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Quality assurance in immunoassay performance – carbamazepine immunoassay format evaluation and application on surface and waste water

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Carbamazepine (CBZ) is one of the most frequently detected pharmaceuticals in water samples. For the determination of this anthropogenic marker, various immunoassay formats were tested and evaluated in order to identify the most suitable one. For these direct competitive assays, the analyte was labelled with the enzyme horseradish peroxidase (HRP) or alkaline phosphatase (AP), and seven substrates with specific detection properties were used. The quality criteria for the standard curves were fulfilled by all HRP assays and the chemiluminescence AP format. Furthermore, intra- and inter-plate coefficients of variation as a measure of the achievable precision were determined for the samples. The application of the AP assays to surface water was unfeasible due to CBZ concentrations below the quantifiable concentration range. Surface as well as waste water samples could be analyzed with the HRP assays. Here, the HRP assay employing the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine yielded the best results.

Introduction

Carbamazepine (CBZ) is a therapeutic pharmaceutical for treating bipolar disorder and controlling epileptic seizures.[1] Annually, 87 tons of the 1014 tons consumed worldwide are taken in Germany. About 30% of the oral intake (daily dose ~1 g) is excreted unmetabolized through faeces (28%) and urine (2%).[2] Therefore, CBZ concentrations in the microgram per liter range are expected in the influent of waste water treatment plants (WWTPs).[2] The CBZ removal rate during waste water treatment is very low with only 7%.[3] The cleavage of CBZ conjugates can be higher than the CBZ removal and therefore, CBZ concentrations in the effluent might be elevated compared to the influent. This phenomenon was observed in 2002 for Berlin's waste water with maximum concentrations of 3.8 μg L^{-1} in influent and 5.0 μg L^{-1} in effluent.[4] Berlin's waste water treatment includes a mechanical and biological purification stage, phosphate elimination, nitrification, and denitrification. CBZ elimination from waste water can be achieved with, *e.g.*, membrane filtration,[5] membrane bioreactors,[6] ozonation,[7,8] and hydrodynamic-acoustic-cavitation.[9]

CBZ concentrations of up to 1 μg L^{-1} were determined in Berlin's surface water.[4] Ternes found CBZ in 24 of 26 surface water samples;[3] even in the brackish water of a lagoon CBZ was detected in the low ng L^{-1} range.[10] In drinking water, the antiepileptic drug was detected in concentrations up to 42 ng L^{-1}.[11] Assuming a daily water consumption of 2 L per adult, the CBZ intake is still considerably below the pharmaceutical dosage. In consequence, the CBZ intake *via* drinking water causes no health risk. The German Federal Environment Agency operates with health orientation values for surface water used for drinking water preparation which stipulate that a concentration below 0.3 μg L^{-1} CBZ is required. CBZ also presents an interesting anthropogenic marker for the water cycle.

The CBZ concentration in water samples can be determined by chromatographic methods such as liquid chromatography-tandem mass spectrometry (LC-MS/MS)[12] or immunochemical methods.[13,14] Only recently a direct competitive enzyme immunoassay (EIA) using the enzyme horseradish peroxidase (HRP) and the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB) has been developed for CBZ yielding a sensitivity of 130 ng L^{-1} and a measurement range of 50–50 000 ng L^{-1}.[14] Other direct immunoassays yielded similar results in regard to sensitivity: for ethinylestradiol 136 ng L^{-1} and caffeine 180 ng L^{-1} were reported.[15,16] An EIA for estradiol employing the enzyme alkaline phosphatase (AP) in combination with a chemiluminescent substrate yielded a very low sensitivity of 2 ng L^{-1}.[17] Competitive EIAs for the protein gliadin relevant in coeliac disease yielded assay sensitivities of 76 μg L^{-1} for HRP TMB, 53 μg L^{-1} for HRP 3-(4-hydroxyphenyl)propionic acid (HPPA), and 12 μg L^{-1} for AP 4-methylumbelliferyl phosphate (MUP).[18]

Specific parameters to compare and evaluate immunoassay formats have been defined in a previous publication and applied to caffeine immunoassays: sensitivity, measurement range, relative dynamic range (RDR), and goodness of fit of the

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standard curves as well as accuracy and precision in terms of intra- and inter-plate coefficients of variation (CVs).[19] The assessment of these criteria for direct competitive immunoassays for CBZ was extended to other luminescence detection techniques such as chemiluminescence. Additionally, the application to water samples is reported here.

Experimental

Reagents and materials

Chemicals and solvents were purchased in the best available quality from Merck KGaA (Darmstadt, Germany), Sigma-Aldrich (Taufkirchen, Germany), Mallinckrodt Baker (Griesheim, Germany), and Serva (Heidelberg, Germany). Terbium(III)chloride hexahydrate (99% purity) was obtained from Acros Organics, Thermo Fisher Scientific (Rockford, IL, USA). The enzymes HRP (EIA grade) and AP (EIA grade) were obtained from Roche (Mannheim, Germany). The CBZ reference standard, the anti-mouse IgG antibody as well as the anti-CBZ antibody were identical to the chemicals described in a previous study.[20] The chemiluminescent HRP substrate Super Signal ELISA Femto Maximum Sensitivity (SSE) was obtained from Pierce Perbio (Thermo Fisher Scientific). For the chemiluminescent AP substrate, "AP juice" (with enhancer at 450 nm, low background) from p.j.k. GmbH (Kleinblittersdorf, Germany) was used.

High-binding microtiter plates (MTPs) with 96 flat-bottomed wells were purchased from Greiner Bio-One (Frickenhausen, Germany); additionally, white Lumitrac 600 MTPs were employed for chemiluminescence measurements. Buffers (phosphate-buffered saline (PBS), tris(hydroxymethyl)aminomethane (TRIS), PBS-based and diethanolamine (DEA)-based washing buffers, sample buffer, and DEA substrate buffer), as well as calibrators were prepared and the same instruments (washer, shaker) were used as described in previous studies.[19,20]

CBZ enzyme conjugate synthesis and coupling ratio analysis

The CBZ derivative (CBZ-triglycine) and HRP conjugate were synthesized as described by Bahlmann *et al.*[14] The *N*-hydroxysuccinimide (NHS)/*N*,*N*′-dicyclohexylcarbodiimide (DCC) activated ester method was used for the synthesis of a CBZ–AP conjugate by analogy with the synthesis described for the CBZ–HRP conjugate. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry measurements were carried out as described by Grandke *et al.*[19] Masses of 45 290 Da and 44 210 Da *m*/*z* for the single-charged species were determined for the CBZ–HRP conjugate and the unmodified HRP, respectively. The analyte derivative minus water has an *m*/*z* of 390 Da. Consequently, the mean coupling ratio was 2.8 molecules of CBZ derivative per HRP molecule. For the AP conjugate, masses of 62 095 Da and 57 931 Da were assigned to the conjugate and the pure enzyme resulting in an average of 10.7 molecules of CBZ derivative per AP molecule.

The protein concentrations of the CBZ–HRP conjugate and the CBZ–AP conjugate were determined to be 9.0 mg mL^{-1} and 0.24 mg mL^{-1}, respectively; according to the Bradford method as described before.[19,21]

Immunoassay procedures

Direct competitive immunoassays were performed employing either the enzyme HRP or AP as label and chromogenic, fluorogenic or chemiluminogenic substrates. Each well was coated with 200 μL 1 mg L^{-1} anti-mouse IgG antibody in PBS buffer (pH 7.6). MTPs were covered with Parafilm® M and shaken at 750 rpm for 18 h. The MTPs were then washed three times with an automatic plate washer. A PBS-based washing buffer (pH 7.6) was used for all washing steps of the HRP immunoassays whereas a DEA-based washing buffer (pH 9.8) was used for all AP assays.

Subsequently, 200 μL anti-CBZ antibody (8.6 μg L^{-1}) in PBS buffer were added to each well and incubated for 60 min. After completion of another washing step, 150 μL calibrator (0 ng L^{-1} to 200 000 ng L^{-1}) or sample were added to each well. 32 calibrators were used to obtain a standard curve with precision profile, while 8 calibrators were applied to determine the CBZ content of 24 samples per plate. The calibrators or samples ($N = 3$) were measured with identical sample distribution on two separate plates.

8 min after adding the sample or the calibrator, 50 μL of CBZ enzyme conjugate were added. The CBZ–HRP conjugate was diluted in sample buffer (pH 9.5). Different concentrations of the enzyme conjugate were used, depending on the substrate employed: 18 μg L^{-1} for TMB and HPPA, and 12 μg L^{-1} for SSE. The CBZ–AP conjugate was diluted in TRIS buffer (pH 8.5). Here, the following concentrations were used: 12 μg L^{-1} for *para*-nitrophenyl phosphate (*p*NPP), 3.5 μg L^{-1} for MUP, 3.0 μg L^{-1} for diflunisal phosphate in complex with terbium (DIFP + Tb) and 2.4 μg L^{-1} for AP juice. After a 30 min incubation period and another washing step, the substrate solution was added. The substrate solutions had the following composition for one MTP, but the actual volumes prepared for the experiments were adjusted to the number of MTPs (at least two and up to four). The substrates TMB, HPPA, *p*NPP and MUP were prepared as described by Grandke *et al.*[19]

(i) For HRP chemiluminescence measurements, equal volumes of SSE substrate components A and B were mixed. 100 μL of this freshly prepared mixture were added to each well. After 5 min the emission at all wavelengths (360–630 nm) was measured and the signal was integrated over a period of 500 ms.

(ii) The AP substrate diflunisal phosphate (DIFP) was synthesized according to Evangelista *et al.*[22] A DIFP stock solution (4 mM) was prepared in 0.1 M NaOH. A 0.5 mM DIFP working solution was prepared in DEA substrate buffer and 100 μL were pipetted into each well. After an incubation period of 45 min, 100 μL 3 mM Tb-EDTA solution in CAPS buffer (125 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 12.6) were added.[23] Both 30 mM stock solutions were prepared in ultrapure water. After 5 min the reaction was stopped by adding 100 μL CAPS buffer. Time-resolved fluorescence was excited at 337 nm (530 nm cutoff filter) and detected at 544 nm. The delay as well as the integration time was set to 400 μs.

(iii) 100 μL AP juice was added to each well. After 90 min the signal was measured as the integrated emission of all wavelengths (360–630 nm) over an interval of 500 ms.

The immunoassays described above were thoroughly optimized. Parameters studied for optimization included buffer composition and pH as well as the concentrations and incubation times of the substrates, enzyme conjugates, and antibodies used.

A 96-channel pipette Liquidator[96] from Steinbrenner Laborsysteme (Wiesenbach, Germany) with tips from Mettler-Toledo (Giessen, Germany) was employed for all pipetting steps; here, a 15 mL surplus of each solution had to be used to ensure a sufficient volume in each well. The SpectraMax M5 multimode reader with corresponding software was employed for all measurements in the top reading mode with an adapter.

Immunoassay evaluation

All calibrators and water samples were analyzed in triplicate on two MTPs and the values were subjected to a Grubbs outlier test ($\alpha = 0.01$). Sigmoidal standard curves were obtained for each MTP by fitting a four-parameter logistic function to the mean signal intensities of each set of 32 calibrators.[20] The measured intensities varied slightly between MTPs and were therefore normalized by dividing the measured intensities by the difference of the upper and lower asymptote of the curve. In consequence, the normalized values of the individual plates were combined, averaged, and fitted to a new unified sigmoidal standard curve using the Origin 8G software (OriginLab, Northampton, USA). Doing so, the standard curves of the different substrates can be compared more easily and effectively. The parameter sensitivity, measurement range, RDR and goodness of fit for the standard curves were determined as described before.[19]

The water samples were analyzed with calibrators on two separate MTPs. Eight calibrators were used to obtain a calibration curve for each MTP. The resulting CBZ concentrations were combined for both MTPs and averaged. Intra- and inter-plate precisions were determined in 6 × 4 replicates as explained previously.[19]

LC-MS/MS

The Agilent 1100 LC system from Agilent Technologies (Waldbronn, Germany) was coupled to an API 4000 triple quadrupole mass spectrometer from Applied Biosystems (Darmstadt, Germany); here, the same settings and injection volumes were used as described before.[19] A binary gradient consisting of 10 mM ammonium acetate and 0.1% acetic acid in water (A) and methanol (B) was used: starting with 80% A, isocratic for 3 min, with a linear decrease to 5% A within 17 min, maintained at 5% A for 8 min, and then increased to 80% within 1 min and kept for 9 min. The acquisition was done in duplicate in the Multiple Reaction Monitoring (MRM) mode. The first transition MRM1, m/z 237 → 194, was used for quantification by integrating the peak area, and the second one, MRM2, m/z 237 → 179, for confirmation.

Water samples

Surface and waste water samples were collected in Berlin, Germany. 24 waste water samples were provided by Berliner Wasserbetriebe from the six WWTPs,[13] one influent and one effluent sample on two different dates. Additionally, 24 surface water samples were collected as grab samples at different rivers and lakes including Landwehrkanal, Teltowkanal, Spree, Dahme, Panke, and Wannsee.

Results and discussion

Quality assessment of immunoassay formats

The evaluation of the immunoassays was performed on the basis of the sigmoidal standard curves obtained for the different enzyme labels and substrates. From these standard curves, the values of the test midpoint, the RDR, and the coefficient of determination were extracted. The precision profile, as the relative error of the concentration, was calculated from the standard deviations of the mean signal for each calibrator. The range with a relative error of the concentration below 30% was assigned the measurement range of the respective assay. For the HRP assays, the chromogenic TMB, the fluorescent HPPA, and the chemiluminescent SSE substrate were employed (Fig. 1). Characteristic standard curves for the AP assays were acquired using four different substrates: *p*NPP, MUP, DIFP + Tb, and AP juice (Table 1).

Sensitivity

The assay sensitivities were compared on the basis of the test midpoints C (point of inflection ≈ IC_{50}). The highest sensitivity, determined as the lowest test midpoint, was found for the HRP substrate SSE (Table 1). No significant variations were found between the test midpoints of the HRP assays. The assays performed with the HRP conjugates provided better sensitivities than the AP label. The test midpoints of the different AP assays were in a similar range with the exception of the less sensitive *p*NPP assay. If the AP label is to be used, then AP juice seems to be the most sensitive substrate as revealed in this study.

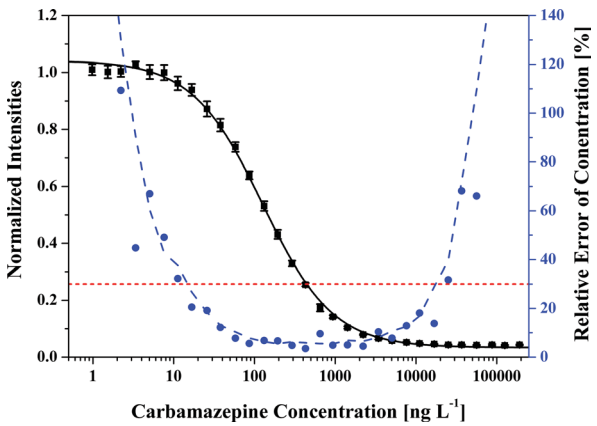

Fig. 1 Standard curve (solid black line, the normalized intensities are depicted as a function of the CBZ concentration), corresponding precision profile (dashed blue line, the relative error of concentration), and measurement range (intersection points at 30% relative error of concentration = dotted red line) are shown for the HRP HPPA immunoassay.

Table 1 Distinctive values of the standard curves: test midpoint C [in ng L^{-1}], measurement range [in ng L^{-1}], relative dynamic range (RDR), and coefficient of determination R^2

Assay name/substrate	Enzyme	Detection	C [ng L^{-1}]	Measurement range [ng L^{-1}]	RDR	R^2
TMB	HRP	Absorbance	147	16.6–19 500	0.98	1.000
HPPA	HRP	Fluorescence	126	14.6–17 400	0.97	0.999
SSE[a]	HRP	Chemiluminescence	121	17.8–27 100	1.00	0.997
*p*NPP	AP	Absorbance	867	131–19 000	0.54	0.998
MUP	AP	Fluorescence	333	186–5170	0.80	0.995
DIFP + Tb	AP	Time-resolved fluorescence	304	175–8540	0.85	0.994
AP juice[b]	AP	Chemiluminescence	201	60.8–93 700	0.99	0.998

[a] Super Signal ELISA Femto Maximum Sensitivity substrate from Pierce Perbio, Thermo Fisher Scientific. [b] AP juice from p.j.k. GmbH.

Measurement range

CBZ quantification is possible within the measurement range determined by the precision profile with a relative error of concentration below 30%. The range ought to cover three orders of magnitude. All HRP immunoassays as well as the AP juice assay fulfilled this requirement. The largest measurement ranges were found for the assays with chemiluminescent substrates. The smallest measurement range was obtained for the MUP assay. The lowest CBZ concentrations quantifiable were three to twelve times higher for the AP assays compared to the HRP assays.

Relative dynamic range

The HRP assays and the AP juice assay exceeded the required value of 0.90 for the RDR. All other AP assays did not reach this value. The *p*NPP assay showed a significantly higher background and as a result a reduced RDR.

Goodness of fit

The coefficient of determination R^2 was used to judge the quality of the fitted function to the data points. R^2 is an indicator for goodness of fit. All the immunoassays performed showed very high R^2 values (>0.990). In order to assess the quality of the standard curves, the normalized standard deviations of the mean signals were also compared for all assay formats. The highest standard deviation for each curve increased from 0.033 for HPPA, 0.045 for TMB, 0.060 for AP juice, 0.066 for SSE, 0.096 for *p*NPP, 0.113 for MUP to 0.150 for DIFP + Tb. Based on the combination of the reasonably low R^2 values and rather high standard deviations for the MUP and DIFP + Tb assay, it can be concluded that the goodness of fit for these standard curves is insufficient.

Summarizing the above-mentioned criteria for the standard curves, the three HRP assays (TMB, HPPA, and SSE) and the AP juice assay are most suitable for the determination of CBZ. The suitability was particularly apparent in the parameter sensitivity, measurement range and RDR.

Quality assessment in water sample analysis

The CBZ concentrations of different waste water (influent and effluent) and surface water in Berlin, Germany, were determined with the immunoassays suitable for CBZ determination and the results were compared to the reference method LC-MS/MS. The CBZ antibody shows a high cross-reactivity (CR), *e.g.*, towards the CBZ metabolites 10,11-epoxy-CBZ and 2-hydroxy-CBZ;[14] therefore, linear correlations between the results for immunoassays and LC-MS/MS are not reliable. In each water sample, different concentrations of cross-reactants are present and their composition can vary.

For the antihistaminic pharmaceutical cetirizine the highest CR was observed; moreover, the CR for this analyte is pH-dependent: 403% (pH 4.5) and 22% (pH 10.5).[24] For immunoassay performances, this is especially important during the competition step. Nevertheless, a buffer with pH 9.5 was used for the HRP assays (CR 50%) for this step, because buffers with pH 10.5 led to lower upper asymptotes for the standard curves,[24] and therefore the RDR would be reduced. If a higher accuracy is desired, a higher pH should be chosen.

Different buffers with different pH values were used for the HRP and AP assays. The TRIS buffer (pH 8.5) was used for the AP assays because the sample buffer (pH 9.5) used for the HRP assays reduced the activity of the AP enzyme and decreased the sensitivity. Therefore, a more pronounced overestimation is expected for the AP assays compared to the assays using the HRP conjugate. The pH values of the samples were determined, but no noticeable differences were detected.

The CBZ concentrations of the water samples were determined with all HRP assays and the AP juice assay. The other AP immunoassays did not fulfil the requirements for the standard curves and were therefore not taken into consideration.

Application on waste water samples

The matrices of the influent and effluent samples are very different because comprehensive purification steps are performed in WWTPs. Therefore, the intra- and inter-plate CVs were determined separately (Fig. 2). All the assays tested here showed an inter-plate CV lower than 20%, even below 10%, with the exception of the SSE assay (12.1% for the influent), thus fulfilling this requirement. The intra-plate CVs should not exceed 10%. This requirement was met by all assays for the influent as well as the effluent; more specifically the highest values were determined as 5.5% and 4.3% for TMB, 8.3% and

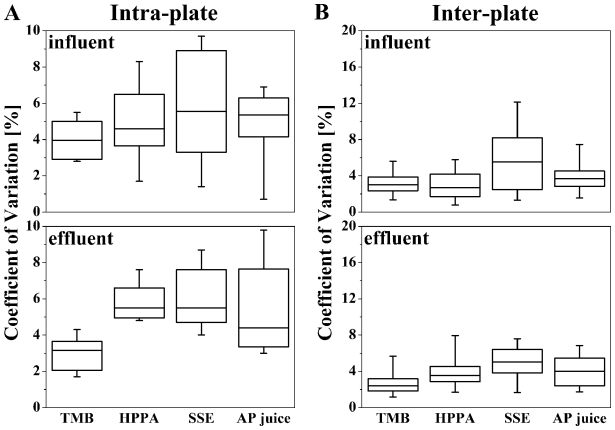

Fig. 2 Intra- (A) and inter-plate (B) coefficients of variation (CVs, %) were determined for the three HRP assays using the chromogenic TMB, the fluorogenic HPPA, and the chemiluminogenic SSE as well as for the immunoassay using the chemiluminogenic AP juice substrate for the application in WWTP influent (top) and effluent (bottom) samples. The ranges of the *y*-axis were chosen based on the requirements for the intra- and inter-plate precision (10 and 20%, respectively).

7.6% for HPPA, 9.7% and 8.7% for SSE, and 6.9% and 9.8% for AP juice, respectively.

The results of the waste water analysis correlate well with the results obtained for the evaluation of standard curves and precision profiles. The TMB assay as well as the AP juice assay show the smallest dispersion ranges (25% to 75% percentile) for the influent, whereas the TMB and HPPA assays are most suitable for the effluent with respect to intra- and inter-plate precision. For waste water screenings, it is desirable that the assay used is suitable for influent and effluent samples. The TMB assay is therefore recommended for either application.

Exemplarily, the applicability is shown for the WWTP Waßmannsdorf; the effluent of this WWTP is discharged directly into the river Teltowkanal. Surface water samples were taken before (Teltowkanal 1) and after (Teltowkanal 2) the input of the WWTP (influent and effluent) (Fig. 3). The CBZ concentrations at Teltowkanal 1 were quantified in the range of 36 to 42 ng L^{-1} with the HRP immunoassays, whereas LC-MS/MS measurement allowed detection, but not quantification of CBZ in this sample (limit of quantification: 100 ng L^{-1}). The AP juice assay was not sensitive enough to detect CBZ in this sample. In the untreated waste water sample CBZ concentrations of 1.2 μg L^{-1} (LC-MS/MS), 1.8 μg L^{-1} (TMB), 1.9 μg L^{-1} (HPPA), 2.0 μg L^{-1} (SSE), and 2.0 μg L^{-1} (AP juice) were determined. The overestimation with immunoassays in the influent is a result of the high CR of the antibody to cetirizine and CBZ metabolites.[24] All immunoassays yielded similar concentrations in the influent. However, in the effluent, the AP juice assay showed an underestimation (1.5 μg L^{-1}) compared to the HRP assays (2.0–2.3 μg L^{-1}). This was also the case for the other monitored WWTP samples.

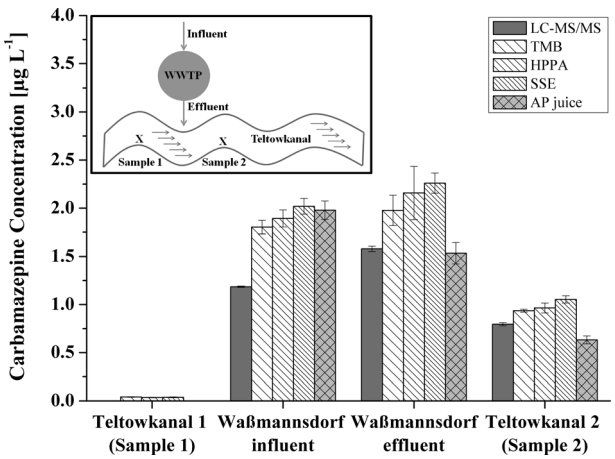

Fig. 3 CBZ concentrations in Teltowkanal (surface water) and WWTP Waßmannsdorf (influent and effluent) measured with four different immunoassays (the three HRP assays using TMB, HPPA and SSE as substrate as well as the AP assay with AP juice) and reference method LC-MS/MS (Inset: sampling positions).

The concentration determined by LC-MS/MS for the Waßmannsdorf effluent (1.6 μg L^{-1}) is ~33% higher compared to the influent. This increase may be the result of the higher degradation of CBZ metabolites (*e.g.*, CBZ-*N*-glucuronide) to CBZ during the waste water treatment[25] than the CBZ degradation itself (7%).[3] The lower overestimation of the HRP assays in relation to the reference method in the effluent is possibly caused by degradation of cetirizine (16%).[26] A reduced CBZ concentration was found for the surface water collected after the waste water discharge (Teltowkanal 2) due to the dilution of the treated waste water. The CBZ contents were 0.8 μg L^{-1} (LC-MS/MS), 0.9 μg L^{-1} (TMB), 1.0 μg L^{-1} (HPPA), and 1.1 μg L^{-1} (SSE). The AP juice assay (0.6 μg L^{-1}) showed an underestimation even to the LC-MS/MS results. Hence, this expensive substrate may only be used for CBZ measurements in influents; but in respect of the limited scope of application it is not recommended.

Application for surface water samples

In 23 out of 24 surface water samples, CBZ was detected with LC-MS/MS (limit of detection: 30 ng L^{-1}) but not reliably quantified. Only half of the surface water samples had CBZ concentrations which fell within the limits of the AP juice measurement range. Therefore, the CBZ concentrations of the surface water samples were only investigated with the HRP assays. The determined CBZ concentrations of Berlin's surface water are shown for four examples (Fig. 4A). The values between the HRP assays did not differ from each other within the measurement uncertainties. CBZ concentrations of 67–73 ng L^{-1} for sample 1 (Rummelsburger Bucht), 94–96 ng L^{-1} for sample 2 (Spree), 120–125 ng L^{-1} for sample 3 (Spandauer Schifffahrtskanal) and 133–142 ng L^{-1} for sample 4 (Landwehrkanal) were determined with the HRP assays.

The lowest intra- and inter-plate CVs were observed for the TMB assay (Fig. 4B, 1.5–8.5%, 1.1–7.7%). The intra-plate CVs were slightly higher for the HPPA assay (1.0–9.1%) and the SSE assay (3.2–9.6%). The results for the inter-plate CVs confirmed this trend: the ranges of 1.4–15% and 1.2–14% were determined for the HPPA and the SSE assay for the surface water samples. The CV values for all assays did not exceed the required thresholds for the intra- (10%) and inter-plate CVs (20%) and

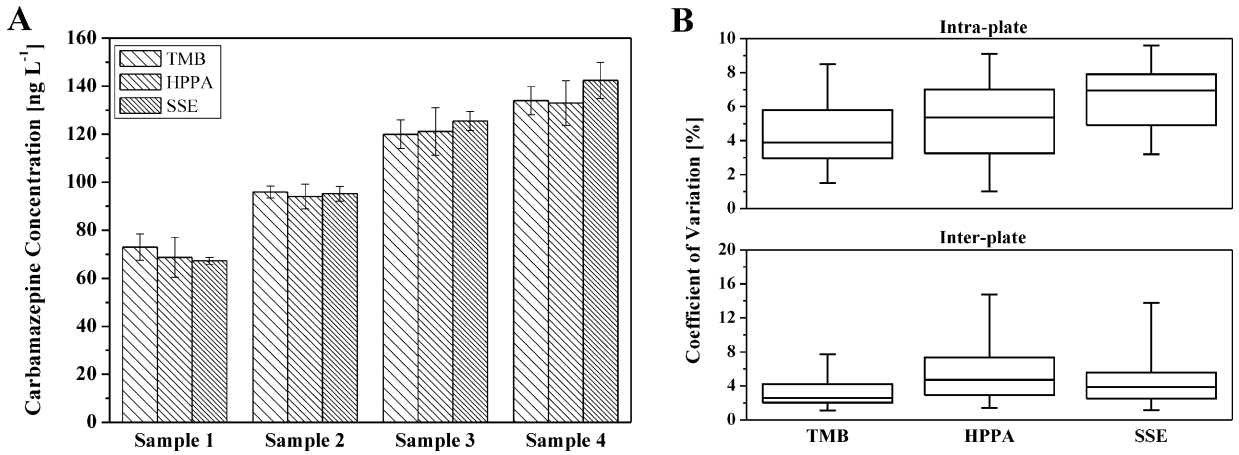

Fig. 4 CBZ concentrations of four surface water samples as determined by all HRP assays (A). The intra- (top) and inter-plate (bottom) coefficients of variation (CVs, %) of selected surface water have been illustrated as box-plots for the HRP assays using the substrates TMB, HPPA, and SSE (B).

can be used for CBZ measurements. However, the TMB assay shows the best performance for the application on surface water.

Conclusions

As revealed by the systematic assessment of the different assays for the determination of CBZ in various water samples, the previously defined quality criteria can be transferred to CBZ assays. Only the accuracy of analysis could not be assessed against a reference method due to the high cross-reactivity of the antibody used. The required thresholds for the quality parameters for the standard curves were met for all HRP assays and the AP juice assay. The sample matrix had a substantial influence on the results obtained for each assay format as the immunoassays using HRP and AP conjugates revealed differing suitability. If the enzyme label AP is required for the assay, the chemiluminescent substrate AP juice is the substrate of choice in accordance with our results: four criteria were fulfilled for the standard curves and the highest precision was obtained. However, the AP juice assay is only suitable for waste water influent samples.

All HRP assays are suitable for the analysis of surface and waste water. For the standard curves, the SSE assay yielded the best results. This substrate is very expensive and requires a costly multi-mode microplate reader for the chemiluminescence detection. However, most laboratories are equipped with readers for absorbance measurement only. In consequence, for a widespread application, the chromogenic HRP substrate is the best choice. In addition, the lowest intra- and inter-plate CVs were obtained for all sample matrices tested with this format. All in all, the HRP TMB immunoassay is recommended for the application on any type of water sample.

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