Technische Universität München

Development of a residue analysis microarray chip for milk

Entwicklung eines Rückstandsanalyse-Mikroarray-Chip für Milch

Wissenschaftliche Arbeit zur Erlangung des Grades

Master of Science

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Abstract

This thesis outlines the development of a residue analysis microarray chip for milk by the example of diclofenac (DF) and sulfamethazine (SMA). Diclofenac is a non-steroidal anti-inflammatory drug with a carboxylic group as a functional group. The MRL for diclofenac in milk and meat is 100 ng/kg. Sulfamethazine is an antibiotic and belongs to the group of the sulfonamides. It has an amino group as a functional group. The MRL for sulfamethazine in milk and meat is 100 µg/kg. The aim of this thesis was to immobilize both molecules with their different functional groups on the same surface. As analysis platform, the automated microarray-chip reader MCR 3 with a flow cell for the simultaneous detection of several analytes via indirect-competitive chemiluminescence microarray immunoassay was used.

Two different immobilization strategies were combined on one microarray chip. Diclofenac was covalently bound to the Jeffamine® surface of the microarray chip by modification of the functional carboxyl group with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysulfosuccinimide (S-NHS). A novel, two-step immobilization process was developed for sulfamethazine, in which first a layer of Poly(ethylene glycol) diglycidyl ether (DE-PEG) as crosslinker and second the sulfamethazine solution was applied. In the meantime, diclofenac was investigated in more detail, therefore singleplex calibrations in water were performed, which resulted in a limit of detection (LoD) of 83.0 ± 11.2 ng/L.

After successful development of the immobilization strategies, the selectivity of the primary antibodies were examined. Affinities of nearly 100% could be determined for the antibodies towards their corresponding analytes. In order to characterize the microarray chip system, duplex calibrations were performed, and recovery rates determined. While duplex calibrations were carried out, the regenerability of the microarray chips could be assessed simultaneously. After 35 regeneration cycles, relative signal reductions of 21% for diclofenac and 9% for sulfamethazine were obtained.

The duplex calibrations were performed in ultra-heat treatment (UHT) milk with a fat content of 1.5% and resulted in LoD values of 0.264 µg/L for diclofenac and 8.0 µg/L for sulfamethazine. The latter was below the corresponding maximum residue limit (MRL) of 100 µg/L, but the LoD of diclofenac was about 2.5 times the corresponding MRL of 100 ng/L. In order to improve the LoD for diclofenac, experiments with longer

antibody-analyte interaction time and longer competition time on the microarray chip were performed. An improvement of the LoD could not be observed.

Finally, experiments were performed to determine the recovery-rates in UHT and fresh milk. With UHT milk as matrix, average recovery-rates of 99% ± 7 for diclofenac and 124% ± 12 for sulfamethazine were found. In fresh milk, the recovery rates were significantly higher at 142% ± 9 for diclofenac and 148% ± 10 for sulfamethazine. In conclusion it can be said that a microarray chip for the simultaneous detection of two chemically diverse molecules was developed. The optimization of reaction conditions for lowering LoD values and adjusting recovery rates will be a project of future investigations.

Kurzfassung

Im Rahmen dieser Arbeit wurde ein Mikroarray-Chip für die Rückstandsanalyse für Milch am Beispiel von Diclofenac (DF) und Sulfamethazin (SMA) entwickelt. Diclofenac ist ein nichtsteroidales Antirheumatikum mit einer Carboxylgruppe als funktionelle Gruppe. Der maximale Rückstandswert (*maximum residue level*, MRL) für Diclofenac in Milch und Fleisch beträgt 100 ng/kg. Sulfamethazin ist ein Antibiotikum und gehört der Gruppe der Sulfonamide an. Es besitzt eine Aminogruppe als funktionelle Gruppe. Der MRL für Sulfamethazin in Milch und Fleisch beträgt 100 µg/kg. Das Ziel dieser Arbeit war es, beide Moleküle mit ihren unterschiedlichen funktionellen Gruppen auf der gleichen Oberfläche zu immobilisieren. Als Analyseplattform wurde der automatisierte MCR 3 mit einer Durchflusszelle für den simultanen Nachweis von mehreren Analyten über einen indirekt-kompetitiven Chemilumineszenz-Mikroarray-Immunoassay verwendet.

Dafür wurden zwei unterschiedliche Immobilisierungsstrategien auf einem Mikrochip kombiniert. Diclofenac wurde durch Modifikation der funktionellen Carboxylgruppe mit 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimid und N-hydroxysulfosuccinimid auf der Jeffamine® Oberfläche des Mikroarray-Chips kovalent gebunden und Einzelkalibrierungen wurden in Wasser durchgeführt. Für Sulfamethazin wurde ein neuartiges, zweistufiges Immobilisierungsverfahren ausgearbeitet, bei dem zuerst eine Schicht Poly(ethylene glycol) diglycidyl ether (DE-PEG) als Vernetzer und anschließend die Sulfamethazin-Lösung aufgetragen wurden. Diclofenac wurde bezüglich der Sensitivität genauer untersucht. Dafür wurden Einzelkalibrierungen in Wasser durchgeführt und ergaben eine Nachweisgrenze von 83.0 ± 11.2 ng/L.

Nach erfolgreicher Entwicklung des Mikroarray-Chips wurden die Selektivitäten der primären Antikörper untersucht und es konnten annähernd 100%-ige Selektivitäten der Antikörper zu den entsprechenden Analyten festgestellt werden. Während der Durchführung von Duplexkalibrierungen konnte gleichzeitig die Regenerationsfähigkeit der Mikroarray-Chips kontrolliert werden. Nach 35 Regenerationszyklen wurden relative Signalreduktionen von 21 % für Diclofenac und 9% für Sulfamethazin bezogen auf das maximale Ausgangssignal der ersten Messungen festgestellt.

Die Duplexkalibrierungen wurden in H-Milch durchgeführt und ergaben Nachweisgrenzen von 0.3 µg/L für Diclofenac und 8.0 µg/L für Sulfamethazin.

Letzteres lag unterhalb des entsprechenden MRL von 100 µg/L. Die Nachweisgrenze für Diclofenac lag jedoch um den Faktor 2.5 über den MRL von 100 ng/L. Um eine Verbesserung der Nachweisgrenze für Diclofenac zu erreichen, wurden Versuche mit längerer Antikörper-Analyt Interaktionszeit und längerer Kompetitionszeit auf dem Mikroarray-Chip unternommen. Eine Verbesserung der Nachweisgrenze konnte nicht festgestellt werden.

Zum Schluss wurden Experimente durchgeführt, um die Wiederfindungsraten in H-Milch und Frischmilch zu bestimmen. In der Matrix H-Milch konnten Wiederfindungsraten von durchschnittlich 99% ± 7 für Diclofenac und 124% ± 12 für Sulfamethazin festgestellt werden. In Frischmilch ergaben sich mit 142 ± 9 für Diclofenac und 148 ± 10 für Sulfamethazin eine leichte Überbestimmung. Zusammenfassend kann gesagt werden, dass ein Mikroarray-Chip für den gleichzeitigen Nachweis zweier chemisch unterschiedlicher Moleküle entwickelt wurde. Die Optimierung der Reaktionsbedingungen zur Senkung der Nachweisgrenze und die Verbesserung der Wiederfindung wird ein Projekt zukünftiger Untersuchungen sein.

List of Abbreviations

AP	Alkaline phosphatase
AFM	Atomic force microscopy
BRT	Brilliant black reduction test
COX	Cyclooxygenase
DCC	Dicyclohexylcarbodiimide
DE-PEG	Poly(ethylene glycol) diglycidyl ether
DMS	Dimethyl suberimidate
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EIA	Enzyme immunoassays
EU	European Union
F_{ab}	Fragment of antigen binding
F_c	Constant fragment
GOPTS	(3 Glycidyloxypropyl) trimethoxysilane
HRP	Horseradish peroxidase
MCR 3	Microarray Chip Reader 3
MIA	Microarray immunoassay
MRL	Maximum residue limit
NSAID	Nonsteroidal anti-inflammatory drug
OI-RD	Oblique-incidence optical reflectivity difference
RIA	Radioimmunoassay
SAM	Self-organized monolayer
S-NHS	N-hydroxysulfosuccinimide
SPRi	Surface plasmon resonance imaging
WR	Working range

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1. Introduction

Pharmaceuticals have been known since the beginning of human history. One of the probably oldest surviving works on pharmaceuticals in medicine comes from the Sumerians in Mesopotamia and is estimated to be 5000 years old.[1] Another famous work is the Ebers Papyrus, which was written in Egypt 3500 years ago.[2] At that time, medicines consisted mainly of plants and medicinal herbs.[3] Knowledge of the effects of medicines was based on observation, coincidence and experience.[4] Modern pharmaceuticals seem to have nothing in common with this, but it was also coincidence that in 1928 Alexander Fleming discovered a mould on a forgotten staphylococcus culture and it was only observation when he realized that the mould would prevented the bacteria from growing.[5]

Today, more than 90 years later, there are antibiotics with the most diverse structures and fields of application.[6] They have saved the lives of countless people but despite this there are also negative effects. Uncontrolled application or presence in ecosystems can lead to the formation of resistant bacteria.[7] It has gone to the point that people inadvertently consume antibiotics. They are omnipresent and have found their way into our food through factory farming.[8] Especially in factory farming, the use of antibiotics is widespread and often unavoidable. In order to prevent infections from spreading rapidly among animals kept in confined spaces, it is sometimes necessary to treat them as a precaution. In treated animals, antibiotic residues settle in tissue, milk, eggs or honey and thus enter our food chain.[9]

Analgesics are another example of drugs that are both a curse and a blessing. They have anti-inflammatory effects and their pain-relieving effects make the healing of diseases or injuries more bearable not only for humans but also for animals. But excessive or uncontrolled use can lead to problems of unimagined dimensions. For example, in the 1990s, residues of diclofenac in the cadavers of treated cattle led to mass deaths of vultures.[10] The drug can also have negative effects on humans for example by affecting the cardiovascular system.[11]

With EU directives, attempts are being made to counteract the uncontrolled and excessive administration of such drugs to protect humans and animals. Maximum residue limits (MRL) have been set for pharmacologically active substances in foodstuffs of animal origin.[12] State-of-the-art methods and techniques are used to comply with and monitor these limits. Requirements include economy and speed. In

the dairy industry, for example, it is important to generate rapid test results, since longer residence times of large quantities of milk are costly. Microbiological inhibitor tests are relatively time-consuming[13], chromatographic methods are cost-intensive[14] and receptor- and enzyme-based methods can only identify individual classes of substances.

The development of analytical microarrays with automated readout systems were a milestone in routine diagnostics in the dairy industry. It was possible to perform rapid and multi-analyte detections. The Microarray Chip Reader 3 is one of these automated readout systems and it revolutionized routine diagnostics with the use of a regenerable immunochip for the rapid determination of 13 different antibiotics in raw milk.[15] The system is based on an automated indirect competitive chemiluminescence microarray immunoassay. In this thesis, the technology of the MCR 3 as a multi-analysis platform was to be used as a basis for the development of a micro-residue chip for milk by the example of diclofenac and sulfamethazine.

2. Theoretical background

2.1 Sulfamethazine and Diclofenac

2.1.1 Classification and characterization of sulfamethazine

The substance group of antibiotics are low-molecular metabolites produced by fungi or bacteria and are mainly used to fight infectious diseases.[16] Even small amounts are sufficient to inhibit growth of other bacteria or to devitalize them by blocking vital metabolic processes. Likewise, the propagation of the microorganisms can be inhibited or completely blocked.[17] Antibiotics are classified according to their chemical structure into aminoglycosides, quinolones, β-lactams, polyketides and sulfonamides.[18]
Sulfamethazine belongs to a substance group called sulfonamides, a group of synthetic compounds which are derivatives of p-aminobenzenesulfonamide (Figure 1).[18] The antimicrobial effectiveness of this substance group is based on the inhibition of the synthesis of bacterial folic acid, an important building block of bacterial DNA-, RNA- and protein biosynthesis. Competitive inhibition occurs due to the structural similarity of the sulfonamides with p-aminobenzoic acid. The activity of the latter can be improved by substitution on the sulfonamide group N1. In the case of substitution of the aromatic amino group N4, the antibacterial activity is lost through a reduction in resorption.[19]

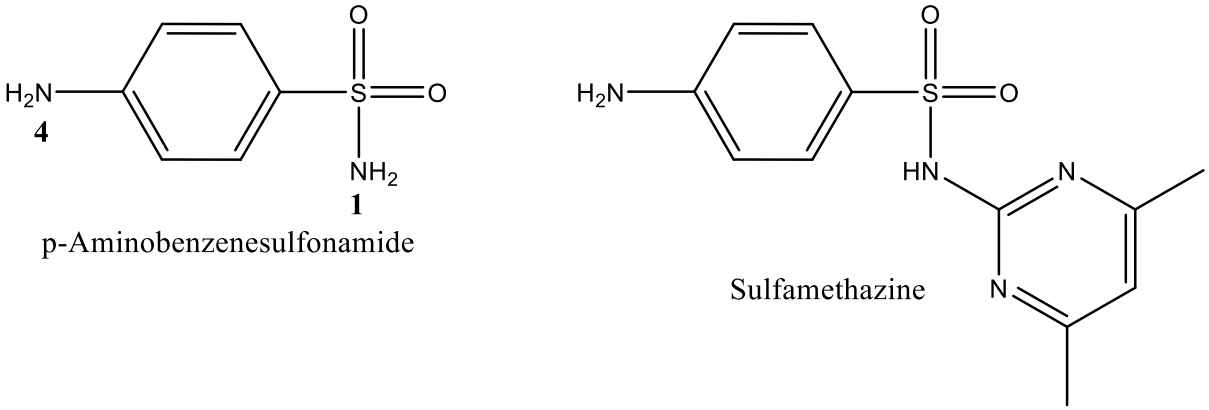


Figure 1: Structures of p-aminobenzenesulfonamide and sulfamethazine.[18]

In veterinary medicine, antibiotics are used to treat bacterial infectious diseases such as diseases of the gastrointestinal tract, the respiratory tract and mastitis.[20, 21] In flock

management antibiotics are also administered prophylactically to the entire flock to prevent the spread of diseases. Sulfonamides are widely used in the dairy industry especially sulfamethazine which is frequently used for the treatment of mastitis.[20] In contrast to other sulfonamides, this drug is used therapeutically as a single substance. It is reported to be the least toxic within the family of sulfonamides while being active against both gram-negative and gram-positive organisms.[22] In addition to the health aspects, some antimicrobials are used as performance-enhancers and growth-promoters in the meat- and dairy industry.[23] This type of application has been banned in the European Union (EU) since 2006[24], but is still used in other parts of the world.[25] In Germany, pharmaceutical companies have been obliged to pass on the number of antibiotics handed out to veterinarians to the German Institute for Medical Documentation and Information (Deutsches Institut für Medizinische Dokumentation und Information DIMDI) since the commencement of the Medicinal Products Act (Arzneimittelgesetz § 47 Abs. 1c AMG) in 2011. The collected data shows a decline in antibiotic consumption in Germany since 2011. According to the Federal Office of Consumer Protection and Food Safety (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit), sulfonamides are one of the most administered group of antibiotics after tetracyclines and penicillins. In 2011, 185 tons of sulfonamides were delivered to veterinarians. After a drop of 122 tons, only 63 tons were recorded in 2018.[26] In addition to strict guidelines for the allocation of antibiotics to food-producing farm animals, the produced food and its raw material have to be tested regularly. The European Commission has set a maximum residue limit (MRL) of 100 µg/Kg for animal products such as milk or meat.[27]

2.1.2 Classification and characterization of diclofenac

Diclofenac is a typical nonsteroidal anti-inflammatory drug (NSAID). NSAIDs have analgesic, anti-inflammatory and antipyretic effects and can therefore be used for a variety of diseases. Their effect is based on the non-selective inhibition of cyclooxygenases (COX), which are responsible for the formation of prostaglandins in the human body and in higher mammals. COX-1 occurs in all tissue types and catalyses the production of prostaglandins, which are involved in processes such as aggregation in blood clotting or the production of gastric acid.[28] COX-2, on the other hand, is increasingly formed in the event of inflammation, pain reactions or tissue

damage.[29] The pain-relieving and antipyretic effects of NSAIDs are based on the inhibition of COX-2, whereas the side effects such as stomach discomfort or disorders of haematopoiesis and blood coagulation are mainly due to the inhibition of COX-1.[30] Diclofenac (Figure 2), which belongs to the phenyl acetic acid derivatives, inhibits both COX-1 and COX-2, with a clear structural preference for COX-2.[31]

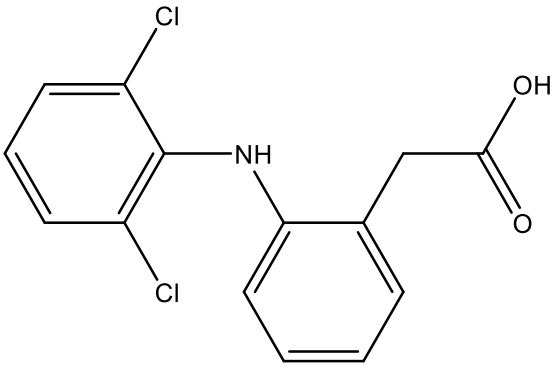


Figure 2: Structure of diclofenac.[32]

DCF is not approved as drug for animals which produce milk for human consumption but is allowed for meat-producing animals.[33] However, the European Commission has set an MRL of 100 ng/L[27]. Studies were carried out on dairy cows, whereby the milk was screened for residues after a three-day intramuscular application of 2.5 mg/Kg diclofenac per day. 12 hours after the last application, diclofenac residues of 3.76 µg/Kg were determined. 108 hours after the last application, no diclofenac could be detected because its concentration had dropped below the detection limit of 0.03 µg/Kg.[34] Therefore there is an interest in food analysis to be able to detect residues of DCF in milk, to detect improper use of diclofenac in dairy cows producing for human consumption.

2.2 Detection methods for sulfamethazine and diclofenac in milk

The detection of drug residues in foods of animal origin can be provided by a wide variety of methods. A distinction is made between microbiological, chemical-physical and immunological assays. These include screening methods that deliver a positive or a negative result and quantitative analysis methods that can identify and quantify the analytes.

2.2.1 Microbiological inhibition test

Microbiological inhibitor tests are some of the simplest and cheapest methods for the detection of antibiotics.[35, 36] These can be carried out without great effort and partially outside of the laboratory.[36] Microorganisms are used to indicate the presence of an inhibitor due to the lack of growth by measurement of a colour change. Well known representatives are the brilliant black reduction test (BRT)[37], Delvotest[36] and the agar diffusion test[38].

The BRT inhibitor test is based on the colour change of the brilliant black redox indicator. In the event of a negative result, this indicator is reduced by the test organisms, which is typically *B. stearothermophilus var. calidolactis* and undergoes a colour change from dark blue to yellow or colourless. In addition to the samples, positive and negative controls are also prepared. Depending on the methodology, the evaluation of the test procedure can be carried out either by comparison with the positive control, whereby all samples which at least correspond to the blue colour of the positive control are evaluated as positive. The second option is the comparison with the negative control, whereby all samples that do not have the yellow tone of the negative control are considered positive. In the agar diffusion test, a test strip soaked with the milk to be tested is placed on a nutrient medium. If the milk contains inhibitors, they diffuse into the nutrient medium during the incubation and prevent the test organisms from growing around the test strip. The advantages of these detection methods are low costs and simple implementation. They are suitable for screening larger quantities of samples and have a wide detection spectrum. Disadvantages are long test times of up to 24 hours.[13] In addition, the test methods are not intended for the identification and quantification of proven inhibitors.[39]

2.2.2 Chemical-physical detection methods

Chemical-physical detection methods can be used to detect both antibiotics and NSAIDs. A distinction is made between colorimetric, spectroscopic, electrophoretic and chromatographic methods, the latter being of the greatest importance. The individual substances are separated by means of thin layer[40, 41], gas[42, 43] or liquid chromatography[44, 45]. In some cases, especially for analytes in complex matrices, extractions such as solid phase extraction are used as sample preparation method.[46]

UV adsorption, fluorescence or mass spectrometry is usually used as detection method. Due to the low detection sensitivity of these methods, they are increasingly used in reference diagnostics and as confirmation tests. Operating such systems requires a lot of maintenance, highly qualified personnel and is quite expensive. Therefore, they are less advantageous in routine diagnostics or sample screening.[39] It becomes problematic when it comes to the detection of several analytes with different molecular structures or sizes. Influences such as the solubility, polarity or the size of the molecules make simultaneous detection and quantification difficult.[47] This is the case with milk, for example. It can contain various residues, such as of antibiotics or anti-inflammatory drugs, and needs to be screened on a regular basis.

2.2.3 Enzyme-linked immunosorbent assay

2.2.3.1 Assay formats

Immunological methods are based on the ability of antibodies to recognize and bind specific molecular structures (= antigens).[48] This can be used to separate unknown analytes or target analytes from complex matrices like milk with the aid of antibodies and subsequently detection. Labeled reagents (antigens or antibodies) are used to make this antigen-antibody binding reaction visible and measurable. Historically, radioactive isotopes such as iodine-125 were initially used in radio immunoassays (RIA).[49] More modern variants are fluorescence-immunoassays (FIA) and enzyme-immunoassays (EIA), which make the antigen-antibody binding reaction visible and measurable via color, chemiluminescence or fluorescence reactions. The most commonly used marker enzymes include horseradish peroxidase (HRP) and alkaline phosphatase (AP).[50]

Enzyme-linked immunosorbent assays are heterogeneous assay formats in which either the antibodies or antigens are immobilized on a solid phase such as Polystyrene[51], polypropylene[52], polycarbonate[53] or glass[15]. There are competitive and non-competitive enzyme-immunoassays (Figure 3). Non-competitive assays are the sandwich assay (A) and the antibody capture assay (B). The former require two different antibodies that recognize the antigen but do not interfere with each other in their binding to the antigen. One, the detection antibody is immobilized on the solid phase. The sample is then added and the antigens can bind to the capturing

antibodies. All unbound components of the sample matrix are washed away. A detection antibody then binds the antigen. The excess is washed away and after the addition of substrate, the reaction can be measured. The more antigen was bound, the higher the measured signal. The sandwich assay is mainly suitable for large molecules or proteins because the enclosed antigen has to be big enough to be bound by two antibodies at different locations.[50]

The antibody capture assay (B) is mainly used when the analyte is an antibody. The antigen is immobilized on the solid phase to which the antibody can bind. A second enzyme-labelled antibody then binds to the constant part of the first antibody. After adding substrate, the reaction can be measured. The more antigen that is bound, the higher the measured signal.

For competitive processes, a distinction can be made between direct and indirect assay. The direct method (C) works as follows. The antibody is bound to the solid phase, enzyme-labelled antigens are added to the sample solution, which then compete directly with the analytes for the binding sites. A measurable signal is initiated by adding substrate and becomes more intense, the less analyte is present in the sample.

At indirect-competitive-immunoassays (D), the antigen is immobilized on the solid phase. It competes with the free antigens in the sample solution for the available binding sites of the specific antibodies added to the sample. The more antigen is in the sample, the fewer free antibodies bind to the immobilized antigens on the solid phase. An enzyme-labelled secondary antibody then binds to the constant part of the capture-antibody and after the addition of the substrate, the reaction can be measured. The intensity of the signal increases with decreasing antigen concentration in the sample. This principle is one of the most common for the detection of small molecules such as antibiotics or NSAIDs.

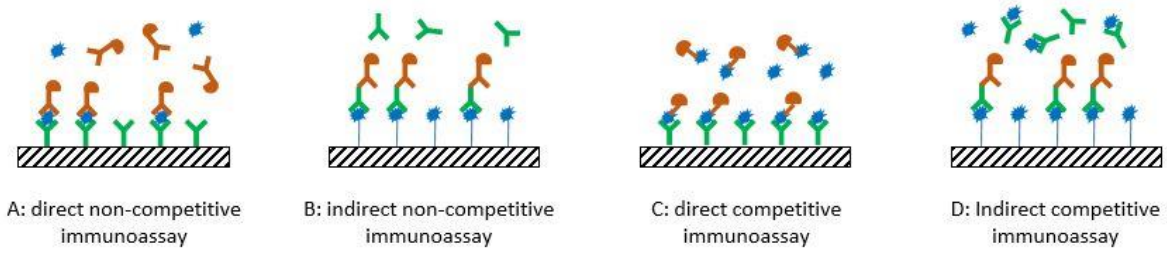


Figure 3: Schematic representation of various immunoassays.

2.2.3.2 Interpretation of immunoassays

The calibrators of an immunoassay form a sigmoidal calibration curve when plotted semi-logarithmically, regardless of the assay format. There is a difference between direct (low concentration = low intensity) and indirect (low concentration = high intensity) formats. Using the example of the indirect assay, the signal intensity is given as a function of the analyte concentration and is shown graphically in Figure 4.

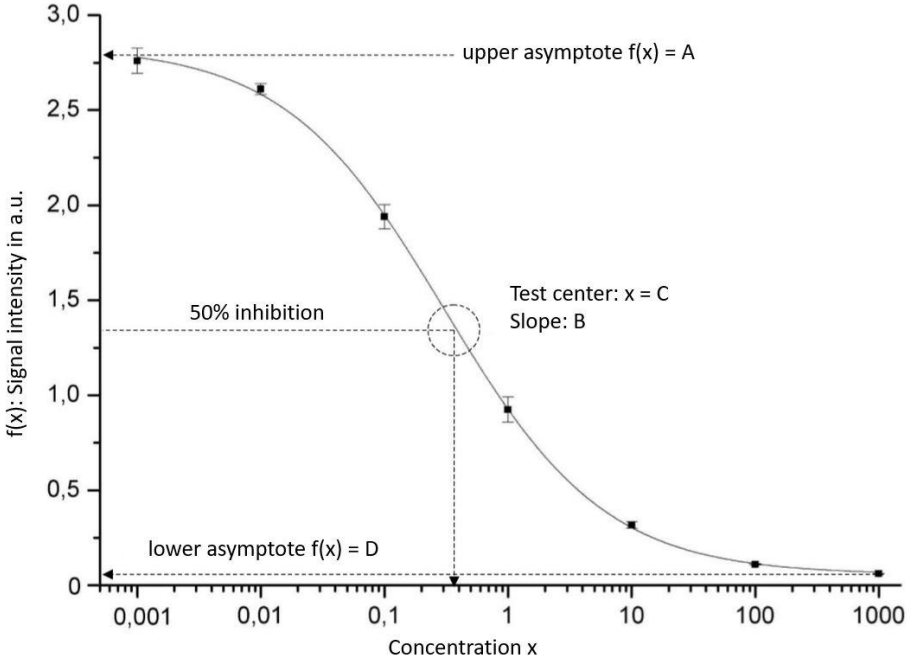


Figure 4: Exemplary calibration curve for an indirect competitive immunoassay.

The dependency between the measured intensity f(x) and the semi-logarithmically plotted analyte concentration x, can be described with the sigmoidal 4-parameter formula (Equation 1).[54] The four parameters describe the upper asymptote (A), the slope at the test centre (B), the test centre (C) and the lower asymptote (D). The parameters can be accessed by an iterative curve fitting process in which the parameters are changed until the best curve fit is obtained.

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

Equation 1: 4-parameter formula.[55]

Assays performed on different days may show different maximum signals but the same sensitivity. Differences can result if for example fresh buffer or antibody solutions are used. In order to be able to compare the assays, the measured intensity is indicated as the relative signal intensity B/B_0 in %. This is calculated with Equation 2 and the corresponding relative standard deviation with Equation 3. Test characteristics such as sensitivity and detection limit can be obtained from the fitting parameters of the 4-parameter formula. The detection limit is determined from the maximal intensity minus three times the standard deviation of the blank value. Blank values are the intensities resulting from measurements without analytes. Sensitivity is defined as the degree at half-maximal signal intensity and the working range (WR) within 20 to 80% in relation to the maximum relative signal intensity.[56]

$$\frac{B}{B_0}[\%] = \left(\frac{\Delta I_{sample}}{\Delta I_{blank}}\right) = \left(\frac{I - I_{min}}{I - I_{max}}\right) * 100$$

Equation 2: Equation for the calculation of the relative signal intensity.

$$\sigma[\%] = \left(\frac{\sigma_{Abs}}{I_{max} - I_{min}}\right) * 100$$

Equation 3: Equation for the calculation of the standard deviation.

2.2.3.3 Antibodies

Antibodies are part of the immune system of vertebrates. The immune system has the task of protecting the organism from viruses, bacteria, toxins, or other foreign substances or defective cells in the body. Antibodies belong to the adaptive immune system and can be divided into five different isotypes: IgM, IgD, IgG, IgA and IgE. They differ in their structure, function and distribution in the body. IgG is the most common immunoglobulin and is distributed in the blood and interstitial fluid.[57] With up to 80%, it is the largest part of the total antibody amount in the blood serum.[58] Immunoglobulin G (Figure 5) consists of two identical light and two identical heavy chains. One heavy chain with one light chain and the two heavy chains are covalently linked via disulfide bridges and non-covalent forces. It forms a Y-shaped and axisymmetric heterotetramer.[59]

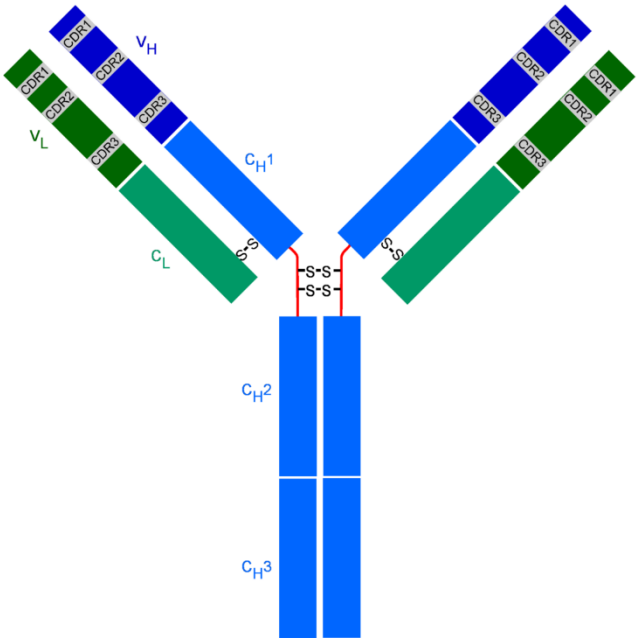


Figure 5: Schematic structure of immunoglobulin G consisting of two heavy- (V_H and C_H) and two light chains (V_L and C_L).[60]

The light chains each consist of a variable and a constant domain, while the heavy chains are each composed of one variable and three constant domains. Between the first and second domains of the heavy chains there is a hinge region that provides spatial flexibility between the constant fragment (F_c) and fragment of antigen binding (F_{ab}) fragments. The F_c fragments are responsible for triggering the biological functions, while the F_{ab} fragments are responsible for the specific and high-affinity antigen recognition and binding. The variable domains form the antigen binding sites, with three short sections being referred to as hypervalent regions.[61]

A basic differentiation is made between monoclonal and polyclonal antibodies. Rats, rabbits, goats, sheep and horses are mainly used to produce polyclonal antibodies. An immunogen is injected into the organism several times at defined intervals. After a few weeks, the polyclonal antiserum can be harvested. It consists of a mixture of antibodies that all recognize different epitopes of an antigen. They are mostly used as secondary antibodies against the actual detection antibody.[62] Monoclonal antibodies, on the other hand, are produced by single cells or clones. They are homogeneous and recognize only a single binding site of an antigen. These antibodies are produced by cell culture technology and can theoretically be produced in unlimited quantities.[63]

Monoclonal antibodies are important as primary antibodies because they can always be reproduced and have a significant influence on the performance of the assay.

In summary, very good specificities regarding the antigen and the strength of the non-covalent bond between antibody and antigen make a good antibody for immunoassays. This characteristics form the basis for optimized immunoassays to work correctly and precisely even at the lowest concentrations.[64]

2.2.3.4 Antigen-antibody interactions

Immunoassays are based on antibody-antigen binding. The underlying binding forces are electrostatic interactions, van der Waals forces, hydrogen bonds and hydrophobic interactions.[65] The equilibrium of this reaction can be influenced by various factors such as assay design, pH, organic solvent content, ionic strength and temperature.[66] The reaction of antibody and antigen can be described using the law of mass action (Figure 6). To simplify the approach, it is assumed that all reactions follow the 1st order, the antibodies show a monovalent binding behaviour, no unspecific binding is present, and no marginal or allosteric effects occur. Under these assumptions, all equilibrium and kinetic constants listed are merely formal constants.[67]

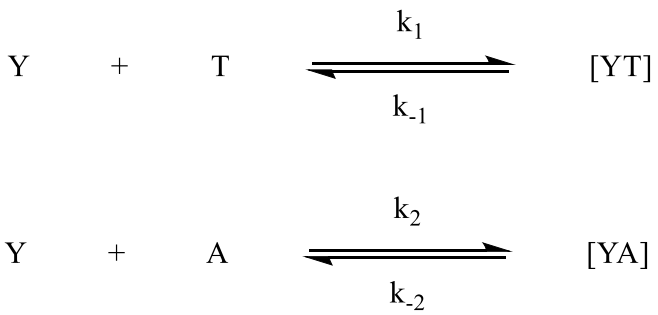


Figure 6: Reversible antibody-antigen binding reactions with Y for antibody, T for tracer, A for analyte, YT and YA for corresponting antibody-tracer/analyte komplex, assotiation- and dissotiations constant k_1, k_2, k_{-1} and k_{-2}.[68]

The equilibrium constants K_T and K_A are differentiated because tracer and analyte are different and therefore have different association and dissociation constants. (Equation 4)

$$K_T = \frac{k_1}{k_{-1}} = \frac{[YT]}{[Y][T]}$$

$$K_A = \frac{k_2}{k_{-2}} = \frac{[YA]}{[Y][A]}$$

Equation 4: Equilibrium constants K_T and K_A for antibody-antigen binding reaction.

The equilibrium constants (= affinity constant) describe the ratio between bound to free antibody and analyte/tracer, which is decisive for all immunoassays. Antibodies with a high affinity to the corresponding antigen have a significantly higher association than dissociation constant. Affinity is defined from a thermodynamic point of view as the strength of the non-covalent binding of an antibody binding site to a monovalent antigen. Polyclonal sera have a heterogeneous composition and may have different affinities to an antigen. Therefore, it is not possible to determine individual affinity constants for antibodies from sera; but an average value of the individual affinities. In principle, the higher the affinity of an antibody to the antigen, the stronger the effect of minimal changes of the antigen concentration.[69]

The number of available antibody binding sites is also crucial in addition to affinity. If the proportion of bound analytes is plotted against the concentration of the analyte using the competitive assay as an example, a dose-response relationship is obtained (Figure 7). This shows how the sensitivity of the assay can be influenced by the antibody concentration. A higher antibody concentration means more bound analyte and lower sensitivity.

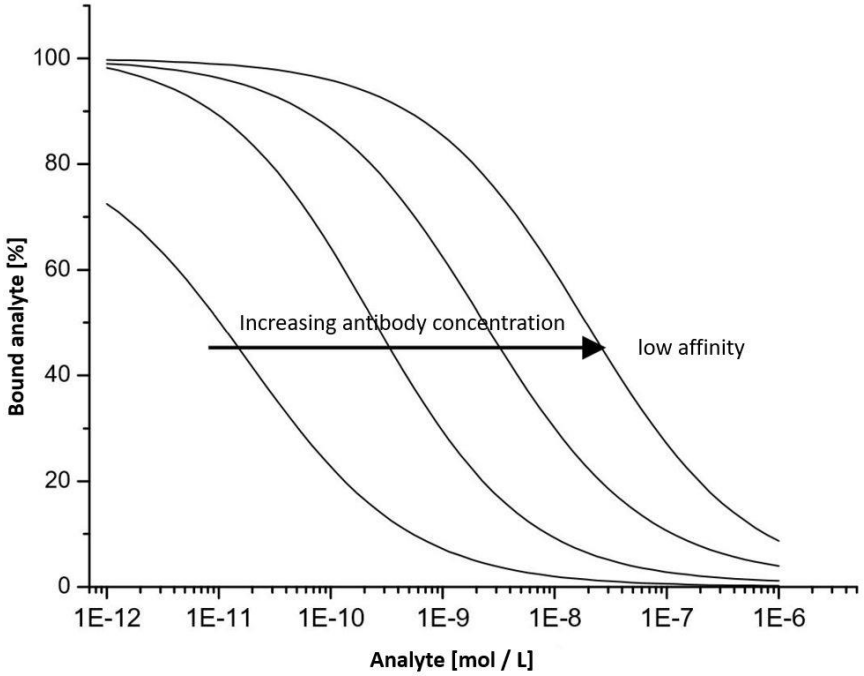


Figure 7: Dose-response relationship between analyte and bound analyte for different antibody concentrations.[70]

There are two starting points for the optimization of an assay, firstly the affinity constant and secondly the concentration of the antibodies. The affinity constant is an integral property of the antibody and cannot be changed, but the concentration can be varied to optimise sensitivity.[71]

2.2.4 Microarray technology

A more modern variant of immunoassays are microarray immunoassays (MIA), which allow a parallel analysis of different analytes. Capturing molecules are immobilized on a carrier surface in the form of arrayed spots. The capture molecules can include haptens[72], antibodies[73], DNA[74] or RNA[75]. The spotting processes are usually performed by robots.

In addition to the areas of application in genome analysis, diagnostics and gene expression, the importance of microarrays in the field of food and environmental analysis is growing.[76, 77] The possibility to detect several analytes such as pharmaceuticals, pesticides, toxins or larger ones e. g. proteins or bacteria in parallel combined with the possibility of a complete automation of the systems has many advantages. An example is the development of the so-called milk chip as a biosensor, which is able to detect 13 different antibiotics in milk within six minutes.[15] This was

made possible by the development of a flow-through microarray based on a glass chip on which an regenerable indirect-competitive chemiluminescence microarray immunoassay is carried out using the fully automated platform Microarray Chip Reader 3 (MCR 3).

2.2.4.1 Surface-chemistry of microarray chips

The surface chemistry of microarray chips has a significant influence on the precision of the affinity analysis. On one hand, the receptor molecules must be able to bind strongly, and on the other hand, unspecific adsorption, e.g. by matrix proteins, needs to be prevented. To ensure this, the microarray chip surface must meet certain criteria. This includes a homogeneous surface quality, a high immobilization yield of the receptor molecules, steric accessibility for the target molecules and a high signal-to-background ratio.[78]

Glass, noble metals, metal oxides, silicones and plastics are suitable substrates as support for microarray chips.[79] Which type of carrier material is used depends on the immobilization strategy, the detection principle and the requirements of the respective application. Glass is a commonly used material for chip manufacturing.[80, 81] Some of the advantages are high physical and chemical stability, excellent optical properties and therefore only imperceptibly interferes with the detection of the measurement signal. In addition, glass is an inexpensive material and the silane surface can be activated relatively easily and in a variety of ways.[82] The activation and modification of the surface comprises several steps and takes place in layers.

The glass surface needs to be thoroughly cleaned, before it is etched with concentrated acids as a pre-treatment for silanization. The glass surface is oxidized and silanol groups are formed. The silanization takes place by covalent binding of (3 glycidyloxypropyl) trimethoxysilanes (GOPTS) to the hydroxyl groups of the glass surface (Figure 8). A self-organized monolayer (SAM) is formed from organosilanes. The driving force is the spontaneous formation of the covalent bonds and the intramolecular interactions between the alkyl chains of the silanes.

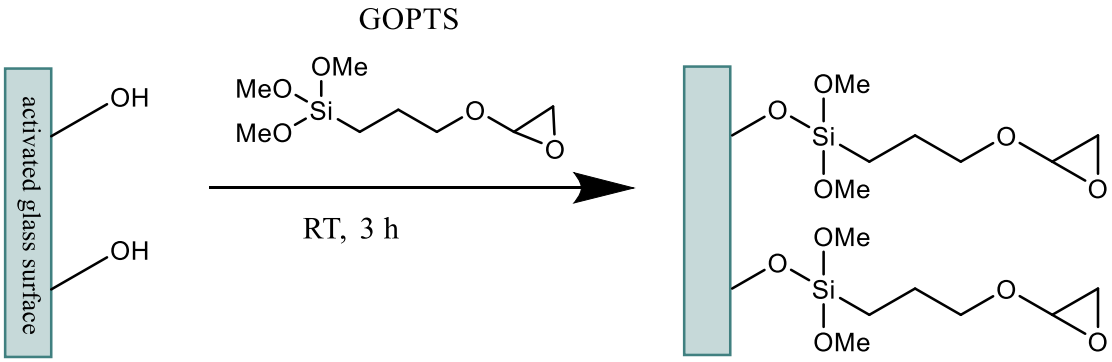


Figure 8: Formation of a self-organized monolayer of organosilanes on the activated glass surface.

The hydrophobic epoxy-surface created in this way has a strong tendency towards non-specific protein binding. To avoid this, another layer consisting of Jeffamine® ED-2003 is applied. Jeffamine® ED-2003 is a polyethylene glycol, which is functionalized with short polypropylene glycol combined with amino groups at the chain ends.[83] The immobilization takes place through the reaction of the amino groups of Jeffamine® with the epoxy groups of the surface (Figure 9). The amino surface created in this way allows the targeted immobilization of capture molecules and prevents non-specific binding.

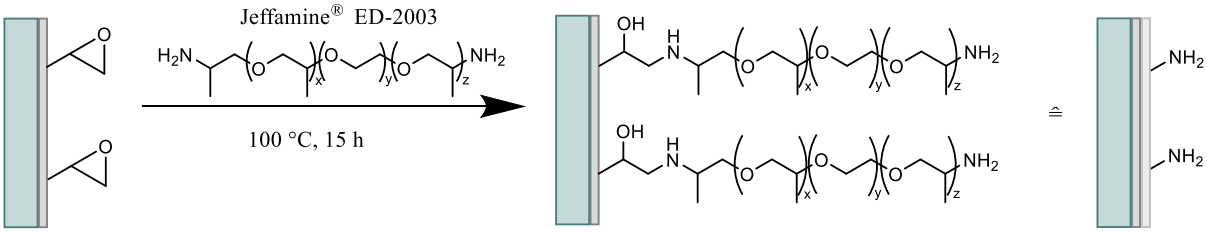


Figure 9: Surface functionalization with Jeffamine® ED-2003.

2.2.4.2 Immobilization

In order to immobilize the capture molecules on the microarray chip, it is essential to ensure the defined and homogeneous placement on the surface in a defined array. This can be achieved by photolithography, in which oligonucleotides and peptides in particular are produced directly on the microarray by stepwise extension.[84]

Another possibility is microspotting. A distinction is made between contact printing and non-contact printing processes. A big advantage of non-contact printing technology, as the name suggests, is that the printing device never has direct contact with the

surface of the microarray. This reduces the possibility of cross-contamination to a minimum. There is also no need to periodically clean the printing device during the spotting process. It is also a quick method which is suitable for high throughput production. The non-contact printing method is implemented e. g. by electrical printing or inkjet printing.[85]

The contact printing process places the spot directly on the surface of the microarray, whereby the needle or split pin has direct contact with the surface. The robustness of the method is a great advantage. Especially with the solid pin, there is no capillary that could clog. This means that saline buffer solutions can easily be used for spotting. Disadvantages are the possibility of contamination caused by the contact between pin and microarray surface. In addition, only low buffer solutions with low vapour pressure can be used to prevent evaporation.[85] Microcontact printing with a solid pin was used to produce the microarrays for this work.

In addition to applying the spotting solution to the chip surface, chemical processes also play an important role in immobilizing the capture molecules. In order to immobilize sulfamethazine, a bond must be established between the amino groups on the surface and the antibiotic. This can be achieved using imidoester dimethyl suberimidate (DMS) as crosslinker. DMS is a well-known crosslinking agent and has amine-reactive imidoester groups on both ends.[86] Despite the fact that DMS is an excellent cross-linker between amino groups, this can lead to problems during immobilization. The cross-linking reaction is shown in Figure 10. Two possible scenarios can arise during the reaction: In one case, the amino group with the rest R1 stands for the immobilized Jeffamine® and the amino group with the rest R2 for sulfamethazine or vice versa. In the second case, two amino groups belonging to sulfamethazine are linked together. The latter would have a negative impact on the immobilization yield.

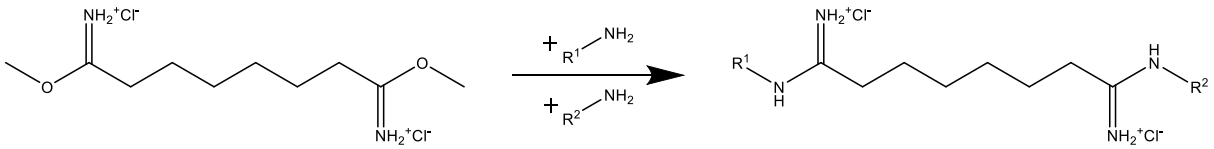


Figure 10: Reaction of DMS with two amino groups.

Another method to immobilize sulfamethazine is to use poly(ethylene glycol) diglycidyl ether (DE-PEG) as crosslinking agent. DE-PEG consists of a linear polyethylene glycol chain which is modified with two epoxide groups on both ends.[87] Each of the epoxide groups can react with an amino group and form a covalent bonding. The reaction is shown in Figure 11.

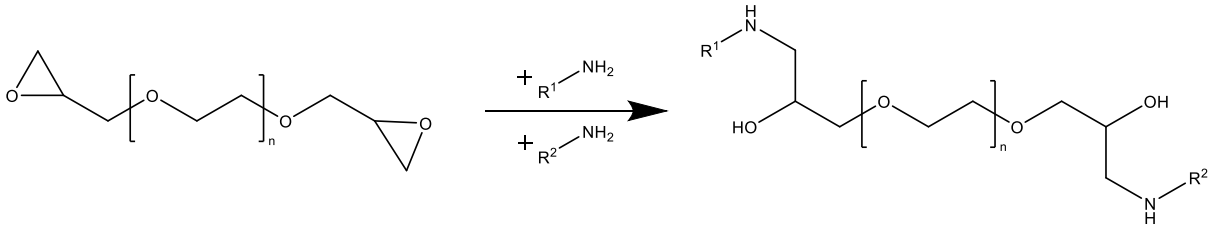


Figure 11: Reaction of DE-PEG with two amino groups.

Diclofenac has a carboxyl group as functional group (Figure 2) which has to be modified to be able to bind to the amino group surface on the microarray chip. There are carbodiimide compounds which are a versatile method to label or crosslink carboxylic acids. The most common ones are dicyclohexylcarbodiimide (DCC) for non-aqueous systems and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) for aqueous solutions.[88] The immobilization by EDC begins with the reaction of the carboxylic acid with EDC to the unstable intermediate o-acylisourea. In combination with n-hydroxysulfosuccinimide (S-NHS) the dry-stable amine-reactive S-NHS ester is formed. The amine-reactive species forms a stable amide bond in presence of primary amines (Figure 12). Thus diclofenac is immobilized.

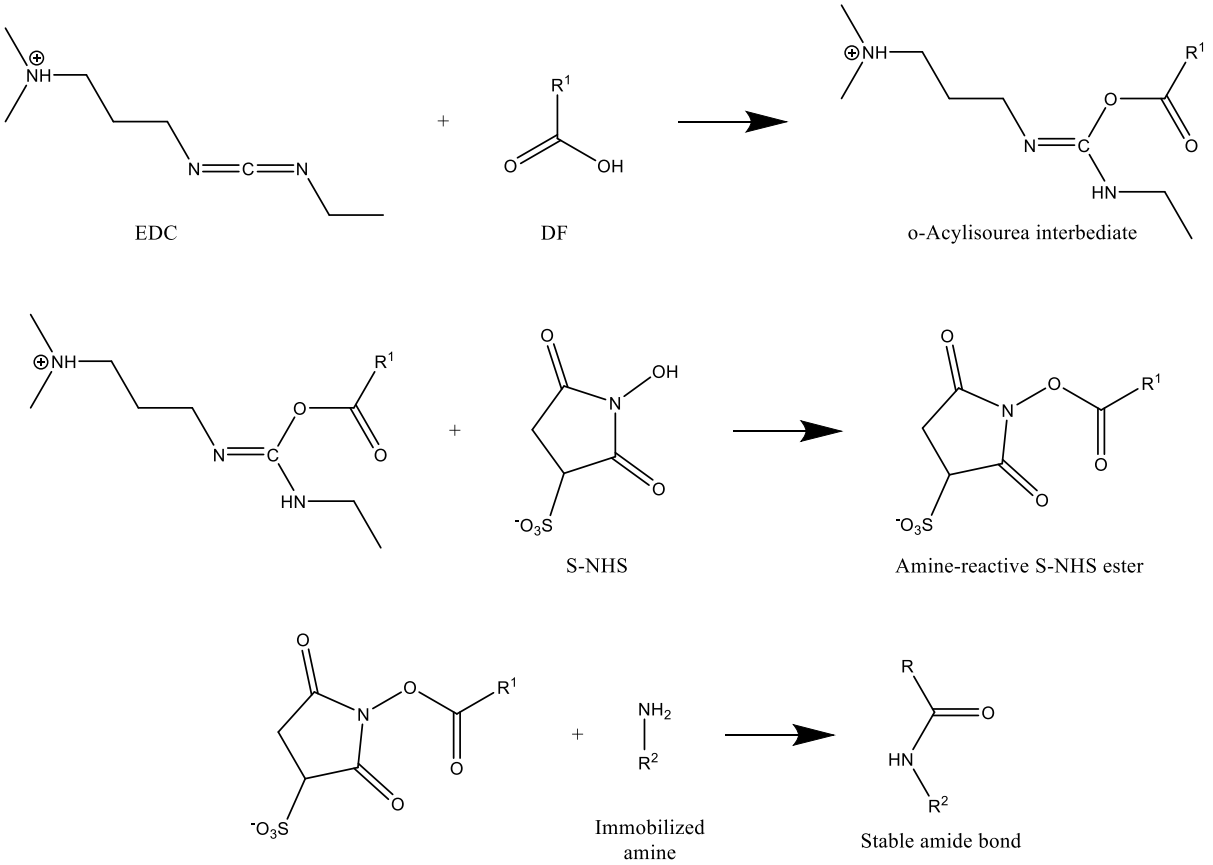


Figure 12: Reaction scheme for the activation of diclofenac with EDC and S-NHS and the formation of a stable amide bond with the immobilized amine.

2.2.4.3 Detection

Various methods have been developed for the detection of microarrays. There are label-free microarrays which are used in special cases such as measurements in the field of protein expression and protein-molecule interactions. Detection can be carried out using the oblique-incidence optical reflectivity difference (OI-RD) microscope[89], atomic force microscopy (AFM)[90] or surface plasmon resonance imaging (SPRi)[91]. In the case of proteins, it can lead to a change in activity through labeling.[92] Label free detections circumvents these problems.

Another method is fluorescence detection, which is a common method especially in the field of DNA and protein microarrays.[93-95] In order to read a microarray using fluorescence, the added reaction partner is labeled with a fluorophore via covalent bonding. Common fluorescent labels are fluorescein, rhodamine-derivatives or other organic dyes.[96, 97] Enzyme-labeled detection antibodies are used in the field of ELISA formats. For the frequently used HRP or AP, the substrates Thyramid or AttoPhos can

be used for the fluorescence reading of immunoassays.[98] The fluorescence signal from stationary microarrays is generated and read out with the aid of fluorescence laser scanners.

With the use of enzyme-labeled reagents there is no need for expensive and complex technology. A simple CCD camera is sufficient to capture photons emitted by an enzyme-catalyzed chemical reaction. The secondary antibody is labeled with the marker enzyme HRP which catalyzes the luminescence reaction. The main steps in this reaction are the oxidation of luminol by hydrogen-peroxide to an excited aminophthalate dianion. The unstable dianion emits light with the wavelength 425 nm during the relaxation into the ground state (Figure 13).[99]

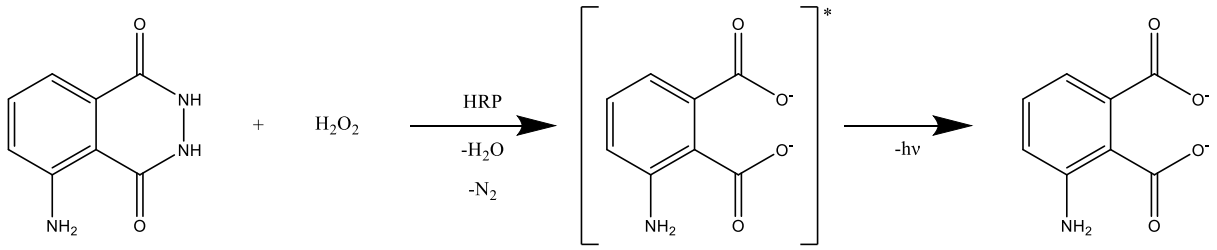


Figure 13: Chemiluminescence reaction of luminol and peroxide catalyzed by HRP.[99]

The amount of light emitted depends on the amount of substrate used, the ambient temperature, the exposure- and incubation time. In a temperature-controlled laboratory and with a constant reading time, the amount of substrate converted is the only variable. This allows conclusions to be drawn about the quantity of the HRP-labeled antibody bound on the microarray chip.

2.3 Microarray Chip Reader 3

The Microarray Chip Reader of the 3rd generation (MCR 3) is a fully automated device for carry- and read out of chemiluminescence microarray immunoassays. The analysis-speed and the possibility of regeneration of the microarray chips make it possible to analyze a large number of samples with little effort and in a short time. The device is designed so that it can be operated as a stand-alone platform, which is not tied to the laboratory and can be used on the field, provided that an external power source is present.

2.3.1 Setup of the MCR 3

The MCR 3 (Figure 14) consists of a robust housing with a hinged cover for closing and a fan for cooling the interior. The individual sub-units are permanently mounted on the base plate with enough space for reagent storage containers.

The subunits comprise the following modules: a pump unit with three motor-driven syringe pumps and integrated rotary valves (1), a unit with four further rotary valves (2), a flow cell (3) with a CCD camera as detection unit (4), two 50 mL antibody syringes (5), a motor-driven 1 mL syringe for sample injection (6), the storage containers for buffer solutions (7) and for detection solutions (8).

There is also a software-based control unit and a waste container, which are not shown in Figure 14. The tubes consist of chemical-resistant PTFE capillaries with an inner diameter of 1.0 or 0.5 mm.

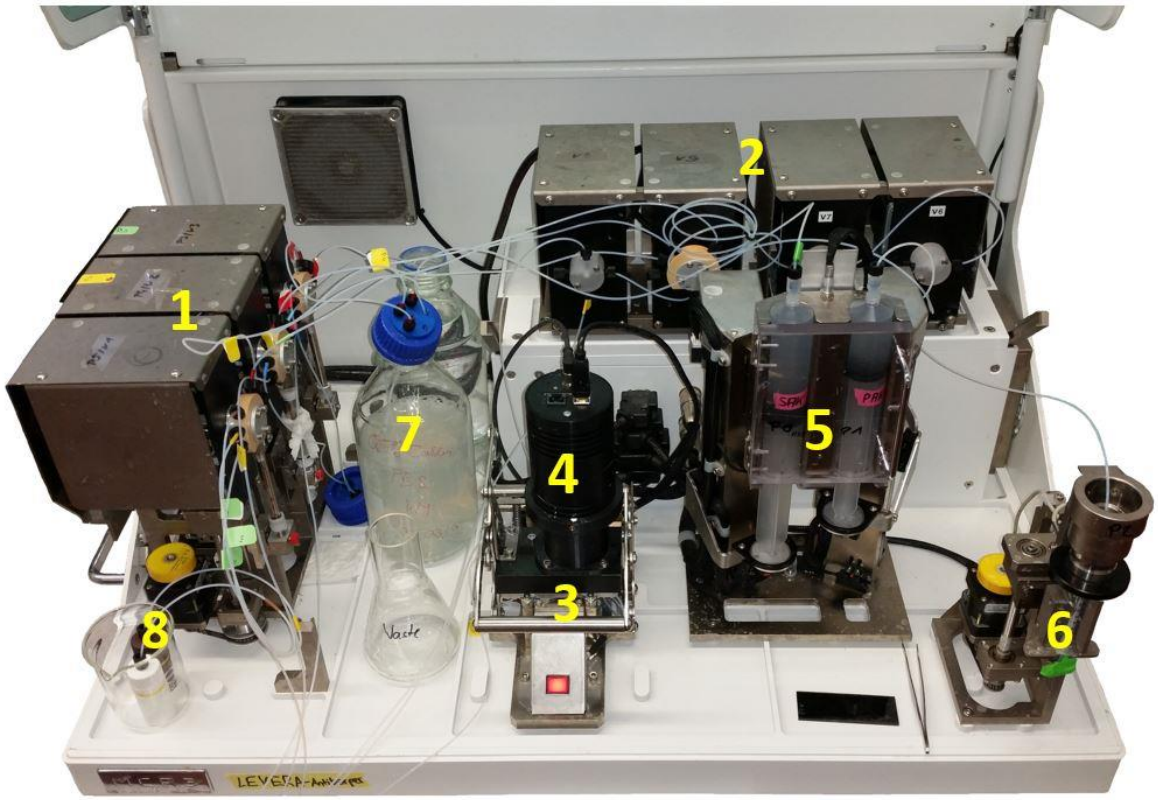


Figure 14: Setup of the MCR 3.

2.3.2 Measurement principle of the MCR 3

The sample of interest is taken up with a 1 mL syringe and dispensed into the device. The sample is then injected into the incubation loop at the same time as the primary

antibody. The incubation loop is a 1.5 meter long tube with an inner diameter of 1.0 mm. The dwell time can be adjusted by incubation times or the flow rate in order to give the antibody enough time to bind to the analyte molecules. After incubation, the mixture is passed into the flow cell and thus through the flow cell of the microarray chip where unbound primary antibodies can bind to the immobilized analyte molecules of the microarray. Primary antibodies which are already bound to analyte molecules from the sample are flushed through the flow cell and end up in the waste container. A wash cycle rinses the tubing and flow cell and thus removes the remaining unbound antibodies. Next, horseradish peroxidase-labeled secondary antibody is passed over the microarray chip and binds to the primary antibody. Another washing step rinses remaining antibody from the tubes and unbound antibodies from the microarray. The detection reagents luminol and hydrogen peroxide are then alternately drawn up in small amounts in the syringe and injected into the flow cell. The alternating pulling up of the reagents is intended to enable the reagents to be mixed as completely as possible. The resulting chemiluminescence signal on the chip surface is recorded by a CCD camera and saved on the internal hard drive. After the detection, the flow cell is rinsed again with running buffer. In order to regenerate the microarray chip, regeneration buffer is passed over the microarray chip followed by a final rinse with running buffer. A detailed description of the measurement protocol is described in chapter 4.2.5.

2.3.3 Data evaluation

A background image has to be taken after inserting a microarray chip. The software MCRVisu automatically subtracts the background from every measurement and saves them as text-files. These files are evaluated with MCRImageAnalyzer. A grid is placed over the chemiluminescence spots to ensure that the individual spots are evaluated separately. The program detects the 10 brightest pixels for each square. The mean value of these 10 pixel forms the signal intensity for a spot. All spots in a row are compared automatically and possible outliers are neglected for further data processing. The mean value and deviation of all valid measured intensities are formed within a row of spots.

3. Objective

The objective of this master's thesis was to develop a residue analysis microarray chip for milk by the example of diclofenac and sulfamethazine.

First, a method needed to be developed to immobilize the two analytes on the microarray chip surface. This posed a particular challenge as the two molecules are chemically very different and therefore two different functional groups had to be bound to the same amino-surface of the chip. Based on this, two different immobilization strategies had to be developed, tested and optimized. In order to do this, the immobilization strategies were first established for each analyte separately before combining them on the same chip surface. After the successful development of both immobilization strategies, both analytes could be combined on a single microarray chip. In addition, selectivity tests for the antibodies had to be carried out to identify possible cross reactivities. The regenerability of the microarrays also had to be examined to ensure that the microarray would deliver reliable and stable CL signals across a certain number of regeneration cycles. After the establishment of these basic components of the assay, calibrations needed to be carried out in milk to determine the LoD, IC_{50} and working range of the microarray. After this was done, improvement experiments for the diclofenac assay had to be carried out in order to decrease the respective LoD. Finally, the recovery for the assay had to be assed. This had to be done in commercially available milk and raw milk to show that the developed microarray is capable of detecting diclofenac and sulfamethazine in certain concentration ranges.

4. Materials and methods

4.1 Materials

4.1.1 Technical equipment

Device	Manufacturer
Calligrapher™ MiniArrayer	*Bio-Rad* (Eschwege, Deutschland)
Drying oven UM 400	*Memmert* (Schwabach, Germany)
Incubator HCP 108	*Memmert* (Schwabach, Germany)
Magnetic stirrer	*Heidolph* (Schwabach, Deutschland)
MCR 3	*GWK* Präzisionstechnik (München, Deutschland)
Microscale	*Mettler* (Columbus, USA)
Pipettes	*Eppendorf* (Hamburg, Deutschland)
Refrigerated/Heating Circulator F12	*Julabo* (Seelbach, Germany)
Scale	*Mettler-Toledo* (Giessen, Deutschland)
Signograph	*Proxxon* (Föhren, Deutschland)
Ultrasonic bath RK 510	*Bandelin Sonorex* (Berlin, Germany)
Vortex Mixer	*Fischer Scientific* (Hamton, USA)

4.1.2 Software

Software	Publisher
BioOdyssey™ Calligrapher 2.0	*Bio-Rad* (Eschwege, Deutschland)
ChemDraw Professional 19.0	*PerkinElmer* (Waltham, USA)
MCRImageAnalyser	*GWK* (München, Deutschland)
MCRVisu	*GWK* (München, Deutschland)
Microsoft Office Professional Plus 2013	*Microsoft* (Redmond, USA)
Origin 9.1	*OriginLab* (Northampton, USA)

4.1.3 Chemicals and standards

Chemicals	Manufacturer
Casein from bovine milk	*Sigma-Aldrich* (Taufkirchen, Germany)
Diclofenac sodium salt (DF)	*Sigma-Aldrich* (Taufkirchen, Germany)
1,4-Dioxane	*Sigma-Aldrich* (Taufkirchen, Germany)
Dimethylsulfoxid (DMSO)	*Sigma-Aldrich* (Taufkirchen, Germany)
Ethanol	*Carl Roth* (Karlsruhe, Germany)
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimid	*Sigma-Aldrich* (Taufkirchen, Germany)
(3-Glycidyloxypropyl)trimethoxysilane (GOPTS)	*Sigma-Aldrich* (Taufkirchen, Germany)
Glycine	*Sigma-Aldrich (*Taufkirchen, Germany)
Hellmanex III	*HelmaAnalytics* (Müllheim, Germany)
Hydrochloric acid (37%)	*Merck* (Darmstadt, Germany)
N-Hydroxysulfosuccinimide sodium salt (S-NHS)	*Sigma-Aldrich* (Taufkirchen, Germany)
Jeffamine ED-2003	*Huntsman* (USA)
Methanol	*Sigma-Aldrich* (Taufkirchen, Germany)
Potassium dihydrogen phosphate	*Sigma-Aldrich* (Taufkirchen, Germany)
Potassium hydrogen phosphate	*Sigma-Aldrich* (Taufkirchen, Germany)
Sulfuric acid (97%)	*Sigma-Aldrich* (Taufkirchen, Germany)
SDS	*Sigma-Aldrich* (Taufkirchen, Germany)
Sodium azide	*Sigma-Aldrich* (Taufkirchen, Germany)
Sodium chloride	*Carl Roth* (Karlsruhe, Germany)
Sodium hypochlorite	*Carl Roth* (Karlsruhe, Germany)
Sodium thiosulfate	*Sigma-Aldrich* (Taufkirchen, Germany)
Sulfamethazine sodium salt (SMA)	*Sigma-Aldrich* (Taufkirchen, Germany)

4.1.4 Materials

Materials	Manufacturer
Centrifuge tubes	*Carl Roth* (Karlsruhe, Germany)
Disposable cannula	*B Braun* (Melsungen, Deutschland)
Eppendorf tubes	*Eppendorf* (Hamburg, Deutschland)
Gloves	*Carl Roth* (Karlsruhe, Deutschland)

Microscope slides (corners grounded)	*Carl Roth* (Karlsruhe, Deutschland)
Pipette-tips	*Carl Roth* (Karlsruhe, Deutschland)
Syringes	*B Braun* (Melsungen, Deutschland)
2-channel PMMA carrier	*IWC* (München, Deutschland)
96 well plate	*Greiner Bio-One* (Frickenhausen, Deutschland)

4.1.5 Buffers and solutions

Ultrapure water was used for the preparation of all buffers.

PBS buffer (10x)

K_2HPO_4, 122 g

KH_2PO_4, 13.85 g

NaCl, 85 g

Water, ad 1000 mL

Running buffer

Casein, 5.0 g

PBS (10x), 100 mL

Water, add 900 mL

Heating to 90 °C while stirring until casein is dissolved

Regeneration buffer

Glycine, 7.51 g

NaCl, 5.87 g

SDS, 1.0 g

Water, ad 1000 mL, pH = 3.0 (HCl)

Spotting buffer

S-NHS, 1.6 mg

EDC, 1.6 mg

PBS (1x), ad 400 µL

4.1.6 Antibody preparation

The lyophilized monoclonal primary antibody anti-diclofenac (12 G5, *Squarix*, Marl, Germany) was reconstituted by adding 1 mL of 0.05% sodium-azide in 1xPBS. The stock solution was stored in the refrigerator.

The monoclonal primary antibody anti-sulfamethazine (4D9, 0.5 mg/mL in 0.09% sodium azide solution) was produced and kindly provided by the Chair of Hygiene and Technology of Milk (LMU Munich, Germany). The stock solution was stored in the refrigerator.

The HRP-labeled anti-mouse IgG (H + L) secondary antibody was purchased from Sigma-Aldrich (RABHRP1, Taufkirchen, Germany) and stored in the freezer at -20 °C. The antibody solutions were freshly prepared every time. Dilutions were prepared in running buffer according to Table 1 if not mentioned otherwise.

Table 1: Dilution factors for the antibody solutions used for measurements at the MCR 3.

Antibody	**Dilution factor**
Anti-sulfamethazine	1:1500
Anti- diclofenac	1:2000
Anti-mouse IgG	1:1000

4.1.7 Preparation and coating of glass slides

Cleaning and activation

The DAPEG coated microarrays were produced in batches of 80. The glass slides were engraved with a sinograph to be numbered and subsequently incubated in a 2% Hellmanex solution with sonication for 30 minutes. Afterwards the Hellmanex solution was renewed and in this solution, the glass slides were incubated overnight. After incubation the glass slides were sonicated for 30 minutes and washed 5 times with a total of 1 L with water by shaking by hand. Next, the glass slides were treated for one hour with a freshly prepared solution consisting of concentrated hydrochloric acid and methanol (1:1). After washing 5 times with 1 L of fresh water, the glass chips were immersed in 97% sulfuric acid for one hour. The sulfuric acid was washed off by

washing 5 times with 1 L of water, and finally the glass slides were dried in a nitrogen stream and placed in the drying oven for 15 minutes at 70 °C.

Silanization

A volume of 600 µL GOPTS was pipetted onto the surface of the glass slide and a second one was placed on top. The so prepared sandwich was incubated for 3 hours at room temperature. Afterwards the sandwich was separated and washed in ethanol. The glass slides were stored in fresh ethanol until continuing the washing procedure that included sonicating for 15 minutes in ethanol, methanol and ethanol respectively. After washing, the glass slides were dried in a stream of nitrogen and placed in the drying oven for 15 minutes at 70 °C.

Jeffamine® coating

The glass slides were coated via the previously explained sandwich method with 600 µL of molten Jeffamine® and placed in the drying oven at 100 °C for overnight incubation. On the next day, the glass slides were separated and washed thoroughly in water and sonicated subsequently for 15 minutes. Afterwards, they were dried in a nitrogen stream and placed in the drying oven for 15 minutes at 70 °C. The so prepared glass slides were stored in a nitrogen flooded desiccator until further use.

4.1.8 Spotting and assembly of microarray chips

The micro-contact printer Calligrapher™ MiniArrayer was used to perform the spotting procedure. The device is controlled by the BioOdyssey™ Calligrapher 2.0 software. The humidity and temperature in the spotting chamber were adjusted to 25 °C and a relative humidity of 55%. To ensure that the contact printing solid pin (SciFlexArrayerS1) is unpolluted, it was washed 5 times with water. The solutions to be spotted were pipetted in a 96 well plate (300 µL per well) which was then placed in the micro-contact printer. For each solution, a row of five spots was spotted on the surface of the modified glass chip.

Immobilization of sulfamethazine

Sulfamethazine was immobilized in two steps. The first step consisted of spotting DE-PEG mixed with water (1:1) and subsequent incubation at 100 °C overnight. Afterwards the excessive DE-PEG was removed by washing five times in water and

drying in a nitrogen stream. The second step included spotting of a sulfamethazine solution (10 mg/mL sulfamethazine in carbonate buffer) on top of the DE-PEG spots. After spotting, the glass chips were placed in an incubator at a temperature of 25 °C and a relative humidity of 55% or left in the spotting chamber for incubation overnight.

Immobilization of diclofenac

300 µL of a diclofenac solution (0.13 mg/mL diclofenac dissolved in 90% dioxan and 10% 1x PBS) was mixed with 100 µL of a freshly prepared spotting buffer. The final diclofenac concentration was 0.10 mg/mL. The spotting solution was incubated for 1 hour at room temperature and spotted subsequently. After the spotting procedure, the glass chips were placed in an incubator at a temperature of 25 °C and a relative humidity of 55% or left in the spotting chamber for incubation overnight.

Immobilization of sulfamethazine and diclofenac

In order to immobilize diclofenac and sulfamethazine on the same microarrays, both immobilization methods had to be combined. First, only the DE-PEG solution was prepared and spotted. The microarray chips were then incubated at 100 °C. overnight. Afterwards the excessive DE-PEG was removed by washing five times in water and drying in a nitrogen stream. Next, the spotting solutions with diclofenac and sulfamethazine were prepared as described previously. When filling the spotting solutions into the cavities of the microtiter plates, it was important that sulfamethazine was in the first place. This ensured that the sulfamethazine solution was spotted on top of to the already pre-spotted DE-PEG layers. The diclofenac spotting solution was in second place and was thus applied directly to the modified glass surface. After the spotting procedure, the glass chips were placed in an incubator at a temperature of 25 °C and a relative humidity of 55% or left in the spotting chamber for incubation overnight.

Assembly of microarray chips

The fully assembled microarray chip persisted of the glass slide, a 2-channel PMMA carrier and a double sided adhesive PE film which keeps the glass slide and carrier together and creates a gap within the cut out areas (Figure 15). These gaps create the flow cells. When assembling the microarray, the PMMA carrier was first joined together with adhesive film. Then, the glass slide was combined with the adhesive film with the

spots on the glass surface facing the PMMA carrier. To finish assembly, the microarray chips were pressed firmly together to tighten the connection and avoid leaking while measuring. As a last step, the microarray chips were filled with 55 µL of PBS and were stored in the refrigerator until use.

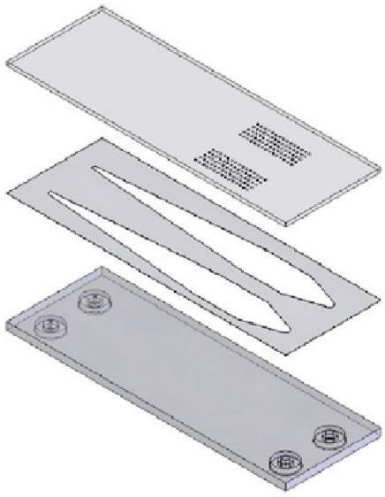


Figure 15: Assembly of microarray-chips with glass slide (top), adhesive PE film (middle) and PMMA carrier (bottom).[100]

4.1.9 MCR 3 measurements

The MCR 3 was controlled by the MCRVisu Software. First, a washing program was carried out twice to flush all tubes, syringes and valves with water. Afterwards the storage vessels for running- and regeneration buffer, as well the detection reagent luminol and hydrogen-peroxide and the 50 mL syringes, containing the primary and secondary antibodies, were filled and installed. A loading program was carried out to fill all tubes with the appropriate solutions. Afterwards, a microarray chip was inserted and a blank program flushed the flow cell with running buffer and a background image was recorded. The background image was subtracted automatically from all subsequent measurements. Before the actual measurements could be carried out, two measurements were executed which were not taken into account in the data evaluation. Each measurement was carried out like described in Table 2 if not mentioned otherwise.

Table 2: Measuring program of the MCR 3.

Step	Volume	Flow/Time
Injection of sample + PAB-solution (1:1)	1000 µL	10 µL/s
Flushing with running buffer	2000 µL	500 µL/s
Injection of SAB-solution	1000 µL	10 µL/s
Flushing with running buffer	2000 µL	500 µL/s
Injection of luminol and hydrogen peroxide	200 µL each	68 µL/s
Image acquisition		60 s
Flushing with running buffer	2000 µL	500 µL/s
Flushing with regeneration buffer	3000 µL	1000 µL/s
Flushing with regeneration buffer	1000 µL	10 µL/s
Flushing with running buffer	2000 µL	500 µL/s

At the end of a day after the last measurement the washing program was carried out once with a 0.1% sodium hypochlorite solution for disinfection purposes and once with a 0.2% sodium thiosulfate solution to neutralize the hypochlorite. At last the washing program was carried out two times with water.

4.2 Methods

In this section the preparations for the different experiments are explained and the order of measurements is explained. Separate stock solutions were prepared in ultrapure water with concentrations of 1.0 mg/mL for diclofenac and 10 mg/mL for sulfamethazine.

4.2.1 Calibration experiments

To prepare the calibration solutions, all dilutions were carried out in the required matrix. For the calibration of diclofenac in water a 1:1000 dilution of the stock solution was prepared in a 15 mL centrifuge tube. The resulting solution with 1000 µg/L was then further diluted six times with 1:10 dilutions each in 2 mL Eppendorf reaction tubes until a concentration of 0.001 µg/L was reached (Figure 16). Thus 7 calibration solutions in the concentration range from 1000 µg/L to 0.001 µg/L were prepared in steps of 10^1. For calibrations in milk, calibration solutions were prepared that contained both,

diclofenac and sulfamethazine. Dilutions of 1:1000 for the stock solution of diclofenac and 1:100 for the stock solution of sulfamethazine were prepared in a 15 mL centrifuge tube. The milk with the spiked concentrations of 1000 µg/L for diclofenac and 100,000µg/L for sulfamethazine were then further diluted according to Figure 17.

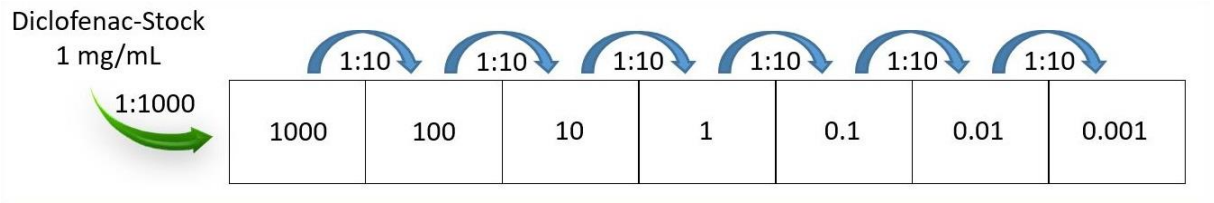


Figure 16: Dilution scheme for the preparation of the calibration solutions in water. The row represents the diclofenac concentration in µg/L.

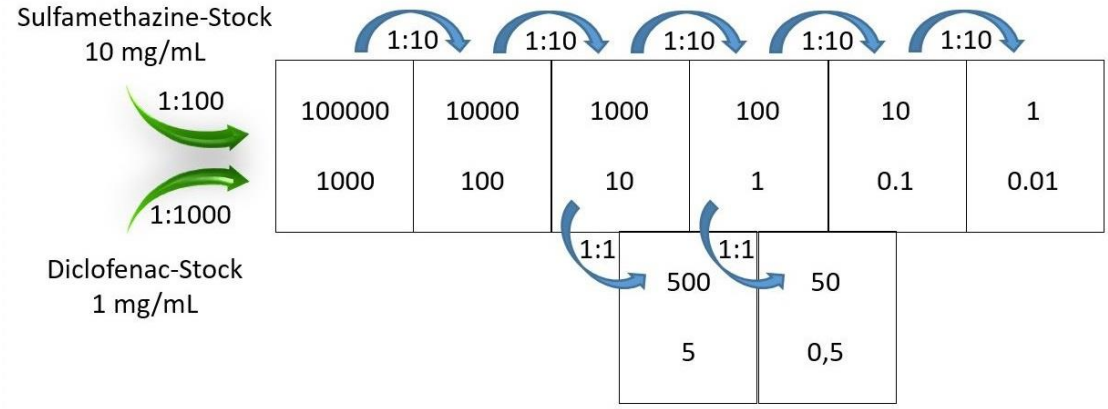


Figure 17: Dilution scheme for the preparation of the calibration solutions in milk. The upper row represents the sulfamethazine concentration and the lower row the diclofenac concentration in µg/L.

With the MCR 3, first three blank measurements were carried out in the corresponding matrix and then the calibration solutions were measured. The measuring sequence of the calibration solutions started with the lowest concentration and was followed by the next higher one. If there were further measurements after the last calibration solution, five blank measurements were carried out first and then further measurements were carried out.

4.2.2 Selectivity measurements

The identification of the selectivity of the antibodies began with measurements using a microarray chip on which diclofenac was immobilized. Subsequently, measurements were made with a microarray chip on which sulfamethazine was immobilized. The

measuring sequence was exactly the same for both microarray chips only with the opposite order of the primary antibody solutions being used.
The measuring principle is explained using the example of the diclofenac microarray chip. The microarray chip was inserted into the MCR 3 and three blank measurements were performed using the anti-diclofenac antibody. The primary antibody syringe was then removed and replaced with one that contained anti-sulfamethazine antibodies. The loading program was performed to rinse residual anti-diclofenac antibodies from the tubes and fill them with anti-sulfamethazine antibodies. Afterwards three more blank measurements were performed.

4.2.3 Improvement of LoD

Calibrations had to be carried out to see whether the measures taken to improve the LoD were successful. Single calibrations were carried out to save time and materials. The calibration solutions were prepared as already explained in chapter 4.2.1. Three calibrations were measured on three microarray chips with three different measuring programs. For each of the calibrations three blank measurements were carried out and afterwards the calibration solutions were measured in increasing concentrations.
The measuring programs were the standard and two modified programs. For this purpose, two copies of the standard program were created and one parameter was modified each. In one case, the flow rate of the primary antibody syringe and the sample syringe was changed from 60 to 10 µL/s. As a result, the antibody-analyte interaction time was extended. In the second modified program, the flow rate of the antibody-analyte solution injected into the flow cell was changed from 10 to 5 µL/s. Due to the reduced flow rate through the flow cell, the competition time on the microarray chip was increased.

4.2.4 Recovery experiments

The recoveries were performed following to the duplex calibrations. There were three times two recoveries each, one in UHT- and one in fresh milk. The concentrations of the five recoveries were adjusted so that they were within 20 to 80% of the maximum CL-signal intensity. The concentrations are shown in Table 3.

Table 3: Concentrations of the solutions for the recovery experiment.

Solution No.	Referring to the maximum CL-signal	Diclofenac in µg/L	Sulfamethazine in µg/L
1	30%	0.50	10
2	40%	0.75	15
3	50%	1.00	25
4	60%	1.50	50
5	70%	2.00	75

To prepare the solutions, separate dilutions of the diclofenac and sulfamethazine stock solutions were prepared in the respective matrices. In each case, a 1:100 and then a 1:1000 dilution were prepared in centrifuge tubes, resulting in concentrations of 10 µg/L for diclofenac and 100 µg/L for sulfamethazine. The solutions were then pipetted into 1-ml reaction tube according to the pipetting scheme shown in Table 4.

Table 4: Pipetting scheme for the preparation of recovery solutions. The values are indicated in µL.

Solution No.	1	2	3	4	5
diclofenac solution	0.075	0.1125	0,150	0,225	0,300
sulfamethazine solution	0,150	0,225	0,375	0,750	1,125
Milk	1,275	1,1625	0,975	0,525	0,075

4.2.5 Data evaluation

The MCRVisu software saved the images as text files with the dark image already subtracted. These text files were evaluated using MCRImageAnalyzer. A grid was placed over the spots to ensure that the individual spots were evaluated separately. The program calculated the mean value of the 10 brightest pixels for each of the squares. Then the mean value and deviation of the spots were calculated for each row. It was possible to have single incorrect or significantly different values, those were neglected in the calculations.

During further data processing the background signal was subtracted from the measured values. The background signal could be observed where no analytes were immobilized and varied for different microarray chips and measurement days. The data were thus ready for comparison or calculation of the relative signal intensity B/B_0 (Equation 2) and the relative standard deviation (Equation 3).

Calibrations were plotted using the relative signal intensities B/B0 and logarithmic analyte concentrations. The sigmoidal curve fitting was performed by Origin 9.1 using sigmoidal logistic curve fitting, which also provided the variables for setting up the curve equation (Equation 1) that was used to calculate the analyte concentrations, working range, and LoD.

5. Results and discussion

5.1 Immobilization strategies

To immobilize diclofenac and sulfamethazine properly, a spotting solution with suitable conditions like the composition of solvents and the concentration of analytes needed to be identified. For a time and effort efficient investigation it was advantageous to immobilize the two analytes on separate microarray chips first.

5.1.1 Immobilization of diclofenac

5.1.1.1 Optimization of the spotting solution

The first task was to investigate which solvent composition would lead to the best immobilization result. Therefore, microarray chips were spotted using different compositions of solvents with constant diclofenac concentration in the spotting buffer. The concentration was adjusted to 3 mg/mL, since in previous studies a diclofenac concentration in the mg/mL range was used for immobilization.[101] The composition of the solvents used for the first experiment is shown in Table 5.

Table 5: Composition of the tested spotting solutions. The numerical values are given in percent.

Buffer name	PBS	Carbonate buffer	Dioxane	DMSO
90% dioxane in PBS	10	-	90	-
75% dioxane in PBS	25	-	75	-
100% DMSO	-	-	-	100
50% DMSO in carb. buffer	-	50	-	50
50% DMSO in PBS	50	-	-	50
PBS	100	-	-	-

The immobilization routine was carried out whereby microarrays with a 6 x 4 matrix of spots were produced. Six rows for the different solutions and four replica per row. The best method is classified so that the signals are as high as possible and the spots can be evaluated as much as possible, therefore blanks were measured. Measurements were done in triplicates.

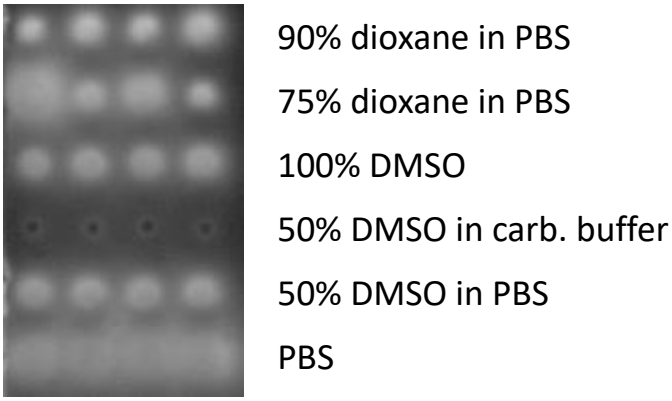


Figure 18: CL image of a blank measurement of the diclofenac microarray. Different spotting solutions were used for the immobilization of diclofenac.

The properties of the spots can be compared in Figure 18, which shows one of the blank measurements. The spotting solution consisting of 100% PBS did not lead to a formation of defined spots. It looks like spots converged, leading the immobilized diclofenac to form a bright band. In the case of 50% DMSO in carbonate buffer, the spots are indicated but not completely developed, they appear to be dark compared to the others. In this row the immobilization of diclofenac was insufficient. The solution consisting of 75% dioxane in PBS seemed to form irregular spot sizes. All other solutions lead to defined spot shapes. Their average spot size is about 0.5 mm.

The absence of CL in the case of 50% DMSO in carbonate buffer can be a result of minimal immobilization efficiency. The carbonate can interfere with the reaction of EDC with the carboxyl group of diclofenac, which is shown in Figure 12. If the carbonate in the solution consumes the EDC it would lead to less modified diclofenac that is available to bind on the surface. The different spot sizes for the solution with 75% dioxane may not have a specific cause but as a precaution, this solution was neglected for further experiments. With the spotting solutions consisting of 90% dioxane, 100% DMSO and 50% DMSO in PBS spots were created which were suitable for further experiments.

Figure 19 shows the mean values of the maximum CL-signals and their deviations in a.u. The 90% dioxane solution had with about 15000 a.u. the highest CL intensity with a relatively small deviation of 1.8%. The least intensity belonged to the solution consisting of 50% DMSO in carbonate buffer, which is a result of insufficient immobilization of diclofenac. The other solutions have medium CL intensities between 12000 and 9000 a.u and a relative standard deviation between 1.1% and 34.9%. The

spotting solution consisting of 90% dioxane in PBS was one of the three solutions that formed defined spots and produced the highest signal intensity with minimal deviation. For these reasons all further experiment were carried out with the spotting solution for diclofenac consisting of 90% dioxane and 10% PBS.

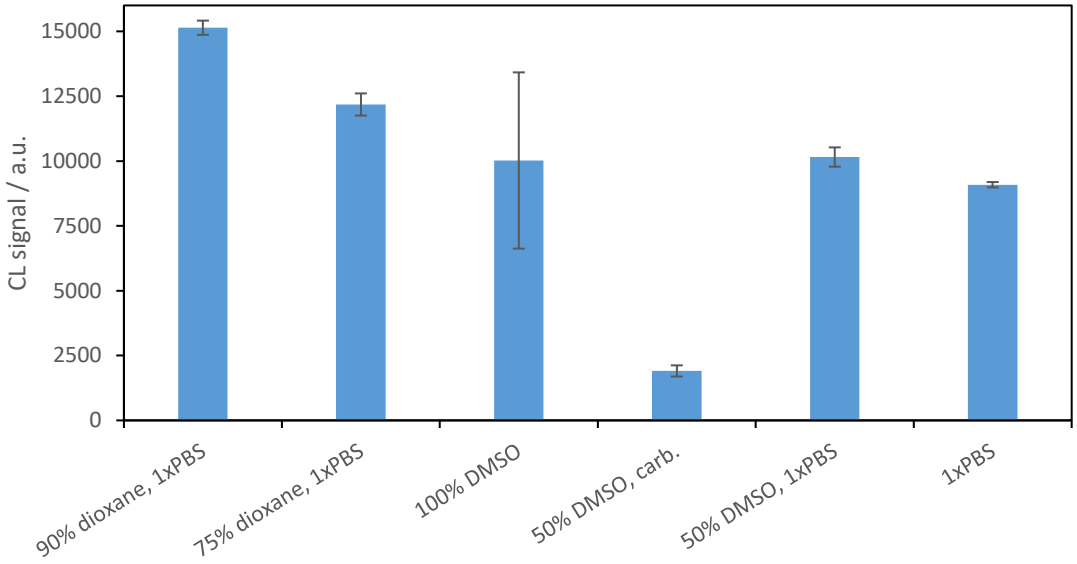


Figure 19: Effect of the different spotting solutions for diclofenac on the maximum CL-signal intensities (m=3).

5.1.1.2 Optimization of the diclofenac concentration for the spotting process

The concentration of diclofenac in the spotting solution can have an impact on the immobilization efficiency and the performance of the microarray as well. To investigate this question, microarrays were produced using spotting solutions with diclofenac concentrations of 1, 2, 3, 4 and 5 mg/mL. Blank measurements in triplicate were carried out. An exemplary CL image of one measurement is shown in Figure 20.

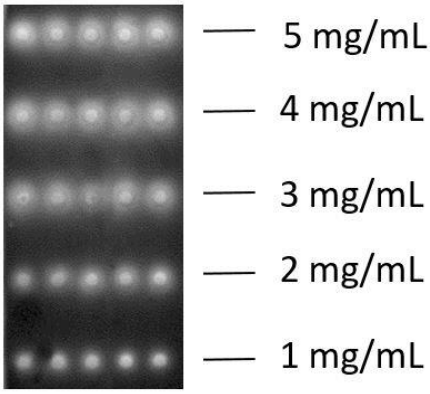


Figure 20: CL image of a blank measurement of a diclofenac microarray. Different diclofenac concentrations (between 1 and 5 mg/mL) were used in the spotting solutions.

The total spot size increased from a diameter of about 0.35 mm to 0.70 mm with increasing diclofenac concentration in the spotting solution. All spots have a bright center with an average diameter of about 0.35 mm. Lower concentrations result in a more defined spot with less corona effect. A reliable microarray, should have defined and homogeneous spots. As can be seen in Figure 20, this strongly depends on the concentration of the spotted analyte. The homogeneity increases and the tendency to form coronas decreases with decreasing diclofenac concentration in the spotting solution. To investigate this trend, new microarrays were produced using spotting solutions with diclofenac concentrations of 0.01, 0.05, 0.1, 0.5 and 1.0 mg/mL. Blank measurements with three replicates were carried out. A CL-image of an exemplary blank measurement is shown in Figure 21.

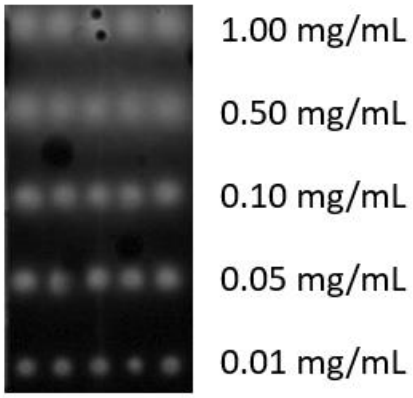


Figure 21: CL image of the blank measurement of diclofenac microarray. Different diclofenac concentrations (between 0.01 and 1.0 mg/mL) were used in the spotting solutions.

The spots for 0.1 mg/mL and below show no corona. The size decreases from a diameter of about 0.6 mm for 1.0 mg/mL to 0.3 mm for 0.01 mg/mL. The spots above a diclofenac concentration of 0.1 mg/mL do not have clear edges, they are fading with increasing distance to their center. The blank CL signal intensities are shown in Figure 22. The CL intensity is decreasing from 16800 to 9500 a.u. with decreasing diclofenac concentration in the spotting solutions.

Of the five different diclofenac concentrations in the spotting buffers, the one with 0.1 mg/mL was rated as best. The shape of the spots is defined, they are sufficiently large but clearly separated from each other and the CL intensity of 13,700 a.u. is sufficiently high. From here on, all diclofenac microarray chips were spotted with a spotting solution containing a diclofenac concentration of 0.1 mg/mL.

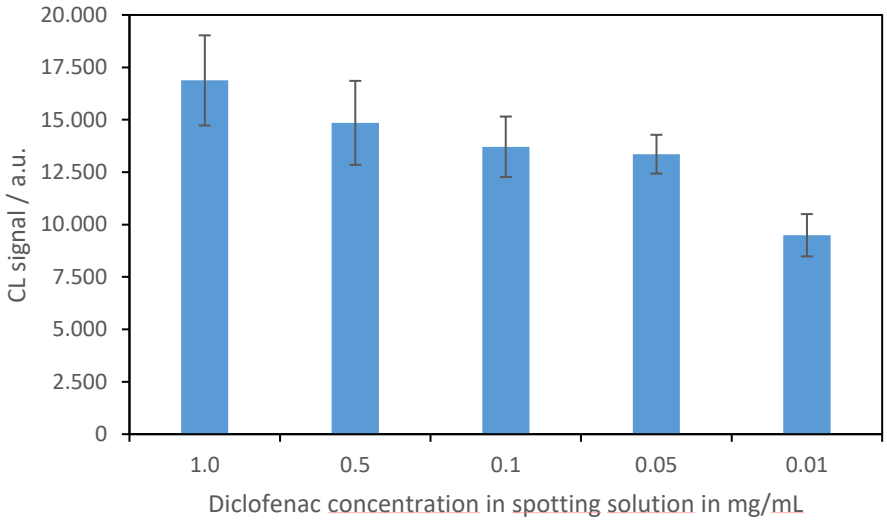


Figure 22: CL-signal intensities for the blank measurements of different diclofenac concentrations in spotting solution (m=3).

5.1.2 Immobilization of sulfamethazine

5.1.2.1 Immobilization using DMS as crosslinker

First attempts to immobilize sulfamethazine were carried out using DMS as crosslinking reagent. The spotting solution was prepared by diluting the sulfamethazine stock solution and subsequent mixing with a DMS solution to achieve final concentrations of 10 µg/mL for sulfamethazine and 50 mM for DMS. After mixing sufficiently the solution was instantaneously spotted on the microarray chip and

afterwards incubated over night at 25 °C at 55% relative humidity. Blank measurements were carried out but no spots could be detected on the microarray (data not shown). The absence of a CL signal can be attributed to the fact that no sulfamethazine has been immobilized. The terminal imidoester groups of DMS are reactive towards primary amino groups. The fact that no sulfamethazine was immobilized lead to the assumption that the reaction between the imidoester and amino groups immediately started when combining the solutions and that the amidine bond formation was already complete before the solution came in contact with the microarray chip. This means that this procedure was not an option for immobilizing sulfamethazine.

Another attempt to immobilize sulfamethazine using DMS as a crosslinking agent consisted of two spotting cycles. The first would spot a 50 mM DMS solution on the microarray chip. A second run would spot the sulfamethazine solution on top of it. The idea behind this experiment was to let the crosslinking agent react with the amino groups first. In the best case, only one of the two imidoesters per crosslinking molecule would bind to the surface. The second one could then react with the amino group of sulfamethazine and thereby immobilize it. Blank measurements were carried out to investigate the immobilization efficiency. The results were hardly visible CL signals. It can be concluded that only a small amount of sulfamethazine was immobilized, which is not suitable for this assay. The immobilization of sulfamethazine using the crosslinking agent DMA was unsuccessful and was not pursued further.

5.1.2.2 Immobilization using DE-PEG as crosslinker

Another attempt to immobilize sulfamethazine was to use DE-PEG as crosslinking agent. It was already used for the immobilization of antibiotics in the production of the milk chip.[15] The difference is that not the entire chip surface was activated with DE-PEG, but only the areas where sulfamethazine should be immobilized. This was done in two spotting cycles immediately to avoid the same problem as previously mentioned. A layer of DE-PEG was applied during the first cycle, and after incubation and washing of the microarray chip, a layer of sulfamethazine (10 mg/ml) was applied in the second spotting cycle. DE-PEG with a molar mass distribution of 500 is a relatively viscous liquid compared to aqueous solutions. The high viscosity could negatively affect the

spotting process, which was prevented by diluting DE-PEG. Five different dilutions with proportions of 10, 20, 30, 40 and 50 vol.-% DE-PEG in ultrapure water were tested to immobilize sulfamethazine. Blank measurements in three replicates were carried out. A CL image of a blank measurement is shown in Figure 23.

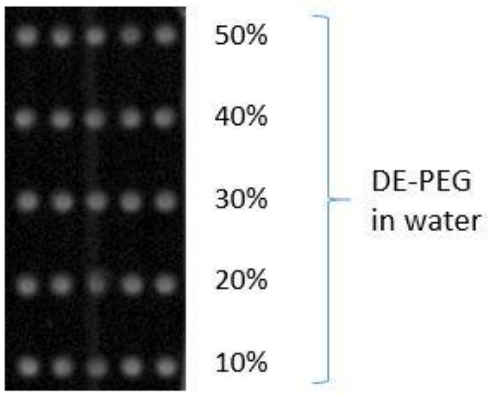


Figure 23: CL image of a blank measurement of a sulfamethazine microarray using varying DE PEG content during the first spotting cycle and a concentration of 10 mg/mL sulfamethazine for the second.

All spots were very similar, had defined shapes, clear edges and a uniform size with a diameter of about 0.5 mm. It could be concluded that a DE-PEG content between 10 to 50% did not affect the size and texture of the spots. To decide which percentage would lead to the best results for further experiments, the CL signal intensities were compared (Figure 19). The highest CL signal with 6000 AU was measured with the 50% DE-PEG solution, while the remaining DE-PEG dilutions had constant intensities of about 5000 AU. In order to be able to guarantee the highest possible CL signals, the DE-PEG solution with the highest signal intensity was chosen. From here on, all sulfamethazine microarray chips were spotted using a 50% DE-PEG solution for the first spotting cycle and a concentration of 10 mg/mL sulfamethazine for the second spotting cycle.

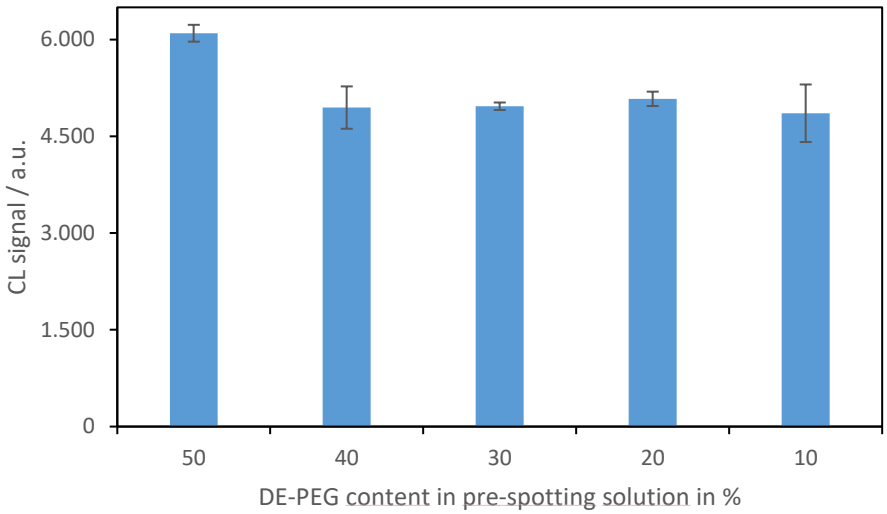


Figure 24: CL signal intensities of the blank measurements for sulfamethazine immobilized using different DE-PEG content during the first spotting cycle (m=3).

5.2 Singleplex calibrations for diclofenac

Once all the immobilization optimizations were done, the singleplex calibrations were carried out on the same microarrays which were manufactured for the development of the immobilization strategy for diclofenac. Five rows with five replicates each were immobilized on the microarrays. The spotting solutions had diclofenac concentrations of 0.01, 0.05, 0.1, 0.5 and 1.0 mg/mL. A total of nine calibrations were carried out, three calibrations each on three different days, on one microarray chip per day. Five blanks were measured between the individual calibrations per microarray chip to ensure a stable maximum CL signal for the next one. The concentrations of the seven calibration points ranged from 0.001 to 1000 µg/L in steps of 10. The matrix was tap water. The results are listed in Table 6. The calibration curves are shown in Figure 25.

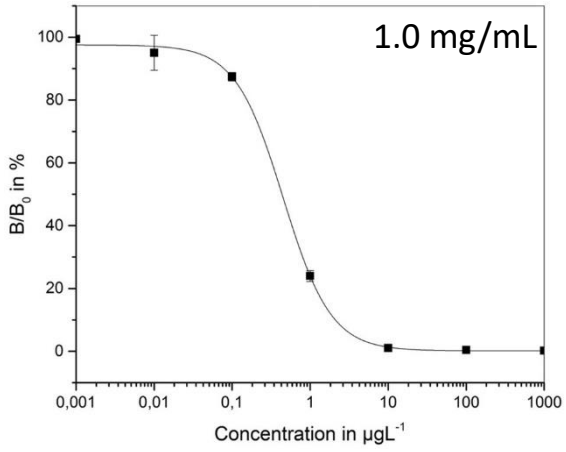
Each immobilized row was evaluated separately. The five resulting calibration curves were very similar. The average LoD for all diclofenac concentrations is 83.0 ± 11.2 ng/L, whereby the deviation is 14%. The average IC_{50} is 462.3 ± 65.7 ng/L and also has a deviation of 14%. The average working range lies between 0.202 ± 0.069 µg/L and 1.155 ± 0.107 µg/L. It could be concluded that concentration differences in the spotting solution from 0.01 to 1.0 mg/mL only had an insignificant effect on the assay performance. This is an important information, since minor errors

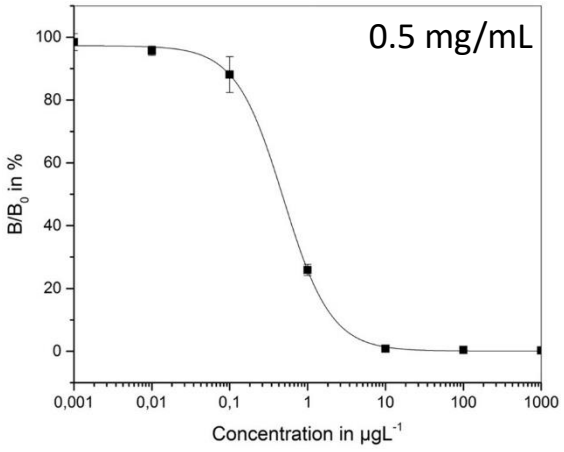
in the manufacturing of the microarray chips due to deviating diclofenac concentrations have no negative effects. Considering the individual rows, the standard deviations are also relatively small. With at least 36 measurements per microarray chip, this indicates a robust microarray, which is capable of a series of regeneration cycles.

In comparison, ELISA can achieve detection limits of up to 6 ng/L.[102] This is a factor of 10 higher than with the developed microarray. The result of 83 ng/L is below the MRL of diclofenac in drinking water, which was set at 100 ng/L.[103] The achieved detection limit would be sufficient to detect violations against the marginal limit. However, the marginal limit is outside the working range and an improvement in the performance of the diclofenac assay would be advantageous.

Table 6: LoD, IC50 and WR of calibrations for diclofenac in tap water (m=9).

Immobilized diclofenac [mg/mL]	LoD [ng/L]	IC_{50} [ng/L]	WR [µg/L]
1.00	66.4 ± 42.3	456.3 ± 65.6	0.175 – 1.193
0.50	81.9 ± 29.0	499.5 ± 102.7	0.202 – 1.275
0.10	80.4 ± 34.8	470.5 ± 101.9	0.189 – 1.206
0.05	95.1 ± 23.4	529.2 ± 295.7	0.314 – 1.101
0.01	91.3 ± 23.5	356.0 ± 53.9	0.128 – 0.998





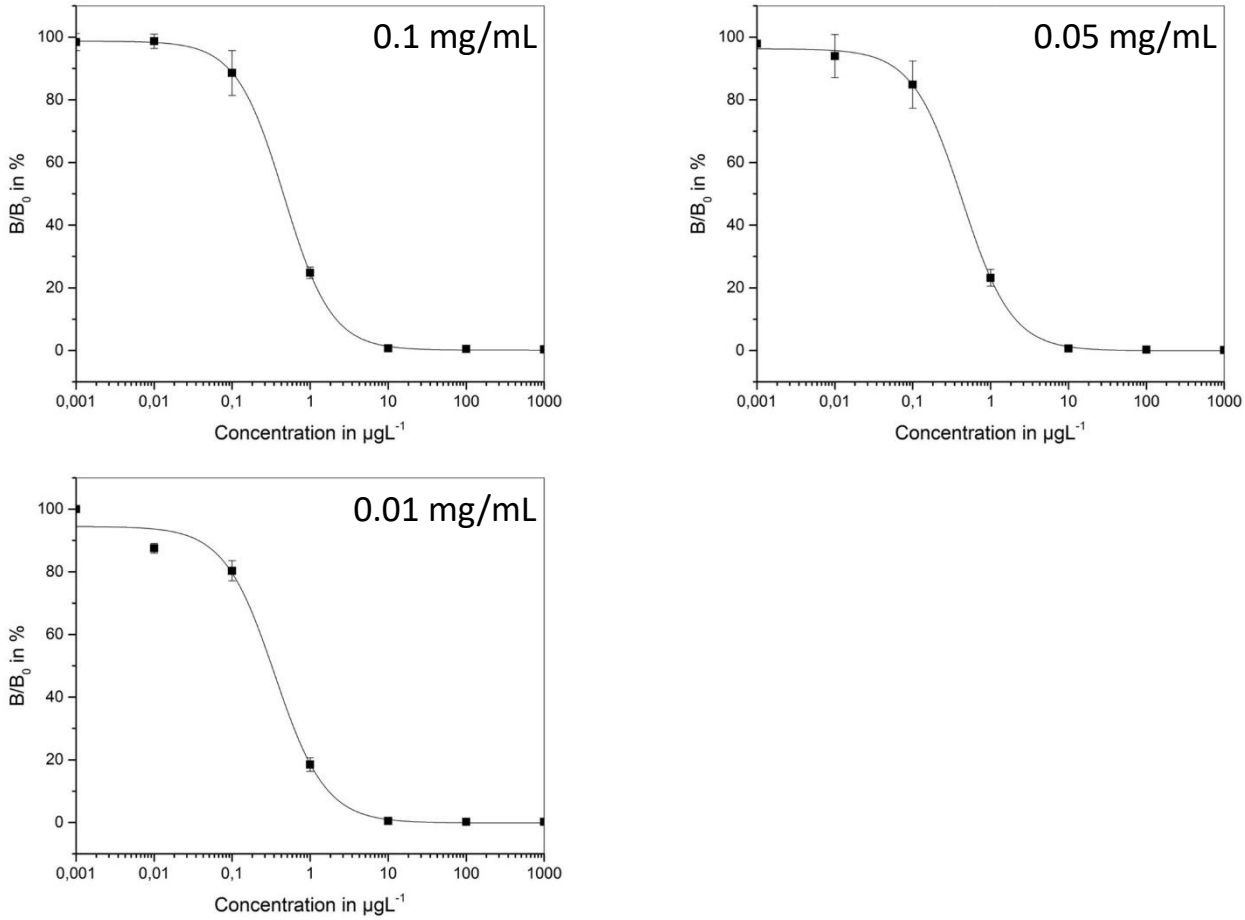


Figure 25: Five calibration curves of diclofenac with different diclofenac concentrations immobilized on the microarray chip.

5.3 Selectivity of the antibodies

In multiplex calibrations it is important to identify the selectivity of the antibodies towards the different analytes. Antibodies with unknown unspecific affinities could lead to false results. The experimental procedure, which was intended to demonstrate the selectivity of the anti-diclofenac and anti-sulfamethazine antibodies, is explained using the diclofenac microarray as an example. The microarray used was originally produced to develop the immobilization strategy. Five rows of diclofenac were immobilized on the microarray. The spotting solutions contained diclofenac concentrations ranging from 0.01 to 1.0 mg/mL.

The MCR 3 was prepared for the measurements as described in the method section. Anti-diclofenac was initially in the primary antibody solution. Three blind measurements were carried out in milk, whereby clear CL signals were measured. The primary antibody syringe was then removed and replaced with a second one containing anti-

sulfamethazine antibodies. The corresponding tubes were rinsed with the fresh antibody solution in order to remove anti-diclofenac residues and to not produce any falsified results. Then three blank measurements were carried out in milk. If the anti-sulfamethazine antibody is selective and does not bind to diclofenac, no CL signal should be detected. However, if the antibody is not completely selective and has a slight affinity for diclofenac, a CL signal would be detected.

This test procedure was carried on a sulfamethazine microarray as well. Just as of diclofenac, the sulfamethazine microarray was originally manufactured to develop the immobilization strategy. Five rows of sulfamethazine were immobilized on the microarray, DE-PEG contents ranging from 10 to 50% being used during spotting. The second test-run was meant to provide information about the selectivity of anti-diclofenac. If the antibody is selective towards diclofenac, it would not bind to the immobilized sulfamethazine and no CL signal would be detectable. The results are shown graphically in Figure 26 for the selectivity of anti-sulfamethazine antibody and in Figure 27 for the selectivity of anti-diclofenac antibody. The results are shown numerically in Table 7.

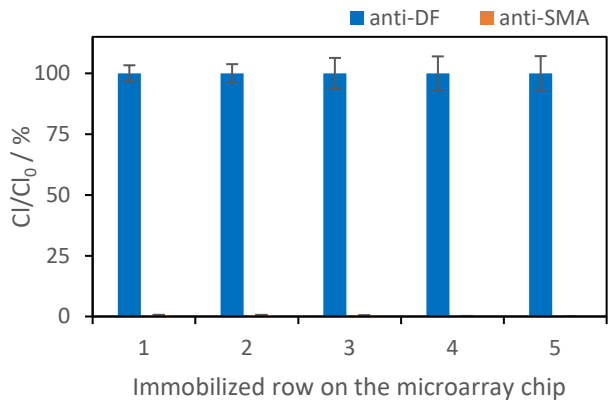


Figure 26: Selectivity testing, diclofenac microarray on selectivity of anti-sulfamethazine antibodies. 1 - 5 represents the different diclofenac concentrations in spotting solution from 0.01 to 1.0 mg/mL (m=3).

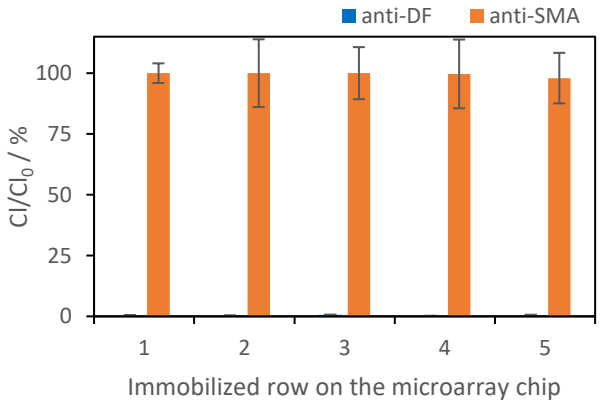


Figure 27: Selectivity testing, sulfamethazine microarray on selectivity of anti-diclofenac antibodies. 1 – 5 represents the different DE-PEG concentrations at first spotting cycle from 10 to 50% (m=3).

Table 7: Selectivity of antibodies in the multiplex system diclofenac and sulfamethazine. The values describe the affinity of the antibodies to diclofenac and sulfamethazine.

	Anti-diclofenac	**Anti-sulfamethazine**
Immobilized diclofenac	100%	0.17 ± 0.22%
Immobilized sulfamethazine	0.47 ± 0.31%	100%

For the interpretation of the measurement results, the mean values of the CL signals were calculated using the first three measurements with the antibodies correlating with the microarray. Each of the five rows were considered separately. The mean values were considered as 100% signal intensity. The error bars represent the standard deviation of the three measurements in percent. Mean values were also calculated for the signal intensities of the following three measurements with the antibodies to be examined in each case and converted into percent.

Figure 26 and Figure 27 shows the CL intensities as a percentage of the highest intensity as CL/CL_0 for each row. It can be clearly seen that almost no CL could be detected using the opposite antibody to the analyte. The numerical values show 0.17% for the selectivity of anti-sulfamethazine to diclofenac and 0.47% for the selectivity of anti-diclofenac to sulfamethazine. These are clear indications for a high selectivity of the antibodies against their corresponding analytes in the duplex immunoassay sulfamethazine and diclofenac.

5.4 Regenerability of the microarray

Regenerability is an important property for the multiple use of a microarray chip. Regeneration means removing the antibodies from the microarray surface. This is done after each measurement using a regeneration buffer, which denatures the primary and secondary antibodies. Denaturation of the antibodies is achieved by lowering the pH and using SDS. A denatured antibody loses its specific affinity for the corresponding antigens. It is important that repeated regeneration does not change the surface of the microarray and thus deteriorates its performance.

The robustness of the microarray was investigated by repeatedly measuring blanks and by comparison of the respective CL intensities. In order not to waste antibodies and time unnecessarily, an independent experiment was not carried out, instead data from the calibration experiments was used. Calibrations with subsequent recoveries were carried out on three microarray chips on three different days. Among others, measurements number 5, 15, 25 and 35 were blank measurements and were used to investigate the regenerability (Figure 28).

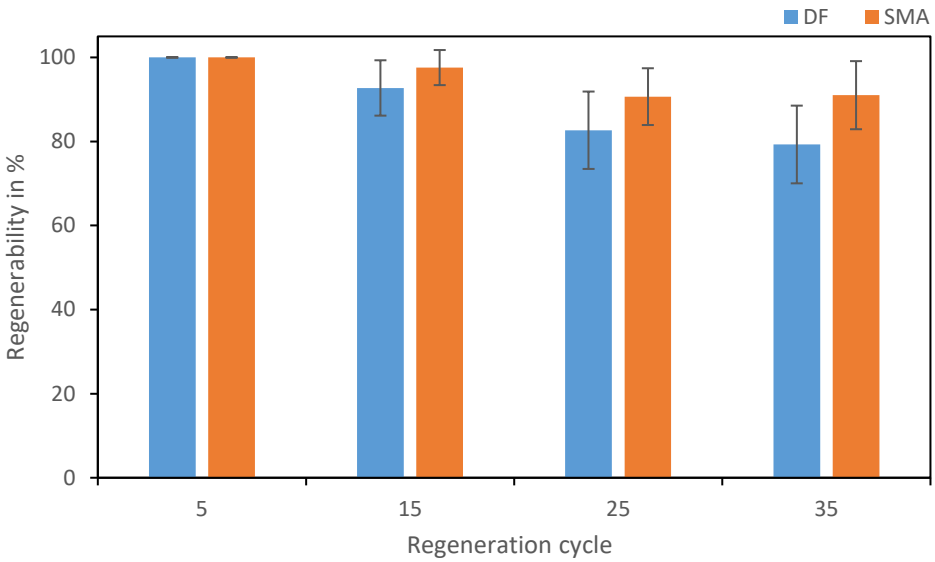


Figure 28: Regeneration of the duplex chip shown in percent of the maximum CL-intensities of blank measurements after 5, 15, 25 and 35 regeneration cycles (m=3).

Figure 28 shows the evaluation of the investigations of the regenerability. The mean values and standard deviations of the 5th, 15th, 25th and 35th depicted in percent based on the maximum CL-signal intensity, which is for measurement number five. The signal loss of the 35th measurement cycle compared to the 5th cycle is 20.7 ± 9.2% for diclofenac and 9.0 ± 8.1% for sulfamethazine. A decrease in the intensity of the CL signal can be explained by loss of immobilized antigen, non-specific binding or by structural changes in the immobilized molecules. Nevertheless, quantitative detection measurements can be carried out successfully within the 35 measurement cycles.

5.5 Duplex calibrations in milk

In order to be able to deduce an analyte concentration in the sample from the measured CL signals, calibrations are necessary. Three duplex calibrations in milk were carried out on three different days on three microarray chips, each with a row of immobilized diclofenac and a row of sulfamethazine. Before the actual calibrations, three blank measurements were carried out. The seven-point-calibrations were then measured. The concentration ranges of the calibrations were tested in advance. Calibrations were carried out for diclofenac in the concentration range from 0.01 to 100 µg/L and for sulfamethazine within 1 to 10000 µg/L.

The calibrations for diclofenac and sulfamethazine are plotted as relative signal intensity B/B_0 against the logarithmic concentration in µg/mL (Figure 29). The LoD, IC_{50} and WR are given in Table 8. The calibrations were reproducible. The maximum CL-signals varied between the individual days by 11% for diclofenac and 32% for sulfamethazine. The difference in CL intensity can be caused by fresh chemiluminescence reagents, buffers or antibody solutions. The use of different microarray chips can also have an impact on the CL-signals. The MRL (100 µg/L) of sulfamethazine is at the upper limit of the working range. This means that sulfamethazine residues in milk can be detected precisely. The LOD of diclofenac (0.264 µg/L) is well above the MRL of 0.1 µg/L. Compared to the calibration in water, the matrix milk leads to an increase of the LoD from 0.083 to 0.264 µg/mL.

Milk contains many proteins and fat which could interfere with the immunoassay.[104] For the same reasons, milk is significantly more viscous than water. This change in viscosity could lead to an increased diffusion coefficient and negatively influence the antibody-analyte interaction or the competition on the chip surface. An attempt was made to increase the sensitivity of the diclofenac assay. Possible solutions could be a longer antibody-analyte interaction time or a longer competition time on the surface of the microarray chip. Experiments and results concerning this question are described in section 5.6.

Table 8: LoD, IC_{50} and WR of calibrations for diclofenac and S sulfamethazine MA in milk (m=3).

Analyte	LoD [µg/L]	IC_{50} [µg/L]	WR [µg/L]
Diclofenac	0.264 ± 0.076	1.005 ± 0.035	0.468 – 2.158
Sulfamethazine	8.0 ± 7.9	24.9 ± 2.8	6.1 – 102.3

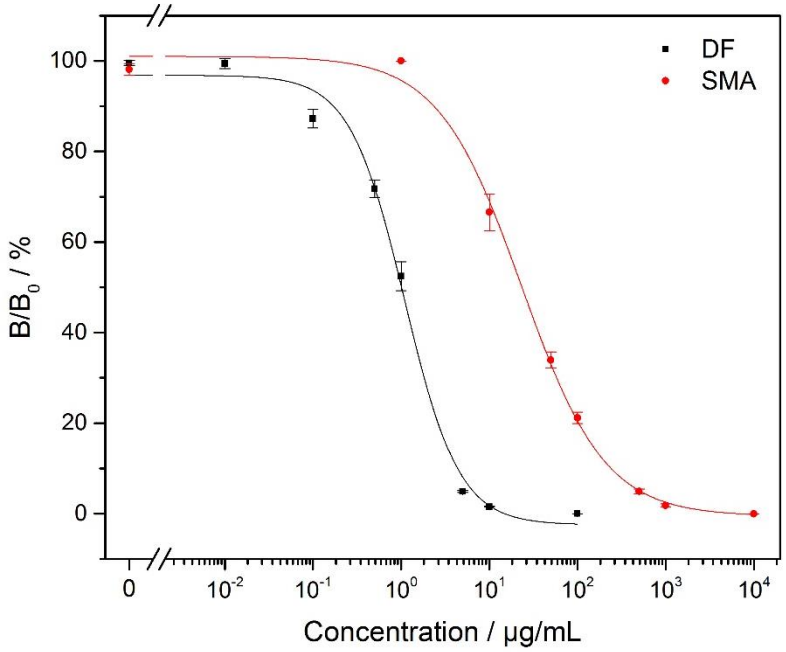


Figure 29: Calibration curves for diclofenac and sulfamethazine in milk (m=3).

5.6 Strategies for the improvement of the LoD in the diclofenac assay

Two different strategies were followed to improve the LoD in the diclofenac assay. On the one hand, the time of the antibody-analyte interaction was extended. The flow rate through the incubation loop was reduced. In the standard assay, the sample and primary antibody solution were injected simultaneously into the incubation loop with a flow rate of 60 µL s. In order to create a longer interaction time, the flow rate was reduced to 10 µL/s. The second strategy was to prolong the competition time on the chip surface. In the standard assay, the sample-antibody mixture was passed from the incubation loop into the flow cell with a flow rate of 10 µL/s. The duration of this step was doubled by reducing the flow rate to 5 µL/s. In order to examine effects on the performance of the developed microarray, three calibrations were carried out in milk. One with the standard assay, one with a longer antibody-analyte interaction time and one with an extended competition time on the microarray chip. A separate microarray was used for each of the three calibrations. One row of each, diclofenac and sulfamethazine were immobilized. The calibrations were carried out simultaneously for diclofenac and sulfamethazine in concentration ranges between 0.001 to 1000 µg / L and 0.1 to 100,000 µg / L.

The calibration curves are shown in Figure 30 for diclofenac and in Figure 31 for sulfamethazine. Since only single calibrations were carried out, no standard deviation could be specified. The graphs clearly show that the curves for diclofenac are almost congruent. For sulfamethazine, the curves show a slight deviation, but are still very close to each other. The gradients in the quasi-linear WR show no significant change for the different assay approaches. Table 9 shows LoD, IC_{50} and WR of the calibrations. These values show only slight deviations between the different assays. There is no trend for a significant change in assay performance. This experiment has thus shown that a longer antibody-analyte interaction or a longer competition time on the chip surface does not improve the LoD.

Table 9: LoD, IC50 and WR of calibrations for diclofenac and sulfamethazine in milk using different assay (m=1).

Analyte	Assay approach	LoD [µg/L]	IC_{50} [µg/L]	WR [µg/L]
DF	Standard assay	0.245	0.979	0.498 – 1.926
	Longer antibody-analyte interaction	0.387	1.175	0.579 – 2.386
	Longer competition time	0.425	1.018	0.476 – 2-176
SMA	Standard assay	7.0	15.3	5-5 – 42.8
	Longer antibody-analyte interaction	4.5	19.4	5.7 – 65.8
	Longer competition time	9.4	28.4	7.5 – 107.6

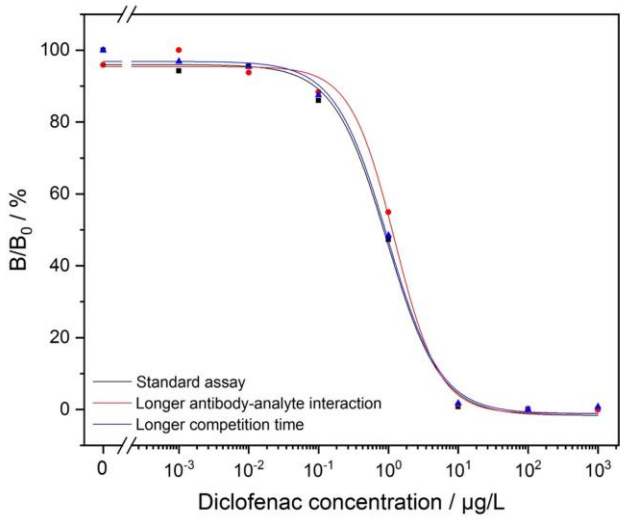


Figure 30: Calibration curves for diclofenac using different assay approaches in milk (m=1).

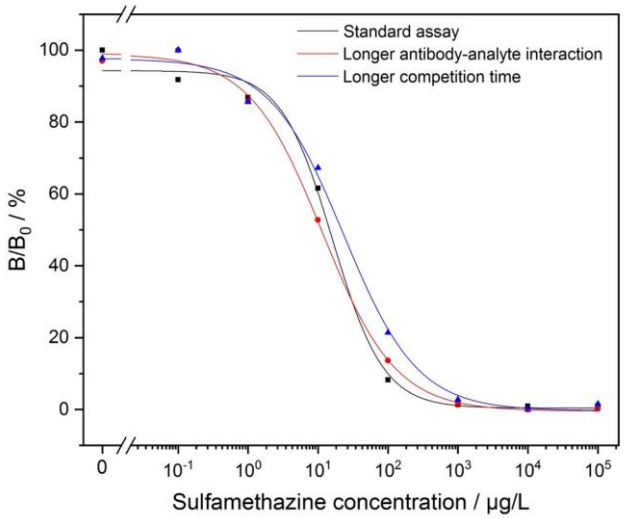


Figure 31: Calibration curves for sulfamethazine using different assay approaches in milk (m=1).

5.7 Recovery experiments

Recovery experiments were carried out in UHT and fresh milk. The UHT milk had a fat content of 1.5% and was purchased in a local store. The fresh milk came directly from a local dairy farm with a size of 50 dairy cows. It was milked on March 1st 2020 and consisted of morning and evening milk. Five samples with different diclofenac and sulfamethazine concentrations were prepared. The concentrations of the samples were chosen so that they lie within 20 to 80% of the maximum CL signal intensity, which is the working range of the calibrations. The recovery-rates were measured immediately after the calibrations. Before that, five blank measurements were carried out in the corresponding matrix. Three identical recovery experiments were carried out on three different microarray chips on three different days. The recovery rates are the percentage ratio of the experimentally determined concentrations to the exact spiked concentrations. The results are shown graphically in Figure 32, Figure 33 and numerically in Table 10. Diclofenac has the best averaged recovery rate of 99 ± 7% in UHT milk. Sulfamethazine shows somewhat higher results in the same matrix with an average of 124 ± 12%. The average recovery rates in fresh milk as a matrix are significantly higher with 142 ± 9% for diclofenac and 147 ± 10% for sulfamethazine. The clear over-determination in fresh milk likely comes from a change of matrix properties. The calibrations were carried out in UHT-treated milk with a defined fat content of 1.5%. The fresh milk was untreated and the exact fat content is unknown. Untreated milk can contain up to 5% fat.[104] Calibrations in this matrix would be necessary to examine the influence of the changed matrix on the performance of the developed assay.

Table 10: Recoveries for diclofenac and sulfamethazine in UHT milk and fresh milk.

DF	c in µg/L	0.50	0.75	1.0	1.5	2.0
	UHT milk	95 ± 8	90 ± 8	99 ± 6	106 ± 5	107 ± 4
	fresh milk	133 ± 2	138 ± 11	143 ± 10	138 ± 11	156 ± 10
SMA	c in µg/L	10	15	25	50	75
	UHT milk	108 ± 17	113 ± 20	132 ± 5	130 ± 8	136 ± 8
	fresh milk	133 ± 21	154 ± 7	152 ± 6	143 ± 9	156 ± 15

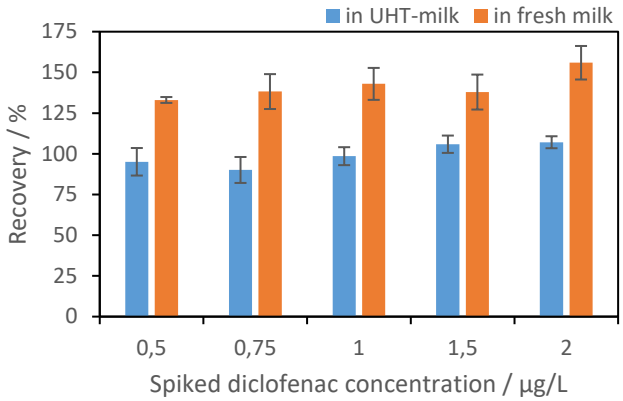


Figure 32: Recoveries for diclofenac in UHT and fresh milk in percent of the exact spiked amount (m=3).

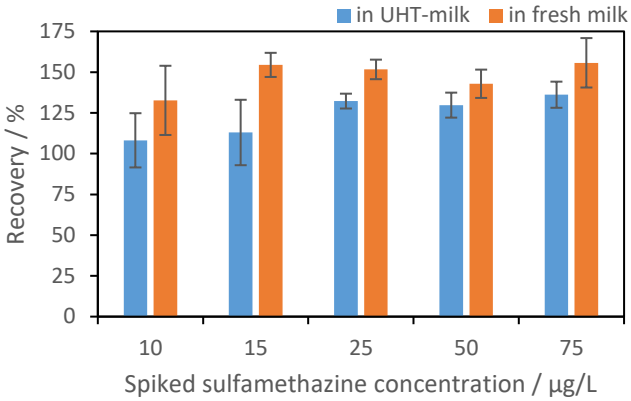


Figure 33: Recoveries for sulfamethazine in UHT and fresh milk in percent of the exact spiked amount (m=3).

6. Conclusion and outlook

In the present work a residue analysis microarray chip for milk was developed using the example of diclofenac and sulfamethazine. The analyses were carried out using the automated MCR 3 with a flow cell for the simultaneous detection of several analytes via an indirect competitive chemiluminescence microarray immunoassay.

Diclofenac and sulfamethazine were successfully immobilized on Jeffamine® modified glass slides by two different methods. The best immobilization results for diclofenac were achieved by using a spotting solution consisting of 90% dioxane and 10% ultrapure water. This solution contained 1.2 mg/mL EDC, 1.2 mg/mL S-NHS and 0.1 mg/mL diclofenac. For sulfamethazine, a two-step immobilization procedure was developed, whereby a 50% DE-PEG solution in UPW was pre-spotted. In a second spotting run a sulfamethazine solution with 10 mg/mL in ultrapure water was applied.

For diclofenac, calibrations were performed in tap water. It was found, that the diclofenac concentration between 0.01 and 1.0 mg/mL in the spotting buffer had no influence on the sensitivity of the calibrations. The average values for the LoD were 83.0 ± 11.2 ng/L, for the IC_{50} 462.3 ± 65.7 ng/L and the working range was between 0.202 ± 0.069 µg/L and 1.155 ± 0.107 µg/L.

The investigation of the primary antibodies for their selectivities showed a high selectivity to the corresponding analytes. The selectivity of the antibodies to the respective other analytes were less than 0.5% relative to the CL intensities. Therefore cross reactivities in the developed assay could be excluded.

The microarray chips had good regenerative abilities. After 35 regeneration cycles, a signal loss of 21% was observed for diclofenac and only 9% for sulfamethazine. Duplex calibrations in UHT milk resulted in a LoD of 0.264 µg/L and an IC_{50} of 1.005 µg/L for diclofenac. Sulfamethazine showed a LoD of 8.0 µg/L and an IC_{50} of 24.9 µg/L. The LoD of sulfamethazine was clearly below the detection limit of 100 µg/L. Diclofenac on the other hand had a LoD 2.5 times higher than the defined MLR in milk of 0.100 µg/L. Attempts to improve the sensitivity of the diclofenac assay failed. One approach was to extend the antigen-analyte interaction time. Another approach was to increase the competition time on the microarray chip. At this point further experiments could be done to improve the performance of the diclofenac MIA. A possible approach could be a change in antibody concentrations. A decrease in antibody concentration could lead to a lower analyte concentration for the same amount of bound analyte and thus

improve the sensitivity of the assay.[71] Instead of decreasing the antibody concentration, an attempt could be made to increase the density of the immobilized analytes on the microarray chips or to increase the surface area of the spots with constant immobilization densities. Recovery tests in UHT milk showed average recovery rates of 99% ± 7 for diclofenac and 124% ± 12 for sulfamethazine. In fresh milk, recovery rates were significantly higher, 142 ± 9 for diclofenac and 148 ± 10 for sulfamethazine. Since the change of the matrix from UHT to fresh milk has led to a significant deterioration of the recovery rates, it would be useful to continue calibrations in fresh milk and to investigate more closely what influence the fresh milk has on the assay.

In conclusion, it can be said that a microarray chip for the simultaneous detection of two chemically diverse molecules was developed. The optimization of reaction conditions for lowering LOD values and adjusting recovery rates will be a project of future investigations.

7. Appendix

7.1 List of tables

7.2 List of Figures

8. References

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9. Declaration of independent work

I hereby declare that the master's thesis at hand has been independently written by myself and that no resources and devices other than those indicated have been used.

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