APPLICATION NOTE

Liquid Chromatography/ Mass Spectrometry

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Analysis of 213 Pesticide Residues in Grapes by LC-MS/MS with Time-Managed MRM

Introduction

The Grape crop is one of the most important fruit crops consumed in the world. Grapes are consumed both as fresh and as processed products, such as wine, jam, juice, jelly, grape seed extract, raisins, vinegar and grape seed oil. A large variety of pesticides are used in grape production throughout its growing season to control pests and diseases in vineyards and to increase crop yield. Pesticide residue is a major concern for the stakeholders of the grape industry, due to more and more stringent regulations and safety standards in most countries. It is also a concern for the general consumers, due to increased demand for safer products. Therefore, to prevent health risks, it is important to monitor the presence of pesticides and regulate their levels in grapes.

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In the European Union (EU), Regulation 396/2005/EC establishes the maximum residue levels (MRLs) of pesticides permitted in products of animal or vegetable origin intended for human or animal consumption.[1] The MRLs for pesticide residues in grapes mostly range between 10 µg/kg and 5000 µg/kg, depending on the pesticide. However, in some cases higher limits are established; for example, 100 mg/kg for fosetyl-aluminium.[1] Regulatory Agencies around the world, as in the EU, have provided similar guidelines. In the United States, tolerances for more than 450 pesticides are stated by the U.S. EPA (Environmental Protection Agency) Office of Pesticide Program and enforced by U.S. FDA (Food and Drug Adminsitration).[2] In China, national standard GB28260-2011 was introduced in 2012, which specifies 181 MRLs for pesticides in food.[3] India has regulations on the residue analysis of pesticides for grape export.[4] In order to determine low levels of pesticides in grapes, highly sensitive, selective and accurate analytical methods are needed. Due to the large number of pesticides potentially used in grape production, the use of multi-residue methods capable of determining a multitude of pesticides in one single run is the most efficient approach. Traditionally, pesticide residues were analyzed mainly by gas chromatography/mass spectrometry (GC/MS) methods,[5, 6] but a GC method is not suitable for ionic and polar compounds, especially for compounds that are thermally labile and could decompose in the GC injection port. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become the method of choice for pesticide analysis, due to its high selectivity and sensitivity, as well as its suitability for a wide range of compounds in various sample matrices.[7-11] Recently, review articles on the behavior and fate of pesticide residues in grapes and on the analytical methods applied for the analysis of pesticide residues in grapes and related products have been published.[11, 12]

In this study, a fast, sensitive and selective multi-residue method has been developed by coupling QuEChERS sample preparation with LC-MS/MS. Using time-managed-MRM™ in the QSight™ triple quadrupole mass spectrometer, the optimum dwell time can be generated automatically for MRM transitions based on the number of co-eluting transitions, expected cycle time, retention time and tolerance time window of the targeted analytes. Such method automation results in improved data quality, better sensitivity, accuracy, and reproducibility, as demonstrated in this study by the results of 213 pesticide residues analyzed in grape samples.

Experimental

Hardware/Software

The Chromatographic separation was conducted by a PerkinElmer UHPLC System and detection was achieved using a PerkinElmer QSight™ 220 triple quadrupole mass spectrometer, equipped with both ESI and APCI ionization sources. All instrument control, data acquisition and data processing were performed using the Simplicity 3Q™ software.

Method Parameters

Sample Preparation

Organic and non-organic grape samples were obtained from local grocery stores in Ontario, Canada. The mixed pesticide standards were obtained from ULTRA Scientific® (North Kingstown, RI). The samples were prepared using Supra-d QuEChERS kits (AOAC 2007.01 method). Grape samples were homogenized by a blender, and then ~10 g of slurry was transferred to a 50-mL tube and followed by the addition of 10 mL of cold acetonitrile. To this mixture, QuEChERS salts (6 g of $MgSO_4$ and 1.5 g of sodium acetate, Part # N9306900) were added, vortexed for 30 min. and centrifuged at 7000 rpm for five min. after further clean-up, using AOAC 2007.01 clean up kit (which containing 1200 mg $MgSO_4$, 400 mg PSA, Part # N9306909). The filtered supernatants were then injected directly onto the column.

LC Conditions and MS Parameter Settings

The LC conditions are shown in Table 1 and the MS source settings are shown in Table 2. Source parameters, including gas flows, temperature and position settings, were optimized for maximum sensitivity. For example, compound-dependent parameters for a partial list of the multiple reaction monitoring (MRM) transitions are shown in Table 3. The MS acquisition method is generated automatically by selecting the pesticides of interest from the built-in compound library in the time-managed-MRM module of the Simplicity software, including both positive and negative analytes, as shown in Figure 1.

Table 1. LC Conditions.

LC Column	Bownlee, SPP Phenyl-Hexyl, Part # N9308485 100 x 2.1 mm, 2.7 µm
Mobile Phase A	5-mM ammonium formate
Mobile Phase B	5-mM ammonium formate in methanol
Mobile Phase Gradient	10% mobile phase B for one min, then ramp to 95% B in 15 min and hold for two min. Re-equilibration: three min.
Column Oven Temperature	40 °C
Auto sampler Temperature	15 °C
Injection Volume	1 µL

Table 2. MS Source Settings.

ESI Voltage (Positive)	5000 V
ESI Voltage (Negative)	–4000V
Drying Gas	140
Nebulizer Gas	350
Source Temperature	325 °C
HSID Temperature	200 °C

Table 3. Optimized MRMs and compound-dependent parameters for selected pesticides.

Compound Name	Polarity	Q1 Mass	Q2 Mass	CE	EV	CCL2
Acetamiprid	Positive	223.2	126.1	-30	25	-49
Acetamiprid-2	Positive	223.2	99.1	-56	25	-73
Azoxystrobin	Positive	404.1	372.1	-18	25	-57
Azoxystrobin-2	Positive	404.1	344.1	-34	25	-71
Boscalid	Positive	343.0	307.0	-25	25	-57
Boscalid-2	Positive	343.0	140.0	-28	25	-60
Chlorantranilprole	Positive	484.0	452.8	-20	25	-66
Chlorantranilprole-2	Positive	484.0	285.8	-18	25	-65
Chlorpyriphos	Positive	350.0	97.0	-32	25	-64
Chlorpyriphos-2	Positive	350.0	198.0	-20	25	-53
Clofentezine	Positive	303.0	138.0	-28	25	-56
Clofentezine-2	Positive	303.0	102.0	-50	25	-75
Cyprodinil	Positive	226.0	93.0	-48	25	-66
Cyprodinil-2	Positive	226.0	77.0	-34	25	-53
Diafenthiuron	Positive	385.2	329.1	-26	25	-62
Diafenthiuron-2	Positive	385.2	278.1	-44	25	-78
Difenoconazole	Positive	406.2	251.1	-32	25	-69
Difenoconazole-2	Positive	406.2	111	-76	25	-109
Difenoconazole-3	Positive	406.2	272.1	-22	25	-60
Dimethomorph	Positive	388.2	301.1	-26	25	-62
Dimethomorph-2	Positive	388.2	165.1	-40	25	-75
Fenhexamid	Positive	302.0	97.0	-32	25	-59
Fenhexamid-2	Positive	302.0	55.0	-60	25	-84
Fludioxonil	Negative	246.6	125.9	40	-25	60
Fludioxonil-2	Negative	246.6	179.9	39	-25	60
Fluopyram	Positive	397.0	173.0	-35	25	-71
Fluopyram-2	Positive	397.0	145.0	-70	25	-103
Imidachloprid	Positive	256.2	209.0	-18	25	-42
Imidachloprid-2	Positive	256.2	175.2	-26	25	-49
Pyrimethanil	Positive	200.0	107.0	-33	25	-50
Pyrimethanil-2	Positive	200.0	82.0	-32	25	-49
Pyraclostrobin	Positive	388.0	194.0	-16	25	-53
Pyraclostrobin-2	Positive	388.0	163.0	-36	25	-71
Spinosad -1	Positive	732.6	142.0	-42	25	-111
Spinosad -2	Positive	732.6	98.1	-100	25	-163
Spirotetramat	Positive	374.2	330.1	-21	25	-56
Spirotetramat-2	Positive	374.2	216.1	-45	25	-78
Spirotetramat-3	Positive	374.2	302.1	-23	25	-58
Spiroxamine	Positive	298.3	144.2	-30	25	-57
Spiroxamine-2	Positive	298.3	100.2	-50	25	-75
Spinetoram	Positive	748.4	142.1	-42	25	-113
Spinetoram-2	Positive	748.4	98.1	-100	25	-165
Trifloxystrobin	Positive	409.0	186.0	-26	25	-64
Trifloxystrobin-2	Positive	409.0	206.0	-20	25	-59
Tebuconazole	Positive	308.0	70.0	-30	25	-58
Tebuconazole-2	Positive	308.0	125.0	-50	25	-76

Results and Discussion

Analytical Challenges for Testing Multi-residues of Pesticides from Food Samples

Traditional MRM method development is not suitable for analysis of a large number of analytes such as pesticide residues in a single run. This is not only because it is time-consuming and labor intensive to manually entering all the mass transitions into a method, but also because the dwell time for each transition cannot be optimized easily. Therefore, the time-managed-MRM feature in Simplicity software is especially helpful in this regard, as this approach results in better data quality by generating an optimum dwell time for each MRM. Figure 1 shows an example of a method generated using time-managed-MRM in this study.

Sample matrix effects are still the main concern for LC-MS/MS, especially for food analysis due to the diversity and complexity of food sample matrices. To overcome sample matrix effects, numerous tools have been widely applied to LC-MS/MS method development, such as sample dilution, use of stable isotope internal standards, sample matrix-matched standard calibration, standard addition method, sample clean-up, use of high efficiency UHPLC column for better separation, and the use of alternative ionization sources.[13] The most common sample matrix effect is discussed in details in the following section.

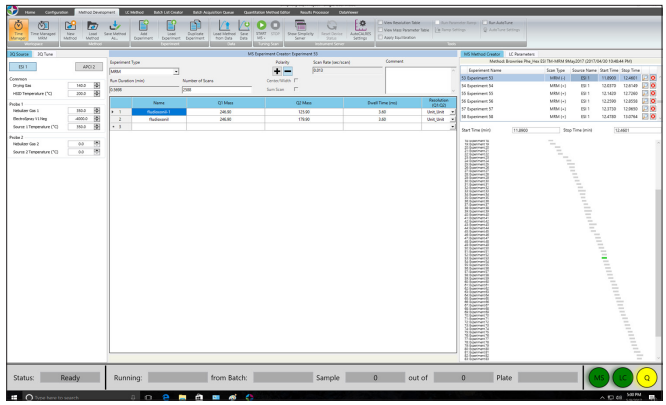

Figure 1. Example of MS method for 500 MRMs for 213 analytes generated by the time-managed MRM module of the Simplicity Software.

Linearity and Sample Matrix Effect

Calibrations were performed by preparing and running seven concentration levels of analytes standards in both neat solution (pure solvent) and grape sample matrix (matrix-matched calibration). Example calibration curves for some of the most frequently found pesticides in grapes in this study are shown in Figure 2. Overall, for all analytes, calibration results showed good linearity over three orders of magnitude (0.1 – 200 µg/L), with regression coefficient ($R^2 \geq 0.98$) for most of the analytes in both neat solution and grape matrix. Quantitative precision (%RSD) for all analytes at 10 and 100 µg/L (not shown), were all found to be between 1.2 to 15.1% (average of five replicate injections).

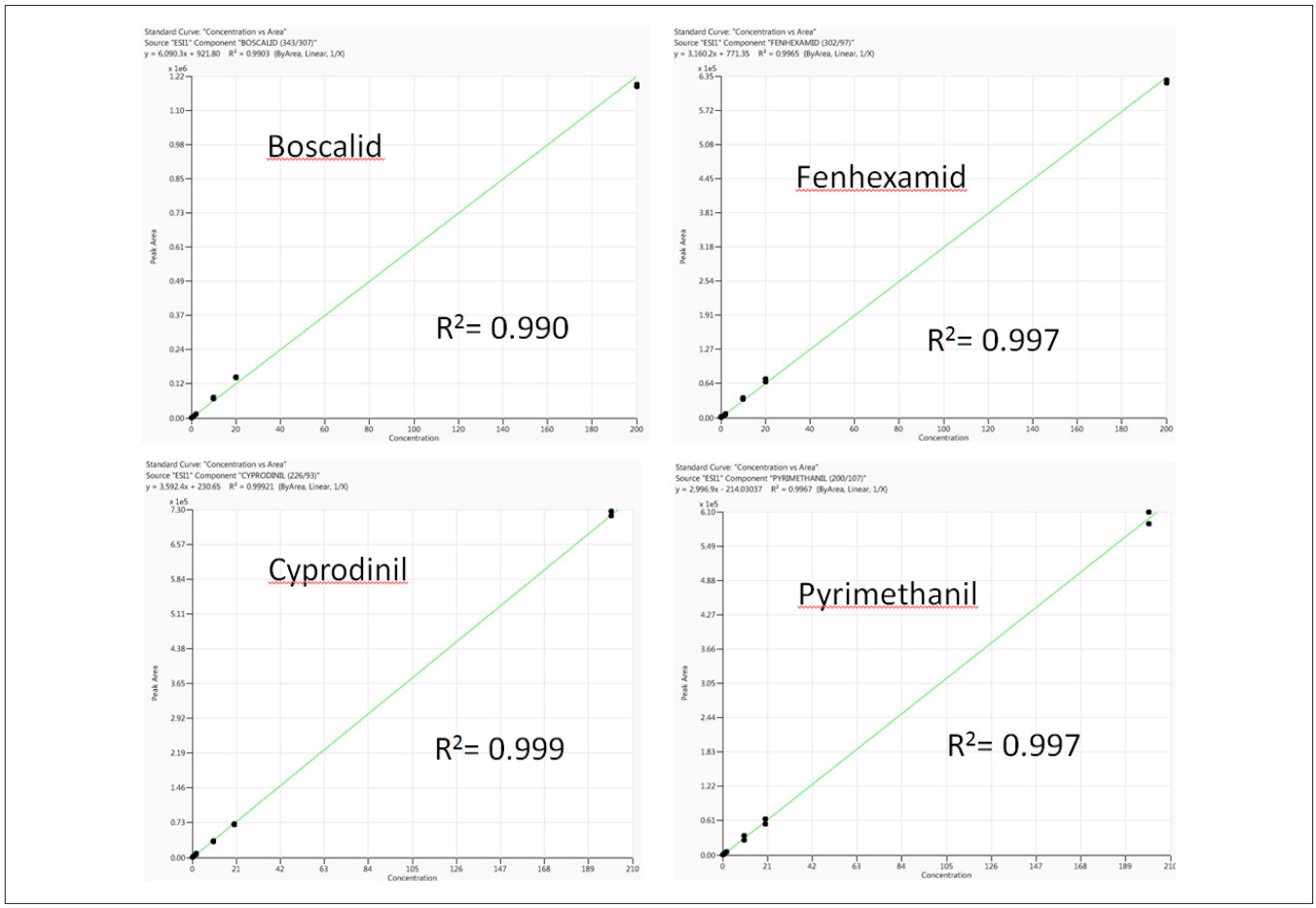

Figure 2. Example calibration curves for some of the most frequently found pesticides in grapes: boscalid, cyprodinil, fenhexamid and pyrimethanil.

Possible sample matrix effects were evaluated by comparison of the responses (peak area in this case) of analytes obtained from neat solution and those obtained from grape sample matrix (spiked at the same concentration, 100 ppb in this case). When the percentage ratio value is greater than 100%, there is a signal-enhancement, whereas a value of less than 100% indicates signal-suppression. As illustrated in Table 3, no significant ion suppression effect was found for the studied compounds, except for diafenthiuron, which showed some ion suppression.

Table 3. Example matrix effects for the pesticides identified from the grape samples in this work.

Pesticides	Peak Area (In Neat Solution)	Peak Area (In Matrix Spiked Solution)	Matrix Effect
Acetamiprid	1024279	1068162	104.3
Boscalid	696490	734080	105.4
Chlorantraniliprole	197766	190834	96.5
Cyprodinil	453221	464004	102.4
Diafenthiuron	957108	692523	72.4
Difenoconazole	1260903	1344640	106.6
Dimethomorph	435381	366000	84.1
Fenhexamid	373104	411145	110.2
Fludioxonil	293046	322095	109.9
Fluopyram	2281108	2379392	104.3
Imidacloprid	305674	304391	99.6
Pyrimethanil	366881	381018	103.9
Pyraclostrobin	1682524	1764409	104.9
Spinosad	666859	698195	104.7
Spirotetramat	517179	485990	94.0
Spiroxamine	1273651	1354261	106.3
Spinetoram	641735	665530	103.7
Trifloxystrobin	2132708	2219020	104.0
Tebuconazole	653128	620261	95.0

Analyte Recovery, Limit of Quantification and Sample Results

The percent recoveries of pesticides were evaluated at a concentration level of 100 µg/kg in two different samples: brand A non-organic and brand G organic grapes. The recoveries of analytes were between 75% to 114%, with an RSD < 10% for most analytes in the studied matrices. An overlay of the total ion chromatograms (TIC) for an organic grape sample fortified at 100 µg/kg before and after the QuEChERS sample preparation is shown in Figure 3.

The limits of quantification (LOQs) were determined based on the signal to noise ratio of ≥10 for the quantifier transitions of all analytes. The identity of each pesticide residue is confirmed by ensuring that the product ion ratios (qualifier vs. quantifier) were within 30% tolerance windows of the expected ratio.[14] The majority of the tested pesticides have a LOQ of ≤ than 1 µg/L in grape matrix with a 1 µL direct injection.

The developed method was applied for the analysis of pesticide residues in a few brands of grapes. Example chromatograms for the positively identified pesticides in sample brand B and F are shown in Figure 4 and 5, respectively. For brand F, it should be noted that both green and red grape samples were analyzed. The determined pesticide concentrations from these samples are summarized in Table 4, along with the corresponding LOQ and EU maximum residue limit (MRL) values for these pesticides.

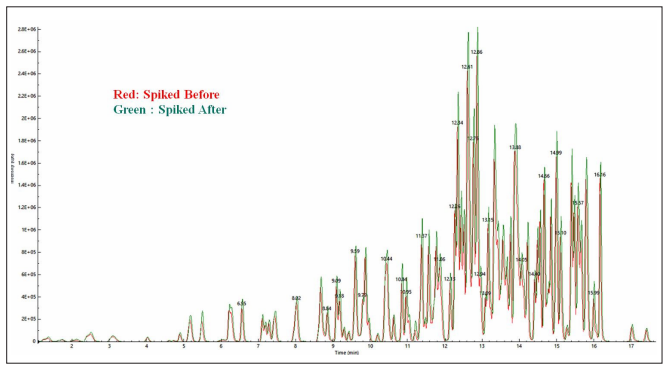

Figure 3. An overlay of the total ion chromatograms (TIC) for an organic grape sample fortified at 100 µg/kg before (red) and after (green) the sample preparation.

#	Pesticide Residues	RT (min)
1	Acetamiprid	8.03
2	Boscalid	12.76
3	Chlorantraniliprole	12.36
4	Cyprodinil	13.48
5	Fenhexamid	12.63
6	Fludioxonil	12.16
7	Pyrimethanil	11.82
8	Spinetoram	17.40
9	Spirotetramat	13.43
10	Trifloxystrobin	14.85
11	Tebuconazole	13.59

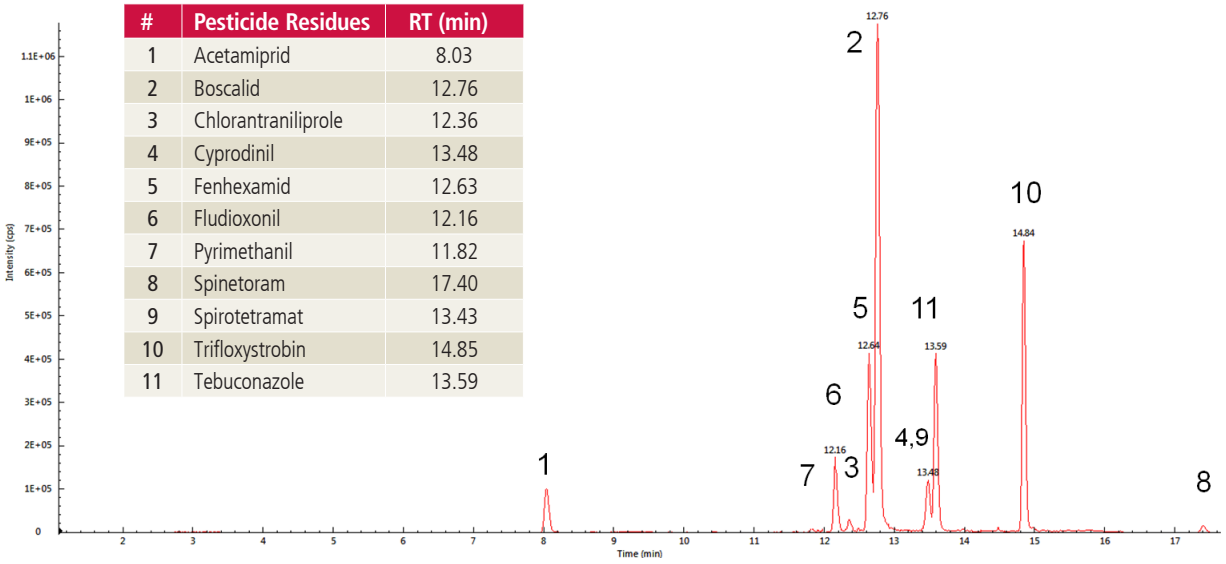

Figure 4. Chromatogram and list of pesticides positively identified in brand B grape sample.

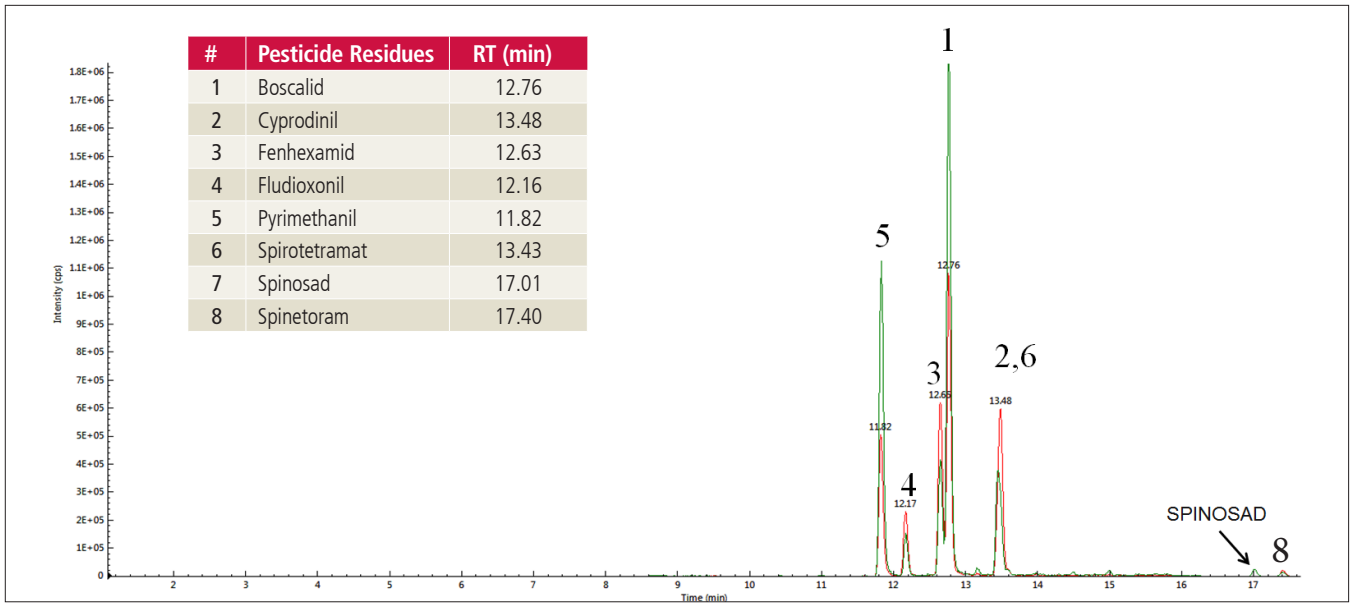

Figure 5. Chromatogram and list of pesticides positively identified in brand F grape samples (green for green grape and red for red grape).

Table 4. Summary results for the positively identified pesticide residues in grapes in µg/kg.

Pesticide	Brand A	Brand B	Brand C	Brand D	Brand E	Brand F*	Brand G	MRL	LOQ
Acetamiprid	17	31						500	0.1
Boscalid	1244	888	2294		1139	1438 (800)	1	5000	0.2
Chlorantraniliprole		29						20	0.2
Cyprodinil		93	51		3	169 (435)		3000	0.2
Diafenthiuron			18					3000	0.2
Difenoconazole	29		27					3000	0.1
Dimethomorph			14	58				3000	0.2
Fenhexamid		319	17	777	1018	340 (553)		15000	1
Fludioxonil		213	66		11	195 (336)		5000	0.2
Fluopyram				1				1500	0.1
Imidacloprid	2							1000	0.2
Pyrimethanil		5	66		271	966 (451)		5000	1
Pyraclostrobin	180							1000	0.2
Spinosad			27		57	46		500	0.2
Spirotetramat		7	7		3	87 (61)		2000	1
Spiroxamine				12	11			600	0.2
Spinetoram		47		6	29	47 (54)		500	0.1
Trifloxystrobin		69	14	4				3000	0.2
Tebuconazole		204						500	0.2

** Initial value is for green grape sample and the value in parenthesis is for red grape sample.*

Conclusions

A LC-MS/MS method for multi-residue pesticides analysis in grapes was developed by coupling a UHPLC system to a QSight 220 triple quadrupole mass spectrometer. This method can be applied for the determination of pesticide residues in grapes, with LOQs well below the limits set by regulatory boards.

The time-managed-MRM module in the Simplicity software was effectively used in this study for monitoring 213 pesticide residues in grapes using the QSight™ LC-MS/MS system. This feature has simplified the creation and optimization of MS methods for monitoring a large number of analytes in food samples.

The QuEChERS sample preparation method utilized in this study demonstrated good recovery (75–114%) and excellent quantitative reproducibility (RSD<10%) for most pesticides.

The developed LC-MS/MS method showed good linearity, with LOQ ≤ 1 µg/kg for most of the 213 pesticides in grape matrix. A number of pesticide residues were identified and quantified with concentrations greater than 1 µg/kg in all the non-organic brands of grapes. However, for the organic grapes tested, only one pesticide (boscalid) was detected at a concentration level of 1 µg/kg.

The same LC-MS/MS method has also been applied successfully to other fruit analyses, such as berries, orange and grapefruit, all with good performance. These results demonstrated the method's applicability and effectiveness in detecting and quantifying pesticide residues in fruit samples.

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