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**SIMULTANEOUS DETERMINATION OF  
LINCOMYCIN AND FIVE MACROLIDE  
ANTIBIOTIC RESIDUES IN HONEY BY LIQUID  
CHROMATOGRAPHY COUPLED TO  
ELECTROSPRAY IONISATION MASS  
SPECTROMETRY**

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Giancarlo Biancotto

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**SIMULTANEOUS DETERMINATION OF LINCOMYCIN AND FIVE MACROLIDE ANTIBIOTIC RESIDUES IN HONEY BY LIQUID CHROMATOGRAPHY COUPLED TO ELECTROSPRAY IONISATION MASS SPECTROMETRY**

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4 **SIMULTANEOUS DETERMINATION OF LINCOMYCIN AND**  
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6 **FIVE MACROLIDE ANTIBIOTIC RESIDUES IN HONEY BY**  
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8 **LIQUID CHROMATOGRAPHY COUPLED TO ELECTROSPRAY**  
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10 **IONISATION MASS SPECTROMETRY**  
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33 A sensitive and specific method based on liquid chromatography-tandem mass  
34  
35 spectrometry (LC/MS/MS), for the simultaneous determination of lincomycin and five  
36  
37 macrolide antibiotics in honey, was developed and validated. The analytes were  
38  
39 extracted with Tris buffer 0.1 M pH 10.5 and cleaned-up by a single solid phase  
40  
41 extraction step on an OASIS HLB column. The chromatographic separation of analytes  
42  
43 was performed on a Synergi Hydro-RP reversed – phase column using a gradient  
44  
45 program of aqueous 0.01 M ammonium acetate pH 3.5 and acetonitrile as the mobile  
46  
47 phase, at a flow rate 0.25 ml min<sup>-1</sup>. Detection of analytes was achieved by positive  
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3 ionisation electrospray in multiple reaction monitoring (MRM) mode. Two  
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5 characteristic transitions were monitored for each substance. Validation was carried out  
6  
7 according to the guidelines laid down by Commission Decision 2002/657/EC to  
8  
9 establish linearity, specificity, decision limit ( $Cc\alpha$ ), detection capability ( $Cc\beta$ ),  
10  
11 repeatability, within-laboratory reproducibility, recovery and ruggedness.  
12

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15 **Keywords:** lincomycin, macrolides, honey, validation, LC/MS/MS  
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## Introduction

American foulbrood (AFB) is one of the most important and destructive diseases of honey bees (*Apis mellifera*) and is caused by the ingestion of the spores of a Gram + bacterium *Paenibacillus larvae* subsp. *larvae* (Shimauki 1997). The spores are highly resistant to heat, desiccation, and chemical disinfectants (Hansen *et al.* 1999) and can remain dormant for many years in beehive, beekeeping equipment, honey and wax. This increases the risk that unwitting manipulation by the beekeeper may transfer AFB spores to new bees-colonies. For this reason AFB has traditionally been controlled by burning, scorching or irradiating contaminated beekeeping equipment (Elzen *et al.* 2002). In order to lessen this financial loss, sulphonamides and antibiotics are used worldwide in apicultural practices to suppress clinical symptoms of AFB, even if these substances have a transient effectiveness and are totally ineffective against the spores of *Paenibacillus larvae* subsp. *larvae* (Kochansky *et al.* 2001, Feldlaufer *et al.* 2001, Mutinelli 2003). Home-made uncontrolled treatments with these drugs increase the risk that residues can be present in beehive products (mainly honey) and that undesired effects on consumers like allergic reactions or bacterial resistance can potentially occur. To avoid these consequences, European Community legislation (Council Directive 96/23/EC) on residues in animal-origin food, considers honey like any other food product, but, differently from the others, in this case even minor traces of antibiotics are not tolerated, since no legal limit (MRL) have been established for any antibiotic drug in honey matrix. As a consequence, analytical methods intended for the detection of residues of antibacterial drug in honey at trace level should be developed and applied to routine samples.

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5 The use of tetracyclines and sulphonamides in beekeeping is known since 1940 (Eckert  
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7 1947, Katznelson *et al.* 1949, Gochnauer 1951, Katznelson *et al.* 1952), and many  
8  
9 analytical methods have been developed to detect the presence of their residues in  
10  
11 honey matrix (Horie *et al.* 1992, Kaufmann *et al.* 2002, Vinas *et al.* 2004, Oka *et al.*  
12  
13 1994), but very little information are available regarding methods for detecting other  
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15 antibiotic drugs like lincomycin, and some macrolide antibiotics that seem to be  
16  
17 increasingly used in beekeeping for the prevention and treatment of brood diseases  
18  
19 (Feldlaufer *et al.* 2001, Kochansky *et al.* 2001, Hitchcock *et al.* 1970, Moffett *et al.*  
20  
21 1970, Peng *et al.* 1996). In fact, currently, there are a few analytical methods reported  
22  
23 for the determination of lincomycin and macrolides in honey and no one for the  
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25 simultaneous determination of these drugs (Thompson *et al.* 2003, Wang 2004, Benetti  
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27 *et al.* 2004).

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31 Therefore, in absence of maximum residue levels in honey, the aim of this work was the  
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33 development of a multi-residue method for the simultaneous detection and confirmation  
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35 of lincomycin and five macrolides (tylosin, erythromycin, tilmicosin, spiramycin and  
36  
37 josamycin) in honey at trace levels using roxithromycin as internal standard. Due to the  
38  
39 complexity of honey matrix the analytical approach was based on a combination of  
40  
41 liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) technique. The  
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43 following analytical parameters of the method were validated according to the  
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45 guidelines laid down by Commission Decision 2002/657/EC using an in-house  
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47 validation: linearity of the standard response both in solvent and in matrix extracts,  
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3 specificity, recovery, repeatability, decision limit ( $CC\alpha$ ), detection capability ( $CC\beta$ ) and  
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5 ruggedness.  
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## 8 9 **Materials and methods**

### 10 *Materials and reagents*

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12 Ammonium acetate, tris(hydroxymethyl)-aminomethane (TRIS) and 30% aqueous  
13 ammonia solution (Sigma, Milan, Italy) were of the highest available purity grade.  
14  
15 Acetonitrile, ethanol, methanol (Carlo Erba, Milan, Italy) were all HPLC grade.  
16  
17 Distilled water was de-ionised by a Milli-Q apparatus from Millipore. Tylosin (TYLO),  
18 spiramycin (SPIRA), erythromycin (ERYTRO), lincomycin (LINCO) and  
19 roxithromycin (ROXI) were purchased from Sigma (Milan, Italy), josamycin (JOSA)  
20 was purchased from Fluka (Milan, Italy) and tilmicosin (TILMI) was purchased from  
21 Eli Lilly (Sesto Fiorentino FI, Italy). A stock solution of  $1000 \text{ mg kg}^{-1}$  for each drug  
22 was prepared by dissolving the appropriate amount of each substance in ethanol (for  
23 TYLO, TILMI, SPIRA, JOSA, ERYTRO) and in methanol (for LINCO). The  
24 appropriate working standard solutions for fortification and external calibration curves  
25 were prepared daily by diluting the stock solution with the reconstitution phase. The  
26 composition of reconstitution phase is ammonium acetate 0.01 M pH 3.5 – acetonitrile  
27 70/30 v/v.  
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29 Samples clean-up was achieved by solid phase extraction (SPE) on OASIS HLB  
30 columns (3 ml / 60 mg) (Waters, Milan, Italy).  
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3 *Fortified samples*

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5 Negative honey samples (5 g) were fortified at 0.2, 0.4, 0.6  $\mu\text{g kg}^{-1}$  of TYLO, TILMI,  
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7 ERYTRO, JOSA, LINCO and 1.6, 3.2, 4.8  $\mu\text{g kg}^{-1}$  of SPIRA.  
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10  
11 *Sample extraction and clean - up*

12  
13 Honey samples were processed according to the following procedure: 5 g of honey  
14  
15 were weighed in a 50 ml-tube and diluted with 35 ml of TRIS buffer (0.1 M, pH 10.5).  
16

17 Then the raw extract was purified and concentrated by OASIS HLB columns (3 ml / 60  
18 mg) (Waters, Milan, Italy). The cartridges were conditioned sequentially with methanol  
19  
20 (10 ml) and water (10 ml). The sample extracts were quantitatively loaded into the SPE  
21  
22 cartridges. The cartridges were washed with 20 ml of a solution of methanol – water (5  
23  
24 – 95, v/v), then the OASIS columns were vacuum – dried for 5 minutes. Finally the  
25  
26 analytes were eluted with 10 ml of a solution of methanol – 30% ammonia (95 – 5, v/v).  
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29 A 100  $\mu\text{l}$  volume of a working solution of ROXI ( $1\text{mg l}^{-1}$ ) was added to each eluate: this  
30  
31 compound is added at this point as internal standard (IS) to compensate the variation in  
32  
33 ionisation efficiency of ESI source during HPLC-MS/MS analysis. The eluates were  
34  
35 then dried under nitrogen stream at 50°C and redissolved in 1 ml of reconstitution-phase  
36  
37 and transferred into vials, 10  $\mu\text{l}$  were injected into the HPLC-MS/MS system.  
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### LC/MS/MS

The HPLC equipment was an Alliance 2695 quaternary solvent delivery system with column heater module and cooling device (Waters, Milford, MA, USA). The chromatographic separations were performed using a Synergi Hydro-RP (150 x 2.0 mm I.D. 4  $\mu$ m) reversed – phase column from Phenomenex maintained at 30°C. The mobile phase consisted of two solutions: A (aqueous 0.01 M ammonium acetate pH 3.5) and B (acetonitrile); the gradient program was: 0-2 min 0 % B, 2-16 min 90% B, 16-18 min 90% B, 18-19 min 0% B, 19-25 min 0% B. The flow rate of mobile phase was 0.25 ml min<sup>-1</sup>. In these conditions the elution profile was in the following order: LINCO (tr = 9.9), SPIRA (tr = 11.4), TILMI (tr = 12.2), ERYTRO (tr = 13.0), TYLO (tr = 13.3), ROXY (tr = 14.2), JOSA (tr = 14.9).

The MS/MS system used was a Quattro Ultima II triple quadrupole mass spectrometer (Micromass, Altrincham, UK), equipped with electrospray ionisation (ESI) ion source. The analysis was performed in the positive ion mode. Collision energies were optimised to choose the best product ions for monitoring each molecule in multiple reaction monitoring (MRM). The chosen MS/MS transitions were listed in table 1.

[Insert Table 1 about here]

### *Calibration and quantification*

To avoid possible variability of the instrument response due to matrix effects, all analytes were quantified by calibration curves prepared daily by processing blank honey samples. The final evaporated extracts were spiked with a mixture of drugs at concentration levels corresponding to 0.1, 0.2, 0.4, 0.6 and 2  $\mu$ g kg<sup>-1</sup> of TYLO, TILMI,

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3 ERYTRO, JOSA, LINCO and 0.8, 1.6, 3.2, 4.8, 16  $\mu\text{g kg}^{-1}$  of SPIRA on honey samples  
4 and with a small volume (100  $\mu\text{l}$ ) of ROXI working standard solution (1  $\text{mg l}^{-1}$ ) used as  
5 internal standard (IS). After stirring, these solutions were evaporated to dryness under  
6 nitrogen stream at 50°C and were re-dissolved in 1 ml of reconstitution phase to be  
7 transferred into vial.  
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### 13 14 15 **Results and discussion**

16  
17 This paper describes the validation procedure of a LC/MS/MS method according to the  
18 guidelines laid down by the Commission Decision 2002/657/EC for the simultaneous  
19 identification and quantification of LINCO and five macrolides (TYLO, TILMI,  
20 ERYTRO, JOSA, SPIRA) in honey. For each compound, all parameters considered in  
21 the validation study were assessed on the basis of the highest intense transition ion, a  
22 part from the analytical limits ( $C_{c\alpha}$  and  $C_{c\beta}$ ) assessed on the basis of the less intense  
23 transition ion.  
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33 The analyte responses were always related to the internal standard response: in the  
34 absence of isotope – labelled form of the considered analytes, particularly suited for MS  
35 detection, ROXI, a macrolide not used in veterinary medicine, was used as the internal  
36 standard added at the end of sample preparation before the LC/MS/MS analysis to avoid  
37 response variability due to variation in the ionisation yield. Figure 1 and figure 2 shows  
38 typical LC/ESI/MS/MS chromatograms of the mixture of standard solution at 0.2  $\mu\text{g kg}^{-1}$   
39 of TYLO, TILMI, ERYTRO, JOSA, LINCO and at 1.6  $\mu\text{g kg}^{-1}$  of SPIRA (in the left  
40 side), a blank honey extract (in the medium) and a fortified honey sample containing 0.2  
41  $\mu\text{g kg}^{-1}$  of TYLO, TILMI, ERYTRO, JOSA, LINCO and 1.6  $\mu\text{g kg}^{-1}$  of SPIRA  
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3 submitted to extraction and clean-up procedure (in the right side). Figure 1 shows  
4 chromatograms obtained by monitoring the quantification transitions, figure 2 shows  
5 chromatograms obtained by monitoring the confirmation transitions.  
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11 [Insert Figure 1 about here]

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13 [Insert Figure 2 about here]

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17 *Linearity of response*

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19 The linearity of the LC/MS/MS response was demonstrated with solvent standard  
20 solutions at 6 calibration levels in the concentration range 0.10 – 2.0  $\mu\text{g kg}^{-1}$  for TYLO,  
21  
22  
23 TILMI, ERYTRO, JOSA, LINCO and in the concentration range 0.80 – 16.0  $\mu\text{g kg}^{-1}$  for  
24  
25 SPIRA. The correlation coefficients ( $R^2$ ) of the standard curves in solvent were at least  
26  
27 0.999 for all analytes considered. The linearity of the LC/MS/MS response was proved  
28  
29 also with standard solutions in matrix extracts to take into account an ion enhancement  
30  
31 matrix effect. The correlation coefficients ( $R^2$ ) of these standard curves were at least  
32  
33 0.995.  
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36  
37 *Specificity*

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39 Specificity of the LC/MS/MS method was established by processing and analysing 20  
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41 different control honey samples (10 wildflower 5 robinia, 5 chestnut). No interference  
42  
43 peaks were found around the retention time of analytes in the matrices under  
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45 investigation. Moreover, blank honey extracts were spiked with a relevant concentration  
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47 of tetracycline, sulphonamides and amphenicols (other substances that may occur in  
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49 honey like residues of chemical treatments to prevent AFB) but no one interfere with  
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3 the identification and quantification of TYLO, TILMI, ERYTRO, JOSA, LINCO and  
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5 SPIRA.  
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9 *Decision limit (CC $\alpha$ ) and detection capability (CC $\beta$ )*  
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11 In the 2002/657/EC European decision CC $\alpha$  and CC $\beta$  replace the limit of detection and  
12 the limit of quantification. These parameters were determined, at first, by the calibration  
13 curve procedure according to the ISO 11843-2. As recommended by the ISO the  
14 number of reference states  $I$  (number of blank honey samples fortified at equidistant  
15 steps with the considered analytes) used in the calibration experiment were 4 (including  
16 the value zero represented by a blank honey sample); the number of preparations  $J$  for  
17 each reference state was 2; the number of repeated measurements  $L$  performed per  
18 preparation was 2. Data collected were elaborated following the experimental design,  
19 indicated in the ISO 11843-2 Part 2, based on the assumptions that the obtained  
20 calibration function is linear and that the standard deviation is linearly dependent on the  
21 net state variable. The general equation is:  
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$$33 Y_{ij} = a + bx_i + \varepsilon_{ij}$$

34 where  
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36

37  $x_i$  is the symbol for the net state variable in state  $i$ ,  
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40  $Y_{ij}$  is the response variable  
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42  $\varepsilon_{ij}$  are random variables which describe the random component of sampling, preparation  
43 and measurement error.  
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45 Results are listed in table 2.  
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47 [Insert Table 2 about here]  
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3 The extrapolated theoretical values for  $CC\alpha$  and  $CC\beta$ , estimated by accurate application  
4 of ISO 11843 part 2 annex B.2, corresponded to very low concentration values. Blank  
5 honey samples were spiked at these  $CC\alpha$  levels and processed according to the  
6 described procedure, but the method was not able to detect or properly identify the  
7 analytes. This is not unusual and has already been described in other publications  
8 (Jülicher *et al.* 1998, Poltzer *et al.* 2001). For this reason another approach,  
9 recommended by SANCO/2004/2726 was used to estimate them: by parallel  
10 extrapolation to x axis at the lowest spiking level ( $0.2 \mu\text{g kg}^{-1}$  for TYLO, TILMI,  
11 ERYTRO, JOSA, LINCO and  $1.6 \mu\text{g kg}^{-1}$  for SPIRA) and by taking into account the  
12 method inter-day repeatability, estimated during recovery and repeatability studies.  
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24 More in detail the decision limit was calculated according to the following equation:

$$25 \quad CC\alpha = C_{(mrpl)} + 2.33 S_{c(mrpl)}$$

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28 Where:

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30 mrpl is the minimum required performance *level* (which is not the Minimum Required  
31 Performance *Limit*) as defined in SANCO/2004/2726

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33  
34  $S_{c(mrpl)}$  is the standard deviation of whole series of recalculated concentrations at the  
35 mrpl over the total number of replicates ( $n=18$ );  
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40 The minimum required performance level (for each compound) was chosen during the  
41 pre-validation studies as the lowest tested concentration at which the method fulfils the  
42 recommended identification criteria (4 identification points).  
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46 Results are listed in table 3.

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48 [Insert Table 3 about here]  
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3 *Recovery and intra/inter-repeatability*  
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5 The absolute recovery and intra and inter-day repeatability of the method were assessed  
6 by spiking three different series of 18 blank honey at 0.2, 0.4, 0.6  $\mu\text{g kg}^{-1}$  for TYLO,  
7 TILMI, ERYTRO, JOSA, LINCO and 1.6, 3.2, 4.8  $\mu\text{g kg}^{-1}$  for SPIRA (six replicates for  
8 each concentration level). Each series was processed in different days by different  
9 operators using different batches of reagents and solutions. The results are summarised  
10 in table 3.  
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17 *Ruggedness*

18 Ruggedness is the characteristic of an analytical method when minor variations in the  
19 procedure produce no significant effects in its performance. The ruggedness test was  
20 conducted by the Youden procedure (Youden *et al.* 1975) cited by Commission  
21 Decision 2002/657/EC. Seven variables were chosen and deliberately altered: the  
22 volume of dilution buffer (TRIS), the pH and molarity of dilution buffer, the methanol  
23 percentage during the washing steps of the SPE purification, the ammonia solution  
24 percentage in elution solvent, the SPE elution volume and the evaporation temperature  
25 of solvents in the final extract.  
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37 As shown in table 4, eight experiments were conducted for the evaluation of seven  
38 selected factors by spiking eight blank honey samples with TYLO, TILMI, ERYTRO,  
39 JOSA, LINCO at 0.2  $\mu\text{g kg}^{-1}$  and SPIRA at 1.6  $\mu\text{g kg}^{-1}$  (first fortification levels used in  
40 the recovery and intra/inter-repeatability study).  
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48 The effect of a particular variable was estimated by subtracting the mean result obtained  
49 with the variable at “high level” indicated by capital letter, from the mean result  
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3 achieved with it at “low level”, marked with the corresponding small letter. For each  
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5 variable, the calculated difference was indicated as  $D_i$ . The standard deviation of the  
6  
7 differences ( $S_{D_i}$ ), was calculated by the formula:  
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9

$$S_{D_i} = \sqrt{2 \times \sum \left( \frac{D_i^2}{7} \right)}$$

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17 When  $S_{D_i}$  is significantly larger than the standard deviation of the method, it means that  
18  
19 the combination of all the chosen factors have an effect on the analytical result. Besides,  
20  
21 by means of a  $t$ -test it is possible to evaluate the influence of each investigated factor  
22  
23 (Vander Heyden *et al.* 1995, Scortichini *et al.* 2005). The experimental  $t$  is given by:  
24  
25

$$t = \frac{\sqrt{n} \times |D_i|}{\sqrt{2} \times S.D.}$$

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33 where  $n$  ( $n = 4$ ) is the number of experiments carried out at each level for each  
34  
35 parameter and  $S.D.$  is the estimate of the method precision obtained from the analysis of  
36  
37 18 spiked samples at the first spiking level during the validation study.

38  
39 The obtained  $t$ -value, for all seven variables, was compared with the 2-tailed  $t$ -critical  
40  
41 value ( $t_{crit}$ ) for  $N-1$  degrees of freedom, where  $N$  ( $N=18$ ) is the number of  
42  
43 determinations used in the estimation of  $S.D.$  at 95% confidence level. If  $t$  is greater than  
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45  $t_{crit}$  the investigated variable shows a significant influence and the method is not  
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47 sufficiently robust against the chosen modification.  
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3 The results in table 5, obtained following the experimental plan showed in table 4,  
4 indicate that the method is robust and minor but still significant fluctuations in the  
5 operative parameters that can occur during the routine application of the method have  
6 not significantly effect in its performance characteristics. In fact the experimental  $t$   
7 values resulted below the 2-tailed  $t$ -critical value for all seven factors:  $t_{crit} = 2,11$  for 17  
8 degrees of freedom at 95% confidence level.  
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19 [Insert Table 4 about here]  
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21 [Insert Table 5 about here]  
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## 25 **Conclusions**

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27 The test carried out to verify the performances of the method demonstrated that it is  
28 extremely sensitive (with a detection capability  $CC\beta \leq 0.26 \mu\text{g kg}^{-1}$  for TYLO, TILMI,  
29 ERYTRO, JOSA, LINCO and  $CC\beta < 2.1 \mu\text{g kg}^{-1}$  for SPIRA), accurate (mean absolute  
30 recoveries  $> 84\%$  with  $CV\% < 12\%$  for all analytes) and specific (no endogenous  
31 interfering substances or interferences due to other possible antibiotics used in  
32 beekeeping) also in within-laboratory reproducibility conditions. The Youden  
33 ruggedness test showed that the method is fairly robust: minor fluctuations in some  
34 potential critical operative parameters, that can occur during the routine application of  
35 the method over a long period of time, do not significantly affect its performance  
36 characteristics. Furthermore, since the method requires only a simple extraction and a  
37 single clean up step, up to 24 honey samples per day can be processed.  
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3 Some recent reports of the mission carried out in the European countries by FVO to  
4 evaluate the control of residues in live animals and animal products  
5 (DG(SANCO)/3389/2001, DG(SANCO)7504/2005) and some notifications reported by  
6 the Rapid Alert System for Food and Feed (RASFF) in 2004 (RASFF Annual Report,  
7 2004) indicate the potential use of some of these substances in beekeeping. Therefore a  
8 validated method for the detection and identification of these analytes is required and  
9 this need is fulfilled by the method reported in this paper.  
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Analytical Chemists, Arlington Va

Multiple reaction monitoring parameters					
Compound	Quantification transition	Cone voltage (V)	Collision energy (eV)	Confirmation transition	Collision energy (eV)
LINCO	407.0 > 126.0	44	29	407.0 > 358.9	19
SPIRA	843.4 > 174.0	40	35	843.4 > 317.8	30
TILMI	869.7 > 174.0	42	45	869.7 > 696.4	42
ERYTRO	734.1 > 158.0	40	32	734.1 > 576.0	20
TYLO	916.2 > 174.0	40	40	916.2 > 772.1	32
JOSA	828.4 > 174.0	38	32	828.4 > 600.2	28
ROXI	837.2 > 679.3	40	24		

Table 1

Level ( <i>I</i> )	Reference state ( $x_i$ )	LINCO				TILMI			
		Preparation ( <i>J</i> ) Measurements ( <i>L</i> )				Preparation ( <i>J</i> ) Measurements ( <i>L</i> )			
<i>I</i> = 4	$\mu\text{g kg}^{-1}$	<i>J</i> = 1		<i>J</i> = 2		<i>J</i> = 1		<i>J</i> = 2	
		<i>L</i> = 1	<i>L</i> = 2	<i>L</i> = 1	<i>L</i> = 2	<i>L</i> = 1	<i>L</i> = 2	<i>L</i> = 1	<i>L</i> = 2
1	0	0,0149	0,0089	0,0111	0,0093	0,004	0,004	0,005	0,002
2	0.2	0,0475	0,0574	0,0505	0,0557	0,344	0,359	0,391	0,376
3	0.25	0,0585	0,0587	0,0611	0,0649	0,426	0,437	0,423	0,418
4	0.3	0,0798	0,0767	0,0710	0,0748	0,501	0,529	0,507	0,545
<b>Cc<math>\alpha</math></b>		<b>0.018</b>				<b>0.0027</b>			
<b>Cc<math>\beta</math></b>		<b>0.032</b>				<b>0.0049</b>			
Level ( <i>I</i> )	Reference state ( $x_i$ )	ERYTRO				TYLO			
		Preparation ( <i>J</i> ) Measurements ( <i>L</i> )				Preparation ( <i>J</i> ) Measurements ( <i>L</i> )			
<i>I</i> = 4	$\mu\text{g kg}^{-1}$	<i>J</i> = 1		<i>J</i> = 2		<i>J</i> = 1		<i>J</i> = 2	
		<i>L</i> = 1	<i>L</i> = 2	<i>L</i> = 1	<i>L</i> = 2	<i>L</i> = 1	<i>L</i> = 2	<i>L</i> = 1	<i>L</i> = 2
1	0	0,008	0,011	0,008	0,005	0,007	0,004	0,004	0,003
2	0.2	0,187	0,183	0,197	0,200	0,087	0,094	0,112	0,104
3	0.25	0,230	0,218	0,232	0,216	0,100	0,110	0,128	0,122
4	0.3	0,279	0,280	0,255	0,277	0,142	0,143	0,148	0,154
<b>Cc<math>\alpha</math></b>		<b>0.008</b>				<b>0.009</b>			
<b>Cc<math>\beta</math></b>		<b>0.014</b>				<b>0.020</b>			
Level ( <i>I</i> )	Reference state ( $x_i$ )	JOSA				SPIRA			
		Preparation ( <i>J</i> ) Measurements ( <i>L</i> )				Preparation ( <i>J</i> ) Measurements ( <i>L</i> )			
<i>I</i> = 4	$\mu\text{g kg}^{-1}$	<i>J</i> = 1		<i>J</i> = 2		<i>J</i> = 1		<i>J</i> = 2	
		<i>L</i> = 1	<i>L</i> = 2	<i>L</i> = 1	<i>L</i> = 2	<i>L</i> = 1	<i>L</i> = 2	<i>L</i> = 1	<i>L</i> = 2
1	0/0	0,003	0,003	0,005	0,004	0,004	0,004	0,003	0,011
2	0.2/1.6	0,085	0,080	0,104	0,100	0,134	0,138	0,130	0,137
3	0.25/2	0,109	0,139	0,122	0,123	0,154	0,163	0,153	0,159
4	0.3/2.4	0,146	0,146	0,148	0,166	0,179	0,182	0,175	0,196
<b>Cc<math>\alpha</math></b>		<b>0.011</b>				<b>0.15</b>			
<b>Cc<math>\beta</math></b>		<b>0.020</b>				<b>0.25</b>			

Table 2



Intra-day and inter-day repeatability for the determination of lincomycin and macrolides in fortified honey samples														
Compound	Spike level ( $\mu\text{g kg}^{-1}$ )	Day 1 (n=6)			Day 2 (n=6)			Day 3 (n=6)			Inter-day (n=18)			
		Mean found ( $\mu\text{g kg}^{-1}$ )	R.S.D. (%)	Recovery (%)	Mean found ( $\mu\text{g kg}^{-1}$ )	R.S.D. (%)	Recovery (%)	Mean found ( $\mu\text{g kg}^{-1}$ )	R.S.D. (%)	Recovery (%)	Mean found ( $\mu\text{g kg}^{-1}$ )	R.S.D. (%)	Recovery (%)	
LINCO	0.2	0.19	12.1	93.5	0.19	9.1	96.1	0.20	3.2	101.1	0.19	8.5	96.9	
	0.4	0.39	7.1	96.0	0.41	5.5	102.2	0.41	5.3	102.7	0.40	6.3	100.3	
	0.6	0.62	3.9	102.2	0.61	7.8	102.2	0.61	3.9	100.9	0.61	5.2	101.8	
	Cc $\alpha$	0.23 $\mu\text{g kg}^{-1}$												
	Cc $\beta$	0.26 $\mu\text{g kg}^{-1}$												
SPIRA	1.6	1.46	6.0	91.2	1.41	10.8	88.1	1.58	2.9	99.0	1.48	8.4	92.8	
	3.2	2.99	4.3	93.5	2.85	12.0	89.0	3.02	2.1	94.5	2.95	7.3	92.3	
	4.8	4.24	4.5	88.3	4.29	3.1	89.3	4.27	6.0	88.9	4.26	4.5	88.8	
	Cc $\alpha$	1.9 $\mu\text{g kg}^{-1}$												
	Cc $\beta$	2.1 $\mu\text{g kg}^{-1}$												
TILMI	0.2	0.15	12.0	76.7	0.19	6.4	93.2	0.18	5.7	91.1	0.17	11.5	87.0	
	0.4	0.34	9.6	85.3	0.39	9.9	96.5	0.37	5.4	93.0	0.37	9.6	91.6	
	0.6	0.51	6.5	84.8	0.57	3.8	95.4	0.57	4.8	95.2	0.55	7.3	91.8	
	Cc $\alpha$	0.23 $\mu\text{g kg}^{-1}$												
	Cc $\beta$	0.25 $\mu\text{g kg}^{-1}$												
ERYTRO	0.2	0.17	10.0	84.4	0.19	3.9	95.7	0.19	3.5	94.8	0.18	8.2	91.6	
	0.4	0.35	8.6	87.1	0.36	1.4	90.2	0.40	4.1	99.2	0.37	7.6	92.2	
	0.6	0.54	3.4	90.7	0.51	2.9	85.6	0.55	5.8	91.5	0.54	5.0	89.3	
	Cc $\alpha$	0.22 $\mu\text{g kg}^{-1}$												
	Cc $\beta$	0.24 $\mu\text{g kg}^{-1}$												
TYLO	0.2	0.18	6.0	90.4	0.17	3.5	86.3	0.17	5.9	85.3	0.17	5.6	87.3	
	0.4	0.37	5.1	91.7	0.36	6.2	89.0	0.34	3.4	86.2	0.36	5.4	89.0	
	0.6	0.51	6.5	84.7	0.55	5.2	91.3	0.53	3.3	87.9	0.53	5.8	88.0	
	Cc $\alpha$	0.22 $\mu\text{g kg}^{-1}$												
	Cc $\beta$	0.24 $\mu\text{g kg}^{-1}$												
JOSA	0.2	0.16	6.2	80.2	0.16	11.8	82.4	0.20	3.0	97.8	0.17	11.6	86.8	
	0.4	0.33	3.5	82.3	0.33	8.3	83.6	0.38	1.8	94.8	0.35	8.2	86.9	
	0.6	0.49	3.0	82.3	0.50	1.6	83.2	0.52	2.4	87.0	0.51	3.4	84.2	
	Cc $\alpha$	0.24 $\mu\text{g kg}^{-1}$												
	Cc $\beta$	0.26 $\mu\text{g kg}^{-1}$												

Table 3

Selected variables	Units	Abbreviation <sup>a</sup>	High level	Low level
Volume TRIS Buffer	ml	A,a	40	30
Molarity TRIS Buffer	M	B,b	0.11	0.09
pH TRIS Buffer	pH	C,c	11.5	9.5
%MeOH in SPE washing solution	%	D,d	5.5	4.5
%NH <sub>3</sub> in SPE elution solvent	%	E,e	5.5	4.5
SPE elution volume	ml	F,f	11	9
Final extract evaporation temperature	°C	G,g	55	45

<sup>a</sup>Upper case letter represents high level, lower case letter represents low level value of variable according to the experiment design for ruggedness studies.

Table 4

Variable	LINCO		SPIRA		TILMI		ERYTRO		TYLO		JOSA	
	Difference (Di) in % recovery absolute value	t-value	Difference (Di) in % recovery absolute value	t-value	Difference (Di) in % recovery absolute value	t-value	Difference (Di) in % recovery absolute value	t-value	Difference (Di) in % recovery absolute value	t-value	Difference (Di) in % recovery absolute value	t-value
Volume												
TRIS Buffer	0.50	0.09	11.0	1.79	1.88	0.26	5.00	0.94	1.38	0.40	3.38	0.47
Molarity												
TRIS Buffer	1.75	0.30	5.92	0.97	1.38	0.19	4.00	0.75	2.13	0.61	3.13	0.44
pH												
TRIS Buffer	3.00	0.52	1.48	0.24	1.12	0.16	0.25	0.05	3.13	0.90	1.38	0.19
%MeOH in SPE washing solution	0.75	0.13	4.86	0.80	0.12	0.02	6.00	1.13	0.38	0.11	0.88	0.12
%NH <sub>3</sub> in SPE elution solvent	1.00	0.17	2.14	0.35	3.12	0.44	0.75	0.14	2.13	0.61	3.13	0.44
SPE elution volume	0.25	0.04	0.27	0.04	2.62	0.37	1.75	0.33	0.38	0.11	0.38	0.05
Final extract evaporation temperature	1.25	0.22	2.67	0.44	0.88	0.12	0.75	0.14	2.38	0.69	0.13	0.02

Table 5

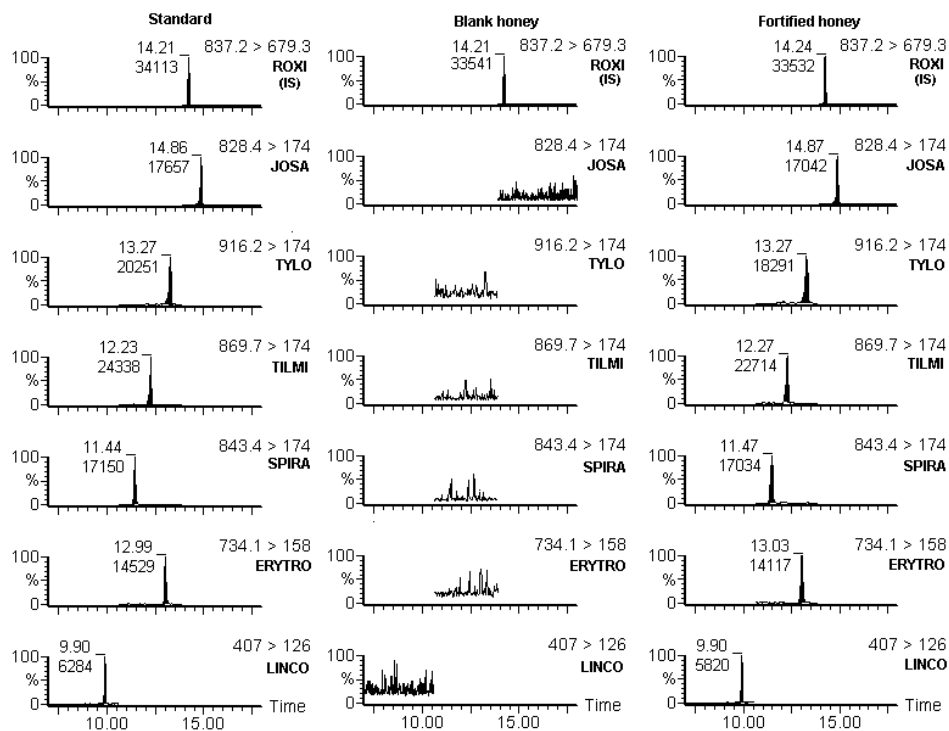


Figure 1

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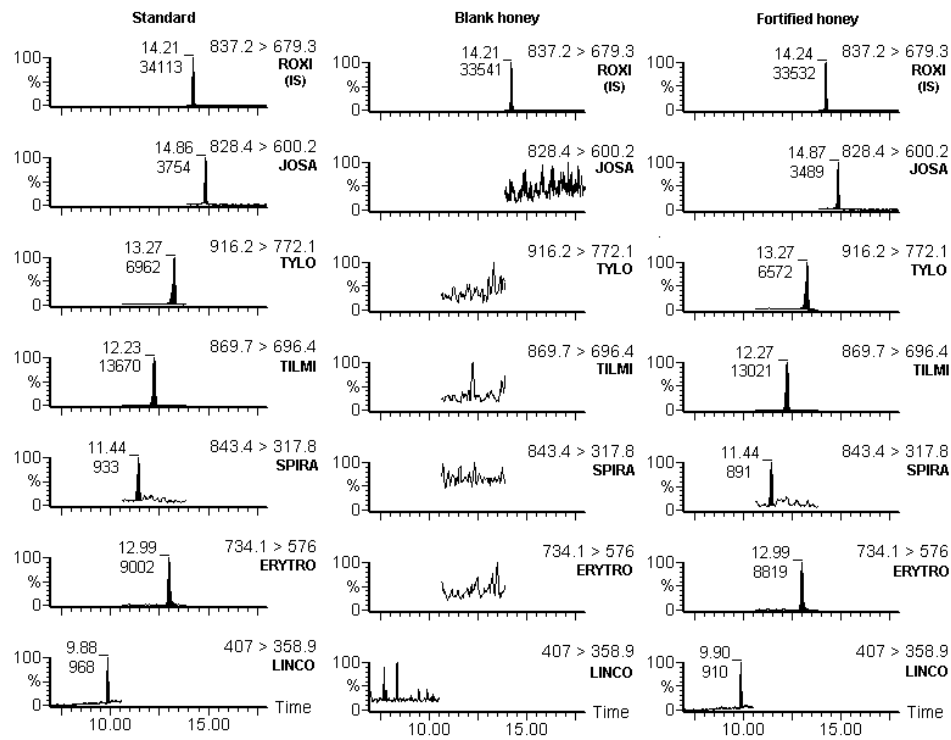


Figure 2

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Table 1: MS/MS conditions for detection, quantification and confirmation of TYLO, TILMI, ERYTRO, JOSA, LINCO and SPIRA in MRM mode.

Table 2:  $CC\alpha$  and  $CC\beta$  of analytes calculated by calibration curve procedure according to the ISO 11843-2. Values reported for each reference state correspond to the ratio between the area of each analyte and the area of the internal standard.

Table 3: validation data for honey spiked samples ( $n = 6$  of each fortification level on each day, three series on three different days)

Table 4: Variables and their levels in the Youden robustness test experimental design (see table 11 of Commission Decision 2002/657/EC)

Table 5: Ruggedness test results

Figure 1: HPLC-ESI-MS/MS chromatograms of a reference standard solution ( $0.2 \mu\text{g kg}^{-1}$  of TYLO, TILMI, ERYTRO, JOSA; LINCO and  $1.6 \mu\text{g kg}^{-1}$  of SPIRA, left side), a blank honey extract (in the medium) and a fortified honey sample at  $0.2 \mu\text{g kg}^{-1}$  of TYLO, TILMI, ERYTRO, JOSA; LINCO and  $1.6 \mu\text{g kg}^{-1}$  of SPIRA (right side).  
Quantification transitions.

Figure 2: HPLC-ESI-MS/MS chromatograms of a reference standard solution ( $0.2 \mu\text{g kg}^{-1}$  of TYLO, TILMI, ERYTRO, JOSA; LINCO and  $1.6 \mu\text{g kg}^{-1}$  of SPIRA, left side), a

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3 *blank honey extract (in the medium) and a fortified honey sample at 0.2  $\mu\text{g kg}^{-1}$  of*  
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5 *TYLO, TILMI, ERYTRO, JOSA; LINCO and 1.6  $\mu\text{g kg}^{-1}$  of SPIRA (right side).*  
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7 *Confirmation transitions.*  
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Precursor ion	Product ion	Dwell (secs)	Cone Voltage (V)	Collision Energy (eV)
916.2	772.1	0.3	40	32
916.2	598.2	0.3	40	35
916.2	174	0.3	40	40

Roxithromycin

Precursor ion	Product ion	Dwell (secs)	Cone Voltage (V)	Collision Energy (eV)
837.2	158	0.3	40	34



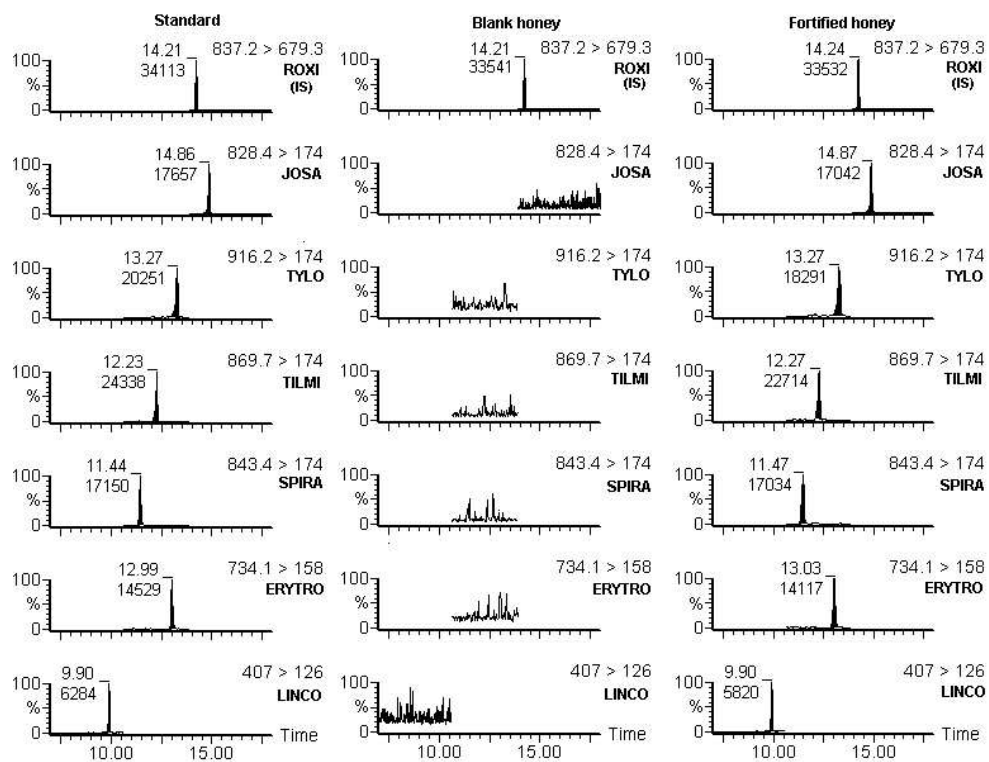


Figure 1: HPLC-ESI-MS/MS chromatograms of a reference standard solution (left side), a blank honey extract (in the medium) and a fortified honey sample submitted to extraction and clean-up procedure (right side)

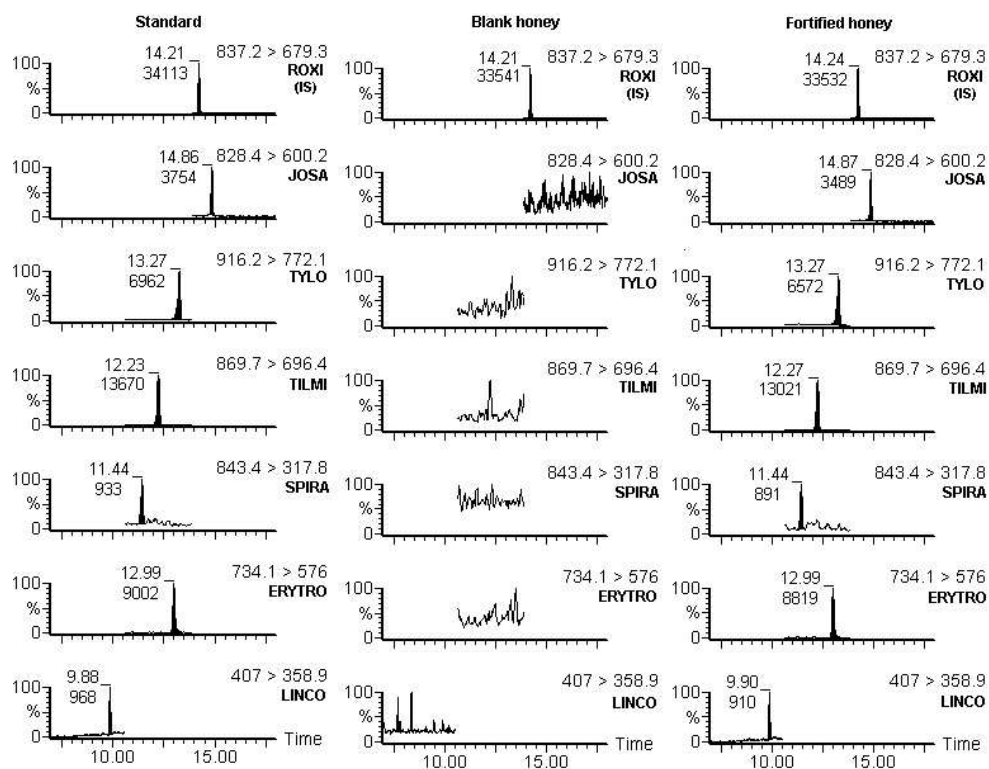


Figure 2: HPLC-ESI-MS/MS chromatograms of a reference standard solution (0.2  $\mu\text{g kg}^{-1}$  of TYLO, TILMI, ERYTRO, JOXA; LINCO and 1.6  $\mu\text{g kg}^{-1}$  of SPIRA, left side), a blank honey extract (in the medium) and a fortified honey sample at 0.2  $\mu\text{g kg}^{-1}$  of TYLO, TILMI, ERYTRO, JOXA; LINCO and 1.6  $\mu\text{g kg}^{-1}$  of SPIRA (right side).