Results are presented from an NMKL (Nordic Committee on Food Analysis) collaborative study of a method for the determination of cholecalciferol (vitamin D₃) in foods. The method is based on the addition of an internal standard (vitamin D₂), followed by saponification and extraction with n-heptane. The fraction that contains vitamin D₂/D₃ is separated by preparative normal-phase liquid chromatography (LC), and the analytes are determined by reversed-phase LC with UV detection at 265 nm. The method was tested by 8 participating laboratories. In this study 6 different matrixes were analyzed for cholecalciferol content: milk, liquid infant formula (gruel), cooking oil, margarine, infant formula, and fish oil. The contents varied from 0.4 to 12 μg/100 g. Three matrixes (milk, gruel, and margarine) were fortified with vitamin D₃. In the other matrixes, vitamin D₃ was added at 3 different levels at the Swedish National Food Administration. The milk was analyzed as a blind duplicate, whereas the other matrixes were analyzed as split-level pairs. The recoveries from the samples with vitamin D₃ added varied from 93 to 102%. The repeatability relative standard deviation (RSDᵣ) values for accepted results varied between 2.2% (fish oil) and 7.4% (cooking oil), whereas the reproducibility relative standard deviation (RSDᵣ) values varied between 6.8% (margarine) and 24% (cooking oil).

Vitamin D includes different sterol derivatives, but only vitamins D₂ and D₃ are important factors in nutrition. Vitamin D₂ (ergocalciferol) is formed by UV irradiation of 7-dehydrocholesterol. In Sweden, milk, margarine, and infant formula are fortified with vitamin D₃, but in Finland both vitamins D₂ and D₃ can be used for fortification.

Current internationally accepted regulatory and compendial methods for the analysis for vitamin D have been summarized (1). Although most of the methods for vitamins D₂ and D₃ are now based on liquid chromatographic (LC) determination, rat and chick bioassay methods are still cited. Some are used only for the determination of vitamin D₃, whereas both vitamins D₂ and D₃ can be determined with other methods. The principal applications for these methods are the determinations of vitamin D in vitamin tablets, vitamin preparations, and feed supplements. Among the AOAC Official Methods, only 2 LC methods (981.17 and 995.05) are described for the determination of vitamin D in fortified milk and infant formula. None of the currently available, internationally validated methods, however, are universally applicable to different types of foods.

In general, LC-based determination of vitamin D require a preliminary alkaline digestion of lipid and solvent extraction of a crude vitamin D-containing fraction. Analytical LC is usually preceded by fractionation of the crude extract, which may be accomplished by various chromatographic techniques (e.g., LC, thin-layer chromatography, and solid-phase extraction). The method used in this collaborative study was based on an earlier method published by Johnsson and Hessel (2), who found that a longer saponification time and a lower saponification temperature were suitable for samples with a low fat content. A modified method was developed for the determination of vitamin D in fatty foods. Both methods incorporated vitamin D₂ as the internal standard (3), and both methods were compared with a reference bioassay method. During saponification, a temperature-dependent and reversible transformation of vitamin D into a previtamin form occurs. Because the extent of the transformation is accepted as equivalent for both vitamins D₂ and D₃, there is no need to take the transformation factor into account (3). Thompson and Plouffe (4) analyzed fatty foods by sequential normal-phase and reversed-phase LC and described how some interfering
substances are rejected in different fractions in the cleanup process. Rychener and Walter (5) also used vitamin D2 as the internal standard and confirmed equal losses of vitamins D2 and D3 during the saponification and extraction step.

This method is primarily intended for the routine determination of vitamin D3 in fortified foods, but it can also be used for the determination of natural vitamin D3 in foods. Alternatively, vitamin D2 can be determined with vitamin D3 as the internal standard. In either case, it is necessary to confirm that the natural content of internal standard in the foods is below the detection limit by assay of the test sample without the addition of internal standard.

Collaborative Study

In a preliminary study, one cooking oil sample commercially fortified with vitamin D3 was sent to 10 laboratories with a draft copy of the standard method and an estimated result obtained at the Swedish National Food Administration (Uppsala, Sweden). Nine laboratories submitted their results from 3–5 replicate analyses with a reproducibility relative standard deviation (RSDr) of 15.0% after exclusion of the results of one laboratory. This RSDr was considered acceptable, making it possible to conduct a full collaborative study. The samples were sent to 11 Nordic and European laboratories that had expressed interest in taking part in the study. For different reasons, 3 laboratories did not analyze the samples, and the minimum number of 8 laboratories participated in the study.

In the collaborative study, the participants were supplied with 6 different test materials. Three matrices (milk, gruel, and margarine) were commercially fortified with vitamin D3; for the other matrices (cooking oil, infant formula, and fish oil), vitamin D3 was added by the Swedish National Food Administration. The milk was analyzed as a blind duplicate, and the other matrices were analyzed as split-level Youden pairs (<5% difference between materials, with the exception of cooking oil at 9.6%). The participants were also requested to determine the concentration of an enclosed vitamin D2 working standard solution. Finally, the participants were requested to prepare in-house standards to determine the LC response factor between vitamins D2 and D3 at 3 different relative concentrations (40 µg vitamin D2; 20, 40, and 80 µg vitamin D3, respectively, diluted to 50 mL with acetonitrile). The response factor for each participant was calculated as the mean of the 3 determinations.

The following samples were used in the study: (1) low-lactose ultra-high temperature (UHT) milk with a durability of 6 months and vitamin D2 label claim of 0.38 µg/100 g (fat content 1.5%); (2) 2 different types of ready-to-serve infant formula (gruel), with vitamin D3 label claims of 1.5 µg/100 g; (3) cooking oil with vitamin D3 added at 4.4 and 4.7 µg/100 g; (4) 2 brands of margarine with vitamin D3 label claims of 7.5 µg/100 g; (5) powdered infant formula with vitamin D3 added at 10.4 and 11.1 µg/100 g; and (6) fish oil with vitamin D3 added at levels of 12.3 and 12.8 µg/100 g. Low-lactose UHT milk is not commercially fortified with vitamin D3. The fish oil used was concentrated oil from deep-sea fish, and it contained 38% omega-3 fatty acids.

The cooking oil, infant formula, and fish oil were analyzed for endogenous vitamin before addition of vitamin D3, and the endogenous concentration was found to be below the detection limit of 0.1 µg/100 g in all 3 matrices. Infant formula (10.0 g) was weighed directly into individual glass bottles before vitamin D3 was added in ethanolic solution, to minimize the heterogeneity problems of dry-blended products. Only 1 mL ethanolic solution was added, to ensure that the infant formula remained a powder. The laboratories quantitatively transferred the powder to a 300 mL Erlenmeyer flask, assuming a weight of 10 g. The additions to the oil samples were performed in volumetric flasks (stock solutions), and the samples were mixed carefully before aliquots were transferred to individual glass bottles. The vitamin D3 content of each oil sample was calculated after the density of the oil had been measured.

The samples and the standard solutions reached the participants within 2 days after mailing, and the laboratories were requested to store the samples in a refrigerator until analysis. No testing order was specified, and all the samples from one matrix had the same number. The method was sent under separate cover just before the samples were mailed.

AOAC Method 2002.05

Determination of Cholecalciferol (Vitamin D3) in Selected Foods

Liquid Chromatography
First Action 2002

[A applicable to the determination of vitamin D3 (0.4–12 µg/100 g) in fortified milk, infant formula, gruel, margarine, cooking oil, and fish oil. Materials tested must not contain measurable levels of endogenous vitamin D2.]

See Table 2002.05 for the results of the interlaboratory study supporting acceptance of the method.

Caution: Observe standard precautions with potassium hydroxide, butylated hydroxytoluene (BHT), and flammable organic solvents. Work in a well-ventilated area, and avoid inhalation of solvent and vapor. Dispose of waste solvents according to local environmental regulations.

A. Principle

After the addition of an internal standard (vitamin D2) and basic hydrolysis, vitamin D3 is extracted with n-heptane. The fraction that contains vitamin D2/D3 is separated by preparative normal-phase liquid chromatography (LC). After evaporation and dilution in acetonitrile-methanol, vitamin D3 is determined by reversed-phase LC with UV detection at 265 nm. A separate test portion is analyzed in parallel to confirm the absence of endogenous vitamin D2.
B. Apparatus

Use volumetric glassware, class A or equivalent. Measure the volumes with volumetric pipets, and use volumetric flasks for all dilutions.

(a) Liquid chromatograph for semipreparative cleanup.—With an autoinjector or a loop injector, a fraction collector, a UV detector, an integrator, and a column thermostat. The fraction collector can be replaced by manual collection. Operating conditions: injection volume, 500 µL; column temperature, 35°C; wavelength, 265 nm; and flow rate, 1.5 mL/min.

(b) LC column for semipreparative cleanup.—Silica, 250 × 4.6 mm, particle size (dp) = 5 µm (Nucleosil 50-5, or equivalent) with precolumn, silica, 30 × 4.0 mm, dp = 5 µm (Nucleosil 50-5, or equivalent).

(c) Liquid chromatograph for quantitative analysis.—With an autoinjector or a loop injector, a UV detector (dual channel or photodiode array), and an integrator or data processor. Operating conditions: injection volume, 100 µL; wavelength, 265 nm; and flow rate, 1.3 mL/min.

(d) LC column for quantitative analysis.—C18, 250 × 4.6 mm, dp = 5 µm (Vydac 201 TP 54, or equivalent) with precolumn, C18, 4.0 × 4.0 mm, dp = 5 µm (LiChroCART 4-4, or equivalent).

(e) UV spectrophotometer.—Calibrated for both wavelength and photometric accuracy. Measure absorbance at 265 nm in 1 cm quartz cuvettes.

(f) Reflux apparatus.—To fit 300 mL Erlenmeyer flasks with ground-glass necks, condenser length ca 50 cm, with heated water bath, or equivalent.

(g) Rotary evaporator.—To fit 50 and 500 mL round-bottom flasks, with heated water bath.

C. Reagents

(a) Solvents.—Methanol, n-heptane, methyl-tert-butyl ether (MTBE), cyclohexane, isopropanol, and acetonitrile.

(b) Ascorbic acid

(c) Ethanol.—Absolute, 99.5%.

(d) Ethanol.—40%. Dilute 400 mL ethanol, (e), to 1 L with water.

(e) Potassium hydroxide (KOH) solution.—50% (w/w). Dissolve 500 g KOH pellets in 500 mL water.

(f) KOH solution.—1M. Dissolve 56 g KOH pellets in water and dilute to 1 L.

(g) Phenolphthalein solution.—1% (w/v). Dilute 1 g phenolphthalein in ethanol, (e), and dilute to 100 mL.

(h) BHT.—2,6-Di-tert-butyl-4-methylphenol.

(i) Cyclohexane–n-heptane (1 + 1).—Dilute 500 mL cyclohexane to 1 L with n-heptane.

(j) Mobile phase 1.—0.5% isopropanol and 2% MTBE in cyclohexane–n-heptane. Mix 5 mL isopropanol and 20 mL MTBE with 1 L cyclohexane–n-heptane (1 + 1), (i).

(k) Mobile phase 2.—Isopropanol–n-heptane (20 + 80). Dilute 200 mL isopropanol to 1 L with n-heptane.

(l) Mobile phase 3.—Methanol–acetonitrile (20 + 80). Dilute 200 mL methanol to 1 L with acetonitrile.

(m) Vitamin D$_2$, ergocalciferol.—Crystalline form for biochemistry, purity of >98%.

(n) Vitamin D$_2$ standard solutions.—(1) Stock standard solution.—1.00 mg/mL. Dissolve 0.100 g vitamin D$_2$ in ethanol, (e), and dilute to 100 mL. (2) Working standard solution.—20 µg/mL. Dilute 2.0 mL stock standard solution to 100 mL with ethanol, (e). (3) Internal standard solution.—0.8 µg/mL. Dilute 4.0 mL working standard solution, (2), to 100 mL with ethanol, (e). (4) Internal standard solution.—0.2 µg/mL. Dilute 1.0 mL working standard solution, (2), to 100 mL with ethanol, (e).
(o) Vitamin D₃, cholecalciferol.—Crystalline form for biotechnology, purity of >98%.

(p) Vitamin D₃ standard solutions.—(1) Stock standard solution.—1.00 mg/mL. Dissolve 0.10 g vitamin D₃ in ethanol, (c), and dilute to 100 mL. (2) Working standard solution.—20 μg/mL. Dilute 2.0 mL stock standard solution to 100 mL with ethanol, (e).

(q) Vitamins D₂ and D₃ working standard solutions.—(1) 5 μg/mL of both vitamins D₂ and D₃.—Transfer 0.5 mL of each stock solution, (n)(1) and (p)(1), to 50 mL round-bottom flask, evaporate to dryness, dissolve residue in cyclohexane—n-heptane (1 + 1), (i), and dilute to 100 mL. (2) 0.8 μg/mL of both vitamins D₂ and D₃.—Dilute 2.0 mL of each working standard solution, (n)(2) and (p)(2), to 50 mL with acetonitrile.

Stock solutions are stable in the dark at −18°C. Discard after 12 months or if purity decreases to <90% as determined by LC. Stock solutions should be brought to room temperature before use. The stability is not affected by repeated cooling (−18°C) and warming (room temperature). Working and internal standards should be prepared monthly and stored at 4–8°C.

D. Determination of Standard Concentration

Determine the concentrations of vitamins D₂ and D₃ standards monthly by spectrophotometry and LC according to the following procedure:

(a) Spectrophotometry.—Scan the working standard solutions, C(n)(2) and C(p)(2), with a spectrophotometer. Record absorbance at 265 nm versus ethanol, C(e). Calculate the concentration (c) according to the following equation:

\[ c, \mu g/mL = (A/E) \times 10,000 \times f_{LC} \]

where \( A \) = absorbance at 265 nm; \( E \) (1%, 1 cm) = 475 for vitamin D₂ and 480 for vitamin D₃ (6); 10,000 = conversion factor; and \( f_{LC} \) = purity value determined by LC, (b).

(b) LC.—Evaporate 1.0 mL internal standard solution of vitamin D₂, C(n)(3), or working standard solution of vitamin D₃, C(p)(3), nearly to dryness, and dissolve residue in 3 mL mobile phase 3, C(l). Analyze the extract by reversed-phase LC according to H. Calculate the purity value (\( f_{LC} \)) of the standard according to the following equation:

\[ f_{LC} = A_{LP}/A_{tot} \]

where \( A_{LP} \) = peak area for vitamins D₂ and D₃, respectively, and \( A_{tot} \) = total area of all vitamin D peaks present in the chromatogram (excluding solvent and void peaks). The purity value should be between 0.96 and 1.0.

E. Saponification

(a) High-fat products (margarine and oils).—With an accuracy of 0.01 g, weigh a test portion containing 5–8 g oil into a 300 mL Erlenmeyer flask with a ground-glass neck, and add 0.5 g ascorbic acid, C(b), 50 mL ethanol, C(c), 2.0 mL vitamin D₂ internal standard solution, C(n)(3), and 20 mL 50% KOH solution, C(e). Should there be a problem in the fractionation stage due to visible residual lipid, the test portion can be reduced to 2 g, if a higher detection limit is acceptable. Add a reflux condenser to the Erlenmeyer flask, and place in a water bath (ca 95°C). Hydrolyze for 30 min after boiling has started. Remove the Erlenmeyer flask from the water bath. Add 50 mL water through the condenser. Let the mixture cool to room temperature.

(b) Infant formula, milk powders, and other solid products.—With an accuracy of 0.01 g, weigh 10 g powder in a 300 mL Erlenmeyer flask with ground-glass neck, and add 0.5 g ascorbic acid, C(b), 60 mL ethanol, C(c), and 10 mL water. Add 2.0 mL vitamin D₂ internal standard solution, C(n)(3), to fortified powders, 2.0 mL C(n)(4) to unfortified powders, and 10 mL 50% KOH solution, C(e). Continue according to (a).

(c) Milk and other liquid products.—With an accuracy of 0.01 g, weigh 50 g milk in a 300 mL Erlenmeyer flask with ground-glass neck, and add 0.5 g ascorbic acid, C(b), 100 mL ethanol, C(c), 2.0 mL vitamin D₂ internal standard solution, C(n)(4), and 25 mL 50% KOH solution, C(e). Continue according to (a), except add 25 mL water instead of 50 mL.

F. Extraction

(a) Solid foods and oils (margarine, oils, infant formula, milk powder, etc.).—Quantitatively transfer the hydrolysate to a 500 mL separatory funnel with 100 mL 40% ethanol, C(d). Add 75 mL n-heptane, and shake the separatory funnel vigorously for 1.5 min. Let the phases separate, and transfer the upper heptane phase to a 250 mL separatory funnel. Repeat the extraction procedure once, and combine the extracts.

Wash the combined heptane phases once with 50 mL 1M KOH solution, C(f), then twice with 50 mL 40% ethanol, and finally with 50 mL portions of water until the heptane phase is free of alkali. Check alkalinity with the phenolphthalein solution, C(g); the water phase should be totally colorless. Shake separatory funnel vigorously for 30 s at every washing step.

Transfer the heptane phase to a 500 mL round-bottom flask. Add some granules of BHT, C(h), and 15 mL ethanol, C(c), and evaporate with a rotary evaporator at ca 45°C. Stop immediately when phase is dry. Dissolve the residue in cyclohexane—n-heptane (1 + 1), C(i). Transfer the extract to a 2 mL volumetric flask, rinsing twice, and dilute to volume. Use this dilution for semipreparative cleanup of vitamin D. At this stage, extracts may be stored at 4–8°C overnight.

(b) Milk and other liquid products.—Quantitatively transfer the hydrolysate to a 500 mL separatory funnel with 50 mL 40% ethanol, C(d). Add 50 mL n-heptane, and shake the separatory funnel vigorously for 1 min. Let the phases separate, and transfer the heptane phase to a 250 mL separatory funnel. Repeat the extraction procedure twice. Then continue with the washing step according to (a).

G. Chromatographic Semipreparative Cleanup

(a) System setup.—Use mobile phase 1, C(j), for the semipreparative cleanup, and equilibrate the system. Check
the stability of the retention time by injecting the working standard solution, \( C(q)(J) \), 2 or 3 times. Normally, the retention times fluctuate less than ±1%. If there is a larger variation, check the pumping unit, the homogeneity of the mobile phase, the injection solution (must be free of water), and the temperature stability. Then repeat the injections of the standard solution, \( C(q)(J) \). Under the above conditions, the retention time for vitamin D2/D3 is about 17 min. The composition of the mobile phase may have to be altered to suit the quality of the column in use.

Determine the collection window from the retention time and the peak volume. The peak volume, i.e., the base width, under the conditions above is ca 3 mL. Collect the fraction in such a way as to ensure that the entire peak volume is collected without incorporating too many interfering components. This can be a problem with some matrixes, notably certain oils.

(b) Mobile phase purity.—Evaporate 5 mL mobile phase 1, \( C(j) \), in the same way as for the test extract and perform a reversed-phase LC run with the analytical system. If there are interfering components eluting at the same time as vitamins D2 or D3, evaporate each solvent present in mobile phase 1, analyze by LC, and change the possibly contaminated batch.

(c) Chromatography.—Inject the test extract, and collect the vitamin D2/D3 fraction in a 50 mL round-bottom flask. Evaporate to dryness with a rotary evaporator at ca 45°C. Dissolve the residue in 0.5 mL mobile phase 3, \( C(l) \). These residues are stable and may be stored at 4–8°C overnight until analysis.

To avoid accumulation of polar lipids, clean the silica column at the end of each run with mobile phase 2, \( C(k) \), for ca 15 min.

H. Chromatographic Determination

Chromatography.—Use mobile phase 3, \( C(l) \), for the quantitative analysis, and equilibrate the system. Check the stability of the retention time by injecting the working standard solution, \( C(q)(2) \), 2 or 3 times. Normally, the retention times fluctuate less than ±1%. Inject test extracts, and measure peak areas with an integrator or data processor. Under the above conditions, the retention time for vitamin D2 is ca 10 min and that for D3, ca 11 min.

The composition of the mobile phase may have to be altered to suit the quality of the column in use. The vitamins D2 and D3 peaks should be free from interfering components as determined by either a wavelength ratio technique (e.g., 265:280 nm) or spectral monitoring, and have a resolution value, \( R_s \), of ≥1.5.

I. Calculations

Calculate the vitamin D3 content (\( C_s \)) of the product according to the following equation:

\[
C_s, \mu g/100 \, g = \frac{AD_3 \times mD_2 \times 100}{AD_2 \times ms \times F}
\]

where \( AD_3 \) = peak area for vitamin D3; \( AD_2 \) = peak area for vitamin D2; \( mD_2 \) = weight of vitamin D2 added to the test portion (\( \mu g \)); \( ms \) = weight of test portion (g); and \( F \) = response factor (D3/D2) at 265 nm.

Determine the response factor, \( F \), by reversed-phase LC according to H. Use the working standard solution, \( C(q)(2) \), for the determination. Calculate \( F \) according to the following equation:

\[
F = \frac{\text{(peak area } D_3/\text{peak area } D_2)}{cD_2/cD_3}
\]

Table 1. Vitamin D3 content (\( \mu g/100 \, g \)) found in the method performance study

<table>
<thead>
<tr>
<th>Sample</th>
<th>Milk</th>
<th>Gruel</th>
<th>Cooking oil</th>
<th>Margarine</th>
<th>Infant formula</th>
<th>Fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>0.407</td>
<td>0.427</td>
<td>1.40</td>
<td>1.37</td>
<td>4.0</td>
<td>4.1</td>
</tr>
<tr>
<td>2</td>
<td>0.470</td>
<td>0.415</td>
<td>1.59</td>
<td>1.53</td>
<td>5.9</td>
<td>6.6</td>
</tr>
<tr>
<td>3</td>
<td>0.409</td>
<td>0.432</td>
<td>1.56</td>
<td>1.40</td>
<td>4.4</td>
<td>5.3</td>
</tr>
<tr>
<td>4</td>
<td>0.336</td>
<td>0.349</td>
<td>1.22</td>
<td>1.09</td>
<td>4.5</td>
<td>4.1</td>
</tr>
<tr>
<td>5</td>
<td>0.453</td>
<td>0.446</td>
<td>1.54</td>
<td>1.42</td>
<td>2.7</td>
<td>3.3</td>
</tr>
<tr>
<td>6</td>
<td>0.744^c</td>
<td>0.446</td>
<td>1.45</td>
<td>1.43</td>
<td>3.5</td>
<td>4.6</td>
</tr>
<tr>
<td>7</td>
<td>0.408</td>
<td>0.440</td>
<td>1.02</td>
<td>1.23</td>
<td>5.8</td>
<td>6.3</td>
</tr>
<tr>
<td>8</td>
<td>0.427</td>
<td>0.434</td>
<td>1.43</td>
<td>1.40</td>
<td>4.2</td>
<td>4.4</td>
</tr>
<tr>
<td>Vitamin D3 added, ( \mu g/100 , g )</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>4.4</td>
<td>4.7</td>
</tr>
</tbody>
</table>

\(^a\) Outlier by the single Grubbs test at \( P = 2.5\% \).

\(^b\) NR = Not reported.

\(^c\) Outlier by the Cochran test at \( P = 2.5\% \).
where \( c_{D_2} \) = concentration of vitamin D2 in the standard solution and \( c_{D_3} \) = concentration of vitamin D3 in the standard solution.

Refs.: J. AOAC Int. 86, 401–405 (2003)

**Results and Discussion**

Results from the collaborative study are shown in Table 1. The participants were asked to perform a single determination for each sample. The collaborative study results were statistically evaluated according to the International Union of Pure and Applied Chemistry (IUPAC) 1987 Harmonized Protocol (7). Outliers were determined for all paired samples with the Cochran, single Grubbs, and double Grubbs tests at \( P = 2.5\% \) (1-tailed). One milk result was excluded after the Cochran test, and 2 margarine results were excluded after the single Grubbs test. No results were excluded after the double Grubbs test. One participant did not report the results for the analysis of the infant formula.

Vitamin D3 was added to 6 of the materials, and the mean values of the recoveries are shown in Table 2002.05. The repeatability relative standard deviation (\( RSD_r \)) and reproducibility relative standard deviation (\( RSD_R \)) values after exclusion of outliers are also listed in Table 2002.05. The results are satisfactory, with \( RSD_r \) values in the range of 2.2–7.4% and \( RSD_R \) values between 6.8 and 24.1%. The \( RSD_R \) values fall within the limits set by the Horwitz equation (8), \( RSD_H, \% = 2^{\frac{1}{4} - 0.5\log C} \), where C is the analyte concentration by weight expressed as a decimal fraction. A comparison between the \( RSD_R \) values and the Horwitz values, \( RSD_H \), is shown in Table 2, with satisfactory results (HORRAT of <2) for all samples.

Average response factors determined by individual participants at 3 different concentrations of vitamin D3 ranged from 1.00 to 1.08, demonstrating that it is important for each laboratory to determine its in-house response factor. The concentration (unknown to the collaborators) of the vitamin D2 standard solution sent to each participant was determined to be 0.711 \( \mu \)g/mL, with purity of 94.9% determined by LC, at the Swedish National Food Administration before dispatch. The mean value of the concentrations reported by the participants was 0.733 \( \mu \)g/mL (\( RSD_R \), 9.2%). No outliers were identified by the single and double Grubbs tests.

Analyses of the working standard solution of unknown concentration were intended as a check of the independent in-house standards with respect to concentration, response factor, and sample analyses. Interlaboratory variation in the analytical results can be attributed to a combination of standard determination, preparative LC, and quantitative LC factors.

The participants were asked to supply one chromatogram obtained by quantitative LC for each matrix and one chromatogram obtained by preparative LC of the cooking oil. The overall results of the analytical LC separations were satisfactory for the analyses of milk, gruel, infant formula, and margarine. Typical examples are shown in Figures 1 and 2 for a vitamin-fortified margarine sample. In the chromatograms from the analyses of cooking oil and fish oil, there were some interferences near the vitamin D peaks. Cooking oil was the most difficult matrix in this study, but quantitative LC peak integration was nevertheless acceptable.

### Table 2. Comparison of Horwitz values with the calculated \( RSD_R \) values

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Relative concn</th>
<th>Horwitz value ( (RSD_H) ), %</th>
<th>( RSD_R ) value, %</th>
<th>( RSD_R/RSD_H )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk(^a)</td>
<td>( 4.2 \times 10^{-9} )</td>
<td>36</td>
<td>9.1</td>
<td>0.25</td>
</tr>
<tr>
<td>Gruel</td>
<td>( 1.4 \times 10^{-6} )</td>
<td>30</td>
<td>12.1</td>
<td>0.40</td>
</tr>
<tr>
<td>Cooking oil</td>
<td>( 4.6 \times 10^{-6} )</td>
<td>25</td>
<td>24.1</td>
<td>0.95</td>
</tr>
<tr>
<td>Margarine(^a)</td>
<td>( 8.4 \times 10^{-6} )</td>
<td>23</td>
<td>6.8</td>
<td>0.29</td>
</tr>
<tr>
<td>Infant formula</td>
<td>( 1.0 \times 10^{-7} )</td>
<td>23</td>
<td>7.1</td>
<td>0.31</td>
</tr>
<tr>
<td>Fish oil</td>
<td>( 1.2 \times 10^{-7} )</td>
<td>22</td>
<td>17.7</td>
<td>0.80</td>
</tr>
</tbody>
</table>

\(^a\) After exclusion of one participant.
Collaborators’ Comments

This method was developed with the intention of applying it horizontally to different food matrixes. In this collaborative study, the types of LC columns were optional, although the eluants were specified. After the comment from one participant that the eluant composition should also be optional, the method was revised accordingly. The selectivities of the LC columns can differ from batch to batch, and other columns and eluants may provide equivalent performance. However, the columns specified in the method have been used in the analyses of a wide variety of matrixes.

According to one participant, the list of extraction solvents should be extended to diethyl ether, pentane, or related solvents. However, we have not included this remark in the method description, because only n-heptane was used in the validation of the method.

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References


Figure 2. Chromatogram from the determination of vitamin D₃ in a margarine sample. The vitamin D₃ content reported was 12.4 μg/100 g.