COLOUR REACTIONS IN CHROMATOGRAPHY

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SUMMARY

Fifteen location reagents, their preparation and use for chromatographic staining have been described.

The results obtained with 25 physiological substances have been summarized in Table I and demonstrate in a number of cases the lack of specificity of the location reagents.

Dyes to make visible, otherwise invisible substances on paper chromatograms are indispensable. However, the results obtained by stain reactions often lead to conclusions which are not justified as a result of the lack of specificity of the reagents used. As one example in this connection may be mentioned that amido black, for instance, which is considered to be a specific reagent for proteins, also gives positive reactions in the presence of amino acids, peptides and a number of lipids.

The description of the performance of the stain reactions in the literature, and the preparation of the reagents needed are often not readily available, incomplete or absent. Therefore, an exact description of the staining procedures with addition of certain important characteristics may be justified and useful.

REAGENTS AND STAINING

250 μg of 25 different physiological substances were applied to small strips of Whatman paper No. 1 and stained by 15 reagents as described below.

(1) Rhodamine 6 G (C.I. No. 752)

Reagent: Rhodamine 6 G in aqueous solution (10 mg%).

Staining: Immerse the dry chromatogram in the solution at 37° for approx. 2 min, rinse for 5 min in running water and view wet under ultraviolet light (long or short wave). Blue or purple spots are observed when anionic phospholipids (PL) such as phosphatidyl inositide (PI), phosphatidyl serine (PS), and phosphatidic acid are present, while yellow or orange spots are produced when other PLs, such as phosphatidyl choline (PC), sphingomyelin (Sphm.), lyso PC, and phosphatidyl ethanolamine (PE) are present[1].

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The fluorescent indicator Rhodamine 6 G is recommended as a very useful general reagent for all classes of lipids. Contrary to other rhodamine dyes it is also effective in detecting lipids on silicic acid-impregnated paper[1].

As an improvement the use of a 0.001% solution of rhodamine 6 G in 0.25 *M* K_2HPO_4 solution instead of distilled water has been proposed. This was suggested to increase the colour intensity when the wet chromatograms are viewed under ultraviolet light[2]. The lower limit of identification is somewhat less than 1 μg[3]. We could not determine any advantage in this modification.

(2) Rhodamine B (C.I. No. 45170)

Reagent: Rhodamine B in aqueous solution (10 mg%).

Staining: As described under (1). Staining of PLs on silicic acid paper by rhodamine B is considered to be less effective than by rhodamine 6 G. For this reason the use of rhodamine 6 G was suggested[1].

(3) Amido black 10 B (C.I. No. 20470) (Syn. Naphtolblauschwarz B.)

Reagent: Saturated solution of amido black in a mixture of methanol–acetic acid (9:1).

Staining: Immerse the chromatogram in the solution at room temperature for 10 min during magnetic stirring. Rinse strip in running water to remove excess dye.

Positive reactions (black spots) are obtained with proteins, but also with amino acids, peptides, some lipids and their degradation products such as phosphoserine, phosphoethanolamine, and ethanolamine.

(4) Sudan black B (C.I. No. 26150)

Reagent: Dissolve 100 mg Sudan black B in 100 ml of a mixture containing: 30 ml methanol, 30 ml isopropanol and 40 ml dist. water.

Staining: Immerse strip in the solution at 37–40° for 2 h and rinse with isopropanol (30%).

(5) Malachite green (C.I. No. 42000)

Reagent: Dissolve 250 mg of Malachite green in 500 ml of dist. water.

Staining: Spray strip or immerse it in solution at room temperature for 15 min. Only lipids and no proteins, peptides or amino acids appear as green spots.

Malachite green is recommended to be used as a specific reagent for lyso-compounds[4]. However, the white spot on a light green background, as described for lyso-compounds[4] could not be reproduced. Sphingosine, for instance, produces a white spot (see Table I).

(6) Nile blue (C.I. No. 51180) (Syn. Nile blue A)

Reagent: Dissolve 0.01 *M* Nile blue in 0.1 *M* sulphuric acid (*i.e.*, 4.15 g in 100 ml 1% H_2SO_4).

Staining: Immerse the strip in the solution for 1 min at room temperature and wash in running water for 30 min.

According to Hack[4], Nile blue produces blue spots with lipids of strong acid function such as cardiolipin, PS, etc., and pink coloured spots with strongly lipophilic compounds such as free fatty acids, fatty aldehydes, neutral fats etc. (Compare Table I.)

TABLE I

LOCATION REAGENTS AND THEIR REACTIONS WITH VARIOUS SUBSTANCES

Location reagents	*1 Serine*	*2 Tyrosine*	*3 Arginine*	*4 Glycyl-glut. acid*	*5 Glycyl-serine*	*6 Alanyl-serine*	*7 Glycyl-glycine*	*8 Glycyl-alanine*	*9 Albumin*	*10 Serum-protein*	*11 Lipid activator (PL-mixture)*
1. Rhodamine 6 G	—	—	—	—	—	—	—	—	(+)	+ r-b	+ r
2. Rhodamine B	(+) fl	(+) fl	—	—	—	—	—	—	(+) fl	—	+ fl
3. Amido black	— bl	+ bl	+ bl	+	+	+	+	+	+ bl	+ bl	—
4. Sudan black	—	—	—	—	—	—	—	—	+ bl ring	+ bl	+ bl
5. Malachite green	—	—	—	—	—	—	—	—	+	+	+
6. Nile blue	—	—	—	—	—	—	—	—	+	+ lt b ring	+ dk b
7. Ciba blue	—	(+)	+	—	—	—	—	—	+	+ y	+ y
8. Ninhydrin	+ b	+ p	+ b	+ y	+ y	+ b	+ y	+ y	+ b	+ p	+ y-b
9. Bromothymol blue	+ y ring	+	+ y ring	+	+ y ring	+ y ring	+ y ring	+ y ring	+	+ o ring	—
10. Oil red O	—	—	—	—	—	—	—	—	(+) lt r	+ brown y	+ y
11. Ammonium molybdate (for P)	—	—	—	—	—	—	—	—	—	+ b ring	+ b ring
12. $FeCl_3$-sulfosalycylic acid (for P)	+ y ring	—	—	+ y ring	+ y ring	+ y ring	+ y ring	+ y ring	—	+	+
13. Phosphomolybdic acid (for choline)	—	—	—	—	—	—	—	—	+	+	+
14. $KMnO^4$	—	+	+	(+)	—	—	—	—	+	+	+
15. Biebrich scarlet	—	-	—	—	—	(+)	—	-	— w ring	+	+

Stains used were supplied by the Chroma Gesellschaft, Schmid & Co., Stuttgart-Untertürkheim, Germany, the substances 1–3 by Serva, Entwicklungslabor Heidelberg, Germany, 4–8 and 12–17 by Fluka, A.G., Buchs, Switzerland, 19 and 20 by Dr. D. Shapiro, Rehovot, Israel, and 25 by Dr. G. H. de Haas, Utrecht, Netherlands.

Abbreviations:

Intensity of reaction: (+), +, ++ and +++. No reaction: —.

b: blue	o: orange	PL: phospholipid
bl: black	p: purple	PE: phosphatidyl ethanolamine
dk: dark	r: red	PC: phosphatidyl choline
fl: fluorescence	w: white	Sphm: sphingomyelin
lt: light	y: yellow	

* pH < 5: o and pH > 5: b

** later b

(7) Ciba blue

Reagent: Saturated solution of Ciba blue in ethanol.

Staining: Analogous to (6).

(8) Ninhydrin (Syn. Indane-trione hydrate)

Reagent: Dissolve 300 mg ninhydrin in a mixture of 90 ml acetone and 10 ml lutidine (2:4 or 2:6).

12 *Etha-nol-amine*	*13* *Phos-pho-serine*	*14* *Phos-pho etha-nol-amine*	*15* *PE (synth. mitoyl) purum*	*16* *PE dipal-puriss.*	*17* *PC (synth.)*	*18* *Cere-bron*	*19* *Sphin-go-sine*	*20* *Sphm.*	*21* *Oleic acid*	*22* *Tri-olein*	*23* *Stearic acid*	*24* *Tripal-mitin*	*25* *Lyso PC*
–	–	–	(+) fl	(+) fl	++ fl	(+) fl	green-ish	–	+++ fl	+ fl	++ r	++ r	+++ fl
(+ ?	–	–	(+) fl	(+) fl	+++ fl	+ fl	+++ fl	(+) fl	++ fl	+ fl	+ fl	+ fl	+++ fl
+	+	+	(+) ?	(+) ?	–	+	–	+	–	–	–	++	–
+	–	–	++ bl	++ bl	+ bl ring	++ bl	++ bl ring	–	–	+++ bl	–	++ bl	+ bl ring
–	–	–	+	+	++	++	w	+	++	++	++	+++	++
–	–	–	–	–	+ dk b ring	+++ dk b	+ lt b ring	+ lt b ring	+++ dk b	+++ r-b	++ b	++ dk b	++ dk b
–	–	–	–	–	–	–	–	–	–	–	–	+ bl	–
+++ *	+++ b	+++ b	++ p	++ p	(+)	(+)	+++ p	–	–	–	–	–	–
–**	++	+++	–	–	+++ y-w	–	–**	++	+++	+	++	yw ring	+++
–	–	–	+ r	+ r	–	–	–	–	–	++ r	–	+ r	–
++ b	+++ b	+++ b	+ b ring	+ b ring	+ b ring	+ b ring	–	+ b ring	–	–	–	–	++ b ring
+++	+	+	+	+	+	+	+	+	–	–	–	–	++
–	–	–	(+)	(+)	++	+	+++	+	–	–	–	–	+++
–	–	–	++	+	–	+++	+++	–	++	++	–	–	–
–	–	+	++	++	++	–	+++	++	–	–	–	++	++

The reagent has to be kept in the refrigarator and renewed after a few days.

The presence of lutidine intensifies the reaction and promotes the development of specific differences in colour (glycine: reddish; proline: yellowish, tryptophane: greyish, etc.).

Some authors prefer a ninhydrin reagent containing a small amount of acetic acid (300 mg ninhydrin in 100 ml *n*-butanol and 3 ml acetic acid) if alkaline solvent systems are used. The ninhydrin test requires a pH of approx. 5.

Staining: Spray the strip and air-dry it in the dark at room temperature or at 60° for 30 min, or at 100° for 10 min. Drying slowly at room temperature is preferable for better separation of the spots. Some investigators avoid development of the spots by heating.

In general, blue or purple spots are obtained but with proline a yellow spot is formed which later changes to purple. Peptides produce purple spots with ninhydrin, or yellow spots which change later also to purple.

This most distinct ninhydrin reaction on all substances with free NH_2-groups can also be positive when N-free reducing substances are present together with NH_3

(ref. 5). The sensitivity of the ninhydrin reaction varies from 0.001 μg for glycine to 0.1 μg for proline.

Stabilization of the ninhydrin spots. Reagent: Add 0.2 ml HNO_3 (10%) in 100 ml ethanol (96%) to 1 ml saturated aqueous cupric nitrate solution.

Staining: Spray strip very lightly and dry at room temperature.

Varycoloured spots. Reagent I: Dissolve 100 mg ninhydrin in 50 ml abs. ethanol and add 10 ml acetic acid, and 2 ml 2,4,6-collidine.

Reagent II: Dissolve 500 mg $Cu(NO_3)_2 \cdot 3\ H_2O$ in 50 ml ethanol. Mix before use 50 parts of reagent I with 3 parts of II.

Staining: Spray and note the colours within 10 min because they change and fade rapidly.

(9) Bromo-thymol blue

Reagent: Dissolve 40 mg bromo-thymol blue in 100 ml *N* NaOH.

Staining: Spray and note wet the spots since they change rapidly.

(10) Oil red O

Reagent: A saturated solution of oil red O in ethanol (60%).

Staining: Immerse strip in the solution at 37° for 15 min and remove excess of colour solution with distilled water and then with ethanol. In our experience washing of the paper in a mixture of ethanol–acetic acid (3:1) as recommended[6] has to be avoided.

(11) Ammonium molybdate reagent (for phosphorus, Dawson [7])

Reagent: 10 ml perchloric acid (approx. 72%) + 20 ml 5 *N* HCl + 40 ml ammonium molybdate (5%) + 130 ml distilled water.

Staining: Spray strip and air-dry (hair dryer). Observe after 10 min under the UV lamp. The positive blue spots may appear later.

(12) Ferrichloride–sulfosalicylic acid reagent (for phosphorus (Wade and Morgan[8]))

Reagent I: $FeCl_3 \cdot 6\ H_2O$ (0.1%) in ethanol (80%).

Reagent II: Sulfosalicylic acid (1%) in ethanol (80%).

Staining: (Strip should not be washed previously.) Spray the dry strip with reagent I. Dry strip at 60° for 30 min. Spray with reagent II. Dry at room temperature over night. The positive reaction is characterized by an ivory-coloured spot on a purple background.

(13) Phosphomolybdic acid reagent (PMA-reaction) (for choline and choline-containing compounds)

Reagent I: Phosphomolybdic acid (1%) in mixture of ethanol–methanol (1:1).

Reagent II: $SnCl_2$ (1%) in 3 *N* HCl (add 250 mg $SnCl_2$ to 6 ml conc. HCl + 19 ml H_2O).

Staining: Spray dry strip with reagent I. If SiO_2-paper was used, the strip has to be rinsed in running water for 15 min first and dried at 100° before continuing. However, the same wash procedure is also necessary for other types of chromatographic papers if they become completely blue in colour.

Immerse the wet strip in reagent II. An intensive blue spot indicates a positive reaction. An unspecific positive test can also be observed with unsaturated PE[1].

The PMA-reaction can also be performed after a previous staining with ninhydrin. In this case the strip has to be washed before the PMA-reaction in running water for 15 min.

The possibility has been mentioned of staining with ninhydrin, phosphomolybdic acid, rhodamine and Nile blue successively[9].

A number of other choline reactions have been described. Reference here be made to the reactions using Reinecke salt[4,10,11], dipikrylamide[12], and J_2 in KJ[13]. A recent method for the quantitative assay of choline by *cis*-aconitic acid[14] may be recommended here.

(14) Potassium permanganate (for unsaturated compounds)

Reagent: $KMnO_4$ (1%) in distilled water.

Staining: The dry chromatogram has to be rinsed in distilled water for 10 min and immersed in the reagent for 1–2 min.

Remove excess reagent by running water. A brown spot indicates a positive reaction.

(15) Biebrich scarlet (C.I. No. 26905)[4] (Syn. Ponceau BS, Crocein scarlet, Scarlet B, Xylidin Ponceau)

Reagent: Biebrich scarlet (0.01 *M*) in sulfuric acid (0.1 *M*) *i.e.*, 556.5 mg in 100 ml 1% H_2SO_4.

Staining: Dip strip in the solution for 30 sec and rinse in running water. A pink-to-red spot indicates a positive reaction for lipids (see Table I).

Reactions 1, 2, 8, 9, 10 and 11 are also suitable for use in thin-layer chromatography on silica gel plates. In reaction 5, a more dilute malachite green solution has to be used. Application of the remaining reactions causes difficulties because of the inability to wash the plates. A technique for a liquid flow through Sephadex gel layers[6] was not successful with our silica gel plates.

The results of the staining procedures are summarized in Table I.

CONCLUSION

It is perfectly clear that quite a number of the staining reactions are far from specific.

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