Microchim Acta
DOI 10.1007/s00604-011-0548-9

ORIGINAL PAPER

Simultaneous determination of four different antibiotic residues in honey by chemiluminescence multianalyte chip immunoassays

Klaus Wutz · Reinhard Niessner · Michael Seidel

Received: 9 November 2010 / Accepted: 14 January 2011

Abstract We are presenting the first method for identification and quantification of antibiotic derivatives in honey samples using regenerable antigen microarrays in combination with an automated flow injection system. The scheme is based on an indirect competitive immunoassay format using monoclonal antibodies bound to the surface of the microarray. The surface of glass slides was coated with epoxy-activated poly(ethylene glycol) and enables direct immobilization of the antibiotic derivatives. The antigen/antibody interaction on the surface of the chip can be detected by chemiluminescence (CL) read-out via CCD camera. The method allows for fast analysis of the four analytes simultaneously and without purification or extraction. An effective data evaluation method also was developed to warrant unambiguous identification of the spots and to establish grey levels of CL intensities. The software developed enables fast and automated processing of the CL images. Dose–response curves were obtained for the derivatives of enrofloxacin, sulfadiazine, sulfamethazine and streptomycin. Spiking experiments revealed adequate recoveries within the dynamic ranges of the calibration curves of enrofloxacin (92%±6%), sulfamethazine (130%±21%), sulfadiazine (89%±20%) and streptomycin (93%±4%).

Keywords Antibiotic microarray · Chemiluminescence detection · Regenerable biochip · Automated flow-injection system · Microarray image evaluation

Introduction

Honey is generally considered as natural and healthy product of animal origin. However, in the recent years there were some publications dealing with the determination of antimicrobial contaminants in bee products. Antibiotics are used in apiculture for the treatment of bacterial diseases, in particular American and European foulbrood [1]. For the effective abatement of the causers *Paenibacillus larvae* and *Melissoccocus pluton*, respectively, drugs based on antibiotic derivatives, e.g. sulfonamides and tetracyclines, have been approved [2, 3]. All sulfonamides inhibit the bacterial synthesis of folic acid due to their structural analogy to *p*-aminobenzoic acid, whereas the group of tetracylines interferes with the ribosomal protein synthesis. The broad activity spectrum of these antimicrobials has led to widely use in veterinary practice since the 1950s implicating the appearance of bacterial resistance. Hence, new synthetic agents, e.g. the class of quinolones, have been developed to replace effectless antibiotics. The use of antibiotics for the treatment of honey bees is illegal in the European Union, but due to the high import quota from other countries, contaminated honey products can be found on the European markets [4]. In the last years another source for the contamination of honey with antibiotic residues has been attracting notice. The plant disease *fireblight* has become a serious problem for fruit-growers. *Fireblight* is caused by the bacterium *Erwinia amylovora* which affects plants from the family of *rosaceae*, e.g. apple and pear. This infection disease leads to dieback of the

Electronic supplementary material The online version of this article (doi:10.1007/s00604-011-0548-9) contains supplementary material, which is available to authorized users.

K. Wutz · R. Niessner · M. Seidel (✉)
Institute of Hydrochemistry and Chair for Analytical Chemistry,
Technische Universität München,
Marchioninistraße 17,
81377 München, Germany
e-mail: michael.seidel@ch.tum.de

Published online: 29 January 2011

blossoms and even can cause death of the entire tree [5]. The associated great economic loss for fruit-growers requires effective abatement of *Erwinia amylovora*. Up to now, agents based on the aminoglycoside streptomycin are the most common treatment of affected plants and orchards [6]. Thus, antibiotic residues can be found in honey bee products due to contaminated collected pollen.

As contamination of food with antibiotic residues poses risks for human health particularly with regard to increasing formation of resistance in bacteria strains to antimicrobials, there is a need for monitoring of antibiotic contamination in honey. Most of the screening methods which have been published are using chromatographic techniques. In particular, liquid chromatography with fluorescence detection or combined with coupled mass spectrometry detection (LC-MS/MS) is used for the determination of antibiotics in food matrices. In most cases, multi-analyte screening methods are only applicable for antimicrobials of similar molecular structures. There were methods published for the determination of several macrolides [7], sulfonamides [8] and tetracyclines [9] in honey. Some chromatographic multi-class methods have been established in the past years which allow identification [10] and also quantification [11] of a broad spectrum of antibiotics in honey. All HPLC based methods require liquid-liquid or solid-phase extraction of the matrix followed by pre-concentration or clean-up of the extract. For multiclass screening there is the additional need of several extraction steps in dependency on polarity and solubility of the various analyte structures. This sample preparation is time-consuming as well as laborious and can lead to loss of analytes along the extraction procedure. The development of an adequate chromatographic separation of a broad variety of antibiotic residues is also a challenge which requires optimization. Additionally, in case of honey strong matrix effects due to the floral ingredients can cause interferences which have to be minimized for chromatographic analysis [12].

Thus, chromatographic methods are not preferable for fast and cost-efficient high-throughput monitoring.

The advantage of immunochemical methods is the usage of specific antibodies to the analyte, which is dedicated for multianalyte screening. Various studies have been concerned with the immunological screening for antimicrobial substances in honey and other food matrices [13–15]. Otherwise, as heterogeneous immunochemical methods are based on the formation of the antigen/antibody complex on solid phase, time-consuming incubation steps are necessary. Because of this, methods based on ELISA formats executed in standard micro-well plates are not applicable for fast screening of high sample amounts. Therefore, at our institute we have been focusing on microarray technique based on multianalyte immunoassays (MIA) in combination with a flow-through principle realized by construction of a flow injection system [16]. Analytical microarrays are a powerful tool for the simultaneous detection of multiple analytes in a single measurement due to separated affinity-binding events at the surface interface, which reduces time and costs for the analysis of contaminations in food [17]. They have been approved for the detection of DNA target molecules [18], as well as for e.g. microorganisms [19, 20] and toxins [21] or pharmaceuticals [22] in food and water samples. For analytical microarray read-out purposes fluorescence [23]- and chemiluminescence (CL) [24]-detection methods are often used [25, 26]. CL microarrays measure the light emitted by an enzyme-assisted chemical reaction. In contrast to fluorescence, there is no background signal, neither from the light source nor from light scattering from the matrix. Therefore, CL is the most sensitive read-out principle for microarrays [27, 28]

Since microarray-immunoassay methods require several incubation and washing steps, miniaturized bioanalysis platforms have been developed which combine microarray assays with fluidic microsystems [16, 29, 30]. Using flow-through microarrays, the microarray is part of a flow cell through which samples and reagents are pumped. In comparison with conventional micro-well plates, flow-through microarrays present thinner diffusion layers enabling efficient mass transport [31]. This reduces the time needed to perform a multianalyte assay. Thus, automated flow-through microarrays allow the analysis of a sample within minutes and ensure reproducible and easy operating.

The design of the platform MCR 3 (Microarray Chip Reader 3), which is used in this study, has been published for the determination of antibiotic residues in milk [32, 33]. The specific antibody/antigen interactions on the regenerable microarray chip surface allow for determination of different analytes in parallel without extraction and separation steps before analysis. The detection is carried out by sensitive CL read-out using horseradish peroxidase (HRP)-labelled antibodies.

Materials and methods

Chemicals and reagents

The water used for all aqueous buffer solutions was deionized and treated by a Milli-Q plus 185 system (Millipore, Schwalbach, Germany, www.millipore.com). All standard chemicals for the production of buffer solutions were obtained from Sigma-Aldrich (Taufkirchen, Germany, www.sigmaaldrich.com). The antibiotic derivatives streptomycin sulfate, sulfadiazine (SDZ) sodium salt, sulfamethazine (SMZ) sodium salt and enrofloxacin were purchased from Sigma-Aldrich. Clinafloxacin hydrochloride was obtained from Axxora (Lörrach, Germany, www.

axxora.com). The positive control N-(2,4-dinitrophenyl)-ethylene diamine (DNPEDA) was purchased from Chem-Pur (Karlsruhe, Germany, www.chempur.de).

For surface modification 3-glycidyloxypropyltrimethoxysilane (GOPTS) and poly(ethylene glycol)diglycidyl ether (diepoxy-PEG) were obtained from Sigma-Aldrich. Diamino-poly(ethylene glycol) (diamino-PEG) was provided by Huntsman Holland (Rozenburg, The Netherlands, www.huntsman.com).

The monoclonal primary antibodies (mAb) used for the detection of norfloxacin (mAb 1F7, reactive with enrofloxacin), streptomycin (mAb 4E2), sulfamethazine (mAb 4D9) and sulfadiazine (mAb 2G6) were produced at the Chair of Hygiene and Technology of Milk (LMU München) [34]. The mouse monoclonal antibody to trinitrotoluene (mAb A1) was obtained from Strategic Diagnostics Inc. (Newark, USA, www.sdix.com). The horseradish peroxidase-labelled anti-mouse IgG produced in horse was purchased from Axxora. The chemiluminescence substrates Westar Supernova ELISA Luminol solution and Westar Supernova ELISA Peroxide solution were obtained from Cyanagen (Bologna, Italy, www.cyanagen.it).

Materials

CL-MIA measurements were performed with the automated microarray chip read-out platform MCR 3 (GWK Präzisionstechnik, München, Germany, www.gwk-munich.com).

For microarray chip production conventional microscope glass slides (26×76×1 mm) were purchased from Carl Roth (Karlsruhe, Germany, www.carlroth.com). The carriers for the microarray flow cells were fabricated from black poly(methyl methacrylate) at the Institute of Hydrochemistry (TU München). The double-sided adhesive foil ARcare 90106 was supplied by Adhesive Research Ireland Ltd. (Limerick, Ireland, www.ahdesiveresearch.com). The production of the laser cuts with the microfluidic measuring channels was carried out by A.L.L. Lasertechnik GmbH (München, Germany, www.all-laser.de).

96-well polypropylene (PP) microtiter plates were obtained from Greiner Bio-One (Frickenhausen, Germany, www.greinerbioone.com).

Microarray surface chemistry

The fabrication of microarray chips was carried out following the standard procedure published by our group formerly [33, 35]. Briefly, for cleaning and activation the glass slides were immersed first in methanol/hydrochloric acid (1:1), then in fuming sulfuric acid. The activated glass slides were silanized by dispensing 0.6 mL GOPTS on one slide and covering it with a second slide ("sandwich format") for 1 h at room temperature. The silanized glass slides were functionalized with 0.6 mL molten diamino-PEG for one sandwich in smelter at 98 °C for 15 h. The resulting diamino-PEG-coated glass slides were washed with water and dried under a nitrogen flow. The diamino-PEG chips were stored at room conditions for a maximum of 4 weeks.

Diepoxy-PEG glass slides were prepared by dispensing 0.6 mL diepoxy-PEG on one diamino-PEG glass slide and covering it with another diamino-PEG glass slide. The slides were incubated in a sandwich format for 15 h at 100 °C. After cleaning with methanol and drying under nitrogen the produced diepoxy-PEG-coated glass slides were directly used for the spotting process.

For spotting, the antibiotic derivatives were dissolved in mixtures of DMSO and carbonate buffer. The carbonate buffer (pH 9.6) contained 15 mM disodium carbonate, 35 mM sodium hydrogen carbonate and 3 mM sodium azide in 1 L of water. For SMZ sodium salt, SDZ sodium salt, clinafloxacin hydrochloride (used for immobilization instead of enrofloxacin) and the positive control DNPEDA a 1:1 mixture of DMSO and carbonate buffer was used, for streptomycin sulfate a 2:3 mixture was prepared. Each of the four antibiotics was spotted in different concentrations ranging between 0.01 mg mL^{-1} and 10 mg mL^{-1}. The positive control was immobilized with a concentration of 0.1 mg mL^{-1}. As negative control a 1:1 mixture of DMSO and carbonate buffer without any additives was used. 200 µL of each spotting solution were given into the cavities of a 96-well PP microtiter plate and spotted on the diepoxy-PEG glass slides. The spotting process was carried out with a BioOdyssey Calligrapher Miniarrayer from Bio-Rad Laboratories (München, Germany) using the Stealth Solid Pin SNS 9 from ArrayIt (Sunnyvale, USA). Two 14×5 clusters were set on one microarray glass chip with a grid spacing of 1,100 µm for the columns and 1,300 µm for the rows, respectively. During the spotting process the chips were cooled to 20 °C and the humidity in the spotting chamber was set to 35%. After spotting the microarray chips were incubated for 15 h at 25 °C and 50% humidity. The deactivation of free binding sides was carried out by sonicating the chips in 1 M Tris–HCl-buffer (pH 8.5) for 15 min. Further, the chips were cleaned by sonicating in water and methanol for 5 min. After drying under a continuous nitrogen flow, the microarray glass slides were connected with plastic carriers by use of a double-sided adhesive foil forming the microfluidic measuring channels.

Sample preparation

For measurement on the MCR 3 platform the honey samples were diluted with phosphate buffered saline (PBS, pH 7.6) consisting of 145 mM sodium chloride, 10 mM potassium dihydrogen phosphate and 70 mM

dipotassium hydrogen phosphate. To get a low viscous, homogeneous liquid sample, 1 g honey was dissolved in 9 g PBS and the solution was vortexed vigorously. The 1 : 10 (w/w) solution of all honey samples with PBS ensures consistent pH of 7.6 for the analysis, which minimizes possible matrix influences associated with acidic pH values and formation of interfering polymeric phenolic compounds [36].

The samples for calibration and determination of the recovery rates were spiked with the four antibiotic derivatives. The antibiotic concentrations of the samples were calculated on the honey content, so no dilution factor has to be taken in consideration for the determination of the recovery. For calibration, samples with 0.01, 0.1, 1, 10, 100, 1,000 and 10,000 μg kg^{-1} of each antibiotic were prepared. The honey used for the measurements was obtained from Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit (Erlangen, Germany) and was tested negative for streptomycin (<2.5 μg kg^{-1}).

Chemiluminescence-microarray-immunoassay measurements

The Chemiluminescence-Microarray-Immunoassay (CL-MIA) measurements on the MCR 3 platform are based on the specific antigen/antibody interaction. The assay format is an indirect competitive immunoassay on a heterogeneous phase. There is a competition between immobilized analytes on the microarray chip surface and the free analytes in the sample. The more antibiotic contaminants are in the sample, the less specific antibodies can bind to the immobilized analytes. The bound antibodies can be detected via HRP-labelled secondary antibodies by chemiluminescence read-out.

One characteristic of the MCR 3 measurement is the flow-through principle. All reagent solutions are pumped over the microarray chip without time consuming incubation steps known from classical microtiter plate immunoassays.

The second characteristic of the MCR 3 is the use of regenerable microarray chips. This implicates the removal of the highly affine antibodies from the chip surface, so one chip can be used for several repeated measurements. For chip regeneration a buffer solution consisting of 10 mM glycin, 100 mM sodium chloride and 0.1% (w/v) sodium dodecyl sulfate in 1 L of water adjusted to pH 3 with hydrochloric acid was used. As running buffer PBS containing 0.5% (w/v) casein was used for all measurements.

The measurement steps were as following: 0.5 mL sample and 0.5 mL of primary antibody solution were injected simultaneously in an incubation loop at a flow rate of 3.6 mL min^{-1}. The primary antibody solution was a cocktail of the four specific monoclonal antibodies anti-SMA, anti-SDA, anti-Streptomycin and anti-Norfloxacin and the anti-trinitrotoluene antibody with a concentration of 0.5 mg L^{-1} (diluted in running buffer). The mixture of sample and primary antibody solution was pumped over the chip at a flow rate of 0.6 mL min^{-1}. After a washing step with 2 mL of running buffer, 1 mL of the secondary antibody solution with a concentration of 1 mg L^{-1} (diluted in running buffer) was given over the chip at a flow rate of 6 mL min^{-1} for the first 0.2 mL and 0.6 mL min^{-1} for the remaining 0.8 mL. Afterwards a second washing step was executed. For the detection of bound antibodies, 0.2 mL of a luminol respectively peroxide solution were mixed and pumped over the fluidic cell at a flow rate of 9 mL min^{-1}. Then the flow was stopped and a picture was taken with an exposure time of 60 s by a highly sensitive cooled CCD camera. All immunochemical assay steps including the chemiluminescence reaction took 8 min. Afterwards, an extended rinsing program was carried out to remove sugar and antibiotic residues in the fluidic system and on the chip. After intensive rinsing of the tubes and the sample syringe with a total volume of 30 mL (running buffer) the chip was treated with 4 mL of the regeneration buffer at a flow rate of 15 mL min^{-1} (3 mL), respectively 0.6 mL min^{-1} (1 mL). Finally, 2 mL of running buffer were pumped over the chip at a flow rate of 30 mL min^{-1}. The overall assay time including rinsing and regeneration steps was less than 14 min.

Data evaluation

The 2D images of the CCD camera were automatically saved as text-files. Before the measurements with one chip were carried out, a background picture was taken. This background noise of the camera was subtracted from the measuring images using LabVIEW 8.2 (National Instruments, USA). These pictures were evaluated with a new image evaluation software *MCRImageAnalyzer* developed for the automated data-processing for CL microarrays in cooperation with GWK Präzionstechnik GmbH, Munich. The calculated chemiluminescence data were transferred to Origin 7.0 (MicroCal Software Inc., Newark, USA) for graphical evaluation. The data of the calibrations were fitted by use of a 4-parameter logistic (4-PL) function, which gave sigmoidal-shaped semi-logarithmic calibration curves [37]. For determination of the recoveries, the obtained CL signal of the sample measurement (SCL_{sample}) was corrected by referencing the blank measurement directly before ($SCL_{reference}$) with the blank measurement, which was done before calibration (SCL_{blank}). This correction method is expressed by Eq. 1

$$SCL_{sample,referenced} = \frac{SCL_{blank}}{SCL_{reference}} \cdot SCL_{sample} \quad (1)$$

The corrected CL signals of the spiked samples were normalized and set in the 4-PL calibration function.

Results and discussion

Data evaluation of chemiluminescence microarrays

The measurements on the MCR 3 platform result in 2D images (2×2 pixel binning mode, 696×520 pixels) of the chip surface obtained by a 16-bit CCD camera. The resolution of one pixel is 41 μm. The immobilized analyte molecules are visible as bright spots where the CL reaction has taken place. The brightness (CL intensity) is described by grey level intensities of single pixels ranging between 0 and 65535 a.u. (saturation). The camera background noise of ca. 2000 a.u. was subtracted from the measurement CL signal of each spot.

Following the indirect competitive immunoassay format, the CL intensity is depending on the amount of free analytes in the sample. For simple and fast quantification of these antibiotic residues, an efficient method for the evaluation of the CL intensities is needed.

Thus, we developed the software *MCRImageAnalyzer* which enables easy and automated processing of the measurement raw data. For recognition of the spots we used a grid pattern (see Fig. 1), where size and distances of the quadratic grid cells are adjustable to the spotting array.

For our investigations we created squares with a size of 25×25 pixels (equivalent to an area of ca. 1 mm^2). In relation to the spot diameters, which vary in dependency on the analyte between 7 and 13 pixels, these cells are large-scaled. The advantage of the big sized grid cells is the entire registration of each spot even if there are drifts in the spotting array, what means that the spots are not immobilized in perfect horizontal or vertical lines.

For calculation of the CL signal of one spot the software detects the ten brightest pixels within the corresponding square. The average value represents the CL signal of one single spot. Defective pixels with grey level intensities in the saturation region are filtered by an implemented threshold to exclude artificial influences on the mean value. As each analyte was spotted in five replicates, the generated CL signal of one analyte is represented by the mean value (MV) of the five single spot signal values and its standard deviation.

The calculation of the signal intensity based on the ten brightest pixels was chosen instead of integration of all pixels within the grid cell, because we found this method more robust regarding the spot morphology. Slight deficiencies in the printing process or non-uniform evaporation can lead to deviations in the detailed shapes of individual spots from the ideal circular form. In cases with inhomogeneous morphologies the summation of the grey level intensities within the corresponding grid cells leads to bigger variances of the mean CL signal for one analyte than the evaluation of the ten brightest pixels (see Supp. Fig. 1 and Supp. Table 1).

Since the quantification of the analyte molecules in the sample is based on the indirect proportional CL intensity, the brightness of the spots is the decisive factor for the analysis. In consequence, the established method is preferable for analytical quantitative CL data evaluation, because the relative standard deviation of the analyte CL intensity is clearly minimized.

The second aim of the data processing was to establish an automated outlier control. In some cases, there are spots with significantly decreased CL intensities compared with the other spots of the same analyte or missing of the whole spot. This phenomenon can be explained by the existence of small bubbles in the measurement flow channel during light exposure. The occurrence of air bubbles in the flow-injection system could not be completely excluded, since in this study we did not use any air trap (see Supp. Fig. 2). Thus, an efficient detection of outlier spots before evaluation of the analyte CL intensities is needed to minimize irregular influences on the dose–response measurements. For this purpose, the algorithm implemented works on basis of the standard deviations. The first calculation step is the determination of the overall relative standard deviation (R.S.D.). Outliers are defined by a certain R.S.D. limit value. For our investigations this limit was set to 20%. Exceeding of the limit value indicates the occurrence of one or more outlier spots in the measured analyte column. Because of

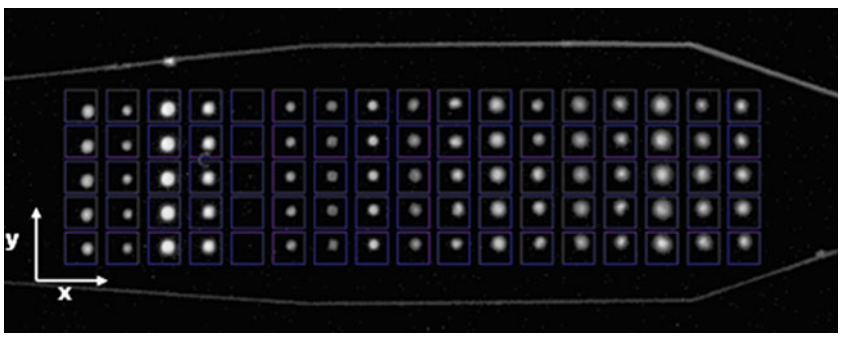

Fig. 1 Grid pattern for the evaluation of the chemiluminescence intensities (different analytes in x-direction, replicates of the same analyte in y-direction). Size of the grid cells is 25×25 pixels

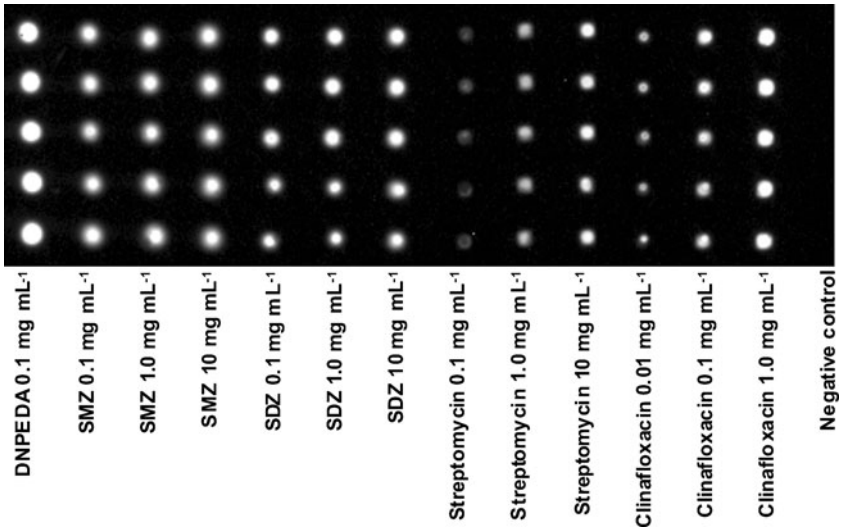

Fig. 2 Characteristic image of the antibiotic microarray. Immobilization in three different spotting concentrations per derivative

this, the second calculation step is the determination of the R.S.D. for the possible combinations x following Eq. 2

$$x = \binom{n}{k} = \frac{n!}{k!(n-k)!} \tag{2}$$

In this equation n represents the number of spots per analyte and k is the number of chosen spots for the R.S.D calculation.

With this method outlier spots can be effectively eliminated by choosing the combination of spots without the outlier. The working principle of the algorithm is explained in Supp. Table 2. The algorithm developed allows the elimination of a second outlier per analyte in rare cases of exceeding the limit value though. All combinations of three spots (n=5, k=3) are evaluated by their corresponding R.S.D. and the combination with

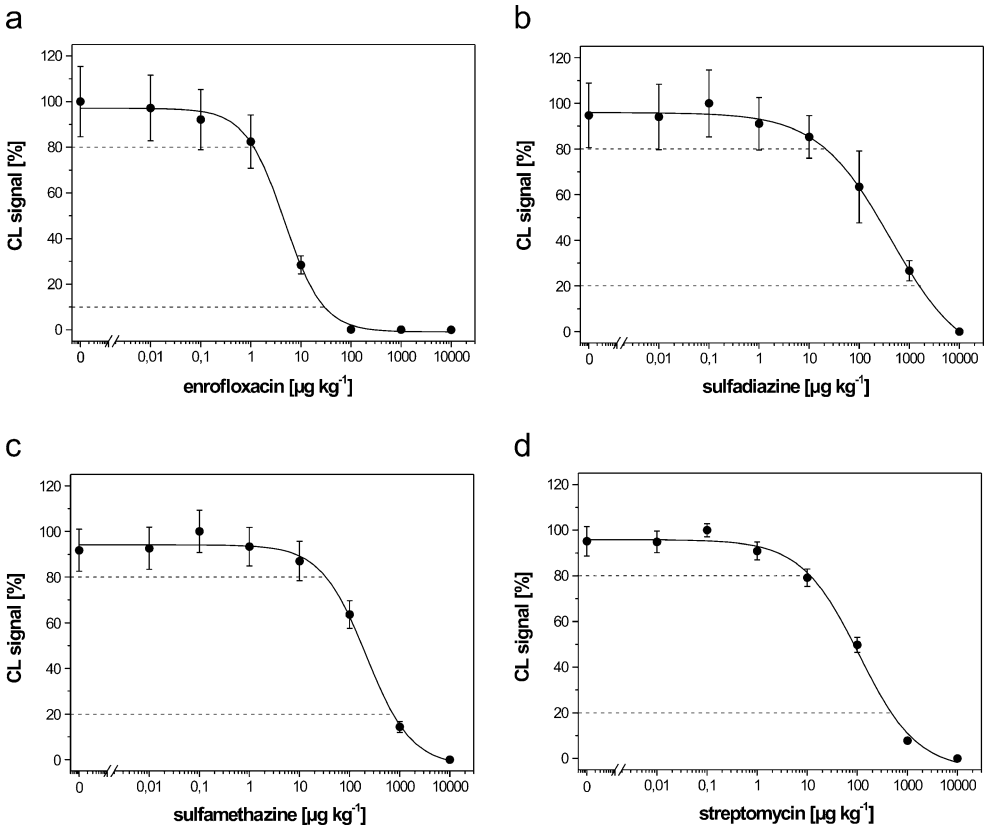

Fig. 3 Dose–response curves for the four antibiotic analytes enrofloxacin (**a**), SDZ (**b**), SMZ (**c**) and streptomycin (**d**) in honey samples. Standard deviation is represented by *error bars* (m=5)

Table 1 Characteristics of the dose–response curves

Antibiotic derivative	IC_{50} [μg kg^{-1}]	WR [μg kg^{-1}]	LOD [μg kg^{-1}]
Enrofloxacin	4.6	1 – 29	4.2
Sulfadiazine	420.5	22 – 1,575	192.6
Sulfamethazine	213.5	34 – 730	81.5
Streptomycin	108.9	12 – 477	15.9

minimal deviation is chosen for calculation of the analyte CL signal.

Spotting adjustment for antibiotic microarrays

The establishment of an appropriate microarray immunoassay for the detection of antibiotic contaminants requires adjustment of the spotting concentrations. On the one hand, the amount of analyte molecules immobilized on the spot surface affects the obtained CL signal and has to be optimized to achieve high signal-to-noise ratios (SNR). On the other hand, the spotting concentration influences the IC_{50} values of the standard calibration curves and for this reason the working ranges. Furthermore, choosing to high spotting concentrations can lead to an excess of analyte molecules on the spot surface detectable by a decrease of the CL signal during the first measurement cycles due to detaching effects. Therefore, different concentrations of the four antibiotics examined were tested for immobilization carrying out blank measurements. The resulting microarray image is depicted in Fig. 2, the spotting sizes and SNR values are shown in Supp. Table 3.

For all immobilized antibiotic derivatives similar trends were obtained. With increasing spotting concentrations enhanced CL signal values could be observed. In case of streptomycin, the spotting concentration of 0.1 mg mL^{-1} showed low SNR of 16 : 1 for the blank measurement and inhomogeneous surface covering densities, whereas higher amounts of streptomycin lead to sufficient CL signals of the spots. The spots measured for the clinafloxacin spotting solution with 0.01 mg mL^{-1} gave small spot sizes compared with the other tested solutions for the immobilization process. For SMZ and SDZ all spotting concentrations were applicable for the further experiments regarding the obtained SNR, but regeneration studies showed strong detaching effects of excessive molecules on the spot surface in case of high spotting concentrations (data not shown). Thus, the spotting concentrations of the antibiotic derivatives selected for calibration were 0.1 mg mL^{-1} for SMZ and clinafloxacin and 1.0 mg mL^{-1} for SDZ and streptomycin, respectively.

Simultaneous dose–response measurements and determination of recovery

Dose–response measurements were carried out for the sulfonamides sulfadiazine (SDZ) and sulfamethazine (SMZ), the aminoglycoside streptomycin and the fluoroquinolone enrofloxacin. The multianalyte ELISA assay on the microarray chips was performed in an indirect competitive format, which is most applicable for small analytes. The first two measurements with one chip were used for complete loading of reagents to obtain maximal CL signal, the third measurement was used for the blank measurement with a honey sample without antimicrobial additives. Afterwards, the standard solutions were measured along increasing analyte concentration. Figure 3 illustrates the resulting dose–response curves. The characteristic standard calibration data for the four determined analytes are shown in Table 1. The working ranges (WR) were defined as 10%–80% of the maximum CL signal in case of enrofloxacin, for the other analytes as 20%–80%.

For determination of the recovery rates, spiking experiments were carried out. Honey samples were prepared with various contents of the four antibiotics. Spiked samples were directly measured after calibration on the microarray chip. Due to an observed signal decrease along chip regeneration cycles a blank measurement was done before analysis of each spiked sample. The obtained CL signal (*SCL*) of the sample measurement was corrected following Eq. 1. To confirm the feasibility of this correction method the determination of the recovery rates was performed as duplicates for each sample on the same chip. In addition, a contaminated honey sample that was

Table 2 Recoveries of the spiking experiments

Antibiotic derivative	Spiking with 1.0 μg kg^{-1} [μg kg^{-1}]	Spiking with 10 μg kg^{-1} [μg kg^{-1}]	Spiking with 100 μg kg^{-1} [μg kg^{-1}]	Contaminated sample (25.5 μg kg^{-1} streptomycin) [μg kg^{-1}]
Enrofloxacin	0.9 / 1.0	8.7 / 9.6	75.7 / 55.9	
Sulfadiazine		11.4 / 8.1	102.6 / 74.5	
Sulfamethazine		12.9 / 22.8	115.9 / 145.8	
Streptomycin		10.9 / 9.6	90.6 / 96.7	32.5 / 32.7

determined to 25.5 μg kg^{-1} streptomycin by LC-MS-MS analysis was examined. The results of the spiking experiments are presented in Table 2.

The dose–response curve for enrofloxacin showed good sensitivity with distinct differences of the CL signal in the range between 1 and 29 μg kg^{-1}, so precise analysis of samples within this contamination level is possible. The calibrations for SDZ, SMZ and streptomycin dynamic ranges allow determination of antibiotic contamination within a broader span.

A high precision of the calibration for enrofloxacin could be confirmed by recovery for the samples spiked with 1.0 μg kg^{-1} and 10 μg kg^{-1}, respectively. The spiked enrofloxacin sample of 10 μg kg^{-1} was in the working range of the multianalyte immunoassay. A recovery of 92%±6% was achieved. The calculated detection limit was 4.2 μg kg^{-1} although a concentration of 1.0 μg kg^{-1} enrofloxacin could be precisely quantified with recovery of 95%±7%. This indicates that the precision of this system depends mainly on the spot quality of the microarray. These differences between the LOD and the analytical sensitivity are also known in other immunoassay assay platforms [38, 39]. A concentration of 100 μg kg^{-1} enrofloxacin was outside of the working range and therefore, the two determined recoveries were 56% and 76%. Thus, samples with high enrofloxacin contamination levels have to be diluted before analysis. SDZ and streptomycin could be quantified precisely at 10 μg kg^{-1} and 100 μg kg^{-1} with recoveries between 75% and 114%. SMZ shows an overestimation. The measurement of a honey sample spiked with 100 μg kg^{-1} sulfamethazine showed recoveries of 130%±21%. Streptomycin had a detection limit of 15.9 μg kg^{-1}. Nevertheless, a spiked concentration of 10 μg kg^{-1} could be quantified with a recovery of 103%± 9%. The recovery of streptomycin in a contaminated real honey sample was 130.4%±0.6% referring to the result of the LC-MS/MS analysis which is comparable to other immunoassays.

Conclusion

With this study we have shown the first time that the flow-through microarray technique can be used for the rapid analysis of antibiotics in honey. A chemiluminescence multianalyte chip immunoassay could be performed to analyse directly antibiotics in honey. No extraction method has to be conducted which simplifies the analysis of honey samples. Four relevant antibiotics for honey (enrofloxacin, streptomycin, sulfamethazine and sulfadiazine) could be quantified simultaneously in 8 min. As result of the high viscosity of honey, samples were diluted 1:10, leading to reduction of sensitivity compared to other food samples like milk which need no dilution steps. Due to the regenerability of the antibiotics microarray each chip could be individually calibrated before the analysis is performed and more than 40 analyses could be done per chip which reduces the costs per analysis and achieve an automated work flow in routine laboratories. Spiking experiments showed with a recovery rate of ±10% a high accuracy of enrofloxacin down to a concentration of 1 μg kg^{-1}. Sulfamethazine, sulfadiazine and streptomycin have a recovery rate between 75% and 146% at 100 μg kg^{-1} as this concentration lies in the dynamic ranges of their calibration curves in honey. Finally, the multianalyte immunoassay has successfully identified a streptomycin contaminated honey product which was approved through LC-MS/MS.

Additionally, a new data processing method for CL microarrays images was examined in this study for rapid microarray analysis in routine laboratories. Each spot was automatically evaluated; outlier could be identified and were excluded for the analysis. This algorithm is important to reduce the variances in the CL-MIA and allows an automated data processing for analysis.

We have shown with this study that a multianalyte immunossay based on an automated flow-through chemiluminescence microarray technique is suitable for the quality control of food sample even for such difficult matrices like honey. Further investigations have to be focused on the analytical requirements associated with 2002/657/EC for validation as effective screening method for routine residue analysis. In particular, more samples have to be analyzed to determine the false non-compliant rate (α-error) and the false compliant rate (β-error).

Acknowledgement This work was supported by the Bayerische Forschungsstiftung (BFS AZ-842-08). We would also thank Prof. E. Märtlbauer and Dr. R. Dietrich (Lehrstuhl für Hygiene und Technologie der Milch, LMU München, Oberschleißheim, Germany) for providing the monoclonal antibodies to enrofloxacin, SDZ, SMZ and streptomycin. Further, we thank Dr. C. Hinkel (Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Erlangen, Germany) for the honey samples and Huntsman Corporation (Rotterdam, The Netherlands) for the free DAPEG samples.

References

1. Bogdanov S (2006) Contaminants of bee products. Apidologie 37:1–18
2. Williams DL (2000) A veterinary approach to the european honey bee (apis mellifera). Vet J 160:61–73
3. Hansen H, Brodsgaard CJ (1999) American foulbrood: a review of its biology, diagnosis and control. Bee World 80:5–23
4. Michaud V (2005) Antibiotic residues in honey - the FEEDM view. Apiacta 40:52–54
5. Eastgate JA (2000) Erwinia amylovora: the molecular basis of fireblight disease. Mol Plant Pathol 1:325–329
6. Vidaver AK (2002) Uses of antimicrobials in plant agriculture. Clin Infect Dis 34:S107–S110

7. Wang J (2004) Determination of five macrolide antibiotic residues in honey by LC-ESI-MS and LC-ESI-MS/MS. J Agr Food Chem 52:171–181
8. Sheridan R, Policastro B, Thomas S, Rice D (2008) Analysis and occurrence of 14 sulfonamide antibacterials and chloramphenicol in honey by solid-phase extraction followed by LC/MS/MS analysis. J Agr Food Chem 56:3509–3516
9. Pena A, Pelantova N, Lino CM, Silveira MIN, Solich P (2005) Validation of an analytical methodology for determination of oxytetracycline and tetracycline residues in honey by hplc with fluorescence detection. J Agr Food Chem 53:3784–3788
10. Lopez MI, Pettis JS, Smith IB, Chu PS (2008) Multiclass determination and confirmation of antibiotic residues in honey using LC-MS/MS. J Agr Food Chem 56:1553–1559
11. Hammel YA, Mohamed R, Gremaud E, LeBreton MH, Guy PA (2008) Multi-screening approach to monitor and quantify 42 antibiotic residues in honey by liquid chromatography-tandem mass spectrometry. J Chromatogr A 1177:58–76
12. Debayle D, Dessalces G, Grenier-Loustalot MF (2008) Multi-residue analysis of traces of pesticides and antibiotics in honey by HPLC-MS-MS. Anal Bioanal Chem 391:1011–1020
13. Heering W, Usleber E, Dietrich R, Martlbauer E (1998) Immunochemical screening for antimicrobial drug residues in commercial honey. Analyst 123:2759–2762
14. Pastor-Navarro N, Gallego-Iglesias E, Maquieira A, Puchades R (2007) Development of a group-specific immunoassay for sulfonamides - application to bee honey analysis. Talanta 71:923–933
15. Adrian J, Pinacho DG, Granier B, Diserens JM, Sanchez-Baeza F, Marco MP (2008) A multianalyte ELISA for immunochemical screening of sulfonamide, fluoroquinolone and beta-lactam antibiotics in milk samples using class-selective bioreceptors. Anal Bioanal Chem 391:1703–1712
16. Seidel M, Niessner R (2008) Automated analytical microarrays: a critical review. Anal Bioanal Chem 391:1521–1544
17. Valdés M, Valdés González A, García Calzón J, Díaz-García M (2009) Analytical nanotechnology for food analysis. Microchim Acta 166:1–19
18. Donhauser SC, Niessner R, Seidel M (2009) Quantification of *E. coli* DNA on a flow-through chemiluminescence microarray readout system after pcr amplification. Anal Sci 25:669–674
19. Magliulo M, Simoni P, Guardigli M, Michelini E, Luciani M, Lelli R, Roda A (2007) A rapid multiplexed chemiluminescent immunoassay for the detection of Escherichia coli 0157: H7, yersinia enterocolitica, salmonella typhimurium, and listeria monocytogenes pathogen bacteria. J Agr Food Chem 55:4933–4939
20. Wolter A, Niessner R, Seidel M (2008) Detection of Escherichia coli 0157: H7, salmonella typhimurium, and legionella pneumophila in water using a flow-through chemiluminescence microarray readout system. Anal Chem 80:5854–5863
21. Taitt CR, Shriver-Lake LC, Ngundi MM, Ligler FS (2008) Array biosensor for toxin detection: continued advances. Sensors 8:8361–8377
22. Knecht BG, Strasser A, Dietrich R, Martlbauer E, Niessner R, Weller MG (2004) Automated microarray system for the simultaneous detection of antibiotics in milk. Anal Chem 76:646–654
23. Nagl S, Schaeferling M, Wolfbeis OS (2005) Fluorescence analysis in microarray technology. Microchim Acta 151:1–21
24. Seidel M, Niessner R (2010) Chemiluminescence to immunoassays. In: Roda A (ed) Chemiluminescence and bioluminescence. R Soc Chem, Cambridge, pp 291–335
25. Heyries KA, Loughran MG, Hoffmann D, Homsy A, Blum LJ, Marquette CA (2008) Microfluidic biochip for chemiluminescent detection of allergen-specific antibodies. Biosens Bioelectron 23:1812–1818
26. Schaferling M, Nagl S (2006) Optical technologies for the read out and quality control of DNA and protein microarrays. Anal Bioanal Chem 385:500–517
27. Roda A, Guardigli M, Michelini E, Mirasoli M, Pasini P (2003) Analytical bioluminescence and chemiluminescence. Anal Chem 75:462A–470A
28. Tsunoda M (2009) Chemiluminescence detection with separation techniques for bioanalytical applications. Bioanal Rev 1:25–34
29. Hartwell SK, Grudpan K (2010) Flow based immuno/bioassay and trends in micro-immuno/biosensors. Microchim Acta 169:201–220
30. Ligler FS (2009) Perspective on optical biosensors and integrated sensor systems. Anal Chem 81:519–526
31. Langer V, Niessner R, Seidel M (2011) Stopped-flow microarray immunoassay for the detection of viable *E. coli* by use of chemiluminescence flow-through microarrays. Anal Bioanal Chem 399:1041–1050
32. Kloth K, Niessner R, Seidel M (2009) Development of an open stand-alone platform for regenerable automated microarrays. Biosens Bioelectron 24:2106–2112
33. Kloth K, Rye-Johnsen M, Didier A, Dietrich R, Martlbauer E, Niessner R, Seidel M (2009) A regenerable immunochip for the rapid determination of 13 different antibiotics in raw milk. Analyst 134:1433–1439
34. Dietrich R, Usleber E, Martlbauer E (1998) The potential of monoclonal antibodies against ampicillin for the preparation of a multi-immunoaffinity chromatography for penicillins. Analyst 123:2749–2754
35. Wolter A, Niessner R, Seidel M (2007) Preparation and characterization of functional poly(ethylene glycol) surfaces for the use of antibody microarrays. Anal Chem 79:4529–4537
36. Jongberg S, Orlien V, Skibsted LH, Weigel S (2009) Elimination of matrix interferences in biosensor analysis of streptomycin in honey. Eur Food Res Technol 228:659–664
37. Findlay JWA, Dillard RF (2007) Appropriate calibration curve fitting in ligand binding assays. AAPS J 9:E260–E267
38. Albrecht C, Kaeppel N, Gauglitz G (2008) Two immunoassay formats for fully automated CRP detection in human serum. Anal Bioanal Chem 391:1845–1852
39. Petersen AB, Gudmann P, Milvang-Gronager P, Morkeberg R, Bogestrand S, Linneberg A, Johansen N (2004) Performance evaluation of a specific IgE assay developed for the advia centaur ((r)) immunoassay system. Clin Biochem 37:882–892