Determination of Sulfonamides in Milk Using Solid-Phase Extraction and Liquid Chromatography-Tandem Mass Spectrometry

Application Note

Pharmaceuticals

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Abstract

The extraction of trace levels of nine nitrogen-containing sulfa drugs (sulfamethoxazole, sulfadiazine, sulfathiazole, sulfamerazine, sulfamethizole, sulfamethazine, sulfamethoxypyridazine, sulfachloropyridazine, and sulfadimethoxine) in milk samples by solid-phase extraction was studied using Agilent SampliQ polymeric strong cation exchange (SCX) cartridges. An Agilent 6410 triple quadrupole LC/MS-MS System was used for the separation and determination of the sulfa drugs. For reversed-phase chromatography, an Agilent ZORBAX Eclipse Plus column (C18, 3.0 mm × 50 mm, 1.8 µm) with a 0.1% formic acid/acetonitrile gradient was used. Overall recoveries from the milk samples ranged from 73 to 99%, with %RSD values less than 10%. Limits of detection ranged from 0.2 to 2.0 ng/mL in milk (S/N = 3) depending on the sulfa drug, below the U.S. Food and Drug Administration acceptable levels in milk.

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Introduction

Since the discovery of the effective antimicrobial properties of sulfonamides in the early 1900s, they have been used to treat a variety of diseases. In the dairy farming industry, sulfa drugs are administered to dairy cattle to prevent infection. This leads to the possibility of the drugs being excreted in the milk and passed on to the consumer. This ingestion can cause a drug resistance, making the drugs ineffective in later uses to treat illness [1,2].

Sulfonamides, commonly known as sulfa drugs, have proven to be effective antimicrobial agents since their discovery in 1929 by Gerhard Domagk. Today, β-lactam antibiotics are much more commonly used to prevent infection than sulfonamides, but sulfonamides are still routinely used in different parts of the world due to their low cost. Over the years, many microorganisms have become resistant to these compounds. Of growing concern are drug-resistant bacteria that may be passed from animals to humans. One major cause of the resistance to these compounds is that feed animals are being fed antimicrobial drugs at low levels to treat diseases. In the 1990s, the United States Food and Drug Administration (FDA) began conducting tests on several milk supplies [3]. If dairy cattle were given sulfa drugs, low levels of these compounds could be found in the milk, leading to allergic reactions in some consumers, as well as an increase in drug-resistant organisms. This application demonstrates a complete solution to the analysis in milk of nine important sulfa drugs: sulfamethoxazole, sulfadiazine, sulfathiazole, sulfamerazine, sulfamethizole, sulfamethazine, sulfamethoxypyridazine, sulfachloropyridazine, and sulfadimethoxine (see Figure 1 for structures and chemical properties).

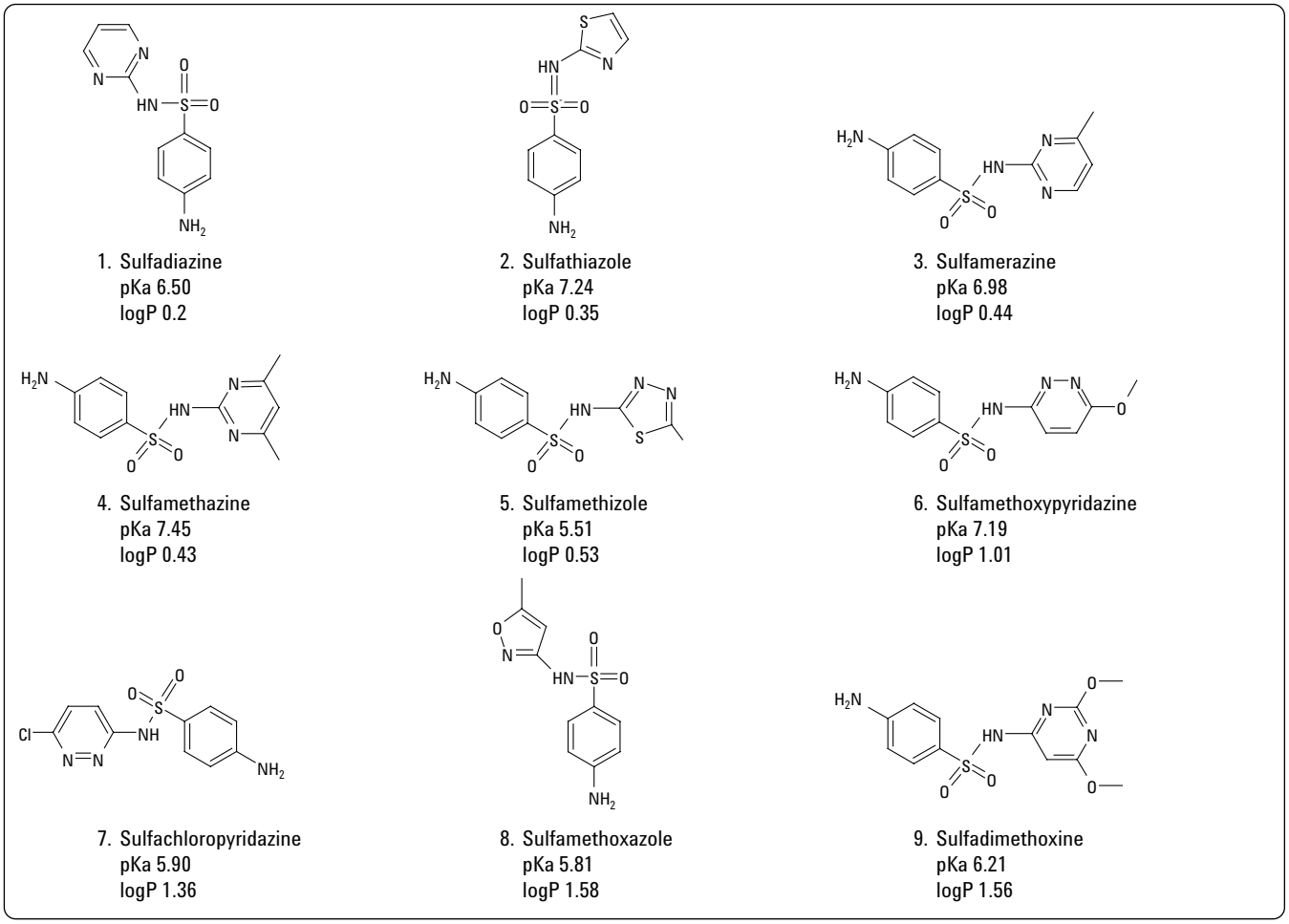

Figure 1. Structures and chemical constants for sulfa drugs used in this study.

Experimental

Materials and Chemicals

Water (EMD Chemicals, Gibbstown, NJ), acetonitrile, and methanol (Burdick and Jackson, Muskegan, MI) were HPLC grade. Sulfa drugs were analytical grade and purchased from Sigma-Aldrich (Saint Louis, MO). The stock solution (~1.19 mg/mL) was prepared in 25 mL of methanol and kept refrigerated for up to 14 days. Working solutions were made daily by dilution of the stock solution in water.

The SPE cartridges were Agilent SampliQ SCX, 3 mL/60 mg p/n 5982-3236), a polymeric cation exchanger with a 30-µm average particle size. The analysis was performed on an Agilent 1200 Series HPLC coupled to a 6410 triple quadrupole mass spectrometer with electrospray ion source. The analytical column was an Agilent ZORBAX Eclipse Plus C18, 3.0 mm × 50 mm, 1.8 µm (p/n 959941-302). Formic acid was purchased from J.T. Baker (Phillipsburg, NJ) (Baker PCS reagent, 90%) for use in mobile phase preparation and for precipitation of proteins and lipids in the milk.

Sample Preparation

20 µL of a 45% solution of formic acid in water (prepared by mixing 10 mL of 90% formic acid with 10 mL of water) solution was added to each 1 mL of whole milk to precipitate proteins and lipids. The milk samples were then centrifuged at 8000 rpm for 10 minutes (Eppendorf 5810R 15 amp, Westbury, NY). Alternatively, the samples may be centrifuged at 3500 rpm for 20 minutes. An aliquot of the supernatant (prepared whole milk extract) was removed and used to load onto SampliQ SCX cartridges.

SPE Purification

After the SPE cartridge was conditioned and equilibrated as described in Figure 2, 5 mL of prepared whole milk extract was loaded onto the column. Care was taken that the flow rate during the load step did not exceed 1.5 mL/min. During both drying steps, the cartridge was dried under vacuum at 15 in Hg for the time indicated. The eluate was dried under nitrogen and then reconstituted in 1 mL of solvent (9:1 water:methanol). The samples were then sonicated (Branson 1200, Danbury, CT) for 5 minutes and analyzed using the Agilent 6410 Triple Quad LC/MS-MS system.

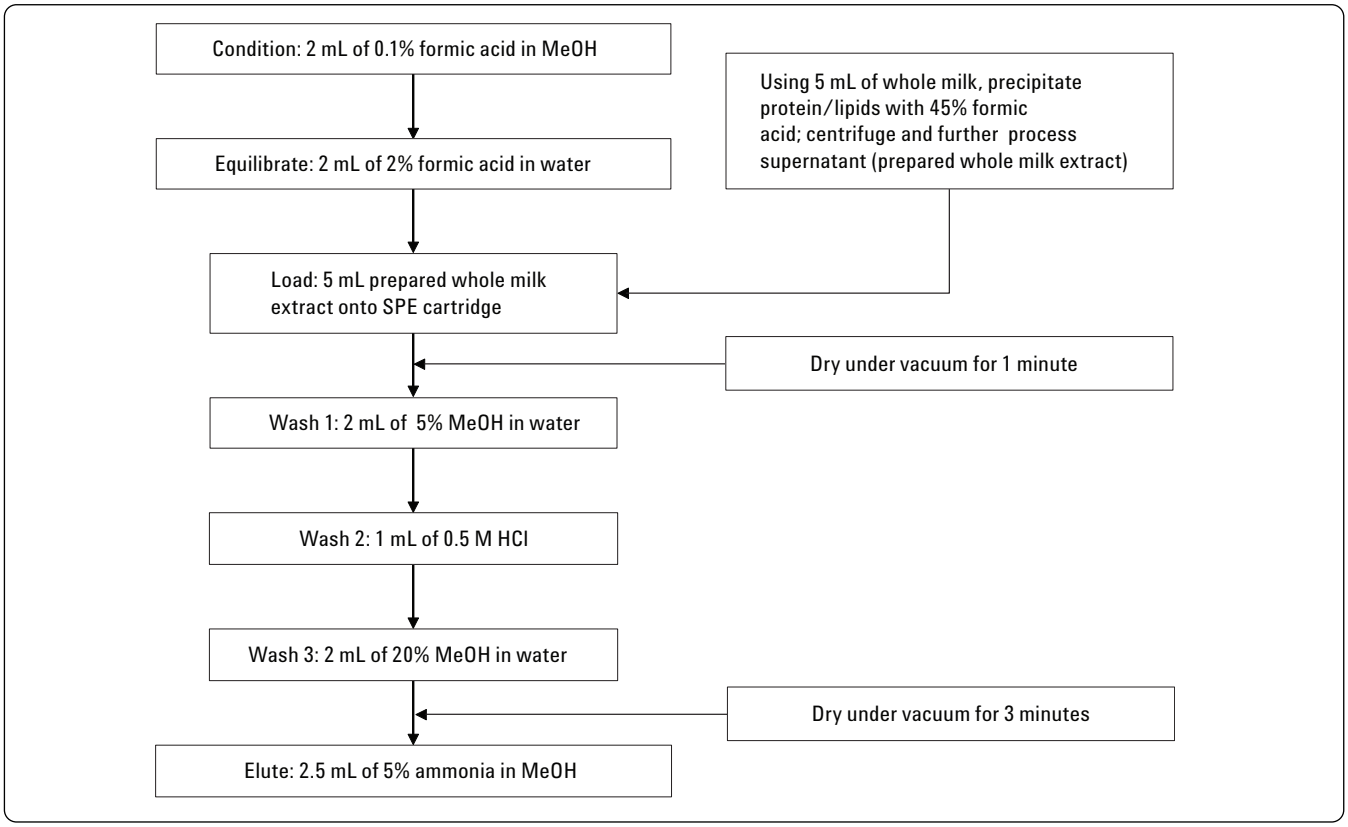

Figure 2. SPE procedure.

Due to the low concentrations of sulfa drugs being analyzed, and the very low LOD that can be achieved with Agilent 6410 Triple Quad LC/MS-MS system, extra care must be taken to keep the SPE manifold system clean to prevent contamination of samples. The manifold system must be thoroughly cleaned between uses or, if the option is available, needle inserts may be newly installed.

Separation and Analysis

The chromatographic and MS/MS experimental setup is shown in Tables 1, 2, and 3.

Table 1. HPLC Setup

Column	Agilent ZORBAX Eclipse Plus C18, 3.0 × 50 mm, 1.8 µm (p/n 959941-302)			
Flow rate	0.42 mL/min			
Column temperature	35 °C			
Injection volume	1.7 µL w/ needle wash; wash for 30 s in flush port with MeOH/H_2O (5:1)			
Mobile phase	A: H_2O/acetonitrile (9:1) w/ 0.1% formic acid B: Acetonitrile w/ 0.1% formic acid			
Run time	8 min			
Post time	3 min			
Gradient	Time	0	3.5	8
	%B	0	0	65

Table 2. MS/MS Conditions

T_R (min)	Compound	Precursor ion	Product ion
1.69	Sulfadiazine	251.2	156.0 108.0
1.93	Sulfathiazole	256.1	156.0 108.0
2.41	Sulfamerazine	265.2	156.0 108.0
3.44	Sulfamethazine	279.2	186.1 124.1
3.89	Sulfamethizole	271.1	156.0 108.0
4.10	Sulfamethoxypyridazine	281.2	156.0 108.0
5.99	Sulfachloropyridazine	285.1	156.0 108.1
6.42	Sulfamethoxazole	254.2	156.1 108.1
7.17	Sulfadimethoxine	311.2	156.0 108.0

Table 3. Conditions for Electrospray Ionization Source

Gas temperature	350 °C
Gas flow	12 L/min
Nebulizer	40 psi
Capillary	4000 V

Results and Discussion

Linearity and Limits of Detection

Solutions used to create external calibration curves were prepared by using a stock solution to spike matrix blanks. Matrix blanks were created by taking the milk through the entire procedure, including the precipitation, centrifugation, and SPE procedures. The results for the calibration curves are summarized in Table 4. The regression results were used to calculate the recoveries. The limits of detection were chosen as the concentration of each drug that gave a signal-to-noise (S/N) ratio greater than 3:1. The limits of detection are given in Table 4.

Table 4. Calibration Curve Regression Analysis for Sulfa Drugs

Compound	Regression equation	R^2	LOD in milk (ng/mL)
Sulfadiazine	y = 282.62x + 225.59	1.0000	0.4
Sulfathiazole	y = 440.38x + 246.43	0.9996	1.0
Sulfamerazine	y = 358.34x + 485.54	0.9998	1.0
Sulfamethazine	y = 539.09x + 576.81	0.9989	1.0
Sulfamethizole	y = 499.57x + 333.03	0.9994	2.0
Sulfamethoxypyridazine	y = 494.61x + 139.66	0.9970	1.0
Sulfachloropyridazine	y = 343.78x + 92.808	0.9999	2.0
Sulfamethoxazole	y = 260.05x – 351.97	0.9901	2.0
Sulfadimethoxine	y = 956.97x + 1420.9	0.9973	0.2

Recovery and Reproducibility

The recoveries and precision for the method were determined at two levels, milk spiked to a concentration of 5 ng/mL and 10 ng/mL. Since the procedure for the SPE used 5 mL of prepared whole milk extract to load the SPE cartridge and the extract was reconstituted in 1 mL of mobile phase, the sample analyzed is five times as concentrated as the milk samples. The analyzed solutions are therefore 25 ng/mL for the samples that were spiked with 5 ng/mL in milk, and 50 ng/mL for the samples that were spiked with 10 ng/mL. The analysis was performed with five replicates at each level. The recovery and reproducibility data are shown in Table 5. The chromatograms for the blank and spiked milk extracts (5 ng/mL) are shown in Figure 3.

Table 5. Recovery and Precision Data for Nine Sulfa Drugs Used in This Study

Compound	Level spiked in milk (ng/mL)	Recovery	RSD (%)
Sulfadiazine	5	74.2	8.3
	10	99.7	5.7
Sulfathiazole	5	76.8	4.4
	10	83.2	4.7
Sulfamerazine	5	73.2	6.3
	10	84.8	0.6
Sulfamethazine	5	78.3	7.5
	10	89.0	3.1
Sulfamethizole	5	78.4	7.0
	10	94.5	5.3
Sulfamethoxypyridazine	5	76.3	6.2
	10	86.9	2.2
Sulfachloropyridazine	5	78.3	9.4
	10	84.3	6.0
Sulfamethoxazole	5	74.0	4.3
	10	87.7	6.4
Sulfadimethoxine	5	75.4	3.1
	10	82.5	5.4

Conclusions

The results of this study show that Agilent SampliQ SCX cartridges can be used as an effective method of purification and enrichment of multiple sulfonamides in complex samples such as whole milk. The impurities remaining after the SPE cleanup step were minimal and did not interfere with the quantitation of the sulfonamides. The levels at which the quantitation was performed are below the levels of sulfonamides that are considered by the FDA as safe in milk for consumption (10 ng/mL). The LOD for the method was also well below these levels (3 ng/mL in milk).

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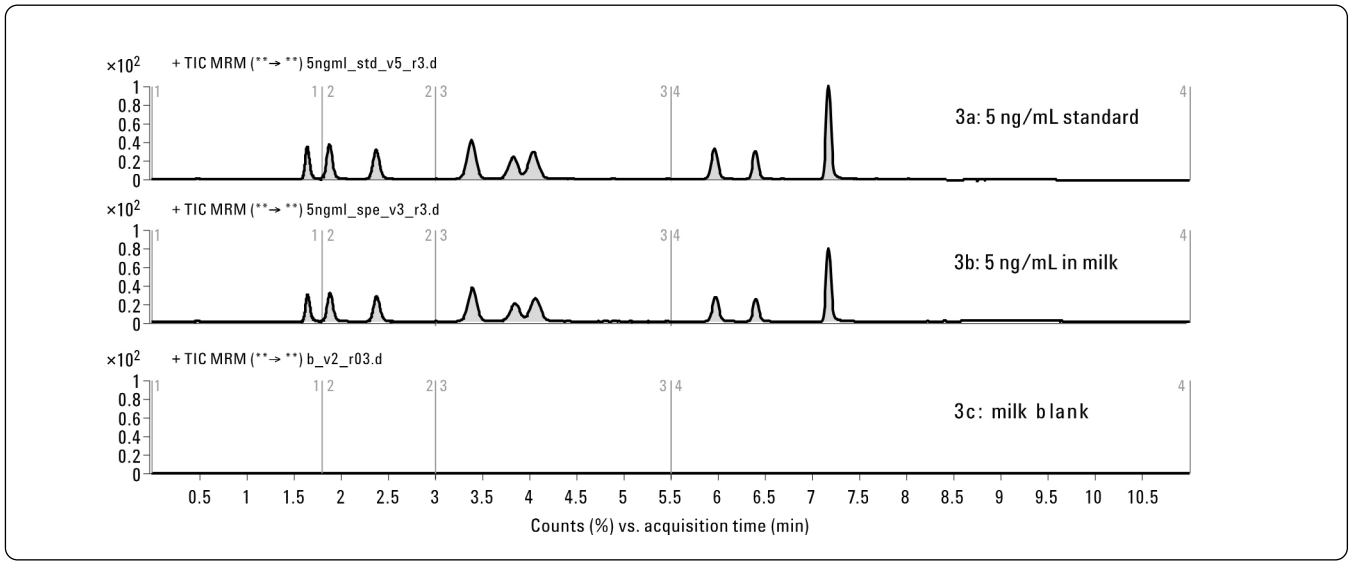

Figure 3. Total ion chromatograms of (3a) milk taken through extraction and cleanup, then spiked with sulfa drugs; (3b) milk spiked at 5 ng/mL, then taken through extraction and SPE cleanup; and (3c) milk blank.

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