50.1.04 - Infant Formulas, Baby Foods, and Enteral Products

AOAC Official Method 992.03
Vitamin E Activity (All-*rac*- -Tocopherol) in Milk-Based Infant Formula

Liquid Chromatographic Method
First Action 1992
Final Action 1996

*Codex-Adopted-AOAC Method**

(Applicable to determination of vitamin E activity in milk-based infant formula.)

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

Mean recovery = 24.14 IU vitamin E/L infant formula
s_r = 2.04; s_R = 2.82; RSD_r = 8.46%; RSD_R = 11.69%

A. Principle

Vitamin E activity in test portions of infant formula is determined by saponification of all-*rac*- -tocopherol, partitioning with organic solvent, separation from product matrix, and quantification by liquid chromatography.

B. Apparatus

(**a**) *Liquid chromatograph (LC)*.—Capable of pressures up to 3000 psi with injector capable of 100 µL injections. Operating conditions: eluent flow rate 2.0 ± 0.2 mL/min; temperature ambient.

(**b**) *Detector*.—Capable of measuring absorbance at 280 nm, with sensitivity 0.02 AUFS.

(**c**) *Precolumn*.—2 mm id 2 cm stainless steel, packed with 40 µm pellicular reversed-phase C_{18} (Alltech 28551 is suitable).

(**d**) *Column*.—4.6 mm id 25 cm stainless steel, packed with 5 µm silica (Hypersil Silica is suitable).

(**e**) *Shaking water bath*.—Capable of maintaining 70 ± 2°C, with variable speed capable of 60 oscillations/min, ca 11 14 in. (28 36 cm) test sample area (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 (99.92 + 0.08, 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*.—0.5 mg/mL all-*rac*- -tocopheryl acetate in hexane. Accurately weigh ca 50 mg all-*rac*- -tocopheryl acetate (USP Reference Standard) into 100 mL low-actinic volumetric flask and dilute to volume with hexane (HPLC grade). Shake well to dissolve. Make fresh every 3 weeks. Store at -20°C in explosion-proof freezer when not in use.

(*2*) *Working standard solution*.—10 µg/mL all-*rac*- -tocopheryl acetate. Pipet 2 mL stock standard solution, (*1*), 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.

(**g**) *Suitability test solution*.—Approximately 15 µg/mL all-*rac*- -tocopherol (USP Reference Standard) and all-*rac*- -tocopheryl acetate (USP Reference Standard) in hexane (HPLC grade).

D. Extraction of Standard and Test Portions

Pipet 10.0 mL working standard solution, **C**(**f**)(*2*), or test portion containing ca 0.095 IU vitamin E activity (10 mL for ready-to-feed formulas) into 150 mL centrifuge tube. Bring test solution volume 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 sample 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 solution 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 into 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**). Inject 100 µL standard or sample into LC.

E. System Suitability Test

Inject 100 µL suitability test solution, **C**(**g**), into LC. Typical peak retention times for tocopherol and tocopheryl acetate are 6.0 and 5.0 min, respectively. Calculate resolution (*R*) factor between tocopherol and tocopheryl acetate as follows:

$$R =$$

where t_1 and t_2 = retention time measured from injection time to elution time of peak maximum of tocopherol and tocopheryl acetate, respectively, and W_1 and W_2 = width of peak measured by extrapolating relatively straight sides to baseline of alcohol and acetate, respectively.

If *R* factor is >1.0, proceed with test portion analysis; if *R* factor is <1.0, decrease amount of isopropyl alcohol added per liter [mobile phase solution, **C**(**a**)] by ca 0.01%. Inject working standard solution, **C**(**f**)(*2*), 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 ±1.5%.

F. Liquid Chromatography

Inject 100 µL standard or test solution into LC.

G. Calculations

Measure peak heights or peak areas of all-*rac*- -tocopherol in test solution and standard chromatograms. Calculate IU per reconstituted quart of vitamin E activity (A) as follows:

$$A = H_{sam}/H_{std} \quad C_{std} \quad 0.001 \text{ IU}/\mu\text{g} \quad 946.33 \text{ mL/quart}$$

where H_{sam} = peak height of test portion solution; H_{std} = peak height of standard; C_{std} = concentration of standard, µg/mL.

Reference:
J. AOAC Int. **76**, 398(1993).

CAS-59-02-9 (-tocopherol)
CAS-7695-91-2 (-tocopheryl acetate)

Revised: March 1997

* Adopted as a Codex Reference Method (Type II) for liquid chromatography of vitamin E (milk based infant formula) in special foods.