Analysis of Streptomycin and Dihydrostreptomycin by LC-MS/MS using ITSP clean-up and a ZIC-cHILIC HPLC column.

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Overview

Streptomycin:

- aminoglycoside antibiotic.
- widely used as veterinary medicine for large animals, along with dihydrostreptomycin.
- used on apple, pear and stonefruit crops to control bacterial diseases.
- used on kiwifruit against *Pseudomonas syringae* pv. actinidiae (PSA) since its discovery in New Zealand in 2011, with risk of contamination of honey via bees used for pollination.
- has been used in beehives against "foulbrood" infections, resulting in a food scare in the United Kingdom in 2002, when it was detected in Chinese honeys.

Current Analytical Approaches for Honey:

- ELISA, detection limits range from 0.004 – 0.05mg/kg for streptomycin plus dihydrestreptomycin, but prone to false positives due to cross-reactions.
- LC-MS/MS on reversed-phase C-18, using ion pairing reagents (e.g. hepta- or nonafluoropentanoic acid).
- LC-MS/MS on HILIC columns (silica-based or zwitterionic).

Introduction

To meet the Action Limits for both compounds in honey (0.05mg/kg in the UK, 0.02 mg/kg in Germany and Switzerland), an analytical method with an MDL of 0.01 mg/kg or better was required.

Use of LC-MS/MS with HILIC avoids the use of ion-pairing reagents which can cause significant ESI signal suppression (80% for streptomycin[1]).

Both silica-based HILIC and zwitterionic HILIC columns (ZIC-*p*HILIC and ZIC-*c*HILIC, Merck SeQuant) were trialled, the former relying on hydrophilic partitioning for retention, the latter also providing electrostatic interaction (Figure 1).

- ZIC-*p*HILIC – sulfobetaine stationary phase (Figure 2).
- ZIC-*c*HILIC – phosphorylcholine stationary phase (Figure 2).

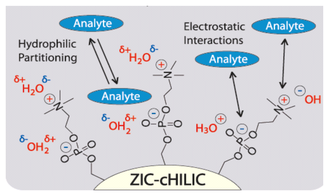


Figure 1: Retention mechanisms on ZIC-cHILIC stationary phase.[1]

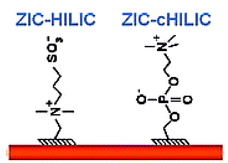


Figure 2: ZIC-*p*HILIC (sulfobetaine) and ZIC-cHILIC (phosphorylcholine) functional groups.

LC-MS/MS

- **Instrument :** AB Sciex 3200 QQQ.
- ESI Parameters: TEM = 400, IS = 3000, compound specific parameters in Table 1.

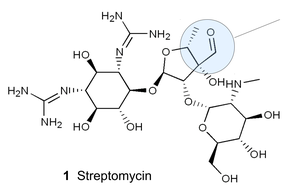
Table 1: ESI-MS/MS parameters.

ESI-MS/MS Parameters (ESI Positive Polarity)					
Compound	Q1 m/z	Q3 (quant) m/z	Q3 (qual) m/z	Period #	Declustering potential
Streptomycin	582.3	263.2	246.2	2	DP = 200
Dihydrostreptomycin	876.6	263.2	246.2	2	DP = 200
Spectinomycin (SMC)	333.2	112.0	122.0	1	DP = 50

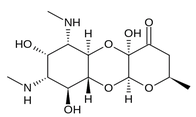
- Injection volume = 30 µL.
- **HPLC Columns:**
  - Column **1**: Ascentis Express HILIC (100 × 2.1mm, 2.7µm particle size).
  - Column **2**: SeQuant ZIC-*p*HILIC (150 × 2.1mm, 5µm particle size).
  - Column **3**: SeQuant ZIC-*c*HILIC (100 × 2.1mm, 3µm particle size).
  - Column **4**: SeQuant ZIC-*c*HILIC (50 × 2.1mm, 3µm particle size).

Table 2: HPLC column gradients.

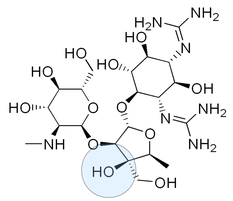
Gradient A (Columns 1-3)			Gradient B (Column 4, 50mm)	
Time (min.)	%B	Flow (mL/min)	Time (min.)	%B
0.00	90	0.4	0.00	95
0.20	90	0.4	0.20	95
3.70	10	0.4	3.60	10
4.90	10	0.4	4.60	10
5.00	90	0.6	5.10	95
8.00	90	0.6	6.50	95
8.10	90	0.4	6.60	95
Mobile Phases : A = 200mM ammonium formate + 0.5% formic acid in water , B = 0.5% formic acid in acetonitrile.				



1 Streptomycin



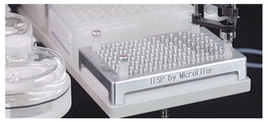
3 Spectinomycin



2 Dihydrotreptomycin

ITSP Method Development

- Initial experiments showed injections of spiked 50g/L honey solutions gave >150% recoveries (cf. solvent standards) similar to the enhancement previously observed[2], possibly due to co-eluting sugars.
- Therefore a clean-up was developed to remove sugars, using weak cation exchange ITSP (Instrument Top Sample Preparation), a miniaturized, robotized SPE.
  ITSP cartridges.
  07-CBA10-20A packed with 10 mg of weak cation exchange (Isolute CBA).
- Developed by Microliter Analytical Supplies Inc., for use on a CTC Analytics robotic autosampler.



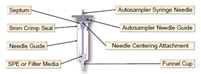


Figure 3: ITSP cartridges.

Since streptomycin positives are rare in honey, spectinomycin was used as a surrogate (SMC), to monitor recovery off the ITSP cartridges for each sample.

- Spectinomycin is more weakly retained, so analyte losses in the rinse step show up as low recovery of spectinomycin first.
- Initially 0.1M HCl used for elution, however signal suppression was observed with HCl present

Table 3: ESI signal suppression by HCl, when using 0.1 M HCl for ITSP elution.

Compound (in-vial conc.)	Peak area, standard, no HCl	Peak area, 0.1M HCl elution	Signal suppression
Streptomycin (10 µg/L)	2.65×10^4	1.04×10^4	61%
Dihydrostreptomycin (10 µg/L)	5.19×10^4	4.00×10^4	23%
Spectinomycin (SMC, 40 µg/L)	3.04×10^4	1.68×10^4	45%

- Elution with 2% formic acid in water avoided HCl.
- Due to precise control of flow rate, ITSP is ideal for cation exchange SPE where too high a flow rate can result in poor recoveries with bench-top SPE.

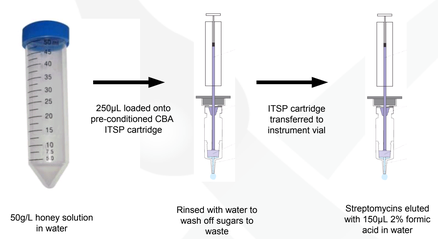


Figure 4: ITSP clean-up for streptomycin and dihydrostreptomycin in honey.

HPLC Method Development

Comparison of Columns.

- Columns 1, 2 and 3 were compared using gradient A (Figures 5-7): (Figure 5).

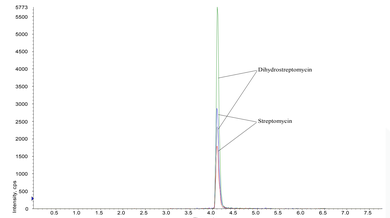


Figure 5: Column 1, Ascentis Express HILIC, gave co-elution of streptomycin and dihydrostreptomycin, as expected for interaction based solely on hydrophilic partitioning (25 µg/L in-vial).

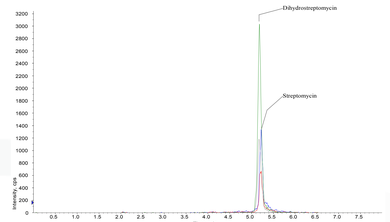


Figure 6: Column 2, SeQuant ZIC-*p*HILIC, gave greater retention than column 1, and 0.03 min separation of streptomycin and dihydrostreptomycin, indicating additional electrostatic interactions with the sulfobetaine stationary phase, likely involving the analytes protonated guinadine and also hydroxyl groups, (25 µg/L in-vial).

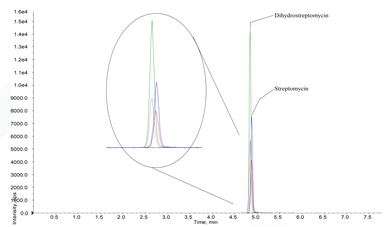


Figure 7: Column 3, SeQuant ZIC-cHILIC, gave similar retention with improved separation (0.04 min.) over column 2, possibly due to greater interaction of the δ – charge on the streptomycin aldehyde group with the more exposed -NH_4^+ on the phosphorylcholine stationary phase (25 µg/L in-vial).

Mobile phase gradient.

- Higher percentages of mobile phase B in the initial composition resulted in greater retention of streptomycin, giving greater ESI sensitivity with elution in mobile phase with a higher aqueous percentage.
- Faster gradients gave better peak shapes, with significantly affecting separation of streptomycin and dihydrostreptomycin, as observed previously for aminoglycoside antibiotics on ZIC-HILIC columns.[3]

Mobile phase composition.

- Increasing the concentration of ammonium formate in mobile phase A gave lesser retention (Figure 8), likely due to ion pairing with the protonated guanidine group.[2]
- 300mM ammonium formate caused significant signal suppression compared with 200mM.
- Lower formate (50 and 100 mM) resulted in poor peak shape, and therefore lower detection limits.

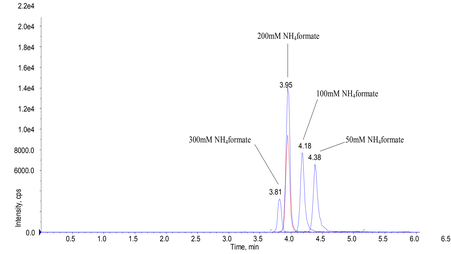


Figure 8: Effect on varying ammonium formate concentration in mobile phase A (aqueous), on retention and sensitivity for streptomycin, column 4 (50mm ZIC-cHILIC), gradient B. Streptomycin = 10 µg/L in-vial.

Method Validation Results

- **Spike recoveries** for 0.5 and 0.05 mg/kg spike experiments were 98-105%, showing that the ITSP clean-up was removing honey matrix previously observed to give signal enhancement.[1]
- Method detection limits were ≤ 0.01 mg/kg, lower than the EU Action Limits.

Table 4: Method detection limits, precision and recoveries for 0.05 mg/kg spikes of streptomycin and dihydrostreptomycin on manuka honey (column 4, gradient B).

Compound	Mean recovery (n=8)	Precision (%Standard deviation)	Method detection limit (EPA calculation)[a]	Method detection limit (from S/N)[b]
Streptomycin (**1**)	105%	5.9%	0.01 mg/kg	0.005 mg/kg
Dihydrostreptomycin (**2**)	101%	10.6%	0.01 mg/kg	0.005 mg/kg

[a] EPA MDL = standard deviation × Student's *t*-distribution value (2.998 for n = 8, 99% 1-sided CI).
[b] Signal-to-noise MDL = 3 × concentration /peak-peak signal-to-noise, for a 0.2µg/L standard.
(both MDL results from qualifier MRMs)

Table 5: Precision and recoveries for 0.5 mg/kg spikes of streptomycin and dihydrostreptomycin on manuka honey (column 4, gradient B).

Compound	Mean recovery (n=9)	Standard deviation	Precision, (RSD)
Streptomycin (**1**)	104%	0.34µg/kg	3.3%
Dihydrostreptomycin (**2**)	98%	0.45µg/kg	4.6%

Discussion

Use of a ZIC-*c*HILIC column gave the best separation (0.04 min.) of streptomycin and dihydrostreptomycin, compared with none on silica-based HILIC, reducing the overlap of the $[M + H + 2]^+$ ion of streptomycin with the $[M + H]^+$ of dihydrostreptomycin to 3%.
This means a 0.1mg/kg residue of streptomycin would result in a dihydrostreptomycin peak less than 1/3 of the MDL, however it would be offset by 0.04 min from the dihydrostreptomycin retention time therefore could be rejected.

References and Acknowledgements

1. Gremilogianni, A.M., Megoulas, N.C. and Koupparis, M. A. *J. Chromatogr. A* 1217 (2010) 6646-51.
2. Yue, D., Hui-yuan, Y., and Wei-dong, X. *Acta Metallurgica Sinica*, 34 (2009) 669-677.
3. Jiang, W., Appelblad, P., Jonsson, T. and Hemstrom, P. *Chromatography Today* May/June 2011, pp26-28.

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