Determination of Vitamin A in Infant Formula and Adult Nutritionals by UPLC-UV: First Action 2011.07

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Vitamin A, a fat-soluble vitamin, is essential for health and plays an important part in vision, bone growth, reproduction, regulating the immune system, cell function, and skin health. Due to the advances in technology and the expansion of its uses, LC technologies are being studied for effectiveness in detecting and quantifying vitamin A in an effort to help determine the amount of vitamin A in various types of samples. For this reason, an Expert Review Panel agreed on June 29, 2011, at the “Standards Development and International Harmonization: AOAC INTERNATIONAL Mid-Year Meeting,” to approve “Determination of Vitamin A in Infant Formula and Adult Nutritionals by UPLC-UV” as AOAC Official MethodSM 2011.07.

To move from First to Final Action status, it was recommended that additional information be generated for all types of infant formulas and adult nutritional formula matrixes at varied concentration levels, as indicated in the standard method performance requirements. International units or retinol equivalents typically represent the concentration of vitamin A in food and supplements. However, for the purpose of this method, the concentration represented is presented in μg/100g.

On March 28, 2011, the AOAC Board of Directors approved an alternative path to achieve Official First Action status for methods selected and reviewed using the AOAC volunteer consensus standards development processes. Under this path, an Expert Review Panel (ERP) vetted by the Official Methods Board selected the method, “Determination of Vitamin A in Infant Formula and Adult Nutritionals by UPLC-UV” for Official First Action status. Methods approved First Action under the alternative path will remain First Action for a period of about 2 years. During this time, methods will be used in laboratories, generating additional information. ERPs will monitor the performance of methods; after about 2 years, they will determine whether the method should be recommended to the Official Methods Board for Final Action.

All methods were reviewed by a primary and secondary expert reviewer. Panel members summarized their reviews, along with the advantages and disadvantages of each method, which were then discussed thoroughly by the entire panel, stakeholders, and observers present. Methods were evaluated for completeness of validation and likelihood of meeting standard method performance requirements (SMPRs), which include appropriateness for the intended use, clarity of the method description, ruggedness, reproducibility, recovery, analytical range, and LOQ.

In November 2010, the Working Group on Vitamin A, chaired by Jonathan DeVries of General Mills/Medallion Laboratories (Minneapolis, MN), recommended the method submitted by Nestlé Research Center (Lausanne, Switzerland) which was approved in June 2011 by the ERP at the “Standards Development and International Harmonization: AOAC INTERNATIONAL Mid-Year Meeting” as AOAC Official MethodSM 2011.07. The method uses alcoholic saponification with potassium hydroxide in the presence of antioxidants to convert retinol esters to retinol; diatomaceous earth cartridges to extract, clean, and concentrate the saponified sample; and normal-phase chromatography with UV detection to separate, detect, and quantitate retinol. To move from First to Final Action status, it was recommended that additional information be generated for all types of infant formulas and adult nutritionals at varied concentration levels as indicated in the SMPRs.

AOAC Official Method 2011.07
Vitamin A in Infant Formula and Adult Nutritionals
UPLC-UV
First Action 2011

[Applicable to the determination of vitamin A (retinol) in infant formula and adult nutritionals Vitamin A is defined as the sum of all-trans retinol, 13-cis retinol, and retinol esters.]


Caution: Potassium hydroxide is extremely corrosive. Avoid any contact with eyes and skin. Wear laboratory safety goggles. Perform work in a fume hood when using solvents. Refer to Material Safety Data Sheets (MSDS) for specific information.
Table 2011.07A. Milk-based infant formula

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Unit</th>
<th>n_d * n_r</th>
<th>Median</th>
<th>SD_r</th>
<th>CV_r, %</th>
<th>r</th>
<th>t, %</th>
<th>SD_R</th>
<th>CV_R, %</th>
<th>IR</th>
<th>IR, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A</td>
<td>μg/100 g</td>
<td>7 * 2</td>
<td>475</td>
<td>6.3</td>
<td>1.3</td>
<td>17.0</td>
<td>3.7</td>
<td>22.7</td>
<td>4.8</td>
<td>63</td>
<td>13.2</td>
</tr>
</tbody>
</table>

A. Principle

The method uses an alcoholic saponification with potassium hydroxide in the presence of antioxidants to saponify vitamin A to retinol in test samples. The extraction uses solid-phase extraction with a diatomaceous earth cartridge followed by elution with n-hexane. The extract is then evaporated to dryness and diluted in n-hexane. Retinol is then analyzed by UPLC with UV detection at 326 nm and quantitated by comparing the peak heights or peak areas of external standards.

B. Apparatus

(a) Glassware.—Standard laboratory, amber, class A.
(b) Membrane filters.—0.22 μm.
(c) Syringe.—Disposable, plastic, 2 mL.
(d) Adapter.—Chromabond.
(e) Diatomaceous earth cartridges.—Chromabond XTR (Macherey-Nagel, Duren, Germany) 70 mL/14 500 mg, or equivalent (with needle and clamp).
(f) Columns.—Chromabond, empty, 70 mL (optional).
(g) Filling material.—Chromabond XTR Btl 14.5 g (optional; fill material ≤0.4 mm in diameter).
(h) Polytron homogenizer.
(i) Water bath.—Equipped with magnetic stirrers and Allihn condenser.
(j) Rotary evaporator.
(k) UPLC system.—Equipped with a binary gradient pump and hexane tetrahydrofuran compatibility kit, sample injector equipped with a 5 μL injection loop, UV/diode array detector, and data software (Waters Corp., Milford, MA).
(l) Analytical column.—Acquity UPLC BEH HILIC 1.7 μm, 2.1 × 100 mm.

C. Reagents

Note: Refer to adequate manuals or safety data sheets approved by local authorities and ensure that safety guidelines are applied before using chemicals.

(a) Absolute alcohol.—GR, ACS, ISO, CAS 64-17-5 (Merck, Geneva, Switzerland; 100983 or equivalent).
(b) 2-Propanol.—For HPLC LiChrosolv, CAS 67-63-0 (Merck 101040 or equivalent).
(c) n-Hexane.—For HPLC LiChrosolv, CAS 110-54-3 (Merck 104391 or equivalent).
(d) Potassium hydroxide pellets.—For analysis, CAS 1310-58-3 (Merck 105033 or equivalent).
(e) All-trans retinol (vitamin A) cryst.—CAS 68-26-8 (Fluka nb. 95144, Buchs, Switzerland, or equivalent).
(f) Sodium sulfide hydrate.—CAS 27610-45-3 (Merck 106638 or equivalent).
(g) Sodium dodecyl sulfate.—CAS 151-21-3 (Fluka nb. 71727 or equivalent).
(h) Sodium sulfate anhydrous.—CAS 7757-82-6, ACS, ISO (Merck 106649 or equivalent).
(i) Sodium 1-pentanesulfonate.—CAS 207605-40-1 (Fluka 76955 or equivalent).
(j) Sodium ascorbate.—CAS 134-03-2 (Merck 500076 or equivalent).
(k) Pyrogallol (optional).—CAS 87-66-1.
(m) Potassium dihydrogen phosphate.—CAS 7778-77-0.
(n) Butylhydroxytoluene (2,6-di-tert-butyl-4-methylphenol) (BHT).—CAS 128-37-0 (Merck 822021 or equivalent).
(o) Takadiastase or amylase.—CAS 9001-19-8 (Merck 86247 or equivalent).

D. Preparation of Reagents

Note: Volumes of glassware are purely indicative and may be modified as long as the proportion of reagents is maintained.

(a) n-Hexane containing 0.05 mg/mL BHT.—Weigh 50 mg BHT into a 1000 mL volumetric flask and make up to the mark with n-hexane. Store solution at room temperature for up to 6 months.
(b) Mobile phase.—1% (v/v) 2-propanol in n-hexane.—Pipet 10 mL 2-propanol into a 1000 mL volumetric flask. Make up to the mark with n-hexane. Store solution in a tightly closed flask at room temperature for up to 2 months.
(c) All-trans retinol standard solutions.—(1) All-trans retinol stock solution, about 150 μg/mL.—Weigh 15 ± 5 mg all-trans retinol crystall into a 100 mL amber glass volumetric flask. Dissolve and make up to the mark with ethanol. Note: Solution is stable for at least 2 weeks at –20°C. Concentration of solution must be determined by spectrophotometry each day of use [see D(d)]. Calculated concentration should be at least 80% of the theoretical, otherwise prepare freshly the stock solution. If all-trans retinol standard is not available, saponified retinyl acetate can be substituted. See G. Standard Preparation Procedure.

(2) All-trans retinol intermediate solution, about 15 μg/mL.—Pipet 5 mL stock solution into a 50 mL amber glass volumetric flask. Make up to the mark with n-hexane. Note: Prepare solution fresh daily.

Table 2011.07B. Recovery: Milk-based infant formula

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Unit</th>
<th>n_d * n_r</th>
<th>Consensus value</th>
<th>n</th>
<th>Uncertainty of consensus value</th>
<th>Recovery</th>
<th>Median of results</th>
<th>Rec., %</th>
<th>SD (Rec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A</td>
<td>μg/100 g</td>
<td>7 * 2</td>
<td>474</td>
<td>41</td>
<td>13</td>
<td>475</td>
<td>100.2</td>
<td>0.033</td>
<td></td>
</tr>
</tbody>
</table>

*Uncertainty of consensus value calculated as SD_μ = 1.2533*SD_μ/sqrt (n), where SD_μ is the standard deviation of reproducibility obtained during proficiency testing and n is the number of participating laboratories.
(3) All-trans retinol working solutions, about 3.0, 1.8, and 0.6 μg/mL. — (a) Approximately 3.0 μg/mL.—Pipet 5 mL intermediate solution into a 25 mL amber glass volumetric flask. Make up to the mark with n-hexane. (b) Approximately 1.8 μg/mL.—Pipet 6.0 mL solution in D(e)(3)(a) into a 10 mL amber glass volumetric flask. Make up to the mark with n-hexane. (c) Approximately 0.6 μg/mL.—Pipet 2.0 mL solution in D(e)(3)(a) into a 10 mL amber glass volumetric flask. Make up to the mark with n-hexane. Note: Prepare these solutions fresh daily.

(d) Determination of the concentration of the all-trans retinol stock solution by spectrophotometry.—Pipet 2 mL all-trans retinol stock solution into a 100 mL amber glass volumetric flask. Make up to the mark with ethanol. (Note: Solution should contain about 3 μg/mL all-trans retinol.) Measure the absorbance (A) at 326 nm against ethanol. Determine the all-trans retinol concentration according to the following formula:

\[
\text{Concentration of stock solution (μg/mL)} = \frac{A_{326 \, \text{nm}} \times 150}{0.549}
\]

where \(A_{326 \, \text{nm}}\) = measured absorbance at 326 nm, 0.549 = theoretical absorbance of all-trans retinol solution in ethanol at 3 μg/mL (\(E^{1\%}_{1\,cm} = 1830\)).

E. Preparation of Test Samples: Reconstitution

(a) Infant formula and adult nutritional powder.—Weigh 50.0 ± 1.0 g powder sample into a 250 mL beaker. Record mass to 0.1 g. Add 100 g water at 40 ± 5°C. Mix with a glass rod or Polytron until the suspension is homogeneous. Note: Add 50 ± 10 mg Takadiastase or amylase if samples contain starch to facilitate handling. Mix well and let stand for 15 min at 40 ± 5°C. Proceed as in F(a).

(b) Liquid products (e.g., ready-to-feed formulas, liquid milk).—Note: Mix sample well if there has been any fat separation. Allow laboratory sample to come up to room temperature. Mix well. Note: Add 50 ± 10 mg Takadiastase or amylase if samples contain starch to facilitate handling. Mix well and let stand for 15 min at 40 ± 5°C. Proceed as in F(a).

F. Preparation of Test Portions and Solutions

Note: QC samples (certified reference materials, in-house reference samples, or spiked samples) must be regularly included and analyzed in duplicate.

If necessary, different sized glassware may be substituted for specific volumes listed during the preparation of test solutions as long as the proper dilution ratios are maintained.

(a) Saponification.—(1) Weigh 30.0 ± 0.1 g sample suspension, E(a) and E(b), into a 250 mL brown glass, flat-bottomed flask with a ground-glass neck. Note: This corresponds to 10.0 g dry test portion, m, or 30.0 ± 0.1 g liquid sample.

(2) Add antioxidant mix (1 g sodium sulfite, 1 g sodium ascorbate). Note: 1 g sodium ascorbate can be replaced with 0.5 g pyrogallol. Hydroquinone (0.5 g) can also be used as an antioxidant instead of sodium sulfite and sodium ascorbate mixture.

(3) Add 7 g potassium hydroxide puriss.

(4) Mix to dissolve.

(5) Add 50 mL absolute ethanol.

(6) Add a magnetic stir rod.

(7) Proceed as in F(a)(8) or F(a)(9).

(8) Hot saponification.—Mount on the flask an adapter for gas introduction and an Allihn condenser. Introduce a slight nitrogen stream. Reflux for 30 min at 85 ± 3°C while stirring in a water bath provided with magnetic stirrers.

(9) Overnight saponification.—Introduce a slight nitrogen stream for about 30 s in order to replace oxygen. Stopper the flask. Place the flask on a magnetic stirrer overnight at room temperature (25 ± 5°C). Note: Ensure thorough stirring of the reaction medium during saponification.

(b) Extraction.—(1) Cool the flask to room temperature.

(2) Transfer quantitatively into a 100 mL amber glass volumetric flask.

(3) Add 2 g sodium 1-pentanesulfonate and make up to the mark with water.

(4) Shake well for 1 min. Note: Sodium 1-pentanesulfonate is expensive and can be replaced by 1.5 g sodium dodecyl sulfate which is much cheaper and less soluble. In principle it is easier to use as aqueous solution (1.5 g sodium dodecyl sulfate in 3.5 mL water).

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Table 2011.07C. Recovery: Infant cereal

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Unit</th>
<th>Median</th>
<th>Normal</th>
<th>n</th>
<th>Consensus value</th>
<th>Uncertainty of consensus value</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A</td>
<td>μg/100 g</td>
<td>475</td>
<td>4.8</td>
<td>20</td>
<td>365</td>
<td>22</td>
<td>0.099</td>
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</tbody>
</table>

Note: Uncertainty of consensus value calculated as \(SD_{quad} = 1.2533^*SD_n/sqrt(n)\), where \(SD_n\) is the standard deviation of reproducibility obtained during proficiency testing and n is the number of participating laboratories.

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Table 2011.07D. Uncertainty: Milk-based infant formula

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Unit</th>
<th>Median</th>
<th>CV%</th>
<th>RSD (Rec.)</th>
<th>Standard uncertainty(a)</th>
<th>Expanded uncertainty(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A</td>
<td>μg/100 g</td>
<td>475</td>
<td>4.8</td>
<td>0.033</td>
<td>28</td>
<td>55</td>
</tr>
</tbody>
</table>

\(a\) Standard uncertainty: \(u = \text{Median}^* \sqrt{CV(\text{Rec})^2 + \text{RSD(Rec)}^2}\)

\(b\) Expanded uncertainty: \(U = 2 * u\), which gives a level of confidence of approximately 95%.
than 2% for retention time and peak response. Repeatable response, and retention time before starting a series curve at least three times and ensure the stability of the system, standards and samples. Make sure the system pressure is stable system to equilibrate for at least 15 min for UPLC before injecting See

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytical column</td>
<td>Acquity UPLC HILIC 1.7 μm, 2.1 × 100 mm</td>
</tr>
<tr>
<td>Column temperature</td>
<td>25°C</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>A: 1% 2-propanol in n-hexane</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.2 mL/min</td>
</tr>
<tr>
<td>Injection volume</td>
<td>4.00 μL</td>
</tr>
<tr>
<td>Injection mode</td>
<td>Partial loop (5 μL loop)</td>
</tr>
<tr>
<td>UV detection</td>
<td>326 nm</td>
</tr>
<tr>
<td>Data rate</td>
<td>2 pts/s</td>
</tr>
</tbody>
</table>

(5) Prepare cartridge by fitting a needle to the Luer connection at the lower end of the cartridge.

(6) Fix the latter by means of a clamp. Note: Too coarse granulometry of the filling material will reduce the extraction recovery. Particle size should not be larger than 0.4 mm diameter.

(7) Pipet 20 mL saponified solution on the top of the cartridge. Start again with 15 mL and another cartridge if the solution is not completely retained by the packing as the solution must be completely retained by the packing. Note: It is also possible to mix the Chromabond XTR filling material in a beaker with 20 mL hydrolysat with a glass rod and then transfer it into an empty cartridge. This improves the dispersion and absorption of the liquid throughout the filling material. In this case, it is not necessary to wait 15 min before starting the elution step.

(c) Internal control plan.—QC samples.—Include QC samples (certified, in-house reference samples, or spiked samples) in every series of analysis and analyze in duplicate. Spiking experiments.—Verify recovery rate by spiking samples. Calculate recovery rate (Rec.) on the samples using the following equation:

\[
\text{Rec.} = \frac{C_s - C_n}{C_s} \times 100
\]

where \( C_s \) = concentration of vitamin in the spiked test portion, \( C_n \) = concentration of vitamin in the nonsampled test portion, \( C_a \) = concentration of vitamin A added to the test portion.

Recovery rate should be higher than 90%.

(2) Operating procedure and determination.—(a) Sequence setup.—Set up (in duplicate) 4 μL of each of the working standard solutions [see D(c)(3)].Inject, sequentially, 4 μL of the reagent blank and test solutions. Include a working standard or a QC sample every 6–8 samples to monitor system stability.

(b) Calibration.—Calculate average of peak area (or height) and SD in the series of analysis. Construct a calibration curve by plotting the peak area (or height) of vitamin A (all-trans retinol) in each of the working standard solutions against concentration in micrograms per milliliter. Calculate the slope (S) and the intercept (I) by linear regression.

(c) Identification.—Identify the all-trans retinol, 13-cis retinol peak on the sample chromatogram by comparison with the retention time of the corresponding peak in the standard solutions (Figures 2011.07A and B).

(3) Calculations and results.—(a) All-trans retinol.—Calculate the mass fraction, \( w \), of all-trans retinol (in micrograms per 100 g sample) by using the following equation:

\[
w_{\text{all-trans retinol}} = \frac{(A_s - I) \times V_0 \times V_i \times 100}{S \times m \times V_i}
\]

where \( m \) = mass of the test portion, in g (10.0 for powders and 30.0 for liquids), \( A_s \) = area (or height) of the all-trans retinol peak in the sample chromatogram, \( S \) = slope of the calibration curve, \( I \) = intercept of the calibration curve, \( V_0 \) = volume in which the saponified solution has been diluted, in mL (100.0), \( V_i \) = aliquot of the saponified solution introduced in the cartridge, in mL (20.0 or 15.0 if the saponified solution is not

Figure 2011.07A. All-trans retinol working solution, about 1.8 μg/mL.
(b) 13 cis Retinol.—Calculate the mass fraction, w, of 13 cis retinol, in micrograms per 100 g sample, by using the following equation:

\[ w_{\text{13-cis retinol}} = \frac{(A_i - I) \times V_0 \times V_2 \times 1830}{S \times m \times V_1 \times 1680} \times 100 \]

where \( m \) = mass of the test portion, in g (10.0 for powders and 30.0 for liquids), \( A_i \) = area (or height) of the 13 cis retinol peak in the sample chromatogram, \( S \) = slope of the all trans retinol calibration curve, \( I \) = intercept of the all trans retinol calibration curve, \( V_0 \) = volume in which the saponified solution has been diluted, in mL (100.0), \( V_1 \) = aliquot of the saponified solution introduced in the cartridge, in mL (5.0), \( V_2 \) = final volume of the test solution, in mL (5.0), 1830 = absorbance coefficient E (1 cm, 1%), 1680 = absorbance coefficient E (1 cm, 1%) of 13 cis retinol.

(c) Expression of results for vitamin A.—Express vitamin A as the sum of all trans retinol and 13 cis retinol in µg/100 g.

\[ \text{Vitamin A, } \mu\text{g/100 g} = w_{\text{all-trans retinol}} + (0.75 \times w_{\text{13-cis retinol}}) \]

**G. Standard Preparation Procedure**

Preparation of all trans retinol standard from all trans retinyl acetate

1. Weigh 20 mg all trans retinyl acetate into a 25 mL tube with screw cap.
2. Add 5 mL ethanol.
3. Mix until dissolution is complete.
4. Add 2 mL 50% KOH aqueous solution and 0.1 g sodium ascorbate.
5. Heat in water bath at 85°C for 30 min.
6. Cool down to room temperature.
7. Add 5 mL water and 10 mL n-hexane.
8. Stopper the tube and shake vigorously for about 30 s.
9. Allow the phases to separate by letting the mixture stand.
10. Remove the lower aqueous layer with a Pasteur pipet.
11. Add 10 mL water.
12. Stopper the tube and shake vigorously.
13. Stopper the tube and shake vigorously.
14. Swirl and filter into a 100 mL amber glass volumetric flask.
15. Rinse the tube with two portions of about 10 mL n-hexane.
16. Dilute to the mark with n-hexane.


**Results and Discussion**

Because approval of this method was achieved under the alternative pathway, approved by the AOAC Board of Directors, the method will remain First Action for approximately 2 years while additional information is generated and laboratories have the opportunity to use the method.

The data provided below were generated during proficiency testing. Using a 10 g test portion, vitamin A can be accurately quantified (LOQ) at 30 µg/100 g.

Table 2011.07A presents a statistical review of the results obtained in the proficiency testing. Repeatability SDs [SD(r)], CVs [CV(r)], repeatability (r), and percent repeatability (r%) are presented. Reproducibility SDs [SD(R)], CVs [CV(R)], reproducibility (R), and percent reproducibility in µg/100 g R% are also presented.

Tables 2011.07B and C present recovery calculations based on the data received from laboratories. Also listed are the values for uncertainty, median results, percent recovery (Rec%), and SD of the recovery [SD(Rec)].

Table 2011.07D presents the uncertainty data. The calculations include the CV for intermediate reproducibility [CV(iR)], RSD of the recovery [RSD(Rec)], standard uncertainty (u), and expanded uncertainty (U).

Based on the results given, the recovery for vitamin A was over 100% for milk-based infant formula. Also notable is that the reproducibility was larger, showing higher between-laboratory than within-laboratory variability.

LOQ

LOQ is the limit at which the analyte can be detected.

**Precision**

In Table 2011.07A the repeatability and intermediate reproducibility (within-laboratory variability) were estimated by replicate analysis (n_i) on different days (n_d).

**Trueness/Recovery**

Analysis of internal reference samples.—Tables 2011.07B and C show results obtained from samples with consensus values obtained from internal proficiency testing. The data show recovery rates obtained during proficiency testing.

Spiking experiments.—The spiking experiments showed recovery in the range of 93–103% for retinol.
Measurement Uncertainty

Measurement uncertainty is estimated using the simplified approach based on existing validation data purposed by Barwick and Ellison (1). The studies were mainly precision and trueness which, if properly planned to cover as many of the uncertainty sources previously identified as possible, provide the necessary data required to calculate measurement uncertainty. Precision and trueness are combined together in Table 2011.07D to obtain the overall uncertainty. As seen in Table 2011.07D, the proficiency samples yield expanded uncertainly for retinol at 11.6% for milk-based infant formula.

References