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Note

Specific detection of pyrrolizidine alkaloids on thin-layer chromatograms

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Pyrrolizidine alkaloids, occurring in various plant species, present a significant hepatotoxic hazard to grazing animals and may enter the human food chain via meat, milk products[1,2], grains, honey[3] and herbal teas.

Thin-layer chromatography (TLC) provides a rapid method for separation and monitoring of these alkaloids[4–6] in biological extracts but methods available for detection on the chromatographic plate are limited. The commonly used alkaloid-sensitive chromogenic spray reagents, such as Dragendorff's reagent and iodoplatinate, are non-specific, producing colors with most classes of naturally-occurring organic bases. The only specific pyrrolizidine alkaloid detection method developed to date is that of Mattocks[6] which is lengthy and somewhat inconvenient, involving spraying of TLC plates with three separate reagents and thorough drying between sprays. This method involves oxidation of the pyrrolizidine alkaloids to their N-oxides by hydrogen peroxide, treatment with acetic anhydride to convert the N-oxides to pyrroles and subsequent reaction with Ehrlich's reagent to give characteristic purple colours.

The method described herein also makes use of the pyrrole-specific Ehrlich's reagent but oxidation of the pyrrolizidine alkaloids to pyrroles is achieved rapidly and directly by treatment with *o*-chloranil, this reagent itself giving an intense, albeit transient, deep blue colour with the alkaloids.

EXPERIMENTAL*

o-Chloranil (tetrachloro-*o*-benzoquinone) was obtained from Aldrich (Milwaukee, WI, U.S.A.) and was dissolved in benzene to give a 1% solution. Ehrlich's reagent was prepared by dissolving *p*-dimethylaminobenzaldehyde (Eastman-Kodak, Rochester, NY, U.S.A.) (2.0 g) in absolute ethanol (100 ml) containing boron trifluoride etherate (2.0 ml)[7]. Acetic anhydride spray reagent was prepared as a 10% solution in benzene.

For TLC, solutions of pyrrolizidine alkaloids, or crude plant extracts, in

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chloroform were applied to silica gel 60 0.25-mm pre-coated plates, either glass- or aluminum-backed (E. Merck, Darmstadt, G.F.R.). The plates were developed with either chloroform–methanol–17% ammonium hydroxide (82.5:15.5:2) (solvent 1) or chloroform–acetone–ethanol–ammonium hydroxide (5:3:1:1)[6] (solvent 2), for a distance of approximately 15 cm.

The plates were dried, sprayed with *o*-chloranil solution (or acetic anhydride solution for N-oxides), dried on a steam-bath for *ca.* 1 min, resprayed with the Ehrlich's reagent and again heated on the steam-bath for 1 min.

RESULTS AND DISCUSSION

The R_F values in two different solvent systems and the levels of detection after TLC are shown in Table I for twelve pyrrolizidine alkaloids of the macrocyclic diester type, one necine base and three N-oxide derivatives.

TABLE I

THIN-LAYER CHROMATOGRAPHY OF PYRROLIZIDINE ALKALOIDS

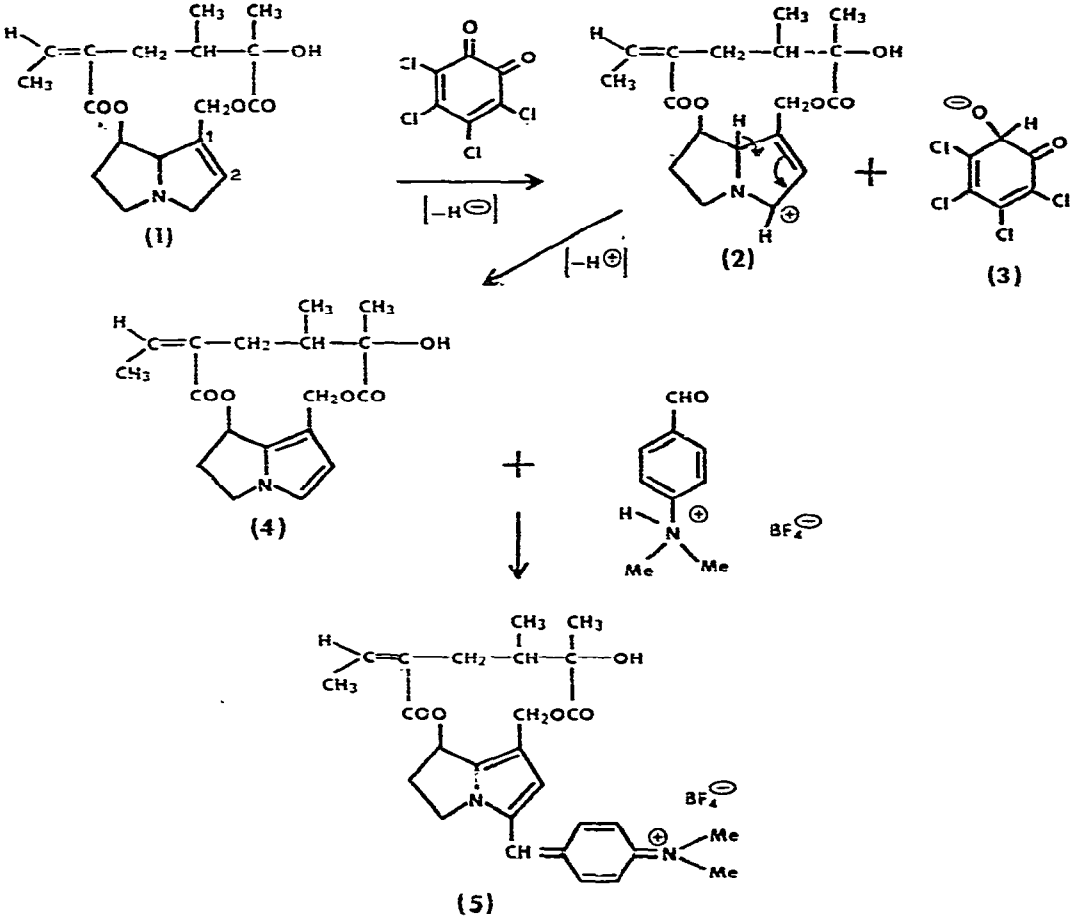
Solvents: 1 = chloroform–methanol–17% ammonium hydroxide (82.5:15.5:2); 2 = chloroform–acetone–ethanol–ammonium hydroxide (5:3:1:1).

Alkaloid	*Detection level (μg)*	R_F	
		Solvent 1	*Solvent 2*
Anacrotine	0.5	0.40	0.61
Integerrimine	2	0.62	0.82
Jacobine	1	0.37	0.79
Jacoline	0.5	0.29	0.52
Jaconine	2	0.61	0.79
Monocrotaline	1	0.39	0.63
Monocrotaline N-oxide	—	0.17	0.05
Platyphylline	25	0.46	0.78
Retronecine	<0.5	0.05	0.21
Retrorsine	2	0.35	0.54
Riddelliine	2	0.34	0.54
Riddelliine N-oxide	—	0.19	0.04
Senecionine	1	0.62	0.82
Senecionine N-oxide	—	0.38	0.15
Seneciphylline	2	0.61	0.82
Spectabiline	2	0.37	0.68

Previous TLC detection methods have involved oxidation of the alkaloids to their N-oxides which are then converted to the corresponding pyrroles. The latter react with Ehrlich's reagent (dimethylaminobenzaldehyde) under acidic conditions to give a characteristic deep purple color. Alkaloids lacking unsaturation in the 1,2-position fail to yield pyrroles and therefore do not react with Ehrlich's reagent. The sequence of three spray reagents (hydrogen peroxide, acetic anhydride and Ehrlich's reagent) required to convert the alkaloids to the pyrroles via their N-oxides, with subsequent detection, makes this method somewhat tedious and time-consuming. Direct oxidation to the pyrroles is obviously advantageous and can be achieved using

o-chloranil. Attempts to use dichlorodicyanobenzoquinone (DDQ) as an alternate oxidizing agent gave erratic results.

On spraying thin-layer chromatograms of pyrrolizidine alkaloids with a 1% solution of *o*-chloranil in benzene intense blue spots on a yellow background develop which fade quite rapidly at room temperature and within 1 min on heating the TLC plate on a steam-bath. On subsequent spraying with Ehrlich's reagent and further heating on a steam-bath the pyrrole-characteristic, stable purple spots develop. The blue color which forms on spraying with *o*-chloranil is probably due to formation of a charge-transfer complex (2 + 3), produced by abstraction of a hydride ion from a pyrrolizidine alkaloid, such as senecionine (1). Loss of a proton from the carbonium ion (2) would then yield the pyrrole (4) which reacts with Ehrlich's reagent to give the highly colored compound (5).



An alternative structure for the initial blue color could be a quinhydrone, formed by reduction of the *o*-chloranil. However, other readily oxidizable compounds which might be expected to lead to formation of the quinhydrone, such as quinols, catechols and various flavanoids, failed to give a blue color with *o*-chloranil. Deep blue aminovinylquinones have been reported to be formed on reaction of nitrogenous bases with chloranil but these are generally only produced with N-ethyl compounds[5]. Moreover, such quinones are relatively stable and reaction at the 2-position of the

pyrrolizidine ring to yield an aminovinylquinone would prevent oxidation to the pyrrole and subsequent reaction with Ehrlich's reagent. The formation of a charge-transfer complex therefore appears to account for the transient blue coloration most satisfactorily.

The detection level after TLC development for pyrrolizidine alkaloids having unsaturation at the 1,2-position ranged from 0.5 to 2 μg, compared to levels of 1 μg and 2 μg respectively for the pyrrolizidine non-specific Dragendorff's and iodoplatinate reagents. The necine base, retronecine, was detectable at levels well below 0.5 μg. Platyphylline, which lacks unsaturation at the 1,2-position and should therefore not be oxidizable to a pyrrole, gave a faint purple spot when applied to a TLC plate at a level of 25 μg and sprayed with *o*-chloranil followed by Ehrlich's reagent. The positive reaction at this high concentration was probably due to the presence of trace amounts of pyrrolizidine alkaloids having 1,2-unsaturation or to partial aerial oxidation of platyphylline to senecionine or its pyrrole.

The three N-oxides failed to react with *o*-chloranil and could therefore not be converted to the pyrroles by this method. However they were readily converted to the pyrroles by spraying with acetic anhydride[6,9] and subsequently detected by Ehrlich's reagent. Since many pyrrolizidine alkaloid-containing plants have a large proportion of the alkaloids in the form of their N-oxides[10], a crude extract of plant material can be analyzed for the presence of both parent alkaloids and N-oxides. Concurrent chromatography of a plant extract on opposite sides of a TLC plate, treatment of one side of the plate with *o*-chloranil spray reagent and the other side with acetic anhydride spray, followed by application of Ehrlich's reagent to the whole plate, provided detection of alkaloids and their N-oxides respectively. The presence of both pyrrolizidine alkaloids and N-oxides has been demonstrated in extracts from *Senecio longilobus*, *S. riddellii*, *S. vulgaris* and *Crotalaria spectabilis* using this procedure. In addition, pyrrolizidine alkaloids of the non-macrocyclic type were shown to be detectable by the *o*-chloranil–Ehrlich's reagent method by examination of extracts of *Amsinckia intermedia* and *Symphytum officinale*, which are known to contain mono- and di-ester pyrrolizidine alkaloids.

The solvent systems used provide useful complementary results for identification of pyrrolizidine alkaloids by TLC since R_F values are quite different in the two systems. In general the alkaloids move more slowly with increasing degrees of hydroxylation. In both systems retronecine and the N-oxides are relatively immobile, reflecting the high polarity of these compounds, enabling the alkaloids and their corresponding N-oxides to be readily distinguished by both R_F values and the different methods required for detection on the TLC plate.

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