ELSEVIER

Journal of Chromatography B, 723 (1999) 127–137

JOURNAL OF CHROMATOGRAPHY B

Confirmation of multiple sulfonamide residues in bovine milk by gas chromatography–positive chemical ionization mass spectrometry

Valerie B. Reeves[1]

U.S. Food and Drug Administration, Center for Veterinary Medicine, 8401 Muirkirk Road, Laurel, MD 20708, USA

Received 6 April 1998; received in revised form 26 November 1998; accepted 26 November 1998

Abstract

Gas chromatography–mass spectrometry (GC–MS) using positive chemical ionization was utilized to confirm the presence of 10 ng ml^{-1} of nine sulfonamides (SFAs) in bovine milk (50 ml). After the addition of a surrogate and hydroxylamine hydrochloride, the SFAs are extracted with ethyl acetate followed by cyclohexyl solid-phase extraction clean-up. The methylamidotrifluoroacetyl derivatives are prepared and analyzed in selected ion monitoring mode. For regulatory confirmation, the required specificity was achieved by monitoring the molecular ion plus three to five fragment ions for each SFA. Retention times for all SFAs were within 0.1 min of their respective standard. The relative ion abundances were within 10% of those obtained with standards diluted to the same concentration, analyzed on the same day. Concentration was critical, especially for the early eluting SFAs, as the enhancement of the relative abundance of the parent was more pronounced in extracted samples then in the standards. The sensitivity of the mass spectrometer for the different SFAs varied greatly. The amount of SFA necessary to obtain spectra that would meet the confirmation criteria varied from 25 ng on column for the least sensitive to less than 3 ng for the more robust.

Keywords: Sulfonamides; Drug residues; Sulfamethazine

1. INTRODUCTION

Sulfonamides (SFAs) were the first clinically useful antibacterial drugs [1] available and are widely used in veterinary medicine as therapeutic, prophylactic, or growth-promoting agents. Improper use of unprescribed drugs or improper withdrawal times can lead to the presence of illegal residues in milk [2]. A limited nationwide survey found detectable levels of illegal SFAs in shelf milk [3]. This is of toxicological and regulatory concern because it has been reported that sulfamethazine (SMZ) produces thyroid tumors in rodent bioassays [4]. The US Food and Drug Administration (FDA) has set a tolerance of 10 ppb (ng ml^{-1}) for sulfadimethoxine (SDM) in milk [5]. A safe level of 10 ppb has been set by the FDA for the following SFAs: sulfachlorpyridazine (SCP), sulfadiazine (SDZ), sulfamerazine (SMR), sulfamethizole (SMTZ), sulfapyridine (SPD), sulfaquinoxaline (SQX), and sulfathiazole (STZ). SMZ is no longer allowed in milk at any level. Structures of these SFAs are shown in Fig. 1. An LC/UV method was developed in our laboratory to quantify these SFAs in milk [6]. This collaboratively validated method [7] is used to determine if the milk contains residues above these concern levels. However, to support regulatory ac-

[1]Tel.: +1-301-8278164; fax: +1-301-8278170; e-mail: vreeves@bangate.fda.gov

0378-4347/99/$ – see front matter
PII: S0378-4347(98)00548-9

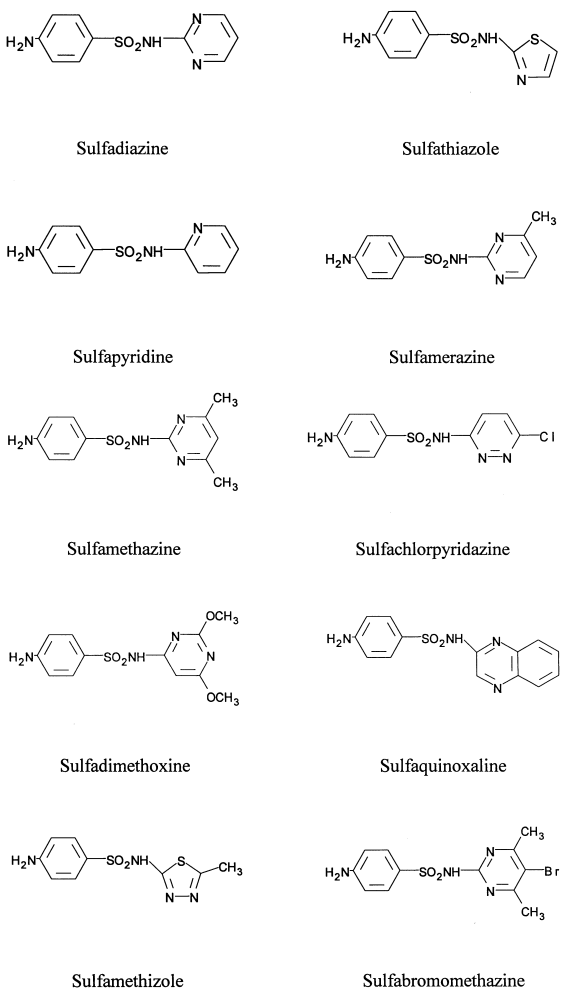


Fig. 1. Chemical structures of sulfonamides analyzed in this study.

tion a method must be able to identify a substance with high specificity as seen with mass spectrometric analysis [8]. Therefore, MS methods are used to confirm the identity of suspect drugs found positive by the one-dimensional chromatographic methods.

Historically SFAs in animal tissue have been methylated and then analyzed after GC separation by electron ionization–mass spectrometry (EI–MS) [9–12]. Analyses of these same derivatives have been accomplished by positive chemical ionization (PCI) GC–MS [13,14]. More recently analyses in tissue have employed discharge-assisted LC–MS [15] and thermospray (TS) LC–MS [16].

With the advent of more sophisticated pumping systems and instrumentation, it has recently been possible to analyze SFAs in milk using LC–TS–MS–MS [17] and atmospheric pressure chemical ionizaton LC–MS [18]. Although to date these methods have not been shown to exhibit the sensitivity necessary for analysis of biologically incurred milk at the level of interest. This report presents our method for the confirmation of nine SFAs in fortified and incurred milk after dosing with SFAs and following the concentration of the individual SFAs in milk until the 10 ng ml^{-1} level is acheived.

2. Experimental

2.1. Apparatus

All GC–MS data were acquired on a 5989A MS with an MS ChemStation data system (DOS series) and direct capillary interface from a 5890-series II GC with a 7673B automatic sampler and split/splitless injector (Hewlett-Packard, Palo Alto, CA). Methane gas pressure was approximately 1 torr optimized with m/z 19>m/z 17 and m/z 28≥m/z 27. Source temperature was 200°C with the quadrupole temperature at 100°C. The MS was tuned in positive ion mode using perfluorotributylamine (PFTBA). Source voltages were adjusted to maximize the signal so that abundance of the PFTBA ion m/z 414>m/z 219 (~2×). All tunes were performed at a GC column temperature of 200°C.

The GC column used for all reported analyses was a DB-1, 30 m×0.25 mm I.D., 0.25 μm film thickness (J&W Scientific, Folsom, CA) with retention gap of fused-silica capillary tubing, deactivated, 1 m×0.25 mm I.D., and glass seal connector. Linear velocity was 32 cm s^{-1} at 200°C. The injection port temperature was 240°C, with a septum purge-on time of 1 min at 0.5 ml min^{-1}. Samples (1 μl) were introduced to the column via a 2 mm I.D. split/splitless liner. The temperature program was 40°C for 1 min, 30°C min^{-1} to 200°C, 6°C min^{-1} to 280°C, and hold for 10 min. The transfer line was set at 280°C. The analyzer was on from 4–23 min.

A standard containing the SFAs of interest was run before the analysis of each sample set. Start and stop times for ion monitoring were set based on the

retention times of the specific SFA while monitoring all molecular ions. While the retention times did not change significantly from day to day, due to relatively small differences in retention times between SFAs, these start and stop times were updated daily. Four to six ions were monitored for each SFA (Table 1). The dwell time per ion in SIM was set at 50 ms. Retention times of the SFAs were monitored to determine the lifetime of the column.

2.2. Reagents

All solvents were Burdick & Jackson HPLC grade obtained from Baxter Diagnostics Inc. (McGraw Park, IL). All reagents were analytical grade and obtained from J.T. Baker (Phillipsburg, NJ) unless stated otherwise. *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide and 1-methylimidazole were obtained from Sigma Chemical Co. (St. Louis, MO). Trifluoroacetic anhydride, dimethyldichlorosilazane, and silylation grade acetonitrile were obtained from Pierce (Rockford, IL). Distilled deionized water (ddH_2O) was prepared to give a resistivity of at least 17 mΩ-cm and further treated with UV irradiation to remove trace organic impurities (Barnstead Co., Boston, MA). Toluene and acetone were dried over anhydrous sodium sulfate before use.

2.3. Standards

Each sulfonamide standard was obtained as the free base from Sigma Chemical Co., except for SQX which was purchased as the sodium salt. Sulfabromomethazine (SBZ) was a gift from Joseph Unruh, USDA, ARS, Eastern Regional Research Center, Philadelphia, PA.

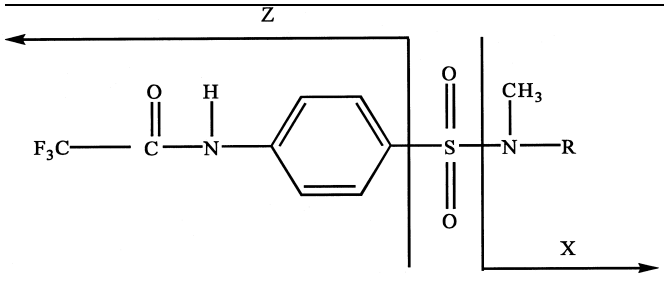
Standard stock solutions were prepared by accurately dissolving approximately 10 mg of SFA standard (SQX standard was corrected for the weight of the salt) in 100 ml of methanol. Working standards were prepared individually, diluted to 10 $\mu g\,ml^{-1}$ in ddH_2O. All standard solutions were prepared every six months. Primary standards were stored at <15°C and working standards at 0–4°C.

2.4. Incurred positive samples

Cows from the USDA dairy herd at Beltsville were dosed individually with 200 mg/quarter of each SFA. The cows were milked at 12 hr intervals and samples collected for analysis by LC [6]. When the

Table 1
Ions monitored for individual sulfonamides

Sulfa	$[M+H]^+$	$[M+C_2H_5]^+$	$[Z+2H]^+$	$[Z+32]^+$	$[XH]^+$	Misc
SPD	360	388	109	137	190	294
STZ	366	394	115	143	190	
SDZ	361	389	110	138	190	150
SMR	375	403	124	152	190	164
SMZ	389	417	138	166	190	
SCP	395		144	172	190	397
SDM	420	449	170	198	190	
SMTZ	381	409	130	158	190	
SBZ	467	(469)	216	(218)	190	138
SQX	411		160	188	190	



level of each SFA reached approximately 40 ng ml^{-1} the samples were aliquoted and stored at −80°C. These samples could then be diluted with either control milk or other incurred milk for analysis at the 10 ng ml^{-1} level of interest.

2.5. Extraction

Five g of hydroxylamine HCl (to aid phase separation) was transferred to a heavy-duty 200 ml screw cap centrifuge bottle. A 50 g milk sample was weighed into the centrifuge bottle. The surrogate, SBZ working standard, was added (amount depending on suspect SFA) to give a final concentration in the injection vial of 50 ng μl^{-1}. The cap was sealed, and the bottle was shaken horizontally on an oscillating shaker for 10 min at approximately 100 cycles min^{-1}. Ethyl acetate (EtOAc, 100 ml) was added and the bottle was shaken as before for 15 min. The bottle was centrifuged for 20 min at 500 *g* at 10°C. The upper (EtOAc) layer was transferred to a 500-ml round bottom flask. The milk was extracted with an additional 50 ml of EtOAc as before, and the extracts are combined. The solvent was removed on a rotary evaporator, and the residue transferred to a 50-ml centrifuge tube with 5 ml of hexane and then three times with 5 ml of 0.1 *M* monobasic potassium phosphate. The tube was capped, vortex mixed for 1 min, and centrifuged for 10 min at 200 *g* and room temperature. The hexane was removed and the samples were washed with an additional 5 ml of hexane. The aqueous layer was loaded onto a preconditioned cyclohexyl solid-phase extraction column, washed with 15 ml ddH_2O, and the SFAs eluted with 5 ml of methanol. The sample was evaporated to dryness under N_2 at 32°C, and the SFAs were transferred to 2-ml screw-cap vials with methanol to a final volume of 800 μl.

2.6. Derivatization

The N^1-methyl-N^4-trifluoroacetyl derivatives were prepared using a modification of the derivatization procedure reported by Mooser and Kock [19] for SFAs in tissue. Standards were prepared from the stock solutions with the following volumes, (a) 50μl for SCP, SDM, SMTZ, and SQX, (b) 10 μl for SMR and SMZ, and (c) 5 μl for STZ, SPD, and SDZ, transferred to a 2-ml screw-cap vial and the solution taken to a final volume of 800 μl. One ml of diazomethane was added and the resultant solution was allowed to sit at room temperature for 15 min. The solution was evaporated and redissolved in 200 μl toluene, 35 μl 10% 1-methylimidazole in toluene, 45 μl acetonitrile, and 75 μl trifluoroacetic anhydride. After heating for 30 min at 60°C, the reaction was stopped by adding 1 ml of 50 m*M* ammonium acetate buffer pH 6.0. The vial was vortex mixed for 1 min and centrifuged at 200 *g* for 10 min. The derivatized SFAs in toluene were transferred to a micro reaction vial. The buffer was re-extracted twice with 150 μl toluene each time. The extracts were combined and the solvent was gently evaporated under N_2 at room temperature. The derivatized standard was dissolved in 200 μl acetone. If the analysis originally used to distinguish the presence of an SFA in milk can give presumed identity, the samples are dissolved in dry acetone to the following concentrations: (a) 20 μl for SCP, SDM, SMTZ, and SQX, (b) 100 μl for SDZ, SMR and SMZ, and (c) 200 μl for SPD, and STZ. If the identity of the SFA is unknown, the sample is dissolved in 20 μl dry acetone and chromatographed. The sample may then be diluted and reanalyzed to the above concentrations, if necessary. Derivatized SFAs may be stored up to 1 week at <15°C before analysis.

3. Results

Table 2 presents a summary of method validation data collected in our laboratory. The average relative abundances for the monitored ions for the individual SFA standards are given. The average differences of the relative abundances of the monitored ions between the standards and the 10 ng ml^{-1} fortified and biologically incurred SFA residues in milk are also shown. For confirmation, the ratios of the monitored ions for the known fortified and the suspect unknown samples must be within 10% of those obtained with standards on the day of analysis. The retention times must be within 0.1 min. It is also necessary to show that the control samples and solvent blanks are not contaminated and therefore would be unable to meet these criteria. Consequently, these samples would be

Table 2
Summary of validation data

m/z	Standard rel. ab. mean±SEM	Fortified % change mean±SEM	Incurred % change mean±SEM	*m/z*	Standard rel. ab. mean±SEM	Fortified % change mean±SEM	Incurred % change mean±SEM
STZ	*n*=3	*n*=6	*n*=5	SCP	*n*=3	*n*=5	*n*=5
394	6.1±0.87	0.00±0.41	0.14±0.08	**397**	5.3±1.92	−1.42±0.59	−1.16±0.07
366	63.8±12.2	−0.78±2.80	2.08±1.24	395	13.4±4.83	−2.26±0.60	−2.72±0.26
190	46.0±5.12	−3.72±2.12	1.72±1.65	190	25.5±1.24	−2.50±2.50	−6.56±1.07
143	19.0±1.13	−0.80±0.35	−1.00±0.78	172	9.9±0.35	−0.40±0.38	−1.06±0.27
115	100	BP	BP	144	100	BP	BP
SPD	*n*=5	*n*=5	*n*=6	SDM	*n*=4	*n*=7	*n*=8
388	6.3±1.32	0.36±0.19	0.05±0.19	449	1.3±0.30	0.37±0.10	0.01±0.10
360	57.3±10.8	0.68±1.44	1.05±	420	11.7±2.42	2.66±0.71	0.36±0.74
294	5.2±0.74	0.40±0.19	0.13±0.20	198	17.6±0.56	0.07±0.45	−0.84±0.36
190	34.0±4.28	0.34±1.53	−2.70±1.02	190	17.3±1.87	−2.33±1,13	−2.74±0.97
137	23.4±0.86	−0.66±0.44	−0.55±1.11	170	100	BP	BP
109	100	BP	BP				
				SMTZ	*n*=2	*n*=3	*n*=5
SDZ	*n*=3	*n*=6	*n*=5	409	5.6±0.35	0.70±0.51	−0.10±0.34
389	4.5±0.95	0.22±0.17	0.34±0.29	381	46.4±5.45	6.43±2.68	−0.22±2.40
361	44.2±7.65	3.07±2.57	4.16±2.25	190	53.1±4.15	−7.10±0.60	−3.20±2.96
190	23.5±4.42	3.98±1.28	3.30±1.51	158	19.1±0.70	0.80±2.54	0.54±1.21
150	5.5±0.46	0.42±0.38	1.78±0.19	130	100	BP	BP
138	19.6±0.95	0.47±0.51	2.18±0.23				
110	100	BP	BP	SQX	*n*=4	*n*=6	*n*=10
				411	5.4±1.54	−0.55±0.24	−0.60±0.26
SMR	*n*=3	*n*=5	*n*=7	190	19.6±2.30	0.07±1.76	0.90±1.37
403	3.2±0.32	0.12±0.17	0.07±0.21	188	26.0±0.39	−0.37±0.34	−0.91±0.37
375	31.5±3.40	2.26±1.42	1.09±1.78	160	100	BP	BP
190	23.1±4.18	2.02±1.31	3.07±0.68				
164	5.6±0.21	−0.50±0.14	−0.21±0.23				
152	21.4±0.22	−0.46±0.74	0.54±0.58			Control	Samples
124	100	BP	BP			mean±SEM	mean±SEM
				SBZ	*n*=6	*n*=6	*n*=26
SMZ	*n*=4	*n*=7	*n*=6	469	14.9±0.95	15.3±2.05	14.2±0.59
417	3.2±0.77	−0.01±0.32	0.12±0.13	467	14.5±0.95	14.6±2.12	13.5±0.61
389	28.4±6.02	1.41±1.61	−1.93±0.62	218	93.8±0.95	94.3±1.04	93.3±0.69
190	22.9±2.49	1.91±0.61	0.70±1.03	216	100	100	100
166	21.7±0.81	−0.19±0.39	−0.33±0.64	190	40.9±1.84	42.6±1.96	44.6±0.84
138	100	BP	BP	138	41.8±2.70	43.2±2.42	40.2±1.29

listed as 'failed to confirm.' For these analyses, SBZ was added as a surrogate to all the samples except those used to test SMTZ. This surrogate was used to follow the extent of extraction and derivatization.

As can be seen, for most of the SFAs, the greatest between day variation in relative abundances was seen for the molecular ion. It was found that the relative abundance of the molecular ion is concentration dependent. This effect was so pronounced in the extracted milk samples that it was necessary that the SFA concentration in the standard and the sample be similiar (Fig. 2). This figure shows the effect of increasing concentration of SPD on the ratio of the molecular ion to the base peak. This phenomena is more dramatic in the SFAs in the first half of the chromatogram (Fig. 3A). There is a break point between SMZ and SCP, where in the second half the relative abundances were fairly consistent over the

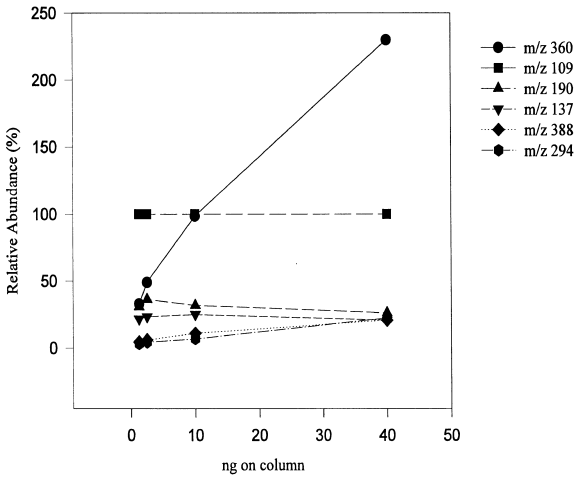


Fig. 2. Relative abundance (m/z 109=100%) of major sulfapyridine ions, in fortified extracted milk, with increasing amount on column.

concentration range studied. Fig. 4 shows the total ion chromatogram of the SIM analysis of 5 ng equivalent on column of STZ, SDZ, SMR, and SMZ from a standard, 10 ng ml^{-1} co-mingled biologically incurred milk, and control milk. Fig. 5 shows the same for 25 ng equivalent on column of SCP, SDM, SMTZ, and SQX. As can be seen, the response of 5 ng on column for the SFAs in figure 4 is similiar to the response for 25 ng on column for the SFAs in figure 5. This effect, along with the different extraction efficiencies, is conspicuous in the biologically incurred milk and most dramatic for the analysis of SQX. The incurred SFAs analyzed in figure 4 have greater than 2×10^6 area counts for 2.5 ng equivalent on column and effective recoveries of 50% or greater. The respective standards for 2.5 ng on column run approximately 2.5 to 4.5×10^6 area counts. In Fig. 5, area counts for biologically incurred SQX are approximately 2×10^5, with an effective recovery of only 11% based on the standard equivalent of 25 ng on column. The approximate recoveries for the other SFAs in Fig. 5 are greater than 50% and the area counts for the other SFAs for incurred samples are more than five times those found for SQX.

Fig. 6 shows the background subtracted scan mass spectrums of the various SFAs used to determine the SIM ions to be monitored. These spectrums were collected when approximately 500 ng of each SFA was injected on column. As stated previously, this pattern is concentration dependent. Peaks at m/z 190, 222, and 236 could be identified in the spectra of all the SFAs. They are attributable to the common trifluoroacylated aniline portion of the molecule. These fragment ions are associated with the halogenated portion of the molecule and result in much broader peaks then the other ions. At concentrations listed above used for the confirmation of the SFAs these ions, except m/z 190, were hard to distinguish from background.

4. Discussion

Regulatory confirmation is based on analogous retention times between standards and unknowns in the chromatographic system. The peak heights for the monitored ion chromatograms must be at least three times that observed for control samples at the same retention time. Further, it is necessary for ion fragmentation patterns of the unknowns to be within a certain percentage of those obtained for pure standards [20]. As an added benefit, the identification of the MI can greatly enhance confirmation. To insure that the MI was present for all SFAs, tuning incorporated adjustment of source voltages to maximize the signal for the PFTBA ion m/z 414.

The specificity required for confirmation of the SFAs is achieved by monitoring the MI and three to five other structure-specific fragment ions. The PCI analysis of the extracted SFAs has been found to be very sensitive to concentration. For relative abundances to be within guidelines, the total ion chromatograph area for the particular SFA in the standard and unknown sample must be similar. At higher concentrations the relative abundance of the MI to the base peak is augmented, sometimes to the point that the MI becomes the base peak. A similiar signal enhancement was previously described by Heller and Schenck [21], which they attributed to a matrix effect in the source. If this happens, diluting the sample to meet the relative abundance criteria as established in the standard is necessary. The abundances of the other ions are not as sensitive to concentration and are matched over a broader concentration range. In those cases where the [$M+$

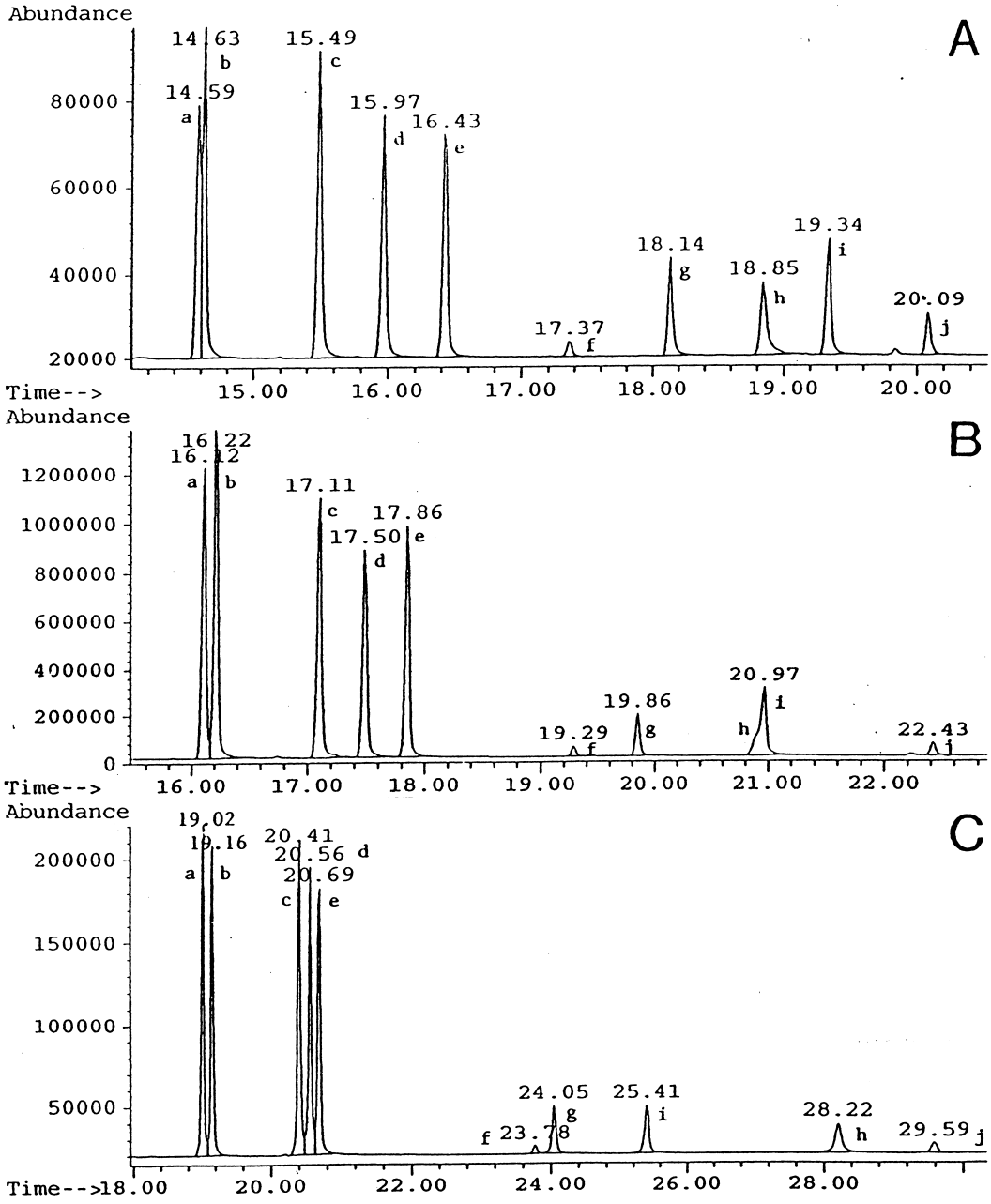


Fig. 3. Retention times on (A) DB-1 column, (B) DB-5MS column, and (C) DB-35MS column for the sulfonamides: (a) sulfapyridine; (b) sulfathiazole; (c) sulfadiazine; (d) sulfamerazine; (e) sulfamethazine; (f) sulfachloropyridazine; (g) sulfadimethoxine; (h) sulfamethizole; (i) sulfabromomethazine; (j) sulfaquinoxaline.

$C_2H_5]^+$ is of sufficient concentration, it may be possible to use this ion rather than the MI for molecular weight identification.

During the study many MS techniques were tried to develop a single assay system that could be applied to the confirmation of the listed SFAs. The

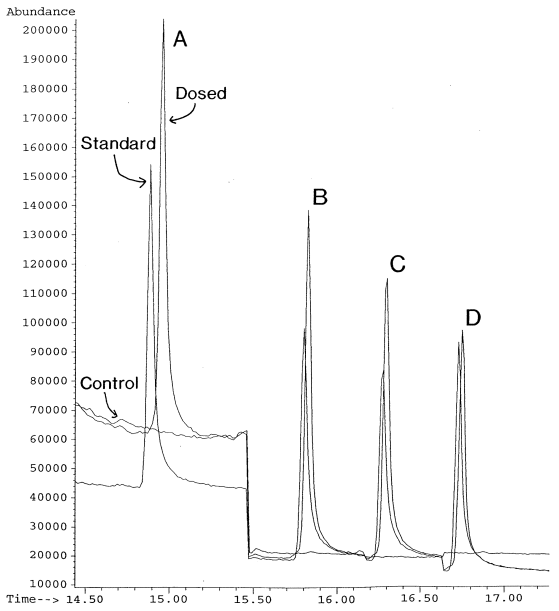


Fig. 4. Overlay of selected ion chromatograms of (A) sulfathiazole, (B) sulfadiazine, (C) sulfamerazine, and (D) sulfamethazine from standard, 10 ng ml^{-1} co-mingled dosed milk, and control milk.

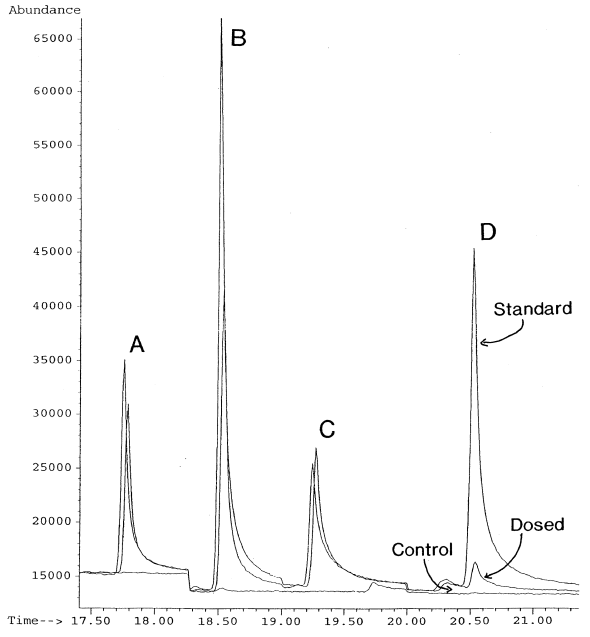


Fig. 5. Overlay of selected ion chromatograms of (A) sulfachloropyridazine, (B) sulfadimethoxine, (C) sulfamethizole, and (D) sulfaquinoxaline from standard, 10 ng ml^{-1} co-mingled dosed milk, and control milk.

credo behind FDA regulatory methods is to develop a procedure with the widest application based on available of instrumentation and practicability of analysis. Mass selective detection MS is the instrumentation most available in State and Federal regulatory laboratories. Because of this, a number of different derivatization techniques were tried but no single procedure was identified that would give sufficient fragmentation for all the SFAs of interest. Non-derivatized SFAs were also analyzed by cool-on-column GC–MS and LC–MS, both particle beam and TS. None of these techniques was able meet the specified criteria. As seen in the procedure by Abián et al. [17], TS–MS results in predominately the detection of $[M+H]^+$ ion. TS–MS–MS[17] analysis results in further fragmentation, but only SMR, SMZ, and SDZ gave sufficient fragmentation ions from the base side of the molecule. And, while sufficient fragmentation ions from LC–APCI–MS [18] seem available to use for confirmation, this procedure was validated with control milk spiked with SFAs at the 100 ng ml^{-1} level. Chromatograms of extracted control milk fortified at 5 ng ml^{-1} show that this method may be extended to action level of SFAs measured in incurred milk, but this will depend on the overall extraction efficiency present in the method.

Typically N^1-methyl-N^4-perfluoroacyl derivatization results in a less polar compound allowing better GC separation. This has been shown, and with the appropriate column (Fig. 3), all the SFAs of interest can be separated. This derivatization reaction results in better electron-capture properties that can be used if a suspect violative SFAs is identified by LC [6]. To determine quickly if the suspect peak is an SFA, the eluant over the identified retention time may be collected directly from the LC and the procedure followed starting at the SPE step. If the suspect peak resulted from an SFA, the EI–MS of the derivatized compound will give a strong signal at m/z 252/251 at the corresponding retention time of the SFA. This will prevent the necessity of the more labor-intensive procedure presented here if the unknown compound is not an SFA.

All data presented here were analyzed on a DB-1, 30 m column. This column was unable to separate the derivatized SPD from STZ and required that they be analyzed separately. The current DB-MS series

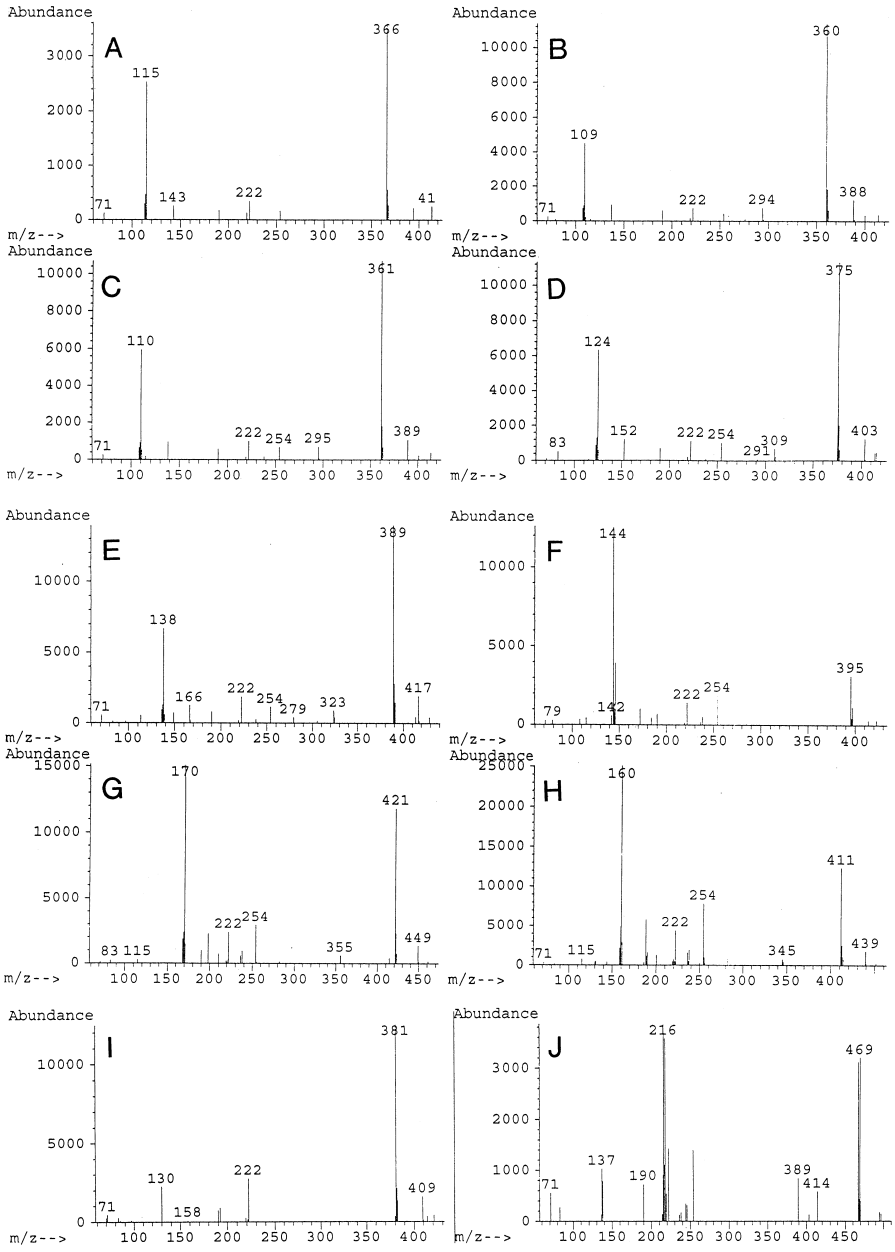


Fig. 6. Background-subtracted scan PICI mass spectra of: (A) sulfathiazole; (B) sulfapyridine; (C) sulfadiazine; (D) sulfamerazine; (E) sulfamethazine; (F) sulfachloropyridazine; (G) sulfaquinoxaline; (H) sulfamethizole; (I) sulfadimethoxine; (J) sulfabromomethazine.

columns (DB-5MS and DB-35MS) are now able to give better separation of these two derivatized SFAs, but these columns also have resolution problems. The DB-5MS column is unable to separate SMTZ from the surrogate, SBZ, and while the DB-35MS can separate all the SFAs, there is lost resolution between several SFAs. The separation of the derivatized SFA standards, monitoring the molecular ion of each SFA over the full time of acquision, is seen in Fig. 3.

Table 3
Compounds tested for interference with method

Albendazole[a]	Dihydrostreptomycin	Minocycline
Amoxicillin	Dimethyl Phosphate	Monensin
Ampicillin[a]	Dimethyl Phosphite	Morantel Tartrate
Amprolium	Diquat Dibromide	Neomycin, Sulfate
Bacitracin, Zinc	Doxycycline	Nitrofurazine
Benzocaine	Fenbendazole[a]	Novobiocin
Ceftiofur	Flumequinn	Oxytetracycline
Cephapirin, sodium	Furazolidone	Phenylbutazone[a]
Chloramine	Gentamicin, Sulfate	Pirlimycin[a]
Chloramphenicol	Ivermectin	Procaine Penicillin G
Chlortetracycline	Lasalocid, Sodium	Spectinomycin
Clorsulon	Leuco Gentian Violet	Tetracycline
Cloxacillin	Leuco Malachite Green	Thiabendazole
Coumaphos	Levamisol	Trichlorfon
Demeclocycline	Lincomycin	Trimethoprim
Dexamethasone	Methylene Blue	Tylosin Tartrate
Dichlorvos	17α-Methyl-Testosterone	

[a] Compounds seen after derivatization.

The procedure was tested for interference from compounds (Table 3) that may be found in raw milk samples. These included beta-lactam, tetracycline, benzimidazole, and aminoglycoside antibiotics, and organophosphate pesticides, among others. Of the compounds tested, only five could be seen after derivatization and none of these compounds interfered with the analysis after extraction of the fortified milk. Milk samples from three different geographical areas of the country were tested, and none were found to contain endogenous peaks that would interfere with the analysis of the SFAs.

Ruggedness testing of the procedure showed a number of variables that are important in the analysis. Recovery of the SFAs through the procedure was sensitive to the effects of drying, temperature, and flow-rate through the SPEs.

The total volume is decreased several places in the procedure, and these steps are listed as critical. The first is the evaporation of the ethyl acetate after extraction. With sufficient vacuum, this step should take 10–12 min. If the samples are kept under vacuum for more than 15 min, the recovery of SFAs will decrease which is especially critical for SCP and SQX. The second reduction in volume occurs after elution of the SFAs from the SPEs with methanol. Originally, a vacuum centrifuge was used to accomplish this, but it was found that at temperatures less than 35°C this required so much time that the recoveries were again lowered. The volumes are also reduced during derivatization. This can be important due to the increased volatility of the SFA derivatives. It is important that whenever the sample is reduced to dryness, it is watched carefully, and solvent is added as soon the sample is dry.

All steps requiring heat (except the production of the trifluoroacetyl derivative) should be done at temperatures less than 35°C. Some SFAs are light sensitive (see labels) and analysis should be carried out under subdued lighting conditions. All of the SFAs should be protected from direct sunlight.

Care must be taken during the steps using the SPE columns. Once the columns are wetted, they should not be allowed to dry except just before elution of the SFAs. A critical area regarding the recovery obtained is the flow-rate through the SPE columns. Too high a flow-rate will yield low recoveries. It has been found that a flow-rate of approximately 1–2 drops per second will give acceptable recoveries. Flow rates higher than this have yielded recoveries resulting in insufficient SFAs available for confirmation.

Milk samples are kept frozen at −80°C. No unusual effects were seen if milk was frozen and then thawed. Experiments using fresh versus frozen milk gave similar results suggesting this is not a significant factor in the analysis.

In conclusion, this method shows applicability

toward confirmation of SFAs at the FDA's current concern levels. It also presents an alternative method of quick screening suspect SFAs after collection from the LC system, minizing the need for the more labor intensive procedure.

Acknowledgements

The author would like to thank the following people: David N. Heller for the development of the macro used to quantitate the relative abundances and the helpful conversations discussing MS analysis in general; Herbert R. Righter for dosing the cows and supplying the milk containing biologically incurred SFAs; and Patricia G. Schermerhorn for the second analyst check of the procedure.

References

[1] J.S. Spinelli, L.R. Enos (Eds.), Drugs in Veterinary Practice, The C.V. Mosby Co, St. Louis, 1978, p. 39.
[2] D.E. Dixon-Holland, S.E. Katz, J. Assoc. Off. Anal. Chem. 74 (1991) 784.
[3] FDA's 1990 Milk Residue Survey. Description of Analytical Methods used for Detecting Sulfonamides and Tetracyclines. Center for Veterinary Medicine, Beltsville, Maryland.
[4] N.A. Littlefield, W.G. Sheldon, R. Allen, D.W. Gaylor, Food Chem. Toxicol. 28 (1990) 157.
[5] 21 CFR 500.640, (1992) 474.
[6] M.D. Smedley, J.D. Weber, J. Assoc. Off. Anal. Chem. 73 (1990) 875.
[7] M.D. Smedley, J. AOAC Int. 77 (1994) 1112.
[8] W.C. Brumley, J.A. Sphon, Biomed. Mass Spectro. 8 (1981) 390.
[9] F.B. Suhre, R.M. Simpson, J.W. Shafer, J. Agric. Food Chem. 29 (1981) 727.
[10] A.J. Malanoski, C.J. Barnes, T. Fazio, J. Assoc. Off. Anal. Chem. 64 (1981) 1386.
[11] R.M. Simpson, F.B. Suhre, J.W. Shafer, J. Assoc. Off. Anal. Chem. 68 (1985) 23.
[12] K. Takatsuki, T. Kikuchi, J. Assoc. Off. Anal. Chem. 73 (1990) 868.
[13] S.J. Stout, W.A. Steller, A.J. Manuel, M.O. Poeppel, A.R. daCunha, J. Assoc. Off. Anal. Chem. 67 (1984) 142.
[14] J.E. Matusik, C.G. Guyer, J.N. Geleta, C.J. Barnes, J. Assoc. Off. Anal. Chem. 70 (1987) 546.
[15] G. Balizs, L. Beneshch-Girke, S. Börner, S.A. Hewitt, J. Chromatogr. B. 661 (1994) 75.
[16] G.K. Kristiansen, R. Brock, G. Bojesen, Anal. Chem. 66 (1994) 3253.
[17] J. Abián, M.I. Churchwell, W.A. Korfmacher, J. Chromatogr. 629 (1993) 267.
[18] D.R. Doerge, S. Bajic, S. Lowes, Rapid Commun. Mass Spectrom. 7 (1993) 1126.
[19] A.E. Mooser, H. Kock, J. AOAC Int. 76 (1993) 976.
[20] J.A. Sphon, J. Assoc. Off. Anal. Chem. 61 (1978) 1247.
[21] D.N. Heller, F.J. Schenck, Biol. Mass Spectro. 22 (1993) 184.