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Simultaneous detection of five antibiotics in milk by high-throughput suspension array technology

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ABSTRACT

A new suspension array technology is proposed for the simultaneous quantitative determination of five antibiotics—tylosin, tetracycline, gentamicin, streptomycin, and chloramphenicol in milk. A novel treatment of milk samples for suspension array with diethyl ether was performed which greatly reduced the interference of the disturbing components in milk on the reaction results with no significant effect on detection sensitivity. Compared with using biotin labeled monoclonal antibody, using of secondary antibody–biotin make the detection sensitivity further improved. The minimum detectable concentration in samples of tylosin, tetracycline, gentamicin, streptomycin, and chloramphenicol were 0.3, 1.5, 4, 20, and 25 ng/ml, respectively, and the working ranges of samples were 6–400, 7–300, 8–200, 90–3000 and 70–8000 ng/ml, respectively. The mean recovery was 89.38–113.73% with a standard deviation within 16.62%. The suspension assay technology is powerful for the fast quantitative analysis of multi-antibiotics residue in milk.

1. Introduction

Antibiotics are used in veterinary and human medicine for the treatment and prevention of microbial infections. Antibiotics for the medication of cows, including those that are lactating, are approved in China. Milk from medicated animals may contain antibiotics residues if withdrawal periods are disobeyed after administration; however, the National Standards of China has no established maximum residue limits (MRLs) yet on the amount of antibiotics in milk. Chloramphenicol (CAP) is a potent and broad-spectrum antibiotic used in veterinary practice, including the treatment of aquaculture species and livestock husbandry [1]. Gentamicin (GM), an aminoglycoside antibiotic known to be useful in the treatment of coliform and other Gram-negative pathogens [2], is applied for the treatment of mastitis by intramammary route among Chinese dairy farmers. In veterinary medicine, streptomycin (STM) and tetracycline (TC) are used against many species of Gram-positive and Gram-negative bacteria in cattle, pigs, sheep, and poultry [3]. Tylosin (TYL) is a macrolide antibiotic that may persist in animal tissues and cause tumors and secretions for extended periods. It is approved for use in mastitis therapy [4]. These antibiotics are widely used by dairy farmers in China for the treatment and prevention of microbial infections in livestock. In Europe, the MRLs for CAP, GM, STM, TC, and TYL in milk are 0.3, 100, 200, 100, and 50 ng/ml, respectively.

For the detection of these antibiotics, a number of different methods based on different technologies have been described, such as chromatography-based methods [5–11], the microbial inhibition assays [4,12] and immunoassays [13–15]. However, these technologies have some drawbacks, including complicated operation of gas or liquid chromatogram with mass spectrometry technology (GS–MS or LC–MS), niggling sample preparation, and time-consuming testing. Microbial inhibition assays require relatively long assay time (4–6 h), while traditional immunoassays such as enzyme-linked immunosorbent assay (ELISA) need more antibodies and are not readily compatible for detecting large numbers of different targets in samples.

As a result of rapidly increasing demand for accurate, cost-effective, and highly flexible multiplexed assay systems, novel techniques have been described for use. Luminex (*x*MAP®) suspension array is a high-throughput and efficient screening technology developed by Luminex Corporation, USA. In this technology, 5.6 μm polystyrene microspheres that bear carboxylate functional groups on the surface are used as vehicles for molecular reactions and are available in 100 distinct sets classified by two kinds of fluorochrome [16]. 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 in theory. It offers several other distinct advantages over traditional methods, such as high-throughput, versatility, flexibility, accuracy, and reproducibility. This technology has been used

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in many fields, for instance, in the detection of cytokines [17,18] and nucleotides [19–21], antibody screening [22] and the assay of substances in food [23,24]. Due to the prominent characteristics of Luminex system, it has the potential for the simultaneous detection of small molecules.

The detection of three kinds of veterinary drugs had been showed in our previous report [25], but the actual sample was not evaluated. In the present work, using of secondary antibody–biotin made the detection sensitivity further improved and avoided the effects caused by the unstability of the biotin labeled monoclonal antibody. A novel treatment of milk samples was also evaluated and five different kinds of antibiotics in milk were detected by suspension array technology.

2. Materials and methods

2.1. Reagents and materials

Carboxylated beads and Bio-Plex™ amine coupling kit were supplied by Bio-Rad, USA. Monoclonal antibodies (mAb) and holoantigen [the targets conjugated with bovine serum albumin (BSA)] for GM and TC were purchased from Jiemen & Baolinmai Bioengineering Co., Ltd. (Shanghai, China). The mAb and holoantigen for CAP were purchased from Huaan Magnech Bio-Tech Co., Ltd. (Beijing, China), the mAb and holoantigen for STM were purchased from Food Safe Bio-Tech Co., Ltd. (Guangzhou, China). The mAb for TYL was purchased from W.H.P.M. Bioresearch & Technology Co., Ltd. (Beijing, China), and the holoantigen of TYL was provided by the same company. Rabbit anti-mouse IgG–biotin conjugate (secAb–biotin) was from Abcam Ltd., UK. 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), streptavidin-R-phycoerythrin (SA-PE), and N-hydroxysulfo-succinimide (S-NHS) were purchased from Pierce (Rockford, IL, USA). All the standard substances of antibiotics and BSA were obtained from Sigma–Aldrich, USA. The milk samples (full milk) to be detected were bought from a local supermarket. Other chemicals were of analytical grade.

2.2. Instruments

Bio-Plex™ suspension array system (Bio-Rad, USA) was employed for analysis and data-processing. THZ-C constant temperature oscillator (Taicang, China), KUBOTA 6930 centrifuge (KUBOTA Inc., Japan), and IKA MS3 digital orbital shaker (IKA Inc., Germany) were employed for the treatment of beads. Polystyrene sterile 96-well microtiter plates were from Costar® (No. 3599, Corning, NY).

2.3. Preparation of milk sample

The milk sample free from antibiotics was divided into two parts: one spiked with antibiotics (TYL, TC, GM, STM, and CAP) at three different concentrations (50, 100 and 200 ng/ml) and the rest without any treatment. Transfer the samples to centrifuge tubes, followed by adding five times volume of diethyl ether in tubes, and then mix them by high-speed vortex for 1 min, centrifuged at 8000 × *g* for 10 min. The precipitate between the two phases (sample and diethyl ether) was dropped carefully. Then the diethyl ether was removed by gentle nitrogen blow-down. After purification by 0.22 μm ultrafiltration membrane, samples were stored at −20 °C before analysis. In this paper, the treated milk sample free from antibiotics was called standard milk, the treated milk sample with spiked antibiotics was called spiked milk, and the treated milk sample for detection was called sample milk.

Table 1
The concentration of each target in mixed working standards (ng/ml).

Number	Concentration gradient of targets				
	TYL	TC	GM	STM	CAP
1	0.0256	0.05	0.4	0.08	0.5
2	0.128	0.25	0.8	0.4	2.5
3	0.64	1.25	1.6	2	12.5
4	3.2	6.25	3.2	10	62.5
5	16	31.25	6.4	50	312.5
6	80	156.25	12.8	250	1562.5
7	400	781.25	25.6	1250	7812.5

2.4. Preparation of the beads

The preparation of the beads was followed by the procedures of our previous report [25]. Five holoantigens were coupled with beads with different codes. The prepared beads were suspended in 0.5 ml of the storage buffer (Bio-Plex™ amine coupling kit), counted by hemocytometer, and stored at 4 °C before use.

2.5. Assay protocol for milk samples

The prepared five different sets of conjugates-coupled beads were mixed with the same proportion for detection. The mixed working standards of the five antibiotics were diluted into seven concentration gradients. The concentrations of each target were shown in Table 1. The five mAbs solution were diluted to an appropriate concentration based on the results of the optimization.

To obtain the standard curve in the milk sample, 5 μL gradients working standards of antibiotics, 5 μL mixed mAbs, 2 μL secAb–biotin, 5 μL standard milk, and 10 μL mixed beads were added into the appropriate wells. Control wells were left free of the mixed working standards of antibiotics.

In sample wells, 5 μL mixed mAbs, 2 μL secAb–biotin, 5 μL spiked milk or sample milk, and 10 μL mixed beads were added. 5 μL spiked milk was used to calculate the recovery and 5 μL sample milk was used for detection.

All wells were complemented with the total volume of 50 μL by reaction buffer (0.01 mol/L phosphate-buffered saline containing 0.1% BSA, 0.05% Tween-20, and 0.02% NaN_3, PBS-TBN). Afterward, the whole plate was vortexed in the dark for 1 h in medium speed at 37 °C for competitive reaction. Then, SA-PE was added and vortexed for 20 min under the same condition. A total of 100 microspheres per region were read out well by well by Bio-Plex™ suspension array to obtain the median fluorescent intensity (MFI). Based on the obtained MFIs for each target, the standard curves could be plotted and the recovery was calculated. The original concentration of antibiotics in the sample was calculated by the detection concentration multiplied by 10 (samples in reaction wells were diluted 10-fold).

2.6. Specificity of the suspension array

To determine the specificity of the suspension array, it was exposed to different kinds of chemicals: florfenicol, thiamphenicol, oxytetracycline hydrochloride, doxycycline hyclate, azithromycin, kanamycin, apramycin sulfate, and neomycin sulfate. Blank control (without competitive substance) was also prepared. Milk spiked with these chemicals (500 ng/ml for all chemicals) was evaluated and the concentrations were calculated by standard curves. The cross-reaction rates were calculated by these values divided by 500 ng/ml.

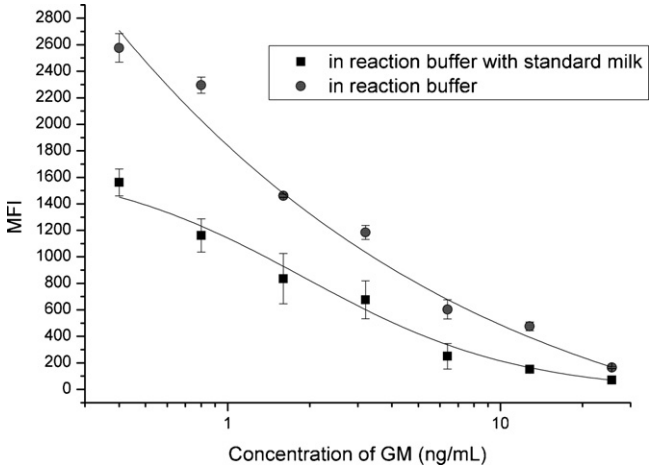

Fig. 1. Standard curves of GM in PBS-TBN and the buffer with standard milk.

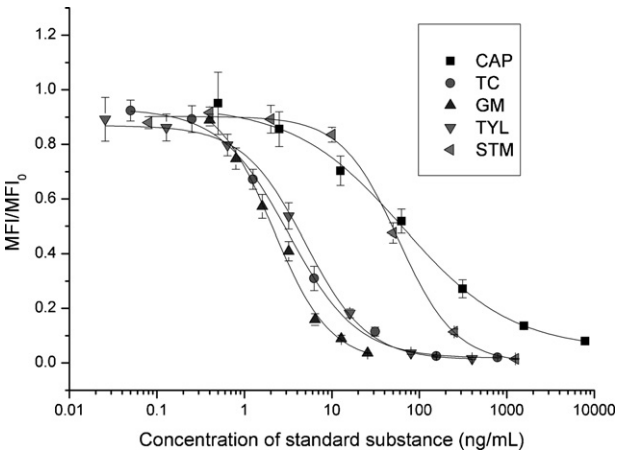

Fig. 2. Standard curves for the five antibiotics in the buffer with standard milk (MFI0: average of MFIs obtained from blank wells).

3. Results and discussion

3.1. Assay development in buffer

For the detection of small molecule by suspension array system, biotinylation for the mAbs were available [25]. Therefore, at the beginning of the present research, mAbs were biotin-labeled and the standard curves were plotted. However, the maximum obtainable MFI was decreased significantly after storage of more than 2 weeks because of the unstability of the biotinylated mAbs.

Taking into account of this, the reaction of secAb–biotin and mAbs was used as an alternative. Compared to the mAb–biotin, secAb–biotin can greatly improve the detection sensitivity and expand the scope which can be distinguished. It also significantly reduced the dose of mAbs due to the amplification system of mAb–secAb. The standard curve obtained by TYL with mAb–biotin shows an inhibitory concentration at 50% binding (IC_{50}) of 52.67 ± 4.76 ng/ml whereas the employment of secAb–biotin significantly reduced the IC_{50} to 4.17 ± 1.32 ng/ml. The maximum obtainable MFI was still stable after 6 months. The present research used secAb–biotin rather than the biotin-labeled one.

PBS-TBN with added BSA, Tween-20, and sodium azide was assessed. Compared with using PBS (Table 2), the reaction stability was enhanced (the MFI obtained from control was higher, the standard deviation of the MFI was smaller) and detection sensitivity was increased to a certain extent. The sodium azide contributed to the storage of this buffer.

3.2. Sample preparation

Initially, 5 μL untreated milk sample was added directly to the wells (the total volume was 50 μL) but the obtainable MFI became unpredictable and the countable beads were much fewer than in the buffer. It indicated that the matrix such as saccharide, fat, and different kinds of proteins in the milk had great influence on the detection results. Pre-treatment of milk sample is therefore necessary.

Different methods to treat milk sample had been described [11,26–31], but all of them was proved to be unsuitable for the detection of suspension array system. de Keizer [23] had detected some sulfonamides in raw milk by Luminex (*x*MAP®) suspension array. Only dilution and filtration of the milk sample took place, which did not made any significant improvement. Compared to different treatments, diethyl ether has efficient effect to denature proteins in milk and consequent filtration is used to remove disturbing fat particles (presented in Section 2.3). Diethyl ether is a volatile organic solvent used to extract free fatty acids from milk [32]. Adding it to milk followed by vortex could also precipitate the protein. The treated samples had no significant effect on the experimental results (Table 3).

The recovery (Table 4) of the different spiked milk samples indicated that the treatment had no significant effect on targets.

Diethyl ether was chosen for the following reasons: (1) it is almost insoluble with water; thus, the treated samples contain almost no organic solvents, so the effect on immunoreactions can be ignored; (2) most antibiotics do not dissolve in diethyl ether so the loss of targets can be avoided; and (3) diethyl ether is highly volatile and lighter than water; therefore, it can float on the samples and removable. The detection results indicated that most interfering components in milk can be removed by this method.

3.3. The optimal concentration of mAb and secAb–biotin in simultaneous detection

Reaction efficiency of antigen with antibody was closely related to their concentration [33]. The macromolecules immune complexes are unable to form if the antigen or antibody is excessive. There has an equivalence zone [34] that the dose of antigen and antibody are in optimum proportions and the huge grid-like aggregate formation of immune complexes can be formed. The chessboard titration was employed to optimize the concentration of mAb and secAb–biotin. Under the same concentration of secAb–biotin, the MFI of different targets presented different trends with the change of mAb concentration (Table 5).

When the dose of secAb–biotin was 80 and 120 ng/well, the obtainable MFI of TYL and GM were reduced under the same dose of mAb. The maximum MFI of each target obtained under 40 ng/well of secAb–biotin was more than 1700, which met the needs of detection. The 40 ng/well was chosen as the optimal dose of secAb–biotin. Based on this result, the optimal dose of different mAbs was selected based on the highest obtainable MFI.

3.4. Standard curves plotting and detection for the five antibiotics in milk

Standard curves of GM in PBS-TBN and the buffer with standard milk are compared and shown in Fig. 1. Although the maximum response (MFI_0, without competition substance in reaction) in the buffer with standard milk was lower than in PBS-TBN (caused by the milk components which were not removed by treatment), it had no significant effect on the sensitivity of detection. The calibration graphs of GM in PBS-TBN and the buffer with standard

Table 2
Effect of different buffers ($\bar{x} \pm s$, $n = 3$).

Buffers	MFI_0		IC_{50} (μg/L)	
	GM	TYL	GM	TYL
ddH_2O	476.33 ± 67.87	1162.33 ± 141.24	13.17 ± 1.94	34.33 ± 3.53
PBS (0.01 mol/L)	971.67 ± 108.12	3445.5 ± 283.75	5.67 ± 1.02	19.67 ± 1.83
PBS-TBN[a] (0.01 mol/L)	1691.5 ± 74.64	4182.5 ± 152.71	3.5 ± 0.84	14.83 ± 1.52

MFI_0: MFI obtained from control.
[a] Compared with PBS, $P < 0.05$ (t-test).

Table 3
The effect of treated sample on reaction results ($\bar{x} \pm s$, $n = 20$).

Targets	MFI_0[a]	IC_{50}[a] (ng/ml)	MFI_0[b]	IC_{50}[b] (ng/ml)
TYL	2968.83 ± 89.71	3.47 ± 0.47	2711.67 ± 94.09	3.92 ± 0.73
TC	2606.83 ± 53.42	2.18 ± 0.38	2447.83 ± 62.43	2.79 ± 0.67
GM	2704.58 ± 53.18	2.25 ± 0.27	1812.67 ± 82.10	2.45 ± 0.54
STM	2552.67 ± 69.25	36.42 ± 2.41	2235.53 ± 113.22	40.28 ± 3.76
CAP	3534.65 ± 47.34	53.87 ± 4.92	3171.34 ± 79.64	57.34 ± 5.33

MFI_0[a]: maximum MFI obtained in reaction buffer; IC_{50}[a]: IC_{50} calculated by standard curve, which is obtained from the reaction buffer.
MFI_0[b]: maximum MFI obtained in reaction buffer with standard milk; IC_{50}[b]: IC_{50} calculated by standard curve, which is obtained from the reaction buffer with standard milk, and compared with IC_{50}[a], $P > 0.05$ (t-test).

Table 4
Recovery in spiked milk ($\bar{x} \pm s$, $n = 20$).

Level spiked (ng/mL)	Recovery (%)				
	TYL	TC	GM	STM	CAP
50	90.45 ± 9.68	89.38 ± 6.26	108.36 ± 10.38	112.38 ± 6.58	105.83 ± 12.78
100	96.03 ± 8.23	96.18 ± 7.82	106.71 ± 12.84	109.94 ± 8.45	113.73 ± 16.62
200	97.34 ± 7.41	92.35 ± 5.51	94.57 ± 14.17	105.27 ± 11.52	108.84 ± 15.82

Table 5
MFI obtained by chessboard titration for targets ($\bar{x} \pm s$, $n = 3$).

Targets	Dose of mAb (ng/well)	MFI			
		Dose of secAb–biotin (ng/well)	40	80	120
TYL	0.05		1278.83 ± 94.08	774.67 ± 125.04	568.5 ± 78.1
	0.1		1945.5 ± 89.93	1367.33 ± 34.01	965 ± 82.13
	0.15		2620.33 ± 107.70	1894.67 ± 71.61	1436.83 ± 67.32
TC	2		2350.5 ± 53.42	3024.67 ± 74.89	3031.33 ± 48.08
	4		1832.33 ± 95.27	2626.83 ± 18.04	3444.67 ± 55.22
	6		1273 ± 73.89	2245.33 ± 20.29	2912.5 ± 76.21
GM	1		907.5 ± 35.36	701 ± 23.76	544.83 ± 16.87
	2		1164.67 ± 42.98	1070.67 ± 66.12	793.5 ± 32.46
	3		1783.5 ± 53.18	1623.67 ± 85.91	1241 ± 36.77
STM	2		1037 ± 85.62	1270 ± 44.37	1252.83 ± 63.4
	4		1945.67 ± 75.69	1732.5 ± 23.87	1955.67 ± 50.73
	6		2300.33 ± 113.22	2580.83 ± 65.28	2940.83 ± 47.26
CAP	2		1832.5 ± 44.57	2565.5 ± 66.53	2557.67 ± 47.83
	4		2420.33 ± 87.15	3525.83 ± 15.01	4590.33 ± 52.85
	6		2720.67 ± 79.85	4227.5.83 ± 30.46	5905.83 ± 102.16

Table 6
The results of detection of antibiotics in sample milk ($\bar{x} \pm s$, $n = 3$, ng/ml).

Number of sample milk	The concentration of targets in sample milk				
	TYL	TC	GM	STM	CAP
1	–	–	58.47 ± 2.89	–	–
2	20.11 ± 3.57	–	87.65 ± 3.56	–	–
3	19.82 ± 6.86	–	111.6 ± 9.32	–	–
4	12.56 ± 2.77	–	29.76 ± 8.23	–	–
5	–	–	66.84 ± 5.28	–	–

"–" Refers to the undetectable concentrations.
The concentration in sample milk was calculated by the detection concentration multiplied by 10 (the samples in reaction wells were diluted 10-fold).

Table 7
The cross-reaction rates of chemicals with mAbs ($\bar{x} \pm s$, $n = 3$).

Chemicals	The cross-reaction rates (%)				
	TYL	TC	GM	STM	CAP
Florfenicol	<1	<1	<1	<1	<1
Thiamphenicol	<1	<1	<1	<1	<1
Oxytetracycline hydrochloride	<1	576.34 ± 14.38	<1	<1	<1
Doxycycline hyclate	<1	60.77 ± 9.76	<1	<1	<1
Azithromycin	<1	<1	<1	<1	<1
Kanamycin	<1	<1	<1	<1	<1
Apramycin sulfate	<1	<1	<1	<1	<1
Neomycin sulfate	<1	<1	<1	<1	<1

milk show the mean IC_{50} at 2.25 and 2.45 ng/ml, respectively. There were no significant differences between them ($P > 0.05$). The standard deviation (SD) of the reaction in the buffer with standard milk was higher than that in PBS-TBN, which could be explained by the matrix interference in milk. All the other four antibiotics showed the same regularity (Table 3). Standard curves for the detection of the five antibiotics simultaneously in the buffer with standard milk were plotted together as shown in Fig. 2.

Five different sample milks were also detected and the concentration of antibiotic was calculated. Twenty blank milk ($MFI_0{}^b$) samples were analyzed and the average MFIs for TYL, TC, GM, STM, and CAP were 2711.67 ± 94.09, 2447.83 ± 62.43, 1812.67 ± 82.10, 2235.53 ± 113.22 and 3171.34 ± 79.64, respectively. The obtained MFIs of sample milks were compared with standard curves and the calculated results were shown in Table 6. GM was found in all the samples. It indicates that maybe the GM residue in milk was a major problem in China.

There are negative logistical correlations between MFI and the concentrations of the antibiotics. All the determination coefficients-R^2 were greater than 0.991, implying good correlation. Owing to the dilution, the minimum detectable concentrations (Min DC) in samples were 10 times the Min DC obtained by detection, at 0.3, 1.5, 25, 20, and 4 ng/ml for TYL, TC, CAP, STM, and GM, respectively. The working ranges of samples were 6–400, 7–300, 70–8000, 90–3000, and 8–200 ng/ml for TYL, TC, CAP, STM, and GM, respectively. The sensitivity of detection for these targets by suspension array system can meet the needs of daily detection of antibiotics residue in milk. The total detection times, including the preparation of samples which took approximately 2 h, also met the needs of rapid detection.

3.5. Specificity of the suspension array detection

The chemicals chosen to estimate the specificity of the suspension array detection include amphenicols (florfenicol and thiamphenicol), tetracyclines (oxytetracycline hydrochloride and doxycycline hyclate), macrolide (azithromycin), and aminoglycoside (kanamycin, apramycin sulfate, and neomycin sulfate). These chemicals have similar structures to the five targets, respectively. Table 7 shows the cross-reaction rates of these chemicals with the five mAbs.

All the cross-reaction rates of the analogues were less than 1% and had no cross-interactions between the different mAbs and beads, except for that of two tetracyclines with the mAb of TC, implying that the recognition site of this mAb was on the common structure of tetracyclines. If the target chemical would be strictly distinguished from the analogues, the mAb of which the recognition site was on the special structure should be selected.

4. Conclusion

A new suspension array technology is proposed for the simultaneous quantitative determination of five antibiotics in milk. The use of secAb–biotin was an improvement compared with the biotinylation for mAbs. The higher signals were obtained and the sensitivity of detection was improved.

For the multi-antibiotics suspension array in milk, pretreatment was necessary to remove the disturbing components in milk. The treatment by diethyl ether and ultrafiltration membrane was proved efficiently and it greatly reduced the interference of the disturbing components in the sample on the reaction results without having significant effect on detection sensitivity.

The suspension array was optimized for the detection of the most applied antibiotics in China's dairy products. The Min DCs in samples were 0.3, 1.5, 25, 20, and 4 ng/ml for TYL, TC, CAP, STM, and GM, respectively. All of them, except CAP, comply with EU MRLs. Compared with chromatography or mass spectrometry [5–11,35], suspension array has certain advantages, including increased sensitivity, broader detection range, and simpler operation. Thus, suspension assay can be suitable for rapid quantitative analysis of the presence of antibiotic residues in milk. Further work should focus on the possible application of such method to other contaminants in milk or food, thus allowing for the development of a suspension array able to meet the needs of food safety.

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