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Rapid and Sensitive Determination of Sulfonamide Residues in Milk and Chicken Muscle by Microfluidic Chip Electrophoresis

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ABSTRACT: A new, rapid, and sensitive method was proposed for the determination of sulfonamide residues in milk and chicken muscle samples by microchip electrophoresis with laser-induced fluorescence detection. Separation of fluorescamine-labeled sulfonamides was accomplished by using a buffer containing 5 mmol/L boric acid and 1% (w/v) polyvinyl alcohol (PVA). The pH, amount of PVA, and concentration of boric acid in the running buffer were found to have great influence on the separation. By optimizing these conditions, the separation of four sulfonamides, sulfamethazine, sulfamethoxazole, sulfaquinoxaline, and sulfanilamide, was achieved within 1 min with limits of detection (S/N = 3) of 0.2−2.3 μg/L, which are well below the maximum residue limit. The proposed method also exhibited very good repeatability; the relative standard deviations for both within-day and between-day measurements were ≤3.0%. With a simplified sample pretreatment protocol, fast determination of sulfonamides in real samples was successfully performed with standard addition recoveries of 93.3−100.8 and 82.9−92.3%, respectively.

KEYWORDS: *microfluidic chip electrophoresis, fluorescence detection, sulfonamides, fluorescamine, polyvinyl alcohol*

INTRODUCTION

Sulfonamides usually refer to a group of antibacterial drugs that possess a *p*-aminobenzensulfonamide framework in their chemical structures. They have been widely used for clinical and veterinary purposes for decades due to the advantages of broad antibacterial spectrum, high efficacy, and low prices. In animal husbandry, sulfonamides are often added to the feed of poultry, pigs, and cattle for the prevention and treatment of gastrointestinal and respiratory diseases[1] or for growth promotion.[2,3] Abuse of sulfonamides or insufficient withdrawal time can lead to accumulation of these drugs in animal tissues. This problem has received increasing public attention because sulfonamides at high levels in foods of animal origin may cause allergy, carcinogenesis, and formation of resistant bacteria in the human body.[4,5] To ensure food safety, many authorities around the world have proposed maximum residue limits (MRL) of sulfonamides that can be allowed in foods. The total sulfonamides in foods of animal origin (including milk) should not exceed 100 μg/kg in the European Union (EU) and China.[5,6] Therefore, the development of rapid, simple, and sensitive methods for the fast screening of sulfonamide residues in foods has practical significance.

The methods that have been reported for the determination of sulfonamides include liquid chromatography (LC),[1,7] high-performance liquid chromatography (HPLC),[8,9] liquid chromatography−mass spectrometry (LC-MS),[10−12] immunoassay,[13,14] and electrochemistry.[5,15] Among them, electrochemical detection cannot provide enough sensitivity for sulfonamide residues analysis. LC-MS has enough sensitivity and is a promising technique for trace analysis, but it is not suitable for on-site fast screening because of the complexity of the equipment. Immunoassay methods, that is, ELISA, are currently widely used, but their analysis time is relatively long. Capillary electrophoresis (CE) has been proved to be a powerful separation technique for sulfonamides in various samples due to the advantages of excellent efficiency and low consumption of samples/solvents. Several separation modes of CE such as CZE,[16−18] MEKC,[19,20] CEC,[21] and CE-MS[22,23] have been successfully adopted in sulfonamide analysis. For example, capillary−UV detection has been used for the separation of 16 sulfonamides in citrate buffer at low pH[16], and pK_a values of various sulfonamides have been determined.[17] Unfortunately, the detection sensitivity was low in these works because of the lower absorption of the sulfonamides. To solve the problem, Lamba et al.[19] combined MEKC with fluorescence detection to separate sulfonamides using phosphate buffer containing sodium dodecyl sulfate. As a result, five sulfonamides were successfully separated, and limits of detection (LODs) in the range of 1.59−7.68 nmol/L were achieved in about 7 min.

Microfluidic analysis, or micro total analysis system, has obtained widespread attention because of the merits of small size, low sample/reagent consumption, rapid analysis speed, high integration, and great potential for portable devices.[24−26] Microfluidic chip electrophoresis is an alternative for conventional capillary electrophoresis with usually better performance. Fan et al.[27] has performed the separation and detection of trimethoprim, sulfadiazine, and sulfamethoxazole in commercial pharmaceutical preparations using a simplified microchip coupled with flow injection. In their work, the simplified microchip was assembled through fused silica capillary and tygon tunings, and the separation was fulfilled within 2.5 min

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with LODs of 0.17, 1.05, and 1.28 μg/mL for trimethoprim, sulfadiazine, and sulfamethoxazole, respectively. Its sensitivity is not adequate for sulfonamide residue analysis in real food samples. Although extensive research has been reported on microchip electrophoretic separation of various samples such as proteins and DNAs, there are few studies related to the detection of sulfonamide residues in foods through microfluidic chip electrophoresis.

In this paper, a simple, sensitive, and reliable microchip electrophoresis method is proposed for the efficient separation and detection of four sulfonamides (Figure 1), sulfamethazine

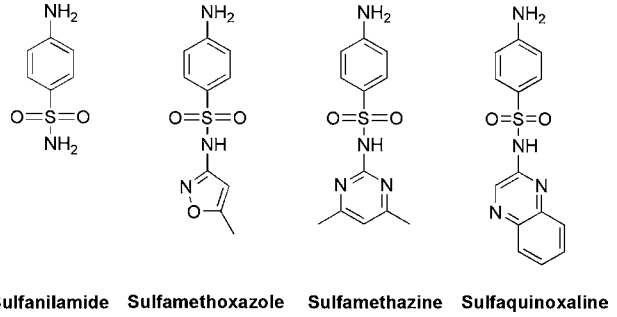

Figure 1. Molecular structures of SAM, SMX, SMZ, and SQX.

(SMZ), sulfamethoxazole (SMX), sulfaquinoxaline (SQX), and sulfanilamide (SAM), in milk and chicken drumstick muscle with laser-induced fluorescence detection.

MATERIALS AND METHODS

Apparatus and Materials. The laser-induced fluorescence detector used in this work was built in a confocal optical configuration. A 405 nm laser diode (Sanyo) equipped with collimation lens was driven by a constant-current laser driver. The laser beam was reflected by a dichroic mirror (430 nm, Shenyang HB Optical Technology Co., Ltd., Shenyang, China) and focused on the microchannel of microchips by a microscope objective (20×, Beijing 7-Star Optical Instruments Co., Ltd., Beijing, China). The fluorescence was collected by the same objective and transported through the dichroic mirror, a long-pass filter (520 nm, Shenyang HB Optical Technology Co. Ltd.), and reflected by an aluminum mirror to a pinhole (500 μm i.i.) arranged in front of a photomultiplier tube (CR 105, Beijing Hamamatsu Photon Techniques Inc. Co., Ltd.), where the fluorescence was transformed to electronic signals. Data were digitized with an NI-6009 data acquisition card (National Instruments, Austin, TX) that connected to a computer to display and store the results. High voltage for the electrophoresis was provided by a high-voltage module (DW-P602, Dongwen High-Voltage Power Supply, Tianjin, China). The power supply, together with a negative pressure-induced injection unit, and the data acquisition card were controlled by a program in Labview (National Instruments). Viscosity was measured using an Ubbelohde viscometer thermostatted with a water bath.

SMZ (98%), SMX (98%), and SAM (99%) were provided by Aladdin Chemistry Co. Ltd. (Shanghai, China). SQX (AR) and fluorescamine were purchased from Sigma-Aldrich (St. Louis, MO). Anhydrous sodium acetate was obtained from Chengdu Chemical Reagent Factory (Chengdu, China). Glacial acetic acid was from Tianjin Guangfu Science and Technology Development Co. Ltd. (Tianjin, China). Anhydrous methanol and acetone were supplied by Tianjin Chemical Reagent Co. Ltd. (Tianjin, China). Boric acid and polyvinyl alcohol (PVA) were obtained from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). All other reagents were of analytical grade and used as received.

Solution Preparation. Stock solutions of sulfonamides (10.00 mg/mL for SMZ, SMX, and SAM; 1.00 mg/mL for SQX) were prepared by dissolving appropriate amounts of sulfonamides in 5 mL of anhydrous methanol and diluted to 10 mL with sodium acetate−acetic acid. These solutions were stored in a refrigerator (4 °C) and were stable for at least 1 month. Fresh fluorescamine solution (3.0 mg/mL) in acetone was prepared daily and stored in the dark at 4 °C. Acetate buffer (pH 3.5) used for derivatization reaction was prepared by the addition of 1.0 mL of 0.10 mol/L sodium acetate to 16.00 mL of 0.10 mol/L glacial acetic acid. The buffer used for the separation of sulfonamides was prepared by mixing 10 mL of 10 mmol/L boric acid solution with 10 mL of 0−2.4% (w/v) PVA solutions. The pH values of the buffer solutions were adjusted to the desired value with a 1.0 mol/L NaOH or HCl solution. All solutions were filtered with a 0.22 μm membrane before use.

Sample Preparation. To match the fast separation of the microfluidic chip, a simplified sample pretreatment protocol[19] was used to speed the sample preparation. Milk and chicken drumsticks were purchased from local supermarkets.

For milk samples, a duplicate set of milk (1.00 mL) was added to two 50 mL polypropylene centrifuge tubes, and one of them was spiked with sulfonamides standards equivalent to 100 μg/L of each. Both samples were acidified with 100 μL of 0.1 mol/L HCl and diluted to 10 mL with methanol to precipitate protein and extract the analytes. The mixture was vortexed for 30 s and sonicated for 5 min, then centrifuged at 4000 rpm at room temperature for 10 min. The supernatant was collected and filtered through a 0.22 μm syringe filter before derivatization.

For chicken samples, the skin and fat of the drumsticks were removed, and the remaining muscle was homogenized in a commercial mincer and stored at refrigerator at −20 °C before use. Duplicate sets of homogenized chicken (1 g) were accurately weighed and transferred to two 50 mL centrifuge tubes, one of which was spiked with sulfonamide standards (100 μg/kg each). Five grams of anhydrous sodium sulfate was added to each of the samples followed by 5 mL of acetonitrile to extract the analytes. The mixtures were filtered under vacuum after being vortexed for 30 s and sonicated for 5 min; the precipitate was washed three times with acetonitrile (5 mL each time). The filtrates were combined together and evaporated to dryness by rotary evaporator at 40 °C. The residue was dissolved in 1.00 mL of ammonium acetate (10 mmol/L, containing 7% v/v acetonitrile), and 1 mL of acetonitrile saturated *n*-hexane was added to the solution. The upper hexane layer was discarded after the mixture was vortexed for 1 min and centrifuged at 4000 rpm at room temperature for 3 min. The lower acetonitrile layer was filtered with a 0.22 μm filtrate membrane before derivatization.

Derivatization. Fifty microliters of sulfonamide working solutions or sample extracts in acetate buffer was transferred to 1.5 mL centrifuge tubes, and 50 μL of 3 mg/mL fluorescamine solution in acetone was added to each tube. The tubes were vortexed and placed on a hot plate at 80 °C for 20 min. The derivatives produced were diluted to the desired concentrations or volumes, if necessary, with running buffer before analysis.

Fabrication and Pretreatment of Microfluidic Chips. The detailed fabrication procedures of cyclic olefin copolymer (COC) microfluidic chips will be reported elsewhere. Briefly, a piece of COC plate was sandwiched between a blank microscope slide and a slide with 80 μm copper wires attached to its inner surface. The assembly was clamped with six small binder clips (three at each long side) and put in an oven at 140 °C for 25 min. After cooling, the COC plate together with copper wires was dipped into concentrated nitric acid to etch copper away to form microchannel. Then, four holes were drilled at the ends of each channel and sealed with another COC plate of the same size by sandwiching them inside a pair of microscope slides with the aid of six binder clips and heating at 122 °C for 10 min. Solution reservoirs were glued at the access holes, which can accommodate about 100 μL of solution. The chip has a cross configuration; the total length of the separation channel is approximately 5 cm, and the sample injection channel is around 1 cm. Prior to use, the chip was flushed with ethanol, distilled water, and buffers each for 2 min before use. When not in use, the chip reservoirs were filled with distilled water, and the whole chip was sealed in a zip-lock plastic bag.

Microfluidic Chip Electrophoretic Separations. Negative pressure pinched sample injection[28] was used in the microchip electrophoresis. Buffer solution was filled in buffer and buffer waste reservoirs in volumes of 100 and 20 μL, respectively. Sample solution (10 μL) was filled in the sample reservoir. To start sample injection, vacuum provided by an air pump was applied to the sample waste reservoir to force the sample solution to flow through the cross for 1 s. Then high voltage (3000 V) was applied over the buffer waste and buffer reservoirs to inject a small portion of sample into the separation channel and initiate the separation (anodic migration). The effective separation length (from cross to detection point) was 2.5 cm unless indicated otherwise. All electropherograms were recorded with a sampling frequency of 50 Hz and smoothed through 10-point moving average for peak integration and noise calculation. The calibration curves were constructed using the peak area versus the concentration of analyte standards. For real sample measurement, standards were diluted with blank solutions containing the same matrix of sample extracts. Standard addition recovery was calculated using equation

$$\text{recovery}\ (\%) = \frac{C_{\text{total}} - C_{\text{sample}}}{C_{\text{spiked}}} \times 100\%$$

where C_{total} is the measured concentration with standards spiked, C_{sample} is the concentration measured in the sample, and C_{spiked} is the concentration of spiked standards.

RESULTS AND DISCUSSION

Derivatization. The derivatization procedure was optimized with regard to the peak area obtained under different conditions. The influence of pH on the derivatization is shown in Figure 2; the maximum peak areas were achieved at pH 3.5

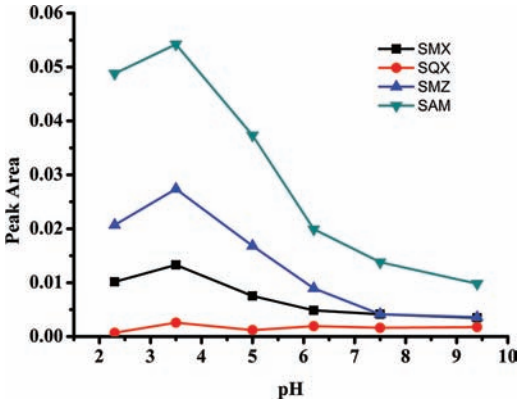

Figure 2. Effect of pH on the derivatization.

for all analytes. Although derivatization of compounds with primary amine groups, such as amino acids, is usually carried out in basic medium, to perform the derivatization of sulfonamides with fluorescamine, an acidic buffer was frequently adopted.[19] The experimental results also demonstrated that elevated temperature was beneficial to the derivatization and larger peak areas could be obtained. The influence of derivatization time was also investigated. At 80 °C, peak areas increased with derivatization time and leveled off after 20 min. Therefore, derivatization in acetate buffer of pH 3.5 at a temperature of 80 °C for 20 min was used in all subsequent experiments.

Influence of Buffer pH. The influence of the pH of the running buffer on separation was tested from pH 5.5 to 9.5. As shown in Figure 3, rapid separation of four fluorescamine-derivatized sulfonamides was achieved at pH 6.5−8.5. At pH >8.5, SQX and SMZ merged together, whereas at pH <6.5, the

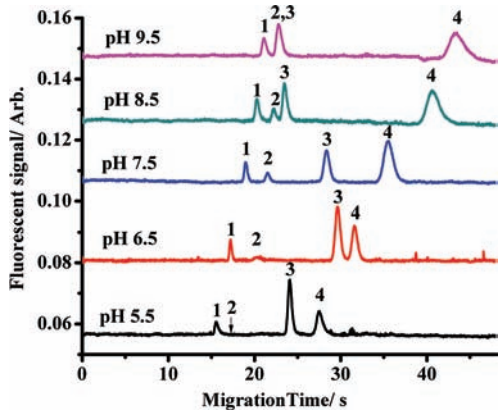

Figure 3. Influence of running buffer pH on sulfonamide separation: buffer composition, 5 mmol/L boric acid and 1.0% PVA; microchip, 5 cm (2.5 cm effective separation length) × 80 μm i.d.; separation voltage, 3000 V; concentration of each analyte, 500 μg/L. Peaks: 1, SMX; 2, SQX; 3, SMZ; 4, SAM.

peak of SQX was too small. In the subsequent experiments, pH 7.5 was chosen as the buffer pH.

Effect of PVA Concentration. PVA was added to the running buffer to act as a viscosity regulator and surface charge/status modifier. PVA was selected in this work because of the abundant hydroxyl groups present on the backbone of this polymer. It is probable that partial anionic polymer chains are formed through complexation of adjacent hydroxyl groups and boric acid in the solution. These in situ formed anionic polymer chains could act as a pseudostationary phase and enhance the resolution of analytes. The higher viscosity provided by the polymer, on the other hand, can minimize the analyte band dispersion caused by diffusion. The effect of PVA concentration on sulfonamide separation was studied with PVA concentration ranging from 0 to 1.2% (w/v), whereas boric acid concentration was maintained at 5 mmol/L and pH at 7.5. As shown in Figure 4 (left panel), without PVA, the migration of analytes was cathodic but the separation was rather poor. With a small amount of PVA in the buffer (0.2%), the migration direction of all analytes switched to anodic and good resolution could be achieved. When PVA was increased to 0.4%, both peak shape and resolution of these substances were improved further; but no evident change in separation was observed, although migration time increased slightly with the content of PVA increased to 1.0%. With 0.4−1.0% PVA, the number of theoretical plates of these compounds were between 3.0×10^5 and 6.7×10^5/m. Further increase of PVA to 1.2% led to a decrease in the number of theoretical plates.

To verify the effect of the viscosity of the buffer on the migration time, viscosities of these buffers were measured using an Ubbelohde viscometer. With increasing amount of PVA, both migration time and solution viscosities increased. However, their increases are not proportional. For migration time, it extended from 30.7 to 42.8 s, about a 40% increase, whereas the increase of viscosity was >200%, from 1.1×10^{-3} to 3.3×10^{-3} Pa s. This result also implies that it is possible to use higher PVA concentration to suppress analyte dispersion through increased viscosity without reducing analysis speed. Therefore, 1.0% PVA was used for the further experiments.

Influence of Boric Acid Concentration. The concentration of boric acid is directly related to the ionic strength of the buffer and might have an influence on the complexation of boric ions with PVA, which may affect the electrophoretic

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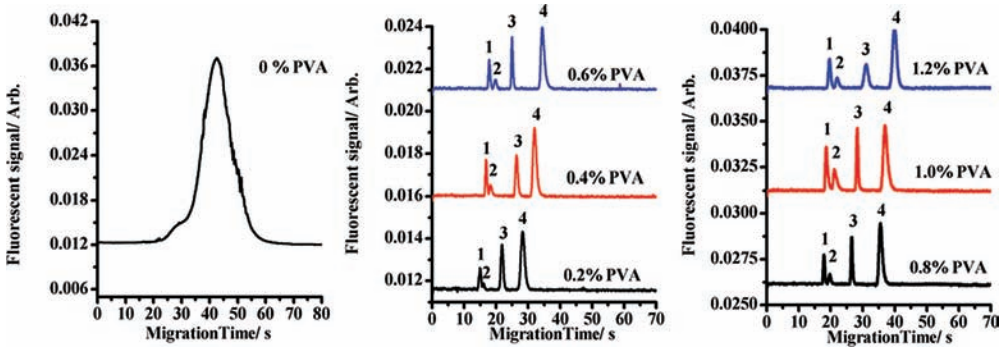

Figure 4. Effect of PVA concentration on sulfonamide separations: buffer pH, 7.5; all other conditions were the same as in Figure 3. With 0% PVA, analytes migrated toward the cathode; with the presence of 0.2% or more PVA, analytes migrated toward the anode. Peaks: 1, SMX; 2, SQX; 3, SMZ; 4, SAM.

separation. The effect of boric acid concentration was examined in the range of 5−25 mmol/L with 1.0% PVA at pH 7.5. The electropherograms in Figure 5 demonstrated that sulfonamides,

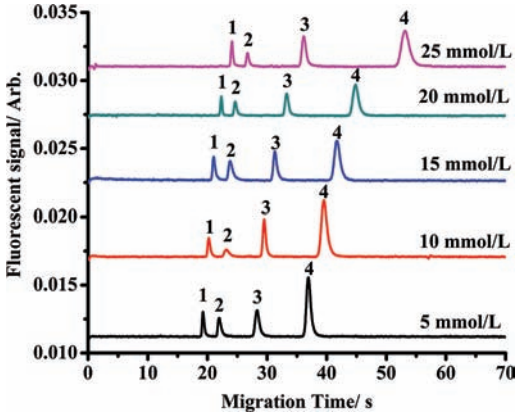

Figure 5. Influence of boric acid concentration on sulfonamide separations: buffer pH, 7.5; all other conditions were the same as in Figure 3. Peaks: 1, SMX; 2, SQX; 3, SMZ; 4, SAM.

particularly SAM, migrated more slowly at higher concentrations of boric acid. Because all analytes migrated anodically, this result could not be explained by the suppression of zeta potential of the channel wall by higher salt concentration. Suppressed zeta potential should enhance the apparent migration speed as a result of reduced electroosmotic flow. A higher degree of cross-linking of PVA due to the PVA−boric acid complexation might be responsible for the slower analyte migration, which could be proved partially by the improvement of resolution between SMZ and SAM along the increase of the concentration of boric acid. However, the increase of resolution is rather moderate; the number of theoretical plates of sulfonamides were between 5.9×10^5 and 1.1×10^6/m. Therefore, 5 mmol/L boric acid was adopted in all other experiments.

Method Validation. Parameters including slope, intercept, and linearity of calibration curves together with LODs, limits of quantitation (LOQs), and within-day and between-day repeatabilities were evaluated. The results are shown in Tables 1 and 2. They were linear up to concentrations well above the MRL of sulfonamides with correlation coefficients (r) >0.995. It should be mentioned that the linear range could be extended further to higher concentrations using a lower gain of the detector. The LODs and LOQs defined concentrations corresponding to signals of 3 and 10 times baseline noise (S/N ratios of 3 and 10), respectively, and were calculated using peak heights. LODs obtained were in the range of 0.2−2.3 μg/L, which were far below the MRL of sulfonamides in foods of animal origin. The relative standard deviations (RSD, n = 5) of retention time within a day for SMX, SQX, SMZ, and SAM were 0.48, 0.91, 0.23, and 0.31%, respectively. The RSDs of peak area were in the range of 0.80−2.7% for these four sulfonamides. The repeatability of the method was also

Table 2. RSDs of Within-Day and Between-Day Measurements of Recoveries of Spiked Sulfonamides (100 μg/L) in Milk and Chicken Muscle Extracts

	RSD (%), within-day (n = 5)		RSD (%), between-day[a] (n = 3)	
analyte	milk (%)	chicken (%)	milk (%)	chicken (%)
SMX	2.8	2.9	2.4	1.7
SQX	0.82	0.79	1.6	1.6
SMZ	3.0	2.2	2.3	1.9
SAM	1.9	2.0	2.7	1.4

[a]Calibrated every day.

Table 1. Method Validation for the Determination of Sulfonamides with Microfluidic Chip Electrophoresis−Fluorescence Detection

	regression equation						RSD (%)	
sulfonamide	slope ($\times 10^{-5}$)	intercept ($\times 10^{-4}$)	correl coeff	LOD (μg/L)	LOQ (μg/L)	linear range (μg/L)	migration time (n = 5)	peak area (n = 5)
SMX	1.69	−0.79	0.997	0.6	2.0	2−200	0.48	2.1
SQX	0.272	−0.20	0.996	2.3	7.7	8−200	0.91	0.80
SMZ	1.59	−1.37	0.996	0.6	2.0	2−200	0.23	2.7
SAM	9.51	−5.38	0.996	0.2	0.6	1−200	0.31	0.94

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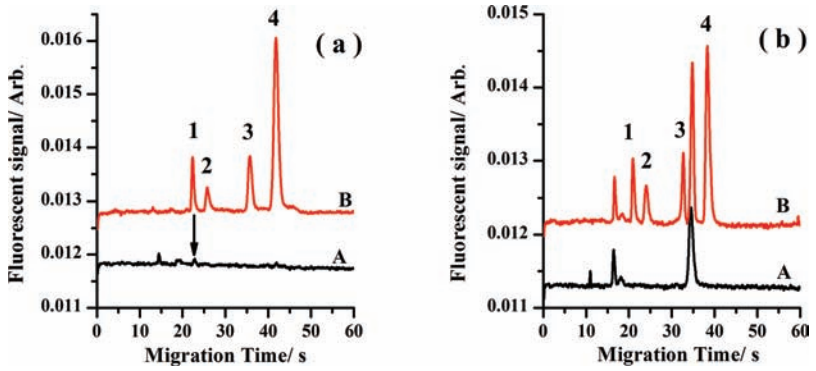

Figure 6. Electropherograms of (a) a milk sample (A) and one spiked with 100 μg/L of sulfonamides (B) and (b) a chicken sample (A) and one spiked with 100 μg/kg sulfonamides (B). Separation buffer: 5 mmol/L boric acid, 1.0% PVA at pH 7.5. Other conditions were the same as in Figure 3. Peaks: 1, SMX; 2, SQX; 3, SMZ; 4, SAM.

Table 3. Content and Standard Addition Recovery of Sulfonamides in Milk and Chicken Muscle Samples

	content		added		total found		recovery	
analyte	milk (μg/L)	chicken (μg/kg)	milk (μg/L)	chicken (μg/kg)	milk (μg/L)	chicken (μg/kg)	milk (%)	chicken (%)
SMX	8.1	nd[a]	100	100	101.4	83.5	93.3	83.5
SQX	nd	nd	100	100	100.8	92.3	100.8	92.3
SMZ	nd	nd	100	100	96.2	82.9	96.2	82.9
SAM	nd	nd	100	100	96.5	84.4	96.5	84.4

[a]nd, not detected.

evaluated through measuring recoveries of four sulfonamides spiked at MRL into both blank milk and chicken muscle extracts (Table 2). Within-day RSDs of sulfonamide recoveries in both extracts were no more than 3.0 and 2.9%, whereas between-day RSDs were less than 2.7 and 1.9%, respectively. These results indicate good precision of the method.

Because fluorescamine derivatization has rather good specificity for sulfonamides,[29] there is little interference from the matrix of milk and chicken muscle samples. As shown in Figure 6a, only three small peaks appeared in the electropherogram of milk extracts, but all four sulfonamides were well resolved from them. For chicken muscle samples, a big peak appeared, but it could be well resolved from sulfonamides, too (Figure 6b). Although the solvents in both kinds of sample extracts were different, migration of sulfonamides exhibited negligible difference in both migration times and peak height/area under the same experimental conditions. The absence of influence from sample matrix was also confirmed by the results obtained in the repeatability study shown in Table 2.

Analysis of Sulfonamides in Food. The established method was used for the determination of sulfonamide residues in milk and chicken muscle samples. For each sample, after the pretreatment and derivatization, it was immediately analyzed using microfluidic electrophoresis. From five batches of milk of different venders, SMX was positively detected in a sample; no other sulfonamides were detected in these samples. The electropherogram of the sample with SMX is shown in Figure 6a, and the amount was calculated to be 8.1 μg/L, much lower than the MRL (100 μg/L). No sulfonamide residues were detected in all chicken muscle samples (Figure 6b). The reliability of the method was evaluated by standard addition. Sulfonamides at MRL were spiked into 1 mL of the milk and 1 g of the chicken muscle sample. These spiked sulfonamides in both milk and chicken samples were determined successfully. The recovery data are summarized in Table 3. In the milk sample, the recoveries for SMX, SMZ, SQX, and SAM were 93.3−100.8%. In the chicken sample, the recoveries were 82.9−92.3% for the four compounds. The relatively lower recovery for the chicken sample may be related to the complicated analyte extraction procedure. These results indicate that the proposed method can be successfully used for the determination of sulfonamides at low concentration levels in real food samples.

The most important merit of the proposed method is its shorter analysis time. The separation could be performed within 1 min, much faster than capillary electrophoresis and HPLC. For example, 6 min was needed for a micellar electrokinetic chromatographic separation for rapid determination of sulfonamides in milk,[19] and the time was even longer (15 min) if field-amplified sample stacking was incorporated.[18] HPLC (/LC) separation required times ranging from 14 to 40 min.[1,8−11] LODs of the present work (0.2−2.3 μg/L) are comparable to those of capillary electrophoresis or HPLC with fluorescence detection[1,9,19] and MS detection,[10,11] far below the MRL of sulfonamides in foods. The plastic microfluidic chips used here are rather cheap and disposable. Compared with normal capillary electrophoresis and HPLC, the device used in the present work has great potential to be miniaturized and can be developed to a portable instrument for on-site screening of sulfonamide residues in foods and related samples.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; LC, liquid chromatography; LC-MS, liquid chromatography−mass spectrometry; LOD, limit of detection; LOQ, limit of quantitation; MRL, maximum residue limit; PVA, polyvinyl alcohol; RSD, relative standard deviation; SAM, sulfanilamide; SMX, sulfamethoxazole; SMZ, sulfamethazine; SQX, sulfaquinoxaline.

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