Analytical Methods

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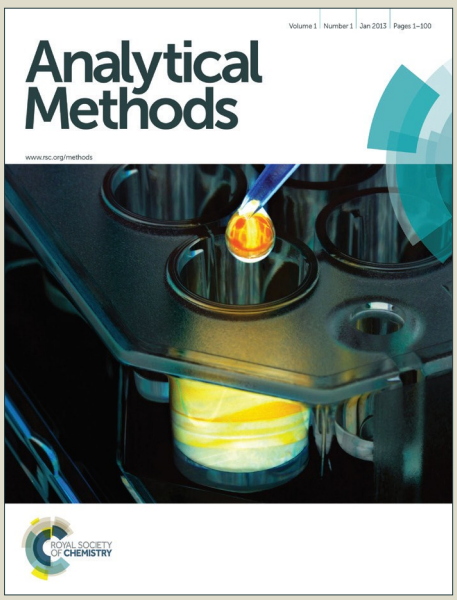

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ARTICLE

Fluorescence Polarization Immunoassays for Carbamazepine – Comparison of Tracers and Formats

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For the antiepileptic drug and anthropogenic marker carbamazepine (CBZ) a fast and cost-effective immunoassay based on fluorescence polarization (FPIA) was developed. The required fluorophore conjugates were synthesized from different fluorescein and carbamazepine derivatives. The most suitable tracer was CBZ-triglycine-5-(aminoacetamido)fluorescein. Additionally, the applicability of the assay in tubes and on microtiter plates was tested. The first format can be performed in a portable instrument and therefore can be applied in field measurements. The measurement of an individual sample can be carried out within 4 min. This assay shows a measurement range of 2.5–1000 µg/L and a test midpoint (or IC_{50}) of 36 µg/L. The FPIA performed on microtiter plates is useful for the assay development and is suitable for a very high throughput (up to 24 samples in 20 min). The test midpoint of this assay is 13 µg/L and the measurement range is 1.5–300 µg/L. Furthermore, this assay requires smaller sample volumes and less reagents, including the crucial amount of antibody. The applicability of both assays to spiked surface water samples was evaluated. The recovery rates vary between 66–110 % on MTPs and 81–140 % in tubes.

Introduction

Pharmaceuticals in the water cycle are an emerging concern.[1, 2] The way that such pollutants enter the environment depends on their pattern of usage and mode of application but, in the case of those coming from human use and excretion, wastewater discharge is a very important source for the aquatic environment.[3] The huge number, which is increasing constantly, and the variety of these compounds, as well as their transformation and degradation products make it difficult and costly to monitor all of them.[4, 5] However, this monitoring is crucial to assess the quality of water resources, since it affects what they can be used for, as drinking water, for recreation, industrial uses or agricultural activities, such as irrigation and livestock watering. A minimum quality is required to maintain aquatic and associated terrestrial ecosystem function. An approach that has been discussed is to track the origin and type of contamination by the fate of anthropogenic markers,[6] *i.e.* indicators of human presence or activity,[7] *e.g.* caffeine.[8]

One proposed marker for wastewater cleaning efficiency and consequently wastewater contamination of surface and ground waters is carbamazepine (CBZ),[9-14] an antiepileptic drug with a yearly consumption of 1,014 tons worldwide.[15] Due to its low degradation rate in most wastewater treatment plants, it enters the water cycle.[16] CBZ was recently one of the most frequently detected pharmaceutical in surface and ground water samples from Danube river in Serbia.[17] Negative effects of this pharmaceutical on health status of aquatic organisms were reported.[18-20]

Instrumental methods like liquid chromatography with tandem mass spectrometry (LC-MS/MS)[21, 22] and gas chromatography MS[23] were developed. The description of the fate of a marker like CBZ can only be achieved by broad screening and long-term monitoring of its concentrations in the water cycle. For this purpose, immunoanalytical techniques are more suited than the instrumental methods due to the feasibility of a cost-effective high-throughput screening. Additionally, these assays are characterized by a high specificity and sensitivity. Heterogeneous enzyme immunoassays such as enzyme-linked immunosorbent assays (ELISA) have been developed for high throughput screenings of CBZ in water samples and their application has been described.[9, 24-26]

The fluorescence polarization immunoassay (FPIA) is a homogeneous format without any washing or long incubation steps. Hence, the FPIA is much faster and easier to perform than heterogeneous assays and can be completed within a few minutes. This assay has been applied to food, diagnostic and envi-

ronmental analysis to determine small compounds, including mycotoxins, drugs and pesticides.[27-38]

The principle of FPIA is based on the polarization difference between an unbound and an antibody-bound fluorophore-labeled analyte (tracer). The analyte and the tracer compete for the analyte-specific binding sites of the antibody. When the analyte concentration is high, most of the labeled molecules remain unbound. When these conjugates are excited by linearly polarized light, the emitted light is mainly depolarized due to the low mass and the fast rotation of the molecules (Figure 1). When few or not any analyte molecules are present, the labeled analyte is completely bound by the antibody. This complex is much bigger and so the emitted light will retain a high degree of polarization.

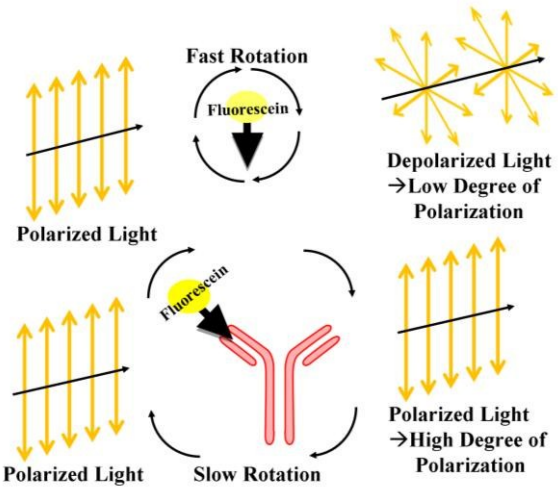

Figure 1: The principle of FPIA.

Usually fluorescein derivatives are used for the synthesis of tracers, because most FPIA instruments are equipped with filters to select the fluorescein excitation and emission wavelengths. These filters are expensive and sometimes cumbersome to change. Additionally, fluorescein tracers show a high quantum yield and are stable.[28] Still there are many different ways of linking fluorescein with the analyte. It has been shown that hapten structure and spacer length influence the performance and especially sensitivity of FPIAs.[27, 32-34] Therefore conjugate design and evaluation is an inherent part of assay optimization.

A standardized CBZ FPIA is already frequently used for the CBZ determination in clinical purposes, where usually concentration of 4 to 12 mg/L need to be quantified.[39] In this study, we developed a CBZ FPIA suitable for measurements of environmental samples, where much lower concentrations of around 1 µg/L have to be detected. Therefore we synthesized different tracers for their application on CBZ FPIA and compared the suitability of different FPIA formats for the CBZ determination in surface water samples (on microtiter plates, MTPs, and in tubes). To our knowledge no CBZ FPIA for the application on surface water was developed before.

Experimental

Reagents and Materials

All solvents and chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany), Merck KGaA (Darmstadt, Germany), Serva (Heidelberg, Germany), and Mallinckrodt Baker (Griesheim, Germany) in the highest available quality. 5-(aminoacetamido)fluorescein (AAF) was obtained from Invitrogen (Carlsbad, CA, USA). Ethylenediamine thiocarbamoyl-fluorescein (EDF) was synthesized as described by Pourfarzaneh *et al.*[40] *N*-hydroxysuccinimide (NHS) and Dicyclohexylcarbodiimide (DCC) were used for the tracer synthesis. The anti-CBZ monoclonal antibody (mouse IgG1, clone B3212M, lot 1C07011) was obtained from Meridian Life Science Inc. (Saco, MN, USA). A Synthesis A10 Milli-Q® water purification system from Millipore (Schwalbach, Germany) was used to obtain ultrapure reagent water for the preparation of buffers and solutions. Black non-binding 96 well MTPs from Greiner Bio-One (Frickenhausen, Germany) were employed for FP measurements on a Synergy H1 multimode plate reader (Bio-Tek, Bad Friedrichshall, Germany). A Sentry® 200 (Ellie, Wauwatosa, WI, USA) portable FP instrument was used for the FPIA measurements in tubes.

Tracer synthesis

The tracers (Figure 2) were synthesized using CBZ-triglycine,[26] dibenz[b,f]azepine-5-carbonyl chloride (DBA), or cetirizine (CET) hydrochloride as hapten. Fluorescein building blocks were AAF, and EDF. The tracers were synthesized using the NHS/DCC method. CBZ-triglycine-AAF was synthesized as described before for a caffeine-AAF tracer.[31] The EDF tracers with the haptens CBZ-triglycine and CET were synthesized according to the following protocol: Approximately 5 µmol of antigen were dissolved in 100 µL DCC solution in dimethylformamide (DMF, 100 µmol/mL) and 100 µL NHS solution (100 µmol/mL in DMF), leading to a ratio of 1:2:2 of antigen to DCC to NHS and a total volume of 200 µL. The reaction mixture was mixed and incubated for 6 h at room temperature. Approximately 1 µmol of EDF was added and incubated for 18 h at room temperature. CBZ-EDF was synthesized by dissolving 2 mg of DBA and 1 mg of EDF in 200 µL DMF and 10 µL triethylamine. The mixture was incubated for 18 h.

The success of the synthesis was confirmed by LC-MS (Agilent 1260 LC system, Agilent Technologies, Waldbronn, Germany coupled to a Triple Quad™ 6500 MS, AB SCIEX, Darmstadt, Germany). The product was cleaned by HPLC (Series 1200, Agilent Technologies) using a C18 pre-column and a Kinetex XB-C18 150×3 mm analytical column with a particle size of 2.6 µm (Phenomenex, Aschaffenburg, Germany). The oven temperature was set to 50 °C and the flow rate was 0.3 mL/min. The solvents were ultrapure water (A) and methanol (B) containing 10 mmol/L ammonium acetate and 0.1 % acetic acid. 70 % solvent A was used at the beginning. After 3 min, solvent B was linearly increased to 95 % within 12 min. After 5 min, the percentage of solvent B was decreased to 30 % within

0.5 min. Then the composition was kept constant until the end of the run (28 min). The fraction of the respective main peak was evaporated to dryness under a current of nitrogen, dissolved in methanol and stored at 4 °C.

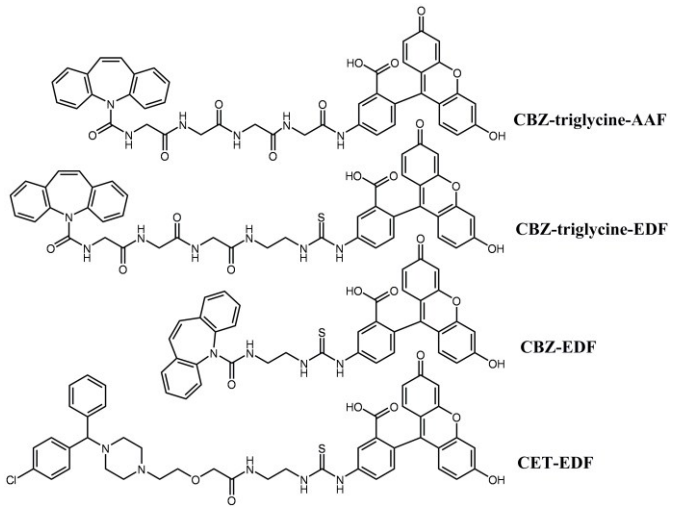

Figure 2: Chemical structures of the synthesized tracers for the application on CBZ FPIA.

CBZ FPIAs

FPIA ON MTPs Into each well, 280 µL borate buffer (2.5 mmol/L disodium tetraborate decahydrate, 0.01 % sodium azide, pH 8.5) with 0.01 % Triton™ X-100 were pipetted. After adding 20 µL of the calibrators or spiked samples, the MTP was briefly shaken on a plate shaker and the background fluorescence measurement was performed with the following filter settings: excitation at 485 nm, emission at 528 nm (at parallel and perpendicular polarizer settings, gain 91). In the measurement of the background fluorescence of the calibrators, no difference between the different CBZ concentrations could be observed. 20 µL of the different tracers, diluted in a PBS (10 mmol/L sodium dihydrogen phosphate, 70 mmol/L disodium hydrogen phosphate, 145 mmol/L sodium chloride, pH 7.6) based tracer stabilization buffer (PBS containing 20 % glycerol and 5 % methanol) were added to each well and shaken for 5 min. Then 20 µL of the anti-CBZ antibody dilution optimized for each tracer in PBS based antibody stabilization buffer (PBS containing 20 % glycerol, 0.2 % sodium azide, 0.05 % TWEEN® 20 and 0.1 % bovine serum albumin) were added. After shaking for 10 min, the fluorescence was measured with the settings described above.

To determine the degrees of polarization, background corrected fluorescence intensities in parallel and perpendicular direction were used. The G-factor was set to 1. A four-parametric logistic function (4PL) was fitted to the mean of the polarization values using the Origin 9.1G software (OriginLab, MA, USA):

$$f(x) = y = \left[\frac{A-D}{\left[1+\left(\frac{x}{C}\right)^{B}\right]}\right] + D$$

where y is the degree of polarization, x is the CBZ concentration, A is the degree of polarization for an infinitely small analyte concentration (upper asymptote), B is the slope at the test midpoint, C is the concentration at the inflection point (test midpoint or IC_{50}), and D is the degree of polarization for an infinitely high analyte concentration (lower asymptote).

For the determination of CBZ concentrations in spiked surface water samples and the determination of calibration curves, 8 calibrators were measured in triplicate on each MTP. The calibrators were prepared by diluting a methanolic stock solution gravimetrically with ultrapure water. The samples were also measured in triplicate.

To determine the measurement range (defined as the highest and the lowest concentration that can be determined with a given precision level of 30 %), 16 calibrators in six-fold determination and the precision profile were used. The precision profile describes the relative error of the CBZ concentration (Δx), calculated from the respective standard deviations of the degree of polarization (StD) and the slope (1st derivative) at each individual calibrator concentration, as described by Ekins:[41]

$$\Delta x = -\frac{StD}{x \cdot \frac{df(x)}{dx}} = -\frac{StD}{B \cdot (D-A)}\left[\left(\frac{C}{x}\right)^{B} + 2 + \left(\frac{x}{C}\right)^{B}\right]$$

Following the "three sigma criterion" that is usually used for instrumental methods to determine the limit of detection, the relative error of the concentration threshold for the determination of the measurement range was set to 30 %.[42]

FPIA IN TUBES In a round-bottom glass tube, 1 mL of borate buffer and 100 µL of calibrator or sample were mixed using a vortexer. The background fluorescence intensities in parallel and perpendicular direction were measured in the portable tube FP reader for each measurement. Afterwards 100 µL of the tracer CBZ-triglycine-AAF, diluted 1:6,000 in tracer stabilization buffer and 100 µL of the monoclonal anti-CBZ antibody, diluted in antibody stabilization buffer (4.5 µg/mL; 450 ng per measurement) were added and the reagents were mixed for 10 s. After an incubation time of 3 min and another short mixing step, FP was measured. For all calculations, the background corrected signals were used.

A calibration curve with 16 calibrators measured in triplicate was used to obtain the calibration curve and the measurement range as described above. The same calibration curve could be used to determine the CBZ concentrations of the samples.

Sample Preparation

Surface water samples were collected in February 2014 from the Teltowkanal, a channel that runs across southern Berlin and that receives wastewater. The samples were collected in the morning, at noon and in the evening on two different days. So in total six different samples were collected. For collecting the samples a spot was chosen from which we knew from previous studies that negligible CBZ concentrations could be expected (Teltowkanal 1).[25] Right after collecting the samples, they were filtered through a folded filter (Sartorius Stedim Biotech,

Göttingen, Germany), 0.1 % sodium azide was added to inhibit the growth of microorganisms, and then the samples were spiked gravimetrically at four different CBZ concentrations: 1, 10, and 100 µg/L. The samples were stored at -20 °C until their usage.

Results and discussion

Optimization and comparison of FPIA using different tracers

The CBZ FPIA optimization for the different tracers was performed using the MTP format because here, a lot of measurements can be performed in a short time. First, the dilutions of the tracers were optimized so that the total fluorescence intensity, the sum of parallel and perpendicular intensity, of the calibration curve is approximately 10 times higher than the total intensity of the buffer. With these conditions, the same gain factor can be used for all measurements. The time dependency of the reaction between the tracers and the antibody were studied. For all tracers the equilibrium was reached after 10 min. The binding affinities of the antibody towards the tracers were investigated by adding different amounts of antibody to the tracers. With these antibody titrations, the maximum degrees of polarization (P_{max}) of the different tracers were determined (Figure 3).

The lowest P_{max} of 135 mP was observed using the tracer CET-EDF. CET was chosen for tracer synthesis, because it shows very high cross reactivity with the used antibody. It was observed, that the cross reactivity is pH-dependent: in acidic environment the cross reactivity is higher than in alkaline.[43] Due to the pKa of 6.30 of fluorescein,[44] an alkaline buffer has to be used for efficient fluorescence. Under alkaline conditions it is expected that the antibody shows a relatively low affinity towards CET-EDF. Consequently the observed low P_{max} can be explained.

No difference between P_{max} of CBZ-EDF and CBZ-triglycine-EDF was observed (225 and 220 mP, respectively). But when small amounts of antibody are used (< 140 ng per measurement), the degree of polarization is higher for CBZ-EDF than for CBZ-triglycine-EDF. The highest P_{max} (260 mP) and the strongest increase of P with small antibody amounts was observed for the tracer CBZ-triglycine-AAF. So the antibody shows the highest affinity towards this conjugate in comparison to the other tracers used in this study.

For the comparison of sensitivity of the tracers, calibration curves using optimized concentrations of all reagents were used (Table 1). The optimum dynamic range (distance between upper and lower asymptote, A-D) was fixed to around 140 mP.

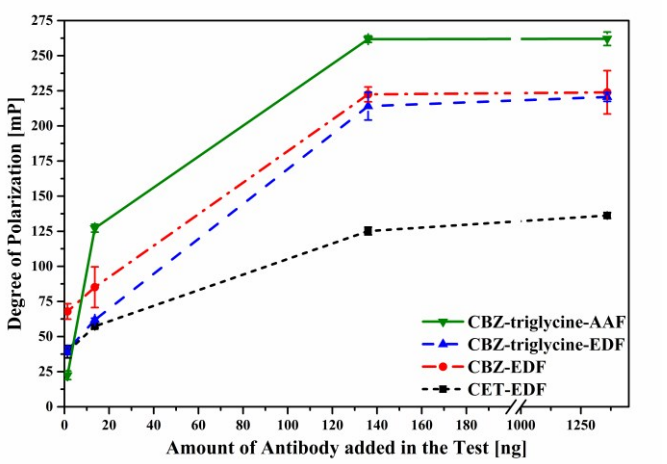

Figure 3: Antibody titration using the tracers CET-EDF (black dotted line), CBZ-EDF (red dash-dotted line), CBZ-triglycine-EDF (blue dashed line) and CBZ-triglycine-AAF (green solid line).

The assays using different tracers were optimized concerning this parameter. Unfortunately, when CET-EDF is used, only a smaller dynamic range of 64 mP could be obtained even when a high amount of antibody was used (136 ng per measurement). This was expected due to the low P_{max} observed for this tracer. Even with twice as much antibody, only a dynamic range of approximately 85 mP could be reached. But with the increasing dynamic range, the test midpoint also increased from 34 to 81 µg/L which is quite high compared to the other tracers. Additionally the slope at the test midpoints increased. It can be summarized that the assay using CET-EDF as tracer is insufficiently sensitive because of the low affinity of the antibody towards this tracer.

During the optimization of the assay using CBZ-EDF, the desired dynamic range of 140 mP could not be reached, even when the upper asymptote almost reached P_{max}. The reason for this is the high value of the lower asymptote (102 mP). This suggests that the affinity of the antibody towards this tracer is higher than towards the free analyte. That means that even high CBZ concentrations cannot suppress the binding of the tracer. Perhaps a similar conjugate was used for the synthesis of the immunogen for the production of this antibody. This would explain the high affinity towards this tracer compared to the other tracers. There is no structural data about the immunogen given by the manufacturer ('immunogen: CBZ-BSA'). Nevertheless, the test midpoint for this tracer is lower (26 µg/L) than that of CET-EDF.

Table 1: Characteristic parameters of the calibration curves of CBZ FPIA using different tracers: mass of antibody used per measurement (m(Ab)), upper and lower asymptote (A and D), test midpoint (C), slope at C (B), dynamic range (DR, A-D), and coefficient of determination R^2

Tracer	m(Ab) [ng]	A [mP]	B	C [µg/L]	D [mP]	DR [mP]	R^2
CET-EDF	136	98.6	1.06	34.4	35.0	63.6	0.998
	272	121	1.23	81.1	36.9	84.1	0.999
CBZ-EDF	45.3	219	1.04	26.4	102	117	0.998
CBZ-triglycine-EDF	30.2	173	1.00	20.6	35.2	138	0.999
CBZ-triglycine-AAF	13.6	151	1.03	12.5	12.7	138	0.999

For both tracers synthesized with CBZ-triglycine, a good dynamic range of 138 mP could be obtained. For CBZ-triglycine-EDF a much smaller value of the lower asymptote was observed than for CBZ-EDF, but the value is similar to the one of CET-EDF. This tracer leads to a slightly more sensitive assay than the tracer described before. The difference between CBZ-triglycine-EDF and CBZ-EDF is the length of the spacer. Thus, the conclusion from previous publications, that the longer the spacer, the higher the sensitivity can be confirmed.[27, 32-34]

For CBZ-triglycine-AAF, the optimum dynamic range was reached, even by using only half of the antibody amount that had to be used for CBZ-triglycine-EDF. This can be explained by the previously shown high affinity of the antibody towards CBZ-triglycine-AAF. Additionally, the lowest lower asymptote was observed. So the background value of the degree of polarization is among other things dependent on the fluorescein derivative used.

The AAF tracer led to the lowest test midpoint of 13 µg/L, *i.e.* this tracer allows for the most sensitive CBZ FPIA assay. At the same time, the lowest antibody amount has to be used when this tracer is applied. There is only a slight structural difference compared to CBZ-triglycine-EDF. The spacer is even shorter for the more sufficient tracer. This would suggest that tracers using AAF as fluorescein derivative are more sensitive. Hatzidakis *et al.* described that the fluorescence intensity of the fluorescein is quenched due to a hapten-to-dye interaction.[45] Therefore we propose that the quenching effect is smaller for the derivative AAF compared to EDF. This suggestion would also explain why an almost 10times higher dilution factor could be used for the preparation of tracer CBZ-triglycine-AAF compared to CBZ-triglycine-EDF leading to similar fluorescence intensity.

Summarizing it can be said that a too high affinity of the antibody towards the tracer is not good, as shown for tracer CBZ-EDF. But if the affinity towards the tracer is too low, also no sensitive assay can be developed as it could be shown for CET-EDF. For the development of an optimum assay with a good sensitivity, the affinity of the antibody towards analyte and tracer should be similar.[45] This criterion is fulfilled for CBZ-triglycine-AAF, which is therefore the tracer of choice and will be used for all further experiments.

Comparison of CBZ FPIA on different formats

The resulting system was applied to two different measurement formats: the multimode plate reader that was used for the experiments described above and a handheld inexpensive tube-based device. For the CBZ FPIA performance in tubes, a higher ratio of total intensities of the tracer and the background of approximately 20 is necessary to reach good signals. After thoroughly optimizing the assay in tubes, the calibration curve and the precision profile were measured and compared to those of the CBZ FPIA performed on MTPs using the same tracer, CBZ-triglycine-AAF (Figure 4).

Characteristic values for the evaluation of immunoassays were previously defined for heterogeneous immunoassays[25, 42] and already applied for homogeneous assays.[31] These parameters include relative dynamic range, sensitivity, goodness of fit, and measurement range. This set of criteria was taken into consideration for the assessment of the assay performance on different formats, besides the relative dynamic range, the normalized dynamic range ((A-D)/A). This parameter was used for the evaluation of different kinds of immunoassays and is especially useful for the comparison of different detection methods, *e.g.* absorbance and fluorescence. Here, only the degree of polarization is used. Therefore the consideration of the dynamic range (A-D) instead of the relative dynamic range is sufficient. The assays in both formats were optimized so that their dynamic ranges were around 140 mP. It should be noted that the calibration curve in tubes is shifted towards higher degrees of polarization.

The calibration curves obtained for both formats fulfilled the requirement for the coefficient of determination ($R^2 > 0.990$) very well (0.999 on MTPs and 1.00 in tubes). The highest standard deviation was 3.42 mP for the assay in tubes and 9.30 mP on MTPs. Normalized to the dynamic range, values of 2.5 % and 6.7 % were determined, respectively. For the assay on MTPs lower pipetting volumes of 20 instead of 100 µL are used. This might be the reason for the slightly higher standard deviations. Additionally, the mixing of the reagents can influ-

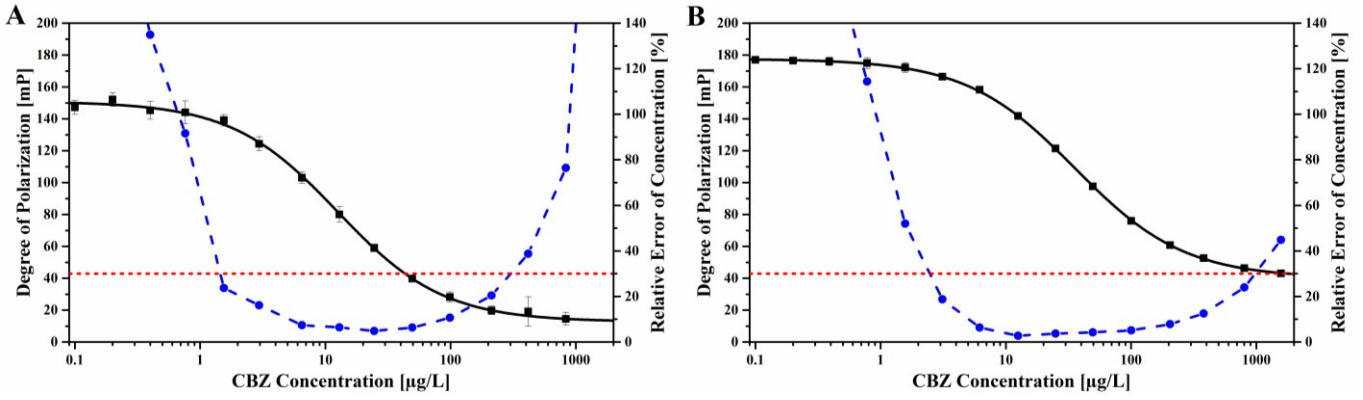

Figure 4: CBZ FPIA calibration curves (black solid lines), precision profiles (blue dashed lines) and measurement ranges (intersection points at 30 % relative error of concentration, dotted red lines) determined on MTP (A) and in tubes (B).

ence the precision of the assay. The reagents in tubes were mixed by using a vortexer, whereas the MTPs were shaken on plate shakers what probably results in slower and less sufficient mixing. Nevertheless, it can be summarized that the goodness of fit of FPIA on both formats is satisfactory.

For heterogeneous assays, the slope B at the test midpoint is sometimes fixed to 1.[25, 42] This was not done for homogeneous assays.[31] But in order to reach a wide measurement range, it is crucial, that the curve has a slight slope. In an optimum manner, it should be 1.0 ± 0.1. This criterion is fulfilled for both formats (1.03 on MTPs and 0.994 in tubes).

One of the most important points regarding the quality of an assay is the sensitivity that is indicated by the test midpoint. Both test midpoints are in the low µg/L range. The assay on MTPs is slightly more sensitive (13 µg/L) than the assay performed in tubes (36 µg/L). Compared to the previously developed ELISA using the same monoclonal anti-CBZ antibody, horseradish peroxidase and a chromogenic substrate, the test midpoints of FPIAs are two orders of magnitude higher (ELISA test midpoint: 147 ng/L).[25] Previously developed FPIAs performed on MTPs showed test midpoints in the range of 0.25 µg/L for azoxystrobin[35] to 207 µg/L for butachlor.[36] For FPIAs in tubes even a wider range of test midpoints was reported: from 0.48 µg/L for ochratoxin A[37], over 517 µg/L for zearalenone[38] up to 2.48 mg/L for sodium benzoate.[46] So the test midpoints of the assays developed in this study are in a middle range compared to values from literature.

The lower limit of detection is lower on MTPs (1.5 µg/L) than in tubes (2.5 µg/L). But when the assay in tubes is used, a wider concentration range of CBZ can be determined (up to 980 µg/L in tubes; up to 310 µg/L on MTPs). The measurement range of the previously developed CBZ ELISA covers a range of three orders of magnitude (16.6-19,500 ng/L).[25] The ranges of the FPIAs developed in this study are narrower.

The reproducibility of the characteristic values for calibration curves of the FPIA on MTP was checked by determining the calibration curve on five MTPs: three MTPs on one day and one MTP on two other days (n = 5). For these experiments the same reagent dilutions were used for all MTPs. All characteristic values, including upper and lower asymptote, dynamic range, test midpoint and slope at the test midpoint, showed coefficients of variations lower than 10 %. Therefore it can be concluded that the calibration curve for the FPIA on MTP is highly reproducible. It seems that as long as the same reagents are used, the calibration curve could probably be transferable from MTP to MTP, so that even more samples can be determined per MTP and therefore an even higher throughput could be achieved.

For the FPIA in tubes a lower tracer dilution of 1:6,000 (1:40,000 on MTPs) and five times more volume had to be used per measurement (100 instead of 20 µL) compared to the procedure on MTP. This means that approximately 33 times as much of the tracer had to be used compared to the execution on MTPs. The antibody, too, had to be used in a 33 times higher amount for FPIA in tubes than on MTPs (450 ng and 13.6 ng, respectively). So the ratio of tracer to antibody is the same for both formats. Therefore it can be concluded that the dynamic range is the same for a constant ratio of antibody to tracer, independent of the format. So the most important factor on how much antibody has to be used, besides the choice of the tracer, is the sensitivity for fluorescence intensities of the applied instrument. Compared to ELISA, eight times more antibody had to be used for FPIA on MTPs (ELISA: 8.6 ng/µL in 200 µL, equal to 1.72 ng per measurement).[25] On the other hand FPIAs do not require the usage of a secondary antibody or an enzyme. These arguments together with the saved working time, makes the CBZ FPIA probably to a cost-effective alternative to ELISA.

Application to surface water

The applicability of the assays for water samples was verified by measuring the CBZ concentration of spiked surface water samples. First, the original samples were measured. For both formats the CBZ concentration could not be quantified, *i.e.* the concentrations in the unspiked samples were lower than the respective lower limit of detection. The sample background fluorescence signals were higher than the fluorescence signal of calibrators: 19 % in tubes and 41 % on MTPs. Therefore a background correction of the fluorescence intensities was performed. The background corrected fluorescence intensities after adding the tracer and the antibody were practically the same for calibration and sample measurements: on MTPs the values were 18,500±600 RFU (relative fluorescence units, mean from all measurements ± standard deviation) for calibrators and 18,100±1,100 RFU for samples; in tubes background corrected fluorescence intensities of 331,000±4,000 RFU for calibrators and 332,000±3,000 RFU for samples were determined. That means that the fluorescence intensity of the tracer is not quenched or enhanced due to matrix compounds. Additionally, it was checked if matrix compounds contained in surface water, *e.g.* metal ions or proteins, have an influence on the polarization properties of the tracer. Therefore the degrees of polarization of the free tracer with calibrators or samples but without antibody were determined (measurements were performed on MTPs). Here, values of 21.4±2.7 mP for calibrators and 20.0±3.3 mP for samples were found. So it can be concluded that the tracer is not influenced by matrix constituents of surface water.

The recovery rates for spiked surface water samples were within a range from 74–110 % for 10 µg/L and 66–110 % for 100 µg/L when the CBZ FPIA on MTPs was applied (Figure 5). The medians were 94 % and 99 % for 10 and 100 µg/L, respectively. Similar recovery ranges were obtained when the CBZ FPIA in tubes was applied for the CBZ determination: 81–136 % for 10 µg/L and 84–107 % for 100 µg/L. The medians were very accurate with 103 and 101 % for 10 and 100 µg/L, respectively. For the spiking values that are within the measurement range, good recovery rates were observed. One spike outside the measurement range was tested (1 µg/L). As expected, poor recovery rates with high deviation were observed for both methods: 32–240 % on MTPs, and 69–226 % in tubes.

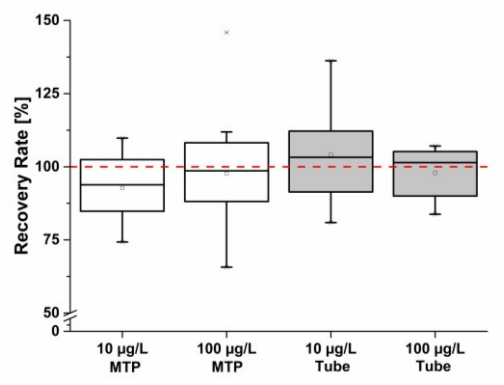

Figure 5: Recovery rates determined for the spiked surface water samples with 10 and 100 µg/L CBZ (n = 18 per concentration level), determined with FPIA on MTPs (empty boxes) and in tubes (grey shading). The red dotted line marks the ideal recovery rate of 100 %.

In previous studies it could be shown that the anti-CBZ antibody used here is applicable for immunochemical determination of CBZ in surface water.[25, 26] The applicability to FPIA for CBZ determinations in surface water was proven due to the good recovery rates within the measurement ranges, no quantifiable CBZ concentrations in blank samples and no changes of fluorescence properties of the fluorescein tracer. Hence it was concluded that there are no matrix effects of surface water on this assay. Both assays appear applicable for the CBZ determination in surface water and they give the opportunity for a fast CBZ quantification in wastewater.

The intra-assay coefficient of variation (CV) for FPIA on MTP was up to 9.3 % for 10 µg/L and 25 % for 100 µg/L. The inter-assay CV for this assay was up to 10 % for 10 µg/L and 18 % for 100 µg/L. The highest spiking value was close to the highest quantifiable concentration of this assay what explains the higher CV values. But all CVs are still lower than 30 %, the limit of the relative error of concentration that was by definition accepted for the measurement range. But the concentrations determined with FPIA in tubes have a higher precision over a wider concentration range. Here the CV for each determined concentration is lower than 15 % for 10 µg/L and 9.5 % for 100 µg/L. The reason for this higher precision in tubes might be the more effective mixing procedure in tubes.

Chun *et al.* also compared FPIAs on different formats for the determination of zearalenone in corn. The authors came to the result that both FPIAs, on MTPs and in tubes can be applied for determination of zearalenone in food samples.[33] In general we agree with the statement on formats, but it still depends on the individual requirements on the measurement system. The main advantage of the assay on MTPs is the high throughput. Here, 24 samples can be determined in triplicate within 20 min, including all pipetting and incubation steps. The total assay time in the portable tube reader is 4 min for one sample in single determination. So the decision which assay format to choose should take into consideration the number of samples and the measurement platform.

Conclusions

FPIAs for CBZ determination were developed. Different tracers were synthesized and tested. We found out that not only the length of the spacer between the analyte and fluorescein derivative is important, but that also the type of fluorescein derivative influences the assay performance.

Different assay formats were studied, which were both successfully applied to surface water samples. For the precise determination of CBZ in individual samples and for field measurements, the performance in the portable tube FP reader is favorable. For high-throughput the performance on MTPs is beneficial. Additionally this format requires only 3 % of the antibody amount, which is often the crucial cost factor of immunoassays. In conclusion, the developed assays can be useful tools for a broad monitoring of water samples.

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