AOAC Official Method 995.05
Vitamin D in Infant Formulas and Enteral Products
Liquid Chromatographic Method
First Action 1995

[Applicable to determination of 8–2600 IU (International Unit; 1 µg vitamin D = 40 IU) vitamin D/qt (1 qt = 0.946 L) in infant formulas and enteral products.]

See Table 995.05A for the results of the interlaboratory study supporting the acceptance of the method.

A. Principle
Test portion is saponified, extracted, and evaporated to concentrate nutrient. Vitamin D is determined by reversed-phase LC equipped with UV detector at 265 nm.

B. Apparatus
(a) Liquid chromatograph (LC).—With UV detector, meeting system suitability requirements. Operate at 27 ± 1°C; higher temperature results in loss of resolution.

(b) LC column.—250 × 4.6 mm id, C18, 5 µm particle size. Operating conditions: injection volume, 250 µL; column temperature, 27°C; wavelength, 265 nm; flow rates, see Table 995.05B for flow rates; stop time, 35 min; retention times: vitamin D2, 19.5 min; vitamin D3, 23 min. (Note: The column must not be end-capped.)

(c) Solid-phase extraction (SPE) column.—Silica; 500 mg/2.8 mL.

(d) Vacuum manifold.—For SPE column, (e).

(e) Evaporator.—With N flow.

(f) Rotary evaporator.

(g) Water bath shaker.—Maintaining 60°C.

(h) Separatory funnel.—250 mL.

(i) Amber volumetric flask.—250 mL.

(j) Reflux apparatus.

C. Reagents
(a) Solvents (HPLC grades).—n-Hexane, dichloromethane, acetonitrile, isopropanol, methanol, ethyl acetate.

(b) Ethanol.—Absolute, pharmaceutical grade.

(c) Phenolphthalein solution.—1%. Dissolve 1 g phenolphthalein in 100 mL absolute ethanol.

(d) Dichloromethane–isopropanol (IPA) solutions.—(1) 99.8 + 0.2 (v/v).—Transfer 2 mL isopropanol into 1 L volumetric flask. Dilute to volume with dichloromethane and mix. (2) 80 + 20 (v/v).—Transfer 200 mL isopropanol into 1 L volumetric flask, dilute to volume with dichloromethane, and mix.

(e) Acetic acid solution.—10%. Transfer 10 mL glacial acetic acid (AR grade) into 100 mL volumetric flask, dilute to volume with H2O, and mix well.

(f) Ethanolic potassium hydroxide (KOH) solution.—Dissolve 140 g KOH pellets (AR grade) in 310 mL absolute ethanol and add 50 mL H2O. Prepare on day of use.

(g) Mobile phase.—Gradient mixture of acetonitrile, methanol, and ethyl acetate. See Table 995.05B for concentrations of mobile phase components.

(h) Vitamin D2 standard solutions.—USP reference standard or traceable secondary standard (NIST, USP, etc). (1) Stock standard solution.—180 µg/mL. Accurately weigh 45 mg vitamin D2 and quantitatively transfer to 250 mL amber volumetric flask. Dilute to volume with absolute ethanol. (2) Working standard solution.—2.88 µg/mL. Transfer 4.0 mL stock standard solution into 250 mL amber volumetric flask, and dilute to volume with absolute ethanol. Store in refrigerator ≤7 days. (3) Internal standard solution.—46 ng/mL. Use to quantitate vitamin D3. Transfer 4.0 mL working standard solution into 250 mL amber volumetric flask and dilute to volume with absolute ethanol. Store in refrigerator ≤7 days.

(i) Vitamin D3 standard solutions.—USP reference standard or traceable secondary standard. Prepare and store in refrigerator ≤7 days. (1) Stock standard solution.—180 µg/mL. (2) Working standard solution.—2.88 µg/mL. (3) Internal standard solution.—46 ng/mL. Use to quantitate vitamin D2.

(j) System suitability standard solution.—Dissolve 125 mg certified vitamin D3 standard and 125 mg certified vitamin D2 standard in 10 mL acetonitrile. Heat solution 45 min at 90°C under reflux, then cool. Transfer 1.0 mL refluxed solution to 100 mL amber volumetric flask and dilute to volume with acetonitrile.

Table 995.05A Interlaboratory study results for determination of vitamin D in infant formulas and enteral products by liquid chromatography

<table>
<thead>
<tr>
<th>Sample</th>
<th>$C^2$</th>
<th>$s_r$</th>
<th>$s_B$</th>
<th>RSD1, %</th>
<th>RSD2, %</th>
<th>r</th>
<th>R</th>
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<tbody>
<tr>
<td>IF liquid</td>
<td>1086.70</td>
<td>112.40</td>
<td>142.13</td>
<td>10.34</td>
<td>13.08</td>
<td>314.72</td>
<td>397.96</td>
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<td>IF liquid</td>
<td>983.50</td>
<td>76.89</td>
<td>135.98</td>
<td>7.82</td>
<td>13.83</td>
<td>214.73</td>
<td>380.74</td>
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<tr>
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<td>35.59</td>
<td>42.61</td>
<td>6.08</td>
<td>7.28</td>
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<td>119.31</td>
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<tr>
<td>IF liquid</td>
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<td>46.27</td>
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<td>9.35</td>
<td>12.67</td>
<td>129.56</td>
<td>175.53</td>
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<tr>
<td>IF liquid</td>
<td>488.10</td>
<td>36.08</td>
<td>57.81</td>
<td>7.39</td>
<td>11.84</td>
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<td>161.87</td>
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<tr>
<td>IF liquid</td>
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<td>20.99</td>
<td>46.46</td>
<td>4.44</td>
<td>9.83</td>
<td>58.77</td>
<td>130.09</td>
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<td>E liquid</td>
<td>357.60</td>
<td>31.03</td>
<td>58.19</td>
<td>8.68</td>
<td>16.27</td>
<td>86.88</td>
<td>162.93</td>
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<td>27.49</td>
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<td>7.94</td>
<td>9.69</td>
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<td>93.94</td>
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<td>0.60</td>
<td>3.87</td>
<td>10.46</td>
<td>0.62</td>
<td>1.68</td>
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<tr>
<td>IF powder</td>
<td>3.96</td>
<td>0.54</td>
<td>0.77</td>
<td>13.71</td>
<td>19.44</td>
<td>1.51</td>
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<tr>
<td>E powder</td>
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<td>23.11</td>
<td>23.11</td>
<td>0.70</td>
<td>0.70</td>
</tr>
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</table>

a IF = infant formula; E = enteral product.
b Mean concentration of vitamin D in IU/QND for liquid formulas (IU = International Unit [1 mcg vitamin D = 40 IU]; QND = quart normal dilution [1 qt = 0.946 L]), and in IU/g for powders.

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Add 4.0 mL internal standard solution to flask. Use vitamin D2 standard solution to be 0.5 IU/mL. Transfer 15.0 mL test portion into 125 mL standard solution, and discard aqueous layer. Add 10% acetic acid solution, shaking, until washing is neutral to phenolphthalein (colorless). Heat in same round-bottom flask.

Place on rotary evaporator and evaporate hexane to dryness at 40°C. Immediately after evaporation, add 2.0 mL 250 = volumes of subsequent dilutions of vitamin D3 standard solutions; 250 = weights of vitamin D3 certified reference standard.

Calculate concentration of vitamin D2 in standards mixture (CSD2) as follows:

\[ CS_{D2} (\text{IU/mL}) = \frac{W \times 4 \times 4 \times 40000}{250 \times 250 \times 250} \times 105 \]

where \( W \) = weight of vitamin D2 certified reference standard, mg; 4 = dilution factor; 40 000 = 1 IU vitamin D/mg; 250 = volumes of subsequent dilutions of vitamin D2 standard solutions; 105 = correction factor for pre-vitamin D.

Calculate concentration of vitamin D2 in test portion from standards mixture (CSD2) as above, using weight of vitamin D3 certified reference standard.

Calculate response ratio of vitamin D2 in test portion (RS_D2) as follows:

\[ RS_{D2} = \frac{PS_{D2}}{PS_{D3}} \]

where \( PS_{D2} \) = peak height of vitamin D2 in standards mixture; and \( PS_{D3} \) = peak height of vitamin D3 in standards mixture.

Calculate response ratio of vitamin D2 in test portion (RT_D2) as follows:

\[ RT_{D2} = \frac{PT_{D2}}{PT_{D3}} \]

where \( PT_{D2} \) = peak height of vitamin D2 in test portion; and \( PT_{D3} \) = peak height of vitamin D3 in test sample.

Calculate concentration of vitamin D2 in test portion (CT_D2) or D3 (CT_D3) in test portion as follows:

\[ CT_{D2} (\text{IU/mL}) = \frac{RT_{D2}}{RS_{D2}} \times CS_{D2} \times U \times D \]

\[ CT_{D3} (\text{IU/mL}) = \frac{RT_{D3}}{RS_{D3}} \times CS_{D3} \times U \times D \]
where \( V_t \) = volume of test portion, mL (usually 15); \( U \) = conversion factor to appropriate units (if necessary); and \( D \) = dilution factor for diluted powders or liquids.

I. System Suitability

For system suitability use standards mixture, \( D(1) \), during routine operation. Resolution factor between vitamin D\(_2\) and vitamin D\(_3\) should be 2.0. Calculate resolution factor as follows:

\[
R = \frac{2(t_2 - t_1)}{w_1 + w_2}
\]

where: \( t_1 \) and \( t_2 \) = elution times for vitamins D\(_2\) and D\(_3\), respectively; and \( w_1 \) and \( w_2 \) = peak widths of vitamins D\(_2\) and D\(_3\). Peak widths are measured as the time interval between the points where the baseline intersects the 2 lines produced by extending the relatively straight sides of the peak.

Separation between these peaks should be sufficient to allow additional peak to be resolved between vitamin D\(_2\) and D\(_3\), namely, pre-vitamin D\(_3\).

To verify that pre-vitamin D\(_3\) is resolved, inject system suitability standard solution, \( C(1) \). The system suitability standard solution contains, in order of elution, pre-vitamin D\(_2\), vitamin D\(_2\), pre-vitamin D\(_3\), and vitamin D\(_3\).

Optimize chromatography by adjusting amount of methanol in mobile phase; decreasing methanol content increases retention times. Maintain column temperature at 27 ± 1°C; increased temperature decreases retention, and vice versa. Six-replicate injections of standard should have <2% relative standard deviation (RSD). RSD values for standards throughout run should be <4%.