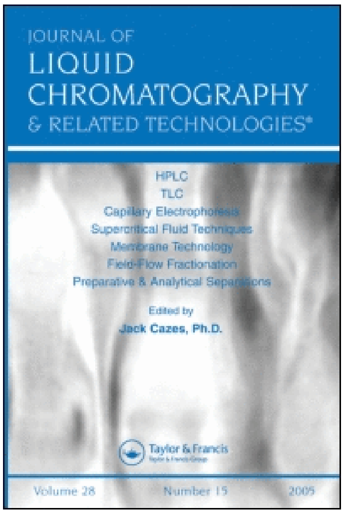

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ABSTRACT

A simple clean up method for sulfonamides in milk, using a solvent extraction and unprecedented solid phase extraction (SPE) was studied. The effects of solid phase's type, solvent's type, and sample's pH on the extraction of sulfonamids were compared. Sample extracts were simply cleaned up and enriched by SPE on LiChrolut EN (ethylvinylbenzene–divinylbenzene-copolymer). Liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry (LC/APCI-MS) was used for the determination of sulfonamides. Milk samples, spiked with 8.2 ng/mL of

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sulfamethazine (SMZ), were assayed with a relative standard deviation (RSD) of 2.8% using isotope labeled internal standards.

Key Words: Sulfonamides; Milk; SPE; LC/MS; APCI.

INTRODUCTION

Sulfonamides are widely used as veterinary drugs for the treatment of infections and the promotion of growth of livestock and fish.[1–5]

Sulfonamide residues in food are an important concern, due to the possibility of risk to human health, such as resistance development, and toxicity.[5,6] Many countries, including Korea, have established maximum allowable residue limits (MRLs) of 100 ng/g for most sulfonamides in edible animal tissues, and 10 ng/mL in milk.[1–4,7]

For the extraction of sulfonamides residues in milk, acetonitrile,[8] chloroform–acetone,[9–11] ethanol,[12] and ethyl acetate[13] were used. As a clean up or enrichment method for sulfonamides in milk, precipitation,[8,14,15] ultrafiltration,[12,16] liquid–liquid extraction (LLE),[9–11,13,14,17] and solid phase extraction (SPE)-ion exchange,[18] C_{18},[17,19] cychlodextrin bonded phase,[18,19] alumina,[18] and cyclohexyl[13] were used.

For the determination of sulfonamides in milk, GC/MS,[13] HPLC/UV,[9–12,15,18] HPLC/fluorescence detection,[8] and LC/MS[14,16,17,19] were used. GC/MS and HPLC/fluorescence detection need derivatization, but LC/MS does not need derivatization.

Most of the present methods for milk[13,17–19] have used multiple clean up procedures, even though selective LC/MS has been used.[17,19] On the other hand, highly specific detection, such as tandem mass spectrometry[14,16] or fluorimetry[8] have been needed for relatively simple clean up procedures.[8,14,16] Without such highly specific detection, only the samples that have a residue level (50–500 ng/mL) above the MRL (10 ng/mL) can be analyzed without severe interference with the results.[9,12,15] Furthermore, in spite of their importance, most of the previous reports did not show the effect of solvents and pH on extraction efficiency. Although M. Y. Haller et al. recently studied the effect of pH, the optimum pH for sulfonamide extraction from animal manure (pH 9) and deionized water (pH 5) were contrary to each other.[20]

In this work, a simple solvent extraction and unprecedented SPE clean up using LiChrolut EN is described for sulfonamide residues in milk at a concentration (8.2 ng/mL) under the MRL. The effects of solid phase type, solvent type, and sample pH on the extraction of sulfonamides were studied. Liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry (LC/APCI-MS) was used for the determination of sulfonamides.

EXPERIMENTAL

Chemicals and Materials

The sulfonamide standards were purchased from Sigma (sulfamethazine, SMZ; sulfadimethoxine, SDM; sulfathiazole, STZ; sulfadiazine, SDZ; sulfamethizole, SMTZ; Sulfapyridine, SP; St. Louis, MO). Isotope enriched SMZ was provided by Cambridge Isotope Laboratories (phenyl-$^{13}C_6$, atomic purity 90%, CLM-3045, $^{13}C_6$-SMZ, Inc., Andover, MA 01810-5413). Ammonium acetate (NH_4AC, 97%, Aldrich corp., St. Louis, MO), sodium phosphate monobasic (NaH_2PO_4, Merck, P.O. Box 4, Darmstadt, Germany), ammonia solution (30%, Junsei chemical Co., Japan), and hydrochloric acid (37%, Showa, Japan) were used as buffers for HPLC or sample. The organic solvents, acetonitrile, ethylacetate, and acetone were pesticide grade (Burdick & Jackson, Muskegon, MI).

LiChrolut EN (200 mg, Merck), C_{18} (500 mg, Waters, Milford, MA 01757), and aminopropyl silane (NH_2, 500 mg, Waters) SPE cartridges were used. A 0.2 μm syringe filter (4 mm Nylon, Whatman Inc., 9 Bridewell, NJ 07014) and a 300 μL vial insert (part No. 5181-1270, Agilent, 2850 Centerville Rd., Wilmington, DE 19808) for 2 mL autosampler vial were used.

Equipment

Reversed-phase liquid chromatographic experiments for the separation of the sulfonamides were performed on a chromatograph equipped with a HP 1050 autosampler and pump (Hewlett-Packard, Washington, DC). Phenomenex ODS2 (250 mm × 2.5 mm × 5 μm, Phenomenex, Torrance, CA 90501-1456) was used as a stationary phase. Samples were separated in an isocratic mode, and the eluents were acetonitrile : water : 0.1 M aqueous ammonium acetate solution (17 : 33 : 50 or 13 : 37 : 50, v/v).

The HPLC conditions were as follows: volume injected, 2 μL; column pressure, 600 psi; and flow rate, 200 μL/min.

A Finnigan LCQ iontrap LC/MS system (Finnigan, San Jose, CA), equipped with an APCI was used. The LC/MS system was operated at a positive-ion, full scan ($m/z = 200 \sim 350$) or a high resolution scan (Zoom Scan) mode. In Zoom Scan modes, the protonated positive ions of each sulfonamide ($[M + H]+$; SDZ, $m/z = 251$; STZ, 256; SMTZ, 271; SP, 250; SMZ, 279; $^{13}C_6$-SMZ, 285; SDM, 311) were scanned within a 10 amu window with a high-resolution, and the scan rages were programmed for the acquisition of different compounds. Atmospheric pressure chemical ionization conditions were: sheath gas (nitrogen) flow rate, 50 (an arbitrary value used in

LCQ); vaporizer temperature, 450°C; discharge voltage, 5.5 kV; tube lens offset voltage, 25 V; capillary temperature, 150°C; and, capillary voltage, 10 V.

For centrifugation, Allegra™ 6R benchtop centrifuge and GH-3.8 swinging-bucket rotor (Beckman Coulter, Fullerton, CA 92834310) were used. Turbo-vap evaporator (Zymark Corp., 68 Elm Street, Hopkinton, MA 01748-9987) and Rotavapor (Buchi, RE 120, CH-9230 Flawil, Switzerland) were used for evaporation of solvents. The pH of sample was measured by Piccolo Plus pH meter (Hanna Instruments, Inc., Woonsocket, RI 02895).

Extraction and Cleanup

The extraction efficienies of solvents (including sample pH) and solid phases were studied using 20 mL of water or milk samples spiked with sulfonamides at a concentration level of 100 ng/mL. On the other hand, for determination, 20 mL of milk sample spiked with sulfonamides at a concentration level of 8.5 ng/mL was used.

To examine the efficiency of SPE, LiChrolut EN, C_{18}, and NH_2 were used. Solid phase extraction cartridges were preconditioned with 15 mL of acetonitrile and water. Twenty milliliter of water sample was passed through SPE cartridges with a flow rate of 3 mL/min. Milk sample, 20 mL, was extracted with 20 mL of acetonitrile by agitating for 5 min, manually, without control of pH. This sample was centrifuged in 1990 *g* for 5 min; then upper organic layer was transferred to a 100 mL round flask and the volume of extract was reduced to 5 mL by rotary evaporation at 40°C. The concentrated organic extract of milk sample was converted to an aqueous medium by adding 100 mL of pure water, and passing through an SPE cartridge with a flow rate of 5 mL/min. After that, the SPE cartridges was washed with 2 mL of pure water, and enriched sulfonamides were eluted using 10 mL of acetonitrile (for LiChrolut EN and C_{18}) or 10 mL of acetic acid/acetonitrile solution (5 : 95, v/v) (for NH_2). The eluates were concentrated to 0.5 mL by Turbo-vap evaporator. To this concentrate, 0.5 mL of pure water was added and the solution was filtered through a 0.2 μm syringe filter to a 2 mL autosampler vial.

To compare extraction efficiency of organic solvents for milk samples, acetonitrile, ethylacetate, and acetone were used. Except for the variation of solvent type and utilization of LiChrolut EN cartridge, the other sample pretreatment procedures were the same as described above.

To compare the effect of pH to extraction efficiency, the pH of milk sample was varied to 2, 4.5, and 10 using hydrochloric acid, sodium phosphate, and aqueous ammonia solution, respectively. Spiked milk sample was extracted with acetonitrile and cleaned up by SPE with a LiChrolut EN cartridge; the other procedures were the same as described above.

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For determination, SF and $^{13}C_6$-SMZ [150 μL (0.1 g) of 1.5 μg/g solution in acetonitrile] were spiked to a 20 mL portion of milk sample as internal standards. After 12 hours, the pH of the sample was adjusted to 4.5, and extracted with acetonitrile and then cleaned up by LiChrolut EN cartridge as described above. The solvent of the eluate was removed to dryness by rotary evaporation. The residue was reconstituted with 250 μL of HPLC eluent and filtered through a 0.2 μm syringe filter to a 300 μL vial insert for a 2 mL autosampler vial.

RESULTS AND DISCUSSION

Optimization of Solvent and Solid Phase Extraction

Selection of Solid Phase

Through the preliminary experiment, the capability of polar (NH_2) and non-polar [C_{18}, LiChrolut EN ethylvinyl–divinyl benzene copolymer (EVDVB)] cartridges for the enrichment and clean up of sulfonamides from pure water and milk was examined. For a water matrix, LiChrolut EN, NH_2, and C_{18} can enrich sulfonamides. On the other hand, sulfonamides in a milk matrix (before loading sample into cartridge, sulfonamides were extracted with acetonitrile and the solvents were changed into aqueous phase) can be efficiently enriched only by LiChrolut EN. These may result from the competition of sulfonamides with interference, to interact with solid sorbents. The acidic or non-polar characteristics of fatty acids in milk may suppress the interaction between sulfonamide and sorbents (NH_2, C_{18}). On the other hand, the interaction between benzene rings on sulfonamide and EVDVB copolymers may enhance the enrichment of sulfonamides, and the high surface area of porous polymers may provide enough capacity for non-polar sulfonamides and lipids. Enriched sulfonamides were selectively eluted from the LiChrolut EN cartridge by acetonitrile. Therefore, the LiChrolut EN cartridge could be utilized as a new and efficient solid phase for the enrichment and clean up of sulfonamides from biological matrices.

Effect of Extraction Solvent

Acetonitrile,[8] acetone,[21,22] ethyl acetate,[13] chloroform–acetone,[9,11] ethanol, and methylene chloride[22] have been used to extract sulfonamides from biological matrices. In this study, the effect of solvents for extraction efficiency of sulfonamides from milk was compared using acetonitrile, ethylacetate, and acetone (Fig. 1). The more polar solvent (acetonitrile) was more effective than the less polar solvents (acetone and ethylacetate). Only

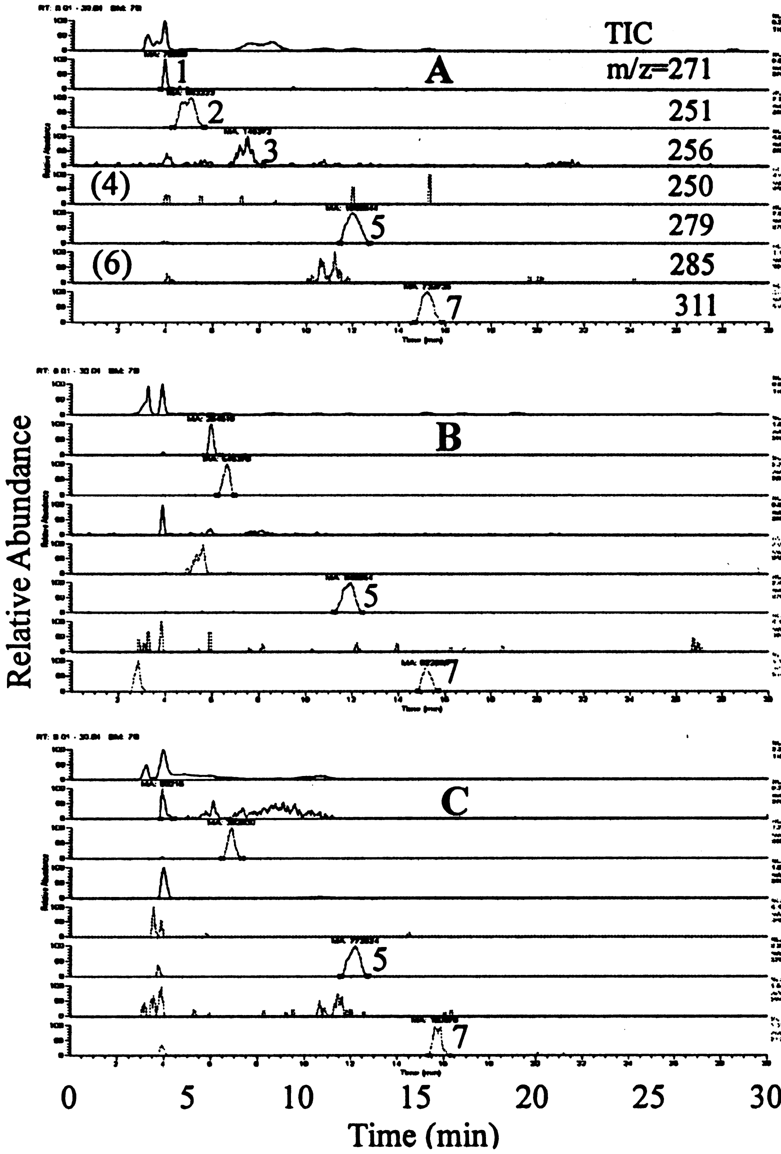

Figure 1. Extracted ion chromatograms of LC/APCI-MS (Full Scan Mode, m/z = 200 ~ 350) for the samples extracted by various solvents. A, acetonitrile; B, ethylacetate; C, acetone. 1, SMTZ; 2, SDZ; 3, STZ; (4, SP*); 5, SMZ; (6, $^{13}C_6$-SMZ*); 7, SDM. *, internal standard (did not spike to the sample for this experiment). Acetonitrile : water : 0.1 M aqueous ammonium acetate solution (17 : 33 : 50, v/v).

acetonitrile could recover most target compounds from the milk. Furthermore, acetonitrile was efficient for removing proteins by coagulation.

Effect of pH on Solvent Extraction

In most of the present work, sulfonamides in milk were extracted after the pH of the sample was not controlled or controlled in the range of pH 3–6.[15,18,19] In this study, the extraction efficiency of sulfonamides by acetonitrile was investigated, changing the pH of the samples to 2, 4.5, and 10, respectively (Fig. 2). The alteration of pH has a dramatic effect on the solubility of sulfonamide in acetonitrile. The best extraction of sulfonamides was done at pH 4.5. This can be explained by the ionization of sulfonamides as a function of pH. Sulfonamides are based on a 4-aminobenzenesulfonamide skeleton and carry a thiazole or pyrimidine system on the sulfonamide nitrogen. The sulfonamide moiety is acidic, while the aromatic amino group imparts basic properties to these drugs. The pKa values of sulfonamides are 5.4 (SMTZ)–7.4 (SMZ). Therefore, in acidic or basic condition, the sulfonamides are in ionized form while, at pH 4.5, most of the sulfonamides are in unionized form. As a result, at pH 4.5, unionized sulfonamides exhibit much higher solubility in relatively non-polar acetonitrile, than in a polar aqueous sample matrix. In addition, the effective extraction of SMZ and SDM on any solvents or under any pH conditions is attributable to the hydrophobic effect of the methyl substituents.

Analysis of Milk Samples

The LC/APCI-MS extracted ion chromatogram of the standard solution and the sample solution of milk separated on an ODS2 column are shown in Fig. 3. The quantification of SMZ in spiked milk sample using isotope labeled internal standard ($^{13}C_6$-SMZ) is shown in Table 1. The fortified level used in this study [8.2 ng/mL (8.8 ng/g)] was lower than the maximum allowed residue limits (MRLs) of 10 ng/mL in milk. The precision of this method for five independent samples was an RSD of 2.8%. The measured concentration (0.0089 μg/g) was different from the prepared concentration (0.0088 μg/g) about 1.7%.

CONCLUSIONS

This work has demonstrated that a simple clean up of each sulfonamide residue in milk (<10 ng/mL) can be performed using acetonitrile extraction at pH 4.5 and SPE on a LiChrolut EN cartridge. To the best of the authors'

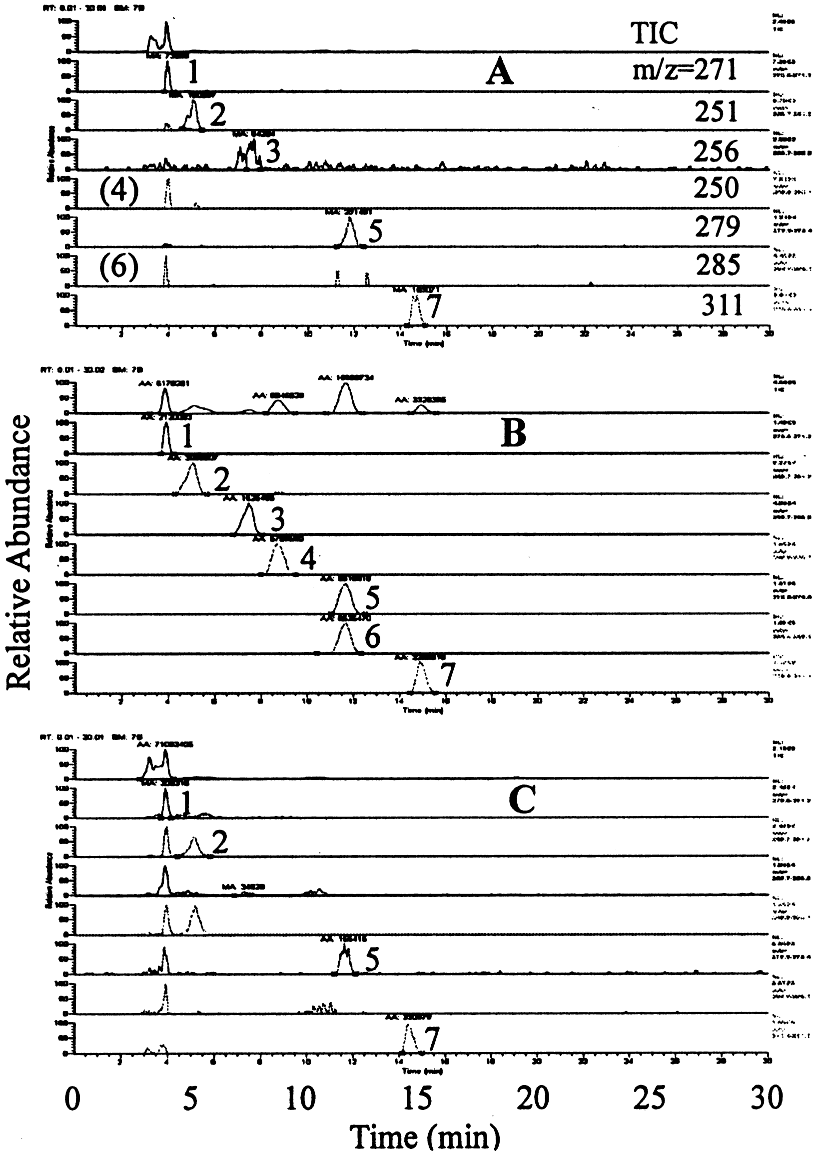

Figure 2. Extracted ion chromatograms of LC/APCI-MS (Full Scan, m/z = 200 ~ 350) for the samples extracted with acetonitrile at different pH conditions. A, pH 2; B, pH 4.5; C, pH 10. 1, SMTZ; 2, SDZ; 3, STZ; (4, SP*); 5, SMZ; (6, $^{13}C_6$-SMZ*); 7, SDM. *, internal standard (spiked only to the sample B). Acetonitrile : water : 0.1 M aqueous ammonium acetate solution (17 : 33 : 50, v/v).

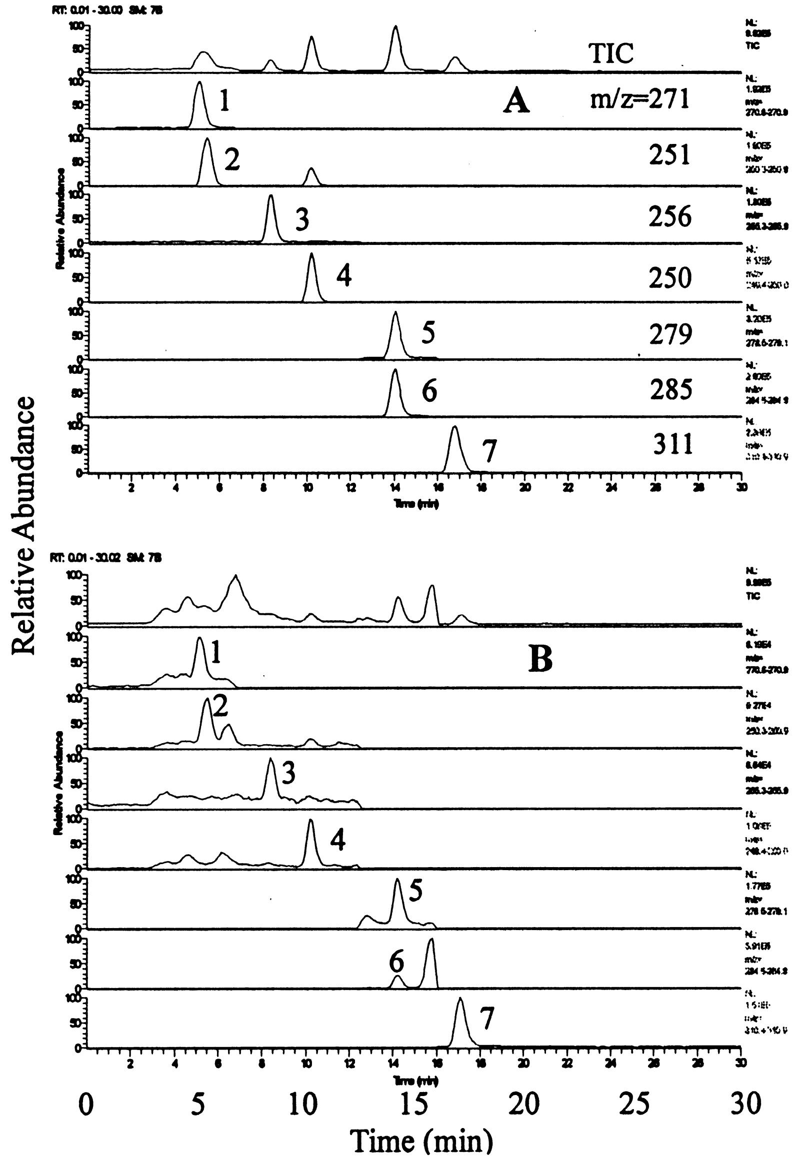

Figure 3. Extracted ion chromatograms of APCI-LC/MS (Zoom Scan Mode) for standard solution (A) and sample solution of milk (B). 1, SMTZ; 2, SDZ; 3, STZ; 4, SP*; 5, SMZ; 6, $^{13}C_6$-SMZ*; 7, SDM. *, internal standard. Concentration of standard solution: 0.7 µg/g. Acetonitrile : water : 0.1 M aqueous ammonium acetate solution (13 : 37 : 50, v/v).

Table 1. Results of determination of sulfamethazine in milk sample by LC/MS.

Sample	Prepared concentration, μg/g	Measured concentration, μg/g ($n = 2$) 1	2	Avg.
1	0.0088	0.0092	0.0087	0.0090
2		0.0086	0.0086	0.0086
3		0.0087	0.0087	0.0087
4		0.0093	0.0090	0.0091
5		0.0093	0.0090	0.0092
			Avg.	0.0089[a]
			SD	0.0002[a]
			RSD, %	2.8[a]
			Difference, %	1.7[a,b]

Note: Five sulfonamides and $^{13}C_6$-SMZ were spiked to milk and cleaned-up.

[a] Result of five independent samples.

[b] Difference from prepared concentration.

knowledge, utilization of a LiChrolut EN for the analysis of sulfonamides in a bio-matrix is unprecedented. Milk samples, spiked with 8.2 ng/mL of SMZ, were assayed with an RSD of 2.8% using isotope labeled internal standards by LC/APCI-MS. The combination of LC/APCI-MS and SPE can provide promising results for the determination of sulfonamides from biological matrices.

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