Analyzing vitamin D in foods and supplements: methodologic challenges[1-4]

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ABSTRACT

This report briefly reviews existing methods for analyzing the vitamin D content of fortified and unfortified foods. The existing chemical methods are similar; all are time consuming, require experienced technicians, and have only been validated for a few materials (eg, dairy products or animal feed materials). This report also describes the lack of standard reference materials with certified values for vitamin D that laboratories need to guarantee the accuracy of existing analytic methods. Recently, the US Department of Agriculture, as part of a project to update the vitamin D values in the National Nutrient Database of Standard Reference, established an analytic methods committee to compare several existing vitamin D methods and to characterize 5 control materials (skim milk, processed cheese, cereal, orange juice, and salmon). Initial relative SDs for the 5 materials ranged from 35% to 50%. Elimination of systematic biases related to the methods and the standards yielded much more satisfactory relative SDs of 7% to 12%. This research has shown that existing methods for analyzing the vitamin D content in foods can produce accurate results. A new, simpler, and faster method, however, would greatly benefit the field. To guarantee accuracy, we need certified reference materials for foods.   *Am J Clin Nutr* 2008; 88(suppl):554S–7S.

INTRODUCTION

This conference has emphasized the increased importance of vitamin D to human health. During the conference, the presenters pointed out that most adults, and increasing numbers of young people, depend on dietary vitamin D as their primary source of this nutrient. Consequently, to evaluate the vitamin D intake of the US population, we need an accurate database with the vitamin D content of foods (1). An accurate database, in turn, requires accurate analytic methods for vitamin D.

This report describes some of the existing vitamin D analytic methods and their strengths and weaknesses. It also discusses the availability of and future needs for food standard reference materials (SRMs) with certified values for vitamin D. Finally, this report summarizes a study conducted by the US Department of Agriculture (USDA) to compare existing methods for analyzing the vitamin D content of foods and to develop analytic values for 5 control materials.

VITAMIN D ANALYTIC METHODS

Historically, the measurement of vitamin D concentrations in foods has presented an enormous analytic challenge. Vitamin D is a complex, highly reactive, and lipophilic molecule. Extracting vitamin D from food materials with all the other lipid components complicates an already difficult separation process and makes detecting vitamin D by ultraviolet molecular absorption highly problematic. Consequently, saponification of the sample is necessary before a sophisticated separation process.

Today, the instrumental methods of choice for analyzing vitamin D in foods include separation by HPLC and detection by either ultraviolet absorption with a diode array (DA) detector or mass spectrometry (MS). Most laboratories prefer DA detection because it is relatively inexpensive and very robust, with a relative precision of <3%. MS detection is more specific and less subject to interferences than DA detection, but it is more expensive and less robust, with relative precisions of ≈10% as a result of instabilities in the ionization process.

The Association of Official Analytic Chemists International (AOACI), the organization responsible for establishing official, legally defensible analytic methods in the United States, has validated 11 methods for vitamin D analysis. Laboratories have used 4 chemical methods (as opposed to microbiological methods) recently:

TABLE 1
Reference materials[1]

Source	Description	Form	Level
National Institute of Standards and Technology	Fortified coconut oil	D_2	Information
SRM 1563	Infant formula	D, Pre-D	Reference
SRM 1846	Baby food composite	D	Information
SRM 2383	Whole-milk powder	D	Information
SRM 8435			
US Pharmacopoeia	Vitamin D in peanut oil	D_3	Certified
European Union			
BCR 122	Margarine	D_3	Certified
BCR 421	Milk powder	D_3	Certified

[1] SRM, standard reference material; BCR, Community Bureau of Reference.

- Method 982.29: vitamin D in mixed feeds, premixes, and pet foods (2);
- Method 992.26: vitamin D_3 (cholecalciferol) in ready-to-feed, milk-based infant formula (2);
- Method 995.05: vitamin D in infant formulas and enteral products (2); and
- Method 2002.05: cholecalciferol (vitamin D_3) in selected foods (milk and cheese) (2).

The methods listed are quite similar. In general, analysts saponify samples to hydrolyze triacylglycerols into fatty acids and glycerol, extract vitamin D_2 and vitamin D_3, collect both vitamin D_2 and D_3 as a single peak by using preparative-scale, normal-phase HPLC, and separate vitamin D_2 and D_3 by using analytic reversed-phase chromatography with DA detection. Variability arises from the different extraction solvents (usually either hexane or petroleum ether) and internal standards used.

The methods listed have 3 major problems. First, they are time consuming and labor intensive and require extreme attention to detail. The quality of the results is directly proportional to the experience of the analyst. The relative standard deviations (RSDs) of the methods are $\approx$10–15%. Because these methods are costly, laboratories tend to process as few samples as possible, even though they should increase the number of analyses to achieve the best relative precision of the means ($RSD_{mean} = RSD_{individual}/\sqrt{n}$, where n is the number of analyses).

Second, researchers have only validated these methods for a limited number of materials, most notably dairy products, which have a high fat content. Researchers need to validate these methods for other foods. This is especially critical because many of the newest fortified foods (eg, orange juice and cereals) have a low fat content. Applying methods developed for high-fat foods to low-fat foods could lead to inaccurate results because the digestion of the micellular forms of vitamin D necessary for solubilization in nonfatty foods is not addressed.

Third, researchers have designed and validated methods only to produce analytic values for vitamin D_3. Vitamin D_2 behaves similarly to vitamin D_3 with respect to saponification, extraction, and separation steps. Thus, vitamin D_2 is a suitable internal standard for Method 2002, as long as no vitamin D_2 is present in the food under investigation. Assuming that the food under investigation contains no vitamin D_2 can lead to inaccuracies in an analysis if vitamin D_2 is present. A method that analyzes only vitamin D_3 produces erroneous results if vitamin D_2 is the fortificant. For these reasons, the ideal method must measure both vitamin D_2 and vitamin D_3 separately.

In recent years, many laboratories have started using MS detection instead of DA detection to provide greater specificity in identifying vitamin D and to reduce the need to separate vitamin D_2 and vitamin D_3 from all the other sample components. Unfortunately, the initial saponification and extraction steps are still necessary. In addition, a mass spectrometer costs at least $100 000, whereas a DA detector costs approximately $10 000. Furthermore, the AOACI has not yet validated any methods based on MS.

REFERENCE MATERIALS

Another major obstacle to vitamin D analysis is the lack of SRMs (**Table 1**) with certified values for vitamin D. Validated methods ensure precision, or agreement, between laboratories. SRMs ensure accuracy. They are accompanied by a certificate of analysis that provides characterization of listed properties, uncertainty limits, information on proper use, and traceability to the standards of the metrological institution that issued them. The National Institute of Standards and Technology (NIST) does not have an SRM with a certified value for vitamin D, although SRM 1846 (infant formula) has a reference value. Three other SRMs have information values for vitamin D. Unfortunately, neither the information nor the reference values have the same guaranteed level of confidence as certified values because neither is accompanied by a certificate of analysis from NIST. In addition, uncertainty intervals are not generated for information values. Like the validated analytic methods, the NIST SRMs have focused on milk products. Assuming that accurate analysis of vitamin D in milk products guarantees accurate analysis of vitamin D in other foods may not be analytically valid.

The US Pharmacopoeia has a certified vitamin D_3 nonmatrix reference standard in peanut oil. This is a pure standard, as opposed to a matrix reference material (such as the SRMs), at a very high concentration, and the peanut oil serves as an appropriate lipid solvent. The European Union had 2 SRMs with certified values for vitamin D, milk powder (BCR 421) and margarine (BCR 122). Unfortunately, these are no longer available. In general, appropriate SRMs for vitamin D in foods other than milk are lacking.

DEVELOPMENT AND CHARACTERIZATION OF CONTROL MATERIALS

Recently, the USDA initiated a project to update the vitamin D content of foods listed in the National Nutrient Database for

TABLE 2
Analytic Methods Committee

Name	Institution
Craig Byrdwell	US Department of Agriculture
Jon DeVries	Medallion Laboratories
James Harnly	US Department of Agriculture
Michael Holick	Boston University Medical Center
Bruce Hollis	Medical University of South Carolina
Ron Horst	Heartland Assays
Mark Lada	Coca Cola Analytic Laboratory
Katherine Phillips	Virginia Polytechnic Institute and State University
Wayne Wolf	US Department of Agriculture

Standard Reference (3). The inspiration for the project was the lack of data in the database derived from analytic measurements and the need for newer and more representative values (1). Given the difficulties with the existing analytic methods for vitamin D and the lack of SRMs, the USDA decided that the first step in the project would be to establish an analytic methods committee to determine the best analytic approach (**Table 2**).

The analytic methods committee made development of control materials its top priority. This development project simultaneously made it possible to compare methods and establish much-needed reference materials. The committee decided to characterize 5 control materials based on the results of 6 laboratories, each using its own method (**Table 3**). The control materials were a single dietary source that had a high natural vitamin D concentration (salmon) and 4 fortified foods with high vitamin D concentrations (skim milk, processed cheese, cereal, and orange juice). The Food Analysis Laboratory and Control Center at the Virginia Polytechnic Institute and State University collected, composited, and shipped all 5 of the control materials in 3 batches.

The initial results disappointed the committee. The interlaboratory RSDs for each of the 5 control materials fell between 35% and 50%. For every control material, ≥2 laboratories reported values that differed by a factor of 2. A major source of error for at least one laboratory was the primary standard (the standard used to prepare all calibration standards). The analytic methods committee identified and corrected other inconsistencies within and between laboratories. Further details on the investigative process to identify the areas of bias between laboratories have been published (6).

After the analytic methods committee thoroughly evaluated the data and corrected all the identified inconsistencies between laboratories, the RSDs for all 5 control materials ranged from 7% to 12%. This level of agreement is within the expected range for full collaborative methods validated by the AOACI, as characterized by the Horwitz criteria (7). This agreement is particularly significant because the committee did not require the participating laboratories to use a specific method. Thus, the final RSDs included the variance of the different methods and the bias between the methods.

The characterization of the control materials was a productive exercise. From this study, the committee obtained a set of control materials with assigned vitamin D values, agreement of methods for 6 analytic laboratories, and expanded knowledge of the chemistry of vitamin D and of the methodology necessary to achieve accurate analytic results for vitamin D in foods.

TABLE 3
Analytic methods[1]

Variable	Laboratory 1	2	3	4	5	6
Method source	AOAC 2002.05, modified (2)	AOAC 982.29, modified (2)	Chen et al (4)	Hollis, modified (5)	Hollis, modified (5)	Extraction from AOAC 992.26 (2) internal standard added and chromatographic method from AOAC 2002.05 (2)
Internal standard (IS)	Dihydrotachysterol	None	^{3}H-Vitamin D_3	Vitamin D_2	^{3}H-Vitamin D_3	Vitamin D_2
Initial extraction solvent	n-Heptane	Hexane	Hexane	Hexane	Hexane	Ether/petroleum ether
Cleanup steps	2	2	3	3	3	2
Quantification	HPLC–UV	HPLC–UV	HPLC–UV + scintillation counting for the IS	HPLC–UV	HPLC–UV + scintillation counting for the IS	HPLC–UV diode array and LC–MS (auxiliary detection)
Further confirmation of data	HPLC/MS	—	—	—	—	LC/MS in SIM mode

[1] AOAC, Association of Official Analytic Chemists International; UV, ultraviolet; LC-MS, liquid chromatography–mass spectrometry; SIM, selected ion monitoring.

The results showed that laboratories can obtain accurate results on the vitamin D content of foods by using existing analytic methods. With proper standards, no systematic bias was observed. However, existing validated methods are not sufficiently precise. Too many analyses are required for an accurate estimate of the mean. Researchers can maximize precision by analyzing increased numbers of samples to establish an acceptable RSD for the mean value.

The approach that the USDA used to evaluate the suitability of current analytic methods for measuring the vitamin D content in foods can serve as a model for analyzing other vitamins and nutrients. Specifically, on the basis of our experience, we recommend forming an analytic methods committee, selecting a series of control materials, and asking participating laboratories to analyze the control materials. This approach will produce a comparison of existing methods and, following evaluation of the data, a set of control materials that can be used to improve the accuracy of future analytic results.

CONCLUSION

In this report, we briefly reviewed the disadvantages of existing methods for analyzing the vitamin D content of foods. We showed that existing methods can produce accurate results, but they are time consuming and expensive. We need a new method to measure vitamin D in foods that is simpler and faster; this will help to ensure that laboratories conduct the number of analyses needed to establish a reasonable RSD for the mean values. We also discussed the lack of food SRMs with certified values for vitamin D. We characterized 5 control materials that are critical to improving the quality of data for vitamin D in the database. We need new SRMs that match a range of foods with detectable vitamin D content or that are fortified with vitamin D.

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