# Methodology for determining neonicotinoid concentrations in insect pollinator hive products (nectar & honey, wax and pollen) using liquid chromatography tandem mass spectrometry

Neonicotinoids (Thiamethoxam (TMX) and Clothianidin (CLO)) were extracted using solid-liquid extraction from homogenised samples.

***Nectar and honey samples.*** Wet samples (0.1g) were spiked with labelled internal standards (Thiamethoxam-D4, Clothianidin D3, QMX). Two ml aliquots of 50:50 methanol water (v:v) were added and the sample was mixed using a vortex for 30 seconds. The samples were then centrifuged at 3000 rpm for 5 minutes. One ml of the aqueous layer was removed and 4ml of HPLC grade water added. The samples were thoroughly mixed again using a vortex.

***Pollen and wax samples*.** Wet samples (0.1g) were spiked with labelled internal standards (Thiamethoxam-D4, Clothianidin D3, QMX). One ml of 80:20 hexane/isopropanol (v:v) were added to each sample and they were briefly vortexed. Samples were placed in a water bath at 45ºC for 15 minutes and were mixed every 5 minutes, using a vortex. 1.8 ml of 50:50 methanol water (v:v) were added to each sample and they were vortexed for 30 seconds. The samples were then centrifuged at 3000 rpm for 5 minutes, 1ml of the aqueous layer removed and 4 ml of HPLC grade water added. The samples were thoroughly mixed again using a vortex.

The extracts from the different matrix types were cleaned using Oasis HLB (60mg, 3cc size, Waters) cartridges. The SPE columns were conditioned with methanol followed by HPLC grade water. The SPE columns were dried under vacuum and then 2 ml of hexane were added and then dried again under vacuum. The SPEs were eluted with 6 ml of acetonitrile.

The extracts were blown down to dryness using a Turbovap (Biotage, Uppsala, Sweden) set at 45$^{o}$C and 10 psi. The extracts were then re-dissolved in 1ml of mobile phase (95% phase A & 5% phase B to match starting eluent at injection time in the LC), mixed using a vortex and added into a LC vial and analysed by LCMS-MS.

***LCMS-MS analyses.*** Analysis was performed using a liquid chromatography coupled to a triple quadrupole 'Quantum Ultra TSQ' mass spectrometer (Thermo Fisher Scientific, Hemel Hemsptead; UK) interfaced with an ion max electrospray ionisation (ESI) and operated with Xcalibur software ™ (V.2.0.7.. Analyte separation (25 µL inj. volume) was performed on a Phenomenex Synergi Fusion column (2.5 µm particle size, 50 mm x 2mm I.D., Phenomenex) using a $H_2O$:MeOH mobile phase gradient.

The analytes were eluted from the column using a programme which mixed different ratios of mobile phase A: 0.1% Acetic acid in water and mobile phase B: 0.1% Acetic acid in Methanol at a rate of 0.3 ml min$^{-1}$. Gradient elution started from 95% A and 5% B, increased to 50% B in 15 min and to 100% B in 5 min, then decreased to 5% of B in 0.1 min and held for 5 min, and returned to initial conditions.

MS/MS was performed in single reaction mode (SRM) using ESI in the positive mode, and two characteristic fragments of deprotonated molecular ion [M + H]+ were monitored for each compound. Both fragments were used for quantification. Other parameters were optimized as follows: Spray Voltage 3000V, Vaporiser Temp

350°C, Capillary Temp 300°C, Collision Cell pressure 1.4 Torr. Argon was used as the collision gas, while nitrogen was used as the desolvation gas.

The quantification was achieved by calculating the response factor of neonicotinoid compounds to their respective internal standards (native to deuterated). Chromatographic peaks were integrated using the ICIS algorithm of Xcalibur™ which was also used to generate linear calibration curves using a 1/X weighting with $R^2$>0.99. The method was linear between ND and 2.5ng/mL and a minimum of 5 standards were used.

The performance of the method was assessed in terms of the limit of detection (LOD), recovery of the internal standards for the analytes and linearity. Recovery for the total procedure was calculated using the labelled standards. For both compounds the method LOD for the entire procedure is 0.4 ng/g and the method LOQ (limit of quantification) is 0.6 ng/g. The LOD was determined using 3 times the signal to noise ratio and the LOQ was calculated as the LOD plus the calculated expanded uncertainty of the method (U). The expanded uncertainty for TMX and CLO was calculated using the Nordtet TR537 handbook. The method expanded uncertainty (which is 2 times the method uncertainty) is 40%.

The method recoveries for the samples analysed so far are:

- Thiamethoxam-D4 and Clothianidin- D3 in nectar/honey samples 91.3%±7.2 and 89.7%±7.2, respectively;
- Thiamethoxam-D4 and Clothianidin- D3 in pollen samples 83.5%±8.2 and 82%±10.1, respectively.

***Quality Control***. In addition to the deuterated standards, for quality control and assurance purposes, we also analysed a blank (i.e. a solvent without matrix) and a matrix matched blank to ensure that no contamination occurred during the sample extraction and clean- up stages for each batch of samples (which contain no more than 16 real samples). Mobile phase blank samples were also injected every four samples to ensure that there was no carryover in the LCMS-MS that could affect the following samples in the analytical run.

An in-house QC was also extracted and analysed with each batch of samples. This was prepared because no certified reference material is commercially available. The QCs were prepared using neonicotinoids free honey, pollen and wax to which a known amount of native TMX and CLO (Sigma) was added. These standards were from a different origin than those used in the standards calibration. The QC analyses followed the same procedure described for the samples.

During the analytical run a traceable NIST certificated standard (CLO and TMX, Spex) was also analysed. Every 8 to 10 samples we analysed a standard, checked for retention time drifts and the instrument sensitivity. Identities of detected neonicotinoids were confirmed by comparing the ratio of MRM transitions in samples and pure standards.

The samples were analysed blind, whereby the analyst was unable to identify the treatment from the sample label. The identification and quantification of the neonicotinoids was double checked.

## References

Magnusson B., Näykki T., Hovind H. , Krysell M. 2012. Nordtest report 537. Handbook for calculation of measurement uncertainty in environmental laboratories. Nordic Innovation. 52 pps.

**This method is accredited to ISO17025:2005**