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Multiplex immunoassay for persistent organic pollutants in tilapia: comparison of imaging- and flow cytometry-based platforms using spectrally encoded paramagnetic microspheres

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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 super-paramagnetic SEMs-based alternative platform transports SEMs with considerably less fluid volume into a measuring chamber. Once there SEMs are held in a monolayer by a magnet. Light-emitting diodes (LEDs) are focused on the chamber to illuminate the SEMs – instead of lasers and they are imaged by a charge-coupled device (CCD) detector, offering a more compact sized, transportable and affordable system. The feasibility of utilising 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 3-plex IMIA were critically compared with the conventional 3-plex flow cytometric immunoassay (FCIA). Both SEM 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-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 the results obtained were critically compared. The lower-cost imaging SEMs-based system performed similarly 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.

Keywords: POPs; PCBs; PBDEs; PAHs; multiplex immunoassay; super-paramagnetic spectrally encoded microspheres; imaging; flow cytometry

Introduction

Several food contamination incidents with persistent organic pollutants (POPs) have occurred in the past, emphasising the demand for a more systematic monitoring of these chemicals which can be hazardous to human health in order to exclude them from the consumer’s shopping basket (Kelly et al. 2007; Malisch 2008). Important POPs are polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and polycyclic aromatic hydrocarbons (PAHs). Especially these POPs attract worldwide attention since they are recognised to be persistent, highly toxic, cause a range of adverse health effects and found to bioaccumulate/biomagnify into higher trophic levels in the food chain (Bernard et al. 2002; Johnson-Restrepo et al. 2005; Martorell et al. 2010; Plaza-Bolaños et al. 2010). One of the main routes of human exposure to POPs is via food (Busstes et al. 2010; Yu et al. 2010; Fromberg et al. 2011). It has been proven that these lipophilic compounds accumulate in fatty food such as fish (Van Leeuwen et al. 2009; Adu-Kumi et al. 2010; Montory et al. 2011). The European Food Safety Authority (EFSA Scientific Committee 2010) 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 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 factor (TEF) value of 0.0001 for humans (Van den Berg et al. 1998), the toxic equivalency quotient (TEQ) is 80 μg PCB77 kg−1 for fish. For the emerging PBDEs’ contaminants, however, still no regulations are set, despite strong evidence of posing health risks.

Tilapia is a newly farmed fish species of which its production and consumption are growing rapidly in Europe and the United States (Food and Agriculture Organization 2012). 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 (Van Leeuwen et al. 2009).

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Current methods of analysis for POPs in fish involve mainly GC-MS (Cocco et al. 2011; Hollosi & Wenzl 2011; Kalachova et al. 2011) 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 (Hoogenboom 2002), but that assay requires special laboratory facilities and handling for cell culturing. Several enzyme-linked immunosorbent assays (ELISAs) were developed for certain groups of POPs such as PCBs (Fránek et al. 2001; Okuyama et al. 2004), PAHs (Matschulat et al. 2005) and PBDEs (Shelver et al. 2008) but not, until recently (Meimaridou et al. 2011), 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 utilised SEMs and a flow cytometer to develop either a single immunoassay for the detection of PAHs (Meimaridou et al. 2010) or a multiplex competitive inhibition immunoassay for the screening of three groups of POPs in fish (Meimaridou et al. 2011).

In the present work, we developed for the first time a 3-plex immunoassay for these POPs using an emerging detection platform for a super-paramagnetic 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 (Figure 1). The main differences between the flow cytometric- and the imaging-based platforms described in this work are illustrated in Figure 1. Both technologies require the same assay process prior to multiplex analyte determination by the reader. In the imaging-based set-up (Figure 1), 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) (Meimaridou et al. 2011) 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-like extraction procedure based on a previously developed protocol (Kalachova et al. 2011). The modified

![Figure 1. Main features of the imaging and the flow cytometric spectrally encoded microspheres (SEMs)-based technologies.](image-url)
sample preparation differs in the composition of the extraction salts and the clean-up step (dispersive solid-phase extraction (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′-tetabromodiphenyl ether (PBDE47), or a cocktail of several POPs belonging to the three target groups (PCBs, PBDEs and PAHs), was performed and the results obtained with both platforms were compared.

Materials and methods

Reagents and 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. Sheler of the USDA (Fargo, ND, 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-phycocerythrin (PE) conjugates were from Prozyme (San Leandro, CO, USA).

The standard solutions of POPs were supplied by AccuStandard (New Haven, CT, USA). Dr. Ehrenstorfer Laboratory (Ausburg, Germany) supplied the 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, Netherlands). The N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were provided by Fluka Analytical (Steilam, Switzerland). Hexane, dichloromethane, magnesium sulfate, sodium chloride and sodium hydrogen carbonate for the preparation of PBS buffer and all other non-specified reagents were from Sigma Aldrich (Zwijndrecht, Netherlands). All solvents were of analytical grade. The dSPE tubes were purchased from Agilent (Eindhoven, Netherlands).

We obtained different frozen and fresh tilapia fillets from the local market. The fresh tilapia fillets (Oreochromis spp. and O. mossambica) were farmed in China and Ecuador, respectively. The frozen tilapia fillets (O. niloticus and tilapia spp.) were farmed in Indonesia and Malaysia. The fat contents of the tilapia fillets indicated on the packages varied from 2% to 3%.

Equipment

The Luminex 100 IS 2.2 flow cytometric system, consisting of a Luminex 100 analyser and a Luminex XY Platform, programmed to analyse a 96-well plate, was supplied by Applied Cytometry Systems (Dinnington, Sheffield, UK). The MagPix® imaging analyser was purchased from Luminex B.V. (Oosterhout, The Netherlands). The super-paramagnetic carboxylated microspheres (MagPlex™ numbers 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 Laboratories B.V. (Veenendaal, Netherlands). The DynaMag™-2 magnetic separator was obtained from Invitrogen Dynal (Oslo, Norway). The test tube rotator was from Sniijders (Tilburg, 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 four-parameter curve fitting was used from GraphPad Prism software GraphPad Software Inc. (La Jolla, CA, USA).

Procedures

POPs-coated super-paramagnetic SEMs protocol

The different POPs–protein conjugates (BaP-BSA, PCB77-OVA and BDE47-BSA) were covalently coupled to unique super-paramagnetic carboxylated microspheres sets (spectral codes 21, 70 and 42, respectively) according to the procedure already described (Meimaridou et al. 2010, 2011) 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 1 h with PBDE47-BSA-coated SEMs (the 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 and IMIA protocols

The initial protocol for the 3-plex FCIA was described previously (Meimaridou et al. 2011) in which the applied standard dilution series of each POP was 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 µl were combined with 40 µl of 50% DMSO in the well of a low-binding 96-wells microplate. In the new optimised 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. After that, the recovered microspheres were washed once and resuspended in 100 µl PBS by gently shaking for 5 min prior to the final measurement in the MagPix analyser. A dilution series of the cocktail of the three POPs (0.01–1000 ng ml⁻¹) was 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 (Kalachova et al. 2011; Meimaridou et al. 2011) which was modified by changing the salt composition (using sodium hydrogen carbonate (NaHCO₃) and magnesium sulphate (MgSO₄) instead of sodium chloride (NaCl) and MgSO₄) during the extraction and by introducing a dSPE clean-up. Spiked samples were prepared by adding different mixed standard solutions in acetone to homogenised tilapia fillet 30 min prior to extraction. One spiking solution contained 2.5 ng BaP, 2 ng PBDE47 and 40 ng PCB77 per g of homogenised tilapia fillet. The second spiking solution was a mixture of 4 PAHs (10 ng g⁻¹ fish of BaP, CHR, BaA and CCP), four 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 two PBDEs (2 ng g⁻¹ PBDE47 and 2,2′,4,4′,5-pentabromodi phenyl ether (PBDE99)). For the POPs extraction, 5 g of homogenised tilapia fillet were 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 MgSO₄ and 2 g of NaHCO₃ or NaCl were added. After 1 min of shaking, the tube was centrifuged for 8 min (7800g) 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 re-dissolved in 5 ml of acetonitrile and this solution was introduced into the dSPE tube. After 1 min of shaking, the dSPE tubes were centrifuged for 5 min (3400g) 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 re-dissolved 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 (Meimaridou et al. 2011), 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 normalised dose–response curves for PCB77, BaP and PBDE47 obtained in both 3-plex FCIAs 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 analysed per 96-well plate.

3-Plex IMIA

In 2010, a super-paramagnetic SEMs-based imaging analyser with planar readout (MagPix) was introduced (Luminex 2010). This system uses the same SEMs principle as the flow cytometric platform but only operates with super-paramagnetic 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 normalised 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 50% 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. These sensitivities were almost similar to those obtained with ELISA using the same antibodies (Fránek et al. 2001; Matschulat et al. 2005); only the sensitivity for PBDE47 was higher in a magnetic particle ELISA (Shelver et al. 2008). 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 with three individual ELISAs or the 3-plex FCIA.

**QuEChERS-like extraction** is a procedure that was initially developed for the detection of pesticides in foods and agricultural products (Lehotay et al. 2005). 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 (Ramalhosa et al. 2009) and for PAHs, PCBs and PBDEs (Kalachova et al. 2011) prior to the analysis with instrumental analytical techniques. In our previous work (Meimaridou et al. 2011) 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% to 15%). 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. The extraction procedure was improved by the addition of NaHCO₃, instead of NaCl, in combination with MgSO₄ (Figure 3). NaHCO₃, when heated or dissolved in water, breaks down to produce carbon dioxide (CO₂) gas. The effervescent effect of the CO₂ produced *in situ* was acting as a dispersant and supported sample homogenisation and separation of the water and organic layers. MgSO₄ 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-up of QuEChERS tilapia extracts to remove any residuals lipids that might act as interferences. DSPE is a simpler and faster clean-up step since the entire procedure takes only 6 min which is in sharp contrast to the handmade silica mini-column that required extra time for column preparation, conditioning and elution steps. Six different homogenised blank tilapia fillets and the same samples spiked with a mixture of BaP, PCB77 and PBDE47 at 2.5, 40 and 2 ng g⁻¹, respectively, were extracted with the QuEChERS-like approach (with NaCl or NaHCO₃) and the dSPE clean-up. The modified extraction performances were tested and compared in combination with the 3-plex IMIA and FCIA (Figures 3 and 4). A more homogenous mixture of fish, salts and ethyl acetate was obtained during the extraction procedure with NaHCO₃ and 1 min of shaking. Moreover, after centrifugation, we observed a clearer ethyl acetate aliquot that implied a better clean-up. Both observations can explain the fact 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 with the extraction with NaCl after the clean-up with dSPE (Figure 3). 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 with 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 fish, 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 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 (two 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. Compared with their performances in buffer, the sensitivities of the assays within the 3-plex IMIA were higher in fish extract. However, in the case of the BaP assay (data not shown), we obtained a significant decrease of the maximum response from $4600 \pm 100$ MFIs in buffer to $2300 \pm 500$ in tilapia extract, clearly indicating a matrix influence of the assay. This matrix effect of the BaP assay was also demonstrated previously (Meimaridou et al. 2010, 2011). The maximum responses for the PCBs and PBDEs assays were less affected. For the PBDE47 assay, we obtained $1130 \pm 60$ MFIs in buffer versus $1600 \pm 200$ MFIs in tilapia extract and for the PCB77 assay $1390 \pm 80$
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 (Meimaridou et al. 2011).

Forty different homogenised tilapia fillets (fresh, frozen, from various species, origins and slightly different fat content) were analysed 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 not shown) were similar to the 3-plex IMIA (Figure 6).

The responses obtained with the blank and the spiked tilapia fillets with the 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 (Poljakova et al. 2000), PCB77 (Bernard et al. 1999, 2002) and PBDE47 (Schechter et al. 2003; Bordajandi et al. 2004; Hites et al. 2004; Johnson-Restrepo et al. 2005; Viganò et al. 2008; Van Leeuwen et al. 2009; Adu-Kumi et al. 2010) are never the only POPs present in food and especially in fish. Therefore, apart from the target compounds, several others contaminants belonging to these PAHs, PCBs and PBDEs groups will be present in contaminated samples. 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 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\(^{-1}\) each of PBDE47 and PBDE99). 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 previously (Meimaridou et al. 2011). In addition to the previous samples, 20 different blank fillets were extracted and analysed as above. The variations in MFIs of the blank and spiked samples in the 3-plex IMIA for the three targets POPs are demonstrated in Figure 6(D–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 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 a few months utilising 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 European Union limit set for smoked fish and smoked fishery products, but when a higher concentration of several PAHs was added, the difference between the MFIs of blanks and spiked samples was clear. In previous work (Kalachova et al. 2011) 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 they are 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 super-paramagnetic 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 with the previously developed 3-plex FCIA 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 being a lower-cost and easy transportable system, and, after further application and validation in a range of different matrices, it 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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