Application Note

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Quantitation of Neonicotinoid Pesticides in Honey with the Agilent 1290 Infinity II LC and Agilent 6470 Triple Quadrupole LC/MS

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Abstract

This application note describes the quantitation of neonicotinoids in bee honey using an Agilent 1290 Infinity II LC in combination with the Agilent 6470 triple quadrupole LC/MS. The described approach is simple and straightforward and does not need any form of sample preparation or cleanup prior to LC/MS analysis. Several commercially available honey samples were spiked at two different concentrations: 5 µg/kg (in accordance with the maximum residue levels) and 20 µg/kg of the individual pesticides, and injected multiple times. All neonicotinoid pesticides were recovered from the matrix and the method showed excellent robustness for extended sequences of the heavy matrix samples.

Introduction

Neonicotinoids are a class of neuro-active insecticides, chemically related to nicotine. They were introduced as an alternative to chlorinated hydrocarbons, organophosphates, carbamates, and pyrethroids. Initially, they were praised for their low toxicity to several beneficial insects, such as bees. Later, however, it appeared that neonicotinoids were potentially harmful to bees due to low-level contamination of pollen and nectar.[1,2] These low levels are not necessarily lethal to bees, but their ability to forage for nectar and to remember where flowers are located is impacted. Due to their association with honeybee colony collapse disorder, the use of imidacloprid, clothianidin, and thiamethoxam is banned in the European Union, except for use in greenhouses, treatment of some crops after flowering, and in winter cereals.[3] For the two other currently approved neonicotinoids, acetamiprid is considered to be low risk, while thiacloprid should be substituted due to its endocrine-disrupting properties. Dinotefuran cannot be applied during blooming. The maximum residue level (MRL) for this compound in fruits and vegetables is 0.01 ng/g. Stricter regulations result in the need for analytical methods capable of analyzing neonicotinoids in other bee-derived products at low levels (typically in the 10 ng/g range). Currently, the EU pesticide database mentions MRLs for neonicotinoids in the range of 0.01 to 200 µg/kg in bees' honey (acetamiprid, clothianidin, flonicamid, imidacloprid, thiamethoxam: at 50 µg/kg; dinotefuran and nitenpyram: at 10 µg/kg, and thiacloprid: at 200 µg/kg).[4] Several analytical approaches to monitor these contaminants are described in literature and the use of LC/MS has gained in popularity.[1] The call for robust methods is intensifying, given the complexity of the matrices involved, as potential matrix interferences can lead to significant signal suppression or enhancement in MS detection particularly when measuring in the µg/kg (ppb) range. An LC/MS method for the quantitation of neonicotinoids in bee honey is described using a 1290 Infinity II LC in combination with the 6470 triple quadrupole LC/MS.

Experimental

Materials

Dinotefuran, nitenpyram, thiamethoxam, flonicamide, clothianidin, imidacloprid, acetamiprid, thiacloprid (structures are given in Figure 1), acetamiprid-d3, clothianidin-d3, imidacloprid-d4, and ammonium formate were purchased from Merck (Darmstadt, Germany). Water (ULC/MS grade), formic acid (99%, ULC-MS grade), and acetonitrile (HPLC-S grade) were from Biosolve (Valkenswaard, The Netherlands).

Five different honey samples were purchased from local supermarkets. They are referred to as honey A–E. The falcon tubes, syringes, and syringe filters (0.22 µm) were from VWR (Radnor, PA, USA).

Sample preparation

A solution of all individual neonicotinoids and the internal standards was made at a concentration of 1 mg/mL in acetonitrile. From these stock solutions, mixtures were prepared containing all neonicotinoids. An internal standard mixture was made at a concentration of 1 µg/mL to add to the standard mixtures and honey samples.

Two grams of a honey sample were weighed in a falcon tube and 10 µL of a 1 µg/mL internal standard mixture was added. Each honey sample was also spiked with 20 µL of a 500 ng/mL mixture of the neonicotinoids, resulting in a concentration of every individual neonicotinoid of 5 µg/kg honey. To obtain samples with a concentration of neonicotinoids of 20 µg/kg honey, 2 g of honey was weighed in a falcon tube, 10 µL of a 1 µg/mL internal standard mixture was added, and 80 µL of a 500 ng/mL mixture of neonicotinoids. The samples were dissolved in 9.97 mL of water and 9.91 mL of water, respectively, and vortex mixed for 30 seconds. Finally, the sample was filtered through a syringe filter directly into an HPLC vial for analysis.

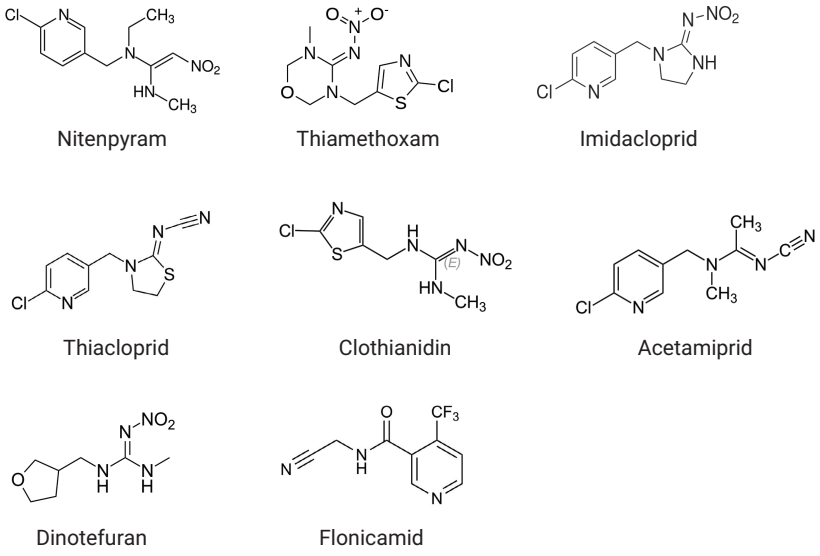

Figure 1. Structures of the neonicotinoids.

A reference solution was prepared containing 2 mL of water, 20 µL of a 500 ng/mL mixture of neonicotinoids, and 10 µL of the 1 µg/mL internal standard mixture to which 9.97 mL of water was added.

Equipment

Analyses were performed on an Agilent 1290 Infinity II LC equipped with the following modules:

- Agilent 1290 Infinity II High-Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B) with 100 µL analytical head and 100 µL sample loop flex
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)

The LC system was coupled to an 6470A triple quadrupole LC/MS system equipped with an Agilent JetStream technology electrospray ion source (G1958-65138). Data acquisition was performed using Agilent MassHunter acquisition software version 10.1. Agilent MassHunter quantitative analysis for QQQ (version 10.0) was used for data handling.

Methods

The general LC/MS parameters are described in Table 1. All compounds were measured in positive ionization mode. The MRM parameters are summarized in Table 2, containing precursor ion, product ions, fragmentor voltage, collision energy, and quantifier (Q) or qualifier (q) ion data.

Table 1. LC/MS parameters.

Agilent 1290 Infinity II LC	
Column	Agilent InfinityLab Poroshell 120 SB-C18, 2.1 × 100 mm × 2.7 µm (p/n 685775-902)
Column Temperature	40 °C
Injection Volume	20 µL
Autosampler Temperature	6 °C
Needle Wash	95% Acetonitrile/5% water
Mobile Phase A	5 mM ammonium formate + 0.1% formic acid in water
Mobile Phase B	0.1% formic acid in acetonitrile/water (95/5)
Flow Rate	0.3 mL/min
Gradient Program	Time %B 0.00 2 3.00 2 10.00 50 10.10 100 15.00 100 15.10 2 18.00 2
Agilent 6470 Triple Quadrupole LC/MS	
Gas Temperature	200 °C
Gas Flow	11 L/min
Nebulizer	25 psi
Sheath Gas Temperature	250 °C
Sheath Gas Flow	11 L/min
Capillary	3,000 V
Nozzle Voltage	1,500 V
Delta EMV +	200
Q1/Q2 Resolution	Unit (0.7 amu)
Cell Accelerator Voltage	5 V

Results and discussion

A representative chromatogram of all neonicotinoids spiked at a concentration of 20 µg/kg in a honey sample is provided in Figure 2.

To check the influence of the matrix on method performance, recovery of the individual neonicotinoids, and signal stability in long sequence runs, a sequence was analysed over 52 hours. The sequence consisted of the alternate injection of different spiked honey samples and a reference solution (being a water-based solution of the neonicotinoids of interest). The sequence consisted of 90 injections in total of which 60 were injections of honey samples (5 different honey samples, spiked at 2 different concentrations, each injected six-fold over the entire time span), 20 were injections of reference sample, and 10 were blanks. After running the sequence, the MS source was very dirty but could readily be cleaned. In these analyses, the entire LC run was sent to the MS source. Even without the use of the diverter valve, robustness was maintained. For this reason, adding compounds eluting in another part of the chromatogram to the method would be possible. From subsequent analyses, it could be concluded that the deposited matrix material had no influence on the instrument performance. In Figure 3, a representative overlay of chromatograms is given of a honey sample spiked with 20 µg/kg of neonicotinoids. Six injections of this sample were performed over the entire sequence of 52 hours.

Comparison of the chromatographic profiles revealed no retention time shift (illustrated in Table 3 through retention time range (minimum–maximum) of all individual compounds over the 52-hour sequence and the %RSD on retention time). No substantial signal loss after the continuous injection of honey samples was observed.

Table 2. MRM parameters.

Compound	Precursor Ion	Product Ion	Fragmentor (V)	CE (V)	Q/q
Thiamethoxam	292.2	211.0	85	4	Q
		181.0	85	16	q
Nitenpyram	271.1	225.1	90	8	Q
		126.1	90	36	q
Imidacloprid	256.1	175.1	90	20	q
		209.0	90	15	Q
Thiacloprid	253.1	186.0	100	10	q
		126.0	100	20	Q
Clothianidin	250.0	169.1	90	7	Q
		132.1	90	15	q
Flonicamid	230.1	203.1	80	15	Q
		174.1	80	15	q
		148.0	80	15	q
Acetamiprid	223.1	126.0	100	15	Q
		56.0	100	15	q
Dinotefuran	203.0	157.0	90	10	q
		129.0	90	10	Q
		113.0	90	10	q
Imidacloprid-d4	260.0	213.0	90	15	Q
		179.0	90	20	q
Clothianidin-d3	253.0	172.1	90	7	Q
		132.1	90	15	q
Acetamiprid-d3	226.1	126.1	100	15	Q
		56.0	100	15	q

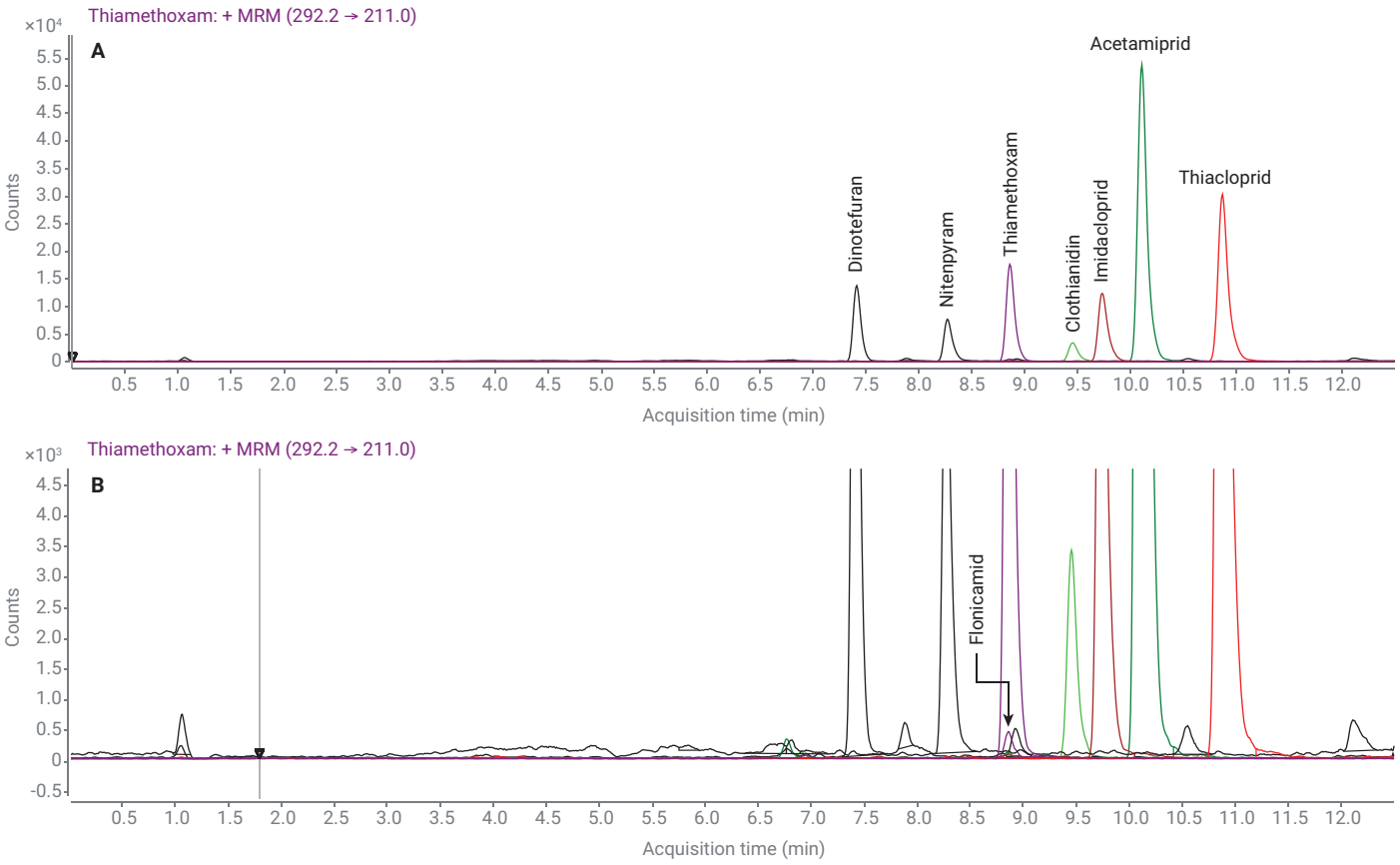

Figure 2. Separation and MRM detection of eight neonicotinoids spiked in a commercial honey sample (A). (B) shows a zoomed-in region of the chromatogram to indicate the detection of flonicamid.

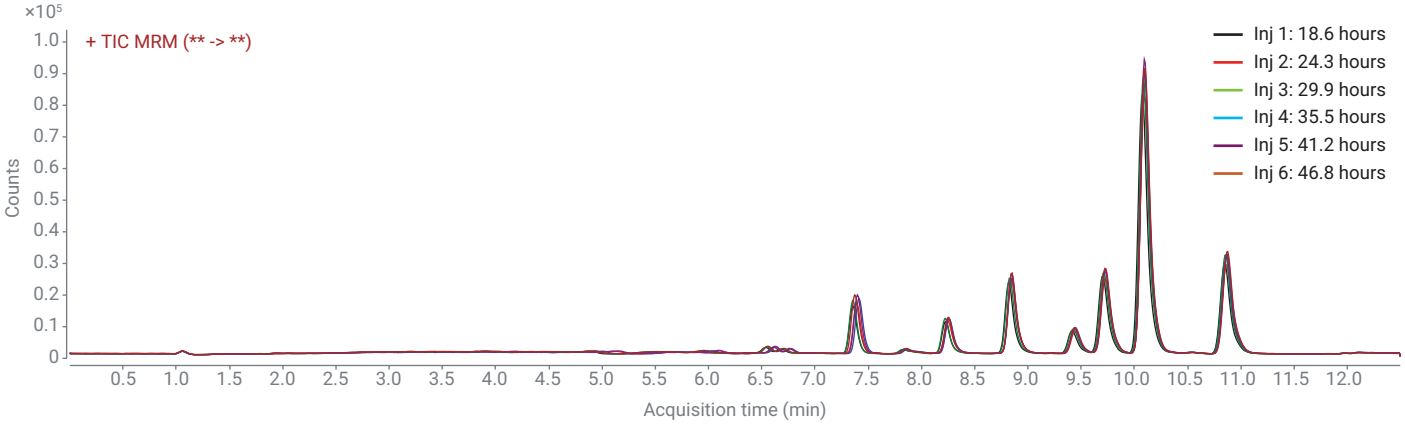

Figure 3. Repeated injection of a honey sample spiked at the 20 µg/kg level performed over a time period of 52 hours.

Table 3. Retention time precision for all individual compounds throughout a 52-hour sequence in different honey samples.

	Dinotefuran	Nitenpyram	Flonicamide	Thiamethoxam	Clothianidin	Imidacloprid	Acetamiprid	Thiacloprid
RT (Min-Max)	7.34 to 7.50	8.23 to 8.28	8.80 to 8.88	8.82 to 8.88	9.42 to 9.48	9.70 to 9.76	10.07 to 10.13	10.84 to 10.90
%RSD on RT	0.34	0.21	0.2	0.19	0.17	0.16	0.15	0.15

Moreover, the peak area for all compounds was stable as is illustrated in Figure 4, where peak areas for the individual neonicotinoids in a spiked honey sample (spiked at 5 and at 20 µg/kg) are plotted over time. Although the %RSD on peak area looks satisfactory, a small trend towards increased peak areas could be observed. Therefore, correction of the peak areas was performed using isotopically labelled internal standard (ISTD), which further improved the data quality (spiking level: 5 µg/kg honey). The %RSD of the peak areas and of the corrected peak areas (peak area of native compound/peak area of internal standard) from the individual injections were calculated and are summarized in Table 4.

Correction for clothianidin, imidacloprid, and acetamiprid was made using their labelled analogue as an internal standard. The use of the labelled analogue for correction is clearly reflected in the %RSD values.

Matrix effects (either signal suppression or enhancement) were evaluated. The recovery was evaluated in five different commercial honey samples after spiking with standards and internal standards. Recovery was calculated against the result of a water sample spiked at the same concentration (i.e., matrix free). None of the samples contained quantifiable amounts of the neonicotinoids of interest. The limit of quantitation (LOQ) in matrix of the individual compounds was

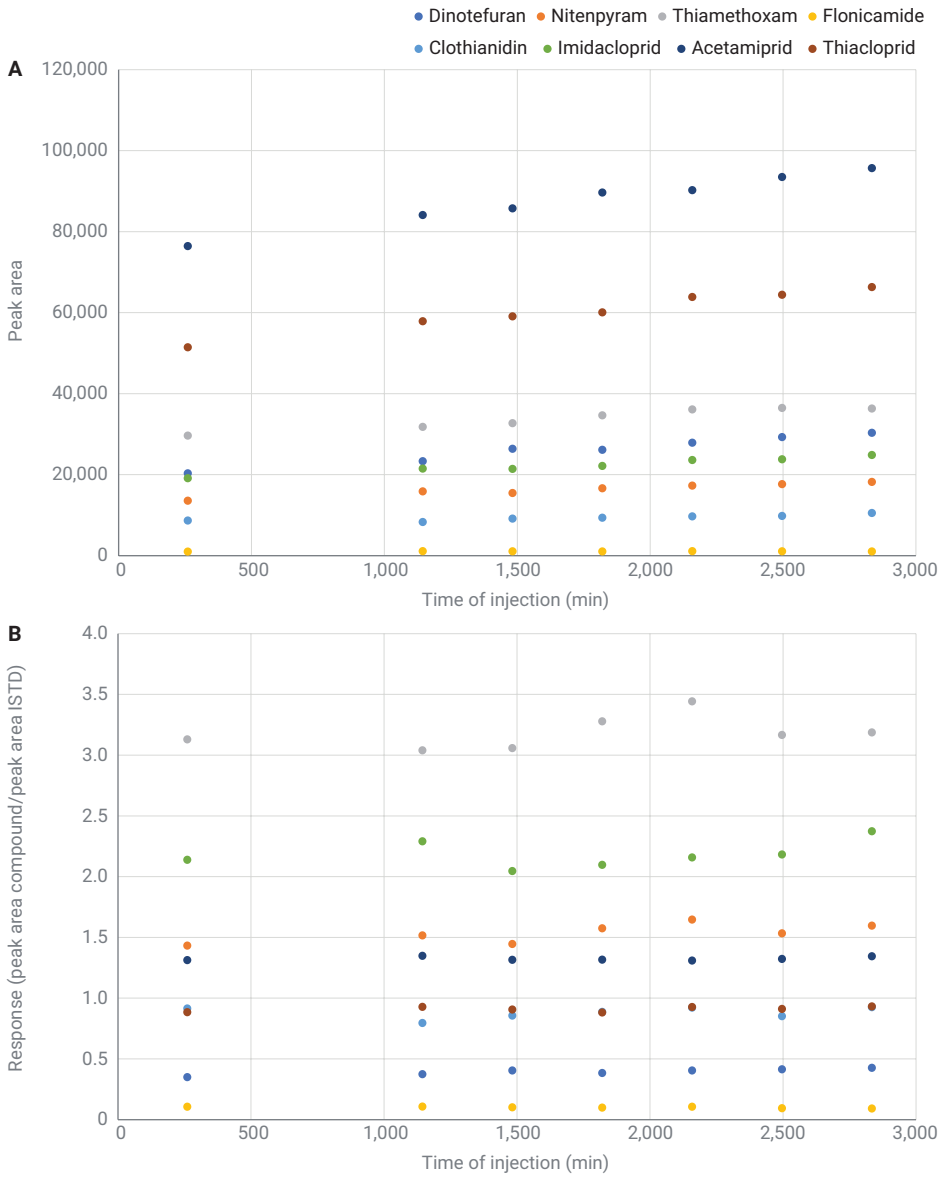

Figure 4. Evolution of peak area (A) and corrected peak area (B) for the individual neonicotinoids in a commercial honey sample spiked at 5 µg/kg. ISTD solution was spiked at 5 µg/kg honey by repeated injection in a 52-hour period.

Table 4. Summary on %RSD calculated on peak area and corrected peak area for the five-fold injection of a honey sample.

	Dinotefuran	Nitenpyram	Flonicamide	Thiamethoxam	Clothianidin	Imidacloprid	Acetamiprid	Thiacloprid
%RSD on Peak Area	13.2%	9.6%	3.7%	7.8%	7.9%	8.6%	7.4%	8.3%
%RSD Corrected Peak Area	2.4%	10.3%	4.8%	6.0%	60%	4.1%	1.7%	2.0%

determined through extrapolation of the signal-to-noise ratio (S/N) obtained for the sample spiked at 5 µg/kg to an S/N = 10. LOQ was 0.5 µg/kg for acetamiprid; 1.5 µg/kg for clothianidin, dinotefuran, and nitenpyram; 2 µg/kg for flonicamid and thiamethoxam; and 1 µg/kg for imidacloprid and thiacloprid. The results of the spiked samples were compared with a reference solution containing the same concentration of the individual neonicotinoids and internal standards. The resulting recoveries for spiking at the 5 µg/kg level are indicated in Table 5. This level is below the maximum residue level of all these neonicotinoids, which typically ranges from 10 to 200 µg/kg. The labeled neonicotinoid used for correction is indicated in brackets. Recoveries between 70 and 130% are indicated in bold. Compounds where a labeled analog standard was used for correction showed better results compared with standards where the isotopically labeled compound was not available.

Clothianidin, imidacloprid, and acetamiprid recoveries ranged between 95 and 112%. Using labelled analogues or a matrix-matched calibration curve would be beneficial in terms of recovery for the other neonicotinoids (dinotefuran, nitenpyram, thiamethoxam, and thiacloprid). In Figure 5, an overlay is presented of the quantifier ions detected in a spiked honey sample and in the reference standard solution. All compounds are annotated and the recovery for every individual compound based on its peak area (i.e., without correction of internal standard) is shown. Recoveries without correction vary from 53 to 105%. It is clear that the matrix effect on ionization is not sufficiently counteracted when the internal standard is not the labeled analog of the compound of interest.

Table 5. Recovery (%) for eight different neonicotinoids spiked at the 5 µg/kg level in five different honey samples (A-E).

	Dinotefuran (ISTD: acetamiprid-d3)	Nitenpyram (ISTD: acetameprid-d3)	Thiamethoxam (clothianidin-d3)	Flonicamid (ISTD: clothianidin-d3)	Clothianidin (ISTD: clothianidin-d3	Imidacloprid (ISTD: imidacloprid-d4)	Acetamiprid (ISTD: acetamiprid-d3)	Thiacloprid (ISTD: acetamiprid-d3)
Honey A	32	93	139	128	112	104	98	79
Honey B	26	78	115	89	96	102	95	74
Honey C	45	70	100	97	104	96	99	79
Honey D	54	64	103	93	101	102	100	71
Honey E	50	75	95	92	100	100	97	75

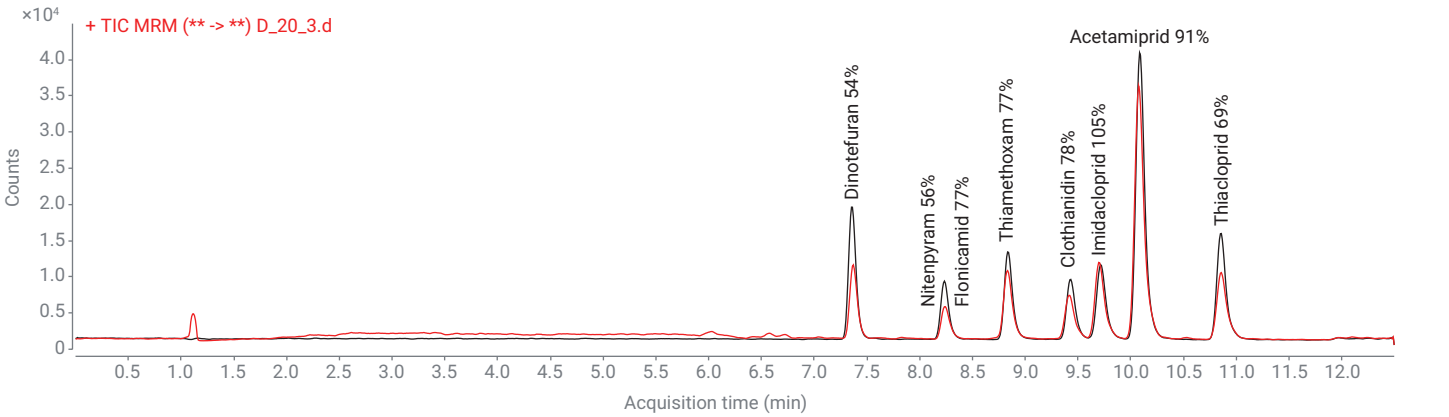

Figure 5. Overlay of a spiked honey sample (5 µg/kg) (red) and a reference standard solution (same concentration in solution) (black) with indication of the recoveries based on uncorrected peak areas of the individual compounds. Acetamiprid-d3 was used for correction of acetamiprid, dinotefuran, nitenpyram, and thiacloprid; clothianidin-d3 was used for correction of thiamethoxam, clothianidin, and flonicamid; and imidacloprid-d4 was used for imidacloprid correction.

Conclusion

The combination of an Agilent 1290 Infinity II LC and Agilent 6470 triple quadrupole LC/MS has proven its status as a workhorse in quantitative analysis. The system can handle long sequences (multiple days) with injection of complex and heavy matrix samples (in this case honey) without loss of performance. Although the ESI source was significantly contaminated by the honey matrix, chromatographic profiles were repeatable and %RSD on peak areas and corrected areas for all individual compounds were below 15% and below 10.5%, respectively, over a 52-hour time span. %RSD on retention time was below 0.5% over the entire sequence, demonstrating the superior retention time precision of the Agilent 1290 Infinity II High-Speed Pump. Concentrations of neonicotinoids as low as 5 µg/kg could be determined with minimum sample preparation. The robustness, selectivity, and sensitivity of the system allowed the direct injection of the samples after simple dilution. Recoveries for compounds where peak area was corrected with an isotopically labeled analog ranged from 95 to 112%. Compounds not corrected by their labeled analog showed recoveries mainly ranging from 70 to 130%. This can be improved using labeled analog internal standards or by using a matrix-matched calibration curve.

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DE44473.0393171296

Printed in the USA, October 15, 2024
5994-4263EN

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