

GENERAL REFEREE REPORTS

Committee on Food Nutrition

Fat-Soluble Vitamins

CHRISTOPHER J. BLAKE

Nestlé Research Center, Quality and Safety Assurance Department, Vers Chez-les-Blanc, 1000 Lausanne 26, Switzerland, Tel: +0041 21 785 8348, E-mail: christopher-john.blake@rdls.nestle.com

Summary

The analysis of the fat-soluble vitamins A, E, D, and K₁ are covered by the AOAC Official Methods in Chapters 45 (vitamins and other nutrients) and 50 (infant formula, baby foods, and enteral products). The collaborative study of the NMKL procedure for vitamin D₃ was published (1). This procedure had been previously approved as AOAC Official Method **2002.02**. However there is still a need for further horizontal food methods of analysis for FSV and a validated method for carotenes.

Since the last review (2) some further developments have been made in the area of sample preparation. Hoeller et al. (3) described a rapid microwave technique for saponification followed by cyclohexane extraction of vitamins A and E from beverages. This merits further study on other matrixes. Rodriguez-Comensana et al. (4) described a rapid procedure for controlling nutritional label content of beta-carotene by direct injection of the drink onto an HPLC-UV system.

A matrix-specific method for supplemented vitamin A palmitate in liquid milk has been approved for First Action through Committee F (5). Retinyl acetate is used as the internal standard.

One of the major problems in analysis of FSV is to optimize the saponification and extraction conditions. Some progress has been made in this area by Paixao and Campos (6) who investigated the intrinsic variability of analyses of fat-soluble vitamins by reversed-phase HPLC-UV. They concluded that methanol and ethanol saponification media without heat provided the best conditions for simultaneous extraction of FSV. Tanumihardjo and Penniston (7) reported the use of 3,4-didehydroretinyl acetate as an internal standard for vitamin A analysis in breast milk by HPLC. The authors report that it can be added to milk before saponification and is carried through the analysis as dehydroretinol (vitamin A₂).

One of the major recent developments is the use of LC/MS as a sensitive and selective means of determining one or more FSV in foods. The single-quadrupole mass spectrometer is capable of providing good sensitivity and quantitative linear calibration over several orders of magnitude. Kalman et al. (8) made a preliminary study of the determination of α -tocopherol in fortified food products using the selective ion monitoring (SIM) mode with deuterium labeled α -tocopherol

as an internal standard. Heudi et al. (9) described a normal-phase isocratic separation of vitamins A, D₃, and E with SIM detection for infant formula. A common saponification/extraction/SPE procedure was used for all 3 vitamins. Both all-*trans*-retinol and 13-*cis*-retinol were quantified to provide the total vitamin A content. The use of the readily-available vitamins D₂ and 5,7-dimethyltolcol as internal standards improved the quantification of vitamins D₃ and E (α -tocopherol), respectively. The use of vitamin A₂ as an internal standard was investigated for analysis of vitamin A but was not implemented due to some difficulties to obtain it sufficiently pure, free of vitamin A.

The area of dietary supplements is becoming increasingly important. A recent study was made (10) for the simultaneous determination of vitamin A and beta-carotene in dietary supplements. Two extraction methods were used: hexane-methylene chloride for soft gel capsules and direct solvent extraction for dietary supplements in tablet form. The LC gradient method could separate all-*trans*-retinol and beta-carotene from *cis*-isomers which have different biological activities.

The large number of recent publications shows the maintained interest in carotenoids analysis, probably due to increasing interest in using carotenoids for food fortification purposes. Reviews of carotenoid analysis were made by Minguez-Mosquera et al. (11), Oliver, and Palou (12), and Schoefs (13). Hayashi et al. (14) described the simultaneous analysis by TLC-densitometry of carotenoids used as colorings in foods. The carotenoids included annatto extract (norbixin), beta-cryptoxanthin (orange color), crocin, crocetin (gardenia yellow), capsanthin (paprika), lycopene (tomato), lutein (marigold), and beta-carotene. The procedure was applied to 294 food materials commercially available in Japan, including confectionery, ice cream, beverages, meat products, dairy products, and noodle dishes. A routine isocratic HPLC method for determination of the major carotenoids in ultra frozen orange juices was recently reported by Meléndez-Martínez et al. (15). Huck et al. (16) monitored carotenoids in vegetables using both HPLC and LC/MS/MS. The latter technique showed clear advantages for unambiguous identification of the various carotenoids.

A study for determination of alpha-carotene, beta-carotene, and lycopene is being set up for dietary supplements under the sponsorship of FDA/NIH. This work is expected to be assigned to Committee E for First Action.

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Selected Study Director Topics

(1) **2001.13** *Vitamins A and E in Foods by HPLC*.—Collaborative study for vitamin E to be re-run using an international collaborative study protocol. Study Directors, Jonathan W. DeVries and Karlene Silvera. No progress.

(2) *Carotene in foods*.—Study Director, Lynn Hagemann. The collaborative study results were not accepted by review committee. The method will not be proposed as First Action. However the study report is being redrafted for publication in *J. AOAC Int.*

(3) *Determination of vitamin K₁ in foods using C30 reversed-phase HPLC, separation of cis- and trans-phyloquinone*.—Study Director, Vacant. No progress.

(4) *Determination of vitamin K₃ in human and pet foods using reversed-phase HPLC*.—Study Director, Vacant. No progress.

Recommendations

(1) **2001.13** *Vitamins A and E in Foods by HPLC*.—Study Directors, Jonathan W. DeVries and Karlene Silvera, Medalion Laboratories. Continue topic to include vitamin E.

(2) *Carotene in foods*.—Study Director, Lynn Hagemann, Nestlé USA. Discontinue topic.

(3) *Determination of vitamin K₁ in foods using C30 reversed-phase HPLC, separation of cis- and trans-phyloquinone*.—No Study Director. Continue topic.

(4) *Determination of vitamin K₃ in human and pet foods using reversed-phase HPLC*.—No Study Director. Continue topic.

Sugars and Sugar Products

MARY AN GODSHALL

Sugar Processing Research Institute, Inc., 1100 Robert E. Lee Blvd, New Orleans, LA 70124, Tel: +1-504-286-4329, Fax: +1-504-282-5387, E-mail: godshall@srcc.ars.usda.gov

Summary

Sugars and Sugar Products has 7 subsections: (1) sugars and syrups, (2) molasses and molasses products, (3) confectionery, (4) honey, (5) maple sap, maple syrup, and maple syrup products, (6) sugar beets, and (7) corn syrups and other starch-derived sweeteners. The subcommittee continues to be under-represented in several areas.

Sugars and Syrups

Topic Advisor, Mary An Godshall. This topic could be included in Sugar and Sugar Products.

Molasses and Molasses Products

Topic Advisor is needed.

Confectionery

Topic Advisor is needed.

Honey

Topic Advisor, Peter Martin, Q.P. Services, Orchard Cottage, Crazyes Hill, Reading RG10 BLU, United Kingdom, Tel: +44-118-940-2212, Fax: +44-118-940-1235, E-mail: honeysci@aol.com. Did not follow up on his plan to list the methods that need to be updated, and is considered inactive at this time.

Corn Syrup and Other Starch-Derived Sweeteners

Topic Advisor, Jennifer Snyder, Corn Refiners Association, Inc., Regulatory & Technical Affairs, 1701 Pennsylvania Ave, NW, Suite 950, Washington, DC 20006, Tel: +1-202-331-1634, E-mail: jsnyder@corn.org. Snyder has been nominated and agreed to serve as Topic Advisor. The AOAC methods within this subsection were mailed to the Corn Refiners Association, Inc., for review, and Snyder, with the assistance of the membership of CRA, sent the following comments: (1) Corn Industry Research Foundation is still referenced rather than CRA and current revision dates are not used. (2) AOAC Method **977.21** Moisture in Corn Syrup: Sections of this method need revision and updating. For example, under section B, Determination, the method describes the use of a pan balance for weighing. Pan balances are obsolete, and their mention should be deleted. (3) AOAC Method **943.05**: The description on the Baume' and DS tables for measurement by refractometer do not reflect if these are IX or Non-IX syrups. (4) AOAC Method **969.39** Glucose Oxidase Method: May be an obsolete method; May have been dropped from the CRA method manual. (5) AOAC Method **979.23**

Saccharides in Corn Syrup (Major) and AOAC Official Method 983.22 Saccharides (Minor) in Dextrose Products: Both are on the list of methods to be updated.

Maple Sap, Maple Syrup, and Maple Syrup Products

A Topic Advisor is needed.

Sugar and Sugar Products

Topic Advisor, Mary An Godshall. There is no new activity to report. The 2002 report recommended updating methods and eliminating obsolete methods. It had been hoped to liaison with the International Commission for Uniform Methods of Sugar Analysis (ICUMSA) so that methods already collaboratively studied and accepted by ICUMSA under the IUPAC harmonized protocol could be rewritten in AOAC format and recognized by AOAC. We were in the process of contacting various Study Directors for this purpose when the issue of the large fee for recognition of methods arose. Since the sugar industry will not be inclined to pay these fees for recognition of their methods by AOAC, this initiative has been halted. There is, therefore, the concern that AOAC methods in this area will continue to fall behind and become obsolete.

Cane and Beet Sugar Products

Topic Advisor, Gillian Eggleston, U.S. Department of Agriculture, SRRC-ARS, 1100 Robert E. Lee Blvd, New Orleans, LA 70124, Tel: +1-504-286-4446, Fax: +1-504-286-4367, E-mail: gillian@srrc.ars.usda.gov. See below for Eggleston's update on oligosaccharide analysis. Oligosaccharides are gaining more importance as markers for various forms of degradation of sugar and syrups during processing. She also reiterated her concern from 2002 that AOAC sugar methods need to be updated.

Selected Study Director Topics

Formaldehyde in Maple Syrup by a Spectrofluorimetric Method

Study Director, Nathalie Martin, Centre ACER, 3600 Blvd Casavant, Saint-Hyacinthe, Quebec, Canada J2S 8E3, Tel: +1-450-773-1105, Fax: +1-450-773-8461, E-mail: nathaliemartin@centraccer.qc.ca. Martin has withdrawn the proposed collaborative study on formaldehyde in maple syrup by a spectrofluorimetric method, in view of the new fee structure imposed by AOAC for collaborative studies.

Recommendations

(1) In view of the fact that many AOAC methods in the area of sugar and sugar products are badly outdated or obsolete, and that the sugar industry will not likely fund their rec-

ognition by AOAC, it is requested that AOAC consider a mechanism for cross-referencing relevant methods to the up-to-date ICUMSA method and remove or surplus outdated AOAC methods.

(2) Continue the review of methods relating to corn syrup and other starch-derived sweeteners and make specific, detailed recommendations in next year's report.

Cane and Beet Sugar Products

GILLIAN EGGLESTON

U.S. Department of Agriculture, SRRC-ARS, 1100 Robert E. Lee Blvd, New Orleans, LA 70124, Tel: +1-504-286-4446, Fax: +1-504-286-4367, E-mail: gillian@srrc.ars.usda.gov

Summary

Separation and Analysis of Oligosaccharides

Despite the trend to more sophisticated (and expensive) instrumental methods for the analysis of oligosaccharides, the traditional methods of paper chromatography and thin-layer chromatography (TLC) still have their place. TLC, in particular, is still a useful technique for the rapid separation of large numbers of samples, requiring little or no prior cleanup. For screening studies, TLC may be used to analyze hundreds of samples in a single day. It has the added advantage of relatively low cost, as no expensive instrumentation is necessary. Developed plates may be scanned using ordinary desktop scanners and analyzed using densitometry software, yielding quantitative results that are perfectly acceptable for many purposes.

High-performance size exclusion chromatography (HPSEC) and gel permeation chromatography (GPC) with laser light scattering (LLS) or refractive index detection allow the separation and direct detection of oligosaccharides and provides molecular weight distribution information. However, LLS can sometimes give erroneous results, because of molecule-molecule interactions and associations which can occur in higher MW oligosaccharides, and refractive index detection of oligosaccharides is very insensitive (detects only in the mg and hundreds of mg range).

Oligosaccharides can be separated by high-performance liquid chromatography (HPLC), using reversed-phase columns, amino-silica columns, or ion-exchange sulfonate resin columns with calcium, lead, or silver counterions. High-performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) is now frequently used to separate and directly detect oligosaccharides at alkaline pH using gradient methods. HPAEC offers high separation resolution of oligosaccharides and even oligosaccharide isomers, coupled with very sensitive detection. However, the mass sensitivity of PAD decreases with an increase in degree of polymerization (DP).

High-performance capillary electrophoresis (HPCE) with laser-induced fluorescence (LIF) detection also provides high resolution of oligosaccharides, but a precolumn derivatization is required to produce spectroscopically active compounds.

HPCE has also been coupled with PAD to analyze oligosaccharide and alditol mixtures.

McPherson and Jane (1), using HPAEC-PAD on enzyme-digested starches, were able to detect maltooligosaccharides up to DP 85. In comparison, in a recent comparative study of oligosaccharides by Kuhn et al. (2) using capillary electrophoresis and matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF MS) and HPAEC-PAD, dextran oligosaccharides up to 45 DP were detected by HPCE and HPAEC-PAD, whereas MALDI-TOF MS allowed detection from DP 4 to DP 60. HPAEC-PAD was observed to be the most sensitive technique, but the separation resolution performance was better in HPCE and MALDI-TOF MS. Another advantage of MALDI-TOF MS is that it can provide accurate mass values, and the exact number of monosaccharides present in an oligosaccharide can be found. Conversely, a disadvantage of MALDI-TOF MS is that it is a destructive technique and, therefore, preparative work cannot be undertaken.

Fluorophore-assisted carbohydrate electrophoresis (FACE) technology is also used to separate and detect oligosaccharides, particularly from glycoconjugates. Analysis involves 4 steps: release, labeling with a fluorescent tag, separation using precast polyacrylamide gels, and imaging. Although FACE technology is simple and reliable, its use is not yet widespread. This may be due to a lack of papers describing specific applications, or possibly due to unfamiliarity with the technique.

Another technique also used to separate and detect oligosaccharides is the automated use of modern planar chromatography. This utilizes HPTLC plates, automated multiple development, completely automated elution systems, and sample positioning and spots detection apparatus, all of which allows reproducible separation of different oligosaccharides and other compounds in complex mixtures. Despite the improvement of TLC and HPLC techniques, gas chromatography (GC) still continues to have a place in oligosaccharide analysis, particularly for structural studies, although prederivatization is required.

Nuclear magnetic resonance (NMR) is a powerful technique used to elucidate the structure of oligosaccharides, especially with the advent of 2-D and various hyphenated methods for the determination of position and configuration of glycosidic linkages.

Many of the separation and analytical techniques mentioned above are used in conjunction with enzymatic or chemical degradation of an unknown oligosaccharide. A typical structural analysis of a complex oligosaccharide may involve partial acid hydrolysis or hydrolysis by specific enzymes, followed by separation and identification of the hydrolytic products. This combination of methods often yields structural information that cannot be obtained by methylation analysis, NMR, or chromatographic techniques alone. In particular, monomer sequence and distribution of branches and other substituents may be obtained in this way.

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Water-Soluble Vitamins

ERIK J.M. KONINGS

Food and Consumer Product Safety Authority/Inspectorate for Health Protection and Veterinary Public Health, PO Box 2168, 5600 CD Eindhoven, The Netherlands, Tel: +31 40 2911500, Fax: +31 40 2911600, E-mail: Erik.Konings@vwa.nl

Summary

Developments within the Committee of European Normalization (CEN) Concerning Water-Soluble Vitamin Analysis

CEN has published 5 standards. For the determination of vitamin B₆ in foods, ENV 14164 specifies an LC method for the determination of vitamin B₆ (the mass fraction of the sum of pyridoxine, pyridoxal, and pyridoxamine, including their phosphorylated derivatives in foods). ENV 14166 specifies a microbiological assay for the determination of the total vitamin B₆ content in foods. EN 14152 describes an LC method for the determination of vitamin B₂. CEN has released LC methods for vitamins B₁ (ENV 14122) and C, (ENV 14130), as well as a microbiological method for folate (ENV 14131).

In support of coming European legislation with harmonized rules on the sale of dietary supplements that contain vitamins and minerals and probable introduction of common safety rules on upper levels of vitamins, the vitamin working group of CEN decided to investigate possibilities for standardized methods of analysis. It appears that due to problems that may occur in sample preparation, standardized methods for water-soluble vitamins in dietary supplements should be prioritized. For example, Kall et al. (1) described the degradation of folic acid during extraction of multivitamin–mineral preparations possibly caused by the presence of copper ions.

The vitamin working group of CEN will elaborate a new European standard for the determination of niacin in foodstuffs by LC. The standard will be based on the work of Ndaw et al. (2). An enzymatic extraction will be used for the separate determination of nicotinic acid and nicotinamide, while for the determination of the sum of nicotinamide and nicotinic acid an acid hydrolysis can be used. Also a new European standard for the determination of biotin will be elaborated. This procedure is based on the work of Lahély et al. (3) and Arella et al. (4). D-Biotin and D-biocytyl are extracted from foods after an enzymatic treatment and quantified by HPLC with post-column derivatization.

Vitamin C

In the reporting year a method for the determination of vitamin C in fruit juices and related products was published (5). The results of this collaborative study were not satisfactory for AOAC First Action due to analyte instability.

Regarding vitamin C, it will be important to have (collaborative tested) studies for the determination of ascorbyl palmitate, because this substance may be used as vitamin C source in foodstuffs. Ascorbyl palmitate is not determined by the usual methods for the determination of vitamin C. A possibly suitable method for the determination of ascorbyl palmitate was published a few years ago by Dieffenbacher and Trisconi (6).

Niacin

Lacroix et al. (7) performed additional data on variability, robustness, and accuracy of AOAC *Peer-Verified Method*SM 1:2000 for the determination of niacin in infant formula by solid-phase extraction/LC (8).

Vitamin B₆

Kall (9) described an LC method for the determination of total vitamin B₆. By application of a mild acid hydrolysis prior to enzymatic digestion with acid phosphatase and -glucosidase and analysis of the 2 digests separately, it was possible to distinguish between free pyridoxine and -glucosylated forms of pyridoxine. Furthermore possible reasons for the difference between total vitamin B₆ values by the LC method and the microbiological technique are discussed.

Folic Acid

In 2003 a collaborative study for the analysis of added folic acid in cereal-based foods was performed by AACC. The method is based on an isocratic reversed-phase LC determination with UV detection preceded by solid-phase extraction. However, this cleanup might introduce chromatographical interferences. In that regard a recently published method by Doherty and Beecher (10) showed the subjection of an appropriate segment of the HPLC column effluent to photolytic conditions and thereby measures folic acid as a fluorescent product, which might be more selective for the determination of folic acid.

Multi-Methods

A rapid, reliable, and convenient liquid chromatographic (LC) procedure for routine compliance control and labeling of several B vitamins in infant formulas has been published by Woollard and Indyk. Riboflavin, riboflavin, pyridoxin, and niacinamide can be determined simultaneously, with consecutive measurement of thiamine under modified elution conditions (11).

Recently an LC method for the separation and determination of several water-soluble vitamins of the B group was published (12). The procedure is based on the use of a new amide-based stationary phase, which avoids the need of using the ion-pair technique, leading to narrower peaks and a simpler mobile phase. The performance of the method seemed good

according to the analysis of 2 certified reference materials. After adaptation of phosphate buffers the method might be useful in combination with MS.

New Analytical Techniques to Quantify Water-Soluble Vitamins

Surface plasmon resonance (SPR).—The Biacore®Q Biosensor system exploits the optical phenomenon of SPR to detect and measure biological interactions at an interface. The interface is a thin gold film on a sensor chip, to which is attached a layer of carboxymethyl dextran. The sensor monitors changes in the resonant angle which shifts when biomolecules bind to the chip surface and change the refractive index on the surface layer. The change in angle is directly proportional to the change of mass on the surface. Assays for biotin and folic acid using the Biacore Biosensor system had already been developed. New assays have recently become available for the quantification of riboflavin (vitamin B₂; 13) and pantothenic acid (vitamin B₅; 14) in foods.

Capillary zone electrophoresis (CZE).—Recently Okamoto et al. (15) described the in-capillary enzyme reaction method for the determination of riboflavin phosphate in a vitamin-enriched drink based on its conversion to riboflavin with alkaline phosphatase. Simultaneously, 3 water-soluble vitamins could be quantitated. Practical utility of a CZE method for the determination of flavin derivatives in foods and beverages was demonstrated by Cataldi et al. (16).

LC/MS.—More work is published where LC/MS is used in the determination of water-soluble vitamins. Thomas et al. (17) described a stable isotope LC/MS method for the quantitative determination of 5-methyltetrahydrofolic acid and folic acid in a variety of commercial citrus juices. It was described that stable isotope dilution assay is a promising tool for the quantification of pantothenic acid and folates (18, 19). Soon results will be published of the determination of pantothenic acid in a range of fortified products by reversed-phase liquid chromatography with electrospray ionization and mass spectrometry detection (20).

Yang and Irudayaraj (21) described the use of near-infrared (NIR) techniques for rapid determination of vitamin C in powdered mixtures and solutions.

Selected Associate Referee Topics

(1) *Vitamin C in Foods, LC Method.*—Study Director, Allan Brause. Repeat collaborative study in fruit juices. Prepare the method protocol for AOAC approval at the earliest opportunity.

(2) *Determination of Calcium Pantothenate in Vitamin Premixes and Tablets Using LC.*—Study Director, Gerald Woollard. Prepare the revised method protocol for AOAC approval at the earliest opportunity.

(3) *Determination of Folic Acid and 5-Methyltetrahydrofolic Acid in Foods by LC.*—Study Director, Erik J.M. Konings. Prepare the method protocol for AOAC approval at the earliest opportunity.

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