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**Immunochemical Determination  
of Caffeine and Carbamazepine  
in Complex Matrices  
using Fluorescence Polarization**

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# **Immunochemical Determination of Caffeine and Carbamazepine in Complex Matrices using Fluorescence Polarization**

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## Abstract

Pharmacologically active compounds are omnipresent in contemporary daily life, in our food and in our environment. The fast and easy quantification of those substances is becoming a subject of global importance. The fluorescence polarization immunoassay (FPIA) is a homogeneous mix-and-read format and a suitable tool for this purpose that offers a high sample throughput. Yet, the applicability to complex matrices can be limited by possible interaction of matrix compounds with antibodies or tracer.

Caffeine is one of the most frequently consumed pharmacologically active compounds and is present in a large variety of consumer products, including beverages and cosmetics. Adverse health effects of high caffeine concentrations especially for pregnant women are under discussion. Therefore, and due to legal regulations, caffeine should be monitored. Automated FPIA measurements enabled the precise and accurate quantification of caffeine in beverages and cosmetics within 2 min. Samples could be highly diluted before analysis due to high assay sensitivity in the low  $\mu\text{g/L}$  range. Therefore, no matrix effects were observed.

The antiepileptic drug carbamazepine (CBZ) is discussed as a marker for the elimination efficiency of wastewater treatment plants and the dispersion of their respective effluents in surface water. The development of a FPIA for CBZ included the synthesis and evaluation of different tracers. Using the optimum tracer CBZ-triglycine-5-(aminoacetamido) fluorescein, CBZ concentrations in surface waters could be measured on different platforms: one sample within 4 min in tubes or 24 samples within 20 min on microtiter plates (MTPs). For this study, a commercially available antibody was used, which led to overestimations with recovery rates up to 140% due to high cross-reactivities towards CBZ metabolites and other pharmaceuticals.

For more accurate CBZ determination, a new monoclonal antibody was produced. In this attempt, methods for improving the monitoring during the production process were successfully applied, including feces screening and cell culture supernatant screening with FPIA. The new monoclonal antibody is highly specific for CBZ and showed mostly negligible cross-reactivities towards environmentally relevant compounds. Measurements at non-equilibrium state improved the sensitivity and selectivity of the developed FPIA due to slow binding kinetics of the new antibody. Additionally, this measure enables for CBZ determination over a measurement range of almost three orders of magnitude. The comprehensively characterized antibody was successfully applied for the development of sensitive homogeneous and heterogeneous immunoassays.

The new antibody made the development of an on-site measurement system for the determination of CBZ in wastewater possible. After comprehensive optimization, this automated FPIA platform allows the precise quantification of CBZ in wastewater samples only pre-treated by filtration within 16 min. Recovery rates of 61 to 104% were observed. Measurements in the low  $\mu\text{g/L}$  range are possible without the application of tedious sample preparation techniques.

Different FPIA platforms including MTPs, cuvettes and tubes were successfully applied. For the choice of the right format, the application field should be considered, e.g. desired sample throughput, usage for optimization or characterization of antibodies or if a set-up for routine measurements is sought for. For high sample throughput and optimization, FPIA performance on MTPs is advantageous. The best results for the application to real samples were obtained using kinetic FP measurements in cuvettes.



## Kurzzusammenfassung

Pharmakologisch wirksame Substanzen sind weitverbreitet im täglichen Leben, unter anderem in Lebensmitteln und in der Umwelt. Die schnelle und einfache Überwachung dieser Substanzen nimmt an Bedeutung stetig zu. Für dieses Anliegen stellt der Fluoreszenz-polarisationsimmunoassay (FPIA) ein geeignetes Hilfsmittel dar und ermöglicht dabei einen hohen Probendurchsatz. Die Anwendung dieses Assays für komplexe Matrices ist limitiert durch mögliche Wechselwirkungen von Matrixbestandteilen mit dem Antikörper oder dem Tracer.

Koffein stellt eine der am häufigsten konsumierten pharmakologisch wirksamen Substanzen dar und kommt in einer Vielzahl von Konsumgütern, wie zum Beispiel in Getränken und kosmetischen Mitteln, vor. Negative gesundheitliche Effekte durch hohen Koffeinkonsum, vor allem für Schwangere, werden diskutiert. Aufgrund dessen und wegen gesetzlicher Regulierungen, sollte der Koffeingehalt verschiedener Produkte überwacht werden. Der automatisierte FPIA ermöglicht eine präzise und genaue Quantifizierung von Koffein in Getränken und kosmetischen Mitteln innerhalb von 2 min. Dank der hohen Sensitivität des Assays im niedrigen  $\mu\text{g/L}$  Bereich, konnten die Proben vor der Messung stark verdünnt werden, wodurch keine Matrixeffekte auftraten.

Das Antiepileptikum Carbamazepin (CBZ) wird als Marker für die Reinigungsleistung von Kläranlagen und die Verteilung der Abläufe in den Oberflächengewässern diskutiert. Die Entwicklung des CBZ-FPIAs beinhaltete die Synthese und den Vergleich verschiedener Tracer. Unter Verwendung des besten Tracers, CBZ-Triglycin-5-(Aminoacetamido) Fluoreszein, konnten CBZ-Konzentrationen in Oberflächengewässern auf verschiedenen Plattformen gemessen werden: eine Probe konnte innerhalb von 4 min in Röhrchen gemessen werden, während 24 Proben auf Mikrotiterplatten (MTPs) innerhalb von 20 min vermessen wurden. Für diese Untersuchungen wurde ein kommerziell erhältlicher Antikörper verwendet. Dies führte auf Grund hoher Kreuzreaktivitäten gegenüber CBZ-Metaboliten und anderen Pharmazeutika zu Überbestimmungen mit Wiederfindungsraten von bis zu 140 %.

Für eine genauere CBZ-Bestimmung wurde ein neuer monoklonaler Antikörper produziert. Dabei wurden Methoden zur Verbesserung der Überwachung des Herstellungsprozesses erfolgreich angewendet. Dies beinhaltete die Untersuchung von Mäusekot und die Anwendung des FPIA für das Screening der Zellkulturüberstände. Der neue monoklonale Antikörper zeigt CBZ gegenüber eine hohe Spezifität und größtenteils vernachlässigbar geringe Kreuzreaktivitäten gegenüber umweltrelevanten Substanzen. Die Sensitivität und Selektivität des entwickelten FPIA konnten auf Grund der hohen Zeitabhängigkeit der Antigen/Antikörper Reaktion durch Messungen vor dem Erreichen des Gleichgewichts verbessert werden. Mit diesem umfangreich charakterisierten Antikörper konnten sensitive homogene und heterogene Immunoassays entwickelt werden.

Der neue Antikörper ermöglichte die Entwicklung eines Vorort-Messsystems für die CBZ-Bestimmung in Abwasser. Dieses automatisierte FPIA-Format erlaubt die präzise Quantifizierung von filtrierten Abwasserproben innerhalb von 16 min. Die Wiederfindungsraten lagen zwischen 61 und 104 %. CBZ-Konzentrationen im niedrigen  $\mu\text{g/L}$ -Bereich konnten bestimmt werden, wobei hierfür keine aufwändigen Probenvorbereitungstechniken erforderlich waren.

Der FPIA wurde erfolgreich auf verschiedenen Messplattformen durchgeführt. Dies beinhaltete MTPs, Küvetten und Röhrchen. Für die Wahl des richtigen Formats, sollte die gewünschte Anwendung wie zum Beispiel der angestrebte Probendurchsatz, die Anwendung zur Assayoptimierung oder Charakterisierung von Antikörpern oder der Wunsch nach Durchführung von Routinemessungen, in Betracht gezogen werden. Für einen hohen Durchsatz und die Optimierung von Assays empfiehlt sich die Verwendung von MTPs. Für die Anwendung auf Realproben wurden mit der kinetischen FP-Messung in Küvetten die besten Resultate erzielt.

## Abbreviations

<i>A</i>	parameter <i>A</i> of sigmoidal curve, representing the maximum signal intensity
AAF	5-(aminoacetamido)fluorescein
<i>B</i>	parameter <i>B</i> of sigmoidal curve, slope at the inflection point
BSA	bovine serum albumin
<i>C</i>	parameter <i>C</i> of sigmoidal curve, inflection point (in concentration units)
CafD	caffeine derivative, 7-(5-carboxypentyl)-1,3-dimethylxanthine
CBZ	carbamazepine
CBZ-AAF	carbamazepine-triglycine-5-(aminoacetamido)fluorescein
CBZ-BSA	carbamazepine-triglycine-bovine serum albumin
CBZ-HRP	carbamazepine-triglycine-horseradish peroxidase
CBZ-OVA	carbamazepine-triglycine-ovalbumin
CET	cetirizine
CR	cross-reactivity
CV	coefficient of variation
<i>D</i>	parameter <i>D</i> of sigmoidal curve, representing the minimal signal intensity
DBA	dibenz[ <i>b,f</i> ]azepine-5-carbonyl chloride
DCC	dicyclohexylcarbodiimide
DF	dilution factor
DiH-CBZ	10,11-dihydro-carbamazepine
DiOH-CBZ	10,11-dihydro-10,11-dihydroxy-carbamazepine
DMF	dimethylformamide
DR	dynamic range
EDF	ethylenediamine thiocarbamoylfluorescein
EDTA	disodium ethylenediaminetetraacetic acid dihydrate
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
Ep-CBZ	10,11-epoxy-carbamazepine
FITC	fluorescein isothiocyanate
FP	fluorescence polarization
FPIA	fluorescence polarization immunoassay
FPIA 1	caffeine FPIA in cuvettes
FPIA 2	caffeine FPIA in MTPs
FRET	fluorescence resonance energy transfer

G	G factor; for calculation of the degree of polarization
GC	gas chromatography
HAT	hypoxanthine-aminopterin-thymidine
9-HMCA	9-hydroxymethyl-10-carbamoylacridan
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IC <sub>50</sub>	analyte concentration at the half maximum signal intensity
<i>I</i> <sub>Par</sub>	fluorescence intensity in parallel direction; for calculation of <i>P</i>
<i>I</i> <sub>Per</sub>	fluorescence intensity in perpendicular direction; for calculation of <i>P</i>
LC	liquid chromatography
LHW	liquid handling workstation
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
MS/MS	tandem mass spectrometry
MTP	microtiter plate
NHS	<i>N</i> -hydroxysuccinimide
OD	optical density
10-OH-CBZ	10,11-dihydro-10-hydroxy-CBZ
OTA	ochratoxin A
OVA	ovalbumin
Ox-CBZ	oxcarbazepine
<i>P</i>	degree of polarization (unit: mP)
<i>P</i> <sub>max</sub>	maximum degree of polarization (unit: mP)
PBS	phosphate buffered saline
PP	precision profile
<i>R</i> <sup>2</sup>	coefficient of determination
RDR	relative dynamic range
RFU	relative fluorescence unit
SBG	sample background
SPE	solid-phase extraction
StD	standard deviation
TMB	3,3',5,5'-tetramethylbenzidine
TRIS	tris-(hydroxymethyl)aminomethane
WWTP	wastewater treatment plant

# 1. Introduction

## 1.1 Immunoassay

Immunoassays are bioanalytical methods that are based on the specificity of the binding between an antibody and its antigen. The application range of these assays includes diagnostics, clinical and biochemical research, food and environmental analysis. The first immunoassay was developed by Yalow and Berson in 1960. They used a radioimmunoassay for the detection of insulin.<sup>1</sup> From then on, the development and improvement of a diversity of immunoassays started and is still ongoing.

Immunoassays are characterized by high sensitivity that can reach the zeptomolar range.<sup>2,3</sup> Due to the high specificity of the antibody-antigen interaction, most types of samples can be measured without any preparation step as they are usually required for instrumental methods. However, immunoassays are typically single-analyte methods. There are some approaches to determine more than one analyte with one antibody. These include the variation of pH values or the determination of sum parameters.<sup>4-8</sup> Both approaches are based on the cross-reactivity (CR) of antibodies against structurally related substances (more about CR in Section 1.3).

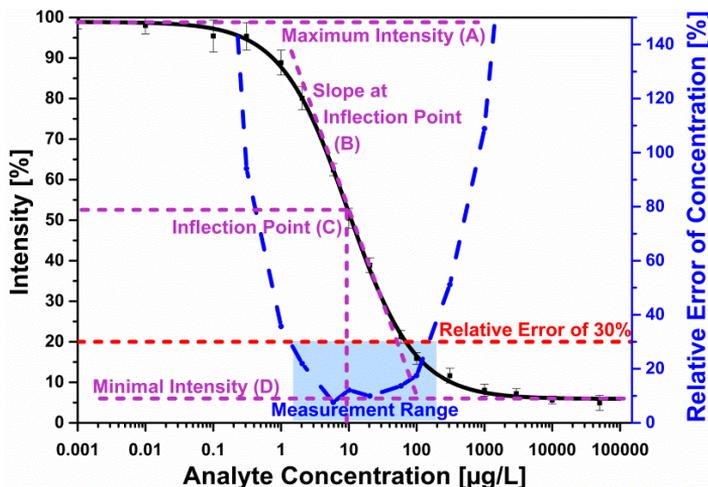
Immunoassays can be divided into different groups and subgroups. One differentiation is the classification into competitive and non-competitive assays. Non-competitive assays, also known as sandwich immunoassays, can be used for the detection of big molecules, e.g. proteins, where two antibodies can bind to the antigen at the same time. Here, the analyte is bound by two antibodies of which one is labeled which enables the detection of the complex. Many pharmaceutically active compounds, like those described in this work, are too small to be bound by two antibodies at the same time. Therefore a conjugated, sensitively detectable analyte is necessary which competes for the binding sites of the antibody with the free analyte from the sample. This type of assay is called competitive immunoassay.<sup>9</sup> The competition of conjugated and free analyte leads to an indirectly proportional sigmoidal calibration curve (*Figure 1*) which can be described using the following four-parameter function:<sup>10</sup>

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

$A$  describes the highest and  $D$  the lowest signal intensity, i.e. the signal intensity at infinitely low or high analyte concentrations, respectively.  $C$  indicates the test midpoint or inflection point at the half maximum intensity ( $\approx$  IC50; in concentration units).  $B$  describes the slope in this point.  $y$  and  $x$  represent the signal intensity and the analyte concentration, respectively. The measurement range can be determined by calculating the relative error of concentration as described by Ekins:<sup>11</sup>

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

StD represents the standard deviation of the signal intensity of each calibration point. Using this so called precision profile, the range with a relative error of concentration lower than 30% was defined as measurement range following the “three sigma criterion” usually used for instrumental methods.<sup>12-16</sup> Other groups describe the working range as the range between 20 and 80% of inhibition, expressed as IC<sub>20</sub> and IC<sub>80</sub>.<sup>17-19</sup>



**Figure 1** Sigmoidal calibration curve (black solid line), precision profile (blue dashed line) and specific parameters for the evaluation of immunoassays are given.

Immunoassays can further be classified into heterogeneous and homogeneous formats. Heterogeneous assays include the immobilization of reagents to a solid phase. The best-known example is the enzyme-linked immunosorbent assay (ELISA). Here, two different competitive formats are known, direct and indirect ELISA. For indirect ELISA, the analyte is coupled to a protein, which is immobilized to the surface of a microtiter plate (MTP). Then the immobilized and the free analyte from the sample compete for the analyte-specific antibody. The more analyte is present in the sample, the less antibody will be bound to the immobilized analyte and vice versa. During the following washing step, all antibodies that are not bound to the immobilized analyte will be washed away. The detection is then performed by the addition of an antibody that binds specifically to the previously bound anti-analyte antibody. This secondary antibody is labeled with an enzyme, which converts, after another washing step, a substrate into a usually chromophore substance, which is then detected through absorbance. The alternative to this format is the direct ELISA. Here, the secondary antibody is immobilized to the surface. In the next step, the anti-analyte antibody binds to the secondary antibody, followed by the competition of the analyte and an enzyme-labeled analyte. After washing away the excess of enzyme-labeled analyte, the detection takes place as described above including the enzymatic conversion.<sup>9</sup>

Between all steps of heterogeneous assays, not bound reagents have to be washed away resulting in three washing steps for the direct ELISA. The incubation steps usually vary between 30 min and 1.5 h, besides the coating step which is typically performed overnight. This makes ELISA a tedious assay format. However, benefits of this method are the outstanding sensitivity and the high throughput; the assay is normally performed on 96-well

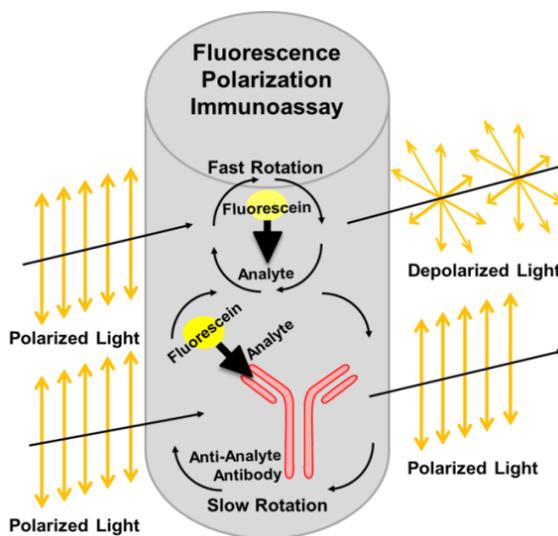
MTPs so that 24 samples can be determined at once in triplicate including an eight-point calibration on each MTP.

Homogeneous immunoassays do not require the immobilization of reagents. They can be performed in one phase and do not require any washing steps what makes them faster and easier to handle than heterogeneous immunoassays. Here, the signal detection is based on the change of specific properties due to the interaction between antigen and antibody of which at least one is typically labeled. Different types of detection can be used, many of them being based on fluorescence, e.g. increasing fluorescence due to conformation changes,<sup>20</sup> fluorescence resonance energy transfer (FRET) based fluorescence quenching,<sup>21</sup> FRET based time-resolved fluorescence measurement,<sup>22, 23</sup> fluorescence polarization (FP, Section 1.2), but also redox quenching can be utilized.<sup>24</sup> There are also homogeneous immunoassays that do not require any labels, because the fluorescence of the antibody itself is influenced while binding the analyte.<sup>25</sup>

## 1.2 Fluorescence polarization immunoassay

The FP immunoassay (FPIA) belongs to the group of homogeneous immunoassays. The first application of FP for the quantification of the antigen-antibody reaction was described by Dandliker and Feigen in 1961.<sup>26</sup> The main advantage of FPIA over commonly used ELISA is the expendability of washing steps what makes the assay much faster and easier to handle. Additionally, the fluorophore-labeled analytes for FPIA are usually much more stable than the enzyme tracers utilized for ELISA. Compared to other homogeneous immunoassays, only the analyte needs to be coupled to a fluorophore, whereas for other homogeneous assays like time-resolved FRET immunoassays the analyte and the antibody have to be labeled.<sup>22</sup>

In general, the detection of FP is based on the mass change of a fluorescent molecule. Small and light molecules rotate faster than big ones. So if a small fluorescent molecule is excited by linearly polarized light, it usually rotates before the light is emitted. Therefore the emitted light has another orientation than the exciting light



**Figure 2** Principle of FPIA.

(Figure 2). This means that the light emitted is depolarized, which corresponds in total to a low degree of polarization. If this small molecule, e.g. a fluorophore-labeled analyte, is bound to a big molecule, e.g. an analyte-specific antibody, the rotation of this complex is much slower and therefore the light will mostly be emitted polarizedly. So if many analyte molecules are present in a sample, most of the fluorophore-labeled analyte, the so-called

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tracer, remains unbound and the degree of polarization will be low. If no analyte is present in the sample, most of the tracer is bound and the degree of polarization will be high.

The degree of polarization is determined by measuring the fluorescence intensities in parallel ( $I_{Par}$ ) and perpendicular ( $I_{Per}$ ) direction to the polarized exciting light. The following formula is used to calculate the degree of polarization ( $P$ ) which is usually given in millipolarization units (mP):<sup>27</sup>

$$P = \frac{I_{Par} - G \cdot I_{Per}}{I_{Par} + G \cdot I_{Per}}$$

The phenomenon of FP is also known as anisotropy  $r$ . It only differs in the way of calculation; for anisotropy, the perpendicular intensity in the denominator is counted twice ( $I_{Par} + 2 \cdot I_{Per}$ ). This denominator term describes also the total fluorescence intensity of polarized light.<sup>27</sup>

$G$  represents the so-called  $G$  factor, which is a measure of the instrument-specific geometry. It is also dependent on the applied wavelength. The  $G$  factor is determined by measuring the intensities in parallel and perpendicular polarizer settings, while the polarizer for the exciting light is rotated by  $90^\circ$  compared to normal polarization measurement. The ratio of the perpendicularly and parallelly measured intensities is the  $G$  factor. This factor was especially important – because rather variable – in times when the instruments for FP measurements were home-built. Nowadays the  $G$  factor is not taken into considerations so much anymore due to the confidence in the accurate instrument design from manufacturers.<sup>27</sup>

The degree of polarization can be influenced by a variety of factors like the binding properties of the antibody and the tracer or analyte. The quantum yield, fluorescence lifetime and size of the tracer show a high impact on the degree of polarization. But also the viscosity and, in consequence, the temperature of the solvent or buffer influence the speed of rotation of the tracer and consequently the measured degree of polarization.<sup>27</sup>

Immunoassays using FP are only one of many application fields for this spectroscopic phenomenon. In general, the change of size of molecules or complexes can be detected as long as one of the components is able to fluoresce itself or is labeled with a fluorophore. FP can be used for investigations of protein-DNA interactions; but also enzymatic reactions can be analyzed by detecting smaller parts of proteins after the enzymatic digestion.<sup>28</sup> Furthermore, FP can be applied for cell imaging.<sup>27</sup>

### 1.2.1 Fluorophore tracer

The most important factors for the successful development of a FPIA are the choices of antibody and tracer. The first one will be discussed later on (Section 1.3). For the development of the optimum tracer, two main aspects have to be taken into consideration: the structure of the hapten (the part of the tracer that represents the analyte) and the kind of fluorophore.

The choice of hapten is crucial, because this part is recognized by the antibody. The structure should be similar to the analyte so that it can be recognized by the analyte-specific antibody. However, the affinity of the antibody towards the hapten should not be higher than towards the analyte, so that the analyte and the tracer can compete for the binding sites of

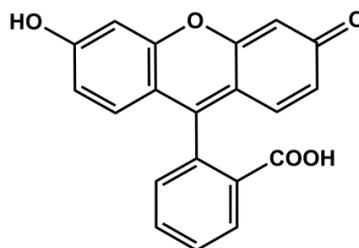
the antibody.<sup>29, 30</sup> It has been reported that the sensitivity of an assay can be increased by connecting the hapten and the fluorophore through a spacer. Usually the application of longer spacers is beneficial.<sup>31-34</sup> Of course, this is only true until a certain degree; the tracer should not get too big. Otherwise, the speed of rotation is reduced and consequently the degree of polarization of free tracer increases.

Fluorophores with high quantum yields are preferred for tracer synthesis. The quantum yield is defined as the ratio of emitted to absorbed photons. Rhodamine dyes, e.g. TAMRA, can reach quantum yields of up to 1, meaning that the same amount of photons that were absorbed are re-emitted.<sup>27, 35</sup> The quantum yield can be affected by different kinds of interactions, inter alia, it can be reduced through coupling to a hapten.<sup>36</sup> But this quenching effect can sometimes be reduced again by a change of tracer conformation through the binding to an antibody.<sup>20</sup>

The degree of polarization depends on the rotation rate of the molecule during the fluorescence lifetime. If the fluorescence lifetime is short, the molecule needs to rotate fast to emit the light depolarized.<sup>27</sup> Quantum dots show longer fluorescence lifetimes than traditional dyes. Thus, they offer the possibility to measure bigger analytes, because the slower rotation due to the larger molecule size can be compensated through the longer fluorescence lifetime. Additionally, they are highly photo- and chemically stable and show high quantum yields. Hence, quantum dots offer new perspectives for application in FPIA, e.g. for the detection of tumor marker proteins.<sup>37</sup>

A large Stokes' shift of the applied fluorophore is desirable to minimize the influence of scattering light during polarization measurement. Metal complexes of e.g. osmium<sup>38</sup> or ruthenium<sup>39</sup> show very high Stokes' shifts of up to 250 nm. Additionally, they are long-wavelength fluorophores, which simplifies the differentiation between the fluorescence from tracer and possible fluorescent matrix compounds. Nile blue, an oxazine dye, can also be used for the development of long-wavelength FPIAs.<sup>40</sup>

There is a wide range of fluorophores that can be and have been applied for FPIAs, including umbelliferyl derivatives,<sup>41</sup> "Alexa Fluor" dyes<sup>42</sup> and the ones mentioned above. Nevertheless, fluorescein (Figure 3) is still the by far most commonly used fluorophore for this kind of assay. In general, it is one of the most popular fluorophores, especially in bioanalysis. Fluorescein, which belongs to the group of xanthenes, was first synthesized by Adolf Baeyer in 1871.<sup>43</sup> The synthesis involved the reaction of phthalic acid and resorcin, which led to the second part of the name fluorescein. 'Fluo' was obviously chosen because of the fluorescence properties. This fluorophore is so popular since it is cheap, not patented and it allows the application in different coupling methods.<sup>27</sup> The disadvantages of fluorescein are its photodegradability and the pH dependence of its fluorescence properties.<sup>27</sup> At neutral pH, the lactone form of fluorescein is predominant, which is not fluorescent. The fluorescent dianion is formed in alkaline medium. The absorption maximum is at 490 nm and the emission maximum at 520 nm. Thus, the Stokes' shift is 30 nm.<sup>27</sup> The fluorescence lifetime is 4.1 ns. The quantum yield is relatively high with 0.93.<sup>44</sup> But the



**Figure 3** Chemical structure of fluorescein.

spectroscopic properties can vary for different derivatives of fluorescein.<sup>45</sup> The most popular derivatives are fluorescein isothiocyanate (FITC)<sup>29, 46-50</sup> and ethylenediamine thiocarbamoyl-fluorescein (EDF).<sup>34, 47, 51-55</sup> But there are a lot more fluorescein conjugates that can be used for tracer synthesis, e.g. 4'-(aminomethyl)fluorescein,<sup>56-59</sup> 5-(aminoacetamido)fluorescein (AAF),<sup>58, 60</sup> fluorescein amine,<sup>60, 61</sup> dichlorotriazinylamino fluorescein,<sup>46, 62</sup> 5-(5-aminopentyl-thioureidyl) fluorescein and fluorescein thiosemicarbazide.<sup>60</sup>

As mentioned before, fluorescein and its derivatives offer many different methods for tracer synthesis. If FITC is applied for tracer synthesis, the direct reaction of FITC and hapten in presence of triethylamine can be used for tracer synthesis.<sup>48-50</sup> Another common and easy way is the *N*-hydroxysuccinimide (NHS)/*N,N'*-dicyclohexylcarbodiimide (DCC) activated ester method.<sup>32, 34, 59</sup> The prerequisites are that the hapten contains a free carboxylic group and the fluorophore offers an amine group. First, the hapten forms a highly reactive ester with DCC which then reacts to an activated NHS ester. The by-product, dicyclohexylurea, is insoluble, precipitates and can be removed by centrifugation. The activated ester can then react with the amino group from the fluorophore; NHS is released.

### 1.2.2 Formats and instrumentation

Different formats for the performance of FPIAs can be utilized: the assay can be performed on MTPs, which enables a high sample throughput due to the possibility to measure theoretically 96 samples at the same time.<sup>31, 48, 63, 64</sup> Cuvettes can also be applied for the assay performance.<sup>50, 57, 59, 65</sup> This offers the possibility of an automatization of the assay. But the sample throughput on this platform is limited, because only one sample can be measured after the other. Thus, FPIAs in cuvettes are valuable for individual samples and on-site measurements. The instruments for the two formats usually use different measurement arrangements: MTP readers typically measure fluorescence in an epifluorescence mode using a dichroic mirror while for cuvette the emission is mostly measured in an angle of 90°.<sup>66</sup>

Typical excitation sources are Xe or Hg arc lamps. For higher intensities, it is also possible to utilize laser or LED, which is only suitable when a fixed wavelength should be used.<sup>27</sup> If Xe or Hg arc lamps are used, the desired excitation wavelength can be selected with a monochromator or filters. Using a monochromator, the selected wavelength can be easily changed and spectral scanning can be performed. This is advantageous when new assays or fluorophores are applied, especially when their spectroscopic properties are not known. But for defined and known wavelengths, the usage of filter is beneficial due to lower losses of the emitted light and therefore an intrinsically higher sensitivity.<sup>66</sup>

The selection of the emission wavelength is necessary to reduce the detection of scattering light which would influence the degree of polarization. Both, monochromator and filter, can be applied for this purpose.<sup>27</sup> In general, filter systems are a better choice for FP measurements, because the transmission efficiency of monochromators depends on the polarization of the light. Additionally, monochromators are more susceptible for scattering light and as mentioned before, this can influence the FP measurement.<sup>67</sup>

Most instruments for FP measurements use fixed polarizers for excitation. The polarizer for the emitted light is usually rotatable to an angle of 90° for the determination of parallel and

perpendicular fluorescence intensities. There are also some instruments that enable the simultaneous determination of both orientations by utilizing T optics. Polarizers can be thin films of stretched polymers, which are cheap, but show low transmission of UV light. They are not very robust, because they only transmit the light polarized in one orientation and absorb the light from all other directions.<sup>67</sup> A pair of birefringent prisms, typically calcite, can also be used as polarizers. All vectors of light, besides the chosen one, are separated or reflected in large angle, so that only the linearly polarized light in the desired orientation is transmitted. Although this kind of polarizer is more expensive, they are advantageous because of their higher robustness and greater transmission. The emitted light is usually detected with photomultiplier tubes or photodiodes.<sup>27</sup>

### 1.2.3 Application to real samples

FPIAs have a wide application range in diagnostics, food and environmental analysis. Usually concentrations in the  $\mu\text{g/L}$  to  $\text{mg/L}$  range can be determined.<sup>68</sup> Most frequently, mycotoxins, pesticides and pharmaceuticals are determined using this method. But also metal ions can be detected indirectly by raising antibodies against their chelate complexes. These complexes can be labeled with a fluorophore so that the application of FPIA is possible.<sup>61, 69</sup>

The applicability of FPIA to different matrices is limited due to interference from scattering light, fluorescent matrix compounds and interactions between matrix and tracer or antibody.<sup>68</sup> To reduce the influence of fluorescent matrix compounds, background correction can be performed, *i.e.* the parallel and perpendicular fluorescence intensities of the sample are subtracted from the respective values of the tracer. Another approach to minimize matrix effects is the development of stopped-flow FPIAs. Here, the initial rate of the reaction, *i.e.* the slope of the degree of polarization over time ( $dP/dt$ ) is measured shortly after mixing the reagents instead of measuring the degree of polarization after the equilibrium of the reaction is reached.<sup>52, 70</sup> This approach is only applicable on instruments for kinetic measurements, where the parallel and perpendicular intensities can be determined simultaneously.

The application range for food samples extends from antibiotics in milk<sup>48, 50</sup> and other animal products<sup>71</sup> over mycotoxins in cereals<sup>56-58</sup> and pesticide on fruits and vegetables<sup>31, 65</sup> to preservatives in candies and beverages.<sup>72</sup> Most of these methods require extraction steps mainly because many samples are solid. But these sample preparations usually only include a single solvent extraction step after homogenization. The extracts are often diluted, mostly due to the instability of the antibody towards used solvents.

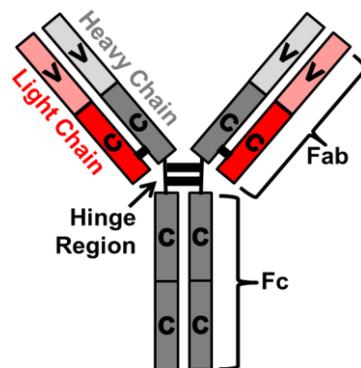
For environmental samples, usually no sample preparation is needed, only if soil samples are investigated.<sup>65, 69</sup> Almost all methods for water analysis found in literature apply spiked samples independent of the type of analyte, including plasticizers<sup>59</sup> and various types of pesticides.<sup>63, 64, 73</sup> Different kinds of water samples were investigated using FPIA like surface, pond, tap, bottled and distilled water, but only one FPIA method for the application to wastewater could be found in literature. Here, surfactants were determined, but the detection was only possible using solid-phase extraction (SPE) for sample clean-up and concentration.<sup>40</sup> The application of FPIA to wastewater is complicated due to the complexity of the matrix. This contains a lot of different ingredients in wide concentration ranges, *e.g.* proteins, salts and pharmaceuticals. Thus, one of the main advantages of homogeneous

assays, the redundancy of washing steps, is at the same time the most problematic issue for the application of FPIA to wastewater.

### 1.3 Antibodies

The most crucial factor for the development and success of immunoassays is the choice of antibody. It influences the sensitivity and specificity of the assay. In general, antibodies have a wide application range in medical therapy, diagnostics, biomedical research, food and environmental analyses. In analytics, they can be applied for quantitative or qualitative analysis and for sample preparation.<sup>74</sup>

The original function of antibodies is the protection of the body from infections. They are produced in B lymphocytes. Antibodies, or immunoglobulins, are glycoproteins that are constructed from four polypeptide chains (*Figure 4*). The two identical heavy chains (~55 kDa) and the two identical light chains (~25 kDa) are connected to each other through disulfide and noncovalent bonds. Each chain consists of one variable (v) and multiple constant regions (c). All chains together form a Y-shaped molecule of approximately 150 kDa. The base of the Y-shaped molecule is called Fc (crystallizable fragment) domain. Each “arm” of the molecule, also referred to as Fab fragment (antigen binding fragment), presents one antigen binding site, formed by the variable regions of the light and heavy chain. The part of an antigen that is recognized by the binding site is called epitope. Fab and Fc are connected through the so called hinge region.



**Figure 4** Scheme of antibody structure.

Depending on the heavy chains, immunoglobulins of mammals can be divided into five groups (IgA, IgD, IgE, IgG and IgM). IgG and IgA can be separated into subclasses, so-called isotypes, due to polymorphisms in the constant regions of the heavy chain.<sup>74</sup> Most antibodies in plasma and extracellular space are IgG (~75%). They can be easily extracted from serum and are the most commonly used antibodies for analytical purposes.<sup>75</sup>

The immune system is able to recognize substances with masses of at least 1000 Da.<sup>76</sup> Small molecules, like the pharmacologically active compounds described in this work, do not elicit an immune response. In order to produce antibodies against these structures, immunogens have to be synthesized by coupling the analyte or a hapten to a carrier protein, mostly making use of free amino, carboxyl or sulfhydryl groups of the protein.<sup>76</sup> One common method for immunogen synthesis is the NHS/DCC method that has been previously described for fluorescein tracer synthesis (Section 1.2.1). This method has been frequently applied, e.g. for the immunogen synthesis of isolithocholic acid,<sup>14</sup> 2,4,6-trinitrotoluene<sup>77</sup> and atrazine.<sup>78</sup> Often a spacer is used between the hapten and the carrier protein to improve the accessibility of the hapten for the antibody. Typical carrier proteins are ovalbumin (OVA), albumin, thyroglobulin, keyhole limpet hemocyanin<sup>76</sup> or bovine serum albumin (BSA).<sup>14, 77, 78</sup>

Antigens usually represent more than one epitope, which can be recognized by the immune system. Therefore different types of antibodies are produced by the immune system against the different epitopes. The mixture of these immunoglobulins is called a “polyclonal antibody”. They are secreted from different B lymphocytes. Polyclonal antibodies are produced by immunizing an animal with the antigen. After a certain time, the serum of this animal contains various antibodies against different epitopes of the antigen and can be used as polyclonal serum. The production of these kinds of antibodies is easy and cheap, compared to monoclonal antibodies.<sup>76</sup> Due to their broad specificity, they are a good choice if the determination of structurally related compounds in a sort of sum parameter is desired. But these mixtures often show reactivity against non-target substances, e.g. the protein that was used for immunogen synthesis.<sup>76</sup> Another drawback is that the sera are not infinitely available and the immunization of another animal usually leads to a completely different mixture of antibodies. Furthermore, the serum components can influence the assay performance, especially for homogeneous immunoassay due to the absence of washing steps.

Monoclonal antibodies consist of antibodies produced by the cell line of a single B lymphocyte and are therefore typically more specific than polyclonal antibodies. They can be produced over and over again with exactly the same properties. Usually Balb/c mice are used for generating monoclonal antibodies. The antigen is administered to the mice in several boosts together with an adjuvant, which enhances the immune response.<sup>76</sup> The immunization progress may be monitored by determining the antibody titer in blood samples taken from time to time. Due to animal welfare considerations, blood samples can only be taken unfrequently. Carvalho *et al.* presented a good alternative by which the animals are not affected: the detection of antibodies in feces.<sup>79</sup> This method offers several advantages: no need for trained staff for blood taking, less stress for the mice and a time-resolved screening of the immunization progress. Especially the last point would improve the whole immunization process, because it would make the decision of the necessity of additional boosts and the right time for the fusion much easier. Despite all these advantages and although the suitability of this method has been shown for different analytes, feces screening has not been established yet.

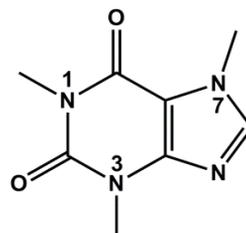
After choosing the mouse with the best antibody titer, antibody-producing B cells from spleen are fused with myeloma cells according to the method developed by Milstein and Köhler in 1975.<sup>80</sup> The cells are then seeded in HAT (hypoxanthine-aminopterin-thymidine) medium. Here, only hybridomas of myeloma and B cells can survive; all other cells do not grow in this medium. After selection of hybridoma cells, normal medium is used for cultivation of cells. Usually the fusion products are distributed over several 96-well MTPs in order to separate the clones from each other. The cell culture supernatants are then investigated regarding the analyte-specific antibodies. For this, indirect ELISA is usually applied. After selecting the antibody with the desired binding properties, a high amount of supernatant is collected and purified to obtain the pure antibody. This can be done by protein A or G chromatography, ion exchange chromatography, ammonium sulfate precipitation or affinity chromatography.<sup>76</sup>

There are many crucial steps during the production of monoclonal antibodies including synthesis of the antigen, choice and immunization of the animal, fusion, rising and separation of the clones, selection of the desired clone and production of a larger amount of

this antibody.<sup>81</sup> Once the anti-analyte antibody is produced, it should be carefully characterized. This includes specificity and affinity. The binding strength to a specific epitope is expressed as the affinity of the antibody. The specificity represents the ability of the antibody to recognize a specific epitope. Antibodies recognize a relatively small component of an antigen. Therefore they can cross-react with similar epitopes of compounds structurally related to the target analyte, but usually with less affinity.<sup>74</sup> Antibodies with a broad CR pattern can be used to detect simultaneously a group of structurally related compounds and for the development of broad-specificity screening immunoassays.<sup>76</sup> But for the accurate and precise determination of one analyte, only low or ideally no cross-reactivities are desired.

#### 1.4 Caffeine in consumer products

Caffeine or 1,3,7-trimethylxanthine (*Figure 5*) is an alkaloid, naturally occurring in plants. It belongs to the group of methylated derivatives of uric acid. Other known representatives are theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine). Caffeine improves cognitive skills, the reaction time and the ability to concentrate.<sup>82</sup> Therefore, caffeine is the most commonly consumed pharmacologically active compound in the world.<sup>83, 84</sup>



**Figure 5** Chemical structure of caffeine.

Caffeine occurs naturally in coffee (0.8-4.0%), tea (2.5-5.5%), the guaraná plant (3.6-5.8%), the cola nut (2.2%), mate (0.5-1.5%) and in cocoa beans (0.2%).<sup>84, 85</sup> The most common source of caffeine consumption is coffee, which can be served in a great number of types, e.g. espresso, latte macchiato or cappuccino, but also instant or decaffeinated coffees are widespread. Caffeine concentrations between 580–7000 mg/L were found in coffees, whereby the different sizes of coffee drinks should be considered.<sup>86</sup> A study of more than 100 coffees found 48–317 mg caffeine per serving.<sup>87</sup> In general, the caffeine concentration of coffee drinks depends on the preparation of the drink and the used beans.<sup>88</sup> Arabica beans contain less caffeine (0.8-1.4%) than Robusta beans (1.7-4.0%).<sup>84</sup> In decaffeinated coffee, not more than 1 g/kg caffeine in dry material are allowed according to the German coffee regulation.<sup>89</sup> Therefore, caffeine is extracted from coffee, typically by using supercritical carbon dioxide. The same method can be applied for caffeine extraction from tea, guaraná and mate.<sup>90-94</sup> But also pressurized liquid extraction, microbial and enzymatic methods can be used.<sup>95, 96</sup>

The extraction of caffeine is performed for the production of decaffeinated products, but also for further utilization of extracted caffeine for other products. Synthetic caffeine is also used for consumer products, e.g. soft drinks to which about 60-130 mg/L caffeine are added.<sup>97</sup> A special case of soft drinks are energy drinks, which are characterized by much higher caffeine concentrations of 300-320 mg/L.<sup>98</sup> For those beverages, the caffeine concentrations need to be labeled regarding the commission directive 2002/67/EC.<sup>99</sup> According to this European directive, all beverages with concentration higher than 150 mg/L have to be labeled with 'high caffeine content' and the concentration has to be given. The only exception to this rule is when the product is based on coffee or tea; this has to be evident in the product name. Additionally, caffeine has to be mentioned in the ingredient list,

e.g. in caffeine-containing flavored beers. Furthermore, caffeine tablets or powders are commercially available, intended for direct intake or dissolution in drinks.

Caffeine is more and more used in cosmetics. It is able to penetrate the skin barrier in different cosmetic formulations.<sup>100</sup> These cosmetics usually contain around 3% caffeine. It prevents excessive fat accumulation, stimulates the degradation of fat in cells and is therefore used in anti-cellulite products. Caffeine can also protect cells from UV radiation and slows down the process of photo-aging. It supports the apoptosis of UV damaged cells and therefore prevents the development of skin cancer. Additionally, caffeine enhances the microcirculation of blood in the skin. It is also used in shampoos, because it is able to penetrate hair follicle and stimulate their growth by inhibiting the activity of 5 $\alpha$  reductase.<sup>101</sup> Furthermore, caffeine is used in pharmaceuticals in doses of 30-200 mg, e.g. as an adjuvant for analgesics.<sup>102</sup>

In consequence of the widespread occurrence of caffeine in daily life, almost everyone consumes it somehow. It has been reported that 85% of US citizens older than 2 years consume at least one caffeinated beverage per day. Here, the overall daily caffeine intake was found to be 165 mg.<sup>103</sup> The average daily consumption of caffeine in Germany is almost twice as high with 313 mg per person.<sup>83</sup>

When caffeine is consumed, it is rapidly absorbed in the gastrointestinal tract and then metabolized in the liver. Only 2% are excreted unchanged in urine. Caffeine is mainly metabolized by cytochrome P450 1A2 to dimethylxanthines. The main metabolite with up to 80% is paraxanthine (1,7-dimethylxanthine).<sup>104</sup>

Caffeine promotes the release of intracellular calcium ions and inhibits phosphodiesterase. At relevant doses, the interaction of caffeine and paraxanthine with adenosine receptors A<sub>1</sub> and A<sub>2A</sub> are of great importance.<sup>83</sup> Caffeine acts as an antagonist of adenosine and therefore promotes the release of several neurotransmitters, e.g. dopamine. This leads to enhancement of blood pressure and lipolysis activity, resulting in an increased energy turnover.<sup>82, 105, 106</sup> Furthermore, the antagonism of the adenosine A<sub>2A</sub> receptor seems to have a positive effect on the prevention of tumors: it has been shown that this caffeine interaction reduces the rate of cancer in mice.<sup>107</sup>

A daily caffeine intake of up to 1000 mg does not lead to any adverse effects.<sup>82</sup> A higher dosage can lead to tachycardia, anxiety, restlessness and tremors. Lethal doses of 5-50 g caffeine are discussed, which is almost impossible to reach through consumption of beverages. And even for caffeine intoxications of 30 g, recovery has been reported.<sup>108</sup> Therefore lethal caffeine overdoses are very rare and only very few cases are known.<sup>102, 108, 109</sup>

Of major concern is the caffeine intake during pregnancy. It can easily pass the placenta barrier. The enzyme activity of a fetus is not fully developed and therefore caffeine is not completely metabolized. Caffeine consumption during pregnancy can lead to reduced birth weight or increase the risk of spontaneous abortion, especially at the beginning of pregnancy.<sup>110-113</sup> To give a complete overview, it should be mentioned that there are also publications claiming that caffeine has no influence on any aspect of reproduction.<sup>114-116</sup> Nevertheless, the advice of the European Food Safety Authority (EFSA) is that pregnant and breast feeding women should consume not more than 200 mg of caffeine per day,

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which is half of the amount proposed for other adults.<sup>117</sup> Anyway, it should be possible for everyone to have an overview of one's own caffeine intake, no matter if due to the possible influence on reproduction or simply because of sleep problems. Furthermore, the compliance of caffeine concentrations with the regulations mentioned before has to be monitored. Additionally, caffeine has been proposed as an indicator for the input of untreated wastewater into in surface waters and as a valuable anthropogenic marker in the water system.<sup>118-121</sup> For all these reasons, easy, fast and accurate methods for the quantification of caffeine are desirable.

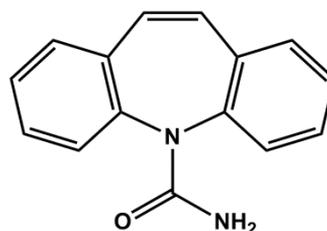
The most common method for caffeine quantification in consumer products is high-performance liquid chromatography (HPLC). This method can be coupled to various detection systems like absorbance, fluorescence,<sup>122-124</sup> or mass spectrometry (MS) detectors.<sup>125-127</sup> Furthermore, other chromatographic methods like thin-layer or gas chromatography (GC) can be applied using different detection systems, e.g. MS, nitrogen/phosphorous or flame ionization detectors.<sup>128-130</sup> Moreover, electrophoresis and electrochemical methods are used for caffeine determination in beverages.<sup>131, 132</sup> Most of these methods require a sample preparation. One of the most frequently used methods is SPE.<sup>123, 124, 133</sup> But also aqueous or liquid-liquid extractions are commonly applied.<sup>122, 130, 134</sup> All these methods are usually time and labor intensive.

One possibility for direct caffeine detection without sample preparation or chromatographic separation is the ambient ionization direct analysis in real time MS.<sup>135</sup> Spectroscopic methods are also applied for the analysis of caffeine-containing consumer products. UV/Vis spectroscopy, surface-enhanced Raman scattering and Fourier transform infrared spectroscopy have proven their suitability for this approach.<sup>136-139</sup> Fluorescence can also be used for caffeine detection, utilizing detection kits, microfluidic devices or test strips.<sup>140, 141</sup> Furthermore, a microbial biosensor was developed for caffeine detection in beverages.<sup>142</sup> Immunoassays for caffeine determination in biological samples were established,<sup>143-145</sup> and also the quantification in consumer products using heterogeneous immunoanalytical methods has been reported.<sup>12, 119, 146</sup>

## 1.5 Carbamazepine in the environment

### 1.5.1 Carbamazepine metabolism

Carbamazepine (CBZ, 5H-dibenzo[*b,f*]azepine-5-carboxamide; *Figure 6*) is an anticonvulsant drug which is widely used for therapy of epileptic seizures, bipolar disorder, schizophrenia, attention deficit hyperactivity disorder, post-traumatic stress and neuropathic pain.<sup>147, 148</sup> In 2014, 38.9 million daily doses of 800-1200 mg were prescribed in Germany.<sup>149, 150</sup> Despite the decline in prescription in Germany of 40% in the last ten years, CBZ is still one of the most frequently used antiepileptic drugs.<sup>150, 151</sup>



**Figure 6** Chemical structure of carbamazepine.

In the human body, 86% of CBZ is metabolized mostly by cytochrome P450 to 10,11-epoxy-CBZ (Ep-CBZ). This is enzymatically hydrolyzed to 10,11-dihydro-10,11-dihydroxy-

CBZ (DiOH-CBZ). Via ring contraction, these both metabolites can form 9-hydroxymethyl-10-carbamoylacridan (9-HMCA). Another less pronounced cytochrome P450 mediated pathway involves the formation of 1-, 2-, and 3-hydroxy-CBZ (1-, 2- and 3-OH-CBZ). Other metabolites are 4-hydroxy-CBZ, 2-hydroxy-1-methoxy-CBZ, 2-hydroxy-3-methoxy-CBZ, acridine, acridone, iminostilbene, 2-hydroxyiminostilbene and 9-acridine-10-carboxaldehyde, but these are produced only in small or trace amounts (less than 2%). During phase II metabolism, most of the hydroxyl metabolites form O-glucuronides. Additionally, N-glucuronides of CBZ and Ep-CBZ are formed.<sup>152</sup>

14% of CBZ are excreted non-metabolized, mostly through feces (93%). The majority of metabolites are eliminated through urine in the following amounts: 32% DiOH-CBZ, 11% CBZ-N-glucuronide, 5.2% 9-HMCA, 5.1% 3-OH-CBZ, 4.3% 2-OH-CBZ, 2-10% 1-OH-CBZ and 1.4% Ep-CBZ. 15% of consumed CBZ are excreted in feces in unidentified form.<sup>152</sup>

### 1.5.2 Carbamazepine in wastewater treatment plants

CBZ and its metabolites find their way into wastewater through human excrements. Therefore, they are frequently found in influents of wastewater treatment plants (WWTPs). In German WWTPs, median concentration of 1.9 µg/L CBZ, 4.0 µg/L DiOH-CBZ, 0.49 µg/L 10-OH-CBZ, 0.17 µg/L 1- and 2-OH-CBZ, 0.15 µg/L 3-OH-CBZ and 0.059 µg/L Ep-CBZ were found.<sup>152</sup> In general, CBZ metabolites, besides DiOH-CBZ, are found in much lower concentrations than the parent compound.<sup>152, 153</sup> In all influent samples from Berlin WWTPs, CBZ was detected with concentrations up to 5.0 µg/L.<sup>4, 154</sup> In Dresden even 5.8 µg/L of the antiepileptic drug were found.<sup>155</sup> CBZ is frequently detected in influent samples all over Europe.<sup>152, 156-159</sup> Also in Canada<sup>160</sup> and China,<sup>161</sup> CBZ was found in the influent of WWTPs, what clearly indicates the ubiquity of this pharmaceutical.

Degradation rates of CBZ during wastewater treatment of less than 30% were reported in the majority of publications dealing with this subject.<sup>149, 162, 163</sup> Hence, it is a suitable example for recalcitrant compounds during conventional wastewater treatment.<sup>164</sup> In many surveys, even an increase of CBZ concentration during wastewater treatment were reported,<sup>4, 156, 157, 159</sup> e.g. Bahlmann *et al.* found an increase of averaged 14% in five of the six Berlin WWTPs.<sup>152</sup> But also the metabolites Ep-CBZ and DiOH-CBZ were detected in higher concentrations in effluent than in influent samples. This might be explained by the partial cleavage of N- and O-glucuronides.<sup>152</sup> Median concentrations of 2.0 µg/L CBZ, 3.4 µg/L DiOH-CBZ, 0.50 µg/L 10-OH-CBZ, 0.14 µg/L 1-/2-OH-CBZ, 0.14 µg/L 3-OH-CBZ and 0.087 µg/L Ep-CBZ were determined in German wastewater effluents.<sup>152</sup> In Berlin WWTPs, CBZ concentrations up to 4.5 µg/L were found, and it was detected in all effluent samples collected in WWTPs of the German capital.<sup>4, 154</sup> In many European countries and also in Israel, concentrations in the low µg/L range were found.<sup>156, 157, 159, 165, 166</sup> CBZ has been detected in almost all effluent samples from North America as well, but mostly in lower concentrations than in Europe.<sup>167, 168</sup> In China, CBZ concentrations up to 50 ng/L were found.<sup>161</sup> CBZ also occurs in sludge samples from WWTPs, but only in very low concentrations due to the low sorption of CBZ.<sup>159, 169, 170</sup>

Currently, not only CBZ, but also many other micropollutants are not effectively removed during conventional wastewater treatment. This leads to a high load of pharmaceuticals and other compounds in surface water.<sup>171</sup> Therefore, the enhancement of cleaning efficiencies of WWTPs is of major concern. There are a lot of different strategies addressed to this

issue. These include activated carbon,<sup>172, 173</sup> membrane filtration,<sup>174</sup> electro dialysis,<sup>175</sup> photolysis,<sup>176, 177</sup> ozonation<sup>172, 178, 179</sup> and advanced oxidation processes. For the latter, methods like corona discharge,<sup>180</sup> hydrodynamic-acoustic-cavitation,<sup>181</sup> or magnetic nanocatalysts<sup>182</sup> can be applied. Almost all of these methods showed removal rates of more than 90% for CBZ. For the evaluation of these treatment methods, special attention should be given to the degradation products. For UV treatment for example, high removal rates of CBZ were determined, but acridine and acridone were formed during photolysis. These both substances show higher ecotoxicity than CBZ itself.<sup>176</sup> For advanced oxidation processes, the formation of these compounds was also described, but only as intermediates.<sup>181</sup> The main ozonation product, 1-(2-benzaldehyde)-4-hydro-(1H,3H)-quinazoline-2-one, is more biodegradable than CBZ and leads therefore to an improvement of the water quality.<sup>178</sup>

In March 2014, the Swiss government decided to implement technical measures on selected WWTPs to reduce the load of micropollutants and toxicity of wastewater. The review of surveys in that field led them to the conclusion that most micropollutants are removed by ozonation and activated carbon by more than 80%. One hundred WWTPs will be upgraded in Switzerland in the next 20 years. It is expected that the costs for water discharge will increase by 6%, what seems to be a low price for better water quality and a healthier aquatic ecosystem. For controlling and monitoring the efficiency of additional purification steps in WWTPs, a limited number of compounds was defined, one of them being CBZ.<sup>183</sup>

### 1.5.3 Carbamazepine in surface waters

Due to the negligible removal rate during conventional wastewater treatment, CBZ enters surface waters and is therefore an excellent indicator for wastewater input into water bodies.<sup>164, 170, 184, 185</sup> Across Europe, CBZ was found among others in Germany,<sup>154</sup> Switzerland,<sup>186</sup> France,<sup>187</sup> Italy,<sup>188</sup> Portugal,<sup>189</sup> Serbia,<sup>190</sup> Austria, Hungary, Croatia, Romania and Ukraine<sup>118</sup> in at least half of all surveyed rivers and lakes. Usually concentrations in the mid ng/L range were found. In Berlin, a peak concentration of 4.5 µg/L CBZ was determined.<sup>4</sup> Also in the United States<sup>191</sup> and China,<sup>161</sup> CBZ was found in surface waters. The antiepileptic drug was even detected in marine systems.<sup>192, 193</sup> CBZ is generally one of the most frequently found micropollutants in environmental samples.<sup>187, 190</sup>

Murray *et al.* reviewed the occurrence and toxicity of 71 compounds and indicated that CBZ is one of the pollutants with the highest priority in fresh water systems.<sup>194</sup> One reason is the low sorption to soil and high resistance to biodegradation.<sup>170, 195</sup> Therefore it shows a high persistence in water bodies. Only radiation from sun seems to promote the removal of CBZ from surface waters.<sup>188</sup> But this takes up to 4 weeks and some transformation products are more toxic than the parent compound.<sup>176, 196</sup>

Pharmaceuticals are made for having an influence on biochemical interactions. Therefore it is not surprising that once they enter the water system, they also affect the health status of aquatic organisms. Chronic toxicity of CBZ on clams, which can be seen as a bio indicator for marine quality, was observed in relevant CBZ concentrations.<sup>197, 198</sup> The toxicity is mainly based on induction of oxidative stress, whereby environmental parameters seem to have an influence on the degree of damage.<sup>199, 200</sup> Negative effects on health status of other

aquatic organisms like bacteria,<sup>165</sup> algae,<sup>201</sup> annelid worms,<sup>202</sup> insects,<sup>203</sup> and fish<sup>204</sup> were also reported.

In wildlife fish, CBZ concentrations in the low ng/g range were found, but not very frequently.<sup>205</sup> But even if those fish are used for food production, the exposure would not be of any hazard for humans. Another way of unintentional human exposure to CBZ is the consumption of contaminated vegetables. They can be contaminated through the irrigation with treated wastewater. The uptake of CBZ has been proven for a variety of vegetables<sup>206-208</sup> and grass for animal feed.<sup>209</sup> It has been reported, that CBZ can negatively influence the growth of plants.<sup>208</sup> For humans, negligible annual CBZ intake of 0.64 µg per person are predicted through the consumption of contaminated vegetables.<sup>207</sup> Another study reports on CBZ concentrations of 1 ng/g in cucumbers, so that the previously mentioned annual consumption would already be reached by eating two cucumbers (300-500 g per cucumber).<sup>206</sup> But this is still negligible compared to the daily dose of around 1000 mg.

Due to the low sorption to soil,<sup>170</sup> CBZ is also frequently found in ground water samples up to 140 ng/L.<sup>187, 190, 210-212</sup> Ground water and water from re-charged aquifers that take in surface water are the common water sources of waterworks so that CBZ can also occur in tap water if no further degradation by water purification processes occur; CBZ has consequently been found in concentrations of a few ng/L.<sup>161, 187, 213-215</sup> But due to these low concentrations, no health risk for humans is expected,<sup>216</sup> not even in combination with the other unintentional sources of CBZ consumption.<sup>217, 218</sup>

#### 1.5.4 Analysis of carbamazepine in environmental samples

For the determination of CBZ in environmental samples including sludge, soil, waste, surface, ground and sea water, LC is most commonly used. GC can also be applied, but in the injector, CBZ is thermally converted to iminostilbene. 10,11-Dihydro-CBZ (DiH-CBZ) reacts in the same way and can therefore be used as an internal standard to compensate this effect.<sup>186, 219</sup> The detection after the chromatographic separation can be performed by UV,<sup>220, 221</sup> pulsed amperometry,<sup>222</sup> photochemically induced fluorimetry,<sup>223</sup> high-resolution MS,<sup>224, 225</sup> but most commonly MS/MS is applied resulting in limits of quantification in the low ng/L range.<sup>152, 158, 191, 215, 226</sup> These instrumental methods are usually multianalyte approaches, e.g. using ultra HPLC coupled to high-resolution MS, up to 72 micropollutants can be determined simultaneously in waste, surface or drinking water.<sup>225</sup>

MS can be utilized for the detection of CBZ in environmental samples without previous chromatographic separation, using laser diode thermal desorption.<sup>160</sup> Capillary electrophoresis with UV detection has been applied for CBZ determination in wastewater.<sup>227</sup> Furthermore, photoinduced fluorometric determination of CBZ in surface, ground and tap water has been developed.<sup>228</sup>

All these methods require sample preparation steps due to the complexity of the matrices and the low concentrations. Most commonly, SPE is applied to pre-concentrate the samples and to reduce matrix compounds.<sup>158, 224, 226</sup> Molecular imprinted polymers can also be applied for this kind of sample preparation.<sup>229</sup> Other methods like solid-bar microextraction were utilized as well.<sup>220</sup> Using SPE and HPLC-MS/MS, limits of quantification of 0.05 ng/L could be reached for CBZ determination in drinking water.<sup>215</sup> For wastewater samples, limits of quantification of 12 ng/L were reported using SPE/LC-MS/MS.<sup>158</sup>

## Introduction

Immunoanalytical methods usually do not require those time-consuming sample clean-ups. ELISA has been applied for the determination of CBZ in waste and surface water without any sample preparation<sup>4, 16, 230</sup> within a quantification range of 0.02-20 µg/L.<sup>13</sup> Of course, SPE can be applied for ELISA to lower the quantification range. With this approach, CBZ concentrations of 3 ng/L could be quantified in surface water.<sup>231</sup> Furthermore, ELISA has been utilized for the determination of CBZ degradation rates during advanced wastewater treatment processes.<sup>181</sup> The applicability of CBZ determination in aquatic organisms has also been proven related to toxicological analyses.<sup>197, 198, 232</sup>

The antibodies that are used for CBZ determinations showed CRs against CBZ metabolites or other pharmaceuticals, e.g. immunoassays for clinical approaches showed overestimations due to Ep-CBZ and the antihistaminic drugs hydroxyzine and cetirizine.<sup>233, 234</sup> For environmental analyses, quite high CRs were determined, the highest being norchlorcyclizine (antihistamine, 114%), Ep-CBZ (63%), cetirizine (50%), hydroxyzine (41%) and cloperastine (cough suppressant, 13%). These values were determined for ELISA at pH 9.5. But some of these CRs are highly pH dependent, most of all cetirizine. This antihistaminic drug, which is not related to CBZ, showed CRs between 22% at pH 10.5 and 400% at pH 4.5.<sup>235</sup> These CRs led to high overestimations for immunoanalytical determination of CBZ in environmental samples.<sup>13, 16, 230</sup> Despite all the advantages of immunoassays compared to instrumental methods, like high throughput or expendability of expensive instruments and sample preparation, these CRs are a big disadvantage of immunoanalytical methods for environmental analysis.

For therapeutic drug monitoring of CBZ, immunoassays are one of the most utilized methods.<sup>236</sup> FPIA in particular is widely used for clinical approaches. There are several automated systems and reagent kits available from different suppliers.<sup>237, 238</sup> The detection limits are around 0.5 mg/L for these methods, which is sufficient regarding a therapeutic drug level in serum of 4-12 mg/L.<sup>239</sup> Until now, no application of FPIA for CBZ determination in environmental samples and for associated concentration in the low µg/L range had been described.

## **2. Aims of the thesis**

Pharmacologically active compounds are frequently present in consumer products and the environment. Hence, methods for efficient monitoring should be available. Fast and easy quantifications, applicable for on-site measurements or high-throughput screenings, can be performed using FPIA. But during the development of applications of this method, many crucial points have to be considered, including assay platform, tracer synthesis, the choice of analyte-specific antibody and the applicability to complex matrices.

The aim of this work was the development, optimization and application of FPIA for pharmacologically active compounds in complex matrices. The analytes caffeine and CBZ were chosen. The first one represents one of the worldwide mostly consumed pharmacologically active compounds, while CBZ represents one of the most frequently detected pharmaceuticals in the environment.

Summarizing, the aims of this thesis are:

1. Development of a FPIA for caffeine determination in consumer products  
including the application on different platforms
2. Development of a FPIA for CBZ determination in environmental samples  
including the comparison of different tracers  
and the application on different platforms
3. Production and characterization of a new CBZ-specific monoclonal antibody

### 3. Results and discussion

#### 3.1 Fluorescence polarization immunoassays for the quantification of caffeine in beverages

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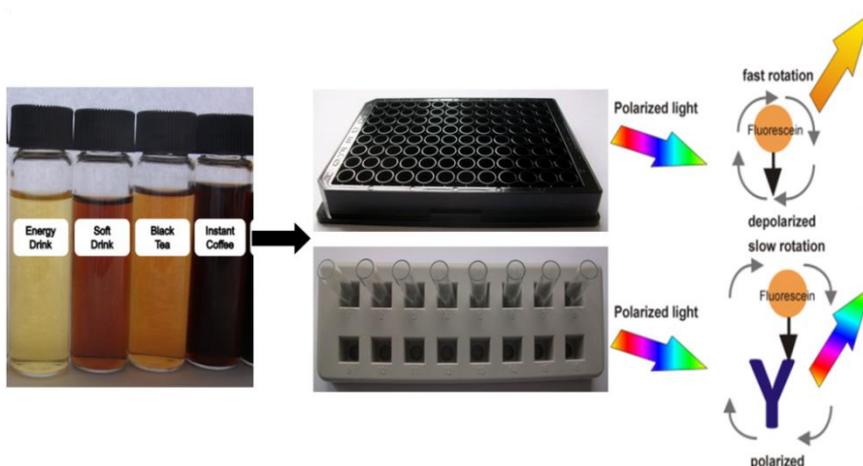
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**Figure 7** Graphical abstract of Fluorescence polarization immunoassays for the quantification of caffeine in beverages.

##### 3.1.1 Abstract

Homogeneous fluorescence polarization immunoassays (FPIAs) were developed and compared for the determination of caffeine in beverages and cosmetics. FPIAs were performed in cuvettes in a spectrometer for kinetic FP measurements as well as in microtiter plates (MTPs) on a multimode reader. Both FPIAs showed measurement ranges in the  $\mu\text{g/L}$  range and were performed within 2 and 20 min, respectively. For the application

on real samples, high coefficients of variations (CVs) were observed for the performance in MTPs; the CVs for FPIAs in cuvettes were below 4%. The correlations between this method and reference methods were satisfying. The sensitivity was sufficient for all tested samples including decaffeinated coffee without preconcentration steps. The FPIA in cuvettes allows a fast, precise, and automated quantitative analysis of caffeine in consumer products, whereas FPIAs in MTPs are suitable for semiquantitative high-throughput screenings. Moreover, specific quality criteria for heterogeneous assays were applied to homogeneous immunoassays.

### 3.1.2 Introduction

Caffeine (1,3,7-trimethylxanthine) is one of the most frequently used psychoactive substances in the world with a yearly consumption of 9300 tons in Germany and a worldwide daily intake of 70–76 mg per person.<sup>83</sup> The main sources of caffeine are coffee, tea, cacao, soft drinks, and energy drinks; there are also caffeine-containing beers and cosmetics. The range of caffeine concentrations in consumer products varies greatly; for example, in teas, it varies between 160 and 333 mg/L.<sup>83</sup> Caffeine concentrations of individual coffee samples are in the range of 267 to 1200 mg/L<sup>83</sup> and depend strongly on the preparation method<sup>123</sup> and the coffee bean; robusta beans contain more caffeine than arabica beans.<sup>240</sup> Concentrations of approximately 20 and 400 mg/L are to be expected for decaffeinated and instant coffees, respectively.<sup>241</sup> In espresso, concentrations of up to 1800 mg/L caffeine were found.<sup>242</sup> Assuming a daily coffee consumption of 2–4 cups (filter coffee), a 70 kg person ingests approximately 280 mg caffeine. An extensive coffee drinker can reach a daily intake of up to 1.050 mg.<sup>105</sup>

More and more adults drink decaffeinated coffee, for example, during pregnancy, because high caffeine consumption can lead to miscarriages.<sup>110</sup> A small market has formed for self-testing of presence or absence of caffeine by dipsticks.<sup>141</sup> On the other hand, for consumer protection, monitoring of the caffeine content is imperative for producers of caffeine-containing consumer products. Furthermore, a fast caffeine determination during the decaffeination process is desirable.

Caffeine concentrations can be determined spectroscopically,<sup>88</sup> by capillary electrophoresis,<sup>131</sup> gas chromatography,<sup>243</sup> and liquid chromatography coupled with mass spectrometry.<sup>119, 244</sup> These methods often include labor-intensive sample preparation steps like extraction, filtration, and evaporation of solvents under reduced pressure. Immunoanalytical methods like enzyme immunoassays (EIAs) do not need such extraction steps. Provided concentrations are clearly higher than the limits of detection; often, simple dilution is sufficient. Different EIAs have been developed and compared for the determination of caffeine in beverages and cosmetics with respect to quality criteria for the assessment.<sup>12, 119</sup> The most suitable EIA using horseradish peroxidase (HRP) as enzyme and 3,3',5,5'-tetramethylbenzidine (TMB) as substrate (enzyme-linked immunosorbent assay, ELISA) showed a very high sensitivity (test midpoint 0.095 µg/L), a wide quantification range (0.033–33 µg/L), and a good applicability to many different sample matrixes. However, several washing steps and long incubation times are required for these heterogeneous EIAs.

In contrast, homogeneous immunoassays like fluorescence resonance energy transfer (FRET) or fluorescence polarization immunoassays (FPIAs) do not require washing steps or

tedious sample preparation.<sup>21, 245</sup> For FRET assays, the antibody and analyte has to be labeled, whereas only the analyte needs a label to perform a FPIA; but the necessary polarizers for FP measurements reduce the signal intensity. These homogeneous assays are usually completed within several minutes; for example, with a FPIA for chlorsulfuron, 10 samples could be analyzed within 7 min without incubation.<sup>246</sup>

FPIAs can be performed in microtiter plates (MTPs) or cuvettes with different instrumental configurations. Generally, the assays performed in cuvettes are faster for individual sample measurements (approximately 2 min), but up to 20 or 30 samples can be measured simultaneously within 10 min in MTPs.<sup>33</sup> The missing (enzymatic) amplification step can lead to a lower overall sensitivity of the assay; for example, the EIA for the determination of the herbicide simazine yielded a 30 times lower detection limit than the FPIA using the same antibody.<sup>54</sup> Usually working ranges in the micrograms per liter to milligrams per liter range are observed for FPIAs;<sup>68</sup> for example, the detection limit of the herbicide chlorsulfuron was 10 µg/L.<sup>246</sup> FPIAs have been used for high-throughput screenings of small-molecule analytes such as the mycotoxins ochratoxin A (OTA), zearalenone, and deoxynivalenol in food-safety control within the following ranges: 5–200, 500–5000, and 100–2000 µg/L, respectively.<sup>68</sup>

Here, we present and compare two novel FPIAs for the fast, easy, and cost-effective determination of caffeine in beverages and cosmetics, one performed with a multimode plate reader, the other in a spectrometer especially developed for FPIA measurements. The concentrations obtained with these assays were verified with LC tandem mass spectrometry (LC-MS/MS) and ELISA using TMB as substrate. Additionally, the applicability of quality criteria from heterogeneous to homogeneous immunoassays was tested.

### 3.1.3 Materials and methods

#### 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 was obtained from Invitrogen (Carlsbad, CA, U.S.A.). The enzyme HRP (EIA grade) was obtained from Roche (Mannheim, Germany). The synthesis of the caffeine HRP conjugate was described before.<sup>119</sup> To obtain ultrapure reagent water for the preparation of buffers and solutions, a Synthesis A10 Milli-Q water purification system from Millipore (Schwalbach, Germany) was used.

All MTPs with 96 flat-bottomed wells were purchased from Greiner Bio-One (Frickenhausen, Germany). Black nonbinding MTPs were employed for fluorescence polarization measurements, whereas clear Microton 600 MTPs were used for ELISAs. The caffeine reference standard used for the preparation of the calibrators was obtained from Sigma-Aldrich (Cat. no. C1778-1VL). The anti-mouse IgG whole molecule antibody (polyclonal, sheep, lot 21481) was purchased from Acris Antibodies (Herford, Germany). The anti-caffeine antibody (monoclonal, mouse IgG2B, clone 1.BB.877, lot L2051502M) was obtained from United States Biological (Swampscott, MA, U.S.A.). The beverages, coffees, tea, and cosmetics were purchased in a local supermarket.

## Synthesis and characterization of the caffeine fluorescein conjugates

The synthesis of a caffeine spacer derivative (CafD) 7-(5-carboxypentyl)-1,3-dimethylxanthine was described elsewhere.<sup>119</sup> For the FPIA application in MTPs, the following protocol was used to synthesize the caffeine fluorescein conjugate: 2.42 mg of CafD were dissolved in *N,N*-dimethylformamide (DMF) and a small amount of *N,N'*-disuccinimidyl carbonate was added. *N*-Hydroxysuccinimide and *N,N*-dicyclohexylcarbodiimide were both dissolved in DMF, and each was added to the CafD solution in a molar excess of 1.2 compared to the amount of CafD. The mixture was shaken for 18 h at 21 °C (750 rpm). Then the reaction mixture was centrifuged for 10 min. The solution containing the activated NHS caffeine ester was mixed with the 5-(aminoacetamido)fluorescein (dissolved in 0.27 mol/L sodium dihydrogencarbonate) in a molar ratio of 1.5:1. After shaking for 18 h at room temperature, the chemical identity of the reaction product was confirmed with high-resolution mass spectrometry (Orbitrap Exactive, Thermo Scientific, Schwerte, Germany; ESI negative). A mass peak of  $m/z = 679.22$  showed that the caffeine fluorescein conjugate had formed.

The product was cleaned by HPLC (Series 1200, Agilent Technologies, Waldbronn, Germany; column: Phen 250 × 3 mm, Sepserv, Berlin, Germany). The oven temperature was set to 40 °C, the flow rate was 0.4 mL/min, and the pressure was 170 bar. The solvents were ultrapure water (A) and methanol (B) containing 10 mmol/L ammonium acetate and 0.1% acetic acid. At the beginning, 80% solvent A was used. After 3 min, the percentage of solvent B was linearly increased to 95% within 17 min. After 28 min, the percentage of solvent B was decreased to 20% within 1 min. Then the composition was kept constant until the end of the run (40 min). The fraction containing the main peak was evaporated to dryness under a current of nitrogen and dissolved in methanol.

For the fluorescein conjugate for the application in cuvette, CafD was coupled to aminopropylamido carboxyfluorescein. This conjugate was obtained from aokin AG (Berlin, Germany).

## Sample preparation

The soft drink, energy drink, and caffeine-containing beer were degassed by shaking, followed by approximately 15 min in an ultrasonic bath. One bag of caffeine powder for soft drinks (2 g, containing 120 mg caffeine) was dissolved in 250 mL water. One bag of tea (Ceylon-Assam black tea, 1.75 g per bag) was brewed with 250 mL of boiling water allowing an infusion time of 10 min. The cosmetic sample (caffeine-containing shampoo) was prepared by dissolving 5.05 g in 1 L ultrapure reagent water. The espresso was prepared in a capsule espresso machine (Nespresso, Ristretto capsule). The instant coffee was prepared by dissolving 2.50 g of the instant coffee granulate in 200 mL boiling water.

A total of  $7.00 \pm 0.05$  g of ground coffee powder per sample (three different types of 100% arabica ground coffee (1, 2, and 3 (decaffeinated), and one 100% robusta ground coffee) were brewed with 250 mL of boiling water. Different preparation methods for all coffees were employed; however, the same masses of ground coffee and water were always used. (i) A filter coffee machine was used. (ii) In a French press, an infusion time of 5 min was allowed. (iii) A Turkish coffee was prepared by pouring hot water on the ground coffee.

## Results and discussion

When the coffee cooled down, it was filtered. (iv) For the preparation of the Italian espresso, an electric espresso machine (De'Longhi, Italy) was used.

For arabica 1, one other preparation method was used: the ground coffee was boiled with water and then refilled gravimetrically with water. Three different approaches were used for this preparation method: 5.00 g of coffee was boiled with 400 mL of water for 10 min, or 7.00 g of coffee was boiled with 250 mL of water for 10 or 30 min. The reference standard for decaffeinated coffee was obtained from FAPAS (Sand Hutton, Great Britain) and prepared as Turkish coffee (iii).

### FPIA in cuvettes (FPIA 1)

The FPIA 1 was performed in the filter-based aokin spectrometer FP 470 (aokin AG), that was developed especially for FPIA measurements. All reagents were pipetted with the aokin Liquid Handling Workstation directly into the round glass cuvette within the spectrometer. The system was controlled by the aokin software mycontrol v.3.4.3.1. The excitation wavelength was set to 470 nm, and the emission was measured at 520 nm. The fluorescence intensities at perpendicular and parallel polarizer settings were measured simultaneously and constantly (kinetic measurement). First, 2.2 mL of reaction buffer (phosphate buffered saline based buffer) were pipetted into the cuvette that contained a stir bar. An  $\sim 1$  g/L methanolic stock solution of caffeine was prepared gravimetrically, and calibrators were obtained by sequential dilution with ultrapure water. A total of 200  $\mu$ L of the calibrator (0–1000  $\mu$ g/L) or sample dilution were added, followed by the addition of 100  $\mu$ L of the caffeine fluorescein conjugate dilution (aokin AG). Afterward, 100  $\mu$ L of the anti-caffeine antibody dilution (aokin AG) were added. The caffeine concentrations were determined by the software over a defined time range (40–80 s after the antibody was added). The degrees of polarization (in millipolarization units mP) for the calibration curve were determined 60 s after the antibody was added. The degrees of polarization were corrected by the background signal and G-factor (0.979). All samples and calibrators were measured in triplicate.

The degrees of polarization were subjected to a Grubbs outlier test ( $\alpha = 0.01$ ). The mean values of the calibrators were fitted to a four-parameter logistic function with the parameters  $A$  (upper asymptote),  $B$  (slope at the test midpoint),  $C$  (concentration at test midpoint), and  $D$  (lower asymptote)<sup>247</sup> using the Origin 8G software (OriginLab, Northampton, U.S.A.).<sup>10</sup> Standard deviations of the mean signals were used to obtain the precision profile according to Ekins by calculating the relative error of each calibrator caffeine concentration.<sup>11</sup> The accordingly determined range with a relative error of the concentration below 30% was assigned the measurement range of the assay.

### FPIA in MTPs (FPIA 2)

All pipetting steps were carried out with 8-channel pipettes from Eppendorf (Hamburg, Germany). A total of 300  $\mu$ L of TRIS buffer (10 mmol/L tris-(hydroxymethyl)aminomethane, 150 mmol/L sodium chloride, pH 8.5) with 0.01% Triton X-100 and 1% methanol were pipetted into each well. After adding 20  $\mu$ L of the calibrators in triplicate (0–1000  $\mu$ g/L) and sample dilutions (6-fold), a background measurement was performed on the monochromator-based multimode reader SpectraMax M5 (Molecular Devices, Biberach an der Riss, Germany) with the following settings: excitation at 492 nm, emission at 520 nm (at

parallel and perpendicular polarizer settings), and a cutoff filter at 515 nm. A total of 10  $\mu$ L of caffeine fluorescein conjugate, diluted in TRIS buffer, was added to each well, followed by 10  $\mu$ L of the anti-caffeine antibody (1.37 mg/L in TRIS buffer). After shaking for 10 min on the plate shaker Titramax 101 from Heidolph (Schwabach, Germany; 750 rpm), the fluorescence was measured with the settings described above. The perpendicular and parallel intensities resulting from the background measurement were subtracted from the respective values. These background-corrected values were then used for the calculation of the degree of polarization. The values were corrected by the G-factor (0.946) of the instrument. The background corrected intensities and the degrees of polarization were subjected to a Grubbs outlier test. The assay was repeated four times, yielding a 6  $\times$  4 determination of the caffeine concentration for each sample. The calibration curve was fitted as described above. A calibration curve with 8 calibrators was used to determine the caffeine concentrations of the samples. These calibrators were measured on each MTP. The measurement range was determined as described above with 16 calibrators in triplicate.

## Reference methods

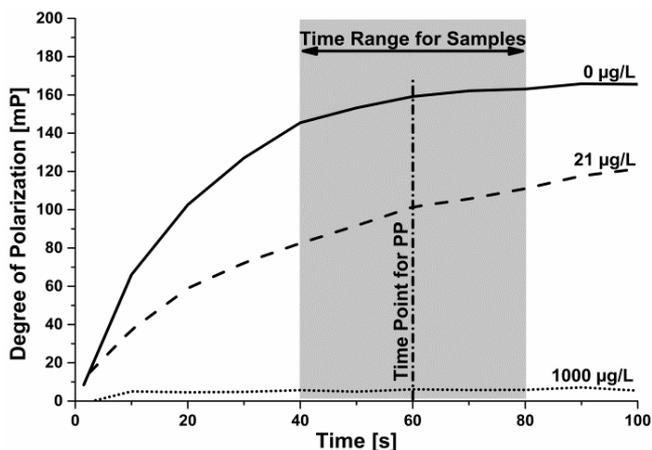
Caffeine determination with the reference methods HRP TMB ELISA and the LC-MS/MS were performed with the same instruments and methods as described before by Grandke *et al.*<sup>12</sup>

### 3.1.4 Results and discussion

#### Comparison of the caffeine FPIAs and applicability of quality criteria

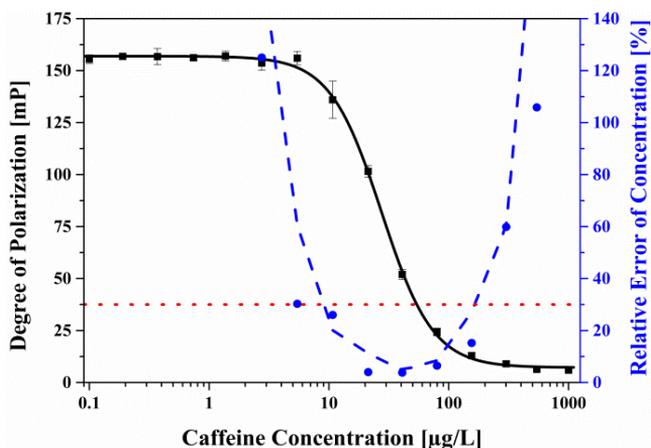
The FPIA in cuvettes (FPIA 1) is a kinetic assay where the degree of polarization can be measured as a function of time (*Figure 8*); here, no incubation step is required as it is a continuous process. One time point was chosen at which the values for the calibration curves and precision profile were determined (60 s after the antibody was added). The caffeine concentrations for real samples were determined over a time range (40–80 s). In contrast, the degrees of polarization for the FPIA in MTPs (FPIA 2) were determined after a defined time of 10 min (end point measurement). The incubation time in MTPs is prolonged compared to measurements in the cuvettes, as it takes longer to reach the equilibration because the circulation is much faster when a stir bar is used than on a plate shaker.

The FPIAs were optimized in regard to the parameters buffer basis, buffer additives, anti-caffeine antibody concentration, and caffeine fluorescein conjugate concentration. Additionally, different types of MTPs (nonbinding and untreated MTPs, different manufacturers) were tested for FPIA 2. Calibration curves with precision profiles for the FPIAs were determined under optimized conditions. Quality criteria for the assessment of caffeine EIAs in respect of the calibration curves (4-PL) had been previously defined and applied to a series of heterogeneous EIAs.<sup>12, 13</sup> The applicability of these criteria to FPIAs was to be evaluated in this study.

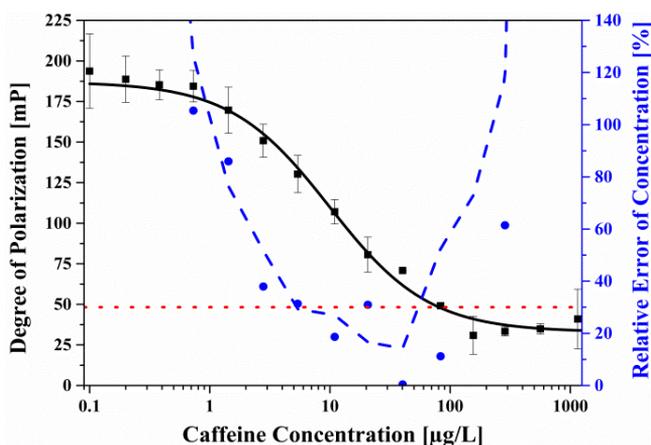


**Figure 8** Kinetic measurement of the degree of polarization after antibody addition shown for three caffeine calibrators (0, 21, and 1000 µg/L) measured with the FPIA in cuvettes. The time range for the determination of caffeine concentrations in samples (40–80 s, gray background) and the time point (60 s, dash-dotted line) at which the values for the calibration curve and the precision profile (PP) were determined are highlighted.

The sensitivity in terms of the test midpoint  $C$  of the calibration curve was determined to 27.4 µg/L for FPIA 1 (Figure 9) and is approximately three times higher than that obtained for FPIA 2 with 9.9 µg/L (Figure 10). Therefore, FPIA 2 is more sensitive than FPIA 1. Compared to that of the ELISA ( $C = 95$  ng/L),<sup>12</sup> the test midpoint of FPIA 2 is relatively high. FPIAs for other analytes showed higher test midpoints: 207 µg/L for butachlor and 165 µg/L for melamine.<sup>32, 63</sup> Hence, the sensitivity of our caffeine FPIAs is comparatively good. Similar dynamic ranges were observed for FPIA 1 and 2 (150 mP and 154 mP, respectively). The relative dynamic ranges (RDRs) were on a normalized scale 0.96 for FPIA 1, and 0.82 for FPIA 2. Accordingly, only FPIA 1 fulfilled the predefined required threshold of 0.90. The calibration curve of FPIA 1 showed a slope  $B$  at the test midpoint of 2.02. The slope of the calibration curve for FPIA 2 was 1.05. The coefficient of determination  $R^2$  is a measure for the goodness of fit. FPIA 1 showed a good  $R^2$  value of 0.999, whereas FPIA 2 ( $R^2 = 0.986$ ) did not reach the required value of 0.990. Additionally, the standard deviations of the measured values were analyzed. The highest standard deviation for FPIA 1 was 9.94 mP, whereas the highest standard deviation for FPIA 2 was 22.95 mP. Overall, a better goodness of fit was obtained for FPIA 1 compared to FPIA 2. Measurement ranges of 8.94–164 µg/L and 5.19–55.5 µg/L were determined for FPIA 1 and FPIA 2 according to the precision profiles. Neither of the ranges covered 3 orders of magnitude, even though the measurement range than that of FPIA 1 was three times wider than FPIA 2. Other FPIAs had shown comparable measurement ranges: 5–200 µg/L for OTA,<sup>68</sup> 32.0–1220 µg/L for the herbicide butachlor.<sup>63</sup> Therefore, a critical assessment of the requirement for this criterion should follow for homogeneous assays. In summary, FPIA 1 fulfilled all quality criteria for the calibration curve with the exception of the measurement range.



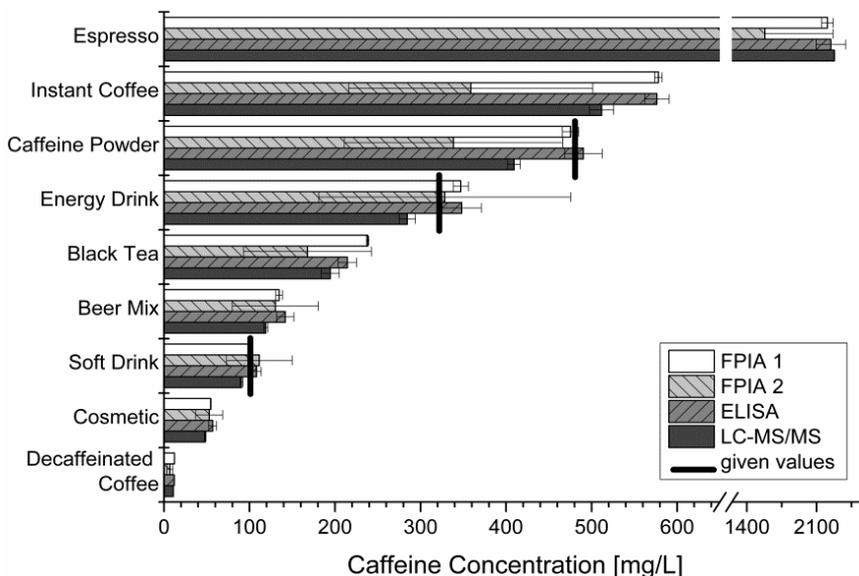
**Figure 9** Calibration curve (black squares and solid line), precision profile (blue circles and dashed line), and measurement range (intersection points at 30% relative error of concentration, dotted red line; 8.94–164  $\mu\text{g/L}$ ) were determined for FPIA 1 in cuvettes ( $A = 157 \text{ mP}$ ;  $B = 2.02$ ;  $C = 27.41 \mu\text{g/L}$ ;  $D = 7.20 \text{ mP}$ ;  $R^2 = 0.999$ ;  $RDR = 0.96$ ).



**Figure 10** Calibration curve (black squares and solid line), precision profile (blue circles and dashed line), and measurement range (intersection points at 30% relative error of concentration, dotted red line; 5.19–55.5  $\mu\text{g/L}$ ) were determined for FPIA 2 in MTPs ( $A = 187 \text{ mP}$ ;  $B = 1.05$ ;  $C = 9.93 \mu\text{g/L}$ ;  $D = 33.0 \text{ mP}$ ;  $R^2 = 0.986$ ;  $RDR = 0.82$ ).

### Assay evaluation for different matrixes

The most common matrixes of caffeine occurrence were selected to compare the suitability of the FPIAs for caffeine determination (Figure 11). For the kinetic FPIA 1, the measurement for one sample takes approximately 2 min. This assay is automated, and eight samples can be measured in triplicate with the liquid handling workstation in one run. FPIA 2 allows a 6-fold determination of eight samples within 20 min, including all pipetting, incubation, and measurement steps. Smaller sample volumes are required for FPIA 2 than for FPIA 1. Additionally, all samples were measured by ELISA and LC-MS/MS.



**Figure 11** Caffeine concentrations of beverages and cosmetics determined with FPIA 1 and 2, ELISA, and LC-MS/MS. Furthermore, the values provided by the manufacturer are depicted for three samples (black lines). For better comparability, the caffeine concentrations are given in milligrams per liter.

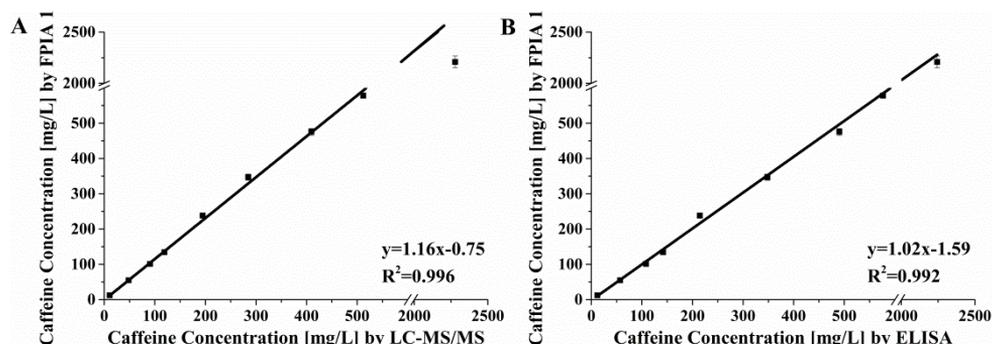
Beverages with high caffeine concentrations (>150 mg/L) need to be labeled as required by Commission Directive 2002/67/EG.<sup>99</sup> Here, a direct comparison is possible between the values provided by the manufacturer and the determined values. The closest agreement for the energy drink was found with 328 mg/L for FPIA 2 compared to the given value of 320 mg/L. The values for ELISA and FPIA 1 were higher with 348 mg/L and 347 mg/L, respectively, whereas the concentration obtained with LC-MS/MS was lower with 285 mg/L. One bag of the caffeine powder (dissolved in 250 mL water) should contain 120 mg caffeine. The caffeine contents calculated from the results of ELISA and FPIA 1 were 122 mg and 119 mg, respectively, and were therefore very close to the value given by the manufacturer. FPIA 2 and LC-MS/MS led to underestimations. FPIA 1 with 101 mg/L, led to the best agreement for the soft drink compared to the expected value of 100 mg/L. Slight overestimations were observed with ELISA (108 mg/L) and FPIA 2 (112 mg/L). LC-MS/MS showed lower concentrations for all three samples than the expected values. The caffeine contents of the shampoo determined with the different methods were all very similar: 9.56 (LC-MS/MS), 11.3 (ELISA), 10.9 (FPIA 1), and 10.5 mg/g (FPIA 2) based on the amount of shampoo. These data correlate well with the values obtained by Carvalho *et al.*<sup>119</sup>

A decaffeinated reference standard was investigated. All determined concentrations were within the satisfactory range of 193–606 g/kg. The closest agreement to the assigned value of 399 mg/kg was found for LC-MS/MS with 390 mg/kg. FPIA 1 (438 mg/kg) and ELISA (428 mg/kg) led to higher values, whereas FPIA 2 led to a lower caffeine concentration (246 mg/kg).

The concentrations determined with FPIA 2 for the energy drink, beer mix, soft drink, and cosmetic showed a good correlation with the data determined for ELISA and FPIA 1. For the other samples (espresso, instant coffee, caffeine powder, black tea, and decaffeinated coffee), a large underestimation was observed compared to the other immunoanalytical methods. The coefficients of variation (CVs) for the FPIA 2 were very high. The CVs for LC-MS/MS, ELISA and FPIA 1 were below 6%, 9%, and 4%, respectively. High precision corresponding to low CVs and the applicability to many different matrixes is desired. Therefore, FPIA 2 is not suitable for the quantitative determination of caffeine in these consumer products yet. This method can be used for fast semiquantitative analysis of many samples.

The intra- and interplate variations of concentrations of real samples as a measure for precision were proposed by Grandke *et al.* to assess the applicability of EIAs.<sup>12</sup> For the FPIAs performed in cuvettes, no intra- and interplate variations could be determined. The FPIAs performed in MTPs showed very high CVs for the real samples, which evidently exceed the desired values of 10% for the intraplate and 20% for the interplate variation. All in all, the parameters for intra- and interplate precision are not applicable.

Additionally, the correlation with LC-MS/MS as reference method was proposed as a measure for accuracy.<sup>12</sup> However, the cross-reactivity of the antibody toward other alkaloids can cause overestimations compared to instrumental methods. Therefore, the HRP TMB ELISA using the same monoclonal antibody was used as immunoanalytical reference method to render the correlation independent of cross-reactivity. For FPIA 1, the following correlation parameters were determined: slope  $m = 1.16$ , intercept  $n = -0.75$ , and coefficient of determination  $R^2 = 0.996$  for LC-MS/MS and  $m = 1.02$ ,  $n = -1.59$ , and  $R^2 = 0.992$  for ELISA (Figure 12). The parameters  $n$  and  $R^2$  show similar results for both linear regressions and are in agreement with the required values ( $R^2 > 0.95$ ,  $n$  near 0). However, the crucial slope parameter  $m$  is significantly better (requirement:  $1.00 \pm 0.05$ ) for the correlation with ELISA.



**Figure 12** Correlation between FPIA 1 and LC-MS/MS (A) and ELISA (B) for caffeine-containing beverages and cosmetics.

For FPIA 2, the parameters for the correlations with LC-MS/MS ( $m = 1.90$ ,  $n = -2.03$ ,  $R^2 = 0.933$ ) and ELISA ( $m = 0.80$ ,  $n = -1.98$ ,  $R^2 = 0.954$ ) did not fulfill all requirements, especially because the slope parameter differed significantly from unity. A notable underestimation was observed for the correlation with ELISA, although the same

monoclonal antibody was used. Altogether, the best correlation was found for FPIA 1 and ELISA, resulting in a highly accurate assay.

### Applicability of FPIA for different ground coffees and preparation methods

The caffeine concentration of different types of ground coffee (arabica and robusta) and preparation methods (filter coffee, French press, Turkish coffee, and Italian espresso) were measured with the newly developed FPIA methods. On the basis of the previous findings, only the results obtained for FPIA 1 are discussed (*Table 1*). Generally, the coffees made of robusta beans showed higher caffeine concentrations (740–850 mg/L) than arabica beans, in agreement with Casal *et al.*<sup>240</sup> The arabica coffees 1 and 2 showed similar caffeine concentrations (390–510 mg/L), and for Arabica 3, the decaffeinated coffee, caffeine concentrations in the range of 15–17 mg/L were determined. For all samples, no preconcentration steps were necessary; on the contrary, the decaffeinated coffee samples had to be diluted as well.

**Table 1** Caffeine concentrations (FPIA 1) and coefficients of variation (CVs) for several preparation methods (filter coffee, French press, Turkish coffee, Italian espresso) determined for different types of coffee (robusta, arabica 1, 2 and 3 (decaffeinated)).

	filter coffee		French press		Turkish coffee		Italian espresso	
	concn [mg/L]	CV [%]	concn [mg/L]	CV [%]	concn [mg/L]	CV [%]	concn [mg/L]	CV [%]
robusta	742±15	2.0	825±6	0.7	761±15	2.0	848±17	2.1
arabica 1	487±4	0.9	387±4	0.9	454±8	1.7	490±4	0.9
arabica 2	452±4	1.0	512±6	1.1	471±10	2.2	465±4	0.9
arabica 3	16.2±0.4	2.7	14.7±0.1	0.9	14.6±0.5	3.4	17.1±0.2	0.9

Comparing the various preparation methods, the French press method revealed opposing results for the different arabica coffees (arabica 1 and 2) because, here, the highest and the lowest caffeine concentrations were determined. All other preparation methods led to relatively similar results. For the robusta coffee, the highest caffeine concentrations were found for the French press and Italian espresso preparation method. No clear correlation between the preparation method and the caffeine concentration could be concluded for different ground coffees.

In addition to the four preparation methods, the influence of the boiling time and the ratio of coffee to water on the extracted caffeine amount was investigated (*Table 2*). A higher ratio of coffee to water yielded lower extracted caffeine amounts (based on the mass of coffee). Moreover, the extracted caffeine amount from ground coffee increased from 12.9 to 14.1 mg/kg with longer boiling times (30 min instead of 10 min). These results confirm the

conclusions made by Bell *et al.*<sup>248</sup> The results obtained for the coffee samples with FPIA 1 are precise as indicated by the good CVs, which are all below 4%.

**Table 2** Caffeine contents and coefficients of variation (CVs) determined for different ratios of ground coffee to water and different boiling times of arabica 1.

mass of arabica 1 [g]	volume of water [mL]	boiling time [min]	concn [mg/kg]	CV [%]
5.0	400	10	13.5±0.2	1.5
7.0	250	10	12.9±0.5	3.5
7.0	250	30	14.1±0.5	3.8

Two caffeine FPIA formats (FPIA 1 in cuvettes and FPIA 2 in MTPs) were developed and carefully optimized. In contrast to previously developed instrumental methods, neither FPIA requires sample preparation steps, which are typically time- and cost-intensive. Also, the measurement time for each sample is much lower for homogeneous assays compared to instrumental methods; for example, the caffeine determination in one sample takes 40 min using LC-MS/MS instead of 2 min with FPIA 1 or 20 min for the measurement of up to 24 samples simultaneously using FPIA 2. Additionally, the instruments for immunoanalytical methods are usually less expensive than equipment needed for instrumental methods like LC-MS/MS. Compared to heterogeneous immunoassays (e.g., ELISA) the FPIA is a mix-and-read assay, so no time-consuming incubation or washing steps are necessary. This makes the homogeneous assay a fast and easy screening method with sufficient sensitivity (but lower than ELISA) for almost all caffeine-containing beverages.

Both FPIAs were assessed with quality criteria previously defined for heterogeneous assays.<sup>12, 13</sup> FPIA 2 did not fulfill the requirements for the quality criteria and showed high coefficients of variation for the caffeine determination in real samples. Because of its high throughput, FPIA 2 is a good screening tool for semiquantitative caffeine determination. FPIA 1 fulfilled almost all quality criteria for the calibration curve. A variety of matrixes were analyzed and led to reliable and accurate caffeine concentrations with FPIA 1. This homogeneous assay represents an automatable method for the fast and easy quantification of caffeine in consumer products.

### 3.1.5 Acknowledgments

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## 3.2 Fluorescence polarization immunoassays for carbamazepine – Comparison of tracers and formats

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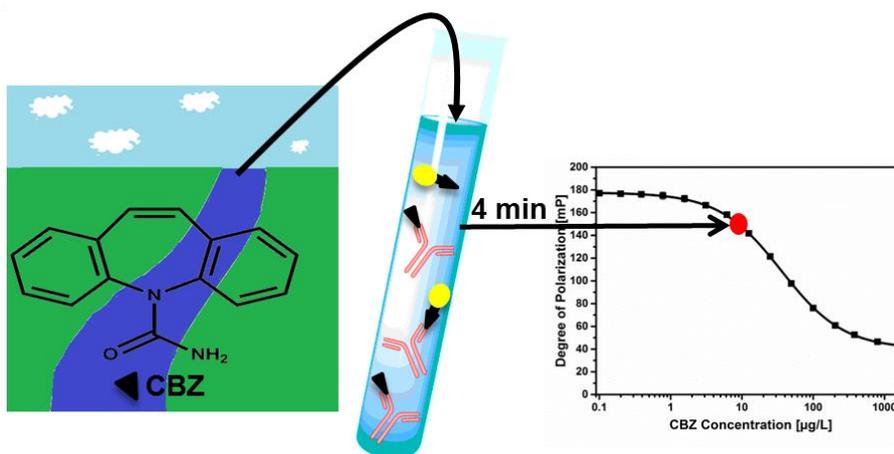
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**Figure 13** Graphical abstract of Fluorescence polarization immunoassays for carbamazepine – Comparison of tracers and formats.

### 3.2.1 Abstract

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 CBZ 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<sub>50</sub>) 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 microtiter plates and 81–140% in tubes.

### 3.2.2 Introduction

Pharmaceuticals in the water cycle are an emerging concern.<sup>249, 250</sup> 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.<sup>159</sup> 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.<sup>225, 251</sup> 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,<sup>252</sup> *i.e.* indicators of human presence or activity,<sup>120</sup> *e.g.* caffeine.<sup>119</sup>

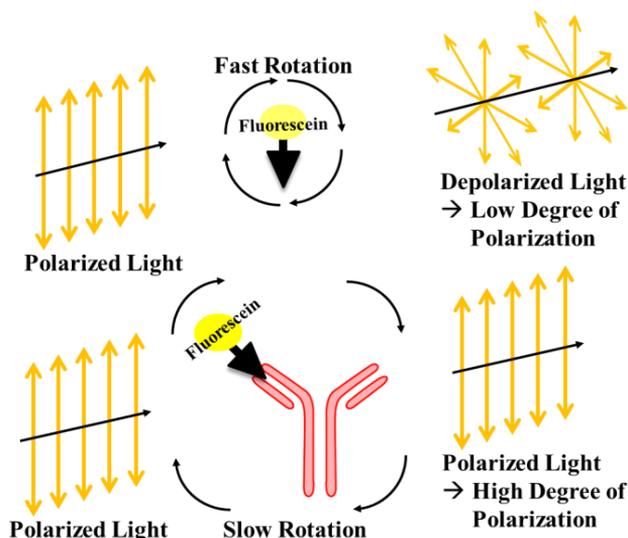
One proposed marker for wastewater cleaning efficiency and consequently wastewater contamination of surface and ground waters is carbamazepine (CBZ),<sup>4, 167, 170, 183, 184, 253</sup> an antiepileptic drug with a yearly consumption of 1,014 tons worldwide.<sup>149</sup> Due to its low degradation rate in most wastewater treatment plants, it enters the water cycle.<sup>152</sup> CBZ is recently one of the most frequently detected pharmaceutical in surface and ground water samples from Danube river in Serbia.<sup>190</sup> Negative effects of this pharmaceutical on health status of aquatic organisms were reported.<sup>165, 201, 232</sup>

Instrumental methods like liquid chromatography with tandem mass spectrometry (LC-MS/MS)<sup>155, 177</sup> and gas chromatography MS<sup>219</sup> 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.<sup>4, 13, 16, 230</sup>

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 environmental analysis to determine small compounds, including mycotoxins, drugs and pesticides.<sup>31-34, 55, 57, 58, 63, 68, 254-256</sup>

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 14). 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 14** 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.<sup>68</sup> 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.<sup>31-34</sup> 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 concentrations of 4 to 12 mg/L need to be quantified.<sup>257</sup> 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.

### 3.2.3 Experimental

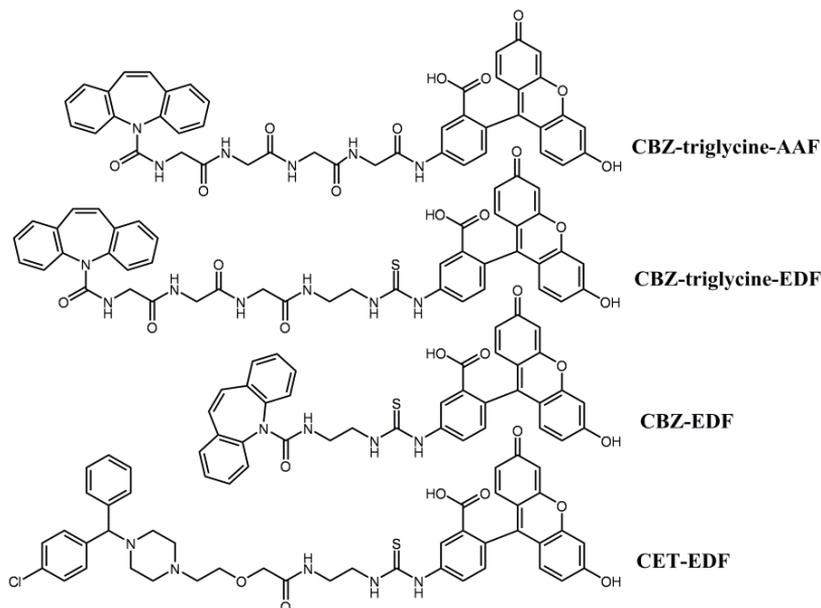
#### Reagents and materials

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.*<sup>258</sup> *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 (BioTek, 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 15*) were synthesized using CBZ-triglycine,<sup>16</sup> 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.<sup>256</sup> The EDF tracers with the haptens CBZ-triglycine and CET were synthesized according to the following protocol: Approximately 5  $\mu\text{mol}$  of antigen were dissolved in 100  $\mu\text{L}$  DCC solution in dimethylformamide (DMF, 100  $\mu\text{mol}/\text{mL}$ ) and 100  $\mu\text{L}$  NHS solution (100  $\mu\text{mol}/\text{mL}$  in DMF), leading to a ratio of 1:2:2 of antigen to DCC to NHS and a total volume of 200  $\mu\text{L}$ . The reaction mixture was mixed and incubated for 6 h at room temperature. Approximately 1  $\mu\text{mol}$  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  $\mu\text{L}$  DMF and 10  $\mu\text{L}$  triethylamine. The mixture was incubated for 18 h.



**Figure 15** Chemical structures of the synthesized tracers for the application on CBZ FPIA.

## Results and discussion

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.

### 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):

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

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 ( $\Delta x$ ), calculated from the respective standard deviations of the degree of polarization (StD) and the slope (1<sup>st</sup> derivative) at each individual calibrator concentration, as described by Ekins:<sup>11</sup>

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

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%.<sup>12</sup>

**FPIA in tubes** In a round-bottom glass tube, 1 mL of borate buffer and 100  $\mu$ L 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  $\mu$ L of the tracer CBZ-triglycine-AAF, diluted 1:6000 in tracer stabilization buffer and 100  $\mu$ L of the monoclonal anti-CBZ antibody, diluted in antibody stabilization buffer (4.5  $\mu$ g/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).<sup>13</sup> 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 three different CBZ concentrations: 1, 10, and 100  $\mu$ g/L. The samples were stored at -20 °C until their usage.

## 3.2.4 Results and discussion

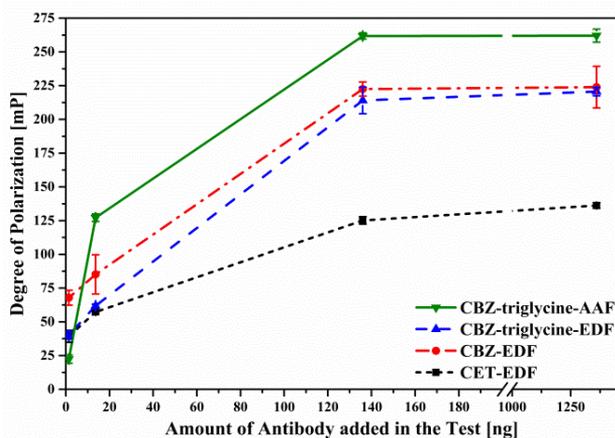
### Optimization and comparison of FPIA using different tracers

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 16).

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.<sup>235</sup> Due to the  $pK_a$  of 6.30 of fluorescein,<sup>259</sup> 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.



**Figure 16** 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).

For the comparison of sensitivity of the tracers, calibration curves using optimized concentrations of all reagents were used (Table 3). The optimum dynamic range (distance between upper and lower asymptote,  $A - D$ ) was fixed to around 140 mP.

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 84 mP could be reached. But with the increasing dynamic range, the test midpoint also increased from 34 to 81  $\mu\text{g/L}$  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.

**Table 3** Characteristic parameters of the calibration curves of CBZ FPIA using different tracers: mass of antibody used per measurement ( $m(\text{Ab})$ ), upper and lower asymptote ( $A$  and  $D$ ), test midpoint ( $C$ ), slope at  $C$  ( $B$ ), dynamic range ( $\text{DR}$ ,  $A - D$ ), and coefficient of determination  $R^2$ .

Tracer	$m(\text{Ab})$ [ng]	$A$ [mP]	$B$	$C$ [ $\mu\text{g/L}$ ]	$D$ [mP]	$\text{DR}$ [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

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  $\mu\text{g/L}$ ) than that of CET-EDF.

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.<sup>31-34</sup>

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.

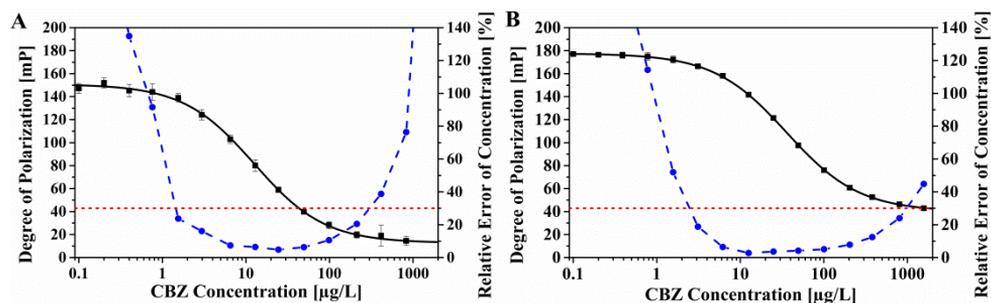
The AAF tracer led to the lowest test midpoint of 13  $\mu\text{g/L}$ , *i.e.* this tracer allows for the most sensitive CBZ FPIA assay. At the same time, the lowest antibody amount has to be used when this tracer is applied. There is only a slight structural difference compared to CBZ-triglycine-EDF. 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.<sup>36</sup> Therefore we propose that the quenching effect is smaller for the

derivative AAF compared to EDF. This suggestion would also explain why an almost 10 times 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.<sup>36</sup> 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 17).



**Figure 17** 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).

Characteristic values for the evaluation of immunoassays were previously defined for heterogeneous immunoassays<sup>12, 13</sup> and already applied for homogeneous assays.<sup>256</sup> 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  $\mu\text{L}$  are used. This might be the reason for the slightly higher standard deviations. Additionally, the mixing of the reagents can influence 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.<sup>12, 13</sup> This was not done for homogeneous assays.<sup>256</sup> 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  $1.0 \pm 0.1$ . 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  $\mu\text{g/L}$  range. The assay on MTPs is slightly more sensitive (13  $\mu\text{g/L}$ ) than the assay performed in tubes (36  $\mu\text{g/L}$ ). 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).<sup>13</sup> Previously developed FPIAs performed on MTPs showed test midpoints in the range of 0.25  $\mu\text{g/L}$  for azoxystrobin<sup>55</sup> to 207  $\mu\text{g/L}$  for butachlor.<sup>63</sup> For FPIAs in tubes even a wider range of test midpoints was reported: from 0.48  $\mu\text{g/L}$  for ochratoxin A<sup>57</sup>, over 517  $\mu\text{g/L}$  for zearalenone<sup>58</sup> up to 2.48 mg/L for sodium benzoate.<sup>72</sup> 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  $\mu\text{g/L}$ ) than in tubes (2.5  $\mu\text{g/L}$ ). But when the assay in tubes is used, a wider concentration range of CBZ can be determined (up to 980  $\mu\text{g/L}$  in tubes; up to 310  $\mu\text{g/L}$  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).<sup>13</sup> 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 1:6000 (1:40,000 on MTPs) and five times more volume had to be used per measurement (100 instead of 20  $\mu\text{L}$ ) 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

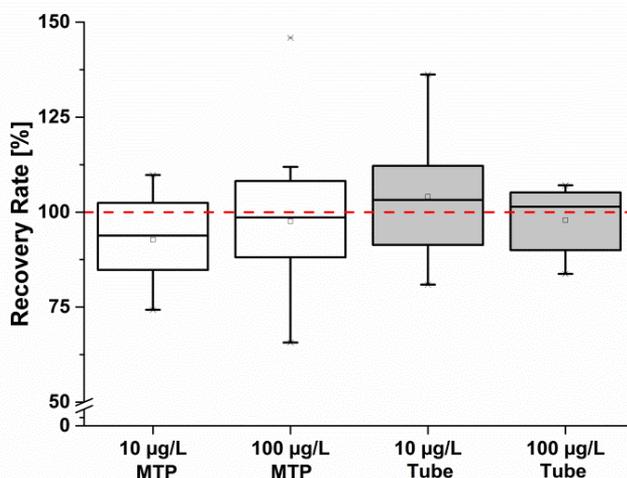
the dynamic range is the same for a constant ratio of antibody to tracer, independent of the format. So the most important factor on how much antibody has to be used, besides the choice of the tracer, is the sensitivity for fluorescence intensities of the applied instrument.

Compared to ELISA, eight times more antibody had to be used for FPIA on MTPs (ELISA: 8.6 ng/ $\mu$ L in 200  $\mu$ L, equal to 1.72 ng per measurement).<sup>13</sup> 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  $18,500 \pm 600$  RFU (relative fluorescence units, mean from all measurements  $\pm$  standard deviation) for calibrators and  $18,100 \pm 1100$  RFU for samples; in tubes background corrected fluorescence intensities of  $331,000 \pm 4000$  RFU for calibrators and  $332,000 \pm 3000$  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  $21.4 \pm 2.7$  mP for calibrators and  $20.0 \pm 3.3$  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 of 74–110% for 10  $\mu$ g/L and 66–110% for 100  $\mu$ g/L when the CBZ FPIA on MTPs was applied (*Figure 18*). The medians were 94% and 99% for 10 and 100  $\mu$ g/L, respectively. Similar recovery ranges were obtained when the CBZ FPIA in tubes was applied for the CBZ determination: 81–136% for 10  $\mu$ g/L and 84–107% for 100  $\mu$ g/L. The medians were very accurate with 103 and 101% for 10 and 100  $\mu$ g/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  $\mu$ g/L). As expected, poor recovery rates with high deviation were observed for both methods: 32–240% on MTPs, and 69–226% in tubes.



**Figure 18** 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.<sup>13, 16</sup> 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. 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.<sup>33</sup> 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.

### **3.2.5 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.

### **3.2.6 Acknowledgements**

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### 3.3 Production and characterization of new monoclonal anti-carbamazepine antibodies and application to fluorescence polarization immunoassay

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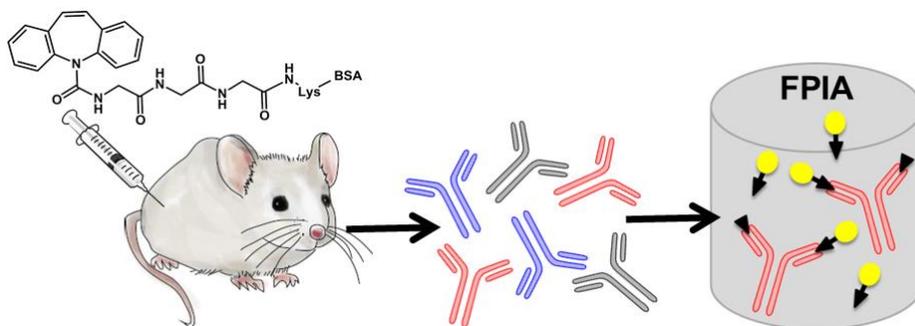
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**Figure 19** Graphical abstract of Production and characterization of new monoclonal anti-carbamazepine antibodies and application to fluorescence polarization immunoassay.

#### 3.3.1 Abstract

Carbamazepine (CBZ) is a widely used antiepileptic drug which also frequently occurs in the environment. A fast, easy and accurate determination is desirable and can be achieved by immunoanalytical methods such as homogeneous fluorescence polarization immunoassay (FPIA). The prerequisite for this is the choice of the optimal antibody. We present a new monoclonal antibody selective for CBZ and methods for a more efficient, transparent, animal-friendly and faster antibody production process including feces screening and supernatant screening with FPIA. The new antibody enables CBZ determination in the concentration range 0.66-110  $\mu\text{g/L}$  within 10 min using a high-

throughput microtiter plate-based FPIA, and between 1.4 and 79 µg/L within 5 min applying an automated cuvette-based FPIA instrument, and at 0.049-36 µg/L using ELISA. Due to low cross-reactivity especially towards the main CBZ metabolite 10,11-dihydro-10,11-dihydroxy-CBZ and other pharmaceuticals like cetirizine or oxcarbazepine (< 1%), this antibody can be applied to medical and environmental analysis; the FPIA can be a tool for process analysis applications.

### 3.3.2 Introduction

Carbamazepine (CBZ) is an antiepileptic drug, which is widely used in the treatment of trigeminal neuralgia, and grand mal seizures. It can also be used for the treatment of psychiatric disorders, e.g. bipolar disorder or borderline personality disorder.<sup>260</sup> The main metabolic pathways of CBZ in humans and the distribution of extracted CBZ were summarized by Bahlmann *et al.*<sup>152</sup> The major degradation pathway is through the transformation by the enzyme cytochrome P450 to 10,11-epoxy-CBZ (Ep-CBZ). This intermediate is then enzymatically hydrolyzed to 10,11-dihydro-10,11-dihydroxy-CBZ (DiOH-CBZ), which represents the major part of excreted CBZ.

Due to the widespread use and a low degradation rate in wastewater treatment plants, CBZ is often used as a marker for wastewater input into surface and ground water.<sup>4, 167, 170, 253</sup> When CBZ enters surface water, it can reveal negative effects on health status of aquatic organisms.<sup>165, 198, 200, 201, 232</sup> If treated wastewater is used for irrigation, pharmaceuticals like CBZ can be taken up by plants. But usually the resulting annual exposure through dietary intake of vegetables is negligible (0.64 µg CBZ per capita) compared to the defined daily dose of 1000 mg.<sup>207</sup> Additional cleaning steps, e.g. ozonation, membrane filtration or hydrodynamic acoustic cavitation would improve the degradation rate in wastewater treatment plants.<sup>174, 181, 183, 261</sup> Therefore, CBZ can be seen as a marker for the cleaning efficiency of wastewater treatment plants.<sup>183</sup>

Immunoassays give a good opportunity for an extensive screening for this marker. Usually these methods are performed on 96 well microtiter plates (MTPs) and thus they are characterized by a very high throughput. The most used immunoassay is the enzyme-linked immunosorbent assay (ELISA), which belongs to the group of heterogeneous assays and shows very high sensitivity. ELISAs have been developed for CBZ and have been successfully applied to water samples.<sup>13, 16</sup> But this assay includes long incubation steps (0.5-18 h) and several washing steps. The fluorescence polarization immunoassay (FPIA) represents a fast alternative. This assay belongs to the group of homogeneous assays, which means that no washing steps are required. Additionally, FPIA usually only requires one short incubation step of a few minutes. FPIA for the determination of CBZ in serum is already used.<sup>257</sup> Recently this assay has been also applied to surface water samples.<sup>262</sup>

The prerequisite for a sensitive and accurate determination with immunoassays is the availability of a highly selective antibody with high affinity to the target analyte. Previously described CBZ immunoassays were performed with a monoclonal anti-CBZ antibody, which showed high cross-reactivity (CR) against CBZ metabolites and related compounds, but also to the antihistaminic drug cetirizine which is not structurally close to CBZ.<sup>4, 234, 235</sup> This leads to overestimations of CBZ levels in water samples, especially during hay fever season, when the antihistamine is present in waters. To avoid this effect, a new, more selective monoclonal antibody against CBZ was desirable.

The common protocol for the production of monoclonal antibodies starts with the immunization of one or more mice. The blood of the mice is examined by ELISA to check the presence of anti-analyte specific antibody. This practice is painful for the animals because usually the blood sample is taken by facial vein puncture, retrobulbar puncture or tail vein puncture. In order to warrant good animal welfare, this test can only be performed at long time intervals. This makes it impossible to find the best moment for re-immunization or the termination of immunization process. A more time-resolved method is therefore desirable. Carvalho *et al.* showed that the extraction and evaluation of antibodies from mouse feces is a good alternative to serum screening.<sup>79</sup> Additionally, this allows a time-resolved evaluation of the immunization progress without hurting the animals. After several boosts, the mouse presenting the highest level of anti-analyte antibodies is selected for further steps of antibody production.

Next, spleen cells from the selected mouse are fused with myeloma cells as described by Köhler and Milstein.<sup>80</sup> After the fusion, the cell culture supernatants have to be tested in order to decide which hybridoma cells are producing the best antibody. This screening is usually performed by ELISA, which is very time-consuming due to long incubation times. As a fast alternative, FPIA could be used as screening method. The applicability of FPIA to antibody-enriched medium has been already shown by Kolosova *et al.*<sup>65</sup> Additionally, FPIA can be used for the characterization of antibody properties.<sup>30</sup>

The goal of this work was to produce a new monoclonal CBZ-specific antibody that could be applied especially to the analysis of water samples without giving a hay fever season dependent overestimation. For the monitoring of the immunization progress, the antibody selection and the antibody characterization, animal-friendly and time-efficient methods should be evaluated for their suitability.

### 3.3.3 Material and methods

#### Reagents and materials

All solvents and chemicals were purchased from Sigma-Aldrich, Merck KGaA, Serva, Mallinckrodt Baker and Toronto Research Chemicals Inc. in the highest available quality. The FP tracer CBZ-triglycine-5-(aminoacetamido)fluorescein (CBZ-AAF) was previously synthesized.<sup>262</sup> CBZ-triglycine and the tracer for ELISA, CBZ-triglycine-horseradish peroxidase (CBZ-HRP) was previously prepared by Bahlmann *et al.*<sup>16</sup> CBZ-triglycine-ovalbumin (CBZ-OVA) was prepared following the same procedure.<sup>16</sup> For the preparation of buffers and solutions, ultrapure water from a Synthesis A10 Milli-Q® water purification system from Millipore was used. The composition of the phosphate buffered saline (PBS) buffer, PBS-based washing buffer, sample buffer, citrate buffer, and 3,3',5,5'-tetramethylbenzidine (TMB) solution were described previously.<sup>12</sup>

During synthesis of the immunogen, a thermomixer compact (Eppendorf) was used. PD-10 desalting columns (GE Healthcare) were used for the purification of the immunogen. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) measurements using a Bruker Reflex III instrument (Bruker-Daltonik) was used to determine the coupling ratio of the immunogen. 96-well clear UV-Star MTPs (Greiner Bio-One) were used for fractionating the synthesized immunogen. Clear high-binding and black non-binding 96-well MTPs from Greiner Bio-One were employed for ELISA and FP

## Results and discussion

measurements, respectively. All assay incubation and shaking steps were performed on the plate shaker Titramax 101 from Heidolph (750 rpm). The MTPs for ELISA were washed using an automated plate washer from BioTek. For the measurements of absorbance (ELISA) and fluorescence polarization (FPIA), eon and Synergy H1 plate readers from BioTek were used, respectively. Both were controlled by the software Gen5 (BioTek). FPIA in cuvettes was performed on the filter-based aokin spectrometer FP 470 (aokin AG). The system was controlled by the aokin software mycontrol™. The excitation wavelength was fixed at 470 nm, and the emission was measured at 520 nm. The fluorescence intensities at perpendicular and parallel polarizer settings were measured simultaneously and continuously (kinetic measurement). For automated measurements, the aokin liquid handling workstation (LHW), which can be connected to the spectrometer, was used.

### Synthesis of immunogen

The *N*-hydroxysuccinimide (NHS)/*N,N'*-dicyclohexylcarbodiimide (DCC) activated ester method was used for the synthesis of the immunogen CBZ-triglycine-bovine serum albumin (CBZ-BSA). For this, 6.8 μmol of the hapten CBZ-triglycine were dissolved in 50 μL dimethylformamide (DMF). Then 20 μL of NHS (46.5 g/L in DMF) and DCC solution (83.5 g/L in DMF) were added. The mixture was shaken for 18 h in a thermomixer at 22 °C and 700 rpm. Then the reaction mixture was centrifuged for 10 min at 20 °C and 14,000 rpm, in order to separate the solution from the precipitate formed. BSA (6.0 mg) was dissolved in 600 μL of a 0.27 mol/L sodium hydrogen carbonate solution. Into that solution, small volumes of the activated ester solution were added every few minutes (12×5 μL). Between the pipetting steps, the reaction mixture was shaken in the thermomixer. After in total 60 μL of the activated ester having been added to the BSA solution, the mixture was shaken for 4 more hours at 22 °C and 700 rpm.

The conjugate was purified using a PD-10 desalting column. The column was first equilibrated with 25 mL 1:10 diluted PBS buffer (pH 7.6). Then the reaction mixture was applied to the column and was then eluted with 7.5 mL of the diluted PBS buffer. The fractions were collected in a MTP (three drops per well) and the absorbance was measured at 280 nm with a reference wavelength of 620 nm. The fractions with an optical density (OD) higher than 0.5 were collected.

The collected fraction was applied on a Zeba™ spin micro desalting column. A dihydroxyacetophenone (DHAP) matrix was used for MALDI-TOF-MS measurement. Masses of 66,454 and 76,635 Da were determined for the BSA and CBZ-BSA conjugate, respectively. CBZ-triglycine minus water has a mass of 390 Da. Consequently, the mean coupling ratio was 26 molecules of CBZ-triglycine per BSA molecule. The protein concentration of the CBZ-BSA (3.2 g/L) was determined using a Bradford assay as described before.<sup>12, 263</sup>

### Antibody production

The production of the anti-CBZ antibodies including the immunization, fusion, cultivation, purification and subtyping was performed at hybrotec GmbH (Potsdam, Germany). All animal experiments were conducted in accordance with animal ethical care regulations and with German law. For the immunization of three Balb/c mice (mouse 1-3), the immunogen CBZ-BSA was used. For the first injection, 100 μg of the conjugate with Freund's adjuvant

were used for each mouse. After 42 days, another 50 µg were injected. Blood samples were tested 48 days after the first injection. The mouse with the highest antibody titer, determined by indirect ELISA using CBZ-OVA, was chosen for the production of monoclonal anti-CBZ antibodies. After another CBZ-BSA injection (day 112), spleen cells of this mouse (mouse 1) were fused with myeloma cells 116 days after the first immunization.

The resulting hybridoma cells were cultivated in eight 96-well MTPs. The presence of anti-CBZ antibodies was tested for the supernatants of all these clones with an indirect, competitive ELISA. 14 clones showed a reaction with CBZ-OVA and five of them gave a reasonably high signal. For further investigations, 0.1% sodium azide was added to the supernatants of the five selected clones. After additional testing, these clones were further cultivated and purified through a protein A column and the subclasses for each of these antibodies were determined (all subisotype IgG1).

The purified antibodies were stored at -20 °C after adding different amounts of glycerol, depending on the antibody concentration. Too low concentrations should be avoided. Therefore 25% instead of 50% glycerol were added for the longtime storage of antibodies from clone 2 and 4, so that all concentrations were higher than 400 mg/L.

### **Feces screening**

Feces samples of all three mice were collected from day 11 after the immunization and then every 7 days. The samples were stored at -20 °C until analysis. The antibodies from these samples were extracted by dissolving the feces in extraction buffer (1.5 mL extraction buffer per 0.1 g feces). The extraction buffer was prepared by dissolving 1% BSA, 1% NaN<sub>3</sub> and 2 tablets protease inhibitor cocktail tablets (Roche) in 100 mL PBS buffer. The mixtures of extraction buffer and feces were shaken for 23 h in centrifugation tubes on a shaking table with 80 rpm at room temperature. Afterwards the mixtures were centrifuged two times for 10 min at room temperature. The supernatants were used to analyze the content of anti-CBZ specific antibodies using direct, competitive ELISA as described later on. Instead of monoclonal anti-CBZ antibodies, the undiluted feces extracts were used. When enough extract was present, a triplicate determination was performed. Unfortunately in some cases not enough feces could be collected and therefore not enough extract could be produced. For some samples, only a single or duplicate determination could be performed.

### **Direct, competitive ELISA**

For direct, competitive ELISA, each well was coated with 200 µL anti-mouse IgG antibody (polyclonal, sheep, lot 21481, Acris Antibodies) at 1 mg/L in PBS buffer. 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 using a PBS-based washing buffer.

Then 200 µL of the respective anti-CBZ antibodies were added to each well and incubated for 1 h. For the feces screening, undiluted feces extracts instead of the monoclonal antibody dilutions were used. For investigations of cell culture supernatants, different dilutions of supernatants were used, so that the upper asymptote was comparable for all clones. The fully optimized assay described here uses the antibody from clone 1 diluted in PBS buffer at a concentration of 7.5 µg/L.

## Results and discussion

After another washing step, 150  $\mu\text{L}$  of different calibrators were added to the respective wells. For the comparison of the sensitivity of the antibodies, only CBZ calibrators were used. For the determination of CRs, calibrators of the different CBZ-related substances were used. Directly after adding the calibrators, 50  $\mu\text{L}$  of the CBZ-HRP conjugate diluted in sample buffer (8.3  $\mu\text{g/L}$ , pH 9.5) were added. For feces screening and investigations on cell culture supernatants, a higher tracer concentration of 16.6  $\mu\text{g/L}$  was chosen. After a 30 min incubation period and another washing step, the TMB substrate solution was added. This solution was prepared according to the following protocol:<sup>264</sup> 21 mL citrate buffer with 8.1  $\mu\text{L}$  hydrogen peroxide (30%) and 525  $\mu\text{L}$  TMB solution were mixed and 200  $\mu\text{L}$  were added to each well. The reaction was stopped after 30 min by adding 100  $\mu\text{L}$  1 mol/L sulfuric acid. Absorbance was measured at 450 nm and referenced to 620 nm.

The precision profile was determined by measuring 16 CBZ calibrators in sixuplicate. For calculations, the software Origin 9.1G (OriginLab) was used. As described by Ekins, the relative errors of concentration were calculated.<sup>11</sup> The concentrations with a relative error of lower than 30% were defined as the measurement range. This value was chosen following the three sigma criterion as described previously.<sup>12</sup>

### FPIA on MTP

For the homogeneous assay on MTPs, 280  $\mu\text{L}$  borate buffer (2.5 mmol/L disodium tetraborate decahydrate, 0.01% sodium azide, pH 8.5) with 0.01% Triton™ X-100 were pipetted into each well. After adding 20  $\mu\text{L}$  of calibrators, a background measurement was performed with excitation at 485 nm and emission at 528 nm (using a polarizer at parallel and perpendicular settings). 20  $\mu\text{L}$  of the tracer CBZ-AAF, 1:40,000, diluted in a PBS-based tracer stabilization buffer,<sup>262</sup> was added to each well and shaken for 5 min. Then 20  $\mu\text{L}$  of anti-CBZ antibody in a PBS-based antibody stabilization buffer<sup>262</sup> were added. For the final assay, 20  $\mu\text{L}$  of the antibody from clone 1 (375  $\mu\text{g/L}$ ) were used. After 10 min of shaking, the fluorescence intensities were measured with the settings described above.

The total fluorescence intensities were determined as the sum of the parallel and double perpendicular intensity. The G factor was set to 1.0. The fluorescence intensities at perpendicular and parallel polarizer settings from the background measurement were subtracted from the respective values. These background-corrected values were then used for the calculation of the degree of polarization. The precision profile for clone 1 was determined as described for ELISA.

### FPIA in cuvettes

For the examination of the cell culture supernatants with FPIA in cuvettes, all steps were performed manually. First, 2 mL borate buffer were pipetted into the round glass cuvette containing a stir bar. 100  $\mu\text{L}$  ultrapure water was added instead of calibrators. Then 100  $\mu\text{L}$  of tracer dilution (1:20,000 in stabilization buffer) were added. Afterwards, small volumes of the cell culture supernatants were pipetted into the cuvette. The degrees of polarization were corrected by the background signals and the G factor (determined for each measurement) and the degree of polarization of the free tracer was subtracted.

The calibration curve and precision profile of the selected antibody (clone 1) was determined automatically using the LHW. All volumes were adapted from the manual measurement described above, besides the calibrator (here: 200  $\mu\text{L}$ ) and the antibody.

Here, 100  $\mu\text{L}$  of a dilution of clone 1 (1500  $\mu\text{g/L}$ ) in stabilization buffer were used. All calibrators were measured in triplicate. The G factor was fixed at 1.10.

### Cross-reactivity

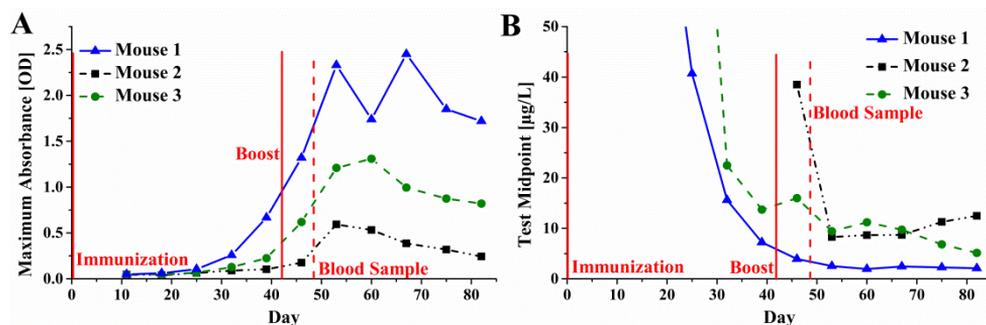
The CR of twelve substances were determined with ELISA and FPIA on MTPs: 10,11-dihydro-CBZ (DiH-CBZ), Ep-CBZ, Oxcarbazepine (Ox-CBZ), DiOH-CBZ, 10,11-dihydro-10-hydroxy-CBZ (10-OH-CBZ), 2-hydroxy-CBZ (2-OH-CBZ), 3-hydroxy-CBZ (3-OH-CBZ), CBZ-triglycine, iminostilbene, opipramol dihydrochloride, loratadine and cetirizine dihydrochloride (CET). Each cross-reactant was determined in triplicate on each MTP and on two MTPs. The molar CRs were determined dividing the molar test midpoint of CBZ by the molar test midpoint of the cross-reactant. The CR towards 2-OH-, 3-OH- and DiH-CBZ were additionally determined on one MTP for ELISA at pH 8.5 (pH of the sample buffer was varied). The CR of DiOH-CBZ, 2-OH-CBZ and CET were also determined on one MTP for cell culture supernatants from clone 1-5 using ELISA.

## 3.3.4 Results and discussion

### Immunization progress

The extraction of antibodies from mice feces was performed for all three immunized mice. With the undiluted extracts, calibration curves were set up with direct, competitive ELISA. The maximum absorbance as a measure for the antibody titer and the test midpoint as an indicator for affinity were determined (Figure 20). The immune response of the three mice differed considerable. After the immunization, nearly no signal could be detected for mouse 2, *i.e.* almost no anti-CBZ antibodies were found in the feces of this mouse; *i.e.* this mouse did not produce anti-CBZ antibodies until the first boost. In feces of mice 1 and 3 an increasing antibody titer was observed even before the first boost (Figure 20A). Moreover the affinity increased strongly (lower test midpoints) before the second dose of the immunogen was administered to the mice (Figure 20B).

The maximum absorbance for all three mice decreased after they had reached their maximum after the first boost. But the test midpoints stayed almost constant at their lowest levels. So the reached affinity seems not to deteriorate again, even when there is no new contact with the immunogen for a while.



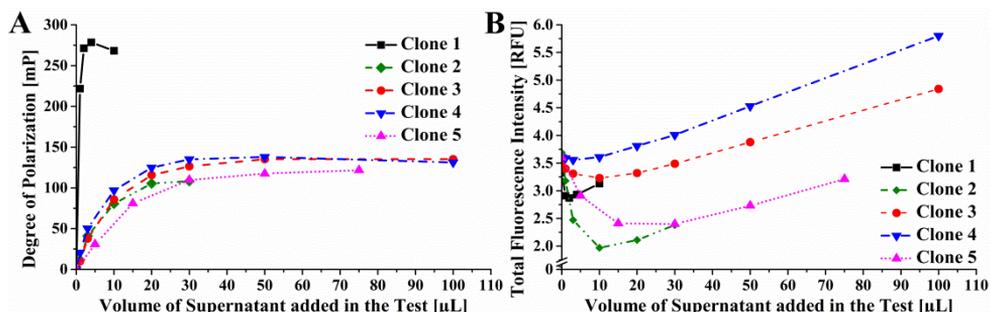
**Figure 20** Maximum absorbance (A) and test midpoints (B) were determined with ELISA for feces samples of the three mice. The day of immunization (day 0), the first boost (day 42, solid red lines) and the day of collecting blood samples (day 48, dashed red line) are given.

For the blood samples collected 48 days after the immunization, the maximum absorbance of different dilutions were determined with indirect ELISA (performed at hybrotec). Blood from mouse 2 showed also the by far lowest absorbance for all dilution factors. Mouse 1 and 3 showed values in a similar range, the results for mouse 1 being a little bit better. So the results from blood and feces screening were in accordance with each other, while much more information can be obtained using the feces method and this without hurting the animals. Mouse 1 was finally selected for spleen removal and fusion of B-cells with myeloma cells.

### Characterization of antibodies in cell culture supernatants

The supernatants of hybridoma cells (8x96) were tested with an indirect ELISA (performed at hybrotec) and the five best clones, showing the highest signals, were selected. All these clones showed also an inhibition by CBZ. The properties of these antibodies in cell culture supernatants were investigated with FPIA. Therefore the assay was performed in cuvettes with an instrument which allows the kinetic observation of the tracer/antibody reaction.<sup>256</sup> Different buffers were tested to select the optimum conditions for FPIA measurements with the selected cell culture supernatants: carbonate buffer pH 9.6, sample buffer pH 9.5, Tris buffer pH 8.5, borate buffer pH 8.5 and PBS buffer pH 7.6. Only buffers with neutral to alkaline pH values were selected because the fluorescence intensity of the fluorescein tracer decreases considerably under acidic conditions. For all clones, besides clone 2, borate buffer led to the highest degree of polarization values using the smallest volume of supernatant. For clone 2, carbonate buffer led to the best results. For a better comparability, borate buffer was used for further experiments.

The maximum degrees of polarization ( $P_{max}$ ) for different supernatants were determined by adding continuously small amounts of supernatant to the buffer containing the CBZ-fluorescein tracer (Figure 21A). For clone 1,  $P_{max}$  was already reached after adding 2  $\mu\text{L}$  of the supernatant. For the other clones,  $P_{max}$  was not reached until 20  $\mu\text{L}$  (clone 2) or 30  $\mu\text{L}$  (clone 3-5) of supernatant had been added.  $P_{max}$  of clone 1 with 280 mP was much higher compared to all other supernatants (100-140 mP). So the by far highest affinity towards the tracer was observed for the antibodies in supernatant of clone 1.

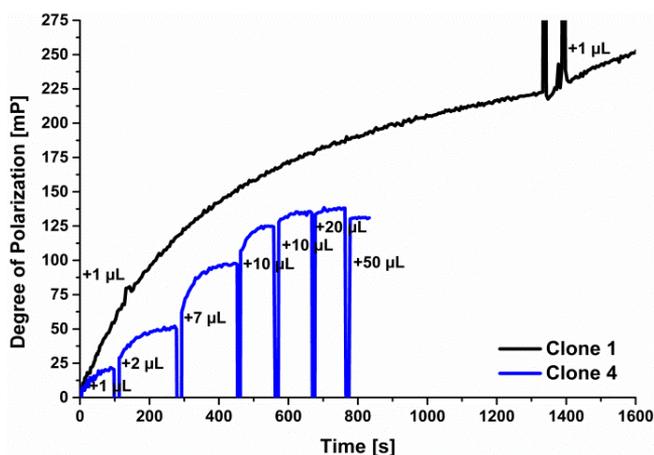


**Figure 21** Degrees of polarization (A) and total fluorescence intensities (B) were measured with FPIA depending on the volume and kind of cell culture supernatant (clone 1-5).

The supernatants showed an intense color due to phenol red that is contained in the cell culture medium used. Therefore it was expected that the fluorescence intensity would increase the more supernatant was added to the assay. This was the case for clone 1, 3

and 4, but not for clone 2 and 5 (*Figure 21B*). Here, first strong decreases of the fluorescence intensities were observed before the intensities increased again. This means that the antibodies in the supernatants significantly reduced the fluorescence intensity of the tracer. After adding a certain volume of the supernatant, the fluorescence intensities increased again due to the high amount of phenol red. So for purified antibodies, it is expected that the fluorescence intensity does not increase again. This can have negative effects on FPIA performance because the measured values are fluorescence intensities and based on them the degree of polarization is determined. So if the measured intensities are low, the relative error increases and therefore also the error of the determined degree of polarization becomes larger.

Another interesting issue is the kinetics of the tracer/antibody interaction. Usually this reaction is finished within a few hundred seconds, e.g. for a previously described caffeine FPIA using the same instrument, the equilibrium was reached after 100 s.<sup>256</sup> Here, similar reaction times were observed: 100 s for clone 3 and 5, and 200 s for clone 2 and 4 (exemplarily shown for clone 4 in *Figure 22*). Antibodies from clone 1 showed a much slower reaction with the tracer (1400 s, *Figure 22*). But much less supernatant is necessary to reach a much higher degree of polarization than for all other supernatants.



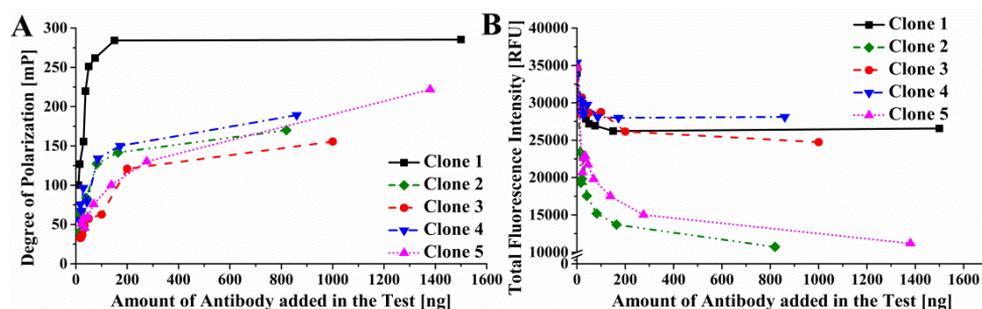
**Figure 22** Kinetic measurements of degrees of polarization of supernatants from clone 1 (black line) and 4 (blue line) were performed; the amount of supernatant addition for each clone is given in the figure (in  $\mu\text{L}$ ) (Explanation on the peaks: when supernatant was added to the assay, the pipette was within the optical pathway and therefore the degree of polarization changed rapidly for a short time).

CR of the antibodies in cell culture supernatants were determined by direct, competitive ELISA for some selected cross-reactants. DiOH-CBZ is the main metabolite of CBZ and is therefore frequently found in wastewater in high concentrations. Compared to the structure of CBZ, this substance shows a change in the central, nitrogen-containing ring. To investigate the influence of changes of other parts of CBZ, the cross reactivity against 2-OH-CBZ was determined. 4.3% of CBZ are excreted as 2-OH-CBZ.<sup>152</sup> CET was chosen because this was one of the main cross-reactants of previously used monoclonal anti-CBZ antibody, although CET is not structurally related to CBZ.<sup>234, 235</sup>

Here, only semi quantitative statements can be made, because these results were only produced to simplify the choice of the right antibody. All antibodies (from supernatants) showed very low CR (< 1%) against DiOH-CBZ and CET. For the latter, antibodies from clone 5 showed a higher CR of approximately 8%. This is still a much lower CR than the one the previously used antibody showed towards this pharmaceutical.<sup>235</sup> Nevertheless this would lead to an overestimation of CBZ determination in water samples. For 2-OH-CBZ, comparable CRs were observed for all antibodies (10-15%) except clone 2 (ca. 45%). It is noticeable that, with two exceptions, all CRs of the antibodies were similar for at least the three tested cross-reactants.

### Characterization and comparison of purified antibodies

After the purification of the selected antibodies, the best antibody was to be carefully chosen. The FPIA on MTPs was used for this evaluation because more measurements can be performed simultaneously. First, different amounts of antibody (constant volumes of antibody dilutions were used with different dilution factors) were added to a constant amount of tracer in order to determine  $P_{max}$  (Figure 23A). Clone 1 showed the by far highest  $P_{max}$  (285 mP) and the lowest amount of antibody had to be employed (160 ng) to reach this level. This  $P_{max}$  is in good agreement with the value obtained before for the antibodies contained in the hybridoma supernatants. For the other antibodies, higher  $P_{max}$  values were obtained compared to the ones obtained for the respective supernatants (150-220 mP). For some antibodies,  $P_{max}$  was not completely reached using 1000 ng antibody per measurement.



**Figure 23** Degrees of polarization (A) and total fluorescence intensities (B) were measured with FPIA depending on the amount of the different purified antibodies added in CBZ FPIA (clone 1-5).

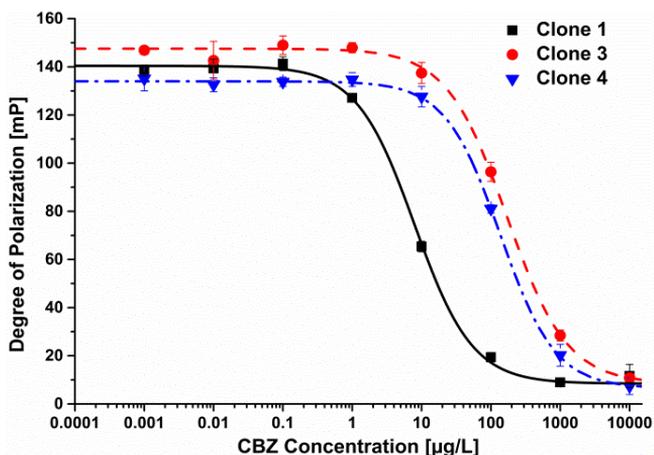
Total fluorescence intensities decreased for all antibodies after the addition of the antibody doses (Figure 23B). The fluorescence intensities showed only slight decreases when clone 3 (29%), 1 (22%) or 4 (21%) were used. However, the antibodies from clone 2 and 5 led to significant decreases of fluorescence intensity of 69 and 68%, respectively. The contrary effect was observed by Tan *et al.*<sup>20</sup> They found that the binding of the tracer to the antibody increased the fluorescence intensity of the tracer. They used this effect and developed a homogeneous increasing fluorescence immunoassay (HiFi). They suggested that the fluorescence of fluorescein is quenched due to the coupled analyte (tetrahydrocannabinol). When the analyte part of the tracer is obscured due to the binding to the antibody, the quenching effect is eliminated and the fluorescence intensity increases. In our study, the opposite effect was observed. That means that the fluorescence of fluorescein is not quenched due to the coupled analyte. But the interaction with the antibody

quenches the fluorescence intensity of the tracer. A reason could be that the conformation of CBZ is changed by the binding to the antibody. Eisold *et al.* observed both effects.<sup>265</sup> Two antibodies were compared that were produced in the same immunization process against a fluorophore: one antibody enhanced and the other antibody quenched the fluorescence intensity of the fluorophore. The idea of developing a homogeneous decreasing fluorescence immunoassay using clone 2 or 5 was not pursued in this study because first experiments with CBZ calibrators showed that the sensitivity of this assay would be quite low.

The results made for the purified antibodies are in good agreement with the assumptions made after the initial examination of the supernatants. As described above, a too strong decrease of the measured values would increase the measurement uncertainty. The tracer concentration could be increased to compensate the effect observed for clone 2 and 5. But this would lead to a reduction of the assay sensitivity. Additionally, first studies on CR performed with the supernatants showed higher non-specific binding for these antibodies. Calibration curves determined for ELISA confirmed that these two antibodies lead to less sensitive methods for the determination of CBZ than the other antibodies. Taking all this together into account, these two antibodies are not suitable for the development of a CBZ FPIA and therefore were not taken into consideration for further evaluation.

The studies on the cell culture supernatants already showed that the reaction times of the antibodies with the tracer vary considerably for different antibodies, especially for clone 1, where it took very long to reach the equilibrium. All other supernatants showed a quite fast reaction. This could be confirmed for purified antibodies using FPIA: for assays on MTPs, the reactions were finished within 5 min for clone 3 and 4, whereas clone 1 did not reach equilibrium before 30 min incubation time. For the standard assay procedure on MTPs, 10 min was chosen as incubation time because it was well reproducible for the procedure of FPIA on MTPs even so as requiring shaking and transfer to the multimode plate reader. Additionally, a longer incubation time would be counterproductive with regard to one of the main advantages of FPIA: the quickness.

Calibration curves for the three remaining antibodies were determined on MTPs. For this the same amount of tracer was used and the antibody concentrations were optimized so that the dynamic range (the distance between upper and lower asymptote of the calibration curve) were in a similar range of  $130 \pm 10$  mP (*Figure 24*). Under these conditions, a good comparability of the curves could be ensured. It should be mentioned that for clone 3 the highest amount of antibody had to be used per measurement (200 ng) to reach the desired dynamic range. Using clone 1, less than one tenth of the amount used of clone 4 was necessary to reach the desired dynamic range (7.5 instead of 86 ng per measurement, respectively). The assay using antibodies from clone 1 showed the best sensitivity with a test midpoint of 7.93  $\mu\text{g/L}$ , whereas clone 3 and 4 showed similar test midpoints of 170 and 137  $\mu\text{g/L}$ , respectively. With regard to sensitivity and the usually most expensive reagent of FPIA, the antibody, clone 1 was chosen for further antibody production and development of FPIA applications.

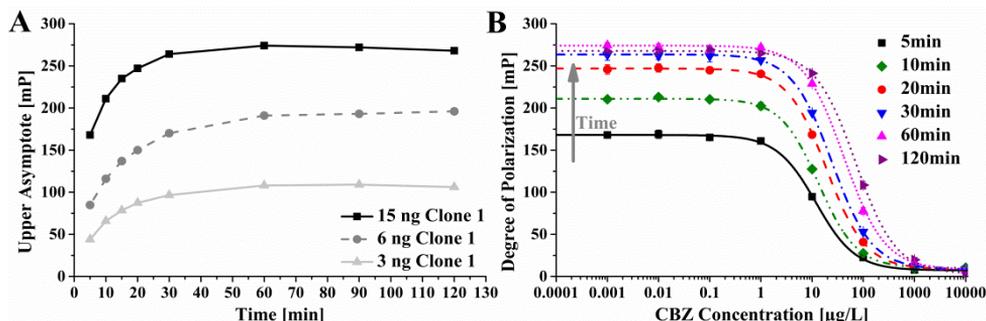


**Figure 24** CBZ FPIA calibration curves for purified antibodies from clone 1, 3 and 4 measured on MTPs after 10 min incubation time (measurements for each calibration point were performed in triplicate).

In addition to the careful examination for their use in FPIA, the antibodies from our clones were compared for their employment in direct competitive ELISA. Again the antibody from clone 1 showed the lowest test midpoint and therefore the highest measurement sensitivity. Consequently, this antibody is our choice also for its application in ELISA.

### Characterization of the selected antibody (clone 1)

**Time dependency** The selected antibody from clone 1 showed a slow reaction with the tracer. Calibration curves of different antibody dilutions (given as mass added per measurement for a better comparability to other formats) over a time range of 120 min were determined on MTPs. The maximum upper asymptote is dependent on how much antibody is used for the assay (*Figure 25A*). 15 ng of this antibody was sufficient to reach almost  $P_{max}$ . When less antibody was used, the values were much lower. The highest upper asymptote of each antibody dilution was reached between 30 and 60 min.



**Figure 25** Time dependency of the upper asymptotes for different amounts of purified antibodies from clone 1 (A) and calibration curves using this antibody (15 ng purified antibody per measurement) were determined after different incubation times (B).

The calibration curve for one antibody dilution (15 ng purified antibody per measurement) was measured after different times: 5, 10, 20, 30, 60, and 120 min (*Figure 25B*). The upper

asymptote increased from 168 mP to 274 mP. After 30 min incubation time the upper asymptote did not increase any more whereas the test midpoint still increased after 30 min from 24 µg/L, over 42 µg/L after 60 min, up to 58 µg/L after 120 min. The time dependent increase of the test midpoints was also strong at shorter incubation times: the test midpoint increased from 12 µg/L after 5 min, to 13 µg/L after 10 min, up to 19 µg/L after 20 min. Therefore the compliance to the defined incubation time is very important. The effect of increasing test midpoint over incubation times was previously observed for ELISA for polyclonal<sup>266</sup> and monoclonal antibodies, whereas the effect was stronger for polyclonals.<sup>267</sup>

The time dependency of the antibody reaction with the enzyme tracer was also studied by direct ELISA. Calibration curves were measured after 15, 30, 45 and 60 min tracer incubation time. Here, the dynamic range increased from 0.35 up to 1.3 OD. The test midpoint varied only between 0.17 and 0.25 µg/L, whereby there was no clear time dependency visible. To keep the assay time short, the incubation time of the 'standard' ELISA was kept (30 min).

The increase of the test midpoints for homogeneous assays, especially after the highest degree of polarization being reached, suggests that the antibody first reacts with the free analyte, which is then slowly replaced by the fluorophore tracer. For the heterogeneous assay the test midpoint does not continuously increase over time, *i.e.* the kinetics of antibody/tracer and antibody/analyte interactions are similar to each other. The interaction with both, analyte and enzyme tracer is slow, but none of them replaces the other due to a longer incubation time. For synthesis of fluorescein and enzyme tracers, respectively, the same hapten had been used. So the different kinetics towards the tracers may be induced by their different size (fluorescein tracer 795 Da, enzyme tracer 44,900 Da<sup>16</sup>). It could also be possible that the slightly higher ratio of hapten coupled to the enzyme of  $1.5 \pm 0.3$ <sup>16</sup> compared to 1:1 coupling of hapten and fluorescein is the reason for the different kinetics.

**Characteristic parameters for CBZ FPIA on MTPs** The measurement ranges of the assays were determined from the evaluation of the precision profile, *i.e.* the relative error of concentration (*Figure 26A*). For a higher sensitivity, only half of the amount of antibody as described before was used for FPIA on MTPs (7.5 ng per measurement). Due to the time dependency of the chosen antibody, the characteristic parameters of the calibration curve were determined after 10, 20, 30 and 60 min (*Table 4*). The dynamic range and the test midpoint increased over time as described previously. Consequently, the lower limit of the measurement range also increased from 0.66 to 1.6 µg/L the longer the incubation. The least sensitive measurement range is comparable to the previously developed CBZ FPIA using the same tracer, but a different antibody (measurement range 1.5-310 µg/L).<sup>262</sup> The upper limit of the measurement range also increased. This gives the opportunity to measure an even wider concentration range, once after 10 min and if concentrations are too high at that moment, the MTP can be measured again after 1 h.

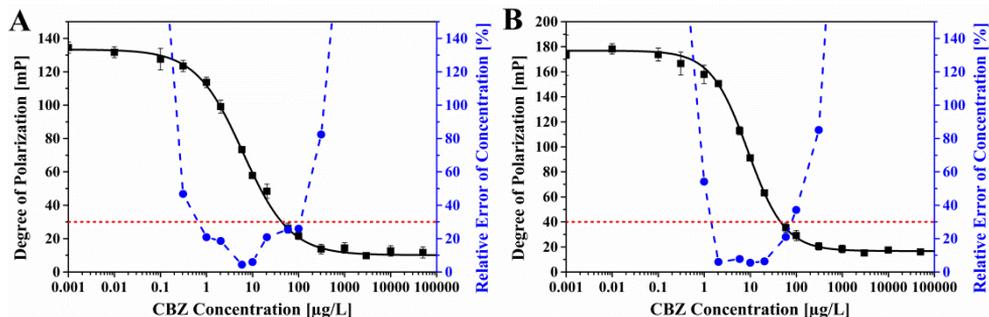
The highest standard deviation for each curve was very low with less than 8 mP. For a better comparability also to other immunoassays, the standard deviations of the degrees of polarization were normalized to the dynamic range. These normalized values decrease over time due to the increasing dynamic range. Nevertheless, the highest relative error was determined to be 5.2 %.

## Results and discussion

**Table 4** Characteristic parameters determined after different incubation times for antibodies from clone 1 under optimized conditions for CBZ FPIA on MTPs including dynamic range, slope, test midpoint, coefficient of determination ( $R^2$ ) and measurement range.

Time [min]	Dynamic Range [mP]	Slope	Test Midpoint [ $\mu\text{g/L}$ ]	$R^2$	Measurement Range [ $\mu\text{g/L}$ ]
10	123	0.85	6.2	0.998	0.66-110
20	155	0.93	7.7	0.999	0.68-98
30	176	0.88	9.7	0.999	1.3-150
60	200	0.94	17	0.998	1.6-380

In previous publications, quality criteria for the evaluation of immunoassays were defined including sensitivity, dynamic range, slope, goodness of fit and measurement range.<sup>12, 13, 256</sup> Almost all these criteria were fulfilled for this assay at all incubation times, besides some slopes at the test midpoints; they should be  $1.0 \pm 0.1$ .<sup>256</sup> The measurement ranges did also not reach the requirement of the width of three orders of magnitude that was stated for heterogeneous immunoassays.<sup>12</sup> But it was already previously discussed that this value should be reduced for homogeneous assays.<sup>256</sup> The measurement ranges determined after different incubation times reached all more than two orders of magnitude width, which is, compared to other FPIAs, rather good.



**Figure 26** CBZ FPIA calibration curves (black solid lines) and precision profiles (blue dashed lines) determined on MTPs after 10 min (A) and in cuvettes after 5 min (B) incubation time using antibodies from clone 1.

**Characteristic parameters for CBZ FPIA in cuvettes** For the determination of a calibration curve and the respective precision profile for CBZ FPIA in cuvettes (Figure 26B), higher amounts of antibody from clone 1 had to be used (150 ng per measurement). Reasons for this are the higher volumes of the reagents that have to be used for this assay format and the higher concentration of the tracer that was necessary to reach a reasonable fluorescence signal on this instrument. Shorter incubation times were chosen, because the mixing in cuvettes is more efficient than on MTPs: in cuvettes a stirring bar is used, whereas the MTPs are incubated on plate shakers.

The tendencies over time of the different characteristic parameters (Table 5) are similar to those determined for FPIA on MTPs. Only the lower limit of the measurement range shows a different behavior: it does not increase so much. Here, the lower limit between the

shortest and the longest incubation increased by 6%, whereas it increased by 140% for FPIA on MTPs. However, the upper limit of the measurement range shows a higher increase in cuvettes. The width of the measurement range reached almost three orders of magnitude after 30 min and is therefore similar to ELISA. In general, the FPIA on MTPs is slightly more sensitive and needs less antibody than the same assay performed in cuvettes.

Besides the lowest CBZ calibrator, which showed normalized standard deviation of 6.4-10%, all other errors were lower than 5.8% normalized to the dynamic range. Almost all characteristic parameters were in good agreement with the previously defined quality criteria.

**Table 5** Characteristic parameters determined after different incubation times for antibodies from clone 1 under optimized conditions for CBZ FPIA in cuvettes including dynamic range, slope, test midpoint, coefficient of determination ( $R^2$ ) and measurement range.

Time [min]	Dynamic Range [mP]	Slope	Test Midpoint [ $\mu\text{g/L}$ ]	$R^2$	Measurement Range [ $\mu\text{g/L}$ ]
5	160	1.1	8.9	1.00	1.4-79
10	221	1.0	11	1.00	1.4-290
15	249	1.0	13	1.00	1.5-210
30	273	1.0	21	1.00	1.5-1200

**Characteristic parameters for CBZ ELISA** Under optimized conditions, the precision profile and the characteristic parameters were determined for ELISA: dynamic range 0.86 OD, slope 1.0, test midpoint 0.32  $\mu\text{g/L}$ , coefficient of determination 0.999 and measurement range 49 ng/L to 36  $\mu\text{g/L}$ . The highest standard deviation of the measured value was 4.4%, normalized to the dynamic range. All requirements for heterogeneous immunoassay quality criteria described by Grandke *et al.* were fulfilled.<sup>12</sup>

ELISA is approximately 20 times more sensitive than the FPIA on MTPs regarding the test midpoint and the lower limit of the measurement range is 14 times lower. At the same time, 5 times as much antibody is used for FPIA on MTP (7.5 instead of 1.5 ng per measurement). Nevertheless, the performance of ELISA requires altogether 20 h, whereas for FPIA on MTPs the same amount of samples can be determined in 20 min, including all pipetting, incubation and measurement steps (incubation time of 10 min).

#### Cross-reactivity of the selected antibody (clone 1)

Under optimized assay conditions, CRs of the antibody were measured with FPIA and with ELISA for twelve substances, most of them structurally related to CBZ (Table 6). For FPIA measurements, the MTP format was used, because here more measurements could be performed in a shorter time. For most of the cross-reactants, the results from FPIA and ELISA are in good agreement. Only some CRs showed differences between the results of both methods, especially for 2-OH-, 3-OH- and DiH-CBZ. It was suggested that this is a result of the different pH values used for the competitive step for the two assay platforms. The effect of different pH values on CRs was previously observed by Bahlmann *et al.*<sup>235</sup> Therefore the CRs for the three mentioned substances were determined again for ELISA

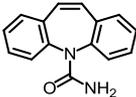
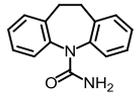
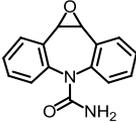
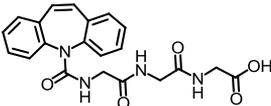
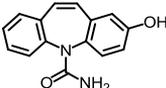
## Results and discussion

but this time at pH of 8.5 (sample buffer was used as described before, adjusted to pH 8.5 instead of 9.5). For 2- and 3-OH-CBZ, the CR of ELISA at pH 8.5 were more similar to FPIA than before at pH 9.5 (23 and 24%, respectively). For DiH-CBZ, an even higher CR of 226% was determined.

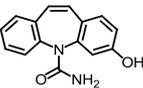
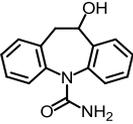
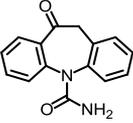
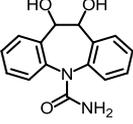
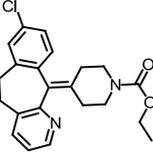
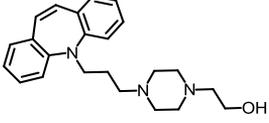
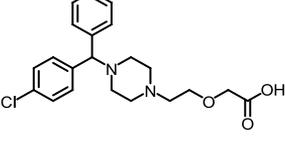
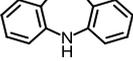
Differences between CRs of single cross-reactants determined by FPIA and competitive ELISA were reported previously. Kolosova *et al.* found that only CRs determined for a direct assay differ from results of FPIA, whereas the results from indirect ELISA were comparable with the homogeneous assay.<sup>268</sup> However, Xu *et al.* also found different CRs using FPIA and indirect ELISA.<sup>73</sup>

CRs determined with ELISA using the cell culture supernatant and the purified antibody, are in very good agreement: for CET and DiOH-CBZ for both types of antibody of clone 1 CR was lower than 1%. The third tested substance, 2-OH-CBZ, showed a CR of 13% for the supernatant and 15% after purification of the antibody. So the results from supernatants can be attributed also to the purified antibody when the same assay type and assay conditions are used.

**Table 6** Molar CRs of the new antibody (clone 1) determined for FPIA (10 min) and ELISA.

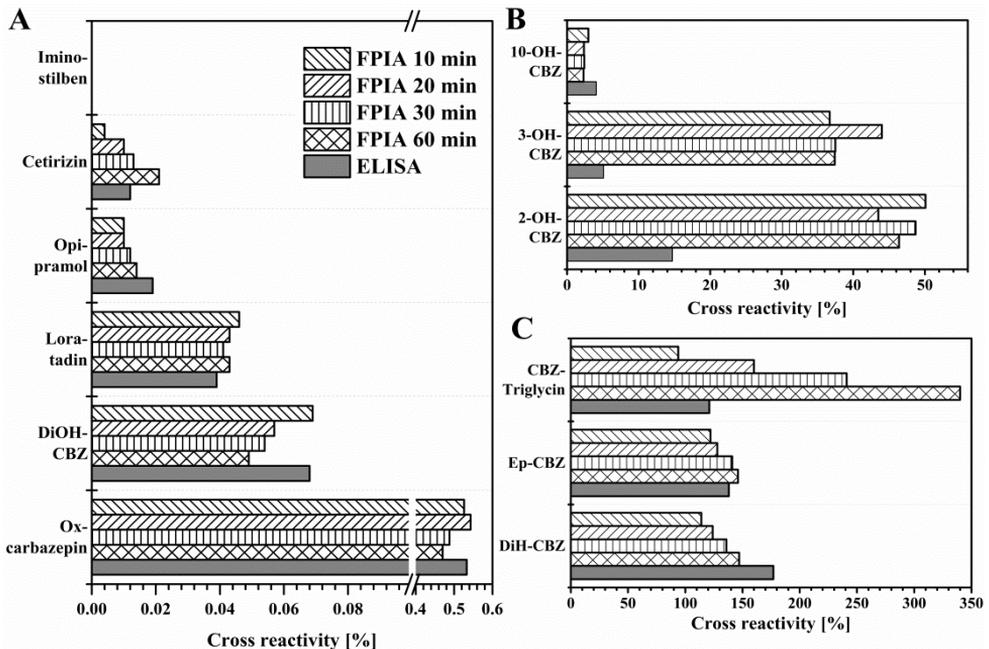
Cross reactant	Chemical structure	CR FPIA [%]	CR ELISA [%]
CBZ		100	100
DiH-CBZ		110	180
Ep-CBZ		120	140
CBZ-triglycine		94	120
2-OH-CBZ		50	15

**Table 6 (continued)** Molar CRs of the new antibody (clone 1) determined for FPIA (10 min) and ELISA.

Cross reactant	Chemical structure	CR FPIA [%]	CR ELISA [%]
3-OH-CBZ		37	5.1
10-OH-CBZ		3.0	4.1
Ox-CBZ		0.53	0.53
DiOH-CBZ		0.07	0.07
Loratadine		0.05	0.04
Opipramol		0.01	0.02
Cetirizine		< 0.01	0.01
Iminostilbene		< 0.01	< 0.01

## Results and discussion

Due to the time dependency of the reaction noted for FPIA, the CR was additionally measured after 10, 20, 30 and 60 min incubation time (Figure 27). For some cross-reactants, an increase of the CR was observed over time: CET ( $< 0.01$  to 0.21%), CBZ-triglycine (94 to 340%), Ep-CBZ (120 to 150%), and DiH-CBZ (110 to 150%). Therefore the strict compliance of the incubation time is very important, because a longer incubation time can lead to higher overestimations. This effect was previously observed for polyclonal antibodies: here, the CR increased with longer incubation times or remained stable.<sup>267</sup> For two cross-reactants the antibody showed a decrease of the CR: DiOH-CBZ (from 0.07 to 0.05%) and Ox-CBZ (from 0.53 to 0.47%). But these differences are so small, that the benefit of a longer incubation time is negligible. For further considerations only the values after the standard FPIA incubation time of 10 min are taken into account.



**Figure 27** CR of the antibody from clone 1 determined after different incubation times of FPIA (10, 20, 30 and 60 min) and ELISA (30 min) for cross-reactants with CR lower than 1% (A), between 1-50% (B) and higher than 90% (C).

After passing the human body, the highest amount of CBZ is excreted as DiOH-CBZ (32%).<sup>152</sup> This is the main metabolite of CBZ, what makes it very valuable that the CR towards this substance is lower than 1% (Table 6). For the metabolite iminostilbene, also a very low CR was observed. CRs against other pharmaceuticals like antihistamines (CET and Loratadine), an antidepressant (Opipramol) and another anticonvulsant (Ox-CBZ) are lower than 1%, too, and therefore negligible.

The CR against 10-OH-CBZ (3.0 %) is negligible, especially when taking into consideration the excretion of this compound of less than 0.1%.<sup>152</sup> The CRs of 3- and 2-OH-CBZ are higher with 37 and 50%, respectively. Associated with the presence in human excretion of 5.1 and 4.3%, respectively, only slight overestimations are expected for the determination of CBZ in water samples.<sup>152</sup>

CBZ-triglycine was used to synthesize the immunogen and tracers for ELISA and FPIA. Therefore it was expected to show a high CR (94%). There is no natural occurrence of this substance. Although a high CR was found against DiH-CBZ (110%), no overestimations are expected due to this compound, because it occurs neither in human metabolism nor has it been found in any kind of water samples.<sup>16</sup> The presence of Ep-CBZ may lead to slight overestimations due to its high CR (120%). The excretion of this substance is approximately one tenth of the CBZ excretion (1.4% compared to 13.8%).<sup>152</sup>

Summarizing it can be said that the antibody only showed CRs towards CBZ related substances. The CRs towards substances with relevant concentrations in human metabolism and consequently in water samples are mostly very low and therefore the possibility for an accurate determination of CBZ in these samples is given when this antibody is used.

### **3.3.5 Conclusion**

A new monoclonal anti-carbamazepine (CBZ) antibody was produced and characterized for the application to FPIA and ELISA. It could be shown that examination of IgG in feces showed good agreement to the conventional serum screening to monitor the immunization progress. This is an animal-friendly alternative to blood sampling, which allows even a better time-resolved monitoring. The properties of antibodies from cell culture supernatants and purified antibodies were determined using FPIA and ELISA. A good agreement between these methods was found. Therefore the application of FPIA should be considered for a more time-efficient cell culture supernatant screening. Additionally, it could be shown that the reaction time, binding properties and also fluorescence quenching varies significantly between different antibodies.

With the finally selected antibody (clone 1), sensitive immunoassays could be established. Using FPIA in cuvettes, CBZ concentrations in the range of 1.4-79 µg/L can be determined after an incubation time of 5 min and with a test midpoint of 8.9 µg/L. This assay allows a fast and automated CBZ determination of single samples. FPIA on MTPs allows a simultaneous determination of 24 samples in a total assay time of 20 min within the concentration range of 0.66-110 µg/L and a test midpoint of 6.2 µg/L. With the ELISA format, a more sensitive, but more time consuming assay could be developed; here, a measurement range of 0.05-36 µg/L and a test midpoint of 0.32 µg/L could be reached. The CR of the purified antibody was determined by ELISA and FPIA. Most of the determined values are in good agreement, but for some cross-reactants, the different pH value used for the assays influence the CR. For DiH-CBZ, the kind of immunoassay (heterogeneous and homogeneous) seems to influence the binding affinity of the antibody. The antibody showed a high time dependency of CRs and the assay performance including characteristic parameters. In general, the determined CRs indicate a good specificity of the antibody and enables for future application to medical and environmental analysis.

The antibody can be requested from the corresponding author. It was assigned the ordering code BAM-mab 01 (CBZ).

### **3.3.6 Acknowledgments**

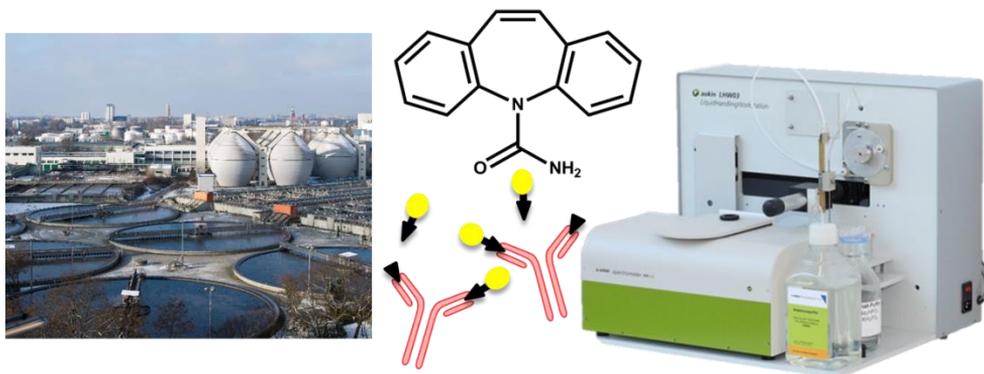
We express our gratitude to K. Hoffmann for the help for ELISA measurements and S. Flemig and S. Ewald for the MALDI-TOF measurements (all BAM). We also thank Marie Schumann for graphical assistance. This work was supported by a grant from the Federal Ministry of Economic Affairs and Energy (BMW; program MNPQ, project no. 22/11).

### 3.4 Application of fluorescence polarization immunoassay for determination of carbamazepine in wastewater

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#### Final Manuscript

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**Figure 28** Graphical abstract of Application of fluorescence polarization immunoassay for determination of carbamazepine in wastewater.

#### 3.4.1 Abstract

Carbamazepine is an antiepileptic drug that can be used as a marker for the cleaning efficiency of wastewater treatment plants. Here, we present the optimization of a fast and easy on-site measurement system based on fluorescence polarization immunoassay and the successful application to wastewater. A new monoclonal highly specific anti-carbamazepine antibody was applied. The automated assay procedure takes 16 min and does not require sample preparation besides filtration. The recovery rates for carbamazepine in wastewater samples were between 60.8 and 104% with good intra- and inter-assay coefficients of variations (less than 15 and 10%, respectively). This automated assay enables for the on-site measurement of carbamazepine in wastewater treatment plants.

#### 3.4.2 Introduction

A large variety of pharmaceuticals enter wastewater treatment plants (WWTPs) where many of them are not efficiently removed. Due to the disposal of treated wastewater into surface water, a high amount of various pharmaceutically active compounds are found in surface waters, what may influence the ecosystem and the natural organization.<sup>269</sup> Through irrigation with treated wastewater, pharmaceutical active compounds can also be found in vegetables.<sup>207, 270</sup> Therefore additional purification steps are discussed since long. Several

approaches like ozonation, hydrodynamic-acoustic cavitation, heterogeneous Fenton-like reactions, production of singlet oxygen and other reactive oxygen species, enhanced biodegradation, pulsed corona discharge and activated carbon filtration have shown high efficiency for reducing the load of micropollutants.<sup>180-183, 271-273</sup> A method for verification and monitoring of the cleaning efficiency directly in the WWTPs would be desirable for an effective control of those additional purification steps. Monitoring of all pharmaceuticals would obviously not be possible due to their large number. Therefore a suitable indicator should be considered.

Carbamazepine (CBZ) has often been reported as a marker for wastewater input into water bodies.<sup>4, 166, 167, 170, 253</sup> This antiepileptic drug is excreted by humans in about 14% of unmetabolized form and enters in this way or through incorrect disposal of pills and tablets via the toilet the water cycle.<sup>152</sup> Once CBZ has arrived in surface water, it can negatively influence the health status of aquatic organisms.<sup>165, 197-201, 232</sup> During common wastewater treatment, mostly less than 30% of CBZ is degraded.<sup>149, 162, 163</sup> On the contrary, even higher CBZ concentrations were found in effluent than in influent samples of WWTPs due to degradation of CBZ metabolites.<sup>149, 152</sup> CBZ is usually found in any wastewater sample, what illustrates the ubiquitous occurrence of this substance.<sup>152, 155, 224</sup> The removal of CBZ from wastewater through additional purification steps has been proven in several studies.<sup>181-183, 271</sup> Therefore, CBZ can be used as a marker for an effective purification of wastewater from micropollutants.

For prompt monitoring of this marker, a system is required that enables for on-site measurements. One approach is the immunoanalytical determination of CBZ. Several studies for CBZ determination in water samples using antibodies for detection have been reported. Heterogeneous enzyme immunoassays have been used, which are very sensitive but do not offer the possibility of an on-site measurement due to several long incubation and washing steps.<sup>4, 13, 16, 230, 231</sup> Fluorescence polarization immunoassay (FPIA) as a homogeneous assay does not require these steps and therefore could prove capability for on-site monitoring. The assay is based on the change of fluorescence polarization of a fluorophore-labeled analyte when it is bound to an analyte-specific antibody. This labeled analyte, the so-called tracer, competes with the analyte from the samples for the antibody binding sites. The principle of this assay has been described in detail many times.<sup>27, 68, 274</sup> For the determination of CBZ, FPIAs have been previously developed for the application to serum and to surface water.<sup>257, 262</sup>

Recently, a new monoclonal anti-CBZ antibody was produced and characterized.<sup>275</sup> This antibody showed low cross-reactivity against other pharmaceuticals like cetirizine, loratadine or opipramol. Cetirizine led to high overestimation of CBZ in water samples in previous studies.<sup>13, 16, 235</sup> Due to its low cross-reactivity towards relevant environmental pollutants, the new antibody offers the opportunity for a more accurate CBZ determination in environmental samples. The applicability of this antibody to wastewater samples using FPIA was to be verified in this study. To the best of our knowledge, this is the first time that a FPIA is used for CBZ determination in wastewater. Actually only one FPIA for wastewater analysis has been reported until now using a pre-concentration by solid-phase extraction.<sup>40</sup> The difficulty with the application of FPIA to this matrix lies in the complexity of wastewater, which contains a lot of different ingredients like salts, proteins and pharmaceuticals in a wide concentration range. Thus, one of the prerequisites for on-site measurements, the

avoidance of washing steps, is at the same time the main problem that needs to be solved for the application of FPIA to this complex matrix.

### 3.4.3 Material and methods

#### Reagents

All solvents and chemicals were purchased from Sigma-Aldrich, Merck KGaA, Serva, AppliChem GmbH and J.T. Baker. The tracer CBZ-triglycine-5-(aminoacetamido)fluorescein (CBZ-AAF) was previously synthesized.<sup>262</sup> Calibrators, dilutions and the following buffers were prepared in ultrapure water (Synthesis A10 Milli-Q® water purification system, Millipore): sample buffer (250 mmol/L glycine, 50 mmol/L sodium chloride, 0.5% disodium ethylenediaminetetraacetic acid dihydrate (EDTA), 35 mmol/L sodium hydroxide, pH 8.5), phosphate buffered saline (PBS, 10 mmol/L sodium dihydrogenphosphate, 70 mmol/L disodium hydrogenphosphate, 145 mmol/L sodium chloride, pH 7.6), tracer stabilization buffer (70% PBS, 20% glycerol, 10% methanol), antibody stabilization buffer (80% PBS, 20% glycerol, 0.2% sodium azide, 0.1% bovine serum albumin, 0.05% Tween20). CBZ calibrators for calibration and spiking were prepared gravimetrically in ultrapure water from a 1.15 g/kg methanolic stock solution.

#### FPIA in cuvettes

For FPIA measurements, aokin spectrometer FP 470 (aokin AG, Berlin, Germany) was used. The optical filter system in this instrument is designed for fluorescein tracer and is able to measure parallel and perpendicular intensities simultaneously and time-resolved. For instrument control and sample evaluation, aokin software mycontrol (ver. 4.1.12) was used. The spectrometer was connected to aokin liquid handling workstation (LHW) for automated assay performance.

For the CBZ FPIA, all steps were performed automatically. Here, the optimized protocol is described. First, 1.7 mL sample buffer were pipetted into the round-bottom cuvette, which contains a magnetic stir bar. The buffer background was measured for 5 s. Next, 100  $\mu$ L CBZ calibrator or sample were added. The pipetting tube was rinsed with 100  $\mu$ L sample buffer so in total a volume of 200  $\mu$ L was added during this step. After measuring the sample background (SBG, 5 s), 100  $\mu$ L tracer dilution, 1:20,000 in tracer stabilization buffer, were added, followed by 100  $\mu$ L sample buffer. Subsequently, the fluorescence intensities of the free tracer were measured (5 s). Then 100  $\mu$ L antibody BAM-mab 01 (CBZ) dilution in antibody stabilization buffer (1.5  $\mu$ g/mL) were added and flushed again with 100  $\mu$ L sample buffer. The total volume in the cuvette after this step was then 2.3 mL. The measurement time, after addition of antibody, was set to 600 s. In total, the assay procedure took 16 min, including automated rinsing of the cuvette.

Sixteen CBZ calibrators in the range of 0.01 to 40,000  $\mu$ g/L were measured in triplicate for setting up the sigmoidal calibration curve and a precision profile determined as the relative error of concentration according to Ekins.<sup>11</sup> The measurement range was defined as the range with relative errors of concentrations less than 30% as described previously.<sup>12, 13, 256</sup> For the calibration and evaluation of sample concentrations with the software mycontrol, point-to-point interpolation is applied. For this, seven CBZ calibrators (2.5-180  $\mu$ g/L) were measured in triplicate. Additionally, a low CBZ calibrator (0.01  $\mu$ g/L) was taken into

## Results and discussion

consideration to have a reference point for CBZ concentrations that are below the calibration range. All samples were measured in triplicate. The concentrations were determined over a time range from 400 to 550 s after the addition of antibody. Single measurements were repeated when the signals were too noisy (e.g. due to air bubbles in the cuvette). Approximately 10% of the sample measurements were repeated.

The degrees of polarization for calibration curves were calculated by using SBG-corrected fluorescence intensities and subtraction of the degree of polarization value of the free tracer. The G factor was fixed to 1.10. For evaluation of degrees of polarization of the free tracer, SBG-corrected fluorescence intensities were used. For samples, the degree of polarization was determined without any correction of fluorescence intensities and the G factor was set to 1. Total fluorescence intensities are given as the sum of parallel and double perpendicular intensity. For calculation of these values for the free tracer, again SBG-corrected intensities were used.

### Sample preparation

The samples were obtained from four Berlin WWTPs, one influent and effluent sample from each. The samples were filtered through folded filters and then through glass fiber syringe filters (1-2  $\mu\text{m}$ , neoLab, Heidelberg, Germany). The samples were stored at 4 °C.

Samples were spiked at levels of 160, 80, 40, 16, 8 and 4  $\mu\text{g/L}$ . Spiking of the samples was performed by adding 1% of CBZ standard in ultrapure water to the sample. The samples with the highest spiking value (160  $\mu\text{g/L}$ ) were diluted with ultrapure water by a factor of 2, 4, 10 and 20.

### LC-MS/MS

The CBZ concentrations of pure samples were determined with LC-MS/MS using an Agilent 1260 Infinity LC system (Agilent Technologies Waldbronn, Germany) with a binary pump, degasser, autosampler, column heater and UV detector coupled to a Triple Quad™ 6500 MS (AB Sciex). A Kinetex C18 precolumn (Phenomenex) and a Kinetex XB-C18 core/shell column (150 mm x 3 mm, 2.6  $\mu\text{m}$ ) were used.

20  $\mu\text{L}$  of the samples were injected. The column oven temperature was set to 55 °C, the flow rate was kept at 400  $\mu\text{L}/\text{min}$ . A binary gradient consisting of (A) water and (B) methanol, both solvents containing 10 mmol/L ammonium acetate and 0.1% (v/v) acetic acid, was used under the following conditions: 20% B, isocratic for 2 min, linear increase to 95% B within 13 min, kept at 95% B for 8 min, return to the initial conditions 20% B within 0.5 min, and kept for 7.5 min.

Electrospray ionization was performed in the positive mode (ESI+) with a source temperature of 400 °C and an ion spray voltage of 4500 V. The following parameters were applied to operate the mass spectrometer: curtain gas 35 psi, collision gas 6 psi, nebulizer gas 62 psi, turbo gas 62 psi, entrance potential 10 V.

For the quantification, the following transition of CBZ was analyzed in selected reaction monitoring mode:  $m/z$  237→194; collision energy 30 V; cell exit potential 14 V, declustering potential 60 V, dwell time 100 ms. Data acquisition and analysis was performed using the software Analyst® 1.6.2 (AB Sciex).

For all samples, the concentrations were within the range from 0.71 to 2.0 µg/L. Only one effluent sample (WWTP 2) showed a significantly higher concentration of 4.0 µg/L. All effluent samples showed higher concentrations than the respective influent samples. These values were included in the calculation of the concentration of spiked samples.

### 3.4.4 Results and discussion

#### Optimization of CBZ FPIA for application to wastewater

The optimization of the FPIA performance for the application to wastewater included assay procedure, reagent concentrations, sample preparation and buffer composition. As the basis for the assay optimization, the previously described method using the same tracer, antibody and FP instrument was used.<sup>275</sup> All pipetting steps were performed automatically using the LHW. This includes not only all pipetting steps, but also the cleaning procedure between measurements. During the first measurements with real samples, it became obvious that this cleaning protocol which has been used for other analytes and matrices is not suitable for this matrix. Usually samples that are measured on this instrument are strongly diluted, e.g. caffeine in beverages, or extraction steps are used prior to the sample measurement.<sup>256</sup> However, water samples usually do not require any clean-up steps besides filtration. And the usage of time-consuming preparation steps should be avoided to offer the possibility of an on-site measurement in WWTPs. The problem was solved by an additional and more intensive cleaning step: the cuvette is cleaned once before the measurement starts (1 mL buffer) and again after each measurement using a larger buffer volume (2.4 mL). This is still a quite easy cleaning procedure which only requires buffer and can therefore be performed automatically using the LHW.

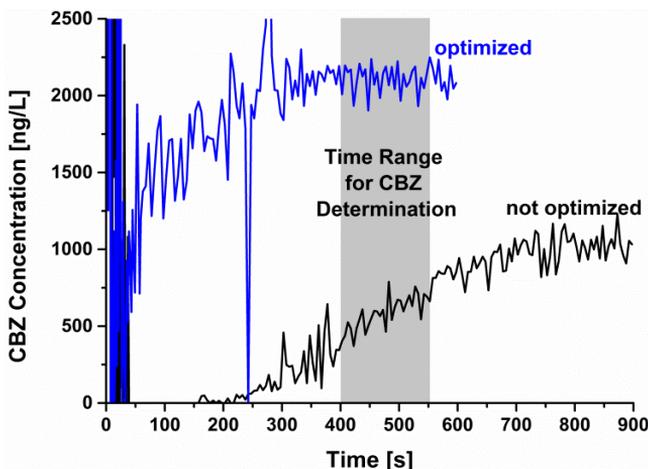
The antibody used in this study is characterized by slow reaction kinetics, but the assay sensitivity was not improved with longer incubation times (tested between 5 and 30 min). Additionally, some cross-reactivities increased with longer incubation times.<sup>275</sup> Hence, the reaction time was shortened to make the assay as quick and accurate as possible. During optimization of the assay, different incubation times were applied. Finally the reaction time could be reduced to 10 min.

At first, borate buffer (25 mmol/L) was used as reaction buffer referring to a previous publication.<sup>275</sup> The concentrations of the samples were determined over a time range which is possible due to the time-resolved measurement capabilities of the spectrometer. For most samples, concentrations increased over time even when samples were diluted by a factor of two (*Figure 29*, black line). To verify if proteins from the matrix influence the assay performance, protein precipitation methods using different ratios of solvents (methanol and acetonitrile) were applied. But no or only very slight precipitates were observed. Furthermore, the solvents affected the antibody properties.

One approach to compensate matrix effects is the adaptation of the reaction buffer. Bahlmann *et al.* used different sample buffers for the determination of CBZ in wastewater with ELISA.<sup>235</sup> Following these recipes, sample buffers with different sodium chloride (50-500 mmol/L) and EDTA contents (0.5-5%) were tested. Glycine concentration (250 mmol/L) and pH value were kept constant at 8.5 due to pH dependent fluorescence of fluorescein and, as previously reported, pH dependency of the antibody performance.<sup>275</sup> The addition of methanol to reaction buffer was also tested. Regarding sensitivity, variance of

## Results and discussion

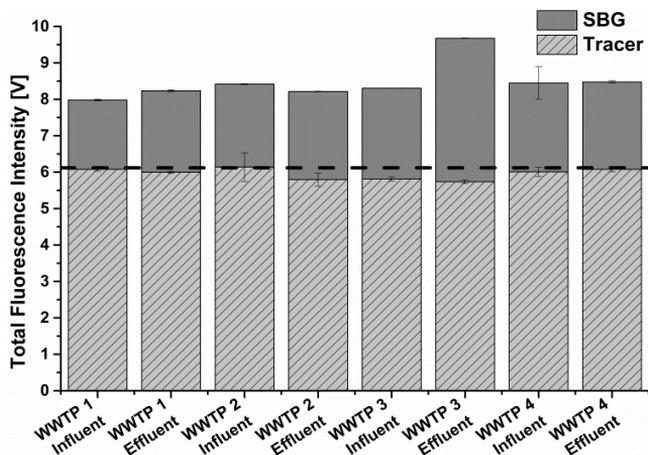
determined concentration over time for real samples and required assay time, the following buffer composition was found to be optimal: 250 mmol/L glycine, 50 mmol/L sodium chloride, 0.5% EDTA and no methanol. Additionally, a reduction of the sample volume from 200  $\mu\text{L}$  (as previously described)<sup>275</sup> to 100  $\mu\text{L}$  improved precision for measurements of real samples. Using this optimized assay protocol (as described in Section 3.4.3), averaged CBZ concentrations reached a plateau after approximately 300 s (*Figure 29*, blue line). Therefore determination was performed within the time range of 400-550 s.



**Figure 29** CBZ concentration determined over time presented for the same wastewater sample (WWTP 4 effluent) using not optimized (black line) and optimized assay conditions (blue line). The gray area marks the time range in which the CBZ concentration is determined using optimized assay conditions.

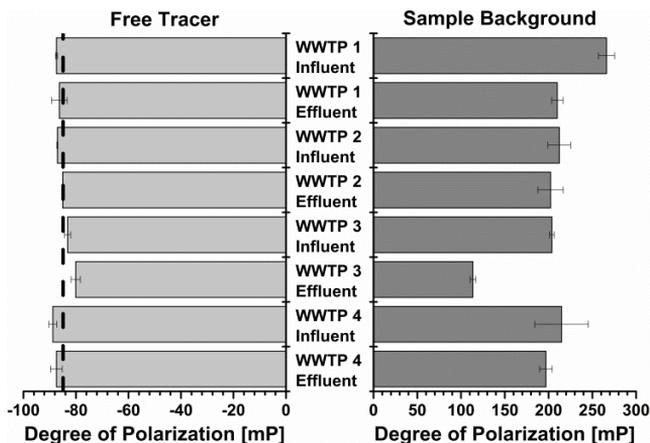
Using these assay conditions, lower antibody concentrations were tested to improve the assay sensitivity, but even when half the amount of antibody was used, the sensitivity regarding the test midpoint did not improve significantly (18.6 instead of 21.7  $\mu\text{g/L}$ ). But at the same time, the maximum degree of polarization decreased strongly from almost 200 mP to 130 mP. Therefore the higher antibody concentration was used for the final assay conditions.

For applicability of the assay to wastewater, it is important to consider the fluorescence intensities of the samples and the possible influence on the fluorescence properties of the tracer. The wastewater samples investigated in this study showed high and - for different samples - widespread total fluorescence intensities of 1.90-3.94 V compared to CBZ calibrators with approximately 0.69 V (*Figure 30*). The total fluorescence intensities of free tracer (SBG corrected) were not influenced by the sample matrix: values of  $5.96 \pm 0.20$  V (CV 3.4%) were determined in the presence of samples and were therefore in good agreement with 6.12 V determined in the presence of calibrators. In agreement with a previous study, the CBZ concentration of calibrators showed no influence on the fluorescence intensities.<sup>262</sup>



**Figure 30** Total fluorescence intensities were determined for SBG (wastewater, dark gray bars) and free tracer (striped light gray bars, SBG corrected). The black dashed line represents the total fluorescence intensity of free tracer for calibrator measurements.

For samples, different degrees of polarization of SBG were determined in the range from 114 to 266 mP (Figure 31; calibrators: 643 mP). But the degrees of polarization for the free tracer were not influenced by them; the values for free tracer measured in the presence of samples are in good agreement with those determined in the presence of calibrators ( $-85.7 \pm 3.0$  mP compared to  $-84.9$  mP). It can be concluded that the strongly fluorescent matrix components do not influence the fluorescence or rotational speed of the free tracer.



**Figure 31** Degrees of polarization determined for sample background (dark gray bars) and free tracer (light gray bars, SBG corrected) during measurement of wastewater samples. The black dashed line represents the degree of polarization of free tracer for calibrator measurements.

### Application of the optimized CBZ FPIA to wastewater

Under optimized assay conditions, the sigmoidal calibration curve and measurement range were determined at different time points of the measurement (400, 500 and 600 s, Table 7). The test midpoints (or  $IC_{50}$ ) as indicator for the assay sensitivity were all in a similar range,

## Results and discussion

while the dynamic ranges, the distance between upper and lower asymptote, increased from 135 to 167 mP. Measurement ranges, determined as the range with relative errors of concentrations less than 30%, increased slightly from 400 to 600 s.

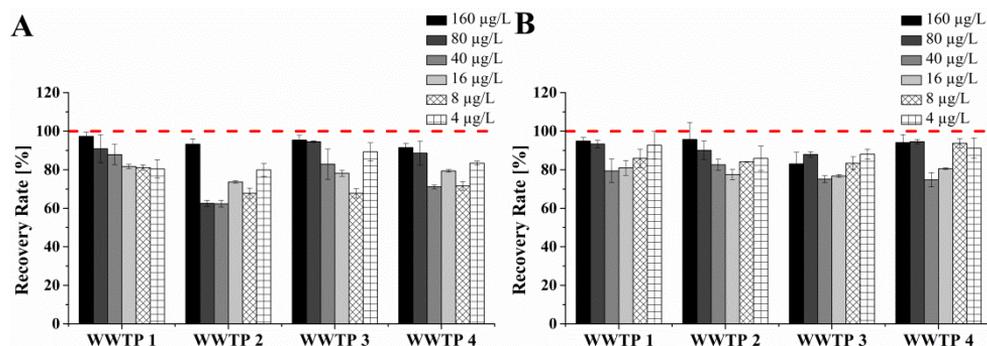
The evaluation of samples was performed using the software that is also used to control the instrument. This software does not use sigmoidal calibration curves, but point-to-point calibration. Hence, we preferred to use a calibration range that is narrower than the one determined via the precision profile. A calibration range between 2.5 and 180 µg/L was used according to the IC<sub>10</sub> and IC<sub>90</sub> values (Table 7).

**Table 7** Characteristic parameters for CBZ FPIA calibration curves after different incubation times: upper and lower asymptote (A, D), slope at test midpoint (B), test midpoint (C), concentration at 10 and 90% degree of polarization (IC<sub>90</sub> and IC<sub>10</sub>), coefficient of determination (R<sup>2</sup>) and measurement range (MR).

Time [s]	A [mP]	B	C [µg/L]	D [mP]	IC <sub>10</sub> [µg/L]	IC <sub>90</sub> [µg/L]	R <sup>2</sup>	MR [µg/L]
400	149	0.986	19.8	15.0	2.13	184	0.999	2.06-394
500	167	0.983	20.5	15.4	2.19	191	0.999	1.73-227
600	182	0.982	21.9	15.1	2.33	205	0.999	1.54-469

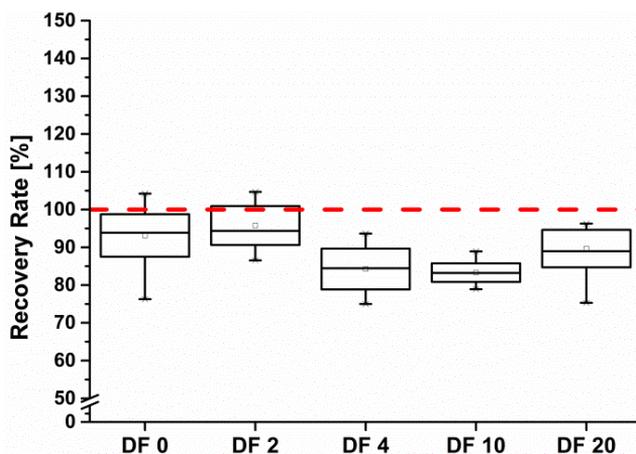
Only one pure sample showed a CBZ concentration within this calibration range. For this effluent sample, a good agreement of concentrations determined by LC-MS/MS (4.01 µg/L) and FPIA (3.83 µg/L) was observed. According to instrumental results, all other samples showed concentrations lower than the calibration limit of FPIA (0.71-2.0 µg/L). No concentrations could be determined. Hence, different spiking values were used within the calibration range (4, 8, 16, 40, 80 and 160 µg/L).

The recovery rates for spiked wastewater samples were all between 60.8 and 104% (mean values 62.3-97.3%; Figure 32). No significant differences between the recovery rates of CBZ in influent and effluent samples or due to spiking values were observed. Intra-assay coefficients of variation (CVs), determined as the variation over time during each measurement, were all except one (14.7%) below 10%. Inter-assay CVs determined as the variation between the values of different measurements were all below 10% (max 9.51%).



**Figure 32** Recovery rates determined for influent (A) and effluent (B) samples from different WWTPs, spiked with 160, 80, 40, 16, 8 or 4 µg/L. The red dashed line marks the ideal recovery rate of 100%.

Most likely slight overestimations are expected when concentrations are determined with immunoassays, the percentage of overestimation depending on identity, amount and number of relevant cross-reactants. For this antibody, only slight overestimations were expected for CBZ determination in wastewater, due to its low cross-reactivity towards matrix-relevant substances.<sup>275</sup> Instead in this study almost only underestimations were observed. Matrix effects could be the reason for the underestimation. This was investigated by diluting the samples with the highest spiking value (160 µg/L) by different dilution factors (Figure 33). The recovery rates were a little bit higher and closer to 100% than for undiluted samples. But no improvements due to higher dilution factors were observed: even for a dilution factor of 20 the recovery rates were between 75 and 96%. The same undiluted samples (DF 0) showed quite similar results with a recovery range of 76-104%.



**Figure 33** Recovery rates determined for eight different wastewater samples, spiked with 160 µg/L CBZ (DF 0) and subsequently diluted by factor (DF) 2, 4, 10 or 20 (n=24). The red dashed line marks the ideal recovery rate of 100%.

Dose-dependent and -independent bindings of CBZ to different proteins have been reported.<sup>276</sup> So the binding of CBZ with components present in wastewater could be a reason for the observed underestimation in real-world samples. One part of the tracer consists of CBZ, so if the binding of CBZ to matrix components should be the reason for underestimation, the tracer would most likely also bind to them. This would influence his fluorescent properties, especially the polarization. But as shown before, the matrix did not influence fluorescence properties of the tracer.

### 3.4.5 Conclusion

For the first time, FPIA was successfully applied to the determination of pharmaceuticals in wastewater. For original samples within calibration range (2.5-180 µg/L), good agreement of CBZ concentrations obtained with instrumental methods were found. For spiked samples, recoveries of 60.8-104% were observed. The percentage of underestimation of CBZ concentration was independent of the type of wastewater (influent or effluent), the spiking value or the dilution factor. The intra- and inter-assay CV were all lower than 15% and 10%, respectively. We could show that a fast and automated FPIA can be utilized for the determination of CBZ as a marker substance in environmental samples. Using this

technique, the success of additional purification steps during wastewater treatment could be monitored on-site without the necessity of laboratory environment or highly trained staff.

### **3.4.6 Acknowledgments**

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### 3.5 Supporting data – Automatization of FPIA on microtiter plates

The automatization of FPIA has been shown for measurements in cuvettes. But also the performance on MTPs can be semi-automatized. Therefore, dispenser directly connected to the MTP multi-mode reader can be applied. Here, two dispensers in combination with the filter-based MTP reader were used to automate the addition of tracer and antibody. All shaking and measurement steps can be performed in the instrument. Thus, only the buffer and the calibrators or samples have to be added manually; all following steps can be performed automatically. The time needed for dispensing the reagents is automatically considered so that the time between the dispensing and the measurement of each well is constant.

#### 3.5.1 Experimental

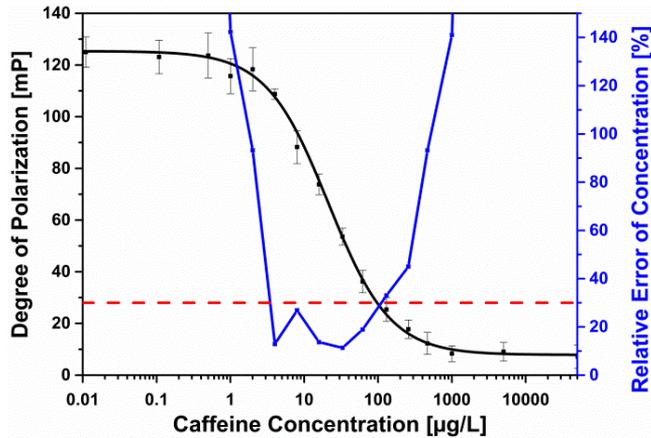
Exemplarily, the caffeine FPIA was optimized for this semi-automatized assay performance. The same caffeine specific antibody and caffeine-fluorescein tracer were used as described previously in this thesis (Section 3.1). The following optimized protocol was used: 280  $\mu\text{L}$  of borate buffer (25 mmol/L disodium tetraborate decahydrate, 0.01% sodium azide, pH 8.5) with 0.01% Triton-X and 20  $\mu\text{L}$  caffeine calibrator (0.01-50,000  $\mu\text{g/L}$ , sixuplicate) were pipetted manually into each well of black nonbinding MTPs from Greiner Bio-One. For this, electronic 8-channel pipettes (Eppendorf) were used. Afterwards, the MTP was slid into the filter-based MTP reader Synergy H1 (Biotek) and the sample background was measured. The obtained fluorescence intensities in parallel and perpendicular orientation were later on subtracted from the respective values for the calculation of the degrees of polarization. Next, 20  $\mu\text{L}$  of caffeine tracer diluted in tracer stabilization buffer<sup>262</sup> were added to each well by one of the dispensers. After shaking the MTP for 5 min, 20  $\mu\text{L}$  of antibody were added (1.52 mg/L, in antibody stabilization buffer<sup>262</sup>) using the second dispenser. The MTP was shaken in the instrument and the fluorescence intensities were measured after 10, 15, 20 and 30 min. The G factor was set to 1, the gain was fixed to 88, the plate mode was chosen and the values were subjected to a Grubbs outlier test.

The precision profile was determined by measuring 16 calibrators. Calibration curves with eight calibrators were measured on five MTPs to determine the reproducibility of this kind of assay procedure. On one MTP, the calibration curve was determined manually, using identical reagents and volumes. Here, the tracer and antibody dilutions were added using a Multipette™ from Eppendorf and the MTP was shaken on a Titramax™ 101 plate shaker (Heidolph). All reagents were used at room temperature so that the temperature of the reagents did not vary between the MTPs.

#### 3.5.2 Results

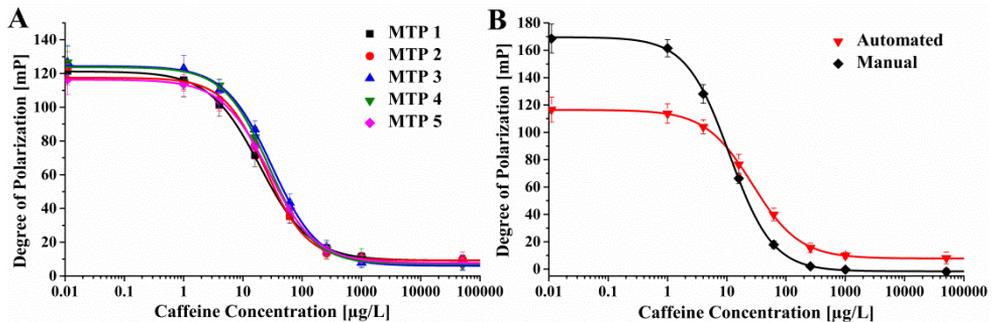
The calibration curve and precision profile were determined after different times (*Figure 34*, exemplarily shown for an incubation time of 10 min). The dynamic range increased by 7.1% from 117 after 10 min to 126 mP after 30 min. After 10 min, the test midpoint was lower with 21.2  $\mu\text{g/L}$  than after 30 min incubation time (32.5  $\mu\text{g/L}$ ). The lower limit of the measurement range, determined as the relative error of concentration lower than 30%, only slightly increased from 3.46  $\mu\text{g/L}$  after 10 min to 5.90  $\mu\text{g/L}$  after 30 min incubation time. But the upper limit and therefore the width of the measurement range increased consistently from 111  $\mu\text{g/L}$  to 1150  $\mu\text{g/L}$ , whereby for the widest range a break was observed; that means that for one calibration point a higher relative error of concentration was observed,

surrounded by values with errors lower than the threshold of 30%. The slope at the test midpoint decreased over time from 1.04, over 0.958 after 15 min and 0.897 after 20 min to 0.822 after 30 min incubation time. The coefficient of determination was good at all measurement times ( $> 0.998$ ). The maximum standard deviation (StD) after 10 min was 8.67 mP, or 7.4% normalized to the dynamic range. For all incubation times, StDs lower than 10.5 mP were observed. Due to the lower test midpoint and the overall relative small variation of characteristic parameters over time, only the performance with an incubation time of 10 min is taken into consideration for further discussion.



**Figure 34** Calibration curve (black line) and precision profile (blue line) determined for the semi-automated performance of caffeine FPIA on MTPs; 10 min incubation time. The threshold of 30% relative error of concentration for the determination of the measurement range is given (red line).

Calibration curves were measured on five MTPs using exactly the same reagents (Figure 35A). The curve progressions were in good agreement. The dynamic range and the slope at the test midpoint showed low variations of 4.1% and 7.0%, respectively. On average, dynamic range of 113 mP and slope of 1.12 were determined. The test midpoint differed between the MTPs from 19.3 to 30.8  $\mu\text{g/L}$  (average 25.6  $\mu\text{g/L}$ ), but it did not consistently increase or decrease in the order the MTPs were measured. All coefficients of determination were higher than 0.998. The StDs were all below 10 mP.



**Figure 35** Caffeine FPIA calibration curves were measured semi-automatically on five MTPs (A) and compared (exemplarily shown for MTP 5) with a calibration curve determined for a manually performed assay (B).

Semi-automated and manual performances of this assay were compared (*Figure 35B*). For this, the identical reagents were used and in general the same protocol was transferred to a manual assay performance. The manually performed FPIA led to a much higher dynamic range (171 mP). So for a manual procedure, a higher antibody dilution could be chosen to reach a similar dynamic range as for the automated assay. This would most likely increase the sensitivity. The test midpoint was lower than for semi-automatic performance (11.1 µg/L compared to 19.3-30.8 µg/L), but regarding the variation between the automatically performed MTPs, the difference seems to be relatively low. The maximum StD was 10.6 mP and therefore insignificantly higher than for semi-automated performance. The coefficient of determination was excellent (1.00).

It can be summarized that FPIAs on MTPs can be applied for semi-automatic performance, which simplifies the already easy assay procedure of FPIA. The reagents can be used for at least six sequential MTPs without any cooling. But the way of performing the assay should be considered during assay optimization, because the characteristic parameters, especially the dynamic range can highly differ. For caffeine FPIA, the slight reduction of sensitivity due to the semi-automated performance is not crucial, because caffeine-containing consumer products show concentrations in the mg/L range and have to be diluted anyway to be measured with this kind of assay. However, for other applications or other analytes, this could be a drawback. Interestingly, the StDs of the measurement points were not significantly reduced by using automated dispensers. But for unexperienced workers, the StDs of the manual procedure might be drastically higher. So the application of the semi-automated assay procedure would probably improve the reproducibility and precision, not only for trained staff.

## 4. Final discussion

The development of FPIAs and the successful application to real samples include the selection of tracer, antibody, assay platform and instrument. Additionally, several steps of the assay procedure have to be optimized, e.g. incubation time, buffer composition, concentrations and volumes of reagents. In this work, FPIAs for the pharmacologically active compounds caffeine and carbamazepine (CBZ) were developed and thoroughly optimized for the application to complex matrices.

### 4.1 Tracers for FPIA

One crucial factor for the successful development of a FPIA is the choice of the tracer. Different hapten and fluorophore structures can be applied, which influence the sensitivity of the assay. In this work, only fluorescein derivatized at the benzoic acid moiety was utilized for tracer synthesis. But in general, coupling through the xanthene part of fluorescein can also be applied for tracer synthesis.

For the CBZ FPIA, different tracer structures were synthesized, purified and verified for their suitability for assay performance. CBZ-triglycine, CBZ and cetirizine (CET) were utilized as hapten structures and were coupled to 5-(aminoacetamido)fluorescein (AAF) and ethylenediamine thiocarbamoylfluorescein (EDF). The first mentioned hapten structure has already proven its suitability for enzyme tracer synthesis for the application to heterogeneous immunoassays.<sup>13, 16</sup> The antibody showed the highest affinity towards the CBZ-triglycine-AAF tracer, visible in the highest maximum degree of polarization. The lowest affinity was observed against the tracer using CET as hapten, which could be explained by the relative low affinity against this pharmaceutical at the chosen alkaline pH value.<sup>235</sup> For EDF tracers using CBZ and CBZ-triglycine as hapten, similar maximum degrees of polarizations were reached. No difference between the reaction times of the antibody and the different tracers was observed.

For the development of sensitive assays, the affinity of the antibody for the tracer and the analyte should be similar.<sup>36</sup> The affinity towards the tracer without the spacer triglycine (CBZ-EDF) was higher than for the free analyte so that the latter could not efficiently replace the tracer even at high concentrations. It has been reported that longer bridges between the analyte and fluorescein improve the sensitivity of FPIA.<sup>31-34</sup> The tracer CBZ-triglycine-EDF has the longest bridge between CBZ and fluorescein. Nevertheless, CBZ-triglycine-AAF yielded higher assay sensitivity. This might be explained by a possible quenching effect of fluorescein within the tracer CBZ-triglycine-EDF. In summary, CBZ-triglycine-AAF was found to be the best tracer and enabled the development of a sensitive CBZ FPIA.

For caffeine tracer synthesis, a caffeine derivative (CafD) using hexanoic acid as spacer was applied as hapten. This derivative has already proven its suitability for protein and enzyme conjugates for the application to heterogeneous immunoassays.<sup>12, 119</sup> AAF and aminopropylamido carboxyfluorescein were utilized as fluorophores. The tracer with the shorter bridge (CafD-AAF) led to a slightly more sensitive assay, different assay platforms having been used for both tracers. Therefore, the direct comparison is not reasonable. In general, both tracers are highly suitable for the performance of caffeine FPIA.

## 4.2 Antibodies for FPIA

Polyclonal and monoclonal antibodies can be used for the development of immunoassays. For heterogeneous assays, both are highly suitable. For homogeneous assays, monoclonal antibodies are preferred, because here, the influence of serum components of polyclonal antibodies might be high due to the absence of washing steps.

For caffeine, a highly suitable monoclonal antibody is available. This antibody showed low cross-reactivities (CRs) against naturally occurring derivatives of caffeine: 12% for theophylline, 0.13% for theobromine and 0.08% for paraxanthine.<sup>119</sup> The applicability of this antibody for caffeine determination in consumer products has been proven for several heterogeneous immunoassays.<sup>12, 119</sup> Here, the suitability of this antibody for homogeneous assays could be demonstrated.

A monoclonal anti-CBZ antibody is commercially available and has been applied for heterogeneous immunoassays for the determination of CBZ in environmental water samples.<sup>4, 13, 16</sup> For first studies concerning the development of CBZ FPIA and the application to surface water, this antibody has been applied. High overestimations of CBZ are expected due to the high CR of this antibody against several CBZ metabolites and other pharmaceuticals.<sup>16, 235</sup> Using CBZ FPIA, recovery rates of up to 140% were observed. For accurate determination of CBZ in environmental samples, the necessity for the production of a new monoclonal antibody with high specificity towards CBZ was given.

### 4.2.1 Improvements for the production process of monoclonal antibodies

The whole production and characterization process of monoclonal antibodies is time and labor consuming. Not always antibodies with the desired specificity can be obtained. Some aspects cannot or can only hardly be improved, e.g. the antibody production in mice, the efficiency of fusion, the growth of cell lines or the final production of antibodies with the desired properties. But for monitoring and screening processes during antibody production, the efficiency and quality can be enhanced using already known methods like feces screening and the replacement of heterogeneous immunoassays by homogeneous ones. The applicability of these methods to the production of the new anti-CBZ antibody has been proven.

#### Feces screening

During the immunization of mice, typically serum samples are taken to study the production of analyte-specific antibodies. Due to the requirement to ensure animal welfare, the distances between the bleedings have to be large. Alternatively, the development of analyte-specific antibodies could be monitored in a time resolved manner by extracting antibodies from feces. It could be proven that results from feces and conventional serum screening are in very good agreement. The deviation between two of the mice were only small during serum screening, but investigation of antibodies from feces showed higher differences between the titer and the affinity of the produced antibodies from both mice. These results facilitated the selection of most suitable mouse for the following fusion.

In a previous study, only the comparability of feces and serum sampling on one specific day was studied.<sup>79</sup> For the new anti-CBZ antibodies, it could be shown that also the development of antibodies in the course of time can be monitored by this method. If feces

of mice are collected, first studies on specificity of the produced antibodies can be performed and how or if the affinity varies over the immunization process. The application of feces screening would lead to more substantiated decisions for additional boosts and the time for spleen removal and fusion. For the immunization process described in this work, the feces screening from mouse 1 showed that the affinity towards CBZ did not change anymore after the second injection of the immunogen and therefore the fusion could have been performed earlier. In particular for this mouse, it is not clear if the boost influenced the titer or affinity, because the upper asymptote was already increasing and the test midpoint decreased before the boost; no change of the curve progression could clearly be observed due to the boost. However, for mouse 2, a clear increase of produced antibodies was observed after the boost. This highlights the different immune response in different animals, even within one species and hence, the necessity of monitoring the immunization progress.

Another advantage of feces screening is of course that this procedure is non-invasive so the mice are less stressed. Combining the results of this thesis and previous studies,<sup>79</sup> the suitability of this monitoring process has been proven for production of antibodies for several analytes. It should be considered to include this method to standard practice for immunization processes.

### **Cell culture supernatant screening**

After fusion of myeloma and B-cells, a large number of different clones is usually obtained. The standard protocol to identify analyte-specific antibodies typically includes ELISA. As a fast alternative, the suitability of FPIA for this purpose could be shown. This method simplifies the selection process due to easier and faster assay performance. Fewer steps have to be performed during the FPIA procedure which reduces the possibility of errors and variations during assay performance. The fluorescence intensities after tracer addition have to increase. After the supernatant is added to the assay, another increase of fluorescence intensity is usually observed due to the cell culture medium, independent of the fact if analyte-specific antibodies are present or not. The presence of antibodies would increase the degree of polarization. For ELISA, there is a signal at the end of the assay or not; it cannot always be determined for sure if no analyte-specific antibodies are present or if just something went wrong during the assay performance. So results of FPIA as supernatant screening method are additionally more reliable.

The application of FPIA offers even more benefits: more information about the antibody properties can be discovered, *e.g.* it is easy to perform the supernatant screening with different buffers at different pH values. Additionally, lower volumes of the supernatants are required. Furthermore, the time dependence of the antibody reaction can be easily monitored also at this stage of antibody production; for the assay performance in cuvettes, time resolved measurements can and have been performed for this purpose. These data were in good compliance with the ones for the finally purified antibodies. Also if FPIA on microtiter plates (MTPs) would be applied, the time dependency of antigen/antibody reaction could easily be detected by multiply measuring the degree of polarization on the same MTP. For those kinds of investigation with heterogeneous assays, it would be necessary to perform the whole assay several times. Additionally, other antibody properties like the influence on tracer fluorescence behavior can be observed already during supernatant screening and therefore further application fields of the antibodies can be

identified, e.g. the development of immunoassays using enhancement or quenching of fluorescence.<sup>20, 265</sup>

Summarizing, the application of this faster and more efficient supernatant screening procedure should be considered for standard screening processes. This should include investigation of all supernatants with FPIA on MTPs. Subsequently, the positive clones should be studied more in detail using FPIA in cuvettes.

#### 4.2.2 Characteristics of the new carbamazepine specific antibody

The new monoclonal CBZ-specific antibody was applied to FPIA and ELISA and showed good characteristic parameters for both kinds of assays. Compared to the previously applied monoclonal antibody, the sensitivity observed for ELISA was slightly inferior. Using the same heterogeneous assay, the test midpoints were 320 and 147 ng/L<sup>13</sup> for the new and old antibody, respectively. Also the measurement range was slightly more advantageous for the old antibody with 0.02-20 µg/L<sup>13</sup> compared to 0.05-36 µg/L for the new antibody. But in general, both antibodies showed excellent applicability for ELISA.

The characteristic parameters for FPIA were slightly better for the new developed antibody. For the comparison, the performance on MTPs is taken into consideration, because for both antibodies this assay format has been carefully optimized. The commercially available antibody showed a test midpoint of 13 µg/L and a measurement range from 1.5 to 300 µg/L. The equilibrium was reached after 10 min. For the same incubation time, the newly produced antibody showed higher sensitivity with a test midpoint of 6.2 µg/L and a measurement range of 0.66-110 µg/L. But for this antibody, the equilibrium is not reached within this incubation time. It takes 60 min until no further increase of the dynamic range could be observed. At equilibrium, the characteristic parameters of the calibration curve are comparable to those of the old antibody: test midpoint of 17 µg/L and measurement range of 1.6-380 µg/L. But a much higher dynamic range was observed after this incubation time than for the old antibody (200 mP instead of 140 mP). The highest observed standard deviation (StD) of measurement points at all measurement times was lower than 8 mP and therefore even slightly lower than for the previously used antibody (9.3 mP) even though only for the latter, the reaction was completed for all considered values.

The time dependency of the new antibody is not necessarily a disadvantage: first, the assay is more sensitive after short incubation times, also compared to the assay using the old antibody with the same incubation time. An increase of assay sensitivity due to shorter incubation times has been also reported for heterogeneous immunoassays.<sup>266, 267, 277</sup> Second, if a wider measurement range is desired, the incubation time can be extended. This enables the determination of CBZ concentrations between 0.66 and 380 µg/L. The width of this range is comparable to those of heterogeneous assays and is usually not reached for FPIAs.

Mostly very low CRs were determined for the newly developed antibody. Especially CRs against other pharmaceuticals were very low. And also the main metabolite of CBZ, DiOH-CBZ is only slightly recognized by the antibody. Therefore, this antibody offers the possibility to determine accurate CBZ concentrations in environmental samples. The antibody showed relevant CRs towards the CBZ metabolites 2-OH- and 3-OH-CBZ (50 and 37%, respectively) what might lead to some overestimation. CRs against these two

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substances seem to be pH dependent: for ELISA at pH 9.5, lower CRs of 15 and 5.1% were observed, respectively; FPIA was performed at pH 8.5. So for ELISA, probably even more accurate results can be expected. ELISA performed with a sample buffer at pH 8.5 showed similar CRs towards these two metabolites compared to FPIA.

For one other analyte, DiH-CBZ, also an increasing CR was determined for decreasing pH values (180% at pH 9.5 and 226% at pH 8.5). But here, the adaption of the pH value leads to an even higher difference between the CRs for heterogeneous and homogeneous assays; a CR of 110% was determined for FPIA. But DiH-CBZ is not relevant for medical and environmental analyses; it is only used as an internal standard for CBZ determination with GC-MS/MS.<sup>186, 219</sup> All other CRs seem to be independent of the chosen pH value for assay performance, at least within this small pH range.

For ELISA, the variation of the pH value during the competition step is easy due to washing steps and thus, the competition is separated from the pH-dependent enzymatic conversion of the substrate. The indirect assay format is in this case even more advantageous, because the enzyme is coupled to the secondary antibody and is therefore not present during the competitive step. For direct ELISA, the enzyme may be destroyed, depending on the pH stability of the applied enzyme.

The pH of the reaction buffer for FPIA cannot be changed without repeated optimization of the assay because of the high pH dependency of the fluorescence of fluorescein. The pH dependency was especially important for the usage of the commercially available antibody. But the CRs against almost all of the cross-reactants decrease with increasing pH value so the applied alkaline pH range is preferable anyway.<sup>235</sup> FPIAs using fluorescein for tracer synthesis can only be performed at alkaline pH values. If other pH ranges are required for the performance of FPIA, other fluorophores have to be considered.

The time dependency of the antigen/antibody reaction indicated the necessity of studying the time dependency of CRs. The new antibody showed some time-dependent CRs, namely for CET, CBZ-triglycine, Ep-CBZ, DiH-CBZ, DiOH-CBZ and Ox-CBZ. For the last two mentioned substances, a slight decrease was observed over time. But the CRs were already so low that no improvement due to longer incubation time would be observed (CR < 0.6%). Maybe the before mentioned discrepancy between the determined CRs for DiH-CBZ by ELISA and FPIA could be at least partly a result of the time dependent increase of CR. The CRs for the four mentioned substances increased over time and therefore confirm the usage of a short incubation time.

The effect of variances of CRs at different incubation times has been described previously for some heterogeneous immunoassays.<sup>267, 278-280</sup> But usually, studies on time dependency of CRs are not performed for ELISA measurements, because for heterogeneous assays, the whole assay has to be performed for each incubation time. Often only the influence of incubation time on assay sensitivity is investigated during assay optimization.<sup>14, 277</sup> Using FPIA, studies of time dependency of antibody reaction with tracer, analyte or cross-reactant can easily be performed and should generally be considered for the characterization of the antibodies. If time-dependent effects are observed with this method, they can or should be verified for heterogeneous assays. Thus, this homogeneous assay could also be used as a tool to improve the sensitivity and selectivity of other immunoanalytical formats.

Summarizing, the newly developed antibody showed a high specificity to CBZ. For both, heterogeneous and homogeneous immunoassays, sensitive assays could be developed. Thus, this antibody is a promising tool for the accurate determination of CBZ in medical and especially environmental analyses.

### 4.3 Formats and instrumentation

FPIA can be performed on MTPs or in cuvettes or tubes, utilizing a variety of instruments. Generally, the FPIA performance on MTPs enables a very high sample throughput, whereas cuvette- and tube-based systems offer fast determination of single samples. The general assay procedure can be applied for different platforms, but the exact protocol usually needs to be adapted. On some instruments, automatization of the assay is possible.

#### 4.3.1 Measurement arrangement

For the selection of excitation and emission wavelength, monochromators or filters can be used. For cuvette- and tube-based systems, only filter-based systems were applied within this work. For FPIA on MTPs, instruments with different measurement settings were utilized.

The caffeine FPIA was developed and optimized on a monochromator-based instrument. This measurement arrangement is especially useful for the development of new assay formats and the application of fluorophores with unknown fluorescence properties. Later in this work, a filter-based MTP reader could be utilized for caffeine FPIA. Using this measurement arrangement, scattering light is more efficiently separated. Additionally, the transmission of light is less dependent on the orientation of the light, which is especially important for FP measurements.<sup>67</sup>

Not all aspects of assay performance on these two instruments can be compared, because both methods were thoroughly optimized for each utilized instrument. Therefore, not all buffer, volumes and reagent concentrations were the same. Furthermore, the measurement settings differ from each other: for measurements on the monochromator-based instrument, the absorption and emission spectra were determined and according to that, the excitation (492 nm) and emission wavelengths (520 nm, cutoff filter at 515 nm) were selected. For the filter-based instrument, filters for application to polarization measurements of fluorescein were used. Therefore, the wavelengths could not be varied ( $\lambda_{\text{excitation}} = 485 \text{ nm}$ ,  $\lambda_{\text{emission}} = 528 \text{ nm}$ ).

The dynamic range of the caffeine FPIA on the filter-based instrument was higher (171 mP, manual performance) than on the monochromator-based instrument (154 mP). The test midpoints and therefore the sensitivities were similar for both measurement arrangements (11  $\mu\text{g/L}$  and 9.9  $\mu\text{g/L}$ , respectively). High differences between the maximum StD of both instruments were observed: when monochromators were utilized, the highest StD was 23 mP, whereas the highest value for filter-based measurements was considerably lower (11 mP). Additionally, the coefficient of determination and consequently the goodness of fit were much better for filter-based instrument ( $R^2 = 1.00$  compared to 0.986). Therefore, it can be concluded that FP measurements on the filter-based MTP reader are more precise than on monochromator-based multi-mode instruments. For applications of new tracers, especially new fluorophores, monochromator-based instruments are recommended.

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Furthermore, these instruments can certainly be utilized in case only semi-quantitative determinations are required.

For the FPIA on MTPs, epifluorescence measurements using dichroic mirrors are performed. In cuvettes, usually an angle of 90° is applied between excitation and detection of emission.<sup>66</sup> Typically, the parallel and perpendicular fluorescence intensities are measured one after the other by rotating the polarizer by 90°. Hence, only values at a certain time of the reaction can be measured. On the aokin spectrometer, T optics are used for simultaneous detection of parallel and perpendicular fluorescence intensities. This kinetic measurement enables the evaluation of the analyte concentration over a time range. The averaged concentration leads to more precise values because variations over time, that might strongly influence the single-point measurement, can be compensated by these kinetic measurements.

### **4.3.2 Automatization**

The filter-based MTP reader can be applied for semi-automatization of assay performance. Here, the tracer and antibody dilutions can be added automatically and all measurement and shaking steps can be performed in the instrument. For the caffeine FPIA, it could be shown that the type of assay performance should already be considered during assay optimization. Here, the automated assay led to a reduced dynamic range (117 mP compared to 171 mP) and sensitivity (21 µg/L compared to 11 µg/L). But the goodness of fit and the precision of measurement points were comparable and good for both assay performances.

The plate mode was chosen for semi-automated assay performance. This means that the time difference between the dispensing to each individual well is considered during measurement of the wells; the speed of dispensing and reading are aligned to each other. Consequently, the incubation time for each well is exactly the same. For caffeine FPIAs, this might not be so important, because the equilibrium of antigen/antibody interaction is reached within a few minutes. But the application of this time-controlled assay performance might increase the precision of CBZ FPIAs using the new antibody which showed a long reaction time and is measured at non-equilibrium state.

One advantage of semi-automated assay performances is the reduction of measurement uncertainties especially for unexperienced experimenters. Additionally, it could be proven that the reagents can be used for a sequence of MTPs and that the resulting calibration curves are in good agreement with each other. So not only the precision on one MTP, but also the reproducibility on different MTPs is high, at least for calibrations of the caffeine FPIA. A future goal should be the application of this automated system to caffeine determination in consumer products. Furthermore, the CBZ FPIA should be optimized to this automated assay format.

A direct comparison of manual tube- and automatized cuvette-based FPIA is not possible at this point, because only CBZ was measured on both platforms and different antibodies were applied. But in general, lower StD were determined for measuring calibration curves in tubes than in cuvettes (lower than 5 mP and 10 mP, respectively; both measured at one fixed incubation time). Caffeine FPIA in cuvettes showed similar maximum StD as the CBZ FPIA performed on the same instrument.

For the measurement of real samples, automated performance in cuvettes seems to be advantageous: for CBZ, coefficients of variation (CVs) of less than 10% were determined between the measurements of individual samples. For caffeine, the CVs were even below 4%. However, for tube-based FPIA, up to 15% variation was observed between the determined CBZ concentrations of real samples. It has to be taken into consideration that an intra-assay CV of up to 15% was observed for CBZ quantification in cuvettes, determined as the error over the measurement time. Nevertheless, for cuvette-based measurements, wastewater samples were used as sample matrix which is highly more complex than surface waters that were applied on tube-based FPIA. It can be summarized that both platforms, cuvette- and tube-based, are highly suitable for FPIA measurements. But for determinations in real samples, the automated cuvette-based system seems to be slightly more precise due the kinetic determination of concentration.

### 4.3.3 Evaluation

One advantage of FPIAs performed in cuvettes or tubes is that one calibration curve can be used for sample evaluation as long as the identical and stable reagents and dilutions are applied. Usually, on each MTP calibration curves are determined. But for both, CBZ and caffeine FPIAs, it could be proven that characteristic parameters of calibration curves are reproducible for different MTPs as long as the identical reagents are used. Variations between the surface of different MTPs do not or only slightly influence the degree of polarization, because a ratio of fluorescence intensities is used for evaluation. For absorbance or fluorescence measurements, variations between MTPs might have a stronger influence on calibration curves, because here, the measured values are directly used for evaluation. For FPIA, characteristic parameters including dynamic range, test midpoint and slope showed CVs lower than 10% for the assays of both analytes. Only the test midpoint of caffeine FPIA showed a higher variation between the MTPs ( $25.6 \pm 4.3 \mu\text{g/L}$ , CV = 17%), although this assay was performed semi-automatically. These results indicate the possibility of transferring calibration curves from MTP to MTP which would increase the already high sample throughput on MTPs.

For immunoassays, typically a sigmoidal calibration curve is applied for the evaluation. The relative error of concentration and the corresponding precision profile have been frequently used for the determination of the measurement range,<sup>12-16, 119</sup> Adopted from the traditional “three sigma criterion” often applied for instrumental methods, the measurement range is mostly defined as the range of concentration with a relative error of concentration lower than 30%. This definition was also applied for FPIAs described in this thesis. Only for the measurements in cuvettes (aokin spectrometer), another kind of calibration was used for real samples. Here, the concentration is determined over a time range by the associated software. This enables the compensation of single outliers. For each measurement point, a point-to-point calibration is used. Therefore, the range of quantifiable concentrations should be defined in a way that the point-to-point calibration is approximately a linear function. Hence, the range between 10 and 90% signal intensity ( $IC_{90}$  and  $IC_{10}$ , respectively) was chosen as quantification range for CBZ FPIA on this instrument. For other immunoassays, similar approaches using IC values have been used for evaluation.<sup>17-19</sup>

#### **4.3.4 Sample throughput and measurement environment**

For caffeine and the commercially available anti-CBZ antibody, fast reaction times were observed. Therefore, a very short overall assay time could be reached which enables a high sample throughput. For caffeine FPIA on the aokin instrument, a measurement time of 2 min was sufficient. The CBZ FPIA in tubes, performed in the portable Sentry FP reader, can be completed within 4 min including an incubation time of 3 min. But for these systems, only one sample can be measured after the other.

Usually, the assay performance takes longer when MTPs are utilized due to less efficient shaking and longer reading times. On the other hand a much higher throughput can be achieved. The incubation time on MTPs was set to 10 min for all analytes. The overall assay time on this platform is approximately 20 min and up to 24 samples can be measured in triplicate within one run. The FPIA performance on MTPs is also highly recommended for the assay optimization, e.g. the evaluation of different tracers and the characterization of antibodies, in particular the determination of CRs. The applicability for these purposes has been proven. Additionally, lower volumes are used and therefore the consumption of reagents can be reduced using MTP-based FPIA; compared to CBZ measurements in tubes, only 3% of the amount of commercially available antibody was required for a single measurement. Especially regarding this reagent, this might have a high impact on cost efficiency of the assay.

The required assay time and consequently the sample throughput depends also on the applied antibody. The measurement time in cuvettes using the new anti-CBZ antibody was set to 10 min, the equilibrium not being reached within this time. The whole assay procedure including all pipetting and cleaning steps requires 16 min. The equilibrium of antibody/tracer reaction was not reached before 60 min reaction time on MTPs, but an incubation time of 10 min enabled the development of a sensitive and specific CBZ FPIA. So also for antibodies showing slow reactions, the assay time can be kept short.

FPIAs on MTPs require a laboratory environment, because of the necessity of single- and 8-channel pipettes, stepper pipettes, reservoirs for the reagents, plate shakers and a relatively large instrument compared to single-measurement systems. Even if the assay is implemented semi-automatically, the performance in a laboratory is beneficial. FPIAs performed in the FP spectrometer Sentry™ require only minimal equipment like pipettes and a Vortex shaker, but the latter can be replaced if necessary by longer manual shaking of the tube. The instrument itself is small, has a very low weight (1.1 kg) and can be battery-operated. Thus, it can be easily transported and applied for field measurements. For example, measurements could be performed directly along a river to monitor the fate of a pharmaceutical compound in surface waters. The only requirements therefore are the availability of pipettes and a cooling system for the antibody (4 °C). Cuvette-based FPIAs can also be easily detached from laboratories. Measurements on the aokin spectrometer can be applied for on-site measurements, especially when the automatized procedure is utilized. In that case, the instrument can be controlled by staff without any scientific background. This indicates the high applicability of this instrument for monitoring of pharmacologically active compounds in WWTPs or generally for process analytical technologies.

## 4.4 Applicability of FPIA to complex matrices

The challenge for the application of FPIA to real samples is that no washing step is required for homogeneous assays. Hence, all matrix compounds have contact with the antibody and the tracer and are present during the measurement step. For FPIA, especially fluorescent compounds are of great concern, because they can directly influence the measured values. Therefore, sample background correction was done for all assays and samples. But still, interactions of matrix compounds could influence the fluorescence properties of the tracer or the binding behavior of antibody.

### 4.4.1 Applicability of caffeine FPIA to consumer products

The application of the caffeine FPIA to consumer products is fairly simple due to the high caffeine concentrations in those samples and the high sensitivity of immunoanalytical methods. For FPIA, concentrations in the low  $\mu\text{g/L}$  range can be measured. Therefore, only dissolving, brewing or degassing had to be done as sample preparation for the different consumer products. Afterwards, the samples had to be diluted with ultrapure water, at least by a factor of 1000 and up to 240,000, even for decaffeinated coffee samples. These high dilution factors indicate that the matrix of these samples cannot or only slightly influence the assay performance.

The applicability of FPIAs to complex matrices can in general be performed on all discussed platforms. MTP is the platform of choice for high sample throughput, whereas cuvettes are desirable for on-site measurements of single samples. For caffeine determinations of real samples, only the cuvette platform (FPIA 1, aokin spectrometer) led to reproducible results. With measurements on MTPs (FPIA 2) only semi-quantitative statements regarding the caffeine content could be made, because the determined concentrations were highly afflicted with errors. No good correlations with reference methods were observed. Here, the monochromator-based multimode MTP reader was used, because at that time only this instrument was available. Maybe the application of the filter-based reader would improve the reliability of the results of this method. Another possible error source for FPIA on MTP is the utilization of small sample and reagent volumes; at lower volumes, the errors of pipetting are higher.

The assay in cuvettes was performed automatically. Using this platform, precise results ( $\text{CV} < 4\%$ ) of caffeine concentrations could be determined for many different consumer products, e.g. different kinds of coffees including decaffeinated coffee, soft drinks, energy drinks, tea and cosmetics. The correlations with instrumental and immunoanalytical reference methods were very good. Summarizing, automatized caffeine FPIA could successfully be applied to a large variety of consumer products, yielding in reliable and accurate caffeine determinations within a measurement time of 2 min.

### 4.4.2 Applicability of carbamazepine FPIA to environmental samples

For samples with low analyte concentrations as they are usually present for CBZ in environmental samples, sample dilution is not applicable. Therefore, the application to these matrices requires a more detailed optimization. In wastewater, high concentrations of salts, proteins, metal ions and a large variety of pharmaceutical compounds are present in wide concentration ranges. The treated and therefore at least partly cleaner wastewater is discharged to surface water, where it is highly diluted. Hence, surface waters are in general

## *Final discussion*

a less complex matrix than wastewater. The aim of the application to environmental samples was the utilization of easy-to-perform sample preparation. At the end, only filtration had to be applied for all samples, using folded paper filters for surface water and glass fiber filter (1-2  $\mu\text{m}$ ) for wastewater.

For FP measurements, the fluorescence signal of samples is a crucial factor regarding the applicability. Surface waters showed increased fluorescence intensities of 20 and 40% compared to CBZ calibrators, depending on the applied platform (tube or MTP, respectively). The variation between the fluorescence intensities of different samples was lower than 10%. For wastewater samples, high variations between the fluorescence intensities of different samples were observed. The fluorescence intensities of samples were higher than for calibrators by 175 to 471%. Furthermore, the degree of polarization varied between different wastewater samples. But thanks to sample background correction, no influence on the fluorescence intensity or degree of polarization of free tracer was observed, not even for highly fluorescent wastewater samples. Despite the complexity of the samples, no influence on the fluorescence properties of the tracer was observed.

However, the matrix compounds could still influence properties of antigen/antibody interaction. One way to overcome matrix effects is the utilization of different buffers. For the application to surface waters, the usage of a common borate buffer was sufficient. To compensate the complexity of the wastewater sample matrix, much more concentrated reaction buffers had to be applied containing glycine, sodium chloride and EDTA.

Recovery rates of CBZ in surface water of 81 to 140% in tubes and 66 to 110% on MTPs were obtained. Within the measurement range, the variations of the results were all lower than the defined threshold of relative error of concentration of 30% (< 15% in tubes, < 25% on MTPs).

The recovery rates for surface and waste water are not directly comparable, because different antibodies were used for the investigation of both matrices. The antibody applied for wastewater samples showed lower CRs for relevant matrix compounds and therefore more accurate results were expected. The recovery rates of CBZ concentrations in wastewater were good, but mostly slightly underestimated (recovery rates: 61-104%), independent of the kind of wastewater or the CBZ concentration in the sample. Even dilution factors of up to 20 did not increase the recovery rates. Low intra- and inter-assay CVs of less than 15 and 10% were observed, respectively. Generally, the new antibody and the utilization of the automated cuvette-based instrument have proven their suitability for application to wastewater. Hence, this assay procedure should be easily adaptable to the less complex matrix of surface waters.

During the optimization of the assay, a strong time dependency of the determined CBZ concentrations of samples was observed. For heterogeneous immunoassays, usually the incubation time of the competitive step is optimized for calibration curves, but for verification of the applicability to real samples, typically no attention is given to the incubation time. Due to the results obtained for FPIA measurements, the influence of incubation time should be considered more carefully for all kinds of antibody-based methods.

Measurement ranges in the low  $\mu\text{g/L}$  range were reached for all CBZ assays. The lowest limits of measurement ranges were 2.5  $\mu\text{g/L}$  for FPIA in tubes and 1.5  $\mu\text{g/L}$  on MTPs for

surface waters. For this matrix, CBZ concentrations in the mid ng/L range are typically expected. For wastewater, the lowest quantifiable concentration was 2.5 µg/L. For one investigated real sample, the CBZ concentration was within this range. Here, the results from FPIA and the reference method LC-MS/MS were in good agreement. But mostly, the CBZ concentrations in this sample matrix are around 1 µg/L. Therefore, spiked samples had to be applied for CBZ studies of environmental samples. For medical analyses, these measurement ranges would be more than sufficient so that serum samples could be even diluted by a factor of approximately 1000 (therapeutic drug level of CBZ: 4-12 mg/L<sup>239</sup>). Hence, it is expected that this assay could be easily applied for diagnostic purposes.

In 2015, Manickum and John indicated the preference of immunoanalytical methods for the determination of hormones in wastewater.<sup>281</sup> This review points out that heterogeneous immunoassays are fairly equally used as LC- and GC-MS/MS for determination of this analyte group in water samples. This illustrates the demand on immunoassays for environmental analyses. Within this thesis, it could be shown that homogeneous immunoassays, especially FPIA, can also be applied for fast and accurate determination of CBZ in water samples. Therefore, this kind of assay may be established for monitoring the fate of pharmacologically active compounds in the water system by offering platforms for on-site measurements or high-throughput screenings.

## 5. Conclusion

Fluorescence polarization immunoassays (FPIAs) for the determination of the pharmacologically active compounds caffeine and carbamazepine (CBZ) were developed. Different platforms including microtiter plates, cuvettes and tubes were applied and compared on different instruments. For the choice of the right format, several factors for the desired application field should be considered: on-site measurements or laboratory environment, routine measurements or optimization of general assay parameters, individual samples or high sample throughput. Generally, all platforms were suitable for FPIA measurements. The most precise analyte determination in real samples could be performed in cuvettes using kinetic measurements.

FPIA enabled the precise and accurate determination of caffeine in the  $\mu\text{g/L}$  range. The assay could be successfully applied to consumer products by simply diluting the samples, including the caffeine determination in decaffeinated coffee. Good correlations with reference methods were found.

The development and optimization of FPIA for CBZ included the synthesis and comparison of different tracers and the application of a commercially available antibody to surface water. Due to high cross-reactivities of this antibody, yielding in overestimations of CBZ in environmental samples, a new monoclonal antibody, highly specific for CBZ was produced.

For this production process, several possibilities for improving this process were successfully applied. During the development of the new monoclonal antibodies it could be proven that feces screening for the monitoring of the immune response and supernatant screening by FPIA are powerful techniques that should be considered by anyone in future immunizations.

The newly developed antibody was comprehensively characterized using ELISA and FPIA and was highly applicable for both formats. Low cross-reactivities were observed for environmentally relevant CBZ metabolites and other pharmaceuticals. Strong time dependency of the reaction of the antibody with tracer, analyte or cross-reactant was observed and the careful study of it could be used for the development of more sensitive and more specific FPIAs. Furthermore, these studies revealed the possibility to determine CBZ over a wider concentration range. Generally, FPIA can be used as a tool for improving the sensitivity and selectivity of immunoassays.

The new anti-CBZ antibody enables for the accurate and precise determination of CBZ in water samples. Hence, an on-site measurement system for monitoring the fate of CBZ in wastewater treatment plants could be developed which can be operated automatically within 16 minutes. Sample preparation could be reduced to filtration. Concentrations in the low  $\mu\text{g/L}$  range could be quantified. This work presents the first application of FPIA to CBZ determination in environmental samples, or more general the first application of FPIA to wastewater without tedious sample preparation.

## 6. Bibliography

1. R.S. Yalow, S.A. Berson; Immunoassay of endogenous plasma insulin in man. *J. Clin. Invest.* 1960, 39, 1157-1175.
2. S. Lee, H. Yu, S.H. Kang; Plasmonic metal scattering immunoassay by total internal reflection scattering microscopy with nanoscale lateral resolution. *Chem. Commun.* 2015, 51, 945-947.
3. S. Lee, S.H. Kang; Gold-nanopatterned single interleukin-6 sandwich immunoassay chips with zeptomolar detection capability based on evanescent field-enhanced fluorescence imaging. *Analyst* 2013, 138, 3478-3482.
4. A. Bahlmann, J.J. Carvalho, M.G. Weller, U. Panne, R.J. Schneider; Immunoassays as high-throughput tools: Monitoring spatial and temporal variations of carbamazepine, caffeine and cetirizine in surface and wastewaters. *Chemosphere* 2012, 89, 1278-1286.
5. X. Yan, H.X. Li, Y. Yan, X.G. Su; Developments in pesticide analysis by multi-analyte immunoassays: A review. *Anal. Methods* 2014, 6, 3543-3554.
6. F. Zhao, C. Hu, H. Wang, L. Zhao, Z. Yang; Development of a MAb-based immunoassay for the simultaneous determination of *O,O*-diethyl and *O,O*-dimethyl organophosphorus pesticides in vegetable and fruit samples pretreated with QuEChERS. *Anal. Bioanal. Chem.* 2015, 407, 8959-8970.
7. M.G. Weller, A. Zeck, A. Eikenberg, S. Nagata, Y. Ueno, R. Niessner; Development of a direct competitive microcystin immunoassay of broad specificity. *Anal. Sci.* 2001, 17, 1445-1448.
8. M. Winklmair, M.G. Weller, J. Mangler, B. Schlosshauer, R. Niessner; Development of a highly sensitive enzyme-immunoassay for the determination of triazine herbicides. *Fresen. J. Anal. Chem.* 1997, 358, 614-622.
9. M.H. Gey; *Instrumentelle Analytik und Bioanalytik*. 3<sup>rd</sup> ed., Springer-Verlag Berlin Heidelberg 2015.
10. R.A. Dudley, P. Edwards, R.P. Ekins, D.J. Finney, I.G.M. McKenzie, G.M. Raab, D. Rodbard, R.P.C. Rodgers; Guidelines for immunoassay data-processing. *Clin. Chem.* 1985, 31, 1264-1271.
11. R. Ekins; The precision profile: its use in RIA assessment and design. *Ligand Q.* 1981, 4, 33-44.
12. J. Grandke, L. Oberleitner, U. Resch-Genger, L.-A. Garbe, R.J. Schneider; Quality assurance in immunoassay performance – Comparison of different enzyme immunoassays for the determination of caffeine in consumer products. *Anal. Bioanal. Chem.* 2013, 405, 1601-1611.
13. J. Grandke, L. Oberleitner, U. Resch-Genger, L.-A. Garbe, R.J. Schneider; Quality assurance in immunoassay performance – Carbamazepine immunoassay format evaluation and application on surface and waste water. *Anal. Methods* 2013, 5, 3754-3760.
14. S. Gärtner, J.J. Carvalho, F. Emmerling, L.-A. Garbe, R.J. Schneider; Polyclonal murine and rabbit antibodies for the bile acid isolithocholic acid. *J. Immunoassay Immunochem.* 2015, 36, 233-252.
15. D.L.D. Lima, R.J. Schneider, V.I. Esteves; Development of an enzyme-linked immunosorbent assay for atrazine monitoring in water samples. *Environ. Sci. Pollut. R.* 2013, 20, 3157-3164.

## Bibliography

16. A. Bahlmann, M.G. Weller, U. Panne, R.J. Schneider; Monitoring carbamazepine in surface and wastewaters by an immunoassay based on a monoclonal antibody. *Anal. Bioanal. Chem.* 2009, 395, 1809-1820.
17. A.P. Deng, M. Himmelsbach, Q.Z. Zhu, S. Frey, M. Sengl, W. Buchberger, R. Niessner, D. Knopp; Residue analysis of the pharmaceutical diclofenac in different water types using ELISA and GC-MS. *Environ. Sci. Technol.* 2003, 37, 3422-3429.
18. J. Chen, I.A. Shanin, S. Lv, Q. Wang, C. Mao, Z. Xu, Y. Sun, Q. Wu, S.A. Eremin, H. Lei; Heterologous strategy enhancing the sensitivity of the fluorescence polarization immunoassay of clinafloxacin in goat milk. *J. Sci. Food Agr.* 2016, 96, 1341-1346.
19. M. Huebner, E. Weber, R. Niessner, S. Boujday, D. Knopp; Rapid analysis of diclofenac in freshwater and wastewater by a monoclonal antibody-based highly sensitive ELISA. *Anal. Bioanal. Chem.* 2015, 407, 8873-8882.
20. C. Tan, N. Gajovic-Eichelmann, R. Polzius, N. Hildebrandt, F.F. Bier; Direct detection of  $\Delta$ 9-tetrahydrocannabinol in aqueous samples using a homogeneous increasing fluorescence immunoassay (HiFi). *Anal. Bioanal. Chem.* 2010, 398, 2133-2140.
21. C. Tan, N. Gajovic-Eichelmann, W.F.M. Stöcklein, R. Polzius, F.F. Bier; Direct detection of  $\Delta$ 9-tetrahydrocannabinol in saliva using a novel homogeneous competitive immunoassay with fluorescence quenching. *Anal. Chim. Acta* 2010, 658, 187-192.
22. G. Wang, J. Yuan, X. Hai, K. Matsumoto; Homogeneous time-resolved fluoroimmunoassay of 3,5,3'-triiodo-L-thyronine in human serum by using europium fluorescence energy transfer. *Talanta* 2006, 70, 133-138.
23. K. Kuningas, T. Ukonaho, H. Pakkila, T. Rantanen, J. Rosenberg, T. Lovgren, T. Soukka; Upconversion fluorescence resonance energy transfer in a homogeneous immunoassay for estradiol. *Anal. Chem.* 2006, 78, 4690-4696.
24. J. Ettlinger, J.A. Schenk, B. Micheel, E. Ehrentreich-Förster, N. Gajovic-Eichelmann; A direct competitive homogeneous immunoassay for progesterone - The redox quenching immunoassay. *Electroanal.* 2012, 24, 1567-1575.
25. T. Li, J.-Y. Byun, B.B. Kim, Y.-B. Shin, M.-G. Kim; Label-free homogeneous FRET immunoassay for the detection of mycotoxins that utilizes quenching of the intrinsic fluorescence of antibodies. *Biosens. Bioelectron.* 2013, 42, 403-408.
26. W. Dandliker, G.A. Feigen; Quantification of antigen-antibody reaction by the polarization of fluorescence. *Biochem. Biophys. Res. Co.* 1961, 5, 299-304.
27. D.M. Jameson, J.A. Ross; Fluorescence polarization/anisotropy in diagnostics and imaging. *Chem. Rev.* 2010, 110, 2685-2708.
28. M.S. Nasir, M.E. Jolley; Fluorescence polarization: An analytical tool for immunoassay and drug discovery. *Comb. Chem. High T. Scr.* 1999, 2, 177-190.
29. S. Zhang, Z. Wang, I.S. Nesterenko, S.A. Eremin, J. Shen; Fluorescence polarisation immunoassay based on a monoclonal antibody for the detection of sulphamethazine in chicken muscle. *Int. J. Food Sci. Tech.* 2007, 42, 36-44.
30. P. Önnarfjord, S. Eremin, J. Emneus, G. Marko-Varga; Fluorescence polarisation for immunoreagent characterisation. *J. Immunol. Methods* 1998, 213, 31-39.
31. M. Li, X.F. Liu, X.D. Hua, W. Yin, Q.K. Fang, M.H. Wang; Fluorescence polarization immunoassay for highly efficient detection of clothianidin in agricultural samples. *Anal. Methods* 2014, 6, 6541-6547.

32. Q. Wang, S.A. Haughey, Y.-M. Sun, S.A. Eremin, Z.-F. Li, H. Liu, Z.-L. Xu, Y.-D. Shen, H.-T. Lei; Development of a fluorescence polarization immunoassay for the detection of melamine in milk and milk powder. *Anal. Bioanal. Chem.* 2011, 399, 2275-2284.
33. H.S. Chun, E.H. Choi, H.-J. Chang, S.-W. Choi, S.A. Eremin; A fluorescence polarization immunoassay for the detection of zearalenone in corn. *Anal. Chim. Acta* 2009, 639, 83-89.
34. Y.J. Sheng, S. Eremin, T.J. Mi, S.X. Zhang, J.Z. Shen, Z.H. Wang; The development of a fluorescence polarization immunoassay for aflatoxin detection. *Biomed. Environ. Sci.* 2014, 27, 126-129.
35. J. Qi, K. Kizjakina, R. Robinson, K. Tolani, P. Sobrado; A fluorescence polarization binding assay to identify inhibitors of flavin-dependent monooxygenases. *Anal. Biochem.* 2012, 425, 80-87.
36. G.I. Hatzidakis, A.M. Tsatsakis, E.K. Krambovitis, A. Spyros, S.A. Eremin; Use of L-lysine fluorescence derivatives as tracers to enhance the performance of polarization fluoroimmunoassays. A study using two herbicides as model antigens. *Anal. Chem.* 2002, 74, 2513-2521.
37. J.N. Tian, L.J. Zhou, Y.C. Zhao, Y. Wang, Y. Peng, S.L. Zhao; Multiplexed detection of tumor markers with multicolor quantum dots based on fluorescence polarization immunoassay. *Talanta* 2012, 92, 72-77.
38. E. Terpetschnig, H. Szmackinski, J.R. Lakowicz; Fluorescence polarization immunoassay of a high-molecular-weight antigen using a long wavelength-absorbing and laser diode-excitabile metal-ligand complex. *Anal. Biochem.* 1996, 240, 54-59.
39. A. Dürkop, F. Lehmann, O.S. Wolfbeis; Polarization immunoassays using reactive ruthenium metal-ligand complexes as luminescent labels. *Anal. Bioanal. Chem.* 2002, 372, 688-694.
40. M.L. Sanchez-Martinez, M.P. Aguilar-Caballo, S.A. Eremin, A. Gomez-Hens; Long-wavelength fluorescence polarization immunoassay for surfactant determination. *Talanta* 2007, 72, 243-248.
41. T.M. Li, J.L. Benovic, J.F. Burd; Serum theophylline determination by fluorescence polarization immunoassay utilizing an umbelliferone derivate as a fluorescent label. *Anal. Biochem.* 1981, 118, 102-107.
42. K.M. Kleman-Leyer, T.A. Klink, A.L. Kopp, T.A. Westermeyer, M.D. Koeff, B.R. Larson, T.J. Worzella, C.A. Pinchard, S.A.T. van de Kar, G.J.R. Zaman, J.J. Hornberg, R.G. Lowery; Characterization and optimization of a red-shifted fluorescence polarization ADP detection assay. *Assay Drug Dev. Technol.* 2009, 7, 56-67.
43. A. Baeyer; Ueber eine neue Klasse von Farbstoffen. *Ber. Dtsch. Chem. Ges.* 1871, 4, 555-558.
44. R. Sjöback, J. Nygren, M. Kubista; Absorption and fluorescence properties of fluorescein. *Spectrochim. Acta A* 1995, 51, L7-L21.
45. X.F. Zhang, J.L. Zhang, L.M. Liu; Fluorescence properties of twenty fluorescein derivatives: Lifetime, quantum yield, absorption and emission spectra. *J. Fluoresc.* 2014, 24, 819-826.
46. Z.-H. Wang, S.-X. Zhang, J.-Z. Shen, S.A. Eremin; Analysis of sulfamethazine by fluorescence polarization immunoassay. *Chinese J. Anal. Chem.* 2007, 35, 819-824.

## Bibliography

47. S.A. Eremin, J. Landon, D.S. Smith, R. Jackman; Development of a polarization fluoroimmunoassay for sulfamethazine using an automated analyser. *Analyst* 1994, 119, 2723-2726.
48. J. Chen, S. Lv, Q. Wang, Z. Xu, J. Yang, Y. Shen, H. Wang, Y. Sun, H. Lei; A specific and high-throughput fluorescence polarization immunoassay for surveillance screening of cinafloxacin in milk. *Food Anal. Methods* 2015, 8, 1468-1476.
49. J. Choi, C. Kim, M.J. Choi; Comparison of capillary electrophoresis-based immunoassay with fluorescence polarization immunoassay for the immunodetermination of methamphetamine using various methamphetamine antibodies. *Electrophoresis* 1998, 19, 2950-2955.
50. N.V. Gasilova, S.A. Eremin; Determination of chloramphenicol in milk by a fluorescence polarization immunoassay. *J. Anal. Chem.* 2010, 65, 255-259.
51. Y.V. Nartova, T.N. Ermolaeva, M.R. Fleisher, R. Abuknesha, S.A. Eremin; Determination of alachlor in food products by a fluorescence polarization immunoassay. *J. Anal. Chem.* 2008, 63, 499-505.
52. B. Sendra, S. Panadero, S. Eremin, A. Gomez-Hens; Kinetic determination of atrazine in foods based on stopped-flow fluorescence polarization immunoassay. *Talanta* 1998, 47, 153-160.
53. W.B. Shim, A.Y. Kolosova, Y.J. Kim, Z.Y. Yang, S.J. Park, S.A. Eremin, I.S. Lee, D.H. Chung; Fluorescence polarization immunoassay based on a monoclonal antibody for the detection of ochratoxin A. *Int. J. Food Sci. Tech.* 2004, 39, 829-837.
54. S.A. Eremin, J.V. Samsonova; Development of polarization fluoroimmunoassay for the detection of s-triazine herbicides. *Anal. Lett.* 1994, 27, 3013-3025.
55. G.S. Furzer, L. Veldhuis, J.C. Hall; Development and comparison of three diagnostic immunoassay formats for the detection of azoxystrobin. *J. Agric. Food Chem.* 2006, 54, 688-693.
56. V. Lippolis, M. Pascale, S. Valenzano, V. Pluchinotta, S. Baumgartner, R. Krska, A. Visconti; A rapid fluorescence polarization immunoassay for the determination of T-2 and HT-2 toxins in wheat. *Anal. Bioanal. Chem.* 2011, 401, 2561-2571.
57. V. Lippolis, M. Pascale, S. Valenzano, A.C.R. Porricelli, M. Suman, A. Visconti; Fluorescence polarization immunoassay for rapid, accurate and sensitive determination of ochratoxin A in wheat. *Food Anal. Methods* 2014, 7, 298-307.
58. E.H. Choi, D.M. Kim, S.-W. Choi, S.A. Eremin, H.S. Chun; Optimisation and validation of a fluorescence polarisation immunoassay for rapid detection of zearalenone in corn. *Int. J. Food Sci. Tech.* 2011, 46, 2173-2181.
59. P. Huang, S. Zhao, S.A. Eremin, S. Zheng, D. Lai, Y. Chen, B. Guo; A fluorescence polarization immunoassay method for detection of the bisphenol A residue in environmental water samples based on a monoclonal antibody and 4'-(aminomethyl) fluorescein. *Anal. Methods* 2015, 7, 4246-4251.
60. M.S. Nasir, M.E. Jolley; Development of a fluorescence polarization assay for the determination of aflatoxins in grains. *J. Agric. Food Chem.* 2002, 50, 3116-3121.
61. D.K. Johnson; A fluorescence polarization immunoassay for cadmium(II). *Anal. Chim. Acta* 1999, 399, 161-172.
62. T.J. Mi, Z.H. Wang, S.A. Eremin, J.Z. Shen, S.X. Zhang; Simultaneous determination of multiple (fluoro)quinolone antibiotics in food samples by a one-step fluorescence polarization immunoassay. *J. Agric. Food Chem.* 2013, 61, 9347-9355.

63. H. Lei, G. Xue, C. Yu, S.A. Haughey, S.A. Eremin, Y. Sun, Z. Wang, Z. Xu, H. Wang, Y. Shen, Q. Wu; Fluorescence polarization as a tool for the detection of a widely used herbicide, butachlor, in polluted waters. *Anal. Methods* 2011, 3, 2334-2340.
64. J.-Y. Yang, Y. Zhang, H. Wang, Z.-L. Xu, S.A. Eremin, Y.-D. Shen, Q. Wu, H.-T. Lei, Y.-M. Sun; Development of fluorescence polarisation immunoassay for carbofuran in food and environmental water samples. *Food Agr. Immunol.* 2015, 26, 340-355.
65. A.Y. Kolosova, J.H. Park, S.A. Eremin, S.J. Kang, D.H. Chung; Fluorescence polarization immunoassay based on a monoclonal antibody for the detection of the organophosphorus pesticide parathion-methyl. *J. Agric. Food Chem.* 2003, 51, 1107-1114.
66. X. Amouretti; Multidetector microplate readers: Filter-based, monochromator-based... or both? *Am. Biotechnol. Lab.* 2007, 25, 22.
67. J.R. Lakowicz; *Principles of Fluorescence Spectroscopy*. 3<sup>rd</sup> ed., Springer-Verlag US 2006.
68. D.S. Smith, S.A. Eremin; Fluorescence polarization immunoassays and related methods for simple, high-throughput screening of small molecules. *Anal. Bioanal. Chem.* 2008, 391, 1499-1507.
69. D.K. Johnson, S.M. Combs, J.D. Parsen, M.E. Jolley; Lead analysis by anti-chelate fluorescence polarization immunoassay. *Environ. Sci. Technol.* 2002, 36, 1042-1047.
70. S.A. Eremin, E.G. Matveeva, A. Gomez-Hens, D. Perez-Bendito; Kinetic determination of 2,4-dichlorophenoxyacetic acid by stopped-flow fluorescence polarization immunoassay. *Int. J. Environ. An. Ch.* 1998, 71, 137-146.
71. Z. Wang, S. Zhang, S. Ding, S.A. Eremin, J. Shen; Simultaneous determination of sulphamerazine, sulphamethazine and sulphadiazine in honey and chicken muscle by a new monoclonal antibody-based fluorescence polarisation immunoassay. *Food Addit. Contam.* 2008, 25, 574-582.
72. L.L. Ren, M. Meng, P. Wang, Z.H. Xu, S.A. Eremin, J.H. Zhao, Y.M. Yin, R.M. Xi; Determination of sodium benzoate in food products by fluorescence polarization immunoassay. *Talanta* 2014, 121, 136-143.
73. Z.L. Xu, Q. Wang, H.T. Lei, S.A. Eremin, Y.D. Shen, H. Wang, R.C. Beier, J.Y. Yang, K.A. Maksimova, Y.M. Sun; A simple, rapid and high-throughput fluorescence polarization immunoassay for simultaneous detection of organophosphorus pesticides in vegetable and environmental water samples. *Anal. Chim. Acta* 2011, 708, 123-129.
74. N.S. Lipman, L.R. Jackson, L.J. Trudel, F. Weis-Garcia; Monoclonal versus polyclonal antibodies: Distinguishing characteristics, applications, and information resources. *Ilar J.* 2005, 46, 258-268.
75. F. Lottspeich, J.W. Engels; *Bioanalytik*. 3<sup>rd</sup> ed., Spektrum Akademischer Verlag 2012.
76. E.P. Meulenber; *Antibodies Applications and New Developments*. Bentham eBooks 2012.
77. S. Ramin, M.G. Weller; Extremely sensitive and selective antibodies against the explosive 2,4,6-trinitrotoluene by rational design of a structurally optimized hapten. *J. Mol. Recognit.* 2012, 25, 89-97.
78. K.V. Singh, J. Kaur, G.C. Varshney, M. Raje, C.R. Suri; Synthesis and characterization of hapten-protein conjugates for antibody production against small molecules. *Bioconjugate Chem.* 2004, 15, 168-173.

## Bibliography

79. J.J. Carvalho, M.A. Walter, Y. Baermann-Stapel, M.G. Weller, U. Panne, J.A. Schenk, R.J. Schneider; Non-invasive monitoring of immunization progress in mice via IgG from feces. *In vivo* 2012, 26, 63-70.
80. G. Köhler, C. Milstein; Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 1975, 256, 495-497.
81. M. Leenaars, C.F.M. Hendriksen; Critical steps in the production of polyclonal and monoclonal antibodies: Evaluation and recommendations. *Ilar J.* 2005, 46, 269-279.
82. M.J. Glade; Caffeine - Not just a stimulant. *Nutrition* 2010, 26, 932-938.
83. B.B. Fredholm, K. Bättig, J. Holmen, A. Nehlig, E.E. Zvartau; Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol. Rev.* 1999, 51, 83-133.
84. H.-D. Belitz, W. Grosch, P. Schieberle; *Lehrbuch der Lebensmittelchemie*. 6<sup>th</sup> ed., Springer-Verlag Berlin Heidelberg 2008.
85. T.W. Baumann, B.H. Schulthess, K. Hänni; Guarana (*Paullinia-Cupana*) rewards seed dispersers without intoxicating them by caffeine. *Phytochemistry* 1995, 39, 1063-1070.
86. B. Desbrow, R. Hughes, M. Leveritt, P. Scheelings; An examination of consumer exposure to caffeine from retail coffee outlets. *Food Chem. Toxicol.* 2007, 45, 1588-1592.
87. I.A. Ludwig, P. Mena, L. Calani, C. Cid, D. Del Rio, M.E.J. Lean, A. Crozier; Variations in caffeine and chlorogenic acid contents of coffees: What are we drinking? *Food Func.* 2014, 5, 1718-1726.
88. A. Santini, R. Ferracane, P. Mikusova, S. Eged, A. Srobarova, G. Meca, J. Manes, A. Ritieni; Influence of different coffee drink preparations on ochratoxin A content and evaluation of the antioxidant activity and caffeine variations. *Food Control* 2011, 22, 1240-1245.
89. Verordnung über Kaffee, Kaffee- und Zichorien-Extrakte. 2001, last amended 2006.
90. H. Peker, M.P. Srinivasan, J.M. Smith, B.J. McCoy; Caffeine extraction rates from coffee beans with supercritical carbon-dioxide. *Aiche J.* 1992, 38, 761-770.
91. D.V. Bermejo, E. Ibanez, G. Reglero, T. Fornari; Effect of cosolvents (ethyl lactate, ethyl acetate and ethanol) on the supercritical CO<sub>2</sub> extraction of caffeine from green tea. *J. Supercrit. Fluid.* 2016, 107, 507-512.
92. W.J. Kim, J.D. Kim, J. Kim, S.G. Oh, Y.W. Lee; Selective caffeine removal from green tea using supercritical carbon dioxide extraction. *J. Food Eng.* 2008, 89, 303-309.
93. M.D.A. Saldana, C. Zetzl, R.S. Mohamed, G. Brunner; Extraction of methylxanthines from guarana seeds, mate leaves, and cocoa beans using supercritical carbon dioxide and ethanol. *J. Agric. Food Chem.* 2002, 50, 4820-4826.
94. J. Tello, M. Viguera, L. Calvo; Extraction of caffeine from Robusta coffee (*Coffea canephora* var. Robusta) husks using supercritical carbon dioxide. *J. Supercrit. Fluid.* 2011, 59, 53-60.
95. D.V. Bermejo, P. Luna, M.S. Manic, V. Najdanovic-Visak, G. Reglero, T. Fornari; Extraction of caffeine from natural matter using a bio-renewable agrochemical solvent. *Food Bioprod. Process.* 2013, 91, 303-309.
96. S. Gokulakrishnan, K. Chandraraj, S.N. Gummadi; Microbial and enzymatic methods for the removal of caffeine. *Enzyme Microb. Technol.* 2005, 37, 225-232.
97. B. Sik; Development and validation of a green high performance liquid chromatographic method for the determination of some artificial sweeteners and caffeine in soft drinks. *Food Anal. Methods* 2012, 5, 1443-1452.

98. A. Gliszczynska-Swiglo, I. Rybicka; Simultaneous determination of caffeine and water-soluble vitamins in energy drinks by HPLC with photodiode array and fluorescence detection. *Food Anal. Methods* 2015, 8, 139-146.
99. Commission Directive 2002/67/EG on the labelling of foodstuffs containing chinine and of foodstuffs containing caffeine. 2002.
100. L. Luo, M.E. Lane; Topical and transdermal delivery of caffeine. *Int. J. Pharm.* 2015, 490, 155-164.
101. A. Herman, A.P. Herman; Caffeine's mechanisms of action and its cosmetic use. *Skin Pharmacol. Physiol.* 2013, 26, 8-14.
102. P. Banerjee, Z. Ali, B. Levine, D.R. Fowler; Fatal caffeine intoxication: A series of eight cases from 1999 to 2009. *J. Forensic Sci.* 2014, 59, 865-868.
103. D.C. Mitchell, C.A. Knight, J. Hockenberry, R. Teplansky, T.J. Hartman; Beverage caffeine intakes in the U.S. *Food Chem. Toxicol.* 2014, 63, 136-142.
104. L.M. Grosso, M.B. Bracken; Caffeine metabolism, genetics, and perinatal outcomes: A review of exposure assessment considerations during pregnancy. *Ann. Epidemiol.* 2005, 15, 460-466.
105. H.G. Mandel; Update on caffeine consumption, disposition and action. *Food Chem. Toxicol.* 2002, 40, 1231-1234.
106. M.A. Heckman, J. Weil, E.G. de Mejia; Caffeine (1,3,7-trimethylxanthine) in foods: A comprehensive review on consumption, functionality, safety, and regulatory matters. *J. Food Sci.* 2010, 75, R77-R87.
107. H. Eini, V. Frishman, R. Yulzari, L. Kachko, E.C. Lewis, C. Chaimovitz, A. Douvdevani; Caffeine promotes anti-tumor immune response during tumor initiation: Involvement of the adenosine A<sub>2A</sub> receptor. *Biochem. Pharmacol.* 2015, 98, 110-118.
108. S. Kerrigan, T. Lindsey; Fatal caffeine overdose: Two case reports. *Forensic Sci. Int.* 2005, 153, 67-69.
109. T. Ishikawa, I. Yuasa, M. Endoh; Non specific drug distribution in an autopsy case report of fatal caffeine intoxication. *Leg. Med.* 2015, 17, 535-538.
110. E.M. Stefanidou, L. Caramellino, A. Patriarca, G. Menato; Maternal caffeine consumption and sine causa recurrent miscarriage. *Eur. J. Obstet. Gyn. R. B.* 2011, 158, 220-224.
111. S. Cnattingius, L.B. Signorello, G. Anneren, B. Clausson, A. Ekbom, E. Ljunger, W.J. Blot, J.K. McLaughlin, G. Petersson, A. Rane, F. Granath; Caffeine intake and the risk of first-trimester spontaneous abortion. *New Engl. J. Med.* 2000, 343, 1839-1845.
112. K.A. Hahn, L.A. Wise, K.J. Rothman, E.M. Mikkelsen, S.B. Brogly, H.T. Sorensen, A.H. Riis, E.E. Hatch; Caffeine and caffeinated beverage consumption and risk of spontaneous abortion. *Hum. Reprod.* 2015, 30, 1246-1255.
113. V. Sengpiel, E. Elind, J. Bacelis, S. Nilsson, J. Grove, R. Myhre, M. Haugen, H.M. Meltzer, J. Alexander, B. Jacobsson, A.-L. Brantsaeter; Maternal caffeine intake during pregnancy is associated with birth weight but not with gestational length: Results from a large prospective observational cohort study. *BMC Med.* 2013, 11, 42.
114. J.D. Peck, A. Leviton, L.D. Cowan; A review of the epidemiologic evidence concerning the reproductive health effects of caffeine consumption: A 2000-2009 update. *Food Chem. Toxicol.* 2010, 48, 2549-2576.

## Bibliography

115. B. Clausson, F. Granath, A. Ekbohm, S. Lundgren, A. Nordmark, L.B. Signorello, S. Cnattingius; Effect of caffeine exposure during pregnancy on birth weight and gestational age. *Am. J. Epidemiol.* 2002, 155, 429-436.
116. R.L. Brent, M.S. Christian, R.M. Diener; Evaluation of the reproductive and developmental risks of caffeine. *Birth Defects Res. B* 2011, 92, 152-187.
117. EFSA NDA Panel; Scientific opinion on the safety of caffeine. *EFSA Journal* 2015, 13, 4102.
118. A. Bahlmann, T. Lochen, T. Schulze, A. Kirschner, W. Brack, R.J. Schneider, M. Krauss; *Chemical and immunochemical analysis of anthropogenic markers and contaminants*. In *Joint Danube Survey 3: A comprehensive analysis of Danube water quality*. International Commission for the Protection of the Danube River (ICPDR) 2015; pp 277-283.
119. J.J. Carvalho, M.G. Weller, U. Panne, R.J. Schneider; A highly sensitive caffeine immunoassay based on a monoclonal antibody. *Anal. Bioanal. Chem.* 2010, 396, 2617-2628.
120. I.J. Buerge, T. Poiger, M.D. Müller, H.R. Buser; Caffeine, an anthropogenic marker for wastewater contamination of surface waters. *Environ. Sci. Technol.* 2003, 37, 691-700.
121. O. Hillebrand, K. Nödler, T. Licha, M. Sauter, T. Geyer; Caffeine as an indicator for the quantification of untreated wastewater in karst systems. *Water Res.* 2012, 46, 395-402.
122. M.A. Rostagno, N. Manchon, M. D'Arrigo, E. Guillamon, A. Villares, A. Garcia-Lafuente, A. Ramos, J.A. Martinez; Fast and simultaneous determination of phenolic compounds and caffeine in teas, mate, instant coffee, soft drink and energetic drink by high-performance liquid chromatography using a fused-core column. *Anal. Chim. Acta* 2011, 685, 204-211.
123. E. Rudolph, A. Färbinger, J. König; Determination of the caffeine contents of various food items within the Austrian market and validation of a caffeine assessment tool (CAT). *Food Addit. Contam. A* 2012, 29, 1849-1860.
124. C.I. Rodrigues, L. Marta, R. Maia, M. Miranda, M. Ribeirinho, C. Maguas; Application of solid-phase extraction to brewed coffee caffeine and organic acid determination by UV/HPLC. *J. Food Compos. Anal.* 2007, 20, 440-448.
125. E. Marchei, D. De Orsi, C. Guarino, S. Dorato, R. Pacifici, S. Pichini; Measurement of iodide and caffeine content in cellulite reduction cosmetic products sold in the European market. *Anal. Methods* 2013, 5, 376-383.
126. D. Perrone, C.M. Donangelo, A. Farah; Fast simultaneous analysis of caffeine, trigonelline, nicotinic acid and sucrose in coffee by liquid chromatography-mass spectrometry. *Food Chem.* 2008, 110, 1030-1035.
127. M.S. Lee, N.L. Huong, N.H. Hoang, A. Shrestha, J.W. Park; Ultra-high performance liquid chromatography with electrospray ionization tandem mass spectrometry for the determination of caffeine in energy drinks. *Anal. Lett.* 2014, 47, 1852-1861.
128. M.J. Ford, M.A. Deibel, B.A. Tomkins, G.J. Van Berkel; Quantitative thin-layer chromatography/mass spectrometry analysis of caffeine using a surface sampling probe electrospray ionization tandem mass spectrometry system. *Anal. Chem.* 2005, 77, 4385-4389.
129. A.A. Rahim, B. Saad, H. Osman, N. Hashim, S. Yahya, K.M. Talib; Simultaneous determination of diethylene glycol, diethylene glycol monoethyl ether, coumarin and caffeine in food items by gas chromatography. *Food Chem.* 2011, 126, 1412-1416.

130. H. Sereshti, S. Samadi; A rapid and simple determination of caffeine in teas, coffees and eight beverages. *Food Chem.* 2014, 158, 8-13.
131. N. Maeso, C. del Castillo, L. Cornejo, A. Garcia-Acicollar, L.F. Alguacil, C. Barbas; Capillary electrophoresis for caffeine and pyroglutamate determination in coffees - Study of the in vivo effect on learning and locomotor activity in mice. *J. Pharm. Biomed. Anal.* 2006, 41, 1095-1100.
132. L. Svorc; Determination of caffeine: A comprehensive review on electrochemical methods. *Int. J. Electrochem. Sci.* 2013, 8, 5755-5773.
133. J. Hackett, M.J. Telepchak, M.J. Coyer; Analysis of total caffeine and other xanthines in specialty coffees using mixed mode solid-phase extraction and liquid chromatography-diode-array detection after microwave digestion. *J. Anal. Toxicol.* 2008, 32, 695-701.
134. A.G. Caudle, Y.F. Gu, L.N. Bell; Improved analysis of theobromine and caffeine in chocolate food products formulated with cocoa powder. *Food Res. Int.* 2001, 34, 599-603.
135. L. Wang, P.Y. Zhao, F.Z. Zhang, A.J. Bai, C.P. Pan; Detection of caffeine in tea, instant coffee, green tea beverage, and soft drink by direct analysis in real time (DART) source coupled to single-quadrupole mass spectrometry. *J. AOAC Int.* 2013, 96, 353-356.
136. A. Belay, K. Ture, M. Redi, A. Asfaw; Measurement of caffeine in coffee beans with UV/vis spectrometer. *Food Chem.* 2008, 108, 310-315.
137. O. Alharbi, Y. Xu, R. Goodacre; Simultaneous multiplexed quantification of caffeine and its major metabolites theobromine and paraxanthine using surface-enhanced Raman scattering. *Anal. Bioanal. Chem.* 2015, 407, 8253-8261.
138. M.M. Paradkar, J. Irudayaraj; A rapid FTIR spectroscopic method for estimation of caffeine in soft drinks and total methylxanthines in tea and coffee. *J. Food Sci.* 2002, 67, 2507-2511.
139. L.M. Magalhaes, S. Machado, M.A. Segundo, J.A. Lopes, R. Pascoa; Rapid assessment of bioactive phenolics and methylxanthines in spent coffee grounds by FT-NIR spectroscopy. *Talanta* 2016, 147, 460-467.
140. W. Xu, T.-H. Kim, D. Zhai, J.C. Er, L. Zhang, A.A. Kale, B.K. Agrawalla, Y.-K. Cho, Y.-T. Chang; Make caffeine visible: A fluorescent caffeine "traffic light" detector. *Sci. Rep.* 2013, 3, 2255.
141. N. Luisier, A. Ruggi, S.N. Steinmann, L. Favre, N. Gaeng, C. Corminboeuf, K. Severin; A ratiometric fluorescence sensor for caffeine. *Org. Biomol. Chem.* 2012, 10, 7487-7490.
142. V.R.S. Babu, S. Patra, N.G. Karanth, M.A. Kumar, M.S. Thakur; Development of a biosensor for caffeine. *Anal. Chim. Acta* 2007, 582, 329-334.
143. J.J. Carvalho, M.G. Weller, U. Panne, R.J. Schneider; Monitoring caffeine in human saliva using a newly developed ELISA. *Anal. Lett.* 2012, 45, 2549-2561.
144. C.E. Cook, C.R. Tallent, E.W. Amerson, M.W. Myers, J.A. Kepler, G.F. Taylor, H.D. Christensen; Caffeine in plasma and saliva by a radioimmunoassay procedure. *J. Pharmacol. Exp. Ther.* 1976, 199, 679-686.
145. S.A. Fickling, S.M. Hampton, D. Teale, B.A. Middleton, V. Marks; Development of an enzyme-linked immunosorbent assay for caffeine. *J. Immunol. Methods* 1990, 129, 159-164.
146. J.J. Carvalho, R.J. Schneider; *Analysis of Caffeine by Immunoassay*. In *Caffeine: Chemistry, Analysis, Function and Effects*. Royal Society of Chemistry 2012; pp 213-229.

## Bibliography

147. Z. Tolou-Ghamari, M. Zare, J.M. Habibabadi, M.R. Najafi; A quick review of carbamazepine pharmacokinetics in epilepsy from 1953 to 2012. *J. Res. Med. Sci.* 2013, 18, S81-S85.
148. P.W. Wang, T.A. Ketter; Clinical use of carbamazepine for bipolar disorders. *Expert Opin. Pharmacother.* 2005, 6, 2887-2902.
149. Y. Zhang, S.-U. Geissen, C. Gal; Carbamazepine and diclofenac: Removal in wastewater treatment plants and occurrence in water bodies. *Chemosphere* 2008, 73, 1151-1161.
150. U. Schwabe, D. Paffrath; *Arzneiverordnungs-Report 2015*. Springer-Verlag Berlin Heidelberg 2015.
151. U. Schwabe, D. Paffrath; *Arzneiverordnungs-Report 2005*. Springer-Verlag Berlin Heidelberg 2006.
152. A. Bahlmann, W. Brack, R.J. Schneider, M. Krauss; Carbamazepine and its metabolites in wastewater: Analytical pitfalls and occurrence in Germany and Portugal. *Water Res.* 2014, 57, 104-114.
153. X.S. Miao, C.D. Metcalfe; Determination of carbamazepine and its metabolites in aqueous samples using liquid chromatography-electrospray tandem mass spectrometry. *Anal. Chem.* 2003, 75, 3731-3738.
154. S. Zuehlke, U. Duennbier, T. Heberer; Determination of polar drug residues in sewage and surface water applying liquid chromatography-tandem mass spectrometry. *Anal. Chem.* 2004, 76, 6548-6554.
155. J. Rossmann, S. Schubert, R. Gurke, R. Oertel, W. Kirch; Simultaneous determination of most prescribed antibiotics in multiple urban wastewater by SPE-LC-MS/MS. *J. Chromatogr. B* 2014, 969, 162-170.
156. N. Vieno, T. Tuhkanen, L. Kronberg; Elimination of pharmaceuticals in sewage treatment plants in Finland. *Water Res.* 2007, 41, 1001-1012.
157. C. Lacey, S. Basha, A. Morrissey, J.M. Tobin; Occurrence of pharmaceutical compounds in wastewater process streams in Dublin, Ireland. *Environ. Monit. Assess.* 2012, 184, 1049-1062.
158. V.L. Borova, N.C. Maragou, P. Gago-Ferrero, C. Pistos, N.S. Thomaidis; Highly sensitive determination of 68 psychoactive pharmaceuticals, illicit drugs, and related human metabolites in wastewater by liquid chromatography-tandem mass spectrometry. *Anal. Bioanal. Chem.* 2014, 406, 4273-4285.
159. A. Jelic, M. Gros, A. Ginebreda, R. Cespedes-Sanchez, F. Ventura, M. Petrovic, D. Barcelo; Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment. *Water Res.* 2011, 45, 1165-1176.
160. D.P. Mohapatra, S.K. Brar, R.D. Tyagi, P. Picard, R.Y. Surampalli; Carbamazepine in municipal wastewater and wastewater sludge: Ultrafast quantification by laser diode thermal desorption-atmospheric pressure chemical ionization coupled with tandem mass spectrometry. *Talanta* 2012, 99, 247-255.
161. M.H. Wu, J.J. Xiang, C.J. Que, F.F. Chen, G. Xu; Occurrence and fate of psychiatric pharmaceuticals in the urban water system of Shanghai, China. *Chemosphere* 2015, 138, 486-493.
162. T.A. Ternes; Occurrence of drugs in German sewage treatment plants and rivers. *Water Res.* 1998, 32, 3245-3260.

163. A. Joss, E. Keller, A.C. Alder, A. Göbel, C.S. McArdell, T. Ternes, H. Siegrist; Removal of pharmaceuticals and fragrances in biological wastewater treatment. *Water Res.* 2005, 39, 3139-3152.
164. R.L. Oulton, T. Kohn, D.M. Cwiertyny; Pharmaceuticals and personal care products in effluent matrices: A survey of transformation and removal during wastewater treatment and implications for wastewater management. *J. Environ. Monitor.* 2010, 12, 1956-1978.
165. B. Ferrari, N. Paxeus, R. Lo Giudice, A. Pollio, J. Garric; Ecotoxicological impact of pharmaceuticals found in treated wastewaters: Study of carbamazepine, clofibrac acid, and diclofenac. *Ecotox. Environ. Safe.* 2003, 55, 359–370.
166. G. Gasser, M. Rona, A. Voloshenko, R. Shelkov, O. Lev, S. Elhanany, F.T. Lange, M. Scheurer, I. Pankratov; Evaluation of micropollutant tracers. II. Carbamazepine tracer for wastewater contamination from a nearby water recharge system and from non-specific sources. *Desalination* 2011, 273, 398-404.
167. E.R.V. Dickenson, S.A. Snyder, D.L. Sedlak, J.E. Drewes; Indicator compounds for assessment of wastewater effluent contributions to flow and water quality. *Water Res.* 2011, 45, 1199-1212.
168. J.H. Writer, I. Ferrer, L.B. Barber, E.M. Thurman; Widespread occurrence of neuro-active pharmaceuticals and metabolites in 24 Minnesota rivers and wastewaters. *Sci. Total Environ.* 2013, 461, 519-527.
169. A. Nieto, F. Borrull, E. Pocurull, R.M. Marce; Occurrence of pharmaceuticals and hormones in sewage sludge. *Environ. Toxicol. Chem.* 2010, 29, 1484-1489.
170. M. Clara, B. Strenn, N. Kreuzinger; Carbamazepine as a possible anthropogenic marker in the aquatic environment: Investigations on the behaviour of carbamazepine in wastewater treatment and during groundwater infiltration. *Water Res.* 2004, 38, 947-954.
171. Y.L. Luo, W.S. Guo, H.H. Ngo, L.D. Nghiem, F.I. Hai, J. Zhang, S. Liang, X.C.C. Wang; A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *Sci. Total Environ.* 2014, 473, 619-641.
172. X. Yang, R.C. Flowers, H.S. Weinberg, P.C. Singer; Occurrence and removal of pharmaceuticals and personal care products (PPCPs) in an advanced wastewater reclamation plant. *Water Res.* 2011, 45, 5218-5228.
173. S.A. Snyder, S. Adham, A.M. Redding, F.S. Cannon, J. DeCarolis, J. Oppenheimer, E.C. Wert, Y. Yoon; Role of membranes and activated carbon in the removal of endocrine disruptors and pharmaceuticals. *Desalination* 2007, 202, 156-181.
174. S.D. Kim, J. Cho, I.S. Kim, B.J. Vanderford, S.A. Snyder; Occurrence and removal of pharmaceuticals and endocrine disruptors in South Korean surface, drinking, and waste waters. *Water Res.* 2007, 41, 1013-1021.
175. W. Pronk, M. Biebow, M. Boller; Electrodialysis for recovering salts from a urine solution containing micropollutants. *Environ. Sci. Technol.* 2006, 40, 2414-2420.
176. E. Donner, T. Kosjek, S. Qualmann, K.O. Kusk, E. Heath, D.M. Revitt, A. Ledin, H.R. Andersen; Ecotoxicity of carbamazepine and its UV photolysis transformation products. *Sci. Total Environ.* 2013, 443, 870-876.
177. M.M. Dong, R. Trenholm, F.L. Rosario-Ortiz; Photochemical degradation of atenolol, carbamazepine, meprobamate, phenytoin and primidone in wastewater effluents. *J. Hazard. Mater.* 2015, 282, 216-223.

## Bibliography

178. U. Hübner, B. Seiwert, T. Reemtsma, M. Jekel; Ozonation products of carbamazepine and their removal from secondary effluents by soil aquifer treatment - Indications from column experiments. *Water Res.* 2014, 49, 34-43.
179. J. Hollender, S.G. Zimmermann, S. Koepke, M. Krauss, C.S. McArdell, C. Ort, H. Singer, U. von Gunten, H. Siegrist; Elimination of organic micropollutants in a municipal wastewater treatment plant upgraded with a full-scale post-ozonation followed by sand filtration. *Environ. Sci. Technol.* 2009, 43, 7862-7869.
180. R. Banaschik, P. Lukes, H. Jablonowski, M.U. Hammer, K.D. Weltmann, J.F. Kolb; Potential of pulsed corona discharges generated in water for the degradation of persistent pharmaceutical residues. *Water Res.* 2015, 84, 127-135.
181. P. Bräutigam, M. Franke, R.J. Schneider, A. Lehmann, A. Stolle, B. Ondruschka; Degradation of carbamazepine in environmentally relevant concentrations in water by hydrodynamic-acoustic-cavitation (HAC). *Water Res.* 2012, 46, 2469-2477.
182. C. Nadejde, M. Neamtu, V.D. Hodoroaba, R.J. Schneider, A. Paul, G. Ababei, U. Panne; Green Fenton-like magnetic nanocatalysts: Synthesis, characterization and catalytic application. *Appl. Catal. B-Environ.* 2015, 176, 667-677.
183. R.I.L. Eggen, J. Hollender, A. Joss, M. Schärer, C. Stamm; Reducing the discharge of micropollutants in the aquatic environment: The benefits of upgrading wastewater treatment plants. *Environ. Sci. Technol.* 2014, 48, 7683-7689.
184. M. Williams, A. Kumar, C. Ort, M.G. Lawrence, A. Hambly, S.J. Khan, R. Kookana; The use of multiple tracers for tracking wastewater discharges in freshwater systems. *Environ. Monit. Assess.* 2013, 185, 9321-9332.
185. K. Ruzicka, M. Zessner, A.P. Blaschke, R. Fenz, M. Clara, H. Kroiss; Evaluating the success of sewer reconstruction by using carbamazepine as anthropogenic marker in groundwater. *Water Sci. Technol.* 2011, 63, 727-732.
186. S. Öllers, H.P. Singer, P. Fässler, S.R. Müller; Simultaneous quantification of neutral and acidic pharmaceuticals and pesticides at the low-ng/l level in surface and waste water. *J. Chromatogr. A* 2001, 911, 225-234.
187. E. Vulliet, C. Cren-Olive; Screening of pharmaceuticals and hormones at the regional scale, in surface and groundwaters intended to human consumption. *Environ. Pollut.* 2011, 159, 2929-2934.
188. P. Calza, C. Medana, E. Padovano, V. Giancotti, C. Minero; Fate of selected pharmaceuticals in river waters. *Environ. Sci. Pollut. R.* 2013, 20, 2262-2270.
189. T.V. Madureira, J.C. Barreiro, M.J. Rocha, E. Rocha, Q.B. Cass, M.E. Tiritan; Spatiotemporal distribution of pharmaceuticals in the Douro river estuary (Portugal). *Sci. Total Environ.* 2010, 408, 5513-5520.
190. T. Radovic, S. Grujic, A. Petkovic, M. Dimkic, M. Lausevic; Determination of pharmaceuticals and pesticides in river sediments and corresponding surface and ground water in the Danube river and tributaries in Serbia. *Environ. Monit. Assess.* 2014, 187, 4092.
191. J.M. Conley, S.J. Symes, S.A. Kindelberger, S.A. Richards; Rapid liquid chromatography-tandem mass spectrometry method for the determination of a broad mixture of pharmaceuticals in surface water. *J. Chromatogr. A* 2008, 1185, 206-215.
192. S. Weigel, K. Bester, H. Hühnerfuss; New method for rapid solid-phase extraction of large-volume water samples and its application to non-target screening of North Sea water

for organic contaminants by gas chromatography-mass spectrometry. *J. Chromatogr. A* 2001, 912, 151-161.

193. K. Wille, M. Claessens, K. Rappe, E. Monteyne, C.R. Janssen, H.F. De Brabander, L. Vanhaecke; Rapid quantification of pharmaceuticals and pesticides in passive samplers using ultra high performance liquid chromatography coupled to high resolution mass spectrometry. *J. Chromatogr. A* 2011, 1218, 9162-9173.

194. K.E. Murray, S.M. Thomas, A.A. Bodour; Prioritizing research for trace pollutants and emerging contaminants in the freshwater environment. *Environ. Pollut.* 2010, 158, 3462-3471.

195. A. Kruglova, P. Ahlgren, N. Korhonen, P. Rantanen, A. Mikola, R. Vahala; Biodegradation of ibuprofen, diclofenac and carbamazepine in nitrifying activated sludge under 12 °C temperature conditions. *Sci. Total Environ.* 2014, 499, 394-401.

196. V. Calisto, M.R.M. Domingues, G.L. Erny, V.I. Esteves; Direct photodegradation of carbamazepine followed by micellar electrokinetic chromatography and mass spectrometry. *Water Res.* 2011, 45, 1095-1104.

197. A. Almeida, R. Freitas, V. Calisto, V.I. Esteves, R.J. Schneider, A.M.V.M. Soares, E. Figueira; Chronic toxicity of the antiepileptic carbamazepine on the clam *Ruditapes philippinarum*. *Comp. Biochem. Physiol. C* 2015, 172, 26-35.

198. R. Freitas, A. Almeida, A. Pires, C. Velez, V. Calisto, R.J. Schneider, V.I. Esteves, F.J. Wrona, E. Figueira, A. Soares; The effects of carbamazepine on macroinvertebrate species: Comparing bivalves and polychaetes biochemical responses. *Water Res.* 2015, 85, 137-147.

199. R. Freitas, A. Almeida, V. Calisto, C. Velez, A. Moreira, R.J. Schneider, V.I. Esteves, F.J. Wrona, A.M.V.M. Soares, E. Figueira; How life history influences the responses of the clam *Scrobicularia plana* to the combined impacts of carbamazepine and pH decrease. *Environ. Pollut.* 2015, 202, 205-214.

200. R. Freitas, A. Almeida, V. Calisto, C. Velez, A. Moreira, R.J. Schneider, V.I. Esteves, F.J. Wrona, E. Figueira, A. Soares; The impacts of pharmaceutical drugs under ocean acidification: New data on single and combined long-term effects of carbamazepine on *Scrobicularia plana*. *Sci. Total Environ.* 2016, 541, 977-985.

201. P. Tsiaka, V. Tsarpali, I. Ntaikou, M.N. Kostopoulou, G. Lyberatos, S. Dailianis; Carbamazepine-mediated pro-oxidant effects on the unicellular marine algal species *Dunaliella tertiolecta* and the hemocytes of mussel *Mytilus galloprovincialis*. *Ecotoxicology* 2013, 22, 1208-1220.

202. A. Pires, A. Almeida, J. Correia, V. Calisto, R.J. Schneider, V.I. Esteves, A.M.V.M. Soares, E. Figueira, R. Freitas; Long-term exposure to caffeine and carbamazepine: Impacts on the regenerative capacity of the polychaete *Diopatra neapolitana*. *Chemosphere* 2016, 146, 565-573.

203. M. Oetken, G. Nentwig, D. Löffler, T. Ternes, J. Oehlmann; Effects of pharmaceuticals on aquatic invertebrates. Part I. The antiepileptic drug carbamazepine. *Arch. Environ. Con. Tox.* 2005, 49, 353-361.

204. R. Triebkorn, H. Casper, V. Scheil, J. Schwaiger; Ultrastructural effects of pharmaceuticals (carbamazepine, clofibrac acid, metoprolol, diclofenac) in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). *Anal. Bioanal. Chem.* 2007, 387, 1405-1416.

205. A.J. Ramirez, R.A. Brain, S. Usenko, M.A. Mottaleb, J.G. O'Donnell, L.L. Stahl, J.B. Wathen, B.D. Snyder, J.L. Pitt, P. Perez-Hurtado, L.L. Dobbins, B.W. Brooks, C.K. Chambliss; Occurrence of pharmaceuticals and personal care products in fish: Results of a national pilot study in the United States. *Environ. Toxicol. Chem.* 2009, 28, 2587-2597.
206. M. Shenker, D. Harush, J. Ben-Ari, B. Chefetz; Uptake of carbamazepine by cucumber plants - A case study related to irrigation with reclaimed wastewater. *Chemosphere* 2011, 82, 905-910.
207. X. Wu, J.L. Conkle, F. Ernst, J. Gan; Treated wastewater irrigation: Uptake of pharmaceutical and personal care products by common vegetables under field conditions. *Environ. Sci. Technol.* 2014, 48, 11286-11293.
208. L.J. Carter, M. Williams, C. Böttcher, R.S. Kookana; Uptake of pharmaceuticals influences plant development and affects nutrient and hormone homeostases. *Environ. Sci. Technol.* 2015, 49, 12509-12518.
209. M. Winker, J. Clemens, M. Reich, H. Gulyas, R. Otterpohl; Ryegrass uptake of carbamazepine and ibuprofen applied by urine fertilization. *Sci. Total Environ.* 2010, 408, 1902-1908.
210. A. Jurado, R. Lopez-Serna, E. Vazquez-Sune, J. Carrera, E. Pujades, M. Petrovic, D. Barcelo; Occurrence of carbamazepine and five metabolites in an urban aquifer. *Chemosphere* 2014, 115, 47-53.
211. R. Lopez-Serna, A. Jurado, E. Vazquez-Sune, J. Carrera, M. Petrovic, D. Barcelo; Occurrence of 95 pharmaceuticals and transformation products in urban groundwaters underlying the metropolis of Barcelona, Spain. *Environ. Pollut.* 2013, 174, 305-315.
212. K. Osenbrück, H.-R. Gläser, K. Knöller, S.M. Weise, M. Möder, R. Wennrich, M. Schirmer, F. Reinstorf, W. Busch, G. Strauch; Sources and transport of selected organic micropollutants in urban groundwater underlying the city of Halle (Saale), Germany. *Water Res.* 2007, 41, 3259-3270.
213. K. Nödler, O. Hillebrand, K. Idzik, M. Strathmann, F. Schiperski, J. Zirlwagen, T. Licha; Occurrence and fate of the angiotensin II receptor antagonist transformation product valsartan acid in the water cycle - A comparative study with selected beta-blockers and the persistent anthropogenic wastewater indicators carbamazepine and acesulfame. *Water Res.* 2013, 47, 6650-6659.
214. H.W. Leung, L. Jin, S. Wei, M.M.P. Tsui, B.S. Zhou, L.P. Jiao, P.C. Cheung, Y.K. Chun, M.B. Murphy, P.K.S. Lam; Pharmaceuticals in tap water: Human health risk assessment and proposed monitoring framework in China. *Environ. Health Persp.* 2013, 121, 839-846.
215. M.Q. Cai, R. Wang, L. Feng, L.Q. Zhang; Determination of selected pharmaceuticals in tap water and drinking water treatment plant by high-performance liquid chromatography-triple quadrupole mass spectrometer in Beijing, China. *Environ. Sci. Pollut. R.* 2015, 22, 1854-1867.
216. P. Houeto, A. Carton, M. Guerbet, A.C. Mauclair, C. Gatignol, P. Lechat, D. Masset; Assessment of the health risks related to the presence of drug residues in water for human consumption: Application to carbamazepine. *Regul. Toxicol. Pharmacol.* 2012, 62, 41-48.
217. V.L. Cunningham, C. Perino, V.J. D'Aco, A. Hartmann, R. Bechter; Human health risk assessment of carbamazepine in surface waters of North America and Europe. *Regul. Toxicol. Pharmacol.* 2010, 56, 343-351.

218. A. Kumar, I. Xagorarakis; Human health risk assessment of pharmaceuticals in water: An uncertainty analysis for meprobamate, carbamazepine, and phenytoin. *Regul. Toxicol. Pharmacol.* 2010, 57, 146-156.
219. Z.R. Yu, S. Peldszus, P.M. Huck; Optimizing gas chromatographic-mass spectrometric analysis of selected pharmaceuticals and endocrine-disrupting substances in water using factorial experimental design. *J. Chromatogr. A* 2007, 1148, 65-77.
220. N. Al-Hadithi, B. Saad, M. Grote; A solid bar microextraction method for the liquid chromatographic determination of trace diclofenac, ibuprofen and carbamazepine in river water. *Microchim. Acta* 2011, 172, 31-37.
221. D. Camacho-Munoz, J. Martin, J.L. Santos, I. Aparicio, E. Alonso; An affordable method for the simultaneous determination of the most studied pharmaceutical compounds as wastewater and surface water pollutants. *J. Sep. Sci.* 2009, 32, 3064-3073.
222. R.M. De Carlo, L. Rivoira, L. Ciofi, C. Ancillotti, L. Checchini, M. Del Bubba, M.C. Bruzzoniti; Evaluation of different QuEChERS procedures for the recovery of selected drugs and herbicides from soil using LC coupled with UV and pulsed amperometry for their detection. *Anal. Bioanal. Chem.* 2015, 407, 1217-1229.
223. C. Gonzalez-Barreiro, M. Lores, M.C. Casais, R. Cela; Simultaneous determination of neutral and acidic pharmaceuticals in wastewater by high-performance liquid chromatography-post-column photochemically induced fluorimetry. *J. Chromatogr. A* 2003, 993, 29-37.
224. R. Bade, N.I. Rousis, L. Bijlsma, E. Gracia-Lor, S. Castiglioni, J.V. Sancho, F. Hernandez; Screening of pharmaceuticals and illicit drugs in wastewater and surface waters of Spain and Italy by high resolution mass spectrometry using UHPLC-QTOF MS and LC-LTQ-Orbitrap MS. *Anal. Bioanal. Chem.* 2015, 407, 8979-8988.
225. F. Wode, C. Reilich, P. van Baar, U. Dünnebier, M. Jekel, T. Reemtsma; Multiresidue analytical method for the simultaneous determination of 72 micropollutants in aqueous samples with ultra high performance liquid chromatography-high resolution mass spectrometry. *J. Chromatogr. A* 2012, 1270, 118-126.
226. D. Hummel, D. Löffler, G. Fink, T.A. Ternes; Simultaneous determination of psychoactive drugs and their metabolites in aqueous matrices by liquid chromatography mass spectrometry. *Environ. Sci. Technol.* 2006, 40, 7321-7328.
227. S.E. Gibbons, C. Wang, Y.F. Ma; Determination of pharmaceutical and personal care products in wastewater by capillary electrophoresis with UV detection. *Talanta* 2011, 84, 1163-1168.
228. V.A. Lozano, G.M. Escandar; Second-order advantage with excitation-emission photoinduced fluorimetry for the determination of the antiepileptic carbamazepine in environmental waters. *Anal. Chim. Acta* 2013, 782, 37-45.
229. A. Beltran, E. Caro, R.M. Marce, P.A.G. Cormack, D.C. Sherrington, F. Borrull; Synthesis and application of a carbamazepine-imprinted polymer for solid-phase extraction from urine and wastewater. *Anal. Chim. Acta* 2007, 597, 6-11.
230. V. Calisto, A. Bahlmann, R.J. Schneider, V.I. Esteves; Application of an ELISA to the quantification of carbamazepine in ground, surface and wastewaters and validation with LC-MS/MS. *Chemosphere* 2011, 84, 1708-1715.
231. C. Wade, E. Otero, B. Poon-Kwong, R. Rozier, D. Bachoon; Detection of human-derived fecal contamination in Puerto Rico using carbamazepine, HF183 Bacteroides, and fecal indicator bacteria. *Mar. Pollut. Bull.* 2015, 101, 872-877.

## Bibliography

232. A. Almeida, V. Calisto, V.I. Esteves, R.J. Schneider, A.M.V.M. Soares, E. Figueira, R. Freitas; Presence of the pharmaceutical drug carbamazepine in coastal systems: Effects on bivalves. *Aquat. Toxicol.* 2014, 156, 74-87.
233. G.A. McMillin, J.M. Juenke, M.J. Johnson, A. Dasgupta; Discordant carbamazepine values between two immunoassays: Carbamazepine values determined by ADVIA Centaur correlate better with those determined by LC-MS/MS than PETINIA assay. *J. Clin. Lab. Anal.* 2011, 25, 212-216.
234. A. Dasgupta, G. Tso, M. Johnson, L. Chow; Hydroxyzine and cetirizine interfere with the PENTINA carbamazepine assay but not with the ADVIA CENTEUR carbamazepine assay. *Ther. Drug Monit.* 2010, 32, 112-115.
235. A. Bahlmann, J. Falkenhagen, M.G. Weller, U. Panne, R.J. Schneider; Cetirizine as pH-dependent cross-reactant in a carbamazepine-specific immunoassay. *Analyst* 2011, 136, 1357-1364.
236. H.M. Neels, A.C. Sierens, K. Naelaerts, S.L. Scharpe, G.M. Hatfield, W.E. Lambert; Therapeutic drug monitoring of old and newer anti-epileptic drugs. *Clin. Chem. Lab. Med.* 2004, 42, 1228-1255.
237. L.S.W. Steijns, J. Bouw, J. van der Weide; Evaluation of fluorescence polarization assays for measuring valproic acid, phenytoin, carbamazepine and phenobarbital in serum. *Ther. Drug Monit.* 2002, 24, 432-435.
238. M.L. Pan, W.Y. Lin, H.Y. Wang, S.C. Tsai, P.F. Hsieh, Y.L.O. Su, P.W. Huang; Determination of carbamazepine: A comparison of the differential pulse voltammetry (DPV) method and the immunoassay method in a clinical trial. *J. Anal. Chem.* 2014, 69, 57-61.
239. H.Y. Wang, M.L. Pan, Y.L.O. Su, S.C. Tsai, C.H. Kao, S.S. Sun, W.Y. Lin; Comparison of differential pulse voltammetry (DPV) - a new method of carbamazepine analysis - with fluorescence polarization immunoassay (FPIA). *J. Anal. Chem.* 2011, 66, 415-420.
240. S. Casal, M. Oliveira, M.R. Alves, M.A. Ferreira; Discriminate analysis of roasted coffee varieties for trigonelline, nicotinic acid, and caffeine content. *J. Agric. Food Chem.* 2000, 48, 3420-3424.
241. J.J. Barone, H.R. Roberts; Caffeine consumption. *Food Chem. Toxicol.* 1996, 34, 119-129.
242. R.R. McCusker, B.A. Goldberger, E.J. Cone; Caffeine content of specialty coffees. *J. Anal. Toxicol.* 2003, 27, 520-522.
243. C. Mesaros, M. Culea, A.M. Iordache, I. Visovan, C. Cozar, C. Cosma; A new caffeine test for diagnosis of cirrhosis by GC/MS. *Asian J. Chem.* 2010, 22, 3608-3614.
244. P.R. Gardinali, X. Zhao; Trace determination of caffeine in surface water samples by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS). *Environ. Int.* 2002, 28, 521-528.
245. M.C. Gutierrez, A. Gomez-Hens, D. Perez-Bendito; Immunoassay methods based on fluorescence polarization. *Talanta* 1989, 36, 1187-1201.
246. S.A. Eremin, I.A. Ryabova, J.N. Yakovleva, E.V. Yazynina, A.V. Zherdev, B.B. Dzantiev; Development of a rapid, specific fluorescence polarization immunoassay for the herbicide chlorsulfuron. *Anal. Chim. Acta* 2002, 468, 229-236.
247. J. Grandke, U. Resch-Genger, W. Bremser, L.-A. Garbe, R.J. Schneider; Quality assurance in immunoassay performance – Temperature effects. *Anal. Methods* 2012, 4, 901-905.

248. L.N. Bell, C.R. Wetzel, A.N. Grand; Caffeine content in coffee as influenced by grinding and brewing techniques. *Food Res. Int.* 1996, 29, 785-789.
249. A. Küster, N. Adler; Pharmaceuticals in the environment: Scientific evidence of risks and its regulation. *Philos. T. Roy. Soc. B* 2014, 369, 20130587.
250. K.E. Arnold, A.R. Brown, G.T. Ankley, J.P. Sumpter; Medicating the environment: Assessing risks of pharmaceuticals to wildlife and ecosystems. *Philos. T. Roy. Soc. B* 2014, 369, 20130569.
251. S.D. Richardson, T.A. Ternes; Water analysis: Emerging contaminants and current issues. *Anal. Chem.* 2014, 86, 2813-2848.
252. A. Pal, Y.L. He, M. Jekel, M. Reinhard, K.Y.H. Gin; Emerging contaminants of public health significance as water quality indicator compounds in the urban water cycle. *Environ. Int.* 2014, 71, 46-62.
253. M. Kahle, I.J. Buerge, M.D. Müller, T. Poiger; Hydrophilic anthropogenic markers for quantification of wastewater contamination in ground- and surface waters. *Environ. Toxicol. Chem.* 2009, 28, 2528-2536.
254. Z. Wang, S. Zhang, I.S. Nesterenko, S.A. Eremin, J. Shen; Monoclonal antibody-based fluorescence polarization immunoassay for sulfamethoxypyridazine and sulfachloropyridazine. *J. Agric. Food Chem.* 2007, 55, 6871-6878.
255. X.J. Wu, J. Zhang, J.C. Yu, G.Y. Cao, Y.G. Shi, Y.Y. Zhang, M.G. Wang; Establishment of norvancomycin fluorescence polarization immunoassay for therapeutic drug monitoring. *J. Antibiot.* 2012, 65, 35-39.
256. L. Oberleitner, J. Grandke, F. Mallwitz, U. Resch-Genger, L.-A. Garbe, R.J. Schneider; Fluorescence polarization immunoassays for the quantification of caffeine in beverages. *J. Agric. Food Chem.* 2014, 62, 2337-2343.
257. W.Y. Lin, M.L. Pan, H.Y. Wang, Y.O. Su, P.W. Huang; Analysis of carbamazepine serum by differential pulse voltammetry (DPV) and comparison with fluorescence polarization immunoassay (FPIA): An animal study. *Med. Chem. Res.* 2012, 21, 4389-4394.
258. M. Pourfarzaneh, G.W. White, J. Landon, D.S. Smith; Cortisol directly determined in serum by fluoroimmunoassay with magnetizable solid-phase. *Clin. Chem.* 1980, 26, 730-733.
259. L.D. Lavis, T.J. Rutkoski, R.T. Raines; Tuning the pK<sub>a</sub> of fluorescein to optimize binding assays. *Anal. Chem.* 2007, 79, 6775-6782.
260. S.K. Bedada, P. Nearati; Effect of resveratrol on the pharmacokinetics of carbamazepine in healthy human volunteers. *Phytother. Res.* 2015, 29, 701-706.
261. T.A. Ternes, J. Stüber, N. Herrmann, D. McDowell, A. Ried, M. Kampmann, B. Teiser; Ozonation: A tool for removal of pharmaceuticals, contrast media and musk fragrances from wastewater? *Water Res.* 2003, 37, 1976-1982.
262. L. Oberleitner, S.A. Eremin, A. Lehmann, L.-A. Garbe, R.J. Schneider; Fluorescence polarization immunoassays for carbamazepine - Comparison of tracers and formats. *Anal. Methods* 2015, 7, 5854-5861.
263. M.M. Bradford; A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976, 72, 248-254.
264. A. Frey, B. Meckelein, D. Externest, M.A. Schmidt; A stable and highly sensitive 3,3',5,5'-tetramethylbenzidine-based substrate reagent for enzyme-linked immunosorbent assays. *J. Immunol. Methods* 2000, 233, 47-56.

## Bibliography

265. U. Eisold, F. Sellrie, J.A. Schenk, C. Lenz, W.F.M. Stöcklein, M.U. Kumke; Bright or dark immune complexes of anti-TAMRA antibodies for adapted fluorescence-based bioanalysis. *Anal. Bioanal. Chem.* 2015, 407, 3313-3323.
266. C. Schneider, H.F. Schöler, R.J. Schneider; Direct sub-ppt detection of the endocrine disruptor ethinylestradiol in water with a chemiluminescence enzyme-linked immunosorbent assay. *Anal. Chim. Acta* 2005, 551, 92-97.
267. M.G. Weller, L. Weil, R. Niessner; Increased sensitivity of an enzyme immunoassay (ELISA) for the determination of triazine herbicides by variation of tracer incubation time. *Mikrochim. Acta* 1992, 108, 29-40.
268. A.Y. Kolosova, J.-H. Park, S.A. Eremin, S.-J. Park, S.-J. Kang, W.-B. Shim, H.-S. Lee, Y.-T. Lee, D.-H. Chung; Comparative study of three immunoassays based on monoclonal antibodies for detection of the pesticide parathion-methyl in real samples. *Anal. Chim. Acta* 2004, 511, 323-331.
269. V. Osorio, A. Larranaga, J. Acena, S. Perez, D. Barcelo; Concentration and risk of pharmaceuticals in freshwater systems are related to the population density and the livestock units in Iberian rivers. *Sci. Total Environ.* 2016, 540, 267-277.
270. E.L. Miller, S.L. Nason, K.G. Karthikeyan, J.A. Pedersen; Root uptake of pharmaceuticals and personal care product ingredients. *Environ. Sci. Technol.* 2016, 50, 525-541.
271. R. Andreozzi, R. Marotta, G. Pinto, A. Pollio; Carbamazepine in water: Persistence in the environment, ozonation treatment and preliminary assessment on algal toxicity. *Water Res.* 2002, 36, 2869-2877.
272. R. Rosal, A. Rodriguez, M.S. Gonzalo, E. Garcia-Calvo; Catalytic ozonation of naproxen and carbamazepine on titanium dioxide. *Appl. Catal. B-Environ.* 2008, 84, 48-57.
273. L. Fernandez, V.I. Esteves, A. Cunha, R.J. Schneider, J.P.C. Tome; Photodegradation of organic pollutants in water by immobilized porphyrins and phthalocyanines. *J. Porphyr. Phthalocya.* 2016, 20, 150-166.
274. C. Maragos; Fluorescence polarization immunoassay of mycotoxins: A review. *Toxins* 2009, 1, 196-207.
275. L. Oberleitner, U. Dahmen-Levison, L.-A. Garbe, R.J. Schneider; Improved strategies for selection and characterization of new monoclonal anti-carbamazepine antibodies during the screening process using feces and fluorescence polarization immunoassay. *Anal. Methods* 2016, 8, 6883-6894.
276. J.J. MacKichan, E.M. Zola; Determinants of carbamazepine and carbamazepine 10,11-epoxide binding to serum-protein, albumin and alpha-1-acid glycoprotein. *Brit. J. Clin. Pharmacol.* 1984, 18, 487-493.
277. M. Nichkova, R. Galve, M.-P. Marco; Biological monitoring of 2,4,5-trichlorophenol (I): Preparation of antibodies and development of an immunoassay using theoretical models. *Chem. Res. Toxicol.* 2002, 15, 1360-1370.
278. R.F. Vining, P. Compton, R. McGinley; Steroid radioimmunoassay - Effect of shortened incubation-time on specificity. *Clin. Chem.* 1981, 27, 910-913.
279. J.J. Pratt, M.G. Woldring; Radioimmunoassay specificity and the first-come, first-served effect. *Clin. Chim. Acta* 1976, 68, 87-90.
280. L. Yannakou, E.P. Diamandis, A. Souvatzoglou; Effect of incubation-time and temperature on the interference of digoxin-like immunoreactive substances in digoxin immunoassays. *Ther. Drug Monit.* 1987, 9, 461-463.

281. T. Manickum, W. John; The current preference for the immuno-analytical ELISA method for quantitation of steroid hormones (endocrine disruptor compounds) in wastewater in South Africa. *Anal. Bioanal. Chem.* 2015, 407, 4949-4970.

## Publications

### Paper (peer-reviewed)

L. Oberleitner, U. Dahmen-Levison, L.-A. Garbe, R.J. Schneider; Application of fluorescence polarization immunoassay for determination of carbamazepine in wastewater, *final manuscript*

L. Oberleitner, U. Dahmen-Levison, L.-A. Garbe, R.J. Schneider; Improved strategies for selection and characterization of new monoclonal anti-carbamazepine antibodies during the screening process using feces and fluorescence polarization immunoassay. *Anal. Methods* 2016, 8, 6883-6894.

L. Oberleitner, S.A. Eremin, A. Lehmann, L.-A. Garbe, R.J. Schneider; Fluorescence polarization immunoassays for carbamazepine – Comparison of tracers and formats. *Anal. Methods* 2015, 7, 5854-5861.

L. Oberleitner, J. Grandke, F. Mallwitz, U. Resch-Genger, L.-A. Garbe, R.J. Schneider; Fluorescence polarization immunoassays for the quantification of caffeine in beverages. *J. Agric. Food Chem.* 2014, 62, 2337-2343.

### Poster

L. Oberleitner, L.-A. Garbe, R.J. Schneider; *Fluorescence polarization immunoassay - Fast screening method for antibody characterization*; Tag der Biotechnologie 2015, Berlin, Germany.

L. Oberleitner, J. Grandke, R.J. Schneider; *Fluorescence polarization immunoassay – Fast alternative to ELISA*; Schnell, schneller, Optik – wie optische Technologien die Lebensmittel- und Umweltanalytik optimieren 2014, Berlin, Germany.

A. Lehmann, L. Oberleitner, S.A. Eremin, R.J. Schneider; *Synthesis and verification of new fluorescence polarization immunoassay tracers for carbamazepine by LC-MS*; International Symposium on Chromatography 2014, Salzburg, Austria.

L. Oberleitner, F. Mallwitz, L.-A. Garbe, R.J. Schneider; *New monoclonal anti-carbamazepine antibody for application in fluorescence polarization immunoassays*; Analytica Conference 2014, Munich, Germany.

L. Oberleitner, J. Grandke, F. Mallwitz, L.-A. Garbe, R.J. Schneider; *Comparison of heterogeneous and homogeneous immunoassays*; Trends in Diagnostics 2013; Tübingen, Germany.

L. Oberleitner, J. Grandke, F. Mallwitz, L.-A. Garbe, R.J. Schneider; *Fluorescence polarization immunoassays for caffeine*; Euroanalysis 2013, Warsaw, Poland.

L. Oberleitner, A. Lehmann, S.A. Eremin, L.-A. Garbe, R.J. Schneider; *Fluorescence polarization immunoassays for carbamazepine*; Pharmaceutical and Biomedical Analysis 2013, Bologna, Italy.

L. Oberleitner, J. Grandke, U. Resch-Genger, L.-A. Garbe, R.J. Schneider; *Application and evaluation of carbamazepine immunoassays*; ANAKON 2013, Essen, Germany.

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