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Fluorimetric Determination of Trace Sulfonamides in Milk Samples

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A precise and reliable fluorimetric method for the determination of four sulfonamide antibiotics (sulfadiazine, sulfaguanidine, sulfamethoxazole, sulfamethazine) in milk samples has been developed. Sulfonamide in presence of *o*-phthalaldehyde produces an isoindole derivative, which can produce strong fluorescence. *N*-butanol was used for the extraction of the derivative to bring down the detection limit largely. Optimum conditions for the determination of sulfonamides were also investigated. Fluorimetric detection was carried out at λ_{ex} of 295 nm and λ_{em} of 425 nm. Linear ranges for sulfonamides determination were 5-500 mg/L. The developed method is practical and can be successfully applied to determine the content of sulfonamides residues in milk samples with satisfactory results.

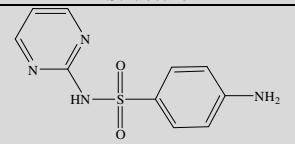
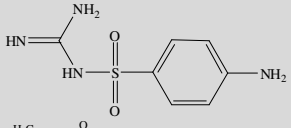
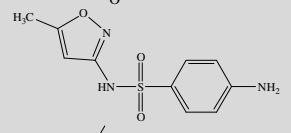
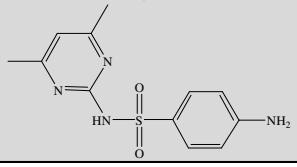
Key Words: Fluorescence, Sulfonamide, *o*-Phthalaldehyde, Derivatization, *N*-butanol.

INTRODUCTION

Sulfonamides (SAs) (Table-1) are a large group of broad-spectrum synthetic antibiotics widely used in veterinary therapy for the treatment and prevention of several bacterial and protozoan infections in food producing animals. Sulfonamides are commonly incorporated into feed and employed as both growth promoters and prophylactics of diseases caused by Leukocytozoa and Coccidian[1]. As a consequence, residues of these drugs may remain in foods of animal origin, especially in milk. The residues of sulfonamides may cause allergic reactions and antibiotic resistance and can be carcinogenic in humans[2]. Therefore, it is very important for the quality control of sulfonamide formulations and their trace determination in foods[3]. To limit the harm sulfonamides cause, European community has adopted for sulfonamides a maximum residue level (MRL) of 100 mg/kg in foodstuffs of animal origin[4].

Bioassays are the most commonly employed methods for determining antibiotics residues in milk because of their simplicity and low cost. Although sensitive, these assays are not specific or quantitative and may give origin to false positives. Several physicochemical identification methods have been developed for the determination of residual sulfonamides including high-performance liquid chromatography (HPLC)[5-8], capillary electrophoresis-mass spectrometry (CE-MS)[9], immunochromatographic assay[10,11], liquid chromatography-tandem mass spectrometry (LC-MS)[1,12,13], fluorescent spectrometry[3,14]. HPLC is the most common method used for the determination of sulfonamides, which exhibits high sensitivity and selectivity. However, the equipment is expensive and hard to handle for small Private enterprises so that it's difficult to be popularized. As a method with high sensitivity and high selectivity, fluorescence analysis has extraordinary superiority

TABLE-1
MOLECULAR STRUCTURE OF INVESTIGATED SULFONAMIDES

Name	Structure
Sulfadiazine (SD), 2-(*p*-aminobenzenesulfonamido)-pyrimidine	
Sulfaguanidine (SG), *N*-(*p*-aminobenzenesulfonamido)-aminoiminomethyl	
Sulfamethoxazole (SMX), 3-(*p*-aminobenzenesulfonamido)-5-methyloxazole	
Sulfamethazine (SMZ), 2-(*p*-aminobenzenesulfonamido)-4,6-dimethylpyrimidine	

for the analysis of drugs. Its pretreatment method is simple, the device is easy to operate and the method is reliable and stable. Díez *et al.*[15] reported the determination of sulfonamides based on sulfadiazine derivatization with fluorescamine. Garcia *et al.*[3] set up a method for determination of sulfathiazole and sulfadiazine by photochemically induced fluorescence and first-derivative fluorescence. A linear relation of the method was found between 0.23 and 3.00 mg/mL of sulfathiazole.

In this study, we found that sulfonamides could produce strong fluorescence after their derivatization with *o*-phthalaldehyde in acidic condition. After the derivatization of sulfonamides in the supernatant of milk after centrifugation, the derivatives have poor polarity so that they could be extracted by *n*-butanol to improve the sensitivity of the method. Compared to the method of fluorescamine, this method exhibits higher sensibility and has lower limit of detection. It has been successfully applied to determine the residues of sulfonamides in milk with satisfactory results.

EXPERIMENTAL

All fluorescence measurements were carried out on a RF-5301 Spectrofluorophotometer (Shimadzu, Kyoto, Japan). A XMTB digital temperature controller (Tianjin, China) and a PHS-25 digital pH meter (Leici, Shanghai, China) were used in present study.

Sulfadiazine, sulfaguanidine, sulfamethoxazole, sulfamethazine and *o*-phthalaldehyde (OPA) were purchased from Aladdin Chemistry Co. Ltd., (Shanghai, China). The reagents of glacial acetic acid, boric acid, orthophosphoric acid, *n*-butanol, ethyl acetate, chloroform, dichloromethane, hydrochloric acid, acetonitrile and lead acetate were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). All reagents were of analytical grade.

The stock standard solutions of sulfonamides were prepared in water at a concentration of 1 g/L. The working standard solution (1 mg/L) was prepared on the day of use. The *o*-phthalaldehyde solution was prepared in methanol at a concentration of 1 g/L. The lead acetate solution was prepared at a concentration of 50 g/L. The acidic acetonitrile was made up by hydrochloric acid (1mol/L) and acetonitrile in proportion of 2:3. Britton-Robinson (B-R) buffer solution (pH = 2.0) was used in this paper. All the stock solution and working solution were stored at 0-4 ºC.

Two milliliters of B-R Buffer solution (pH = 2.0) was placed into a 10 mL test tube. Then the supernatant of milk containing 1-500 µg/L of sulfonamide in final volume and 0.5 mL of *o*-phthalaldehyde (1 g/L) was added into the tube. The volumes were completed to the mark with distilled water. Obtained solution was mixed thoroughly and heated in a 70 ºC water bath for 50 min.

After the solution was heated, the solution containing the derivative was transferred into a 20 mL separatory funnel and extracted twice using 2 mL of *n*-butanol. The organic layers are merged and determined by the spectrofluorophotometer. The fluorescence intensity was measured at λ_{ex} / λ_{em} = 295 nm /425 nm.

Sample preparation: All milk samples were analyte-free and spiked with 100 mg/L of the four sulfonamides according to the conditions required by the experimental design. The milk samples were mixed with the solution of the acidic acetonitrile in proportion of 5:3 and then centrifuged for 15 min at 4000 rpm. The step was repeated for a second time. Then the supernatant was filtered and prepared for the determination.

RESULTS AND DISCUSSION

Fluorescence emission spectra of *o*-phthalaldehyde, sulfadiazine, sulfadiazine-*o*-phthaldehyde system and the extracted system are shown in Fig. 1. It can be seen from Fig. 1 that sulfadiazine can produce strong fluorescence intensity with *o*-phthalaldehyde existing.

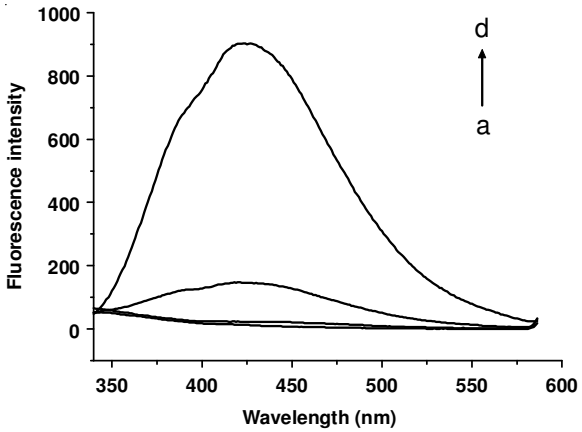


Fig. 1. Fluorescence spectra of sulfadiazine/OPA/*n*-butanol; C (SD) = 0.2 mg/L; C (OPA) = 0.1 mg/L; pH = 2; l = 1 cm; λ_{ex} = 295 nm; a = OPA-*n*- butanol; b = SD; c = SD-OPA; d = SD-OPA-*n*-butanol

Effect of acidity: The reagent of o-phthalaldehyde alone produces strong fluorescence intensity in basic environment which is confirmed in present study.

The experimental results showed that ΔF reached the maximum in the pH range of 1.5-2.5 (Fig. 2). So we selected pH = 2.0 of B-R buffer solution for further study. As the volume of the buffer solution added from 1.0 to 4.0 mL, the fluorescence intensity reached the maximum and remained constant. 2.0 mL was used for the following experiments.

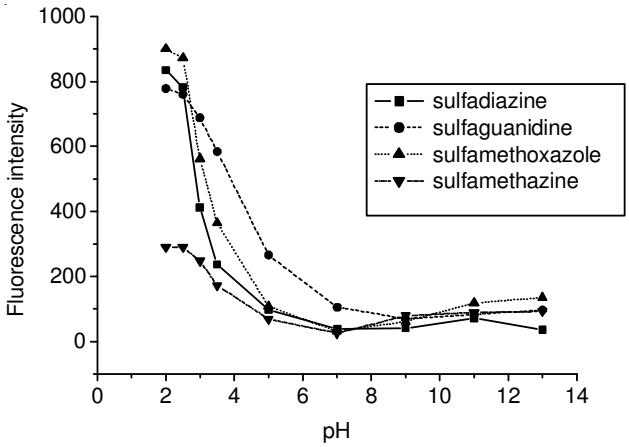


Fig. 2. Fluorescence intensity of sulfonamides in B-R buffer solution with different pH values; C (SAs) = 0.2 mg/L; C (OPA) = 0.1 mg/L; *l* = 1 cm; λ_{ex} = 295 nm; Adding B-R Buffer Solution (pH = 2.0) was the best condition

Effect of reagent concentration: Fig. 3 shows the effect of *o*-phthalaldehyde concentration on the quantitativeness of its reaction with sulfonamides under study. It was found that, when various concentrations of *o*-phthalaldehyde solution were added to a certain concentration of sulfonamides (1 mg/L), 0.1-0.5 mL of *o*-phthalaldehyde (1 g/L) solution is found to be sufficient for the quantitative determination of sulfonamides. When the concentration of sulfonamides in the tube was 1 mg/L, adding 0.5, 0.1, 0.1, 0.5 mL 1g/L of *o*-phthalaldehyde are the best conditions for sulfadiazine, sulfaguanidine, sulfamethoxazole and sulfamethazine, respectively. As a screening method, we choose to add 0.5 mL 1 g/L of *o*-phthalaldehyde for all the samples.

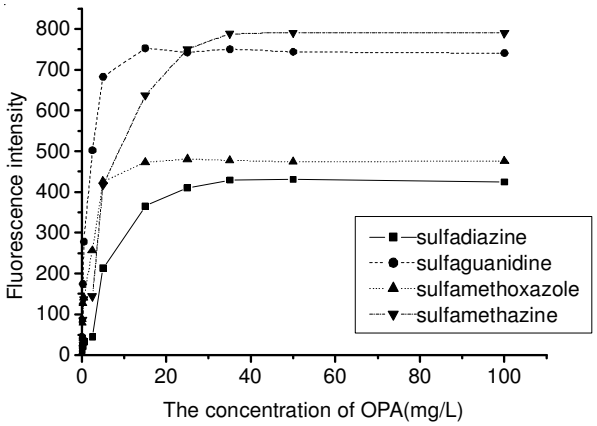


Fig. 3. Fluorescence intensity of sulfonamides adding *o*-phthalaldehyde with different concentrations; C (SAs) = 0.2 mg/L; pH = 2; l = 1 cm; λ_{ex} = 295 nm

Effect of temperature: The fluorescence intensity was closely related to the heating temperature. The fluorescence intensity increased with the increasing of the temperature. Experiments showed that the fluorescence intensity reached the maximum when the solution was heated in a 70 ºC water bath (Fig. 4).

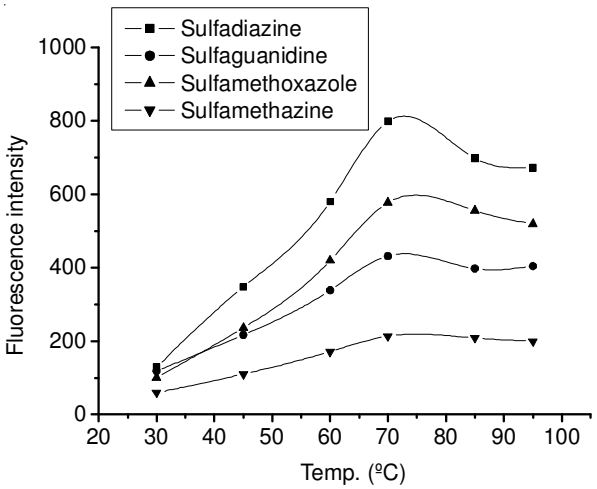


Fig. 4. Fluorescence intensity of sulfonamides heating in different temperatures (low sensitivity); C (SAs) = 0.2 mg/L; C (OPA) = 0.1 mg/L; pH = 2; l = 1 cm; λ_{ex} = 295 nm

Effect of time: The reactions were completed within 50, 40, 50 and 40 min in a 70 ºC water bath for sulfadiazine, sulfaguanidine, sulfamethoxazole and sulfamethazine, respectively (Fig. 5). The fluorescence intensity reached its highest value and remained constant for 24 h, then decreased slowly. Hence 50 min in a 70 ºC water bath was selected for the four sulfonamides and then measured them in 24 h.

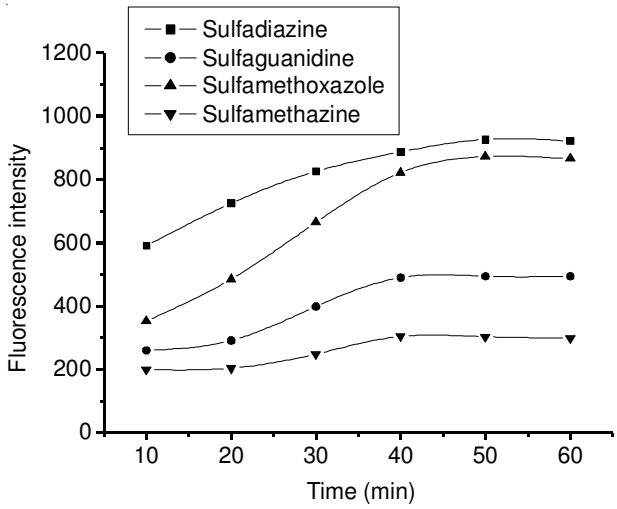


Fig. 5. Fluorescence intensity of sulfonamides after heating for different period of time (low sensitivity); C (SAs) = 0.2 mg/L; C (OPA) = 0.1 mg/L; pH = 2; l = 1 cm; λ_{ex} = 295 nm

Effect of extract solvent: Ethyl acetate, chloroform, dichloromethane and *n*-butanol were tried as extractants to extract the derivatives, respectively. Experiments showed that using 2 mL of *n*-butanol to extract the derivative twice could lead to the best result.

Influence of coexisting substance: Series experiments of coexist substances had been carried out on the fluorescence of sulfonamides-*o*-phthalaldehyde-*n*-butanol system under the optimum conditions. Several substances were examined for interference (Table-2). Table-2 shows the possible coexisting substances have little effect on the system.

TABLE-2
INFLUENCE OF COEXISTING SUBSTANCE

Coexisting substance	Concentration (mg/L)	ΔF (%)
NaCl	10	-2.37
$CaCl_2$	10	1.54
Glucose	10	-0.75
Lactose monohydrate	10	2.60
Peptone	10	-0.54
Ascorbic acid	10	-1.68

Analytical applications: Under the optimal experimental conditions, the standard curves of the determination of sulfonamides were obtained, respectively. The linear equation, the coefficient of the correlation, the linear range, the detection limit and the relative standard deviation for each drug were tabulated in Table-3. As can be seen from the table, the present method is highly sensitive and reproducible.

The developed method was applied to the determination of four sulfonamides in milk (Table-4). From Table-4 it can be seen that the developed method can be easily performed and affords good precision and accuracy when applied to real samples.

TABLE-3
ANALYTICAL PARAMETERS FOR THE DETERMINATION OF SULFADIAZINE, SULFAGUANIDINE, SULFAMETHOXAZOLE AND SULFAMETHAZINE USING *o*-PHTHALALDEHYDE REAGENT

Drug	The linear range (μg/L)	R^2	F = a × C + b		Detection limit (μg/L)	RSD (n=5)
SD	5-700	0.9983	1.2426	6.839	1.33	1.05
SG	5-700	0.9989	1.0596	3.059	1.67	0.23
SMX	5-500	0.9994	1.8594	6.7895	2.2	2.18
SMZ	5-700	0.9979	1.1269	0.8877	1.67	1.85

TABLE-4
DETERMINATION OF SULFADIAZINE (SD), SULFAGUANIDINE (SG), SULFAMETHOXAZOLE (SMX) AND SULFAMETHAZINE (SMZ) IN MILK SAMPLES

Drug	Taken (μg/L)	Obtain (μg/L)	Average recovery (%)	RSD (%) (n=5)
SD	100.00	95.50	95.5	2.81
	200.00	192.82	96.41	0.8
SG	100.00	98.87	98.87	1.18
	200.00	198.63	99.32	1.77
SMX	100.00	94.14	94.14	1.01
	200.00	196.60	98.3	0.51
SMZ	100.00	97.92	97.92	0.12
	200.00	193.12	96.56	0.23

Conclusion

The amino group of sulfonamides can react with *o*-phthalaldehyde to form a derivative of isoindole[16,17], which can produce strong fluorescence at λ_{ex} = 295 nm (Fig. 6).

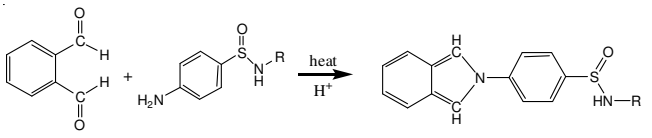


Fig. 6. The possible mechanism of sulfonamides reacting with *o*-phthalaldehyde

In summary, a precise and reliable fluorescence method has been developed. Compared with the existing literature, the method using *o*-phthalaldehyde as fluorescent derivation agent without a thiol has higher sensitivity than fluorescamine[15] and is more environmental friendly[17]. The method with high sensitivity is practical and has been successfully applied to determine the content of sulfonamide residues in milk samples with satisfactory results.

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