Confirmatory Method for the Determination of Streptomycin and Dihydrostreptomycin in Honey by LC-MS/MS

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2011-346.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	17-Oct-2011
Complete List of Authors:	Bohm, Detlef; Federal Office of Consumer Protection and Food Safety, NRL for Contaminants and Residues Stachel, Carolin; Federal Office of Consumer Protection and Food Safety, NRL for Contaminants and Residues gowik, petra; Federal Office of Consumer Protection and Food Safety, NRL for Contaminants and Residues
Methods/Techniques:	Chromatography - LC/MS, Clean-up, Extraction, Method validation
Additives/Contaminants:	Veterinary drug residues - antibiotics
Food Types:	Honey
Abstract:	<p>The method was specifically developed for the determination and confirmation of streptomycin and dihydrostreptomycin in different types of honey. The method is simple, rapid, sensitive and was validated for streptomycin and dihydrostreptomycin in accordance with Commission Decision 2002/657/EC. After extraction with phosphate buffer and a pH change, the clean-up was performed by way of SPE with polymeric phase. The LC-MS/MS analysis was carried out testing two different HILIC columns for the separation of the analytes and using a triple quadrupole mass spectrometer in positive ESI mode to measure the transitions of the substances in MRM mode. For the quantification of both substances matrix calibration curves in the linear range of 5 – 80 µg/kg were used. The calculated validation parameters for streptomycin and dihydrostreptomycin like CCα (11.8 and 11.5 µg/kg), CCβ (18.9 and 19.9 µg/kg) and the recovery (97 and 101 %) respectively the relative within-laboratory reproducibility RSDωR (16.4 and 20.8 %) at the recommended concentration of 40 µg/kg fulfil the requirements of Commission Decision 2002/657/EC.</p> <p>Keywords: streptomycin, dihydrostreptomycin, honey, LC-MS/MS, validation, CD 2002/657/EC, CCα, CCβ</p>

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

--

SCHOLARONE™
Manuscripts

For Peer Review Only

1
2
3 1 **Confirmatory method for the determination of streptomycin and**
4
5
6 2 **dihydrostreptomycin in honey by LC-MS/MS**
7
8
9
10 3

11 4 D. A. Bohm*, C. S. Stachel and P. Gowik
12

13 5

14
15 6 *Federal Office of Consumer Protection and Food Safety (BVL,)*
16

17 7 *National Reference Laboratories for Contaminants and Residues,*
18

19 8 *Mauerstr. 39-42, 10117 Berlin, Germany*
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Abstract

The method was specifically developed for the determination and confirmation of streptomycin and dihydrostreptomycin in different types of honey. The method is simple, rapid, sensitive and was validated for streptomycin and dihydrostreptomycin in accordance with Commission Decision 2002/657/EC. After extraction with phosphate buffer and a pH change, the clean-up was performed by way of SPE with polymeric phase. The LC-MS/MS analysis was carried out testing two different HILIC columns for the separation of the analytes and using a triple quadrupole mass spectrometer in positive ESI mode to measure the transitions of the substances in MRM mode. For the quantification of both substances, matrix calibration curves in the linear range of 5 – 80 $\mu\text{g/kg}$ were used. The validation parameters which were established for streptomycin and dihydrostreptomycin such as $\text{CC}\alpha$ (11.8 and 11.5 $\mu\text{g/kg}$), $\text{CC}\beta$ (18.9 and 19.9 $\mu\text{g/kg}$) and the recovery (97 and 101 %) respectively the relative within-laboratory reproducibility RSD_{WR} (16.4 and 20.8 %) at the recommended concentration of $<40\mu\text{g/kg}$ fulfil the requirements of Commission Decision 2002/657/EC.

Keywords: streptomycin, dihydrostreptomycin, honey, LC-MS/MS, validation, CD 2002/657/EC, $\text{CC}\alpha$, $\text{CC}\beta$

Introduction

Streptomycin is an aminoglycoside antibiotic that is particularly active against aerobic gram-negative bacteria. The toxicology of streptomycin is well-studied and several cases of allergic reactions have been reported. As a veterinary drug, streptomycin is authorised for use in cattle and pigs, but it is not allowed for the treatment of bees (European Commission 2010). Residues of streptomycin have to be controlled in various food products such as meat, milk, apples and honey. As a pesticide this substance can be used for the treatment of fire blight, which is amongst common diseases in apples. Streptomycin may be found in honey,

because bees may transfer it from the apple tree to the beehive. However, it may also originate from an illegal treatment of the bees with this antibiotic substance. Dihydrostreptomycin is a derivative of streptomycin and was included into the method, because according to the National Residue Control Plan, both aminoglycosides have to be analysed in honey. In the last few years some methods have been published for the determination of streptomycin in food of animal origin (Berrada et al. 2010; Bogialli et al. 2005; Gallina et al. 2005; Gaudin et al. 2005; Gaugain-Juhel et al. 2009; Granja et al. 2009; Hammel et al. 2008; Lee et al. 2007; McGlinchey et al. 2008; Turnipseed et al. 2009; Van Bruijnsvoort et al. 2004; Zhu et al. 2008).

This paper describes a method with SPE clean-up step and LC-MS/MS measurement without fluoric components, which are known to possibly cause unfavourable effects such as peak suppression. In combination with the use of the HILIC column, satisfactory chromatograms are obtained.

As part of our responsibility as German National Reference Laboratory for residues of veterinary drugs, we developed a method which is suitable to confirm and quantify streptomycin and dihydrostreptomycin in honey samples. This serves to detect cases of misuse of these substances in the beehive. Therefore the method was validated in accordance with Commission Decision 2002/657/EC (European Commission 2002).

Materials and Methods

Chemicals

Streptomycin (STR) and dihydrostreptomycin (DSTR) were purchased from Ehrenstorfer (Augsburg, Germany). LC-grade acetonitrile (ACN) and methanol (MeOH) were obtained from LCS (Amsterdam, The Netherlands). LC-grade water was bought from Biosolve (Valkensward, The Netherlands). Hydrochloric acid (HCl), sodium hydroxide (NaOH), potas-

sium dihydrogen phosphate (KH_2PO_4), acetic acid, formic acid, trichloroacetic acid (TCA) and titriplex III ($\text{Na}_2\text{EDTA} \times 2 \text{H}_2\text{O}$) were purchased from Merck (Darmstadt, Germany). Solid-phase extraction (SPE) was performed by means of Oasis HLB cartridges (200 mg, 6 ml) from Waters (Milford, MA, USA).

Equipment

A vortexer and a shaker from Heidolph (Schwabach, Germany), an analytical balance from Mettler Toledo (Greifensee, Switzerland), a high-volume centrifuge from Heraeus (Hanau, Germany), an evaporator from Zymark (Idstein, Germany), a pH meter from WTW (Weilheim, Germany) and a vacuum station from Supelco (Deisenhofen, Germany) were used for sample preparation and extraction.

Standard solutions

Stock solutions (S0) at concentrations of 1 mg ml^{-1} were prepared by dissolving 10 mg of streptomycin and dihydrostreptomycin in 10 ml of water each. To prepare the working solutions, the stock solutions were diluted with water, resulting in concentrations of $100 \mu\text{g ml}^{-1}$ (S1) and $1 \mu\text{g ml}^{-1}$ (S3). The working solution S3 was used for preparing the samples for matrix calibration as well as for the spike samples.

Solutions for sample preparation and mobile phases

For the phosphate buffer solution, 1.36 g of KH_2PO_4 were dissolved in approximately 950 ml of H_2O . The pH value was adjusted to 4 with 1 N HCl using a pH meter. Then 0.15 g of $\text{Na}_2\text{EDTA} \times 2 \text{H}_2\text{O}$ and 20 g of TCA were added. The solution was filled up to 1000 ml with H_2O . The solutions of 3 % formic acid in MeOH, 5 N NaOH in water, 0.05 % formic acid in water (mobile phase component A) and 0.05 % formic acid in ACN (mobile phase component B) were prepared by mixing the equivalent volumes. The reconstitution solution was prepared by mixing the components of mobile phases A and B at a ratio of 30/70 (v/v).

86 *Preparation of honey samples*

87 Eight different laboratory samples of nearly 500 g of honey each were used. This quantity was
88 necessary to produce a sufficient number of representative samples to carry out the analyses
89 for the eight runs of the validation study. The eight samples were different with regard to the
90 origin and colour of the honey. Four honey samples came from forest plants (heath, forest
91 blossom, robinia, lime) and four samples came from field plants (spring blossom, rape, buck-
92 wheat, sunflower). Four of these eight honey samples had a dark colour and four had a light
93 colour. The samples were stored in a refrigerator at approximately +4 °C to avoid any kind of
94 thermal stress. The laboratory samples were divided into 1 g +/- 1 % test samples. The test
95 samples were spiked with different volumes of the S3 working solution mix of streptomycin
96 and dihydrostreptomycin (1 µg ml⁻¹). Before the next step, a waiting time of 10 min was to be
97 respected.

98 *Extraction and clean-up procedure*

99 Twenty ml of phosphate buffer were added to the sample of 1 g of honey. Then the sample
100 was vortexed for approximately 1 min, shaken for 10 min und treated in an ultrasonic bath for
101 5 min. After centrifugation at 3800 g for 10 min at 5 °C, the supernatant was decanted and
102 filtered through filter paper into a 50 ml centrifuge tube. Then the extract was adjusted with
103 30 % NaOH to pH 7.5 and was controlled with a pH meter. The extract was centrifuged and
104 decanted a second time. The SPE cartridge was conditioned with 6 ml of methanol and 6 ml
105 of water. Then the entire extract solution was applied directly onto the cartridge. After the
106 rinsing step with 3 ml of water, the cartridge was air-dried for 10 min. The analyte was eluted
107 with 6 ml of a mix solution of 3 % formic acid in methanol. Then the eluate was concentrated
108 to dryness in a TurboVap evaporator (operation mode „sensor and time“; water bath at 50 °C,
109 nitrogen stream at 0.4 bar). The dry residue was reconstituted in 500 µl of a mobile phase mix
110 (component A/B = 30/70, v/v) using a vortex mixer. The solution was transferred into vials

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

and centrifuged for 10 min at 4000 rpm. The clear solution was filtered through a micro filter, carefully transferred into dark glass vials with inserts and analysed by LC-MSMS.

LC-MS/MS analysis

The LC-MS/MS system consisted of the LC instrument 1100 from Agilent Technologies (Waldbronn, Germany) with a binary pump, an autosampler, a degasser, a column oven and a system controller. The LC was coupled to a triple mass spectrometer QTRAP 3200 from AB SCIEX (Darmstadt, Germany). As analytical columns for the separation of the analytes, the HILIC “Atlantis” (150 mm x 2.1 mm, 3 µm particle size) and the HILIC “Mono-Chrom 5 MS” from Varian (150 x 2 mm), each with adequate guard, were used.

The separation of the analytes was performed by applying a gradient of components A (water with 0.05 % formic acid) and B (ACN with 0.05 % formic acid) at an oven temperature of 30 °C and a flow rate of 0.4 ml min⁻¹ without splitting. The injection volume was 20 µl. The samples were kept in the autosampler at a temperature of 10 °C. The gradient started with 90 % of component B for 1 min and then decreased to 10 % within 5 min. This composition was kept for 4 min, then increased to 90 % of component B within 1 min. With the following equilibration time of 9 min, the resulting total run time was 20 min. The instrument parameters for the mass spectrometry measurement by means of the software “Analyst”, version 1.5.1, were as follows: ESI+; scan type = MRM or MRM-S; dwell-time = 100 ms or variable; resolution Q1 and Q3 = unit; gas = nitrogen; gas 1 = 40 psi; gas 2 = 50 psi; curtain gas = 30 psi; collision gas = high; ion spray voltage = 5500 V; source temp. = 500 °C; CXP = 4 V.

The mass spectrometry parameters applied for the substances streptomycin and dihydrostreptomycin with regard to the transitions from precursor to product ions are shown in Table 1.

137 *Validation of the method*

138 Validation was performed in accordance with Commission Decision 2002/657/EC
139 (European Commission 2002) on the basis of an in-house validation concept. The preparation
140 of the specific study design and the calculation of the validation experiment data were carried
141 out with the help of “InterVal” (Uhlig et al. 2003).

142 For the design of the validation study, it is necessary to select relevant factors accord-
143 ing to the requirements of the samples and the laboratory to consider major changes which
144 may occur during routine analysis. In this study for STR and DSTR in honey the seven factors
145 “origin of honey”, “colour of honey”, “operator”, “MS/MS measurement type”, “LC column”,
146 “lot of cartridge” and “duration before measurement” were varied on two levels (Table 2).
147 This results in an experimental plan of 8 different factor-level-combinations or runs (Table 3).
148 Eight different types of honey were used for the 8 runs. Commission Decision 2002/657/EC
149 (European Commission 2002) requires a validation around the MRL for authorised drugs and
150 at concentrations as low as possible for Group-A-substances. For STR and DSTR in honey no
151 MRLs exist, since these antibiotics are not allowed for the treatment of bees in Europe. There
152 are no regulations for non-authorised substances in food-producing animals concerning the
153 concentration levels to be considered during validation. Therefore the validation study of the
154 method was accomplished with a view to the recommended concentration of $40 \mu\text{g kg}^{-1}$ for
155 streptomycin in honey (CRL Guidance Paper 2007). For each of the 8 runs the analyses were
156 performed on 6 concentration levels for STR and DSTR respectively plus an additional matrix
157 blank sample.

158 To determine the recovery, 6 samples for each run were spiked with 5, 10, 20, 40, 60
159 and $80 \mu\text{l}$ of the working mix solution of $1 \mu\text{g ml}^{-1}$ for STR and DSTR to obtain concentration
160 levels of 5, 10, 20, 40, 60 and $80 \mu\text{g kg}^{-1}$ based on 1 g of sample. A total number of 56 sam-
161 ples including the matrix blank samples were analysed for the validation study.

162

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Confirmation and quantification

The confirmation of the substances streptomycin and dihydrostreptomycin was performed on the basis of the retention times and the ratio of the intensities of the two most abundant product ions obtained from the precursor ion with a signal-to-noise ration of $\geq 3:1$ (Table 1). The areas of the most intensive product ions of streptomycin (m/z 263.2) and dihydrostreptomycin (m/z 263.3) were used for the quantification. The quantitation of the spiked recovery samples was carried out with the help of matrix calibration curves on 6 concentration levels. These matrix calibration samples were spiked with 0, 5, 10, 20, 40, 60 and 80 μl of the working mix solution of $1\text{ }\mu\text{g ml}^{-1}$. The samples of the calibration curve were prepared in the same manner as the recovery samples. Due to the use of 1 g of matrix, sample levels of 0, 5, 10, 20, 40, 60 and 80 $\mu\text{g kg}^{-1}$ honey were achieved. The matrix calibration curves of the quantification for streptomycin ($r = 0.999$) and dihydrostreptomycin ($r = 0.999$) were linear over the concentration range from 0 to 80 $\mu\text{g kg}^{-1}$ (Figure 1).

Results and discussion

The selectivity of the LC-MS/MS method was investigated by analysing blank matrix samples. The extracted ion chromatograms of the blank samples (Figure 2) did not show any signals for either streptomycin or dihydrostreptomycin nor any other interfering compounds at the corresponding retention time. The chromatogram of a spiked sample at half the recommended concentration (CRL Guidance Paper 2007), i.e. 20 $\mu\text{g kg}^{-1}$ for streptomycin and dihydrostreptomycin, showed a satisfactory peak shape for both transitions (Figure 3). In Table 4 the results of the validation parameters according to Commission Decision 2002/657/EC (European Commission 2002) are reported. All method performance parameters, e.g. the decision limit $CC\alpha$, the detection capability $CC\beta$, the repeatability s_r , the within-laboratory reproducibility S_{wR} and the recovery were calculated and fulfil the requirements of the above-mentioned Decision.

The method has proven to be robust. The applicability and ruggedness of the method were tested by examining the influence of different factors (Tables 2 and 3). At the displayed single measurement curves of the 8 runs for streptomycin and dihydrostreptomycin (Figure 4), no outliers were identified by means of the Grubbs test for single values and for curve functions. All curves lay within the prediction interval of the 8 curves. The validation study was performed within 2 weeks, hence the analytical system can be considered as being stable for this time period at least.

The method can be applied to STR and DSTR in honey (1 g sample) in a concentration range of 5 to 80 $\mu\text{g kg}^{-1}$. A sequence should be limited to 24 samples and should include the unknown samples, samples for quality control and samples for matrix and standard calibration.

The confirmation of the substances was possible in all samples considering the ratio of the two fragment ions and the retention time. The preparation of a standard calibration curve is not sufficient for the quantification of both substances. Therefore the preparation of a matrix calibration curve is necessary for quantitative purposes. The linearity was proven by matrix calibration in a range from 5 to 80 $\mu\text{g kg}^{-1}$. Experiments to monitor the stability of the analytes in matrix and in solution are ongoing.

Conclusions

The use of matrix calibration curves is necessary and suitable for quantification. With a small number of experiments (56 analyses), the validation study was performed in accordance with the legal requirements. The calculated relevant method performance parameters, e.g. the decision limit $CC\alpha$, the detection capability $CC\beta$, the repeatability, the within-laboratory reproducibility and the recovery, are in the ranges required by Commission Decision 2002/657/EC (European Commission 2002) and the CRL Guidance Paper (2007).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

The method is applicable for the confirmation and quantification of streptomycin and dihydrostreptomycin in honey in the framework of official residue control according to Council Directive 96/23/EC (European Commission 1996) and Regulation (EC) No 882 (European Commission 2004).

Acknowledgements

Special thanks to Ms. S. Mönch, Ms. M. Jüsgen and Mr. A. Petruck for their assistance.

References

Berrada H, Molto JC, Manes J, Font G. 2010. Determination of aminoglycoside and macrolide antibiotics in meat by pressurized liquid extraction and LC-ESIMS. J Sep Sci. 33(4–5):522–529.

Bogialli S, Curini R, Di Corcia A, Lagana A, Mele M, Nazzari M. 2005. Simple confirmatory assay for analyzing residues of aminoglycoside antibiotics in bovine milk: hot water extraction followed by liquid chromatography–tandem mass spectrometry. J. Chromatogr. A 1067: 93

CRL Guidance Paper 2007. CRLs view on state of the art analytical methods for national residue control plans. Published on the EU-RLs web sites.

European Commission 1996: Council Directive 96/23/EC on measures to monitor certain substances and residues thereof in live animals and animal products. Off J Eur Comm L. 125: 1 - 28

European Commission. 2002. Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of the analytical methods and the interpretation of the results. Off J Eur Comm L. 221:8-36

European Commission 2004: Regulation (EC) No 882/2004 of the European Parliament and of the Council on official controls performed to ensure the verification of compliance

- 240 with feed and food law, animal health and animal welfare rules.
- 241 Off J Eur Comm L. 165: 1 - 141
- 242 European Commission. 2010. Commission Regulation (EC) 37/2010 on pharmacologically
- 243 active substances and their classification regarding maximum residue limits in foodstuff of
- 244 animal origin. Off J Eur Union L. 15:1-72
- 245 Gallina A, Benetti C, Biancotto G, Baggio A, Manzinello C, Dainese N, Mutinelli F. 2005.
- 246 Antibiotics residues in honey: validation procedure. *Apiacta* 40: 45
- 247 Gaudin V, Cadieu N, Sanders P. 2005. Results of a European proficiency test for the detection
- 248 of streptomycin/dihydrostreptomycin, gentamicin and neomycin in milk by ELISA and
- 249 biosensor methods. *Anal. Chim. Acta* 529: 273
- 250 Gaugain-Juhel M, Delepine B, Gautier S, Fourmond M.P, Gaudin V, Hurtaud-Pessel D,
- 251 Verdon E, Sanders P. 2009. Validation of a liquid chromatography-tandem mass spectro-
- 252 metry screening method to monitor 58 antibiotics in milk: a qualitative approach.
- 253 *Food Addit Contam A.* 26: 1459-1471
- 254 Granja R, Nino A, Zucchetti R, Nino R, Patel R, Salerno A. 2009. Determination of strepto-
- 255 mycin residues in honey by liquid chromatography-tandem mass spectrometry.
- 256 *Anal. Chim. Acta* 637:64
- 257 Hammel Y.A, Mohamed R, Gremaud E, LeBreton M.H, Guy P.A. 2008. Multi-screening
- 258 approach to monitor and quantify 42 antibiotic residues in honey by liquid
- 259 chromatography-tandem mass spectrometry. *J. Chromatogr. A* 1177:58
- 260 Lee J.B, Chung H.H, Chung Y.H, Lee K.G. 2007. Development of an analytical protocol for
- 261 detecting antibiotic residues in various foods. *Food Chem.* 105 (4):1726
- 262 McGlinchey T.A, Rafter P.A, Regan F, McMahon G.P. 2008. A review of analytical methods
- 263 for the determination of aminoglycoside and macrolide residues in food matrices.
- 264 *Anal. Chim. Acta* 624:1
- 265 Turnipseed S.B, Clark S.B, Karbiwnyk C.M, Andersen W.C, Miller K.E, Madson M.R. 2009.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

266 Analysis of aminoglycoside residues in bovine milk by liquid chromatography electrospray
267 ion trap mass spectrometry after derivatization with phenyl isocyanate.
268 J. of Chromatogr. B, 877: 1487–1493

269 Uhlig S, Gowik P, Radeck W. 2003. Performance of a matrix-comprehensive in-house valida-
270 tion study by means of an especially designed software. Anal. Chim. Acta 483:351

271 Van Bruijnsvoort M, Ottink S.J.M, Jonker K.M, De Boer E. 2004. Determination of strepto-
272 mycin and dihydrostreptomycin in milk and honey by liquid chromatography with tandem
273 mass spectrometry. J. Chromatogr. A 1058:137

274 Zhu W, Yang J, Wei W, Liu Y, Zhang S. 2008. Results of a European proficiency test for the
275 detection of streptomycin/dihydrostreptomycin, gentamicin and neomycin in milk by.
276 ELISA and biosensor methods. J. Chromatogr. A 1207: 29

Figure 1. Matrix calibration curve with 0 – 80 $\mu\text{g kg}^{-1}$ for streptomycin ($r = 0.999$) and dihydrostreptomycin ($r = 0.999$)

254x190mm (96 x 96 DPI)

Figure 2. Total ion and product ion chromatograms for streptomycin and dihydrostreptomycin of blank honey sample

254x190mm (96 x 96 DPI)

Figure 3. Total ion and product ion chromatograms for streptomycin and dihydrostreptomycin of honey sample spiked with 20 µg kg⁻¹

254x190mm (96 x 96 DPI)

Figure 4. Single measurement values with curves and prediction interval (fat line) of streptomycin and dihydrostreptomycin resulting from the 8 runs of the validation study

254x190mm (96 x 96 DPI)

Table 1. LC-MS/MS parameter.

Analyte	RT ^a (min)	Transition (m/z)	DP ^b (V)	EP ^c (V)	CE ^d (V)	CEP ^e (V)
Streptomycin (MonoChrom)*	8.0*	582.2 > 263.3	111	10	41	27
(Atlantis)**	8.7**	582.2 > 246.2	111	10	49	27
Dihydrostreptomycin	8.0*	584.3 > 263.2	108	7	40	27
	8.7**	584.3 > 263.2	108	7	51	27

^a Retention Time^b Declustering Potential^c Entrance Potential^d Collision Energy^e Cell Entrance Potential

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table 2. Design of validation study: factors and levels.

Factors	Levels
Origin of honey	field - wood
Colour of honey	light - dark
Operator	A - B
MS/MS measurement type	MRM – MRM-S
LC column	Monochrom - Atlantis
Lot of cartridge	Lot A – Lot B
Duration before measurement	directly – after 12 h

Table 3. Design of validation study: factor-level-combinations of 7 factors on 2 levels.

Run		Factor-level-combination					
No.	Origin	Colour	Operator	MS/MS type	LC column	Lot of cartridge	Duration before meas.
3	field	light	B	MRM	Monochrom	Lot A	after 12 h
6	wood	dark	A	MRM	Monochrom	Lot A	directly
5	wood	dark	B	MRM	Atlantis	Lot B	after 12 h
4	field	light	A	MRM	Atlantis	Lot B	directly
1	field	dark	B	MRM-S	Monochrom	Lot B	directly
8	wood	light	A	MRM-S	Monochrom	Lot B	after 12 h
7	wood	light	B	MRM-S	Atlantis	Lot A	directly
2	field	dark	A	MRM-S	Atlantis	Lot A	after 12 h

Table 4. Method performance parameters.

Analyte	CC α ($\mu\text{g kg}^{-1}$)	CC β ($\mu\text{g kg}^{-1}$)	RSD $_r$ (%) ^a	RSD $_{wR}$ (%) ^b	Recovery (%)
STR	11.8	18.9	6.5	16.4	97
DSTR	11.5	19.9	5.2	20.8	101

^a repeatability

^b within-laboratory reproducibility

For Peer Review Only

FIGURE CAPTIONS

Figure 1. Matrix calibration curve with 0 – 80 $\mu\text{g kg}^{-1}$ for streptomycin ($r = 0.999$) and dihydrostreptomycin ($r = 0.999$)

Figure 2. Total ion chromatogram (TIC) and product ion chromatograms for streptomycin and dihydrostreptomycin of blank honey sample

Figure 3. Total ion chromatogram (TIC) and product ion chromatograms for streptomycin and dihydrostreptomycin of honey sample spiked with 20 $\mu\text{g kg}^{-1}$

Figure 4. Single measurement values with curves and prediction interval (fat line) of streptomycin and dihydrostreptomycin resulting from the 8 runs of the validation study