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Analysis of 510 Pesticide Residues in Honey and Onion on an Agilent 6470 Triple Quadrupole LC/MS System

Pesticides residue workflow for high sugar content and high water content samples

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Abstract

This application note extends the previously developed LC/MS pesticides workflow covering over 510 targets in a single analysis using Agilent 1290 Infinity II LC system coupled to a 6470 triple quadrupole LC/MS. The workflow applicability was expanded to honey (high sugar content) and onion (high water content) food matrices. The sample preparation protocol, based on QuEChERS extraction followed by dSPE cleanup, was optimized and used to extract these two different types of matrices.

Matrix effect in terms of ion suppression and enhancement on 510 pesticides was also assessed and discussed. Workflow performance was evaluated according to SANTE/12682/2019 based on method sensitivity, limit of quantitation (LOQ), calibration curve linearity, and recovery and precision using matrix-matched calibration standards. Method precision was evaluated using intralaboratory recovery repeatability (RSD_r) and interlaboratory reproducibility (RSD_{iR}). The method performance demonstrated here provided further evidence on the robustness and transferability of the Agilent LC/MS pesticides workflow across multiple food commodity groups, multiple analysts, and multiple instruments/labs.

Introduction

Pesticides play a significant role in agriculture by mitigating insects, rodents, fungi, and weeds to improve the crop and food production. Regulatory organizations like the World Health Organization (WHO), the Food and Agricultural Organization (FAO), the U.S. Environmental Protection Agency (EPA), and the European Union (EU) have developed and established internationally accepted maximum residue limits (MRL) to protect food consumers from adverse effects of pesticides.

Previously, a comprehensive LC/MS/MS workflow was developed for the reliable analysis of 510 pesticide residues in different types of food commodity groups: tomato (high water content), wheat (high starch content), olive oil (high oil content).[1] It combined a simple and robust sample preparation protocol, a highly sensitive and accurate triple quadrupole LC/MS system, optimized chromatographic separation and MS detection, and full quantitation and results interpretation protocols to meet regulatory requirements for a wide range of pesticide compounds. This workflow is now extended and verified on two additional matrices: honey (high sugar content) and onion (high water content) based on SANTE/12682/2019.[2]

This efficient analytical workflow will accelerate lab throughput and productivity by streamlining the routine process of pesticide analysis.

The LC/MS/MS method and a method protocol with details on sample preparation, acquisition, and data analysis steps are available from Agilent.[3]

Experimental

Chemicals and reagents

Agilent LC/MS-grade acetonitrile (ACN) (part number 5191-4496), methanol (MeOH) (part number 5191-4497), and water were used for the study. LC/MS-grade formic acid and ammonium formate were purchased from Sigma-Aldrich. All other solvents used were HPLC-grade from Sigma-Aldrich.

Standards and solutions

The pesticide standards, including 10 custom standard mix, were from Agilent Ultra (Rhode Island, USA) as listed in Table 1.[4]

An intermediate standard mix comprised of 510 targets at a concentration of 1,000 µg/L was prepared in ACN from stock standard solutions and used for the rest of experiment.

Solvent calibration standards were prepared in ACN for the matrix effect assessment.[2] The intermediate standard solution 1,000 µg/L was diluted appropriately to make the seven calibration concentration levels of 1, 2, 5, 10, 25, 50, and 100 µg/L in ACN. Calibration standard solutions must be freshly prepared and stored in the refrigerator at 4 °C if not immediately used.

Sample preparation

Organic honey and brown onion were purchased from local grocery stores. The onion was chopped and prefrozen at −20 °C. The frozen onion was then homogenized using a domestic blender and stored in the refrigerator at −20 °C.

The following products and equipment were used for sample preparation:

- Agilent BondElut QuEChERS EN extraction kits (part number 5982-5650CH)

- Agilent BondElut QuEChERS EN dispersive SPE kits (part number 5982-5056)

- Geno/Grinder (SPEX, Metuchen, NJ, USA)

- Centrifuge (Eppendorf, Centrifuge 5804R and 5430R)

- Vortexer and multitube vortexer (VWR, Plainfield, NJ, USA)

Table 1. Agilent Ultra custom premixed pesticide standards.

Vendor	Part Number	Part Description	Analyte Concentration	Matrix	No. of Vials	Total No. of Analytes
Agilent Ultra (Rhode Island, USA)	5190-0551	LC/MS pesticide comprehensive test mix	100 µg/mL	Acetonitrile	8	254
	CUS-00000635	Custom pesticide test mix 1	100 µg/mL	Acetonitrile	1	27
	CUS-00000636	Custom pesticide test mix 2	100 µg/mL	Acetonitrile	1	26
	CUS-00000637	Custom pesticide test mix 3	100 µg/mL	Acetonitrile	1	27
	CUS-00000638	Custom pesticide test mix 4	100 µg/mL	Acetonitrile	1	28
	CUS-00000639	Custom pesticide test mix 5	100 µg/mL	Acetonitrile	1	25
	CUS-00000640	Custom pesticide test mix 6	100 µg/mL	Acetonitrile	1	26
	CUS-00000641	Custom pesticide test mix 7	100 µg/mL	Acetonitrile	1	28
	CUS-00000642	Custom pesticide test mix 8	100 µg/mL	Acetonitrile	1	29
	CUS-00000643	Custom pesticide test mix 9	100 µg/mL	Acetonitrile	1	30
	CUS-00004663	Custom pesticide test mix 10	100 µg/mL	Acetonitrile	1	26

A 5 ±0.1 g portion of honey, and 10 ±0.1 g homogenized fresh onion were weighed into a 50 mL conical polypropylene tube, respectively. Prespiked QC samples were made by fortifying an appropriate volume of intermediate pesticide standard solution (1,000 µg/L) into matrix blank to generate the level of 10 µg/kg. After spiking standard into the matrix, the samples were capped tightly, vortexed, and equilibrated for 15 to 20 minutes to allow the analytes infiltrating the sample matrix. An aliquot of 8 mL water was added to the honey sample. An aliquot of 10 mL ACN was added to the tubes. QuEChERS extraction EN salts, including the ceramic homogenizer, were then added into the matrix. Tubes were capped and vigorously shaken using Gino Grinder. All the samples were then centrifuged at 4,000 rpm for 5 minutes. An aliquot of 6 mL supernatant was transferred to the 15 mL dSPE tube. Tubes were capped and vortexed for 3 minutes and then centrifuged for 5 minutes. The supernatant was then ready for instrument analysis. The preparation procedure is illustrated in Figure 1.

Preparation of matrix-matched calibration standards

Matrix-matched calibration standards were prepared by postspiking the intermediate standard solution into matrix blank. Preparation of matrix-matched calibration levels was identical to solvent standards preparation, except using matrix blank instead ACN solvent blank.

Instrumentation

The chromatographic separation was conducted by ZORBAX RRHD Eclipse Plus C18, 2.1 × 150 mm, and 1.8 µm column (part number 959759-902) installed on An Agilent 1290 Infinity II LC system, which included:

– Agilent 1290 Infinity II high-speed pump (G4220A)

– Agilent 1290 Infinity II autosampler (G4226A)

– Agilent 1290 Infinity II thermostatted column compartment (G1316C)

An Agilent 6470 LC/TQ mass spectrometer equipped with an Agilent Jet Stream (AJS) electrospray ion source was operated in dynamic MRM (dMRM) mode. The LC/TQ autotune was performed in unit and wide modes. All data acquisition and processing were performed using the Agilent MassHunter software (version 10.1 or higher).

The LC system conditions and TQ parameters used in the workflow is available in the previous study.[1,3]

Results and discussion

Development of LC/TQ method

The development of dMRM method for 510 pesticide compounds, was optimized using the MassHunter optimizer software by flow injection.[1] Two or three target-specific MRM transitions were selected per pesticide (except for EPTC and procymidone where only one transition was stable enough to be monitored) to meet the regulatory requirements for the identification and confirmation by LC/MS/MS.[2]
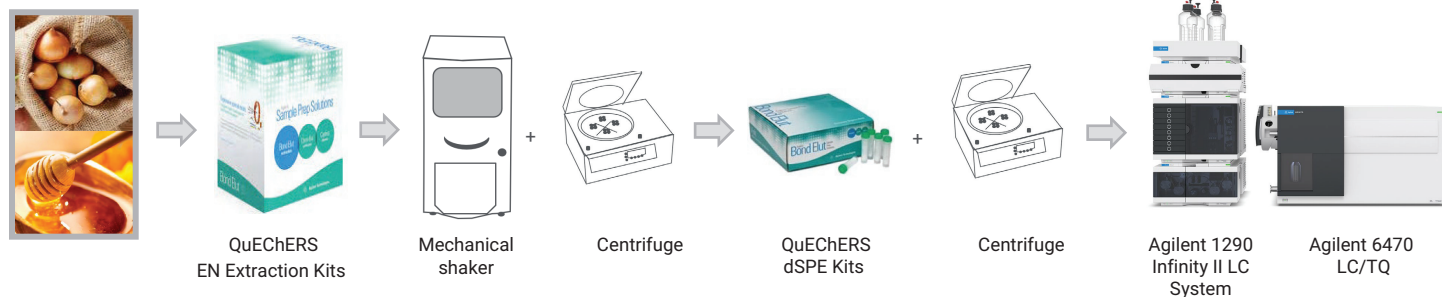

Figure 1. Sample preparation procedure for honey and onion samples.

QuEChERS EN Extraction Kits — Mechanical shaker — + Centrifuge — QuEChERS dSPE Kits — + Centrifuge — Agilent 1290 Infinity II LC System — Agilent 6470 LC/TQ

Figure 2 shows a representative MRM chromatogram for all 510 pesticides postspiked QC at 10 µg/L in honey matrix blank extract. The symmetric sharp peaks demonstrate the efficient chromatographic separation of targets within the retention time window.

Compounds including acephate, omethoate, methamidophos and pymetrozine showed split peaks in both honey and onion marices because of solvent effects on early eluted analytes caused by high organic sample injection. Split peaks were also observed for: brodifacoum, difenoconazole, etaconazole, halfenprox, iprovalicarb, orbencarb, phosphamidon, propamocarb, resmethrin, thiobencarb, thiofanox sulfone, and triadimenol because of

unresolved isomers. Peak fronting, tailing or broadening were consistently found in butachlor, cycloprothrin, dimethachlor, imazamox, oxadixyl, pretilachlor, and tridemorph in honey and onion. Potential possible causes resulting in the aforementioned peak shape distortation include analytes feature on LC column, separation conditions, solvent effect and sample matrix impact. No further optimization was necessary for such large panel pesticides (>500) analysis in this study, plus peak integration was not impacted and integration was consistent. If the peak shape of these compounds is critical, the Agilent application note from Katerina Mastovska can be used for further evaluation.[5]

Matrix effect assessment

Matrix effect (ME) was assessed at the initial method development stage. ME was obtained by the ratio of target response in matrix-matched standards to that in corresponding solvent standards. More than 20% signal suppression or enhancement in the matrix is classified as significant ME, which can be compensated by matrix-matched calibration curve.[2] In this study, ME at 10 µg/L, which is the MRL for all 510 pesticides, was used for method evaluation.

Approximately 15% of 510 targets in honey showed significant ME at 10 µg/L. For analytes with significant ME in the honey matrix, most of them showed ion suppression. As for onion, significant ME was observed for around 70% of all 510 pesticides at 10 µg/L, and a majority of compounds demonstrated significant ion suppression as well.
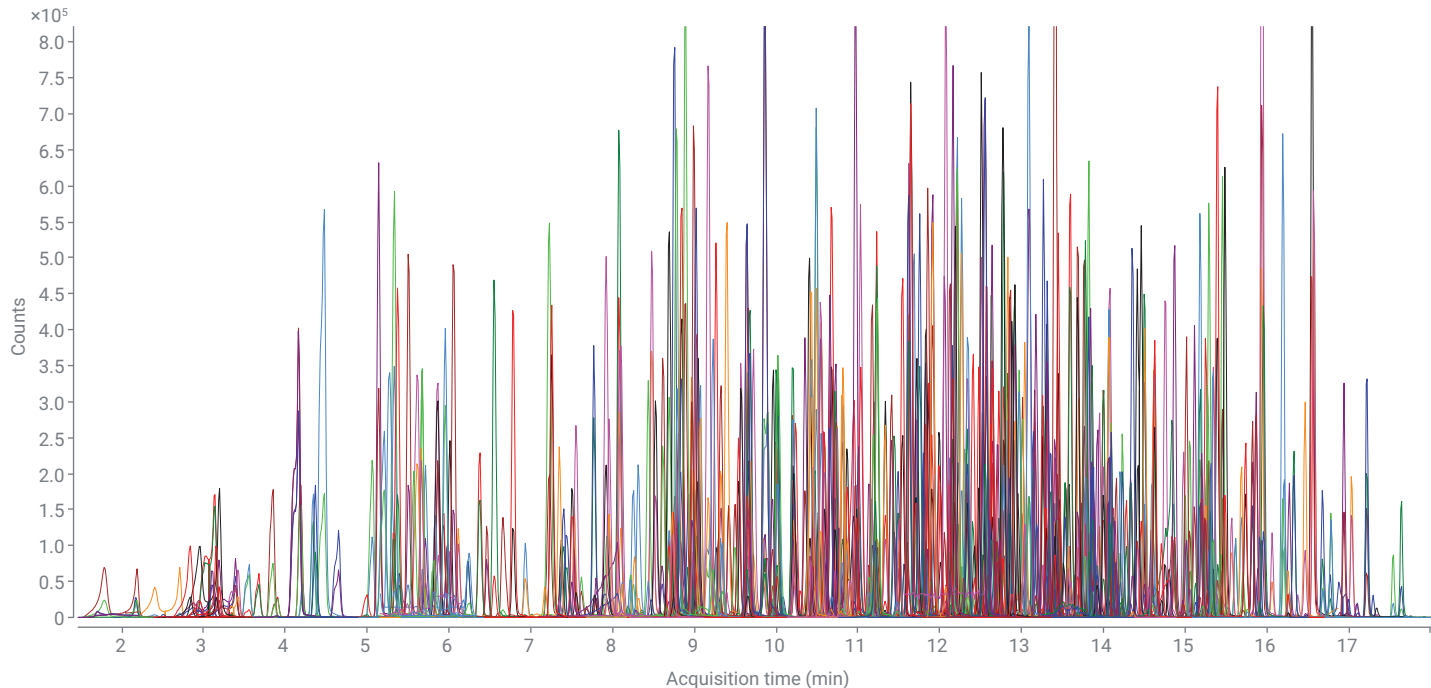

Figure 2. Representative MRM chromatogram of 510 pesticides postspiked QC at 10 µg/L in honey matrix extract.

Some targets showed similar ME in different matrices, while others showed quite different ME in onion and honey. For example, the ME of sulfadiazole in onion was insignificant, while was ~40% ion suppression in honey (Figure 3A). Similarly, the ME of benoxacor in honey was insignificant, but was ~47% in onion (Figure 3B). However, ME of dimoxytrobin was equivalently insignificant in both honey and onion (Figure 3C). Based on the result of the ME study, it is highly recommended to use matrix-matched calibration standards to achieve more reliable and consistent quantitation results.

Verification of workflow performance

The workflow performance was evaluated based on calibration curve linearity, method sensitivity, precision, and recovery according to SANTE guidelines.[2] Two batches of analyses including intra and interlaboratory were carried out for each matrix. The batch run for each sample matrix included solvent blank, matrix-matched calibration standards, matrix blank and prespiked QCs. Six replicates of prespiked QCs were prepared for per batch.

1) Calibration curve linearity: The linearity of calibration curve for all targets was evaluated using matrix-matched standards from 1 to 100 µg/L. Various regression models were evaluated to determine the best linearity response function, and the best calibration model was with Type: Linear, Origin: Ignore, Weight: $1/x^2$. A few compounds showed better linearity with $1/x$ weight. Overall, more than 95% targets met the calibration curve linearity requirement of $R^2 \geq 0.99$ from LOQ to 100 µg/L, while a range from LOQ to 50 µg/L was applied for some compounds due to saturation at 100 µg/L. The accuracy of linearity was also assessed based on the deviation of back calculated concentration from true concentration at all calibration levels. For example, more than 98% of 510 compounds met the deviation of 20% at calibration level 4 in both honey and onion extract.

2) Instrument limit of detection (LOD) and workflow limit of quantification (LOQ): Instrument LOD and the analytical workflow LOQ were used to evaluate the method sensitivity. Instrument LOD was established based

on matrix-matched lowest calibration standard at signal-to-noise ratio (S/N) of three and up, defined by the peak height and auto-RMS algorithm embedded in Agilent MassHunter Quantitative analysis software. The instrument method provided LOD ≤10 µg/L for over 99% of 510 targets in honey and onion, demonstrating the high sensitivity of Agilent 6470 LC/TQ mass spectrometer. Furthermore, 85% of 510 targets in honey and 82% of 510 tragets in onion showed LOD ≤1 µg/L.The analytical workflow LOQ was obtained from the prespiked samples at lowest level providing S/N of 10 and up, with additional consideration of target selectivity in sample matrix, and analyte recovery and reproducibility in matrix.[2] Repeatability was evaluated based on six replicates of prespiked QCs at 10 µg/kg, and %RSD was less than or equal to 20%. Figures 4A and 4B show the LC/MS/MS MRM chromatograms overlay of 6-benzylaminopurine, bensulide, and pyridalyl for six technical replicates at prespiked QC 10 µg/kg in honey and onion, respectively. This indicates high sensitivity and good precision at MRL level across two matrices.
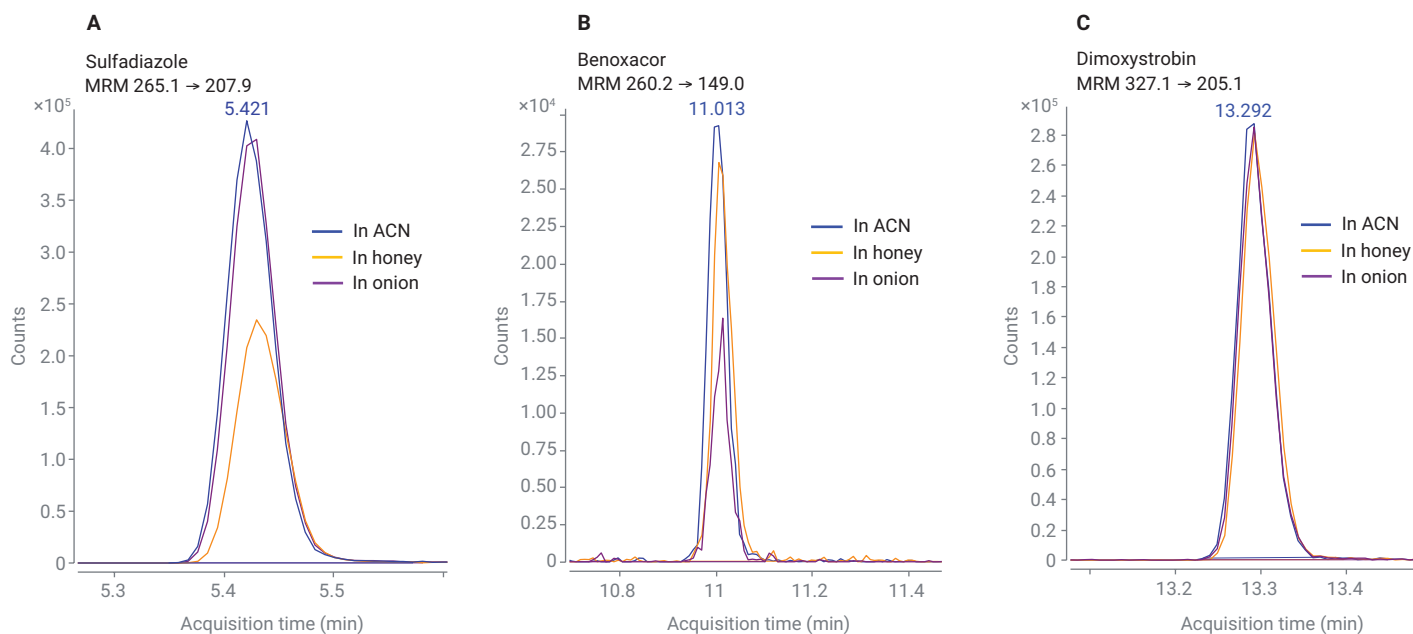

Figure 3. LC/MS/MS MRM chromatograms overlay of sulfadiazole (A), benoxacor (B), and dimoxytrobin (C) at 10 ppb in ACN solvent standard, honey, and onion extract.
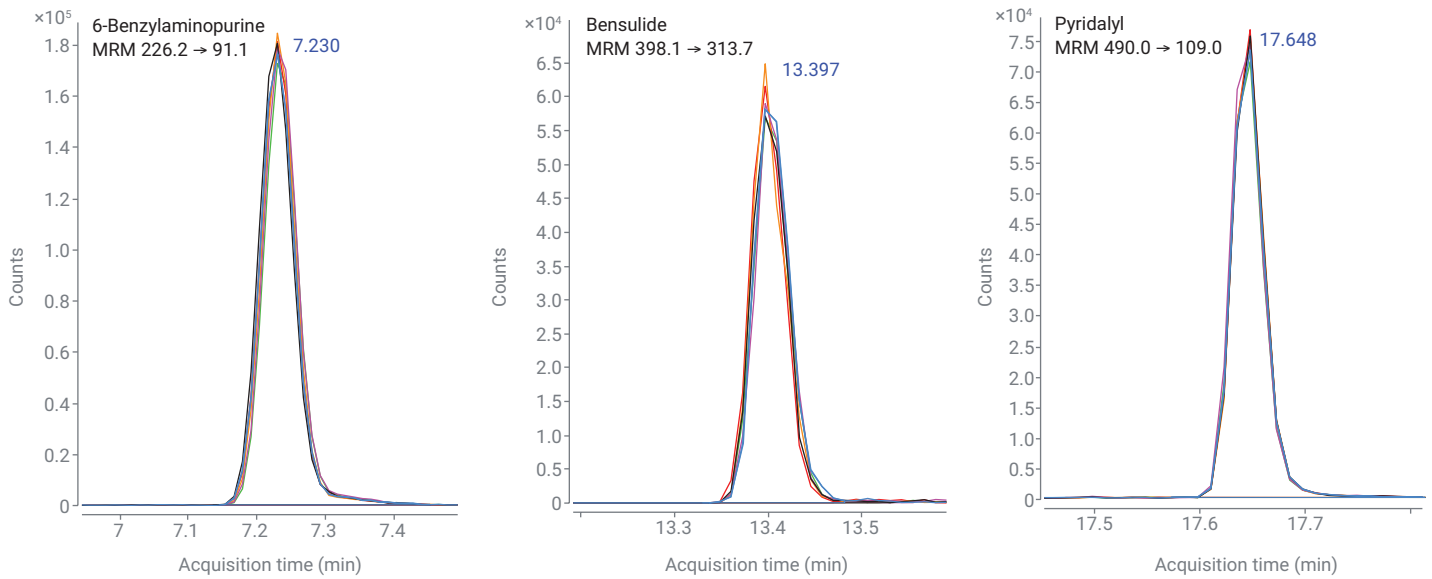
Figure 4A. LC/MS/MS MRM chromatograms overlay of 6-benzylaminopurine, bensulide and pyridalyl for six techincal replicates at 10 µg/kg (prespiked QC) in honey matrix.
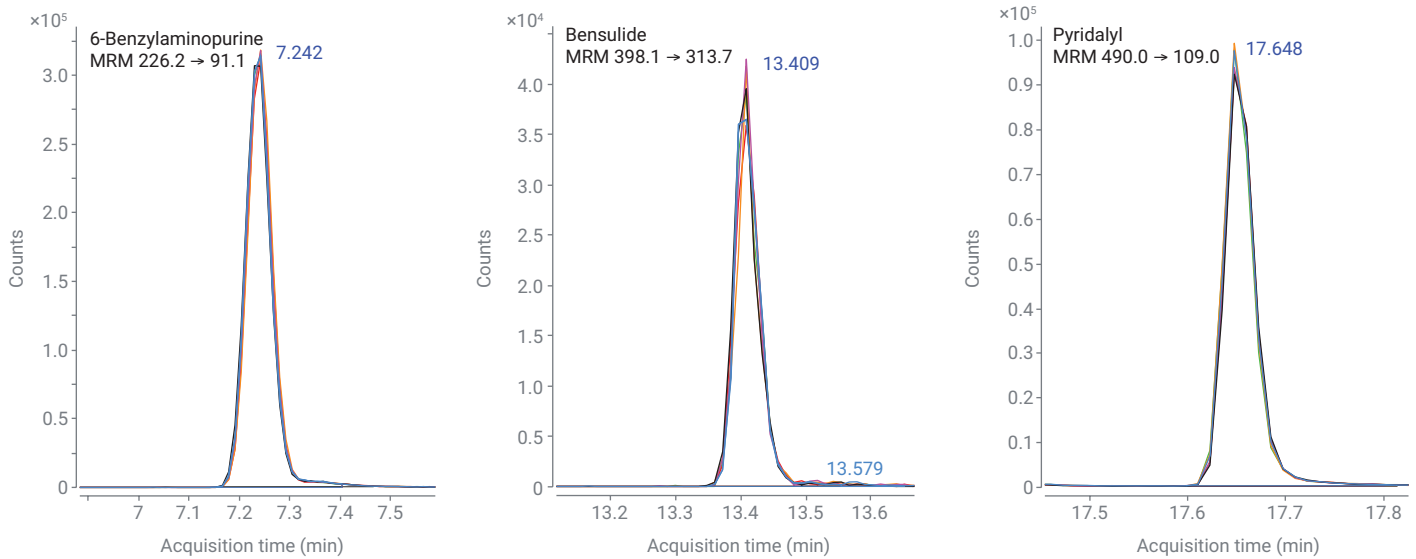

Figure 4B. LC/MS/MS MRM chromatograms overlay of 6-benzylaminopurine, bensulide and pyridalyl for six technical replicates at 10 µg/kg (prespiked QC) in onion matrix.

3) Method precision and recovery:

Method precision was demonstrated using intralaboratory study (RSD_r) and interlaboratory study (RSD_{iR}) based on technical replicates of prespiked QC at 10 µg/kg in two matrices. RSD_r % was calculated based on the recoveries of six replicates of prespiked QCs within a batch (intralaboratory). RSD_{iR} % was calculated based on the recoveries of 12 replicates of QCs across two batches, prepared by different personnel in different labs, and run on two different LC/TQ instruments.

Over 91% targets in honey and over 95% targets in onion provided ≤20% in RSD_r, demonstrating good method consistency.

Interlaboratory reproducibility study was conducted in consideration of potential introduction of variations caused by personnel, LC/TQ systems, sample preparation products, analytical columns, and sample matrix. Among 510 targets, over 90% targets in honey and 92% of targets in onion gave out ≤20% RSD_{iR}. The results were comparable with those from the previous study on tomato, wheat, and olive oil.[1] These results confirm the precision of workflow performance across different commodity groups of sample matrices under different experimental conditions.

Variation of retention time (RT) for all targets within the batch was also monitored to evaluate the precision of the chromatographic method. RT tolerance of all targets for each matrix was within ±0.1 minutes. Figure 5 shows TIC overlay of triplicate injections of postspiked QC at 10 µg/L in honey matrix extract, which confirms the reproducibility of the elution profile and of the MS detection.

Targets recovery is a critical parameter for the quantitative analytical workflow method evaluation. Prespiked QCs at 10 µg/kg was used to evaluate the targets recovery in the two matrices. Recovery was calculated based on the ratio of analyte response in prespiked QCs (n = 6) to that in corresponding level of matrix-matched standard. According to SANTE/12682/2019, acceptable average recoveries should be within the range of 40 to 120% if they are consistent (RSD_r ≤20%). Based on these criteria, the average recovery results of 88% targets in honey and 93% targets in onion met the acceptance criteria. Furthermore, 70 to 120% recovery was achieved for ≥84% of targets in both matrices. Figure 6A and 6B shows the recovery distribution of 510 targets in honey and onion.
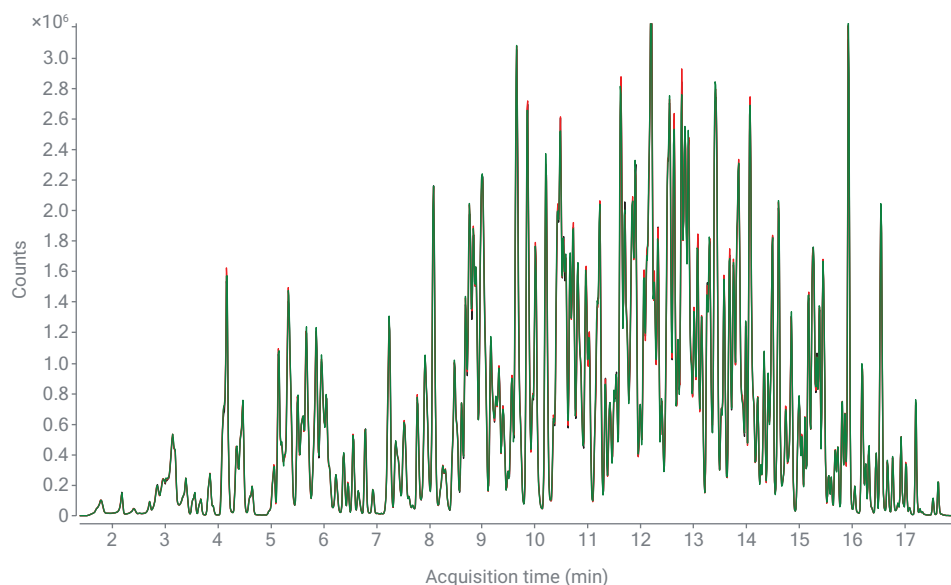

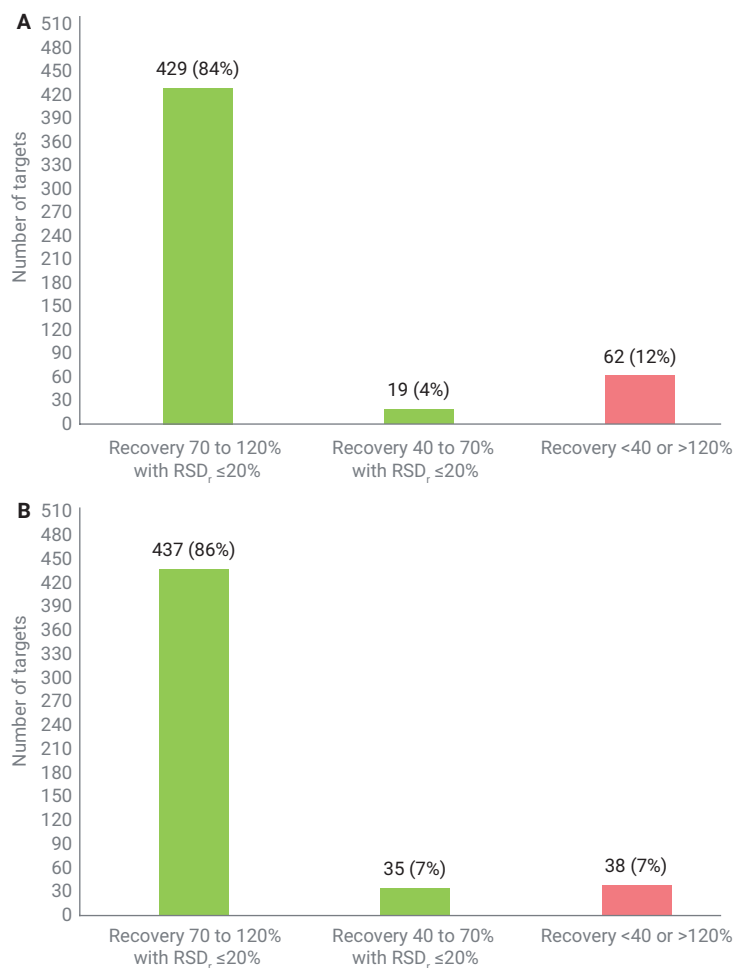**Figure 5.** TIC overlay of triplicate injections of postspiked QC at 10 µg/L in honey matrix extract.

Figure 6. Recovery distribution of all 510 targets in honey (A) and onion (B) at prespiked QC 10 µg/kg.

4) Robustness assessment: Instrument method robustness is important for reliable analysis as part of routine, day-to-day laboratory testing. Here, a method robustness study was designed to run 15 injections of postspiked QCs at 50 µg/L in honey and onion extract each day, for three consecutive days. The result of these representative compounds in the honey matrix is displayed in Figure 7. The calculated concentrations based on the calibration curve from each batch (or day) were observed with RSD <4%. The 33 pesticides demonstrated were selected to represent compounds with different features, including different pesticides classes and different polarities, to cover the entire retention window. The results demonstrate excellent instrument method robustness for sustainable and reliable, day-to-day, routine analyses.
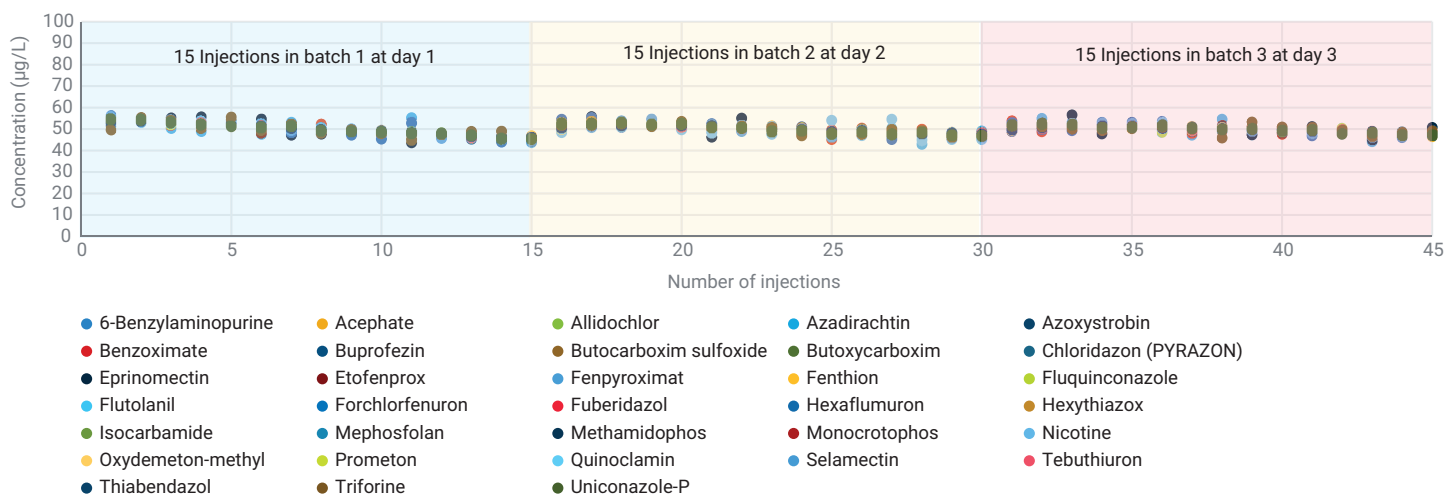

Figure 7. Calculated concentrations of 33 representative compounds for repeated three consecutive days in honey extract spiked at 50 µg/L.

Conclusion

The workflow method sensitivity offered a sub-10 ng/mL limit of detection (LOD) for 99% of 510 analytes. Calibration curves were plotted from LOQ to 100 µg/L, where over 95% of analytes demonstrated linearity $R^2 \geq 0.99$. Fortified quality control samples (matrix-spiked QC) at 10 µg/kg in honey and onion were used to assess extraction efficiency and method recovery. Method precision was evaluated using intralaboratory recovery repeatability (RSD_r) and interlaboratory reproducibility (RSD_{iR}). 88% targets in honey and 93% targets in onion fell within 40 to 120% recovery, with RSD_r within the limit of 20% in honey and onion.

An analytical workflow for the sensitive and reliable quantitation of 510 pesticide residues in honey and onion matrices was described and evaluated in this study. The dMRM method was applied from the previous study for the aqcusition of all targets.[1] QuECheRS extraction and dSPE cleanup were used to prepare honey and onion samples.

The Agilent 1290 Infinity II LC coupled to the Agilent 6470 triple quadrupole LC/MS is easily and readily scalable to Agilent 6495 for achieving additional sensitivity when needed. The separation using Agilent ZORBAX RRHD Eclipse Plus C18 column offered a 20-minute chromatography and even RT distribution for all targets.

The workflow performance was verified in honey and onion matrices based on matrix-matched calibration curve linearity, instrument LOD and workflow LOQ, recovery and precision. The results across intra- and interlaboratory studies were reproducible for honey and onion. In alignment with previous study, the developed pesticides workflow is applicable for more than 500 pesticide residues analysis in various food matrices.

References

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