16.16.07

AOAC Official Method 973.64
Urine Stains on Foods and Containers
Thin-Layer Chromatographic Method II
First Action 1973
Final Action 1977

(Applicable to residues on materials with significant amounts of extractable interfering substances.)

A. Apparatus

(**a**) *Soxhlet extractor.*—250 mL extraction flask; extraction chamber 39 (id) 115 mm with top joint standard taper 45/50 and bottom joint standard taper 24/40; 35 90 mm thimbles; condenser joint standard taper 45/50 (Thomas Scientific, No. 4406-E34, or equivalent).

(**b**) *Kuderna-Danish concentrator.*

(**c**) *Thin-layer apparatus.—See* **945.75B**(**t**) (*see* 16.1.01).

B. Reagents

(**a**) *Tryptophan solution.*—1 mg/mL 50% aqueous acetone (used as longwave fluorescent marker with R_f approximately that of urea in developing solvent).

(**b**) *Developing solvent.*—*n*-Butyl alcohol–CH_3COOH–H_2O (10 + 5 + 5); prepare fresh daily.

C. Determination

On previous day prepare plates for overnight drying. Equilibrate tanks ca 1 h before extracting test products.

(**a**) *Soxhlet extraction.*—Extract 18 g test portion with 60 mL acetone 1 h at 3–4 min/siphon. Transfer extract to 100 mL glass-stoppered graduate, dilute to volume, and take aliquot equivalent to 9 g test portion. Concentrate as in (**c**).

(**b**) *Alternative extraction.*—Place 18 g test portion in 50 mL beaker or Erlenmeyer. Add 1 mL acetone/g sample and boil gently 5 min, avoiding excessive loss of acetone. Decant through glass wool pad into 100 mL glass-stoppered graduate. Repeat acetone boil and decanting 3 additional times, and dilute to 100 mL. Concentrate aliquot equivalent to 9 g as in (**c**).

(**c**) *Concentration.*—Concentrate aliquot to ca 0.2 mL in Kuderna-Danish concentrator as follows: Evaporate to ca 5 mL on steam bath in Kuderna-Danish concentrator fitted with 3-ball Snyder column and volumetric flask or graduated collection tube; 20 mesh boiling chip is necessary. Remove collection tube from concentrator and fit tube with 2-ball micro-Snyder or micro-Vigreux column. Evaporate to slightly less than desired volume, permit condensate to drain into tube, and remove column. Alternatively, place empty 13 mL graduated conical centrifuge tube in beaker of boiling water. When tube is hot, slowly transfer portions extract, using syringe fitted with long needle, to evaporation tube. Let each portion evaporate before next is added. Evaporate to ca 0.20 mL. Chromatograph without appreciable delay.

(**d**) *Preparation of TLC tank.*—Add ca 150 mL saturated $NaHSO_4$ solution to lined tank; then add ca 15 g additional solid $NaHSO_4$ to bottom of tank. Place empty solvent trough in bottom of tank and cover tank.

(**e**) *Preparation of thin-layer plates.*—(*1*) *Brinkmann-Desaga.*—Add 15 g cellulose, **945.75C**(**g**) (*see* 16.1.01), to 100 mL H_2O in blender operating at ca 90 V setting of variable transformer. Use small spatula to work powder into H_2O. Turn variable transformer to 120 V (line voltage) and blend 1.5 min. Apply slurry as 0.375 mm layer to 5 plates and air dry plates overnight.

(**f**) *Spotting of plates.*—Spot test portion concentrate as band ca 25 mm long on line 15 mm up and 15 mm in from edge of plate. Wash sides of evaporation tube with ca 50 L acetone and transfer wash to test portion band area. Repeat 50 L washings and transfers until last transfer is colorless (ca 4 transfers). Spot 1 L each of urea standard solution, **945.75C**(**aa**) (*see* 16.1.01), allantoin standard solution, **945.75C**(**d**) (*see* 16.1.01), and indoxyl sulfate standard solution, **945.75C**(**m**) (*see* 16.1.01), and tryptophan solution, **B**(**a**), ca 10 mm apart along line 15 mm to left of center of plate and 15 mm from bottom of plate.

(**g**) *Development of plates.*—Place plate in trough containing ether in lined tank presaturated with ether. Let ether travel to top of plate. Remove plate and let air dry. Immediately draw intersecting lines to divide plate into 4 equal squares. Dry plate 5 min in 80°C forced-draft oven. Remove plate from oven and promptly place in dry solvent trough in TLC tank with spotted band down. Close tank and let stand 20 min. Slide top aside just enough to introduce long-stem funnel into solvent trough. Slowly add 20 mL developing solvent, **B**(**b**), to trough. Close lid and develop in dark to line of first direction. Dry plate 5 min in 80°C forced-draft oven.

Rotate warm plate to place chromatographed standards in upper left quarter of plate and promptly place in dry trough in tank. Let stand 20 min without touching any liquid in closed tank. Then slide cover aside just enough to introduce long-stem funnel into solvent trough and slowly add 20 mL developing solvent. Let front move to line in this second dimension. Dry plate 5 min in 80°C forced-draft oven.

(**h**) *Color development.*—Spray plate with pDMAB reagent, **945.75C**(**r**) (*see* 16.1.01), until distinctly moist but not shiny wet and again heat 5 min in 80°C forced-draft oven. Strong yellow-to-orange area at R_f 0.75–0.80 is urea. Pale yellow smaller spot at R_f 0.45–0.50 is allantoin. Mark each area as color develops, since colors fade from one step to next. Place under longwave black light in darkened room and check for pale yellow fluorescent area between urea and allantoin. Spray saturated CH_3COONa solution, **945.75C**(**s**) (*see* 16.1.01), (ca 1–2 mL/plate) in space between urea and allantoin until yellow of both has faded. Let plate air dry ca 10 min in hood (do not heat), and check plate under longwave black light. Weak fluorescent pink-to-orange color against very pale blue fluorescent background confirms presence of urinary indican.

References: *JAOAC* **56,** 637(1973); **57,** 689(1974).

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