Calibration of Quadrupole Systems

3_5_002F

What causes shifts in calibration?

- Environmental changes
  - Temperature or humidity changes
  - Power supply changes over time
- Changing Resolution Settings and or Ion Energies

Ion Energies

- Important to work within the calibrated range of the instrument!
  - Just as you do not want to quantitate greater than the range of your quantitation curve, you do not want to work outside your mass calibration range.

3_5_002F

Tune → calibration → default.cal

Instrument Calibration Curves

A triple-quadrupole instrument relies on three calibrations for each MS1 and MS2, to give a maximum of six calibration curves:

Static calibration accurately "parks" the quadrupole mass analyser on a specific mass of interest (e.g. Tuning, SIR and MRM).

Scanning calibration enables peaks acquired in a scanning acquisition to be mass measured accurately (e.g. Full Scan).

Scan speed compensation calibration compensates for "lag time" in the system when the instrument is scanned at different rates.

A separate mass spectrum of the reference solution is acquired for each selected calibration type. MassLynx can perform all of the needed calibrations in one step.

3_5_002F

reference file

Calibration - Firmware vs Software

- There is a hardware calibration that is set up by the factory and the engineers that will be accurate to within +/- 0.2 Da across the mass scale.

  For some applications this firmware calibration will be sufficient.

- For situations where higher mass accuracy is required, or fast scan speeds are needed, the user can perform a software calibration through MassLynx and refine the mass calibration of the system.

  This can be useful in identification of unknowns and in insuring the correct masses are being selected for MRM and SIR analyses.

3_5_002F

Calibration Procedure

- A mass spectrum of a reference solution is acquired. The peaks in the acquired spectrum are matched against a table of expected masses which are stored in a Reference File.
- Each peak in the Reference File is matched to a corresponding peak in the acquired spectrum file.
- A calibration curve is created that 'adjusts' the masses of the acquired spectral peaks to the expected masses of peaks as listed in the reference file.
- Typically a Syringe Pump capable of delivering a slow, steady flow rate of 5 or 10 µL/min is used with an Infusion Kit consisting of a fused silica capillary tubing with appropriate fittings.

3_5_002F

I can make a reference file.

More on the Reference Solution

- A reference solution is used that produces multiple ions over a broad mass range.

  The mass spectrometer can be calibrated over this mass range.

  Examples of commonly used reference solutions are solutions of:

  PEG, PPG

  NaI/CsI, NaI/RbI

  Horse Myoglobin, Poly Alanine

3_5_002F

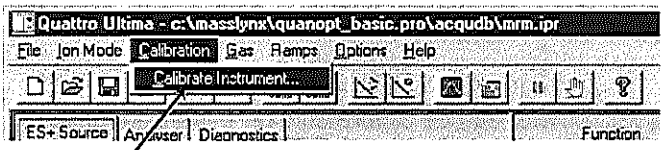
Reference File

- A file containing information on the spectral peaks produced by a reference solution that MassLynx will search for and attempt to match during calibration.
- This file lists both the mass (m/z) and expected intensity of these peaks. (Intensity info not usually used when calibrating LC/MS systems.)
- Reference Files are located in the *Ref* folder under MassLynx.
- Reference files are text files that can be viewed in Notepad.
- Reference filenames must have a '.ref' extension.

3_5_002F

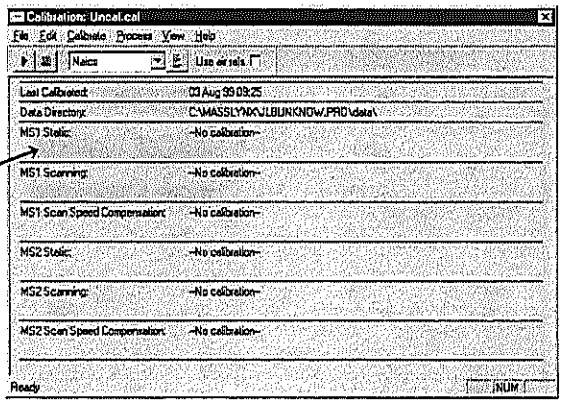
Mass Calibrations are Controlled From the Calibration Dialog Box.

To Display this dialog box on EPCAS Systems:



From Tune Page Select Calibrate Instrument

Calibration Dialog Box Should Appear

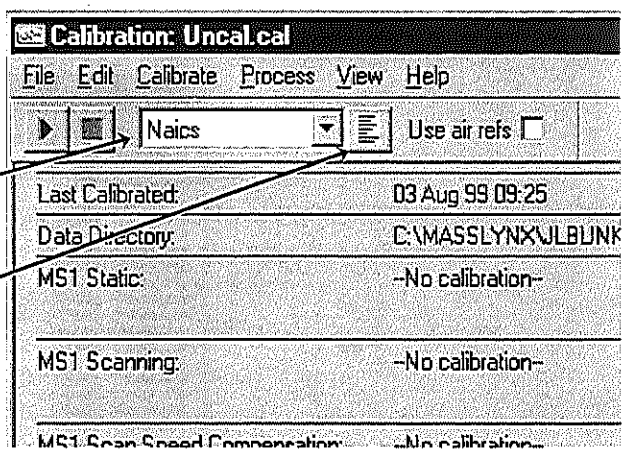


3_5_002F

In Preparation for Calibration: Check the Reference File

From the 'Drop Down' menu Choose Appropriate Reference File

Click here to open this 'Reference File' in 'Notepad'



3_5_002F

Calibration Reference Files

Calibration Reference Files Are Stored in: C:\MASSLYNX\REF

Examples of Calibration Reference Files ->

Myo.ref	Naics.ref	Pegh.ref
Myo1500.ref	Naics1.ref	Pegh1000.ref
Myo1500n.ref	Naics2.ref	Pegh2000.ref
Myoneg.ref	Naics3.ref	peghna.ref
Myotrp.ref	Naics4.ref	peghnam.ref
	Naineg.ref	peghnh4.ref
Pigb.ref	Nairb.ref	ppghna.ref
		ppghnh4.ref

Horse Myoglobin and Pig

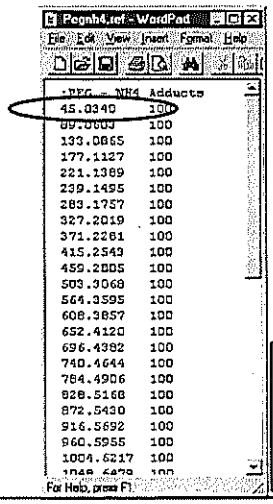
NaI with Cs or Rb

PEG or PPG

3_5_002F

The PegNH4 Reference File

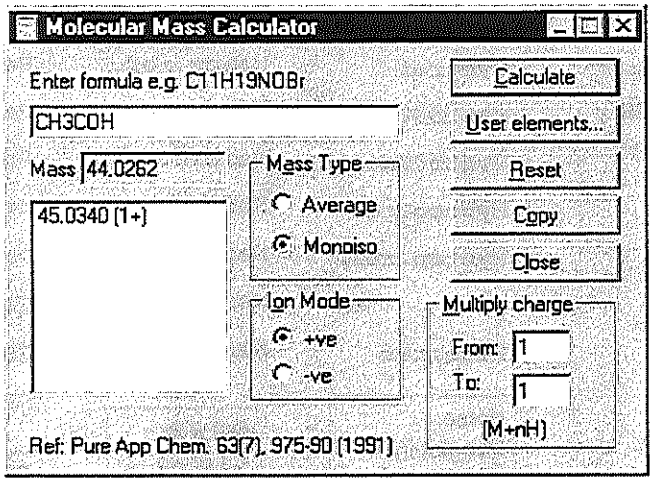
Mixture of PEG 200/300/400/600/1000 at 2/2/4/8/16 ng/µL respectively in 2mM Am. Acetate/Acetonitrile



"100's" are Intensity Weighting Factors Not Used with LC/MS Systems

3_5_002F

Adding a reference mass for PEG fragments



3_5_002F

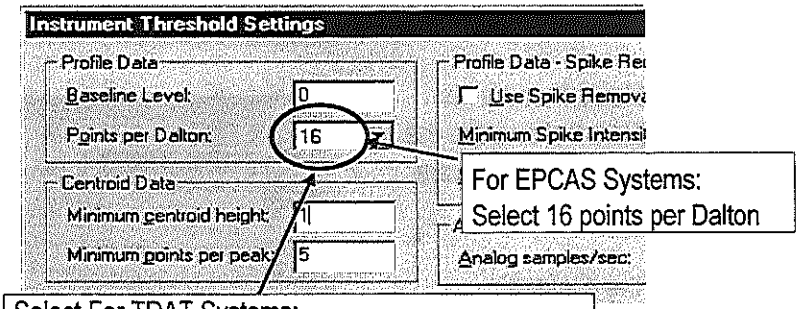
Mass Calibration Overview

- Select and prepare Reference Solution
- Setup Syringe/Pump and Correct Probe
- Remove any Existing Software Calibration
- Tune Mass Spectrometer for Maximum Sensitivity to Ions in the Reference Solution
- Check Parameters to be Used in the Mass Calibration
- Start Acquisition / Mass Calibration Procedure
- Review and Edit (if needed) the Calibration

3_5_002F

Step 1: Setting up the Instrument for Calibration

Check Sampling Rate: On the instrument tune page, select the 'Options'/'Set Instrument Threshold' menu item to display this dialog box. (On TDAT systems it is on the Acquisition Control Panel).



Select For TDAT Systems:

16 points/Dalton to scan at 500 amu/sec

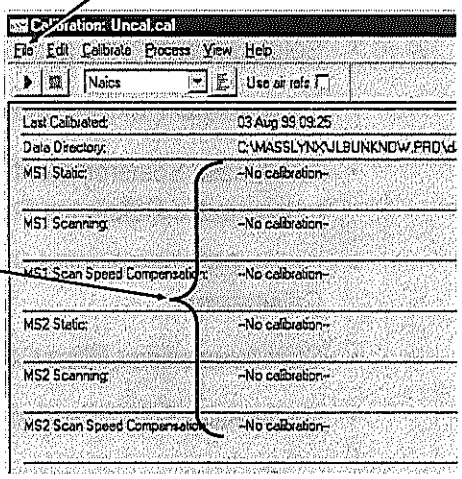
8 points/Dalton to scan at 1000 amu/sec

3_5_002F

Step 2 –

Check Calibration - Open the Calibration Dialog Box and Make Sure You are Starting with No Calibration

The Calibration 'Uncal' should be a blank calibration (no software calibration) which you can load in (use "File/Open" menu item).



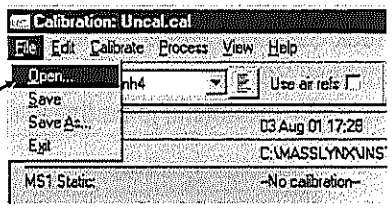
It is important that when a calibration is performed that the Mass Spectrometer **Does Not** have a Software Calibration already in place.

3_5_002F

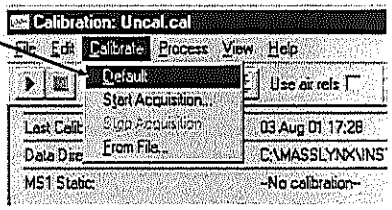
Check Calibration (cont)

If There is a Software Calibration in Place either:

Load the 'Uncal' calibration using 'File'/'Open'



Or Use the 'Default' menu item and use 'File/Save As' to store the default and get rid of the software calibration.

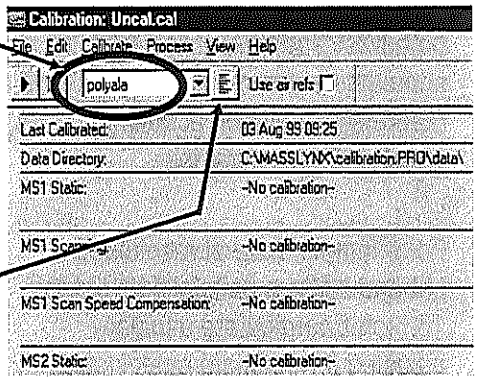


Do not 'Save' the 'Default' calibration using the same name as the current software calibration. Enter another name.

3_5_002F

Step 3 - Select Proper Reference File

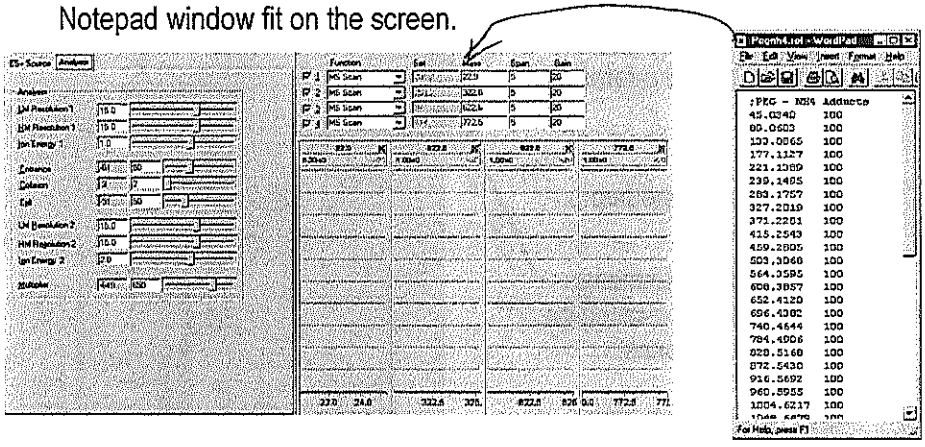
- Choose the correct reference file for the reference solution you are using for calibration by using the 'drop-down' menu.
- You may will probably need to consult this file during the calibration process. You can display this reference file with 'Notepad', then . . .



3_5_002F

Step 3 (cont) : Have a Copy of the Reference File Handy

- Resize the 'Notepad' window so both the Tune Page window and the Notepad window fit on the screen.



- Alternatively you can print out a copy of the reference file from "Notepad".

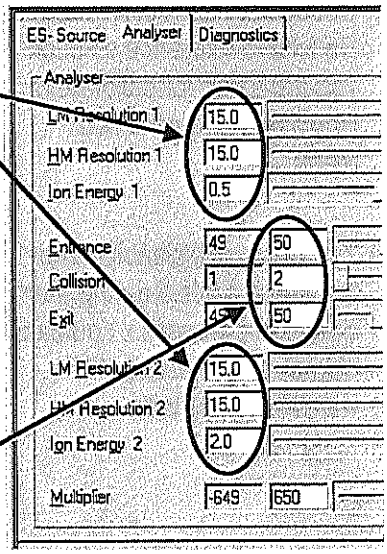
3_5_002F

Step 4 - Setup the Analyzer for Calibration

- Tune page resolution settings for both MS1 and MS2 (LM, HM & IE) should be set to match settings normally used when acquiring spectra of your samples.

  For example: for unit resolution, LM and HM are usually both set to 15. Ion Energies are usually adjusted for optimum peak shape.

- Collision Cell settings should be set for full scan acquisition.



3_5_002F

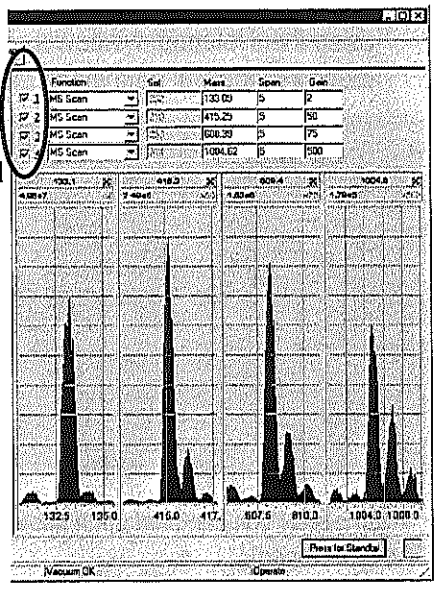
Step 5 - Tuning the Mass Spectrometer

- The mass spectrometer will be tuned by optimizing sensitivity to the peaks produced by the reference solution.

- Start the syringe pump.

- Enable all four functions for MS Scan by clicking on the left-most boxes.

  This will allow you to evaluate four different peaks simultaneously.



3_5_002F

Step 5 (cont.) - Viewing four peaks simultaneously

	Function	Set	Mass	Span	Gain
☑ 1	MS Scan	252	133.09	5	2
☑ 2	MS Scan	210	415.25	5	50
☑ 3	MS Scan	253	608.39	5	75
☑ 4	MS Scan	51	1004.62	5	500

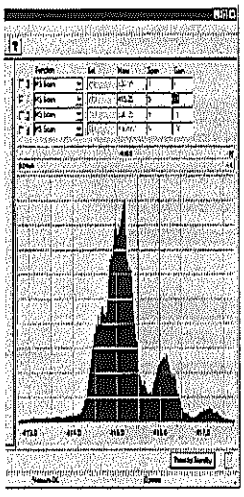
- Select four peaks representative of the range being calibrated and enter them into the *Mass* column. Make sure you check a mass near the top and bottom of the mass range over which you plan to calibrate.
- For each peak, set the Span to 5 and enter an appropriate Gain value to view the peak.

3_5_002F

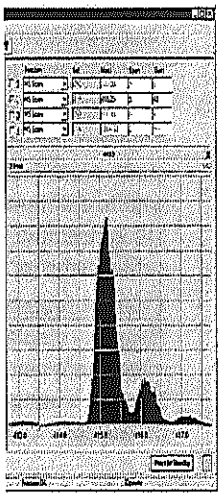
Step 5 (cont.) - Peak Optimization

- Optimize the Capillary and the Cone Voltage as best possible for all peaks. (sensitivity and interference removal)
- Remember that the absolute optimum conditions for one peak may completely obliterate another....the goal here is to find conditions amenable to all peaks.
- Also check the most intense peak in the reference solution to make sure the system is not saturated.

Cone = 25V

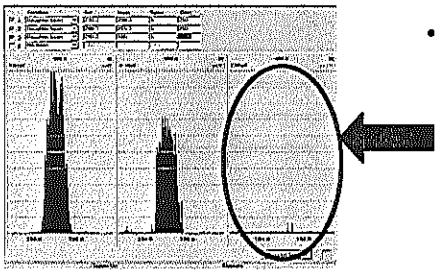


Cone = 50V

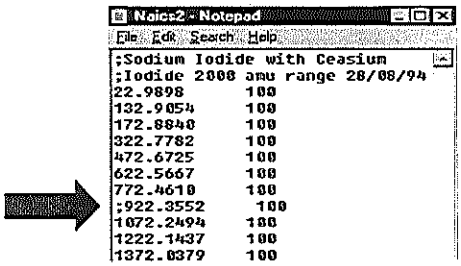


3_5_002F

Step 6 - Editing the Reference File



- If you cannot find certain peaks, they can be 'commented' out of the Reference File by placing a semi-colon in front of the peak. This peak will then be excluded from the calibration.

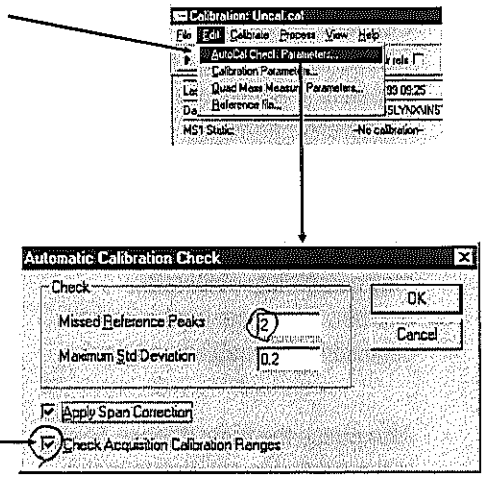


- Be sure to save (File, Save) the Reference File. This is especially important if you have commented out specific peaks.

3_5_002F

Step 7 - Setup Instrument Calibration Parameters - AutoCal Check

- *From the Calibration window . . . .*
- *Missed Reference Peaks -* number of consecutive (*not* total) peaks allowed to miss before failing calibration.
- *Apply Span Correction -* An extra correction that helps if mass scale that you are working with is different to the one that the instrument was originally calibrated over.
- *Check Acquisition Calibration Ranges -* displays a reminder message when a user attempts to run a sample outside the instrument calibration range.

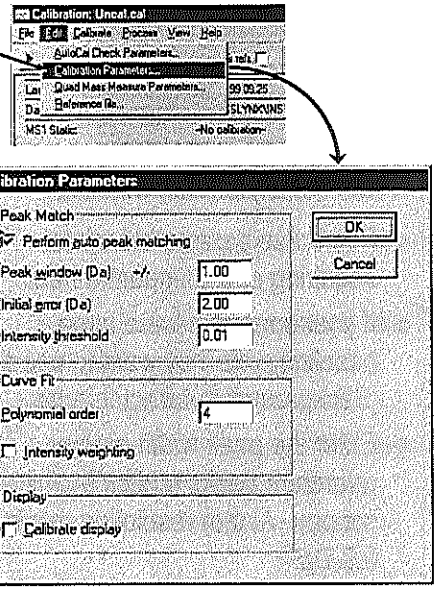


3_5_002F

Step 8a - Setup Calibration Parameters

From the Calibration window:

- ***Auto Peak Match*** – Perform auto peak matching Check the box to enable the software to automatically match peaks in the reference file with those in the acquired file.
- **Peak Window –** Specifies the maximum allowable difference between the peaks in the reference file and corresponding peaks in the acquired file.
- ***Initial Error*** –Tries to match the most central peak in the calibration range within a 2 Da window.

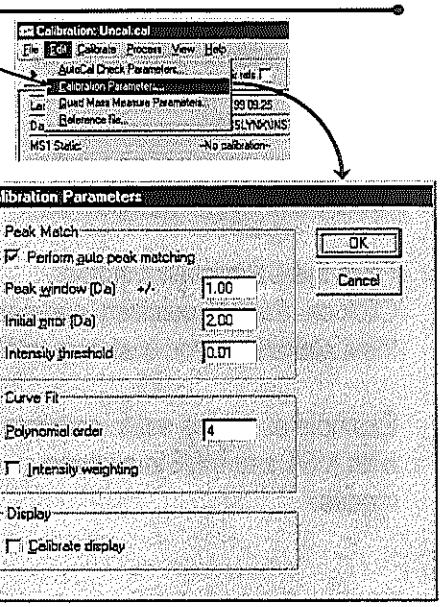


3_5_002F

Step 8b - Setup Calibration Parameters

From the Calibration window:

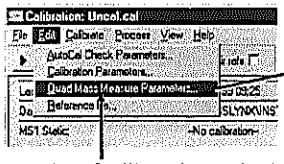
- ***Intensity Threshold*** – Usually set to 0.01. (Will ignore peaks below 0.01% of base peak.)
- ***Polynomial Order*** - Mathematical expression for the curve. For quadrupoles, it is usually either 4 or 5.
- ***Intensity Weighting*** - Only used in EI mode (EI gives reproducible fragmentation patterns).

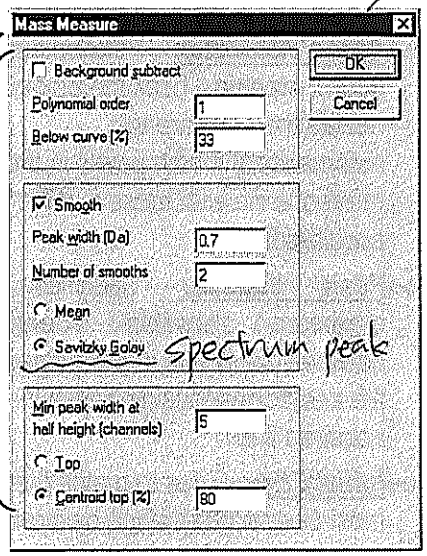


3_5_002F

Step 9 - Setup Instrument Calibration Parameters - Mass Measure

recommend option.





spectrum peak

From the Calibration window:

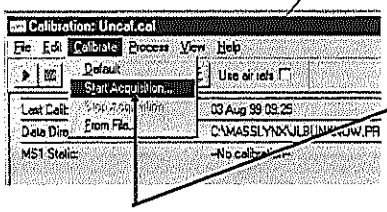
- Use these parameters when acquiring in Continuum mode.
- If your spectra have a large baseline, you may want to use the 'Background' subtract feature.
- Enter the peak width of your reference peaks (check on the scope). At unit resolution this is 0.7 Da.

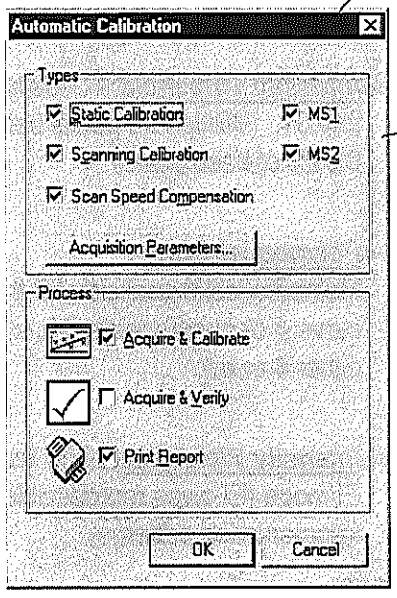
3_5_002F

Step 10 - Start Calibration Acquistion

recommend option

check all





From the Calibration window,
Click on 'Start Acquisition' and select the Calibrations you wish to perform.

Example at right is set to calibrate both MS1 and MS2 in all three modes.

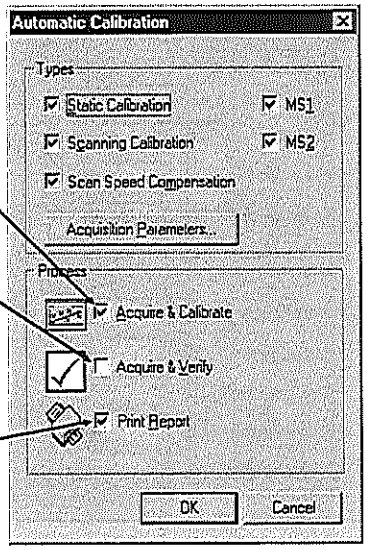
3_5_002F

Step 10 - Start Calibration Acquisition (cont.)

Acquire & Calibrate – Spectra will be acquired for each of the selected modes. The masses obtained in the spectra will be compared against those in the reference file. A calibration will be performed and applied to the mass spectrometer.

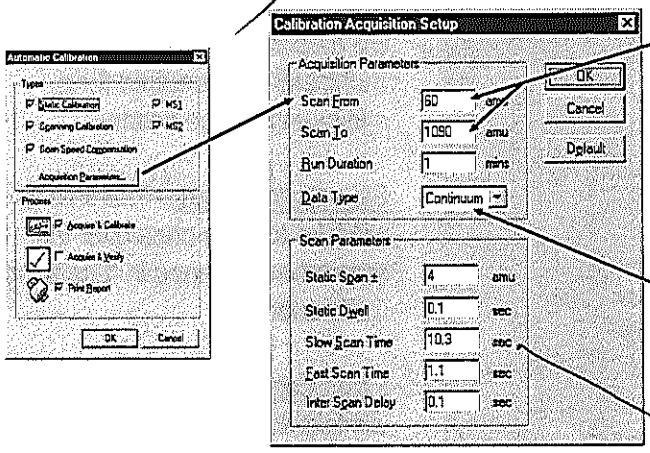
Acquire & Verify – Spectra will be acquired for each of the selected modes. The masses obtained in the spectra will be compared against those in the reference file to check the calibration currently applied to the mass spectrometer.

Print - Click here to get a printed report.



3_5_002F

Step 11 - Setup Calibration Acquisition



Mass Range – Enter the mass range over which the calibration is to be performed.

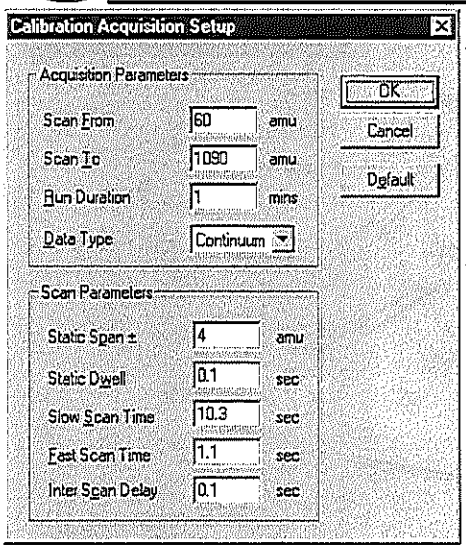
Continuum Scan is normally used

19.8

(Choose the mass range so the low end is just below (5-20 amu) the lowest ref mass and the high end is just above the highest ref mass)

3_5_002F

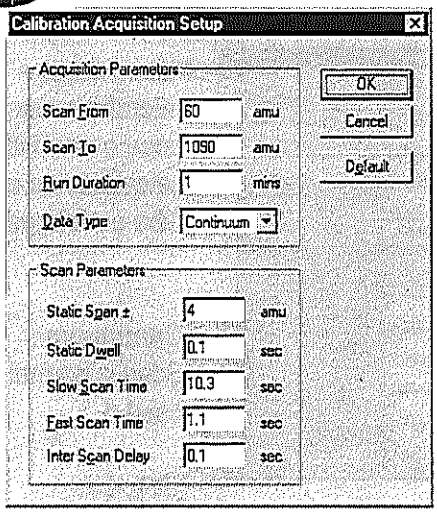
Step 11 - Setup Calibration Acquisition (cont.)



- Static Span - during static calibration, the quadrupole will park on the expected masses (as listed in the Reference File) and look for the peak within the static span window above and below the mass (here ±4 amu).
- Static Dwell - how long the quadrupole will sit on each mass
- Inter Scan Delay - defines the time between the completion of one scan and the initiation of another a value of 0.1 s is sufficient.
- Typical values are shown for the 'Static Span', 'Static Dwell' and 'Interscan Delay'.

3_5_002F

Step 11 - Setup Calibration Acquisition (cont.)



Note for this example, that a 'Slow Scan Time' of 10.3 seconds means:

60/10.4 = 5.8 scans

At least 3 slow scans are preferable.

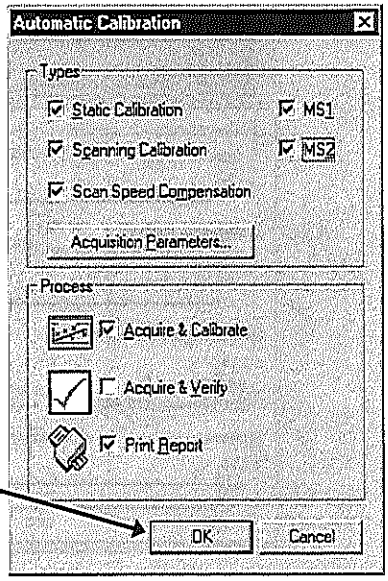
Run Duration – Total time data will be acquired for each mode. Sufficient time required for a minimum of three scans.

Slow Scan Speed – The time it will take to scan once over the entered mass range in 'Scanning Calibration'

Fast Scan Speed – The time it will take to scan once over the entered mass range in 'Fast Scan Compensation'.

Step 12 - Acquiring for Calibration

- After all the parameters (mass range, run duration, etc.) are entered, Click *OK* and you will return to window shown to the right.
- This brings you back to the Automatic Calibration window (shown to the right), Click *OK* to begin acquisition



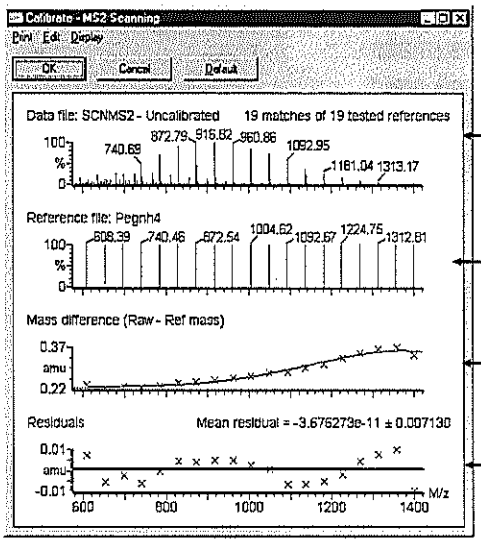
3_5_002F

Step 13 – Check Calibration Reports

- If you have selected the 'Print Report' option, MassLynx will print out a report on each of the calibrations (MS1 Static, MS1 Scanning, etc.) as they are completed.
- Check the reports to see how the calibration is proceeding. An example of a report is shown on a following page. The report contains:
  - –1 Acquired Spectrum
  - –2 Reference Peak Masses
  - –3 Differences between masses of acquired peaks and the masses listed in the reference file. The calibration line is also shown.
  - –4 Residuals Deviations of the acquired peaks from the calibration line.

3_5_002F

The Calibration Report



3_5_002F

Acquisition of Spectra for Calibration

For each Calibration type a spectra will be acquired and stored in a '.raw' file.

The following file names will be used for these spectra:

Static MS1 = StatMS1.RAW
Static MS2 = StatMS2.RAW

Scanning MS1 = ScnMS1.RAW
Scanning MS2 = ScnMS2.RAW

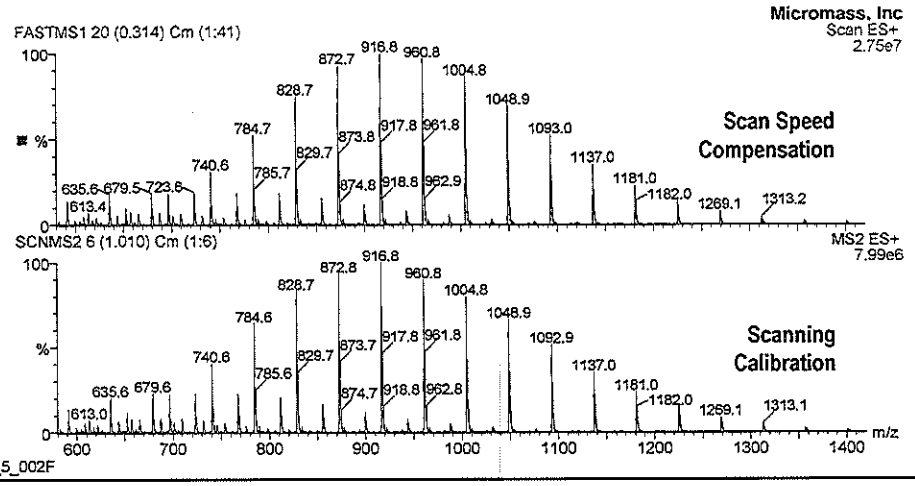
Scan Speed Compensation MS1 = FastMS1.RAW
Scan Speed Compensation MS2 = FastMS2.RAW

More on this later:

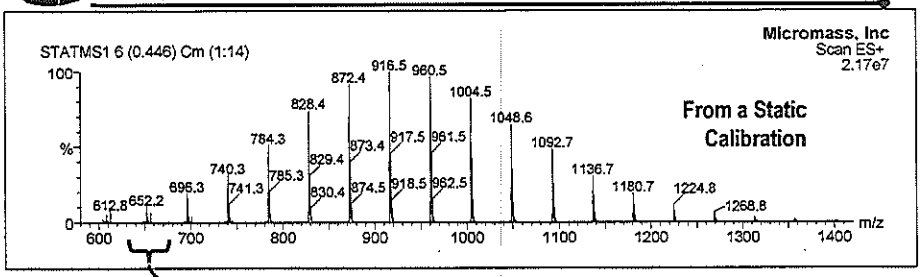
3_5_002F

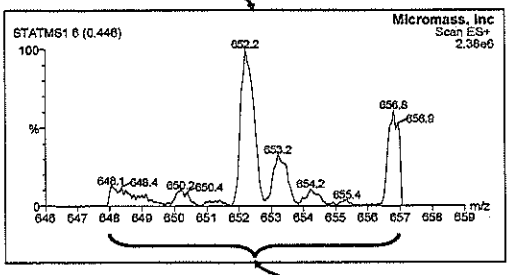
Examples of spectra acquired for a calibration

For the Scanning Calibration (SCNMS1 or SCNMS2) or the Scan Speed Compensation (FASTMS1 or FASTMS2) spectra are acquired at the scan speeds specified on the 'Acquisition Parameters' dialog box.



Example of spectrum acquired for a calibration





For the static calibration, spectra are acquired only for a small mass range around each peak.

For the 652 peak, spectra measured only over the range from 648 to 657 Da

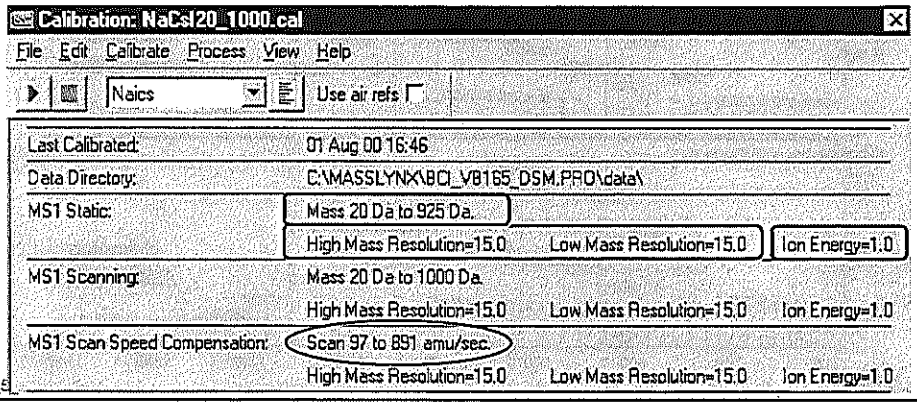
3_5_002F

Step 15 - Checking the Calibration

Once a calibration has been successfully performed, the screen will look like this. Given for each calibration will be:

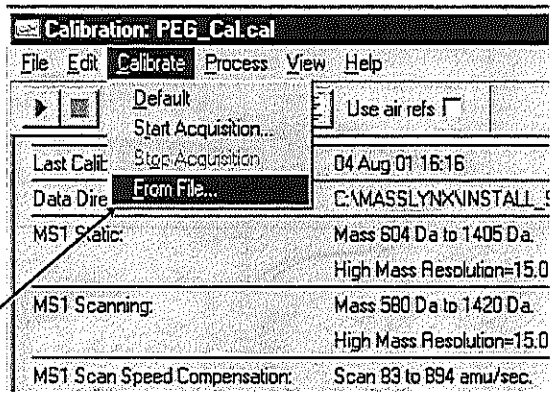
Mass Range Resolution Ion energy

Also the slowest and fastest scan rates that this calibration is valid for are given.



3_5

Step 16a - Reviewing Calibration



- To review the curves for each type of calibration, click on the *'Calibrate, From File'* menu item.

3_5_002F

Step - Browsing in files for Viewing Curves

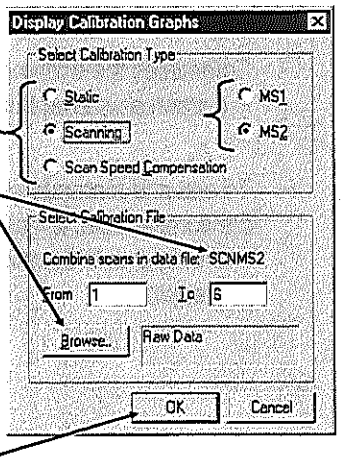
To view curves:

1) Select which calib type (Static, Scanning, Scan Compensation) and which quad (MS1 or MS2)

2) 'Browse In' the a file:

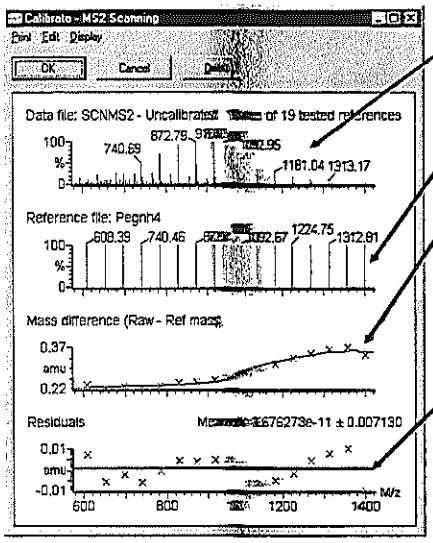
MS1 = StatMS1.RAW
MS2 = StatMS2.RAW
MS1 = ScnMS1.RAW
MS2 = ScnMS2.RAW
Scan Speed Compe MS1 = FastMS1.RAW
Scan Speed Compe MS2 = FastMS2.RAW

3) Click on 'OK' and owing calibration report should ap



3_5_002F

Step - Viewing Calibration Results & Curves



1) Peaks found in the infused reference solution.

2) Reference File peaks.

3) Difference between the mass found and the mass in the Reference File along with the calibration line.

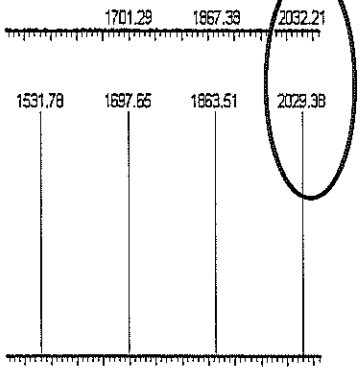
4) Residuals. Deviation of the mass differences from the calibration line.

3_5_002F

Step 16d – Editing Calibration

The peaks here are such low intensity that they cannot be seen until you zoom into this area.

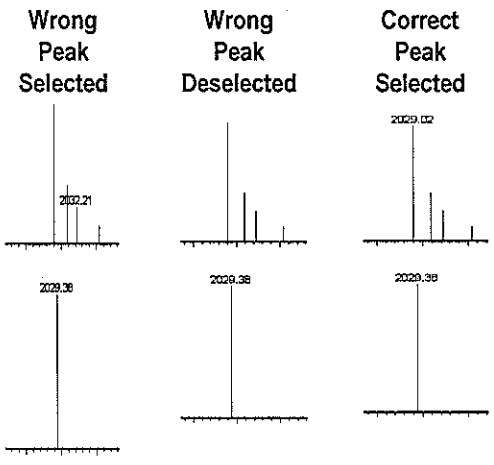
- Sometimes the wrong peak is chosen as shown here.
- To edit and manually choose the correct peak, left click and drag over the peak of interest.



3_5_002F

Step 16e - Editing the Calibration

- Use 'left click' and drag to magnify the region of interest.
- 'Right click' on the incorrectly detected peak to deselect it.
- 'Right click' on the correct peak to select it and then click *OK* and the calibration will include the change.



3_5_002F

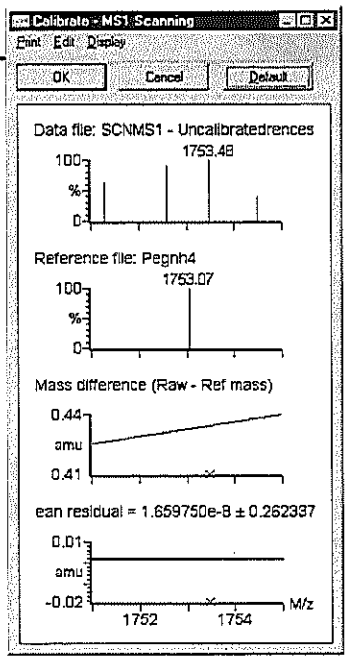
Split Peaks

If you end up with a situation similar to that shown on the right where you have two peaks to choose from where one is too high and one is too low while the rest of your peaks fall pretty close to the line, you will have to either:

1) Try and increase the peak signal and repeat the calibration

2) Use neither one of the peaks

3) Try increasing the peak width used in by the mass measure procedure.



3_5_002F

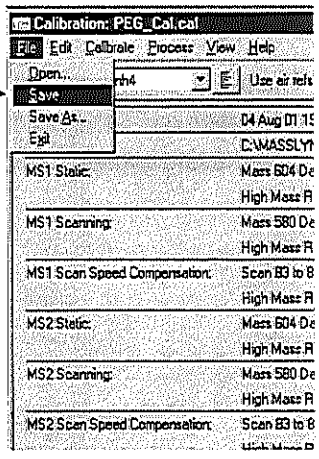
Step 17 - Save the Calibration

If the calibration is successful make sure you save the calibration

It is best to not save the calibration under the name 'Uncal' (use 'Save As').

The calibration is now the one in place on the mass spectrometer.

'Saved' calibrations can be used at a later date by loading them back into the system using the 'File'/'Open' menu item.



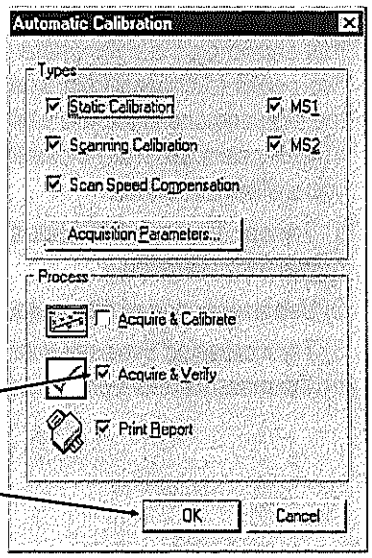
3_5_002F

Step 18 - Verifying a Calibration

A calibration can also be verified.

With the calibration that is to be verified in place on the mass spectrometer, use the same setup as that used for the calibration. Note that the same resolution and ion energies should be used.

From the acquisition dialog box, select the 'Acquire & Verify' option and click on 'OK'.



3_5_002F

Step 18 - Verifying a Calibration - Filenames

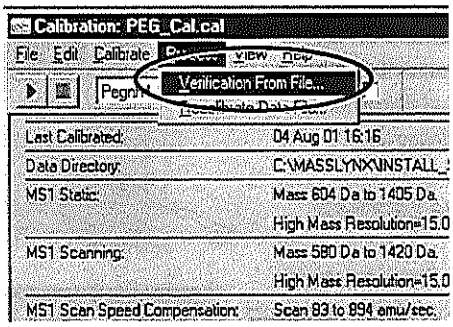
Spectra will be acquired and peak masses compared against reference file masses just like in the calibration procedure.

The mass differences will be reported to see how good the current calibration is. The spectra acquired for the verification process will be stored using slightly different filenames.

Static MS1 =	StatMS1V
Static MS2 =	StatMS2V
Scanning MS1 =	ScnMS1V
Scanning MS2 =	ScnMS2V
Scan Speed Compensation MS1 =	FastMS1V
Scan Speed Compensation MS2 =	FastMS2V

3_5_002F

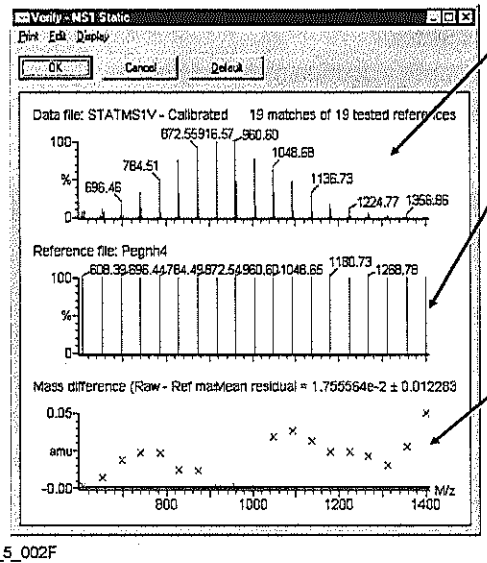
Step 19 - Reviewing a Calibration Verification



- If it is necessary to review the curves for each type of verification, then from the window menu, click on *'Process, Verification From File'* and 'browse in' the appropriate file. (Similar to reviewing a calibration as shown in Step 16b.)

3_5_002F

Verification Report is Similar to a Calibration Report



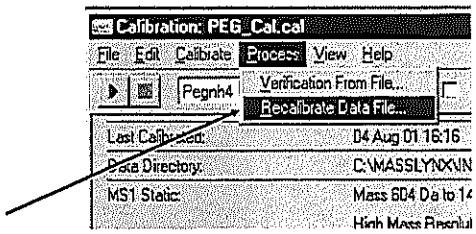
- The top graph shows the peaks found in the infused reference solution.
- The middle graph shows the Reference File peaks.
- The lower graph shows the difference between the mass found (acquired using the calibrated system) and the mass in the Reference File. The mass differences should be less than 0.1 amu.

3_5_002F

A Calibration Can Be Applied to Previously Acquired Data

Data that was acquired on an uncalibrated instrument can be corrected using a calibration performed at a later time.

Select 'Recalibrate Data File'.



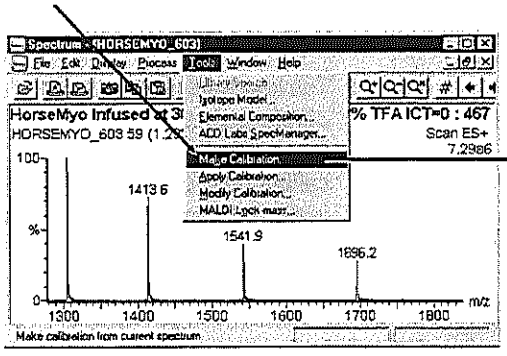
The dialog box at the right should appear. 'Browse In' the file needing a calibration and click on okay. You will have to find the appropriate '.raw' file directory in the 'Data' directory of the appropriate project.

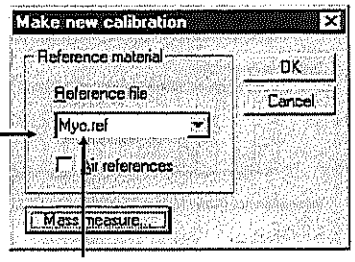
In the above example, the 'PEG_Cal' calibration will be applied to the selected data file.

3_5_002F

A Calibration Can be Made Using A Previously Acquired Spectrum

An acquired spectrum of a reference compound can be used to create a calibration that can be applied to other spectra.
In the 'Spectrum' window display the acquired spectrum of the reference compound and select 'Tools'/'Make Calibration'



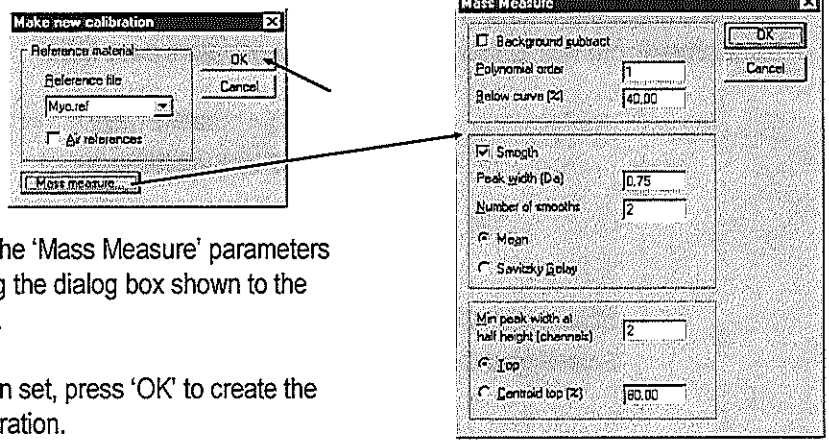


Make sure proper reference file is selected.

3_5_002F

Calibration from a Previously Acquired Spectrum (cont.)

'Mass Measure' parameters should be set in a manner similar to that used in the full calibration.



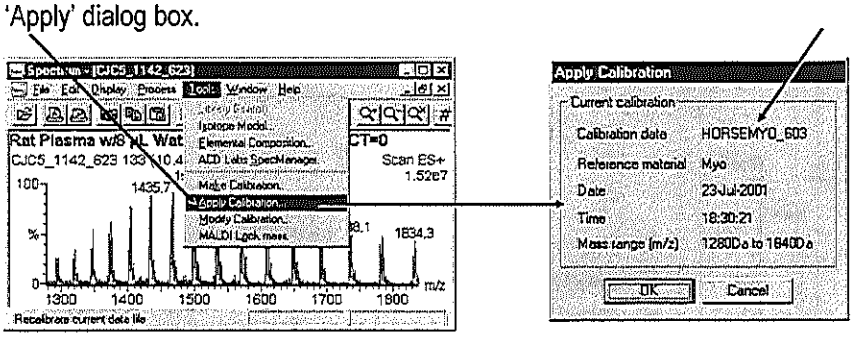
Set the 'Mass Measure' parameters using the dialog box shown to the right.

When set, press 'OK' to create the calibration.

3_5_002F

Calibration from a Previously Acquired Spectrum (cont.)

To apply the calibration just created, display in the 'Spectrum' window the spectrum you wish calibrated and select 'Tools'/ 'Apply Calibration'. Note the name of the file used to create the calibration should be displayed in the 'Apply' dialog box.



This procedure should only be used when both files were acquired using exactly the same mass spec settings (LM, HM, IE, mass range, scan speed, etc.).

3_5_002F

Save Calibration Spectra for Later Practice

It may be a good idea to save/move the calibration data folders off into another folder for practice later. For example, you can load 'Uncal' and practice changing the mass measure parameters. Then choose the 'Recalibrate From File' option and select one of your saved files.

Static MS1 = StatMS1.raw
Static MS2 = StatMS2.raw

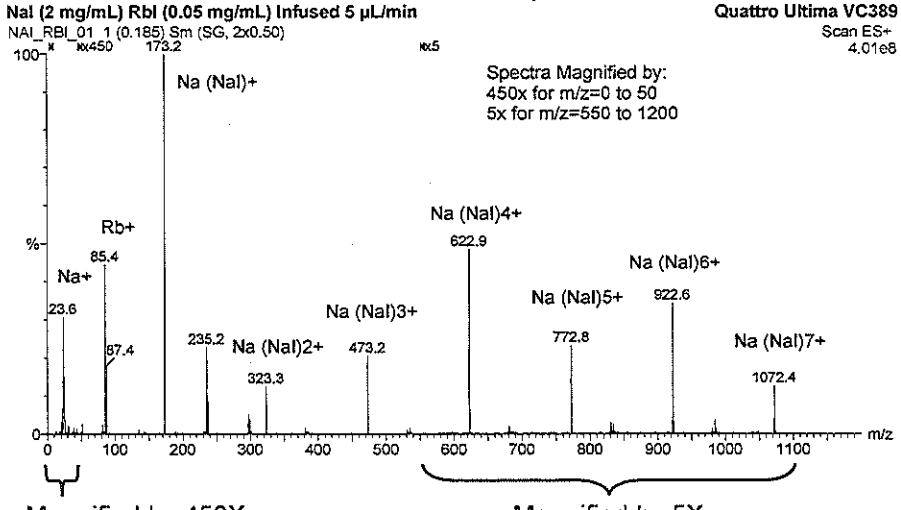
Scanning MS1 = ScnMS1.raw
Scanning MS2 = ScnMS2.raw

Scan Speed Compensation MS1 = FastMS1.raw
Scan Speed Compensation MS2 = FastMS2.raw

3_5_002F

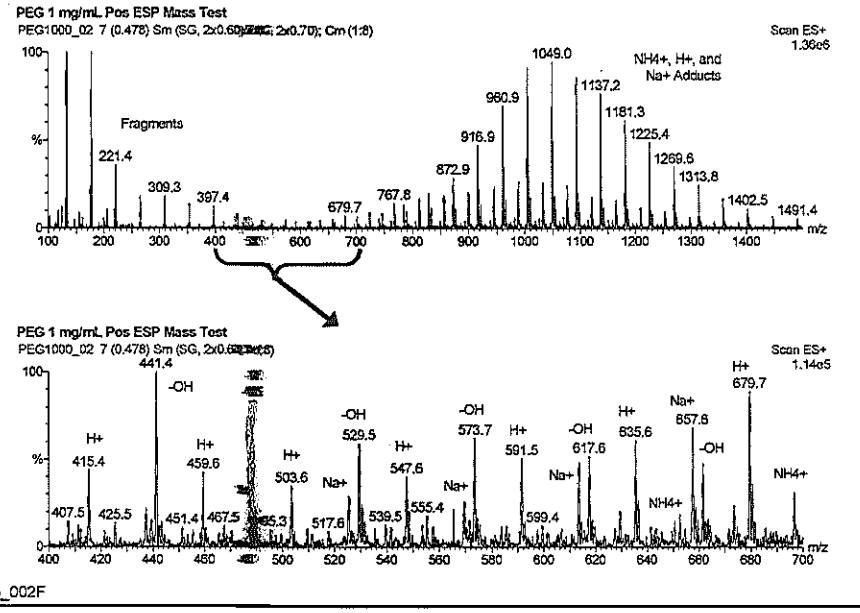
Example of NaI RbI Spectrum

2 mg/mL NaI with 0.05 mg/mL RbI in 50/50 IPA/Water
Infused at 10 µL/min



3_5_002F

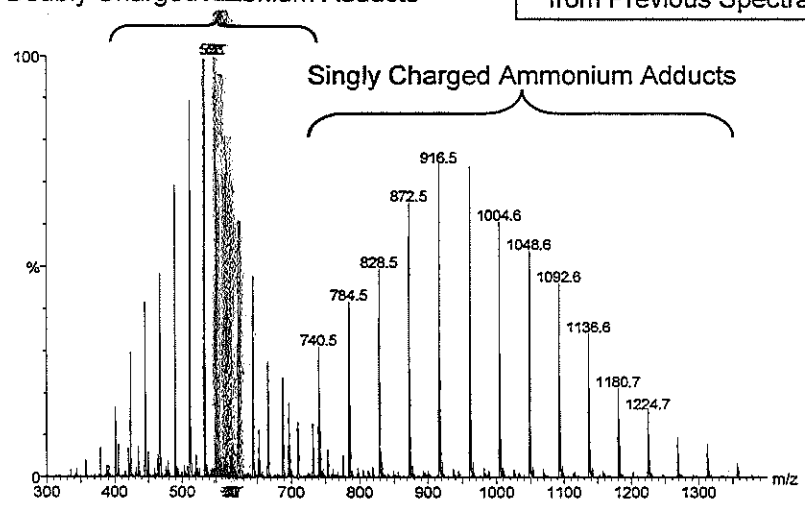
Example of PEG 1000 Spectrum



3_5_002F

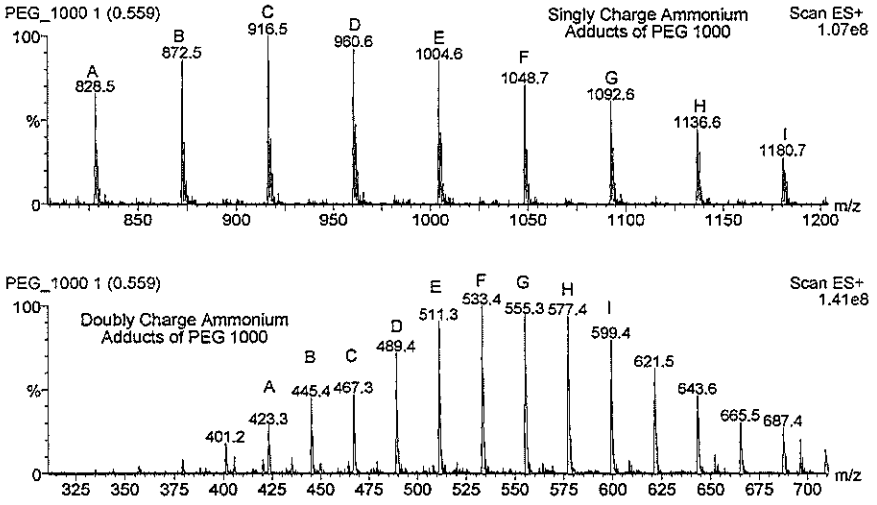
PEG1000 with Ammonium Acetate

Different Cone Voltage from Previous Spectra



3_5_002F

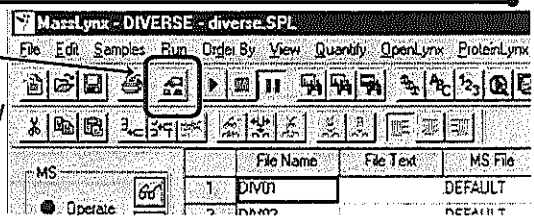
PEG 1000 with Ammonium Acetate Spectrum from m/z=800-1200 (top) and m/z=310-710 (bottom)

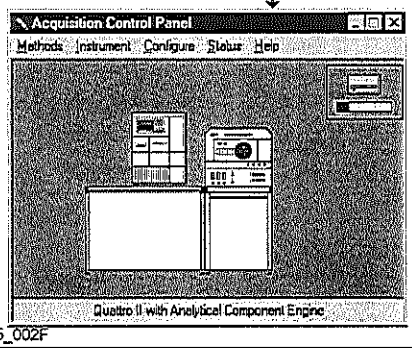


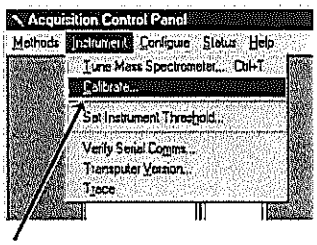
3_5_002F

To Display the Calibration dialog box on TDAT Systems:

1) Click on the Acquisition Control Panel Button on the MassLynx Sample List Window and the Acquisition Control Panel should appear.



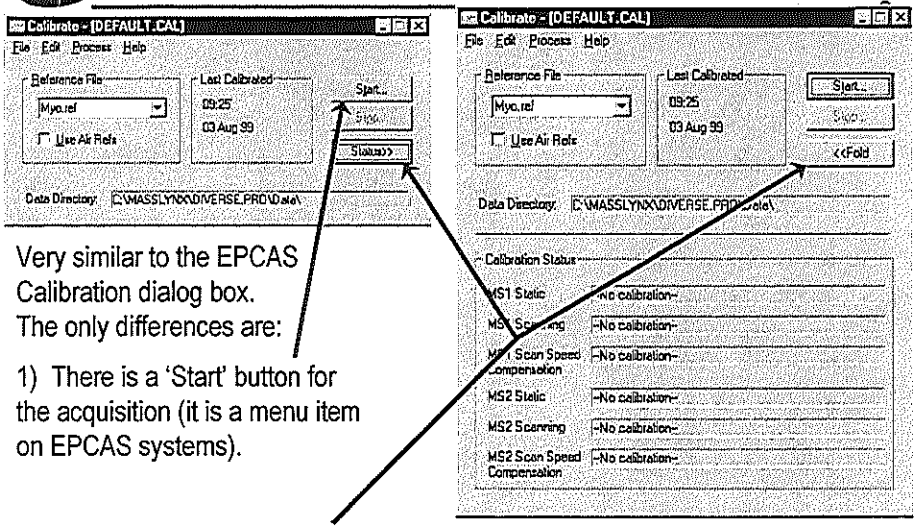




2) From the Control Panel, select the menu item: "Instrument/Calibrate".

3_5_002F

Calibration Dialog Box for TDAT Systems



Very similar to the EPCAS Calibration dialog box.
The only differences are:

1) There is a 'Start' button for the acquisition (it is a menu item on EPCAS systems).

2) There is a 'Fold/Status' button to provide 'Compact' and 'Expanded' view of the dialog box.

3_5_002F