

GENERAL REFEREE REPORTS

Committee on Food Nutrition

Sugars and Sugar Products

MARY AN GODSHALL

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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, maple syrup products; (6) sugar beets; and (7) corn syrups and other starch-derived sweeteners. This subcommittee is currently under-represented in several areas, and attempts are being made to correct this situation.

Honey

Topic Advisor Peter Martin, Q.P. Services, Orchard Cottage, Crazies Hill, Reading RG10 BLU, UK, Tel: 44-118-940-2212, Fax: 44-118-940-1235, E-mail: honeysci@aol.com. The stable isotope method for honey (998.12, "Plant Sugars in Honey, Internal Standard Stable Carbon Isotope Ratio Method") is written incorrectly and Martin requests help rewriting it. (See also Study Director report on Stable Isotope Ratio Methods, below.) Martin also reports that with the adoption of the new Codex Standard, some AOAC methods are out of date. He plans to compile a list of the methods that need to be updated.

Corn Syrup and Other Starch-Derived Sweeteners

A Topic Advisor is needed. Melanie O'Donnell, Corn Refiners Association, Inc., 1701 Pennsylvania Ave, NW, Suite 950, Washington, DC 20006, Tel: +1-202-331-1634, Fax: +1-202-331-2054, E-mail: modonnell@corn.org, has been recommended. She has agreed to review the existing methods in the *Official Methods of Analysis*, 17th Ed. The Corn Refiners Association, like many professional organizations, has its own book of methods called the "Analytical and Microbiological Methods of the Member Companies," so it is possible that some of the AOAC corn sweetener methods may require updating.

Maple, Sap, Maple Syrup, and Maple Syrup Products

A Topic Advisor is needed.

Sugar and Sugar Products

The International Commission for Uniform Methods of Sugar Analysis (ICUMSA) is the organization within the sugar industry that is concerned with adopting official meth-

ods of analysis. ICUMSA follows the harmonized protocol for conducting collaborative studies. Methods are reviewed and updated periodically and older methods repealed. Because of this active pursuit of methods for the sugar industry within the ICUMSA organization, it is noted that many of the methods for sugar products listed in the OMA (subsections 1, 2, and 6) are out of date. ICUMSA held its 23rd Session June 3–5, 2002, in Pune, India. A number of collaborative studies were reported, which may be of interest to AOAC. All are found in the Reports of 2002 ICUMSA 23rd Session and are listed below. Copies of the reports are available upon request to the General Referee.

Collaborative tests reported at the 23rd Session of ICUMSA:

- (1) Polarization of sugar products without wet lead clarification
- (2) Determination of insoluble matter in white sugar
- (3) Determination of acid beverage floc in white cane sugar—10 day floc test
- (4) Determination of acid beverage floc in white beet sugar—24 h test
- (5) Comparative study of 2 color methods for highly colored specialty sugars and syrups using conventional pH adjustment and using TEA buffer
- (6) Determination of anticaking agents in powdered sugar
- (7) Sulfite analysis in brown sugar using an enzyme method
- (8) Reducing sugars in molasses using Layne and Eynon constant volume procedure
- (9) Total reducing sugars in molasses after hydrolysis using Layne and Eynon constant volume procedure
- (10) Modified Ofner method for reducing sugars in white sugar
- (11) Knight and Allen method for reducing sugars in white sugar
- (12) Hexokinase method for glucose + fructose in white sugar

Polarimetric Methods that Use Lead Acetate

A review of methods in Sugars and Sugar Products reveals that several AOAC methods use lead acetate for clarification in polarization measurement. For some years now, there has been an effort in the sugar industry to eliminate the use of lead salts in clarification and to substitute less toxic reagents, such as aluminum salts, or to use filter aid filtration with polarimeters that read sugar degrees in the near infrared region, where solution color does not interfere as much. AOAC methods in the OMA (16th Ed.) that use lead acetate include:

- (1) **925.46:** Sucrose in sugars and syrups, polarimetric methods

(2) **925.47**: Sucrose in sugars and syrups, polarimetric method before and after inversion with invertase

(3) **896.02**: Sucrose in sugars and syrups, double dilution method

(4) **930.36**: Sucrose in sugars and syrups, from reducing sugars before and after inversion

(5) **970.57**: Sucrose in molasses, polarimetric methods

(6) **948.23**: Reducing substances (unfermentable) in molasses, titrimetric method

(7) **920.190**: Sugars (reducing) in maple products as invert sugar

(8) **942.20**: Sucrose in sugar beets

It is recommended that a Study Director be appointed to review polarimetric methods and efforts should be made to update to methods that do not use lead salts as clarifying agents.

Cane and Beet Sugar Products

Topic Advisor Gillian Eggleston, SRRC-USDA-ARS, 1100 Robert E. Lee Blvd, New Orleans, LA 70124, Tel: +1-504-286-4446, Fax: +1-504-286-4367, E-mail: gillian@src.ars.usda.gov. In Chapter 44 of OMA, many AOAC methods for measuring sucrose in sugars and syrups are based on polarimetric methods, e.g., **925.46**. Three out of the 4 clarifying agents recommended in **925.46** include lead compounds. Because of the environmentally unsafe nature of lead compounds, and the high costs to safely dispose of them after use, there has been a great drive, over the last 10 years in the international sugar industry, to stop using them. Instead, clarifying agents based on aluminum compounds are being used, or in highly colored sugars to use celite as the clarifying agent and measure the clarified solution at 880 nm (instead of the standard 589 nm) for improved accuracy. It is recommended that the AOAC polarimetric methods be updated and brought into line with ICUMSA methods.

In general, the AOAC methods for measuring sucrose and invert sugars in cane or beet products are based on polarimetric and chemical methods. More modern methods of LC and GC should be included to give the users of AOAC methods more accurate options. ICUMSA has both LC and GC methods and the acceptance of Kevin Schaffler's ICUMSA method, using ion chromatography, on trace glucose and fructose analysis in sugar products, was a step in the right direction.

Eggleston recently demonstrated that an ion chromatography method (also known as HPAEC-PAD) that she developed can simultaneously detect ethanol, mannitol, and oligosaccharides in cane products (1). These compounds are sensitive indicators of different types of enzymatic and microbial deterioration in cane. This method could be applied to other agricultural and food commodities, especially sugarbeets. She would be interested in undertaking an interlaboratory study of this method, if there is enough interest.

She co-organized an American Chemical Society symposium in 2002 titled "Oligosaccharides in Food and Agriculture," and is currently editing a book based on the symposium, which includes up-to-date methodologies to measure oligosaccharides (2). Oligosaccharides are currently a very hot

topic, particularly their application as prebiotic nutrients to stimulate the growth of bifidobacteria in the human intestine. Much interest is being shown in the analysis of oligosaccharides for many applications, and robust and accurate analytical methods are essential for future progress. AOAC should be providing these. There are a number of techniques to measure oligosaccharides. Included here is an adapted review section written by Eggleston for the introductory chapter of the aforementioned book.

Separation and Analysis of Oligosaccharides (2)

Until the last 15 years, paper chromatography and thin-layer chromatography (TLC) were the most frequently used methods to separate oligosaccharides, but these have largely been replaced with more rapid and powerful separation and analytical techniques. High-performance size exclusion chromatography (HPSEC) and gel permeation chromatography (GPC) with laser light scattering (LLS) or refractive index detection allows the separation and direct detection of oligosaccharides and provides molecular weight distribution information.

Oligosaccharides can be separated by liquid chromatography (LC) including the use of reversed-phase and calcium columns. 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, one stated problem of using PAD to detect oligosaccharides of increasing DP (degree of polymerization) is that the mass sensitivity of PAD decreases with the increase of DP (3).

High-performance capillary electrophoresis (HPCE) with laser-induced fluorescence (LIF) detection also provides high separation resolution of oligosaccharides, but a precolumn derivitization is required to produce spectroscopically active compounds. HPCE can also be coupled with PAD to separate oligosaccharide and alditol mixtures.

McPherson and Jane (1999), using HPAEC-PAD on enzyme digested starches, were able to detect maltooligosaccharides up to DP 85 (4). In comparison, in a recent comparative study of oligosaccharides by Kuhn et al. (1999; 5) of capillary electrophoresis, 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. Drawbacks of MALDI-TOF MS are that quantitation is usually limited by poor reproducibility, and it is a destructive technique; therefore, preparative work cannot be undertaken.

Fluorophore assisted carbohydrate electrophoresis (FACE) technology is also being currently 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, quantitative accuracy is limited and also limited by available standards.

Another technique also being currently used to separate and detect oligosaccharides includes the automated use of modern planar chromatography. Furthermore, despite the improvement of LC 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 also still seen as a powerful technique to elucidate the structure of oligosaccharides.

Selected Study Director Topics

Visual Appearance of Sugar and Sugar Products

Study Director Mary An Godshall, Sugar Processing Research Institute, Inc., 1100 Robert E. Lee Blvd, New Orleans, LA 70124, E-mail: godshall@srrc.ars.usda.gov. SD recommends that **954.10**, Color of Raw Cane Sugars, be repealed [*JAOAC* **37**, 292(1954)]. The method is not scientifically valid, according to accepted modern methods of color measurement of raw cane sugar, studied and reported for many years by the International Commission for Uniform Methods of Sugar Analysis (ICUMSA). Modern methods of raw sugar solution color analysis are standardized at pH 7.0, 0.45 μ membrane filtration, and read at 420 nm. This method, which does not adjust pH, reads at 560 nm, and uses filter aid filtration, is based on obsolete methods used before the days of standardized color measurement. Color measurement at 560 nm gives much lower values than at 420 nm, and in a collaborative test showed poor precision (6).

Other methods for the measurement of the color of raw cane sugar are available. ICUMSA Method GS1/3-7 (The Determination of Raw Sugar Solution Color at pH 7.0 and the Determination of Solution Colors of Partly Refined and Brown Sugars and Colored Syrups at pH 7.0) underwent a collaborative test using IUPAC protocols in 1990 with satisfactory results for raw sugar (6). Subsequently, a collaborative test on colored specialty sugars was performed, again with satisfactory results (7).

A second method was developed and tested in 1998, using a pH 7.0 buffer (3-(N-morpholino) propanesulphonic acid) (MOPS). The collaborative study was satisfactory and the method was accepted as ICUMSA Method GS1-8, The Determination of Raw Sugar Solution Color at pH 7.0 by the MOPS Method (8).

The raw data and statistics for both methods were published in the referenced proceedings and can be made available to the AOAC Statistics Committee. It is recommended that these methods be adopted as ICUMSA-AOAC methods, after the appropriate documentation is provided. The individuals who conducted the various tests referenced above are agreeable to having the work presented to AOAC.

Chromatographic Methods for Sugar and Sugar Products

Study Director Kevin Schaffler, Sugar Milling Research Institute, University of Natal, King George V Ave, Durban 4001, South Africa, Tel: +27-31-261-6882, Fax: +27-31-261-6886, E-mail: kschafler@smri.org. SD recommends that **996.04**, "Sugars in Cane and Beet Final Molasses, Ion Chromatographic Method," (cross referenced to ICUMSA Official Method No. GS7/8/4-24), progress to Final Action. This method received First Action in 1996. Because of changes in General Referees during the 1998 period, it is possible this was overlooked. In a survey conducted by Schaffler on the use of this method, it was noted that it is in routine use in several sugar industry laboratories (9).

Schaffler also recommends that **2000.17**, "Determination of Trace Glucose and Fructose in Raw Cane Sugar, High-Performance Anion-Exchange Chromatography," (cross referenced to ICUMSA Official Method No. GS1/2/3-4) receive Final Action. In a survey conducted by Schaffler on the use of this method, it was noted that the method is in routine use in several sugar industry laboratories (10).

Schaffler further informs that **2000.17** was also tested for glucose and fructose in white sugar, but the Statistics Committee felt the HORRAT for white sugar were too high (average 2.8). For this reason, the method was accepted only for raw sugar. The method was published in *J. AOAC Int.* (January/February 2002), in which the First Action recommendation includes the analysis of refined beet sugar and refined cane sugar, along with raw cane sugar (9). Schaffler notes that other AOAC methods have been accepted with higher HORRAT, illustrated by an AOAC collaborative study of an LC method for food products, in which it was decided that the HORRAT was exceeded due to the use of different instruments and columns (11).

Formaldehyde in Maple Syrup by a Spectrofluorimetric Method

Study Director Nathalie Martin, Centre ACER, 3600 Boul. Casavant Ouest, Saint-Hyacinthe, Quebec, Canada J2S 8E3, Tel: 450-773-1105, Fax: 450-773-8461, E-mail: nathaliemartin@centraccer.qc.ca. SD reports that a manuscript, Spectrofluorimetric Determination of Formaldehyde in Maple Syrup by L. Lagacé, J. Dumont, G. Brazeau, A. Soucy, J. Haché, and V. Marquis, has been submitted to *J. AOAC Int.* It is proposed that this method may replace **964.21**, "Formaldehyde in Maple Syrup, Spectrophotometric Method."

As justification, Martin offers the following: To control microbial growth in the tap holes of maple trees, paraformaldehyde was used with a tolerance for residue of 2 mg/kg in maple syrup set by Health Canada and the U.S. Food and Drug Administration. Since long term damage to maple trees is associated with this practice, paraformaldehyde is no longer recommended for maple syrup production. Registration of the pesticide was not renewed in 1990 and the tolerance of 2 mg/kg residue was revoked in 1999 by the U.S. Environmental Protection Agency. This new regulation status of no tolerance of paraformaldehyde residue in maple syrup has

revived interest in formaldehyde determination since a certain amount of formaldehyde can be naturally present in maple syrup without using paraformaldehyde in the tap holes. When the use of paraformaldehyde was introduced, a method for formaldehyde determination in maple syrup was adopted as AOAC Method **964.21**, Formaldehyde in maple syrup, spectrophotometric method. However, this method was later described as tedious and found to have a low recovery and a great variability. As yet, no method has been adopted as an official method to replace the original one. The method we are proposing (Spectrofluorimetric determination of formaldehyde in maple syrup) is a quick and simple method for the determination of formaldehyde in maple syrup with suitable performance. In this method, formaldehyde reacts with Fluoral P to form a complex which is chemically extracted from maple syrup by isobutanol and determined by spectrofluorimetry. Performance, as gauged by the limits of detection (0.16 mg/kg) and quantitation (0.21 mg/kg) as well as recovery (over 79%) and variability (1.9 to 16.1%, depending on fortification level and class of syrup) were superior to the current official AOAC standard method. A limited collaborative test with 3 laboratories showed good performance. We recommend that the new method undergo a collaborative study process (AOAC Official Methods Program) in order to replace the old method.

Sugar Alcohols

Study Director Jeff Rohrer, Dionex Corp., 500 Mercury Dr, Sunnyvale, CA 94088, E-mail: Jeff.Rohrer@dionex.com. SD served as the AOAC representative for the CEN/TC275/WG 2, a European committee reviewing methods for intense sweeteners and sugar alcohols. He reviewed the proposed methods and the report and resolutions from that meeting. He reports that, with his job responsibilities, he does not foresee having the time to initiate and conduct collaborative studies. In a February 2002 interim report, previous GR Raffaella Bernetti recommended that the topic of sugar alcohols be transferred to the General Referee, Food Additives, Committee C.

Stable Isotope Ratio Methods

Study Director Réal Paquin, Laboratoire de Spectrométrie de Masse de Rapports Isotopiques, MAPAQ-DLEAA, 2700 Einstein, C2.105, Sainte-Foy (Québec) G1P 3W8, Canada, Tel: 418-266-4440 #232, Fax: 418-266-4440, E-mail: rpaquin@hertz.phy.ulaval.ca. SD reports that he submitted corrections to Bernetti that were included in the latest edition of the *Official Methods of Analysis of AOAC INTERNATIONAL*, 17th Ed. At that time, he suggested rewriting those methods concerning stable isotope ratio analysis, and he feels that this still should be done. Paquin is presently writing a manuscript for an internal standard method for maple syrup, and he intends to submit it as an official method. He also reports that he has done much work on new methods of combustion for the analysis of carbon isotope ratio analysis and is now ready to begin publishing the results of this research. It would

be of interest to include these newest methods of sample preparation in official methods.

Paquin states that minor errors still exist in the latest edition of OMA: **984.23**, "Corn Syrup and Cane Sugar in Maple Syrup, Carbon Ratio Mass Spectrometric Method" and **998.12**, "C4 Plant Sugars in Honey, Internal Standard Stable Carbon Isotope Ratio Method." He will send the modifications at a later date. With this in mind, **998.12**, which received First Action in 1998, will not be recommended for Final Action until the necessary modifications are made.

Recommendations

(1) The topic of sugar alcohols be transferred to the General Referee, Food Additives.

(2) **996.04** be progressed to Final Action (Sugars in Cane and Beet Final Molasses, Ion Chromatographic Method). In a recent survey conducted by Schaffler on the use of this method, it was noted that it is in routine use in several sugar industry laboratories (9).

(3) **2000.17** be progressed to Final Action (Determination of Trace Glucose and Fructose in Raw Cane Sugar, High-Performance Anion-Exchange Chromatography). In a survey recently conducted by Schaffler on the use of this method, it was noted that the method is in routine use in several sugar industry laboratories (9).

(4) Repeal **954.10** (Color of Raw Cane Sugars). The method is no longer scientifically valid, based on modern methods of color measurement of raw cane sugar. This method, which does not adjust pH, reads at 560 nm, and uses filter aid filtration, is based on obsolete methods used before the days of standardized sugar solution color measurement.

(5) Pursue alternative, modern methods of raw sugar solution color measurement in cooperation with ICUMSA. Appoint a Study Director for this topic.

(6) Appoint a Study Director to review AOAC polarimetric methods that use lead clarification, and make efforts to update to methods that do not use lead salts as clarifying agents.

(7) Conduct a collaborative study on the new spectrofluorimetric method for the determination of formaldehyde in maple syrup.

(8) The SNIF-NMR method for beet or cane sugar in maple syrup (**2000.19**), which received First Action in 2000, be progressed to Final Action.

References

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- (5) Kuhn, R., Weide, F., & Schollaert, W. (1999) *BIOforum* 1–2, 29–32
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- (8) Urquhart, R.M. (1998) General Subject 1, Raw Sugar, ICUMSA Proc. 22nd Session, pp 90–107
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Nonvitamin Micronutrients

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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 (including choline, inositol), and any compounds with antioxidant or antimicrobial potential. Other conditionally essential, or pseudo-vitamin dietary components also are attracting increasing attention such as the important lipid carrier L-carnitine. These analytes are currently under-represented in the *Official Methods of Analysis* (OMA) and it is hoped that activities in these areas will increase. In the case of amino acids, it is disappointing that there has been no replacement of **960.47** (Amino Acids in Vitamin Preparations, Microbiological Assay) with a chromatographic procedure.

A new method for isoflavones in soy-based foods (**2001.10**) was approved during 2001 for First Action by the Food Nutrition Committee E and Official Methods Board. These phytoestrogenic flavonoids are naturally occurring in a variety of plants with high levels in soybeans and have been connected with inhibition of certain cancers, reduction in menopausal symptoms and improvements to bone density. At least 15 isoflavones are found in food, usually as glycosides, while aglycones are found in fermented soy products. The new method is based on hydrolysis of glycoside esters and determination as isoflavone glycosides and the corresponding aglucones.

Methods for many of the botanical extracts have been transferred to the new Committee K which specializes in such validations. One method, for ginsenosides in panax species, has remained with Committee E. The method is based upon LC determination of extracted analytes (R_{g1} , R_c , R_f , R_{g2} , R_{b1} , R_c , R_{b2} , and R_d) using UV detection. The ginsenosides have been collectively well-researched but are also believed to have some individual nutritional properties. Standardized extracts need a reliable method of analysis to substantiate label claims,

so a validated method is currently under review for First Action status.

Nucleotides play important roles in major biochemical functions and recent evidence suggests that dietary nucleotides are semi-essential 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 (CMP, UMP, AMP, GMP, and IMP). A complicating problem for such products is the potential conversion of nucleotides to nucleosides during production. As far as analytical methodology is concerned, the 2 main approaches would seem to be either simultaneous determination of both nucleotides and nucleosides, or conversion of nucleotides to nucleosides during sample preparation and target the latter as the sum of nucleotide and nucleosides. A protocol LC method for determining total nucleoside content in milk and infant formulas is currently being drafted. Study Director Bruce Molitor has contributed the following summary of this approach:

The addition of nucleotides to nutritional formulas and the quantitative confirmation of fortified amounts can be a complex determination. Most proteins contain inherent amounts of nucleotides and/or nucleosides. Alkaline phosphatase present in the milk proteins can reduce nucleotides to nucleosides during the manufacturing process. Adenosine deaminase in the protein can convert adenosine to inosine. Hydrolyzed proteins can contain large amounts of nucleic acid components as background amounts. This method can evaluate total nucleotides and total nucleosides, and track conversion of adenosine to inosine. The first step involves enzymatic hydrolysis to their corresponding nucleosides of adenosine, cytidine, guanosine, inosine, and uridine. Samples are then covalently bonded to a boronic acid gel, which virtually eliminates all nonsugar, non-nucleotide, and non-nucleoside components prior to LC quantification. This LC quantification method has already been extensively used to evaluate American, Asian, and European breast milk; infant and adult nutritionals; soy formulas, cow's milk, and hydrolyzed proteins; various processing stages during manufacturing; and multiple international infant nutritionals. Since no current AOAC method exists for quantification of nucleotides, a collaborative international AOAC of this method will be used to verify this as a universal method that is accurate, precise, rugged, and appropriate for routine use.

The antioxidant potential of foods and dietary supplements is an important nutritional property to gauge the potential benefit against oxidative damage and age-related diseases. Rather than attempting to sum the antioxidant properties of all compounds, an assessment is better achieved using one of various indicating reagents. A common reagent for free radical detection is 2,2-azino-bis(3-ethylbenzothiazoline)-6-sulphonate (ABTS), a well-known substrate for enzymatic peroxide tests. The reaction product is a green colored radical cation. Another is the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). A collaborative study for total antioxidant capacity of foods using DPPH is currently at the protocol submission

stage. The following has been submitted by Topic Advisor Aruna Prakash:

Antioxidants such as phenolic compounds, flavonoids, carotenoids, vitamin C, vitamin E, and phytoestrogens are present in fruits, vegetables, and whole grains. Scientific evidence suggests that antioxidants in food scavenge free radicals and inhibit oxidative reactions, thereby reducing risk for chronic diseases. The qualitative analysis of foods to determine its free radical scavenging capacity is necessary in order to find its efficacy and nutritional value and also help us understand the functional properties of food. Antioxidant capacity and antioxidant activity are used interchangeably. The free radical scavenging activity of antioxidants in foods have been substantially investigated using various methods and reported in the literature. The antioxidant activity of food is determined by reacting the sample with the stable DPPH radical in 50% aqueous methanol for 4 h at 35°C. DPPH radical is purple in color and has an intense absorption at 517 nm in 50% aqueous methanol solution. On reaction with an antioxidant, the purple color of the DPPH solution changes to pale yellow. This change in absorbance is related to the antioxidant activity. A common reference standard, (S)-(-)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), a water soluble analogue of vitamin E is used. Antioxidant activity of foods is expressed as Trolox equivalents (TE)/100 g sample. Antioxidant activity for a wide variety of food samples can be conveniently determined using the rapid, simple DPPH method.

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. While commercial RID kits are available, they are generally quite variable in response. An affinity LC method based on specific binding of bovine IgG with immobilized Protein G has been validated within the laboratory of the prospective Study Directors, Don Otter and Colin Hughes, and is currently at the protocol development stage. Biosensor technology may provide an alternative approach to IgG analysis and a method is currently under development based on the interaction between an anti-bovine-IgG ligand and analyte utilizing an optical transducer.

The General Referee suggests 3 nonvitamin micronutrients, carnitine, inositol, and conjugated linoleic acid (CLA) need to be strategically targeted for collaborative study since they are not covered in the *Official Methods of Analysis* 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 [*Food Chem.* **66**, 121–127(1999)] will hopefully be subjected to collaborative study in the near future. Similarly, inositol is considered a con-

ditionally essential pseudo-vitamin and although microbiological or gas chromatographic techniques are generally used, neither approach has been subjected to the highest level of validation. However, a Nestlé sponsored interlaboratory trial is currently evaluating a GLC-based assay for inositol. Acceptable performance may result in submission of this technique to the official methods program. Conjugated linoleic acid has attracted much attention recently due to the reportedly beneficial biological properties of some of its 56 geometrical and positional isomers [Roach et al. (2002) *Anal Chim. Acta.* **465**, 207–226]. A gas chromatographic approach will undoubtedly be sought to provide a validated methodology for CLA supplements and dairy products, in which the principal active isomer is thought to be the 9*c*,11*t*-18:2 and 10*c*,12*t*-18:2 forms.

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 OMB level.

Selected Study Director Topics

Determination of Ginsenosides (ginseng saponins) in Dry Root Powder from Panax Ginseng, Panax Quinquefolius and Selected Commercial Products by LC Method

Study Directors Ebenezer Asafu-Adjaye, FDA, Rockville, MD 20857, and Siu Kay Wong, Hong Kong Government Laboratory, Hong Kong. Study completed and waiting First Action approval by Methods Committee and Official Methods Board. The study may be in violation of the Harmonized Protocol for Collaborative Studies.

2001.10 Determination of Isoflavones in Soy and Foods Containing Soy by Extraction, Saponification, and LC

Study Directors Stephen P. Klump and John L. MacDonald, Ralston Analytical Laboratories, Saint Louis, MO, E-mail: sklump@ralston.com. Study completed and method approved First Action.

Nucleotides and Nucleosides in Infant Formulas and Milk

Study Director Bruce Molitor, Ross Abbott Laboratories, Columbus, OH, E-mail: Bruce.Molitor@RossNutrition.com. New method; study protocol in process.

Nucleotides in Infant Formulas

Study Director to be appointed.

Antioxidant Activity in Foods

New Study Director to be appointed.

Determination of IgG Levels in Milk and Colostrum

Study Directors Donald Otter and Colin Hughes, New Zealand Dairy Research Institute, Palmerston North, New Zealand, E-mail: don.otter@nzdri.org.nz and colin.hughes@nzdri.org.nz. New method; study protocol in progress.

Recommendations

(1) Determination of Ginsenosides (ginseng saponins) in Dry Root Powder from *Panax Ginseng*, *Panax Quinquifolius* and Selected Commercial Products, LC Method: Move to First Action and continue study.

(2) **2001.10**, Determination of Isoflavones in Soy and Foods Containing Soy by Extraction, Saponification, and LC: First Action approved by the OMB.

(3) Nucleotides in Infant Formulas and Milk: Study Director Bruce Molitor now appointed. Prepare the method protocol for AOAC approval at the earliest opportunity.

(4) Nucleotides in Infant Formulas: SD (Christine Rose Sallin, Nestle, Lausanne, Switzerland) to be appointed.

(5) Antioxidant Activity in Foods: Appoint new Study Director and prepare the method protocol for AOAC approval at the earliest opportunity.

(6) Determination of IgG Levels in Milk and Colostrum: Study Directors Donald Otter and Colin Hughes to be appointed. Prepare the method protocol for AOAC approval at the earliest opportunity.

Fat-Soluble Vitamins

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Summary

Fat Soluble Vitamins: Review of Recent Analytical Developments

The analysis of the individual fat-soluble vitamins A, E, D, and K₁ by LC are covered by the AOAC Official Methods in Chapters 45 (vitamins and other nutrients) and 50 (infant formula, baby foods, and enteral products). These methods are rather product-specific and often dated. Modern methods generally focus on LC separations which allow multi-analyte determinations and detection of individual isomers. This advance in methodology has a greater opportunity to address biopotency differences between various vitamins.

Recent 'horizontal' versions of these methods, applicable to all of the 9 sectors of the food matrix, are under study. One such LC method has been evaluated for vitamins A and E (1) and given First Action approval (**2001.13**) for vitamin A, but vitamin E requires a second collaborative study. A joint

NMKL-AOAC method for vitamin D has recently been approved as Official Method **2002.02**. An LC method for β -carotene has been collaboratively studied for a variety of foods and the statistical analysis of the results has been made. A study report should be available in the near future.

The Committee of European Normalization (CEN) has released LC methods for vitamins A, D, and E and β -carotene similar to the AOAC procedures. However, their validation was not always up to internationally accepted protocols and covered only selected foods. CEN (2-5), ISO (6), and ISO/IDF (7) issued LC methods specifically for vitamins A and D in skimmed milk powder. A product-specific method for vitamin A in supplemented liquid milks is being proposed for adoption as a First Action method (F-26) which avoids the use of saponification.

International standard methods are currently lacking for vitamin K₃ (used mostly in pet foods) and for some pro-vitamin carotenoids like β -cryptoxanthin. Methods for nonvitamin carotenoids with nutritional significance, such as lycopene, are also in need of further validation. In addition a method for the separation of *cis*- and *trans*-phyloquinone (K₁) using a C30 reversed-phase column has been reported (8) which has potential regulatory use. The natural biologically active form of vitamin K₁ (phyloquinone) is the *trans*-isomer. The inactive *cis*-isomer is found in synthesized vitamin K₁. There is a need for reliable data on vitamin K content because of recent changes in U.S. legislation on nutritional labeling. Rodrigo et al. (9) described the analysis of the various tocopherols using normal-phase LC in infant formulas to assess total vitamin E although such separations are now possible using C30 reversed-phase columns (10).

A number of new developments to speed up sample preparation have been reported. An on-line supercritical fluid extraction (SFE) procedure/immobilized lipase hydrolysis was proposed by Turner et al. (11) for the determination of vitamins A and E esters in dairy and meat products. This technique should also be applicable to other fat-soluble vitamins. The method was collaboratively studied for vitamins E and A and β -carotene (12) for a wide variety of food matrices. It was reported that sample throughput was at least 12 per day, about double that of the conventional LC methods. This promising technique should be further examined.

Another approach involves direct solvent extraction avoiding saponification (13, 14) which is applicable to vitamin E in margarine and reduced fat products and for total vitamin E and β -carotene in reduced-fat mayonnaise. A similar approach was also reported for extraction of *all-trans*-retinyl palmitate, β -carotene, and vitamin E in fortified foods (15). However it was reported by Paixao and Stamford (16) that this technique gave poor recoveries of vitamins A, D, and E from milk-based products and these authors obtained better recoveries using a classical overnight saponification followed by solvent extraction.

Chase (17) described the use of accelerated solvent extraction (ASE) to reduce extraction time. ASE was combined with matrix solid-phase dispersion (MSPD) for the analysis of vitamin K₁ in medical foods. Coupling the 2 techniques automates

the MSPD. The vitamin K₁ in the ASE was analyzed by reversed-phase liquid chromatography.

Since the extraction methods are similar for the vitamins A, D, and E, it would be advantageous to determine several vitamins in one chromatographic run. Eitenmiller and Landen (18) reviewed multi-analyte methods for FSV. A novel approach was described by Gomis et al. (19) in which A, D₂, D₃, E, K₁, retinyl acetate, retinyl palmitate, tocopherol acetate, and provitamins D₂ and D₃ milk were separated simultaneously by reversed-phase fused-silica microcolumn chromatography with UV detection. Recoveries of each vitamin spike were in the range 89–107%, however this method needs further validation. Qian and Sheng (20) described the simultaneous determination of vitamins A, D, and E and pro-vitamin D₂ in animal feeds by a one-step extraction and LC analysis.

Some attempts have been made to automate sample preparation and LC separation using robotics. Gamiz Gracia et al. (21) described an automated analysis of vitamins A and E based on AOAC procedures.

Although not in routine or regulatory use for the fat-soluble vitamins (FSV), liquid chromatography–mass spectrometry (LC–MS) and tandem LC–MS/MS are beginning to be recognized as important tools for the future. The analytes are not easily ionized so the harsher treatment of APCI (atmospheric pressure chemical ionization) is currently preferred to ESI (electrospray ionization). Several papers describe the analysis of individual vitamins—tocopherols and carotenoids (22) and vitamin E (23). Conditions for vitamin K₁ in foods are also reported in supplier application notes (Shimadzu Application 036). If a multi-analyte method can be developed, significant productivity gains will be possible. A difficulty is to find suitable internal standards for each vitamin. A possible approach to analyze FSV could be to combine the SFE extraction with SPE cleanup and LC–MS or tandem LC–MS/MS analysis.

Scheiber et al. (24) described the simultaneous determination of carotenes and tocopherols in vitamin supplemented juices and nectars by LC–UV. The method allowed the complete separation of α -, β -, γ -, and δ -tocopherol, α -tocopherol acetate, *all-trans* α -carotene, *all-trans* β -carotene, and the 9- and 13-*cis* isomers of β -carotene in about 50 min. Majchrzak et al. (25) reported the determination of the carotenoid profile and retinol content of baby foods.

Capillary electrophoresis (CE) is as yet poorly exploited for analysis of fat soluble vitamins.

Sanchez and Salvado (26) described the use of micellar and microemulsion electrokinetic chromatography for the analysis of both water and fat-soluble vitamins.

Very few applications of NIR/FTIR have been described for analysis of vitamins in food products. However, an interesting application is for controlling FSV in vitamin premixes. Shi et al. (27) described the quantitative determination of vitamin E by NIR.

Flow-injection analysis is particularly suitable where a large number of analyses are required for quality control or monitoring purposes. An on-line microwave-assisted saponification of vitamin A to retinol and direct in-line spec-

trophotometric/FIA determination was reported by Perez and Haswell (28).

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Selected Study Director Topics

2001.13 Vitamins A and E in Foods by LC

Study Director Jonathan W. de Vries. Vitamin A approved as First Action **2001.13**. Vitamin E was not approved [*J. AOAC Int.* **85**, 424–434(2002)].

Collaborative study for vitamin E will be re-run using an international collaborative study protocol. Study Directors Jonathan W. de Vries/Karlene Silvera.

Carotene in Foods

Study Director Lynn Hagemann. Continue with preparation of study report. Submit to Food Nutrition Committee at earliest opportunity.

Determination of Cholecalciferol in Selected Foodstuff by LC

In the absence of a GR for FSVs until the current appointment, Harvey Indyk (GR nonvitamin micronutrients) has acted as GR for this study. The Study Director is Anders Staffas (NMKL). The principle of the method is saponification of food product, extraction of vitamin D₃ and vitamin D₂ (internal standard) into *n*-heptane. The fraction containing vitamin D₂/D₃ is separated by preparative normal-phase LC, and then determined quantitatively by reversed-phase LC with UV detection at 265 nm. This procedure provides a more general horizontal technique, applicable to a wider range of food products than current AOAC methods. This method was validated in a study by the Nordic Committee for Food Analysis using the Harmonized Protocol. It therefore becomes a joint AOAC–NMKL method. The revised study report is now under review for recommendation to First Action approval.

Determination of Vitamin K₁ in Foods Using C30 Reversed-Phase LC, Separation of *cis*- and *trans*-Phylloquinone

Study Director Vacant. New topic.

Determination of Vitamins K₃ in Human and Pet Foods Using Reversed-Phase LC

Study Director Vacant. New topic.

Recommendations

(1) Vitamins A and E in Foods by LC: Collaborative study for vitamin E to be re-run using an international collaborative study protocol.

(2) Carotene in Foods: Continue with preparation of study report. Submit report to Food Nutrition Committee at earliest opportunity.

(3) Determination of Cholecalciferol in Selected Foodstuff by LC: The revised study (NMKL procedure) for cholecalciferol should be recommended for acceptance as a joint NMKL–AOAC method as First Action.

(4) Determination of Vitamin K₁ in Foods Using C30 Reversed-Phase LC: Separation of *cis*- and *trans*-phyloquinone. New topic.

(5) Determination of Vitamins K₃ in Human and Pet Foods Using Reversed-Phase LC: New topic.

Water-Soluble Vitamins

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Summary

In the 2001 General Referee Report, it was stated that progress in establishing new vitamin methods for foods is rather slow. This situation has not been changed. In general, microbiological assays (MBA) are the primary reference methods in the *Official Methods of Analysis*, and should be changed for chromatographic separations preferably or other modern techniques. Apart from AOAC, the Committee of European Normalization (CEN) is also in progress with the standardization of methods of analysis for vitamins. However, there is some question whether the validation procedures are comparable. Because validation procedures are expensive and time consuming, it should be encouraged to align validation activities around the world in order to obtain good analytical methods in an effective way.

Dietary Supplements

Vitamins in food supplements are a large and growing market that cannot be ignored. In nearly all European countries, vitamins in food supplements fall under food law instead of, e.g., pharmaceutical regulations. The importance of economical and safety aspects (e.g., vitamins A, D, and B₆) in food supplements is higher than for food. The degree of standardization in methods of analysis for vitamins in food supplements is less than for food (e.g., companies and commercial laboratories use a large number of different methods leading to a wide range of results). Considering these observations, members of CEN Working Group 9 have decided to start ac-

tivities to standardize methods of analysis. Collaboration with industry will be sought.

In the *Official Methods of Analysis*, few methods are available for the analysis of vitamins in food supplements (932.16, 936.14, 940.33, 944.12, 948.26, 970.65, 975.43, 989.09). Furthermore, the available methods are outdated: 932.16 (Vitamin D₃ in Poultry Feed Supplements: Chick Bioassay); 975.43 (Identification of RRR- or all-rac-alpha-Tocopherol in Drugs and Food or Feed Supplements, Polarimetric Method).

Vitamin B₆

Currently, the FDA is running a collaborative study on the determination of vitamin B₆ in infant formula based on the method of Bergaentzle et al. [*Food Chem.* (1999)]. However, this method falls under the scope of the Infant Formula and Medical Diets Committee.

Within CEN, the group is working on 3 methods for vitamin B₆ (2 LC and one microbiological method). The microbiological method might be withdrawn in the future because it is rarely used within European laboratories. In one LC method (ENV 14164), after dephosphorylation, pyridoxamine is transformed into pyridoxal, which is then reduced to pyridoxine. Pyridoxine is then quantified fluorimetrically. This method is based on the method published by Bergaentzle et al. [*Food Chem.* (1999)]. β-Glycosylated forms are excluded, while in the other method (doc. CEN/TC 275/WG 9 N 121rev) vitamin B₆ is the sum of the individual dephosphorylated vitamins pyridoxine, pyridoxal, and pyridoxamine calculated as pyridoxine including the β-glycosylated forms. This analysis is based on the work of Bognár et al. [*Z. Lebensm. Unters. Forsch.* (1985 and 1995)]. This procedure may be considered as an official method.

Folic Acid

So far, only microbiological analysis, which measures total folate, is accepted as reference method for determining the level of this vitamin (992.05, Folic acid in infant formula; CEN: EN 14131 Determination of folate by microbiological assay). Apart from the urgent need to measure dietary folates with a higher specificity, one would like to be able to measure the 'added' folic acid in order to enforce the allowed additions and the accompanying maximum intake level of 1 mg folic acid per day. Within CEN, efforts are employed to set up a collaborative study on folic acid (used for enrichment) and 5-methyltetrahydrofolic acid (the most abundant natural folate form) based on an LC method [*J. AOAC Int.* **82**, 119–127(1999)] and the Harmonized Protocol.

Biotin

Indyk and Woollard are interested in looking at a biotin method for foods using BIA techniques. However, there are some technical issues. Collaboration with other scientists will be sought. CEN is working on a draft for the determination of biotin in foods. D-biotin and D-biocytyl are extracted from food after an enzymatic treatment and quantified by LC with postcolumn derivatization. This procedure is based on the

work of Lahély et al. [*Food Chem.* (1999)] and Arella et al. [*Ann. Fals. Exp. Chim.* (2000)].

Vitamin B₁₂

Indyk et al. [*J. AOAC Int.* (2002)] have published a paper on the determination of vitamin B₁₂ in milk products and selected foods by optical biosensor protein-binding assay. The proposed method was compared with reference microbiological and radioisotope protein-binding methods for a range of food samples.

Other Vitamins

In Europe, methods for the determination of vitamins B₁ (prEN 14122) and B₂ (prEN 14152) are about to be adopted as final drafts. However, these methods do not meet the AOAC or ISO 5725 validation criteria. This is also the case for a method to determine vitamin C (prEN 14130).

Selected Study Director Topics

Vitamin C in Foods, LC Method

Study Director Allen Brause, Analytical Services of Columbia Inc., 9151 Rumsey Rd, Suite 190, Columbia, MD 21045, E-mail: atplabs@netscape.net. A collaborative study was completed in 1995, which was extended to other food products. This collaborative study will be repeated at a later date after consultation with the International Fruit Juice Union. The completed method is not satisfactory for AOAC First Action because of analyte instability but will be reported in *J. AOAC INTERNATIONAL*. Additional foods will also be withdrawn from study (as originally intended) because LC–UV techniques are not suitable for low level detection.

Determination of Niacin in Foods by Capillary Electrophoresis and Liquid Chromatography: Acid and Alkaline Extraction

Study Director Norbert Strobel, Australian Government Analytical Laboratories, 51-65 Clarke St, S. Melbourne, Victoria 3205, Australia. The original Study Director, Craige Trenerry, has moved to another laboratory but it was expected that this study would continue. However, there was no response from the Study Director for 2 years. So this study will be canceled. In France a collaborative study for the determination of niacin in foods will be finalized by the end of 2002. With this method niacin vitamins are extracted from food with an enzymatic treatment and quantified by LC with a fluorimetric detection after a postcolumn derivatization with UV irradiation. However, this collaborative study does not follow the harmonized protocol.

Determination of Calcium Pantothenate in Multivitamin Premixes by LC

Study Director Gerald A. Woollard, Auckland Hospital, Department of Clinical Biochemistry, Park Rd, Auckland, New Zealand, E-mail: geraldw@adhb.govt.nz. The study would be extended to include certain supplemented products.

Further progress is dependent upon charges imposed by AOAC for collaborative studies. Sponsorship of this study may be required.

Recommendations

- (1) Vitamin C in Foods, LC Method: Repeat collaborative study in fruit juices. Prepare the method protocol for AOAC approval at the earliest opportunity.
- (2) Determination of Niacin in Foods by Capillary Electrophoresis and Liquid Chromatography: Acid and Alkaline Extraction: Discontinue study.
- (3) Determination of Calcium Pantothenate in Vitamin Premixes and Tablets Using LC: Prepare the revised method protocol for AOAC approval at the earliest opportunity.
- (4) Determination of Folic Acid and 5-Methyltetrahydrofolic Acid in Foods by LC: Appoint Erik J.M. Konings as SD. Prepare the method protocol for AOAC approval at the earliest opportunity.