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Determination of β-adrenergic agonists by hapten microarray

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ABSTRACT

The use of highly active β-agonists as growth promoters is not appropriate because of the potential hazard for human and animal health. To investigate the residue level of these β-agonists, hapten microarrays were employed for clenbuterol (CLB), ractopamine (RAC) and salbutamol (SAL) residue analysis. CLB, RAC and SAL conjugates were immobilized on the slides, which were precoated by agarose film to construct hapten microarrays, and then the corresponding monoclonal antibodies of these β-agonists and the standards or samples were introduced for indirect competitive immunoassay. Finally, Cy3-labeled secondary antibody was employed to indicate the antigen–antibody complex. The fluorescence intensity of each spot was imaged and recorded, and the calibration curve of each analyte was obtained by plot fluorescence intensity against different standard concentrations. Compared to the ELISA, the hapten microarray method was more sensitive, which got the detection limits 0.09 μg/L for CLB, 0.50 μg/L for RAC, and 0.01 μg/L for SAL. What's more, with the recovery rate between 96.5% and 106.4%, and the coefficient of variation below 10%, the proposed hapten microarray method was shown to be both quantitative and reproducible.

1. Introduction

β-Adrenergic agonists are a group of synthetic compounds that share some similarity in their chemical structure and function. Clenbuterol (CLB), ractopamine (RAC) and salbutamol (SAL) are some of the examples of β-adrenergic agonists, the chemical structures of them are shown in Fig. 1. β-Adrenergic agonists are repartitioning agents used to increase feeding efficiency and carcass leanness, and also promote animal growth. Clenbuterol, which acts as nutrient repartitioning agent in livestock by diverting nutrients from fat deposition in animals to the production of muscle tissues [1], is the most effective β-agonist that had been widely used before. However, it has been banned for feeding animals all over the world in recent years because it has been proved very toxic to human beings. Since CLB is prohibited to feed animals, RAC and SAL, as the functional analogues of CLB, are used as the substitute of CLB to promote growth. Due to economic benefits incurred by the application of β-agonists, illegal abuse of β-agonists with adverse health effects have been reported worldwide [2–5], and the residue problem has come to be an increasing public health concern. To ensure food safety, several countries such as China and some European countries have set strict regulations for these three β-agonists as zero tolerances in animal foods [6].

In order to comply with these strict regulations, a lot of analytical methods have been validated to assay for agonist residues in different levels of sensitivity, selectivity and other characteristics in animal foods, such as instrumental measurements, immunological methods, and biosensors [7–11]. However, instrumental measurements are limited not only because of their low screening efficiency and complicated operation procedures, but also they are too expensive and time-consuming, thus they are always served as an effective reference method for the confirmatory purposes only. Enzyme linked immunosorbent assay (ELISA) is an excellent survey tool for its high sensitivity and selectivity. These important characteristics make immunoassay an attractive tool for food testing by regulatory agencies to ensure food safety. However, the conventional immunoassay requires a relatively long assay time, troublesome liquid-handling procedures, and large reagent consumption [12,13]. Immunoassay is traditionally performed as individual test, however in many cases it is necessary to perform high-throughput and parallel analysis. To address this requirement, microarray-based immunoassay technologies have been developed by integrating the advantages of both microarray technology and ELISA. A number of research groups have developed immunoassays potentially useful for antibiotics multiplexed analysis. For example, Weller and co-workers have developed a parallel affinity sensor array (PASA)-based ELISA for the rapid automated analysis of 10 antibiotics in milk [14]. And Ye et al. have reported microarray-

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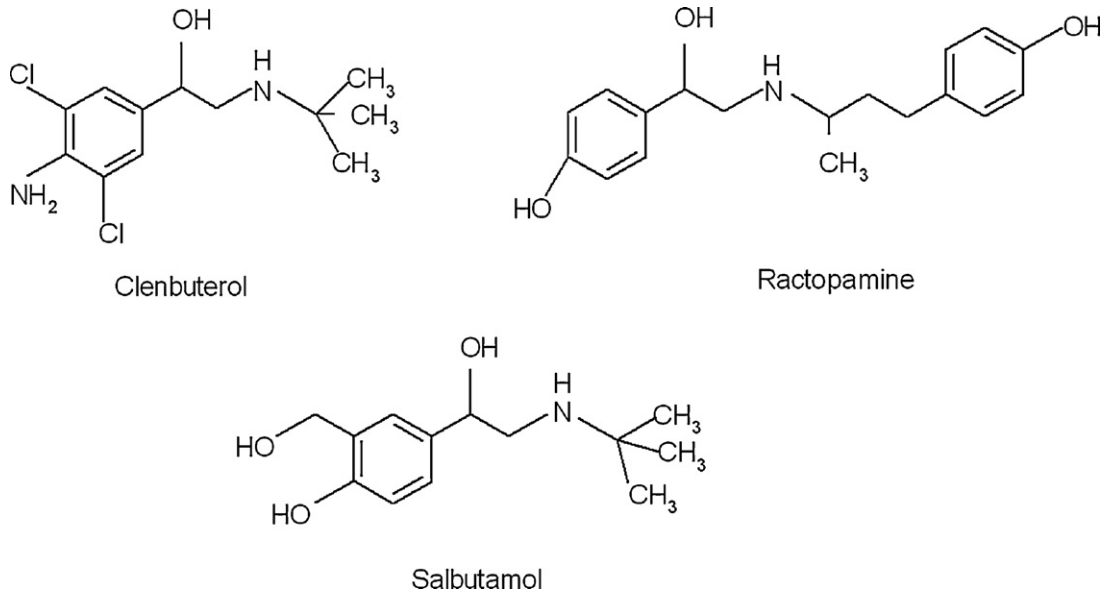

Fig. 1. Chemical structures of clenbuterol, ractopamine and salbutamol.

based methods for sensitive detection of veterinary drug residues [15–17]. It was also effectively employed to study the interaction between residue drugs and antibodies [18–20].

In this paper, an approach to detect three typical β-adrenergic agonists simultaneously by hapten microarray-based on indirect competitive immunoassay was presented. Microscope glass slides coated with agarose were used for the construction of hapten microarrays. Bovine serum albumin (BSA) was used as carrier to anchor the haptens on the agarose coated slides. Monoclonal antibodies against three β-agonists allowed the simultaneous detection of the respective analytes. The β-agonists in sample or standard solutions competed for the antibodies with the conjugates immobilized on the slides. The antigen–antibody binding on slide was detected using Cy3 dye labelled secondary antibody. Thus, the three β-agonists can be quantitatively analyzed by detecting the fluorescence signal with laser confocal scanner.

2. Experimental

2.1. Materials

Clenbuterol hydrochloride, ractopamine hydrochloride, $NaIO_4$, agarose, CLB standard, RAC standard, BSA and butane-1,4-diol diglycidyl ether were purchased from Sigma–Aldrich (St. Louis, MO). The monoclonal antibodies for CLB, RAC, SAL, and the SAL–BSA conjugate, SAL standard were from Guangzhou FuSai biology Co. Ltd (Guangzhou, China). Cy3-labeled goat anti-mouse IgG was from Rockland (Burlingame, PA).

All monoclonal antibodies and Cy3-labeled antibody solutions were prepared with phosphate buffered saline (PBS, 0.01 mol/L phosphate buffer containing 0.8% NaCl at pH 7.4). The standard stock solutions (1 mg/mL) were prepared by dissolving 10 mg of RAC, CLB and SAL in 10 mL PBS, respectively, and the working standard solutions (0.01 μg/L, 0.1 μg/L, 1 μg/L, 10 μg/L, 100 μg/L) were prepared by diluting the stock solutions.

2.2. Synthesis and identification of the CLB–BSA, RAC–BSA conjugates

The synthesis of CLB–BSA was performed by diazotization methods according to the method described by Petruzzelli and coworkers [21] with little modifications. Briefly, 10 mg of CLB was dissolved in 8 mL of 0.25 M HCl and cooled to 0 °C. After 20 min, 0.5 mL of 0.2 M $NaNO_2$ was added dropwise to this mixture under stirring in the dark at 4 °C until the starch iodide paper test turned to dark purple. After diazotization, this solution was added to BSA solution which have 45 mg of BSA dissolved in 5 mL of 0.1 M carbonate buffer (0.1 M Na_2CO_3 and 0.1 M $NaHCO_3$, pH 9.5). During this operation, the pH was maintained at 9.5 by addition of 1 M NaOH. The reaction mixture was incubated at 4 °C for 8 h. The conjugated CLB–BSA was purified by dialysis against 0.15 M NaCl for two days, during dialysis, the NaCl solution should be replaced with fresh solution for a few times. Precipitates were removed by centrifugation (4000 rpm, 10 min).

RAC–BSA conjugate was prepared by the activated epoxy method, according to a former described procedure [22]. In brief, 100 mg BSA was dissolved in 3 mL distilled water and the pH was adjusted to 10.8, then 30 μL butane-1,4-diol diglycidyl ether was added and kept stirring for 24 h at room temperature. 60 mg RAC dissolved in 2 mL of 0.1 M NaOH with 400 μL N,N-dimethyl formamide for improving RAC solubility. Subsequently, this solution was added to the epoxy-activated BSA solution. The reaction mixture was stirred to react for another 24 h at 4 °C. The conjugated RAC–BSA was purified by dialysis against 0.15 M NaCl for two days, during dialysis, the NaCl solution should be replaced with fresh solution for a few times. Precipitates were removed by centrifugation (4000 rpm, 10 min).

After synthesis and purification, the conjugated CLB–BSA and RAC–BSA were identified by UV–vis spectrophotometer and SDS-PAGE, which are different methods in principle. While the hapten was anchored to BSA, its characteristic absorption peak will shift. According to this principle, UV–vis absorption spectra of the conjugates, BSA, CLB and RAC under the concentration of 0.5 mg/mL were measured one by one at the wavelength ranged from 250 nm to 310 nm, each test was repeated three times under the same condition.

The SDS-PAGE electrophoresis was performed under the condition as follows: 3.7% stacking gel and 10% separating gel, the stacking voltage and separating voltage were 70 V and 120 V, respectively. After the electrophoresis, gels were stained overnight in 0.25% kenacid blue R, 50% (v/v) methanol, 7% (v/v) glacial acetic and destained by shaking in 50% methanol and 7% glacial acetic acid, the destained gels were photographed on white film.

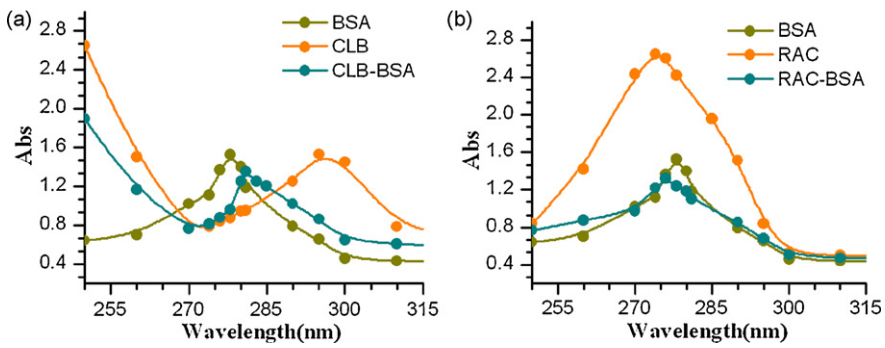

Fig. 2. The UV spectrograms of two β-agonists, conjugates and BSA. (a) The characteristic absorption peak of CLB, BSA and CLB–BSA were 295 nm, 278 nm and 281 nm, respectively and (b) the characteristic absorption peak of RAC, BSA, and RAC–BSA were 274 nm, 278 nm and 276 nm, respectively.

2.3. Preparing activated agarose film-coated glass slides

The glass slides were first 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. 1% agarose solution was prepared and boiled for 5 min. Subsequently, 2 mL of agarose solution was poured over each slide, which had preheated at 60 °C. After gelation, the slides were dried at 60 °C in a dryer overnight. The dried slides can be stored at room temperature for further use. Before immobilization, the agarose film-coated glass slides were activated by immersion in 20 mM $NaIO_4$ for 30 min at room temperature [23]. After activation, the slides were thoroughly rinsed with deionized distilled water and then dried.

2.4. Hapten microarray construction

The BSA conjugates of three β-agonists were used as probes to construct hapten microarray. BSA used as negative control and three BSA conjugates were suspended in PBS containing 20% glycerol and subsequently printed robotically onto the activated agarose film-coated slides with a distance of 600 μm between the centers of adjacent spots by the contact printing robot (SpotBot, Telechem International, CA) with micro-spotting pin (SMP4, Telechem International, CA). The spots printed on each slide were arrayed as twelve subarrays in two columns, each subarray with 4×5 pattern of three different kinds of β-agonist conjugates and negative control (BSA). Printing was performed in a cabinet at 25 °C and 60% humidity.

Hapten conjugates covalent immobilization were carried out at 37 °C overnight through a Schiff's base reaction between the amino of the conjugate and the aldehyde group on the surface of the activated agarose film-coated glass. After immobilization, the excess aldehyde group of the glass slide was blocked by PBS with 1% (w/v) BSA solution for 30 min, followed by a rinse with PBST (PBS supplemented with Tween-20) and PBS alternately for 2 min each and then dried. The proposed hapten microarrays were stored in dry condition for future use.

2.5. Hapten microarray analysis

10 μL of CLB, RAC, SAL corresponding monoclonal antibodies and 20 μL of standard samples with appropriate concentration or unknown samples were simultaneously added to the subarrays for immunoreaction. The immunoreaction was carried out by incubating the glass slide at 37 °C in a humidified chamber for 30 min. After antigen–antibody reaction, the excess antibodies were removed and the slides were washed with PBST and PBS each for 2 min and dried, and Cy3-labeled goat anti-mouse secondary antibody was introduced to indicate the antigen–antibody bindings. The incubation condition was the same as mentioned above. Finally, the slide was washed and dried for scanning.

2.6. Signal recording and data analysis

After washed and dried, the slides were imaged on the Axon 4000B (Axon Instruments, Foster City, CA) with 5 μm resolution using a Cy3 optical filter. The laser power and photomultiplier tube voltage (PMT) were set to gain optimum signal intensities. The original 16-bit tiff images were quantified with Genepix software 5.0 (Axon Instruments, Foster City, CA). In quantitative analysis, it is assumed that the strength of fluorescent signal of each spot presents 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 calibration

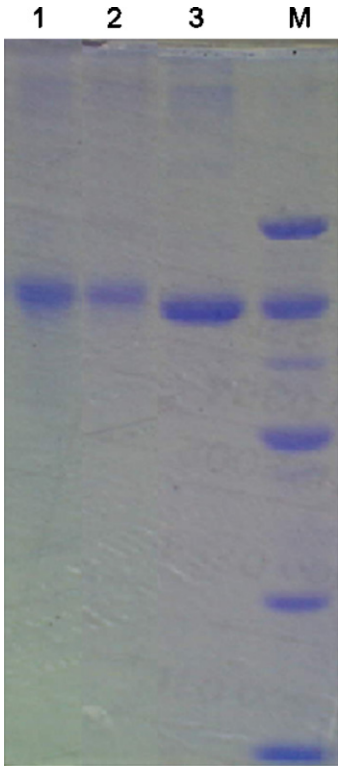

Fig. 3. SDS-PAGE of BSA, CLB–BSA and RAC–BSA (1, RAC–BSA; 2, CLB–BSA; 3, BSA; M, Marker).

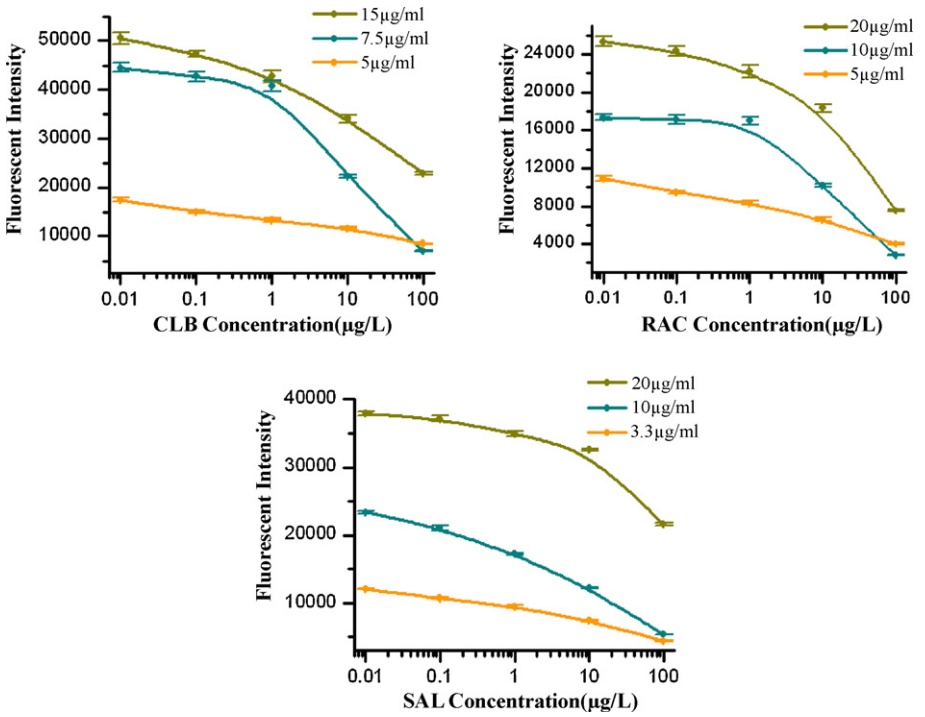

Fig. 4. Optimization of CLB, RAC and SAL monoclonal antibody concentration. The optimized concentration of antibodies of CLB, RAC and SAL were 7.5 μg/mL, 10 μg/mL and 10 μg/mL, respectively.

curve was carried out by plotting the relative fluorescence intensity against the negative solution and five standards containing each analyte in a concentration ranging from 0.01 μg/L to 100 μg/L. Thus, the residues in unknown samples can be analyzed according to the calibration curve.

3. Results and discussion

3.1. Identification of the conjugates

UV spectrum scanning is the simplest method for identification of the β-agonist conjugates. The waveforms and the characteristic absorption peak of UV spectrums between free β-agonists and their corresponding conjugates are obviously different. The results are shown in Fig. 2. The characteristic absorption peak of CLB, RAC and BSA were 295 nm, 274 nm and 278 nm, respectively, after conjugation, the characteristic absorption peak of CLB–BSA and RAC–BSA were 281 nm and 276 nm, respectively. Comparing with the change before and after the conjugation, it can be primarily confirmed that the BSA conjugates were successfully prepared. The conjugates were also confirmed by SDS-PAGE electrophoresis. One BSA molecule can combine a mount of hapten molecules, which will significantly increase the molecule weight of the conjugates. Thus, conjugates migrate at a slower rate than BSA on native SDS-PAGE gel. Typical results shown in Fig. 3 demonstrated that the BSA conjugates were successfully prepared.

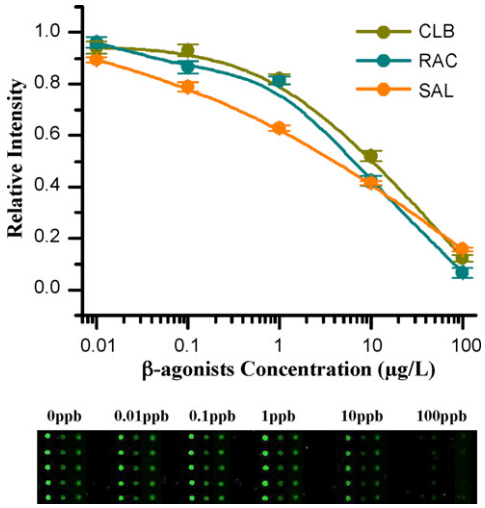

Fig. 5. Calibration curve of CLB, RAC and SAL. Using the mean detection fluorescence intensity of five spot replicates of each agonist as the one in this concentration, and then they were divided by the intensity which was obtained by the solution without any agonist standards. The calibration curves were obtained with the relative intensity against agonists concentration (μg/L).

Assuming that the molar absorbance of haptens was the same for the free and conjugated forms, according to the absorbance additivity principle, the coupling ratios (hapten/protein) of the conjugates could be approximately estimated directly by the molar absorbance [24]. The molar extinction coefficient was calculated according to the formula: K = absorbance (A)/hapten, the CLB/BSA coupling ratio was calculated by the formula: $C_{CLB}/C_{BSA} = (A_{CLB-BSA295} \times K_{BSA278} - A_{CLB-BSA278} \times K_{BSA})/(A_{CLB-BSA278} \times K_{CLB295} \times K_{CLB})$. The RAC/BSA coupling ratio was also calculated by the formula: $C_{RAC}/C_{BSA} = (A_{RAC-BSA274} \times K_{BSA278} - A_{RAC-BSA278} \times K_{BSA})/(A_{RAC-BSA278}$

Table 1
The midpoints, working range, detection limits of hapten microarray system for CLB, RAC and SAL determination.

Analytes	Detection limits (μg/L)	Working range (μg/L)	IC_{50} (μg/L)
CLB	0.09	0.09–90.95	9.82
RAC	0.50	0.50–90.89	9.13
SAL	0.01	0.01–69.42	5.31

IC_{50}: the half maximal inhibitory concentration (IC_{50}), it is the concentration of hapten required to effect a 50% inhibition in binding of an antibody to the immobilized hapten–BSA conjugate.

Table 2
Results of the spike samples determination and the mimic samples determination.

Analytes	Spike (μg/L)	Spike samples determination (n = 5)			mimic samples determination (n = 10)		
		Average found (μg/L)	Recovery rate (%)	CV (%)	Average found (μg/L)	Recovery rate (%)	CV (%)
CLB	5	4.95	98.43	7.17	5.10	102.00	5.12
	50	51.58	103.16	4.85	51.42	102.84	3.82
RAC	5	5.24	106.4	9.95	4.99	99.86	4.72
	50	48.22	96.52	4.93	50.53	101.05	3.73
SAL	5	5.24	104.8	4.07	5.03	100.62	5.99
	50	49.16	98.32	3.95	49.94	99.87	5.32

$\times K_{RAC274} \times K_{RAC}$). Follow these two formulas, as a result, the coupling ratios of CLB and RAC with BSA are 41:1 and 23:1, respectively.

3.2. Optimization the competitive immunoassay conditions

The concentration of antibody is an important factor for the quantitative analysis. In the competitive immunoassay, the antibody will competitively bind to the immobilized conjugates or free residues lies in samples or standards. The antibody concentration that is neither too high nor too low led to poor selectivity and sensitivity. The standard solutions containing three β-agonists were diluted in 10-fold, ranging from 100 μg/L to 0.01 μg/L. Different antibody concentrations against these β-agonists were tested for the competitive immunoassay.

As indicated in Fig. 4, the fluorescence intensity was affected by the concentration of antibodies and the concentration of β-agonists, however, when the antibody concentration for CLB, RAC and SAL were 7.5 μg/mL, 10 μg/mL and 10 μg/mL, respectively, good selectivity and excellent detection fluorescence intensity were achieved. In addition, secondary antibody in this study was used only for indicating the antigen–antibody complex anchored on the spots, therefore, good fluorescence signal value is the main consideration for optimization of secondary antibody concentration. In this experiment, the optimal Cy3-labeled secondary antibody concentration was 2 μg/mL.

3.3. Calibration curves

For multiplexed immunoassays, there is a potential problem of cross-interaction among the different assays. Before the calibration curves for the three analytes were set up simultaneously, the interaction among these three kinds of antigen–antibody immunoassays were tested. The test result showed that no cross-interaction was observed.

According to the optimized conditions, three conjugates were printed on the same glass slide. The standard curve is generated by plotting the relative fluorescence signal intensities obtained for each of the standards on the vertical axis against the corresponding β-agonists concentrations on the horizontal axis with Origin 7.0 software (Origin Lab, Northampton, MA). The calibration curves for CLB, RAC and SAL are shown in Fig. 5. The limits of detection (LODs) were obtained on the basis of the usual 3σ definition as 3 times the standard deviation of the blank samples. The working range can be calculated from the calibration curve and they are shown in Table 1.

3.4. Performance testing of the optimized test systems

The percentage recovery of spike analyte is one of the important parameters for evaluating the performance of the hapten microarray system. In proposed hapten microarray immunoassay system, the recoveries were obtained by determining five spike samples with 5 μg/L and 50 μg/L of CLB, RAC and SAL each in PBS. The results are shown in Table 2.

Table 3
Reproducibility performance of proposed hapten microarray.

Analytes	Inter-spot (%)	Inter-well (%)	Inter-plate (%)	Batch-to-batch (%)
CLB	2.98	7.13	9.19	17.15
RAC	3.24	6.78	9.62	18.54
SAL	2.78	4.95	9.31	14.57

The reproducibility is another important parameter in determining the β-agonists residue in samples. Samples determined in five duplicates from which coefficients of variation (CV, defined as the ratio of the standard deviation to the mean) can be calculated. The CVs of inter-spot (among spot replicates), inter-well (among subarrays), inter-plate (among microarrays), and batch-to-batch, with five repetition tests each, were shown in Table 3. These values are in accordance with the required accuracy in trace analysis (CV < 15%). Though the CV of batch-to-batch is a little higher then the CVs of inter-well and inter-plate, if the calibration curves are set up for every series of tests, the detection accuracy will be improved.

Sample testing is the final purpose for developing the hapten microarray system. The sample testing ability is undoubtedly the most important parameter to evaluate the performance of the proposed system. Like other drugs and substances used in food animals to enhance growth or control diseases, β-agonists may leave residues in the animal tissues especially in kidney. For the positive actual samples are strictly controlled, in this approach, we spiked standards into pig urine to substitute for actual samples. Different concentrations of these three β-agonists were added to 75 pig urine samples. The experimental results are shown in Table 2. The found concentrations were in accordance with CLB, RAC and SAL spiked very well with the average recovery rate from 99.86% to 102.84%.

4. Conclusions

In this study, we developed a simple and high efficient method for simultaneously determination β-adrenergic agonist residues by hapten microarray-based on indirect competitive immunoassay. The hapten microarray system, combining the advantages of microarray technology and ELISA, can perform high-throughput and parallel analysis with high sensitivity and selectivity. The result indicates that simultaneous determination of β-adrenergic agonist residues with an excellent performance can be achieved by utilizing the system. Take CLB, RAC and SAL determination as examples, the three detection limits for three analytes were 0.09 μg/L for CLB, 0.5 μg/L for RAC and 0.01 μg/L for SAL. These values are in accordance with the required accuracy in trace analysis. Therefore, the hapten microarray system has the potential for customs, entry–exit inspection, and quarantine, quality control, or food safety supervised domain applications and should be able to extend to detect of not only kinds of veterinary drug residues in parallel but also various substances of considerable interest for food safety and environmental monitoring.

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