) biosensor-based assay. The SPR biosensor, apart from the limited multiplexing capacity, has high contamination risks due to the adsorption of hydrophobic compounds to its tubing and microfluidics. The use of the SEM-based flow cytometer eliminated this problem since all bioreactions are performed outside the flow cytometer and for each measurement new SEMs are used. The development of this assay turned out to be challenging due to the different configuration of the assay and the loss of rTTR's thyroxine (T4) binding sites during the conjugation processes to the microspheres or to a fluorochrome and the low sensitivity for the natural ligand (T4) in the final format of the assay.

In conclusion, the necessity of better monitoring of the presence of environmental contaminants in the food chain is emphasized in **Chapter 6** and all the challenges and achievements of the work described in this thesis are summarized, with an eye on future developments in the field of POPs bioanalytical screening in food and environmental samples.

Samenvatting

Binnen het in dit proefschrift beschreven onderzoek werden verschillende bioanalytische systemen met multiplex capaciteit, meerdere biochemicalïen en monstervoorbereidingen onderzocht, met als doel het gecombineerd kunnen detecteren van drie groepen van persistente organische verontreinigingen in vis. Dergelijke systemen werden ook onderzocht voor het bepalen van de potentie van deze verontreinigingen om het transporteiwitsysteem van schildklierhormoon te verstoren.

Voor het ontwikkelen van de bioanalytische (mono- of multiplex) screeningsmethoden werden relevante doelstoffen, beschikbare biochemicalïen (antilichamen en transporteiwitten) en andere reagentia (zoals de eiwitconjugaten van de doelstoffen), geschikte buffers en verschillende detectiesystemen geselecteerd. Voor het onderzoek van de schildklierhormoon verstorende stoffen werden de schildklierhormoontransporteiwitten recombinant transthyretine (TTR) en thyroxine bindend globuline (TBG) gebruikt. Voor het meten van de persistente organische verontreinigingen in vis werden verschillende monoklonale (Mab) en polyklonale (Pab) antilichamen, opgewekt tegen de drie doelstoffen, geselecteerd. Achtergrondinformatie over al deze genoemde elementen, die essentieel is voor de ontwikkeling van multiplex screeningsmethoden, wordt samen met een kort overzicht van de verschillende bestaande monstervoorbewerkingsprocedures en instrumentele/bioanalytische methoden voor de specifieke analyse van persistente organische verontreinigingen, beschreven in de algemene introductie (Hoofdstuk 1).

De toepassing van het flowcytometrische systeem met fluorofoor- gecodeerde microdeeltjes werd onderzocht tijdens de ontwikkeling van een monoplex immunochemische test voor de analyse van verschillende polycyclische aromatische koolwaterstoffen (PAKs) in visextracten (Hoofdstuk 2). Een Mab opgewekt tegen benzo(a)pyreen (BaP) werd gebruikt in deze flowcytometrische immunochemische test (FCIT). Naast BaP, konden ook andere PAKs worden gemeten die door de Europese Autoriteit voor Voedselveiligheid (EFSA) zijn aangewezen als geschikte indicatoren voor PAKs contaminatie in voedsel. PAKs werden gemeten in diverse visextracten (variërend in soort, vetgehalte en contaminatieprofiel) na dezelfde monstervoorbereidingprocedure als gebruikt voor de analyse met gaschromatografie-massaspectrometrie (GC-MS). De resultaten van de FCIT voor PAKs waren in goede overeenstemming met GC-MS resultaten na de analyse van met verschillende PAKs gecontamineerde gerookte karpereextracten en de analyse van tarwebloem.

Als vervolg op het succes van de monoplex FCIT voor het screenen van de lipofiele PAKs werd onderzocht of hetzelfde systeem kon worden uitgebreid naar een driplex voor de screening van polychloor bifenyleen (PCBs) en polybroom difenylethers (PBDEs) in vis met verschillende vetgehalten en profielen van organische verontreinigingen (Hoofdstuk 3). Visextracten werden bereid met een eenvoudigere procedure gebaseerd op een

modificatie van de “QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe)” methode die oorspronkelijk was ontwikkeld voor de extractie van pesticiden.

Alle testen werkten gelijk in het monoplex of driplex systeem in buffer (met 25% DMSO), na een eliminatiestap van de ongewenste kruisreactie met één van de Pabs. De prestaties van de mono- en multiplex FCIT werden vergeleken met eerder ontwikkelde ELISAs gebruik makend van dezelfde biochemicalïën en werden gelijkwaardig bevonden. Echter, de op QuEChERS gebaseerde extractieprocedure was nog steeds relatief bewerkelijk, vooral indien vismonsters met hoge vetgehalten moesten worden geëxtraheerd.

Daarom werd in Hoofdstuk 4 het gebruik van een disperse vaste fase extractie opzuivering gecombineerd met een aangepast QuEChERS protocol, door het toepassen van natriumcarbonaat als één van de zouttoevoegingen, dat optimaal bleek voor de extractie van de drie beoogde groepen van contaminanten uit magere vis. In deze studie werd de driplex FCIT omgezet naar een goedkoper en transporteerbaar op “imaging spectral- gecodeerde microdeeltjes (SEM)” gebaseerd systeem. Een eerste binnenlaboratorium validatie met 40 verschillende blanco en verrijkte tilapiafiletmonsters werd uitgevoerd met de flowcytometer en met het op “imaging SEM” gebaseerde systeem en de resultaten waren overeenkomstig.

Om de mogelijkheden van de op SEM gebaseerde systemen voor andere biochemicalïën te onderzoeken, werd een op flowcytometrie gebaseerde test met transporteiwitten voor schildklierhormonen ontwikkeld (Hoofdstuk 5), voor de bepaling van de schildklierhormoontransport versturende potentie van verontreinigingen, en vergeleken met een eerder ontwikkelde biosensortest gebaseerd op “surface plasmon resonantie” (SPR). De SPR biosensor heeft, naast de gelimiteerde multiplex capaciteit, een hoog risico voor contaminatie ten gevolge van de adsorptie van hydrofobe stoffen aan het microkanaal systeem. Door het gebruik van de op SEMs gebaseerde flowcytometer werd dit probleem geëlimineerd omdat alle biochemische reacties buiten de flowcytometer werden uitgevoerd en omdat voor iedere meting nieuwe SEMs werden gebruikt. De ontwikkeling van deze test bleek een uitdaging vanwege het verschil in uitvoering van de test en het verlies van de bindingsplaatsen van rTTR voor thyroxine (T4) tijdens het conjugeren aan de microdeeltjes of aan de fluorofoor, resulterend in een lage gevoeligheid voor het natuurlijke ligand (T4) in de uiteindelijke test.

In de conclusies (Hoofdstuk 6) wordt de noodzaak van een betere monitoring voor de aanwezigheid van milieucontaminanten in de voedselketen benadrukt en worden alle uitdagingen en successen van het in dit proefschrift beschreven onderzoek samengevat met oog voor toekomstige ontwikkelingen in het veld van de bioanalytische screening van persistente organische verontreinigingen in voedsel- en milieumonsters.

Περίληψη

Στόχος της παρούσας εργασίας ήταν η ανάπτυξη και η εφαρμογή μιας γρήγορης, απλής και φθηνής βιοαναλυτικής μεθόδου ταυτόχρονης ανίχνευσης διαφόρων δύσκολων βιοδιασπόμενων (εμμόνων) οργανικών ρύπων (persistent organic pollutants) στα τρόφιμα και κυρίως στα ψάρια.

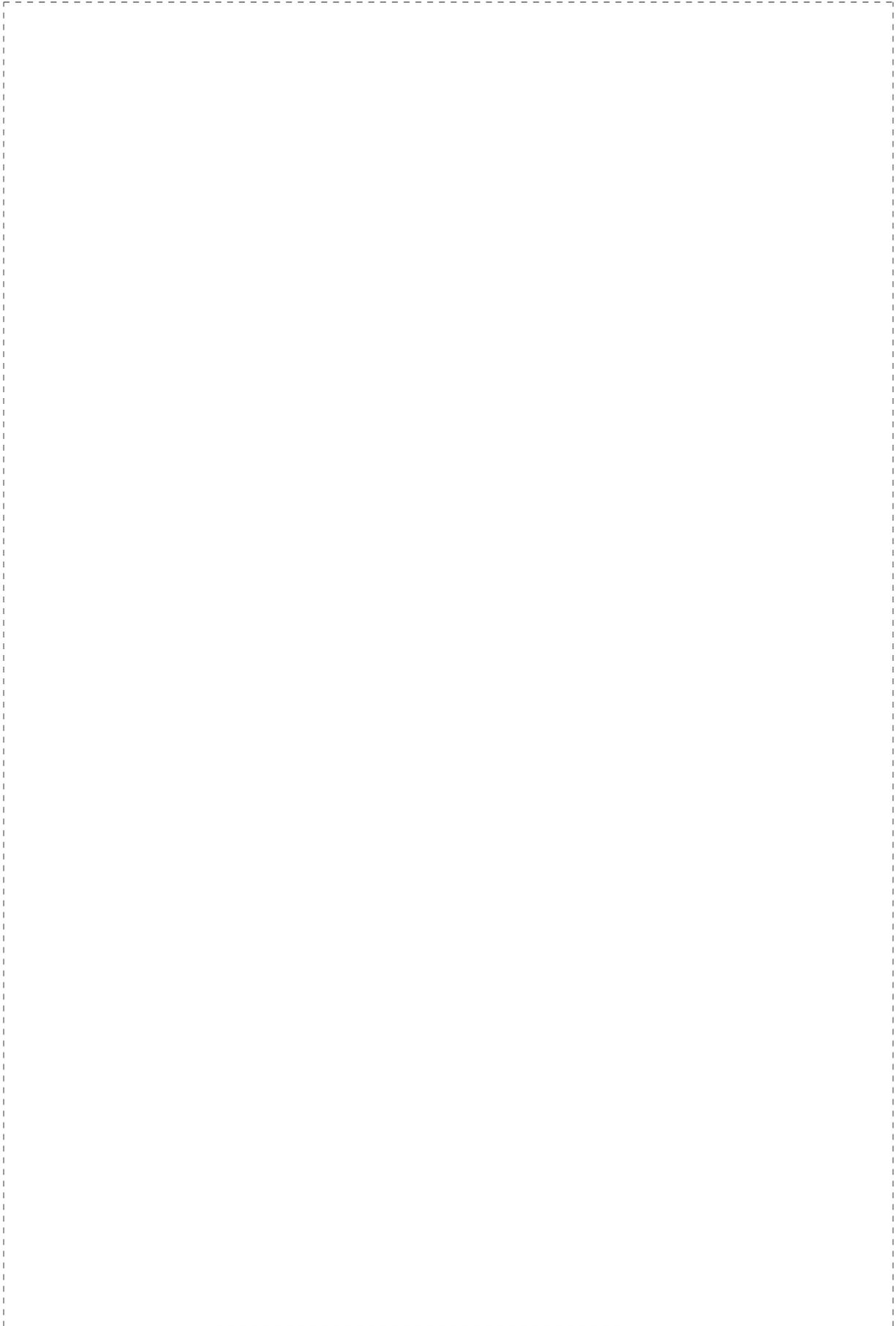
Για το σκοπό αυτό χρησιμοποιήσαμε την τεχνολογία xMAP® του οίκου Lumipex που βασίζεται σε σύγχρονα επιστημονικά ευρήματα και μεθόδους κυτταρομετρίας ροής, μικροσφαιρίδια, λέιζερ, ψηφιακή επεξεργασία σήματος και παραδοσιακή μεθοδολογία χημείας, συνδυασμένα με ένα μοναδικό τρόπο. Βασισμένη σε σχεδιασμό ανοιχτής αρχιτεκτονικής, η τεχνολογία xMAP® μπορεί να τροποποιηθεί ώστε να πραγματοποιεί ευρύ φάσμα βιολογικών εξετάσεων γρήγορα, οικονομικά και με ακρίβεια. Αρχικά, τα μικροσκοπικά σφαιρίδια χρωματίζονται και κατατάσσονται σε 100 διαφορετικές κατηγορίες ή αλλιώς οικογένειες. Κάθε μία οικογένεια μικροσφαιριδίων μπορεί να επιστρωθεί με μία ουσία/αντιδραστήριο που επιτρέπει την ανίχνευση ενός συγκεκριμένου αντίστοιχου παράγοντα σε μια βιολογική εξέταση. Κατόπιν, μέσα στον αναλυτή Lumipex τα ειδικά λέιζερ διεγείρουν και αναγνωρίζουν την χαρακτηριστική εσωτερική χρώση που ταυτοποιεί το κάθε μικροσφαιρίδιο καθώς και την εξωτερική χρώση που έχει "αιχμαλωτιστεί" κατά τη διάρκεια της βιοχημικής διαδικασίας. Η τεχνολογία αυτή χρησιμοποιείται ευρέως στην κλινική ανάλυση αλλά οι εφαρμογές της στην ανάλυση τροφίμων είναι ελάχιστες. Κάποια από τα οφέλη και πλεονεκτήματα της τεχνολογίας xMAP® είναι το μειωμένο κόστος και χρόνος εργασίας, τα γρηγορότερα και επαναλήψιμα αποτελέσματα λόγω της κινητικής υγρής φάσης και ο μικρότερος απαιτούμενος αρχικός όγκος δείγματος.

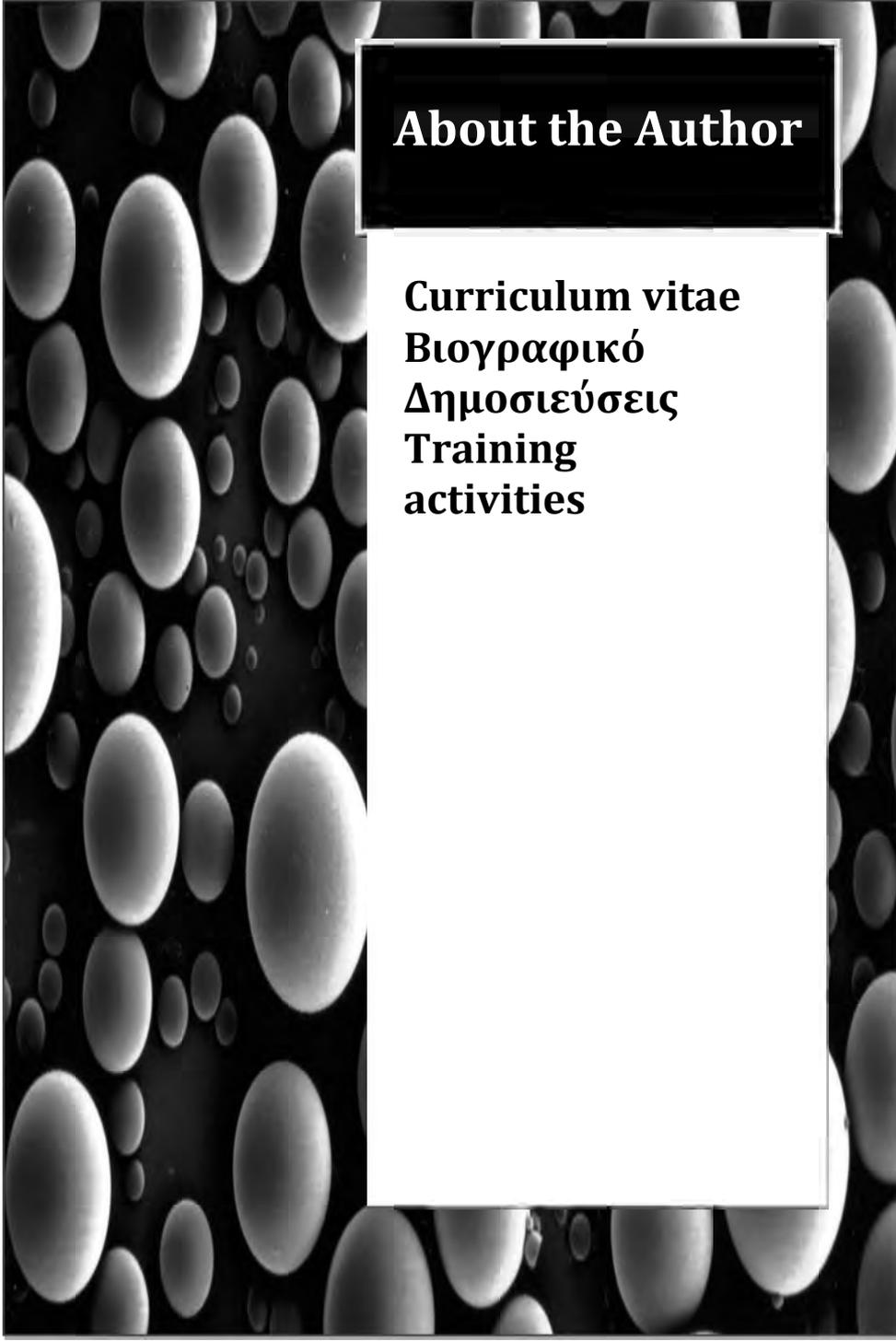
Οι έμμονοι οργανικοί ρύποι έχουν τοξικές ιδιότητες, ανθίστανται στη διάσπαση, βιοσυσσωρεύονται και μεταφέρονται, μέσω του αέρα, νερού και μεταναστευτικών ειδών, διαμέσου διεθνών συνόρων και αποτίθενται μακριά από τον τόπο έκλυσης τους, όπου συσσωρεύονται σε χερσαία και υδάτινα οικοσυστήματα. Υπάρχουν διάφοροι προβληματισμοί σχετικά με την προστασία της υγείας, ιδιαίτερα στις αναπτυσσόμενες χώρες, που προέρχονται από τοπική έκθεση σε έμμοτους οργανικούς ρύπους. Ειδικότερα προβληματισμοί για τις επιπτώσεις στις γυναίκες και, μέσω αυτών, στις μελλοντικές γενιές, για τα Αρκτικά οικοσυστήματα και ιθαγενείς κοινότητες που διατρέχουν ιδιαίτερο κίνδυνο λόγω της βιομεθέθυνσης έμμοτων οργανικών ρύπων και για τη μόλυνση των παραδοσιακών τροφών τους που είναι πια θέμα δημόσιας υγείας. Έχοντας επίγνωση την ανάγκη παγκόσμιας δράσης για τους έμμοτους οργανικούς ρύπους, στις 7 Φεβρουαρίου 1997 το Συμβούλιο Διοίκησης του Προγράμματος «Περιβάλλον» των Ηνωμένων Εθνών έβγαλε την απόφαση 19/13 C περί ανάληψης διεθνούς δράσης για την προστασία της ανθρώπινης υγείας και του περιβάλλοντος με μέτρα που θα μειώνουν ή/και εξαλείφουν την εκπομπή και απελευθέρωση έμμοτων

οργανικών ρύπων. Οι έμμονοι οργανικοί ρύποι αποτελούν μια πολυάριθμη ομάδα χημικών ουσιών. Σε αυτή την εργασία επικεντρωθήκαμε σε τρεις σημαντικές ομάδες στα πολυχλωροδιφαινύλια (PCBs), πολυβρωμοδιφαινυλαιθέρες (PBDEs) και στους πολυκυκλικούς αρωματικούς υδρογονάνθρακες (PAHs). Οι πολυκυκλικοί αρωματικοί υδρογονάνθρακες δεν ανήκουν στην κατηγορία των εμμόνων οργανικών ρύπων γιατί βιοδιασπώνται εύκολα αλλά έχουν παρόμοιες χημικές και τοξικές ιδιότητες με τα PCBs και τα PBDEs και πάντα τα εργαστήρια αναλυτικής χημείας ελέχουν την παρουσία τους στα τρόφιμα και όχι μόνο.

Εντός του πεδίου εφαρμογής της παρούσας έρευνας, πολλοί παράμετροι μελετήθηκαν: όπως διάφορες βιοαναλυτικές πλατφόρμες τεχνολογίας xMAP με δυνατότητες να αναλύουν ταυτόχρονα πολυάριθμες χημικές και μη ουσίες, διαφορετικές διαδικασίες απομόνωσης των χημικών ουσιών από τα τρόφιμα και η χρήση ποικίλων αντισώματων και πρωτεϊνών για τη ταυτόχρονη ανίχνευση των τριών ομάδων των έμμονων οργανικών ρύπων (POPs) σε διάφορα ψάρια.

Εν κατακλείδι, στην παρούσα εργασία, αναπτύξαμε μια μέθοδο για την ταυτόχρονη ανίχνευση πολυχλωροδιφαινυλίων (PCBs), πολυβρωμοδιφαινυλαιθέρων (PBDEs) και των πολυκυκλικών αρωματικών υδρογονάνθρακων (PAHs) στα ψάρια σε συνδυασμό με δύο διαφορετικές απλουστευμένες γενικές διαδικασίες καθαρισμού, ανάλογα με την λιποπεριεκτικότητα των ψαριών. Τεχνικά μίγματα PCBs (Aroclors)) μπορεί να ανιχνευθούν επίσης σε χαμηλά επίπεδα σε δείγματα ψαριών με απλό, εύκολο, γρήγορο και φθηνό τρόπο σε σχέση με τις υπάρχουσες τεχνικές.





About the Author

**Curriculum vitae
Βιογραφικό
Δημοσιεύσεις
Training
activities**

Curriculum Vitae



Anastasia Meimaridou was born in Kavala on the 16th of September 1978 and grew up in Thessaloniki (GR) the sea-shell of Thermaikos Gulf. After high school she studied Food Technology and Science in Alexandrio Highest Technological Institute of Thessaloniki. During her 4-years bachelor studies she obtained a strong background on food engineering and identified all critical control points (CCPs) throughout the processing line. Since she was a child she always wanted to discover the “world” and that was her motivation in 2002 for doing her Bachelor thesis in the Instituut voor Agrotechnologisch Onderzoek (ATO) B.V. in Wageningen. Some of the results of her BSc thesis “Predicting sensory properties of “vla” (custards) by measuring its physicochemical characteristics” (back then she had no clue how delicious vla can be) were used for few publications. That time she firstly discovered Wageningen and life in the Netherlands. On her way back home she thought she will never live again in the Netherlands. Back to Greece she did an internship at the Greek State’s Water Company EYATH S.A., for the Water quality department and she got involved in the sampling and analysis of water. Directly after her studies she worked as a sales manager in the Greek company SIVVAS SA that imports machinery for meat processing small-scale industries and slaughterhouses. She was in contact with the clients to consult them about the design of their processing lines according their needs and the EU regulations and give them technical support during the machinery installation and after sales support. Realizing that there was more knowledge to be gained she participated and succeeded in the Greek State’s Study scholarship (IKY) exams, thus obtaining full funding for her MSc on Food Safety in Wageningen University in 2004. During her masters she got familiar with Rikilt- Food safety institute and worked there from August 2005 till the end of 2012. Her research including also her PhD thesis was on developing simple, cheap and fast screening methods to detect several chemical contaminants (such as dioxin-like polychlorinated biphenyls, polycyclic aromatic hydrocarbons, growth promotor etc) in foods. Her thesis was part of a collaborative EU project named Confidence (<http://www.confidence.eu/>). Whilst working in this project she gained a broad range of scientific experience. She also had the opportunity to participate in scientific conferences and present and substantiate her work all the while meeting people and broadening her scientific network through people working in academic, research field in Europe as well as the USA. It was rather difficult to leave Rikilt after such a long time, but the members of RnAssays BV in Utrecht gave her a warm welcome on the 2nd of January 2013 where she currently works as a head of the R&D department. In this function she is responsible for the development, validation and application of diagnostic tests for safety and quality control in both primary and secondary food production sector.

Βιογραφικό



Η Αναστασία Μείμαριδου γεννήθηκε στην Καβάλα στις 16 Σεπτεμβρίου του 1978 και μεγάλωσε στη Θεσσαλονίκη το κοχύλι του Θερμαϊκού Κόλπου. Μετά το λύκειο σπούδασε Τεχνολογία Τροφίμων στο Αλεξάνδρειο ανώτατο τεχνολογικό Ίδρυμα (ΑΤΕΙ) Θεσσαλονίκης. Κατά τη διάρκεια της τετράχρονης φοίτησης στα ΑΤΕΙ απέκτησε ένα ισχυρό υπόβαθρο στην τεχνολογία των τροφίμων και τον εντοπισμό όλων των κρίσιμων σημείων ελέγχου (CCPs) σε όλη τη γραμμή επεξεργασίας. Από παιδί πάντα ήθελε να ανακαλύψει τον "κόσμο" και έχοντας αυτό σαν κίνητρο το 2002 έκανε την πτυχιακή της στο Instituut Agrotechnologisch Onderzoek (ATO) BV στο Wageningen. Μερικά από τα αποτελέσματα της πτυχιακή εργασία της "Πρόβλεψη των οργανοληπτικών ιδιοτήτων του "νla" (κρέμες) με τη μέτρηση των φυσικοχημικών χαρακτηριστικών του" (τότε δεν είχε ιδέα πόσο νόστιμο μπορεί να είναι το νla) χρησιμοποιήθηκαν για δημοσιεύσεις. Εκείνη την εποχή ανακάλυψε για πρώτη φορά το Wageningen και τη ζωή στην Ολλανδία. Στο ταξίδι πίσω στο σπίτι της, σκέφτηκε ότι ποτέ δεν θα ξαναζούσε στην Ολλανδία. Μετά την επιστροφή στην Ελλάδα έκανε την πρακτική της στην Εταιρεία Ύδρευσης και Αποχέτευσης (ΕΥΑΘ) του Ελληνικού Δημοσίου Α.Ε., για το τμήμα ελέγχου της ποιότητας του νερού. Αμέσως μετά τις σπουδές της εργάστηκε ως υπεύθυνη πωλήσεων στην SIVVAS Α.Ε. που εισάγει μηχανήματα για τις βιομηχανίες επεξεργασίας κρέατος μικρής κλίμακας και τα σφαγεία. Συνειδητοποιώντας ότι υπήρχε περισσότερη γνώση που πρέπει να αποκτηθεί συμμετείχε και πέτυχε στις εξετάσεις του Ιδρύματος Κρατικών Υποτροφιών, αποκτώντας έτσι την πλήρη χρηματοδότηση για τις μεταπτυχιακές σπουδές της στην Ασφάλεια των Τροφίμων στο Πανεπιστήμιο Wageningen το 2004. Τον Αύγουστο του 2005 ξεκίνησε την μεταπτυχιακή της διατριβή στο RIKILT κρατικό Ολλανδικό ινστιτούτο για την ασφάλεια τροφίμων όπου εργάστηκε ως ερευνητρια έως το τέλος του 2012. Η έρευνά της όπως επίσης και η διδακτορική της διατριβή ήταν σχετικά με την ανάπτυξη απλών, φθηνών και γρήγορων μεθόδων ελέγχου για την ανίχνευση διαφόρων χημικών ρυπαντών (όπως διοξίνες, πολυκυκλικοί αρωματικοί υδρογονάνθρακες, ορμονών κ.λπ.) στα τρόφιμα. Η διδακτορική διατριβή της ήταν μέρος ενός μεγάλου Ευρωπαϊκού προγράμματος Confidence (<http://www.confidence.eu/>). Κατά την χρονική αυτή περίοδο είχε την ευκαιρία να συμμετάσχει σε επιστημονικά συνέδρια και να παρουσιάσει και να τεκμηριώσει την εργασία της. Ήταν αρκετά δύσκολο να φύγει από το RIKILT μετά από τόσο μεγάλο χρονικό διάστημα, αλλά τα μέλη της RnAssays BV στην Ουτρέχτη της έδωσαν ένα θερμό καλωσόρισμα στις 2 Ιαν. του 2013, όπου εργάζεται σήμερα ως επικεφαλής του τμήματος έρευνας και ανάπτυξης. Σε αυτή τη λειτουργία είναι υπεύθυνη για την ανάπτυξη, την επικύρωση και την εφαρμογή των διαγνωστικών tests για την ασφάλεια και τον έλεγχο της ποιότητας τόσο στην πρωτογενή όσο και δευτερογενή τομέα παραγωγής τροφίμων.

Lists of Publications/ Δημοσιεύσεις

- Multiplex immunoassay for Persistent Organic Pollutants in Tilapia: Comparison of Imaging- and Flow Cytometric spectrally encoded paramagnetic microspheres in – platforms. Anastasia Meimaridou , Willem Haasnoot, Weilin L. Shelver , Milan Franek , Michel W.F. Nielen to be published in Food additives and contaminants.Part A,2013
- Microsphere-based Bioassay to Determine Thyroid Hormone-Like Activity of Chemical Contaminants. Anastasia Meimaridou, Willem Haasnoot, Michel W.F. Nielen to be resubmitted
- Multiplex Screening of Persistent Organic Pollutants in Fish Using Spectrally Encoded Microspheres Anastasia Meimaridou, Kamila Kalachova,, Michel W F Nielen. Anal Chem 83(22):8696-8702.2011
- Color encoded microbeads-based flow cytometric immunoassay for polycyclic aromatic hydrocarbons in food. Anastasia Meimaridou, Willem Haasnoot, ... Michel W F Nielen. Anal Chim Acta 672(1-2):9-14 2010
- Biosensor discovery of thyroxine transport disrupting chemicals. Gerardo R Marchesini, Anastasia Meimaridou, Albertinka J Murk. Toxicol Appl Pharmacol 232(1):150-60 2008

Overview of completed Training activities



Discipline specific activities

- Cell Toxicology, Postgraduate education in Toxicology, Leiden 2008
- Pathobiology, Postgraduate education in Toxicology, Utrecht, 2008
- NWO courses Netherland organization for scientific research, Luteran 2008-2011
- 9th workshop on (Bio)sensors and bioanalytical microtechniques in environmental and clinical analysis, Montreal University, Montreal, 2009
- Xth International Conference on AgriFood Antibodies (ICAFA), Chester University, Wageningen, 2009
- 4th International Symposium on Recent Advances in Food Analysis (RAFA), Institute of chemical technology Prague/international Association of Environmental analytical Chemistry, Prague (2009&2011)
- Screening Europe, Select Biosciences, Barcelona
- ASSET (Food integrity and traceability Conference), Queen's University Belfast, 2010
- CHAINS 2011, NWO, Netherland organization for scientific research, Utrecht 2011
- Advanced PhD course "CONTaminants in Food and Feed: Inexpensive DETection for Control of Exposure (CONFfIDENCE)", VLAG, Wageningen, 2010

General courses

- Radiation Course, Wageningen University, Wageningen, 2008
- Teaching and supervising students, Wageningen University, Wageningen, 2009
- Time management course, RIKILT, Wageningen 2009
- Career perspectives, Wageningen University Wageningen, 2001
- Scientific writing, CENTA, Wageningen, 2008
- Working with Endnote, Wageningen University Library, Wageningen (2008-2011)
- Young researcher in the EU research activities, Institute of Chemical Technology, Prague, 2011
- Publish & Perish, RIKILT, 2011

Optionals

- EU-Confidence meetings, 2008-2011
- Organic colloquia, 2008-2011
- Internal training in RIKILT, 2008-2011
- Open days EU-Confidence, 2008-2011
- Preparation PhD research proposal, 2008

