# Gas Chromatography as a Reference Method for Moisture Determination by Near-Infrared Spectroscopy

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# Gas Chromatography as a Reference Method for Moisture Determination by Near-Infrared Spectroscopy

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**Near-infrared spectroscopic (NIRS) methods were developed to determine moisture in freeze-dried drug products with gas chromatography as the reference measurement. The gas chromatography method was optimized to provide a relative standard deviation of 1.1%. Because NIRS calibration models are limited by the quality of the reference measurement, all aspects of the GC technique were studied, including water standard preparation, operating conditions, and sample treatment. Vials of a freeze-dried drug product were prepared with water contents from 0.1 to 5.7% (w/w). NIR spectra were collected over the spectral range of 1100−2500 nm for these vials. Calibration models were established from these spectra by PLS regression analysis. Second-derivative spectra were used and spectral range and number of PLS factors were optimized for the lowest standard error of prediction (SEP). The best calibration was built with second-derivative spectra in the spectral range of 1850−1960 nm with three PLS factors and provided a SEP of 0.07% water (w/w). These results indicate that GC is a competitive reference method and that NIRS can be used for water determination in cases where other moisture analysis techniques are not compatible.**

Many methods have been developed for water determination, including Karl Fischer titration (KF), loss on drying, and chromatographic procedures.[1−5] However, each has pros and cons in terms of accuracy, speed, ease of operation, and compatibility with certain analytes. For example, although Karl Fischer titration is widely used, it consumes the sample and exposes the operator to toxic reagents. It also takes several minutes to complete one assay with no commonly available autosampler. Some analytes are not compatible with KF or loss on drying techniques because they react with the titrant, are volatile, or degrade. In addition, all methods are susceptible to ambient moisture if strict precautions are not taken. To determine water in a wide variety of sample types, it is necessary to consider each of the techniques that are available.

Near-infrared (NIR) spectroscopy has gained increasing recognition for rapid and nondestructive analysis.[6] NIR spectroscopy is especially suitable for moisture determination[7−9] because water has strong absorption bands in the near-infrared region which provide the sensitivity needed for accurate determinations. It is desirable to have NIR methods for water determination that are not only rapid, nondestructive, and easy to use but also accurate. Since the NIR method is a secondary method for quantitative analysis, an accurate and dependable reference method is needed.

NIR methods with KF titration as the reference have been reported.[8,9] In previous studies[10,11] we have also investigated this approach. Our KF method was optimized to minimize the influence from ambient moisture. For hygroscopic drug substances, an NIR calibration model[10] was built with a standard error of prediction (SEP) of 0.11% (w/w) in the range of 0.5−11.4% water (w/w). Using the same KF reference technique, an NIR method[11] was also constructed for the freeze-dried drug formulation which provided an SEP of 0.05% (w/w) in the range of 0.1−5.3% water (w/w).

In this report, a gas chromatographic method has been explored as the reference measurement. This approach is most applicable for products that are incompatible with Karl Fischer titration and when large batches of samples are analyzed when a calibration model is being built. Direct or indirect water determination by GC has been previously reported.[3−5] Driscoll reported a GC method to detect low-level water with a far-ultraviolet detector.[5] Loeper determined water with a headspace GC method.[12] Chen also reported a gas chromatographic method of water after reaction with triethyl orthoformate.[4] For our drug product formulation, we chose to use direct detection of water with a TCD detector with an emphasis on accuracy through procedure optimization and protection against ambient moisture.

## EXPERIMENTAL SECTION

**Apparatus and Materials.** Diffuse reflectance spectra of samples were obtained with an NIRSystems 6500 spectrophotometer (Perstorp Analytical, Silver Springs, MD) equipped with a

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rapid content analyzer (RCA). The software package NSAS, accompanying this instrument, was used to collect spectra. Multivariate regression analysis was performed with the software package GRAMS/32 enhanced with an add-on subroutine PLSplus/IQ[2] (Galactic Industries Corp., Salem, NH).

Gas chromatographic analyses were performed on a HP 5890 Series (Hewlett Packard, Wilmington, DE). Plastic syringes of 3 and 10 mL (Becton Dickinson & Co., Franklin Lakes, NJ) with special 10 cm long 22-gauge needles (Metrohm, Herisau, Switzerland) and glass syringes of 10 and 100 $\mu$L (Hamilton, Reno, NV) were used to transfer solvent or solution. GC vials were clear glass vials (Part No. 5180-4197, Hewlett Packard) and 11 mm seals with TFE-faced rubber liners (Alltech Associates, Inc., Deerfield, IL). A microgram MT5 balance (Mettler-Toledo, Inc., Hightstown, NJ) was used to check for leakage of the GC vials.

A freeze-dried product formulation of LY333328, an antibiotic under development at Eli Lilly and Co., was used in this study. This product is contained in 10 mL clear glass bottles (Wheaton, Glass Division, Millville, NJ). A moisture-tight closure was obtained with Diakyo D777-3 Gold Butyl stoppers (The West Co., Lionville, PA) and an aluminum crimp seal. Each bottle contains 175 mg of formulation which normally forms a whole, porous "plug". The moisture levels of these formulations are adjusted from 0.1 (w/w) to 5.7% (w/w) in a glovebox by controlling the relative humidity. Anhydrous HPLC-grade methanol (Mallinckrodt Chemical Inc., Chesterfield, MO) was used as the solvent because relatively high solubility was achieved for this drug product.

**Moisture Measurement by GC.** Water determination by the GC method was optimized to address the issues of solubility and peak tailing in addition to ambient moisture. The vial contents would not totally dissolve in 5 mL of methanol so a sonicating step was applied to break up particles and increase total surface area, ensuring the recovery of water. Comparison to KF titration showed that acceptable recovery of water was achieved in methanol by sonicating the solution for 5 min.

Chromatographic peak tailing of water occurred during the initial development of the GC method. Decomposition of components in the formulation was causing materials to bleed onto the column. After reducing the temperature of the injection port to 195 °C, this phenomenon was almost unnoticeable (see Figure 1c for detail). Elimination of peak tailing helped ensure reproducible integration of the water peak. Centrifuging the sample solution before transferring to the GC vial and frequent replacement of the injection port liner helped to improve the peak shape by preventing the accumulation of materials.

The precision of the GC method was evaluated by measuring samples in sequence and over a period of time. A sample with 0.168% water (w/w) in methanol was divided into five autosampler vials. A relative standard deviation of 1.1% was obtained when these vials were analyzed for water level over three days. The same relative standard deviation was obtained when these vials were injected in the same GC run.

**Standard Preparation.** Accurate and precise standard preparation is essential and can be very difficult because methanol is hygroscopic. As an example, a stock solution of 2.694 (±0.001)% (w/w) was prepared by injecting 0.4452 g of water into 16.0792 g of methanol in a 20 mL clear glass bottle (Wheaton) with a Diakyo

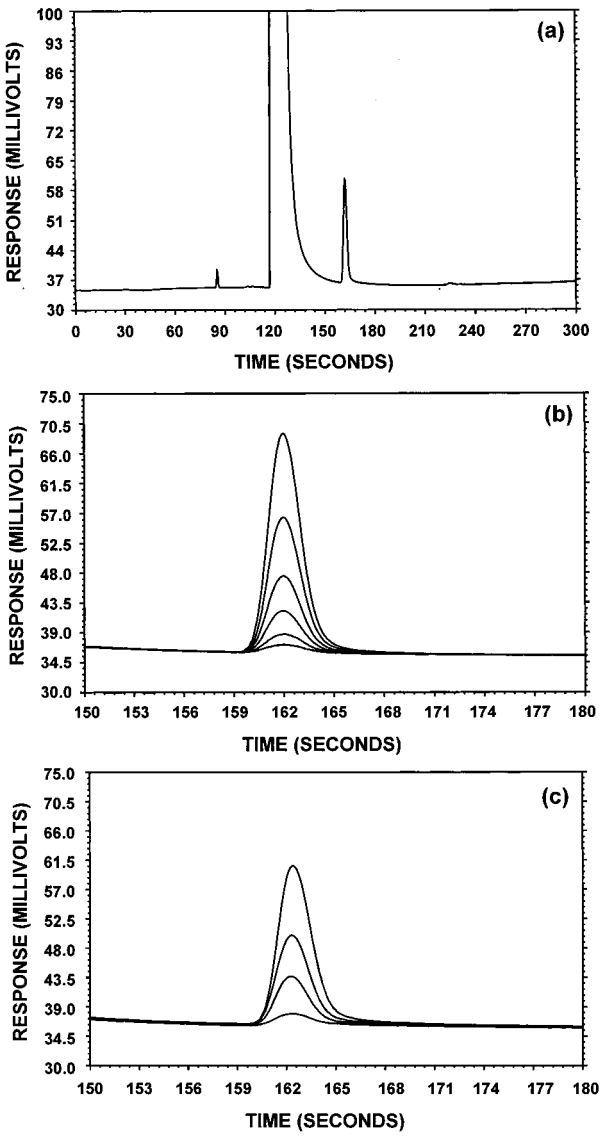


Figure 1. Chromatograms of entire time interval (a), water standards (b), and three samples (c). The corresponding water levels are (b) 0.0, 0.0145, 0.0472, 0.0951, 0.1757, and 0.2901% (w/w) in methanol and (c) 0.0056, 0.0631, 0.1140, and 0.2104% (w/w) in methanol or 0.146, 1.637, 3.038, and 5.500% (w/w) in drug product vials.

D777-3 Gold Butyl stopper (The West Co.). A set of five standards was prepared from this stock solution inside a glovebox. These standards, from 0.0145 to 0.2901% (w/w) water, were prepared by injecting 5.861−110.604 mg of the water stock standard into the appropriate amount of methanol in a GC vial.

**Sample Preparation.** Samples were reconstituted with about 5 mL of methanol introduced with a plastic syringe and 30 cm long needle. Samples were handled inside the glovebox with relative humidity below 1%. A net weight increase of these sample vials was recorded and used for the future calculation of moisture content. The samples were then sonicated for 5 min to extract water. After centrifuging, about 1 mL of the supernatant of these samples was transferred into each individual GC vial and sealed tightly. Finally, a microgram balance was used to check whether each GC vial was completely sealed.

In a typical run, standards and samples were injected sequentially on the GC instrument. The results were recorded electroni-

Table 1. Experimental Conditions of Gas Chromatography

| parameters | conditions |
|---|---|
| detector | TCD |
| column | DBWAX, 30 m, 1 $\mu$m film, 0.53 mm i.d. |
| inject temperature | 195 °C |
| detector temperature | 300 °C |
| oven temperature | 60 °C |
| oven temperature program | 60−160 °C over 5 min |
| carrier gas flow rate | 6 mL/min |
| auxiliary gas flow rate | 15 mL/min, total |
| reference gas flow rate | 50 mL/min, total |
| split flow | 6 mL/min |
| split ratio | 1:2 |
| purge flow | 4 mL/min |

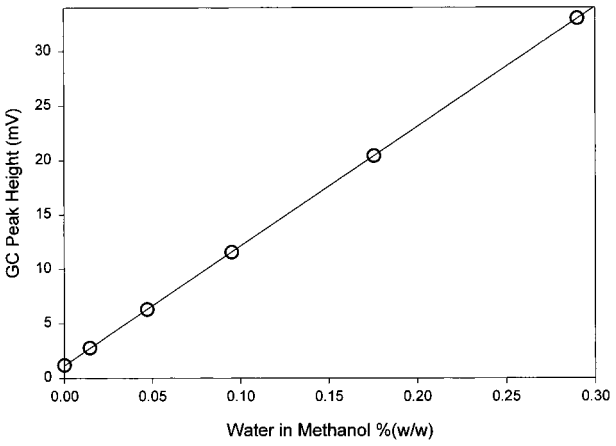


Figure 2. Standard calibration curve for water in methanol by gas chromatography obtained based on peak height.

cally, and the water peaks were measured for peak height and peak area. Both measurements were evaluated for calibration and moisture determination of samples. Peak heights were chosen for the rest of the study because they provided consistent results, possibly due to less reproducible area integration caused by a slight peak tailing effect (see Figure 1c).

## RESULTS AND DISCUSSION

Water determination with GC and NIRS was completed for 37 samples. Three spectra were collected for each sample, and then the vial was analyzed by GC for water on the basis of the experimental conditions listed in Table 1, and the preparation scheme described previously.

**GC Analysis.** A typical chromatogram of a sample with 2.5% water is presented in Figure 1a where the water peak appears at about 162 s following the large peak of methanol. Figure 1b presents the chromatograms of six water standards. These standards provide a calibration curve with a linear regression coefficient $R^2$ of 1.0000, a slope of 109.6909, and a *y*-intercept of 1.153 based on the water peak height. This *y*-intercept corresponds to the 0.01% water residue in the original methanol solvent. On the other hand, when peak area is used, the corresponding linear regression coefficient, slope, and *y*-intercept are 0.9998, 2586.8322, and 37.157, respectively. Figure 2 shows the standard calibration curve for water in methanol by GC based on peak height. It covers a range of water from 0.0 to 0.29% (w/w) added to the methanol solvent.

Table 2. Water Levels by the GC Method Based on Peak Height for Samples in the Calibration Set

| sample | water (%, w/w) | sample | water (%, w/w) |
|---|---|---|---|
| 1 | 0.292 | 13 | 3.851 |
| 2 | 0.146 | 14 | 3.843 |
| 3 | 1.529 | 15 | 3.649 |
| 4 | 1.563 | 16 | 3.687 |
| 5 | 1.570 | 17 | 3.722 |
| 6 | 1.637 | 18 | 5.687 |
| 7 | 3.086 | 19 | 4.623 |
| 8 | 3.038 | 20 | 5.599 |
| 9 | 3.037 | 21 | 0.590 |
| 10 | 3.917 | 22 | 0.745 |
| 11 | 3.856 | 23 | 0.138 |
| 12 | 3.945 | | |

Table 3. Water Levels by the GC Method Based on Peak Height and by Karl Fischer Titration for Samples in the Prediction Set

| | water (%, w/w) | | | water (%, w/w) | |
|---|---|---|---|---|---|
| sample no. | by GC | by KF | sample no. | by GC | by KF |
| 1 | 0.255 | 0.356 | 8 | 3.944 | 3.860 |
| 2 | 0.215 | 0.327 | 9 | 3.704 | 3.645 |
| 3 | 1.535 | 1.506 | 10 | 3.684 | 3.628 |
| 4 | 1.540 | 1.594 | 11 | 5.500 | 5.418 |
| 5 | 3.066 | 2.894 | 12 | 4.780 | 4.680 |
| 6 | 3.867 | 3.769 | 13 | 0.746 | 0.785 |
| 7 | 3.903 | 3.811 | 14 | 0.562 | 0.646 |

The amount of water in each sample was calculated from the above calibration curve based on peak height and is presented in Table 2 for the calibration set and Table 3 for the prediction set. All these samples span a water range of 0.14−5.69% (weight of water in vial per total weight of vial contents). Figure 1c illustrates three representative chromatograms of these samples. For the prediction set, Karl Fischer titration was also performed and a standard error of deviation of 0.10% (w/w) was obtained for water level difference by these two methods. This level of difference is within the accuracy range of KF and GC methods and supports the validity of the GC method.

**Calibration Model Development.** To build an NIR method for water determination, we first randomly selected 14 samples representing each target moisture level for use as the prediction set. The remaining 23 samples were used as the calibration set. From the calibration set, calibration models by partial least squares (PLS) were built and used to predict the water levels in the prediction set. These models were constructed with varying conditions including spectral range, number of PLS factors, and data pretreatment process. Performance was judged by the value of SEP when calibration models were used to quantify water in the samples in the prediction set.

There are many algorithms available to correlate spectral information to the concentration of an analyte of interest. Largely due to the strong absorption bands of water, most work on water determination has been done with simple linear regression or more subtle factor-based multivariate regression. In some cases,[13] simple linear regression can account for most of the spectral variations related to water if the water range is narrow, the

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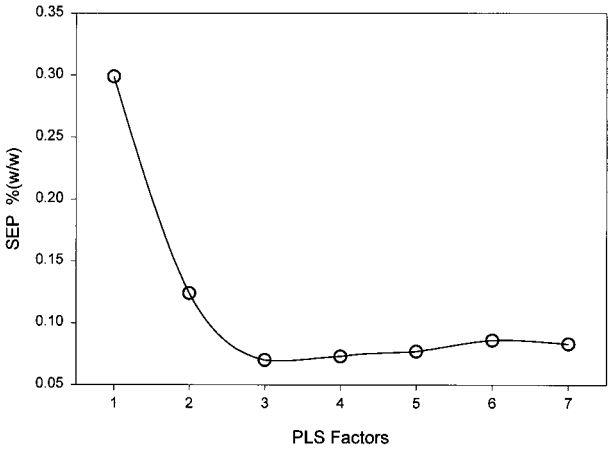


Figure 3. SEP vs PLS factors in the range of 1850−1960 nm with second-derivative spectra.

reference method itself is not so accurate, or the target accuracy requirement is not strict. In cases where better accuracy of water determination is desired, however, a multivariate regression method is needed to reach the accuracy limit by the reference method. PLS regression has previously been applied to build a calibration model for water in a freeze-dried drug formulation and bulk drug material.[10] These studies closely approached the accuracy limit set by the reference method of Karl Fischer titration. In this study PLS regression is also applied to build the NIR method for water determination.

We have found that NIR methods built with the strong water absorption bands at 1920 nm perform best. In addition, derivative calculation as a spectral pretreatment also helps to achieve better results. In this study, we tested several spectral regions including some narrow regions with only a few spectral data points (2 nm/one point). The spectral range of 1850−1960 nm was finally selected based on the performance of the resulting calibration models. When spectral pretreatment was used with a second-derivative calculation, a plot of SEP vs PLS factors was obtained (see Figure 3). It clearly indicates that a calibration model built with three PLS factors performs best in terms of the value of SEP. Therefore, the optimum calibration model was determined to be within the spectral range 1850−1960 nm using second-derivative spectra and three PLS factors.

Figure 4 illustrates the performance of this calibration model. In Figure 4a, a concentration correlation plot is presented for the calibration data set. Regression analysis ($R^2$ 0.9953) results in a slope of 0.9883 and a *y*-intercept of 0.026% (w/w) which demonstrates the closeness of these two methods. For the prediction set, the concentration correlation between the water levels by the GC method and the NIRS method when the above calibration model is used is illustrated in Figure 4b. The slope and the *y*-intercept of regression analysis ($R^2$ 0.9985) are 0.9868 and 0.031% (w/w), respectively. The corresponding SEP of this calibration model is 0.070% (w/w). Obviously, the NIRS method provides quite accurate results which approach closely the accuracy limit of the GC method. The residual plot of the prediction set is presented in Figure 5, which further illustrates the difference in water levels of these samples by these two methods. Only 3 out of 14 samples are in the region of ±0.1−0.2% (w/w) while the

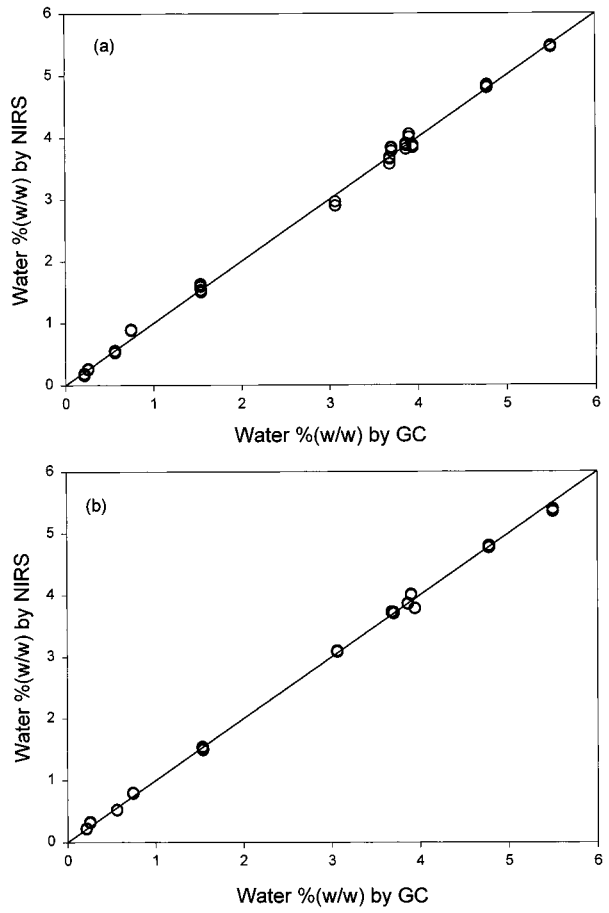


Figure 4. Concentration correlation plot for calibration (a) and prediction (b) data sets by PLS with three factors in spectral range of 1850−1960 nm.

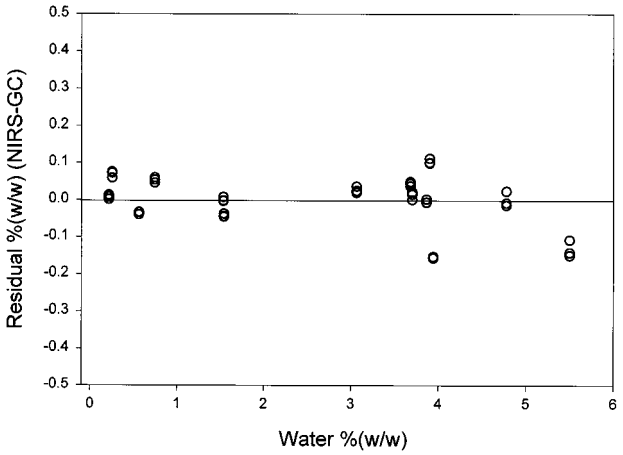


Figure 5. Residual plot for prediction data set by PLS with three factors in spectral range of 1850−1960 nm.

remaining 11 samples are in the region below 0.1% (w/w) deviation.

## CONCLUSION

To determine water in freeze-dried drug products, one can use a GC method as a reference to build an NIRS method. An SEP as low as 0.07% (w/w) was obtained for the NIRS method in the range of 0.1−5.7% water (w/w). Central to this work are the efforts that must be taken with the GC method to assure that the

low SEP can be reached by the NIRS method. Careful preparation of water standards and an optimized sample preparation and handling procedure are described for this GC method. In addition, proper application of PLS regression makes it possible to reach the most accurate and precise NIRS method.

Although the GC method requires more time and careful experimentation to test a single vial, there are some real advantages to the use of GC as a reference for NIRS. Foremost are cases where thermal and titration methods cannot be used due to the sample chemistry. In such cases, another primary method such as GC is required. It is especially attractive to co-develop an NIRS method so that the difficult primary technique can be avoided during future routine testing.

Even when simpler primary methods are available, GC might be an overall time saver if an NIRS method is being considered. This is because GC is most amenable to automating the analysis of the large numbers of vials tested during initial NIRS method development. Finally, in some cases such as very low water levels, GC might prove to be a significantly more precise primary method. With some extra work during calibration, this higher quality measurement can be carried forward in the form of an easy to use NIRS method.

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