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High-performance liquid chromatography–thermospray mass spectrometry of ten sulfonamide antibiotics

Analysis in milk at the ppb level

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ABSTRACT

Ten sulfonamide antibiotics including sulfanilamide (SNL), sulfamethazine (SMZ), sulfamethizole (SMTZ), sulfachloropyridazine and sulfaquinoxaline (SQX), were analyzed by thermospray (TSP) mass spectrometry on-line with a high-performance liquid chromatography–UV detection system. Except for the pairs SMZ–SMTZ and sulfadimethoxine–SQX, the standards were resolved in both the UV and TSP profiles. Co-eluting compounds could be differentiated in TSP by their different relative molecular masses. The $[M + H]^+$ ion was the base peak for all the standards except SNL, which showed an $[M + NH_4]^+$ ion. Collision-induced dissociation of the $[M + H]^+$ ions afforded daughter ion spectra characterized by common ions at m/z 92, 108 and 156, and ions derived from the amine substituent ($[MH - 155]^+$). TSP detection limits [signal-to-noise ratio (S/N) > 3] were below 20 ng (scan mode), 2 ng (selected reaction monitoring, daughter ions from $[M + H]^+$) and 400 pg (selected ion monitoring). UV detection limits were *ca.* 2 ng ($S/N > 5$). Results obtained from the multi-residue analysis of spiked cow milk samples at the low ng ml level are presented.

INTRODUCTION

Sulfonamides are a group of antibacterial agents widely used in veterinary practice for the treatment of infections and to promote growth of food-producing animals. The presence of residues of these compounds can be detected in meat [1] and milk [2,3] from treated animals. Owing to the possible carcinogenic character of some members of this group [4,5], and the potential development of resistance through ingestion that could make sulfonamides ineffective as therapeutic drugs [6], analysis for these compounds has become necessary in food surveys. For some sulfonamides the US Food and Drug Administration has established limits that oscillate between 0 and 100 ppb (w/w) in edible animal tissues and between 0 and 10 ppb in milk [7].

Current analytical methods for sulfonamides are based on gas chromatographic (GC) or liquid chromatographic (LC) techniques. Various methods using GC with electron-capture detection [8] or high-performance LC (HPLC) with UV detection [9] that afford detection limits below the 100 ppb level have been published recently. In addition, mass spectrometry (MS) was suggested or used for confirmation of the target compounds by these workers.

HPLC methods offer the advantage over GC that they require no previous derivatization of the sample. Some techniques that use LC–MS interfaces such as HPLC–MS using direct liquid introduction (DLI) [10] and supercritical fluid chromatography coupled to TSP or moving-belt interfaces [11] have

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been shown to be useful for analyses for sulfonamides in meat and some biological fluids such as plasma and urine. Recently, Pleasance *et al.* [12] showed the utility of HPLC–ionspray MS for the determination of 21 sulfonamides and described the application of parallel HPLC–diode-array UV detection and HPLC–ionspray tandem mass spectrometry (MS–MS) to the characterization of sulfadimethoxine in salmon flesh. Finally, LC–electrospray MS using capillary LC and capillary zone electrophoresis has also been applied by Perkins *et al.* [13] to the analysis of sulfonamide standards.

Among the LC–MS techniques, HPLC–thermospray (TSP) MS could be considered as one of the most suitable methods for the determination of medium- and high-polarity compounds. TSP has been applied previously with good results to the determination of sulfonamides in meat [14]. Although the eluent composition with TSP is restricted to a relatively narrow range of buffers and solvents, the spectrometric information obtained often easily compensates for the loss of chromatographic separation or resolution. Further, MS–MS techniques can be applied in order to enhance structural information. The inherent high selectivity of the MS–MS technique often allows the design of methods with minor sample clean-up requirements.

In this paper, we describe the use of HPLC–TSP-MS and HPLC–TSP-MS–MS for the determination of ten sulfonamides (Fig. 1). This list includes the more important ones in terms of the violation rates of the established maximum levels, *i.e.*, sulfamethazine, sulfadimethoxine, sulfathiazole and sulfachloropyridazine [8].

The application of TSP to the determination of these compounds in milk at the [illegible] ng/ml level is presented. Some approaches to the direct analysis of milk by TSP have been previously investigated in

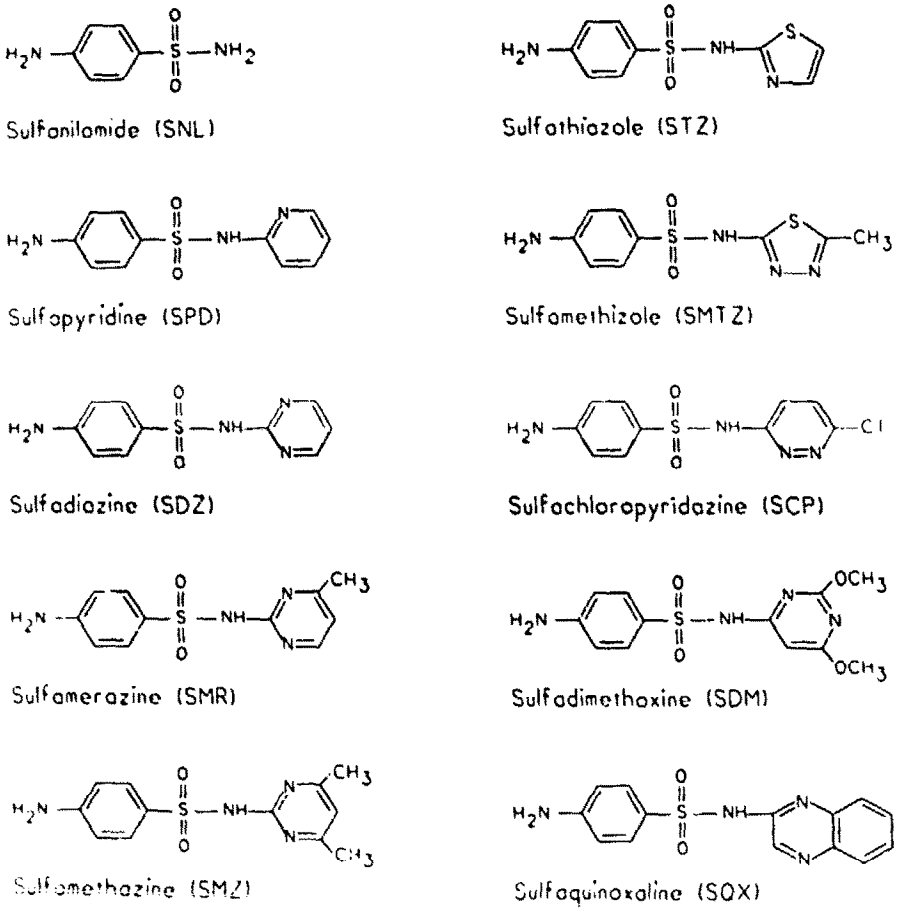

Fig. 1. Structures of the ten sulfonamides.

this laboratory for a group of three sulfonamides [15]. In this work, we also tested the use of on-line preconcentration and clean-up methods in order to obtain a rapid and specific procedure for multi-residue analysis.

EXPERIMENTAL

Standards and reagents

Sulfanilamide (SNL), sulfapyridine (SPD), sulfadiazine (SDZ), sulfamerazine (SMR), sulfamethazine (SMZ), sulfathiazole (STZ), sulfamethizole (SMTZ), sulfachloropyridazine (SCP), sulfadimethoxine (SDM) and sulfaquinoxaline (SQX) (see Fig. 1) were obtained from Sigma (St. Louis, MO, USA). Standard solutions at 1 mg/ml in methanol were prepared and stored below 0°C.

Ammonium acetate was obtained from Fluka (Ronkonkoma, NY, USA). Water was purified through a Milli-Q system (Millipore–Waters, Bedford, MA, USA). Other reagents and solvents were of analytical-reagent or chromatographic grade.

Preparation of milk extracts

A 5-ml volume of control and spiked (5–500 ng/ml) milk (commercially available whole cow milk) were acidified with concentrated hydrochloric acid (100 μl) and sonicated for 15 s. Proteins were removed by centrifugation at 3000 *g* for 10 min. The precipitate was washed with 2 ml of water and recentrifuged. Lipids in the aqueous phase were extracted twice with 5 ml of hexane. Phase separation was helped by centrifugation for *ca.* 1 min at 1500 *g*. The extracted aqueous sample was evaporated to dryness at low pressure, the residue was dissolved in methanol and the solution was centrifuged. The methanolic phase was evaporated to dryness and the residue was dissolved in 3 ml of water. Aliquots of 50–500 μl of this solution were injected.

Chromatography

A Spectra-Physics (San Jose, CA, USA) SP8700XR programmer and pumping system, a Rheodyne (Cotati, CA, USA) Model 7125 injector with a 20-μl sample loop and a 5-μm Spherisorb ODS-2 reserved-phase column (15 × 0.46 cm I.D.) (Phase Separations, Norwalk, CT, USA) were used. The column exit was connected to a Spectra 100 variable-wavelength detector (Spectra-Physics) equipped with a Model 9550-0150 high-pressure microbore cell (2 mm path length, 250 nl) capable of operating up to 7000 p.s.i. (1 p.s.i. = 6894.76 Pa) (Linear Instrument, Reno, NV, USA). A Shimadzu (Kyoto, Japan) C-R3A Chromatopac integrator was used as a recorder for the UV detector.

Two solutions were used for chromatography. Solvent A was a 0.1 *M* ammonium acetate buffer (1% formic acid) and solvent B was acetonitrile (ACN)–water (70:30) containing 0.1 *M* ammonium acetate (1% formic acid).

Sulfonamides were chromatographed at a flow-rate of 1 ml/min, with a gradient programme starting at 100% solvent A. After injection a linear gradient up to 20% solvent B in 30 s was used. After 1 min, solvent B was increased to 75% in 10 min.

A schematic diagram of the system used for the analysis of spiked milk extracts is shown in Fig. 2. The gradient programmer (PROG), HPLC pump (PUMP A), injector (INJ), analytical column (COL 2) and solvents are the same as described before, except for the use of a 500-μl sample loop. The analytical column (COL 2) was preceded by a precolumn unit (COL 1) from Brownlee Labs. (Santa Clara, CA, USA). The precolumn consisted of an MPLC holder and a disposable 3-cm cartridge filled with 10-μm RP-18 stationary phase, and was used as a preconcentration and clean-up system. The precolumn flow could be directed to either the analytical column or waste by means of a Rheodyne Model 7040 switching valve (V2). Another Model 7040 valve (V1) was used in order to backflush the precolumn system when needed. Continuous eluent flow through the analytical column to the mass spectrometer during the preconcentration and clean-up steps or during precolumn backflushing was assured by means of an independent HPLC unit (PUMP B) (Model 6000A, Waters, Milford, MA, USA). Samples were injected (100–500 μl) under the initial HPLC conditions and after a 3-min wash of the precolumn the flow was directed to the analytical column and the solvent gradient indicated before was started.

Thermospray mass spectrometry

A Finnigan (San Jose, CA, USA) TSQ 70 triple quadrupole mass spectrometer equipped with a Finnigan TSP source and interface was used. The exit of the UV detector was connected to the TSP

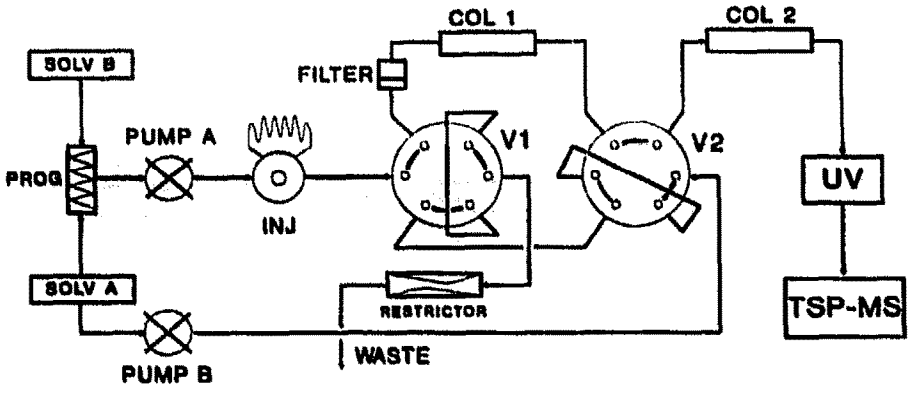

Fig. 2. Scheme of the HPLC system used for the analysis of milk samples. Valve position is depicted as it is during TSP analysis (COL 1 on-line with COL 2). The valve V1 allows backflushing of the precolum COL 1; valve V2 switches the exit of COL 1 between WASTE (during preconcentration and clean-up) and the entry of COL 2 (during TSP analysis). For details, see text.

interface through a 0.5-μm on-line HPLC filter.

The interface temperature was set at 80–85°C. The source and manifold temperatures were kept at 250 and 70°C, respectively. For the MS–MS experiments, argon at *ca.* 0.4 mTorr (1 Torr = 133.322 Pa) (manifold readout) was used as the collision gas. Collision energies of 40–50 eV were used for most of the work.

RESULTS AND DISCUSSION

The structures of the compounds assayed are depicted in Fig. 1. The on-line UV and TSP profiles obtained using our chromatographic conditions are shown in Fig. 3. As can be seen in the UV profile, except for the pairs SMZ–SMTZ (8.4 min) and SDM–SQX (12.1 min), complete chromatographic resolution was obtained. The chromatographic peaks in the TSP profile show some tailing that is not observed in the corresponding UV chromatogram. This loss of resolution on the TSP profile was not the result of the presence of dead volumes due to the on-line UV detection system and thus should be explained by band diffusion and adsorption–des-

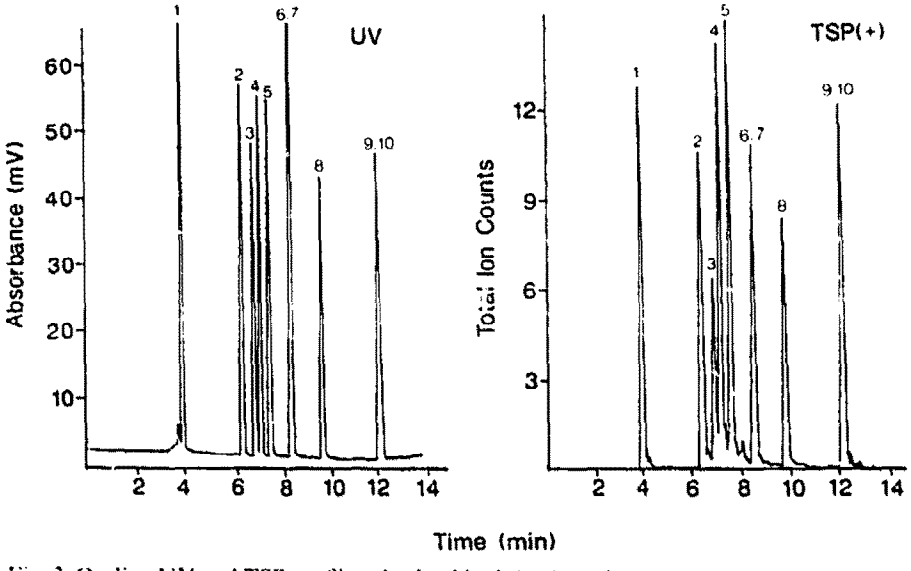

Fig. 3. On-line UV and TSP profiles obtained by injection of 400 ng of each sulfonamide (40 ng of SQX). HPLC conditions as indicated under Experimental. For these chromatograms COL 1 in Fig. 2 was bypassed and no preconcentration and clean-up steps were used. UV detection at 254 nm. Full-scan (100–300 u) MS detection of positive ions; filament off. 1 = SNL; 2 = SDZ; 3 = STZ; 4 = SPD; 5 = SMR; 6 = SMTZ; 7 = SMZ; 8 = SCP; 9 = SDM; 10 = SQX.

orption processes in the TSP interface. These adsorption–desorption phenomena could also be responsible for some spurious TSP signals, such as that observed at 8 min in the profile.

Acetonitrile was shown to be a better organic modifier than methanol for this application (Fig. 4). Although in general methanol affords better ionization efficiencies than ACN when these solvents are used at medium or high concentrations in TSP, in this instance methanol-based eluents showed more background noise and instability during the gradient mixing process. In addition, TSP chromatographic peaks were sharper using ACN as the organic modifier.

The use of an interface temperature gradient was not necessary for good performance because the ACN concentration was kept low during the chromatography and most of the compounds eluted in a narrow range of ACN percentage (between *ca.* 15 and 35%).

The TSP mass spectra of these compounds were very simple (Table I). Except for SNL, all the spectra showed a base peak due to the $[M + H]^+$ ion and very little if any fragment ion signals. SNL showed an $[M + NH_4]^+$ adduct (base peak) in addition to the protonated molecule. Other low-abundance signals were observed in the spectra. SMZ, SCP, SDM and SQX showed signals at $[MH-155]^+$ that also were observed in the collision-induced dissociation (CID) spectra as shown below. Further, SPD showed a signal at *m/z* 186 that could arise from the loss of SO_2 from the $[M + H]^+$ ion at *m/z* 250.

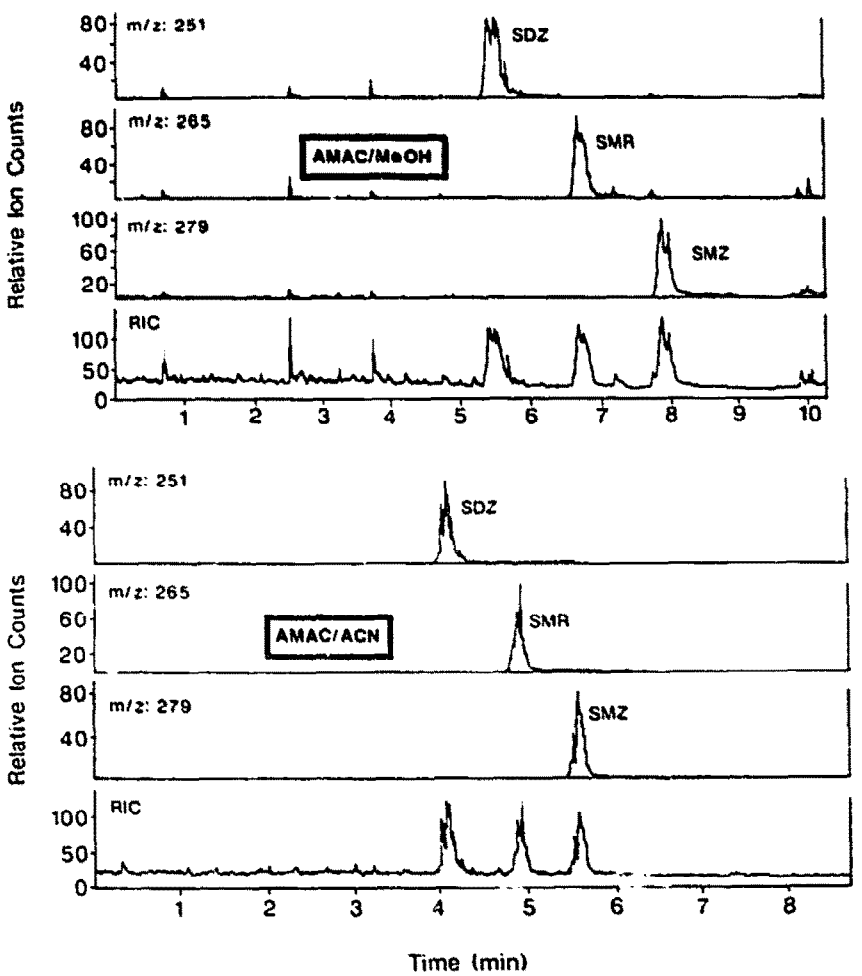

Fig. 4. HPLC–TSP-MS of SMZ, SMR and SPD using (a) MeOH- and (b) ACN-based eluents. The eluent gradient programs were (a) from 10 to 27% MeOH in 4 min and (b) from 12 to 25% MeCN in 4 min. Other conditions as decribed under Experimental.

TABLE I

TSP MASS SPECTRA AND RETENTION TIMES (t_R) OF THE TEN SULFONAMIDES

Compound	t_R (min)[a]	M_r	Base peak	Other ions[b]
SNL	3.9	172	$[M + NH_4]^+$	173[c] (25)
SPD	7.2	249	$[M + H]^+$	186 (2)
SDZ	6.4	250	$[M + H]^+$	–
SMR	7.6	264	$[M + H]^+$	–
SMZ	8.5	278	$[M + H]^+$	124[d] (2)
STZ	6.9	255	$[M + H]^+$	–
SMTZ	8.4	270	$[M + H]^+$	–
SCP	9.8	284	$[M + H]^+$	130[d] (3)
SDM	12.1	310	$[M + H]^+$	156[d] (2)
SQX	12.2	300	$[M + H]^+$	146[d] (7)

[a] HPLC–TSP-MS conditions as described under Experimental and in Fig. 3.

[b] The relative abundance (%) is given in parentheses after the m/z value of the ion.

[c] $[M + H]^+$ ion.

[d] $[MH - 155]^+$ signal ($m/z = M_r - 154$). See also Fig. 5 and Table II.

As the ten compounds have different relative molecular masses, co-eluting sulfonamides can easily be differentiated by their different $[M + H]^+$ base peaks. For this reason, chromatographic resolution and separation are not as critical parameters as in UV methods and therefore it is not neccesary to use time-consuming HPLC procedures or more than one injection per sample as described previously for the multi-residue analysis of these compounds [9].

Full-scan detection limits were in the range 5–20 ng of the compounds injected on-column (scan range 150–330 u, $S/N > 3$ for the ion trace at $[M + H]^+$). These values are slightly over the 2 ng detection limit ($S/N > 5$) estimated for the UV analysis using our chromatographic system. UV detection limits are determined by the high absorption of the eluent and the baseline drift at high detector sensitivities owing to the ammonium acetate buffer and the ACN gradient programming. Further, and for comparative purposes, it should be taken into account that the reduced path length of the microbore UV cell results in the loss of about two thirds of the sensitivity of a conventional cell.

Selected ion monitoring of the $[M + H]^+$ signals ($[M + NH_4]^+$ for SNL) afforded detection limits of *ca.* 400 pg on-column (multiple ion detection of the ten ions, 2 s per cycle, $S/N > 5$). These detection limits could be lowered to the 100 pg level by programming the ions to be monitored at the different elution zones (one to three ions monitored at the same time).

CID of the $[M + H]^+$ ion ($[M + NH_4]^+$ for SNL) gives tandem mass spectra characterized by four major signals at m/z 92, 108, 156 and $M_r - 154$ (Table II and Fig. 5). These signals are equivalent to those previously observed in DLI [10], chemical ionization [8,16,17] ionspray [12] and electrospray [13] MS–MS of these compounds. Brumley *et al.* [17] using high-resolution mass measurements and, more recently, Pleasance *et al.* [12] using the data obtained from granddaughter ion experiments suggested some possible structures and formation pathways for these ions. In addition to these major daughter ion peaks, signals corresponding to the $[MH - 93]^+$ and $[MH - 66]^+$ ions are observed in some of the spectra with low relative abundance and can be assigned to the $[O_2SNHR]^+$ and $[MH - H_2SO_2]^+$ ions [10,12,13,16,17].

Collision-induced ions at m/z 92, 108 and 156 are common to the sulfonamides when TSP is used as the ionizing technique. It is interesting that, for SNL, the signal at m/z 156, also present in its electrospray tandem mass spectra [13], is not observed in the CID spectra of the $[MH]^+$ ion when ionspray, a closely related ion evaporation-based technique, is used [12]. These common daughter ions can be used for group recognition in MS–MS. In this way, selected reaction monitoring in the parent ion mode (Q3 focuses on the fragment at m/z 156 and Q1 focuses alternatively on the different $[M + H]^+$ parent ions) affords additional specificity to the analysis. The fragment at m/z 156 was selected as the daughter ion to be monitored because of its high relative abundance in all ten of the sulfonamides.

Optimum collision gas pressure (determined as that which provides a maximum signal at 50 eV collision energy) was found between 0.3 and 0.5 mTorr (manifold readout) for this fragmentation. Lower or higher pressures afforded a low CID fragment intensity owing to a small number of collisions or to ion scattering, respectively. Characterization of the actual gas pressure was shown to be only approximate, and the average 0.4 mTorr gas pressure selected for the experiments oscillated as far as 0.05–0.07 mTorr around the central value during normal TSP operation.

TABLE II

DAUGHTER IONS OBSERVED ON THE CID MASS SPECTRA OF THE $[M + H]^+$ IONS FROM THE SULFONAMIDES

Collision energy, 50 eV; collision pressure, 0.5 mTorr. See references in text and Fig. 5 for the ion structures.

Compound	Parent m/z = $M_r + 1$[a]	$[H_2NPh]^+$ m/z = 92	$[H_2NPhO]^+$ m/z = 108	$[H_2NPhSO_2]^+$ m/z = 156	$[H_3NR]^+$ m/z = $M_r - 154$	$[O_2SNHR]^+$ m/z = $M_r - 92$	$[MH - H_2SO_3]^+$ m/z = $M_r - 65$
SNL[b]	100	6	3	21	–	–	–
SPD	100	11	5	32	4	–	5
SDZ	100	20	10	48	7	2	–
SMR	100	11	8	35	10	5	–
SMZ	100	9	5	30	14	9	1
STZ	100	21	9	69	2	–	–
SMTZ	75	17	9	100	2	–	–
SCP	100	13	6	48	3	–	–
SDM	100	8	5	70	[c]	–	5
SQX	100	11	9	82	6	–	–

[a] For SNL, $m/z = M_r + 18$.
[b] Also shows the $[M + NH_4 - NH_3]^+$ ion at $m/z = M_r + 1$ with 13% relative abundance.
[c] Indicates that this ions is isobaric with the CID ion $[H_2NPhSO_2]^+$.

The effect of the collision energy on the total peak area for each sulfonamide is shown in Fig. 6. All the compounds showed a maximum signal level between 30 and 60 eV. Below 30 eV, the response decreases rapidly, except for SNL (for which the selected parent is the $[M + NH_4]^+$ ion), which shows intense fragmentation ions at energies as low as 10 eV.

Owing to ion scattering in the collision cell and to the non-quantitative fragmentation processes, detection limits in the MS–MS mode are higher than those obtained in the MS mode (2 ng on-column when monitoring the ten $[M + H]^+$ parents).

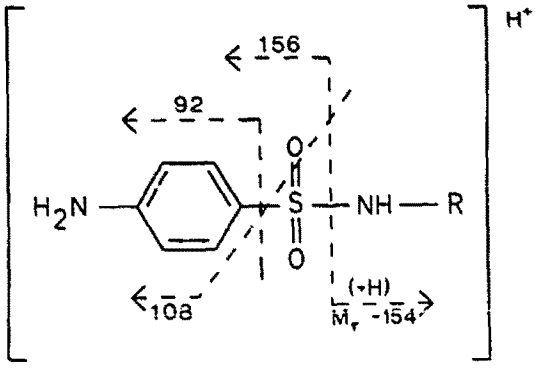

Fig. 5. Visualization of the possible fragment structures derived from the CID of the $[M + H]^+$ ions of the ten sulfonamides.

Analysis of milk

The detection limits are low enough to allow the multi-residue analysis of these compounds at the 10 ppb levels required for milk surveys. Although some work has been done on their determination in meat by TSP [14], no data have been reported for milk except for our preliminary report where diluted milk samples were injected into the HPLC–TSP-MS system without prior clean-up [15]. Current methods for the extraction of sulfonamides from milk utilize large volumes of organic solvents such as diethyl ether and ethyl acetate and are not suitable for automation of analysis. In order to overcome these problems, some workers have shown the utility of solid cartridge extraction columns for sample clean-up [18,19]. Extensive clean-up is required owing to the low levels to be measured and the presence of co-eluting compounds in the milk that also show a response using the UV detector.

In Fig. 7, the ion traces obtained in the analysis of a milk sample spiked at 20 ng/ml are shown. The extraction procedure described under Experimental uses minimum sample and organic solvent volumes and hence it should be possible to process in parallel a large number of samples rapidly. Although the clean-up is not good enough for HPLC–UV detection, the sensitivity and specificity of the HPLC–TSP-MS technique allowed the analysis of milk

samples using this simple procedure. Initially, the extraction procedure used included only protein precipitation and lipid extraction. An aliquot of the aqueous phase was injected directly into the HPLC system. Using this method, rapid degradation of the TSP response occurred owing to the deposition of solid materials in the interface tip. This residue, probably from milk carbohydrates and remaining proteins, was eliminated using the methanol extraction as described under Experimental.

The preconcentration and clean-up system shown in Fig. 2 was used with good results. The system could be easily automated and needs only two different solvent solutions. A continuous flow through COL 2 is assured by means of PUMP B when the exit of the precolumn is directed to waste. In this way, it is possible to maintain a more stable TSP performance and to stabilize COL 2 with the initial eluent composition during the injection and clean-up steps. The Spectra-Physics pump used for chromatography was very sensitive to pressure changes and some flow-rate problems affecting proper TSP interface performance were observed when the eluent of PUMP A was directed to COL 2 from the "waste" position. These problems were eliminated when a restrictor (an old 10-μm analyt-

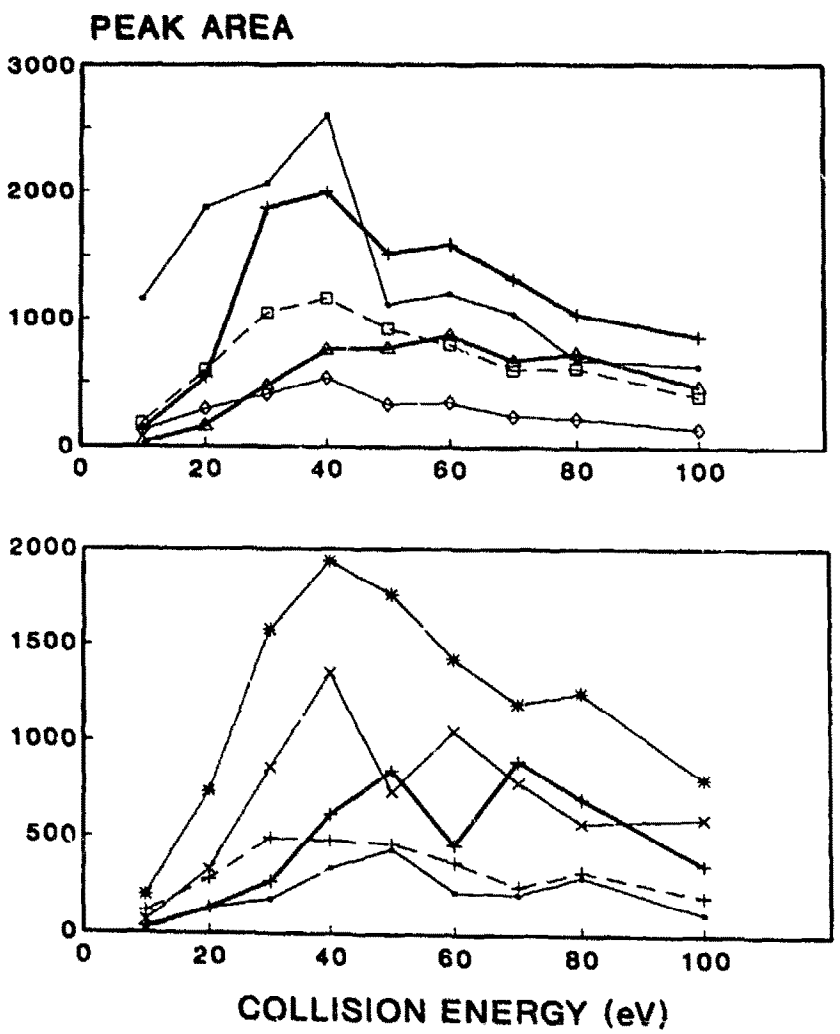

Fig. 6. Effect of collision energy on total peak area obtained in the SRM analysis (parent ion mode using the ion at m/z 156 as the common daughter ion) of the ten sulfonamides. Top: ■ = SNL; + = SPD; ▲ = STZ; ▽ = SMTZ; △ = SMZ. Bottom: ■ = SQX; + (solid line) = SDM; * = SDZ; × = SMR; + (dashed line) = SCP.

ical column), situated in the waste exit, was used to smooth the pressure difference between the two positions of valve V2. In some instances, the use of sample loops and injection volumes of up to 1 ml was tested without observing any detrimental effect in the TSP analysis. Precolumn concentration allows further sample clean-up by retaining lipid residues that can be washed out after each analysis.

Also, the method eliminates polar compounds from the sample that could plug the interface or interfere with the ionization processes. Hence, after precolumn washing, the common Na^+ (or Cl^- in the negative-ion mode)-derived adducts usually present in the spectra of compounds from salt-containing matrices were not observed. An unresolved problem of the on-line precolumn concentration method is that the washing step results in the loss of SNL. This compound, the most polar of the group assayed, showed low retention characteristics in the precolumn system and eluted (the UV detector was connected to the waste exit) as a broad peak in the first 5 min even when the eluent contained no ACN. In contrast, no losses of SDZ or STZ, the next eluting compounds, were detected under these conditions during a 10-min elution period.

As can be seen in Fig. 7, matrix-derived background noise and peak interferences are not important in determining the overall sensitivity of the TSP technique. Thus, HPLC–MS–MS procedures offer no advantage over HPLC–MS for trace detec-

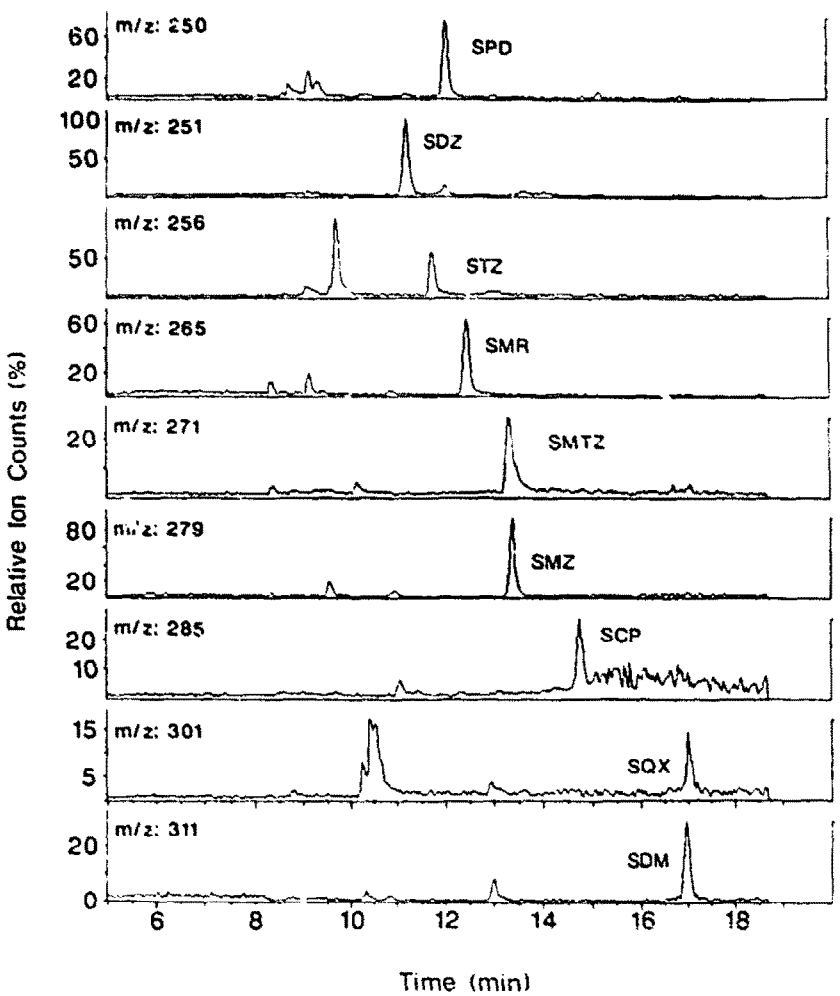

Fig. 7. HPLC–TSP-MS analysis (selected ion monitoring) of a cow milk sample spiked with 20 ng ml of each sulfonamide (SNL is lost in the clean-up step).

tion in milk (actually, MS–MS shows higher detection limits than SIM analyses) but can be used when the amounts of the target compounds are at the high ppb level and a more specific characterization of the compounds is required.

CONCLUSIONS

The ten sulfonamide antibiotics tested in this work can be separated and characterized by HPLC–TSP-MS and HPLC–TSP-MS–MS. Co-eluting sulfonamides SMTZ and SMZ and SDM and SQX are readily resolved by their different relative molecular masses. The sensitivity of the technique allows its application to the multi-residue analysis of these compounds in biological matrices with minor sample preparation. The technique allows the detection of nine of these compounds in milk at the low ppb level using a simple and rapid clean-up procedure, and could be easily adapted to other HPLC–UV sample preparation methods for compound confirmation and/or characterization using HPLC–MS or HPLC–MS–MS.

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