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THE VAN URK-SALKOWSKI REAGENT - A SENSITIVE AND SPECIFIC CHROMOGENIC REAGENT FOR SILICA GEL THIN-LAYER CHROMATOGRAPHIC DETECTION AND IDENTIFICATION OF INDOLE DERIVATIVES

AXEL EHMANN
Department 01 Botany and Plant Pathology, Michigan State University, East Lansing, Mich. 48824 *(U.S.A.)*
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SUMMARY

The chromogenic reagent described has been tested with seventy-nine indole derivatives and found to be very sensitive and indole-specific. The lower limit of detection on silica gel thin-layer plates was between 25 and 5'0 ng for most indoles. Phenols and hydroxy-, and amino-benzoic acids, hydroxy-, and methoxy-cinnamic acids did not yield chromophores with the exception of p-amino-benzoic acid and p-l1\rtroxy-cinnamic acid which gave yellow and pink chromophores at concentrations g .: than I and 2 ,ug. Although many of the C-3 substituted indoles such as indole- 3-acetic acid and tryptamine had colors in the reddish-yiolet-blue color region, most exhibited sufficient color differentiation to allow their identification by thin-layer chromatography. The procedure was simple and required only 10 min from the time of spraying the thin-layer plate until full color development was reached. The colors had a wide spectral range from yellow of the indole-3-glyoxylamide chromophore to blue of the melatonin chromophore, and were extremely stable.

INTRODUCTION

Silica gel thin-layer chromatography (TLC) has become a powerful technique in the purification, separation and possible identification of natural and synthetic indole derivatives [l-4]: The advantages over paper chromatography are short developing times, inertness of the silica gel layer towards corrosive spray reagents and minimal aone spreading of the chromatographing compounds, resulting in a l0-20-fold decrease of the detection limits[5].
The indole compounds have been visualized on TLC plates by one of the following chromogenic reagents: (a) Salkowski reagent[6-13] (strong mineral acid plus oxidant); (b) Ehrlich reagent[5,14-34] <p-:dimethylaminobenzaldehyde-HCl with or without oxidant); (c) van Urk reagent [35-42] (p-dimethylaminobenzaldehyde-H2SO4 and oxidant); (d) Renz and Loew reagent43-49 (p-dimethylaminocinnamaldehyde-HCl);

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(e) Adamkiewicz reagent50-57 (formaldehyde-HCI); (f) Maickel and Mille agent[58-61] (o-phthalaldehyde-HCI). , The last two reagents give strongly yellow fluorescing indole condensation products, which makes them the most sensitive reagents available. Their use is limited because extracts (especially from plant material) contain many non-indolytlic yellow fluorescing substances, and the visible yellow-orange colors are not diagnostic for indole derivatives.
The Renz and Loew[43-47] reagent is claimed to be more sensitive for indoles on TLC[48,49,62] than the Ehrlich[14-21] or van Urk[35-37] reagents. A comparative study of the three reagents on TLC with a number of biologically important indoles, such.' indole-3-acetic acid (Iaa), tryptophan (Trp) and indole-3-acetyl esters, has shown[63], that the p-dimethylaminocinnamaldehyde *(P-DMAC)* reagent is 3-8 times less sensitive for most of the indoles. In addition the *p-DMAC* reagent develops a yellow to red background within 12 h which makes the subsequent identification of the colored indole condensation products difficult.

The Ehrlich and van Urk reagents are, to date, the most specific chromogenic reagents for indole derivatives., but color development is slow~ (3-8 h) and the colors are not stable, due to the mineral acid retained on the silica gel layer. We hay reported a modified van Urk spray reagent procedure25 which resulted in considerable color stability, but color development was slow (5-8 h). Color development with the Salkowski[6-13] reagent is rapid (15-30 min), but the colors change quickly to non-diagnostic brown tones. The sensitivity is about 10-fold less than for the Ehrlich and van Urk reagents, and has poor specificity for indoles except for Iaa and some Iaa derivatives.

A spray reagent has now been developed that has a high sensitivity and specificity for indole compounds, gives rapid color development and color stability of the indole condensation products.

EXPERIMENTAL

Materials 1 *Sources of in doles.* The (indole-3-acetyl)-myo-inositols, di-O-, and tri-O-(indole-3-acetyl)-myo-inositols, (indole-3-acetyl)-myo-inositolglycosides, 2-0-, 4-0-, and 6-0-- (indole-3-acetyl)-D-glucopyranoses, N-(p-coumaryl)-tryptamine and N-ferulyltryptamine were isolated from sweet Corn kernels of *Zea mays25,29-31.* The 1-0-(indole-3 acetyl)-p-D-glucopyranoside was a gift from Dr. D. Keglevic (Institute "Ruder] Boskovic", Bijenicka 54, Zagreb, Yugoslavia). ;

Other indoles were obtained from the following sources: 5-benzyloxy-6-' methoxyindole, 6-benzyloxy-5-methoxyindole, N-acetyl-indole, indole-3-acetonitrile;
and 5-methoxytryptophol from Regis (Morton Grove, Ill., U.S.A.); l-methylindole, from Eastman-Kodak (Rochester, N.Y., U.S.A.); ethylindole-3-acetate, indole-3- propionic acid, indo~-3-butyric acid and indole-3-acetyl-L-aspartic acid from Cal-biochem (La Jolla, Calif., U.S.A.); tryptophol, gramine, tryptamine HCI and bufotenine from Sigma (St. Louis, Mo., U.S,A.). All other indoles were obtained from Aldrich (Milwaukee, Wisc., U.S,A.). '

Indole standards. The indoles were dissolved in either absolute ethanol, 50%: ethanol, 2-propanol or chloroform to give 1- or 2- µg/ µl solutions. From these stock solutions serial dilutions containing 10, 25, 100, 200 and 500 ng/µl were prepared

Solvents and reagents. Methanol, ethanol, 2-propanol, 2-butanone, ethylacetate and chloroform were reagent grade and further purified with activated charcoal and fractional distillation. The purity was monitored by UV. Water was distilled, deionzed, and redistilled- in an all-glass distillation apparatus. The reagents were made a follows. (A) van Urk[37] reagent: 1 g p-dimethylaminobenzaldehyde (Aldrich) decolorized with activated carbon and recrystallized from ethanol-water (m.p. *74.5°),* was dissolved in 50 ml conc. HCl (specific gravity 1.190) and 50 ml absolute ethanol was added; this reagent is stable for several months at room temperature when stored in a brown glass bottle. (B) Salkowski[6] reagent (as modified by Tang and Bonner[9]) 2.03 g FeCI3. 6 H2O were dissolved in 500 ml water and 300 ml conc. H2SO4 (specific gravity. 1.840); this reagent is stable indefinitely.

Spray reagent. The new TLC spray reagent used, was made up of reagent A and B (1 :3). The spray reagent may be kept at room temperature for several weeks

Silica gel TLC plates. Precoated silica gel G TLC glass plates with or without fluorescent indicator and a layer thickness of 0.25 room were used throughout this study (E. Merck, Elmsford, N.Y., U.S.A.).

TLC solvent systems. The indoles listed in Table I were chromatographed in one of the following solvent systems: (1), butanone-ethyl acetate-ethanol-water (3:5:1 :1); (2) propanol-water (8:2); (3), propanol-water-28 % ammonium hydroxide (8:1 :1); (4), chloroform-methanol-water (84:14:1).

Methods

Visualization of indoles. After one-, or two-dimensional TLC of indole standards or partially purified extracts containing indolylic compounds in the appropriate. solvent system the plate was dried at *45°* until all traces of solvent had evaporated (5-10 min). The dry plate could then be examined under UV for fluorescing and/or quenching spots. Spraying of the TLC plate was done in a fume hood, using a glass atomizer such as a Desaga standard glass atomizer (100-ml capacity; Brinkman Westbury, N.Y., U.S.A.) connected to an air line. The plate was sprayed evenly in an upright position until the silica gel layer became transparent. If the plate was accidentally over sprayed the excess reagent on the silica gel layer could be removed with a paper towel.

The plate was heated in a 100° oven for 5 min, then removed from the oven and allowed to cool to room temperature. Heating up to 10 min had no adverse effect on the indole condensation products, but heating for more than 10 min caused a graying of the silica gel background. The plate was immersed in distilled water (2-3 1 per 20 X 20-cm plate), agitated periodically for 1 min. The plate was washed two more times as before. Thorough washing of the TLC plate was necessary to assure the complete removal of acids since it was found that incomplete washing often resulted in yellowing of the silica gel within a few weeks.

The plate was removed from the last water wash and blotted with a dry paper towel. At this time the colors of the indole condensation products were evaluate. (Table I; wet-plate color reading). The plate was then dried at *45°* (20-30 min). The colors of the indole condensation products were evaluated once more (Table I; dry-plate color reading). The colors of the indole condensation products are extremely stable and fade resistant. We have kept TLC plates at room temperature in the dark

TABLE I

COLOR REACTIONS AND LOWER LIMITS OF DETECTION OF INDOLE AND INDOLE DE CHROMATOGRAPHED ON SILICA GEL TLC AND SPRAYED WITH THE VAN URK-SALKWOSKI REAGENT

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Color region, color name and page number from the Horticultural Colour Chart[66]. For each compound the color. name for wet-plate color reading is followed by the name for dry-plate color reading, if the color name does not have a page number, the color could not be matched with one of the 200 color plates, and a descriptive name was chosen. Limit of detection: see *Methods.*

Substitution	*Name of compound*	*Color region*	*Color Name(page no.)*	*Limits of Detection (ng)*
None	Indole	reddish violet	Royal Purple (174)	25
			gray with reddish cast	25
N-l	1-Methylindole	Reddish violet	Violet Purple(16l)	25
		orange	Mars Orange (104)	50
	I-Acetylindole	reddish violet	Royal Purple (174)	25
			reddish grey	25
	I-Indoleacetic acid	reddish violet	Doge Purple (96)	25
		reddish violet	LilacPurple(115)	25
C-2	2-Methylindole	violet red	Peony Purple (95)	25
		greenish yellow	Naples Yellow (121)	50
	2-Phenylindole	bluish violet	Dauphin's Violet (117)	25
- -		yellowish green	Fern Green (186)	25
	Ethyl indole-2-carboxylate	red Victoria	Violet (97)	25
		bluish violet	Dauphin's Violet(117)	25
C-3	3-Methylindole	green	IvyGreen(200)	25
			greyish-blue	25
	Indole-3-methanol	violet red	Peony Purple (95)	50
		reddish violet	Pansy Violet (116)	50
	Indole-3-ethanol	blue	Princes Blue (98)	50
		greenish	blue Capri Blue (52)	50
	3-Acetylindole	reddish violet	Mauve (80)	1000
			light grey	1500
	Indole-3-carboxaldehyde	reddish violet	Mauve (80)	300
		orange red	Orient Pink (124)	600
	Indole-3.acetic acid	bluish violet	Aconite Blue (180)	25
		violet blue	Sea Blue (119)	25
	Indole-3-propionicacid	violet blue	Sea Blue(119)	25
		violet blue	Sea Blue (119)	25
	Indole-3-butyricacid	violet blue	Sea Blue (119	25
		green.	Carnation Green (194)	25
	Indole-3-pyruvic acid	yellowish green	Fern Green (186)	100
		yellowish green	Sage Green (198)	100
	Indole-3-lactic acid	blue	Princes Blue (98)	25
		violet blue	Sea Blue(119)	25
	Indole-3-acetamide	violet blue	Sea Blue (119)	50
		violet blue	Sea Blue (119)	50
	Indole-3-g1yoxylamide	reddish violet	Petunia Purple (32)	25
		yellow	Straw Yellow (67)	50
	Indole-3-acetic acid	hydrazide blue.	Oriental Blue (47)	50
		violet blue	Sea Blue (119)	50
	Indole-3-acrylicacid	red	Oxblood Red(19l)	25
			light siena	25
	Indole-3-acetone	yellowish green	Fern Green (186)	50
		yellowish green	Fern Green (186)	50

.

Table 1 *(continued)*

Substitution	*Name of compound*	*Color region*	*Color Name(page no.)*	*Limits of Detection* (ng)
	Indole-3-acetonitrile	bluish violet	Dauphin'sViolet(117)	25
		violet blue Sea	Blue (119)	25
	Gramine	reddish violet	Pansy Violet (116)	50
	bluish violet	Methyl Violet (39)		50
	Tryptamine	blue	Princes Blue (9S)	25
		Blue	Oriental Blue (47)	25
	Ethyl indole-3-acetate	blue	Princes Blue (9S)	25
		violet blue	Sea Blue (119)	25
	1-.O-(indole-3-acetyl)-.β-o-glucopyranose	bluish violet	AconiteBlue(I80)	50
		violet blue	SeaBlue(119)	50
	2-0-(indole-3-acetyl)-o-glucopyranose	bluish violet	AconiteBlue(180)	50
		violet blue	SeaBlue(119)	50
	4-0-(indole-3-acetyl)-o-glucopyranose	bluish violet	AconiteBlue(180)	50
	violet blue	SeaBlue(119)		50
	. 6-0-(indole-3-acetyl)-o-glucopyranose	bluish violet	Aconite Blue (180)	50
		violet blue	Sea Blue (119)	50
	2-0-(indole-3-acetyl)-myo-inositol	bluish violet	AconiteBlue(180)	50
		violet blue	Sea Blue (119)	50
	1-dl (4)-O-(indole-3-acetyl)-myo-inositol	bluish violet	Aconite Blue(180)	50
		violet blue	SeaBlue(119)	50
	5-0-.β-L-arabinopyranosyl-l-0-(indole-3-acetyl)-myo-inositol	bluish violet	Aconite Blue (180)	75
		violet blue	Sea Blue (119)	75
	5-0-.β-L-arabinopyranosyl-2-0-(indole-3-acetyl)-myo-inositol	bluish violet.	Aconite Blue (180)	75
		violet blue	SeaBlue(119)	75
	5-0-.β-L-galactopyranosyl-l-0-(indole-3-acetyl)-myo-inositol	bluish violet	Aconite Blue (180)	75
		violet blue	Sea Blue (119)	75
	5-0-.B-L-galactopyranosyl-2-0-(indole-3-acetyl)-myo-inositol	bluish violet	Aconite Blue (180)	75
		violet blue	Sea Blue(119)	75
	Di-O-(indole-3-acetyl)-myo-inositol	bluish violet	Aconite Blue (180)	40
		violet blue	SeaBlue(119)	40
	Tri-O.(indole-3-acetyl)-m}'o-inositol	bluish violet	Aconite Blue (180)	30
		violet blue	SeaBlue(119)	30
	DL-Tryptophan	violet blue	Sea Blue (119)	25
		blue	Oriental Blue (47)	25
	N-Acetyltryptophan	blue	Princes Blue (9S)	25
		violet blue	Sea Blue (119)	25
	Glycyl-L-tryptophan	violet blue	Sea Blue (119)	25
		blue	Cerulein Blue (46)	25
	L- Tryptophyltyrosine	violet blue	Sea Blue (119)	25
		blue	Butterfly Blue (S5)	25
	Indole-3-acetyl-L-aspartic acid	reddish violet	Cobalt Violet (S7)	50
		bluish violet	Moorish Blue (163)	50
	5-Methylindole	reddish violet	Dahlia Purple (17S)	25
			light umbra	25
	5-Fluoroindole	reddish violet	Dahlia Purple (17S)	25
		reddish orange	Garnet Brown (192)	50
	5-Nitroindole	reddish violet	Plum Purple (179)	25
		reddish violet	MagnoliaPurple(114)	25
	5-Hydroxyindole	reddish violet	Plum Purple (179)	25
			greyish-brown	50

Table 1 *(continued)*

Substitution	*Name of compound*	*Color region*	*Color Name(page no.)*	*Limits of Detection* (ng)
C-2, C-3,C-5	5-Chloro-2-methylindole-3-acetic acid	reddish violet	Rose Purple (140)	75
		reddish violet	Pastel Mauve (127)	100
	5-Methoxy-2-methylindole-3-acetic acid	reddish violet	Pansy Violet (116)	25
		bluish violet	Pastel Lavender (129)	25
	EthyI2-ethoxy-5-hydroxy-indole-3-		light umbra	50
	Carboxylase	yellowish green	Pod Green (120)	25
	2,3,5- Trimethylindole		light brown rose	100
		reddish orange	Orient Pink (124)	100
C- -.3, C-6	Reserpine	green	Cyprus Green (59)	300
		Yellowish green	Pod Green (120)	300
	Rescinnamine	bluish green	Verdigris (88)	600
		yellowish green	Pod Green (120)	600

For more than two years with little or no fading of the original dry-plate colors. However the silica gel background tended to become slightly grey-yellow if the plates were stored for more than six months. This could be eliminated by covering the dry TLC plate with permanent, invisible mending tape (Highland-Brand No. 6200; 3M . 0., St. Paul, Minn., U.S.A.) or similar tape. TLC plates covered with this tape have been kept for more than one year with no detectable color changes of the indole condensation products, and have retained a white silica gel background;

Purity and identity of indole standards. In order to evaluate the color reaction of each indole standard listed in Table I the indoles were chromatographed at 1-; 5-, 10- and *20-υg* concentrations in one of the four solvent systems to assure a minimum migration of 6 cm of each indole from the origin (solvent front, 10 cm). After the plates were sprayed and processed as described above, they were examined for possible secondary chromogenic spots. In addition each indole standard was analyzed by gas-liquid chromatography and the identity confirmed by mass spectrometry (GLC-MS)[65].

Colors of the indole condensation products:. Each indole standard with the exception of the indole derivatives isolated from mature sweet corn karyopses (see *Sources of indoles)* was spotted at 1-, 5-, 10- and *20-μg* concs. on a 5 x 10-cm TLC plate. The corresponding spot areas were 20, 30 and 60 mm2. The plate was sprayed and processed as described above. After the third water wash of the plate and removal of excess water the color of the indole condensation product was matched by eye with one of the 200 plates of 64 full hues, 60 tints, 38 shades and 38 grayed hues of the Horticultural Color Chart[66]. The 200 plates had been arranged according to color families, and the matching could be done routinely in less than 1 min (Table I; wet-plate color reading). After the plate had been dried the color was matched again (Table I; dry-plate color reading). In addition each color was rematched three more times after 6, 12, and 24 h to evaluate any color changes which might have occurred. The color evaluation of the indole condensation products of the indole derivatives isolated from mature sweet com of *Zea mays* was done the same way except that the indoles were first chromatographed on a 20 X 20-cm TLC plate at 1-, 5-, and *10υg* concentrations in solvent 1.

Limits of detection. The indoles were chromatographed at concentrations ranging from 10-1500 ng in the appropriate solvent systems and a minimum spot migration of 6 cm. The limit was determined as the smallest amount of indole to give. a 5-7mm^2 detectable color spot.

RESULTS AND DISCUSSION

The colors of 79 indole condensation products with p-DMAB on silica gel acid TLC and their limits of detection are listed in Table I. Most of the indoles can readily. be detected at the 25-50-ng level which makes this reagent a very sensitive and indole specific chromogenic reagent. Certain phenols and aromatic acids are known to give positive color reactions with p-DMAB-HCI (Ehrlich reagent)[67]. A number of hydroxy:. and aminobenzoic acids, hydroxy-, and methoxycinnamic acids were examined and found that only p-aminobenzoic acid (yellow) and p-coumaric acid (pink) give a positive color reaction. However the limit of detection for these compounds is about 40-80 times (1-2 *μg)* higher than for most of the indoles tested.

The indoles in Table I are arranged according to their substitution(s) on the indole ring system. It has been reported that condensation of the indole derivative with p-DMAB occurs at the free C-2 position and results in a violet-blue color product if C-3 has a -CH2-R group[68]. A structural analysis of a number of indole condensation products by low- and high-resolution MS has shown that condensation of p-DMAB can also occur at C-3, N-1, and to a limited extent on C-5 and C-6 of the. indole ring system (see Table I, I-methylindole-2-carboxylic acid, carbazole and 1I-phenylcarbazole)[69].

Although many of the C-3 substituted indoles such as IAA and its esters tryptamine and its derivatives have colors in the- reddish-violet-blue color region., most exhibit sufficient color differentiation to allow their identification on TLC. We have chosen the Horticultural Colour Chart to illustrate this color differentiation (Table I, color region and color name). Frequently it is possible to identify tentatively 18 endogenous indole derivatives from plant extracts by their colors and *RF* values on TLC, and the subsequent identification by GLC-MS confirms the great diagnostic' :~ value of the chromogenic spray reagent.

Another advantage of the spray reagent for TLC is the relatively simple procedure. It takes about 10 min from the time of spraying until the wet-plate colors can be evaluated, and less than 1 hr for the permanent color evaluation. The majority of: the colors of the indole condensation products are extremely stable. TLC plates of some 40 indole standards at 1- and *5-p.g* concentrations and developed in solvent systems (1) and (4) have been kept for more than two years with virtually no fading , or change of the original dry-plate colors.

Some indoles, such as indole, I-methylindole, l-acetylindole, 2-methylindole and indole-3-acrylic acid exhibit a characteristic color change as the plate dries (see Table 1). It was found that this color shift and or fading is reversed to the original, wet-plate color by rewetting the plate. It is thus possible to evaluate the wet-colors of those indoles which in the dry-plate state are no longer diagnostic. The colors of some of the indoles have been reversed as often as ten times in one day with no apparent loss of color or color intensity. 3

An important aspect of this reagent is the finding that it can also be used as

a colorimetric reagent with a sensitivity and specificity for many indoles equal to that on TLC. The use *of* this reagent for the colorimetric determination of indole derivatives will be published in a separate communication..

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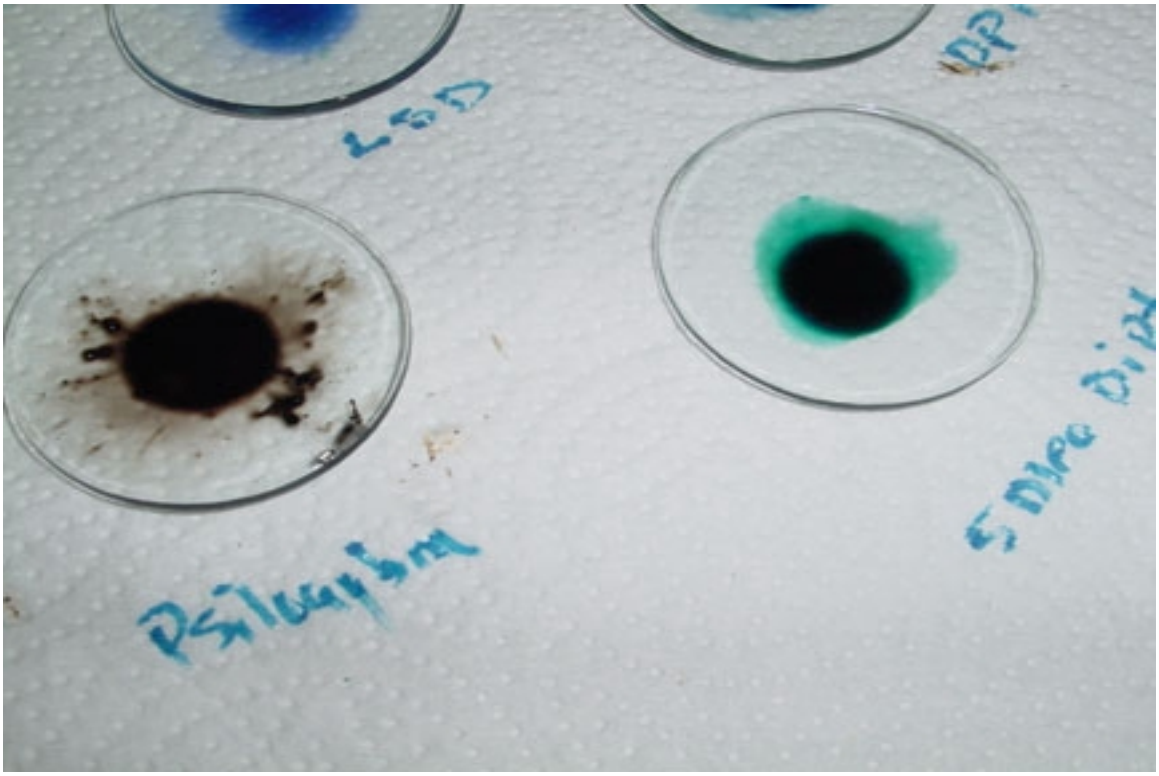
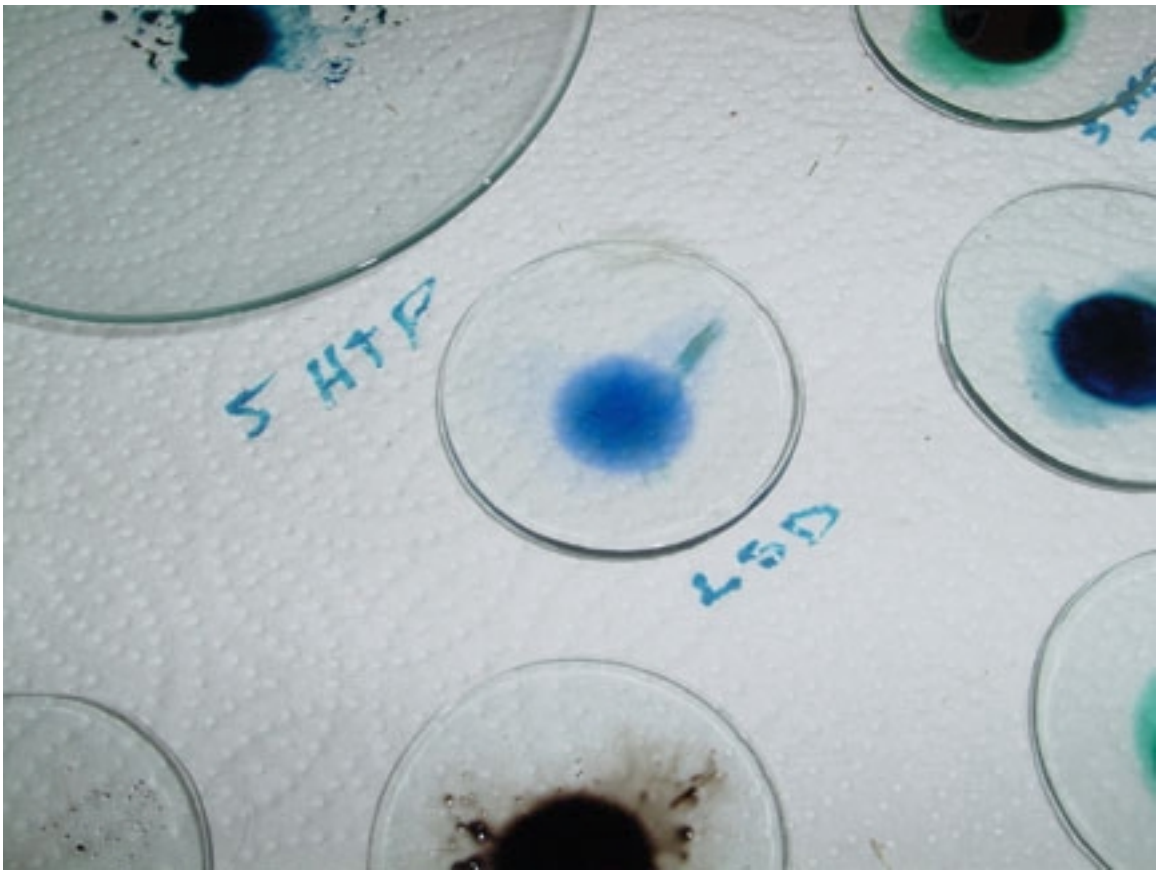
-------- End of Journal Article ------

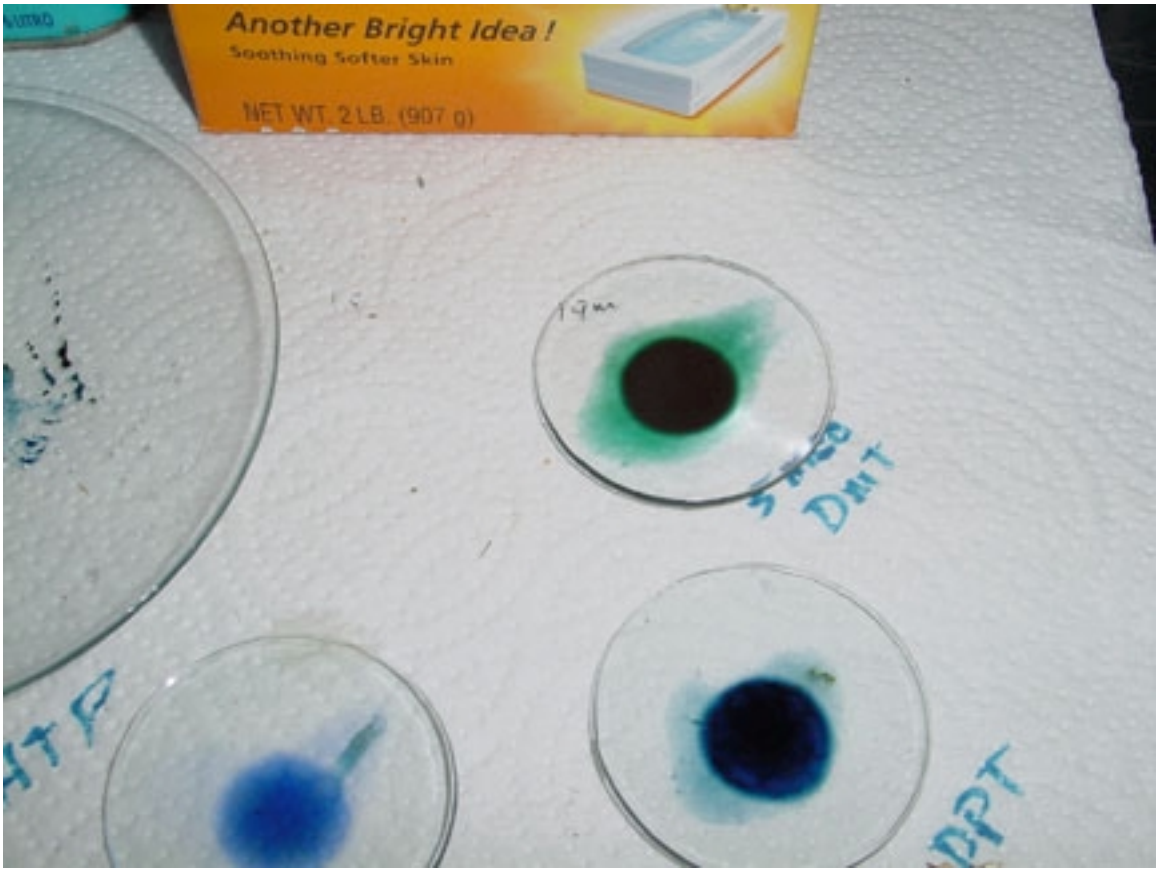
In order to better use the information here, color photographs of tryptamines of interest are included below. These are not indicated in the document, so these are provided for reference. Color references are provided via common household items.



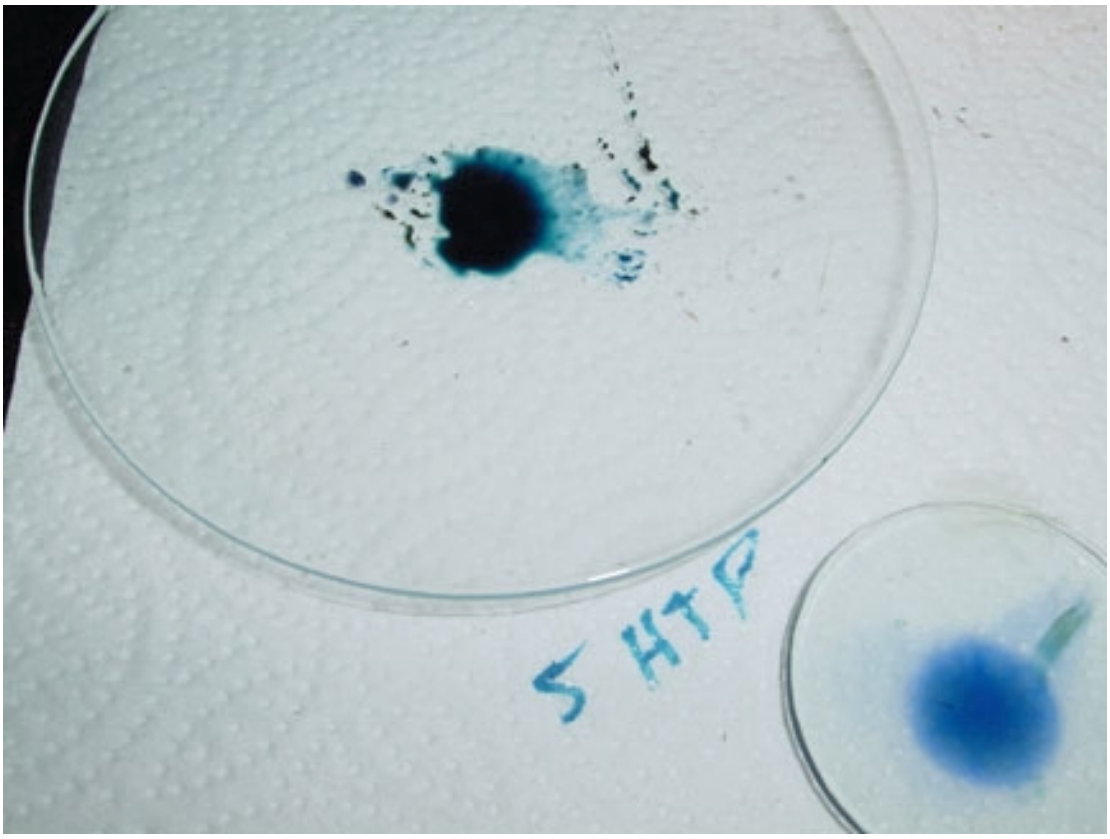
Psilocybin and 5meo-DMT



5 – HTP and d-Lysgeric Acid, Diethylamide (LSD-25)



5-Meo DMT, DiPropyl Tryptamine (DPT)



5-HTP , LSD –25

Van Urk –Salkowski Reagent colors for tryptamines:

LSD	SkyBlue
5MEO-DMT	Deep Green
DPT	Deep Blue
5-MEO-DIPT	Blue-Green
5-HTP	Blue-Gray