

50.1.25

AOAC Official Method 999.15 Vitamin K in Milk and Infant Formulas Liquid Chromatographic Method First Action 1999

(Applicable to the determination of total vitamin K₁ [phyloquinone] in infant formula and milk [fluid, ready-to-feed, and powdered] containing >1 µg vitamin K₁/100 g solids.)

See Table 999.15 for the results of the interlaboratory study supporting the acceptance of the method.

Caution: Avoid skin contact with zinc dust.

A. Principle

Following enzymatic digestion of fat and precipitation of fatty acids, vitamin K is extracted with hexane. Vitamin K is separated by HPLC with post-column reduction and quantitated by fluorescence detection with an external standard.

Phylloquinone geometric isomers are quantitated either as a single unresolved peak with a C₁₈, or may be estimated selectively with a C₃₀ column ($k'_{trans} < k'_{cis}$), under the reversed-phase conditions employed.

(Note: Menaquinone-4 [MK-4] will also be present in milk and infant formulas incorporating milk fat, while 2',3'-dihydrophyloquinone may be present at appreciable levels in infant formulas containing hydrogenated soya or canola oils. If required, both may be estimated against appropriate standards. The order of elution is MK-4 < K₁ < 2',3'-dihydroK₁ [relative retention times ca 0.6, 1.0, and 1.2, respectively].)

B. Apparatus

(a) **HPLC system.**—Manual or automated isocratic system incorporating a fluorescence detector (λ_{ex} 243 nm, λ_{em} 430 nm) exhibiting a Raman spectra signal:noise specification of >200:1 (water). Since UV detection is insensitive to vitamin K at the working concentration levels, configure with single-mode fluorescence detection only to minimize extra-column band-broadening. For multi-detector LCs, configure with the fluorescence detector in the first position. Ensure the absence of leaks.

(b) **Column.**—Any C₁₈ column (monomeric or polymeric) containing 5 µm spherical particle silica with ≥10% carbon loading, preceded by a guard column of similar packing.

(c) **Post-column reductor.**—20 × 4 mm stainless steel post-column assembly placed between analytical column and fluorescence detector (Part No. WAT084550, Waters, or equivalent). Alternative devices, such as an empty (30–50 mm) LC column can be used.

(d) **Spectrophotometer.**—UV/Vis spectrophotometer, digital readout to 0.001 A.

(e) **Waterbath.**—37 ± 1°C.

(f) **Centrifuge.**—Fixed or swing-arm rotor.

(g) **Mechanical shaker.**—Orbital or wrist-action.

(h) **Vortex mixer.**

(i) **pH meter.**—Accurate to 0.01 pH, with calibration buffers.

(j) **Test tubes.**—200 × 24 mm with ground-glass stoppers.

(k) **Vials.**—Amber-tinted glass vials (1.8 and 8.0 mL), with Teflon-sealed caps.

(l) **Volumetric flasks.**—Low-actinic volumetric flasks, 100 mL.

C. Reagents

Use AR grade reagents unless otherwise stated.

(a) **Water.**—Purified to >18 MΩ resistivity.

(b) **Nitrogen.**—Cylinder N (low O content).

(c) **Lipase.**—From *Candida rugosa*, ca 1000 units/mg (Type VII, Sigma L-1754). Other sources of lipase can be used from *Pseudomonas* and *Rhizopus* species with the correct activity of enzyme.

(d) **Monobasic potassium phosphate (KH₂PO₄).**

(e) **Potassium hydroxide solution.**—40% (w/v). Dissolve 40 g KOH, (j), in water and dilute to 100 mL.

(f) **Phosphate buffer.**—0.8M. Dissolve 54.0 g KH₂PO₄, (d), in 350 mL water. Adjust pH to 7.9–8.0 with 40% KOH solution and dilute to 500 mL.

(g) **Ethanol.**

(h) **Methanol.**—LC grade.

(i) **Reagent alcohol.**—Mix ethanol, (g), with methanol, (h), (95 + 5, v/v).

(j) **Potassium hydroxide.**

(k) **Zinc chloride.**

(l) **Sodium acetate.**—Anhydrous.

Table 999.15 Interlaboratory study results for vitamin K₁ collaborative data

Parameter	Sample ^a							
	1	2	3	4	5	6	7	8
No. of labs ^b	31	28	33	33	33	33	32	33
No. of outliers	1	5	0	0	0	0	1	0
Mean, µg/100 g	0.49	6.63	118.07	32.24	78.69	49.64	90.94	94.62
s _r , µg/100 g	0.04	0.21	5.00	1.54	2.04	2.54	4.04	5.38
RSD _r , %	9.03	3.23	4.24	4.77	2.59	5.11	4.44	5.68
2.8 s _r , µg/100 g	0.12	0.60	14.01	4.31	5.71	7.11	11.32	15.05
s _R , µg/100 g	0.05	0.39	6.50	2.14	3.40	3.80	4.14	6.41
RSD _R , %	10.94	5.81	5.50	6.63	4.33	7.66	4.56	6.78
2.8 s _R , µg/100 g	0.15	1.08	18.19	5.98	9.53	10.65	11.60	17.95

^a Samples 1 and 2 = unfortified, whole liquid UHT milk and goat milk powder, respectively; 3 = milk-based infant formula, oil-filled; 4 and 7 = whey-based infant formula, partially oil-filled; 5 = soy-based infant formula, oil-filled; 6 = whey-based infant formula, oil-filled; 8 = NIST SRM 1846.

(m) *Acetic acid*.—Glacial.
 (n) *Potassium carbonate*.—Anhydrous.
 (o) *Zinc powder*.—<60 μm, e.g., Merck 108774 and BDH 102964L. Use freshly opened bottle.
 (p) *Hexane*.—HPLC grade.
 (q) *Dichloromethane*.—HPLC grade.
 (r) *Isopropanol*.—HPLC grade.
 (s) *Mobile phase*.—To dichloromethane, (q),-methanol, (h), (100 + 900, v/v), add 5 mL methanol containing ZnCl₂ (1.37 g; k), anhydrous sodium acetate (0.41 g; l), and glacial acetic acid (0.30 g; m). Filter through 0.45 μm filter and degas.
 (t) *Zinc post-column reductor*.—Dry-pack the 20 × 4 mm assembly B(c), with Zn powder, (o). Minimize voids by sequentially adding small amounts with frequent gentle tapping. With longer columns, use extra care. Re-prepare reductor column before each analytical schedule. Reduction efficiency of Zn powder should be monitored over time and replaced every 1–2 years as required.
 (u) *Vitamin K₁ (phylloquinone)*.—USP grade. Conduct all operations must be conducted under low incandescent light conditions and in low-actinic volumetric flasks. (1) *Stock solution*.—ca 1.0 mg/mL. Dissolve ca 100 mg, weighed accurately, in 100.0 mL isopropanol (r) with warming at ca 30°C and standing. Store under N, (b), at –10°C for up to 6 months. (2) *Intermediate I*.—ca 50 μg/mL. Dilute 5.0 mL stock with methanol to 100.0 mL and store under N at –10°C for up to 1 month. (3) *Intermediate II*.—ca 2.5 μg/mL. Dilute 5.0 mL Intermediate I with methanol to 100.0 mL. Prepare daily. (4) *Working standards*.—ca 6.25, 12.50, 18.75, 25.00, and 31.25 ng/mL. Dilute 0.25, 0.50, 0.75, 1.00, and 1.25 mL, respectively, Intermediate II with methanol, (h), to 100.0 mL. Prepare daily and calculate accurate concentrations as follows: Accurate concentrations for working standards are calculated after estimation of phylloquinone purity from absorbance (248 nm) of a solution prepared by evaporating 5.00 mL Intermediate I under N flow and re-dissolving in 25.0 mL hexane (a^{1%} = 419):

$$\text{Theoretical } A_{248} = \frac{W}{100} \times \frac{5}{100} \times \frac{5}{25} \times 419 \times 100$$

where W = weight of phylloquinone in stock standard (g)
 Purity factor (PF) = measured A₂₄₈/theoretical A₂₄₈.

$$K_1 \text{ in Intermediate II } (\mu\text{g/mL}) = \text{PF} \times \frac{W}{100} \times \frac{5}{100} \times 5 \times \frac{106}{100}$$

Accurate vitamin K₁ concentrations in the 5 working standards (ng/mL) are then calculated by multiplying PF by 2.5, 5.0, 7.5, 10.0, and 12.5, respectively.

D. Procedure

Perform all steps of assay under incandescent lighting in the absence of direct sunlight.

(a) *Preparation of test solution*.—(1) *Digestion*.—Weigh 1.0 g powder or 10.0 g liquid into test tube. Include both a reagent blank and a quality control test portion in each analytical schedule. Dissolve powder in ca 15 mL warm water (<40°C) with Vortex mixer (for liquids, add 5 mL warm water). Add 5.0 mL phosphate buffer to each solution and mix. Add 1.0 g lipase powder and Vortex mix. Stopper securely and shake until well dispersed (30–60 s). Incubate at 37 ± 2°C for 2 h. Shake each tube for 15 s at 20 min intervals. Cool

to ambient temperature by standing in water. Add 10 mL reagent alcohol and mix. Add 1.0 g K₂CO₃ and mix.

(2) *Extraction*.—(Note: Take precautions to prevent concentration of extracts through uncontrolled solvent evaporation.) Add 30.0 mL hexane, stopper, and shake vigorously for ≥10 min using mechanical shaker. Let stand in the dark, preferably refrigerated, until layers separate. If this is slow (>10 min) or incomplete, centrifuge at ca 1000 rpm (ca 200 g) for 10 min (decant a portion of supernatant into smaller centrifuge tube if necessary). Transfer 1.00 mL (fortified products) or 5.00 mL (nonfortified products) of upper hexane phase into vial. Evaporate under N flow to dryness. (Note: larger extract volumes may be required, with the option of rotary evaporation, dependent on the sensitivity of the fluorescence detector.) These dried extracts may be held for 3–5 days in the dark at <0°C under N prior to LC analysis. Re-dissolve residue in 1.00 mL methanol. Seal vial and Vortex mix. If using autosampler, use a low volume vial (or insert) to minimize evaporation into the headspace.

(b) *HPLC determination*.—(1) *Set-up*.—Prior to connection of post-column Zn reductor assembly, establish stable operating LC conditions over ca 30 min through equilibration of column with mobile phase (ca 1 mL/min) and set fluorescence detector to λ_{ex} 243 nm and λ_{em} 430 nm (gain and sensitivity will be detector dependent). Install Zn reductor assembly between analytical column and detector and equilibrate system for a further 30–60 min, ensuring the absence of leaks (anhydrous LC eluent conditions are required to minimize loss of reducing potential of Zn). Do not recycle mobile phase. Inject a standard (20–50 μL) and set flow rate in order to elute phylloquinone between 8–15 min, at typically 1.0–1.5 mL/min (retention may also be manipulated by modification of dichloromethane content in eluent between 8–12%). Allow total run time of ca 5 min beyond elution of K₁.

(2) *Calibration and analyses*.—Inject methanol (standard blank) and confirm absence of chromatographic activity at retention time for phylloquinone. Inject lowest level working standard and ensure repeatable response, with signal:noise ≥10. Inject each working standard and ensure linear response. Sequentially inject reagent blank and test extracts and include a working standard after every 4–6 test solutions to monitor system stability. When analytical schedule is complete, flush system with methanol. Store analytical column in methanol when not in use. Dismantle and empty post-column assembly, soak in 5M HCl to clean frits, and rinse thoroughly with water and methanol. Dry thoroughly before repacking with Zn.

(c) *Calculation*.—Construct a 5-level calibration using either peak area or height and calculate slope (S) by linear regression with forced zero. Calculate vitamin K₁ (cis- and trans-) content in test samples as follows:

$$\text{Vitamin } K_1 (\mu\text{g}/100 \text{ g}) = \frac{A'}{S} \times \frac{30}{V} \times \frac{100}{Wt} \times \frac{1}{1000}$$

where A' = peak area (or height) of phylloquinone in test extract; Wt = weight of test portion (g); V = volume, 1.0 mL (fortified) or 5.0 mL (nonfortified); S = slope of calibration graph.

Calibration and calculations may be achieved through data processing within the instrument or off-line.

Reference: *J. AOAC Int.* **83**, 121(2000).