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Identification and determination of pirlimycin residue in bovine milk and liver by high-performance liquid chromatography–thermospray mass spectrometry

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Abstract

Determinative and confirmatory methods of analysis for pirlimycin (I) residue in bovine milk and liver have been developed based on HPLC–thermospray (TSP) MS. Milk sample preparation consisted of precipitating the milk proteins with acidified acetonitrile followed by a solvent partitioning with a mixture of *n*-butyl chloride and hexane, extraction of I from the aqueous phase into methylene chloride (MC), and solid-phase extraction clean-up. For liver, samples (2 g) were extracted with 0.25% trifluoroacetic acid in acetonitrile. The aqueous component was released from the organic solvent with *n*-butyl chloride. The aqueous solution was reduced in volume by evaporation, basified with ammonium hydroxide, then extracted with MC. The MC was evaporated to dryness and the dried residue reconstituted in 2.0 ml of 0.1 *M* ammonium acetate for analysis. A chromatographically resolved stereoisomer of I with TSP-MS response characteristics identical to I was used as an internal standard (I.S.) for quantitative analysis based on the ratio of peak areas of I to I.S. in the protonated molecular-ion chromatogram at *m/z* 411.2.

The method for milk was validated by the analysis of control milk samples spiked with I at concentrations from 0.05 to 0.8 μg/ml. The overall recovery of pirlimycin across this concentration range was 95.4% ± 8.7%. The limit of quantitation (LOQ) and limit of confirmation (LOC) of the method were validated to be 0.05 μg/ml and 0.10 μg/ml, respectively.

The method for liver was validated by the analysis of control liver samples spiked with I at concentrations ranging from 0.025 to 1.0 μg/g. The overall recovery of pirlimycin was 97.6% ± 5.1% in this concentration range. The validated limit of quantitation (LOQ) and limit of confirmation (LOC) of the method were 0.025 μg/g and 0.10 μg/g, respectively.

Four diagnostic ions for I were monitored for confirmation: the pseudo-molecular ions $(M+H)^+$ at *m/z* 411.2 (^{35}Cl) and *m/z* 413.2 (^{37}Cl), and fragment ions at *m/z* 375.2 and 158.1. Confirmatory criteria were defined for these assays.

Keywords: Pirlimycin

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1. Introduction

The development and validation of an appropriate method of analysis for measuring trace residues of drugs in consumable tissues and products of food producing animals is an important and necessary step in the drug approval process [1–4]. The method may be categorized as either screening, determinative (quantitative) or confirmatory. Screening methods are generally developed to give a fairly quick semi-quantitative indication of the presence (or absence) of a given residue above (or below) some threshold concentration established in the screening test. Determinative methods are generally more labor-intensive but quantitatively more accurate, measuring the concentration of a residue in a sample within the reference standard curve concentration range of the method. As an example, the inhibition of the growth of an organism by antibiotic residues, which is the basis for the microbiological cylinder-plate assay, gives quantitative results by the degree of inhibition as a function of the concentration of antibiotic in the test matrix. This methodology is often used because of the technique's high sample volume through-put, relative ease of sample preparation, and turn-around times of less than 24 h. Such methods, by their very nature, are generally antibiotic-class specific and lack the ability to select a single substance as the end point response. High-performance liquid chromatography (HPLC) procedures are often favored because they exhibit greater specificity for a single entity since chromatographic and detector-response characteristics can be quite unique for a given analyte. However, HPLC methods, with UV detection, etc., are not considered confirmatory since matrix components often co-elute and a response in the elution region of the expected analyte is not, therefore, definitively diagnostic for a given residue.

A confirmatory method of analysis is required to precisely identify which of the possible drugs is present in a 'violative' sample as detected by either a screening test or a determinative method. The confirmatory method must be highly specific to verify the presence or absence of a given drug residue. Some characteristic response, whether instrumental (physical), chemical or biological, must be developed which is unique for the specific drug of interest. Thus, a diagnostic qualitative component in the confirmatory procedure is absolutely essential, whereas a quantitative component is not necessarily required. The confirmatory method should possess a level of sensitivity near that of the determinative method, although it need only be used (in a regulatory sense) at the allowable tolerance (R_m) or maximum residue level (MRL) for the given drug residue.

Perhaps the most powerful tool available to the analytical chemist for the identification and verification of any compound is the mass spectrometer (MS). Since each chemical entity has a unique chemical structure, in terms of the elements present, the spacial arrangement of those elements, and its chemical and physical properties, each will generally possess a unique molecular mass and characteristic MS molecular fragmentation pattern [5,6]. The power of this instrument to precisely identify a compound is multiplied many-fold when coupled to a separation technique such as HPLC. However, coupling the HPLC to the MS has been no small technological challenge since one must introduce liquid into the MS at flow-rates up to 2 ml/min, volatilize the solvents in a chamber held at vacuums near those required in the analyzer section of the MS and carry the excess vapors away without overwhelming the analyzer. This problem was essentially solved for many substances by Vestal et al. [7,8], with the introduction of the electrically heated thermospray (TSP) interface in the early 1980s, providing the analytical chemist with a practical commercially available HPLC–MS tool.

Since the introduction of commercially available TSP interfaces, many practitioners of the technique have published countless papers utilizing HPLC–TSP-MS for a variety of analytical problems. A recent paper by Tyczkowska et al. [9], which reported the analysis of penicillin residue in milk, is a relevant example to our own confirmatory needs. This method had separate HPLC procedures for the determinative and

confirmatory analysis of penicillin residue in milk. Although much of the methodology was not applicable to pirlimycin, the general practicality of the thermospray HPLC–MS technique was demonstrated.

We therefore set out to develop HPLC–TSP-MS confirmatory procedures for pirlimycin in bovine milk and liver that were highly specific in a qualitative sense and appropriately sensitive in a quantitative sense. Furthermore, the methods needed to be useful and acceptable as regulatory confirmatory methods suitable for adaptation in appropriately equipped government and industrial laboratories.

2. Experimental

2.1. Materials and reagents

Acetonitrile, *n*-butyl chloride, hexane, methanol, and methylene chloride were all chromatographic grade or better (Baxter Healthcare, Burdick and Jackson, Muskegon, MI, USA). Ammonium hydroxide (29%), glacial acetic acid and hydrochloric acid (37%) were all analytical reagent grade (Mallinckrodt, Paris, KY, USA). Trifluoroacetic acid (99%) was obtained from Aldrich, Milwaukee, WI, USA. Triethylamine (TEA) (99%) was obtained from Aldrich. HPLC Water or Milli-Q Water (MQW) was obtained from on-line Millipore filter to a purity >14 MΩ cm (Milli-Q Plus 4 stage system, Model ZD40 115 84, Millipore, Milford, MA).

Pirlimycin hydrochloride and iso-pirlimycin hydrochloride were obtained as Control Reference Standard (Issue B) and from Upjohn inventory, respectively (The Upjohn Company, Kalamazoo, MI, USA).

2.2. Solutions

Various solutions were prepared as follows: milk extractant (ME): 0.015 *M* HCl in acetonitrile; milk partition solution (MPS): *n*-butyl chloride–hexane (1:1); liver extractant (LE1): 0.25% TFA in acetonitrile; liver wash solution (LE2): 15% water (MQW) in LE1; 0.1 *M* ammonium acetate, pH 6.8 (AA): 24 ml of glacial acetic acid, 0.41 moles, was added to 1000 ml of MQW in a 1000-ml beaker and the pH was adjusted to 6.8 with ca. 24–25 ml of concentrated ammonium hydroxide; 15% ammonium hydroxide (15AH): concentrated NH_4OH was diluted 1:1 with MQW; 0.15 *M* ammonium hydroxide (015AH): 1.0 ml of concentrated ammonium hydroxide was added to 100 ml of MQW in a 125-ml Erlenmeyer flask; injection diluent (ID): 20% acetonitrile in 0.1 *M* AA; pirlimycin stock solution (PSS): 11.5 mg of pirlimycin-HCl (868 μg/mg potency as the free base—FB) standard was weighed into a 20-ml screw-cap vial and 10.0 ml of MQW was added to dissolve. This provided a concentration of 1.00 mg of pirlimycin FB per ml; for the milk assays, a series of dilutions was prepared giving concentrations of pirlimycin FB at 120.0, 80.0, 40.0, 20.0, 8.0, 4.0, 2.0, 1.0 and 0.50 μg/ml. For the liver assays, a series of dilutions giving concentrations of pirlimycin FB at 20.0, 10.0, 5.0, 2.0, 1.0 and 0.50 μg/ml. Iso-pirlimycin internal standard stock solution (IST): 11.3 mg of iso-pirlimycin HCl internal standard was weighed into a 20-ml screw-cap vial and 10.0 ml of MQW to give a stock solution concentration of 1.00 mg of iso-pirlimycin FB per ml. For the milk assays, two dilutions were prepared with a FB concentration of 50 μg/ml and 5 μg/ml (MIS-5). For the liver assays, a 1:100 dilution was prepared to give a concentration of 10 μg/ml (LIS-10). SPE wash solution (WS): 20% acetonitrile in MQW with 50 μl of ammonium hydroxide per 100 ml. SPE elution solution (ES): 5% TEA in methanol.

2.3. Milk and liver samples

Control (antibiotic free) milk and liver samples were obtained from six Holstein dairy cows for the preparation of fortified samples as well as for the assessment of the blank-sample response performance (background noise and interference characteristics). Biologically incurred residue samples were obtained from various cows treated intramammarily with an aqueous gel formulation of pirlimycin hydrochloride, PIRSUE.

2.4. Milk sample preparation procedure

Into a 12 × 75 mm polypropylene snap-cap tube was added 100 μl of MIS-5 (500 ng of I.S.) followed by 1.0 ml of the milk sample. For pirlimycin-fortified control milk samples, 100 μl of the appropriate dilution was also added at this time. Then 3.0 ml of ME was added to the tube, the tube capped and vigorously shaken for 15 s, followed by centrifugation at 1200 *g* for 3 min. The supernatant was decanted into a 17 × 100 mm polypropylene snap-cap tube and 5 ml of MPS was added. The tube was tightly capped, vigorously shaken for 5–10 s, and centrifuged at 1000 *g* for 2 min. Most of the upper organic layer(s) was removed with a pipet and discarded. To the bottom aqueous layer was added 50 μl of concentrated ammonium hydroxide and the tube was briefly swirled to mix, then 3.0 ml of methylene chloride was added. The tube was tightly capped and vigorously shaken for 30 s then centrifuged at 1200 *g* for 5 min. The upper aqueous phase was carefully removed and discarded with a dispo-pipet. The methylene chloride solution was evaporated to dryness with a nitrogen stream with the water-bath set at 60°C (N-EVAP, Organomation, South Berlin, MA, USA).

The dried residue was taken-up in 2.0 ml of 015AH and this basic solution was carefully transferred to the top of a 3-ml C_{18} SPE column (pre-conditioned by successive washes with methanol and 015AH) and slowly drawn through the column under gentle vacuum at 1 to 3 drops/s. The tube was rinsed with 2.0 ml of WS then air was drawn through the column under full vacuum for 1 min. Elution was accomplished with 2.0 ml of ES into a 12 × 75 mm glass tube. The tube was blown dry with a nitrogen stream (N-EVAP). Then 1.0 ml of AA was filtered into the tube and vortex-mixed for 10 s, then 100 μl of the sample was injected by the autosampler into the HPLC–MS system and the 8-min selected ion monitoring (SIM-MS) acquisition started.

2.5. Liver sample preparation procedure

Into a 40-ml glass centrifuge tube was weighed 2.0 ± 0.2 g of ground liver followed by 100 μl of LIS-10 (1000 ng of I.S.). For pirlimycin-fortified control liver samples, 100 μl of the appropriate pirlimycin dilution was added. A 10-ml volume of LE1 was added to the tube and the mixture was homogenized with a tissue homogenizer (Polytron, Brinkmann Instruments) at medium-high speed for 30 s. The sample was vacuum-filtered through an empty SPE-type 75-ml reservoir containing a filter frit into a clean 50-ml glass-stopper centrifuge tube. The extraction tube and filter cake were rinsed twice with 5 ml of LE2 and these washes were filtered into the 50-ml tube. The combined filtrate was then partitioned with 25 ml of *n*-butyl chloride. The tube was capped, vigorously shaken for 10 s, then centrifuged at 500 *g* for 1 min. The bottom aqueous layer (ca. 1 ml) was transferred to a 20-ml glass vial, then 4.0 ml of MQW added to the organic phase. The tube was stoppered, vigorously shaken for 5–10 s, then centrifuged at 500*g* for 1 min. Most of the upper organic layer was decanted and discarded. The bottom aqueous layer (about 5 ml) was transferred to the 20-ml vial containing the first aqueous isolate. The glass vial was set in a nitrogen evaporator with the water-bath set at 80°C and the aqueous volume was reduced to 1.5–2.0 ml. The sample was cooled to ambient temperature and basified with 1.0 ml of 15AH. This solution was extracted with 15 ml of methylene chloride (MC). The MC layer was transferred to a clean 20-ml glass vial and evaporated (N-EVAP) to dryness. The residue was reconstituted in 2.0 ml of ID, then 100 μl injections of this solution were made into the HPLC–MS system. The mass spectrometer was set for an 8-min SIM-MS acquisition.

2.6. Liquid chromatography–mass spectrometry

The HPLC equipment consisted of a Waters 600-MS quaternary gradient pump and a Waters 715 Ultra-Wisp with 96-position autosampler tray (Millipore Corp., Waters Chromatography Division, Milford, MA, USA). The HPLC column was a Keystone Scientific CPS-Hypersil-2 (end-capped cyano), 5 μm, 250 mm × 4.6 mm, with integral 10 mm × 4 mm cyanopropyl (CN) guard column (Keystone Scientific, Bellefonte, PA,

USA). The mobile phase was 0.1 *M* NH_4OAc–CH_3CN (70:30) at 1.1 ml/min.

The thermospray mass spectrometer consisted of a Vestec-Nermag Vaporizer, Thermospray source, and a Nermag Resolver R 10–10 L 2000 amu quadrupole instrument (Delsi-Nermag USA, Delsi, Houston, TX) and was set in the positive ion mode. The thermospray controller was obtained from Vestec (Houston, TX, USA). The data system was the Teknivent Vector/Two GC/LC/MS Workstation (Teknivent, Maryland Heights, MO, USA). The instrument was tuned to the TSP response for pirlimycin at *m/z* 411.19 (the pseudomolecular ion, MH^+), *m/z* 375.21 (MH^+–HCl), *m/z* 158.12 (4-ethyl pipecolic acid · H^+, a hydrolytic fragment), and a solvent related ion at *m/z* 59.06 ($CH_3CN \cdot NH_4^+$).

2.7. Milk method standard curve

A series of standards containing pirlimycin FB at various concentrations from 0.05 μg/ml to 1.2 μg/ml and the internal standard at 0.5 μg/ml were prepared to generate the standard curve for quantitative analysis. Injections of 100 μl of each solution were made into the HPLC–TSP-MS system with the autosampler to give pirlimycin (I) in the amounts of 5, 10, 20, 40, 80, and 120 ng on column, respectively, and iso-pirlimycin (II) in the amount of 50 ng on column for all cases. This produced a pseudo-biphasic linear curve with a low-end range of 0.05 μg/ml to 0.20 μg/ml, and a high-end range of 0.20 μg/ml to 1.20 μg/ml. However, a weighted linear regression based on a weighting factor of $1/x^2$ (1 over concentration squared) produced a single full-range highly linear calibration curve.

2.8. Liver method standard curve

A series of standards containing pirlimycin at various concentrations from 0.10 to 1.0 μg/ml and the internal standard at 0.5 μg/ml were prepared to generate the calibration curve for the quantitative analysis of samples containing pirlimycin FB at 0.10 μg/ml to 1.0 μg/g. Injections of 100 μl of each solution were made into the HPLC–TSP-MS system to give pirlimycin (I) on-column amounts of 10, 25, 50, and 100 ng, respectively, and iso-pirlimycin (II) on-column amounts of 50 ng on column for all cases. For the method extension to samples containing pirlimycin at concentrations <0.10 μg/g, a standard curve with 2.5, 5, and 10 ng on column was used. As in the milk method, a weighted linear regression based on a weighting factor of $1/x^2$ (1 over concentration squared) produced a single full-range highly linear calibration curve.

2.9. Quantitative calculation

The areas of the peaks in the ion chromatograms for I and II were integrated and the peak-area ratios of *m/z* 411.2 for I to II were computed. A plot of the concentration of I on the X-axis versus the *m/z* 411.2 peak-area ratio on the *y*-axis generated the quantitative calibration curve. The concentration of pirlimycin was calculated by the expression:

$$R = \text{pirlimycin}_{\text{conc.}} \times \text{slope} + y\text{-intercept}$$

where:

$$R = \frac{\text{peak area of } m/z \text{ 411.4 for pirlimycin}}{\text{peak area of } m/z \text{ 411.4 for iso-pirlimycin}}$$

3. Results and discussion

3.1. TSP-MS of pirlimycin

Thermospray is a soft ionization technique for generating gas-phase ions and is basically categorized as a chemical ionization process, especially when operated in the filament-off mode [10,11]. As such, ionization in the positive-ion mode generally occurs by proton or ammonium ion (when ammonium salts are used as buffers in the mobile phase) addition to either the molecular species or a stable fragment. The various steps in the mechanisms of TSP ionization have been described by Vestal [11]. This gentle technique generally transfers low amounts of energy to the analyte which results in minimal fragmentation of the molecular adduct into smaller ions, unless the molecule is thermally labile or affected by the ion-chemistry that occurs in the droplets as they pass through the heated MS source.

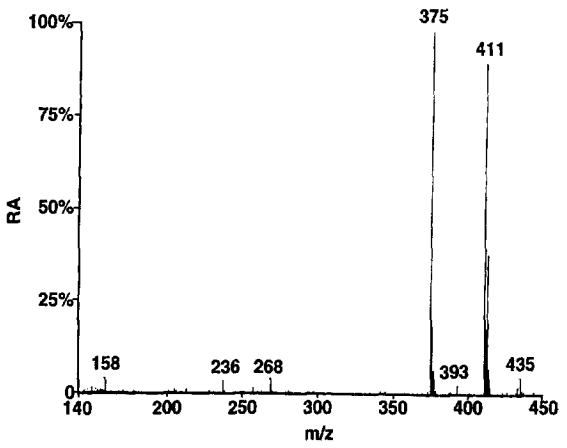

Fig. 1. Thermospray mass spectrum of pirlimycin.

Pirlimycin is readily analyzed by TSP-MS and undergoes a partial thermal/hydrolytic fragmentation to generate, in addition to the monochloro pseudomolecular ion doublet at m/z 411.2 and 413.2, fragment ions at m/z 375.2 (thermal loss of HCl) and m/z 158.1 (hydrolytic cleavage of the amide bond to produce protonated ethylpipecolic acid) as shown in Figs. 1 and 2. The intensity of the m/z 375 ion relative to the m/z 411 ion is readily altered by changing the vaporizer and/or source temperatures. The mass spectrometer is, therefore, set-up to detect these four ions by the selective ion monitoring (SIM) technique for the detection and confirmation of pirlimycin.

3.2. Internal standard selection for quantitative analysis

Quantitative analysis of an analyte at the residue level is often best facilitated with the aid of an internal standard (I.S.). The I.S. should have several essential characteristics: (a) it should not interfere with any phase of the procedure for the analyte, either in extraction, work-up, or detection; (b) it should mimic as close as possible the behavior of the analyte during all phases of the procedure; (c) it should be distinguishable from the analyte in the detection step; and (d) it should have some consistent measurable relationship to the analyte as the concentration or amount of analyte varies. Because the TSP technique can produce a significant variability in response as the solvent composition changes [11,12] such as during gradient elution, the I.S. should have a TSP response comparable to the analyte and not be so far removed chromatographically from the analyte that vastly different solvent compositions occur when gradient elution is necessary. The ideal I.S. is a stable isotopically labeled analog of the

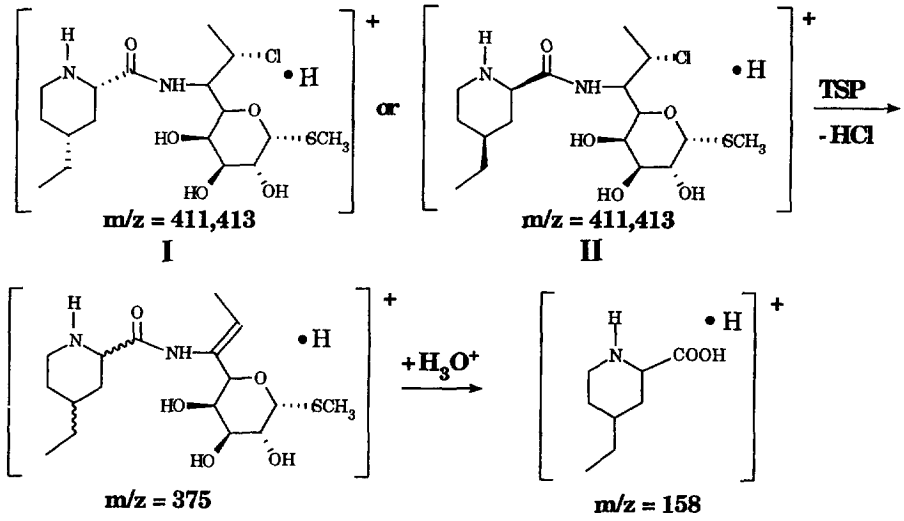

Fig. 2. Thermospray fragmentation ions of pirlimycin (I) and iso-pirlimycin (II).

analyte, such as the deuterated penicillin used in the Tyczkowska et al. [9] procedure. However, stable-isotope labeled compounds are not always readily available for general procedures.

During the early synthetic studies for the preparation of pirlimycin, I, one of the major side products was the *cis/trans*-isomer, II, resulting from the non-stereospecific hydrogenation of the aromatic ring. This isomer is readily separated from I by simple crystallization. This isomer was also found to be well resolved from I during reversed-phase HPLC, as shown in Fig. 3. It also provided an identical TSP mass spectrum (Fig. 2) with identical TSP-MS response compared to I. Therefore, II is a suitable I.S., since one can monitor the same four ions (*m/z* 158, 375, 411, and 413) for qualitative confirmation and select the pseudo-molecular ion at *m/z* 411 for quantitation. It has also proven to meet all other I.S. criteria as outlined above.

3.3. Milk method sample preparation

The isolation of pirlimycin residue from milk was initiated by a protein precipitation step with acetonitrile containing 0.015 *M* hydrochloric acid. This precipitant ensured a slightly acidic aqueous medium to keep the pirlimycins (I and II) as the protonated species to enhance their water solubility. Once the denatured proteins and fat were removed by centrifugation, the clear supernatant was treated with a hydrophobic solvent mixture of *n*-butyl chloride–hexane (1:1) to take up the acetonitrile and release the water and the water soluble components. Both I and II were extracted into methylene chloride (MC) from the base adjusted aqueous solution (both are secondary amines), providing a colorless extract containing the free bases. This extract was then evaporated to dryness and the residue was taken up in dilute ammonium hydroxide for processing through a C_{18} SPE column as a final sample purification. A set of six samples was typically processed in 1.5 h.

3.4. Milk method validation

The method validation was designed to simultaneously address both quantitative and qualitative analytical requirements, since the quantitative aspect of the method is inseparable from the primary qualitative confirmatory objective because analyte extraction efficiency and instrument response characteristics must be calibrated before a legitimate qualitative decision can be made. This method was initially evaluated at a concentration of 0.4 μg/ml, which was established as the R_m (tolerance) for pirlimycin in milk in the US [13], as well as one-half and twice R_m as required by FDA Guidelines [4]. However, the validation was extended to a concentration of 0.05 μg/ml to increase the utility and acceptability of the method.

The first-step validation of this method was

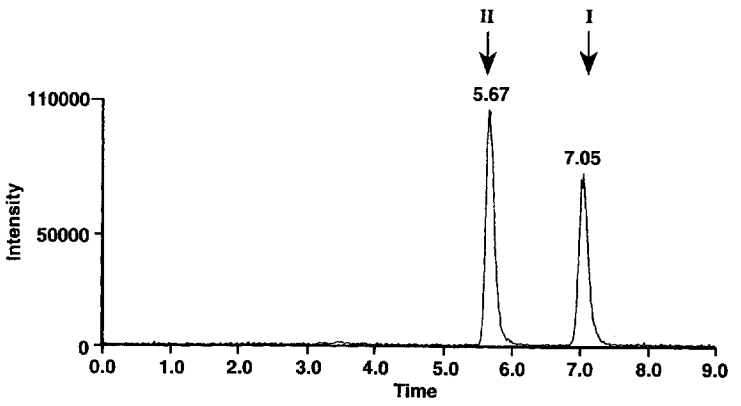

Fig. 3. Reconstructed ion chromatogram at *m/z* 411 for the HPLC–TSP-MS of pirlimycin (I) and iso-pirlimycin (II).

performed on five sets of fortified control milk samples at three concentrations ranging from 0.20 μg/ml to 0.80 μg/ml, a non-fortified control and two samples of incurred residue that were previously quantitatively analyzed by the *M. luteus* microbiological cylinder-plate determinative procedure [14]. The quantitative analysis was based on the ratio of the peak-area responses for pirlimycin to the I.S. for the principal pseudo-molecular ion at m/z 411.2. The quantitative results are summarized in Table 1 as Set 1 data.

The second-step validation was performed with milk samples fortified with pirlimycin at concentrations of 0.05, 0.10 and 0.20 μg/ml, a non-fortified control and two samples of incurred residue that were previously quantitatively analyzed by the *M. luteus* microbiological cylinder-plate determinative procedure [14]. The mass spectrometer was set with a dwell time of 200 ms for m/z 411, compared to dwell times of 4 ms for m/z 158, 375, and 413, to enhance the sensitivity of the method for quantitation. The results are presented in Table 1 as Set 2 data.

Across the six spiked samples and four incurred residue samples, the day-to-day coefficient of variation (C.V.) of the determination of pirlimycin concentration in the range 0.05–0.80 μg/ml was ≤6%. The within day C.V. of the recovery of pirlimycin from the spiked samples was ≤5%. The slope of the concentration added regressed on the concentration found was 1.057, the intercept was −0.015, and the linear regression correlation (R^2) was 0.9997. The overall recovery of the method was computed to be 95.4% ± 8.7% across the entire concentration range.

This two-step validation was performed at different times with separate calibration curves for each step based on the operational concentration range of each. Initially, the standard curves did not appear to be linear extensions of each other due to slightly different slopes and y-intercepts. When the entire data set was merged, the resultant linear regression analysis indicated that the linearity of the combined standards was preserved, as shown in Table 2, where the linear correlation (R^2) was 0.9987. This simple evaluation of the linear relationship may, however, overlook statistical errors that would indicate non-linearity. Weighted linear regression with a back-calculation of the standard concentration and a co-calculated accuracy parameter provides an assessment of the linearity fit for each of the standard concentrations in the

Table 1
Summary of recovery of pirlimycin at a concentration range of 0.05 μg/ml to 0.80 μg/ml from milk determined by HPLC–TSP-MS

Fortified concentration (μg/ml)	Set	n	Concentration recovered (mean ± S.D.) (μg/ml)	C.V. (%)	Mean recovery (%)
0	1	5	0.01 ± 0.01		
0.05	2	5	0.042 ± 0.003	7.5	84.0
0.10	2	5	0.085 ± 0.003	3.9	85.0
0.20	2	5	0.196 ± 0.013	6.5	98.0
0.20	1	5	0.202 ± 0.004	1.8	101.1
0.40	1	5	0.401 ± 0.017	4.2	100.2
0.80	1	5	0.833 ± 0.050	6.0	104.2
Fortified mean:				5.0	95.4 ± 8.7
Incurred samples					
B121-4	2	5	0.122 ± 0.007	5.7	
B121-5	2	5	0.054 ± 0.005	9.6	
592-3	1	5	0.466 ± 0.032	6.9	
2788-3	1	5	0.401 ± 0.027	6.7	
Overall:				5.9	

Table 2
Calibration curve data comparison of non-weighted and weighted linear regression

Standard concentration (μg/ml)	Peak-area ratio	Non-weighted regression[a]		Weighted regression[b]	
		Calculated concentration (μg/ml)	Accuracy (%)	Calculated concentration (μg/ml)	Accuracy (%)
0.025	0.0376	0.036	144.0	0.025	100.0
0.050	0.0998	0.062	124.0	0.052	104.0
0.10	0.2150	0.111	111.0	0.103	103.0
0.20	0.4310	0.202	101.0	0.199	99.5
0.30	0.6280	0.285	95.0	0.286	95.3
0.20	0.4120	0.194	97.0	0.190	95.0
0.40	0.8590	0.382	95.5	0.389	97.3
0.80	1.8160	0.785	98.1	0.813	101.6
1.20	2.8480	1.219	101.6	1.270	105.8

[a] $R^2 = 0.9987$, y-intercept = − 0.048, slope = 2.376.
[b] $R^2 = 0.9999$, y-intercept = − 0.018, slope = 2.256.

data set. Table 2 provides an assessment of the standards when either no weighting factor or a weighting factor of $1/x^2$ was applied to the calculation. These results demonstrated that a weighting factor of $1/x^2$ provided the best standard curve over the entire concentration range of 0.025 to 1.2 μg/ml, based on the accuracy calculation falling within $100 \pm 5\%$ for all standards. Therefore, if one chooses to generate a single standard calibration curve over this concentration range, a $1/x^2$ weighting factor should be applied. However, simple non-weighted linear regression standard curves are appropriate for separate concentration ranges of 0.025 to 0.20 μg/ml and 0.20 to 1.2 μg/ml.

3.5. Milk method ruggedness

An important issue in the acceptability and adaptability of an analytical method which may be used by other laboratories is the method's sensitivity to minor or subtle changes which may influence its performance. Several parameters were examined to assess the ruggedness of the method, beginning with the potential lot-to-lot variability of the solid-phase extraction (SPE) columns used in the procedure. Triplicate samples of control milk fortified at 0.4 μg/ml were tested on each of three lots of 3 ml C_{18} SPE columns. The recoveries were $98.9\% \pm 2.5\%$, $97.7\% \pm 7.4\%$, and $103.0\% \pm 2.3\%$, respectively. Analysis of variance procedures were used to test the hypothesis that the recoveries for the three lots were the same. The results of this test demonstrated that they were not significantly different, either by a pairwise comparison (all $p > 0.05$) or by ANOVA ($p = 0.406$).

The potential effect of varying the SPE wash solution used to rinse the column after sample loading, which is normally done with 2.0 ml of 20% acetonitrile in water, was also tested. Triplicate samples of control milk fortified with pirlimycin at 0.4 μg/ml were processed using a volume wash of 3.0 ml rather than 2.0 ml. Another three samples were processed where the concentration of acetonitrile was raised from 20 to 25%, in case this solution might be inaccurately prepared. Recoveries of $99.6\% \pm 1.1\%$ and $107.2\% \pm 13.9\%$, respectively, were obtained. To evaluate the effect of these deviations, these recoveries were compared with the nine samples used to evaluate the lot-to-lot effect of the SPE columns, which were used as controls. There was no evidence of significant difference between these variations of the method and the controls ($p = 0.964$ and $p = 0.134$ for 3 ml–20% and 2 ml–25% washes, respectively).

The length of time that the dried SPE eluent is left in the N-EVAP solvent evaporation system (under a stream of nitrogen and a water bath

temperature of 60°C after solvent evaporation) was tested to check for deleterious effects on the integrity of the final sample. Triplicate samples of 0.4 μg/ml pirlimycin fortified control milk were processed by the standard procedure, but left in the N-EVAP system for times of 10, 30, and 60 min after drying. The results demonstrated that there was no loss or noticeable deterioration of sample integrity by comparison of the peak-area integrations for the samples to the standards. However, there appeared to be an apparent increase in the quantitative evaluation based on the relative peak areas of pirlimycin to I.S. A 95% confidence interval was computed for each of the three drying times. If 100% was not included in the intervals, then this could be taken as statistical evidence at the 5% level that the drying time significantly changed the recovery rate. Since both 30 and 60 min recoveries were significantly greater than 100% and the lower 95% confidence limit was above 100%, these results indicated that the samples should not be left in the N-EVAP for more than 10–15 min after dryness is attained.

There were no apparent environment-sensitive, or unstable, solutions during the course of sample preparation in which sample integrity would suffer if the sample could not be processed to completion within a rigid time frame. Furthermore, since the entire procedure from start to finish typically takes 75 to 90 min for six samples processed at a time, the critical concern is the stability of the final solution that is to be analyzed on the HPLC–MS system. In this regard, several samples that were analyzed within a few hours of preparation were re-analyzed after seven days storage at 2–4°C. These results demonstrated that there was minimal loss of sample integrity within the bounds of the variability of the method (<10%), where the ratio of the results at the two time points was not far from 1.0 (1.07). Further, when the previously assayed samples were regressed onto the assay of the stored samples, the slope was not significantly different from 1.0 (0.987) and the intercept was not significantly different from 0.0 (0.020) at the 0.05 level. Thus, one could prepare several samples on a given day with assurance that they would not require immediate assay, but could be analyzed the next day or stored at 4°C for several days if necessary.

3.6. Liver method sample preparation

The extraction of pirlimycin residue from liver was accomplished with acetonitrile containing a small amount of trifluoroacetic acid (LE1). Once the denatured proteins, cellular debris and fat were removed by filtration, the filter cake was further extracted (washed) with 15% aqueous LE1 (LE2). The clear combined filtrate was treated with *n*-butyl chloride, a hydrophobic solvent that provided a phase separation and concurrent concentration of the analytes into an aqueous phase. The organic phase was extracted with water to remove additional pirlimycin residue, a step which significantly increased the recovery of the method. The combined aqueous phases were basified with ammonium hydroxide and the pirlimycin and iso-pirlimycin free bases were then extracted into methylene chloride. To facilitate this extraction and improve the recovery of pirlimycin, the volume of the aqueous phase was first reduced to about 2 ml prior to the addition of ammonium hydroxide. The methylene chloride extract was then evaporated to dryness and the residue was taken-up in 2.0 ml of 20% ACN in 0.1 *M* ammonium acetate for final analysis. A set of six samples was processed in about 2 h.

3.7. Liver method validation

The method for pirlimycin residue in liver was first evaluated at a concentration of 0.5 μg/g, which was recently approved as the tolerance (R_m) for pirlimycin in liver [13], one-half R_m and twice R_m (as required by FDA Guidelines [4]), and at 0.10 μg/g, near the estimated limit of quantitation (LOQ) for this method (see below). This concentration range is expected to encompass the MRL as well. A second evaluation for liver samples fortified from 0.025 to 0.10 μg/g was also done to enhance the acceptability and utility of this method for conducting residue depletion studies.

The first-step validation of this method was performed with five sets of fortified control liver samples (obtained from six different cows) at four concentrations ranging from 0.10 to 1.0 μg/g and two samples of incurred residue that were previously analyzed by a microbiological procedure [14]. A standard curve was generated for each set of samples consisting of pirlimycin (free base equivalent) solution standards at concentrations of 0, 0.10, 0.25, 0.50, and 1.0 μg/ml, and each containing iso-pirlimycin at 0.50 μg/ml.

The quantitative results of the analysis of the six sets of liver samples are summarized in Table 3 as Set 1 data. The estimated limit of detection (eLOD) and estimated limit of quantitation (eLOQ) were calculated from the blank control samples in terms of the standard deviation (σ) of the quantitative estimates (S_o) at the retention times of the analytes following established procedures [15,16], where $LOD = S_o + 3\sigma$ and $LOQ = S_o + 10\sigma$. The quantitative estimate (i.e. integrated signal) of S_o is empirically produced by performing a forced integration of baseline noise. The S_o was estimated to be 0.007, which produced an eLOD of 0.03 μg/g and an eLOQ of 0.08 μg/g.

The second-step evaluation was done with control-liver samples fortified at 0.025, 0.050, and 0.10 μg/g of pirlimycin and with two biologically incurred residue samples containing pirlimycin <0.10 μg/g. The calibration standard curve was limited to the concentration range 0.025 μg/ml to 0.10 μg/ml. The results of the second-step validation are presented in Table 3 as Set 2 data.

When these data were combined with the first-run data, the overall performance of the assay was characterized by a linear relationship from 0.025 μg/g to 1.0 μg/g with a slope of 0.981 (not significantly different from 1), a y-intercept of -0.003 (not significantly different from 0) and a linear correlation of 0.994 (R^2). The overall recovery of the method was computed to be 97.6% ± 5.1%. As was indicated for the milk method, the linearity of the standard calibration curve over the entire concentration range of 0.025 μg/ml to 1.0 μg/ml is reasonably preserved with a weighted ($1/x^2$) regression. Although a non-linear polynomial curve fit would

Table 3
Summary of recovery of pirlimycin at a concentration range of 0.025 to 1.0 μg/g from bovine liver determined by HPLC–TSP-MS

Fortified concentration (μg/g)	Set	n	Concentration recovered (mean ± S.D.) (μg/g)	C.V. (%)	Mean recovery (%)
0	1	6	0.012 ± 0.007		
0.025	2	3	0.024 ± 0.004	16.7	96.0
0.050	2	3	0.053 ± 0.008	15.1	106.0
0.10	2	3	0.108 ± 0.009	8.3	108.0
0.10	1	6	0.095 ± 0.005	5.3	95.0
0.25	1	6	0.232 ± 0.035	15.2	92.7
0.50	1	6	0.477 ± 0.024	5.1	95.3
1.0	1	6	0.987 ± 0.060	6.1	98.7
Fortified mean:				9.4[a]	97.6 ± 5.1[a]
Incurred samples					
12	2	3	0.081 ± 0.001	1.2	
37	2	3	0.063 ± 0.002	3.2	
176	1	6	0.323 ± 0.021	6.4	
189	1	6	0.672 ± 0.052	7.8	
Overall:			8.0[a]		

[a] Weighted average.

also describe this calibration curve, the use of a weighted regression is recommended for practical application of this method to simplify the calculation of residue concentration below 1.0 μg/g.

3.8. Liver method ruggedness

The degree of evaporation of the aqueous sample was tested to determine whether keeping this solution from complete evaporation is required for preserving the integrity of the sample preparation. Three samples of an incurred residue liver were prepared side-by-side, except that the volume of one was reduced to 2.5 ml and the other two were completely evaporated to dryness prior to the addition of 15% ammonium hydroxide. These samples provide a comparison of the extremes for this step, evaporation of the solution to only 2.5 ml or to dryness and allowing the dried sample to remain at 80°C for 15 min. The results demonstrated that there was no loss or appreciable deterioration of sample integrity by comparison of the peak areas for the samples to the standards, nor by the equivalent quantitative result obtained for the three samples. The difference is within the variability of the method.

The 'long-term stability' of various intermediate solutions in this procedure was not rigorously tested since the entire procedure, from start to finish, typically takes about 2 h for experienced personnel to process six samples at a time. Therefore, a formally tested stopping-point in the procedure was not identified. The recommendation, therefore, is that the sample should be processed to the end without lengthy delays along the way. However, the stability of the final solution that is to be analyzed on the HPLC–MS system needed evaluation.

Final sample and standard solution stability was tested with samples and standards that were first analyzed within a day of preparation and then were re-analyzed after 12 days storage at 4–5°C. The results demonstrated that there was no significant change in sample integrity within the bounds of the variability of the method (C.V. = 9.1%), where the correlation between the results at the two time points was not significantly different from 1.0. Thus, one could prepare several samples on a given day with assurance that they would not require immediate assay, but could be analyzed the next day or stored at 4°C for several days.

3.9. Qualitative performance of the methods as confirmatory procedures

The thermospray process is influenced by many variables, not the least of which are co-eluting constituents present in a given sample which impact on the chemistry of the thermospray ionization process. In addition, minor flow-rate fluctuations and composition changes in the mobile phase cause changes in the thermal energy imparted to the solutes during solvent evaporation which impacts upon the response of an analyte. These variables lead to differences in the relative abundance (RA) of the various fragment ions, which are produced by a combination of thermal and chemical processes, from one injection to another across a series of standards and samples. However, if the sample preparation is effective in removing matrix constituents and an isocratic mobile phase is used in conjunction with a highly stable flow-rate, then direct comparison of external standards to samples will provide acceptable results.

The confirmatory criteria were applied to all spiked and incurred residue samples throughout the determinative methods validation sets to measure the performance of the method and to establish the lower limit of confirmation (LOC). The regulatory application of the confirmatory methods will likely be limited, however, to those samples measured by the determinative method to contain pirlimycin residue at or above the R_m or the MRL.

The qualitative confirmation for the presence or absence of pirlimycin in milk and liver was provided by the SIM of four ions at m/z 158.1, 375.2, 411.2, and 413.2. The qualitative diagnosis was made for both pirlimycin and the internal standard at the appropriate HPLC retention times by comparing standards to samples within a given sample-standard set. Not only were all four ions observed for both compounds as appro-

priate to the sample, the relative intensity, or relative abundance (RA), of a given ion between I and II *within each chromatographic run* for either standards or samples were approximately the same within defined variability limits. The acceptability of this criteria must be absolutely coupled to the HPLC retention time characteristics, which in itself is a highly selective technique.

Three principal criteria for the confirmation of pirlimycin were defined as follows:

(1) Observation of peak-area responses above a signal-to-noise ratio (S/N) of 3:1 for the four ions m/z 413, 411, 375, and 158 for pirlimycin. The minimum acceptable S/N ratio for each ion may be obtained from three times (3 ×) the area of the integration of the pirlimycin retention-time window in each RIC for a control milk sample analysis, or from a 'clean' peak-free area of the chromatogram identified as a suitable background area.

(2) The appearance of the peaks in all four RICs should be at the appropriate HPLC retention time (within 0.2 min, or 12 s) relative to a known external standard.

(3) The relative abundances (RAs) of m/z 158, 375, 411 and 413 (relative to the base peak) for pirlimycin in the samples must not differ by more than ± 10% from the average of external pirlimycin standards, using either the 0.4 μg/ml (or 0.8 μg/ml) standard for the milk method or the 0.5 μg/ml (or 1.0 μg/ml) standard for the liver method, obtained just before and after the sample by the bracketing technique. The RAs are determined from averaged background-subtracted spectra obtained for the pirlimycin response in the TIC chromatogram in each sample and standard, respectively, and the variability measured by a simple arithmetic difference of the RAs.

3.10. Milk method confirmation

For the confirmation of pirlimycin residue in milk samples determined to be violative (concentration $\geq R_m$ or ≥MRL) by the determinative phase of the analysis, the samples should be re-analyzed by bracketing the sample with external pirlimycin standards at a concentration approximately equal to the residue if the RA difference for any ion in the sample exceeds the 10% criteria relative to the standards acquired during the quantitative run. This will provide the most reliable confirmatory data for the assessment of the relative abundances of the various ions for the sample relative to contemporary external reference standards. The application of these criteria for the confirmation of pirlimycin residue are illustrated by the multiple analyses of eight sets of milk samples: five control-fortified with pirlimycin at 0.05, 0.10, 0.20, 0.40, and 0.80 μg/ml and three biologically incurred residue samples. The SIM spectra and RAs were compared to pirlimycin standards run immediately before and after each sample. The RICs of an incurred residue sample containing pirlimycin at 0.28 μg/ml are presented in Fig. 4. The average

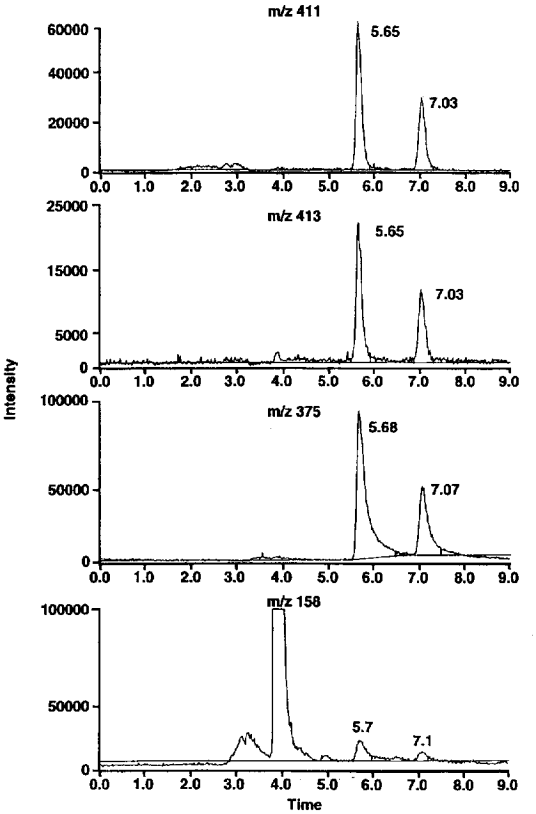

Fig. 4. Reconstructed ion chromatograms of an incurred-residue milk sample containing pirlimycin at 0.28 μg/ml and iso-pirlimycin at 0.50 μg/ml.

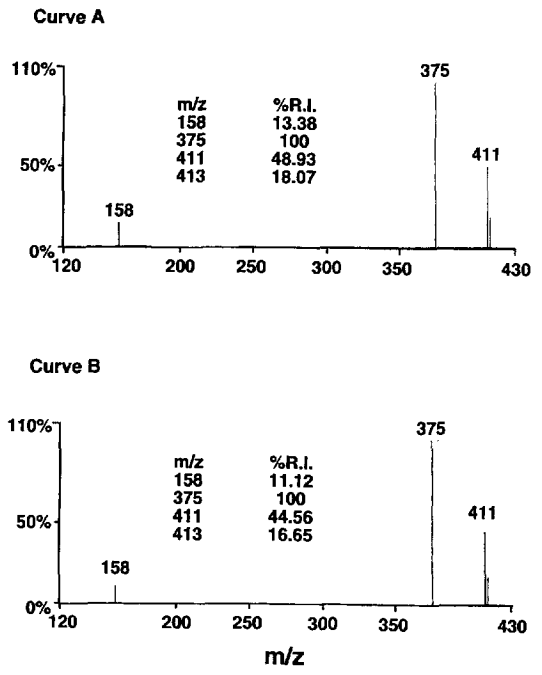

Fig. 5. Thermospray mass spectrum of the pirlimycin peak in a 0.4 μg/ml standard (curve A) and an incurred-residue milk sample containing pirlimycin at 0.28 μg/ml (curve B).

background subtracted spectra for both a contemporary 0.4 μg/ml standard and the 0.28 μg/ml incurred-residue sample are presented in Fig. 5. A summary tabulation of the confirmatory procedure criteria is presented in Table 4. All criteria were met for the confirmation of pirlimycin residue at a concentration ≥0.20 μg/ml and for three of the five samples at 0.10 μg/ml. The data show that the method has a validated limit of confirmation (LOC) of 0.10 μg/ml, although the intensity of the m/z 158 ion was always borderline acceptable for the low-end concentration samples. Based on all other criteria (excluding the RA of the m/z 158 ion), pirlimycin was readily observed and confirmed down to a concentration of 0.05 μg/ml.

The selectivity of the method was tested on milk samples spiked with the following eight antibiotics that are potentially present in milk samples whose source and history may be unknown: ampicillin, amoxicillin, cephapirin, erythromycin, novobiocin, penicillin, sulfadimethoxine, and ceftiofur. These drugs were added to raw control milk at a concentration of 0.1 μg/ml in each case, a concentration at or above the

Table 4
Confirmatory analysis of pirlimycin in bovine milk

Sample ID	Set	n	Number of samples meeting criteria					
			[$S/N > 3$][a] (all ions)	[R_t Std ± 0.2 min][b]	[±10% RA for four ions][c]			
					411	413	375	158
0.05 Fort.	2	5	1	5 (var ± 0.02)	5	5	3	1
0.10 Fort.	2	5	5	5 (var ± 0.03)	5	5	5	3
0.20 Fort.	1	5	5	5 (var ± 0.01)	5	5	5	5
0.40 Fort	1	5	5	5 (var ± 0.08)	5	5	5	5
0.80 Fort	1	5	5	5 (var ± 0.06)	5	5	5	5
Inc. 1[d]	2	5	5	5 (var ± 0.05)	5	5	5	5
Inc. 2[e]	1	3	3	3 (var ± 0.07)	3	3	3	3
Inc. 3[f]	1	3	3	3 (var ± 0.03)	3	3	3	3

[a] Criterion 1.
[b] Criterion 2. (var ± n) = observed variation, in min, of the R_t of I in samples.
[c] Criterion 3.
[d] Incurred sample concentration = 0.12 μg/ml.
[e] Incurred sample concentration = 0.28 μg/ml.
[f] Incurred sample concentration = 0.77 μg/ml.

tolerance or minimum acceptable level for these drugs. The RICs for pirlimycin in these samples fortified at both 0.2 μg/ml and 0.4 μg/ml were identical to samples containing none of the eight antibiotics, indicating that the method will perform as a specific confirmatory procedure for pirlimycin residue in milk even in samples contaminated with other residues.

3.11. Liver method confirmation

The confirmatory criteria for all liver samples analyzed are summarized in Table 5. On first analysis, all of the 0.10 μg/g spikes and two of the six 0.25 μg/g fortified control samples in the first-set validation failed the confirmatory requirements. All other samples were positive for the confirmation of pirlimycin residue. As in the milk method, the limiting criterion was the strength of the m/z 158 ion in the 0.10 and 0.25 μg/g fortified samples. The other criteria were met for these samples.

A test of the capability of the confirmatory method for liver samples containing pirlimycin <0.50 μg/g that failed confirmation using the initial determinative run was conducted with a 0.25 μg/ml fortified sample. The sample was re-injected at the same volume as before (50 μl) and at twice the volume (100 μl) and immediately bracketed with a 0.25 μg/ml standard. In addition, a 1 ml aliquot of the final sample solution was concentrated 2-fold to 0.50 ml utilizing the N-EVAP and 50 μl, 100 μl, and 200 μl injections made. The overall results of the re-assessment of confirmation show that the confirmation of this sample was affirmed at the increased injection amounts where the response of m/z 158 met the confirmatory criteria.

Confirmatory analysis of the liver samples examined in the second-step validation was also done. These results, summarized in Table 5 as Set 2 data, show that in this set of samples, appropriate confirmatory data were acquired for both the fortified control samples at 0.10 μg/g

Table 5
Confirmatory analysis of pirlimycin in bovine liver

Sample ID	Set	n	Number of samples meeting criteria					
			[$S/N > 3$][a] (all ions)	[R_t Std ± 0.2 min][b]	[±10% RA for four ions][c]			
					411	413	375	158
0.025 Fort.	2	3	0[d]	3	3	3	3	0
0.050 Fort	2	3	1[d]	3	3	3	3	1
0.10 Fort	2	3	3	3	3	3	3	3
0.10 Fort.	1	6	1[d]	6	4	4	6	6
0.25 Fort	1	6	4[d]	6	6	6	6	6
0.50 Fort	1	6	6	6	6	6	6	6
1.0 Fort	1	6	6	6	6	6	6	6
Inc. 12[e]	2	3	3	3	3	3	3	3
Inc. 37[f]	2	3	3	3	3	3	3	2
Inc. 176[g]	1	6	6	6	6	6	6	6
Inc. 189[h]	1	6	6	6	6	6	6	6

[a] Criterion 1.
[b] Criterion 2.
[c] Criterion 3.
[d] S/N <3 for m/z 158.
[e] Incurred sample concentration = 0.08 μg/g.
[f] Incurred sample concentration = 0.06 μg/g.
[g] Incurred sample concentration = 0.32 μg/g.
[h] Incurred sample concentration = 0.67 μg/g.

and incurred sample 12, calculated to contain 0.08 μg/g. By the data presented in this paper, the validated LOC of the method is 0.10 μg/g.

For the confirmation of pirlimycin residue in liver samples determined to be violative (concentration $\geq R_m$ or MRL) by the quantitative phase of the analysis, samples should be re-analyzed by bracketing each sample with external pirlimycin standards at a concentration approximately equal to the residue if either the intensity of any of the ions is $<S/N = 3$ or the RA difference for any ion in the sample exceeds the 10% criteria relative to the standards acquired during the quantitative run. This will provide the most reliable confirmatory data for the assessment of the relative abundances of the various ions for the sample relative to contemporary external reference standards.

3.12. Observations of the TSP technique

The ruggedness of the instrumentation was assessed by several observational parameters. The HPLC–TSP-MS technique exhibited suitable ruggedness within the context of the analytical instrumentation utilized. The retention time of the components on the HPLC column during a given run of standards and samples never varied by more than about 6 s. The signal strength of the standards and samples from run to run did vary to a significant degree. However, the relative peak-area ratios of I to II were always reproducible within a given run. The failure of the confirmatory method at concentrations below about 0.10 were usually attributed to the weak and variable response of the m/z 158 ion, while all other criteria were met. Reasons for the differences in comparing one sample set to another are thought to be related to slight variabilities in the operating temperatures of the TSP probe and source, although the condition of the probe tip appeared to have the greatest impact on the overall intensity/abundance of the m/z 158 ion. No single variable, or set of variables, that would a priori allow one to adjust this ion intensity was identified. One potential signal enhancement remedy would be to adjust the dwell times of the acquisition in favor of m/z 158 if absolute confirmation of pirlimycin in low-level samples required the acceptance of m/z 158 according to the above criteria. The impact of dwell time differences was illustrated above in the milk method discussion.

The thermospray vaporizer performance represented the weakest link in the overall method where the integrity and condition of the TSP probe was the most critical factor for successful analysis. Each new vaporizer assembly was tested before use to ensure proper performance, and of three Nermag-Vestec vaporizers tested, all were found suitable for this analysis. However, performance deterioration may be evident when, after time, non-volatile substances may deposit within or around the vaporizer orifice resulting in increased back-pressure and increased operating temperatures with subsequent loss of sensitivity and stability of the ion-flux. Another loss of performance was produced when the tip operating temperature was set too high (at or above the 'take-off' temperature), a condition which often leads to either signal enhancement of some of the ions (or loss of signal if it gets to high above), but degrades the accuracy of signal integration resulting in less reliable quantitative data due to an unstable ion-flux of the eluting analytes.

4. Conclusions

Determinative and confirmatory methods of analysis for pirlimycin residue in bovine milk and liver have been developed based on HPLC–TSP-MS technology. Six samples can be processed and analyzed in approximately 3 h. These methods provide a specific and simultaneous quantitative and qualitative analysis of pirlimycin residue in milk at concentrations of 0.05 to 0.8 μg/ml and in liver at concentrations of 0.025 to 1.0 μg/g. The methods are linear with a correlation (R^2) of 0.999 over the pirlimycin concentration ranges evaluated. The qualitative confirmatory criteria for parent pirlimycin residue based on its HPLC characteristics and the subsequent detection of four diagnostic ions by SIM TSP-MS, relative to an internal standard, have also been established. These methods, therefore, should be

suitable as the regulatory methods for the analysis of pirlimycin residue in bovine milk and liver.

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