Design and Development Challenges of Natural Products Certified Reference Solutions

Authors: Tamara Tarbox, Isil Dilek, Derrell Johnson, and Mitzi Rettinger

Abstract

Laboratories often encounter multiple technical challenges in natural products testing of botanicals such as Ginkgo biloba, Ginseng, and Green tea. These phytochemicals offer significant handling issues due to inherent problems with hygroscopicity or sensitivity to air and/or light, which makes preparation of an accurate and consistent reference standard problematic. Weighing accuracy of milligram amounts of phytochemicals can be negatively affected by balance environment and weighing technique. Without consideration of proper diluent selection and controls in weighing, handling and solution preparation, accuracy and stability of phytochemical solution reference standards can be impacted. This poster will discuss these factors and their influence on the design, preparation, and certification of selected multi-component phytochemical solution standards.

Introduction

There are several factors critical to production of a high quality natural product reference standard including raw material handling, characterization and potency; certification and qualification of solutions; and homogeneity and stability of the solution. Certified phytochemical reference standard solutions prepared in a diluent that promotes stability and packaged under argon in flame-sealed ampoules can be stable for many years. Accurate results depend on accurate reference standards.

Cerilliant addresses the challenges associated with making certified phytochemical reference standards by ensuring the following:

- High purity, well-characterized components
- High purity diluents and/or stabilizers, compatible with the compound(s)
- Pre-formulation solubility and accelerated stability studies
- Environmental controls to ensure integrity of the compounds e.g. glove boxes
- Preparation using accurate, calibrated, and qualified balances
- Accuracy in solvent addition using a gravimetric approach
- Traceability of all components to container and lot
- Analysis to verify accuracy, homogeneity, and consistency
- Appropriate packaging and storage
- Assessment of shelf life

Neat Material Certification

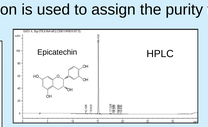
Numerous techniques may be utilized for characterization activities. Testing plans are tailored to each raw material and special consideration is given to the material's intrinsic properties.

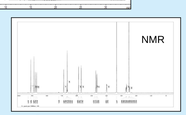
- Purity, potency and impurities
  - Mass balance – orthogonal approach
    - Multiple techniques for chromatographic purity and residuals
    - Based on ISO Guide 34; used by NIST
  - Chromatographic purity
    - Use of two techniques and different columns – values must agree within 0.5%
    - Purity and related substances – resolution of known impurities
  - Residual impurities
    - Residual water by KF – USP <921>
    - Residual solvent by GC/HS – validated Cerilliant method or USP <467>
    - Residual inorganic content – micro ROI based on USP <281>
    - NMR evaluation, EA and other techniques possible
- Identity – multiple techniques and comparison to literature references
  - 1D and 2D NMR – Proton, Carbon-13, other nuclei
  - GCMS, LCMS, LCMSMS, QTOF
  - Other techniques as needed: EA, optical rotation, DSC, melting point, TGA

All of the information collected from the neat material characterization is used to assign the purity factor for the neat material using a mass balance equation.

Compound	Chrom. Purity (%)	Residual Solvent Content (%)	Residual Water Content (%)	Trace Inorganic Content (%)	Purity Factor for Quantitative Use (%)	PF Difference from Chrom Purity (%)
(-)-Epigallocatechin 3-gallate	99.5	None detected	3.55	<0.2	95.9	-3.55
(+)-Catechin	99.5	<0.05	4.83	<0.1	94.7	-4.84
(-)-Gallocatechin	99.6	0.10	10.3	<0.1	89.3	-10.3
(-)-Catechin 3-gallate	95.1	13.4	2.34	0.50	79.7	-16.3
Ginkgolide A	99.7	0.07	4.66	<0.1	95.0	-4.73
Ginkgolide C	99.4	<0.05	5.89	<0.1	93.5	-5.89
Kaempferol	99.6	0.22	5.49	<0.2	93.8	-5.76
Quercetin	99.2	6.91	8.44	<0.2	90.7	-8.50

*Neat material certification performed by PhytoLab





Mass Balance Equation

$$PurityFactor = \left[\left[100 - (wt\%Solvents) - (wt\%H_2O) - (wt\%Inorganics)\right] * \frac{ChromPurity}{100}\right]$$

wt%Solvents: the weight percentage of residual solvents present in the neat material
wt%H_2O: the weight percentage of water present in the neat material
wt%Inorganics: the weight percentage of inorganic content in the neat material
ChromPurity: based on the chromatographic purity of the specified primary purity method; either GC or HPLC

Cerilliant® Analytical Reference Standards
a SIGMA-ALDRICH company

Pre-Formulation Studies

Useful Product Development Tool: Pre-Formulation Study

- Potential formulations evaluated for:
  - Solubility – several solvent systems investigated at once
  - Compatibility with analysis technique(s)
  - Stability in solvent
- Soluble formulations are dispensed into ampoules for accelerated stability testing
- Stability is determined by measuring purity of samples stored at different temperatures such as: sub-freezer, freezer, refrigerator, ambient, and 40°C

G-015, Ginseng Ginsenosides Mix Pre-Formulation Study

- Three formulations, 100 µg/mL each component:
  - (80:20) Acetonitrile:Water
  - (60:40) Water:Methanol
  - (60:40) Water:Methanol with 5% 1M HCl
- Acetonitrile-containing formulation:
  - Solubility issues, no accelerated stability
- Acid-containing formulation:
  - Initial purity value significantly lower
  - At temperatures ≥ refrigerate, rapid degradation
  - Sub-freezer samples did not appear to degrade

.

Ginseng Ginsenoside Mix, G-015, Pre-Formulation Study Results Summary
(Sum of %Peak Area for Eight Components)

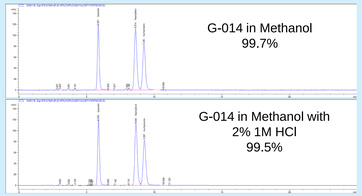
Timepoint	Storage Condition				
	Sub-freezer	Freezer	Refrigerate	Room Temp.	40°C
G-015, 100 µg/mL each Component, in (60:40) Water:Methanol with 5% 1M HCl (% Peak Area)					
Initial	88.9	88.9	88.9	88.9	88.9
1 week	89.5	88.7	71.9	20.0	9.01
2 weeks	89.3	88.3	Not analyzed due to degradation noted at 1 week timepoint		
4 weeks	89.6	87.0			
G-015, 100 µg/mL each Component, in (60:40) Water:Methanol (% Peak Area)					
Initial	97.3	97.3	97.3	97.3	97.3
1 week	97.5	97.4	97.1	96.2	96.6
2 weeks	97.4	97.3	97.3	97.4	94.5
4 weeks	96.7	96.2	96.1	96.8	No sample

Temperatures Ranges
Sub-freezer: -70°C to -80°C
Freezer: -10°C to -25°C
Refrigerate: 2°C to 8°C
Room Temperature: 15°C to 30°C

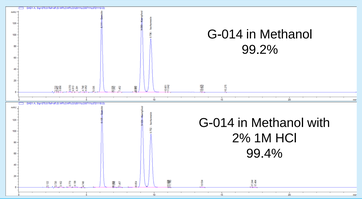
G-014, Ginkgo Biloba Flavonoids Mix Pre-Formulation Study

- Three formulations, 100 µg/mL each component:
  - Acetonitrile
  - Methanol
  - Methanol with 2% 1M HCl
- Acetonitrile-containing formulation:
  - Solubility issues, no accelerated stability
- Methanol formulations:
  - Minimal differences in purity values over 2 weeks
  - Acid formulation had higher purity at 40°C
  - All other purity values higher in Methanol without use of additives

Ginkgo Biloba Flavonoids Mix, G-014, Pre-Formulation Study Chromatograms for Initial Timepoint Analysis



Ginkgo Biloba Flavonoids Mix, G-014, Pre-Formulation Study Chromatograms for 2 Weeks at 40°C Analysis



Certified Reference Standard Preparation

To ensure accurate preparation of the reference standard solution, strict manufacturing controls are used. In addition to specific material properties (such as hygroscopicity, light or oxygen sensitivity) other considerations must be made regarding each step of the process. Robust manufacturing practices are critical to the accuracy and lot-to-lot consistency of each product.

Considerations made for reference standard preparation include:

- Material and equipment requirements
  - Hygroscopicity, static potential, sensitivity to air or light
  - Room selections, environmental controls – glove box
- Gravimetric preparation – higher precision than volumetric
  - Weight/weight
  - Better flexibility in determining batch size
  - Traceability with weigh tapes
- Dispensing
  - Equipment checks, line purges, segregation
  - Single use volumes flame-sealed under inert atmosphere
  - Sampling plans – allows batch homogeneity verification

Factors Affecting Weighing Accuracy

Tongs vs. gloved hands
Balance equilibration time
Sample and solvent temperature vs. ambient temperature
Air currents, drafts around the balance, and additional vibrational forces on the pan can significantly affect the repeatability of weighing

Hygroscopic materials are handled in glove box – inert atmosphere, relative humidity ≤ 5%

Balance environment and weighing technique can significantly influence reference accuracy

Analytical Verification and Certification

Analytical verification and certification, or release testing, comprises accuracy, consistency, homogeneity, and purity testing of the solution via HPLC/UV under various columns and run conditions. Accuracy of the prepared concentration is verified by comparison to a calibration curve or standard – a primary source or certified second source. This comparison of multiple independent preparations also includes previous lots where available which ensures lot-to-lot consistency of reference standard preparations. Homogeneity is confirmed across each batch of reference solution prepared. The solution purity is evaluated for consistency with the neat material to rule out degradation and contamination.

Release Testing Data for Natural Products Reference Standards

Catalog Number	Name	Number of Components	Accuracy: % Difference from Curve	Homogeneity: %RSD of Samples	Solution Purity (Sum of Components)
G-013	Ginkgo Biloba Terpene Lactones Mix	5	-0.76 to 0.73	0.15 to 1.40	94.9%
G-014	Ginkgo Biloba Flavonoids Mix	3	-0.86 to -0.21	0.26 to 0.33	99.9%
G-015	Ginseng Ginsenosides Mix	8	-1.30 to 0.73	0.78 to 2.50	98.1%
G-016	Green Tea Catechin Mix	8	-3.41 to -0.29	0.50 to 1.33	99.7%

Stability Testing

- Solution stability or expiration (shelf life) is established through long-term stability studies:
  - Solution purity/concentration evaluated at regular intervals – up to five years to assign expiration dates
  - Properly designed & prepared solution standards stable for years vs. weeks or months which is typical for most lab preparations
  - Most accurate information, but requires years to complete

Real-Time Stability of Anthocyanins

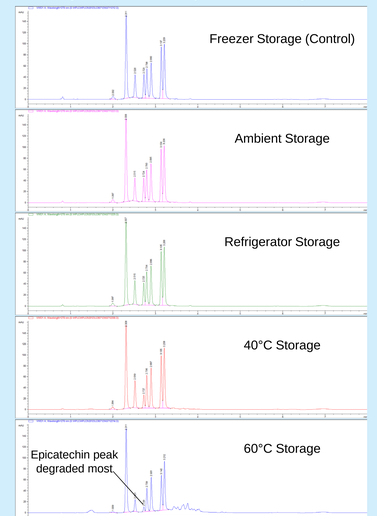
Catalog Number	Name	Neat Material Purity (%)	Solution Standard Purity (%) Release Testing	Solution Standard Purity (%) 4 years
C-069	Cyanidin-3-glucoside	94.6	95.2	93.1
C-070	Cyanidin-3-galactoside	94.6	93.4	82.3
P-057	Petunidin-3-glucoside	91.9	91.1	92.1
P-058	Petunidin-3-galactoside	92.4	90.2	88.6

- Accelerated stability studies are a useful tool in estimating shelf life:
  - Comprehensive, formalized approach needed to predict long-term stability
  - Predictive stability study performed for G-016 using Arrhenius equation

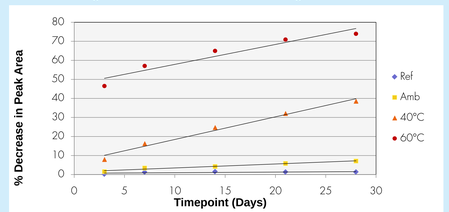
Predictive Stability Study of G-016

Time points	3, 7, 14, 21, 28 days
Storage Temperature	Freezer (as control), Refrigerator, Ambient, 40°C, 60°C
Rate (k) Plots	Degradation vs. Time for each Temperature (T)
Arrhenius Plot	Rate (1/k) vs. Temperature (1/T) for Epicatechin
Prediction from Model:	≤ 5% degradation in 4.4 years of Freezer Storage

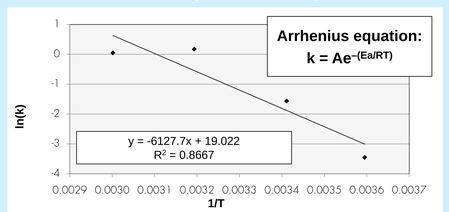
Predictive Stability Study of G-016
Chromatograms for 4 Week Timepoint at Each Storage Condition



Green Tea Catechin Mix, G-016, Epicatechin Peak Degradation Rate Curves for Four Storage Conditions*



Arrhenius Plot for Epicatechin in G-016 Determined from Degradation at Four Temperatures



*Time points and storage conditions the same as the predictive study.

Conclusions

Certified reference solutions offer significant advantages over neat reference materials, especially with regard to ease of use and handling of sensitive materials. Despite the challenges associated with handling and characterizing natural product materials, the use of a variety of development tools enables the formulation of accurate, consistent, and stable reference solutions.

Through the use of fully characterized neat materials, a mass balance approach to determining purity factor, pre-formulation studies, careful material handling and manufacturing controls, and reference standard verification against appropriate calibrators, natural product reference standards have successfully been developed.