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Improvement of the starch–iodide method for detection of imides and other NH-containing compounds on thin-layer chromatograms

The best methods currently available for the chromatographic detection of ninhydrin-negative nitrogenous compounds such as imides, amides and cyclic peptides involve chlorination followed by detection of the N-chlorinated areas by spraying with a chlorine-sensitive chromogenic reagent, after removal of the background chlorine by aeration. A chromogenic reagent widely used with paper chromatograms consists of a solution containing potassium iodide and starch[1–3], but this reagent has not proved sufficiently sensitive for some of the more micro-scale TLC systems now in use. Also since it is a somewhat viscous aqueous solution it is difficult to spray it evenly and to avoid distortion of delicate silica gel thin layers. These defects greatly impair definition and render the method unreliable. A solution of benzidine in 50 % methanol has proved far more sensitive[4,5] and reliable as a chromogenic spray, particularly for thin-layer chromatograms and it has been particularly valuable for the detection of the cyclic imides produced from pyrrole rings in the micro-degradation technique of RÜDIGER developed for the micro-scale investigation of tetrapyrrole structure[6–8]. However, benzidine is a potent carcinogen and its use as a spray reagent constitutes a particularly insidious health hazard unless complex and very inconvenient precautions are observed.

In our efforts to find a safe and satisfactory replacement for the benzidine spray, we returned eventually to the iodide–starch reagent and found that a simple modification makes it almost as effective as benzidine. Starch is simply included in the thin-layer material from the beginning so that the plates, after chromatography, chlorination and aeration, need only be treated with potassium iodide. This can be sprayed on in 75 % acetone much more finely and evenly than the iodide–starch solution so that definition is improved to the same level as that obtainable with the benzidine spray. Furthermore, the acetone solution does not disrupt silica gel thin layers, these being in any case far more stable in the presence of the added starch which acts as an effective binder. The level of sensitivity approaches, thought it still does not quite equal, that obtainable with benzidine and has proved completely satisfactory for use in conjunction with RÜDIGER's microdegradation technique and the various refinements thereof. The presence of the starch in the Silica Gel G layers does not affect chromatography of the pyrrole-derived imides in the solvent systems normally used for their separation[6–8], and the technique of performing the chromic acid degradation of microgram quantities of tetrapyrroles directly on the starting point on the thin-layer chromatograms prior to chromatography[6,7] is also unaffected.

The modification also leads to improved detection limits and much better definition with other compounds, such as peptides, on thin-layer chromatograms and the presence of starch in the thin layers does not seem to be detrimental to their chromatography, although this should be checked out for each chromatographic system before use.

Experimental details

A 0.5 % solution of soluble starch is made in the usual manner by mixing the starch with a little cold water, stirring in boiling water and filtering the cooled solution.

The adsorbent for the thin-layer plates is slurried in this starch solution rather than in water, but the plates are otherwise made in the usual manner except that they should not be heated above 90° during activation. Silica Gel G layers are activated at 90° for at least 1 h. As mentioned above, such layers are much firmer and easier to work with than layers made without starch.

Chromatographic procedures are carried out as usual. After chromatography, care must be taken to thoroughly evaporate off any positive-reacting components of the chromatographic system such as ammonia. Any polar components, particularly water, must also be thoroughly dried off, otherwise sensitivity will be impaired.

Detection is achieved by first chlorinating with 1 % *tert.*-butyl hypochlorite in cyclohexane sprayed evenly over the chromatogram. (As reported by MAZUR *et al.*[3] for paper chromatograms, chlorination with this reagent is more convenient and results in better sensitivity than chlorination with chlorine gas.) The excess hypochlorite is removed by aerating the chromatogram in a stream of cool dry air (*e.g.* in a partly open fume cupboard). This usually takes 15–30 min and can be monitored by spotting aliquots of the subsequent iodide spray onto previously assigned blank regions of the chromatogram to check the background. When this is negative or very low, the chromatogram is immediately sprayed with a fresh 1 % solution of analytical grade potassium iodide in acetone–water (3:1). Positive substances immediately appear as brown spots. The intensity of the spots can be increased if desired by allowing the acetone to evaporate off briefly and then moistening the chromatogram evenly with a finely atomised spray of water acidified with a little hydrochloric acid. The spots then assume the blue-black hue characteristic of the starch–iodine complex in aqueous solution.

In order to achieve maximum sensitivity and definition it is necessary to control or monitor the aeration step carefully to ensure that the background is adequately cleared but that aeration is not prolonged unnecessarily beyond that point, as this results in excessive breakdown of the labile N-chloro groups. Such breakdown mainly takes place by hydrolysis, so that sensitivity is also dependent on careful initial removal of any moisture from chromatograms before spraying and on the dryness of the air during aeration. It is essential to use a fresh solution of iodine-free potassium iodide for the visualization spray, otherwise a heavy background is obtained.

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