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Original Article

Development and validation of control materials for the measurement of vitamin D_3 in selected US foods

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ABSTRACT

As part of the United States Department of Agriculture's (USDA) National Food and Nutrient Analysis Program (NFNAP), food composition data for vitamin D in the USDA National Nutrient Database for Standard Reference are being updated and expanded, focusing on high priority foods and validated analytical methodology. A lack of certified reference materials and analytical methods validated for these key foods required the development of five matrix-specific control composite materials (CC) (canned salmon and vitamin D_3 fortified cereal, orange juice, milk, and cheese). Each of six experienced laboratories (research and commercial) analyzed vitamin D_3 in five subsamples of each CC in five separate analytical batches, with one subsample of each material in each run. Research laboratories performed recovery studies, mass spectrometric analysis, and other studies to validate quantitation in each matrix. Initial results showed a wide disparity between the six laboratories (RSDs of 26–46%). Extensive collaboration resolved several problems related to calibration standards, extraction solvents and the internal standard, achieving final values with RSDs of approximately 10%, validated by mass spectrometry tests that confirmed lack of matrix interferences in these foods.

1. Introduction

There has been increased interest recently by the scientific community in the role of vitamin D in health beyond preventing rickets or osteomalacia. Evidence suggests that raising levels of the circulating form of vitamin D in serum may result in improved bone health, oral health, and colon cancer prevention (Bischoff-Ferrari et al., 2006). While sunlight induces cutaneous vitamin D synthesis thus increasing serum levels, there are multiple factors that can reduce an individual's exposure to sunlight (Holick, 2007; Calvo et al., 2004), making reliance on foods or supplements with added vitamin D more important (Chen et al., 2007). Vitamin D occurs in foods naturally primarily as vitamin D_3 (cholecalciferol) (Holick, 2007) and also as vitamin D_2 (ergocalciferol) in plants and 25-hydroxyvitamin D_3 in animal products such as meat and eggs (Ovesen et al., 2003; Mattila et al., 1996). In the USA, vitamin D_3 is used as a fortificant for most foods (e.g., milk, orange juice, cheese, cereals), although vitamin D_2 is sometimes used (primarily in soy and vegetarian products).

Many epidemiological studies of vitamin D are based on calculated intake using consumption data from dietary surveys and vitamin D concentrations from food composition databases (Affenito et al., 2007; Gilmore et al., 2008). Population estimates of dietary intakes in the US are estimated through the dietary component of the National Health and Nutrition Examination Survey (NHANES) using the Food and Nutrient Database for

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Dietary Surveys (FNDDS) (US Department of Agriculture, 2006). The source of nutrient data for the FNDDS is the United States Department of Agriculture's (USDA) National Nutrient Database for Standard Reference (SR) (US Department of Agriculture, 2007), which is maintained by the Nutrient Data Laboratory (NDL) at the Beltsville Human Nutrition Research Center, an institute of the Agricultural Research Service (Beltsville, MD). The SR contains data for 7500 foods, approximately 2700 of which are used in the FNDDS. Current epidemiological work on vitamin D intake, whether through NHANES or through other surveys, is impaired by the lack of a complete, and well verified, database of vitamin D values for foods commonly eaten in the US. SR currently contains vitamin D values for only 594 foods and just 87 of those are analytical values. Thus far, data on vitamin D has been presented in SR as total vitamin D in IU/100 g because IU is the unit required under the current US labeling regulations, and most of the data on vitamin D to date have been provided by industry or based on US standards of identity. Beginning in 2009, vitamin D data will be reported in SR as micrograms D_2 or D_3, and total vitamin D in micrograms will be calculated from the sum of specific forms, with eventual provision of values for 25-hydroxyvitamin D for some foods. Currently, 25-hydroxy vitamin D values (Ovesen et al., 2003) are included in the UK food tables for meats (Chan et al., 1995).

To support these research needs, NDL has initiated an update and expansion of food composition data for vitamin D in SR (Holden et al., 2008), using accurate, validated sampling and analytical methodology. This work is being conducted as part of NDL's National Food and Nutrient Analysis Program (NFNAP) (Pehrsson et al., 2000; Haytowitz et al., 2002, 2008). The foods likely to supply the most vitamin D in the US diet were identified as fish (a natural contributor) and the following vitamin D_3 fortified foods: orange juice, ready-to-eat breakfast cereals, fluid milk, margarines, sliced American cheese, and yogurt; therefore, these foods were given the highest priority for new chemical analyses, focusing on vitamin D_3 (Holden et al., 2008).

The NFNAP protocol requires that analytical methods used are verified for accuracy and acceptable precision and that control samples and/or certified reference materials (CRMs) are included in all assay batches to provide validation of individual datasets and continuity of results across time, laboratories, and methodology (Phillips et al., 2006). Usually CRMs with a known nutrient content can be included to evaluate the accuracy of results from prospective analytical laboratories. In the case of vitamin D, however, there is a lack of CRMs for the primary food sources (Phillips et al., 2007), which presents a major obstacle to validating methodology and laboratories for the foods planned for analysis. Existing CRMs include only infant formula (NIST SRM 1846; National Institute of Standards and Technology (NIST), Gaithersburg, MD), margarine (BCR122; Institute for Reference Materials and Methods (IRMM), Geel, Belgium), and powdered milk (BCR 421). The vitamin D value for the NIST Infant Formula is a reference value, not a certified concentration, indicating less confidence. BCR 421 has a certified value, but the material is over 10 years old and has recently been discontinued. Dry matrices (e.g., powdered milk and infant formula) may not be analytically equivalent to their fluid counterparts, because water content and nutrient concentration can affect selection and performance of the extraction, separation, and quantitation. Margarine (BCR 122) is dissimilar to the primary food matrices to be assayed in NFNAP. The peanut oil matrix and vitamin D_3 concentration (80,000 IU/100 g) in the commercially available USP standard are not equivalent to the foods to be assayed. Therefore, a set of matrix-matched control materials was needed for the NFNAP.

Additionally, preliminary data obtained during prior NFNAP phases for available CRMs analyzed along with a limited number of food samples submitted to major commercial laboratories raised uncertainty about the adequacy of precision and accuracy of measurements. The CRM results showed high variability and often deviated significantly from the certificate values; also, replicate analyses of vitamin D in control materials prepared for the NFNAP study (Phillips et al., 2006) showed poor repeatability and obvious errors in many cases (e.g., a high value in a mixed vegetable control material) (Holden et al., 2008; Holden et al., unpublished data). These observations generated fundamental uncertainty about the reliability of existing standard methods for the range of food matrices representing primary sources of vitamin D in the US and motivated studies to validate measurements using the control materials prior to the planned analysis of foods for NFNAP.

Historically, the determination of vitamin D in foods has presented an enormous analytical challenge because the chemistry of this vitamin is complex and the methods are detailed and time consuming. There are 11 methods validated by AOAC International (Gaithersburg, MD), the US organization responsible for establishing official methods that are legally defensible. Three chemical methods have been published since 1990: Method 992.26—vitamin D_3 (cholecalciferol) in ready-to-feed milk-based infant formula (AOAC, 2007b), Method 995.05—vitamin D in infant formulas and enteral products (AOAC, 2007a), and Method 2002.05—cholecalciferol (vitamin D_3) in selected foods (milk and cheese) (AOAC, 2007d). One older method, Method 982.29—vitamin D in mixed feeds, premixes, and pet foods also has applicability (AOAC, 2007c).

All the methods are quite similar. In general, samples are saponified to hydrolyze the lipids, vitamin D_2 and D_3 are extracted, then both vitamin D_2 and D_3 are collected as a single peak using preparative scale normal-phase high performance liquid chromatography (HPLC) and vitamin D_2 and D_3 are separated using analytical reversed-phase chromatography with diode array detection. Variations arise from the different extraction solvents used (usually either hexane or ether/petroleum ether) and the use of internal standards (IS).

There are two major problems with existing standard methods for fortified foods. First, they are long, labor-intensive, and require extreme attention to detail. This creates the potential for error and poor precision, resulting in a tendency to run as few samples as necessary, and also requiring a skilled analyst. Consequently, the relative standard deviation (RSD) of data generated by these methods tends to be high and is at odds with the inclination to run fewer samples. More, not fewer, samples are needed to achieve assayed concentrations with an acceptable level of confidence when there is relatively low precision. Second, the methods were only validated for vitamin D_3 fortified dairy products, and not other types of food. Expansion of the applicability of the methods is mandatory for reliable overall food composition data. This is especially critical since many of the newest fortified foods (e.g., orange juice and cereals) have different matrix characteristics that might affect extraction and separation of vitamin D_3. Also, naturally occurring vitamin D (e.g., in meat, fish, eggs) may not be extracted as efficiently as it is from fortified foods, as well as also being present as 25-hydroxy vitamin D (Jakobsen et al., 2004; Mattila et al., 1996) or, in foods such as mushrooms, as vitamin D_2 (ergocalciferol) (Mattila et al., 2002).

The primary goal of this study was to prepare and characterize control materials for vitamin D_3 in specific food matrices to enhance the quality of analytical measurements of vitamin D in key foods for the NFNAP, to ensure the accuracy and consistency of new SR data, and to harmonize results from methods among a subset of laboratories.

2. Materials and methods

2.1. Study design

Canned salmon and vitamin D_3 fortified skim milk, orange juice, ready-to-eat breakfast cereal, and processed cheese were selected as representative food matrices for the control materials, based on the identification of key foods for vitamin D analysis in the NFNAP (Holden et al., 2008). A homogeneous composite of each food was prepared using methods similar to those described previously (Phillips et al., 2006) and portioned into approximately 200 subsamples, to allow for five replicate analyses by each of six laboratories as well as a stock of aliquots to be retained as control material for future analyses of NFNAP foods. Composites were prepared at a central facility (the Food Analysis Laboratory Control Center (FALCC) at Virginia Tech), which then distributed subsamples to each laboratory and stored the remaining aliquots. The participating laboratories included two major US commercial laboratories offering vitamin D analysis of foods, the USDA Food Composition and Methods Development Laboratory (FCMDL; Beltsville, MD) (formerly the USDA Food Composition Laboratory), and three university/research laboratories with the necessary experience in analyzing vitamin D in foods.

After evaluation of the initial dataset, the four research laboratories (C, D, E, and F) collaborated to determine characteristics of the optimal analytical methodology, including additional analyses to further validate accuracy of the results.

2.2. Preparation of control composites (CCs)

Foods were procured locally (Blacksburg, VA), with all packages of each product coming from a single lot, as follows: skim milk fortified with vitamins A and D (seven 3.78 L jugs); a multi-grain ready-to-eat breakfast cereal fortified with vitamins and minerals, including vitamin D_3 (six boxes, 454 g each); pasteurized processed American cheese slices fortified with vitamin D_3 (four packages, 681 g each); refrigerated orange juice fortified with calcium and vitamins D_3, A, B1, C, and E (14 cartons, 1.89 L each); canned Alaskan red sockeye salmon (12 containers, 421 g each). Composites were prepared using methods previously tested to yield homogeneous composites (Phillips et al., 2006). Briefly, the skim milk and orange juice CCs were prepared by thorough stirring. The canned salmon (drained), cereal, and processed cheese composites were homogenized using a stainless steel industrial food processor (Robot Coupe® Blixer BX6V; Robot Coupe USA, Jackson, MS); the cheese was frozen with liquid nitrogen and ground in liquid nitrogen. Precautions were taken to avoid contamination of the composites during preparation and to protect them from ultraviolet light. No plastic utensils were allowed to contact the composites, and the ambient temperature was maintained at 21–24 °C.

Subsamples were dispensed into glass jars with Teflon™-lined screw caps immediately after composite preparation, blanketed with nitrogen and sealed. Liquids were portioned into 125 mL straight-sided glass bottles (I-Chem; Fisher Scientific, Pittsburgh, PA) (90–110 mL/jar) and solids into 30 mL straight-sided glass jars (10–20 g/jar). Each container was surrounded with aluminum foil and stored at -60 ± 5 °C.

2.3. Initial interlaboratory analyses

Five subsamples of each CC were sent frozen, on dry ice, via overnight express delivery to each of the six laboratories. Each laboratory checked the samples upon receipt to ensure lack of thawing or leakage during shipment. Laboratories were instructed to assay the subsamples for vitamin D in five separate analytical batches, with one subsample of each material in each batch. The laboratories were not instructed as to the method of analysis to use but were given the food descriptions; each facility used its typical methodology and reported results considered valid for vitamin D. The method characteristics cited by each facility are summarized in Table 1. FCMDL served as the reference laboratory due to the additional method validation performed there, as described below.

Results are reported on a μg/100 g sample basis.

2.4. Method used by FCMDL (reference laboratory)

2.4.1. Chemicals and standards

HPLC or spectrophotometric grade solvents were purchased from Fisher Scientific, Inc. (Fairlawn, NJ) and were used without further purification, except 100% ethanol was obtained from Sigma-Aldrich (St. Louis, MO) (#459828). Vitamin D standards were purchased from Sigma-Aldrich: vitamin D_3 (#C9756), 98% purity; vitamin D_2 (#E5750), 97% purity.

2.4.2. Extraction

All samples were stored at -60 ± 5 °C, under nitrogen and in darkness prior to analysis. Samples were extracted using ethyl ether/petroleum ether specified in AOAC method 992.26 (AOAC, 2007b). The method was modified to include 0.5 μg vitamin D_2 (1.0 mL of a 0.5 μg/mL solution, in ethanol) added as an IS to most samples (milk, orange juice, cereal, processed cheese, blank). The canned salmon contained a higher level of endogenous vitamin D_3; therefore, 2 μg (4.0 mL of 0.5 μg/mL solution) were added to those samples.

The weight of the analytical portion taken for most samples (except canned salmon) was calculated to contain approximately 0.3 μg (12 IU) of vitamin D_3, based on estimates from existing food composition data (e.g., label claims). The sample was weighed into a 250 mL Erlenmeyer flask with a ground glass neck, 400 mg ascorbic acid (as antioxidant) was added, and the IS solution was added by volumetric pipette. Ethanol (15 mL) was added, and the sample was swirled thoroughly to mix. KOH was added, as a solid to milk and orange juice samples and as a 1 M aqueous solution to solid samples, as follows: (1) skim milk ~30 mL = ~29.5 g, 7.5 g KOH; (2) orange juice 30 mL = ~30.5 g, 7.5 g KOH; (3) cereal ~9 g, 135 mL 1 M KOH; (4) canned salmon ~10 g, 135 mL 1 M KOH; (5) processed cheese ~9 g, 135 mL 1 M KOH. The flask was swirled until the solid KOH was dissolved in the liquid sample or the solid sample was thoroughly suspended in liquid. The mixture was put onto a refluxing condenser and lowered into a water bath at 75 °C. After 30 min, the sample was removed and placed into ice water to rapidly cool to room temperature. The sample was transferred to a 500 mL separatory funnel, with a 5 ml ethanol rinse. Ethyl ether (130 mL) was added to the funnel, which was then stoppered and shaken vigorously for at least 1 min. Next, 130 mL of petroleum ether were added to the funnel, which was again stoppered and shaken vigorously for at least 1 min. The shaking during extraction needed to be sufficiently vigorous to avoid incomplete extraction of vitamin D_3. The samples were allowed to stand at room temperature until separated. Swirling aided separation of the two layers. The lower layer was drained and discarded. Deionized (D.I.) water (50 mL) was added to the flask, which was then stoppered and shaken for >30 s. The samples were again allowed to stand to separate. The lower layer was drained to waste. Another 50 mL of D.I. water was added to the flask, which was stoppered and shaken for >30 s. The samples were allowed to stand to separate and the lower layer was drained to waste. Ethanol (15 mL) was added to the flask and shaken, and then a third wash of 50 mL D.I.

Table 1
Methods cited by laboratories for the analysis of vitamin D

	Laboratory					
	A	B	C	D	E	FCMDL[a]
Method source	AOAC 2002.05[b] (modified)	AOAC 982.29[c] (modified)	Chen et al. (1990)	Hollis (2005) (modified[d])	Hollis (2005) (modified)	Extraction from AOAC 992.26[e] (internal standard added) and chromatographic method from AOAC 2002.05[b]
Internal standard	Dihydrotachy-sterol	None	^{3}H-Vitamin D_3[f]	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	N/A	N/A	Recovery studies on skim milk[g]	N/A	LC/MS in SIM mode

All methods included initial saponification of the samples. N/A, not applicable.
[a] USDA Food Composition and Methods Development Laboratory (Beltsville, MD) (reference laboratory, as described in text).
[b] AOAC (2007d).
[c] AOAC (2007a).
[d] Modification: use of vitamin D_2 instead of ^{3}H-vitmain D_3 as the internal standard.
[e] AOAC (2007b).
[f] Tritiated vitamin D_3 as previously published (Holick et al., 1980).
[g] As described in text.

water was added to the flask, stoppered, shaken and allowed to separate. The lower layer was drained to waste. The remaining ether layer was then collected in a 500 mL flat-bottom round flask. This solution was then decanted into a clean 500 mL round bottom flask, which was put onto a rotary evaporator (Buchi, Flawil, Switzerland) and taken to dryness with the water bath at 45 °C. Acetone (50 mL) was added to the flask, and it was again taken to dryness. The sample was then dissolved in 10 mL ethyl ether, with swirling, and transferred to a pre-rinsed 50 mL centrifuge tube. The round bottom flask was rinsed with two more 10 mL portions of ethyl ether, which were combined in the centrifuge tube. The ether solution was evaporated to dryness under ultra high purity N_2 on an N-Evap evaporator (Organomation; Northborough, MA). The sample was reconstituted in 1.0 mL hexane.

Even with a large amount of KOH, high fat samples such as salmon and processed cheese did not saponify completely, resulting in an oily extract. This condition did not interfere with the analyses, since the oily mixture contained mostly diacylglycerols, which do not hinder the chromatography of vitamin D.

2.4.3. High performance liquid chromatography

The two chromatographic separations from AOAC Official Method 2002.05 (AOAC, 2007d) were used. The first was a normal-phase (NP) preparative HPLC separation on a 25.0 cm × 4.6 mm, 5 μm, silica column (Inertsil®; GL Sciences, Torrance, CA). It was conducted on an Agilent 1200 system consisting of a quaternary pump with membrane degasser, autosampler with extended volume injection option, diode array detector (DAD) SL, and 35900E analog-to-digital converter for acquisition of signal from an Alltech ELSD 800 (Alltech Associates, Deerfield, IL) evaporative light scattering detector (ELSD). There were two solvent programs used, one for standards and one for samples. Both programs started with isocratic mobile phase 1, consisting of 0.5% isopropanol:2.0% methyl *t*-butyl ether:48.75% cyclohexane:48.75% *n*-heptane. For standards, only isocratic mobile phase 1 was used for 25 min. For samples, isocratic mobile phase 1 was used for 25 min, after which the column was washed with mobile phase 2, consisting of 20% isopropanol:80% *n*-heptane. The gradient for samples was as follows: 0–25 min, 100% mobile phase 1; 25–35 min, linear gradient to 100% mobile phase 2; 35–55 min, 100% mobile phase 2; 55–75 min, linear gradient back to mobile phase 1; 75–85 min, equilibrate in 100% mobile phase 1. It was important to allow sufficient time for the column to re-equilibrate between runs, to avoid inconsistent retention times. The flow rate was 1.3 mL/min throughout. The injection volume was 450 μL, which allowed two injections plus waste for each 1.0 mL sample. Flow after the DAD went to the fraction collector or ELSD. The diverter valve on the fraction collector sent eluate to the ELSD except during the fraction time window. The vitamin D eluted at ~17.5 min, and the fraction was collected from 16 to 19 min in a 13 mm test tube. The fraction time was adjusted slightly as needed, based on the elution time of analyte in a standard solution. Fractions were taken to dryness by inserting the test tube into a 50-mL-long neck round-bottom flask on a rotary evaporator. The fractions were reconstituted in 650 μL mobile phase 3, consisting of 20% methanol/80% acetonitrile. The reconstituted fraction was transferred to two autosampler vials containing limited volume inserts, which allowed four 100 μL injections plus waste for each fraction collected.

The reversed-phase (RP) HPLC was carried out using a Thermo Separation Product (Thermo Fisher Scientific Corp., San Jose, CA) chromatograph consisting of a P4000 quaternary pump with membrane degasser, AS3000 autosampler, UV6000 DAD, and a UV2000 dual channel detector operated in single channel mode at 265 nm. The solvent system was isocratic mobile phase 3 for 20 min on an Inertsil® ODS-2 column, 25.0 cm × 4.6 mm, 5 μm particle size (GL Sciences) at a flow rate of 1.3 mL/min. The outlet from the DAD was split so that 0.6 mL/min went to the mass spectrometer source, and 0.7 mL/min went to waste, or to a second mass spectrometer.

Quantification was based on integration of the areas under the peaks in the UV 265 nm chromatogram from the DAD, which was the observed absorption maximum for vitamin D in the 210–400 nm range, using vitamin D_2 as an IS for analysis of vitamin D_3. For routine analysis of unknown samples, the vitamin D_2 peak area would be monitored to ensure the absence of vitamin D_2 in each sample. The quantification was based on the response factor (RF) determined from the ratio of the integrated

area from vitamin D_3 to that of vitamin D_2 in a standard solution composed of equal amounts, 0.8 μg/mL each, of vitamin D_2 and D_3. RF = (area D_3std/area D_2std). Each sequence of runs had five standard runs and sixteen sample runs. The samples were quantified using the average RF for the five standard runs obtained on the same day as the sample runs. The ratio of IS to vitamin D_3 in the samples was very close to the same as the ratio of internal to vitamin D_3 in the RF calibration standard.

2.4.4. Mass spectrometry

Tandem sector quadrupole mass spectrometry was used as an auxiliary detection method on the RP-HPLC system. The mass spectrometer was a TSQ 7000 mass spectrometer (Finnigan MAT, now Thermo Fisher Scientific Corp.) operating in Q3 SIM mode, using the $[M+H]^+$ at m/z 397.3 and the $[M+H-H_2O]^+$ ion at m/z 379.3 for the vitamin D_2 IS, and the $[M+H]^+$ at m/z 385.3 and the $[M+H-H_2O]^+$ ion at m/z 367.3 for vitamin D_3, with a scan time of 0.5 s per ion and 1.0 m/z peak width. The total area for each analyte was the sum of the integrated areas of the $[M+H]^+$ and the $[M+H-H_2O]^+$ ions. The RP-HPLC was coupled to the TSQ 7000 via an atmospheric pressure chemical ionization (APCI) source, with the vaporizer heater at 250 °C, the sheath and auxiliary gases at 40 psi and 10 mL/min, respectively, and the corona current at 5.0 μA. Flow after the DAD was split via a tee, with 0.63 mL/min going to the APCI source of the TSQ7000 mass spectrometer, and 0.67 mL/min going to waste or to a second mass spectrometer. In some experiments (e.g., processed cheese), data were also obtained on an LCQ Deca XP ion trap mass spectrometer (Thermo Electron Corp., now Thermo Fisher Scientific Corp.), in parallel, operated in full scan mode. In those experiments, APCI was also performed on the ion trap mass spectrometer using the same parameters as listed for the TSQ7000 instrument.

2.5. Additional method validation

Prior to analysis of the control samples at FCMDL, both AOAC 2002.05 (AOAC, 2007d) and AOAC 992.26 (AOAC, 2007b) methods were carried out on a wide variety of commercially obtained food samples, including milk, infant formula, orange juice, breakfast cereal, diet supplement drinks, supplement pills, and others to determine which method was most appropriate for the widest variety of sample types. The method was optimized for the CC matrices being studied based on this preliminary testing.

The hexane extraction in AOAC 2002.05 (AOAC, 2007d) produced an intractable emulsion from some samples, such as orange juice, so the ethyl ether/petroleum ether extraction of AOAC 992.26 (AOAC, 2007b) was chosen for the control samples. The chromatographic method from AOAC 2002.05 was chosen because this used lower flow rates and therefore less solvent than AOAC 992.26. Separate experiments were conducted to determine the optimal percentage of methanol in the acetonitrile mobile phase, the optimal flow rate, and best conditions for atmospheric pressure chemical ionization mass spectrometry (APCI-MS). The results of these experiments are planned to be reported elsewhere. It was determined that the conditions in the chromatographic separation cited in AOAC 2002.05 were not optimal, but the standard AOAC method conditions were used for the control materials because the method was previously validated as an official method.

Standard addition studies were performed at one of the research laboratories (laboratory D) to verify the accuracy of quantitation of vitamin D_3 from foods (milk) in the exact manner in which it would be determined in unknown samples. For this work, 60 ng of vitamin D_3 standard (99% purity, #95220, Sigma-Aldrich) were added to the sample prior to extraction, and vitamin D_3 was quantified using the methodology of that laboratory, with five replicates assayed using vitamin D_2 as the IS. Percent recovery was calculated as the difference between the assayed concentration in the spiked sample and the expected concentration, with the expected concentration taken to be the assayed concentration in the unspiked sample plus the quantity of added vitamin D_3, as a percent of expected concentration.

Laboratory D also performed standard addition studies and analyzed BCR 421 CRM (milk powder) (IRMM). The milk powder was weighed into the extraction vessel and nine parts by weight water were added prior to analysis, to simulate fluid milk in the extraction. Results were corrected for the assayed moisture content of 2.45 g/100 g to compare accurately to the certified values, which are given on a dry mass basis.

2.6. Statistical analyses

Means, standard deviations, RSD, and 95% confidence intervals (CI) were calculated using Microsoft Excel® 2000 (Microsoft Corp., Redmond, WA). Data were subjected to an analysis of variance (ANOVA) and the Tukey test for multiple comparisons, with $\alpha = 0.05$, using SAS® (version 8.2 (TS2M0)) (SAS Institute, Cary, NC).

3. Results and discussion

3.1. Initial interlaboratory analyses

Initial data from the six laboratories for the five CCs are summarized in Fig. 1. An unexpected outcome was an unacceptable disparity in values obtained from state-of-the-art analytical methods performed by experienced laboratories. The between-laboratory RSD ranged from 26.4% (cereal) to 45.6% (orange juice) and was 30.6%, 30.7%, and 36.4%, respectively, for cheese, milk, and canned salmon.

A striking feature of this initial dataset was the markedly higher values from laboratory E, for all CCs. It was hypothesized that a possible discrepancy in calibration standards might explain the deviation (e.g., either purity of the original standard or the accuracy of the prepared solution). In fact, when values were normalized to the FCMDL values set at 100%, the other facilities (with the exception of laboratory A), had an internally consistent ratio across materials, and all results from laboratory E were systematically high by approximately the same ratio (Fig. 1). Three research laboratories (C, E, and FCMDL) then exchanged the standard used by laboratory C (vitamin D_3, >99% purity, Aldrich (Milwaukee, WI, V380-8) in 100% ethanol, with purity verified by HPLC). When FCMDL and laboratory E used this standard to calibrate the previous analyses, there was no correction to the data from FCMDL, but results from laboratory E were reduced by a factor of 0.58, based on the response of the standard which revealed a problem with the lamp in the UV detector at laboratory E. The adjusted data from laboratory E were reasonably in range with those from other facilities and therefore only these corrected values from laboratory E were further considered, to separate effects due to fundamental methodology differences (e.g., extraction and detection parameters) from the source/preparation of standards.

The results for the five CCs are summarized in Table 2. Adequate within-laboratory precision was considered a first premise for acceptable methodology, regardless of whether the precision resulted from inherent method characteristics or the skill with which the method was implemented by a given facility. Within-laboratory RSDs (Table 2) were <10% except for cheese,

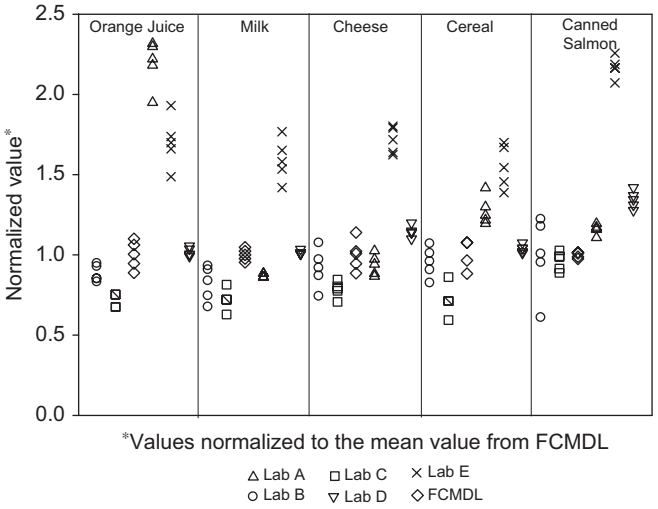

Fig. 1. Initial data for vitamin D_3 assayed in five control materials at six laboratories, normalized to the mean value from FCMDL for each material (vitamin D_3 fortified orange juice, 1.30 μg/100 g; milk, 1.08 μg/100 g; cheese, 7.22 μg/100 g; cereal, 3.37 μg/100 g; and canned salmon, 22.2 μg/100 g).

skim milk, and canned salmon from laboratory B (13–24%) and cereal from laboratory C (13%). For orange juice and processed cheese, four facilities had RSDs < 7%; for skim milk and cereal, laboratory D had the lowest RSDs (1.2% and 2.4%, respectively); for canned salmon, FCMDL had the lowest variability (1.8%). These data suggested that an intra-laboratory RSD of < 10% was routinely achievable, and an RSD < 5% was possible for all matrices. All of the laboratories using an IS reported values with RSDs < 10% with only one exception (cereal by laboratory C). These data suggest that an IS was important for achieving optimal precision.

The mean assayed vitamin D_3 concentration differed among laboratories for each material ($p < 0.0001$). Between-laboratory RSDs ranged from 12.4% (processed cheese) to 47.0% (orange juice). However, paired comparison of means showed that differences were not statistically significant among subsets of the laboratories (see Table 2). Interestingly, these were cases with low within-laboratory RSDs, indicating a high level of confidence overall. Because consensus does not necessarily imply accuracy, the significant differences among subsets of laboratories raised the question of which values were accurate, as well as what is the practical significance of the magnitude of the disparity in measured concentrations. The question of accuracy was addressed by the additional studies described below.

3.2. Additional validation studies

Selected ion monitoring (SIM) mass spectrometry data were obtained in parallel with the UV data by FCMDL for each of the foods. Both the SIM data and UV data indicated the presence of a species that was chromatographically overlapped with the vitamin D_3 peak in processed cheese, which interfered with the quantification of vitamin D_3 by UV detection. The full-scan UV–vis spectrum also differed from that of vitamin D_3 standard, and confirmed the presence of the interferent. Therefore, quantification was performed using the SIM data, since the UV data were conclusively shown to be compromised by the interfering species. However, the MS data showed a larger standard deviation than UV data, which accounts for the larger confidence interval shown around the value reported by FCMDL for processed cheese in Fig. 2. Additional experiments were carried out that employed an

Table 2
Results of interlaboratory analyses of vitamin D_3 concentration (μg/100 g) assayed in control composites at six laboratories (mean values[a] are shown, with low, high results and relative standard deviation indicated in parentheses)

Matrix	Laboratory						Between-lab mean (%RSD)
	A	B	C	D	E[b]	F	
Orange juice	2.84^{A} (2.53, 3.00; 6.7%)	$1.14^{B,C}$ (1.08, 1.23; 5.9%)	0.94^{C} (0.88, 0.98; 5.9%)	1.32^{B} (1.28, 1.37; 2.7%)	1.28^{B} (1.13, 1.45; 9.1%)	1.30^{B} (1.15, 1.43; 8.6%)	1.47 (46.8%)
Processed cheese	6.77^{B} (6.25, 7.40; 7.0%)	$6.60^{B,C}$ (5.35, 7.75; 13.4%)	5.66^{C} (5.10, 6.10; 6.4%)	8.26^{A} (7.94, 8.65; 1.5%)	7.18^{B} (6.80, 7.55; 4.9%)	$7.22^{A,B}$ (6.39, 8.22; 9.5%)	6.95 (12.3%)
Skim milk	0.94^{B} (0.93, 0.95; 1.5%)	$0.88^{B,C}$ (0.73, 1.00; 13.2%)	0.78^{C} (0.68, 0.88; 9.1%)	1.09^{A} (1.08, 1.11; 1.2%)	$0.99^{A,B}$ (0.88, 1.10; 8.5%)	1.08^{A} (1.03, 1.13; 3.7%)	0.96 (12.6%)
Cereal	4.30^{A} (4.03, 4.78; 7.0%)	3.21^{B} (2.78, 3.60; 9.9%)	2.42^{C} (2.00, 2.90; 13.2%)	3.50^{B} (3.40, 3.62; 2.4%)	3.04^{B} (2.73, 3.33; 8.6%)	3.37^{B} (2.98, 3.63; 9.4%)	3.31 (18.6%)
Salmon	$25.7^{A,B}$ (24.6, 26.5; 2.8%)	22.0^{B} (13.5, 27.1; 24.5%)	21.3^{B}(19.7, 22.8; 6.0%)	29.8^{A} (28.3, 31.5; 4.1%)	27.9^{A} (26.7, 29.0; 3.0%)	22.2^{B} (21.6, 22.5; 1.8%)	24.8 (14.2%)

[a] Mean values in the same row with the same letters did not differ significantly ($\alpha = 0.05$).
[b] Mean of original values after adjustment factor of 0.58 was applied for error in calibration standard, as described in text (only adjusted values are included in the statistical analysis).

ion trap mass spectrometer, operated in full scan mode, in parallel, to characterize the interfering species, to be reported elsewhere. APCI-MS confirmed that interferents were not present at the same retention times as the analytes in other samples, so UV data for all other samples were reported.

One of the research laboratories observed problems during application of routine methodology to the orange juice and cereal, and performed additional work to modify the procedure to overcome interferences before the results were reported. This situation highlights the caveat that while a standard method of analysis used might be generally reliable, it may not be possible to apply it universally to other matrices without matrix-specific modifications and confirmation of the absence of interfering components.

Recovery of added vitamin D_3 from skim milk, quantified using the proposed optimal methodology characteristics verified that factors such as the IS concentration, preparation, or detection that can affect the precision and accuracy of quantitation in unknown samples did not bias the data. Mean recovery for five replicates was 103% (range 96–106%). Results from laboratory D for the CRM, BCR 421 powdered milk, 14.6 μg/100 g agreed well with the certified range of 13.5–15.1 μg/100 g.

These results combined with the detailed studies conducted by FCMDL suggested that the values that were in agreement as reported by laboratories D, E (adjusted), and FCMDL for the skim milk CC (mean 1.05 μg/100 g; 42 IU/100 g had the highest accuracy and that those from the other three laboratories (mean 0.85 μg/100 g; 34 IU/100 g) were biased low (19% difference in means). Taking the density to be 1032 g/L (976 g/quart) for milk, this difference represents 2.06 μg/L (78 IU/quart).

3.3. Characteristics of optimal methodology

Standard methods that employ vitamin D_2 as the IS require that a sample be tested without addition of the IS to confirm the absence of endogenous vitamin D_2. Some solvents (e.g., hexane) were not suitable for all matrices. For example, the hexane extraction of orange juice produced an intractable emulsion layer for some laboratories, which was overcome by addition of a portion of ethanol to force the emulsion back into solution. On the other hand, the ether/petroleum ether extraction solvent was found to be suitable for all foods tested in this study, as well as other food matrices not reported here, without the need for additional treatment. Standards of known purity, corrected for purity of the specific standard, and carefully prepared are critical. Fluid samples must be weighed, not measured by volume. Additionally, it is important to screen the vitamin D peak in each food by LC–MS to establish the absence of interfering species. This potential limitation must be recognized by many laboratories not having access to LC–MS, when attempting to develop or apply the method to a previously untested food.

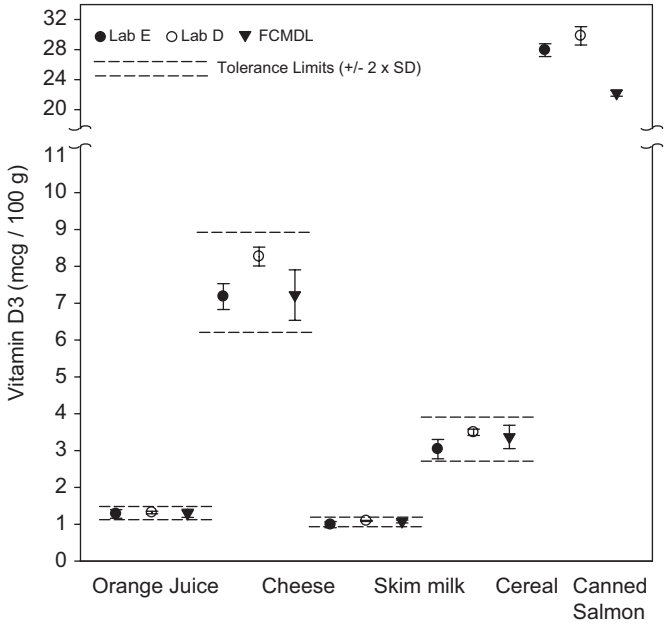

Fig. 2. Validated results for vitamin D_3 in control materials, from three laboratories. Each bar represents the mean of values from three laboratories. SD = standard deviation.

3.4. Tolerance limits

Confidence intervals for validated mean vitamin D_3 concentrations were calculated using the data from the three research laboratories which agreed statistically, had acceptable within-laboratory precision, and were considered valid based on the method testing described above. Using the values from laboratories D, E, and F, tolerance limits were calculated as ±2 S.D. of the unweighted mean. Fig. 2 illustrates these final results, showing the corresponding mean and tolerance interval for the vitamin D_3 concentrations in the skim milk, orange juice, processed cheese, and cereal CCs. These values will be used to validate subsequent datasets. Interlaboratory RSDs for each of these materials among the three laboratory mean values were 4.9%, 1.9%, 8.2%, and 7.0%, respectively. No tolerance limits were established for canned salmon, due to statistically significant differences among laboratory means, with differences of ~7.5 μg/100 g as well as a high interlaboratory RSD (15%) among the three research facilities. Further work remains to determine optimal parameters for canned salmon and validation of results for vitamin D3 as well as of other forms of vitamin D in that matrix.

4. Conclusions

Vitamin D_3 concentrations in four control materials (vitamin D_3 fortified milk, cereal, cheese, and orange juice) were successfully characterized for the purpose of routine quality control in USDA's NFNAP, based on the results from three experienced laboratories and additional validation studies. Initial results identified a significant lack of agreement between experienced laboratories using standard methodologies. Harmonization of the results produced control materials with useful uncertainty limits for vitamin D_3 concentration. These materials will be implemented for ongoing quality control in assay of key foods in the NFNAP as part of the process of updating food composition data in the USDA Nutrient Database for Standard Reference (US Department of Agriculture, 2006). Due to the limited supply and the intent of the development of these materials for the NFNAP, they are not planned to become CRMs for general distribution.

This research demonstrated the intense need for CRMs for vitamin D in food matrices, including validation of measurements for endogenous vitamin D in meats and fish, including other forms of vitamin D (e.g., 25-hydroxyvitamin D) as reported by other researchers (Ovesen et al., 2003; Purchas et al., 2007; Jakobsen et al., 2004; Mattila et al., 1995). The results also indicate that other studies of vitamin D in foods require similar validation of methodology and development/implementation of matrix-matched control materials to ensure accuracy and precision of the data.

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