Application Note

Food Testing and Agriculture

Comprehensive LC/MS/MS Workflow of Pesticide Residues in Food Using the Agilent 6470 Triple Quadrupole LC/MS System

Pesticides residue workflow in high water content, high oil content, and high starch content samples

Authors

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Abstract

A comprehensive LC/MS/MS workflow was developed for the quantitation of 510 pesticide residues with the intention to accelerate and simplify routine laboratory food testing. Compound transitions and optimized parameters were developed based on the Agilent Pesticide Dynamic MRM Database, which has over 750 pesticides including curated parameters for fast and easy transfer into the analytical method. The workflow includes sample preparation, chromatographic separation, mass spectrometry (MS) detection, data analysis, and interpretation. The workflow applicability was demonstrated using an Agilent 1290 Infinity II LC system coupled to an Agilent 6470 triple quadrupole LC/MS on three food matrices with different content types: tomato (high water content), wheat (high starch content), and olive oil (high oil content). For sample preparation of the tomato and wheat samples, an Agilent QuEChERS kit was used with dSPE cleanup. Extraction was performed with the QuEChERS kit followed by Agilent Captiva EMR—Lipid cleanup for preparing olive oil samples.

Workflow performance was evaluated and verified according to SANTE/12682/2019 based on limit of detection (LOD) and limit of quantitation (LOQ), calibration curve linearity, and recovery and precision using matrix-matched calibration standards from 1 to 100 µg/L. Over 95% of analytes demonstrated linearity with R^2 ≥0.99, with calibration curves plotted from LOQ to 50 or 100 µg/L. Method precision was assessed using recovery repeatability (RSD_r) and intralaboratory reproducibility (RSD_{iR}). It was assessed at three levels of fortified quality control (QC) samples at 1, 5, and 10 µg/kg in three matrices. RSD_r and RSD_{iR} at 10 µg/kg for 90% of compounds were within the limit of 20%. The method performance across tomato, wheat, and olive oil matrices demonstrated the method applicability for quantitative analysis of multiresidue pesticides in high water, high oil, and high starch contents with potential implication for use on other food matrices.

Introduction

Pesticides used to protect crops from disease or harmful organisms during production, storage, and transportation have potential toxicity. Pesticide residues remaining in or on commodities such as vegetables, fruits, herbs, honey, oil seeds, cereals, and food of animal origin can cause adverse health effects and environmental concerns as well. Organizations including 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 published policy statements to guide agricultural organizations on the proper use of pesticides. For example, according to EU regulation, a maximum residue level (MRL) is the highest level of a pesticide residue legally tolerated in or on food or animal feed when pesticides are applied.[1] The amount of pesticide residues allowed in food must be as low as possible to ensure food safety for consumers. Ten µg/kg (10 ppb) is the MRL for most pesticides except for explicitly prohibited compounds.

This points to the demand and need for highly sensitive analysis methods of multiresidue pesticides in food matrices.

High performance liquid chromatography coupled to triple quadrupole mass spectrometry (LC/TQ) is a widely accepted modern technique that works with a broad range of pesticides for quantitative analysis. This is because of its high sensitivity, selectivity, and accuracy that ensure high quality data for meeting MRL requirements in complex food matrices. A comprehensive LC/MS/MS workflow has been developed for an accurate and reliable analysis of more than 500 pesticide residues in various plant origin food matrices. This workflow, including sample preparation, chromatographic separation, and MS detection targets quantitation and results interpretation, helps streamline routine pesticide analysis, and therefore accelerates lab throughput and productivity.

The LC/TQ method and a method protocol with details on sample preparation, acquisition, and data analysis steps are available from Agilent.[2]

Experimental

Chemicals and reagents

Agilent LC/MS-grade acetonitrile (ACN), methanol (MeOH), 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 ready-to-use and custom premixed pesticide standards were acquired from the vendors listed in Table 1.[3]

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. Working standard solutions at 50 µg/L and 500 µg/L were diluted from the intermediate standard solution and used for the preparation of prespiked QCs.

Solvent calibration standards were prepared in ACN for the purpose of matrix effect assessment.[1] Serial dilutions were done from 1000 µg/L

Table 1. 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
Accustandard (Connecticut, USA)	ACCU S-85870-R1-10X	Custom pesticide test mix #10	100 µg/mL	Acetonitrile	1	26

intermediate standard to prepare seven calibration concentration levels of 1, 2, 5, 10, 25, 50 and 100 µg/L into Eppendorf tubes. Calibration standard solutions must be prepared freshly and stored in the refrigerator at 4 °C if not used immediately.

Sample preparation

Pesticide-free and organically labeled fresh tomato, wheat powder, and olive oil were obtained from local grocery stores. The tomato was homogenized using a domestic blender and stored in the refrigerator at 4 °C if it was unable to be analyzed immediately.

The following products and equipment were used for sample preparation:

- Agilent QuEChERS EN extraction kits (part number 5982-5650CH)
- Agilent universal QuEChERS dispersive SPE kits (part number 5982-0028)
- Agilent Captiva EMR—Lipid 6 mL cartridges (part number 5190-1004)
- Agilent positive pressure manifold PPM-48 processor (part number 5191-4101)
- Geno/Grinder (SPEX, Metuchen, NJ, USA)
- Centrifuge (Eppendorf, Centrifuge 5804R and 5430R)
- Vortexer and multitube vortexer (VWR, Plainfield, NJ, USA)

Ten ±0.1 g of homogenized fresh tomato, 2 ±0.1 g of dry wheat powder, and 5 ±0.1 g of olive oil were weighed into a 50 mL tube, respectively. Prespiked QC samples were fortified by spiking an appropriate amount of pesticide working standard solution to make low QC at 1.0 µg/kg (LQC), mid QC at 5.0 µg/kg (MQC), and high QC at 10.0 µg/kg (HQC) solutions. After spiking standard into the matrix, the samples were capped tightly, vortexed, and equilibrated for 15 to 20 minutes. It was recommended to add water to the dry wheat powder before extraction to improve the extraction efficiency of low moisture commodities. QuEChERS extraction followed by universal dSPE cleanup was applied for tomato and wheat sample preparation, while Captiva EMR—Lipid cleanup was used for olive oil sample preparation with assistance from the Agilent positive pressure manifold PPM-48 processor for eluting. The preparation procedure is illustrated in Figure 1.

Preparation of matrix-matched calibration standards

Matrix-matched calibration standards (postspiked standards) were used and prepared for the assessment of workflow performance in this study. Matrix blank was prepared using unfortified blank samples of tomato, wheat, and olive oil. Preparation of matrix-matched calibration levels was identical to solvent standards preparation by replacing ACN solvent with matrix blank accordingly. The matrix-matched standards were used to evaluate the matrix effect by comparing responses in the corresponding solvent standards.[1]

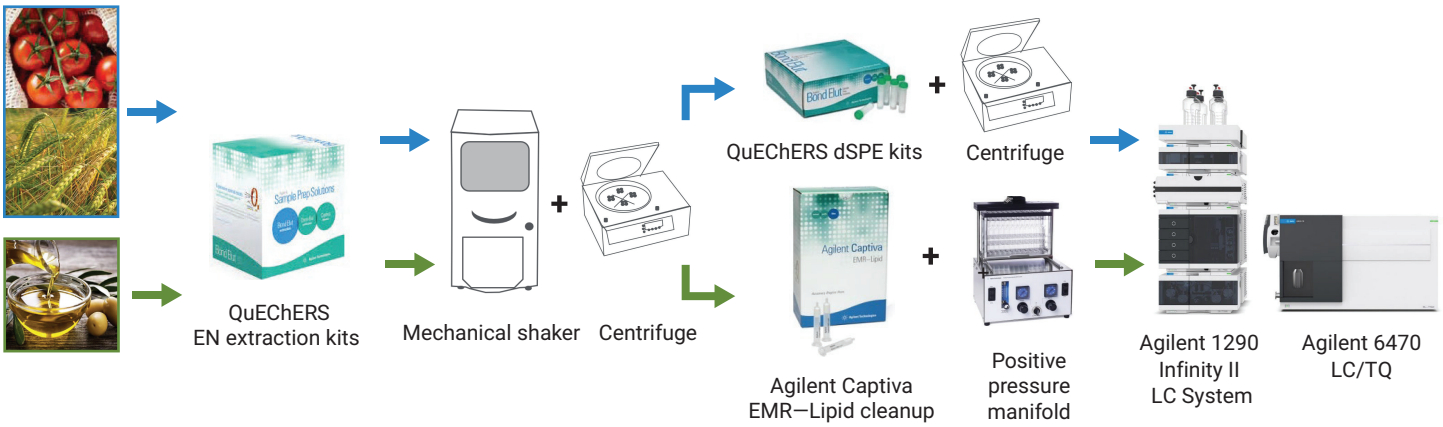

Figure 1. Sample preparation procedure for tomato, wheat, and olive oil samples.

Instrumentation

Chromatographic separation was performed using an Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm column (part number 959759-902) installed on an Agilent 1290 Infinity II LC system.

The individual modules of the 1290 Infinity II LC system included:

- Agilent 1290 Infinity II high-speed pump (G4220A)
- Agilent 1290 Infinity II autosampler (G4226A)
- Agilent 1290 Infinity II thermostatted column compartment (G1316C)

The LC system conditions are listed in Table 2.

An Agilent 6470 LC/TQ mass spectrometer 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 8.0 or higher). The 6470 LC/TQ parameters are shown in Table 3.

Results and discussion

Development of LC/TQ method

A major part of this work was the development of dynamic MRM transitions for 510 pesticide compounds. For each compound, MRM transitions, as well as fragmentor voltages, collision energies, and ionization polarity were optimized using Agilent MassHunter optimizer software by flow injection. The four most abundant product ions per compound were selected automatically. More than 1,000 MRM transitions from 510 pesticides were stored in the dMRM method. Depending on the fragmentation behavior of the individual compound, 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). This was done to satisfy regulatory requirements for identification and confirmation by LC/MS/MS.[1] The two most abundant fragments were defined as primary transitions that were acquired over the retention time window and subsequently used as the quantifier and qualifier ion.

The chromatographic method was optimized using the ZORBAX RRHD Eclipse Plus C18 column, which resulted in good separation and distribution of 510 pesticide residues within a 20-minute HPLC gradient. The 0.4 mL/min flow rate offered effective desolvation of target ions using the AJS ion source. A dMRM method with a cycle time of 500 ms was used. Typical chromatographic peak widths observed were between 8 to 12 seconds. Figure 2A shows a representative MRM chromatogram for all 510 pesticide targets postspiked at 10 µg/L in olive oil matrix extract. The dMRM statistics diagram with the concurrent MRMs plot and min/max dwell time is captured in

Table 2. 1290 Infinity II LC conditions.

Parameter	Value
Column	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm (p/n 959759-902)
Column Temperature	40 °C
Injection Volume	2 µL
Autosampler Temperature	10 °C
Mobile Phase A	5 mM ammonium formate in water with 0.1 % formic acid
Mobile Phase B	5 mM ammonium formate in MeOH with 0.1 % formic acid
Mobile Phase Flow Rate	0.4 mL/min
Gradient Program	Time/min %A %B 0 95 5 3 70 30 17 0 100 20 0 100
Postrun	3 minutes
Needle Wash	Standard wash: flush port (12 s)

Table 3. Agilent 6470 LC/TQ parameters.

Parameter	Value
Software Version	Agilent MassHunter version B.08
Ionization Mode	Simultaneous positive/negative ESI with Agilent Jet Stream (AJS)
Scan Type	Dynamic MRM
Cycle Time	500 ms (Total MRMs = 1,023 Min/Max Dwell = 0.90 ms/248.28 ms)
Stop Time	20 minutes
MS1/MS2 Resolution	Unit/Wide
Gas Temperature	200 °C
Gas Flow	9 L/min
Nebulizer	35 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	2,500 (+)/3,000 (–) V
Nozzle Voltage	0 V

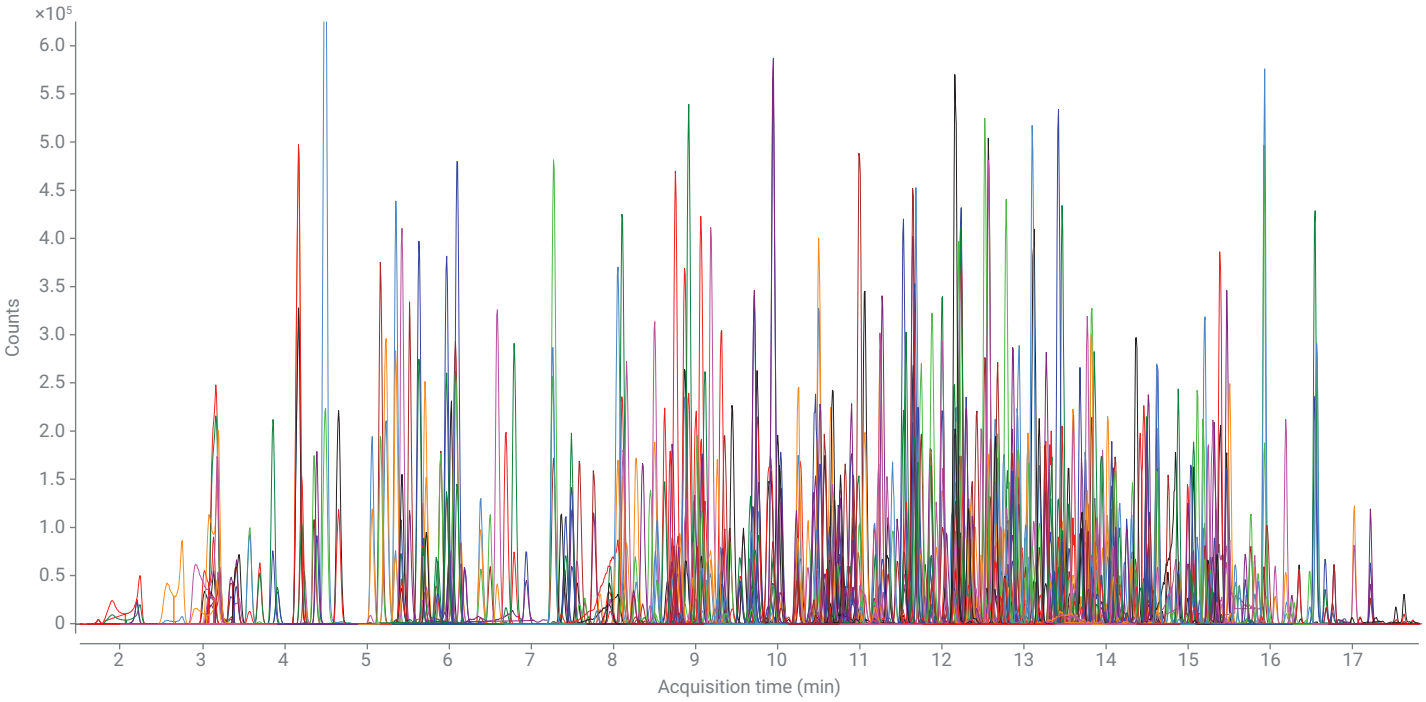

Figure 2A. Representative MRM chromatogram of 510 pesticides postspiked at 10 µg/L in olive oil matrix extract. The symmetric sharp peaks demonstrate the efficient chromatographic separation of targets within the retention time window.

Figure 2B. This shows that the dMRM method accurately quantifies more than 500 individual analytes in a relatively short LC run.

The full list of 510 compounds in the dMRM method, together with retention time, collision energy, fragmentor voltage, and MRM transitions is available in the method. Some compounds including acephate, brodifacoum, difenoconazole, etaconazole, halfenprox, iprovalicarb, omethoate, orbencarb, propamocarb, pymetrozine, resmethrin, thiobencarb, thiofanox sulfone, and triadimenol showed split peaks in all three matrices. Other compounds including butachlor, cycloprothrin, dimethachlor, imazamox, methamidophos, oxadixyl, pretilachlor, and tridemorph, showed peak tailing or broadening in all three matrices.

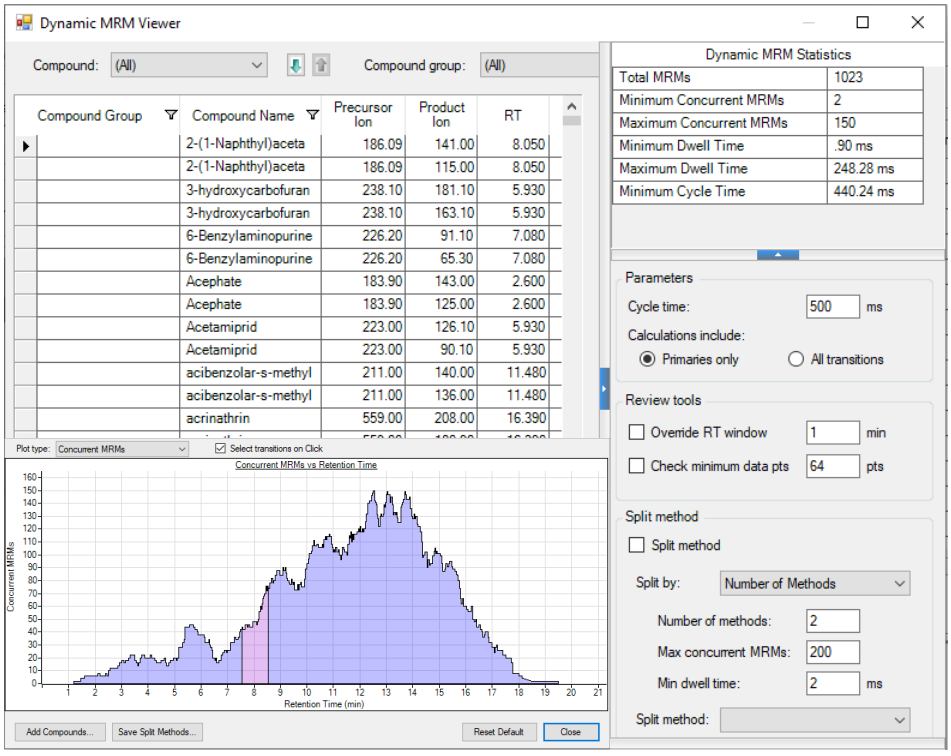

Figure 2B. 510-Compound acquisition method shown in Dynamic MRM Viewer software illustrating efficient management of more than 1,000 MRMs.

Matrix effect assessment

Matrix effects (ME) caused by sample matrix are frequent and behave in terms of suppression or enhancement of the MS detection system response.[1] ME was assessed by the ratio of target response in matrix-matched standards to that in corresponding solvent standards. Typically, there is no strict requirement on acceptance ME criteria, because ME can be corrected by the matrix-matched calibration curve. However, ME is an important parameter for method sensitivity and reliability assessment, and less than 20% signal suppression or enhancement is usually considered as insignificant ME.[1] In this study, ME was investigated using seven levels of matrix-matched calibration standards in comparison to the corresponding same levels of solvent standards. ME at calibration level 4 (10 µg/L), which is the MRL for all 510 pesticides in this study, was considered in the final compilation.

70% to 90% of 510 targets in tomato showed insignificant ME at 10 µg/L. For analytes with relatively significant ME in the tomato matrix, most of them showed matrix enhancement. For the dry wheat powder, insignificant ME was observed for 90% to 95% of total 510 targets at 10 µg/L. As for olive oil, insignificant ME was obtained for 70% to 85% of all 510 pesticides at 10 µg/L. Due to the complexity of oil matrix, more targets were negatively impacted by ion suppression. Based on the result of ME at 10 µg/L in tomato, wheat, and olive oil, matrix-matched calibration standards were finally used to compensate ME in this study.

As an example, the calibration curve of 2-(1-naphthyl)acetamide in solvent calibration standards and matrix-matched standards is plotted in Figure 3. This demonstrates good agreement across solvent standards and tomato, wheat, and olive oil matrices.

Verification of workflow performance

The workflow performance criteria was verified based on linearity, method sensitivity, recovery, and precision. Considering the dilution factor of 1:5 and 1:2 introduced for wheat and olive oil during sample preparation, the final result was corrected accordingly, based on dilution factors. Two batches of analyses were carried out for each matrix. The batch run for each sample matrix included solvent blank, matrix-matched calibration standards, matrix blank, postspiked QCs, and prespiked QCs. At least six technical replicates were prepared for prespiked QCs per level.[1] Each were injected into MS at least once.

1. Linearity: A calibration curve for the majority of targets was generated using matrix-matched standards from the defined LOQ to 100 µg/L, while the range from LOQ to 50 µg/L was applied to some of the compounds due to saturation at 100 µg/L. To determine the best linearity response function, various regression models were evaluated, and the best calibration model was with Type: Linear, Origin: Ignore, Weight: $1/x^2$, while a few compounds showed better linear regression with Weight: 1/x. More than 95% targets met the calibration curve linearity requirement of $R^2 \geq 0.99$.

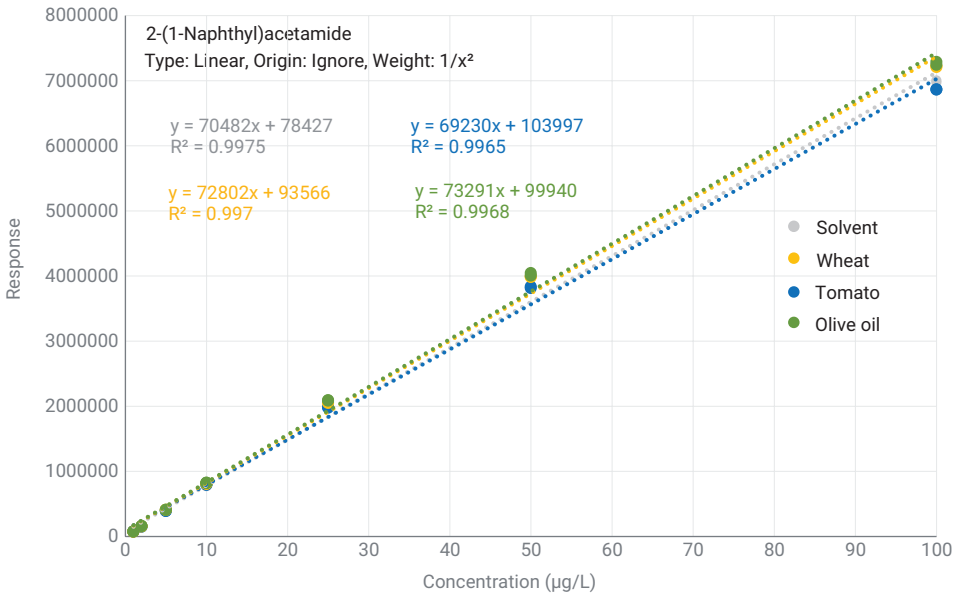

Figure 3. Overlay of calibration curve in solvent standards, tomato, wheat, and olive oil matrices.

2. Limit of quantification (LOQ) and instrument limit of detection (LOD):
A sensitive workflow for pesticide residue analysis is beneficial for users to perform routine operations following various regulatory guidelines. Workflow LOQ and instrument LOD were used to evaluate the method sensitivity. Instrument LOD was established based on matrix-matched calibration standards for signal-to-noise ratio (S/N) of three and up, while workflow LOQ was obtained from the prespiked samples going through the entire workflow procedure for S/N of 10 and up. The S/N was defined using the peak height and auto-RMS algorithm embedded in Agilent MassHunter Quantitative Analysis software. For defining LOQ, additional assessments including target selectivity in sample matrix and precision of analyte response and analytes recovery, were also considered. This is because LOQ is more important for quantitative methods. According to the guidance across the European Union (EU), the lowest spiking level within calibration range meeting the identification and method performance criteria was claimed as LOQ in this study.[1] Precision was obtained from six replicates of prespiked QCs, and %RSD was less than or equal to 20%. Figures 4A and 4B show an MRM chromatogram overlay of 2-(1-naphthyl)acetamide and acetamiprid for six technical replicates at pre-spiked QC 1 µg/kg and 5 µg/kg, respectively. This indicates high sensitivity and good precision at LOQ level across three matrices.

3. Method precision and recovery:
Method precision was estimated using recovery repeatability (RSD_r) and intralaboratory reproducibility (RSD_{iR}) based on the variation of recovery values from technical replicates of pre-spiked QC at 10 µg/kg in two batches across three matrices. RSD_r was determined by calculating percent relative standard deviation (%RSD) of recovery using six technical preparations of HQC within a batch. RSD_{iR} was measured as %RSD of recovery from a total of 12 technical preparations of HQC across two batches.

Typically, the acceptable RSD_r limit at 10 ppb is 20%. The RSD_r values of more than 91% of all targets in three different matrices were within 20%, demonstrating consistent behavior with each technical preparation. These results confirmed the high repeatability of analyte recovery using Agilent Universal QuEChERS dSPE and Agilent Captiva EMR—Lipid sample preparation.

Intralaboratory reproducibility for three matrices was assessed in two batches with the consideration of potential variables for the sample preparation and analysis, including different lots of sample matrix and consumables for extraction, different analytical columns and different days. RSD_{iR} was obtained for all matrices from total 12 technical preparations conducted in two batches. Among 510 targets, results of more than 90% of targets were within 20% RSD_{iR}. These results confirm the precision of workflow performance across different experimental conditions.

Variation of retention time (RT) for all targets in different batches across three matrices was also monitored to evaluate the chromatographic method precision. RT tolerance of all targets in three different matrices was within ±0.1 minutes. The precision results of RT confirm the reliability of the elution profile and MS detection.

Recovery was used in this experiment to evaluate the capability of a quantitative analytical workflow for more than 500 pesticides.[1] Three levels of prespiked QCs were used to evaluate analytes recovery across three different matrices, including 1, 5, and 10 µg/kg. Recovery was calculated based on analytes responses ratio between prespiked QCs and corresponding matrix-matched calibration levels. Mean recovery at each spiking level was obtained for six technical replicates. Given to the MRL for the majority of pesticides, the recovery results of 10 µg/kg spiking level were used to report workflow recovery performance. According to SANTE/12682/2019, mean recoveries can be accepted within the range of 40 to 120% if they are consistent (RSD_r ≤20%). Based on these criteria, the mean recovery results for 92%, 82%, and 86% of targets in tomato, wheat, and olive oil at 10 µg/kg met acceptance criteria, respectively.

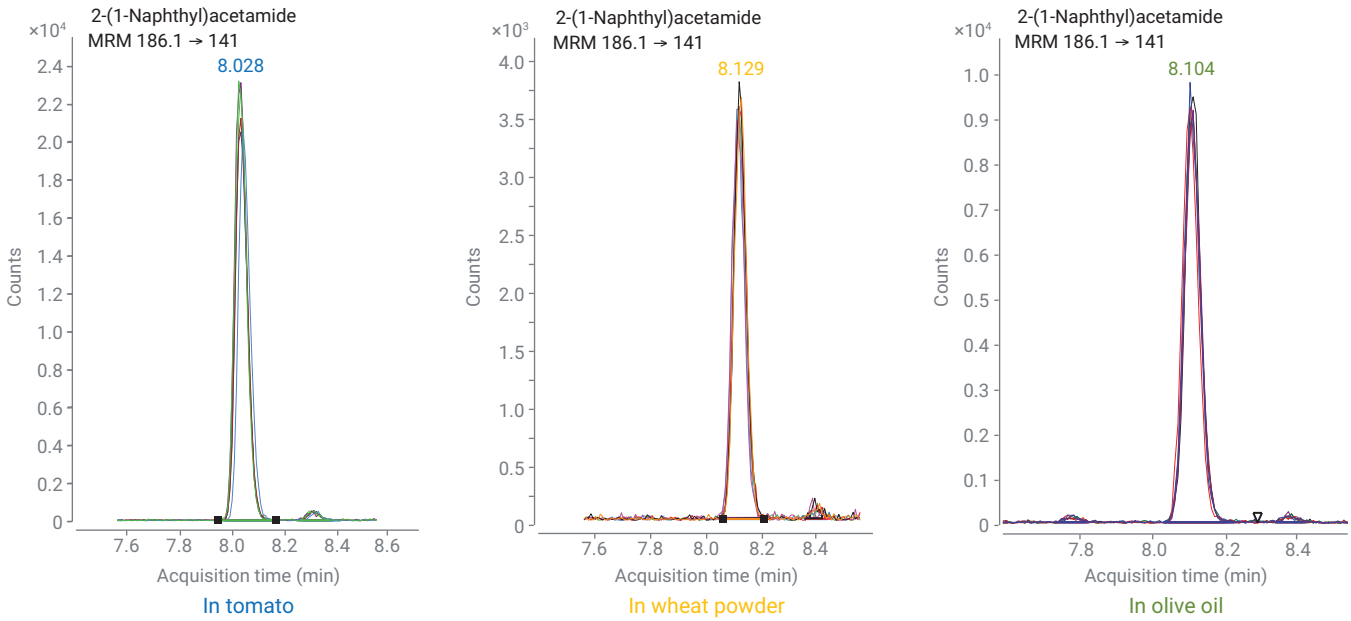

Figure 4A. MRM chromatograms overlay of 2-(1-naphthyl)acetamide for six techincal replicates at 1 µg/kg (prespiked QC) in three matrices.

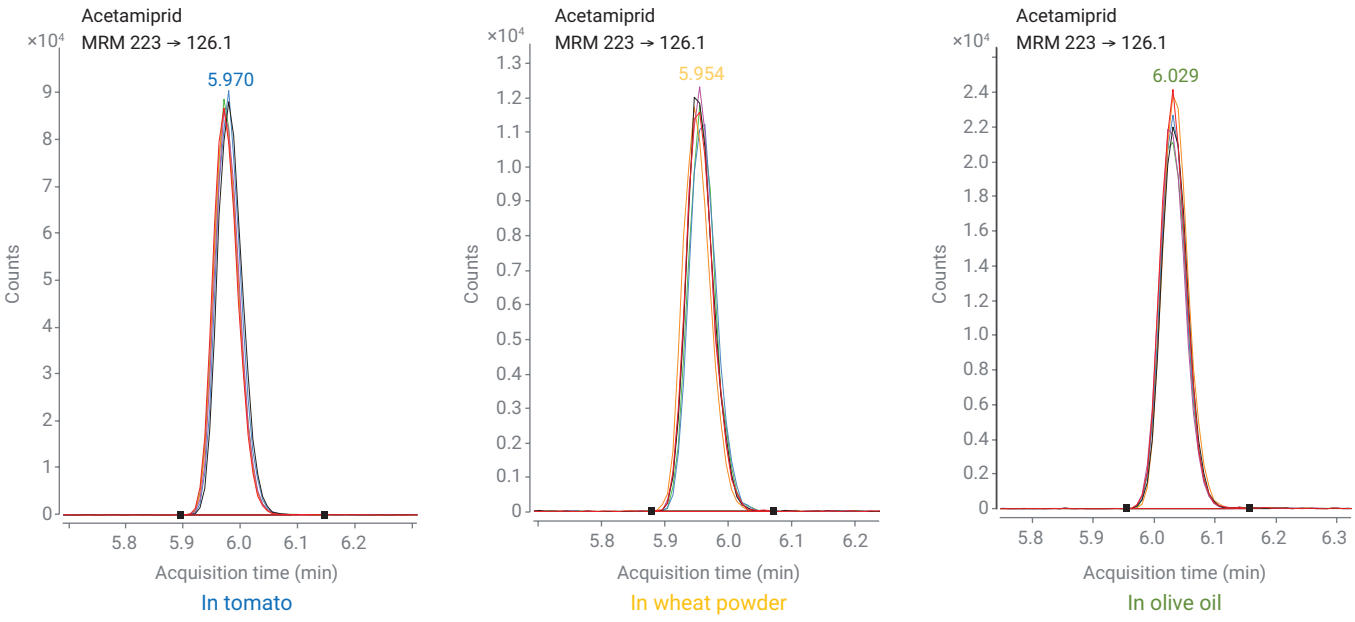

Figure 4B. MRM chromatograms overlay of acetamiprid for six technical replicates at 5 µg/kg (prespiked QC) in three matrices.

4. Robustness assessment

Robustness is the ability of a system and a method to produce a reliable response and result when a long run is required in the laboratory. In this study, robustness was evaluated by two days' (48 hours) continuous injection of olive oil extract spiked with pesticides at 50 µg/L. Nine compounds were selected to represent different classes of pesticides from fungicide, insectidie, herbicide, acaricide, and nematicide. The retention time window of these nine compounds covers from 12.5 to 15.0 minutes, the busiest window where the number of concurrent MRM is 150 (the maximum concurrent MRM). The large concurrent MRM transitions resulted in decreased dwell time for each compound within this window. Thefore, these nine compounds with shorter dwell times were selected to evaluate the performance of the dynamic MRM method in a long run. The analyte responses of nine representative compounds over >100 injections are displayed in Figure 5. Over two days' continuous running, the analyte responses were observed in good consistency with RSD <3.5%. This demonstrates that the use of dMRM mode can produce consistent responses with very short dwell time, which supports the reliable method robustness for the large number of sample injections.

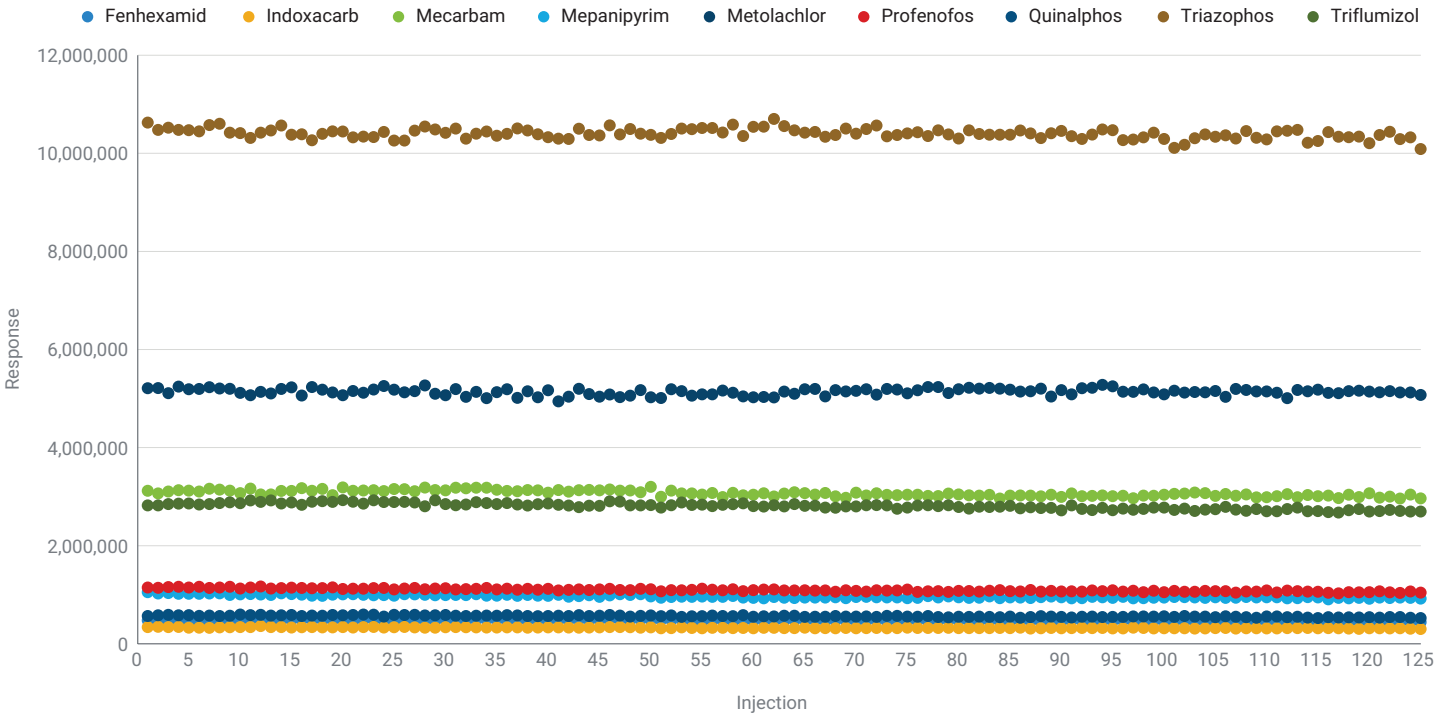

Figure 5. Response of representative compounds for 48 hours of continuous injections in olive oil extract spiked at 50 µg/L.

Conclusion

This study describes a highly sensitive and reproducible workflow for the fast and reliable quantitation of 510 pesticide residues in tomato, wheat, and olive oil matrices. The dMRM method was created and developed based on Agilent Pesticide Database including over 750 pesticides that can be saved to any name for customization by re-optimization of compounds in the database or addition/deletion of those present. The simplified sample preparation protocols included extraction with the Agilent QuECheRS kit followed with Agilent Bond Elut universal dSPE cleanup to prepare tomato and wheat powder samples. QuECheRS extraction followed with Agilent Captiva EMR—Lipid cleanup was used to prepare olive oil samples, providing highly efficient, selective, and reproducible pesticides extraction and complex food matrix cleanup.

The Agilent 1290 Infinity II LC coupled to the Agilent 6470 Triple Quadrupole LC/MS was used for over 500 pesticide residues analysis, which is easily and readily scalable to Agilent 6495 for achieving additional sensitivity if desired. The 20-minute LC gradient method using an Agilent ZORBAX RRHD Eclipse Plus C18 column offered good chromatographic separation and even RT distribution of all targets. LC/TQ data acquisition was in the dMRM mode with fast polarity switching for the most efficient use of instrument cycle time.

The workflow performance was verified in three different matrices based on matrix-matched calibration curve linearity, instrument LOD and workflow LOQ, recovery, and precision. The results in alignment across two batches demonstrate the applicability of the quantitative analytical workflow for more than 500 pesticide residues in high water, high oil, and high starch content with possibility to extend to various other food matrices.

References

1. SANTE/12682/2019: Analytical quality control and method validation procedures for pesticide residues analysis in food and feed.
2. Quantitative Analysis of Multi-Residue Pesticides in Food Matrices Using Agilent 6470 Triple Quadrupole LC/MS System – Method Protocol, **2020**.
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