Analyze Sulfonamide Residues in Pork with Agilent Poroshell 120 EC-C18, 4 µm Columns

Application Note

Food Testing and Agriculture

Authors

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Abstract

An HPLC method for the simultaneous determination of sulfonamide residues in pork was developed and validated. The analytes were extracted and cleaned with Agilent Bond Elut Plexa PCX solid phase extraction, separated on Agilent Poroshell 120 EC-C18, 4 µm HPLC columns, and quantified by liquid chromatography coupled with diode array detection. The dynamic calibration range for all the sulfonamides was obtained from 10 to 1,000 µg/kg. Overall recoveries ranged from 70.2 to 89.9%, with RSD values between 2.7 and 7.5%. We compared performance of different columns, including Poroshell 120, 4 µm and 2.7 µm, and Agilent ZORBAX Eclipse Plus, 3.5 µm and 5 µm.

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Introduction

Sulfonamides (Figure 1) are one of the oldest groups of pharmacologically active substances used in veterinary medicine. The discovery of sulfonamides in 1935 started a new era in the therapy of a wide range of bacterial diseases and some protozoan infections. However, direct allergic or toxic reactions have been described after administration of therapeutic doses of sulfonamides to humans.

To protect consumers from risks related to drug residues, maximum residue limits (MRLs) have been established by law in many countries. The existing EU MRL for all drugs of the sulfonamide group is 100 µg/kg in all food-producing species [1]. MRLs in the China regulation of GB 29694-2013 for 13 sulfonamide residues in animal- derived food are also 100 µg/kg [2].

The objective of this work was to develop a multiresidue method that would be simple and fast for routine regulatory analysis of sulfonamide antibiotic residues in pork. The method relies on a simple SPE step using a polymer sorbent (Agilent Bond Elut Plexa PCX), and an HPLC separation using a Poroshell 120, 4 µm column.

Materials and Methods

Reagents and chemicals

All reagents were HPLC or analytical grade. Acetonitrile was from J&K Scientific Ltd, Beijing. The standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Pork samples were purchased from a local supermarket in China. Standard solutions (1.0 mg/mL) were made in methanol individually and stored in a freezer at –20 °C. A combined working solution was made in water and also stored at –20 °C. The spiked solutions were then made daily by appropriately diluting the combined working solution in water.

Sample preparation

Weigh pork sample (5 ± 0.05 g) into a 50 mL polypropylene centrifuge tube. Add anhydrous sodium sulfate (5 ± 0.05 g) and 20 mL acetic ether to the tube. Vortex thoroughly for 2 minutes and then centrifuge at 5,000 rpm at 4 °C for 5 minutes. Transfer the supernatant into a new 50 mL polypropylene centrifuge tube and add another 20 mL acetic ether to the sediment. Centrifuge the solution again and transfer supernatant to the same tube. Evaporate the mixed supernatant to dryness at 40 °C under nitrogen. Add hydrochloric acid (4 mL of 0.1 mol/L) to dissolve the residues and transfer these to a 15 mL centrifuge tube. Add another 3 mL 0.1 mol/L hydrochloric acid to fully dissolve the residues.

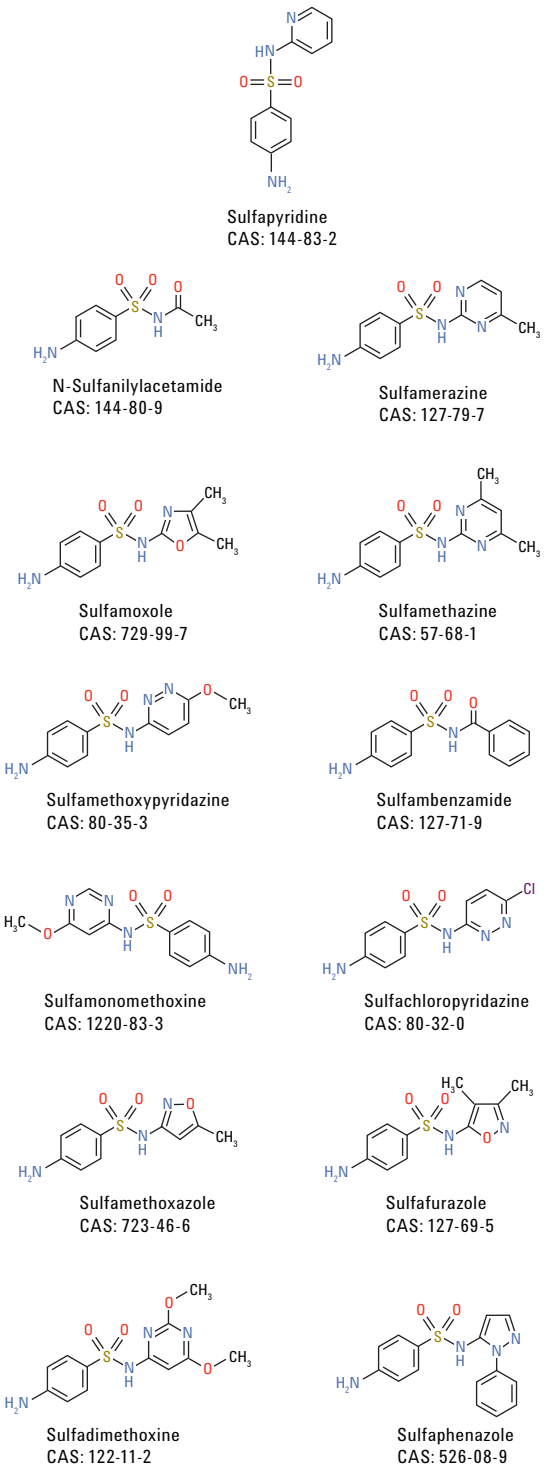

Figure 1. Sulfonamide compounds used in this study.

Add 3 mL *n*-hexane to the tube and vortex thoroughly for 30 seconds, then centrifuge at 5,000 rpm at 4 °C for 5 minutes. Discard the layer of *n*-hexane. Add a second 3 mL aliquot of *n*-hexane to the sample tube and repeat the above procedure. The lower layer of the liquid is then ready for SPE.

Solid phase extraction

The SPE procedure is shown in Figure 2. SPE cartridges (Agilent Bond Elut Plexa PCX, 60 mg, 3 mL, p/n 12108603) were preconditioned with 3 mL methanol and then equilibrated with 3 mL 0.1 mol/L hydrochloric acid in water. The sample solution was then loaded onto the Bond Elut Plexa PCX cartridges and passed through under gravity (about 1 mL/min). The cartridges were washed with 3 mL 0.1 mol/L hydrochloric acid in water and 2 mL methanol. A full vacuum (Agilent Vac Elut 20 manifold, p/n 12234101) was applied to the cartridge for 5 minutes to completely dry the resin. The compounds were eluted with 6 mL 5% ammonia in methanol at 1 mL/min. The eluent was dried under nitrogen at 40 °C. The residue was reconstituted in 1.0 mL of initial mobile phase. The sample was then vortex mixed and ultrasonicated to completely dissolve the residue and centrifuged at 5,000 rpm using an Eppendorf MiniSpin Plus. The liquid was finally transferred to a 2 mL chromatography vial for HPLC analysis.

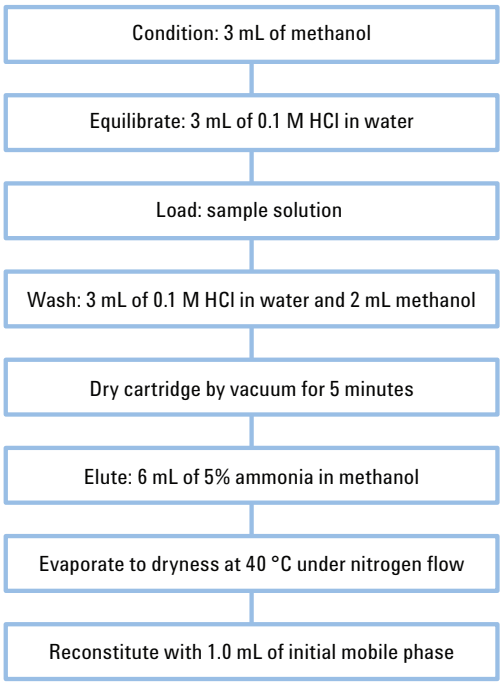

Figure 2. Pork sample cleanup and enrichment using Agilent Bond Elut Plexa PCX solid phase extraction.

Conditions

Columns:	Agilent Poroshell 120 EC-C18, 4.6 × 100 mm, 4 µm (p/n 695970-902), Agilent Poroshell 120 EC-C18, 4.6 × 150 mm, 4 µm (p/n 693970-902), Agilent Poroshell 120 EC-C18, 4.6 × 100 mm, 2.7 µm (p/n 695975-902), Agilent ZORBAX Eclipse Plus C18, 4.6 × 100 mm, 3.5 µm (p/n 959961-902), Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 5 µm (p/n 959993-902)
Mobile phase:	A) 0.1% formic acid in water B) acetonitrile

Gradient (for 100 mm columns)

Time (min)	B (%)
0	16
2	16
4	20
9	40
9.1	90
12	90
12.5	16
15	16

Gradient (for 150 mm columns)

Time (min)	B (%)
0	16
3	16
6	20
13.5	40
14.0	90
18.0	90
18.5	16
22.5	16

Column temperature:	30 °C
Injection volume:	10 µL (for 100 mm columns) 15 µL (for 150 mm columns)
Flow rate:	1.0 mL/min
DAD wavelength:	270 nm
Instrument:	Agilent 1290 Infinity LC with Agilent 1260 Infinity Fluorescence Detector

Results and Discussion

Separation

An optimized gradient method separated the 13 sulfonamide compounds very well on a Poroshell 120 EC-C18, 4 µm column, shown in the upper chromatogram of Figure 3. The separation was achieved in 10 minutes. The blank pork sample (mid chromatogram) and the sample spiked with standards mixture (lower chromatogram) were tested on the same column. It was apparent that several interferential substances in the sample matrix could easily be separated from the target compounds. A wash step was followed after the elution to reduce column contamination.

The method was run on Poroshell 120, 4 µm and 2.7 µm superficially porous particle columns, as well as a 3.5 µm totally porous particle column with the same configuration of 4.6 × 100 mm. The 2.7 µm column gave much better resolution and higher efficiency than the 4 µm column. The peak width at half height ($W_{1/2}$) of the 4 µm column increased by 22% over that of 2.7 µm column. However, the backpressure on the 4 µm column decreased by 47% compared to the 2.7 µm column. The Poroshell 120, 4 µm column is more suitable for use on a 400 bar HPLC, while the 2.7 µm column is suitable for 600 bar HPLC.

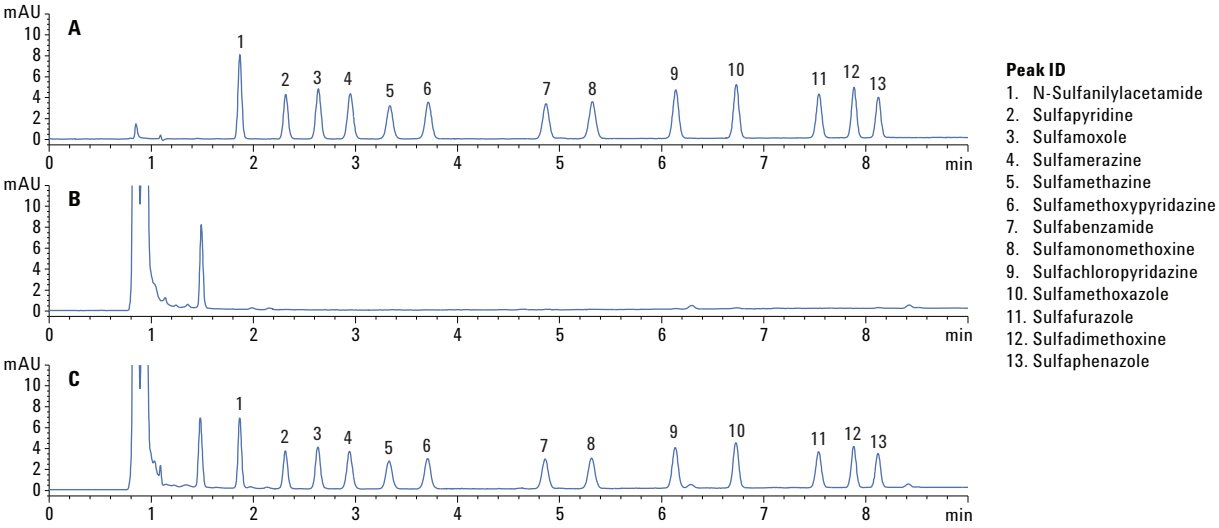

Figure 3. Sulfonamide separations using an Agilent Poroshell 120 EC-C18, 4.6 × 100 mm, 4 µm column. A) sulfonamide standards mixture (0.1 µg/mL); B) blank pork sample; C) sample spiked with standards mixture (10 µg/kg).

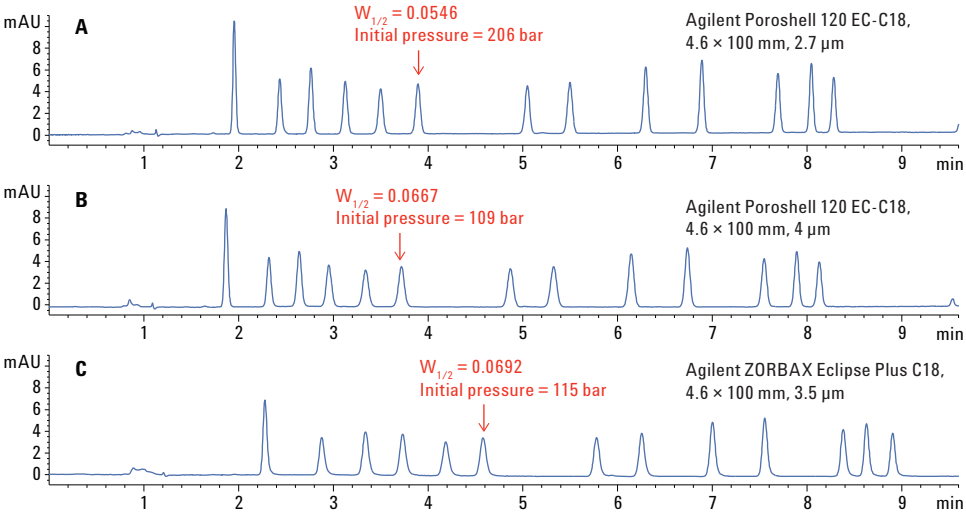

Figure 4. Comparison between Agilent Poroshell 120 EC-C18, 4.6 × 100 mm, 4 µm and 2.7 µm columns, and an Agilent ZORBAX Eclipse Plus C18, 3.5 µm column.

Compared to a ZORBAX 3.5 µm totally porous particle column, the Poroshell 120, 4 µm superficially porous particle column provided slightly higher efficiency with almost the same backpressure. The peak width at half height ($W_{1/2}$) of the 4 µm column dropped by 3.7% compared to the 3.5 µm column, with a 5.5% slightly lower backpressure. In addition, because the Poroshell 120, 4 µm is a 600 bar column, it should have longer lifetime than the totally porous 3.5 µm column, which is only a 400 bar column.

The method was also run on Poroshell 120 EC-C18, 4 µm and ZORBAX Eclipse Plus C18, 5 µm columns with the same configuration of 4.6 × 150 mm (Figure 5). The separation on both columns was excellent and all compounds were resolved. However, the overall peak shapes were significantly sharper on the 4 µm column. The peak width at half height ($W_{1/2}$) with the 5 µm column increased by 33% compared to the 4 µm column, which lead to a higher signal-to-noise (S/N) ratio with the 4 µm column. The backpressure of the Poroshell 120, 4 µm column was increased by over 50% compared to the 5 µm column, but was still significantly low and suitable to any HPLC instrument.

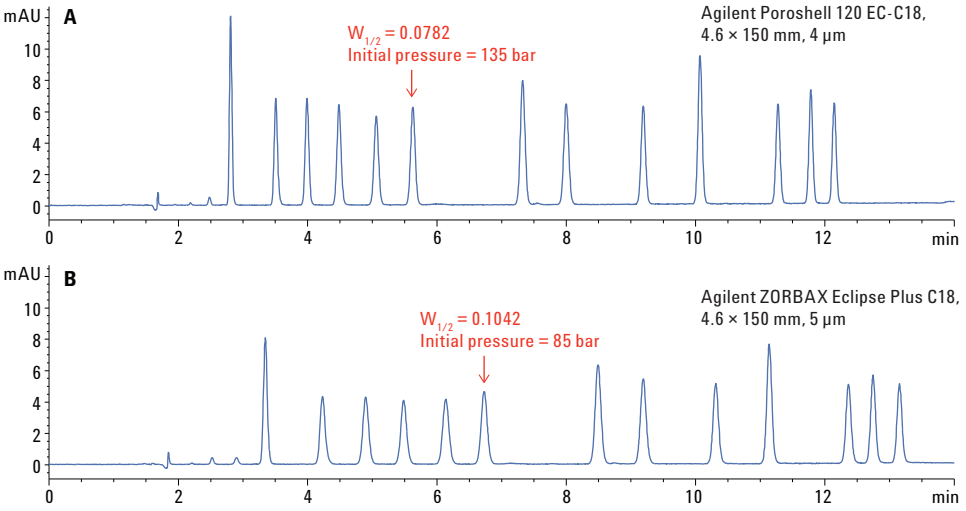

Figure 5. Comparison between Agilent Poroshell 120 EC-C18, 4.6 × 150 mm, 4 µm and Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 5 µm columns.

Linearity and limits of detection

Solutions used to create external calibration curves were prepared by using a combined working solution to spike matrix blanks (10, 40, 200, 500, and 1,000 µg/kg). Matrix blanks were created by taking the pork sample through the entire procedure, including pretreatment and SPE procedures. The limits of detection (LODs) were chosen as the concentration of each compound that gave a S/N ratio greater than 3:1. All of the LODs for the compounds were below 5 µg/kg. The results for the calibration curves are shown in Table 1.

Table 1. Linearity of sulfonamides in a pork sample.

Sulfonamides	Regression equation	Correlation coefficient (R^2)
N-Sulfanilylacetamide	y = 0.048x + 0.430	0.999
Sulfapyridine	y = 0.036x – 0.114	0.999
Sulfamoxole	y = 0.038x – 0.101	0.999
Sulfamerazine	y = 0.038x – 0.914	0.998
Sulfamethazine	y = 0.036x – 0.109	0.999
Sulfamethoxypyridazine	y = 0.036x – 0.111	0.999
Sulfabenzamide	y = 0.038x – 0.069	1.000
Sulfamonomethoxine	y = 0.038x – 0.151	0.999
Sulfachloropyridazine	y = 0.040x – 0.189	0.999
Sulfamethoxazole	y = 0.038x – 0.080	0.999
Sulfafurazole	y = 0.038x – 0.120	0.999
Sulfadimethoxine	y = 0.038x – 0.097	0.999
Sulfaphenazole	y = 0.033x – 0.090	0.999

Recovery and reproducibility

The recovery and repeatability for the method were determined at three levels. The analysis was performed with six replicates at each level. The recovery and reproducibility data are shown in Table 2. The chromatograms of spiked and blank pork extracts (10 µg/kg) are shown in Figure 3.

Table 2. Recoveries and reproducibility of sulfonamides in pork.

Sulfonamides	Spiked level (µg/kg)	Recovery (%)	RSD (n = 6) (%)
N-Sulfanilylacetamide	10	78.54	3.19
	40	75.26	3.72
	80	76.52	2.68
Sulfapyridine	10	80.63	3.16
	40	81.01	3.49
	80	78.49	3.92
Sulfamoxole	10	84.54	4.18
	40	85.21	4.46
	80	80.87	3.26
Sulfamerazine	10	72.86	3.81
	40	73.49	2.98
	80	70.79	3.05
Sulfamethazine	10	84.95	4.76
	40	89.92	3.49
	80	84.32	3.08
Sulfamethoxypyridazine	10	80.89	2.99
	40	81.64	3.51
	80	86.78	3.29
Sulfabenzamide	10	78.65	5.19
	40	74.74	3.64
	80	80.75	6.26
Sulfamonomethoxine	10	79.85	3.79
	40	80.83	3.68
	80	74.47	4.92
Sulfachloropyridazine	10	72.85	3.84
	40	73.63	3.91
	80	72.49	7.48
Sulfamethoxazole	10	75.85	3.64
	40	78.76	3.93
	80	76.91	3.28
Sulfafurazole	10	70.17	4.79
	40	71.64	2.91
	80	68.49	3.14
Sulfadimethoxine	10	82.79	4.93
	40	83.97	3.46
	80	86.43	3.82
Sulfaphenazole	10	75.91	5.56
	40	76.67	5.95
	80	70.19	3.76

Conclusions

The HPLC method developed with the Agilent Poroshell 120, 4 µm column is a reliable technique for the simultaneous quantification of sulfonamides in pork. Both the Poroshell 120, 4 µm column and the 2.7 µm column are effective for the separation of multiple sulfonamides. The 2.7 µm column gives much better resolution and higher efficiency than the 4 µm column. Nevertheless, the back pressure on the 4 µm column is only about half of that on the 2.7 µm column. The Poroshell 120, 4 µm column is more suitable for use on a 400 bar HPLC, while the 2.7 µm column is suitable for 600 bar HPLC. The Poroshell 120, 4 µm column offers a scalable complement to the Poroshell 120, 2.7 µm Fast LC column, with predictable scalability between the two column families. Because of the lower backpressure, it is an excellent bridge to the Fast LC superficially porous column technologies, which offer high resolution at low backpressures.

Compared to a 3.5 µm totally porous particle column, the 4 µm superficially porous particle column provides a slightly higher efficiency with almost the same backpressure. The Poroshell 120, 4 µm column could easily replace the 3.5 µm totally porous particle column. In addition, the Poroshell 120, 4 µm column provides much higher efficiency than the ZORBAX 5 µm column. However, with higher backpressure, the Poroshell 120, 4 µm column is still suitable for any instrument.

Moreover, the results of this study show that Agilent Bond Elut Plexa PCX can be used as an effective method for purification and enrichment of multiple sulfonamide residues in a complex matrix of pork. The recovery and reproducibility results based on matrix-spiked standards are excellent for sulfonamide residue determination in pork subject to regulation. The impurities and matrix effects are minimal and do not interfere with the quantification of any target compound.

References

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2. Anon. *GB 29694-2013, Determination of sulfonamide residues in animal products*; China Standard. General Administration of Quality Supervision, Inspection and Quarantine, Beijing, China, **2013**.

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