

September 4, 2009

Jacqueline Richardson  
Contract Specialist  
Food & Drug Administration  
OC/OM/OAGS/DAO  
Room 2090  
5630 Fishers Lane  
Rockville, MD 20857

Re: Written Report of FDA/NIH/AOAC Contract Number HHSF223200810042C

Dear Ms. Richardson:

This letter is in response to the deliverable of a final report required in the FDA/NIH/AOAC contract, solicitation number REQ1049192, and it details AOAC's completion of all work under this current contract as part of the FDA/NIH dietary supplements initiative. In brief, all the contract requirements have now been satisfied, with the following delivered:

1. one Presidential Task Force on Dietary Supplements (PTFDS) meeting,
2. four Ingredient Ranking Subgroup (IRS) meetings, and
3. five Expert Review Panel (ERP) meetings.

It should be noted that AOAC exceeded contract requirements with regard to the number of IRS meetings; only one IRS meeting was required, as the contract was originally based on an ingredient ranking held in 2006. However, soon after the contract signing, NIH officials elected to have the IRS rank all ingredients anew to determine the contract's scope of work. Four IRS meetings were required to determine those ingredients for which ERPs should be held. The scope of work for the contract was subsequently modified on February 11, 2009, to include Vitamin D, Vitamin B (B6 and B12), milk thistle, and anthocyanins (cranberries).

Black cohosh and turmeric, both of which were included in the original contract, were no longer contract deliverables due to re-ranking and their lower scores.

### ***IRS meetings***

Under the current contract, the IRS first met at the AOAC annual meeting on September 21, 2008, in Dallas, Texas, chaired by Leila Saldanha contractor for National Institutes of Health, Office of Dietary Supplements. NIH officials explained that the IRS should include a method's purpose in its prioritization recommendations. It was also recommended that the IRS' mission statement be changed so that the group not only prioritize "dietary supplement ingredients for which validated analytical methods are needed," but also provide "the scope and need for the method for top-ranking ingredients to assist the expert review panels in determining the fitness-for-purpose."

At a follow up meeting in December 2008, at AOAC headquarters in Gaithersburg, Maryland, the IRS triaged some 120 ingredients into clearly defined categories to simplify and provide for a larger measure of efficiency in the ranking process in this and future meetings. Thirty ingredients were triaged as No. "1s," being important enough that they needed little or no discussion in terms of justification/method needs. These ingredients were subsequently ranked, based on the

established PTFDS criteria, using available sales and market trend data, as well as on whether methods and reference materials are generally available, whether a major NIH clinical trial is planned or underway, and whether there are FDA safety concerns.

The IRS also dropped 50 ingredients at this time as lacking justification for ranking.

The IRS met again in January 2009 at AOAC headquarters, and 40 more ingredients were reviewed (triaged as No. 2s in December and requiring further discussion); 13 ingredients were moved up to No. 1s and included with those triaged No. 1s in the December meeting. Of those No. 1s, they were then ranked, and a list of the 20 ingredients developed of those ingredients with the highest ranking.

Finally, in February, with the IRS meeting by conference call, the list of the top 20 dietary supplement ingredients was further refined, with green tea, lutein, and bitter orange being voted by the IRS off the list. These three ingredients were determined to be in-process, with appropriate methods already undergoing validation.

The IRS also approved in the February meeting a justification form, required of new ingredients that would be submitted for future IRS ranking. Such justifications required for ingredient ranking have been sometimes missing in the past, and without these justifications, ingredients are now removed from the list or table in the ranking process.

### ***Expert Review Panels***

For those five highest ranked ingredients--Vitamin D, Vitamin B (B6 and B12), milk thistle and anthocyanins (cranberries) -- calls for methods and experts were issued, and literature searches were conducted of appropriate methodology. The result: ERPs made up of scientists with long experience with these materials, and appropriate technologies, scrutinized the methods found in the literature searches and narrowed the search to one or a small number mostly to be successful.

***Vitamin B6.***—On April 1, 2009, the ERP for Vitamin B6, chaired by Karen Phinney, National Institute of Standards and Technology, provided in-depth review of Vitamin B6 methods as part of the FDA/NIH initiative on dietary supplements. The panel determined that because the term B6 includes numerous compounds, only pyridoxine hydrochloride, the most frequently encountered compound, would be selected. Not satisfied with one method as a whole, the ERP recommended a method, combining the best components of three different methods: a method from Pharmavite for sample preparation; an LC-UV/MS method from Pei Chen of the U.S. Department of Agriculture with recommendation of the addition of a fluorescence detector for added sensitivity; and a method from DSM (B6 in tablets) for chromatographic separation.

Based on fitness-for-purpose, panel members identified which form(s) of Vitamin B6 should be considered as part of the method selection process. Vitamin B6 has numerous vitamers that may be present in matrixes such as food and serum. Some supplements use pyridoxal-5'-phosphate or other forms. Rather than trying to address all possible forms of B6, it was recommended that the ERP focus its efforts where significant progress could be made in terms of identifying a method for a single-laboratory validation (SLV). Thus, the panel selected pyridoxine HCl as the analyte of interest.

After further discussion of the remaining methods, the ERP determined that none of the submitted methods was ideally suitable in its present form for an SLV. Therefore, the group considered

three aspects of the most promising methods: sample preparation, chromatography, and detection. The ERP agreed by consensus that the sample preparation strategies outlined in the Pharmavite method are suitable and robust. The method provides detailed sample preparation instructions for different types of matrixes that might be encountered, including tablets and softgels. The panel recommended the inclusion of EDTA in the extraction liquid to reduce problems from metal complexation in products containing minerals.

**Vitamin B12.**— Similarly, the Vitamin B12 ERP, chaired by Richard van Breemen (professor, University of Illinois Chicago), combined the best aspects of various methods to develop a candidate method. The panel selected a method using ultraperformance liquid chromatography (UPLC) coupled with triple quadrupole MS, developed by Baiyi Lu et al. of China's Zhejiang University, but modified with the sample preparation selected for Vitamin B6, as the best candidate method.

This method uses UPLC columns with 1.7 µm diameter packing material and MS/MS detection with selected reaction monitoring (SRM). The sensitivity of cyanocobalamin detection is outstanding, and the analysis time is rapid (10 minutes total). Methotrexate is used as an internal standard. Samples for analysis include milk powder and rice flour, so additional development of the sample preparation method would be needed for analysis of dietary supplements.

The ERP recommended it for the analysis of cyanocobalamin, with the addition of the following procedures from other methods:

- During MS/MS detection, the doubly protonated molecule of cyanocobalamin formed during electrospray ionization should be selected for analysis, and at least two product ions should be monitored for the identification of cyanocobalamin and for the detection of possible interfering substances. These product ions should include  $m/z$  359 for quantitative analysis and  $m/z$  147 for quality control.
- The sample preparation method from Pharmavite (selected for Vitamin B6) should be used.
- An internal standard should be used, such as methotrexate (or a superior compound).
- Simultaneous analysis of pyridoxine (Vitamin B6) should be carried out.
- Detector options should include UV, fluorescence, or MS/MS.

**Vitamin D.**— On June 8, 2009, the ERP for Vitamin D, chaired by Erik Konings of Nestlé Research Center (Lausanne, Switzerland), evaluated 20 of the most promising and potential candidate methods for Vitamin D analysis in dietary supplements.

After in-depth review, the ERP agreed that none of the methods as a whole met the criteria required by the fitness-for-purpose statement and criteria developed by the panel. Therefore, the ERP developed a candidate method comprising key components of several methods. The candidate method would require a method development study, followed by an SLV, and if successful, a full collaborative study.

Based on fitness-for-purpose, panel members identified methods from BASF (Vitamin D Determination in Coated Products by HPLC Stability-Indicating) and Nestlé (Determination of Vitamins A, D, E, and K in Vitamin Premixes by HPLC) as having suitable sample preparation steps for premixes. The method from BASF was also suitable for determining previtamin D forms and used hot water heptane extraction.

The ERP agreed by consensus that the following methods were suitable for sample preparation: Nestlé for hot extraction; Huang et al. and Dimartino for cold saponification (overnight); AOAC (995.05) and GMI for hot saponification; and Xue et al. for enzymatic digestion.

***Cranberry.***—On July 21, 2009, the ERP for cranberry, chaired by David Cunningham, Ocean Spray, provided in-depth review of 16 cranberry methods as part of the FDA/NIH initiative on dietary supplements. Cranberries have been studied extensively to investigate their potential health benefits. The beneficial properties of cranberries have been ascribed to anthocyanins and proanthocyanidins. Anthocyanins are present in cranberries and other fruits as glycosides, which can be hydrolyzed to anthocyanidins.

The ERP agreed by consensus that a method by Paula Brown of the British Columbia Institute of Technology, “Single-Laboratory Validation of the Determination of Select Anthocyanins in Raw Cranberry and Cranberry Products by High-Performance Liquid Chromatography with Ultraviolet Detection,” met the criteria required by the fitness-for-purpose statement and criteria developed by the panel for a suitable candidate method. Advantages of the candidate method include use of HPLC-DAD instrumentation readily available to most laboratories; determination of five cranberry anthocyanins (four major and one minor); good separation with reasonable run time; simple non-SPE sample preparation; highly efficient extraction solvent (MeOH-HCl); and use of a commercially available mixed anthocyanin reference standard. In addition, the original SLV, conducted according to AOAC guidelines, was suitable for the four major anthocyanins. Thus, the candidate method was recommended by the ERP for further validation, with recommendations specific to the column, matrixes, and source of reference standards.

***Milk Thistle.***—On July 22, 2009, the ERP for milk thistle, chaired by Mark Anderson, Triarco, reviewed nine methods for the analysis of milk thistle in dietary supplements.

Based on fitness-for-purpose, the ERP reached consensus on a method from INA, with modifications, for further evaluation and validation. The method, “Silymarins in Milk Thistle by HPLC” (INA Method 115.000), is supported by existing validation data from three laboratories. Data available included linearity, limit of detection, LOQ, precision, and extraction efficiency. Samples evaluated were milk thistle extract and fruit material after being defatted. The ERP also recommended the method for its fitness-for-purpose, fast chromatography, and baseline separation of eight compounds. However, peak identification is based only on retention time, and, therefore, a confirmation of analyte identity is needed, and the method is applicable only to extracts and ground milk thistle fruit. The range of matrixes must be expanded.

The ERP recommended modifications to the candidate method, including: (1) Investigate the use of a shorter, polar-imbedded column with smaller particle size (i.e., YMC ODS-AQ 3  $\mu$  3.0 x 100 mm) to possibly increase peak resolution and shorten the run time. (2) Replace the phosphoric acid in the mobile phase with a weak organic acid, such as formic or acetic, making the method adaptable to MS. (3) Compare the extraction efficiency of each flavonolignan from seed powder with and without the hexane fat extraction step to determine if the step may be eliminated from the sample preparation protocol.

Please call me with any questions or comments.

Sincerely yours,

A handwritten signature in black ink, appearing to read 'Robert Rathbone', written in a cursive style.

Robert Rathbone  
Sr. Director of Publications, and Method Validation Programs

cc: Joseph Betz  
Mitchell Smith

Attachments