
50.1.03 - Infant Formulas, Baby Foods, and Enteral Products

AOAC Official Method 992.06
Vitamin A (Retinol)
in Milk-Based Infant Formula

Liquid Chromatographic Method
First Action 1992

*Codex-Adopted-AOAC Method**

(Applicable milk-based infant formulas containing >500 IU vitamin A per reconstituted quart.)

Results of the interlaboratory study supporting the acceptance of the method (milk-based liquid, ready-to-feed):

Mean recovery = 2658 IU vitamin A/L infant formula
s_r = 129.6; s_R = 279.0; RSD_r = 4.9%; RSD_R = 10.5%

A. Principle

Vitamin A in test portion of infant formula is saponified, partitioned with organic solvent, separated from sample matrix, and quantified by liquid chromatography.

B. Apparatus

(**a**) *Liquid chromatograph (LC)*.—Capable of pressures up to 20 700 kPa (3000 psi), with injector capable of 100 µL injections. Operating conditions: eluent flow rate 1.5 ± 0.2 mL/min; temperature ambient.

(**b**) *Detector*.—Capable of measuring absorbance at 336 nm, with sensitivity 0.1 AUFS.

(**c**) *Column*.—4.6 mm id 15 cm stainless steel, packed with 5 µm silica-based cyano group stationary phase (Sepralyte CN, Analytichem International, Harbor City, CA, USA, is suitable).

(**d**) *Spectrophotometer*.—Capable of measuring absorbance at 325 nm.

(**e**) *Shaking water bath*.—Capable of maintaining 70 ± 2°C, variable speed capable of 60 oscillations/min, with test portion area ca 11 14 in. (28 36 cm) (Precision Scientific Model 25 is suitable).

(**f**) *Glassware*.—(*1*) 125 mL separatory funnels. (*2*) 5 mL volumetric flasks. (*3*) 100 mL low-actinic volumetric flasks.

C. Reagents

(**a**) *Mobile phase solution*.—Hexane-isopropyl alcohol (100 + 0.25, v/v), HPLC grade solvents. Degas 2-5 min under vacuum.

(**b**) *Wash solution*.—H_2O-absolute ethanol (3 + 2, v/v).

(**c**) *Extraction solution*.—Hexane-methylene chloride (3 + 1, v/v), HPLC grade solvents.

(**d**) *Saponification solution*.—10.5M potassium hydroxide (KOH). Dissolve 673 g KOH in 1 L H_2O.

(**e**) *Antioxidant solution*.—1% pyrogallol. Dissolve 5.0 g pyrogallol (1,3,5-trihydroxybenzene, 98%, Aldrich is suitable source) in 500 mL absolute ethanol.

(**f**) *Standard solutions*.—(*1*) *Stock standard solution*.—10 mg/mL retinyl palmitate in hexane. Quantitatively transfer equivalent of 0.1 g retinyl palmitate with hexane (HPLC grade) into 100 mL low-actinic volumetric flask, dilute to volume with hexane, and shake well to dissolve. Make fresh every 2 weeks. Store at -20°C in explosion-proof freezer when not in use.

(*2*) *Intermediate standard solution*.—Pipet 2 mL of stock standard solution, (*1*), into 250 mL volumetric flask and dilute to volume with hexane.

(*3*) *Working standard solution*.—Approximately 1.6 µg/mL retinyl palmitate. Pipet 2 mL intermediate standard solution, (*2*), into 100 mL low-actinic volumetric flask. Evaporate to dryness under nitrogen. Dissolve residue in antioxidant solution, **C**(**e**), and dilute to volume. Prepare fresh daily.

D. Extraction of Standards and Test Portions

Pipet 10.0 mL working standard solution, **C**(**f**)(*3*), or test portion containing ca 20 IU vitamin A activity (10 mL for ready-to-feed formulas) into 150 mL centrifuge tube. Bring test portion to 10 mL with H_2O, if necessary. To standard tubes, add 10 mL H_2O, 20 mL antioxidant solution, **C**(**e**), and 5 mL saponification solution, **C**(**d**). To test portion tubes, add 30 mL antioxidant solution and 5 mL saponification solution. Cap tubes and swirl briefly to mix. Place tubes in 70°C shaking H_2O bath (ca 60 oscillations/min) for 25 min. Remove tubes and place in ice 5 min, or until contents cool to room temperature.

Quantitatively transfer contents to separate 125 mL separatory funnels. Wash remaining test portion or standard from tube into funnel with 30 mL H_2O. Pipet 30.0 mL extraction solvent, **C**(**c**), into funnel and shake ca 2 min. When layers separate, discard aqueous (lower) layer. Add 30 mL wash solution, **C**(**b**), to funnel and shake very gently 30 s, venting frequently. Let phases separate and discard aqueous layer. Repeat wash step 3 . Pipet 20.0 mL portion from funnel to 50 mL tube and evaporate to dryness under nitrogen. Transfer residues quantitatively to separate 5 mL volumetric flasks and dilute to volume with mobile phase solution, **C**(**a**).

E. Determination of Standard Concentration

Pipet 2 mL of intermediate standard solution, **C**(**f**)(*2*), into 50 mL volumetric flask and dilute to volume with hexane. Transfer portion of this solution into 1 cm cell path length cuvet and measure absorbance at 325 nm. Calculate concentration of working standard solution, C_{std}, as follows:

$$C_{std} =$$

where A_{325} = absorbance of working standard solution at 325 nm; = 975, extinction coefficient of retinyl palmitate in hexane at 325 nm; and b = 1 cm, cell path length.

F. System Suitability Test

Inject 100 µL of saponified working standard solution into LC. Typical retention times for 13-*cis*-retinol and *trans*-retinol are 7.5 and 9.0 min, respectively. Calculate R factor between 13-*cis*-retinol and *trans*-retinol as follows:

$$R =$$

where t_1 and t_2 = retention time measured from injection time to elution time of peak maximum of 13-*cis*-retinol and *trans*-retinol, respectively, and W_1 and W_2 = width of peak measured by extrapolating relatively straight sides to baseline of 13-*cis*-retinol and *trans*-retinol, respectively.

If *R* factor is <1.3, increase amount of isopropyl alcohol added per liter [mobile phase solution, **C**(**a**)] by ca 0.05%. Inject saponified working standard solution 5 . Calculate reproducibility of replicate injections in terms of standard deviations (per USP), which should be 2%. Typical relative standard deviation values for peak height are ±1.3%.

G. Liquid Chromatography

Inject 100 µL standard or test solution into LC.

H. Calculations

Since 13-*cis* vitamin A palmitate is not readily available, standard curve for all-*trans* vitamin A palmitate is used to determine biological potencies for both, correcting for 13-*cis* vitamin A palmitate, at 0.75 potency relative to all-*trans* vitamin A palmitate. This is based on assumption that relative molar absorptivities of both isomers are virtually equal at 336 nm.

Measure peak areas of *cis*- and *trans*-isomers of retinol in both test solution and standard chromatograms. Multiply peak area under 13-*cis* vitamin A palmitate curve by 0.75. Sum the 2 areas to represent total peak area. Calculate IU per reconstituted quart of vitamin A activity (*V*) as follows:

$$V =$$

where A_{sample} = total peak area of test solution; $A_{standard}$ = total peak area of standard; $C_{standard}$ = concentration of working standard solution, µg/mL; 1/0.55 = IU/µg retinyl palmitate; and 946.33 = mL/quart.

Reference:
J. AOAC Int. **76**, 399(1993).

CAS-68-26-8 (vitamin A)

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* Adopted as a Codex Reference Method (Type II) for liquid chromatography of vitamin A (retinol) in infant formula and follow-up formula.