Phylloquinone (vitamin K_1) and Dihydrophylloquinone Content of Fats and Oils

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ABSTRACT: Assessment of vitamin K (VK) dietary intakes has been limited by the incompleteness of VK food composition data for the U.S. food supply, particularly for VK-rich oils. The phylloquinone (VK-1) and 2′,3′-dihydrophylloquinone (dK) concentrations of margarines and spreads (n = 43), butter (n = 4), shortening (n = 4), vegetable oils (n = 6), and salad dressings (n = 24) were determined by RP-HPLC with fluorescence detection. Each sample represented a composite of units or packages obtained from 12 or 24 outlets, which were geographically representative of the U.S. food supply. Butter, which is derived from animal fat sources, had less VK-1 compared to vegetable oil sources. The VK-1 and dK of the margarines and spreads increased with fat content and the degree of hydrogenation, respectively. In some margarines or spreads and in all shortenings, the dK concentrations were higher than the corresponding VK-1 concentrations. As the fat content of salad dressings increased, the VK-1 concentrations also increased. Fat-free foods had <1 μg/100 g of either form of the vitamin. No dK was detected in the salad dressings or oils tested. Some margarines, spreads, and salad dressings may be significant sources of vitamin K in the U.S. food supply.

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KEY WORDS: Dihydrophylloquinone, fats, HPLC, hydrogenation, margarine, oils, phylloquinone, vitamin K.

Vitamin K functions as a coenzyme and is involved in the synthesis of a number of proteins involved in blood clotting and bone metabolism. It may also have a role in the prevention of osteoporosis and atherosclerosis (1). The National Academy of Sciences has established an Adequate Intake (AI) for vitamin K of 90 μg/d for women and 120 μg/d for men.

Since the publication of a revised provisional table of the phylloquinone (vitamin K_1) content in some foods (2,3), there has been a major expansion in the food composition data available for this fat-soluble vitamin. Several European countries (4,5), as well as one U.S database (6), have since incorporated phylloquinone into their national food tables. However, the food composition data for vitamin K is far from complete. Weaknesses of the existing food composition data for vitamin K include the limited sample sizes used, poor geographical representation, and incomplete data on the effects of processing (2,4). Although phylloquinone is the predominant dietary source of vitamin K, other forms of the vitamin exist but have yet to be systematically analyzed.

In 1993, samples of core foods in the U.S. food supply were obtained from the Food and Drug Administration's (FDA) Total Diet Study (TDS) and were analyzed for phylloquinone using HPLC procedures (7). It was concluded from these data that certain oils derived from vegetables or seeds contained large concentrations of phylloquinone, second in dietary importance only to the green, leafy vegetables. When these phylloquinone-rich vegetable oils are hydrogenated, the phylloquinone is converted to 2′,3′-dihydrophylloquinone, with an overall loss of total vitamin K (defined as the sum of phylloquinone and dihydrophylloquinone concentrations) associated with the processing (8). To date, the dihydrophylloquinone content of foods has not been systematically studied nor has it been included in any of the food composition databases.

The National Food and Nutrient Analysis Program (NFNAP), sponsored by the National Institutes of Health and the U.S. Department of Agriculture, is an initiative designed to provide a comprehensive update to the National Nutrient Databank (9). This program will provide analytical nutrient estimates for approximately 1,000 representative foods and selected ingredients and will add data for selected new components to the database, including phylloquinone and dihydrophylloquinone. The phylloquinone and dihydrophylloquinone concentrations of various fats and oils in the U.S. food supply are presented in the current study.

EXPERIMENTAL PROCEDURES

Selection of fats and oils. As part of the NFNAP, foods are prioritized for analysis. The Key Foods approach (10) was used to select the foods to be analyzed. This approach combines food composition data with food consumption data to determine important food contributors of nutrients of public health significance in the diet. Margarines and spreads, vegetable oils, and salad dressings were shown to be the major sources of fat in the diet. According to regulations, margarine must contain at least 80% fat (21CFR166.110; see http://www.access.gpo.gov/nara/cfr/index.html). Lower-fat margarine-type products may be labeled as spreads, vegetable oil spreads, or margarines, provided that an additional approved descriptor such as light, low-fat, reduced-fat, or fat-free has been added to the name. Also, spreads are frequently reformulated, so samples obtained only a few years ago may

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no longer represent those found in the marketplace today. Therefore, these products were among the first foods sampled under the NFNAP. Although reduced-fat and fat-free salad dressings are not important sources of fat, they were sampled at the same time. To monitor changes in the fats used in formulating these products and to stay abreast of changes in the marketplace, these products will be sampled again under the NFNAP protocol.

NFNAP sampling plan. Food samples used in this study were obtained as part of the NFNAP, which has been described in detail elsewhere (9). The goal of this program is to obtain national estimates of the levels of nutritional components for common foods consumed in the United States. A three-stage sampling plan was employed. The first stage was to identify the counties where sampling would occur. This was accomplished by first dividing the conterminous United States into four regions of roughly equal population. Within each region, three Consolidated Metropolitan Statistical Areas (CMSA) were selected. However, because all counties were not included in a CMSA, Generalized Consolidated Metropolitan Statistical Areas (gCMSA) were used. The gCMSA were defined as the standard CMSA or as individual counties for areas not in a CMSA. The gCMSA within a region were sorted in descending order by population, and a probability proportional to size (PPS) systematic sample of size three was selected. Once the gCMSA were selected, the counties that made up each gCMSA were sorted in descending order by their "urbanicity" (11) and a systematic sample of size two was selected. Urbanicity is a measure of how urban a county is, based on the population of the largest cities or towns in the county. Sorting counties within gCMSA by urbanicity ensured that the sample contained both "more urban" and "less urban" areas.

The second stage was to select a sample of grocery store outlets from within each county selected in the first stage. To achieve a self-weighting sample, outlets were selected proportional to their value of annual sales; larger outlets, in terms of annual sales, had higher probabilities of selection. Only stores with annual sales of U.S. $2,000,000 or more were included, as it was felt they would have the diversity of products needed for this study. The selection process consisted of drawing a systematic sample of size one, proportional to the outlets' annual value of sales, from each selected county. This procedure gave higher probabilities of selection to larger outlets. Alternative outlets were also drawn in each county in case the primary selected outlets were inaccessible or products were unavailable.

The goal of the third sampling stage was to select specific food products (brands and package sizes) for nutrient analysis from food types (e.g., cheese pizza, margarines, and spreads) identified by the Nutrient Data Laboratory (NDL). Product information was obtained from Nielsen Market Research Scantrack data, which were obtained from checkout price scanners. Consequently, this information excluded products sold in stores without such equipment. To maximize the likelihood of having the selected products available in all outlets, we restricted the product sampling universe to products with at least 1% market share. Actual product selection was done proportional to the number of pounds of the product consumed in the United States. This was operationalized by selecting a sequential sample proportional to market share and package size of the product using Chromy's method (12). The number of samples chosen for each product was based on the relative importance of the food in the food supply, the desired statistical results, and the number of nutrient analyses the NDL could afford to perform. Once specific food products were identified for inclusion in the sample, they were purchased from each of the sampled outlets.

After purchase by agents at the designated retail outlets, food samples were shipped overnight to the Food Analysis Laboratory Control Center (FALCC) at Virginia Polytechnic Institute and State University in Blacksburg, Virginia, in appropriate containers. Refrigerated products were packed with cooler packs in insulated containers. At the FALCC, the samples were logged in and evaluated to make sure the proper food items were purchased and were received in good condition. Any improper samples or samples received in poor condition were repurchased. Samples were then composited and homogenized. Margarine and spread samples were received and processed in the same way at the University of Maryland in College Park. Aliquots of composite food samples were placed in glass jars with Teflon-lined screw caps and were shipped on dry ice to the vitamin K laboratory at Tufts University, Boston, Massachusetts, where they were stored at −80°C until analysis. Low-fat and fat-free margarines were stored at 2–8°C to prevent separation of the oil and water phases upon freezing.

Reagents and standards. The extraction and chromatography solvents used were all of HPLC grade (Fisher Scientific Inc., Springfield, NJ). Phylloquinone, zinc chloride, and sodium acetate were purchased from Sigma Chemical Co. (St. Louis, MO); zinc (−200 mesh) was purchased from Johnson Matthey Electronics (Ward Hill, MA); purified 2′,3′-dihydrophylloquinone was a gift from J. Pyrek, University of Kentucky Mass Spectrometry Facility; and the internal standard, $K_{1(25)}$, was a gift from Hoffman-LaRoche (Basel, Switzerland). Primary and secondary stock solutions were diluted to known concentrations in methanol and characterized spectrophotometrically and chromatographically. Glassware and utensils used in sample processing and extraction were washed in acetone to minimize contamination of samples from carryover fluorescent material. All extractions and analyses were performed under yellow light to minimize any degradation due to light exposure.

Extraction of phylloquinone and dihydrophylloquinone. The phylloquinone and dihydrophylloquinone contents of the food samples were determined using a modification of the procedure described by Booth and Sadowski (13). All samples were analyzed in duplicate (including extraction). If the CV of duplicates was greater than 15% (for samples with phylloquinone concentrations >5 μg/100 g), the assay was repeated. A control sample of baby food peaches was run with each batch of foods. A compilation of results from this sample over a 4-mon period gave a mean ± SD result of 4.3 ± 0.7 μg/100 g. Over this same period, the average within-run CV for this sample was 7.8%. This method had a lower limit of

detection of 14 pg per injection (equivalent to 0.006 μg/100 g of sample). Recovery of the internal standard averaged 63% (range: 55–70%). These recovery rates correspond to those previously reported for our laboratory (7,8,13).

Briefly, a 0.1-g aliquot of each of the oils, shortenings, margarines, and spreads (with the exception of fat-free spreads) was weighed into a glass culture tube and dissolved in hexane, and an appropriate amount (equivalent to the amount of phylloquinone projected for each sample) of internal standard was added. Because these foods would dissolve in hexane, no extraction was necessary. The volume of hexane used was adjusted, and dilutions were made to obtain a target of approximately 5 ng of phylloquinone or dihydrophylloquinone for subsequent solid-phase extraction (SPE) on a silica column. For samples of fat-free spreads, butter, and all salad dressings, which would not dissolve in hexane, an extraction procedure was used. The sample (0.1 to 0.5 g) was weighed directly into a 50-mL polypropylene centrifuge tube. Ten milliliters of water and an appropriate amount of internal standard (equivalent to the amount of phylloquinone projected for each sample) were added, followed by 15 mL of 2-propanol/hexane (3:2, vol/vol). The mixture was vortexed for 2 min and then further dispersed by sonication (continuous output at 50% duty cycle, output control 4 for 60 s) using a Branson Model 350 Sonifier Cell Disruptor with a 1/8-in. tapered microtip (Branson Ultra Sonics Corp., Danbury, CT). Phase separation was achieved by centrifugation at $1800 \times g$ for 5 min. The upper (hexane) phase was aspirated into a glass culture tube and evaporated to dryness under reduced pressure in a centrifugal evaporator (model Speed Vac SC210A; Savant Instruments, Farmingdale, NY). The residues were reconstituted with hexane.

All hexane solutions were further processed by SPE on 3-mL (500-mg) silica columns (J.T.Baker, Chicago, IL). Each column was preconditioned by washing with 4 mL of hexane/diethyl ether (96.5:3.5, vol/vol) followed by 4 mL of hexane. After the sample was applied to the column, it was washed with 4 mL of hexane. The fraction that contained phylloquinone was eluted with an 8-mL wash of hexane/diethyl ether (96.5:3.5, vol/vol). After evaporation to dryness in the centrifugal evaporator, the samples were further processed by SPE on 3-mL (500-mg) C_{18} columns (J.T.Baker).

Each residue from a silica column was dissolved in 200 μL of 2-propanol by heating at 45°C for 10 min. The C_{18} columns were conditioned by washing with 3 mL of methanol/methylene chloride (80:20, vol/vol) followed by 3-mL washes of methanol and deionized water. The sample extracts were applied to the conditioned columns. The columns were then washed with 3 mL of methanol/water (95:5, vol/vol) followed by 3 mL of acetonitrile. The fraction that contained phylloquinone was eluted with a 6 mL wash of methanol/methylene chloride (80:20, vol/vol). The eluant was evaporated to dryness in the centrifugal evaporator. The residue was reconstituted initially with 30 μL of methylene chloride followed by 170 μL of methanol with 10 mM $ZnCl_2$, 5 mM CH_3COOH, and 5 mM CH_3COONa (5.5 mL of an aqueous solution of 2.0 M $ZnCl_2$, 1.0 M CH_3COOH, and 1.0 M CH_3COONa was added to the methanol to give a final volume of 1.0 L). The reconstituted residues were transferred to amber sample vials with glass inserts (300 μL) (Chromacol Ltd., Trumbull, CT) and sealed with crimp caps. All vials were centrifuged for 5 min at $1800 \times g$ before placing them on the HPLC instrument.

HPLC analytical conditions. A reversed-phase C_{18} column and an HPLC instrument were used to determine concentrations of phylloquinone and dihydrophylloquinone in the foods. The chromatographic system consisted of a 2690 Separations Module (Waters, Milford, MA) equipped with a vacuum degasser and a model RF-10AXL Shimadzu Fluorescence Detector (Shimadzu Instruments, Columbia, MD). The analytical column (150×3 mm) was packed with 5 μm BDS Hypersil C_{18} (Keystone Scientific, Bellefonte, PA). Fluorescent derivatives of the injected quinones were produced online using a postcolumn reactor (2.0×50 mm) dry-packed with zinc (−200 mesh). The excitation wavelength was 244 nm, and the emission wavelength was 430 nm. The mobile phase consisted of two solvents. Solvent A was methanol with 10 mM $ZnCl_2$, 5 mM CH_3COOH, and 5 mM CH_3COONa prepared as described above. Solvent B was methylene chloride. The 2690 separations module was programmed to perform the following gradient elution procedure: (i) pump a 90:10 (A/B) mixture at 0.60 mL/min from injection for the first 11.50 min; (ii) at 11.50 min, change the flow rate to 0.80 mL/min and the composition to 70:30 (A/B); (iii) at 19.50 min, change the composition to 90:10 (A/B); (iv) at 23.50 min, change the flow rate to 0.60 mL/min; and (v) at 24.0 min, end the cycle.

Standard curves were prepared from each calibrator injection. The fluorescence responses for phylloquinone, dihydrophylloquinone, and $K_{1(25)}$ were linear with the slope of the lines bisecting zero. Therefore, we routinely performed single-point calibrations, forcing the slope of the line through zero. Quantitation was achieved by direct comparison of peak area ratios (phylloquinone or dihydrophylloquinone to $K_{1(25)}$) generated from the calibration standard with those generated by the sample. Peak integration and sample concentration calculations were performed using Waters Millennium^{32} software, version 3.05.01.

RESULTS AND DISCUSSION

The phylloquinone and dihydrophylloquinone data presented in Table 1 are from samples obtained from the NFNAP (9) and provide representative estimates of the vitamin K content of selected fats and oils in the U.S. food supply.

Consistent with earlier reports, plant oils, margarines, spreads, and salad dressings derived from plant oils were higher in phylloquinone concentrations compared to animal fat sources such as butter (4,7,14,15). However, it should be noted that two samples of 70% spreads, which were manufactured using corn oil, had very low levels of vitamin K. The phylloquinone concentration of corn oil was higher in the current study than has been reported by others (15,16), whereas

TABLE 1
Phylloquinone and Dihydrophylloquinone Contents of Fats and Oils

Product	Storage (°C)	*N*	Phylloquinone (μg/100 g) Mean	SD	Range	Dihydrophylloquinone (μg/100 g) Mean	SD	Range
Butter	−80	4	10.1	2.7	7.7–13.1	ND[a]		ND–0.7
Margarine, 80%	−80	8	93.0	45.8	50.8–163	108	50.2	68.9–182
Vegetable oil spread, 70%	−80	12	47.4	22.2	0.1–61	83.5	39.0	0.1–105
Vegetable oil spread, 37–60% squeeze	−80	2	155	37.8	128–182	ND		
Vegetable oil spread, 37–60% tub	−80	8	93.4	18.3	52.8–107	29.1	8.6	21.8–49.7
Vegetable oil spread, 37–60% stick	−80	2	70.9	12.7	61.9–79.9	77.9	7.3	72.7–83.0
Vegetable oil spread, <40% stick or tub	2–8	8	70.0	24.1	28.1–96.4	31.6	20.3	16.0–71.4
Vegetable oil spread, 0% tub	2–8	3	0.1		ND–0.1	0.3	0.2	0.1–0.4
Shortening	−80	4	43.0	27.9	12.4–78.8	132.1	66.2	33.5–175
Oils								
Corn	−80	2	7.9	4.4	4.8–11.1	ND		
Olive	−80	2	60.2	14.3	50.1–70.3	ND		
Vegetable	−80	2	52.7	27.1	33.5–71.8	ND		
French salad dressing								
Regular	−80	2	100	29.1	97.9–121	ND		
Lite	−80	2	25.5	0.3	25.3–25.7	ND		
Fat-free	−80	2	0.4	0.2	ND			
Italian salad dressing								
Regular	−80	2	69.3	28.5	49.1–89.4	ND		
Lite	−80	2	6.3	2.0	4.9–7.7	ND		
Fat-free	−80	2	1.5	0.3	1.3–1.7	ND		
Ranch salad dressing								
Regular	−80	2	125	10.5	118–133	ND		
Lite	−80	2	34.8	20.4	20.4–49.2	ND		
Fat-free	−80	2	2.3	0.1	2.2–2.4	ND		
Thousand island salad dressing								
Regular	−80	2	70.4	1.7	69.1–71.6	ND		
Lite	−80	2	21.2	0.3	21.0–21.4	ND		
Fat-free	−80	2	3.7	0.9	3.1–4.3	ND		

[a]ND, not detected.

the phylloquinone concentrations of olive oil samples were consistent with other reports (4,7,14,16). In the 1993 U.S. provisional table of the phylloquinone content of foods (2), only one type of margarine was included. The phylloquinone content of hard-stick margarine ranged from 4 to 97 μg/100 g, with a median value of 51 μg/100 g. In the provisional United Kingdom database for phylloquinone, the average phylloquinone content of six brands of margarine from the United Kingdom was 43 μg/100 g, with a range of 12 to 78 μg/100 g (4,17). Overall, these phylloquinone concentrations were narrower in range than those reported in Table 1. However, the data presented in Table 1 were generated from a nationwide sampling program and reflect the current variation among brands within the U.S food supply. Since the 2–12 composites per fat level included analytical samples of different brands, it was not possible to determine the within-brand variation attributable to production or location. It was assumed that the magnitude of variability for these sources—packages or units within a brand over a short time span—would have limited effects owing to the highly formulated nature of the product. However, variability in the phylloquinone content of oil sources from location to location cannot be eliminated as a confounding factor.

The phylloquinone content of oils is known to vary with the plant species: Soybean, canola, and olive oils are rich sources of phylloquinone, whereas corn and peanut oils are not (16). The phylloquinone content of oils also can vary with exposure to fluorescent light and storage (16). The data presented in Table 1 suggest that additional sources of variation include the percentage of fat of the margarine or spread and the degree of hydrogenation. Within the 37–60% fat category of spreads, the squeeze type (which is not hydrogenated) had consistently higher phylloquinone concentrations compared to those in the stick or tub categories, which were hydrogenated. A reported change in phylloquinone concentrations in margarine over a 10-yr period within the United Kingdom is indicative of changes in the types of oils used in food manufacturing (4). Similarly, the phylloquinone concentration of representative samples of stick margarines and spreads collected as part of the 1993 FDA-TDS was lower (33 μg/100 g) (7) than the average phylloquinone concentration of stick margarines and spreads collected in 1998–2000, as reported in Table 1 (70.9 μg/100 g). Monitoring the food supply on a regular basis will continue to allow changes in the predominant types of oils and the processes used in food manufacturing to be identified, and these will be critical for maintaining current vitamin K composition databases for purposes of dietary assessment.

A representative chromatogram of margarine within the 80% fat category indicates the presence of both phylloquinone and dihydrophylloquinone (Fig. 1). Fats and oils also have been systematically analyzed for phylloquinone (14) and dihydrophylloquinone (18) in the Finnish food supply. In the Finnish study only hard margarines for industry contained dihydrophylloquinone, in contrast to the considerable amounts of dihydrophylloquinone found in U.S. shortenings and fat-containing margarines and spreads (with the exception of squeeze margarine). The limited presence of dihydrophylloquinone in the Finnish study is indicative of efforts by the Finnish margarine industry to reduce the hydrogenation of margarine, hence *trans* FA. The nationally representative data for the United States that we report here are consistent with another report of the phylloquinone and dihydrophylloquinone concentrations in a local sampling of margarines and oils in the United States (15). A unique feature of the study by Cook *et al.* (15) was the measurement of both *cis* and *trans* isomers of phylloquinone. However, these authors reported that the levels of *cis* isomers were generally lower than 10% of the total phylloquinone; thus, they were unable to quantitate this isomer consistently. Because of the reported low levels of *cis*-phylloquinone, we chose not to quantitate it and focused instead on total concentrations of phylloquinone and dihydrophylloquinone.

The biological role of dihydrophylloquinone was recently examined in a metabolic study of healthy young men and women who had been maintained on a low-vitamin K diet. Relative to equivalent amounts of phylloquinone, dihydrophylloquinone was not as effective in restoring the biochemical markers of vitamin K status to normal range (19,20). Therefore, the presence of dihydrophylloquinone in margarines reduces the contribution of this food source in one's overall vitamin K status. Because shortening is also used in baking, it is plausible that baked goods contain considerable amounts of dihydrophylloquinone. These data will be presented in a future report.

Only two samples of each type of salad dressing were tested, but as each sample was a composite of 24 bottled salad dressings collected from different locations within four geographical regions in the United States, these samples are representative of the salad dressings available to U.S. consumers. As expected, and consistent with the corresponding concentrations in margarines and spreads, the phylloquinone concentrations of salad dressing increased with the percentage of fat. No dihydrophylloquinone was detected in any of the salad dressings tested. In the 1993 FDA-TDS (7), regular French salad dressing had an average phylloquinone content of 51 μg/100 g, which is about half of the average concentration reported in the current study. Low-calorie Italian salad dressing was reported to have an average of 2.9 μg/100 g in the 1993 FDA-TDS (7), whereas an average of 6.3 μg/100 g was reported in the current study for "lite" Italian salad dressing. It is unlikely that these differences were due to differences in analytical methodology or standardization, because the same methods and standards were used in both studies. It is more reasonable to assume that the observed differences are the result of differences in the phylloquinone content of the oils used in the manufacturing processes, as observed when comparing margarines collected at different time points.

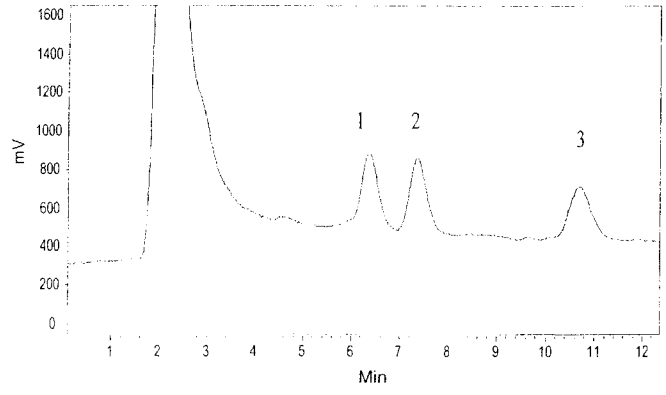

FIG. 1. HPLC chromatogram of a representative sample of an 80% fat margarine. Peak 1 corresponds to phylloquinone, peak 2 corresponds to 2′,3′-dihydrophylloquinone, and peak 3 corresponds to the internal standard, $K_{1(25)}$.

In summary, fats and oils can provide significant amounts of phylloquinone and dihydrophylloquinone in the U.S. diet. For example, one tablespoon of a margarine or spread provides approximately 9.5% of the AI for men and 12.6% for women, depending on the level of fat. Two tablespoons of regular salad dressing provide approximately 25 and 33% of the AI for men and women, respectively. Reduced-fat salad dressings provide lower amounts. The phylloquinone concentrations of margarines, spreads, and salad dressings are dependent on the fat content, whereas the levels of dihydrophylloquinone depend on the degree of hydrogenation. The phylloquinone content of fats and oils does vary widely, presumably owing to the type of oil used in the manufacturing process. These nationally representative food composition data for phylloquinone and dihydrophylloquinone will update and expand upon those in the provisional tables. They will be made available in future releases of the USDA Nutrient Database for Standard Reference, which is available on the NDL web site at http://www.nal.usda.gov/fnic/foodcomp.

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