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Determination of tylosins A, B, C and D in bee larvae by liquid chromatography coupled to ion trap-tandem mass spectrometry

J. Bernal[a,*], Mª.T. Martín[a], L. Toribio[a], R. Martín-Hernández[b], M. Higes[b], J.L. Bernal[a], M.J. Nozal[a]

[a] *IU CINQUIMA, Analytical Chemistry, University of Valladolid, 47071 Valladolid, Spain*
[b] *Centro Apícola Regional, Bee Pathology Laboratory, Consejería de Agricultura, Marchamalo, 19180 Guadalajara, Spain*

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ABSTRACT

A LC–MS/MS method has been developed to simultaneously quantify tylosins A, B, C and D in bee larvae, compounds currently used to treat one of the most lethal diseases affecting honey bees around the world, American Foulbrood (AFB). The influence of different aqueous media, temperature and light exposure on the stability of these four compounds was studied. The analytes were extracted from bee larvae with methanol and chromatographic separation was achieved on a Luna C_{18} (150 × 4.6 mm i.d.) using a ternary gradient composed of a diluted formic acid, methanol and acetonitrile mobile phase. To facilitate sampling, bee larvae were initially dried at 60 °C for 4 h and afterwards, they were diluted to avoid problems of pressure. MSD-Ion Trap detection was employed with electrospray ionization (ESI). The calibration curves were linear over a wide range of concentrations and the method was validated as sensitive, precise and accurate within the limits of quantification (LOQ, 1.4–4.0 ng/g). The validated method was successfully employed to study bee larvae in field tests of bee hives treated with two formulations containing tylosin. In both cases it was evident that the minimal inhibitory concentration (MIC) had been reached.

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1. Introduction

American Foulbrood (AFB) is an infectious disease of *Apis mellifera* honey bee larvae [1] that affects apiculture worldwide. This disease is caused by the Gram-positive spore-forming *Paenibacillus larvae* subsp. *Larvae* [2], which can produce over one billion spores in each infected larva. The spores are extremely heat stable and resistant to chemical agents, and only the spores are capable of inducing the disease. Infection can be transmitted to larvae by nurse bees or by spores remaining at the base of a brood cell. Although the larvae stages of worker bees, drones and queens are susceptible to infection, infected queens and drone larvae are rarely observed under natural conditions. Moreover, the susceptibility of larvae to AFB diminishes as the larva age [3]. AFB is a highly contagious disease and exchanging combs containing the remains of diseased brood is the most common means by which the disease spreads from colony to colony [4]. In addition, the spread of the disease may also be facilitated by feeding or robbing spore laden honey or bee bread, packaged bees and the introduction of queens from infected colonies. Similarly, wax used to produce the comb foundations and that is contaminated with *P. larvae* spores can also spread the disease.

In geographical areas where AFB is more prevalent, antibiotic treatment appears to offer an alternative to the burning of diseased colonies. The antibiotic oxytetracycline hydrochloride (OTC) is one of the most commonly employed to treat AFB and it has been used worldwide for decades to control the disease. Indeed, the accumulation of this antibiotic in honey has been determined in several studies [5–7]. However, tetracycline resistant strains have been identified in the USA, Canada and Argentina [8–13], and so other antibiotics, such as tylosin and lincomycin have also been proposed to control AFB [14–19].

When the incidence of AFB is high or during epidemics (such as the epidemic affecting some Spanish regions in 2008), therapeutic approaches like the use of antibiotics must be combined with prophylactic actions to help professionals control the appearance of the disease and avoid having to assume the high cost associated with the burning of affected beehives. For this reason, it is interesting to examine the potential to use alternative antibiotics for disease treatment. To be effective, such antibiotics must reach the larvae at a dose sufficiently high as to prevent the growth of the bacteria for long enough periods of time. Subsequently, the treatment must be reapplied to maintain the inhibitory concentration in the targets (larvae). In previous laboratory assays, we found that the inhibitory

* Corresponding author at: IU CINQUIMA, Analytical Chemistry Group, Faculty of Sciences, University of Valladolid, E-47011 Valladolid, Spain.
Tel.: +34 983 423280; fax: +34 983 186347.
E-mail address: jose.bernal@qa.uva.es (J. Bernal).

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dose of tylosin for Spanish strains of *P. larvae* is 0.12 μg/ml and no resistance to tylosin was detected with any isolate of the Foulbrood pathogen [20]. These values indicated that very low concentrations of tylosin were required to inhibit the growth of *P. larvae* in field conditions, as suggested elsewhere [14].

The tylosin series of macrolides (Fig. 1) comprises several related substances of which tylosin A (TA) is the major component. Other minor constituents include tylosin B or desmycosin (TB), tylosin C or macrocin (TC) and tylosin D or relomycin (TD). These four compounds each contribute to the potency of tylosin [21], although tylosin A has the strongest activity, and all of them can be found in the technical product. These natural products exhibit distinct antibacterial activity against numerous gram-positive bacteria and mycoplasma, binding to their ribosomes and inhibiting protein synthesis [22].

In order to optimize the dose, it is necessary to develop an analytical method to determine the true concentration of tylosin in bee larvae after application of this antibiotic in field conditions. Several studies and even reviews have been published on the use of LC–MS/MS or LC–MS to analyze tylosin in several matrices, like water [23–26], honey [27–31] and food matrices [32–35], but not in bee larvae. In most of these studies only tylosin A was analyzed, although tylosin B was also studied in one report [28], and tylosins A, B, C and D have been determined in honey [27] and water [23]. As there are no prior references to studies in bee larvae, we set out to define a procedure to prepare the samples and to optimize a sensitive LC–MS/MS (MSD-Trap) method [27] in order to detect the lowest amounts of tylosins in this matrix. We also performed some studies to assess the influence of stressful (alkaline, oxidative and acidic) media and laboratory conditions (temperature, light) on tylosin stability. Although there have been some attempts to define the pH, light and temperature stability of these compounds [36–39], these have only been performed on tylosin A.

Finally, we successfully validated the method developed by analyzing samples of bee larvae obtained from field tests on honey bee colonies infected with AFB and treated with three formulations, two of them containing different amounts of tylosin, with the aim of finding a useful dose.

2. Materials and methods

2.1. Materials and chemicals

Tylosins A, B and C were obtained from Professor Hoogmartens (Katholieke Universiteit Leuven, Leuven, Belgium), Tylosin D was obtained from the European Council Pharmacopeia (Strasbourg, France) and the Tylosin technical product (tylosin tartrate$_{TP}$) was generously donated by Laboratorios Calier S.A. (Barcelona, Spain). Formic acid and roxithromycin (internal standard, IS) were purchased from Sigma–Aldrich Chemie Gbmh (Steinheim, Germany). HPLC grade methanol and acetonitrile were both supplied by Labscan Ltd. (Dublin, Ireland), and analytical grade glacial acetic acid, sodium hydroxide and *hydrochloric acid* (37%, m/v) were obtained from Merck (Darmstadt, Germany). Syringe filters (17 mm Nylon 0.45 μm) were purchased from Nalgene (Rochester, NY, USA) and deionised water was obtained from a Milipore Mili-RO plus system together with a Mili-Q system (Bedford, MA, USA).

A 5810R refrigerated bench-top Eppendorf centrifuge (Hamburg, Germany) was also used. Finally, a drying oven from Selecta (Barcelona, Spain) was used in the stability tests.

2.2. Preparation of standard solutions

Individual standard stock solutions (drugs and roxithromycin) were prepared in methanol at a concentration of 200 mg/L. An intermediate standard solution was prepared by combining portions of each standard stock drug solution, and this intermediate standard solution was diluted daily with water to produce a set of working standards.

Matrix-based standards were made by extracting 2 g of the larvae obtained from beehives not treated with tylosin tartrate$_{TP}$ but that were spiked with the antibiotics studied. This material was reconstituted from the dried residue with 1 mL of a 1:1 (v/v) methanol:water mixture. All the standards and stock solutions were kept in the dark at +4 °C until analysis and they were stable for over one month.

2.3. Stability of the compounds

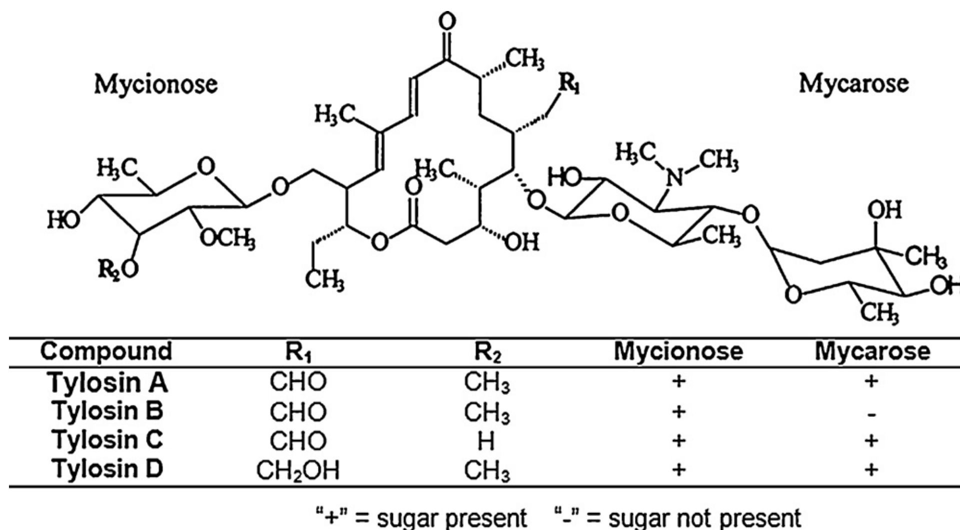
Several tests were performed to check the stability of the four compounds in an aqueous media under different conditions. Firstly, the stability of the tylosins was checked in acidic, oxidative and alkaline media. The influence of light exposure under the precise experimental conditions of the laboratory and temperature on the stability of the compounds was also studied by exposing solutions of the four compounds to different conditions.

2.4. LC–MS/MS system

An Agilent Technologies 1100 series LC/MSD Trap XCT (Palo Alto, CA, USA) instrument was used with electrospray ionization in positive ion mode. The LC instrument was equipped with a vacuum degasser, a quaternary solvent pump and an autosampler with a column oven. The system was controlled by an Agilent ChemStation for LC Rev. A. 10.02 and MSD Trap Control version 5.2. Data analysis was performed by Quant Analysis for LC/MSD Trap 1.6 and Data Analysis for LC/MSD Trap 3.2, all of them form Agilent Technologies (Palo Alto, CA, USA).

A Luna 5 μm C_{18} (2) 100 Å (150 × 4.60 mm i.d.) analytical column was used for LC separation, and it was protected by a C_{18} guard column (4 × 2.0 mm i.d., both from Phenomenex, Torrance, CA, USA). After the optimization study, the mobile phase components used were 1% formic acid in water (solvent A), methanol (solvent B) and acetonitrile (solvent C), applied at a flow rate of 0.5 mL/min in a gradient mode as follows: (i) 0 min (A–B–C, 63:37:0, v/v/v); (ii) 10 min (A–B–C, 80:0:20, v/v/v); (iii) 18 min (A–B–C, 74:0:26, v/v/v); (iv) 20 min (A–B–C, 64:0:36, v/v/v); (v) 22 min (A–B–C, 63:37:0, v/v/v); (vi) 32 min (63:37:0, v/v/v). The injection volume and column temperature were 50 μL and 25 °C respectively. All ESI-MS analyses were performed using multiple reaction monitoring (MRM) in ultra scan mass range mode, scanning from *m/z* 100 to 1000. The ESI-MS/MS detection conditions were optimized for each compound, and with the exception of the capillary voltage and some fragmentation parameters (Table 1), the other parameters studied have the same effect on the sensitivity of each tylosin. The optimal MS/MS conditions were set as follows: Drying gas (N_2) temperature 350 °C; Drying gas (N_2) flow of 9 L/min; Nebulizer pressure at 40 psi; Trap drive 35; Skimmer 40 v: Octopole RF amplitude 130 V; Capillary exit 105.0; Max. Accumulation time 200 ms; ion charge control (ICC) 200,000; and Delay 5 ms.

Among the other MS/MS parameters the transitions monitored for each compound are summarized in Table 1. Matrix matched calibration curves with an internal standard were used for quantification, where relative response factors for the most intense MS/MS transition of each antibiotic were compared to the intensity of the transition monitored for roxithromycin. These calibration curves were constructed by plotting the ratios between the signal (areas) of each tylosin and the internal standard in the *y*-axis, against the concentration values in the *x*-axis. A weighting factor of $1/x^2$ was applied to the linear regression analysis and a second MRM transition was monitored for confirmatory purposes.



Compound	R_1	R_2	Mycionose	Mycarose
Tylosin A	CHO	CH_3	+	+
Tylosin B	CHO	CH_3	+	-
Tylosin C	CHO	H	+	+
Tylosin D	CH_2OH	CH_3	+	+

Fig. 1. Structure of the four macrolide antibiotics TA, TB, TC and TD.

2.5. Honey bee-larvae samples

Irrespective of the sampling day, 48 h old honey bee larvae were obtained aseptically from the Bee Pathology Laboratory of the "Centro Apícola Regional" (CAR) at Marchamalo (Guadalajara, Spain), which has an experimental apiary where a group of honey bee colonies were treated with tylosin tartrate$_{TP}$ for performing a dose-finding study. The treatment consisted of adding two different amounts of tylosin tartrate$_{TP}$ to the bee food, while control samples were obtained from bee hives that were fed with a placebo.

All the larvae bee samples were screened for the four tylosins using LC–MS/MS. The samples from untreated apiaries were used to prepare the calibration standards and also, to generate fortified replicates to validate the method. The average weight of the bee larvae was around 3 mg/larvae.

2.6. Field trials with tylosin application

The apiary was located in Marchamalo (Guadalajara, Spain) and the Langsthrot hives used in the experiment (carried out at the end of September) were single brood chamber colonies, infected with AFB, which contained around 30,000 worker honey bees, one queen, 5–6 brood combs and food stores (pollen and honey).

Fifteen homogeneous honey bee colonies of similar strength and size, in terms of the brood and number of adult bees, were randomly divided into 3 different groups and each was assigned an identification number. The honey bee colonies in each group were fed with one of the three different formulations in order to find an adequate dose. Formulation A consisted of 500 g of "synthetic honey", made by mixing 80% of sugars (60% fructose and 40% glucose) with 20% water, to which 400 mg of tylosin tartrate$_{TP}$ was added (numbered 2 and 4). Formulation B had the same composition as A but it contained only of 200 mg tylosin tartrate$_{TP}$ (numbered 1 and 3). Finally, the third formulation had the same composition but no tylosin tartrate$_{TP}$ was added and it was used as a placebo.

The food was placed in the honey bee colonies on September 22nd and whether the food had been totally consumed was assessed after 48 h (September 24th, day 1), some food remaining in only two bee hives did (20%). At this point, 48 h old bee larvae were collected in an aseptic way (2–7 g of bee larvae per colony) in order to measure the amount of tylosin and to check if the minimum inhibitory dose against *P. larvae* had been reached (as determined in laboratory studies). Sampling of the larvae was repeated on September 26th and 28th (days 3 and 5). Hence, larvae 48 h old bee larvae were sampled 24 h after applying the treatment (first day of the study), on the third and on the fifth day of the study. Thus, bee larvae of the same age (48 h) and similar weight were sampled. The larvae samples were washed three times with deionised water to eliminate any tylosin residues on their surface, and they were then frozen and stored until they were analyzed.

2.7. Sample preparation

The solid–liquid extraction conditions were selected after the optimization studies described below. The samples of bee larvae were thawed at room temperature and then dried for 4 h at 60 °C. Subsequently, 100 mg of the dried bee larvae sample was transferred with 2 mL of methanol to an Eppendorf vial. The mixture was then centrifuged for 15 min at 8000 rpm and 10 °C, and the supernatant obtained was diluted with deionised water at a ratio of 1/2 (v/v) and then passed through a syringe filter. An aliquot of 50 μL was finally injected onto the LC–MS/MS system.

2.8. Method validation

Validation was carried out following the VICH guidelines [40,41], the IUPAC technical report of 2002 [42] and the SANCO/10476/2003 document [43], determining the limits for selectivity, quantification and detection, as well as the linearity, precision and trueness. To check the selectivity of the proposed

Table 1
ESI-MS/MS parameters.

Compound	Quantification transition	Confirmation transition	Capillary voltage (V)	Fragmentation cut off	Fragmentation amplitude (V)
TA	916.4 > 174.4	916.4 > 772.4	4000	247.3	1.3
TB	772.4 > 174.4	772.4 > 156.4	3500	208.5	0.9
TC	902.4 > 174.4	902.4 > 460.4	4000	243.6	1.2
TD	918.4 > 174.4	918.4 > 425.4	4000	248.0	1.4
Roxithromycin	837.4 > 158.4		3750	226.1	1.0

method, a set of non-spiked larvae bee samples were injected onto the LC–MS/MS system. The detection (LOD) and quantification limits (LOQ) were determined by injecting a number of these non-spiked larvae bee extracts ($n = 20$) and measuring the magnitude of the background analytical response. The LOD and LOQ were estimated to be 3 and 10 times the signal-to-noise ratio.

Matrix-matched standard calibration curves were used to quantify the macrolides in bee larvae in order to avoid any effect of the sample treatment, Accordingly, larvae bee samples were spiked with varying amounts of TA, TB, TC and TD across two different analytical ranges: from 4 to 500 ng/g for TA, TB, TC and TD; and from 500 to 2000 ng/g for TA. This additional analytical range for TA was studied to consider the possible result of the most unfavourable situation in terms of treatment application, since tylosin A is the major constituent of the $tylosin_{TP}$ added to the bee food. These spiked honey samples also contained roxithromycin (100 ng/g) as an IS. The extracts obtained were considered as standards to obtain the calibration plots. As such, the concentration versus the ratio of the areas between the macrolides and the IS peak was plotted to prepare the matrix matched calibration curves for each individual set of standards.

Reproducibility and intermediate precision experiments were carries out concurrently by analysing repeated samples but using larvae samples on the same day ($n = 5$). A calibration curve was established in each run and five replicates of each spiking level were analyzed. It must be pointed out that 3 concentrations were tested for TB, TC and TD, while another level was also assayed for TA for the reason mentioned above. The recovery of the 4 tylosins were determined in 5 replicates at 3 concentrations (low, medium and high levels) for TB, TC and TD, and at 4 concentrations for TA.

3. Results and discussion

3.1. Stability tests

3.1.1. Stability in aqueous media

The stability of TA, TB, TC and TD in aqueous media was tested by usual pharmaceutical practices. Firstly, the four compounds were dissolved in HCl 0.1 M, NaOH 0.1 M or H_2O_2 (1% (v/v) in water), working solutions that were made by diluting an aliquot of each of solutions with deionised water. The macrolide concentration in the four working solutions was 300 μg/L. Once the solutions had been prepared they were sequentially injected onto the LC–MS/MS system after different periods of time. After each sequence of injections, a reference standard of each compound at the same concentration in methanol was injected to compare the peak area, symmetry and retention time of the four compounds (see Tables 2 and 3).

When these compounds were first dissolved in HCl and then in deionised water, only TA and TB were detected. Indeed, the peak area of TB increased notably while that of TA represented only 10% that of TB, in accordance with previous studies [28]. Hence, it appears that all the compounds except TB could be broken down in a strong acid medium. Indeed, only TB could be detected when the two working dilutions of HCl were taken into consideration, even though the peak area was lower than that obtained previously. These results suggest that the macrolides could be broken down into compounds other than TB under these circumstances, as reported previously [44].

In alkaline and oxidative media, no significant variations in the area, symmetry and retention time of the peaks were observed in any of the experiments, and hence, the macrolides appeared to be sufficiently stable in both media.

Table 2
Chromatographic parameters from TA, TB, TC and TD mixture firstly dissolved in HCl or NaOH and afterwards diluted in H_2O, HCl or NaOH respectively.

		HCl 0.1 M–H_2O			HCl 0.1 M–HCl 0.1 M			NaOH 0.1 M–H_2O			NaOH 0.1 M–NaOH 0.1 M			Reference[a]
Time (min)		30	60	120	30	60	120	60	120	180	60	120	180	
TA	Area	74,017	64,345	66,534	ND	ND	ND	597,684	624,356	602,345	593,452	612,323	605,839	645,368
	Symmetry	1.25	1.17	1.21	ND	ND	ND	0.98	1.04	1.03	1.02	1.01	1.05	1.01
	Tr (min)	20.6	20.7	20.7	ND	ND	ND	21.0	21.0	21.1	21.1	21.1	21.1	20.9
TB	Area	1,573,266	1,465,422	1,487,555	313,933	326,455	354,667	691,231	712,313	689,030	687,593	695,463	693,848	700,374
	Symmetry	1.20	1.17	1.19	1.51	1.33	1.41	1.07	1.03	1.05	1.08	1.05	1.06	0.99
	Tr (min)	15.2	15.2	15.2	15.2	15.3	15.3	15.6	15.7	15.7	15.7	15.7	15.7	15.5
TC	Area	ND	ND	ND	ND	ND	ND	413,241	423,434	419,845	451,263	448,756	457,839	467,580
	Symmetry	ND	ND	ND	ND	ND	ND	0.92	0.96	1.00	1.10	1.02	1.04	0.97
	Tr (min)	ND	ND	ND	ND	ND	ND	17.3	17.2	17.2	17.1	17.2	17.1	17.0
TD	Area	ND	ND	ND	ND	ND	ND	435,892	456,373	445,321	423,439	434,542	429,588	488,715
	Symmetry	ND	ND	ND	ND	ND	ND	0.88	0.96	0.91	0.93	0.87	0.95	1.00
	Tr (min)	ND	ND	ND	ND	ND	ND	20.1	20.0	20	20.0	20.1	20.0	19.9

ND: not detected.
[a] Data for the mixture in aqueous solution as reference.

Table 3
Chromatographic parameters from TA, TB, TC and TD mixture firstly dissolved in H_2O_2 afterwards diluted in H_2O or H_2O_2 respectively.

		H_2O_2 1%–H_2O		H_2O_2 1%–H_2O_2 1%		Reference[a]
Time (min)		60	120	60	120	
TA	Area	598,271	617,352	602,345	608,393	654,377
	Symmetry	0.97	0.95	0.96	0.98	1.00
	RT (min)	20.7	20.6	20.5	20.5	20.9
TB	Area	637,488	664,632	645,382	656,473	703,341
	Symmetry	0.95	0.98	0.99	0.98	1.01
	RT (min)	15.2	15.2	15.1	15.0	15.6
TC	Area	416,535	435,267	431,257	424,353	472,612
	Symmetry	1.02	0.98	1.00	1.01	0.99
	RT (min)	16.8	16.7	16.7	16.6	17.1
TD	Area	423,617	441,323	436,489	456,722	485,423
	Symmetry	1.00	0.97	0.99	1.02	0.98
	RT (min)	19.7	19.7	19.6	19.6	19.8

[a] Data for the mixture in aqueous solution as reference.

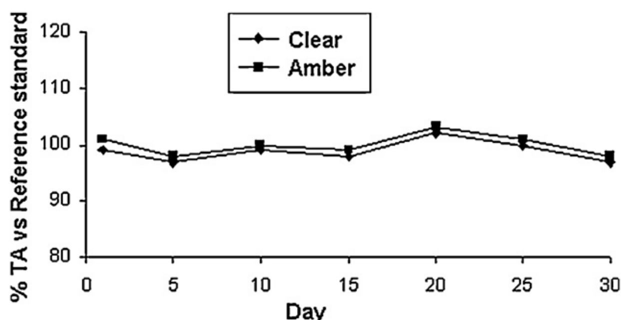


Fig. 2. Influence of light exposure on the TA peak area (300 μg/L) obtained from two identical solutions stored in clear and amber vials. The chromatographic and MS/MS conditions are described in detail in Section 2.4 and Table 1.

3.1.2. Influence of light and temperature

We tested the influence of temperature and light on the stability of the four macrolides. Accordingly, 4 identical solutions of the macrolides were prepared at 300 μg/L by dissolving the compounds in methanol, and the working concentrations reached by diluting in deionised water. Each of these solutions was stored under different conditions, whereby one solution was stored in a refrigerator at 4 °C, two were stored at room temperature (25 °C) in a clear or amber vial, and the fourth was stored at 60 °C. In addition, a reference standard was prepared each day over 30 days at the same concentration to compare the results (Figs. 2 and 3, where the ratio between the signals obtained from the compound and the reference standard are shown). As the effect on the four macrolides was very similar, one example was chosen for each parameter controlled.

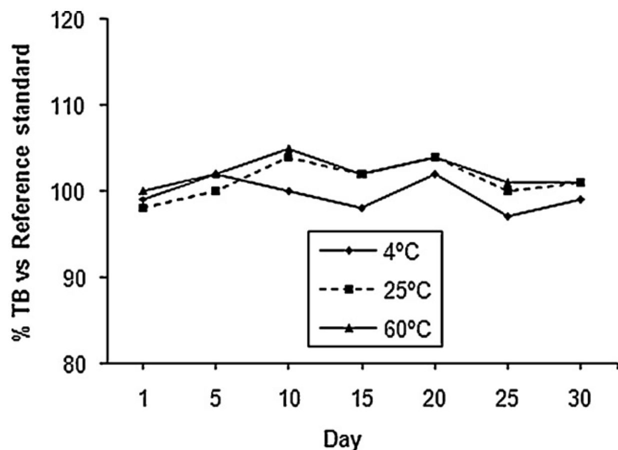


Fig. 3. Influence of temperature on the TB peak area (300 μg/L) for three aliquots stored at 4 °C, 25 °C and 60 °C. The chromatographic and MS/MS conditions are described in detail in Section 2.4 and Table 1.

The influence of light exposure on TA was evaluated by comparing the results obtained from the two identical solutions stored in clear and amber vials (Fig. 2) at the laboratory working conditions. Only a slight variation was observed between these values and it was always close to 100%. Thus, it appeared that light exposure under the tested experimental conditions did not have any significant effect on the stability of the macrolides, in accordance with earlier data [36]. These previous studies indicated that in the dark, tylosin A was stable, and that its concentration fell by ~10% when exposed to the light, although it must be pointed out that the light exposure assays presented in this publication, were not the same than in our tests. Hence, tylosin A does not appear to be very unstable when exposed to light under the storage and precise experimental conditions used in this work, as its concentration remained stable.

When the results obtained for TB were examined, the ratios at the three temperatures assayed were always close to 100% (between 95 and 100%), despite some minor variations in relation to the reference standard (Fig. 3). Thus, these compounds did not appear to be strongly affected by variations in temperature between 4 °C and 60 °C, or by the light conditions of the laboratory.

3.2. Sample preparation

An initial experiment was carried out on control bee larvae, samples that were ground in a mortar and to which an aliquot of the four macrolides was added and allowed for stand overnight. The larvae were then transferred to an Eppendorf vial, centrifuged and the supernatant was injected onto the LC–MS/MS system. This procedure produced peaks with a good shape and resolution, and it was therefore possible to determine the tylosins in the bee larvae by the LC–MS/MS method developed. However, the signal and recovery of TB and TC from this matrix was lower than that obtained for TA and TD, a difference that was not observed in honey samples. In addition, the pressure of the system increased significantly following some injections (>300 bar) and as such, it was necessary to consider incorporating a clean-up step.

Bee larvae must be ground in a mortar to obtain something like a "pap", although due to the high water content and viscosity it is difficult to obtain a constant weight and reproducible aliquots. Hence, we introduced a drying step given that tylosins had previously been shown to be stable up to 60 °C. Accordingly, we performed tests in which the temperature varied between 30 °C and 60 °C for 4 h. While the mass of the bee larvae clearly decreased with the increase in temperature (70% of the mass was lost at 60 °C), the recovery was not affected by this process. Indeed values were around 95% for the

added TA and TD, although they fell to ≈60% for TB and TC. Hence, 60 °C was selected as the drying temperature.

The drying time was also studied by assessing the effect of drying the larvae mass for 1, 2, 4, 8 and 24 h on the recovery of the macrolides. The results showed that excessive mass was lost when dried for longer than 4 h, close to 90% was lost after 8 h and whole bee larvae sample was completely dried up after 24 h. Thus, after such long periods it was impossible to obtain adequate larvae material for a correct analysis and indeed, recovery of all the compounds decreased drastically at drying times longer than 4 h.

Once the drying stage had been defined, we assessed the effectiveness and the influence of the solvent on macrolide extraction, while also attempting to improve the recovery of TB and TC. In previous tests, adequate recovery was obtained with individual solvents and methanol. To enhance the recovery of TB and TC, some mixtures containing methanol were compared with the results of using methanol alone, including: methanol–acetic acid (95:5, v/v), methanol–hydrochloric acid (95:5, v/v) and methanol–sodium hydroxide (95:5, v/v). The extracts obtained with these solvents were injected into the chromatographic system and the recovery of each compound was determined for each solvent (Fig. 4). There was no significant variation in the recovery, which was always higher for TA and TD, and lower for TB and TC. The trend was similar in all cases and thus, any of the solvents could be used. However, since methanol was the easiest option and produced the highest recovery, it was finally selected as the extraction solvent. Using an average mass of 100 mg of dried larvae, 2 mL of methanol was sufficient to transfer the sample to the centrifuge tube and to obtain the highest recovery.

Subsequently, the extract was centrifuged for 15 min at 10 °C and 8000 rpm, and the supernatant recovered was injected onto the system. Optimization of this clean-up step clearly diminished the build up in pressure observed previously, although after a day of work the pressure in the system increased slightly despite using a pre-column and syringe filter. To avoid this problem, the stabilization time between injections was increased and the supernatant was diluted prior to injection. The sample was diluted in deionised water and three different dilutions of the extracts were tested: 1/2, 1/5 and 1/10 (v/v). The increase in pressure was considerably reduced when the extract was diluted 1/2, without significantly affecting recovery (≈95% TA, TD and ≈60% TB, TC). Higher dilutions did not reduce the pressure further, while the limits of detection worsened. Hence, an optimal dilution of 1/2 was finally selected.

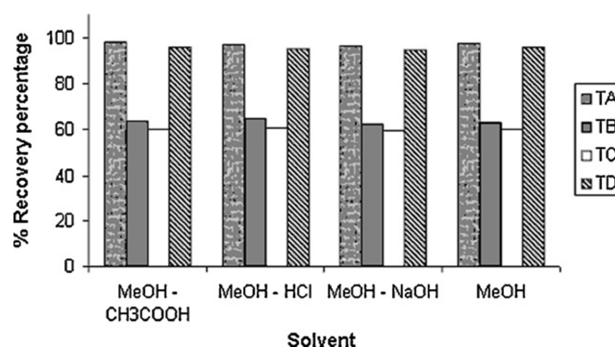


Fig. 4. Percentage recovery (%) obtained for bee larvae samples spiked with TA, TB, TC and TD and using different extraction solvents. The chromatographic and MS/MS conditions are described in detail in Section 2.4 and Table 1.

3.3. LC conditions

The LC conditions used here are based on those previously selected and optimized in a study to separate and analyze TA, TB, TC and TD in honey [27]. The mobile phase flow rate was reduced to 0.5 mL/min as higher flow rates were not suitable for MS/MS, and subsequently, the gradient was slightly modified to maintain the separation between the compounds and the analysis time. Likewise, the length of the last stage of the gradient was also modified to stabilize the baseline for the following analyses. Nevertheless, the overall chromatography run was slightly shorter than that used in the only work where tylosins A, B, C and D have been determined [23], although the matrix was different (water) and three more

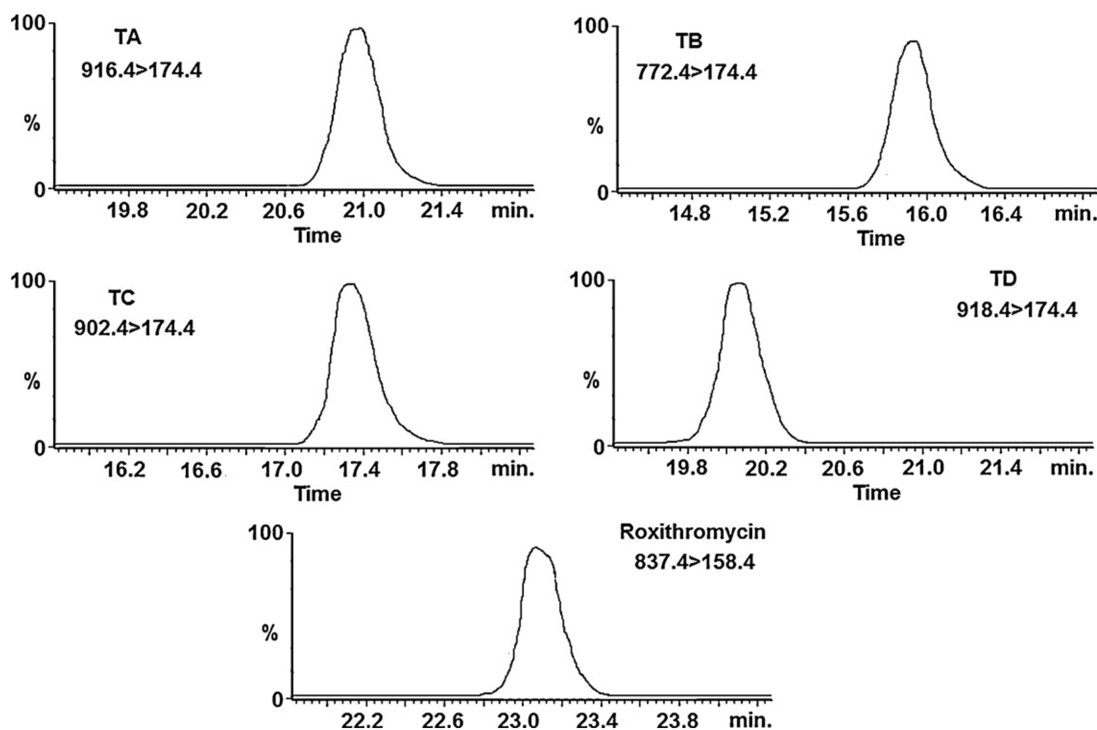


Fig. 5. MRM chromatograms obtained from bee larvae treated with a sample spiked with 150 ng/g of each tylosin and 100 ng/g of roxithromycin (IS). The chromatographic and MS/MS conditions are described in detail in Section 2.4 and Table 1.

Table 4
LOD, LOQ and calibration data values ($n = 5$) obtained for tylosins on spiked bee larvae samples.

Compound	LOD (ng/g)	LOQ (ng/g)	Analytical range (ng/g)	$b \pm S_b$	$a \pm S_a$	$R^2 \pm S_{y/x}$
TA	0.5	1.6	4–500	0.0881 ± 0.0011	−0.1512 ± 0.2165	0.9998 ± 0.2331
			500–2000	0.0397 ± 0.0009	0.0289 ± 0.2917	0.9993 ± 0.3278
TB	1.1	3.5	4–500	0.0719 ± 0.0013	−0.1273 ± 0.1930	0.9991 ± 0.2815
TC	1.3	4.0	4–500	0.0694 ± 0.0012	−0.1196 ± 0.2051	0.9992 ± 0.2092
TD	0.4	1.4	4–500	0.0902 ± 0.0010	−0.1611 ± 0.2279	0.9996 ± 0.2218

$s_{y/x}$: standard deviation of the vertical distances of the points from the line. a: intercept with the y-axis. s_a: standard deviation of the intercept. b: slope of the line. s_b: standard deviation of the slope.

compounds were analyzed. It should be noted that total separation of the four tylosins was achieved, although this was not necessary for their determination due to the use of a MSD-Trap and different MS/MS transitions. Nevertheless, this enables other detectors to be used, such as DAD, which often requires the full separation of the compounds being analyzed, although it is more economical and is usually available in most analytical laboratories. The injection volume was also adjusted after testing several volumes between 10 and 100 μL, and it was set at 50 μL. No significant improvement in the signal to noise ratio was observed with higher injection volumes but rather, a loss of symmetry and peak shape was evident. To check the suitability of these conditions to analyze the tylosins in a different matrix, a spiked larvae bee sample was tested. In this case, the MRM chromatograms obtained (Fig. 5) had good peak shapes that were fully separated. As such, it was not deemed necessary to change any other chromatography parameters.

3.4. MS/MS optimization

To establish the optimal MS/MS conditions for each compound, the individual standard solutions were introduced directly into the MS/MS in the infusion mode in methanol (1 mg/L) using a syringe infusion pump at a flow-rate of 5 μL/min. In ESI-MS, the drugs show intense $[M+H]^+$ on their full-scan spectra. These ions were selected as precursor ions to obtain product ions in MRM mode. To reach maximum sensitivity for each tylosin, a flow injection method, performed by introducing 20 μL of a mixed standard solution of the four analytes (1 mg/L) at 0.5 mL/min into the mass spectrometer in the initial chromatography mobile phase, was used to optimize of the following parameters: drying gas temperature, drying gas flow, nebulizer gas pressure, trap drive, capillary exit, capillary voltage, skimmer, delay time, ion charge control (ICC), fragmentation amplitude and isolation width (the optimal conditions defined are given in Section 2.4).

3.5. Validation of the procedure for LC–MS/MS

To check the selectivity of the method proposed, a set of non-spiked larvae bee samples were injected onto the LC–MS/MS system and there was no interference of the target compounds at the elution times. The LOD and LOQ were determined experimentally for each of the tylosins as indicated in Section 2.8 (summarized in Table 4). Both limits were slightly higher for TB and TC but these values were all still good enough to determine traces of tylosins in bee larvae, and they were lower than those obtained when analysing honey using LC–MS [27] (LOD between 2 and 3 ng/g and LOQ between 6 and 9 ng/g).

It is well known that matrix effects (ion suppression and ion enhancement) can strongly influence the LC–MS analysis due to ionization competition between co-eluting compounds in a chromatography system. Several tests were carried out using standard solutions and matrix matched calibration curves, comparing the MS signals for each tylosin. The area obtained for the same concentrations in both calibration curves ($n = 5$) were approximately 25% lower when analyzing larvae bee samples, which might suggest that ion suppression occurred. To solve this problem, matrix-matched standard curves were employed.

As matrix-matched standard calibration curves were used to quantify macrolides in honey to avoid the effects of the sample treatment, we assessed whether this method was suitable for this new matrix. Larvae samples were spiked with different amounts of TA, TB, TC and TD from 4 to 500 ng/g and from 500 to 2000 ng/g for TA. The graphs obtained were straight lines with an intercept not significantly different from zero ($p < 0.05$), they were linear across the range studied, and the lack of bias was confirmed (Table 4).

Repeatability and intermediate precision experiments were performed (summarized in Table 5), and as can be deduced by analysing the RSD values for both parameters obtained (RSD < 5%), the method was precise. Finally, the recovery and trueness were studied on spiked larvae bee samples at three (TB, TC and TD) and

Table 5
Precision and recovery data.

Compound	Spiking level (ng/g)	Precision studies ($n = 5$)				Recovery studies ($n = 5$)		
		Repeatability		Intermediate precision				
		Measured concentration (ng/g)	RSD (%)	Measured concentration (ng/g)	RSD (%)	Measured concentration (ng/g)	RSD (%)	Recovery (%)
Tylosin A	12	12	1.3	12	2.3	12	1.9	98.1
	204	198	2.1	202	2.0	199	1.8	97.5
	612	604	3.0	601	2.1	599	1.6	97.8
	1814	1801	2.8	1799	2.7	1756	2.1	96.8
Tylosin B	13	8	1.2	8	3.1	8	1.8	61.5
	205	127	1.4	134	2.8	129	2.2	62.9
	619	383	1.3	378	1.6	375	2.3	60.5
Tylosin C	15	9	2.6	9	2.3	9	1.9	62.5
	210	123	1.9	129	2.0	125	2.5	59.6
	593	371	2.1	376	2.2	368	1.9	61.7
Tylosin D	10	10	1.7	10	2.4	10	1.4	98.2
	207	200	1.9	195	2.8	198	2.1	95.6
	609	598	1.3	600	2.1	587	2.5	96.3

Table 6
Concentrations (ng/g) of TA, TB, TC and TD in bee larvae samples from treated beehives.

Day	Sample	TA	TB	TC	TD	Total
1	Placebo	<LOD	<LOD	<LOD	<LOD	<LOD
	1	206	90	46	113	455
	2	450	120	97	163	830
	3	136	83	37	140	396
	4	510	160	70	164	904
3	Placebo	<LOD	<LOD	<LOD	<LOD	<LOD
	1	110	106	33	17	266
	2	200	116	47	100	463
	3	116	110	20	37	283
	4	206	103	37	77	423
5	Placebo	<LOD	<LOD	<LOD	<LOD	<LOD
	1	46	50	13	7	116
	2	73	43	17	10	143
	3	63	23	20	20	126
	4	67	37	27	13	144

<LOD: under limit of detection. Placebo: formulation without tylosin tartrate$_{TP}$. Samples 1, 3: Formulation B (200 mg tylosin tartrate$_{TP}$). Samples 2, 4: Formulation A (400 mg tylosin tartrate$_{TP}$).

Table 7
Mean value (%) obtained for TA, TB, TC and TD in tylosin$_{TP}$ and in the analyzed bee larvae samples ($n = 5$).

Compound	Tylosin$_{TP}$		Samples	
	Mean value	RSD (%)	Mean value	RSD (%)
TA	86.26	0.89	50.44	26.11
TB	7.99	1.13	21.23	57.90
TC	1.80	0.97	11.48	27.54
TD	3.45	1.12	16.84	49.51

four different levels (TA, see above). The percentage recovery of TA and TD ranged between 94 and 98%, and it was close to 60% for TB and TC. The RSD values were also lower than 5% in all cases (Table 5).

3.6. Application of the method

As stated above, the samples analyzed in this study were obtained from three different groups of honey bee colonies in experimental apiaries treated with different formulations as a result of a dose-finding study. These samples of honey bee larvae were analyzed according to the procedure described and validated here, and the results are summarized in Table 6. Residues of the four tylosins were evident in all the larvae bee samples obtained from the colonies treated with tylosin tartrate$_{TP}$. The larvae bee samples obtained on day 1 had more tylosin residues than those obtained on day 3, and in all the cases the lowest amounts were detected in the samples obtained on day 5. The amount of tylosin tartrate$_{TP}$ added to the formulations (A, B) was related to the amount of tylosin detected, although the relation between the tylosin recovered was not the same as that between the amount of tylosin tartrate$_{TP}$ in both formulations, especially on day 5. For example, the amount of total tylosins found on the first day of the assay in samples from beehives treated with Formulation A was practically twice that obtained from those beehives treated with Formulation B, which perfectly reflects the amount of tylosin tartrate$_{TP}$ added initially. This ratio was not maintained on day 3, although there was still a large difference between the concentrations found in the larvae bee samples from beehives treated with the distinct Formulations. By contrast, on the fifth day of the study, there were practically no differences between the larvae samples in terms of tylosin concentration. Indeed, the concentrations of each of the tylosins were not directly related with the formulation employed, especially for days 3 and 5 of the study (Table 6). These differences could be attributed to the different social behaviour of the bee hives in terms of their food storage instinct.

The %RSD values and the proportion of each compound obtained differed after the analysis of tylosin$_{TP}$ standards (as observed in Table 7), possibly due to an influence of the matrix. The data in Tables 6 and 7 also indicate that residues of the four tylosins were present in all the samples from treated honey bee colonies, and that the sum of the percentages of TB, TC and TD was close to 50% of the total tylosin recovered. If we consider the minimum inhibitory doses of tylosin as 0.12 μg/mL (theoretically around 0.36 μg/larvae), both formulations (A and B) gave rise to higher concentrations in the larvae over the period tested. However, the treatment should be repeated weekly to maintain the pharmacological effects if the strains of *P. larvae* are still active, particularly since on day five the concentrations of tylosin were close to the minimum inhibitory dose. These data are in accordance with previous studies on the action of tylosin on *P. larvae* strains in field conditions [14,19]. Nevertheless, although the effectiveness of the treatment has been proven since the minimum inhibitory dose was reached, further field experiments should be performed to optimize the dose, using more beehives and different locations.

4. Conclusions

For the first time a LC–MS/MS protocol has been developed to measure residues of tylosins A, B, C and D in bee larvae. These compounds were seen to be unstable under acidic conditions, while light exposure, under the laboratory working conditions, and temperature do not significantly influence their stability. The validated data demonstrate that this method is consistent and reliable, with low %RSD values, little bias and good recoveries for TA and TD. The limits of quantification for the analytes are suitable to measure tylosin residues in bee larvae.

Residues of TB, TC and TD were found in all the larvae bee samples analyzed, and thus, it is necessary to measure all the tylosins and not only tylosin A to avoid erroneously quantifying tylosin by up to 50%. However, a direct relationship between the quantities of tylosin tartrate$_{TP}$ administered and the amounts of tylosins detected was not observed. Finally, the effectiveness of the treatment was also demonstrated, which must be repeated weekly until the *P. larvae* strain is inactivated.

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