Identifying and Quantifying Metabolites in Blood Serum and Plasma

Colin Vitols, Aalim Weljie

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Blood serum and plasma are biofluids that are increasingly important in NMR-based metabolomics analysis. In this note we discuss several approaches to analyzing serum using Chenomx NMR Suite, focusing on relaxation-edited NMR (CPMG) and physical separation of protein and metabolites using ultrafiltration. The CPMG method is simpler to apply, but the spectra are easier to analyze when protein is removed from a sample.

Introduction

Metabolite analysis of fluids from the circulatory system provides a view of the metabolic state of an organism. Unlike urine analysis, which measures an organism's waste products, serum or plasma analysis measures homeostatic levels of metabolites throughout the organism.

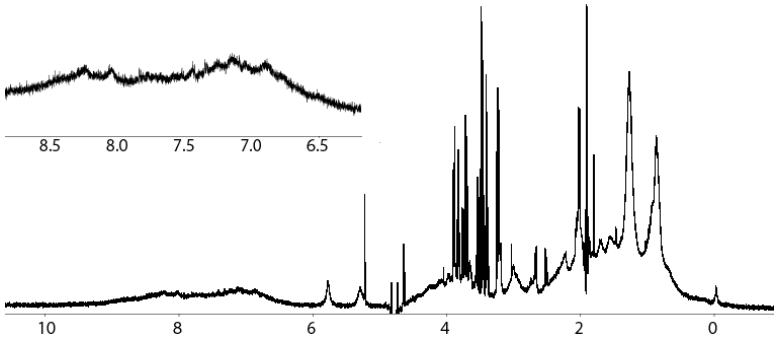

Figure 1. NMR spectrum of a human serum sample acquired with a NOESY-presaturation pulse sequence

In theory, traditional chemometric approaches such as spectral binning (also known as "spectral bucketing") and pattern recognition are applicable to serum spectra. Practically, though, this can be problematic due to the substantial area contributed by protein resonances. The NMR spectrum of a serum sample includes both sharp, narrow peaks from small molecule metabolites and broad peaks from proteins and lipids. As a result, many methods of analyzing serum spectra rely on methods for removing the effects of protein resonances, after which standard analysis techniques may be used (see [1], [2] and Analysis Methods).

Several methods exist for removing removing protein resonances from serum spectra. NMR editing techniques use pulse sequences designed to exclude detection of specific types of signals. For example, relaxation editing via pulse sequences like CPMG can be used to detect only small molecules. Ultrafiltration techniques, using appropriately-selected filters, can take advantage of molecular size differences to separate proteins from dissolved metabolites in serum samples before acquiring spectra.

NMR-Edited Spectra

The most noticeable effect of the high protein content on the spectrum of a typical serum sample is baseline distortion. A serum spectrum acquired using a standard NOESY-presaturation pulse sequence is a combination of both protein and metabolite resonances (Figure 1).

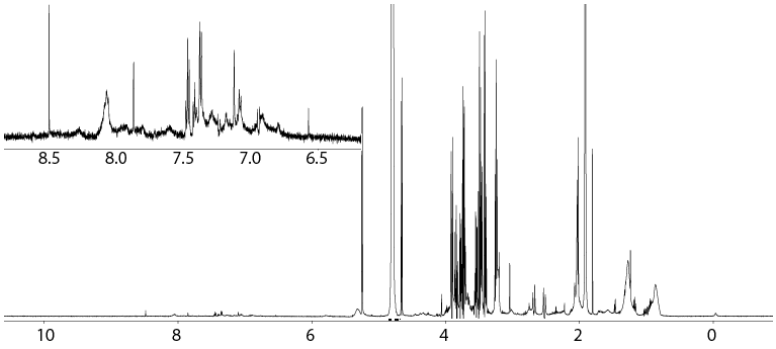

Figure 2. NMR spectrum of the same serum sample acquired with a CPMG relaxation-edited sequence

A common method for removing the contribution of the broad protein resonances is an NMR-edited pulse sequence such as CPMG, which allows the resonances from larger molecules to relax before detecting the longer-lived resonances from small molecules. These CPMG or "relaxation-edited" spectra have improved baselines (Figure 2), and contributions from weaker signals in the aromatic region are clearly visible (inset, 6.0 to 9.0 ppm, Figure 2).

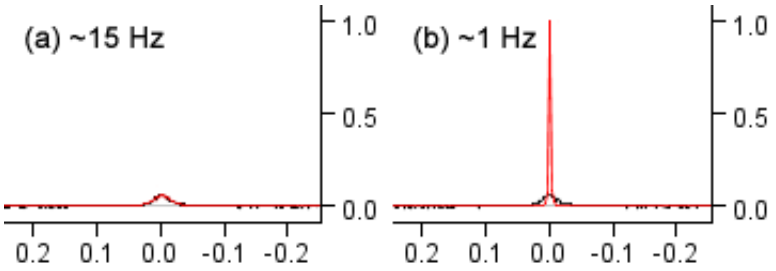

Figure 3. In this CPMG spectrum, the native DSS linewidth (a) must be adjusted to reflect the much narrower lines of other metabolites (b)

Relaxation-edited spectra remove protein resonances while retaining the lineshape and position of the residual metabolite peaks. As a result, chemometric approaches have been proven successful when applied to this type of data [3]. Chenomx NMR Suite can prepare your data for this type of analysis using the spectral binning functions in the Profiler module.

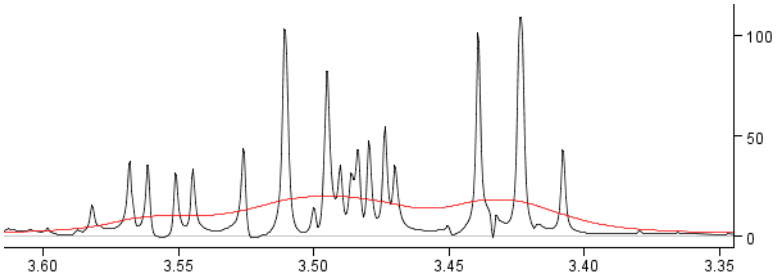

Figure 4. The library signature of glucose does not reflect the observed lineshapes, since the internal standard peak has broadened due to protein binding

Relaxation-edited experiments do not remove the proteins themselves from serum samples, so signals from molecules that bind to proteins can still be visibly affected. For example, the trimethylsilyl group of DSS interacts non-specifically with proteins (Figure 3). The apparent linewidth of the DSS signal in the serum sample shown is ~15 Hz, much larger than the linewidth for other metabolites such as glucose, which does not interact with proteins (Figure 4). The effects of protein-metabolite interaction on metabolite signal lineshape varies, ranging from practically unaffected to strongly broadened due to tight binding with the protein.

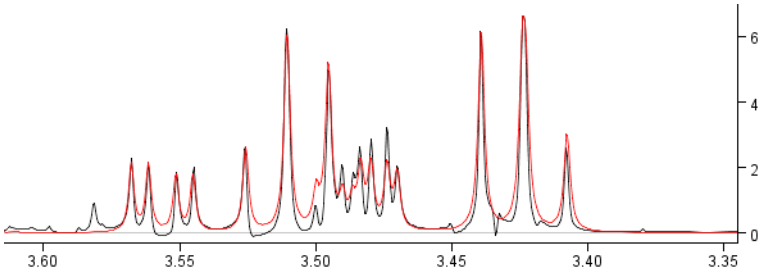

Figure 5. The adjusted CSI linewidth of ~1 Hz provides an excellent match between the library signature of glucose and the experimental spectrum

Before applying targeted profiling to relaxation-edited spectra in Chenomx NMR Suite, you must adjust the apparent linewidth of the chemical shape indicator (CSI) using the Processor module (Figure 3). The first spectrum requires a trial-and-error approach; in the example shown, the linewidth was adjusted from ~15 Hz to ~1 Hz. You can determine the suitability of the final linewidth in this case based on how well the library signature of glucose matched the actual peaks (Figure 5). Subsequent spectra in a dataset often requires similar adjustments of the linewidth of the CSI peak. Due to this approximation of the CSI linewidth, absolute quantification requires confirming metabolite concentrations externally. Spiking the sample with a known amount of one or more metabolites can provide the necessary reference data. Relaxation-edited spectra can provide relative concentrations by normalizing all measured values to that of one of the native metabolites in the sample. Relative concentrations determined this way can also be a valuable source of data, and do not require any sample spikes.

Diffusion-ordered spectroscopy (DOSY) allows editing spectra in the other direction, filtering out signals from small molecules and leaving only the broad signals from larger molecules like lipids and proteins. You can use custom compound signatures created using the Signature Builder module of Chenomx NMR Suite to quantify these broad signals.

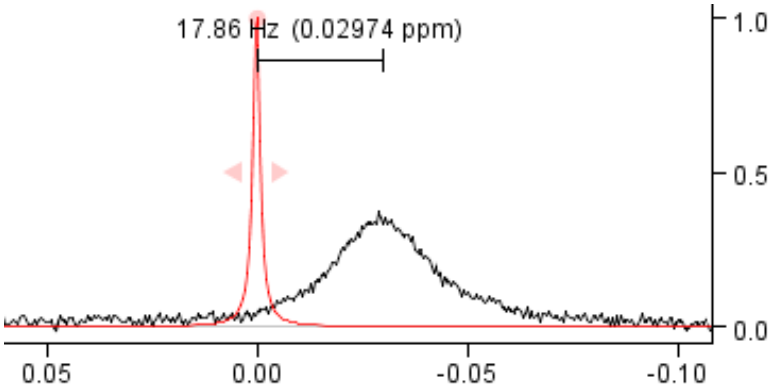

Figure 6. Interaction of DSS with serum protein can result in shifts of position as well as linewidth effects. In this case, the DSS peak has shifted 0.03 ppm upfield

Targeted binning requires an accurate chemical shift reference; however, interactions between the CSI and proteins can induce positional shifts as well as linewidth effects (Figure 6). In the example shown, the actual position of the DSS signal is ~0.03 ppm upfield of the true 0.00 ppm position. You can determine the required adjustment using the position of other compounds with well established chemical shifts at pH 7.0 (such as lactate and glucose). The default peak positions for compounds in the Chenomx libraries correspond to a pH of 7.0. Thus, you can estimate the degree of shift occurring in a sample at the same pH based on these default positions.

Protein Removal

You can also remove protein resonances from a serum spectrum by removing the proteins themselves from the serum sample. For many studies, lipids, proteins and other macromolecules are not interesting, and may be removed from the serum samples. Eliminating these signals allows analyzing the samples using standard techniques, including NOESY-presaturation pulse sequences (see Analysis Methods). Among the numerous extraction methods available [4], ultrafiltration provides the most reliable quantification, and acetonitrile precipitation provides the best balance between ease of use and number of metabolites observed.

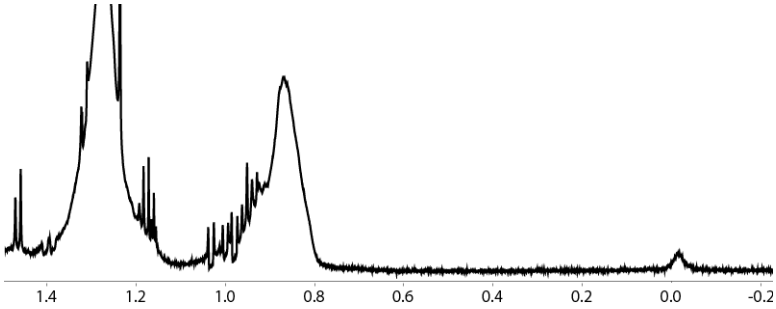

Figure 7. The upfield region of the earlier serum sample run with the CPMG pulse sequence

The size difference between macromolecules and small molecule metabolites provides an excellent basis for size-based filtration, or ultrafiltration, of the serum. The easiest and most reliable method is to pass the sample through a molecular weight filter with an appropriate cut-off, separating the proteins from the metabolites.

For most serum samples, a 3 kDa molecular weight cut off (MWCO) micro-centrifuge filter is enough to separate metabolites from proteins. Wash the filters with water at room temperature before use to remove small molecule membrane preservatives such as glycerol, which may interfere with the analysis. Also, adding a rinsing step can improve recovery of metabolites from the protein component. For example, after filtering a 500 µL serum sample, adding 100 µL of D_2O to the protein component and re-centrifuging can almost entirely recover metabolites that are not tightly bound to proteins.

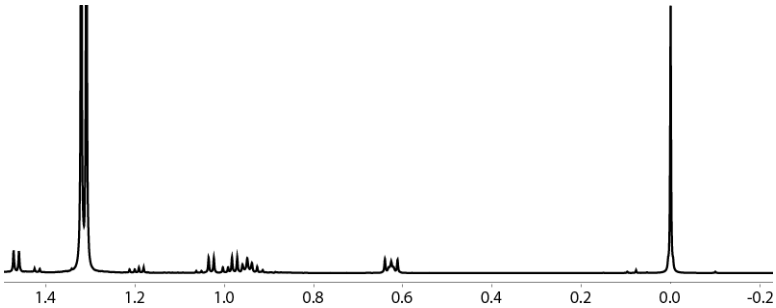

Figure 8. The upfield region of a mouse serum sample filtered using a 3 kDa molecular weight filter

The CPMG spectrum of a serum sample has a very broad DSS peak and a number of baseline distortions near 0.9 and 1.3 ppm (Figure 7), and off-resonance effects from the CPMG pulse sequence distort the lineshapes of the metabolite signals. The NOESY spectrum of a sample acquired after molecular weight-based separation is much cleaner, with a better baseline and improved DSS lineshape characteristics (Figure 8). This is important for accurate quantification in Chenomx NMR Suite, although spectra acquired with the CPMG method can also be analyzed with some adaptations, as discussed earlier.

Another option for removing proteins is chemical precipitation using acetonitrile or other organic solvent. Adding the organic solvent to serum and thoroughly mixing

makes the protein precipitate out of solution; centrifugation helps to separate the solid out of the sample. This method allows extracting metabolites bound to proteins, but absolute quantification may not be consistent from sample to sample. Although precipitation is not as reliable as ultrafiltration, it has some advantages in terms of ease of processing and sensitivity [4].

Special Considerations for Plasma

Anticoagulants such as heparin or citrate are often present in plasma samples, and may interfere with NMR spectral analysis. For example, large amounts of non-deuterated citrate in a plasma sample can saturate the NMR receiver during acquisition. Also, signals overlapping the citrate resonances in such samples may be difficult to identify or quantify. Heparin is a large molecule, so relaxation-edited pulse sequences or ultrafiltration as described earlier can remove resonances associated with heparin. The choice of heparin counter-ion is important, as common ions such as lithium, sodium and ammonium can affect the ionic strength of the sample matrix, and thus alter the appearance of the spectrum.

Conclusion

Analyzing NMR spectra of blood serum or plasma requires dealing with the effects of proteins and other large molecules. Various methods exist that can reduce the effects of these large molecules on sample spectra, including NMR-edited pulse sequences to remove the problem signals, and ultrafiltration to remove the large molecules themselves. In combination with targeted profiling with Chenomx NMR Suite, these techniques allow accurate identification and quantification of a variety of small molecule metabolites.

More details on the experimental techniques described in this note are available. Please contact us at support@chenomx.com for more information.

References

[1] R Rosewell and C Vitols. **March 2006.** *Identifying Metabolites in Biofluids*. *Chenomx Inc.*

[2] D Chang, J Newton, C Vitols, and AM Weljie. **April 2006.** *Statistical Analysis of Targeted Profiling Data*. *Chenomx Inc.*

[3] LH Lucas, CK Larive, PS Wilkinson, and S Huhn. **2005.** *Progress toward automated metabolic profiling of human serum: Comparison of CPMG and gradient-filtered NMR analytical methods*. *J Pharm Biomed Anal.* 39(1-2):156-163

[4] CA Daykin, PJ Foxall, SC Connor, JC Lindon, and JK Nicholson. **2002.** *The comparison of plasma deproteinization methods for the detection of low-molecular-weight metabolites by ^{1}H nuclear magnetic resonance spectroscopy*. *Anal Biochem.* 304(2):220-230

Analysis Methods

Table 1. Methods of analyzing serum data using Chenomx NMR Suite

Technique	Analysis Method(s)	Comments
Raw spectrum	Standard binning, Targeted binning	• Not very useful due to protein baseline issues
NMR-edited	Standard binning, Targeted binning	• Ideal for rapid global analysis • Difficult to identify or quantify metabolites at low concentrations due to baseline artifacts

Technique	Analysis Method(s)	Comments
		• Shifting of DSS peak position due to protein binding may affect targeted binning • Metabolites bound to proteins will be edited out of the spectrum
Ultrafiltration	Standard binning, Targeted binning, Targeted profiling	• Highest quality spectra • Identify and quantify metabolites at low concentration • Metabolites strongly bound to proteins may be difficult to quantify accurately
Precipitation	Standard binning, Targeted binning, Targeted profiling	• Identify and quantify all metabolites in a spectrum • Results may be difficult to reproduce from sample to sample