Trace level quantitation of pesticide residues in fresh fruits using LC-MS/MS

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**Keywords**
TraceFinder, pesticide residues, grape, apple, QuEChERS, LC-MS/MS, TSQ Quantis

**Application benefits**
- No cleanup, acetonitrile extract dilute-and-shoot method
- Sub-ppb level sensitivity (e.g. 0.001 mg/kg)
- Low cost, simple, sensitive and rugged method
- Compliance with the EU and FSSAI MRLs

**Goal**
The objective was to provide an analytical solution for the trace level quantitation of 160 pesticides (parent, isomers and metabolites) in table grapes and in apple using liquid chromatography-tandem mass spectrometry. The optimized method was validated in accordance with the EU SANTE guidelines and further evaluated for compliance with the Food Safety and Standards Authority of India (FSSAI) as well as European Union (EU) MRLs.

**Introduction**
In India, the commercial cultivation of grapes and apples requires frequent applications of pesticides throughout the growing season to control a variety of pests and diseases. Consequently, the occurrence of pesticide residues is a primary concern for the stakeholders of both crops. The minimization of
pesticide residues, especially in grapes, is challenging because besides direct application, pesticide residues may also occur in grapes from indirect sources such as soil, contaminated agro-inputs (e.g., manures, fertilizers, growth regulators, irrigation water, etc.), and drift from adjoining fields of other crops. In India in 2017, grape production was 2683 metric tons, whereas apple production was 2242 metric tons.¹ Until recently, Indian food testing laboratories analyzed pesticide residues in class-specific groups using a combination of GC- and HPLC-based methods. This approach required several days to complete the analysis. However, the present situation demands rapid methods and shorter turn-around times. Currently, 282 pesticides are registered in India under the Central Insecticide Board and Registration Committee (CIBRC) for their crop management.² There are 51 chemicals registered and recommended for grapes as per APEDA.³

The European Commission (EC) and FSSAI have set the maximum residue levels (MRLs) for pesticides and their metabolites in grape and apples.⁴,⁵ Therefore, it is important to develop a fast, robust, sensitive, and cost-effective method able to produce results for LC-amenable pesticides that comply with the new MRLs recently set by the FSSAI.

For extraction of residues, the AOAC version of the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method was selected as it is widely used for the analysis of pesticide residues in fruit.⁶ The instrument method plays an important role in delivering accurate and precise results to meet the regulatory requirements. Therefore, the Thermo Scientific™ TSQ Quantis™ triple quadrupole mass spectrometer system was used for data acquisition with instrument control, and the data processing, carried out using Thermo Scientific™ TraceFinder™ software.

This work aimed to develop an analytical solution validated in accordance with the EU SANTE/11813/ 2017 guidelines.⁷ This method was applied to real samples to demonstrate the workflow, which meets the SANTE guideline requirements in terms of identification of incurred residues.

**Experimental**

**Chemicals and apparatus**

- Acetonitrile, Optima™ LC/MS Grade, Fisher Scientific™
- Methanol, Optima™ LC/MS Grade, Fisher Scientific™
- Water, Optima™ LC/MS Grade, Fisher Scientific™
- Formic acid (85%), Fisher Scientific™
- Acetic acid (100%), Fisher Scientific™
- Ammonium formate, LC/MS Grade, Fisher Scientific™
- Anhydrous magnesium sulfate, Fisher Scientific™
- Sodium acetate, Fisher Scientific™
- LC/MS pesticides mix reference standards, Restek™ P/N 31971
- Analytical balance (Aczet, CY2202, San Diego, CA) and precision balance (Aczet, CY205C, San Diego, CA)
- Vortex mixer (Thermo Scientific, P/N 88880017TS, also known as 88880017)
- Refrigerated centrifuge (Thermo Scientific™ Sorvall™ ST8 ventilated benchtop centrifuge)
- Variable volume micropipettes (Thermo Scientific)
- QuEChERS Salts (AOAC 2007.01) Mylar Pouch 6 g magnesium sulfate (anhydrous), 1.5 g sodium acetate 50 pk Thermo Scientific™ (P/N 60105-341)
Sample preparation
The grape and apple samples collected from a local market were homogenized using a rotating blade chopper (Model: Maharaja Whiteline) to get a uniform slurry. Homogenized sub-samples were extracted using the AOAC Official Method 2007.01 QuEChERS procedure, which is outlined below.

Sample extraction and cleanup:
• Weigh 15 g homogenized sample into a 50 mL extraction tube.
• For the recovery experiment, spike the samples before the addition of the extraction solvent.
• Add 15 mL of acetonitrile (containing 1% acetic acid).
• Shake vigorously and vortex for 1 minute on a vortex mixer at 2500 rpm.
• Add 6 g anhydrous MgSO₄ and 1.5 g sodium acetate to the tube and again mix vigorously for 1 minute on a vortex mixer at 2500 rpm.
• Centrifuge at 5000 rpm for 5 min.
• Dilute the supernatant with water (1:4 ratio, v:v).
• Transfer the extract into an LC vial for instrumental analysis.
• Inject 5 µL of extract into the LC-MS/MS.
• For the recovery experiment: screen the samples for the target list of analytes and check for any residues of analytes of interest. If there is no detection of target pesticides, use this sample as a blank, for spiking, and for the preparation of matrix-matched calibration standards.

LC-MS/MS analysis
The Thermo Scientific™ Vanquish™ Flex UHPLC system was coupled with the Thermo Scientific™ TSQ Quantis™ quadrupole tandem mass spectrometer with a heated electrospray ionization (HESI) source. The optimized LC-MS/MS conditions are detailed in Table 1.

<table>
<thead>
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<th>Table 1. LC-MS/MS instrument conditions</th>
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<td>Column: Thermo Scientific™ Hypersil GOLD™ (100 mm x 2.1 mm x 1.9 µm) (P/N 25002-102130)</td>
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<td>Sample compartment temp.: 10 °C</td>
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<td>Column oven temp.: 25 °C</td>
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<td>Injection volume: 5 µL</td>
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<td>Mobile phase:</td>
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<td>A: 2 mM ammonium formate + 0.1% formic acid in water/acetoniitrile (90:10, v/v)</td>
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<tr>
<td>B: 2 mM ammonium formate + 0.1% formic acid in water/acetoniitrile (10:90, v/v)</td>
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<td>Negative: 2500 V</td>
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<td>Sweep gas: 1 Arb</td>
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<td>Vaporizer temp.: 350 °C</td>
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<td>Source position: Vertical between M and Horizontal1</td>
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</table>
Data acquisition and processing

The data acquisition was performed by using the instrument conditions in Table 1. The data acquisition and processing were carried out using Thermo Scientific™ TraceFinder™ software version 4.1. The data was acquired in t-SRM mode, which includes two or more transitions per analyte. The target list of analytes given in Table 2 (Appendix) with their SRM transition, collision energies, and retention time (min). For data processing, the ion ratio (±30%), retention time (±0.1 min), linearity (>0.99 with residuals ±20%), recovery (70–120%) and precision (±20%) were set as performance criteria by the EU SANTE guidelines.7

Results and discussion

LC-MS/MS analysis

The liquid chromatographic method was optimized, which offered excellent separation for the target analytes and absence of an isobaric interference from the matrix. The extracted ion chromatogram (XIC) is shown in Figure 1 for 160 compounds at 0.01 mg/L.

The data points/scans per peak depended on the dwell time required to monitor the transition. In this method, automatic optimized dwell time is <10 ms per transition, which offered at minimum 12–15 points per peak. Here ametryn has more than 12 points per peak as shown in Figure 2. The optimized instrument conditions provided excellent selectivity, repeatability, and reproducibility.
Identification
The data was processed automatically using user-defined parameters. Color-coded flags indicate whether results pass or fail the user-defined acceptance criteria set in the processing method. The results that passed user-defined criteria (based on SANTE guidelines) are shown in green (Figure 3), which minimizes the time required for review. A red flag indicates further investigation is required and dependent on the reason provided by the flag. As per SANTE guidelines, identification of dimethoate is shown with two transitions (230.0→199.0 and 230.0→125.0) at the same retention time (4.68 min) and ion ratio 50.27% (39.53–73.41%) in comparison with a neat standard (39.53–73.41%). The linearity for dimethoate provided correlation coefficient >0.999 with <20% residuals (Figure 3).

Method performance
The linearity was plotted in the range of 0.0005 to 0.025 mg/L with a 1/x weighting factor and linear equation. The linear curve offered excellent correlation coefficients (>0.99) with <20% residuals for all the target analytes in both solvents as well as in both matrices. The lowest calibration level (0.0005 mg/kg) showed good sensitivity with ≥10:1 signal-to-noise ratio. The high sensitivity was enabled after dilution, without the need for cleanup. Since the extracts are diluted four-fold before analysis, the limit of quantitation (LOQ) with acceptable recoveries and precision was 0.005 mg/kg except carbofuran and 3-hydroxy carbofuran in both matrices. The recovery experiments were carried out at 0.005 (LOQ) and 0.01 (2 × LOQ) mg/kg to demonstrate the method accuracy and precision. Average recoveries were observed in the range of 76–116% with <15 % RSD (Table 3), which were within acceptance criteria (recovery 70–120% and precision <20%) of SANTE guideline criteria. Also, the optimized method was tested for repeatability of results obtained for a long sequence of injections for grape (n=50) and apple (n=55) based on a typical 24-hour schedule of a commercial food testing laboratory. The time required to process the data through TraceFinder software was approximately 90 minutes considering manual revision. The repeatability was <15% for an area without internal standard correction and the retention time variation < ±0.05 min in both matrices. The repeatability of response for selected pesticides in grape and apple is shown in Figure 4.

Figure 3. (A) Extracted ion chromatogram (XIC) for quantifier ion of dimethoate (B), identification based on selection confirmed with ion ratio, and (C) calibration curve
Conclusion

This application note describes a sensitive, robust, and low-cost method for the quantification and identification of 160 pesticides at low mg/kg levels in grape and apple. Using this approach, at least 70 injections (standards, samples, blank) could be completed in a day (24-hour cycle) to increase the sample throughput of commercial food testing laboratories. The validation data fully meets the requirement of the EU SANTE guidelines. TraceFinder software is used for data acquisition and data processing. The color-coded flagging of results outside of the acceptance criteria enables faster processing of the data and automatically identifies results in need of further investigation. The overall outcome is reduced time for reviewing data, with an overall increase in efficiency and productivity. The optimized method meets the EU and FSSAI MRLs for the LC-amenable pesticides in grape and apple.

References


Application of the method to real samples

This method was applied to grape and apple samples (n=5 each) collected from the local market and analyzed for pesticides residue analysis. After the data review, there were no residues detected in apple samples. In the grape samples, a total of 10 different pesticides were identified and quantified. The concentration observed in grape samples were 0.015–2.2 mg/kg. However, spirotetramat and carbendazim were monitored as parent compounds. The detail list with their concentrations is given in Figure 5.

Figure 4. Area repeatability shown for dicrotophos and dimethoate in grape (n=50), and for isoproturon and carbendazim in apple (n=55)

Figure 5. Incurred residue found in grape sample
## Appendix

### Table 2 (part 1). List of pesticides with MRM transitions used

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Compound</th>
<th>RT (min)</th>
<th>Polarity</th>
<th>Precursor (m/z)</th>
<th>Product Ion (m/z)</th>
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*Parent compound
** Only metabolite
**Table 2 (part 2). List of pesticides with MRM transitions used**

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*Parent compound  
** Only metabolite
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Table 3 (part 1). Method validation data (recovery and % RSD)
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Table 3 (part 4). Method validation data (recovery and % RSD)