

Reference Standards

One of the largest sources of variance in analytical results between laboratories when it comes to testing botanicals and dietary supplements can be traced to the use of reference standards. Every laboratory (hopefully) uses reference standards during an analysis, but the quality of the reference standard, how the reference standard is used, and in fact the very definition of a reference standard can vary significantly from laboratory to laboratory.

A reference standard is a type of reference material. The ISO definition of a reference material is a “Material or substance one of whose property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials” (1). The National Institute of Standards and Technology (NIST) uses this definition as well. The FDA defines a reference standard as “usually has the highest metrological quality found at a given location in a given organization, from which measurements made there are derived. Generally, this refers to recognized national or international traceable standards such as National Institute of Standards and Technology (NIST) thermometers and weights” (2).

Often we refer to “primary” and “secondary” reference standards. A primary reference standard is “a highly purified compound that serves as a reference material in all volumetric and mass titrimetric methods. The accuracy of a method is critically dependant upon the properties of this compound. Important requirements for a primary standard are:

1. High purity. Established methods for confirming purity should be available.
2. Stability toward air.
3. Absence of hydrate water so that the composition of the solid does not change with variations in relative humidity.
4. Ready availability at moderate cost.” (3)

Reference standards are used to calibrate instruments, such as HPLCs. They can be qualitative (such as for calibrating NMR chemical shifts or UV spectrophotometer monochrometers), but usually we think of reference standards as being used for quantitative purposes – calibrating a detector response against a concentration, for example.

In order for a reference standard to be useful, we need to be certain of two important properties: the identity of the reference material, and the purity of the reference material. Identity is often verified through a number of different techniques, such as mass spectroscopy to verify the molecular weight, NMR to verify the structure, melting point,

infrared spectrum, and optical rotation if it is a chiral molecule. Often two or more identity methods are used to prevent the possibility of mis-identification or a “false positive.” Often these techniques can give us some information about the purity as well, but in general they are non-quantitative techniques.

In addition to identity, purity is of critical importance to a reference standard. Reference standards should be of the highest purity reasonably possible. Even more important is knowing what the purity is to a high degree of certainty. Impurities in a reference standard fall in one of three categories: Complex organic molecules, inorganic molecules, and volatiles. Complex organic molecule impurities are often related to the reference compound, but not necessarily. Techniques such as HPLC with various detectors such as UV, ELSD, and MS are used to try to estimate the amounts of these often unknown components. These methods should be validated, and at least two different chromatographic techniques should be used to demonstrate there is no co-elution. Volatiles include water and residual solvents. Water in particular can be problematic, as the amount of water in a reference standard can change depending on the climate and storage conditions. The storage conditions need to be specified for the reference standard, and it is important that the water content was determined after storage at these conditions or it might not reflect the content at the time of use.

Inorganics are often overlooked when determining the purity of botanical reference standards, but they can be significant. We often think of organics as heavy metals, but these can include salts as well, which can contain various combinations of sodium, calcium, potassium, sulfates, nitrates, phosphates, and other inorganic ions. The salt content can be significant in some standards, and unless techniques such as residue on ignition, ICP-MS, and/or x-ray fluorescence are used to determine these constituents, the purity assignment may have little meaning.

Knowing the purity of the reference standard is important, but it is also important to use the highest purity material available. This can prevent things like co-elution of impurities, which will affect the calibration. Volatile components can change over time in a reference standard, so if a material has a high amount of water or solvent present, the manufacturer of the reference standard should make every effort to dry the material without decomposing it.

Unfortunately, many compounds sold as reference standards for botanicals do not really meet the requirements outlined above – including those touted as “primary” reference standards. Further complicating matters is the fact that these materials are often very expensive and available in small quantities (10 mg or less). As a result, not only are errors introduced due to uncertainties or inaccuracies in the purity assignments, but weighing errors can begin to become significant when trying to weigh out 5 mg of reference standard. One manufacturer even recommends weighing the vial of reference

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standard first, rinsing the contents into a volumetric flask, drying the vial under nitrogen, and re-weighing the empty vial in order to get all of the material out of the vial. This is an excellent technique if your primary concern is getting your money's worth out of the standard and not wasting any, but it is a terrible technique if you are concerned with the accuracy of your results.

Why is this an inaccurate technique? Statistically, you are now relying on 2 weighings (the before and after rinsing weights), each of which has some component of random error, in order to get the weight of your standard. At best then, the random error associated with your standard weight would increase by $\sqrt{2}$, or 1.4. It is actually much worse than that though, because the vial itself weighs much more than the material that you are trying to weigh out. As weight increases, the absolute random error on the balance also increases, even though the relative random error decreases. To illustrate this point, in the table below are 10 replicate weighings of a 1.5 mL amber HPLC vial, of the type often used for reference standards. The balance used was calibrated electronically, and the calibration verified using NIST-traceable calibration weights. The 10 replicate weighings were performed over a roughly 2 hour time span – about the amount of time that you might take between the initial weighing of the full vial, and the weighing of the emptied, cleaned and dried vial.

<i>Weighing</i>	<i>Weight (mg)</i>
1	2402.08
2	2402.09
3	2402.16
4	2402.28
5	2402.16
6	2402.18
7	2402.15
8	2402.16
9	2402.16
10	2402.23
Average:	2402.17
Standard Deviation:	0.0585
RSD:	0.0024%

The RSD is well within the balance specifications. The difference between the highest and lowest values, however, is 0.20 mg. This isn't too significant when compared to the

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average of 2402.17 mg, but it is very significant when you are trying to measure a difference of only ~5 mg. This would be a 4% variation due solely to the weighing of the standard! If the moisture content of the vial (due to absorption of humidity) is different between the initial weighing and the final weighing, this error would be even more. Reference standards, therefore, should always be directly weighed out, unless the material is an oil or paste that makes direct weighing impossible.

Because of the different standards of evidence used to assign purities to reference standards by different suppliers, the supplier of the reference standard, the lot number, and its assigned purity should always be noted. Until additional authentic reference standards are available from NIST, USP, and/or NRC, reference standards for dietary supplements will always be a source of variance in laboratory results.

References:

- (1) General Terms in Metrology, 2nd ed., BIPM/IEC/IFCC/ISO/IUPAC/IUPAP/OIML, International Organization for Standardization (ISO), 1993.
- (2) ORA Laboratory Procedure, Food and Drug Administration, "Measurement Traceability ORA-LAB.5.6, version 1.3, 2006.
- (3) Douglas A. Skoog, Donald M. West, F. James Holler, "Fundamentals of Analytical Chemistry," Saunders College Publishing: Fort Worth, 1996.