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Simultaneous detection of sulfamethazine, streptomycin, and tylosin in milk by microplate-array based SMM–FIA

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Abstract

This paper presents an approach to simultaneously detect sulfamethazine, streptomycin, and tylosin in milk by indirect competitive multianalyte Fluorescence immunoassay (FIA). Microscope glass slides modified with agarose were used for the preparation of small molecule microarrays (SMMs). Bovine serum albumin (BSA) conjugates of the haptens were immobilized on glass slides. The system consists of four glass slides containing 96 wells formed by an enclosing hydrophobic mask, which precisely matches a standard microplate. All liquid handling and sample processing were fully automated as 96-wells ELISA format. Monoclonal antibodies against sulfamethazine, streptomycin, and tylosin allowed the simultaneous detection of the respective analytes. Antibody binding was detected by a second antibody labeled with Cy5 generating fluorescence, which was scanned with chip scanner. The detection limits for three analytes were 3.26 ng/ml (sulfamethazine), 2.01 ng/ml (streptomycin), and 6.37 ng/ml (tylosin), being far below the respective MRLs. The system proved to be the first SMM–FIA platform having the potential to test for numerous antibiotics in parallel, such being of considerable interest for the control of safety in the food industry.

Keywords: FIA; Microarray; Drug residue; Sulfamethazine; Streptomycin; Tylosin

1. Introduction

Veterinary drug are widely used in domestic animals for the prevention and treatment of infectious diseases and for growth promotion. The improper application can lead to the contamination of foodstuffs at the farm level. The veterinary drug substances in food result in the risk of undesirable health effects for the consumer. Therefore the regulatory authorities worldwide have enacted maximum residue limits (MRLs) for a number of veterinary drugs in food (Commission Regulation (EC), 1990; Food & Drug Administration, 2004). These reasons make it important to effectively control and detect veterinary drug residues in animal food. Various methods for the detection of antibiotic residues have been established, such as microbiological (Nouws et al., 1999), chromatographic (Schenck & Callery, 1998), and immunoassay methods (Strasser, Dietrich, Usleber, & Märtlbauer, 2003). However, microbiological tests are time consuming, lack sensitivity for diverse groups of antibiotics, and do not allow substance identification. Chromatographic methods are expensive and thus restricted to confirmatory purposes. Enzyme linked immunosorbent assay (ELISA) and fluorescence immunoassays (FIA) are excellent survey tools because of their high-throughput, user friendliness, and field portability. These important characteristics make immunoassays attractive tools for food testing by regulatory agencies to ensure food safety. Immunoassay is traditionally performed as individual test, however in many cases it is necessary to perform a panel of tests on each sample (detection of drug residues). To address this requirement, microarray-based immunoassay technologies have been developing utilizing microarray platform (multianalyte analysis) and classic immunoassay

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(multi-samples analysis). A numbers of research groups have developed immunoassays potentially useful for antibiotics multiplexed analysis. Michael et al. (Bertram et al., 2004) developed a parallel affinity sensor array (PASA)-based ELISA for the rapid automated analysis of 10 antibiotics in milk. Claus et al. (Erik, Martin, Jens, Leif, & Claus, 2003) investigated factors that influence the sensitivity of the immunomicroarray for pesticide analysis. Randox system Evidence (Randox Laboratories Ltd., Crumlin, UK) developed a biochip technology for multi-analyte detection. Zuo and Ye (2006) described the small molecule microarrays (SMMs) as analytical tools for drug residue detection. The SMMs are the array-based detection systems that use small molecules as probes immobilized on a variety of surfaces (Falsey, Li, & Lam, 2000; MacBeath, Koehler, & Schreiber, 1999; Reddy & Kodadek, 2005; Ye, Anthony, & Joydeep, 2003). It was effectively employed to study the interaction between small molecules and proteins.

In this paper, we report a microplate-array-based SMM–FIA system to simultaneously detect sulfamethazine, streptomycin, and tylosin in milk. In this system, we constructed SMM by printing these drug small molecules which had conjugated with carrier protein onto four agarose film-coated modified glass slides according to 96-well plate, so these drug small molecules covalently bound to glass slides by carrier protein and retain their ability to interact specifically with corresponding antibodies in solution. The indirect competitive immunoassay format was employed to quantitatively detect drug residues. The optimization and performance of this system are discussed.

2. Experimental

2.1. Microplate-array based SMM–FIA assay setup

A scheme of the microplate-array-based SMM–FIA system is depicted in Fig. 1. The slide frame with sealing mat provides 24 separate locations for individual microarrays on a single glass in the standard 8×12 configuration at 9 mm centers (Fig. 1a). Four glass slides each having 24 wells are assembled to a standard microplate containing 96 wells formed by an enclosing hydrophobic frame (Fig. 1b). The 96-well microarray format is compatible with automated operation systems of the standard ELISA. After reaction and washing, the slides are disassembled out, and scanned with a ScanArray Lite laser confocal scanner (PerkinElmer Life Science, Boston, MA, USA).

2.2. Reagents and chemicals

Sulfamethazine monoclonal antibody (mAb) CH2027, streptomycin mAb CH 2025, and tylosin mAb CH 2023 were purchased from Biodesign (Monrovia, ME). The hapten BSA conjugates for sulfamethazine, streptomycin, and tylosin were delivered by Biodesign together with the antibodies. Sulfamethazine, streptomycin sulfate, tylosin hemitartrate dehydrate, $NaIO_4$, and agarose were from Sigma (St. Louis, MO, USA). Cy5-labeled goat anti-mouse antibody (PI-2000; lot N0904) was purchased from Rockland (Burlingame, PA). All chemicals and solvents were purchased from Sigma and Gibco-BRL (Gaithersburg, Maryland), unless stated, otherwise, and used without additional purification. All solutions were prepared in deionized and sterilized water.

All monoclonal antibody and Cy5-labeled antibody solutions were prepared with phosphate-buffered saline (PBS; 80 mM potassium phosphate buffer with 145 mM NaCl, pH 7.4) containing 0.5% BSA. The washing buffer was a PBST buffer (PBS buffer containing 0.05% Tween 20). Each antibiotics stock (1 mg/mL in PBS) was 10-fold serially diluted (100, 10, 1.0 and 0.1 ng/mL) into PBS buffer.

2.3. Preparing activated agarose film-coated glass slides

The glass slides were cleaned ultrasonically in succession with a 1:10 dilution of detergent in warm water for 5 min, repeatedly rinsed in distilled water and 100% methanol followed by drying in oven at 60 °C. A 1% agarose solution was prepared by adding 100 mg agarose to 10 ml deionized distilled water, mixing and boiling for 5 min. Then 2 ml of the agarose solution was poured over each of the silanized glass slides (Dako, Glostrup, Denmark) which were preheated at 60 °C. After gelation of the agarose, the slides were dried at 37 °C in a dryer overnight. The dried slides can be stored at 4 °C for future use. Before immobilization of the hapten BSA conjugates, the agarose films were activated by immersion in 20 mM $NaIO_4$ in 0.1 M phosphate-buffered saline (PBS), pH 7.4, for 30 min at room temperature, then thoroughly rinsed with deionized distilled water and dried.

2.4. Chip production and microarraying

The veterinary drug small molecules were first conjugated them with carrier protein such as BSA. The BSA conjugates and BSA (as negative control) were suspended in PBS buffer containing 20% glycerol and printed on activated agarose film-coated glass slides by ProSys 5510 spotting workstation (Cartisian Technologies, Ann Arbor, MI) with 150 μm diameter and 250 μm spacing. The robot-mounted print head consists of four CMP3 Chipmaker pin (TeleChem International, Sunnyvale, CA), precisely arranged as a line with spacing of 9 mm. This print head can simultaneously and precisely print same solutions to four designated well locations of slide. Twenty-four subarrays in a 7×4 pattern of three different kinds of conjugates and negative control (BSA) were printed on each slide in three columns. Each conjugate was printed with seven spot replicates. Printing was performed in a cabinet at 25 °C and 60% humidity. Covalent hapten conjugate surface immobilization was established through a Schiff's base reaction at 37 °C overnight. Following immobilization, the microarray substrate surface chemistry was blocked by incubating the slides for 60 min in PBS supplemented with 1% (w/v) BSA

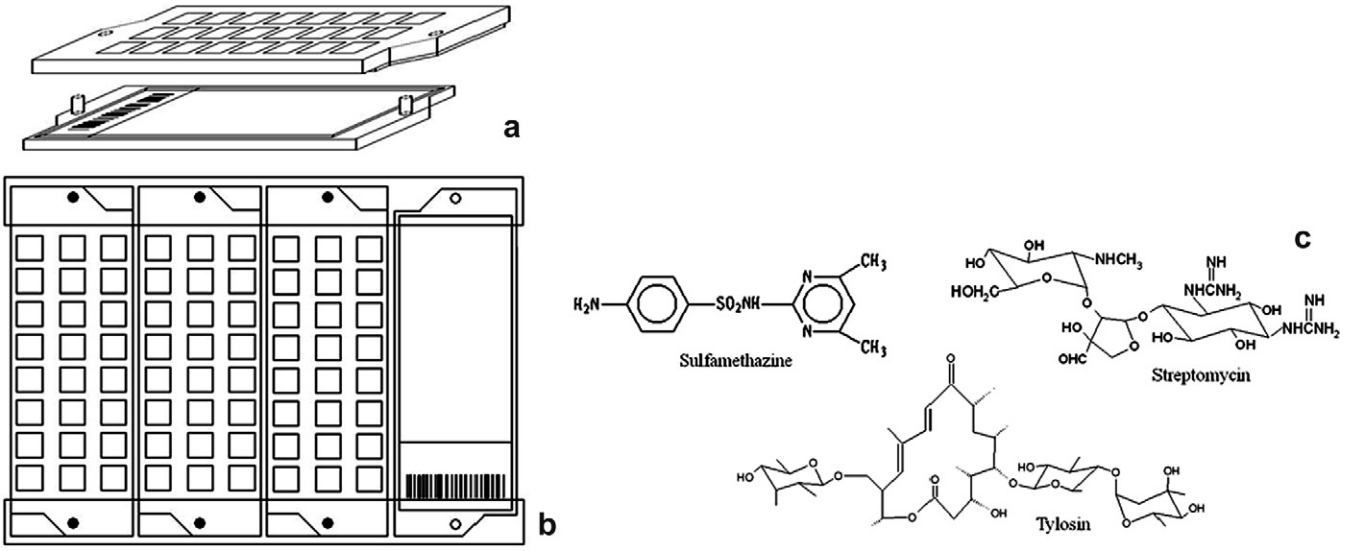

Fig. 1. A scheme of the microplate-array-based ELISA system. (a) Slide and home-made hydrophobic frame having 24 locations for individual microarray. (b) Assembled Microplate, containing 96 wells formed by four enclosing hydrophobic frames on 4 slides. (c) Structures of sulfamethazine, streptomycin, and tylosin in this study.

and 0.1% (v/v) Tween 20, followed by a rinse in PBST buffer thoroughly. The processed microarray substrates were stored in dry conditions until use.

2.5. Microplate-array-based SMM–FIA analysis

Four spotted glass slides each having 24 discrete subarray sites are assembled to a standard microplate containing 96 wells formed by an enclosing hydrophobic frame with sealing mat (Fig. 1b). The protocol of the ELISA procedure that was used for all tests is summarized as follows: 10 μl of the appropriate monoclonal antibodies mixture was added to each well of a 96-well array plate. The typical concentrations of the antibodies were 0.5–3.5 mg/ml. The different concentration of antibiotics standard solutions of 20 μl were then applied to appropriate wells for standard curve, while 20 μl of the samples were added to each of the test wells. The array plate was then maintained at 37 °C in a humidified chamber for 30 min. Following primary monoclonal antibody binding, the excess antibody solution was removed from each well. This was followed by washing each well three times with the PBST buffer. Secondary antibody (Cy5-labeled goat anti-mouse antibody) was applied next to probe for the detection of antibodies. It was carried out by incubating each well with 20 μl of 1:200 dilution of Cy5 labeled secondary antibody at 37 °C for 30 min. The detection solution was then removed, and each well was again rinsed three times with the PBST buffer. Finally, the prepared microarray plates were allowed to air dry before imaging.

2.6. Scan and data analysis

After FIA reaction, four glass slides were disassembled, and then scanned for the acquiring cy5 emissions of the bound secondary antibody with a ScanArray Lite laser confocal scanner (ScanLite, Packard Biochip Technologies, Meriden, CT). The fluorescent signal on each spot was quantified using the Genepix Pro5.0 software (Axon Instruments, USA). In quantitative analysis, it is assumed that the strength of fluorescent signal of each spot is representative of the amount of labeled secondary antibody associated with that spot. The amount of labeled secondary antibody on each spot relies upon the amount of primary antibody bound on that spot. For each spot, pixel intensities within the spot image were summed. The average value and standard deviation of pixel intensities for each spot was calculated and the local background level was subtracted from the sum of the signal intensity. The intensity of the signal can differ from one spot to another because of variability of the concentration of spotted conjugates, concentration of reagents across the wells, and surface conditions. To reduce these variables, we spotted each conjugate in septuple and averaged the intensities for each conjugate. A standard curve was carried out using six wells of one slide. The negative solution and five standard samples containing each analyte in a concentration in the range of 0.1–1000 ng/ml were processed. With the remaining 18 wells of the slide, samples could be tested.

3. Results and discussion

3.1. Optimization of printing and immobilization

The spotting buffer can influence aspects of array quality, such as the immobilization capacity of agarose-coated slide, and the spot morphology. We tested four spotting buffer, PBS, PBSG (PBS + 20% glycerol), Protein Printing Buffer (Telechem International, Sunnyvale, CA), and water. The streptomycin-BSA was separately diluted by these four kinds of buffer described above in four different concentrations,

0.1, 0.2, 0.5 and 0.8 mg/ml. Meanwhile, we also printed BSA beside as negative control. The experimental results show that PBSG buffer, as spotting buffer resulted in the highest signals as well as the best morphology.

The optimum hapten conjugates concentrations for printing the slides were determined by FIA test. Hapten conjugates were diluted into PBSG buffer at concentrations of 0.1, 0.2, 0.4 and 0.8 mg/ml, and spotted on agarose-coated slides. The competitive immunoassay was enough sensitive when the conjugates were printed at a concentration of 0.8 mg/ml (data not shown), and this concentration of the conjugates were used in all subsequent experiments.

Blocking is an essential step in immunoassay to avoid high background signals due to nonspecific binding of primary and secondary antibodies to the agarose-coated slide surface. In immobilized conjugate-based, competitive SMM–FIA, strong nonspecific binding of the primary antibody may prevent effective competition by the analyte and, thus, result in lower assay sensitivity. It is important to block the active sites on the activated agarose-coated slide surface after immobilization of hapten conjugates. In this experiment, BSA (1% in PBS buffer) and glycin (1% in PBS buffer) were employed as blocking reagents. Blocking capacities were evaluated on the basis of the standard curves, background and maximum signal intensities. The use of BSA resulted in effective blocking in our experiments. For BSA not only quenches the unreacted aldehydes groups on the agaroses, but also forms a molecular layer of BSA that reduces nonspecific binding of other proteins in subsequent steps (MacBeath & Schreiber, 2000).

3.2. Optimization of reagent concentrations for a competitive assay

The antibiotics concentrations in solution are quantified by the competitive binding of monoclonal antibodies to either antibiotics conjugates immobilized or antibiotics in solution. In the competitive SMM–FIA, the concentration of antibody is an important factor for quantitative analysis. The assay performance is very highly sensitive to the amount of antibody. The sensitivity of a competitive immunoassay such as RIA or ELISA, is limited by the affinity constant (K) of the antibody and/or by the sensitivity of the detection system employed. The maximum sensitivity that can be obtained with a particular antibody is reached at an antibody concentration at, or slightly below, K^{-1}, provided that the detection system is sensitive enough sufficient to allow for such a high antibody dilution (Elkins, 1991). The effect of antibody concentration on assay sensitivity in the plate array assay was investigated for sulfamethazine mAb, streptomycin mAb, and tylosin mAb, respectively. The relation between signals of spot with antibiotics concentration of 0.1 ng/ml for different dilutions per antibody was used for evaluation. The antibody concentrations in the study were from 500 ng/ml to 10,000 ng/ml, and the best working concentrations for sulfamethazine mAb, streptomycin mAb, and tylosin mAb were 500, 750, and 3500 ng/ml, respectively. The fluorescence signals of spots in absence of analyte was measured for six dilutions (1:10, 1:100, 1:200, 1:500, 1:1000, 1:2000) of Cy5 labeled secondary antibody. The dilution of 1:200 seemed to be close rather approach to its maximum response/noise ratio.

3.3. Sensitivity and calibration curves

The plate array is a competitive FIA for the quantitative measurement of sulfamethazine, streptomycin, and tylosin. Each well of the microplate is pre-spotted with hapten conjugates. These immobilized hapten bind competitively to specific antibodies with the antibiotics in the standards and samples added to the plate. Cy5-labeled secondary antibody was applied next to probe for the detection of antibodies. In principle, the fluorescent signal at the corresponding location is decreased when a tested substance is present in the sample because the drug in the sample competes with drug immobilized on the array for the anti-drug antibody. Within the linear measurement range, the fluorescence signal produced is reciprocally proportional to the amount of each antibiotic in the standard or sample. This method can therefore be used for quantitative determination of the presence of substances in a sample. The standard solutions containing three veterinary drugs were diluted with 10-fold, ranging from 0.1 ng/ml to 1000 ng/ml. As the operation protocol described, instead of adding unknown sample, those five different concentrations of the drug standard solutions were added to compete with the conjugates which immobilized on the surface of each reaction well for the veterinary drug antibodies. So, together with the dilution buffer (PBS) which used as blank control, there are six different concentrations of drug standards ranging from 0 ng/ml to 1000 ng/ml for standard curves. The standard curve is generated by plotting the average fluorescence signal intensities obtained for each of the standards on the vertical (Y) axis vs. the corresponding antibiotics concentrations on the horizontal (X) axis with Origin® 7.0 software (OriginLab, Northampton, MA, USA). Each calibration curve for sulfamethazine, streptomycin, and tylosin obtained from each individual experiment is shown in Fig. 2. The detection limit (LOD) was defined as the concentration, which is equivalent to 30% inhibition (IC30). The limit of the working range at the high concentrations was defined as an inhibition of 70% (IC70). The detection limits for three analytes were 3.26 (sulfamethazine), 2.01 (streptomycin), and 6.37 (tylosin), being far below the respective MRLs. The IC50 value represents the concentration of the analyte resulting in a 50% decrease in the maximal corrected assay signal in the competitive FIA system. Table 1 lists IC50, LOD, working ranges, and MRLs for the antibiotics tested after optimization of the assays. Calibration curves in all-in-one experiment for sulfamethazine, streptomycin, and tylosin were prepared by mixing working solutions with concentrations ranging from 0.1 to 1000 ng/ml for each analyte with the respective antibody. Competitive assay standard curves

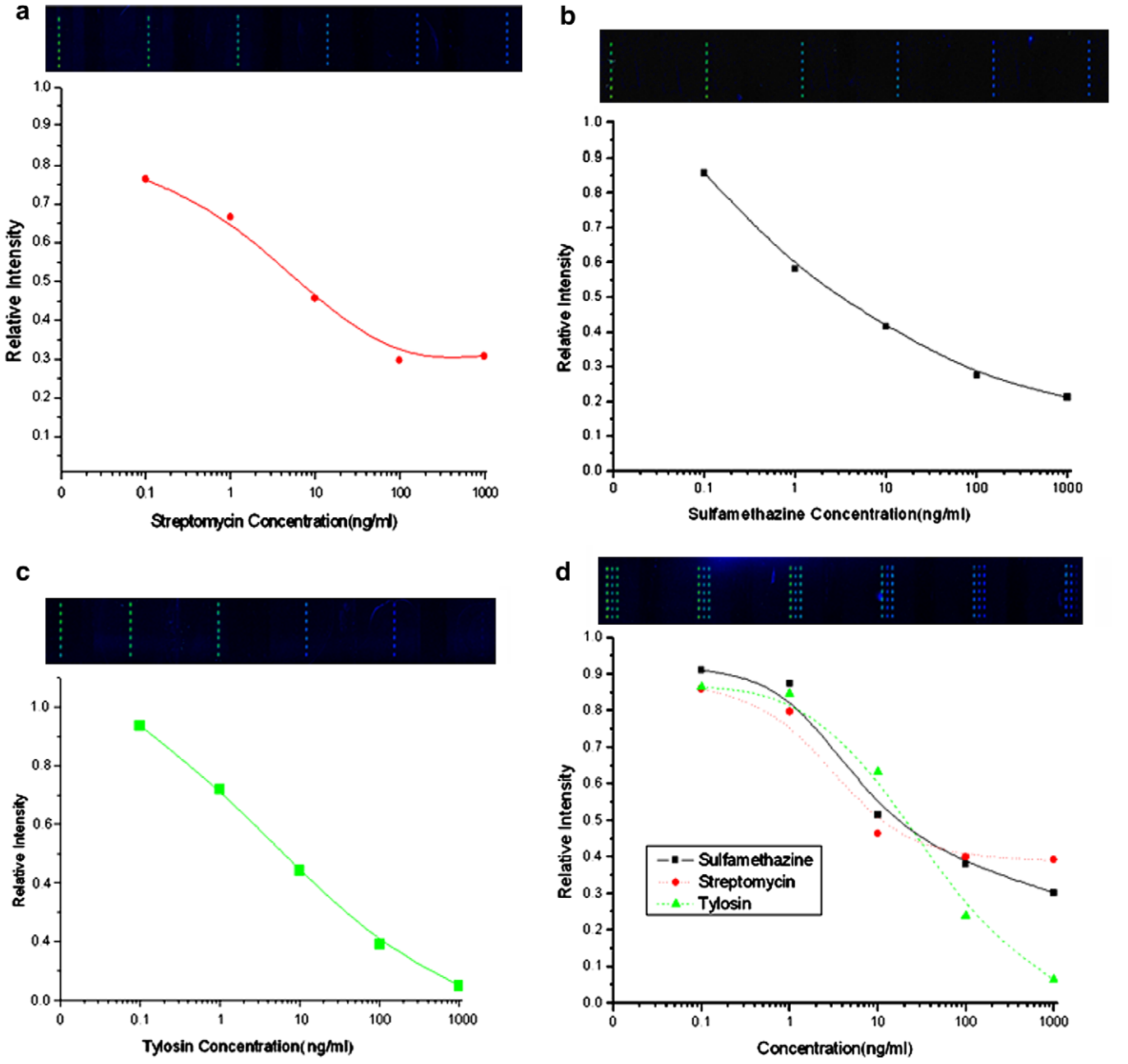

Fig. 2. Calibration curves of sulfamethazine (a), streptomycin (b) and tylosin (c), respectively, Calibration curves for sulfamethazine, streptomycin, and tylosin (mixed) (d).

were constructed using a 4-parametric fit. Fig. 2d illustrates characteristic calibration curves for the three analytes selected for this study. The MRL concentration was in the working range for analytes, thus providing a clear positive readout. The influence of parameters such as temperature, humidity, and variations in spotting made it difficult to produce chips in mass with the same properties. Therefore, it was necessary to perform a calibration for every slide or every series of tests. It is recommended that the standards for a calibration curve and the unknown samples for determination should be spotted on a single slide for each set of experiments.

Table 1
Working range and maximum residue limits (MRL) for microplate-array

Antibiotics	LOD (ng/ml)	IC_{50} (ng/ml)	Working range (ng/ml)	MRL (ng/ml)
Sulfamethazine	3.26	11.85 ± 0.64	3.26–53.2	100
Streptomycin	2.01	7.18 ± 0.1	2.01–26.4	200
Tylosin	6.37	21.62 ± 0.28	6.37–36.5	50

3.4. Precision of assay

The precision of plate array assay was evaluated by three different levels: inter-spot, inter-well, and inter-assay.

Seven spot replicates, eight well replicates of samples containing 0, 0.1, 1, 10, 100 and 1000 ng/ml of sulfamethazine, streptomycin, and tylosin, were run on a single plate array. The coefficients of variation (CVs) of inter-spot, inter-well were shown in Fig. 3. The sample of 10 ng/ml was repeatedly analyzed ($n = 10$) using different plate array. The coefficients of variation of inter-assay were 18.6%, 17.3% and 13.8% for sulfamethazine, streptomycin, tylosin, respectively.

3.5. Validation of the microplate-array based SMM–FIA with samples

To demonstrate how the microplate-array based FIA could be used to simultaneously detect sulfamethazine, streptomycin, and tylosin in milks, we conducted an equivalency study of some numbers of spiked milk samples and food samples. The antibiotics were also detected in the same samples using the traditional ELISA method. The spiked samples containing high, medium, and low levels of sulfamethazine, streptomycin, and tylosin, were determined. The experimental results are shown in Table 2. The recoveries were found to be in range of 79.5% to 119%. Two hundred of milk samples from the supermarkets were analysized and compared by these two methods, respectively, and no positive hit was found.

This method performs the determination of only three antibiotics, but microarray methods could be developed for high-throughput application by adding more small molecule compounds. Novel food additives improperly application is rather difficult to avoid. High-throughput detection is of more importance for food safety, and this method would require further development for these purposes. The flexible nature of our SMM–FIA system allows probes to be easily added or modified without significantly increasing costs. This method proved to be flexible, easy straightforward to update for newly used drug residues, simple to perform, fast, and safe. It is therefore an attractive alternative to GS–MS in routine practice for stock-breeding, aquaculture, poultry farm.

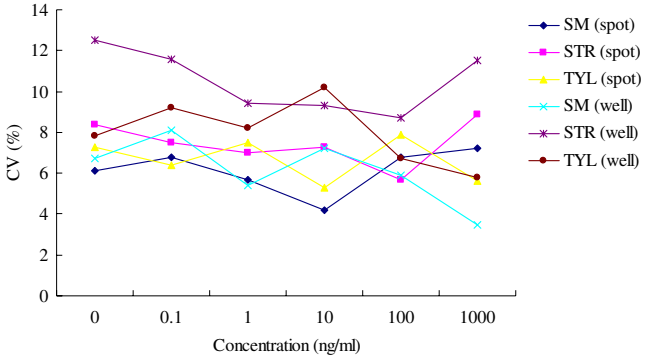

Fig. 3. Coefficients of variation (CV) for the spots ($n = 7$) and the wells ($n = 8$).

Table 2
Comparison of spiked concentration test results with ELISA based on microplate-array

Antibiotics	Spiked (ng/ml)	Detected (ng/ml)	Recovery rate (%)
Sulfamethazine	1	<3.26	ND
	10	11.3	113
	50	55.8	111.6
Streptomycin	1	<2.01	ND
	10	11.9	119
	20	15.9	79.5
Tylosin	1	<6.37	ND
	10	8.1	81
	50	>36.5	ND

4. Conclusion

Sensitive, selective, rapid, and reliable detection and determination of antibiotics are necessary for the protection of food supply in the agricultural industry. Gas and liquid chromatography with various detectors and different types of spectroscopy are the most common analytical techniques presently used for their analysis. However, these techniques are time consuming, expensive, and required in a need of highly trained personnel, and they are available only in sophisticated laboratories. Immunoassays allow quick and inexpensive analysis of pollutants in the laboratory with high affinity and specificity. Monoclonal antibodies against many antibiotics are already available and in use. Enzyme linked immunosorbent assay (ELISA) has been widely used in food monitoring. Although suitable for screening a large number of samples, ELISA is undesirable for a panel of tests on each sample. The goal of this paper was to develop a novel multianalyte–multisample immunoassay method for antibiotics using microplate-based SMM–FIA. The results have demonstrated that the SMM–FIA immunoassay is rapid and sensitive. And the framework presented should illustrate the potential for the economical and highly efficient detection of diverse drug residues.

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