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Simultaneous detection of four nitrofuran metabolites in honey using high-throughput suspension array technology

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Abstract: In this study, a new suspension array technology is proposed for the simultaneous quantitative detection of four major metabolites [3-amino-5-methylmorpholino-2-oxazolidinone (AMOZ), 3-amino-2-oxazolidinone (AOZ), semicarbazide (SEM), and 1-aminohydantoin (AHD)] of nitrofurans in honey samples. The analytes were coupled to bovine serum albumin before coupling to the microspheres. After coupling, a mixture of biotinylated antibodies specific for the four analytes, along with the analyte standard solutions or samples, were added to the microtiter tubes. Then, the microtiter tubes were incubated at 37°C for 45 min before streptavidin-R-phycoerythrin was added. Analyte residues were quantitatively analyzed by detecting the mean fluorescence intensity. The detection ranges for the four analytes were 0.02 - 40 μg/kg and the detection limit of AMOZ, AOZ, SEM, and AHD in honey samples were 0.087, 0.031, 0.055, and 0.131μg/kg, respectively. The recovery rates ranged from 76.6% to 106.1% for fortified samples at levels of 0.25, 1.0, 5.0, and 20μg/kg with coefficient of variation values <15%. Finally, this method was compared to commercially available kits using fortified and 'blind' honey samples and the measurements obtained using these two methods correlated well. These results indicate that the suspension array method is suitable for detecting AMOZ, AOZ, SEM, and AHD, as well as other potential drug residues in animal tissues.

Keywords: Suspension array; drug residue; beads; median fluorescent intensity (MFI); nitrofuran metabolites

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1. Introduction

Nitrofurans containing furaltadone (FTD), furazolidone (FZD), nitrofurazone (NZF), and nitrofurantoin (NFT) are a class of broad-spectrum antibiotics that were widely used commercially as veterinary therapeutics or feed additives to treat bacterial diseases in bees, cattle, swine, and poultry. Because of concerns about their potency as carcinogens and mutagens in human consumers[1], nitrofurans have been banned from use in animal husbandry in the European Union (EU) since 1995[2]. Additionally, the use of nitrofurans in animal husbandry is also strictly prohibited in the United States[3] and China[4]. In accordance with European Directive 96/23/EC[5] and Decision 657/2002/EC [6], a definitive minimum required performance limit (MRPL) was finally set for these drugs (markers = metabolites of nitrofurans) at 1 μg/kg in March 2003[7].

Nitrofurans detection and quantification is typically performed using LC-MS/MS methods[8-11], which provides unambiguous confirmatory data in accordance with EU requirements[6]. LC-MS/MS methods can be used for multiple-residue screening, but the need for extensive sample pretreatment, specialized equipment, and highly trained personnel make most chromatographic methods poorly suited for screening purposes. Therefore, LC-MS/MS methods require expensive equipment and considerable amounts of time, and less practical for screening purposes. Enzyme-linked immunosorbent assay (ELISA) based methods, which are widely used in screening approaches for nitrofuran metabolites, can detect only one component at a time[11-15]. Therefore, the development of new technologies is needed to allow analyses that are miniaturized, integrated, highly sensitive, and high-throughput. Additionally, screening methods should be easy to use and handle, have low costs, a short running time, the possibility of automation, good specificity, and a detection capability with an error probability <5%.

MultiAnalyte Profiling (xMAP) technology from Luminex (Austin, TX, USA) is a new platform for robust multiplexed immunochemical detection. It is an emerging technology that uses small carboxylated polystyrene microspheres that are internally dyed with red and infrared fluorophores[16]. By varying the ratio of the two fluorophores, up to 100 different color-coded microsphere sets can be distinguished, and each microsphere set can be coupled to a different biological probe. The microspheres are detected and

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characterized by a dedicated flow cytometer[17], using a red laser (635 nm) for excitation and emission wavelengths that are measured between 645 and 669 nm and >712 nm. After the microspheres are classified, the reporter signal is measured. The general reporter molecule used is streptavidin-R-phycoerythrin (SA-PE) which is excited by a green laser (532 nm) and has an emission that can be measured at 580 nm[18]. Each microsphere can be coupled to a different biological probe which, in principle, makes it possible to simultaneously measure 100 different biomolecular interactions in a single sample.

This technology has been used in many fields, for instance, in the detection of cytokines [19,20], mycotoxins[21], nucleotides[22-24], antibodies[25] and food substances[26]. Because of the prominent characteristics of the Luminex system, it can potentially be used for the simultaneous detection of small molecules. In this study, we established a novel suspension array method for the simultaneous detection of AMOZ, AOZ, SEM, and AHD. Firstly, AMOZ, AOZ, SEM, and AHD were conjugated to a carrier protein and then the conjugates were covalently bound to small carboxylated polystyrene microspheres by the carrier protein. Next, a mixture of the biotinylated antibodies specific for the four analytes along with the analyte standard solutions or samples were added to the microtiter tubes. Then, the microtiter tubes were incubated at 37°C for 45 min before SA-PE was added. Analyte residues were quantitatively analyzed by detecting the mean fluorescence intensity. Finally, this suspension array method that we have developed was compared to ELISA methods, and no significant difference was observed between the two methods. The suspention array method can be developed for the simultaneous detection of other antibiotics and potentially in other animal samples.

2. Materials and methods

2.1 Reagents and instruments

Carboxylated beads, numbers 19, 37, 57, and 26, and a Bio-Plex™ amine coupling kit were supplied by Bio-Rad, USA. Monoclonal antibodies (mAb) that recognize AOZ, SEM, AMOZ, or AHD were prepared in our lab. N,N-dimethylformamide (DMF) and N,N′-dicyclohexylcarbodiimide (DCC) were purchased from Sinopharm Chemical

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Reagent Co. (Shanghai, China). Biotinyl-N-hydroxy-succinimideester (BNHS), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), and N-hydroxy-sulfosuccinimide (Sulfo-NHS) were supplied by Pierce (USA). Commercial kits for AMOZ, SEM, AOZ,or AHD were purchased from Huaan Magnech Bio-Tech Co. (Beijing, China). All standard antibiotic substances and BSA were obtained from Troody Technology Co. (Shanghai, China). The honey samples used for detection were purchased from a local supermarket. A Luminex 200 suspension array system (Luminex, USA) was used for analysis and data processing.

2.2 Preparation of immunogens and coating antigen

The compounds 4-{[5-(morpholinomethyl)-2-oxooxazolidin-3-ylimino] methyl}benzoic acid (CPAMOZ), 3-{[(3-carboxyphenyl)methylene]-amino}-2-oxazolidinone (CPAMOZ), 3[(3-Carboxyphenyl)methylene]-hydrazinecarboxamide (CPSEM), and 1-[(4-carbo-benzylidene)-amino]-imidazolidin-2,4-dione (CPAHD) were derivatized from AMOZ, AOZ, SEM, and AHD with 4-CBA, respectively, according to a previously published method[27]. Then, CPAMOZ, CPAMOZ, CPSEM, and CPAHD were conjugated to the carrier protein BSA via an active ester method[28]. The carboxylic acid on CPAMOZ, CPAOZ, CPSEM, or CPAHD was activated with DCC and NHS to produce an active ester, which then reacts with the amine groups on BSA to form an amide bond.

2.3 Monoclonal antibody preparation

Monoclonal antibodies were prepared according the method developed by Kohler [29] with minor modifications. Briefly, 60 μg of each immune antigen (CPAMOZ-BSA, CPAOZ-BSA, CPSEM-BSA, or CPAHD-BSA) emulsified with Freund's complete adjuvant was subcutaneously injected into four 6- to 8-week-old female BALB/c mice. Three weeks later, these mice were immunized with a subcutaneous injection of 80 μg of each antigen emulsified in Freund's incomplete adjuvant. The final injection into the peritoneal cavity of a selected mouse with higher antibody titers was performed with each immune antigen without adjuvant. Three days later, these mice were sacrificed and the

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spleens were collected for hybridoma production. Briefly, spleen cells were fused with SP2/0 cells at a 5:1 ratio with 50% PEG6000. After fusion, the cells were selected using selection medium containing 20% FCS and hypoxantin-azaserin solution in microtiter polystyrene plates. The growing hybridoma cells were screened for antibody production using an indirect competitive ELISA method, and the positive hybridomas were subcloned by a limiting dilution method. Stable antibody-producing clones were then expanded until monoclones were obtained. Then, monoclonal antibodies were produced in mouse ascites and purified with saturated ammonium precipitation.

2.4 Biotinylation for the four mAbs

Biotinylation of the four mAbs was performed according to the manufacturer's instructions. Briefly, BNHS was diluted to 10 mM by DMF and the four mAbs were diluted to 2 mg mL^{-1} using 0.2 mol L^{-1} $NaHCO_3$, pH 8.5. The two solutions were mixed at a 1:30 ratio (by volume) and stirred for 30 min for biotinylation of the four mAbs at room temperature. After labeling, excess non-reacted and hydrolyzed biotin reagent in aqueous solution were dialyzed with 10 mM phosphate-buffered saline (PBS), pH 7.2. Next, mAbs were aliquotted and stored at -20°C until subsequent use.

2.5 Bead preparation

Covalent coupling of the capture antibodies to the microspheres was performed following the procedures recommended by Luminex. In brief, the stock solutions for the microspheres were dispersed in a sonification bath (Sonicor Instrument Corporation, Copiaque, NY, USA) for 1 min. An aliquot of 1.25×10^6 microspheres was resuspended in microtiter tubes (Eppendorf, Hamburg, Germany) containing 0.1 M sodium phosphate buffer, pH 6.1 (phosphate buffer), to a final volume of 100 μL. This suspension was sonicated until a homogeneous distribution of the microspheres was observed. Solutions of Sulfo-NHS and EDC, both at 50 mg mL^{-1}, were prepared in phosphate buffer, and 10 μL of each solution was sequentially added to stabilize the reaction and activate the microspheres. This suspension was incubated for 20 min at room temperature and then resuspended in 500 μL PBS (pH 7.4) containing 7 μg of CPAMOZ-BSA, CPAOZ-BSA,

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3 CPSEM-BSA, or CPAHD-BSA. The mixture was incubated for 2 h in the dark at room temperature with continuous shaking. Microspheres were then incubated with 250 μL PBS with 0.05% Tween 20 for 4 h. After aspiration, the beads were blocked with 1 mL PBS with 1% BSA and 0.1% sodium azide. Microspheres were counted with a hemacytometer and stored at a final concentration of 10^6 microspheres per mL in the dark at 4°C. To confirm the coupling of the protein, 2000 beads of each set were taken and mixed with a saturating concentration of specific biotinylated mAbs. After a 1-h reaction in a vortexer at 37°C and filtration of the excess mAbs, sufficient SA-PE was added to each vial for a nonreversible coupling reaction for 30 min with biotinylated mAbs that can capture cognate antigens on beads. Then, 100 microspheres were read out and median fluorescent intensity (MFI) values were determined using a Luminex suspension array system.

2.6 Sample preparation

Samples were prepared according to a method previously described by Pimpitak [15] with minor modifications. Briefly, 1 g honey sample was homogenized and fortified with 10 μL of the four compounds-AMOZ, SEM, AOZ, and AHD-at different concentrations in 4 mL deionized water. Then 0.5 mL 1 M HCl and 100 μL 40 mM 4-nitrobenzaldehyde in DMSO were added to the homogenized tissue solutions. Each sample was thoroughly mixed and incubated for 3 h in a water bath at 55°C. Subsequently, samples were cooled to room temperature and the pH was adjusted by adding 5 mL 0.1 M dipotassium hydrogen phosphate and 0.4 mL 1 M sodium hydroxide. The samples were extracted with 6 mL ethyl acetate and centrifuged (3500×*g* for 10 min) before removing the ethyl acetate supernatant, which was removed by evaporation under a stream of nitrogen using a heating block at 45°C. The resulting residues were dissolved in 2 mL 1:1 (v/v) mixture of hexane and 0.1 M PBS at pH 7.4. The buffer phase, containing the derivative, was separated by centrifugation at 3000×*g* for 10 min and collected for Luminex 200 analysis. Four separate extractions were performed and each sample was measured in triplicate. The concentrations of analyte were calculated based on the standard curve run on the same plate. The precision and recovery of the Luminex 200 analysis were also

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determined from the fortification experiments that used un-spiked samples as blanks

2.7 Suspension array detection procedure

Analyte standard stock solutions were prepared in DMF at concentrations of 50 μg/mL. For the calibration curves, the necessary standard solutions were prepared from these stock solutions by serial dilutions in PBS with 0.05% Tween, 1% BSA, and 0.1% sodium azide (PBS-TBN).

For this assay, 25 μL standard solution and sample extracts or blank solution (negative control) were added to each microtiter tube. During the initial testing, each biotinylated antibody was tested separately to check for cross-reactivity. Next, 15 μL biotinylated antibody cocktail was added to each microtiter tube. Subsequently, 10 μL mixture of 4000 beads coupled to different analytes was added and the microtiter tubes were incubated for 45 min and shaken at a medium speed at 37°C for the competitive antigen-antibody reaction. Microtiter tubes were then centrifuged at 12,000×*g* for 10 min). Beads in the microtiter tubes were washed with PBS-TBN, pH 7.4, to remove the sample and unbound antibodies. SA-PE was then added to the microtiter tubes and incubated for 30 min while shaking at a medium speed at 37°C. The microtiter tubes were then washed using PBS with 1% BSA and 0.5% Tween 20, pH 7.4, and HPE-buffer was added at a final volume of 100 μL. Then, the mixture in the tubes was transferred to a 96-well plate. The fluorescence intensity of the beads was measured using a Luminex analyzer to determine the MFIs.

2.8 Optimization of the amounts of mAbs

To optimize the amounts of the four biotinylated mAbs that were added, serial dilutions of four biotinylated mAbs were added for 1000 beads in triplicate. The optimal amounts of added antibodies were determined based on the MFI obtained.

2.9 Assay validation

The limit of detection (LOD) was defined as the lowest amount of analyte in a sample that could be detected, but not necessarily exactly quantified, which was based on the mean value of 20 blank samples plus the mean standard deviation × 3 according to the

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guidelines of Decision 657/2002/EC [6]. The accuracy and precision of this method were indicated by the recovery and coefficient of variation (CV), respectively. Recovery % = (concentration measured/concentration fortified) × 100%. Blank samples were simultaneously fortified with 0, 0.25, 1, 5, or 20 μg/kg AMOZ, AOZ, SEM, or AHD, and the recovery was calculated after measurement of these samples. To assess the CV, the assay was repeated three times. The mean recovery and CV values were calculated for honey samples. The precision of the suspension array method was analyzed by repeated analyses of the fortified samples and comparisons of the intra- and inter-assay CVs. Intra-assay CVs were measured based on three replicates of each fortified concentration, and the inter-assay CV was based on results on five different days.

To determine the specificity of the suspension array, it was exposed to different types of chemicals: tetracycline, oxytetracycline, chlortetracycline, chloramphenicol, and methylsulfadiazine at a concentration of 200 μg/mL were used to test for cross-reactivity. Blank controls (without competitor) were also prepared.

2.10 A comparison of the suspension array method with commercially available kits for detecting AMOZ, AOZ, SEM, and AHD spiked into honey samples

60 fortified honey samples at different concentrations of AMOZ, AOZ, SEM, and AHD and fifty honey samples with unknown concentrations of these compounds were assayed by suspension array and commercial kits simultaneously. Each of the fortified concentrations were prepared in three replicates. Based on the MFIs and standard curves that we obtained, the concentrations detected in the fortified samples could be calculated. Values of measured and real concentrations of fortified samples were compared.

3. Results and discussion

3.1 The optimal concentration of biotinylated monoclonal antibody used in the simultaneous detection assays

The concentrations of *a*ntigen and antibody have great effects on their reaction efficiencies. The macromolecule-immune complexes are unable to form if the amount of antigen or antibody is excessive. There is an equivalence zone in which the dose of

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antigen and antibody are present in optimal proportions and huge grid-like aggregates of immune complexes can be formed. In this study, serial dilutions of biotinylated mAbs were prepared and a 'checker board' titration was employed to optimize the concentrations of biotinylated mAbs. Using a constant amount of beads, the MFI values first increased and then reached a plateau when the concentration of biotinylated mAbs increased. The concentration of biotinylated mAbs at the slope of the curve was chosen as an optimal concentration. The working concentrations of biotinylated mAbs for AMOZ, AOZ, SEM, and AHD were 0.23, 0.42, 2.13, and 1.84 μg/mL, respectively. As shown in Figure 1, we also found that different biotinylated mAbs were associated with different MFI values at which the value reached a plateau. Based on the beads coupling kit instructions, 7 μg CPAMOZ-BSA, CPAOZ-BSA, CPSEM-BSA, or CPAHD-BSA were used for the coupling reaction along with 100 μL carboxylated microspheres (1.25×10^{7} mL^{-1}). We analyzed the coupling rates of CPAMOZ-BSA, CPAOZ-BSA, CPSEM-BSA, and CPAHD-BSA, and the inter-molecular repulsion effects and the incomplete recognition between the specific mAbs and cognate Ags immobilized on beads resulted in different maximum MFIs values for different conjugates.

3.2 Standard curve generation and the detection of AMOZ, AOZ, SEM, and AHD in honey

In this study, a high-throughput suspension array method was established for the simultaneous detection of AMOZ, AOZ, SEM, and AHD residues in honey. The AMOZ, AOZ, SEM, and AHD residues were derivatized into NPAMOZ, NPAOZ, NPSEM, and NPAHD, respectively, for detection using the high-throughput suspension array method that we developed. Standard solutions of the four analytes were diluted in PBS-TBN at concentrations of 0, 0.02, 0.078, 0.313, 1.25, 5, 20, and 40 μg/mL. Finally, the concentration of NP-analyte was determined and converted into analyte concentration using the following formula: $C_{analyte} = \frac{M_{analyte}}{M_{NPanalyte}} \times C_{NPanalyte}$; where $C_{NPanalyte}$ is the concentration of NPAMOZ, NPAOZ, NPSEM, or NPAHD detected in the sample;

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3 $M_{analyte}$ is the molecular weight of AMOZ, AOZ, SEM, or AHD; $M_{NPanalyte}$ is the molecular weight of NPAMOZ, NPAOZ, NPSEM, or NPAHD; and $C_{analyte}$ is the concentration of AMOZ, AOZ, SEM, or AHD calculated from the formula used to detect samples. The standard curve for the high-throughput suspension array is shown in Figure 2. The 50% inhibitory concentration (IC_{50}) of NPAMOZ, NPAOZ, NPSEM, and NPAHD calculated from the standard curve was 0.76, 0.244, 0.65, or 1.29 μg/mL, respectively. The IC_{50} value is an important index of the sensitivity of the suspension array method. The sensitivity indicates the minimum detectable concentrations for the suspension array-based detection, and the lowest detection limits (LDLs) for NPAMOZ, NPAOZ, NPSEM, and NPAHD in dilution buffer were 0.02 μg/mL.

3.3 Assay validation

The LODs of the suspension array method for AMOZ, AOZ, SEM, and AHD in honey samples were 0.087, 0.031, 0.055, and 0.131 μg/kg, respectively. The LODs for the four analytes in honey samples were below 0.2 μg/kg, which is lower than the MRPL for the metabolites of nitrofurans residues established by the European Commission. The honey samples were fortified with AMOZ, AOZ, SEM, and AHD at concentrations of 0.25, 1.0, 5.0, and 20 μg/kg and the mean recovery and CV values for the analytes in honey samples are shown in Table 1. Compared to the previously reported ELISA methods, the LOD values for the four analytes were comparable. For example, in previous reports the LODs values of AMOZ were 0.1 and 0.16 μg/kg[15, 30]. O'Mahony et al. developed a biochip screening assay for the detection of four nitrofuran metabolites in honey in which the detection capabilities ranged between 0.15 and 0.24 μg/kg[31]. These findings indicated that the suspension array technique was suitable for the rapid and simultaneous screening of the four main nitrofuran metabolites in honey.

3.4 Specificity of the suspension array detection

All of the individual analyte-specific mAbs were tested with a complete mixture of the four analyte-specific microsphere sets to test whether cross-interactions between the assays could be observed. Table 2 shows that the final selection of reagents did not show

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any remarkable cross-reactivity between the assays. Otherwise, NPAMOZ, NPAOZ, NPSEM, NPAHD, Tetracycline, Oxytetracycline, Chlortetracycline ,Chloramphenicol, and Methylsulfadiazine at the concentration of 200 μg/mL, were chosen to estimate the specificity of the suspension array. Based on Table 3, we found that there was no cross-reactivity with the other chemicals. The average MFI of the selected chemicals was not significantly different from the corresponding average MFI of the blank control. This finding indicated that the method developed herein could be used to simultaneously detect the four analytes in honey and potentially in other animal-derived foods.

3.5 A comparison of the suspension array and commercially available kits for detecting AMOZ, AOZ, SEM, and AHD spiked into honey samples

A spiking experiment is a useful method for demonstrating the veracity of a detection method. To demonstrate the applicability of a suspension array method to the evaluation of levels of residual AMOZ, AOZ, SEM, and AHD in honey samples, fortified samples were simultaneously analyzed using the suspension array and ELISA methods. We obtained 60 fortified honey samples at different concentrations (0, 0.25, 1.0, 5.0, or 20 μg kg^{-1}) and purchased 50 'blind' honey samples from a supermarket and analyzed both using the suspension array and commercially available kits (Table 4). Generally, there was a good correlation between positive samples, in the range of 0.25-20 μg/kg, obtained using these techniques. However, the detection ranges of suspension array are much broader than that of the traditional ELISA. In our analysis of 50 'blinded' honey samples using the suspension microarray and commercial kits simultaneously, no positive samples were detected by either method. Such concordant results indicated that the data obtained using these two methods were well correlated. Therefore, this comparison demonstrated the veracity of the suspension microarray method for detecting veterinary drug residues in foodstuffs.

4. Conclusion

Currently, ELISA-based screening tests for nitrofuran metabolites (AMOZ, AOZ, SEM, or AHD) are broadly used as an effective tool in regulatory, residual, and industrial laboratories. Development of the suspension array method enables simultaneous and

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rapid screening for AMOZ, AOZ, SEM, and AHD residues in food samples. The detection capability of the assay for honey samples was sufficiently lower than the MRPL of 1 μg kg^{-1}, demonstrating that the suspension array assay developed herein is suitable for use in screening analyses. Collectively, this evaluation of the suspension array method for simultaneous AMOZ, AOZ, SEM, and AHD detection in blind and fortified samples demonstrated the applicability of this technique to monitoring for AMOZ, AOZ, SEM, and AHD contamination in honey and potentially other types of food.

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Table 1 Mean recoveries of AMOZ, AOZ, SEM and AHD from honey samples using an optimized suspension array method (n = 4).

	Concentration fortified (μg/L)	Recoverys (100%)	CV%	
			Intra-assay	Inter-assay
AMOZ	0.25	93.2	8.2	13.2
	1.0	86.5	6.3	12.7
	5.0	94.9	3.8	9.3
	20	80.4	2.6	8.1
AOZ	0.25	91.4	7.2	12.1
	1.0	106.1	6.8	11.7
	5.0	90.1	6.6	9.6
	20	95.8	4.1	7.1
SEM	0.25	98.3	9.2	14.1
	1.0	103.2	7.8	10.7
	5.0	77.6	4.2	8.6
	20	77.9	3.1	8.1
AHD	0.25	90.4	5.2	10.1
	1.0	89.6	3.7	8.7
	5.0	82.2	2.2	5.6
	20	76.6	3.4	4.8

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Table 2 Average (n=3) respones (MFI) obtained with the suspension array method in buffer using the individual and the mixed biotinylated antibodies.

beas / MFI / antibodies	Beads-AMOZ	Beads-SEM	Beads-AOZ	Beads-AHD
Mixed antibodies	11083.67±397.32	3835.45±187.38	10953.67±345.62	5226.56±232.56
Anti-AMOZ	10051.50±275.56	16	13	15
Anti-SEM	13	3736.54±205.87	25	12.5
Anti-AOZ	11.5	32	9129.25±302.36	13
Anti-AHD	18	12	17	4968.86±294.23

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Table 3 Average responses (MFI) obtained with the suspension array method for cross-reaction experiment

Chemicals (200 μg L^{-1})	MFI ($\bar{X} \pm S$, n=3)			
	Beads-AMOZ Anti-AMOZ	Beads-AOZ Anti-AOZ	Beads-SEM Anti-SEM	Beads-AHD Anti-AHD
Blank control	10051.56±532.32	9072.43±303.43	3836.35±138.49	4982.50±289.34
NPAMOZ	15	8984.35±289.12	3689.00±201.24	4756.78±264.72
NPAOZ	10354.67±478.65	27	3607.59±132.67	5105.35±312.30
NPSEM	9284.50±408.56	8903.59±356.64	13	4965.15±272.83
NPAHD	10284.45±389.40	9006.45±376.34	3998.43±142.87	21
Tetracycline	10084.83±500.67	9124.56±337.78	3748.65±98.96	5001.89±322.19
Oxytetracycline	9684.75±423.16	9087.83±343.38	4024.15±231.90	4932.32±279.54
Chloramphenicol	9883.58±453.45	8906.72±325.78	3824.43±211.32	4983.48±232.93
Chlortetracycline	9984.39±401.67	9012.15±317.38	4004.32±198.54	5005.36±268.48
Methylsulfadiazine	10004.59±486.16	8972.17±342.65	3904.67±241.33	4897.82±252.83

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Table 4 Samples detection for AMOZ, AOZ, SEM, and AHD by suspension array and commercial kits

Analytes	Fortification ($\mu g\ kg^{-1}$)	Real detection concentration ($\mu g\ kg^{-1}$)	
		Suspension array ($\mu g\ kg^{-1}$)	Commercial kits($\mu g\ kg^{-1}$)
AMOZ	0	0.01	0.02
	0.25	0.23	0.26
	1.0	0.87	1.02
	5.0	4.75	4.43
	20	16.08	18.32
AOZ	0	0	0
	0.25	0.23	0.23
	1.0	1.06	0.95
	5.0	4.51	4.20
	20	19.12	17.28
SEM	0	0	0.02
	0.25	0.25	0.27
	1.0	1.03	1.22
	5.0	3.88	4.05
	20	15.58	14.98
AHD	0	0.01	0
	0.25	0.23	0.22
	1.0	0.87	0.79
	5.0	4.11	4.03
	20	15.32	16.02

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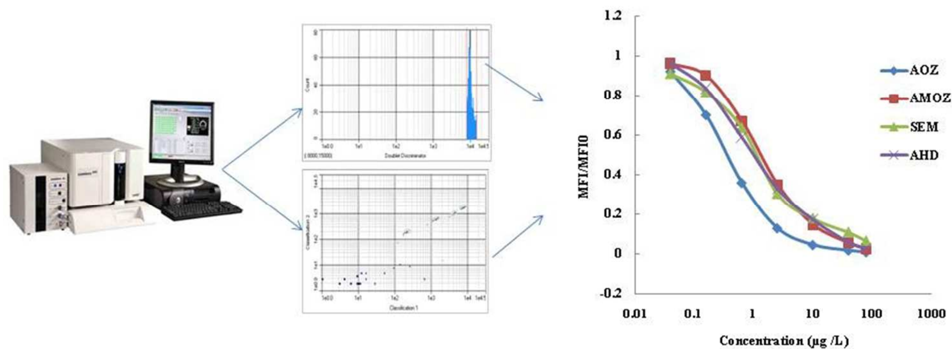

Graphical Abstract.

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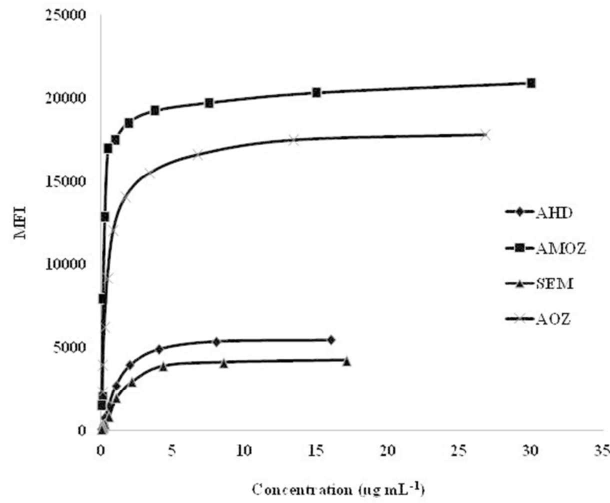

Fig. 1. The relationship between obtained MFIs and the concentrations of biotinylated mAbs for AMOZ, AOZ, SEM, and AHD.

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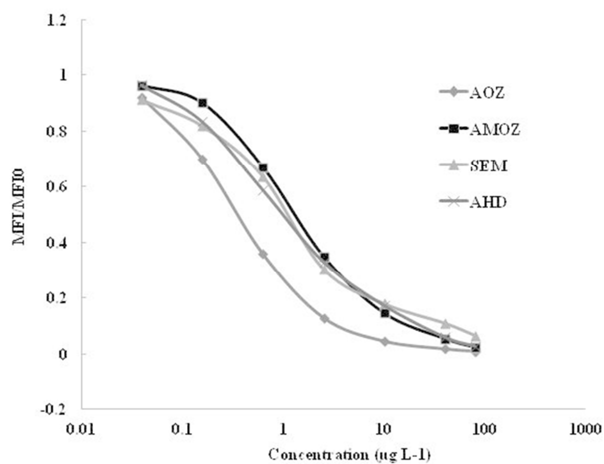

Fig. 2. The standard curves for NPAMOZ, NPAOZ, NPSEM, and NPAHD in the buffer PBS-TBN (MFI_0: average of MFIs obtained from blank wells).

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