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Potential and pitfalls of chromatographic techniques and detection modes in substance identification for systematic toxicological analysis

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Abstract

The potential and the constraints of thin-layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC) towards substance identification, together with their detection modes, are considered. The latter include colour reactions on the plate, molecular masses through chemical ionization mass spectrometry (MS) and diode-array UV spectrophotometry. Evaluations are carried out by the mean list length approach. Not surprisingly, GC–MS and HPLC–diode array detection qualify as the two most powerful combinations. However, one does not necessarily need to have access to these sophisticated detection modes: the identification power of TLC and colour reactions plus GC or HPLC retention indices is high and even a suitable combination of TLC and colour reactions remains a valuable tool. After analysis, the findings for the unknown substance(s) must be matched against databases containing the behaviour of reference substances. The search process for the computerized retrieval of potential candidates must allow the handling of all possible combinations of identification techniques applied.

1. Introduction

Systematic toxicological analysis (STA) can be defined as the logical chemical–analytical search for an unknown substance of toxicological relevance. As such, it represents a most important aspect of all toxicological analyses, such as clinical, forensic, environmental, occupational, workplace and traffic toxicology, and also in drugs of abuse testing and doping. First, it has to be established whether suspicious substances can be detected (screening); then the identity of the detected compounds must be established beyond reasonable doubt (identification or confirmation).

With regard to the actual analysis, toxicologists may choose from a series of analytical techniques and systems, such as thin-layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC), and an array of immunoassay tests. The chromatographic techniques may be combined with appropriate detection modes, such as colour reactions, element-specific detections, UV and diode-array detection (DAD) and mass spectrometry (MS) which can provide additional identification parameters.

However simple as STA may appear at first sight, it poses many difficulties. As the ultimate

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aim of STA is to exclude all relevant substances except one, the major issues are not to miss any substance in the screening phase (false negatives) and to make sure that the substances found are properly identified (false positives). In order to achieve this, the following prerequisites are to be considered: (a) the utility of the various analytical systems towards STA needs to be established and the best choices can then be designated as recommended systems; and (b) reference substances are to be run in these systems so that databases can be compiled of their appropriate parameters (R_F values, retention indices, ultraviolet and mass spectra, etc.). Identifications can then be performed by comparing the behaviour of an unknown substance in the recommended systems with that of the reference substances in the databases. Of course, these databases should be up to date and as comprehensive as possible with respect to hazardous substances and their metabolites, but they should also contain data on therapeutic medicines, endogenous compounds, omnipresent contaminants (*e.g.*, plasticizers), etc. This explains why it is impossible for individual laboratories to develop and maintain their own databases. Instead, they must have access to large databases that have been developed for interlaboratory use.

In this paper we discuss the potential of TLC, GC and HPLC towards STA, taking into account their retention parameters and their detection modes. The latter include colour reactions on the plate for TLC, molecular masses through chemical ionization MS for GC and diode-array detection for HPLC. In addition, the possibilities for identification by means of computerized database searches are considered.

2. The mean list length approach (MLL)

In order to evaluate the suitability of a given analytical technique or system for STA, we developed the MLL concept so that its identification power can be expressed in a concise and objective way [1]. In this statistical approach, it is established for a given analytical parameter in a given system [*e.g.*, a retention index (RI) in a GC system] how many substances from a large population would qualify for identification. The number of substances that qualify is called the list length. If this exercise is repeated for all RI values in that GC system and the individual list lengths are averaged, the so-called mean list length (MLL) for that system is obtained. The shorter the MLL, the better is that system for STA. This can be done for individual systems, but also for any combination of systems, for example, a TLC system (R_F) plus a GC system (RI), two TLC systems (two R_Fs), a TLC system plus a GC system plus an HPLC system (RI), a TLC system (R_F) plus a colour reaction on the plate, a GC system (RI) plus a molecular mass via GC–MS or a TLC system plus an HPLC system plus a diode-array spectrum. The ultimate is reached when an MLL value of 1.00 is found, which means that each individual substance in the data set can be unequivocally identified against the background of all other substances in that set. It will be clear that an MLL of 1.00 can never be obtained by a single system and also that the number of systems required will increase with increasing number of substances in the data set.

Application of the MLL concept has shown that in order for chromatographic techniques to be suitable for STA, the following criteria should be met [1,2]: (1) the relevant substances should show proper migration and be evenly spread over the entire chromatographic range; (2) the retention parameters should be standardized in such a way that good reproducibility is obtained on an interlaboratory scale; and (3) when more than one chromatographic system is used, there must be a low correlation between these systems.

3. Evaluation of TLC, GC and HPLC systems

In recent years, extensive evaluations of TLC and GC systems have been carried out on an interlaboratory basis, which also resulted in extensive databases. Because it is customary to perform a sample work-up on the basis of a pH-dependent extraction, TLC systems were selected that handle either acidic and neutral

drugs (A/N systems) or basic and neutral drugs (B/N systems). This resulted in the recommendation of four TLC systems for A/N drugs and seven for B/N drugs [3]. Table 1 gives an overview of these systems, with their corresponding interlaboratory reproducibilities. The correlations between these systems are given in Table 2. To compensate for variations in the experimental conditions, the R_F values observed are to be corrected by means of reference substances that are run on the same plate as the unknown. In this way, interlaboratory variations can be decreased considerably. The reference substances for each recommended system are also listed in Table 1. The databases for the above TLC systems contain about 1800 substances [3].

For GC, a dimethylsilicone column, either packed or capillary, appears to be the best for screening in STA. Retention is expressed as Kováts retention indices [4], but to cover all substances in one run and to obtain a good spread over the entire chromatogram, temperature-programmed runs are recommended. Under the latter circumstances, there is an almost linear relationship between the carbon number of the *n*-alkanes and the net retention time [not log(retention time) as is the case in the isothermal mode]. Here too, variations in interlaboratory conditions can be compensated for by using a mixture of reference substances of toxicologically relevant compounds. Details can be found in ref. 5. The database in ref. 5 contains data on *ca.* 6000 substances. However, even though GC lends itself better than TLC to screening because of its high separation efficiency (particularly in capillary GC) and good reproducibility, the dimethylsilicone system is the only recommended system. This is due to the fact that other GC systems are all highly correlated with the dimethylsilicone system, so that relatively little additional information is gained by applying a second one [5,6]. Hence it would be a waste of time and effort to undertake the enormous task of setting up a database for a second system.

As for HPLC, its good separation power and general applicability, including thermolabile and non-volatile compounds, seem to make it an attractive technique for STA. Moreover, HPLC offers a wide variety of separation modes and possibilities to vary the stationary and mobile phases. However, difficulties in producing stationary phase materials with sufficient batch-to-batch reproducibility (in addition to brand-to-brand reproducibility) has long been a serious drawback. However, the situation has improved recently and a first HPLC screening system has been recommended [7,8]. It is based on reversed-phase (RP) C_{18} or C_8 columns, using a gradient of 25 m*M* triethylammonium phosphate buffer (pH 3.0) and acetonitrile as organic modifier. Retention is expressed as retention indices based on linear interpolation between consecutive homologues of nitroalkanes (nitromethane to 1-nitrooctane, RI values between 100 and 800). Again, mixtures of reference drugs are used to correct for variations in experimental conditions [9]. A database is under development. As in TLC, it will be no problem to find a second or third HPLC system with low correlations with the RP system described above. It remains to be seen, however, to what extent the former can comply with the other prerequisites of providing a good spread of the substances of interest over the entire run and providing good interlaboratory reproducibility.

4. Evaluation of combinations of systems

When one wishes to evaluate chromatographic systems and to express the utility of the retention parameter in an MLL value, the statistical calculation program is relatively simple [1,10], even when more than one system is being used or when different chromatographic techniques are applied (*e.g.*, a TLC system and an HPLC system). However, evaluating the identification power of detection modes, either as stand-alone technique or in combination with chromatographic systems, is much more complicated. Fortunately, recent developments in our laboratory have now provided calculation programs capable of handling such diverse parameters as

Table 1
Recommended TLC systems

	Solvent[a]	Adsorbent	Reference compounds[b]	hR_F[c]	Error window[d]
(1)	Chloroform–acetone (80:20)	Silica	Paracetamol	15	7
			Clonazepam	35	
			Secobarbital	55	
			Methylphenobarbital	70	
(2)	Ethyl acetate	Silica	Sulfathiazole	20	8
			Phenacetin	38	
			Salicylamide	55	
			Secobarbital	68	
(3)	Chloroform–methanol (90:10)	Silica	Hydrochlorothiazide	11	8
			Sulfafurazole	33	
			Phenacetin	52	
			Prazepam	72	
(4a)	Ethyl acetate–methanol–conc. ammonia[e] (85:10:5)	Silica	Sulfadimidine	13	11
			Hydrochlorothiazide	34	
			Temazepam	63	
			Prazepam	81	
(4b)	Ethyl acetate–methanol–conc. ammonia[e] (85:10:5)	Silica	Morphine	20	10
			Codeine	35	
			Hydroxyzine	53	
			Trimipramine	80	
(5)	Methanol	Silica	Codeine	20	8
			Trimipramine	36	
			Hydroxyzine	56	
			Diazepam	82	
(6)	Methanol–*n*–butanol (60:40); 0.1 mol/l NaBr	Silica	Codeine	22	9
			Diphenhydramine	48	
			Quinine	65	
			Diazepam	85	
(7)	Methanol–conc. ammonia[e] (100:1.5)	Silica impregnated with 0.1 mol/l KOH and dried	Atropine	18	9
			Codeine	33	
			Chlorprothixene	56	
			Diazepam	75	
(8)	Cyclohexane–toluene–diethylamine (75:15:10)	Silica impregnated with 0.1 mol/l KOH and dried	Codeine	6	8
			Desipramine	20	
			Prazepam	36	
			Trimipramine	62	
(9)	Chloroform–methanol (90:10)	Silica impregnated with 0.1 mol/l KOH and dried	Desipramine	11	11
			Physostigmine	36	
			Trimipramine	54	
			Lidocaine	71	
(10)	Acetone	Silica impregnated with 0.1 mol/l KOH and dried	Amitriptyline	15	9
			Procaine	30	
			Papaverine	47	
			Cinnarizine	65	

[a] Eluent composition in v/v. Saturated systems are used except for systems 5 and 6, which are used with unsaturated solvent tanks. System 4 is split: 4a for acidic and neutral substances and 4b for basic and neutral substances.
[b] Solutions of the four reference compounds at a concentration of approximately 2 mg/ml of each substance.
[c] Database R_F values times one hundred from ref. 3.
[d] The error window for each system is based on multiplying by three the interlaboratory standard deviation of measurement of hR_F values.
[e] Conc. ammonia contains 25% NH_3.

Table 2
Correlation coefficients for pairs of recommended TLC systems 1–10

	1	2	3	4	5	6	7	8	9	10
1	–									
2	0.820									
3	0.890	–								
4	0.530	0.748	–	–						
5		0.464	0.593	0.460	–					
6				0.436	0.614	–				
7				0.700	0.745	0.552	–			
8				0.593	−0.128	−0.045	0.228	–		
9				0.723	0.748	0.472	0.728	0.342	–	
10				0.710	0.750	0.655	0.771	0.206	0.820	–

colour reactions on the plate, molecular masses and full-scan diode-array spectra [11]. They were applied as follows.

4.1. Test set

Evaluations were made with a test set of 99 basic and neutral drugs, which were selected to represent various classes/structures of toxicologically relevant drugs. The substances are listed in Table 3, together with their respective chromatographic and detection parameters. UV spectra are not given for technical reasons.

4.2. TLC systems

Three systems, recommended for B/N drugs [3], were used: TLC 1, ethyl acetate–methanol–25% ammonia (85:10:5) on silica gel GF_{254}, standard deviation (S.D.) of measurement 3.8; TLC 2, methanol on silica gel GF_{254}, S.D. 2.8; and TLC 3, cyclohexane–toluene–diethylamine (75:15:10) on silica gel GF_{254}, S.D. 3.0.

Systems 1 and 3 were run in paper-lined tanks, presaturated with solvent vapour for 30 min; system 2 was run in unsaturated tanks. Plates for system 3 were impregnated with KOH prior to development by dipping in 0.1 *M* KOH in methanol and letting the methanol evaporate for at least 24 h. Plates were 20 × 10 cm and were developed over the shortest distance of the plate to 7 cm over the starting points. Developments were carried out at ambient temperatures (20–24°C) and relative humidities of 30–70%. No heat activation of the plates was applied. They were stored in cabinets at ambient temperature and relative humidity.

After development, the plates were dried with a cold hair blower until the smell of the solvents had disappeared. Then a total of four colour reactions were carried out on the same plate in sequence and after each step the colour was noted and encoded by means of a colour chart [12]. The colour reactions (CR) were taken from the Toxi-Lab Drug Compendium [13] and consisted of the following steps: CR 1, expose to formaldehyde vapour, then dip in concentrated sulphuric acid containing 0.1% ammonium vanadate (Marquis–Mandelin reaction) and observe the colour; CR 2, dip in water (exothermic reaction) and observe the colour; CR 3, observe fluorescence under UV light of 366 nm and observe the colour; and CR 4, dip in modified Dragendorff reagent and observe the colour.

Observed R_F values on the plate were corrected by means of reference mixtures run on the same plate, as described in ref. 3. Observed colours were encoded numerically as described in ref. 12, using a colour wheel with eight reference colours.

4.3. GC system

The GC system consisted of an HP-1 fused-silica dimethylsilicone column (12.5 m × 0.53 mm I.D., film thickness 0.88 μm). The tempera-

Table 3
Test set of basic and neutral drugs in the MLL evaluation of combined systems, with their respective chromatographic and detection parameters

Substance	TLC 1					TLC 2					TLC 3					GLC RI	M_r	HPLC RI
	hR_F	Colour codes				hR_F	Colour codes				hR_F	Colour codes						
Acebutolol	33	0	0	0	3	13	0	0	0	3	0	0	0	0	3	2811	336	311
Aminophenazone	62	5	0	0	3	70	0	0	0	3	21	5	5	0	3	1895	231	243
Amitriptyline	69	3	4	1	3	27	4	3	1	3	50	3	4	1	3	2194	277	440
Amitriptyline M/nortriptyline	46	3	3	7	3	87	3	3	3	3	28	3	3	2	3	2215	263	400
Amphetamine	43	1	1	7	3	12	1	1	7	3	26	1	1	7	3	1125	135	238
Atenolol	22	5	5	0	3	14	5	5	0	3	0	5	5	0	3	2385	266	224
Atropine	24	0	0	0	3	5	0	0	0	3	5	0	0	0	3	2190	289	287
Benperidol	60	5	5	0	3	62	5	5	0	3	3	5	5	0	3	3433	381	371
Bromazepam	63	1	1	3	3	73	1	1	3	3	6	1	1	3	3	2665	316	378
Caffeine	52	0	0	0	6	59	0	0	0	6	3	0	0	5	6	1800	194	265
Carbamazepine	56	2	1	7	3	79	2	2	7	3	2	2	2	7	3	2285	236	380
Chlordiazepoxide	52	0	0	2	3	76	0	0	2	3	2	0	0	2	3	2797	300	357
Chloroquine	46	0	0	0	4	4	0	0	0	3	14	0	0	0	3	2605	320	265
Chlorphenamine	46	0	0	0	3	12	0	0	0	3	35	0	0	0	3	1996	275	348
Chlorpromazine	70	3	3	0	3	25	3	3	0	3	45	3	3	0	3	2495	319	452
Clobazam	75	0	0	0	3	84	0	0	0	3	8	0	0	0	3	2558	301	484
Clomipramine	72	0	7	8	3	26	0	7	8	3	53	0	7	8	3	2415	315	470
Clonazepam	67	0	0	7	3	85	0	0	7	3	0	0	0	7	3	2823	316	451
Clopenthixol	44	4	4	2	3	45	4	4	2	3	7	4	4	2	3	3400	401	456
Clorazepic acid	68	0	0	7	3	83	0	0	1	0	3	0	0	1	0	2457	315	464
Cocaine	77	0	0	0	3	35	0	0	0	3	45	0	0	0	3	2187	303	345
Cocaine M/benzoylecgonine	2	0	0	0	3	22	0	0	0	3	0	0	0	0	3	2570	289	295
Codeine	35	7	7	0	3	21	7	7	0	3	6	7	7	0	3	2375	299	250
Demoxepam	41	0	0	1	3	81	0	0	2	3	0	0	0	1	3	2529	287	388
Diamorphine	49	5	5	0	3	26	5	5	0	3	15	5	5	0	3	2615	369	327
Diazepam	76	0	0	1	3	82	0	0	1	3	27	0	0	1	3	2428	285	520
Diazepam M/nordazepam	69	0	0	1	3	82	0	0	1	3	3	0	0	1	3	2490	271	464
Diphenhydramine	65	1	1	5	0	27	1	1	5	0	44	1	1	5	3	1870	255	390
Dipyridamole	44	4	4	0	0	82	4	4	0	0	0	4	4	0	0	1640	505	387
Disopyramide	60	0	0	0	3	9	0	0	0	3	7	0	0	0	3	2505	339	327
Doxepin	63	4	4	0	0	24	5	5	0	0	48	5	5	0	0	2220	279	730
Droperidol	58	5	5	0	3	71	5	5	0	3	2	5	5	0	3	3430	379	369
Ephedrine	25	1	0	0	3	10	1	0	0	3	5	0	0	0	3	1365	165	218
Ethosuximide	66	0	0	6	0	84	0	0	6	0	5	0	0	6	0	1205	141	284
Flecainide	49	1	1	5	3	28	1	1	5	3	6	1	1	5	3	2250	414	410
Flumazenil	61	0	0	0	3	76	0	0	0	3	3	0	0	0	5	2660	303	362
Flunarizine	88	4	4	7	3	83	4	4	5	3	45	4	4	7	3	3035	404	571
Flunitrazepam	74	0	0	7	3	80	0	0	7	3	10	0	0	7	3	2600	313	459
Flupenthixol	46	2	2	1	3	50	2	2	1	3	6	2	2	1	3	3058	435	487
Flurazepam	71	0	0	1	3	52	0	0	1	3	30	0	0	1	3	2780	388	392
Glibenclamide	11	0	0	5	3	90	0	0	5	3	0	0	0	5	3	9999	494	623
Gliclazide	9	0	0	0	3	84	0	0	0	3	0	0	0	0	3	1456	494	538
Glutethimide	80	0	0	0	3	86	0	0	0	3	31	0	0	0	3	1830	217	430
Haloperidol	76	0	9	0	3	51	0	9	0	3	11	0	9	0	3	2930	376	409
Hydroxyzine	54	0	0	0	3	57	0	0	0	3	10	0	0	0	3	2849	375	435
Imipramine	67	8	8	1	3	21	8	8	1	3	48	0	8	1	3	2230	280	437
Imipramine M/desipramine	40	8	7	8	3	7	8	7	8	3	19	8	7	8	3	2235	266	423
Ketamine	79	0	0	0	3	68	0	0	0	3	37	0	0	0	3	1840	238	294
Ketazolam	74	0	0	0	3	83	0	0	0	3	14	0	0	0	3	2444	369	583

Table 3 (*continued*)

Substance	TLC 1		TLC 2		TLC 3		GLC RI	M_r	HPLC RI
	hR_F	Colour codes	hR_F	Colour codes	hR_F	Colour codes			
Labetalol	29	3 3 5 3	32	3 3 5 3	0	3 3 5 3	1230	328	350
Levomepromazine	76	3 4 0 3	32	3 4 0 3	47	3 3 0 3	2525	162	440
Lidocaine	80	4 2 2 3	72	3 3 2 3	35	3 3 2 3	1870	234	278
Loprazolam	40	0 0 0 3	26	0 0 0 3	1	0 0 8 3	3258	465	379
Lorazepam	43	0 0 1 0	82	0 0 1 0	1	0 0 1 3	2410	321	422
Maprotyline	36	6 6 7 3	6	3 3 2 3	18	3 3 2 3	2356	277	440
Medazepam	78	0 5 7 3	79	0 5 7 3	41	5 5 7 3	2235	271	395
Metamizole	2	0 0 5 3	85	0 0 5 3	0	0 0 0 3	1990	351	289
Methadone	77	7 7 8 3	16	7 7 8 3	59	7 7 8 3	2145	310	441
Methamphetamine	42	1 1 0 3	9	1 1 0 3	28	1 1 0 3	1175	149	246
Methaqualone	78	0 0 0 3	79	0 0 0 3	36	0 0 0 3	2135	250	450
Metoclopramide	51	3 4 0 3	17	3 3 5 3	1	5 5 0 3	2620	300	308
Metoprolol	44	5 5 7 3	20	5 5 7 3	10	5 5 7 3	2035	267	317
Mianserin	68	7 5 7 3	48	5 5 7 3	39	5 5 7 3	2210	264	390
Midazolam	60	0 0 7 3	69	0 0 7 3	6	0 0 7 3	2575	326	386
Morphine	20	5 5 0 3	18	5 5 0 3	0	5 5 0 3	2445	285	200
Nifedipine	71	5 5 0 3	79	6 6 8 3	1	4 3 8 3	2170	346	503
Nitrazepam	64	0 0 7 3	84	0 0 7 3	0	0 0 7 3	2740	281	430
Opipramol	38	1 1 8 3	35	2 2 8 3	6	2 2 8 3	3050	363	387
Orphenadrine	68	2 2 7 3	25	1 1 7 0	48	1 1 7 3	1935	269	416
Oxazepam	45	0 0 1 3	82	0 0 1 3	0	0 0 1 3	2325	287	441
Paracetamol	45	5 5 2 3	77	0 0 2 3	0	0 0 2 3	1665	151	234
Pentazocine	70	5 5 2 3	34	5 5 5 3	16	5 5 2 3	2280	285	357
Periciazine	51	4 4 7 3	46	2 2 0 3	4	2 4 7 3	3260	366	405
Perphenazine	42	4 4 5 3	40	4 4 0 3	7	4 4 5 3	2207	404	438
Pethidine	60	0 0 0 3	34	0 0 0 3	37	0 0 0 3	1754	247	334
Phenazone	45	0 0 0 3	66	0 0 2 3	4	0 0 0 3	1850	188	303
Pindolol	43	7 7 0 3	18	7 7 0 3	2	6 6 0 3	2245	248	277
Pipamperone	43	0 0 0 3	33	0 0 0 3	1	0 0 0 3	3040	375	286
Prazepam	81	0 0 7 3	84	0 0 7 3	36	0 0 7 3	2648	325	648
Procainamide	39	0 0 0 3	17	0 0 0 3	1	0 0 0 3	2200	235	202
Prochlorperazine	55	4 4 0 3	26	2 4 0 3	34	4 4 0 3	2955	374	462
Promazine	62	4 4 0 3	18	1 1 5 3	38	1 1 0 3	2315	284	418
Promethazine	65	4 4 0 3	30	1 1 5 3	36	1 1 0 3	2267	284	411
Propoxyphene	82	5 5 8 3	50	5 5 8 3	58	5 5 8 3	2190	339	438
Propranolol	49	8 8 1 3	21	8 8 2 3	6	8 8 2 3	2147	259	370
Propyphenazone	74	0 5 0 3	81	5 5 0 3	32	5 5 0 3	1920	230	422
Quinidine	49	0 0 7 3	30	0 0 7 3	4	0 0 7 3	2790	324	316
Quinine	45	0 0 7 3	26	0 0 7 3	2	0 0 7 3	2800	324	398
Strychnine	32	5 2 0 3	8	5 2 0 3	8	5 4 0 3	3116	344	292
Sulpiride	34	0 0 0 3	17	0 0 0 3	0	0 0 0 3	3102	341	240
Temazepam	62	0 0 1 3	82	0 0 1 3	8	0 0 1 3	2595	301	466
Terfenadin	74	3 3 8 3	45	3 3 8 3	13	3 3 8 3	3436	472	567
Thioridazine	67	7 7 0 3	20	7 7 0 3	42	7 7 0 3	3115	371	504
Tocainide	44	1 0 0 3	42	1 1 0 3	2	1 1 0 0	1714	193	251
Trazodone	66	7 5 5 3	64	7 5 5 3	10	7 5 5 3	3330	372	358
Triazolam	44	0 0 0 3	68	0 0 0 3	1	0 0 0 3	3080	343	452
Trimethoprim	45	2 2 2 3	45	2 2 2 3	0	2 2 2 3	2558	290	345
Verapamil	73	5 5 0 3	43	5 5 0 3	23	5 5 0 3	3150	455	454
Zopiclone	47	0 0 0 3	42	0 0 0 3	4	4 0 0 3	3062	389	314

hR_F values from ref. 3. Colour codes (CC) (according to ref. 12): 1 = yellow, 2 = orange, 3 = brown, 4 = red, 5 = purple, 6 = black, 7 = blue, 8 = green, 0 = no spot observed. GLC RI values from ref. 5. HPLC RI values (according to ref. 5): determined jointly in the laboratory of the authors and at the Department of Forensic Medicine, Cracow, Poland (Dr. M. Klys). UV spectra: not shown, but measured in conjunction with the HPLC RI values.

ture programme was 120°C for 2 min, increased at 10°C/min to 215°C and then at 8°C/min to 300°C, with 5 min at the final temperature. Flame ionization or nitrogen–phosphorus detection was used. Retention times were converted into retention indices as described in ref. 5, using reference mixtures of drugs as calibrators. The S.D. was 25.

4.4. HPLC system

The HPLC system consisted of an RP-Select B C_8 column (12.5 cm × 4.6 mm I.D.), run in a gradient mode from 100% B to 70% A–30% B in 30 min, where solvent A was acetonitrile and solvent B was 0.025 *M* triethylammonium phosphate buffer (pH 3.0). A Hitachi–Merck L-3000 diode-array detector was used. Retention times were converted into retention indices based on nitroalkanes [9], using reference mixtures of drugs as calibrators [7]. The S.D. was 7. Spectral comparisons were made over the range 200–360 nm (65 diodes).

4.5. MLL calculations

MLL calculations were performed for single systems and detection modes as described in ref. 1. For the evaluation of combinations of systems and their corresponding detection modes we used the recently extended calculation models [11].

Table 4 gives the MLL values for single systems and detection modes. As expected, the three TLC systems have low identification powers (IP), owing to their limited separation ef-

Table 4
MLL values for single systems and single detection modes

System	MLL	Detection	MLL
TLC 1	19.60	CC 1	14.99
TLC 2	14.09	CC 2	16.36
TLC 3	22.12	CC 3	15.60
GC	7.20	M_r	1.40
HPLC	9.80	DAD	10.13

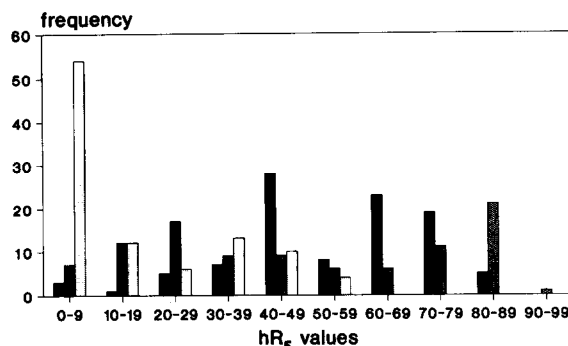

Fig. 1. Frequency distributions of hR_F values in TLC systems 1, 2 and 3. Black bars, TLC 1, ethyl acetate–methanol–ammonia (85:10:5); hatched bars, TLC 2, methanol; screened bars, TLC 3, cyclohexane–toluene–diethylamine (75:15:10).

ficiencies and low reproducibilities. However, the importance of a good spread of the substances over the chromatographic run can be clearly seen from Fig. 1: in TLC 2 the R_F values are evenly spread across the entire run, whereas the R_F distributions in TLC 1 and TLC 3 are skewed. TLC 2 also has the best reproducibility with an S.D. of 2.8, compared with 3.8 and 3.0 for the other two systems. This results in an MLL of 14.09 for TLC 2. Of the chromatographic techniques, GC offers the best IP with an MLL of 7.20, but HPLC is close behind at 9.08. This indicates an effective gradient and a good separation efficiency for the RP-HPLC system on a column only 12.5 cm long. With regard to the detection modes, it is obvious that the molecular mass (M_r) is highly discriminative. However, as the present test set of 99 substances contains a few entries with the same M_r, the MLL does not reach the ideal value of 1.00. On the other hand, it is interesting that the combination of four colour reactions results in an MLL of about 15 and that the MLL of the DAD spectra is of the order of 10. Hence these detection modes provide about the same IP as the corresponding separation techniques.

By using the recently extended mathematical models [11], we could also assess the IPs of combinations of systems and/or detection modes. Table 5 gives the results for combina-

Table 5
MLL values for combinations of single systems with their appropriate detection modes

System and detection	MLL	System and detection	MLL
TLC 1–CC 1	9.46	GC–M_r	1.10
TLC 2–CC 2	7.61	HPLC–DAD	3.52
TLC 3–CC 3	9.88		

tions of single systems and their corresponding detection modes. Interestingly, the combination of TLC R_F values with four colour codes now results in MLL values between 7 and 10, indicating that a simple, rapid TLC test plus a few colour reactions provides the same IP as a GC or HPLC retention index. The highest IP is obviously provided by GC–M_r, but it should be noted that even for this limited test set GC–M_r is unable to provide unequivocal identification for all 99 substances.

A substantial gain in IP is obtained when two separation systems are applied, together with their appropriate detection modes, as demonstrated in Table 6. When the information on R_F values in two TLC systems and the subsequent colour codes is utilized, MLL values of the order of 2 are obtained, whilst the inclusion of GC–M_r or HPLC–DAD, or both, results in MLLs close to 1. Still, even the combination of GC–M_r and HPLC–DAD does not result in an MLL of 1.00.

The MLL values for combinations of three separation systems and detection modes are given in Table 7. The use of all three TLC systems and the color codes now results in an MLL value of 1.35. Combinations including GC–M_r or HPLC–DAD, or both, are even better but there is only one combination that provides the ideal MLL value of 1.00, namely TLC 2–CC plus GC–MS plus HPLC–DAD.

Hence these MLL evaluations provide an objective assessment of the potential of the various systems and detection modes for the identification of unknown substances, leading to some valuable and interesting conclusions. Obviously, HPLC–DAD and GC–M_r are very powerful techniques, but even for this limited test set additional information from a suitable TLC–CC system is necessary to yield unambiguous identification. This contrasts with the widely held belief in analytical toxicology that identification can be achieved by applying two techniques based on different physico-chemical principles. Clearly, the latter is a serious oversimplification, even when MS is included. Although we utilized molecular masses derived from MS, we have good reasons to believe that the same is true if MS information is used in the form of electron impact (EI) mass spectra. More detailed calculations on EI-MS are in progress.

On the other hand, for those workers who do not have access to the more powerful coupled detection modes, such as DAD and MS, it is good to see that identifications can be approached very well on the basis of TLC–CC plus GC and HPLC retention indices alone. This is demonstrated in Table 7, in which the MLL values in parentheses were obtained by excluding M_r and DAD parameters: the best combination

Table 6
MLL values for combinations of two systems and their appropriate detection modes

System and detection	System and detection			
	TLC 2–CC	TLC 3–CC	GC–M_r	HPLC–DAD
TLC 1–CC	2.17	2.65	1.04	1.24
TLC 2–CC	–	2.20	1.04	1.19
TLC 3–CC	–	–	1.06	1.18
GC–M_r	–	–	–	1.02

Table 7
MLL values for combinations of three systems and their appropriate detection modes

System and detection	System and detection		
	TLC 3–CC 3	GC–M_r [a]	HPLC–DAD [a]
TLC 1–CC 1 plus			
TLC 2–CC 2	1.35	1.02 (1.06)	1.08 (1.11)
TLC 3–CC 3	–	1.04 (1.19)	1.12 (1.16)
GC–M_r [a]	–	–	1.02 (1.06)
TLC 2–CC 2 plus			
TLC 3–CC 3	–	1.02 (1.08)	1.08 (1.10)
GC–M_r [a]	–	–	1.00 (1.04)
TLC 3–CC 3 plus			
GC–M_r [a]	–	–	1.02 (1.08)

[a] Values in brackets have been obtained by omitting M_r and DAD parameters.

of TLC 2–CC plus GC and HPLC now gives an MLL value of 1.04, which is only marginally higher than 1.00 when DAD and M_r are included. The values for combinations of two TLC–CC systems with either GC or HPLC RIs are not much higher, between 1.06 and 1.19, respectively. Moreover, even those laboratories which can only afford TLC (*e.g.*, in developing countries) should not despair: the combination of the present three TLC systems and the colour codes already has a very good identification power with an MLL of 1.35.

5. Computerized identification

After having selected the most suitable systems and detection modes for STA on the basis of their MLL values, databases need to be established on an interlaboratory scale. As explained, these bases are to contain data on a great many substances of toxicological relevance. When unknown substances are encountered, their parameters are then to be compared with those in the database to find possible matches. It will be clear that this is to be done with the aid of a bench-top computer that is directly accessible in the laboratory. We are currently developing such a system that is capable of handling TLC–CC, GC–MS (allowing both EI and CI mass spectra) and HPLC–DAD. This system is called MTSS and will be commercially available through Merck (Darmstadt, Germany) from mid-1994. It will contain systems for acidic and neutral substances and also for basic and neutral substances. Users may add their own data to the built-in databases and may create databases on their own.

The system works as follows. After having run the unknown sample in any combination of analytical systems and developing mode one chooses, the parameters found are entered into the computer, together with the data for the reference mixtures used to calibrate the chromatographic systems. The latter allow the computer to correct the R_F values and/or to calculate the RI values. After pressing the "Search" key, the computer then compares the values for the unknown(s) with the values in the databases to find possible matches within the allowed error windows. It can do this for up to five unknown spots or peaks per sample and it automatically checks all possible configurations [14] for combinations of spots and peaks. It is also able to deal with situations where the number of spots and peaks do not match (*e.g.*, two TLC spots and three HPLC peaks). Finally, the computer prints out a list of substances that give acceptable matches for all the analytical parameters entered, in decreasing order of possi-

bility. Also, a similarity index is given for each listed substance, indicating the difference between the measured data and those in the database. Obviously, when data for only one analytical system are entered, the list of candidates will be very long, but the length of the list is drastically decreased when data for a multitude of systems are entered (as already seen under the MLL evaluations). Of course, the ultimate is reached when the final list contains only one substance with an acceptable similarity index. If the list continues to contain more than one candidate, it means that additional systems and detection modes must be applied. The best choice is strongly dependent on the substances involved.

Future versions of the MTSS may include additional HPLC systems and immunoassay systems. Work on the evaluation of the latter is in progress.

The use of a computerized search is indispensable in cases in which no prior information is available, such as the so-called general unknowns. However, it should be stressed that also in cases that appear to be routine and where abundant prior information is available, the MTSS may be very valuable: it may hint at a substance that is not seen very often but whose data are similar to those for a better known substance. Also, even when the identity of at least one toxicant is already known, the computer will check the likelihood of the presence of all other substances in the database, so that no substance is overlooked. This satisfies the ultimate aim of STA, to exclude all substances except the one(s) present.

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