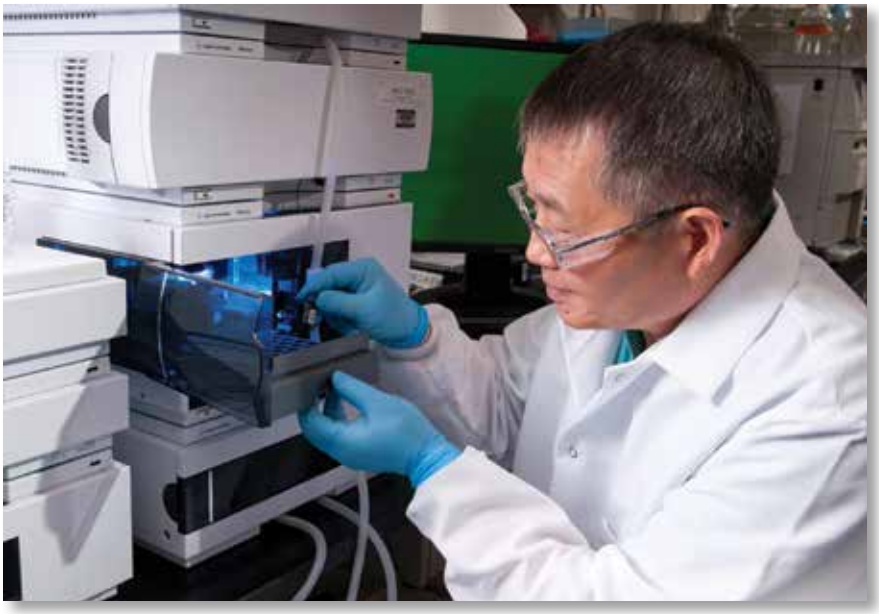
by Neil H. Mermelstein

Assuring **Stevia Quality**

The leaves of the plant *Stevia rebaudiana* (Bertoni) have been used as a natural sweetener for hundreds of years. In recent years, extracts of the leaves have been introduced as natural, non-caloric sweeteners for foods and beverages. The extracts are composed of sweet-tasting compounds called steviol glycosides. Of the nine commonly present glycosides, rebaudioside A (also known as rebiana) and stevioside are primarily responsible for the sweet taste, being more than 200 times as sweet as sucrose.



Researcher inserts a sample into an autosampler *for analysis by high-performance liquid chromatography.*
Photo courtesy of U.S. Pharmacopeial Convention.

In December 2008, the U.S. Food and Drug Administration (FDA) stated that it had no objection to self-determinations by Cargill Inc. and Whole Earth Sweetener Co. that rebaudioside A derived from the stevia plant is generally recognized as safe (GRAS) for use as a general-purpose sweetener at 95% purity or above. As soon as the FDA's acceptance was announced, PepsiCo Inc. began marketing products containing Whole Earth Sweetener's *Pure Via*™ brand of stevia-based sweetener and Coca-Cola began using Cargill's *Truvia*® brand. Since then, other ingredient companies have developed other stevia-based sweeteners, ranging from purified rebaudioside A to other high-purity steviol glycosides and made self-determinations that they are GRAS, also with no objection from the FDA. Consequently, an increasing number of stevia-sweetened food and beverage products are being marketed.

The safety of stevia-based sweeteners was discussed in the article "Ensuring the Safety of Sweeteners from Stevia," by Robert S. McQuate, in the April 2011 issue of *Food Technology*. This month's Food Safety & Quality column discusses efforts to develop standardized methods for stevia analysis.

Analytical Methods

Analysis of stevia extracts is difficult since steviol glycosides are very similar in structure, making it difficult to separate them chromatographically, and do not absorb strongly in the ultraviolet (UV) region. Typically, analysis of stevia is done by high-performance liquid chromatography (HPLC). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has published several revisions to its original 2006 monograph that used an HPLC method with an NH_2 column and UV detection. The most current JECFA monograph, published in 2010, requires a minimum of 95% total steviol glycosides content, measured as the sum of nine named glycosides (rebaudiosides A, B, C, D, and F, stevioside, rubusoside, steviolbioside, and dulcoside A). It uses HPLC with UV detection at 210 nm using a 68/32 mixture of acetonitrile and pH 2.6 phosphate buffer, a 4.6-mm x 250-mm C18 column, and a flow rate of 1.0 mL/min. This flow rate, coupled with an approximately 30-min run time, results in about 30 mL of mobile phase used per run.

The U.S. Pharmacopeial Convention (USP) published its first monograph for a stevia-based ingredient—high-purity rebaudioside A—in 2009. The most recent (2012) version of the monograph uses an HPLC method with an NH_2 column, a mobile phase of 13% (v/v) acetate buffer in

acetonitrile, and UV detection. At a flow rate of 1.5 mL/min with a run time of up to 66 min, it requires approximately 100 mL of mobile phase per run.

Analytical instrument manufacturers have issued application notes on stevia analysis over the past several years, using various techniques and chromatography columns. For example, Thermo Fisher Scientific Inc. and Dionex (www.thermoscientific.com/dionex) describe use of HPLC with UV and charged aerosol detection, saying that charged aerosol detection has the advantage of distinguishing additional components in the sample that are not UV-absorbing. ESA Biosciences Inc. (www.esainc.com) also describes use of HPLC with UV and charged aerosol detection that allows for additional components from stevia to be detected in foods and beverages. ChromaDex Corp. (www.chromadex.com) describes an HPLC method that is compatible with additional detection techniques, including mass spectrometry (LC/MS) and evaporative light-scattering (ELS) detection. Shimadzu Corp. (www.shimadzu.com) also describes use of reversed-phase HPLC with UV and ELS, saying that because the ELS response is independent of the light-absorbing properties of molecules, it can reveal sample components that UV detectors miss and provide a more accurate profile of relative component abundance.

PureVia ***and*** **Truvia** ***are the most widely used brands of stevia-based sweeteners****, available both as tabletop sweeteners and in a variety of food and beverage products.*

Efforts at Standardization

USP has been working with outside groups in industry and its own in-house research and development laboratories to develop standardized methods and supporting reference materials for analysis of stevia. USP publishes its methods as monographs in its *Food Chemicals Codex*.

Jeffrey C. Moore, Senior Scientific Liaison at USP, said that a critical issue has been the standardization of HPLC purity measurement toward a method that is not only accurate but also reproducible enough to allow multiple laboratories around the world to analyze the same samples and obtain similar results. One challenge in this regard, he said, has been finding the right balance between newer

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Assuring Stevia Quality continued...

analytical technologies that may have better performance and other technology that is widely available and affordable throughout the world where stevia is produced and used.

When USP published its first monograph on high-purity rebaudioside A, it adopted a validated isocratic HPLC procedure that used an Agilent Technologies *Zorbax*® NH_2 column. It was considered one of the most reliable and commonly available HPLC columns available at that time for stevia analysis, Moore said, but had downsides, including stationary phase stability issues, retention time precision issues, and the need for a time-consuming column equilibration step. As other, better columns became available for stevia analysis, a revision was made to the monograph in 2010 to use a newer NH_2 column, the Phenomenex *Cosmosil*™ *Sugar-D* column, which resolved many of these issues. Since 2010 industry has moved toward isocratic C18 HPLC methodologies, given their greater availability in quality control laboratories.

JECFA published a C18 method in 2010 to meet this need, Moore added, but concerns have been raised about the capability of that methodology to sufficiently resolve the critical pair in the separation—rebaudioside A and stevioside—as well as the approaches for peak identification and quantification used in the monograph. USP recently addressed the separation and peak identification challenges in a new C18 method recently developed and validated by USP's labs. The method uses a gradient elution to improve the resolution of the analyzed glycosides and a new qualitative USP reference standard containing all nine steviol glycosides to aid in peak identification. The method specifies use of HPLC with UV detection at 210 nm, a 25-cm × 4.6-mm column packed with 5-µm reversed-phase C18 silica stationary phase, a column temperature of 32°F, a flow rate of 0.5 mL/min, and an injection volume of 15 µL.

This new HPLC method, along with specifications harmonized with JECFA's current monograph, was the basis for a new steviol glycosides monograph that USP proposed in its December 2011 *FCC Forum* for public comment. A solution to the quantitation challenge has also been developed, Moore said, based on comments submitted during the forum and lab

Stevia Organizations & Conferences

The European Stevia Association (www.eustas.org) is a nonprofit organization whose goals are the promotion and coordination of all activities focusing on research and health regarding *Stevia rebaudiana* and related compounds to show that they are safe for human consumption. In July 2007 the organization submitted an application to the European Commission's Scientific Committee for use of Stevia leaves as a novel food, and in September 2007 it submitted an application for approval of steviol glycosides as a food additive. The organization holds an annual Stevia symposium; the most recent conference was July 3–4, 2012, in Leuven, Belgium. The next symposium is tentatively scheduled to take place in summer 2012 in Toulouse, France.

Fi Conferences (www.fi-stevia.com) is an organization hosting conferences on various subjects, including stevia. It held its fourth conference on stevia, "Stevia 2012: Capitalising on European Regulatory Approval," April 12–13, 2012, in London, England. The conference brought together leading experts to address the challenges the food and beverage industry faces in incorporating stevia into reduced-sugar products. The next conference is tentatively scheduled for spring 2013.

The Global Stevia Institute (www.globalsteviainstitute.com) is an organization created to provide accurate, credible, and consistent information and resources regarding stevia to health professionals, consumers, and the food industry. The institute provides a resource library of science-based stevia information to help health professionals and manufacturers understand the benefits of stevia in food applications.

The International Stevia Council (www.internationalsteviacouncil.org) is a trade association whose mission is, among other things, to be a trusted knowledge partner for regulatory bodies on the science behind the safety of stevia, establish and ensure accurate analytical methods for measuring the purity of stevia extracts, and ensure industry-wide access to accurate analytical methods and standards for measuring steviol glycosides content. In May 2011 the council launched its independent Proficiency Testing Program for stevia products to address the challenge of ensuring that analytical methods and standards employed throughout the industry are appropriate and also meet minimum levels of accuracy when measuring steviol glycoside content. The program allows participants to benchmark the performance of their stevia analytical methods and standards and analysts' competency.

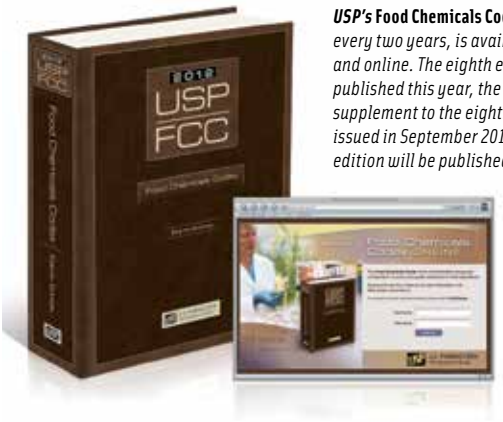
The Japan Stevia Association (www.stevia.gr.jp) is a trade association that promotes the manufacturing and marketing of sweetener from stevia.

The World Stevia Organisation (www.wso-site.com) is an international nonprofit organization that advances the practical applications of stevia and low-calorie natural sweeteners, promotes the nutritional and health benefits of stevia, and exchanges information through international conferences and publications. The organization's next world conference on Stevia, "Stevia Tasteful 2013—Food & Beverages Formulation: The Subtle Balance," will be on June 13, 2013, in Paris, France.

How *Food Chemicals Codex* Monographs Are Approved

USP publishes *Food Chemicals Codex* (*FCC*), a compendium of internationally recognized standards and analytical methods for determining the purity and identity of food ingredients. New editions are published every two years, with supplements issued in between. The *FCC* includes more than 1,100 monographs on foods, food-grade chemicals, processing aids, flavoring agents, vitamins, and functional food ingredients and is relied on internationally for purchasing agreements between suppliers and customers.

Proposed monographs and revisions to existing monographs are posted on the *FCC Forum* website (www.usp.org/food-ingredients/fcc-forum) for public review and comment for a 90-day period. The comments are then forwarded to the Food Ingredients Expert Committee (a group of independent scientific experts) for review and response, after which the final monograph is published in the *FCC*. The *FCC Forum* is available in June and December. The next forum will be available online on December 31, 2012, and comments will be accepted until March 31, 2013. The target date for publication of the third supplement to the eighth edition of *FCC* is September 1, 2013.



***USP's* Food Chemicals Codex**, *published every two years, is available in print and online. The eighth edition was published this year, the third supplement to the eighth edition will be issued in September 2013, and the ninth edition will be published in 2014.*

investigations at USP. The approach uses a single external quantitative reference standard, *USP Rebaudioside A*, and relative response factors (RRFs) established by USP's labs to quantitate the remaining eight steviol glycosides. The RRFs were established by determining the ratio of absorbance to concentration for each analyte relative to rebaudioside A. From an analytical chemistry perspective, the approach is more accurate, Moore said, than using the molecular weight correction factor approach used by other testing standards, including JECFA's.

All of these improvements have been put together in a new series of proposals for public comment this year, he added. USP published the new proposal for rebaudioside A in its June 2012 *FCC Forum* and expects to publish the updated proposal for steviol glycosides in its December 2012 forum for another round of public comments. USP hopes to publish final versions of both monographs in September 2013 in the third supplement to the eighth edition of *Food Chemicals Codex*.

USP is looking forward to setting standards for other stevia sweeteners and is particularly interested in enzymatically modified stevia sweeteners, Moore said. Enzymatically adding more sugars to the backbone of steviol glycosides provides a different sweetness profile that some find superior to non-modified stevia. From an analytical perspective, they are much more difficult to characterize, he said—instead of nine steviol glycosides to measure, there is a significant increase in the number of glycosides to analyze. Another challenge is that companies are developing sweeteners that are composed predominantly of other steviol glycosides besides rebaudioside A and stevioside, and USP hopes to develop standards for such compounds in the future. **FT**

***Neil H. Mermelstein**, a Fellow of IFT, is Editor Emeritus of Food Technology • nhmermelstein@comcast.net*