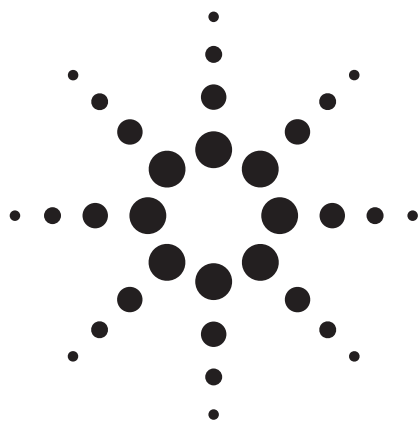




# Agilent 6530 Accurate-Mass Q-TOF LC/MS System with Agilent 1290 Infinity LC for Multi Plant Growth Regulator Analysis from Grapes

## Application Note

Food Analysis

### Author

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### Abstract

The Agilent 6530 Quadrupole Time of Flight LCMS system with Jet stream ESI ion source coupled with Agilent 1290 Infinity UHPLC and Dispersive C18 ODS sample clean up shows excellent sensitivity and outstanding mass accuracy for screening of Multi class PGRs in Grape samples.

The Q-TOF is an excellent system for identifying compounds using accurate mass in full scan MS and MS/MS mode. Accurate mass leads to chemical formula which can also give structural information when forming product ions in MS/MS.

A simple and inexpensive sample clean up technique based on a dispersive solid phase (C18) adsorption is effective in removing background matrix from Grape sample to screen multiclass PGRs using Q-TOF.

Outstanding mass accuracy; all PGRs detected with less than 1 ppm mass accuracy for 50 ppb and 100 ppb and less than 5 ppm accuracy for 5 ppb concentration in grape matrix.



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## Introduction

This document describes an effective, sensitive LC/MS/MS method with a simple, fast and economic sample preparation technique for the screening of 12 Plant Growth Regulators (PGRs) in grape samples.

Plant hormones or phytohormones are chemicals that regulate plant growth. Manmade compounds called PGRs are used to regulate the growth of cultivated plants, weeds, and plants and plant cells grown in vitro. Synthetic PGRs are used in different techniques such as cutting, grafting, and micro-propagation.

PGRs activate cellular responses such as cell death. Some have the ability to adversely affect human cancer cells. European Union (EU) has listed these compounds, therefore they require measurement.

Using accurate mass in full scan (mass range) mass spectrometry, compound empirical formulas can be determined for the purpose of identification. A high degree of spectral resolution allows for selective identification among coeluting compounds. Isotope ratios are an additional tool that helps identify compounds with high carbon numbers as well as those that contain elements such as chlorine and sulfur. Selective MS/MS with quadrupole time-of-flight mass spectrometry (Q-TOF) is implemented to analyze for structural elucidation. Because the Agilent Q-TOF also has very accurate mass at the MS/MS level, it is easier to determine the structure of the product ion that corresponds as the substructure of the precursor ion, and thereby reduces the number of possible structures.

Agilent Q-TOF is capable of acquiring full scan MS data at the rate of 20 spectra/sec. The resulting large amount of data representing a possibly large number of compounds must be converted to useful information. The Agilent Molecular Features Extractor (MFE), which is a standard component of MassHunter Qualitative Analysis software carries out the following steps:

- Remove persistent chemical background
- Resolve coeluting interferences
- Recognize and group isotopic clusters
- Visualize 2D/3D
- Search database and identify chemicals (accurate mass, isotope matching)

## PGRs detected in positive mode

- |                         |                            |
|-------------------------|----------------------------|
| 1 Chlormequat           | 6 Indol acetic acid (IAA)  |
| 2 d4 chlormequat (ISTD) | 7 6 Benzyl adenine         |
| 3 Daminozide            | 8 Indol Butyric acid (IBA) |
| 4 Zeatin                | 9 Forchlorfenuron          |
| 5 Kinetin               | 10 Paclobutrazol           |

## PGRs detected in negative mode

- |                   |                       |
|-------------------|-----------------------|
| 1 Gibbrellic acid | 3 2, 4 Dichlorphenoxy |
| 2 Absciscic acid  | acetic acid (2, 4-D)  |

## New Method

The following are benefits of the new method:

- Easy to use Rugged UHPLC method and robust interfacing with Agilent 6530 Accurate-Mass Q-TOF LC/MS system with Jetstream ESI source
- Sample Prep Easy, fast, and economic dispersive C18 sample cleanup
- Robustness Significantly improved method stability; ion suppression effects reduced to minimum
- Mass accuracy Outstanding; all PGRs detected with less than 1 ppm mass accuracy for 50 ppb and 100 ppb and less than 5 ppm accuracy for 5 ppb concentration in grape matrix.

## Methods and Operations

This method for analysis of PGRs is based on LC/MS/MS with Quadrupole Time-of-Flight (Q-TOF) systems. The sample is prepared by a simple dispersive C18 cleanup process followed by direct injection. Separation is performed with fast UHPLC and screening with ESI–LC/MS/MS (Targeted MS/MS mode).

## Analysis steps

1. Liquid phase extraction followed by dispersive C18 cleanup.
2. Internal standard d4-Chlormequat spiked in sample and standards.
3. UHPLC analysis in gradient mode.
4. Detection by Jetstream ESI-LC/MS/MS (Targeted MS/MS mode).

## Sample Preparation Steps

A 5-g amount of homogenized grape sample was added to 40 mL Methanol: Water (80:20). The mixture was shaken at ambient, and 0.8 mL supernatant liquid was mixed with 200 mg of C18 ODS SPE Bulk. The mixture was vortexed and centrifuged. A 0.4-mL amount was diluted to 1 mL with methanol and 2 µL were injected for LC/MS/MS analysis.

## LC/MS/MS Method

The total run time required to determine 10 PGRs in positive mode is 16 min. Two microliters of the sample extract were injected for LC/MS/MS. Method details in positive mode are listed below.

|                     |                                                     |                  |                  |
|---------------------|-----------------------------------------------------|------------------|------------------|
| Column:             | Agilent ZORBAX Extended C18 2.1 mm × 100 mm, 1.8 μm |                  |                  |
| Flow rate:          | 0.2 mL/min                                          |                  |                  |
| Column temperature: | 40 °C                                               |                  |                  |
| Mobile phase A:     | Water (0.5% Formic acid)                            |                  |                  |
| Mobile Phase B:     | Methanol                                            |                  |                  |
|                     | Time                                                | % Mobile phase A | % Mobile phase B |
|                     | 0                                                   | 95               | 5                |
|                     | 7                                                   | 10               | 90               |
|                     | 9                                                   | 10               | 90               |
|                     | 12                                                  | 81               | 5                |

## Mass Spectrometer Settings

| No | Compound        | Prec $m/z$ | Ret time | CE  |
|----|-----------------|------------|----------|-----|
| 1  | Chlormequat     | 122.0733   | 1.261    | 23  |
| 2  | Chlormequat-d4  | 126.0909   | 1.261    | 25  |
| 3  | Daminozide      | 161.0919   | 1.311    | 6   |
| 4  | Zeatin          | 220.1193   | 4.051    | 13  |
| 5  | Kinetin         | 216.0884   | 5.211    | 15  |
| 6  | 6-Benzyladenine | 226.1091   | 6.261    | 20  |
| 7  | IAA             | 176.0702   | 6.621    | 4.5 |
| 8  | IBA             | 204.1013   | 7.561    | 8   |
| 9  | Forchlorfenuron | 248.0594   | 8.121    | 11  |
| 10 | Paclobutrazole  | 294.1371   | 8.695    | 15  |

## Internal Reference Mass

The wide in-scan dynamic range facilitates continuous, automated introduction of a reference mass compound at a very low concentration. The result is outstanding mass accuracy without significant spectral interference from the reference mass.

Mass observed in positive mode- 121.050873  $m/z$  and 922.009798  $m/z$

Mass observed in negative mode- 112.985587  $m/z$  and 1033.988109  $m/z$

## Ten PGRs in Positive Polarity

See Figure 1 below for the typical chromatogram.

The total run time required to determine three PGRs in negative mode is 6 min. Two microliters of the sample extract were injected for LC/MS/MS. Method details in negative mode are listed below,

|                     |                                                             |                  |                 |
|---------------------|-------------------------------------------------------------|------------------|-----------------|
| Column:             | Agilent ZORBAX Eclipse Plus RRHD C18 2.1 mm × 50 mm, 1.8 μm |                  |                 |
| Flow rate:          | 0.2 mL/min                                                  |                  |                 |
| Column temperature: | 40 °C                                                       |                  |                 |
| Mobile phase A:     | Water (0.5% formic acid)                                    |                  |                 |
| Mobile phase B:     | Methanol                                                    |                  |                 |
|                     | Time                                                        | % Mobile Phase A | %Mobile Phase B |
|                     | 0                                                           | 81               | 19              |
|                     | 2.5                                                         | 10               | 90              |
|                     | 4.5                                                         | 10               | 90              |
|                     | 6                                                           | 81               | 19              |

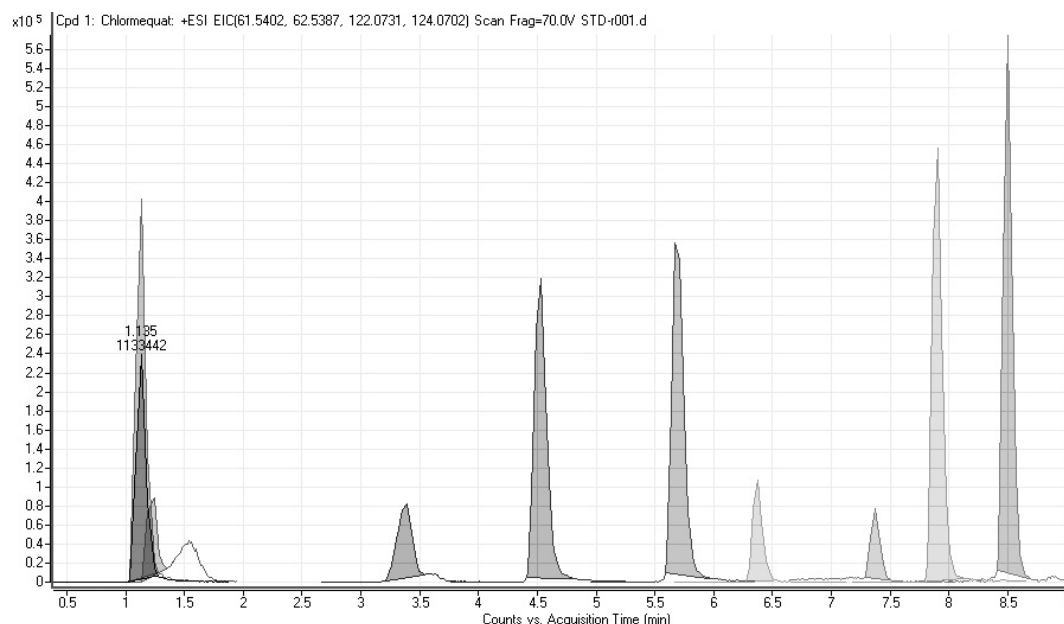


Figure 1. Typical chromatogram, elution pattern Chlormequat, d4-chlormequat, Daminozide, Zeatin, Kinetin, 6-Benzyladenine, IAA, IBA, Forchlorfenuron, Paclobutrazol.

## Three PGRs in Negative Mode

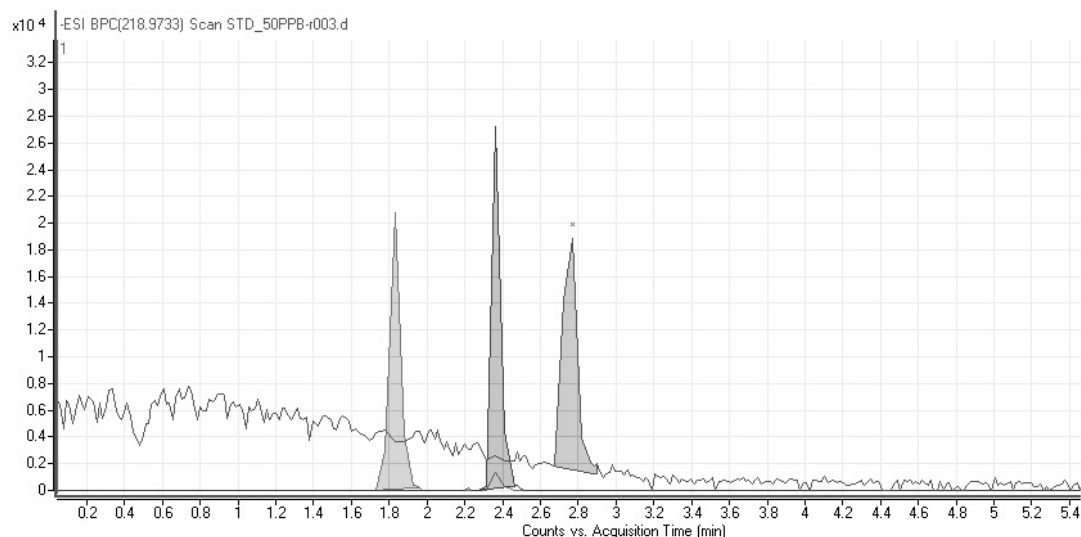


Figure 2. Typical chromatogram at 50.0 ppb concentration, elution pattern Gibberellic acid, Absciscic acid and 2-4-D.

## Mass Spectrometer Settings

| No. | Compound                                | Prec $m/z$ | Ret time | CE |
|-----|-----------------------------------------|------------|----------|----|
| 1   | Gibberellic acid (GAAA)                 | 345.1344   | 1.823    | 18 |
| 2   | Abciscic acid (ABA)                     | 263.1284   | 2.353    | 7  |
| 3   | 2-4-Dichlorophenoxy acetic acid (2-4-D) | 218.9617   | 2.705    | 3  |

## Results

Targeted PGRs were identified within less than 5 ppm mass accuracy for standards as well as standards spiked and extracted from grape samples by applying the algorithm of the Molecular Feature Extractor program to all data files. The database for known PGRs was prepared with MF and RT and screened for all grape samples.

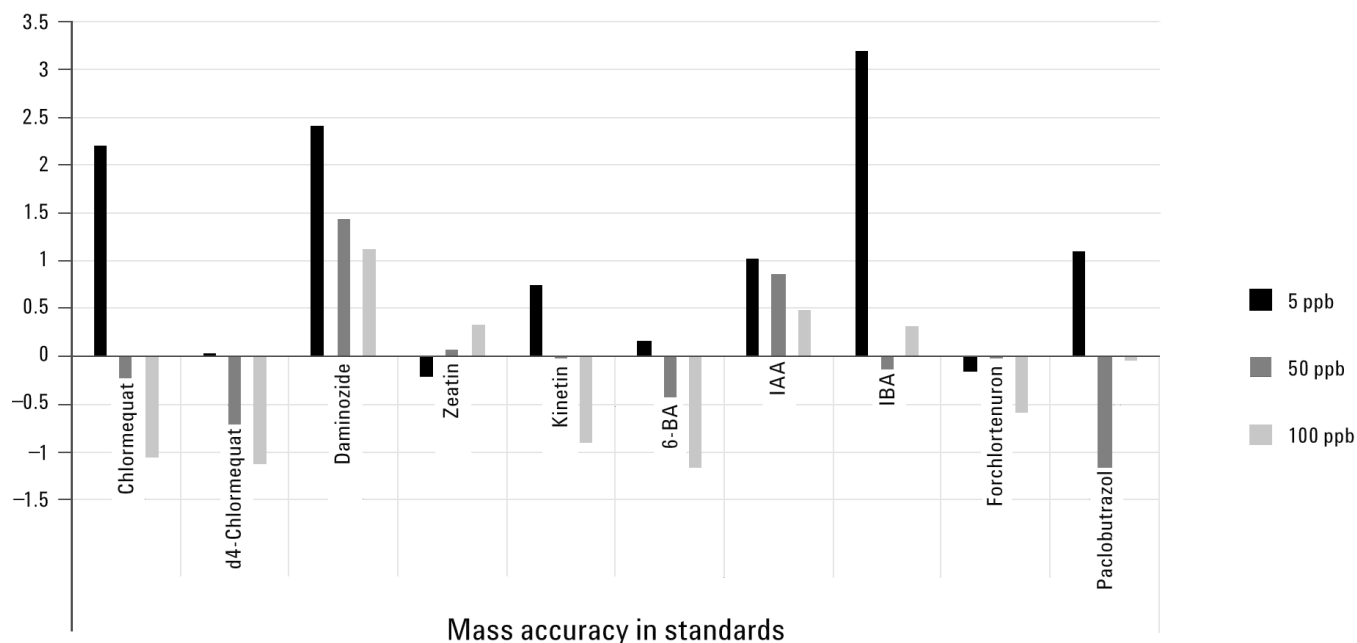


Figure 3. Mass accuracy for standard PGRs in positive mode.

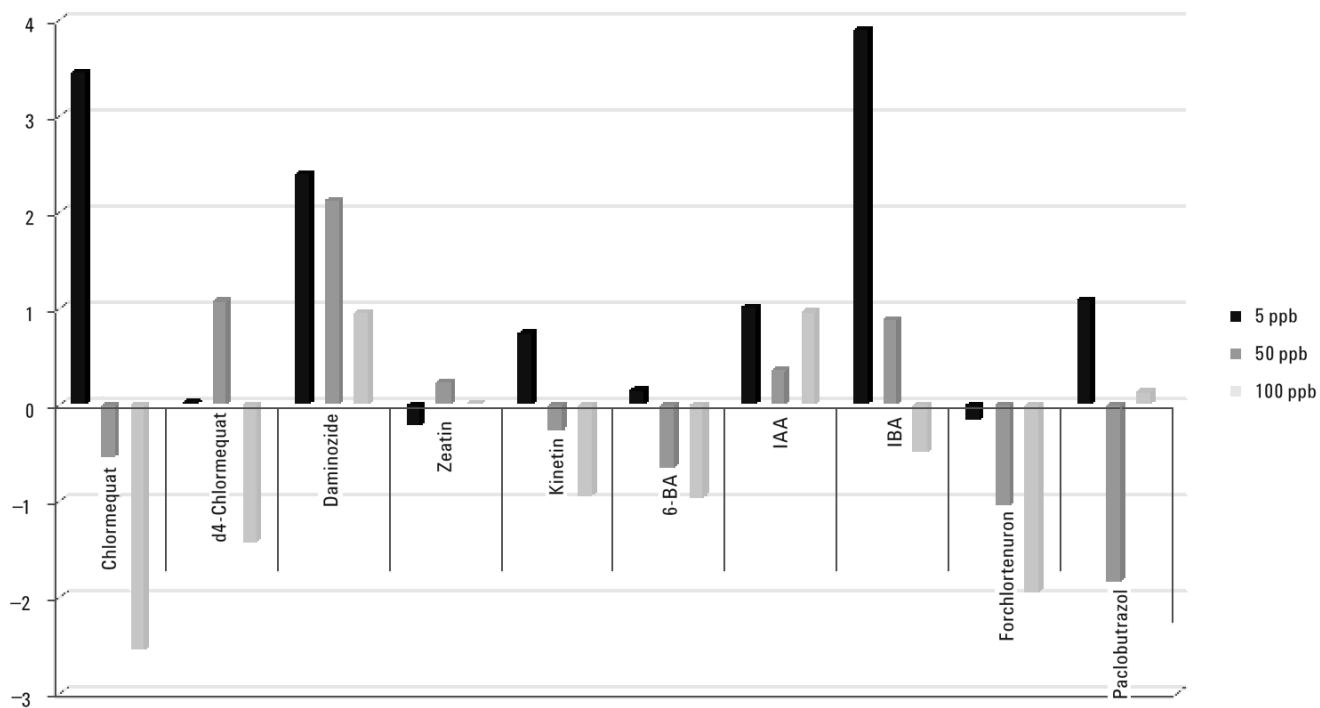


Figure 4 Mass accuracy for standard PGRs spiked and recovered from grape samples in positive mode.

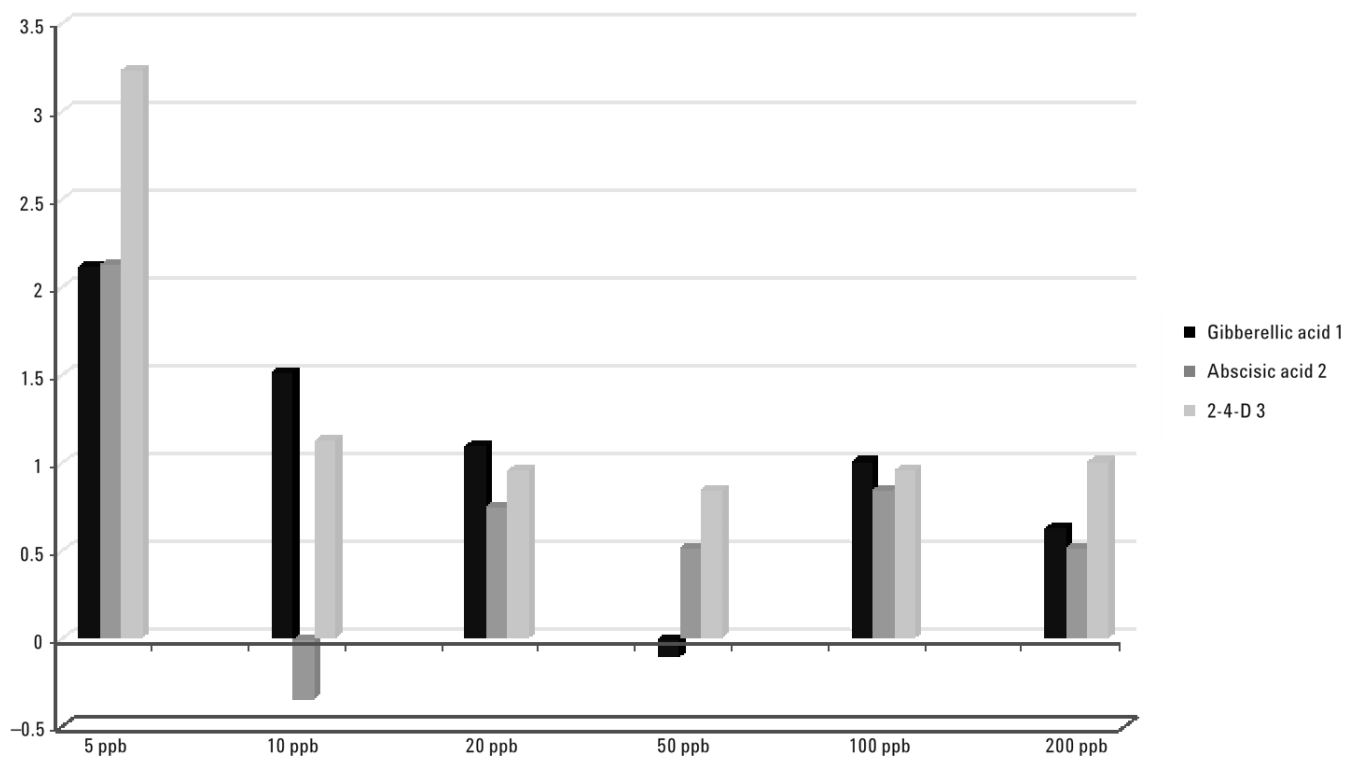


Figure 5. Mass accuracy for standard PGRs in negative mode.

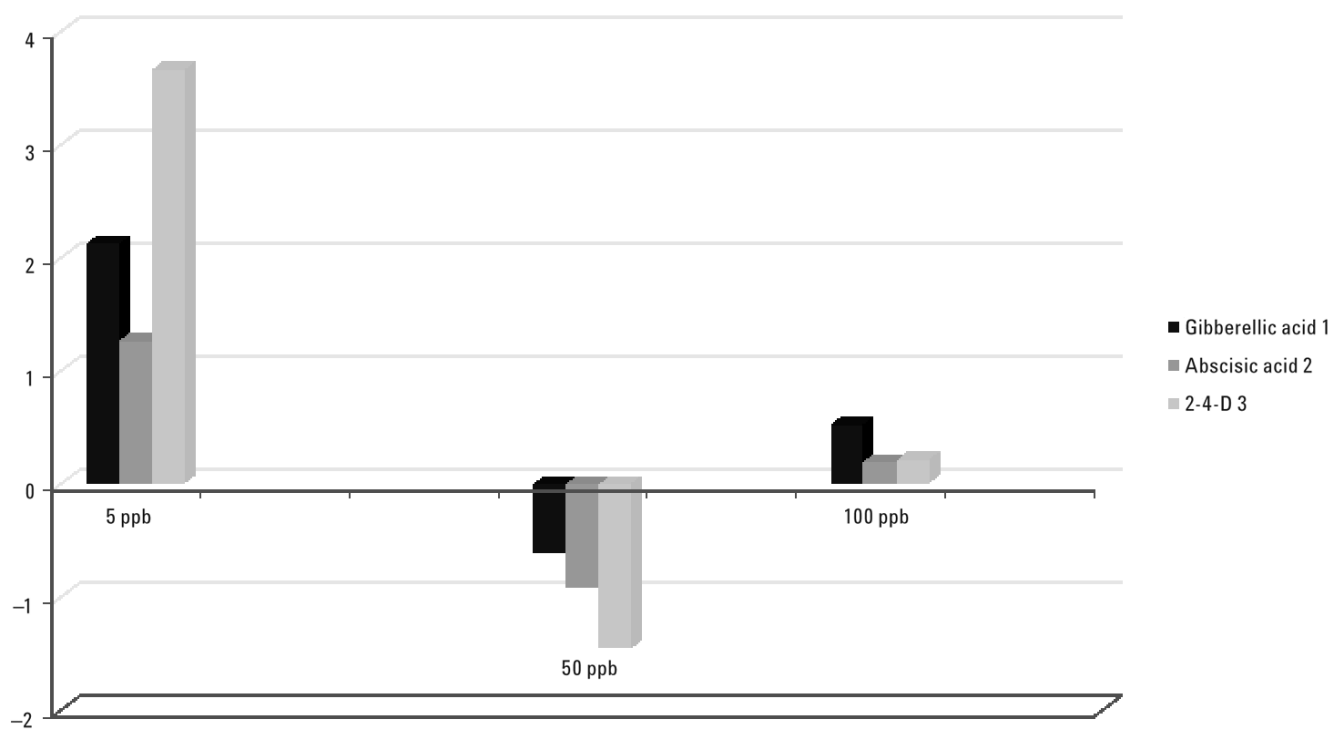


Figure 6. Mass accuracy for standard PGRs spiked and recovered from grape samples in negative mode.

Q-TOF has a high degree of spectral resolution in MS/MS mode. Accurate mass of product ions can determine their chemical formula and possible structures. Figure 7 shows the accurate mass MS/MS fragmentation of Forchlorfenuron. The chemical formula for each product ion is derived based on the possible arrangements of C, H, N and O. Knowing the structure of Forchlorfenuron, the structure of the fragment ions can be proposed using their corresponding chemical formula.

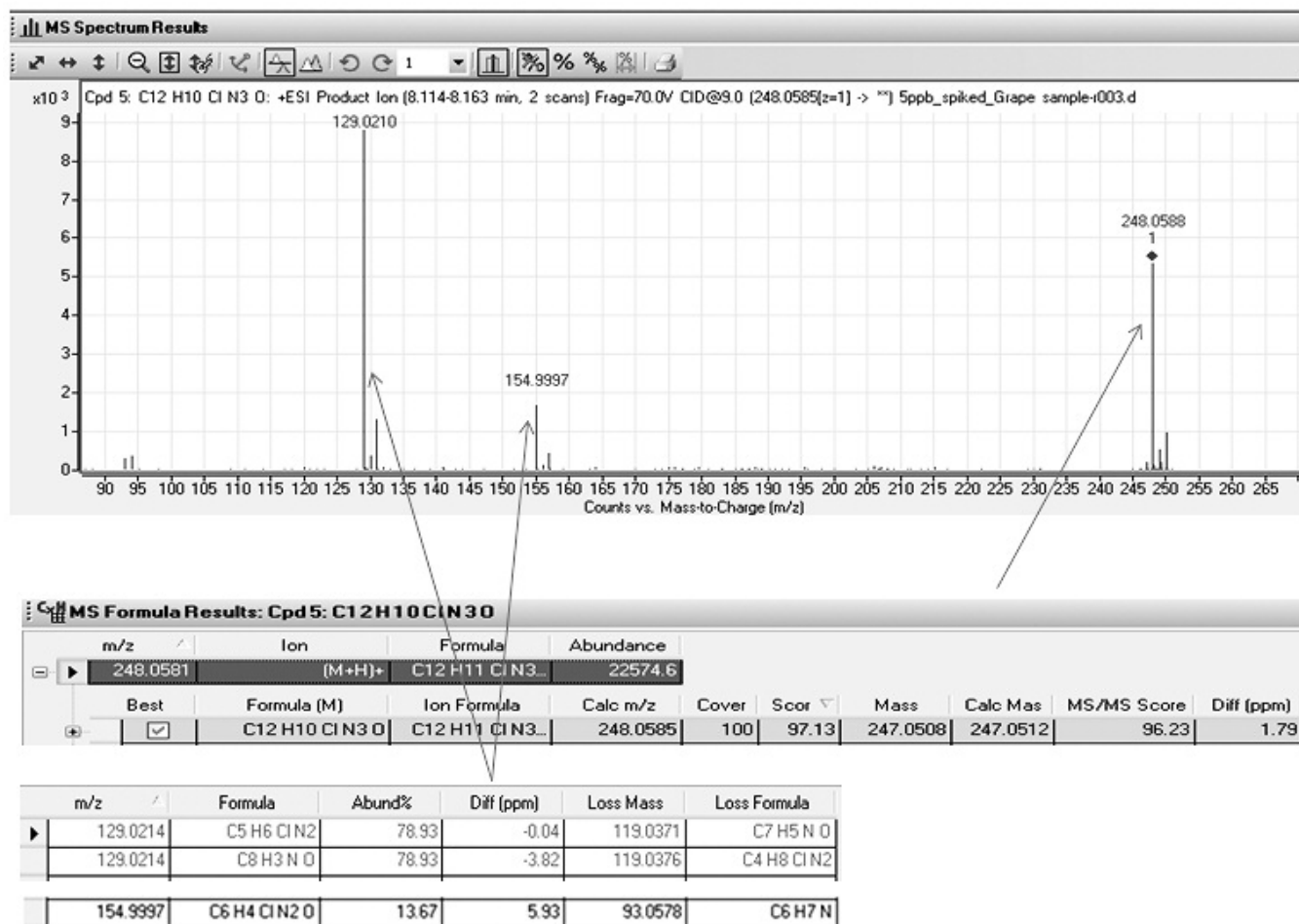


Figure 7. Targeted MS/MS scan of forchlorfenuron producing product ions that may be used for structural elucidation as well as quantitation.

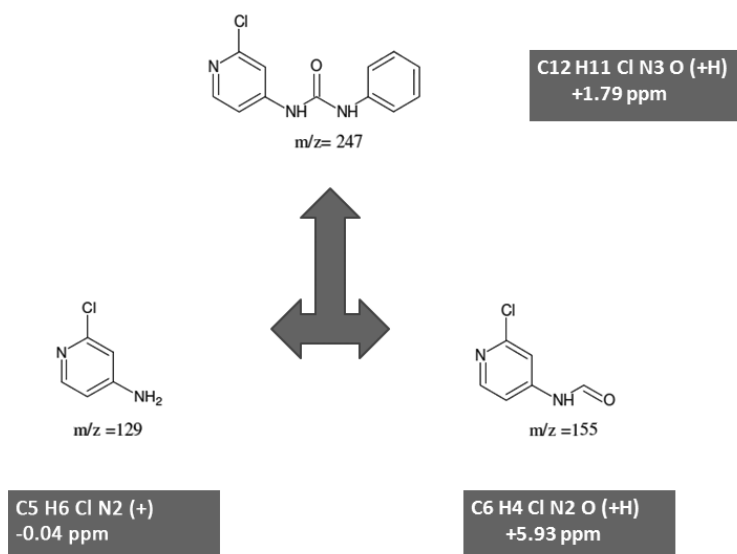


Figure 8. Chemical formulas of the product ions.

## MFG-Key differentiating features

Paclobutrazole (PBZ) MF  $C_{15}H_{20}ClN_3O$  (293.1295  $m/z$ ) when analyzed using QqQ shows major three fragments 70.1  $m/z$ , 60.2  $m/z$  and 57.2  $m/z$  (Figure 9). Similar transitions shown by another ion of mass 293.1990  $m/z$  at same retention time which may be coming from blank or Grape samples.

As per Figure 10 and 11 only differentiating factors is of low intensity of Quant ion and different Ion ratio comparing to standard. Low Intensity of Quant Ion (294.1 > 70.1) and very high intensity of Qual ion (294.1 > 60.2) in sample shows that Paclobutrazol is not present in sample but some time it will be misleading as we cannot differentiate between Paclobutrazol ion 294.1368  $m/z$  and 294.2063  $m/z$  similar ion coming from grape sample/blank as it shows similar MRM transitions at same retention time.

Low resolution system like QQQ will not be able to differentiate between these two ions.

When same Grape sample spiked with standard Paclobutrazol and analyzed using high resolution LCMS system that is, Q-TOF and extracted mass spectra for standard Paclobutrazol ion (M+H) 294.1368  $m/z$  and other ion that is, 294.2063 and generated formula using "Generate Formulas from Spectrum Peaks" and found that,

1. By using Q-TOF we could able to resolve both ions i.e Paclobutraol ion 294.1368  $m/z$  and similar ion 294.2063  $m/z$  correctly.
2. Formula generated for 294.1368  $m/z$  represents exact formula of Paclobutrazol with mass accuracy of 0.11 ppm as shown in Figure 12
3. For 294.2063  $m/z$  ion different formula generated with mass accuracy of 0.39 ppm, as per Figure 13.
3. High mass accuracy and best resolution of Agilent G6530 helps to differentiate between targeted Paclobutrazol ion and unwanted but similar ion eluting at same retention time which will be difficult to resolve on QQQ.

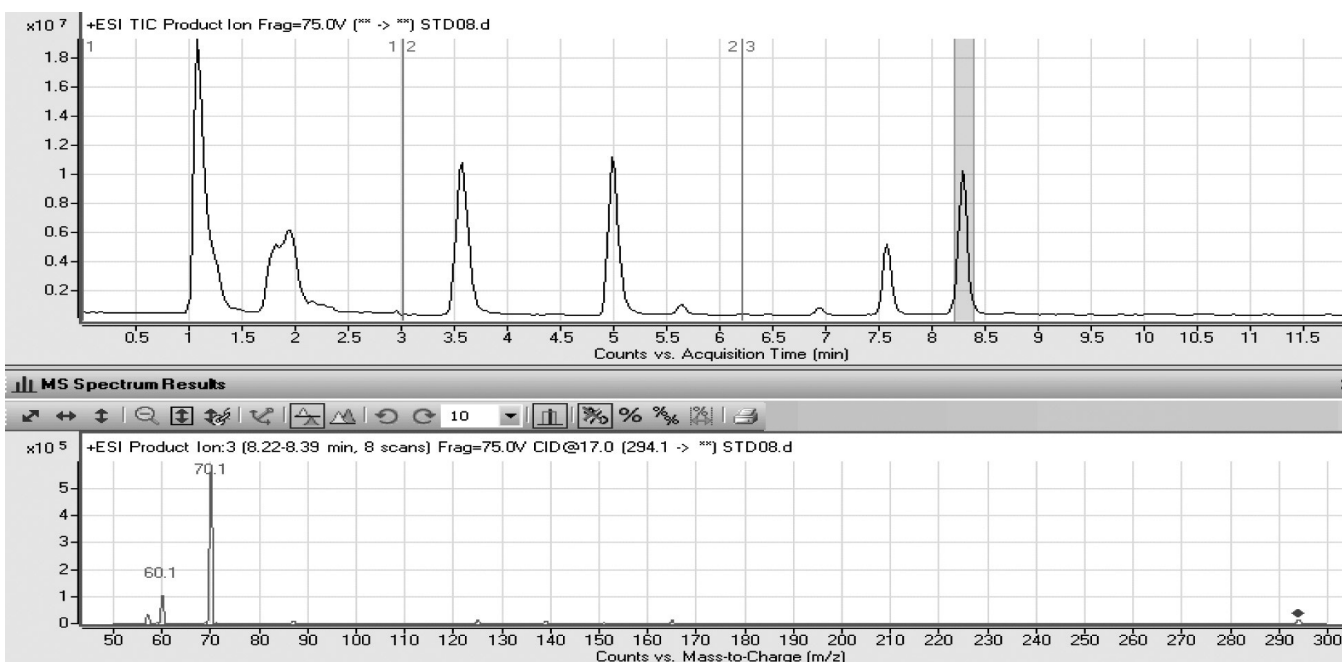


Figure 9. Product ion spectra of Paclobutrazol using Agilent 6460 QqQ.

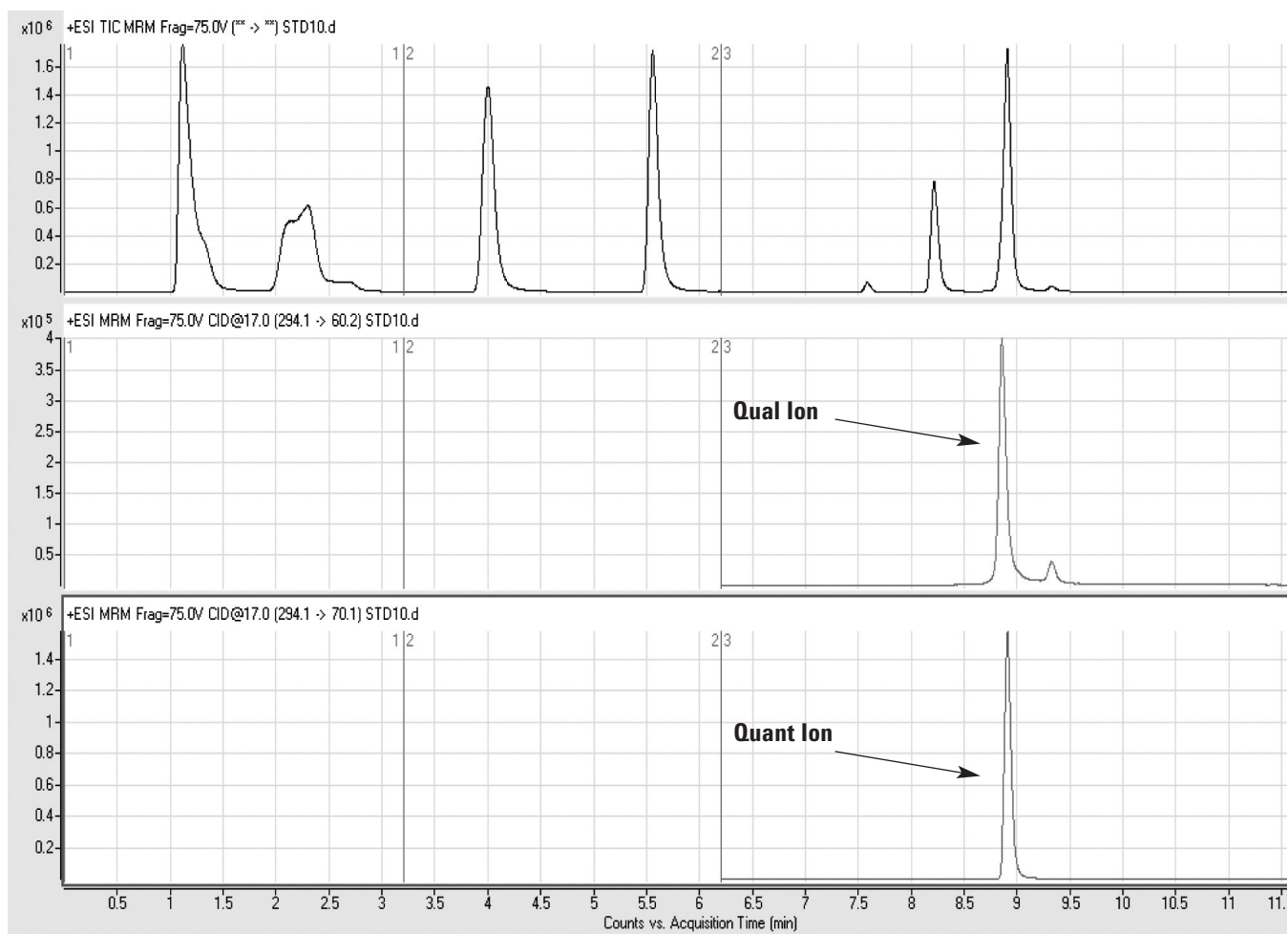


Figure 10. MRM spectra of Paclobutrazol standard using Agilent 6460 QqQ.

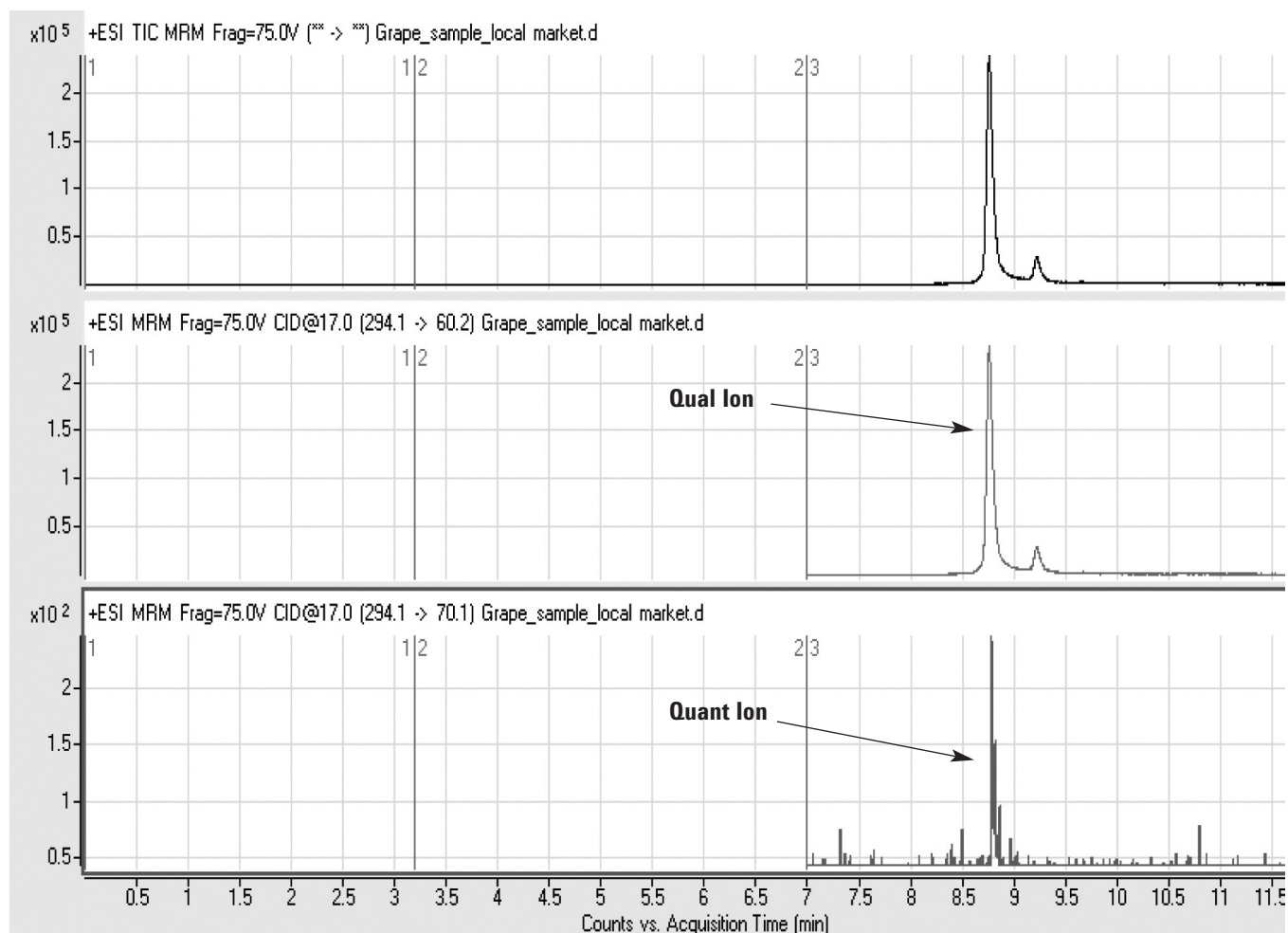
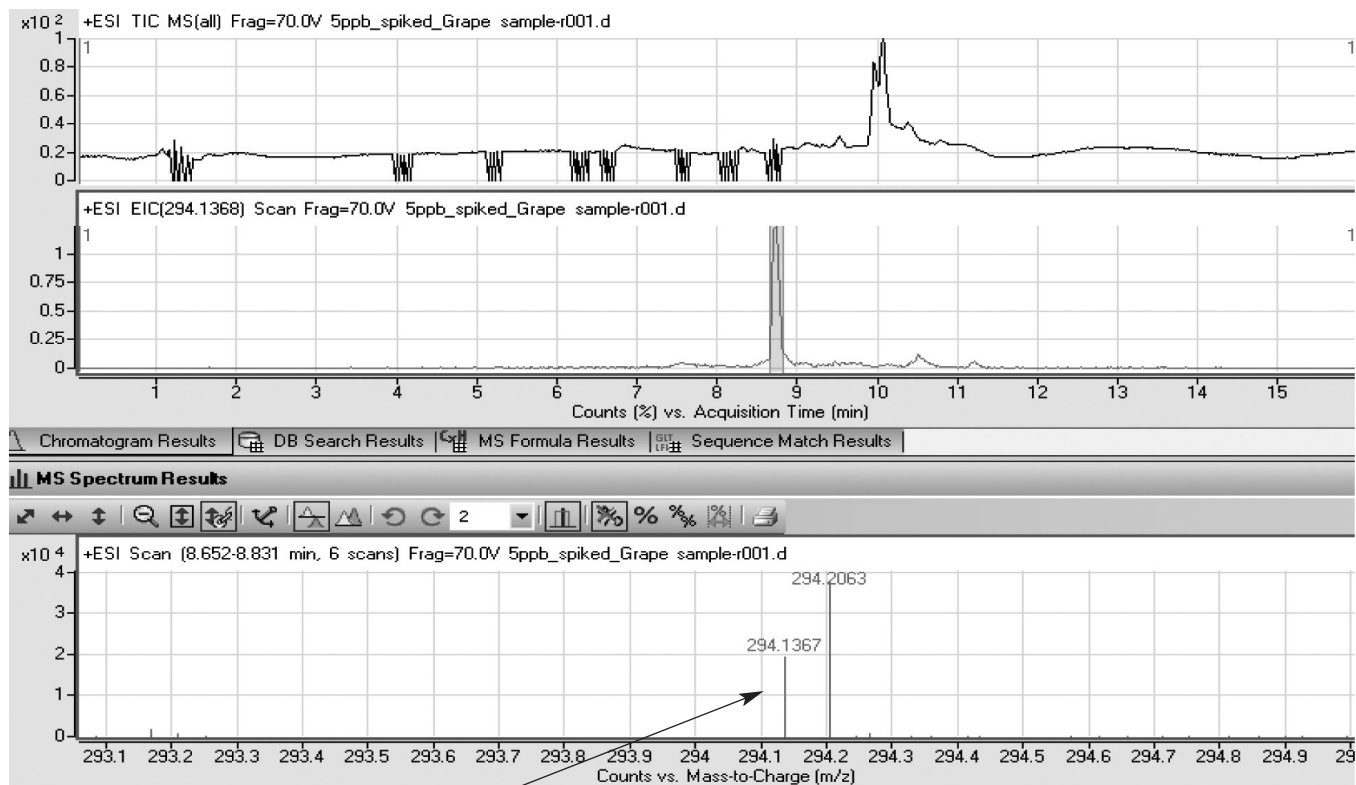


Figure 11. MRM spectra of 293.1990  $m/z$  ion from Grape sample using Agilent 6460 QqQ.

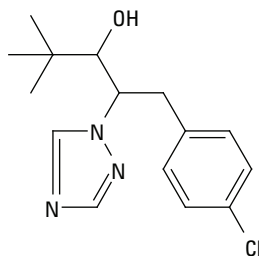


**MS Formula Results: +Scan(8.652-8.831 min)**

| m/z      | Ion                | Formula                                            | Abundance |
|----------|--------------------|----------------------------------------------------|-----------|
| 294.1367 | (M+H) <sup>+</sup> | C <sub>15</sub> H <sub>21</sub> ClN <sub>3</sub> O | 19453.7   |

| Best                                | Formula (M)                                        | Ion Formula                                        | Calc m/z | Score | Cross Score | Mass     | Calc Mass | Diff (ppm) | Abs Diff (ppm) | Abund Match |
|-------------------------------------|----------------------------------------------------|----------------------------------------------------|----------|-------|-------------|----------|-----------|------------|----------------|-------------|
| <input checked="" type="checkbox"/> | C <sub>15</sub> H <sub>20</sub> ClN <sub>3</sub> O | C <sub>15</sub> H <sub>21</sub> ClN <sub>3</sub> O | 294.1368 | 91.39 |             | 293.1295 | 293.1295  | 0.11       | 0.11           | 90.65       |



Paclobutrazol (C<sub>15</sub>H<sub>20</sub>ClN<sub>3</sub>O) 293.1295 m/z

Figure 12. EIC spectra of 294.1368 m/z from Grape sample using Agilent 6530 Q-TOF shows formula C<sub>15</sub>H<sub>20</sub>ClN<sub>3</sub>O with mass accuracy 0.11 ppm which matches to Paclobutrazol.

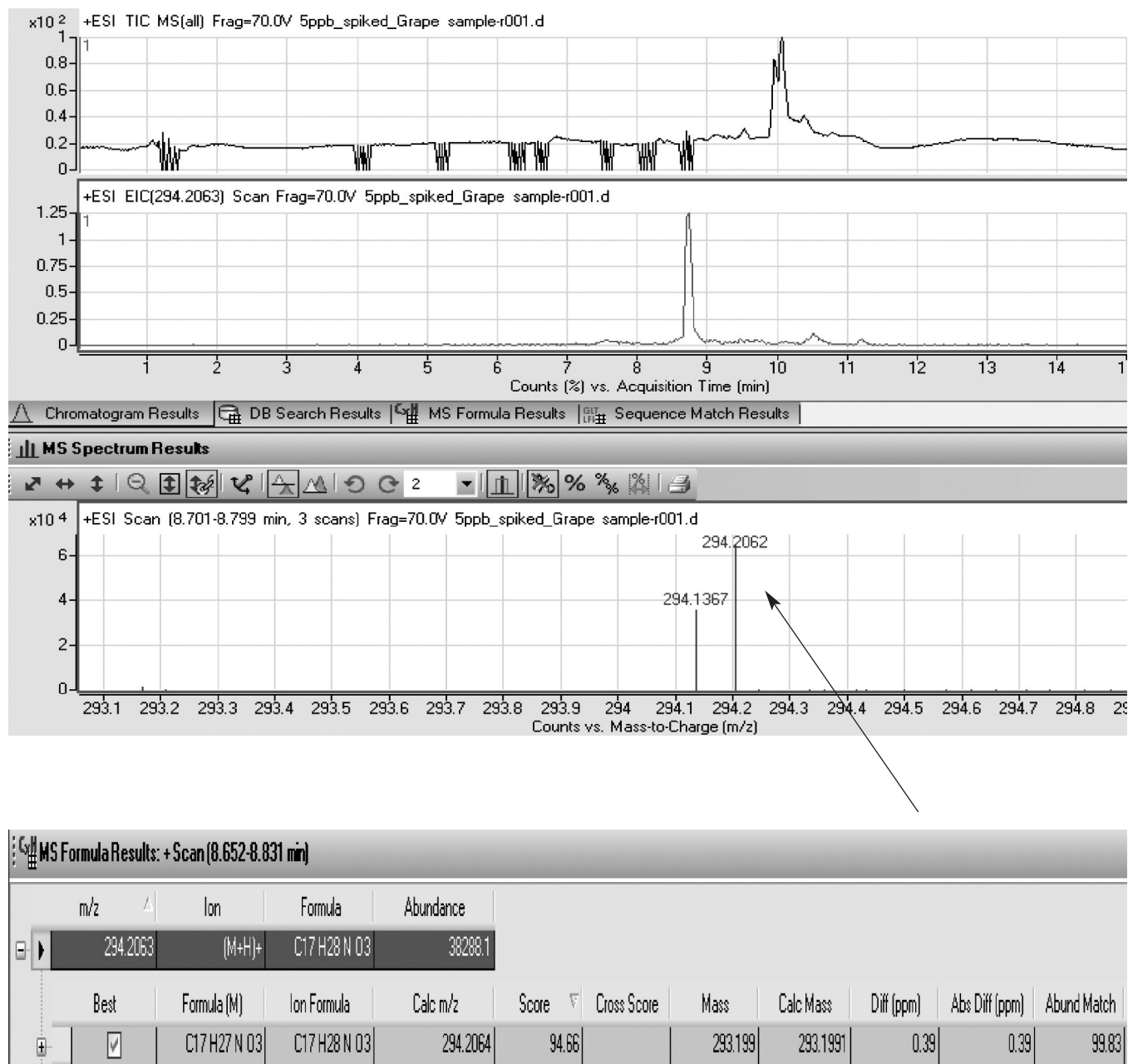


Figure 13. EIC of 293.2063 m/z from Grape sample using Agilent 6530 Q-TOF shows different formula C<sub>17</sub>H<sub>27</sub>NO<sub>3</sub> with 0.39 ppm mass accuracy.

## Conclusions

Q-TOF provides unambiguous identification or conformation of small molecules with mass accuracy previously available only from expensive and hard-to use instruments, such as FT-MS. The outstanding mass accuracy also dramatically reduces the range of possible identities from small to large molecules.

The Q-TOF is an excellent system for identifying compounds using accurate mass in full scan MS and MS/MS mode. Accurate mass leads to chemical formulas, which can also give structural information for product ions in MS/MS. Much data is acquired using this system to study samples that may contain large amounts of known and unknown compounds. It is important therefore to have algorithms like the Molecular Feature Extractor (MFE) that can filter usable features out of the chemical background. These features are generated from spectra as a result of removing random background signals and finding clusters of isotope.

The Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight LC/MS system with Jetstream ESI ion source coupled with Agilent 1290 Infinity LC and dispersive C18 ODS sample cleanup shows excellent sensitivity and outstanding mass accuracy for screening of multiclass PGRs in grape samples.

## Acknowledgements

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