Application Note

Food Testing and Agriculture

Quantitation of 764 Pesticide Residues in Tomato by LC/MS according to SANTE 11312/2021 Guidelines

Authors

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Abstract

A comprehensive, quantitative LC/MS/MS workflow was developed for the quantitation of 764 pesticide residues within a 20-minute LC runtime to accelerate and simplify routine laboratory food testing. Compound transitions and optimized parameters were developed based on the Agilent Pesticide Dynamic MRM Database, including curated parameters for fast and easy transfer into the analytical method. The workflow included sample preparation, chromatographic separation, mass spectrometry (MS) detection, and 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 tomato samples. For sample preparation, an Agilent QuEChERS extraction kit was used without further cleanup.

Workflow performance was evaluated and verified according to SANTE 11312/2021 based on instrument limit of detection (LOD), calibration curve linearity, recovery, and precision using matrix-matched calibration standards from 0.5 to 100 µg/L. Over 98% of analytes demonstrated linearity with R^2 ≥0.99, with calibration curves plotted from 0.5 to 50 or 100 µg/L. Method precision was assessed using recovery repeatability (RSD_r). At 10 µg/kg level, RSD_r values of 96% of compounds were within the limit of 20%. The mean recoveries of the six technical replicates were within the limits of 40 to 120% for 95% of target analytes.

Introduction

Pesticides play an import role in the agriculture and food industry to improve crop yield and food production. Residues of pesticides remaining in or on commodities such as fruits, vegetables, or cereals can cause adverse health effects as well as environmental concerns. Regulatory agencies have set maximum residue levels (MRLs) for hundreds of pesticides and their metabolites. Most MRLs are set at low ppb levels, which poses significant challenges especially if hundreds of analytes are screened and quantified simultaneously in complex food matrices. In Europe, pesticide testing laboratories adhere to the SANTE 11312/2021 guideline. This guideline ensures a consistent approach controlling MRLs legally permitted in food or animal feed. Due to the huge number of pesticides, the analysis is very elaborate. Often, multiple analytical approaches and laboratory intensive workflows are involved. These workflows lead to high operating costs and slow turnaround times.

A comprehensive LC/MS/MS workflow has been developed for an accurate and reliable analysis of over 760 pesticide residues in tomato. 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. Details of sample preparation procedures and instrumentation setup are discussed in conjunction with the data analysis parameters enabling the quantification and confirmation of pesticide residues.

Experimental

Chemicals and reagents

Agilent LC/MS grade acetonitrile (ACN), methanol (MeOH), water, and ammonium formate were used for this study. LC/MS grade formic acid was purchased from VWR. All other solvents used were HPLC grade and purchased from VWR.

Standards and solutions

The following ready-to-use and custom premixed pesticide standards were acquired:

- Agilent LC/MS Pesticide Comprehensive test mix (part number 5190-0551)
- Agilent Custom Pesticide test mix (part number CUS-00000635 – CUS-00000643)
- Agilent Custom Organic Standard (part number CUS-00004663)
- AccuStandard Custom Pesticide Standard (part number S-96086-01 – S-96086-10), amchro GmbH, Hattersheim, Germany

Additional single standards, either as standard solutions or as powders, were purchased from AccuStandard (amchro GmbH, Hattersheim, Germany) and LGC (LGC Standards GmbH, Wasel, Germany)

When single standards were purchased as powders, single stock solutions with a concentration of 1,000 mg/L were prepared in acetone and stored at –20 °C.

Two intermediate standard mixes (mix 1 and mix 2) at concentrations of 1,000 µg/L were prepared in ACN from stock standards and used for the rest of the experiments. Working standards at 500 µg/L were diluted from mix 1 and mix 2 and used for the preparation of prespiked QC samples.

A separate internal standard mixture (IS mix) containing five stable isotope labeled compounds (atrazine d_5, chlorpyrifos d_{10}, dichlorvos d_6, dimethoate d_6, and malathion d_6) was prepared in ACN at a concentration of 1,000 µg/L.

Solvent calibration standards were prepared for both standard mixes in ACN for matrix effect assessment.[1] Serial dilutions were done from mix 1 and mix 2 to prepare eight calibration concentration levels of 0.5, 1, 2, 5, 10, 25, 50, and 100 µg/L. Calibration standards were freshly prepared and stored in a refrigerator at 4 °C if not used immediately.

Sample preparation

Pesticide free and organically labeled tomatoes were obtained from local grocery stores. The tomato was homogenized using a domestic blender and stored in the refrigerator at 4 °C before analysis.

The following products and equipment were used for sample preparation:

- Agilent QuEChERS EN extraction kits (part number 5982-5650CH)
- Vortex mixer (VWR International GmbH, Darmstadt, Germany)
- Centrifuge Universal 320 R (Andreas Hettich GmbH, Tuttlingen, Germany)

Samples of 10 ±0.1 g of homogenized tomato were weighed into a 50 mL tube. Prespiked QC samples were fortified by spiking 200 µL of working standards (500 µg/L) to give a final concentration of 10 µg/kg. Then, 100 µL of IS mix (1,000 µg/L) was added to the matrix blank and QC samples to give an internal standard concentration of 10 µg/L. After spiking, the samples were capped tightly, vortexed, and equilibrated for 15 to 20 minutes. QuEChERS extraction was then performed and, after centrifugation, the ACN extract was directly used for LC/MS/MS analysis. The preparation procedure is illustrated in Figure 1.

Preparation of matrix-matched calibration standards

Matrix-matched calibration standards (post spiked standards) were used and prepared for the assessment of workflow performance in this study. A matrix blank was prepared using an unfortified blank sample of tomato. Preparation of matrix-matched calibration levels was identical to the solvent standards preparation by replacing ACN solvent with a matrix blank. The matrix-matched standards were used to evaluate the matrix effect by comparing responses in the corresponding solvent standards.[1]

Instrumentation

Chromatographic separation was performed using an Agilent ZORBAX Rapid Resolution High Definition (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 (G7120A)
- Agilent 1290 Infinity II Autosampler (G7167B)
- Agilent 1290 Infinity II Multicolumn Thermostat Column Compartment (G7116B)

The LC system conditions are listed in Table 1.

Table 1. LC Conditions.

Parameter	Value
Column	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 150, 1.8 µm (part number 959759-902)
Column Temperature	40 °C
Injection Volume	2 µL
Autosampler Temperature	6 °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
Flow Rate	0.4 mL/min
Gradient	Time (min) A (%) B (%) 0 95 5 3 70 30 17 0 100 20 0 100
Postrun Time	3 min
Needle Wash	Step Time (sec) Solvent 1 7 ACN Seat back flush and needle wash 2 7 MeOH Seat back flush and needle wash 3 7 Water Seat back flush and needle wash

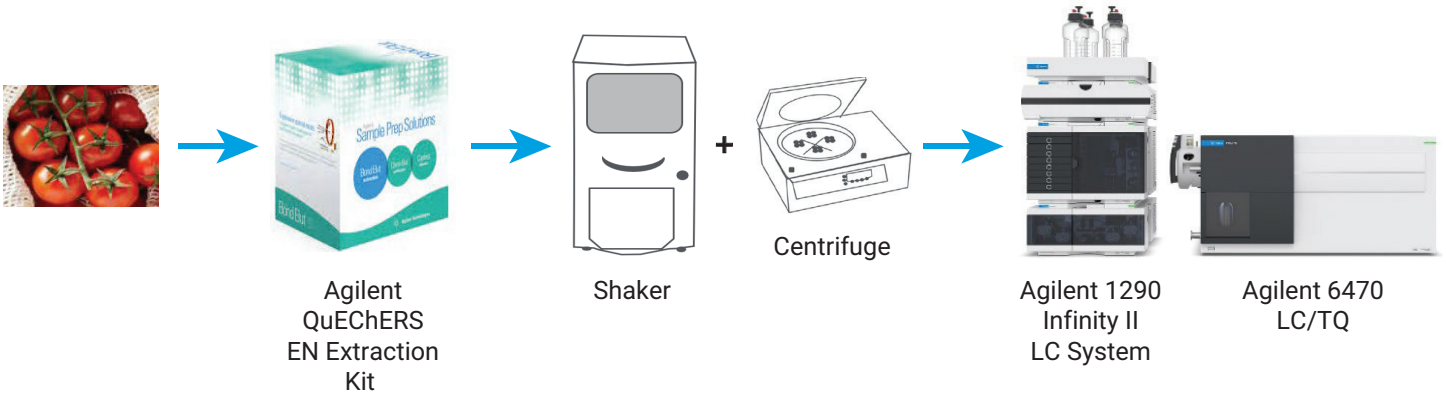

Figure 1. Sample preparation procedure.

An Agilent 6470 triple quadrupole LC/MS (G6470BA, LC/TQ) 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). The 6470 LC/TQ parameters are shown in Table 2.

Table 2. Agilent 6470 LC/TQ parameters.

Parameter	Value
Ionization Mode	Simultaneous positive/negative ESI with Agilent Jet Stream (AJS)
Scan Type	Dynamic MRM (dMRM)
Cycle Time	490 ms
Stop Time	20 min
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 V (+)/3,000 V (–)
Nozzle Voltage	0 V
Total MRMs	1,590
Max Concurrent MRMs	151
Min/Max Dwell Time	0.52 ms/242.30 ms

Results and discussion

Development of LC/TQ method

A major part of this work was the development of dynamic MRM transitions for 764 pesticide compounds based on the Agilent Pesticide Dynamic MRM Database (G1733CA). 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. Approximately 1,600 MRM transitions from 764 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 procymidone, where only one transition was stable enough to be monitored). This targeting 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 Agilent ZORBAX RRHD Eclipse Plus C18 column. This column provided good separation and distribution of 764 pesticide residues within a 20-minute HPLC gradient (Figure 2). 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 490 ms was used. Figure 3 shows a representative MRM chromatogram for all 764 pesticide targets postspiked at 10 µg/L in tomato extract.

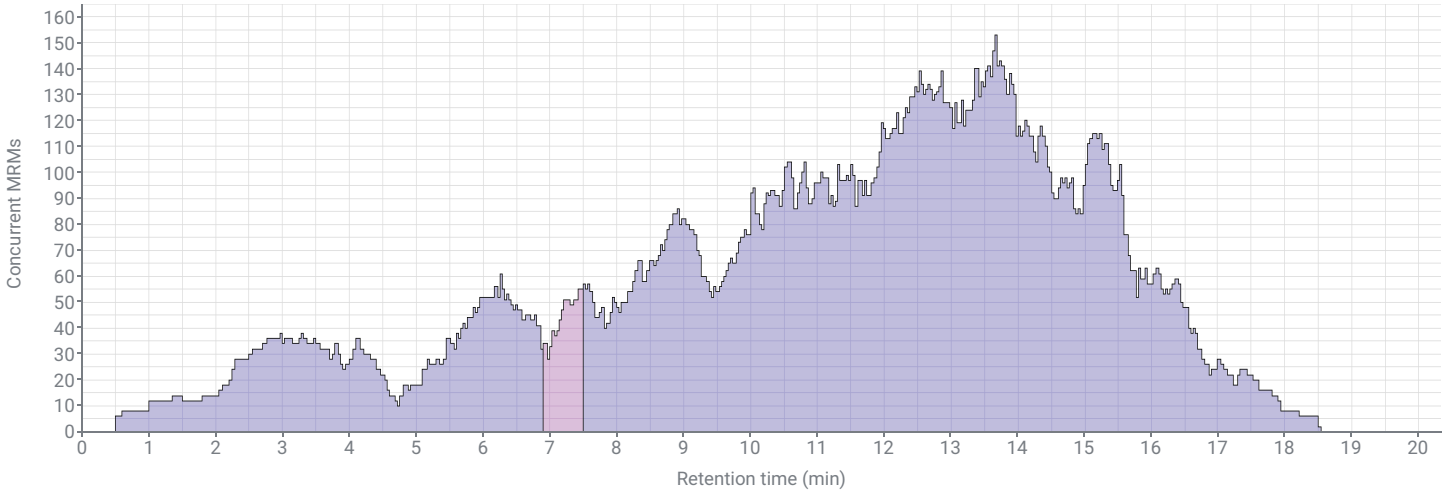

Figure 2. Overview of monitored MRMs over RT.

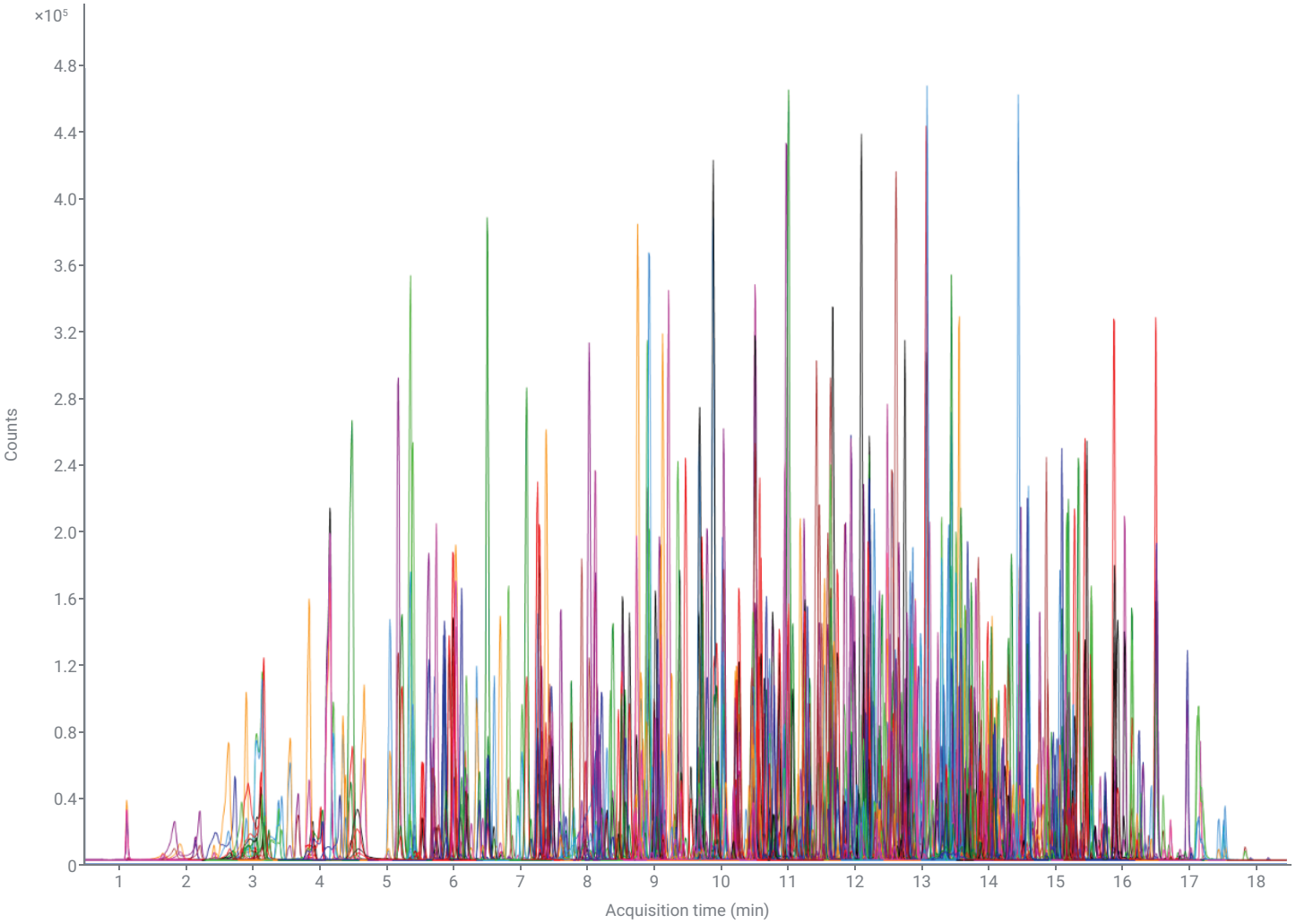

Figure 3. Overlay of MRM chromatograms of 764 pesticides postspiked at 10 µg/L in tomato extract.

Typical chromatographic peak widths were between 8 to 12 seconds. The selected cycle time of 490 ms ensured that sufficient data points were collected across the chromatographic peaks for reproducible quantitation and confirmation of results. Figure 4 shows the example of benodanil, where 16 data points were collected across the peak for both quantifier and qualifier transitions.

Matrix effect assessment

Matrix effects (ME) caused by sample matrix are frequent and cause 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 of 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 were investigated using a 10 µg/L standard in tomato extract (postspiked standard) and the response was compared to the corresponding solvent standard. The 10 µg/L standard was chosen as this is the MRL for all 764 pesticides in this study.

In 72% of the 764 targets in tomato, insignificant ME were shown at 10 µg/L. For analytes with relatively significant ME in the tomato extract, the numbers of compounds with ion enhancement and ion suppression were comparable.

Based on the results of matrix effect assessment, matrix-matched calibration standards were used to compensate matrix effects in this study.

Verification of workflow performance

The workflow performance criteria were verified based on linearity, method sensitivity, recovery, and precision. The batch included solvent blank, matrix-matched calibration standards, matrix blank, and prespiked QCs. Six technical replicates were prepared for the prespiked QCs.

Linearity

Calibration curves were generated for mixes 1 and 2 using matrix-matched standards ranging from 0.5 to 100 µg/L using eight calibration points. For some compounds, the highest calibration point was removed 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. More than 98% targets met the calibration curve linearity requirement of R^2 ≥0.99.

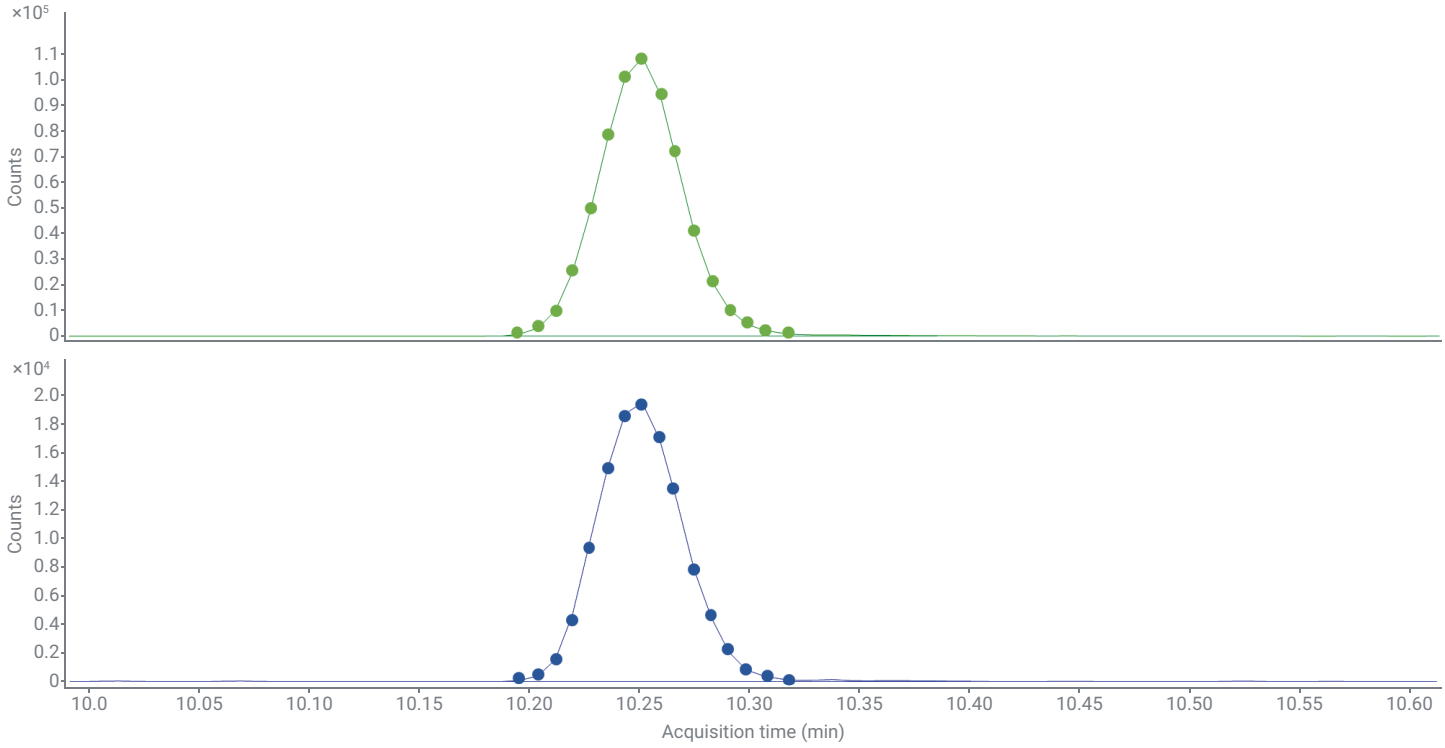

Figure 4. Data points per peak for quantifier and qualifier transitions for benodanil.

Instrument limit of detection (LOD)

A sensitive workflow for pesticide residue analysis is beneficial for users to perform routine operations following various regulatory guidelines. Instrument LODs 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 10 and up. The S/N was defined using the peak height and peak-to-peak algorithm embedded in Agilent MassHunter Quantitative Analysis software. The noise region was manually chosen and had a length of 0.2 minutes (0.1 minutes before and after the chromatographic peak).

More than 99% of target compounds showed an instrument LOD of ≤10 µg/L and even at a concentration level of 1 µg/L, more than 92% of compounds had an S/N of 10 and up (Figure 5). These results demonstrate the high sensitivity of the 6470 LC/TQ against such a complex matrix such as a tomato QuEChERS raw extract.

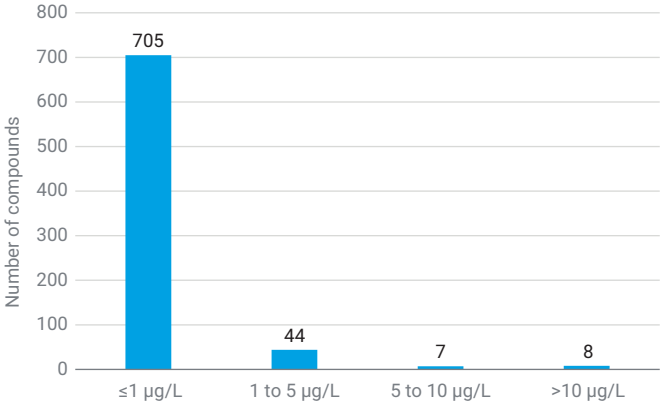

Figure 5. Instrument limit of detection in tomato QuEChERS raw extract.

Method precision and recovery

Method precision was estimated using recovery repeatability (RSD_r) based on the variation of recovery values from technical replicates of prespiked QC samples that were spiked at 10 µg/kg. The RSD_r was determined by calculating percent relative standard deviation (%RSD) of recovery using these six technical preparations. Typically, the acceptable RSD_r is 20% or less. The RSD_r values of more than 96% of all targets were within 20%, demonstrating consistent behavior with each technical preparation. These results confirmed the high repeatability of this workflow. Example chromatograms of the six technical replicates for acetamiprid, benodanil, and dimethoate are given in Figure 6.

Variation of retention time (RT) for all targets in different batches across three matrices was also monitored to evaluate the chromatographic method precision. The 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 764 pesticides.[1] Recovery was calculated based on analyte response ratios between prespiked QCs and corresponding matrix-matched calibration levels. Mean recovery at 10 µg/kg level was obtained for six technical replicates. According to SANTE 11312/2021, 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 more than 95% of targets in tomato QuEChERS raw extract at 10 µg/kg met the acceptance criteria. The vast majority of compounds were within the recovery range of 70 to 120% with RSD_r ≤20% and only 31 compounds (4%) had a recovery rate down to 40% or lower (Figure 7).

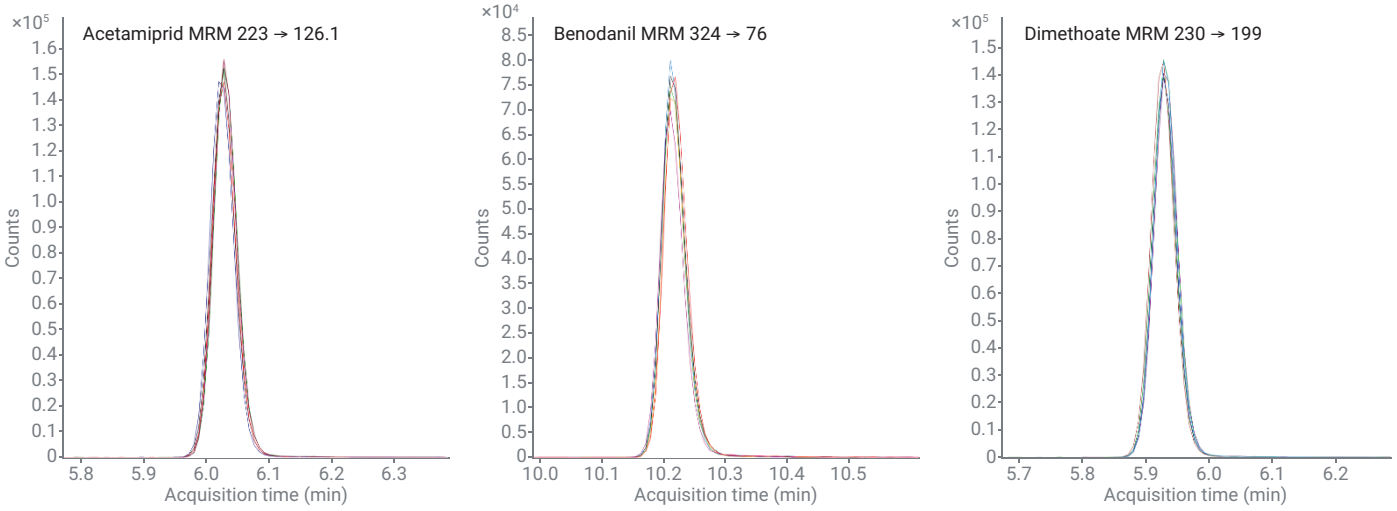

Figure 6. MRM chromatogram overlays for six technical replicates at 10 µg/kg in tomato extract.

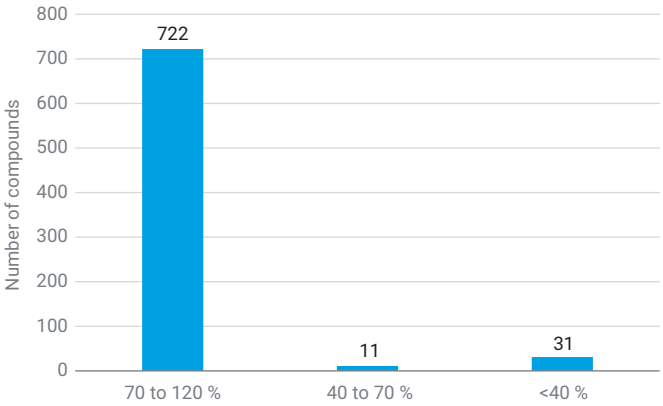

Figure 7. Recovery rates in tomato QuEChERS raw extract (RSDr ≤20%).

Conclusion

This study demonstrates the applicability of a sensitive and reproducible workflow for the fast and reliable quantitation of 764 pesticide residues in tomato QuEChERS raw extract conforming to the SANTE 11312/2021 guidelines. The simple sample preparation protocol exploits the Agilent QuEChERS kit for facile extraction without requirement for further sample cleanup.

The Agilent 1290 Infinity II LC coupled to the Agilent 6470B triple quadrupole LC/MS with matrix-matched calibration was used to successfully quantify 764 pesticide residues. The Agilent ZORBAX RRHD Eclipse Plus C18 LC column with a 20-minute reversed-phase gradient delivered good chromatographic separation and even RT distribution of all target compounds.

To achieve the most efficient use of instrument cycle time, the LC/TQ data acquisition was acquired in the dMRM mode with fast polarity switching. The dMRM method was developed based on the Agilent Pesticide Dynamic MRM Database, which covers more than 750 compounds. This database is readily customizable, allowing the addition or removal of compounds or transitions as required.

The overall workflow performance was assessed for linearity, instrument LOD, recovery, and precision, demonstrating its suitability for the quantitation of 764 pesticide residues in a QuEChERS raw extract.

References

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2. Quantitative Analysis of Multi-Residue Pesticides in Food Matrices Using Agilent 6470 Triple Quadrupole LC/MS System – Method Protocol, **2022**.
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