# AssureNMR

- Screening Software and System Suitability Test

User Manual

Version 2.1 003

Innovation with Integrity

NMR

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P/N: B7150A2-10

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This license agreement is also available as a PDF file:

*<TopSpin installation directory>/classes/doc/English/pdf/topspin_license.pdf*

# 1 Introduction

The AssureNMR software package is a diverse collection of tools for streamlining analysis by NMR (nuclear magnetic resonance) spectroscopy. AssureNMR has two modules: AssureSST (System Suitability Test) and AssureNMR. The SST module is designed to monitor and maintain instrument performance. SST runs a set of user-selected tests, compares the results to user-defined specifications, and generates a dated summary report. AssureNMR is the suite of tools for analyzing spectra. AssureNMR automation simplifies data acquisition and analysis using parameter sets and analysis methods customized for the material of interest, from purified raw materials to natural products to biofluids. Reference NMR spectral databases (SBASEs) can be used in the software to identify mixture components. Reference SBASEs of common chemicals are included with the AssureNMR software. Users may add an unlimited number of compounds to these databases. Additionally, the software can detect and report the presence of unknowns in the sample. Reports designed for both non-technical and advanced users are automatically generated to allow quick assessment of the results and to provide a permanent record of the analysis. AssureNMR may be used for pharmaceuticals, fine chemicals, polymers, dietary supplements, biofluids, food products, petroleum and many other materials. The AssureNMR software package allows for the testing and detection of any compounds containing NMR active nuclei (including $^{1}H$, $^{2}H$, $^{11}B$, $^{13}C$, $^{19}F$, $^{29}Si$ and $^{31}P$) using standard NMR techniques. AssureNMR interfaces with the Bruker TopSpin software package, making use of IconNMR for automation.

AssureSST and AssureNMR are flexible enough to be used in production or research facilities. AssureNMR automates data acquisition, analysis, and reports so that non-NMR spectroscopists can use NMR for testing. AssureNMR and SST use predefined procedures, making it easy to stay within standard operation procedures (SOPs). Therefore, these tools can be used to meet GLP requirements. AssureNMR's novel application of NMR to screening has been awarded a patent:

Kimberly L. Colson, Joshua M. Hicks, Christian Fischer "Method and Apparatus for Automated Raw Material Screening" United States Patent 8,248,072.

## 1.1 AssureSST (System Suitability Test) Highlights

**Summary of Features**

- The System Suitability Test runs acquisition and analysis of NMR standard tests in automation. Available tests include: $^{1}H$ lineshape, $^{1}H$ sensitivity, $^{13}C$ sensitivity, $^{19}F$ sensitivity, $^{31}P$ sensitivity, and temperature calibration.
- The System Suitability Test can also run acquisition and analysis of user-defined tests in automation. The tests can be either lineshape tests or sensitivity tests.
- Shim sets from successful $^{1}H$ lineshape experiments are stored and recalled as the starting shim set for queued samples. This reduces routine maintenance of the spectrometer shims.
- Automated stop criteria halt acquisition upon failure to meet specifications.
- Automated report generation documents the SST results.

**Software Design**

The AssureSST module was designed to monitor instrument performance on a regular basis. This is achieved via IconNMR, which runs experiments to test performance and temperature regulation periodically as specified by the user. The AssureSST module can work either in a standalone mode where the user can use the normal IconNMR submission interface or in conjunction with the AssureNMR module. If AssureSST determines that the system does not meet specifications, data acquisition through IconNMR or AssureNMR will halt until all specifications are met.

## 1.2 AssureNMR Highlights

**Summary of Features**

- AssureSST is used by AssureNMR to check instrument performance.
- Automated data acquisition is accomplished with IconNMR through user-defined parameter sets.
- Automated qualitative analysis of spectra is based on NMR spectral databases, either supplied with the software or developed by the user.
- Qualitative analysis can also be based on statistical models including SIMCA and quantile plots, built by the user in AssureNMR.
- Tools for matching and analysis can be used interactively through the interactive analysis window.
- Quantification can be performed on various nuclei (including $^{1}H$, $^{2}H$, $^{11}B$, $^{13}C$, $^{19}F$, $^{29}Si$, and $^{31}P$).
- Quantification can be based on peak integration or partial least squares (PLS) regression.
- Absolute or relative concentrations can be determined.
- Calculations requiring averages over multiple spectra are available.
- Complete Reduction to Amplitude Frequency Table (CRAFT)[1] is available for use in quantification and targeted fingerprinting.
- HSQC spectra can be analyzed in automation.
- Automated report generation includes (1) a summary quality control report (QCReport.pdf) which typically reports a pass or fail result and (2) a detailed expert report (ExpertReport.pdf) which summarizes the details of the analysis.
- Custom reports are available.
- The software is GLP/GMP compatible.
- Security features can be customized.

**Software Design**

The AssureNMR and SST software is built on three components: (1) TopSpin, (2) IconNMR, and (3) the AssureNMR software itself, as shown schematically below. Successful completion of a System Suitability Test releases IconNMR for general sample submission. Spectra of submitted samples are collected with the acquisition and processing functions of TopSpin. The sample spectrum (or spectra) is (are) then passed to the AssureNMR software for analysis and generation of reports.

[1] K Krishnamurthy, Magn Reson Chem. 2013 51(12):821-9.

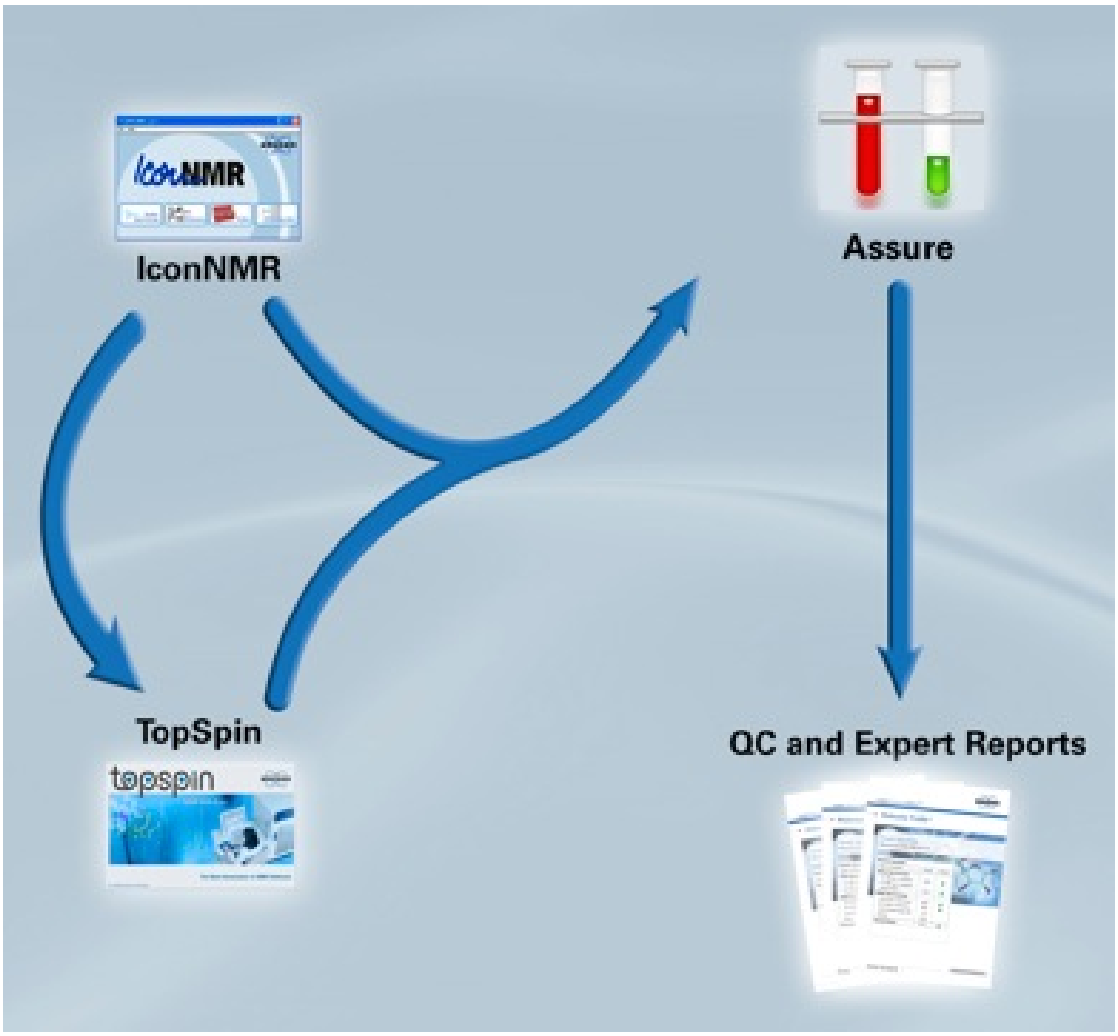



*Figure 1.1: AssureNMR software interaction with TopSpin and IconNMR.*

**Work Flow**

The system is designed to allow sample submission by a novice user. The novice user must only be able to prepare an NMR sample and submit the sample via the AssureNMR IconNMR interface. An experienced user is required to initially prepare the AssureNMR Method, SBASEs and statistical models that will be used for the material analysis and reporting as shown in the figure below. The following figure shows the progression of the software components. When utilized in full automation the workflow utilizes quantification calibration, an AssureNMR Method, data quality check, and spectral databases as shown in the last figure (“Automation Flowcharts and Components”).

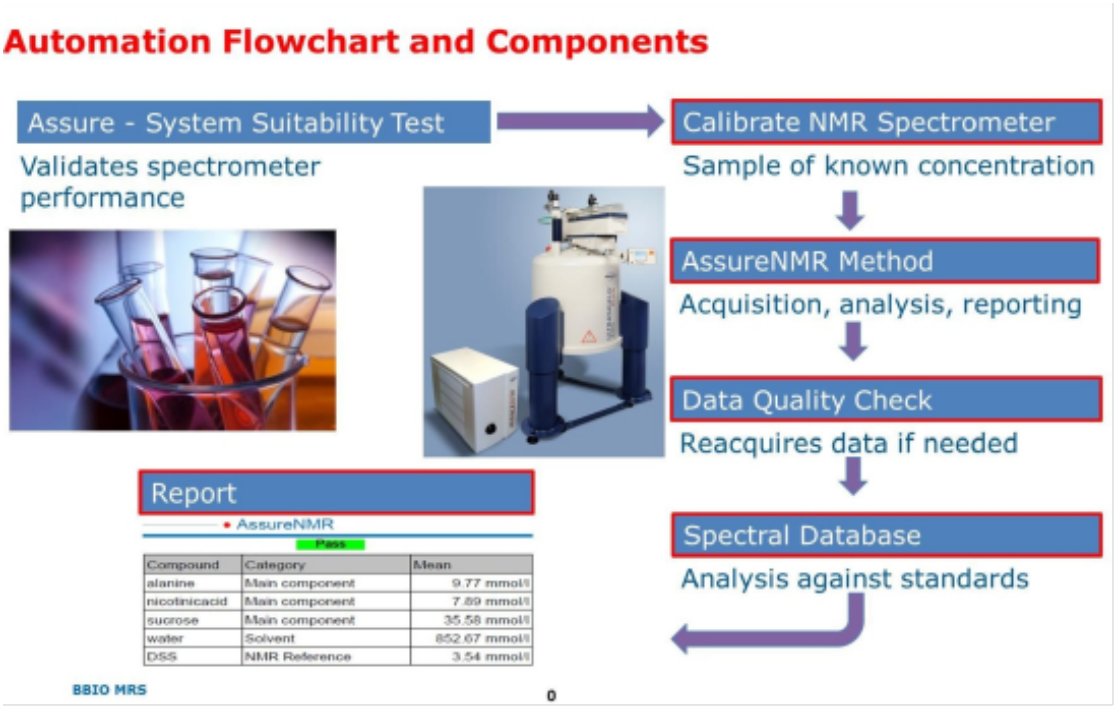



*Figure 1.2: Roles of the NMR savvy and NMR novice users when using AssureNMR for material validation.*

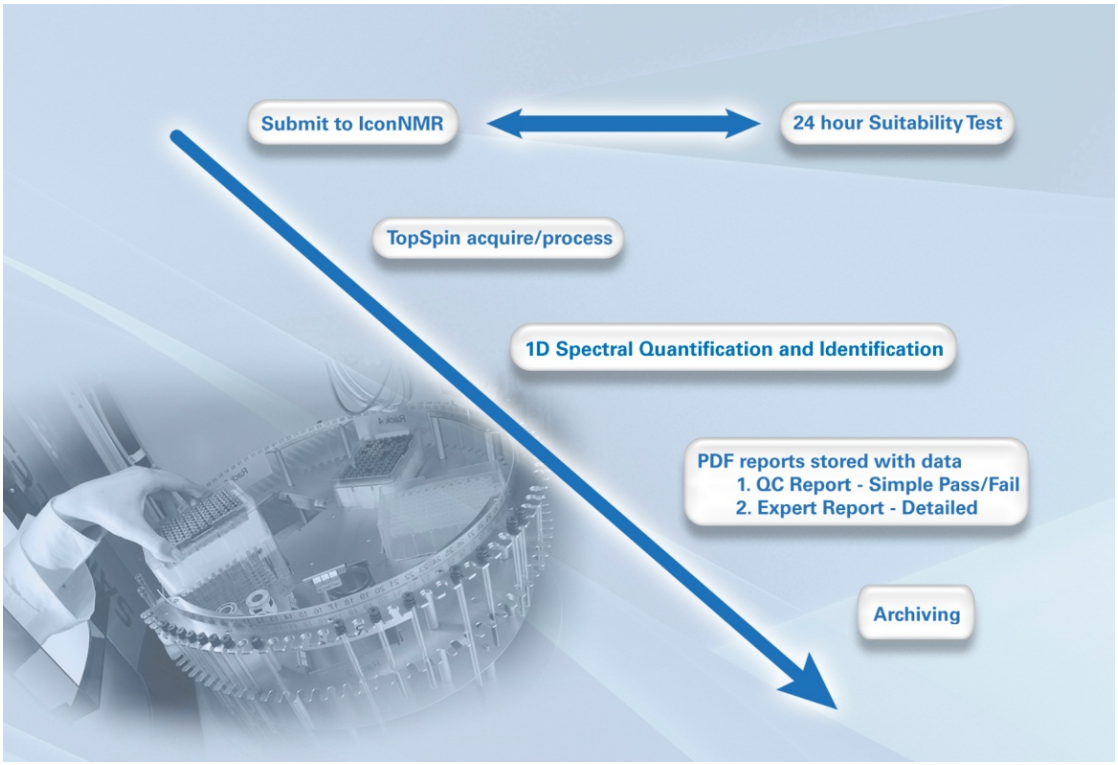



*Figure 1.3: Workflow of the AssureNMR software.*

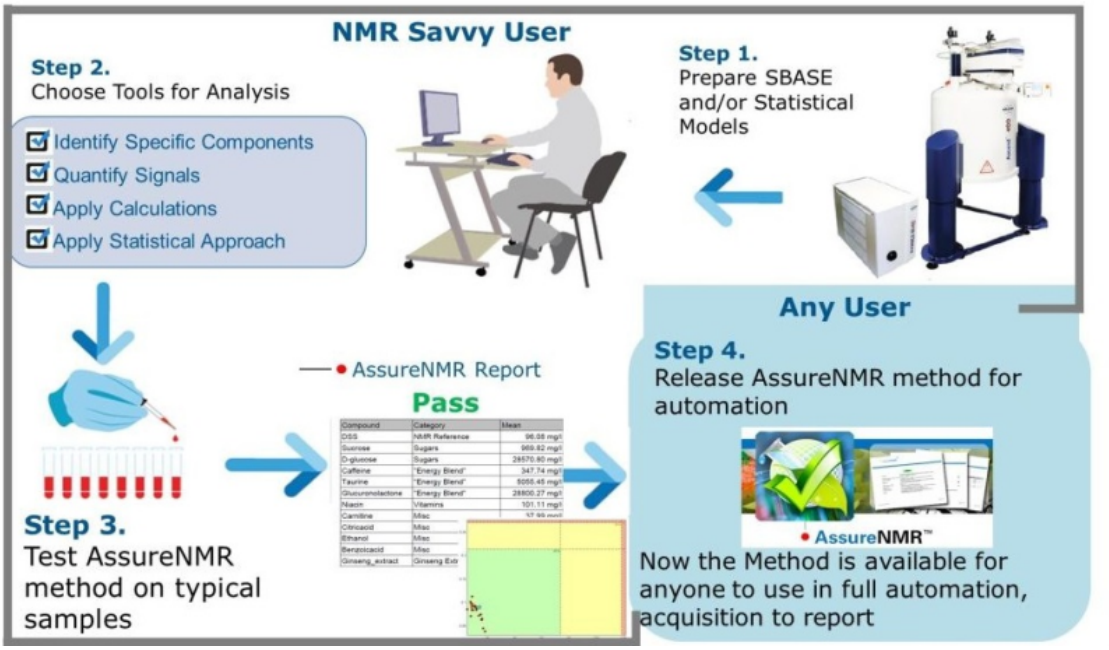



*Figure 1.4: Detailed workflow in full automation*

### User Interface

Using IconNMR, the acquisition interface can be tailored to the user. For example, the access-limited user will observe the simple submission interface shown in **Error! Reference source not found**.

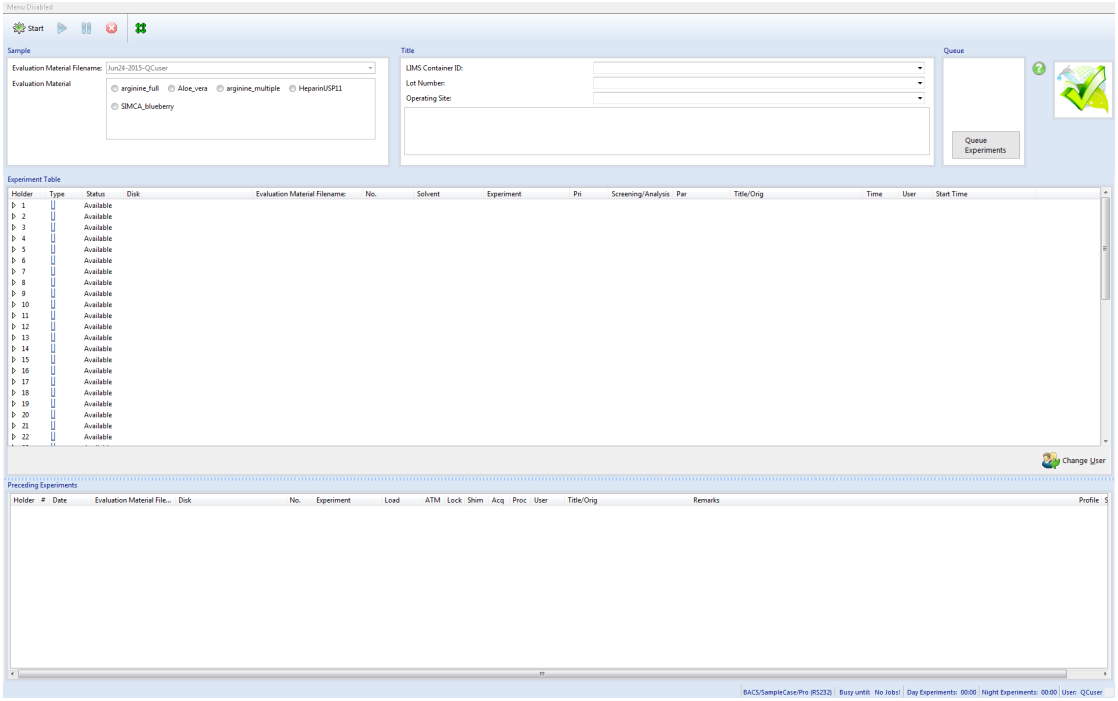



*Figure 1.5: IconNMR sample submission window for access-limited user.*

## 1.3 Example Data, Methods and Tutorials

To assist the user in learning how to use AssureNMR several datasets,SBASES, methods and tutorials are provided on the DVD. Example Data Sets will also be made available on the Bruker BioSpin software download site.

Refer to the following datasets for applications related to the following File Menu entries:

- MATCH
- QUANTIFY
  - ‘Poloxomer’ contains spectra of different poloxomers and an AssureNMR quantMethod for automated analysis of the spectra
  - ‘Polyvinylalcohol_1H’ contains spectra of different polyvinyl alcohol samples and an AssureNMR quantMethod for automated analysis of the spectra
- CHEMOMETICS
- PROFILE
  - ‘ProfileNMR’ contains spectra of reference, pass and fail antibodies. A standard spectrum and buffer spectrum are also included.

## 1.4 Requirements for AssureSST and AssureNMR 2.0

**Computer and Operating System:**

Operating System Requirements:

- Microsoft Operating System Windows 7 64-bit
- CentOS 7.1 64-bit

Current Software:

- TopSpin 3.5 pl6
- IconNMR 5.0 pl7
- AssureNMR 2.1

Software licenses are required; licenses may be obtained by contacting:

*license.NMR@bruker.com*

As of this writing, AssureNMR 2.1 was validated with the following software versions.

- TopSpin 3.5 pl6 that includes IconNMR 5.0.7 build 19

Please contact Bruker for an updated listing of validated software versions.

The software is provided in different bundles with different licenses:

- AssureSST (standalone)
- AssureNMR Launch for Component Identity (includes AssureSST, AssureNMR SBASE manipulation and matching tools, and AssureNMR SBASE Set 1)
- AssureNMR Ascent for Quantification and Automation (includes everything in AssureNMR Launch plus tools for quantification and analysis in automation plus the Residual Solvent SBASE (73 compounds) and AssureNMR SBASE Set 2 (60 additional compounds))
- AssureNMR Summit for Statistics (includes everything in AssureNMR Ascent plus chemometric tools, a Test Sample Set, and an additional desktop license)
- Aloe vera (method plus custom SBASE at 400 and 600 MHz)
- Heparin (method plus custom SBASE at 400, 500, and 600 MHz)

**Spectrometer hardware:**

- Avance III console or later

- Temperature regulation through B(S)VT and B(S)CU-05 or BCU I
- High resolution probe equipped with ATMA
- Sample changer with position recognition (SampleCase, SampleJet, SampleXpress, BACS-60, or BACS-120)

**NMR Reference Standards for AssureSST**

One set of NMR Reference standards is required for the AssureSST package. NMR standard reference samples may be purchased through the Bruker Online Store at www.bruker.com. The details for 5mm NMR Standard Reference Samples to be used with the System Suitability Tests option are found in Table 1.1. The full list of AssureSST samples for different instrument configurations are listed in Chapter 13. Note to use the reference samples on the SampleJet, the samples must be topped with a two-part cap (Z130278).

| Test | Sample | Bruker P/N | Automation Position (default) |
|---|---|---|---|
| 1H Lineshape | 1% Chloroform in Acetone-d6 | Z10248 | 1 |
| 1H Sensitivity | 0.1% Ethylbenzene in CDCl3 | Z10120 | 2 |
| 13C Sensitivity | 10% Ethylbenzene in CDCl3 | Z10153 | 3 |
| 19F Sensitivity | 0.05% Trifluorotoluene | Z10234 | 4 |
| 31P Sensitivity | 0.0485 M Triphenylphosphate | Z10201 | 5 |
| Temperature calibration | 99.8% Methanol-d4 | Z10627 | 6 |

*Table 1.1:* List of 5 mm standard samples for the System Suitability Test typically used for room temperature probes, 300-600 MHz, including the sample type, the reorder number, and the default sample position in automation.

**NMR Validation Standards for AssureNMR**

One set of AssureNMR Test Samples, as described in Table below, is supplied with AssureNMR Summit. These certified validation standard samples may also be purchased through the Bruker Online Store. Note to use the Test Samples on the SampleJet, the samples must be topped with a two-part cap (Z130278).

| Test | Sample | Bruker P/N |
|---|---|---|
| 0% Adulterant | 0.1 M Alanine in $D_2O$ with 0% Lysine and 0.1% DSS | 1835533 |
| 4% Adulterant | 0.1 M Alanine in $D_2O$ with 4% Lysine and 0.1% DSS | 1835534 |
| 5% Adulterant | 0.1 M Alanine in $D_2O$ with 5% Lysine and 0.1% DSS | 1835535 |
| 6% Adulterant | 0.1 M Alanine in $D_2O$ with 6% Lysine and 0.1% DSS | 1835536 |

*Table 1.2:* List of 5 mm AssureNMR Sample Set 2.0 Test Samples including the sample type and reorder number. Sample tubes are 8 inches long.

| Test | Sample | Bruker P/N |
|---|---|---|
| 0mM Adulterant | 0.1 M Alanine in D2O with 0mM Lysine and 0.45mM DSS | B160038 |

| | | |
|---|---|---|
| 4mM Adulterant | 0.1 M Alanine in D2O with 4mM Lysine and 0.45mM DSS | B160039 |
| 5mM Adulterant | 0.1 M Alanine in D2O with 5mM Lysine and 0.45mM DSS | B160040 |
| 6mM Adulterant | 0.1 M Alanine in D2O with 6mM Lysine and 0.45mM DSS | B160041 |

*Table 1.3:* List of 5 mm AssureNMR Sample Set 2.1 Test Samples including the sample type and reorder number. Sample tubes are 7 inches long.

## 1.5 Installation

**TopSpin, IconNMR, AssureSST, AssureNMR, and AssureNMR SBASE**

Proceed as described in the TopSpin Installation Guide. IconNMR, AssureNMR, and the AssureNMR SBASEs can be selected from the customized installation menu during the Bruker TopSpin installation.

## 1.6 Default Home Directories

AssureNMR and IconNMR use specific directories for the AssureNMR modules. The default directories are listed below for Windows 7 and CentOS 7.

**Windows 7**

| | | |
|---|---|---|
| User Directory | %APPDATA% | C:\Users\<name>\AppData\Roaming |
| Shared Directory | %ALLUSERSPROFILE% | C:\ProgramData |

**CentOS**

| | |
|---|---|
| User Directory | /usr/Bruker |
| Shared Directory | /etc/opt/Bruker |

**AssureNMR**

| | |
|---|---|
| Default settings | (%APPDATA%\Bruker or <user home dir>/.Bruker) \Assure |
| Quant Methods | (%APPDATA%\Bruker or /usr/Bruker) \Assure |
| SBASEs | (%APPDATA%\Bruker or <user home dir>/.Bruker) \Databases \SBASE |

**AssureNMR and IconNMR**

| | |
|---|---|
| Logfiles | (%APPDATA% or <user home dir>/.Bruker) \rawMaterialScreening.log |
| Released Quant Methods | (%ALLUSERSPROFILE% or /opt/etc/) \Bruker\Raw Material Screening |

## 1.7 Computer Security

Before installing TopSpin on a PC, the PC administrator must decide which access rights PC users will have to the files of the TopSpin installation.

The policy of the TopSpin installer program is: During the installation process the installer program will inherit the existing access rights from the installation directory (Under Windows, this refers to the ACL: Access Control List security descriptors). In a second step, the installer program will give write or execute rights to a number of files that need these rights during program execution.

Therefore, it is recommended that the following installation procedure be performed by the PC administrator who must have Administrator/NMRSuperUser rights with respect to the operating system:

- Create a PC user group "NMRUser". The accounts of all PC users who should be allowed to run TopSpin must be in this group.
- Create the directory for the installation of TopSpin, e.g. C:\Bruker.
- Assign the desired access rights to this directory, e.g. "read only" and "execute". As an aid, you may inspect the default access rights of the directory "C:\Program Files" under Windows 7. These are typical for any software and might also be suitable for you.
- Install TopSpin in the chosen directory. All TopSpin files will inherit the previously set rights of the directory. At the end of the installation process, the TopSpin installer program will modify the access rights of some files and directories as required during TopSpin run time (write/execute access) for the "NMRUser" group. These are:
- classes\lib\topspin_py
- conf\global
- conf\instr\spect
- conf\instr\topshim\parameters\user
- conf\instr\topshim\solvents\user
- prog\au\bin
- prog\curdir
- prog\logfiles
- savelogs

Except for the GNU subdirectory of TopSpin, which requires read/execute access for everyone, solely the groups NMRUser and NMRSuperUser will be granted access to needed TopSpin directories and files. So besides the users inherited from the installation directory, only users belonging to these two groups will have access to the TopSpin installation.

Using these guidelines, your TopSpin installation will be well protected against any inadvertent damage.

## 1.8 Support

Software support is available from your local Bruker office or via e-mail from the following address:

*NMR-Support@bruker.com*

The Bruker www server www.bruker.com provides additional information such as downloadable upgrades for your AssureSST, AssureNMR, TopSpin and IconNMR installation.

# Introduction

# 2 Assure – System Suitability Test (SST)

## 2.1 Overview

AssureSST runs a user-selected set of experiments to monitor instrument performance. AssureSST is configured and controlled through the IconNMR automation software package which uses TopSpin to acquire and evaluate data. Upon completion of the SST, a PDF report is generated summarizing the specifications tested and the results obtained.

## 2.2 AssureSST Configuration

AssureSST parameters on the spectrometer are accessed through the IconNMR Configuration window (command “iconc” from the TopSpin command line). In the resulting window, select **AssureSST** in the pane on the left. The AssureSST section has three tabs (1) **System Suitability Test (SST)**, which allows the user to activate SST, schedule tests, and print reports (2) **SST Standard Tests**, which allows the user to select from the standard tests and set the specifications for SST, and (3) **SST User Tests**, which allows the user to set up the acquisition parameter set and analysis method for their own tests. Setup for AssureNMR is discussed in Chapter 7.

The SST (up to six individual standard experiments plus up to four user-defined experiments) as a whole must pass before any other samples queued in IconNMR can be acquired while under AssureNMR operation. The six standard tests are covered in the chapter *SST Standard Tests Tab [▷ 24]*. The user-defined tests are covered in section *SST User Tests [▷ 28]*. The user can modify this behavior through the IconNMR Configuration window, by selecting the **Fail Safe/Error Handling** window under Automation from the pane on the left. Unchecking the box beside ‘Stop the run when ‘Assure’ System Suitability Test reports specification failure’ allows queued samples to run even after the SST fails.

Any changes to the System Suitability Test (e.g. changing a specification or turning off one of the tests) immediately invalidates the previous suitability run. Thus, after a change, the test will be automatically queued when starting IconNMR even if the last successful SST was within the time specified.

It is possible to queue the SST at any time as NMR SuperUser (Chapter *Running IconNMR: Supervisor [▷ 121]*).

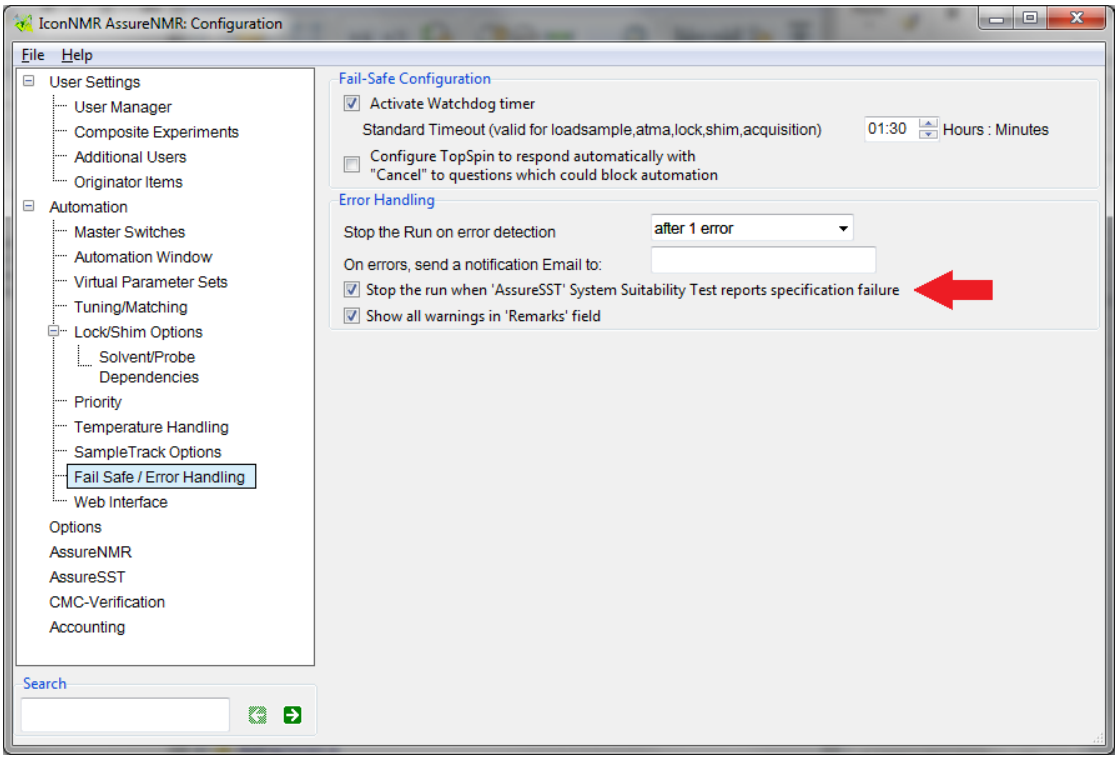



*Figure 2.1: Error Handling options that affect AssureSST.*

## 2.2.1 System Suitability Test (SST) Tab

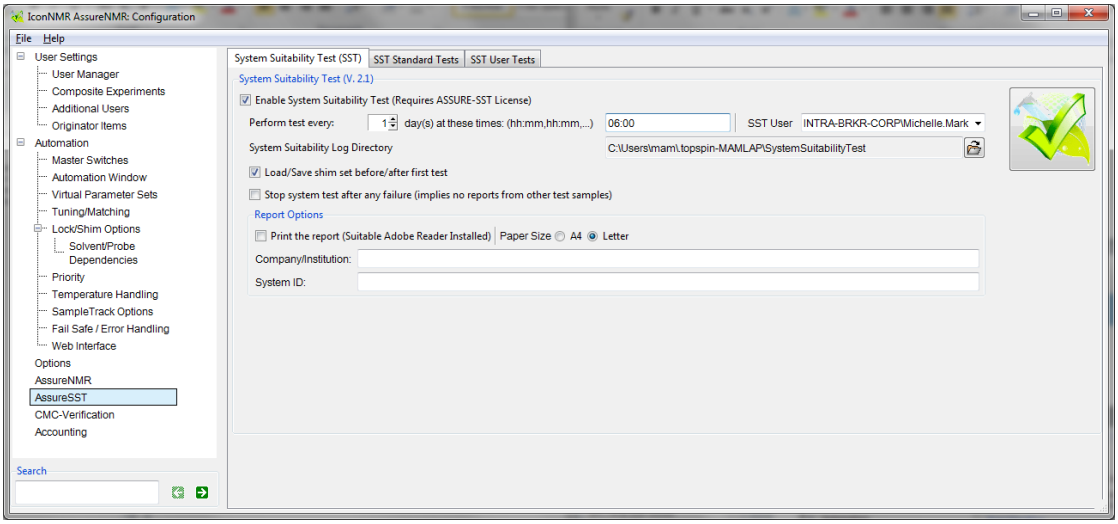



*Figure 2.2: The System Suitability Test (SST) tab in the AssureSST section of the IconNMR Configuration window.*

To activate AssureSST, check the first box on the **System Suitability Test (SST)** tab, 'Enable System Suitability Test (Requires ASSURE-SST License)'. Note that the AssureSST license is included with AssureNMR.

The System Suitability Test is automatically queued for acquisition as required to meet the time set in the entry for 'Perform test every: x day(s) at these times: (hh:mm,hh:mm,…)'. Note the times use a 24-hour format. If other samples are queued (e.g. for AssureNMR analysis) at the time when the SST is required, then the data acquisition of the queued samples will wait until the SST has been performed successfully. Strategies and flexibility in the SST were

designed to accommodate experiments which might exceed the available time. Experiments that run past the set time for the next SST will remain in the queue until sufficient time is available to run the sample(s).

The option to 'Load/Save shim set before/after first test' provides a mechanism for AssureSST to adjust to shim changes over time. This is done through a default shim file that is used for all samples including the system suitability test samples. Once a $^{1}$H Lineshape suitability test has been completed successfully, the shim set is updated by writing to 'IconNMRShimSet.probeName' where the probeName extension is the name from the edprobe table for the current probe. For more information on probe identification, type "help edprobe" in TopSpin.

When 'Load/Save shim set before/after first test' is checked, it loads the shims saved as 'IconNMRShimSet.probeName' before shimming. In this case, the solvent dependent shim files in the **Solvent/Probe Dependencies** are not used.

- To use this feature, check the box 'Load/Save shim set before/after first test'.
- Save the desired starting shims by typing "wsh IconNMRShimSet.probeName" from the TopSpin command line.

The SST as a whole is the criterion for a properly functioning spectrometer for the purposes of the AssureNMR software. As a result, failure of any component test is reported after the complete set of required tests is measured. For example, if the system administrator requires four tests ($^{1}$H Lineshape, $^{1}$H Sensitivity, $^{13}$C sensitivity and Temperature) for the SST, then all four tests must be completed before a final 'system pass' or 'system fail' result is obtained. Optionally, the user may select 'Stop system test after any failure' to immediately halt the acquisition after any test fails. When this box is checked then the tests will stop after the first failed system suitability test and all subsequent system suitability tests will be cancelled until the issue is resolved and AssureSST is restarted.

- Check 'Stop system test after any failure' to halt the SST after any failed test.

Automatic generation of a PDF report (see example in Chapter *Reports [▶ 150]*) for SST results occurs when the 'Print the report' option is active. The administrator can customize the 'Company/Institution' and 'System ID' in the report by filling in the corresponding fields.

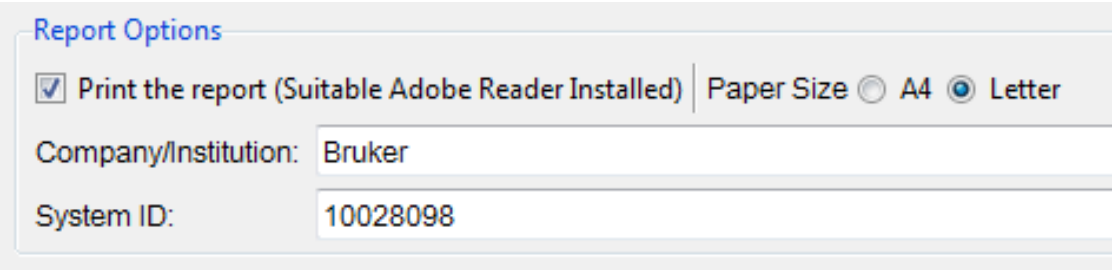



*Figure 2.3: Report Options for AssureSST*

Results from the SST are recorded in a log file written to the SST directory selected in the IconNMR Configuration window. Each of the experiments chosen on the **SST Standard Tests** tab and the **SST User Tests** tab will be run during the SST. An example log file from an SST is shown in Chapter *Log File from the System Suitability Test [▶ 149]*.

The 'Activate Single Peak Linewidth Check' enables continuous monitoring of lineshape for every sample acquired with IconNMR for spectral quality control. When used with the proper processing AU (proc_assureshim) and a sample that has an NMR reference signal at 0 ppm, the system uses the halfwidth of the reference to determine whether the spectrum is of a high enough quality to be passed on for analysis. The threshold cutoff for half height should be at or below the value specified as the 'Peak test cutoff frequency' (in Hertz). A sample with a larger half width will be re-shimmed and re-acquired. Two consecutive sample failures results in automatic queuing of the System Suitability Test.

**See also**

- Examples [▶ 149]

## 2.2.2 SST Standard Tests Tab

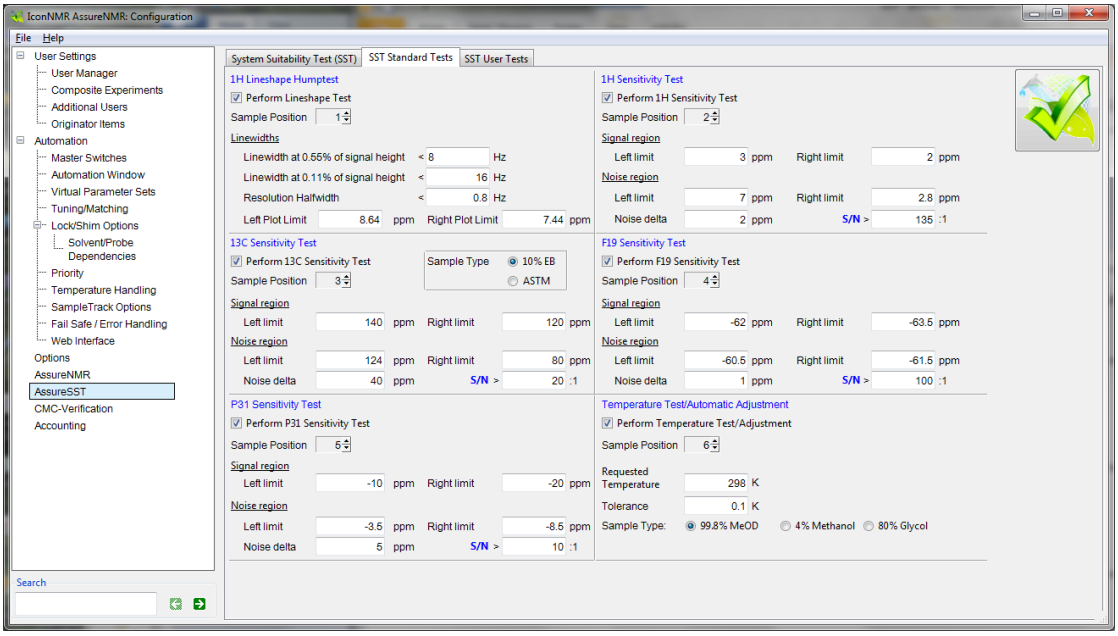



*Figure 2.4: The SST Standard Tests tab in the AssureSST section of the IconNMR Configuration window.*

The **SST Standard Tests** tab gives the user access to a set of six preset tests. These tests can be turned on and off, the sample positions can be specified, details for the analyses can be input, and criteria for passing the tests can be set from this tab. The details for each test are in the following sections.

### 2.2.2.1 $^{1}$H Lineshape

Also referred to as the humptest, this test automatically measures and determines the $^{1}$H lineshape using the GLP $^{1}$H lineshape standard sample, chloroform in acetone. (See Chapter 13 for the correct sample.) The width of the chloroform line at 0.55% height and 0.11% height is calculated with a double exponential fit along the left and right side of the signal. The resolution test is also performed and evaluates the width of the chloroform signal at half height. These values are compared with the specifications set in this window. The test is passed if the results are less than or equal to the defined values.

- Check the ‘Perform Lineshape Test’ box to require this experiment to run as part of the SST.
- Set the ‘Sample Position’ to the holder position of the standard sample in the sample changer (SampleCase, SampleJet, SampleXpress, BACS).
- Enter the desired values for an acceptable lineshape test for the (1) ‘Linewidth at 0.55% of signal height’, (2) ‘Linewidth at 0.11% of sample height’ and (3) the ‘Resolution Halfwidth’ (linewidth at 50% of signal height).

The lineshape and sensitivity values are specific to the probe. When the probe is changed, the values must be updated to correspond to the current probe in the SST Standard Tests tab of the IconNMR Configuration window. If the probe has been used previously, stored parameters will be loaded for that probe.

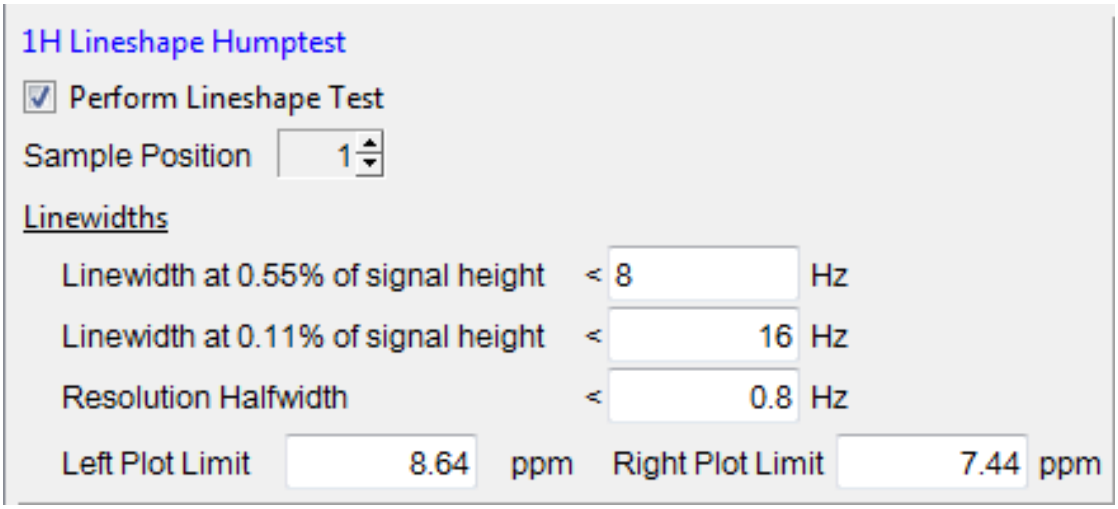



*Figure 2.5: $^{1}$H Lineshape Humptest parameters on the SST Standard Tests tab.*

### 2.2.2.2 $^{1}$H Sensitivity

This test automatically measures and determines the $^{1}$H sensitivity. The $^{1}$H sensitivity standard sample is 0.1% ethylbenzene in chloroform-d for all probes. The height of the biggest signal between the signal limits is calculated. A noise window of width 'Noise delta' in ppm is shifted in 25 steps along the spectrum between the noise limits. Each time, the noise value is determined and the signal-to-noise (S/N) ratio is calculated with respect to the height of the biggest signal within the signal limits. The best value must meet the specification defined in the S/N box.

- Check the 'Perform $^{1}$H Sensitivity Test' box to require this experiment to run as part of the SST.
- Set the 'Sample Position' to the holder position of the standard sample in the sample changer (SampleCase, SampleJet, SampleXpress, BACS).
- Enter the desired 'Signal region' (Left and Right limits) to be used for the signal peak.
- Enter the desired 'Noise region' (Left and Right limits).
- Enter the 'Noise delta' (width of the noise range) in ppm.
- Enter the 'S/N' requirement for a successful test.

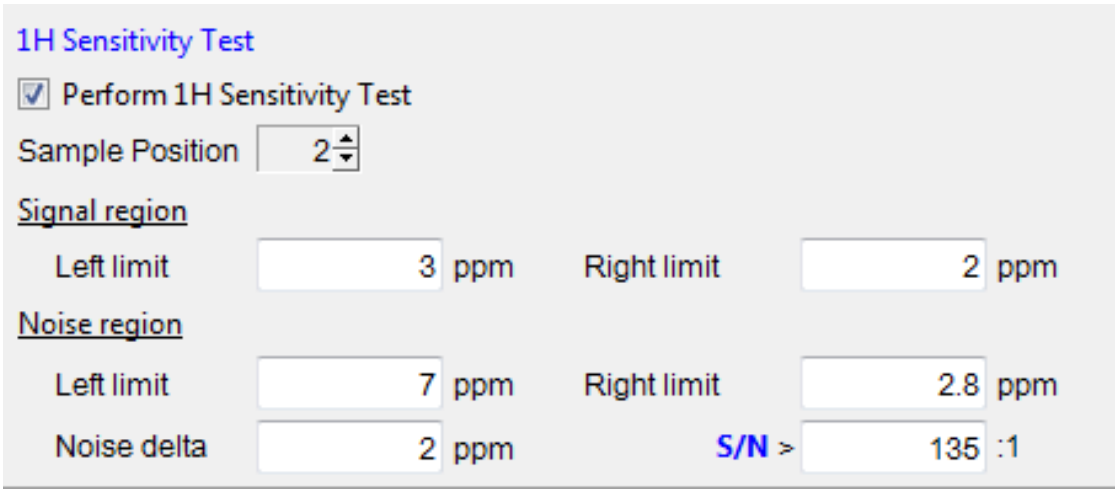



*Figure 2.6: $^{1}$H Sensitivity Test parameters on the System Suitability Test (SST) tab.*

### 2.2.2.3 $^{13}$C Sensitivity

This test automatically measures and determines the $^{13}$C sensitivity. The typical sample used for the $^{13}$C Sensitivity Test is 10% ethylbenzene in cholorform-d for all probes. The ASTM (American Society for Testing and Materials) sample (40% p-Dioxane in benzene-d6) may also be used. The height of the biggest signal within the signal limits is calculated. A noise window of 'Noise delta' ppm is shifted in 25 steps along the spectrum between the noise limits. Each time, the noise value is determined and the signal-to-noise ratio is calculated with respect to the height of the biggest signal. The best value must meet the specification defined in the S/N box.

- Check the 'Perform $^{13}$C Sensitivity Test' box to require this experiment to run as part of the SST.
- Set the 'Sample Position' to the holder position of the standard sample in the sample changer (SampleCase, SampleJet, SampleXpress, BACS)
- Select the Sample Type, either 10% ethylbenzene or the ASTM sample.
- Enter the desired 'Signal region' (Left and Right limits) to be used for the signal peak.
- Enter the desired 'Noise region' (Left and Right limits).
- Enter the 'Noise delta' (width of the noise range) in ppm.
- Enter the 'S/N' requirement for a successful test.

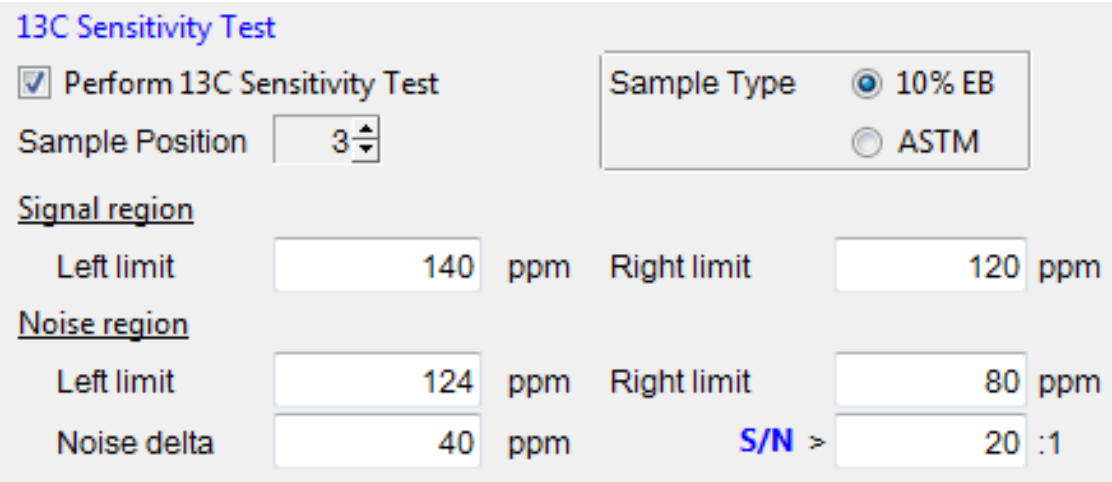



*Figure 2.7: $^{13}$C Sensitivity Test parameters on the SST Standard Tests tab.*

### 2.2.2.4 $^{19}$F Sensitivity

This test automatically measures and determines the $^{19}$F Sensitivity. The typical sample used for the $^{19}$F Sensitivity Test is 0.05% trifluorotoluene in chloroform-d for all probes. The height of the biggest signal between the signal limits is calculated. A noise window of 'Noise delta' ppm is shifted in 25 steps along the spectrum between the noise limits. Each time, the noise value is determined and the signal-to-noise ratio is calculated with respect to the height of the biggest signal. The best value must meet the specification defined in the S/N box.

- Check the 'Perform $^{19}$F Sensitivity Test' box to require this experiment to run as part of the SST.
- Set the 'Sample Position' to the holder position of the standard sample in the sample changer (SampleCase, SampleJet, SampleXpress, BACS).
- Enter the desired 'Signal region' (Left and Right limits) to be used for the signal peak.
- Enter the desired 'Noise region' (Left and Right limits).
- Enter the 'Noise delta' (width of the noise range) in ppm.
- Enter the 'S/N' requirement for a successful test.

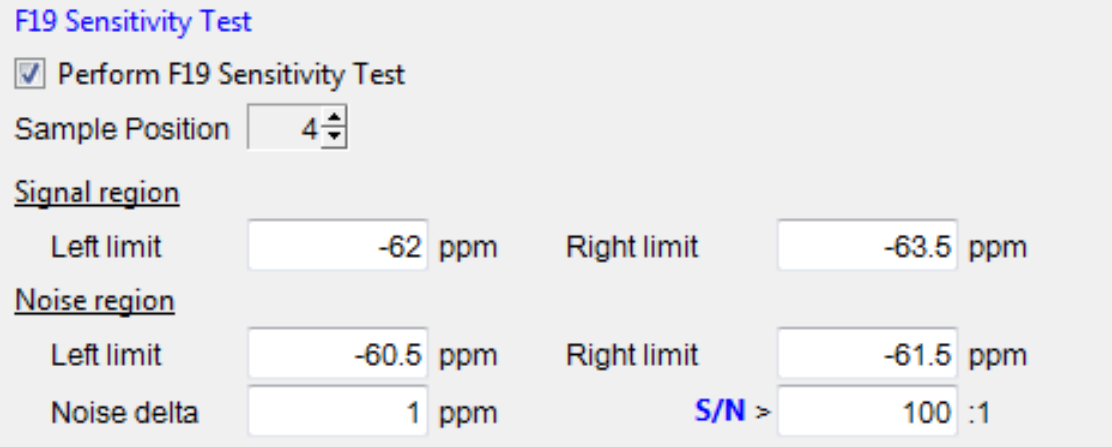



*Figure 2.8: $^{19}$F Sensitivity Test parameters on the SST Standard Tests tab.*

### 2.2.2.5 $^{31}$P Sensitivity

This test automatically measures and determines the $^{31}$P Sensitivity. The typical sample used for the $^{31}$P Sensitivity Test is 0.0485 M triphenylphosphate in acetone-d6 for all probes. The height of the biggest signal between the signal limits is calculated. A noise window of 'Noise delta' ppm is shifted in 25 steps along the spectrum between the noise limits. Each time, the noise value is determined and the signal-to-noise ratio is calculated with respect to the height of the biggest signal. The best value must meet the specification defined in the S/N box.

- Check the 'Perform $^{31}$P Sensitivity Test' box to require this experiment to run as part of the SST.
- Set the 'Sample Position' to the holder position of the standard sample in the sample changer (SampleCase, SampleJet, SampleXpress, BACS).
- Enter the desired 'Signal region' (Left and Right limits) to be used for the signal peak.
- Enter the desired 'Noise region' (Left and Right limits).
- Enter the 'Noise delta' (width of the noise range) in ppm.
- Enter the 'S/N' requirement for a successful test.

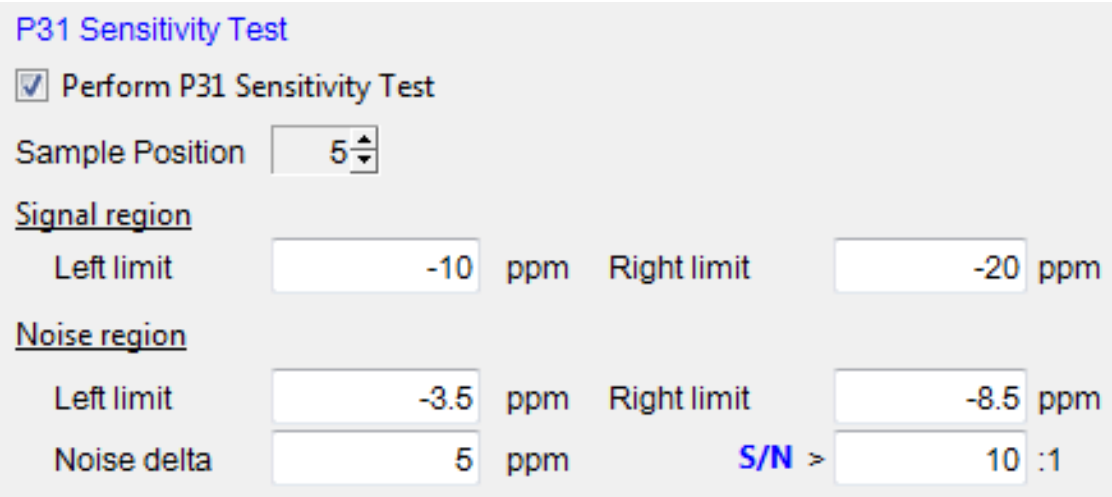



*Figure 2.9: $^{31}$P Sensitivity Test parameters on the SST Standard Tests tab.*

#### 2.2.2.6 Temperature Test with Automatic Adjustment

This test automatically measures and, if necessary, adjusts the temperature to the temperature specified in 'Requested Temperature'. The experiment is designed to run after the other components of the SST. In the example below, the temperature is measured with the 99.8% Methanol-d4 Temperature Calibration Standard which has a linear range from 282 K to 330 K. The method will attempt to adjust the temperature to the set point five times before failing. The final observed temperature after adjustment is recorded in the status parameters under the entry for USERA1.

- Check the 'Perform Temperature Test/Adjustment' box to require this experiment to run as part of the SST.
- Set the 'Sample Position' to the holder position of the desired temperature calibration standard in the sample changer (SampleCase, SampleJet, SampleXpress, BACS).
- Enter the 'Requested Temperature' and 'Sample Type'.

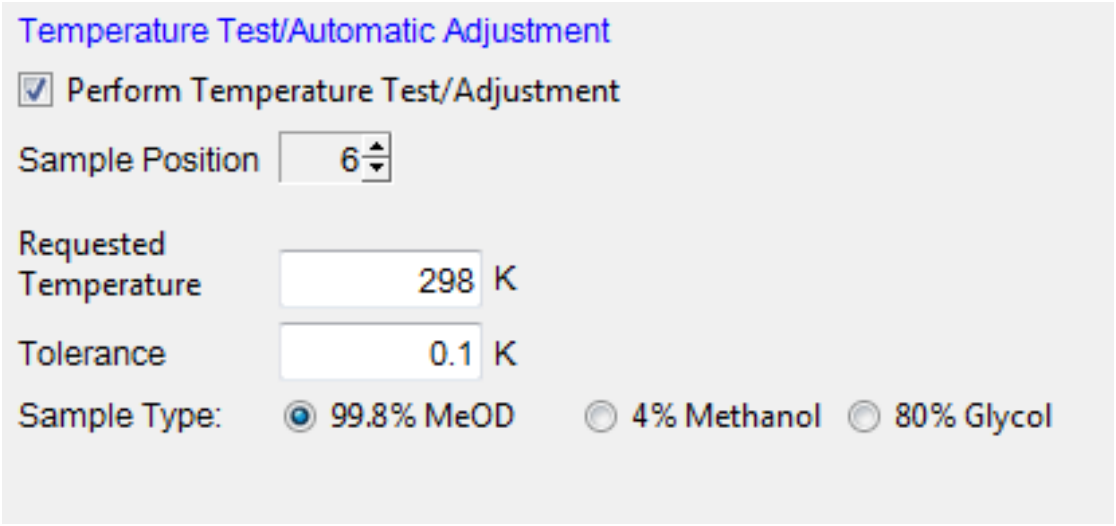



*Figure 2.10: Temperature Test parameters on the SST Standard Tests tab.*

### 2.2.3 SST User Tests

SST also allows users to specify their own tests. For analysis purposes, the tests are classified as lineshape tests or sensitivity tests. The user must supply information about the solvent, the parameter set to use, and the sample position. In the parameter set, the acquisition au program (AUNM) must be au_zgglp and the processing au program (AUNMP) must be proc_1dglp. The user must also supply the information for the analysis – linewidths and plot region for the lineshape tests, signal and noise regions for the sensitivity tests – interactively on the **SST User Tests** tab.

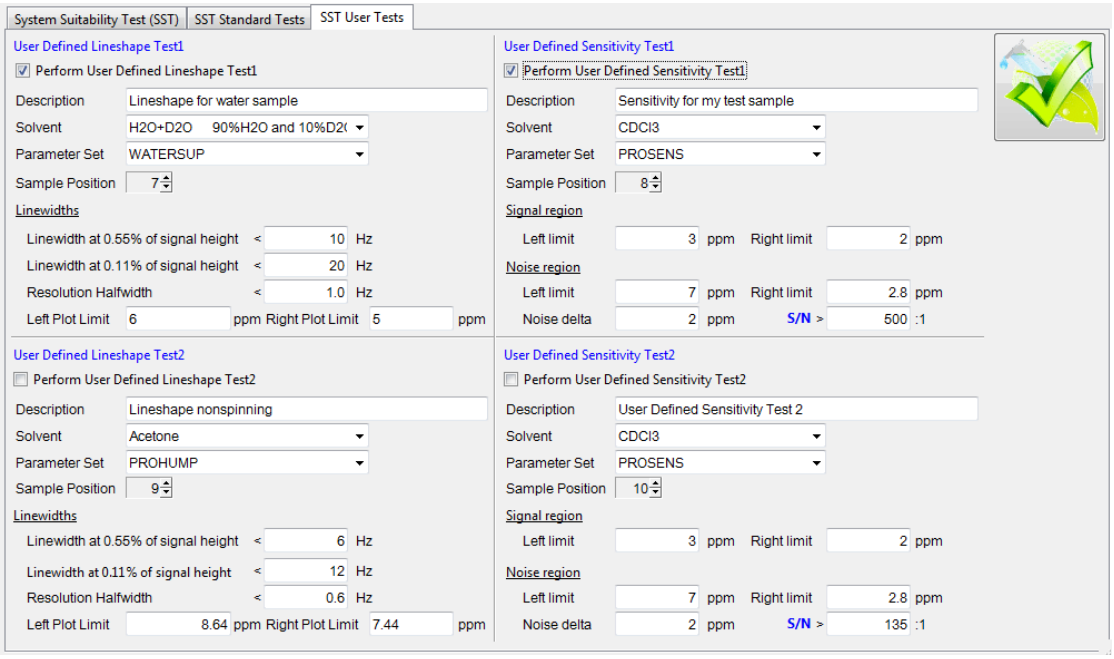



*Figure 2.11: SST User Tests tab for SST, with one lineshape test and one sensitivity test activated.*

## 2.3 Data Organization and Final PDF Report

AssureSST automatically places the SST data in the first directory listed in the Data Directories window for the SST user in the **User Manager** of the IconNMR Configuration window. The generated data directory name uses the following date-stamped format *SST_{YYYY_MM_DD_HH_MM_SS}*. Each directory will contain all of the data for a queued System Suitability Test.

Within the data directory for the last experiment that makes up the SST, a PDF report (see the example in Chapter 9.2) is stored which summarizes which tests were run, the criteria, and the results of each test including a pass or fail notation.

# 3 AssureNMR Easy Workflow

AssureNMR provides a way to streamline analysis of your samples by automating the process, from data acquisition to processing to analysis to reporting. This chapter outlines how to use AssureNMR in automation, once an analysis method has been developed and the experimental parameters selected. The following five chapters will present the details of how to set up AssureNMR. Specific items covered here are sample submission, spectrum quantification and identification, and report generation.

## 3.1 IconNMR Submission Interface

AssureNMR is intended to be used with AssureSST. When AssureSST is activated (by checking 'Enable System Suitability Test (Requires ASSURE-SST License)' in the IconNMR Configuration/AssureSST/**System Suitability Test (SST)** tab), starting the IconNMR automation window automatically queues AssureSST to confirm the instrument performance. Successful completion of the SST is required before the queued samples can run. Samples may be queued either for standard IconNMR automation or for AssureNMR acquisition and analysis as described below. AssureNMR acquisition requires activation of AssureNMR by checking 'Activate AssureNMR Automation' on the **AssureNMR** tab.

Submitting samples for AssureNMR is done through the IconNMR easy setup mode interface. This user interface allows only minimal user interaction to facilitate compliance with SOPs and to prevent the alteration of key parameters of the instrument configuration and the acquisition. To submit a sample, simply fill in each of the active fields:

- Evaluation Material Filename: name of parent directory where data is stored
- Evaluation Material: name of predefined combination of acquisition parameters and AssureNMR analysis parameters (quantMethod), specific for the material in the sample. Select by clicking one of the radio buttons.
- LIMS Container ID: the IconNMR Originator Item for tracking the sample, entered by the user. A barcode reader can be used if available.
- Lot Number: the IconNMR Originator Item for tracking the lot, entered by the user.
- Operating Site: the IconNMR Originator Item that specifies the facility where the NMR spectrum was acquired.

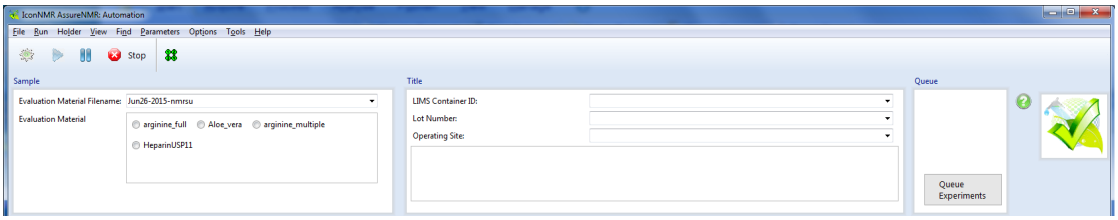



*Figure 3.1: IconNMR easy setup mode submission interface.*

Once all of the fields have been populated, selecting the 'Queue Experiment' button will result in a window which prompts the user to place the sample in the identified sample position. Once the sample is in the automation robot at the designated position the user must select the 'Submit' button at the bottom of the page to release the sample to automation control.


*Figure 3.2: AutoSampler Position window identifies the sample position for the queued sample.*

IconNMR oversees all of the processes in automation, from data acquisition and processing in TopSpin to the analysis and report generation by AssureNMR, to ensure that the evaluation is complete from sample submission to spectral analysis.

## 3.2 Parameter Sets and Pulse Programs

IconNMR utilizes parameter sets to acquire and process NMR data. Parameter sets used with AssureNMR are defined in the IconNMR configuration window (see section *IconNMR Configuration [▶ 108]*). Parameter sets named ASSURE*, PROF* and CMC*, released with the TopSpin software, are typically good starting points for the development of parameter sets for use in AssureNMR. It is essential to configure the prosol tables for probe dependent parameters prior to using the parameter sets. Additionally, parameter sets must be tested before use in AssureNMR to verify the parameter set is fit for the purpose of the analysis to be performed. A relaxation time experiment may need to be conducted to assist in the

establishment of proper relaxation delay parameters to achieve quantitative results. Conducting experiments and literature searches assist in determining the pulse sequences that provides the desired level of accuracy and precision.

## 3.3 Identification and Quantification of Spectral Components

AssureNMR makes two parallel evaluations of each spectrum. The spectrum is matched against an NMR spectral database (SBASE) to identify compounds and integrated to quantitate both known and unknown signals. Statistical models can also be used to determine whether a sample falls within a particular group. The parameters for this analysis are contained in a method file (extension .quantMethod), specified indirectly through the 'Evaluation Material' entered above. Chapter *Quantification in AssureNMR [▷ 73]* explains in detail how to set up a quantMethod. Chapter *Chemometric Modeling in AssureNMR [▷ 125]* explains in detail how to use the statistical tools available in AssureNMR.

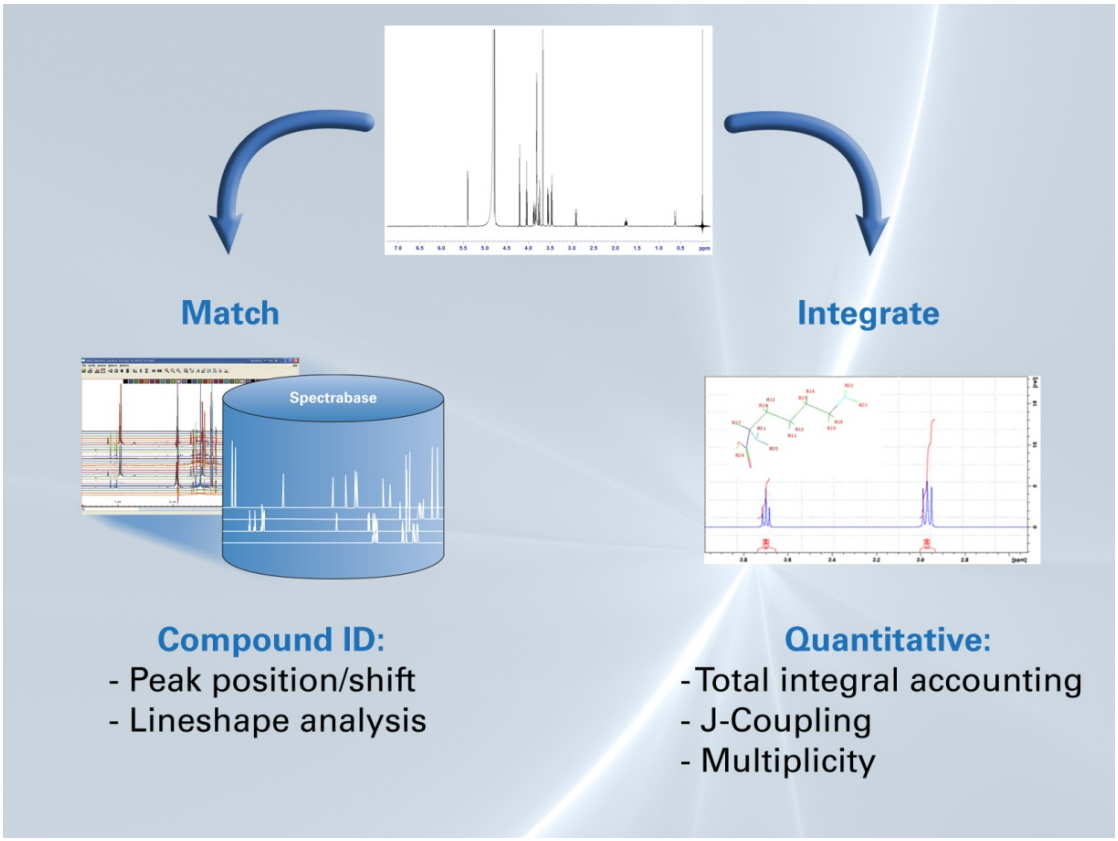



*Figure 3.3: Summary of the parallel, complementary spectral analysis techniques used in the AssureNMR software package. Spectra are evaluated based on qualitative (left side) and quantitative (right side) properties of the constituents.*

## 3.4 Access to Final Reports

The AssureNMR software generates a basic one page report (QCReport.pdf) and a longer, more detailed report (ExpertReport.pdf) for the analysis which are stored with the experiment (in the directory 'Evaluation Material Filename'/experiment number). Both reports contain information about the instrument, the original spectrum, the analysis results, and time. The reports can be accessed by right-clicking the entry in the 'Preceding Experiments' window of IconNMR.

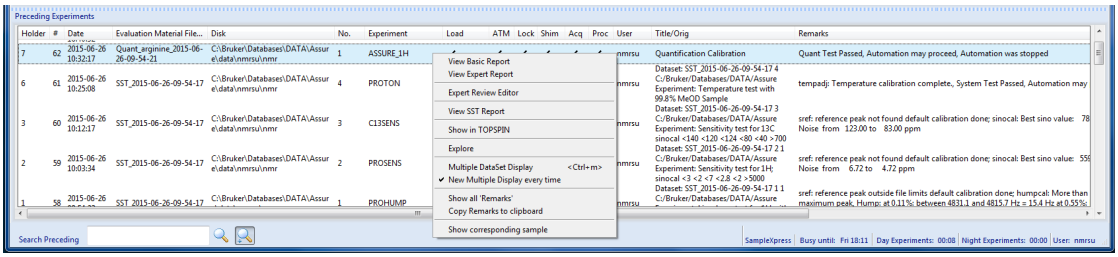



*Figure 3.4: Access to the reports via IconNMR Preceding Experiments window.*

The QC report shows a simple pass or fail result for the spectrum based on the criteria defined in the quantMethod and is designed to provide a summary to accompany the material, certifying that the material has been analyzed. The more detailed expert report contains information on all of the steps of the analysis and is designed to allow detailed evaluation of the data without the need to access the NMR spectra directly. A sample of each of these reports can be found in Chapter *Reports [▶ 150]*.

This completes the information required to submit samples for automated analysis, complete with reports!

# 4 Interactive Analysis with AssureNMR

## 4.1 General Features

AssureNMR is the interface and analysis module of the software, responsible for analysis during automation, with tools for method development and interactive analysis outside automation. This chapter focuses on the tools available outside automation. The AssureNMR software has been implemented in Java. The results of the analysis are available in a flexible reporting format including summary reports (QCReport.pdf), extended reports (ExpertReport.pdf), user-defined reports (CustomReport.pdf), and spreadsheets (ExcelReport.xls, Excel format).

To launch AssureNMR outside automation, click on the AssureNMR icon:

The AssureNMR splash screen appears briefly:


*Figure 4.1: AssureNMR splash screen.*

Then the AssureNMR interactive analysis window appears. The window features a row of pulldown menus (which will be discussed in the following sections), a row of icons for the common tasks of opening files, printing, and manipulating spectra in the viewer window, a browser window with tabs for data and SBASEs, and a viewer window to display spectra. The data browser on the left allows the user to open files from the system (**System** tab) or from any registered SBASE (**SBASEs** tab). Data can be opened by dragging and dropping the experiment from the data browser into the viewer window.

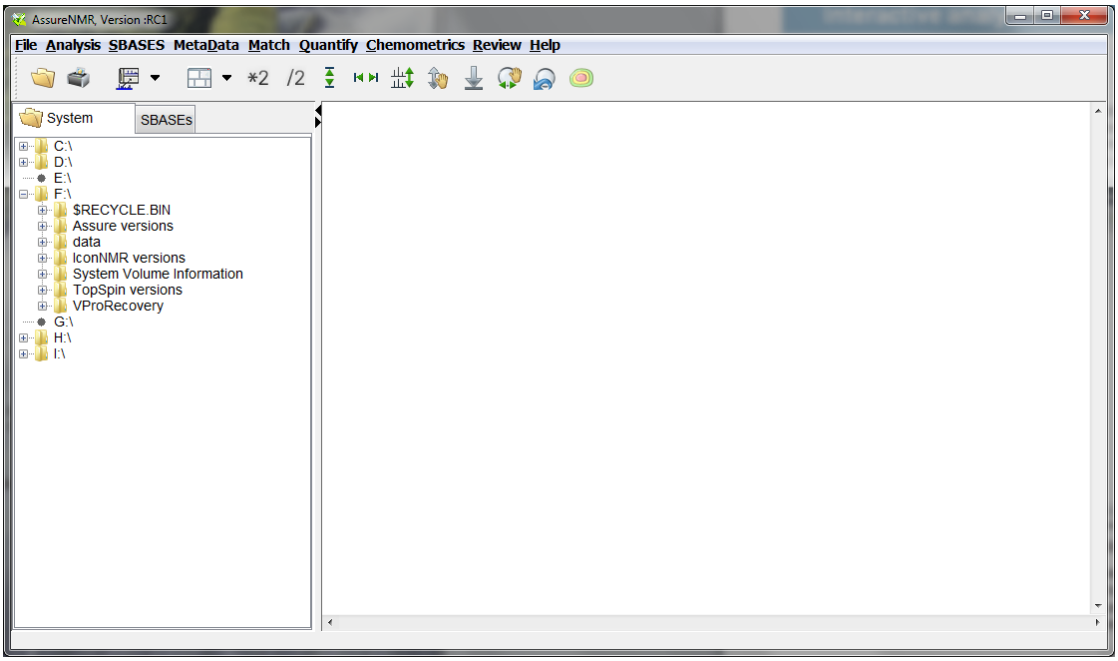



*Figure 4.2: AssureNMR interactive analysis window.*

The icons across the top operate as follows:

 Open File: window pops up, prompting for location and file name.

 Print: 'Print window as pdf' window pops up and prompts for location and file name. A pdf file is created and opens automatically in the default pdf viewer.

 Overlay: Pulldown menu to specify what the program should do when additional spectra are opened: **new window**, **overlay**, or **replace last**.

 Vertical Splitting of Upper and Lower Half: Pulldown menu to choose how new windows will appear, with options **vertical splitting**, **vertical splitting of upper and lower half**, **horizontal splitting**, and **horizontal splitting of left and right half**.

 Larger by Factor of 2: scales up the intensity of the spectrum currently displayed in the viewer window by a factor of 2.

 Smaller by Factor of 2: scales down the intensity of the spectrum currently displayed in the viewer window by a factor of 2.

 Reset Vertical Scale: resets the intensity scaling of the spectrum displayed in the viewer window to the original value, typically such that the largest peak just fills the window.

 Reset to Full Display: resets the horizontal scale to the full range. For a 1D spectrum, this is the full spectral width.

 Proportional Shift: when more than one spectrum is overlaid in the viewer window, this option can be used to spread out the spectra. Left click on the icon, then left click and hold in the viewer window while moving the mouse up and down to set the separation between the spectra.

Smooth Vertical Offset: allows adjustment of the vertical offset of the spectrum displayed in the viewer window with the mouse. Left click on the icon, then left click and hold in the viewer window while moving the mouse to move the spectrum up and down.

Reset Vertical Offset: restores the vertical offset to its original value.

Smooth Horizontal Expand: Left click the icon then left click and hold while moving the mouse sideways to expand the spectrum horizontally about the center point.

Undo Last Zoom: undoes only the last horizontal zoom, so the spectrum returns to its previous expansion.

Set Contours of 2D Spectra: brings up a window for setting the contours for a 2D spectrum.

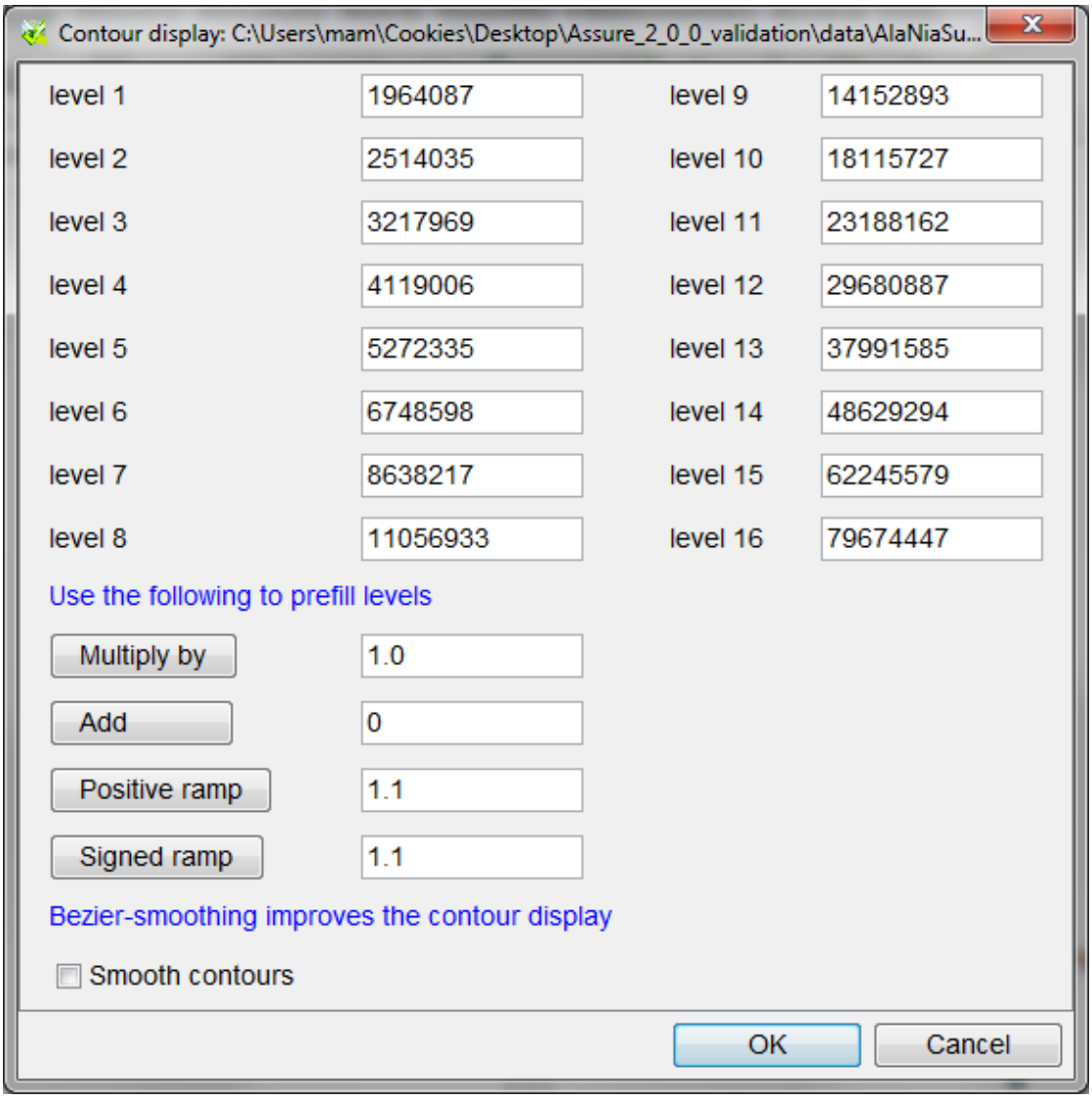
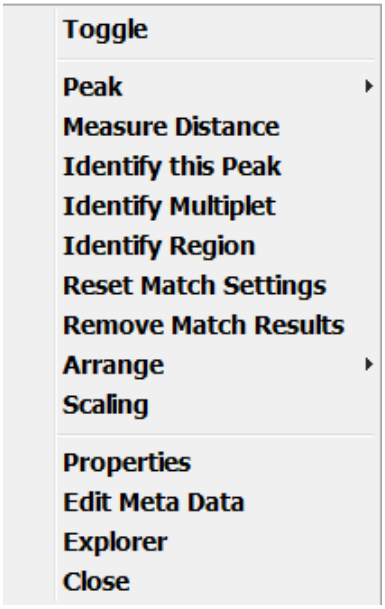
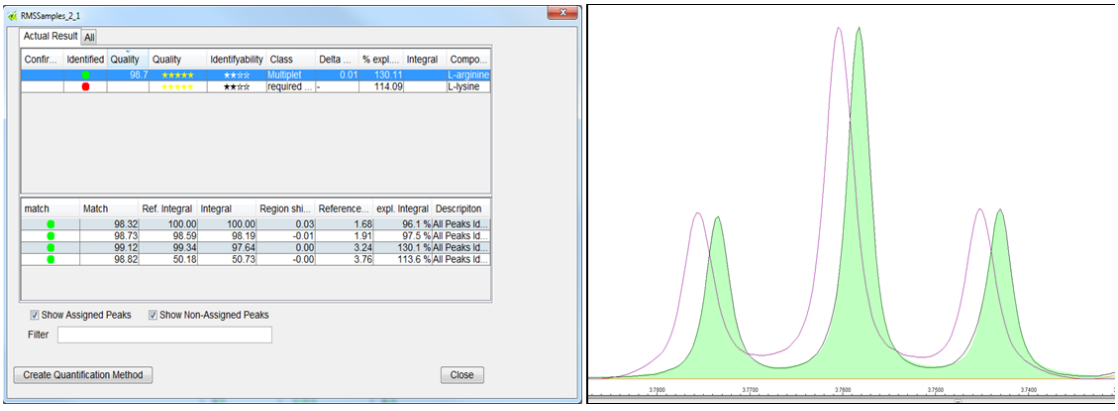



*Figure 4.3: Contour display window.*

Once a spectrum is loaded in the viewer window, clicking and holding the left mouse button allows the user to specify a region to expand; releasing the left mouse button displays the expanded region. The middle mouse button allows the user to adjust the vertical scale of the spectrum. A right mouse click brings up the following menu:




**Toggle** allows the user to shift between different spectra when various spectra (1D and 2D) are open simultaneously. The **Peak** options are **Display Options, Add Singlet**, and **Undo Latest Peak Change**. **Measure Distance** provides a convenient way to measure coupling constants. **Identify this Peak, Identify Multiplet** and **Identify Region** performs a search against the active SBASE for possible matches to the spectrum on the screen for the peak, the multiplet or region selected. The first time one of these commands is run the program will ask for the SBASE designation. To change the SBASE designation, select **Reset Match Settings** and select one of the identify options. For faster matches against the same SBASE, the Match results are saved and are used for future 'Identify' executions. To clear the match memory select **Remove Match Results**. Selecting one of the Identify options opens a MATCH panel that is shown and described in the MATCH section on this manual.




**Arrange** and **Scaling** affect the spectrum's display. **Properties** opens a window with information about the displayed spectrum. **Edit Meta Data** opens a window for editing the MetaData of the current spectrum. **Explorer** opens a window to the directory for the processed data (for example pdata/1). **Close** closes the displayed spectrum. These tools facilitate interaction with the spectrum in the viewer window.

## 4.2 File Menu

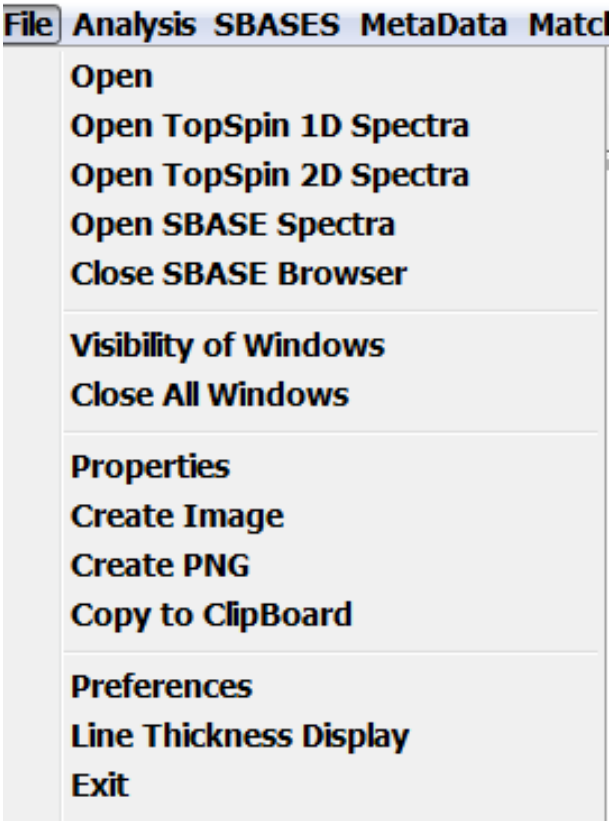



*Figure 4.4: File pulldown menu.*

The first section of the **File** menu deals with opening files. The **File** dropdown menu allows the user to open files, including general system files, TopSpin 1D Spectra, TopSpin 2D Spectra, and SBASE Spectra. From the **File** dropdown, the user can also turn on and off the SBASEs tab in the browser window.

The next section allows the user to specify the **Visibility of Windows** in the viewer window. The user can also **Close All Windows** in the viewer window. Note: to close just one of the objects in the viewer window, right click on the color-coded square for that spectrum, in the upper right hand corner of the viewer window. This produces a pulldown menu with the option to **Close** the spectrum.

In the next section, **Properties** displays a new window with information about the spectra displayed in the viewer window. **Create Image** saves the display in the viewer window as a scalable vector graphics (svg) file. **Copy to ClipBoard** captures the image in the viewer window for easy pasting into another application.

The **Preferences** command opens up a window where the user can specify preferences that apply throughout the AssureNMR interactive analysis interface (like the default PDF viewer, fonts, etc.). **Line Thickness** allows the user to specify how thick (in points) the line used to plot the spectrum will be. For display through a projector or capture to presentations, sometimes thicker lines are better. The default is one point. **Exit** closes AssureNMR. Below **Exit**, there is a list of recently displayed spectra for quick access.

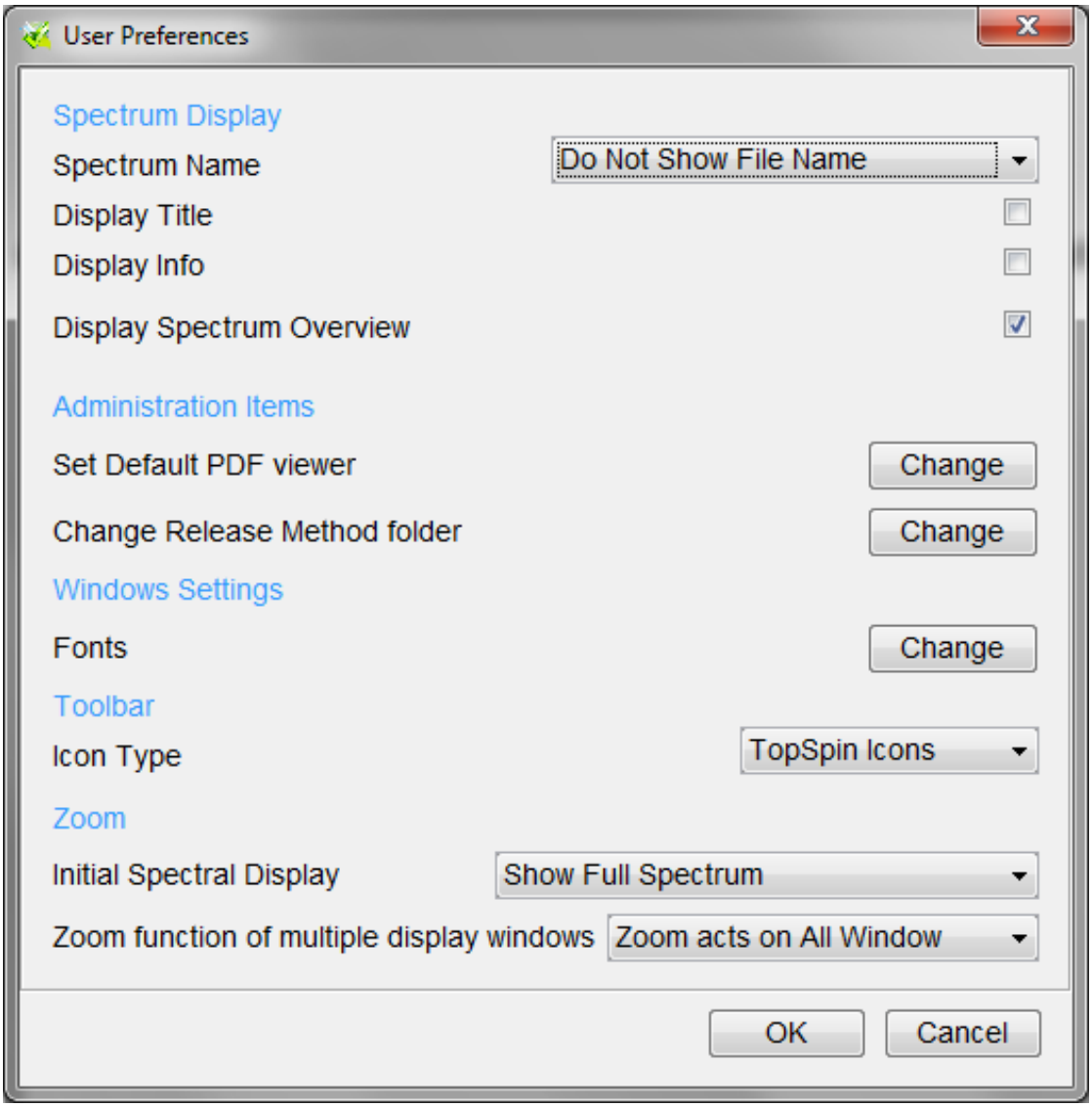



*Figure 4.5: User Preferences window.*

## 4.3 Analysis Menu

The **Analysis** pulldown menu offers tools for analyzing the peaks in the spectrum. **Peak Analysis** has three options, **Pick All Peaks Above Threshold**, **Pick Peaks Manually**, and **Integrate All Peaks**. The **Peak Report** launches a summary table for the current peaks, including the position, intensity, and any annotations. **Align 1D Spectra** gives the user the chance to adjust the chemical shift referencing when a collection of 1D spectra are open to provide the best alignment. Alignment is based on the peaks in the region displayed.

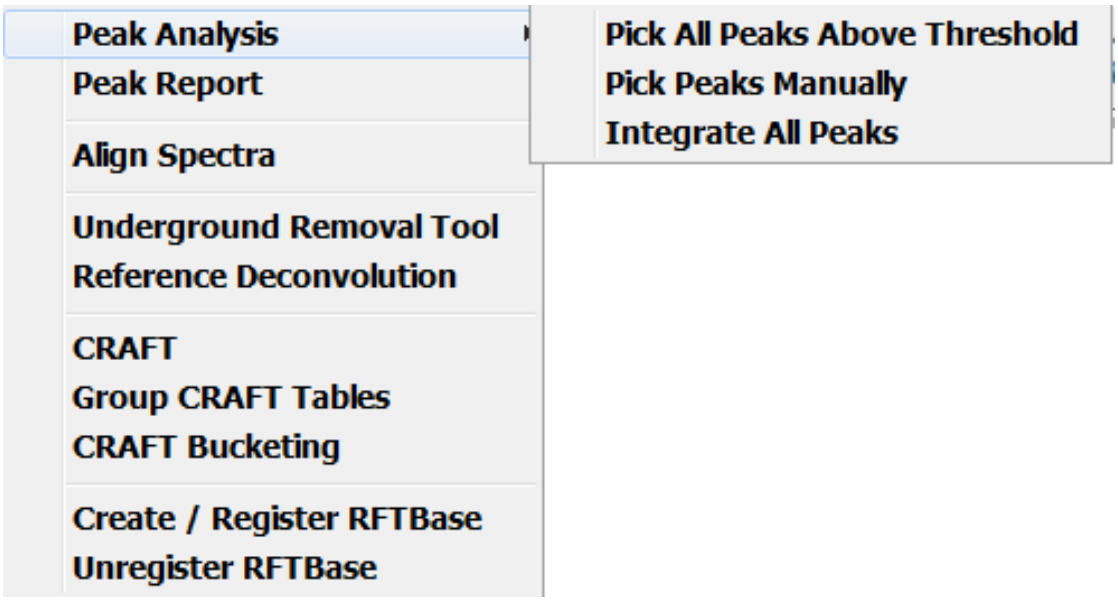



*Figure 4.6: Peak Analysis pulldown menu under Analysis.*

**Underground Removal Tool** and **Reference Deconvolution** are post-processing manipulations of the spectrum in the viewer window. When the user selects the **Underground Removal Tool**, a window pops up and prompts for the region to correct, the filter width, and a noise region to establish the baseline. The moving minimum filter subtracts the minimum value in the search region (+/- filter width) from the current value. This tends to remove broad “underground” signals ie - peaks broader than the filter width. The corrected spectrum is displayed on screen. **Reference Deconvolution** aims to restore any symmetry issues with the spectral peaks (for example, due to shimming problems). A singlet at 0 ppm is required, e.g. TSP. Two parameters are required:

- Regularization exponent: sets the amount of peak smoothing to apply. The higher the exponent, the larger the effect on the signal intensity. A good starting value is 0.
- Line shape factor: used to modify the line width. Any factor < 1 will introduce additional noise. A good starting value is 1.

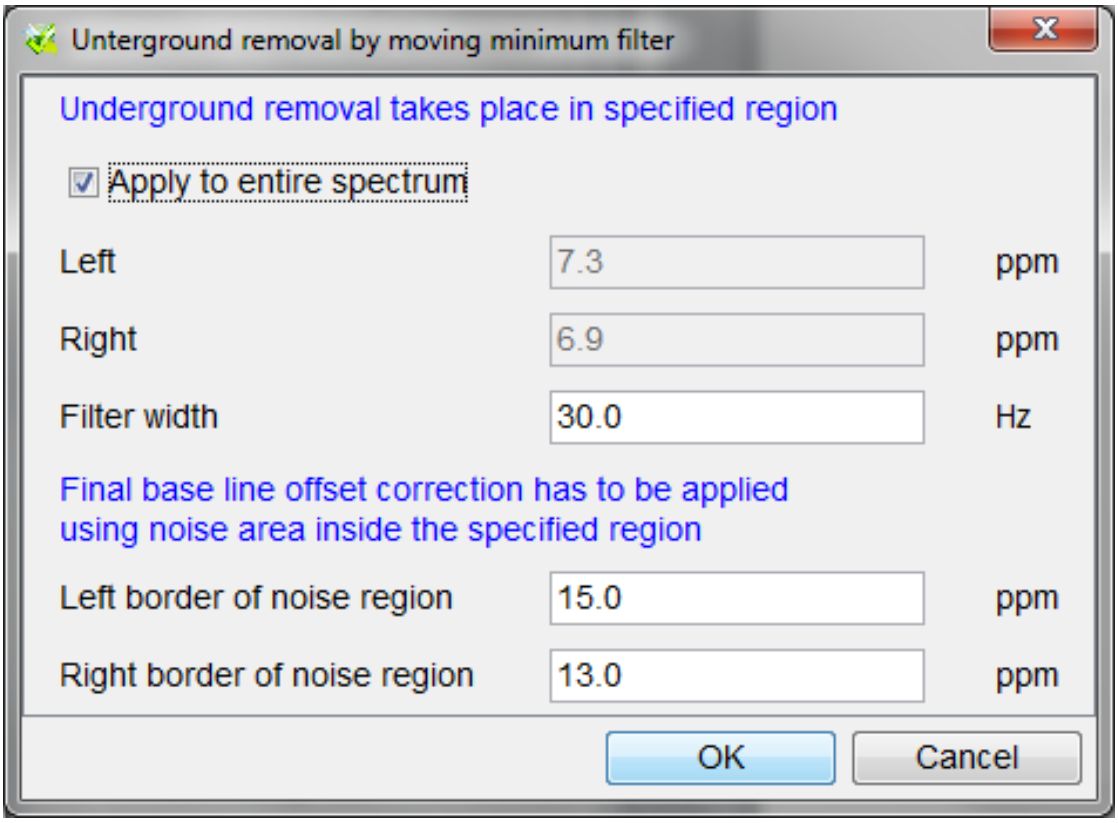



*Figure 4.7: Underground removal by moving minimum filter window.*

### 4.3.1 CRAFT

Complete Reduction to Amplitude Frequency Table (CRAFT) [2] functionality is also available under the Analysis file menu. Briefly, the CRAFT algorithms use a Bayesian analysis to construct frequency models of the time-domain FID data. The models can be displayed visually as Fourier Transformed (FT) spectra or in a tabular format of frquencies and amplitudes. Because the analysis is performed on the time-domain data, this methodology is particularly useful for quantification of spectra having poor baseline and/or phasing, as this does not affect the amplitudes of the frequencies. Moreover, the CRAFT amplitudes may provide more accurate quantification of overlapping peaks or shoulders of large peaks, as the corresponding frequencies are often adequately resolved in the digitization of the time-domain data despite peak overlap in the FT'd spectrum.

To utilize CRAFT, load a spectrum in the AssureNMR window. Selecting **CRAFT** will present a pop-up selection panel (see Figure below) which enables a choice of origin for the regions of interest (ROI): A) Manually, B) Automatically, C) From Excel List, D) RF Table, E) RFT Group. Dividing the spectrum into ROIs speeds up the Bayesian processing and also allows the user to eliminate parts of the spectra that do not contain peaks and/or are not required for quantification, further improving the calculation efficiency. ROIs may be selected manually, in automation, from an excel list, from a RF Table or RFT Group. The latter three options (C, D, or E) are to allow for consistency of ROI selection for a large dataset group or between separate spectra. An example employing the use of an RF Table will be explored later.

The analysis can be targeted towards either specific analytes or specific regions of the spectra, e.g. the aromatics.

[2] K Krishnamurthy, Magn Reson Chem. 2013 51(12):821-9.

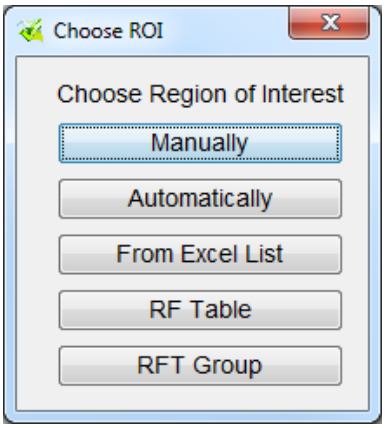



*Figure 4.8: CRAFT Choose ROI pop-up selection panel.*

The second option (B) will divide the full spectrum into ROIs automatically, eliminating regions where there are no peaks present. Most often, however, the user will want to use the first option (A) to allow for manual ROI selection. This brings up a popup window instructing the user to left click/drag to define the regions and click <Stop> to finish and begin the analysis.

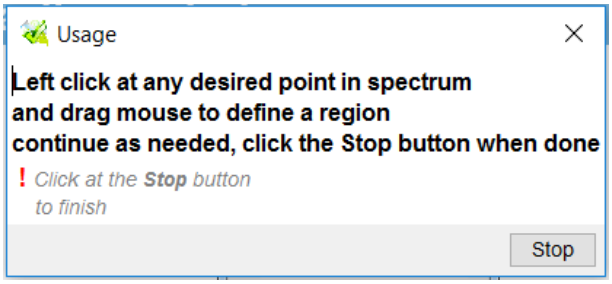



*Figure 4.9: Manually selecting ROIs.*

A few tips regarding ROI selection- first, avoid starting/stopping an ROI mid-peak or multiplet. This may cause visual anomalies when the ROIs are stitched back together into the spectrum. Note, however, that this is a visual effect only and does not impact the quantification via the amplitude. Also, keep the ROI region size to ~1 ppm at most. To prevent excessive calculation time, the number of models per ROI is limited, so each ROI should contain no more than ~60 expected signals.

Once the ROIs are selected, another pop-up with the following options will appear:

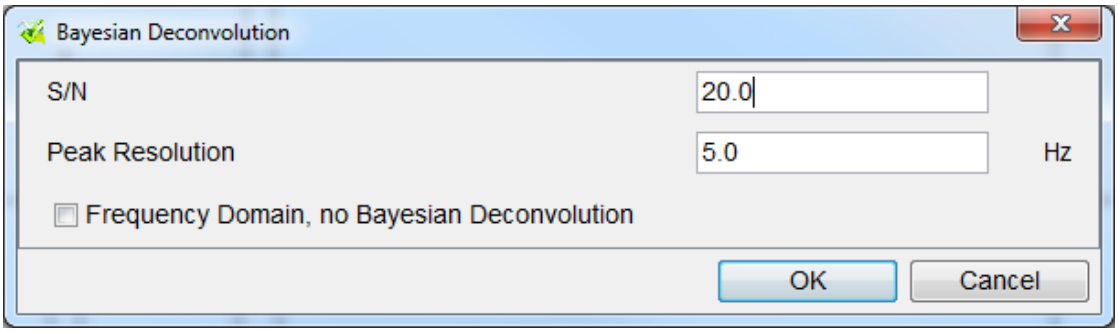



*Figure 4.10: CRAFT analysis parameters.*

The first parameter controls the minimum S/N level for the Bayesian modeling. Please note that good S/N is a requirement for good CRAFT results, 5 should be considered a minimum to prevent anomalous models and the default of 20 is optimal. This value should be decreased only if there are specific peaks you wish to quantify that are not captured with the default parameters. Similarly, the peak resolution defines the distance between frequency models that will be considered distinct by the Bayesian analysis. 5 Hz is a good default value

for most analyses and should be lowered only if the value does not capture specific, overlapping signals. Lowering these values will also increase the computation time, so it may be advantageous when lowering these values to focus on only the specific regions of the spectra that are not adequately modeled with the higher values. Clicking the option for no Bayesian Deconvolution will not perform the full CRAFT analysis, but rather apply an automated peak detection and fitting in the frequency domain. Additionally, if the ROIs are defined automatically, the user will be given the option to omit the residual lock solvent signal from the analysis via a check box.

Let's run a demo analysis on a simple spectrum of ibuprofen as an example. In this case, we will manually select an ROI of the aromatics for a quick analysis and use the default parameters (option A):

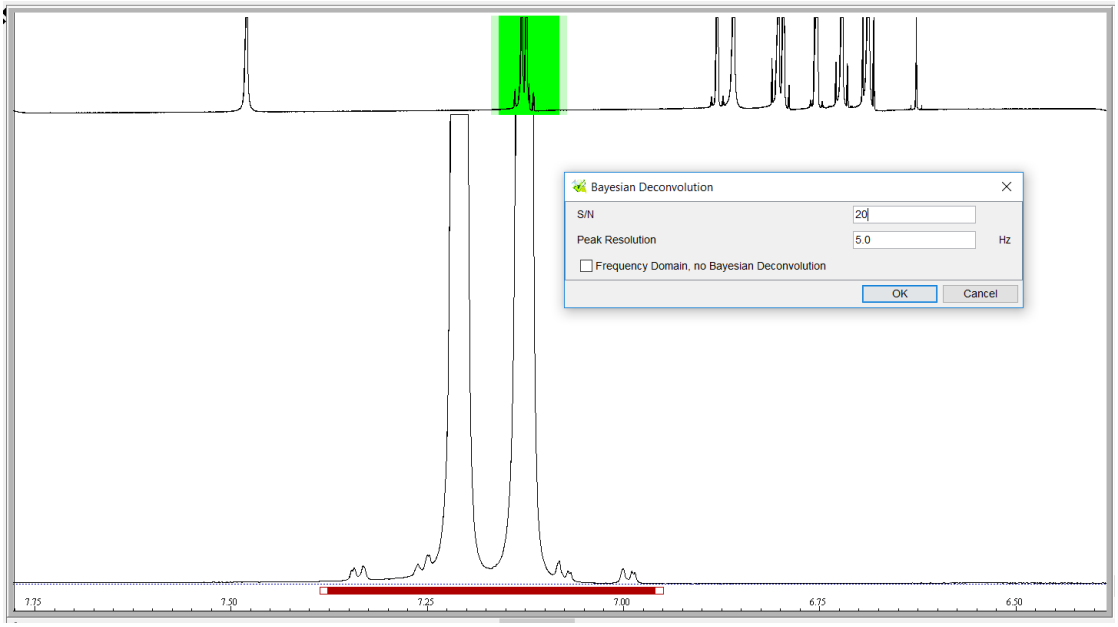



*Figure 4.11: CRAFT analysis results for ibuprofen aromatics.*

The analysis will proceed in background mode and when complete, a message will be displayed indicating the computation time and the number of peaks (or models) found:

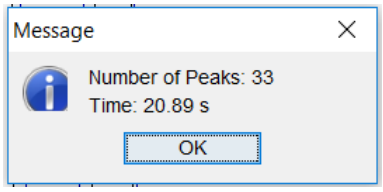



*Figure 4.12: CRAFT analysis completion message.*

Next the user will be given the option to display individual peaks. This can be very useful to visualize the individual models found during the Bayesian analysis in order to select the relevant ones for the desired quantification. The Bayesian analysis proceeds by creating models for the individual frequencies that make up each ROI. The model creation process continues until the residual signal of the difference between the modelled data and the orginal data reaches a defined threshold and/or a specified maximum number of models have been created. Because the analysis is modelling individual frequencies, a multiplet will contain multiple models, e.g. a doublet will have two models, which can be visualized as peaks in the Fourier transformed data. Here are the modeled peaks of ibuprofen aromatics:

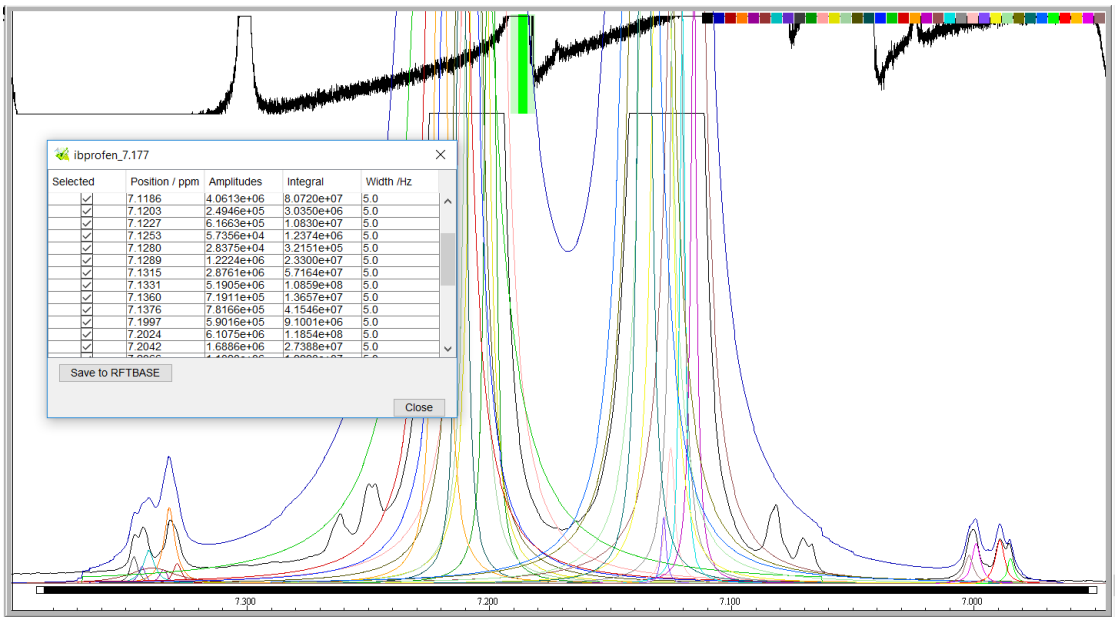
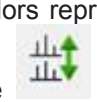



*Figure 4.13: CRAFT analysis results displaying individual peaks for ibuprofen aromatics.*

The black spectrum is the original FT data, the blue is the CRAFTed data, and the red is the residual (or difference). The other colors represent the individual frequency models created from the CRAFT analysis. Note, the  button can be used to separate the models vertically for better visualization if needed, e.g.:

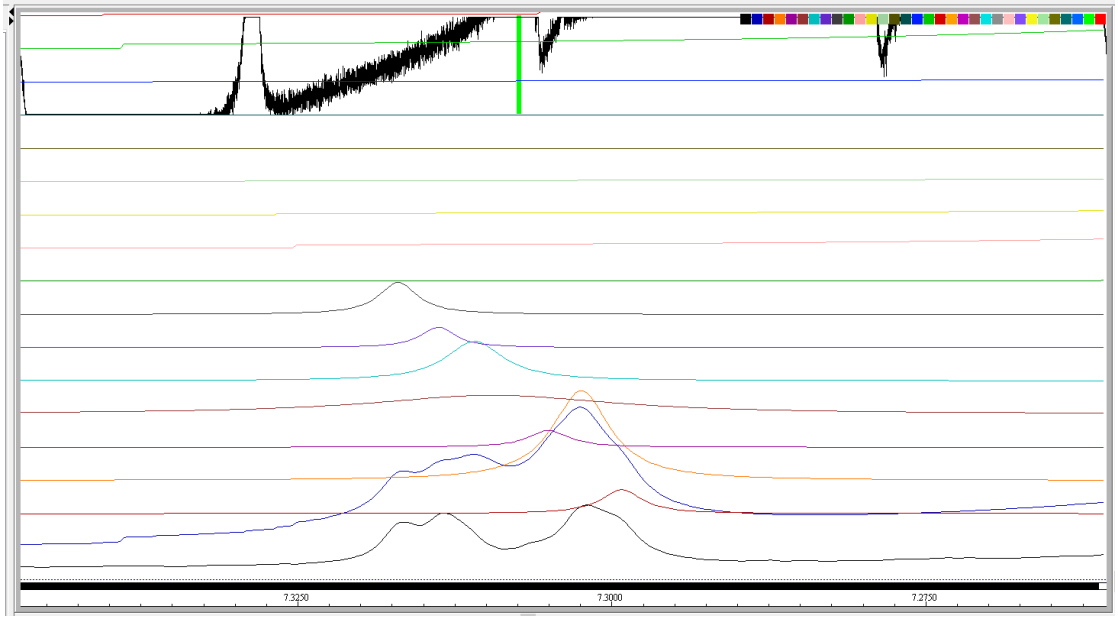



*Figure 4.14: CRAFT analysis results ibuprofen aromatics using vertical offset, zoomed in to upfield 13C satellite region.*

The models are also listed individually in a pop-up table that can be copy/pasted to a spreadsheet or saved to an RFTbase for quantification of other datasets. By default, all the models are selected to copy to the RFTbase, however any can be deselected prior to saving. Note that it is not necessary to include all the models for an entire multiplet for relative quantification between datasets, as long as the same models are compared for each spectrum analyzed. Proper peak alignment is important in these cases, so the tools available in AssureNMR should be utilized to improve consistency between spectra. The amplitudes

may also be used to calculate absolute quantification information, provided a sample of known concentration has been analyzed via the CRAFT methodology and the amplitude value calibrated accordingly.

Next we will use the automatically selected ROIs to analyze the entire ibuprofen spectrum. To perform this analysis, choose the second option, “Automatically“ (B), from the “Choose ROI“ menu. Again, we will use the default parameters, but click the option to “Remove Solvent“.

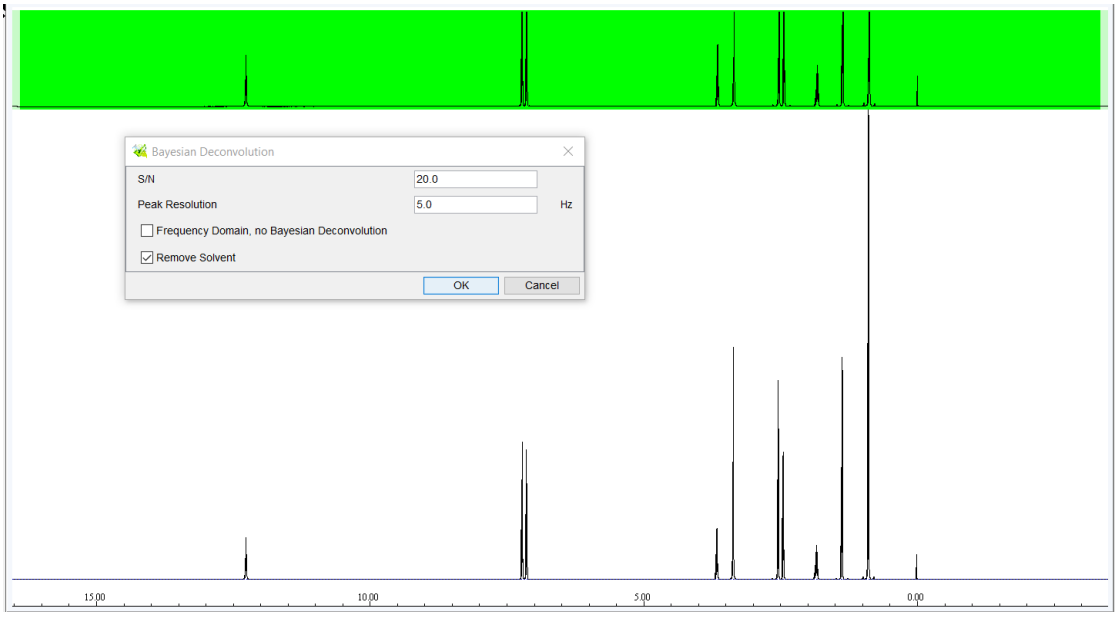



*Figure 4.15: CRAFT analysis for the complete ibuprofen spectrum.*

Because the analysis is performed on the entire spectrum, it will take slightly longer than for a targeted ROI of a smaller region. Below is an expansion of the results for the aliphatic region. Note that because the solvent was removed from the analysis, a closely neighboring signal was not analyzed. If this is undesirable, simply repeat the analysis without the “Remove Solvent” box checked.

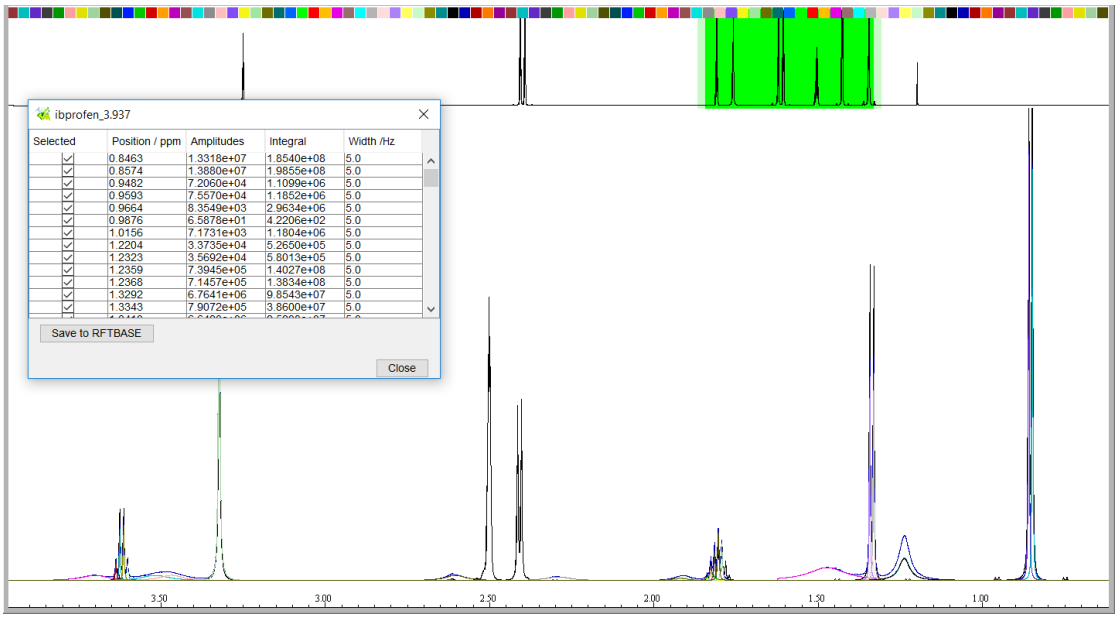



*Figure 4.16: CRAFT analysis results for the complete ibuprofen spectrum.*

Also some broad models can be observed flanking a few of the peaks. These are artifacts that can arise at the edges of the ROIs from the digital filtering of the FID. However, these models do not affect the accuracy of the amplitude of the peaks of interest. They can either

be ignored when performing the quantification, or, if this is to serve as as a reference, these undesired models should be deselected in the table prior to saving to an RFTBASE. If these broad models appear to be interfering with the creation of a model for a peak of interest (say a carbon satellite in the spectrum above), simply increase the width of the ROI to allow these artifacts (should they arise) to occur outside the area of interest for analysis.

The next option in the “Choose ROI” menu (option C), is to import them from an Excel list. Below is an example of the proper formatting to use in the Excel list:

| | A | B | C |
|---|---|---|---|
| 1 | from | to | |
| 2 | 4.4 | 4.288 | |
| 3 | 4.23 | 4.08 | |
| 4 | 2.9 | 2.77 | |
| 5 | | | |
| 6 | | | |

*Figure 4.17: Example of ROIs from Excel spreadsheet (option C).*

Moving away from the simple example of ibuprofen, CRAFT analysis can also be very useful for complex mixtures with overlapping signals. For example, the results picture below are for a sample of blueberry extract using an ROI targeting a particularly overlapped region of aromatic signals.

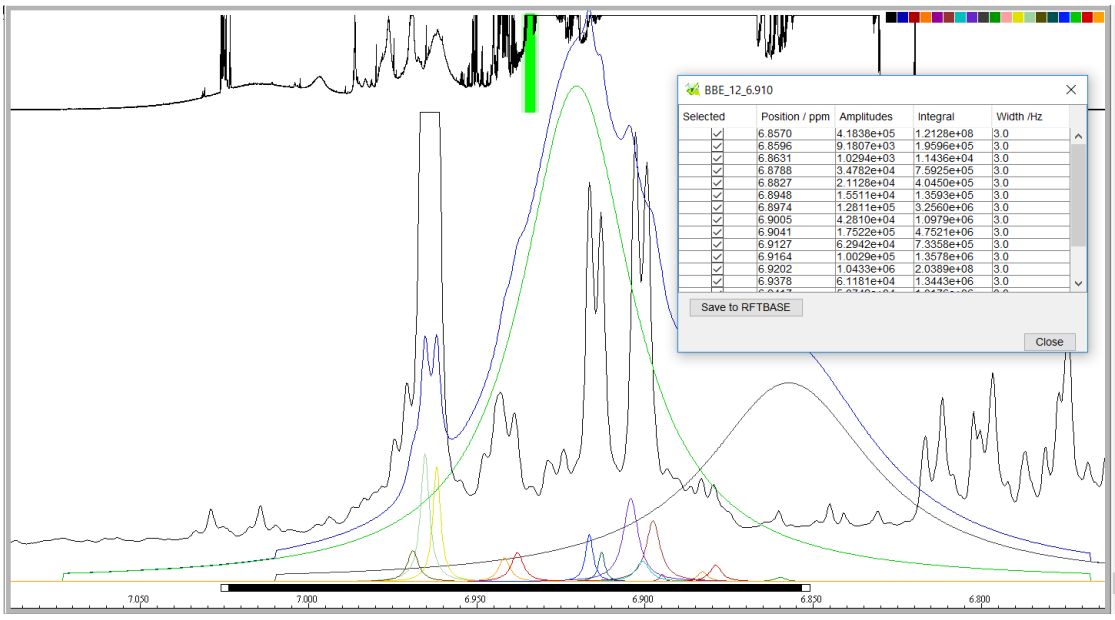



*Figure 4.18: CRAFT analysis results for the blueberry extract spectrum.*

One can see from the individual models displayed above that CRAFT can obtain pure amplitude estimates for many of the components even in this highly crowded region of the spectrum.

Now let’s use CRAFT to create an RFTbase from a reference spectrum of chlorogenic acid and use this to analyze an extract from a variety of blueberrry. (Detailed instructions on the creation and registration of RFTbases, which is not specific to CRAFT alone, can be found in Chapter 5). Below is the CRAFTed reference spectrum of chlorgenic acid (using a S/N of 5). For this example, we have chosen to focus on the doublet at ~6.1 ppm for quantification.

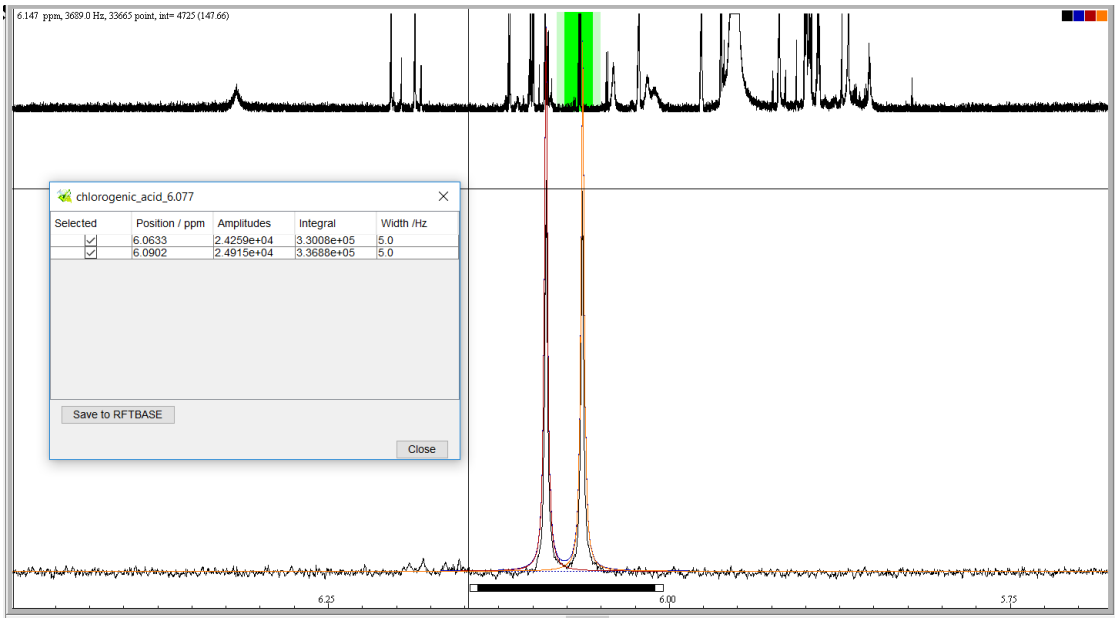



*Figure 4.19: CRAFT targeted analysis of chlorgenic acid for the blueberry extract spectrum.*

Both peaks of the doublet were selected and saved to an RFTBASE called “test“ using only the confirmed peaks:

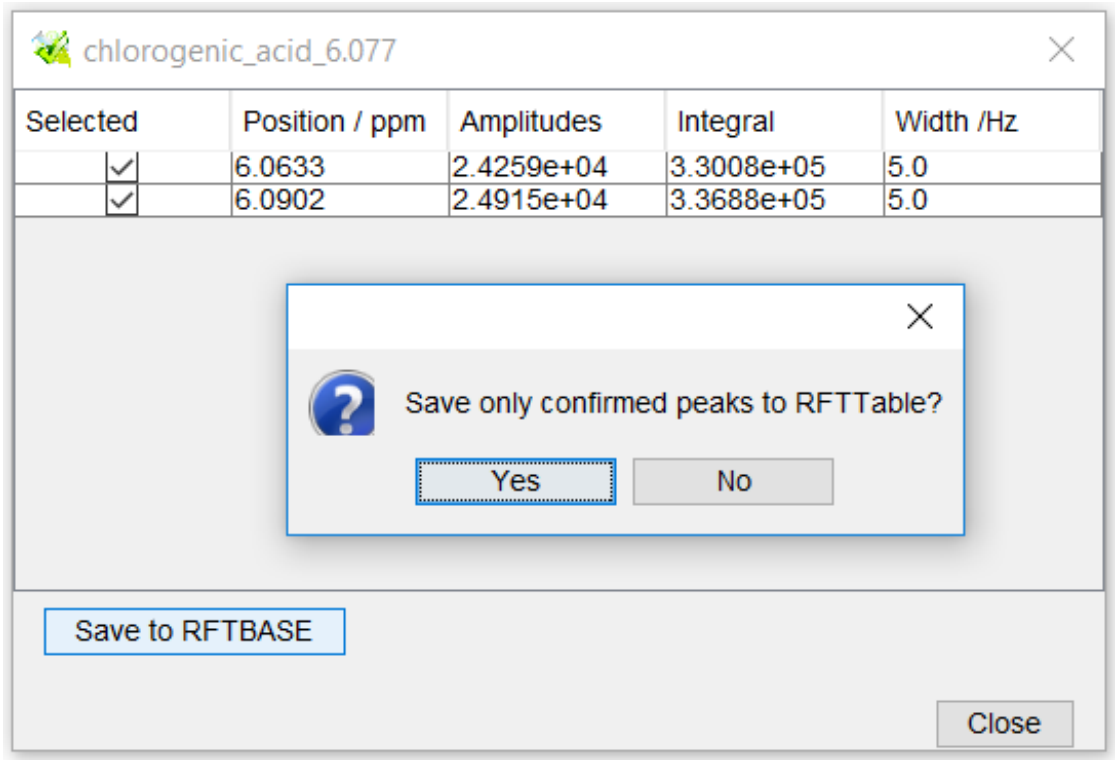



*Figure 4.20: Creation of a CRAFT RFT Table.*

Next a spectrum of blueberry extract targeted for analysis was opened and a CRAFT analysis using the RT table (option D) just created was initiated. Below are the results of this analysis:

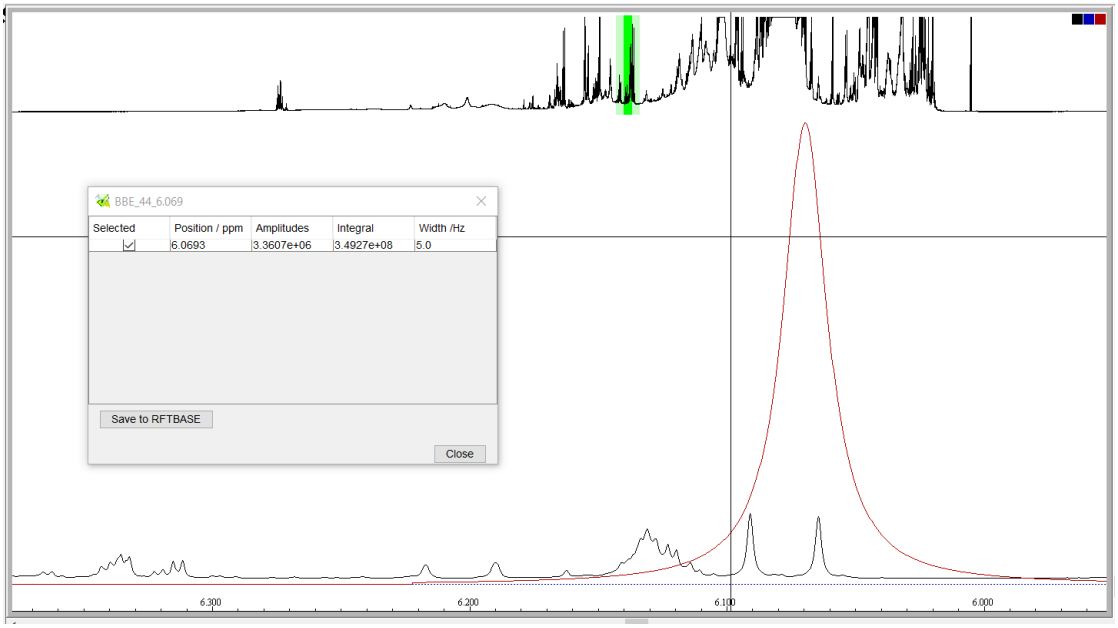



*Figure 4.21: Use of a CRAFT RFT Table for a targeted analysis of chlorgenic acid in blueberry extract.*

The model shown is the sum of the chlorgenic acid peaks found within the ROI of the sample spectral data corresponding to those of the standard stored in the RFTBASE. The extracted amplitudes may be compared directly to obtain a quantitative measure of chlorgenic acid in the blueberry extract sample. More analyses can easily be added to the RFTBASE and/or pulled together in a group from different databases if there are additional components to be tracked in the samples. “Group CRAFT Tables” may be found under the “Analysis” menu. The RFT Group created in this way may then be used for ROI selection (option E).

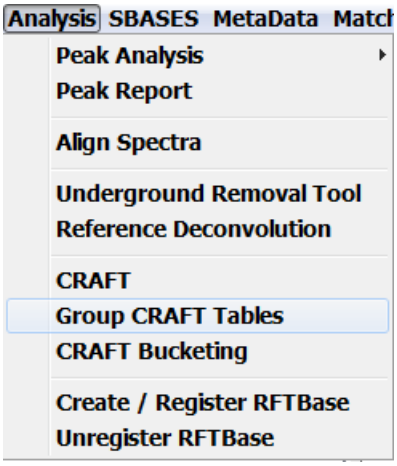



*Figure 4.22: CRAFT-related options in the pulldown menu under Analysis.*

It may also be of interest to use the bucket table capabilities of AssureNMR to specify general spectral regions to focus upon or ignore for an untargeted analysis of a series of spectra. Section 8.1 describes in detail how to create new bucket tables based on integration - the process is similar for the CRAFT bucketing tables. For this example, we will use a series of different blueberry extract spectra. The analysis will target a portion of the aromatics in the sample containing signals from key antioxidents, while excluding the regions of the spectra containing the sugar protons, which are of less interest.

The regions of interest for CRAFT bucketing are controlled by the ROIs used, and the bucketing window allows for additional control of excluding some of these regions (or portions within them) for the bucketing analysis itself.

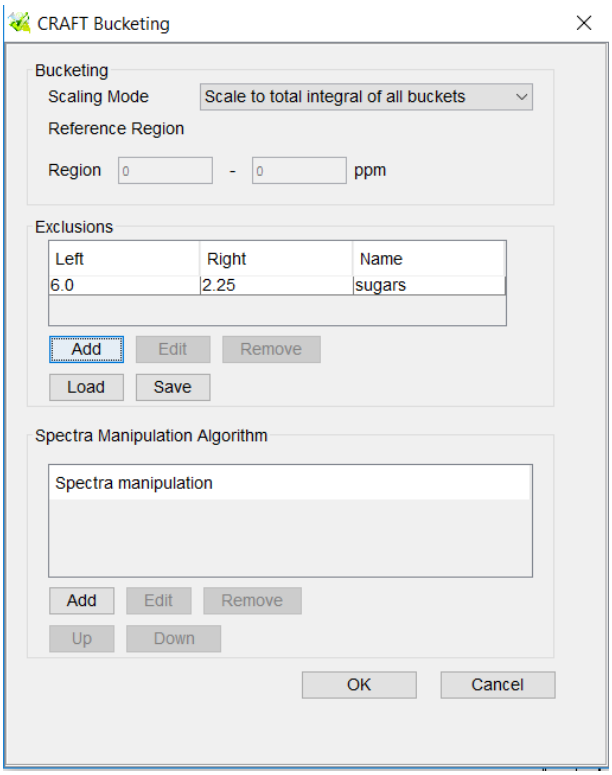



*Figure 4.23: CRAFT Bucketing pop-up window.*

Once the CRAFT bucketing is complete, it can be subjected to all the same AssureNMR chemometric tools that are available for integral-based buckets. For instance, the quantile plot of the CRAFT bucketing for the blueberry sample aromatics is shown below:

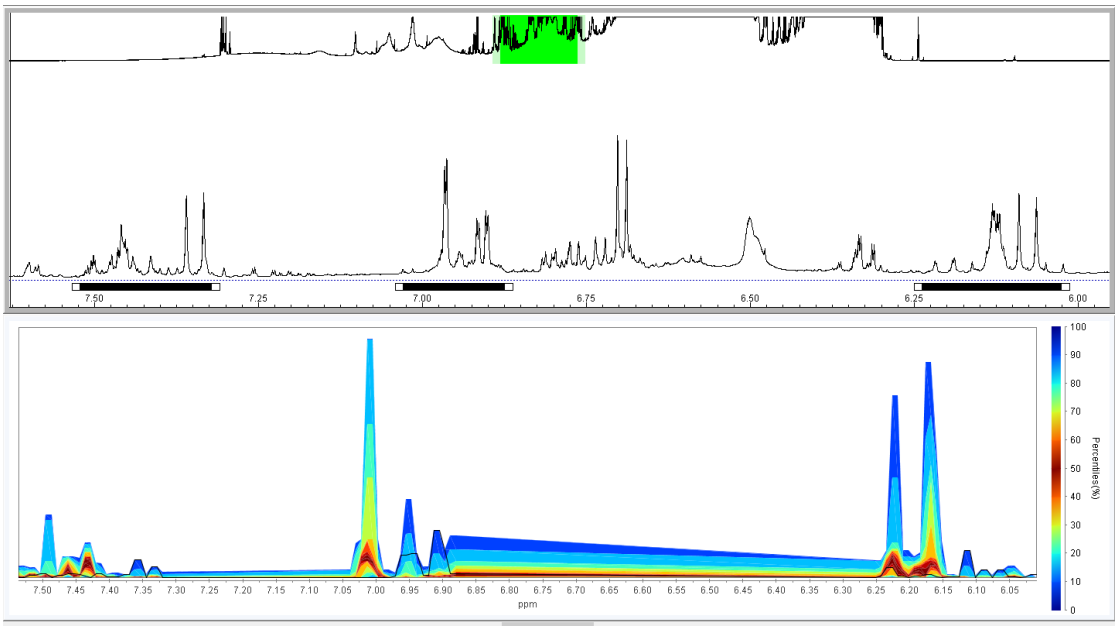
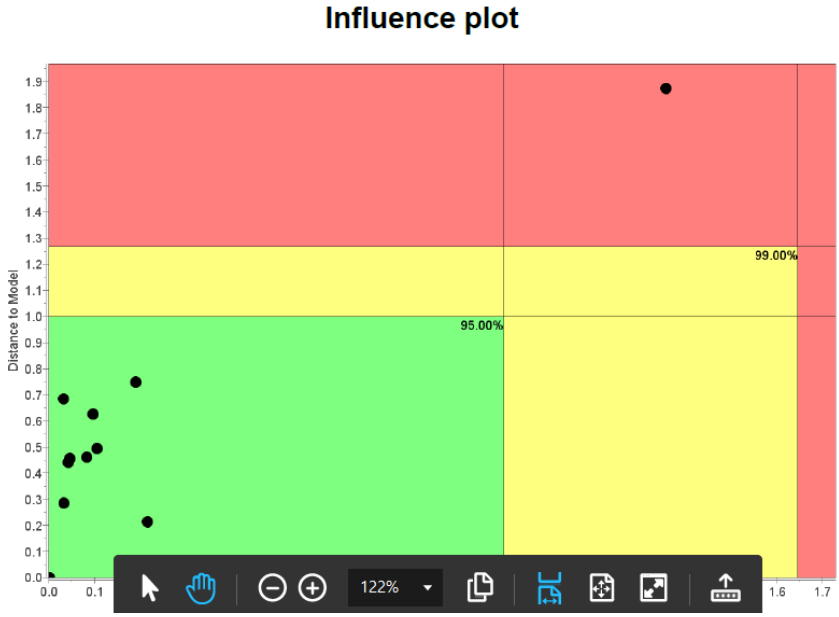



*Figure 4.24: Quantile plot results from CRAFT Bucketing for blueberry extract samples.*

Some of the peaks show quite a bit of variation between the samples, indicating that it may be a key component for distinguishing among the blueberry varieties. PCA or other statistical models may also be constructed from the CRAFT bucketing results. Below is the influence plot of a SIMCA model built from the CRAFT bucket table of the blueberry samples. Most of the samples cluster well, based on this analysis of three aromatic regions, but one clear outlier is present that may be interesting to consider for closer analysis.

**Scaling mode: No scaling**
**Number of PCA components: 8**
**Explained variance: 97.4%**
**$Q^2$: 0.12**
**Minimum confidence level: 95.0%**
**Maximum confidence level: 99.0%**




PCA plot results from CRAFT Bucketing for blueberry extract samples.

As you can see, the CRAFT tools provide an alternate way to approach the quantification of a single data set or series of data. The methodology's approach of using the time-domain data has particular strengths for overlapping peaks and data with baseline and phasing issues that may give poor results with FT data-based methods such as integration and peak deconvolution.

## 4.4 SBASES Menu

Within the **SBASES** pulldown menu, there are options for registering and unregistering SBASEs, creating a new SBASE, to import an SBASE entry from CMC-assist, and to open the preparation module. An SBASE must be registered before it can be used in AssureNMR. Importing spectra from CMC-assist and preparing spectra for an SBASE is explained in detail in Chapter 5.

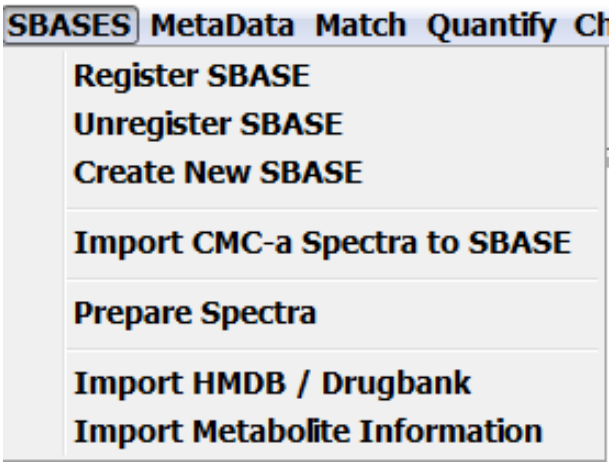



*Figure 4.25: SBASES pulldown menu.*

To register the SBASEs delivered with AssureNMR (assuming the default directory installation), select **Register SBASE** and browse to:

C:\Bruker\Databases\SBASE\AssureNMRsbase1

**Unregister SBASE** makes that reference spectral database unavailable for use in AssureNMR, although the user can decide to leave the files on the computer. **Create a New SBASE** allows the user to build their own reference spectral database.

Note: To open and close the SBASEs tab in the browser window, go to the **File** pulldown menu (**Open/Close SBASE Browser**).

### 4.4.1 HMDB and DrugBank

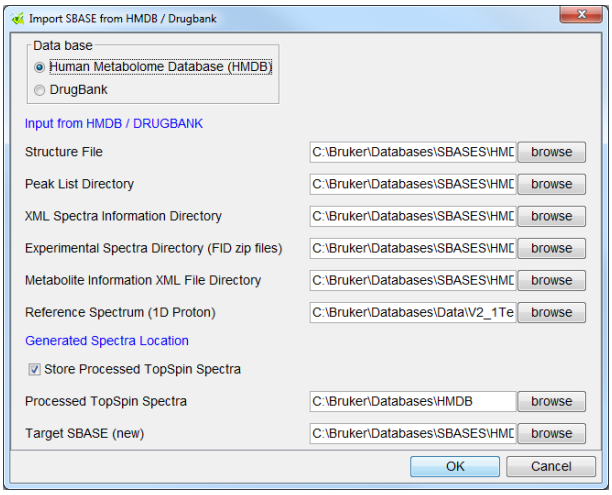
Two public databases, The Human Metabolome Database (HMDB, *http://www.hmdb.ca/*) and Drug and Target Database (Drugbank, *http://www.drugbank.ca/*) are in a format suitable that may be used with AssureNMR. Permissions to use the databases are granted through their developers. These databases are offered to the public as a freely available resource. Use and re-distribution of the data, in whole or in part, for commercial purposes requires explicit permission of the authors and explicit acknowledgment of the source material (HMDB or DrugBank) and the original publication (see below). We ask that users who download significant portions of the database cite the HMDB and DrugBank papers in any resulting publications. Please see the provider's website for additional information. Refer to the tutorial section of this manual for instructions of how to import these databases for use within AssureNMR.

HMDB Citations:

1. Wishart DS, Tzur D, Knox C, et al., HMDB: the Human Metabolome Database. Nucleic Acids Res. 2007 Jan;35(Database issue):D521-6. 17202168
2. Wishart DS, Knox C, Guo AC, et al., HMDB: a knowledgebase for the human metabolome. Nucleic Acids Res. 2009 37(Database issue):D603-610. 18953024
3. Wishart DS, Jewison T, Guo AC, Wilson M, Knox C, et al., HMDB 3.0 — The Human Metabolome Database in 2013. Nucleic Acids Res. 2013. Jan 1;41(D1):D801-7. 23161693"

DrugBank Citation:

Wishart DS, Knox C, Guo AC, Shrivastava S, Hassanali M, Stothard P, Chang Z, Woolsey J. DrugBank: a comprehensive resource for in silico drug discovery and exploration. Nucleic Acids Res. 2006 Jan 1;34(Database issue):D668-72. 16381955.




## 4.5 MetaData Menu

The **MetaData** menu allows the user to associate additional information with the spectra (i.e. supplier, concentration, source, purification method, etc.). The spectra can be the original TopSpin files, SBASE entries, or spectra associated with a bucket table. The information can be entered by the user (**Edit Meta Data**) or read in from an Excel spreadsheet (**Import Meta Data from Excel**). The metadata for a set of spectra can also be exported to an Excel spreadsheet (**Export Meta Data to Excel**). Metadata are particularly helpful when dealing with a large number of samples. The MetaData window allows sorting for easy access to specific spectra. All metadata are stored with the spectrum. For spectra in TopSpin format the metadata are also available in TopSpin (under the “Sample Info” tab).

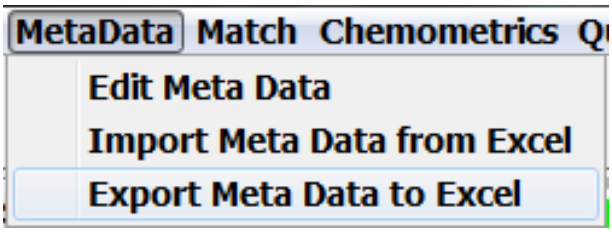



*Figure 4.26: MetaData pulldown menu.*

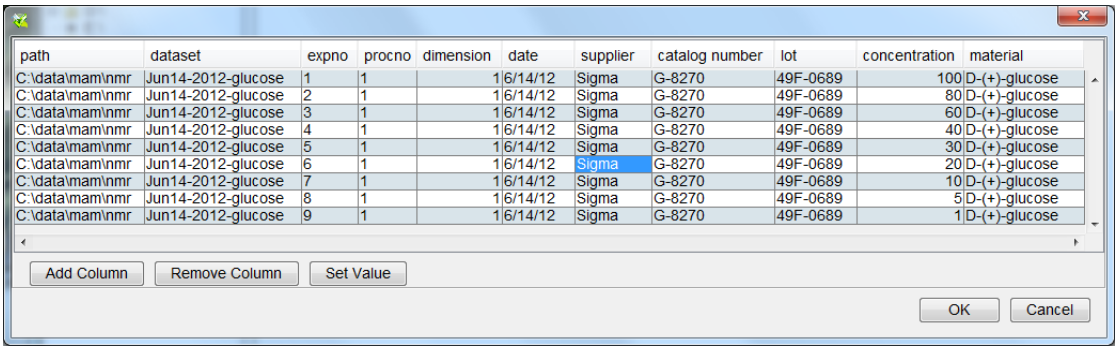



*Figure 4.27: Example of metadata associated with spectra in AssureNMR.*

## 4.6 Match Menu

The **Match** menu contains tools for matching the spectrum on screen to an SBASE, matching several spectra at the same time, and specifying additional information for the JRES spectra.

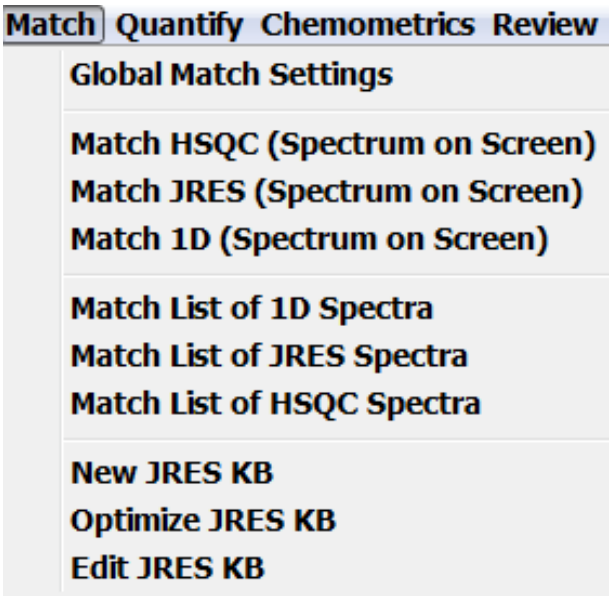



*Figure 4.28: Match pulldown menu.*

In the first section, the user can match the spectrum on screen against an SBASE, to identify components in the sample. The spectrum must be loaded into the viewer window. All visible peaks are used for the match. The contour levels can be adjusted as desired; the lowest contour is taken as the noise level.

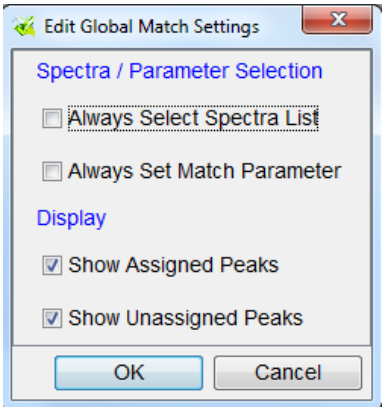



*Figure 4.29: Global Match Settings*

'Global Match Settings' options under 'Spectra / Parameter Selection' provide options for 'Identify this Peak' match functions performed from the interactive window. Selecting 'Always Set Spectra List' and "Always Set Match Parameter" allows the user to select the options everytime the 'Identify this Peak' is called. When these options are not selected, the program asks for the spectra list and parameters the first time "identify this peak' is called on the active spectrum. 'Reset at match' removes the default match selection for the active spectrum and initiates a dialog to reselect the spectral list and match parameters.

'Display' in 'Global Match Settings' are used to filter the resulting match results according to 'Assigned Peaks' and 'Unassigned Peaks'. This is particularly useful when working with mixtures where it is desirable to know which identified metabolites resonances remain to assigned.

There are separate options for different types of spectra: **Match HSQC (Spectrum on Screen)**, **Match JRES (Spectrum on Screen)**, and **Match 1D (Spectrum on Screen)**. The user has the opportunity to specify the spectra from SBASEs to match against and the parameters for matching. The spectra used to match against are automatically analyzed. This information is stored with the SBASE spectra and is available for further matches. Because of this initial step, the first match match calculation may take more time.

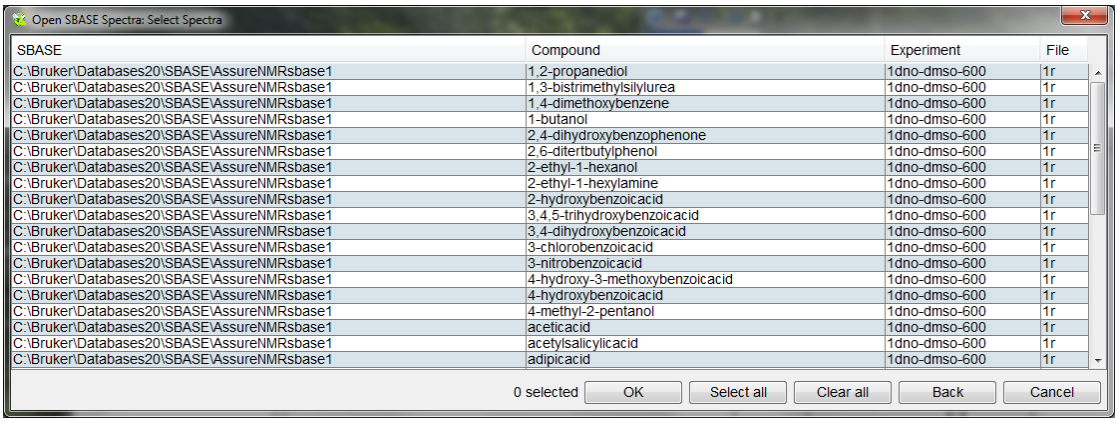



*Figure 4.30: Open SBASE Spectra Select Spectra window.*

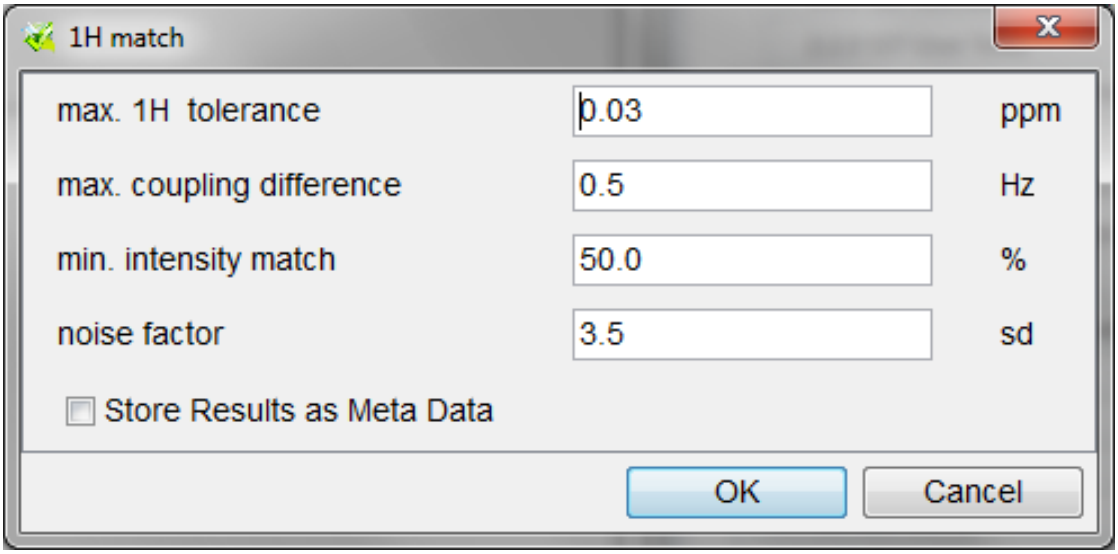



*Figure 4.31: 1H match window to specify match parameters.*

After the calculation runs, a window containing the results for each SBASE entry will appear. Clicking on the row for the compound entry in the top part of the window causes the details for each region of that compound to be displayed in the bottom part of the window. In addition, that SBASE entry will be superimposed on the spectrum displayed in the viewer window. This makes it convenient for the user to inspect the match.

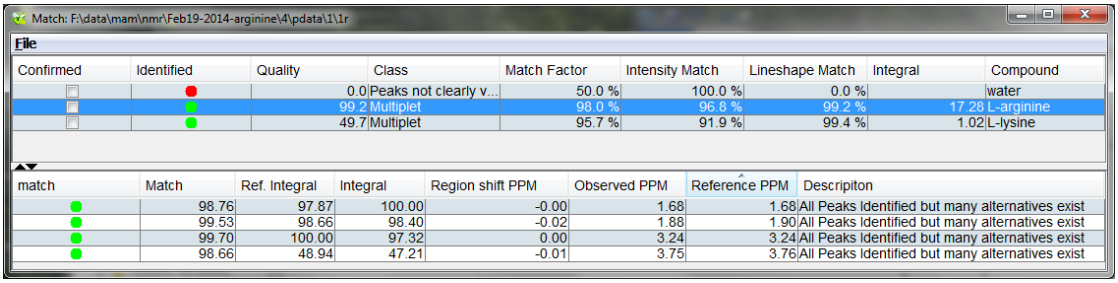



*Figure 4.32: Match results window for a single spectrum.*

The commands **Match List of 1D Spectra**, **Match List of JRES Spectra**, and **Match List of HSQC Spectra** run much like the match spectrum on screen options, except now the first step is to specify the list of spectra to match. Again, the user is prompted for the SBASE spectra to match against and the parameters for the match. After the calculation runs, a table pops up, summarizing the results for each spectrum. Clicking on the row for that spectrum in

the table brings up the summary table by compound for that spectrum and opens the spectrum in a new window within the viewer window. This enables the user to explore the match as for matching a spectrum on screen.

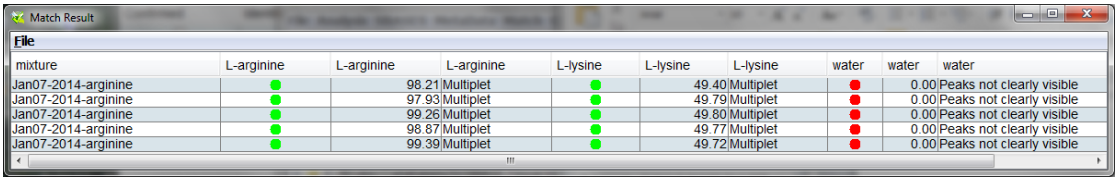



*Figure 4.33: Match Result window for a list of spectra.*

When matching JRES spectra, it can be helpful to provide more information. The menu commands **New JRES KB**, **Optimize JRES KB**, and **Edit JRES KB** let the user create a knowledgebase for the JRES match. In the first step, the SBASE spectra to match against are selected. In the next step, the details of the match are specified, compound-by-compound in the upper part of the window, region-by-region for the compound in the lower part of the window. For each compound, the user can specify:

- Experiment: the spectrum to use for matching
- Quant Ref: which compound (if any) to take as the quantification standard
- is Expected: compounds that are expected are matched first. Note that the peaks matched are then no longer available for further matches.
- min Match [%]: the minimum match factor required to consider the compound a match
- min. LS Match[%]: the minimum lineshape match required
- min Quality[%]: the minimum quality factor required for a match
- check Int. Ratio: option to check the intensity ratio of the peaks while matching
- 1H Shift [ppm]: allowed $^{1}H$ chemical shift tolerance for a match
- max. Coup. diff [Hz]: allowed coupling tolerance for a match
- overlap allowed: Sometimes multiplets are not identified due to overlap with other peaks. Activating this option allows something to match even when a specified fraction of the signals (next parameter) are missing.
- % overlapped signals: extent to which signals can be overlapped and thus not identified and still give a match. For example, at 0%, all the signals must be identified.
- ignore small regions: Sometimes it may be helpful to ignore regions with low intensity. Checking this ignores regions with signal intensity less than that set by the next parameter.
- % integral: This is the signal threshold for this compound, defined relative to the highest signal.
- Noise Factor: multiplicative factor for noise level to set threshold for signals detection. When set to -1, the software automatically determines the threshold.
- LOD[%]: limit of detection, compared to the highest signal, corrected for the number of nuclei contributing to the signal
- Comment: open field for entering information

For each region of the compound, the user can specify:

- rel. Integral[%]: fraction of signals allowed to be missing due to overlap for the region
- Identify: check if this this region will be identified
- Peak Assignment Required: check if this region must be identified and assigned for a successful match
- Clear Cut Peaks: peak pick again to focus on stronger peaks

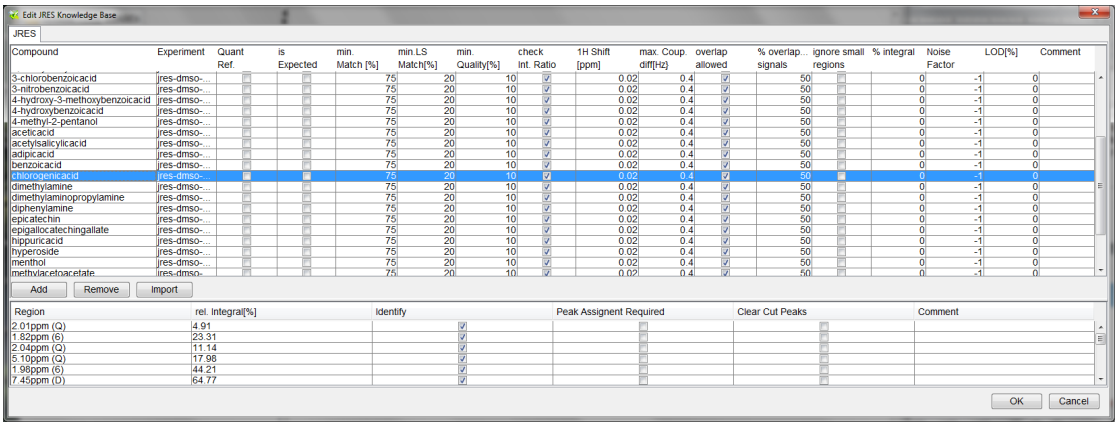



*Figure 4.34: Edit JRES Knowledge Base.*

The command **New JRES KB** lets the user create a new knowledge base. **Edit JRES KB** lets the user edit an existing knowledge base. **Optimize JRES KB** automatically sets the parameters for the knowledge base, one compound at a time. The user must supply examples of spectra with and without the compound of interest.

## 4.7 Quantify Menu

When AssureNMR runs in automation, a quantification method is used to give the parameters for the analysis and report. Automated chemometric analysis is called through the quantification method. All of the tools for building quantification methods (quantMethods) and running analysis interactively can be found in the **Quantify** menu. The details of building a quantMethod are found in Chapter *Quantification in AssureNMR [▶ 73]*.

For customers with AssureNMR Launch licences, 'Calibration Standard Definition' may be used. Refer to Chapter *Quantification in AssureNMR [▶ 73]* for more information.

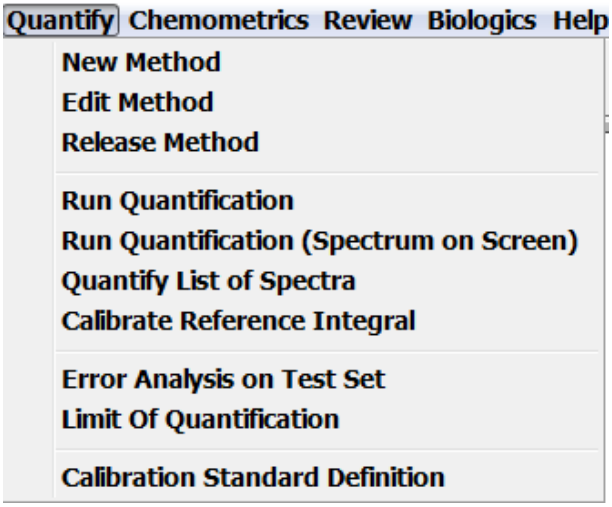



*Figure 4.35: Quantify pulldown menu.*

## 4.8 Chemometrics Menu

The **Chemometrics** menu facilitates the statistical analysis of spectra. The **BucketTable** menu provides tools for generating, organizing, and analyzing bucket table. The **Quantile Plot** option shows the quantile plot for the currently loaded bucket table. The **SIMCA Outlier Detection** menu includes options to **Load SIMCA Model**, **Create New SIMCA Model**, and

**Classify Spectra** against a model. The **Multi-Class** menu allows the user to **Create Model** and **Classify** spectra. The **PLS Regression** menu reveals tools to **Load PLS Model**, **Calibrate New PLS Model**, **Prediction** of values and **Validation** of the model. The Chemometrics tools in AssureNMR are covered in more detail in Chapter *Chemometric Modeling in AssureNMR [▷ 125]*.

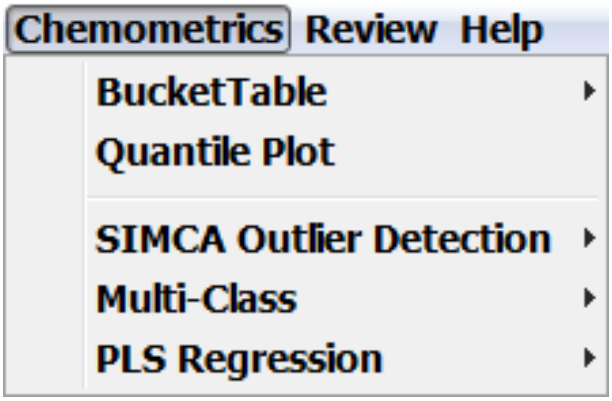



*Figure 4.36: Chemometrics pulldown menu.*

## 4.9 Review Menu

The **Review** menu provides tools for checking results. AssureNMR features a tool with which the expert user can revise the peak assignments from the automated analysis, called the **Expert Review Editor**. Peaks can be ignored, reassigned, or added. Then the spectrum can be reanalyzed. Manual changes to the analysis are documented in the revised reports. **Validate Result Checksum** provides a way to be sure the results have not been changed. **Method Material Information** provides a place to store information about the types of samples expected for the method. More details are provided in the following sections.

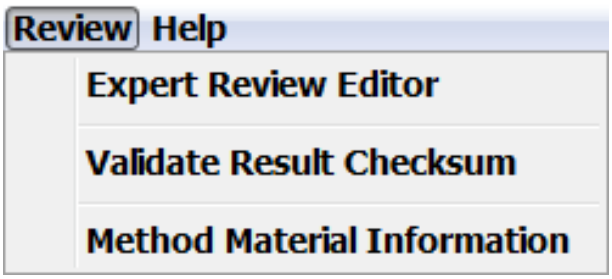



*Figure 4.37: Review pulldown menu.*

### 4.9.1 Expert Review Editor

Whenever an analysis runs in AssureNMR, the information necessary for review is stored in a file called “ReviewInfo” in the output directory. In automation, this output directory is the data directory (NAME/EXPNO). In the interactive analysis window, it is the directory specified as the Result Directory. When **Expert Review Editor** is selected from the Review menu, the user is prompted to find the appropriate ReviewInfo file. The software then loads the spectrum from the previous analysis, complete with peak markers, peak labels for compounds in the quantMethod, analysis regions, and integrals.

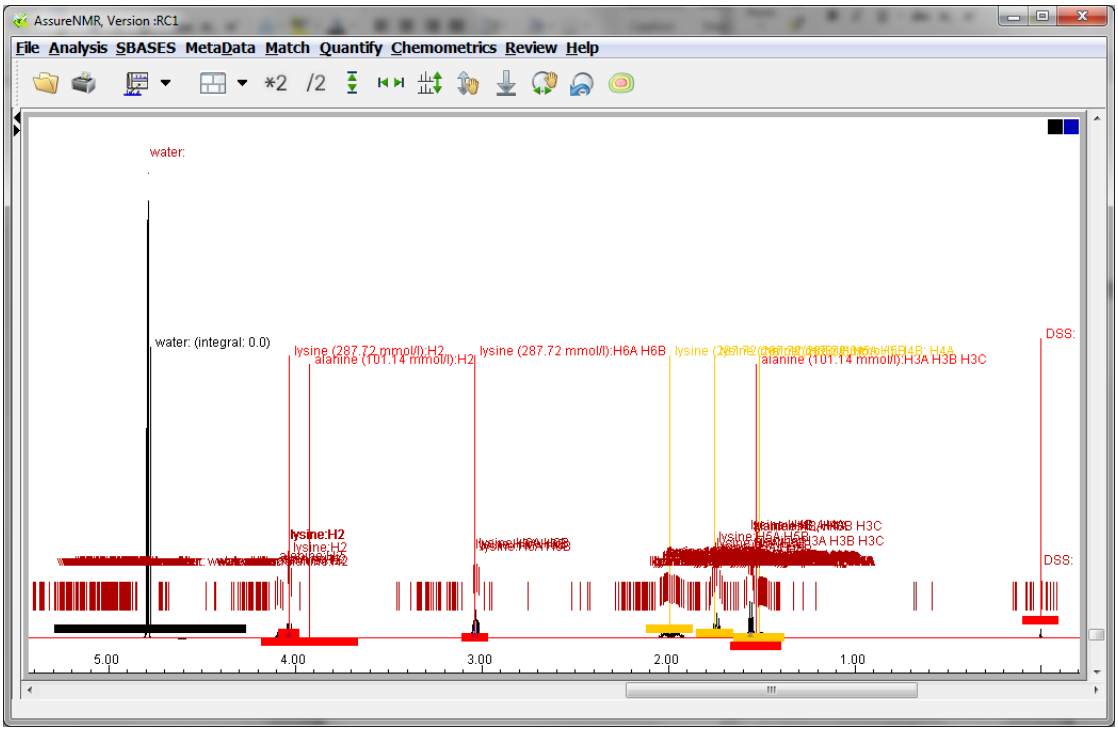



*Figure 4.38: AssureNMR interactive analysis window after loading the ReviewInfo, showing peak markers, peak labels, assignment region bars, and shaded integrals.*

The functions of the Expert Review Editor are accessed with a right click in the window containing the spectrum. A menu pops up:


As you scroll down the menu, the different options are highlighted and can be selected with a click of the left mouse button. For **Add Peak**, the cursor position when you brought up the menu with the right mouse click is the reference position. A menu will pop up, allowing you to assign the new peak using the compounds identified in the method and make a comment:

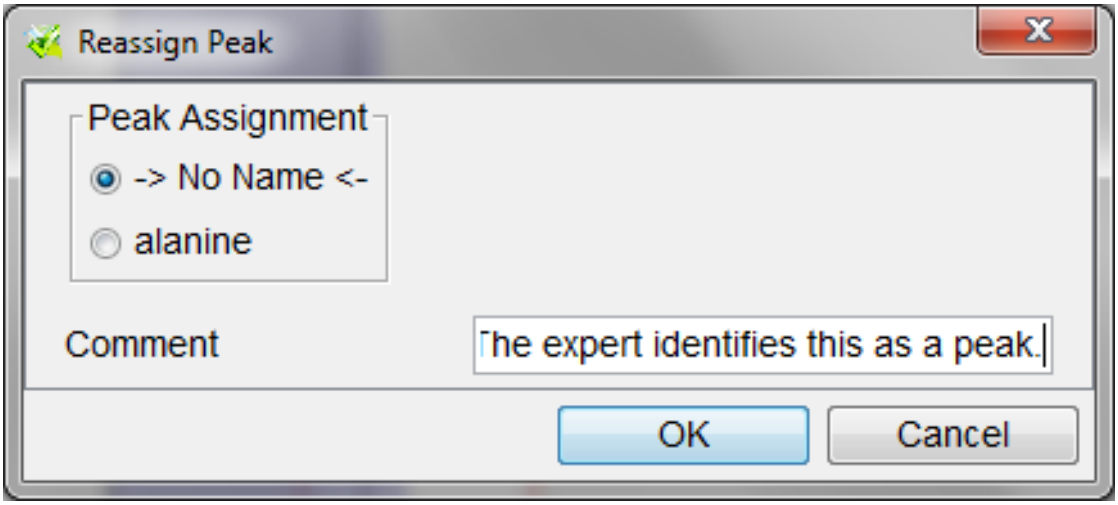
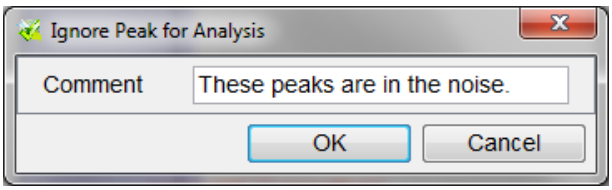
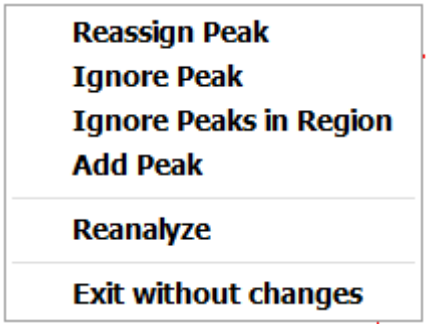



*Figure 4.39: Reassign Peak window in the Expert Review Editor.*

For **Ignore Peaks in Region**, the left mouse button is active to draw a box around the peaks you would like to ignore. Again, a window pops up to allow the expert user to make a comment, explaining the change:

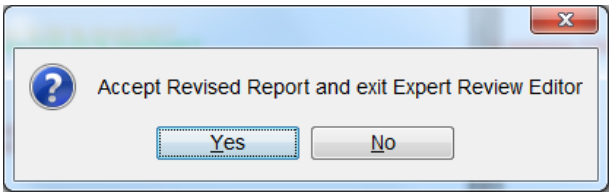



When you right click with the cursor over a peak marker, a longer menu pops up:




**Reassign Peak** lets the expert change the assignment from the one determined in automation to another compound in the method. **Ignore Peak** causes the peak to be ignored in the next analysis. As above, the expert is prompted for a comment in both cases. **Ignore Peaks in Region** allows the expert to draw a box around a group of peaks to ignore. **Add Peak** allows the expert to identify and assign a peak that was not included in the previous analysis.

**Reanalyze** runs the quantMethod with the changes to the peak interpretations. The QC Report and Expert Report pop up automatically for inspection. The new analysis is also displayed in the viewer window. A window pops up, giving the expert the option to accept or reject the changes after inspection of the results:




The Assure output files including the review information are stored in the results directory with the extension Rev1 (ExpertReport_Rev1.pdf, QCReport_Rev1.pdf, ExcelReport_Rev1.xls, monitor_Rev1.csv, RMSChecksum_Rev1, and ReviewInfor_Rev1). For further reviews, the extension is incremented (Rev2, Rev3, etc.).

**Exit without changes** allows the expert to leave without keeping the changes made during review, without running the quantMethod.

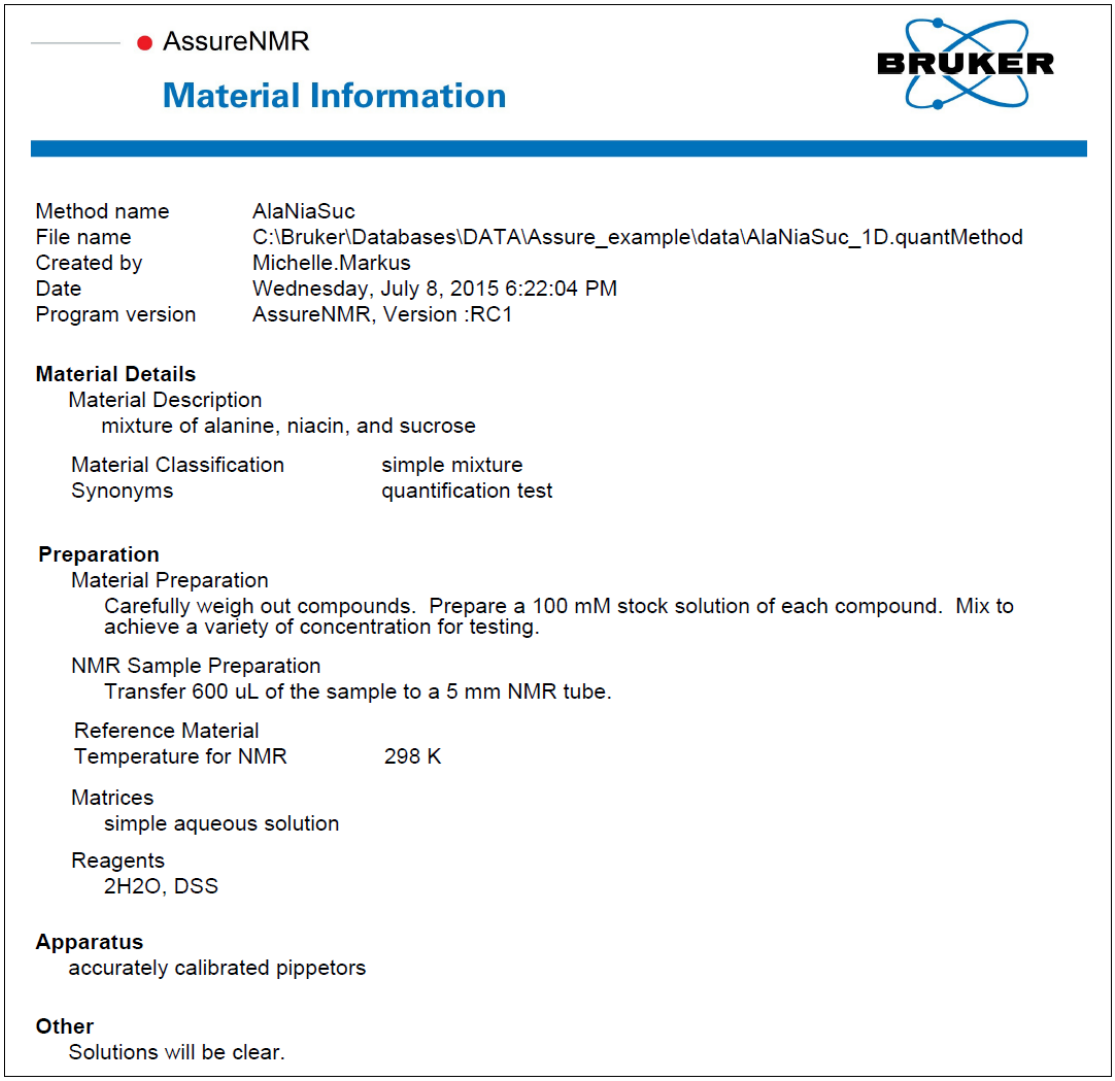
The Expert Review Editor can also be invoked from the IconNMR AssureNMR: Automation window during data acquisition. For details, see Chapter 7.

### 4.9.2 Validate Result Checksum

**Validate Result Checksum** confirms that the results of a calculation have not been changed. It also reports stored information about the calculation, including the user, the program version, the date, and the host.

### 4.9.3 Method Material Information

When building an analysis method (Chapter 6), the user has the option to specify information about the samples expected for that method. The command **Review/Method Material Information** allows the user to see that information. The user is prompted to specify which method they would like to look at. A pdf file summarizing the information opens up.



AssureNMR

**Material Information**

BRUKER

| | |
|---|---|
| Method name | AlaNiaSuc |
| File name | C:\Bruker\Databases\DATA\Assure_example\data\AlaNiaSuc_1D.quantMethod |
| Created by | Michelle.Markus |
| Date | Wednesday, July 8, 2015 6:22:04 PM |
| Program version | AssureNMR, Version :RC1 |

**Material Details**

Material Description
mixture of alanine, niacin, and sucrose

| | |
|---|---|
| Material Classification | simple mixture |
| Synonyms | quantification test |

**Preparation**

Material Preparation
Carefully weigh out compounds. Prepare a 100 mM stock solution of each compound. Mix to achieve a variety of concentration for testing.

NMR Sample Preparation
Transfer 600 uL of the sample to a 5 mm NMR tube.

| | |
|---|---|
| Reference Material | |
| Temperature for NMR | 298 K |

Matrices
simple aqueous solution

Reagents
2H2O, DSS

**Apparatus**
accurately calibrated pippetors

**Other**
Solutions will be clear.

*Figure 4.40: Material Information report.*

## 4.10 Help Menu

The **Help** menu has an option to display the manual that came with the Assure software (**Manual**). **About** shows the version of the Assure software running and provides contact information for Bruker software support. **License Info** gives details about the components of the software that are used. **AssureNMR License** opens the pdf file detailing the license agreement under which Bruker provides the software.

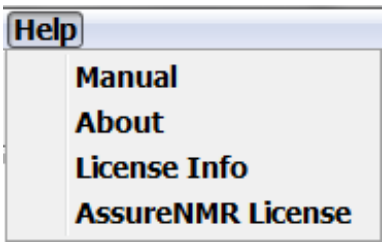



*Figure 4.41: Help pulldown menu.*

# 5 SBASEs in AssureNMR

The AssureNMR software utilizes SBASEs for the identification of components within a material. An SBASE (spectral database) contains the reference spectra for the components to be identified. Information stored within the SBASE can be read into a quantMethod within AssureNMR.

Typically, the user sets up one SBASE for each type of sample (for example, the alanine test samples for AssureNMR). This SBASE contains entries for each component in the samples (for the test samples: alanine, lysine, water, and DSS). Because the user needs to create SBASEs for the materials they screen, this chapter explains that process. AssureNMR comes with an SBASE that includes many common compounds. Custom SBASEs can be generated for users through Bruker Analytical Services. Also see chapter *Importing a Spectrum from CMC-assist [▶ 71]* that addresses how to directly import a compound from CMC_assist into Aussre NMR.

For some applications, generating an SBASE entry can be as easy as picking the peaks of interest, removing noise from the spectrum, and saving. But for more general method development, it is useful to have more information associated with the SBASE entry. Then the first step in generating an SBASE is to make assignments and correlate them with the chemical structure of the compound. Once the assignments are made, this information is stored with the spectrum and can be imported directly into AssureNMR.

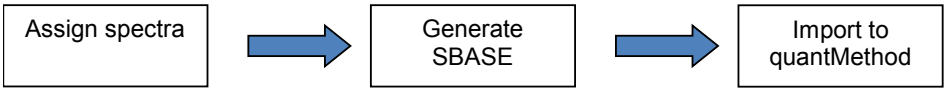
## 5.1 SBASE Registration and Requirements

AssureNMR is delivered with an SBASE. In order to use an SBASE, it must be registered in the AssureNMR software. Whenever an SBASE is transferred from one computer to another, it also needs to be registered on the local machine. To register an SBASE, use the **SBASES** pulldown menu (Chapter *SBASES Menu [▶ 51]*) and select **Register SBASE**, then navigate to the SBASE parent directory. For example, the SBASE provided with AssureNMR is typically found in C:\Bruker\Databases\SBASE\AssureNMRsbase1. (See Chapter for default directories.)

SBASEs registered with AMIX versions 3.9.4 or earlier must be unregistered via AMIX and re-registered with AssureNMR or AMIX version 3.9.7 or greater.

In the event that an SBASE is updated in the same directory as the existing SBASE (more compounds, more data, changes in keys, or changes in nomenclature), it is necessary to unregister the previous SBASE from within the AssureNMR before proceeding to register the updates.

The flow of data into a quantMethod can be summarized in the schematic below. This flow is designed to maximize the speed of importing the data and maintaining the consistency across all databases.




The following data can be used to generate an SBASE entry for a compound:

- 1D proton spectrum - The minimal amount of data required for proton-only screening is the 1D proton experiment. All of the multiplicity and coupling assignments can be made in this spectrum. Peak annotation is important when importing the atom count for quantification.

- 2D-HSQC spectrum - Screening with carbon requires the 2D-HSQC experiment. This can be a standard HSQC or multiplicity edited HSQC (ed-HSQC).
- Molecular structure file (optional) – The structure can be used for peak annotation, but it is not absolutely necessary for the SBASE entry. The benefit of annotating with a saved molecular structure file is that the peak IDs reported in the quantification method correspond to the assignments from the structure file.
- Additional types of spectra can be added to an SBASE as needed for the user's application. Some examples include 1D $^{13}C$, 2D JRES spectra, 2D HSQC spectra, 1D $^{19}F$ spectra, 1D $^{31}P$ spectra, and 1D $^{2}H$ spectra. Note it is important to make annotations for JRES spectra because this information is used in the JRES match.

**See also**

Default Home Directories [▷ 18]

## 5.2 Generating an SBASE

The generation and registration of SBASEs can be done with the AssureNMR software, which invokes the AMIX Preparation window. This requires that AMIX version 3.9.11 or greater is installed (no additional license required). More detailed information on all the tools for generating an SBASE can be found in the AMIX User's Manual.

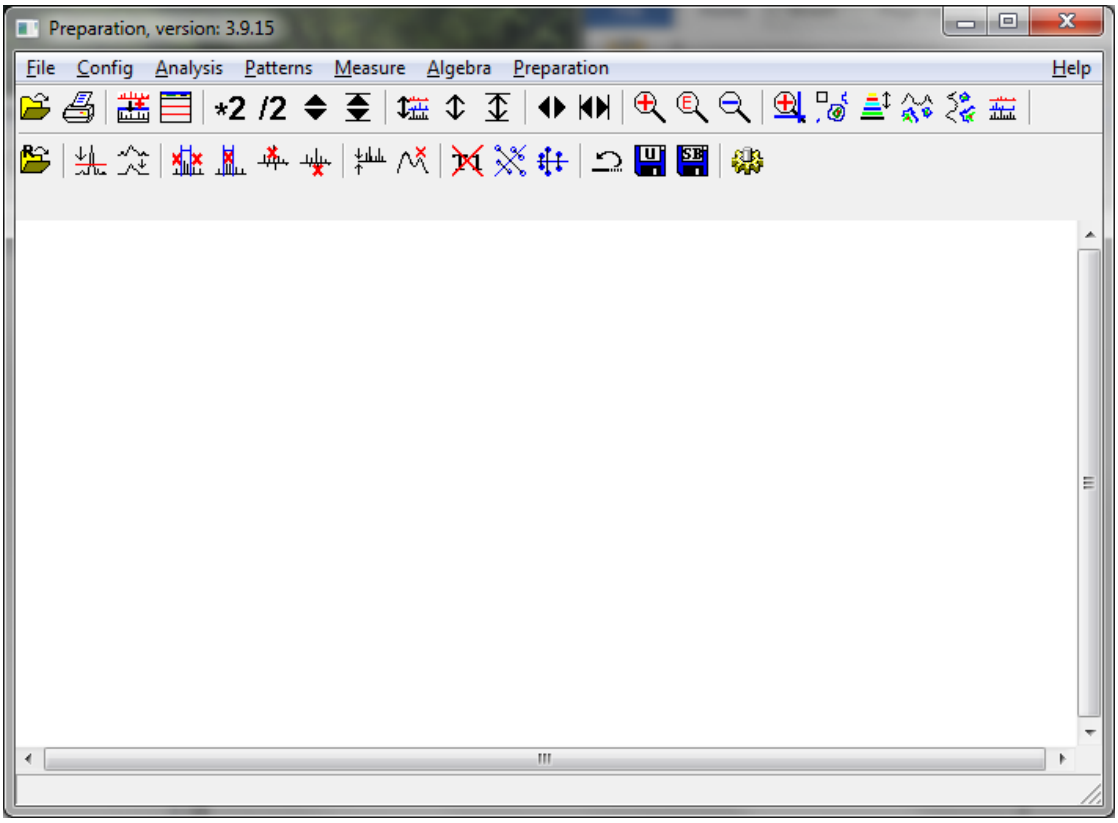



*Figure 5.1: The SBASE Preparation window of AMIX can be accessed through AssureNMR.*

To create a new SBASE, from the Preparation window, using the **Preparation** pulldown menu, select **Spectra bases**, **Create new**:

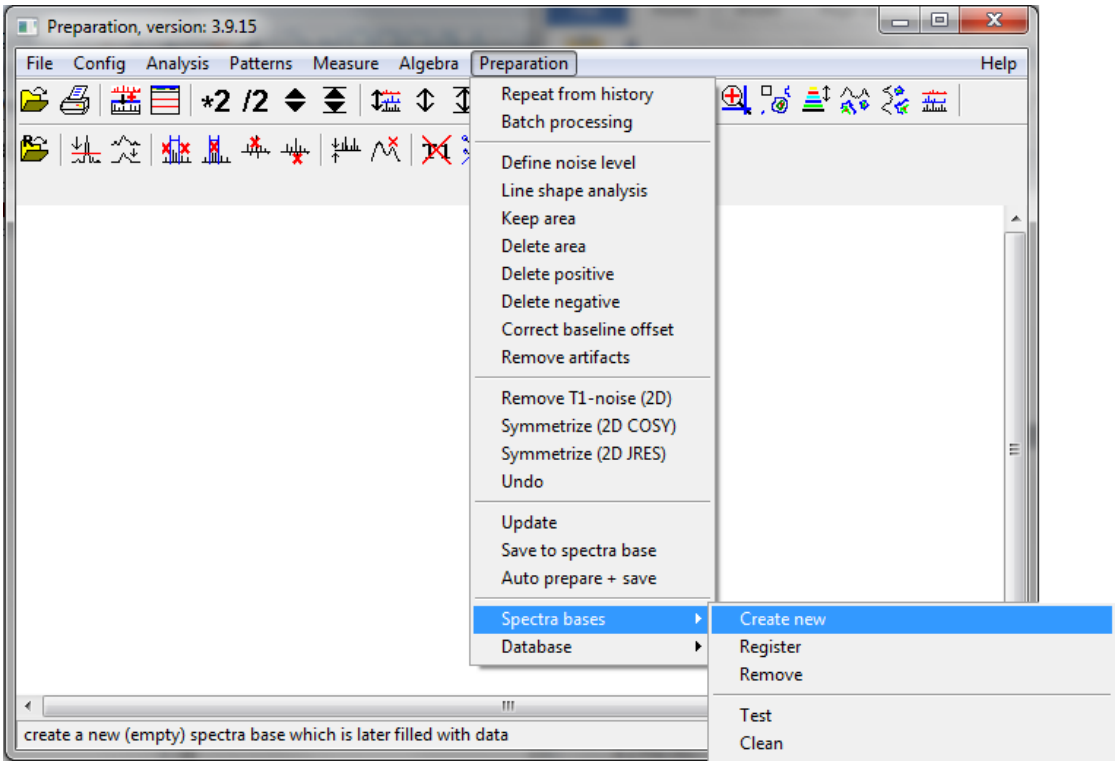



*Figure 5.2: Tools in the Preparation pulldown menu. The Preparation pulldown has all of the tools necessary to import a spectrum into an SBASE.*

Then specify the directory path:

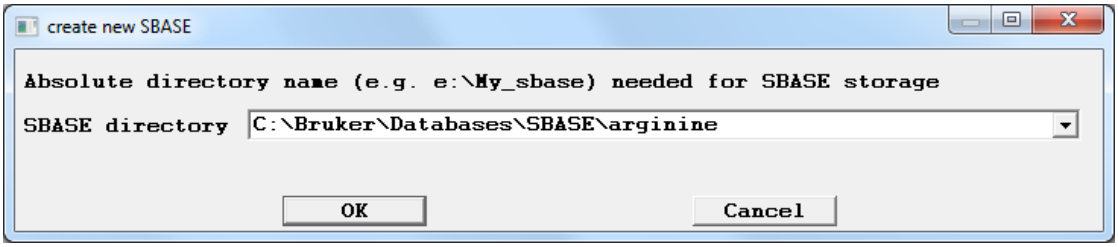



*Figure 5.3: Create new SBASE window, where the user can specify the location for the SBASE.*

Note: A new SBASE can also be created from the AssureNMR interactive analysis window, under **SBASES**/**Create New SBASE**, as mentioned in the chapter *SBASES Menu [ 51]*.

## 5.3 Analyzing the Spectra

To create an entry in the SBASE for a compound, load a representative spectrum of the compound into the Preparation window using **File**/**Open TopSpin 1D file** or **Open TopSpin 2D file**. There are three layers of spectral notation required when assigning peaks to maximize the information available for importing compounds automatically: peaks, annotations, and multiplets. These are described in following sections, using lysine as an example.

### 5.3.1 Picking Peaks

Be sure to check the referencing of all spectra before picking peaks. This can be performed through the **Measure** pulldown menu, **Simple Calibration (1D, 2D NMR)**.

The tools for picking peaks within a spectrum are found in the **Analysis** menu. Peaks can be quickly picked using the option **Auto peak pick** as shown in the figure below. The resulting picked peaks are identified with tick marks above the peaks.

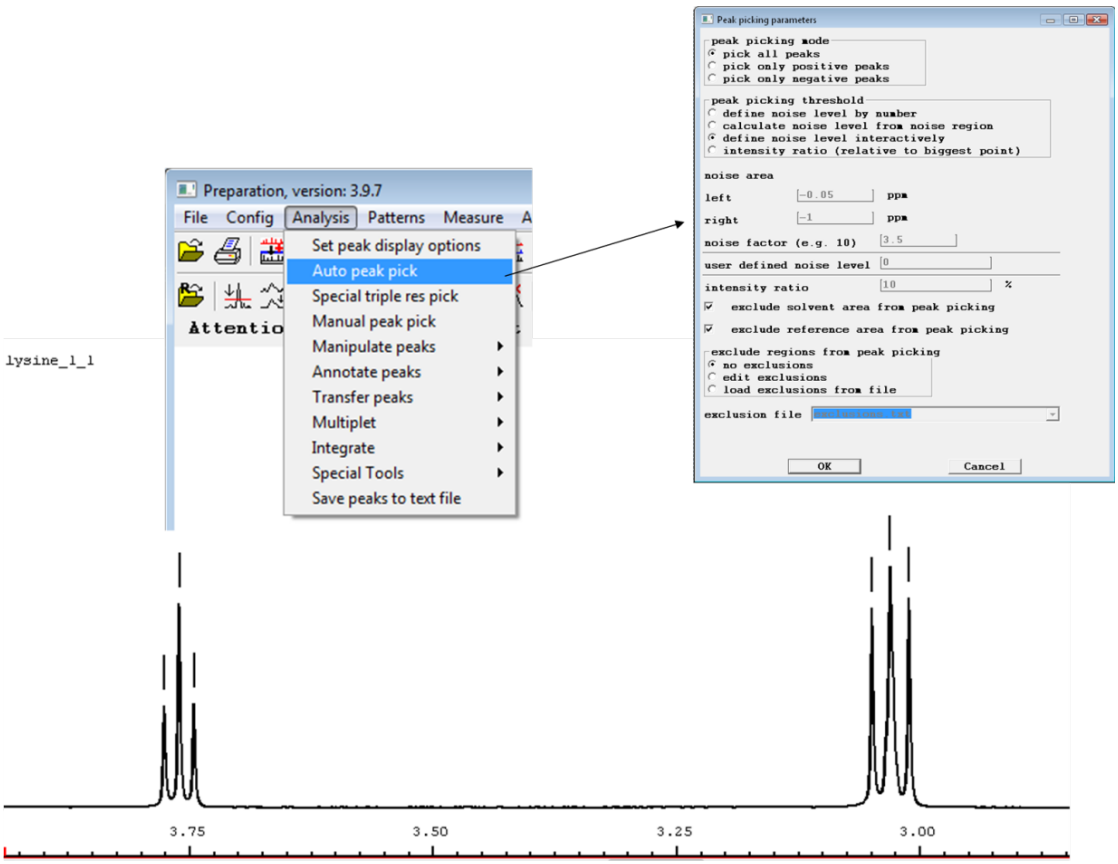



*Figure 5.4: Action and window arguments for automated picking peaks in the Preparation window. The results are indicated by tick marks, shown here for the Hα (ca. 3.75 ppm) and Hε (ca. 3.05 ppm) resonances of lysine.*

It is also possible to pick the peaks manually, using the **Manual peak pick** option. This is useful for adjusting the peak list determined automatically.

## 5.3.2 Annotating Peaks

To annotate peaks from the molecular structure, open the molecule in the Preparation window in addition to the spectrum. To open the molecular structure, use the **File** pulldown, **Open molecule**. This prompts for the path to the .mol file. Once the molecule is open, select from the **Analysis** pulldown **Annotate peaks**, **Annotate from molecule**. Then atoms in the structure can be selected with the mouse and their labels associated with picked peaks in the spectrum, as shown in the figure below. When using the **Annotate from molecule** tool, it is important to remember these key points:

1. Annotating with multiple atoms in the structure can be done by selecting all atoms, then the peak.
2. It is only necessary to select one peak within a multiplet when annotating.
3. Simple annotation or changes can be made with the **Annotate peaks** tool.

It is not necessary to annotate the peaks from the molecule. **Annotate peaks/Annotate peaks** allows the user to enter text for the peak annotations.

It is absolutely necessary to annotate the correct number of atoms for quantification of two protons by filling the field with: **H,H**

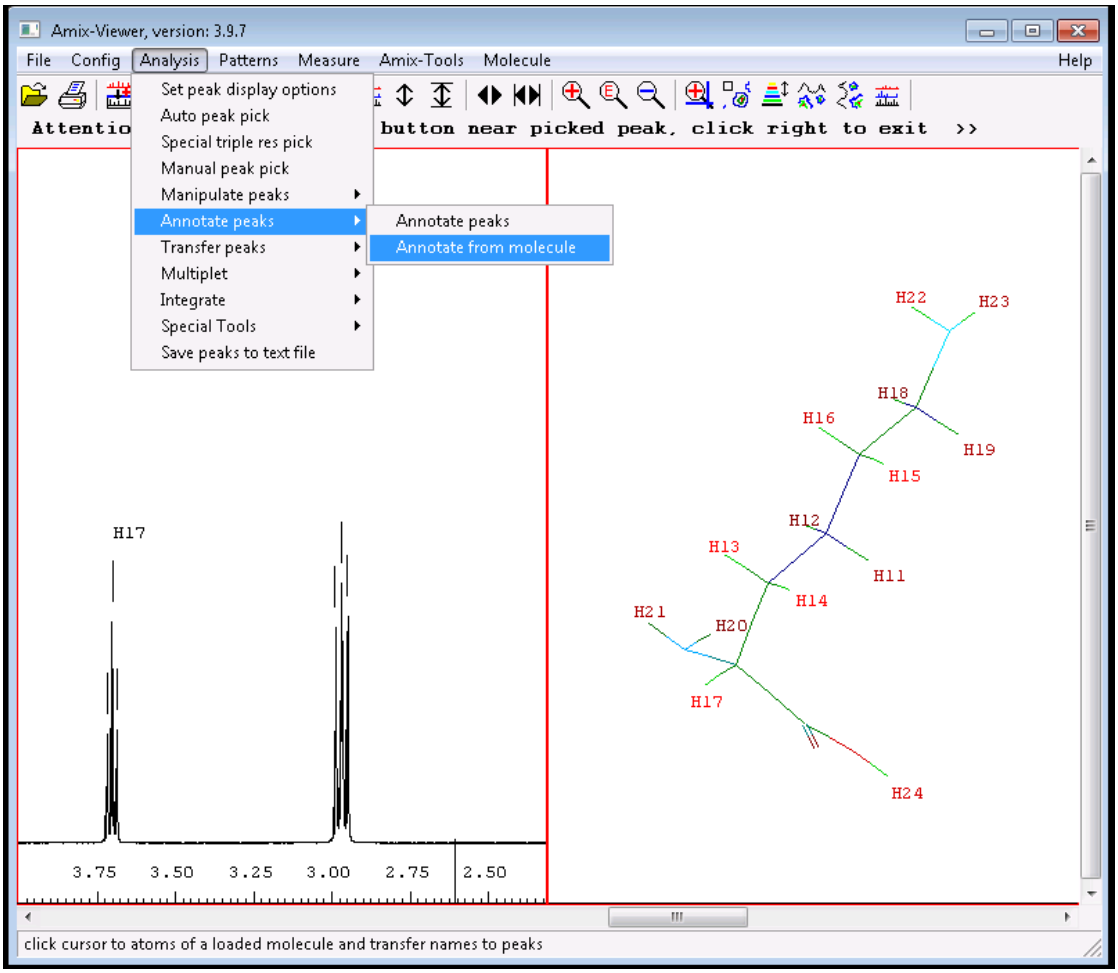



*Figure 5.5: An example of the Annotate from molecule tool and the resulting annotation in lysine proton H17 (Hα).*

## 5.3.3 Defining Multiplicity

Multiplicity is defined by grouping picked peaks. It is therefore necessary that all of the peaks in the multiplet are picked in order for the multiplicity to be properly assigned. The figure below shows the definition of the lysine triplet using the drag-n-drop tool in the multiplicity editor. (**Analysis** pulldown, **Multiplet/Interactive multiplet define (1D NMR)**) Note that the preparation module will measure the coupling constants and allow the user to define the multiplicity once the peaks have been defined. For complex multiplets, the  icon allows the user to build up multiplets of multiplets.

Correct multiplicity assignment is critical to the success of your quantification routine

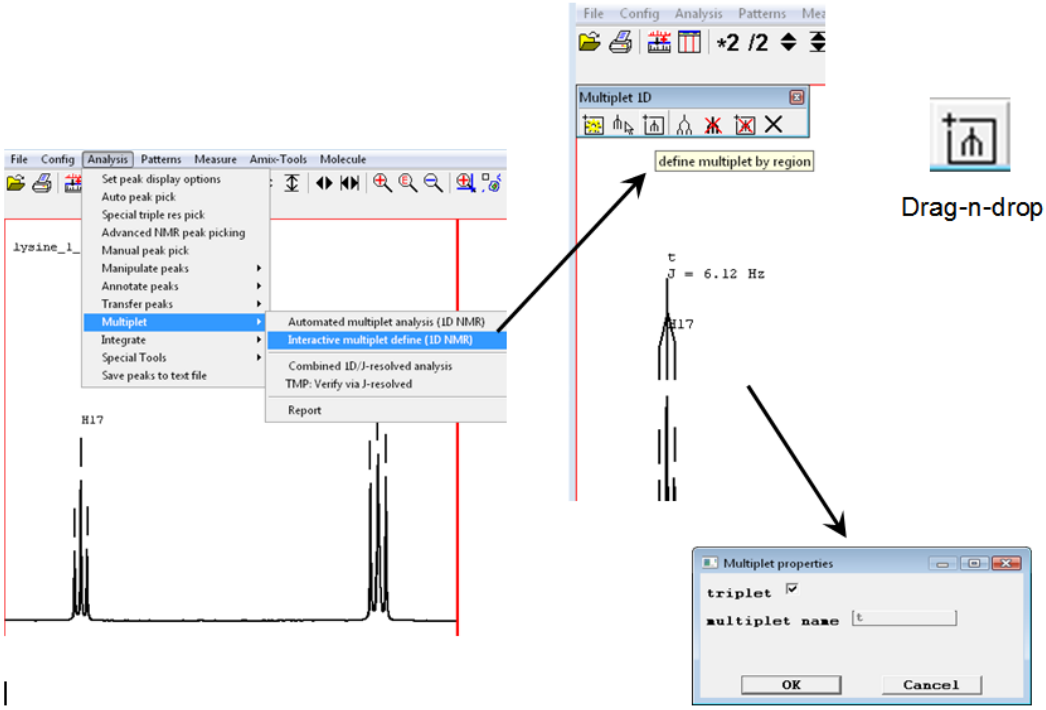



*Figure 5.6: Tools to assign multiplets. Note these tools automatically measure the coupling constants.*

**Notes on spectral analysis:**

- Once all of peaks have been picked, annotated and correctly grouped into multiplets, the spectrum is ready to be converted into an SBASE entry.
- In the case of $^{13}C$ data, it is only necessary to peak pick and annotate the HSQC. Annotations should be made for both $^{1}H$ and $^{13}C$ on each peak. The preparation module can identify either normal or multiplicity edited HSQC.
- The peaks, annotations, and multiplicities are saved into the spectrum directory when the spectrum is closed. The spectral assignments will reopen with the spectrum.
- It is critical to use similar experimental conditions when acquiring data for the SBASE for each reference material. Conditions such as solvent, pulse sequence, and temperature must be identical between the reference library/knowledgebase and the material data.

## 5.4 Creating the SBASE Entry

Once the NMR spectra are analyzed, there is further data preparation to reduce the SBASE file size and to eliminate any signals that are not part of the target molecule. Data preparation is done in the Preparation window. All of the information from the previous spectral analysis sections is carried into the SBASE.

To prepare a spectrum, load it into the Preparation window. Then, the steps flow in order, either using the **Preparation** pull down menu or the icons in the second row of the Preparation window. The steps are explained in the following sections.

### 5.4.1 Removing Signals Below a Noise Threshold

**Define noise level** from the **Preparation** pulldown menu (icon: ) allows the user to specify the noise level in one of several ways:

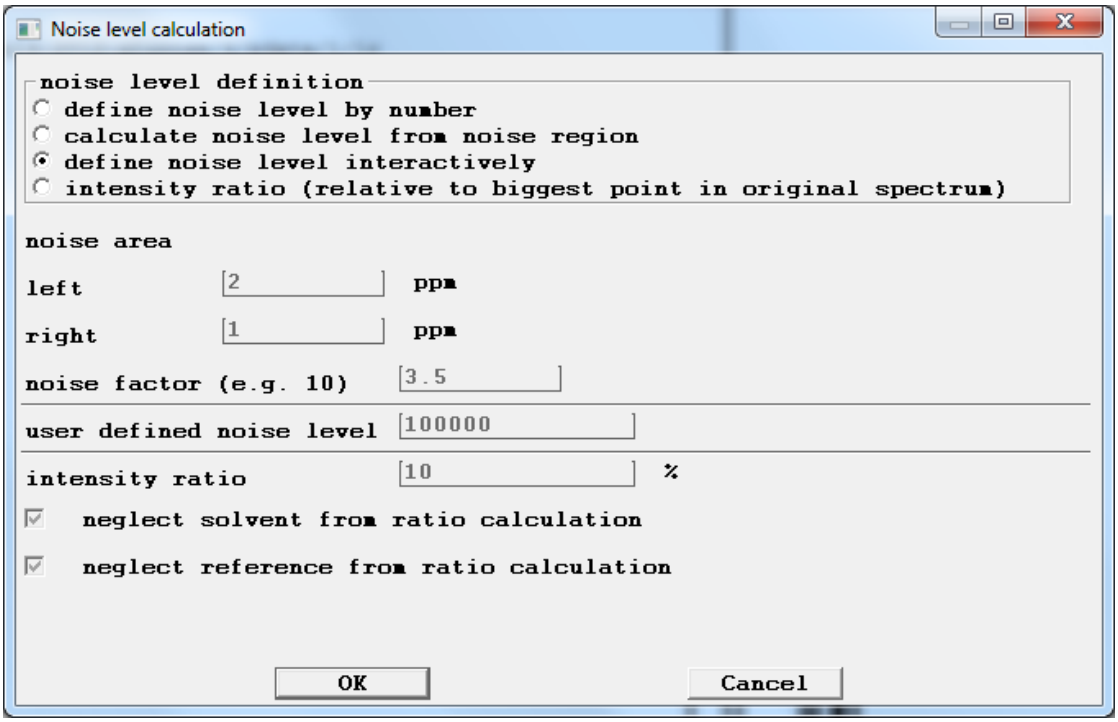



*Figure 5.7: Noise level calculation window, which provides several options for specifying the noise level in a spectrum.*

The option to define the noise level interactively is especially convenient. Based on the noise level chosen, the **Line shape analysis** option (icon:  ) removes all the signals below the noise level.

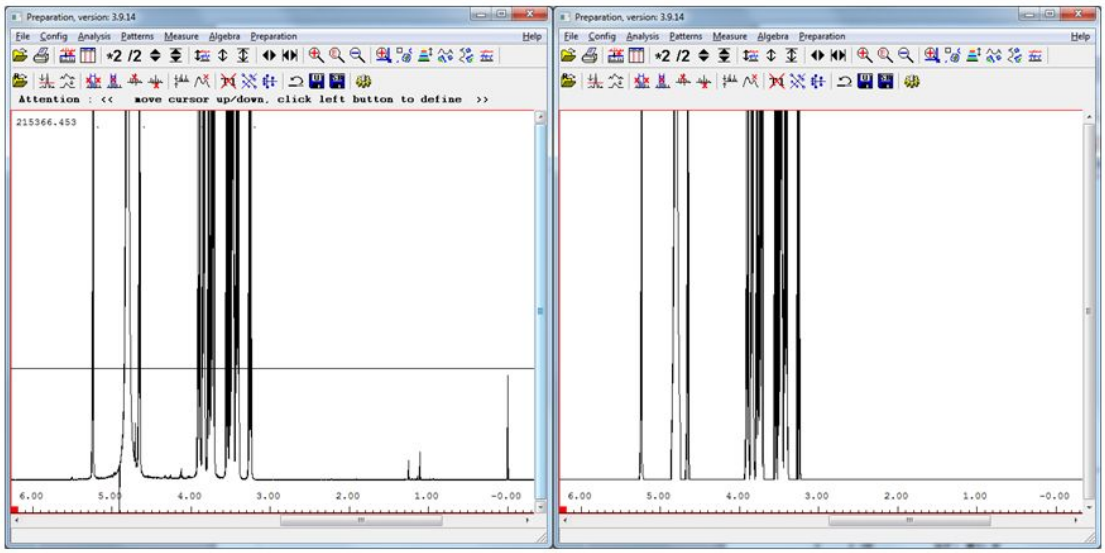



*Figure 5.8: Demonstration of setting the noise level interactively (left panel) and then applying Line shape analysis to remove signals below the noise level (right panel).*

## 5.4.2 Removing Unwanted Signals

There are several options for deleting unwanted signals. **Delete area** (icon:  ) opens a drag and drop tool which allows removal of signals not associated with the component of interest (e.g. water, TSP, etc.). Left click the mouse to begin drawing a box around the

unwanted signal, then release to define the region and delete peals within it. **Keep area** (icon: ) keeps the peaks in the selected region and deletes everything else. **Delete negative** (icon: ) deletes negative peaks. An additional window offers the option to delete negative peaks across the whole spectrum or within an interactively defined region. **Delete positive** (icon: ) deletes positive peaks. Again, the user can specify whether to apply this to the whole spectrum or an interactively defined region.

### 5.4.3 Correcting Artifacts

**Remove artifacts** supplies tools to improve the appearance of the spectrum:

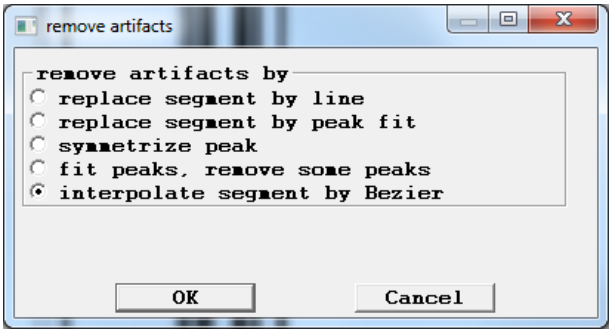



*Figure 5.9: The remove artifact window, showing the different calculations available.*

Note these corrections are not required to produce a good quality SBASE; they are mainly for cosmetic improvement. Also, these tools can potentially distort the reference spectrum. Hence, these tools should be applied with caution.

### 5.4.4 Saving Spectra to the SBASE

**Save to spectral base** (icon: ) opens a window which prompts the user for the SBASE for the spectrum, the compound name, and the spectrum name:

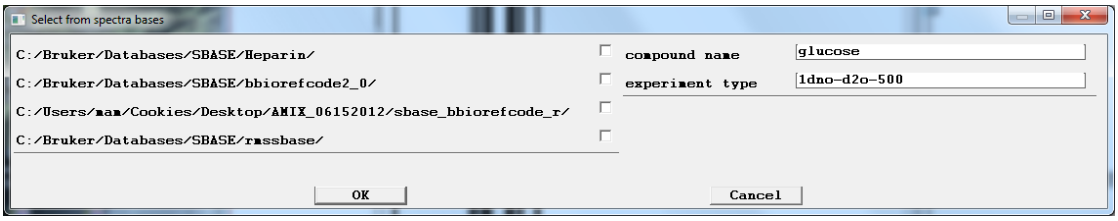



*Figure 5.10: Select from spectra bases window. This window appears when saving spectra to an SBASE.*

It is very useful to give the experiment type a consistent descriptive name. All spectra within the same SBASE must use the same names to facilitate matching. For example, in the name shown in the figure above, the first part specifies the pulse sequence used (1dno denotes a 1D $^{1}$H noesy experiment), the middle segment (d2o) specifies the solvent, and the last part specifies the field strength (here 500 MHz). This is the naming convention used in the AssureNMR SBASEs.

### 5.4.5 Importing a Molecular Structure File

Individual compounds can be represented by a molecular structure file (.mol format). The structure provides information on the molecular weight of the compound. As discussed in section *Annotating Peaks [▷ 66]*, the molecular structure file can be used to annotate the

spectra. The file can also be displayed within the AssureNMR method development window (using **File**/**Open**). Therefore, it is useful to keep the molecular structure file in the SBASE with the spectra for that compound.

To save a structure to the SBASE, in the **Preparation** pulldown, select **Spectra bases**, **Import coordinate file**, as shown in the following figure.

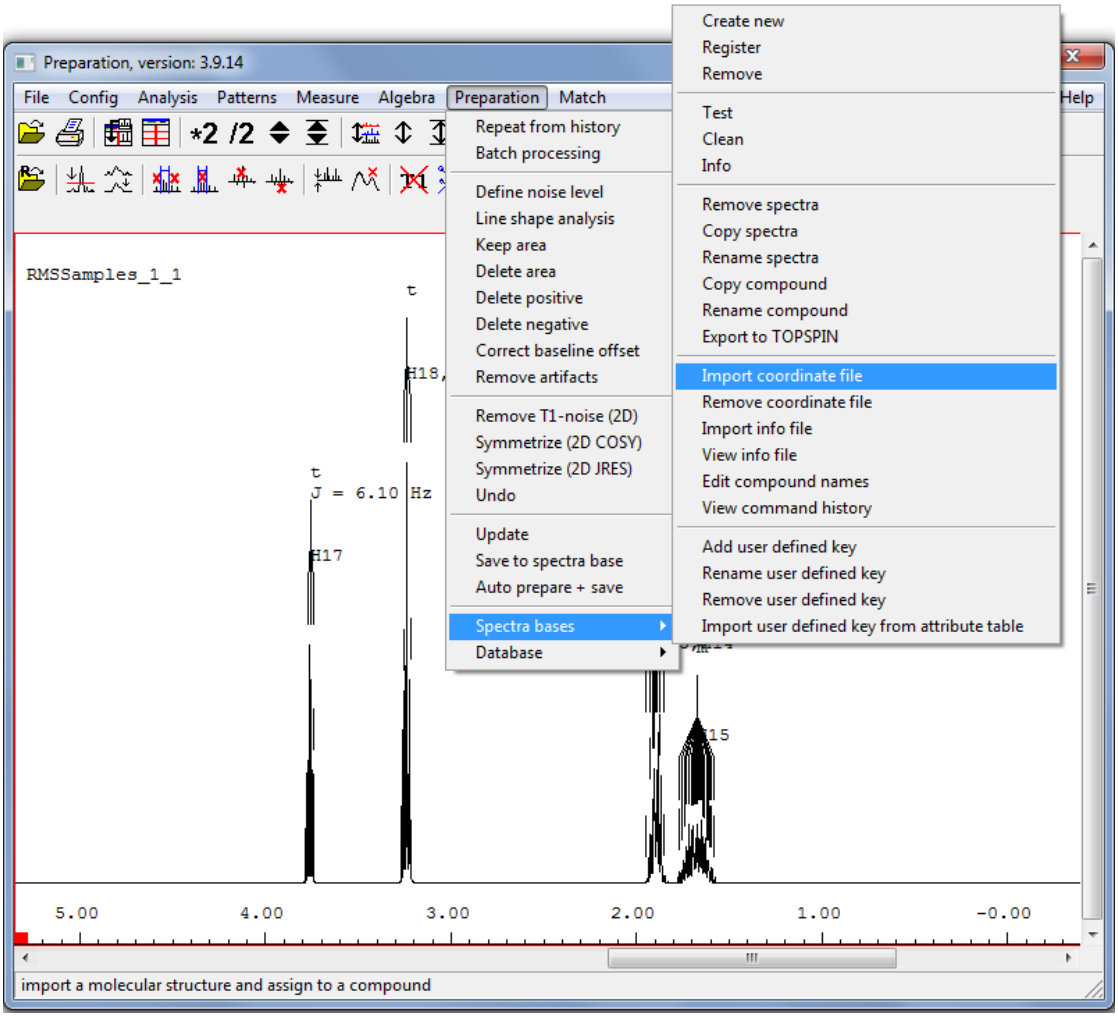



*Figure 5.11: Path to Import coordinate file from the Preparation pulldown menu.*

A series of windows will prompt for the filename and path to the molecular structure file, the SBASE, and the compound.

The SBASE is especially important for applications where matching is important, such as a quantMethod that reports and identifies unknowns. For these applications, it is important to provide as much detail as possible in the SBASE. For other applications, for example, an analysis based on region integration, the SBASE does not require as much information.

## 5.5 Importing a Spectrum from CMC-assist

If the spectrum for a compound of interest for a reference spectral database has already been analyzed using the Bruker CMC-assist software, there is no need to analyze it again using the AMIX Preparation window. The information from the CMC-assist analysis can be directly imported to the SBASE. From the AssureNMR interactive analysis window, select **SBASES/Import CMC-a Spectra to SBASE**. The user will first be prompted for the spectra to import, then the SBASE to import. The SBASE entry will be created with the dataset name as the new compound name. If the user would like a different compound name, this can be changed using the AMIX Preparation window, under **Preparation/ Spectra bases/Rename**

**compound**. Any structure coordinate file from the CMC-assist analysis will also be imported to the SBASE. If the structure is already present when additional spectra are added to the same compound, the structure coordinate file is not changed.

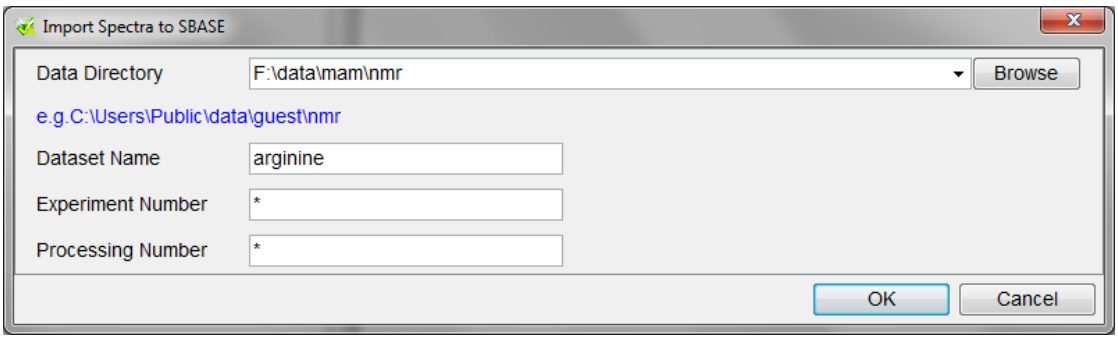



*Figure 5.12: Import Spectra to SBASE prompt for importing spectra analyzed in CMC-assist.*

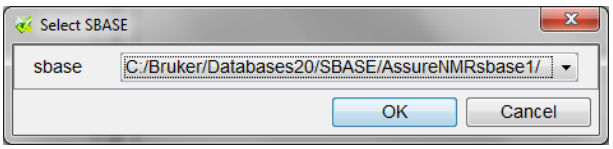



*Figure 5.13: Select SBASE prompt for spectra imported from CMC-assist.*

# 6 Quantification in AssureNMR

In AssureNMR, the material-specific analysis in automation is based upon a material-specific quantification method or quantMethod. Processed data from TopSpin is passed to the AssureNMR software, which then utilizes a spectral database (SBASE) and a knowledge base (implicit within the quantMethod) for identification and quantification of individual components in the sample. Evaluation is based on two criteria: spectral matching and quantification. Positive identification from both criteria gives confidence in the identification of a constituent and is reported as the result of the analysis. Within the quantMethod, it is also possible to set thresholds for different components, allowing samples containing contaminants below a certain level to pass while those containing larger amounts fail. The method specifies what information to report, including the units for concentration and the results of custom calculations. These features are described in detail here. A SIMCA model for outlier detection, a PLS regression for quantification, and multiclassification can also be specified in the quantMethod. The chemometric features will be described in the chapter *Chemometric Modeling in AssureNMR [▶ 125]*.

**Matching**

Spectral matching is done by projecting the test spectrum onto previously acquired spectrum of a known, pure sample stored within the SBASE. The criterion for spectral matching is that the test spectrum and the pure reference spectrum must both have intensity at the same location. Subroutines to determine the multiplicity and lineshape are then used to ensure that the signals observed in the test spectrum are consistent with the structural properties reflected in the pure reference database spectrum. The search region can be specified by the user.

**Quantification**

Quantification of each component observed in a spectrum requires a compiled list of specific chemical shifts detailing the peak shape and the atom count at each of these signal regions. The presence of a compound will be confirmed when all of the signal regions possess integrals and the integrals are in proportion to their respective atom count. The figure below illustrates for lysine how the areas of non-exchangeable peaks maintain a relative atom integral balance. All intensity is accounted for and there is no ± threshold for the expected integral.

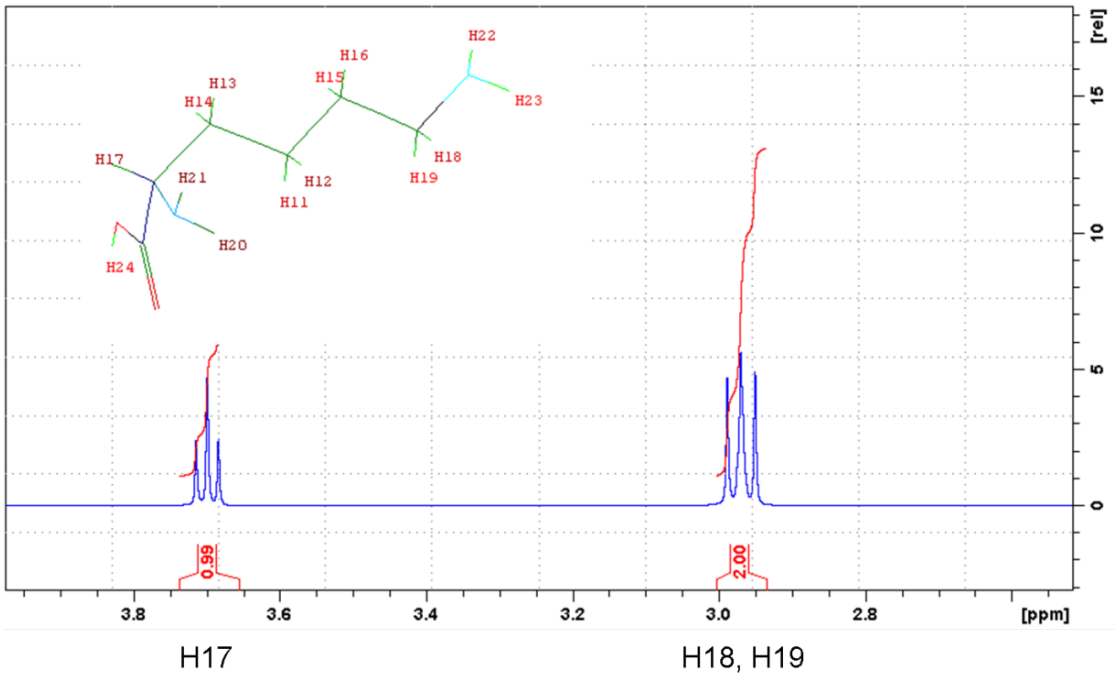



*Figure 6.1: Atom integral balance of lysine. Spectrum shows Hα (H17 – ca. 3.7 ppm) and Hε (H18, H19 – ca. 2.95 ppm). Integrals of relative intensity are indicated.*

## 6.1 Quantify Pulldown Menu

The **Quantify** pull down menu provides access to all the tools necessary for developing quantMethods and running them on spectra outside automation.

The examples in this chapter use $^{1}H$ and $^{13}C$, but AssureNMR also supports $^{2}H$, $^{11}B$, $^{19}F$, $^{29}Si$ and $^{31}P$.

The **Quantify** pulldown menu has options to create new methods (**New Method**) and to edit existing methods (**Edit Method**). **Release Method** is important for running in automation. Once a quantMethod is ready to use, it is released for use in automation. This provides a mechanism to protect the quantMethod from further changes for consistent analysis in automation.

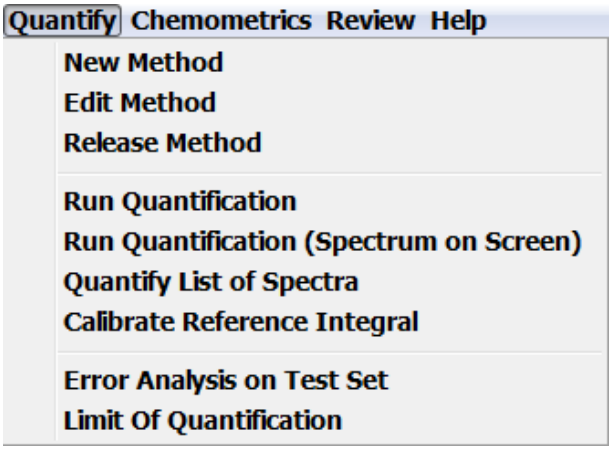
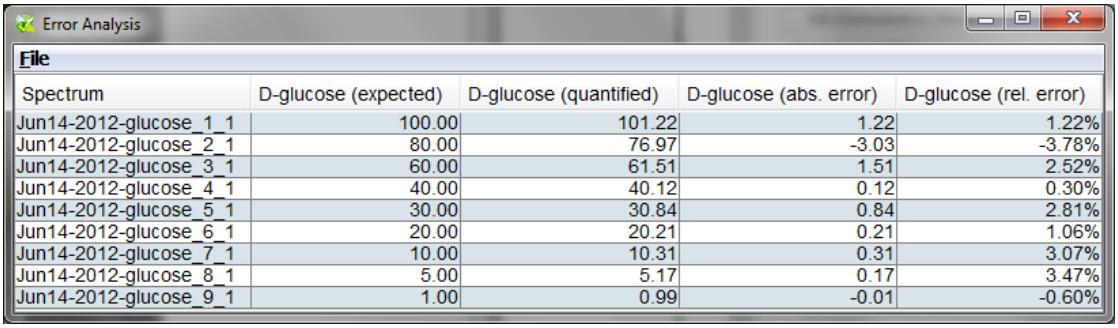



*Figure 6.2: Quantify pulldown menu.*

**Run Quantification** brings up a dialog box that prompts for the quantMethod to be used, the spectrum to be analyzed, an optional additional spectrum, and the directory for the results. **Run Quantification (Spectrum on Screen)** is similar, except that it no longer prompts for the spectrum. In this mode, details of the analysis of the spectrum will be displayed in the viewer window, which is very useful for interactive development of the quantMethod. **Quantify List of Spectra** prompts for a list of spectra and a quantMethod to provide batch analysis of spectra. One report, including averages over all the spectra and analysis for each individual spectrum, is created. A summary table for all the spectra in the list also appears. The option **Calibrate Reference Integral** calibrates a reference spectrum as an external concentration standard. The user defines the molar concentration of a known species and a peak to integrate to generate the standard integral. Then this standard integral can be used to quantify constituents in other spectra which were collected on the same instrument with the same probe and the same pulse sequence. The calculated concentration employs the PULCON principle (Wider and Dreier, (2006) *J. Am. Chem. Soc.***128**:2571-2576).

**Error Analysis on Test Set** provides a tool for comparing measured values from AssureNMR to reference values for a set of spectra. The reference values are supplied by the user and must be stored as metadata for the spectra, with the column heading matching the compound name in the quantMethod. (See chapter *MetaData Menu [ 53]* for metadata information.) **Error Analysis on Test Set** prompts the user for the file containing the method and the results directory and the spectra to quantify, just like **Quantify List of Spectra**. The output is similar, too, with a summary report that gives the averages and the results for each spectrum. A summary table for all the spectra pops up. In addition, there is a table that provides the difference between the value calculated in AssureNMR and the reference value stored in the metadata, both as an absolute value and as a percentage of the reference value.


Error Analysis

File

| Spectrum | D-glucose (expected) | D-glucose (quantified) | D-glucose (abs. error) | D-glucose (rel. error) |
|---|---|---|---|---|
| Jun14-2012-glucose_1_1 | 100.00 | 101.22 | 1.22 | 1.22% |
| Jun14-2012-glucose_2_1 | 80.00 | 76.97 | -3.03 | -3.78% |
| Jun14-2012-glucose_3_1 | 60.00 | 61.51 | 1.51 | 2.52% |
| Jun14-2012-glucose_4_1 | 40.00 | 40.12 | 0.12 | 0.30% |
| Jun14-2012-glucose_5_1 | 30.00 | 30.84 | 0.84 | 2.81% |
| Jun14-2012-glucose_6_1 | 20.00 | 20.21 | 0.21 | 1.06% |
| Jun14-2012-glucose_7_1 | 10.00 | 10.31 | 0.31 | 3.07% |
| Jun14-2012-glucose_8_1 | 5.00 | 5.17 | 0.17 | 3.47% |
| Jun14-2012-glucose_9_1 | 1.00 | 0.99 | -0.01 | -0.60% |

*Figure 6.3: Error Analysis table, comparing calculated and reference values.*

**Limit of Quantification** estimates the limit of quantification for a method using representative spectrum, typically acquired with the same experimental parameters on a sample of the matrix without the compound of interest (a blank). The user is prompted for the method file, the compound for LOQ determination, parameters for the simulation, and a blank spectrum. The calculation produces a pdf file that shows the results and the parameters used.

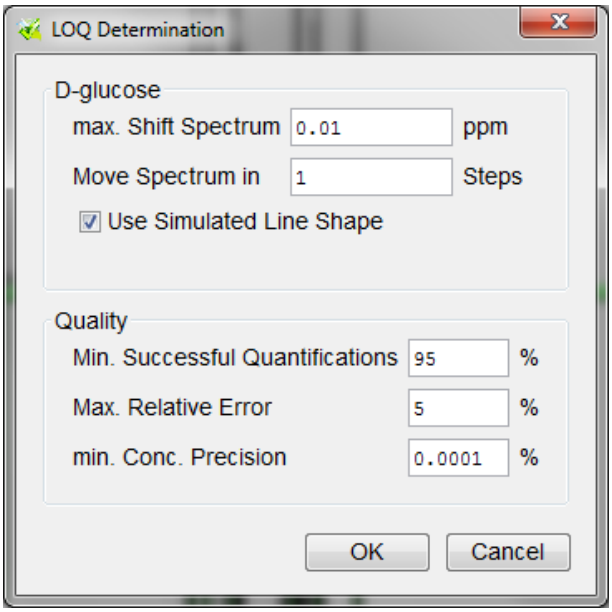
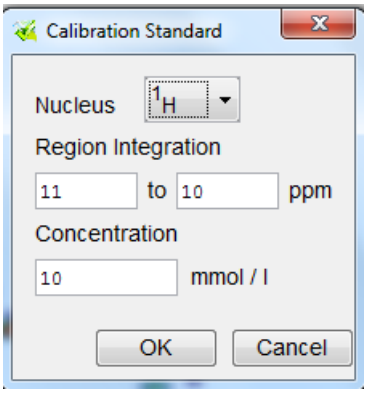



*Figure 6.4: Parameters for the LOQ Determination.*

**Calibration Standard Definition** enables an AssureNMR Launch user to gain minimal access to the quantification features of AssureNMR without the Ascent or Summit license. Selecting this command initiates a dialog window to set a quantitative calibration reference from the active spectrum. This generates an AssureNMR simple method that identifies a region used as an internal quantitative standard.




## 6.2 Overview for Editing a Method

The quantification method defines how a spectrum is evaluated by defining what the expected compounds are, what the known adulterants are, which signals are irrelevant (e.g. solvent), and which information should appear in the final report – concentration units, pass/fail based on adulterant threshold requirements, results of user-defined calculations, results of chemometric tests, etc.

To edit a method, start with a spectrum open in the viewer window. From the **Quantify** pulldown, **New Method** immediately gives the user the option to import compounds from an SBASE, Match Result (if available), RTFBases or to continue without using a database entries.

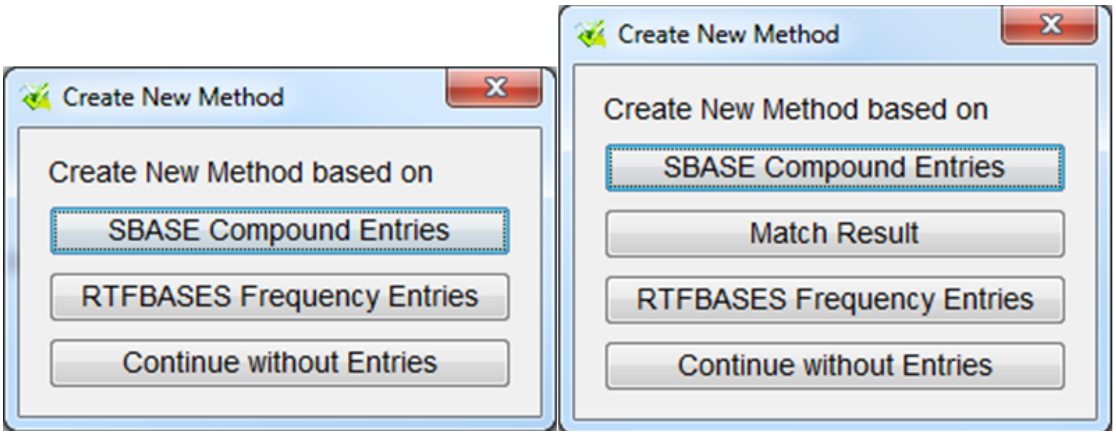



*Figure 6.5: Two possible windows appear for importing compounds from a Database that differ depending on the presence or absence of a prior Match analysis.*

Once the SBASE is selected, available compounds are conveniently listed on the left. The nuclei and spectra to use for matching are set up under 'Experiment Types'. When making a new method based on Match results, the user is given the option to import 'Approved Compounds', 'Matched Compounds', 'Plausible Compounds' and 'All Compounds'.

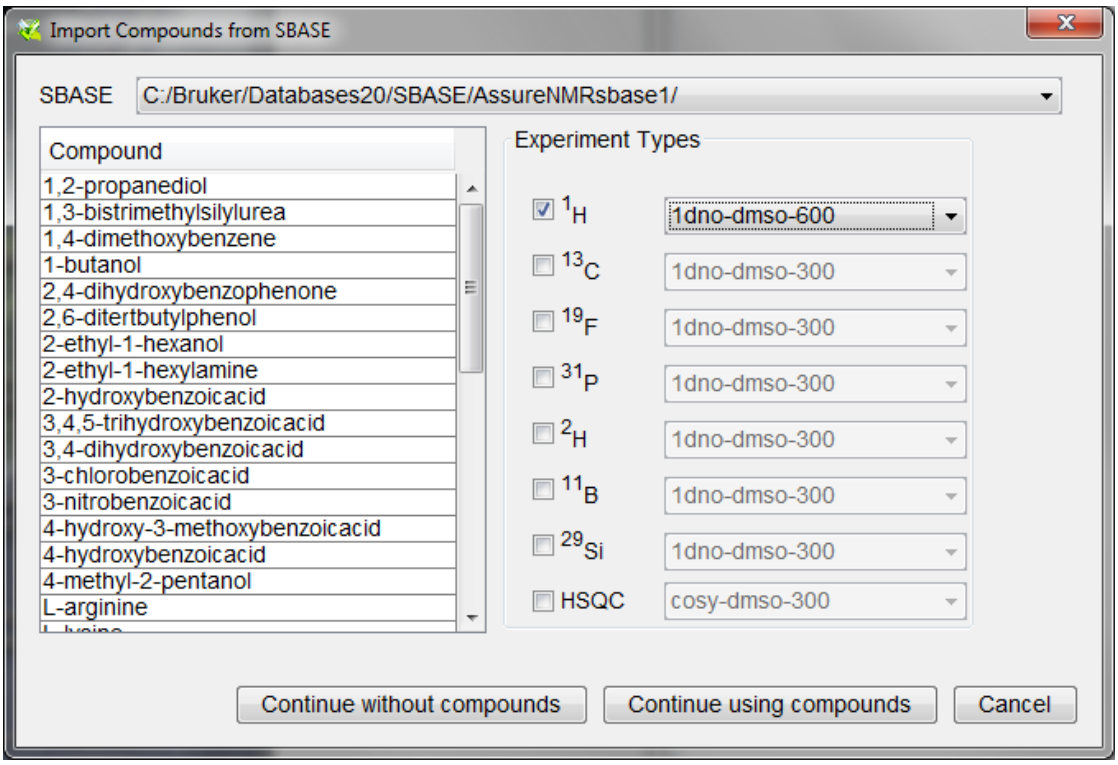



*Figure 6.6: Window for importing compounds from an SBASE.*

The next step is to select some key parameters for analysis.

The Calibration Method options include:

- External (SST): reference concentration will be taken from a standard calibrated during the system suitability test
- External (User Defined): reference concentration will be taken from a dataset specified by the user
- Internal (Fixed Concentration): reference concentration is given by a fixed signal that has a constant concentration from spectrum to spectrum

- Internal (Variable Concentration): reference concentration is given by a fixed signal whose concentration may vary from spectrum to spectrum
- Total Integral: reference concentration is given by the sum of all quantified compounds except NMR Reference and Solvent.

For concentration reporting, the user can choose from relative concentrations (mol % reference and mol % total integral) or percent by weight (g % reference, g % sample weight, g % total integral) and absolute concentrations (mmol/l, mol/l, mg/l, g/l). g % sample weight requires additional information about the weight and volume used to make the NMR sample – this is prompted by the software. Absolute concentrations require an external calibration standard. This is configured in the quantMethod and, for acquisition in automation, the IconNMR Configuration window.

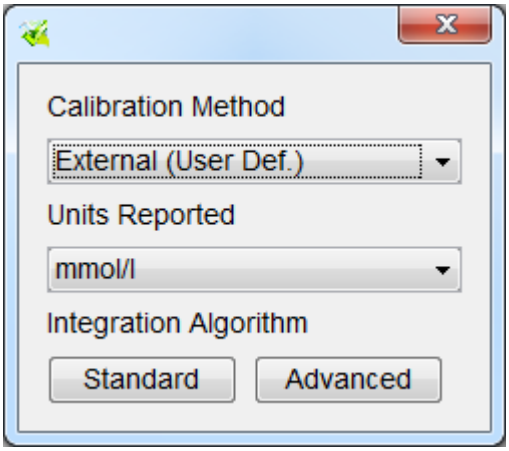



*Figure 6.7: Basic information to specify when setting up a new method.*

The user must also specify the Integration Algorithm. In the Standard algorithm, lineshapes are specified using traditional patterns identified in spectroscopy – singlets, doublets, and triplets. In the Advanced algorithm, the lineshapes have a more general mathematical specification. Note the advanced algorithm corresponds to the peak fitting used in the Bruker FoodScreener and IVDr (research use code) products. For metabolomics applications use of the 'Advanced' algorithm is recommended. For other appications and when uncertainty calculations are required the 'Standard' algorithm is recommended.Once the algorithm is selected, the user is in an interactive editing mode, with icons across the top of the screen and quick access to parameters through a panel of the left. The icons are the same for both algorithms. The **General** parameters and **Compound Description** in the parameter panel are also the same for both algorithms. The details for peak fitting (**Basic Signal Description** etc.) are different depending on the algorithm. These topics will be explored in detail in the following sections.

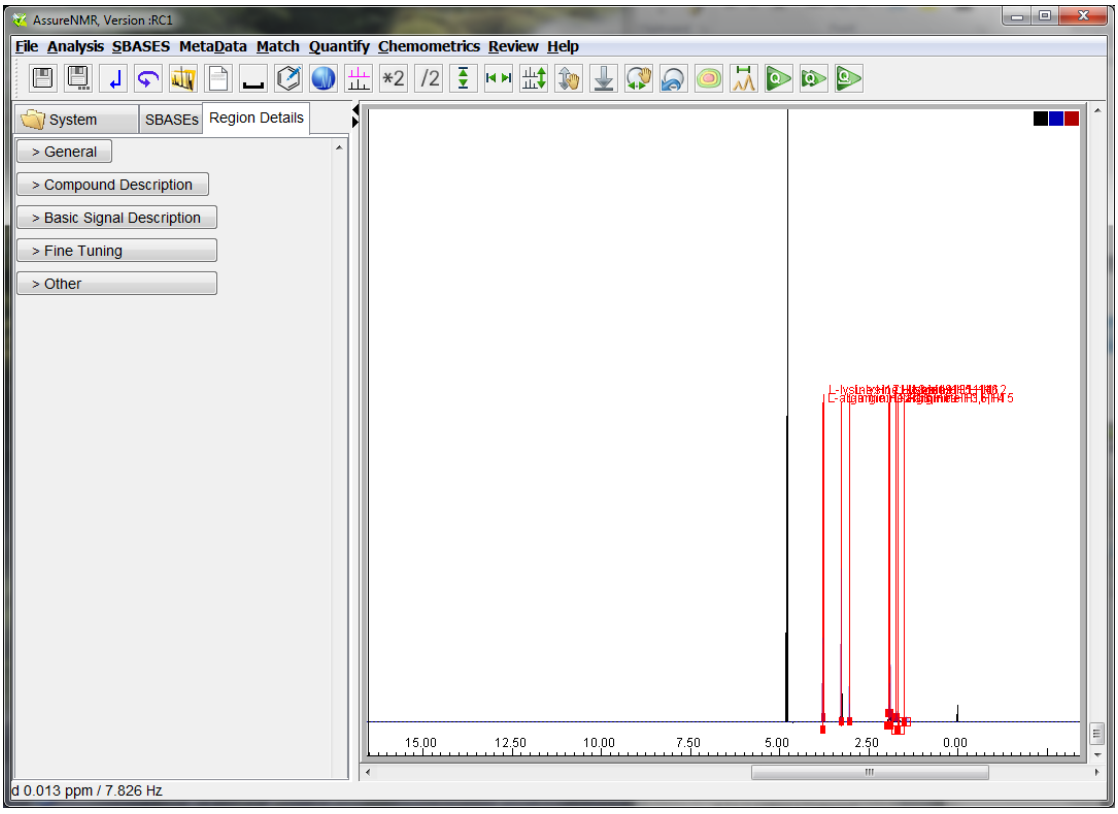



*Figure 6.8: Interactive editing mode.*

The spectrum can be expanded by left clicking with the mouse and drawing a box around the desired region. Right clicking in the spectrum viewer brings up a menu with three options – (1) **Measure Distances**, which lets the user draw a line segment between two points in the spectrum and reports the distance in ppm and Hz. (2) **Peaks/Display options**, which lets the user decide whether or not to display markers and names for each peak picked in the spectrum, and (3) **Zoom out**, which extends the boundaries of the current region by half the width on each side, doubling the window. This is quite useful when the user has been exploring a peak in detail and then wants to confirm there are no other features in the nearby regions. If compounds have been imported, the preliminary regions for integration are shown as bars on the spectrum. These integration regions can be adjusted by pulling on the white squares on the ends of the bars with the left mouse button depressed. Hovering over the bar with the mouse and right clicking brings up the option to **Test Region** – this runs the integration for the region with the current parameters. The option **Quantify On/Off** is also available. This lets the user decide whether to include the current region in the quantitative analysis. The squares in the upper right hand corner of the viewer window allow the user to adjust elements of the display. For example, hovering over the black box and rolling with the mouse wheel adjusts the height of the spectrum. Right clicking on the box brings up a menu to adjust the display of the spectrum: **Shift Horizonally**, **Shift Vertically**, **Properties**, **Close** and **Close All Overlays**.

## 6.3 Icons for Editing a Method Interactively

The icons allow quick access to different functions while examining the spectrum. They are:

Save: saves the quantMethod to the name previously chosen.

Save As: saves the quantMethod. A window pops up to prompt for the path and name.

Return: exit interactive editing mode. The user will be prompted to save the method.

 Undo: undo the last command.

Exchange Spectrum: allows the user to change the spectrum currently displayed in the viewer window without leaving the interactive editing mode.

 Edit Method: opens up the tab and table form of the quantMethod for editing. This provides access to features such as the Equation Builder and Chemometrics. This mode of editing is described in more detail in Chapter 6.6.

New Region: allows the user to add a region for analysis. Left click on the icon, then left click and hold while moving the mouse to define the new region. Click “Stop” in the instruction window when done. Then the user is prompted to identify the region – it can be added as a new compound or as a new region for a compound already in the method. For a new compound, the user will be prompted for key information, such as name, classification, and molecular weight.

 Import SBASE Compound: allows the user to add a new compound from an SBASE. A window prompts for the SBASE, compound, and spectrum to use.

 Import Compound from CMC-a: allows the user to import a compound directly from a spectrum previously analyzed in CMC-assist, without creation of an SBASE entry. Note the new compound name will be the dataset name of the spectrum.

 Show Compound Spectrum: displays the SBASE spectrum for the compound currently named under **Compound Description**, superimposed on the spectrum in the viewer window.

 Larger by Factor of 2: scales up the intensity of the spectrum currently displayed in the viewer window by a factor of 2.

 Smaller by Factor of 2: scales down the intensity of the spectrum currently displayed in the viewer window by a factor of 2.

 Reset Vertical Scale: resets the intensity scaling of the spectrum displayed in the viewer window to the original value, typically such that the largest peak just fills the window.

 Reset to Full Display: resets the horizontal scale to the full range. For a 1D spectrum, this is the full spectral width.

 Proportional Shift: when more than one spectrum is overlaid in the viewer window, this option can be used to spread out the spectra. Left click on the icon, then left click and hold in the viewer window while moving the mouse up and down to set the separation between the spectra.

 Smooth Vertical Offset: allows adjustment of the vertical offset of the spectrum displayed in the viewer window with the mouse. Left click on the icon, then left click and hold in the viewer window while moving the mouse to move the spectrum up and down.

 Reset Vertical Offset: restores the vertical offset to its original value.

 Smooth Horizontal Expand: Left click the icon then left click and hold while moving the mouse sideways to expand the spectrum horizontally about the center point.

 Undo Last Zoom: undoes only the last horizontal zoom, so the spectrum returns to its previous expansion.

 Set Contours of 2D Spectra: brings up a window for setting the contours for a 2D spectrum.

 Measure Distance: after clicking this icon, the user can draw a line segment with the mouse. The horizontal distance will be reported in ppm and Hz the upper left corner of the viewer window.

Run Quantification: AssureNMR will run the current quantMethod on the spectrum displayed in the viewer window. First, a window pops up and asks whether a report should be created, then the analysis runs. The results will be displayed on screen. If selected, the report will automatically open in the default pdf viewer.

Quantify List of Spectra: AssureNMR will run the current quantMethod on a list of spectra. A series of windows prompt the user for the source of the spectra (TopSpin or an SBASE) and the spectra to analyze. After the quantification runs, a summary table with the results for each spectrum appears.

Quantify Active Region: tests the analysis parameters only for the region currently selected in **Compound Description**/'Compound Name' and displayed in the viewer window. The results are displayed in the viewer window.

## 6.4 Editing a Method Based on the Standard Algorithm

The key options for fitting the peaks in the spectra are available in the parameter panel on the left when editing the quantMethod. The rest of this section goes through the headings of the parameter panel for the standard algorithm for analysis. The chapter *Editing a Method in the Tab and Table Mode [▶ 89]* goes through the panel for the advanced algorithm.

### 6.4.1 General Section

Clicking on **General** in the parameter panel brings up options that affect the analysis for all compounds. The units for reporting the concentration can be selected from a pulldown menu. Note that Quantification "Standard" with "External" require an external calibration standard that is either assigned through "(SST)" or "Imported" with "(User Defined)". The spectral database can also be selected from the pulldown menu – the list includes all the spectral databases currently registered. The checkboxes turn on specific features of AssureNMR analysis. When 'Identify Unassigned Peaks' is selected, the software will note any peaks that were not accounted for in the lists for compounds analyzed in the spectrum and compare those peaks against the currently selected spectral database. Any matches are reported as possible assignments for those peaks. When 'Check for Non-Assigned Peaks' is selected, the software will keep track of the unassigned peaks it finds. These will be marked on the summary spectrum in the Expert Report. If these peaks are above a threshold (available to the user through the tab and table edit mode), the spectrum will be flagged with "Fail" as the overall result. When 'Check for Unbalanced Integrals' is selected, the software checks for intensity remaining after peak fitting (using symmetric shapes). Any remaining intensity is considered an additional peak. At the bottom of the **General** list is the **Details** button. This brings the user into the tab and table mode for editing the quantMethod. (See chapter *Chemometrics Tab [▶ 104]*).

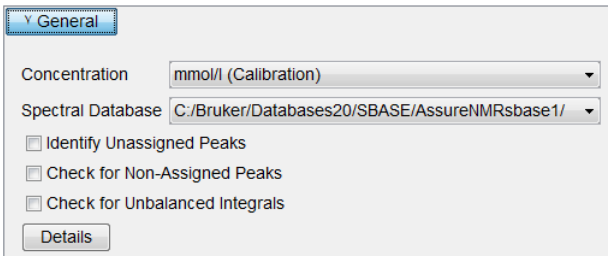



*Figure 6.9: Items under the General heading in the parameter panel. (These are the same for both the standard and advanced algorithms).*

## 6.4.2 Compound Description

The next heading is Compound Description. This opens up to provide information about each compound in the quantMethod. The compounds currently described in the quantMethod can be seen in the pulldown menu under 'Compound Name'. Once the user selects a compound, a comment for that compound and the molecular weight can be specified in the fields below. The **Edit** button brings up a window with more options for the compound. The **Delete** button removes the compound from the quantMethod.

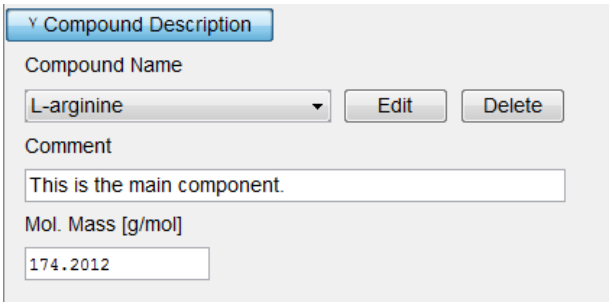



*Figure 6.10: Items under the Compound Description heading in the parameter panel. (These are the same for both the standard and advanced algorithms.)*

In the Edit Compounds window, **Import Molecule** lets you select a structure file (in mol file format) for the compound. AssureNMR automatically determines the formula and the Average Mass (molecular weight) from this file. The Average Mass can also be entered directly. The 'Type' pulldown lets the user specify the type based on classification for the AssureNMR analysis (described in more detail in Chapter 6.6.1.1). Next are the options to specify concentration thresholds, 'Apply Min. Concentration', followed by the value to compare against, and 'Apply Max. Concentration', also followed by the value. Finally, there is an option specifically pertaining to the matching of HSQC spectra, 'HSQC rel. Integrals', with options Strict, Standard, Weak, Plausible(Overlap). These options set how strictly the relative intensity of the peaks, based on the multiplicity, will be enforced, since sometimes the transfer efficiency is different for different peaks.

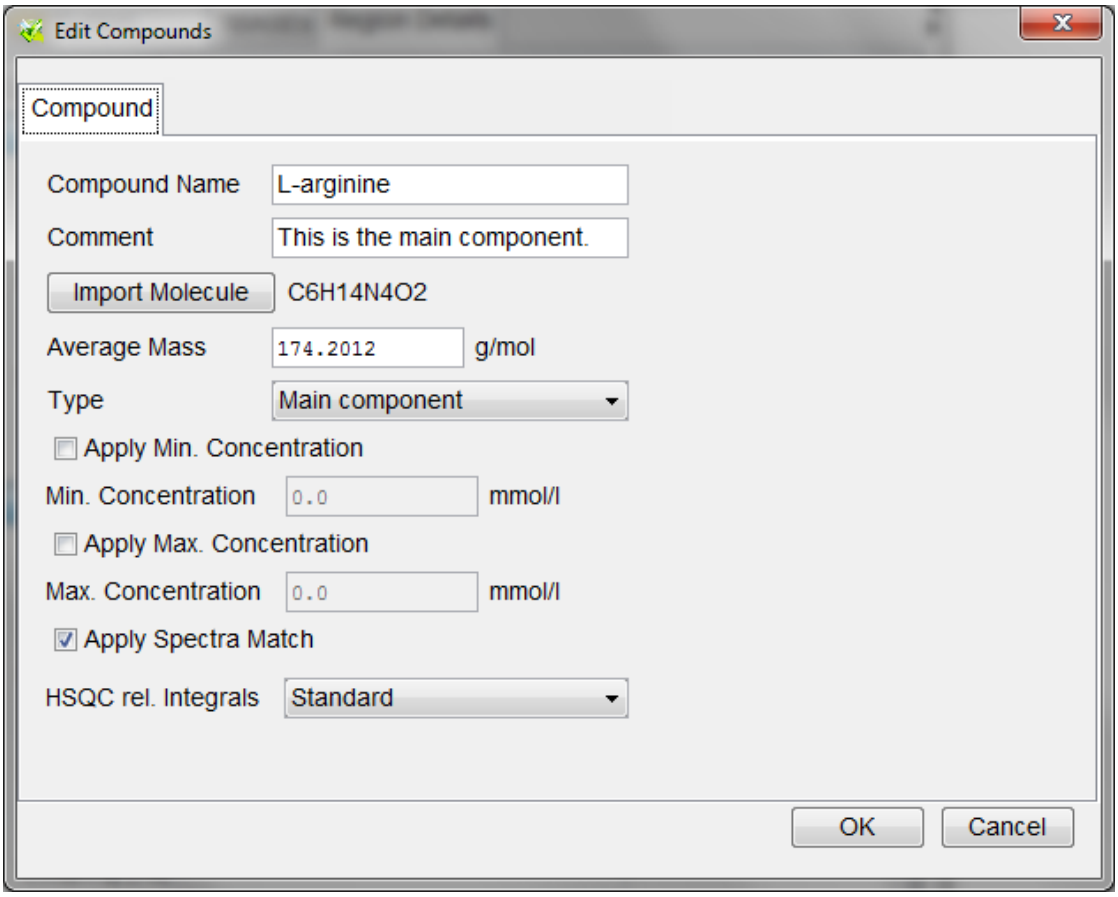



*Figure 6.11: Edit Compounds window.*

### 6.4.3 Basic Signal Description

Under the **Basic Signal Description** heading, there are options pertaining to each region for the compound selected under **Compound Description**. First, there is a pulldown menu to select which signal to focus on ('SelectedSignal'). Next is a checkbox near 'Quantify'. This allows the user to select this signal to be used to quantify this compound. Note if multiple regions are selected for the same compound, by default, the program reports the average. The behavior can be set on the General tab in the tab and table mode of editing (Chapter *General Tab [▸ 91]*). 'Count' refers to the number of nuclei contributing to the signal (for example, 3 protons for a methyl group). The 'Lineshape' must be selected from the pulldown menu. The details for the lineshapes are explained in Chapter *Lineshapes Available [▸ 97]*. The check box above the lineshape, 'Keep Intensity Ratio', specifies whether the ratio of peaks expected for the given lineshape should be strictly enforced when fitting. The 'Search Range [ppm]' specifies the chemical shift region where the signal must be found. The values can be adjusted by typing in the boxes, using the up and down arrows on the right side of the boxes, or interactively in the viewer window, by adjusting the boxes on the ends of the bar for this signal with the mouse. For lineshapes with splitting, the coupling constants are specified next. The table of Position and Intensity shows the lines AssureNMR will use for fitting. The position is in Hz, relative to the center of the multiplet as zero. The buttons below the table allow the user to adjust the peaks. **Import From Region** uses the peaks in the search range to add peaks to the table. **Adjust Peaks** allows the user to use the mouse to adjust peaks for fitting to correspond to the spectrum in the viewer window. Clicking activates this mode; it should be clicked again to turn it off. This option is not active for the 'S' (singlet) lineshape. The **Add**, **Remove**, and **Pick Peaks** buttons only become active for a special lineshape called 'M(user def.)' for "multiplet, user defined". For this lineshape, the user can specify how many peaks there are in the signal, the relative intensities expected, and the distance in Hz

between the components. (See also Chapter *Lineshapes Available [▷ 97]*.) Once the parameters have been chosen, the user can test them in the spectrum window by hovering over the bar for the region, clicking to bring up the menu, and selecting **Test Region**.

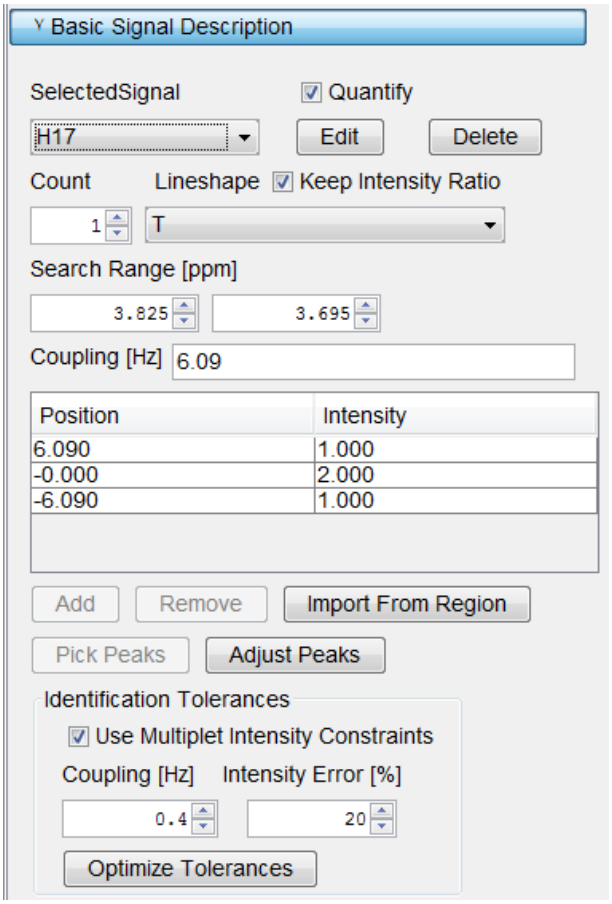



*Figure 6.12: Items under the Basic Signal Description heading. (These are specific for the standard algorithm.)*

The 'Identification Tolerances' refer to parameters for the matching to the SBASE required before the peaks are fit. The user has the option to 'Use Multiplet Intensity Constraints'. If this checkbox is active, the tolerances for the coupling between the peaks and the intensity ratios can be specified. These tolerances can be set automatically by the program, based on the current spectrum, using the **Optimize Tolerances** button.

## 6.4.4 Fine Tuning

The **Fine Tuning** heading includes options to adjust and optimize the peak fitting. Options become active when 'Fine Tuning On' is checked. 'Fit Base Line' accounts for the baseline before fitting peaks. 'Peak Top' weights the peak top more heavily in judging the quality of the fit. 'Fit Must not Exceed Line Shape' requires that the fitted contour never exceed the experimental spectrum. 'Amplitude is Positive' considers only postiive points in the spectrum for fitting. 'Fixed Amplitude' forces the fit to use the highest point as the amplitude regardless of the linewidth.

'Peak Pick Again' repicks the peaks in the current region; it must be checked for the options below to be active. 'Shoulder Detection' accounts for shoulder at the edges of the peak and 'Clear Minimal Peaks' removes the weakest peaks in the region. The 'Noise Factor [sd]' gives the multiplicative factor for the noise standard deviation, above which a peak will be considered a real signal. This factor is specified in the box.

The ‘Peak Shape Parameter’ has five options: Unconstrained, Global Spectrum, Region, Reference Signal, and User Defined. This parameter specifies what information the program uses to determine the lineshape – i.e. the lorentz to gauss ratio. If **User defined** is selected, the boxes below become active and the user can specify the peak shape through the boxes to the right of ‘Peak Width’ and ‘Lorentz [%]’.

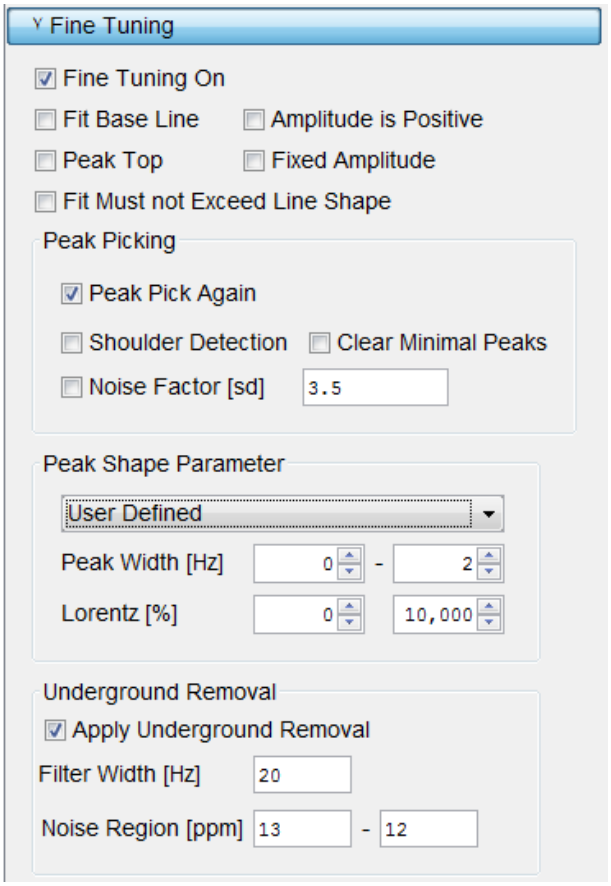



*Figure 6.13: Items under the Fine Tuning heading. (These are specific to the standard algorithm.)*

‘Underground Removal’ is a way to get rid of broad background signals in the spectrum. When it is activated, it uses the ‘Filter Width [Hz]’ to establish a window for averaging. Essentially, peaks broader than the filter width are removed. The ‘Noise region [ppm]’ identifies a region of the spectrum containing only noise, used to set the baseline zero.

## 6.4.5 Other

The **Other** heading gives the user a few more options to customize the interpretation of the peaks. The first section gives ‘Integral Corrections’. The peak can be corrected according to its $T_1$ value (for example, if $T_1$ relaxation times have been measured). When ‘Apply Integral Scaling’ is checked, the integral will be scaled by the number in the ‘Factor’ box. An offset can also be specified.

The option under ‘Region Integration Options’ is implemented for region integration only (Basic Signal Description/Lineshape/Region). When ‘Sum all Points if Minimum S/N is reached’ is checked, the program examines the ‘Min S / N Factor’. If any of the points in the specified region of the spectrum are above the minimum signal-to-noise factor, then the region is integrated. Otherwise, the program returns zero for the region.

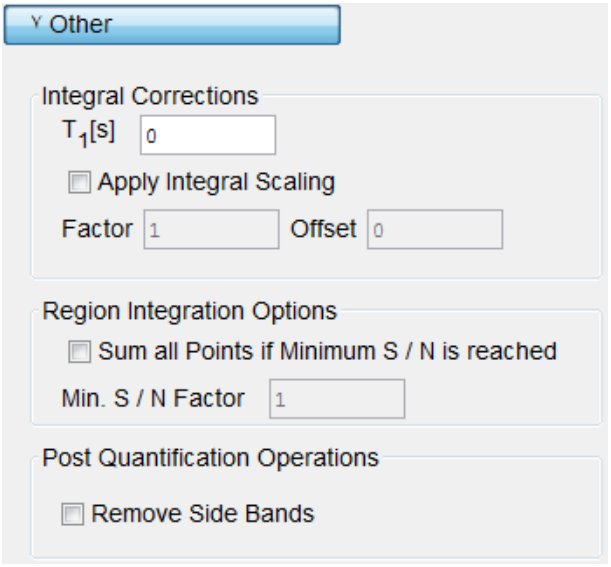



*Figure 6.14: Items under the Other heading. (These are specific to the standard algorithm.)*

In the 'Post Quantification Operations' section, checking 'Remove Side Bands' searches for peaks near the noise level, symmetrically arranged about the main peak, and removes them.

## 6.5 Editing a Method Based on the Advanced Algorithm

The advanced algorithm takes advantage of the same **General** information (Chapter *General Section [▶ 81]*) and **Compound Description** (Chapter *Compound Description [▶ 82]*) as the standard algorithm, so these will not be described again here. The details for the peak description are somewhat different and are described below.

### 6.5.1 Basic Signal Description (Advanced Algorithm)

The items under the **Basic Signal Description** heading for the advanced algorithm are similar to those for the standard algorithm (Chapter *Basic Signal Description [▶ 83]*). The 'SelectedSignal' can be set through the pulldown menu. Checking 'Quantify' turns on quantification for that signal. **Edit** lets the user change the Region Name. **Delete** removes this signal form the analysis. 'Count' gives how many nuclei contribute to the signal. Then the options diverge. There is a check box to designate a signal a 'Main Signal'. A main signal should always be present in the material under analysis. 'Type of Signal' replaces 'Lineshape', with the following options:

- Singlet: fits a single peak to the strongest signal in the region.
- Binomial: expects a multiplet with the ratio of the peak intensities given by binomial coefficients (1:1 for a doublet, 1:2;1 for a triplet, etc.).
- Roof Effect: expects a multiplet with a typical ratio that has been distorted by higher order splittings to give a "roof effect" – outer components of the multiplet have lower intensity than inner components.
- Symmetric: expects a multiplet with the peaks symmetric about the center. There may be an odd or even number of peaks.
- Peak based: allows the user to specify a distribution of peaks in the area with arbitrary intensity ratios and spacings.

The ‘Multiplicity’ refers to the number of peaks to be fit. The ‘Coupling [Hz]’ is the first order coupling for the multiplet. ‘Center[ppm]’ gives the peak position; ‘Line Width [Hz]’ is the starting linewidth for fitting, and ‘Gauss [%]’ is the percentage of gaussian lineshape shape to start with in the fitting. The checkbox ‘Use Exponent’ applies exponential linebroadening to the shape for fitting, with the exponent specified on the right.

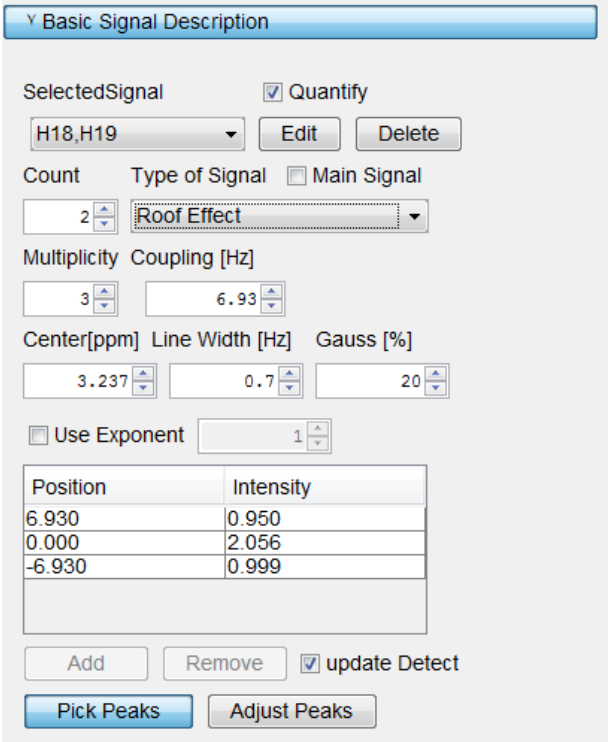



*Figure 6.15: Items under the Basic Signal Description heading for the advanced algorithm.*

The peak table with columns ‘Position’ and ‘Intensity’ lists each peak that will be fit for this SelectedSignal, with the position given in hertz from the center of the multiplet and the intensity given as the ratio between the peaks. To change this table, click on **Pick Peaks** or **Adjust Peaks**. Note the **Add** and **Remove** buttons become active after **Adjust Peaks** is selected when the type of Signal is ‘Peak based’. These options are context based and will only be available for some combinations of parameters. Once the parameters have been chosen, the user can test them in the spectrum window by hovering over the bar for the region, clicking to bring up the menu, and selecting **Test Region**. The shaded area that appears shows the fit. When the ‘update Detect’ checkbox is selected, any adjustments made to the peak table and parameters will also be used for the peak detection.

## 6.5.2 Parameters for Detection

The program first searches for the peaks of the selected signal, then fits them. The **Parameters for Detection** heading contains the information necessary for the search. First is the region of the spectrum to search (‘Search range [ppm]’). Next is the ‘Noise Factor’. Any signal stronger than the noise factor times the noise level in the spectrum is considered a real signal. The ‘Coupling Tolerance [Hz]’ gives the accepted tolerance for the observed coupling in the multiplet.

Sometimes reprocessing the spectrum can improve the detection of the peaks of interest. In the ‘Processing Parameter’ window, the first option is to apply ‘Line Broadening [Hz]’ with the specified value. When ‘Apply Underground Removal’ is checked, broad signals are removed using the value in the ‘Filter Width [Hz]’ box.

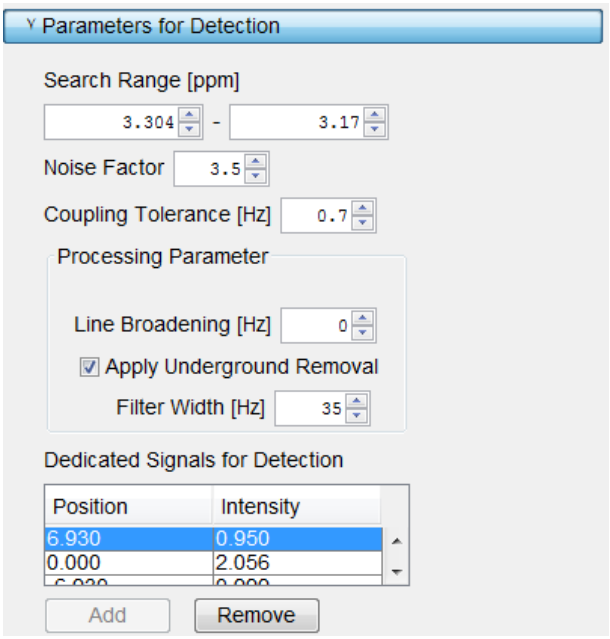



*Figure 6.16: Items under the Parameters for Detection heading. (These are specific for the advanced algorithm.)*

The last section of this heading is the 'Dedicated Signals for Detection' table. This is similar to the peak table above; the position is given in hertz compared to the center of the multiplet and the intensity as the ratio between the peaks. If one of the peaks in the multiplet is very weak or always obscured by overlapping peaks, it may be a good idea to remove it from this table. On the other hand, if a key peak of the multiplet is not listed, it may be added.

### 6.5.3 Parameters for Fitting

The details for fitting the spectrum in the region for the current SelectedSignal are found under the **Parameters for Fitting** heading. The 'Fitting Window [ppm]' gives the tolerance on the position of the top of the peak being fit. The next tolerances ('Coupling Tolerance [Hz]', 'Amplitude Tolerance [%]' and 'Shift Tolerance [ppm]') are typical tolerances for fitting the spectrum.

Under 'Processing Parameter', there are values required for reprocessing the spectrum before fitting, including adjusting resolution by exponential multiplication ('Line Broadening [Hz]'), changing the digitization of the spectrum by adding points ('Interpolation Factor'), and removing broad baseline ('Apply Underground Removal').

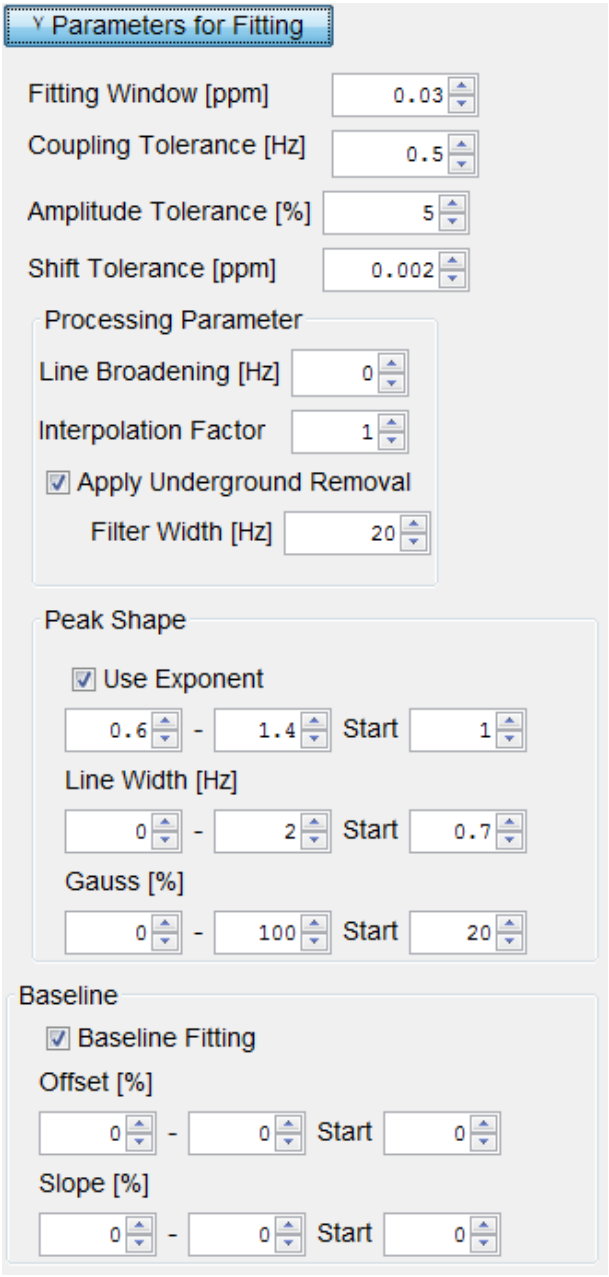



*Figure 6.17: Items under the Parameters for Fitting heading. (These are specific to the advanced algorithm.)*

The details for the fit curve come under ‘Peak Shape’. The curve can be weighted by exponential multiplication (‘Use Exponent’). The fit requires the linewidth (‘Line Width [Hz]’) and shape (‘Gauss [%]’). For these parameters, the user specifies a range of allowed values and the starting value. The program will search within those ranges for the best fit.

Under ‘Baseline’, the first option is to turn on ‘Baseline Fitting’. For baseline fitting, the user must specify the ranges and starting values for the offset and slope.

## 6.6 Editing a Method in the Tab and Table Mode

The main features for fitting peaks are available through the interactive parameter panel, as described above. But certain advanced features, such as the user-defined equations, specifying the calibration for absolute concentrations, and adding chemometric models, are

only available though a more text-based interface. The Edit Method icon (  ) brings you to this interface, as does the **Details** button under the **General** section of the parameter panel. This interface features a window with seven tabs. The features available through each tab are described in the following sections.

### 6.6.1 General Tab

The **General** tab allows the user to specify the details for quantification.

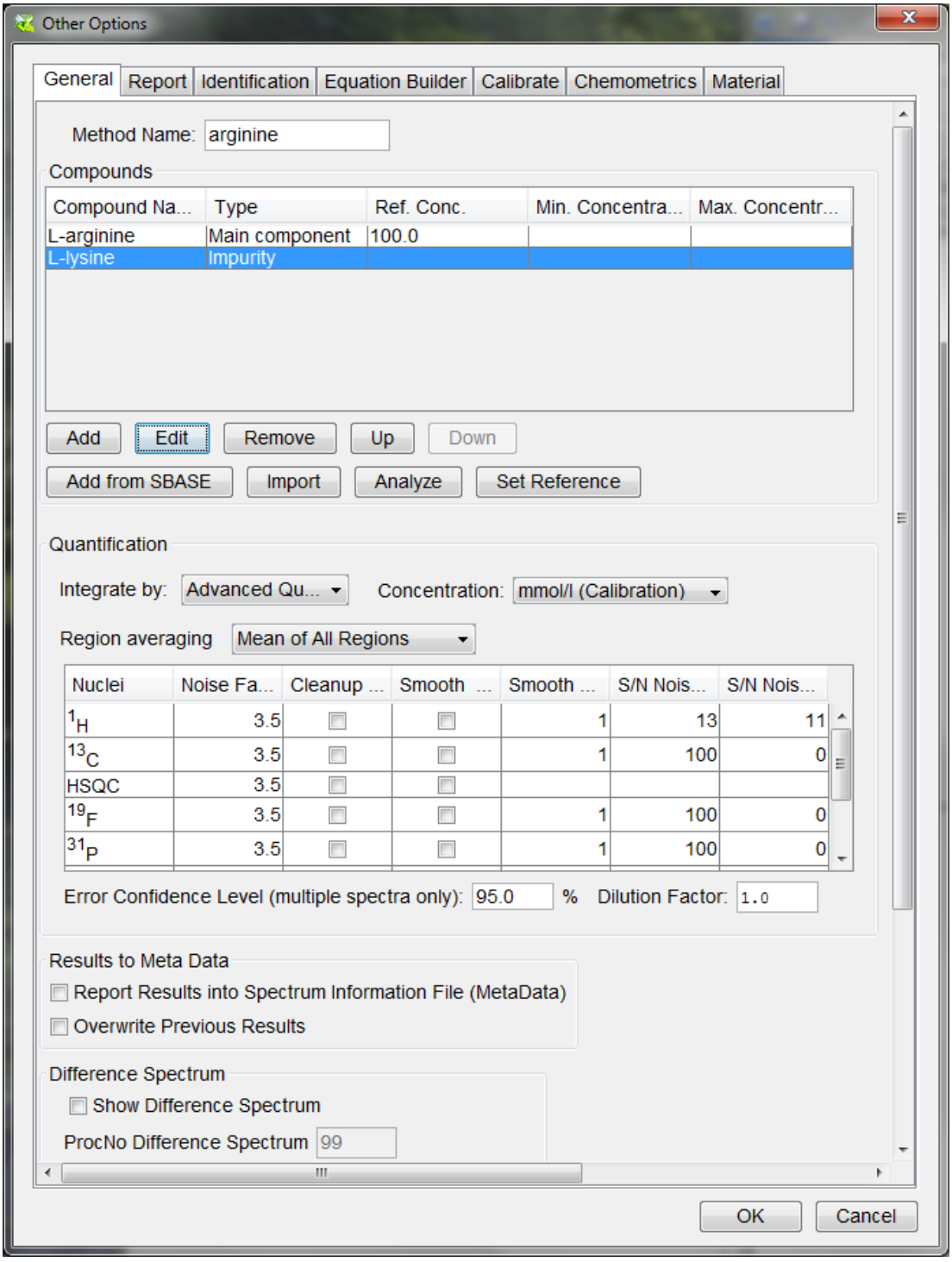



*Figure 6.18: The General tab of the tab and table interface for editing a quantMethod.*

Specifically, the **General** tab provides access to the following parameters:

- Method Name: name of the method, typically the same as the root of the filename for saving the method.
- Compounds window: this window is used to set the compound definitions of known constituents in the sample to be analyzed.

- Quantification window
  - Integrate by: choose whether the method should integrate by advanced quantification, peak fit, region integration, or peak intensity (height).
  - Concentration: specifies how to report the concentration. Relative concentrations can be reported as molar ratios (mol % reference and mol % total integral) or percent by weight (g % reference, g % total integral). Absolute concentrations require calibration (mmol/l, mol/l, mg/l, g/l). The weight percent of the sample (g % of sample weight) requires calibration and will prompt the user for the weight of the original sample and the volume of the NMR sample.
  - Region averaging: when more than one region in the same compound is selected for quantification, the user can select whether the program reports the mean of all regions or the minimum concentration.
  - Error Confidence Level: used with the multi-sample acquisition tool. Here the limit is used to calculate the error for quantifying a set of spectra. The error is calculated from the standard deviation and *t*-test using this confidence limit.
  - Dilution Factor: the dilution of the NMR sample from the sample of interest. This allows the original sample's concentration to be reported directly, correcting for dilution.
- The following options are specified in the table. This allows the user to set different values for different nuclei (or spectra, as in the HSQC row).
  - Noise Factor [sd]: number of standard deviations (sd) above noise to define real peaks for any signal.
  - Cleanup Peaks: used for noisy spectra. It picks peaks above a very low height threshold (typically 5 points) and then takes into account the peak widths to smooth the spectrum and remove the smallest peaks.
  - Smooth (PP): changes the smoothing algorithm used for Cleanup Peaks. When the option is on, the software smooths the spectrum using a Savitzky-Golay filter of the width in hertz specified in the next column. Then peak picking is performed on the smoothed spectrum. This typically results in fewer peaks picked.
  - Smooth Width [Hz]: width for the filter for the Smotth (PP) option for Cleanup Peaks. Typically, the width is chosen to be larger than any peaks expected in the spectrum. For small molecule spectra, 5 Hz may be a good starting value.
  - S/N Noise Left [ppm]: left boundary of the region to be used to calculate the noise. This is used specifically for the threshold option for region integration (found under the **Other** heading/'Region Integration Options' in the interactive interface or on the Edit compound region window in the tab and table interface).
  - S/N Noise Right [ppm]: right boundary of the region to be used to calculate the noise. This is used specifically for the threshold option for region integration (found under the **Other** heading/'Region Integration Options' in the interactive interface or on the Edit compound region window in the tab and table interface).
- Results to Meta Data window
  - Report Results into Spectrum Information File (MetaData): when checked, the main result of the quantMethod is stored with the other metadata for the spectrum.
  - Overwrite Previous Results: when checked, the option above has overwrite permissions, in case there is already a value stored in the metadata.
- Difference Spectrum window
  - Show Difference Spectrum: when checked, the difference spectrum between the recorded data and the fitted spectrum will be created, displayed in the viewer window after running a quantMethod, and stored with the experimental data in the same dataset name.
  - ProcNo Difference Spectrum: number for the directory within the dataset name where the difference spectrum will be stored.

Further explanation for many of these parameters is provided in the following sections.

#### 6.6.1.1 Compounds Window

The Compounds window (at the top in the figure below) is where the user builds up information about the compounds to be analyzed for a particular material. To add compounds, the user can either **Add** or **Add from SBASE**. Clicking on **Add** produces the window shown below; this window can also be reached by highlighting the compound of interest and clicking the **Edit** button:

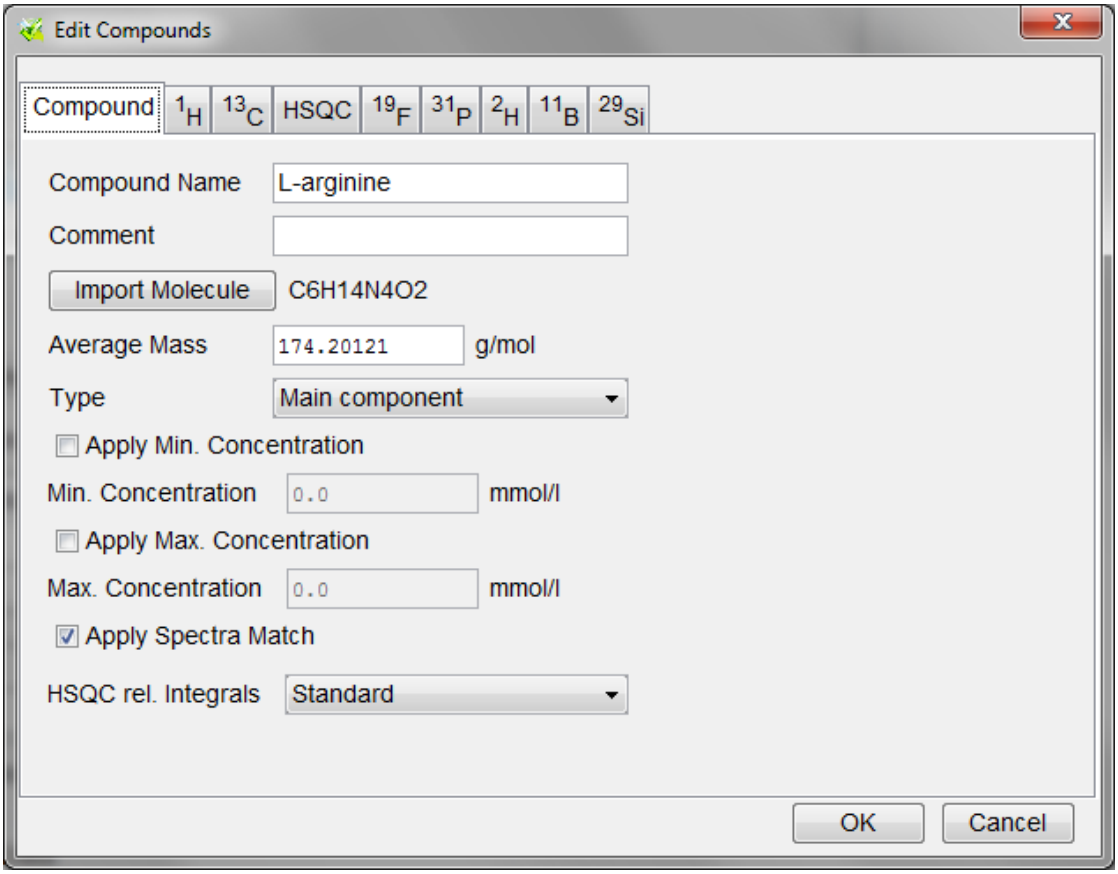



*Figure 6.19: The Edit Compounds window, Compound tab.*

In the **Compound** tab, the parameters are:

- Compound Name
- Comment
- Import Molecule: This button lets the user import a mol file. The program will automatically calculate the molecular formula and molecular weight (average mass) from the structure.
- Average Mass: molecular weight of the compound
- Type: this is the category of component. The nomenclature for the types can be changed using the Report tab of the Edit Method window. The type affects the way in which the compound is analyzed as follows:

| | | Failure on Quantify | | Failure on Match | |
|---|---|---|---|---|---|
| | Full spectral integral | $^{1}$H | $^{13}$C | $^{1}$H | $^{13}$C |
| Main component | Yes | Yes | No | Yes | Yes |
| Additive | Yes | Threshold | Threshold | Threshold | Threshold |
| Adulterant | Yes | Threshold | Threshold | Threshold | Threshold |
| Impurity Group 1 | Yes | No | No | No | No |
| Impurity Group 2 | Yes | No | No | No | No |
| Impurity Group 3 | Yes | No | No | No | No |
| Solvent | No | No | No | No | No |
| NMR Reference | No | No | No | No | No |

*Table 6.1:* How the two-pronged analysis is evaluated for different compound types.

- Apply Min. Concentration: set a minimum required concentration; below that amount, the sample will fail to meet specifications.
- Apply Max. Concentration: set a maximum concentration; above that amount the sample will fail to meet specifications.
- Apply Spectra Match: matches the compound in the test spectrum to the reference spectrum in the SBASE for positive identification; default: on
- HSQC rel. Integrals: specifies how strictly to enforce the relative intensity of the peaks, accounting for multiplicity. The options are Strict, Standard, Weak, Plausible(Overlap). This accounts for the fact that sometimes the transfer efficiency is different for different peaks.

The other tabs contain tables for the individual nuclei and the HSQC spectra ($^{1}$H, $^{13}$C, HSQC, $^{19}$F, $^{31}$P, $^{2}$H, $^{11}$B, or $^{29}$Si) for a compound. These tabs feature tables with the following headings:

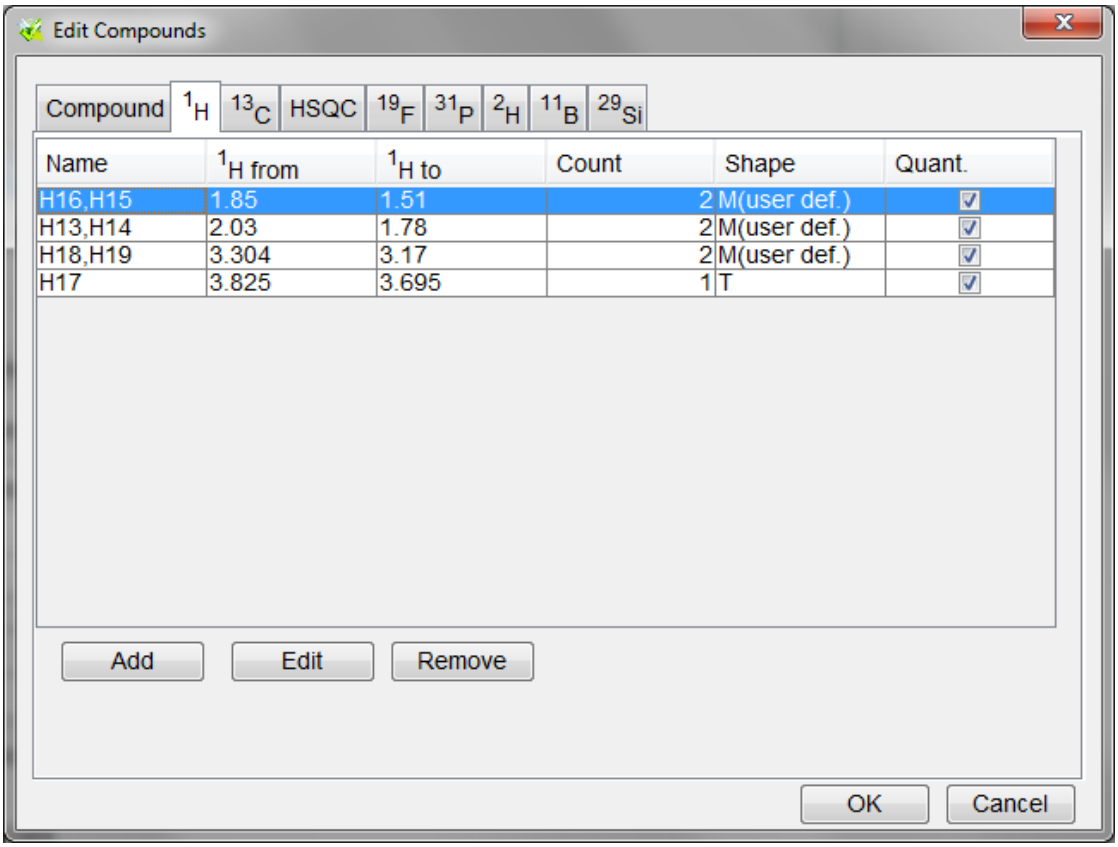



*Figure 6.20: Edit Compounds window, $^{1}H$ tab.*

- Name: identifier for the signal under consideration
- $^{1}H$ from, $^{1}H$ to: the region where signal is expected in the NMR spectrum
- Count: the number of atoms contributing to the signal in that area
- Shape: the lineshape to be used for integration in that signal area
- Quant.: When checked, the region is used to quantify the compound. If multiple regions are selected, the average is reported. Regions might be turned off when there is significant overlap, for example.

The **Edit** button brings the user one level deeper, where additional parameters for the integration can be specified.

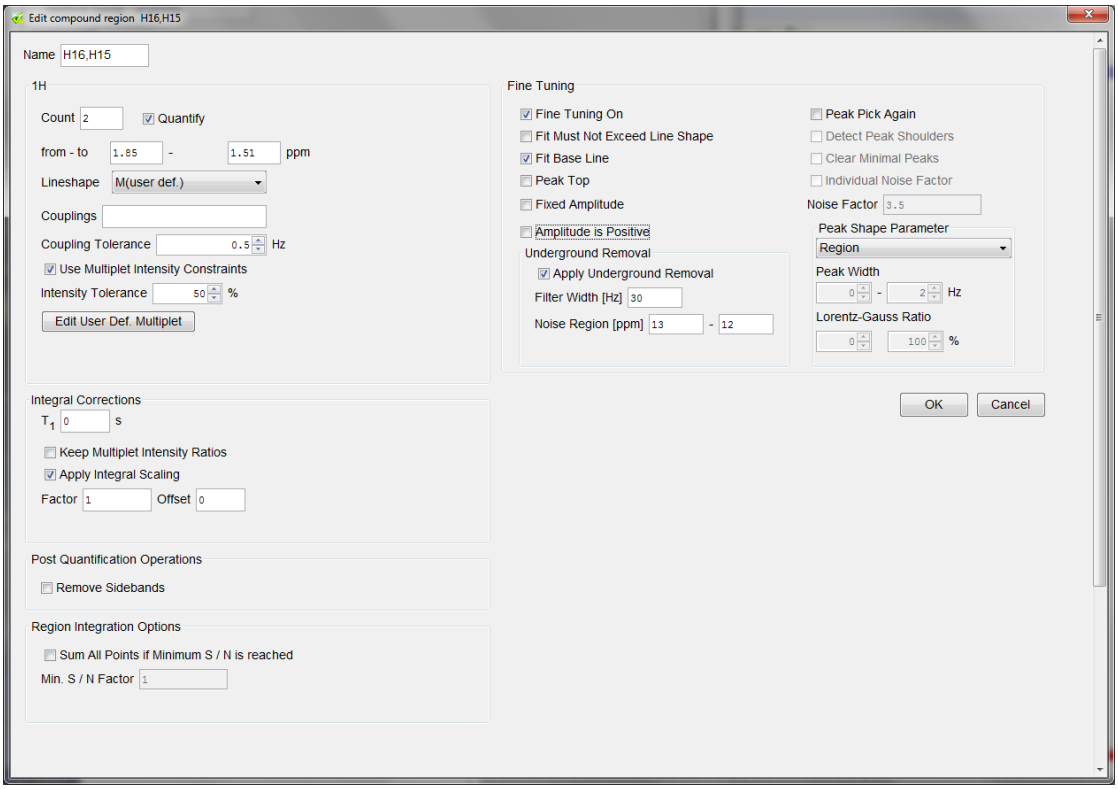



*Figure 6.21: The Edit compound region window specifies analysis information for an individual multiplet peak.*

Again, the atom count, region to analyze, and lineshape are available. In addition, the following parameters can be specified:

- Couplings: value in hertz for the scalar coupling constants for a multiplet
- Coupling Tolerance: how much a coupling can vary between observed and SBASE and still match
- Use Multiplet Intensity Constraints: sets a fixed ratio between components of the multiplet
- Intensity Tolerance: how much intensity can vary within a multiplet and still match
- Edit User Def. Multiplet: only active when M(user def.) is selected as the peak shape

Integral Corrections are available, using $T_1$ relaxation information or a scaling factor. The $T_1$ relaxation time indicates how long it takes the magnetization of a particular signal to return to alignment with the external magnet field after inversion; typically we allow a relaxation delay of 5 times $T_1$ between scans in an experiment for good recovery of magnetization and thus full intensity in each scan. If $T_1$ varies between locations in the molecule, different intensities will be observed for the signals, especially if the relaxation delay is less than 5 times the longest $T_1$. By entering $T_1$, the AssureNMR software will compensate for this effect. Alternatively, the integrals can be scaled by any value by checking 'Apply Integral Scaling' and specifying the factor and offset.

In the 'Post Quantification Operations' window, there is an option to remove sidebands. This option searches for symmetric peaks about the main peak and removes them.

Under 'Region Integration Options', there is the option to sum all points if minimum S/N is reached. The minimum signal-to-noise ratio is specified below, as a factor times the spectrum's signal-to-noise ratio. When this is active, the region will only be included in the analysis if at least one point in the region is stronger than the signal-to-noise cutoff. This only applies to analysis by region integration. The noise region is specified on the **General** tab itself. (See the discussion in Chapter *General Tab [▸ 91]*.)

'Fine Tuning' refines the peak fitting. It must be activated (box checked) for the other options to be active. The user can specify that the fit must never exceed the observed spectrum, or that the baseline should be fit. 'Peak Top' weights the peak top more heavily in judging the quality of the fit. 'Fixed Amplitude' forces the fit to use the highest point as the amplitude regardless of the linewidth. 'Amplitude is Positive' forces the program to use positive peaks only.

The 'Underground Removal' window lets the user clean up broader baseline peaks. The filter width (roughly the width of the peaks to be removed) and the region to take as noise at the baseline must be specified.

'Peak Pick Again' repicks the peaks in the current region; it must be checked for the options below to be active. 'Detect Peak Shoulders' accounts for shoulders and 'Clear Minimal Peaks' removes the weakest peaks in the region. The 'Noise Factor' can be adjusted for this region for the new peak picking.

Finally, under 'Peak Shape Parameter', the user can specify the expected linewidth and the lineshape (as the ratio of Lorentzian to Gaussian components). These are specified as a range; the fitting process will select the actual value.

#### 6.6.1.2 Lineshapes Available

For each compound, for each region analyzed, the lineshape must be specified. The options are:

- S: singlet
- D: doublet
- T: triplet
- Q: quartet
- Quin: quintet
- Hex: hextet
- Sept: septet
- DD: doublet of doublets
- DT: doublet of triplets
- DQ: doublet of quartets
- AB: strong coupling
- ABX(AB): strong coupling between A and B with weak coupling to X

**Special Patterns:**

- M(sum of peaks): defines any set of peaks that can't be described as a defined coupling. In the case that another pattern from another compound exists in the same region, the defined multiplet should be extracted first and the remaining signals will be integrated.
- M(user defined): allows the user to define coupling patterns explicitly such as those in lysine in the example SBASE which are not true triplets, but exhibit strong coupling.
- M(auto defined): uses information in the SBASE to define the expected coupling pattern and lineshape.
- M(moving): similar to the user-defined multiplet above, with the additional feature that it slides through the chemical shift range specified to find the best fit.
- Lineshape: uses information in the SBASE to specify the lineshape only.
- Region: integrates a region. It is only to be used when there is never any other signal than the one of interest in the specified region.

It is particularly important to accurately define the peak shape for peak discrimination and accurate integration. Please use the command Test Region in the interactive editing mode to find the best shape.

### 6.6.1.3 Adding a Compound from the SBASE

On the **General** tab, in the Compound window, perhaps the best way to add a compound to the method is the **Add from SBASE** button. The Import Compounds from SBASE window opens and is described in Chapter *Overview for Editing a Method [▶ 76]*. When a compound is added from the SBASE, information is incorporated automatically, ensuring a precise match in compound and SBASE names and reducing the input burden on the user. This procedure automatically populates the information visible in the Edit Compound window. It is a good idea for the user to verify this information.

### 6.6.1.4 Analyzing a Method

A feature of the quantification building interface is the ability to evaluate the probability of success in identifying and quantifying the compounds in the method. By selecting the **Analyze** button from the **General** tab, a report is generated which evaluates the provided data and reports any conflicts that may hinder the success of the method. The figure below shows the results of the analysis of the arginine quantification method.

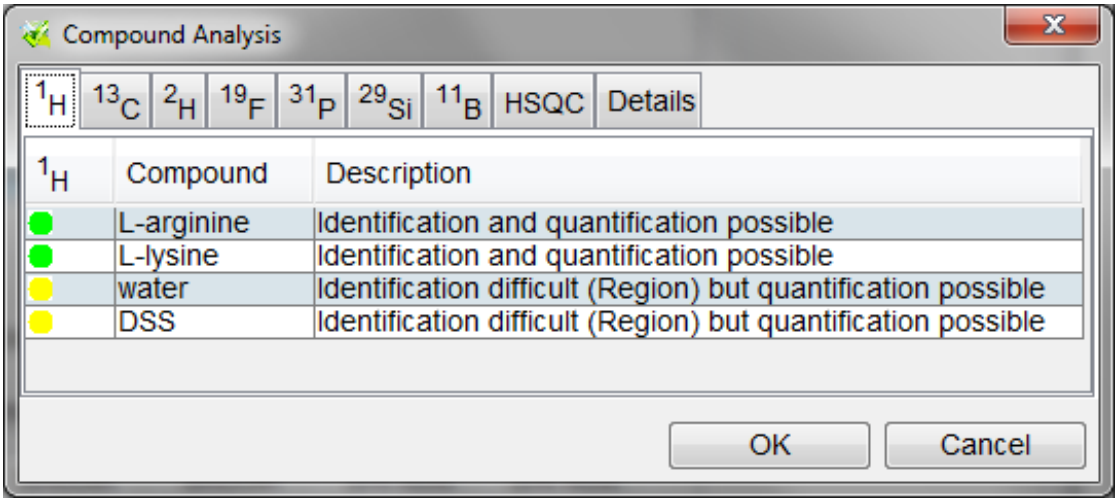



*Figure 6.22: Analysis results of the arginine quantification method. Tabs are available for $^{1}H$, $^{13}C$ and detailed reports. A green status ball suggests that the compound will be suitable and the yellow status ball suggests the compound may have difficulties for the identification and quantification as defined.*

### 6.6.1.5 Concentration Measurements

There are nine choices for reporting the integration results as concentrations. Four (molar % of reference, molar % of total integral, weight % of reference, weight % of total integral) correspond to relative internal quantification to the main component as summarized in the following table:

| | Internal Composition | |
|---|---|---|
| | **Absolute** | **Relative** |
| % Molar | $\frac{\frac{\int \text{Lysine}}{\#\text{of Protons}}}{\left(\frac{\int \text{Lysine}}{\#\text{of Protons}} + \frac{\int \text{Arginine}}{\#\text{of Protons}}\right)}$ | $\frac{\frac{\int \text{Lysine}}{\#\text{of Protons}}}{\frac{\int \text{Arginine}}{\#\text{of Protons}}}$ |
| % Gram | $\frac{\frac{\int \text{Lysine} * \text{MW}}{\#\text{of Protons}}}{\left(\frac{\int \text{Lysine} * \text{MW}}{\#\text{of Protons}} + \frac{\int \text{Arginine} * \text{MW}}{\#\text{of Protons}}\right)}$ | $\frac{\frac{\int \text{Lysine} * \text{MW}}{\#\text{of Protons}}}{\frac{\int \text{Arginine} * \text{MW}}{\#\text{of Protons}}}$ |

*Table 6.2:* Summary of quantification methods available based on the relative concentrations to the main component as exemplified with lysine as the adulterant and arginine as the main component.

The remaining five options (mmol/l (Calibration), mol/l (Calibration), mg/l (Calibration), g/l (Calibration) and g % of sample weight) correspond to absolute quantification, as seen in the table below. For these methods, a spectrum for an external standard of known concentration must be acquired and calibrated through AssureNMR in automation or manually through the AssureNMR interface.

| **Absolute Composition** | |
|---|---|
| Internal Reference | External Reference |
| Formulation | PULCON |

*Table 6.3:* Summary of absolute quantification methods.

### 6.6.1.6 Details of the Integration: Quantification and Detection

The AssureNMR package identifies noise by dividing the spectrum into 16 equidistant regions, or a minimum of 512 points are used. The region fulfilling the following criteria is then used:

- Mean and median are similar.
- Skewness is close to zero.
- No real peaks in the region – no peaks above noise.
- Region with most Gaussian-like distribution.

Noise is then defined as the mean plus a factor (F) times the standard deviation:

Noise = Avg + (F * STD)

Peak identification is then based on the criteria of which F is used and the user can select LOQ (Limit of Quantification) and LOD (Limit of Detection) for the appropriate spectral evaluation scheme. In a composite experiment, composed of a $^{1}H$ spectrum for quantification and a $^{13}C$ spectrum for matching, the user would select the LOQ for the $^{1}H$ spectrum and the LOD for the $^{13}C$ spectrum. For example, the 'Noise Factor' (F) (entered on the **General** tab, see chapter *General Tab [▶ 91]*) would be set to 10 for the LOQ on $^{1}H$ and 3.0 for the LOD on $^{13}C$.

## 6.6.2 Report Tab

The **Report** tab allows the user to configure the automatically generated reports. The 'Report Format 'can be Pass or Fail, Number, or Analyzed, as appropriate for the material. When 'Number' is selected, the boxes below become active, allowing the user to specify the display 'Precision', the 'Unit', and a 'Value Name'.

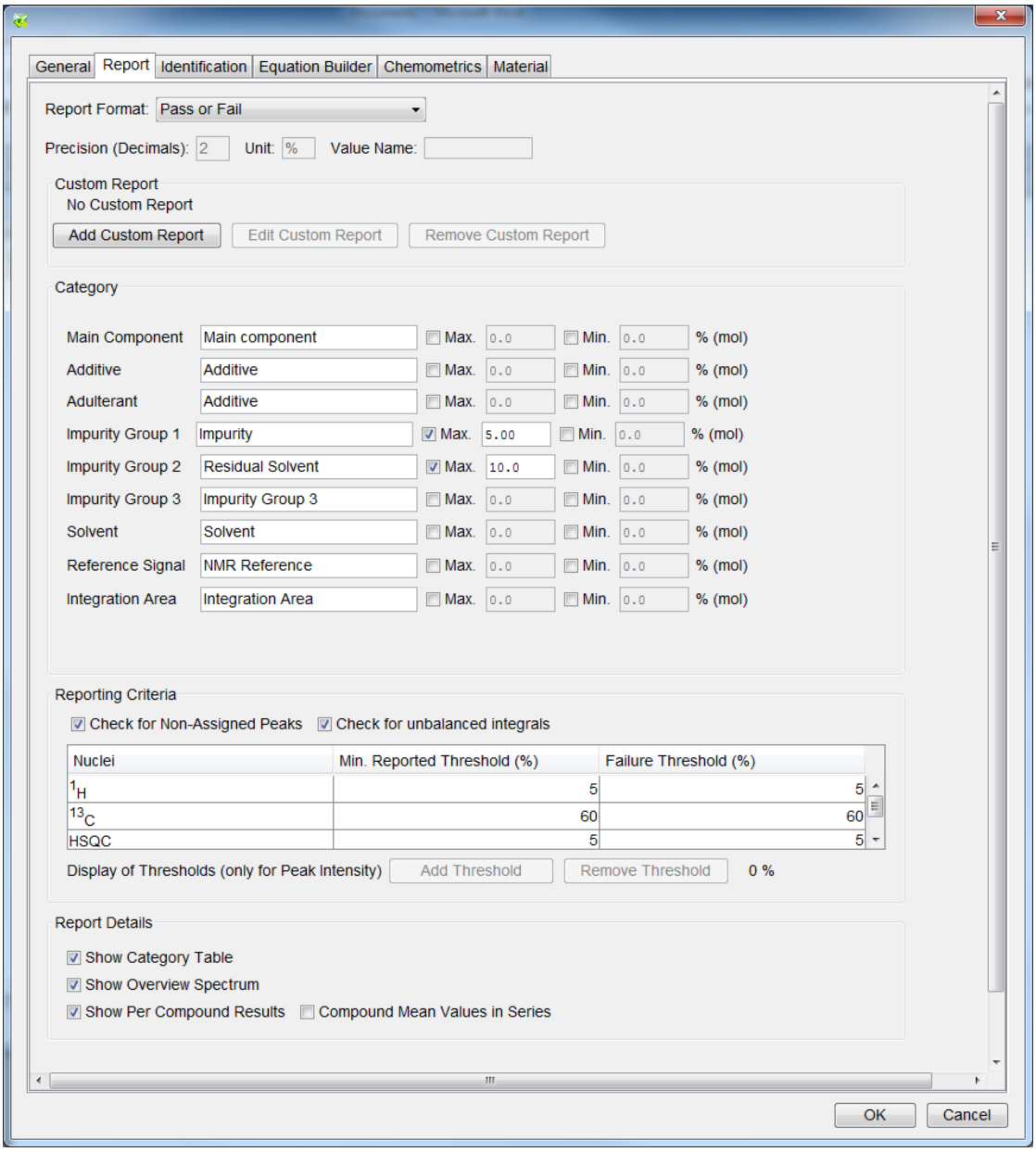



*Figure 6.23: Report tab.*

A Custom Report can be added. Custom reports can be created using the Eclipse BIRT to generate .rptdesign templates. Custom reports are also available from Bruker.

The ‘Category Types’ window allows the user to rename the compound type categories. Note the analysis will still be performed according to the table in chapter *Compounds Window [▷ 93]*. It is also possible to set maximum and minimum allowed levels for each category.

The ‘Reporting Criteria’ window allows the user to specify the details for total integral accounting. Specifically:

- Check for Non-Assigned Peaks: the software uses total integral accounting to keep track of all the signals in the spectrum. The Compounds table may not account for all the signals in the spectrum. With the check for non-assigned peaks on, the software will report any non-assigned peaks detected.
- Check for unbalanced integrals: the software carefully fits shapes of peaks. An unbalanced integral may indicate another signal under the main peak. With the check for unbalanced integrals on, any asymmetry will be reported.
- Minimum Reported Threshold: the level of integration relative to the main component at which any signal NOT defined in ‘Edit Compound List’ is reported (when Check for non-assigned peaks is checked.)
- Failure Threshold: level of integration relative to the main component at which any signal NOT defined in ‘Edit Compound List’ is reported and gives a failure in the final report.
- Display of Thresholds (for Peak Intensity only): This option is only active when peaks are evaluated by height using the Peak Intensity option on the General Tab. Then, threshold lines can be added to the displays of the spectrum at the specified percentages of the height of the reference.

### 6.6.3 Identification Tab

The figure below shows the **Identification** tab of the method. This tab gives access to the following parameters:

- Spectral Database: SBASE which is used to match compounds in the method.
- Exp. Type: name of the experiment as stored in the SBASE

Be careful to specify the correct SBASE and the correct experiment type, otherwise your quanMethod will fail because it cannot match.

- Min. Match Factor (%): the minimum level of confidence from a match at which the presence of a compound from SBASE is reported.
- Max. Shift (ppm): plus and minus search region for SBASE matching.
- Identify Unassigned Peaks: when checked, AssureNMR will use this SBASE to identify any peaks that were not previously identified in the quantMethod.

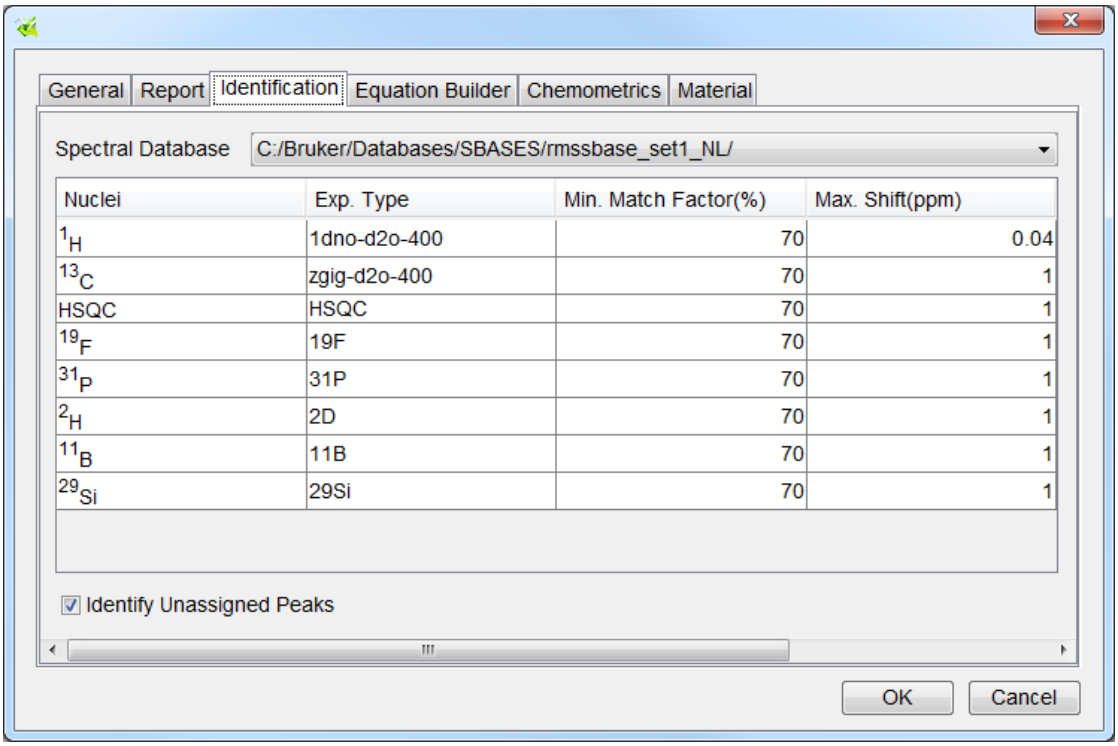



*Figure 6.24: Identification tab.*

### 6.6.4 Equation Builder Tab

In the case that the standard AssureNMR calculations are not sufficient, it is possible for the user to define their own calculation routine. An example using the equation builder for tire rubber analysis is presented in the chapter *Equation Builder [▷ 154]*.

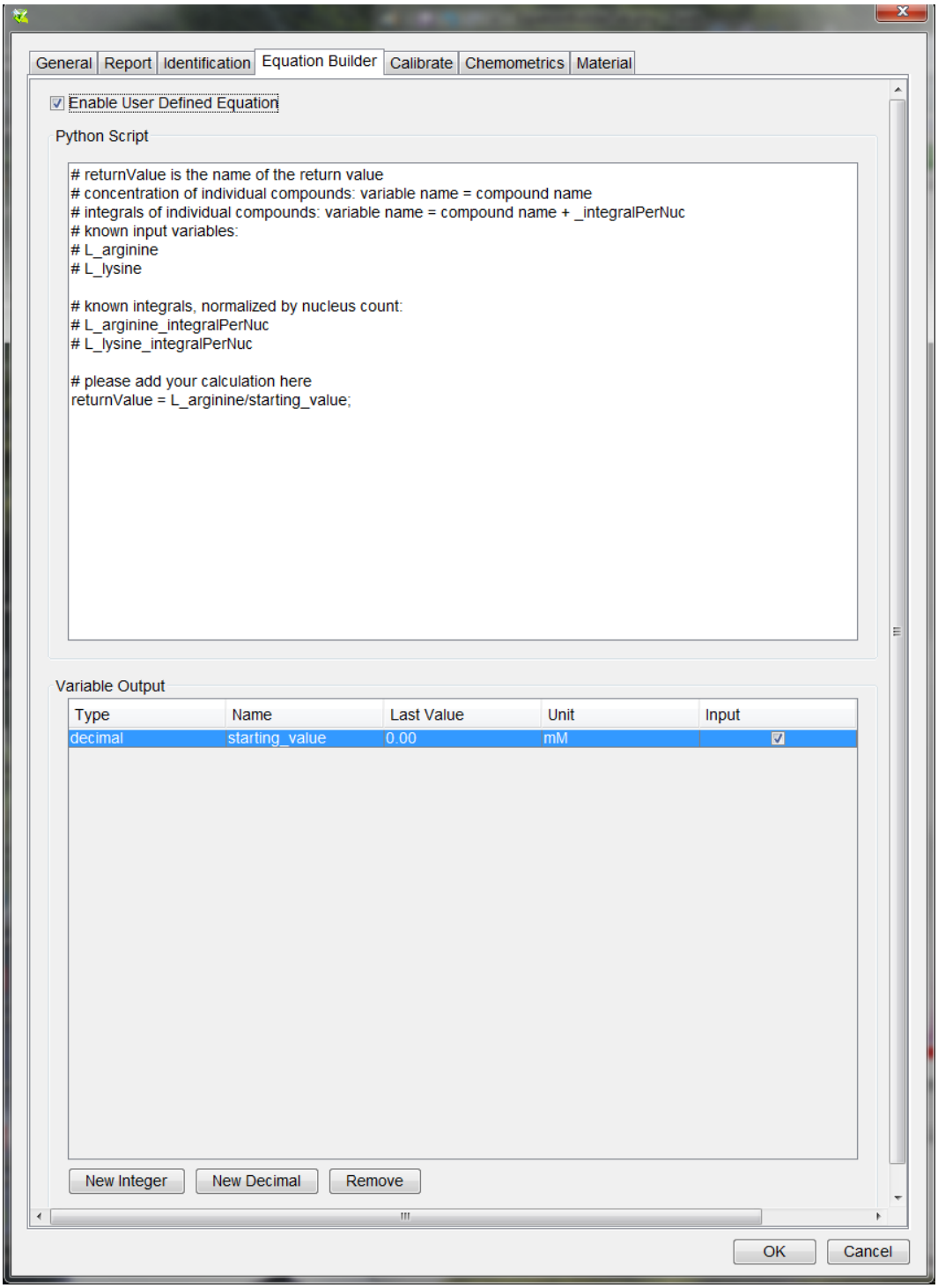



*Figure 6.25: Equation Builder tab.*

The ‘Enable User Defined Equation’ must be turned on. Code for the calulation can be entered into the ‘Python Script’ window. Below, the ‘Variable Output’ window gives the user the opportunity to define their own parameters for inputting and outputting values to and from the equation builder. To define input and output variables:

- New Integer: creates a user defined integer
- New Decimal: creates a user defined decimal
- Remove: deletes a user defined parameter

When ‘Input’ is checked, a dialog box will open when the quantMethod is run so that the user can specify the value. In automation, the user will be prompted for the value through the IconNMR easy setup interface.

### 6.6.5 Chemometrics Tab

The **Chemometrics** Tab allows the user to incorporate models developed with the tools from the Chemometrics pulldown (described in more detail in Chapter 8) into the analysis and reporting.

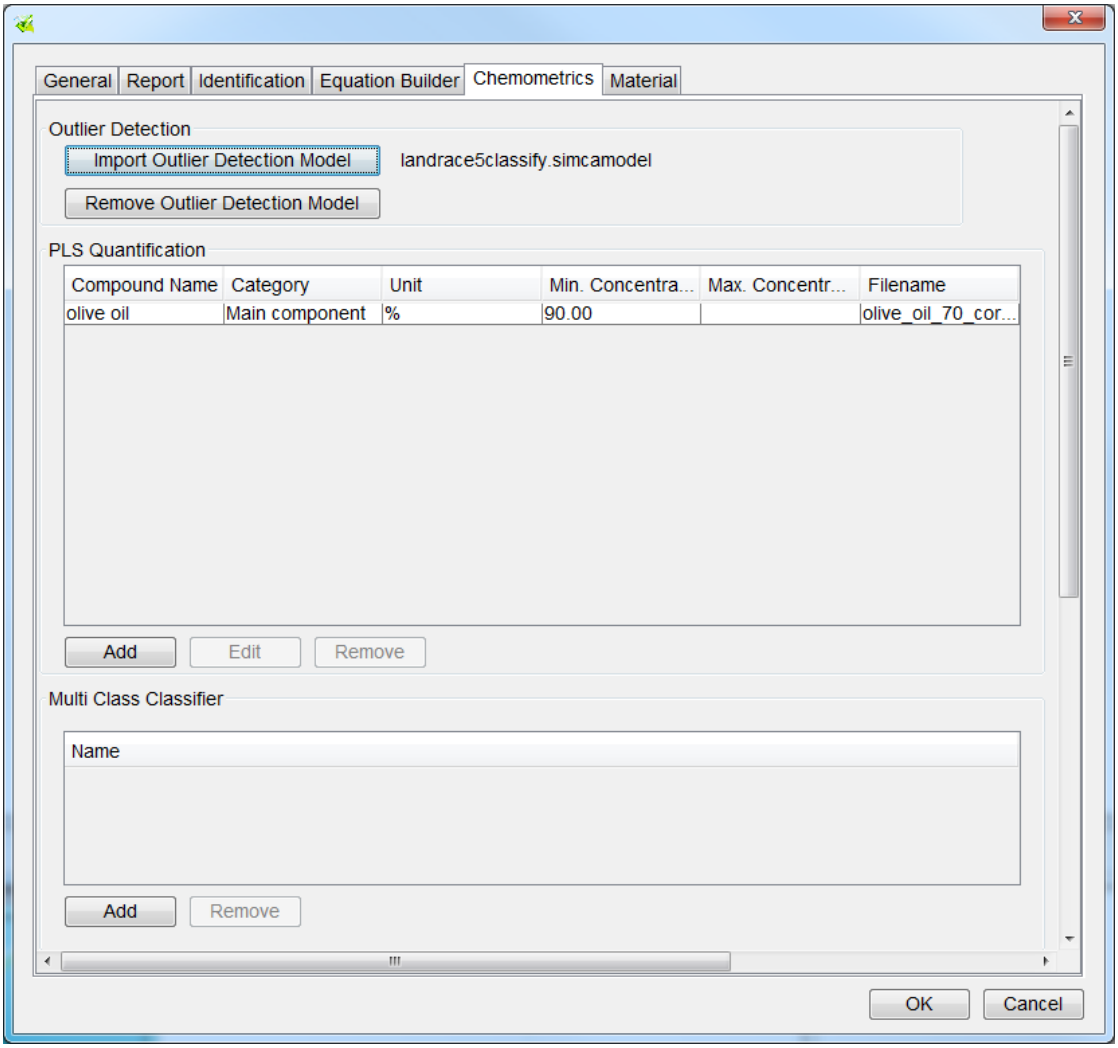



*Figure 6.26: Chemometrics tab.*

- Outlier Detection – allows the user to specify a SIMCA outlier detection model. With a SIMCA outlier detection model, samples can be classified in automation.
- PLS Quantification – allows the user to use make a prediction based on PLS regression to quantify a compound. The user must specify:
  - Compound Name
  - Type (as described in Table 6.1)
  - Unit (for the calculated quantity)
  - Min. Concentration: optional threshold
  - Max. Concentration: optional threshold
  - Filename: file containing the PLS regression model

- Multi Class Classifier: allows the user to classify the spectrum based on a previously built multiclassification model. The user must **Add** the model name. The field used for classification will be displayed in the table under 'Name'.

### 6.6.6 Material Tab

The **Material** tab gives the user a place to record information about the samples expected for the quantMethod. Text can be entered directly into the boxes.

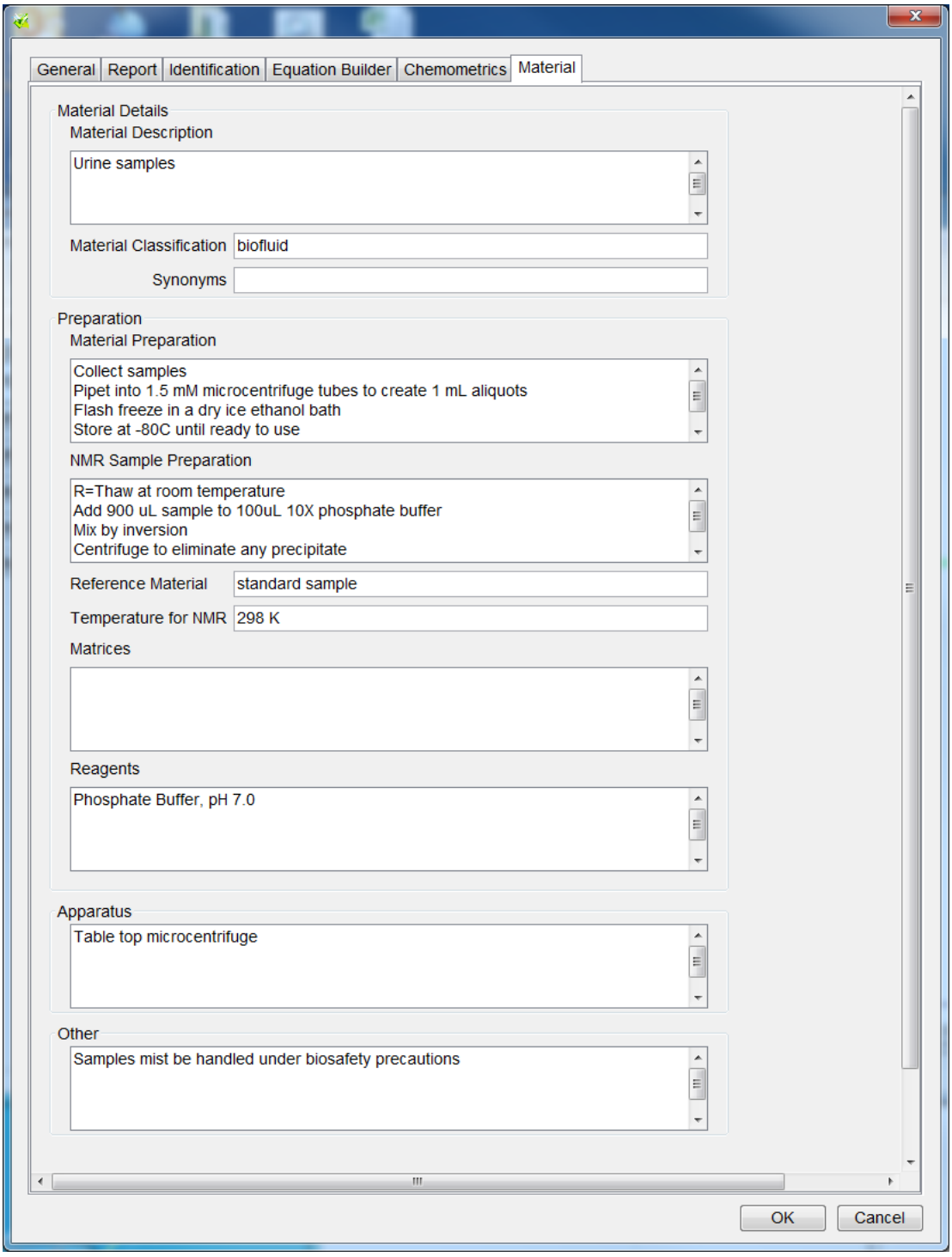



*Figure 6.27: Material tab.*

# 7 AssureNMR in Automation

The AssureNMR software is designed so that it may be used in a variety of environments with primary operators who are unfamiliar with the operation of an NMR spectrometer. For this reason, the software is designed around two different groups of users with different privileges on the system: (1) the QCuser and (2) the NMRSuperUSer and/or NMRUser. The QCuser is generally configured to have minimal access to software functions while the NMRSuperUser has broad access to NMR software functions.

TopSpin is typically set up with three levels of user privilege: the NMRSuperUser, the NMRUser, and access-restricted accounts. The first two users and their corresponding groups are set at the TopSpin installation, as described in the chapter *Installation [▷ 18]*. The QCuser for AssureNMR is an access-restricted account. Generally, we create a default account named “QCuser” on the computer, as shown in the examples in this chapter.

## 7.1 Starting TopSpin

TopSpin configuration must be done as the NMRSuperUser. TopSpin is started using the desktop icon.

In TopSpin 3.0 and above it is possible to launch directly into IconNMR without opening the TopSpin interface. This is useful for open access instruments when it is important to limit user access. To set up TopSpin so that IconNMR launches directly from the TopSpin desktop, open TopSpin as the QCuser. Under the menu flow item Manage, the item list Commands has a selection for Setup Commands Executed at TopSpin Start.

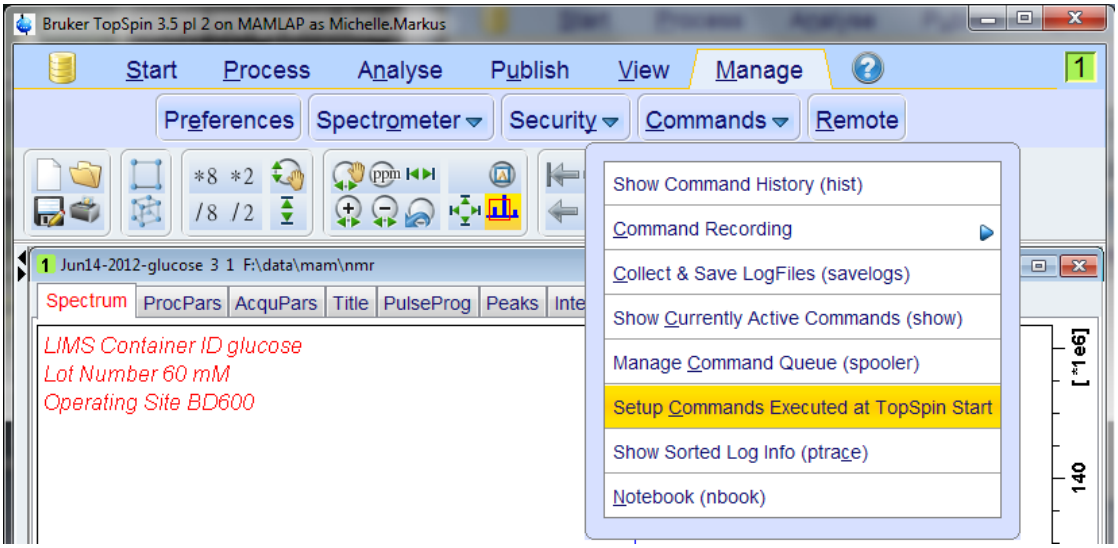



*Figure 7.1: Setting up TopSpin to automatically start the IconNMR acquisition.*

This opens a text file containing commands executed at startup. To launch into IconNMR directly, add the line “icona”. Then, close TopSpin. Now, double-clicking the TopSpin Icon causes TopSpin to open in the background with IconNMR in the foreground.

## 7.2 GxP Requirement

The Assure-System Suitability Test (AssureSST) performs operational performance validation and therefore may be used as part of GxP compliance. However, the default installation and successful run of the AssureSST is not a de facto GxP validation. It is necessary to configure AssureSST according to your sites requirements. In order for the instrument to be brought to full GxP compliance, contact Bruker or an outside party to arrange for complete instrument validation by a certified GxP engineer.

AssureNMR software is validated with only specific versions of released TopSpin software. Verify that the specific version levels of both AssureNMR and TopSpin in use have been validated together by contacting Bruker before using on an instrument for GxP applications.

## 7.3 IconNMR Configuration

This section covers the settings within IconNMR important for AssureNMR. More detailed instructions on all of the tools within IconNMR are found in the Reference Manual, which can be found in the IconNMR **Help** menu.

Access to IconNMR Configuration requires the NMRSuperUser's password. This is a security feature which prevents users from altering instrument functions. The configuration window can be accessed by typing "iconc" into the TopSpin command prompt line. The IconNMR Configuration opens, as shown below. To get to the Assure specific settings, select **AssureNMR** from the browser window on the left. Note the system suitability test settings are also available from this browser. (Select **AssureSST**. This was discussed in chapter *AssureSST Configuration [▶ 21]*.) There are two tabs under AssureNMR, **AssureNMR** and **Assay Setup**.

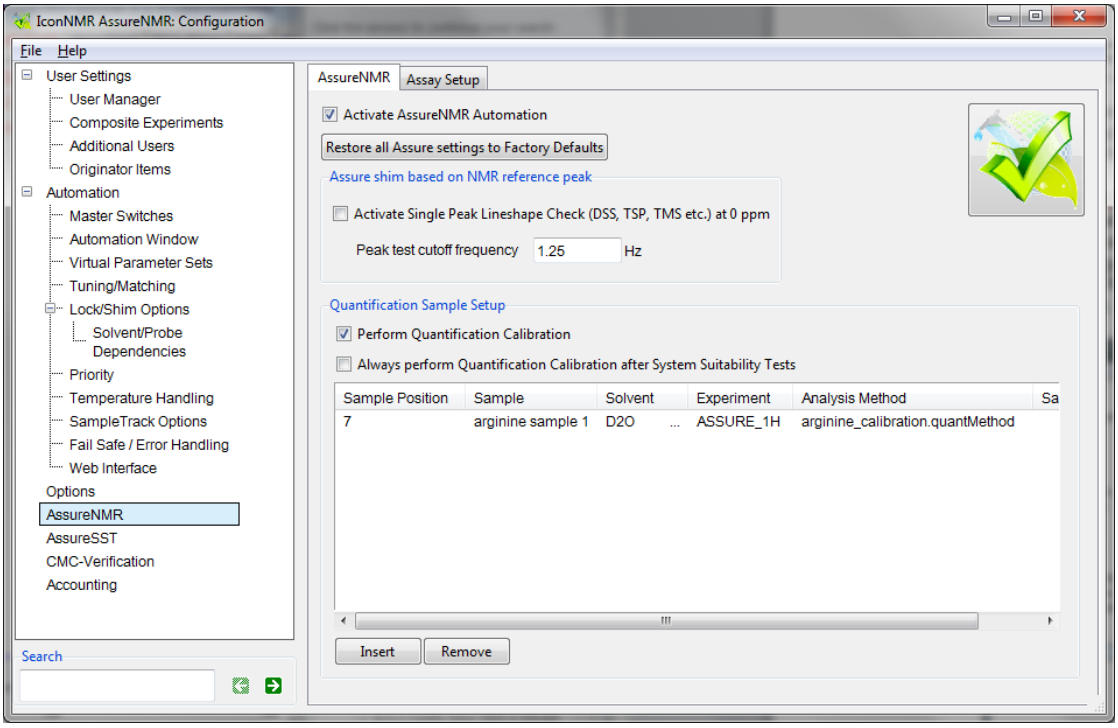



*Figure 7.2: AssureNMR selections in the IconNMR AssureNMR Configuration window.*

### 7.3.1 AssureNMR Tab

On the **AssureNMR** tab, there is a checkbox to 'Activate AssureNMR Automation'. This makes the Assure module active during IconNMR automation. 'Restore all Assure settings to Factory Defaults' resets the parameters to return the system to a defined state.

The 'Assure shim based on NMR reference peak' window is the place to activate a test for spectral quality control. When used with the proper processing AU (proc_assureshim) and a sample that has an NMR reference signal at 0 ppm, the system uses the halfwidth of the reference to determine whether the spectrum is of a high enough quality to be passed on for analysis. The threshold cutoff for half height should be at or below the value specified as the 'Peak test cutoff frequency' (in hertz). A sample with a larger half width will be re-shimmed and re-acquired. Two consecutive sample failures results in automatic queuing of the System Suitability Test.

The 'Quantification Sample Setup' window contains the information for running a standard sample that will be used as a reference for absolute quantification (concentration options mmol/l (Calibration), mol/l (Calibration), mg/l (Calibration), g/l (Calibration), and % weight of sample). A quantification standard of known concentration which can be run periodically is defined by its position in the rack. It is possible to have different quantification standards available in the rack. The spectral name is defined as well as the solvent, parameter set, and quantMethod to be used to analyze the sample. A checkbox turns on the option to run the sample after every SST.

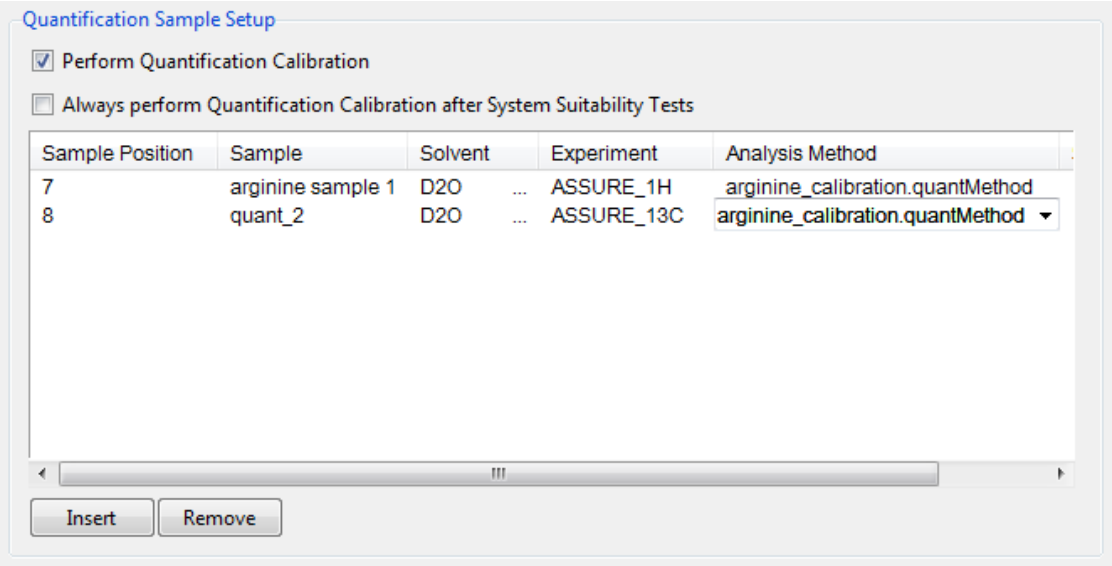



*Figure 7.3: Quantification Sample Setup section of the AssureNMR tab.*

After running the quantification standard, IconNMR will copy the reference integral file generated by AssureNMR to each subsequently acquired spectrum. IconNMR will continue to use the most recent reference integral file.

### 7.3.2 Assay Setup Tab

Each substance to be tested will be evaluated based on the parameters entered in the Assay Setup tab. Here, the user specifies the evaluation material name, the solvent, the experimental parameter sets to use for acquisition and processing, and the quantMethod to use for analysis. The 'Solvent' may be any solvent listed in the solvent table. The experiment may be any parameter set from the 'Experiment List' or 'Composite Experiment' listing for the current user in IconNMR. The 'Analysis Method' may be chosen from any quantMethod that has been released. (See chapter *General Tab [▷ 91]*.)

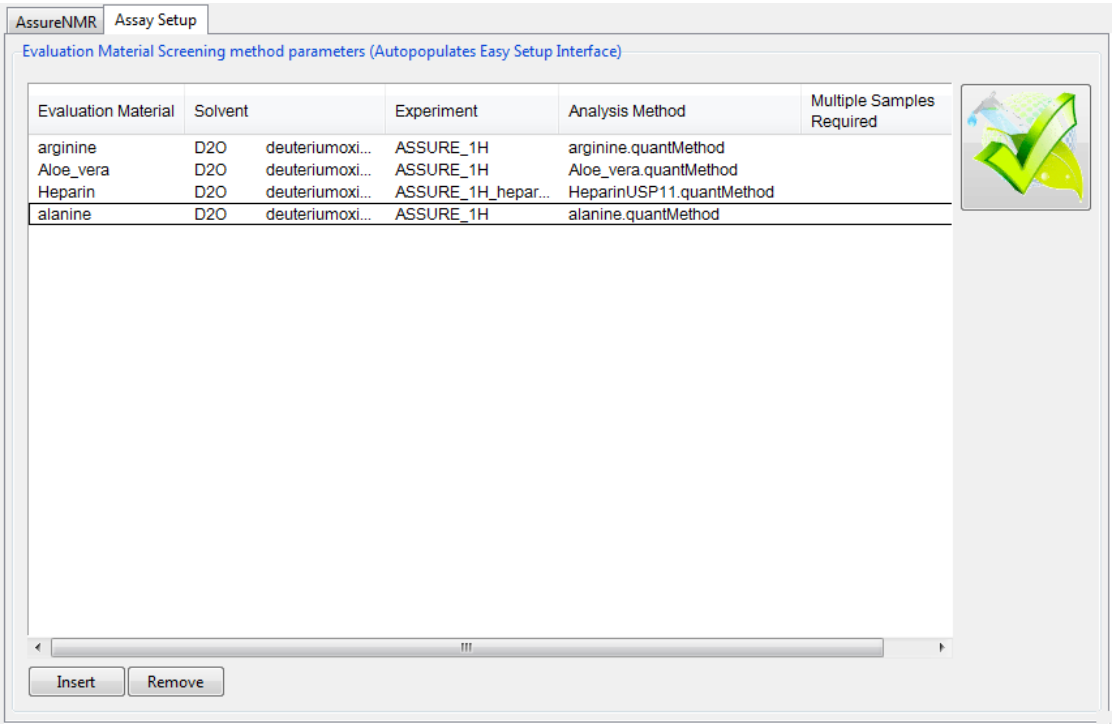



*Figure 7.4: Assay Setup tab for AssureNMR in IconNMR Configuration.*

Select **Insert** to add a new raw material.

- Evaluation Material – arginine: Type in a name for the raw material in the first column. This is the name that will appear in the IconNMR easy setup dialog.
- Solvent – D2O: Left click in the cell for a Solvent and select a solvent from the pull down list. The choices are automatically populated from the list of lock solvents from the local configuration.
- Experiment – Assure_1H: Left click in the cell for the Experiment and select an experiment from the pull down list. This list populates from the local configuration.
- Analysis Method – arginine.quantMethod: Left click in the cell for the Analysis Method and select an Analysis Method from the list. This list populates from the release folder of the AssureNMR software.
- Multiple Samples Required – checking this box allows the user to run multiple samples together, for example, to find the average quantification over multiple samplings. When Multiple Samples Required is activated, IconNMR prompts for the number of samples using the easy setup interface:

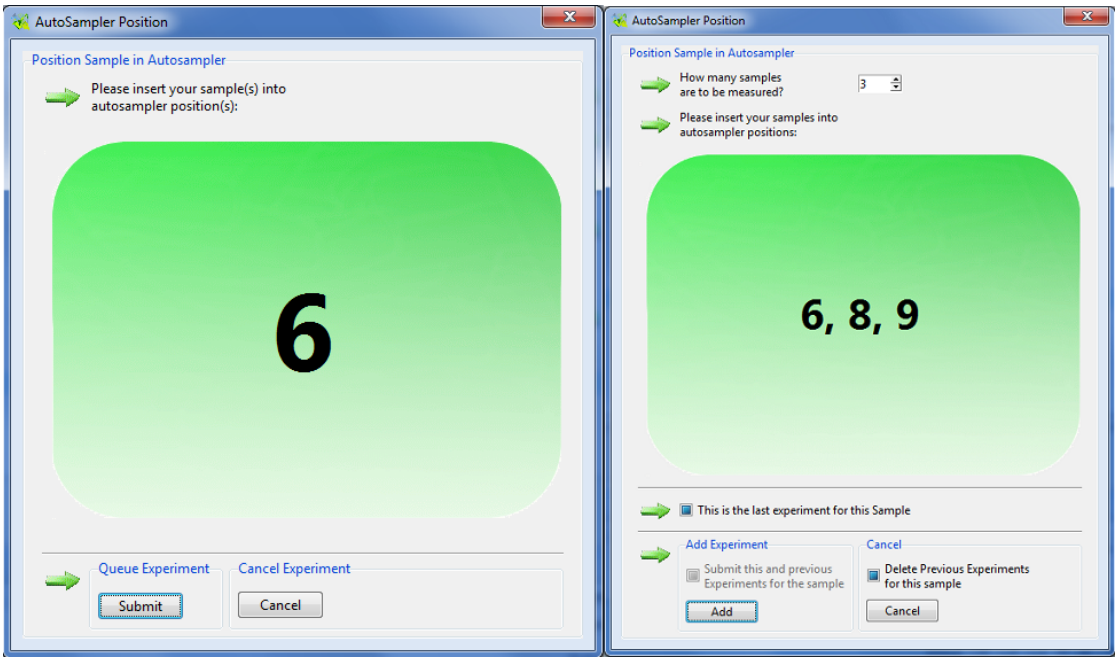



*Figure 7.5: AutoSampler Position window for single samples (left) or multiple samples (right), prompting the user where to place samples in the sample changer. Note this is the appearance of the window with 'Allow multiple experiment selection per sample' active in the Automation/Automation Window of IconNMR Configuration. The window is somewhat simplified when this option is not active.*

### 7.3.3 Additional IconNMR Configuration Settings That Affect AssureNMR

In the IconNMR Configuration window, selecting the **User Manager** from the browser on the left, brings up options where the supervisor can limit many of the actions of the user.

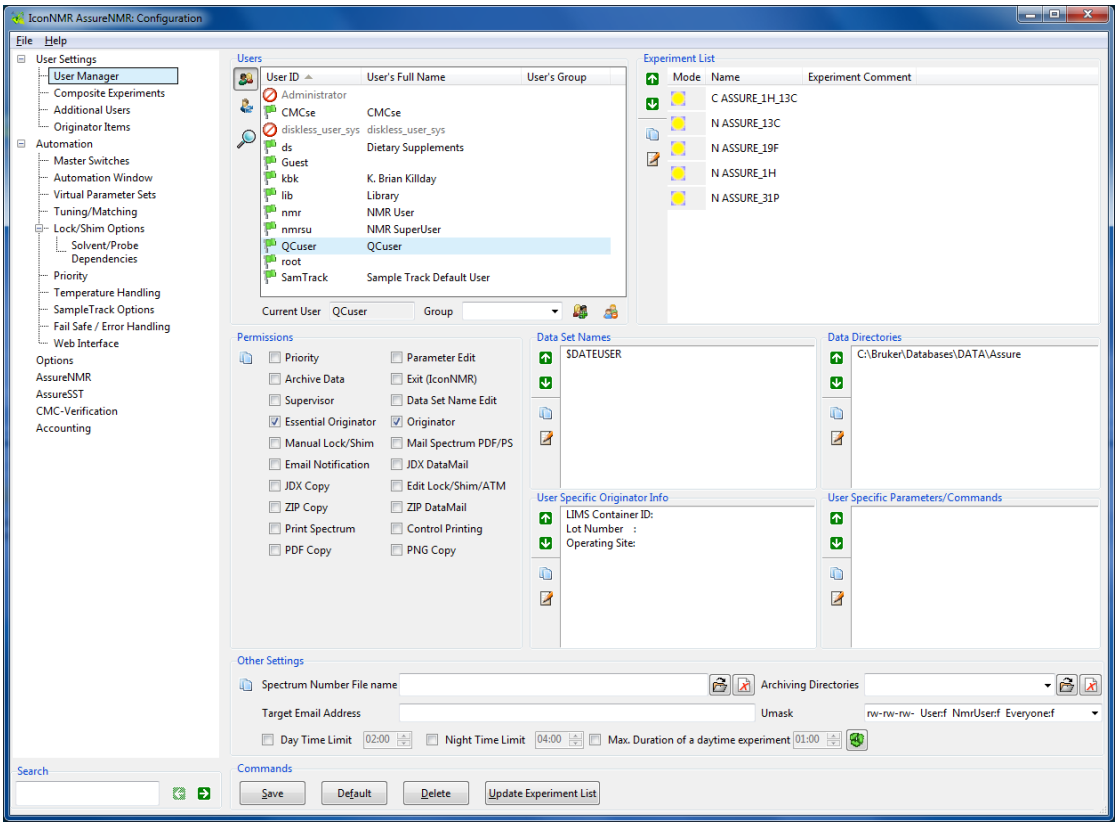



*Figure 7.6: IconNMR screen showing the User Manager interface and current settings for the access-limited user, QCuser.*

### Experiment List

Robust and reliable parameter sets are required for any experiments entailing a high degree of automation, reproducibility, and precision. The parameter sets supplied for the AssureNMR software were designed and tested for this purpose and the use of these parameter sets is highly recommended.

Access to the parameter sets is granted through the **User Manager**, the Experiment List window, including any composite experiments that may be available. Parameter sets that are used for the screening mode are defined in the **AssureNMR** section of the IconNMR configuration.

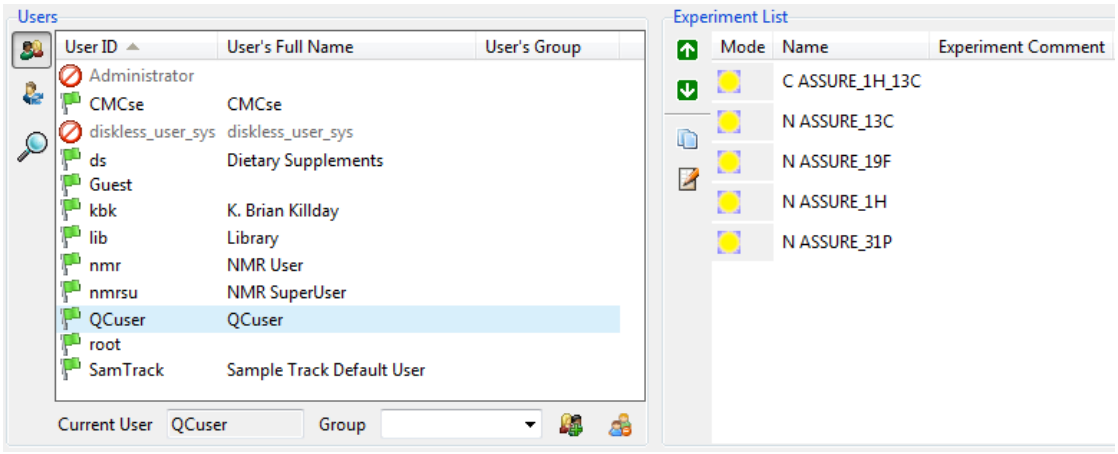



*Figure 7.7: Top of the IconNMR Assure Configuration window, looking at the User Manager options.*

**User Permissions**

QCuser (or any user of AssureNMR) must have permission for:

- ‘Essential Originator’
- ‘Originator’

Restrictions on QCuser are done by not allowing the following permissions:

- Supervisor
- Exit (IconNMR)
- Parameter Edit
- Manual Lock/Shim
- Edit Lock/Shim/ATM

All other possibilities are defined by the NMRSuperUser preferences.

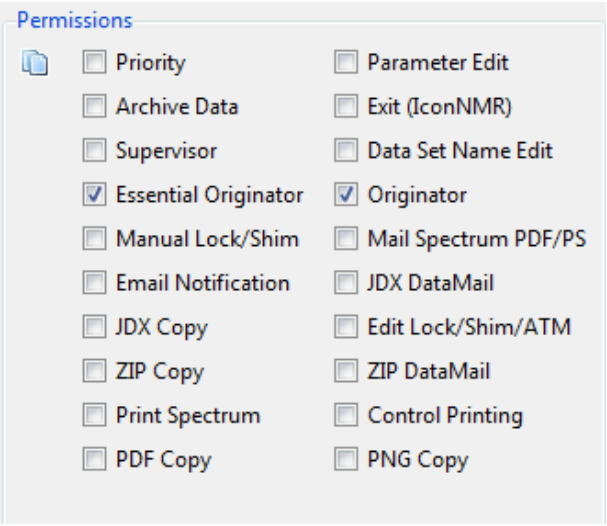



*Figure 7.8: Permissions in the User Manager section of the IconNMR Assure Configuration window.*

### Data Options and Archiving

QCuser should not have access to edit submission parameters of samples under the User Specific Parameters/Commands window (below), so this window is normally empty for such users. If archiving of data is desired, check the box 'Archive Data' under **Permissions** in the **User Manager** and designate an 'Archiving Directory'.

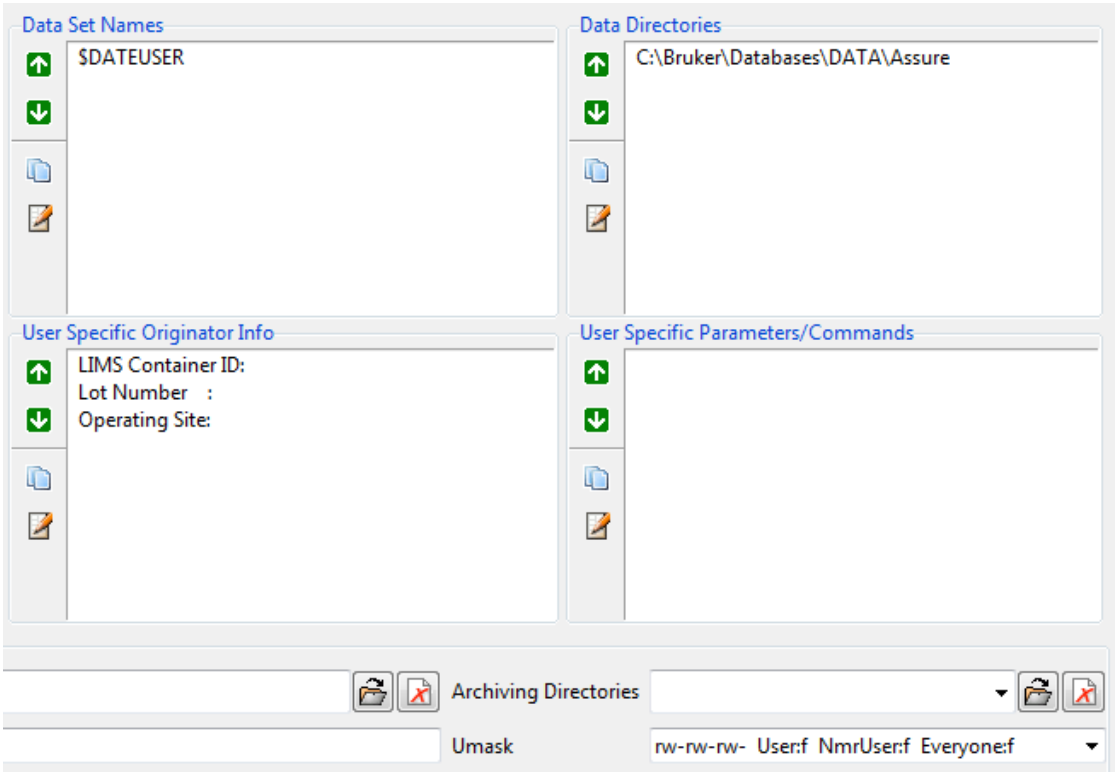



*Figure 7.9: User specific options which will be displayed in pulldown menus in IconNMR acquisition; found in the IconNMR Configuration window under User Manager.*

### IconNMR Originator Items

IconNMR originator items are used in the AssureNMR software for users to input tracking information regarding the samples. These items are included with the spectrum and final report.

Originator items, including 'LIMS Container ID', 'Lot Number' and 'Operating Site', may be customized to reflect user preferences. The system administrator may add 'Standard Originator Values' to any of these 'Originator Items' by selecting the originator item and loading a list of selectable values or by using the 'Add new' button.

- Enter the **Originator Items** window from the **IconNMR Configuration** window
- Select the desired originator item in the window 'Set Standard Originator Values for Originator Item' from the pulldown menu.
- A list of values may be loaded by left clicking on the 'Load list' button and selecting the desired text file.
- Alternatively, type an entry into the window at the very bottom of the screen and click the 'Add new' button to add the new entry.

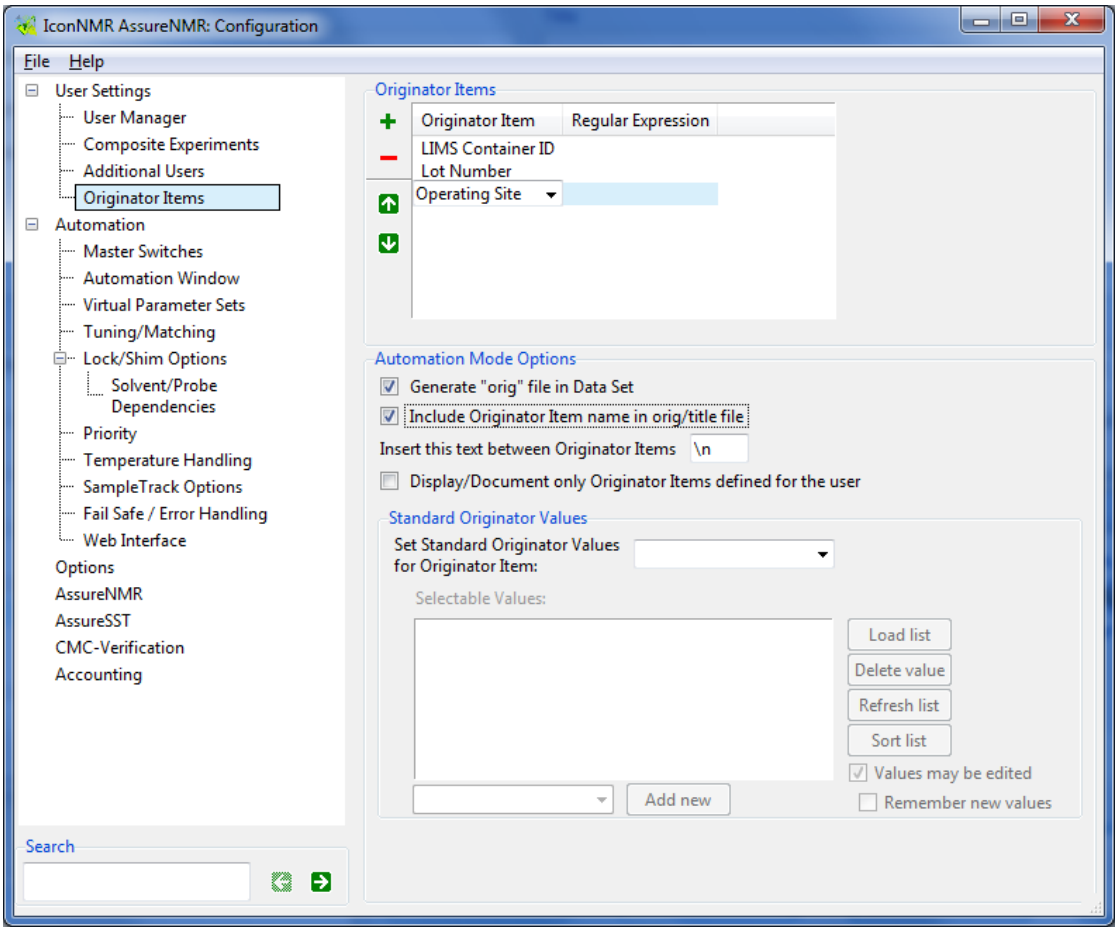



*Figure 7.10: Originator Items in IconNMR Configuration.*

### Configuring the Master Switches

Additional configurations used in the AssureNMR package are found under the **Automation** heading in the IconNMR Configuration browser).

- Enter the IconNMR Configuration window
- Select **Master Switches** from the browser window.
- Turn on ‘Eject last sample in queue’.
- Turn off ‘Never Rotate the Sample’.
- Turn on ‘Start run at user login’.

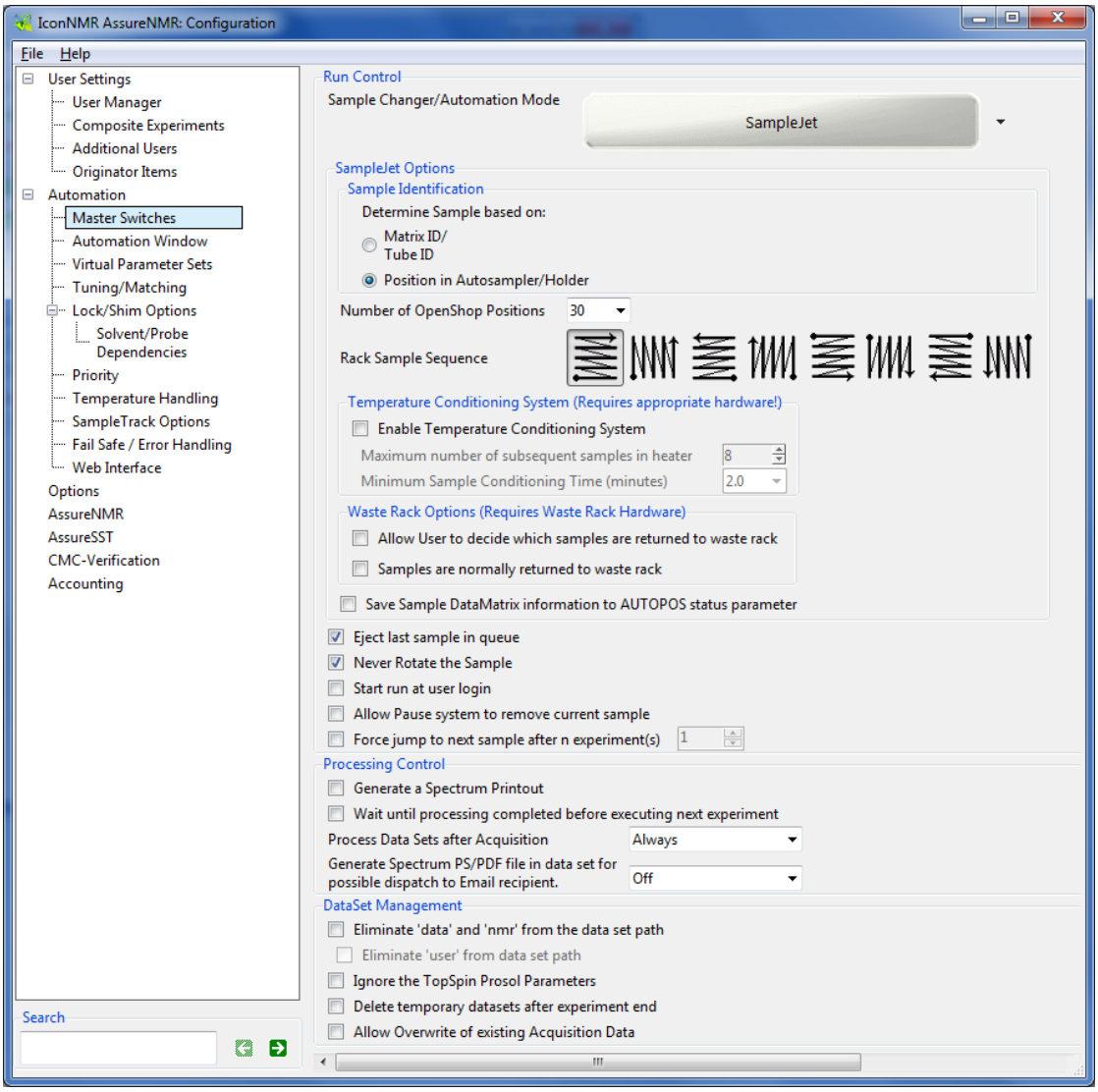



*Figure 7.11: Master Switches in IconNMR Configuration.*

### Shimming Options

Shimming routines can be specified the **Lock/Shim Options** window of IconNMR Configuration. The recommended shimming routine, 'TOPSHIM tuneb', first adjusts for possible solvent changes (tuneb) then carries out a 1D TopShim. Other shimming options such as tunebxyz or convcomp (especially for cryoprobes) may be useful. For more details on shimming options, see the TopShim guide which is available from the TopSpin Help menu.

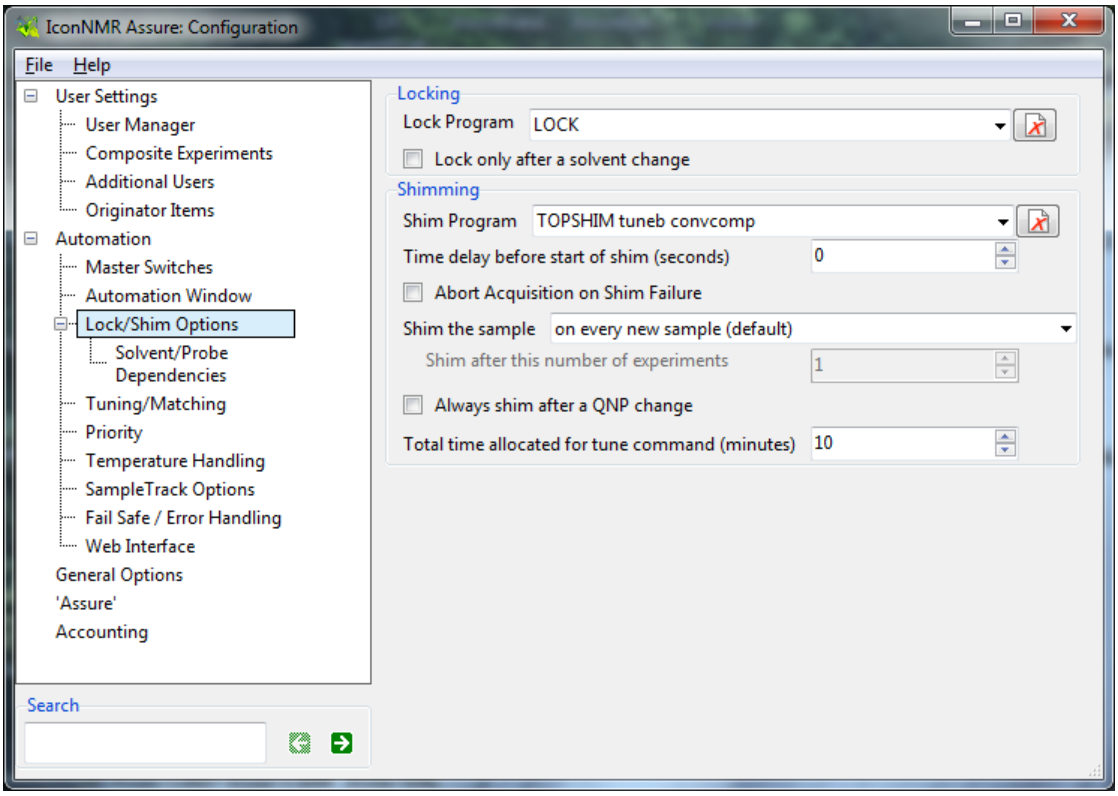



*Figure 7.12: Lock/Shim Options in IconNMR Configuration.*

### Temperature Handling

Optimal results are obtained only when temperature handling is engaged.

- Enter the IconNMR Configuration window.
- Choose **Temperature Handling** under Automation in the IconNMR Configuration browser.
- Under ‘Temperature Handling’:
  - Select ‘Temperature Handling (On/Off), Valid only for BACS, SampleCase, SamplePro, SampleXpress, Manual Mode, LC, MAS, and SampleJet (Post Insertion available on SixPack and NMR Case)’.
  - Select ‘Abort Acquisition on temperature control failure’.
- Under POST INSERTION Set/Check:
  - Select ‘Set & Check Temperature after Sample Insertion’.
  - In the ‘Temperature Setting after Sample Insertion’ box, choose ‘according to first experiment’s TE parameter.
  - In the ‘Post-Insertion Temperature Set/Check Routine’ box, choose ‘TESET;TEREADY 180 0.1’. This sets the temperature, then waits 180 seconds after the temperature unit reports the temperature is within 0.1 degrees of the set temperature to begin acquisition. The best values for the temperature tolerance and equilibration time are probe and site specific and must be determined individually.

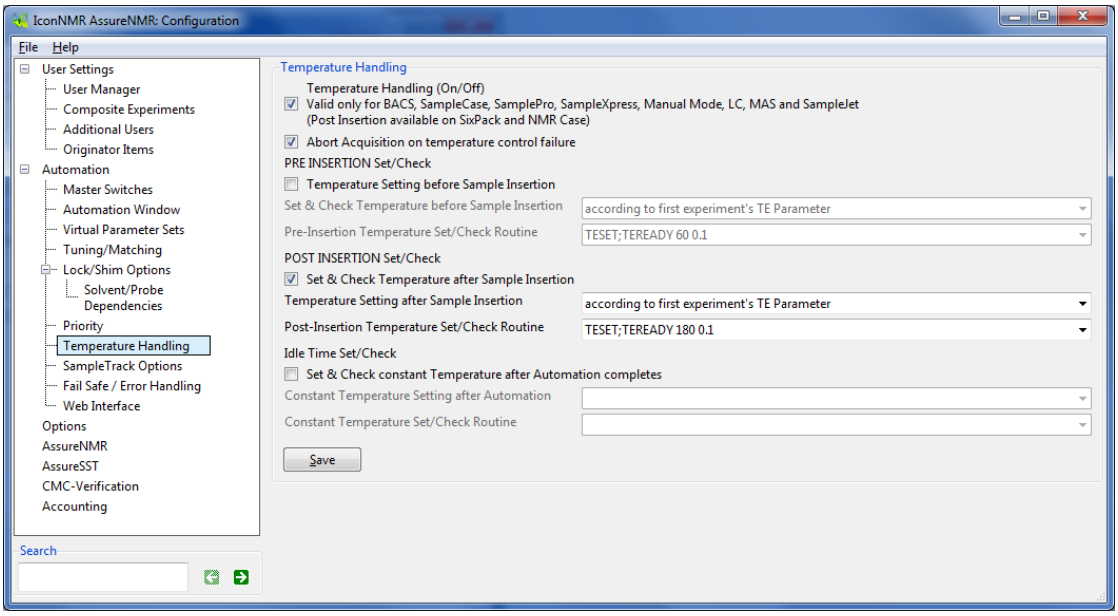



*Figure 7.13: Temperature Handling in IconNMR Configuration.*

### Fail Safe/Error Handling

It is possible to have the system continue to run even if the Assure-SST produces a failure. Deactivating ‘Stop the run when ‘Assure’ System Suitability Test reports specification failure’ will allow the system to continue running even after an SST failure, with an e-mail alert sent to the defined e-mail address entered in the **Error Handling** window.

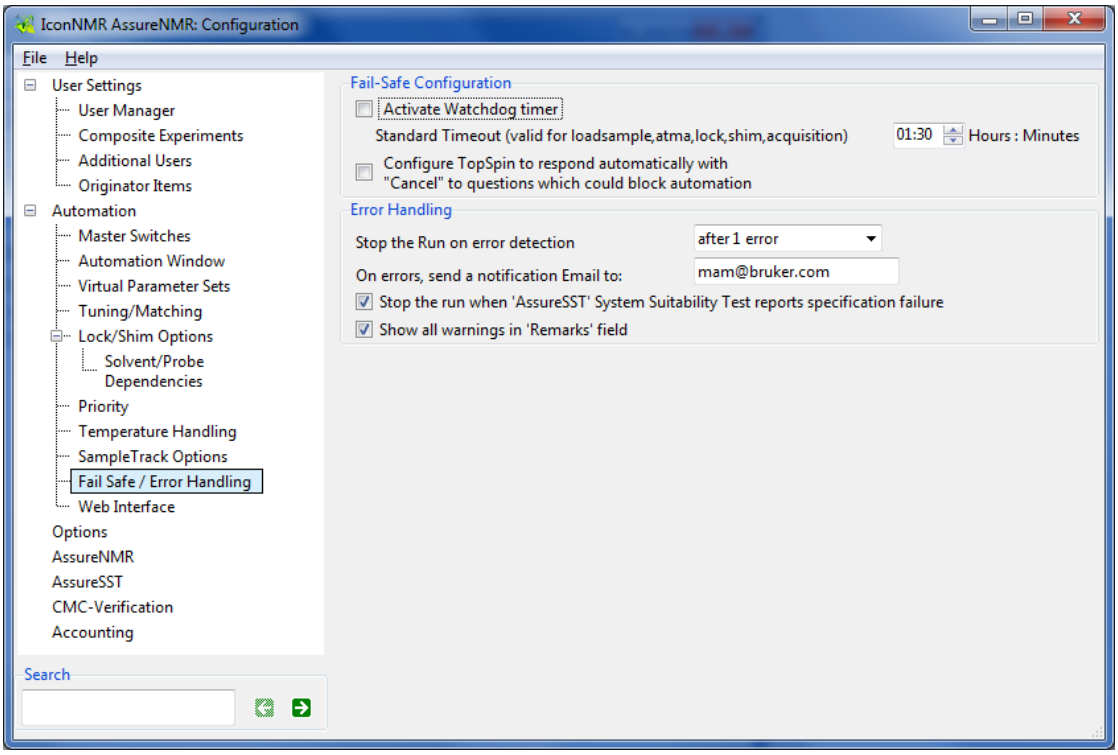



*Figure 7.14: Fail Safe / Error Handling in IconNMR Configuration.*

### 7.3.4 Saving IconNMR Configuration Settings

To save the IconNMR Configuration parameters, from the IconNMR Assure Configuration window, select **File** then **Save** or **Save as**. This will create an Icon Configuration File (extension .icf). This file contains most of the configuration parameters including the Assure settings, such as the specified Evaluation Materials. Note that user settings, composite experiments, and originator items are not included at this time. To recall the parameters, select **Load** from the **File** pulldown. The .icf file can be a convenient way to restore the list of Evaluation Materials after a software upgrade or to transfer the list to another system.

Note, the user may need to reset the SST Log Directory, especially when changing computers, to save the log file correctly.

## 7.4 Running IconNMR: Access-Limited User

**Screening Samples using the AssureNMR Software**

When the QCuser starts TopSpin (while logged onto the computer as QCuser), the following user interface will appear. The System Suitability Test (SST) automatically populates the Experiment Queue and the experiments are listed according to holder number. If the SST has not been run at the time chosen in the IconNMR Configuration window then acquisition will begin immediately on the SST. The QCuser may enter new samples for testing without waiting for the SST to finish.

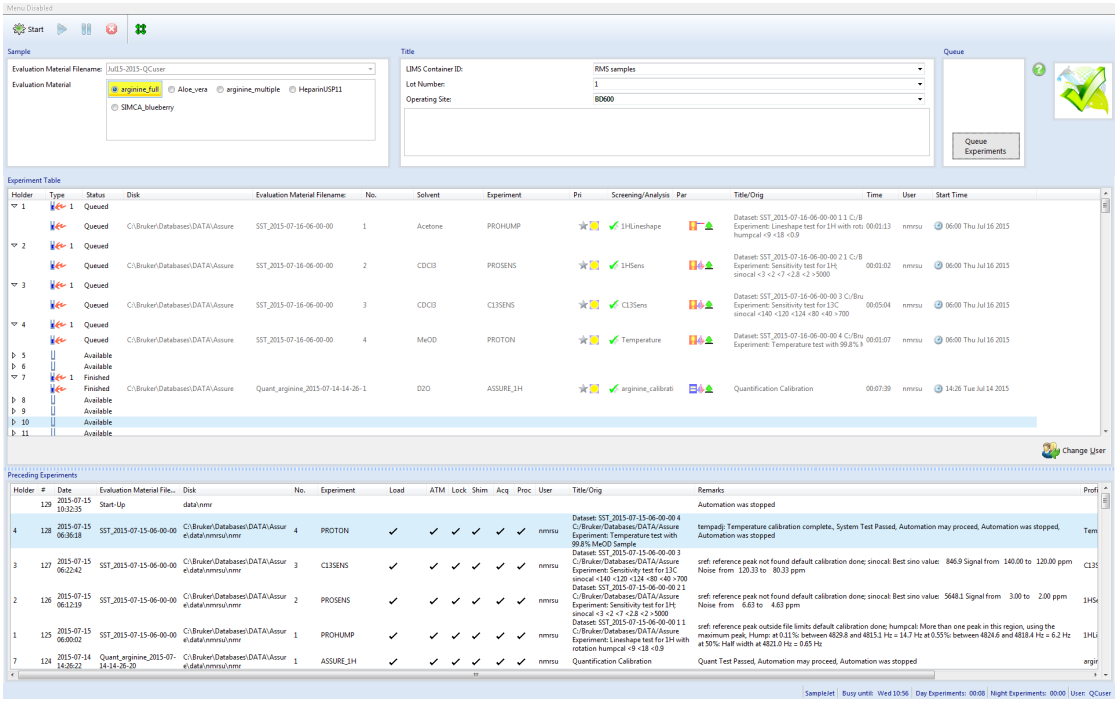



*Figure 7.15: QCuser window interface.*

Notice that several of the traditional options during IconNMR acquisition are not accessible to the QCuser, such as stopping the system, canceling, or deleting experiments.

The purpose of the QCuser is for submitting samples only. This is by design to keep the user from changing conditions important to the automation run following standard operationg procedures (SOPs), as for example, in a GLP environment. Similarly, the QCuser has limited access to the functions in the TopSpin interface.

The only means to stop the automation is to use the **Change User** button l the lower right hand corner of the Experiment Table window. This will allow the NMRSuperUser to logon and change the run as needed.

### Submitting a Sample

To submit samples, the QCuser only needs to complete the flow at the top of the AssureNMR Automation window. IconNMR prompts the QCuser with next available sample position at the end.

To submit samples to queue:

In the ‘Sample’ window:

- Select an ‘Evaluation Material Filename’ from the pulldown menu. These are the Data Set Names selected in the **User Manager** window within IconNMR Configuration.
- Select the ‘Evaluation Material’ from the list by clicking its radio button. These are the Evaluation Material names set up in the **Assay Setup** tab in the AssureNMR window within IconNMR Configuration.

In the ‘Title’ window:

- Enter a ‘LIMS Container ID’ number.
- Enter a ‘Lot Number’.
- Select an ‘Operating Site’ from the pull down menu.

In the ‘Queue window’, click on the **Queue Experiments** button.

Place the sample in the holder as instructed by the software.

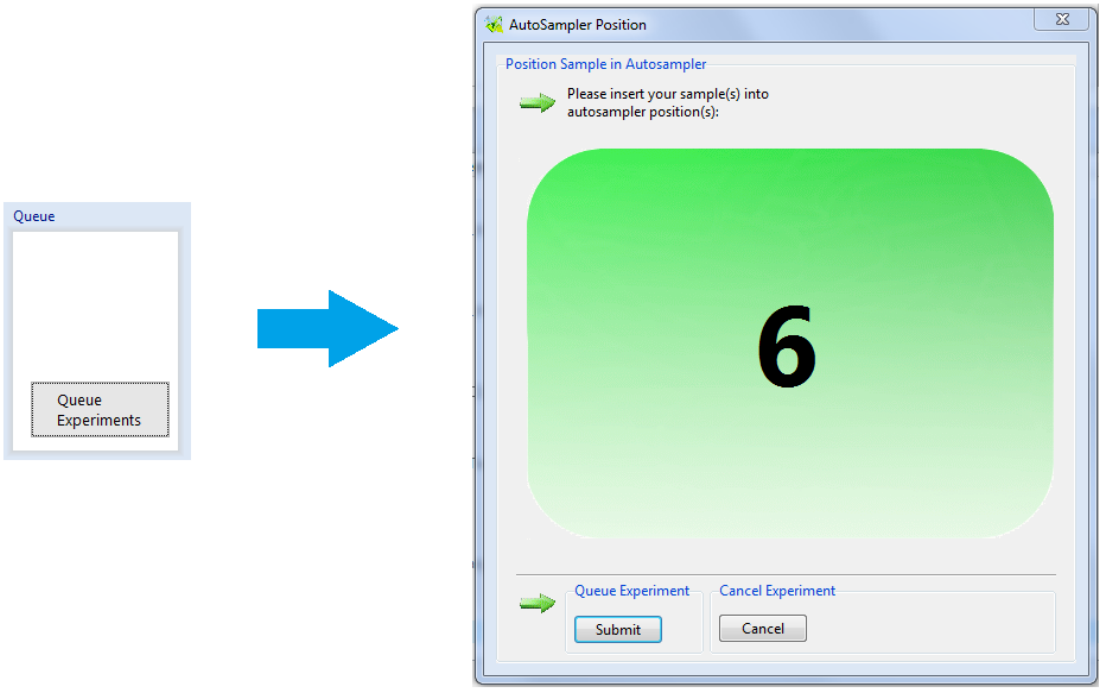



*Figure 7.16: Routine sample submission for the Qcuser.*

Data is acquired and processed automatically for each sample in the queue. The data is analyzed in the background using AssureNMR and once the analysis is complete two reports are generated including a (1) QC report and an (2) expert report. Examples of each of these reports are in Chapter *Reports [▷ 150]*. These reports indicate a pass or fail for the sample screened; other options are available when setting up the quantMethod (Chapter *Report Tab [▷ 100]*).

These reports are stored in the data directory for each sample. For example, using the traditional Bruker data tree structure, the reports are found in the following directory:

*C:\Bruker\TopSpin\data\[user]\nmr\[dataset name]\[expno]*

Access to these documents is also available by right clicking on the spectrum history in the IconNMR acquisiton window's Preceding Experiments window as shown in the following figure.

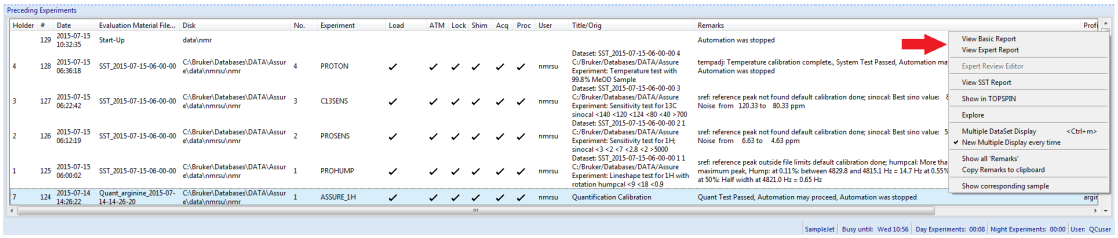



*Figure 7.17: Access to reports from IconNMR Assure Acquisition window for the QCuser.*

## 7.5 Running IconNMR: Supervisor

The administrator (or any user given 'supervisor' permission in IconNMR Configuration/User Manager window) has access to all of the tools available to the limited access user in IconNMR plus three additional features. The supervisor can:

- **Delete entries in** '**Experiment Queue**' – removes all submitted samples.
- **Delete entries in** '**Preceding Experiments**' – clears the history in the Preceding Experiments window.
- **Run System Suitability Test Now** – sets the Suitability Test to start immediately (or after the current experiment finishes).
- **Run Quantification Calibration Now** – sets the calibration standard to start immediately (or after the current experiment finishes).

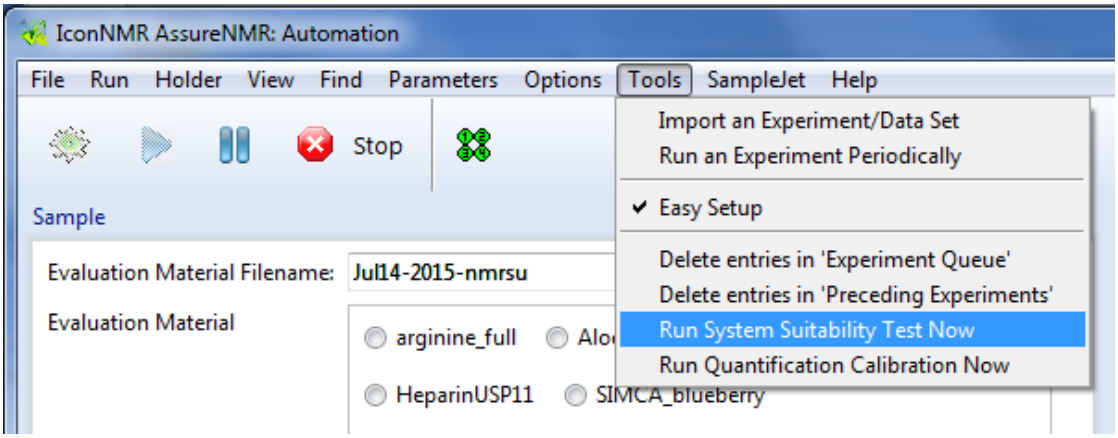



*Figure 7.18: Tools available to users with supervisor privileges.*

## 7.6 Batch Submission

A list of samples can be submitted from the File dropdown menu, using a spreadsheet in the application independent, comma separated files (csv) format. Chapter *csv File for Batch Submission [▷ 158]* contains an example csv file for submission. The format for filling the dialog boxes is shown below:

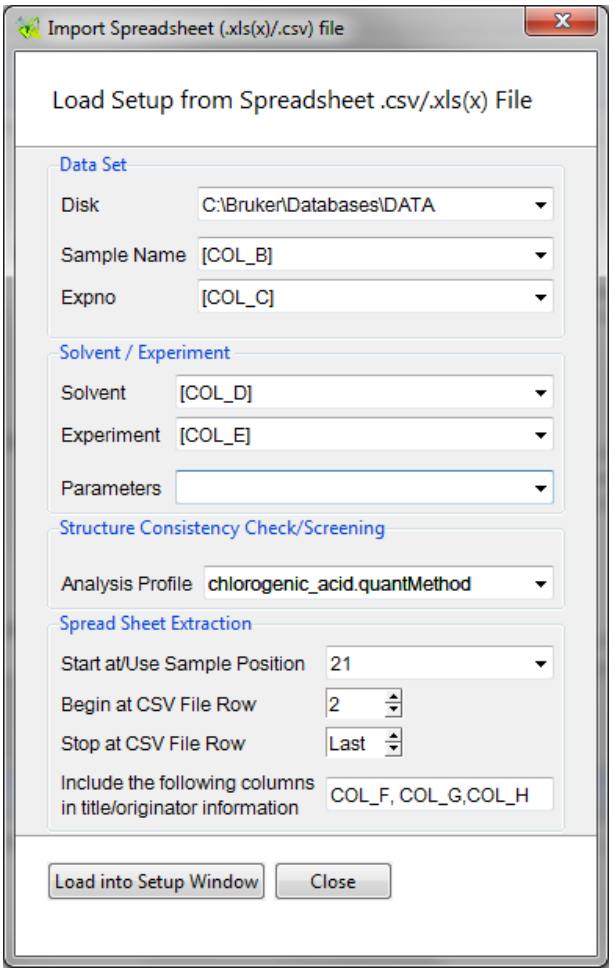



*Figure 7.19: Loading setup from csv file.*

## 7.7 Using Barcodes

AssureNMR can take advantage of features in IconNMR to handle samples with barcodes. The user must have a sample changer with a barcode reader. The following settings must be adjusted in the IconNMR Configuration window, under the Master Switches section:

- Under Master Switches, select the correct sample changer under ‘Sample Changer/ Automation Mode’. In this example, it is set to the SampleJet.
- Under SampleJet Options/Sample Identification, select ‘Determine Sample based on MatrixID/ Tube ID to use barcodes’. The exact wording will be slightly different for different sample changers.
- For the SampleJet, select the appropriate ‘Scan Search Priority’ based on where you are loading the samples.

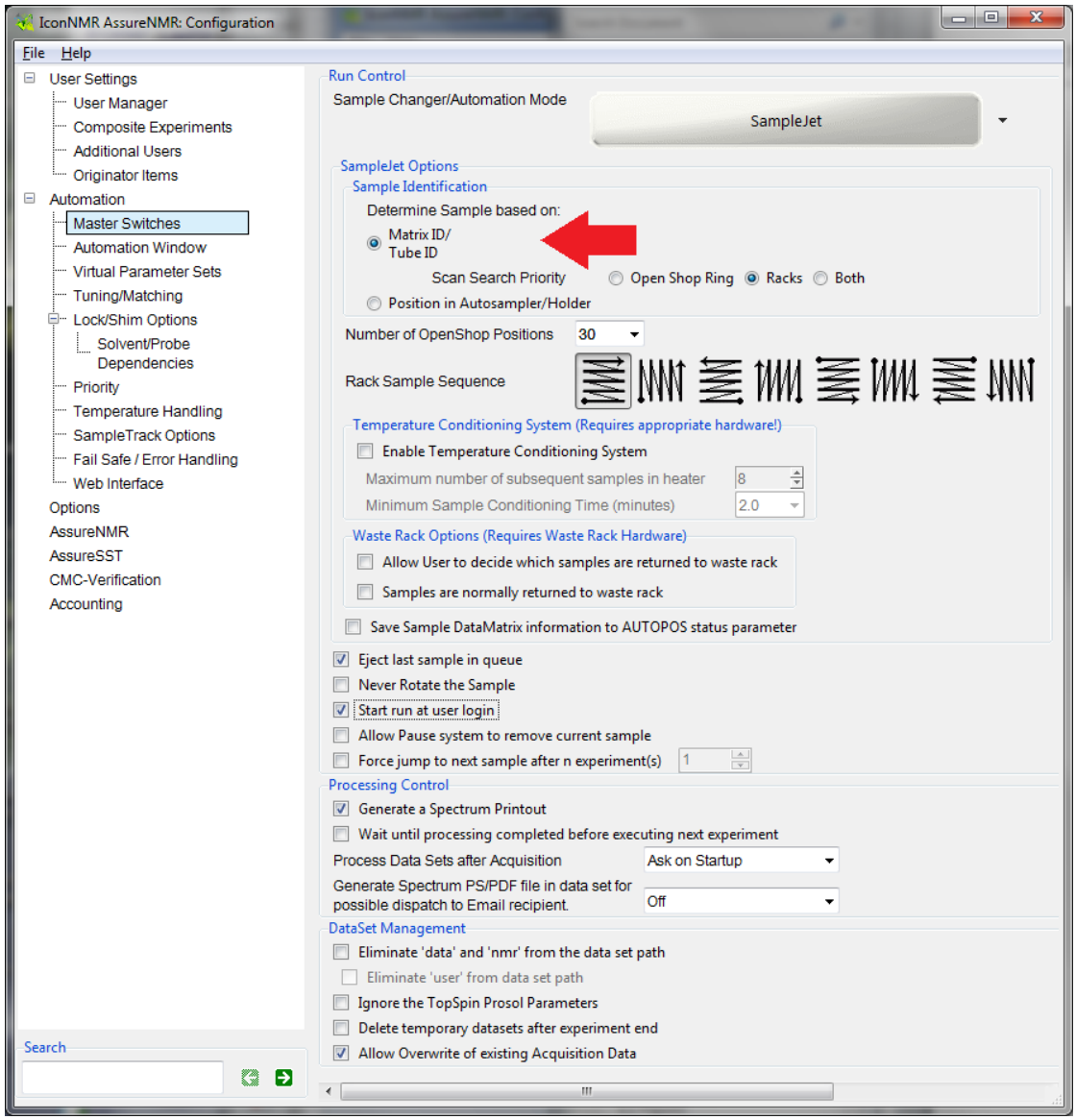



*Figure 7.20: One of the key windows in IconNMNR Assure Configuration for setting up barcodes.*

After adjusting the settings, **Save** from the **File** menu of the main IconNMR Configuration window and exit all instances of IconNMR so that the new settings are available when IconNMR is started next time.

Barcodes cannot be used to find SST samples. The SST samples must be placed in the holder positions specified in the AssureSST window of the IconNMR AssureNMR configuration.

In this configuration, samples with barcodes can be loaded into the sample changer without regard to the specific holder position. The sample is then queued using the easy setup interface. The barcode number must be entered. During acquisition, the sample changer will check the barcodes on the loaded samples until the correct one is found for acquisition.

## 7.8 Viewing AssureNMR Progress During Acquisition

Upon successful completion of a screening test, IconNMR displays the results of each step of the acquisition in the Preceding Experiments window. Any error results in an X and the line for that experiment becomes red. A failure is triggered by instrument errors, processing errors, or report generation errors. For samples which also fail the threshold limits as defined in the quantMethod, a failure error will also be generated.

Full details of the failure can be found in the remarks field in the Preceding Experiments window or displayed in a new window by clicking on the experiment in this window and selecting **Show all** '**Remarks**'.

# 8 Chemometric Modeling in AssureNMR

For complex mixtures, it may not be practical or desirable to analyze against spectra of pure components from an SBASE. For these situations, tools to generate chemometric models are available in AssureNMR. From the method development window, the user can build quantile plots or SIMCA models to distinguish outliers, that is, samples that do not fall within the model to a specified confidence level. A spectrum can be classified by comparing against a set of models using multiclassification. PLS regression can be used to predict the quantity of a specific material that may itself be a pure material or a complex mixture. These features are available through the **Chemometrics** pulldown menu:

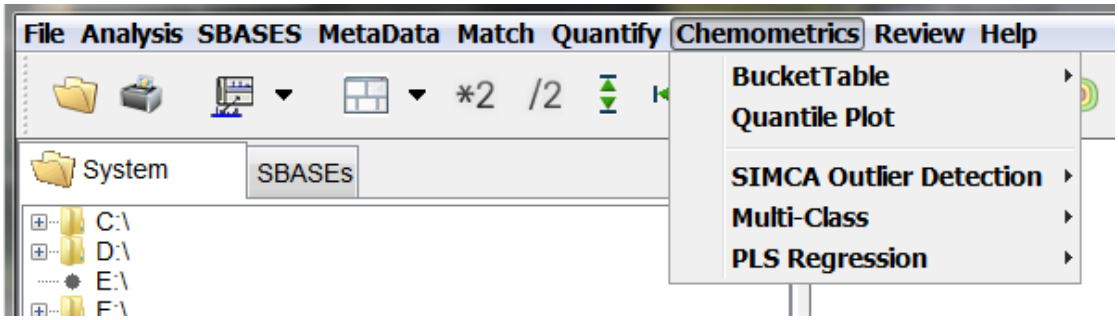



*Figure 8.1: Chemometrics pulldown menu.*

The bucket table provides the necessary information about the spectra for chemometric analysis. It specifies which spectra should be included and which regions of the spectra are important. Before creating a bucket table, it is recommended that users check each NMR spectrum used for chemometrics (using AssureNMR or TopSpin) to ensure all NMR spectra are properly phased and referenced before continuing. This will greatly improve the results from the bucket table and decrease potential outliers due to processing.

## 8.1 Bucket Tables

The first step in any chemometric analysis is to set up the bucket table, which specifies which spectra to use for the model and how to partition them for analysis. The **BucketTable** menu contains the necessary tools:

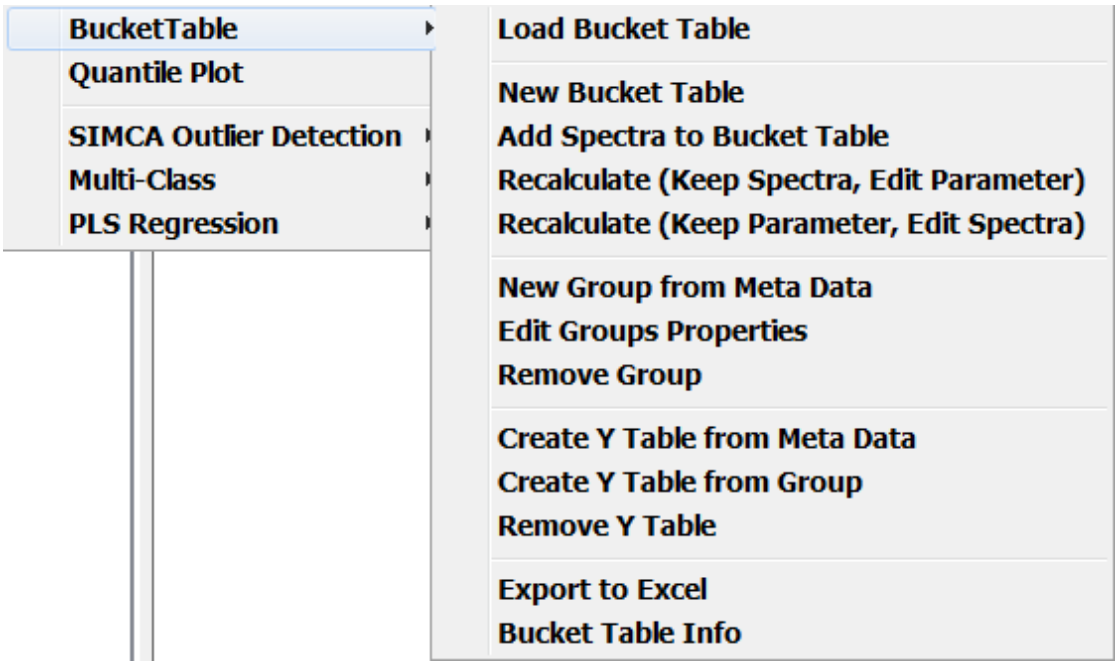



*Figure 8.2: Bucket Table pulldown menu.*

### 8.1.1 Creating and Editing Bucket Tables

**Load Bucket Table** allows the user to bring in a previously established bucket table. **New Bucket Table** takes the user through a series of windows, prompting for the information to set up the table. First select the spectra:

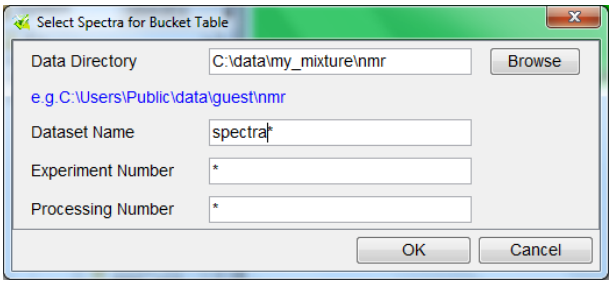



*Figure 8.3: Select Spectra for Bucket Table window.*

The window prompts for the ‘Data Directory’, the path to the data from TopSpin. Then the user can specify ‘Dataset Name’, ‘Experiment Number’, and ‘Processing Number’. An asterisk (*) can be used as a wildcard. After choosing the spectra, the Bucketing window prompts for information about the bucketing:

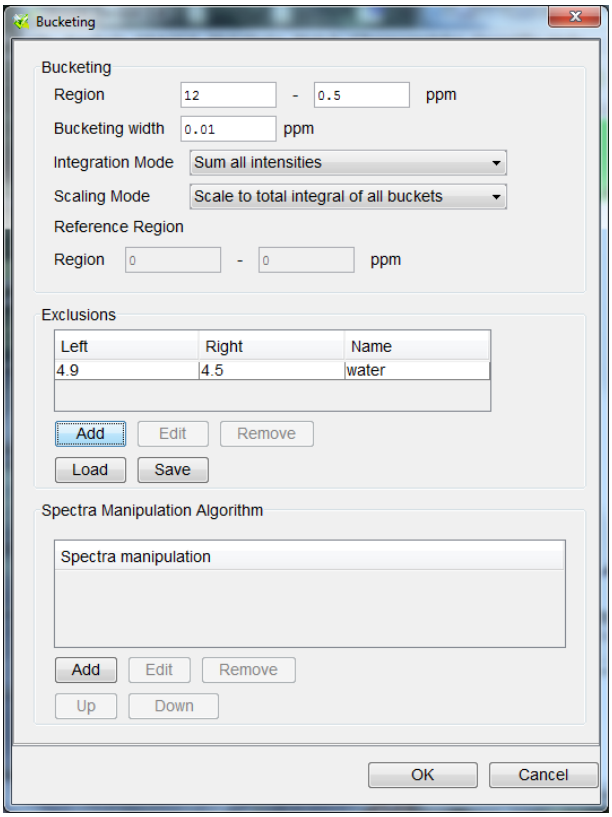



*Figure 8.4: Bucketing window, specifying parameters for the bucket table.*

'Region' refers to the chemical shift range in the 1D spectrum to use. The 'Bucketing width' is the size of the partitions (buckets, in ppm) within this chemical shift range. Bucketing widths for biological samples are commonly in the range of 0.01-0.04 ppm. It is recommended not to select a bucketing width of less than 0.005 as small shifts in the NMR spectra can cause errors in the data analysis. The 'Integration Mode'allows the user to select how the bucket intensity is analyzed. Various options are available. By default, 'Sum all intensities' is chosen. Options are available to analyze only the absolute, positive or negative intensities. The 'Scaling Mode'allows the user to transform their bucket intensities to a particular scaling factor to compare the NMR spectra uniformly. The default option is 'Scale to total integral of all buckets' which takes individual bucket intensities and divides them by the total spectral intensity. Other options are: No scaling, Scale to reference region, Scale to biggest bucket, and Scale to biggest bucket (absolute value). The 'No scaling' option is recommended if the user knows that the sample concentration and experimental parameters are identical in all NMR spectra. 'Scale to reference region' works well when there is a reference signal. The region can be defined in the option below from left to right (in ppm).

The Exclusions section allows the user to specify regions of the spectrum to exclude from the analysis, as for example solvent peaks or chemical shift reference signals. The **Add** button brings up the following window:

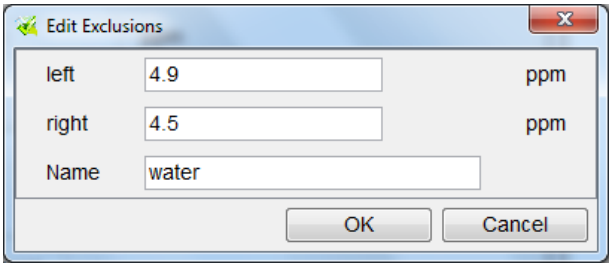



*Figure 8.5: Edit Exclusions window.*

The ‘Name’ appears in the bucket table report, thus helping the user keep track of the exclusions. Exclusions can be highlighted in the Exclusion section of the Bucketing window and then edited (**Edit** button) or removed (**Remove** button). If an exclusion file was created previously, it can be loaded into a bucket table (**Load** button). After the exclusion areas have been created or modified, the new exclusion file can saved for future use (**Save** button).

Spectra manipulation algorithms are special post-processing techniques for complex NMR spectra that might contain noisy, broad underground signals or asymmetric lineshapes. This can cause inaccuracies in the signal integration and can influence the statistical analysis. The various filtering techniques allow modifications of the NMR spectra to reduce the broadness and correct the asymmetry for better bucket table calculations. This section gives a brief introduction to the application of these techniques. Users should consult a reference such as *Data Preprocessing For Chemometric and Metabonomic Analysis* by David E. Axelson for more information. Five options are available in AssureNMR for spectra manipulation:

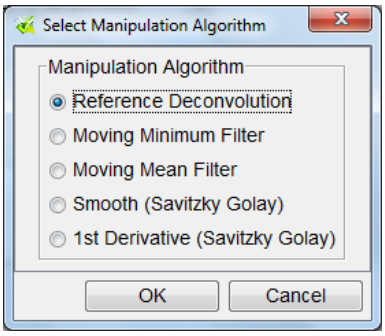



*Figure 8.6: Select Manipulation Algorithm window.*

- Reference deconvolution aims to restore any symmetry issues with the spectral peaks (for example, due to shimming problems). A singlet at 0 ppm is required, e.g. TSP. Two parameters are required:
  - Regularization exponent: sets the amount of peak smoothing to apply. The higher the exponent, the larger the effect on the signal intensity. A good starting value is 0.
  - Line shape factor: used to modify the line width. Any factor < 1 will introduce additional noise. A good starting value is 1.
- The moving minimum filter subtracts the minimum value in the search region (+/- filter width) from the current value. This tends to remove broad “underground” signals. To reconstruct the base line, a signal free region is required (noise region). Required parameters:
  - Filter width: defines the size of the window to use for calculating the minimum or mean intensity respectively in that region. This will then replace the old value with the new calculated value.
  - Left border of noise region
  - Right border of noise region
- Smoothing of spectra can be done using either the moving mean or the Savitzky Golay filter. Both filters are applied to noisy spectra to reduce random noise in the instrument signal. Preprocessing of these signals enhances the signal versus noise and allows improvement of visual resolution. In the case of the moving mean filter, all points are substituted by the mean value in the search region. One parameter is required:
  - Filter width: defines the size of the spectral window to use
- The Savitzky Golay filter is similar to the moving mean filter but less aggressive: It assumes a polynomial peak which helps to preserve the properties of the peak. Again, one parameter is required:
  - Filter width: defines the size of the window to use for calculating a polynomial. The filter will replace the central point with the new calculated value. For best results, the filter width must be in the range of the peak width at half height.

Once the necessary information is specified, the bucket table is saved:

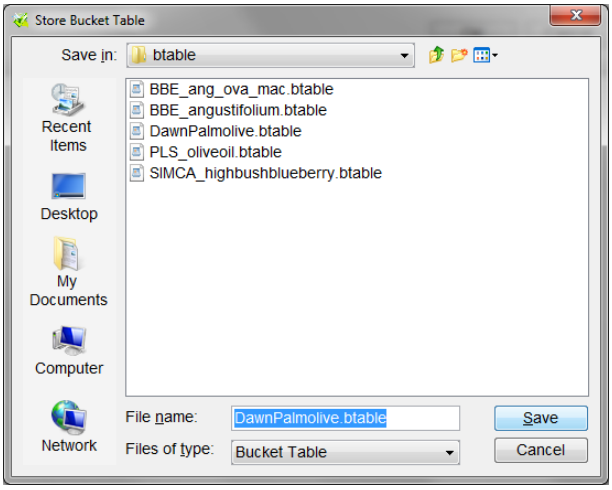



*Figure 8.7: Store Bucket Table window.*

After a brief pause, AssureNMR notifies the user that the bucket table has been created:


*Figure 8.8: Message after successfully storing a bucket table.*

Once a bucket table has been loaded, spectra can be added with the **Add Spectra to Bucket Table** option from the BucketTable pulldown menu. The bucket table can be edited using **Recalculate (keep Spectra, edit Parameters)** and **Recalculate (keep Parameter, edit spectra)** options from the menu.

## 8.1.2 Groups

Typically, the bucket table includes all the spectra available for the current analysis. In some studies, these spectra belong to different classes or groups. It may be of interest to classify future spectra according to these groups, using the multiclassification tools (Chapter 8.4). For this application, it is important to make the group information available to AssureNMR. The best practice is to store information about the samples that give rise to the spectra in the metadata (Chapter 4.5).

- **New Group from Meta Data**: Use this option to create groups for analysis directly from the metadata. A window will pop up and prompt the user for the name of the column containing group information.
- **Edit Groups Properties**: Once the groups are established, the multiclassification analysis will generate some plots featuring symbols for each spectrum. The user can set the color and the marker used for each group, to make it easy to distinguish between spectra in different groups.
- **Remove Group**: As analysis continues, the user may decide they are no longer interested in one of the possible classifications of the spectra. The group (metadata column heading) can be removed from the groups available for analysis.

### 8.1.3 Y Tables

The BucketTable pulldown menu provides options for handling Y tables:

- **Create Y Table from Meta Data**: This option pertains to the PLS regression modeling and uses the metadata to set the dependent or response Y variables. These values are typically imported from Excel spreadsheets
- **Create Y Table from Group**: This option is convenient for PLS discriminant analysis (PLS-DA). The groups can be used to set the Y variables, which are integer labels assigned to the different groups.
- **Remove Y Table**: This will remove the selected Y variables that have been added to the bucket table.

### 8.1.4 Examining the Bucket Table

After the bucket table is created, it can be exported (**Export to Excel**) for convenient inspection and analysis outside AssureNMR. When less detail is required, the user can view and save a summary of the bucket table in PDF format (**Bucket Table Info**). The summary will include the bucket table parameters, NMR spectral names, any spectra manipulation algorithms, metadata and also a quantile plot of all the NMR spectra provided.

## 8.2 Quantile Plots

The quantile plot is a graphical representation of the variance in the bucket table and can be used as a visual test. It calculates and displays the distribution of the intensities for the NMR spectra in the bucket table. The plot is shown as an NMR spectrum where the range of the upper to lower limit of the intensities is color-coded. To create a quantile plot, select **Quantile Plot** from the **Chemometrics** menu. A window will prompt the user for the location to save the quantile plot in .png format. In addition, the user has the option to select an NMR spectrum for comparison to the quantile plot. If no spectrum is chosen, a quantile plot will be calculated using the bucket table provided; no comparison spectrum will appear on the plot.

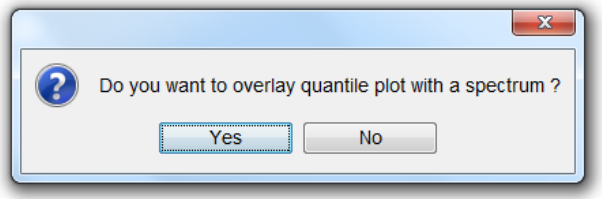



*Figure 8.9: The user has the option to overlay a spectrum on the quantile plot.*

The quantile plot and a legend of the distribution in percentages are shown below. The black spectrum is the test spectrum for comparison to the quantile plot. Outlying spectral areas that do not fit the distribution can be identified by inspection.

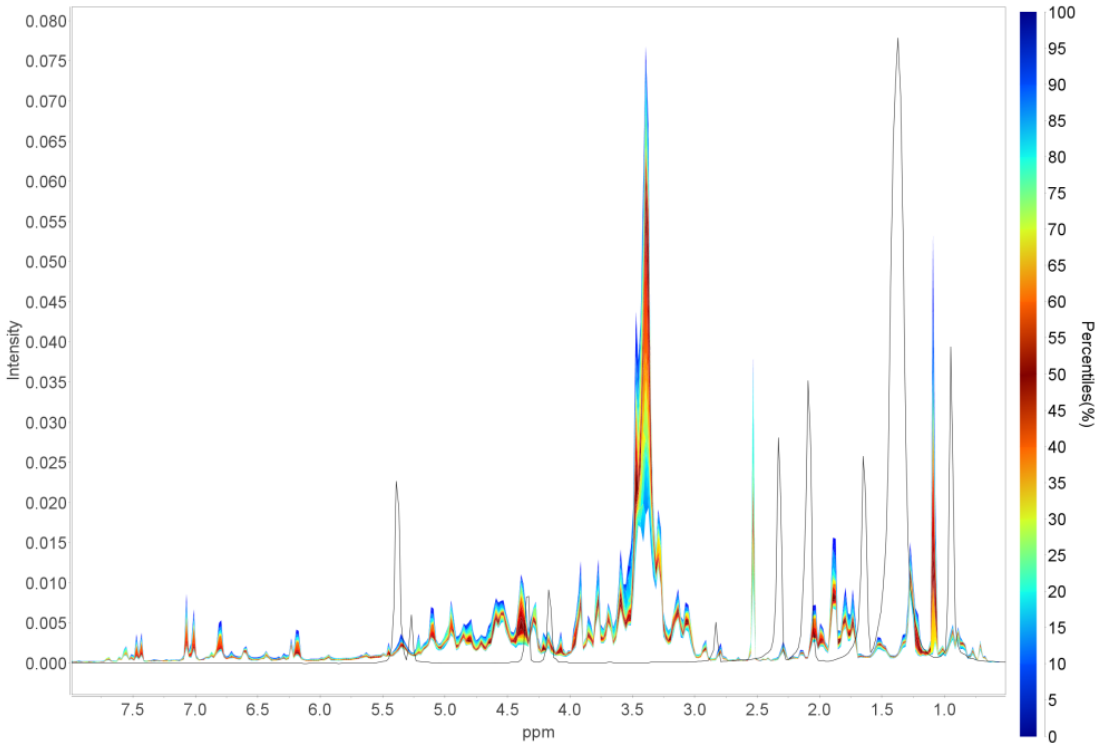



*Figure 8.10: A quantile plot (in color) with a test spectrum superimposed (black line).*

## 8.3 Building and Testing Against a Statistical Class Model

Soft independent modeling of class analogies (SIMCA) outlier detection is a statistical method that allows users to develop a classification model with their NMR spectra and use it to detect whether an NMR spectrum is in their class model (similar to the others) or outside (an outlier). This can be used for sample identification, for example, to identify the source of complex raw materials – geographical location, species or variety, growing conditions – as the data allow. The SIMCA model is developed using principal component analysis (PCA), which is a data reduction technique. PCA analyzes the whole dataset and searches for the major variations seen in the data which are represented by principal components. This method provides a better understanding of which variables are most influential in the user's data to classify their group.

Several options are available from the **SIMCA outlier detection** pulldown menu.

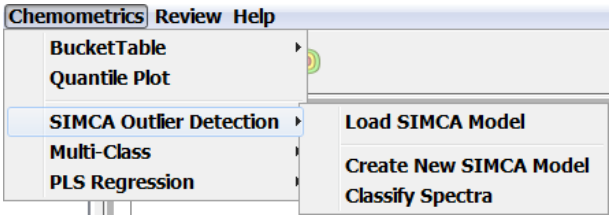



*Figure 8.11: SIMCA outlier detection pulldown menu.*

### 8.3.1 Load SIMCA Model

This option allows users to load SIMCA models made previously in AssureNMR to classify their samples.

### 8.3.2 Create New SIMCA Model

Upon selecting **Create NEW SIMCA Model**, the software prompts the user for a bucket table, if one has not been previously loaded, and the location to store the model (.simcamodel file). Then, a window appears with options to set parameters for the model.

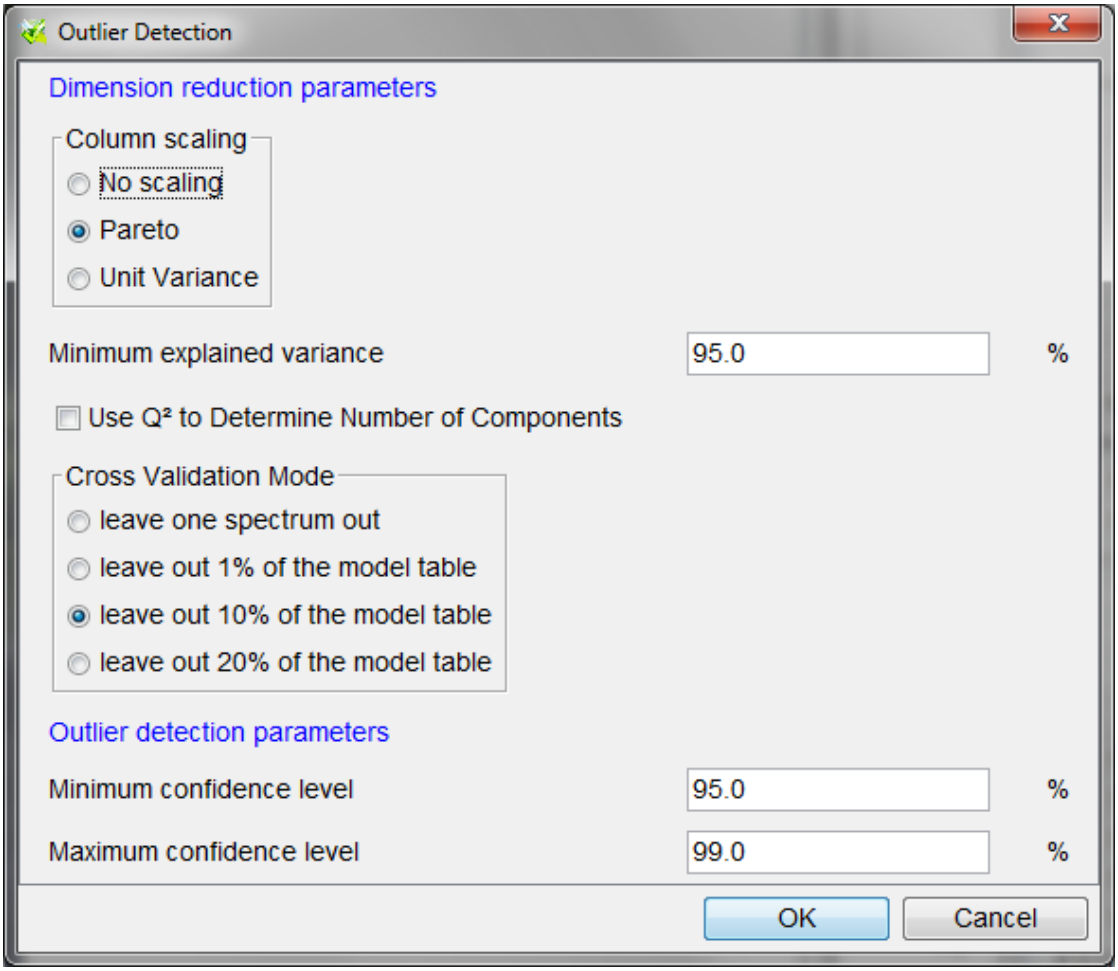



*Figure 8.12: Outlier Detection window.*

#### 8.3.2.1 Outlier Detection Window

The ‘Column scaling‘ radio buttons allow the user to scale the buckets (intensities) in the table so that all the spectra are comparable to each other. The type of scaling is dependent on the user’s experimental parameters and results can be greatly influenced by this option. If the samples are similar to each other and the NMR spectra were all acquired similarly, then ‘No scaling’ will be preferred. This preserves the natural variance in the dataset and focuses on the dominant effects that are seen. ‘Unit Variance’ is preferred if the samples vary and therefore large variances are due to experimental effects. This will allow all the columns to be equally weighted during the model calculation. Pareto scaling (‘Pareto’) is the medium between the two scaling functions and decreases large fluctuations on the dominant effects while still capturing the mild effects in the NMR spectra.

‘Minimum explained variance’ allows users to define how much of the spectral data must be explained in the classification model. The default value is 95.0%. An alternative way to determine how many components to use in the model is based on the quality assessment factor, $Q^2$. By selecting ‘Use $Q^2$ to Determine Number of Components’, the program will add components until the $Q^2$ for the model stops increasing.

The Cross Validation Mode window lets the user specify how to do the cross validation. the results of the cross validation are reported in the pdf file created for the model.

Under ‘Outlier detection parameters‘, the ‘Minimum (and Maximum) confidence levels’ entry fields allow the user to choose confidence limits. These limits are used to classify whether a NMR spectrum is within the limit of the model or is an outlier. The default parameter for most models is between 95-99%. Minimum and maximum confidence levels of 95% and 99%, respectively, are the default values.

### 8.3.2.2 The SIMCA Report

A SIMCA report is generated after the SIMCA model parameters are set to allow the user to review the model. The influence and Hotelling plots are generated to provide a visual illustration of the model space with the confidence levels displayed. The explained variance is plotted as a function of the number of components, as are the variance ($R^2$) and the quality assessment ($Q^2$). The distance of each NMR spectrum to the model center and the model space is listed in a table.

The influence plot shows spectra in a diagram where the vertical axis is a measure of how far away a spectrum is from the model space (off model distance). If a spectrum is in the upper part of this display, it is most likely not in the model space. The horizontal axis is a measure of how far away a spectrum is from the model center after being projected into model space. The two lines displayed inside the plot are the confidence limits. Spectra inside these limits (here, the lower left area in the green) belong to the model. A moderate outlier area is also defined (yellow area) which is between the minimum and maximum confidence area. Any NMR spectra that are outside the confidence areas (red zone) are outliers and have strong influence on the model. If there are outliers in the creation of the SIMCA model, it is recommended that the user investigate why such a case occurred. This might cause inaccurate classification of other spectra.

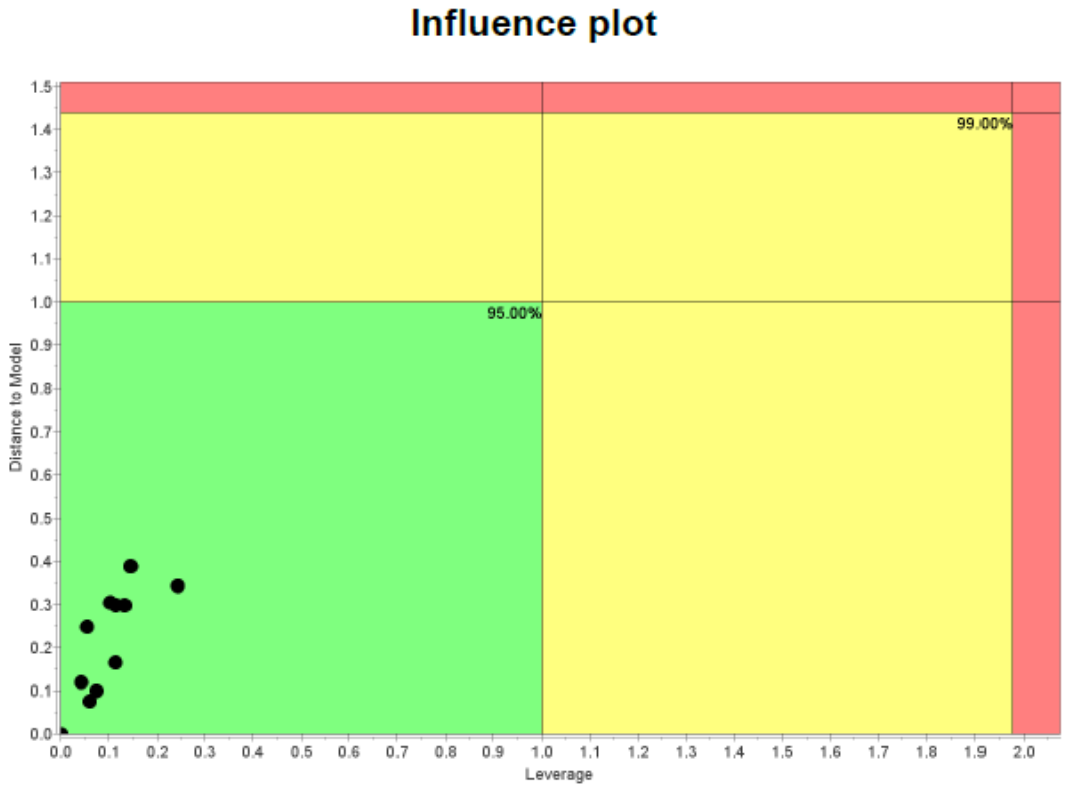



*Figure 8.13: Influence plot for a bucket table. Each spectrum is represented by a black dot. All spectra in this bucket table are within the 95% confidence limit (green region) and reasonably close to the model center. No outliers are detected in this model.*

The Hotelling plot is the distance of individual objects (NMR spectra) to the center of the model space. This is shown with a vertical axis with the minimum and maximum confidence lines defined. This is the same as the horizontal axis in the influence plot. The horizontal axis of the Hotellings plot is the spectrum number (spectra are numbered from 1..n).

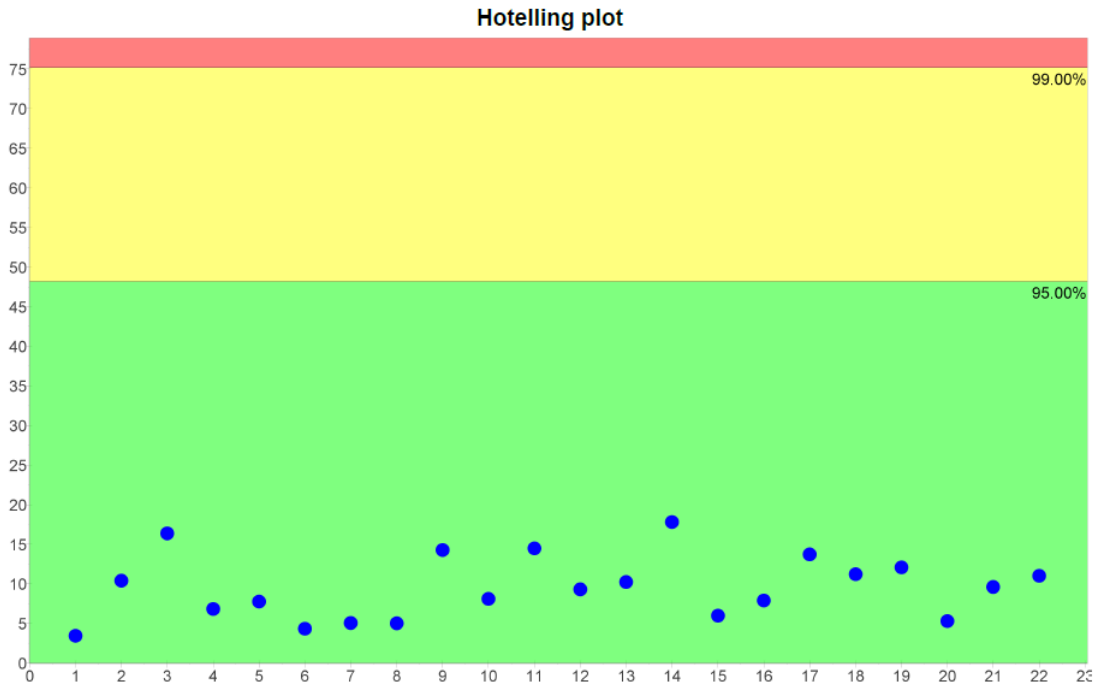
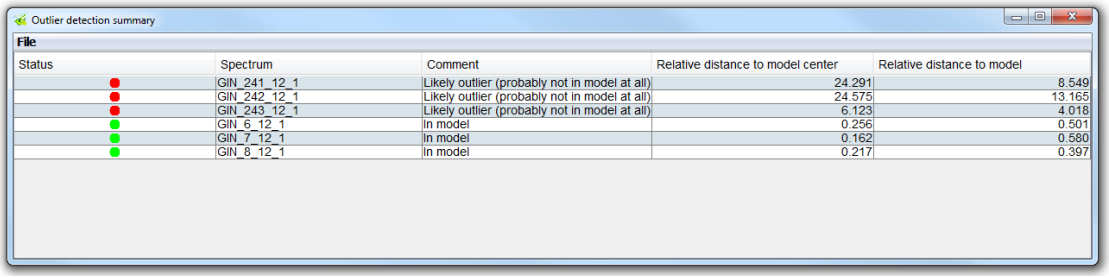



*Figure 8.14: Hotelling plot for a SIMCA model. Note that all spectra are within the 95% confidence limit (green region). No outliers are detected in this model.*

## 8.3.3 Classify Spectra

This option allows users to compare NMR spectra against a SIMCA model. To classify an NMR spectrum, choose **Classify Spectra** from the **SIMCA outlier detection** pulldown menu. A window will prompt for the NMR spectra to be compared with the SIMCA model. After the calculation, a window will appear which shows the results of the NMR spectra selected (green - in model, red - out of the model). The relative distance to model center and relative distance to model will be reported as well.


Outlier detection summary

File

| Status | Spectrum | Comment | Relative distance to model center | Relative distance to model |
|---|---|---|---|---|
| ● | GIN_241_12_1 | Likely outlier (probably not in model at all) | 24.291 | 8.549 |
| ● | GIN_242_12_1 | Likely outlier (probably not in model at all) | 24.575 | 13.165 |
| ● | GIN_243_12_1 | Likely outlier (probably not in model at all) | 6.123 | 4.018 |
| ● | GIN_6_12_1 | In model | 0.256 | 0.501 |
| ● | GIN_7_12_1 | In model | 0.162 | 0.580 |
| ● | GIN_8_12_1 | In model | 0.217 | 0.397 |

*Figure 8.15: Outlier detection summary window.*

Selecting one of the NMR spectra that was used in the classification test brings up a quantile plot that will be overlaid with the classification model to determine the spectral differences. The outliers, calculated from the scores and loadings, will also be displayed in the viewer window in AssureNMR.

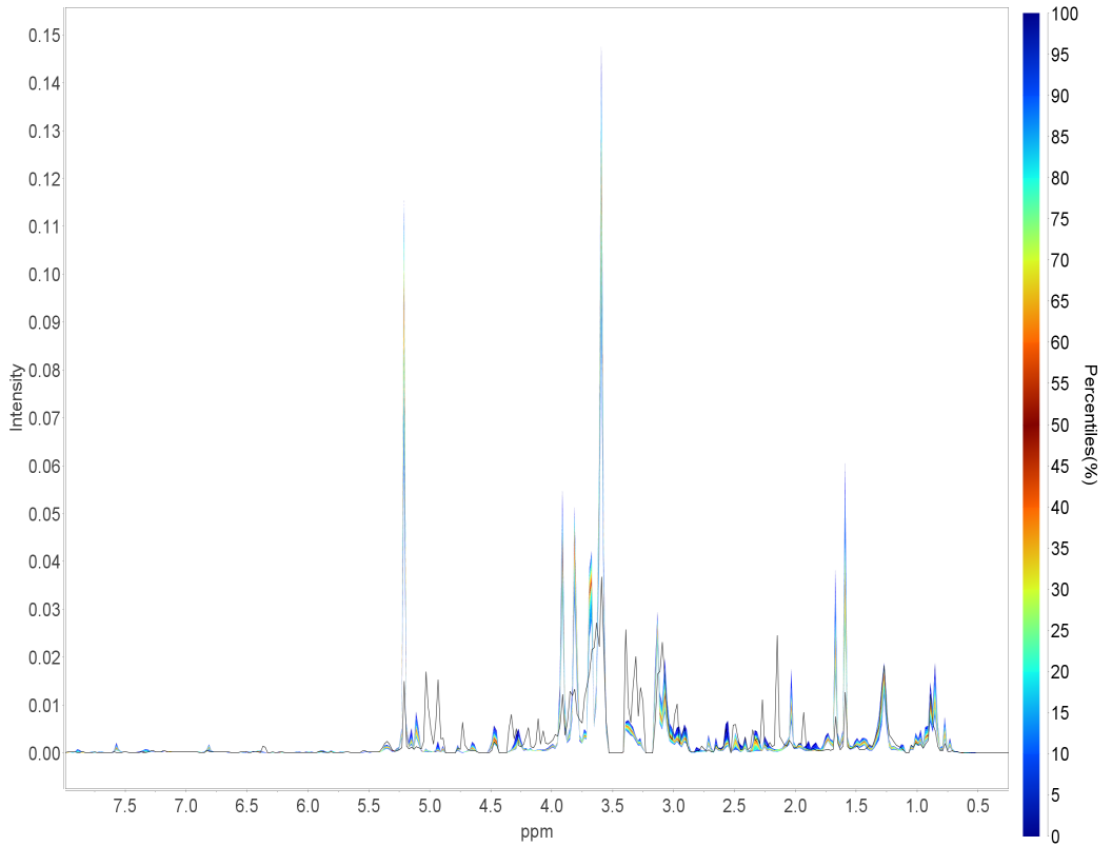



*Figure 8.16: Viewing outliers against the quantile plot.*

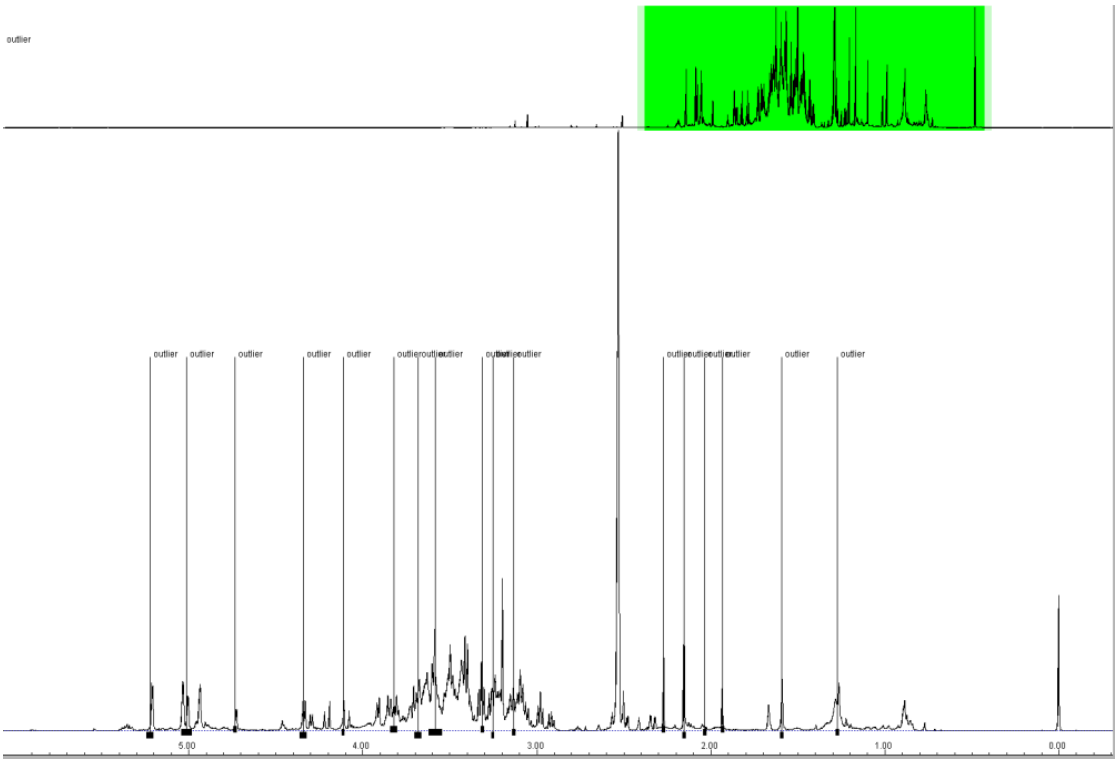



*Figure 8.17: Viewing outliers in the viewer window.*

A PDF report will be generated which displays the influence and Hotelling plots of the classification model. Additional influence plots will be generated to show where each of the selected NMR spectra lies compared to the model.

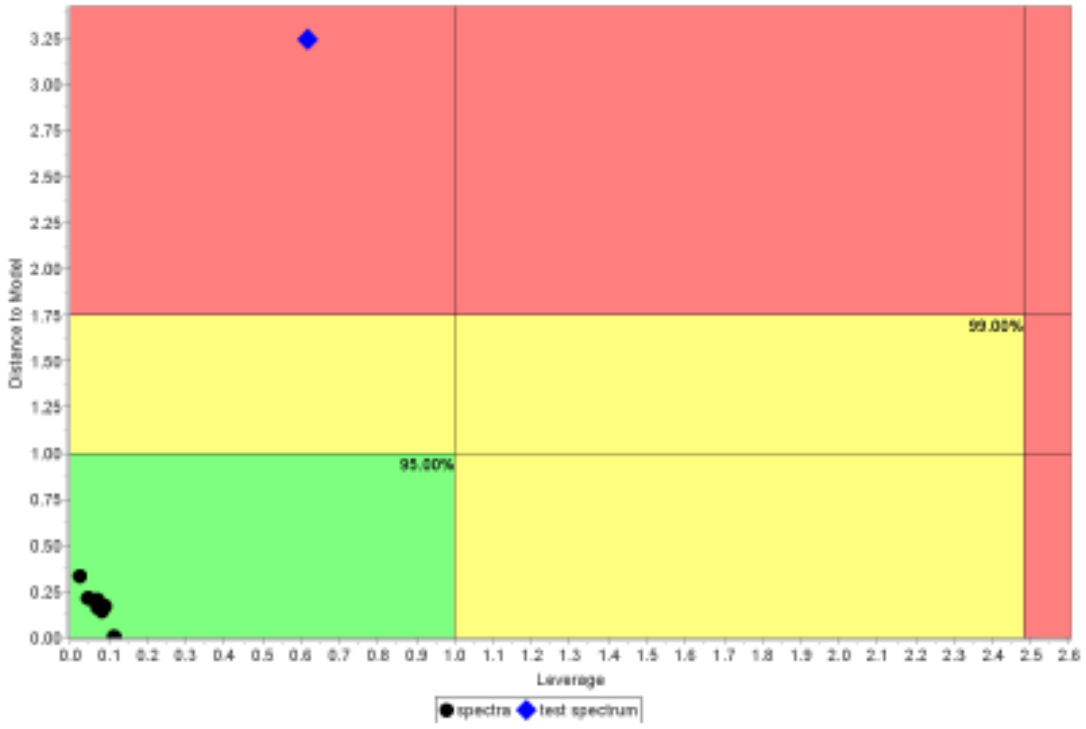



*Figure 8.18: Influence plots for classified spectra. The model spectra are represented by black balls; the test spectrum is represented by a blue diamond.*

The SIMCA model that is saved can now be attached to a quantification method to classify samples in automation. (Please see Chapter 6.6.6 for more details.)

## 8.4 Multiclass Classification

A SIMCA model is good for outlier detection – testing if a spectrum from a new sample fits in with a previously developed model for a class of sample. But sometimes it is useful to classify a sample into one of a handful of known classes. For example, a plant leaf extract known to be from the genus *Vaccinium* might be assigned to a particular species or a sample of dishwashing detergent could be identified by brand. Then it is useful to test against all of the classes at once. This is multiclass classification. As with other chemometric tools, it is important to have a collection of representative samples from each class, for data acquisition and model building. AssureNMR will optimize the model parameters automatically.The following commands are available from the AssureNMR Chemometrics menu, under **Multi-Class**.

### 8.4.1 Create Model

When the user selects **Create Model** from the Multi-Class pulldown menu, the software prompts for a bucket table if one has not previously been loaded. Then it prompts for the location to store the multiclass model. The next window prompts for the group (already defined in the bucket table) – this defines the overall category, Brand, and the classes available to classify new samples, Dawn or Palmolive. A minimum number of 4 spectra per class is necessary.

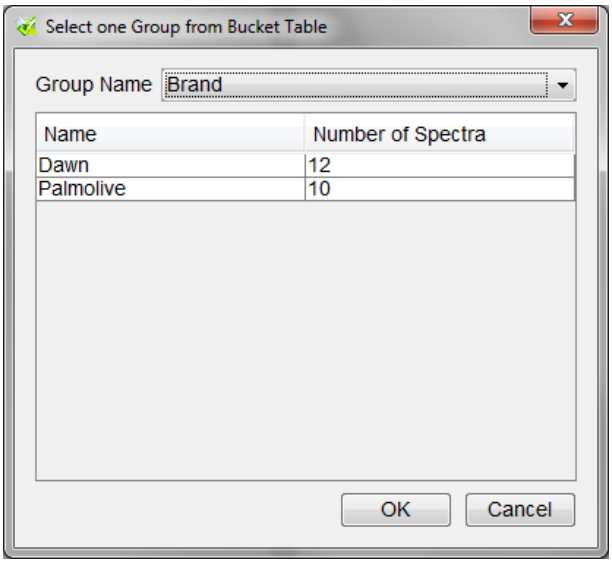



*Figure 8.19: Selecting the category for multiclass classification.*

Then a window comes up, prompting for the parameters for the model. The first window lets the user select the classification method(s). The choices are:

- PCA-ANOVA: principal component analysis – analysis of variance: combines principal component analysis to get a new basis set for the model with ANOVA to assess the statistical significance.
- PLS-DA: partial least squares discriminant analysis: supervised analysis, assigning an integer value to each group to enforce membership
- Multi-SIMCA: generates a SIMCA model for each class
- k-Nearest Neighbors: the test spectrum is assigned to the class of the k - most similar spectra. Similarity is calculated by Euclidian distance.

The Classificator Options window supplies a few key parameters: the scaling for the data, the minimum explained variance required for the principal component-based models, and the number of nearest neighbors to poll in the k-Nearest Neighbors method.

The Confusion Matrix window specifies whether to show inconclusive results, where there were multiple or no assignments. It also defines the minimum assignment probability required to report an assignment.

The Cross Validation window specifies the details for the cross validation, specifically, how to select data for the cross validation and how many Monte Carlo steps to use.

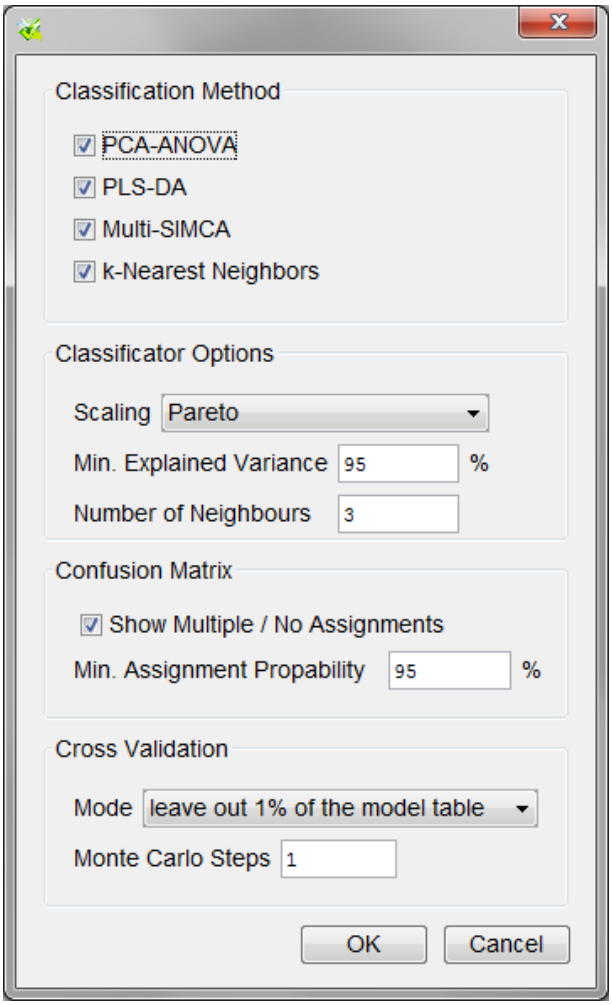
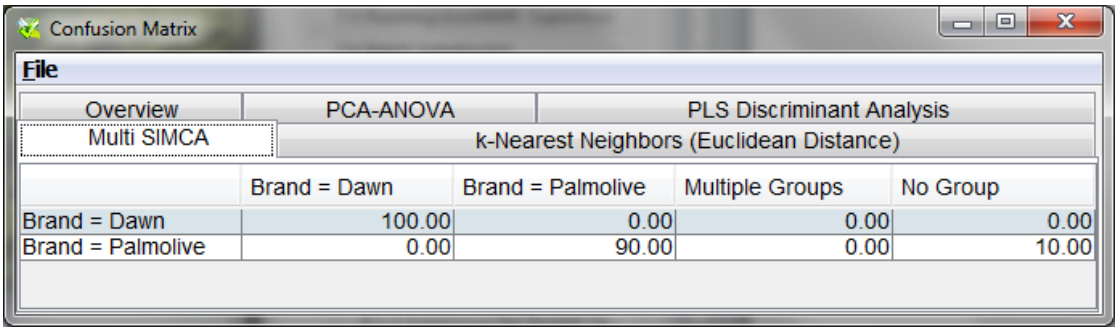



*Figure 8.20: Parameters for building a multiclass model.*

When the calculation is finished, the Confusion Matrix window pops up, summarizing the results, and a pdf report opens. In the Confusion Matrix window, there are tabs for the overview and for each model selected. The tab for the model shows the confusion matrix for that model. A perfect model would have 100% along the diagonal for each class with no examples assigned to multiple groups and no examples without an assignment to a group. The overview tab summarizes which model performs best for each class.




| | Brand = Dawn | Brand = Palmolive | Multiple Groups | No Group |
|---|---|---|---|---|
| Brand = Dawn | 100.00 | 0.00 | 0.00 | 0.00 |
| Brand = Palmolive | 0.00 | 90.00 | 0.00 | 10.00 |

*Figure 8.21: Confusion Matrix window for multiclass classification.*

The report summarizes the parameters used to build the models and shows the results in more detail for each model. The information displayed is specific to each model. It includes the confusion matrix in all cases and scores plots for PCA-ANOVA and PLS-DA and influence plots for multi-SIMCA.

### 8.4.2 Classify

Once the model has been built, it is available to classify spectra. The **Classify** command under the Multi-Class menu prompts the user for the model to use and the spectra to classify. The results are displayed in the classification window, with one tab for each classification method and one row for each spectrum. Clicking on the row for the spectrum brings up a table with each class tested against, the probability for the classification with respect to that class, and the quantile plot for the test sample spectrum against the validated members of the class for easy visual inspection to identify the differences.

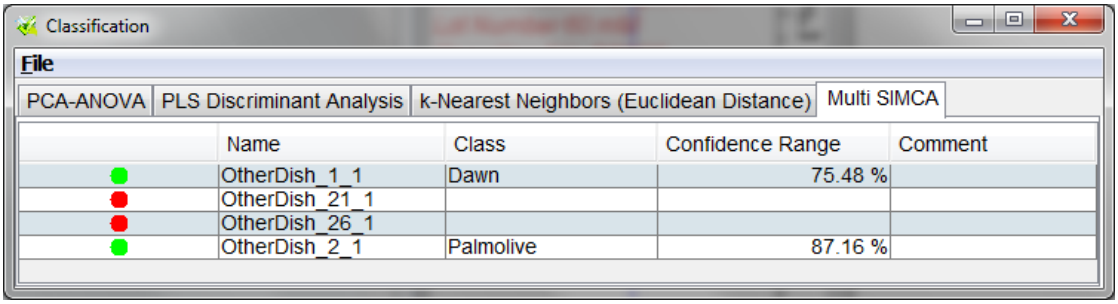



*Figure 8.22: Classification window from a multiclass classification.*

## 8.5 PLS Regression

Partial least squares (PLS) regression is another statistical tool for chemometrics in AssureNMR. PLS allows users to analyze or predict a set of dependent variables (Y-variables) from a set of independent variables or predictors (X-variables). The creation of a Y-table is required for this method. For example, a user can analyze various groups of samples that have different concentrations (Y-variables). By developing a PLS model from samples with known concentrations, they can predict the concentration of their unknown samples based on their regression model. Selecting **PLS Regression** from the Chemometrics pulldown menu opens a new menu with the necessary tools.

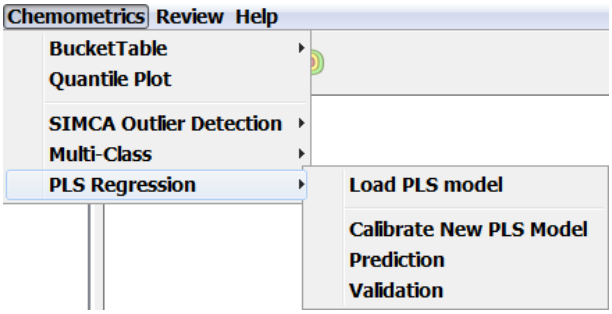



*Figure 8.23: PLS Regression pulldown menu.*

### 8.5.1 Load PLS model

This tool allows the user to open a previously saved PLS model. Once the model is open, it can be used for prediction.

### 8.5.2 Calibrate New PLS Model

In the PLS calibration, AssureNMR selects the number of PLS components for the regression model. The number of components will be automatically adjusted to find the optimum number to use. The objective when selecting the number of components is to maximize the prediction (the cross validated explained variance, $Q^2$) while obtaining a lower root mean square error of calibration cross-validated (RMSEC (Cross Validated)) value. In addition, the PLS

components selected should maximize the explained variance ($R^2$) in the X table (spectra data) and Y table (response variables). A PLS model summary report, in PDF format, is generated for each PLS model calibration. The summary report contains the Number of PLS factors, $R^2$, $Q^2$, RMSEC (Cross Validated), explained variance, range of Y values, and graphs of RMSEC CV as a function of the number of PLS factors, measured Y versus predicted Y*, $T_1/U_1$ scores plot, distances to model X and Y for each spectrum, the variable importance of projection (VIP) across the chemical shift range of the spectrum, and the leverages for each spectrum. The number of PLS factors is determined from the RMSEC CV values. If the RMSEC CV value when the next factor is added is not statistically significantly lower than the current value, the current number of PLS factors is used.

AssureNMR

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## PLS Model Summary

| | |
|---|---|
| **Number of PLS Factors:** | 12 |
| **$R^2$:** | 1 |
| **$Q^2$:** | 1 |
| **RMSEC (Cross Validated)** | 0.3108 |
| **Model Range:** | 0 - 100 |

*Figure 8.24: Results from calibration of a PLS model.*

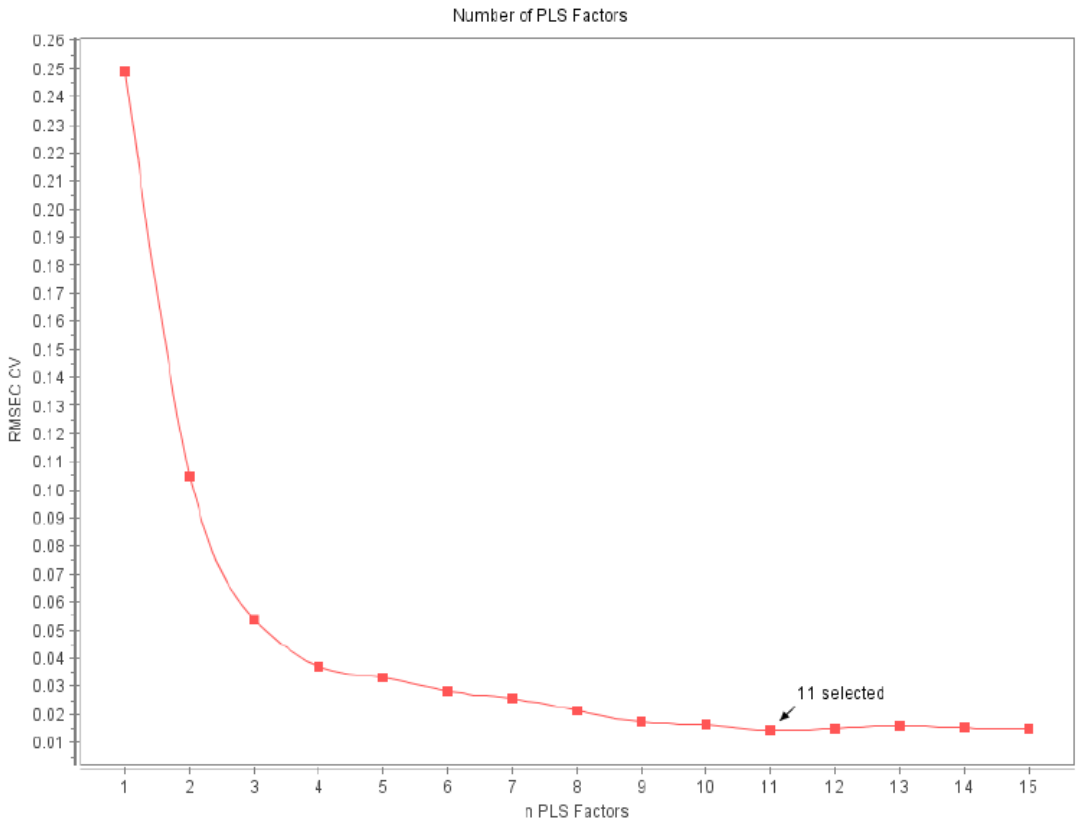



*Figure 8.25: The root mean square error of calibration-cross validation (RMSEC (Cross Validation)) as a function of the number of PLS factors selected.*

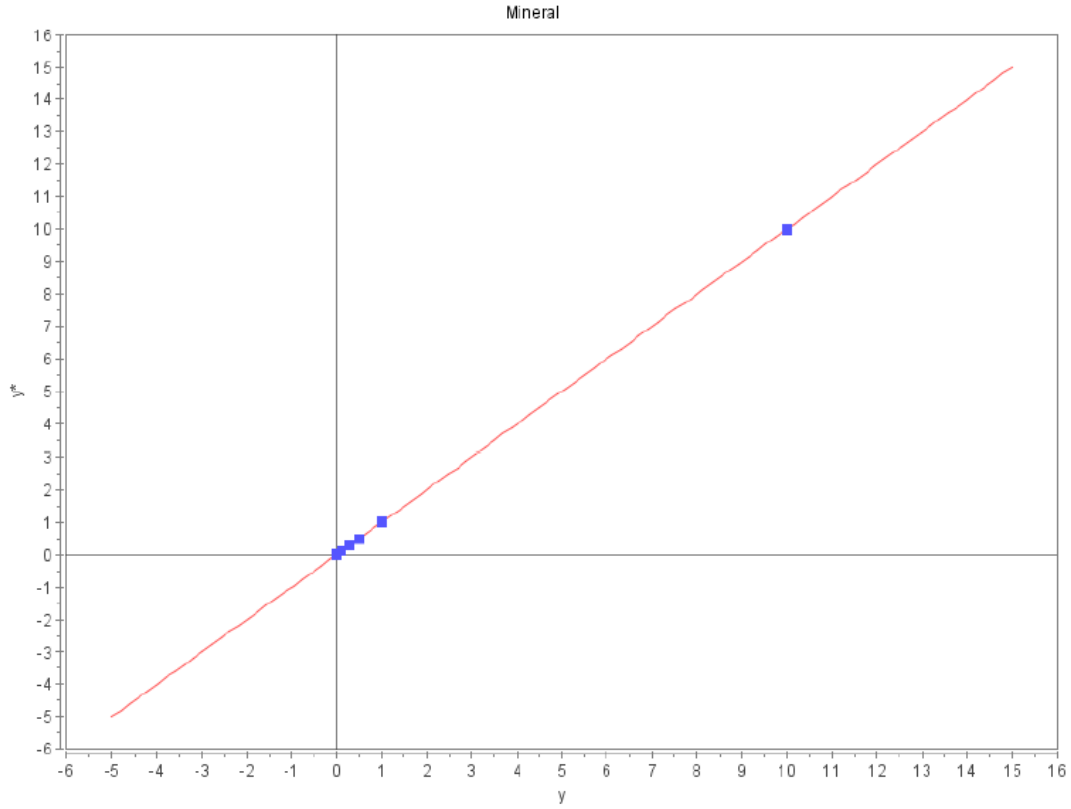



*Figure 8.26: Y/Y* graph of Y-values predicted from the PLS model (Y*) verus the measured Y-values (Y).*

The Y/Y* graph compares the measured Y-values (horizontal axis) with the predicted values from the PLS model (vertical axis). A well calibrated model, with the optimal number of PLS components, enough spectra in the X data, and no outliers, should give a straight diagonal, demonstrating the agreement between the measured and the predicted Y-values. For best results, there should be enough spectra for the X data to properly reflect the Y table and preferrably mulitple spectra for each Y value to assess the variance in the dataset. A high RMSEC (Cross Validation) indicates potential outliers in the dataset or the lack of data for proper prediction. A low $Q^2$ indicates caution is in order - careful inspection of the dataset is recommended.

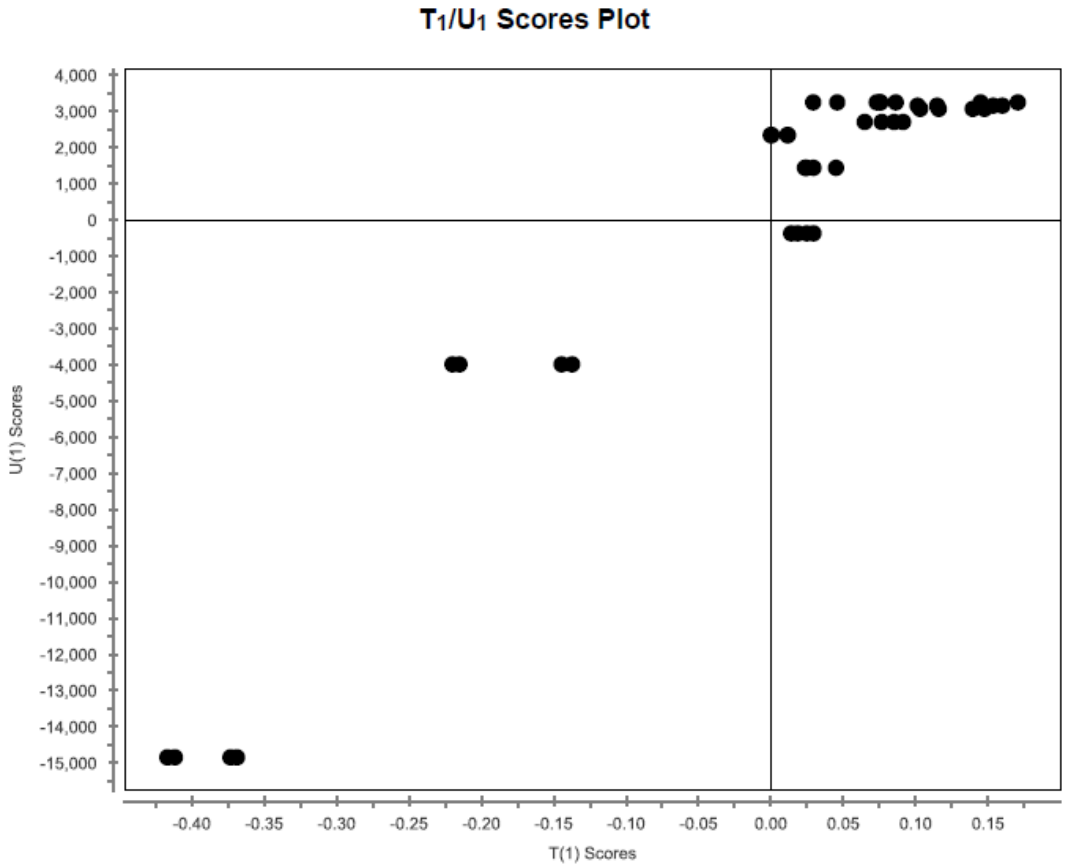



*Figure 8.27: The $T_1/U_1$ scores plot from the PLS model.*

The $T_1/U_1$ scores plot displays the correlation between the X and Y variables. Each black dot represents a sample spectrum and its placement in the X space compared to the Y space. In an ideal case, the $T_1/U_1$ scores show a perfect diagonal. However, in reality, the data are more spread out and this spread shows how strong the correlation is. In some cases, the correlation is not linear and a curvature is seen. Many reasons can explain this behavior such as improper scaling of the X or Y data or differences in data processing.

The PLS model that is saved can now be attached to a quantification method to predict samples in automation. (Please see Chapter 6.6.6 for more details.)

## 8.5.3 Prediction

With this tool, the PLS model can be used to predict the Y value for a test NMR spectrum. For example, one could predict the concentration of a sample from a PLS model created with a Y table of known concentration values. The user is asked to provide the directory for the NMR spectra to be used for prediction.

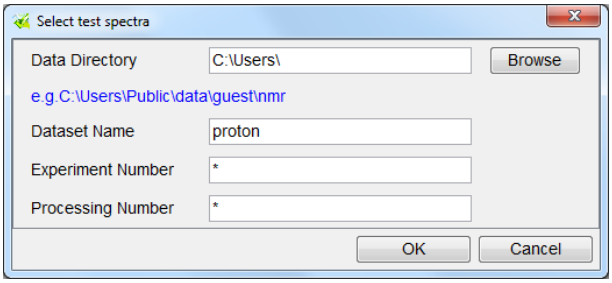



*Figure 8.28: Select test spectra window, for PLS prediction.*

The results of the prediction will show the spectrum file name, the predicted Y value for the group, and the RMSECCV error. The results can also be exported to an Excel spreadsheet or a PDF document by selecting the option under the **File** pulldown menu.

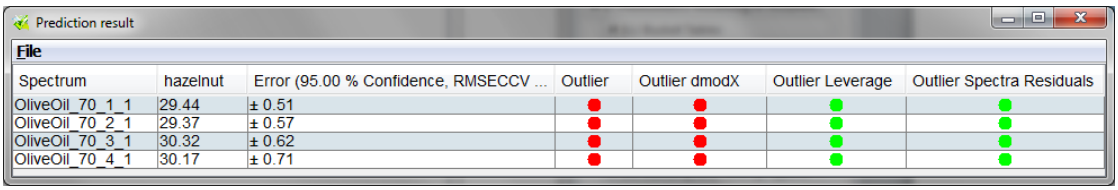



*Figure 8.29: Results from the PLS prediction.*

## 8.5.4 Validation

To ensure that the PLS regression model for the predictor variable matrix X (NMR spectra data) and the response variable Y (Y-table group) is significant, validation using a permutation test is available. A permutation test fits randomly selected subsets of the data to test the model's statistical significance. Since the user is required to input the response variables (Y-table), there is the possibility that the modeled predicted Y value ($Q^2$) was a chance correlation. Therefore, the permutation test can be used to randomize the Y-table for the spectra and new $Q^2$ values are calculated. The new $Q^2$ values are then compared to the original $Q^2$ values. A p-value (probability value) calculation will be performed according to the number of $Q^2$ values that are higher than the original model. If $p < 0.05$ (observed chance is less than 5%, alpha=0.05), then the model is considered significant.

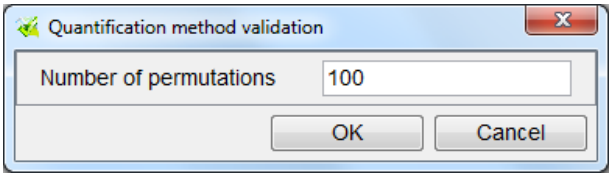



*Figure 8.30: Prompt for the number of permutations for PLS model validation.*

It is advisable to choose an adequate number of permutation tests ($n \geq 100$) to ensure that enough tests are done to validate the original model. A PDF report will be generated after a successful permutation test. The reference prediction $Q^2$ value is shown with the number of permutation models that had better results. A histogram will also be generated as a visual representation of all the $Q^2$ values in the permutation test in comparison with the $Q^2$ of the model. A p-value calculation will be also be done to show if the model is significant ($p < 0.05$). Users should be cautious about their model if many higher $Q^2$ values are generated from the permutation test.

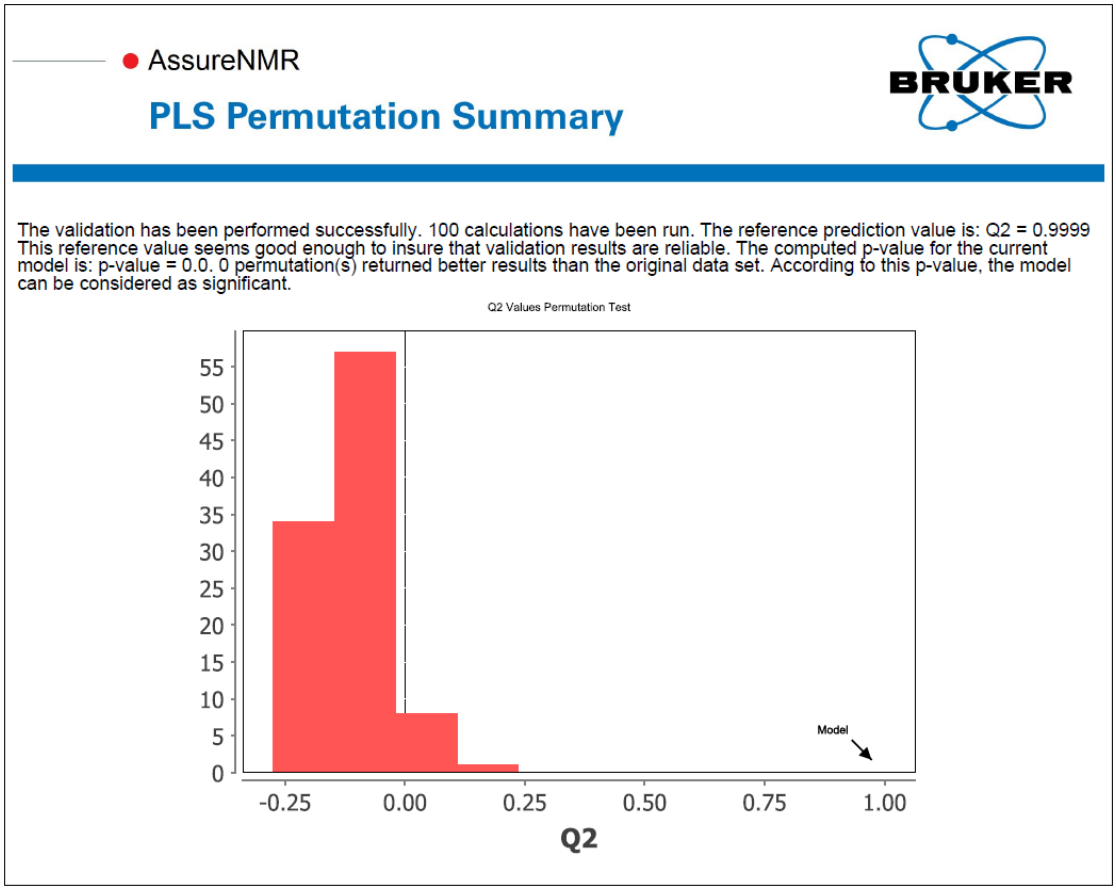



*Figure 8.31: Results of PLS model validation.*

# 9 Biologics

## 9.1 Biopharmaceuticals (Biologics)

Biopharmaceuticals, or biologics, are pharmaceutical drug products manufactured utilizing biological sources. These include materials that may be very large in size such as monoclonal antibodies (mAbs) or they may be smaller proteins such as heparin. AssureNMR has been designed so that it may be useful in applying common approaches to analyzing biologics. For example, the sodium heparin monograph USP 35 and Pharm.Eur 333 call for analysis of a 1D 1H spectrum for various specific components and residual solvents. Details on this approach using AssureNMR is discussed in the section *Heparin quantMethod [▶ 159]*. One-dimensional 1H NMR analysis for mAbs using the PROFILE approach is addressed in section *PROFILE [▶ 145]*[3] Two-dimensional approaches utilizing the evaluation of a 2D HSQC or 2D-HMQC of heparin are discussed in the literature.[4] AssureNMR methods may be designed to evaluate 2D HSQC spectra as addressed in section 6 Quantification.

## 9.2 PROFILE

PROtein Fingerprint by Lineshape Enhancement (PROFILE)[5] is an 1D approach to characterize formulated mAbs using 1D 1H NMR. The method is based on 1H NMR pulsed field gradient stimulated echo experiment to generate a high resolution structural fingerprint of the formulated protein therapeutic. This fingerprint is compared to reference spectral fingerprints to detect relatively small changes in formulated antibody samples. AssureNMR software enables the use of this approach through the Biologics pulldown menu on already acquired spectra as a post processing analysis. Example data, a parameter set, a pulse program and information are provided in ‚Profile' in Example Data Sets. See section *PROFILE NMR [▶ 172]* for a tutorial on using the features in the Biologics menu.

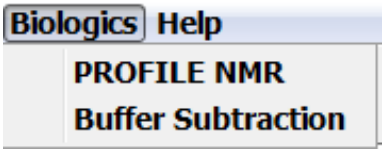



*Figure 9.1: AssureNMR Main Menu with Biologics pulldown selected.*

Selecting PROFILE NMR initiates the acknowledgement window. The references cited provide details of experimental conditions used by the authors at the date of publication and information of this approach in the evaluation of protein therapeutics.

[3] L Poppe et al., Anal. Chem. 2013, 85, 9623−9629; L. Poppe et al., Anal. Chem. 2013, 85, 9623.
[4] Aubin, Marino, RonzoniI. Guerrini M, Guglieri S, Naggi A, Sasisekharan R, Torri G. Low molecular weight heparins: structura differentiation by bidimensional nuclear magnetic resonance spectroscopy. Semin Thromb Hemost 2007; 33(5): 478-87.II. M Guerrini, A Naggi, S Guglieri, R Santarsiero, G Torri, Anal. Biochemistry 2005, 337 35-47. III. Y. Aubin, D. Freedberg, A. Keire; Biophysical Characterization of Proteins in Developing Biopharmaceuticals.IV. L W Arbogast, R G Brinson, and J P.Marino:The tools are there – explore! Resulting spectrum may be used. PROFILE utilizes an automated phase correction routine and li Anal. Chem. 2015, 87, 3556−3561.
[5] L Poppe et al., Anal. Chem. 2013, 85, 9623−9629; L. Poppe et al., Anal. Chem. 2013, 85, 9623.

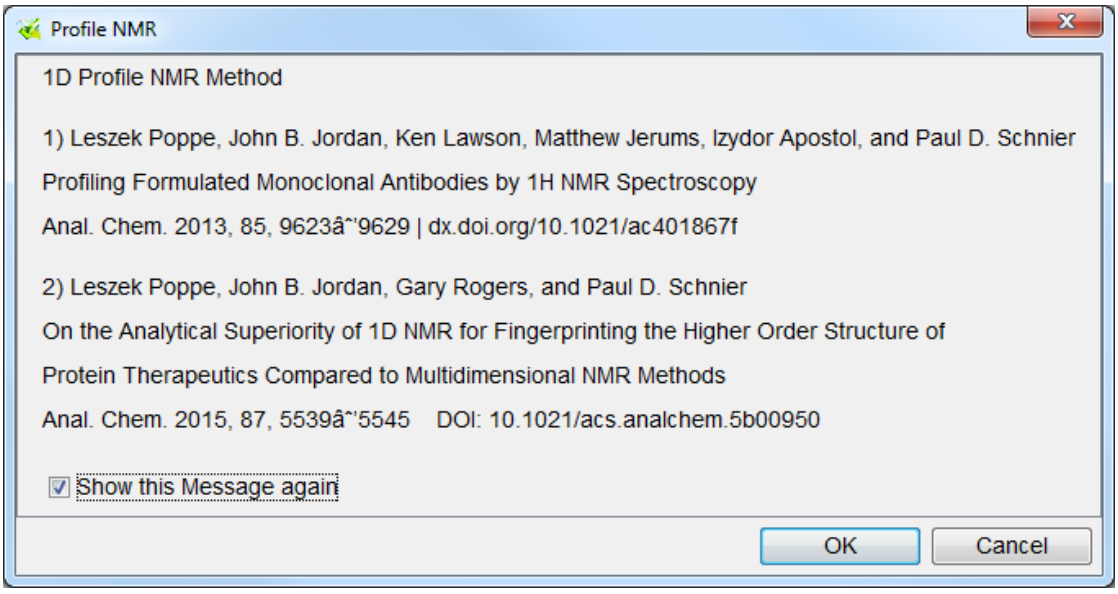



*Figure 9.2: Acknowledgements for the PROFILE method used in AssureNMR*

The PROFILE workflow proceeds by (1) selecting reference spectra, (2) selecting spectra to test against the reference spectra and then (3) comparitive results are displayed. Meta data tags may be used to assist in the selection of spectra. To comapre results PROFILE utilizes an (1) automated phase correction routine, (2) broadening factor, (3) aligns spectra, (4) calculates the featureless component of the spectrum, (5) subtracts the featureless component, and (6) generates the fingerprint spectra. The fingerprint spectra are then subjected to an autocorrelation analysis. For example, the autocorrelation analysis proceeds as follows. If 5 reference spectra and 5 test spectra are chosen, 10 autocorrelations are performed. This results in 20 autocorrelation spectra and 25 cross correlation spectra. The result panel is displayed.

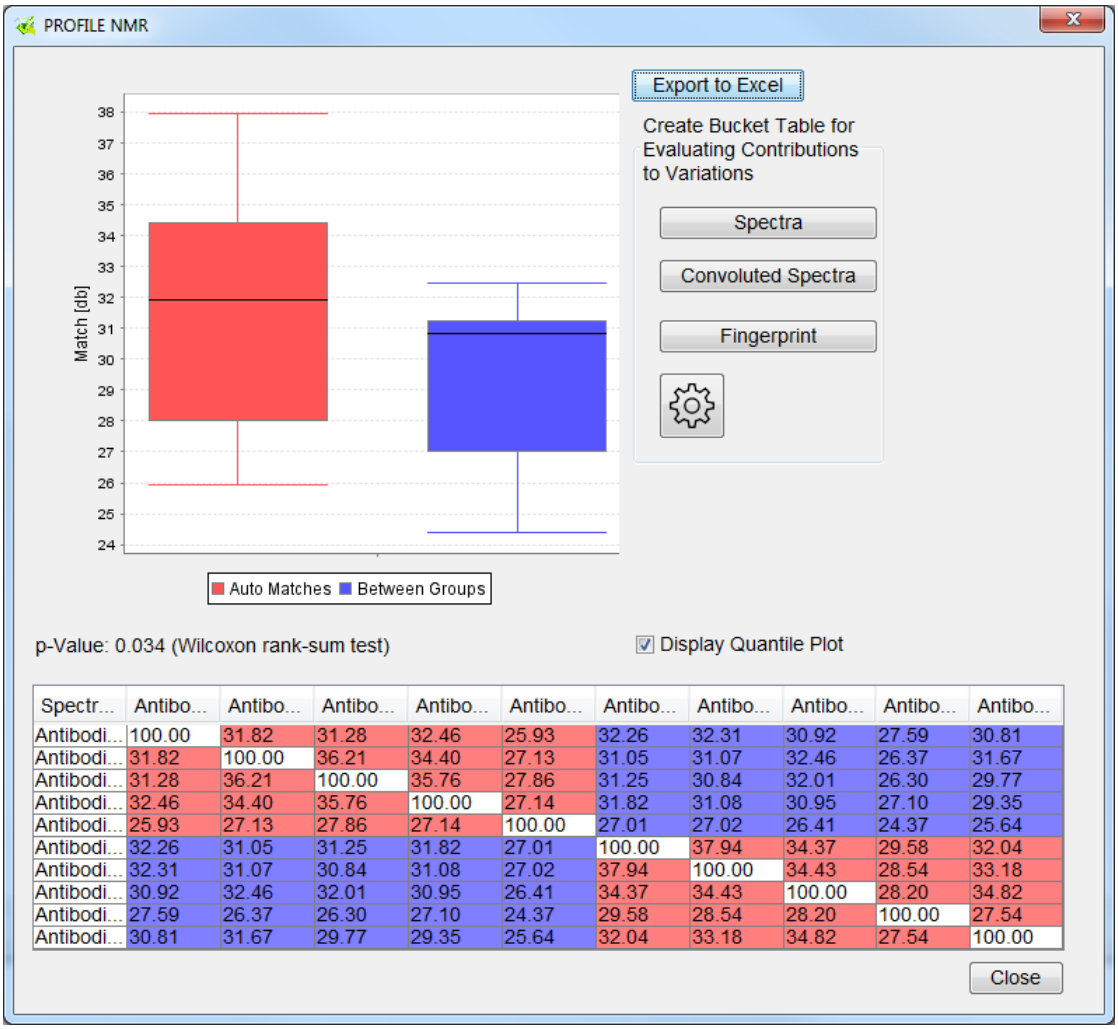



*Figure 9.3: PROFILE results panel*

The Match display shows a whisker plot that enables a rapid visual assessment of the correlation results. The AutoMatch and Between Groups display may be used to assess the similarity of the materials. The calculated probablility, p-value, based on two-sided “Wilcoxon Ranksum Test“ is also displayed.

Clicking any cell in the autocorrelation matrix displays the spectra at various points in the analysis.

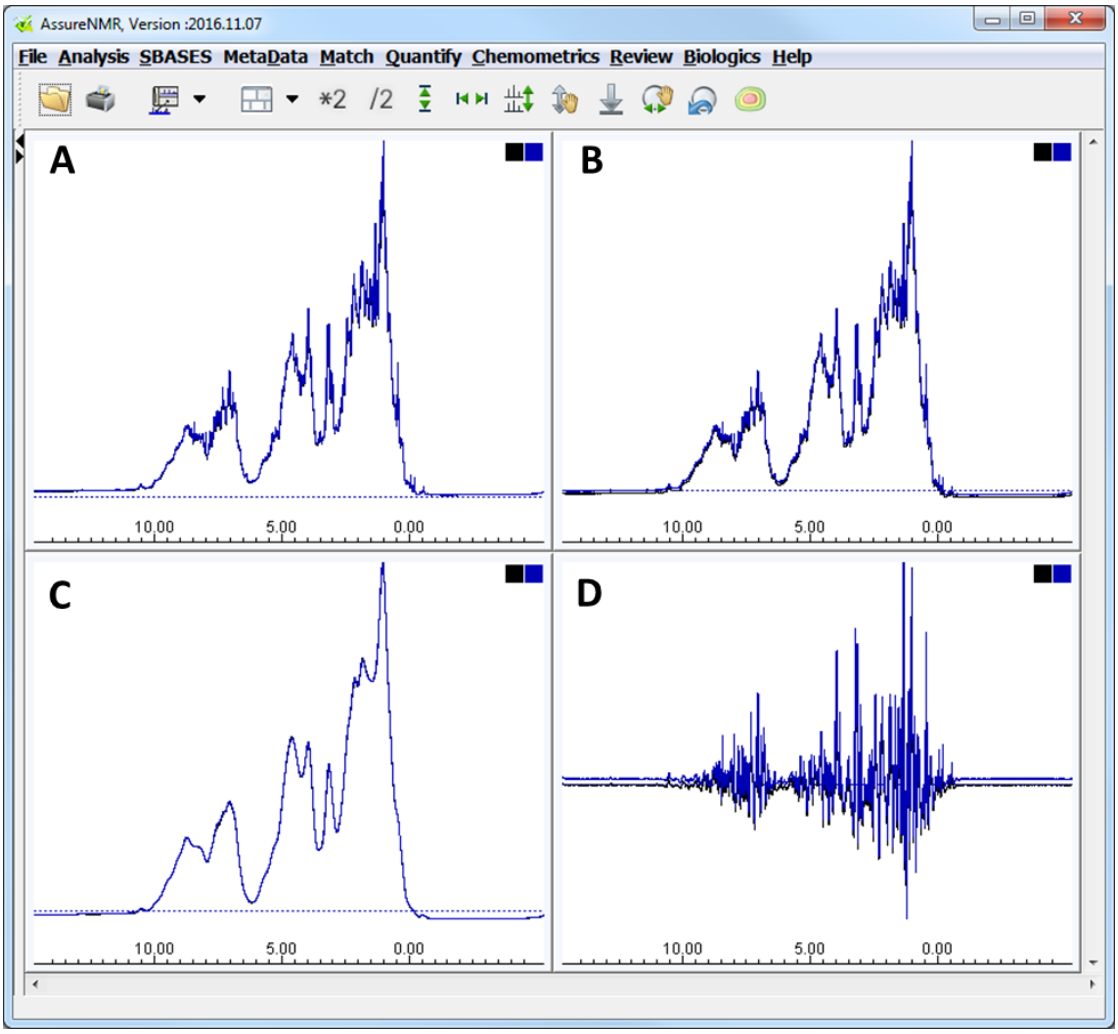



*Figure 9.4: Reference spectra (black) and test spectra (blue) at various stages of the profile analysis. A - Original Spectra; B - Phased Spectra; C - Bottom-Left calculated featureless component spectra; D - PROFILE Fingerprint spectra (used in the results panel)*

### 9.2.1 Preparing PROFILE Results for Further Analysis

Results from PROFILE analysis may be prepared for further use in AssureNMR or other analysis packages from the PROFILE Results Panel. **Export to Excel** converts the autocorrelation results to an excel table. For evaluating contributions to the variance in the spectra, bucket tables may be created from the phased and aligned spectra (**Spectra**), the convoluted spectra (**Convoluted Spectra**), and the PROFILE fingerprint spectra (**Fingerprint**).

### 9.2.2 BufferSubtraction

Preprocessing of the spectra for PROFILE analysis by subtraction of the buffer is available in AssureNMR. **Buffer Subtraction** creates a new spectrum with a new processing number (PROCNO) by subtracting a buffer spectrum from an antibody spectrum. The resulting spectrum may be used for PROFILE analysis. See the tutorial in section Tutorials →*PROFILE NMR [▸ 172]* for details.

# 10 Examples

## 10.1 Log File from the System Suitability Test

**Filename: SST_2015-06-24-16-35-30_log.txt**

### System Suitability Report ###

### IconNMR Version: Version 5.0.3 Build: 11 ###

###

### RESULT: PASSED ###

###

#

# Output of System Tests Follows:

#

#1H Lineshape Humptest

Experiment: Dataset: SST_2015-06-24-16-35-30 1 1 C:/Bruker/Databases/DATA/Assure

Specification result of humpcal: ok

Linewidth at 0.55% of signal height = 6.9 Hz ( < 9Hz) ok

Linewidth at 0.11% of signal height = 14.5 Hz ( < 18Hz) ok

Halfwidth = 0.69 Hz ( < 0.9Hz) ok

#

#1H Sensitivity Test

Experiment: Dataset: SST_2015-06-24-16-35-30 2 1 C:/Bruker/Databases/DATA/Assure

Specification result of sinocal: ok

Best sino value found = 5263.5 :1 ( > 5000 :1) ok

#

#13C Sensitivity Test

Experiment: Dataset: SST_2015-06-24-16-35-30 3 C:/Bruker/Databases/DATA/Assure

Specification result of sinocal: ok

Best sino value found = 832.3 :1 ( > 700 :1) ok

#

#Temperature Test

Dataset: SST_2015-06-24-16-35-30 4 C:/Bruker/Databases/DATA/Assure

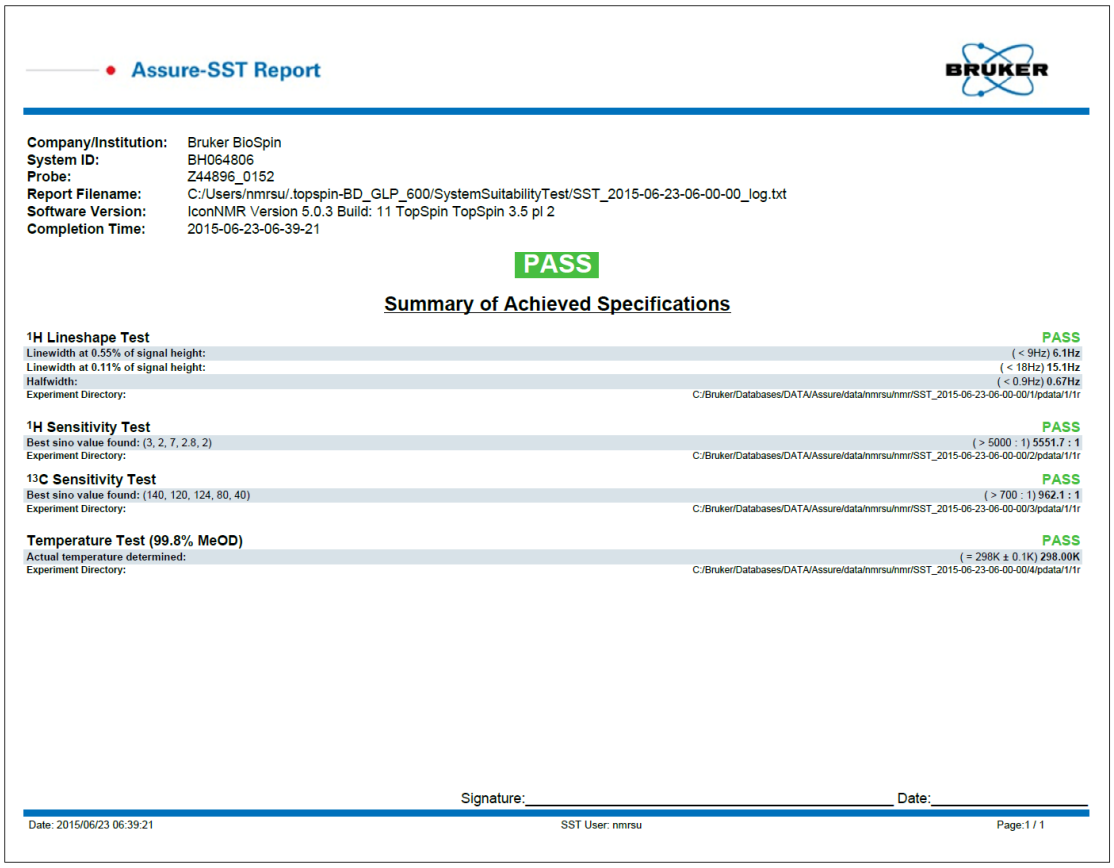
Experiment: Temperature test with 99.8% MeOD Sample

Specification result of temperature: ok

Actual temperature determined to be 298.00 on 2015-06-24-17-13-08 during system suitability test

## 10.2 Reports

### SST Report:



Assure-SST Report

BRUKER

**Company/Institution:** Bruker BioSpin
**System ID:** BH064806
**Probe:** Z44896_0152
**Report Filename:** C:/Users/nmrsu/.topspin-BD_GLP_600/SystemSuitabilityTest/SST_2015-06-23-06-00-00_log.txt
**Software Version:** IconNMR Version 5.0.3 Build: 11 TopSpin TopSpin 3.5 pl 2
**Completion Time:** 2015-06-23-06-39-21

PASS

**Summary of Achieved Specifications**

**$^{1}$H Lineshape Test** PASS
Linewidth at 0.55% of signal height: ( < 9Hz) 6.1Hz
Linewidth at 0.11% of signal height: ( < 18Hz) 15.1Hz
Halfwidth: ( < 0.9Hz) 0.67Hz
Experiment Directory: C:/Bruker/Databases/DATA/Assure/data/nmrsu/nmr/SST_2015-06-23-06-00-00/1/pdata/1/1r

**$^{1}$H Sensitivity Test** PASS
Best sino value found: (3, 2, 7, 2.8, 2) ( > 5000 : 1) 5551.7 : 1
Experiment Directory: C:/Bruker/Databases/DATA/Assure/data/nmrsu/nmr/SST_2015-06-23-06-00-00/2/pdata/1/1r

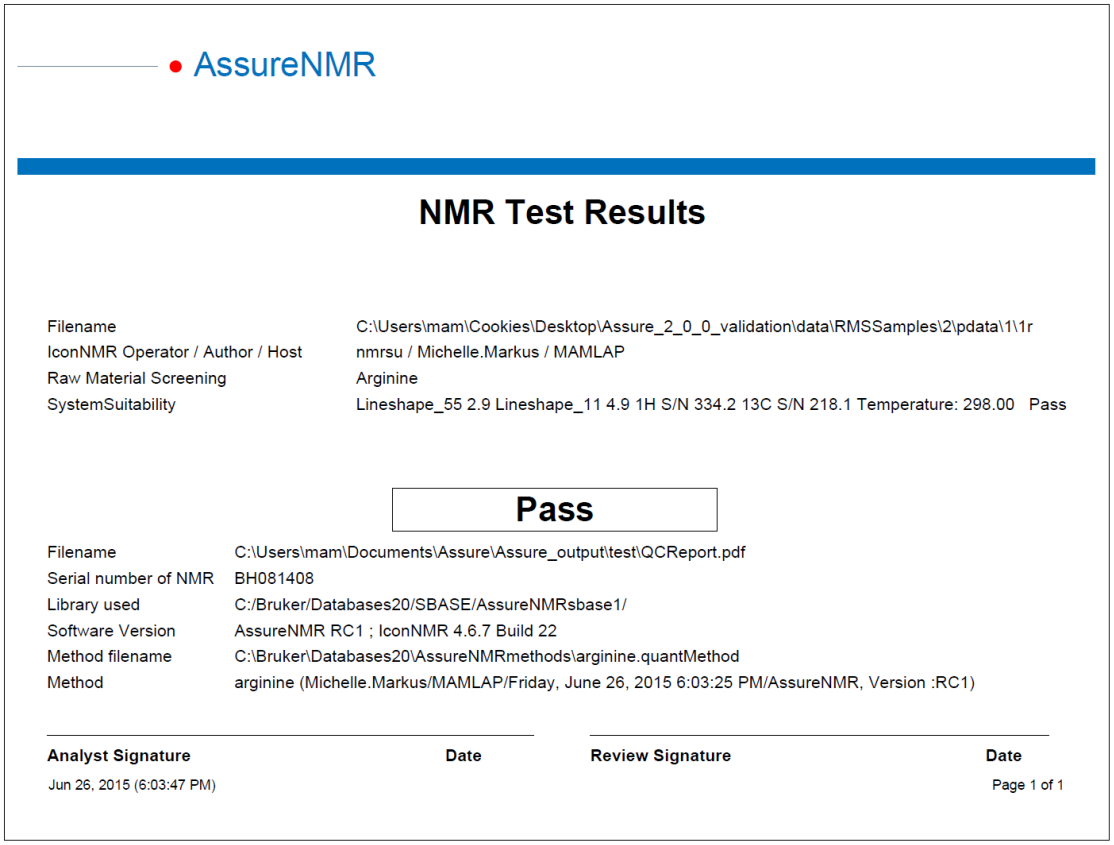
**$^{13}$C Sensitivity Test** PASS
Best sino value found: (140, 120, 124, 80, 40) ( > 700 : 1) 962.1 : 1
Experiment Directory: C:/Bruker/Databases/DATA/Assure/data/nmrsu/nmr/SST_2015-06-23-06-00-00/3/pdata/1/1r

**Temperature Test (99.8% MeOD)** PASS
Actual temperature determined: ( = 298K ± 0.1K) 298.00K
Experiment Directory: C:/Bruker/Databases/DATA/Assure/data/nmrsu/nmr/SST_2015-06-23-06-00-00/4/pdata/1/1r

Signature:_____ Date:_____

Date: 2015/06/23 06:39:21 SST User: nmrsu Page:1 / 1

**QC Report:**



AssureNMR

## NMR Test Results

| | |
|---|---|
| Filename | C:\Users\mam\Cookies\Desktop\Assure_2_0_0_validation\data\RMSSamples\2\pdata\1\1r |
| IconNMR Operator / Author / Host | nmrsu / Michelle.Markus / MAMLAP |
| Raw Material Screening | Arginine |
| SystemSuitability | Lineshape_55 2.9 Lineshape_11 4.9 1H S/N 334.2 13C S/N 218.1 Temperature: 298.00 Pass |

**Pass**

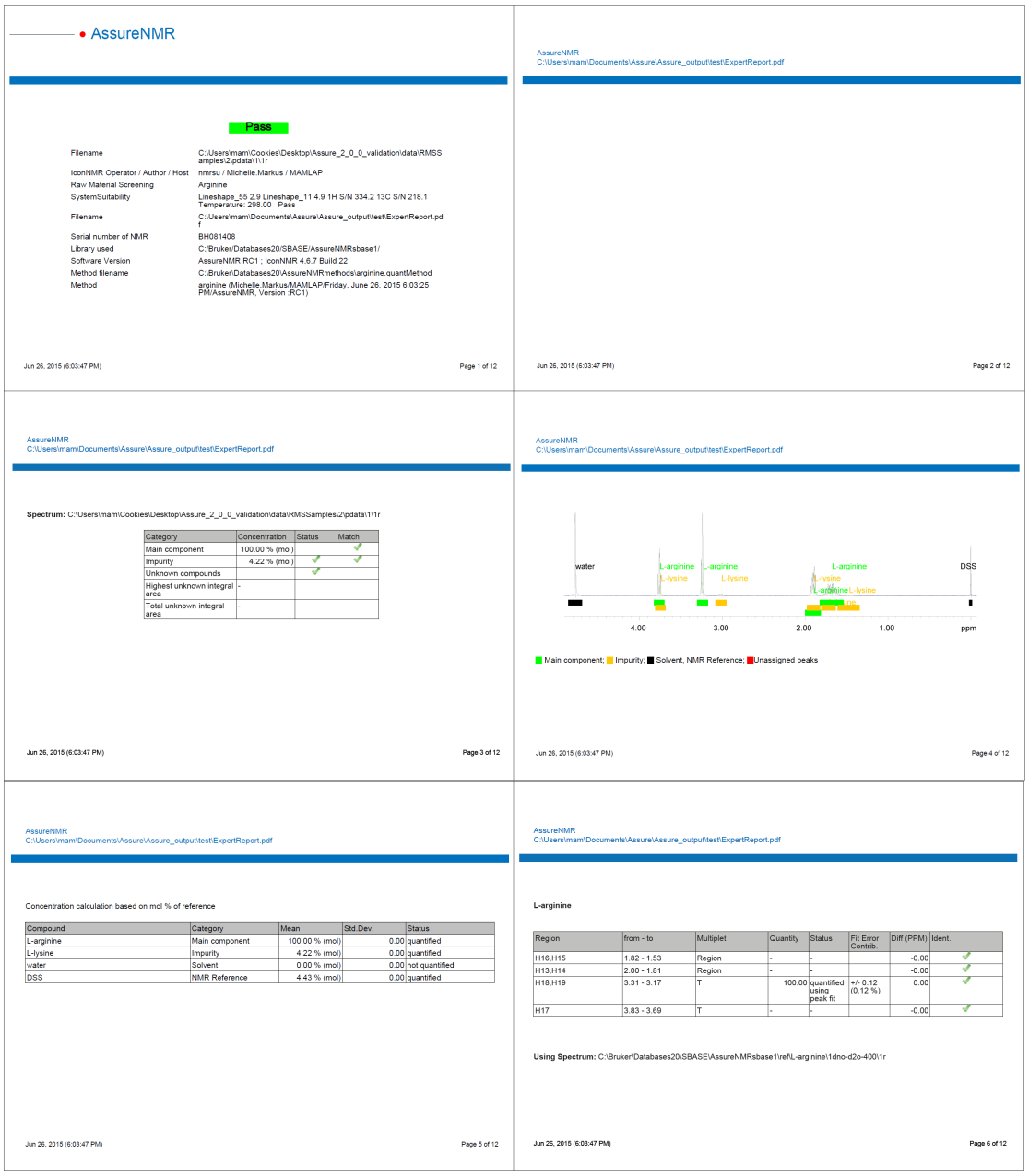
| | |
|---|---|
| Filename | C:\Users\mam\Documents\Assure\Assure_output\test\QCReport.pdf |
| Serial number of NMR | BH081408 |
| Library used | C:/Bruker/Databases20/SBASE/AssureNMRsbase1/ |
| Software Version | AssureNMR RC1 ; IconNMR 4.6.7 Build 22 |
| Method filename | C:\Bruker\Databases20\AssureNMRmethods\arginine.quantMethod |
| Method | arginine (Michelle.Markus/MAMLAP/Friday, June 26, 2015 6:03:25 PM/AssureNMR, Version :RC1) |

**Analyst Signature** **Date** **Review Signature** **Date**

Jun 26, 2015 (6:03:47 PM)

Page 1 of 1

## Expert Report:



AssureNMR

**Pass**

| | |
|---|---|
| Filename | C:\Users\mam\Cookies\Desktop\Assure_2_0_0_validation\data\RMSSamples\2\pdata\1\1r |
| IconNMR Operator / Author / Host | nmrsu / Michelle.Markus / MAMLAP |
| Raw Material Screening | Arginine |
| SystemSuitability | Lineshape_55.2.9 Lineshape_11 4.9 1H S/N 334.2 13C S/N 218.1 Temperature: 298.00 Pass |
| Filename | C:\Users\mam\Documents\Assure\Assure_output\test\ExpertReport.pdf |
| Serial number of NMR | BH081408 |
| Library used | C:/Bruker/Databases20/SBASE/AssureNMRsbase1/ |
| Software Version | AssureNMR RC1 ; IconNMR 4.6.7 Build 22 |
| Method filename | C:/Bruker/Databases20/AssureNMRmethods/arginine.quantMethod |
| Method | arginine (Michelle.Markus/MAMLAP/Friday, June 26, 2015 6:03:25 PM/AssureNMR, Version :RC1) |

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AssureNMR
C:\Users\mam\Documents\Assure\Assure_output\test\ExpertReport.pdf

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AssureNMR
C:\Users\mam\Documents\Assure\Assure_output\test\ExpertReport.pdf

**Spectrum:** C:\Users\mam\Cookies\Desktop\Assure_2_0_0_validation\data\RMSSamples\2\pdata\1\1r

| Category | Concentration | Status | Match |
|---|---|---|---|
| Main component | 100.00 % (mol) | | ✓ |
| Impurity | 4.22 % (mol) | ✓ | ✓ |
| Unknown compounds | | ✓ | |
| Highest unknown integral area | - | | |
| Total unknown integral area | - | | |

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AssureNMR
C:\Users\mam\Documents\Assure\Assure_output\test\ExpertReport.pdf




Main component; Impurity; Solvent, NMR Reference; Unassigned peaks

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AssureNMR
C:\Users\mam\Documents\Assure\Assure_output\test\ExpertReport.pdf

Concentration calculation based on mol % of reference

| Compound | Category | Mean | Std.Dev. | Status |
|---|---|---|---|---|
| L-arginine | Main component | 100.00 % (mol) | 0.00 | quantified |
| L-lysine | Impurity | 4.22 % (mol) | 0.00 | quantified |
| water | Solvent | 0.00 % (mol) | 0.00 | not quantified |
| DSS | NMR Reference | 4.43 % (mol) | 0.00 | quantified |

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AssureNMR
C:\Users\mam\Documents\Assure\Assure_output\test\ExpertReport.pdf

**L-arginine**

| Region | from - to | Multiplet | Quantity | Status | Fit Error Contrib. | Diff (PPM) | Ident. |
|---|---|---|---|---|---|---|---|
| H16,H15 | 1.82 - 1.53 | Region | - | - | | -0.00 | ✓ |
| H13,H14 | 2.00 - 1.81 | Region | - | - | | -0.00 | ✓ |
| H18,H19 | 3.31 - 3.17 | T | 100.00 | quantified using peak fit | +/- 0.12 (0.12 %) | 0.00 | ✓ |
| H17 | 3.83 - 3.69 | T | - | - | | -0.00 | ✓ |

**Using Spectrum:** C:\Bruker\Databases20\SBASE\AssureNMRsbase1\ref\L-arginine\1dno-d2o-400\1r

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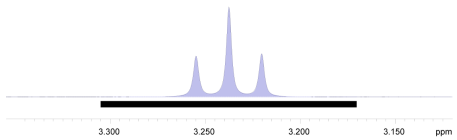



Observed: black line; Integration: blue; Search region: black bar





**L-lysine**

| Region | from - to | Multiplet | Quantity | Status | Fit Error Contrib. | Diff (PPM) | Ident. |
|---|---|---|---|---|---|---|---|
| H11,H12 | 1.61 - 1.34 | Region | - | - | | 0.00 | ✓ |
| H15,H16 | 1.80 - 1.63 | Region | - | - | | 0.00 | ✓ |
| H13,H14 | 1.98 - 1.82 | Region | - | - | | 0.01 | ✓ |
| H18,H19 | 3.08 - 2.95 | M(user def.) | 4.22 | quantified using peak fit | +/- 0.07 (1.56 %) | 0.00 | ✓ |
| H17 | 3.81 - 3.68 | T | - | not identified, but minimum intensity | | 0.01 | ✓ |

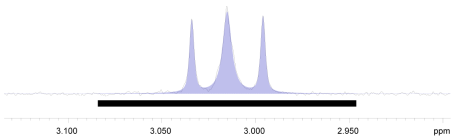
**Using Spectrum:** C:\Bruker\Databases20\SBASE\AssureNMRsbase1\ref\L-lysine\1dno-d2o-400\1r












Observed: black line; Integration: blue; Search region: black bar



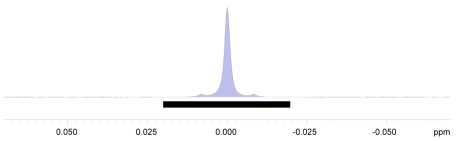


**DSS**

| Region | from - to | Multiplet | Quantity | Status | Fit Error Contrib. |
|---|---|---|---|---|---|
| methyls | 0.02 - -0.02 | Region | 4.43 | quantified using region integration | < 0.1 % |







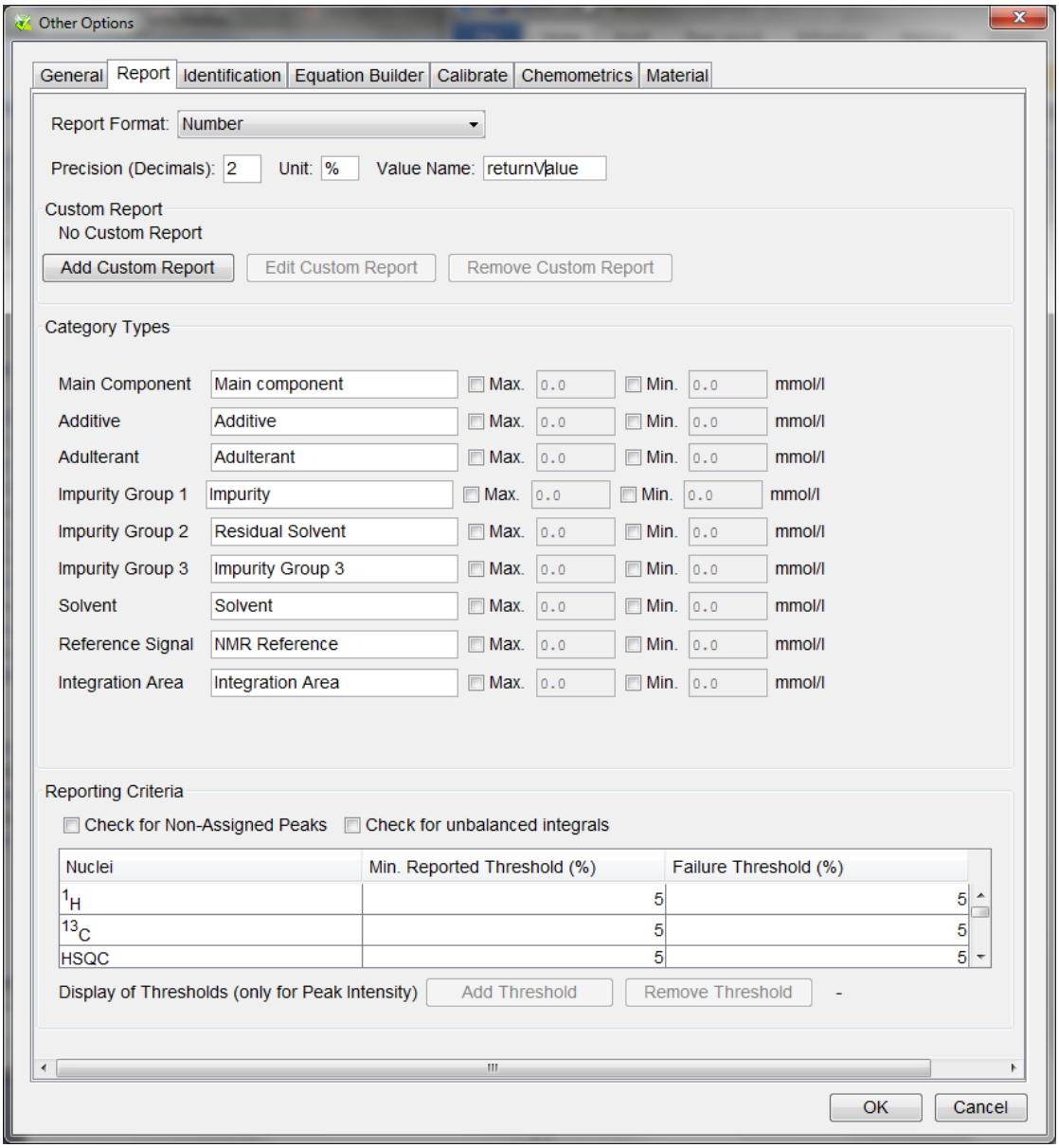

Observed: black line; Integration: blue; Search region: black bar

## 10.3 Equation Builder

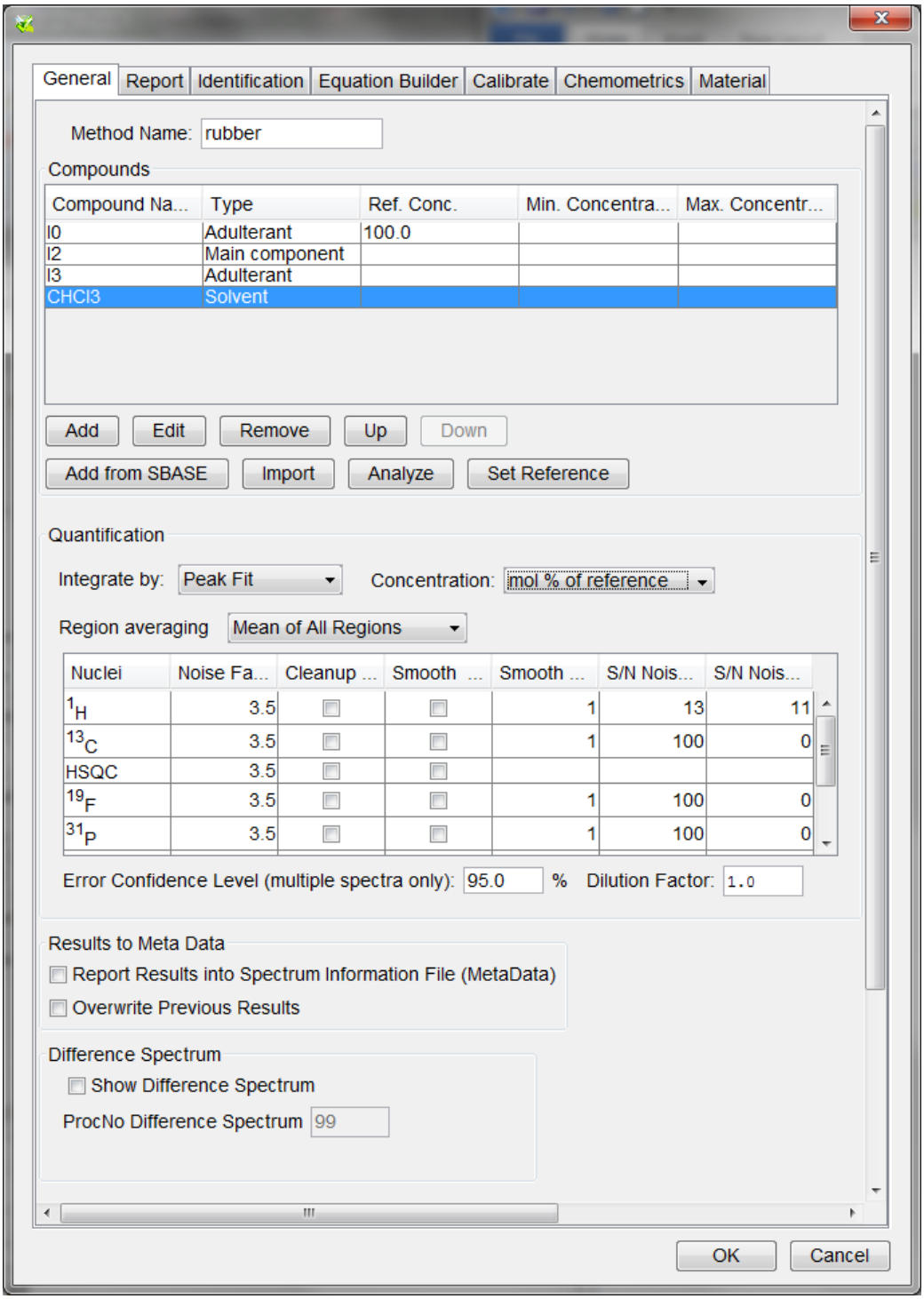
This section works through an example using the equation builder. The method reflects the screening of tire rubber set by ISO 21461. It determines the aromaticity of oil in rubber components according to the following equation:

$$\%H_{Bay} = \frac{I_{8.3-9.5\,ppm}}{\left(I_{6.0-9.5\,ppm} - I_{CHCl3}\right) + I_{0.2-5.8\,ppm}} \times 100$$

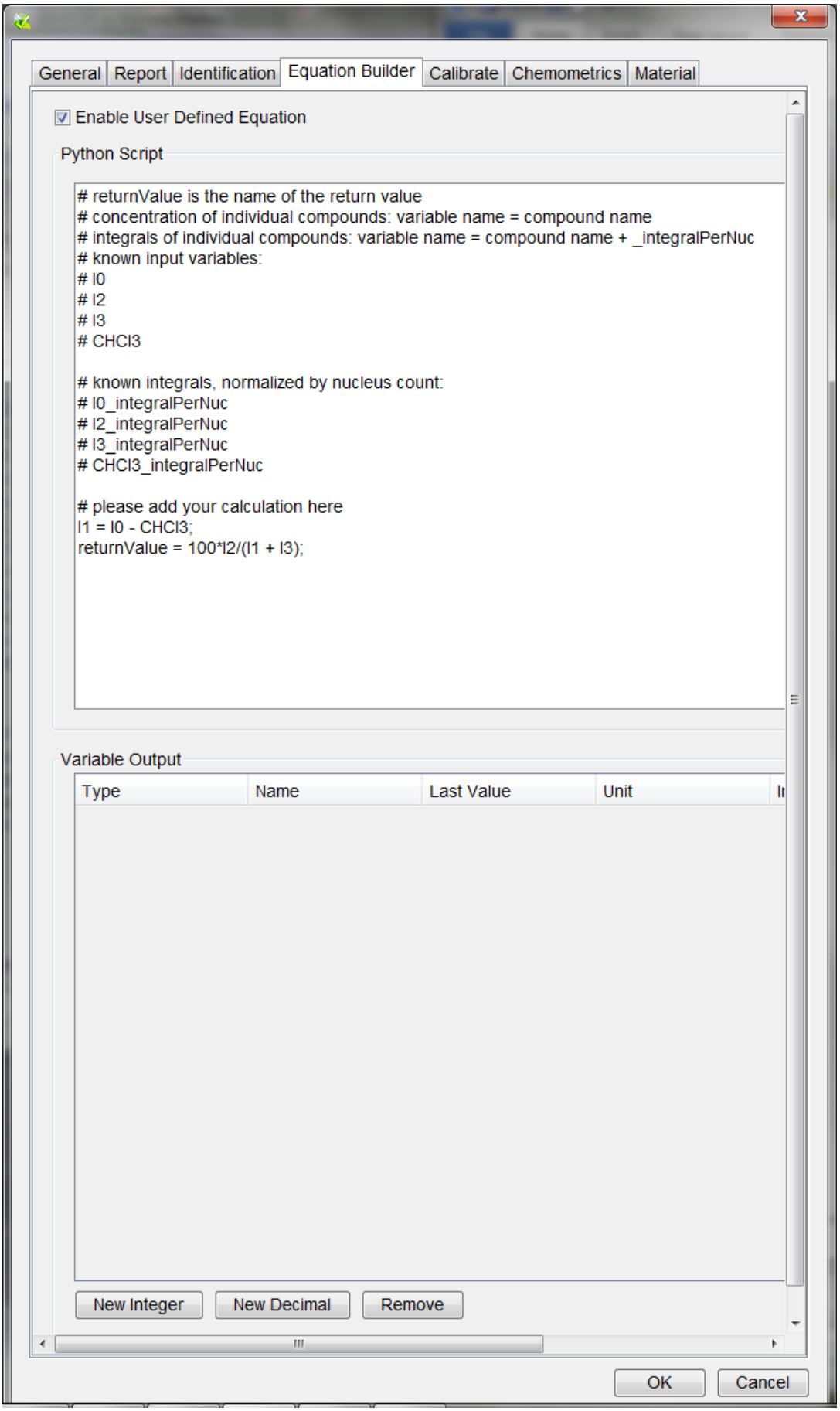
- Open a 1D $^{1}H$ spectrum in the viewer window.
- Select **New Method** from the Quantify pulldown menu. Click ‘Continue without compounds’ then ‘Standard’ to get into an empty method.
- Go into the text and tab editing mode, either by clicking ‘Details’ under the general heading or by selecting the  icon.
- To report the value from the user calculation, go to the Report tab and select the ‘Report Format’ **Number** and set the ‘Value Name’ to “returnValue”.




- Under the **General** tab, set up the compounds. Four compounds are defined:
  I0: Count 1, from-to 9.5-6.0 ppm, Lineshape: Region, check ‘Quantify’
  I2: Count 1, from-to 9.5-8.3 ppm, Lineshape: Region, check ‘Quantify’
  I3: Count 1, from-to 5.8-0.2 ppm, Lineshape: Region, check ‘Quantify’
  CHCl3: Count 1, from-to Singlet at 7.2 ppm, Lineshape: Singlet, check ‘Quantify’




- Go to the **Equation Builder** tab. The user calculation is defined as a Python script. The integrals of all defined compounds are available by their compound name. “returnValue” is displayed on the reports. Enter the equation as seen in the window below.


General
Report
Identification
Equation Builder
Calibrate
Chemometrics
Material
Enable User Defined Equation
Python Script
# returnValue is the name of the return value
# concentration of individual compounds: variable name = compound name
# integrals of individual compounds: variable name = compound name + _integralPerNuc
# known input variables:
# I0
# I2
# I3
# CHCl3
# known integrals, normalized by nucleus count:
# I0_integralPerNuc
# I2_integralPerNuc
# I3_integralPerNuc
# CHCl3_integralPerNuc
# please add your calculation here
I1 = I0 - CHCl3;
returnValue = 100*I2/(I1 + I3);
Variable Output
Type
Name
Last Value
Unit
New Integer
New Decimal
Remove
OK
Cancel

- Click ‘OK’ to exit tab and table editing.
- Use the  (Save As) icon to save the method.
- Use the  (Return) icon to exit.

## 10.4 csv File for Batch Submission

Below is the image of a csv file for batch submission of samples. Notice, the first line MUST have some character and cannot contain information for a sample.

```
,
21, "extract1", 11, "DMSO", "ASSURE_1H", "blueberry_leaf", 1, "Billerica"
22, "extract2", 11, "DMSO", "ASSURE_1H", "blueberry_leaf", 2, "Billerica"
23, "extract3", 11, "DMSO", "ASSURE_1H", "blueberry_leaf", 3, "Billerica"
24, "extract4", 11, "DMSO", "ASSURE_1H", "blueberry_leaf", 4, "Billerica"
25, "extract5", 11, "DMSO", "ASSURE_1H", "blueberry_leaf", 5, "Billerica"
26, "extract6", 11, "DMSO", "ASSURE_1H", "blueberry_leaf", 6, "Billerica"
27, "extract7", 11, "DMSO", "ASSURE_1H", "blueberry_leaf", 7, "Billerica"
28, "extract8", 11, "DMSO", "ASSURE_1H", "blueberry_leaf", 8, "Billerica"
29, "extract9", 11, "DMSO", "ASSURE_1H", "blueberry_leaf", 9, "Billerica"
30, "extract10", 11, "DMSO", "ASSURE_1H", "blueberry_leaf", 10, "Billerica"
31, "extract11", 11, "DMSO", "ASSURE_1H", "blueberry_leaf", 11, "Billerica"
32, "extract12", 11, "DMSO", "ASSURE_1H", "blueberry_leaf", 12, "Billerica"
```

# 11 Methods Available From Bruker

Bruker has developed quantMethods for a variety of materials that we make available to customers. These are provided to the user as is. It is the user's responsibility to validate that these methods perform as expected in their hands and meet their requirements on their instrument. Some of the methods (poloxomer, tire rubber, molar substitution, cell culture media) are generally available in the standard starting set of AssureNMR methods and can be modified for site specific needs. A software license is not needed for these methods. Other methods (Heparin and Aloe vera) require a purchased license (see your sales representitive). The licensed methods are also customizable to meet site specific needs. Example spectra, methods, example reports and additional infomation may be found in the Example Data Sets. These quantMethods are described in the following sections.

## 11.1 Aloe vera quantMethod

The *Aloe vera* method (Aloe_vera.quantMethod) is based on the published method of Jiao *et al.* (*J. of the AOAC International*, **93**:842-848, 2010). Concentrations (in g/L and % weight) for major components including glucose, malic acid, acetic acid, lactic acid, and acetylated polysaccharides are reported. Concentrations are also reported for possible additives including glycerol and diethylene glycol and preservatives such as potassium sorbate and benzoic acid. Samples should be prepared as described in the reference (40 mg Aloe vera powder to 1 mL solvent). The method is flexible enough to analyze samples prepared in $^{2}H_2O$ or buffer (150 mM $KH_2PO_4$, pH 7.4, 0.2 mM $NaN_3$, 0.01% TSP). The user must provide their own standard to calibrate the concentration measurements. This AssureNMR method requires a purchased license.

## 11.2 Heparin quantMethod

The heparin method (HeparinUSP11.quantMethod) is based on the USP monograph for heparin sodium (*Pharmacopeial Forum*, **35**(5):1-7, 2009). Heparin is identified based on four distinct signals called out in the monograph. Specified regions are searched for contaminants above thresholds specified as a percentage of the average intensity of signals 1 and 2. Residual solvents including acetic acid, methanol, ethanol, and methyl isobutyl ketone are accounted for. Oversulfated chondroitin sulfate (OSCS) fails the test on signals above 4% in range 2 specified in the monograph. Samples should be prepared as specified in the USP monograph. This AssureNMR method requires a purchased license.

## 11.3 Molar Substitution quantMethod

The molar substitution method (MolarSubstitution.quantmethod) is based on the European Pharmacopoeia monograph for hydroxypropylbetadex (European Pharmacopoeia 5.0, entry 01/2005:1804). The quantMethod follows the monograph's method for determining the number of hydroxypropyl groups per anhydroglucose units, expressed as molar substitution. The molar substitution is calculated from the ratio of the signal from the methyl group in the hydroxypropyl unit and the signal from the glycosidic proton of the anhydrogluycose units. Samples should be prepared as specified in the monograph. An example 1H spectrum for cyclodextrin is available in the Example Data Sets. This AssureNMR method is unlicensed.

## 11.4 Poloxamer quantMethod

The poloxamer method (poloxamer.quantMethod) is based on the European Pharmacopoeia monograph for poloxamers (European Pharmacopoeia 6.0, entry 01/2008:1464). The method determines the oxypropylene: oxyethylene ratio, based on characteristic signals in the spectrum. Samples should be prepared as specified in the monograph for this application to poloxamer. Alternatively, this method may be used as a starting point for samples requiring region analysis and calculations to be performed based on integrals of the regions. The calculations performed can be found in the AssureNMR method in the Equation Builder tab. An example 1H spectrum for poloxamer is available in the Example Data Sets. This AssureNMR method is unlicensed.

## 11.5 Tire Rubber quantMethod

The method for tire rubber (rubber.quantMethod) is based on the international standard for rubber (Rubber – determination of the aromaticity of oil in vulcanized rubber compounds, ISO 21461, first edition 2006-04-01). Based on a 1D $^{1}$H spectrum, areas are calculated for various regions of the spectrum. The aromatic area is corrected for any chloroform signals that might be present. Then the percentage of bay regions hydrogens is calculated. This provides an indication of the aromatic character of the sample. Samples should be prepared as described in the ISO document. An example spectrum may be found in the Example Data Sets. This AssureNMR method is unlicensed.

## 11.6 Cell Culture Media

The method for Cell Culture Media (DMEMB_500MHz_advanced.quantMethod) is based Dulbecco's Modified Eagle Medium (DMEM) and provides for a targeted analysis of more than 30 components. Based on a 1D 1H spectrum at 500MHz from media dissolved in pH 7 phosphate buffer in D2O, compounds were selected from the BBIOREFCODE spectral database at pH 7 and 600MHz. The corresponding 1H spectrum included in the example set was acquired using a cpmgpr1d pulse sequence. This method was generated using the advanced quantification algorthm on DMEM. This method may serves as a starting point for culture media methods. This AssureNMR method is unlicensed.

# 12 Definitions

ANOVA – Analysis of variance

BIRT – Business Intelligence and Reporting Tools

GLP – Good Laboratory Practice

GMP – Good Manufacturing Practice

GxP – Stands for Good X Practice where X can mean laboratory, manufacturing,

pharmaceutical, etc.

ICH – International Conference on Harmonisation (of Technical Requirements for Registration of Pharmaceuticals for Human Use)

Knowledgebase – composite library of compounds which contains detailed molecular structure information such as coupling constants, multiplicity, and atom count

LIMS – Laboratory information management system

LOD – Limit of detection

LOQ – Limit of quantification

NMR – Nuclear magnetic resonance

PCA - Principal component analysis

PLS regression – Partial least squares regression - a statistical method to analyze or predict a set of dependent variables (Y-variables) from a set of independent variables or predictors (X-variables)

QA – Quality Analysis

QC – Quality Control

quantMethod – method which contains details of how the spectrum or spectra will be evaluated including limits for defining failure, quantification method, peak identification and limits, and SBASE to be matched

SBASE – Spectral database of purified compounds to be used by the match algorithm for identification of compounds in screening mode.

SIMCA - Soft independent modeling of class analogies – SIMCA outlier detection is a statistical method that develops a classification model based on a set of reference data. This model can be used to detect whether test data (an NMR spectrum) is in the respective class model (similar to the others) or outside (an outlier).

SOP – Standard operating procedure

SST - System Suitability Test – A set of up to six experiments run periodically to evaluate the continued performance of the instrument.

# Definitions

# 13 Assure-SST Reference Standards

| 300 MHz / 5mm Room Temperature | |
|---|---|
| **Description** | **Part No.** |
| Lineshape - 3% Chloroform | Z10230 |
| 1H Sensitivity - 0.1% Ethylbenzene, 40mm filling | Z10901 |
| 13C Sensitivity - 10% Ethylbenzene | Z10153 |
| 19F Sensitivity – 0.05% Trifluorotoluene | Z10234 |
| 31P Sensitivity – 0.0485 M Triphenylphosphate | Z10201 |
| NMR Thermometer 99.8% Methanol-d4 | Z10627 |
| | |
| **400 – 900 MHz / 5mm Room Temperature** | |
| **Description** | **Part No.** |
| Lineshape - 1% Chloroform | Z10248 |
| 1H Sensitivity - 0.1% Ethylbenzene, 40mm filling | Z10901 |
| 13C Sensitivity - 10% Ethylbenzene | Z10153 |
| 19F Sensitivity – 0.05% Trifluorotoluene | Z10234 |
| 31P Sensitivity – 0.0485 M Triphenylphosphate | Z10201 |
| NMR Thermometer 99.8% Methanol-d4 | Z10627 |
| | |
| **400 – 900 MHz / 5mm CryoProbe** | |
| **Description** | **Part No.** |
| Lineshape - 0.3% Chloroform, 40mm filling | Z10903 |
| 1H Sensitivity - 0.1% Ethylbenzene, 40mm filling | Z10901 |
| 13C Sensitivity - 10% Ethylbenzene | Z10153 |
| 19F Sensitivity – 0.05% Trifluorotoluene | Z10234 |
| 31P Sensitivity – 0.0485 M Triphenylphosphate | Z10201 |
| NMR Thermometer 99.8% Methanol-d4 | Z10627 |
| | |
| **600 – 900 MHz / 1 mm Room Temperature** | |
| **Description** | **Part No.** |
| Lineshape - 3% Chloroform | Z100926 |
| 1H Sensitivity - 0.1% Ethylbenzene | Z100927 |
| 13C Sensitivity - 10% Ethylbenzene | Z100928 |
| 31P Sensitivity – 0.0485 M Triphenylphosphate | Z100934 |

| | |
|---|---|
| NMR Thermometer 4% Methanol | Z100935 |
| | |
| **600 – 900 MHz / 1.7mm CryoProbe** | |
| **Description** | **Part No.** |
| Lineshape - 1% Chloroform | Z10717 |
| 1H Sensitivity - 0.1% Ethylbenzene | Z10718 |
| 13C Sensitivity - 10% Ethylbenzene | Z10723 |
| 19F Sensitivity – 0.05% Trifluorotoluene | Z10728 |
| 31P Sensitivity – 0.0485 M Triphenylphosphate | Z10722 |
| NMR Thermometer 99.8% Methanol-d4 | Z10734 |

# 14 Tutorials

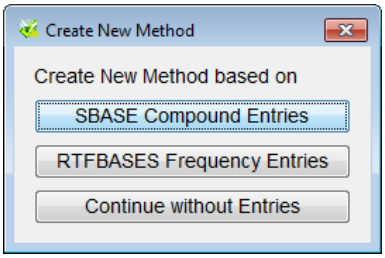
Tutorials are included to assist your effective use of AssureNMR. We aim to keep these up-to-date with new versions of software. If you find an inconsistency between a tutorial in this manual and with this AssureNMR software version please report it to:

*https://www.bruker.com/service/information-communication/helpdesk.html*

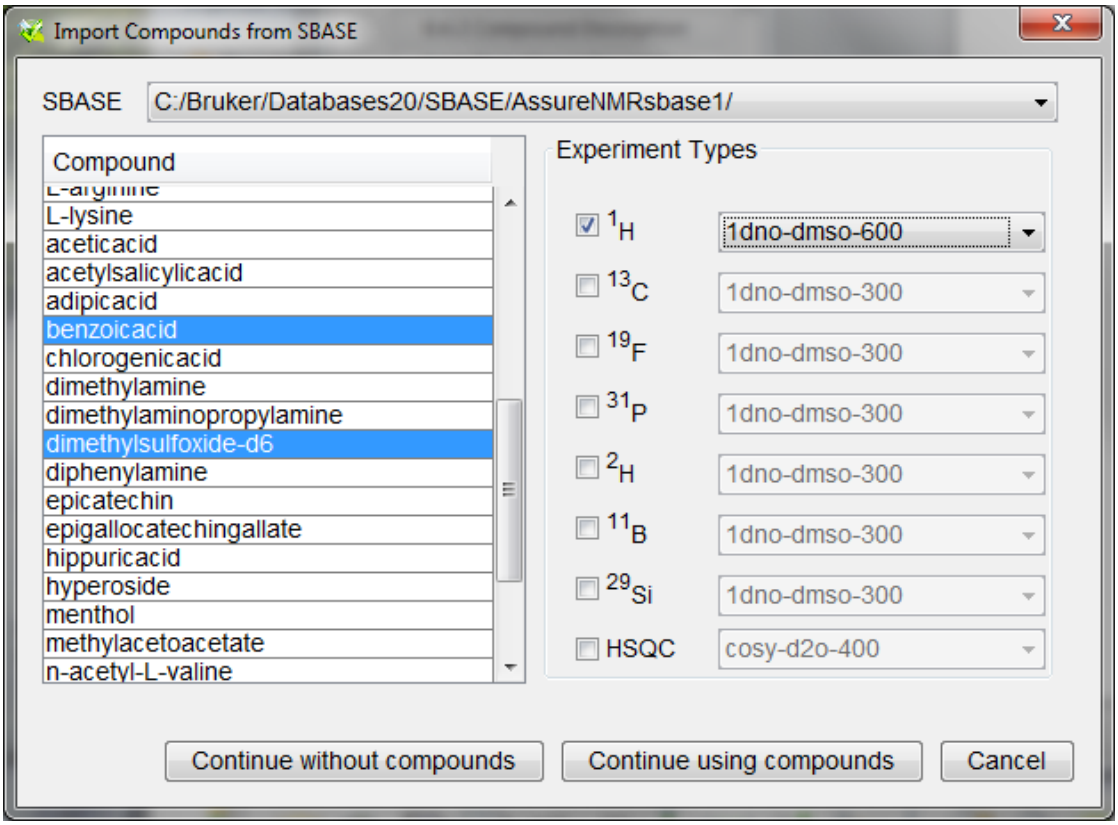
## 14.1 Making a quantMethod for Benzoic Acid

As described in this chapter, there are many important details for quantification. To get an idea of the actual flow of developing a quantMethod, this section goes through the steps to make a quantMethod for a compound in AssureNMRsbase1, benzoic acid.

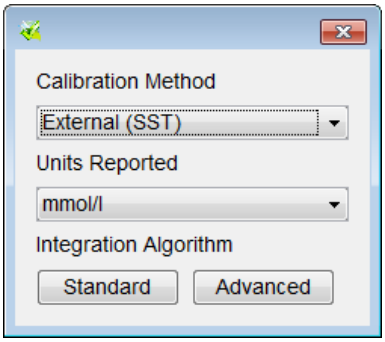
- Launch AssureNMR by clicking on  .
- Load a 1D spectrum. In the best case, this spectrum should be for a sample containing benzoic acid. (Use the **File** menu or the **System** browser on the left of the viewer window.)
- Go to the **Quantify** pulldown menu and select **New Method**.
- Select the origin of the SBASE compounds from the list:




- Select SBASE Compound Entries. This will automatically launch the ‘Import Compounds from SBASE’ window:

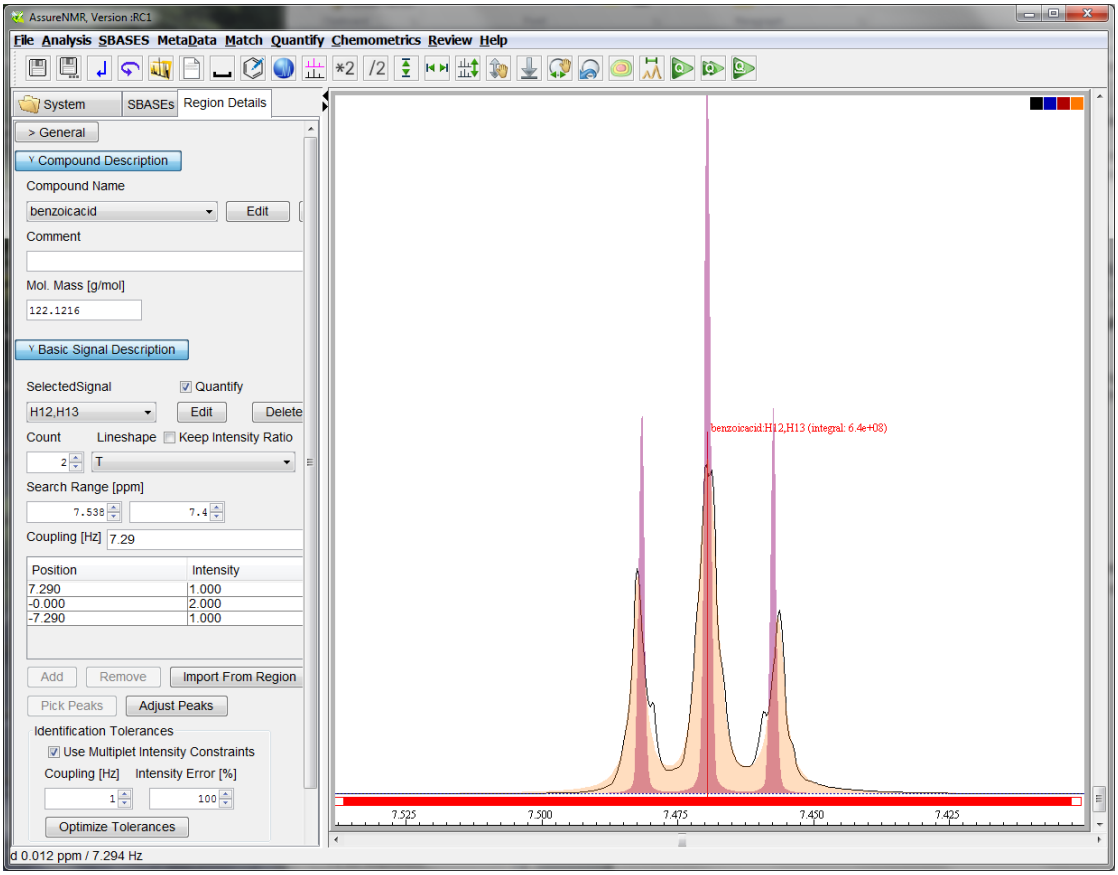



  - Set the SBASE to AssureNMRsbase1.
  - Select benzoicacid and dimethylsulfoxide-d6 from the Compound menu. Note: to select a second compound, use Ctrl-left click with mouse.
  - Make sure $^{1}H$ is checked under Experiment Types.
  - Select a suitable experiment at the appropriate field strength from the pull down menu.
- Select the button **Continue using compounds**.
- In the next window that pops up, select the calibration method and the concentration units to use and the integration algorithm. For this method we will select mol % for the units and the 'Standard' algorithm (notice with this unit, there is no need for calibration method, so it is grayed out.)




- The software goes into interactive editing mode, with parameters available through the window on the left and the information about the imported compounds displayed on the spectrum in the viewer window.
- Under the **Compound Description** heading, select Compound Name "benzoicacid".
- Under the **Basic Signal Description** heading, select SelectedSignal "H14". Adjust the red bar under the signal to just cover that signal. Left click in the spectrum viewer window and select **Zoom Out** - it may help you see the region. When you are ready, hover over the red bar, click, and **Test Region**. If the fitting works, go on to the next region.

- For H12,H13, set the lineshape to “T” for triplet. Right click in the window to bring up the menu and select **Measure Distances**. Set the Coupling [Hz] to the distance between the main triplet lines. Under Identification Tolerances, set the Coupling [Hz] to 1.0. Again, **Test Region**.




- For H10,H11, turn off quantification (uncheck “Quantify”) for this region.
- Under the **Compound Description** heading, select Compound Name “dimethylsulfoxide-d6”.
- Click the **Edit** button. Set the ‘Type’ for this compound to ‘Solvent’, then click **OK**.
- From the icons, select ▶ (Run Quantification). When the window pops up to ask if you want to create a report, click **Yes**. The one page QCreport and multipage ExpertReport should pop up in the pdf viewer. The analysis will also be displayed on the spectrum.
- Save the method to the desired filename by clicking the  icon.

Now you have a quantMethod!

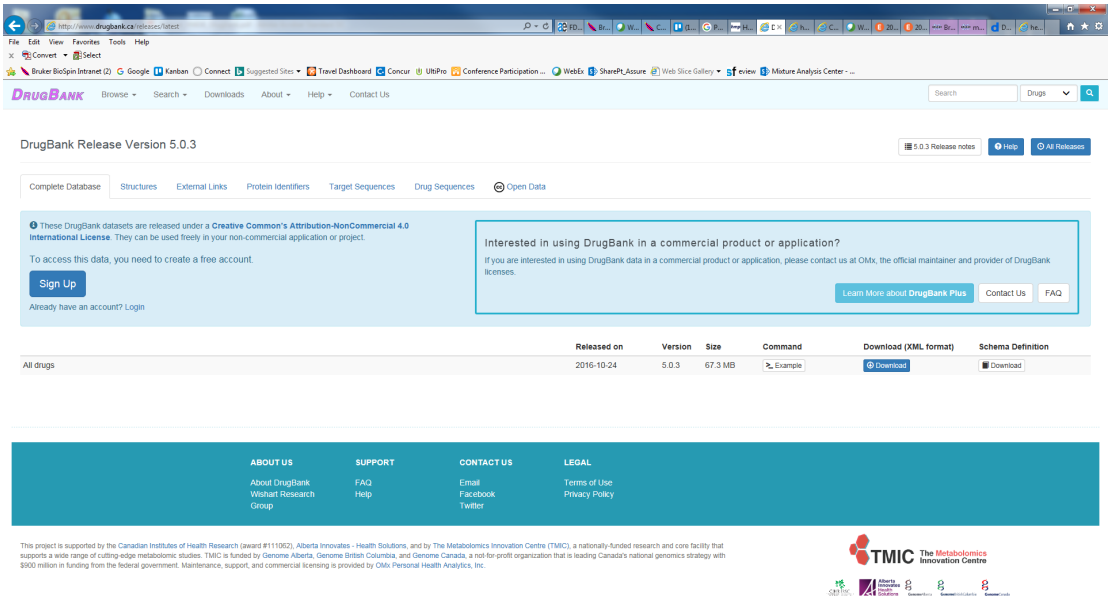
This example gives the flavor of method development. Start with information available for the components of interest – from the SBASE or previously analyzed spectra. Optimize the fitting for each peak of interest. You do not have to use all the peaks in the compound. Then save the method. Typically, there will be more compounds in a quantMethod. For example, the user may want to account for the water line and any material used as an NMR chemical shift reference (DSS, TSP, etc.). Once you have a method, you may want to specify a different concentration method or report format. The tools are there – explore.

## 14.2 HMDB and DrugBank Tutorial

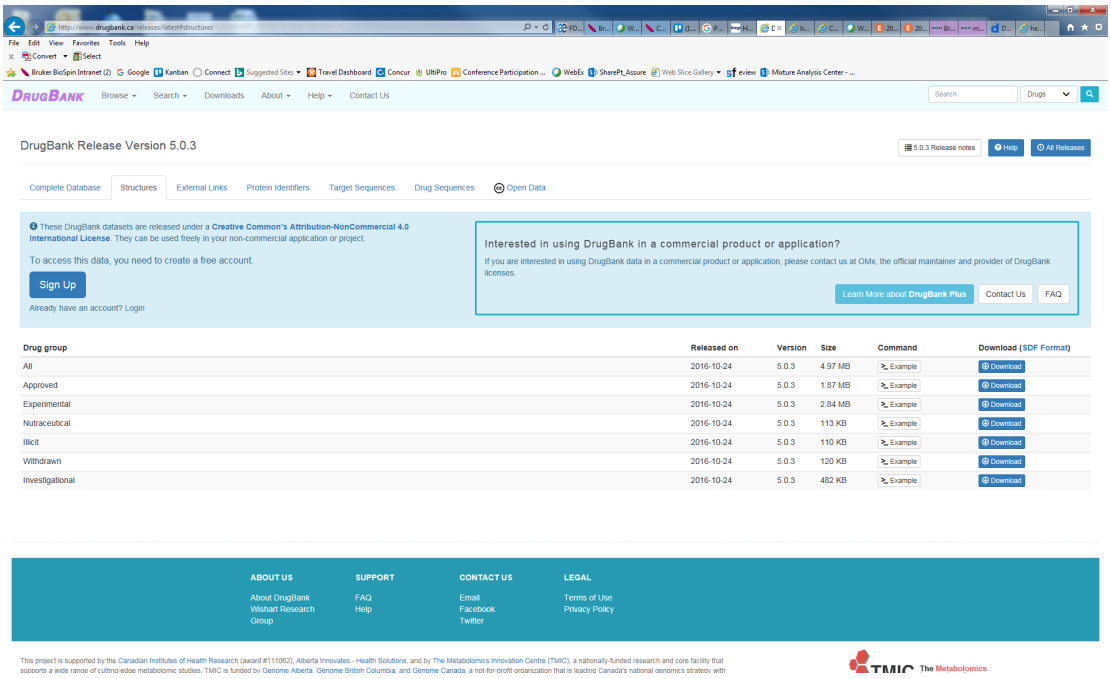
This tutorial demonstrates how to download data from HMDB and DrugBank and then import this data into AssureNMR to be used for searches against a NMR spectral database (SBASE). As of this writing, DrugBank was in the process of changes to their licensing which may change the method of importing and use of DrugBank in AssureNMR. Contact Bruker for updated instructions concerning DrugBank import if you have a DrugBank license and experience difficulties in importing it into AssureNMR.

**Step 1: Drugbank Download (skip this section if you want to import HMDB only)**

1. Open *www.drugbank.ca* in a web browser
2. Navigate to “Downloads”
3. Download: “Full Database”: all drugs

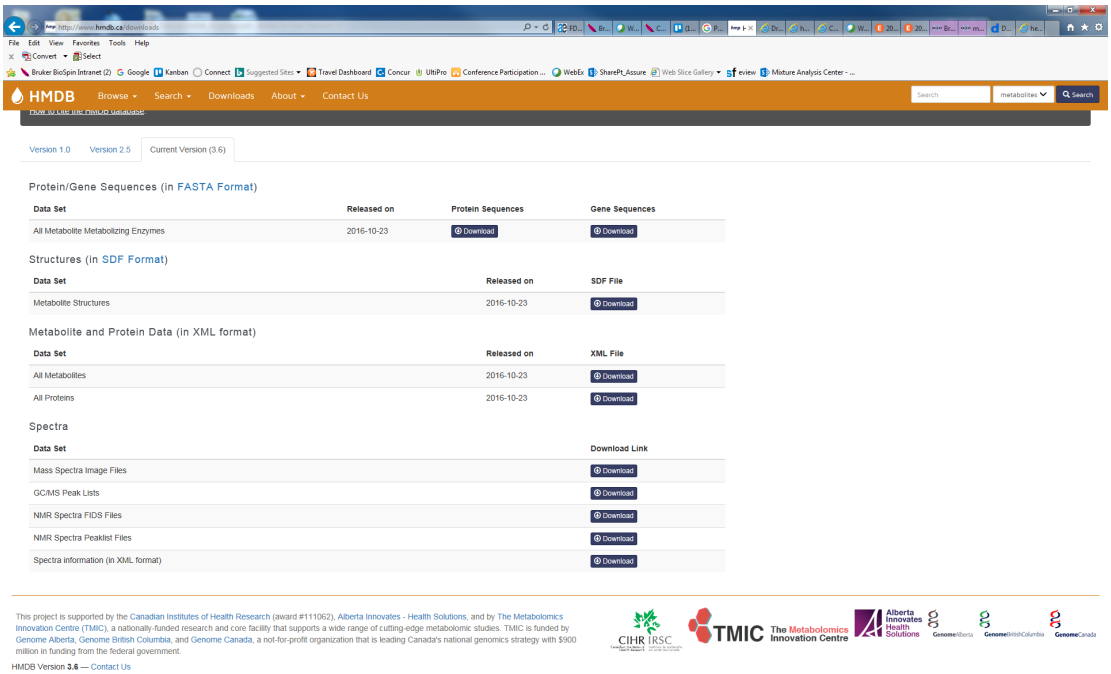



1. Click on Structures and download respective set of molecules, e.g. all Drugs




## Step 2: HMDB Download (skip this section if you want to import DrugBank only)

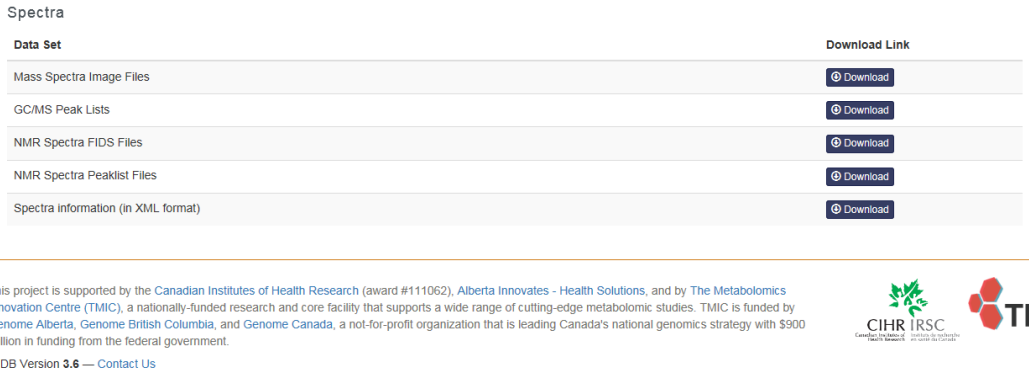
1. Open *www.hmdb.ca* in a web browser
2. Navigate to “Downloads”
3. Download the following items:
   - From the “Structures” section download “Metabolite Structures” and Extract this file (zip).
   - From the “Metabolite and Protein Data (in XML format)” section download “All Metabolites” and extract these into single folder




## Step 3: Download Spectral Information (HMDB + DrugBank)

Spectra information is available at www.hmdb.ca. If you already have downloaded these file for the HMDB import you can skip this step.

1. Navigate to “Downloads”
2. Download the following files
   - NMR Spectra FIDS Files
   - NMR Spectra Peaklist Files
   - Spectra information (in XML format)


Spectra

| Data Set | Download Link |
|---|---|
| Mass Spectra Image Files | Download |
| GC/MS Peak Lists | Download |
| NMR Spectra FIDS Files | Download |
| NMR Spectra Peaklist Files | Download |
| Spectra information (in XML format) | Download |

This project is supported by the Canadian Institutes of Health Research (award #111062), Alberta Innovates - Health Solutions, and by The Metabolomics Innovation Centre (TMIC), a nationally-funded research and core facility that supports a wide range of cutting-edge metabolomic studies. TMIC is funded by Genome Alberta, Genome British Columbia, and Genome Canada, a not-for-profit organization that is leading Canada's national genomics strategy with $900 million in funding from the federal government.

HMDB Version **3.6** — Contact Us

### Step 4: Unzip the downloaded zip files into separate directories

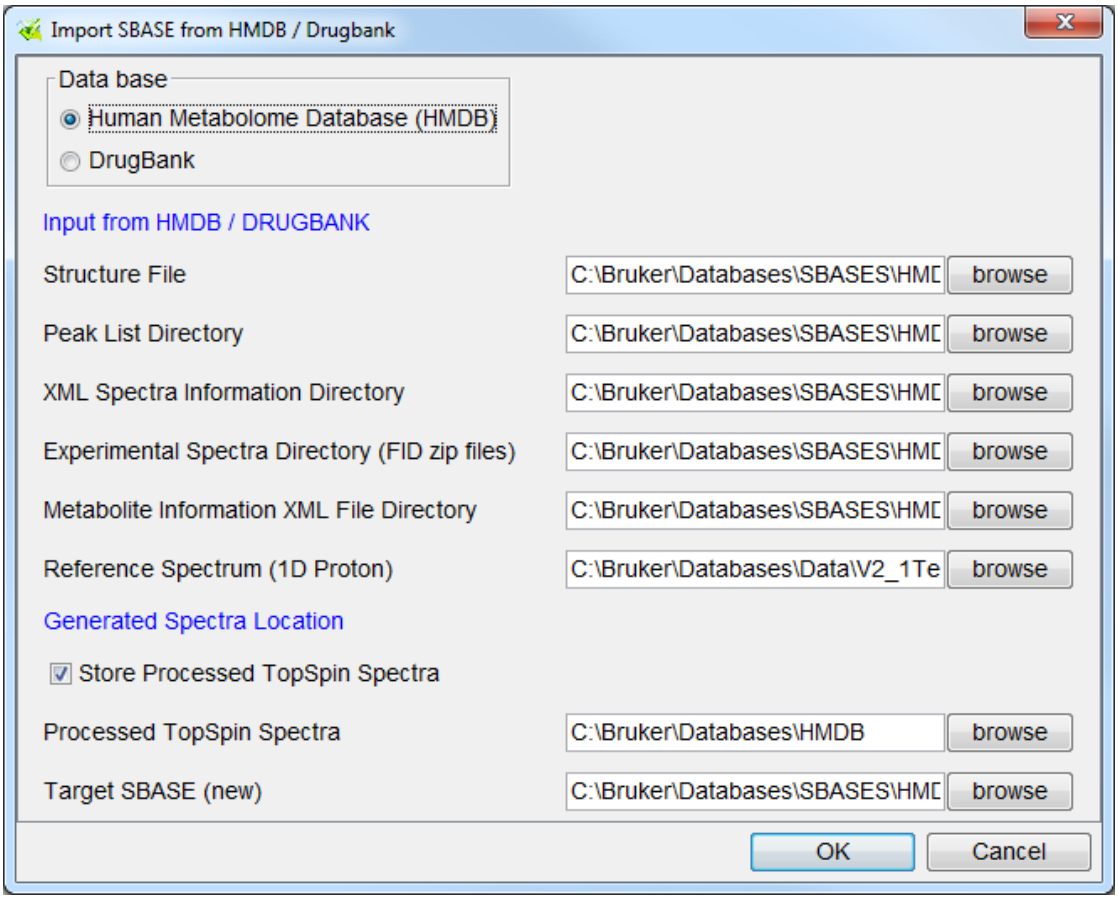
Note: Because some operating system will slow down if there are many files in one directory you should extract these zip files into separate directories. Please do not unzip all individual spectrum zip files from “NMR Spectra FID Files”, Assure will do it.

### Step 5: Import Data in AssureNMR

Open AssureNMR and perform the following steps:

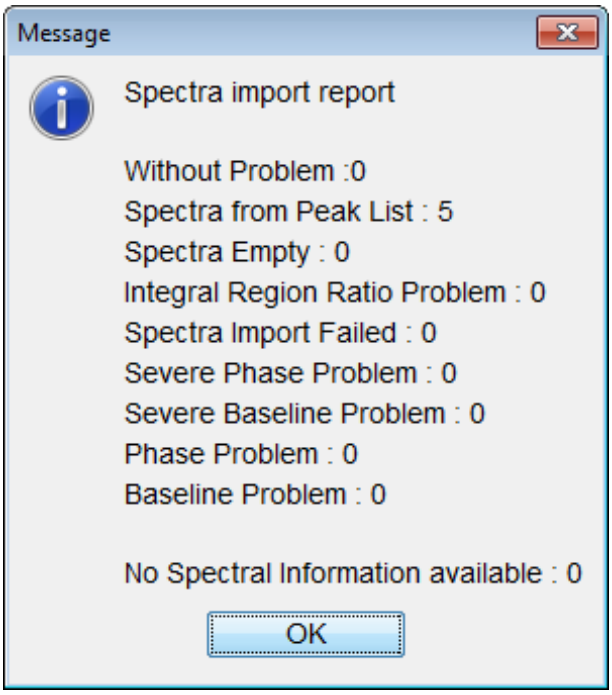
1. Open your latest installed TopSpin, it is required to process the data from HMDB / Drugbank.
2. In AssureNMR: SBASES->Import HMDB/Drugbank

Note: The program will note if the TopSpin version is not correct and indicate which version should be opened. **Import will take 4-12h.** The spectra are processed as part of the import.




Entries in the AssureNMR Import window include:

- Structure File: Single SDF file from HMDB or Drugbank
- Peak List Directory: downloaded “ hmdb_nmr_peak_lists”
- XML Spectra Information Directory: “hmdb_spectra_xml)”
- Experimental Spectra Directory: “hmdb_fid_files”
- Metabolite Information XML File Directory: “hmdb_metabolites””
- Reference Spectrum (1D Proton): Please select a typical proton 1D spectrum., It will be used by TopSpin when processing the HMDB fid’s. This can be any 1D proton spectrum (one of the exam1D_1H datasets would be OK if you have no other data on your compouter.) It is required because for some spectra only peak lists exist.
- You should store the processed TopSpin Spectra, in sum 4GB of data. Select an empty directory.
- Target SBASE(new): Click browse, navigate to your SBASE directory (default will be C: \Bruker\, then enter new name in entrybox. Click Open.
- Click OK. The copyright notice will pop up. You are obligated to obtain appropriate licenses from DrugBank/HMDB. Bruker assumes no responsiblity for improper use of data provided by DrugBank and HMDB. Click yes if you agree.
- Once finished (this could take many hours) a pdf and pop-up with the import statistics will be on the screen. Close them. Note that there should be more „Without Problems“ and „Spectra for Peak List“ than „Spectra Empty“ or „Spectra Import Failed.“ (See window below)




- To register this SBASE (required for use) please click „Yes“ in next pop-up.
- Navigate to the File pull-down menu and close the SBASE browser (if already open) and re-open it. Test that the spectra open.

## 14.3 PROFILE NMR

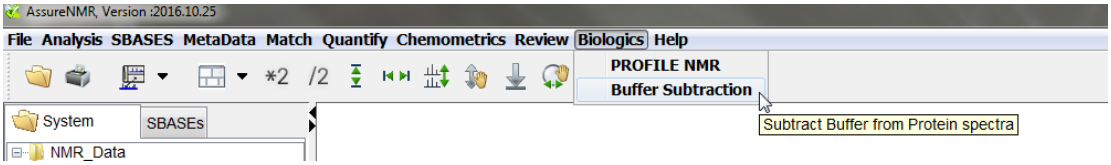
This short tutorial demonstrates how to use ProfileNMR for analyzing Biologics. Two commands are available:

- Buffer Subtraction
- ProfileNMR

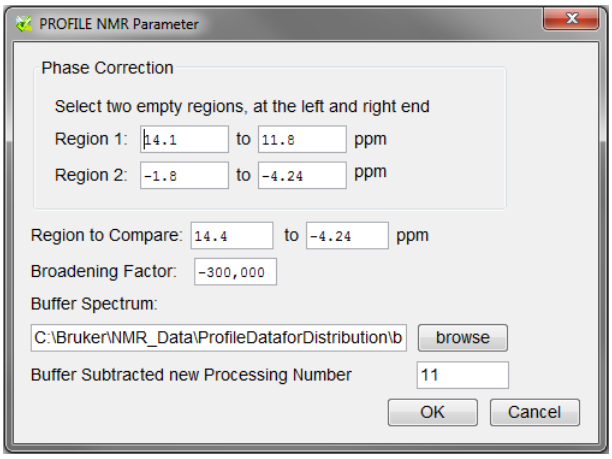
**Buffer Subtraction**

Buffer Subtraction allows one to subtract a buffer spectrum from a spectrum or series of spectra of a protein or other such sample. After subtraction, the resulting spectrum is stored using a new processing number.

The example data provided for this tutorial are grouped in a folder “buffdecon” and include a reference spectrum of the buffer and a single protein spectrum.




After selecting the “Buffer Subtraction” method from the tool bar as above, a dialogue box appears to set appropriate settings for the subtraction:




*Phase correction*

Requires two regions, left and right of your spectrum without signals. The regions shown here work for the example data

*Region to compare*

The region where your signals of interest occur.

*Broadening factor*

Value is used to compare spectra and calculate the fingerprint. Details are explained in the publications on ProfileNMR method.

*Buffer Spectrum*

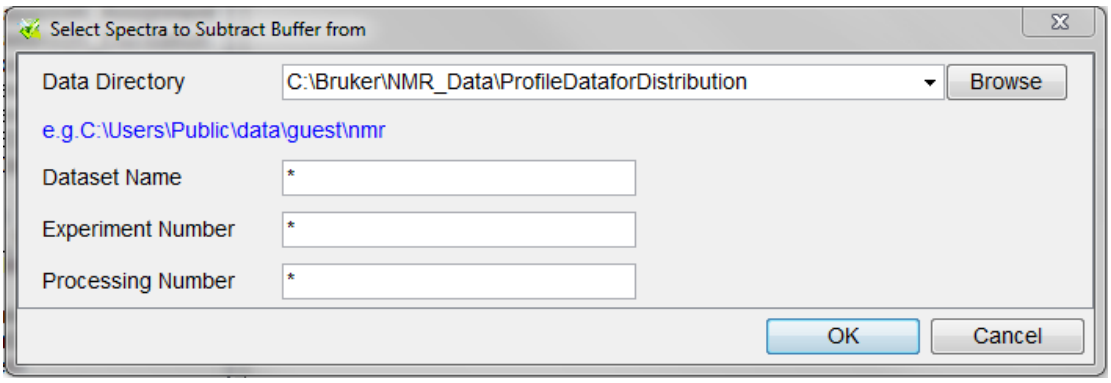
Use the browse button to locate the spectrum of the buffer reference. The standard AssureNMR file selection dialogue opens, and you can select the single spectrum which contains only the buffer signals to subtract out.

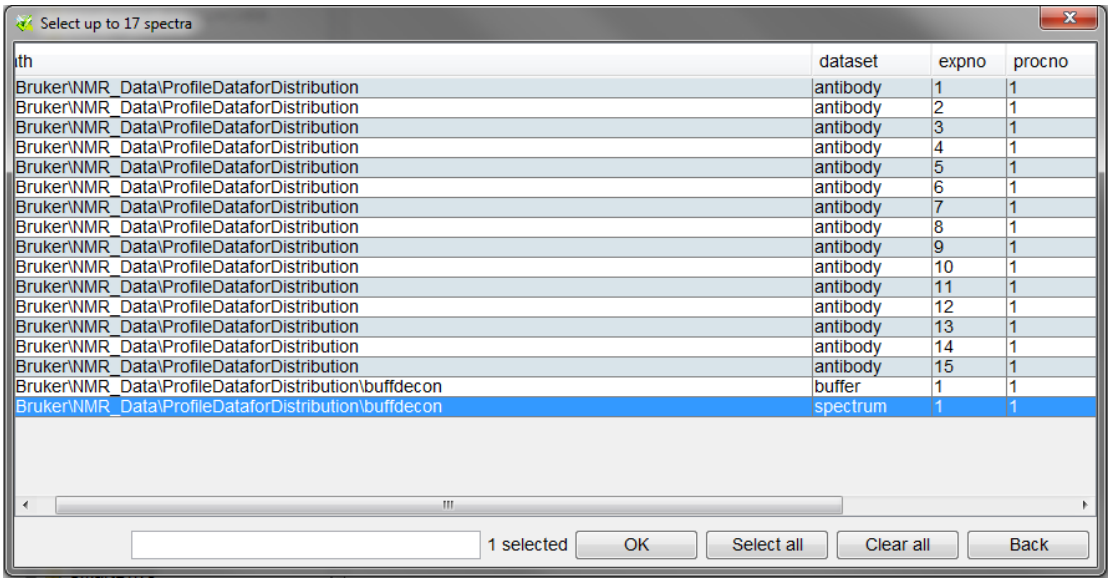
*Buffer Subtracted new Processing Number*

The new processing number (PROCNO) is the location where the subtracted spectrum is stored.

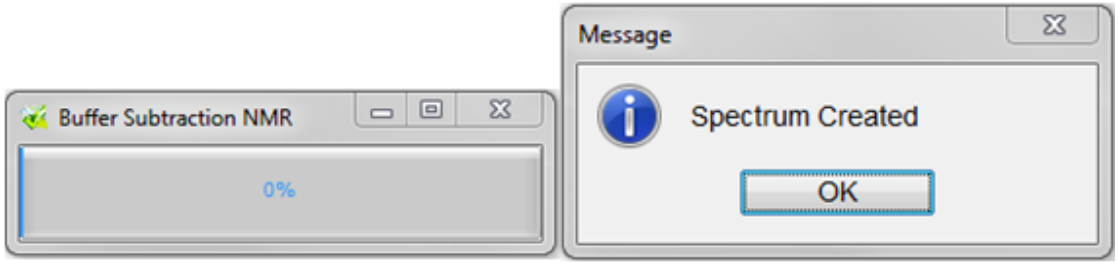
After closing the set-up dialoge with the “OK” button, an additional file selection dialogue opens.

From here you are able to select any number of spectra from which to subtract the buffer reference spectrum. In this case only the single protein spectrum of the provided example data was selected.



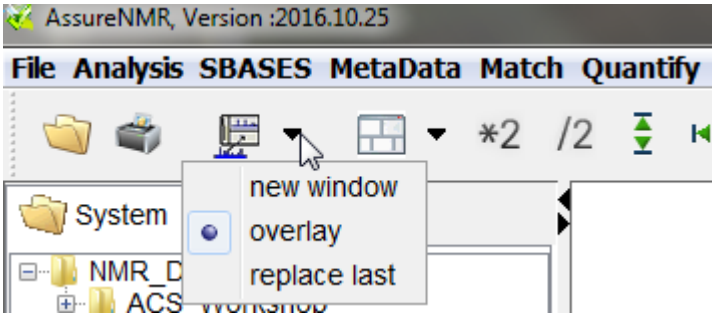



This calculation can take several minutes

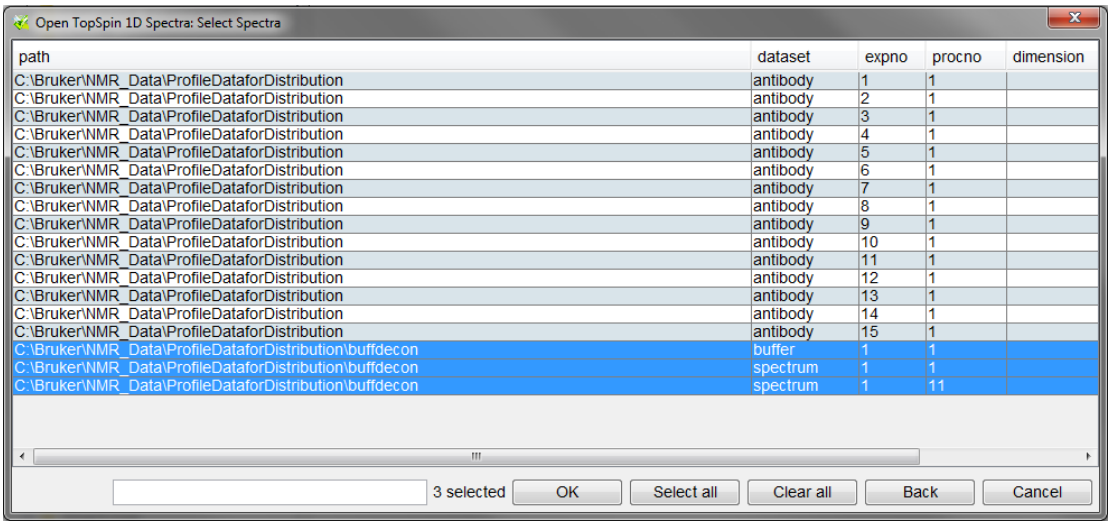



Once calculated, it is possible to compare the spectra using standard commands to display spectra.

It is important that the display mode be set to “overlay”

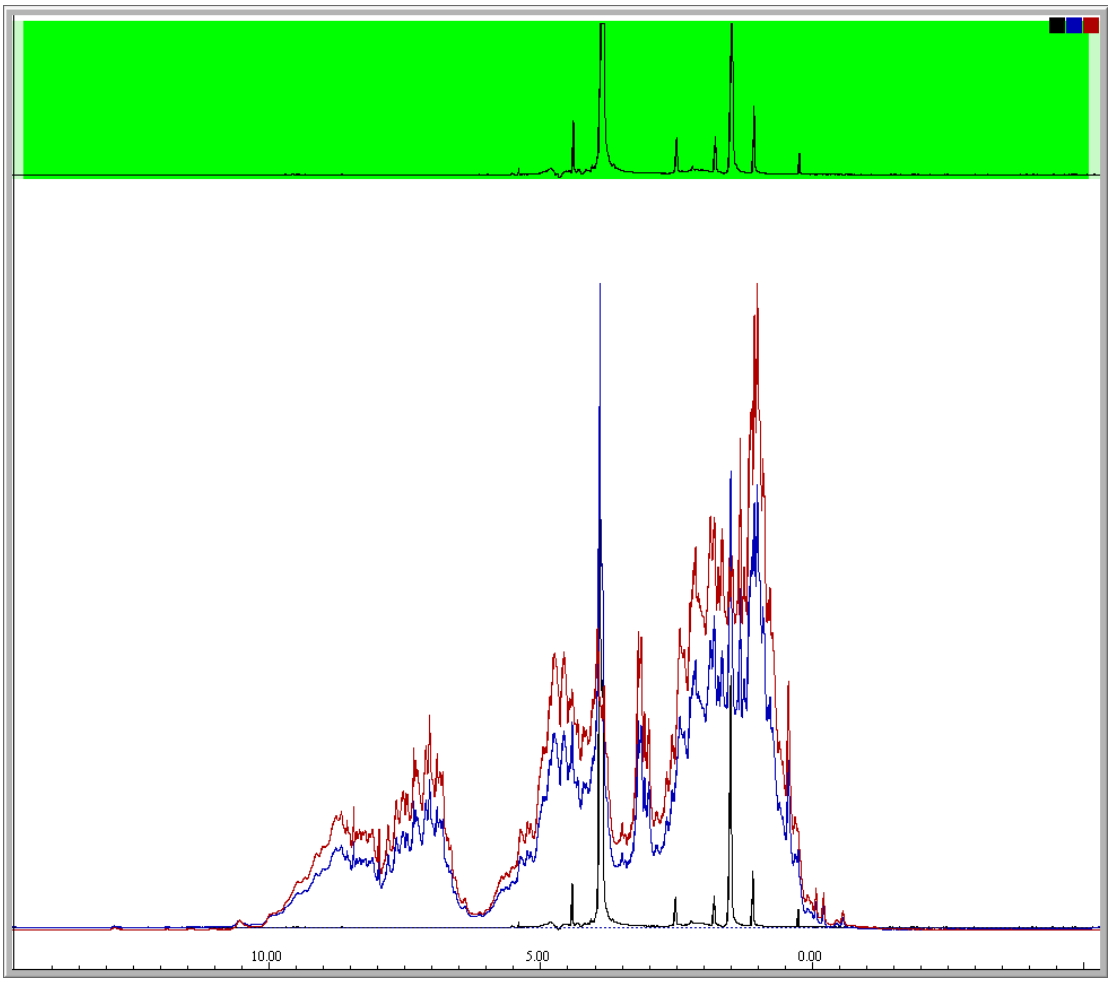



Use the *“File” → “Open TopSpin 1D spectra”* option from the menu bar and select the spectra

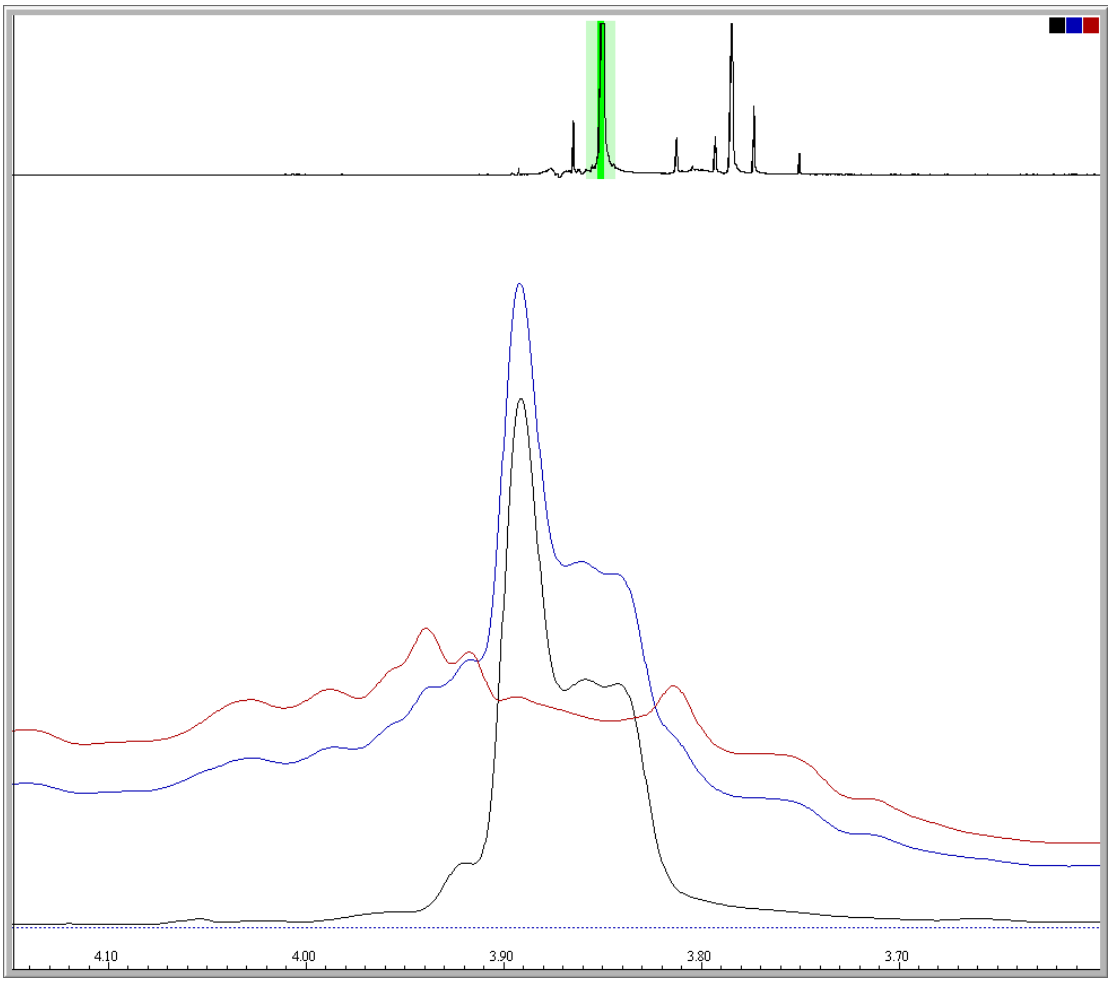



If necessary, use the right mouse click on the specta and select one of the commands in “Arrange” to either separate into different windows, or combine into one depending on user preference.

As shown below, the buffer spectrum (black), the acquired protein spectrum (blue) and subtracted spectrum (red).




The zoom tools by clicking and dragging can be used to examine the results more closely.

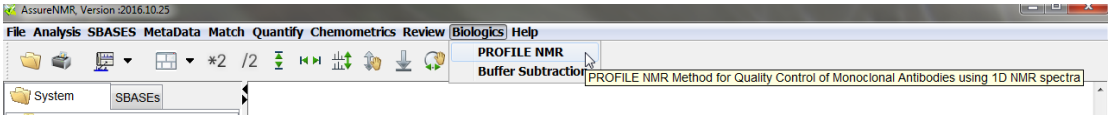



**ProfileNMR**

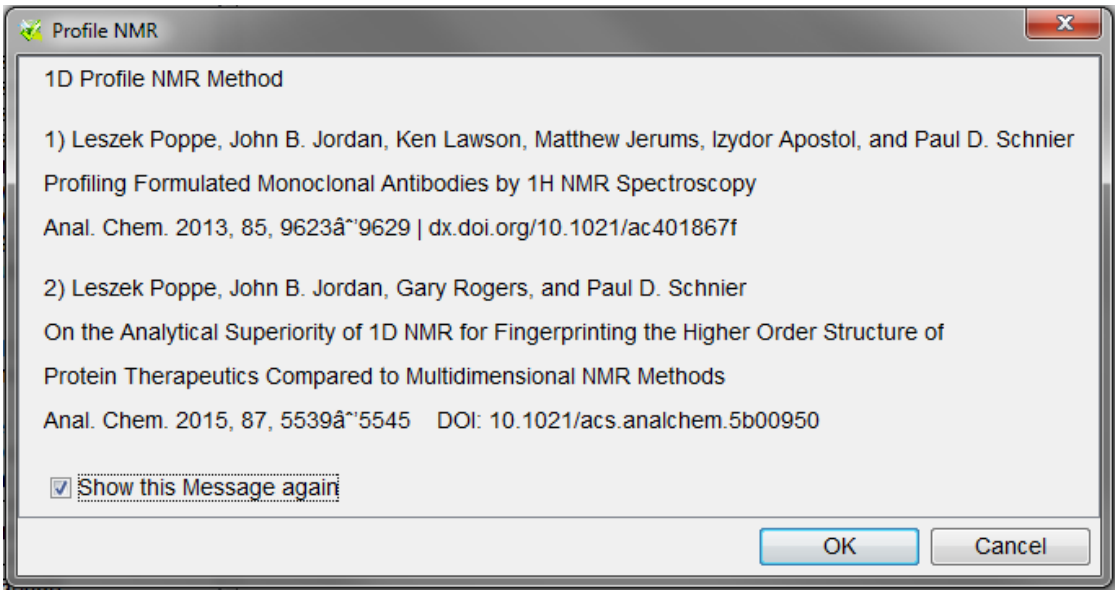
The ProfileNMR method allows comparision of a series of spectra to a set of “reference” spectra.

The example data set for this tutorial include 5 spectra each of a reference antibody, an antibody that “passes” and an antibody that “fails”. All 15 data sets are in the “antibody” directory. For reference, the meta data for these files has been updated to include which group each spectrum belongs to.

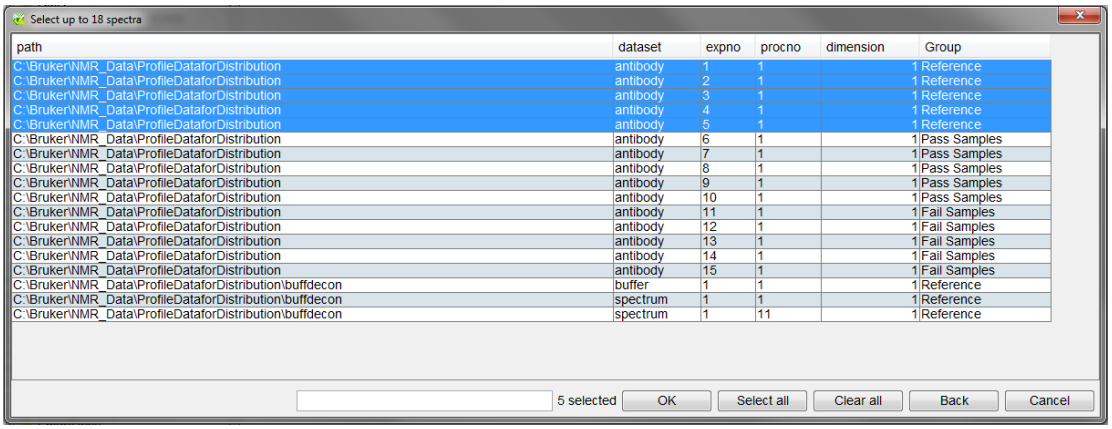
To start the ProfileNMR method, select it from the pull down menu.



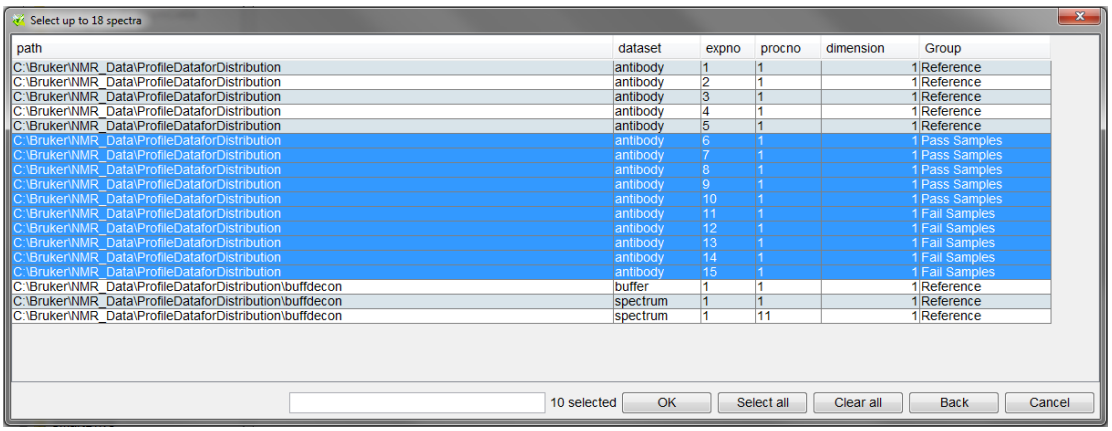

The first time the method is run, a dialogue box with the published articles from which the method was designed is presented. The user has the option to show or not show this message again.



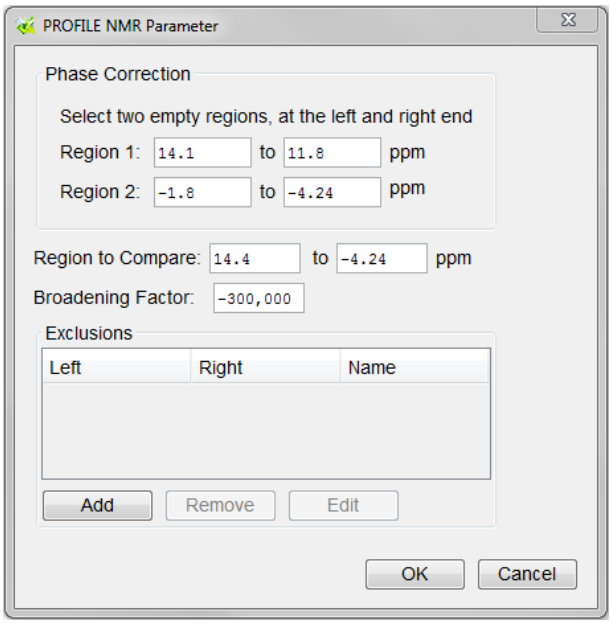

The user is prompted to identify the spectra of the reference material using the standard dialogue boxes for file selection. For the tutorial data set, the first 5 spectra are the reference as indicated by the “Group” parameter from the meta data.




The file selection dialogue continues, and is asking now for the spectra to test against the reference set. The remainder of the “antibody” data were chosen in this example.




A dialogue pops up asking for some imput parameters.




*Phase correction*

Requires two regions, left and right of your spectrum without signals. The regions shown here work for the example data

*Region to compare*

The region where your signals of interest occur.

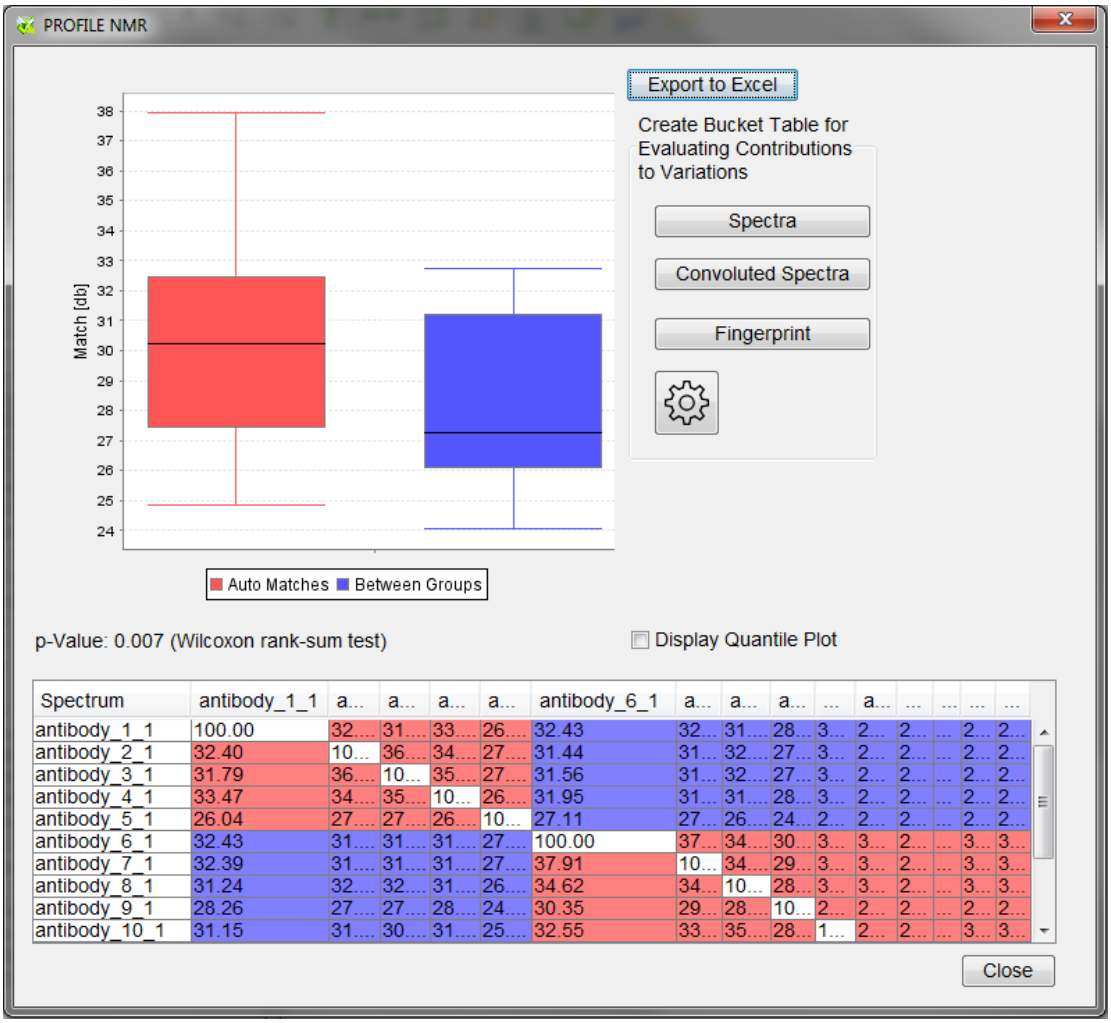
*Broadening factor*

Value is used to compare spectra and calculate the fingerprint. Details are explained in the publications on ProfileNMR method which were shown upon first starting the method.

*Exclusions*

If region(s) of the spectra are to be excluded from the comparison, they can be added in the box as necessary with the respective buttons.

Once completed, the results are displayed in a new window.




| Spectrum | antibody_1_1 | a... | a... | a... | a... | antibody_6_1 | a... | a... | a... | ... | a... | ... | ... | ... | ... |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| antibody_1_1 | 100.00 | 32... | 31... | 33... | 26... | 32.43 | 32... | 31... | 28... | 3... | 2... | 2... | ... | 2... | 2... |
| antibody_2_1 | 32.40 | 10... | 36... | 34... | 27... | 31.44 | 31... | 32... | 27... | 3... | 2... | 2... | ... | 2... | 2... |
| antibody_3_1 | 31.79 | 36... | 10... | 35... | 27... | 31.56 | 31... | 32... | 27... | 3... | 2... | 2... | ... | 2... | 2... |
| antibody_4_1 | 33.47 | 34... | 35... | 10... | 26... | 31.95 | 31... | 31... | 28... | 3... | 2... | 2... | ... | 2... | 2... |
| antibody_5_1 | 26.04 | 27... | 27... | 26... | 10... | 27.11 | 27... | 26... | 24... | 2... | 2... | 2... | ... | 2... | 2... |
| antibody_6_1 | 32.43 | 31... | 31... | 31... | 27... | 100.00 | 37... | 34... | 30... | 3... | 3... | 2... | ... | 3... | 3... |
| antibody_7_1 | 32.39 | 31... | 31... | 31... | 27... | 37.91 | 10... | 34... | 29... | 3... | 3... | 2... | ... | 3... | 3... |
| antibody_8_1 | 31.24 | 32... | 32... | 31... | 26... | 34.62 | 34... | 10... | 28... | 3... | 3... | 2... | ... | 3... | 3... |
| antibody_9_1 | 28.26 | 27... | 27... | 28... | 24... | 30.35 | 29... | 28... | 10... | 2... | 2... | 2... | ... | 2... | 2... |
| antibody_10_1 | 31.15 | 31... | 30... | 31... | 25... | 32.55 | 33... | 35... | 28... | 1... | 2... | 2... | ... | 3... | 3... |

The table shows the match factors between the spectra. Self matches are set to 100 for easy handeling. Row and columns show short spectra names and may be resized as required. The nomenclature is SampleName_Expno_Procno.

This table can be exported to Microsoft Excel for further analysis by using the “Export to Excel” button in the top right of the window.

The distribution of match factors are displayed as box whisker plots in the upper left corner. Red box whisker shows the distribution of “Auto Matches”, which mean match factors within the reference group, and within the tested spectra. The blue whisker box shows the distribution of match factors between the two groups. The color code for the box whisker plot and the table is the same.

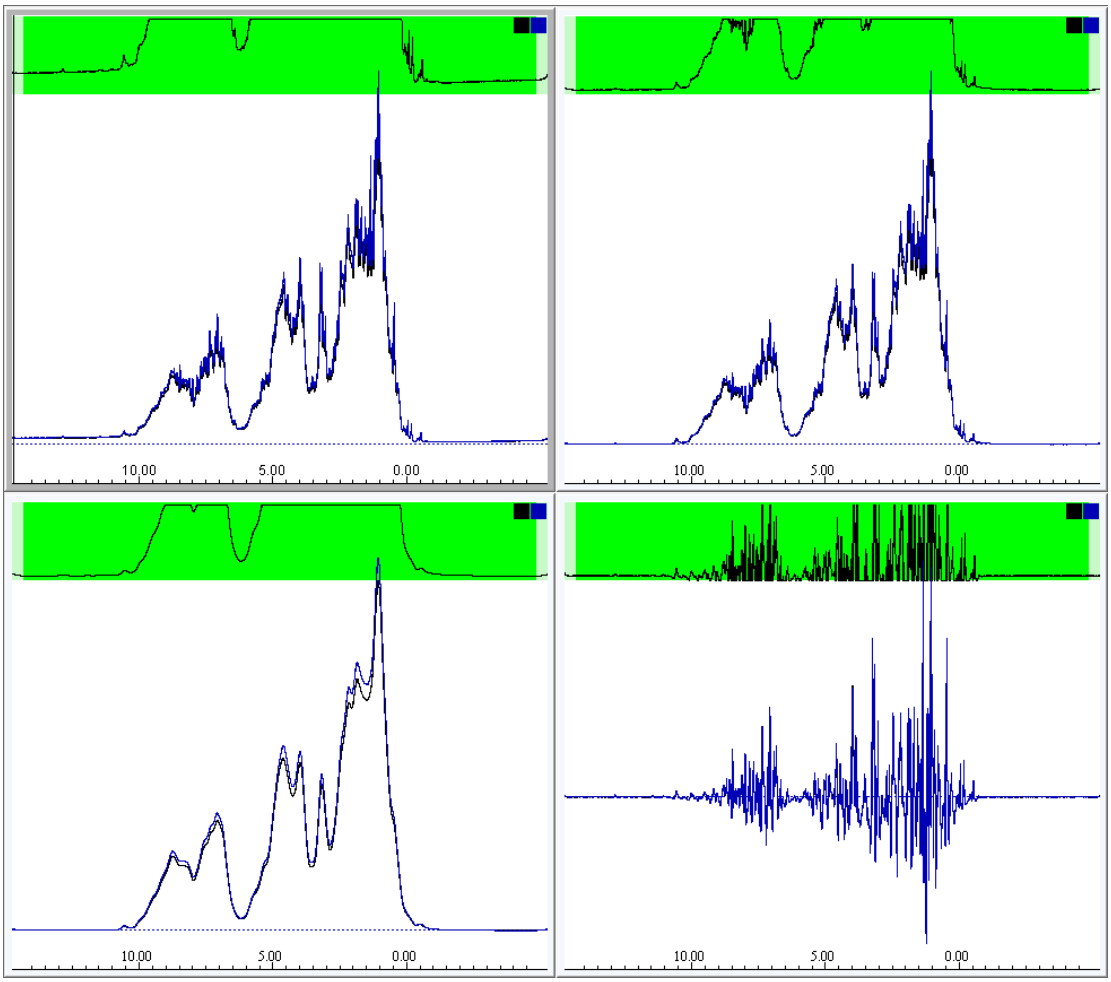
Left clicking on a cell in the table will result in both spectra being loaded into the AssureNMR window as shown below.

The upper left box contains the two original spectra, one in blue, one in black.

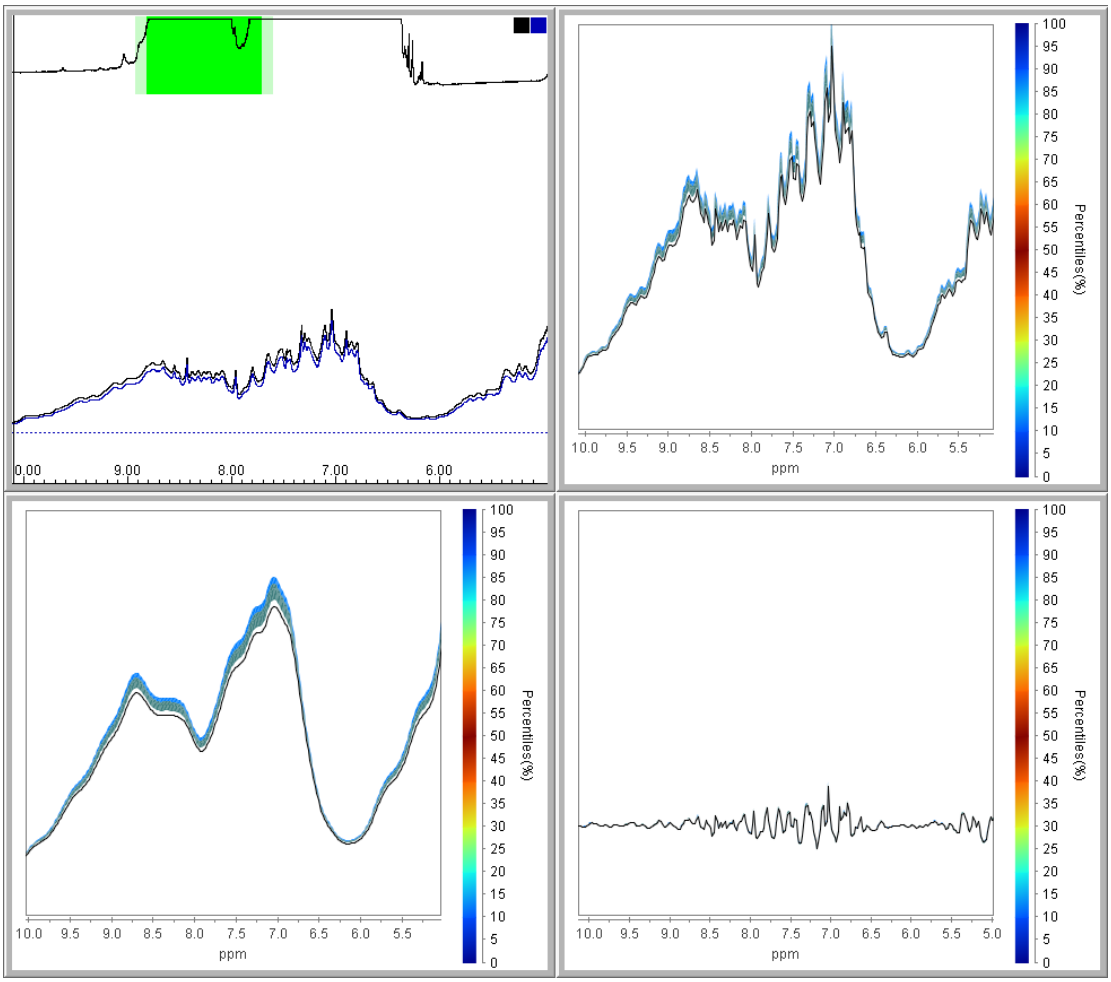
The upper right box contains the “phased” spectra. Reference (black) Test (blue)

The lower left box contains the “Convoluted” spectra. Reference (black) Test (Blue)

The lower right box contains the “Fingerprint” spectra. Reference (black) Test (Blue)

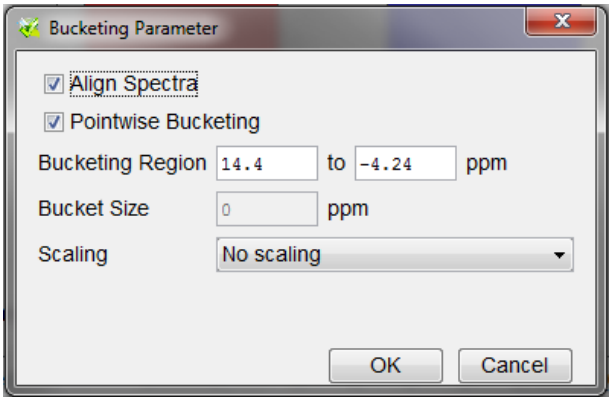



Zooming in on the spectra will show details, and if desired a “quantile plot” can be shown if the option is selected in the Results Window.

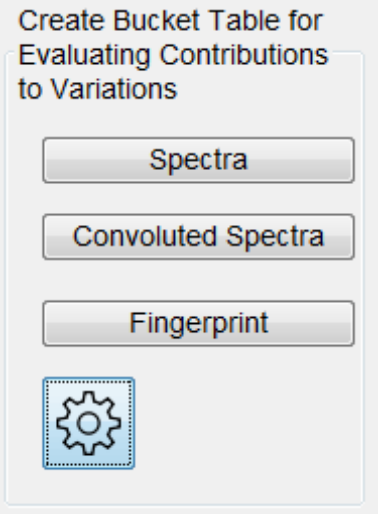



**Further Analysis**

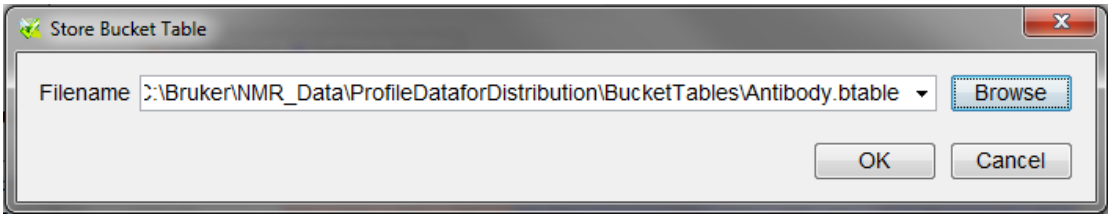
The data can be prepared for further analysis by bucketing (binning). The settings are available via the  button.



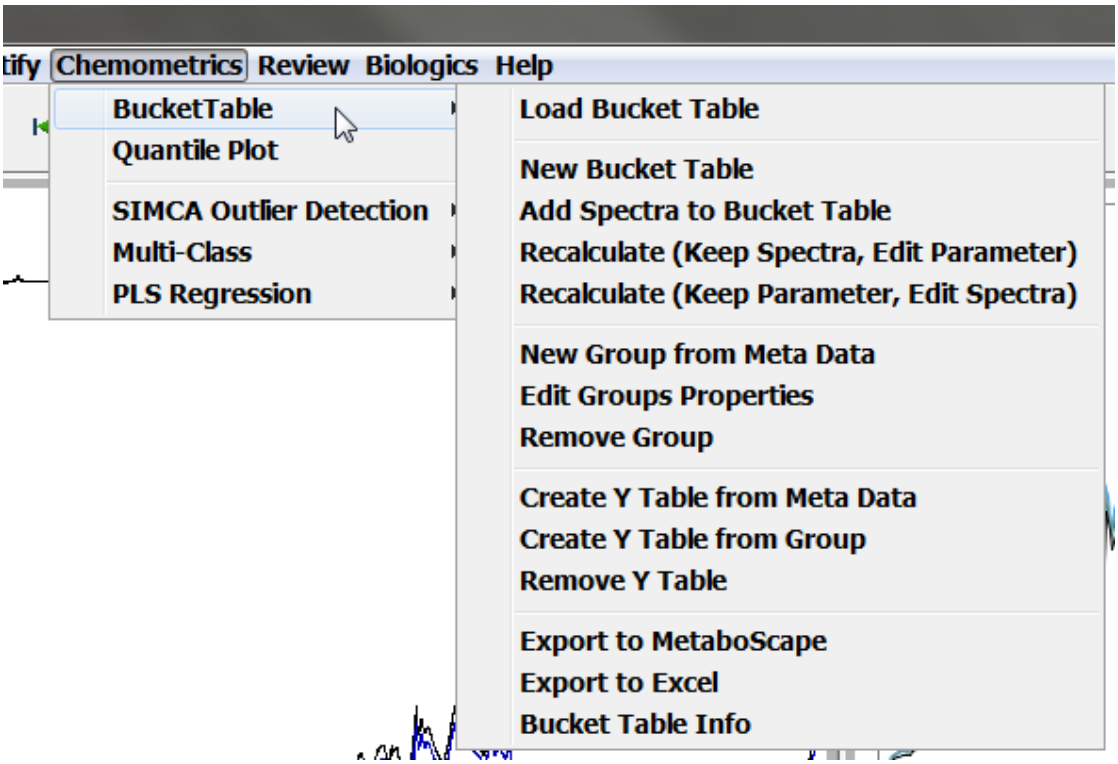

And then the user can decide which type of data to create the bucket table for (Spectra, Convoluted Spectra, or Fingerprint) with the respective button.




The user is then prompted for the storage location of the bucket table.




After creation of the bucket table, it can be loaded into AssureNMR with the commands which are available in the menu bar under “Chemometrics”




for example “Export to Excel”. Please note that Excel supports up to 16k columns, if you choose “pointwise bucketing” the bucket table will be exported in .csv format.

# Tutorials

# 15 License Features

License features provided for the AssureNMR Summit version of software include:
ASSURE_SUMMIT2.0
ASSURE_ASCENT2.0
ASSURE_LAUNCH2.0
ASSURE-SST

SBASE-10-0-0 (RMS SBASE 1)
SBASE-10-0-1 (RMS SBASE 2)
SBASE-11-3-0 (Residual Solvent SBASE)

SMP_CMCA
AMIX3.0
SBASE-1-1-1 (AMIX example SBASE 1-1-2)

# License Features

# 16 Optimizing Hardware Settings

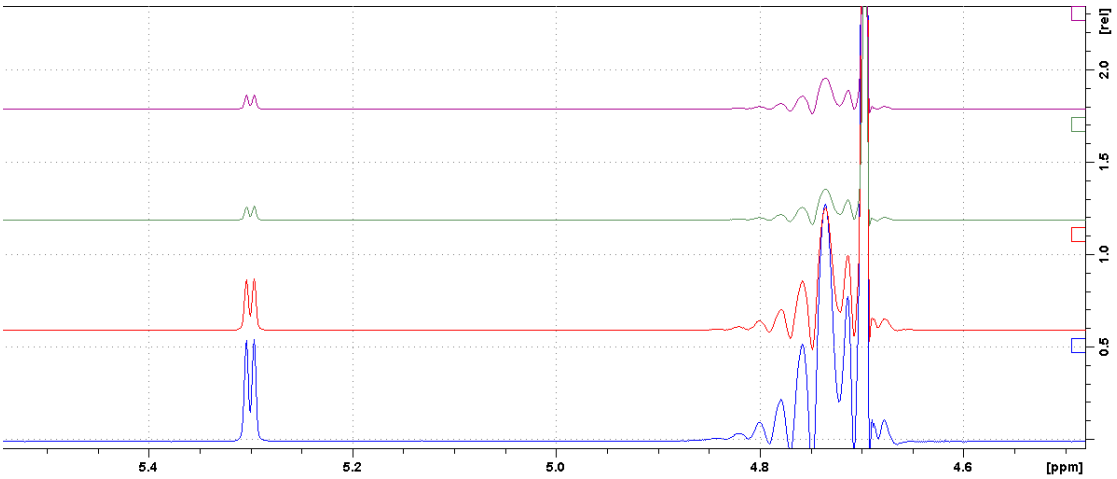
Optimal results using AssureNMR are provided when the spectrometer hardware settings are is optimized.

Optimizing DE, the delay prior to sampling of points.

Manually set optimal de parameter by setting de and running rga

Change de until rg stabilizes at high value

- de=6.5, rga=16, S/N=212
- de=10, rga=16, S/N=200
- de=15, rga=64, S/N=342
- de=20, rga=128, S/N=366
- de=25, rga=128, S/N=368




**Optimizing FILCOR:**

The FILCOR is a parameter that is part of edscon. There is no easy method to set FILCOR, it must be determined empirically.

With 2mM sucrose in 90% H2O standard sample, lock, tune, shim per usual

Call in parameterset WATERSUP

Turn on BASEOPT mode

Acquire data as usual, optimizing o1, etc…

Transform and phase with apk0 (this corrects with only 0-order phase correction)

If additional 1-order phase correction is necessary, adjust, record number, and input this as FILCOR according to the equation:

FILCOR=phc1*DW/180

Re-run to confirm that only apk0 is needed

PROBLEMS:

FILCOR is probe dependent, but edscon is not, so users must remember to go adjust FILCOR every time probe is changed

Even when set, a small amount of phc1 may be needed. APK takes no more time than APK0, and possibly less time! Just run APK.

# 17 Contact

**Manufacturer**

Bruker BioSpin GmbH
Silberstreifen 4
D-76287 Rheinstetten
Germany
*http://www.bruker.com*

WEEE DE43181702

**NMR Hotlines**

Contact our NMR service centers.

Bruker BioSpin NMR provides dedicated hotlines and service centers, so that our specialists can respond as quickly as possible to all your service requests, applications questions, software or technical needs.

Please select the NMR service center or hotline you wish to contact from our list available at:

*https://www.bruker.com/service/information-communication/helpdesk.html*

Phone: +49 721-5161-6155

E-mail: *nmr-support@bruker.com*

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