

Development and validation of single residue method for dithianon in apple and apple juice using LC-MS/MS

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Keywords

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Goal

The objective of this work is to develop and deliver a complete solution that can be implemented for routine analysis of dithianon in apple and apple juice by using LC-MS/MS.

Introduction

Apple is the primary fruit crop grown in temperate geographical zones in the northern part of India (Jammu and Kashmir, and Himachal Pradesh). A substantial share of apple production is from these two states only. To meet the current market demand, a large yield of high quality apples is required. Therefore, many chemicals are used to control the diseases and attacks from insect pests. For example, the fungal infections such as apple scab (*Venturia inaequalis*), powdery mildew (*Podosphaera leucotricha*), and apple canker (*Nectria galligena*) are prevented in their early development phase by use of fungicides.

Dithianon is a broad-spectrum fungicide for the reduction of scab (*Venturia spp.*) on apples and is a registered product for foliar spray applications in grapes, pome fruits, stone fruit, berries, spinach, lettuce, brassica crops, solanaceous crops, and rice.¹ Frequent applications of dithianon can result in residues on the apples, which poses a danger to consumer health. Therefore, the European (EC) and the Food Safety and Standards Authority of India (FSSAI) have set maximum residue limits (MRLs) for dithianon in apple at 3.0 and 0.1 mg/kg, respectively.^{2,3}

Recently, the European Reference Laboratory for Single Residue Methods (EURL) established a method for dithianon residue analysis in fruits and vegetables by use of the modified version of QuEChERS followed by LC-MS/MS analysis.⁴ Dithianon is a base-sensitive molecule, but the EURL method improved the stability of dithianon through acidification and addition of antioxidants during extraction. Similarly, Jang *et al.* reported the use of acidified acetonitrile for extraction of dithianon followed by LC-MS/MS analysis in red pepper.⁵ The fact that very few laboratories (<30%) reported dithianon results for the European Proficiency Test (EUPT-SRM 11) indicates that dithianon is a very challenging analysis.⁶ Hence, there is a definite requirement for sample preparation as well as instrument conditions optimization that can deliver accurate and precise results to meet the regulatory needs.

Our objective was to develop and validate a single residue method for dithianon analysis in apple and apple juice by using a QuEChERS technique⁷ and a Thermo Scientific™ TSQ Quantis™ LC-MS/MS system. The data acquisition and processing were carried out by using Thermo Scientific™ TraceFinder™ software. The optimized method was validated as per the SANTE guidelines.⁸

Experimental

Chemicals, apparatus, and consumables

- Acetonitrile, Optima™ LC/MS Grade, Fisher Scientific™ (P/N A955-4)
- Water, Optima™ LC/MS Grade, Fisher Scientific™ (P/N W6-4)
- Acetic acid glacial (certified ACS), Fisher Scientific™ (P/N A38S-500)
- Stock solutions of dithianon (1000 µg/mL), as well as further working standard solutions, prepared in acetonitrile (1% acetic acid, (v/v))
- Calibration standards prepared in the range of 0.0005–0.10 µg/mL
- 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 (2007.01) Mylar Pouch 6 g Magnesium Sulfate (Anhydrous), 1.5 g sodium acetate 50 pk Thermo Scientific™ (P/N 60105-341)

Sample preparation

Blank (dithianon residue free) apple and apple juice were purchased from local retail stores and used for validation experiments. Apple matrix is already acidic with a high amount of antioxidants. Hence the homogenization was performed without acidification. An acidified extraction solvent was used for extraction to maintain the stability. The pesticides residue extraction was done by following QuEChERS 2007.01 without cleanup.⁷

- Weigh 15 g homogenized sample into a 50 mL QuEChERS extraction tube (*Note: for recovery/QC, spiked before extraction*).
- Add 15 mL acetonitrile (containing 1% acetic acid).
- Vortex for 1 minute using a vortex mixer at 2500 rpm (approximately).
- Add QuEChERS 2007.01 salts (6 g MgSO₄, 1.5 g sodium acetate) to the tube and vortex for 1 min using a vortex mixer at 2500 rpm (approximately).
- Centrifuge at 4000 rpm for 5 min at ambient temperature.
- Filter 1 mL acetonitrile extract through a PTFE membrane filter into the LC vial.
- Inject 5 µL to LC-MS/MS for analysis.
- Prepare a blank sample by following the above protocol; the matrix-matched calibration standards were prepared in the range of 0.0005 to 0.10 mg/kg by spiking an appropriate amount of dithianon into the blank extract of sample.

LC-MS/MS analysis

Analysis was performed using the Thermo Scientific™ Vanquish™ Flex Binary UHPLC system coupled with the Thermo Scientific™ TSQ Quantis™ triple quadrupole mass spectrometer. The specific instrument conditions and selective reaction monitoring (SRM) parameters for dithianon are given in Table 1 and Table 2. Thermo Scientific™ TraceFinder™ 4.1 software was used for instrument control, data acquisition, data processing, data review, and reporting.

Table 1. LC-MS/MS optimized parameters

Liquid chromatography method	
Instrumentation:	Vanquish Flex UHPLC
Column:	Thermo Scientific™ Hypersil GOLD™ 100 × 2.1 mm, 1.9 μm
Column temp.:	40 °C
Injection volume:	5 μL
Mobile phase:	A: 0.01% Acetic acid in water B: 0.01% Acetic acid in acetonitrile
Flow rate:	400 μL/min
Gradient program:	0–0.5 min, 10% B 0.5–5 min, 10–90% B 5–6 min, 90% B 6–6.25 min, 90–10% B 6.25–10 min, 10% B phase
Mass spectrometry method	
Instrumentation:	TSQ Quantis triple quadrupole tandem mass spectrometer
Polarity:	Negative
Ion spray voltage:	2500 V
Capillary temp.:	325 °C
Vaporizer temp.:	290 °C
Sheath gas:	50 arb
Aux gas:	10 arb
Sweep gas:	1 arb
Collision gas:	1.5

Table 2. SRM parameters for dithianon

Q1	Q3	RF lens	CE
296	263.9	108	22.43
296	238.0	108	22.13

Data acquisition and processing

The data acquisition and processing were performed using TraceFinder 4.1 software. The data was acquired in selective reaction monitoring (SRM) mode in negative polarity. The identification and quantitation of dithianon were performed in alignment with SANTE guidelines.⁸

Results and discussion

LC-MS/MS analysis

During dithianon optimization, the highest intensity observed was for m/z 296, the molecular ion without an adduct formation. This compound is an exceptional case where no $[M-H]$ adduct is formed in negative polarity due to the formation of a radical ion. The product ions (m/z 263.85 and 238) were selected by optimizing the collision energy. The optimized compound specific parameters are given in Table 2. The ion m/z 264.85 was used for quantification and the ion m/z 238 used for identification. As per the SANTE guidelines, the ion ratio between the two ions was compared with the same ratio for a standard to verify the identity of the compound.⁸

Reversed-phase chromatography was used for the analysis. The diluted acetic acid (0.01% v/v) was used in both mobile phases, which provided excellent stability and ionization of dithianon during analysis. The detailed chromatography and mass spectrometry parameters are given in Table 1. Optimized gradient chromatography offered excellent retention as well as symmetrical and sharp peak shape (Figure 1A). Direct injection of dithianon extract in pure acetonitrile showed excellent sharp and symmetrical peak shape with <20 s peak width and avoided poor peak shapes that result on dilution with water (Figure 1B).

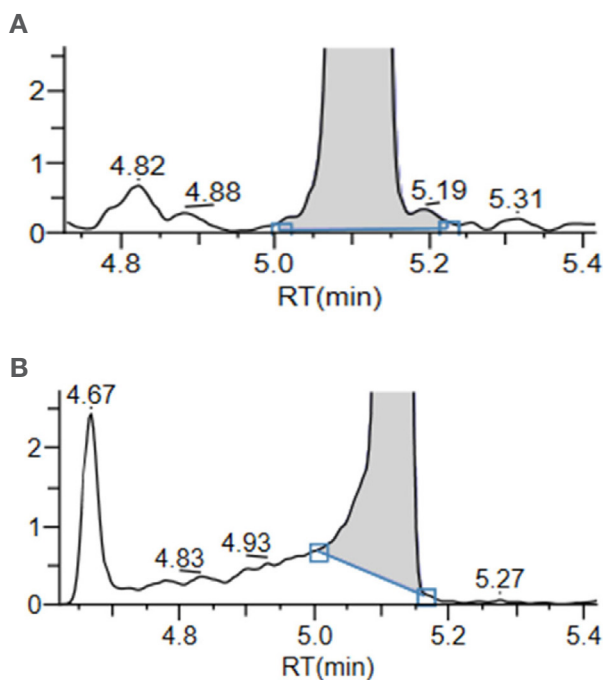


Figure 1. Effect of diluent on dithianon peak shape (A) 1% acetic acid in acetonitrile/water (1:1, v/v) and (B) 1% acetic acid in acetonitrile

Sample preparation

Dithianon is an unstable molecule in neutral and basic conditions. EURL has reported that acidic conditions improved the stability of dithianon during analysis.⁴ Apple matrix is acidic with a pH between 3.5 and 4. So here we preferred acetic acid instead of strong acids like HCl and H₂SO₄.

Using the QuEChERS 2007.01 method, the final extract remains in acidic conditions, which indirectly offered excellent stability to dithianon. Primary, secondary amine (PSA) cleanup was not used because it removes acidic compounds and causes the pH of the extract to increase. This extraction approach was assessed by analysis of apple and apple juice blank samples (n=10) spiked at 0.001 mg/kg before extraction and the recovery and RSD calculated against matrix-matched standards (extracts spiked after extraction). The results for both sample types are shown in Table 3.

Identification and quantitation

Identification of dithianon was based on % difference of the ratio of product ions 264 and 238 observed at 4.89 (±0.1) min for both matrices (apple and apple juice) compared to the standard. The observed ion ratio was 17.43% (11.8–21.99%) in apple and 19.13% (11.8–21.99%) in apple juice at 0.001 mg/kg, which was within 30% of the solvent standard shown in Figure 2. The matrix-matched calibration was plotted in the range of 0.0005 to 0.10 mg/kg by following a linear curve fitting with a 1/x weighting factor (Figure 2). In Figure 2, the calibration line was zoomed/rescaled to show the lower calibration points.

Table 3. Stability and repeatability of dithianon residue content during analysis.

Number of Injections	% Recovery		Ion ratio	
	Apple	Apple Juice	Apple	Apple Juice
1	85.64	95.65	16.34	15.88
2	85.01	95.57	17.60	16.77
3	86.42	96.76	17.66	16.75
4	81.68	90.00	17.95	17.24
5	81.68	89.24	18.28	17.01
6	82.17	88.85	16.93	16.46
7	85.77	91.03	17.05	17.23
8	76.41	92.36	17.50	16.05
9	79.39	86.24	17.19	16.01
10	80.12	85.23	17.70	16.36
%RSD	3.96	4.36		

Method performance

The method validation was performed following SANTE guidelines.⁸ The ion ratio found in both matrices and solvent standard were within 30% of acceptance criteria. The matrix effect observed was in the range of 40% to 50% in both apple and apple juice when compared with the 0.05 mg/L solvent standard. Therefore, matrix-matched calibration standards were required for accurate quantitation of the samples. Calibration coefficients of determination (R²) >0.99 and residuals (% difference) within <15%. The method offered 0.001 mg/kg as the limit of quantification (LOQ) with >10:1 signal-to-noise ratio, identification and confirmation criteria in both matrices with both matrices with >80% recovery and <15% RSD (n=6). The recovery experiment was

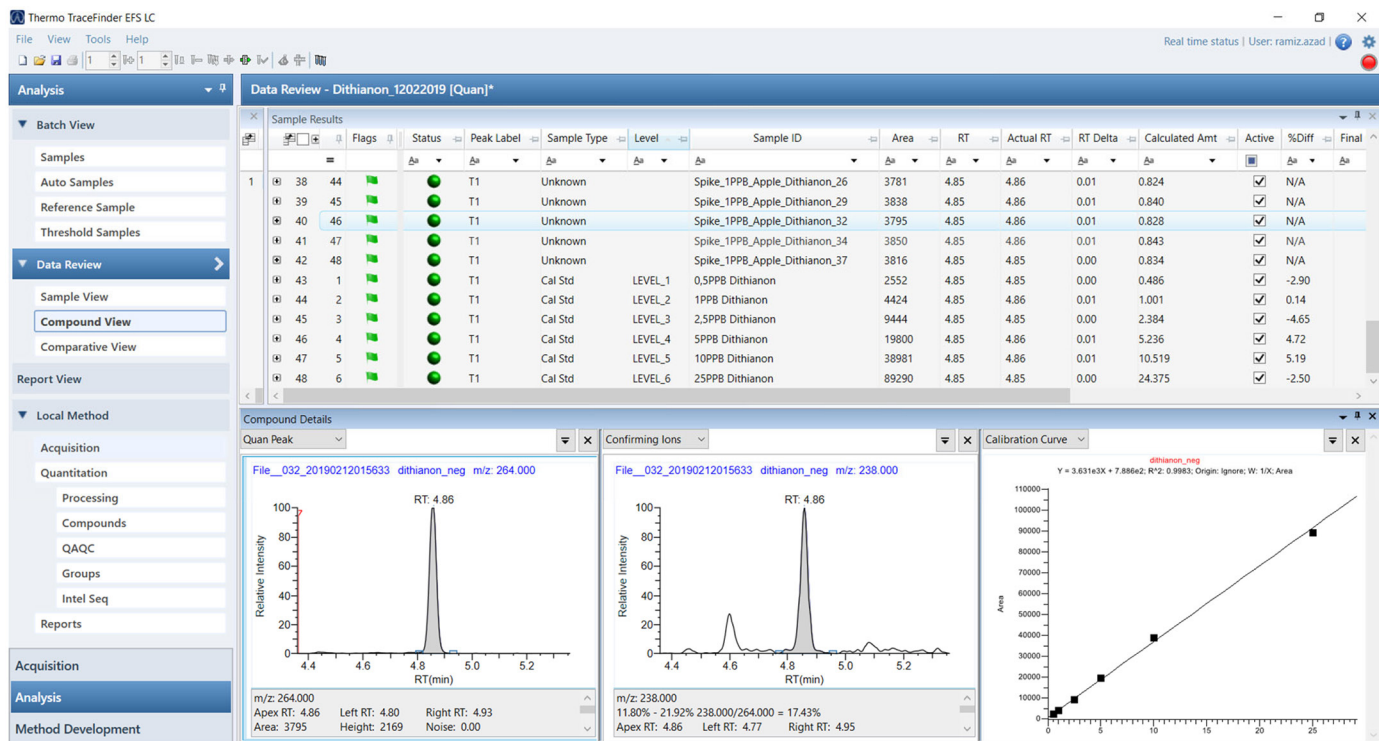


Figure 2. Extracted ion chromatogram of dithianon spiked at 0.001 mg/kg in apple showing two transitions

carried out at four different levels, i.e., 0.001 (LOQ), 0.01 (reporting limit), 0.05, and 0.10 mg/kg with six replicate each in apple and apple matrix. Average recoveries were within 80% to 115% with <15% RSD for all levels, which is within the SANTE acceptance criteria (70% to 120%). The results presented in Table 4 indicate the fulfillment of the SANTE guideline requirements. Also, the method showed stable retention times (± 0.1 min) for a validation batch with excellent area repeatability (<4.2% RSD) of 16 injections for dithianon in the matrix (Figures 3 and 4).

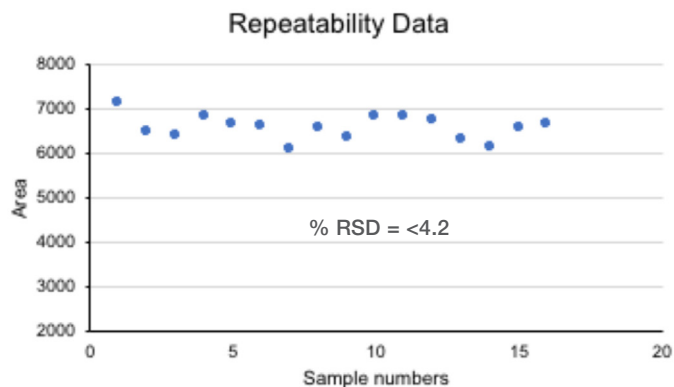


Figure 3. Area repeatability for dithianon in apple matrix (n=16)

Table 4. Method validation data for dithianon (recovery and precision at three different levels)

Matrix	LOQ (mg/kg)	% Recovery (%RSD)			
		0.001 (mg/kg)	0.01 (mg/kg)	0.05 (mg/kg)	0.10 (mg/kg)
Apple juice	0.001	87 (13)	107 (2.5)	108 (4.0)	94 (4.3)
Apple fruit	0.001	84 (16)	114 (10.6)	103 (9.0)	93 (6.3)

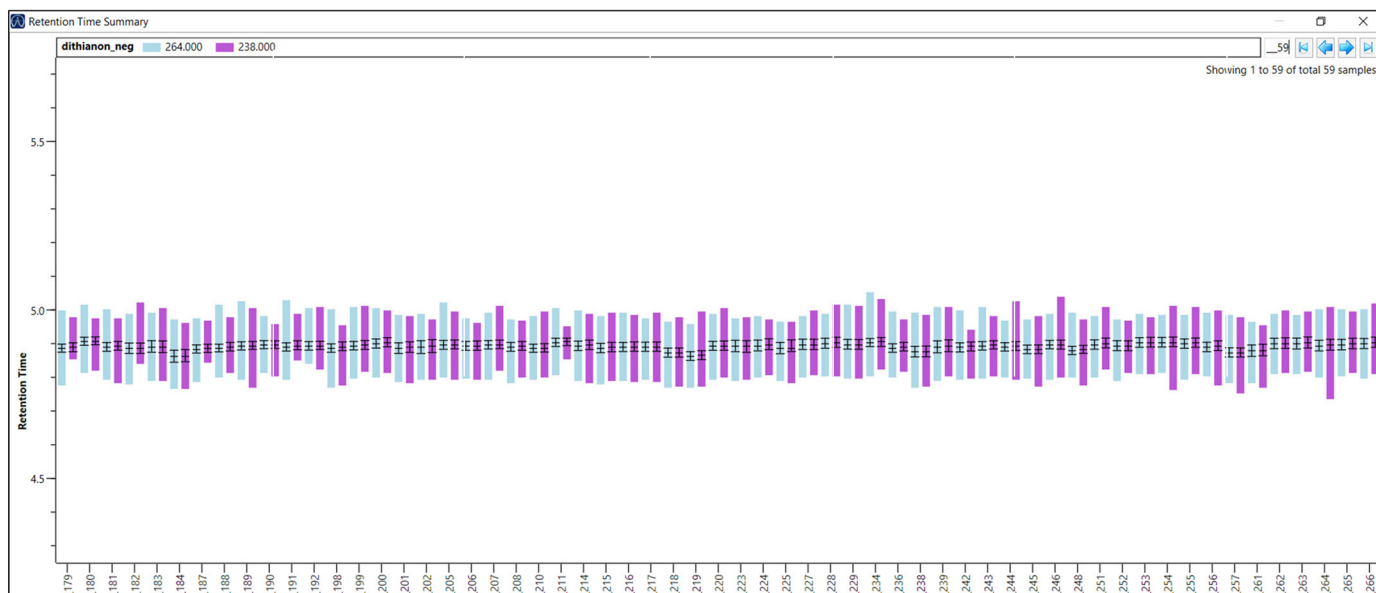


Figure 4. Retention time (min) repeatability for dithianon in apple matrix (n=59)

Conclusion

This application note provided a simple solution for trace level dithianon residue analysis (0.001 mg/kg) in apple and apple juice using a modified QuEChERS method followed by LC-MS/MS using the TSQ Quantis mass spectrometer. This optimized method fulfills the EU and FSSAI MRLs compliance requirement by achieving an excellent lower limit of quantitation (0.001 mg/kg) without compromising the data quality. This approach offered excellent stability for dithianon throughout the analysis. The method provided highly sensitive, robust, accurate, and precise results for dithianon residue in apple and apple juice. This method also demonstrated a simple way for identification and quantitation of dithianon as per the regulatory SANTE guideline requirements. The optimized protocol could result in high throughput in commercial food testing laboratories.

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