Chemical Metrology for Food and Nutrition Assessment

Metrology and Physical Constants
International School of Physics “Enrico Fermi”

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Chemical Measurements in Food

- Nutrients (proximates, vitamins, elements)
- Phytochemicals
- Allergens
- Additives and Contaminants
  - Colors
  - Flavors
  - Hormones
  - Drug Residues
  - Toxic Metals, MeHg
  - Mycotoxins
  - Preservatives
  - GMOs
  - PCBs, Pesticides, etc.
Nominal Concentrations of Measurands in Foods

- **1 g/g**
  - proximates
  - dietary fiber
  - minerals

- **1 mg/g**
  - GMOs
  - trans fatty acids
  - caffeine

- **1 μg/g**
  - nitrates
  - vitamins
  - allergens
  - toxic elements (lead, mercury)

- **1 ng/g**
  - pesticides
  - marine biotoxins (okadaic acid, yessotoxins)
  - veterinary drug residues

- **1 pg/g**
  - mycotoxins (aflatoxin, ochratoxin)
  - polycyclic aromatic hydrocarbons (PAHs)
  - dioxins and dioxin-like PCBs
Current Prominent Food Issues

- Allergens
- Carbohydrate/Fiber
- Fat content
- Trans Fats (Natural vs. Incurred)
- Genetically-Engineered Foods (GMOs)
- Mycotoxins and Phycotoxins
- Hormones and Veterinary Drug Residues
- Methylmercury, PAHs, PCBs, Pesticides, PBDEs, Perfluorinated Compounds
- “Functional” Foods
- Food Safety/Security
Label on processed foods must specify:

- serving size in common household units
- number of servings per container
- total number of calories derived from all sources and derived from fat in each serving
- amount of total fat, saturated fat, cholesterol, sodium, total carbohydrate, dietary fiber, sugars, total protein, vitamin A, vitamin C, calcium, and iron contained in each serving
- any other vitamin or mineral or other nutrient required to assist the consumer in maintaining a healthy diet
The food matrix organizational scheme can be used to select one or two food matrices representing each sector, for development of a series of reference materials representing all foods. In some sectors, several samples may be necessary to account for differences in all the types of protein, fat, or carbohydrate.


Careful selection of two foods or food products from each sector will cover the entire range of carbohydrate, protein, and fat, as well as other food attributes.

NIST Food-Matrix SRMs in 2012

1. SRM 1588c Organics in Fish Oil
   SRM 3274 Fatty Acids in Botanical Oils
   SRM 3275 Fatty Acids in Fish Oils
2. SRM 2384 Baking Chocolate
3. SRM 2387 Peanut Butter
4. SRM 1546 Meat Homogenate
   SRM 1845a Whole Egg Powder*
5. SRM 3287 Blueberries
   SRM 2383a Baby Food Composite*
   SRM 3233 Fortified Breakfast Cereal*
6. SRM 1849 Infant/Adult Nutritional Formula
   SRM 1548a Typical Diet
   SRM 1544 Fatty Acids in a Frozen Diet Composite
   SRM 1549a Whole Milk Powder*
7. SRM 1566b Oyster Tissue
   SRM 2385 Slurried Spinach
   SRM 3234 Soy Flour*
   SRM 3290 Dry Cat Food*
8. SRM 1946 Lake Superior Fish Tissue
   SRM 1947 Lake Michigan Fish Tissue
   SRM 1974c Mussel Tissue*
   SRM 3252 Protein Drink Mix*

* in preparation
SRM 1846 Infant Formula vs. SRM 1849a Infant/Adult Nutritional Formula

- Proximates
- Fatty acids
- **Vitamins C, B₂, B₆, and niacin**
- Other water-soluble vitamins, tocopherols, and vitamins D and K
- Ca, P, Mg, Fe, Zn, Cu, Na, K, I, Cl
- **5 Certified; 38 Reference (issued 1996)**

- Proximates
- Fatty acids, cholesterol
- **Vitamins C, B₁, B₂, B₃, B₅, B₆, B₁₂ folic acid, biotin, choline, carnitine**
- Vitamins A, D, E, and K
- Ca, P, Mg, Fe, Zn, Cu, Na, K, Mn, Cr, Mo, Se
- Amino acids
- Nucleotides
- **42 Certified; 44 Reference**

*bold = certified values*
*normal typeface = reference values*
## Certified Values for Elements in SRM 1849a Infant/Adult Nutritional Formula

<table>
<thead>
<tr>
<th>Element</th>
<th>Mass Fraction (mg/kg)</th>
<th>Mass Fraction (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>5253 ± 51</td>
<td>Molybdenum</td>
</tr>
<tr>
<td>Copper</td>
<td>19.78 ± 0.26</td>
<td>1.707 ± 0.040</td>
</tr>
<tr>
<td>Chromium</td>
<td>1.072 ± 0.032</td>
<td>Phosphorus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3990 ± 140</td>
</tr>
<tr>
<td>Iodine</td>
<td>1.29 ± 0.11</td>
<td>Potassium</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9220 ± 110</td>
</tr>
<tr>
<td>Iron</td>
<td>175.6 ± 2.9</td>
<td>Selenium</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.812 ± 0.029</td>
</tr>
<tr>
<td>Magnesium</td>
<td>1648 ± 36</td>
<td>Sodium</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4265 ± 83</td>
</tr>
<tr>
<td>Manganese</td>
<td>49.6 ± 1.0</td>
<td>Zinc</td>
</tr>
<tr>
<td></td>
<td></td>
<td>151.0 ± 5.6</td>
</tr>
</tbody>
</table>

- **NIST ICP-OES**
- **Collaborating Labs**
- **Manufacturer**
- **NIST ICP-MS**
- **NIST-INAA**
# Certified Values for Vitamins in SRM 1849a Infant/Adult Nutritional Formula

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Mass Fraction (mg/kg)</th>
<th>Mass Fraction (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic Acid</td>
<td>784 ± 65</td>
<td></td>
</tr>
<tr>
<td>Thiamine</td>
<td>12.57 ± 0.98</td>
<td></td>
</tr>
<tr>
<td>Riboflavin</td>
<td>20.37 ± 0.52</td>
<td></td>
</tr>
<tr>
<td>Niacinamide</td>
<td>109 ± 10</td>
<td></td>
</tr>
<tr>
<td>Pantothenic Acid</td>
<td>68.2 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>13.46 ± 0.93</td>
<td></td>
</tr>
<tr>
<td>Folic Acid</td>
<td>2.293 ± 0.062</td>
<td></td>
</tr>
<tr>
<td>Biotin</td>
<td>1.99 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>Choline Ion</td>
<td>1090 ± 110</td>
<td></td>
</tr>
</tbody>
</table>

- **NIST LC/abc**
- **Collaborating Laboratories**
- **Manufacturer**
- **NIST ID-LC/MS**
- **NIST LC/MS**
SRMs for Dietary Supplements

Cover image: Ginkgo leaves overlaid with ginkgo leaf “fingerprint” showing ginkgolides and bilobalide. Chromatogram generated using liquid chromatography with an evaporative light scattering detector courtesy of Catherine A. Rimmer (NIST). Photo courtesy of Lane C. Sander (NIST).
Why SRMs for Dietary Supplements?

- 68% of the US population use dietary supplements to increase their daily intake of nutrients and/or for perceived health benefits
- In 2007, US consumers spent nearly $24 billion on dietary supplements (only 32% of global market)
- Concerns:
  - Consumer safety (contamination, adulteration)
  - Mislabeling of product
  - Accuracy of the product label

Source: Nutrition Business Journal
Dietary Supplement SRMs Currently Available since 2001

**Ephedra sinica Suite**
(alkaloids, caffeine, toxic elements)

**Ginkgo biloba Suite**
(Ginkgolides, flavonoids, toxic elements)

**Bitter Orange Suite**
(adrenergic amines, caffeine)

**Botanical Oils**
(omega-3 and omega-6 fatty acids)

**Saw Palmetto Suite**
(Fatty acids, phytosterols, carotenoids, tocopherols)

**Carrot Extract in Oil**
(carotenoids, tocopherols)

**Multivitamin Tablets**
(15 vitamins, 18 elements)
Alkaloids in *Ephedra sinica* Stapf Aerial Parts (Three Analytical Methods)

- **LC-UV**
  - Terbutaline used as internal standard (IS)
  - Excellent precision
  - Matrix interferences may limit accuracy

- **LC-MS**
  - Ephedrine-d$_3$ used as IS
  - Best precision for ephedrine

- **LC-MS/MS**
  - Ephedrine-d$_3$ used as IS
  - Best precision for ephedrine
  - Overall precision for non-IDMS poor compared to LC-UV

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>LC-UV Avg</th>
<th>RSD (%)</th>
<th>LC-MS Avg</th>
<th>RSD (%)</th>
<th>LC-MS/MS Avg</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synephrine</td>
<td>0.37</td>
<td>10.6%</td>
<td>0.60</td>
<td>10.5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norephedrine</td>
<td>0.13</td>
<td>2.5%</td>
<td>0.20</td>
<td>5.5%</td>
<td>0.14</td>
<td>4.4%</td>
</tr>
<tr>
<td>Norpseudoephedrine</td>
<td>0.12</td>
<td>1.5%</td>
<td>0.20</td>
<td>8.7%</td>
<td>0.19</td>
<td>4.9%</td>
</tr>
<tr>
<td>Ephedrine</td>
<td>11.21</td>
<td>1.0%</td>
<td>11.37</td>
<td>2.2%</td>
<td>10.26</td>
<td>1.2%</td>
</tr>
<tr>
<td>Pseudoephedrine</td>
<td>2.67</td>
<td>2.2%</td>
<td>2.75</td>
<td>4.0%</td>
<td>3.06</td>
<td>3.4%</td>
</tr>
<tr>
<td>Methylephedrine</td>
<td>0.29</td>
<td>3.5%</td>
<td>0.38</td>
<td>4.3%</td>
<td>0.35</td>
<td>4.7%</td>
</tr>
<tr>
<td>Methylpseudoephedrine</td>
<td>0.06</td>
<td>5.5%</td>
<td>0.03</td>
<td>7.8%</td>
<td>0.02</td>
<td>12.2%</td>
</tr>
</tbody>
</table>
Alkaloids in Bitter Orange Fruit (Four Analytical Methods)

- **LC-UV**
  - Terbutaline used as internal standard (IS)
  - Excellent precision
  - Matrix interferences prevent measurement (except synephrine)
  - Matrix interferences limit accuracy

- **LC-FL**
  - Terbutaline used as IS
  - Matrix interferences are reduced; all alkaloids could be determined

- **LC-MS**
  - Synephrine-d$_3$ used as IS
  - Best precision for synephrine

- **LC-MS/MS**
  - Synephrine-d$_3$ used as IS
  - Best precision for synephrine

- Overall precision for non-IDMS poor compared to LC-UV and LC-FL

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>LC-UV avg</th>
<th>RSD (%)</th>
<th>LC-FL avg</th>
<th>RSD (%)</th>
<th>LC-MS avg</th>
<th>RSD (%)</th>
<th>LC-MS/MS avg</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>synephrine</td>
<td>9.24</td>
<td>1.9%</td>
<td>9.14</td>
<td>0.7%</td>
<td>8.95</td>
<td>1.4%</td>
<td>9.13</td>
<td>1.7%</td>
</tr>
<tr>
<td>n-methyltyramine</td>
<td>0.18</td>
<td>1.2%</td>
<td>0.17</td>
<td>2.9%</td>
<td>0.18</td>
<td>10.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>octopamine</td>
<td>0.13</td>
<td>1.1%</td>
<td>0.13</td>
<td>6.3%</td>
<td>0.11</td>
<td>18.7%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tyramine</td>
<td>0.02</td>
<td>8.1%</td>
<td>0.03</td>
<td>7.0%</td>
<td>0.05</td>
<td>14.6%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hordenine</td>
<td>0.01</td>
<td>20.4%</td>
<td>0.01</td>
<td>20.4%</td>
<td>0.02</td>
<td>20.4%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Dietary Supplement SRMs in Progress

**Green Tea Suite**
(catechins, caffeine, theanine)

**Vaccinium Berry Suite**
(organic acids, flavonols, anthocyanidins, procyanidins)

**Kudzu Suite**
(isoflavones)

**Soy Suite**
(isoflavones)

**Red Clover Suite**
(isoflavones)

**St. John’s Wort Suite**
(hypericin, hyperforin, flavonoids, toxic elements)

**Black Cohosh Suite**
(triterpene glycosides)

**Pomegranate**
(organic acids, punicaligins, anthocyanidins)

**Tumeric**
curcumin
Ginkgolides in SRM 3248 Ginkgo-Containing Tablets

Use of LC columns with different selectivity and different MS detection modes
SRM 3280 Multivitamins/Multielement Tablets

- Target analytes:
  - fat soluble vitamins (4)
  - water soluble vitamins (6)
  - carotenoids (2)
  - biotin
  - folic acid
  - Vitamin B$_{12}$
  - chloride
  - elements (17)

- For the vitamins and carotenoids, isotope dilution LC/MS and/or LC/MS/MS methods developed

**Supplement Facts**

<table>
<thead>
<tr>
<th>Serving Size 1 Tablet</th>
<th>Each Tablet Contains</th>
<th>%DV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Niacin 20 mg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Vitamin B$_{6}$ 2 mg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Folic Acid 400 mcg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Vitamin B$_{12}$ 6 mcg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Biotin 30 mcg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Pantothenic Acid 10 mg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Calcium 162 mg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Iron 18 mg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Phosphorus 109 mg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Iodine 150 mcg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Magnesium 100 mg</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>Zinc 15 mg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Selenium 20 mcg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Copper 2 mg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Manganese 2 mg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Chromium 120 mcg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Molybdenum 75 mcg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Chloride 72 mg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Potassium 80 mg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Boron 150 mcg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Nickel 5 mcg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Silicon 2 mg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Tin 10 mcg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Vanadium 10 mcg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Lutein 250 mcg</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Lycopene 300 mcg</td>
<td>100%</td>
</tr>
</tbody>
</table>

*Daily Value (%DV) not established.*
LC-UV Method for Water-Soluble Vitamins

Complementary separations of water-soluble vitamins in SRM 3280 Multivitamin/Multielement Tablets

YMC C18 Pro
Phosphate buffer (pH 3.1):ACN

Cadenza CD-C18
Ammonium formate buffer (pH 4):MeOH
LC/MS Determination of Water-Soluble Vitamins in SRM 3280 Multivitamin/Multielement Tablets

- Multimode ionization (ESI + APCI)
- Isotope dilution MS for all analytes
LC-UV Determination of Fat-Soluble Vitamins in Multivitamin Tablets (SRM 3280)

Intermediate C\textsubscript{18} Column

C\textsubscript{30} Column
## Certified Values for Vitamins in SRM 3280 Multivitamin/Multielement Tablets

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Mass Fraction (mg/g)</th>
<th>Mass Fraction (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>42.2 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>1.06 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>Riboflavin</td>
<td>1.32 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>Niacinamide</td>
<td>14.10 ± 0.23</td>
<td></td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>7.30 ± 0.96</td>
<td></td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>1.81 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>21.4 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>Folic acid</td>
<td></td>
<td>394 ± 22</td>
</tr>
<tr>
<td>Biotin</td>
<td></td>
<td>23.4 ± 3.2</td>
</tr>
<tr>
<td>Ergocalciferol</td>
<td></td>
<td>9.13 ± 0.71</td>
</tr>
<tr>
<td>Phylloquinone</td>
<td></td>
<td>22.8 ± 2.2</td>
</tr>
<tr>
<td>Trans-β-carotene</td>
<td></td>
<td>420 ± 100</td>
</tr>
<tr>
<td>Total β-carotene</td>
<td></td>
<td>514 ± 87</td>
</tr>
</tbody>
</table>

*Note: The data reflects the mass fraction values (mg/g) and mass fraction values (μg/g) for various vitamins in SRM 3280 Multivitamin/Multielement Tablets. The symbols indicate different analytical methods used for measurement.*
Use of Independent Methods for Value Assigning SRMs for Organic Constituents

Use of Independent Analytical Methods to Exploit Differences in

- Extraction
  - Techniques
    - Soxhlet
    - Ultrasonic
    - Pressurized Fluid
    - Supercritical Fluid
    - Microwave-assisted
    - Mechanically agitated
  - Parameters
    - Solvent
    - Temperature
    - Pressure
    - pH

- Cleanup
  - Off-Line Approaches
    - Liquid-Liquid Extraction
    - Column Chromatography
    - Liquid Chromatography
    - Solid Phase Extraction (SPE)
    - Solid Phase Microextraction (SPME)

- Isolation
  - Instrumental Approaches
    - Gas Chromatography (GC)
    - Liquid Chromatography (LC)
    - Ion Chromatography
    - Electrophoresis
    - Multidimensional Separation

- Enrichment
  - GC
  - LC

- Separation
  - GC
  - LC
  - MS
  - MS/MS
  - FID
  - ECD
  - Flame photometric
  - AED
  - Absorbance
  - Fluorescence
  - Electrochemical
  - ELSD
  - CAD

- Detection
  - Calibration
    - External Standard
    - Internal Standard
    - Isotope Dilution
    - Standard Addition
  - Model
    - Linear Regression
    - Slope/Intercept
    - Zero Intercept
    - Bracketed Calibration
    - Exact Matching
    - Nonlinear Calibration

Minimize the possibility of undetected bias in resulting certified concentrations

MS/MS provides excellent specificity
SRM 1845a Whole Egg Powder

Pyridoxamine (B₆-PM)
- 169 → 77
- 172 → 79
- 169 → 134
- 172 → 136
- 172 → 152
- 124 → 80
- 128 → 84

Niacin (B₃-OH)
- 124 → 52
- 128 → 53
- 124 → 56
- 124 → 78
- 128 → 81

Thiamine (B₁)
- 266 → 42
- 269 → 42
- 266 → 81
- 269 → 123

Niacinamide (B₃-NH₂)
- 123 → 53
- 123 → 78
- 123 → 81
- 123 → 80

Pantothenic Acid (B₅)
- 220 → 41
- 220 → 43
- 220 → 72
- 224 → 76

Riboflavin (B₂)
- 377 → 43
- 377 → 172
- 377 → 198
- 377 → 202

Pyridoxal (B₆-PL)
- 168 → 41
- 168 → 67
- 168 → 94
- 168 → 150

Pyridoxine (B₆-PN)
- 170 → 77
- 170 → 80
- 170 → 134
- 170 → 152

Melissa Phillips, NIST
ID LC-MS/MS Determination of Folic Acid and 5-Methyl-THF in Food SRMs

<table>
<thead>
<tr>
<th>SRM</th>
<th>Folate</th>
<th>Value type</th>
<th>Value (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRM 3233 Fortified Breakfast Cereal</td>
<td>Folic acid</td>
<td>Preliminary</td>
<td>15.80 ± 0.78</td>
</tr>
<tr>
<td>SRM 1849a Infant/Adult Nutritional Formula</td>
<td>Folic acid</td>
<td>Certified</td>
<td>2.293 ± 0.062</td>
</tr>
<tr>
<td>SRM 1849a</td>
<td>5-MTHF</td>
<td>Reference</td>
<td>0.0839 ± 0.0031</td>
</tr>
<tr>
<td>SRM 1549a Whole Milk Powder</td>
<td>5-MTHF</td>
<td>Preliminary</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>SRM 1845a Whole Egg Powder</td>
<td>5-MTHF</td>
<td>Preliminary</td>
<td>0.84 ± 0.04</td>
</tr>
</tbody>
</table>

Johanna Camara, NIST
Sample Preparation for Inorganic Analysis for SRM 3280

• X-ray Fluorescence
  – Borate fusion glass beads

• ICP-OES
  – Open Beaker Digestion

• ICP-MS
  – Microwave digestion
Open Beaker Digestion for ICP-OES (Difficult and Not Recommended)

1. 0.35 g of ground tablets from each of 6 bottles
2. 10 mL HNO\textsubscript{3} + 10 mL H\textsubscript{2}O
3. Spike with In and Sc internal standards
4. Cover and heat at 200 °C overnight
5. 2 mL HF + 5 mL HClO\textsubscript{4}, heat to near dryness
6. Repeat step 5 three times
7. 1 mL HClO\textsubscript{4} and dilute to 100 g with 1.5 % HNO\textsubscript{3}
8. Dilute as necessary, split into 2 aliquots, spike one with analyte(s) for standard additions
Microwave Digestion for ICP-MS (much easier)

- 0.45 g of ground tablets from each of 6 bottles into Teflon microwave vessels
  - CEM MARSXpress microwave system
- 9 mL HNO$_3$ + 1 mL HF
- 1200 W, 100% power, 15 min. ramp to 200 °C, hold 20 min., cool 15 min.
- Spike with Rh internal standard, dilute to 100 mL with 1.5% HNO$_3$, split and spike one portion with multielement solution for standard additions
Single tablets analyzed

Nominal level

Copper

mass fraction (mg/g)
Iron

mass fraction (mg/g)

Nominal level
17. NIST PGAA
19. NIST XRF
18. NIST INAA
20. GMA
21. USDA ICP

Single tablets analyzed
INSTRUCTIONS FOR USE
Prior to removal of a test portion for analysis, at least 15 tablets must be ground to obtain a homogeneous sample. A test portion for analysis is then removed from this powder. NIST analysts used either of two methods to grind pellets to a powder prior to analysis: Thirty tablets were ground in a disk mill, which involved shaking in an orbital pattern for 6 min. Batches of 15, 20, or 30 tablets were ground for 10 min using an automated mortar and pestle. (Note that 10 min of shaking in a ball mill did not grind the tablets, particularly the coating material, as finely as did the other techniques.) For certified values to be valid, test portions of the powder equal to or greater than 0.6 g to 2 g for carotenoids and fat-soluble vitamins, 0.3 g to 2 g for water-soluble vitamins, and 0.25 g to 4.5 g for elements should be used. Test portions should be analyzed as received and results converted to a dry-mass basis by determining moisture content (described below) on a separate test portion.
## Elements in SRM 3280 Multivitamin/Multielement Tablets

<table>
<thead>
<tr>
<th>Certified Values</th>
<th>Mass Fraction (mg/g)</th>
<th>Reference Values</th>
<th>Mass Fraction (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boron</td>
<td>0.141 ± 0.007</td>
<td>Antimony</td>
<td>0.159 ± 0.008</td>
</tr>
<tr>
<td>Calcium</td>
<td>110.7 ± 5.3</td>
<td>Cobalt</td>
<td>0.81 ± 0.01</td>
</tr>
<tr>
<td>Chlorine</td>
<td>53.0 ± 2.3</td>
<td>Lanthanum</td>
<td>0.70 ± 0.01</td>
</tr>
<tr>
<td>Chromium</td>
<td>0.0937 ± 0.0027</td>
<td>Nickel</td>
<td>8.4 ± 0.8</td>
</tr>
<tr>
<td>Copper</td>
<td>1.40 ± 0.17</td>
<td>Selenium</td>
<td>17.6 ± 0.8</td>
</tr>
<tr>
<td>Iron</td>
<td>12.35 ± 0.91</td>
<td>Silicon</td>
<td>2010 ± 10</td>
</tr>
<tr>
<td>Magnesium</td>
<td>67.8 ± 4.0</td>
<td>Sodium</td>
<td>330 ± 20</td>
</tr>
<tr>
<td>Manganese</td>
<td>1.44 ± 0.11</td>
<td>Strontium</td>
<td>29.8 ± 0.2</td>
</tr>
<tr>
<td>Molybdenum</td>
<td>0.0707 ± 0.0045</td>
<td>Tin</td>
<td>11.1 ± 0.9</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>75.7 ± 3.2</td>
<td>Titanium</td>
<td>5400 ± 300</td>
</tr>
<tr>
<td>Potassium</td>
<td>53.1 ± 7.0</td>
<td>Vanadium</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>Zinc</td>
<td>10.15 ± 0.81</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **INAA**
- **PGAA**
- **XRF**
- **ICP-MS**
- **ICP-OES**
- **Collaborating Labs**
Why are Nutritional Biomarkers Important?

- Identifying individuals with vitamin deficiencies
- Not everyone responds the same way to nutrient exposure
- Difficult to quantify intake based on diet and self-reporting
- Understanding biochemical pathways
- Population surveys and public health policies

Data is meaningful *only* if the measurement methods used are accurate.
Why is Vitamin D Important?

- Vitamin D is essential for maintaining calcium homeostasis
- Both Calcium and vitamin D are needed for bone health
- Vitamin D deficiency associated with rickets and osteomalacia
- Potential link between vitamin D deficiency and increased disease risk

Low levels are blamed for many of our ills. But how much is really enough?
Vitamin D

Vitamin D occurs primarily in two forms – vitamin D$_2$ and vitamin D$_3$

- **Sunlight**
  - Production of vitamin D$_3$ in skin

- **Food**
  - Contains vitamin D$_2$ or D$_3$
  - Hydroxylation in liver to 25(OH)D

- **Dietary Supplements**
  - Contains vitamin D$_2$ or D$_3$
  - Hydroxylation in kidney to 1,25(OH)$_2$D
Measurement Techniques for Vitamin D

- Immunoassay
  - Antibody specificity is high, cross-reactivity may occur
  - No independent confirmation of analyte identity
- Gas Chromatography (GC-MS)
- Liquid Chromatography
  - LC-UV
  - Mass Spectrometry (LC-MS)
  - Tandem Mass Spectrometry (LC-MS/MS)
Mass Spectrometry-Based Methods for Determination of Vitamin D Metabolites

- The 3-epimer of 25(OH)D$_3$ co-elutes with 25(OH)D$_3$ on C18 columns
- 25(OH)D$_3$ and 3-epi-25(OH)D$_3$ have the same MS/MS fragmentation patterns
- Initially the 3-epimer was thought to be only found in infants (Singh et al.)
LC-MS/MS Methodology for 25(OH)D

Add water and ISTDs* → Equilibrate 1 hr, adjust pH to 9.8 → Extract with hexane:ethyl acetate →

Dry with \( \text{N}_2 \), dilute with methanol → LC-MS/MS analysis

* The internal standards were \(^2\text{H}_3\text{-25(OH)D}_2\) and \(^2\text{H}_3\text{-25(OH)D}_3\)
NIST LC-MS/MS Methodology – 25(OH)D₃

SRM 972 Level 1

- APCI MS using cyano column with methanol:water mobile phase
- 3-epi-25(OH)D₃ fully resolved from 25(OH)D₃ (separation based on work of Lensmeyer et al.)
- Labeled 3-epi-25(OH)D₃ now available for use as internal standard
- Method approved by JCTLM as Reference Measurement Procedure

Susan Tai et al., *Analytical Chemistry*, 2010
NIST LC-MS/MS Methodology – 25(OH)D₂

SRM 972 Level 3

SRM 972 Level 4

~ 65 nmol/L

~ 6 nmol/L
The “Epi” Question?

C-3 Epimers Can Account for a Significant Proportion of Total Circulating 25-Hydroxyvitamin D in Infants, Complicating Accurate Measurement and Interpretation of Vitamin D Status

Ravinder J. Singh, Robert L. Taylor, G. Satyanarayana Reddy and Stefan K. G. Grebe

The 3-epimer of 25(OH)D₃ appears to be present in nearly all adult sera but its concentration varies.
Design of SRM 972 Vitamin D in Human Serum

Level 1
65 ± 15 nmol/L 25-hydroxyvitamin D$_3$ (“normal”)

Level 2
Blend of “normal” serum and horse serum to obtain approximately half the level of 25-hydroxyvitamin D$_3$ in the “normal” pool (35 ± 5 nmol/L)

Level 3
“Normal” serum spiked with an equivalent amount of 25-hydroxyvitamin D$_2$

Level 4
“Normal” serum spiked with 3-epi-25-hydroxyvitamin D$_3$

Goal was to have serum pools that presented different analytical challenges
## Assigned Values for SRM 972

<table>
<thead>
<tr>
<th>Level</th>
<th>25(OH)D$_2$</th>
<th>25(OH)D$_3$</th>
<th>3-epi-25(OH)D$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1</td>
<td>0.59 ± 0.20</td>
<td>23.2 ± 0.8</td>
<td>1.35 ± 0.04</td>
</tr>
<tr>
<td>Level 2</td>
<td>1.67 ± 0.08</td>
<td>12.0 ± 0.6</td>
<td>0.74 ± 0.02</td>
</tr>
<tr>
<td>Level 3</td>
<td>25.8 ± 1.9</td>
<td>18.1 ± 1.1</td>
<td>1.04 ± 0.03</td>
</tr>
<tr>
<td>Level 4</td>
<td>2.35 ± 0.21</td>
<td>32.3 ± 0.8</td>
<td>36.9 ± 1.1</td>
</tr>
</tbody>
</table>

Certified and reference values obtained from combination of results from multiple methods: LC-MS (NIST), LC-MS/MS (NIST) and LC-MS/MS (CDC). Certified values are shown in bold. All data in ng/g.
Human Nutritional Assessment
SRMs – In Progress

- SRM 3949 Folate Vitamers in Frozen Human Serum
- SRM 3950 Vitamin $B_6$ metabolites in Frozen Human Serum
- SRM 3951 Vitamin $B_{12}$ in Frozen Human Serum
- SRM 2378 Fatty Acids in Frozen Human Serum