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JOURNAL

OF THE

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Changes in Official Methods of Analysis Made at the Eightieth Annual Meeting, October 10–13, 1966

The following changes in the Methods of the Association become effective, as provided in Section 5 of the Bylaws, on the thirtieth day from the date of publication of this report, February 15, 1967. References are to the 10th Edition, 1965.

The number following each compound for which a determination is given is the registry number assigned to it by the *Chemical Abstracts Service*. This is a unique number for that compound; it will be useful in identifying the compound in a chemical information system which is in the process of development. Different salts or derivatives of a given compound as well as different stereochemical isomers will receive different numbers.

The following abbreviations were added to pp. xix–xx and are being used starting with changes made at the 80th Annual Meeting:

amt	amount
amp.	ampere(s)
com.	commercial, commercially
compd	compound
est.	estimate
estd	estimated
estg	estimating
estn	estimation
ir	infrared
ng	nanogram(s), 0.001 μg
qual.	qualitative, qualitatively
quant.	quantitative, quantitatively
sep.	separate, separately
uv	ultraviolet

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1. AGRICULTURAL LIMING MATERIALS

The official, final action EDTA titration methods, **1.021–1.023**, were revised as follows:

(A) The equation in **1.023(a)** was changed to read: "% Ca = (Titer EDTA std soln for Ca) × ml EDTA std soln × 2/g sample."

(B) The equation in **1.023(b)** was changed to read: "% Mg = (Titer EDTA std soln for Mg) × [(ml EDTA std soln in Ca + Mg titn) − (ml EDTA std soln in Ca titn)] × 2/g sample," and in line 2 of the same section, "Mg.) Pipet" was changed to read: "Mg.) For Ca + Mg, pipet."

(C) The equation in **1.023(c)** was changed to read: "% Mg = (Titer EDTA std soln for Mg) × ml EDTA std 0.1% soln × 2/g sample."

2. FERTILIZERS

(1) The official, final action method for sampling solid fertilizers, **2.001**, was revised as follows to include a sampling pattern which was adopted as official, first action:

(a) *Bagged fertilizers.—Official, Final Action.*—Use slotted single or double tube, or slotted tube and rod, with solid cone tip at one end. Take sample as follows: Lay bag horizontally and remove core diagonally from end to end. From lots of 10 bags or more, take core from each of 10 bags. When necessary to sample lots of <10 bags, take 10 cores but at least 1 core from each bag present. For small packages (10 lb or less) take 1 entire package as sample.

(b) *Bulk fertilizers.—Official, First Action.*—Use double tube compartmented trier, such as 524 Grain Probe or Missouri trier (available from Seedboro Equipment Co., 618 W. Jackson Blvd., Chicago, Ill. 60606) with solid cone tip; effective sampling length not <4′ and sepd compartments not >6″ long (or trier of other design that has been demonstrated to draw representative vertical core). Draw 10 vertical cores distributed in std concentric sampling pattern of such design that each core represents approx. equal fractions of lot:

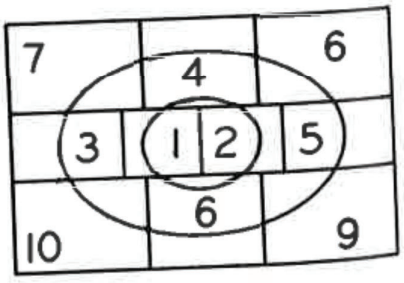


Fig. 1—Sampling pattern.

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trimmed cheese into 4 L beaker. Add ca 700 ml ca 55° filtered H_2O. Set beaker under mechanical stirrer, **36.001(c)**, and stir 15 min., maintaining mixt. at 55°. Add 100 ml dispersing soln, stir, and adjust mixt. to pH 8 with NH_4OH or dil. HCl (1 + 2). Rinse sides of beaker free of adhering cheese particles with ca 60° H_2O. Maintain pH 8 by addn of NH_4OH and keep adding ca 60° H_2O to dil. cheese mixt. to ca 3 L. If foaming occurs, place wet Patapar paper, split to accommodate stirrer blades, over top of beaker to break foam. Continue stirring until cheese becomes finely dispersed.

Cool dispersion to 40° and adjust to pH 8 with NH_4OH or HCl (1 + 2). Add 300 ml pancreatin soln (except use 600 ml pancreatin soln for ricotta). Let mixt. digest at about but not >40° with continued stirring ca 1.5 hr. Maintain pH 8 by addn of NH_4OH.

After digestion with pancreatin, place beaker on hot plate and heat to 65–68°; continue mechanical stirring. Adjust to pH 6.0 ± 0.2 with HCl (1 + 2). Carefully adjust stirrer blades close to bottom of beaker to pick up any cheese particles which settle. Continue stirring 15 min. or until cheese appears completely solubilized. Rinse inside of beaker, stirrer blades, etc., with ca 65° H_2O and filter thru ruled paper, using ca 65° H_2O and later alcohol to rinse beaker. If filtration becomes slow (e.g., cream cheese), let paper clear, wash with alcohol, and use addnl paper. Mixt. will filter more easily if 60 mesh screen (ca 5 cm diam.) is placed under paper and small amt mixt. is allowed to suck dry before filtering is continued. Examine paper microscopically.

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(4) The first sentence of the official, first action method for determining filth in cheese containing mold, plant tissues, and spices, **36.014(g)**, was revised to read as follows:

Disperse cheese as in **36.014(c)** or first par. of *Determination* in method for determining filth in cheese, item (3) above.

37. MICROBIOLOGICAL METHODS

The following method for the detection and identification of Salmonella from dried whole egg, dried egg yolk, and dried egg white was adopted as official, first action:

Method described is minimal. Depending upon history of sample, addnl types of examinations may be applied. Use *Identification of Enterobacteriaceae*, P. R. Edwards and W. H. Ewing, Burgess Publishing Co., Minneapolis, Minn., 2nd Ed., 1962, as guide for further study of isolated microorganisms.

37.X23 Testing Urease-Negative Cultures

(*See* introduction to *Media and Reagents, Changes in Methods, This Journal* **49**, 247 (1966).)

Sizes of culture media containers (test tubes, flasks, and petri dishes) are specified in prepn of each medium. Different size containers may be used if they give identical results. All media containers must have covers, caps, or plugs which prevent contamination but maintain aerobic conditions unless otherwise directed.)

(a) *Lactose broth, 0.5%.*—Dissolve 5.0 g polypeptone or peptone, 3.0 g beef ext., and 5.0 g lactose in 1 L H_2O. Dispense 225 ml portions in 500 ml screw-cap bottles or flasks, and autoclave 15 min. at 121°. Final pH, 6.8 ± 0.1.

(b)(*1*) *Selenite cystine broth.*—Suspend 5.0 g tryptone or polypeptone, 4.0 g lactose, 10.0 g anhyd. Na_2HPO_4, 4.0 g $NaHSeO_3$, and 0.01 g L-cystine in 1 L H_2O and mix thoroly. Heat with frequent agitation. Dispense 10 ml portions in sterile 16 × 150 mm test tubes. Heat 10 min. in flowing steam. *Do not autoclave.* Final pH, 7.0 ± 0.1. Medium is not sterile. Use same day as prepd.

(*2*) *Selenite cystine broth (North and Bartram).*—Prep. as in (*1*), using 5.0 g polypeptone or 4.0 g tryptone, 4.0 g lactose, 4.0 g $NaHSeO_3$, 5.5 g anhyd. Na_2HPO_4, 4.5 g anhyd. KH_2PO_4, and 1 ml (10 mg) 1% L-cystine soln prepd by dissolving 1.0 g L-cystine in 15 ml 1*N* NaOH and dilg to 100 ml with sterile H_2O.

(c) *Tetrathionate broth (with iodine and brilliant green).*—Suspend 5.0 g polypeptone, 1.0 g bile salts, 10 g $CaCO_3$, and 30 g $Na_2S_2O_3 \cdot 5H_2O$ in 1 L H_2O, mix thoroly, and heat to boiling. (Ppt will not dissolve completely.) Cool to <45° and store at 5–8°. Prep. I-KI soln by dissolving 5 g KI in 5 ml sterile H_2O, adding 6 g resublimed I, dissolving, and dilg to 20 ml with sterile H_2O. Prep. brilliant green soln by dissolving 0.1 g dye in sterile H_2O and dilg to 100 ml. On day medium is used, add 20 ml I-KI soln and 10 ml brilliant green soln per 1 L basal broth. Resuspend ppt by gentle agitation and aseptically dispense 10 ml portions in 20 × 150 or 16 × 150 mm sterile test tubes. *Do not heat medium after addn of I-KI and dye solns.*

(d) *Brilliant green agar.*—Use com. dehydrated medium contg 3.0 g yeast ext., 10 g

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proteose peptone No. 3 or polypeptone, 5.0 g NaCl, 10 g lactose, 10 g sucrose, 0.08 g phenol red, 0.0125 g brilliant green, and 20 g agar. Suspend medium in 1 L H_2O, mix thoroly, and heat with occasional agitation. Boil 1 min. to dissolve. Autoclave 1 L portions 12 min. at 121°. (Addnl heating decreases selectivity of medium; less heating increases selectivity.) Cool to 45–50° and pour 20 ml portions into 15 × 100 mm petri dishes. Let dry ca 2 hr with covers partially removed; then close plates. Final pH, 6.9 ± 0.1.

(e) *Salmonella Shigella agar.*—Suspend 5.0 g beef ext., 5.0 g polypeptone or proteose peptone, 10 g lactose, 8.5 g bile salts mixt., 8.5 g Na citrate, 8.5 g $Na_2S_2O_3.5H_2O$, 1.0 g ferric citrate, 13.5 g agar, 0.00033 g brilliant green, and 0.025 g neutral red in 1 L H_2O, and mix thoroly until homogeneous. Heat with occasional agitation and boil 1–2 min. until ingredients dissolve. Cool to 45–50° and pour 20 ml portions into 15 × 100 mm petri dishes. Let dry ca 2 hr with covers partially removed; then close plates. Final pH, 7.0 ± 0.1. *Do not autoclave.*

(f) *Bismuth sulfite agar (Wilson and Blair).*—Suspend 10 g polypeptone or peptone, 5.0 g beef ext., 5.0 g dextrose, 4.0 g $Na_2HPO_4.12H_2O$, 0.3 g $FeSO_4.7H_2O$, 8.0 g $Bi_2(SO_3)_3$ indicator, 0.025 g brilliant green, and 20 g agar in 1 L H_2O, mix thoroly, and heat with occasional agitation. Boil ca 1 min. to obtain uniform suspension. (Ppt will not dissolve.) Cool to 45–50°. Suspend ppt by gentle agitation and pour 20 ml portions into 15 × 100 mm petri dishes. Let dry ca 2 hr with covers partially removed; then close plates. Final pH, 7.6 ± 0.1. *Do not autoclave.* Selectivity of plates decreases 48 hr after prepn.

(g) *Triple sugar iron agar (TSI agar).*—Suspend ingredients (*1*) or (*2*) in 1 L H_2O, mix thoroly, and heat with occasional agitation. Boil ca 1 min. until ingredients dissolve. Fill 16 × 150 mm tubes 1/3 full and cap or plug so that aerobic conditions are maintained during use. Autoclave 12 min. at 121°. Before medium solidifies, place tubes in slanted position so that deep butts (ca 1¼″) and adequate slants (ca 2″) are formed on solidification.

(*1*) 20 g polypeptone, 5.0 g NaCl, 10 g lactose, 10 g sucrose, 1 g dextrose, 0.2 g $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.2 g $Na_2S_2O_3$, 0.025 g phenol red, and 13 g agar. Final pH, 7.3 ± 0.1.

(*2*) 3.0 g beef ext., 3.0 g yeast ext., 15 g peptone, 5.0 g proteose-peptone, 1.0 g dextrose, 10 g lactose, 10 g sucrose, 0.2 g $FeSO_4.7H_2O$, 5.0 g NaCl, 0.3 g $Na_2S_2O_3$, 0.024 g phenol red, and 12 g agar. Final pH, 7.4 ± 0.1.

(h) *Tryptone broth.*—Prep. as in (j), *Media and Reagents, This Journal* **49**, 248 (1966), using 16 or 20 × 150 mm test tubes.

(i) *Methyl red–Voges-Proskauer (MR-VP) medium.*—Prep. as in (k), *Media and Reagents, This Journal* **49**, 248 (1966), using 16 or 20 × 150 mm test tubes.

(j) *Simmon's citrate agar.*—Dissolve 2.0 g Na citrate, 5.0 g NaCl, 1.0 g K_2HPO_4, 1.0 g $NH_4H_2PO_4$, 0.2 g $MgSO_4$, 0.08 g bromothymol blue, and 15 g agar in 1 L H_2O, and heat gently with occasional agitation. Boil 1–2 min. until ingredients dissolve. Fill 13 or 16 × 150 mm test tubes 1/3 full and cap or plug so that aerobic conditions are maintained during use. Autoclave 15 min. at 121°. Before medium solidifies, place tubes in slanted position so that deep butts (ca ¾ or 1¼″, resp.) and adequate slants (ca 1½ or 2″, resp.) are formed on solidification.

(k)(*1*) *Urea broth.*—Dissolve 20 g urea, 0.1 g yeast ext., 9.1 g KH_2PO_4, 9.5 g Na_2HPO_4, and 0.01 g phenol red in 1 L H_2O. *Do not heat.* Sterilize by filtration and aseptically dispense 1.5–3 ml portions in 13 × 100 mm sterile test tubes. Final pH, 6.8 ± 0.1.

(*2*) *Rapid urea broth.*—Prep. as in (*1*), using 0.091 and 0.095 g phosphate salts, resp.

(l) *Malonate broth.*—Dissolve 1.0 g yeast ext., 2.0 g $(NH_4)_2SO_4$, 0.6 g K_2HPO_4, 0.4 g KH_2PO_4, 2.0 g NaCl, 3.0 g Na malonate, 0.25 g dextrose, and 0.025 g bromothymol blue in 1 L H_2O, heating if necessary until dissolved. Dispense 3 ml portions in 13 × 100 mm test tubes and autoclave 15 min. at 121°. Final pH, 6.7 ± 0.1.

(m)(*1*) *Lysine iron agar (Edwards and Fife).*—Dissolve 5.0 g gelysate or peptone, 3.0 g yeast ext., 1.0 g dextrose, 10 g L-lysine, 0.5 g ferric ammonium citrate, 0.04 g $Na_2S_2O_3$, 0.02 g bromocresol purple, and 15 g agar in 1 L H_2O, heating until dissolved. Dispense 4 ml portions in 13 × 100 ml test tubes and cap or plug so that aerobic conditions are maintained during use. Autoclave 12 min. at 121°. Before medium solidifies, place tubes in slanted position so that 1½″ butts and 1″ slants are formed on solidification. Final pH, 6.7 ± 0.1.

(*2*) *Lysine decarboxylase broth (Falkow).*—Dissolve 5.0 g gelysate or peptone, 3.0 g yeast ext., 1.0 g dextrose, 5.0 g L-lysine, and 0.02 g bromocresol purple in 1 L H_2O, heating until dissolved. Dispense 5 ml portions in 16 × 125 mm screw-cap test tubes. Autoclave, loosely capped, 15 min. at 121°. Screw caps tightly

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for storage and after inoculation. Final pH, 6.5–6.8.

(n) *Motility test medium (semisolid medium).*—Dissolve 3.0 g beef ext., 10 g peptone or gelysate, 5.0 g NaCl, and 4.0 g agar in 1 L H_2O and heat gently with occasional agitation. Boil 1–2 min. to dissolve. If medium is to be stored, dispense 20 ml portions into screw-cap containers, replacing caps loosely. Autoclave 15 min. at 121°. Cool to 45°. To store, screw caps on tightly and refrigerate at 5–8°. To use, remelt in boiling H_2O or flowing steam and cool to 45°. Aseptically dispense 20 ml portions in 15 × 100 mm petri dishes and let solidify with dish completely covered. Use plates same day as prepd. Final pH, 7.4 ± 0.1.

(o) *Potassium cyanide (KCN) broth.*—Dissolve 3.0 g proteose peptone No. 3 or polypeptone, 5.0 g NaCl, 0.225 g KH_2PO_4, and 5.64 g Na_2HPO_4 in 1 L H_2O. Autoclave 15 min. at 121°. Cool and refrigerate at 5–8°. Final pH, 7.6 ± 0.1. Dissolve 0.5 g KCN in 100 ml cold (5–8°) sterile H_2O. Using sterile bulb pipet or sterile syringe (*do not pipet by mouth*), aseptically add 15 ml cold KCN soln per L cold, sterile basal broth. Mix thoroly with gentle agitation and aseptically dispense 1–1.5 ml portions in sterile 13 × 100 mm test tubes. Using aseptic technic, immediately stopper tubes with No. 2 corks impregnated with paraffin. Prep. corks by boiling in paraffin ca 5 min. Place corks in tubes so that paraffin does not flow into broth but forms good seal between rim of tube and cork. Medium stored at 5–8° is usually stable ca 2 weeks.

(p)(*1*) *Phenol red carbohydrate broth.*—Dissolve 10 g trypticase or proteose peptone No. 3, 5.0 g NaCl, 1.0 g beef ext. (optional), and 0.018 g phenol red in 1 L H_2O and heat with gentle agitation until dissolved. Dissolve 5 g dulcitol, 10 g lactose, or 10 g sucrose (as specified in title of test) in this basal broth. Dispense 2.5 ml portions in 13 × 100 mm test tubes contg inverted 6 × 50 mm fermentation tubes. Autoclave 10 min. at 118° (12 psi). Final pH, 7.3 ± 0.1. Alternatively, dissolve ingredients, omitting carbohydrate, in 800 ml H_2O with heat and occasional agitation. Dispense 2.0 ml portions in 13 × 100 mm test tubes contg inverted fermentation tubes. Autoclave 15 min. at 118° and let cool. Dissolve carbohydrate in 200 ml H_2O and sterilize by passing soln thru bacteria-retaining filter. Aseptically add 0.5 ml sterile filtrate to each tube of sterilized broth after cooling to <45°. Shake gently to mix. Final pH, 7.4 ± 0.1.

(*2*) *Purple carbohydrate broth.*—Prep. as in (*1*), using as basal broth 10 g proteose peptone No. 3 or gelysate, 5.0 g NaCl, and 0.015 or 0.020 g bromocresol purple. Final pH, 6.8 ± 0.1.

(q) *MacConkey agar.*—Suspend 3.0 g proteose peptone or polypeptone, 17 g peptone or gelysate, 10 g lactose, 1.5 g bile salts No. 3 or bile salts mixt., 5.0 g NaCl, 0.03 g neutral red, 0.001 g crystal violet, and 13.5 g agar in 1 L H_2O and mix thoroly until homogeneous. Heat, with occasional agitation, and boil 1–2 min. until ingredients dissolve. Autoclave 15 min. at 121°. Cool to 45–50° and pour 20 ml portions into 15 × 100 mm petri dishes. Let dry at least 2 hr with plates covered. Do not use wet plates. Final pH, 7.1 ± 0.1.

(r) *Brain heart infusion broth.*—Prep. as in (h), *Media and Reagents, This Journal* **49**, 248 (1966), dispensing 5 ml portions in 16 × 150 mm test tubes.

37.X17 ***Diagnostic Reagents***

(a) *Kovac's reagent for indole test.*—Dissolve 5 g *p*-dimethylaminobenzaldehyde in 75 ml amyl alcohol and slowly add 25 ml HCl.

(b) *Voges-Proskauer (VP) test reagents.*—(*1*) *Alpha-naphthol soln.*—5%. Dissolve 5.0 g α-naphthol in 100 ml absolute alcohol.

(*2*) *Potassium hydroxide soln.*—40%. Dissolve 40 g KOH in H_2O and dil. to 100 ml.

(c) *Sodium hydroxide soln.*—1*N*. Dissolve 42.11 g 95% reagent NaOH in sterile H_2O and dil. to 1 L.

(d) *Hydrochloric acid soln.*—1*N*. Dil. 89 ml to 1 L with sterile H_2O.

(e) *Methyl red indicator.*—Dissolve 0.10 g Me red in 300 ml alcohol and dil. to 500 ml with H_2O.

(f) *Sterile physiological saline soln.*—Dissolve 8.5 g NaCl in H_2O and dil. to 1 L. Autoclave 15 min. at 121° and cool to room temp.

(g) *Formalized physiological saline soln.*—Add 6 ml HCHO soln (36–38%) to sterile saline soln, (f), mix, and store in tightly stoppered containers.

(h) *Salmonella polyvalent somatic (O) antiserum.*—(*Identification of the Salmonella Serotypes,* No. 168, Difco Labs., 920 Henry St., Detroit, Mich. 48201, July 1963, p. 5.) Contains agglutinins for at least somatic (O) antigens: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 19, 22, 23, 24, 25, and Vi. They are

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agglutinins for somatic (O) groups: A, B, C_1, C_2, D, E_1, E_2, E_4, F, G, H, I, and Vi.

(i) *Salmonella individual somatic (O) antisera.*—(*See* reference in (h).) For at least each of the somatic (O) groups listed in (h).

(j) *Salmonella polyvalent flagellar (H) antiserum.*—(*See* p. 11 of reference in (h).) Contains agglutinins for at least the following flagellar (H) antigens: a b, c, d, eh, en complex (enz$_{15}$, enx), fg, fgt, gm, gmq, gms, gp, gpu, gq, gst, gt, i, k, lv, lw, lz$_{13}$, lz$_{28}$, mt, r, y, z, z_4z_{23}, z_4z_{24}, z_4z_{32}, z_6, z_{10}, z_{29}; 1, 2; 1, 5; 1, 6; and 1, 7.

(k) *Salmonella "Spicer-Edwards" flagellar (H) antisera.*—(From p. 10 of reference in (h).) Consists of 7 pooled or polyvalent antisera which react as follows:

Salmonella H Antisera Spicer-Edwards and H Antigens with Which Each Reacts

	Salmonella H Antisera Spicer-Edwards			
H Antigens	1	2	3	4
a	+	+	+	−
b	+	+	−	+
c	+	+	−	−
d	+	−	+	+
eh	+	−	+	−
G Complex[a]	+	−	−	+
i	+	−	−	−
k	−	+	+	+
r	−	+	−	+
y	−	+	−	−
z	−	−	+	+
z_4 Complex[b]	−	−	+	−
z_{10}	−	−	−	+
z_{29}	−	+	+	−

H Antigens	Salmonella H Antisera
enx, enz$_{15}$	en complex
lv, lw, lz$_{13}$, lz$_{28}$	L complex
1, 2; 1, 5; 1, 6; 1, 7	1 complex

[a] The G complex component of Salmonella *H* antisera Spicer-Edwards 1 and 4 reacts with antigens fg, fgt, gm, gms, gmt, gp, gpu, gq, gst, ms, and mt.
[b] The z_4 complex component reacts with z_4z_{23}, z_4z_{24}, and z_4z_{32}.
(From Difco Laboratories, Detroit, Mich.)

(l) *pH Test paper.*—Min. range 6.0–7.6 with max. gradations of 0.4 pH for each color change.

Detection

37.X18 Preparation of Sample Cultures

Open sample container aseptically and weigh aseptically 25 g sample into sterile, empty, wide-mouth, screw-cap pint jar. Add ca 15 ml sterile lactose broth, **37.X16(a)**. Stir with sterile glass rod, sterile spoon, or sterile tongue depressor to smooth suspension. Add 3 addnl portions of lactose broth, 10, 10, and 190 ml for total of 225 ml. Stir after each addn until sample is suspended without lumps. Cap jar securely and let stand at room temp. 60 min. Mix well by shaking, and det. pH with test paper, **37.X17(l)**. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1*N* NaOH or HCl, **37.X17(c)** or **(d)**, capping jar securely and mixing well before detg final pH. Loosen jar cap ca ¼ turn and incubate 24 ± 2 hr at 35°.

37.X19 Isolation

(a) *Growth on selective media.*—Shake gently incubated sample mixt., **37.X18**, and transfer 1 ml to 10 ml selenite cystine broth, **37.X16(b)(1)** or **(2)**, and addnl 1 ml to 10 ml tetrathionate broth, **37.X16(c)**. Incubate 24 ± 2 hr at 35°.

Streak 3 mm loopful of incubated selenite cystine broth on selective media plates of brilliant green agar, **37.X16(d)**. Salmonella Shigella agar, **37.X16(e)**, and $Bi_2(SO_3)_3$ agar, **37.X16(f)**. Repeat with 3 mm loopful of incubated tetrathionate broth. Incubate plates 24 ± 2 hr at 35°.

(b) *Appearance of typical Salmonella colonies.*—(*1*) *On brilliant green agar.*—Colorless, pink to fuchsia, translucent to opaque, with surrounding medium pink to red. Some *Salmonella* appear as transparent green colonies if surrounded by lactose- or sucrose-fermenting organisms which produce colonies that are yellow-green or green.

(*2*) *On Salmonella Shigella agar.*—Uncolored to pale pink, opaque, transparent or translucent, but some strains produce black-centered colonies.

(*3*) *On bismuth sulfite agar.*—Brown, grey to black, sometimes with metallic sheen. Surrounding medium is usually brown at first, turning black with increasing incubation time. Some strains produce green colonies with little or no darkening of the surrounding medium.

If plates do not have typical or suspicious colonies or do not contain growth, incubate addnl 24 hr.

37.X20 Treatment of Typical or Suspicious Colonies

(a) *Inoculation of triple sugar iron (TSI) agar.*—Pick with needle 2 or more typical or suspicious colonies, if present, from each brilliant green, Salmonella Shigella, and $Bi_2(SO_3)_3$ agar plates having growth. Inoculate TSI agar

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slant, 37.X16(g), with portion of each colony by streaking slant and stabbing butt. Store picked selective plates at 5–8° or at room temp. (ca 26°).

(b) *Presumptive positive reactions.*—Incubate TSI slants 24 ± 2 hr at 35°. Presumptive positive Salmonella cultures have alk. (red) slants and acid (yellow) butts, with or without H_2S (blackening of agar). (H_2S-negative TSI cultures which otherwise appear to be Salmonellae cannot be excluded from further examination.)

If TSI fails to give a typical Salmonella reaction, pick addnl suspicious colony from selective medium plate not giving presumptive positive culture and inoculate TSI slant as in (a).

(To detect lactose-fermenting Arizona organisms, test all TSI cultures (including yellow slants) isolated from H_2S-positive $Bi_2(SO_3)_2$ agar colonies, using lysine iron agar, 37.X16(m)(*1*), as in 37.X23(a). Cultures that are lysine decarboxylase-positive and H_2S-positive are tested further to det. if they are Arizona sp., 37.X25(f)(*2*).)

(c) *Selection for identification.*—Apply biochemical and serological identification tests to 3 presumptive positive TSI agar cultures picked from selective agar plates streaked from selenite cystine broth and to 3 presumptive positive TSI agar cultures picked from selective agar plates streaked from tetrathionate broth.

If 3 presumptive positive TSI agar cultures are not isolated from 1 set of selective agar plates, test other presumptive positive TSI agar cultures, if isolated, by biochemical and serological tests. A minimum of 6 TSI cultures are examined for each 25 g sample tested.

Identification

37.X21 *Cultures*

Pure cultures on TSI agar are required for inoculation of biochemical test media.

(a) *Pure cultures.*—Proceed to 37.X22.

(b) *Mixed cultures.*—Streak any culture that appears to be mixed on MacConkey's agar, 37.X16(q), or brilliant green agar, 37.X16(d). Incubate 24 ± 2 hr at 35°.

(c) *Appearance of Salmonella colonies.*—(*1*) *On MacConkey's agar.*—Typical colonies appear transparent and colorless, sometimes with dark centers. *Salmonella* will clear areas of pptd bile caused by other organisms sometimes present in medium.

(*2*) *On brilliant green agar.*—*See* 37.X19(b)(*1*).

Pick with needle at least 2 typical or suspicious colonies and inoculate TSI slants by streaking the slant and stabbing the butt as in 37.X20(a). Retest purified cultures as in 37.X20(b), and proceed with identification.

37.X22 *Subcultures*

(a) *Urease test.*—Subculture two 3 mm loopfuls of presumptive positive TSI agar culture to urea broth, 37.X16(k)(*1*), and incubate 24 ± 2 hr at 35° or inoculate rapid urea broth, 37.X16(k)(*2*), and incubate 2 hr in H_2O bath at 37 ± 0.5°. Discard all cultures that give positive test (purple-red color). *Salmonellae* are urease negative (no change in orange color of medium).

(b) *Serological flagellar (H) screening test.*—To reduce no. of presumptive positive TSI agar cultures carried thru identification tests, perform serological flagellar (H) screening test by transferring one 3 mm loopful of each urease-negative TSI agar culture to either:

(*1*) Brain heart infusion broth, 37.X16(r), (for test on same day) and incubate at 35° until visible growth occurs (ca 4–6 hr); or

(*2*) Tryptone broth, 37.X16(h) (for test on following day), and incubate 24 ±2 hr at 35°.

To 5 ml of each of the 6 broth cultures add ca 2.5 ml formalized physiological saline soln, 37.X17(g). Select 2 formalized broth cultures and test with Salmonella flagellar (H) antisera, 37.X17(j) or (k), as in 37.X31 or 37.X32.

If selected formalized broth cultures are positive, perform addnl tests on these cultures, beginning with 37.X23, except step 37.X23(c)(*4*) may be omitted.

If both formalized broth cultures are not positive, perform test on the 4 remaining other broth cultures (37.X22(b)(*1*) or (*2*)) to obtain, if possible, 2 positive cultures for addnl testing, 37.X23.

If all urease-negative TSI cultures from sample are Salmonella serological flagellar (H) test negative, then perform addnl tests, beginning with 37.X23, on all cultures.

37.X23 *Testing Urease-Negative Cultures*

Transfer 1 loopful of presumptive positive TSI agar culture to each of following media:

(a) *Lysine iron agar,* 37.X16(m)(*1*). —Streak slant and stab butt. Replace tube cap *loosely* and incubate 48 ± 2 hr at 35°. Examine at least every 24 hr. *Salmonellae* give alk. reaction of purple color thruout medium (final color is slightly darker than original

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purple color of medium). If H_2S is produced, butt of medium is blackened. Negative test is purple or red slant and yellow butt.

If liquid medium is preferred, inoculate tube of lysine decarboxylase broth, **37.X16(m)**(*2*). Close tube cap *tightly* after inoculation and incubate 96 ± 2 hr at 35°. Examine at least every 24 hr. *Salmonellae* give alk. reaction of purple color thruout broth (final color is slightly darker than original purple color of medium). Sometimes tubes which have yellow color after 8–12 hr of incubation change to purple later. Negative test is permanent yellow color thruout broth.

(Lysine iron agar is incubated loosely capped so that aerobic conditions are maintained, while lysine decarboxylase broth is incubated tightly closed to exclude air.)

(b) *Phenol red dulcitol broth,* **37.X16(p)** (*1*).—Incubate 48 ± 2 hr at 35°. Examine at least every 24 hr. Most *Salmonellae* give positive test indicated by gas formation (displacement of liquid in inverted tube) and acid reaction (yellow). Negative test is alk. reaction (red) and no gas formation.

(Purple broth base with dulcitol, **37.X16**(p) (*2*), may be substituted. Positive test is acid reaction (yellow) and gas. Negative test is alk. reaction (purple).)

(c) *Tryptone broth,* **37.X16(h)**.—Incubate 24 ± 2 hr at 35° and test as follows:

(*1*) Transfer 3 mm loopful, excluding all solid particles, to KCN broth, **37.X16(o)**. Heat rim of tube to form good seal when restoppered. Incubate 48 ± 2 hr at 35°. *Salmonellae* do not grow in this broth as shown by lack of turbidity (negative test).

(*2*) Transfer 3 mm loopful to malonate broth, **37.X16**(l), and incubate 48 ± 2 hr at 35°. *Salmonellae* give negative test as shown by green color (unchanged). Positive test (alk. reaction) is shown by blue color.

(*3*) Transfer 5 ml to empty test tube and add 0.2–0.3 ml Kovac's reagent, **37.X17(a)**. Positive test for indole is shown by deep red in reagent on surface of broth. Most *Salmonellae* are indole negative.

(*4*) To remainder of 24-hr tryptone broth culture (ca 5 ml) add ca 2.5 ml formalized physiological saline soln, **37.X17(g)**. Refrigerate formalized broth at 5–8° if test is to be performed on another day. Perform Salmonella serological flagellar (H) test, **37.X31**, or "Spicer-Edwards" flagellar (H) test tube test, **37.X32**, using formalized broth culture as flagellar (H) antigen to be tested.

(d) *Tests indicating absence of Salmonella.* —Discard, as not *Salmonella,* cultures that show either:

(*1*) Positive indole test (red) and negative *Salmonella* serological flagellar (H) test.

(*2*) Positive KCN broth test (growth) and negative lysine decarboxylase test (yellow).

(f) *Classification.*— Classify as *Salmonella* sp. cultures that have all characteristics shown in Table **37.X24**. If 1 TSI culture from 25 g sample is classified as *Salmonella* sp., further testing of other TSI cultures from same 25 g sample is unnecessary.

(e) *Testing of TSI agar cultures.*—Use Salmonella serological somatic (O) test, **37.X29**.

(g) *Special cases.* — Cultures that contain demonstrable *Salmonella* antigens as shown by positive *Salmonella* serological somatic (O) test and positive flagellar (H) test but do not have biochemical characteristics of *Salmonellae* should be purified as in **37.X21(b)** and retested, beginning with **37.X22**.

37.X24 ***Characteristics of Salmonella***

Test or substrate	Results
Urease, **37.X22(a)**	Negative (orange-red)
Lysine decarboxylase, **37.X23(a)**	Positive (alk.; purple thruout medium)
Phenol red dulcitol broth, **37.X23(b)**	Positive (yellow and gas)
KCN broth, **37.X23(c)**(*1*)	Negative (no growth)
Malonate broth, **37.X23(c)**(*2*)	Negative (unchanged green)
Indole test, **37.X23(c)**(*3*)	Negative (no red color)
Polyvalent flagellar test, **37.X22(b), 37.X23(c)**(*4*)	Positive (visible agglutination)
Polyvalent somatic test, **37.X23(e)**	Positive (visible agglutination)

37.X25 *Additional Biochemical Tests*

Perform addnl tests on cultures that do not give identical test results as in **37.X24** and do not classify as *Salmonella* sp. Transfer 1 loopful of culture from each unclassified TSI agar slant to each of following media:

(a) *Phenol red lactose broth,* **37.X16(p)** (*1*).—Incubate 48 ± 2 hr at 35°. Examine inoculated broth at least every 24 hr. Positive test is shown by gas formation (displacement of liquid in inverted tube) and acid reaction (yellow). *Salmonellae* give negative test shown by alk. reaction (red) and no gas formation.

Discard, as not *Salmonella,* cultures that give positive phenol red lactose broth test, except cultures that also give positive malonate broth test. Cultures that are phenol red lactose broth positive or negative and malonate

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broth positive are tested further to det. if they are *Arizona* sp., **37.X25(f)**(*2*).

(Purple lactose broth, **37.X16(p)**(*2*), may be substituted. Positive test is acid reaction (yellow) and gas. Negative test is alk. reaction (purple) and no gas formation.)

(**b**) *Phenol red sucrose broth,* **37.X16(p)**(*1*).—Incubate and read as in (**a**) above. Discard, as not *Salmonella,* cultures that give positive test. (Purple sucrose broth may be substituted and read as in (**a**) above.)

(**c**) *Methyl red-Voges-Proskauer (MR-VP) medium,* **37.X16(i)**.—Incubate 48 ± 2 hr at 35°.

(*1*) Perform Voges-Proskauer (VP) test at room temp. by transferring 1 ml 48-hr culture to test tube and adding 0.6 ml α-naphthol soln, **37.X17(b)**(*1*), and 0.2 ml 40% KOH soln, **37.X17(b)**(*2*). Shake after each addn. To intensify and speed reaction, add few creatine crystals to test medium. Read results 4 hr after adding reagents. Positive VP test is development of eosin pink color. *Salmonella* give negative test.

(*2*) Incubate remainder of MR-VP medium addnl 48 ± 2 hr at 35°. Perform Me red test by transferring 5 ml culture to test tube and adding 5-6 drops Me red soln, **37.X17(e)**, and read results immediately. *Salmonellae* give positive test (red). Negative test is indicated by yellow color.

(**d**) *Simmon's citrate agar,* **37.X16(j)**.—Inoculate by streaking slant and stabbing butt. Incubate 96 ± 2 hr at 35°. *Salmonellae* usually give positive test shown by growth and color change from green to blue (alk.). Color change usually appears first on slant and then spreads thru medium. Negative test is indicated by no or very little growth and no change in color of medium.

(**e**) *Tests indicating absence of Salmonella.*—Discard, as not *Salmonella,* cultures that give the following 3 results:

(*1*) Positive KCN broth test (growth),

(*2*) Positive Voges-Proskauer test (red), and

(*3*) Negative Me red test (yellow).

(**f**) *Classification.*—Classify cultures according to results listed in Table **37.X26**. If 1 TSI culture from 25 g sample is classified as *Salmonella* sp., further testing of other TSI cultures from same 25 g sample is unnecessary.

(*1*) *Salmonella* sp.—Cultures that have reaction patterns of Table **37.X26**, column A or B.

(*2*) *Arizona* sp.—Cultures that have reaction pattern of Table **37.X26**, column C.

(*3*) *Non-Salmonella* sp.—Discard, as not *Salmonella,* cultures that give results listed in any 1 subdivision of Table **37.X27**. (*See* p. 238.)

37.X28 Summary of Classification of Non-Salmonella Cultures

Classify, by performing addnl tests described in *Identification of Enterobacteriaceae,* any culture that is not clearly identified as *Salmonella* sp. or *Arizona* sp. by classification schemes in Tables **37.X24** and **37.X26** or not elimi-

37.X26 ***Characteristics of Salmonella and Arizona***

Test or Substrate	Classification[a]		
	Salmonella sp.		*Arizona* sp.
	A	B	C
Urease test, **37.X22(a)**	–	–	–
Lysine decarboxylase, **37.X23(a)**	alk.	alk.	alk.
Phenol red dulcitol broth, **37.X23(b)**	AG	AG or A or –	–
KCN broth, **37.X23(c)**(*1*)	NGr	NGr	NGr
Malonate broth, **37.X23(c)**(*2*)	–	–	+
Indole test, **37.X23(c)**(*3*)	–	–	–
Polyvalent flagellar test, **37.X22(b)**, **37.X23(c)**(*4*) or Spicer-Edwards flagellar (H) test, **37.X32**	+	+	+ or –
Polyvalent somatic test, **37.X23(e)**	+ or –	+	+ or –
Phenol red lactose broth, **37.X25(a)**	–	–	AG or A or –
Phenol red sucrose broth, **37.X25(b)**	–	–	–
Voges-Proskauer test, **37.X25(c)**(*1*)	–	–	–
Methyl red test, **37.X25(c)**(*2*)	+	+	+
Simmon's citrate, **37.X25(d)**	+	+ or –	+

[a] – = negative test or no acid or gas; NGr = no growth; + = positive test; A = acid; AG = acid and gas; alk = alkaline.

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37.X27 *Characteristics of Non-Salmonella Cultures*

Test(s) or Substrate(s)	Results
(a) Urease test, **37.X22(a)**	Positive (purple-red)
(b) Indole test, **37.X23(c)(3)**	Positive (red)
Polyvalent flagellar test, **37.X22(b)**, **37.X23(c)(4)** or Spicer-Edwards flagellar (H) test, **37.X32**	Negative (no agglutination)
(c) Lysine decarboxylase test, **37.X23(a)**	Negative (yellow)
KCN broth, **37.X23(c)(1)**	Positive (growth)
(d) Phenol red lactose broth[a], **37.X25(a)**	Positive (gas and acid)
(e) Phenol red sucrose broth, **37.X25(b)**	Positive (gas and acid)
(f) KCN broth, **37.X23(c)(1)**	Positive (growth)
Voges-Proskauer test, **37.X25(c)(1)**	Positive (red)
Methyl red test, **37.X25(c)(2)**	Negative (yellow)

[a] Malonate broth positive cultures are tested further to det. if they are *Arizona* sp., **37.X25(f)(2)**.

nated from these groups by test reactions listed in Table **37.X27**.

If neither of 2 TSI cultures carried thru biochemical tests, **37.X23–37.X27**, confirm as *Salmonella*, perform biochemical tests, beginning with **37.X23**, on all other urease-negative TSI cultures from same 25 g sample.

Salmonella Serological Tests

Dil. and pretest all *Salmonella* serological antisera with known test cultures to insure reliability of results with unknown cultures. CAUTION: Handle viable cultures carefully to prevent contaminating environment.

37.X29 Polyvalent Somatic (O) Slide or Plate Test

Using wax pencil, mark off 2 sections ca 1 × 2 cm on inside of glass or plastic petri dish. Place ½ of 3 mm loopful of culture from 24- or 48-hr TSI agar slant on dish in upper part of each marked section. Add 1 drop saline soln, **37.X17(f)**, to lower part of each marked section. With clean, sterile transfer loop or needle, emulsify culture in saline soln for 1 section and repeat for other section. Add 1 drop *Salmonella* polyvalent somatic (O) antiserum, **37.X17(h)**, to a section of emulsified culture and mix with clean, sterile transfer loop or needle. Tilt mixt. in both sections back and forth 1 min. and observe against dark background. Any degree of agglutination is positive reaction.

Classify polyvalent somatic (O) test as:

(a) *Positive.*—Agglutination in culture-saline-serum mixt. and no agglutination in culture-saline mixt.

(b) *Negative.*—No agglutination in culture-saline-serum mixt. (Polyvalent somatic antisera does not contain agglutinins for antigens of some *Salmonellae* isolated from foods. Negative somatic reactions occur with the *Salmonellae* serotypes whose corresponding agglutinins are not contained in the antisera, i.e., *S. cerro*, group K(18); *S. minnesota*, group L(21); *S. alachua*, group O(35).)

(c) *Non-specific.*—Both mixts agglutinate. Requires addnl testing as in *Identification of Enterobacteriaceae*.

37.X30 Determination of Somatic Grouping (Optional)

Perform serological somatic (O) test on culture as in **37.X29**, using individual group somatic (O) antiserum (including Vi), **37.X17(i)**, instead of *Salmonella* polyvalent somatic (O) antiserum. Repeat test, using each group somatic antiserum or until culture reacts with specific group antiserum.

Suspend cultures positive with Vi antiserum by emulsifying growth from slant surface in 1 ml physiological saline soln, **37.X17(f)**, to make heavy suspension. Heat in boiling H_2O 20–30 min. and let cool. Retest heated suspension, using somatic group D, C_1, and Vi antisera. Vi-positive cultures which react with somatic group D antiserum are probably *Salmonella typhi*, and Vi-positive cultures which react with somatic group C_1 antiserum are probably *Salmonella paratyphi C*. For these cultures to be classified as *Salmonella* sp., they must have characteristics of *Salmonellae* as in Table **37.X24** or **37.X26**. Heated Vi-positive cultures which do not react with any individual somatic serum but continue to react with Vi antiserum probably belong to *Citrobacter* group and are not *Salmonella*. Confirm conclusion by biochemical tests listed in Table **37.X26**.

Cultures that give positive somatic (O) test with any individual somatic (O) antiserum are recorded as positive for that somatic (O) group; cultures that do not react with any individual somatic (O) antisera are recorded as negative for individual group somatic (O) test.

37.X31 Polyvalent Flagellar (H) Test Tube Test

Place 0.5 ml appropriately dild *Salmonella* polyvalent flagellar (H) antiserum, **37.X17(j)**,

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in 10 × 75 or 13 × 100 mm serological test tube and add 0.5 ml antigen to be tested: formalized brain-heart infusion broth, **37.X22(b)**(*1*), or 0.5 ml formalized tryptone broth culture, **37.X22(b)**(*2*) or **37.X23(c)**(*4*). If formalized culture contains granular particles, pellicles, or sediment, also prep. saline control by mixing 0.5 ml formalized saline soln, **37.X17(g)**, with 0.5 ml formalized tryptone broth culture in same size serological test tube. Incubate mixts 1 hr in H_2O bath at 50°. Observe preliminary results at 15-min. intervals and read final results at 1 hr.

Classify polyvalent flagellar (H) test as:

(a) *Positive.*—Agglutination in culture-formalized saline-serum mixt. and no agglutination in culture-formalized saline mixt.

(b) *Negative.*—No agglutination in culture-formalized saline-serum mixt. (Polyvalent flagellar antiserum does not contain agglutinins for antigens of some *Salmonellae* isolated from foods. Negative flagellar reactions occur with *Salmonellae* serotypes whose corresponding agglutinins are not contained in the antisera (i.e., *S. simsbury*, z_{27}; *S. wichita*, z_{37}; *S. chittagong*, z_{35}).

(c) *Non-specific.*—Both mixts agglutinate. Requires addnl testing as in *Identification of Enterobacteriaceae*.

Cultures that give typical biochemical results as *Salmonellae* but do not agglutinate in *Salmonella* flagellar (H) antisera must be tested to det. if sufficient flagellar (H) antigens are present. Test motility of culture as follows:

Inoculate petri dish contg motility test medium, **37.X16(n)**, with 3 mm loopful TSI culture by stabbing medium once, 10 mm from edge of plate to depth of 2–3 mm. (Do not stab to bottom of plate with inoculum.) Do not inoculate any other portion of plate. Incubate 24 hr at 35°. When organism has migrated 40 mm or more toward other side of plate, it is sufficiently motile to retest.

Transfer 3 mm loopful of growth which migrated farthest from inoculation point into tube of tryptone broth, **37.X16(h)**. Incubate 24 ± 2 hr at 35°. Retest this culture by adding equal amt formalized physiological saline soln, **37.X17(g)**, and repeat *Salmonella* serological flagellar (H) test, **37.X31** or **37.X32**.

Incubate cultures that are not motile after first 24 hr incubation for addnl 24 hr at 35°. If still negative, incubate 5 days at 25° before classifying as non-motile (flagellar (H) antigen not detected).

Cultures that are non-motile or cultures that are *Salmonella* serological flagellar (H) test-negative, when retested, are classified according to results of other tests in *Identification of Enterobacteriaceae*.

37.X32 "Spicer-Edwards" Flagellar (H) Test Tube Test

(Alternative to polyvalent flagellar (H) test tube test, **37.X31**, to det. presence or absence of flagellar (H) antigens.)

Test each culture, using each of the 7 "Spicer-Edwards" flagellar (H) antisera, **37.X17(k)**. Perform test as in **37.X31**, using 1 of the 7 "Spicer-Edwards" (H) antisera for each test instead of *Salmonella* polyvalent flagellar (H) antiserum. Since there are 7 "Spicer-Edwards" antisera, each culture must be tested 7 times.

Positive agglutination indicates presence of flagellar (H) antigen. Identify by comparing pattern of agglutination reactions obtained with agglutinins known to be present in each of the 7 "Spicer-Edwards" (H) antisera. Results of these reactions are shown in table of **37.X17(k)**.

If culture produces positive agglutination when tested with each of the 4 "Spicer-Edwards" antisera 1, 2, 3, and 4 (4 plus pattern), then results indicate presence of non-specific antigen other than *Salmonella* antigen or presence of more than single *Salmonella* H antigen which cannot be identified with this antisera until antigens are sepd.

38. MICROCHEMICAL METHODS

No additions, deletions, or other changes.

39. VITAMINS AND OTHER NUTRIENTS

(1) The official, final action method for vitamin C (ascorbic acid), **39.040–39.041**, was revised as follows and the revised method was adopted as official, first action:

(A) Substitute following for **39.040(a)** and preceding parenthetical phrase:

(Applicable to detn of reduced ascorbic acid. Not applicable in presence of ferrous Fe, stannous Sn, cuprous Cu, SO_2, sulfite, or thiosulfate. *See* NOTE.)

Principles

Ascorbic acid reduces oxidation-reduction indicator dye, 2,6-dichloroindophenol, to colorless soln. At end point, excess unreduced dye is rose pink in acid soln. Vitamin is extd and titrn performed in presence of HPO_3-HOAc or HPO_3-HOAc-H_2SO_4 soln to maintain proper acidity for reaction and to avoid autoxidation of acid at high pH.

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Exhibit 1061
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Changes in Official Methods of Analysis Made at the Eightieth Annual Meeting, October 10–13, 1966

The following changes in the Methods of the Association become effective, as provided in Section 5 of the Bylaws, on the thirtieth day from the date of publication of this report, February 15, 1967. References are to the 10th Edition, 1965.

The number following each compound for which a determination is given is the registry number assigned to it by the *Chemical Abstracts Service*. This is a unique number for that compound; it will be useful in identifying the compound in a chemical information system which is in the process of development. Different salts or derivatives of a given compound as well as different stereochemical isomers will receive different numbers.

The following abbreviations were added to pp. xix–xx and are being used starting with changes made at the 80th Annual Meeting:

amt	amount
amp.	ampere(s)
com.	commercial, commercially
compd	compound
est.	estimate
estd	estimated
estg	estimating
estn	estimation
ir	infrared
ng	nanogram(s), 0.001 μg
qual.	qualitative, qualitatively
quant.	quantitative, quantitatively
sep.	separate, separately
uv	ultraviolet

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1. AGRICULTURAL LIMING MATERIALS

The official, final action EDTA titration methods, **1.021–1.023**, were revised as follows:

(A) The equation in **1.023(a)** was changed to read: "% Ca = (Titer EDTA std soln for Ca) × ml EDTA std soln × 2/g sample."

(B) The equation in **1.023(b)** was changed to read: "% Mg = (Titer EDTA std soln for Mg) × [(ml EDTA std soln in Ca + Mg titn) − (ml EDTA std soln in Ca titn)] × 2/g sample," and in line 2 of the same section, "Mg.) Pipet" was changed to read: "Mg.) For Ca + Mg, pipet."

(C) The equation in **1.023(c)** was changed to read: "% Mg = (Titer EDTA std soln for Mg) × ml EDTA std 0.1% soln × 2/g sample."

2. FERTILIZERS

(1) The official, final action method for sampling solid fertilizers, **2.001**, was revised as follows to include a sampling pattern which was adopted as official, first action:

(**a**) *Bagged fertilizers.—Official, Final Action.*—Use slotted single or double tube, or slotted tube and rod, with solid cone tip at one end. Take sample as follows: Lay bag horizontally and remove core diagonally from end to end. From lots of 10 bags or more, take core from each of 10 bags. When necessary to sample lots of <10 bags, take 10 cores but at least 1 core from each bag present. For small packages (10 lb or less) take 1 entire package as sample.

(**b**) *Bulk fertilizers.—Official, First Action.*—Use double tube compartmented trier, such as 524 Grain Probe or Missouri trier (available from Seedboro Equipment Co., 618 W. Jackson Blvd., Chicago, Ill. 60606) with solid cone tip; effective sampling length not <4′ and sepd compartments not >6″ long (or trier of other design that has been demonstrated to draw representative vertical core). Draw 10 vertical cores distributed in std concentric sampling pattern of such design that each core represents approx. equal fractions of lot:

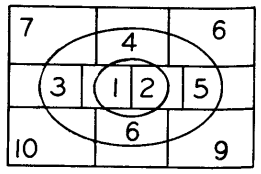


Fig. 1—Sampling pattern.

Bulk shipments may be sampled at time of loading or unloading by passing National Plant Food Institute type of sampling cup thru entire stream of material as it drops from belt or chute. Make sampling such as to assure not <10 equal-timed-spaced passes thruout transfer operation.

(c) *Preparation of sample.—Official, Final Action.*—Place composite sample in airtight container and deliver entire sample to laboratory. If composite sample is reduced in field, use riffle.

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(2) The official, first action method for slow-release nitrogen, *This Journal* **49**, 206 (1966), was adopted as official, final action.

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(3) The following sections of the official, first action atomic absorption method, **2.086–2.091**, were revised to read as shown and the method for calcium was adopted as official, first action:

2.086 ***Apparatus***

Atomic absorption spectrophotometer.—Several com. models are available. Since each maker's design is somewhat different, with varying requirements of light source, burner flow rate, and detector sensitivity, only general outline of operating parameters is given in Table **2.087**. Operator must become familiar with settings and procedures adapted to his own equipment and use this table only as guide to concn ranges and flame conditions.

2.087 ***Operating Parameters***

Element	Wavelength, Å	Flame	Range μg/ml	Remarks
Ca	4227	Rich Air-C_2H_2	2–20	1% La, 1% HCl
	4227	Rich N_2O-C_2H_2	2–20	Requires special burner
Cu	3247	Air-C_2H_2	2–20	
Fe	2483	Rich Air-C_2H_2	2–20	
Mg	2852	Rich Air-C_2H_2	0.2–2	May need La
Mn	2795	Air-C_2H_2	2–20	
Zn	2138	Air-C_2H_2	0.5–5	

2.088 ***Reagents***

(Do not use <2 ml pipets or <25 ml vol. flasks. Auto-dilutors may be used. Prep. std solns in 0–20 μg range fresh daily.)

(**a**) *Calcium stock soln.*—25 μg Ca/ml. Dissolve 1.249 g $CaCO_3$ in min. amount 3*N* HCl. Dil. to 1 L. Dil. 50 ml to 1 L.

(**b**) *Calcium standard solns.*—0, 5, 10, 15, and 20 μg Ca/ml contg 1% La and ca 1% HCl. To 25 ml vol. flasks add 0, 5, 10, 15, and 20 ml Ca stock soln. Add 5 ml La stock soln and dil. to 25 ml.

(**c**) *Copper stock soln.*—1000 μg Cu/ml. Dissolve 1.000 g pure Cu metal in min. amount HNO_3 and add 5 ml HCl. Evap. almost to dryness and dil. to 1 L with 0.1*N* HCl.

(**d**) *Iron stock soln.*—1000 μg Fe/ml. Dissolve 1.000 g pure Fe wire in ca 30 ml 6*N* HCl with boiling. Dil. to 1 L.

(**e**) *Lanthanum stock soln.*—50 g La/L ca 5% HCl. Dissolve 58.65 g La_2O_3 (99.99%, Alfa Inorganics, Beverly, Mass. 01915, or equiv.) in 250 ml HCl, adding acid slowly. Dil. to 1 L.

(**f**) *Magnesium stock soln.*—1000 μg Mg/ml. Place 1.000 g pure Mg metal in 50 ml H_2O and add slowly 10 ml HCl. Dil. to 1 L.

(**g**) *Manganese stock soln.*—1000 μg Mn/ml. Dissolve 1.582 g MnO_2 in ca 30 ml 6*N* HCl. Boil to remove Cl and dil. to 1 L.

(**h**) *Zinc stock soln.*—1000 μg Zn/ml. Dissolve 1.000 g pure Zn metal in ca 10 ml 6*N* HCl. Dil. to 1 L.

(**i**) *Other standard solns.*—Dil. aliquots of solns (**c**), (**d**), (**f**), (**g**), and (**h**) with 0.5*N* HCl to make at least 4 std solns of each element within range of detn.

2.089 ***Preparation of Sample Solutions***

(**a**) *Inorganic materials and mixed fertilizers.*—Dissolve 1.00 g well-ground sample in 10 ml HCl in 150 ml beaker. Boil and evap. soln nearly to dryness on hot plate. CAUTION: *Do not bake residue.* Redissolve residue in 20 ml 2*N* HCl, boiling gently if necessary. Filter thru fast paper into 100 ml vol. flask, washing paper and residue thoroly with H_2O. Measure absorption of soln directly, or dil. with 0.5*N* HCl to obtain solns within ranges of instrument. If Ca is to be detd, add sufficient La stock soln to make final diln 1% La (i.e., 5 ml La to 25 ml flask, 20 ml to 100 ml flask, etc.).

(**b**) *Fertilizers containing organic matter (tankage, corncobs, cottonseed hulls, etc.).*—Place 1.00 g sample in 150 ml beaker (Pyrex or equiv.). Char on hot plate and ignite 1 hr at 500° with muffle door propped open to allow free access of air. Break up cake with stirring rod and dissolve in 10 ml HCl as in (**a**).

(**c**) *Fertilizers containing fritted trace ele-*

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net wt <3 lb and 12″ sieve for larger containers. Drain 2 min., and reweigh sieve and mushrooms to det. drained wt mushrooms.

Rinse container, and use rinsings and several addnl portions H_2O to rinse mushrooms on sieve (ca 500 ml total). Combine drained liquid with rinsings and filter thru ruled paper. Examine residue on paper microscopically and det. total no. maggots in liquid.

Place 100 g drained mushrooms in high speed blender (*see* (d)). Add 300 ml H_2O and blend 30–45 sec. at 3000–3500 rpm. Attain proper speed quickly by boosting setting to 1.5–2 × desired setting on variable transformer for few sec. at start. Fragments of mushrooms after blending should not be >3–5 mm long. Pour mixt. into nested set of 8″ Nos. 20, 40, and 140 sieves. Rinse tissue 2–3 min. with spray of tap H_2O from aerator. Discard material on No. 20 sieve. Transfer residue from No. 40 sieve to 600 ml beaker with H_2O and bring total vol. to ca 100 ml. Add 5 ml satd aq. crystal violet soln and heat to boiling. Pour stained mixt. into No. 40 sieve. Wash mushroom tissue, and maggots, if any, to edge of sieve and remove excess stain with tap H_2O from aerator. Using wash bottle contg NaOCl soln, (b), and gentle spray of tap H_2O from aerator, alternately spray tissue with H_2O and NaOCl soln until stain has been removed from mushroom tissue. Wash tissue into 600 ml beaker and transfer to ruled paper, using vac. Avoid obscuring maggots with mushroom tissues. (Not more than 2–3 papers should be necessary.)

Transfer residue from No. 140 sieve to 600 ml beaker with H_2O and repeat staining, bleaching, and filtering as above.

Examine papers for maggots and other extraneous materials at 10–30×. Maggots are stained dark violet. Det. no. maggots in 100 g drained mushrooms and add to this value no. in proportionate amt of drained liquid calcd as follows:

(100/total g drained mushrooms) × total no. maggots in liquid.

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(2) The following Joint AOAC–ASBC (American Society of Brewing Chemists) method for the determination of aphids in hops was adopted as official, first action:

Apparatus and Reagents

(a) *Flotation solns.*—Satd $Na_2B_4O_7$ soln, 100 g borax/L H_2O.

(b) *White gasoline.*—*See* 36.002(o).

(c) *Iodine stain.*—Dissolve 0.5 g I and 1.5 g KI in 25 ml H_2O.

(d) *Blender.* — "Intensifier" Twin Shell Blender, Patterson-Kelly Co., East Stroudsburg, Pa. 18301, or equiv.

Determination

Mix 10 g representative sample in 100 ml satd borax soln in 2 L Wildman trap flask. Bring to *slow* boil. Do *not* let mixt. boil onto sides of flask; avert by keeping boiling to min. and by washing down sides of flask with H_2O if product boils onto sides. Boil 1.5 hr, and cool to room temp. Fill flask to 1600 ml with H_2O, and 35 ml gasoline, and stir vigorously 10 sec. Fill flask with H_2O, let stand 30 min., and trap off. Perform second trapping, using 25 ml gasoline, stirring 10 sec., and letting flask stand 15 min. Wash neck of flask with 95% isopropyl alcohol. Pour trappings onto ruled paper(s), add 10–12 drops I stain, and examine microscopically. If excess plant tissue is present in trappings, pour trappings thru 5″ No. 10 sieve held over paper. Wash plant tissue on sieve with alcohol onto filter paper to remove any adhering insects.

Count as aphid any whole aphid or part contg head. Count individually aphid cast skins and other insects.

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(3) The following method for the determination of filth in cheese was adopted as official, first action to replace 36.014(d), (e), and (f):

(Not applicable to cheese contg herbs, spices, or mold thruout.)

Reagents and Apparatus

(a) *Dispersing soln.*—Dissolve 20 g Na_4-EDTA in 100 ml H_2O for each detn.

(b) *Pancreatin soln.*—Mix 40 g pancreatin with 400 ml ca 37° H_2O (not >40°). Use 70 g pancreatin and 700 ml ca 37° H_2O for ricotta. Let stand 30 min., stirring intermittently. Centrifuge at ca 1500 rpm and filter supernatant liquid thru 9 cm S&S No. 8 paper.

(c) *Patapar paper (vegetable parchment).*—Paterson Parchment Paper Co., Bristol, Pa. 19007, or equiv.

Determination

Prep. cheese for sampling by trimming and discarding thin layers to remove all "old" cut surfaces and to keep paraffin coating and mold out of sample. Cut and break up 225 g trimmed cheese into 4 L beaker. Add ca 700 ml ca 55° filtered H_2O. Set beaker under mechanical stirrer, 36.001(c), and stir 15 min., maintaining mixt. at 55°. Add 100 ml dispersing soln, stir, and adjust mixt. to pH 8 with NH_4OH or dil. HCl (1 + 2). Rinse sides of beaker free of adhering cheese particles with ca 60° H_2O. Maintain pH 8 by addn of NH_4OH and keep adding ca 60° H_2O to dil. cheese mixt. to ca 3 L. If foaming occurs, place wet Patapar paper, split to accommodate stirrer blades, over top of beaker to break foam. Continue stirring until cheese becomes finely dispersed.

Cool dispersion to 40° and adjust to pH 8 with NH_4OH or HCl (1 + 2). Add 300 ml pancreatin soln (except use 600 ml pancreatin soln for ricotta). Let mixt. digest at about but not >40° with continued stirring ca 1.5 hr. Maintain pH 8 by addn of NH_4OH.

After digestion with pancreatin, place beaker on hot plate and heat to 65–68°; continue mechanical stirring. Adjust to pH 6.0 ± 0.2 with HCl (1 + 2). Carefully adjust stirrer blades close to bottom of beaker to pick up any cheese particles which settle. Continue stirring 15 min. or until cheese appears completely solubilized. Rinse inside of beaker, stirrer blades, etc., with ca 65° H_2O and filter thru ruled paper, using ca 65° H_2O and later alcohol to rinse beaker. If filtration becomes slow (e.g., cream cheese), let paper clear, wash with alcohol, and use addnl paper. Mixt. will filter more easily if 60 mesh screen (ca 5 cm diam.) is placed under paper and small amt mixt. is allowed to suck dry before filtering is continued. Examine paper microscopically.

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(4) The first sentence of the official, first action method for determining filth in cheese containing mold, plant tissues, and spices, 36.014(g), was revised to read as follows:

Disperse cheese as in 36.014(c) or first par. of *Determination* in method for determining filth in cheese, item (3) above.

37. MICROBIOLOGICAL METHODS

The following method for the detection and identification of Salmonella from dried whole egg, dried egg yolk, and dried egg white was adopted as official, first action:

Method described is minimal. Depending upon history of sample, addnl types of examinations may be applied. Use *Identification of Enterobacteriaceae,* P. R. Edwards and W. H. Ewing, Burgess Publishing Co., Minneapolis, Minn., 2nd Ed., 1962, as guide for further study of isolated microorganisms.

37.X23 Testing Urease-Negative Cultures

(*See* introduction to *Media and Reagents, Changes in Methods, This Journal* **49**, 247 (1966).)

Sizes of culture media containers (test tubes, flasks, and petri dishes) are specified in prepn of each medium. Different size containers may be used if they give identical results. All media containers must have covers, caps, or plugs which prevent contamination but maintain acrobic conditions unless otherwise directed.)

(a) *Lactose broth, 0.5%.*—Dissolve 5.0 g polypeptone or peptone, 3.0 g beef ext., and 5.0 g lactose in 1 L H_2O. Dispense 225 ml portions in 500 ml screw-cap bottles or flasks, and autoclave 15 min. at 121°. Final pH, 6.8 ± 0.1.

(b)(*1*) *Selenite cystine broth.*—Suspend 5.0 g tryptone or polypeptone, 4.0 g lactose, 10.0 g anhyd. Na_2HPO_4, 4.0 g $NaHSeO_3$, and 0.01 g L-cystine in 1 L H_2O and mix thoroly. Heat with frequent agitation. Dispense 10 ml portions in sterile 16 × 150 mm test tubes. Heat 10 min. in flowing steam. *Do not autoclave.* Final pH, 7.0 ± 0.1. Medium is not sterile. Use same day as prepd.

(*2*) *Selenite cystine broth (North and Bartram).*—Prep. as in (*1*), using 5.0 g polypeptone or 4.0 g tryptone, 4.0 g lactose, 4.0 g $NaHSeO_3$, 5.5 g anhyd. Na_2HPO_4, 4.5 g anhyd. KH_2PO_4, and 1 ml (10 mg) 1% L-cystine soln prepd by dissolving 1.0 g L-cystine in 15 ml 1*N* NaOH and dilg to 100 ml with sterile H_2O.

(c) *Tetrathionate broth (with iodine and brilliant green).*—Suspend 5.0 g polypeptone, 1.0 g bile salts, 10 g $CaCO_3$, and 30 g $Na_2S_2O_3 \cdot 5H_2O$ in 1 L H_2O, mix thoroly, and heat to boiling. (Ppt will not dissolve completely.) Cool to <45° and store at 5–8°. Prep. I-KI soln by dissolving 5 g KI in 5 ml sterile H_2O, adding 6 g resublimed I, dissolving, and dilg to 20 ml with sterile H_2O. Prep. brilliant green soln by dissolving 0.1 g dye in sterile H_2O and dilg to 100 ml. On day medium is used, add 20 ml I-KI soln and 10 ml brilliant green soln per 1 L basal broth. Resuspend ppt by gentle agitation and aseptically dispense 10 ml portions in 20 × 150 or 16 × 150 mm sterile test tubes. *Do not heat medium after addn of I-KI and dye solns.*

(d) *Brilliant green agar.*—Use com. dehydrated medium contg 3.0 g yeast ext., 10 g

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proteose peptone No. 3 or polypeptone, 5.0 g NaCl, 10 g lactose, 10 g sucrose, 0.08 g phenol red, 0.0125 g brilliant green, and 20 g agar. Suspend medium in 1 L H_2O, mix thoroly, and heat with occasional agitation. Boil 1 min. to dissolve. Autoclave 1 L portions 12 min. at 121°. (Addnl heating decreases selectivity of medium; less heating increases selectivity.) Cool to 45–50° and pour 20 ml portions into 15 × 100 mm petri dishes. Let dry ca 2 hr with covers partially removed; then close plates. Final pH, 6.9 ± 0.1.

(e) *Salmonella Shigella agar.*—Suspend 5.0 g beef ext., 5.0 g polypeptone or proteose peptone, 10 g lactose, 8.5 g bile salts mixt., 8.5 g Na citrate, 8.5 g $Na_2S_2O_3.5H_2O$, 1.0 g ferric citrate, 13.5 g agar, 0.00033 g brilliant green, and 0.025 g neutral red in 1 L H_2O, and mix thoroly until homogeneous. Heat with occasional agitation and boil 1–2 min. until ingredients dissolve. Cool to 45–50° and pour 20 ml portions into 15 × 100 mm petri dishes. Let dry ca 2 hr with covers partially removed; then close plates. Final pH, 7.0 ± 0.1. *Do not autoclave.*

(f) *Bismuth sulfite agar (Wilson and Blair).*—Suspend 10 g polypeptone or peptone, 5.0 g beef ext., 5.0 g dextrose, 4.0 g $Na_2HPO_4.12H_2O$, 0.3 g $FeSO_4.7H_2O$, 8.0 g $Bi_2(SO_3)_3$ indicator, 0.025 g brilliant green, and 20 g agar in 1 L H_2O, mix thoroly, and heat with occasional agitation. Boil ca 1 min. to obtain uniform suspension. (Ppt will not dissolve.) Cool to 45–50°. Suspend ppt by gentle agitation and pour 20 ml portions into 15 × 100 mm petri dishes. Let dry ca 2 hr with covers partially removed; then close plates. Final pH, 7.6 ± 0.1. *Do not autoclave.* Selectivity of plates decreases 48 hr after prepn.

(g) *Triple sugar iron agar (TSI agar).*—Suspend ingredients (*1*) or (*2*) in 1 L H_2O, mix thoroly, and heat with occasional agitation. Boil ca 1 min. until ingredients dissolve. Fill 16 × 150 mm tubes 1/3 full and cap or plug so that aerobic conditions are maintained during use. Autoclave 12 min. at 121°. Before medium solidifies, place tubes in slanted position so that deep butts (ca 1¼″) and adequate slants (ca 2″) are formed on solidification.

(*1*) 20 g polypeptone, 5.0 g NaCl, 10 g lactose, 10 g sucrose, 1 g dextrose, 0.2 g $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.2 g $Na_2S_2O_3$, 0.025 g phenol red, and 13 g agar. Final pH, 7.3 ± 0.1.

(*2*) 3.0 g beef ext., 3.0 g yeast ext., 15 g peptone, 5.0 g proteose-peptone, 1.0 g dextrose, 10 g lactose, 10 g sucrose, 0.2 g $FeSO_4.7H_2O$, 5.0 g NaCl, 0.3 g $Na_2S_2O_3$, 0.024 g phenol red, and 12 g agar. Final pH, 7.4 ± 0.1.

(h) *Tryptone broth.*—Prep. as in (j), *Media and Reagents, This Journal* **49**, 248 (1966), using 16 or 20 × 150 mm test tubes.

(i) *Methyl red–Voges-Proskauer (MR-VP) medium.*—Prep. as in (k), *Media and Reagents, This Journal* **49**, 248 (1966), using 16 or 20 × 150 mm test tubes.

(j) *Simmon's citrate agar.*—Dissolve 2.0 g Na citrate, 5.0 g NaCl, 1.0 g K_2HPO_4, 1.0 g $NH_4H_2PO_4$, 0.2 g $MgSO_4$, 0.08 g bromothymol blue, and 15 g agar in 1 L H_2O, and heat gently with occasional agitation. Boil 1–2 min. until ingredients dissolve. Fill 13 or 16 × 150 mm test tubes 1/3 full and cap or plug so that aerobic conditions are maintained during use. Autoclave 15 min. at 121°. Before medium solidifies, place tubes in slanted position so that deep butts (ca ¾ or 1¼″, resp.) and adequate slants (ca 1½ or 2″, resp.) are formed on solidification.

(k)(*1*) *Urea broth.*—Dissolve 20 g urea, 0.1 g yeast ext., 9.1 g KH_2PO_4, 9.5 g Na_2HPO_4, and 0.01 g phenol red in 1 L H_2O. *Do not heat.* Sterilize by filtration and aseptically dispense 1.5–3 ml portions in 13 × 100 mm sterile test tubes. Final pH, 6.8 ± 0.1.

(*2*) *Rapid urea broth.*—Prep. as in (*1*), using 0.091 and 0.095 g phosphate salts, resp.

(l) *Malonate broth.*—Dissolve 1.0 g yeast ext., 2.0 g $(NH_4)_2SO_4$, 0.6 g K_2HPO_4, 0.4 g KH_2PO_4, 2.0 g NaCl, 3.0 g Na malonate, 0.25 g dextrose, and 0.025 g bromothymol blue in 1 L H_2O, heating if necessary until dissolved. Dispense 3 ml portions in 13 × 100 mm test tubes and autoclave 15 min. at 121°. Final pH, 6.7 ± 0.1.

(m)(*1*) *Lysine iron agar (Edwards and Fife).*—Dissolve 5.0 g gelysate or peptone, 3.0 g yeast ext., 1.0 g dextrose, 10 g L-lysine, 0.5 g ferric ammonium citrate, 0.04 g $Na_2S_2O_3$, 0.02 g bromocresol purple, and 15 g agar in 1 L H_2O, heating until dissolved. Dispense 4 ml portions in 13 × 100 ml test tubes and cap or plug so that aerobic conditions are maintained during use. Autoclave 12 min. at 121°. Before medium solidifies, place tubes in slanted position so that deep butts (ca 1¼″) and adequate slants (ca 2″) are formed on solidification. Final pH, 6.7 ± 0.1.

(*2*) *Lysine decarboxylase broth (Falkow).*—Dissolve 5.0 g gelysate or peptone, 3.0 g yeast ext., 1.0 g dextrose, 5.0 g L-lysine, and 0.02 g bromocresol purple in 1 L H_2O, heating until dissolved. Dispense 5 ml portions in 16 × 125 mm screw-cap test tubes. Autoclave, loosely capped, 15 min. at 121°. Screw caps tightly for storage and after inoculation. Final pH, 6.5–6.8.

(n) *Motility test medium (semisolid medium).*—Dissolve 3.0 g beef ext., 10 g peptone or gelysate, 5.0 g NaCl, and 4.0 g agar in 1 L H_2O and heat gently with occasional agitation. Boil 1–2 min. to dissolve. If medium is to be stored, dispense 20 ml portions into screw-cap containers, replacing caps loosely. Autoclave 15 min. at 121°. Cool to 45°. To store, screw caps on tightly and refrigerate at 5–8°. To use, remelt in boiling H_2O or flowing steam and cool to 45°. Aseptically dispense 20 ml portions in 15 × 100 mm petri dishes and let solidify with dish completely covered. Use plates same day as prepd. Final pH, 7.4 ± 0.1.

(o) *Potassium cyanide (KCN) broth.*—Dissolve 3.0 g proteose peptone No. 3 or polypeptone, 5.0 g NaCl, 0.225 g KH_2PO_4, and 5.64 g Na_2HPO_4 in 1 L H_2O. Autoclave 15 min. at 121°. Cool and refrigerate at 5–8°. Final pH, 7.6 ± 0.1. Dissolve 0.5 g KCN in 100 ml cold (5–8°) sterile H_2O. Using sterile bulb pipet or sterile syringe (*do not pipet by mouth*), aseptically add 15 ml cold KCN soln per L cold, sterile basal broth. Mix thoroly with gentle agitation and aseptically dispense 1–1.5 ml portions in sterile 13 × 100 mm test tubes. Using aseptic technic, immediately stopper tubes with No. 2 corks impregnated with paraffin. Prep. corks by boiling in paraffin ca 5 min. Place corks in tubes so that paraffin does not flow into broth but forms good seal between rim of tube and cork. Medium stored at 5–8° is usually stable ca 2 weeks.

(p)(*1*) *Phenol red carbohydrate broth.*—Dissolve 10 g trypticase or proteose peptone No. 3, 5.0 g NaCl, 1.0 g beef ext. (optional), and 0.018 g phenol red in 1 L H_2O and heat with gentle agitation until dissolved. Dissolve 5 g dulcitol, 10 g lactose, or 10 g sucrose (as specified in title of test) in this basal broth. Dispense 2.5 ml portions in 13 × 100 mm test tubes contg inverted 6 × 50 mm fermentation tubes. Autoclave 10 min. at 118° (12 psi). Final pH, 7.3 ± 0.1. Alternatively, dissolve ingredients, omitting carbohydrate, in 800 ml H_2O with heat and occasional agitation. Dispense 2.0 ml portions in 13 × 100 mm test tubes contg inverted fermentation tubes. Autoclave 15 min. at 118° and let cool. Dissolve carbohydrate in 200 ml H_2O and sterilize by passing soln thru bacteria-retaining filter. Aseptically add 0.5 ml sterile filtrate to each tube of sterilized broth after cooling to <45°. Shake gently to mix. Final pH, 7.4 ± 0.1.

(*2*) *Purple carbohydrate broth.*—Prep. as in (*1*), using as basal broth 10 g proteose peptone No. 3 or gelysate, 5.0 g NaCl, and 0.015 or 0.020 g bromocresol purple. Final pH, 6.8 ± 0.1.

(q) *MacConkey agar.*—Suspend 3.0 g proteose peptone or polypeptone, 17 g peptone or gelysate, 10 g lactose, 1.5 g bile salts No. 3 or bile salts mixt., 5.0 g NaCl, 0.03 g neutral red, 0.001 g crystal violet, and 13.5 g agar in 1 L H_2O and mix thoroly until homogeneous. Heat, with occasional agitation, and boil 1–2 min. until ingredients dissolve. Autoclave 15 min. at 121°. Cool to 45–50° and pour 20 ml portions into 15 × 100 mm petri dishes. Let dry at least 2 hr with plates covered. Do not use wet plates. Final pH, 7.1 ± 0.1.

(r) *Brain heart infusion broth.*—Prep. as in (h), *Media and Reagents, This Journal* **49**, 248 (1966), dispensing 5 ml portions in 16 × 150 mm test tubes.

37.X17 **Diagnostic Reagents**

(a) *Kovac's reagent for indole test.*—Dissolve 5 g *p*-dimethylaminobenzaldehyde in 75 ml amyl alcohol and slowly add 25 ml HCl.

(b) *Voges-Proskauer (VP) test reagents.*—(*1*) *Alpha-naphthol soln.*—5%. Dissolve 5.0 g *a*-naphthol in 100 ml absolute alcohol.

(*2*) *Potassium hydroxide soln.*—40%. Dissolve 40 g KOH in H_2O and dil. to 100 ml.

(c) *Sodium hydroxide soln.*—1*N*. Dissolve 42.11 g 95% reagent NaOH in sterile H_2O and dil. to 1 L.

(d) *Hydrochloric acid soln.*—1*N*. Dil. 89 ml to 1 L with sterile H_2O.

(e) *Methyl red indicator.*—Dissolve 0.10 g Me red in 300 ml alcohol and dil. to 500 ml with H_2O.

(f) *Sterile physiological saline soln.*—Dissolve 8.5 g NaCl in H_2O and dil. to 1 L. Autoclave 15 min. at 121° and cool to room temp.

(g) *Formalized physiological saline soln.*—Add 6 ml HCHO soln (36–38%) to sterile saline soln, (f), mix, and store in tightly stoppered containers.

(h) *Salmonella polyvalent somatic (O) antiserum.*—(*Identification of the Salmonella Serotypes*, No. 168, Difco Labs., 920 Henry St., Detroit, Mich. 48201, July 1963, p. 5.) Contains agglutinins for at least somatic (O) antigens: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 19, 22, 23, 24, 25, and Vi. They are

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agglutinins for somatic (O) groups: A, B, C_1, C_2, D, E_1, E_2, E_4, F, G, H, I, and Vi.

(i) *Salmonella individual somatic (O) antisera.*—(*See* reference in (h).) For at least each of the somatic (O) groups listed in (h).

(j) *Salmonella polyvalent flagellar (H) antiserum.*—(*See* p. 11 of reference in (h).) Contains agglutinins for at least the following flagellar (H) antigens: a b, c, d, eh, en complex (enz_{15}, enx), fg, fgt, gm, gmq, gms, gp, gpu, gq, gst, gt, i, k, lv, lw, lz_{13}, lz_{28}, mt, r, y, z, z_4z_{23}, z_1z_{24}, z_4z_{32}, z_6, z_{10}, z_{29}; 1, 2; 1, 5; 1, 6; and 1, 7.

(k) *Salmonella "Spicer-Edwards" flagellar (H) antisera.*—(From p. 10 of reference in (h).) Consists of 7 pooled or polyvalent antisera which react as follows:

Salmonella H Antisera Spicer-Edwards and H Antigens with Which Each Reacts

	Salmonella H Antisera Spicer-Edwards			
H Antigens	1	2	3	4
a	+	+	+	–
b	+	+	–	+
c	+	+	–	–
d	+	–	+	+
eh	+	–	+	–
G Complex[a]	+	–	–	+
i	+	–	–	–
k	–	+	+	+
r	–	+	–	+
y	–	+	–	–
z	–	–	+	+
z_4 Complex[b]	–	–	+	–
z_{10}	–	–	–	+
z_{29}	–	+	+	–
H Antigens	Salmonella H Antisera			
enx, enz_{15}	en complex			
lv, lw, lz_{13}, lz_{28}	L complex			
1, 2; 1, 5; 1, 6; 1, 7	1 complex			

[a] The G complex component of Salmonella *H* antisera Spicer-Edwards 1 and 4 reacts with antigens fg, fgt, gm, gms, gmt, gp, gpu, gq, gst, ms, and mt.
[b] The z_4 complex component reacts with z_4z_{23}, z_4z_{24}, and z_4z_{32}.
(From Difco Laboratories, Detroit, Mich.)

(l) *pH Test paper.*—Min. range 6.0–7.6 with max. gradations of 0.4 pH for each color change.

Detection

37.X18 Preparation of Sample Cultures

Open sample container aseptically and weigh aseptically 25 g sample into sterile, empty, wide-mouth, screw-cap pint jar. Add ca 15 ml sterile lactose broth, **37.X16(a)**. Stir with sterile glass rod, sterile spoon, or sterile tongue depressor to smooth suspension. Add 3 addnl portions of lactose broth, 10, 10, and 190 ml for total of 225 ml. Stir after each addn until sample is suspended without lumps. Cap jar securely and let stand at room temp. 60 min. Mix well by shaking, and det. pH with test paper, **37.X17(l)**. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1*N* NaOH or HCl, **37.X17(c)** or **(d)**, capping jar securely and mixing well before detg final pH. Loosen jar cap ca ¼ turn and incubate 24 ± 2 hr at 35°.

37.X19 Isolation

(a) *Growth on selective media.*—Shake gently incubated sample mixt., **37.X18**, and transfer 1 ml to 10 ml selenite cystine broth, **37.X16(b)**(*1*) or (*2*), and addnl 1 ml to 10 ml tetrathionate broth, **37.X16(c)**. Incubate 24 ± 2 hr at 35°.

Streak 3 mm loopful of incubated selenite cystine broth on selective media plates of brilliant green agar, **37.X16(d)**. Salmonella Shigella agar, **37.X16(e)**, and $Bi_2(SO_3)_3$ agar, **37.X16(f)**. Repeat with 3 mm loopful of incubated tetrathionate broth. Incubate plates 24 ± 2 hr at 35°.

(b) *Appearance of typical Salmonella colonies.*—(*1*) *On brilliant green agar.*—Colorless, pink to fuchsia, translucent to opaque, with surrounding medium pink to red. Some *Salmonella* appear as transparent green colonies if surrounded by lactose- or sucrose-fermenting organisms which produce colonies that are yellow-green or green.

(*2*) *On Salmonella Shigella agar.*—Uncolored to pale pink, opaque, transparent or translucent, but some strains produce black-centered colonies.

(*3*) *On bismuth sulfite agar.*—Brown, grey to black, sometimes with metallic sheen. Surrounding medium is usually brown at first, turning black with increasing incubation time. Some strains produce green colonies with little or no darkening of the surrounding medium.

If plates do not have typical or suspicious colonies or do not contain growth, incubate addnl 24 hr.

37.X20 Treatment of Typical or Suspicious Colonies

(a) *Inoculation of triple sugar iron (TSI) agar.*—Pick with needle 2 or more typical or suspicious colonies, if present, from each brilliant green, Salmonella Shigella, and $Bi_2(SO_3)_3$ agar plates having growth. Inoculate TSI agar slant, **37.X16(g)**, with portion of each colony by streaking slant and stabbing butt. Store picked selective plates at 5–8° or at room temp. (ca 26°).

(b) *Presumptive positive reactions.*—Incubate TSI slants 24 ± 2 hr at 35°. Presumptive positive Salmonella cultures have alk. (red) slants and acid (yellow) butts, with or without H_2S (blackening of agar). (H_2S-negative TSI cultures which otherwise appear to be Salmonellae cannot be excluded from further examination.)

If TSI fails to give a typical Salmonella reaction, pick addnl suspicious colony from selective medium plate not giving presumptive positive culture and inoculate TSI slant as in (a).

(To detect lactose-fermenting Arizona organisms, test all TSI cultures (including yellow slants) isolated from H_2S-positive $Bi_2(SO_3)_2$ agar colonies, using lysine iron agar, **37.X16(m)**(*1*), as in **37.X23(a)**. Cultures that are lysine decarboxylase-positive and H_2S-positive are tested further to det. if they are Arizona sp., **37.X25(f)**(*2*).)

(c) *Selection for identification.*—Apply biochemical and serological identification tests to 3 presumptive positive TSI agar cultures picked from selective agar plates streaked from selenite cystine broth and to 3 presumptive positive TSI agar cultures picked from selective agar plates streaked from tetrathionate broth.

If 3 presumptive positive TSI agar cultures are not isolated from 1 set of selective agar plates, test other presumptive positive TSI agar cultures, if isolated, by biochemical and serological tests. A minimum of 6 TSI cultures are examined for each 25 g sample tested.

Identification

37.X21 Cultures

Pure cultures on TSI agar are required for inoculation of biochemical test media.

(a) *Pure cultures.*—Proceed to **37.X22**.

(b) *Mixed cultures.*—Streak any culture that appears to be mixed on MacConkey's agar, **37.X16(q)**, or brilliant green agar, **37.X16(d)**. Incubate 24 ± 2 hr at 35°.

(c) *Appearance of Salmonella colonies.*—(*1*) *On MacConkey's agar.*—Typical colonies appear transparent and colorless, sometimes with dark centers. *Salmonella* will clear areas of dptd bile caused by other organisms sometimes present in medium.

(*2*) *On brilliant green agar.*—*See* **37.X19(b)**(*1*).

Pick with needle at least 2 typical or suspicious colonies and inoculate TSI slants by streaking the slant and stabbing the butt as in **37.X20(a)**. Retest purified cultures as in **37.X20(b)**, and proceed with identification.

37.X22 Subcultures

(a) *Urease test.*—Subculture two 3 mm loopfuls of presumptive positive TSI agar culture to urea broth, **37.X16(k)**(*1*), and incubate 24 ± 2 hr at 35° or inoculate rapid urea broth, **37.X16(k)**(*2*), and incubate 2 hr in H_2O bath at 37 ± 0.5°. Discard all cultures that give positive test (purple-red color). *Salmonellae* are urease negative (no change in orange color of medium).

(b) *Serological flagellar (H) screening test.*—To reduce no. of presumptive positive TSI agar cultures carried thru identification tests, perform serological flagellar (H) screening test by transferring one 3 mm loopful of each urease-negative TSI agar culture to either:

(*1*) Brain heart infusion broth, **37.X16(r)**, (for test on same day) and incubate at 35° until visible growth occurs (ca 4–6 hr); or

(*2*) Tryptone broth, **37.X16(h)** (for test on following day), and incubate 24 ±2 hr at 35°.

To 5 ml of each of the 6 broth cultures add ca 2.5 ml formalized physiological saline soln, **37.X17(g)**. Select 2 formalized broth cultures and test with Salmonella flagellar (H) antisera, **37.X17(j)** or **(k)**, as in **37.X31** or **37.X32**.

If selected formalized broth cultures are positive, perform addnl tests on these cultures, beginning with **37.X23**, except step **37.X23(c)**(*4*) may be omitted.

If both formalized broth cultures are not positive, perform test on the 4 remaining other broth cultures (**37.X22(b)**(*1*) or (*2*)) to obtain, if possible, 2 positive cultures for addnl testing, **37.X23**.

If all urease-negative TSI cultures from sample are Salmonella serological flagellar (H) test negative, then perform addnl tests, beginning with **37.X23**, on all cultures.

37.X23 Testing Urease-Negative Cultures

Transfer 1 loopful of presumptive positive TSI agar culture to each of following media:

(a) *Lysine iron agar,* **37.X16(m)**(*1*). —Streak slant and stab butt. Replace tube cap *loosely* and incubate 48 ± 2 hr at 35°. Examine at least every 24 hr. *Salmonellae* give alk. reaction of purple color thruout medium (final color is slightly darker than original

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purple color of medium). If H_2S is produced, butt of medium is blackened. Negative test is purple or red slant and yellow butt.

If liquid medium is preferred, inoculate tube of lysine decarboxylase broth, **37.X16(m)**(*2*). Close tube cap *tightly* after inoculation and incubate 96 ± 2 hr at 35°. Examine at least every 24 hr. *Salmonellae* give alk. reaction of purple color thruout broth (final color is slightly darker than original purple color of medium). Sometimes tubes which have yellow color after 8–12 hr of incubation change to purple later. Negative test is permanent yellow color thruout broth.

(Lysine iron agar is incubated loosely capped so that aerobic conditions are maintained, while lysine decarboxylase broth is incubated tightly closed to exclude air.)

(**b**) *Phenol red dulcitol broth,* **37.X16(p)** (*1*).—Incubate 48 ± 2 hr at 35°. Examine at least every 24 hr. Most *Salmonellae* give positive test indicated by gas formation (displacement of liquid in inverted tube) and acid reaction (yellow). Negative test is alk. reaction (red) and no gas formation.

(Purple broth base with dulcitol, **37.X16**(p) (*2*), may be substituted. Positive test is acid reaction (yellow) and gas. Negative test is alk. reaction (purple).)

(**c**) *Tryptone broth,* **37.X16(h)**.—Incubate 24 ± 2 hr at 35° and test as follows:

(*1*) Transfer 3 mm loopful, excluding all solid particles, to KCN broth, **37.X16(o)**. Heat rim of tube to form good seal when restoppered. Incubate 48 ± 2 hr at 35°. *Salmonellae* do not grow in this broth as shown by lack of turbidity (negative test).

(*2*) Transfer 3 mm loopful to malonate broth, **37.X16(l)**, and incubate 48 ± 2 hr at 35°. *Salmonellae* give negative test as shown by green color (unchanged). Positive test (alk. reaction) is shown by blue color.

(*3*) Transfer 5 ml to empty test tube and add 0.2–0.3 ml Kovac's reagent, **37.X17(a)**. Positive test for indole is shown by deep red in reagent on surface of broth. Most *Salmonellae* are indole negative.

(*4*) To remainder of 24-hr tryptone broth culture (ca 5 ml) add ca 2.5 ml formalized physiological saline soln, **37.X17(g)**. Refrigerate formalized broth at 5–8° if test is to be performed on another day. Perform Salmonella serological flagellar (H) test, **37.X31**, or "Spicer-Edwards" flagellar (H) test tube test, **37.X32**, using formalized broth culture as flagellar (H) antigen to be tested.

(**d**) *Tests indicating absence of Salmonella.* —Discard, as not *Salmonella,* cultures that show either:

(*1*) Positive indole test (red) and negative *Salmonella* serological flagellar (H) test.

(*2*) Positive KCN broth test (growth) and negative lysine decarboxylase test (yellow).

(**f**) *Classification.*—Classify as *Salmonella* sp. cultures that have all characteristics shown in Table **37.X24**. If 1 TSI culture from 25 g sample is classified as *Salmonella* sp., further testing of other TSI cultures from same 25 g sample is unnecessary.

(**e**) *Testing of TSI agar cultures.*—Use Salmonella serological somatic (O) test, **37.X29**.

(**g**) *Special cases.* — Cultures that contain demonstrable *Salmonella* antigens as shown by positive *Salmonella* serological somatic (O) test and positive flagellar (H) test but do not have biochemical characteristics of *Salmonellae* should be purified as in **37.X21(b)** and retested, beginning with **37.X22**.

37.X24 ***Characteristics of Salmonella***

Test or substrate	Results
Urease, **37.X22(a)**	Negative (orange-red)
Lysine decarboxylase, **37.X23(a)**	Positive (alk.; purple thruout medium)
Phenol red dulcitol broth, **37.X23(b)**	Positive (yellow and gas)
KCN broth, **37.X23(c)**(*1*)	Negative (no growth)
Malonate broth, **37.X23(c)**(*2*)	Negative (unchanged green)
Indole test, **37.X23(c)**(*3*)	Negative (no red color)
Polyvalent flagellar test, **37.X22(b), 37.X23(c)**(*4*)	Positive (visible agglutination)
Polyvalent somatic test, **37.X23(e)**	Positive (visible agglutination)

37.X25 ***Additional Biochemical Tests***

Perform addnl tests on cultures that do not give identical test results as in **37.X24** and do not classify as *Salmonella* sp. Transfer 1 loopful of culture from each unclassified TSI agar slant to each of following media:

(**a**) *Phenol red lactose broth,* **37.X16(p)** (*1*).—Incubate 48 ± 2 hr at 35°. Examine inoculated broth at least every 24 hr. Positive test is shown by gas formation (displacement of liquid in inverted tube) and acid reaction (yellow). *Salmonellae* give negative test shown by alk. reaction (red) and no gas formation.

Discard, as not *Salmonella,* cultures that give positive phenol red lactose broth test, except cultures that also give positive malonate broth test. Cultures that are phenol red lactose broth positive or negative and malonate broth positive are tested further to det. if they are *Arizona* sp., **37.X25(f)**(*2*).

(Purple lactose broth, **37.X16(p)**(*2*), may be substituted. Positive test is acid reaction (yellow) and gas. Negative test is alk. reaction (purple) and no gas formation.)

(**b**) *Phenol red sucrose broth,* **37.X16(p)** (*1*).—Incubate and read as in (**a**) above. Discard, as not *Salmonella,* cultures that give positive test. (Purple sucrose broth may be substituted and read as in (**a**) above.)

(**c**) *Methyl red–Voges-Proskauer (MR-VP) medium,* **37.X16(i)**.—Incubate 48 ± 2 hr at 35°.

(*1*) Perform Voges-Proskauer (VP) test at room temp. by transferring 1 ml 48-hr culture to test tube and adding 0.6 ml α-naphthol soln, **37.X17(b)**(*1*), and 0.2 ml 40% KOH soln, **37.X17(b)**(*2*). Shake after each addn. To intensify and speed reaction, add few creatine crystals to test medium. Read results 4 hr after adding reagents. Positive VP test is development of eosin pink color. *Salmonella* give negative test.

(*2*) Incubate remainder of MR-VP medium addnl 48 ± 2 hr at 35°. Perform Me red test by transferring 5 ml culture to test tube and adding 5–6 drops Me red soln, **37.X17(e)**, and read results immediately. *Salmonellae* give positive test (red). Negative test is indicated by yellow color.

(**d**) *Simmon's citrate agar,* **37.X16(j)**.—Inoculate by streaking slant and stabbing butt. Incubate 96 ± 2 hr at 35°. *Salmonellae* usually give positive test shown by growth and color change from green to blue (alk.). Color change usually appears first on slant and then spreads thru medium. Negative test is indicated by no or very little growth and no change in color of medium.

(**e**) *Tests indicating absence of Salmonella.* —Discard, as not *Salmonella,* cultures that give the following 3 results:

(*1*) Positive KCN broth test (growth),

(*2*) Positive Voges-Proskauer test (red), and

(*3*) Negative Me red test (yellow).

(**f**) *Classification.*—Classify cultures according to results listed in Table **37.X26**. If 1 TSI culture from 25 g sample is classified as *Salmonella* sp., further testing of other TSI cultures from same 25 g sample is unnecessary.

(*1*) *Salmonella* sp.—Cultures that have reaction patterns of Table **37.X26**, column A or B.

(*2*) *Arizona* sp.—Cultures that have reaction pattern of Table **37.X26**, column C.

(*3*) *Non-Salmonella* sp.—Discard, as not *Salmonella,* cultures that give results listed in any 1 subdivision of Table **37.X27**. (*See* p. 238.)

37.X28 ***Summary of Classification of Non-Salmonella Cultures***

Classify, by performing addnl tests described in *Identification of Enterobacteriaceae,* any culture that is not clearly identified as *Salmonella* sp. or *Arizona* sp. by classification schemes in Tables **37.X24** and **37.X26** or not elimi-

37.X26 ***Characteristics of Salmonella and Arizona***

	Classification[a]		
	Salmonella sp.		*Arizona* sp.
Test or Substrate	A	B	C
Urease test, **37.X22(a)**	–	–	–
Lysine decarboxylase, **37.X23(a)**	alk.	alk.	alk.
Phenol red dulcitol broth, **37.X23(b)**	AG	AG or A or –	–
KCN broth, **37.X23(c)**(*1*)	NGr	NGr	NGr
Malonate broth, **37.X23(c)**(*2*)	–	–	+
Indole test, **37.X23(c)**(*3*)	–	–	–
Polyvalent flagellar test, **37.X22(b), 37.X23(c)**(*4*) or Spicer-Edwards flagellar (H) test, **37.X32**	+	+	+ or –
Polyvalent somatic test, **37.X23(e)**	+ or –	+	+ or –
Phenol red lactose broth, **37.X25(a)**	–	–	AG or A or –
Phenol red sucrose broth, **37.X25(b)**	–	–	–
Voges-Proskauer test, **37.X25(c)**(*1*)	–	–	–
Methyl red test, **37.X25(c)**(*2*)	+	+	+
Simmon's citrate, **37.X25(d)**	+	+ or –	+

[a] – = negative test or no acid or gas; NGr = no growth; + = positive test; A = acid; AG = acid and gas; alk = alkaline.

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37.X27 *Characteristics of Non-Salmonella Cultures*

Test(s) or Substrate(s)	Results
(a) Urease test, **37.X22(a)**	Positive (purple-red)
(b) Indole test, **37.X23(c)**(*3*) Polyvalent flagellar test, **37.X22(b)**, **37.X23(c)**(*4*) or Spicer-Edwards flagellar (H) test, **37.X32**	Positive (red) Negative (no agglutination)
(c) Lysine decarboxylase test, **37.X23(a)**	Negative (yellow)
KCN broth, **37.X23(c)**(*1*)	Positive (growth)
(d) Phenol red lactose broth[a], **37.X25(a)**	Positive (gas and acid)
(e) Phenol red sucrose broth, **37.X25(b)**	Positive (gas and acid)
(f) KCN broth, **37.X23(c)**(*1*)	Positive (growth)
Voges-Proskauer test, **37.X25(c)**(*1*)	Positive (red)
Methyl red test, **37.X25(c)**(*2*)	Negative (yellow)

[a] Malonate broth positive cultures are tested further to det. if they are *Arizona* sp., **37.X25(f)**(*2*).

nated from these groups by test reactions listed in Table **37.X27**.

If neither of 2 TSI cultures carried thru biochemical tests, **37.X23**–**37.X27**, confirm as *Salmonella*, perform biochemical tests, beginning with **37.X23**, on all other urease-negative TSI cultures from same 25 g sample.

Salmonella Serological Tests

Dil. and pretest all *Salmonella* serological antisera with known test cultures to insure reliability of results with unknown cultures. CAUTION: Handle viable cultures carefully to prevent contaminating environment.

37.X29 *Polyvalent Somatic (O) Slide or Plate Test*

Using wax pencil, mark off 2 sections ca 1 × 2 cm on inside of glass or plastic petri dish. Place ½ of 3 mm loopful of culture from 24- or 48-hr TSI agar slant on dish in upper part of each marked section. Add 1 drop saline soln, **37.X17(f)**, to lower part of each marked section. With clean, sterile transfer loop or needle, emulsify culture in saline soln for 1 section and repeat for other section. Add 1 drop *Salmonella* polyvalent somatic (O) antiserum, **37.X17(h)**, to a section of emulsified culture and mix with clean, sterile transfer loop or needle. Tilt mixt. in both sections back and forth 1 min. and observe against dark background. Any degree of agglutination is positive reaction.

Classify polyvalent somatic (O) test as:

(a) *Positive.*—Agglutination in culture-saline-serum mixt. and no agglutination in culture-saline mixt.

(b) *Negative.*—No agglutination in culture-saline-serum mixt. (Polyvalent somatic antisera does not contain agglutinins for antigens of some *Salmonellae* isolated from foods. Negative somatic reactions occur with the *Salmonellae* serotypes whose corresponding agglutinins are not contained in the antisera, i.e., *S. cerro*, group K(18); *S. minnesota*, group L(21); *S. alachua*, group O(35).)

(c) *Non-specific.*—Both mixts agglutinate. Requires addnl testing as in *Identification of Enterobacteriaceae*.

37.X30 *Determination of Somatic Grouping (Optional)*

Perform serological somatic (O) test on culture as in **37.X29**, using individual group somatic (O) antiserum (including Vi), **37.X17(i)**, instead of *Salmonella* polyvalent somatic (O) antiserum. Repeat test, using each group somatic antiserum or until culture reacts with specific group antiserum.

Suspend cultures positive with Vi antiserum by emulsifying growth from slant surface in 1 ml physiological saline soln, **37.X17(f)**, to make heavy suspension. Heat in boiling H_2O 20–30 min. and let cool. Retest heated suspension, using somatic group D, C_1, and Vi antisera. Vi-positive cultures which react with somatic group D antiserum are probably *Salmonella typhi*, and Vi-positive cultures which react with somatic group C_1 antiserum are probably *Salmonella paratyphi C*. For these cultures to be classified as *Salmonella* sp., they must have characteristics of *Salmonellae* as in Table **37.X24** or **37.X26**. Heated Vi-positive cultures which do not react with any individual somatic serum but continue to react with Vi antiserum probably belong to *Citrobacter* group and are not *Salmonella*. Confirm conclusion by biochemical tests listed in Table **37.X26**.

Cultures that give positive somatic (O) test with any individual somatic (O) antiserum are recorded as positive for that somatic (O) group; cultures that do not react with any individual somatic (O) antisera are recorded as negative for individual group somatic (O) test.

37.X31 *Polyvalent Flagellar (H) Test Tube Test*

Place 0.5 ml appropriately dild *Salmonella* polyvalent flagellar (H) antiserum, **37.X17(j)**, in 10 × 75 or 13 × 100 mm serological test tube and add 0.5 ml antigen to be tested: formalized brain-heart infusion broth, **37.X22(b)**(*1*), or 0.5 ml formalized tryptone broth culture, **37.X22(b)**(*2*) or **37.X23(c)**(*4*). If formalized culture contains granular particles, pellicles, or sediment, also prep. saline control by mixing 0.5 ml formalized saline soln, **37.X17(g)**, with 0.5 ml formalized tryptone broth culture in same size serological test tube. Incubate mixts 1 hr in H_2O bath at 50°. Observe preliminary results at 15-min. intervals and read final results at 1 hr.

Classify polyvalent flagellar (H) test as:

(a) *Positive.*—Agglutination in culture-formalized saline-serum mixt. and no agglutination in culture-formalized saline mixt.

(b) *Negative.*—No agglutination in culture-formalized saline-serum mixt. (Polyvalent flagellar antiserum does not contain agglutinins for antigens of some *Salmonellae* isolated from foods. Negative flagellar reactions occur with *Salmonellae* serotypes whose corresponding agglutinins are not contained in the antisera (i.e., *S. simsbury*, z_{27}; *S. wichita*, z_{37}; *S. chittagong*, z_{35}).

(c) *Non-specific.*—Both mixts agglutinate. Requires addnl testing as in *Identification of Enterobacteriaceae*.

Cultures that give typical biochemical results as *Salmonellae* but do not agglutinate in *Salmonella* flagellar (H) antisera must be tested to det. if sufficient flagellar (H) antigens are present. Test motility of culture as follows:

Inoculate petri dish contg motility test medium, **37.X16(n)**, with 3 mm loopful TSI culture by stabbing medium once, 10 mm from edge of plate to depth of 2–3 mm. (Do not stab to bottom of plate with inoculum.) Do not inoculate any other portion of plate. Incubate 24 hr at 35°. When organism has migrated 40 mm or more toward other side of plate, it is sufficiently motile to retest.

Transfer 3 mm loopful of growth which migrated farthest from inoculation point into tube of tryptone broth, **37.X16(h)**. Incubate 24 ± 2 hr at 35°. Retest this culture by adding equal amt formalized physiological saline soln, **37.X17(g)**, and repeat *Salmonella* serological flagellar (H) test, **37.X31** or **37.X32**.

Incubate cultures that are not motile after first 24 hr incubation for addnl 24 hr at 35°. If still negative, incubate 5 days at 25° before classifying as non-motile (flagellar (H) antigen not detected).

Cultures that are non-motile or cultures that are *Salmonella* serological flagellar (H) test-negative, when retested, are classified according to results of other tests in *Identification of Enterobacteriaceae*.

37.X32 *"Spicer-Edwards" Flagellar (H) Test Tube Test*

(Alternative to polyvalent flagellar (H) test tube test, **37.X31**, to det. presence or absence of flagellar (H) antigens.)

Test each culture, using each of the 7 "Spicer-Edwards" flagellar (H) antisera, **37.X17(k)**. Perform test as in **37.X31**, using 1 of the 7 "Spicer-Edwards" (H) antisera for each test instead of *Salmonella* polyvalent flagellar (H) antiserum. Since there are 7 "Spicer-Edwards" antisera, each culture must be tested 7 times.

Positive agglutination indicates presence of flagellar (H) antigen. Identify by comparing pattern of agglutination reactions obtained with agglutinins known to be present in each of the 7 "Spicer-Edwards" (H) antisera. Results of these reactions are shown in table of **37.X17(k)**.

If culture produces positive agglutination when tested with each of the 4 "Spicer-Edwards" antisera 1, 2, 3, and 4 (4 plus pattern), then results indicate presence of non-specific antigen other than *Salmonella* antigen or presence of more than single *Salmonella* H antigen which cannot be identified with this antisera until antigens are sepd.

38. MICROCHEMICAL METHODS

No additions, deletions, or other changes.

39. VITAMINS AND OTHER NUTRIENTS

(1) The official, final action method for vitamin C (ascorbic acid), **39.040**–**39.041**, was revised as follows and the revised method was adopted as official, first action:

(A) Substitute following for **39.040(a)** and preceding parenthetical phrase:

(Applicable to detn of reduced ascorbic acid. Not applicable in presence of ferrous Fe, stannous Sn, cuprous Cu, SO_2, sulfite, or thiosulfate. *See* NOTE.)

Principles

Ascorbic acid reduces oxidation-reduction indicator dye, 2,6-dichloroindophenol, to colorless soln. At end point, excess unreduced dye is rose pink in acid soln. Vitamin is extd and titrn performed in presence of HPO_3-HOAc or HPO_3-HOAc-H_2SO_4 soln to maintain proper acidity for reaction and to avoid autoxidation of acid at high pH.

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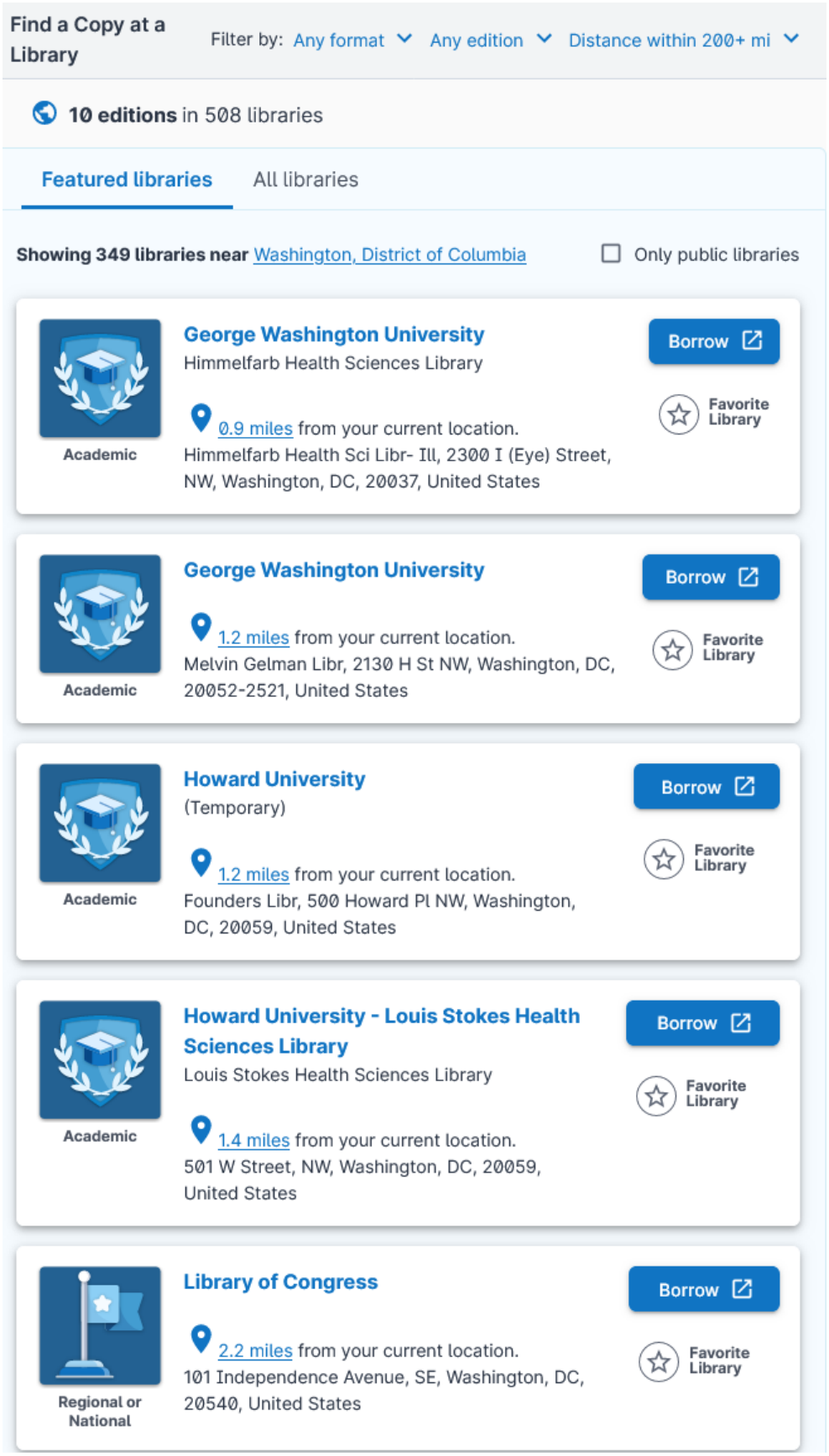


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