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Quantification and confirmation of identity of analytes in various matrices with in-source collision-induced dissociation on a single quadrupole mass spectrometer

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Abstract

Mass spectrometry is often used for the quantification of target analytes. When liquid chromatography (LC) is interfaced with mass spectrometry, it is generally accepted that tandem mass spectral (MS/MS) techniques are required to quantify an analyte with confirmation of identity in a complex matrix. In such applications LC is generally used to deliver the sample to the mass spectrometer with minimal separation, and the bulk of the separation is downloaded to the tandem mass spectrometer. However, LC is also a powerful separations tool, and a tandem mass spectrometer is not required to gain fragmentation data. In-source collision-induced dissociation (IS-CID) on a single stage mass spectrometer will generate rich fragmentation patterns that are often used for qualitative identification and rarely for quantitative confirmation of identity. Quantification with confirmation of identity on a single stage quadrupole mass spectrometer can be done by concurrently monitoring for the expected parent and fragment ions using multiple selected ion recording (SIR) channels. Although such methods have been used to quantify analytes with confirmation of identity in simple matrices, only isolated examples of such work with complex matrices are published. We will extend upon the previous work and discuss the utility of using single quadrupole mass spectrometers for the quantification of an analyte with concurrent confirmation of identity in complex matrices. The analysis of methomyl in spinach extract demonstrates the ability of simple LC/MS to separate out interferences and give accurate relative abundances of fragments. Sulfometuron methyl was quantified accurately out of apple matrix at a level of 200 pg/μL, even with a calibration curve run in pure solvents. β-Lactam antibiotics and lincomycin were analyzed out of bovine milk extract. The limits of detection (LOD) for the β-lactam antibiotics ranged from 15.5 to 105 pg of material on-column, and although these LODs were higher than observed with a triple quadrupole mass spectrometer, they were actually superior to those observed on an ion trap mass spectrometer. Lincomycin gave an LOD of 0.83 pg on-column, despite the fact that trifluoroacetic acid (TFA) was required to give adequate chromatography. In our final example, we demonstrate the quantification of ritonavir out of blood plasma extract. The LOD of ritonavir under these conditions was 2.2 pg on-column. For both lincomycin and ritonavir we achieve confirmation of identity even at the lowest concentrations we ran. (Int J Mass Spectrom 222 (2003) 281–311)

Keywords: Mass spectrometry; Liquid chromatography; Single quadrupole; Quantification; Confirmation of identity; Biological matrix; Methomyl; Sulfometuron methyl; β-Lactam antibiotics; Lincomycin; Ritonavir

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1. Introduction

Mass spectrometry (MS) is a tool often used for the quantification of target analytes. Although liquid chromatography/mass spectrometry (LC/MS) has been used for quantification of analytes in simple matrices [1,2], complex matrices often require additional chemical separation of the analyte from interferences in the matrix. The ability of a tandem mass spectrometer to perform chemical separations via ion isolation is attractive in these cases, and it has been generally accepted that the quantification (with confirmation of identity) of analytes in complex matrices requires tandem mass spectral (MS/MS) techniques [3–5]. For this kind of work the LC is often used only to deliver the sample to the mass spectrometer with minimal separation (the use of short columns with minimal separation allow for faster sample-to-sample cycle times), and the bulk of the separation is performed with the tandem mass spectrometer.[1]

However, LC is also a powerful separations tool. When high-throughput of samples is not required, most of the separation can be performed with the LC instrument, where solvent and column selection can provide for highly selective separations. If it is desired to quantify several analytes out of a single sample, an optimized LC separation can deliver each analyte as discrete peaks, allowing the mass spectrometer to focus on the signals of interest in each chromatographic peak.

It should also be noted that obtaining fragmentation spectra from an analyte does not require an MS/MS instrument. Single stage (usually quadrupole) MS instruments are able to fragment parent ions in their source (in-source collision-induced dissociation, or IS-CID) and then analyze the fragments produced. IS-CID with single stage instruments is often used for qualitative confirmation of a target analyte [6–14], and in isolated cases it has been used for quantitative confirmation of a target analyte [1,2,15].

[1] The tandem instruments most often used for this work are triple quadrupoles (TQs), quadrupole ion traps (QITs), quadrupole-time of flight (Q-TOF) instruments and Fourier transform ion cyclotron resonance (FT-ICR, often shortened to FT) instruments.

The previous considerations suggest that quantification with concurrent confirmation of identity can be done with a single stage instrument. Qualitative confirmation can be done using full scan methods, but the ultimate sensitivity suffers in such methods. The key to doing quantitative confirmation is to concurrently monitor for the expected parent and fragment ions using multiple selected ion recording (SIR) channels. The LC method provides the separation while the observation of fragments provides confirmatory evidence of identity. Sensitivity is increased by summing together the individual data channels into a pseudo-total ion channel, and further enhanced by using dwell times of 0.1–0.2 s to maximize the number of data points across the chromatogram.

This kind of quantification with single quadrupole mass spectrometers has been recently demonstrated for the determination of antibiotics in aqueous environmental samples [1]. An aqueous environmental matrix is comparatively simple, and it is still believed that quantification with a single quadrupole instrument would be unworkable with more complicated matrices [3]. However, this belief may not be entirely justified. Two previous studies have explored quantification with single quadrupole instruments on samples in complex matrices. One demonstrated the determination of gentian violet in catfish muscle [15] and the other β-lactam antibiotics in kidney, liver, meat and plasma [16]. The observed limits of quantification for gentian violet were as low as 0.5 ng/μL (which still corresponds to 500 pg on-column for a 1 μL injection) and IS-CID was used in this case to give quantitative confirmation of the analyte. The limits of quantification for the β-lactam antibiotics were on the order of 50 pg/μL in the various matrices, but this required injections of 100 μL to achieve (5 ng of material on-column!). For the β-lactam antibiotics only the protonated parent ions were monitored: IS-CID was not used in that study. Although both results are promising, they are isolated and have not been followed with additional work.

In this paper we will extend beyond the previous work and discuss the utility of using single quadrupole mass spectrometers for the quantification and

concurrent confirmation of pesticides (specifically methomyl and sulfometuron methyl) in plant extracts, β-lactam antibiotics and lincomycin in bovine milk extracts and ritonavir from human blood plasma extracts. The ability of single quadrupole mass spectrometers to quantify and concurrently confirm the identity of analytes with high sensitivity in this variety of matrices will be demonstrated.

2. Determination of methomyl and sulfometuron methyl in plant matrices

It is critical in the quantification of an analyte to observe a reproducible signal that provides a confirmation of identity. It is also critical to see this confirmatory signal with high sensitivity. We will present work on methomyl in this section that illustrates the ability of a single quadrupole mass spectrometer to obtain highly reproducible confirmatory data in a complex matrix, and further work with sulfometuron methyl that illustrates the sensitivity limits reachable with a single quadrupole instrument.

2.1. Materials and reagents

RODI Water (18 MΩ) was generated in-house. Methanol, acetonitrile and formic acid were obtained from Sigma-Aldrich. The methomyl standards (5.0 and 100 pg/μL) and the associated spinach extract (2 g sample matrix/mL) were acquired from the Florida Department of Agriculture. The samples were all dissolved in methanol. A 100 pg/μL solution of methomyl in spinach was made by dilution of the 5.0 ng/μL standard with the spinach extract. A symmetry C18 (2.1 mm × 150 mm, 5 μm, 100 Å) column was used to chromatograph the methomyl samples. Sulfometuron methyl and the associated apple extract were acquired from the Washington State Department of Agriculture. A 2.65 mg/μL standard in dichloromethane was prepared to generate the standards for quantification, and a 200 pg/μL solution in apple extract was prepared to test our ability to quantify in matrix. An Xterra C18 (2.1 mm × 50 mm, 5 μm, 100 Å) column was used to chromatograph the sulfometuron methyl samples.

2.2. Instrumentation

Experiments were performed with an Alliance 2690 quaternary liquid chromatograph coupled to a ZMD 4000 single quadrupole mass spectrometer (Waters, Milford, MA). Instrument control and data acquisition was done with Masslynx software (version 3.2 build 6).

2.3. LC/MS methodology

Methomyl samples were chromatographed with a water/acetonitrile gradient. Mobile phase A was water and mobile phase B was acetonitrile. From 0 to 20 min the gradient ramped linearly from 10% B to 70% B, and from 20 to 22 min the gradient ran linearly from 70% B back to 10% B. The column was then re-equilibrated from 22 to 27 min at 10% B. The flow rate throughout the gradient was 0.2 μL/min. The column temperature was set to 42 °C and the autosampler temperature set to 5 °C. 5 μL of sample was injected for each run, corresponding to 0.5 ng of methomyl on-column. Methomyl eluted in 6.28 ± 0.02 min under these conditions. Ten replicate injections of the 100 pg/μL methomyl standard were made, and then 10 replicate injections of the 100 pg/μL methomyl in spinach extract.

Methomyl was electrosprayed in the positive ionization mode. The needle voltage was set at 3.5 kV and the rf lens to 0.2 V. The source block and desolvation temperatures were 150 and 350 °C. Nitrogen gas was used for the nebulization (100 L/h) and desolvation (400 L/h). The extractor lens was set to 5 V. Three channels were monitored: *m/z* 163.1 (cone voltage at 22 V) for the parent; *m/z* 106.1 and *m/z* 88.1 (cone voltage 18 V) for the characteristic fragments. The dwell time for each channel was 0.3 s with a mass window of 0 *m/z*.

Sulfometuron methyl samples were chromatographed with a water/acetonitrile gradient. Mobile phase A was water, mobile phase B was acetonitrile

and mobile phase C was water with 1% formic acid. Throughout the gradient we held mobile phase C at 10% of the composition, so the actual concentration of formic acid throughout the gradient was 0.1%. A and B percentages were adjusted so that from 0 to 4 min the gradient ramped linearly from 15% organic (75% A, 15% B, 10% C) to 90% organic (0% A, 90% B, 10% C). From 4 to 8 min that composition was held constant at 90% organic, and from 8 to 8.1 min the gradient ramped from 90% organic back to 15% organic. From 8.1 to 14 min the column was re-equilibrated at 15% organic. The flow rate was 0.25 μL/min up to 10.1 min, and 0.4 μL/min from 10.1 to 14 min to reduce that time needed for re-equilibration. The column temperature was set to 30 °C and the autosampler temperature set to 5 °C. 5 μL of sample was injected for all runs. Sulfometuron methyl eluted in 7.20 ± 0.02 min under these conditions.

Sulfometuron methyl was electrosprayed in the positive ionization mode. The needle voltage was set to 2.90 kV and the rf lens to 0.4 V. The source block and desolvation temperatures were 150 and 350 °C. Nitrogen gas was used for the nebulization (100 L/h) and desolvation (300 L/h). The extractor lens was set to 5 V. For the full scan experiments, we did low/high cone voltage switching between 15 and 60 V to observe parent and fragment ions. For the quantification experiments two channels were monitored: *m/z* 365.0 (cone voltage at 15 V) for the parent and *m/z* 150.0 (cone voltage at 60 V) for the characteristic fragment. The dwell time for each channel was 0.1 s with a mass window of 0 *m/z*.

For the quantification study, sequential dilutions of the stock 2.65 mg/μL solution were made in water (we did not possess enough apple extract to do all the dilutions with it). Triplicate injections of standards were made at the following levels (in the given order): 1.3, 2.6, 5.2, 10.3, 41.4, 165.3, and 662.5 pg/μL. Duplicate blank injections were made before running the standards and after the standards were complete. We also performed six replicate injections of sulfometuron methyl in apple extract at a concentration of 200 pg/μL. These were classified as analyte injections in the software, which allows us to evaluate how well the calibration curve we obtained will quantify a real sample. The injection volume for all samples was 5 μL. Thus, the on-column amounts of material injected ranged from 6.5 pg with the 1.3 pg/μL level to 3312.5 pg with the 662.5 pg/μL level. 1000 pg was injected on-column from the apple matrix.

2.4. Results and discussion

Methomyl is actually a fairly fragile compound, and it falls apart easily even at low cone voltages. Three major fragments (*m/z* 88.1, *m/z* 106.1 and *m/z* 122.1) can be observed in addition to the parent (*m/z* 163.1), as shown in Fig. 1. EPA and FDA guidelines require the observation of three characteristic ions for target confirmation [3,17], and since *m/z* 122.1 has the smallest abundance, we chose to omit it from further consideration. Table 1 presents the relative abundances of *m/z* 88.1, *m/z* 106.1 and *m/z* 163.1 normalized to the *m/z* 88.1 fragment for the 10 replicate injections out of methanol and the 10 replicate injections

Table 1
Methomyl confirmation in spinach: reproducibility of IS-CID in standards and matrix[a]

	Relative abundance (%)					
	Standard			Spinach		
	m/z 88	*m/z* 106	*m/z* 163	*m/z* 88	*m/z* 106	*m/z* 163
	100	51	34	100	55	34
	100	56	36	100	62	36
	100	54	34	100	55	30
	100	51	32	100	65	35
	100	55	31	100	63	36
	100	52	30	100	65	37
	100	54	30	100	69	38
	100	54	30	100	71	37
	100	54	30	100	72	38
	100	55	30	100	51	31
Mean	100	53.6	31.7	100	62.8	35.2
Standard deviation	–	1.7	2.2	–	7.16	2.78
CV (%)	–	3.2	7.0	–	11.4	7.9

[a] Ten replicate injections of each sample, 0.5 ng methomyl on-column in each injection. Intensities normalized on *m/z* 88.

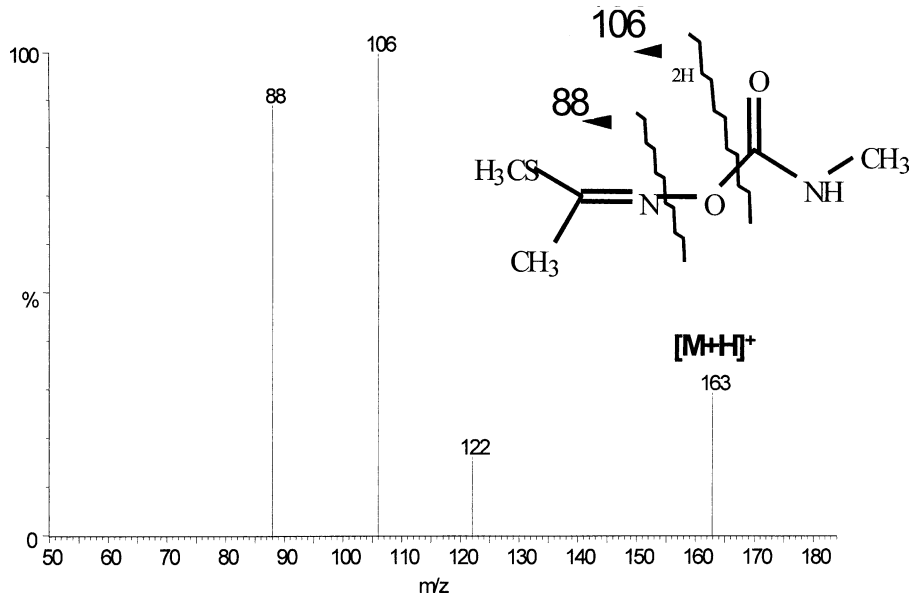


Fig. 1. Characteristic ions observed from methomyl with electrospray in the positive ionization mode. *m/z* 122 arises from the loss of acetonitrile.

out of spinach extract. Even at 0.5 ng of methomyl on-column, the relative abundances are stable and reproducible. Furthermore, the relative abundances observed in spinach extract agree well with the relative abundances observed in methanol. This indicates that LC can effectively separate methomyl from interferences in the spinach extract and eliminate the need for an initial stage of MS to isolate the analyte ion.

In contrast to methomyl, sulfometuron methyl is a robust compound that requires a considerable cone voltage before it will fragment significantly. At low cone voltage we observe mainly the protonated parent molecular ion at *m/z* 365, but also some of the sodiated parent molecular ion at *m/z* 387 (Fig. 2). At high cone voltage we generate one major fragment (at *m/z* 150) from sulfometuron methyl. Although other peaks are seen in the high cone voltage spectrum, Fig. 3 illustrates how we use selected ion chromatograms to differentiate between characteristic fragment signals and spurious background signals. This compound presents an interesting quandary, since it only generates one significant fragment ion. No third ion is seen that would allow us to satisfy the EPA three-ion guideline. Inspection of the sulfometuron structure in Fig. 2 easily explains the predominance of the *m/z* 150 fragment—the neutral lost in this process is a stable sulfonamide compound, while the ion is a resonance stabilized acyl cation. The ability to form two very stable products probably lowers the activation energy for this fragmentation pathway considerably, leaving this pathway highly favored over other possibilities. This behavior illustrates how the chemical properties of the analyte will dictate how it behaves within a mass spectrometer, and how this behavior can sometimes require some flexibility in applying standards such as the three-ion rule to confirm the identity of a target.

With the knowledge that *m/z* 150 was the characteristic fragment of sulfometuron methyl, we ran a quantification study on this compound as described in Section 2.3. Fig. 4 shows the typical selected-ion chromatograms for the 2.6 pg/μL (13 pg on-column) standard. The characteristic fragment from sulfometuron methyl is clearly seen in conjunction with the parent molecular ion at the expected retention time, and this confirms that this signal does indeed arise

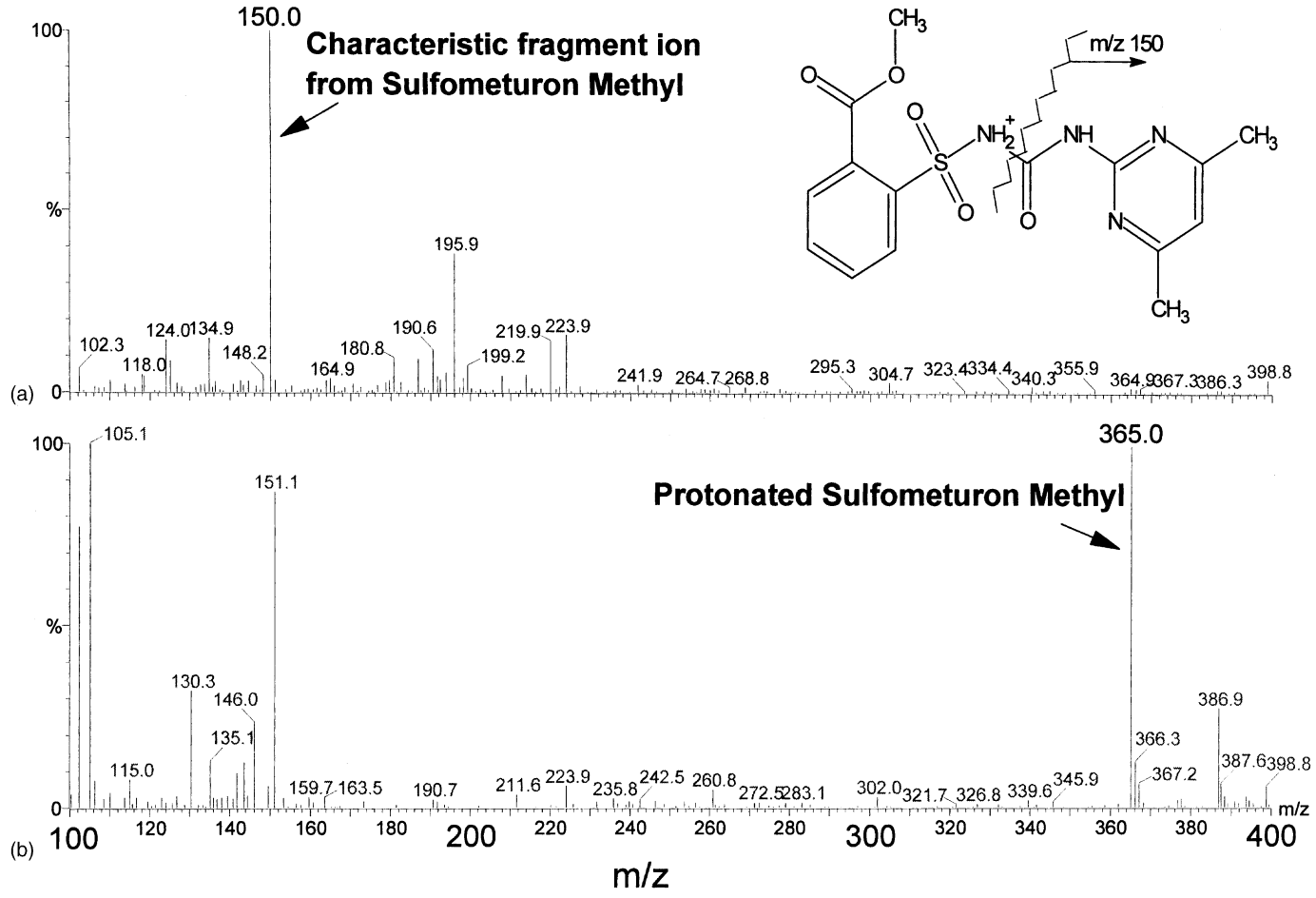


Fig. 2. Background-subtracted full scan positive ionization electrospray spectra of sulfometuron methyl at (a) a cone voltage of 60 V and (b) a cone voltage of 15 V. *m/z* 195.9 and *m/z* 151.1 are a background ions (see Fig. 3).

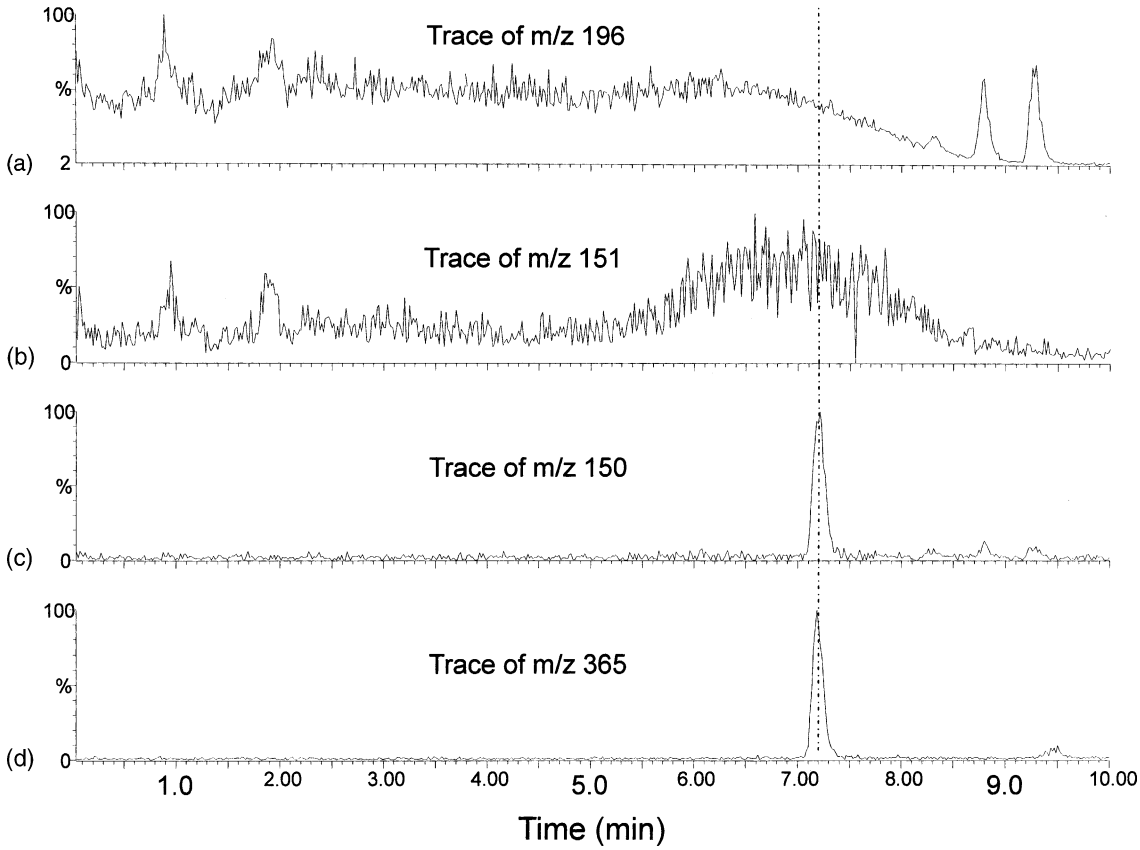


Fig. 3. Selected ion chromatograms for (a) *m/z* 196 at high cone voltage (60 V); (b) *m/z* 151 at low cone voltage (15 V); (c) *m/z* 150 at high cone voltage (60 V); and (d) *m/z* 365 at low cone voltage (15 V). The simultaneous observation of both *m/z* 365 and *m/z* 150 demonstrates that they are related and that *m/z* 150 is not a background ion. In contrast to this, neither *m/z* 196 nor *m/z* 151 show any response above the baseline at the expected retention time, indicating that they are truly background ions that are not completely subtracted from the spectra in Fig. 2.

from sulfometuron methyl. Fig. 5 shows the calibration curve derived from these data, and we should note that we enhanced our sensitivity by summing the signals from both *m/z* 365 and *m/z* 150 into a combined ion chromatogram. The curve was linear, with a correlation constant of 0.9998. Even at the lowest level we ran, the points fit very well to the curve. It is impressive to note that these results were obtained without the use of an internal standard to normalize our signal responses. To insure that we are not seeing a background signal, we compared in an overlay the 1.3 pg/μL (6.5 pg on-column) standard with a blank run (Fig. 6). At this point our combined signal had a signal-to-noise (S/N) ratio of 3:1, which we take to be a limit of detection (LOD).

Although the standards were diluted in water, we did make an "analyte" sample of sulfometuron methyl in apple extract at a known concentration of 200 pg/μL. Comparing the concentration calculated from our calibration curve with the known concentration gives an indication of how the matrix will affect our quantitative results. The calculated concentrations for the six replicate runs are shown in Table 2, along with the average concentration (216 pg/μL) and the standard deviation of these calculated concentrations. Even though the concentration in matrix is calculated

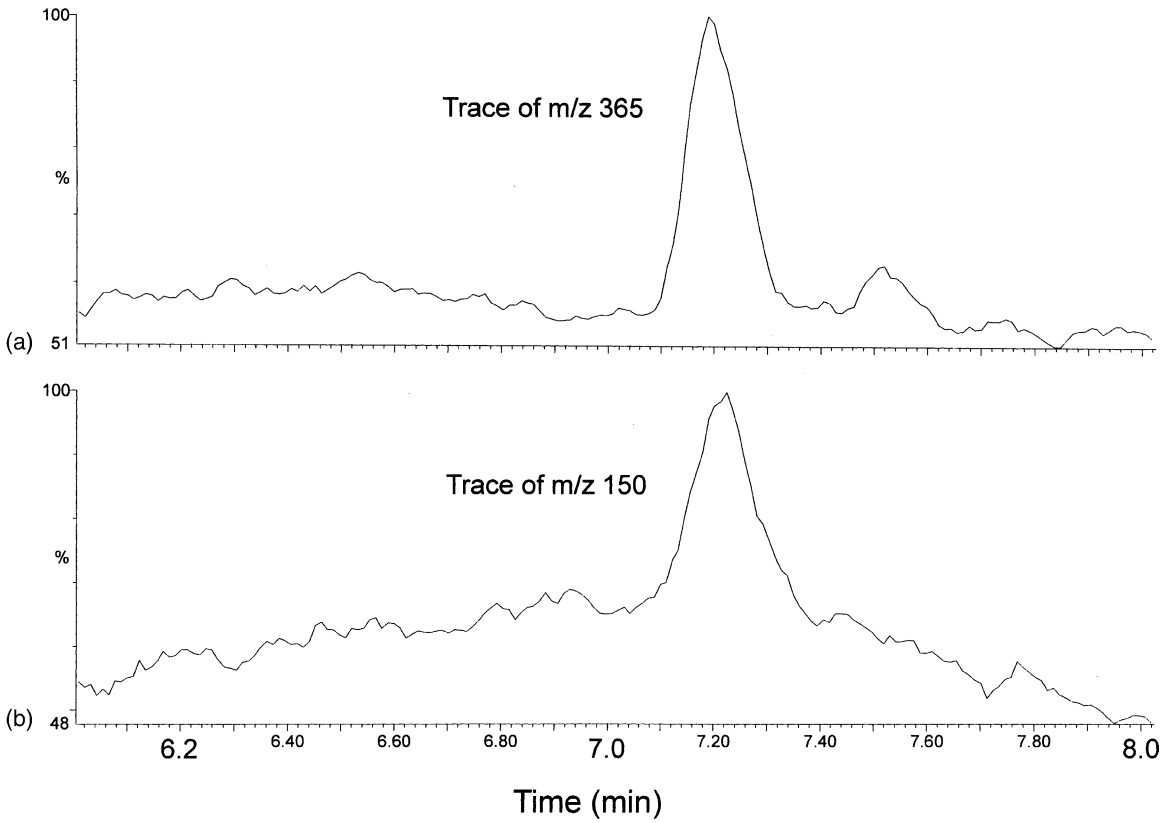


Fig. 4. Selected ion recording for (a) *m/z* 365 and (b) *m/z* 150. For this injection 13 pg of sulfometuron methyl was injected on-column. Note how the appearance of the expected fragment ion at the correct retention time confirms the detected signal is from sulfometuron methyl.

from a calibration curve in plain solvent, the calculated concentration is in good agreement with the actual concentration. This indicates that LC can effectively separate sulfometuron methyl from interferences in the apple extract and eliminate the need for an initial stage of MS to isolate the analyte ion.

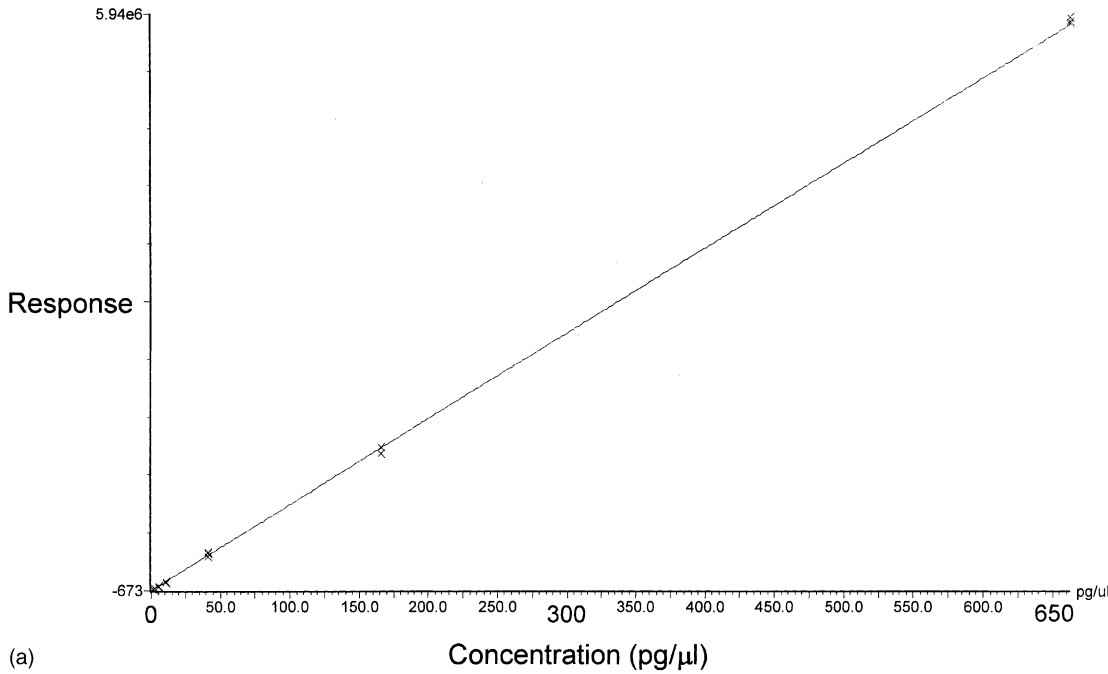
Table 2
Signal response of sulfometuron methyl "analyte" (200 pg/μL) calculated from calibration curve[a]

	Calculated concentration (pg/μL)
Injection 1	220
Injection 2	201
Injection 3	229
Injection 4	215
Injection 5	213
Injection 6	216
Mean	216
Standard deviation	9

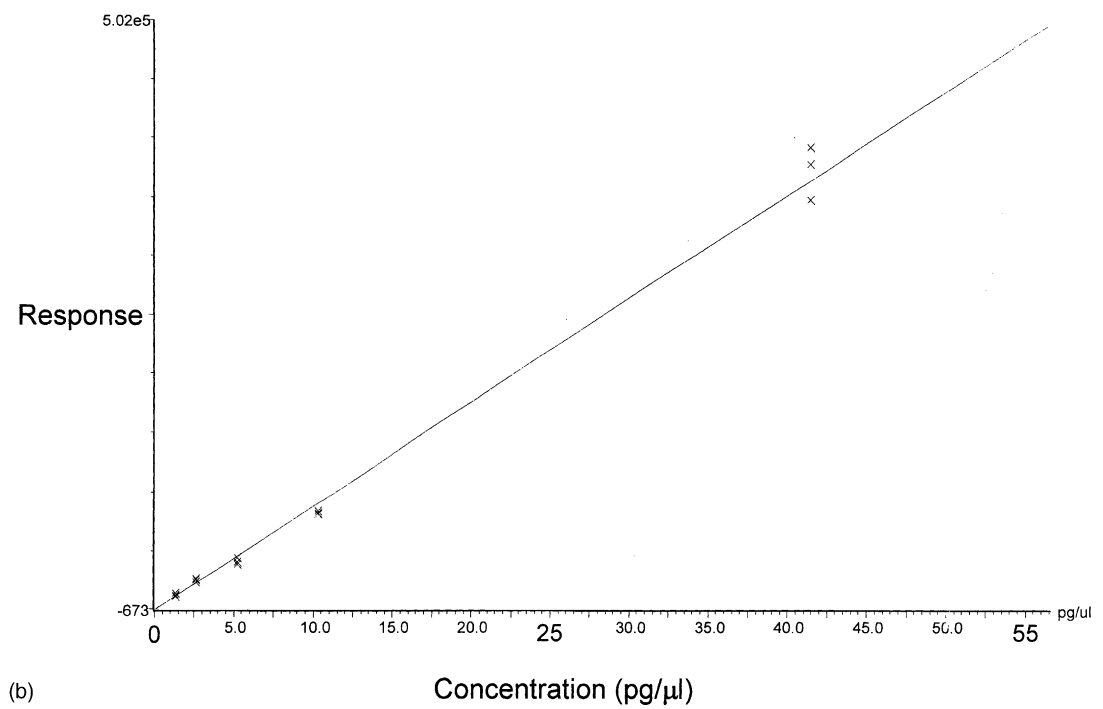
[a] Observed signal is sum of intensities of *m/z* 150 and *m/z* 365.

3. Determination of β-lactam antibiotics and lincomycin in bovine milk extract

We have seen how IS-CID can provide a reproducible signal that allows for quantification with confirmation of identity in plant matrices. In this section we will extend these results by demonstrating the quantification with confirmation of a series of β-lactam antibiotics and lincomycin in bovine milk extract. This work will further illustrate the sensitivity and specificity that can be achieved with a single quadrupole mass spectrometer, and compare favorably to results obtained on ion traps or triple quadrupoles.



(a)



(b)

Fig. 5. Derived calibration curve for sulfometuron methyl from the quantification study. The concentrations of the standards ranged from 1.3 to 662.5 pg/µL (6.5–3312.5 pg on-column). The full range of the curve is shown in (a), while (b) shows a close up of the low standards from 1.3 to 41.4 pg/µL. The fit is linear ($y = 8853.4x - 673.08$) and the coefficient of variation is 0.9998. The origin was excluded from the fit and we used $1/x$ weighting. No internal standard was used to normalize the response. To increase our sensitivity, the quantified response was the sum of the intensities of *m*/*z* 150 and *m*/*z* 365. We used the area of the chromatographic peak from each ion to evaluate our signal intensity.

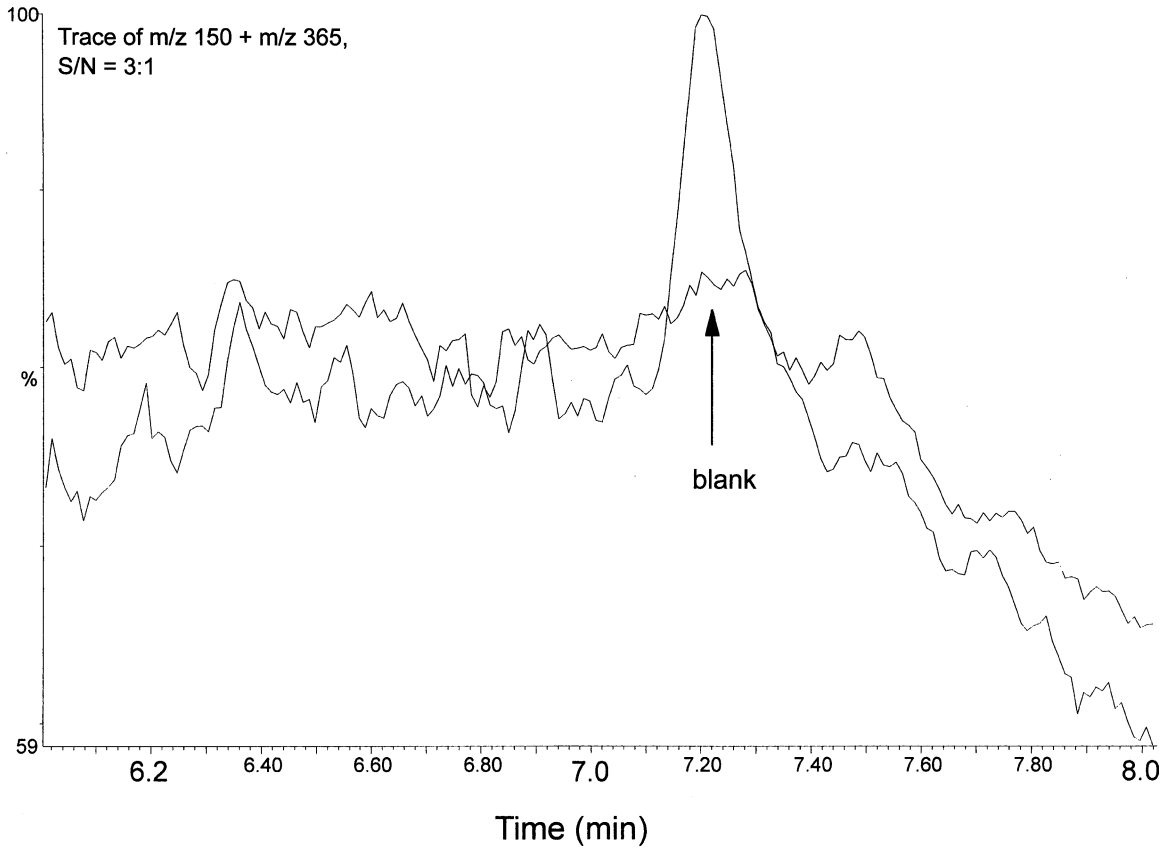


Fig. 6. Overlaid chromatograms of sulfometuron methyl (6.5 pg injected on-column) and a blank injection. Both traces are a combined ion chromatogram of *m*/*z* 150 and *m*/*z* 365. The observed peak in the trace from the sulfometuron methyl injection has a S/N ratio of 3:1. No signal from sulfometuron methyl is observed in the blank injection.

3.1. Materials and reagents

RODI Water (18 MΩ) was generated in-house. Methanol, acetonitrile formic acid and trifluoroacetic acid (TFA) were obtained from Sigma-Aldrich. The β-lactam antibiotics (ampicillin, amoxicillin, cloxacillin sodium and cephapirin sodium), lincomycin and the associated milk extract were acquired from the USFDA in Denver. The milk extract was prepared following the procedure of Moats and Harik-Khan [18]. A stock solution (in 50:50 methanol:water) of the mixed β-lactam antibiotics was made with the following concentrations: 2.19 mg/μL ampicillin, 2.09 mg/μL amoxicillin, 2.15 mg/μL cloxacillin and 2.11 mg/μL cephapirin. For lincomycin a stock solution of 1.13 mg/μL in water was prepared. An Xterra C18 (2.1 mm × 50 mm, 5 μm, 100 Å) column was used to chromatograph the β-lactam antibiotics and the lincomycin samples.

3.2. Instrumentation

Experiments were performed with an Alliance 2690 quaternary liquid chromatograph coupled to a ZMD 4000 single quadrupole mass spectrometer (Waters, Milford, MA). Instrument control and data acquisition was done with Masslynx software (version 3.3).

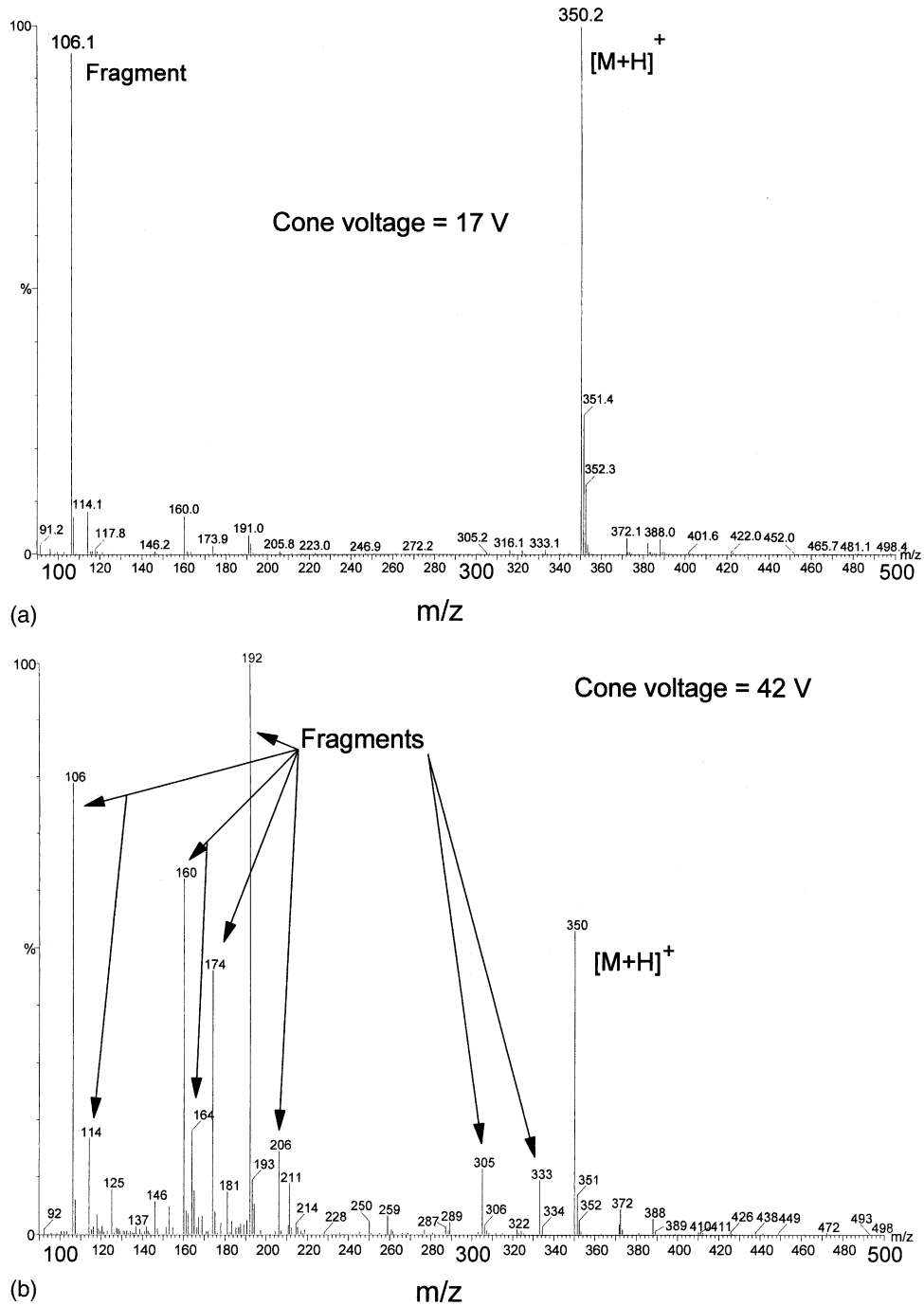


Fig. 7. Background-subtracted full scan positive ionization electrospray spectra of ampicillin at (a) a cone voltage of 17 V and (b) a cone voltage of 42 V.

3.3. LC/MS methodology

The β-lactam antibiotics were chromatographed with a water/methanol gradient containing 0.1% formic acid. Mobile phase A was water with 0.1% formic acid and mobile phase B was methanol with 0.1% formic acid. During the gradient the flow rate was 0.25 μL/min. From 0 to 7 min the gradient ramped linearly from 5% B to 95% B, and from 7 to 10 min the composition was held at 95% B. From 10 to 11 min the composition was ramped from 95% B back to 5% B. At 11.1 min the flow rate was increased to 0.35 μL/min to hasten column re-equilibration, and the column was then re-equilibrated from 11 to 17 min at 5% B. The column temperature was set to 30 °C and the autosampler temperature set to 4 °C.

The β-lactam antibiotics were electrosprayed in the positive ionization mode. The needle voltage was set at 3.6 kV and the rf lens to 0.3 V. The source block and desolvation temperatures were 150 and 350 °C.

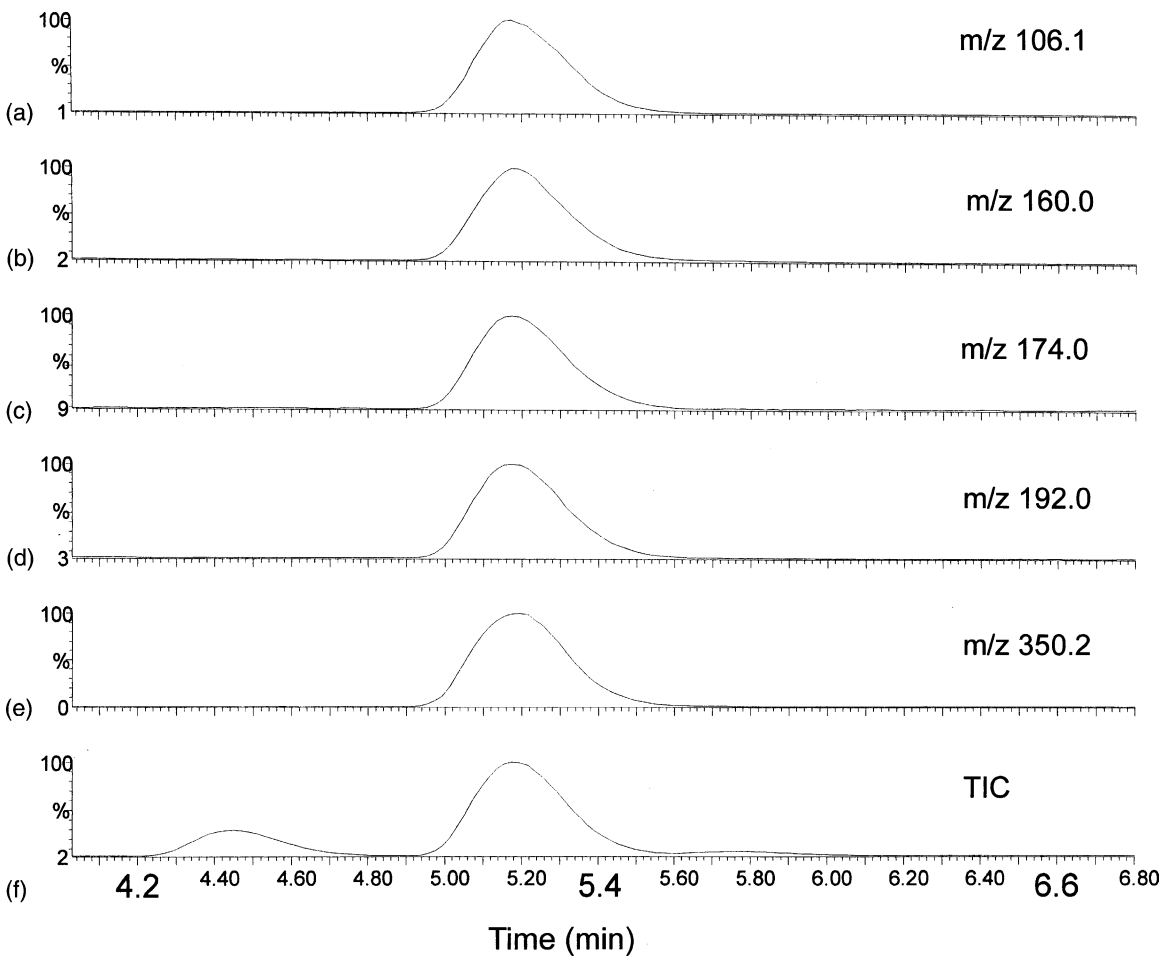


Fig. 8. Selected ion chromatograms for (a) *m*/*z* 106.1; (b) *m*/*z* 160.0; (c) *m*/*z* 174.0; (d) *m*/*z* 192.0 and (e) *m*/*z* 350.2. The trace at the bottom is (f) the total ion chromatogram. 8.5 ng of ampicillin was placed on-column in this injection. The simultaneous appearance of all these masses along with the parent ion (*m*/*z* 350.2) at the appropriate retention time confirms that these are characteristic fragments from ampicillin and not background ions. The use of these characteristic fragments gives high specificity for the detection of a particular species: the TIC detects another peak at 4.4 min, but by following the traces of the characteristic fragments, it is obvious that this peak is not ampicillin.

Nitrogen gas was used for the nebulization (100 L/h) and desolvation (400 L/h). The extractor lens was set to 5 V. Full scan spectra were taken from 105 *m*/*z* to 500 *m*/*z*. We used low/high cone voltage switching, and as a result two data channels were collected, one at 17 V on the entrance cone and the other at 42 V on the entrance cone.

To determine the retention time for each compound, we diluted the stock solution by a factor of 100 and injected 1 μL of this sample. This corresponded to about 21 ng of each antibiotic on-column. Fig. 7 shows an example of the low and high cone voltage spectra of ampicillin, illustrating well how manipulating the voltages in the source can nicely fragment a parent ion into characteristic fragments. Fig. 8 further illustrates how we confirm which ions are characteristic fragments of the parent, and Table 3 lists the parent and fragment ions observed for each of the four antibiotics. Amoxicillin eluted in 1.67 ± 0.02 min, cephapirin in 5.00 ± 0.02 min, ampicillin in 5.63 ± 0.02 min and

Table 3
Parent and fragment ions observed from β-lactam antibiotics at a cone voltage of 17 and 42 V[a]

	Amoxicillin	Ampicillin	Cephapirin	Cloxacillin
17 V				
Parent ion(s) (*m*/*z*)	366.0 (100)	350.2 (100)	424.3 (100)	436.1 (90) 438.1 (35)
Fragment ions (*m*/*z*)	349.0 (80) 207.8 (15) 160.1 (15) 114.0 (65)	305.2 (40) 191.8 (25) 174.4 (15) 160.1 (45) 114.0 (70) 106.1 (75)	364.3 (6) 112.0 (10) 140.9 (4)	282.3 (10) 277.1 (35) 279.1 (10) 266.1 (30) 244.1 (10) 226.1 (40) 228.1 (15) 160.0 (100) 113.9 (30)
42 V				
Parent ion(s) (*m*/*z*)	366.0 (0)	350.1 (25)	424.3 (10)	436.1 (0) 438.1 (0)
Fragment ions (*m*/*z*)	349.0 (7) 321.0 (7) 275.0 (5) 234.0 (5) 211.0 (25) 208.0 (25) 179.9 (15) 160.0 (15) 114.0 (100)	333.0 (5) 305.2 (40) 259.0 (6) 211.1 (25) 191.8 (100) 174.1 (60) 164.1 (25) 160.0 (65) 114.0 (35) 106.0 (95)	364.3 (8) 320.3 (10) 292.1 (100) 253.0 (65) 226.1 (18) 181.0 (30) 152.0 (55) 141.0 (35) 112.0 (18)	408.3 (6) 410.3 (3) 339.9 (5) 341.9 (3) 331.1 (8) 299.1 (45) 301.1 (15) 277.1 (55) 279.1 (35) 250.1 (7) 252.1 (3) 220.0 (15) 222.0 (10) 205.9 (10) 207.9 (5) 182.0 (70) 178.0 (30) 180.0 (15) 160.1 (100) 114.0 (25)

[a] Value in parenthesis indicate normalized intensity (%) of each ion signal.

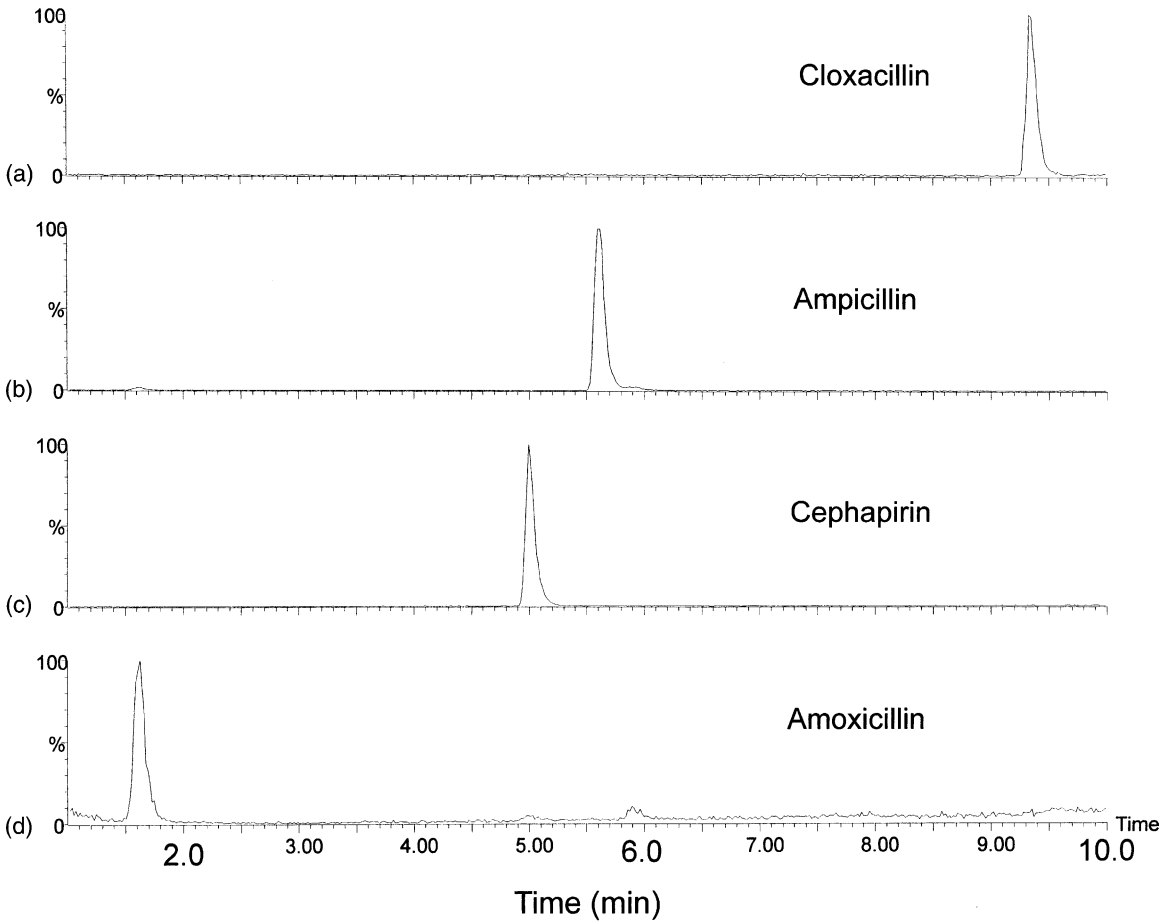


Fig. 9. Selected ion chromatograms for (a) *m*/*z* 436 (parent ion of cloxacillin); (b) *m*/*z* 350 (parent ion of ampicillin); (c) *m*/*z* 424 (parent ion of cephapirin) and (d) *m*/*z* 366 (parent ion of amoxicillin).

cloxacillin in 9.35 ± 0.02 min under these conditions. Fig. 9 shows the four selected ion chromatograms for the parent ion of each antibiotic.

With these full scan results in mind we then designed our quantification experiment. Standards were prepared by sequential dilutions from the stock solution, and all dilutions were done with the milk extract. The standard of highest concentration was made by diluting the initial stock solution by a factor of 200, and the approximate concentration of each antibiotic in this standard was 10.7 ng/μL. Sequential dilutions of this highest level standard by a factor of 2 through 10 additional iterations generated the rest of the standards. The complete list of concentrations for each level is shown in Table 4.

We used the same chromatographic method as for the characterization runs, and the volume for all injections was 5 μL. The standards were run in order of lowest level to highest level, and triplicate injections were made at each level. Duplicate blank injections were made before running the standards and after the standards were complete. The tuning conditions were the same as for the characterization runs, but instead of full scans we used multiple SIRs to quantify with confirmation. Table 5 summarizes the mass spectral data collection parameters, and we should note that the

Table 4
Concentration (pg/μL) of β-lactam antibiotics in each level of the calibration standards[a]

Level	Ampicillin	Amoxicillin	Cloxacillin	Cephapirin
1	10.7 (53.5)	10.2 (51.0)	10.5 (52.5)	10.3 (51.5)
2	21.4 (106.9)	20.4 (102.1)	21.0 (105.0)	20.6 (103.0)
3	42.8 (213.9)	40.8 (204.1)	42.0 (210.0)	41.2 (206.1)
4	85.5 (427.7)	81.6 (408.2)	84.0 (420.0)	82.4 (412.1)
5	171.1 (855.5)	163.3 (816.4)	168.0 (839.8)	164.8 (824.2)
6	342.2 (1711)	326.6 (1633)	335.9 (1680)	329.7 (1648)
7	684.4 (3422)	653.1 (3266)	671.9 (3359)	659.4 (3297)
8	1369 (6844)	1306 (6531)	1344 (6719)	1319 (6594)
9	2738 (13688)	2612 (13062)	2688 (13438)	2638 (13188)
10	5475 (27375)	5225 (26125)	5375 (26875)	5275 (26375)
11	10950 (54750)	10450 (52250)	10750 (53750)	10550 (52750)

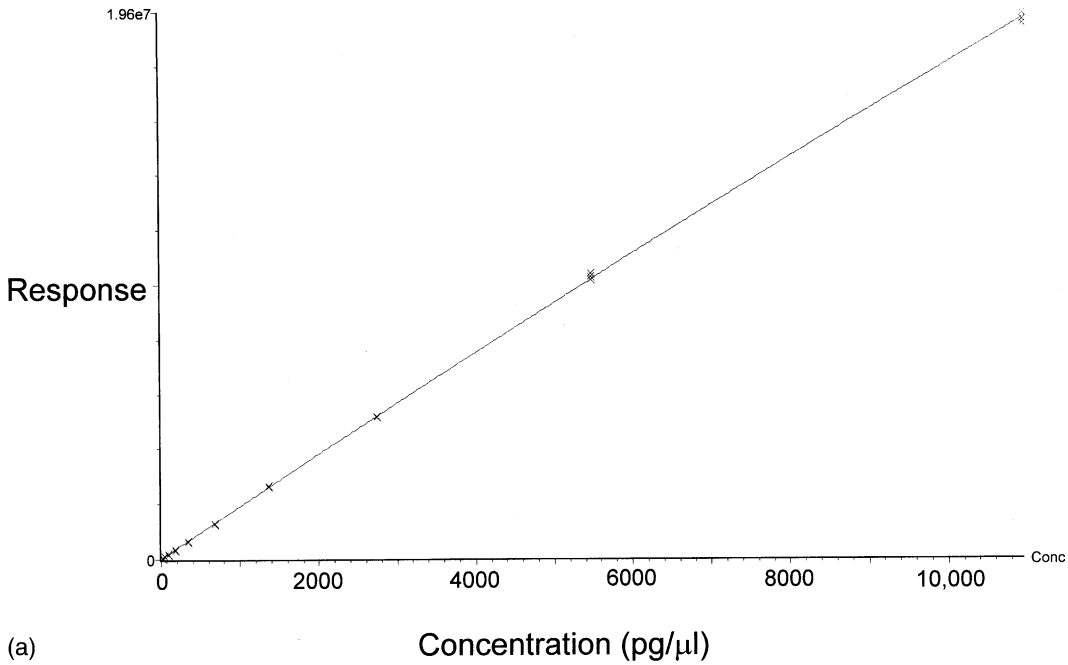
[a] Value in parenthesis is the actual amount injected on-column (pg) with a 5 μL injection.

mass window of each channel was 0 *m*/*z*. An example of the derived calibration curve for ampicillin is shown in Fig. 10. The response for each point is the sum of the intensities of *m*/*z* 160.1, *m*/*z* 190.8 and *m*/*z* 350.0. A comparison of the lowest level to a blank injection is shown in Fig. 11. Table 6 shows the limit of quantification in milk extract observed for each β-lactam antibiotic.

Lincomycin was chromatographed with a water/acetonitrile gradient containing TFA. Mobile phase A was water with 0.05% TFA and mobile phase B was acetonitrile with 0.035% TFA. The flow rate was 0.3 μL/min throughout the experiment. From 0 to 7 min the gradient ramped linearly from 5% B to 95% B, and from 7 to 10 min the composition was held at 95% B. From 10 to 10.1 min the composition was ramped from 95% B back to 5% B. The column was then re-equilibrated from 10.1 to 15.5 min at 5% B. The column temperature was set to 30 °C and the autosampler temperature set to 4 °C.

Table 5
Mass spectral collection parameters for quantification of β-lactam antibiotics

Window	Time (min)	Compound	Ion observed (*m*/*z*)	Cone voltage (V)	Dwell time (s)
1	0.0–3.5	Amoxicillin	366.3	15	0.2
			349.0	15	0.2
			207.9	40	0.2
			159.9	40	0.2
2	3.5–7.0	Cephapirin	424.3	15	0.12
			364.0	35	0.12
			292.0	40	0.12
		Ampicillin	350.0	15	0.12
			211.1	40	0.12
			191.8	35	0.12
			173.8	40	0.12
			160.1	40	0.12
3	7.0–11.0	Cloxacillin	436.1	15	0.15
			438.1	35	0.15
			277.1	40	0.15
			279.1	40	0.15
			160.0	37	0.15



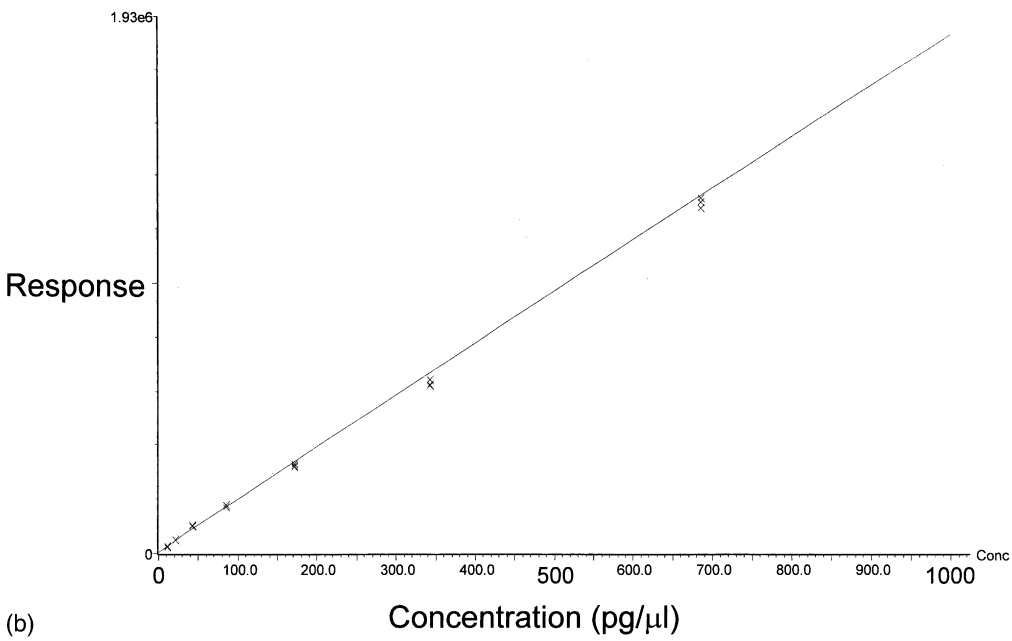


Fig. 10. Derived calibration curve for ampicillin from the quantification study. The concentrations of the standards ranged from 10.7 to 10,950 pg/μL (53.5–54,750 pg on-column). The full range of the curve is shown in (a), while (b) shows a close up of the low standards from 10.7 to 685 pg/μL. The fit is quadratic ($y = -0.096354x^2 + 1878.8x + 5401.2$) and the coefficient of variation is 0.9999. The origin was excluded from the fit and we used $1/x$ weighting. No internal standard was used to normalize the response. To increase our sensitivity, the quantified response was the sum of the intensities of *m*/*z* 160.1, *m*/*z* 191.8 and *m*/*z* 350.0. We used the area of the chromatographic peak from each ion to evaluate our signal intensity.

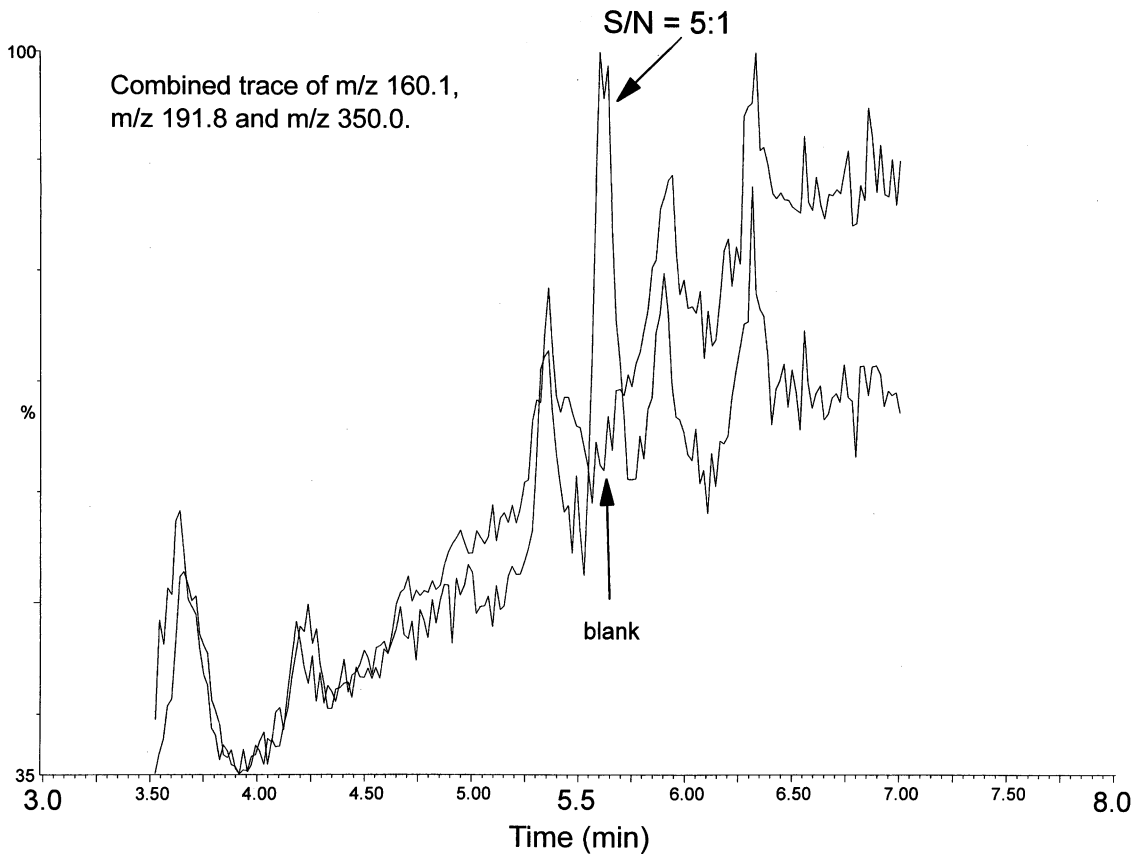


Fig. 11. Overlaid chromatograms of ampicillin (53.5 pg injected on-column) and a blank injection. Both traces are a combined ion chromatogram of *m*/*z* 160.1, *m*/*z* 191.8 and *m*/*z* 350.0. The observed peak in the trace from the ampicillin injection has a S/N ratio of 5:1. No signal from ampicillin is observed in the blank injection.

Table 6
Limits of detection[a] for β-lactam antibiotics in bovine milk extract

Compound	Ions used for quantification (*m*/*z*)[b]	Limit of detection (pg of compound on-column)
Amoxicillin	159.9, 207.9, 349.0	51.0
Ampicillin	160.1, 191.8, 350.0	32.1
Cephapirin	292.0, 364.0, 424.3	15.5
Cloxacillin	160.1, 436.1, 438.1	105

[a] Limit of detection is defined as the concentration where we observe a S/N ratio of 3:1.

[b] The signal used for quantification is the sum of the intensities of the listed ions for each antibiotic.

Lincomycin was electrosprayed in the positive ionization mode. The needle voltage was set at 3.1 kV and the rf lens to 0.1 V. The source block and desolvation temperatures were 120 and 350 °C. Nitrogen gas was used for the nebulization (100 L/h) and desolvation (400 L/h). The extractor lens was set to 3 V. Full scan spectra were taken from 100 *m*/*z* to 800 *m*/*z*. We used low/high cone voltage switching, and as a result two data channels were collected, one at 27 V on the entrance cone and the other at 52 V on the entrance cone.

To determine the retention time for lincomycin, we diluted the stock solution by a factor of 50 and injected 1 μL of this sample. This corresponded to 22.6 ng of lincomycin on-column. Fig. 12 shows the low and high cone voltage spectra of lincomycin, again

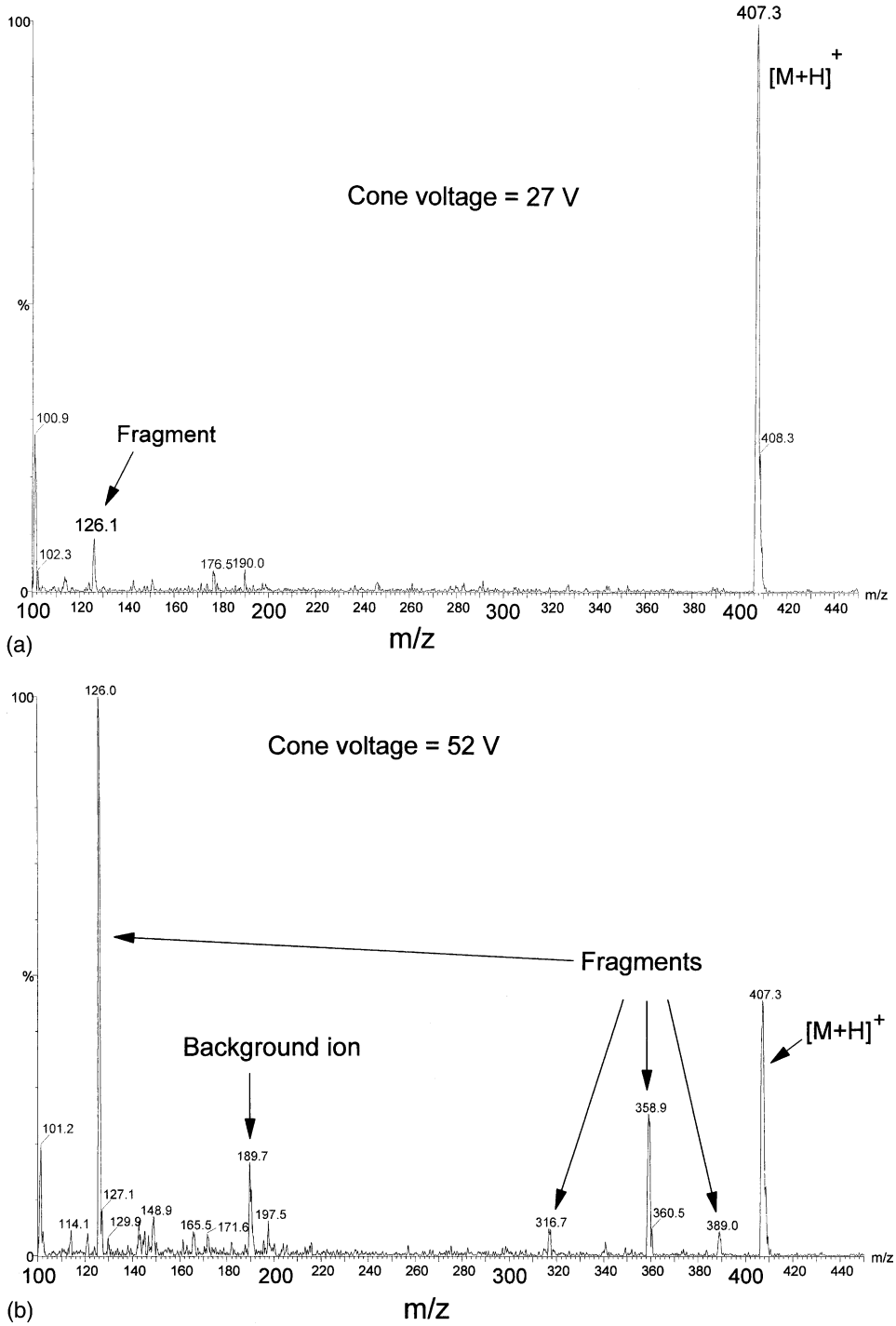


Fig. 12. Background-subtracted full scan positive ionization electrospray spectra of lincomycin at (a) a cone voltage of 27 V and (b) a cone voltage of 52 V.

illustrating well how manipulation of the voltages in the source can nicely fragment a parent ion into characteristic fragments. Lincomycin exhibits a parent ion at *m/z* 407.3 and two major fragments at *m/z* 358.9 and *m/z* 126.0. Two minor fragments are observed at *m/z* 316.7 and *m/z* 389.0. The ion at *m/z* 189.7 is a background ion, since it exhibits no chromatographic peak co-eluting with the parent ion at *m/z* 407.3. Lincomycin eluted at 4.01 ± 0.02 min under these conditions. Fig. 13 shows the selected ion chromatograms for the parent ion and two major fragment ions.

For the quantification study, sequential dilutions of the stock solution were made with the milk extract. Three channels were monitored: *m/z* 407.0 (cone voltage 25 V) for the parent and *m/z* 358.9 (cone voltage 50 V) and *m/z* 126.0 (cone voltage 60 V) for the fragment ions. The dwell time for each channel was 0.2 s with a mass window of 0 *m/z*. Triplicate injections of the standards were made at the following levels (in the given order): 1.1, 2.2, 4.4, 8.8, 17.7, 35.3, 70.6 and 141.3 pg/μL. Triplicate blank injections were made before running the standards and duplicate blank

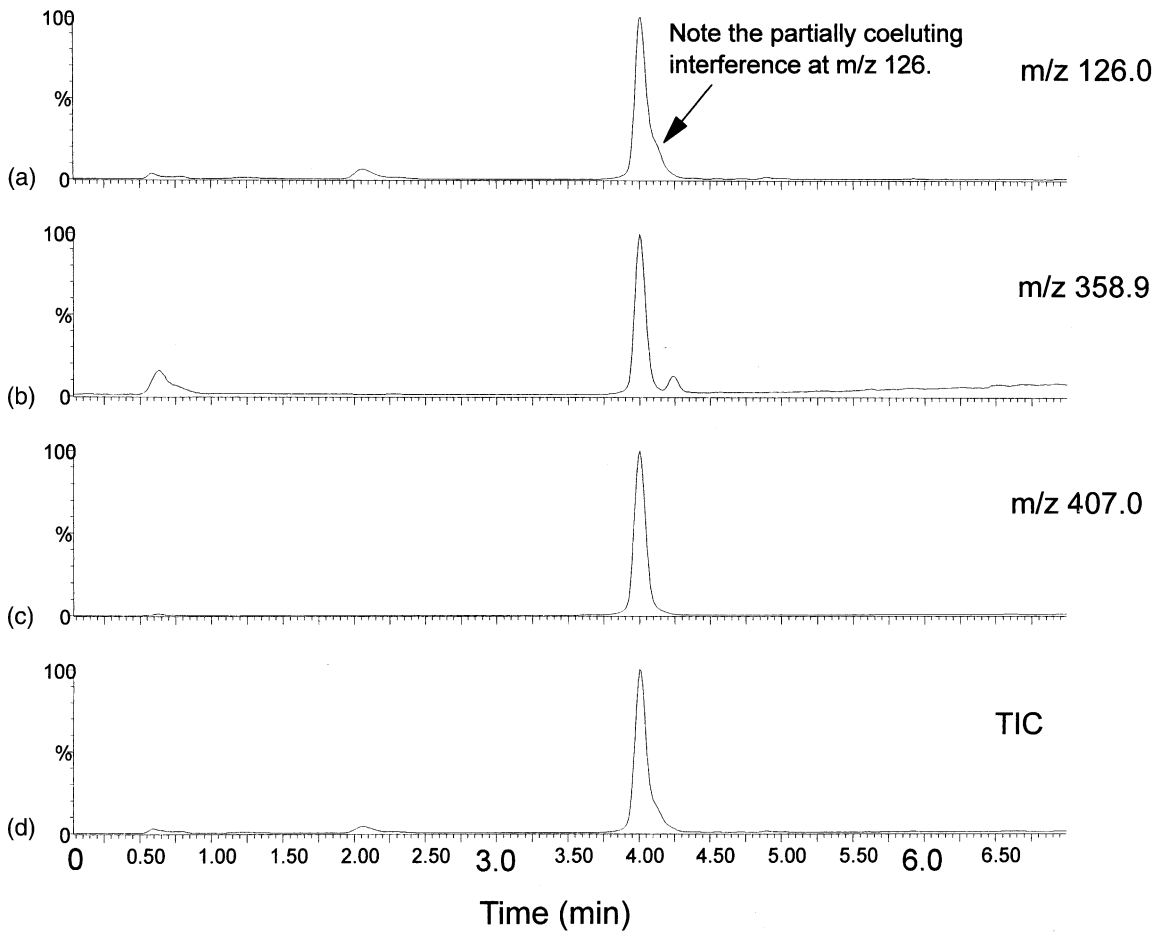


Fig. 13. Selected ion chromatograms for (a) *m/z* 126.0; (b) *m/z* 358.9 and (c) *m/z* 407.0. The trace at the bottom is (d) the total ion chromatogram. 1.4 ng of lincomycin was placed on-column in this injection. The simultaneous appearance of *m/z* 126.0 and *m/z* 358.9 along with the parent ion (*m/z* 407.0) at the appropriate retention time confirms that these are characteristic fragments from lincomycin and not background ions. Note that there is a partially coeluting interference at *m/z* 126. This does not hinder the ability to use *m/z* 126 for confirmation of identity.

injections were made after the standards were complete. We also ran three replicate injections of lincomycin in milk extract at a concentration of 11.3 pg/μL. These three injections were run between the 8.8 and 17.7 pg/μL standards, and bracketed by duplicate blank injections. We classified them as analyte injections in the software, which allows us to evaluate how well the calibration curve we obtained will quantify a real sample. The injection volume for all samples was 10 μL. Thus, the on-column amounts of material injected ranged from 11 pg with the 1.1 pg/μL level to 1413 pg with the 141.3 pg/μL level.

3.4. Results and discussion

The quantification of β-lactam antibiotics in milk has been investigated previously on both ion trap [19] and triple quadrupole mass spectrometers [20]. We should note that the ion trap work we cite used an older, non-orthogonal source design. In the ion trap work, the LOD for most of the antibiotics was 1000 pg of material on-column. Even at these levels, a 500 ms collect time for the analytes was insufficient to avoid: (1) dramatic alterations of relative abundances; (2) the inability to observe key ions and (3) the loss of diagnostic ions among the chemical noise at nearby *m*/*z* values. Furthermore, amoxicillin gave little fragmentation in the ion trap. MS/MS of the protonated parent only gave *m*/*z* 349 (loss of ammonia). MS^3 was required to get more fragmentation data and satisfy the three ion rule [3,17].

In contrast to this, we found our results from a single quadrupole mass spectrometer to be 10–100 times more sensitive than the results from an ion trap (see Table 6). For each β-lactam antibiotic we were able to find three ions to monitor, and there was no problem with losing signals from key ions in the chemical noise. IS-CID also gave ample fragmentation for all of the β-lactam antibiotics. Even with amoxicillin we are able to generate nine fragment ions, while an ion trap required the use of MS^3 experiments to yield enough fragments to satisfy the three-ion rule. The ability of IS-CID to generate all the needed fragments in a single step considerably simplifies the experimental method. Furthermore, the extra time required to do an MS^3 experiment (vs. an MS or MS/MS experiment) can lengthen the cycle time and reduce the number of points obtained in a given data channel when operating on chromatographic time scales. This factor probably contributes significantly to the decreased sensitivity of an ion trap relative to a linear single quadrupole instrument.

Our results also compare well with those from a triple quadrupole mass spectrometer. Since the triple quadrupole is, like the single quadrupole, a linear instrument, it should also exhibit much higher sensitivity than an ion trap. Indeed, the prevailing view would suggest that a triple quadrupole instrument should far outperform a single quadrupole instrument [3–5]. Surprisingly, the actual difference in sensitivity is less than an order of magnitude. For ampicillin, amoxicillin and cloxacillin the LODs from a triple quadrupole are 4.5 pg on-column, 6 pg on-column and 12 pg on-column, respectively. For these three compounds, the triple quadrupole instrument is about 7.5 times as sensitive as a single quadrupole instrument. The ability to do true MS/MS with the triple quadrupole does give an advantage in sensitivity, but it is not as large as one might expect. And even with the use of MS/MS, an ion trap exhibits far less sensitivity than the single quadrupole instrument.

It is interesting to note that quantification of the β-lactam antibiotics required quadratic fits for all of the tested species, as illustrated in Fig. 10. The fits were very lightly quadratic in all cases. Even over concentration ranges of 1–2 orders of magnitude a quadratic model is required to fit the data, and the curves exhibited an upward facing concavity, which is the opposite of what we would expect if the source were being saturated with analyte. A likely explanation for this is poor recovery of the analyte at the low concentrations, due to adsorption of the analyte to the container walls.

Although we ran lincomycin with the same milk extract that was used for the β-lactam antibiotics, we had to use TFA in the mobile phase to get a reasonable retention time for lincomycin. Since TFA (due to its ion-pairing capabilities [21]) will generally suppress

the ionization of cations, we expected that our LOD for lincomycin would be much higher than the values we observed with the β-lactam antibiotics.

Lincomycin generated two major fragments at high cone voltage. However, inspection of Fig. 13 shows that despite all the chromatography, we did have an interference at *m*/*z* 126 that partially co-elutes with lincomycin. Fortunately, even at the lowest concentrations we ran (see Fig. 14), the peak from lincomycin at *m*/*z* 126 could be picked out as a partially resolved peak on the shoulder of the interference, which allowed us to qualitatively confirm the presence of lincomycin at this low level with three ions. But the elevated baseline from this interference compromises our ability to accurately measure the peak area of the signal, and we chose to use only the signal from *m*/*z* 407.0 and *m*/*z* 358.9 to quantify the observed amount of lincomycin in each injection. Fig. 15 shows the calibration curve derived from these data, and again our sensitivity was enhanced by summing the signals from both *m*/*z* 407.0 and *m*/*z* 358.9 to give a combined response. The curve was quadratic, with a correlation constant of 0.9988. Even at the lowest level we ran, the points fit very well to the curve. The calibration

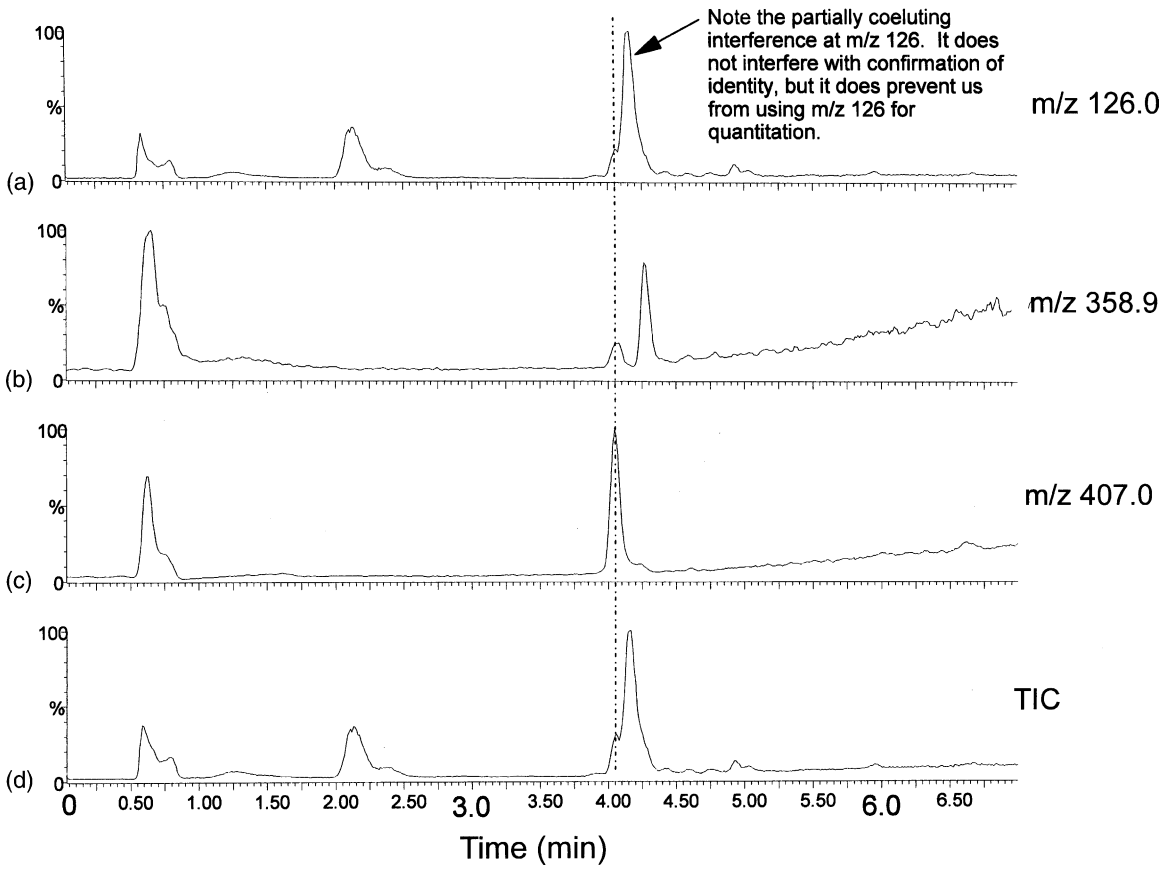


Fig. 14. Selected ion chromatograms for (a) *m*/*z* 126.0; (b) *m*/*z* 358.9 and (c) *m*/*z* 407.0. The trace at the bottom is (d) the total ion chromatogram. 22.1 pg of lincomycin was placed on-column in this injection. The simultaneous appearance of *m*/*z* 126.0 and *m*/*z* 358.9 along with the parent ion (*m*/*z* 407.0) at the appropriate retention time confirms that these are characteristic fragments from lincomycin and not background ions. Now the partially coeluting interference at *m*/*z* 126 is much larger than the signal from lincomycin. Although this does not hinder the ability to use *m*/*z* 126 for confirmation of identity, it will degrade the results if *m*/*z* 126 is used for quantitative purposes, particularly at these lower concentrations.

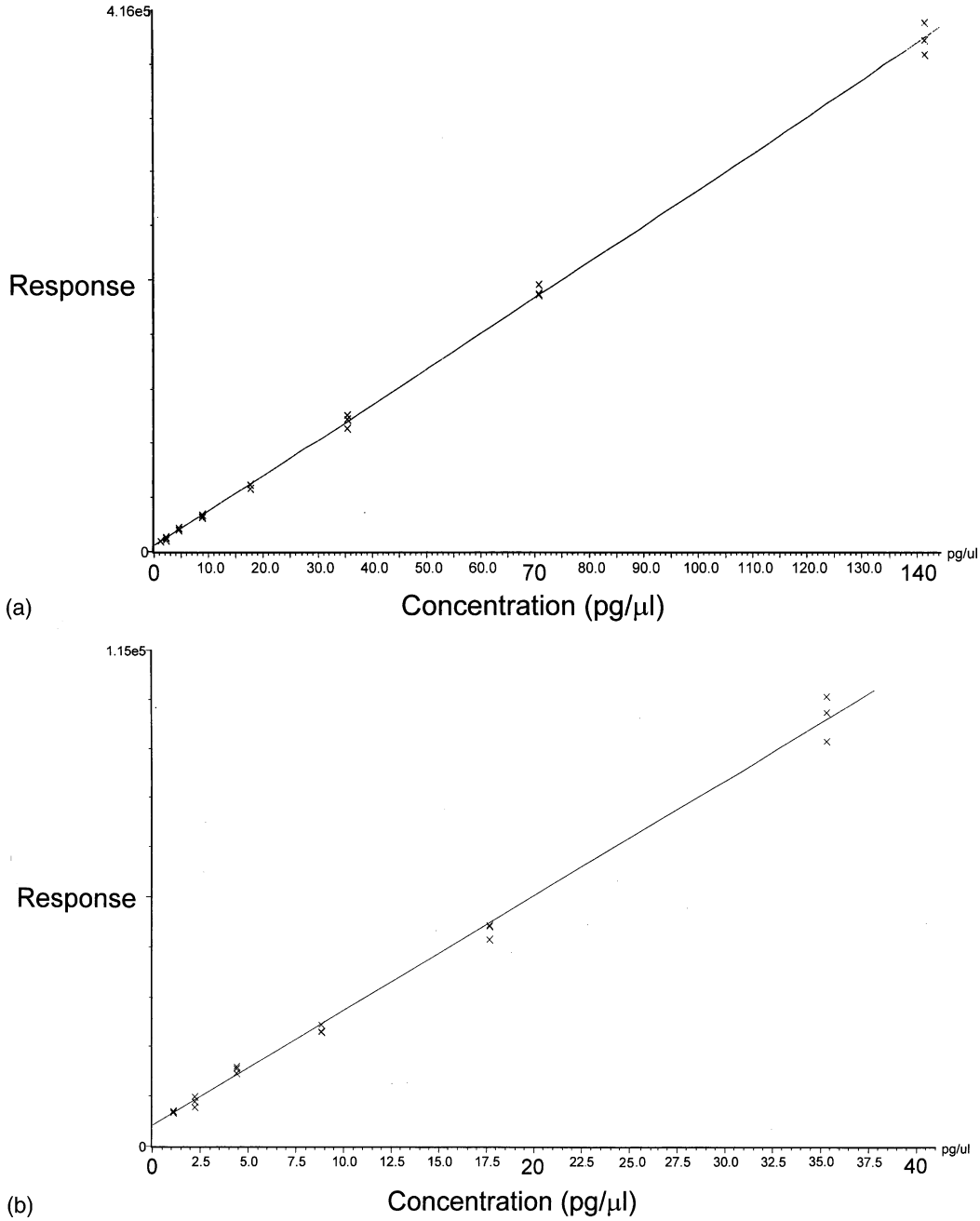


Fig. 15. Derived calibration curve for lincomycin from the quantification study. The concentrations of the standards ranged from 1.1 to 141.3 pg/μL (11–1413 pg on-column). The full range of the curve is shown in (a), while (b) shows a close up of the low standards from 1.1 to 35.3 pg/μL. The fit is quadratic ($y = 0.746252x^2 + 2665.77x + 4758.67$) and the coefficient of variation is 0.9988. The origin was excluded from the fit and we used $1/x$ weighting. No internal standard was used to normalize the response. To increase our sensitivity, the quantified response was the sum of the intensities of *m*/*z* 407.0 and *m*/*z* 358.9. We used the area of the chromatographic peak from each ion to evaluate our signal intensity.

curve exhibited an upward facing concavity, the opposite of what we would expect if the ESI source were being saturated with analyte. Again, the likely explanation for this is poor recovery of the analyte at the low concentrations, due to adsorption of the analyte to the container walls.

To insure that we are not seeing a background signal, we compared in an overlay the 1.1 pg/μL (11 pg on-column) standard with a blank run (Fig. 16). At this point our combined signal had a S/N ratio of 40:1! Because of our use of TFA in the mobile phase, we were rather amazed at the size of the signal at this level. In fact, we have no points at lower concentrations because we expected that the ion-pairing characteristics of TFA would suppress our signal. The addition of TFA to the mobile phase did lengthen to retention time of lincomycin considerably, indicating that it was pairing to some extent with the protonated lincomycin in solution. This suggests that the large signal we saw for lincomycin was probably suppressed compared to what it would have been without TFA. Apparently lincomycin is highly efficient at protonating in solution, and ESI appears to be an extremely sensitive source for analyzing lincomycin via MS. Assuming that a S/N

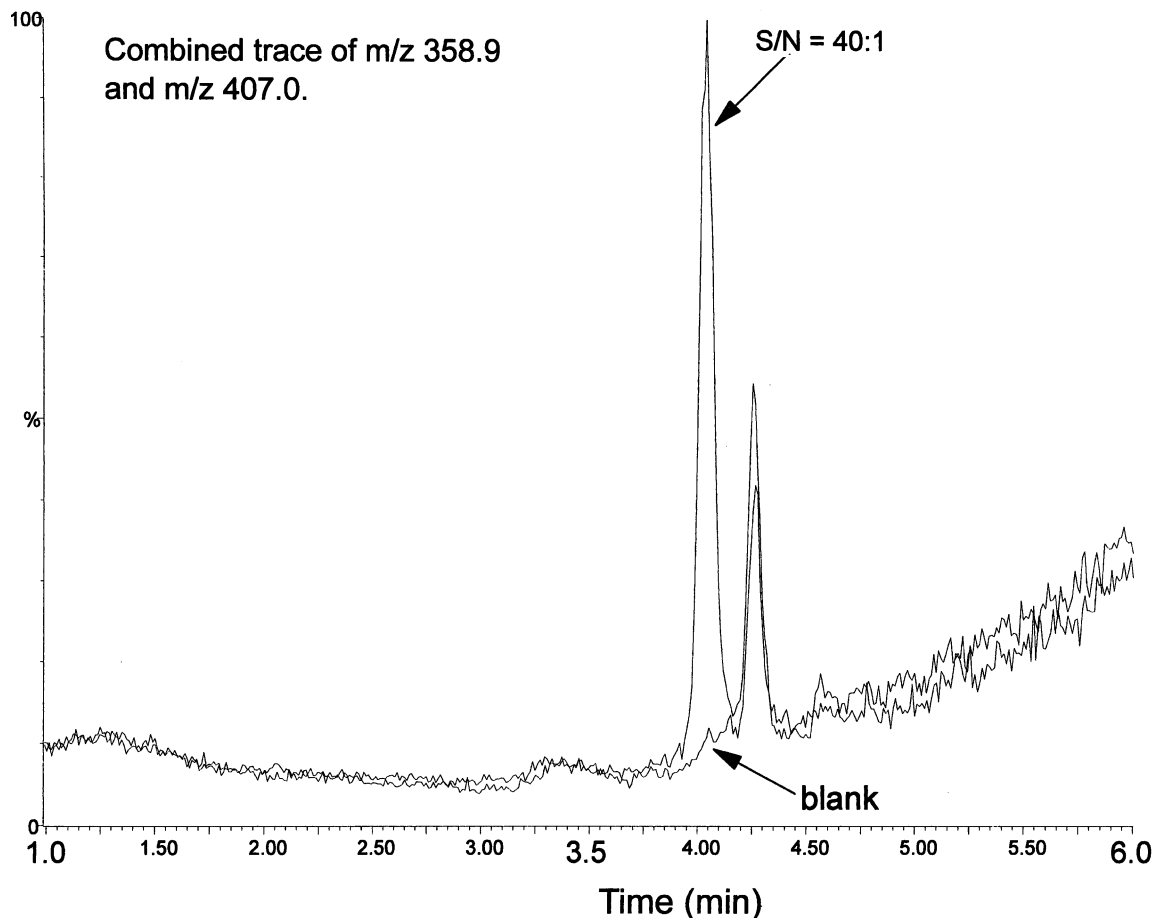


Fig. 16. Overlaid chromatograms of lincomycin (11 pg injected on-column) and a blank injection. Both traces are a combined ion chromatogram of *m/z* 358.9 and *m/z* 407.0. The observed peak in the trace from the lincomycin injection has a S/N ratio of 40:1. No signal from lincomycin is observed in the blank injection.

ratio of 3:1 is the LOD, we can extrapolate that the LOD for lincomycin is 0.83 pg of material on-column.

To test further our ability to quantify lincomycin in milk extract, a sample of "unknown" concentration was prepared by dilution in the milk extract by one of the co-authors and run as though it was an analyte sample. The calculated concentration of that sample was 10.6 pg/μL, with an uncertainty of ±0.6 pg/μL. The actual concentration of that sample was 11.3 pg/μL, so we were in error by −6.2%. This is excellent agreement, and it is very significant that our calculated value was below the actual value. If we were receiving spurious signals from matrix interferences, it would likely manifest itself as a systematic error in our measurements, and we would expect our calculated concentration to be higher than the actual concentration. Since our calculated concentration was lower, this indicates that we had no interferences contributing to our observed signal. Even in a matrix as challenging as milk extract, we are able to accurately quantify, with confirmation of identity, the analyte with adequate chromatography and the use of multiple SIR channels. Tandem MS methods were not required.

4. Determination of ritonavir in blood plasma extract

Plant and milk extracts are challenging matrices often encountered in environmental monitoring. However, in clinical applications blood and tissue extracts will be encountered instead. In this section we build on the work of the previous sections and demonstrate the quantification with confirmation of ritonavir from a blood plasma extract. Amprenavir (in the same drug class as ritonavir) has been quantified in serum and plasma with LC/MS/MS methods [22], and simple LC/MS has been used to characterize the structure of ritonavir metabolites in liver extracts [23], but we have found no studies that illustrate the use of multiple selected ion recordings on a single quadrupole mass spectrometer to quantify, with confirmation, ritonavir in a blood plasma extract.

4.1. Materials and reagents

RODI Water (18 MΩ) was generated in-house. Acetonitrile and formic acid were obtained from Sigma-Aldrich. Ritonavir and the associated blood plasma extract were acquired from the National Jewish Medical and Research Center in Denver. A stock solution of 1.0 ng/μL in water was prepared. An Xterra C18 (2.1 mm × 50 mm, 5 μm, 100 Å) column was used to chromatograph the ritonavir samples.

4.2. Instrumentation

Experiments were performed with an Alliance 2690 quaternary liquid chromatograph coupled to a ZMD 4000 single quadrupole mass spectrometer (Waters, Milford, MA). Instrument control and data acquisition was done with Masslynx software (version 3.3).

4.3. LC/MS methodology

Ritonavir was chromatographed with a water/acetonitrile gradient containing 0.1% formic acid. Mobile phase A was water, mobile phase B was acetonitrile and mobile phase C was 1% formic acid in water. Throughout the gradient we held mobile phase C at 10% of the composition, so the actual concentration of formic acid throughout the gradient was 0.1%. A and B percentages were adjusted so that from 0 to 7 min the gradient ramped linearly from 5% organic (85% A, 5% B, 10% C) to 75% organic (15% A, 75% B, 10% C), and from 7 to 10 min the gradient ramped linearly from 75% organic (15% A, 75% B, 10% C) to 90% organic (0% A, 90% B, 10% C). From 10 to 12 min the composition was held at 90% organic. At 12.1 min the composition was stepped from 90% organic back to 5% organic. The column was then re-equilibrated from 12.1 to 15 min at 5% organic. The flow rate was 0.3 μL/min. The column temperature was set to 30 °C and the autosampler temperature set to 4 °C.

Ritonavir was electrosprayed in the positive ionization mode. The needle voltage was set at 3.15 kV and the rf lens to 0.55 V. The source block and desolvation

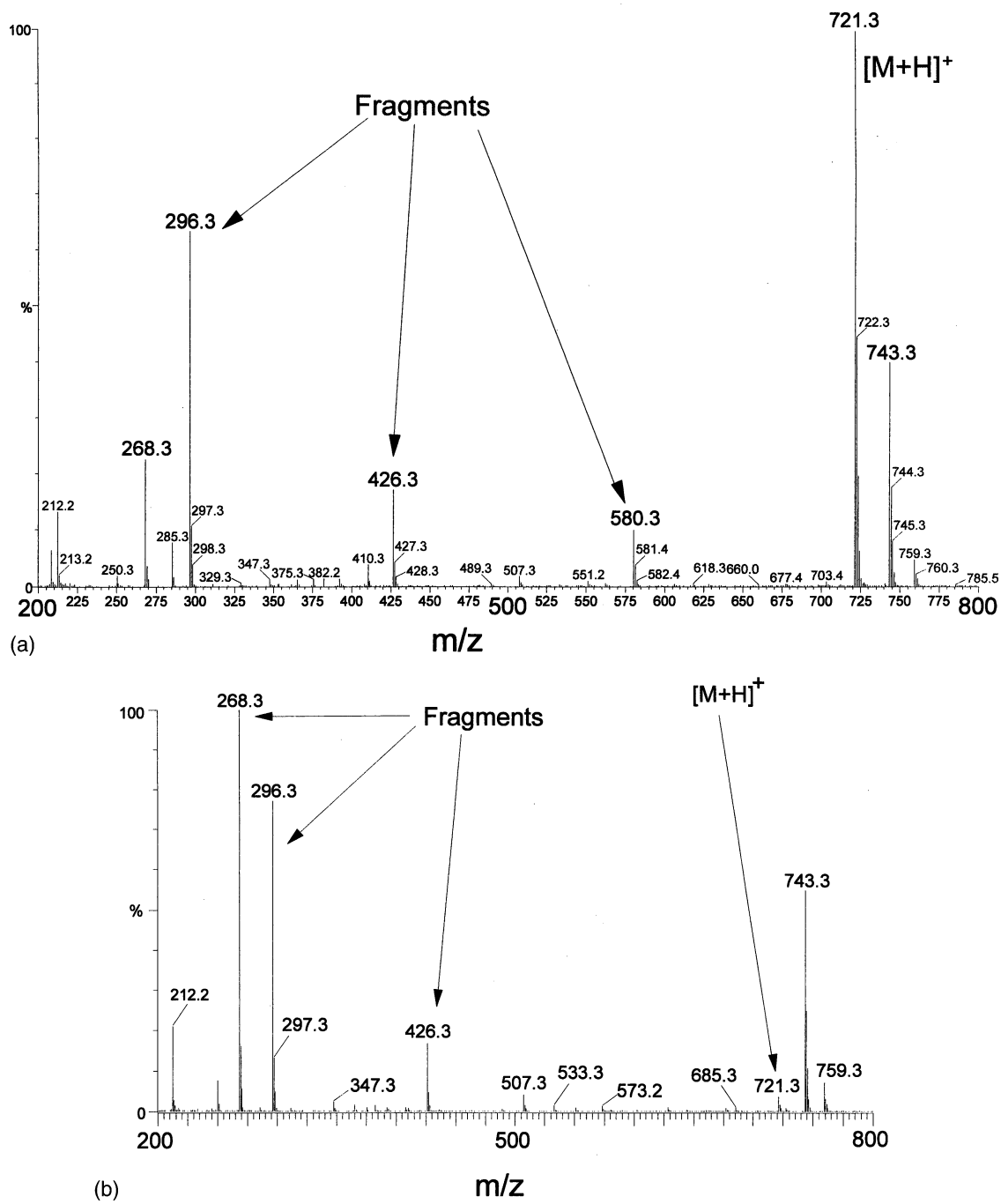


Fig. 17. Background-subtracted full scan positive ionization electrospray spectra of ritonavir at (a) a cone voltage of 37 V and (b) a cone voltage of 62 V.

temperatures were 120 and 350 °C. Nitrogen gas was used for the nebulization (100 L/h) and desolvation (400 L/h). The extractor lens was set to 5 V. Full scan spectra were taken from 200 *m/z* to 800 *m/z*. We used low/high cone voltage switching, and as a result two data channels were collected, one at 37 V on the entrance cone and the other at 62 V on the entrance cone.

To determine the retention time for ritonavir, we injected 2 μL of the stock solution. This corresponded to 2.0 ng of ritonavir on-column. Fig. 17 shows the low and high cone voltage spectra of ritonavir, once more illustrating well how manipulation of the voltages in the source can nicely fragment a parent ion into characteristic fragments. Ritonavir exhibits a parent ion at *m/z* 721.3 and a significant sodiated parent ion at *m/z* 743.3. Three major fragments are observed at *m/z* 426.3, *m/z* 296.3 and *m/z* 268.3, and a minor fragment is observed at *m/z* 580.3 at the low cone voltage, but it disappears at the high cone voltage. Other ions are from the background, since they exhibit no chromatographic peak co-eluting with the parent ion at *m/z* 721.3. Ritonavir eluted at 10.21 ± 0.02 min under these conditions. Fig. 18 shows the TIC, selected ion

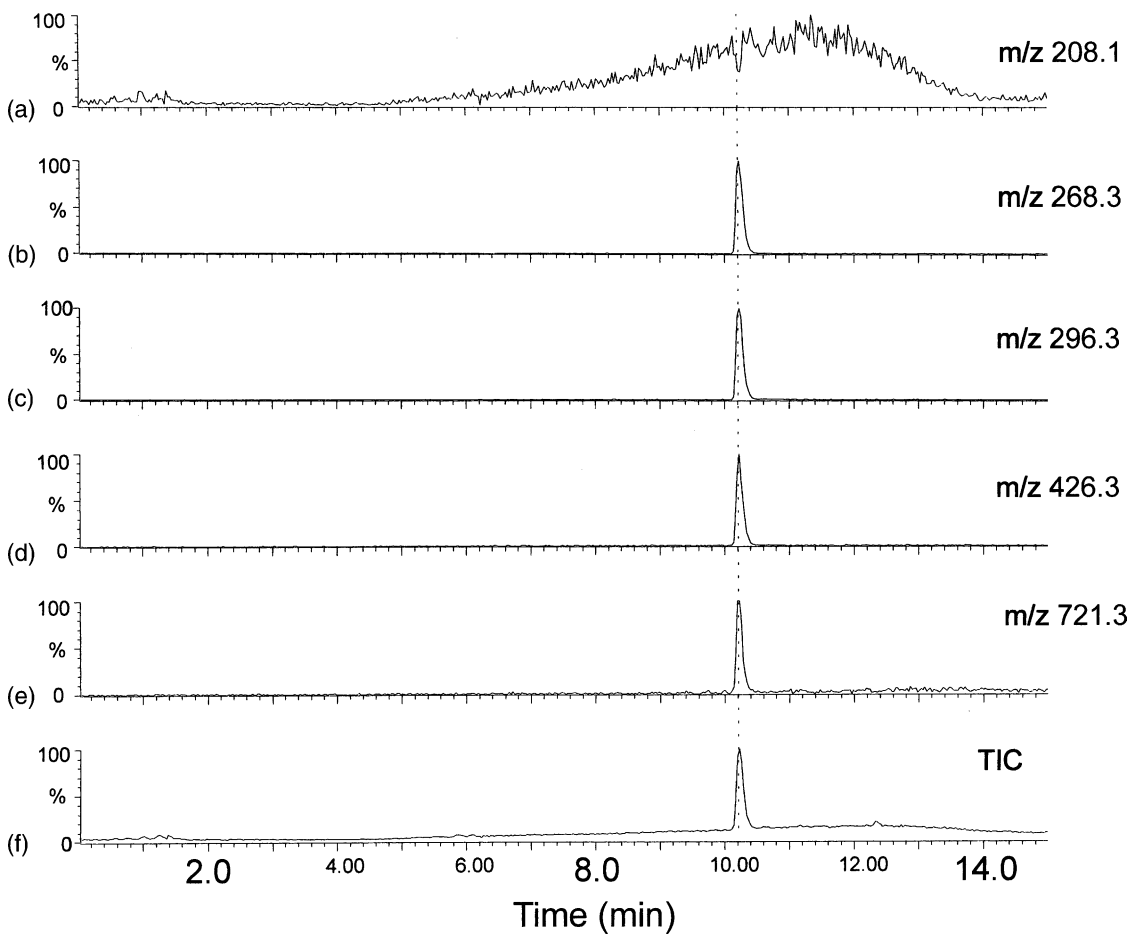


Fig. 18. Selected ion chromatograms for (a) *m/z* 208.1; (b) *m/z* 268.3; (c) *m/z* 296.3; (d) *m/z* 426.3; and (e) *m/z* 721.3. The trace at the bottom is (f) the total ion chromatogram. 2.0 ng of ritonavir was placed on-column in this injection. The simultaneous appearance of all these masses (except *m/z* 208.1) along with the parent ion (*m/z* 721.3) at the appropriate retention time confirms that these are characteristic fragments from ritonavir. On the other hand, *m/z* 208.1 must be a background ion.

chromatograms for the parent ion, three major fragment ions, and a background ion.

With these full scan results in mind we then designed our quantification experiment. Standards were prepared by sequential dilutions from the stock solution, and all dilutions were done with the blood plasma extract. The standard of highest concentration was made by diluting the initial stock solution by a factor of 5. The concentration of ritonavir in this standard was 200 pg/μL. Sequential dilutions of this highest level standard were made to generate standards at 100, 50, 20, 10, 5.0 and 1.0 pg/μL. We used the same chromatographic method as for the characterization runs, and the volume for all injections was 5 μL. Thus, the on-column amounts of material injected ranged from 5.0 pg with the 1.0 pg/μL level to 1000 pg with the 200 pg/μL level. The standards were run in order of lowest level to highest level, and triplicate injections were made at each level. Duplicate blank injections were made before running the standards and after the standards were complete. The tuning conditions were the same as for the characterization runs, but instead of full scans we used multiple SIRs to quantify with confirmation. Table 7 summarizes the mass spectral data collection parameters, and we should note that the mass window for each channel was 0 *m/z*. Even at the lowest level we ran, we were able to observe three characteristic ions (see Fig. 19). The derived

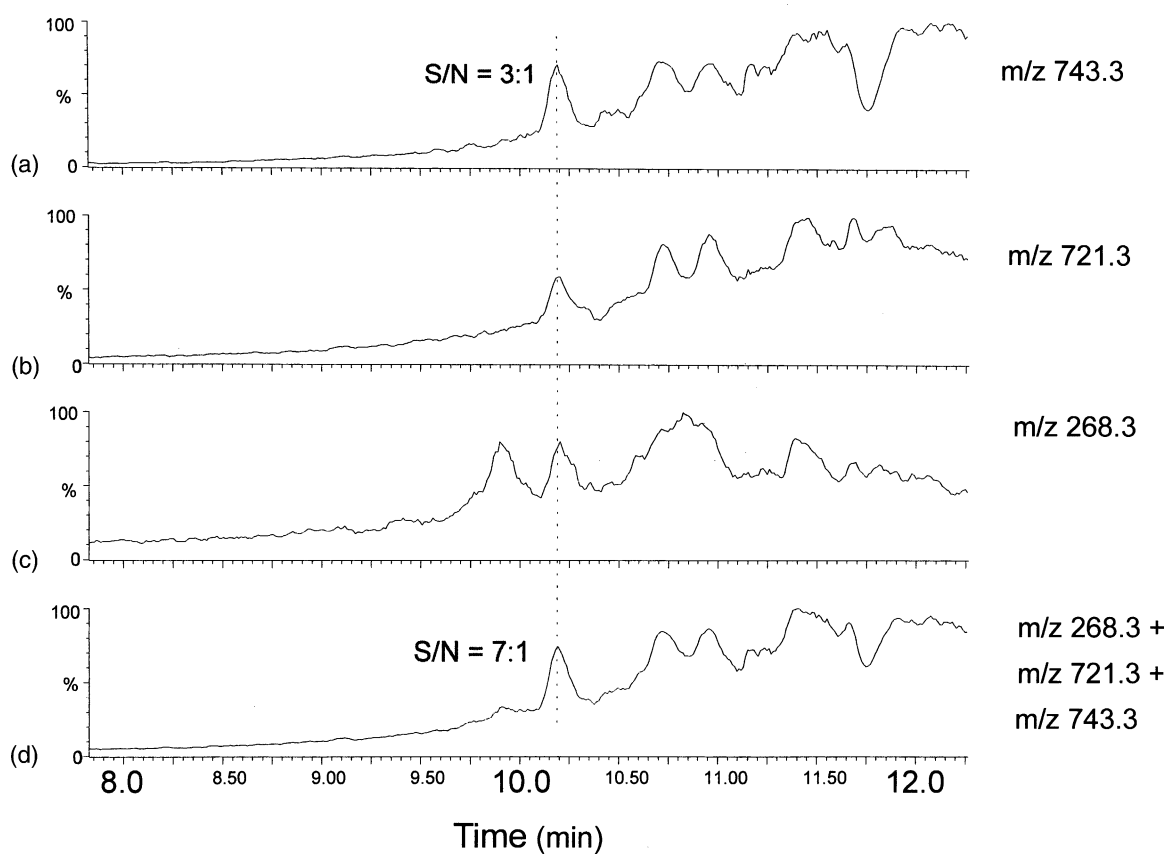
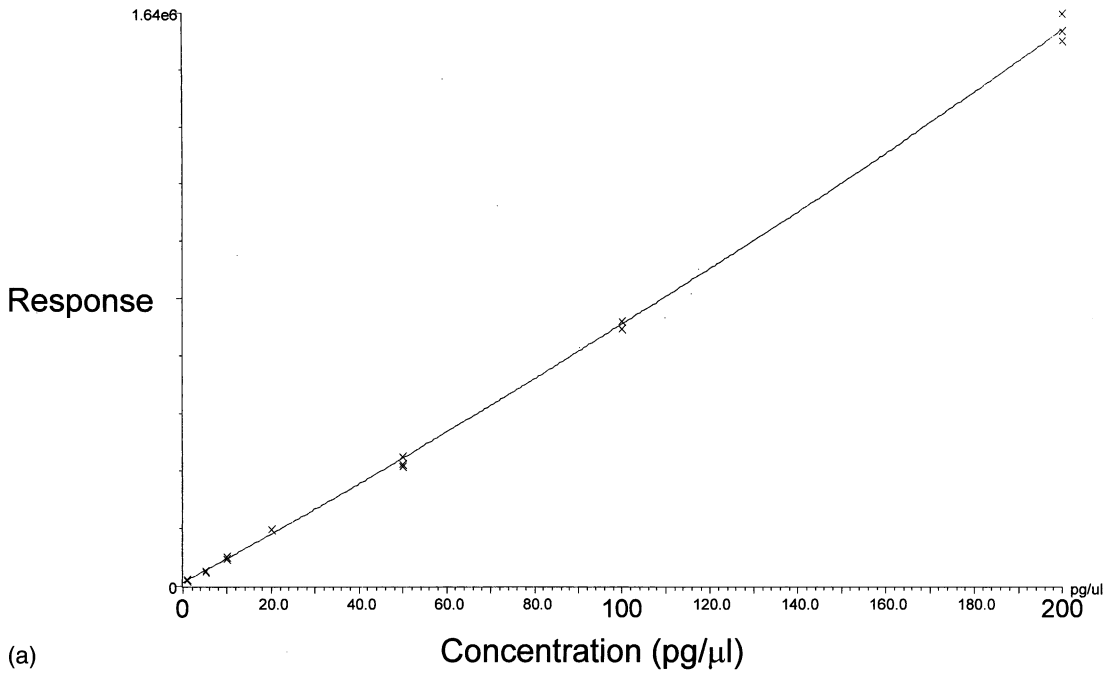
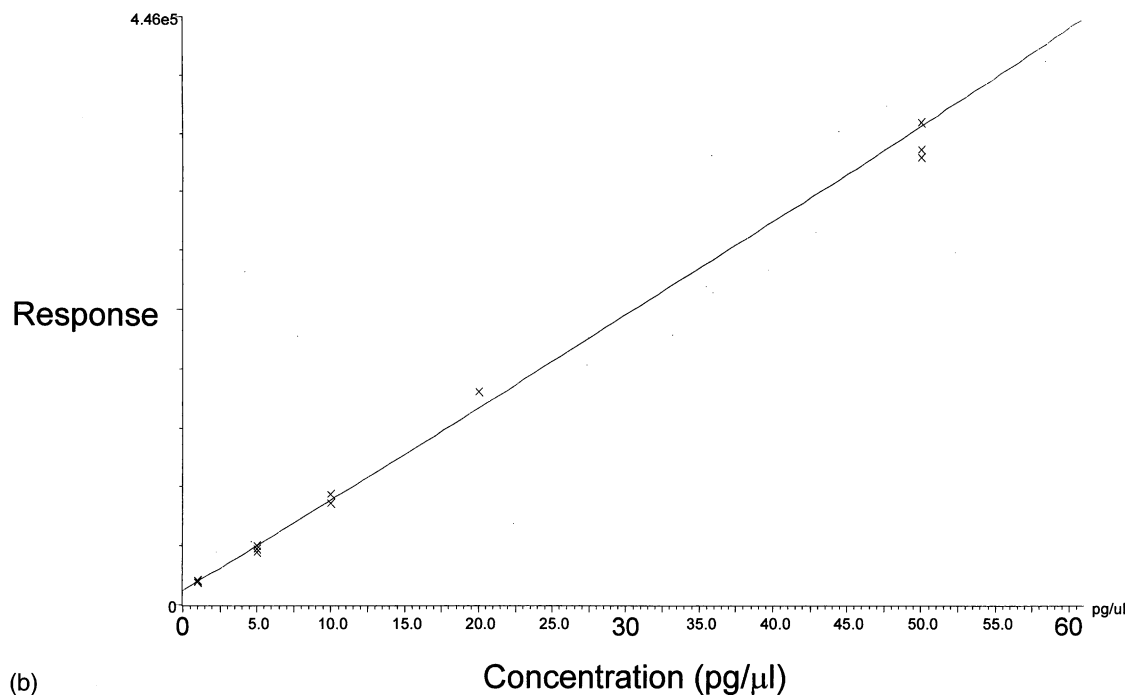


Fig. 19. Selected ion chromatograms for (a) *m/z* 743.3; (b) *m/z* 721.3; and (c) *m/z* 268.3. The trace at the bottom is (d) the combined ion chromatogram from *m/z* 268.3, *m/z* 721.3 and *m/z* 743.3. 5 pg of ritonavir was placed on-column in this injection. The simultaneous appearance of each of the three masses in the individual selected ion chromatograms confirms that this signal is from ritonavir. Summing these channels into a combined ion chromatogram approximately the doubles the S/N ratio of the signal.



(a)



(b)

Fig. 20. Derived calibration curve for ritonavir from the quantification study. The concentrations of the standards ranged from 1.0 to 200 pg/μL (5–1000 pg on-column). The full range of the curve is shown in (a), while (b) shows a close up of the low standards from 1.0 to 50.0 pg/μL. The fit is quadratic ($y = 5.42072x^2 + 6816.27x + 10795.8$) and the coefficient of variation is 0.9992. The origin was excluded from the fit and we used $1/x$ weighting. No internal standard was used to normalize the response. To increase our sensitivity, the quantified response was the sum of the intensities of *m*/*z* 268.3, *m*/*z* 721.3 and *m*/*z* 743.3. We used the area of the chromatographic peak from each ion to evaluate our signal intensity.

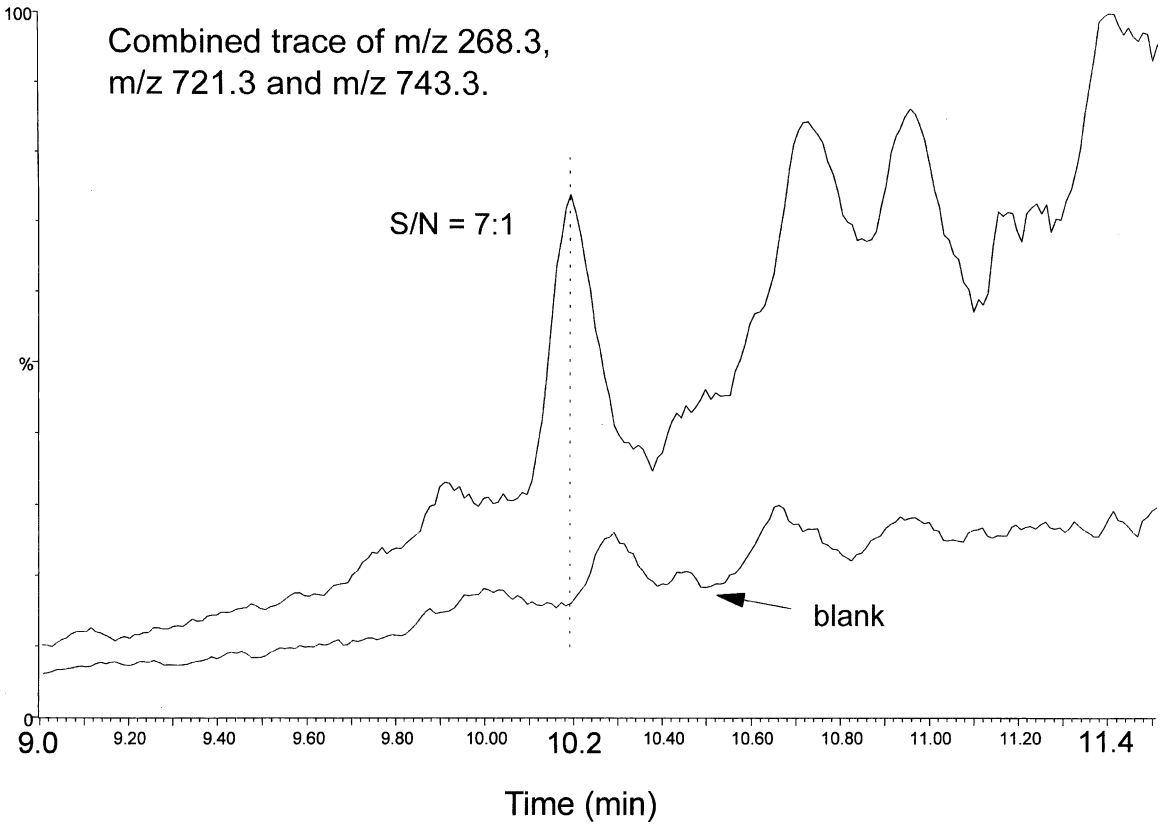


Fig. 21. Overlaid chromatograms of ritonavir (5 pg injected on-column) and a blank injection. Both traces are a combined ion chromatogram of *m/z* 268.3, *m/z* 721.3 and *m/z* 743.3. The observed peak in the trace from the ritonavir injection has a S/N ratio of 7:1. No signal from ritonavir is observed in the blank injection.

calibration curve for ritonavir is shown in Fig. 20. The response for each point is the sum of the intensities of *m/z* 268.3, *m/z* 721.3 and *m/z* 743.3. A comparison of the lowest level to a blank injection is shown in Fig. 21.

Table 7
Mass spectral collection parameters for quantification of ritonavir

Window	Time (min)	Ion observed (*m/z*)	Cone voltage (V)	Dwell time (s)
1	0.0–15.0	268.3	62	0.11
		296.3	62	0.11
		426.3	62	0.11
		721.3	37	0.11
		743.3	62	0.11

4.4. Results and discussion

The quantification of amprenavir (a relative of ritonavir) in blood extract has been investigated previously on a triple quadrupole mass spectrometer [22]. In that work, the LOD for amprenavir was 500 pg of material on-column. In contrast to this, we found the single quadrupole mass spectrometer to be highly sensitive to ritonavir. We were again able to find three ions to monitor, and there was no problem with losing signals from key ions in the chemical noise, even at the lowest level we ran. The lowest concentration we ran was 1.0 pg/μL (5.0 pg on-column), and we could observe the confirmatory signal from each ion in the three individual SIR channels at this level. We

enhanced our sensitivity by looking at the combined ion chromatogram of *m*/*z* 268.3, *m*/*z* 721.3 and *m*/*z* 743.3, and this combined chromatogram gave a signal with a S/N ratio of 7:1. To insure that we are not seeing a background signal, we compared in an overlay the 1.0 pg/μL (5.0 pg on-column) standard with a blank run (Fig. 21). No interferences were observed. Assuming that a S/N ratio of 3:1 is the LOD, we can extrapolate that as little as 2.2 pg of ritonavir on-column could have been detected.

When comparing our results to those from amprenavir, it appears that the LOD observed with the triple quadrupole instrument is anomalously high. However, the source design of their instrument is older and this may contribute significantly to the apparent lack of sensitivity. It is also quite possible that amprenavir has a lesser tendency than ritonavir to pick up protons in solution, even though they have similar structures.

Ritonavir (like the β-lactam antibiotics and lincomycin) required a quadratic fit for the calibration curve. The fit was rather strongly quadratic in this case, but even at the lowest concentration the points fit very well to the curve. The calibration curve exhibited an upward-facing concavity, the opposite of what we would expect if the ESI source were being saturated with analyte. A likely explanation for this is poor recovery of the analyte at the low concentrations, due to adsorption of the analyte to the container walls.

5. Conclusions

Previous work [1,2] has demonstrated that it is possible to use liquid chromatography in conjunction with single quadrupole mass spectrometers to quantify for an analyte with simultaneous confirmation of identity. Confirmation is achieved by concurrently monitoring for both parent and characteristic fragment ions via multiple SIR channels. Sensitivity is increased by summing together the individual data channels into a pseudo-total ion channel, and further enhanced by using dwell times of 0.1–0.2 s to maximize the number of data points across the chromatogram. However, it is still generally believed that quantification with a single quadrupole instrument would be unworkable with more complicated matrices [3].

This belief does not appear to be well justified. Other results have suggested that quantification with confirmation on a single quadrupole instrument can also be done in more complex matrices [15,16], but these reports are isolated and have not been expanded upon. In the present work we have undertaken quantification studies of a wide variety of analytes in a wide variety of complex biological matrices, and demonstrated that single quadrupole instruments are quite capable of quantification with confirmation with all the analytes and in all the matrices we tested. In many cases we were able to detect (with confirmation of identity) less than 10 pg of analyte on-column with a single quadrupole mass spectrometer. In the specific case of bovine milk extract, we found that a single quadrupole instrument was able to provide quantification with confirmation with a higher sensitivity (at least 10 times greater) than an ion trap, while a triple quadrupole was about 7.5 times more sensitive than the single quadrupole instrument.

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