CHROM. 5402

Thin-layer chromatography of caffeine and related methylxanthines

In connection with a research project on the metabolism of caffeine by microorganisms[1], we have tested a number of methods for the separation of methylxanthines by thin-layer chromatography[2–14]. Finding them wanting with respect to either convenience, speed, resolution, or sensitivity, we have developed a new procedure, which we are successfully using to isolate and identify radioactive metabolites in fungi and which is undoubtedly also applicable to other biochemical systems and to quantitative analysis of foods and beverages as well as pharmaceuticals during and after processing.

Reference compounds

Xanthine (X), hypoxanthine (H), 7-methylxanthine (7), 1-methylxanthine (1) and theobromine (3,7-dimethylxanthine, 37) were from Pfalz and Bauer, Flushing, New York*. Paraxanthine (1,7-dimethylxanthine, 17) was a gift of Mrs. R. N. WARREN, London Hospital Medical College, London, Great Britain, which is gratefully acknowledged. Theophylline (1,3-dimethylxanthine, 13) (aminophylline, theophylline$_2$ ethylenediamine), was from Sigma Chemical Corp., St. Louis, Mo. Caffeine (1,3,7-trimethylxanthine, C) was from J. T. Baker Chemical Co., Phillipsburg, N.J.

Solutions of 1 mg of each of the above compounds per ml were prepared with 0.25 *N* NaOH, except in the case of caffeine, which gradually decomposes in NaOH solution, and aminophylline, which is poorly soluble. Caffeine was dissolved in chloroform (1 mg/ml) and aminophylline in 95% aqueous ethanol (1.17 mg/ml).

Solvents

All solvents were 99+ mole % pure (spectroquality, Matheson Coleman and Bell, Los Angeles, Calif.). Three solvent systems are recommended: chloroform–methanol (4:1); ethyl acetate–methanol–ammonium hydroxide (8:2:1) (concentrated (28%) ammonia, Allied Chemicals Division, Morristown, N.J.); *n*-butanol, saturated with a 2.8% aqueous solution of ammonium hydroxide.

Procedure

50 ml of solvent were placed in a flat-bottom chromatography jar 23 × 23 × 8 cm. Prescored glass plates (Uniplates, Analtech, Inc., Newark, Del.), 20 × 20 cm, precoated with layers of Silica Gel GF 0.25 mm thick, were split in half. A finish line was scored in the layer with a dissecting needle, 17 cm from the bottom edge. By means of Drummond disposable pipettes, 5 μl (5 μg) of each reference compound was applied to a spot, 2 cm from the bottom edge. The plates were developed without prior activation. Development in the butanol system took 4¾ h; in the other two solvent systems, about 1 h.

The zones were located in short-wave (254 mμ) UV light, after the solvent had evaporated from the plates. As little as 0.25 μg (*ca.* 1 nmole) of any purine could be detected in the dark (Chromato-Vue Cabinet. Ultra-Violet Products, Inc., San Gabriel, Calif.) by the quenching of the background fluorescence. The location of the

* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

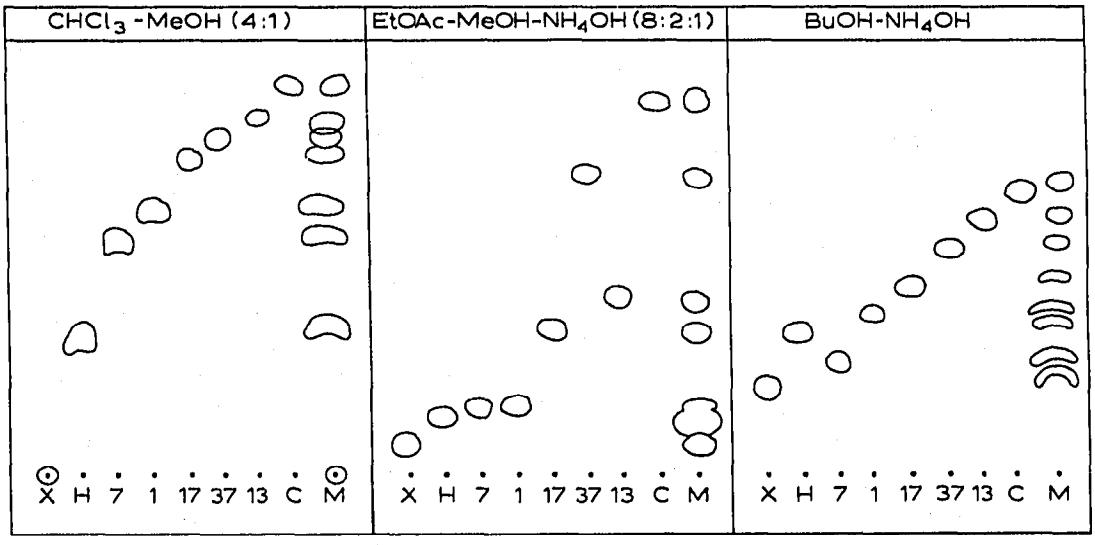

Fig. 1. Thin-layer chromatograms of xanthine derivatives in three solvent systems. Abbreviations, see under *Reference compounds*; M = mixture.

zones was marked with a dissecting needle. A permanent record was prepared by copying the chromatogram, inverted on the glass plate of a photocopier (A. B. Dick 675, A. B. Dick Company, Chicago, Ill.).

Results

As can be seen from Fig. 1, the chloroform–methanol system separates the more polar compounds better than the less polar ones. The reverse is true of the ethyl acetate-containing solvent system. Butanol–ammonium hydroxide, although much slower, has the advantage that a mixture (M) of all eight compounds can be neatly resolved at once. The mobility of hypoxanthine in the last system is greater than that of 7-methylxanthine, but lower in the first two systems. The order of mobilities for theobromine and theophylline is also reversed in the ethyl acetate-containing solvent system.

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