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Combined Information from Retardation Factor (Rf) Values and Color Reactions on the Plate Greatly Enhances the Identification Power of Thin-Layer Chromatography in Systematic Toxicological Analysis

REFERENCE: Hegge, H. F. J., Franke, J. P., and de Zeeuw, R. A., **"Combined Information from Retardation Factor (Rf) Values and Color Reactions on the Plate Greatly Enhances the Identification Power of Thin-Layer Chromatography in Systematic Toxicological Analysis,"** *Journal of Forensic Sciences*, JFSCA, Vol. 36, No. 4, July 1991, pp. 1094–1101.

ABSTRACT: A numerical color coding system has been developed to describe the colors of spots obtained after using location reagents in thin-layer chromatography (TLC). This system makes color reactions on the plate amenable to computer handling, so that the retardation factor (Rf) values plus color reactions can be used for identification of unknown substances in toxicological analysis. It is based on a series of four color reactions carried out in sequence on the same plate, and encoding of the observed color is done by means of a wheel of reference colors. The combined information of Rf values plus color reactions resulted in an eightfold increase in identification power in comparison with the information provided by the Rf value alone. Moreover, a single TLC system now provides a three- to four-times higher identification power than a single gas chromatography system like OV-1. The identification power of TLC can be enhanced even further by running two or more systems in parallel.

KEYWORDS: toxicology, chromatographic analysis, drug identification, systematic toxicological analysis, thin-layer chromatography, color reactions, substance identification, drugs

When screening for potentially harmful substances in systematic toxicological analysis, it is essential to know the identification power (IP) of the analytical techniques used, whether using gas chromatography (GC), thin-layer chromatography (TLC), or mass spectrometry (MS). To compare the suitability of techniques (or individual systems within a technique, for example, OV-1 versus OV-17 in GC), the mean list length (MLL) concept was developed [*1,2*]. The MLL is the mean number of candidates out of a certain population that would qualify for identification when a given technique or system—alone or in combination with others—is used to detect and identify an unknown substance. The smaller the number of substances qualifying, the better the technique or system. The ultimate is reached with an MLL of 1.00, since all substances in the population can then be identified unequivocally. Thus, the MLL gives an objective criterion for defining

Presented at the 42nd Annual Meeting of the American Academy of Forensic Sciences, Cincinnati, OH, 19–24 Feb. 1990. Received for publication 24 Sept. 1990; accepted for publication 9 Nov. 1990.

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the identification power of a single analytical system, combinations of systems, or even combinations of different techniques. So far, the MLL concept has been applied to evaluate GC, TLC, and ultraviolet (UV) spectrometry [3]. With regard to TLC, for example, it has been shown that the use of two or more systems instead of one can greatly enhance the IP; however, the choice between the systems has to be made carefully [3,4].

On the other hand, the IP of TLC is relatively low, because of the intralaboratory and interlaboratory variability of retardation factor (Rf) values, even when these are corrected by using reference substances [4]. However, the IP of TLC may be enhanced when—apart from the Rf values—colors produced by location reagents are taken into account, but so far, it has been rather difficult to handle the information provided by the color reactions objectively, to combine it with Rf information, and to make it amenable to computer handling. Recently, the authors suggested a numerical system for the encoding of color reactions on the plate [5] which showed interesting potential. The aim of the present study was the optimization of the encoding procedure, especially in preventing mismatching of colors, as well as the evaluation of the gain in IP that can be obtained when utilizing Rf values together with the color reactions on the plate.

Materials and Methods

The classical TLC plates used for solvent systems 1 through 4 (Table 1) were 20 by 10-cm glass plates, coated with silica gel with a fluorescence indicator (Kieselgel 60 F_{254}, Merck, Darmstadt, Germany). For solvent system 5 (Table 1), Toxi-Gram A glass fiber plates impregnated with silica were used (Toxi-Lab, Inc., Irvine, California).

The TLC solvent systems are described in Table 1. For these systems, Rf data for 100 drugs frequently encountered in analytical toxicology were taken from the literature [4] or from the "Toxi-Lab Drug Compendium" [6].

The following reagents, prepared in the following manner, were used for the color reactions:

A1—Twenty-five mL of 37% formaldehyde solution was prepared in a 250-mL jar containing a standoff. The top of the standoff was kept free of liquid, and the jar was kept tightly capped. Strong formaldehyde vapors in the jar were es-

TABLE 1—*Description of the TLC solvent systems.*

TLC 1	ethyl acetate/methanol/25% ammonia (85:10:5) saturated chamber silica gel (Merck)
TLC 2	methanol unsaturated chamber silica gel (Merck)
TLC 3	methanol/*n*-butanol (60:40); 0.1*M* NaBr unsaturated chamber silica gel (Merck)
TLC 4	cyclohexane/toluene/diethylamine (75:15:10) saturated chamber KOH-impregnated plate silica gel (Merck)
TLC 5	ethyl acetate/methanol/water/30% ammonia (87:3:1.5:0.5) unsaturated chamber silica gel (Toxi-Lab)

sential: the solution was replaced with fresh formaldehyde solution about once a week.

AII—Mandelin's reagent. To 150 mL of concentrated sulfuric acid in a 500-mL Pyrex flask, 250 mg of ammonium metavanadate was added and stirred under gentle warming until completely dissolved. The solution was cooled to room temperature and diluted with an additional 100 mL of concentrated sulfuric acid.[2]

AIII—Modified Dragendorff's reagent. This reagent consisted of 5 g of potassium iodide, 2 g of iodine, and 0.8 g of bismuth subnitrate dissolved in 10.5 mL of glacial acetic acid, 0.5 mL of 36% hydrochloric acid, and 239 mL of water. The reagent was slightly opaque. It was capped tightly.

The color reactions utilized were those described by Toxi-Lab [*6*] and were carried out in the following way: After development of the plate, the solvent was allowed to evaporate in an oven or on a hot tray. Then, four color stages were carried out in sequence on the same plate.

Color Stage 1

The plate was put on the standoff in the jar with reagent AI for 2 min. Then the plate was put on a warm plate for 5 to 10 s to remove *some* of the formaldehyde fumes. Next, the plate was dipped slowly in and out of reagent AII and allowed to drip dry over an open jar for 20 to 30 s. Observations were recorded.

Color Stage 2

The plate was dipped in water once, quickly, then held above the jar for several seconds to let the colors develop. Then it was dipped once again. Phenothiazines develop a pink color and imipramine a blue color in this stage. Dipping was continued until morphine or codeine in the reference mixtures turned tan. Observations were recorded.

Color Stage 3

The plate was redipped several times in the same jar of water, then viewed in the dark under long-wave UV light (366 nm). Observations were recorded.

Color Stage 4

The plate was placed in reagent AIII and dipped in and out of the solution several times. Observations were recorded.

Note that waiting longer than about 2 min between the color stages may result in reduced visualization of some drugs (for example, cocaine, nicotine).

All the reagents and solvents were of analytical grade (Merck) except the 30% ammonia, which was from Baker (Deventer, The Netherlands). The drugs were of pharmaceutical grade and were dissolved in ethyl acetate, at 1 mg/mL. The spotting volumes were 5 μL. The observed Rf values on the plate were corrected using drug reference mixtures for solvent systems 1 through 4, as described by Moffat et al. [*4*]. For solvent system 5, the same correction approach was applied using a mixture of strychnine (Rf^c = 11), amitriptyline (Rf^c = 54), and methaqualone (Rf^c = 90) as references.

For the encoding of colors, a number of color coding wheels were developed, with the

[2]The Toxi-Gram A plates come preimpregnated with ammonium metavanadate, so this compound can be omitted if AII is used with Toxi-Gram A plates only.

numbers for the colors positioned in a circular way. The individual colors were selected from a commercially available system [7]. Four wheels, with 32, 14, 12, and 10 different colors, respectively, were evaluated. The wheel with 10 colors was eventually selected for routine use and consisted of the following colors (with their respective RAL codes in parentheses): white (9016), yellow (1016), orange (2003), brown (8023), red (3018), violet (4008), black (9017), blue (5015), turquoise (5018), and green (6018). This color wheel is depicted in Fig. 1.

Results and Discussion

Selecting the Systems and the Color Reactions

To investigate the IP of TLC Rf values in combination with color reactions on the plate, four generally accepted and widely used classical TLC systems were selected from those recommended for analysis of basic and neutral drugs [4]. A fifth, a so-called dedicated system, was added—the Toxi-Lab A system for basic and neutral drugs on silica-impregnated glass fibers. This system is widely used in the United States.

The color reactions had to meet the following demands: they had to (1) be fast and easy to perform; (2) give reproducible results; (3) be independent of the TLC system and plate; (4) be applicable in sequence on the same plate, so that for one spot a series of color parameters is obtained; (5) produce a large variety of colors; and (6) show low correlation between the color reactions.

After searching the literature and evaluating a multitude of color reactions, we finally selected the reactions recommended for the Toxi-Lab system [6]. All four reactions proved to be suitable in all systems used, with one exception, which could be easily corrected for: in solvent system 3, the presence of the ion-pairing agent sodium bromide disturbed the colors. To avoid this, the plates had to be immersed in water for 30 s to wash out the sodium bromide. After the plate had been dried, the color reactions could be applied in sequence, as described. This procedure slightly lowered the sensitivity of the color reactions but did not affect the colors as such. It was also noted that the presence of potassium hydroxide in system 4 caused an exothermic reaction when the plate was placed in sulphuric acid in color stage 1. This did not affect the colors or the sensitivity, but the sulphuric acid had to be refreshed earlier when potassium hydroxide was present (after the reagent turned from yellow to green).

Color stages 1 through 3 showed a variety of colors within each series and a relatively low correlation between the series. On the other hand, color stage 4, using the modified Dragendorff's reagent, showed essentially one color, namely brown. However, this reaction was included because it permitted the detection of a number of substances that did not react in color stages 1 through 3. It will be noted that fluorescence quenching under short-wavelength UV light was not incorporated into the present scheme, because fluorescence quenching is considered a detection reaction rather than an identification reaction. Hence, it is not very useful for discerning between substances.

Encoding the Colors

In order for colors to be amenable to computer handling, it is obvious that some type of numerical coding must be developed and that the colors observed on the plate must be encoded accordingly. Further, the coding system has to be set up in such a way that it allows fast and reliable identification of unknown substances. To meet these prerequisites, the following difficulties had to be overcome by the authors:

1. There can be interchangeability or mismatching of colors. When different persons have to name colors or assign numerical codes to given colors, there is a wide variety in

their answers. This also occurs when the same person is asked to assign names or codes to the same colors on different occasions.

2. Colors may be dependent on the substance concentration and may also show within-day and day-to-day variation.

3. One spot may show more than one color; for example, the center may differ from the rim.

4. Although thousands of colors exist, for reasons of feasibility, one has to limit the number of colors and to make a selection that includes the most appropriate colors. On the other hand, this may result in situations in which an observed color on the plate does not exactly match the selected color on the chart.

5. The interrelationships between the color codes and the other parameters (for example, the Rf values) in the MLL calculations and in the identification program are complex.

A number of coding charts were developed and evaluated using student panels. Mismatching of colors was to some extent person-related (some degree of color blindness may have been involved). On the other hand, mismatching appeared to occur more frequently with some colors than with others. This important finding led us to the principle that interchangeable colors should have interrelated codes and that these codes should have sequential numbers. This principle was initially worked out to the extent that interchangeable colors were put together in groups [5], under the assumption that mismatching between colors belonging to different groups would not occur. However, in more extensive investigations, it was found that such was not always the case. Therefore, the colors were rearranged in a circular form in such a way that the sequence in which the colors are positioned reflects the possibilities for interchangeability; for example, orange is between yellow and brown, turquoise between blue and green, and so forth. The optimum sequence was again evaluated by means of student panels, which were asked to encode sets of certain colors (described below).

For the encoding of colors using a color wheel, the following routine was developed.

- The phenomenon of a spot showing more than one color could be solved satisfactorily by coding the predominating color only. In most cases, this color was present in the center of the spot.
- When the observed color on the plate deviated somewhat from the colors on the wheel, the best match was sought and the color was coded as such. When no spot was observed in one of the color stages, a value according to the background was given, namely, white in color stages 1, 2, and 4 and black in color stage 3.
- To solve the problem of the concentration-dependent behavior of colors, the colors were made independent of the color intensity. For instance, a black color in a low intensity appearing to be grey was encoded as black. In the same way, a pink spot was encoded as red.

Calculation of MLL Values

For determination of the IP of the four color reactions, as such and in combination with TLC Rf values, the MLL approach was used [2]. In the MLL approach, for a given data set, the average number of drugs that come into consideration for identification is calculated. When MLL values are calculated by means of retention parameters or UV maxima, two assumptions are made:

- When measuring the analytical parameter of a substance, the values will show a known (usually Gaussian) fluctuation around a certain mean value.
- The reproducibility of an analytical system can be described as the mean standard deviation of all substances tested in that system.

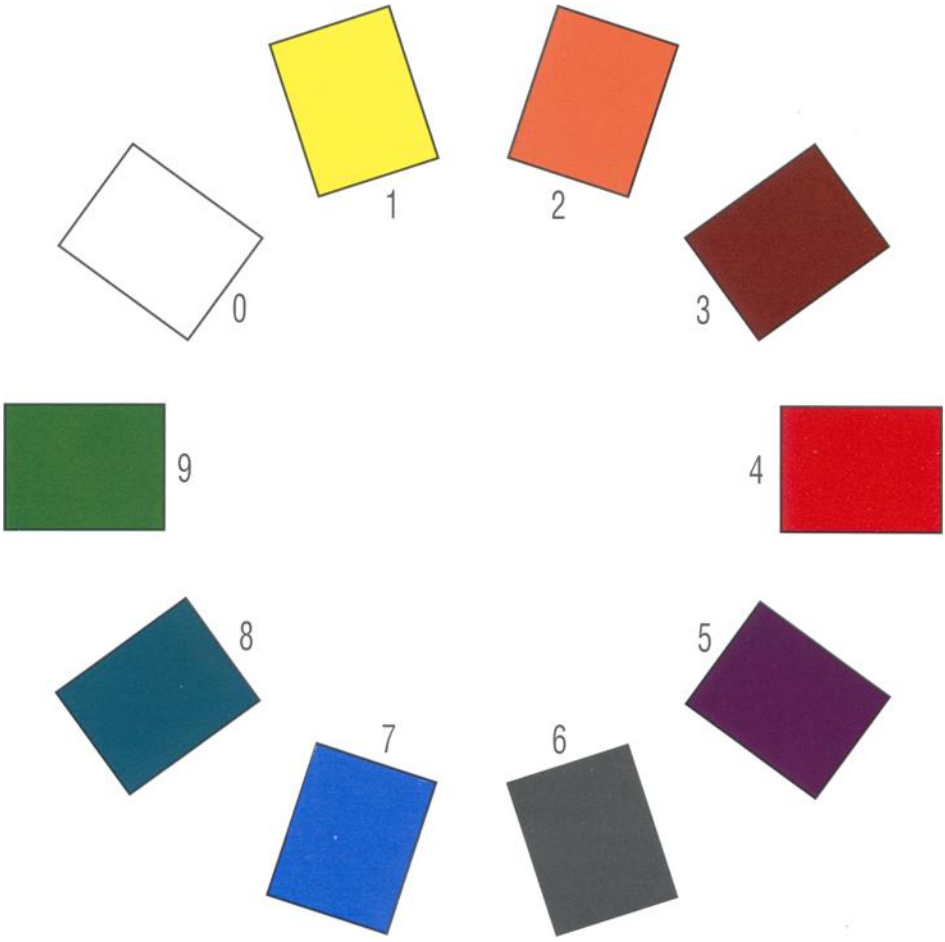

FIG. 1—*Color coding wheel with 10 colors.*

For the color reactions the same assumptions were made. To determine the standard deviation of a color reaction using a given color coding wheel, 20 different substance spots were encoded by a panel of 20 persons. For each spot, the mean value and standard deviation were calculated. For the 20 spots, the standard deviations were averaged to get the standard deviation for a given color coding wheel. Then, for a set of 100 substances, the colors were encoded and MLL calculations were performed. For interpretation of the results, it must be realized that the lower the MLL the better the system, and that the optimum value is 1.00. An MLL of 1.00 indicates that each substance in the data set can be identified unequivocally.

Table 2 gives the standard deviations of the individual color codings and the results of the MLL calculations for the combination of the four color reactions. With the wheel with 32 colors, mismatching occurs more often, resulting in relatively large standard deviations and, hence, in a large MLL value. The MLL values of the other color coding wheels are of comparable magnitude.

Table 3 shows the results of the MLL calculations for Rf values alone in the five TLC systems, and for Rf values in combination with the colors observed in the four color stages but using different coding wheels. These data clearly show that color reactions greatly enhance the identification power of the TLC systems since MLL values are reduced by a factor of 8! When comparing the four color wheels, the one with 14 colors comes out best, followed by the wheels with 10, 12, and 32 colors. When two TLC systems are used, giving two Rf values and two times four color reactions, the combinations of TLC systems 2 and 5 and 3 and 4 come out best, both combinations having an MLL of 1.10. This still means that not all substances of this set of 100 substances can be identified unequivocally, even when the best combination of two TLC systems and color codes are applied. The resulting MLL value of 1.10 is still slightly higher than the ultimate value of 1.00.

TABLE 2—*Mean standard deviation (SD) for each individual color stage and the MLL value for the combination of color stages using different color coding wheels.*

Number of Colors on Coding Wheel	Mean SD in Color Stage				MLL
	1	2	3	4	
32	1.23	2.02	1.04	1.15	7.06
14	0.88	0.98	0.34	0.31	3.82
12	0.65	0.72	0.28	0.02	3.91
10	0.38	0.35	0.20	0.11	3.91

TABLE 3—*MLL values of single TLC systems, based on Rf values alone and on Rf values plus color values in the four color stages, encoded using different color wheels.*

TLC System		Standard Deviation of Rf[a]	MLL for Rf Alone	MLL for Rf with Color Coding Wheel			
No.	Solvent			10	12	14	32
1	EtAc/MeOH/NH_4OH	3.7	18.1	1.88	1.83	1.60	2.42
2	MeOH	2.7	14.4	1.64	1.72	1.55	2.01
3	MeOH/BuOH; NaBr	3.0	13.4	1.65	1.77	1.56	1.81
4	Cyclohexane/toluene/DEA	2.3	13.1	1.55	1.61	1.53	2.02
5	Toxi-Gram A	3.0	12.9	1.70	1.70	1.43	2.20
Average			14	1.7	1.7	1.5	2.1

[a]From Ref *4*.

When TLC is compared with gas chromatography, it is important to note that a single TLC system with the four color stages now provides a three- to four-times higher identification power (see Table 3) than the standard GC system on OV-1/SE-30 [*8*], for which a MLL value of about 8 was established on packed columns [*2*] and one of about 6 on capillary columns[3] for a similar set of 100 substances. Moreover, the IP of TLC can be enhanced even further by using two or more systems in parallel, especially when systems are chosen that have little correlation. Gas chromatography does not offer this additional advantage, since virtually all GC systems are highly correlated [*8*]. The correlations between TLC systems 1 through 4 in Table 1 are relatively low, with system 5 being comparable to system 1. Thus, a combination of two of systems 1 through 4 using classical plates or of system 5 using Toxi-Gram A plates and any of systems 2 through 4 using classical plates can be considered a good choice for running two or more systems in parallel.

Another important factor in the selection of a color coding wheel is the ease and speed of operation. Encoding 20 spots with the wheel with 32 colors took some 45 min, whereas encoding the same spots with the wheel with 10 colors took less than 15 min. Obviously, offering a larger variety of colors delays the encoding and introduces more mismatching. Therefore, in view of the IP values obtained and the ease of operation, a wheel with some 10 colors appears to be a suitable compromise, although it should be noted that the wheel in Fig. 1 may still be open for improvement. This is because evaluating color wheels in general practice is rather time-consuming. Such evaluations are presently being carried out, in relation to setting up a large database of Rf values and color reactions which can be used for computerized drug identification.

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