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IMPROVED FIELD TESTS FOR TOXIC PYRROLIZIDINE ALKALOIDS

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ABSTRACT.—Two new qualitative field tests for unsaturated pyrrolizidine alkaloids (PAs) and their *N*-oxides are described. The tests are sensitive and able to detect all the potentially hepatotoxic PAs, except otonecine-based alkaloids. They do not respond to most saturated PAs. The first test, primarily for PA *N*-oxides, is particularly easy to perform in the field and can be extended to detect basic PAs with lower sensitivity. The second test is an improvement on an earlier *N*-oxide test and now detects both *N*-oxides and basic PAs. Practical details are given for testing both fresh and dried leaves, roots, woody material, seeds, and plant-based foodstuffs such as flour.

The sensitivity of the tests has been assessed using pure PAs and *N*-oxides, and a range of fresh and dried plant samples has been tested. A simple test for PA *N*-oxides only has proved adequate to identify the majority of plants containing 0.005% or more of PAs using samples of 0.1-0.5 *g*. High levels of PA *N*-oxides were found to persist for more than 20 years in dried plant materials.

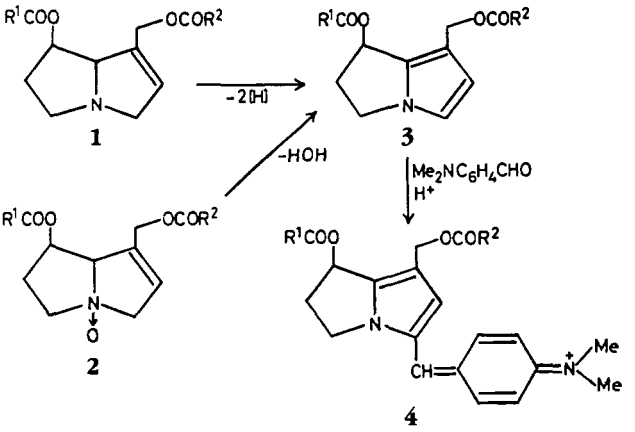
Plants containing hepatotoxic pyrrolizidine alkaloids (PAs) are present in most parts of the world (1) and often cause poisoning of grazing livestock (2). Animals may also become poisoned by feeding on hay or silage contaminated with PA-containing plants (3-6). PA poisoning is a considerable economic problem with losses due to the premature death of livestock and to the general debilitating effects of chronic PA intoxication; very large numbers of animals may be affected (7).

PAs have also caused human fatalities. Large outbreaks of poisoning have occurred when cereal crops have been contaminated with PA-containing seeds, and poisonings by herbal teas or medicines containing pyrrolizidines continue to be reported from time to time (8-10). A number of PA-containing plants have been used for food or medicinal purposes (11).

Analytical techniques available for the identification, separation, and quantitative analysis of PAs in the laboratory include colorimetric analysis using the Ehrlich reaction (12,13), tlc (14,15), nmr (16), hplc (17,18), and capillary gcms (3,19). A need remains for a simple test to detect potentially toxic PAs in plants and plant products which can be performed under field conditions. An earlier "field test" (20) detected principally the *N*-oxides of unsaturated PAs, i.e. those having an unsaturated pyrrolizidine nucleus. Two improved tests are now described. Both can be carried out easily, literally "in the field." The first is a very simple test with high sensitivity for PA *N*-oxides but permitting basic PAs to be detected also, if required. The second test is similar to the earlier procedure (20); it enables the detection both of PA bases and of *N*-oxides, with similar sensitivities.

In both tests the alkaloid [**1**] or *N*-oxide [**2**] is converted to a pyrrolic derivative [**3**] which then reacts with Ehrlich reagent to give a magenta-colored compound [**4**] (Scheme 1). *N*-oxides are converted to pyrroles using iron (II) complexes (21,22) and the basic alkaloids are dehydrogenated to pyrroles with *o*-chloranil (15). Because the hepatotoxic PAs are unsaturated (23), these tests can detect most toxic PAs. The only exceptions are otonecine esters, such as senkirkine and petasitenine (24), which are not converted to pyrroles by the reagents used.

It is common for several different PAs to occur together in the green parts of a plant, and they are usually accompanied by varying amounts of their corresponding *N*-oxides (25-27). Thus, simple field tests for *N*-oxides should be adequate to indicate most po-



SCHEME 1. Dehydrogenation of a pyrrolizidine alkaloid [**1**] or dehydration of a pyrrolizidine N-oxide [**2**] to a dihydropyrrolizine (pyrrolic) compound [**3**], which gives a magenta derivative [**4**] with Ehrlich reagent (acidified 4-dimethylaminobenzaldehyde).

tentially toxic PA-containing plants. However, both of the new tests can also detect basic PAs if necessary, and the second test permits rough comparisons of the relative amounts of PA bases and *N*-oxides.

EXPERIMENTAL

CHEMICALS.—Ascorbic acid, 4-dimethylaminobenzaldehyde, $FeSO_4$, and sodium nitroprusside were analytical grade, from BDH Chemicals Ltd., Poole, England. The remaining chemicals were of laboratory reagent grade, from various sources. *o*-Chloranil (tetrachloro-*o*-benzoquinone) was from Aldrich Chemical Co., Gillingham, England.

TEST 1. FIELD TEST FOR UNSATURATED PA *N*-OXIDES.—*Apparatus*.—The main requirements (with suggested dimensions) are as follows. Pestle and mortar (8 cm diam.); filter funnel (5 cm diam.); fast filter papers (Whatman no. 4, 9 cm); test tubes (12×1.5 cm); dispensing pipettes (0.1 ml; 1 ml); clean sand; and a plastic wash bottle containing H_2O or methylated spirit (denatured EtOH) for rinsing apparatus. Also if possible a hot water bath (about 70°); in the field a hot motor radiator might be used.

Reagents (maximum useful life indicated where appropriate).—Ascorbic acid (ASC), approx. 5% aqueous solution (life 24 h). Nitroprusside (NP) reagent (22): to a 5% w/v aqueous solution of sodium nitroprusside (10 ml) is added 0.1M NaOH (0.1 ml) (life 24 h). Ehrlich reagent: 4-dimethylaminobenzaldehyde (5 g) is dissolved in a mixture of HOAc (60 ml), H_2O (30 ml), and 60% perchloric acid (10 ml) (life, 1 week if kept dark).

Procedure.—Fresh plant material consisting of leaves, flowers, or soft stems (about 0.2-1 g) is ground in a mortar with ASC solution (about 8 ml) and a small amount of sand (28) until it is thoroughly disintegrated. The liquor is filtered through a fluted paper, and two equal portions of filtrate (sample and blank) are transferred to test tubes. NP reagent (0.1-0.2 ml) is mixed with the sample, and the tube is heated for 0.5-1 min at about 70°. Ehrlich reagent (1 ml) is added to each tube, and both tubes are heated for a further 1 min. The development of a magenta color in the sample compared with the blank indicates the presence of an unsaturated PA *N*-oxide. Alternatively, the NP and Ehrlich reactions can be carried out at ambient temperature. At 20-25° the NP reaction requires up to 20 min and the Ehrlich reaction up to 10 min for completion; however, more than half the maximum color should develop after 5 min with each reagent.

Tests on other materials.—Dried plant materials, herbarium specimens, and herbal teas are treated the same way as fresh plants. Flour and other powdered meals can be treated similarly without the need for sand. Woody stems, roots, and seeds are first pulverized, e.g. by hammering (in a thick polythene bag) before being ground with sand and ASC solution.

TEST 1A. EXTENSION OF TEST 1 TO INDICATE UNSATURATED BASIC PAs.—Additional requirements are a small separating funnel, $CHCl_3$, and the following reagents: An aqueous solution containing K_2CO_3 (10%) and NaCl (20%). A solution of *o*-chloranil (0.5% w/v) in acetonitrile (life, 8 h).

Procedure.—A further portion of the filtered plant extract is diluted with its own volume of the K_2CO_3 solution and shaken with 2 ml of $CHCl_3$ in a separating funnel. The $CHCl_3$ (lower) layer is run off; *o*-chloranil reagent (0.1-0.2 ml) is added to it, and the solution is heated for about 0.5 min, then shaken with a few drops of ASC solution to dispel the (orange) chloranil color. Ehrlich reagent (1 ml) is then added, and heating is continued for 1 min with shaking to mix the phases. A magenta color in the upper (aqueous) phase indicates the presence of an unsaturated PA.

Simplified test for basic PAs in materials (e.g. seeds) that are free from chlorophyll.—The sample is crushed, ground with a few ml of $CHCl_3$ and the extract is filtered, then treated with *o*-chloranil and Ehrlich reagent as described above.

TEST 2. FOR UNSATURATED PAs AND *N*-OXIDES.—*Apparatus.*—As for test 1, plus a small separating funnel (20 ml). A source of heat (about 70°) is essential.

Reagents.—MeOH containing ethanediol (ethylene glycol) (5% v/v). Light petroleum (bp 80-100°). $FeSO_4$ (2% w/v) in MeOH (life, 8 h). *o*-Chloranil (0.5% w/v) in acetonitrile (life, 8 h). Ascorbic acid (ASC): a saturated solution in MeOH (life, 24 h). Ehrlich reagent: 4-dimethylaminobenzaldehyde (5 g), dissolved in a mixture of EtOH (75 ml), HOAc (25 ml), and 60% perchloric acid (1 ml) (life, 1 week if kept dark).

Procedure.—Fresh plant material (about 0.2-1 g) is ground in a mortar with a small amount of clean sand and MeOH/glycol mixture (8-10 ml) until it is thoroughly disrupted. Dried plant tops, roots, and seeds should first be pulverized, as in Test 1. The extraction of alkaloids from dry material is much improved if it is left in contact with the solvent for 5 min before filtration.

The mixture is filtered through a fluted paper, and the filtrate is shaken with its own volume of light petroleum in a separating funnel. The green petroleum layer is discarded and the MeOH phase is shaken with further petroleum as many more times (usually two) as are needed to remove most of the chlorophyll. Three equal portions of the MeOH solution are transferred to test tubes (blank designated B; samples designated A, and N). Chloranil reagent (0.1-0.2 ml) is mixed with A; $FeSO_4$ reagent (0.1-0.2 ml) is mixed with N. If the latter forms a green precipitate, more $FeSO_4$ (up to a maximum of 1 ml) must be added. The tubes are heated at 70-80° for 1 min. A few drops of methanolic ASC is added to A to dispel the orange color. Ehrlich reagent (1 ml) is added to each tube, and heating is continued for a further 1 min. A magenta color in A compared with B indicates the presence of an unsaturated basic PA. A magenta color in N indicates an unsaturated PA *N*-oxide.

Practical points.—It may be convenient to make up a "Field Test Kit" with all the necessary apparatus and solutions in a fitted wooden case, similar to that described by Culvenor and Fitzgerald (28).

For semi-quantitative tests, a stable dye solution might be carried in a sealed tube with a color visually similar to that prepared in the laboratory from a known concentration of a PA. A suitable magenta color is given by a dilute solution of acid fuchsine with a trace of crystal violet if needed to add blueness. (This solution is *not* spectroscopically identical to the Ehrlich color, having λ max 550 nm). The test sample is diluted with measured volumes of EtOH until its color matches this standard. From the degree of dilution, the approximate level of PAs in the sample can be estimated.

Tests on pure PAs and N-oxides.—To evaluate the field tests, solutions (3-50 μg/ml) of pure PA *N*-oxides or basic PAs were prepared in 5% aqueous ascorbic acid (for Test 1) or MeOH (for Test 2). The color forming reactions were carried out on 1-ml lots of the appropriate solutions, and the resulting colored solutions were diluted (EtOH) to 4.0 ml or multiples thereof. The absorbance was measured in a 10 mm cuvette at λ max (565-570 nm) against the corresponding blank using a Pye-Unicam SP500 spectrophotometer.

Tests on plant materials.—Various plant samples were subjected to the tests. For each test, 8 ml of solvent was used for the extraction. Color reactions were carried out on 1-ml portions of the filtered extracts, and absorbances were measured as described above.

RESULTS AND DISCUSSION

The above tests are not primarily intended for quantitative analysis, and measurements of color intensity are only given here as a guide to the sensitivity of the tests and their relative response towards different PAs.

Applications of Tests 1 and 2 to some pure PA *N*-oxides and Test 2 to pure basic PAs gave the results shown in Table 1. All the PAs listed gave a characteristic magenta color that was linearly related to the amount of alkaloid within the range tested (3-50 μg). The only hepatotoxic PAs that gave no color in these tests were otonecine-based alkaloids such as senkirkine. Most non-toxic PAs with a saturated base moiety, e.g. hyg-

TABLE 1. Application of Tests 1 and 2 to Solutions of Pure Pyrrolizidine Alkaloids and *N*-oxides

Alkaloid	Mol Wt	Test 1[a]		Test 2[b]	
		absorbance/10μg[c]	ε	absorbance/10μg[c]	ε
Anacrotine	351	—	—	0.25	36,000 (A)[d]
Heliotrine	313	—	—	0.39	49,000 (A)
Heliotrine *N*-oxide	329	0.29	38,000	0.21	27,000 (N)
Lasiocarpine	411	—	—	0.24[e]	40,000 (A)
Lasiocarpine *N*-oxide	427	0.34	58,000	0.27	46,000 (N)
Monocrotaline	325	—	—	0.41	54,000 (A)
Monocrotaline *N*-oxide	341	0.48	65,000	0.33	45,000 (N)
Retrorsine	351	—	—	0.33	46,000 (A)
Retrorsine *N*-oxide	367	0.38	56,000	0.40	59,000 (N)
Riddelliine	349	—	—	0.34	47,000 (A)
Rosmarinine	353	—	—	0.02	3,000 (A)
Senecionine *N*-oxide	351	0.41	57,000	0.44	62,000 (N)
Supinine	283	—	—	0.21	24,000 (A)

[a]Using solutions of PA *N*-oxides (3-50 μg/ml) in 5% aqueous ASC.
[b]Using MeOH solutions of PAs or *N*-oxides (3-50 μg/ml).
[c]From slope of curve: absorbance at 568 nm in 4 ml dilution, plotted against wt (μg) of compound.
[d]Sample designation A=Chloranil reagent added, N=$FeSO_4$ reagent added.
[e]Required up to 4 min heating with chloranil for full color: 1 min gave about 85% of maximum.

rophylline gave no color but rosmarinine (which has a hydroxylated base) gave a weak color, as previously observed with a similar colorimetric procedure (12).

The results in Table 1 indicate the range of sensitivity which can be expected in these tests. Unsaturated PAs gave a color with an absorbance of 0.021-0.048/μg, after dilution to 4.0 ml, so the original undiluted solutions (about 2.1 ml) would have absorbances of ca. 0.04-0.09/μg, depending on the alkaloid. A color with an absorbance of 0.1 is easily distinguished visually as a positive result. Supposing a plant sample (0.5 g) was extracted with 8 ml of solvent and that 1 ml of the extract was tested giving a color with absorbance 0.1, this would represent ca. 9-20 μg of alkaloids in the whole extract or $1.8\times10^{-3}-4\times10^{-3}$% in the plant.

A number of plant materials from species of *Crotalaria*, *Heliotropium*, and *Senecio* known to contain PAs (1,2) were tested. They included both fresh leaves and a range of dried plants and seeds that had been kept for long periods.

In Test 1 for *N*-oxides, all the samples gave a color, often very intense. Results with very old samples showed that PA *N*-oxides can persist for many years in dried plant materials; e.g., 0.1 g of *Senecio retrorsus* leaf 22 years old gave a color with absorbance 16.7 in 4 ml.

Extraction with aqueous ascorbic acid gave excellent results with fresh plant parts. The filtered extracts were largely free from color so that removal of chlorophyll (as in Test 2) was not necessary. Fresh leaves (0.5 g) of *Senecio jacobaea, Senecio squalidus,* or *Senecio vulgaris* gave strong *N*-oxide reactions (absorbance 1.1-3.6 in 4 ml) but much weaker colors in Test 1A for basic PAs. Aqueous extraction was less effective with oily seeds (*Crotalaria*) however, and these were also the only samples to contain a very small proportion of *N*-oxides compared with basic PAs. Test 1A for basic PAs gave good results with such seeds. Thus, two *Crotalaria spectabilis* seeds (30 mg) crushed in $CHCl_3$ (3 ml) gave a strong color in this test.

In Test 2, the same plant samples gave distinct magenta colors in both parts of the test. *N*-oxide levels in plant tops ranged from 3 to 33 times higher than basic PA levels,

whereas amounts of basic PAs were 20 times higher than *N*-oxides in *Crotalaria* seeds (*C. spectabilis, C. juncea,* and *C. nana*).

Dry samples, particularly seeds, gave best results if they were steeped in the extraction solvent for a few min before being filtered. If filtration followed immediately after grinding, results could be decreased by up to 60%. This did not occur with fresh leaf samples.

Ethanediol (glycol) was included in the extraction solvent (MeOH) to improve the subsequent partition of chlorophyll into light petroleum. It could be omitted, as in the earlier test (20), but more shakings with petroleum were then necessary to clear the green color. The petroleum extraction (and the glycol) was not neccessary for chlorophyll-free samples.

Plants known to contain no PAs gave negative results in all the tests.

The test samples were always compared with their corresponding controls containing the plant extract and Ehrlich reagent. This was a safeguard in case the plant contained substances such as pyrroles and indoles that would react with Ehrlich reagent (20); these would give similar amounts of magenta color in both the sample and control tubes.

In Test 1, the blank from fresh (but not from dried) *S. squalidus* showed a magenta color, weaker than that given by the *N*-oxide test but having the same spectrum, indicating that the plant contained a PA-related pyrrole as well as PAs. This pyrrole was labile in absence of ascorbic acid and so gave a much weaker color in the Test 2 blank.

Of the tests described here, Test 1 is the quickest and easiest to perform and represents a considerable improvement over the previous field test (20) in convenience as well as in sensitivity. This test can even be performed without the need for a source of heat, although this takes longer. PAs in plants are nearly always accompanied by a proportion (often large) of *N*-oxides, and in practice an *N*-oxide test rarely fails to indicate a plant containing potentially toxic PAs. The main exceptions appear to be *Crotalaria* seeds in which nearly all the PAs exist in the basic form. For these, the quickest test is 1A used on a simple $CHCl_3$ extract of the seeds.

Test 2 incorporates improvements on the original field test (20) and provides a more sensitive and reproducible test for basic PAs than Test 1A.

These tests, although essentially qualitative, can be made sufficiently quantitative to enable comparisons of alkaloid levels in similar samples from different plants, from different parts of the same plant, and to select high-yield PA source plants in the field. Test 2 can be used to compare roughly the ratio of *N*-oxides to basic PAs in a series of samples.

Those PAs that are otonecine esters, and most nonhepatotoxic PAs, can only be detected by alternative tests, such as those described by Culvenor and Fitzgerald (28) employing Mayers reagent or silicotungstic acid. However, such tests are not specific for pyrrolizidines but respond to a wide range of plant alkaloids.

The results of the field tests for PAs and *N*-oxides should be confirmed in the laboratory using established analytical methods (3,12-19) to identify and quantify the alkaloids present.

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