# Determination of Sulfonamide Antibiotics in Bovine Liver Using Agilent Bond Elut QuEChERS EN Kits by LC/MS/MS

Application Note

Food


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## Abstract

This paper presents an analytical method for the determination of nine sulfonamide antibiotic residues in bovine liver: sulfadizine, sulfathiazole, sulfamerazine, sulfamethizole, sulfamethazine, sulfamethoxypyridazine, sulfachloropyridazine, sulfamethoxazole, and sulfadimethoxin. The procedure involves a rapid and efficient pretreatment with Bond Elut QuEChERS kits. The homogenized liver sample was initially extracted in a buffered aqueous/1% acetic acid acetonitrile system with an extraction and partitioning step after the addition of salts. Finally, the sample was cleaned up using dispersive solid-phase extraction (dispersive-SPE). The final extracts were analyzed by the sensitive and selective determination of all compounds in a single run using LC-ESI-MS-MS operating in positive ion multiple reaction monitoring (MRM) mode. Sulfapyridine was selected as the internal standard. The accuracy of the method, expressed as recovery, was between 53 and 93%. The precision, expressed as RSD, was between 2.1 and 16.8%. The established 5 ng/g limits of quantification (LOQ) were much lower than the respective Maximum Residue Limit (MRL) for sulfonamide in animal food products (20-100 ng/g).


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## Introduction

Sulfonamides (SAs) are a very important class of antibacterial compounds widely used in veterinary practice for therapeutic, prophylactic, and growth-promoting purposes. Residues of SAs may remain in animal tissues if adequate withdrawal time is not observed or if the SAs have been improperly administered. The maximum residue limit (MRL) in the European Union countries and United States for SAs in animal muscle tissue is 100 ng/g, while in Japan it is 20 ng/g. [1]

The QuEChERS EN method is an important variation, which has been officially accepted by the European Committee for Standardization and is widely applied to pesticide analysis in foods of plant origin [2]. The original EN buffered method was designed mostly for multiresidue pesticide analysis in plant food products. In summary, the method uses acetonitrile extraction followed by the salting out of water from the sample using anhydrous magnesium sulfate ($MgSO_4$), NaCl, and buffering citrate salts to induce liquid-liquid partitioning. For cleanup, a dispersive solid-phase extraction (dispersive-SPE) is conducted using a combination of primary secondary amine (PSA) to remove fatty acids from other components and anhydrous $MgSO_4$ to reduce the remaining water in the extract. After mixing and centrifuging, the upper layer is ready for analysis. Fatty dispersive-SPE, which contains 25 mg C18EC per mL of ACN extract, is employed to remove more lipids from the matrix when using fruits and vegetables with fats and waxes.

Food matrices from animal origin contain a substantial amount of proteins and lipids. Therefore, they are very different from food matrices of plant origin such as fruits and vegetables. In this study, a Bond Elut QuEChERS EN buffered extraction kit (p/n 5982-5650) and EN fatty dispersive-SPE 15 mL kit (p/n 5982-5165) were tested for the analysis of sulfonamide antibiotics in bovine liver. Because of the differences in food matrices and the chemical properties of the target analytes, modifications to the method were also investigated to achieve efficient extraction and cleanup.

## Experimental

### Reagents and Chemicals

All reagents and solvents were HPLC or analytical grade. Methanol (MeOH) was from Honeywell (Muskegon, MI, USA). Acetonitrile (ACN), dimethyl sulfoxide (DMSO), and glacial acetic acid (HAc) were from Sigma-Aldrich (St Louis, MO, USA). Ammonium acetate ($NH_4OAc$) was from Fisher Chemicals (Fair Lawn, NJ, USA). Formic acid (FA) was from Fluka (Sleinheim, Germany). The sulfonamides standards and the internal standard were from Sigma (St Louis, MO, USA).

### Solutions and Standards

A 1 M ammonium acetate stock solution was made by dissolving 19.27 g $NH_4OAc$ powder in 250 mL Milli-Q water. The solution was stored at 4 °C. A 5 mM ammonium acetate solution in water, pH 3, was made by adding 5 mL of 1 M ammonium acetate stock solution into 1 L of Milli-Q water. The pH was then adjusted to 3 by adding glacial acetic acid and monitoring with a pH meter. A 1:1 MeOH/ACN solution was made by combining 500 mL of MeOH and ACN, and mixing well. A 1% acetic acid in ACN solution was prepared by adding 5 mL of glacial acetic acid to 500 mL of ACN, and mixing well. A 1:1 ACN/$H_2O$ solution with 0.1% FA was prepared by combining 50 mL of ACN and Milli-Q water, then adding 100 µL of formic acid. A 1:9 MeOH/$H_2O$ solution with 0.1% FA was prepared by combining 10 mL of MeOH and 90 mL of Milli-Q water, then adding 100 µL of formic acid.

Standard and internal standard (IS) stock solutions (1.0 mg/mL for all) were all made in DMSO and stored at 4 °C. Six of the sulfonamides are light-sensitive, so the stock

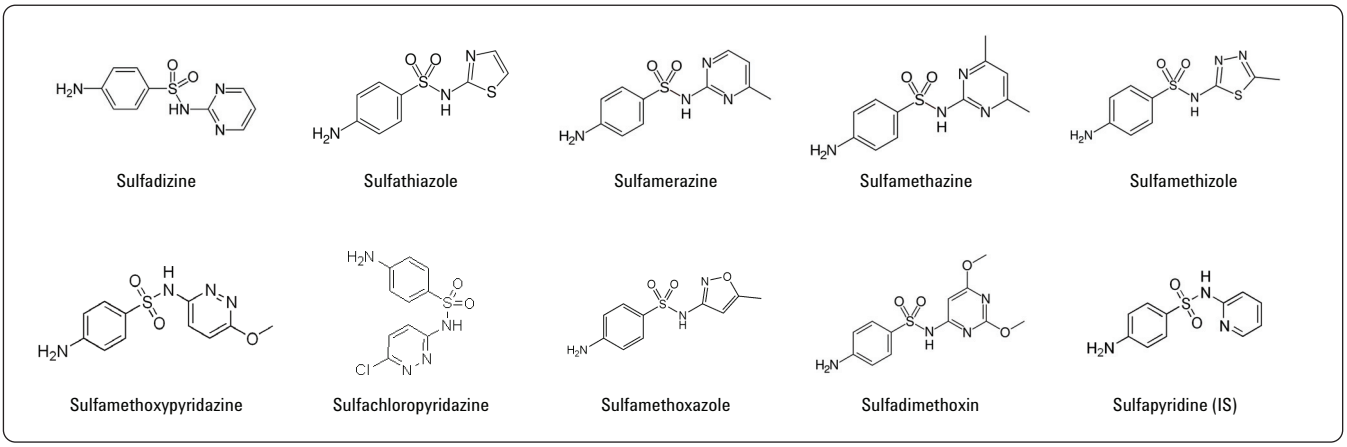


*Figure 1. Chemical structures of the quinolone antibiotics investigated in this study.*

solutions were kept in amber vials and wrapped in aluminum foil. Three combined QC spiking solutions of 0.2, 4, and 16 µg/mL were made fresh daily in 1:1 ACN/$H_2O$ containing 0.1% FA. A 20 µg/mL standard spiking solution in 1:1 ACN/$H_2O$ containing 0.1% FA was made for the preparation of calibration curves in the matrix blank extract. Due to light sensitivity of certain sulfonamides, all of the combined solutions were kept in amber vials and wrapped in aluminum foil. A 20 µg/mL IS spiking solution of Sulfapyridine was made in 1:1 ACN/$H_2O$ containing 0.1% FA.

## Equipment and Material

- Agilent 1200 Series HPLC with Diode Array Detector (Agilent Technologies Inc., CA, USA)
- Agilent 6410 Triple Quadrupole LC/MS system with Electrospray Ionization (Agilent Technologies Inc., CA, USA)
- Agilent Bond Elut QuEChERS EN Extraction kit p/n 5982-5650 (Agilent Technologies Inc., DE, USA)
- Agilent Bond Elut QuEChERS EN fatty dispersive-SPE kit for 15 mL p/n 5982-5156 (Agilent Technologies Inc., DE, USA)
- CentraCL3R Centrifuge (Thermo IEC, MA, USA)
- Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)
- 2010 Geno Grinder (Spex SamplePrep LLC, Metuchen, NJ, USA)
- Multitube Vortexer (Henry Troemner LLC, Thorofare, NJ, USA)

## Instrument conditions

**HPLC conditions**

| | | | |
|---|---|---|---|
| Column | Agilent ZORBAX Solvent Saver HT Eclipse Plus C18 50 × 3.0 mm, 1.8 µm (p/n: 959941-302) | | |
| Flow rate | 0.3 mL/min | | |
| Column Temperature | 30 °C | | |
| Injection volume | 10 µL | | |
| Mobile Phase | A: 5 mM ammonium acetate, pH 3.0 in H2O | | |
| | B: 1:1 MeOH/ACN | | |
| Needle wash | 1:1:1:1 ACN/ MeOH/ IPA/ H2O with 0.2% FA. | | |
| Gradient | Time | % B | Flow rate (mL/min) |
| | 0 | 15 | 0.3 |
| | 0.2 | 15 | 0.3 |
| | 6.0 | 60 | 0.3 |
| | 6.01 | 100 | 0.3 |
| | 7.0 | STOP | |
| Post run | 3.5 min | | |
| Total cycle time | ~11 min. | | |

**MS conditions**

| | |
|---|---|
| Polarity | positive |
| Gas Temperature | 325 °C |
| Gas Flow | 8 L/min |
| Nebulizer | 50 Psi |
| Capillary | 4000 V |

Other conditions relating to the analytes are listed in Table 1.

*Table 1. Instrument Acquisition Data for the Analysis of 9 Sulfonamide Antibiotics by LC/MS/MS.*

| Analyte | MRM channels (m/z) | Fragmentor (V) | CE (V) | RT (min) |
|---|---|---|---|---|
| Sulfadizine | 1) 251.1 → 108.0 | 100 | 25 | 2.1 |
| | 2) 251.1 → 156.0 | | 13 | |
| Sulfathiazole | 1) 256.0 → 156.0 | 94 | 13 | 2.3 |
| | 2) 256.0 → 92.1 | | 29 | |
| Sulfamerazine | 1) 265.1 → 92.1 | 125 | 29 | 2.9 |
| | 2) 265.1 → 108.1 | | 25 | |
| Sulfamethizole | 1) 271.0 → 156.0 | 112 | 9 | 3.7 |
| | 2) 271.0 → 92.1 | | 29 | |
| Sulfamethazine | 1) 279.1 → 124.0 | 116 | 21 | 3.8 |
| | 2) 279.1 → 92.1 | | 33 | |
| Sulfamethoxypyridazine | 1) 281.1 → 156.0 | 128 | 13 | 3.9 |
| | 2) 281.1 → 92.1 | | 29 | |
| Sulfachloropyridazine | 1) 285.0 → 156.0 | 106 | 9 | 4.5 |
| | 2) 285.0 → 92.1 | | 29 | |
| Sulfamethoxazole | 1) 254.1 → 92.1 | 113 | 25 | 4.8 |
| | 2) 254.1 → 108.0 | | 21 | |
| Sulfadimethoxin | 1) 311.1 → 156.0 | 141 | 17 | 6.0 |
| | 2) 311.1 → 92.1 | | 37 | |
| Sulfapyridine (IS) | 250.1 → 92.1 | 113 | 29 | 2.7 |

1) Quantifier transition channel 2) Qualifier transition channel

## Sample preparation

Sample preparation includes sample homogenization, extraction and partitioning, and dispersive-SPE cleanup. Since the main focus of existing QuEChERS methodology has been the extraction of pesticides from plant and vegetable matrices, certain modifications were adapted in order to optimize results for the determination of sulfonamides in bovine liver. These modifications will be discussed in detail later.

Bovine liver was purchased from a local grocery store. It was then washed and chopped into small pieces. The chopped liver sample was homogenized thoroughly with a food grinder, then stored at -20 °C. Two gram (±0.05 g) amounts of homogeneous sample were placed into 50 mL centrifuge tubes. Sample tubes were centrifuged 30 s to move any sample sticking to the inside wall of tube to the bottom. Samples were then fortified with appropriate QC spiking solutions (50 µL) when necessary. Then 50 µL of IS spiking solution (20 µg/mL of sulfapyridine) were added. After vortexing sample for 30 s, 8 mL of Milli-Q water were added. Tubes were then vortexed another 10 s for mixing. Ten milliliters of 1% AA in ACN were added to each tube. Tubes were capped and shaken by a 2010 Geno Grinder for 30 s. An Agilent Bond Elut QuEChERS EN extraction salt packet (p/n 5982-5650) was added to each tube. Sample tubes were capped tightly and shaken vigorously for 1 min by the 2010 Geno Grinder at 4 °C.

A 6 mL aliquot of the upper ACN layer was transferred into an Agilent Bond Elut EN QuEChERS fatty dispersive-SPE 15 mL tube (p/n 5982-5156). This 15 mL dispersive-SPE tube contained 150 mg of PSA, 150 mg of C18EC, and 900 mg of anhydrous $MgSO_4$. The tubes were tightly capped and vortexed for 2 min. The 15 mL tubes were centrifuged at 4000 rpm for 5 min. A 4 mL amount of extract was then transferred into another tube and dried by $N_2$ flow at 40 °C. Samples were reconstituted into 800 µL of 1:9 MeOH/$H_2O$ solution with 0.1% FA. After vortexing and sonicating for 10 min, the sample was filtered by a 0.22 µm Cellulose Acetate Spin Filter (p/n 5185-5990). The clear filtered sample was transferred into an autosampler vial. The samples were capped and vortexed thoroughly, in preparation for LC/MS/MS analysis.

See Figure 2 for the flow chart of the extraction procedure for a bovine liver sample.

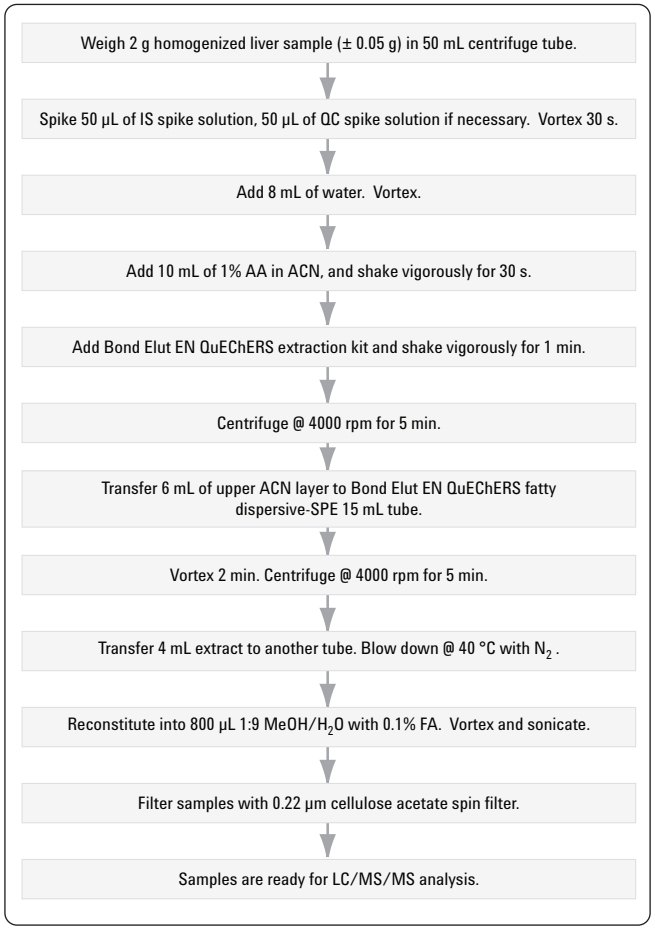


*Figure 2. Flow chart of QuEChERS procedure for the determination of sulfonamides in bovine liver.*

## Results and Discussion

### Method Optimization in the Liver Matrix

As mentioned previously, modifications of the QuEChERS EN method were investigated relative to extraction efficiency. An EN buffered extraction system provides a solution with a pH of 5.0 – 5.5, which illicits neutral sulfonamide analytes (pKa ~6-7). PSA sorbent used in dispersive-SPE can strongly interact with acid compounds and remove various co-extractive interferences, such as polar organic acids, sugars, and fatty acids. However, it may also interact with target compounds and cause the loss of analytes. Therefore, the QuEChERS fatty EN dispersive-SPE kit with PSA was compared to the dispersive-SPE kit without PSA. A previous study [4] showed that the addition of acid to acetonitrile can inhibit the absorption of PSA, weakening the attraction of PSA to the compounds of interest. Therefore, a 1% AA in ACN was evaluated in addition to standard ACN in the first partitioning step.

To evaluate the original EN and modified method, a 50 ng/g of fortified liver sample was extracted with the following procedures:

1. EN buffered extraction kit with ACN and dispersive-SPE kit with PSA (25 mg PSA and C18EC per mL)
2. EN buffered extraction kit with ACN and dispersive-SPE kit without PSA (25 mg C18EC per mL)
3. EN buffered extraction kit with 1% AA in ACN and dispersive-SPE kit with PSA (25 mg PSA and C18EC per mL).

The corresponding matrix blanks were extracted at the same time, then post-spiked with the same amount of sulfonamide standards. Neat solution post-spiked at the same concentration was also compared to the matrix post-spiked samples.

The results are shown in Table 2. The results of method 1 and method 2 show that PSA does contribute to the matrix cleanup during the dispersive-SPE step. The matrix effect values for all of the analytes in method 1 were lower than those in method 2 indicating that the sample processed by method 1 was cleaner than the sample processed by method 2. This was also demonstrated by the color of the ACN extract. After the extraction step, the ACN extract was a brownish-red color. Using PSA in the dispersive step produced an ACN extract that was light yellow in color. Removing PSA from the dispersive step maintained the previously observed brownish-red ACN extract. Unfortunately, the presence of PSA in the dispersive step also caused the loss of certain analytes and produced very low recovery for sulfachloropyridazine (30%) and sulfamethizole (15%). Method 3 produced substantially better recoveries for all the analytes relative to methods 1 or 2. The matrix effect values show that the sample processed by method 3 is as clean as the sample processed by method 2, but not as clean as the sample processed by method 1. The addition of acid in the ACN partitioning step impedes the performance of PSA in the dispersive step preventing the loss of analytes. It also decreases the interaction of PSA with other matrix interferences, producing a greater matrix effect. This is also shown by the color of the ACN extract. Although PSA was used in the dispersive step, the presence of acidified ACN extract elicited a light brown-red color, rather than the light yellow extract in method 1.

*Table 2. Method Optimization Results for the Analysis of Sulfonamides in Bovine Liver*

| | Method 1 | | Method 2 | | Method 3 | |
|---|---|---|---|---|---|---|
| **Analytes** | **Recovery** | **Matrix effect** | **Recovery** | **Matrix effect** | **Recovery** | **Matrix effect** |
| Sulfadiazine | 91.9 | -33.0 | 85.2 | -65.2 | 85.6 | -57.9 |
| Sulfathiazole | 39.9 | -35.9 | 42.0 | -57.3 | 87.7 | -65.7 |
| Sulfamerazine | 77.0 | -19.3 | 43.9 | -23.9 | 89.0 | -51.7 |
| Sulfamethizole | 15.3 | -33.6 | 46.5 | -46.9 | 63.2 | -49.8 |
| Sulfamethazine | 85.7 | -23.1 | 51.4 | -31.6 | 87.3 | -42.0 |
| Sulfamethoxy-pyridazine | 76.6 | -32.6 | 49.0 | -31.7 | 86.1 | -49.1 |
| Sulfachloro-pyridazine | 29.6 | -38.5 | 51.1 | -42.3 | 84.8 | -50.6 |
| Sulfamethoxa-zole | 60.0 | -40.9 | 53.4 | -46.9 | 87.5 | -54.5 |
| Sulfadimethoxin | 67.4 | -35.3 | 56.9 | -43.0 | 89.6 | -51.9 |

| | |
|---|---|
| Method 1 | EN buffered extraction kit + ACN + Fatty dispersive-SPE kit (25 mg PSA + 25 mg C18EC + 150 mg $MgSO_4$ per mL) |
| Method 2 | EN buffered extraction kit + ACN + Dispersive-SPE kit without PSA (25 mg C18EC + 150 mg $MgSO_4$ per mL) |
| Method 3 | EN buffered extraction kit + 1% AA ACN + Fatty dispersive-SPE kit (25 mg PSA + 25 mg C18EC + 150 mg $MgSO_4$ per mL) |

$$\% \text{ Recovery} = \frac{Response_{\text{extracted sample}}}{Response_{\text{post-extracted spiked sample}}} \times 100$$

$$\% \text{ Matrix Effects} = \left( \frac{Response_{\text{post-extracted spiked sample}}}{Response_{\text{non-extracted neat sample}}} - 1 \right) \times 100$$

Method 3 was selected for the final study. In this study, liver sample was extracted by the EN buffered extraction kit (p/n 5982-5650) with 1% AA in ACN. After centrifuging, the ACN extract was further cleaned by EN fatty dispersive-SPE 15mL tube (p/n 5982-5156). Figure 3 shows the MRM chromatograms of the liver control blank and 100 ng/g fortified liver extract. The liver control blank chromatogram indicated that the target analytes were free from any interference.

## Linearity and limit of quantification (LOQ)

The linear calibration range for all the sulfonamide antibiotics was 5 - 400 ng/g. Matrix blanks were prepared for evaluation. Calibration curves, made from spiked matrix blanks, were made at levels of 5, 10, 50, 100, 200, 300 and 400 ng/g for each analyte. The sulfapyridine was used as an internal standard at 200 ng/g. The calibration curves were generated by plotting the relative responses of analytes (peak area of analyte / peak area of IS) versus the relative concentration of analytes (concentration of analyte/concentration of IS). The 5 ng/g limits of quantification (5 ppb) of the sulfonamides is far below the MRLs for residues of these antibiotics in animal food products (20 - 100 ng/g). Table 3 shows the regression equation and correlation coefficient ($R^2$). Linear regression fit was used with $1/x^2$ weight. Results indicated excellent linearity for all of analytes calibration curves over a broad quantification range.

*Table 3. Linearity of Sulfonamide Antibiotics in Bovine Liver*

| Analytes | Regression Equation | $R^2$ |
|---|---|---|
| Sulfadiazine | Y = 1.6585X + 0.0002 | 0.9963 |
| Sulfathiazole | Y = 1.3899X + 0.0002 | 0.9942 |
| Sulfamerazine | Y = 3.5019X – 0.0001 | 0.9962 |
| Sulfamethizole | Y = 2.3064X + 0.0001 | 0.9963 |
| Sulfamethazine | Y = 4.3780X – 0.0004 | 0.9977 |
| Sulfamethoxypyridazine | Y = 4.4044X + 0.0003 | 0.9941 |
| Sulfachloropyridazine | Y = 1.5869X – 0.0005 | 0.9971 |
| Sulfamethoxazole | Y = 1.9047X – 0.0001 | 0.9936 |
| Sulfadimethoxin | Y = 4.5106X + 0.0020 | 0.9922 |

## Recovery and Reproducibility

The recovery and reproducibility were evaluated by fortifying sulfonamides standards in homogenized liver sample at levels of 5, 100, and 400 ng/g. These QC samples were quantified against the matrix spiked calibration curve. The analysis was performed in replicates of six at each level. The recovery and reproducibility (shown as RSD) data are shown in Table 4. The results show that all of the sulfonamides were somewhat low but still at acceptable recoveries (average of 77.8%) and good precision (average of 7.2% RSD). Samples were concentrated during the procedure to optimize sensitivity, which also caused additional matrix effects that possibly contributed to a higher RSD of target compounds at low levels.

*Table 4. Recovery and Repeatability of Sulfonamides in Fortified Liver Homogenate*

| | 5 ng/g fortified QC | | 100 ng/g fortified QC | | 400 ng/g fortified QC | |
|---|---|---|---|---|---|---|
| Analytes | Recovery | RSD (n=6) | Recovery | RSD (n=6) | Recovery | RSD (n=6) |
| Sulfadiazine | 73.9 | 15.6 | 90.0 | 13.7 | 81.9 | 5.3 |
| Sulfathiazole | 62.9 | 16.8 | 75.3 | 8.4 | 67.9 | 5.8 |
| Sulfamerazine | 77.6 | 11.5 | 92.8 | 6.6 | 82.0 | 4.2 |
| Sulfamethizole | 62.8 | 4.7 | 60.7 | 6.5 | 53.0 | 2.1 |
| Sulfamethazine | 87.4 | 6.9 | 90.0 | 10.7 | 83.4 | 3.4 |
| Sulfamethoxy-pyridazine | 81.8 | 9.4 | 84.8 | 8.1 | 76.4 | 2.9 |
| Sulfachloro-pyridazine | 84.2 | 10.0 | 78.6 | 6.3 | 73.8 | 3.6 |
| Sulfamethoxazole | 85.9 | 7.6 | 82.3 | 5.9 | 78.1 | 3.3 |
| Sulfadimethoxin | 77.8 | 8.4 | 80.9 | 4.9 | 75.6 | 3.3 |

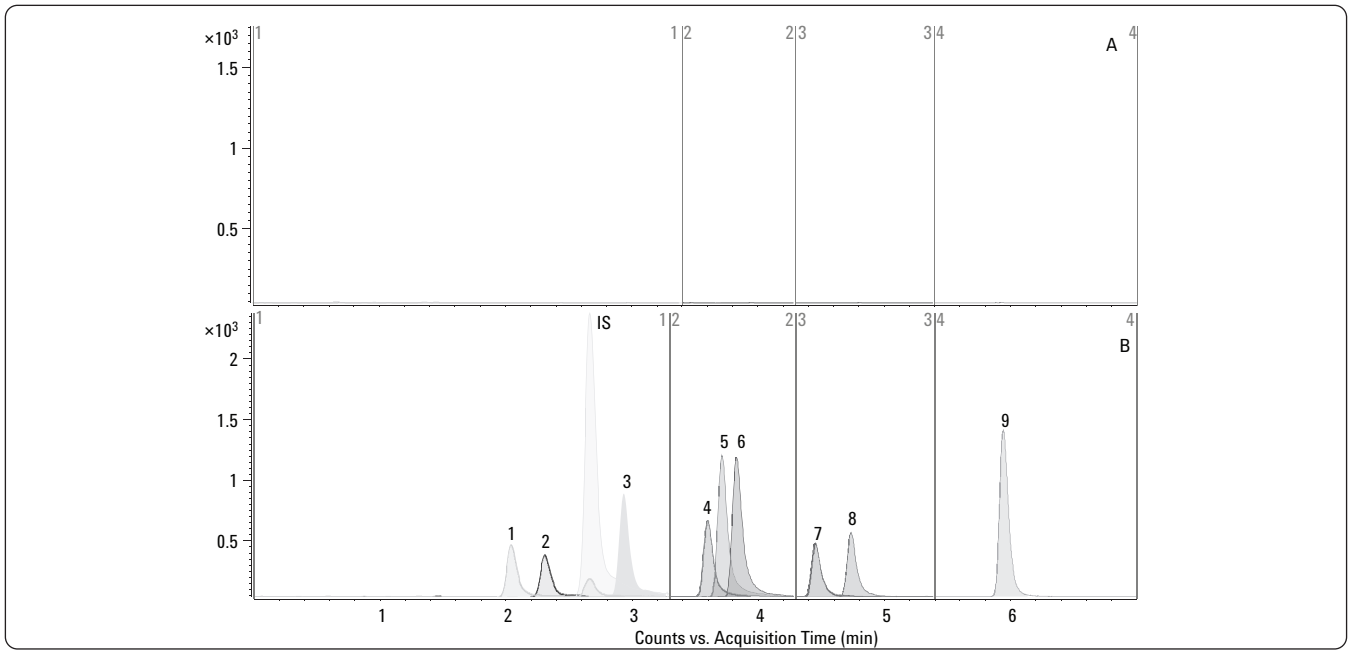


*Figure 3. LC/MS/MS Chromatograms of A) liver blank extract, and B) 100 ng/g fortified liver extract. Peaks identification: 1. sulfadizine, 2. sulfathiazole, 3. sulfamerazine, 4. sulfamethizole, 5. sulfamethazine, 6. sulfamethoxypyridazine, 7. sulfachloropyridazine, 8. sulfamethoxazole, 9. sulfadimethoxin, IS (internal standard), sulfapyridine.*

## Conclusions

The Agilent Bond Elut EN Buffered Extraction kit and Bond Elut EN fatty dispersive-SPE kit provide a simple, fast, and effective method for the purification of sulfonamide antibiotics in bovine liver. When compared to other sample preparation methods, such as LLE and SPE, QuEChERS methodology is easy, fast, low cost and does not require automation. In addition, it is labor saving and a "greener" technology. The recovery and reproducibility, based on matrix-spiked standards, were acceptable for multiresidue sulfonamide determination in bovine liver. The impurities and matrix effects from liver were minimal and did not interfere with the quantification of any target compound. The LOQs of the quinolones were much lower than their regulated MRLs in animal food products (20-100 ng/g). This modified QuEChERS procedure is a very promising methodology for the quantitative analysis of sulfonamides in food products of animal origin. This application demonstrates great potential of Bond Elut QuEChERS extraction and dispersive-SPE kits, and extend far beyond plant matrices to bio-matrices, such as animal food products and bio-fluid.

## References:

[1] Sun H., Ai L., Wang F., Quantitative Analysis of Sulfonamide residues in Natural Animal Casings by HPLC, Chromatographia 2007, 66, Sept, (no. 5/6), 333-337.

[2] European Council Regulation 2377/90/EC of 26 June 1990 laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin, OJ L, 1990, p. 224.

[3] European Committee for Standardization/Technical Committee CEN/TC 275 (2007), Foods of plant origin: Determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and cleanup by dispersive SPE-QuEChERS method. European Committee for Standardization, Brussels.

[4] Zhao L., Stevens J., Determination of quinolone antibiotics in bovine liver using Agilent Bond Elut QuEChERS kits by LC/MS/MS, Agilent application note, 5990-5085EN.

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