A simple and rapid immunochromatography test based on readily available filter paper modified with chitosan to screen for 13 sulfonamides in milk

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ABSTRACT

In this study, we developed a novel, simple, rapid, and low-cost colloidal gold-based immunochromatography method, with filter paper replacing nitrocellulose membrane as the substrate. To obtain adequately immobilized protein, chitosan was used to functionalize the filter paper. After conditions and parameters were optimized, the novel immunochromatography method was applied for detection of sulfonamide residues in milk. Quantitative detection was accomplished using a smartphone and Photoshop software (Adobe Inc., San Jose, CA), allowing us to screen 13 sulfonamides with a limit of detection ranging from 0.42 to 8.64 µg/L and recovery ranging from 88.2 to 116.9% in milk. The proposed novel method performed similarly to the conventional method that uses a nitrocellulose membrane as the transport medium, and it had lower cost and better usability because of the inexpensive and easily available filter paper.

Key words: filter paper, chitosan, lateral flow device, sulfonamides

INTRODUCTION

Antibiotics are widely used in dairy farming and milk production to inhibit growth of bacteria and promote the growth of dairy cows (Li et al., 2019; Rana et al., 2019). Sulfonamides (SA), derivatives of *p*-aminobenzenesulfonamide, are one of the most widely used types of antimicrobial drugs because of their high efficiency and cost effectiveness (Baran et al., 2011). They have

broad-spectrum antibacterial properties. They can affect bacterial reproduction by inhibiting bacterial folate metabolism and interfering with synthesis of bacterial nucleic acids and proteins (Dmitrienko et al., 2014). However, their ubiquitous use in veterinary practice leads to residues in foodstuffs and the environment, including milk, meat, and rivers. Furthermore, the human intake of SA residues through foods over time leads to adverse effects, allergies, poisoning, and even cancer (Wang et al., 2017). According to the European Commission, the Food and Drug Administration, and the Chinese Ministry of Agriculture, the maximum residue level of SA is 100 µg/kg in animal foods (European Commission, 1990; Chinese Ministry of Agriculture, 2002; US Food and Drug Administration, 2003). Therefore, the detection of SA residues in milk consumed by the human has become a function for public health agencies.

Colloidal gold immunochromatography (ICA) is an effective and powerful tool for point-of-care food safety testing of because it is rapid, simple, sensitive, and portable. Colloidal gold ICA has been widely applied in the screening of antibiotic residues in dairy products (Chen et al., 2017). Nitrocellulose membranes (NCM) are important elements of and are generally used in ICA devices. Nitrocellulose membranes provide the substrate for the immune response and color development of ICA devices, due to their uniform pore size, smooth surface, and high protein immobilization capability (Apilux et al., 2013). However, their relatively high cost and complex manufacturing processes have led to their manufacture being monopolized by a few companies, which limits usage of NCM in developing and remote areas. Therefore, other affordable and available easily materials, such as paper and cotton thread, have been proposed as alternatives for ICA devices (Teerinen et al., 2014; Jia et al., 2017).

Filter paper (FP) consists of a cellulose fiber web and has high mechanical stability and tensile strength;

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it is a common material used in filtration and separation applications in scientific research. It has received considerable attention in microfluidic platforms because of its abundance, low cost, hydrophilic properties, biocompatibility, and ease of modification (He et al., 2015; Ilacas et al., 2019). Paper-based ELISA is the classic paper analytical device: it retains the sensitivity and specificity of traditional ELISA and has the advantages of rapid processing, low solution use, and cost effectiveness of FP (Wang et al., 2012; Busin et al., 2018; Yang et al., 2018). In addition, FP shows excellent capillary action for transporting liquids without any external equipment and it facilitates mass manufacturing. Based on the above characteristics, we considered using FP as a substitute for NCM in ICA devices. However, FP without modification has poor capacity to adsorb protein, which affects the analytical performance of ICA strips. Hence, it is essential to modify filter paper to improve the adsorption capacity for protein.

Chitosan (**CS**), a high-molecular-weight linear biopolymer, is obtained from deacetylation of chitin, which is widely abundant on earth. Chitosan is considered a functional material because of its biocompatibility, antibacterial activity, and adsorption properties (Ravi Kumar, 2000). It is a structural polysaccharide with a similar structure to cellulose, although it has better mechanical properties and binding affinities with proteins than cellulose. Hence, it is often used to modify FP or cotton thread (both consisting of cellulose) to enhance the wet strength of substrate, the stability of the hydrophilicity, and the ability to immobilize proteins (Li et al., 2018; Ma et al., 2018). In this work, we used CS to modify the test and control lines on FP so that the protein could be firmly bonded to the cellulose surface by electrostatic force, hydrogen bonds, and hydrophobic interactions (Li and Peng, 2015; Lim et al., 2015).

In this study, we established a filter paper-based immunochromatographic assay (**FP-ICA**) to detect SA residues in milk as model analytes. The FP modified with CS showed excellent ability to bind protein and has additional advantages of cost effectiveness and ease of fabrication compared with NCM. As shown in Figure 1, we used the typical gold nanoparticles (**GNP**) as the label-conjugated antibodies in FP-ICA to permit visual analysis. In addition, we performed a quantitative analysis by calculating and analyzing the colorimetric ratio of the test and control zones using a smartphone and Photoshop software (Adobe Inc., San Jose, CA). Eventually, to validate the developed FP-ICA, we established a traditional ICA based on nitrocellulose membranes (**NCM-ICA**) and compared the two devices.

MATERIALS AND METHODS

Chemicals and Instrumentation

Sulfonamide antigen (3.914 mg/mL) and anti-SA antibody (2.33 mg/mL) were prepared in our laboratory. Goat anti-mouse IgG (17 mg/mL) was purchased from Arista Biologicals (Allentown, PA). Chitosan (95% deacetylation) and BSA were purchased from Sigma-Aldrich (St. Louis, MO). Thirteen sulfonamides, sulfadimethoxine (**SDM**), sulfachloropyridazine (**SCP**), sulfamethoxydiazine (**SMD**), sulfapyridine (**SPY**), sulfamonomethoxine (**SMM**), sulfaquinoxaline (**SQX**), sulfadiazine (**SDZ**), sulfaquinoxaline (**SIM**), sulfaethoxypyridazine (**SEP**), sulfamethazine (**SM2**), sulfamethoxypyridazine (**SMP**), sulfamerazine (**SMR**), and sulfanitran (**SAN**), and other standards were provided by the National Institution for Food and Drug (Beijing, China). All solvents and other chemicals were of analytical reagent grade.

Whatman chromatography paper No. 3MM Chr (pure cellulose paper, **WC 3MM**) and Whatman qualitative paper No. 1 (**WQ 1**), No. 2 (**WQ 2**) and No. 4 (**WQ 4**) were purchased from GE Healthcare (Little Chalfont, UK). Xinxing quantitative paper (**XQ**) was obtained from Hangzhou Special Paper Co. Ltd. (Hangzhou, China). Scotch tape was purchased from local stationery shop in Beijing, China. The absorbent pad, sample pad, and polyvinyl chloride (PVC) cards were obtained from Kinbio Tech Co. Ltd. (Shanghai, China). Ultrapure water was purified using a Milli-Q system from Millipore Corp. (Bedford, MA). Ultrasonic equipment was supplied by Zhixin Instrument Co. Ltd. (Shanghai, China). An XYZ dispensing platform (HM3035) and programmable strip cutter ZQ4000 were obtained from Kinbio Tech Co. Ltd. (Shanghai, China). The shooting box was acquired from our laboratory.

Synthesis of GNP and GNP-Antibody Conjugates

Gold nanoparticles with an average diameter of 40 nm were prepared according to a published method (Hermanson, 2013). Briefly, 1 mL of 1% trisodium citrate solution was added rapidly to 50 mL of boiling 0.02% HAuCl₄ solution with stirring. After the color stabilized, the GNP solution was cooled and stored at room temperature for future immunoassay testing.

The GNP-antibody conjugates were prepared according to a published protocol (Peng et al., 2014). Initially, diluted antibody (containing 0.5% BSA) was added to 1 mL of GNP solution (pH 8.0). After 10 min of incubation, 100 μ L of 10% BSA (wt/vol) was added to the

mixture for 10 min and the mixture was then centrifuged at $8,600 \times g$ for 10 min at 4°C . Subsequently, the complex was resuspended in $200 \mu\text{L}$ of PBS (0.01 M , pH 7.4) containing Tween-20, polyethylene glycol (PEG) 20000, and sucrose. After 3 min of sonication, the conjugates were stored at 4°C until use.

Assembling of Strips

The filter paper was initially coated with 0.5 mg/mL CS (pH 6.5) and dried at room temperature for 15 min. The amino group in CS has a positive charge and binds to proteins with a negative charge under weakly acidic conditions. Then, the antigen and goat anti-mouse IgG (2.2 mg/mL), respectively, were spotted on the test (T) and control (C) lines to combine CS on the filter paper, with a dispensed volume of $0.5 \mu\text{L/cm}$. The prepared filter paper was dried at 45°C for 2 h. The sample pad was treated with phosphate buffer (0.02 M , pH 7.4) containing 1% BSA (wt/vol) and 0.5% polyvinylpyr-

rolidone (wt/vol) and then dried at 60°C for 2 h. Then, the sample pad, filter paper, absorbent pad, and PVC card with a layer of scotch tape were assembled as an FP-ICA strip. In order to simulate the smooth side of commercial NC membrane, it was necessary to paste a layer of scotch tape on the PVC card of the FP-ICA strip. The assembly of the NCM-ICA strip was the same as the process for the FP-ICA strip, except that the PVC card did not include a layer of scotch tape because the commercial NC membrane was already smooth. Finally, the assembled strips were cut to a width of 3.5 mm and stored dry at room temperature.

FP-ICA Testing and Comparison with Traditional NCM-ICA

All milk samples were obtained from local cattle farms. Initially, milk samples with different concentrations of SDM were diluted 6 times in 0.01 M PBS (pH 7.4) and mixed with GNP-antibody conjugates for 3

Figure 1. Preparation of colloidal gold test strips with chitosan-functionalized filter paper as substrate used to detect sulfonamides in milk.

Figure 2. Optimized conditions of filter paper immunochromatography assay (FP-ICA), showing effect of (A) type of filter paper, (B) content of chitosan (CS), (C) concentration of anti-sulfonamide antibody (Ab), and (D) immobilizing concentration of coating antigen (Ag) sulfadimethoxine. Filter papers: WQ No. 1, No. 2, No. 3, No. 4, and WC No. 3MM (pure cellulose paper) were from Whatman, GE Healthcare, Little Chalfont, UK; XQ (Xinxing quantitative paper) was from Hangzhou Special Paper Co. Ltd. (Hangzhou, China). Error bars represent SD.

min. Then, the mixed solution was transmitted to the test strip. After 10 min, the resulting images were captured by smartphone. Finally, the colorimetric results were analyzed using Photoshop software to obtain the green inverted values ratio of the T and C lines. All experiments were performed in triplicate. Under the same conditions, traditional NCM-ICA was compared with FP-ICA to demonstrate the feasibility of FP-ICA. The remaining 12 sulfonamides were detected using the same procedure.

Statistical Analysis

Using OriginPro 8.5 software (<https://www.originlab.com>), the calibration curve was established by the operation of sigmoidal fit, and the 4-parameter equation was obtained. Then, the analytical performance of sensitivity, specificity, and accuracy for FP-ICA was obtained by the equation. One-way ANOVA method in IBM SPSS Statistics 19 (IBM Corp., Armonk, NY) was used for the stability test of FP-ICA. The stability of the strip was estimated by the *P*-value.

RESULTS AND DISCUSSION

Optimization of FP-ICA

Optimization of FP. To demonstrate the effect of FP on color intensity, several commonly used filter papers, including WC 3MM, WQ 1, WQ 2, WQ 4, and XQ, were considered in this study. As shown in Figure 2a, FP differing in purity of cellulose and pore size exhibited different color intensities. The WQ 4 with a pore size of 20 μm resulted in higher intensity of color compared with other FP with smaller pore size. The WC 3MM FP, which is made of pure cellulose, exhibited the highest color intensity. Therefore, WC 3MM was chosen for further experiments.

Optimization of Chitosan Content. As shown by infrared spectroscopy (Supplemental Figure S1; <https://doi.org/10.3168/jds.2020-18987>), chitosan can cross-link with FP. Furthermore, CS has high adsorption to proteins through hydrophobic and electrostatic contacts and slightly inhibits surface hydrophilicity of FP (Bordenave et al., 2007; Orelma et al., 2011; Bekale et al., 2015). Hence, the color intensity band on the T and

C zones is closely related to the CS content. We studied the effect of CS using contents of 0, 0.25, 0.5, 0.75, and 1 $\mu\text{g}/\text{cm}$. As shown in Figure 2b, color intensity increased with increasing CS content up to 0.75 $\mu\text{g}/\text{cm}$. However, intensity decreased at CS content $>0.75 \mu\text{g}/\text{cm}$, likely because the greater content of CS resulted in more nonspecific adsorption and increased background. Therefore, we chose a CS content of 0.75 $\mu\text{g}/\text{cm}$ for further experiments.

Optimization of Content of Anti-SA Antibody.

The use of a suitable concentration of the anti-SA antibody would enhance sensitivity and reduce the cost of FP-ICA. Antibody contents of 1.75, 3.5, 5.25, and 7 μg were used in the study. As shown in Figure 2c, the results indicated that intensity increased with increasing concentration of anti-SA antibody. However, intensity was weak at an antibody concentration greater than 5.25 μg because the background value was enhanced by the increased antibody content. Therefore, an anti-SA antibody content of 5.25 μg was chosen for further experiments.

Optimization of Concentration of Coating Antigen. After obtaining the optimal concentration of the anti-SA Ab, we needed to establish an appropriate concentration of the coating antigen in the T zone to improve the color intensity. Antigen concentrations of 0.4, 0.8, 1.2, and 1.6 $\mu\text{g}/\mu\text{L}$ were used in the study. As shown in Figure 2d, intensity increased with increasing concentration of antigen in general. However, immobilizing antigen at 0.8 $\mu\text{g}/\mu\text{L}$ resulted in higher intensity than antigen at 1.2 $\mu\text{g}/\mu\text{L}$. Hence, considering cost and color intensity, an antigen concentration of 0.8 $\mu\text{g}/\mu\text{L}$ was chosen.

Analytical Performance

Sensitivity of FP-ICA and NCM-ICA. After defining the optimal conditions, milk samples containing different concentrations of SDM in range of 0 to 24 $\mu\text{g}/\text{L}$ were diluted 6 times using 0.01 M PBS (pH 7.4) and detected by FP-ICA and NCM-ICA. Results are shown in Figure 3a and b; cut-off values for FP-ICA and NCM-ICA were 12 and 6 $\mu\text{g}/\text{L}$, respectively, for detection by the naked eye. The quantitative analysis using Photoshop is shown in Figure 3c and d. The resulting calibration curves of FP-ICA and NCM-ICA were $Y = 0.916 + 0.487/[1 + (X/0.745)^{2.504}]$ and $Y = 0.442 + 0.049/[1 + (X/0.604)^{1.968}]$, where Y is the green inverted value ratio of the T line and C line, and X is the concentration of SDM; the coefficients of determination (R^2) were 0.994 and 0.989, respectively. The linear ranges of FP-ICA and NCM-ICA were from 2.42 to 7.74 $\mu\text{g}/\text{L}$ and from 1.80 to 7.32 $\mu\text{g}/\text{L}$, respectively.

The limit of detection (LOD) was estimated as 10% inhibition (IC_{10}) of the standard curve (Sun et al., 2017). Hence, the LOD for FP-ICA and NCM-ICA were 1.86 and 1.20 $\mu\text{g}/\text{L}$ in milk, respectively.

Specificity of FP-ICA. The specificity of FP-ICA was estimated in a cross-reactivity test with 30 other antibacterial agents. The results are shown in Supplemental Table S1 and Supplemental Figure S2 (<https://doi.org/10.3168/jds.2020-18987>). The anti-SA antibody reacted negligibly with drugs other than the 12 sulfonamides (SCP, SMD, SPY, SMM, SQX, SDZ, SIM, SEP, SM_2 , SMP, SMR, and SAN). Therefore, we can consider this antibody to have broad-spectrum activity against sulfonamides. The FP-ICA established in this study could simultaneously detect 13 SA in milk samples (Table 1), with LOD ranging from 0.42 to 8.64 $\mu\text{g}/\text{L}$.

Accuracy and Precision of FP-ICA. To confirm the practical applicability of the FP-ICA, recovery was tested in milk samples spiked with different concentration of sulfonamides. Five of the 13 SA (SMD, SAN, SDZ, SMM, and SM_2) were tested and results are shown in Table 2. The average recoveries for SA in milk ranged from 88.2 to 116.9%, with a relative standard deviation (RSD) $<18\%$.

Stability of FP-ICA. We tested the stability of the FP strip using the Arrhenius equation $\left(k = AT^m e^{-\frac{E_0}{RT}}\right)$,

where k = specific reaction rate; AT^m = preexponential factor, E_0 = apparent activation energy; R = gas constant; and T = thermodynamic temperature, by placing the strips at 55°C for 2 consecutive weeks (Wang et al., 2013). The intensity values were almost constant, as shown in Supplemental Figure S3 (<https://doi.org/10.3168/jds.2020-18987>). According to one-way ANOVA, the homogeneity of variance was $P = 0.659$, and the single factor analysis resulted in $P = 0.409$, indicating no decline in intensity values from 0 to 14 d. This means the strip could be stored stably for 1 yr at room temperature.

Method Validation. To validate the developed FP-ICA, we tested 10 milk samples that were negative for sulfonamides, 3 of which were randomly spiked with a certain concentration of SDM, confirmed by instrument, and detected using the developed FP-ICA and NCM-ICA. The results showed that both methods had good consistency (Table 3). The performance of the developed FP-ICA was similar to that of the conventional NCM-ICA, but the cost was lower and the preparation process was simpler for FP than NCM. The cost of FP is about 10% of the cost of NCM for similar lengths, and it is easier to obtain and use than NCM.

Figure 3. Appearance of test strips and calibration curves of filter paper immunochromatography assay (FP-ICA) and nitrocellulose membrane immunochromatography assay (NCM-ICA) for detection of sulfadimethoxine (SDM) in milk. (A) FP-ICA and (B) NCM-ICA at different concentrations of SDM; calibration curve of (C) FP-ICA and (D) NCM-ICA. Error bars represent SD.

Therefore, the developed FP-ICA uses readily available components and is practical. The method is based on a broad-spectrum antibody, and its purpose is to prevent missed detection of sulfonamides to the extent possible. After samples positive for sulfonamides are detected, the type and residual amount of the sulfonamide need to be verified using an instrument method.

CONCLUSIONS

The proposed FP-ICA using filter paper functionalized with chitosan as an analytical membrane exhibited good performance for detecting SA residues in milk, sufficient to meet testing standards of legal limits. The chitosan-modified filter paper greatly enhanced the

Table 1. Cross-reactivity of sulfonamide mAb with different sulfonamides

Sulfonamide	Adjusted R ²	LOD ¹ (µg/L)	Linear range (µg/L)	Cross-reactivity (%)
Sulfadimethoxine	0.994	1.86	2.42–7.74	100
Sulfachloropyridazine	0.979	2.94	3.72–12.30	65
Sulfamethoxydiazine	0.999	2.88	3.42–10.80	73
Sulfapyridine	0.992	3.06	4.02–9.72	71
Sulfamonomethoxine	0.999	2.40	3.96–22.20	47
Sulfaquinoxaline	0.996	3.00	6.18–73.80	21
Sulfadiazine	0.953	8.64	14.94–97.10	12
Sulfaquinoxaline	0.988	1.11	2.22–24.42	60
Sulfaethoxypyridazine	0.991	0.42	1.02–20.40	97
Sulfamethazine	0.997	1.26	2.28–15.60	75
Sulfamethoxypyridazine	0.987	1.74	2.82–15.06	68
Sulfamerazine	0.960	1.38	2.52–18.84	65
Sulfanitran	0.995	1.44	2.16–8.88	101

¹Limit of detection.

Table 2. Recovery of sulfonamides in spiked milk samples by filter paper immunochromatography assay (FP-ICA)

Sulfonamide	Spiked (µg/L)	Measured (µg/L)	Recovery (%)	RSD ¹ (%)
Sulfamethoxydiazine	2.4	2.49 ± 0.41	103.9	16.4
	4.8	4.41 ± 0.40	91.8	8.9
	7.2	6.50 ± 1.14	90.3	17.6
Sulfanitran	2.4	2.58 ± 0.22	107.8	6.8
	4.8	4.95 ± 0.54	103.2	10.7
	8.4	7.79 ± 1.26	92.7	16.6
Sulfadiazine	21	19.70 ± 1.44	109.5	7.4
	54	54.12 ± 7.38	100.2	13.6
	80	88.92 ± 12.36	98.8	13.9
Sulfamonomethoxine	4.8	4.50 ± 0.37	93.5	8.2
	8	9.12 ± 0.48	101.1	5.6
	18	20.58 ± 3.18	114.2	15.6
Sulfamethazine	3	3.48 ± 0.53	116.9	15.1
	8	7.93 ± 0.43	88.2	5.4
	15	15.90 ± 2.29	106	14.4

¹Relative standard deviation.

ability to immobilize proteins by electrostatic force, hydrogen bonds, and hydrophobic interactions. Thus, FP can be used as a substitute for conventional NCM in ICA. Compared with NCM-ICA, the FP-ICA strip is cheaper and more readily available. Hence, this cost-effective, simple, and rapid paper-based analytical device has potential for use in residue determination, especially in remote areas and low-resource regions. Further studies will focus on detection of other analytes in different samples and using different materials to functionalize the filter paper.

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Table 3. Validation assessment (10 milk samples with negative sulfonamides, 3 of which were randomly spiked with a certain concentration of sulfadimethoxine) of filter paper immunochromatography assay (FP-ICA)

Sample	Spiked (µg/L)	Detected (µg/L ± SD)	
		FP-ICA strip	NCM-ICA ¹ strip
1	—	—	—
2	—	—	—
3	—	—	—
4	3.0	3.21 ± 0.21	2.73 ± 0.38
5	5.0	4.76 ± 0.32	4.20 ± 0.26
6	—	—	—
7	—	—	—
8	7.0	7.13 ± 1.02	6.64 ± 1.16
9	—	—	—
10	—	—	—

¹Nitrocellulose membrane immunochromatography.

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