APPNOTE

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Automated Sugaring-Out Assisted Liquid-Liquid Extraction and Determination of Neonicotinoids in Honey Samples using a Robotic Autosampler and LC-MS/MS Platform

Fred D. Foster, Megan C. Harper, Nicole C. Kfoury, and Jacqueline A. Whitecavage

GERSTEL, Inc., 701 Digital Drive, Suite J, Linthicum, MD 21090, USA

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Sample Preparation, LC-MS/MS, High Throughput Lab Automation, Neonicotinoids

Abstract

Honeybees are experiencing high mortality in the United States and worldwide. Neonicotinoids, a class of commonly used insecticides, have been found in honey samples suggesting that bees and other pollinators are being exposed to these neurotoxic chemicals. Pesticide exposure has been identified as one of the stressors causing increased mortality and as a possible cause of colony collapse disorder in bees.

Here we show that a robotic sampler can be used to automate the extraction and determination of neonicotinoid compounds from honey samples. Automating the entire workflow from liquid-liquid extraction to LC-MS/MS analysis results in high throughput. The GERSTEL MPS robotic^{PRO} sampler performs syringe transfer of all liquids involved in the liquid-liquid extraction as well as controlled mixing and centrifugation of the sample extracts. The resulting extracts are introduced into an Agilent Ultivo LC-MS/MS instrument for detection and quantification.

Introduction

In liquid-liquid extraction (LLE), compounds are generally extracted from a liquid aqueous sample using a liquid organic solvent that is immiscible with the sample and therefore forms a separate liquid phase, that can subsequently be aspirated for analysis. Salting-out assisted liquid-liquid extraction (SALLE) relies on introducing an inorganic salt to the aqueous sample before adding a water miscible organic extraction solvent to enable phase separation of otherwise miscible sample and solvent types, resulting in a bilayer system after extraction [1]. Sugaring-out assisted liquid-liquid extraction (SULLE), uses sugar, such as naturally contained in honey, to induce partitioning [2]. Taking advantage of the honey sample matrix enables the quick extraction of neonicotinoid compounds using a LC-MS/MS amenable solvent such as acetonitrile.

As a result of this study, we were able to show that an automated SULLE method performed by the GERSTEL MPS robotic^{PRO} sampler could successfully be used for a variety of neonicotinoids in honey samples. The analytes isolated from the honey samples using the procedure were introduced to an Agilent Technologies 1260 HPLC coupled with an Agilent Ultivo Triple Quadrupole Mass Spectrometer with Jet stream electrospray source. The recoveries of the neonicotinoid compounds extracted from honey samples were found to be 104% for acetamiprid, 81.5% for clothianidin, 94.1% for imidacloprid, 82.4% for thiamethoxam, and 92.3% for thiocloprid. Accuracy data averaged 105% (range: 99.3% - 108%) and precision data averaged 2.28% RSD (range: 1.64% - 3.60%) for all neonicotinoid compounds extracted from honey samples.

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Experimental

Materials

Acetamiprid, imidacloprid, and thiocloprid standards were purchased from MilliporeSigma. Clothianidin and thiamethoxam standards were purchased from LGC Standards Ltd. Standard stock solutions at concentrations of 1 mg/mL were prepared by dissolving known amounts of each standard with the appropriate volume of acetonitrile. Combined intermediate analyte stock solutions were prepared by combining the analyte stock solutions with (1:1) acetonitrile: water, resulting in appropriate concentrations for the neonicotinoid compounds for method evaluation.

Deuterated analogues, d_3-clothianidin and d_4-thiamethoxam, were purchased from LGC Standards Ltd. The deuterated analogue, d_4-imidacloprid, was purchased from MilliporeSigma. Standard stock solutions for d_3-clothianidin and d_4-imidacloprid were prepared by dissolving known amounts of each standard with the appropriate volume of acetonitrile resulting in 1 mg/mL stock solutions. The d_4-thiamethoxam standard was purchased as a 100 µg/mL solution in acetone. Combined intermediate internal standard stock solutions were prepared by combining the internal standard stock solutions with (1:1) acetonitrile: water resulting in appropriate concentrations for method evaluation. Table 1 shows which deuterated internal standards were used for the quantitation of the respective analytes.

A raw and unfiltered honey sample was pre-screened using the automated SULLE-LC-MS/MS method and determined to be free of both the targeted neonicotinoids and the deuterated internals standards used in the method. Calibration standard and QC honey samples were prepared by making appropriate dilutions of the combined intermediate analyte stock solutions and adding them to the analyte-free honey to reach the concentrations listed in Table 1. Calibration standards were prepared using a dilution ratio strategy from the high concentration sample of 1:2:5:2:5:2:5. The high, middle, and low QC samples were prepared using a dilution ratio strategy from the high concentration sample of 1:10:10. Table 1 lists the concentrations for the highest calibration standard and the limit of quantitation found during this study.

Six different raw and unfiltered honey samples were purchased from a local market. All blank honey samples were extracted both with and without internal standard.

All other reagents and solvents used were reagent grade.

Table 1: Mass spectrometer acquisition parameters.

Compound Name	Precursor Ion [m/z]	Product Ion [m/z]		Dwell [ms]	Fragmentation Voltage [V]		CE [V]		Ret Time [min]	High Std Conc. [ng/mL]	LOQ [ng/mL]
acetamiprid[2]	223	125.9	98.9	30	110	110	20	35	2,805	2820	2,82
clothianidin[1]	250	169	131.9	30	100	100	10	10	2,695	2820	2,82
d_3-clothianidin	253	131.9	125.9	30	100	100	10	20	2,692	-	-
d_4-imidacloprid	260	213.1	179.1	30	90	90	15	20	2,743	-	-
d_4-thiamethoxam	296	215	183	30	90	90	10	20	2,589	-	-
imidacloprid[2]	256	209	175	30	100	100	15	20	2,744	2820	2,82
thiamethoxam[3]	292	211	181	30	100	100	10	25	2,589	2820	2,82
thiocloprid[2]	253	186	172	30	100	100	10	10	2,938	2820	2,82

1 - Internal Standard d^3-clothianidin
2 - Internal Standard d^4-imidacloprid
3 - Internal Standard d^4-thiamethoxam

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Instrumentation

All automated Prep Sequences were performed using a MPS robotic^{PRO} sampler with the GERSTEL ^{quick}MIX and centrifuge options as shown in Figure 1. All analyses were performed using an Agilent 1260 HPLC with an Agilent Zorbax RRHD, Eclipse Plus C18 column, (2.1 x 50 mm, 1.8 µm) and an Agilent Ultivo Triple Quadrupole Mass Spectrometer with Jet stream electrospray source. Sample injections were made using the GERSTEL LC-MS tool into a 6 port (0.25 mm) Cheminert C2V injection valve outfitted with a 2 µL stainless steel sample loop.

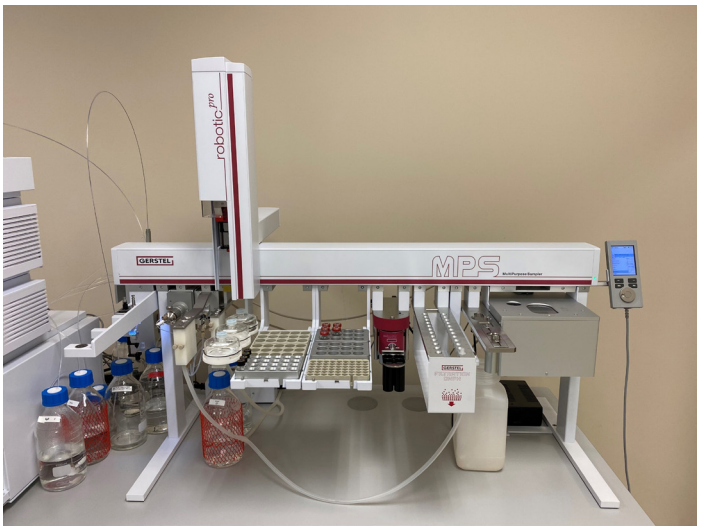

Figure 1: MPS robotic^{PRO} sampler with the GERSTEL ^{quick}MIX and centrifuge options.

Honey Sample Pretreatment

1. Weigh a 2 gram sample of honey into a 10 mL autosampler vial.
2. Pipette 10 µL of a 14.1 µg/mL working internal standard into the sample and cap with a magnetically transportable cap.

Automated MPS Prep Sequence for Neonicotinoids in Honey

1. The MPS adds 4 mL of a (6:4) acetonitrile:water mixture to each vial.
2. The MPS mixes each vial for 1 minute at 1500 rpm.
3. The MPS centrifuges each 10 mL vial for 5 minutes at 3000 rpm.
4. The MPS adds 1 mL of the resulting supernatant to a 2 mL autosampler vial.
5. The MPS centrifuges each 2 mL vial for 3 minutes at 3000 rpm.

Automated MPS Sample Introduction

1. Using the GERSTEL LCMS Tool, the MPS injects the extract into a 2 µL stainless steel sample loop (loop over-fill technique).

LC Method Parameters

Pump	Gradient (800 bar)	
	Flow rate = 0.3 mL	
Mobile Phase	A – 0.1 % formic acid in water	
	B – acetonitrile	
Gradient	Initial	2% B
	0.5 min	2% B
	1.0 min	50% B
	4.0 min	65% B
	4.1 min	98% B
	6.0 min	98% B
	6.1 min	2% B
Run time	10 minutes	
Injection volume	2.0 µL (loop over-fill technique)	
Column Temperature	45 °C	

Mass Spectrometer Parameters

Operation	Electrospray positive mode
Gas Temperature	300 °C
Gas Flow (N_2)	5 L/min
Nebulizer pressure	45 psi
Sheath Gas Flow (N_2)	11 L/min
Sheath Gas Temperature	350 °C
Capillary voltage	4000 V
Nozzle voltage	500 V
Delta EMV	0 V

The mass spectrometer acquisition parameters are shown in Table 1 with qualifier ions.

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Results and Discussion

Figure 2 shows a representative overlay of mass chromatograms for the neonicotinoid compounds and the deuterated internal standards obtained from an extracted low QC honey sample.

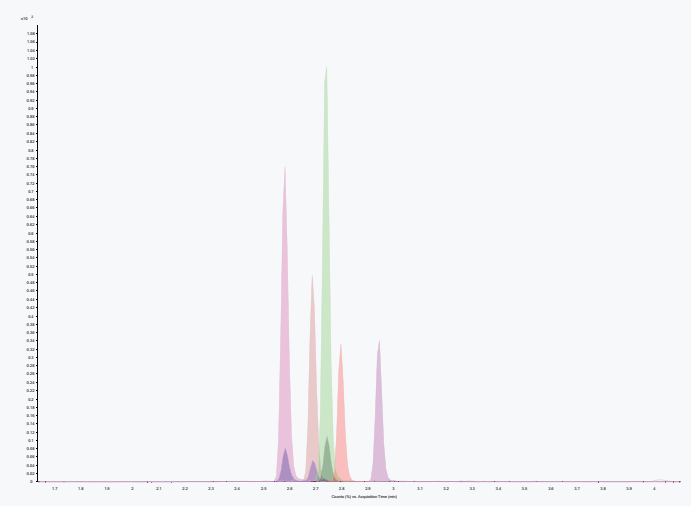

Figure 2: Overlay mass chromatogram for extracted low QC sample.

The lower limit of quantitation for this method was found to be 2.82 ng/g for each target neonicotinoid as shown in Table 1. Representative calibration curves for acetamiprid, imidacloprid, and thiamethoxam, are shown in Figure 3. Regression analysis for all neonicotinoids monitored within this method resulted in R^2 values greater than 0.995.

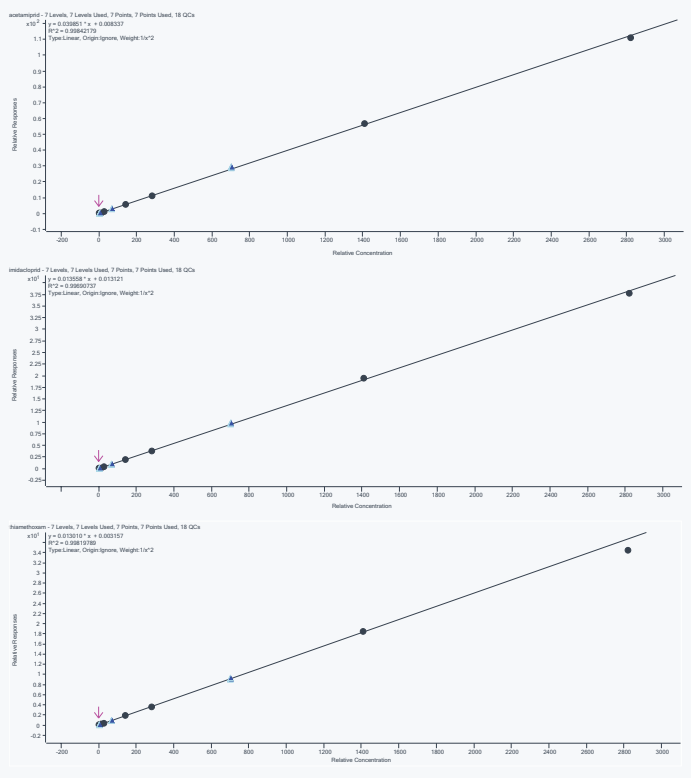

Figure 3: Calibration curve results for acetamiprid, imidacloprid, and thiamethoxam.

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The accuracy and precision of the method were evaluated for all neonicotinoid compounds using QC samples at high, middle, and low concentrations. Table 2 shows the resulting accuracy and precision data for all neonicotinoids. Accuracy data averaged 105% (range: 99.3% - 108%) and precision data averaged 2.28% RSD (range: 1.64% - 3.60%) for all neonicotinoid compounds extracted from honey samples.

Table 1: QC sample % accuracy and % precision results.

Acetamiprid honey								
Name	Final Conc.	Accuracy	Name	Final Conc.	Accuracy	Name	Final Conc.	Accuracy
QCL - 1	7,57	107	QCM - 1	79,0	112	QCH - 1	738	105
QCL - 2	7,62	108	QCM - 2	74,6	106	QCH - 2	731	104
QCL - 3	7,98	113	QCM - 3	75,5	107	QCH - 3	726	103
QCL - 4	7,46	106	QCM - 4	76,5	109	QCH - 4	739	105
QCL - 5	7,90	112	QCM - 5	76,7	109	QCH - 5	748	106
QCL - 6	7,48	106	QCM - 6	77,4	110	QCH - 6	731	104
mean	7,67	109	mean	76,6	109	mean	736	104
SD	0,220	3,12	SD	1,54	2,18	SD	7,65	1,08
%CV	2,87	2,87	%CV	2,01	2,01	%CV	1,04	1,04

Clothianidin honey								
Name	Final Conc.	Accuracy	Name	Final Conc.	Accuracy	Name	Final Conc.	Accuracy
QCL - 1	7,93	112	QCM - 1	76,5	109	QCH - 1	731	104
QCL - 2	6,64	94	QCM - 2	75,4	107	QCH - 2	698	99
QCL - 3	8,34	118	QCM - 3	74,2	105	QCH - 3	700	99
QCL - 4	7,92	112	QCM - 4	75,3	107	QCH - 4	717	102
QCL - 5	8,24	117	QCM - 5	71,4	101	QCH - 5	685	97
QCL - 6	8,50	121	QCM - 6	72,9	103	QCH - 6	719	102
mean	7,93	112	mean	74,3	105	mean	708	100
SD	0,668	9,48	SD	1,86	2,64	SD	16,80	2,38
%CV	8,43	8,43	%CV	2,50	2,50	%CV	2,37	2,37

Imidacloprid honey								
Name	Final Conc.	Acc.	Name	Final Conc.	Acc.	Name	Final Conc.	Acc.
QCL - 1	6.66	94.5	QCM - 1	73.0	104	QCH - 1	730	104
QCL - 2	6.60	93.6	QCM - 2	69.6	98.7	QCH - 2	715	101
QCL - 3	6.82	96.7	QCM - 3	72.0	102	QCH - 3	718	102
QCL - 4	6.27	88.9	QCM - 4	70.8	100	QCH - 4	727	103
QCL - 5	6.76	95.9	QCM - 5	70.2	99.6	QCH - 5	713	101
QCL - 6	6.80	96.5	QCM - 6	72.7	103	QCH - 6	720	102
mean	6.65	94.3	mean	71.4	101	mean	720	102
SD	0.207	2.94	SD	1.38	1.96	SD	6.71	0.951
%CV	3.12	3.12	%CV	1.94	1.94	%CV	0.931	0.931

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Table 1 (cont.): QC sample % accuracy and % precision results.

Thiamethoxam honey								
Name	Final Conc.	Accuracy	Name	Final Conc.	Accuracy	Name	Final Conc.	Accuracy
QCL - 1	7,77	110	QCM - 1	71,4	101	QCH - 1	709	101
QCL - 2	7,60	108	QCM - 2	72,6	103	QCH - 2	711	101
QCL - 3	8,00	113	QCM - 3	72,6	103	QCH - 3	709	101
QCL - 4	7,05	100	QCM - 4	69,7	98,8	QCH - 4	701	99,5
QCL - 5	7,43	105	QCM - 5	73,3	104	QCH - 5	695	98,6
QCL - 6	7,28	103	QCM - 6	73,5	104	QCH - 6	721	102
mean	7,52	107	mean	72,2	102	mean	708	100
SD	0,343	4,87	SD	1,44	2,04	SD	8,76	1,24
%CV	4,56	4,56	%CV	2,00	2,00	%CV	1,24	1,24

Thiocloprid honey								
Name	Final Conc.	Accuracy	Name	Final Conc.	Accuracy	Name	Final Conc.	Accuracy
QCL - 1	8,02	114	QCM - 1	78,1	111	QCH - 1	708	100
QCL - 2	8,10	115	QCM - 2	75,2	107	QCH - 2	704	99,9
QCL - 3	8,01	114	QCM - 3	77,9	111	QCH - 3	689	97,7
QCL - 4	8,13	115	QCM - 4	78,2	111	QCH - 4	721	102
QCL - 5	7,93	112	QCM - 5	75,6	107	QCH - 5	713	101
QCL - 6	8,26	117	QCM - 6	77,4	110	QCH - 6	708	100
mean	8,08	115	mean	77,1	109	mean	707	100
SD	0,115	1,63	SD	1,31	1,86	SD	10,7	1,51
%CV	1,42	1,42	%CV	1,70	1,70	%CV	1,51	1,51

Representative stacked mass chromatograms for acetamiprid, imidacloprid, and thiamethoxam from an extracted LOQ honey standard (A) compared to extracted raw and unfiltered honey samples of Brand W (B), Brand X (C), Brand Y (D), Brand Z (E), Brand Z organic (F), and Brand Z with honeycomb (G) are shown in Figures 4-6 A-G. None of the samples evaluated in this study were found to contain any of the targeted neonicotinoids.

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Figure 4: Representative stacked mass chromatograms for acetamiprid from an extracted LOQ honey standard (A) compared to extracted raw and unfiltered honey samples of Brand W (B), Brand X (C), Brand Y (D), Brand Z (E), Brand Z organic (F), and Brand Z with honeycomb (G).

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Figure 5: Representative stacked mass chromatograms for imidacloprid from an extracted LOQ honey standard (A) compared to extracted raw and unfiltered honey samples of Brand W (B), Brand X (C), Brand Y (D), Brand Z (E), Brand Z organic (F), and Brand Z with honeycomb (G).

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Figure 6: Representative stacked mass chromatograms for thiamethoxam from an extracted LOQ honey standard (A) compared to extracted raw and unfiltered honey samples of Brand W (B), Brand X (C), Brand Y (D), Brand Z (E), Brand Z organic (F), and Brand Z with honeycomb (G).

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To assess the recovery of neonicotinoid compounds from extracted honey samples, the resulting peak areas from the extracted mid-level QC honey samples were compared to those from three replicate injections of a spiked recovery standard having concentrations equivalent to an extracted mid-level QC honey sample. The recovery results of all neonicotinoid compounds and deuterated internal standards when extracted from honey samples are shown in Table 3. Together with the data from the extracted QC samples, this data shows that the MPS robotic^{PRO} sampler can be used to determine neonicotinoid compounds from honey samples using an automated SULLE-LC-MS/MS method.

Table 3: Recovery results from extracted honey samples.

Acetamiprid					
Name	Resp.	Int Std.	Name	Resp.	Int Std.
Rec Std	63443	22597	QCM - 1	69344	21957
Rec Std	66758	23884	QCM - 2	67148	22518
Rec Std	67061	23717	QCM - 3	67468	22376
			QCM - 4	68839	22511
			QCM - 5	69488	22668
			QCM - 6	68791	22247
mean	65754	23399	mean	68513	22379
SD	2007	700			
%CV	3,05	2,99			
%Recovery	104	95,6			

Clothianidin					
Name	Resp.	Int Std.	Name	Resp.	Int Std.
Rec Std	12931	12487	QCM - 1	11268	10657
Rec Std	14166	13552	QCM - 2	11182	10737
Rec Std	13571	13289	QCM - 3	11056	10785
			QCM - 4	11111	10680
			QCM - 5	10727	10863
			QCM - 6	10982	10895
mean	13556	13109	mean	11054	10770
SD	618	555			
%CV	4.56	4.23			
%Recovery	81.5	82.2			

Table 3 (cont.): Recovery results from extracted honey samples.

Imidacloprid					
Name	Resp.	Int Std.	Name	Resp.	Int Std.
Rec Std	22863	22597	QCM - 1	22025	21957
Rec Std	23822	23884	QCM - 2	21542	22518
Rec Std	23284	23717	QCM - 3	22130	22376
			QCM - 4	21904	22511
			QCM - 5	21885	22668
			QCM - 6	22216	22247
mean	23323	23399	mean	21950	22379
SD	481	700			
%CV	2.06	2.99			
%Recovery	94.1	95.6			

Thiamethoxam					
Name	Resp.	Int Std.	Name	Resp.	Int Std.
Rec Std	19533	19958	QCM - 1	16332	17531
Rec Std	20423	20952	QCM - 2	16551	17453
Rec Std	20315	21189	QCM - 3	16419	17325
			QCM - 4	16088	17691
			QCM - 5	16810	17572
			QCM - 6	17070	17794
mean	20090	20700	mean	16545	17561
SD	486	653			
%CV	2.42	3.16			
%Recovery	82.4	84.8			

Thiocloprid					
Name	Resp.	Int Std.	Name	Resp.	Int Std.
Rec Std	68725	22597	QCM - 1	68662	21957
Rec Std	77596	23884	QCM - 2	67819	22518
Rec Std	78095	23717	QCM - 3	69802	22376
			QCM - 4	70431	22511
			QCM - 5	68606	22668
			QCM - 6	68909	22247
mean	74805	23399	mean	69038	22379
SD	5272	700			
%CV	7.05	2.99			
%Recovery	92.3	95.6			

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Conclusions

As a result of this study, we were able to show:

- Neonicotinoid compounds in honey samples can be successfully extracted using an automated sugaring-out assisted liquid-liquid extraction method and determined using the Agilent Ultivo Triple Quadrupole Mass Spectrometer.
- This method was readily automated using the GERSTEL MPS robotic^{PRO} sampler.
- Linear calibration curves resulting in a R^2 values of 0.995 or greater were achieved for all neonicotinoid compounds.
- The automated SULLE-LC-MS/MS method proved to be accurate and precise. Accuracy data averaged 105% (range: 99.3% - 108%) and precision data averaged 2.28% RSD (range: 1.64% - 3.60%) for all neonicotinoid compounds extracted from honey samples.
- The recovery of the neonicotinoid compounds extracted from honey samples was found to be 104% for acetamiprid, 81.5% for clothianidin, 94.1% for imidacloprid, 82.4% for thiamethoxam, and 92.3% for thiocloprid.

References

[1] GERSTEL Application Note No. 230, *Automated Salting-out Assisted Liquid-Liquid Extraction and Determination of Bisphenol A in Beverage Samples using a Robotic Autosampler and LC-MS/MS Platform*, **2022**.

[2] W. Chen, S. Wu, J. Zhang, F. Yu, J. Hou, X. Miao, and X. Tu, **2019** "*Matrix-Induced Sugaring-Out: A Simple and Rapid Sample Preparation Method for the Determination of Neonicotinoid Pesticides in Honey*", Molecules, 24:2761.