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IDENTIFICATION POWER OF THIN-LAYER CHROMATOGRAPHIC COLOUR REACTIONS AND INTEGRATION OF COLOUR CODES IN A DATABASE FOR COMPUTERIZED IDENTIFICATION IN SYSTEMATIC TOXICOLOGICAL ANALYSIS

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SUMMARY

A system has been developed that makes colour reactions on thin-layer chromatographic (TLC) plates amenable to computer handling. As a result, corrected R_F values and colour reactions can now be used for the identification of unknown substances by means of computerized retrieval from large databases. The system is based on a series of four colour reactions carried out in sequence on the same spot and by numeric encoding of the observed colour by means of a colour reference chart. Other identification parameters such as retention indices in gas chromatography (GC) and UV absorption maxima can also be introduced in the identification program. The utilization of colour reactions in combination with corrected R_F values considerably increased the identification power of TLC in that the so-called Mean List Length parameter could be reduced by *ca.* 50–70% compared with using corrected R_F values alone. The application of a single TLC system with colour reactions now provides about the same identification power as a single GC system and can be further increased by using two or three different TLC systems in parallel.

INTRODUCTION

Systematic toxicological analysis (STA) consists in screening for potentially harmful substance(s) whose presence is (are) uncertain and its (their) identity unknown. Hence, it forms the qualitative part in areas such as clinical intoxication, forensic analysis, drug abuse analysis, doping and environmental pollution. In each area one must be able to detect and distinguish between a great many possible substances, and a variety of analytical methods may be applied, such as gas chromatography (GC), thin-layer chromatography (TLC), immunoassays, UV spectrometry and mass spectrometry. In order to identify an unknown substance properly against a background of a great many others, it is clear that one must have access to a large computerized database containing reference data for all these substances in the various analytical techniques to be applied.

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In recent years, considerable attention has been given to the evaluation of analytical techniques and systems (GC, TLC, UV, etc., are called techniques, whereas within techniques such as GC one can distinguish different systems such as OV-1, OV-17) with regard to their individual suitability for STA. To compare the suitability of techniques and/or systems, the Mean List Length (MLL) concept was developed[1,2]. In this concept, the MLL is the mean number of candidates that would qualify for identification when a given technique/system alone or in combination is used for the identification of an unknown substance. The smaller the number of substances qualifying for identification, the better are the techniques or systems. The optimum is reached with a MLL of 1.00, which means that all substances in a given set can be identified unequivocally. Thus, the MLL gives an objective criterion to establish the identification power (IP) of a single system, combinations of systems or even combinations of different techniques. So far, the MLL concept has been extensively applied in GC, TLC and UV spectrometry[3]. A computerized identification program utilizing these three techniques and with a database of about 1600 substances has recently become available[4]. With regard to TLC it could be shown, for example, that the use of more than one system could greatly enhance the IP as reflected by a shortening of the MLL[3], yet that the choice between the systems had to be made carefully[5].

Previously, these TLC investigations using the MLL concept were carried out on the basis of the R_F values alone, as observed by fluorescence quenching. It is well recognized that TLC offers the extra advantage of applying colour reactions on the plate and that this may add additional IP. However, the incorporation of colour reactions proved to be a very difficult problem.

EXPERIMENTAL

Thin-layer plates coated with silica gel (0.25 mm) on 20 × 10 cm glass plates with fluorescence indicator (Kieselgel 60 F_{254}; Merck, Darmstadt, F.R.G.), for use with solvent systems 1–4. For solvent system 5 Toxi-Gram A glass-fibre plates (Toxi-Lab, Irvine, CA, U.S.A.) were used.

The following TLC systems were applied, with their standard deviations (S.D.) in R_F units in parentheses:

TLC 1: ethyl acetate–methanol–25% ammonia (85:10:5) in saturated tanks (S.D. = 3.7);

TLC 2: methanol in unsaturated tanks (S.D. = 2.7);

TLC 3: methanol–*n*-butanol (60:40) containing 0.1 *M* sodium bromide in unsaturated tanks (S.D. = 3.0);

TLC 4: cyclohexane–toluene–diethylamine (75:15:10) in saturated tanks (S.D. = 2.3); the plates were impregnated with 0.1 *M* potassium hydroxide solution in methanol and dried;

TLC 5: ethyl acetate–methanol–water (87:3:1.5) containing 5 ml/l of 30% ammonia solution (S.D. = 4.2).

All solvents were of analytical reagent grade (Merck), except the 30% ammonia, which was from Baker (Deventer, The Netherlands). Drugs were of pharmaceutical grade and were dissolved in ethyl acetate at 1 mg/ml.

Most of the TLC data for systems 1–4 were taken from Moffat *et al.*[5], and those

for system 5 were from the Toxi-Lab Drug Compendium[6]. Data not available from these references were determined and are the means of at least four determinations. R_F values were expressed as corrected R_F values (R_F^c values). These corrections were made with the standard reference samples described in ref. 5, or with the standards present on the Toxi-Gram A plates.

The following colour reactions were carried out in sequence:

CR 1: exposure of the plate to formaldehyde vapour and submersion in concentrated sulphuric acid–ammonium vanadate;

CR 2: dipping of the plates in water;

CR 3: observation of fluorescence at 366 nm;

CR 4: dipping of the plates in iodine–bismuth subnitrate–potassium iodide solution (modified Dragendorff reagent).

The colour reactions were carried out after development of the TLC plate in one of the above systems 1–5 after evaporation of the solvent in an oven or on a hot-tray (Maxim Warming Tray; Toxi-Lab). The exact procedure was as follows: (1) about 25 ml of formaldehyde were placed in a beaker, which was put in a closed chromatography jar. The plate was exposed to formaldehyde vapour for 2 min and dried for 5–10 s on the hot-tray (stage 2), then the plate was slowly submersed in sulphuric acid–ammonium vanadate solution (250 ml of 95–97% sulphuric acid + 200 mg of ammonium vanadate) and removed after 5 s. The latter was repeated twice after 20 s and the colour was observed. (2) The plate was quickly submersed in 250 ml water and immediately removed. After drying in air for about 1 min, the plate was dipped in and out the water again two or three of times until the colours were stable. (3) The plates were drip-dried and observed under UV-light (366 nm). (4) The plates were submersed in modified Dragendorff reagent (5.0 g of potassium iodide, 2.0 g of iodine, 0.2 g of bismuth subnitrate, 0.5 ml of concentrated hydrochloric acid, 10.5 ml of glacial acetic acid and 239 ml of water) for about 1 min, after which the colours were observed.

These colour reactions are essentially from the Toxi-Lab system[6], but were found to be equally well applicable to the Merck glass plates. It should be noted, however, that the Toxi-Gram plates are supplied preimpregnated with ammonium vanadate, so that the latter can be omitted from CR 1 when working with Toxi-Grams.

The colour reactions were carried out with the plate in an appropriate holder or clamp. The colours observed were compared with a chart containing 21 different colours that had been coded numerically (see Fig. 1) and were encoded on the basis of their closest match. In this way, each substance received for colour codes (CC 1–CC 4), one for each colour reaction carried out. The colour codes and the R_F^c values provided the imput for the database.

MLL calculations were carried out with these parameters for a set of 99 basic and neutral drugs, using the program Color Tox, written in Turbo Pascal under MS-DOS. The substances with their respective R_F^c values and colour codes are listed in Table I.

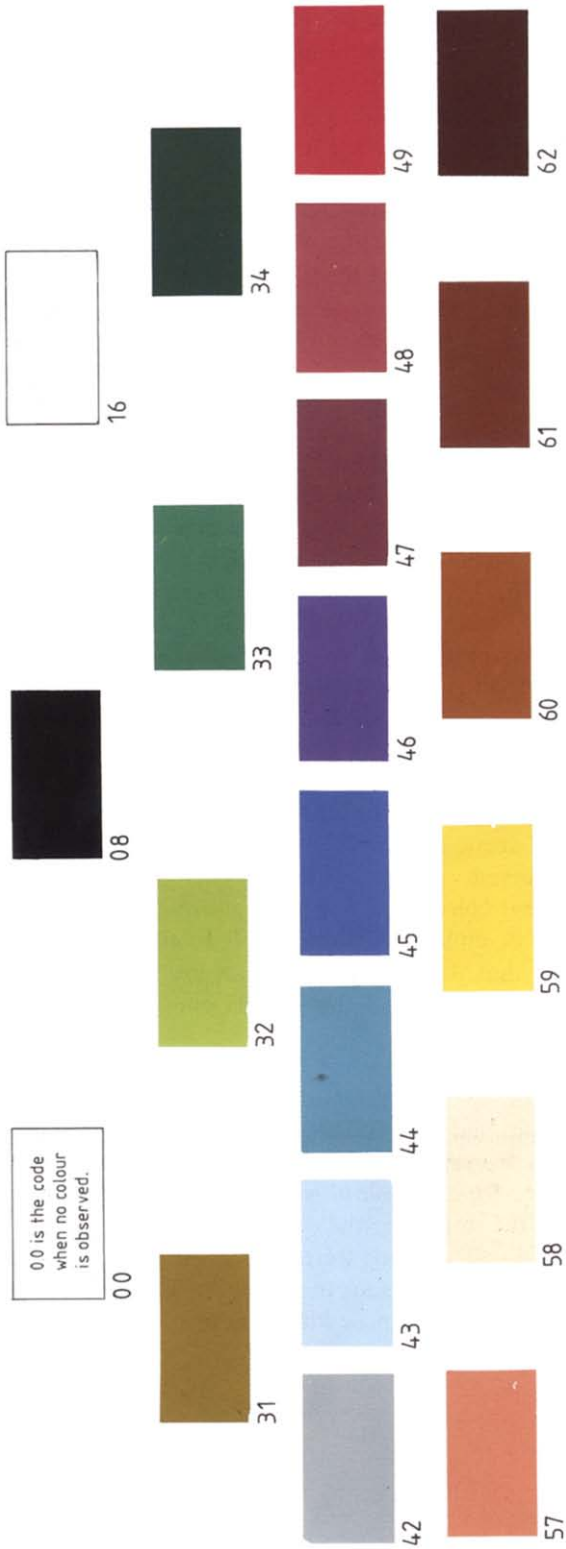

Fig. 1. Colour coding chart.

TABLE I

R_F^c VALUES AND COLOUR CODES FOR THE 99 BASIC AND NEUTRAL DRUGS INVESTIGATED

R_F^c values are expressed as hR_F^c values; NA indicates not analysed in this system; MMDA = 2-methoxy-4,5-(methylenedioxy)amphetamine; ND means that the four colour reactions were negative and that the spot in system 5 was therefore not detectable.

No.	*Substance*	*TLC system*					*Colour code*			
		1 R_F^c	*2* R_F^c	*3* R_F^c	*4* R_F^c	*5* R_F^c	*1* CC	*2* CC	*3* CC	*4* CC
1	Amitriptyline	70	26	51	55	58	60	60	60	60
2	Amphetamine	44	12	75	15	32	61	33	44	60
3	Atropine	25	6	28	6	5	43	0	0	60
4	Benzatropine	36	6	NA	26	11	59	0	44	60
5	Caffeine	50	60	55	3	64	0	0	0	42
6	Carbamazepine	57	78	75	4	70	0	0	44	60
7	Chlordiazepoxide	52	76	77	2	65	0	0	0	61
8	Chlormethiazole	76	74	84	44	81	0	0	0	60
9	Chlorpromazine	70	25	45	49	57	46	48	0	60
10	Cimetidine	51	72	54	0	22	43	0	0	60
11	Clomipramine	72	26	54	54	57	0	45	32	60
12	Clorazepic acid	69	82	87	3	81	0	0	33	61
13	Cocaine	77	35	30	47	79	0	0	0	60
14	Codeine	35	20	22	6	24	46	57	0	60
15	Desipramine	41	7	71	20	24	0	45	0	60
16	Desmethyldiazepam	69	82	83	4	81	0	0	33	61
17	Dextromethorphan	50	10	48	44	26	45	0	0	60
18	Dextromoramide	78	71	78	40	86	0	0	0	61
19	Dextropropoxyphene	80	50	63	59	82	8	8	34	60
20	Dextrorphan	46	10	50	11	19	61	60	32	61
21	Diamorphine	51	26	33	15	30	47	57	8	60
22	Diazepam	76	82	85	23	90	0	0	33	61
23	Dihydrocodeine	27	11	19	8	12	46	60	45	61
24	Diphenhydramine	68	28	48	45	55	59	0	45	60
25	Dipipanone	84	30	72	66	85	46	0	16	60
26	Dipyridamole	41	82	83	0	60	47	58	34	60
27	Disopyramide	60	9	7	7	32	0	0	0	60
28	Doxepine	65	24	45	52	52	49	48	0	60
29	Doxylamine	60	12	NA	41	38	45	48	33	60
30	Ephedrine	27	10	64	5	14	34	33	44	60
31	Flunitrazepam	76	79	82	10	80	0	0	45	60
32	Flurazepam	72	52	45	30	64	0	0	34	60
33	Haloperidol	74	51	75	10	64	43	0	45	60
34	Hydrocodone	31	11	13	4	18	45	0	0	60
35	Hydromorphone	18	12	14	3	10	46	59	0	60
36	Hydroxyzine	53	56	65	9	52	0	59	44	60
37	Imipramine	67	21	47	49	50	0	45	0	60
38	Ketamine	87	80	68	37	85	0	0	0	60
39	Lidocaine	80	70	69	35	88	57	0	45	60
40	Lorazepam	45	82	82	1	75	59	0	33	61
41	Maprotiline	35	6	71	17	15	62	62	0	61
42	Meclofenoxate	67	46	NA	26	15	45	48	44	60
43	Medazepam	78	79	83	40	82	48	59	59	60
44	Mepyramine	56	21	33	39	49	48	0	45	60
45	Metamphetamine	42	9	63	28	22	61	31	43	60
46	Methadone	77	16	60	61	66	45	0	3	60

(*Continued on p. 666*)

TABLE I (*continued*)

No.	*Substance*	*TLC system*					*Colour code*			
		1 R_F[c]	*2* R_F[c]	*3* R_F[c]	*4* R_F[c]	*5* R_F[c]	*1* *CC*	*2* *CC*	*3* *CC*	*4* *CC*
47	Methapyrilene	64	18	24	43	52	48	46	44	60
48	Methaqualone	78	78	84	37	90	0	0	0	63
49	MMDA	43	9	74	17	28	57	59	0	61
50	Metoprolol	42	19	NA	8	25	46	42	45	60
51	Metronidazole	46	80	66	2	ND	0	0	0	0
52	Mianserine	70	49	52	39	66	0	0	44	60
53	Morphine	20	18	23	0	15	46	60	0	60
54	Morphine-6-acetate	48	25	27	6	30	47	57	8	60
55	Nadolol	20	14	NA	1	1	49	49	45	60
56	Nicotine	61	39	22	39	40	0	0	44	60
57	Nomifensine	64	53	51	8	64	60	48	44	60
58	Nortriptyline	45	9	71	27	30	47	0	46	60
59	Orphenadrine	70	23	49	48	52	60	0	44	60
60	Oxazepam	47	81	82	0	68	61	0	44	60
61	Oxycodone	60	30	33	23	62	43	0	0	60
62	Papaverine	69	74	74	8	72	46	57	0	60
63	Pentazocine	72	33	72	15	61	57	57	33	60
64	Pethidine	62	34	40	37	50	61	0	45	60
65	Phenazopyridine	70	80	NA	1	88	48	48	45	60
66	Phendimetrazine	64	50	41	36	55	61	0	44	60
67	Phenethylamine	54	44	NA	28	22	61	31	44	60
68	Pheniramine	46	14	26	35	21	0	0	0	60
69	Phenmetrazine	46	34	45	14	39	61	0	45	60
70	Phentermine	48	11	78	26	34	61	31	32	60
71	Phenylpropanolamine	28	12	75	4	23	34	33	44	60
72	Phenyltoloxamine	66	30	NA	39	55	49	0	0	60
73	Pindolol	43	18	75	2	32	60	46	0	61
74	Prazepam	81	85	89	36	95	0	0	44	60
75	Procainamide	39	16	33	1	20	0	0	34	60
76	Procaine	70	33	42	6	64	0	0	34	60
77	Prochlorperazine	54	23	26	33	ND	0	0	0	0
78	Procyclidine	72	19	68	63	55	61	61	16	60
79	Promethazine	65	30	44	37	27	34	49	46	60
80	Propranolol	52	20	79	7	36	34	8	61	61
81	Psilocin	50	12	48	5	33	61	47	33	60
82	Quinidine	52	30	63	4	25	0	0	44	60
83	Quinine	42	27	65	2	25	0	0	44	60
84	Strychnine	33	8	11	8	12	48	57	0	60
85	Temazepam	63	82	82	8	81	59	0	0	60
86	Theophylline	10	72	66	1	40	0	0	0	42
87	Thioridazine	67	19	55	43	ND	0	0	0	0
88	Timolol	48	21	74	6	30	45	0	0	60
89	Tiothixene	44	25	NA	9	25	0	0	33	60
90	Trazodone	68	66	58	9	60	43	0	45	60
91	Triamterene	30	50	NA	1	31	59	59	44	60
92	Triazolam	43	69	65	1	39	0	0	45	60
93	Trifluoperazine	56	30	29	33	37	32	48	0	60
95	Trihexyphenidyl	82	44	75	65	39	47	47	34	60
96	Trimeprazine	76	31	46	55	72	33	48	0	60
97	Trimipramine	80	36	56	62	78	0	0	45	60
98	Tripelenamine	68	22	34	44	51	59	31	45	60
99	Verapamil	74	44	61	23	62	45	0	45	60

RESULTS AND DISCUSSION

The colour reactions

The colour reactions had to meet the following demands: (1) fast and easy to perform; (2) reproducible; (3) independent of TLC system and plate; (4) applicable in sequence on the same plate, so that a series of colour parameters is obtained; and (5) low correlation between the colour reactions, yet a large variety of colours within a given colour reaction.

After searching the literature and evaluating a multitude of colour 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 that could be easily corrected for: in system 3, the presence of the ion-pairing agent sodium bromide disturbed the colours. To avoid this, the plates had to be immersed in water for 30 s to wash out sodium bromide. After drying the plate, the colour reactions could be applied in sequence as described. This procedure slightly lowered the sensitivity of the colour reactions but did not affect the colours 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 CR 1. This did not affect the colours 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).

Colour reactions 1–3 showed a variety of colours within each series and a relatively low correlation between the series. On the other hand, CR 4, the modified Dragendorff reagent, showed essentially one colour, namely brown. However, this reaction was included because it permitted the detection of a number of substances that did not react with CR 1–3. It will be noted that fluorescence quenching under short-wavelength UV light was not incorporated in the present scheme, because fluorescence quenching is to be considered a detection reaction rather than an identification reaction. Hence, it is not very useful to discern between substances.

Encoding the colours

In order for colours to be amenable to computer handling, it is obvious that some type of numerical coding must be developed and that the colours observed on the plate have to 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. When trying to meet these prerequisites, the following difficulties had to be overcome: (1) interchangeability or mismatching of colours: when different persons have to name colours or assign numerical codes to given colours, 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 colours on different days. (2) Colours may be dependent on substance concentration and may also shown within-day and day-to-day variety. (3) One spot may show more than one colour; *e.g.*, the centre may differ from the rim. (4) Although thousands of colours exist, for reasons of feasibility one has to limit the number of and to make a selection that includes the most appropriate colours. On the other hand, this may result in situations where an observed colour on the plate does not exactly match the selected colours on the chart. (5) How to interrelate the colour codes with the other parameters (*e.g.*, the R_F^c values) in the ML calculations and in the identification program.

This finally resulted in the colour coding chart depicted in Fig. 1, which contains 21 codes, subdivided into six groups which differ by eight digits. The first three groups contain only one code each:

00 when no spot is seen;
08 for black spots;
16 for white spots; as "no spot" is coded by 00, this means that for code 16 the white spot must appear against some kind of a background (CR 3);
31–34 for various shades of green;
42–49 for grey to blue to violet to red;
57–62 for orange to yellow to brown.

The underlying reasons are the following: (1) mismatching of colours was to some extent person-related (colourblindness). On the other hand, mismatching between certain colours occurred more frequently than with others. Therefore, interchangeable colours should have interchangeable codes and the latter should have successive numbers. This principle was utilized to divide the standardized colours into groups. Within a group, colours are interchangeable, between groups they are not. The results were evaluated in-house by various panels of students, in the end leading to the above-mentioned groupings. The largest group (42–49) contained eight colours, whereas the first three groups contained only one. Because the largest difference in CC *within* a given group never exceeded seven, the differences in CC *between* groups was always eight or more, and when this occurred the colours were considered not te be interchangeable. (2) Within-day, day-to-day and concentration-dependent variations were found to occur, but always within a certain group. However, when the concentration of a spot was such that is was clearly overloaded, the sample was re-run again at a lower concentration. This usually provided a more accurate $R_F{}^c$ value and a better colour coding. (3) The phenomenon of a spot showing more than one colour could usually be solved satisfactorily by coding the predominating colour only. In most instances, the latter was present in the centre of the spot. (4) When the observed colour on the plate deviated from the colours on the chart, the best match was sought and the colour was coded as such. (5) When calculating MLL values by means of retention parameters or UV maxima, two assumptions were made: (i) when measuring the analytical parameter of a substance the values will show a known (usually Gaussian) fluctuation around a certain mean value; and (ii) the reproducibility of an analytical system can be described as the mean standard deviation of all substances tested in that system. These two assumptions cannot be used for colour codes as there is no mean colour. However, the principle of gathering interchangeable colours in groups offers a means of calculating the probability that interchange actually occurs. This can be expressed by

$$p(X_{ij}) = Z^{\mathrm{CC}(ij) - \mathrm{CC}(ik)} \tag{1}$$

where $p(X_{ij})$ is the probability of the colour of substance j being interchanged with the colour of substance k, i is the number of the colour reaction (CR 1–CR 4), $\mathrm{CC}(ij) - \mathrm{CC}(ik)$ is the difference in colour code (CC) between substances j and k in reaction i and Z is an experimentally determined value between zero and one. By using $p(X_{ij})$, MLL values could be calculated for the identification power of the set of colour reactions alone and for combinations of $R_F{}^c$ values and colour reactions.

The value for Z was experimentally established by calculating the MLL values with only the CC as input and for different values of Z ranging from 0.01 to 1.0. With $Z = 1$, the colour reactions give no information, as for every value of CC(ij) − CC(ik) the value of $p(X_{ij})$ will remain 1. Fig. 2 shows that for Z values lower than 1 the MLL value decreases rapidly until it reaches a plateau of about 4 with a Z value of 0.6. Z values below 0.6 had virtually no further impact on MLL. A Z value of 0.6 indicates that the probability of a given colour being mismatched with a code on the chart is 60% for neighbouring numbers. As the latter appeared to be overwhelmingly the case in practice, a Z value of 0.6 was adopted to calculate $p(X_{ij})$ and also the MLL values.

Finally, it should be noted that although the above colour coding system assumes that no mismatching can occur between groups, this principle is not fully enforced in the identification program. In the latter, mismatching between groups is accepted, albeit at a low probability of 0.1% for all substances and codes. This means, for example, that a substance q with an encoded colour of 16 (white) in the database still receives a 0.1% probability in the identification of an unknown if for the latter the colour is considered to be grey on the plate and coded as 42. If the principle of no interchangeability between colour groups were fully enforced. substance q would be automatically rejected as a candidate for the grey spot, and would never be considered again, even if all other colour codes, R_F^c values, etc., were correct. This low probability precaution has also been built in as a safety measure in the ToxAnalysis program[4] in anticipation of unexpected interferences and outliers[1,2].

Calculation of MLL values

With the data listed in Table I and the application of eqn. 1 for the colour reactions, MLL values were calculated for single systems and for combinations of systems, for R_F^c values alone and for R_F^c values plus colour codes. The results are given in Table II and graphically depicted in Fig. 3. It can be clearly deduced that colour reactions add considerable identification power to TLC, as MLL values are reduced by *ca.* 50–70%. Also, using a single TLC system with colour codes provides

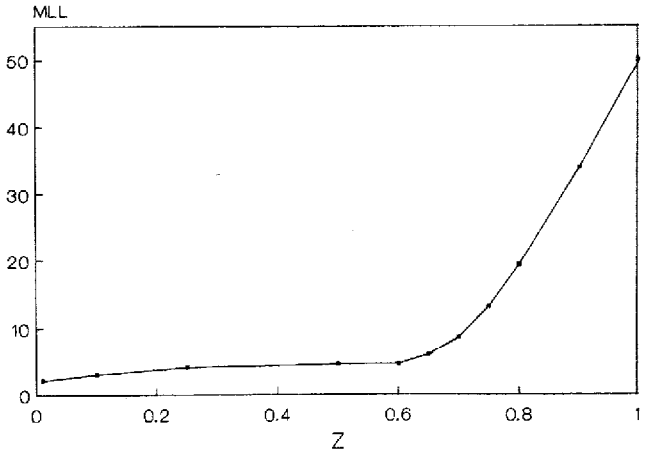

Fig. 2. Relationship between Mean List Length (MLL) and Z. For details, see text.

TABLE II

MLL VALUES FOR TLC SYSTEMS ALONE AND IN COMBINATION WITH COLOUR CODES

Data set for 99 basic and neutral substances (Table I). Values in italics are the lowest in the respective columns and indicate highest identification power.

TLC system	*MLL*		*TLC system*	*MLL*		*TLC system*	*MLL*	
	R_F^c	R_F^c *and CC*		R_F^c	R_F^c *and CC*		R_F^c	R_F^c *and CC*
1	22.33	7.13	1 + 2	6.87	2.13	1 + 2 + 3	2.80	1.39
2	16.68	5.59	1 + 3	6.54	2.19	1 + 2 + 4	3.01	1.31
3	*14.53*	*5.35*	1 + 4	7.00	2.18	1 + 2 + 5	4.63	1.68
4	16.10	5.40	1 + 5	10.96	3.20	1 + 3 + 4	2.48	1.25
5	18.79	6.03	2 + 3	5.21	2.18	1 + 3 + 5	3.36	1.53
			2 + 4	4.70	1.76	1 + 4 + 5	3.53	1.53
			2 + 5	7.39	2.62	2 + 3 + 4	*2.01*	*1.22*
			3 + 4	*3.96*	*1.75*	2 + 3 + 5	2.84	1.55
			3 + 5	5.78	2.21	2 + 4 + 5	2.72	1.28
			4 + 5	5.42	2.03	3 + 4 + 5	2.21	1.32
Mean	17.69	5.90		6.38	2.23		3.00	1.41

about the same identification power as R_F^c values in two systems, whereas R_F^c values in two systems with colour codes is as good as a combination of three R_F^c values. Further, another important practical advantage of the colour reactions in TLC becomes clear when a mixture is being analysed: the various spot patterns obtained with the different TLC systems can now be interrelated via their colour reactions so that the number of so-called configurations can be reduced substantially[1].

It can also be seen that not all substances in this set of 99 substances can be identified unequivocally, even when the best combination of systems 2 + 3 + 4 plus

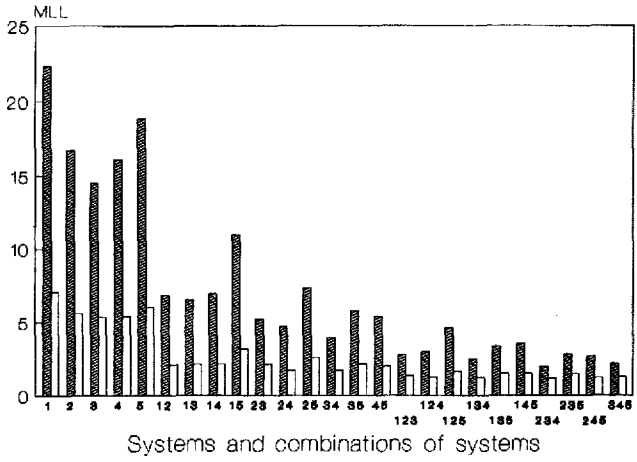

Fig. 3. Mean List Length (MLL) values calculated with R_F^c alone (hatched bars) and with R_F^c combined with colour codes (CC) (open bars). For details, see text.

colour codes are applied. The resulting MLL value of 1.22 is still slightly higher than the ultimate value of 1.00. On the other hand, when the computer lists the number of cancidates that come into consideration for identification, it will do so in ranking order on the basis of probability and similarity index, which indicate the closeness between observed data and listed data in the database[4]. This rank order is of additional help in the final identification process.

When TLC is compared with GC it is interesting that a single TLC system with the colour codes now provides a slightly better identification power than the standard GC system on OV-1[7], for which an MLL value of about 8 was established[3].

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