

16.16.11

AOAC Official Method 993.27 Mammalian Feces in Ground Black Pepper Alkaline Phosphatase Detection Method First Action 1993 Final Action 1996

(Applicable to determination of mammalian feces in ground black pepper.)

Results of the interlaboratory study supporting acceptance of the method:

Black pepper, 10 particle spike/10 g pepper: Mean recovery = 122.5%; s_r = 3.8; s_R = 4.3; RSD_r = 30.7%; RSD_R = 34.8%

Black pepper, 20 particle spike/10 g pepper: Mean recovery = 120.3%; s_r = 5.4; s_R = 7.2; RSD_r = 22.5%; RSD_R = 29.9%

A. Principle

Most mammalian intestinal tracts contain alkaline phosphatase (AKP). At test conditions, pH 9.5 and 42°C, AKP hydrolyzes phosphate radicals from substrate/pH indicator phenolphthalein diphosphate to produce light pink to red-purple color from free phenolphthalein.

B. Apparatus

(a) *Water bath*.—Maintaining 42 ± 1°C.

(b) *Hot plate/stirrer and stir bars*.—See 945.75B(n) (see 16.1.01); use 41 mm ovoid stir bars or equivalent.

(c) *Petri plates*.—Disposable plastic, or glass; 150 × 20 or 150 × 15 mm.

(d) *Weighing boats*.—8.1 × 8.1 × 1.9 cm, 100 mL capacity, or similar size.

C. Reagents

(a) *Magnesium chloride solution*.—Dissolve 0.203 g $MgCl_2 \cdot 6H_2O$ and dilute to 500 mL with H_2O . Solution has indefinite shelf life.

(b) *Phenolphthalein diphosphate*.—Tetrasodium salt, ca 95%. Store in desiccator at <0°C.

(c) *Stock test reagent*.—Dissolve 19.0 g borax ($Na_2B_4O_7 \cdot 10H_2O$) and 6.28 g anhydrous Na_2CO_3 in 1 L H_2O with stirring. Add 0.94 g phenolphthalein diphosphate, (b), and stir while adding 2 mL $MgCl_2$ solution, (a). Reagent is stable ca 4 months at room temperature. Discard reagent if not colorless and ca pH 9.5 (degraded phenolphthalein diphosphate produces pink color in test reagent).

(d) *Agar*.—Bacto agar (Difco).

(e) *Liquid test agar*.—Measure equal volumes (half total test agar volume needed) of stock test reagent, (c), and H_2O into separate beakers. Beaker for H_2O must be large enough for 2 × volume of H_2O measured. Place beaker with H_2O on hot plate/stirrer, add stir bar, and with rapid stirring add enough agar to H_2O to yield 2% agar solution (1.5 g agar/75 mL H_2O). Continue stirring and heat to boil (avoid foam-over). Place cover glass over beaker to prevent heat loss. When agar begins to foam, add measured stock test reagent, pouring reagent down side of beaker to prevent agar from coming out of solution. Stir rapidly over heat ca 1 min. Prepare fresh daily and maintain at 42°C; use 150 mL/plate.

D. Determination

Prepare 4 plates for each 10 g test portion of ground black pepper and 1 plate for positive control.

Prepare liquid test agar, C(e), and maintain in water bath at 42 ± 1°C. Weigh 10 g ground black pepper from each well-mixed test portion. Add pepper, stir bar, and ca 300 mL H_2O to 400 mL beaker. Place beaker on stirrer and stir contents rapidly 10 min. Move beaker around on stirrer to ensure that all pepper particles are suspended. (*Note:* Failure to suspend all pepper particles will result in inadequate color leaching which interferes with detecting positive results.)

Transfer beaker contents to 8 in. (20 cm) No. 50 sieve. Rinse beaker and stir bar into sieve using gentle stream of cool water from water aerator as in 945.75B(a) (see 16.1.01). Direct gentle spray of cool water onto pepper until water through sieve is clear of any pepper particles (30 s). Quantitatively transfer sieve retainings to 90 mm Hirsch funnel, 945.75B(k) (see 16.1.01), containing 11 cm filter (medium porosity, ca 8–11 μm particle retention, Whatman No. 1 or 2 is suitable) over wire mesh screen. Vacuum-filter, continuing 10 min after most visible liquid has been removed.

Pour ca 150 mL 42°C liquid test agar into each plate. Carefully remove filter from funnel, loosen pepper particles using metal spatula, and distribute pepper from each test portion equally on agar surface of 4 plates. Scrape residual pepper particles from filter into plates.

Mix contents of each plate well to distribute particles evenly. Scrape spatula on side of 1 plate to remove any adhering material. (*Note:* Equal particle distribution among and within dishes is essential; concentrations of pepper particles could physically block visibility of positive color.)

Prepare positive control by thinly scattering some ground, known rodent feces evenly on one plate.

Allow test agar to gel completely (no agar flows as dish is slightly tipped), with lids removed, ca 20 min. Small fan may be used to hasten gelling.

E. Reading Results

When agar is firm, examine agar surface such that line of sight is perpendicular to plate while plate is held at 45°C to white background surface, with light source directed onto plate from above. Rotate plate as needed to view agar clearly, checking for pink to red-purple (positive) spots. Positive spots in test plates should be same color as spots in positive control plate. Mark positive spots on plate lid, using grease pencil, and mark lid and bottom together, using waterproof marker, so lid can be replaced in same orientation.

Place plates in 42°C water bath and incubate 15 min. Remove plates from bath, wipe inside lid to remove fog. Hold lid so bottom edge of lid is 2–3 mm above top edge of plate base while plate is read and marked. Replace lid. Repeat 15 min incubation and marking of positive spots.

Spots that appear and then are not seen on subsequent checks, and spots that appear anywhere in agar are counted. Record number of spots as fecal particles/10 g test portion.

(*Note:* Amount, intensity, and range of color [light pink to red-purple] will vary, depending on fecal particle size, species, and diet of animal. Particles <250 μm can be identified.)

Reference: *J. AOAC Int.* 77, 1146(1994).