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Determination of lincomycin and tylosin residues in honey using solid-phase extraction and liquid chromatography-atmospheric pressure chemical ionization mass spectrometry

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Abstract

An analytical method for the determination of residues of the antibiotic drugs lincomycin and tylosin in honey was developed. The procedure employed a solid-phase extraction for the isolation of lincomycin and tylosin from diluted honey samples. The antibiotic residues were subsequently analyzed by reversed-phase HPLC with atmospheric pressure chemical ionization mass spectrometric detection. Average analyte recoveries for lincomycin and tylosin ranged from 84 to 107% in replicate sets of honey samples fortified with drug concentrations of 0.01, 0.5, and 10 μg/g. The method detection limits were determined to be 0.007 and 0.01 μg/g for lincomycin and tylosin, respectively.

Keywords: Honey; Solid-phase extraction; American foulbrood disease; Lincomycin; Tylosin; Antibiotics

1. Introduction

American foulbrood (AFB) is a disease of honey bees (*Apis mellifera* L.) caused by the spore-forming bacteria, *Paenibacillus larvae* ssp. *larvae* [1]. It is considered to be one of the most widespread and lethal diseases affecting honey bees in the world [2]. The infective spores are particularly resilient, being highly resistant to heat, desiccation, and chemical disinfectants [3] and, moreover, can remain virulent for decades [4]. In the western hemisphere, clinical symptoms of AFB are routinely suppressed by prophylactic applications of antibiotics. As early as the 1940s, sodium sulfathiazole was registered for the control of brood diseases in the United States but its use was later banned because residues of the drug continued to be found in honey many months after application [5]. While numerous other drugs have been evaluated for efficacy in controlling AFB [6–10], oxytetracycline (OTC) is the only drug currently approved for control of AFB in Canada and the US [11,12]. The emergence of OTC-resistant strains of *P. larvae larvae* in the US [13], Argentina [14] and recently in western Canada [15,16], poses a severe threat to the livelihood of

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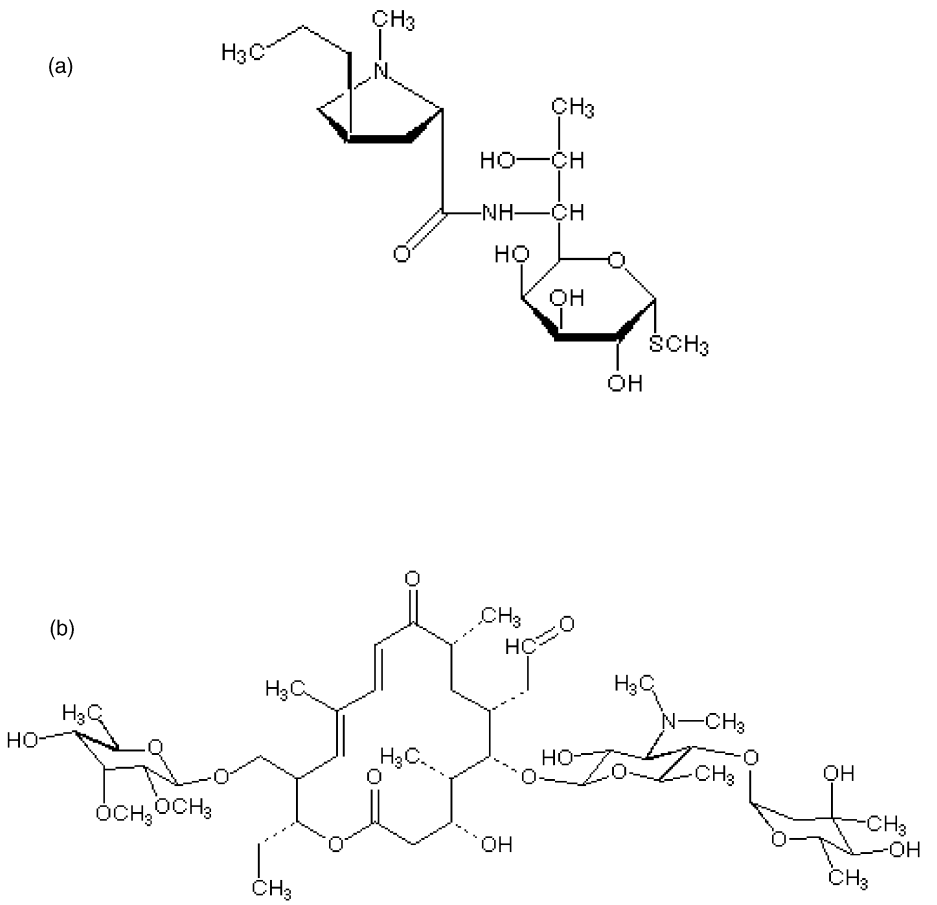

Fig. 1. Structure of (a) lincomycin and (b) tylosin.

beekeepers worldwide. As a result, recent studies have examined the effectiveness of other antibiotic drugs against both OTC-susceptible and OTC-resistant strains of *P. larvae larvae* [17–20]. Two antibiotics are being proposed as registered alternatives to OTC. The first, lincomycin (Fig. 1a), is a lincosamide that is derived from *Streptomyces lincolnensis*. The second, tylosin (Fig. 1b) is a macrolide antibiotic produced by *Streptomyces fradiae*. Both drugs are currently approved in Canada for specified prophylactic and therapeutic disease treatment in livestock [21].

While AFB is not transmitted to humans and does not affect the quality or safety of honey used for human consumption, the use of antibiotics to control brood diseases does create public health concerns regarding potential drug residues in honey. Moreover, the potential stability of compounds such as tylosin in sugar syrup [22] or honey [23–25] underscores the need to test for residual levels of antibiotics. There are numerous microbiological assays designed to detect the presence of antibiotic residues. These tests, while frequently very sensitive, are often limited by a lack of specificity towards a given compound. Chromatographic techniques are therefore commonly used as a means of confirming and quantifying the presence of targeted antibiotics.

Because neither tylosin nor lincomycin is approved for apicultural use in Canada or the US, very little information is available regarding methods for detecting their residues in honey. There are no reported methodologies for the chromatographic determination of lincomycin in honey, and very few for other foods or

animal tissues in general. Gas chromatography with nitrogen-selective detection has been employed for the determination of the trimethylsilyl derivative of lincomycin in porcine and bovine kidney samples [26] as well as fish tissue extracts [27]. A reversed-phase HPLC procedure was developed for the determination of lincomycin in various matrices including milk, muscle, liver and kidney [28], and Luo et al. used ion-pair reversed-phase HPLC for the analysis of lincomycin residues in salmon muscle and skin [29].

Limited information is available concerning the analysis of tylosin residues in honey and other sugar matrices. During their investigation of tylosin for the control of AFB in Argentina, Alippi et al. also performed field studies of the degradation of tylosin in honey [9]. Honey was diluted with acetonitrile and filtered prior to analysis by reversed-phase HPLC with ultraviolet (UV) detection. Using this procedure, a method detection limit of 2 μg/g was reported. As part of dosing experiments, Kochansky et al. studied the comparative stability of OTC and tylosin in sugar syrup using HPLC [22]. The syrup solution, which contained 50–70% sucrose in water (w/v), was analyzed by reversed-phase HPLC without any prior cleanup. The authors noted that the relatively high concentrations of tylosin (200 mg/l of syrup) they were working with precluded any interferences. They further commented that the simplified analytical procedure would likely require modification to permit its use with honey.

Unlike lincomycin, there are many chromatographic methods reported for the analysis of tylosin in various foods and animal tissues. Kanfer et al. thoroughly reviewed the chromatographic analysis of a series of macrolide antibiotics including tylosin [30]. Since the publication of that review, several other methods have been reported. HPLC with UV detection has been employed for the determination of tylosin in various animal tissues [31–35] and milk [36]. Delepine et al. developed a liquid chromatography–mass spectrometry (LC–MS) technique that included a particle beam interface and negative chemical ionization for the analysis of tylosin in bovine muscle [37]. The same group later used particle beam LC–MS with both negative and positive chemical ionization to confirm five macrolides including tylosin in bovine muscle [38]. LC–MS with electrospray ionization has been recently utilized for the determination of seven macrolides including tylosin in poultry muscle [39]. Liquid chromatography–tandem mass spectrometry with electrospray ionization (LC–ESI–MS/MS) has been used to detect tylosin in edible swine tissues [40], as well as tylosin and other macrolide residues in bovine muscle, liver and kidney [41].

Studies are presently being conducted in the Province of Alberta by Agriculture and Agri-Food Canada to evaluate various formulations and dosages of tylosin and lincomycin for their AFB treatment efficacy, effects on bee mortality and residual persistence in honey. The LC–MS procedure described herein was developed to support the latter objective of these studies, and the potential registration of these products for therapeutic disease control in honey bees.

2. Experimental

2.1. Materials and reagents

Tylosin tartrate (872 units tylosin per mg) and lincomycin hydrochloride (872 units lincomycin per mg) were purchased from Sigma Aldrich Corporation (St. Louis, MO, USA).

Methanol (EM Science, Gibbstown, NJ) and acetonitrile (Mallinckrodt Baker Inc., Phillipsburg, NJ) were HPLC grade. Trifluoroacetic acid (>99% purity) was purchased from Sigma Aldrich. Reagent water was generated using a Barnstead Nanopure water purification system.

Sodium carbonate and sodium hydrogen carbonate were obtained from Fisher Scientific (Fairlawn, NJ). A buffer solution was prepared by taking 100 mM $NaHCO_3$ (29.4 g in 3.5 l of reagent water) and adding 100 mM Na_2CO_3 (5.29 g in 0.5 l of reagent water) until the pH reached 9.0.

Bond Elut C18 SPE tubes (6 ml with 500 mg of sorbent; Varian Inc., part number 12102052) and a 12-port vacuum manifold were used for all extractions.

2.2. Honey samples

Honey samples were obtained from a local beekeeping operation verified as having colonies never treated with antibiotics. These samples were used as negative controls (blanks), and for the preparation of matrix-based standards and drug-fortified controls.

2.3. Preparation of matrix-based standard solutions

Individual drug stock standard solutions containing 1000 μg/ml of each active ingredient were prepared in 25 ml of 1:1 (v/v) methanol:water. An intermediate standard solution was prepared by combining equal portions of each drug stock standard solution. This intermediate standard solution was subsequently diluted with 1:1 (v/v) methanol:water to produce a set of working standards with concentrations ranging between 0.05 and 50 μg/ml. Matrix-based standards were prepared by extracting 5 g samples of blank honey according to the procedure described in the following section and reconstituting the dried residue with 1 ml of the appropriate working standard solution. Matrix-based standards were prepared in this manner in order to determine the absolute recovery of tylosin and lincomycin in fortified honey samples as part of the method validation procedure.

2.4. Sample preparation

All honey samples including positive and negative controls were processed according to the following procedure. Bulk debris was removed from the sample by liquefying the honey with mild heat (water bath set at 60 °C) and centrifuging for 10 min at 3400 rpm (2400 × *g*). Wax and bulk debris was found to settle above the honey and could be easily removed. Five grams of pre-cleaned honey was then thoroughly mixed with 10 ml of the Na_2CO_3–$NaHCO_3$ buffer solution until a homogeneous solution was obtained. The diluted sample was loaded onto an SPE cartridge that had been preconditioned with 5 ml of methanol followed by 5 ml of reagent water immediately prior to use. The honey solution was drawn through the SPE cartridge at a flow rate of approximately 2 ml/min with the application of mild vacuum. The bulk of the sample matrix including sugars was washed from the SPE column using 4 ml of 5:95 (v/v) methanol:water followed by 4 ml of 30:70 (v/v) methanol:water. Both rinses were discarded and the drugs were eluted from the SPE cartridge with 1 ml of methanol followed by 1 ml of acetonitrile. The collection tube was placed in a water bath maintained at 63 °C and the extract evaporated to dryness under a gentle stream of nitrogen gas. The residue was finally reconstituted with 1 ml of a 1:1 (v/v) mixture of water and methanol.

2.5. Analysis by LC–MS

All analyses were performed using an HP1050 liquid chromatograph (Hewlett-Packard GmbH, Waldbronn, FRG) interfaced to a VG Platform II quadrupole mass spectrometer (Fisons Instruments, Altrincham, UK). Chromatographic separations were achieved using a 150 mm × 2.1 mm reversed-phase Zorbax C8 column packed with 5 μm particles (Agilent Technologies, Mississauga, Ont., Canada) that was preceded by a 4 mm × 2.0 mm i.d. C8 guard cartridge (Phenomenex, Torrance, CA). Three solvents were employed in the gradient elution program. Solvent A consisted of methanol while solvents B and C consisted of 0.1% (v/v) trifluoroacetic acid in acetonitrile and 0.1% (v/v) trifluoroacetic acid in water, respectively. It was found that a low concentration of trifluoroacetic acid in both the acetonitrile and water components of the mobile phase yielded improved chromatographic peak shapes. The initial mobile phase composition of 10:22.5:67.5 A:B:C was changed to 10:90:0 A:B:C over 7 min and held at this composition for an additional 3 min. The mobile phase composition was subsequently returned to the initial conditions over 5 min and allowed to equilibrate for a further 5 min prior to the next injection. The mobile phase flow rate was held fixed at 0.5 ml/min throughout the gradient elution program. The LC column temperature was maintained at 40 °C and an injection volume of 25 μl was employed for all chromatographic runs.

The LC was interfaced to the MS via an atmospheric pressure chemical ionization (APcI) probe. The probe was operated at a temperature of 550 °C while the ion source was maintained at 150 °C. High purity nitrogen was used as the APcI sheath and drying gases, the flow rates of which were set to 90 and 175 l/h, respectively. Quantitative analyses were performed using selected ion recording (SIR). Prior to choosing ions for SIR, full scan mass spectra (100–1000 amu) were collected for each compound at various cone voltages. In the SIR mode, the MS software permits time-programmable control of both the cone voltage and the mass-to-charge value being monitored. This allows for the simultaneous acquisition of signal for

Table 1
Selected ion recording parameters

Compound	Time (min)	Cone voltage (V)	*m/z*	Function
Lincomycin	0–2.75	50	126.1	Confirmation ion
		20	407.2	Quantitation ion
Tylosin	2.75–10	70	174.1	Confirmation ion
		30	916.5	Quantitation ion

each of the quantitation and confirmation ions under optimal conditions. The SIR parameters employed for the detection of lincomycin and tylosin are summarized in Table 1. Each ion was monitored over a span width of ±0.3 amu with a dwell time of 50 ms.

3. Results and discussion

Ions formed in the MS source must pass through a tiny orifice in the sample cone which isolates the high vacuum region of the analyzer from the source chamber. While the potential applied to this cone assists in the transmission of ions towards the mass analyzer, it also affects the appearance of the resulting mass spectrum. Under APcI conditions with the cone voltage set at 20 V, the full scan mass spectra of lincomycin (shown in Fig. 2a) is dominated by the presence of the $[M+H]^+$ parent ion. Similarly, the mass spectrum of tylosin (Fig. 2c) at a cone voltage of 30 V exhibits a base peak corresponding to the $[M+H]^+$ parent ion. However, as the cone voltage is increased, more kinetic energy is imparted to the parent ions and they are more likely to fragment as a result of collision with nitrogen molecules present in the source (sometimes called within-source collision-induced dissociation, or CID). As the cone potential is increased above 20 V, the $[M+H]^+$ parent ion of lincomycin fragments to yield predominantly a single product ion at *m/z* 126 (Fig. 2b). Similarly, increasing the cone potential above 30 V for tylosin results in the fragmentation of the $[M+H]^+$ parent ion largely to a single ion at *m/z* 174 (Fig. 2d). The mass spectrometer data system permits the collection of SIR data at alternating cone voltages. Therefore, it is possible to maximize the signal of the quantitation ion ($[M+H]^+$) for each drug and also obtain data for a confirmation ion at a higher cone voltage.

The SPE procedure used for the isolation of lincomycin and tylosin from honey was based largely upon the method employed by the USDA in their evaluation of tylosin as a potential antibiotic for the control of OTC-resistant AFB. As part of this work, antibiotic residues were extracted from honey using SPE and subsequently determined using a microbiological disk assay [42]. The microbiological assay is not susceptible to interference from co-extracted compounds provided that they do not possess antimicrobial properties. In any sort of chromatographic procedure, co-extracted compounds may result in chemical interference during detection. Because both confirmatory fragment ions utilized in the LC–MS analysis are relatively low in mass, there is a higher potential for interference due to co-extracted materials. The SPE procedure originally employed by the USDA researchers was therefore modified to enhance the separation of lincomycin and tylosin from other compounds present in the honey matrix. An additional wash step employing 4 ml of 30:70 (v/v) methanol:water was used to elute other compounds while still leaving the analytes retained on the SPE adsorbent. This second wash was visibly observed to remove the bulk of the colored material retained on the SPE adsorbent after the initial rinsing with 4 ml of 5:95 (v/v) methanol:water.

3.1. Analytical method validation

Aliquots of antibiotic-free honey were fortified at drug concentrations of 0.01, 0.5, and 10 μg/g. Replicate samples of each fortified honey control were analyzed to determine the percent recovery for each drug using the SPE procedure. The unfortified honey was analyzed as a negative control to confirm that neither drug was present in the blank honey and that no interferences were observed. The average recovery for each drug is given in Table 2.

Table 2
Recovery of tylosin and lincomycin in fortified honey samples

Level of fortification (μg/g)	Number of replicates analyzed	Percent recovery ± S.D.	
		Lincomycin	Tylosin
0.01	10	105 ± 24	107 ± 34
0.5	8	103 ± 10	97 ± 8
10	7	102 ± 13	84 ± 9

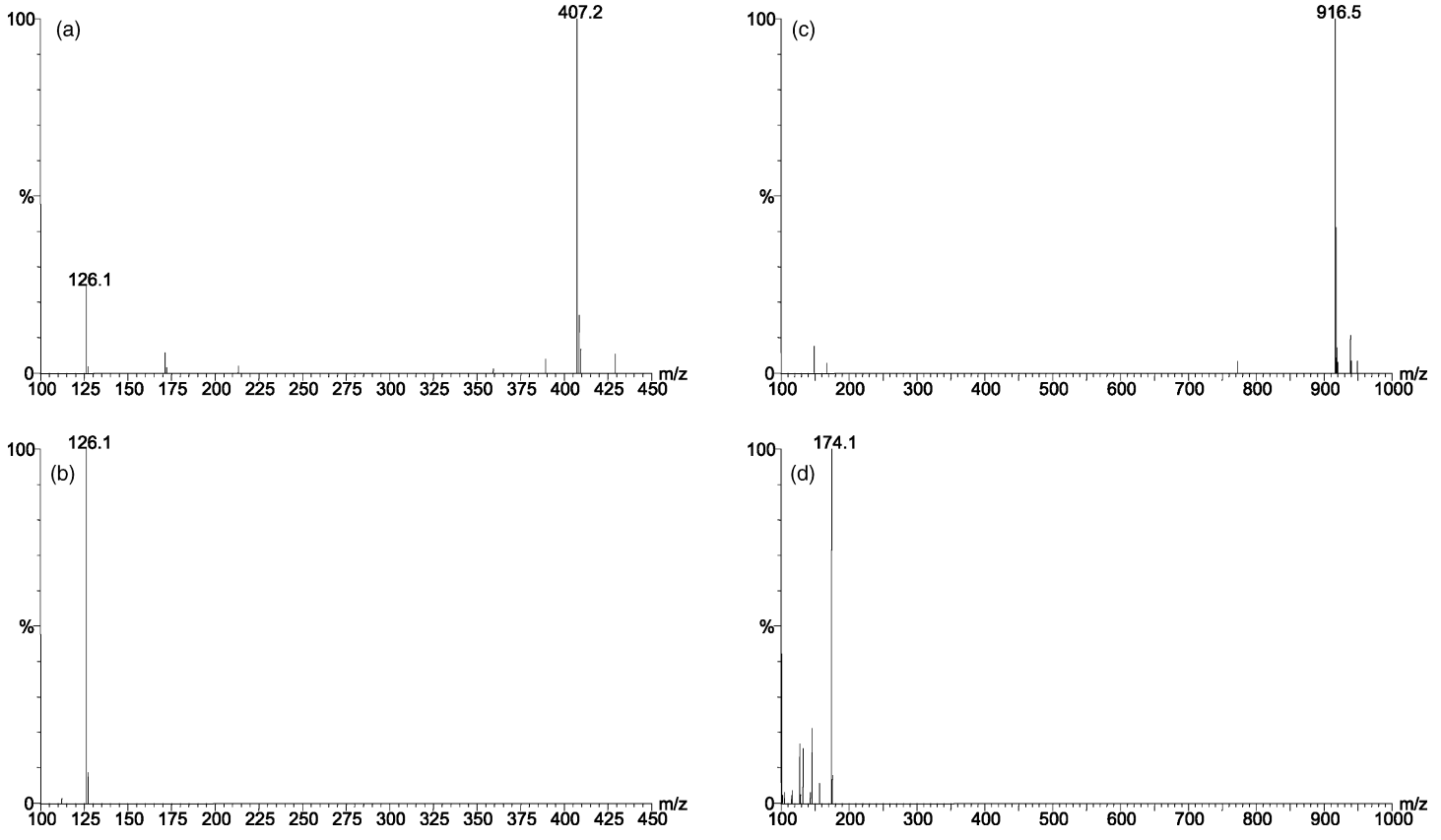

Fig. 2. Full scan APcI mass spectra of lincomycin and tylosin at various cone voltages (CV). (a) Lincomycin at CV = 20 V; (b) lincomycin at CV = 50 V; (c) tylosin at CV = 30 V; and (d) tylosin at CV = 70 V.

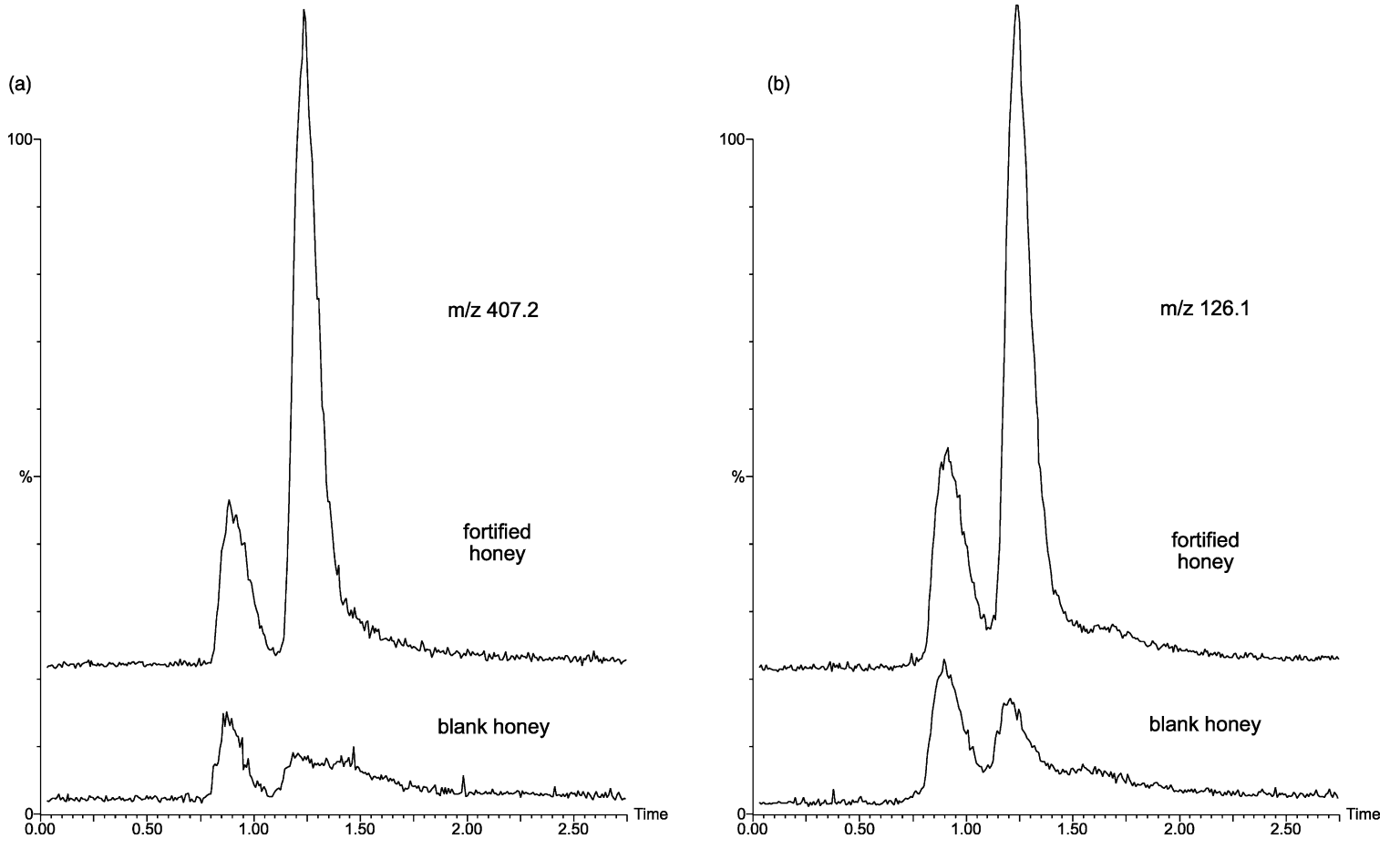

Fig. 3. Reconstructed ion chromatograms of (a) quantitation ion (*m*/*z* 407.2) and (b) confirmation ion (*m*/*z* 126.1) for blank control honey and control honey fortified with 0.01 μg/g of lincomycin.

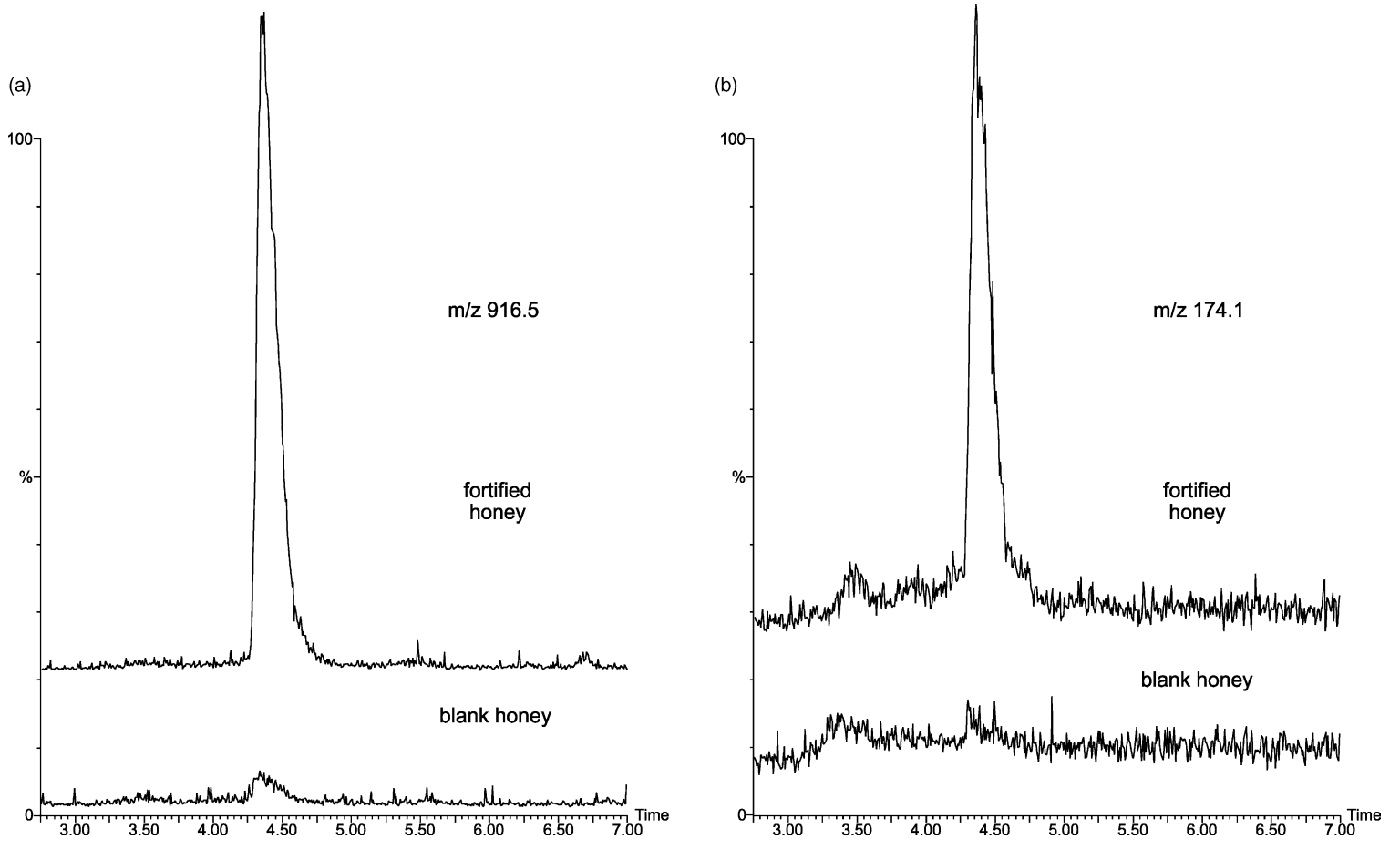

Fig. 4. Reconstructed ion chromatograms of (a) quantitation ion (*m*/*z* 916.5) and (b) confirmation ion (*m*/*z* 174.1) for blank control honey and control honey fortified with 0.01 μg/g of tylosin.

During the initial method validation, it was noted that if reagent-based standard solutions (i.e. standards prepared only in a 1:1 (v/v) mixture of water and methanol) were used for quantitation, consistently elevated recoveries were observed for lincomycin. The average recovery for samples fortified at 0.5 μg/g was observed to be 132 ± 10% (eight samples) when quantitation was performed using reagent-based standards versus an average recovery of 103 ± 10% (eight samples) obtained with matrix-based standards. There were no differences in the average recoveries observed for tylosin regardless of whether quantitation was performed using reagent- or matrix-based standards. The recoveries determined for each drug (102–105% for lincomycin and 84–107% for tylosin) are acceptable for determining residues over a concentration range that spans three orders of magnitude.

The method detection limit was statistically determined for each drug by analyzing a series of 10 replicate samples of honey fortified at approximately 0.01 μg/g. These replicate samples were processed in separate batches on different days along with other honey samples and therefore demonstrate the method performance over time. With a set of seven or more replicate samples fortified at a concentration within one to five times the estimated detection limit, the method detection limit can be calculated from:

$$\mathrm{MDL} = t_{n-1,\alpha=0.01} \times s$$

where "$t_{n-1,\alpha=0.01}$" is the student's *t*-value for a 99% confidence level based on "*n*" replicates processed and "*s*" is the standard deviations of the measured values [43]. Based on the analysis of 10 replicate samples fortified with 0.01 μg/g of each drug, the method detection limit for lincomycin and tylosin were calculated to be 0.007 and 0.01 μg/g, respectively.

Figs. 3 and 4 show the reconstructed ion chromatograms (RICs) that were obtained for lincomycin and tylosin, respectively in blank and fortified control honey samples. The concentration of each drug in the fortified honey control was 0.01 μg/g.

4. Conclusions

Liquid chromatography coupled with mass spectrometry is rapidly becoming the method of choice for the determination of antibiotic residues in foods. As demonstrated in the analytical procedure described herein, LC–MS permits the rapid and sensitive detection of both lincomycin and tylosin in honey extracts. While other groups have utilized LC–MS for the determination of tylosin in various matrices, little has been reported on the determination of lincomycin using this technique. One recent study described the use of lincomycin as an internal standard for the determination of clindamycin in human serum and bone tissue using LC–APcI–MS [44]. Similarly, lincomycin was employed as an internal standard for the analysis of clindamycin in animal plasma by LC–ESI–MS/MS [45]. Our study, however, details the first reported use of LC–MS specifically for the trace analysis of lincomycin residues. LC–MS provides a significant advantage over the use of HPLC coupled with UV absorption for the detection of lincomycin since the latter technique is hindered by the fact that the drug absorbs at low wavelengths (below 220 nm) and has a weak molar absorptivity [28]. This necessitates sufficient isolation of lincomycin from potential interferences by selective cleanup and/or chromatographic separation.

The use of a confirmation ion (*m*/*z* 126 and 174 for lincomycin and tylosin, respectively) provides additional confidence in the identification of drug residues. The RICs were all found to be free of interference as verified by comparing the relative peaks areas in the RICs for the quantitation and confirmation ions with that obtained from the injection of a standard solution of the drugs. Therefore, the SPE procedure was found to be a simple and effective means of simultaneously extracting lincomycin and tylosin from honey and isolating the drugs from the bulk of the sample matrix. It must be noted, however, that the SPE cleanup procedure does not eliminate the need to employ matrix-based standards for quantitation, in particular for lincomycin. The use of matrix-based standards was found to provide superior accuracy based on the analysis of fortified honey samples.

This LC–MS method was developed in support of field trial studies being carried out to evaluate the potential use of lincomycin or tylosin for the control of AFB disease in honeybees. It could also be used in the future for monitoring residues of these drugs in honey should their application in apiculture receive regulatory approval. The method reported herein provides the sensitivity and precision required for residue

analysis and also permits the simultaneous determination of two drugs from different chemical families.

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