

50.1.24

AOAC Official Method 999.14
Choline in Infant Formula and Milk
Enzymatic Colorimetric Method
First Action 1999

(Method is applicable to the determination of choline in milk and infant formula containing 45–175 mg solids/100 g. Method does not apply to powdered infant formula/milk containing more than 100 mg vitamin C/100 g solids because of ascorbate suppression of color development.)

See Table 999.14 for the results of the interlaboratory study supporting the acceptance of the method.

A. Principle

The product is acid digested at 70°C, to release most of the bound choline. Following pH adjustment, residual choline phospholipids are cleaved with phospholipase D and free choline is subjected to choline oxidase with liberation of H₂O₂. In the presence of peroxidase, phenol is oxidized and a quinoneimine chromophore is formed with 4-aminoantipyrine. Absorbance is measured at 505 nm and choline content calculated by interpolation from a multilevel calibration. For this procedure, choline is defined and reported as the hydroxide.

B. Apparatus

(a) *Spectrophotometer*.—Digital readout to 0.001 absorbance at 505 nm. Glass cuvettes (10 mm) or flow-through cell.

(b) *Covered water baths*.—37 ± 2°C and 70 ± 4°C.

(c) *pH meter*.—Reading to 0.01 unit, with calibration buffers, 4.0 and 7.0.

(d) *Pipets*.—Calibrated glass, or preferably auto-pipetors for 0.100 and 3.00 mL.

(e) *Volumetric flasks*.—10, 50, and 100 mL and 1 L.

(f) *Digestion vessels*.—Conical flasks, 100 or 150 mL, with stoppers. Alternatively, boiling tubes (dimensions ca 150 × 25 mm) with stoppers can be used, with suitable rack. Either vessel should permit insertion of a pH probe for post-digestion pH adjustment.

(g) *Test tubes*.—10 mL with glass stoppers, with rack. Alternatively disposable polypropylene or polystyrene plastic tubes fitted with screw-capped lids may be used.

(h) *Filter paper*.—Medium speed, porosity 1.4–2.9 microns, Whatman No. 2, or equivalent.

C. Reagents

If substituting enzymes from another source than that specified, ensure that the declared activity (units/mg) are similar. Store enzymes and 4-aminoantipyrine at 4°C or as instructed by the supplier.

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

(b) *Hydrochloric acid*.—1.0M. Measure 85 mL concentrated HCl into 1 L volumetric flask and dilute to the mark with water. Standardization is not required.

(c) *Sodium hydroxide solution*.—50% w/v. Dissolve 50 g NaOH in ca 80 mL water in graduated cylinder with cooling, and dilute to 100 mL.

(d) *Choline bitartrate*.—Sigma C-2654 or equivalent. Dry ca 0.7–1.0 g choline bitartrate at 102°C to constant weight. Store desiccated at ambient temperature. (1) *Stock choline standard*.—2500 µg/mL as choline hydroxide. Add 523 mg dry choline bitartrate to 100 mL volumetric flask. Dissolve in water and dilute to the mark. Store refrigerated at 4 ± 2°C. Do not use after 1 week. (2) *Working choline standard*.—250 µg/mL as choline hydroxide. Pipet 10 mL stock solution, (1), and dilute to 100 mL with water. Prepare fresh daily.

(e) *Tris (hydroxymethyl)aminomethane (Trizma) buffer*.—0.05 M (pH 8.0). Add 6.057 g tris (hydroxymethyl)aminomethane (Sigma T-1503 or equivalent) to 500 mL water in 1 L volumetric flask. Bring pH to 8.0 with 1M HCl, (b), and dilute to the mark with water. Store at 4°C. Solution is stable for 1 month.

(f) *Phospholipase D*.—Sigma Type VI, P-8023, from *Streptomyces chromofuscus*, 150 units/mg. Unit definition: One unit will liberate 1.0 µmol choline from L-α-phosphatidyl choline (egg yolk) per hour at pH 5.0 at 30°C. This form of phospholipase D is preferred over others due to its activity for sphingomyelins and lysophospholipids.

(g) *Choline oxidase*.—Sigma, C-5896, from *Alcaligenes species*, 10 units/mg. Unit definition: One unit will form 1.0 µmol H₂O₂ with oxidation of 1.0 µmol choline to betaine aldehyde per minute at pH 8.0 at 37°C.

Table 999.14 Interlaboratory study results for choline in milk and infant formula

Parameter ^a	Sample ^b							
	1	2	3	4	5	6	7	8
No. of labs	28	29	29	29	29	28	29	26
Average, mg/100 g	87.66	73.22	173.02	47.13	99.71	132.68	109.39	124.94
s _r , mg/100 g	3.03	2.03	5.95	1.52	2.23	3.07	2.01	3.16
RSD _r , %	3.46	2.77	3.44	3.23	2.24	2.32	1.84	2.53
2.8s _r , mg/100 g	8.49	5.68	16.66	4.26	6.25	8.60	5.62	8.86
s _R , mg/100 g	4.55	4.19	8.04	2.99	5.26	5.85	4.66	6.54
RSD _R , %	5.19	5.73	4.64	6.34	5.27	4.41	4.26	5.23
2.8s _R , mg/100 g	12.75	11.74	22.50	8.37	14.72	16.39	13.06	18.31

^a 1, 3, 4 = Milk/whey-based infant formulas; 2 = milk-based follow on; 5 = soy-based infant formula; 6 = NIST SRM 1846; 7 and 8 = whole milks. Samples are variously vitaminized, oil-filled, lecithinated, and supplemented with choline chloride.

^b All values excluding invalid data; no outliers detected.

(h) *Peroxidase*.—Sigma Type I, P-8125, from horseradish, 80 units/mg. Unit definition: One unit will form 1.0 mg purpurogallin from pyrogallol in 20 s at pH 6.0 at 20°C.

(i) *4-Aminoantipyrine*.—Sigma A4382 or equivalent.

(j) *Phenol*.

(k) *Chromogenic reagent*.—Into a 100 mL volumetric flask, sequentially weigh: 75–100 units phospholipase, (f); 100–120 units choline oxidase, (g); 250–280 units peroxidase, (h); 15 mg 4-aminoantipyrine, (i); and 50 mg phenol, (j). Dissolve and dilute to volume with 0.05M Trizma buffer, (e), (pH 8.0). Prepare fresh daily.

D. Protocol

Perform all steps of assay under incandescent lighting; avoid fluorescent lighting or direct sunlight.

(a) *Hydrolysis*.—Weigh 5.00 g test portion into 100 mL conical flask (or boiling tube). Add 30 mL 1.0M HCl, C(b), stopper, and mix by shaking until well dispersed. Place flasks into water bath at 70°C for 3 h, shaking occasionally. Loosen or remove stoppers occasionally to avoid excessive pressure buildup. Cool to ambient temperature, adjust pH to 3.5–4.0 with 50% NaOH, C(c), and quantitatively transfer discolored solution to 50 mL volumetric flask. Dilute to the mark with water, filter through filter paper, and collect filtrate after discarding the first 5–10 mL. Ensure filtrate is free of particulates; otherwise refilter. Filtrate is stable and may be stored in the dark at 4°C for up to 3 days. Return filtrate to ambient temperature before proceeding with assay.

For each test solution, separately label two 10 mL test tubes as test solution (tube 1) and blank (tube 2). Into each tube, dispense a 0.100 mL aliquot of filtrate.

(b) *Preparation of standard curve*.—Pipet 2, 4, 6, and 8 mL aliquots of working standard, C(d)(2), (250 µg/mL) into four 10 mL volumetric flasks and dilute to the mark with water. The fifth level calibrant is the undiluted working standard.

Prepare a 5-level standard curve (50, 100, 150, 200, and 250 µg/mL) by dispensing 0.100 mL of each standard into separately labeled 10 mL test tubes. A reagent blank tube (0 µg/mL) is also included by substituting 0.100 mL water in place of choline solution.

(c) *Enzymatic determination*.—To each blank (tube 2), add 3.00 mL water. Add 3.00 mL chromogenic reagent, C(k), to each test solution (tube 1), the 5 standards, and the reagent blank.

Place test tubes in a covered water bath at $37 \pm 2^\circ\text{C}$ for 15 min to develop a pink/red color. Remove from water bath and cool to ambient temperature for 15 min. Without delay, transfer contents of each tube into 10 mm optical cells, or use a flow-through cuvette.

Zero the spectrophotometer at 505 nm against water and measure the absorbance of the standards (A_{std}), test solutions (A'), blanks (A_{bl}), and reagent blank (A_{reag}).

(d) *Calculation*.—Create a standard calibration of net absorbance ($A_{\text{std}} - A_{\text{reag}}$) versus choline concentration (µg/mL) using linear least mean squares regression analysis with forced origin.

Subtract the absorbance of the blanks and reagent blank from each test solution to eliminate nonspecific spectral background:

$$\text{Net absorbance of test solution, } A = A' - A_{\text{bl}} - A_{\text{reag}}$$

Determine the choline concentration in test solution, expressed as choline hydroxide:

$$\text{Choline hydroxide, mg/100 g} = \frac{A}{S} \times \frac{V}{1000} \times \frac{100}{W}$$

where A = net absorbance of test solution; S = slope of standard curve; V = volume in mL of the hydrolysates (50 mL); W = weight in grams of test portion (5.00 g); 100 = conversion to 100 g basis; 1000 = conversion µg to mg. Report data to one decimal place.

Reference: *J. AOAC Int.* **83**, 131(2000).