AOAC Official Method 974.38
Clostridium perfringens in Foods
α-Toxin Estimation Method
First Action 1974
Final Action 1979

(Applicable to examination of outbreak foods in which presence of large numbers of vegetative cells are suspected but which may no longer be viable.)

A. Apparatus

(**a**) *Centrifuge.*—High-speed, preferably refrigerated, with 250 mL bottles.

(**b**) *Seitz filter.*—100–250 mL with sterilizing filter pads.

(**c**) *High-speed blender.*—With blending vessels.

(**d**) *Vacuum flask.*—Sidearm 1 L Erlenmeyer fitted with 1-hole rubber stopper to receive 200 mm glass tubing with 125 cm of 6 mm od (3 mm id) rubber tubing attached.

(**e**) *Tubing.*—Stainless steel thin wall (No. 9 surgical), 3 (od) × 180 mm (Tubesales, 175 Tubeway St, Forrest Park, GA 30297, USA).

(**f**) *Dialysis tubing.*—1.21 in. (30.7 mm) flat width (Fisher Scientific Co., 15 Jet View Dr, Rochester, NY 14624, USA).

B. Reagents

(**a**) *N-2-Hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES) buffer solution.*—Dissolve 6.0 g HEPES (Calbiochem Novabiochem Corp., No. 391338) and 11.7 g NaCl in 500 mL H_2O. Adjust to pH 8.0 with 3M NaOH and store at 4°C.

(**b**) *Lecithovitellin solution.*—Mix one egg yolk with 250 mL saline solution, (**e**), and clarify by centrifuging 20 min at 14 000 × *g* at 4°C. Filter-sterilize supernate with Seitz filter and store at 4°C.

(**c**) *Saline agar base.*—Add 15.0 g purified agar (Difco Laboratories [No. 0142]) and 8.5 g NaCl to 1 L H_2O. Adjust to pH 7.0, heat to dissolve agar, dispense in 100 mL portions, and autoclave 15 min at 121°C.

(**d**) *Washed red blood cells.*—Wash packed human red blood cells 3 times by mixing with 4 volumes saline solution, (**e**). Centrifuge 10 min at low speed (2500 rpm) to sediment cells. Remove supernate with vacuum flask. Resuspend cells in additional saline solution and repeat these steps twice. After final wash, mix cells with equal volume saline solution. Use sterile precautions.

(**e**) *Sterile saline solution.*—Dissolve 8.5 g NaCl in 1 L H_2O. Adjust to pH 7.0, dispense 250 mL portions into Pyrex containers, and autoclave 15 min at 121°C.

(**f**) *Polyethylene glycol solution.*—30%. Dissolve 120 g polyethylene glycol (Carbowax Compound 20M, Union Carbide Corp., PO Box 8361, S Charleston, WV 25303, USA) in 400 mL H_2O.

(**g**) *Antiserum.*—*Clostridium perfringens* Type A diagnostic serum.

C. Preparation of Hemolysin Plates

Melt 100 mL saline agar base, **B**(**c**), cool to 50°C, and add 11 mL washed red cells, **B**(**d**). Mix thoroughly and dispense 7 mL into 15 × 100 mm sterile plastic Petri dishes. Dry plates overnight at room temperature and store at 4°C. Just before use, cut test wells by applying vacuum to sterile stainless steel tube, **A**(**e**), and plunging tube into agar. Using template, space 9 test wells 3 cm apart and 2 cm from edge, and place 2 additional wells 3 cm apart near center of plate.

D. Toxin Extraction

Homogenize 25 g food (do not include fat) in 100 mL HEPES buffer solution, **B**(**a**), 1 min in high-speed blender. Centrifuge homogenate 20 min at 14 000–20 000 × *g* at 5°C. Filter supernate through Whatman No. 31 paper, or equivalent, to remove fat (chill extract centrifuged without refrigeration 1 h at 4°C before filtering). Discard solids. Rinse Seitz filter pad with 15 mL saline solution. Discard saline solution and filter-sterilize extract, rinsing filter pad with 10 mL saline solution.

E. Concentration

Soak 90 cm dialysis tubing 1 h in H_2O. Tie one end and fill with saline solution. Check for leaks and rinse out twice with saline solution. Transfer sterile extract to dialysis sack and concentrate to <10 mL by dialyzing 4–5 h against 400 mL 30% polyethylene glycol, **B**(**f**), at 4°C. Rinse outside of sack with tap H_2O and collect concentrated extract in sterile tube.

F. Toxin Testing

Adjust volume of concentrated extract to 10 ± 0.5 mL with saline solution. Set up 10 sterile 13 × 100 mm test tubes and add 0.5 mL saline solution to all tubes except first and last. Add 0.5 mL extract to first and second tubes. Mix extract and saline solution in second tube and transfer 0.5 mL to third tube, etc., to serially dilute extract from 0 to 1 + 255. Change pipet after 3 dilutions to prevent excessive carry-over. Mix 0.25 mL extract, 0.25 mL saline solution, and 0.1 mL antiserum, **B**(**g**), in last tube. Fill one peripheral well of duplicate hemolysin plates with each dilution of extract, using fine-tipped Pasteur pipet. Fill one center well of each plate with extract–antiserum mixture and the other with saline solution. Add 0.5 mL lecithovitellin solution, **B**(**b**), to remainder of diluted extract in each tube, including extract–antiserum mixture. Mix well and incubate tubes and plates (in plastic bag) 24 h at 35°C.

G. α-Toxin Titer

After incubation, refrigerate plates 2 h at 4°C. Measure hemolytic zone (width from edge of well in mm). Last 3 dilutions before end point should exhibit ca 1 mm reduction in width for each 2-fold dilution. If not, repeat α-toxin test. Hemolytic zone 1 mm in width is end point of titration.

Table 974.38 Correlation between population levels of *C. perfringens* and amount α-toxin produced in food[a]

α–Toxin titer[b]		Estimated *C. perfringens* population/g
HI plate	LV test	× 10^6
Undiluted	Undiluted	1.2
1 + 1		2.5
1 + 3	1 + 1	6.5
1 + 7	1 + 3	9.5
1 + 15	1 + 7	25
1 + 31	1 + 15	55
1 + 63	1 + 31	80
1 + 127	1 + 127	150
1 + 255	1 + 255	210

[a] Based on viable counts obtained with 6 strains in chicken broth.

[b] Dilution which produces 1 mm zone of hemolysis in HI plate or one + reaction in LV test.

Examine extract–lecithovitellin mixture in tubes for lecithinase activity and record results. Maximum reaction (+ + + +) is white pellicle 4–5 mm thick over clear liquid. Activity decreases with dilution to (+) reaction (opaque solution with no pellicle). This dilution is end point of lecithovitellin test. Hemolytic and lecithinase activities neutralized by antiserum are due to α-toxin.

H. Population Estimate

Compare titer of α-toxin present in extract with data in Table **974.38** to estimate population of *C. perfringens*. Hemolysin plate titer is preferred for this because lecithovitellin test is less sensitive with some food extracts.

Reference: *JAOAC* **57**, 91(1974).