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Sulfamethazine, Sulfothiazole and Albendazole Residue Dosage in Food Products Determined by Liquid Chromatography/Tandem Mass Spectrometry†

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There is a growing demand for analytical techniques for the detection of a wide variety of residues from synthetic molecules in matrices such as soil, water, air and food. These techniques have to meet the requirements of speed and sensitivity as well as the ability to handle any matrix with minimal sample clean-up. Features of mass spectrometry combined with liquid chromatography can fulfill these requirements as is shown by this work which reports the use of ion spray ionization coupled with tandem mass spectrometry for the detection of some drug residues. In particular, the direct use of existing LC methods, originally conceived for use with some other sort of detector, is demonstrated.

There is a growing demand for analytical techniques for the detection of residues of a wide variety of synthetic molecules in different matrices such as soil, water, air and food in order to address environmental and toxicological problems related, for example, to improper handling procedures in pesticide treatment of crops or in the sanitary care of livestock (drugs and hormone residues). For these applications, the matrix often interferes seriously with the detection of the analyte (pesticide, drug, etc.) which is usually present at trace levels.

The analytical techniques developed to date still face two main challenges: the introduction of new molecules with unanticipated chemical and physical properties and the requirement for the development of fast sensitive analytical methods, particularly those that can handle any matrix with minimal sample clean-up. The latter is compounded by the requirements for ever decreasing detection limits and often by the use of reduced sample sizes.

The present work reports the use of atmospheric pressure ionization (API) coupled with tandem mass spectrometry (MS/MS) for the detection of some drug residues. Specifically we provide examples employing the ion spray (nebulizer-assisted electrospray) technique with sample introduction by high-performance liquid chromatography (HPLC). The mild conditions of HPLC and ion spray make these techniques ideal for the separation and ionization respectively of such compounds. This paper will show that their combination with the highly sensitive and specific detection afforded by MS/MS provides the capabilities necessary for their sensitive detection.

EXPERIMENTAL

Sample handling

Drug residues were extracted from foods using standard methods as reported here, without any special modification for the present experiments. For sulphonamide residues in honey, 5 g of honey were dissolved in 50 mL water containing 30% NaCl and the solution extracted with 60 mL dichloromethane. The organic phase was passed through a BakerBond Florisil SPE cartridge (J. T. Baker Inc., Phillpsburg, NJ, USA) and sulphonamides were eluted from the column with 10 mL methanol. The methanolic solution was evaporated to dryness using a rotary evaporator and the residue is dissolved in 1 mL of an aqueous solution of acetonitrile (50%) containing 10 mM ammonium acetate and acidified to pH 4.5 with formic acid.[1,2]

Two honey samples were prepared by spiking with 143 and 222 ng/g of either sulfamethazine or sulfothiazole. Assuming a 100% recovery in the extraction procedure, the final extracts should contain 71 and 111 pg/μL, respectively, of the spiked drug.

For albendazole and metabolite residues in goats milk, 10 g of milk were extracted with 40 mL acetonitrile. To this extract was added 40 mL hexane, then 40 mL dichloromethane and the mixture was shaken. After separation of the aqueous layer, the organic phase was evaporated to dryness using a rotary evaporator. The residue was redissolved in 1 mL of 0.2 N HCl in ethanol, 5 mL hexane added, and the mixture shaken using a vortex mixer. A 0.3 mL aliquot of the aqueous phase (containing HCl) was neutralized with 2 mL of $KHCO_3$ solution (2%) and cleaned up using a BioLabo SPE C2 cartridge (Lida Manufacturing Corp., Kenosha, WI, USA), which had been sequentially pre-activated with ethyl acetate, ethanol and water. Albendazole and its metabolites were eluted with 2 mL of ethyl acetate and the eluate evaporated to dryness under nitrogen. The residue was finally dissolved in 0.3 mL of an aqueous solution of acetonitrile (50%) containing 10 mM ammonium acetate and acidified to pH 4.5 with formic acid.[3,4]

Two samples of goats milk were spiked with 143 and 222 ng/g of albendazole. Assuming a 100% recovery in the extraction procedure and no metabolism in the time between spiking and the completion of sample preparation, the final extracts should contain 1.43 and 2.22 ng/μL respectively, of the spiked drug.

Standard solutions of the drugs (sulfothiazole, sulfamethazine and albendazole (Sigma Chemicals Co., St Louis,

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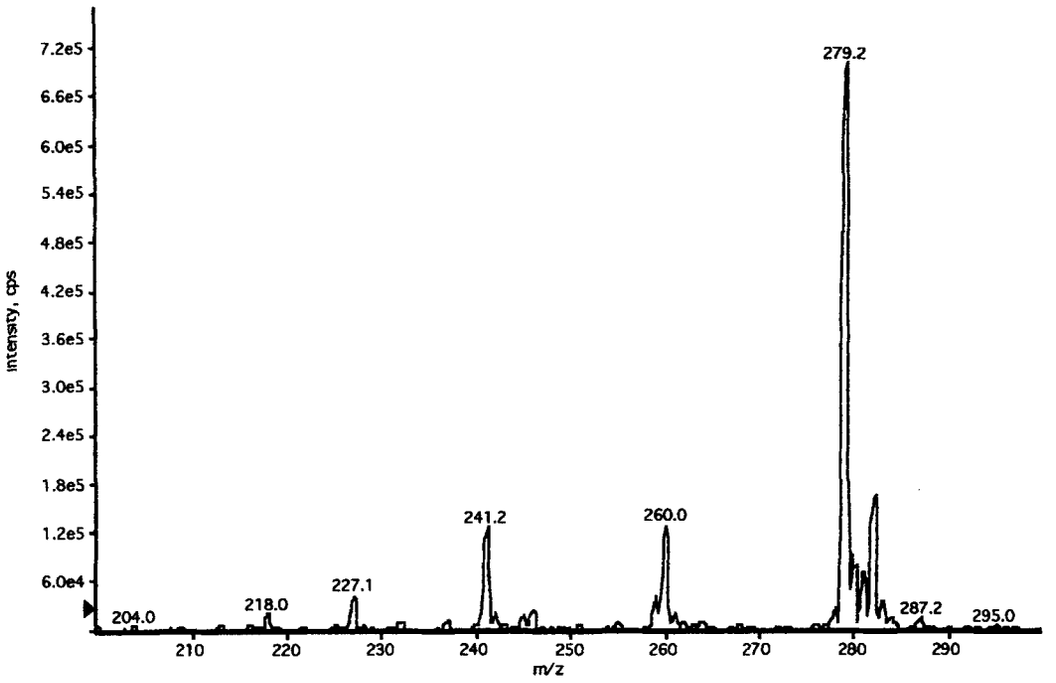

Figure 1. Positive-ion ion spray spectrum for sulfamethazine at a concentration of 500 pg/μL introduced by flow injection.

USA)) and the metabolite, albendazole sulfone (Merck & Co. Inc., Rahway, NJ, USA), were made up at a concentration of 0.5 ng/μL in an aqueous solution of acetonitrile (50%) containing 10 mM ammonium acetate and acidified to pH 4.5 with formic acid. In addition to individual standards, mixture solutions containing sulfathiazole and sulfamethazine at 12.5 to 100 pg/μL, albendazole and albendazole sulfone at 16 to 125 pg/μL, were also prepared in an aqueous solution of acetonitrile (50%) containing 10 mM of ammonium acetate acidified to pH 4.5 with formic acid. All the solvents and chemicals used were from Merck (Darmstadt, Germany). Solvents were HPLC grade.

Instrumentation

A Perkin-Elmer Sciex API 300 bench-top triple quadrupole mass spectrometer (Perkin-Elmer Sciex, Toronto, Canada) with a standard API-IonSpray™ ionization source was employed for this study. The ion spray ion source (pneumatically-assisted electrospray) was operated in the positive-ion

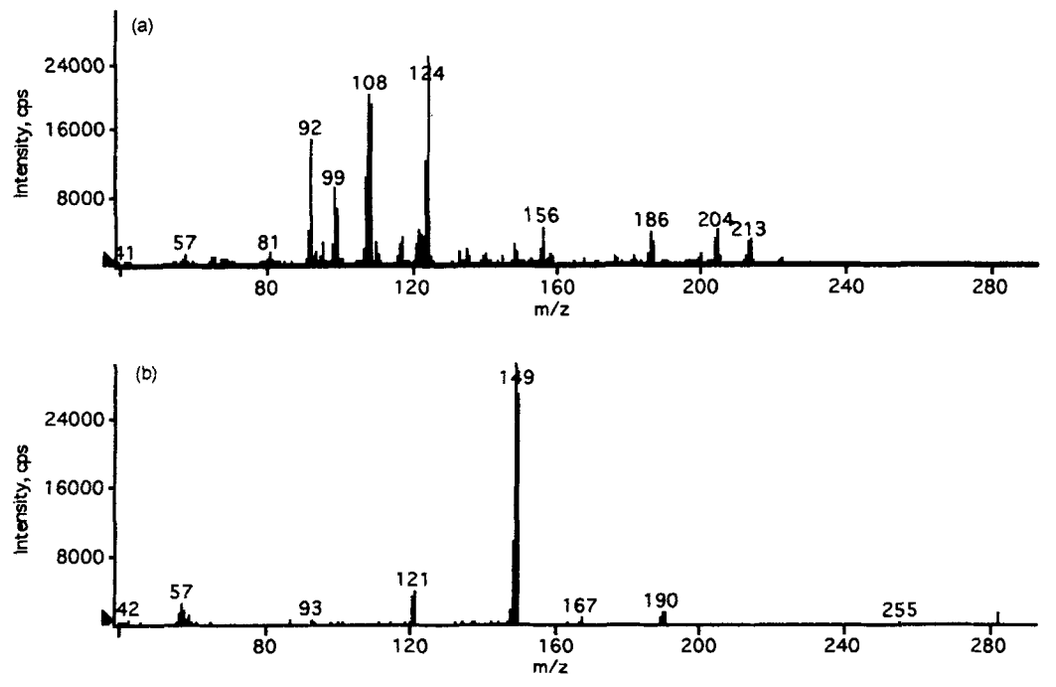

Figure 2. MS/MS spectra in positive-ion mode of (a) sulfamethazine and (b) the contaminant di-butyl phthalate from fragmentation of the 279.2 Th precursor ion using a CAD energy of 40 eV.

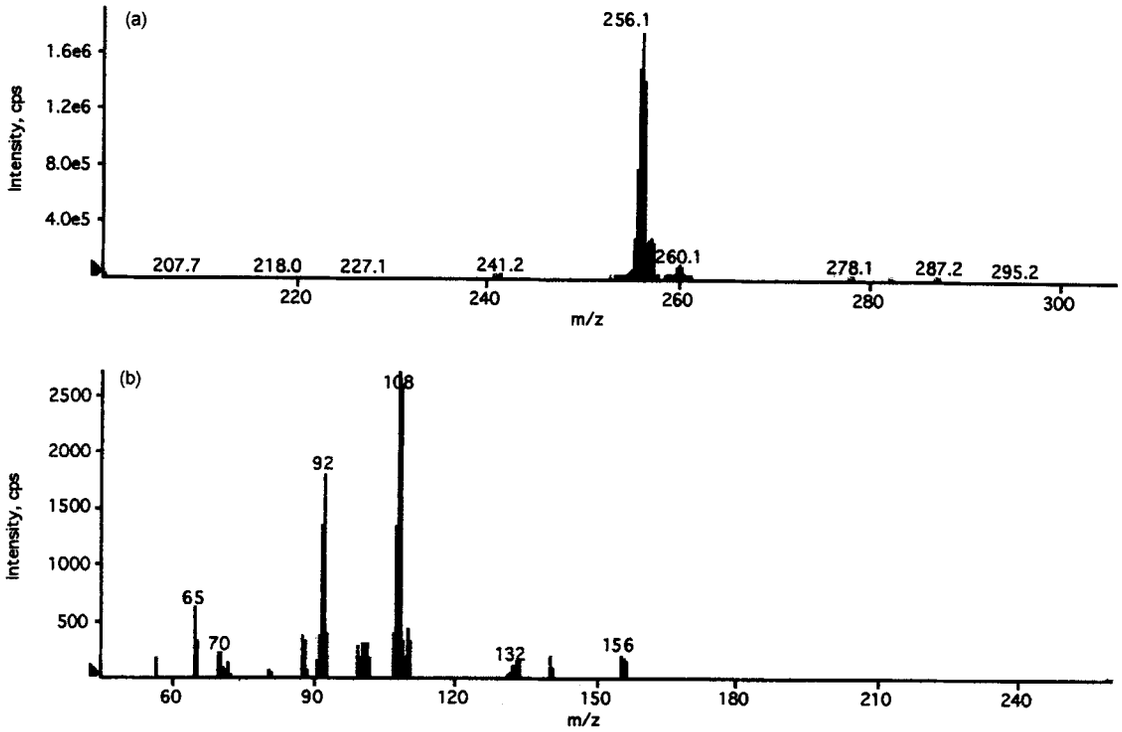

Figure 3. Positive-ion MS (a) and introduced MS/MS (b) spectra for sulfathiazole at a concentration of 500 pg/μL introduced by flow-injection. The MS/MS spectrum was obtained by fragmentation of the 256.2 Th precursor ion using a CAD energy of 40 eV.

mode with a spraying needle voltage of 5000 V and the nebulizer gas (air) flow set at 0.8 L/min. The interface curtain gas (nitrogen) flow rate was 1 L/min and the orifice voltage (defined as the cluster-breaking voltage) was set at 40 V. Acquired data were processed with either Multiview 1.1.1 (spectral information data processing) and MacQuan 1.4 (quantitative data processing) proprietary software packages.

The MS and MS/MS experiments were both run with a resolution of 0.8 mass units (measured at half-height) for both the analytical quadrupoles. Collision-activated decomposition (CAD) experiments used the closed-design Q2 collision cell operating with nitrogen as collision gas at a pressure of 8 m torr and a collision energy of 40 eV (lab.

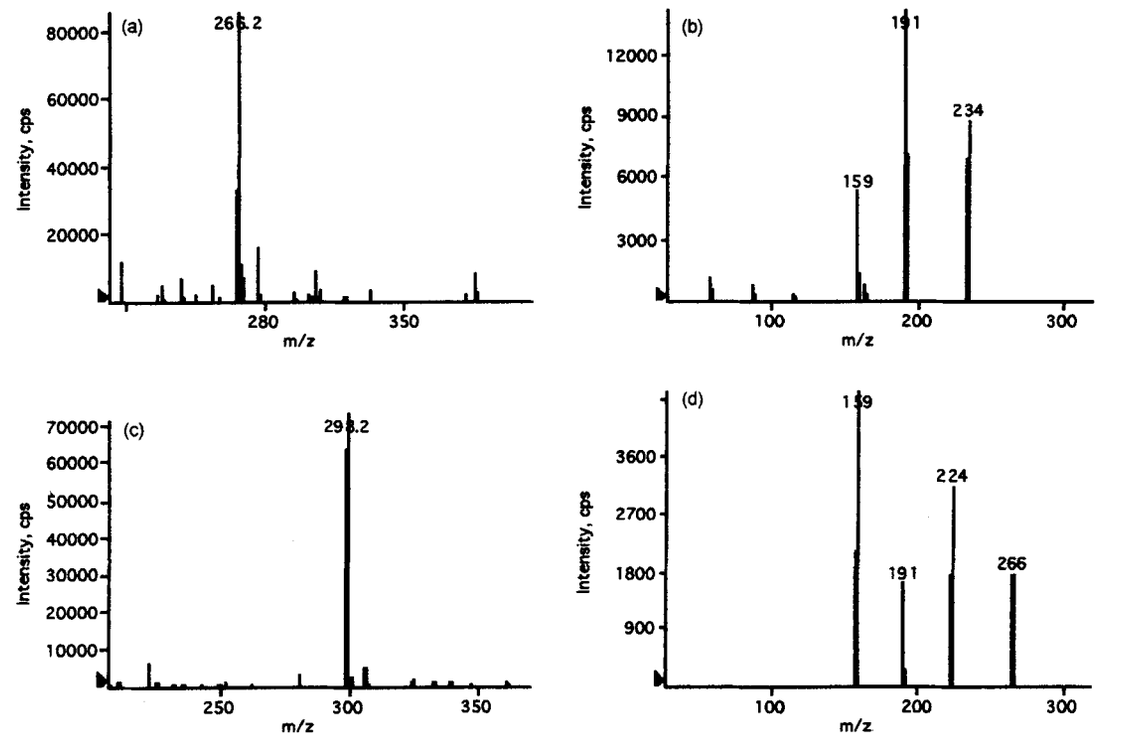

Figure 4. Positive-ion MS (a) and MS/MS (b) ion spray spectra for albendazole and the corresponding spectra (c), (d) for its metabolite, albendazole sulfone. The MS/MS spectra were obtained by fragmentation of the 266.2 and 298.2 Th precursor ions respectively, using a CAD energy of 40 eV.

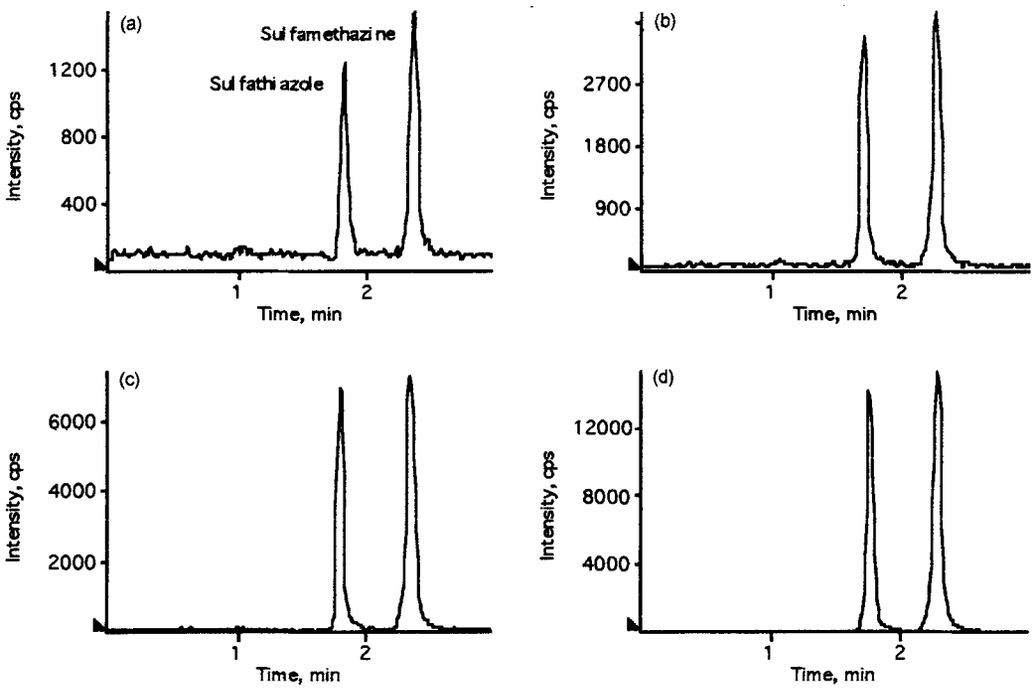

Figure 5. LC/MRM chromatographic tracings for sulfothiazole (transition 256.2>108 Th, RT=1.7 min) and sulfamethazine (transition 279.2>124 Th, RT=2.3 min) using an isocratic LC method with 20% acetonitrile (80% eluent A+20% eluent B). 20 μL was injected on a 4.6 mm-column. Solution concentrations were (a) 12.5, (b) 25, (c) 50 and (d) 100 pg/μL.

frame) for all analytes.

A Model 22 Harvard Infusion pump (Harvard Apparatus, South Natick, MA, USA) was used to introduce a 10^{-4} M polypropylene glycol (PPG) solution for mass calibration or resolution adjustment of both analytical quadrupoles. All other experiments used a Perkin-Elmer LC-200 quaternary-pump (Perkin-Elmer, Norwalk, CT, USA) equipped with a 7125 Rheodyne valve (Rheodyne, Cotati, California, USA) fitted with a 20 μL loop-injector. Samples for MS and MS/MS experiments were injected in the flow-injection mode by connecting the valve output directly to the ion spray source and using a flow rate of 60 μL/min of an aqueous solution of acetonitrile (50%) containing 10 mM ammonium acetate and acidified to pH 4.5 with formic acid.

For the LC/multiple-reaction monitoring (MRM) experiments, the HPLC column was a Supelco C18, 4.6×75 mm (Supelco, Bellefonte, PA, USA). The column was operated at a flow rate of 1 mL/min using different mixtures of aqueous 10 mM ammonium acetate acidified to pH 4.5 with formic acid (eluent A), and acetonitrile (eluent B) either at fixed concentrations or by using gradients as described below. In spite of the fact that the ionization source, when

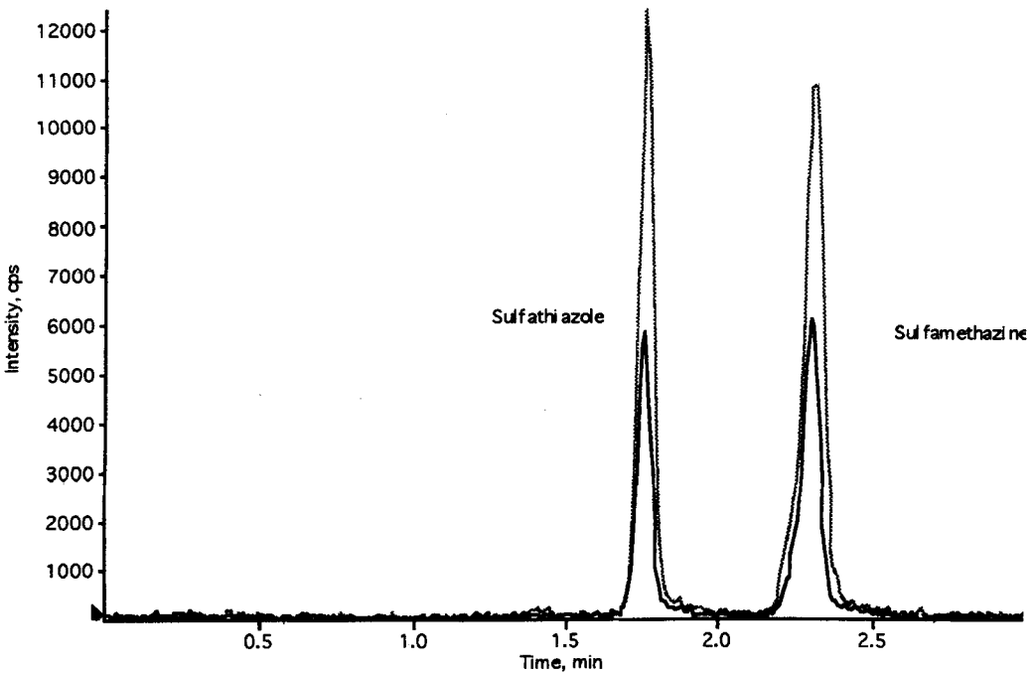

Figure 6. LC/MRM chromatographic tracings for sulfothiazole and sulfamethazine in a honey sample spiked with 222 ng/g of both of the drugs. Tracing of a two-fold-diluted solution is superimposed.

Table 1. Quantitation report for sulfamethazine. Sample 7 is reported to contain 46.5 pg/μL, which corresponds to 93 ng/g in the original sample. Sample 8 is reported to contain 72.5 pg/μL, which corresponds to 145 ng/g in the original sample. The filenames refer to the undiluted extracts, and to the twice-diluted ones.

Sulfamethazine
No Internal Standard
279.2→124.0
Linear
Intercept = −2590.341
Slope = 770.784
Correlation Coeff. = 1.000
Use Area

Filename	Filetype	Accur.	Nom. Conc.	Calc. Conc.	Area	Height
ss-LcMrm-12.5	Standard	92.7	12.5	11.6	6638	1473
ss-LcMrm-25	Standard	105.4	25.0	26.3	17 715	3751
ss-LcMrm-50	Standard	99.2	50.0	49.6	35 632	7331
ss-LcMrm-100	Standard	100.0	100.0	100.0	74 476	15 500
Sample 7-undil.	Sample	n/a	n/a	46.5	33 268	4628
Sample 7-2 × .dil.	Sample	n/a	n/a	24.0	15 930	2374
Sample 8-undil.	Sample	n/a	n/a	72.5	53 280	10 354
Sample 8-2 × .dil.	Sample	n/a	n/a	36.8	25 801	5666

used in the 'Turbo Ion Spray' configuration, can accept the whole LC eluent flow, it was decided to split the eluent downstream the column by a factor of 1:25 before entering the source. Because ion spray-MS response is dependent on the concentration of the analyte and not on the absolute amount, solvent splitting does not deleteriously affect the sensitivity toward the analyte. A second benefit provided by such a split is that any concomitant matrix material makes the ion source 25 times less dirty than with a full flowrate.

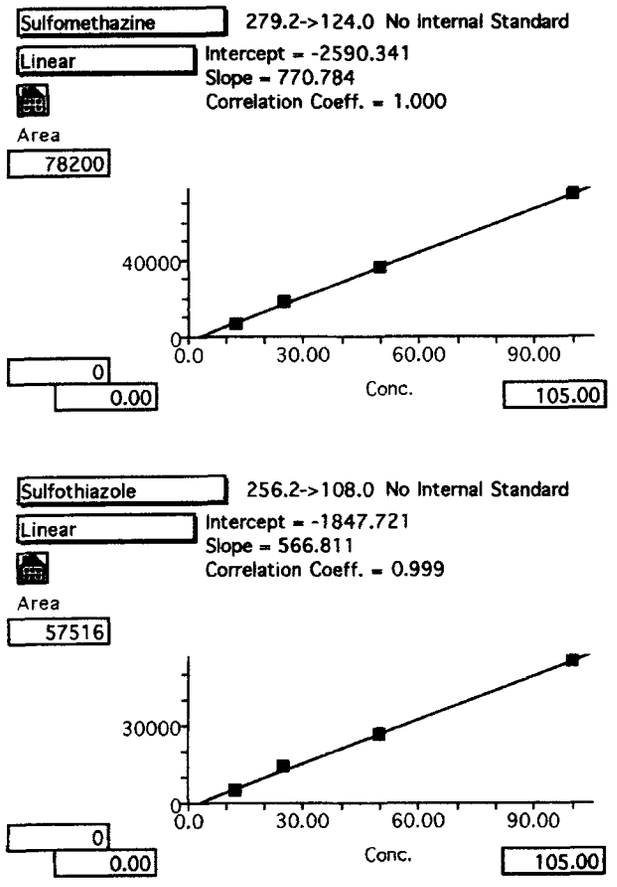

Figure 7. Calibration curves for sulfamethazine and sulfothiazole.

RESULTS AND DISCUSSION

Figure 1 shows the positive-ion ion spray spectrum for sulfamethazine recorded at a concentration of 500 pg/μL. The major ion species is the protonated molecular ion $[MH]^+$ at 279.2 Th. By coincidence, an ion at 279.2 Th is well-known in LC/MS, since it originates from the ubiquitous plasticizer di-butyl phthalate. Consequently, the monitoring of any low level dosage of sulfamethazine by conventional mass spectrometry is prevented because of the high background signal provided by such a contaminant having the same mol. wt. as the analyte. In this case, the use of MS/MS becomes mandatory.

Figure 2 displays MS/MS spectra in the positive-ion mode of both sulfamethazine and the contaminant ion (di-butyl phthalate) obtained, in each case, by fragmentation of the common 279.2 Th precursor ion using a CAD energy of 40 eV. By comparison of the spectra, the fragmenttion patterns are seen to be totally different, which means that quantitation by MS/MS is now viable since no prominent fragment ion of one spectrum matches any ion belonging to the other spectrum.

Figure 3 shows the MS and MS/MS spectra in positive-ion mode for sulfathiazole at a concentration of 500 pg/μL. The MS/MS spectrum records fragmentation of the 256.2 Th precursor ion using the previous CAD energy of 40 eV. Figure 4 shows either the positive-ion MS and MS/MS ion-spray spectra for albendazole and its metabolite, albendazole sulfone. MS/MS spectra were also produced in the positive-ion mode by fragmentation of the 266.2 and 298.2 Th precursor ions, respectively.

The sensitivity and specificity in any analytical determination are respectively affected by the concentration step built into the extraction procedure and by the purity of the extract itself. In the latter case, no other impurities should compete with the known analytes in the chosen analytical technique. A high purity level is unlikely to occur in real life because extracts usually contain co-extractants from a wide variety of species. Depending on the specificity offered by the analytical technique, a more or less extended purification step may be required to adequately detect the analytes.

Usually, however, the time required for the *de novo* development of a method to thoroughly separate analytes is incompatible with the control laboratory demands where

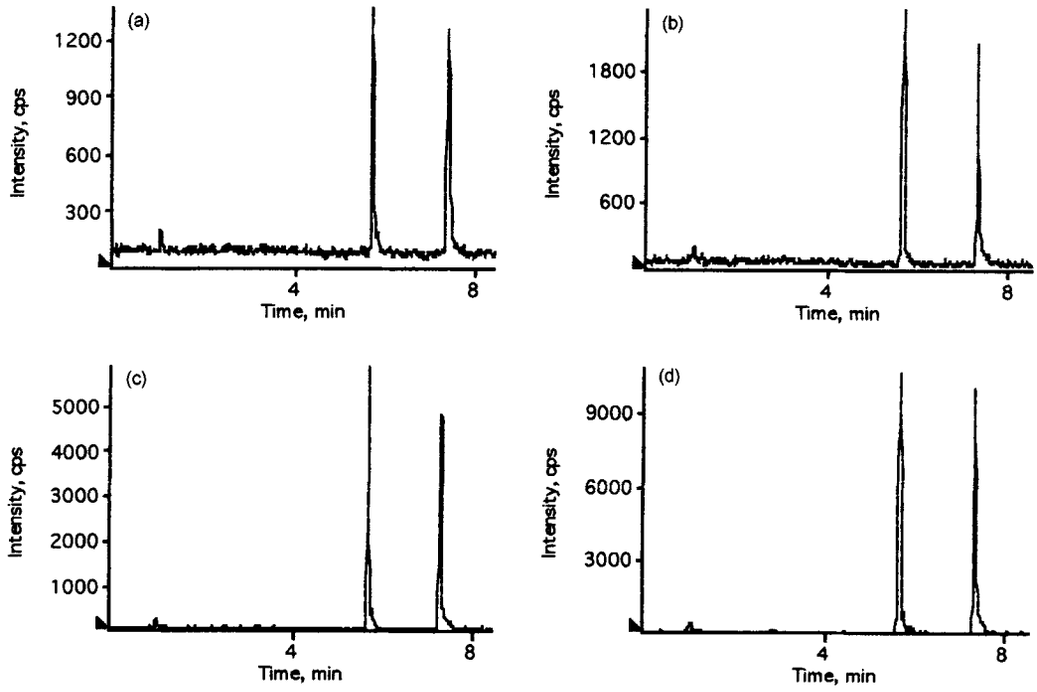

Figure 8. LC/MRM chromatographic tracings for albendazole sulfone (transition 298.2>159 Th, RT=5.4 min) and albendazole (transition 266.2>191 Th, RT=7.2 min). Solution concentrations are (a) 16, (b) 31, (c) 62 and (d) 125 pg/μL.

quick response is an important requirement. Therefore instead of time consuming refinement, by development of the chromatographic method, the selectivity offered by the MS/MS technique can be used to locate residues in real-life samples using a very simple LC method.

Figure 5 shows the MRM chromatographic traces for sulfamethazine and sulfothiazole using an isocratic LC method with 20% acetonitrile (80% eluent A+20% eluent B). With a 20 μL injection on a 4.6 mm-column, a detection limit better than 5 ppb, which corresponds to 10 ng/g expressed as the original concentration before the extraction step (10 mL extract from 5 g sample), is easily achievable. Figure 6 displays the trace from a honey sample spiked with 222 ng/g of sulfamethazine and sulfothiazole. With the LC/MRM techniques, only the analytes are visible. Any other co-extractant, if present, is hidden since the method

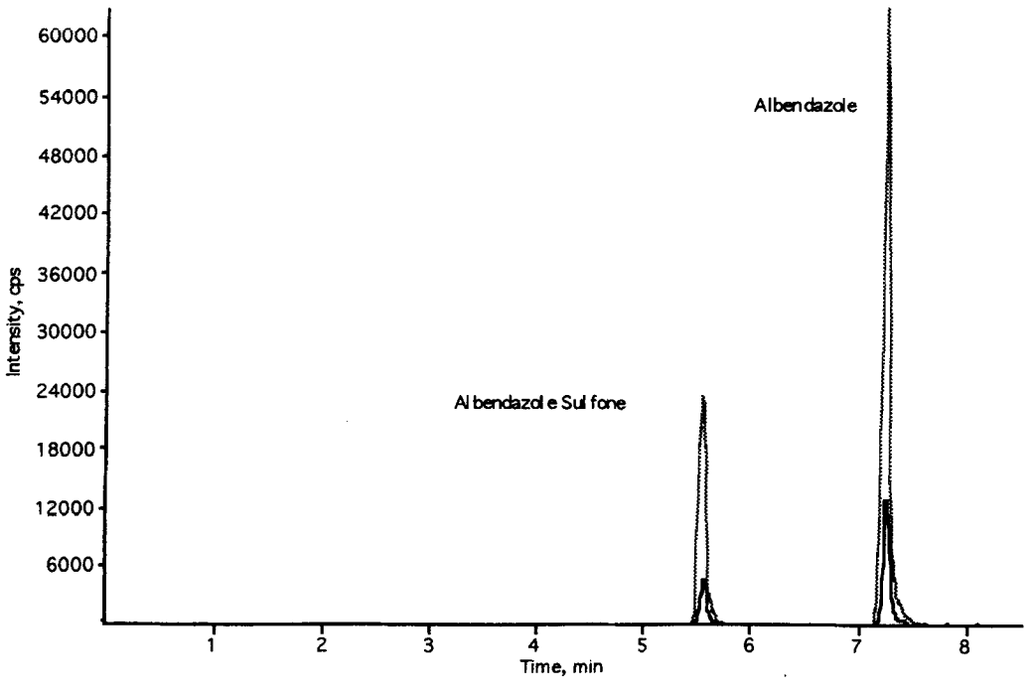

Figure 9. LC/MRM chromatographic tracings for albendazole sulfone and albendazole in a goat milk sample spiked with 143 ng/g of albendazole. Superimposed tracings refer to 20 μL of undiluted and five-fold diluted extracts. Related data are reported in Table 2.

Table 2. Quantitation report for albendazole. Sample 3 is reported to contain 745 pg/μL which corresponds to 74.4 ng/g of unmetabolized drug in the original sample. Sample 4 is reported to contain 855 pg/μL which corresponds to 85 ng/g in the original sample. The filenames refer to the undiluted extracts, and to the five times-diluted ones.

Albendazole
No Internal Standard
266.2→191.0
Linear Thru' Zero
Intercept=0.0
Slope=392.251
Correlation Coeff.=0.998
Use Area

Filename	Filetype	Accur.	Nom. Conc.	Calc. Conc.	Area	Height
as-LcMrm-16	Standard	86.1	15.7	13.5	5305	1161
as-LcMrm-31	Standard	72.0	31.2	22.5	8811	1999
as-LcMrm-62	Standard	96.6	62.5	60.4	23 686	4774
as-LcMrm-125	Standard	102.8	125.0	128.5	50 409	9993
Sample 3-undil.	Sample	n/a	n/a	744.6	292 089	62 483
Sample 3-5 × .dil.	Sample	n/a	n/a	150.7	59 118	12 793
Sample 4-undil.	Sample	n/a	n/a	854.7	335 262	70 501
Sample 4-5 × .dil.	Sample	n/a	n/a	182.8	71 694	14 020

exclusively monitors the transition between the protonated molecular ion of the analyte and one of its more prominent fragment ions. In order to assess the result in Fig. 6, a twice-diluted sample was then injected and the tracing obtained superimposed on the same Fig. 6, proving that no matrix effect occurs. An example of the quantitation report for sulfamethazine is illustrated in Table 1 and Fig. 7 and shows the calibration curves obtained for sulfamethazine and sulfothiazole.

In the case of albendazole and albendazole sulfone, Fig. 8 shows the traces of the LC/MRM experiments on standard solutions (16 to 100 pg/μL) injected using a 20 μL loop and analysed using a gradient LC method starting at 10% acetonitrile (90% eluent A+10% eluent B) and increasng to 60% acetonitrile (40% eluent A+60% B) over 10 min. A detection limit around 5 ppb or better is expected for these compounds, when using a 4.6 mm LC column. Figure 9 displays the results from a goats milk sample spiked with 143 ng/g of albendazole. Again, in order to assess the absence of any matrix effect, a five-fold dilution step was made and the resulting solution was also injected. The new trace is overlaid on Fig. 9. A quantitation report for albendazole is shown in Table 2. Again, the diluted samples report values that are proportional to the undiluted ones.

CONCLUSION

The aim of this work was to investigate how easy it is to interface an LC method, originally conceived for a classical detector (e.g. UV, fluorescence, etc), to a mass spectrometer, without any further time-consuming refinement but, at the same time, with some improved sensitivity. As expected, the MS/MS capabilities, implemented through MRM, provide an outstanding specificity which allows the transfer of almost any LC method to MS interfacing.

The reported sensitivity is related either to the i.d. of the LC column or to the concentration step introduced with the extraction procedure. Therefore, performance can be further improved either by switching to an LC column with a smaller i.d. and/or by changing the concentration factor during the extraction procedure.

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