

GENERAL REFEREE REPORTS

Committee on Food Nutrition

Water-Soluble Vitamins

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Summary

Simultaneous Determination of Vitamins.—Klejdus et al. (1) described a simultaneous determination of 10 water- and 10 fat-soluble vitamins in pharmaceutical preparations by liquid chromatography-diode-array detection (LC-DAD). A combined isocratic and linear gradient allowed separation of vitamins in 3 distinct groups: polar, low-polar, and nonpolar. The method was applied to pharmaceutical preparations, fortified powdered drinks, and food samples, for which results were in good agreement with values claimed.

Heudi et al. (2) described a separation of 9 water-soluble vitamins by LC-UV. The method was applied for the quantification of vitamins in polyvitaminated premixes used for the fortification of infant nutrition products. The repeatability of the method was evaluated at different concentration levels and coefficients of variation were <6.5%. The concentrations of vitamins found in premixes with the method were comparable to the values declared.

A disadvantage of the methods mentioned above is that sample composition has to be known in advance. According to European legislation, for example, foods might be fortified with riboflavin phosphate or thiamin phosphate, vitamers which are not included in the simultaneous separations described.

Vitamin B₂.—Viñas et al. (3) elaborated an LC analysis of riboflavin vitamers in foods. Vitamin B₂ can be found in nature as the free riboflavin, but in most biological materials it occurs predominantly in the form of 2 coenzymes, flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD). Several methods usually involve the conversion of these coenzymes into free riboflavin before quantification of total riboflavin. According to the authors, there is growing interest to know flavin composition of foods. The described method separates the individual vitamers isocratically. Accuracy of the method is tested with 2 certified reference materials (CRMs).

Vitamin B₅.—Methods for the determination of vitamin B₅ in foods are limited because of their low sensitivity and poor selectivity. Pakin et al. (4) proposed a post-column derivatization of pantothenic acid as a fluorescent compound and used this principle in a specific and sensitive method for the determination of free and bound pantothenic acid in a large variety of foods. A French laboratory invited European

laboratories to participate in a series of collaborative studies for this method, which will be carried out in 2005/2006.

A more sophisticated method was described by Mittermayer et al. (5). They developed an LC-mass spectrometry (LC/MS) method for the determination of vitamin B₅ in a wide range of fortified food products. Application of the method to various samples showed consistent results with those obtained by microbiology.

Vitamin B₆.—Method 2004.07, an LC method for the analysis of vitamin B₆ in reconstituted infant formula, was published by Mann et al. (6). In contrast with this method, which quantifies vitamin B₆ after converting the phosphorylated and free vitamers into pyridoxine, Viñas et al. (7) published an LC method which determines 6 vitamin B₆ related compounds, the 3 B₆ vitamers, their corresponding phosphorylated esters, and a metabolite. Accuracy was determined using 2 CRMs. Results were within the certified ranges.

Vitamin C.—Franke et al. (8) described an extensive study to vitamin C and flavonoid levels of fruits and vegetables consumed in Hawaii. Vitamin C was determined by measuring ascorbic acid in its reduced state by LC and coulometric detection along with UV absorbance detection at 245 nm. No attempts were made to assess levels of dehydroascorbic acid. Most recent research revealed that cell uptake of dehydroascorbic acid is unlikely to play a major role, which may explain the very low vitamin C activity of orally administered *L*-dehydroascorbic acid in rats (9). The food levels found by Franke et al. (8) are variably lower, higher, or equal in comparison to other studies.

Iwase (10) described a method for the determination of ascorbic acid in foods using *L*-methionine for the pre-analysis sample stabilization. Electrochemical detection was used for the quantification. Traditionally, metaphosphoric acid was proven to be a useful dissolving agent for the determination of ascorbic acid. However, it dissolves in water very slowly, it is hygroscopic, and accurate weighing is not easy. Adjustment at pH 2–3 takes a long time. It appeared to be possible to replace metaphosphoric acid by 0.2% phosphoric acid. Methionine played an important role on the stability of ascorbic acid. The method seemed to be applicable to the routine analysis of ascorbic acid in foods.

Folic Acid.—Microbiological analysis of total folate in foods is often considered as the golden standard compared to other methods based on, for example, LC. Koontz et al. (11) showed results of total folate concentrations measured by microbiological assay in a variety of foods. Samples were submitted in a routine manner to experienced laboratories that regularly perform folate analysis fee-for-service basis in the United States. Each laboratory reported the use of a microbiological method similar to the AOAC Official Method for the determination of folic acid. Striking was, the use of

3 different pH extraction conditions by 4 laboratories. Only one laboratory reported using a tri-enzyme extraction. Results were evaluated. Results for folic acid fortified foods had considerably lower between-laboratory variation, 9–11%, versus >45% for other foods. Mean total folate ranged from 14 to 279 $\mu\text{g}/100\text{ g}$ for a mixed vegetable reference material, from 5 to 70 $\mu\text{g}/100\text{ g}$ for strawberries, and from 28 to 81 $\mu\text{g}/100\text{ g}$ for wholemeal flour. One should realize a large variation in results, which might be caused by slight modifications in the microbiological analysis of total folate in foods or the analysis in various (unfortified) food matrices. Furthermore, optimal combination of enzymes and reaction conditions may vary depending on the composition of the food. Padrang and Laborde (12) showed recently that treatment with α -amylase had no significant effect on measured folate in spinach, although addition of protease significantly increased the release of folate.

LC/MS applications gain increasing attention because of their specificity. Rychlik (13) used stable isotope dilution assays for the determination of the folate content of broccoli and bread. Compared to data in the literature and food data bases, amounts were significantly lower. Pawlosky et al. (14), however, found comparable values for 5-methyltetrahydrofolic acid and folic acid by HPLC analysis with fluorescent detection and HPLC/MS. Among samples analyzed were CRMs and broccoli. Besides folic acid, other water-soluble vitamins were also determined by LC/MS/MS by Leporati et al. (15). The method was applied to the quantitative analysis of the natural content of vitamins in typical Italian pasta samples, as well as in fortified pasta samples produced for the U.S. market.

Biotin.—A paper from Staggs et al. (16) included the assertion that existing biotin data in food composition tables are inaccurate because the majority are based on bioassays with all relevant disadvantages. Data in most cases are overestimated with consequences for recommendations for dietary biotin intake. An HPLC/avidin-binding assay was used to analyze 87 foods to support the hypothesis mentioned.

Reference Materials

The National Institute of Standards and Technology (NIST) has developed a suite of food-matrix standard reference materials (SRMs) characterized for nutrient concentrations (17, 18). They are essential to demonstrate accuracy of analytical methods. To demonstrate that a method is applicable to a variety of foods, AOAC INTERNATIONAL's Task Force on Methods for Nutrition Labeling developed a triangle in which foods could be organized based on their fat, protein, and carbohydrate content. Their belief is that one or 2 foods within a sector are representative of other foods within that sector, and if an analytical method provides accurate results for the test foods, it should also provide accurate results for the other foods in the same sector. Assigned values for vitamins in NIST's food-matrix SRMs and an RM that was prepared by Agriculture Canada are Slurried Spinach and Peanut Butter, which were available from 2003. Others were available before

2003: Meat Homogenate, Coconut Oil, Infant Formula, Baby Food Composite, Baking Chocolate, and Whole Milk Powder. In a multi-year interagency collaboration among NIST, the National Institutes of Health's Office of Dietary Supplements, and the U.S. Food and Drug Administration (FDA) Center for Food Safety and Applied Nutrition and Center for Drug Evaluation and Research, SRMs for dietary supplements will be developed. The goal of this collaboration is to provide SRMs for 6–8 dietary supplements, including a multivitamin/multielement supplement, over the next 5 years.

Furthermore, CRMs for vitamins are available from the Institute of Reference Materials and Measurements (IRMM) in Geel (Belgium). They include a Pig Liver, Milk Powder, Vegetable Mix, Margarine, Brussels Sprouts, and a Wholemeal Flour.

Extension of AOAC Method for the Liquid Chromatographic Analysis of Vitamin B₆ in Infant Formula to the Determination of Vitamin B₆ in Baby Food (AOAC 2004.07), Biscuit, Cereals, Yeast, Tube-Feeding Solution, Chocolate Powder, and Powdered Milk

From last year's report it could be concluded that most of AOAC Official Methods of analysis in the area of water-soluble vitamins are outdated. HPLC is used in few of the methods. It is clear that replacement of these methods would be time consuming and expensive. Meanwhile, it would be sensible to look at other appropriate information available. In 2004 a method from the FDA for the LC analysis of vitamin B₆ in infant formula achieved First Action status (6). This work was based on a method described by Reitzer-Bergaentzle et al. (19).

The European Committee for Standardization (CEN) used precision data from a collaborative study described by Bergaentzle et al. (20) and was based on the method described in 1993, which was the basis for FDA's method.

Total vitamin B₆ is quantified by converting the phosphorylated and free vitamers into pyridoxol. Pyridoxol is determined by ion pair reversed-phase LC using fluorescence detection. The method subjected to an AOAC collaborative study involved 4 milk-based and 4 soy-based infant formulas, each in blind duplicate. Statistical parameters included mean RSD_r% of 4.97 and 4.63, mean RSD_R% of 8.27 and 8.43, mean HorRat values of 0.472 and 0.475, and RSD_r:RSD_R ratios of 0.601 and 0.549, respectively, for fortified milk- and soy-based infant formulas.

Table 1 shows the precision data for the determination of vitamin B₆ performed and described by Bergaentzle et al. (20) and included in CEN ENV 14164.

As described in the results of the collaborative study, 12 participants received 8 different samples. For each foodstuff tested, all the laboratories were asked to perform the analysis in triplicate. The participants in this collaborative study first familiarized themselves with the method during a pretrial test, for which foodstuffs of a very similar nature were proposed. The precision data for the determination of vitamin B₆ were established in the interlaboratory test according to

Table 1. Precision data for the determination of vitamin B₆

Sample ^a	1	2	3	4	5	6	7	8
Year of interlaboratory test	1993	1993	1993	1993	1993	1993	1993	1993
Number of laboratories	12	12	12	12	12	12	12	12
Number of laboratories after elimination of outliers	11	10	11	12	11	11	12	11
Number of results retained	32	29	31	36	33	33	35	33
Mean value (mg/100 g)	0.06	0.14	0.22	0.53	0.55	0.67	1.50	3.28
Repeatability standard deviation	0.01	0.02	0.02	0.04	0.02	0.03	0.10	0.09
Repeatability relative standard deviation RSD _r (%)	18	13	10	8	4	4	6	3
Reproducibility standard deviation (mg/100 g)	0.02	0.05	0.07	0.14	0.07	0.08	0.18	0.43
Reproducibility relative standard deviation RSD _R (%)	30	35	30	26	13	12	12	13
HorRat	1.7	2.3	2.1	2.1	1.1	1.1	1.1	1.4
Mean results of AOAC microbiological method (1990; only 4 labs)	0.10	0.19	0.24	0.54	0.53	0.60	1.61	3.55

^a 1 = Baby food, 2 = biscuit, 3 = cereal B, 4 = yeast, 5 = tube-feeding solution, 6 = chocolate powder, 7 = cereal A, 8 = powdered milk.

ISO 5725 carried out by DGCCRF (Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes) in France.

As can be seen from the HorRat, vitamin B₆ amounts in 8 different matrixes could be determined with acceptable precision data. The Official Methods Board recommended to extend the existing AOAC First Action method for the determination of vitamin B₆ in infant formula with 8 other matrixes.

Update on European Committee Standardization (Working Group on Vitamins and Carotenoids)

The working group had its last meeting in June 2005. Topics discussed were classified in:

Status of work.—The working group has finished a standard (prEN 14663) for the determination of vitamin B₆ in foods by HPLC. Vitamin B₆ vitamers pyridoxine, pyridoxal, and pyridoxamine are extracted from food by acid hydrolysis and dephosphorylated and deglycosylated enzymatically using acid phosphatase and β-glucosidase. The individual vitamers (pyridoxine, pyridoxal, pyridoxamine) are separated by HPLC and quantified by fluorescence detection. This method was successfully validated for infant food, potato puree, vegetables with ham, and multivitamin drink. After approval of individual member states, this standard will be published.

New items on the work program.—The working group discussed a draft of a method for the determination of biotin in foods and a draft of a method for the determination of niacin (vitamin B₃) in foods. Both are HPLC methods. The working group agreed that these methods are suitable for elaboration as European Standard. Drafts will be included in the work program for further discussion and improvement.

Periodical review of existing CEN standards.—European Standards are, 5 years after publication, due for review. Existing standards for the determination of Vitamin D (EN 12821), Vitamin E (EN 12822), and Vitamin A (EN 12823) are reviewed by the working group. All reactions on the reviews are evaluated. Revisions like extension of the Standard

for vitamin D with additional validation data of other matrixes, or an improved text for the purity determination of vitamin A will be included in revised versions of the Standards.

Possible future work.—(a) *Astaxanthin/cantaxanthin.*—The working group identified the need for possible future standards for the determination of astaxanthin and cantaxanthin in, e.g., salmon. It can be used to differentiate between wild salmon, conventionally raised and organically raised salmon by analyzing the ratio of the configurational isomers of astaxanthin in salmon flesh. Several suitable methods are identified. However, none have been interlaboratory tested. The working group feels the need to combine all the good aspects of these methods into one, which, e.g., could be used for a future collaborative trial. A subgroup works on a proposal for the next meeting. (b) *Vitamins in supplements.*—The working group has identified several methods of analysis for supplements containing vitamins. However, none have been interlaboratory tested. The working group feels the need to combine all the good aspects of these methods into one, which, e.g., could be used for a future collaborative trial. In early 2006 some new data are expected from a collaborative trial organized for UK laboratories. The Food Standards Agency commissioned LGC to develop in consultation with supplement manufacturers, guidelines for the analysis of supplements and where possible, generic procedures for control purposes. On a next meeting of the working group it will be decided to start a subgroup to come up with a proposal for a “combined” method.

Evaluation of Biacore Test Kits for the Determination of Riboflavin (Vitamin B₂), Pantothenic Acid (Vitamin B₅), Cyanocobalamin (Vitamin B₁₂), Folic Acid, and Biotin in Foods and Supplements by Optical Biosensor Immunoassay

Biacore submitted applications to obtain *Performance-Tested Methods*SM status of 4 test kits for the determination of

vitamin B₂, vitamin B₅, vitamin B₁₂, and biotin, respectively. All kits are used together with a Biacore Q, Qflex[®].

The purpose of the AOAC Research Institute *Performance-Tested Methods*SM program is to provide an independent third-party review of producers' test kit performance claims. Upon application and independent testing, test kits found to be in conformance with their claims will be granted *Performance-Tested Methods*SM.

All kits use Biacore's Surface Plasmon Resonance (SPR) biosensor technology and will be used for the analysis in foods, especially fortified products and supplements. The sensitivity of the SPR assay technology coupled with the specificity of the ligand-binding protein interaction allows a rapid assay with a minimum of sample preparation required.

Biacore's folic acid kit is a *Performance-Tested Method*SM, already.

After performance testing, all 5 test kits (including folic acid) will proceed to AOAC's Official MethodsSM Program. Vitamins mentioned will be collaboratively tested in different matrixes, such as infant formula, breakfast cereals, fortified drinks, and dietary supplements.

Selected Study Director Topics

Biacore methods for the determination of riboflavin (vitamin B₂), pantothenic acid (vitamin B₅), cyanocobalamin (vitamin B₁₂), folic acid, and biotin in foods and supplements by optical biosensor immunoassay will be Study Director Topics in 2005/2006. Pathik Vyas of AgriQuality in New Zealand will be assigned as Study Director for these topics.

Recommendations

Extend the scope of the AOAC Official Method for the LC analysis of vitamin B₆ in infant formula to also include the determination of vitamin B₆ in baby food, biscuit, cereals, yeast, tube-feeding solution, chocolate powder, and powdered milk.

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Nonvitamin Micronutrients

HARVEY INDYK

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Summary

Nonvitamin micronutrients encompass innumerable nutritionally active substances present in foods at low levels that may have beneficial and protective properties. These include nonvitamin carotenoids, essential fatty acids, amino acids, phospholipid components, and conditionally essential components such as carnitine, choline, inositol, and any compounds with antioxidant, growth promoting, antimicrobial, or immune potential. These analytes are grossly underrepresented in the *Official Methods of Analysis* (OMA).

The General Referee urges that the nonvitamin micronutrients, carnitine, inositol, and nucleotides need to be strategically targeted for collaborative study because they are not covered in OMA by any technique. Carnitine is available through limited de novo synthesis, although deficiency is recognized, particularly in infants. Thus, infant formulas are commonly supplemented with carnitine, and reliable analytical techniques are needed. A published enzymatic methodology will hopefully be subjected to collaborative study in the near future. Similarly, inositol is considered a conditionally essential pseudovitamin and although microbiological, gas and liquid chromatographic techniques

are generally used, these approaches have not been subjected to the highest level of validation. Nucleotides play important roles in major biochemical functions, and recent evidence suggests that dietary nucleotides are semiessential for newborns. The nucleotides and nucleosides are present in human milk at relatively high levels, so bovine milk-based infant formulas are increasingly supplemented with the 5'-monophosphate nucleotides, and it is unfortunate that no current AOAC method exists for quantification of nucleotides.

Colostrum immunoglobulins, specifically IgG, confer passive immunity to the neonate until the immune system is developed. There has been an increase in the global availability of colostrum-based functional foods and supplements, which are claimed to improve gastrointestinal health and stimulate the immune system. In the absence of a reference analytical method, it is becoming increasingly important to standardize analysis techniques for IgG in such materials. Although commercial radial immunodiffusion (RID) kits are available, they are generally variable in response. An affinity high-performance liquid chromatography (HPLC) method based on specific binding of bovine IgG with immobilized Protein G has been validated within the laboratory of the prospective Study Director Don Otter and is currently at the protocol development stage. Biosensor technology can provide an alternative approach to IgG analysis, and an antibody-based optical method is also slated for collaborative study under Study Director Leyton Gapper.

Where other organizations involved with method validation are active in these analyte areas, it may be timely to consider AOAC policy regarding joint adoption in order to avoid duplication of scarce scientific resources. Major difficulties may occur when validation protocols do not entirely meet the AOAC INTERNATIONAL, ISO 5725, and

IUPAC harmonized protocols. Whether such studies need either to be repeated in full using OMA protocol, or perhaps, preferably, publish the method within OMA at a lower level of validation, requires clarification at the Official Methods Board level.

Selected Study Director Topics

No research activity or method updates this year.

Recommendations

Determination of IgG in Colostrum Products—Affinity LC Method.—Study Director Donald Otter, Fonterra Research Centre, Palmerston North, New Zealand, E-mail: don.otter@fonterra.com. Seeking collaborators. Continue study. This is a new method study and is to be funded by Fonterra, New Zealand.

Determination of IgG in Colostrum Products—Biosensor Method.—Study Director Leyton Gapper, Fonterra Research Centre, Palmerston North, New Zealand, E-mail: leyton.gapper@fonterra.com. Seeking collaborators. Continue study. This is a new method study and is to be funded by Fonterra, New Zealand.

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