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Small Molecule Microarrays for Drug Residue Detection in Foodstuffs

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Microarrays have been used as tools for analyzing biological compositions at different levels. In this study, we proposed a small molecule microarray (SMM) method for detection of three veterinary drug residues, chloramphenicol, clenbuterol, and tylosin, in foodstuffs simultaneously and quantitatively. The small drug molecules were immobilized on the surface of the modified glass slides. Then the mixture of drug corresponding antibodies and standards or samples was added to the reaction area. After incubation, the antigen−antibody binding was detected using cy5 labeled secondary antibody. The calibration curves of the residues were drawn, and they indicated the lowest detection limit the linearity range. The detectable concentrations of the three residues are lower than the maximum residue levels (MRLs). No cross reactivity was found among the three residues. The coefficient of variation of the spot intensities was below 5% in a subarray, and below 15% among subarrays. The spike sample test and the comparison of detection results by SMMs and ELISA demonstrated the accuracy of the proposed SMMs method.

KEYWORDS: Small molecule microarrays; veterinary drug residue; immunoassay

INTRODUCTION

Veterinary drugs are widely used in domestic animals for prevention and treatment of infectious diseases and for growth promotion. Inappropriate use of the veterinary drugs may produce toxic or allergic reactions in susceptible individuals who eat the poultry or meat that contains veterinary drug residues. Regulations on the maximum residue levels (MRLs) of inhibitory substances in foodstuffs have been decreed world-wide for ensuring the safety and quality of the tissues. For example, the MRL standards in the European Union are 0 μg/L for chloramphenicol and clenbuterol, and 50−200 μg/L for tylosin. Similar regulations were also issued in other countries (*1*−*3*).

To comply with these regulations, various technologies for detection of the veterinary drug residues in animal food have been developed, such as microbiological (*4*), chromatographic (*5*), and immunochemical methods (*6*, *7*). However, low sensitivity and sample capacity as well as the time-consuming property limited their wide application. The enzyme linked immunosorbent assay (ELISA) based method (*8*), which is widely employed as a screening approach, can detect only one component at a time. The instrument-based method (*9*), which serves as an effective reference method, was also limited by its low screening efficiency and complicated operation procedures.

Developments of biological and chemical technologies have made the analysis miniaturized, integrated, high-sensitivity, and high-throughput. One of the analytical breakthroughs was high-throughput screening using microarrays for the simultaneous analysis of thousands of samples (*10*). Small molecule microarrys (SMMs) are array-based detection systems that use small molecules as probes immobilized on a variety of surfaces (*11*, *12*). Nowaday, the SMMs research is mainly concentrated on two aspects (*13*): construction of SMMs and its application. The immobilization of small molecules is critical in the SMM construction procedure. Several immobilization strategies have been developed including covalent immobilization with Staudinger ligation (*14*), photoactivatable reaction with a diazirin-based photoaffinity linker (*15*), noncovalent binding with C_8F_{17} tail (*16*), and *in situ* synthesis (*17*). The SMM method has been used in the discovery of ligands for a variety of protein targets (*18*−*20*), screening inhibitors of enzymes (*21*), and activity-based profiling of enzymes (*22*−*24*). SMMs were also effectively employed as analytical tools for protein identification (*25*) and toxin detection (*26*).

In this study, we established a novel SMM assay method for simultaneous detection of chloramphenicol (CAP), clenbuterol (CL), and tylosin (TYL). The molecular structures of these three analytes are depicted in **Figure 1**. We constructed the SMM by printing these small drug molecules which had been conjugated with carrier protein onto the modified glass slides. The small drug molecules covalently bind to the glass slides by the carrier protein and retain their ability to interact specifically with the corresponding antibodies in solution. After immobilization, the mixture of the antibodies to the three drugs and the three drug standard solutions or sample was added to the array reaction area. The drug molecules in sample or

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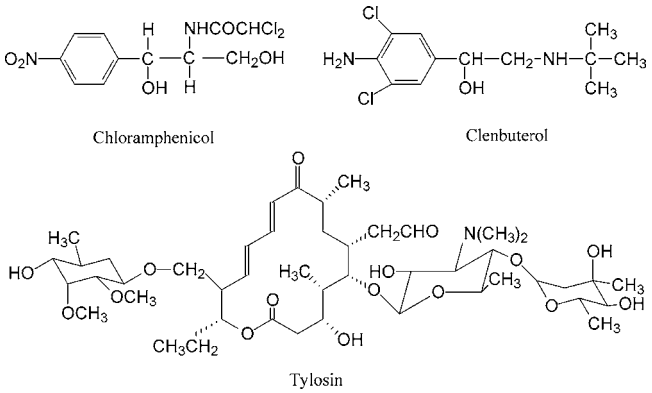

Figure 1. Molecular structures of three analytes determined in this study

standard solutions competed for the drug antibody with the drug conjugates which had immobilized on the slides. After incubation for antigen−antibody reaction, the antigen−antibody binding was detected using fluorescence labeled secondary antibody. Thus the drug residue in food can be quantitatively analyzed by detecting the fluorescence signal. Compared with the methods mentioned above, the SMMs method showed the great advantages of large sample capacities, high throughput, and low reagent consumption.

MATERIALS AND METHODS

Materials. The modified glass slides (CSS-100) were purchased from CEL Associates (Pearland, TX). The monoclonal antibody (mAb) to clenbuterol was purchased from Affiland (ANS-LIEGE, Belgium). The mAb to chloramphenicol, mAb to tylosin, and tylosin−BSA conjugate were purchased from Biodesign (Monrovia, ME). The chloramphenicol−BSA and clenbuterol−OVA were from Bio-Den (Hangzhou, China). The standards of chloramphenicol and tylosin were from Sigma (St. Louis, MO), and the clenbuterol standard was from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The cy5 conjugated affinity purified goat-anti-mouse IgG from Rockland (Burlingame, PA) with a fluorochrome/protein (F/P) labeling ratio of 11.2 was used as secondary antibody. All chemicals and solvents were purchased from Sigma and Gibco-BRL (Gaithersburg, MD), unless stated otherwise, and used without additional purification. All solutions were prepared using deionized and sterilized water.

Probe Preparation. The immobilization strategy of veterinary drug small molecules was to conjugate them first with carrier proteins such as BSA and OVA and then print these conjugates onto modified glass slides. In this study the probes were three drug conjugates, chloramphenicol−BSA, clenbuterol−OVA, and tylosin−BSA. These conjugates were diluted with spotting buffer and printed at the final concentration of 0.8 mg/mL.

Construction of SMMs. Glass slides which had been chemically modified with aldehyde groups were used as the substrate to covalently bind drug molecule conjugates at the designated locations. Twelve subarrays in a 4 × 4 pattern of three different kinds of conjugates and negative control (BSA) were printed on each slide in two columns by the contact printing robot (PixSys 5500, Cartesian Technologies Inc., CA) with a steel microspotting pin (Model SMP4, Telechem International, CA). Each conjugate was printed with four spot replicates and a spot space of 500 μm. Printing was performed in a cabinet at 25 °C and 60% humidity. These conditions were constantly monitored by a thermosensor and a humidifier. First, the printed slides were incubated at 37 °C for 3 h for the purpose of immobilization, followed by incubation in the blocking buffer for 30 min to block the nonspecific active groups on the microarray substrate surface. Then the slides were thoroughly washed with phosphate buffered saline (PBS, 0.01 mol/L phosphate buffer containing 0.8% NaCl at pH 7.4) containing 0.05% Tween 20 (PBST, PBS buffer containing 0.05% Tween 20), briefly rinsed with PBS twice, and then dried by spinning. Finally the microarrays were stored in dry conditions until use.

Operation Protocol. Twelve individual reaction areas were formed by the hydrophobic borders for analysis in a parallel way on each slide as shown in **Figure 2**. Five microliters of incubated buffer (PBS + 0.05% Tween 20 + 0.05% BSA) (*27*) was added to each reaction area. A 20 μL mixture of three residue corresponding antibodies and samples was then added to the girded reaction area (shown in **Figure 2** partial enlargement view). The slide was then incubated at 37 °C in a humidified chamber for 30 min and then rinsed once with PBST and twice with PBS. After spin drying, 10 μL of secondary antibody (Cy5 conjugated goat anti-mouse IgG) was added to each reaction area and then incubated again in a humidified and photophobic chamber at 37 °C for 30 min. Finally, the slides were washed again and scanned for the presence of the fluorescence label conjugated with the secondary antibody by using a laser confocal scanner (ScanLite, Packard Biochip Technologies, Meriden, CT). The analog fluorescent signal was converted to digital signals by data analysis software (Quantarray 3.0, Packard Biochip Technologies). The schematic flowsheet of the operation protocol is shown in **Figure 2**.

RESULTS AND DISCUSSION

To obtain the optimum competitive microarray reaction conditions, the impact of several assay parameters including different spotting buffer and blocking buffer was studied. The detecting limits, reproducibility, microarray sensitivity, and precision were also investigated in this study.

Spotting Buffer Comparison. The spotting buffer influences the probe capacity of a surface, the stability of printed probes, and the spot morphology (*28*). The effect of different spotting buffers including PBS with 20% glycerin, 3× standard saline citrate (SSC: 0.3 mol/L NaCl, 30 mmol/L sodium citrate, pH 7.4), 2× Telechem's Micro Spotting Solution (Telechem International, CA), and PBS buffer without additives on the microarray performance were compared. Chloramphenicol−BSA and BSA used as the negative control were separately diluted by these four buffers under different concentrations of 0.1 mg/mL, 0.2 mg/mL, 0.5 mg/mL, and 0.8 mg/mL. The results of the experiment are shown in **Figure 3**. From this figure, we can clearly observe that, when 3× SSC was used as spotting buffer, there was almost no fluorescence signal. On the contrary, strong fluorescence signals appeared when 2× Telechem's Micro Spotting Solution was used, but the selectivity was poor. The spot of BSA which was used as negative control also had strong signals, and there was no gradient of fluorescence signals with varying probe concentration. The results indicate that signals for 2× Telechem's Micro Spotting Solution might be false positive signals. These two kinds of spotting buffer were used very well in DNA microarrays, but according to the result, we found it not suitable for this small molecule microarray. When the PBS with 20% glycerin and without glycerin was used, the fluorescence signal intensity increased with increasing probe concentrations. The fluorescence signal intensity using PBS with 20% glycerin is significantly greater than that using PBS without glycerin, and showed a more obvious gradient than that using PBS without glycerin. Glycerin might serve as a stabilizer and keep the moistening conditions during printing and immobilization in this section.

Influence of Different Blocking Buffer on the Fluorescence Signal on SMMs. For the immobilization of probes on the slide surface, the glass slides should modified with appropriate groups for coupling the probes. The high sensitivity implies a high signal to noise ratio and stringent limitation to the acceptable background signal due to nonspecifically bound reactant. A high signal to noise ratio and low background is usually achieved by thorough blocking of the nonspecific active groups on the slides with an inert or an irrelevant protein. In this study, we used PBS with 1% glycin and PBS with 1% BSA as blocking

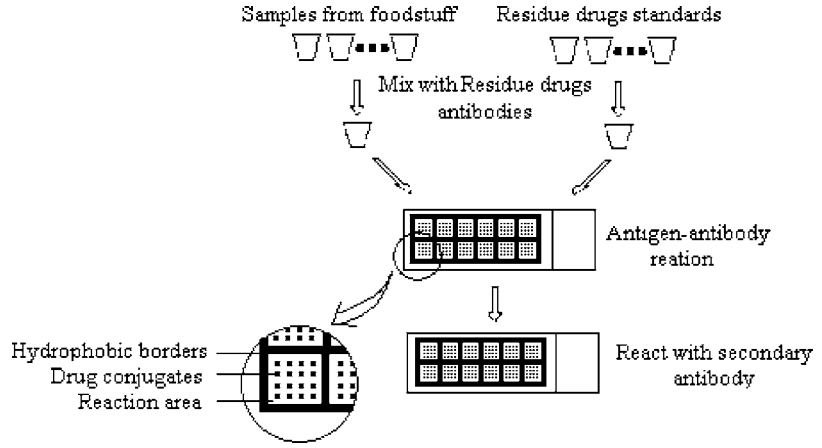

Figure 2. SMM operation protocol using competitive immunoassay principle.

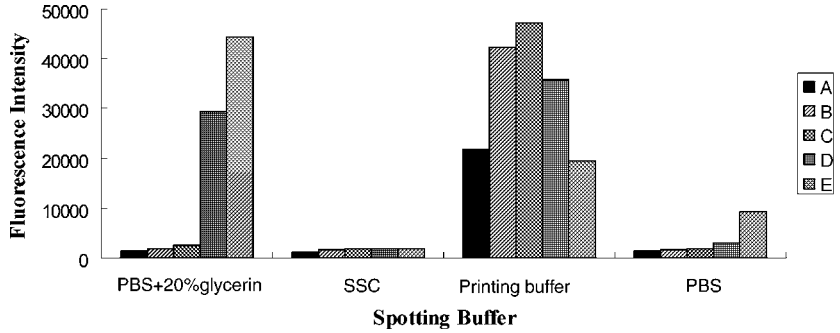

Figure 3. Four different kinds of spotting buffer PBS with 20% glycerin, 3× SSC, 2× Telechem Micro Spotting Solution, and PBS without other additives were compared for their impact on the microarray performance. Columns indicate the fluorescence signal intensities. **A**–**E** indicate different kinds of probes. **A**: BSA as negative control. **B**: 0.1 mg/mL chloramphenicol–BSA. **C**: 0.2 mg/mL chloramphenicol–BSA. **D**: 0.5 mg/mL chloramphenicol–BSA. **E**: 0.8 mg/mL chloramphenicol–BSA.

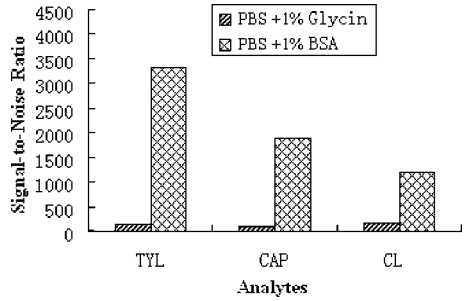

Figure 4. Influence of different blocking buffer on the fluorescence signal on protein chips. After blocking by these two buffers, antibodies were added to react with the drug conjugate without adding standards. Then the secondary antibody was added. By detect the fluorescence signal, we got the signal to noise ratio.

buffers. **Figure 4** shows the influence of different blocking buffers. The results show that the signal to noise ratio by blocking with PBS with 1% BSA was apparently higher than the signal to noise ratio by blocking with PBS with 1% glycin. This indicates that PBS with the supplementation of 1% BSA resulted in lower background and in higher signal to noise ratio. BSA not only quenches the unreacted aldehydes groups on the slide but also forms a molecular layer of BSA that reduces nonspecific binding of other proteins in subsequent steps (*29*).

Calibration Curves, Detection Limit, and Reproducibility. The calibration curves of three residues were drawn before sample testing. The tylosin standard was diluted with PBS into 300 μg/L; chloramphenicol and clenbuterol were diluted with PBS into 30 μg/L. Then an isometric mix of the above three kinds of solutions gives standard solution 4. Then a 10-fold dilution of standard solution 4 gives standard solution 3. Standard solution 2 and standard solution 1 may be deduced by analogy. Standard solution 0 used as blank control was only composed of PBS. As described in Materials and Methods, those five different concentrations of drug standard solutions were added to compete with the conjugates immobilized on the surface of each reaction area for the drug antibodies. The optimal antibody condition was found to be a mixture with 10 000 μg/L of mAb to chloramphenicol and clenbuterol, 5000 μg/L of mAb to tylosin.

The antigen−antibody binding was detected using fluorescence labeled secondary antibody. The label of the secondary antibodies gave a detectable fluorescent signal by laser induction. In this experiment, the optimal secondary antibody concentration was 2000 μg/L. Follow the operating protocol, after scanning, we can get the resulting image. The resulting laser scan image of the calibration curves drawn experimentally is shown in **Figure 5A**. The spot fluorescence signal intensities determined by the software Quantarray 3.0 was the mean of the intensities of numerous pixels calculated for each spot. The average of the four replicates was taken as the intensity value of each drug. The calibration curves were drawn by fitting the relative intensity with the different concentration of each drug standard as shown in **Figure 5B**.

The detection limit of the method depends on the detectable minimum concentration of the veterinary drug residues. Based on the assumption of a coefficient of variation of 10% at zero analyte concentration and the usual 3σ definition, the detection limit was defined as the concentration which is equivalent to 30% inhibition (IC_{30}). The limit of the working range at high concentrations was defined as an inhibition of 85% (IC_{85}) (*30*).

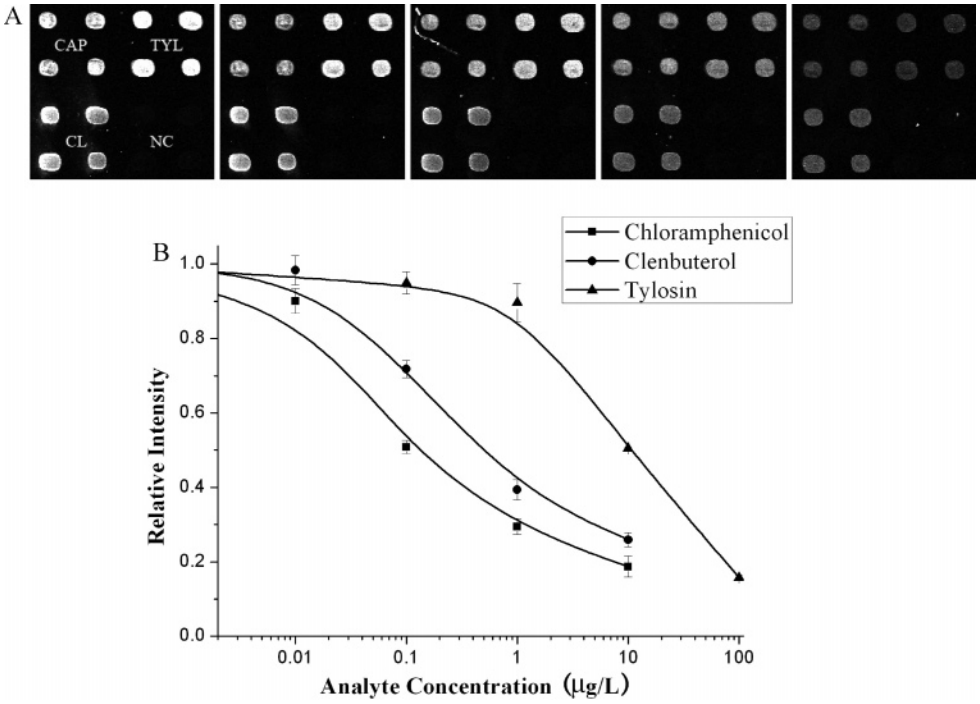

Figure 5. **A**: An image of the laser scan result. From left to right, the concentration of each drug standard was increased 10-fold. In each subarray, the four spots in the top left corner were chloramphenicol–BSA, the four spots in the bottom left corner were clenbuterol–OVA, the four spots in the top right corner were tylosin–BSA, and the last four spots in the bottom right corner were BSA used as negative control. **B**: Calibration curves for three analytes. Using the mean detection fluorescence intensity of four spot replicates of each drug as the one in this concentration, then they were divided by the intensity which was obtained by the solution without any drug standards. The calibration curves were obtained with the relative intensity against antibiotic concentration (μg/L).

Table 1. Midpoints, Working Range, and Maximum Residue Limits (MRL) for All Used Antibodies and Secondary Antibodies after Optimization and Comparison of Spiked Concentration against Found Concentration

		sample						
analytes		0	1	2	3	IC_{50} (μg/L)	working range (μg/L)	MRL (μg/L)
chloramphenicol	spike (μg/L)	0	0.01	0.1	1	0.14	0.03–1.21	0
	found (μg/L)	<0.03	0.018	0.2	1.92			
clenbuterol	spike (μg/L)	0	0.023	0.23	2.28	0.53	0.01–5.18	0
	found (μg/L)	<0.01	0.028	0.17	5.59			
tylosin	spike (μg/L)	0	1	10	100	10.53	0.88–37.7	50–200
	found (μg/L)	<0.88	2.57	19.36	58.49			

Table 2. Coefficients of Variation (CV) for Spot Intensities in Subarray and among Subarrays

	CV (%)	
analytes	in subarray	among subarrays
chloramphenicol	4.1	13.5
clenbuterol	4.6	12.8
tylosin	2.4	13.1

The midpoints, working range, and maximum residue limits (MRL) are shown in **Table 1**. The experimental results show that there was no cross-reactivity among the three drugs.

The reproducibility and stability of SMMs were examined, and the results of the coefficients of variation (CVs) in or among subarrays are shown in **Table 2**. It indicates that the coefficients of variation of the spot intensities were below 5% in a subarray and 15% among subarrays.

Quantitative Analysis. The calibration curves show that the decrease in fluorescent signal was proportional to the amount of drug concentration in the sample within the linear measurement range. Samples from foodstuffs were pretreated before testing, and then the samples were analyzed according to the operating protocol. After scanning, the mean fluorescence intensity was determined. The sample was regarded as safe if the relative intensity was below the detection limit. The concentration of the residue was calculated using the curve equation when the residue concentration was in the linear measurement range. But if the residue drug concentration was higher than the maximum of the working range, it was first diluted 10-fold; if it was still on the high side, the last step was repeated until the concentration was in the linear measurement range. The final concentration of the sample was obtained by conversion using the detected value. The method can therefore be used for both qualitative and quantitative determination of the presence of substances in a sample. Fifty blind samples (20 from milk, 10 from cheese, 10 from chicken, and 10 from pork) were detected following this protocol, and three positive samples were found.

Comparisons. One of the useful methods for demonstrating our detecting method's veracity was spike experiments. The results of the spike experiment are shown in **Table 1**, and a plot of the spiked concentrations against the found concentra-

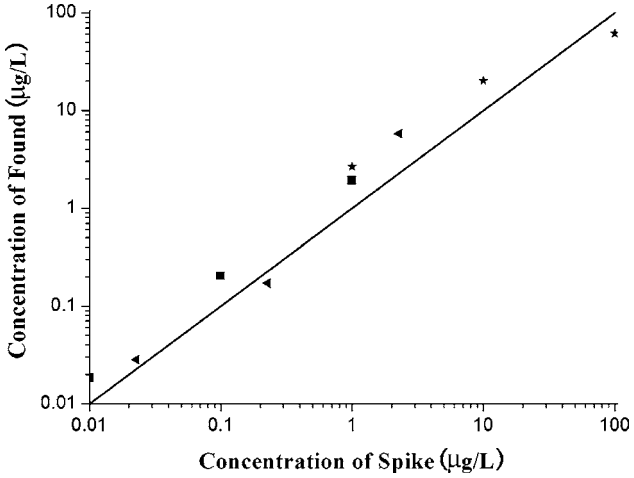

Figure 6. Spiked concentration (μg/L) against found concentration (μg/L) for quantification of three residues, each containing three analytes. Spiked samples were detected by SMMs, and then the results were compared with spiked concentration.

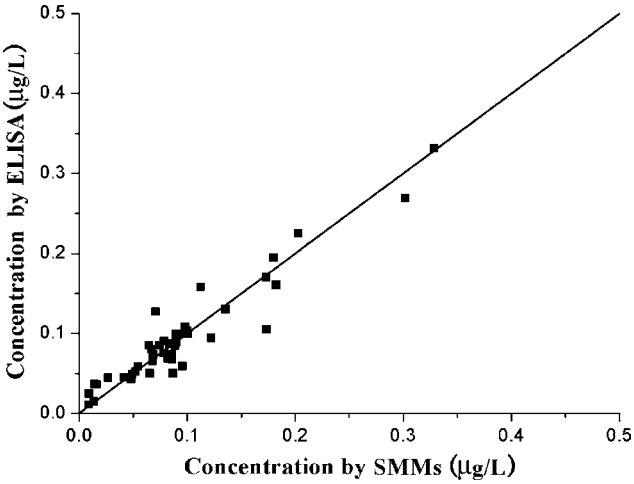

Figure 7. Comparison of the found concentration (μg/L) obtained from the SMM method and the ELISA method for the quantification of the veterinary drug residue in 50 blind samples. Fifty blind samples were detected respectively with the SMM method and the ELISA method; the curve with the found drug concentration obtained by the ELISA method was plotted against the found drug concentration obtained by the SMM method. The straight line in this figure is a reference line, and the equation of this line is $Y = X$.

tions is shown in **Figure 6**. The correlation coefficient (R^2) value generated by the spike concentration and found concentration of these three drug residues was 0.968. The results obtained from the SMM and ELISA methods agree with each other, thus verifying the accuracy of this method. Those 50 blind samples were analyzed again by the traditional ELISA method using commercial ELISA kits as parallel to validate the veracity of the SMM method. Chloramphenicol ELISA kits were bought from R-Biopharm (Darmstadt, Germany), and we operated strictly by following the instructions of the kit. The absorbance was recorded using a microtiter plate reader set (BIO-RAD Laboratories, Richmond, CA) at 450 nm. After analyzing, we noticed that there were also three positive samples. Comparing the results obtained from these two methods, we can draw a figure using the concentration obtained by SMMs against the concentration obtained by ELISA. It is shown in **Figure 7**. From **Figure 7** we can find that the data spots are nearly distributed on both sides of the standard line ($Y = X$), that is to say, the data obtained from these two methods are very similar and with no significant differences. Therefore it also demonstrated the veracity of the SMM method for detecting veterinary drug residues in foodstuffs.

Conclusion. The SMM system based on antigen−antibody reaction can be used to detect the veterinary drug residues of chloramphenicol, clenbuterol, and tylosin in foodstuffs. The three residues can be determined simultaneously within 2 h. PBS with 1% BSA was the better blocking buffer compared to PBS with 1% glycin. The detection limits for these three residues are lower than the MRLs. The results of the experiment showed that there was no cross-reactivity among the three drug residues and the coefficient of variation of the spot intensities was below 5% in a subarray and below 15% among subarrays. Comparisons of both spike sample and unknown sample detection results demonstrated the high accuracy of the microarray method. The proposed microarray system can be used to quickly analyze the residue concentrations of chloramphenicol, clenbuterol, and tylosin simultaneously in foodstuffs. Its accuracy and quick response should be suitable for customs, entry-exit inspection and quarantine, quality control, or food safety supervised domain application.

The proposed method should be able to be extended to detection of not only more kinds of veterinary drug residues but also various substances including pigment, toxins, pesticides, and chemical additives in foodstuffs.

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