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GC/MS Method for the Detection of Terbufos, Diazinon and Parathion in Blood

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Reference to any commercial materials, equipment, or process does not in any way constitute approval, endorsement, or recommendation by the Food and Drug Administration.

Abstract

Scope

This is a multi-residue method for the detection of the organophosphate pesticides, terbufos, diazinon and parathion in blood by gas chromatography and mass spectrometry (GC/MS). This is a qualitative screening method intended to isolate and concentrate organophosphate pesticides from sample extracts for GC/MS confirmation. This method also allows for adequate separation and detection of 42 other organophosphorus, carbamate and organochlorine pesticides.

In this multi-residue method, pesticides are extracted from blood samples in acetonitrile and isolated using a dual-layer, ENVI-Carb-II™/PSA solid-phase extraction procedure. The pesticides are subsequently separated and detected using gas chromatography and detected using electron-impact mass spectrometry.

Table 1. Instrument and Method Limits of Detection

	A	B	C	D
		Instrument LOD*	**Method LOD****	**Method Assurance Levels*****
		This is the lowest concentration of analyte, which can be detected in solvent. Procedure: Solvent is spiked and injected into GC/MS directly	This is the lowest concentration of analyte which can be detected in blood. Procedure: Blood is spiked and processed though the method's extraction protocol	These levels can be observed with a higher degree of confidence vs. the method LOD levels
	Terbufos	0.2 ppm	0.3 ppm	1 ppm
	Diazinon	0.2 ppm	0.2 ppm	1 ppm
	Parathion	0.2 ppm	0.2 ppm	1 ppm

*Instrument limits of detection (LODs) are specific to the type of instrument used and therefore, can impact the method LODs. Hence, instrument and method LODs may need to be established in-house by each collaborating laboratory.

**Method levels of detection are levels of the analytes that can be detected that have been reported in cases of chronic exposure in animals. The ability of the method to detect analytes at these low levels may make the method suitable for research purposes in addition to diagnostic analyses.

***The method assurance levels are in the appropriate range for identifying the analytes at concentrations in the blood which could be related to adverse clinical signs in cases of acute toxicity (i.e. when clinical signs appropriate for acute exposure to these insecticides are present in animals).

Method validation/evaluation/verification:

In-house method validation data and evaluation by an independent laboratory (Vet-LIRN) in collaborative multi-laboratory studies

Attachments

Targeted-SOP-for-Pro...

1.3MB

Materials

Apparatus

- Balance
- Centrifuge (VWR International, Clinical 200 or equivalent)
- Dual-layer Supelclean ENVI-Carb-II/PSA solid phase extraction cartridge, 500mg/500mg, 20mL capacity (Supelco, P/N 54217-U or equivalent)
- GC/MS (ThermoFisher Scientific, 1310 Trace GC and TSQ 8000 triple quadrupole MS or equivalent)
- Autosampler (ThermoFisher Scientific TriPlus RSH autosampler or equivalent)
- GC autosampler vials (VWR, P/N 46610-726 or equivalent), inserts (VWR, P/N 46610-708 or equivalent) and caps (Agilent technologies, 5181-1270 or equivalent)
- Glass test tubes, 50 mL (VWR, P/N 89090-930 or equivalent)
- Glass storage bottle with screw caps, 1,000 mL
- Glass vials, amber with screw cap, 5 mL
- Graduated cylinders
- Nitrogen evaporator with heating block (Thermo Scientific Reacti-Therm;TS-18822 or equivalent)
- Nitrogen gas
- Pipettes or pipettors
- Polypropylene tubes with screw caps -15 mL (VWR P/N 21008-214 or equivalent)
- Spin-X centrifuge tube filters, 0.22 µm pore size nylon membrane (VWR, P/N 29442-760 or equivalent)
- Vortex mixer

Reagents and Standards

- Acetonitrile, Optima LCMS grade (Fisher Chemical P/N A9554 or equivalent)
- Bovine whole blood, Innovative grade US origin, in K_2EDTA (Innovative Research Inc, IR1040N or equivalent)
- Pesticide Analytical Standard Solutions
- Diazinon – 100 ppm (Chem Service, Inc., P/N S11621U1 or equivalent)
- Parathion – 100 ppm (Chem Service, Inc., P/N S12819A1 or equivalent)
- Terbufos – 100 ppm (Chem Service, Inc., P/N S13510M1 or equivalent)
- Sodium sulfate (Na_2SO_4), anhydrous ACS grade (Amresco P/N 97062438 or equivalent)
- Toluene, Optima (Fisher Chemical P/N T291SK4 or equivalent)

Safety warnings

Safety Precautions:

Read and follow all procedure and guidelines found in the Safety Data Sheets for the chemicals to be used during this procedure. This will include wearing appropriate gloves, safety glasses, and lab coat during standard and sample preparations. Preparation of standards and samples must be performed in a chemical hood.

Safety Considerations

- Unless otherwise specified within, this procedure must be performed in accordance to the Certificate of Hazard Assessment posted on the entry door to the laboratory in which the procedure is performed.
- Wear gloves, safety glasses, and lab coat during standard and sample preparations and analyses. Preparation of standards and samples must be performed in a chemical hood.
- All standard and sample solutions shall be disposed in a container properly labeled for hazardous waste disposal.

Sample Disposal:
All standard and sample solutions shall be disposed in a container properly labeled for hazardous waste disposal.

Preparation of Pesticide Standards and Spiking Solutions

1 Preparation of 20 ppm terbufos, diazinon and parathion mixed pesticide standard solution

1.1 Record manufacturer, manufacturer part number, lot number, and expiration date for all standards and reagents in laboratory notebook.

1.2 Pipet 1.50 mL of acetonitrile:toluene (3:1) into a 5 mL glass vial.

1.3 Transfer 0.75 mL of each of the 100 ppm terbufos, 100 ppm diazinon and 100 ppm parathion. Label on the 5 mL glass vial should include contents, expiration date, laboratory notebook reference, analyst initials and hazard communication designations. The expiration date for the solution should be the earliest expiration date of the two reagents/standards used.

1.4 Cap and vortex mix well.

1.5 The 20 ppm mixed standard solution is stable for up to 5 weeks when stored at refrigeration temperature and for 24 hours at room temperature.

Extraction Solution and Standard Diluent Preparation

2 Preparation of acetonitrile:toluene (3:1) solution:

2.1 Transfer 600 mL of acetonitrile to 1L graduated cylinder.

2.2 Add 200 mL of toluene to the 1L graduated cylinder containing the acetonitrile.

2.3 Transfer contents to a properly labeled 1L glass storage bottle and store at room temperature.

2.4 Label should include contents, expiration date, laboratory notebook reference, analyst initials and hazard communication designations. The expiration date for the solution should be the

earliest expiration date of the two reagents/standards used.

Preparation of Pesticide Standards and Spiking Solutions

3 Preparation of 1 ppm terbufos, diazinon, and parathion mixed standard solution:

3.1 Record all pertinent information regarding the standard stock solution in laboratory notebook.

3.2 Aliquot 2.00 mL of acetonitrile:toluene (3:1) into a 5 mL glass vial.

3.3 Transfer 106 µL of the 20 ppm terbufos, diazinon, and parathion mixed standard solution prepared above. Label on the 5 mL glass vial should include contents, expiration date, laboratory notebook reference, analyst initials and hazard communication designations. The expiration date for the solution should be the earliest expiration date of the two reagents/standards used.

3.4 Cap and vortex mix well.

3.5 The 1 ppm mixed standard solution is stable for up to 3 weeks when stored at refrigeration temperature and for 24 hours at room temperature.

Control Samples

4 **Negative control:** Negative control blood must be a blood sample that is absent of pesticides. Transfer 2 mL of blood to a properly labeled, 15 mL polypropylene tube. Proceed to step 6.2.

5 **Positive control**: Positive control blood is negative control blood that is fortified with a known concentration of pesticides. Transfer 2 mL of blood to a properly labeled test tube and add 106 µL of the 20 ppm terbufos, diazinon, and parathion mixed standard solution to obtain a spike level of 1 ppm. Proceed to step 6.2.

Procedure for Samples

6 Sample Extraction Procedure:

6.1 Aliquot 2 mL of blood sample into a properly labeled 15 mL polypropylene tube. Record sample ID, sample volume and any other pertinent information in laboratory notebook.

6.2 Add 4 mL acetonitrile to the sample and vortex mix for approximately 00:01:00 . 1m

6.3 Centrifuge sample at approximately 4,000 RPM (~ 1500 RCF) for 00:10:00 10m

6.4 Aliquot and transfer supernatant to properly labeled 15 mL polypropylene tube.

6.5 Add an additional 4 mL of acetonitrile to the remaining pellet from step 6.4. Vortex mix for approximately 00:01:00 . 1m

6.6 Centrifuge sample at approximately 4,000 RPM (~ 1500 RCF) for 00:10:00 10m

6.7 Aliquot supernatant and combine with supernatant from step 6.4.

6.8 Add 1 g of Na_2SO_4 to the combined supernatants and vortex mix for approximately 00:01:00 to dry the extract. Allow Na_2SO_4 to settle before proceeding. 1m

6.9 Using a disposable glass pipet, transfer all of dried acetonitrile extract to a 15 mL polypropylene tube. (Stopping point: This extract is stable for 24 hours under refrigeration conditions).

6.10 Proceed with SPE clean-up procedure.

7 SPE Clean-up Procedure:

7.1 Condition a multi-layer supelclean ENVI-Carb-II/PSA SPE cartridge (20 or 6 mL capacity) with 5 mL acetonitrile:toluene (3:1). Collect the eluate as waste in a properly labeled waste beaker.

7.2 Load the acetonitrile extract from above (step 6.9) into the SPE cartridge and collect the eluate in a properly labeled waste beaker.

7.3 Elute pesticides from SPE cartridge with 15 mL acetonitrile:toluene (3:1) into a properly labeled 50 mL glass test tube.

7.4 Place test tube containing SPE extract in a nitrogen evaporator heating block (set at approximately 40^{o}C) and evaporate eluate to dryness under low nitrogen gas flow. (Stopping point: Dried residue is stable for 24 hours under refrigeration conditions)

7.5 Reconstitute residue with 0.5 mL acetonitrile:toluene (3:1), vortex well to reconstitute entire residue and transfer to spin-x microcentrifuge tubes with nylon filters.

7.6 Vortex tubes for 00:00:30 . Transfer the filtrate to a properly labeled GC/MS autosampler vial containing a vial insert. (Stopping point: This solution is stable for 24 hours under refrigeration conditions)

30s

7.7 Proceed to GC/MS analysis

GC/MS Analysis for the Detection of Pesticides

8 Instrument Parameters:

8.1 Inject 1 µL of the reconstituted sample extract into a GC/MS that is equipped with a triple quadrupole mass spectrometer under conditions found in Table 1.

Initial results have shown that the use of an ion-trap mass spectrometer has significantly higher instrument and method LODs when compared to using triple quadrupole MS.

Table 1: Instrument Parameters for GC/MS for the Detection of Terbufos, Diazinon, and Parathion in Blood Samples

A	B	C
GC Parameters		
	Initial Oven Temp and Hold	40 °C for 0.5 min
	Final Oven Temp and Hold	300 °C for 1.0 min
	Injector Temp	275 °C (splitless)
	Carrier Gas Flow	1.4 ml/min
	Injection Volume	1 µL
	Column	ThermoFisher TR-5 MS
	Column Size	30 m X 0.25 mm
	Column Film Size	0.25 µL
	Rinse for Autosampler	Toluene
MS Parameters		
	Transfer Line Temp	325 °C
	Ion Source Temp	310 °C
	Ionization Mode	EI
	Scan Range	50-650 m/z
	Quadrupole Temp	
	MS Type	Triple Quadrupole

9 Acceptance Criteria to Determine the Presence of the Pesticide:

Note

All criteria must be met for reporting the presence of the pesticide.

9.1 The retention time needs to be within 3% of the retention time of the positive controls or the 1 ppm standard solution. (See Table 2 for more information)

Table 2: Retention Time (RT) Information for Pesticides

A	B	C	D
	Pesticide	Approximate RT* (Minutes)	Relative RT** (Minutes)
	Terbufos	13.20	1.000

	A	B	C	D
		Diazinon	13.17	1.012
		Parathion	14.60	1.121
		Myristic Acid (FA)	12.73	0.978
		Palmitic Acid (FA)	14.20	1.091

* RT can differ with changing column
**Notes: The relative retention time is the comparison of the approximate retention time of one pesticide relative to the retention time of another. This value can serve as a predictor of anticipated retention times for other analytes in a given GC run. For example, if a GC analysis of the pesticides indicated that the retention time of terbufos for that run is 12.98 minutes, then multiplying 12.98 minutes by 1.012 would provide an approximate retention time to be expected for diazinon at 13.14 minutes.

9.2 The signal-to-noise (S/N) ratio for pesticide needs to be greater than 3 RMS.

9.3 All observed ions need to be present (See Table 3 for more information).

Table 3: Ions Monitored for the Detection of Terbufos, Diazinon, and Parathion

	A	B
	Pesticide	Observed Ions
	Terbufos	**231** 103 153
	Diazinon	**137** 179 152 199
	Parathion	**109** 139 291

* Major ions monitored to detect each pesticide are listed in bold above.

9.4 The relative ion intensities for secondary ions monitored need to be within 30% of the major ion observed for each pesticide when compared to ions in the positive control sample or the 1 ppm mixed standard
solution.

9.5 High concentration of fatty acids may interfere with the method performance and therefore need to be monitored. See Appendix 1 for details.

Appendix 1

10 Terbufos linear fit for analysis

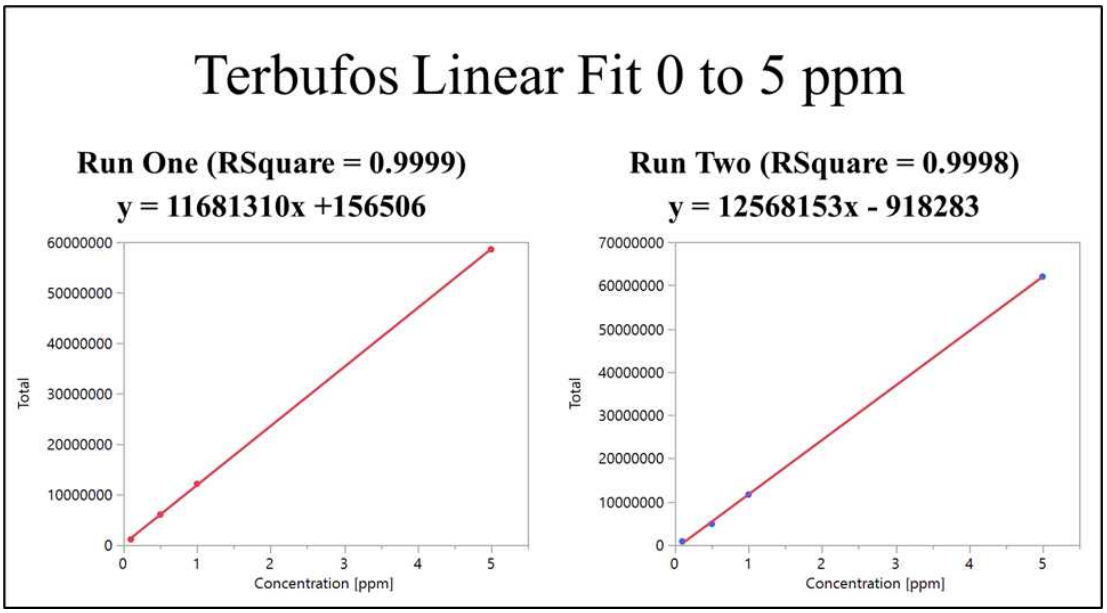

Figure 1: A statistically significant linear fit was found between the concentration of the standard and the total intensity observed in the GC-MS results for Terbufos between 0 and 5 ppm.

11 To reduce the false negative observations, expanding the acceptance criteria ranges was explored and resulted in the following:

1. The retention time shall be within 3% of the retention time of the positive controls or the 1 ppm standard solution.
2. The signal-to-noise (S/N) ratio for terbufos shall be greater than 3 RMS.
3. The ions 231 m/z, 103 m/z and 153 m/z shall be present for terbufos.
4. One of the relative intensities for ion 103 m/z or 153 m/z shall be within 30 % of the major ion o

12 An “Alert Criteria” was established to determine if a false positive result could be suspected for this method. This concern stems from the possibility of co-eluting peaks with m/z values of 103 and 153 from
blood matrix interferences. For this particular method that could not be successfully achieved due to co-elution peaks (103 and 153) from the blood matrix.

1. The “Alert Criteria” is the ratio of the sum of the S/N ratios of the fatty acids myristic and palmitic acid divided by the S/N of terbufos peak. See equation 1 below.

Equation 1: Calculated Ratio for “Alert Criteria”

$$\text{Alert Criteria} = \frac{\text{S/N for Myristic Acid peak} + \text{S/N for Palmitic Acid peak}}{\text{S/N for Terbufos peak}}$$

13 Studies were completed to determine what could influence intensities or the amount of terbufos after extraction and clean-up procedures. The first study included examiningdifferent lot
numbers of blood determine their effects on terbufos intensities. These findings were significant.

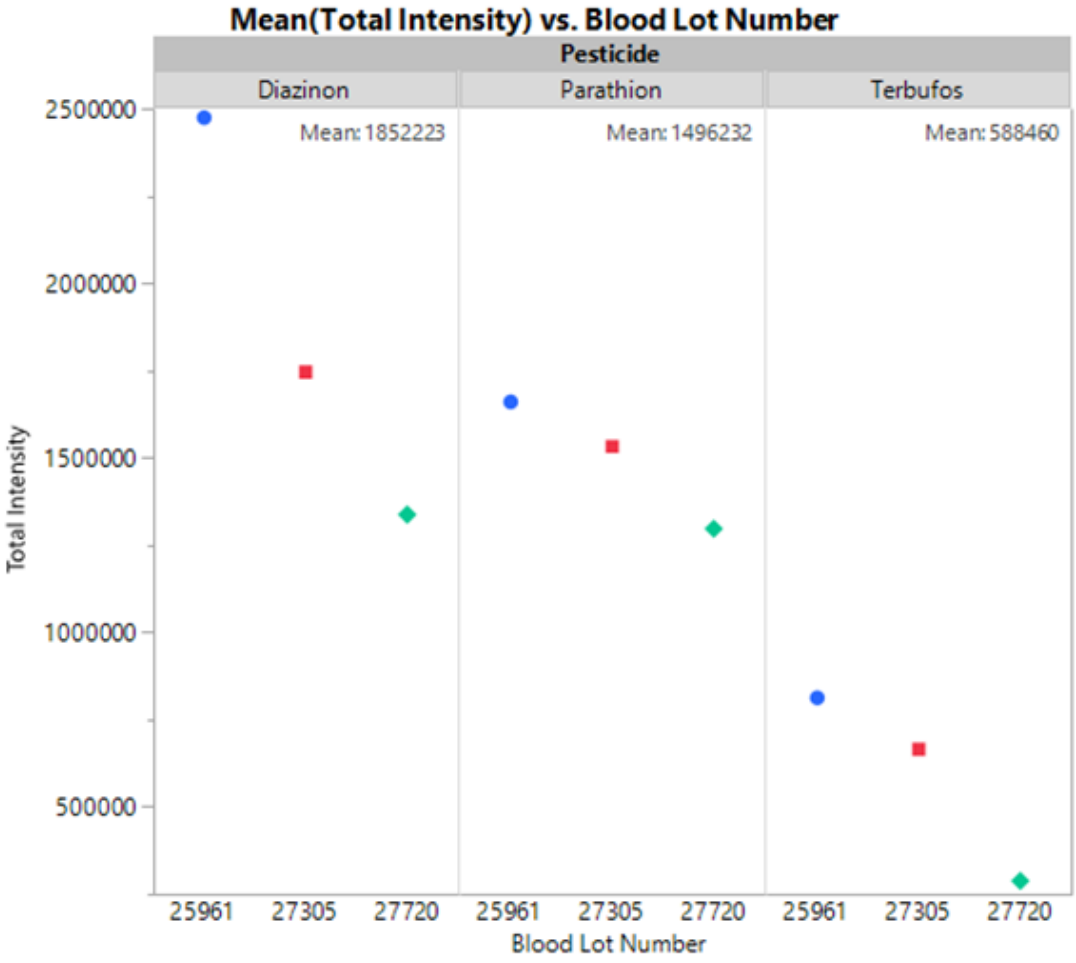

Figure 2: The “Total Intensity” versus “Blood Lot Number” by “Pesticide”

The intensities were then correlated with the S/N ratio for myristic acid. This correlation was also found to be significant.

14

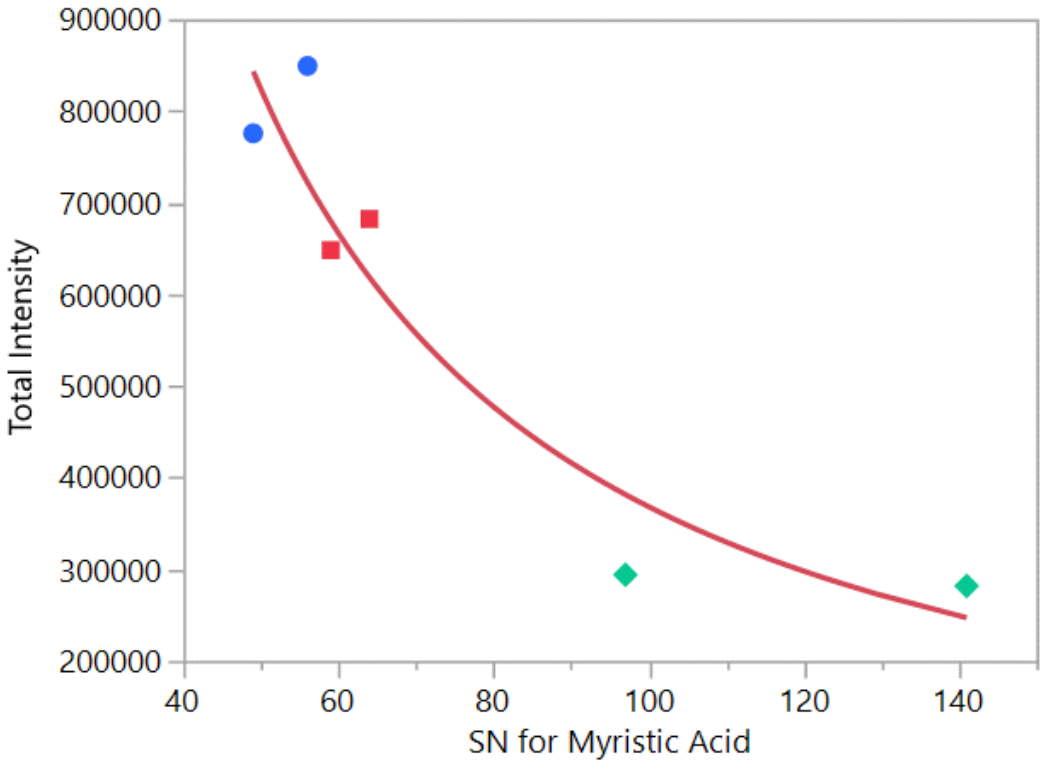

Figure 3: The "Total Intensity" versus "S/N for myristic acid" for terbufos.
(Log to Log Fit) R^2 = .8929 with an F-Ratio of 0.0045
Different colors and shapes are different blood lot numbers obtained from the vendor.

15 A main effects screening design for water and its removal was completed to determine their influences on the process. All samples were spiked with 0.3 ppm of terbufos, diazinon, and parathion. However, the identification of terbufos was the focus. This study showed the amount of water and the amount of Na_2SO_4 used was significant to fatty acid removal. The lot number of the blood control and time the Na_2SO_4 was added were found not to be significant in the removal of fatty acids.

Further statistical examination revealed differences in identification probabilities related to the sum of the S/N ratio of the fatty acids, however there were samples that did not correlate with these criteria, apparently due to baseline variations. To account for these differences in the baseline, the S/N ratio for terbufos was introduced into the equation to eliminate baseline variability.

Figure 4: The “Fatty Acid to Terbufos Ratio (SN)” versus “Identification” for Terbufos with one-way analysis. Since all samples were spiked with terbufos, an indicator of "absent" is incorrect while "present" is correct.

16 In summary, the removal of key saturated fatty acids appears to be important in identifying terbufos at low levels in bovine, blood samples. Levels of fatty acids can fluctuate by the amount of water, amount of sodium sulfate, and the blood from the particular animal. The levels can fluctuate from sample to sample due to what may carry forward into the clean-up step of the work-up procedure.

17 Possible solutions to help reduce the fatty acid concentrations during the preparation of the sample:

- Switch from sodium sulfate to magnesium sulfate as previous research has shown sodium sulfate is not as effective drying agent for acetonitrile solutions.
- Change the extraction/elution solvents during the process.Solutions in published literature include using acetone, acetone:toluene (65:35), and acetone:cyclohexane (various compositions) with the current SPE cartridges containing PSA.

Appendix 2. Pesticide SOP TOX.103 Software and Instrument Details

18 The figures are examples for using Xcalibur at the Purdue University ADDL. These screen shots are subject to change and are not representative of other software.

19 **GC/MS Instrument Control Method:** Pesticide Screen Tox.103 20 min

- Inject 1 µL of reconstituted sample extract into GC/MS (GC/MS operating system is equipped with Trace 1310 gas chromatography/TSQ 8000 mass spectrometer and TriPlus RSH autosampler) under the conditions found in Table 1 using the Xcalibur software system. These conditions should currently be in the method entitled "TOX.103 20 min."
- If method "TOX.103 20 min" cannot be found or needs to be modified, then follow the instructions below.

19.1

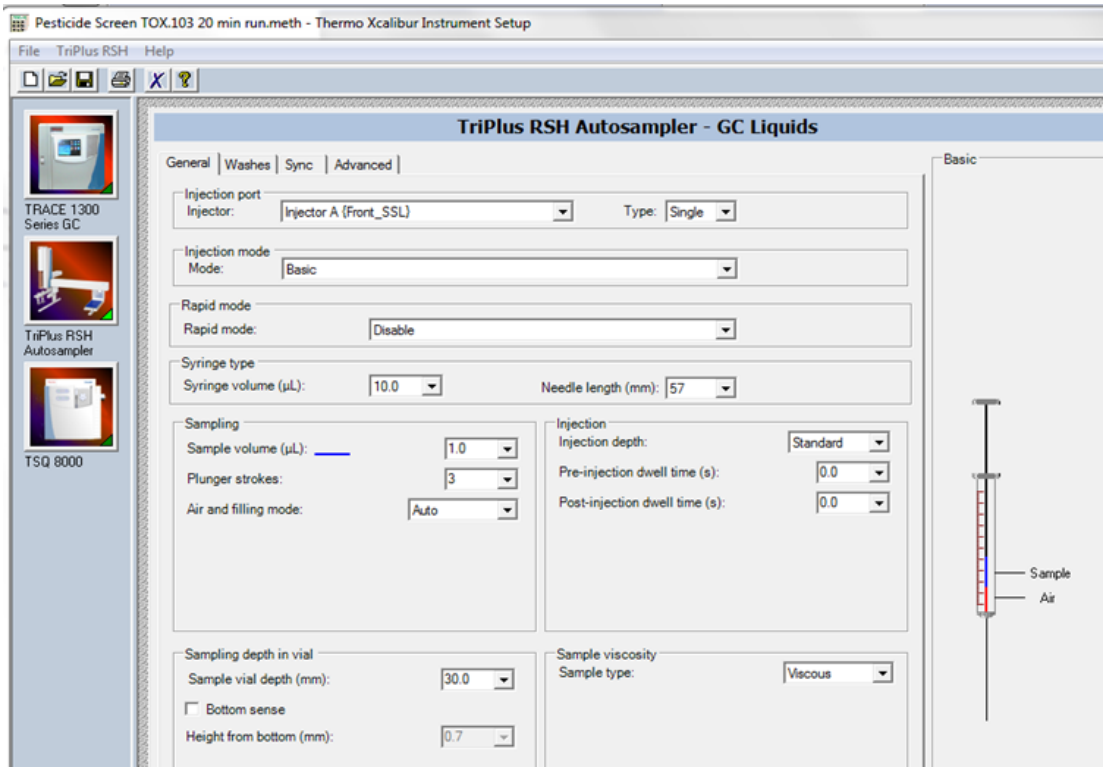

Figure 5. Auto-Sampler Settings

19.2

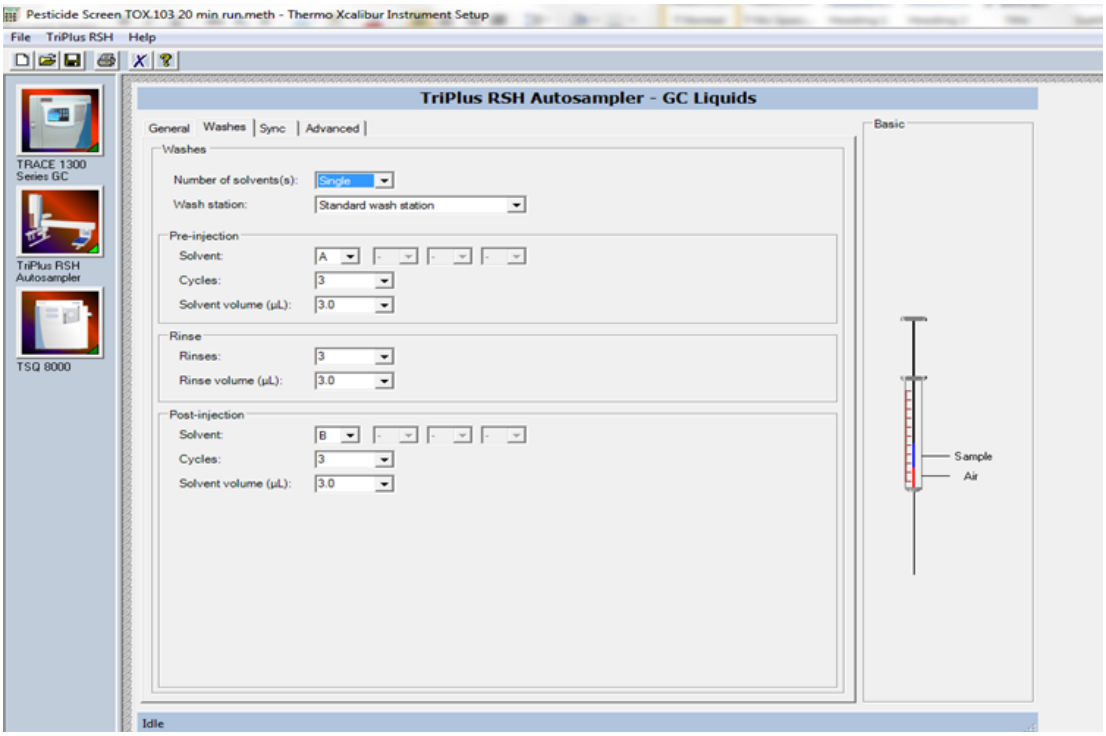

Figure 6. Wash Station Settings

19.3

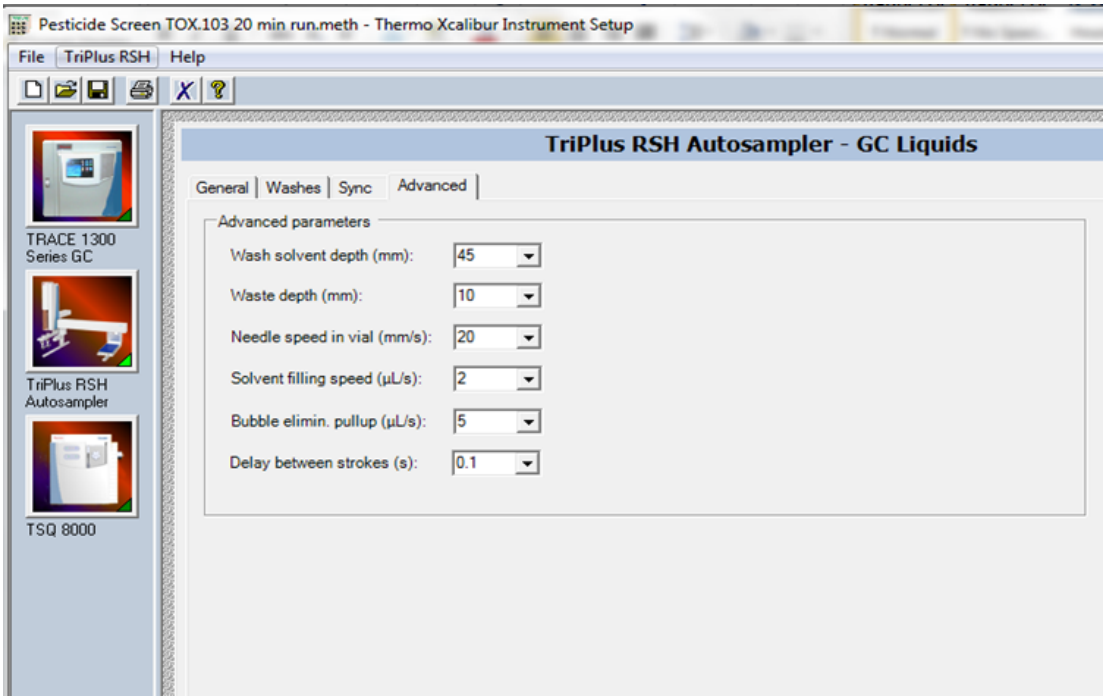

Figure 7. Advanced Wash Settings

20 GC Conditions and Settings

20.1

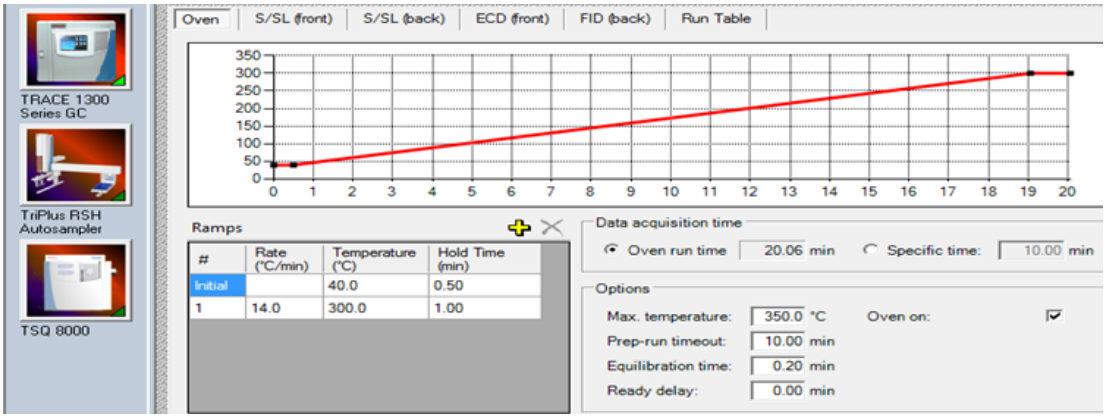

Figure 8. GC Oven Temperature Parameters

20.2

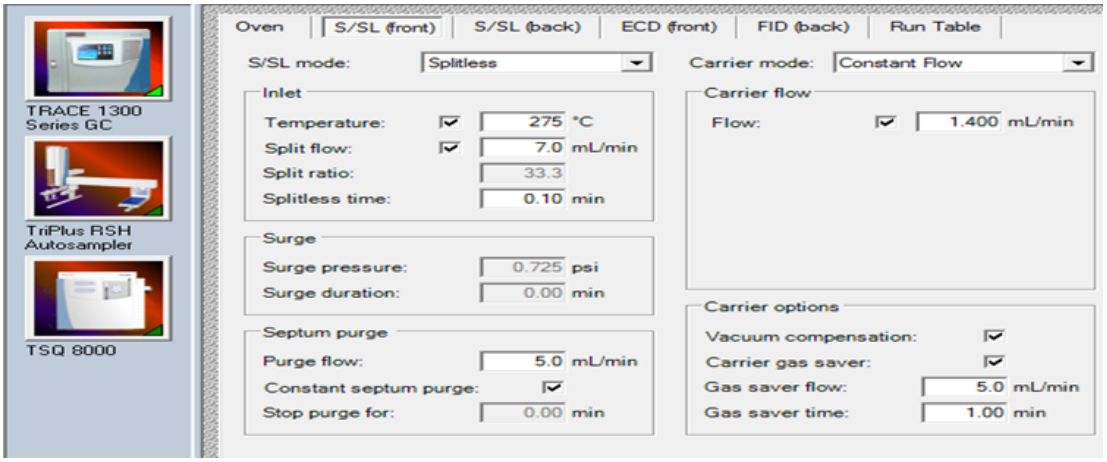

Figure 9. GC Injector Port Settings

21 MS Conditions and Settings

21.1

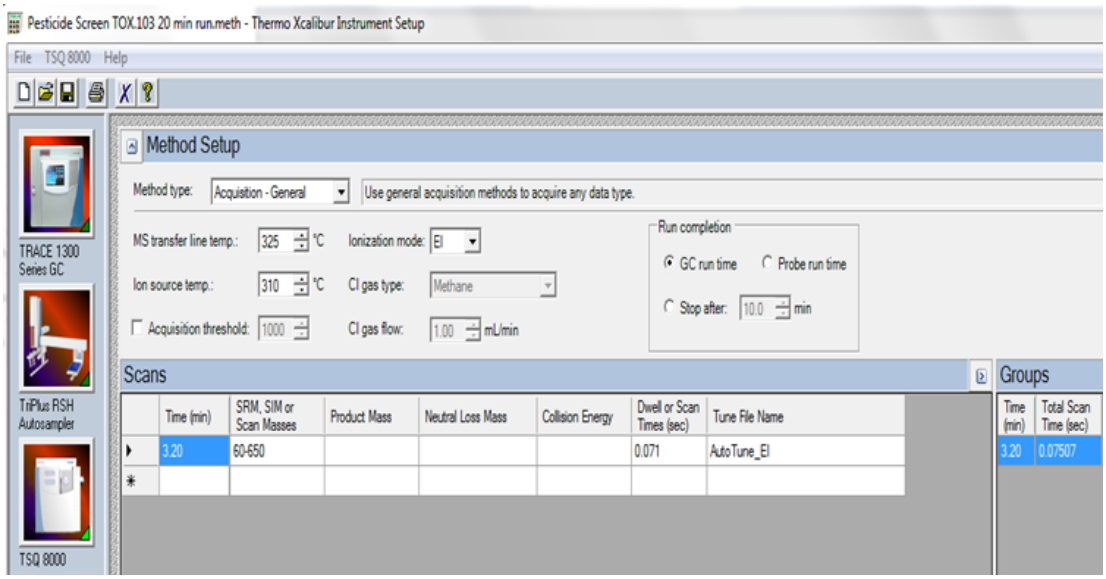

Figure 10. MS Settings

22 Analyzing Samples in System Control (Xcalibur)

22.1 Open “Xcalibur” by double-clicking the “Xcalibur” icon. Double-click on “Qual Browser” to create the data file for your analysis.

22.2 With Qual Browser open, go to “File” --> “Open.” The “Open Raw File” window will be displayed.

22.3 Right click inside the list of files and create a new folder (“New” --> “Folder”). Name the data file with the date and any descriptors. Once the data folder is created, exit out of Qual Browser

22.4 To enter samples into the sample set, click on “Sequence Setup”. Enter the pertinent information about the samples to be analyzed. See Figure 11 for placement details.

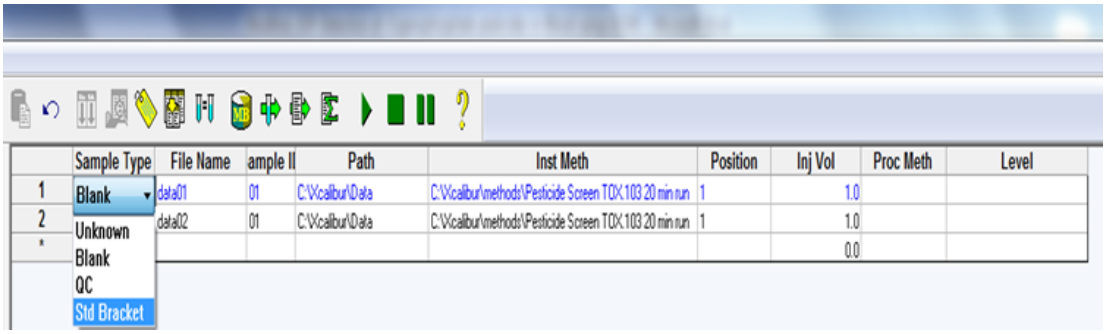

Figure 11. Sample Set Sequence Setup

22.5 Sample type: Select one from the drop-down box
- Unknown: This is selected for all samples and extracted samples.
- Blank: This is for all blank vials (e.g. containing acetonitrile: toluene (3:1) method blank vials).
- QC: This is for positive, negative, and internal control samples.
- Std Bracket: This is for neat/pure standard injections.

22.6 File name: Enter name of sample, standards or control vials used (cannot use spaces).

22.7 Path: Select the data folder previously created in Qual browser.

22.8 Instrument Method: Select “Pesticide Screen Tox.103 20 min run”

22.9 Position: Enter the position number for the vial placed in the auto sampler tray

22.10 Injection Volume: 1.0 (1 µL).

22.11 Once sample set information has been entered, go to “File” --> “Save As” --> and type in the name of the method to save the method file in XCalibur --> Methods --> FDA Vet-LIRN Grant Method Files. Make sure to include the date, description, and notebook number and pages.

22.12 To start the analysis and run the entire sequence, select the “Run Sequence” icon. A window will open --> select “OK” to begin the analysis.

“Run Sequence” icon

23 **Viewing and Processing Data using Qual Browser in Xcalibur: Viewing and Processing Data using Qual Browser in Xcalibur**

23.1 Go to “Road Map View” in XCalibur and double-click on the “Qual Browser” icon. Select “File" --> “Open Sequence” --> then select the sequence file for that sample set.

23.2 The vials injected for that sequence will be listed. Select the file you want to open and the chromatogram and mass spectrum window will open.

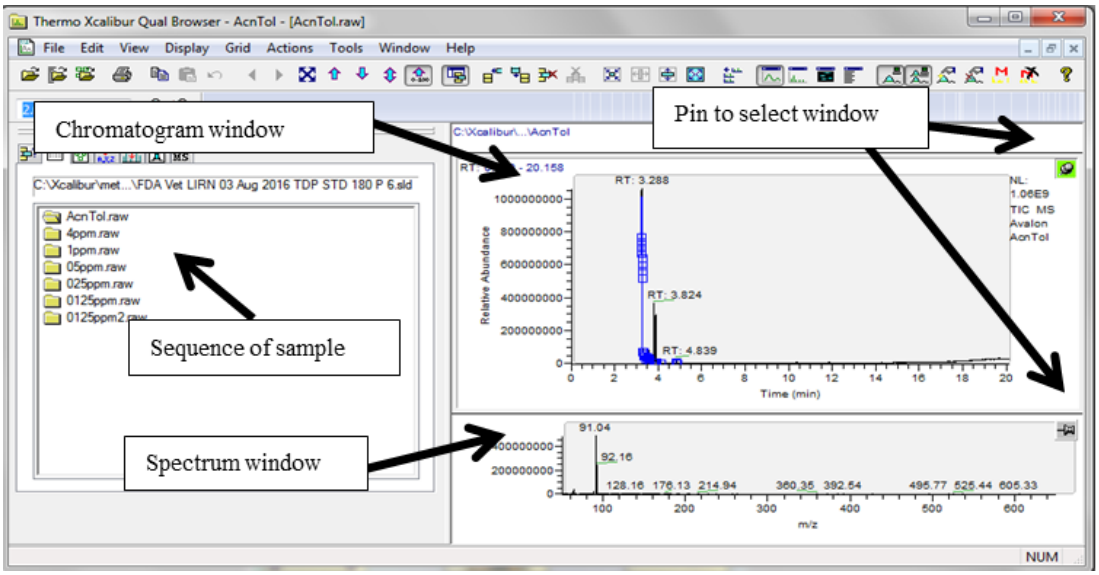

Figure 12. Qual Browser for Processing Data

23.3 Click and select the pin (turns green on selection) on the top right corner of the chromatogram to allow viewing and zooming of the chromatographic data.

23.4 In order to process the data, right click in the chromatogram pane -->select “Peak Detection” --> “Set Peak Detection Algorithm and Detect in this Plot” --> “ICIS.”

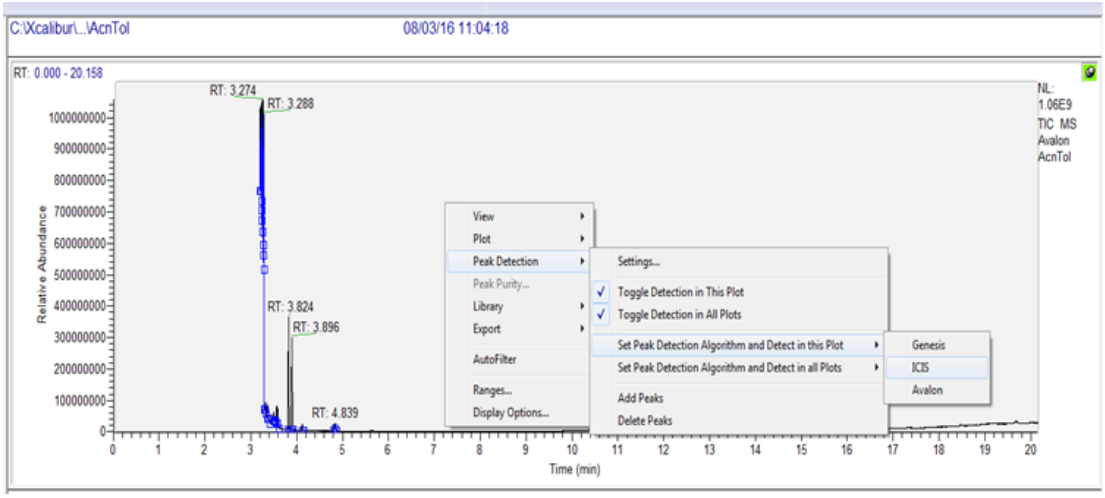

Figure 13. Example for Setting Detection Algorithm

23.5 Then, right click in the top window (chromatogram) and select “Display Options.” Another window will be opened for the display options --> select the tab labeled “Labels.” Check the boxes for “Signal-to-Noise,” and “Retention Time” --> Then select “OK.”.

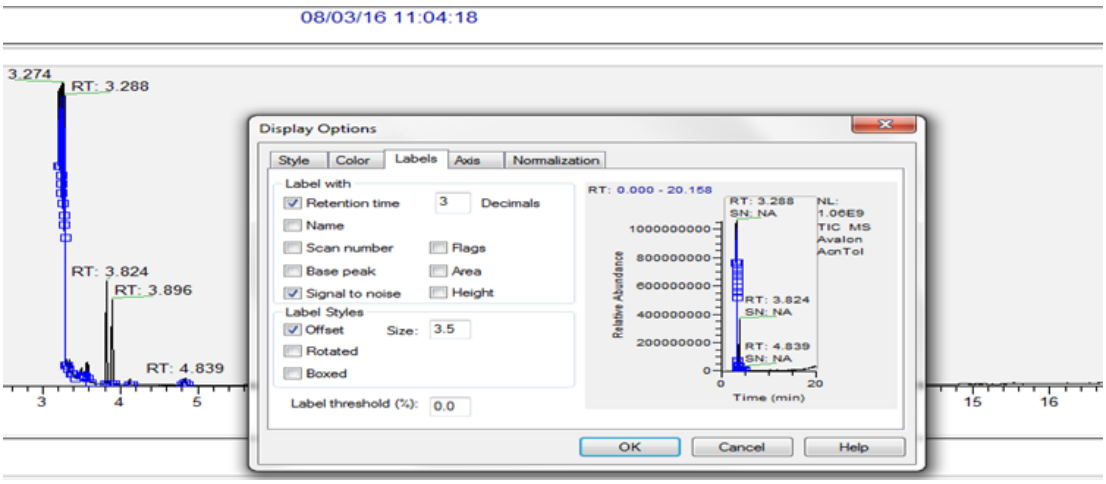

Figure 14. Example for Setting Display Options

23.6 Zoom-in on peak areas of interest by clicking-dragging the mouse over that area

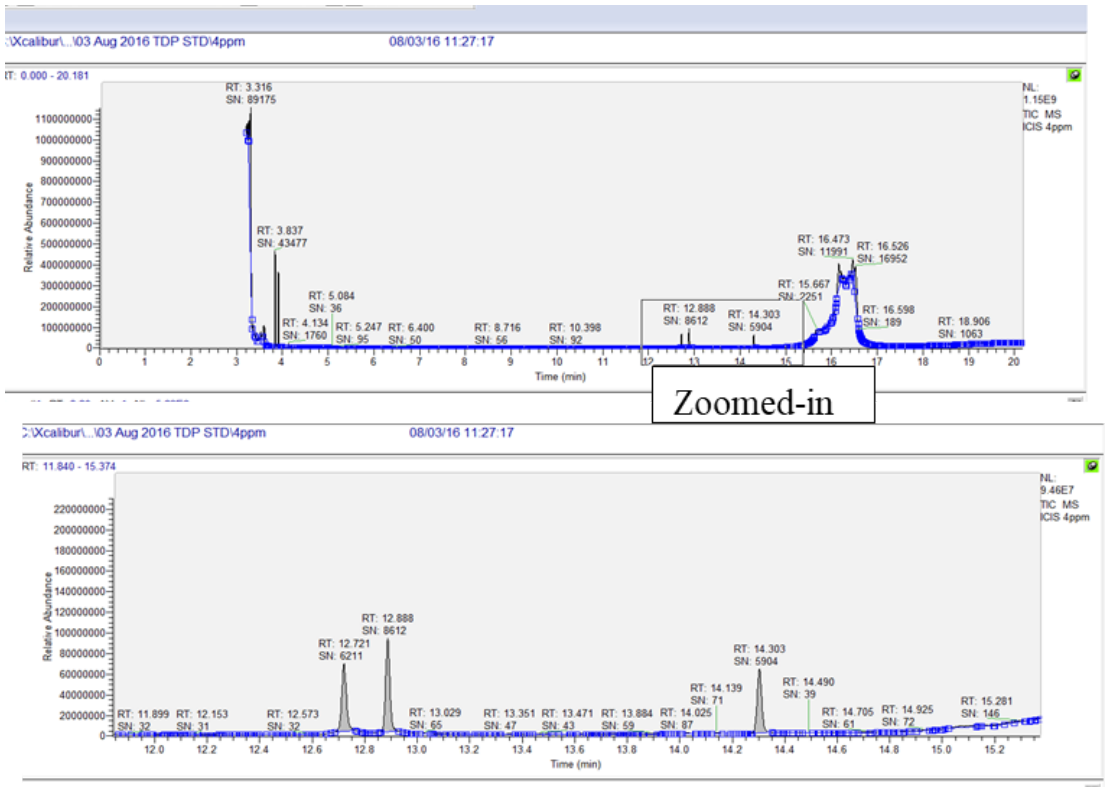

Figure 15. Example Showing Peak Zooming

23.7 When peak detection is selected, “Detection tab” button appears (letter ‘I’ in the button stands for ICIS detection algorithm selected earlier). Select the detection tab, check “Manual noise region” and enter the retention time range to be used for calculating signal-to-noise region.

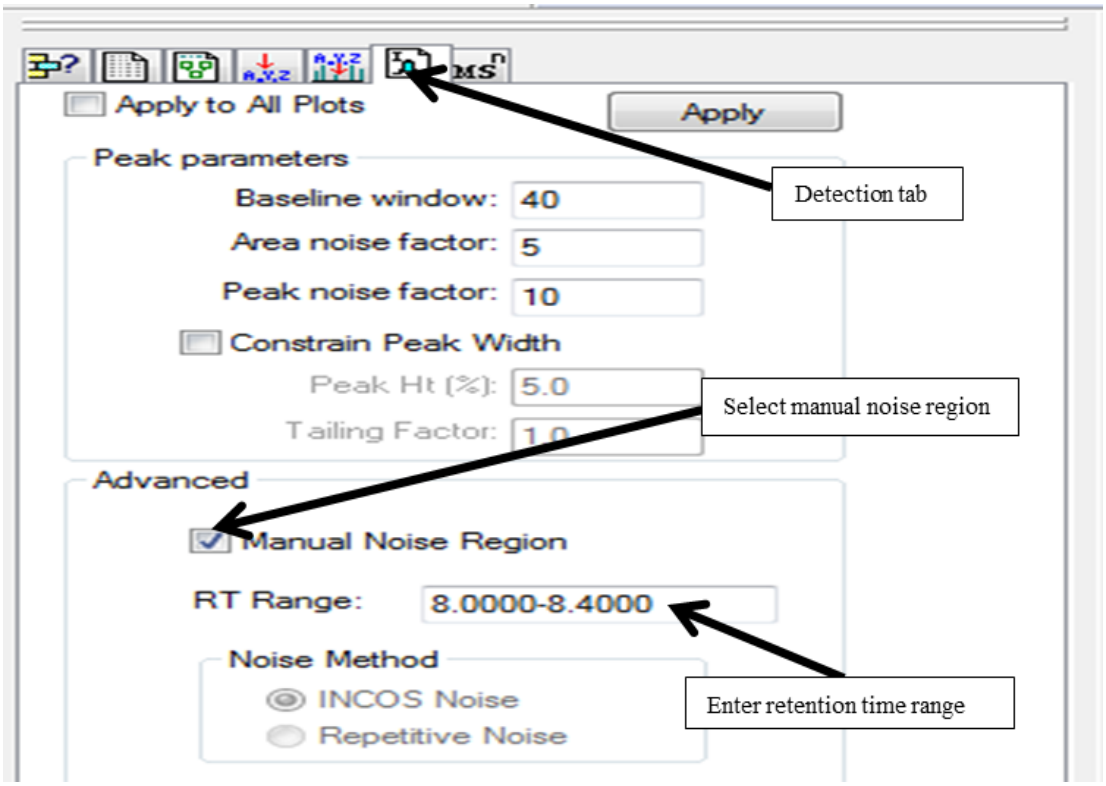

Figure 16. Example for Setting Manual Noise Region

24 Viewing and Processing Data using Qual Browser in Xcalibur: ·Viewing and Processing MS Data

24.1 Select the MS window by clicking on the pin (turns green on selection) on the top right corner of the MS window (bottom window) to allow matching the spectrum and the major ions in the spectral list for confirming identity of the analytes of interest.

24.2 Left click over the peak of interest from the chromatogram and match the major ions to be monitored for the analyte of interest from the spectral window. For example, peak seen at RT 12.72 min, selection of this peak shows the spectrum with presence of the major ions m/z 231, m/z 103 and m/z 153 (circled in blue in the bottom window of the image below).

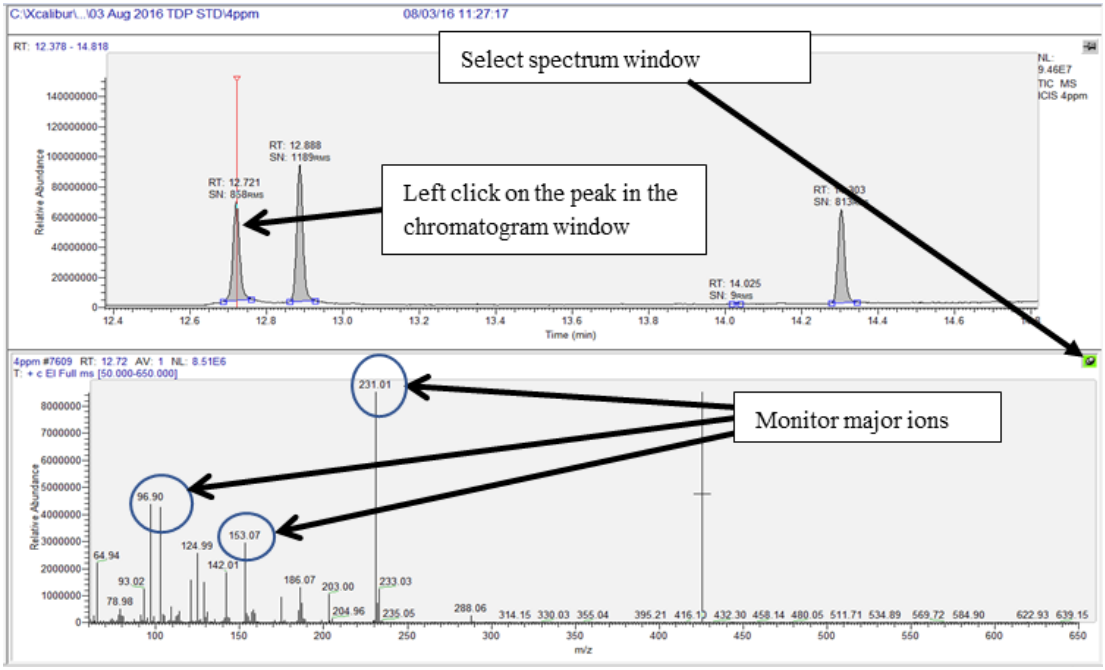

Figure 17. Example for Selecting Peaks of Interest

24.3 With the MS window still selected and showing the mass spectrum left click and select “Ranges”.

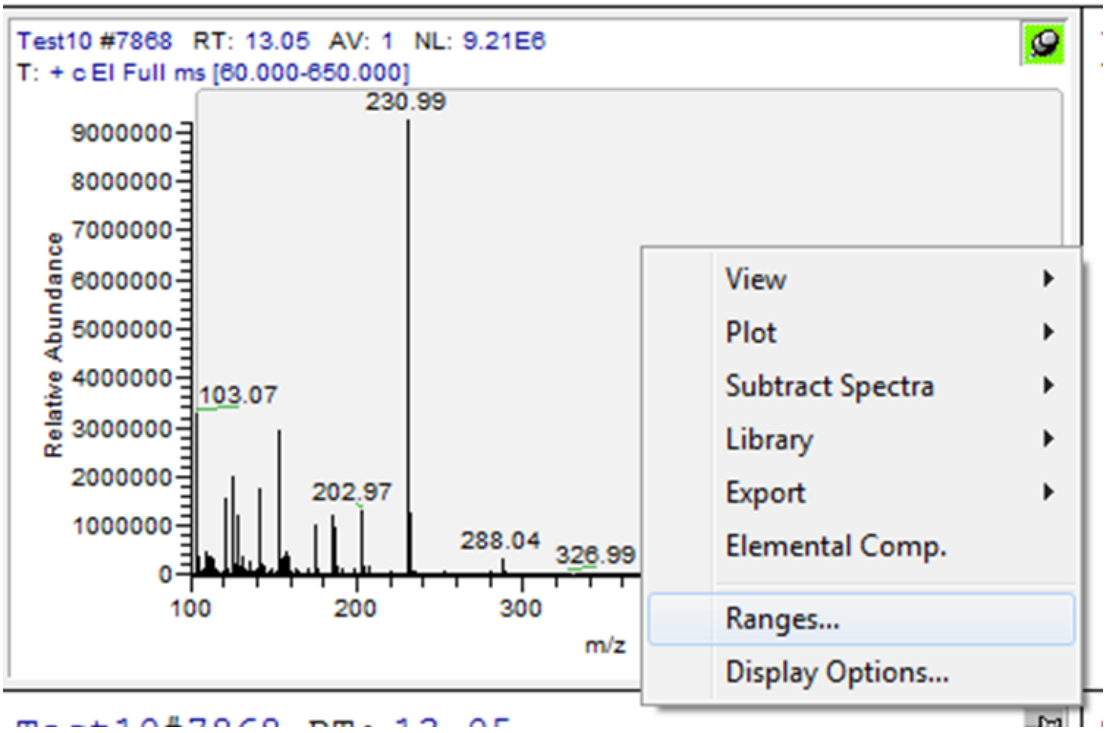

Figure 18. Example for Selecting Mass Ranges

24.4 The “Spectrum Ranges” window open up. Change the lower mass range from “60.00 – 600.00” to “100.00 – 600.00” and select “OK” to eliminate any low m/z ions interfering with analyte identification.

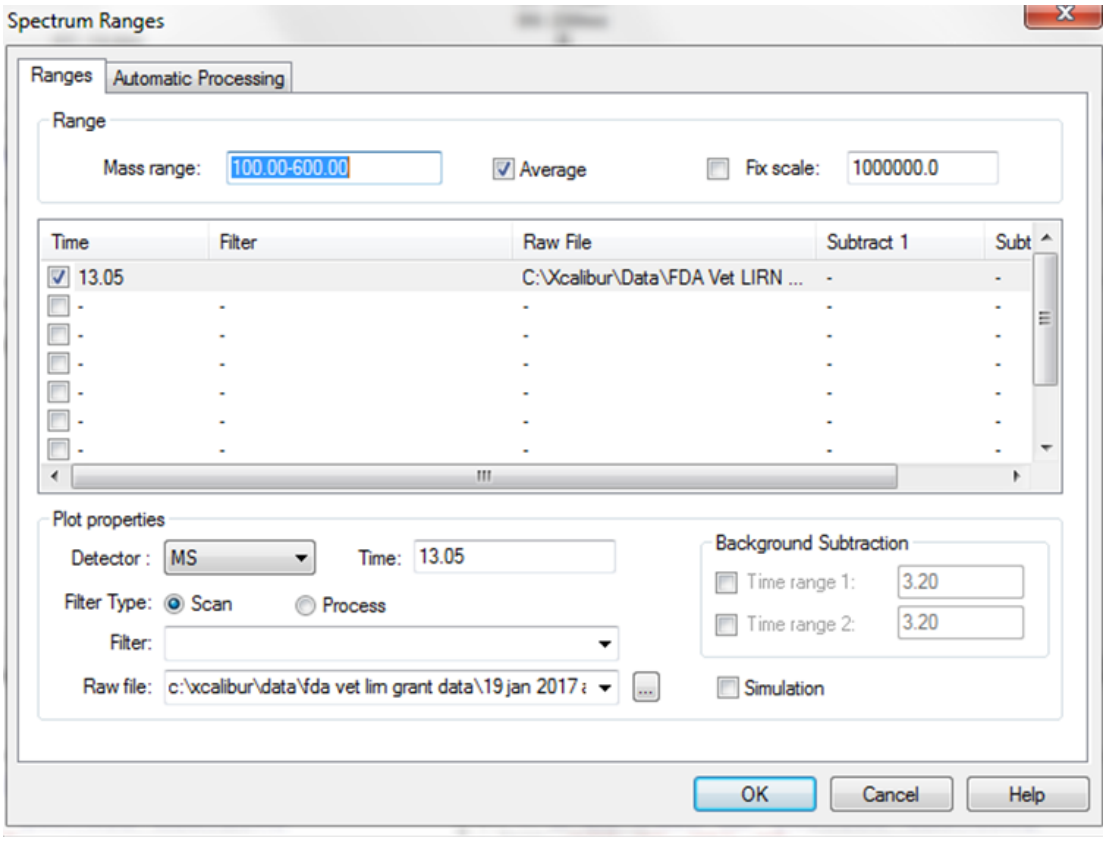

Figure 19. Spectrum Ranges Window

24.5 In next window, select "Grid" --> "Insert cells" --> "Below" to add another window below the spectrum window.

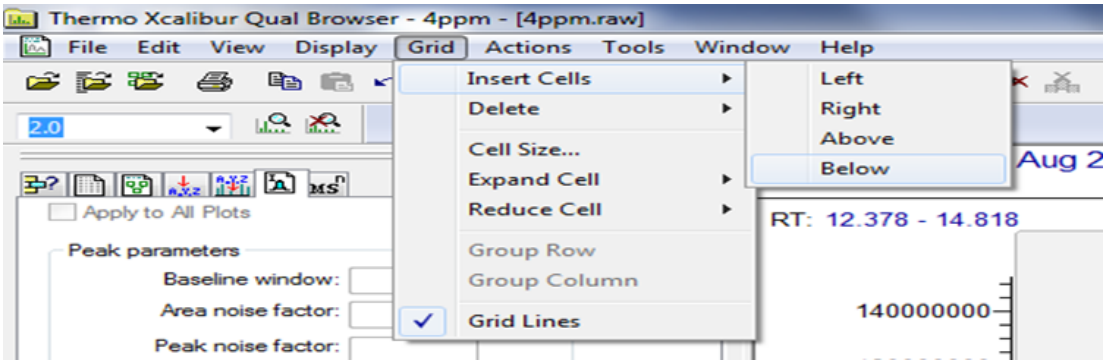

Figure 20. Adding Spectrum Windows

24.6 Select the third window, right click and select --> “View” --> “Spectrum list” to view the relative intensities in percentage for the major ions monitored.

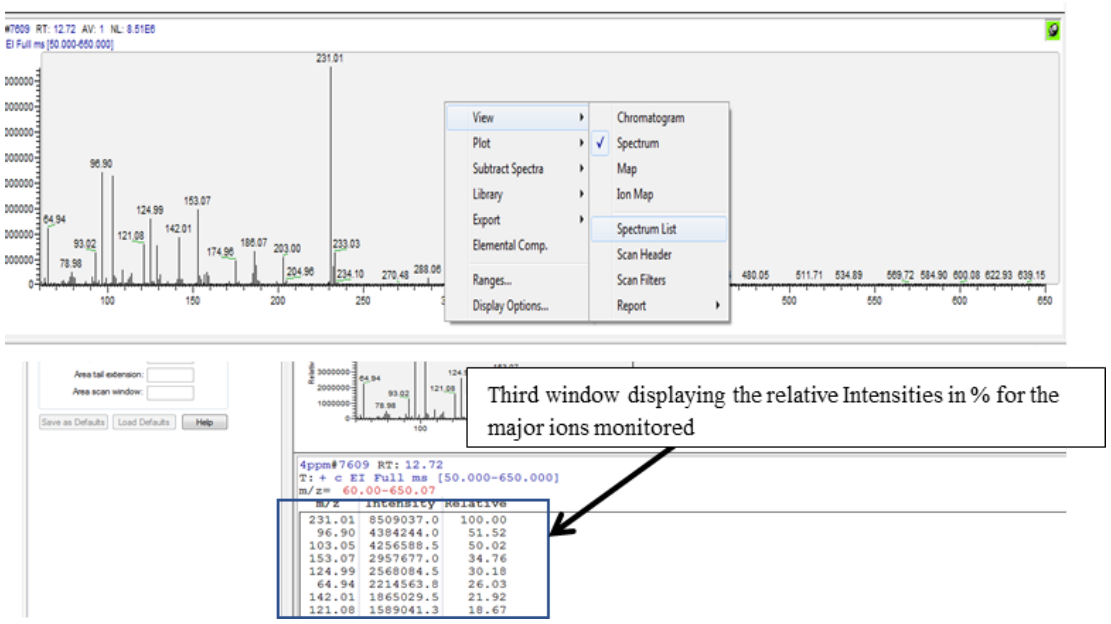

Figure 21. Viewing Relative Intensities of Ions

24.7 Print the data in the peak windows for chromatogram, spectrum and spectral list by selecting “File”
--> “Print” --> “All cells in selected window” --> "One Page” --> “OK”.

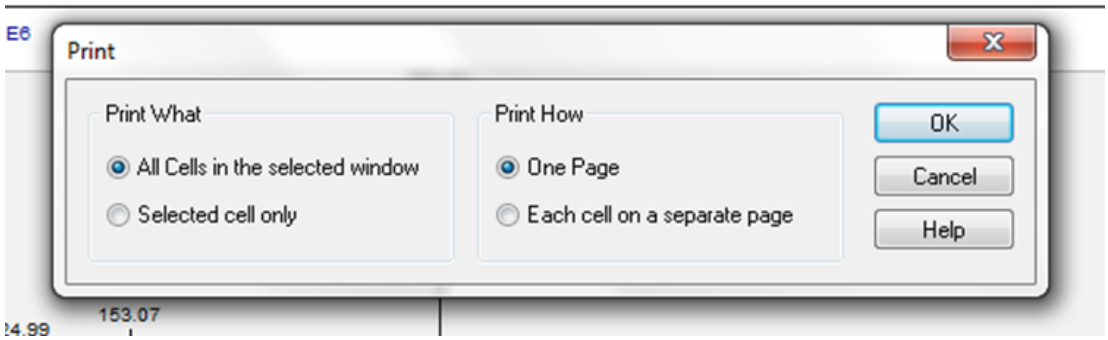

Figure 22. Printing Chromatograms and Spectral Data

24.8 Process the MS data for all the analytes by selecting the peak of interest in the chromatogram window as shown in steps above.