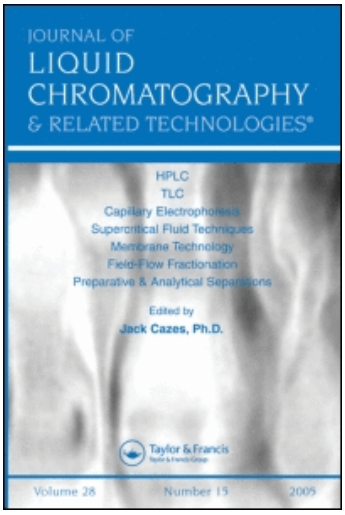

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Liquid Chromatographic Analysis of Multiple Sulfonamide Residues in Chicken Muscle Using Pre-Column Derivatization and Fluorescence Detection

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LIQUID CHROMATOGRAPHIC ANALYSIS OF MULTIPLE SULFONAMIDE RESIDUES IN CHICKEN MUSCLE USING PRE-COLUMN DERIVATIZATION AND FLUORESCENCE DETECTION

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ABSTRACT

A rapid liquid chromatographic (LC) method is described for the quantitation of sulfadiazine, sulfamethazine, sulfadimethoxine, and sulfaquinoxaline residues in chicken muscle. The sulfonamides are extracted with chloroform, partitioned into hydrochloric acid, and submitted to pre-column derivatization with fluorescamine. LC analysis of the fluorescent derivatives is performed on a C_{18} column using a mobile phase of acetonitrile /20 mM phosphate buffer pH 4, (34/66, v/v), containing 20 mM octanesulfonate sodium salt. Owing to the sensitivity and selectivity of the fluorescence detection, residue

levels of as low as 3 ng/g for sulfadiazine, 4 ng/g for sulfamethazine, 9 ng/g for sulfadimethoxine, and 40 ng/g for sulfaquinoxaline could be readily determined in chicken muscle. Overall recoveries were found to be 77.7±4.8% for sulfadiazine, 84.6±4.2% for sulfamethazine, 92.3±4.1% for sulfadimethoxine, and 82.5±7.0% for sulfaquinoxaline. The linearity of the method was quite acceptable in the range examined (5-100 ng/g for sulfadiazine and sulfamethazine, 15-150 ng/g for sulfadimethoxine, and 50-300 ng/g for sulfaquinoxaline).

INTRODUCTION

Sulfonamides are widely used in food-producing animals for the prevention and treatment of diseases. However, improper use of these antibacterials can leave residues in edible animal products. Owing to the potential impact of such residues on human health, maximum residue levels that oscillate between 0 and 100 ppb have been established,[1-3] whereas food surveys for monitoring of violatile sulfonamide residues are often made.[4]

Large-scale screening applications require multiresidue methods that are rapid, accurate, selective, and provide low detection limits. Several multiresidue LC methods for analyzing sulfonamides in animal tissues have been reported, but most are based on ultraviolet detection which does not have sufficient selectivity to eliminate matrix interferences without resorting to time-, labor-, and material-intensive sample purification.[5-9] Post-column derivatization and detection at 450 nm has been also applied for enhancing the selectivity of the analysis, but this approach still requires significant handling for sample cleanup,[10] or for setting up and optimizing the rather expensive equipment needed.[11]

Pre-column derivatization with fluorescamine and fluorescence detection has been suggested,[12] in the past, as a very efficient means of increasing the selectivity and sensitivity of sulfonamides analysis by LC. This most promising approach, which eliminates extensive clean-up and/or chromatographic separation of the fluorescent derivatives from the excess reagent, has been recently adopted in the development of rapid LC methods for the determination of multiple sulfonamide residues in animal tissues.[13,14]

These methods give useful information for trace analysis of sulfonamide residues, but conflict each other as far as the optimum pre-column derivatization conditions and some performance characteristics concern.

The objectives of this study were a re-examination of the conditions that influence the pre-column derivatization with fluorescamine, and the establishment of the most suitable LC conditions for separation of the fluorescent derivatives in order to develop an optimized LC multiresidue method for rapid, sensitive, accurate, and precise quantitation of some sulfonamides, representative of those commonly used in a commercial basis, in chicken muscle.

EXPERIMENTAL

Instrumentation

LC was carried out on a Gilson system consisting of a Model 802 manometric module, a Model 302 piston pump, a Model 121 fluorometric detector, a Model TC 831 HPLC-Technology column oven (Macclesfield, UK), and a model N1 variable-span recorder (Villiers-le-Bel, France). Injections were made using a Rheodyne 7125 sample injector equipped with a 20-μL loop.

Homogenization of the samples was performed using an Ultra-Turrax (Janke & Kunkel, GmbH, Germany) high speed blender, and centrifugation using a Centra-MP4 IEC centrifuge (Needham Heights, MA, USA).

Reagents

Analytical-grade reagents including chloroform, hydrochloric acid, phosphoric acid, sodium dihydrogen phosphate, and octanesulfonate sodium salt, and HPLC-grade methanol and acetonitrile were obtained from Merck AG (Darmstadt, Germany). Sodium acetate Suprapur-grade was purchased from Merck-Schuchard (Munchen, Germany), whereas, HPLC-grade water was produced using a Milli-Q (Millipore, Bedford, MA, USA) purification system.

Fluorescamine reagent (0.1%) was prepared by dissolving 10 mg of Fluram (Sigma, St. Louis, MO, USA) in 10 mL of acetonitrile. The reagent was prepared fresh weekly and stored in the dark at 4 °C when not in use.

Standard sulfadiazine, sulfamethazine, sulfadimethoxine, and sulfaquinoxaline compounds were all purchased from Sigma (St. Louis, MO, USA). Stock solutions of the individual sulfonamides (100 μg/mL) were prepared by weighing ca 10 mg of each and dissolving in and diluting to 100 mL with methanol. Aliquots of these stock solutions were appropriately diluted with 3 N hydrochloric acid to give standard working solutions for the calibration

curve study in the range 4.8-300 ng/mL. Spiking solutions for the precision and accuracy study were also prepared from stock solutions by diluting with 0.03 M sodium dihydrogen phosphate (range 0.3-18 μg/mL).

Extraction Procedure

The extraction procedure used in this investigation was based on that reported by Takeda and Akiyama.[13] A 3-g ground tissue sample was mixed with 30 mL chloroform to be further homogenized for 2 min. Following centrifugation for 5 min at 3000g, the supernatant was filtered through filter paper, and a 10-mL aliquot was added to 1 mL 3 N hydrochloric acid. The two-phase system was vortexed for 1 min, and centrifuged for 5 min at 2000g. The aqueous layer was collected and a 250-μl volume was submitted to the derivatization procedure.

Derivatization Procedure

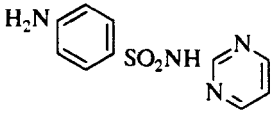
The collected aqueous extract was mixed with an equal volume (250-μL) of 3.8 M sodium acetate solution to be further mixed, under vortexing, with 100 μL of fluorescamine reagent. The reaction was left to proceed for 20 min at ambient temperature before a 20-μL aliquot, which was equivalent to 0.0083 g of the sample, be injected into the LC system.

Derivatization of standard sulfonamides was also performed using 250-μL volumes of each of the working solutions and mixing them with 250 μL 3.8 M sodium acetate and 100 μL of fluorescamine reagent as above.

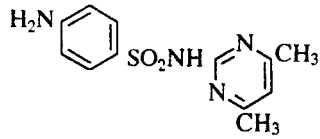
LC Analysis

LC analysis was performed at 30 °C on a reverse phase Hichrom column, 250x4.6 mm, packed with Nucleosil 120 C_{18}, 5 μm, whereas a mobile phase consisting of acetonitrile/20 mM sodium dihydrogen phosphate, pH 4, (34/66, v/v) and containing 20 mM octanesulfonate sodium salt was used to elute, isocratically, the fluorescent sulfonamide derivatives. The mobile phase was passed through 0.45 μm filter before use, degassed using helium, and delivered at a rate of 1.2 mL/min.

Detection was made using an excitation wavelength of 405 nm and an emission wavelength of 495 nm. Recordings were made at a chart speed of 2 mm/min and a 0.02 RFU sensitivity setting.



Sulfadiazine



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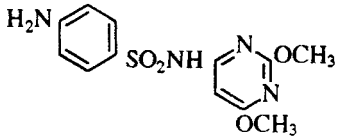
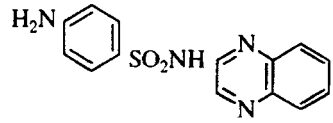
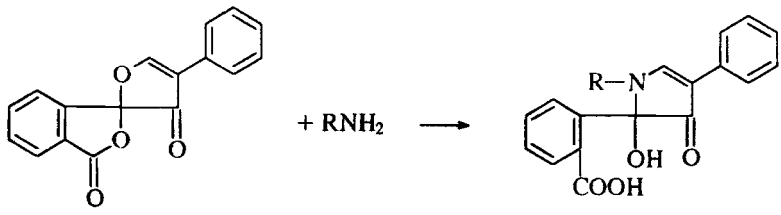


Sulfadimethoxine



Sulfaquinoxaline



Fluorescamine

Sulfonamide derivative

Figure 1. Chemical structures of investigated sulfonamides and their reaction with fluorescamine.

Calculation

Standard calibration curves were constructed by derivatization and LC processing five replicates of each of the four series of the working solutions of standard sulfonamides. The recorded peak heights, y (mm), were plotted versus the quantity, x (ng), of each sulfonamide injected, and the slope (b), intercept (a), and least square fit of each calibration curve were computed according to the equation $y=a+bx$. The concentration (ppb) of each sulfonamide in tissue samples was determined by reference to corresponding calibration curve and multiplication by appropriate dilution factor.

Table 1

Peak Heights (mm) of Sulfadiazine (SDZ), Sulfamethazine (SMZ), Sulfadimethoxine (SDX), and Sulfaquinoxaline (SQX) Derivatives with Fluorescamine as a Function of the Reaction Time and the Derivatizing Reagent Concentration

	0.02% Fluorescamine				0.05% Fluorescamine				0.10% Fluorescamine			
Reaction Time, Min	SDZ	SMZ	SDX	SQX	SDZ	SMZ	SDX	SQX	SDZ	SMZ	SDX	SQX
5	35.8	46.4	46.5	35.4	64.3	64.3	85.5	61.0	89.9	75.2	115.7	86.1
10	43.2	51.0	61.0	49.2	77.0	68.7	108.9	80.0	96.0	75.0	131.2	93.2
20									107.7	79.7	141.4	100.8
35	65.9	59.0	106.0	75.0	79.3	64.7	120.5	82.9	96.9	75.4	139.2	100.1
40	70.1	61.4	102.0	75.3	76.6	62.1	128.7	90.2	79.3	63.3	139.3	93.0
90	52.9	47.1	94.3	65.3								
125					72.7	56.6	106.5	73.1	83.8	62.9	124.7	88.2

RESULTS AND DISCUSSION

Optimization of Fluorescamine Derivatization

Fluorescamine, a fluorogenic reagent specific for primary amines, reacts directly with sulfonamides at ambient temperature to form pyrrolinone derivatives (Fig. 1), which upon excitation at 405 nm emit strong fluorescence at 495 nm.[12] Separation of any excess reagent, that might interfere with the quantitation of the fluorescent derivatives is not required, since fluorescamine and its hydrolysis products, unlike other fluorogenic reagents, are nonfluorescent.[15] These properties have made the fluorescamine reaction attractive for the identification and quantitation of many compounds, notably those of biological importance.[16] Analytical parameters for optimal reaction conditions of sulfonamides with fluorescamine prior to LC can be found in recent literature; however, complete agreement as to the exact pH value, time, and amount of fluorescamine needed for optimal reactivity does not exist.[13,14]

Using serially diluted sodium acetate solution for the derivatization reaction, optimum reactivity was found in the pH range 3.0-3.4 (3.5-3.8 M sodium acetate solution), decreasing at both lower and higher pH values. It was further observed, that the purity of the sodium acetate reagent could become the critical parameter in the LC analysis of the produced fluorescent derivatives; considerable interferences appeared when not highly purified sodium acetate was used.

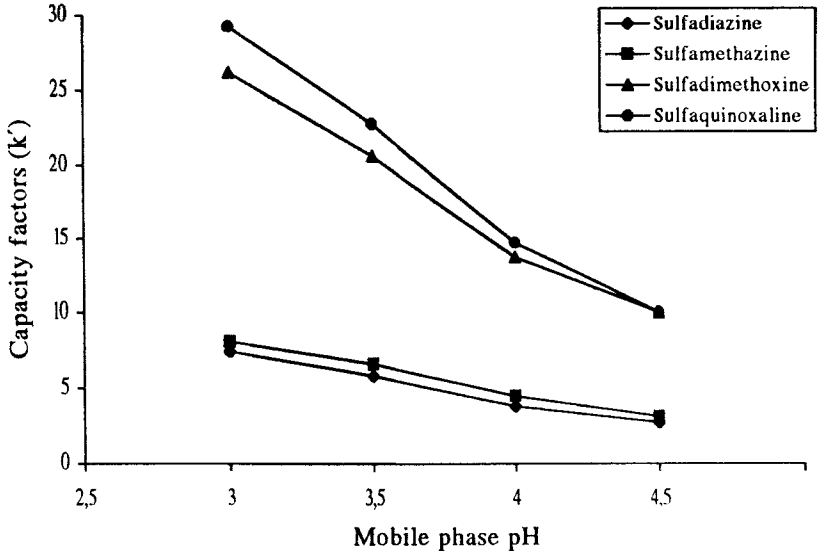


Figure 2. Capacity factors (k') of fluorescamine derivatives of sulfonamides versus mobile phase pH. Conditions: mobile phase, acetonitrile-0.02 M phosphate buffer (34:66, v/v); column, 250x4.6 mm, C_{18} 5 μm; temperature, 30 °C; flow rate, 1.2 mL/min; wavelength, ex 405 nm em 495 nm.

These results lend support to previous findings[13] indicating that the fluorescence yield at pH 3.75 is lower than that at 3.0, but oppose others suggesting optimum reactivity at pH 3.6.[14]

Using a 3.8 M sodium acetate solution to attain a pH value of 3.4 for the derivatization mixture, the reaction time and the amount of fluorescamine needed for optimal derivatization were also investigated. Table 1 shows the effect of reaction time on peak heights of the produced sulfonamide derivatives under three different concentrations of fluorescamine reagent. It becomes evident that the reaction yield largely depends on both the reaction time and the concentration of the fluorescamine reagent used. Optimal reactivity for all sulfonamides was observed after 20-min reaction using the 0.1% fluorescamine reagent. When the reaction was left to proceed for less than 5 min, as in the method of Tsai and Kondo[14] where it proceeds for only 1 min, the yield was low, even using 0.1% fluorescamine reagent. On the other hand, when the reaction was carried out with 0.02% fluorescamine, as in the procedure of Takeda and Akiyama,[13] the yield was very low, even if the reaction was left to proceed longer than 20 min.

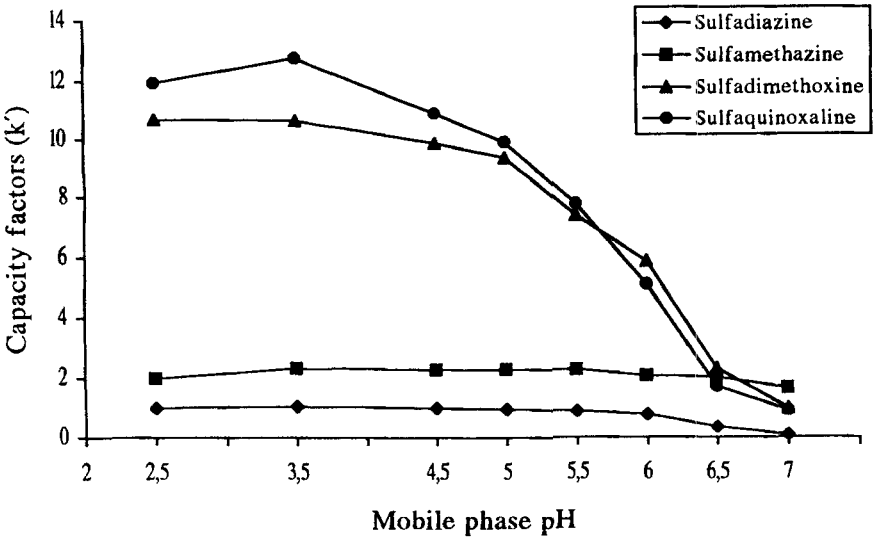


Figure 3. Capacity factors (k') of underivatized sulfonamides versus mobile phase pH. Conditions: mobile phase, acetonitrile-0.02 M phosphate buffer (18:82, v/v); column, 250x4.6 mm, C_{18} 5 μm; temperature, 35 °C; flow rate, 1.0 mL/min; wavelength, 272 nm.

LC Analysis

Literature LC conditions have described reverse phase separations of the fluorescamine derivatives of sulfonamides, using mobile phases of either acetonitrile-2% acetic acid, pH 2.5, at 55 °C or acetonitrile-10 mM phosphate buffer, pH 5.3, at ambient temperature. None of these conditions was found to be the most suitable to our LC system; the former ones caused a gradual baseline rise during each run unless column temperature had been set below 40 °C, a temperature where the compounds eluted too late, while the latter resulted in chromatograms with small but undesirable peak tailing.

Initial experiments towards optimizing LC conditions were made using acetonitrile/20 mM potassium dihydrogen phosphate (34/66, v/v) mobile phases of varying pH. LC runs were all performed at 30 °C, a temperature slightly higher than the ambient temperature, to avoid decreasing the fluorescence intensity of the sulfonamide derivatives due to elevated temperature. In Fig. 2 the observed capacity factors, k', of the derivatives of sulfonamides as a function of mobile phase pH are presented. The larger, apolar derivatives of sulfadimethoxine and sulfaquinoxaline gave the highest k' values. On the other hand, slight changes in the mobile phase pH had great impact on k' values of all

compounds, the retention behavior being mainly governed by the ionization state and polarity; all k′ values lowered with increasing pH from 3 to 4.5, a behavior accounted for by the presence of the carboxylic group which should be in a dissociated form in the derivatized molecules. Contribution to this ionization state by other groups should be excluded at this pH range, since the acidic NH linkage adjacent to the sulphonyl group has a pK_a ranging from 6.0 for sulfadimethoxine to 7.6 for sulfamethazine.[11] Additional support to these literature values may be given by Fig. 3 where the capacity factors of underivatized sulfonamides versus mobile phase pH are presented.

Fig. 2 shows that the sulfonamide derivatives remain well resolved when the pH of the mobile phase is less than 4.5; however, the retention of the late eluted compounds is high at these conditions. Attempts to decrease their retention by increasing the concentration of acetonitrile in the mobile phase resulted in loss of resolution. Considering, finally, the ionization state of the analytes, the use of octanesulfonate, a negatively charged ion-pairing reagent, for decreasing their retention was examined. The retention of such charged solutes has, in many instances, been decreased by addition to the mobile phase of an anionic ion-pair reagent, the decrease being the result of reduced availability of the octadecylsilica surface which becomes negatively charged and displaces similarly charged ions reducing, thus, their retention.[17]

The dependence of the retention of sulfonamide derivatives on the concentration of octanesulfonate reagent was investigated at pH 4.0 using mobile phases containing 0-20 mM of the ion-pair reagent. Fig. 4 shows that, the addition of octanesulfonate markedly decreases the retention of all analytes without affecting their resolution. The decrease was higher with 20 mM of octanesulfonate, a concentration which was finally selected as peak shapes were also substantially improved.

Calibration Curves and Detection Limits

In Fig. 5 a chromatogram of a mixture of standard sulfonamides, derivatized according to the described conditions, is presented. Chromatographic peaks are well resolved, the retention times being 8.5, 9.5, 22.0 and 24.0 min for sulfadiazine, sulfamethazine, sulfadimethoxine, and sulfaquinoxaline derivatives, respectively. The linearity of the fluorescence intensity of the fluorescamine derivatives was evaluated in the concentration range of 0.04-0.83 ng/20 μL for sulfadiazine and sulfamethazine, 0.12-1.25 ng/20 μL for sulfadimethoxine, and 0.42-2.5 ng/20 μL for sulfaquinoxaline. Regression analysis of the data, obtained by running five replicates of the derivatized standard working solutions, showed the response to be linear in the range examined for each sulfonamide ($y=0.8+213.9x$, $r=0.9997$ for sulfadiazine;

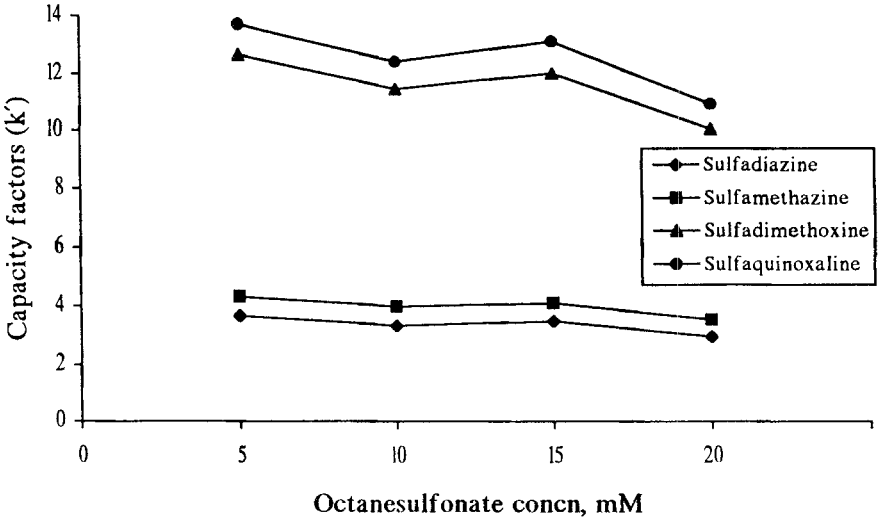


Figure 4. Capacity factors (k') of fluorescamine derivatives of sulfonamides versus concentration of octanesulfonate in the mobile phase. Conditions: mobile phase, acetonitrile-0.02 M phosphate buffer, pH 4, (34:66, v/v) in presence of octanesulfonate; other LC conditions as in Fig. 2.

y=0.7+158.0x, r=0.9998 for sulfamethazine; y=1.7+55.9x, r=0.9989 for sulfadimethoxine; y=0.8+15.1x, r=0.9995 for sulfaquinoxaline, where *y* represents peak height in mm and *x* the sulfonamide quantity in ng, relative to the fluorescamine derivative injected). In standard solutions, with a 20-μL injection volume, 2.4, 3.4, 8.0 and 33.5 ng/mL were the lowest concentrations that could be detected. These correspond to residue concentrations of as low as 3 ng/g for sulfadiazine, 4 ng/g for sulfamethazine, 9 ng/g for sulfadimethoxine, and 40 ng/g for sulfaquinoxaline, which could be readily determined in chicken tissue (peak to noise ratio, 3/1) due to absence of any interfering peaks in sample chromatograms (Fig. 5). The detection limits achieved are much better than those reported by other workers, but cannot reach those (0.003-0.006 ng/g) stated by Takeda and Akiyama[13] which, however, have been incorrectly calculated.

Precision and Accuracy

To evaluate the accuracy and the precision of the method, series of 3-g tissue samples were spiked with standard sulfonamides (50 μL of 0.3-18 μg/mL spiking solutions) at 5 fortification levels ranging from 5 to 100 ng/g for

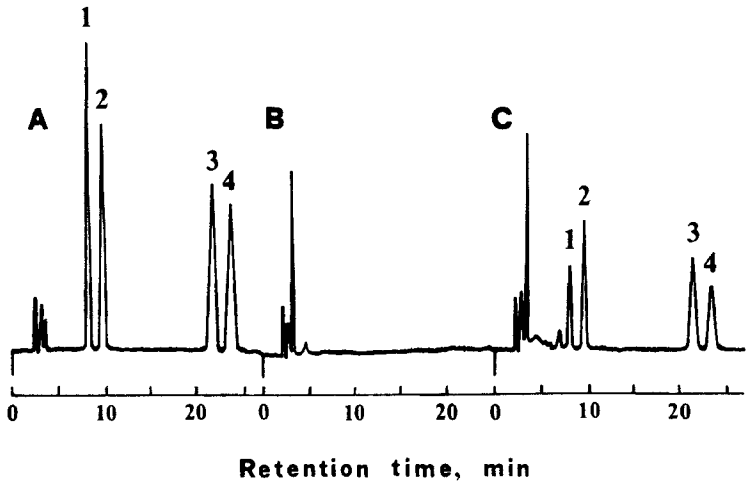


Figure 5. Typical chromatograms of (A) standard solution containing 30 ng/mL sulfadiazine (1) and sulfamethazine (2), 50 ng/mL sulfadimethoxine (3), and 200 ng/mL sulfaquinoxaline (4), relative to the fluorescamine derivative injected, (B) a blank muscle tissue sample, (C) muscle tissue sample fortified with 10 ppb of sulfadiazine, 20 ppb of sulfamethazine, 30 ppb of sulfadimethoxine, and 100 ppb of sulfaquinoxaline. LC conditions: mobile phase, acetonitrile-0.02 M phosphate buffer, pH 4, (34:66, v/v) containing 20 mM octanesulfonate sodium salt; column, 250x4.6 mm, C_{18} 5 μm; temperature, 30 °C; flow rate, 1.2 mL/min; wavelength, ex 405 nm em 495 nm; recorder sensitivity, 0.02 RFU; chart speed, 2 mm/min; injection volume, 20 μL.

sulfadiazine and sulfamethazine, 15 to 150 ng/g for sulfadimethoxine, and 50 to 300 ng/g for sulfaquinoxaline. Five replicates were analyzed at each fortification level. Least-squares and regression analysis of the data presented in Tables 2 and 3, showed that the relationship between "added" and "found" was adequately described by a linear regression for each of the four sulfonamides tested, ($y=0.32+0.777x$, $r=0.9956$ for sulfadiazine; $y=1.50+0.846x$, $r=0.9966$ for sulfamethazine; $y=1.49+0.825x$, $r=0.9913$ for sulfadimethoxine; $y=0.28+0.923x$, $r=0.9958$ for sulfaquinoxaline).

Therefore, the slopes of these regression lines could be used as estimates of the overall recovery for sulfadiazine (77.7±4.8%), sulfamethazine (84.6±4.2%), sulfadimethoxine (92.3±4.1%), and sulfaquinoxaline (82.5±7.0%) determination in chicken muscle.

Table 2

Precision and Accuracy Data for the Determination of Sulfadiazine and Sulfamethazine Residues in Chicken Muscle

	Sulfadiazine		Sulfamethazine	
Sulfonamide Added, ppb	Mean Concn.[a] Found, ppb	Mean Rec.[b], %	Mean Concn.[a] Found, ppb	Mean Rec.[b], %
5.0	4.5 ± 0.31	89.5 (6.9)	4.8 ± 0.05	96.7 (1.0)
15.0	11.7 ± 0.29	77.8 (2.5)	13.4 ± 0.71	89.3 (5.3)
35.0	27.6 ± 0.68	78.9 (2.5)	33.1 ± 1.07	94.6 (3.2)
70.0	54.4 ± 2.26	77.7 (4.2)	54.8 ± 2.24	85.4 (3.7)
100.0	78.4 ± 5.39	78.4 (6.9)	86.0 ± 3.83	86.0 (4.4)

[a] Mean of 5 replicates ± SD.
[b] Values in parenthesis represent RSD %.

Table 3

Precision and Accuracy Data for the Determination of Sulfadimethoxine and Sulfaquinoxaline Residues in Chicken Muscle

	Sulfadimethoxine		Sulfaquinoxaline	
Sulfonamide Added, ppb	Mean Concn.[a] Found, ppb	Mean Rec.[b], %	Mean Concn.[a] Found, ppb	Mean Rec.[b], %
15.0	13.2 ± 0.86	87.9 (6.9)		
30.0	27.8 ± 0.86	92.5 (3.1)		
50.0			37.9 ± 1.83	75.8 (4.8)
70.0	66.0 ± 1.98	94.3 (3.0)		
100.0	94.0 ± 5.13	94.0 (5.5)	77.8 ± 6.02	77.8 (7.7)
150.0	137.3 ± 2.14	91.5 (5.2)	140.1 ± 1.46	93.4 (1.0)
200.0			165.7 ± 5.6	82.8 (3.4)
300.0			243.0 ± 9.72	81.0 (4.0)

[a] Mean of 5 replicates ± SD.
[b] Values in parenthesis represent RSD%.

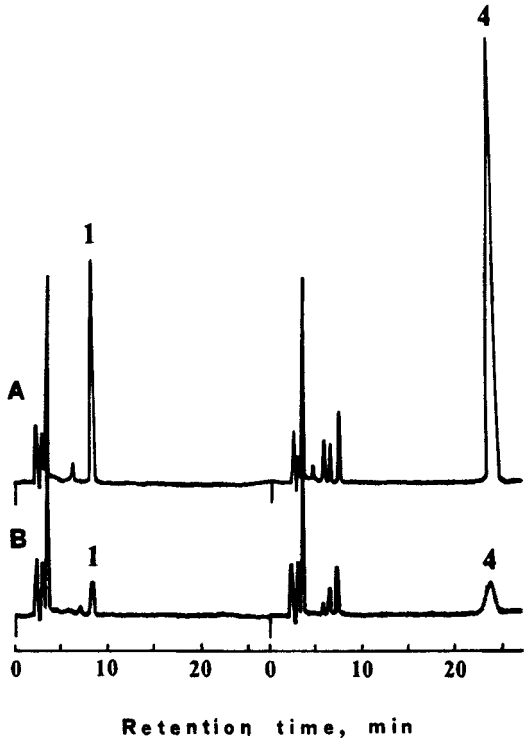


Figure 6. Chromatograms of muscle tissue samples from sulfadiazine (1) or sulfaquinoxaline (4) medicated broilers 48 h (A) and 96 h (B) after drug withdrawal; LC conditions as in Fig. 5.

Application

To validate the method with real samples, a trial was undertaken to quantitate residues in muscle tissue of four broilers, each two administered with water sulfadiazine at 80 mg/L and sulfaquinoxaline at 45 mg/L for 5 days and 3 days, respectively. Analysis data showed that both compounds could be detected in muscle tissue, 48 h (26.9 ng/g for sulfadiazine; 733.9 ng/g for sulfaquinoxaline) and 96 h (3.8 ng/g for sulfadiazine; 53.9 ng/g for sulfaquinoxaline), after drug withdrawal (Fig. 6). Extractable metabolites could be seen in muscle tissue from sulfaquinoxaline-medicated broilers; their chromatographic behavior indicated a substantial increase in polarity over the parent compound. When the method was further applied to liver and kidney samples, significant residue levels could also be found (Fig. 7).

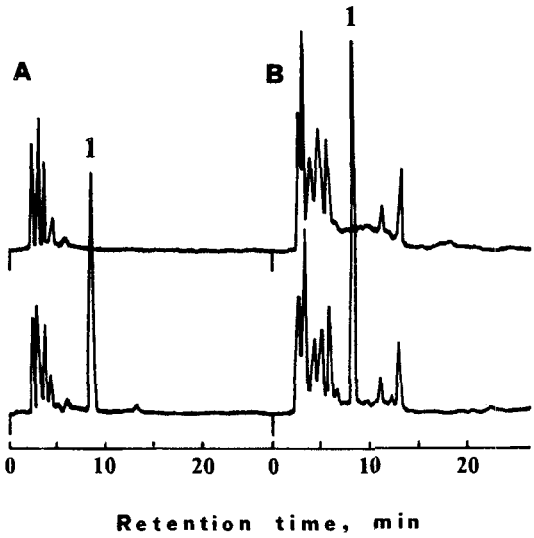


Figure 7. Chromatograms of kidney (A) and liver (B) samples from sulfadiazine (1) medicated or not broilers; LC conditions as in Fig. 5.

CONCLUSION

The results of the present study suggest that pre-column derivatization with fluorescamine may be an efficient and reliable means for enhancing the sensitivity and selectivity of the detection of multiple sulfonamide residues in chicken tissues.

The precision of the measurement is not affected due to the pre-column derivatization, whereas, there are considerable savings in terms of time-, labor- and material-requirements, compared to classical procedures. Considering that the derivatization step is easily amenable to automation through use of suitable LC autosamplers, the method may be proved in the future particularly useful for regulatory purposes.

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REFERENCES

1. K. Takatsuki, T. Kikuchi, J. Assoc. Off. Anal. Chem., **73**, 886-892 (1990).

2. US Code Fed. Reg., Title 21, Sections 556.620-556.690 (1991).

3. Official Journal of the European Communities, No L 156, Brussels, p. 6 (1994).

4. M. Horie, K. Saito, Y. Hoshino, N. Nose, N. Hamada, H. Nakazawa, J. Food Hyg. Soc. Japan, **31**, 171 (1990).

5. N. Haagsma, C. Van De Water, J. Chromatogr., **333**, 256-261 (1985).

6. S. Horii, C. Momma, K. Miyahara, T. Maruyama, M. Matsumoto, J. Assoc. Off. Anal. Chem., **73**, 990-992 (1990).

7. A. R. Long, L. C. Hsieh, M. S. Malbrough, C. R. Short, S. A. Barker, J. Agric. Food Chem., **38**, 423-426 (1990).

8. Y. Ikai, H. Oka, N. Kawamura, J. Hayakawa, M. Yamada, K.-I. Harada, M. Suzuki, H. Nakazawa, J. Chromatogr., **541**, 393-400 (1991).

9. Y. S. Endoh, Y. Takahashi, M. Nishikawa, J. Liq. Chromatogr., **15**, 2091-2110 (1992).

10. L. V. Bui, J. Assoc. Off. Anal. Chem., **76**, 966-976 (1993).

11. M. M. L. Aerts, W. M. L. Beek, U. A. Th. Brinkman, J. Chromatogr., **435**, 97-112 (1988).

12. J. A. F. de Silva, N. Strojny, Anal. Chem., **47**, 714-718 (1975).

13. N. Takeda, Y. Akiyama, J. Chromatogr., **558**, 175-180 (1991).

14. C.-E. Tsai, F. Kondo, J. Liq. Chromatogr., **18**, 965-976 (1995).

15. H. Nakamura, Z. Tamura, Anal. Chem., **52**, 2087-2092 (1980).

16. S. Udenfriend, S. Stein, P. Bohlen, W. Dairman, W. Leimgruber, M. Weigele, Science, **178**, 871 (1972).

17. N. A. Botsoglou, A. B. Spais, Chromatographia, **33**, 174-176 (1992).

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