A Validated Atmospheric Pressure Chemical Ionization Method for Analyzing Sulfonamides in Pork Muscle

Application

Food

Author

Ralph Hindle
Access Analytical Labs
#3, 2616 - 16 Street N.E.
Calgary, AB. T2E 7J8
Canada

Agilent contact:
Chin-Kai Meng
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

Abstract

This application note presents a simple method for the analysis of sulfonamide antibiotics in pork muscle. Samples were extracted with acidified methanol, centrifuged, and a portion of the extract was diluted with water. This dilution was analyzed directly by HPLC mass spectrometry using chemical ionization, with all compounds eluting in less than 5 minutes. Using an internal standard, recoveries for seven sulfonamides ranged from 84%–118% at a spiking level of 50 ppb (ng/g). The statistically derived detection limit was 10–25 ppb. A comparison was made to the cleaned extracts using solid phase extraction, as well as a comparison of mass selective detector settings for both screening (maximum sensitivity) and confirmation (greater fragmentation). The enhanced sensitivity of the Agilent quadrupole mass selective detector allows this dilution cleanup technique to be used in labs where high throughput is required.

Introduction

Meat, edible organs, animal feed, and animal waste may contain antibiotics, growth hormones, and other chemicals that can enter the food supply. These compounds are added to maintain animal health, to increase animal growth rate, and to reduce stress. Human exposure can result from eating contaminated meat, or contacting runoff and leaching from manure and compost. Health specialists warn that there may be reduced options for effectively treating disease with antibiotics, such as penicillin and sulfa drugs, since antibiotic-resistant strains of bacteria may develop from the low-level exposure.

Sulfonamides are broad-spectrum antimicrobials used in both humans and animals. The maximum residue limit (MRL) in Canada for sulfonamides in meat is 100 ppb (ng/g), and 10 ppb in milk, while the MRL in the European Union is 100 ppb for both of these matrices. The Canadian Food Inspection Agency method for sulfonamides in meat tissue calls for extraction in ethyl acetate, partitioning with glycine buffer, followed by a pH-adjusted back extraction into methylene chloride [1]. Extracts are evaporated, reconstituted, then separated by thin layer chromatography (TLC), derivatized, and quantitated by densitometry. Alberta Agriculture has improved the quantitative and qualitative aspects by using liquid chromatography/mass spectrometry (LC/MS) with atmospheric pressure chemical ionization (APCI) for the final analysis [2]. There are a number of

extraction steps in the Alberta method, and a faster method would greatly benefit laboratories monitoring the food supply for residues.

The goal of this method was to reliably quantitate the sulfa drugs at one-half of the regulatory limit or lower, with minimal sample preparation, and a maximum injection cycle time of 10 minutes. Maximum sensitivity is generally obtained by forming as many parent ions $[M+H]^+$ as possible and minimizing fragmentation. Due to the operational complexity of triple quadrupole instruments, it is also desirable to confirm positive findings on a single quadrupole. This could be achieved by using collision induced dissociation (CID) to enhance fragment ions characteristic of the compounds.

Experimental

Chemicals and Materials

All sulfonamide standards were purchased from Sigma Aldrich Canada, with a minimum purity of 99%. Stock solutions were prepared at 2 mg/mL in acetone, with the exceptions of sulfadiazine and the sodium salt of sulfaquinoxaline. Three mL of 0.2N NaOH was added in order to completely dissolve these compounds. Standard solutions at different concentrations were prepared for spiking and quantitation by diluting with de-ionized water.

Internal standard (IS): sulfachloropyridazine (SCPD) at 2 mg/mL in de-ionized water.

HPLC-grade methanol and acetonitrile were purchased from Caledon Labs (Georgetown, Ontario).

Formic acid (min. 98%), was purchased from EM Science.

Acidified methanol was prepared by adding about 100 µL of 98% formic acid to 100-mL methanol.

Ultra-Turrax T8 homogenizer with 8-mm diameter dispersing element, 50-mL polypropylene centrifuge tubes, and 13-mm polyvinylidene fluoride (PVDF) syringe filters (0.2 µm), were purchased from VWR Scientific.

Oasis HLB (3 cc, 60 mg) solid phase extraction (SPE) cartridges were purchased from Waters.

Sample Preparation

1. For pork muscle, 3 g samples were weighed directly into 50-mL polypropylene centrifuge tubes.
2. The samples were homogenized for 3 minutes with 10 mL acidified methanol using the Ultra-Turrax homogenizer.
3. The samples were then centrifuged for 10 minutes, and the supernatant decanted into a clean test tube.
4. The samples were then re-extracted with a further 10 mL acidified methanol, and centrifuged again.
5. The supernatants were combined, and 1 mL IS (2 mg) was added to the combined extract.
6. The extract was diluted with de-ionized water 1 in 4 (250 µL extract + 750 µL water), filtered through a 0.2 µm PVDF filter into an autosampler vial, and analyzed directly by LC/MS.

By adding an accurately known amount of IS to the combined extracts, there is no need to measure the final volume of the extract. The IS calculations performed by the ChemStation measure the relative amounts of the analytes and IS. This corrects for any concentration or dilution effects in the samples.

Sample extracts were also taken through SPE cleanup cartridges in order to compare with the dilution-only extracts. The 60-mg Oasis HLB cartridges are prewashed by eluting 1.5 mL acidified methanol, followed by 1.5 mL de-ionized water. The 1 mL extract was diluted to 10 mL with de-ionized water, eluted through the cartridges, and the eluant was discarded. The sulfa drugs were then eluted with 1.5 mL acidified methanol. This eluant was evaporated to near dryness under nitrogen. Samples were reconstituted in 1 mL of 25% methanol in water, filtered, and analyzed by LC/MS.

A further comparison was done by evaporating 1 mL methanol extract to near dryness, and reconstituting it in 1 mL of 25% methanol in water without the SPE cleanup. This gave the sample extract the proper solvent composition for HPLC analysis, but without the dilution step to negatively affect the detection limits (DL) of the compounds.

LC/MS Conditions

The LC/MS system was made up of Agilent Technologies 1100 Series solvent degasser, binary pump, autosampler, column oven, diode array detector, and quadrupole mass selective detector (MSD) (Table 1).

Compound Identification and Confirmation

In general, the goal of a monitoring method for target analytes is to separate the compounds from potential interferences and maximize sensitivity on the instrument. Using mass spectrometry (MS), maximum sensitivity is achieved by the production of a single ion, for example, the protonated parent ion $[M+H]^+$ in LC electrospray ionization (ESI) or APCI in target ion mode. However, once a positive is detected, a confirmation must be made as to whether the suspect peak is actually the target analyte, or simply a co-eluting compound that produces the same ion. There are a number of ways to perform the confirmation: re-extract the sample with a different solvent system; further clean up the sample to a higher final concentration, to allow detection of additional confirmation ions or analysis in scan mode; derivatize and analyze by gas chromatography/mass spectrometry (GC/MS); or re-analyze the extract on a triple quadrupole LC/MS/MS. All of these techniques are useful, but the drawback is the additional time and expense involved, especially with LC/MS/MS.

Table 1. LC/MSD Conditions

HPLC	
Column	Zorbax Eclipse XDB-C8, 150 mm × 4.6 mm, 5 µm (p/n 993967-906)
Solvent A	0.1% Formic acid in water
Solvent B	0.1% Formic acid in acetonitrile
Gradient	t_0 = 20% B t_1 = 20% B t_3 = 90% B $t_{6.5}$ = 90% B Post time = 1.5 min
Flow rate	1.0 mL/min
Injection volume	50 µL
Column temp	30 °C
MSD	
Source	APCI (positive ion mode)
Ion dwell time	8 Ions at 63 ms each
Fragmentor	70 V
Drying gas	6.0 L/min
Nebulizer pressure	60 psi
Drying gas temperature	350 °C
Vaporizer temperature	400 °C
Capillary voltage	3000 V
Corona current	4 µA

The Agilent 1100 MSD has the capability of acquiring up to four separate MS signals during the same run, where each signal can be made up of a number of selected ions (SIM) or a full scan spectrum. For example, Signal 1, with a low fragmentor voltage to maximize parent ion response, can include each of the $[M+H]^+$ ions in the target list, while Signal 2, at higher fragmentor voltages can acquire the confirmatory fragment ions. For analytes expected at higher concentrations, Signal 1 could acquire in SIM mode for quantitation, while Signal 2 could be set for scan mode for identification. Figure 1 demonstrates the former example, with the Fragmentor set to 70 V for Signal 1 (MSD1), and 200 V for Signal 2 (MSD2).

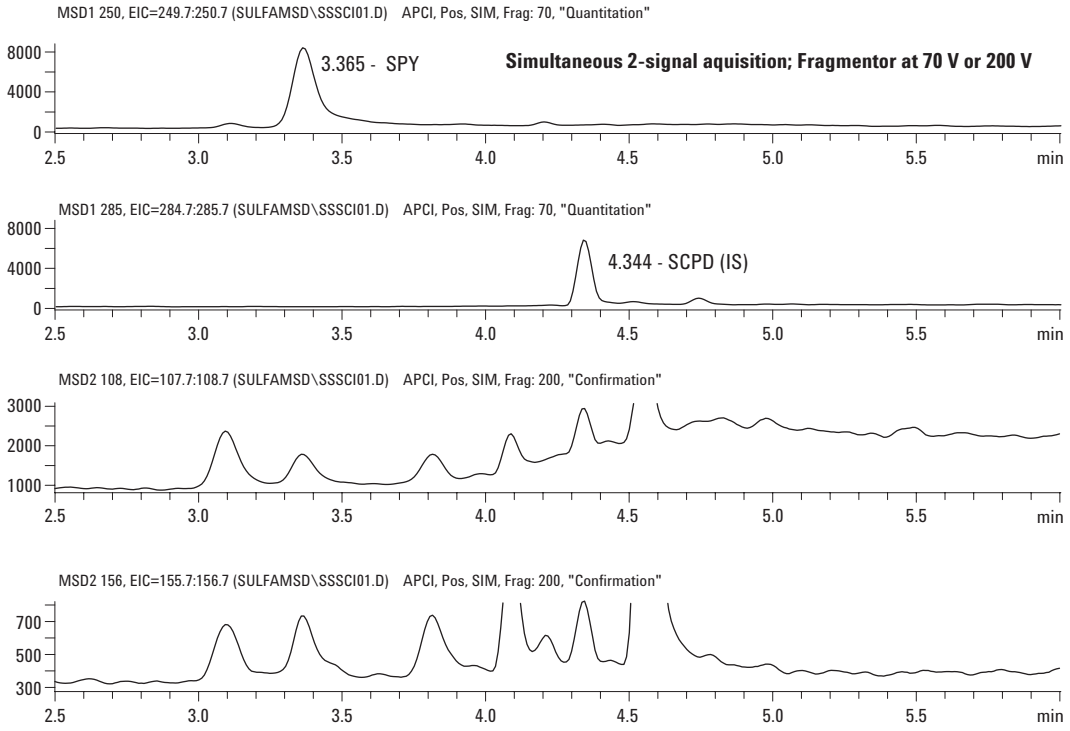


Figure 1. Dual MSD acquisition signals (Masses 108 and 156 are class-specific fragments for sulfonamides).

Table 2 shows the mass spectra for the sulfonamides using various fragmentor voltages. Masses 108 and 156 are class-specific fragments for sulfonamides ($H_2N^+=[C_6H_4]=O$ and $H_2N^+=[C_6H_4]=SO_2$, respectively), and, as such, are very useful diagnostic ions, when acquired along with the protonated molecular ion.

Table 2. APCI Spectra of Sulfonamides, Using Various Fragmentor Voltages

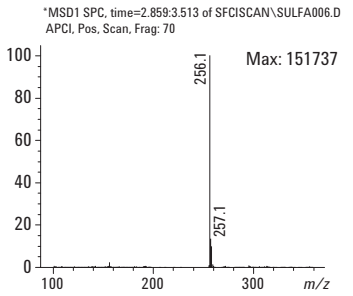
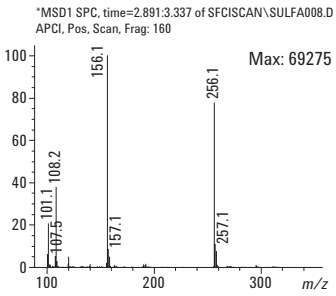
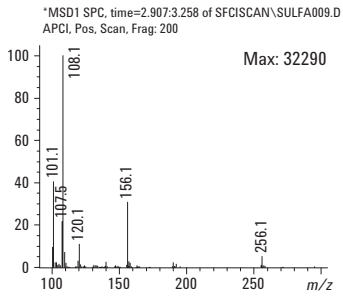
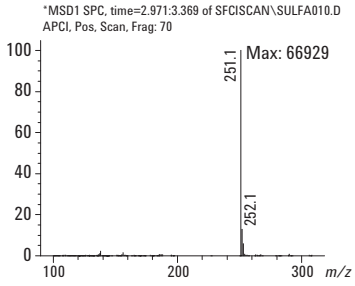
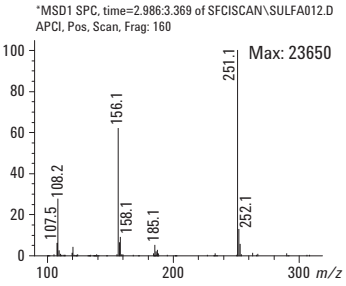
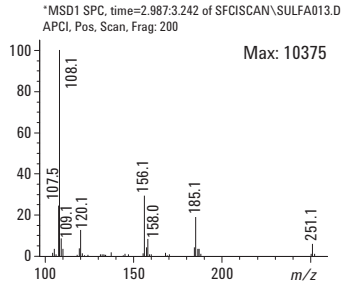
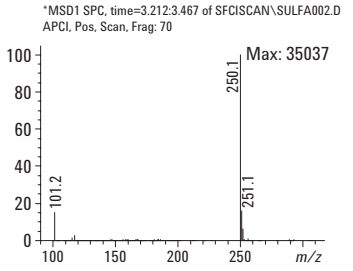
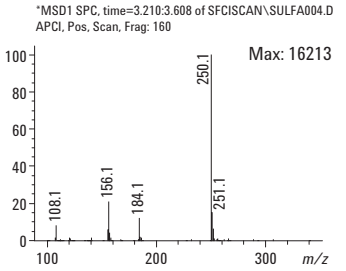
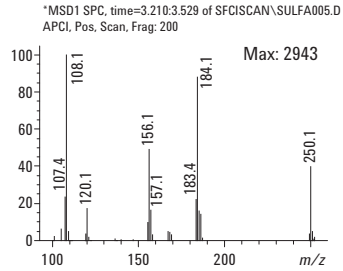
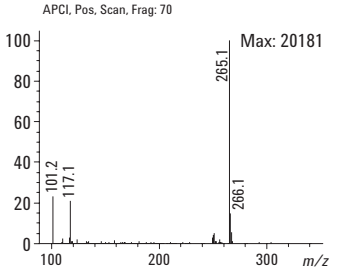
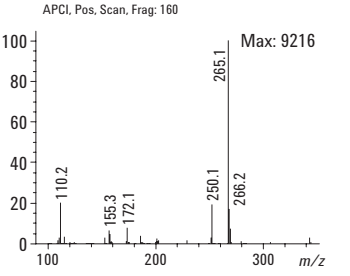
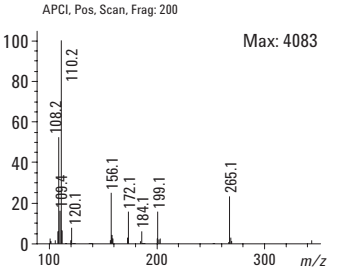
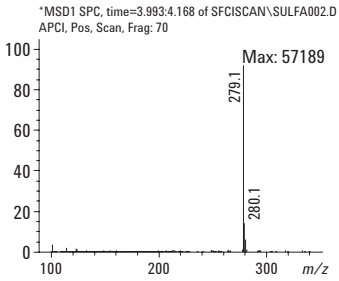
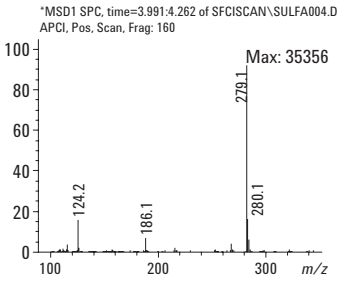
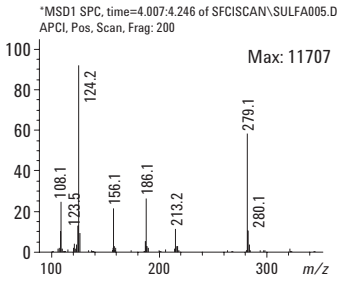
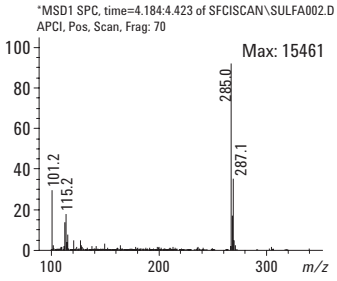
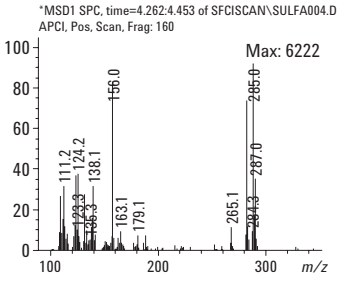
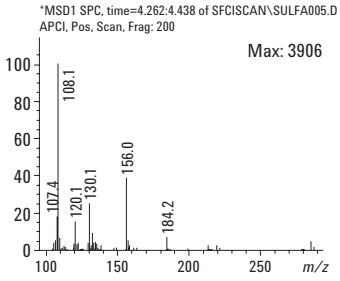
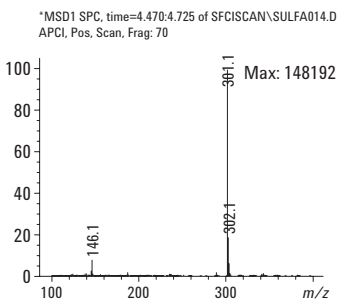
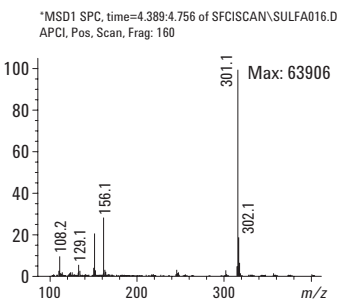
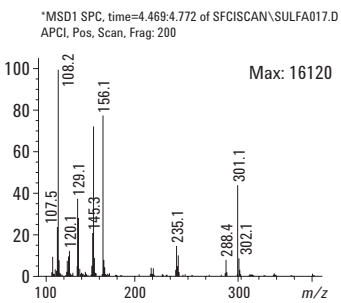
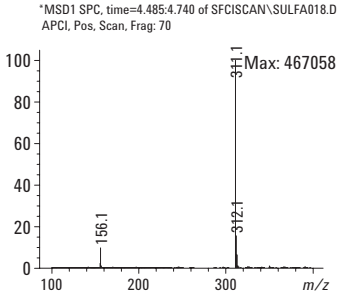
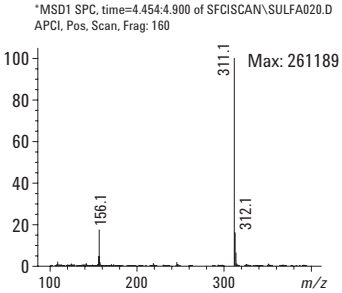
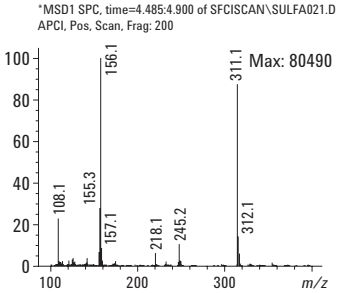
Compound	Fragmentor 70 V	Fragmentor 160 V	Fragmentor 200 V
Sulfathiazole (STZ) $C_9H_9N_3S_2O_2$ MW = 255 RT = 3.05 min			
Sulfadiazine (SDZ) $C_{10}H_{10}N_4SO_2$ MW = 250 RT = 3.09 min			
Sulfapyridine (SPY) $C_{11}H_{11}N_3SO_2$ MW = 249 RT = 3.33 min			
Sulfamerazine (SMR) $C_{11}H_{12}N_4SO_2$ MW = 264 RT = 3.78 min			

Table 2. APCI Spectra of Sulfonamides, Using Various Fragmentor Voltages (Continued)

Compound	Fragmentor 70 V	Fragmentor 160 V	Fragmentor 200 V
Sulfamethazine (SMZ) $C_{12}H_{14}N_4SO_2$ MW = 278 RT = 4.06 min			
Sulfachloropyridazine (SCPD); istd $C_{10}H_9N_4SO_2Cl$ MW = 284 RT = 4.31 min			
Sulfaquinoxaline (SQ) $C_{14}H_{12}N_4SO_2$ MW = 300 RT = 4.51 min			
Sulfadimethoxine (SDMX) $C_{14}H_{12}N_4SO_2$ MW = 310 RT = 4.54 min			

Chromatography

While complete separation of target compounds is not always necessary when using mass spectral detection, it is, however, essential when common ions are present. For example, the protonated molecular ion of SPY is 250 mass units. Due to the naturally-occurring C^{13} isotope, ions 251 coexist with the parent ions 250. Separating SPY from SDZ (m/z = 251) was, therefore, important when trying to optimize the chromatographic conditions, and was achieved as shown in Figure 2. While this results in a slightly longer chromatographic run than would otherwise be necessary, there is more consistent integration of the peaks during data analysis; the chromatogram is easier to interpret; and the amount of SDZ is not underestimated due to co-elution of SPY in the standard mix.

A recently published application shows four sulfonamides were analyzed with an injection cycle time of 1.1 minutes, using a 2-position 10-port valve, two analytical columns in parallel, and a second binary pump [3]. Since most labs do not have such high sample volume requirements, the method described in this application note was developed using more conventional techniques, without the additional hardware costs. Conditions were set up to provide good chromatographic separation in a relatively short time of 6 minutes (total cycle time was 10 minutes).

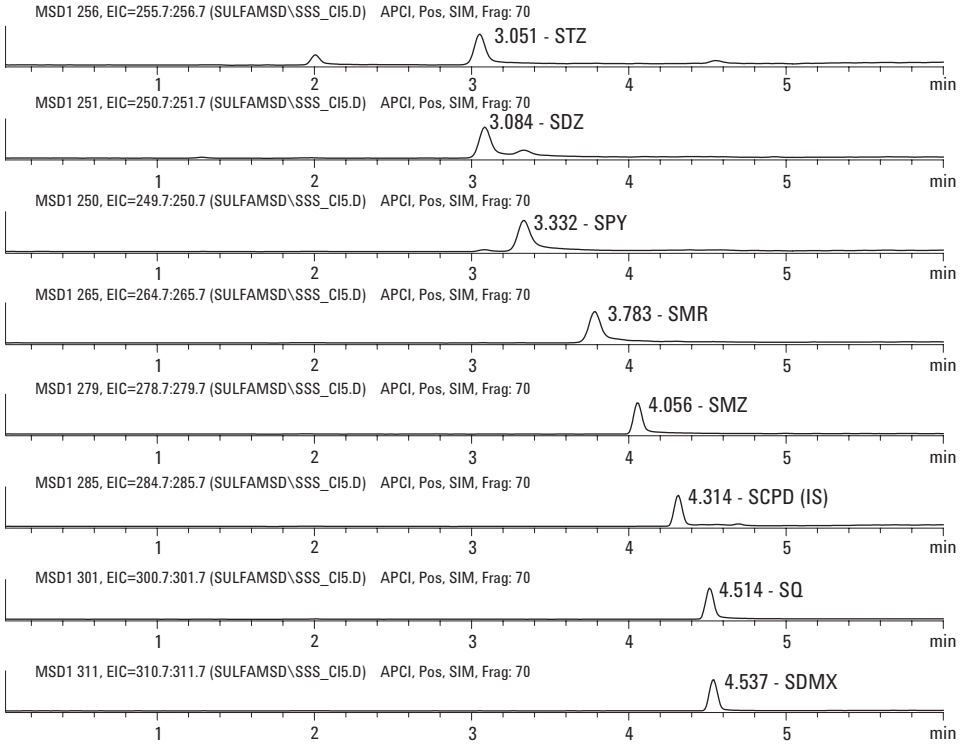


Figure 2. Sulfonamide standard mix, 500 pg each (SIM).

Sample Cleanup

The total ion chromatograms (TIC) in Figure 3 show that there is considerable matrix background from the samples. A simple solvent exchange was performed, where 1 mL of extract was evaporated under nitrogen, and reconstituted in 25% methanol in water. One of the problems with solvent exchange only is the amount of matrix material that is injected onto the HPLC column. Peak shape can be negatively affected by overloading, and eventually the performance of the column will deteriorate. All of this matrix material is also introduced into the MSD. Frequent cleaning and maintenance may be required for the MSD, further reducing productivity.

In order to develop a high-throughput method, keep the number of required steps to a minimum. The Agilent liquid chromatography/mass selective detector (LC/MSD) has enough sensitivity to allow simple dilution of the extracts with water to act as a cleanup technique. This eliminates the need for costly SPE cartridges and analyst time to further prepare the samples. Minimal sample handling can also improve recoveries, since losses are possible at each step.

The third chromatogram in Figure 3 shows how the use of SPE cleanup techniques can remove the majority of co-extracted materials, allowing for a more concentrated final extract and ultimately lower DLs. This also results in a simpler chromatogram for integration and interpretation.

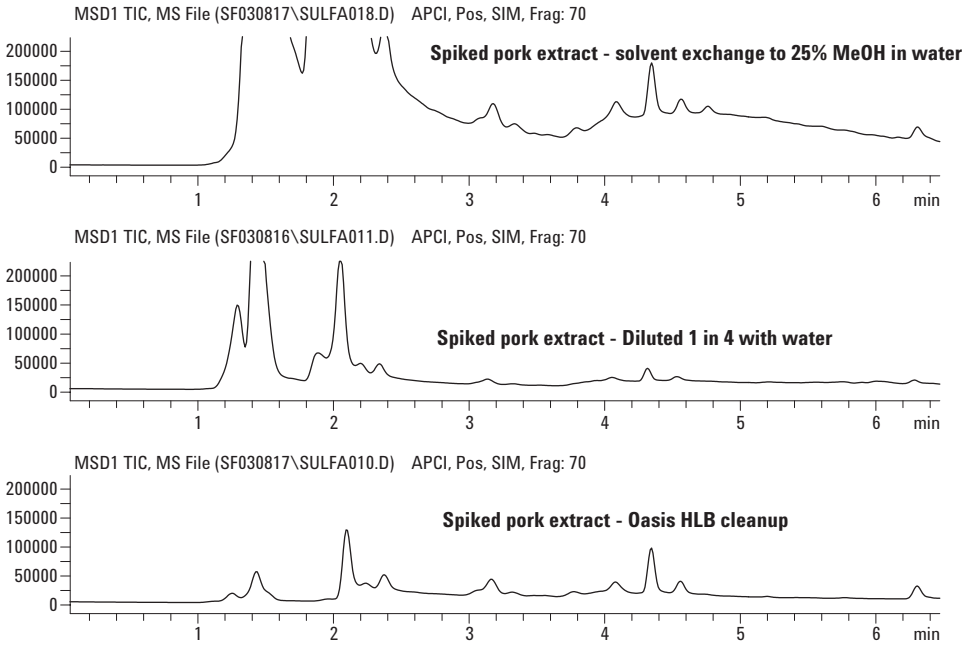


Figure 3. TIC comparisons of various cleanup techniques.

However, where the goal of a method is to screen large numbers of samples to find potential violations of MRLs, a simple dilution technique may be preferred. Dilution could offer enough cleanup for good chromatographic separation, while remaining concentrated enough to meet DL requirements. The second chromatogram in Figure 3 shows a much improved baseline. Figures 4 through 6 show the same analyses with all the target ions in SIM mode.

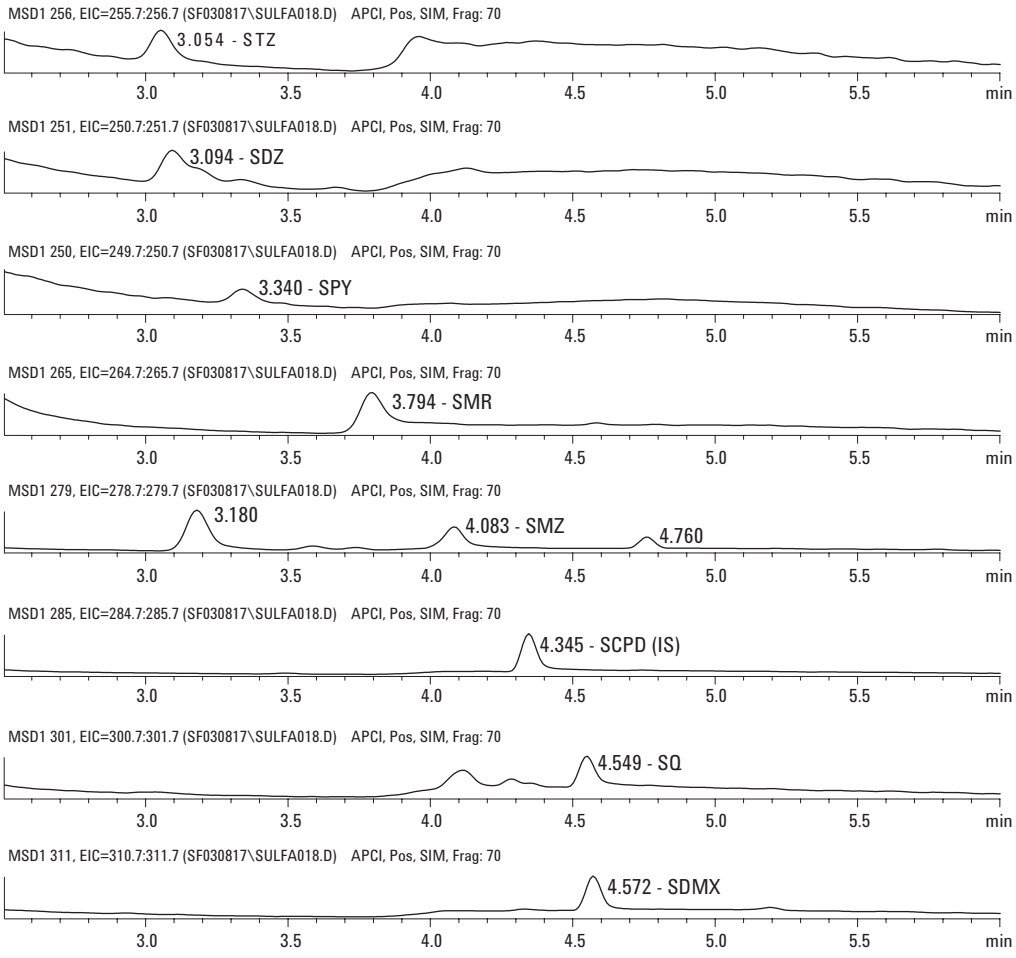


Figure 4. Solvent exchange only (SIM)

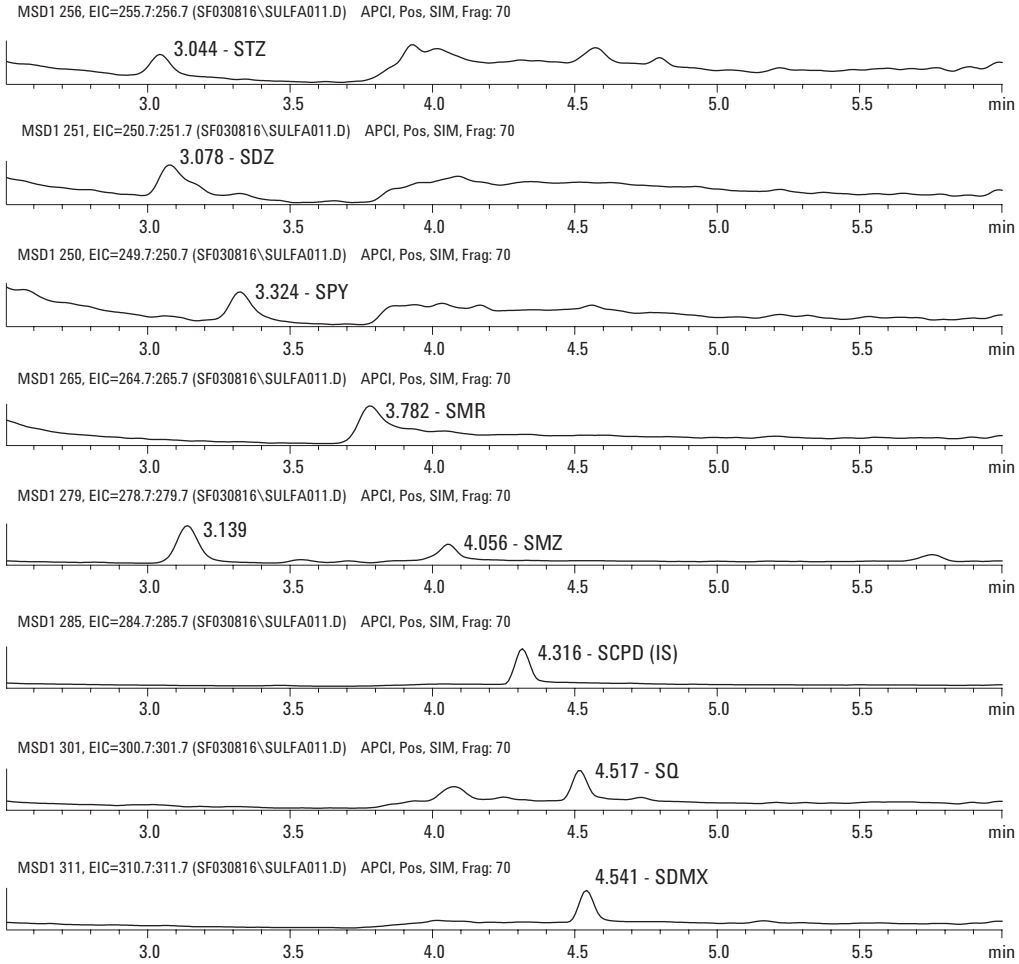


Figure 5. Diluted 1 in 4 with water.

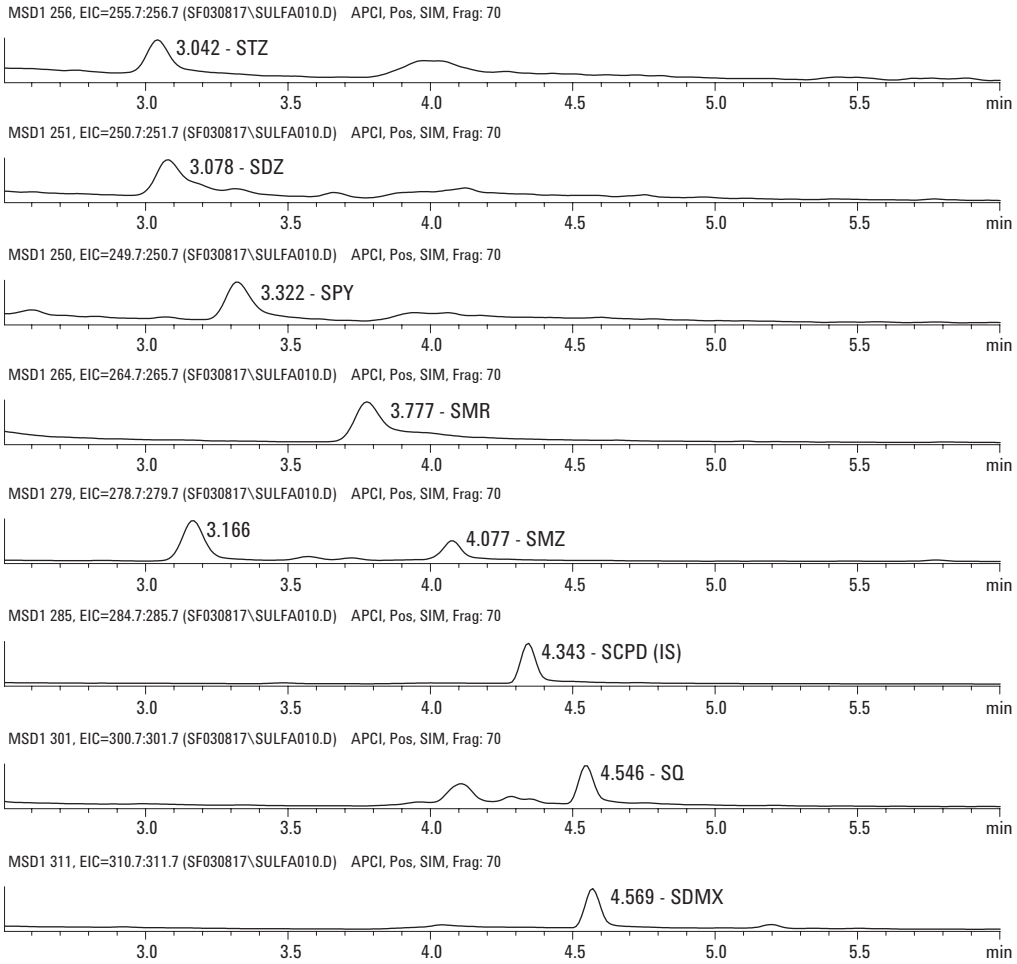


Figure 6. After HLB cleanup (SIM).

Results and Discussion

The recoveries obtained for seven samples spiked at a level of 50 ppb (150 ng of each sulfonamide in 3 g sample) appear in the following tables. The spiking solutions were added before homogenization and allowed to stand for at least 30 minutes before extraction. SMR (sulfamerazine) was added separately at 300 ng per sample before homogenization, and could be used as a surrogate. Results in Table 3 were obtained by simply diluting the extracts 4-fold with water (recovery 84%–118%), while results in Table 4 are from extracts taken through SPE cleanup (recovery 79%–104%).

In both cases, a five-point IS calibration with SCPD was used, with 20 to 200 pg of each target compound injected, plus 2,000 pg SCPD. The five standards were injected both before and after the set of seven spikes, and the curves were created by using the average responses of the two sets of standards. Peak height was used to measure response, as there was less variability compared to peak area, due to the noticeable tailing of these compounds. The linearity results (R^2) are tabulated in Tables 3 and 4.

Table 3. Recoveries of Sulfonamides by Diluting Extracts 1 in 4 with Water

	Amount recovered (ng)							
Description	**STZ**	**SDZ**	**SPY**	**SMR**	**SMZ**	**SCPD(IS)**	**SQ**	**SDMX**
Pork spike 1	167	172	164	317	151	2,000	148	130
Pork spike 2	168	197	68	343	164	2,000	169	137
Pork spike 3	160	183	158	315	157	2,000	133	121
Pork spike 4	158	189	167	336	156	2,000	138	129
Pork spike 5	151	169	154	295	169	2,000	133	129
Pork spike 6	147	161	144	322	143	2,000	120	112
Pork spike 7	144	72	141	272	151	2,000	124	125
Amount spiked (ng)	150	150	150	300	150	2,000	150	150
Mean	156	178	157	314	156	2,000	138	126
SD (Precision)	9	13	11	24	9	–	17	8
MDL (SD × t-stat) ng	29	40	34	77	28	–	53	26
LOQ (SD × 10) ng	94	126	108	245	88	–	167	82
RSD (SD × 100/Mean)	6	7	7	8	6	–	12	7
Accuracy (%)	104	118	104	105	104	100	92	84
Linearity (R^2)	0.9997	0.9996	0.9997	0.9972	0.9996	1.0000	0.9984	0.9992
t-stat (N=7)	3.14	3.14	3.14	3.14	3.14	3.14	3.14	3.14

Table 4. Recoveries of Sulfonamides Using Oasis HLB Cleanup Cartridges

	Amount recovered (ng)							
Description	**STZ**	**SDZ**	**SPY**	**SMR**	**SMZ**	**SCPD(IS)**	**SQ**	**SDMX**
Pork spike 1	161	157	132	273	149	2,000	139	126
Pork spike 2	154	156	132	293	157	2,000	153	131
Pork spike 3	149	158	124	267	155	2,000	132	113
Pork spike 4	145	152	122	279	144	2,000	119	111
Pork spike 5	151	162	127	294	149	2,000	127	121
Pork spike 6	136	147	127	274	136	2,000	116	108
Pork spike 7	148	161	128	275	155	2,000	124	116
Amount spiked (ng)	150	150	150	300	150	2,000	150	150
Mean	149	156	127	279	149	2,000	130	118
SD (Precision)	8	5	4	10	7	–	13	8
MDL (SD × t-stat) ng	24	17	11	33	23	–	40	26
LOQ (SD × 10) ng	76	53	36	104	73	–	128	82
RSD (SD × 100/Mean)	5	3	3	4	5	–	10	7
Accuracy (%)	99	104	85	93	100	100	87	79
Linearity (R^2)	0.9994	0.9994	0.9997	0.9979	0.9998	1.0000	0.9989	0.9989
t-stat (N=7)	3.14	3.14	3.14	3.14	3.14	3.14	3.14	3.14

Table 5 summarizes the comparison of recoveries when diluted with water versus using Oasis HLB cartridge cleanup. Generally there is a greater difference in recoveries for the early eluting compounds, as one might expect. Since the samples are loaded onto the cartridge with a mostly aqueous phase (10% methanol in water), the water-soluble matrix components would tend to pass through the cartridge to waste. Because these early eluting compounds were removed prior to injection on the HPLC column, the chromatograms are cleaner with more reproducible chromatography, as shown by the smaller standard deviations in recoveries. The results from the HLB cleanup exhibited smaller standard deviations and lower minimum detection levels (MDLs).

Conclusion

A fast and sensitive single quadrupole LC/APCI/MS method was developed and validated for detection of sulfonamide residues in pork. The DL ranged from 10 to 25 ng/g of tissue when analyzed by simple dilution of the extracts, and 4 to 13 ng/g when SPE cleanup is used. Instrumental conditions allow injection cycle-time of 10 minutes using typical columns and conditions for most labs.

References

1. TLC-Densitometric Procedure for Sulfonamide Residues in Animal Tissue, SUL-SP08, Canadian Food Inspection Agency, Saskatoon, Saskatchewan, Canada; 2001/04.
2. Sulfonamides in Tissue by LC/MS, Alberta Agriculture, Edmonton, Alberta, Canada, Standard Operating Procedure TX-0278-01.
3. Mark Stahl, “High-throughput analysis with the Agilent 1100 Series high-throughput LC/MS system”, Agilent Technologies, publication 5988-9638EN. www.agilent.com/chem

For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.

Table 5. Comparison of Recoveries Obtained by Dilution vs Oasis HLB Cleanup

Description	STZ	SDZ	SPY	SMR	SMZ	SCPD(IS)	SQ	SDMX
Accuracy % (1 in 4 dilution)	104	118	104	105	104	100	92	84
SD (Precision)	9.4	12.6	10.8	24.5	8.8	–	16.7	8.2
MDL (ng)	29	40	34	77	28	–	53	26
Accuracy % (HLB cleanup)	99	104	85	93	100	100	87	79
SD (Precision)	7.6	5.3	3.6	10.4	7.3	–	12.8	8.2
MDL (ng)	24	17	11	33	23	–	40	26

www.agilent.com/chem

Printed in the USA
October 20, 2003
5989-0182EN

Agilent Technologies