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Determination of sulfonamides by liquid chromatography, ultraviolet diode array detection and ion-spray tandem mass spectrometry with application to cultured salmon flesh[a]

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ABSTRACT

Ion-spray mass spectrometry was investigated for the analysis of 21 antibacterial sulfonamide drugs. All of the sulfonamides analyzed gave positive ion mass spectra with abundant protonated molecules and no fragmentation. Tandem mass spectrometry (MS–MS) using collision-induced dissociation provided structural information, allowing the identification of common fragmentation pathways and the differentiation of isomeric and isobaric sulfonamides. A reversed-phase high-performance liquid chromatographic method was developed, using gradient elution and ultraviolet diode-array detection (DAD), enabling the separation of 16 of the sulfonamides. Combined liquid chromatography (LC)–MS was accomplished using the ion-spray interface. Analyses of a mixture of sulfonamide standards were performed with gradient elution and the mass spectrometer configured for full-scan acquisition, selected-ion monitoring, or selected-reaction monitoring.

Procedures for the analysis of sulfadimethoxine (SDM), a representative sulfonamide used in the aquaculture industry, are described. The presence of SDM in cultured salmon flesh was confirmed at levels as low as 25 ng/g by a combination of LC–DAD and LC–MS–MS.

INTRODUCTION

Sulfonamides are antibacterial compounds commonly used for the prevention and treatment of diseases in livestock production. A major concern with the use of these compounds is that residues may be present in food products if proper withdrawal times for treated animals have not been strictly enforced. Such residues may pose a health threat to consumers through allergic or toxic reactions, or through induction of antibiotic resistance in pathogenic organisms. For this reason, regulatory agencies

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have established tolerance levels (0.1 ppm in most countries) for these drugs in food products.

Concerns over these issues in Canada have recently focused public attention on the aquaculture industry, where antibiotics are used to control bacterial infections in farmed fin-fish such as salmon. Some of the sulfonamides currently used to treat salmon include sulfamerazine, Romet-30 (a potentiated sulfonamide containing sulfadimethoxine and ormetoprim) and Tribrissen (sulfadiazine and trimethoprim). Sulfonamide residues may be monitored by a variety of analytical techniques such as bioassay, colorimetric assays, thin-layer chromatography, gas chromatography (GC), high-performance liquid chromatography (LC) and mass spectrometry (MS) [1]. Instrumental methods provide the advantages of ease of automation, high sensitivity and increased specificity. Spectroscopic methods are particularly important for confirmation of structures.

GC and GC–MS have been used for confirmatory analyses of antibiotics such as sulfonamides. However, due to their low volatility and thermally labile nature, chemical derivatization is usually required. Several methods have been reported that are based on derivatization followed by GC with detection either by electron capture [2] or MS with selected-ion monitoring in the electron ionization (EI) [3] and chemical ionization (CI) [4] modes. GC–MS and tandem mass spectrometry (MS–MS) have also been used for the confirmation of methylated sulfonamides in bovine and porcine tissue [5]. Unfortunately, such analyses are time-consuming as they require extensive sample clean-up prior to the additional derivatization step.

The mass spectral behaviour of underivatized sulfonamides under EI and more recently CI has been well documented [6–7]. Chemical ionization with isobutane as the reagent gas has also been used for the tandem MS of protonated sulfonamides by collision-induced dissociation with mass-analyzed ion kinetic energy spectrometry (CID–MIKES) [7]. In a more recent investigation [8], very similar CID spectra of sulfonamides were obtained using ammonia as CI reagent gas, on a hybrid instrument. While both of these studies demonstrated that direct confirmation of sulfonamide residues in animal tissue at a level of 0.1 ppm could be achieved by MS–MS without chromatography, the authors stressed the problems of possible interferences. Due to the complex nature of the matrices involved, even after clean-up procedures, the most reliable method will likely be one combining chromatographic separation prior to spectroscopic detection.

LC is the most commonly used instrumental method for the analysis of antibiotics and antimicrobial agents in biological matrices [9,10]. Several authors have reported multi-residue LC methods with UV detection for the determination of sulfonamides, other antibiotics and growth promoters in feed stuffs [11], animal tissue [12,13] and other food products [14–16]. Sensitive LC methods for the determination of sulfonamides in cultured fish tissue have also been described previously [17–20].

Despite the selectivity of the extraction and cleanup methods employed, LC with UV detection at single wavelengths is still prone to interferences which can result in reduced confidence in analyses. The combination of LC with UV diode-array detection (DAD) [21] and with MS have tremendous potential for this type of analysis. In 1982, Henion *et al.* [22] described the use of an atmospheric pressure ionization (API) source for the LC–MS analysis of five sulfonamides in racehorse urine and plasma by LC–MS. While clearly demonstrating the potential of LC–MS and also of

LC–MS–MS, the prototype heated nebulizer interface used in this investigation provided full-scan detection limits only in the μg range.

Ion spray (ISP) is a recently developed API technique [23] that is proving to be well suited to the LC–MS analysis of trace levels of polar and thermally labile compounds [24,25]. The mechanism of both ISP and electrospray, a related technique [26] is believed to involve ion evaporation [27] although nebulization plays an important role in the former process [23]. A recent review has detailed the use of API-MS as a detection system for the separation sciences [28]. This paper will present LC–DAD and LC–MS–MS methods that have been developed for the analysis of sulfonamides and related agents, and demonstrate their application to the determination of sulfadimethoxine (SDM) in salmon flesh.

EXPERIMENTAL

Chemicals

All of the sulfonamide standards listed in Table I were obtained from Sigma (St. Louis, MO, USA) and used as received, without purification. Trifluoroacetic acid (TFA) and formic acid were obtained from BDH (Poole, UK). HPLC-grade acetonitrile was purchased from Anachemia (Lachine, Canada). A Milli-Q water purification system (Millipore, Bedford, MA, USA), equipped with ion-exchange and carbon filters, was used to further purify glass-distilled water.

Extraction of sulfadimethoxine from salmon flesh

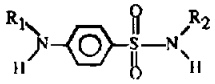
The extraction procedure used in this investigation was based on those reported by Weiss *et al.* [17] and Nose *et al.* [20], Salmon flesh (40 g) was mixed with acetone (200 ml) and homogenized with a Brinkmann Polytron for 3 min. After addition of sufficient quantities of Celite (10 g) and sodium sulphate (20 g) the homogenate was again blended for a further 2 min. This mixture was vacuum-filtered and the filter cake was washed with acetone (3 × 15 ml). After transferring the filtrate to a round-bottomed flask, the acetone was removed on a rotary evaporator at 40°C. The residue was re-dissolved in 100 ml of dichloromethane; 50 ml of this solution was mixed with 100 ml of 0.1 *M* sodium hydroxide and shaken vigorously. This mixture was transferred to a 250-ml centrifuge jar and centrifuged for 20 min at 12 *g* and 10°C. The aqueous layer was transferred to a round-bottomed flask and neutralized with hydrochloric acid. This aqueous extract was freeze-dried overnight. The remaining water was removed on a rotary evaporator at 40°C the following day. The residue was dissolved in 5.0 ml of aqueous 25% methanol, and the solution was filtered through a 0.45-μm nylon syringe filter.

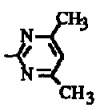
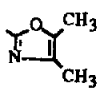
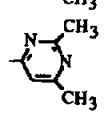
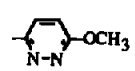
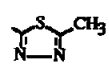
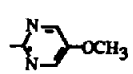
Liquid chromatography

Analyses were performed on a Hewlett-Packard Model HP1090M liquid chromatograph equipped with a variable-volume (1–25 μl) injector and autosampler, a ternary DR5 solvent-delivery system, a built-in HP1040A diode-array detector and a HP79994A data system. For the analysis of sulfonamide mixtures, separations were achieved on a 25 cm × 2.1 mm I.D. column packed with 5-μm Vydac 201TP stationary phase (Separations Group, Hesperia, CA, USA). Aqueous acetonitrile containing 0.1% TFA was used as the mobile phase at a flow-rate of 200 μl/min with

TABLE I

NAMES, STRUCTURES, RETENTION TIMES, UV AND PARTIAL MS–MS SPECTRAL DATA FOR SULFONAMIDES EXAMINED IN THIS STUDY

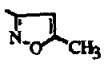
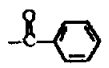
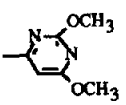
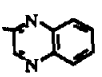


Name	Code	R_1	R_2	Mol.wt.	t_R[a] (min)
Sulfaguanidine	SGN	H		214	3.60
Sulfanilamide	SNL	H	H	172	3.74
Sulfacetamide	SMD	H	$-COCH_3$	214	6.49
Sulfadiazine	SDZ	H		250	6.50
Sulfapyridine	SPR	H		249	7.36
Sulfamerazine	SMR	H		264	7.51
Sulfathiazole	STH	H		255	7.97
Sulfamethazine	SMT	H		278	8.51
Sulfamoxole	SMX	H		267	8.63
Sulfisomidine	SID	H		278	9.24
Sulfamethoxypyridazine	SMP	H		280	10.59
Sulfamethizole	SMZ	H		270	10.77
Sulfameter	SME	H		280	10.89
Succinylsulfathiazole	SRTZ	$HOOC(CH_2)_2CO^-$		355	12.08
Sulfachloropyridazine	SCP	H		284	12.98

λ_{max}[b]	*f*	*c*	*a*	*b*	*d*	*e*	Other
263	122(1)	156(75)	60(12)	108(32)	92(38)		
261	–	–	–	156(8)	NA	92(11)	139(2)
272	149(1)	–	156(61)	60(1)	108(16)	92(23)	173(60) 65(1)
270	185(2)	158(5)	156(100)	96(9)	108(21)	92(16)	
245 264 312	184(21)	–	156(88)	95(8)	108(18)	92(13)	232(3) 167(4)
245 265	199(3)	172(22)	156(100)	110(40)	108(28)	92(15)	
260(S)[c] 285	190(1)	–	156(100)	101(6)	108(19)	92(15)	139(1)
245 265(WS)[c] 310(WS)[c]	213(7)	186(42)	156(100)	124(85)	108(25)	92(20)	
272	–	–	156(100)	113(36)	108(21)	92(12)	
260 285(S)[c]	–	186(22)	156(60)	124(100)	108(13)	92(1)	
270	215(3)	188(3)	156(60)	126(30)	108(15)	92(10)	
255(WS)[c] 275	–	178(1)	156(100)	116(8)	108(19)	92(14)	
270	215(12)	188(8)	156(100)	126(45)	108(21)	92(12)	
260 285	–	–	256(100)	–	208(1)	192(41)	156(16) 108(7)
270	–	–	156(100)	130(8)	108(12)	92(9)	

(*Continued on pp. 160 and 161*)

TABLE I (*continued*)

Name	Code	R_1	R_2	Mol.wt.	t_R[a] (min)
Sulfamethoxazole	SMO	H		253	14.86
Sulfisoxazole	SIX	H		267	15.57
Phthalylsulfathiazole	PST			403	15.65
Sulfabenzamide	SBD	H		276	16.79
Sulfadimethoxine	SDM	H		310	18.48
Sulfaquinoxaline	SQO	H		300	18.05

[a] Retention time on Vydac 201TP52 column with a linear gradient of the aqueous mobile phase from 5–40% acetonitrile (containing 0.1% TFA) in 20 min; flow-rate of 200 μl/min.
[b] λ_{max} obtained under isocratic conditions with a mobile phase of aqueous acetonitrile (80%) containing 0.1% TFA.
[c] S = Shoulder; WS = weak shoulder.

a linear gradient of 5–40% acetonitrile in 20 min. An injection volume of 5 μl was used. Detection was at 270 nm (10-mm bandwidth) with acquisition of UV spectra in the peak-triggered mode. For the determination of SDM in salmon extracts, isocratic separations were achieved on a 25 cm × 4.6 mm I.D. column packed with 5-μm Supelcosil LC 18DB stationary phase (Supelco, Santa Clara, CA, USA), using an injection volume of 20 μl. Aqueous 35% acetonitrile containing 0.1% formic acid was used as the mobile phase at a flow-rate of 1 ml/min. Multi-wavelength detection with the DAD system was used at the following wavelengths (and bandwidths): 250 (4), 260 (4), 265 (10), 270 (4), 280 (4) and 290 (4) nm.

LC–MS–MS

All LC–MS experiments were performed on a SCIEX API III triple quadrupole mass spectrometer (Thornhill, Canada), equipped with an API source and an IonSpray interface. A Macintosh IIx computer was used for instrument control, data acquisition and data processing. Polypropylene glycols were used to calibrate the mass scale and to adjust the resolution to unity (40% valley definition) over the mass range 50–500 dalton. For LC–MS experiments the column effluent was connected to the fused-silica transfer line (50–75 μm I.D.) of the ISP interface via a Valco sub-μl injection valve with interchangeable loops. This injector was used to optimize the LC–MS system (0.1-μl loop) and for all flow-injection experiments (1.0 μl loop). For

λ_{max}[b]	*f*	*c*	*a*	*b*	*d*	*e*	Other
270	188(6)	159(4)	156(100)	99(9)	108(24)	92(18)	
270	–	–	156(100)	113(59)	108(19)	92(18)	139(1) 175(1)
285 260	–	–	304(24)	–	256(27)	–	149(70) 156(100) 386(26)
235	– –	–	156(100)	–	108(9)	92(7)	174(12)
269	245(4)	218(4)	156(100)	156(0)	108(9)	92(5)	173(4)
249 269 395(WS)[c]	235(1)	208(1)	156(100)	146(18)	108(9)	92(5)	

simultaneous LC–DAD–MS acquisitions the column effluent was split using a zero-dead-volume "T" connector, with approximately one quarter of the flow being fed to the mass spectrometer (again via the post-column injector). The split ratio was determined by the length of a fused-silica capillary (50 μm I.D.) connecting the "T" to the DAD flow cell. Identical LC conditions to those described above were used for the analysis of sulfonamide mixtures. For the confirmation of SDM in salmon flesh by LC–MS, the 2.1 mm I.D. (201TP) column was used with 100 μl/min aqueous 35% acetonitrile containing 0.1% formic acid and with the end of the column connected directly to the ISP interface.

The ISP voltage was maintained at approximately 5.6 kV for all LC–MS analyses. High-purity air at an operating pressure of 90 p.s.i. (approximately 2 l/min) was used as the nebulizing gas. A dwell time of 5 ms/dalton was used for full-scan LC–MS analyses. For single- and multiple-ion monitoring LC–MS experiments dwell times of 200 and 100 ms/dalton were employed, respectively. MS–MS measurements were based on CIDs within the r.f. only quadrupole at a collision energy of 35 eV (aboratory frame). Both parent- and daughter-ion LC–MS–MS scans were performed. Argon was used as the target gas at an indicated thickness of $2.5 \cdot 10^{14}$ molecules/cm^2. For comparative MS–MS work, CID product ion spectra of the standards were acquired over a range of collision energies (0–50 eV) and at several target-gas thicknesses ($0.1–5.0 \cdot 10^{14}$ molecules/cm^2). Preliminary MS–MS experiments with the

standards indicated that the masses of the product ions were well separated; it was possible therefore to obtain an appreciable gain in sensitivity, for the less-intense product ions, by operating at peak widths on the third quadrupole (Q_3) corresponding to more than 1 dalton wide. The first quadrupole (Q_1), however, was maintained at unit mass resolution. Selected fragmentation pathways were confirmed by grand-daughter-ion experiments, whereby dissociations of the protonated molecules were induced in the source region, prior to entering the mass analyzer, by increasing the declustering voltage applied to the sampling orifice. Product ions generated in this way were then subjected to further fragmentation in conventional parent/daughter MS–MS experiments.

RESULTS AND DISCUSSION

ISP–MS

Table I gives the names, abbreviations and structures of the 21 sulfonamides examined in this study. All of these compounds provided very simple ISP mass spectra, containing only a protonated molecule, $[M + H]^+$, and no fragment ions of significant intensity. Good-quality spectra could be obtained by flow-injection analysis (FIA) in the full-scan mode with as little as 1 ng of compound injected. Fig. 1a presents the full-scan, background-subtracted ISP mass spectrum obtained from flow injection of 50 ng SDM into a 50-μl/min flow of aqueous acetonitrile (50%) containing 0.1% TFA. The fact that most of the iron current is associated with the $[M + H]^+$ ion suggests that ISP should be ideal for trace-level determinations using selected-ion monitoring (SIM). This is demonstrated in Fig. 1b, which shows the response obtained from duplicate injections of standard solutions of SDM. These results indicate a FIA–SIM detection limit of around 10 pg (signal-to-noise ratio = 3). Very similar sensitivities

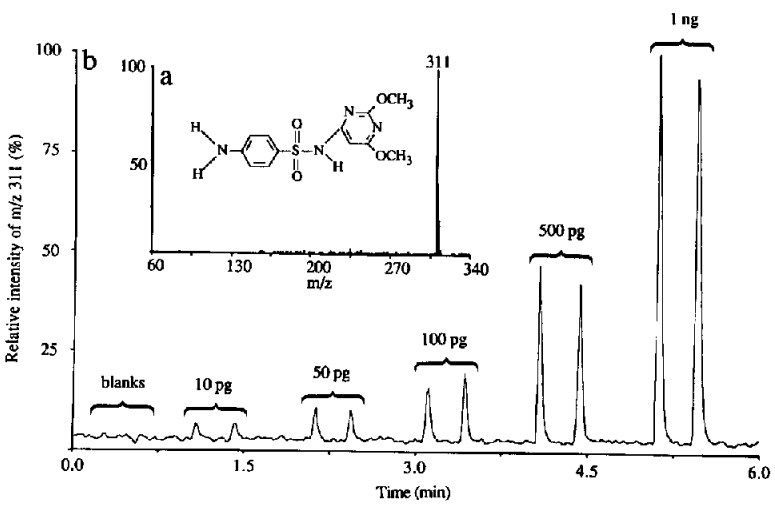


Fig. 1. Flow-injection ISP–MS analysis of sulfadimethoxine (SDM). (a) Positive ion, background-subtracted ISP mass spectrum of 50 ng of SDM; (b) duplicate 1-μl injections of a series of dilutions using SIM of the protonated molecule (*m/z* 311). Conditions: 50 μl/min aqueous acetonitrile (50%) containing 0.1% TFA.

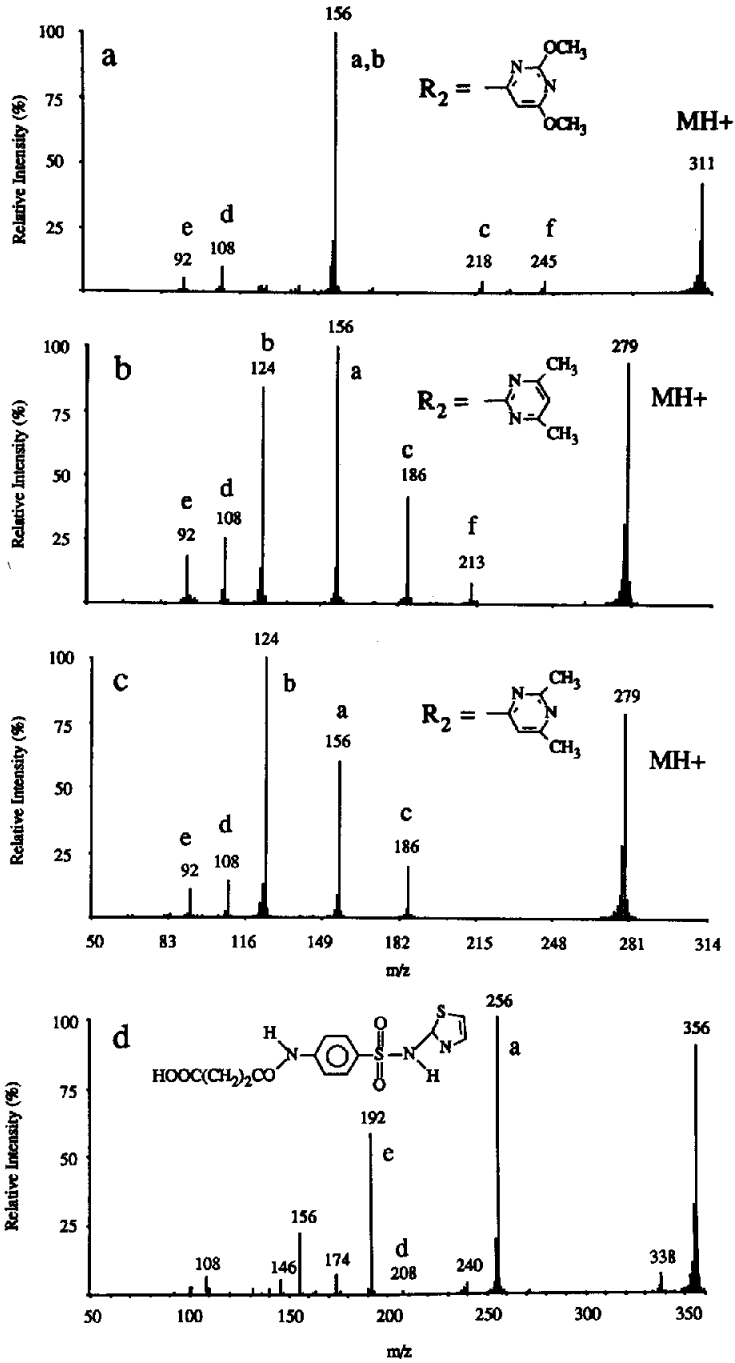


Fig. 2. Positive-ion ISP-MS–MS product ion spectra of the protonated molecules of standard compounds obtained by flow injection. (a) SDM (*m/z* 311); (b) SID (*m/z* 279); (c) SMT (*m/z* 279); and (d) STZ (*m/z* 356). Conditions: as Fig. 1 except with a collision energy of 35 eV (laboratory frame) and using argon as the collision gas at an indicated thickness of $3.0 \cdot 10^{14}$ molecules/cm^2.

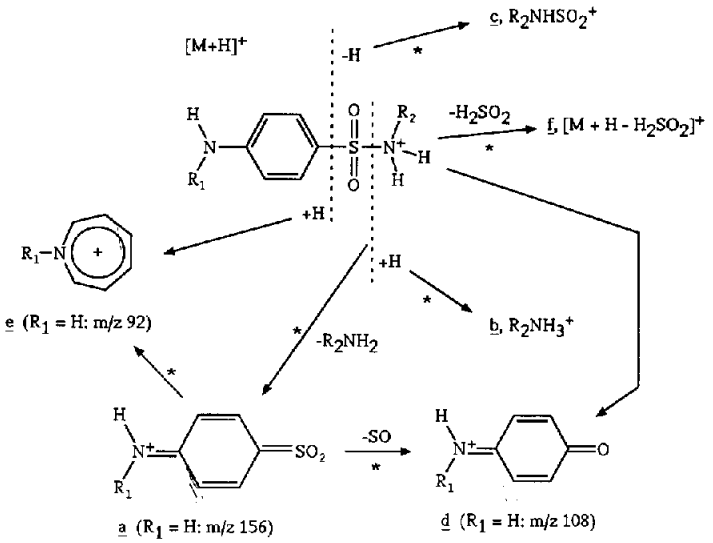


Fig. 3. Proposed fragmentation scheme for the collison-induced dissociation of the protonated sulfonamides. Pathways denoted with an asterisk are supported by granddaughter-ion experiments using SDM, SID and SMT (see Experimental section).

were obtained for the other sulfonamides in Table I, with the exception of the parent sulfonamide, sulfanilamide (SNL), the response of which was approximately 5 times lower.

The lack of structural information provided by the simple ISP mass spectra can be remedied by the use of MS–MS techniques. Table I lists the most abundant product ions observed for the 21 standard sulfonamides using the triple quadrupole API III mass spectrometer, all under identical CID conditions. The resolution on Q_3 was reduced to provide additional sensitivity for the less-abundant product ions. It should be noted that this results in a somewhat fragmented appearance of peaks in the CID spectra presented; the latter is also due, in part, to the nature of the detection system used, which performs ion counting instead of the more usual continuous analog acquisition. In general, the ISP product ion spectra are less complex than those previously reported on sector instruments using CI [7,8]. Some representative product ion spectra of sulfonamides obtained by FIA–ISP are shown in Fig. 2. These spectra are similar to the CID spectra reported by Henion *et al.* [22], generated by corona discharge atmospheric pressure CI using a prototype heated nebulizer interface.

Fig. 3 illustrates fragmentation mechanisms proposed to rationalize the CID spectra of the sulfonamides. Charge localization in the $[M+H]^+$ ion through protonation of the sulfonamide nitrogen helps to explain the observed fragmentations. The abundant product ion *a* (*m/z* 156 for $R_1 = H$) is characteristic of all the sulfonamides analyzed and likely arises from the cleavage of the sulfur–nitrogen bond and loss of the neutral species R_2NH_2. Charge retention on the nitrogen with hydrogen abstraction results in ion *b*, corresponding to $R_2NH_3^+$, *i.e.*, $[M+H-155]$. In the case of SDM, ions *a* and *b* both coincidentally appear at *m/z* 156 (see Fig. 2a). For the isomeric sulfonamides SMT and SID (Fig. 2b and c), ion *b*, due to the protonated

pyrimidyl moiety, appears at *m/z* 124. Two other product ions, *d* and *e* observed at *m/z* 108 and 92, are common to all of the sulfonamides and may be rationalized by the losses of SO and of SO_2 from the ion at *m/z* 156, with the latter assigned to a ring-expanded azatropylium ion. An additional characteristic product ion at *m/z* 65, which presumably results from the loss of HCN from *m/z* 92, was also reported in the heated nebulizer CID spectra [22]. However, under the present conditions, this ion was observed only occasionally. Fragment ion *c*, at *m/z* 218 in the spectrum of SDM and at *m/z* 186 in the spectra of both SMT and SID, is assigned to the loss of neutral aniline (93 dalton) from $[M+H]^+$. This loss is also observed in the majority of the sulfonamides (Table I). It should be noted that while SMT and SID, and another isomeric pair SMP and SME, can be differentiated by the relative intensities of the ions described above, an additional ion at *m/z* 213 is observed in the spectrum of SMT which does not appear in that of SID. This ion, designated as *f*, has been reported previously in the CID–MIKES spectra of protonated sulfonamides generated with CI, where it was assigned to $[M+H-H_2SO_2]$ [7]. Selected-fragmentation pathways indicated in Fig. 3 were confirmed by granddaughter-ion experiments in which dissociations of the protonated molecules were induced in the source region, prior to conventional MS–MS experiments in the triple quadrupole.

Additional evidence for the above assignments was obtained from CID of the ^{37}Cl isotope peak of the protonated molecule of the chlorinated sulfonamide SCP, at *m/z* 287. The product ion spectrum was identical to that obtained for the ^{35}Cl protonated molecule, indicating that the R_2 group is not involved in the major fragmentation pathways discussed above. The two sulfonamides which contain an R_1 group, STZ and PST, also show analogous product ions and losses. The product ion spectrum of STZ is presented in Fig. 2d, and product ion from the corresponding loss of H_2NR_2 is seen at *m/z* 256 with those from the subsequent losses of SO and SO_2 at *m/z* 208 and 192, respectively.

The identification of the common fragmentation pathways shown in Fig. 3 opens up the possibility of using MS–MS techniques to screen extracts for putative sulfonamides, and this approach is discussed later. Although isomer distinction could be enhanced to some degree by proper selection of target-gas thickness and collision energy, the use of combined LC–MS should provide the necessary additional selectivity via retention time.

LC–MS

While we have found excellent sensitivity and quantitation for standard solutions of sulfonamides using FIA (Fig. 1b), the technique has a number of drawbacks in the analysis of real-world complex extracts containing salts and other endogenous material. The simultaneous introduction of many possible sources of interferences into the ion source with the analyte effectively prevents the use of FIA for quantitative analyses. Although the use of MS–MS techniques in conjunction with FIA can provide additional selectivity to filter out many potential interferences, there still remains the problem of other components influencing the ionization efficiency. Effective sample clean-up is necessary for quantitative work, and the easiest way to accomplish this without introducing additional sample handling steps is to provide a chromatographic separation prior to ionization.

Recent advances in bonded stationary phase technology have resulted in the

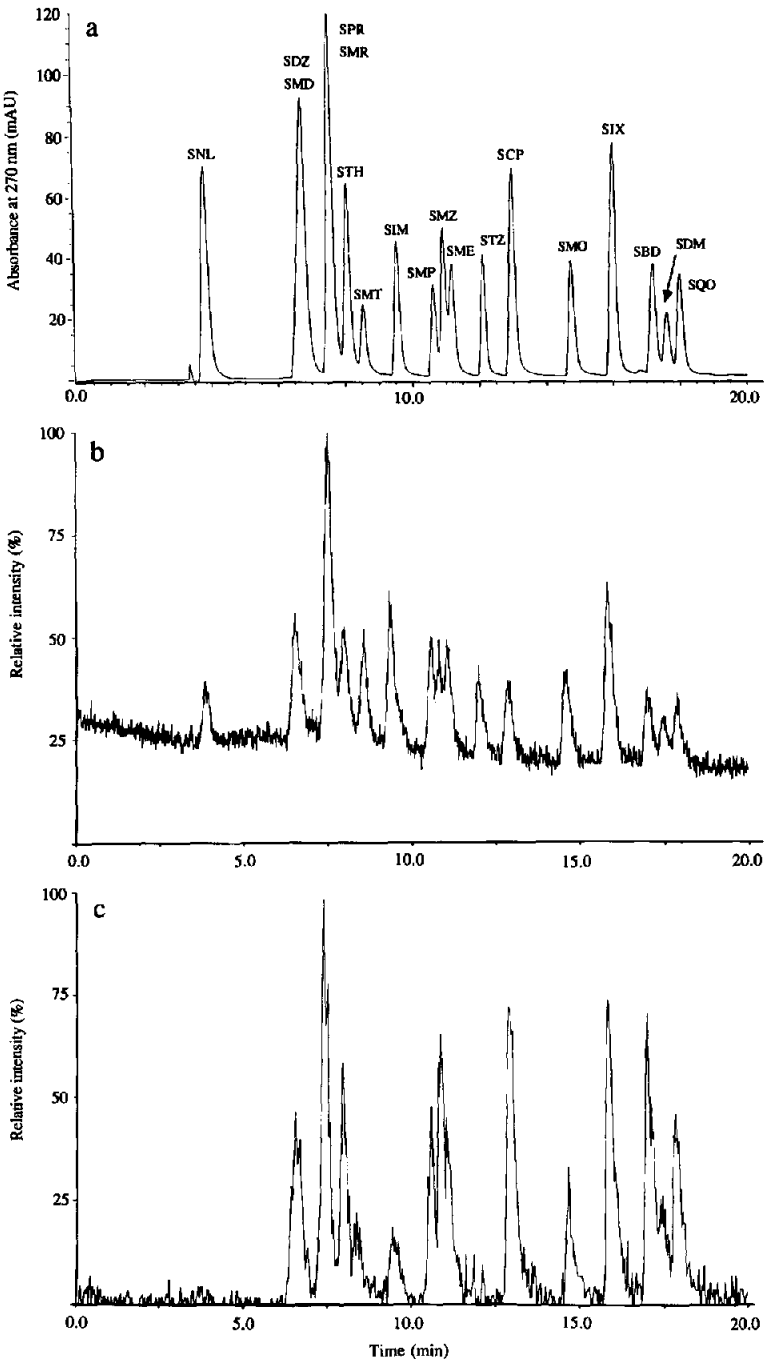


Fig. 4. Separation of a mixture of 18 sulfonamides by reversed-phase HPLC with simultaneous detection using (a) DAD with 270 nm (bandwidth = 4 nm) and (b) ISP-MS with multiple-ion monitoring of protonated molecules. A separate LC–MS–MS parent-ion scan experiment was conducted to produce the data in (c) where Q_1 was scanned from 160 to 400 dalton with Q_3 set at m/z 156. Conditions: 25 cm × 2.1 mm I.D. Vydac 201 TP52 column and a mobile phase of aqueous acetonitrile containing 0.1% TFA, with a linear gradient of 5–40% acetonitrile in 20 min and a flow-rate of 200 μl/min split 1:4 post-column between DAD and MS, respectively; 5-μl injection volume.

development of a number of multi-residue, reversed-phase LC methods for the analysis of several classes of antibiotics [9]. One recent study reported the separation of a wide range of sulfonamides using a small-particle-size (3 μm) C_{18} stationary phase packed in a capillary column (30 cm × 0.35 mm I.D.). Although 20 sulfonamides could be resolved adequately, peak tailing was evident for the late-eluting drugs and the analysis required more than 1 h [10].

For the present study, several commercially available columns packed with different stationary phases (aminopropylsilica and octadecylsilica) were examined as potential candidates for a general LC method. It quickly became apparent that, for such a complex mixture of closely related compounds, gradient elution in conjunction with a C_{18} stationary phase would be required. Mass spectral detection can also reduce the reliance on the complete resolution of all individual components, provided that these are not isobaric. In the present study complete baseline resolution remained an objective, but greater emphasis was placed on the development of an LC method capable of presenting a wide range of sulfonamides in suitable form to the ISP interface.

The sensitivity of the ISP process is directly related to the degree of ionization of the analyte in solution. The nitrogen of the central sulfanilamide bond is amenable to protonation which can be enhanced by increasing the acidity of the analyte solution. Lowering the pH of the mobile phase can also improve peak shape of basic compounds in reversed-phase LC by reducing adsorption on silica-based stationary phases, although this does lead to lower capacity factors, k', values. Both formic and trifluoroacetic acids were evaluated as modifiers in the aqueous acetonitrile mobile phase. The latter has the added advantage of being an effective ion-pairing agent, thus compensating for the lower pH by increasing retention.

Fig. 4a shows LC–UV analysis of a mixture of 18 sulfonamide standards on a 2.1 mm I.D. column, using 0.1% TFA and a linear-gradient elution profile. Only 16 peaks were observed in the trace due to the coelution of SDZ with SMD and SPR with SMR. The retention times of all of the 21 sulfonamides analyzed under this elution profile are listed in Table I. Using DAD, complete UV spectra of the individual sulfonamides were obtained to help confirm peak identities. The observed maximum wavelength, λ_{max} values for each of the sulfonamides examined are given in Table I. SPR and SMR could not be differentiated in this way as they possessed almost identical UV spectra.

The LC–MS reconstructed-ion chromatogram (RIC), acquired simultaneously using multiple-ion monitoring of 16 protonated molecules (two isobaric pairs), is presented in Fig. 4b. The correspondence between the UV and RIC traces is excellent, and this close correspondence allows the rapid confirmation of individual chromatographic peaks by mass as well as retention time.

The ability to screen samples for putative sulfonamides by selective LC–MS–MS techniques is demonstrated in Fig. 4c, which shows the full-scan total-ion current (TIC) trace from an analysis of the same mixture of sulfonamides using a parent-ion scan mode. For this analysis, the post-column split was removed and the 200-μl/min flow from the column was fed directly to the ISP interface. In this experiment Q_3 was adjusted to transmit only m/z 156 product ions, formed from the CID (in Q_2) of parent ions scanned by Q_1 (160–400 dalton), *i.e.*, only those protonated molecules which give rise to an m/z 156 product ion gave a response at the detector. Not all of the sulfonamides provided an intense m/z 156 product ion, and this is shown by the lack of

a response for SNL and also for STZ, which contains an R_2 group. The neutral-loss mode is another MS–MS mode in which the triple quadrupole mass spectrometer may be used. In this configuration Q_1 and Q_3 are scanned together, with Q_1 leading Q_3 by some neutral mass loss characteristic of the compound(s) of interest. In the case of the sulfonamides this was achieved using the $[M+H-155]^+$ fragmentation discussed earlier, although fewer of the sulfonamides give this particular fragmentation than that yielding the product at *m/z* 156.

The sensitivity of the more selective LC–MS–MS method can be increased by using selected-reaction monitoring (SRM). In this configuration, both Q_1 and Q_3 may be linked to monitor individual parent/daughter CID reactions. The LC–MS–MS detection limit for SDM, monitoring the dissociation of *m/z* 311 to *m/z* 156, was found to be approximately 200 pg on-column. This is approximately an order of magnitude less sensitive than by LC–MS with SIM.

As mentioned above, trimethoprim (TMP) and ormetoprim (OMP) are commonly used as potentiators in commercial formulations with sulfonamides, and it would be useful if LC–MS could be used in a multi-residue approach for the determination of both classes of compound. Fig. 5a shows the SIM LC–MS response obtained from the analysis of a 1:1 mixture of TMP and OMP, with 50 ng of each injected on-column, under the same gradient conditions as used for the sulfonamides. The two compounds gave approximately equal molar responses under the ISP ionization process, with TMP eluting slightly before OMP. As for the sulfonamides, only protonated molecules are observed in their ISP spectra. Good-quality CID spectra with abundant product ions were obtained for both compounds, and these are presented in Fig. 5b and c. The product ion at *m/z* 123, which is assigned to a product

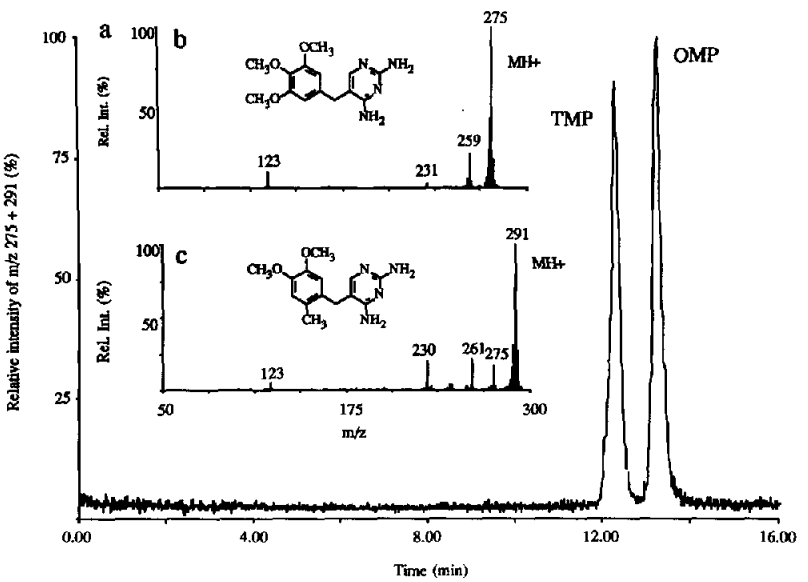


Fig. 5. (a) Analysis of a 1:1 mixture of trimethoprim (TMP) and ormetoprim (OMP) (50 ng each injected) by LC–MS using selected-ion monitoring of protonated molecules (*m/z* 291 and 275, respectively). The insets show the positive-ion ISP-MS–MS product ion spectra of OMP (b) and TMP (c). LC–MS and MS–MS conditions as in Figs. 4b and 2, respectively.

ion containing the pyrimidine moiety plus the bridging carbon atom, appears in the spectra of both TMP and OMP and could be used as a target dissociation in an SRM methodology.

Determination of SDM in salmon flesh

An opportunity to test the effectiveness of the LC–DAD and LC–MS methods, described above, arose when samples of farmed Coho salmon suspected of containing SDM were made available for analysis. Initial measurement of suspect flesh extracts, using LC with single-wavelength UV detection, showed a peak at the correct retention time for SDM and indivated levels in the different samples ranging from 25 to 1800 ng/g (ppb). The main objective of our subsequent analyses was to confirm the peak identity with DAD and MS.

A specific extraction method for SDM could be used in this case. The method selected is similar to those reported in the literature and is detailed in the Experimental section. Recoveries of SDM spiked into control flesh samples over the 100–1000 ng/g level were approximately 60% (unpublished results). These results are consistent with those previously reported by other workers, who have suggested that the poor recoveries may be due to the higher levels of cholesterol found in salmonids than in other species of fish [18]. Clearly there is a need to improve the extraction and cleanup procedures, and these are currently under investigation.

For the present experiments, a rapid isocratic procedure was developed which gave a retention time of 7.3 min ($k' = 3$) for SDM. Excellent peak shape and separation of SDM from other endogenous substances was achieved, using a base-deactivated column (Supelcosil LC18DB) and a mobile phase of aqueous 35% acetonitrile with 0.1% formic acid. Fig. 6a shows the analysis of a control salmon flesh extract spiked with 83 ng/g SDM, using UV absorption detection at 265 nm with a 10-nm bandwidth. This method gave an excellent detection limit of 0.03 μg/ml in salmon flesh extract. This corresponds to approximately 13 ng/g in the flesh with correction for a 60% recovery factor. The quantitation aspects were not investigated further at this stage since the primary objective at present was qualitative analysis.

At trace residue levels the DAD is not sufficiently sensitive in the full-scan mode to provide useful UV spectra for confirmation of identity. However, by acquiring at several characteristic wavelengths simultaneously, it is possible to conform that a compound has the correct spectral features in a broad sense. The UV spectrum of SDM is given in Fig. 6b, and indicates the six additional wavelengths that were selected for acquisition: 240, 250, 260, 270 and 290 nm (all with 4-nm bandwidths). Fig. 6c shows the multi-wavelength data set for the same spiked control flesh sample (83 ng/g) as in Fig. 6a. The peak area ratios in the six traces match exactly the absorbance ratios measured in the UV spectrum in Fig. 6b, at the different wavelengths indicated.

An LC–MS method was then established for the rapid analysis of SDM. Fig. 7a shows the LC–ISP-MS analysis of an SDM standard solution (1.1 μg/ml) using similar isocratic conditions, with SIM of the protonated molecule at m/z 311. The inset in Fig. 7a shows a typical calibration curve for standard solutions over the 0.08–80 μg/ml range. A detection limit of 0.04 μg/ml was estimated.

Fig. 7b and c shows the LC–MS and LC–DAD analyses, respectively, of an extract of a salmon flesh sample estimated by the latter technique to contain 25 ng/g (ppb). Confirmation of the SDM was clear and unequivocal with the LC–MS data.

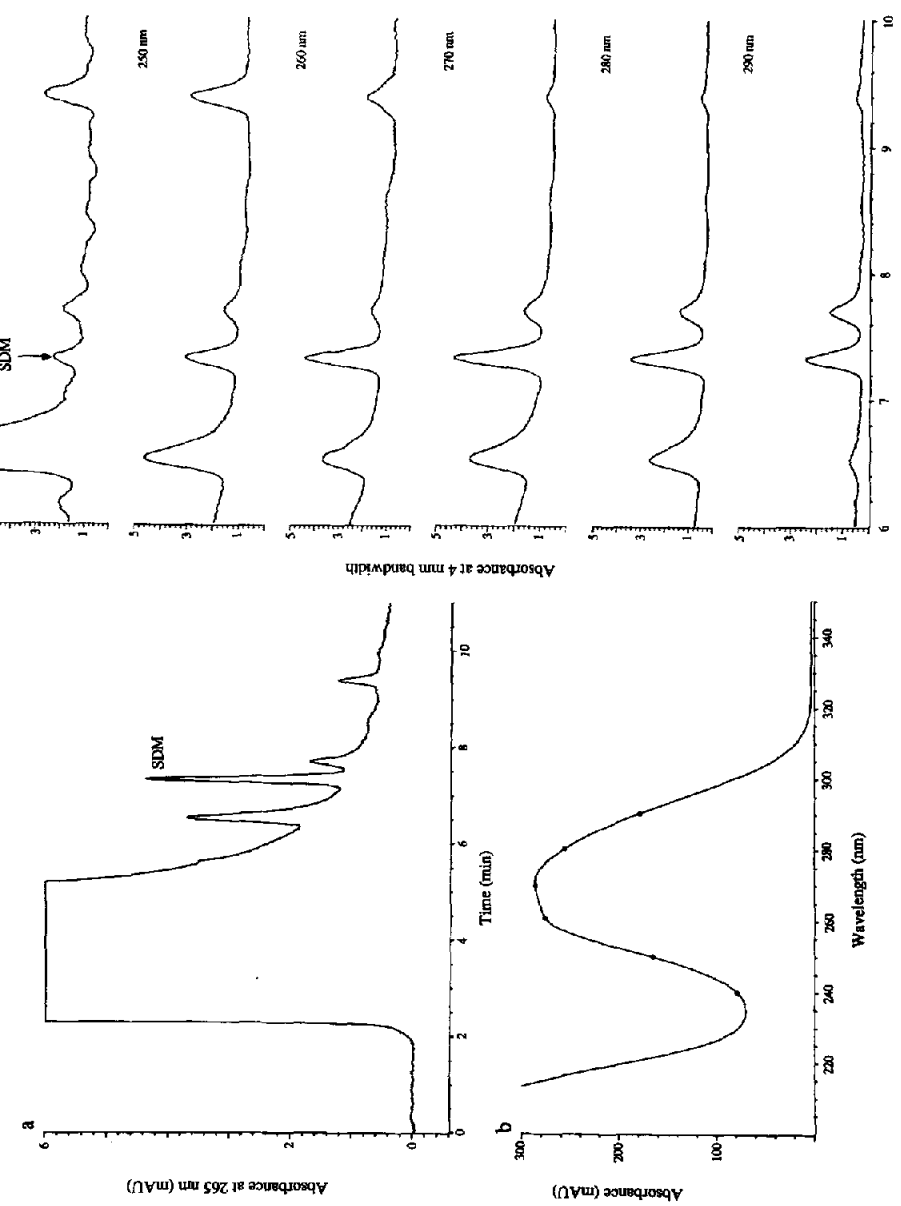


Fig. 6. Isocratic LC–DAD analysis of an extract of salmon flesh spiked at the 83 ng/g level with SDM. The first trace (a) shows the chromatogram with detection at 265 nm using a 10-nm bandwidth. The UV spectrum of SDM acquired for a standard is given in (b) to indicate the wavelengths (designated by dots) selected for the acquisition of the multi-wavelength data set (c) [acquired simultaneously with the trace in (a)]. Conditions: 25 cm × 4.6 mm I.D. Supelcosil LC18DB column; 1 ml/min aqueous acetonitrile (35%) containing 0.1% formic acid; 20-μl injection volume.

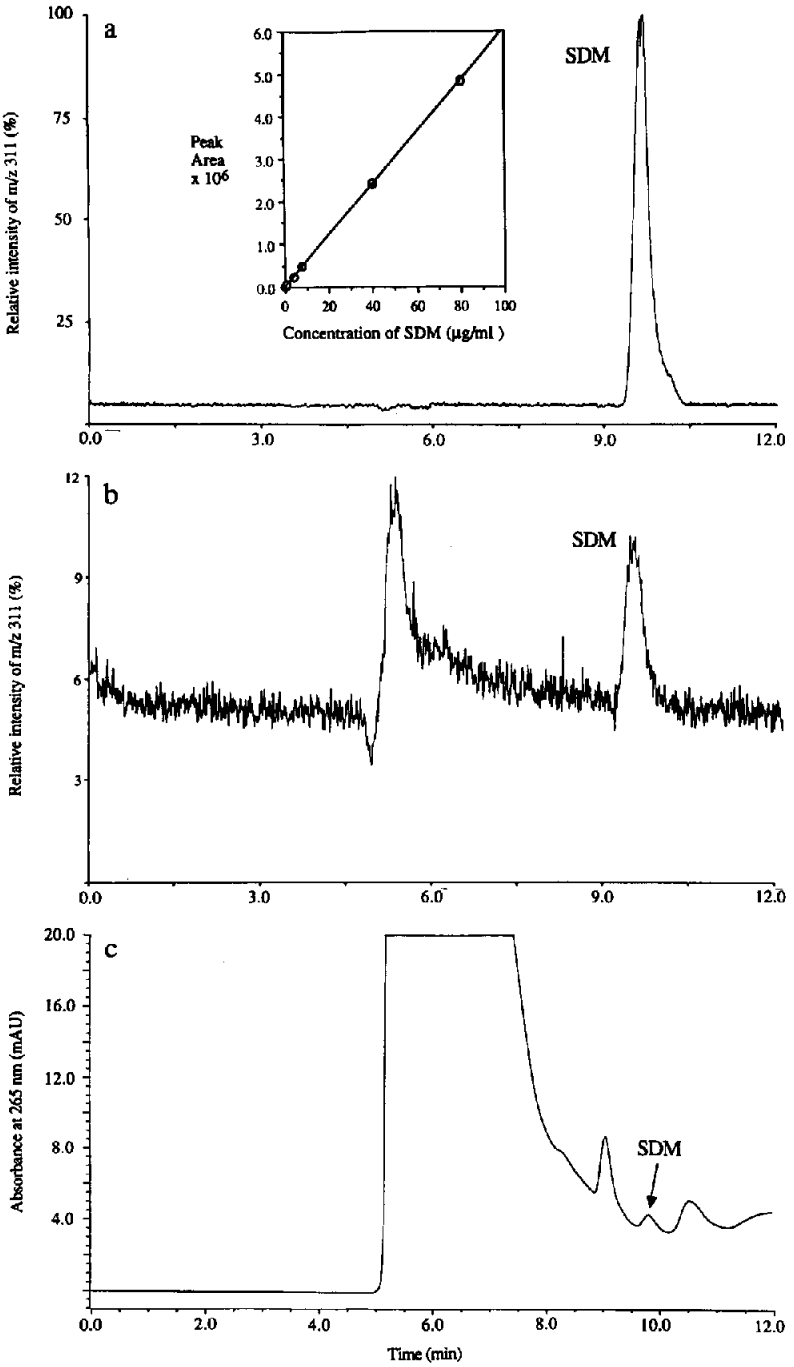


Fig. 7. (a) Analysis of SDM standard (1.1 μg/ml) by LC–MS with selected-ion monitoring of the protonated molecule, m/z 311; (b) analysis of suspect salmon flesh extract by LC–MS; (c) LC–DAD analysis of salmon flesh extract confirming the presence of SDM. The inset in (a) shows the linear relationship between LC–MS peak area and concentration of SDM. Conditions: 25 cm × 2.1 mm I.D. Vydac 201TP52 column; 100 μl/min aqueous acetonitrile (35%) containing 0.1% formic acid; 10-μl injection volume.

With the present extraction and cleanup procedure, it is possible to confirm SDM in flesh at the 10 ppb level. At these low levels the LC–DAD analysis was complicated by closely eluting endogenous substances, but the multi-wavelength data set did provide additional conformatory evidence for the presence of SDM. Improvements in the extraction procedure, together with the use of gradient elution, should provide significantly lower detection limits. Both of these improvements are under development.

CONCLUSIONS

There is considerable urgency for the development of reliable instrumental methods of analysis for antibiotics in the flesh of farmed fish. Since LC is the tool most widely used for routine monitoring of these compounds, it is important to develop confirmatory methods based on the combination of LC with spectroscopic methods. Mass spectrometry is a preferred method due to its inherent sensitivity and selectivity. It has been shown here that LC–ISP–MS–MS provides the key to confidant detection and identification of trace level sulfonamides in fish tissue. It has also been shown that UV DAD, in either the full-scan or the multiwavelength acquisition modes, is a useful technique for additional confirmation of sulfonamide identity. The combination of LC, DAD, and ISP-MS–MS techniques, provides a powerful approach to the analysis of sulfonamides and other antibiotics in fish tissue.

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