SOP Title:	GC/MS Method for Detection of Terbufos, Diazinon and Parathion in Blood

Version: 2019-10-22 Purdue University. Christina Wilson-Frank

Scope

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

Safety Considerations

- **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.

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

Table 1. Summary of Target Analytes and Their Limits of Detection

	Instrument LOD* This is the lowest concentration of analyte which can be detected in solvent. Procedure: Solvent is spiked and injected into GC-MS directly (not processed though method's steps such as extraction, SPE clean up, filtration and centrifugation)	**Method LOD**** This is the lowest concentration of analyte which can be detected in blood. Procedure: Blood is spiked and processed though the method's steps (extraction, clean up by SPE, centrifugation and filtration) and injected into GC-MS	**Method Assurance Levels***** These levels can be observed with a higher degree of confidence when compared to 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) can impact the method limits of detection (Method LODs) and therefore, need to be established in each collaborating laboratory.

** Method LODs allow diagnosticians to address recent or chronic exposure of the animal. The animal may or may not be exhibiting clinical signs related to exposure to these insecticides. Ability of the

method to detect analytes at such low levels also makes the method suitable for research purposes in addition to diagnostic analyses.

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

Abbreviations

- TDP: Terbufos, Diazinon, Parathion
- ACN: Acetonitrile
- LOD: Limit of Detection
- RT: Retention Time
- RPM: Revolutions per Minute
- RCF: Relative Centrifugal Force
- SPE: Solid-Phase Extraction
- BMT: Blind Method Test

Preparation for Analyzing Samples

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

- **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, 1000 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)

- **Extraction Solution and Standard Diluent Preparation**

  - Acetonitrile: Toluene (3:1)
    - Transfer 600 mL of acetonitrile to 1L graduated cylinder.
    - Add 200 mL of toluene to the 1L graduated cylinder containing the acetonitrile.
    - Transfer contents to a properly labeled 1L glass storage bottle and store at room temperature.
    - The expiration date for the solution would be same as the expiration date of the reagents used (earliest expiration date of the two reagents used).

- **Preparation of Pesticide Standards and Spiking Solutions**

  - Preparation of the mixed 20 ppm standard solution:

    - Record all pertinent information regarding the standard stock solution in the laboratory notebook.
    - Aliquot 1.50 mL of acetonitrile:toluene (3:1) into a properly labeled, 5 mL glass vial.
    - Transfer 0.75 mL of each of the 100 ppm terbufos, 100 ppm diazinon and 100 ppm parathion into the 5 mL glass vial containing the acetonitrile:toluene (3:1).
    - Cap and vortex mix well.
    - Label vial with contents, expiration date, laboratory notebook reference, analyst initials and NFPA designations.
    - TDP standards at 20 ppm concentration are stable for up to 5 weeks when stored at refrigeration temperature or for 24 hours at room temperature.

- Preparation of the mixed 1 ppm standard solution

  - Record all pertinent information regarding the standard stock solution in the laboratory notebook.
  - Aliquot 2.00 mL of acetonitrile:toluene (3:1) into a properly labeled 5 mL glass vial.
  - Transfer 0.106 mL (106 µL) of the 20 ppm TDP mixed standard solution prepared above.
  - Cap and vortex mix well.
  - Label vial with contents, expiration date, laboratory notebook reference, analyst initials and NFPA designations
  - TDP standards at 1 ppm concentration are stable for up to 3 weeks when stored at refrigeration temperature or for 24 hours at room temperature.

- **Control Samples**

  - Negative Controls
    Blood with no known exposure to pesticides (i.e. no detectable pesticides in the sample) are used as negative control samples. Use 2 mL of blood as negative control for sample extraction procedure without spiking the samples with TDP standards.

  - Positive Controls
    Add 0.106 mL (106 µL) of the 20 ppm TDP mixed standard solution to approximately 2 mL of blood to obtain a spike level of 1 ppm.

- **Procedure for Samples**

  - Sample Extraction Procedure
    - Aliquot 2 mL of blood into a 15 mL polypropylene tube labeled appropriately. Record sample ID and sample volume in the laboratory notebook.
    - Add 4 mL acetonitrile to the sample and vortex mix for approximately 1 minute.
    - Centrifuge sample at approximately 4,000 RPM (~ 1500 RCF) for 10 minutes.
    - Aliquot supernatant to a properly labeled 15 mL polypropylene tube.
    - Add an additional 4 mL of acetonitrile to the remaining pellet. Vortex mix for approximately 1 minute.
    - Centrifuge sample at approximately 4,000 RPM (~ 1500 RCF) for 10 minutes.
    - Aliquot supernatant and combine with supernatant from step 4.
    - Add 1 g of Na_2SO_4 to the combined supernatants and vortex mix for approximately 1 minute to dry the extract. Allow Na_2SO_4 to settle before proceeding.
    - Using a disposable glass pipet, transfer all of dried acetonitrile extract to a 15 mL polypropylene tube. (Extract is stable for 24 hours under refrigeration conditions)
    - Proceed with SPE clean-up procedure.

- SPE Clean-up Procedure
  - 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 beaker.
  - Load the acetonitrile extract from above into the SPE cartridge and collect the eluate as waste in a properly labeled beaker.
  - Elute pesticides from SPE cartridge with 15 mL acetonitrile:toluene (3:1) into a 50 mL glass test tube.
  - Place test tube in a nitrogen evaporator heating block (set at approximately 40˚C) and evaporate eluate to dryness under low nitrogen gas flow. (Evaporated sample is stable for 24 hours under refrigeration conditions)
  - 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.
  - Vortex tubes for 30 seconds. Transfer the filtrate to properly labeled GC/MS autosampler vials with insert. (Stable for 24 hours under refrigeration conditions)
  - Proceed to GC/MS analysis

GC/MS Detection of Pesticides

- Instrument Conditions

Table 2a: GC Instrument Method

Carrier gas	Helium at 1.4 mL/min
Injector port temperature	275˚C (splitless)
Injection volume	1 µL
Column	ThermoFisher TR-5MS; 0.25 mm i.d. x 30 m Film thickness: 0.25 µm
Column temperature	40˚C (0.5 min) → 14˚C/min → 300˚C (1.0 min)

Table 2b: Triple Quadrupole MS Instrument Method

Ionization mode	Electron impact ionization (70 eV)
Ion source temperature	310˚C
Transfer line temperature	325˚C
Mass scan range	50 – 650 m/z Dwell time: 0.071 sec

- Acceptance Criteria for Determining the Presence of the Pesticide
  (Note: All criteria must be met for reporting the presence of the pesticide)

  - The retention time shall be within 3% of the retention time of the pesticides in the positive control sample or the 1 ppm standard solution. (See Table 3 for retention time information)
  - The signal-to-noise (SN) ratio for each pesticide shall be greater than 3.
  - All observed ions listed in Table 4 shall be present.
  - The relative intensities of at least one of the qualifier ions shall be within 30% of the relative intensity of the target ion (base peak) observed.

Table 3: Pesticide Retention Times

Pesticide	Approximate RT*	Relative RT**
Terbufos	13.02 min	1.000
Diazinon	13.17 min	1.012
Parathion	14.60 min	1.121

* The approximate retention time can differ slightly with GC injector port or column maintenance.
**Notes: The relative retention time is the ratio of the retention time of an analyte peak relative to that of another analyte which is used as a reference peak obtained under identical conditions. The relative retention times for the pesticides in this method are ratios calculated with respect to terbufos. For example, if the experimental retention time for terbufos is 13.00 min, then one would multiply that retention time (13.00 min) by 1.012 to estimate an approximate retention time of 13.27 min for diazinon.

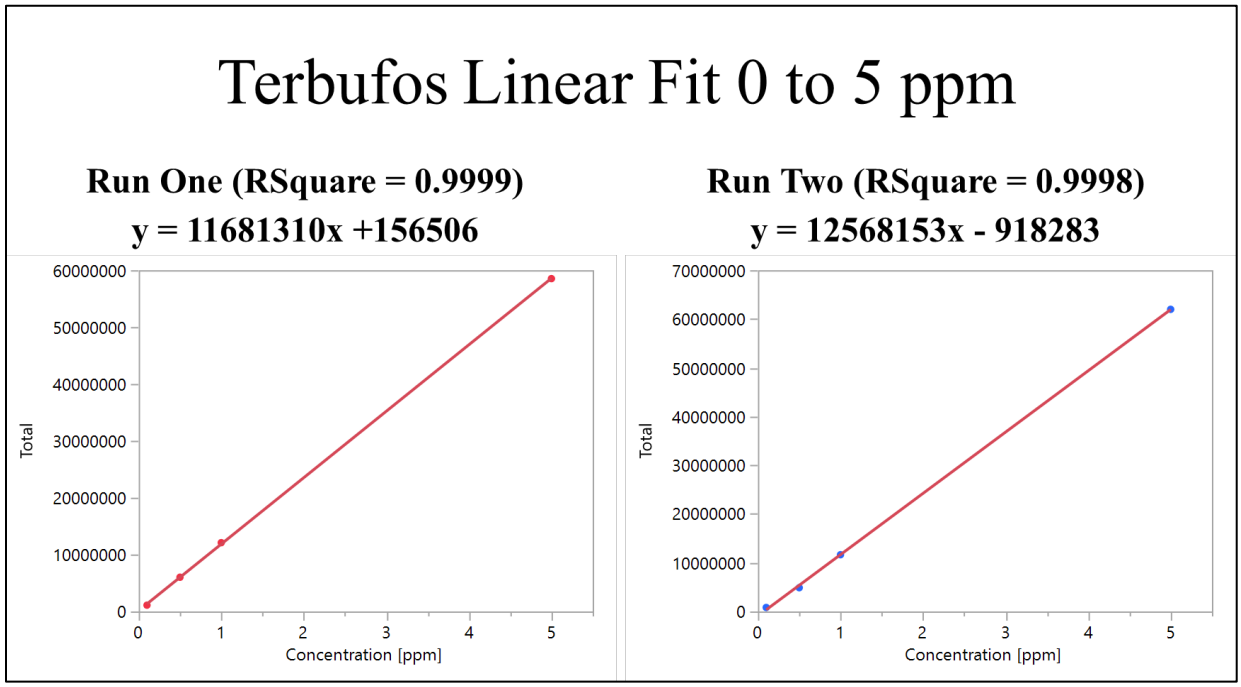
Table 4. Target Ions and Qualifier Ions for Pesticides

Pesticide	Target Ion (m/z) ***Base Peak***	Qualifier Ions (m/z)
Terbufos	231	153, 103
Diazinon	137	199, 179, 152
Parathion	109	291, 139

Appendix One for Pesticide SOP

A couple of studies discussed below have shown inadequate false negative percentages when terbufos was known to be present at the 0.3 ppm level in bovine blood samples. The identification of terbufos at low levels (limit of detection is 0.3 ppm in blood) may be influenced by the removal or lack of removal of saturated fatty acids such as myristic acid, palmitic acid and possibly others. (Mysristic acid has an approximate retention time of 12.73 minutes and palmitic acid has an approximate retention time of 14.20 minutes). When there is adequate removal of the fatty acids, there is improved recovery of terbufos and vice versa. It is evident that a linear correlation exists between intensity and concentration.

Figure 1: A statistically significant linear fit was found between the concentration of the standard and theoretical intensity observed for terbufos between 0 and 5 ppm.



To reduce these false negative observations, the acceptance criteria was expanded to the following:

- The retention time shall be within 3% of the retention time of the pesticides in the positive control sample or the 1 ppm standard solution.
- All observed ions need to be present (231 m/z, 103 m/z, and 153 m/z).
- The relative intensities of at least one of the qualifier ions (103 m/z or 153 m/z) shall be within 30% of the relative intensity of the target ion (base peak) observed (231 m/z).

Upon investigation, these acceptance criteria could not be successfully achieved due to co-elution of matrix interference peaks from the blood samples due to interference with observations of qualifier ions 103 m/z and 153 m/z). However, an "Alert Criteria" might be used

to determine if a false result should be suspect. This "Alert Criteria" the ratio of the sum of the SN ratios fatty acids (myristic and palmitic acids) divided by the SN of the terbufos peak.

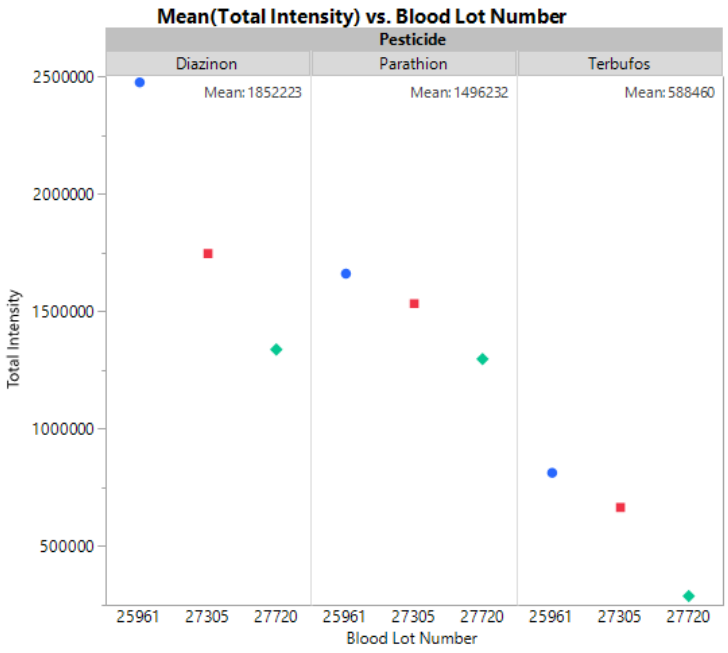
Equation 1: Calculated Ratio for "Alert Criteria"

$$\text{Alert Criteria} = \frac{\text{SN for myristic acid peak} + \text{SN for palmitic acid peak}}{\text{SN for terbufos peak}}$$

If this calculation is greater than 7.5, the result should be considered suspect and samples and standards re-extracted and re-analyzed.

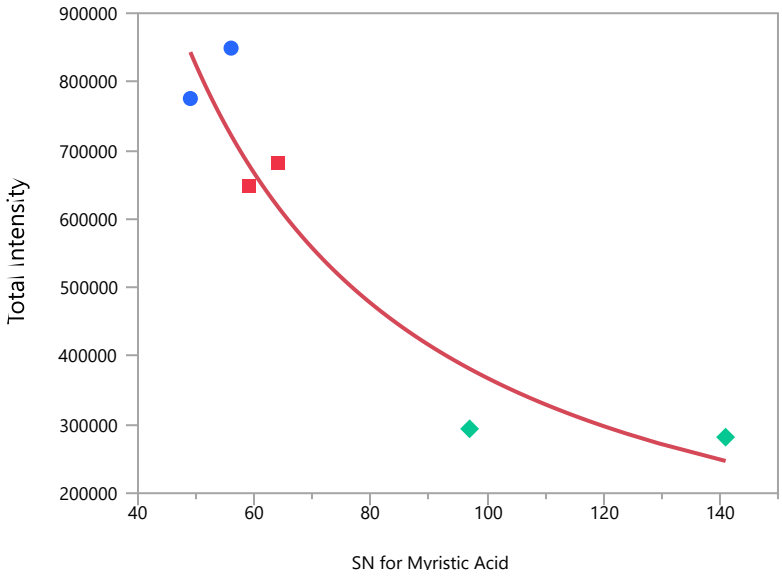
Studies were completed to determine what could influence intensities or the amount of terbufos after extraction and clean-up procedures. Extracting and analyzing differing lot numbers of blood samples was completed to determine if different lot numbers influenced terbufos intensities. The results were significant.

Figure 2: Total Peak Intensity versus Blood Lot Number by Pesticide



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

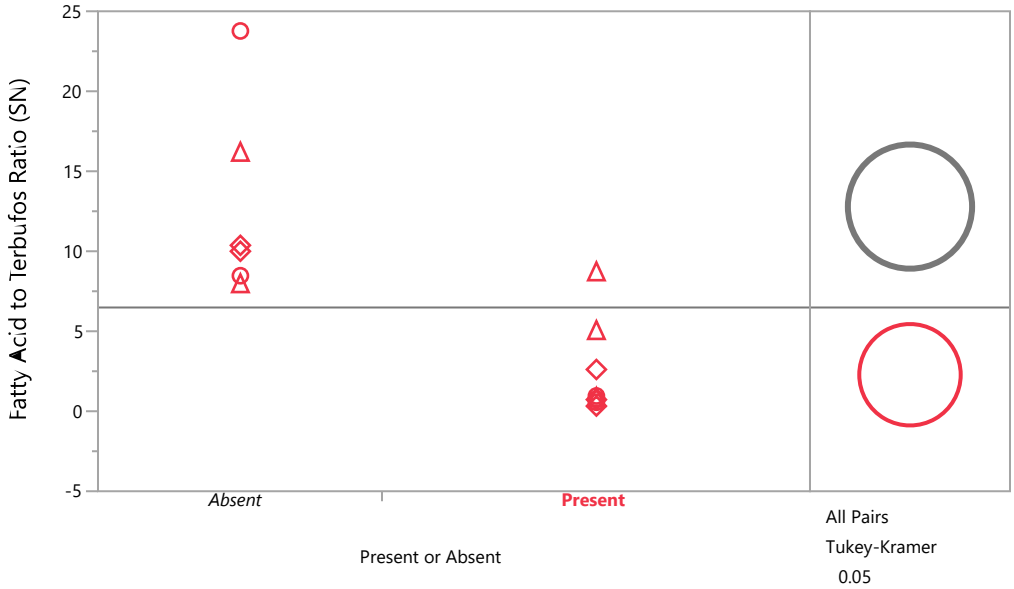
Figure 3: Terbufos Intensity versus SN for Myristic Acid (Log to Log Fit)



Different Colors and Shapes are different Blood Lots obtained from the vendor.
RSquare = .8929 with an F-Ratio of 0.0045

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 revealed that the amount of water and the amount of Na_2SO_4 used was significant for fatty acid removal. The lot number of blood 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 SN ratio of the fatty acids, however there were samples that did not make sense with this criteria, apparently due to baseline variations. To account for these differences in the baseline, the SN ratio for terbufos was introduced into the equation to remove baseline variability.

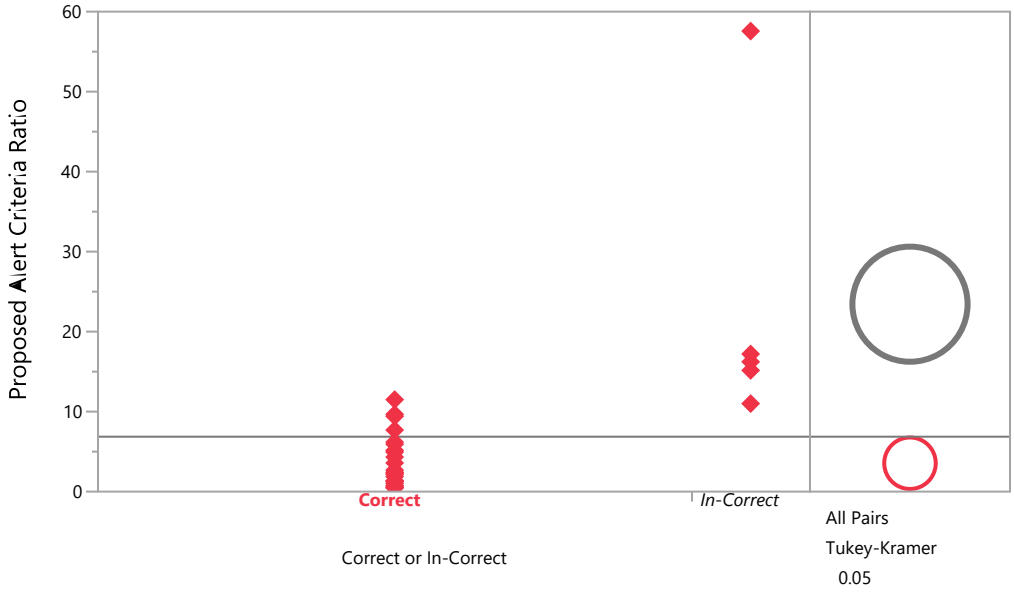
Figure 4: Fatty Acid to Terbufos SN Ratio versus Terbufos Identification



All samples were spiked with terbufos, therefore, an indication of "absent" is a reflection of an in-correct result while an indication of "present" reflects a correct identification of terbufos.

This criteria was also examined for the multi-laboratory BMT study, which used a different lot number of blood fortified with different concentrations of terbufos. The results were similar to the above study showing significant differences between the proposed "Alert Criteria" ratio (fatty acid to terbufos SN ratio. This ratio was also effective for correct identification when terbufos was not present in the blinded sample (see Figure 5).

Figure 5*: *Fatty Acid to Terbufos* SN *Ratio versus* Terbufos *Identification



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 and can depend on the amount of water, amount of Na_2SO_4 added in the extraction process and possibly individual variation in animal blood specimens. The levels can fluctuate from sample to sample due to what may carry forward into the clean-up step of the work-up procedure.

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 (at various percentages) with the current SPE cartridges containing PSA.

Appendix Two for Pesticide SOP TOX. 103 20 Min
(Details for Purdue University Method using Xcalibur Software)

Note: The following screen shots are examples for using Xcalibur at the Purdue University ADDL. These screen shots may change in the future and are not representative of other software.

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.

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.
  - Auto-Sampler Settings
    - Set the injection volume to 1 µL (See Figure 6).

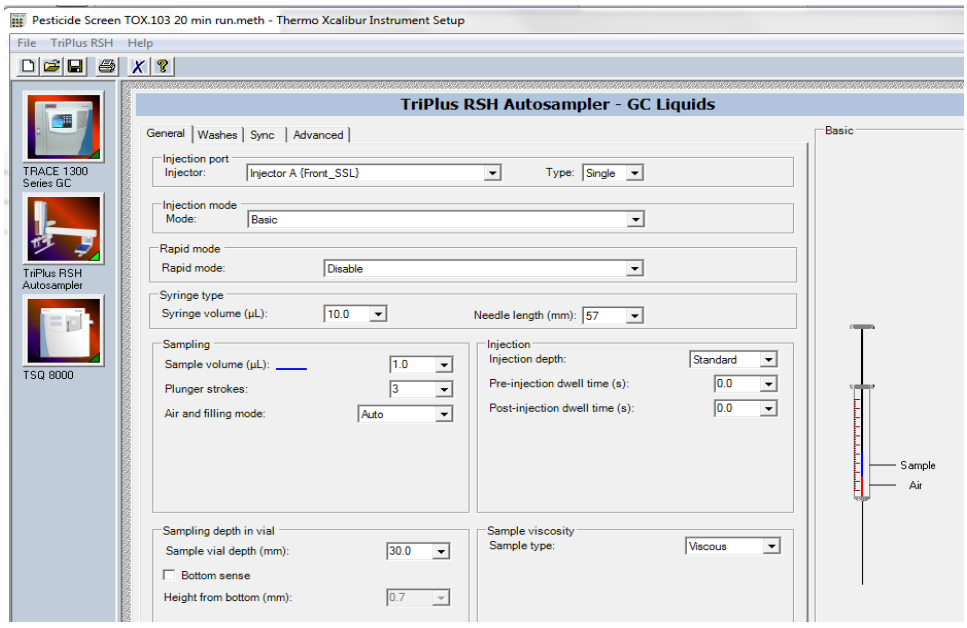


Figure 6

- Set the wash station settings as seen in Figure 7.

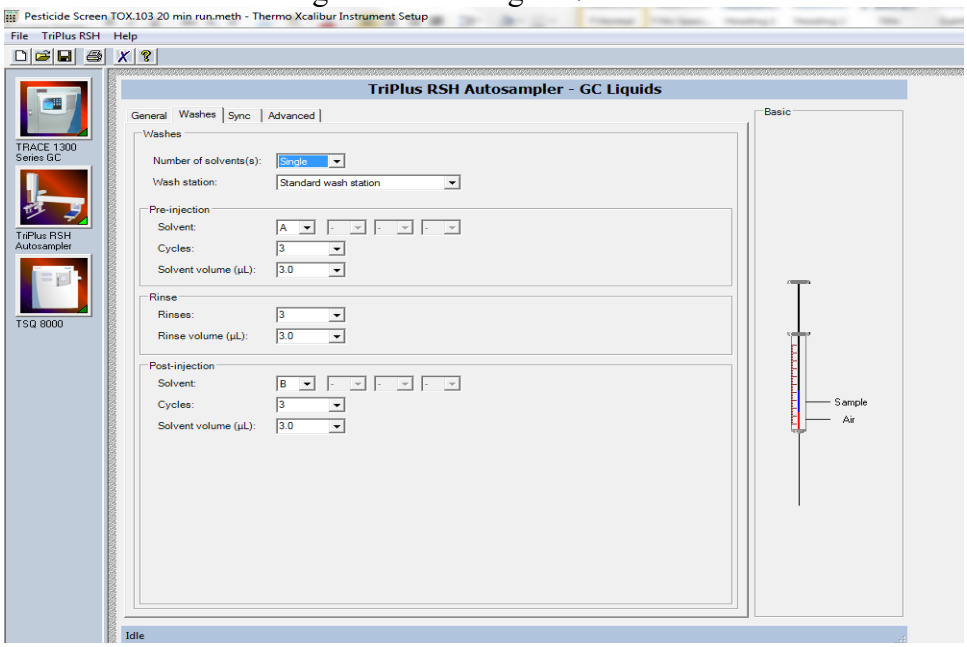


Figure 7

- The advanced wash settings should be set to the values seen in Figure 8.

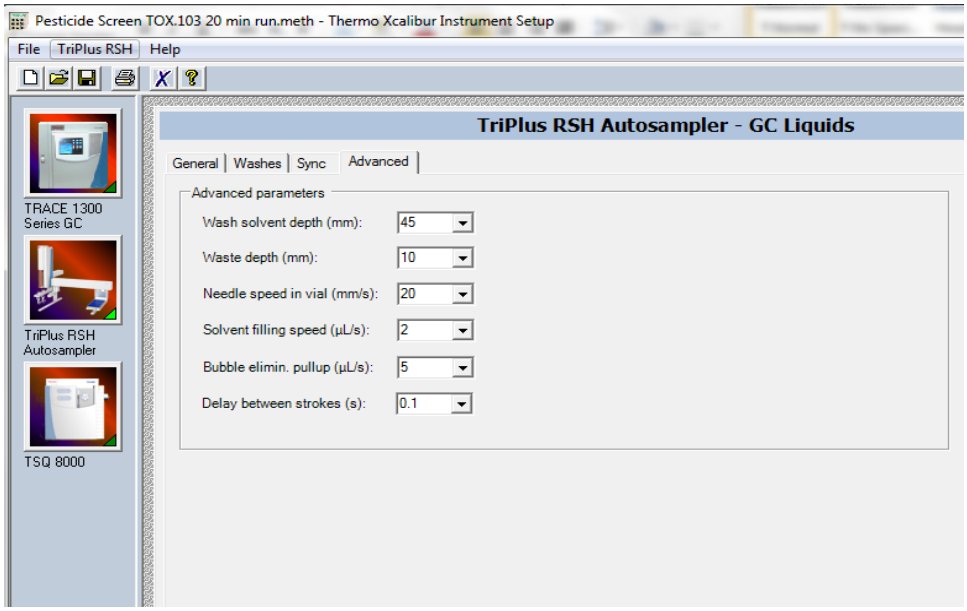


Figure 8

- GC Conditions and Settings
  - Under the oven tab, the initial temperature should be set at 40°C and held for 0.5 minutes. The temperature should then be increased to 300°C at a rate of 14°C/minute. Once the temperature reaches 300ºC, that temperature should be held for 1.0 minute. This will provide a run time of 20.06 minutes. (See Figure 9

for details)

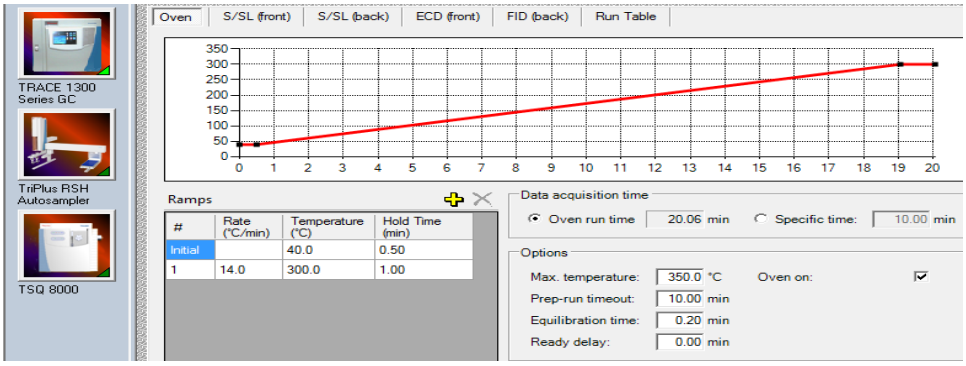


Figure 9

- Set the front injector port so that it is in "splitless" mode at a temperature of 275°C with a carrier flow of 1.4 mL/min. (See Figure 10 for details)

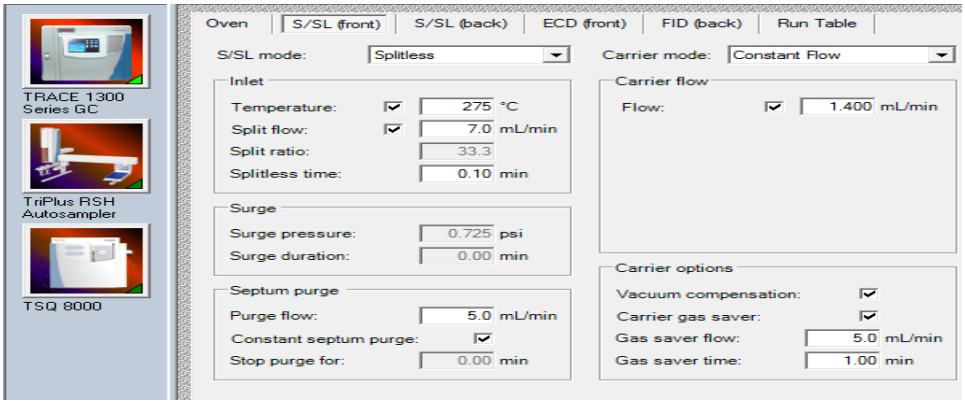


Figure 10

- MS Conditions and Settings
  - Set MS transfer line temperature to 325°C and the ion source temperature to 310°C. (See Figure 11 for details)

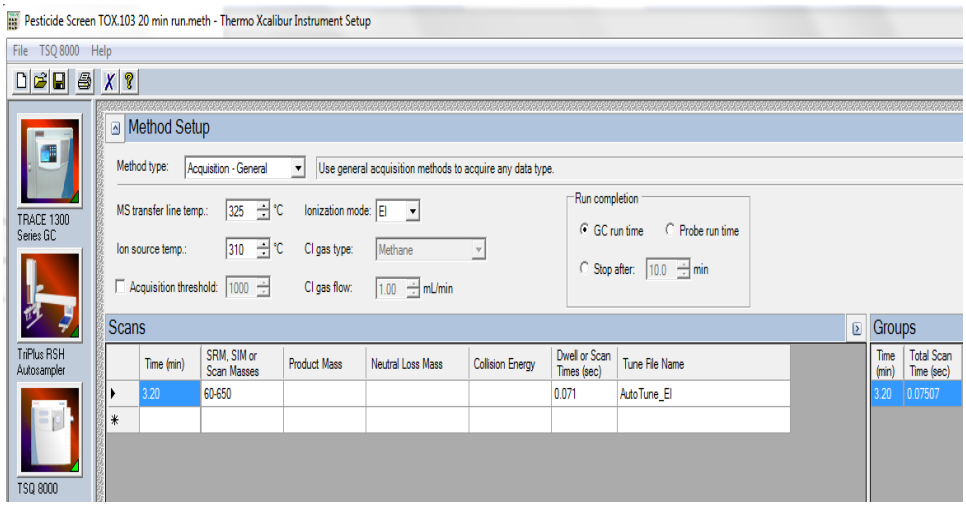


Figure 11

- Analyzing Samples in System Control (Xcalibur)
  - Open "Xcalibur" by double-clicking the "Xcalibur" icon. Double-click on "Qual Browser" to create the data file for your analysis.
  - With Qual Browser open, go to "File" → "Open." The "Open Raw File" window will be displayed.
  - 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.
  - To enter samples into the sample set, click on "Sequence Setup". Enter the pertinent information about the samples to be analyzed. See Figure 12 for placement details.

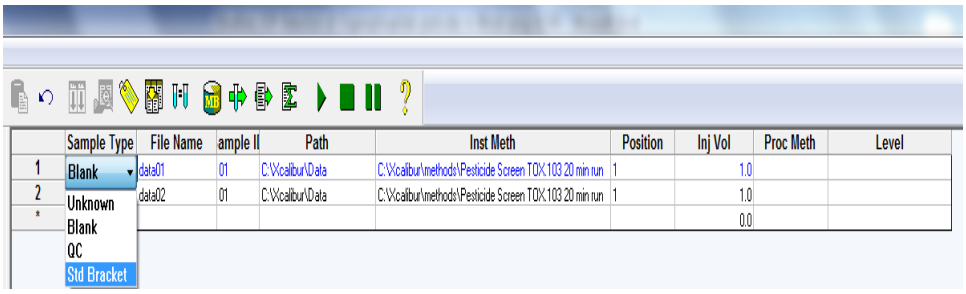


Figure 12

  - 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.

- File name: Enter name of sample, standards or control vials used (cannot use spaces).
- Path: Select the data folder previously created in Qual browser.
- Instrument Method: Select "Pesticide Screen Tox.103 20 min run"
- Position: Enter the position number for the vial placed in the auto sampler tray
- Injection Volume: 1.0 (1 µL).

- 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.
- To start the analysis and run the entire sequence, select the "Run Sequence" icon  . A window will open → select "OK" to begin the analysis.

Viewing and Processing Data using Qual Browser in Xcalibur

- Viewing GC Chromatogram prior to MS Processing
  - 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.
  - 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.

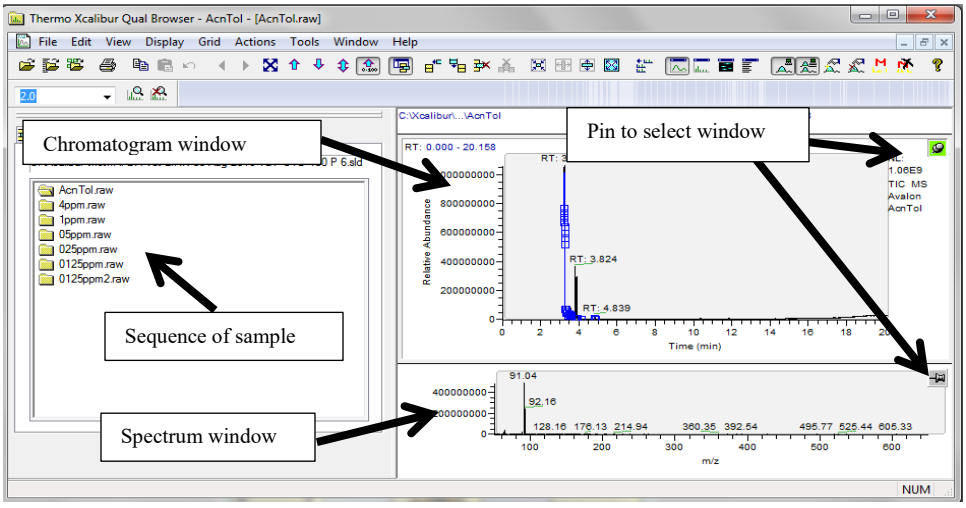


Figure 13

- 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.
- 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."

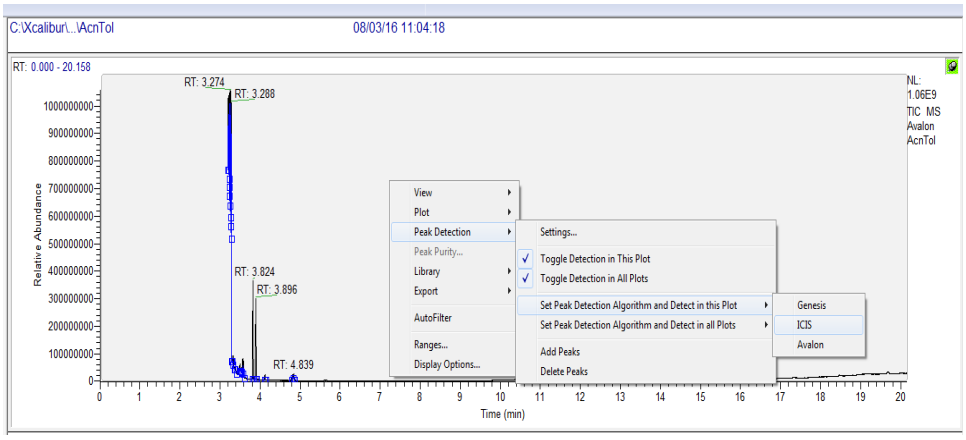


Figure 14

- 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.".

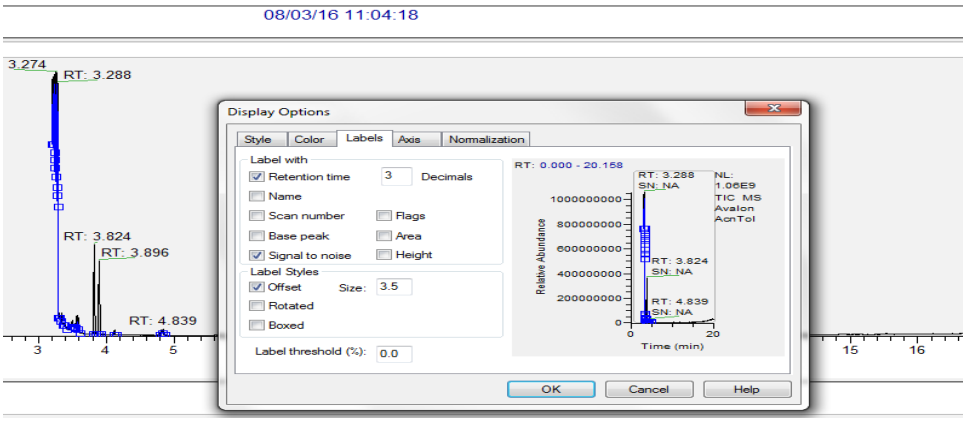
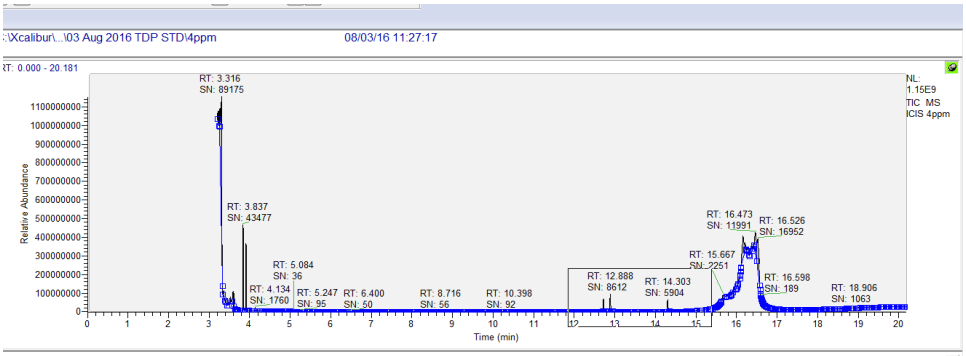


Figure 15

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



Zoomed-in

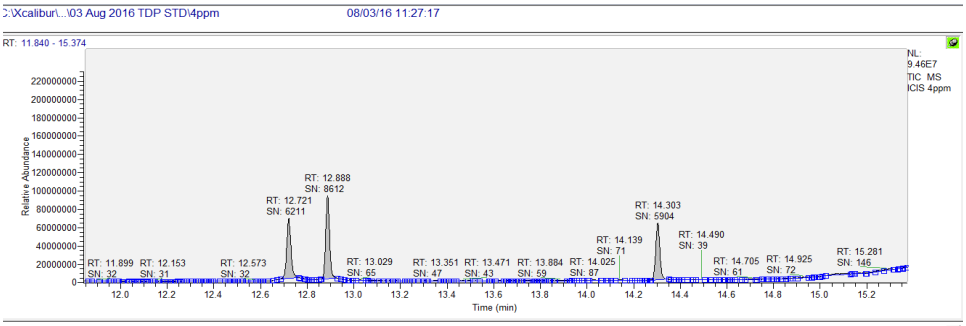


Figure 16

- 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.

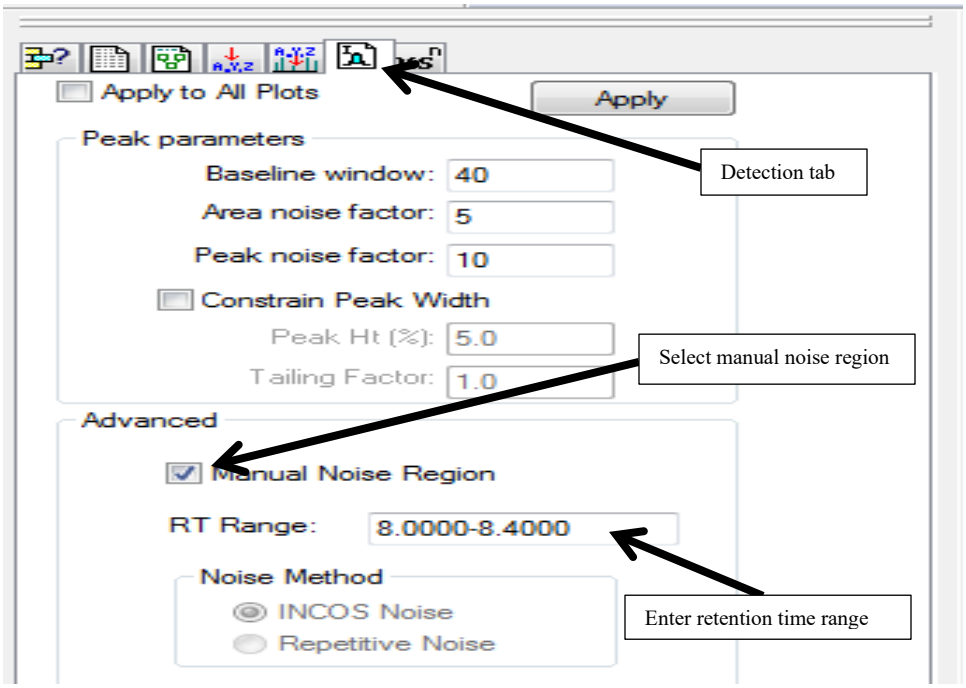


Figure 17

-
- **Viewing and Processing MS Data**
  - 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.
  - 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).

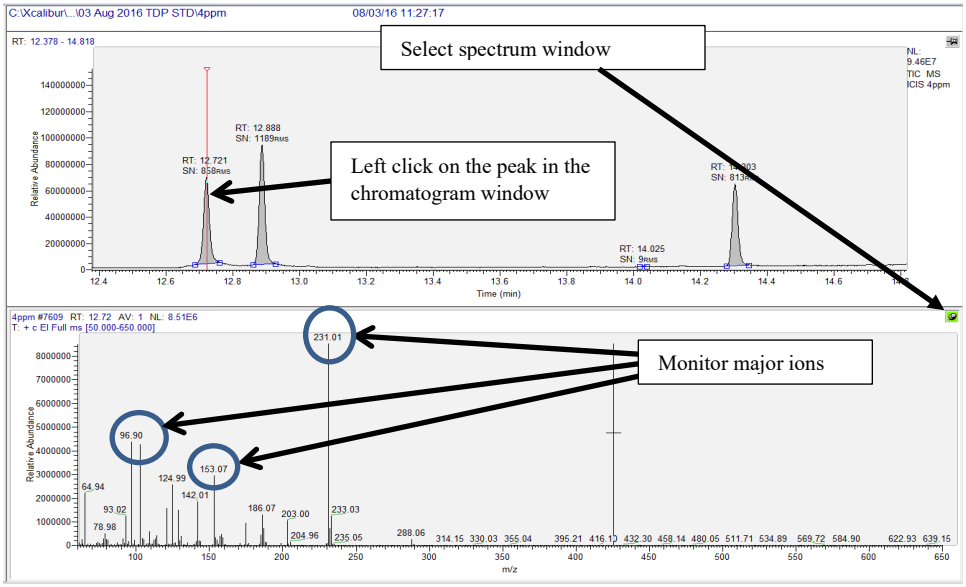


Figure 18

- With the MS window still selected and showing the mass spectrum left click and select "Ranges".

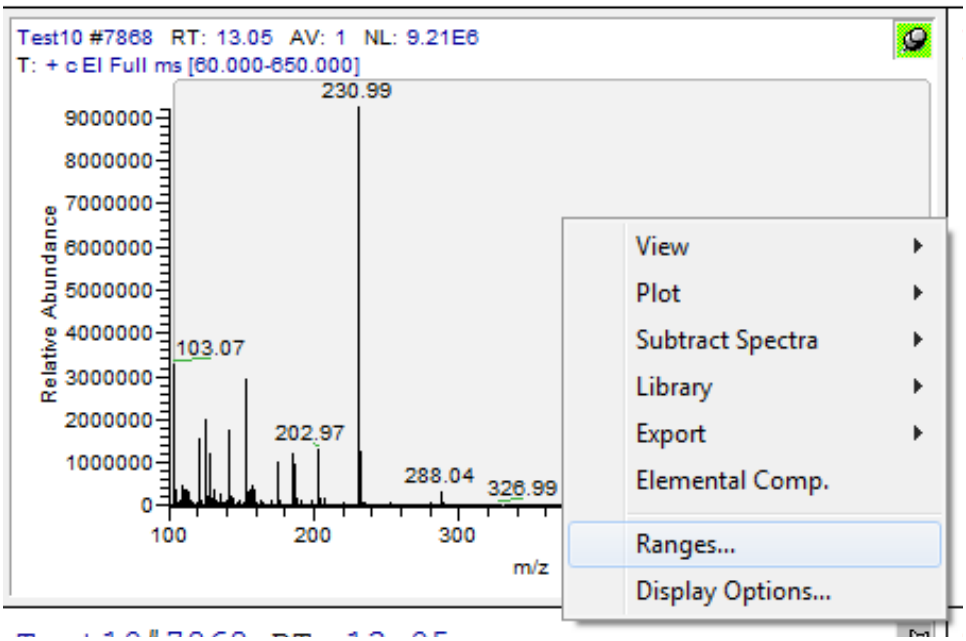


Figure 19

- "Spectrum Ranges" window will pop up; change the lower mass range from "60.00 – 600.00" to "100.00 – 600.00" and press → "OK" to eliminate any low m/z ions interfering with analyte identification.

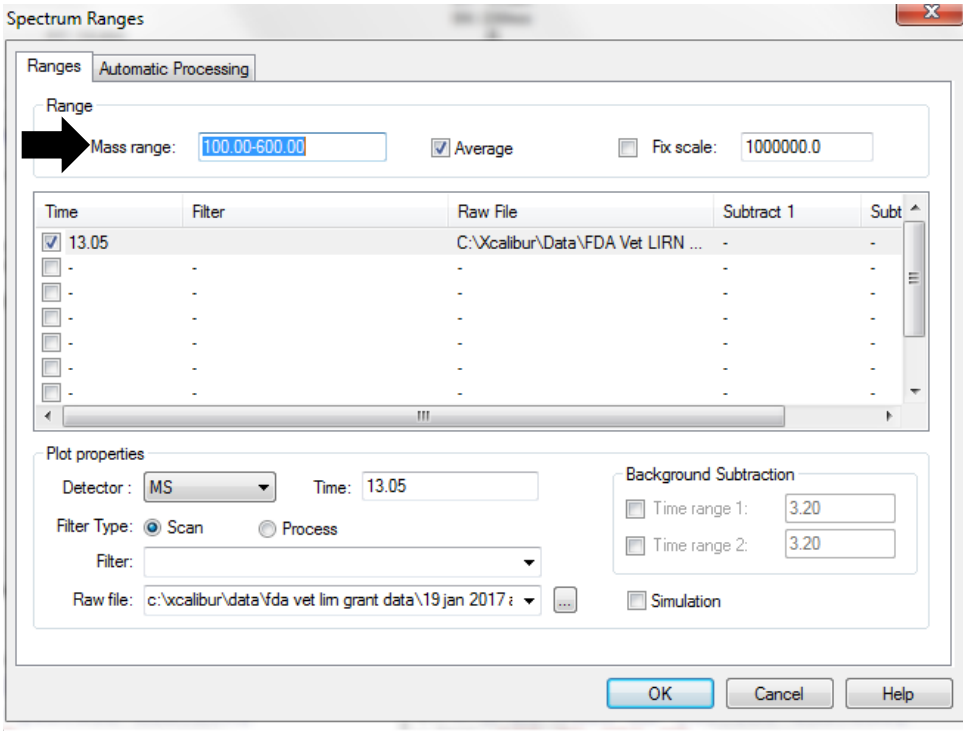


Figure 20

- Select Grid → Insert cells → below, to add another window below the spectrum window.

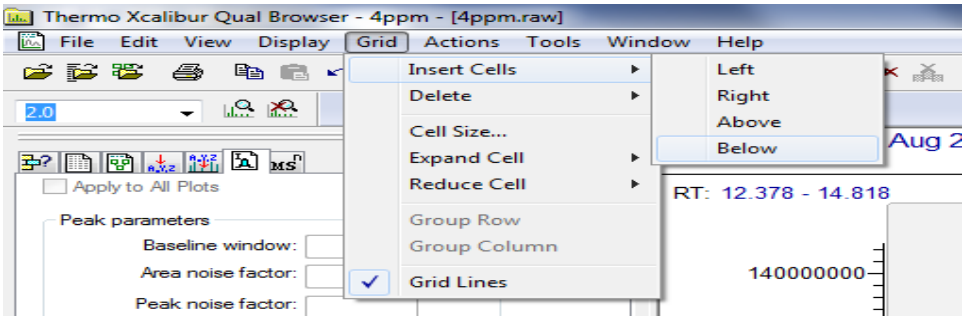


Figure 21

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

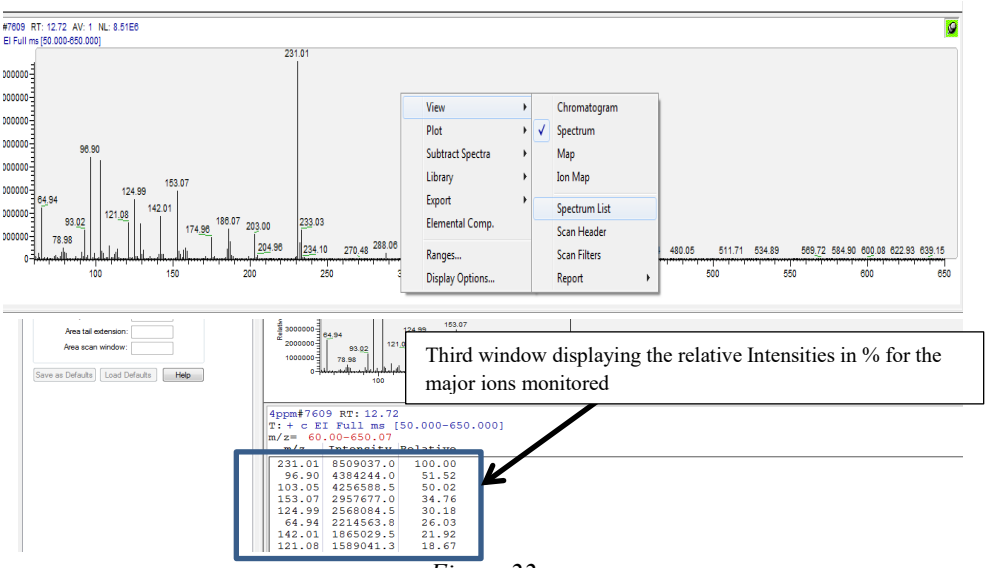


Figure 22

- 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".

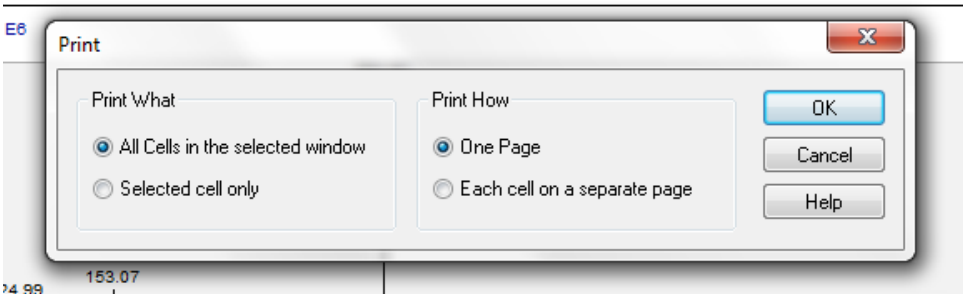


Figure 23

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