Determination of Sulfonamide Residues in Chicken Muscle by Agilent Bond Elut QuEChERS AOAC Kit and HPLC-FLD

Application Note

Food

Authors

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Abstract

An HPLC-Florescence detection (FLD) method has been developed and validated for the determination of nine sulfonamides in chicken muscle, after precolumn derivatization with fluorescamine. The analyzed drugs include sulfadiazine, sulfathiazole, sulfamerazine, sulfamethazine, sulfamethizole, sulfamethoxypyridazine, sulfachloropyridazine, sulfamethoxazole and sulfadimethoxine with sulfapyridine as an internal standard. The derivatives were separated on an Agilent ZORBAX Eclipse Plus C18 column (4.6 mm × 75 mm, 3.5 µm) using a gradient elution with a binary system of methanol–0.05 M acetate buffer (pH 4.5) and fluorescence detection at excitation and emission wavelengths of 406 and 496 nm, respectively. The method employs a multiresidue sample preparation procedure based on QuEChERS (quick, easy, cheap, effective, rugged and safe) which was adopted from the Association of Analytical Communities (AOAC) Official method 2007.01 for extraction and cleanup. The recoveries ranged from 76.8% to 95.2% with relative standard deviation from 1.5% to 4.7% at the 50, 100 and 150 ng/g fortification levels. The limits of detection and quantification ranged from 0.02 to 0.39 and 0.25 to 1.30 ng/g respectively.

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Introduction

Sulfonamides are a broad spectrum of antimicrobial drugs used mainly in veterinary practice for prophylactic, therapeutic or growth promoting purposes [1]. They are the treatment of choice for disease control of coccidiosis in poultry management [2-3]. Their use in human therapy has since become limited due to the advent of antibiotics [4]. There is a health risk associated with consumption of animal products contaminated with sulfonamide residues. The residues are usually the result of inappropriate administration or withdrawal from these drugs. The presence of sulfonamide residues can trigger adverse side effects such as allergic reactions in hypersensitive individuals and are potential carcinogens in the long term. Furthermore, prolonged exposure to sulfonamide residues may give rise to an increase in drug-resistant bacteria [5]. In order to protect consumers from risks related to the drug residues, maximum residue limits (MRL) have been established by law in many countries. In Europe (EU Regulation 1999), Canada and USA (FDA Regulation 1991) the MRL for the total sulfonamide concentration in edible tissue is 100 µg/kg while it is 20 µg/kg in Japan [6-7].

The AOAC QuEChERS method has been widely applied in the analysis of pesticides in food since it was introduced by USDA scientists [8]. In general, there are two major steps: extraction and dispersive SPE cleanup. The method uses a single-step buffered acetonitrile (1% HOAc) extraction while simultaneously salting out from the aqueous sample using anhydrous magnesium sulphate ($MgSO_4$) to induce liquid-liquid partitioning. For cleanup, a dispersive solid phase extraction (dSPE) step is employed using a combination of primary secondary amine (PSA) to remove fatty acids and other components, and anhydrous $MgSO_4$ to reduce the remaining water in the extract. Other sorbents may be added in this step, such as graphitized carbon black (GCB), to remove pigments and sterol, or C_{18} to remove more lipids and waxes.

This application note presents a method for the determination of sulfonamide drugs in chicken muscle with HPLC-FLD after a precolumn derivatization with fluorescamine, which is a fluorogenic reagent specific for primary aliphatic and aromatic amines [9–10] such as the sulfonamides in the study (Figure 1). The method includes sample preparation with Bond Elut AOAC Buffered Extraction kit (p/n 5982-5755) and Bond Elut AOAC Fatty Dispersive SPE 15 ml kit (p/n 5982-5158).

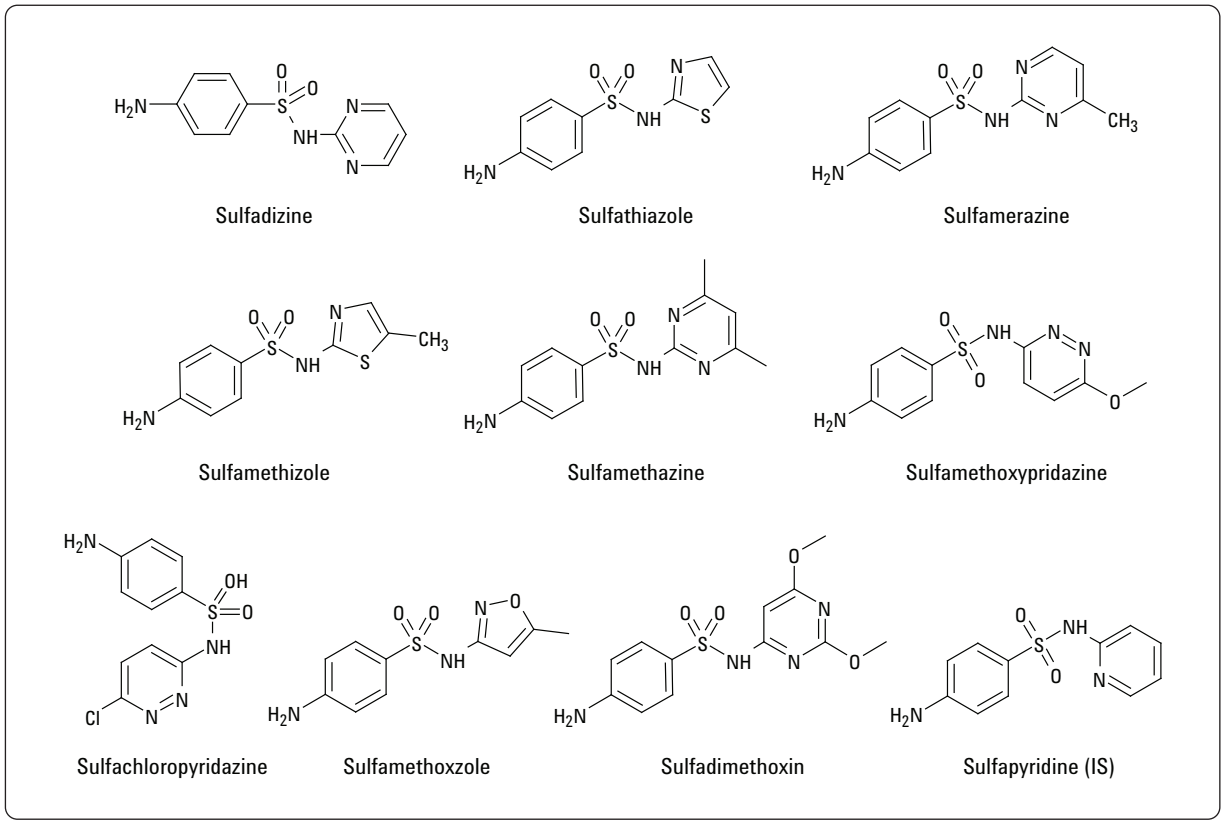

Figure 1. Chemical structures for the sulfonamide drugs used in the study.

Experimental

Reagents and Chemicals

All reagents were analytical or HPLC grade. Methanol (MeOH) was purchased from Merck KGaA (Darmstadt, Germany) while acetonitrile (ACN), acetone and glacial acetic acid (HOAc) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate (NaOAc) was from Saarchem Analytical (Krugersdorp, South Africa). Fluorescamine (98%) and sulfonamide drugs including the internal standard were purchased from Sigma-Aldrich (St. Louis, MO, USA). The water used was from a MilliQ system (Milford, Mass, USA).

Solutions and Standards

A stock solution of 0.05 M sodium acetate was prepared by dissolving 4.1 g NaOAc in 1.0 L of ultrapure water and filtered through a Whatman membrane filter (47 mm diameter and 2 µm pore size). The pH was adjusted using HOAc. Fluorescamine reagent (0.02%) was prepared by dissolving 20 mg Fluram in 10 mL of acetone. The solution was stored at 4 °C. A 1% HOAc in ACN solution was prepared by diluting 10 mL HOAc to 1.0 L with ACN.

Standard and internal standard primary stock solutions (1 mg/mL) were prepared in ACN and stored at –20 °C. From the primary stock solution, 10 µg/mL standard mixtures also in ACN were prepared for the calibration curves. All working solutions were prepared daily by serial dilution in 0.05 M NaOAc (pH 3.5). All the solution vials were wrapped with aluminium foil because some of the sulfonamide drugs are light-sensitive.

Equipment and Material

The analysis was performed on an Agilent 1200 Series HPLC (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a binary pump and a fluorescence detector (FLD) set at λ_{ex} = 405 nm and λ_{em} = 495 nm. Separation of the compounds was achieved on an Agilent ZORBAX Eclipse Plus C18 column (4.6 mm × 75 mm, 3.5 µm, p/n 959933-902). The data was processed by HPLC 2D Chemstation software.

Extraction and cleanup were carried out with an Agilent Bond Elut Buffered QuEChERS AOAC Extraction kit, p/n 5982-5755 and an Agilent Bond Elut QuEChERS AOAC Dispersive SPE kit, p/n 5982-5158, (Agilent Technologies).

A Jenway 3510 pH meter (Jenway, London, UK) monitored the pH of the solutions, and a Kenwood grinder (Kenwood, Grahamstown, South Africa) homogenized the chicken sample.

Instrument conditions

HPLC conditions

Table 1. HPLC Conditions Used for Separation and Analysis

Column:	Agilent ZORBAX Eclipse Plus C18 4.6 × 75 mm, 3.5 µm	
Flow rate:	1 mL/min	
Column temperature:	25 °C	
Injection volume:	5 µL	
Mobile phase:	A = 0.05 M Sodium Acetate pH 4.5 B = MeOH	
Gradient:		
	T (min)	% B
	0	35
	35	41
	50	55
Detection:	Ex = 405 nm	Em = 495 nm

Sample preparation

The chicken muscle was purchased from a local food store, minced and deep frozen until analysis.

Extraction

Figure 2 outlines the methodology used in the QuEChERS experiments. A 2-g portion of chicken muscle homogenate was placed into a 50-mL centrifuge tube from the Bond Elut QuEChERS AOAC Extraction kit. The tube was centrifuged for 20 s. Samples were then spiked with appropriate spiking solutions to yield 50, 100, and 150 ng/g sample concentrations for recoveries and reproducibility studies. A 100-µL IS spiking solution was added to all the samples except the blank. After shaking vigorously for 1 min, 8 mL Milli-Q water was added followed by further shaking for 30 s. Next, 10 mL 1% HOAc in ACN was added followed by the Agilent Bond Elut QuEChERS AOAC Extraction salt packet (p/n 5982-5755). The packet contained 6 g of anhydrous $MgSO_4$ and 1.5 g of anhydrous NaOAc. The sample tubes were hand shaken vigorously for 1 min then further centrifuged at 4000 rpm for 5 min.

Dispersive SPE cleanup

A 6-ml aliquot of the upper ACN layer was transferred into a Bond Elut QuEChERS AOAC Dispersive SPE 15 mL tube. This SPE tube contained 400 mg of PSA, 400 mg of C18EC, and 1200 mg of anhydrous $MgSO_4$. The tubes were then centrifuged at 4000 rpm for 5 min. Next, 4 mL of the extract was transferred to a test tube and dried with N_2 gas at 35 °C. Samples (200 µL) were reconstituted into 600 µL of 0.05 M NaOAc (pH 3.5).

Derivatization

Aliquots of 400 µL working standard mixtures of sulfonamides, dissolved in 0.05 M acetate buffer (pH 3.4), were filtered through a 0.45 µm PVDF syringe filter then transferred

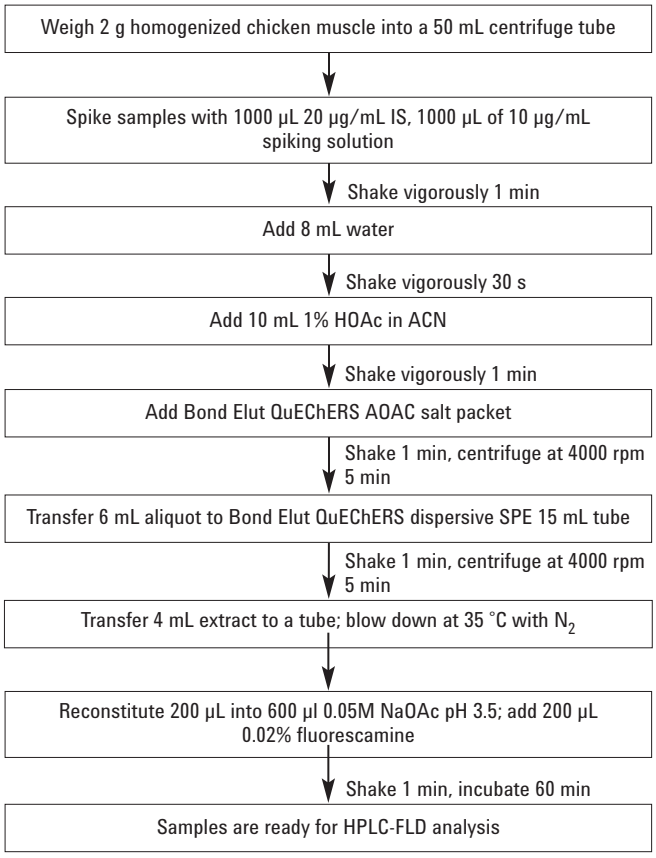

Figure 2. Flow chart for the QuEChERS AOAC sample preparation procedure.

to reaction vials. A 200 µL 0.02% w/v amount of fluorescamine solution in acetone was added. The mixtures were shaken for 1 min and the reaction left to proceed for 60 min at ambient temperature. Aliquots of 10 µL of the derivatized solutions were directly injected into the liquid chromatograph.

Results and Discussion

Derivatization of sulfonamide drugs

Fluorescamine is a fluorogenic reagent specific for primary aliphatic and aromatic amines that produce fluorophors of a high fluorescence yield [9]. This reagent and its hydrolysis products do not fluoresce, which eliminates the extensive cleanup step. Fluorescamine was therefore used in this application note to derivatize sulfonamides in the precolumn mode. The results indicated that the reaction time is the most important factor. The reaction was complete within 60 – 100 min and for reproducibility 60 min was the chosen time. The derivatised sulfonamides were detected with a single pair of wavelengths, λ_{ex} = 405 nm and λ_{em} = 495 nm.

Chromatographic results

The chromatogram of the standard mixture of these sulfonamide derivatives is shown in Figure 3. Figure 4 is the chromatogram for the blank chicken muscle extract, and Figure 5 is that of the spiked chicken muscle. All chromatograms of standards, blanks, and spiked extracts were run using the conditions outlined in Table I.

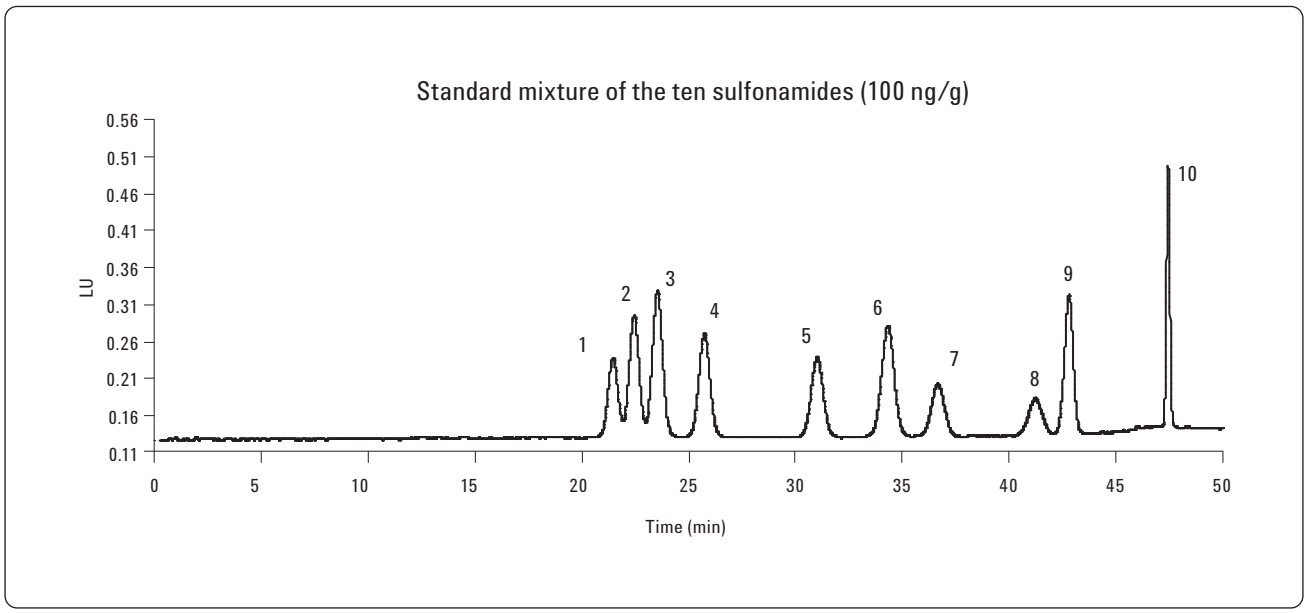

Figure 3. Chromatogram of the standard mixture of the sulfonamides (100 ng/g): 1. Sulfadiazine; 2. Sulfathiazole; 3. Sulfapyridine (IS); 4. Sulfamerazine; 5. Sulfamethazine; 6. Sulfamethizole; 7. Sulfamethoxypyridazine; 8. Sulfachloropyridazine; 9. Sulfamethoxazole; 10. Sulfadimethoxine.

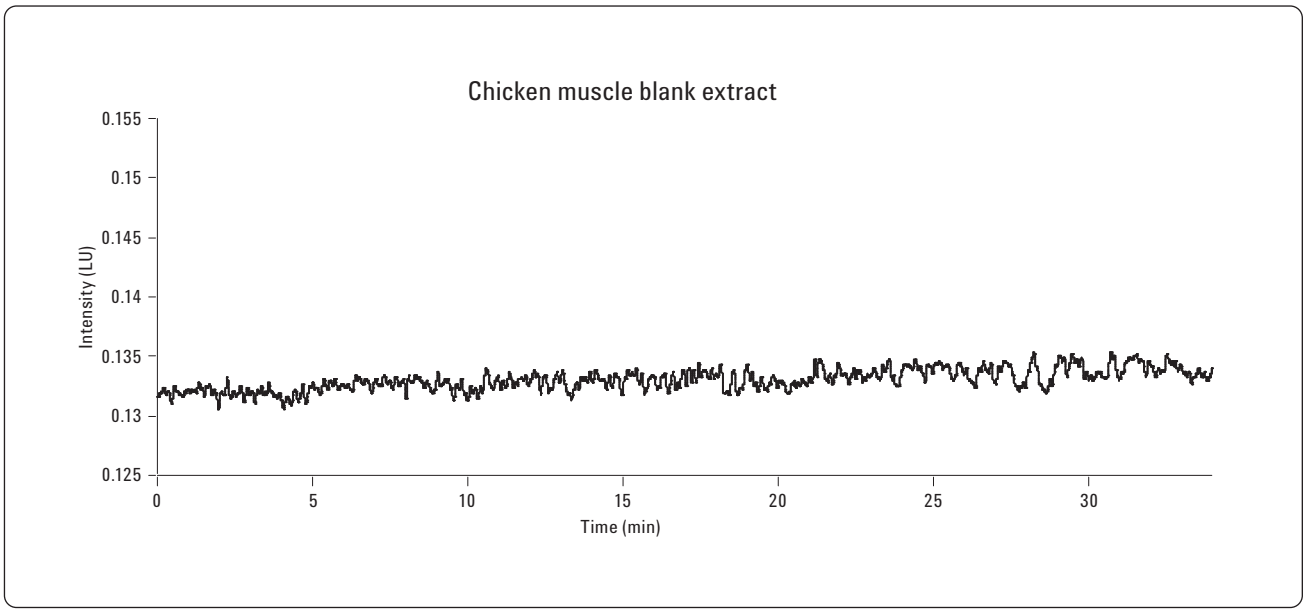

Figure 4. Chromatogram of the blank chicken muscle extract.

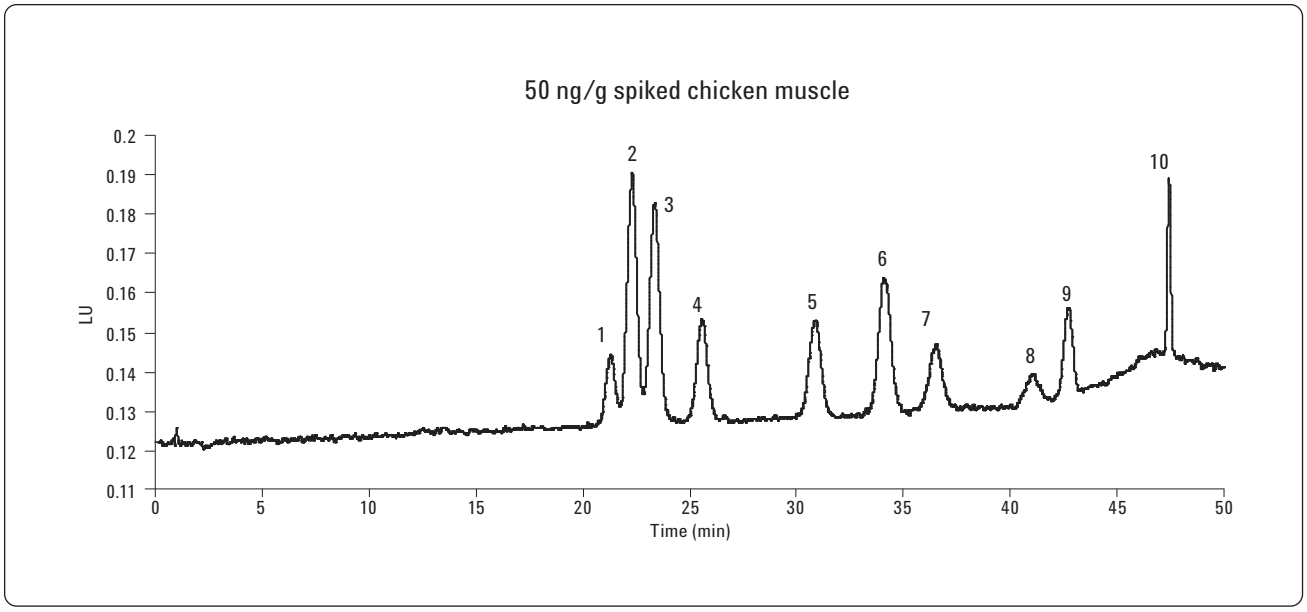

Figure 5. Chromatogram of the spiked chicken muscle extract at 50 ng/g level: 1. Sulfadiazine ; 2. Sulfathiazole; 3. Sulfapyridine (IS); 4. Sulfamerazine; 5. Sulfamethazine; 6. Sulfamethizole; 7. Sulfamethoxypyridazine; 8. Sulfachloropyridazine; 9. Sulfamethoxazole; 10. Sulfadimethoxine.

Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ)

Linearity

The linear calibration curves were obtained by plotting the relative responses of analytes (peak area of analyte/peak area of IS) verses the relative concentration of analytes (concentration of analyte/concentration of IS). They were generated by spiking the sample blanks at levels of 10, 50, 100, 150, 200, 300 and 400 ng/g.

Limits of Detection and Quantification

The limits of detection and quantification were estimated from the concentration of sulfonamides required to give signal-to-noise ratios of 3 and 10 respectively. Table 2 shows the regression equation, correlation coefficients, and very acceptable limits of detection and quantification.

Recovery and Reproducibility

The recovery and reproducibility (RSD) were evaluated on spiked samples at MRL (100 µg/kg), half MRL (50 µg/kg) and one and a half times the MRL (150 µg/kg). The analysis was performed in replicates of six (n = 6) at each level. Table 3 shows the recoveries and RSD values for the nine sulfonamides.

Table 2. Linearity, LOD and LOQ for the Nine Sulfonamides

Sulfonamide	Regression equation	R^2	LOD ng/g	LOQ ng/g
Sulfadiazine	Y = 0.4154x + 0.0112	0.9995	0.26	0.87
Sulfathiozole	Y = 1.0231x – 0.0757	0.9991	0.02	0.27
Sulfamerazine	Y = 0.6735x + 0.0184	0.9993	0.14	0.46
Sulfamethazine	Y = 0.6735x + 0.0042	0.9996	0.08	0.26
Sulfamethizole	Y = 0.9751x + 0.0115	0.9995	0.30	1.00
Sulfamethoxypyridine	Y = 0.4713x – 0.0069	0.9994	0.24	0.80
Sulfachloropyridazine	Y = 0. 2769x + 0.0190	0.9992	0.33	1.10
Sulfamethoxazole	Y = 0.6996x + 0.0421	0.9991	0.39	1.30
Sulfadimethoxine	Y = 0.5008x + 0.0329	0.9991	0.08	0.25

Table 3. Recovery and Repeatability for Sulfonamides in Spiked Chicken Muscle (n = 6)

Sulfonamide	Level of spiking (ng/g)					
	50		100		150	
	%Recovery	%RSD	%Recovery	%RSD	%Recovery	%RSD
Sulfadiazine	77.8	2.6	78.1	2.1	78	1.9
Sulfathiazole	83.0	3.9	88.2	2.4	85.2	2.2
Sulfamerazine	85.7	2.9	85.5	3.1	88.4	1.6
Sulfamethazine	80.3	3.1	81.3	2.2	80.3	1.5
Sulfamethizole	95.2	4.1	89.5	2.6	87.2	2.5
Sulfamethoxypyridazine	91.4	2.3	90.7	2.1	90.5	1.7
Sulfachloropyridazine	78.1	3.0	89.4	2.6	80.7	2.2
Sulfamethoxazole	76.8	3.5	87.2	2.5	89.1	1.5
Sulfadimethoxine	90.3	4.7	92.8	2.2	89.2	2.0

Conclusions

A simple and fast mulitiresidue method based on Bond Elut QuEChERS AOAC Method 2007.01 and HPLC-FLD with pre-column derivatization has been developed for the simultaneous determination of nine sulfonamide residues in spiked chicken muscle. The recoveries were good with excellent RSD and the LOQs were well below the MRL in animal food products. This method can therefore be recommended for residue control purposes.

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