INFANT FORMULA AND ADULT NUTRITIONALS

Infant Formula and Adult/Pediatric Nutritional Methods Approved First Action Using the AOAC Voluntary Consensus Standards Process

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New Path to Official MethodsSM

On March 28, 2011, the AOAC Board of Directors approved a new path to achieve Official First Action status for methods selected and reviewed using the AOAC volunteer consensus standards development process (Figure 1). This approach places an expanded and high-level responsibility on expert review panels (ERPs) which now have the authority—for the first time—to approve methods as AOAC First Action Official MethodsSM. ERPs established by stakeholder panels, and whose members are thoroughly vetted by the Official Methods Board (OMB), evaluate methods against standard method performance requirements (SMPRs) developed by stakeholder bodies. A method approved First Action will remain First Action for a period of 2 years. During this time, the method will be used in laboratories, and method users will be asked to provide feedback. An ERP will monitor the performance of the method, and after the 2-year period, the ERP will determine whether the method should be recommended to the OMB for Final Action.

Standard Method Performance Requirements

SMPRs, based on the fitness-for-purpose statement developed by stakeholders, establish the minimum performance that stakeholders expect of a method. SMPRs provide detailed information about analytical requirements for a test method.

SMPRs are developed through voluntary stakeholder consensus by those who use and need methods, and reflect the analytical requirements of the user community, taking into account technological considerations, compliance expectations, and other issues deemed priority by stakeholders and examined carefully by topic-specific working groups who are experts in the field. The process by which SMPRs are developed ensures transparency, openness, balance of interest, due process, appeals, and voluntary consensus. In developing SMPRs, AOAC typically forms smaller working groups from a larger stakeholder body to address specific analytical issues. Working groups provide technical expertise in establishing SMPRs, which set the criteria that methods must meet. After working groups reach agreement, recommendations such as SMPRs (for example, sensitivity, specificity, analytical range, and LOD) are provided to the stakeholder panel for approval.

Once SMPRs are adopted by stakeholders, candidate methods (which are received through calls for methods and literature searches) are reviewed by ERPs to determine if they meet the performance requirements specified in the SMPR. It is possible that several methods of analysis could potentially meet the requirements of an SMPR, and be approved by an ERP. Multiple methods would provide analysts with more freedom to select and use a method that best fits their laboratory. The SMPR approach is less prescriptive than the traditional Official MethodsSM program and more similar to the Codex criteria-based approach, which involves compliance with a set of criteria without endorsement of specific methods. However, the new AOAC system does allow for the designation of “dispute resolution” methods where it is necessary to specify a single “reference” method. Dispute resolution methods are identified as such in the applicability statement of the method.

Infant Formula Initiative

On April 26, 2010, AOAC reached an agreement with the International Formula Council (IFC) on a 2-year project to create SMPRs for methods for infant and adult nutritionals nutrients: vitamin A, vitamin D, vitamin B12, folate, inositol, vitamin E, nucleotides, ultra trace minerals (selenium, chromium, molybdenum), carnitine, iodine, and pantothenic acid (vitamin B5). Methods are urgently needed for dispute resolution because a variety of methods is used around the world for these products.

AOAC formed an advisory panel in the summer of 2010 to identify and prioritize the infant formula nutrients most needing SMPRs. The advisory panel decided to organize the nutrients into four groups of about five nutrients for the sake of project management. The groups were organized to maximize complementary methods that might use similar procedures, and to represent different areas of expertise so that the work of subject matter experts in one specific area could be better managed over the 2-year contract.

The nutrient priorities and groupings were forwarded to the Stakeholder Panel on Infant Formula and Adult Nutritional (SPIFAN), which was charged with organizing working groups. Methods for vitamins A and E were considered similar enough to organize into one logical unit. SPIFAN then organized individual working groups for vitamins B12 and D, folate, and inositol. The five working groups met at AOAC headquarters on November 8–12, 2010, to draft SMPRs for each of the nutrient methods groups. The resulting draft SMPRs were posted on the AOAC website in February 2011, for a public comment period. All comments received were carefully considered and reconciled. The final draft SMPRs were reviewed and approved by SPIFAN on April 4–6, 2011 (Appendix 1).

At the same time the SMPRs were being drafted, the nutrient-specific working groups also evaluated a total of approximately
80 (narrowed down from about 150) of the most promising methods for further evaluation, and recommended potential candidate methods most likely to meet fitness-for-purpose and the draft SMPRs for vitamins A, B₁₂, and D, folate, and inositol. High-technology methods using LC-MS/MS were most often chosen. In early 2011, the method developers for each of the selected candidate methods were notified and asked to prepare an informative presentation on their method for review by the stakeholder panel and the ERP. In cases where a method developer could not be identified, an advocate for the method was recruited to prepare an informative presentation.

In April 2011, SPIFAN reviewed the presentations for the most promising methods that were selected by the working groups in order to assess their potential for further evaluation and possible adoption as First Action by an ERP.

After the SPIFAN review of the informative presentations, the candidate methods were transferred to the ERP for consideration. The ERP recruited a primary and a secondary reviewer for each candidate method to determine the worthiness and fitness-for-purpose of each method, using the SMPR as the standard. The primary and secondary reviewers summarized the advantages and disadvantages of their assigned method, and its compliance to the SMPR. Candidate methods were then discussed thoroughly by the entire panel, stakeholders, and observers present. The methods were evaluated for completeness of validation and likelihood of meeting SMPRs (appropriateness for the intended use, clarity of the method description, ruggedness, reproducibility, recovery, analytical range, LOQ, etc.).

In April 2011, SPIFAN reached consensus on fitness-for-purpose statements for nucleotides and ultra trace minerals, AOAC issued a call for methods, and working groups developed draft SMPRs, which were posted for public comment. Also in April, each working group chair provided background and examined analytical challenges, regulatory requirements, and existing methodology for nucleotides and ultra trace minerals in an effort to reach consensus on the expected performance of each candidate method.

During the “Standards Development and International Harmonization: AOAC INTERNATIONAL Mid-Year Meeting,” in June 2011, the ERP adopted 11 methods for First Action (folate and vitamins A, B₁₂, and D) and made recommendations for additional information needed, when needed, to advance the methods from First to Final Action status.

In September 2011, during the AOAC Annual Meeting, SPIFAN approved SMPRs and First Action methods for ultra trace minerals and nucleotides, as well as approved one First Action method for inositol.

Methods Granted First Action with Changes Noted for Final Action Approval

Folate


The second method, “Total Folates in Various Foods by Trienzyme Extraction and UPLC-MS/MS Quantitation,” was submitted by General Mills/Medallion Laboratories. The method uses ultra-high performance LC-MS/MS to measure total folates of seven chemical forms. The method was approved by the ERP as Official Method 2011.06, with modifications to include purity assessment of the standards and a second product ion.

Vitamin A

The Working Group on Vitamin A, chaired by Jonathan DeVries of General Mills/Medallion Laboratories, recommended two methods for adoption as First Action methods. The first method was submitted by the Nestlé Research Center, and is a UPLC method that uses alcoholic saponification with potassium hydroxide in the presence of antioxidant to extract retinol and convert retinol esters to retinol. Diatomaceous earth cartridges are employed to clean up and concentrate the saponified extracts, and normal-phase chromatography with UV detection to separate, detect, and quantitate retinol. This method was adopted as Official Method 2011.07.

The second method, submitted by General Mills/Medallion Laboratories, was a previously AOAC-approved method for vitamin A in foods: AOAC Official Method 2001.13 “Determination of Vitamin A (Retinol) and E (alpha-Tocopherol) in Foods by Liquid Chromatography: Collaborative Study” [J. AOAC Int. 85, 424 (2002)]. In AOAC method 2001.13,
standards and samples are saponified in a basic ethanol–water solution to convert fats to fatty acids, and retinol esters to retinol. Although already approved as AOAC Official Method 2001.13, the ERP agreed that the method should be approved for infant formula and adult nutritionalas as Official Method 2011.15, and still undergo the same evaluation process as all other methods for this project. The ERP also recommended that additional information be collected for all types of infant formulas and adult nutritional matrixes at varied concentration levels, as indicated in the SMPRs, prior to adoption as a Final Action method.

Vitamin B₁₂

The Working Group on Vitamin B₁₂, chaired by Esther Campos-Giménez, Nestlé Research Center, recommended adoption of “Determination of Vitamin B₁₂ in Milk Products and Selected Foods by Optical Biosensor Protein-Binding Assay” [Indyk, H.E., Persson, B.S., Caselunghe, M.C.B., Moberg, A., Filonzi, E.L., & Woollard, D.C. (2002) J. AOAC Int. 85, 72–81] as Official Method 2011.16. This method is similar to the company’s folate method (2011.05). Both methods use SPR optical biosensor, which measures refractive index changes on a chip surface due to binding events. Stakeholders expressed concern over the availability of the relatively uncommon SPR instrument and commercial kit. However, the ERP deemed the method scientifically sound. [The method was previously approved as Official Method 2011.01 (Vyas, P., & O’Kane, A.A. (2011) J. AOAC Int. 94, 1217–1226), but not in terms of the SPIFAN project for dispute resolution methods.]

The Working Group on Vitamin B₁₂ also recommended two methods that were similar enough to be considered together. They are “Determination of Vitamin B₁₂ in Food Products by Liquid Chromatography/UV Detection with Immunoaffinity Extraction: Single-Laboratory Validation” [Campos-Giménez, E., Fontannaz, P., Trisconi, M-J., Kilinc, T., Gimenez, C., & Andrieux, P. (2008) J. AOAC Int. 91, 786–793], and “Determination of Vitamin B₁₂ in Baby Food (Milk Formulas) Using HPLC After Purification on an Immunoaffinity Column,” submitted by Central Laboratories Friedrichsdorf GmbH (CLF; a wholly owned subsidiary of the DANONE Group). Both methods are based on extraction, immunoaffinity column cleanup, and LC/UV quantitation. The methods were adopted as Official Methods 2011.08 and 2011.09, respectively, and the ERP recommended that they be combined into a single method for consideration of Final Action approval.

The working group also recommended “Determination of Vitamin B₁₂ by HPLC” submitted by Abbott Nutrition. This method uses SPE to concentrate sample extracts, size exclusion and reversed-phase chromatography to separate vitamin B₁₂, and visible detection at 550 nm to measure vitamin B₁₂ concentrations. The ERP adopted the method as Official Method 2011.10, and suggested that additional clarification on standardization, calibrations, and precision would be helpful when the method is considered for Final Action status.

Vitamin D

The ERP approved three of the most promising methods recommended by the Working Group on Vitamin D, chaired by Donald Gilliland of Abbott Nutrition. The first method, “Application of Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS) for the Measurement of Vitamin D in Foods and Nutritional Supplements” [J. AOAC Int. 94, 211(2011)], was submitted by Covance Laboratories. The method uses UPLC-MS/MS to measure vitamin D in various foods and nutritional supplements. The ERP adopted the method as Official Method 2011.11.

The working group also recommended “Analysis of Vitamin D in Foods by Ultra-Pressure Liquid Chromatography with Tandem Mass Spectrometry Detection (UPLC-MS/MS),” submitted by General Mills/Medallion Laboratories. This method uses saponification and determination by UPLC-MS/MS. The ERP adopted the method as Official Method 2011.12.

The third method, “Simultaneous Determination of Vitamin D₂ and D₃ by LC-MS/MS for Infant Formula and Adult Nutritional,” submitted by Abbott Nutrition, uses an internal isotope standard that is saponified with the test sample. Vitamin D is extracted using liquid–liquid extraction, and the sample is evaporated under nitrogen. The dried sample is then reconstituted, filtered, and analyzed by LC-MS/MS. The ERP adopted this method as Official Method 2011.13.

Inositol

In November 2010, the Working Group on Inositol, chaired by Harvey Indyk, Fonterra Co-operative Group Ltd, and Karen Schimpf, Abbott Nutrition, recommended the development of (1) a hybrid GC method that combines two GC methods, and (2) a hybrid LC method that combines two LC methods. The hybrid GC method combines a method submitted by CLF, “Gas Chromatographic Determination of Inositol in Foodstuffs,” and one submitted by Nestlé Research Center, “Inositol by Gas Chromatography.” The first GC method uses flame ionization detection (FID) to measure free myo-inositol and inositol. The second GC method uses a GC/FID with overnight hydrolysis (16 h) to determine bound inositol.


At the time, the ERP agreed that none of the individual methods met the requirements of the SMPR applicability statement for inositol. However, the ERP requested that the working group evaluate the methods separately (four individual methods vs two hybrid methods). The ERP also requested more information for the GC methods. The ERP did not vote on the candidate methods for inositol, and agreed to re-examine these methods at the AOAC Annual Meeting in September 2011, when additional information would be available.

Subsequently, in September 2011, an ERP approved Official Method 2011.18 for determination of myo-inositol (free and bound as phosphatidylinositol) in infant formula and adult/pediatric nutritional formula. The HPLC method, submitted by Abbott Nutrition, has been in use since 1994. In addition, the ERP endorsed continuation of research for the method.
**Nucleotides**

The Working Group on Nucleotides, chaired by Brendon Gill, Fonterra Co-operative Group Ltd, recommended two methods for ERP review and possible approval. In “A Liquid Chromatographic Method for Routine Analysis of 5’-Mononucleotides in Pediatric Formula,” submitted by Fonterra Co-operative Group Ltd, the sample is dissolved in a high-salt solution to inhibit protein and fat interactions. Nucleotides are then extracted using strong anion exchange SPE, and then are analyzed by reversed-phase LC using photodiode array detection. Quantitation is by internal standardization against thymidine 5’-monophosphate. Advantages of the method, approved as *Official Method 2011.20*, are security of internal standard, moisture correction of calibration standards, and a simple mobile phase system.

In “Development and Application of an HILIC-MS/MS Method for the Quantitation of Nucleotides in Infant Formula,” submitted by Kinjo Gakuin University [J. Agric. Food Chem. (2010) 58, 9918–9924], and approved as *Official Method 2011.21*, the sample is dissolved in water followed by centrifugal ultrafiltration to remove compounds with a molecular weight higher than 3000. Isotopically labeled internal standards are used from the beginning of sample preparation, and a HILIC column is used for LC separation. Both milk-based and soy-based infant formula matrixes were studied. The method is highly selective, sensitive, and accurate (stable isotope internal standards), with good precision. It is quick and uses a simple sample preparation step (centrifugal ultrafiltration) for easy operation.

Both methods were approved by the ERP with recommendations to optimize the methods. For example, for the latter method, it was recommended to evaluate for robustness and optimizing chromatography to reduce the run time.

**Ultra Trace Minerals**


Advantages of the method are as follows: time to result is less than 8 h in many samples, addition of an internal standard up front provides good precision data, use of the latest collision/reaction cell technology for minimal interference, and better LOQ than required by the SMPRs. In addition, the method uses equipment/procedures common for major minerals (ICP-AES) and total iodine (ICP-MS). It has been implemented in routine use at Abbott Nutrition for about 1 year, without issues. The method uses a 2-step digestion process to maximize carbon removal.

In addition to First Action approval, the ERP recommended to examine optimizing the method from a 2- to 1-step digestion process and check for nickel in sample matrixes.

**First to Final Action Recommendations**

In addition to approving 15 First Action methods, the ERPs recommended collection of additional information to advance these First Action methods to Final Action status: evaluate methods against all of the types of infant formula identified in the SMPRs; evaluate the precision and accuracy of methods using samples representing the different types of infant formula identified in the SMPRs; properly characterize standards and clarify calibration procedures; demonstrate proof of performance (in terms of proprietary methods and chips) tested with either reference materials or by system suitability; demonstrate reproducibility if ERPs determine it to be necessary; test all future analyses on ready-to-feed (reconstituted) materials; and if possible, include information on rice-based infant formula.

**Conclusions**

Manuscripts were prepared from the available information, and submitted to the ERPs for review and publication for each of the 15 methods described above and approved as AOAC First Action *Official Methods*, during the “Standards Development and International Harmonization: AOAC INTERNATIONAL Mid-Year Meeting” in June 2011, and the AOAC Annual Meeting in September 2011. The first collection of seven manuscripts to be reviewed and approved through this peer-review process is published in this issue.

Under the SPIFAN project, the ERPs recommended that all First Action methods should undergo further study in a single-laboratory validation (SLV) using centrally prepared testing materials. Once the SLVs are complete, data will be reviewed by ERPs in an effort to select one dispute resolution method per nutrient, which will also be published in subsequent issues of this journal.

Details are yet to be finalized at this writing, but dispute resolution methods most likely will undergo further study to generate additional information for consideration as Final Action status.