Development of Standard Reference Materials for the Analysis of Dietary Supplements: The Story Continues

Katherine E. Sharpless, Ph.D., Lane C. Sander, Ph.D., Stephen A. Wise, Ph.D.,
Agnes NguyenPho, Ph.D., and Joseph M. Betz, Ph.D.

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In 2004, we published an article in HerbalGram that described a collaboration among the National Institute of Standards and Technology (NIST), the National Institutes of Health, Office of Dietary Supplements (NIH-ODS), and the Food and Drug Administration (FDA) that was then in its infancy [1]. This article provides an update of this program, which is directed at production of Standard Reference Materials (SRMs) that can be used in support of the Dietary Supplement Health and Education Act of 1994 [2] and requirements imposed by the Good Manufacturing Practices (GMPs) published by the FDA. GMPs were issued in June of 2007, with a phase-in period staggered over three years. As of June 2010, all companies, including those with fewer than 20 employees, are required to comply with GMPs. Among other things, the GMPs require dietary supplement manufacturers to establish specifications to evaluate the identity, purity, strength, and composition of ingredients used in their products; set limits on contaminants (e.g., pesticides, bacteria, toxic elements) and adulterants; and evaluate their finished products. The GMPs require that manufacturers follow processes for selection and use of appropriate analytical methods and reference materials. Manufacturers must verify that the methods are appropriate for their intended use, then use those methods to determine whether their specifications are being met. The first SRM developed in this program was issued in 2006. By the end of 2010, there will be 28 dietary supplement-related SRMs available from NIST.

Between 2008 and 2009, sales of herbal dietary supplements in the U.S. increased by almost 5 %, totaling more than $5 billion in 2009 [3]. This represents about 1/5 of the U.S.'s entire $24 billion dietary supplement industry, which includes non-botanical dietary supplements such as vitamin and mineral supplements [4]. The increase in sales in a tough economy is somewhat surprising, but it is possible that people are "stocking up on pills that they think can spare them expensive doctor visits" [5]. With increasing sales and more than half of the U.S. population reporting that they take dietary supplements [6], it is important to maintain the quality and safety of these products.

Because of a recognized lack of publicly available, validated analytical methods for dietary supplements — and a lack of reference materials for validation of analytical methods — NIH's Office of Dietary Supplements (ODS) was mandated by Congress to fund development of analytical methods and reference materials for dietary supplements [7]. "Analytical methods" are used to measure the amount of an element or chemical compound in something – to measure iron in spinach, for example. "Reference materials" are a homogeneous substances that can be used for calibration of an instrument or for assessing whether or not an analytical method is working properly. A certified reference material (CRM) is a reference material that is provided with a Certificate of Analysis, which, in the case of the dietary supplement materials being discussed

here, is a document that shows the chemical composition of the material (e.g., calcium or vitamin C content). CRMs from the NIST are known as Standard Reference Materials (SRMs). A Certificate of Analysis for an SRM contains a description of the material, its intended use, an expiration date, storage and handling recommendations, description of the analytical methods used for characterization, and certified values. Depending on their intended use, SRMs can be used to calibrate an instrument or to determine whether an analytical method is working properly. (A single CRM cannot be used for both calibration and quality control.) If an analyst measures the CRM and obtains a result that falls within the uncertainty limits of the certified value, he/she can have confidence in the method of analysis and that results for similar samples being analyzed will be accurate [8]. The use of SRMs as quality control samples has been demonstrated to improve the quality of analytical results [9-11].

The natural-matrix dietary supplement SRMs being produced by NIST are not intended to represent what a "good" dietary supplement product should look like, and the existence of such a material does not imply that the botanical in question is either safe or effective for use as a dietary supplement. NIST's dietary supplement SRMs are intended for the same purposes as NIST's other natural-matrix SRMs, namely: (1) to validate the reliability and precision of new analytical methods, and (2) to provide quality control for routine analyses whereby the SRM is analyzed at appropriate regular intervals as part of a laboratory's quality assurance protocol or a company's good manufacturing practices (GMPs). Natural-matrix SRMs that are similar to the other samples being analyzed by the laboratory can be used to validate the complete analytical process including extraction, isolation of the analytes of interest from the matrix, and separation and detection.

NIST's SRMs and industry's use of them fits into a larger measurement (metrology) picture. NIST is a non-regulatory agency within the U.S. Department of Commerce and is responsible for building the foundation for measurements in the U.S.; these measurements support national and international commerce [12]. In the case of dietary supplements, this foundation is established by assigning values to concentrations of active and/or marker compounds and toxic elements (arsenic, cadmium, lead, and mercury) in dietary supplement SRMs. (Quantifiable levels of pesticides in the SRMs that have been issued so far have not been found. If and when they are detected in future SRMs, values will be assigned.) Manufacturers measuring these analytes in their products can link their results – make them traceable – to the SRMs maintained by NIST [13].

NIST participates in intercomparisons among other nations' National Metrology Institutes (NMIs) to ensure that results traceable to the CRMs produced by NMIs throughout the world are equivalent. For example, if NIST and other NMIs have proven their abilities to measure cadmium in a plant material in one of these intercomparisons and make this capability available to analytical chemists in their respective countries through a CRM, laboratories within that country who are measuring cadmium and analyzing the CRM as a control – and obtaining the correct result – should all be accurately measuring cadmium in their products [14].

NIST assigns certified values to SRMs in three ways: (1) using a single primary method with confirmation by other methods, (2) using two or more independent, critically evaluated methods, or (3) using one method at NIST and different methods by outside collaborating laboratories [15]. Reference or information values may be assigned in cases where NIST has not made

measurements, where NIST made measurements using a single analytical technique, or where there is less confidence in the value for a technical reason.

The First Suite of Dietary Supplement SRMs

Because of safety concerns, our initial efforts focused on ephedra (*Ephedra sinica* Stapf.) as the dietary supplement of highest priority for reference material development. (This effort was begun prior to FDA's ban of ephedra in dietary supplements which went into effect April 12, 2004.) In the U.S. dietary supplement marketplace, ephedra was used in weight loss and body-building products. Because of the different analytical challenges presented by the various different matrices, NIST developed a suite of ephedra-containing SRMs consisting of: ground and sieved plant material (SRM 3240 *Ephedra sinica* Stapf, Aerial Parts); a natural extract (SRM 3241*Ephedra sinica* Stapf, Native Extract); the extract used to prepare SRM 3241 fortified to contain nominally 8% total ephedrine alkaloids (SRM 3242 *Ephedra sinica* Stapf, Commercial Extract); and two "finished product" materials containing ephedra – one a mixture of ground caplets and the contents of capsules (SRM 3243 Ephedra-Containing Solid Oral Dosage Form) and the other a mixture of chocolate-flavored protein drink mixes (SRM 3244 Ephedra-Containing Protein Powder) [16]. The plant and extract materials were all prepared from the same source plant material, for which a voucher specimen was obtained. Herbarium sheets are deposited at the Missouri Botanical Garden and are available through their website [17].

The suite of ephedra SRMs has served as the model for the other botanical dietary supplement materials in NIST's portfolio, i.e., most of the other suites also consist of plant material, an extract, and a finished product. A plant material can be difficult to analyze because compounds of interest may be incorporated in the plant's cell walls, and there are many, many other compounds present from which the analyte of interest must be isolated. An extract may simply require dissolution in a suitable solvent (e.g., water or alcohol). Finished products, while often containing extracts, may be difficult to analyze because of interferences from other ingredients present in mixed-botanical products.

After packaging, samples were selected for analysis, including testing to assess bottle-to-bottle homogeneity. For the suite of ephedra SRMs, the ephedra alkaloids were the primary focus [18]. A chromatographic profile or "fingerprint" of the ephedrine alkaloids as well as other plant constituents is provided for use as a reference, and the Missouri Botanical Garden performed microscopy. NIST also determined caffeine, synephrine (see "Bitter Orange-Containing Reference Materials," below), and toxic elements (i.e., arsenic, cadmium, mercury, and lead) in some of these materials. Collaborating laboratories provided results for these analytes as well as for the nutritional composition (fat, protein, carbohydrate, individual amino acids, vitamins, and minerals) of the protein powder.

Ginkgo-Containing Reference Materials

Ginkgo is largely used today to prevent or treat memory loss, Alzheimers, and other forms of dementia [19]. NIST's suite of ginkgo-containing materials consists of SRM 3246 *Ginkgo biloba* (Leaves), SRM 3247 *Ginkgo biloba* Extract, and SRM 3248 Ginkgo-Containing Tablets [20]. All three of these materials are linked to a common source. And unlike most of the "finished product"

SRMs, SRM 3248 consists of a single type of tablets with no other botanical ingredients. Values in these materials were assigned for ginkgolides (terpene lactones), flavonols, cadmium, lead, and mercury [21]. A thin-layer chromatogram is provided for the ginkgolides and flavonols as well as for ginkgolic acid.

Saw Palmetto-Containing Reference Materials

The suite of saw palmetto-containing materials consists of SRM 3250 *Serenoa repens* (Saw Palmetto) Fruit and SRM 3251 *Serenoa repens* Extract. These two SRMs were not prepared from the same raw material. Because most finished products containing saw palmetto contain either ground "berries" or a saw palmetto extract by itself or mixed with other oils, a "finished product" SRM was not prepared. Saw palmetto products are typically used as a treatment for benign prostate hyperplasia [22]. The two SRMs have been characterized for their phytosterols and fatty acid content, including both free fatty acids and those occurring as triglycerides [23]. Values for beta-carotene have also been assigned in the extract.

Green Tea-Containing Reference Materials

Green tea can be consumed as a beverage and in dietary supplements. The green tea SRM suite consists of SRM 3254 *Camellia sinensis* (Green Tea) Leaves, SRM 3255 *Camellia sinensis* (Green Tea) Extract, and SRM 3256 Green Tea-Containing Solid Oral Dosage Form. Values are assigned for catechins, theanine [24], and caffeine in these materials.

Bitter Orange-Containing Reference Materials

After ephedra-containing products were removed from the marketplace, many manufacturers used bitter orange as a replacement in their products. Although *Citrus aurantium* is known as bitter orange, bitter orange in the dietary supplement industry is often a mixture of different immature citrus fruits that are combined in proportions to provide a target concentration of synephrine. SRM 3258 Bitter Orange (Fruit) consists of such a mixture. SRM 3259 Bitter Orange Extract is an extract of immature citrus fruits that provides a nominal synephrine concentration of 6 %. SRM 3260 Bitter Orange-Containing Solid Oral Dosage Form is a mixture of ground tablets and capsules' contents. These materials have been characterized for the citrus alkaloids [25] as well as toxic elements [21].

***Vaccinium* Berry-Containing Reference Materials**

Berries are traditional foods that are also used as ingredients in dietary supplements. This suite consists of seven different materials, some of which are characterized for nutrients as well as organic acids [26]. All materials will be characterized for anthocyanidins with fingerprints provided for procyanidins in the future. These materials are SRM 3281 Cranberry (Fruit), SRM 3282 Low-Calorie Cranberry Juice Cocktail, SRM 3283 Cranberry Extract, SRM 3284 Cranberry-Containing Solid Oral Dosage Form with Mixed Botanicals, SRM 3285 Mixed Berry-Containing Solid Oral Dosage Form (i.e., bilberry, blueberry, and cranberry), SRM 3287 Blueberry (Fruit), and SRM 3291 Bilberry Extract. These materials are currently characterized for organic acids. Because of their use as traditional foods, the blueberries, cranberries, and cranberry juice are

characterized for sugars and nutrient elements (e.g., calcium, iron, sodium, etc.). The blueberries are also characterized for fiber, two water-soluble vitamins, amino acids, and other nutrients.

Oils for Omega-3 and Omega-6 Fatty Acid Measurement

Health effects of omega-3 and omega-6 fatty acids are increasingly being studied, and dietary supplement products that contain these materials are very popular. SRM 3274 Botanical Oils Containing Omega-3 and Omega-6 Fatty Acids (Flax, Borage, Evening Primrose, Perilla) consists of ampoules of the four individual oils with values assigned for fatty acids. A comparison of selected fatty acid concentrations in these four oils is provided in reference [27]. SRM 3275 Fish Oils Containing Omega-3 and Omega-6 Fatty Acids consists of three individual oils: a concentrate high in docosahexaenoic acid (DHA), anchovy oil high in DHA and eicosapentaenoic acid (EPA), and a concentrate containing 60 % long-chain omega-3 fatty acids. SRM 1588c Organics in Fish Oil, expected to be available later this year, is menhaden oil that will have values assigned for contaminants such as pesticides, polychlorinated biphenyl congeners (PCBs), and brominated flame retardants as well as fatty acids.

Non-Botanical Dietary Supplement Reference Materials

A number of other non-botanical dietary supplement SRMs have been prepared as part of this program. SRM 3280 Multivitamin/Multielement Tablets has values assigned for 13 vitamins, 2 carotenoids, and 24 elements [29]. We are currently in the process of assigning values for four additional elements and vitamin B_{12} in this material. SRM 3278 Tocopherols in Edible Oils is a mixture of oils (sunflower, soy, canola, safflower) that were combined to provide similar levels of gamma- and alpha-tocopherol. Delta-, gamma-, and alpha-tocopherol are naturally occurring in edible oils but one form typically predominates in each type of oil; beta-tocopherol is also sometimes present but at very low levels. Four oils were blended to give comparable levels of all three of the main tocopherols so that the SRM can be used for quality assurance of tocopherol measurements in all types of oils.

Reference Materials Currently in Preparation

St. John's wort has been used for a number of indications, including "melancholia," since medieval times [28]. This suite of SRMs consists of SRM 3262 *Hypericum perforatum* (St. John's Wort) Aerial Parts, SRM 3263 *Hypericum perforatum* (St. John's Wort) Carbon Dioxide Extract, SRM 3264 *Hypericum perforatum* (St. John's Wort) Methanol Extract, and SRM 3265 St. John's Wort-Containing Tablets. Scientific studies have tried to determine which compounds in St. John's wort might be responsible for its biological activity, with the focus on hyperforin and hypericin. Hyperforin is concentrated in a supercritical fluid carbon dioxide extraction and hypericin is concentrated in the methanol extract, thus the two different extract SRMs will provide quality assurance for measurement of both types of products. Values will be assigned for hyperforin, hypericin, pseudohypericin, and toxic elements in these SRMs, which are expected to be available in 2011.

Suites of SRMs for soy, kudzu, red clover, and American black cohosh are currently being characterized; with plans for production of SRMs for eleuthero, Asian ginseng, pomegranate,

turmeric, and yohimbe. (National Research Council Canada, the NMI in Canada, is preparing American ginseng CRMs.) SRMs for iodine and iodate in table salt and calcium in supplements are also being planned. In addition, NIST is beginning production of solutions containing compounds of interest to the dietary supplement community: selected catechins, flavonols, ginsenosides, isoflavones, organic acids, and terpene glycosides. These materials can be used for instrument calibration

Analytical Quality Assurance

SRMs can be used by dietary supplement manufacturers to build a quality assurance program in compliance with GMPs, by researchers for verifying the accuracy of their analyses, and by the FDA for monitoring marketed products and for enforcement actions when necessary. For measurement of toxic and nutrient elements, a broad range of SRMs (and CRMs in general) are available for matrices ranging from calibration solutions to botanicals to soils and sediments. For active and marker compounds, the range is more limited, but it is continuing to expand.

While the use of SRMs has been shown to improve the quality of analytical results, participation in a quality assurance or proficiency testing program can be similarly effective [9-11]. NIST has conducted quality assurance programs for the measurement of contaminants in environmental samples and micronutrients in human serum for more than 20 years. Participants in these programs have improved the comparability of their between-laboratory results as well as their own within-laboratory precision. NIST and NIH-ODS recently established a quality assurance program for the analysis of dietary supplements with the expectation of helping labs that measure dietary supplements to realize these same goals [30].

Katherine E. Sharpless, Lane C. Sander, and Stephen A. Wise are employees of the National Institute of Standards and Technology. Agnes NguyenPho is employed by the U.S. Food and Drug Administration's Center for Drug Evaluation and Research. Joseph M. Betz is employed by the Office of Dietary Supplements, National Institutes of Health.

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