Analysis of Soy Dietary Supplements Using NMR Spectroscopy

Application Note

Author
David Russell
Agilent Technologies, Inc.
Santa Clara, CA USA

Abstract
Three commercially available dietary supplements containing soybean extract were investigated using NMR spectroscopy. One-dimensional $^1$H NMR analysis, requiring less than 3 minutes per sample, allowed absolute quantification of key flavonoid compounds. Structural verification of genistin, a major flavonoid component in soybeans, was accomplished by comparison to a standard sample. Other components were identified based on spectroscopic signatures.

Once the significant spectroscopic features were identified, automated processing was used to reduce the collected data sets to a spreadsheet containing the concentration of the components of interest plus peak width information to confirm the validity of each measurement for every sample. This allowed statistical analysis and comparison of the combined data to show the variation between samples and between the different sample types. If performed manually, data interpretation would take at least 5 minutes per spectrum.
Introduction

The FDA current Good Manufacturing Practice (cGMP) guidelines for dietary supplements stipulate that manufacturers must provide verification that all products meet established identity, purity, strength, and composition requirements. The guidelines do not stipulate which analytical method(s) are required to comply with dietary supplement cGMP guidelines. Instead, the FDA requires manufacturers to conduct analytical tests such that each botanical component can be authenticated using a valid scientific method.

The evaluation of botanical extracts and dietary supplements using NMR spectroscopy provides researchers with a flexible and powerful tool to comply with regulatory requirements. NMR analysis requires minimal sample preparation and allows a survey spectrum, or chemical fingerprint, to be obtained in just a few minutes. The data obtained from these spectra can then be used to simultaneously establish potency and verify structural data on multiple compounds contained in a sample. If more detailed chemical fingerprint information is required, it can be obtained by acquiring further data sets, such as a 1D $^{13}$C spectrum or a heteronuclear single quantum coherence (HSQC) NMR spectrum.

This application note demonstrates the analysis of three over-the-counter dietary supplements containing soybean extracts using the Agilent comprehensive portfolio of NMR solutions. Soy supplements are commercially marketed as “A natural way to replenish the aging body’s declining estrogen levels, ...relieve menopausal symptoms, such as hot flashes, as well as decrease the risk of heart disease and osteoporosis, without promoting breast cancer.”

Isoflavonoids are a subclass of flavonoid phenolic compounds found in soybean extracts that have the basic C6-C3-C6 structure shown in Figure 1.

Experimental

Standard samples of isoflavonoids genistin (Figure 1, structure 1) and genistein (Figure 1, structure 2) were purchased from Sigma. Two milligrams of each standard were dissolved in DMSO-$d_6$ and transferred to an 8 inch NMR tube for analysis. Two milligrams of each standard were dissolved in DMSO-$d_6$ and transferred to an 8 inch NMR tube for analysis.

Three different soybean dietary supplements were purchased from a local health food store. One product contained only soybean extract: soybean supplement A consisted of 80 mg of soy isoflavones per capsule. The other two products contained soybean extract plus additional components: soybean supplement B, consisted of soybean, wild yam, black cohosh, dong quai, and additional herbs; and soybean supplement C, consisted of 50 mg of soy isoflavones plus black cohosh root extract, wild yam, sage leaf, chasteberry, vervain, astragalus, and motherwort.

Sample preparation

One capsule of each dietary supplement was extracted with 3 mL of DMSO-$d_6$ for 20 minutes at room temperature. After extraction, the samples were filtered through a 0.45 μm nylon membrane and a 0.5 mL aliquot was transferred to an 8 inch NMR tube for analysis. The extract solution obtained from soybean supplement C became highly viscous, precluding simple filtration. For this soybean supplement the extract solution was centrifuged and a 0.5 mL aliquot of the supernatant was then transferred to an NMR tube for analysis. Fifteen replicates were prepared for each soy dietary supplement (A, B, and C) for a total of 45 samples.
The samples were analyzed using an Agilent Direct Drive 500 MHz NMR Spectrometer equipped with a 5 mm OneNMR Probe. Parameters used for the analyses are described in Table 1. ¹H NMR spectra were acquired for each sample. Each sample was automatically tuned, locked, and shimmed before data acquisition.

Results and Discussion

The key spectroscopic feature that distinguishes isoflavonoids from other flavonoids is the isolated proton signal arising from the hydrogen atom at position 2. This proton displays a singlet resonance in the NMR spectrum between 8.0 and 8.5 ppm. Isoflavonoids commonly have a hydroxyl group located at positions 5, 7, and 4'. In addition, glycosylation, when present, usually occurs at position 7 (as in genistin), or position 4'.

Genistin is an isoflavonoid glycoside found in a number of plants including soybeans (Glycine max) and kudzu (Pueraria lobata). Genistein is the aglycone analog of genistin. Genistein is not naturally found at high abundance in soybeans, but it can be formed when harsh processing conditions are used. Figure 2 shows the 1D ¹H NMR spectrum of genistin, with assignments.

Analysis of standard samples

Assignment of the NMR spectra obtained for the two reference compounds is straightforward based on classic NMR techniques. The diagnostic aromatic signals are observed for each sample as well as higher-field signals observed for the exchangeable hydroxyl protons. The resonances in the 3-5 ppm range observed for the genistin sample are consistent with the presence of the carbohydrate moiety in this compound.

These data can now be used to identify and verify the presence of genistin and genistein signals observed in the spectra of the soy dietary supplements. One advantage of NMR quality control is that data for the standards need only be acquired once. Stored standard spectra can be compared with data collected on incoming raw materials to confirm the identity of a raw material or to verify the presence of a specific compound in finished products.
Analysis of purchased supplements

The proton spectra for soybean supplements A-C are displayed in Figure 3. Each spectrum represents a chemical fingerprint for each of the purchased products. $^1$H NMR fingerprints can be used to establish the identity of raw materials, to analyze finished products, and to test for adulteration or contamination.

A simple visual inspection shows that similar data are observed for each sample. The aliphatic region displays resonances consistent with the presence of lipids, such as linoleic acid, and the side chain resonances of the expected phytosterols. The carbohydrate components, including polysaccharides, starch, and the side chain sugars from soy saponins, appear in the heavily congested region between 3 and 4 ppm. The signals observed near 5 ppm are primarily assigned as the resonances from the anomeric protons of the various carbohydrate moieties.

The primary focus of this study will be those resonances observed in the high frequency region of the spectrum, between 6 and 8.5 ppm. This region contains the aromatic resonances associated with the isoflavone components of the samples and is shown in Figure 4.

Three large signals are observed at approximately 8.04, 7.20, and 7.13 ppm in each sample. Inspection of the coupling pattern for these signals implies that they are part of a 1,3,4-trisubstituted aromatic system and allows their assignment as that of the A ring in daidzin (Figure 1, structure 3), a known major isoflavanoid component in soybeans. The H$_2$ proton resonance expected for daidzin appears as a sharp singlet at 8.35 ppm.
The third significant component expected in processed extracts of soybeans is glycitin (Figure 1, structure 5). Glycitin displays a resonance for the H2 proton similar to that of daidzin, while the H5 and H8 resonances both appear as singlets. These resonances are easily identified at 7.48 and 7.30 ppm, respectively.

**Quantitation of isoflavones**

Once resonance assignments for the compounds of interest are accomplished, accurate and precise quantitation of each flavonoid is readily obtained from a normal proton experiment. In those cases where a definitive assignment is not available, quantitation of an unknown is still possible as long as a resonance that represents a known number of protons can be identified. In the case of isoflavonoids, each singlet observed in the 8.2-8.5 ppm range represents an H2 resonance; as a result, quantitation of the associated impurity is still achievable.

The Agilent qNMR method for quantitation relies on the established reproducibility and linearity of the NMR spectrometer. This is accomplished by carrying out a single, one-time calibration of the spectrometer using any sample of known concentration. The results recorded for that calibration can be used to scale any subsequent data set for changes in solvent, tip angle, and receiver gain to produce an accurate absolute intensity measurement of the unknown. This procedure eliminates the need for expensive natural product standards and daily recalibration of the instrument.

Integrals of the $^1$H resonances corresponding to genistin, daidzin, glycitin, and genistein were measured for each sample and the resulting integral values were then converted to concentration measurements during data processing. Figure 5 shows the overall quantitation results.

![Figure 5. Quantitative results from analysis of 45 soy dietary supplements showing the concentration of genistin, daidzin, glycitin, and genistein as determined by 1D $^1$H NMR analysis.](image-url)
Verification of the data set

Review of the line width measurements in the raw data spreadsheet indicated that for soybean supplement C 12 the lineshape was almost twice as wide as any other sample in the entire study (Figure 6). Therefore, the data obtained for this sample should be carefully reviewed before it is included in any analysis. This ability to internally test the quality of the data as an intrinsic part of the experiment significantly increases the confidence of the results obtained by NMR analysis.

Figure 6. Comparison of the measured linewidth for the H2 proton in genistin in each sample in the study, indicating an anomalous result that should be reviewed further before inclusion.
Investigation of genistein created during processing or storage

A comparison of the spectra acquired for the test samples to those recorded for the genistin and genistein standard samples indicates that genistin is a major component of all three soy extracts. In contrast, those resonances indicative of genistein are present only at low levels. This is consistent with the fact that genistein is a decomposition product created by processing and it is not present at high concentrations in raw soybeans.

A comparison of the ratio of genistin to genistein in the sample set provides insight into the conditions used to produce the soybean extract. Figure 7 shows the genistin/genistein ratio, after normalization, for each sample. The results indicate that similar raw material was used to make soy supplements B and C, but both the relative concentration and variability in soy supplement A is significantly higher and indicates that the extract used to manufacture soy supplement A was treated more harshly than that used for soy supplements B and C.

Figure 7. Comparison of the genistin/genistein ratio for soy supplements A, B, and C, normalized within each series of samples.
**Statistical analysis of results**

Visual inspection and comparison of the spectral results obtained for each sample in a study such as this would be inefficient. Statistical multivariate analysis tools can be used to display the collective results graphically, thereby allowing relationships between the various samples and data sets to be easily understood.

**Targeted analysis**

For those data sets where only a restricted number of variables are to be considered for interpretation, simple visualization of the relationship between those variables is often sufficient for reducing the data set to a result.

Since the spectra of the soy supplements were already translated to a series of isoflavonoid components and concentrations, those data can be used as the input for simple bivariate analysis. An example of this approach is shown in Figure 8. Even using a simple method such as this, discrimination between the three different types of samples is obvious. This kind of analysis would be very useful for monitoring the consistency of a given soybean supplement during processing and manufacturing, or as a tool to monitor the production of isoflavonoids when soybean plants are grown under various conditions.

![Figure 8. Bivariate analysis of soybean supplements A, B, and C comparing variation in the concentration of genistin and daidzin in each preparation.](image-url)
Untargeted analysis

Principal component analysis (PCA) is a common method used to quickly investigate variance in very large data sets. In essence, PCA allows visualization of the quantitative difference in a collection of data.

PCA analysis is the method of choice for exploratory studies where analysis of the entire complement of a complex mixture is used to compare samples. It allows a collection of related spectra to be characterized according to the degree of similarity between them. As such, it provides a rapid and effective way to identify classes of spectra, and to relate these classes to properties of interest. Using the NMR spectra obtained for soybean supplements A, B, and C as an example, PCA analysis can be used to compare the normalized intensity of every data point in the aliphatic portion of the spectra between 0.0 and 2.4 ppm. As shown in Figure 9, the PCA results can readily discriminate between the three types of samples without the use of any specific structural information, peak analysis, binning, or inclusion of isoflavonoid resonances.

Figure 9. PCA analysis of soybean supplements A, B, and C comparing point-by-point variation between the spectra in the region of 0.0 to 2.4 ppm.
Conclusion

A number of advantages are realized when analyzing dietary supplements using NMR spectroscopy: acquisition of a chemical fingerprint, the availability of quantitative information for a number of different compounds in a single sample, and structural verification data in one simple experiment. This information can be used for raw material quality control, for process and final product control, and adulterant screening. The Agilent portfolio of NMR instruments and software products enables high-quality data collection for these applications and includes automated data reduction, and statistical analysis using state-of-the-art software tools.

References


2. Crouch, R., and Russell, D. Easy, Precise and Accurate Quantitative NMR. Agilent publication 5990-7601EN.