Application Note

Food Testing and Agriculture

Quantification of up to 764 Pesticide Residues in Tomato According to SANTE 11312/2021 Guidelines

Using the Agilent 6546 LC/Q-TOF System

Authors

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Abstract

For the quantitation of pesticides according to SANTE 11312/2021 guidelines, a quantitative LC/MS/MS workflow was developed using an Agilent 6546 LC/Q-TOF system. An accurate mass retention time database containing 764 pesticides, their MS/MS transitions, and corresponding retention times was generated.[1] The workflow uses a 20-minute LC runtime and provides a fast and simple solution for routine laboratory food testing analysis. As proof-of-concept, the workflow was used for the quantification of 368 randomly chosen target pesticides, and was based on the method described in "Quantitation of 764 Pesticide Residues in Tomato According to SANTE 11312/2021 Guidelines Using the Agilent 6470 Triple Quadrupole LC/MS System".[2] Compound transitions and optimized parameters were developed based on the Agilent MassHunter Pesticide Dynamic MRM Database, which contains entries for more than 760 pesticides. The workflow includes sample preparation, chromatographic separation, mass spectrometry (MS) detection, data analysis, and interpretation. The workflow was implemented on an Agilent 1290 Infinity II LC system coupled to a 6546 LC/Q-TOF and was applied to tomato samples. Sample preparation was carried out by the use of an Agilent QuEChERS extraction kit 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 ranging from 0.5 to 100 µg/L. Over 91% of analytes demonstrated linearity with R^2 ≥0.99, from calibration curves plotted from 0.5 to 100 µg/L. Method precision was assessed using recovery repeatability (RSD_r). At the 10 µg/kg level, RSD_r values of more than 93% 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 96% of target analytes.

Introduction

Pesticides are used in the food industry and in agriculture to increase the yield of food and crops. However, residual pesticides remaining in or on commodities such as crops, fruits, or vegetables might lead to adverse health effects as well as environmental concerns. Therefore, regulatory agencies have set maximum residue levels (MRLs) to limit the application of hundreds of pesticides and their metabolites. The high number of pesticides, combined with the low MRLs (often in the ppb range) pose a challenge for the simultaneous analysis of hundreds of analytes. The SANTE 11312/2021 guidelines were established to define a standard for analytical laboratories analyzing pesticides in Europe to ensure a consistent approach for controlling MRLs legally permitted in food or animal feed. The high number of pesticides makes the analysis very laborious, and often demands multiple analytical approaches, leading to high operating costs and slow turnaround times.

This application note describes the development of a comprehensive LC/MS/MS workflow for the accurate and reliable analysis of over 760 pesticide residues in tomato. It includes sample preparation, chromatographic separation, MS detection, target quantitation, and the interpretation of results. This workflow helps to streamline routine pesticide analysis and accelerate lab throughput and productivity.

Experimental

Chemicals and reagents

Agilent LC/MS grade acetonitrile (ACN), methanol (MeOH), water, and ammonium formate were used for the study. LC/MS-grade formic acid was purchased from VWR International GmbH. All other solvents used were HPLC grade, 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 kit (part number 5190-0551)
- Agilent Custom pesticide test mixes (part numbers CUS-00000635 through CUS-00000643)
- Agilent Custom Organic Standard (part number CUS-00004663)
- AccuStandard Custom Pesticide Standard (part numbers S-96086-01 through S-96086-10), obtained from amchro GmbH, Hattersheim, Germany

Additional single standards, either as standard solution or as powders, were purchased from AccuStandard (obtained from amchro GmbH, Hattersheim, Germany) and LGC Standards GmbH (Wesel, Germany).

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

An intermediate standard mix (mix 1) containing 368 pesticides at a concentration of 1,000 µg/L was prepared in ACN from stock standards and used for the rest of the experiments. Mix 1 was 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 yielding a concentration of 1,000 µg/L.

A solvent calibration standard was prepared for mix 1 in ACN for matrix effect assessment.[1] Serial dilutions were created from mix 1, to prepare nine calibration concentration levels of 0.2, 0.5, 1, 2, 5, 10, 20, 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 sieved tomatoes were obtained from local grocery stores.

The following products and equipment were used for sample preparation:

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

10 ±0.1 g of homogenized tomato samples were weighed into 50 mL tubes. QC samples were spiked with 100 µL of mix 1 and IS mix (1,000 µg/L) to give a final concentration of 10 µg/kg. After spiking, the samples were capped tightly and vortexed, followed by an equilibration step for 15 to 20 minutes. A QuEChERS extraction was then carried out, and the resulting extract was subsequently used for LC/MS/MS analysis. The preparation procedure is illustrated in Figure 1.

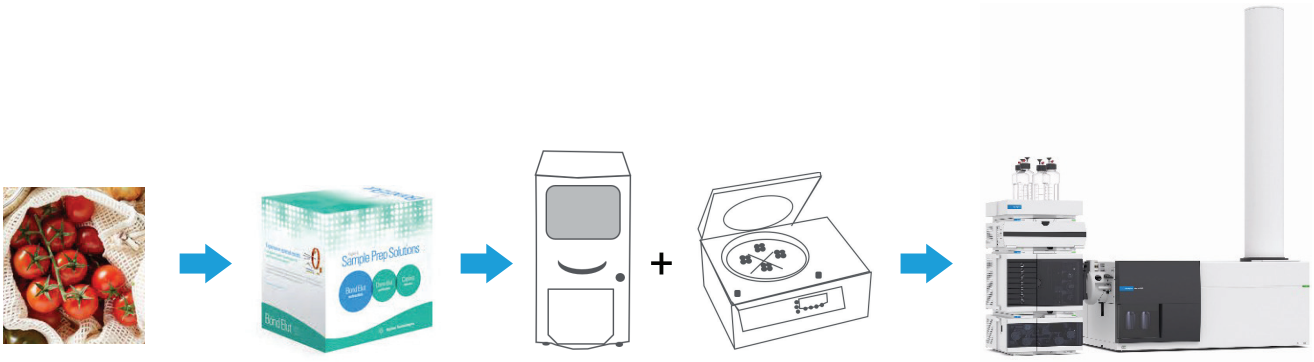

Figure 1. Sample preparation procedure.

Preparation of matrix-matched calibration standards

Matrix-matched calibration standards (postspiked standards) were prepared and used to assess the performance of the conducted workflow. As a matrix blank, an unfortified blank sample of tomato was prepared. The matrix-matched calibration standards were prepared according to the solvent standards, but varied in replacement of the 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

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

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.

An Agilent 6546 Q-TOF LC/MS with an Agilent Jet Stream (AJS) electrospray ion source was operated in All Ions mode. Data acquisition and procession were performed using Agilent MassHunter software version 11. The 6546 LC/Q-TOF parameters are shown in Table 2.

Table 1. Parameters of the LC method applied in this study.

Parameter	Value
Column	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 150, 1.8 µm (p/n 959759-902)
Column Temperature	40 °C
Injection Volume	2 µL
Autosampler Temperature	5 °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

Table 2. Parameters of MS method applied in this study.

Parameter	Value
Ionization Mode	Positive/negative ESI with Agilent Jet Stream (AJS)
Acquisition Type	AI Ions
Cycle Time	0.5 sec
Stop Time	20 min
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

Results and discussion

Development of MS/MS transitions on the LC/Q-TOF

A major focus of this work included the development of a database containing the MS/MS transitions mirroring the dynamic MRM transitions for the 764 pesticides of the application note "Quantitation of 764 Pesticide Residues in Tomato According to SANTE 11312/2021 Guidelines Using the Agilent 6470 Triple Quadrupole LC/MS System".[1,2] Therefore, pesticide standards were measured in the All Ions mode at four different collision energies (0, 10, 20, and 40 eV) with a cycle time of 0.5 seconds.

The applied chromatography led to peak widths ranging from 8 to 12 seconds. With the selection of a 0.5-second cycle time, sufficient data points were acquired for reproducible quantitation and confirmation of the results. The acquisition of fludioxonil with 14 data points and two associated MS/MS transitions is illustrated in Figure 2.

Matrix effect assessment

Matrix effects (MEs) caused by sample matrices often lead to signal suppression or enhancement during MS detection.[1] To determine ME, the ratio of target response in matrix-matched standards to that of corresponding solvent standards was calculated. There are no strict requirements regarding ME criteria, since ME can be corrected by the matrix-matched calibration curve. Nevertheless, it acts as an important parameter for method sensitivity and reliability assessment. Generally, an ME of less than 20% signal suppression or enhancement is considered insignificant.[1] MEs were investigated using a 10 µg/L standard in tomato extract (postspiked standard) since that concentration corresponds to the MRL for the pesticides in this study. The response was compared to the response of the corresponding solvent standard.

Fifty-five percent of the tested compound targets in tomato showed insignificant ME 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.

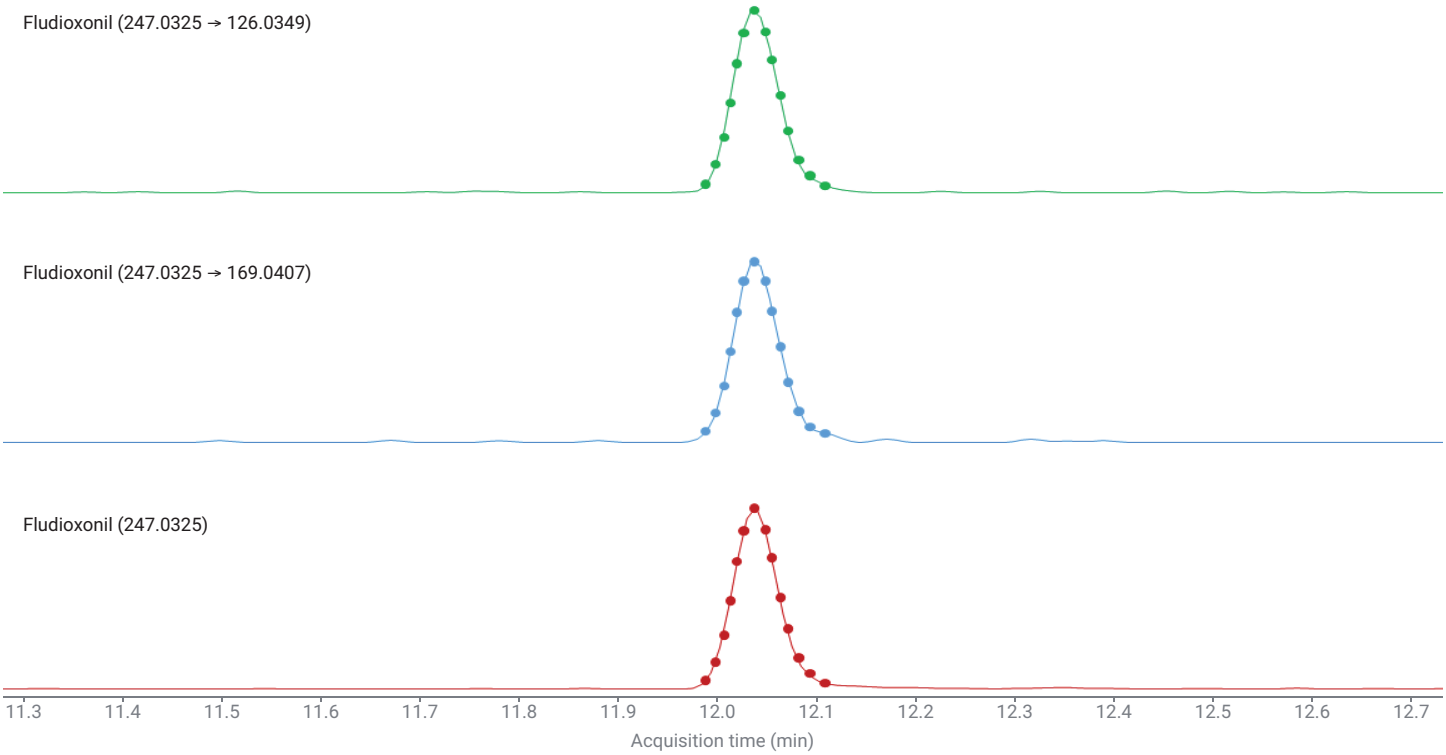

Figure 2. Data points per acquisition for the qualifier and quantifier transitions shown for fludioxonil.

Verification of workflow performance

The workflow performance was evaluated based on the criteria of linearity, method sensitivity, recovery, and precision. The batch included solvent blank, matrix-matched calibration standards, matrix blank, and prespiked QCs. For the latter, six technical replicates were prepared.

Linearity

Matrix-matched standards of mix 1 were used to generate calibration curves ranging from 0.5 to 100 µg/L, using nine calibration points. The following regression model was used for the calibration of the linearity response function: linear, origin: ignore, weight: 1/x. Ninety-one percent of the target compounds met the calibration curve linearity requirement of R^2 ≥0.99.

Instrument limit of detection

For the application of routine pesticide analysis in a regulated field, it is crucial to implement a sensitive workflow. Therefore, the instrument LOD was used to evaluate the method sensitivity. The LOD was established based on matrix-matched calibration standards for a signal-to-noise ratio (S/N) of ≥10. The S/N was obtained by using the peak height and RMS algorithm embedded in Agilent MassHunter Quantitative Analysis software. The timeframe for the noise region was manually chosen, and had a length of 0.2 minutes (0.1 minutes before and after the chromatographic peak).

Ninety-one percent of target compounds showed an instrument LOD of ≤10 µg/L, and even at a concentration level of 1 µg/L, approximately 70% of compounds had a S/N of ≥10 (Figure 3). These results demonstrate the sensitivity of the 6546 LC/TOF for a complex matrix such as tomato QuEChERS raw extract.

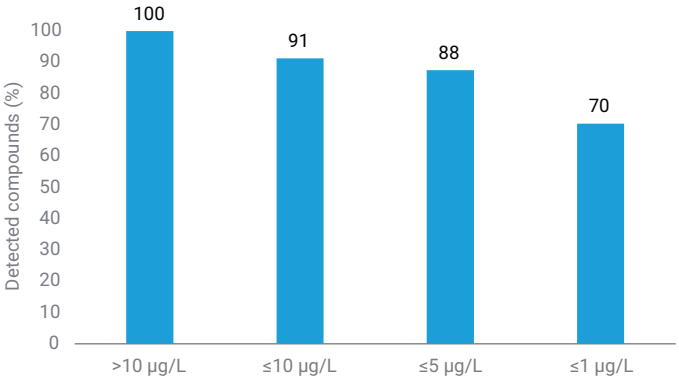

Figure 3. Limit of detection for a S/N of 10.

Method precision and recovery

The method precision was determined by use of the recovery repeatability (RSD_r) based on the variation of recovery values from technical replicates of prespiked QC samples spiked at 10 µg/kg. The RSD_r was determined by calculation of percent relative standard deviation (%RSD) of recovery using six technical preparations. The typical acceptable RSD_r accounts to ≤20%. Ninety-three percent of all targets in this study were within 20% of RSD_r and thus demonstrated consistent behavior with each technical preparation, highlighting the high repeatability of this workflow. Example chromatograms of the six technical replicates for dimethoate are given in Figure 4 with the corresponding coelution scores.

Recovery was used in this experiment to evaluate the capability of a quantitative analytical workflow for the detection of 368 target pesticides.[1] For the calculation of recovery, the ratio of the analyte response between prespiked QCs and corresponding matrix-matched calibration levels was calculated. The mean recovery at the 10 µg/kg level was obtained for six technical replicates. Mean recoveries are sufficient within the range of 40 to 120% if they are consistent (RSD_r ≤20%) according to SANTE 11312/2021. The mean recovery results for approximately 96% of targets in tomato QuEChERS raw extract at 10 µg/kg matched those criteria.

LC Screener Tool analysis

The Agilent MassHunter Quantitative Analysis software features an inbuilt LC Screener Tool, which enables rapid analysis of compounds, including the distinction between target and suspect compounds. The software displays positively identified analytes (highlighted in green), analytes which need to be reviewed (in orange), and entries which could not be identified (red). The criteria and parameters for analytes can flexibly be defined in the method. In Figure 5, the LC Screener Tool is depicted for the analysis of grape matrices (obtained from the local grocery store) which were prepared according to the calibration for the tomato matrices.

Since the calibration was carried out with tomato matrix, the measured concentration might deviate from the actual one. The analysis of grapes resulted in hits of already quantified analytes, but also yielded hits for compounds deposited as suspects in the database, such as santonin (Figure 5). With the calibration of a compound with a similar chemical structure, it was possible to obtain an inherent calibration, and thus a final concentration for the found suspect, as shown in Figures 5 and 6. The LC Screener Tool is therefore a useful tool for reviewing thousands of analytes in a simple and fast procedure, and even enables a quantification for suspects.

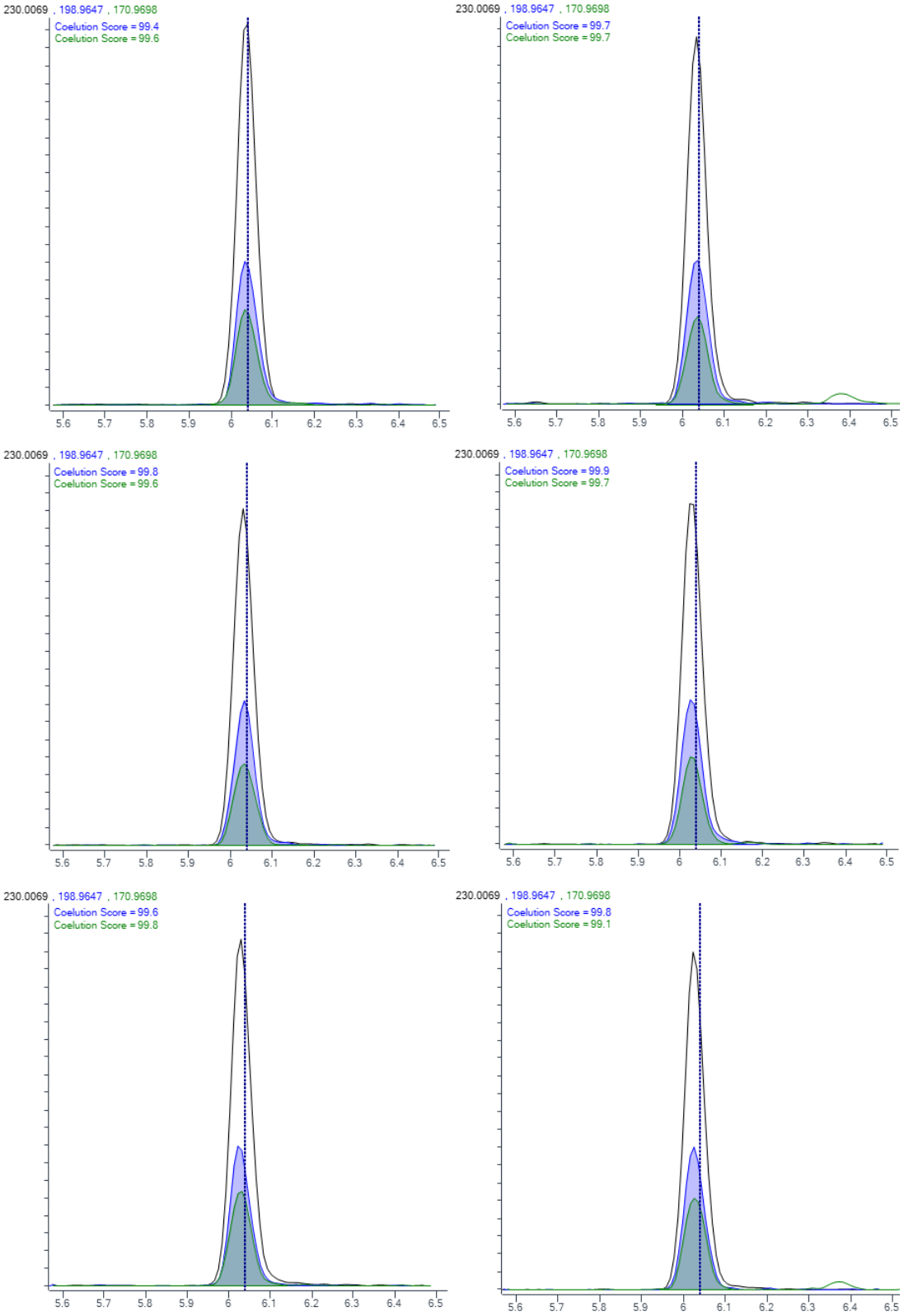

Figure 4. Dimethoate qualifiers with their corresponding coelution scores, acquired from six technical replicates of prespiked tomato.

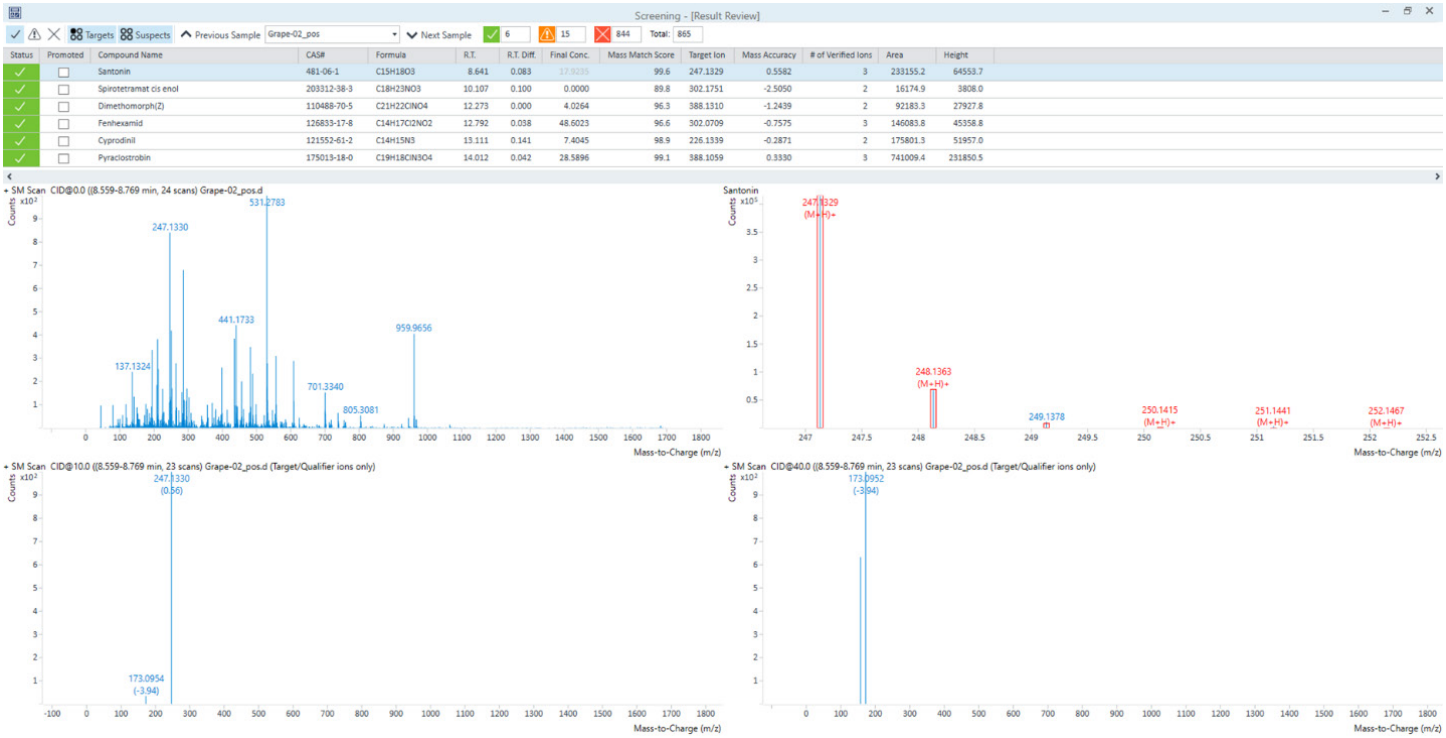

Figure 5. Agilent MassHunter Quantitative Analysis software LC Screener Tool for fast analysis of targets and suspects in grape matrix with quantitation results based on tomato matrix calibration. The quantification of the suspect santonin through inherent calibration is highlighted.

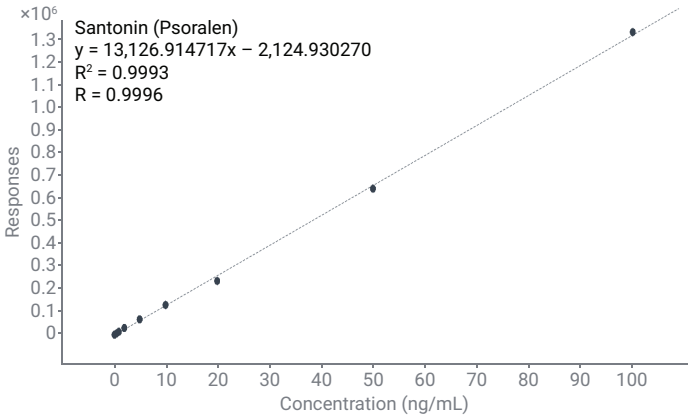

Figure 6. Inherent calibration of santonin based on the linear calibration of psoralen in grape matrix.

Conclusion

In this study, a sensitive and reproducible workflow was developed for the analysis and quantification of pesticide residues in tomato QuEChERS with an Agilent 6546 LC/Q-TOF. This generated a customizable database, containing more than 760 pesticide entries and their corresponding MS/MS transitions.

For sample preparation, the extraction protocol included the Agilent Bond Elut QuEChERS EN extraction kit. The chromatographic separation was carried out by the Agilent 1290 Infinity II LC system, with the Agilent ZORBAX RRHD Eclipse Plus C18 column, within a 20-minute gradient. The Q-TOF acquisition was measured in positive or negative All Ions mode for four different collision energies. The workflow performance was verified based on matrix-matched calibration curve linearity, instrument LOD, recovery, and precision. The results show the applicability of the workflow to quantify randomly chosen target compounds in spiked tomato, and could be matched to the database developed in this study.

References

1. SANTE 11312/2021: Analytical Quality Control and Method Validation Procedures for Pesticide Residues Analysis in Food and Feed.
2. Quantitation of 764 Pesticide Residues in Tomato According to SANTE 11312/2021 Guidelines Using the Agilent 6470 Triple Quadrupole LC/MS System, *Agilent Technologies application note*, publication number 5994-5847EN, **2023**.

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