Electronic Supplementary Material

Methiocarb metabolites are systemically distributed throughout corn plants grown from coated seeds

Andrea Lentola[1,2], Chiara Giorio[3], Sara Bogialli[1], Marco Roverso[1], Matteo Marzaro[4], Vincenzo Girolami[4], Andrea Tapparo[1*]

[1] Dipartimento di Scienze Chimiche, Università degli Studi di Padova, via Marzolo 1, 35131 Padova, Italy
[2] Laimburg Research Centre, Laimburg 6, 39040 Ora, Bolzano, Italy
[3] Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW, United Kingdom
[4] Dipartimento di Agronomia Animali Alimenti Risorse Naturali e Ambiente, Università degli Studi di Padova, viale dell'Università 16, 35020 Legnaro, Padova, Italy

* Corresponding author: andrea.tapparo@unipd.it

S1. Chemicals and reagents

Pure compounds for the preparation of standard solutions of thiamethoxam, N-desmethyl thiamethoxam, thiamethoxam-d_3, clothianidin, clothianidin-d_3, thiacloprid, thiacloprid-d_4, thiacloprid amide, methiocarb, methiocarb-d_3, methiocarb sulfoxide and methiocarb sulfone were obtained from Sigma-Aldrich (purity >99%, isotopic enrichment >97% for deuterated standards, Pestanal®).

Methanol (99.8%, HiPerSolv Chromanorm®, VWR) and acetonitrile (99.9%, LiChrosolv®, VWR) were of HPLC grade and water was purified using a Millipore Milli-Q® equipment.

Analytical grade magnesium sulfate anhydrous (99%, AnalaR NORMAPUR®, VWR) and sodium acetate trihydrate (99.0 %, Fluka) were used in the sample preparation step. Primary-secondary amine (PSA) sorbent was obtained from Sigma-Aldrich (Supelco® analytical). 2-chloro-5-(chloromethyl)pyridine, 2-chloroethylamine HCl, triethylamine and KSCN used for thiacloprid imine synthesis, were obtained from Aldrich® chemistry and Fluka® analytical.

Thiacloprid imine, methiocarb phenol, methiocarb sulfoxide phenol and methiocarb sulfone phenol were synthesized to be used as analytical standards.

S2. Synthesis of primary standards

Thiacloprid imine was synthesized using a two-step procedure available in the literature.[1] Briefly, 2-chloro-5-(chloromethyl)pyridine, 2-chloroethylamine HCl and triethylamine were mixed in acetonitrile for 40 h at 25°C. The intermediate 1-[(6-chloro-3-pyridinyl)methyl]-2-chloroethyl-amine was isolated from the crude reaction product by preparative chromatography and it was mixed with KSCN for 3 h at 95°C in a water/acetonitrile (50:50) solution. Finally, preparative chromatography was used to purify the synthetized thiacloprid imine.

Methiocarb phenol, methiocarb sulfoxide phenol and methiocarb sulfone phenol were synthesized according to a procedure reported in the literature.[2] Briefly, 5 mg of methiocarb, methiocarb sulfoxide, and methiocarb sulfone pure standards were dissolved in 5 mL of methanol in separated volumetric flasks. 1 mL of NH_3 1 M was added and the solution was placed in an ultrasonic bath at 50°C for 30 minutes. In the end, 200 µL of a formic acid 5 M were added to neutralize the base.

The products obtained were characterized by ^{1}H-NMR (Bruker 300, only thiacloprid imine) and HRMS (Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer). Their purity was determined by comparing the area of the main peak and total peak areas of the impurities, using a Shimadzu Prominence UFLC-XR chromatograph (SIL 20AC-XR autosampler; CTO-20A column oven; SPD-M20A UV–vis diode-array detector, set at λ = 202 nm) equipped with a Kinetex Biphenyl column (2.6 µm, 100x2.1 mm, Phenomenex).

S3. UHPLC-HRMS method optimization and validation

In order to identify the insecticide degradation products, a suspect screening analysis of guttation drop samples was performed. The acquisition method was a full-scan-data dependent MS^2 in both ESI+ and ESI- polarities. The analyte identification was based on the accurate mass of the pseudo molecular ion acquired in the full-scan mode, the characteristic isotopic pattern, and the structure was confirmed by the MS^2 spectra. Once the AI degradation products were identified, a list of target compounds was built for the MS^2 target analyses with Normalized Collision Energy (NCE) optimized for each analyte (Table S1).

Table S1. Parameters for quantification of active ingredients and metabolites by UHPLC-MS^2.

Analyte[a]	Retention Time (min)	MSX ID	ionization	NCE (eV)	Q1 precursor ion (*m/z*)[b]	Q3 quantification ion (*m/z*)
thiacloprid imine (a)	9.80	1	ESI+	30	228.0357	126.0097
methiocarb sulfoxide hydroxy[c]	10.40	1	ESI+	30	258.0795	185.0626
clothianidin urea[c]	10.49	1	ESI+	30	206.0149	131.9669
desnitro thiamethoxam[c,d]	10.67	1	ESI+	30	247.0415	131.9691
thiamethoxam nitroso[c]	10.85	1	ESI+	30	276.0316	131.9673
methiocarb sulfoxide phenol (a)	11.09	2	ESI+	40	185.0631	170.0382
thiacloprid amide hydroxy[c]	11.16	2	ESI+	40	287.0364	126.0098
thiamethoxam (a)	11.34	2	ESI+	20	292.0266	211.0631
methiocarb sulfoxide (a)	12.00	3	ESI+	15	242.0845	185.0632
thiacloprid amide (b)	12.44	3	ESI+	15	271.0415	254.0142
thiamethoxam urea[c]	12.51	3	ESI+	15	265.0520	176.9699
clothianidin (b)	12.65	3	ESI+	15	250.0160	169.0542
methiocarb sulfone phenol (b)	13.19	4	ESI-	35	199.0434	184.0211
thiamethoxam N-desmethyl (b)	13.29	4	ESI+	10	278.0109	131.9660
thiacloprid SO[c]	13.35	4	ESI+	35	237.0538	237.0538
thiacloprid hydroxy[c]	13.51	4	ESI+	35	269.0258	126.0108
thiacloprid olefin	13.70	4	ESI+	30	251.0151	126.0106
methiocarb sulfone (c)	14.12	5	ESI+	10	258.0795	122.0719
thiacloprid (c)	14.61	5	ESI+	35	253.0309	126.0098
methiocarb phenol (d)	18.29	6	ESI-	10	167.0536	152.0307
methiocarb (d)	18.75	7	ESI+	10	226.0896	169.0670
thiamethoxam-d3 (a)	11.34	2	ESI+	20	295.0454	214.0818
clothianidin-d3 (b)	12.65	3	ESI+	15	253.0348	172.0728
thiacloprid-d4 (c)	14.61	9	ESI+	35	257.0560	126.0096
methiocarb-d3 (d)	18.75	10	ESI+	10	229.1085	169.0666

[a](a), (b), (c) and (d) indicate which internal standard has been used for the quantification.
[b]thiamethoxam urea was detected as the ammonium adduct, all the other compounds were detected as $[M+H]^+$ in positive or $[M-H]^-$ in negative mode.
[c]analytical standard not available. The quantification is based on the calibration curve of the closest analyte for which the standard was available.
[d]desnitro thiamethoxam was never detected in real samples

Method performance acceptability criteria from EU guidelines were used for assessment.[3] For validation experiments, blank samples obtained from plants treated only with fungicides were used as blank matrices both for leaves and guttations. Grounded leaf samples (four replicates) were spiked at two concentration levels (0.060 and 0.60 µg g^{-1}). The repeatability of the method was determined as the intraday relative standard deviation of these recoveries. Matrix matched calibration solutions were prepared using leaf extracts spiked after extraction. The calibration curve consisted of six points for each tested analyte equivalent to 0.030, 0.060, 0.30, 0.60, 1.5 and 3.0 µg mL^{-1} together with 0.15 µg mL^{-1} of IS mixture. The matrix effect was evaluated by comparing the slopes of the matrix-matched calibration curves with those obtained by diluting the analytes in a water/methanol 80:20 solution, as described in other work[4]. Linearity was evaluated in both conditions: an F-test between linear and polynomial regression models applied to the obtained calibration functions was used to assess their linearity.[5] The sensitivity of the method was calculated in terms of method detection and quantification limits (MDL and MQL, respectively), assessed from the matrix-matched calibration curves.[6,7] For guttation samples, recovery experiments were not performed, but matrix-matched calibration curves were prepared at 0.5, 1, 5, 10, 25, 50 and 100 ng mL^{-1} together with 2.5 ng mL^{-1} of IS mixture. MDL and MQL values were calculated using the same method used for leaf samples.

The results for method validation are summarized in Table S2. For all the analytes in both matrices, no significant differences were observed between the linear and polynomial regression models ($p > 0.072$ for leaf samples, $p > 0.055$ for guttation samples) and so the linear function was used for sample quantification. The matrix effect was considered acceptable for both guttation and leaf samples (Table S2). Considering that the sample preparation applied to guttation samples is a simple dilution, the only potential bias is the matrix effect. Therefore, these results proved that the guttation matrix (xylem sap) does not require clean-up steps prior to injection in order to obtain a proper quantification in UHPLC-ESI-HRMS. Also, the leaf extraction method used fitted the requirements of accuracy and precision for pesticide residue analysis[3].

MDLs for guttation samples were between 0.27 and 8.7 ng mL^{-1}, while MQLs were between 0.81 and 26 ng mL^{-1}. Considering that the typical concentrations of systemic insecticides in guttation drops are in the range of µg mL^{-1} the first days after sowing and few ng mL^{-1} in the following month,[8,9] the MDL and MQL obtained fulfilled the sensitivity required for the quantification of insecticides and their degradation products in guttation drops. MDLs for leaf samples were between 0.81 and 26 ng g^{-1} and the MQL were between 2.4 and 79 ng g^{-1}. In conclusion, the optimized method allowed us to quantify the target list of analytes both in guttation and leaf samples and it was applied to real samples to assess the presence of insecticides and their degradation products in corn leaves and guttations.

Table S2. Performance of the validated methods for the quantification of insecticides and their degradation products in guttation drop and leaf samples.

Analyte	Guttations				Leaves							
									Recovery (%)			
	R^2	Matrix effect[a] (%)	MDL (ng/mL)	MQL (ng/mL)	R^2	Matrix effect[a] (%)	MDL (ng/g)	MQL (ng/g)	0.060 µg/g		0.60 µg/g	
									Mean	RSD	Mean	RSD
Methiocarb	0.9997	18	8.7	26	0.9975	11	26	79	110	8.6	122	5.3
Methiocarb phenol	0.9984	13	1.1	3.4	0.9907	22	3.4	10	95	6.8	82	10
Methiocarb sulfoxide	0.9814	6.5	2.9	8.6	0.9932	-21	8.6	26	98	8.9	98	3.2
Methiocarb sulfoxide phenol	0.9930	-9.5	2.6	8.0	0.9966	14	7.9	24	86	3.3	101	4.5
Methiocarb sulfone	0.9901	1.6	5.1	16	0.9915	-10	15	47	71	2.1	93	5.5
Methiocarb sulfone phenol	0.9952	-14	1.3	4.0	0.9931	-21	4.0	12	126	4.2	108	4.2
Thiacloprid	0.9933	5.2	2.4	7.3	0.9895	-7	7.2	22	101	3.0	109	6.0
Thiacloprid amide	0.9880	17	1.9	5.7	0.9943	-19	5.7	17	97	2.6	69	16
Thiacloprid imine	0.9963	-4.2	0.49	1.5	0.9932	-22	1.5	4.5	113	10	84	3.0
Clothianidin	0.9998	1.9	3.4	10	0.9895	13	10	31	79	2.4	91	4.7
Thiamethoxam	0.9804	4.3	1.0	3.1	0.9977	6	3.1	9.3	71	3.3	113	5.5
N-desmethyl thiamethoxam	0.9980	18	1.0	3.1	0.9494	-9	21	64	110	4.6	69	4.8

[a] The matrix effect was evaluated by comparing the slopes of the matrix-matched calibration curves with those obtained by diluting the analytes in a water/methanol 80:20 solution

S4. Thiamethoxam and its metabolites in corn plants grown in pots

Seven guttation samples were collected from plants treated with thiamethoxam. For this insecticide, but also for the other analytes, a significant concentration trend over time was not observed, likely because of the high sample-to-sample variability. The active ingredient (AI) was always detected, and its mean concentration was 5.0±1.6 µg mL^{-1}. Also, clothianidin was identified in all the analyzed samples with a mean value of 1.33±0.31 µg mL^{-1}. Clothianidin is a well-known thiamethoxam metabolite, but it is also a widely used systemic insecticide. Other metabolites identified were clothianidin urea, N-desmethyl-thiamethoxam, thiamethoxam nitroso and thiamethoxam urea. The insecticide and its degradation products were detected also in corn leaves: the AI was always detected, and its mean concentration was 7.4±3.5 µg g^{-1}. Clothianidin was the main metabolite, followed by thiamethoxam urea. All the other thiamethoxam metabolites previously identified in corn guttations were identified also in corn leaves in this study (Table S3).

Corn seedlings, grown from coated seeds, are also a potential exposure route to systemic insecticides for non-target insects, birds and small herbivore mammals that may eat these young plants. Also, un-buried coated seeds are dangerous from this point of view in particular for birds.[10] The results obtained from corn leaf analysis revealed that neonicotinoids were present in corn seedling at high concentrations (µg g^{-1} level). This is due to the high amount of AI applied to the seeds with respect to the low weight of the whole plant during the first weeks after sowing. Clothianidin and thiamethoxam urea were the main degradation products identified in plants coated with

thiamethoxam. Clothianidin is considered moderately toxic for some bird species (e.g. clothianidin LD_{50} for Japanese quail, *Coturnix japonica*, is 423 μg g^{-1}).[10]

Table S3. Mean concentrations of thiamethoxam and its metabolites detected in corn guttations and leaves. For calculations, when concentration was below MDL it was considered zero and when it was below MQL the MDL value was assigned.

Analyte	Guttations (μg mL^{-1})					Leaves (μg g^{-1})				
	mean	SD	median	1st quartile	3rd quartile	mean	SD	median	1st quartile	3rd quartile
thiamethoxam	5.0	1.6	4.6	4.4	5.5	7.4	3.5	7.3	5.5	8.0
clothianidin	1.33	0.31	1.32	1.26	1.39	2.0	1.0	1.8	1.4	2.6
clothianidin urea	0.036	0.018	0.031	0.023	0.043	0.055	0.037	0.042	0.030	0.070
thiamethoxam urea	1.49	0.53	1.80	1.08	1.90	0.49	0.37	0.44	0.20	0.67
N-desmethyl thiamethoxam	0.014	0.007	0.017	0.008	0.019	0.059	0.053	0.021	0.011	0.110
thiamethoxam nitroso	0.045	0.022	0.044	0.032	0.059	0.019	0.009	0.019	0.013	0.023
thiacloprid	1.02	0.57	0.93	0.75	1.20	4.7	3.8	3.3	2.4	6.0
thiacloprid amide	<0.0057	/	/	/	/	0.23	0.28	0.11	0.066	0.26
thiacloprid imine	0.0025	0.0001	0.0025	0.0025	0.0026	0.033	0.029	0.029	0.017	0.036
thiacloprid amide hydroxy	0.0025	0.0009	0.0024	0.0021	0.0028	0.039	0.033	0.030	0.012	0.060
thiacloprid SO	0.0046	0.0033	0.0038	0.0026	0.0058	0.0047	0.0022	0.0041	0.0033	0.0056
thiacloprid hydroxy	0.018	0.012	0.018	0.012	0.024	0.020	0.017	0.013	0.007	0.027
thiacloprid olefin	0.117	0.079	0.124	0.085	0.157	0.0084	0.0077	0.0062	0.0045	0.0094

S5. Thiacloprid and its metabolites in corn plants grown in pots

For thiacloprid, four guttations samples were analyzed. Thiacloprid was always detected and its mean concentration was 1.02±0.57 μg mL^{-1}. In addition, five metabolites were identified: thiacloprid amide, thiacloprid hydroxy, thiacloprid olefin, thiacloprid imine, thiacloprid hydroxyl amide and thiacloprid with a sulfur atom substituted by an oxygen atom (thiacloprid SO). In corn leaves, thiacloprid mean concentration was 4.7±3.8 μg g^{-1}. In addition, all its metabolites were also detected, the main one being thiacloprid amide but its concentration is much lower if compared to the active ingredient (Table S3).

Few data are available about the toxicity of thiacloprid metabolites and so it is difficult to assess their effects on non-target animals.[11] However, thiacloprid amide is reported to have 15.6 times lower mortality than the parent compound against the pest *Aphis craccivora*.[12] In addition, among the identified metabolites, a modification of the cyano group for thiacloprid often occurred. This may lead to an inversion of selectivity between insects and mammals.[13]

The use of thiacloprid as seed-coating insecticide replacing thiamethoxam, clothianidin and imidacloprid that are currently banned, does not circumvent the environmental problem associated with contamination of guttation drops. In fact, albeit thiacloprid is less toxic for honeybees, the high

content of the active ingredient and its metabolites may constitute a relevant exposure route for wild insects feeding on these contaminated water sources.

S6. References

(1) Latli, B.; D'Amou, K.; Casida, J. E. Novel and Potent 6-Chloro-3-Pyridinyl Ligands for the A4β2 Neuronal Nicotinic Acetylcholine Receptor. *J. Med. Chem.* **1999**, *42* (12), 2227–2234. https://doi.org/10.1021/jm980721x.

(2) Tian, F.; Qiang, Z.; Liu, W.; Ling, W. Methiocarb Degradation by Free Chlorine in Water Treatment: Kinetics and Pathways. *Chem. Eng. J.* **2013**, *232*, 10–16.

(3) Guidance Document on Analytical Quality Control and Method Validation Procedures for Pesticide Residues and Analysis in Food and Feed. SANTE/11813/2017. EUROPEAN COMMISSION DIRECTORATE GENERAL FOR HEALTH AND FOOD SAFETY 2017.

(4) Walorczyk, S. Validation and Use of a QuEChERS-Based Gas Chromatographic–Tandem Mass Spectrometric Method for Multiresidue Pesticide Analysis in Blackcurrants Including Studies of Matrix Effects and Estimation of Measurement Uncertainty. *Talanta* **2014**, *120*, 106–113.

(5) Brüggemann, L.; Quapp, W.; Wennrich, R. Test for Non-Linearity Concerning Linear Calibrated Chemical Measurements. *Accreditation Qual. Assur.* **2006**, *11* (12), 625–631.

(6) Evard, H.; Kruve, A.; Leito, I. Tutorial on Estimating the Limit of Detection Using LC-MS Analysis, Part I: Theoretical Review. *Anal. Chim. Acta* **2016**, *942* (Supplement C), 23–39. https://doi.org/10.1016/j.aca.2016.08.043.

(7) Hubaux, A.; Vos, G. Decision and Detection Limits for Calibration Curves. *Anal. Chem.* **1970**, *42* (8), 849–855. https://doi.org/10.1021/ac60290a013.

(8) Girolami, V.; Mazzon, L.; Squartini, A.; Mori, N.; Marzaro, M.; Bernardo, A. D.; Greatti, M.; Giorio, C.; Tapparo, A. Translocation of Neonicotinoid Insecticides from Coated Seeds to Seedling Guttation Drops: A Novel Way of Intoxication for Bees. *J. Econ. Entomol.* **2009**, *102* (5), 1808–1815. https://doi.org/10.1603/029.102.0511.

(9) Reetz, J. E.; Schulz, W.; Seitz, W.; Spiteller, M.; Zühlke, S.; Armbruster, W.; Wallner, K. Uptake of Neonicotinoid Insecticides by Water-Foraging Honey Bees (Hymenoptera: Apidae) Through Guttation Fluid of Winter Oilseed Rape. *J. Econ. Entomol.* **2015**, tov287. https://doi.org/10.1093/jee/tov287.

(10) Gibbons, D.; Morrissey, C.; Mineau, P. A Review of the Direct and Indirect Effects of Neonicotinoids and Fipronil on Vertebrate Wildlife. *Environ. Sci. Pollut. Res.* **2015**, *22* (1), 103–118.

(11) Akkoc, H.; Acar, A.; Toprak, G.; Uyar, E. Effects of Sublethal Doses of Thiacloprid, a Neonicotinoid Insecticide, on Learning and Memory Performance of Mice. *Int. J. Pharmacol.* **2020**, *16* (1), 72–78.

(12) Liu, Z.; Dai, Y.; Huang, G.; Gu, Y.; Ni, J.; Wei, H.; Yuan, S. Soil Microbial Degradation of Neonicotinoid Insecticides Imidacloprid, Acetamiprid, Thiacloprid and Imidaclothiz and Its Effect on the Persistence of Bioefficacy against Horsebean Aphid Aphis Craccivora Koch after Soil Application. *Pest Manag. Sci.* **2011**, *67* (10), 1245–1252.

(13) Tomizawa, M.; Casida, J. E. Selective Toxicity of Neonicotinoids Attributable to Specificity of Insect and Mammalian Nicotinic Receptors. *Annu. Rev. Entomol.* **2003**, *48* (1), 339–364.