Reference Standards in the Analysis of Botanicals

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Challenges with Botanical Reference Materials

• Complex mixtures of many different constituents
• Constituents
  – Ubiquitous
  – Characteristic
• Some constituents may be used as marker compounds for identity and quantitation
• Concentration of markers is usually low, similar to impurities in synthetic drugs
• Content of markers is inherently variable
How content is defined?

- “Activity” may depend on synergistic action of several constituents
- Safety may depend on compensating action of different constituent
- Ingredient is considered to be the whole Extract or plant material, not just a few markers, but...

*markers are all we can chemically measure*
Problems of the pure markers

- Not available
- Not pure enough
- Difficult to isolate
- Difficult to purify
- Very expensive
Examples of Pure Markers in USP RS Catalog

- Rutin
- Valerenic Acid
- Vitexin
- Formononetin
- Eleutheroside B
- Parthenolide
- Berberine
- Silybin
Problems using only one marker

• Easy adulteration

• Different Extracts or plants from different origins may have different concentrations of the same marker.

• Determination of many compounds are usually required, therefore:

  – *we need numerous expensive reference standards!*
Saw Palmetto: An ideal case
Alternatives to Expensive or Unavailable Marker Compounds

- Standardized and Quantified Extracts
- Surrogate compounds
- Problem: If the exact marker is not used, location of right compound in the chromatogram is a challenge
USP Approach to the Problem: three components linked to each other

- USP Monograph with a System Suitability Requirement
- Reference Standard Material (Extract)
- Reference Chromatogram
Use of a Reference Chromatogram in System Suitability Requirements

- USP characterizes reference extracts and assigns identity to relevant peaks.
- Each lot of reference extract is accompanied of one reference chromatogram.
- System suitability requirements are met if a chromatogram similar to that provided with each lot of reference standard is obtained by the analyst.
- The analyst now is able to identify the peaks by comparison with the reference chromatogram.
What is a similar chromatogram?

- Similar: permits differences in retention times but allows the identification of the relevant peaks by recognizing their relative abundance and elution order.

- System suitability allows modification of chromatographic system parameters (flow, column dimensions, proportions of solvents, gradient steps) in order to achieve appropriate separation.
Asian Ginseng Extract, Laboratory B
Asian Ginseng two tablets,
Examples of quantified extracts

**GINKGO TERPENE LACTONES** 50 mg

Do not dry. Each mg of this material contains 0.47 mg of bilobalide, 0.28 mg of ginkgolide A, 0.17 mg of ginkgolide B, 0.041 mg of ginkgolide C and 0.011 mg of ginkgolide J on the as is basis. Keep container tightly closed. Protect from light. Store in a freezer. This material is hygroscopic.

CAT. NO. 1291559  USP ROCKVILLE, MD  LOT I0K042
Examples of quantified extracts
Another alternative: Surrogate Standards

5 mg = $ 300
St. John wort method, run @ 588 nm
St. John wort method, run @ 270 nm
St. John wort method, run @ 588 nm
Uses of surrogate standards

• For quantitation (response factors)

• Relative retention times may vary from column to column especially if gradient are used.

• Relative response factors need multi laboratory validation
Three approaches:

- Pure Markers
  - Expensive, Candidates difficult to acquire
  - Definitive quantitation and peak identification

- Reference Standard Extracts
  - Easier to obtain, harder to characterize
  - Provide a solution to peak loci identification in complex mixtures

- Surrogate Standards
  - Readily available
  - Useful for quantitation using response factors, combined with a Reference Standard Extract and Reference Chromatograms for peak identification
Why are Reference Standards important?

How does USP develop a RS?

How are RSs used?

How should I store and handle my USP RS?

What RS are there for DS?

What are USP Certified Reference Materials (CRMs)?
What Is a Reference Standard?

A Reference Standard is a highly characterized specimen of a drug substance, excipient, major impurity, degradation product, food ingredient, or performance calibrator.

Most are intended for use in compendial methods; however some Reference Standards are available for customer convenience as a service to the industry.

When a Reference Standard is required within compendial methods, it is used to assure that products are of the appropriate identity, strength, quality, and purity.

Established and released under the authority of the USP Board of Trustees and Council of Experts.
There are many important reasons to use Reference Standards:

- Reference Standards provide users with confirmation of the identity of the article they are testing as well as of known impurities.

- Reference Standards are an integral part of performing compendial tests properly. In many cases, these materials allow users to assess the purity of manufactured materials.

- Reference Standards provide users with confirmation of the presence and content of known impurities and potential contaminants from Economically-Motivated Adulteration.
The reference materials relate directly to methods in the USP publications:
More than 3000 Reference Standards for use in the full range of *USP–NF* tests and procedures

Highly characterized specimens of:

- drug substances
- excipients
- impurities
- degradation products
- food ingredients
- dietary supplements
- compendial reagents
- performance test tablets

Rigorously tested by USP, industry, and government scientists
Reference Standard Development Process

Bulk Material Received at USP

- FDA
- USP
- Health Canada
- Industry Lab
- Industry Lab

USP RSE
(Test protocol development, Data Review and Evaluation, Purity Assignment, Internal Balloting)

Packaging, Labeling, QC

QA Review

Inventory
Procurement

- Candidate bulk material obtained from industry
- Bulk supplier provides Certificate of Analysis (CoA)
  - Data and methodology to support CoA
  - MSDS, handling, storage and stability data as available
- For donated bulks:
  - Tax deductible donation
  - Supplier receives Evaluation Package
  - House bulk traceable to USP RS
Collaborative Testing

- A minimum of three to five laboratories—USP, FDA, Health Canada, supplier, and/or other laboratory.

- Collaborators thoroughly characterize each RS candidate using compendial and non-compendial tests.

- Collaborators may or may not perform the same tests.
Test Methods Typically Employed:

- Appearance
- Identification Tests (e.g., NMR, IR, UV, HPLC, etc.)
- Indirect purity tests (e.g. Melting range, Specific rotation, Refractive index, etc)
- Direct purity tests
  - Chromatographic purity
  - Inorganic contaminants determination (ROI, etc.)
  - Volatiles (water, solvents)
- Functional group analysis (titration, UV/Vis, Elemental Analysis, etc.)
- Assays against another well-characterized standard
Data Evaluation and Internal Review

- Analyze Data/Value Assignment
  - Mass balance approach
    100% - % sum of all impurities (w/w)
  - Impurities including
    - Organic impurities by chromatography (e.g., process impurities), inorganic impurities (e.g., catalyst, salt, etc), volatile impurities (residual solvents, water)

- Prepare RS Candidate Evaluation Package
  - Demonstrate suitability for use in monograph tests
  - Create label text
    - Package size (sufficient quantity for the monograph uses), handling directions (hazard level, light sensitive, hygroscopic), storage conditions (room temperature, refrigerator, freezer)

- Review of RS material by an internal scientific panel and approval by a USP committee of experts
Echinacoside Case study

**Substance:** Echinacoside

**USP Proposed Lot:** F0

**USP Bulk Lot No.:** B100409

Submitted for NMR analysis at University of Illinois at Chicago (UIC)
NMR Operator: José G. Napolitano

The candidate lot was evaluated as described below.
HPLC impurity test results of Echinacoside before investigation

<table>
<thead>
<tr>
<th>Column/date of the vial was opened</th>
<th>RSL</th>
<th>India</th>
<th>China</th>
</tr>
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<tbody>
<tr>
<td>Waters Symmetry/Jan 10, 2012</td>
<td>3.0%, Sample was tested on Jan 12, 2012</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Waters Symmetry/Jan 10, 2012</td>
<td>7.83%, Sample was tested on June 7, 2012</td>
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<td>--</td>
</tr>
<tr>
<td>Waters Symmetry/July 5, 2012</td>
<td>0.75%, sample was freshly prepared</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>(Brand new) Phenomenex Prodigy ODS3/ July 9, 2012</td>
<td>3.58, sample was freshly prepared</td>
<td>--</td>
<td>--</td>
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<tr>
<td>YMC Pack ODS-AQ/Dec. 30, 2012</td>
<td>--</td>
<td>0.78%, Sample was tested on Jan 3, 2012</td>
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<tr>
<td>Waters Symmetry/Dec. 30, 2011</td>
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<td>2.83%, Sample was tested on May 23, 2012</td>
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<td>Phenomenex Prodigy ODS3/ Mar 8, 2012</td>
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<td>--</td>
<td>0.44, Sample was tested on April 24, 2012</td>
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<td>Waters Symmetry/Aug. 27, 2012</td>
<td>--</td>
<td>--</td>
<td>0.8, sample was freshly prepared</td>
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</table>
LC/MS spectra (0.1% TFA as Mobile Phase A)

11.05 minute peak

[M+Na]^+

12.04 minute peak

[M+Na]^+
11.05 minute peak

12.04 minute peak
Figure S1. The 1D $^1$H-NMR spectrum of Echinacoside USP in methanol-$d_4$ (800 MHz, 298 K).
Complete analysis of spectra

**Table S1.** Calculated $^1$H NMR parameters for Echinacoside USP in methanol-$d_4$ (800 MHz, 298 K).

<table>
<thead>
<tr>
<th>atom</th>
<th>$\delta$(ppm)</th>
<th>$\Delta\nu_{12}$(Hz)</th>
<th>coupling</th>
<th>$J$(Hz)</th>
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</thead>
<tbody>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>H-1</td>
<td>4.390</td>
<td>1.57</td>
<td>H-1 / H-2</td>
<td>7.90</td>
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<tr>
<td>H-2</td>
<td>3.397</td>
<td>1.69</td>
<td>H-2 / H-3</td>
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<tr>
<td>H-3</td>
<td>3.813</td>
<td>1.78</td>
<td>H-3 / H-4</td>
<td>9.34</td>
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<tr>
<td>H-4</td>
<td>5.013</td>
<td>1.86</td>
<td>H-4 / H-5</td>
<td>10.05</td>
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<tr>
<td>H-5</td>
<td>3.767</td>
<td>2.09</td>
<td>H-5 / H-6a</td>
<td>2.36</td>
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<tr>
<td>H-6a</td>
<td>3.943</td>
<td>2.12</td>
<td>H-5 / H-6b</td>
<td>5.61</td>
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<tr>
<td>H-6b</td>
<td>3.637</td>
<td>2.17</td>
<td>H-6a / H-6b</td>
<td>-11.60</td>
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<tr>
<td>Glucose $^3$</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>H-1</td>
<td>4.299</td>
<td>1.48</td>
<td>H-1 / H-2</td>
<td>7.77</td>
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<tr>
<td>H-2</td>
<td>3.210</td>
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<td>H-2 / H-3</td>
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<td>H-3</td>
<td>3.342</td>
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<td>H-3 / H-4</td>
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<td>9.73</td>
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<td>H-5 / H-6b</td>
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<td>H-6b</td>
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<td>2.06</td>
<td>H-6a / H-6b</td>
<td>-11.95</td>
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<td>Rhamnose</td>
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<tr>
<td>H-1</td>
<td>5.185</td>
<td>1.83</td>
<td>H-1 / H-2</td>
<td>1.78</td>
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<td>H-2</td>
<td>3.921</td>
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<td>H-2 / H-3</td>
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<td>H-5 / Me-6</td>
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<tr>
<td>Me-6</td>
<td>1.086</td>
<td>1.86</td>
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</table>

**3-OH-Tyrosol**

<table>
<thead>
<tr>
<th>atom</th>
<th>$\delta$(ppm)</th>
<th>$\Delta\nu_{12}$(Hz)</th>
<th>coupling</th>
<th>$J$(Hz)</th>
</tr>
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<tbody>
<tr>
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<td>H-2 / H-6</td>
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<td>H-6</td>
<td>6.577</td>
<td>1.64</td>
<td>H-5 / H-6</td>
<td>8.01</td>
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<tr>
<td>H-7a</td>
<td>2.805</td>
<td>2.20</td>
<td>H-7a / H-7b</td>
<td>-13.72</td>
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<tr>
<td>H-7b</td>
<td>2.787</td>
<td>2.39</td>
<td>H-7a / H-8a</td>
<td>8.56</td>
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<tr>
<td>H-8a</td>
<td>3.730</td>
<td>1.92</td>
<td>H-7a / H-8b</td>
<td>6.35</td>
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<tr>
<td>H-8b</td>
<td>4.035</td>
<td>1.90</td>
<td>H-7b / H-8a</td>
<td>6.29</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-2</td>
<td>7.061</td>
<td>1.48</td>
<td>H-2 / H-5</td>
<td>0.28</td>
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<td>H-5</td>
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<td>H-2 / H-6</td>
<td>2.10</td>
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<td>H-6</td>
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<td>1.57</td>
<td>H-5 / H-6</td>
<td>8.17</td>
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<tr>
<td>H-7</td>
<td>7.602</td>
<td>1.61</td>
<td>H-7 / H-8</td>
<td>15.84</td>
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<tr>
<td>H-8</td>
<td>6.281</td>
<td>1.34</td>
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</table>

**Diagram:**

- Glucose
- 3-OH-Tyrosol
- Rhamnose
“Residual” Solvents in Echinacoside

Calculated $^1$H NMR Spectrum

Observed $^1$H NMR Spectrum Methanol-$d_4$, 800 MHz
Stability of Echinacoside exposed to light

Figure S21. Monitoring the isomerization of Echinacoside to cis-Echinacoside. The 1D $^1$H-NMR spectra of Echinacoside USP in methanol-$d_4$ (600 MHz, 298 K) after one week (red) and two weeks (blue) of storage without light protection. Red arrows indicate the presence of new $^1$H resonances due to the impurity cis-Echinacoside (not detected during the analysis of fresh samples at 800 MHz and 600 MHz).
Stage 1: Test Protocol

Ensure test protocol is:
- Robust
- Test appropriate
- Outcome based

Address elements that could impact quality
Take advantage of accumulative knowledge
Stage 2: Draft RSCEP

- Ensure RSCEP fulfills purpose (clear, concise, logical)
- Address any issue (all data supports conclusion)
- Eliminate alternative interpretations and conclusions
- Inspection of number/data transcription
Stage 3: Balloting

Approve reference standard candidate for intended use

Intent is not verification of material purity value

Reviewer only receives RSCEP (Executive Summary, Analytical Summary, Monograph)
Packaging, QC testing, QA review, External review, and Continuing Suitability for Use

- Determine packaging configuration
  - Humidity control/actinic light/O2 sensitive
- QC testing/QA approval/Release
- Approval of suitability for compendial use by Expert Committee
- Continuing Suitability for Use (CSU) studies
There are two main types of USP Reference Standards:

Standards with Quantitative Applications
- Assays (for drug substances and for formulations)
- Limit tests (e.g., Impurity Reference Standards)

Standards with only Qualitative Applications
- Identification tests
- Elution markers
- System Suitability tests
Proper Reference Standard Use

- RSs are provided solely for use as required in pharmacopeial tests and assays
- Suitability for other non-official application(s) rests with the purchaser
- For quantitative standards
  - Purity of 100.0% unless labeled otherwise
- Value assigned for assay and limit standards
- Use the current or valid lot
  - How do I determine the current lot?
From the USP Catalog...

<table>
<thead>
<tr>
<th>Catalog Number</th>
<th>Description</th>
<th>Current Lot</th>
<th>Purity Value/Conc.</th>
<th>Change Code</th>
<th>Previous Lot Valid Use Date</th>
<th>CAS Number</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>1009923</td>
<td>Acetyltriethyl Citrate (500 mg)</td>
<td>H0I339</td>
<td></td>
<td></td>
<td>G0E085 (08/11) F-1 (01/07) F (05/02)</td>
<td>[77-89-4]</td>
<td>$199</td>
</tr>
<tr>
<td>1011007</td>
<td>Acitretin (200 mg)</td>
<td>F0E266</td>
<td>0.998mg/mg</td>
<td></td>
<td></td>
<td>[55079-83-9]</td>
<td>$465</td>
</tr>
</tbody>
</table>

Current Lot Good until end of use date assigned

Lot G0E085 valid until August 31, 2011
How to Dry a RS

- Use a clean and dry vessel
- Do not use original container as a drying vessel
- Do not dry a specimen repeatedly at temperatures above 25
Reference Standard Storage

How to Store a RS

- Use original stoppered containers
- Keep away from heat
- Protect from light
- Avoid humid storage areas
- When special conditions are necessary, directions are given on the label
Label text

- Provides storage and use information
- Calculation value for standards with quantitative applications.
- Safety warnings

Label text takes precedence over compendium or catalog

Additional documentation provided where necessary (technical sheets or typical chromatograms)

REBAUDIOSIDE A – an Example of FCC Standard

- Appearance
- NMR
- IR
- Mass Spectral (MS) analysis
- Water Content
- Elemental Analysis
- Chromatographic Purity by HPLC
- Residual Solvents
- Specific Rotation
- Residue on Ignition
- Vapor Sorption Analysis
REBAUDIOSIDE A – an Example of FCC Standard - Reference Standard Label Text

Information included in the Label Text:

- Name and Quantity
- Safety warnings
- Required information for controlled substances
- Directions for use
- Calculation value for quantitative use standards
- Accompanying Technical Sheets (e.g., typical chromatogram)
Powdered Bilberry Extract—an Example of Reference Standard

Uses in Powdered Bilberry Extract Monograph

<table>
<thead>
<tr>
<th>Monograph</th>
<th>ID</th>
<th>Content of Anthocyanosides and Anthocyanidins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Powdered Bilberry Extract</td>
<td>TLC, HPLC</td>
<td>HPLC</td>
</tr>
</tbody>
</table>
Powdered Bilberry Extract

- Appearance
- IR
- HPLC
- TLC
- Water Content
- Residue on Ignition
- Vapor Sorption Analysis
Information included in the Label Text:

- Name and Quantity
- Safety warnings
- Required information for controlled substances
- Directions for use
- Calculation value for quantitative use standards
- Accompanying Technical Sheets (e.g., typical chromatogram)
Powdered Bilberry Extract—Typical Chromatogram

1 - Delphinidin-3-O-galactoside chloride
2 - Delphinidin-3-O-glucoside chloride
3 - Cyanidin-3-O-galactoside chloride
4 - Delphinidin-3-O-arabinoside chloride
5 - Cyanidin-3-O-glucoside chloride
6 - Petunidin-3-O-galactoside chloride
7 - Cyanidin-3-O-arabinoside chloride
8 - Petunidin-3-O-glucoside chloride
9 - Delphinidin chloride
10 - Peonidin-3-O-galactoside chloride
11 - Petunidin-3-O-arabinoside chloride
12 - Peonidin-3-O-glucoside chloride
13 - Malvidin-3-O-galactoside chloride
14 - Peonidin-3-O-arabinoside chloride
15 - Malvidin-3-O-glucoside chloride
16 - Cyanidin chloride
17 - Malvidin-3-O-arabinoside chloride
18 - Petunidin chloride
19 - Peonidin chloride
20 - Malvidin chloride
USP Certified Reference Materials (CRMs)

- USP CRMs are a new generation of USP Reference Standards
- CRMs undergo additional metrologically-based testing and statistical analysis to meet both USP's criteria and ISO guidelines
- CoA complies with ISO Guide 31 data...
  - Certified property value with uncertainty
  - Expiration date/period of validity for assigned value
- Certified values meet ISO 17025 documentation requirement for testing labs
Questions
Thank You