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Chromatography in analytical toxicology — state of the art and future perspectives

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Abstract: The aims of systematic toxicological analysis are considered with respect to the principal techniques available for quantitative and qualitative purposes. The use of thin-layer (TLC), gas–liquid (GLC), capillary GLC and high-performance liquid chromatography (HPLC) is discussed, with special reference to the need for standardized systems for toxicological analysis. The selectivity of correlations presented as mean list length values, derived from archives of Kováts retention indices in GLC and of R_f-values in up to eight TLC systems, is illustrated by some typical examples. The dependence of Kováts retention indices on column packing materials and on load capacity in CGLC is discussed and the particular difficulties of comparable standard systems in HPLC are considered. Conclusions are drawn concerning the established 'workhorse' techniques, such as TLC and GLC. The need to adapt and standardize other powerful analytical methods, such as HPLC, MS and GLC–MS, is examined with regard to the requirements of toxicological analysis.

Keywords: *Systematic toxicological analysis; drug identification; gas–liquid chromatography; thin-layer chromatography; high-performance liquid chromatography; mass spectrometry; Kováts retention index.*

Introduction

In order to assess adequately the applicability of chromatographic techniques in analytical toxicology, it is helpful to consider the exact goals to be achieved and also to appreciate the conditions under which these techniques will be applied and by whom.

The two primary goals in toxicological analysis are: first, to detect and identify potentially toxic compounds in a given complex matrix; second, to quantitate them. This applies to all analyses in clinical and forensic toxicology and to a lesser extent to environmental and occupational toxicology, where the identity of the toxicant or toxicants is sometimes known. However, the character of these two aims is quite different, namely qualitative *versus* quantitative analysis, and this should be borne in mind when considering the potential uses and pitfalls of chromatographic techniques.

Furthermore, it should be realized that in the daily practice of toxicological analysis a great number and variety of compounds are encountered, that fast and efficient round-

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the-clock service must be provided, and that a variety of instruments and techniques must be kept on stand-by service. This explains the need for relatively simple, straightforward and flexible equipment and procedures in this area, as well as the fact that toxicological analysts can hardly afford to specialize too much in a given technique or methodology. Thus simplicity, flexibility and speed are the major factors determining the practical usefulness of chromatographic techniques in this domain.

Quantitative Analysis

The aims of quantitative analysis in toxicology are quite similar to those in other areas of bioanalysis. It is therefore not surprising to see that gas–liquid chromatography (GLC) and high-performance liquid chromatography (HPLC) are very often used in this area. The vast array of detection techniques available with each technique may yield increased selectivity and/or sensitivity, provided that they can be applied in a simple and flexible way. A few words on the applicability of thin layer chromatography (TLC) may seem to be appropriate. Although quantitative TLC cannot compete with GLC and HPLC in terms of precision, it usually provides a rapid, semi-quantitative answer which may be life-saving. TLC allows a number of reference solutions of known concentration to be run side-by-side with the unknown sample on the same plate. The human eye can then well serve as a first detection mode, especially where rapid action is required. Contemporary TLC scanners, although rather costly, can provide more precise results with relative standard deviations of 10% or less.

Qualitative Analysis

Systematic toxicological analysis (STA) describes the search for a potentially harmful substance, whose presence may be unsuspected and identity unknown. Toxic substances can be divided into five major groups [1]: (1) gaseous and volatile substances; (2) inorganic cations (metals); (3) inorganic anions (e.g. fluoride, cyanide, nitrite); (4) nonvolatile organic substances, which can be further sub-divided into acidic, basic, neutral, amphoteric and quaternary ammonium compounds; (5) miscellaneous.

The relatively nonvolatile organic substances will be emphasized in this section, since they are most frequently encountered in STA and require extensive use of chromatographic techniques. However, it should be realized that even within this class of compounds, the number of substances to be considered is very large, and involves a wide variety of chemical structures. Furthermore, various complicating factors arise: (a) metabolites of exogenous compounds may be present; (b) endogenous compounds from the biological matrix are almost always encountered; (c) intoxications with more than one substance (multi-drug intoxications) are currently very common. Although some drugs are obviously more frequently encountered than others, it is indicative that in 40 000 intoxications dealt with by the Poison Center in Munich, a total of over 8000 different substances were involved [2].

Screening in STA by Means of Chromatographic Techniques

TLC and GLC

TLC and GLC on conventional, packed columns have been by far the most frequently used screening techniques in STA. Detection methods include fluorescence quenching and/or spray reagents in TLC, and flame ionization, nitrogen–phosphorus or electron

capture detection, singly or in combination, in GLC. It is customary to use a combination of two or more systems, such as: (a) using a single TLC or GLC system, but with different detection modes; (b) using more than one TLC or GLC system with appropriate detection modes; (c) using TLC *and* GLC systems with appropriate detection modes. In order to compensate for variations in experimental conditions, R_f-values in TLC are usually corrected by means of concurrent reference susbtances, where retention in GLC is usually expressed as relative retention or, preferably, as a Kováts retention index [3]. Identification is then attempted by matching the retention value of the unknown compound(s) with that of known substances, available as a set of reference values in a data collection. Clearly, when more than one chromatographic system has been used, the matches should be in agreement for all systems.

Although this approach may seem relatively straightforward, two basic questions arise:

(i) How to select the most suitable chromatographic systems for STA, either alone or in combination with one or more different chromatographic systems;

(ii) How to unequivocally identify an unknown compound in the presence of thousands of others; or on the other hand, how to establish the absence of a certain component.

In recent years, various concepts for evaluating systems have been developed, such as the Discriminating Power (DP) of Moffat *et al.* [4–7], the Information Content (IC) of Massart *et al.* [8, 9] and the Separation Quotient (SQ) of Müller *et al.* [10, 11]. These three approaches are system-directed in that they provide information about the efficacy of individual systems and combinations thereof. Though quite helpful in evaluating systems, they have some limitations: They are *a priori* not well suited for substance identification; the reproducibility (intra- and inter-laboratory) is not taken into account or all systems are assigned the same, fixed reproducibility; they do not provide sufficient flexibility to work with combinations of three and more systems.

In order to overcome these limitations, the concept of Identification Power was developed primarily for the identification of unknown substances, but also for system evaluations. In using this concept [12] it became apparent that chromatographic systems, and TLC systems in particular, could vary to such an extent that for adequate evaluation and identification the individual reproducibility had to be taken into account.

The criterion for the comparison and ranking of systems was taken as the number of substances that each could identify from a given number of substances in a databank. Starting with a population of 100 basic drugs to check the feasibility of the concept, some drawbacks became readily apparent. For example, with a single TLC system the number of substances that can be identified out of a set of 100 will be very small, if not zero, even if the substances are well distributed over the entire R_f-range. In real STA situations where a population of several thousand has to be considered the IP-values will rapidly become zero.

Therefore a more sophisticated approach to the IP-concept has been developed, where the number of other substances from the total population is determined — i.e. not including the given substance — that would qualify for identification on the basis of their retention behaviour, taking into account the reproducibility of the system [13, 14]. The number of such substances that qualify is called the 'list length'. Taking this parameter for all substances in the population, we obtain the so-called 'mean list length' (MLL) for a given system or combination of systems. The shorter the MLL, the more effective the system for STA, as indicated in Table 1. Assuming a constant standard

Table 1
Mean list length values (MLL) for some TLC systems and a GLC system, calculated for a population of 100 basic drugs for two probabilities of correctness

		TLC_1	TLC_2	TLC_3	TLC_4	TLC_5	TLC_6	TLC_7	TLC_8	GLC
	SD	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	20
MLL(a)										
95%		15.31	18.63	18.54	15.93	16.22	19.95	16.85	14.76	6.70
99%		38.18	39.74	42.09	36.40	40.24	38.73	34.33	36.92	8.60
	SD	1.00	1.68	1.18	1.30	2.87	1.61	1.88	1.38	10
MLL(b)										
95%		6.39	12.91	8.67	8.64	18.55	13.06	12.50	8.43	3.95
99%		23.27	35.60	29.56	29.03	42.58	30.47	30.00	26.86	4.75

MLL(a): Fixed SD for all TLC systems of 2.5 R_f-units, and for GLC 20 RI-units.
MLL(b): Experimentally determined intra-laboratory S.D. for each system as indicated.
TLC_1: methanol in unsaturated chambers.
TLC_2: acetone in saturated chambers on KOH-impregnated plates.
TLC_3: butanol–methanol (40:60 v/v) in unsaturated chambers.
TLC_4: same as TLC_3, but also containing 0.1 M NaBr.
TLC_5: chloroform–methanol (90:10 v/v) in saturated chambers on KOH-impregnated plates.
TLC_6: chloroform–methanol (90:10 v/v) saturated with NaBr, on NaBr-impregnated plates in saturated chambers.
TLC_7: cyclohexane–toluene–diethylamine (75:75:10 v/v/v) in saturated chambers on KOH-impregnated plates.
TLC_8: ethyl acetate–cyclohexane–methanol (70:15:15 v/v/v), saturated with NaBr on NaBr-impregnated plates in saturated chambers.
GLC: Packed columns with SE 30 or OV-1, as described in [15].

deviation, TLC system 8 turns out to be the best choice, followed by system 1. The experimentally determined reproducibilities vary markedly, and when taken into account they clearly exert a large impact on the Identification Power, such that TLC system 1 now appears as the best with TLC system 8 ranking second. Furthermore, the GLC system seems to be much better suited for STA than any single TLC system.

Another factor of interest is the probability of correctness desired for identification. Since the reproducibility of TLC is limited, the MLL values increase markedly if 99% probability is desired. As a consequence, the identification process for a single spot, e.g. in TLC system 5, may list more than 50 possible candidates out of the set of 100. By contrast, the GLC system yields much shorter lists, usually no longer than 10 possible candidates.

MLL-values for combinations of systems are given in Table 2, calculated with the so-called 'loss function' which gives a probability of about 95% [14]. Assuming a fixed standard deviation, the best TLC-combination is formed by systems 7 and 8, whereas the best choice for a TLC–GLC combination is system 8. However, when using the experimentally-determined reproducibilities, TLC system 1 with the lowest standard deviation gives the best results in combination with TLC system 7 or with GLC. Though TLC system 1 and TLC system 3 have low MLL-values as single systems, their combination has a relatively high MLL-value because they are highly correlated. This is quite explicable in view of their composition. On the other hand TLC system 1 is poorly correlated with TLC system 7, and also with the ion-pair systems 4, 6 and 8, so that low MLL-values are observed for these combinations. For the combination of three TLC

Table 2
MLL-values for combinations of two systems calculated for a population of 100 basic drugs

	TLC_1	TLC_2	TLC_3	TLC_4	TLC_5	TLC_6	TLC_7	TLC_8	GLC
TLC_1	—	6.61	11.68	5.40	6.00	5.71	4.61	4.35	2.43
TLC_2	2.60	—	7.36	6.05	6.67	5.89	6.23	5.59	2.75
TLC_3	4.62	3.45	—	5.66	6.77	6.27	5.02	4.89	2.58
TLC_4	2.05	2.89	2.46	—	4.81	5.90	5.07	6.18	2.31
TLC_5	3.27	5.89	4.33	3.12	—	5.81	4.90	4.25	2.35
TLC_6	2.06	3.54	2.39	2.88	4.70	—	5.32	6.80	3.19
TLC_7	1.98	3.96	2.39	2.55	4.29	3.01	—	4.21	2.44
TLC_8	2.01	2.84	2.34	2.80	3.08	3.45	2.46	—	2.15
GLC	1.16	1.75	1.29	1.30	1.75	1.74	1.51	1.27	—

The upper right-hand corner of the table contains MLL-values for TLC systems with a fixed S.D. of 2.5 R_f units and a GLC system with a S.D. of 20 RI-units. The lower left-hand corner contains MLL-values calculated with experimentally determined S.D. values as given in Table 1. The underlined data indicate the best combination. For description of systems see also Table 1.

methods, systems 1, 4 and 7, or 1, 7 and 8, yield the lowest MLL, the value for both being 1.15.

Apparently, for combinations of systems the reproducibility also plays an important role, in addition to separation selectivity and the degree of correlation between systems. Moreover, these results illustrate how far performance is away from the ideal situation of a MLL value of 1.00, which would be the case if all compounds out of the present set of 100 could be identified unequivocally.

The fact that system evaluations have so far been mainly carried out for TLC and GLC can be explained by the fact that these techniques have been extensively studied and that no major new developments are to be expected in terms of systems. For GLC, SE-30 or OV-1 appear to be the best column systems for screening in STA. However, even though GLC lends itself quite well to both screening and identification, it has also been recommended that either SE-30 or OV-1 be the *only* GLC system for STA [6, 15]. This is because other systems such as OV-17, Carbowax or DEGS are all highly correlated with the SE-30 or OV-1 system, so that the extra gain in information given by a second GLC system becomes extremely low [6].

TLC on the other hand provides much better opportunities for finding two or more systems with little intercorrelation, as shown above and in the literature [5]. Thus the application of a variety of TLC systems usually yields a considerable gain in information, despite the intrinsically low reproducibility of the technique as such.

Once optimum chromatographic systems have been selected on the basis of an adequate evaluation of their usefulness for STA, one can start building up a databank by running reference substances in these systems. Obviously, such a bank should contain reference data on parent drugs, metabolites, endogenous compounds and other frequently encountered substances, such as certain plasticizers, antioxidants, polychlorinated biphenyl compounds and so on. The more, the better. A substance not present in the databank as a reference cannot of course be identified; indeed, a misidentification may result instead. Furthermore, such substances by their absence from the databank may lead to unreasonable estimates of the mean list length.

Furthermore, as the number of toxicologically relevant substances increases, it becomes impossible for individual laboratories and institutes to set up and maintain their own databank of references. Instead, there should be an easily accessible and reliable

international databank for toxicological analysis. However, it seems extremely difficult to set up such an institution or to find an international body willing to undertake this endeavour.

Nevertheless, some positive results have been obtained, thanks to the enthusiasm of some individual workers. For GLC a data collection comprising some 1300 compounds exists [16], while TLC data are available on some 600 basic drugs in four systems and on some 200 acidic and neutral drugs in another four systems [17]. These databases will be expanded in the future.

Finally, some remarks on multiple drug intoxications, which are occurring more and more frequently. Their proper identification involves a special and often quite serious difficulty when using more than one TLC and/or GLC system, since it is not usually known which spot or peak in the one system corresponds with data in the other system, and vice versa. In such cases all possible combinations or configurations must be examined to identify the possibilities. The IP-concept based on the MLL approach has in fact been designed to carry out such a search automatically, as illustrated in the following example.

STA was applied to an unknown sample from a case. Application of TLC system 1 (Table 1) revealed two spots with R_f-values 26 and 64, respectively. These values were transformed to the corrected R_f^c-values 25.32 and 65.74 respectively [12]. Gas chromatography on OV-1 yielded two peaks with retention indices (RI) 1795 and 2656, respectively. These data were compared by computer with the reference values in the databank, yielding lists of potential candidates for each peak and spot observed. These are depicted in Fig. 1 for the two peaks found in GLC, and in Fig. 2 for the two spots found in TLC, together with their probabilities at the 95% confidence level. Clearly, a visual comparison of the data to find correct matches between Figs 1 and 2 is quite difficult, not least because two interpretations need to be considered: (I) compound 1 has R_f^c 25.32 and RI 1795 and compound 2 has R_f^c 65.74 and RI 2656; (II) compound 1 has R_f^c 25.32 and RI 2656 and compound 2 has R_f^c 65.74 and RI 1795.

Automatic search of the two configurations provides the answers very rapidly, as seen in Figs 3 and 4 for configurations I and II respectively. It is interesting to note that for each configuration, answers with a high confidence level are found. Although the likelihood of the combination diamorphine (i.e. heroin) + caffeine would seem to be high, the value of the approach is demonstrated by the fact that a second possibility is listed, pethidine (i.e. meperidine) + clemizole. Additional confirmation or elimination tests are required to arrive at a reliable identification. In this case this was achieved by a positive Marquis color reaction, coupled with data from the gas chromatogram, which also showed evidence of traces of acetylcodeine and monoacetylmorphine, natural congeners of heroin. Mass spectrometry further confirmed the presence of heroin and caffeine in the sample.

There is another interesting feature of the IP concept. As may be noted from Fig. 2, diamorphine is not listed as a possible candidate to match RI 2656, since its reference value in the databank is 2615. Indeed the discrepancy of 41 RI-units is rather large, but may have been attributable to the fact that the GLC data was obtained from a capillary column, as discussed below. Anyway, the possibility that the peak with RI 2656 is due to heroin could be rejected at the 95% confidence level. However, diamorphine is not automatically rejected as a possible candidate, indeed the combination of RI 2656 with R_f^c 25.32 appears to be highly characteristic, so that in the end the correct identification is still achieved, even at a confidence level of 99.56%.

```
GASCHROMATOGRAPHYVALUE :  1795
CANDIDATES ON IDENTIFICATION WITH PROBABILITY :
PHYSOSTIGMINE                          PROBABILITY =   4.90 %
MEPROBAMATE                            PROBABILITY =   4.89 %
ALPHAPRODINE                           PROBABILITY =   4.85 %
QUINALBARBITONE                        PROBABILITY =   4.80 %
METHOIN                                PROBABILITY =   4.80 %
CHLORPROPAMIDE                         PROBABILITY =   4.80 %
ETHOTOIN                               PROBABILITY =   4.75 %
BETAPRODINE                            PROBABILITY =   4.75 %
SPARTEINE                              PROBABILITY =   4.68 %
PHENANTHRENE                           PROBABILITY =   4.43 %
PHENIRAMINE                            PROBABILITY =   4.43 %
COTARNINE                              PROBABILITY =   3.97 %
LOBELINE                               PROBABILITY =   3.70 %
CAFFEINE                               PROBABILITY =   3.70 %
PIPOBROMAN                             PROBABILITY =   3.56 %
NICOUMALONE                            PROBABILITY =   3.56 %
NARCOBARBITAL                          PROBABILITY =   3.12 %
MECLOFENOXATE                          PROBABILITY =   2.24 %
BETAMEPRODINE                          PROBABILITY =   1.84 %
METHOHEXITONE                          PROBABILITY =   1.71 %
PRILOCAINE                             PROBABILITY =   1.59 %
PACHYCARPINE                           PROBABILITY =   1.59 %
FENMETRAMIDE                           PROBABILITY =   1.59 %
MEOSTIGMINE                            PROBABILITY =   1.47 %
SUCCINYLCHOLINE                        PROBABILITY =   1.36 %
TRIMEPERIDINE                          PROBABILITY =   1.06 %
CARISOPRODOL                           PROBABILITY =   1.06 %
THYMOXAMINE                            PROBABILITY =   0.89 %
DIAZINON                               PROBABILITY =   0.89 %
TETRAHYDROZOLINE                       PROBABILITY =   0.81 %
ETHIONAMIDE                            PROBABILITY =   0.73 %
MYRISTIC ACID                          PROBABILITY =   0.60 %
GLUTETHIMIDE                           PROBABILITY =   0.60 %
BUTETHAMATE                            PROBABILITY =   0.60 %
ANTHRACENE                             PROBABILITY =   0.60 %
PETHIDINE                              PROBABILITY =   0.44 %

GASCHROMATOGRAPHYVALUE :  2656
CANDIDATES ON IDENTIFICATION WITH PROBABILITY :
TEMAZEPAM                              PROBABILITY =   8.63 %
CLOBAZAM                               PROBABILITY =   8.63 %
FENTANYL                               PROBABILITY =   8.42 %
BROMAZEPAM                             PROBABILITY =   8.28 %
FLUNITRAZEPAM                          PROBABILITY =   7.97 %
METHYLTESTOSTERONE                     PROBABILITY =   7.13 %
PRAZEPAM                               PROBABILITY =   6.65 %
MALOXONE                               PROBABILITY =   6.39 %
TRIMETHOPRIM                           PROBABILITY =   5.87 %
CLEMIZOLE                              PROBABILITY =   5.61 %
METOCLOPRAMIDE                         PROBABILITY =   3.78 %
TRIFLUOPERAZINE                        PROBABILITY =   3.54 %
PHENAZOCINE                            PROBABILITY =   3.30 %
INDOMETHACIN                           PROBABILITY =   3.08 %
GALLAMINE                              PROBABILITY =   2.65 %
TESTOSTERONE                           PROBABILITY =   1.74 %
ACEPROMAZINE                           PROBABILITY =   1.45 %
PHENACAINE                             PROBABILITY =   1.32 %
BENZETHIDINE                           PROBABILITY =   1.32 %
```

Figure 1
Computer printout of the possible candidates matching the retention index values of 1795 and 2656, respectively, as found for the two unknown peaks in a gas chromatogram on OV-1. Probabilities relate to the 95% confidence level.

Capillary gas–liquid chromatography (CGLC)

CGLC would seem to be a particularly valuable technique for STA, because of its much higher separation efficiency, especially since new generations of glass and fused silica capillary columns have become available which are claimed to have good stability, flexibility and load capacity. However, recent investigations have shown that the special character of CGLC may create some problems. First, there is the stationary phase. Although the newer capillary columns are made from relatively inert, high purity materials, deactivation of active sites (silanol groups) appears to be a prerequisite, so that the major manufacturers of capillary columns have their own deactivation and coating procedures. As a result one can buy different brands of capillary columns with a

```
RF-VALUE :  26
RFC-VALUE :   2.5319148936170E+001
CANDIDATES ON IDENTIFICATION WITH PROBABILITY :
QUININE                                    PROBABILITY =  11.04 %
PHENMETRAZINE                              PROBABILITY =  11.04 %
DOXEPIN                                    PROBABILITY =  11.04 %
DIPHENHYDRAMINE                            PROBABILITY =  11.04 %
PROMETHAZINE                               PROBABILITY =   9.83 %
DIAMORPHINE                                PROBABILITY =   9.83 %
CLOMIPRAMINE                               PROBABILITY =   9.83 %
ETHOPROPAZINE                              PROBABILITY =   6.74 %
BROMODIPHENHYDRAMINE                       PROBABILITY =   6.74 %
PETHIDINE                                  PROBABILITY =   4.92 %
ORPHENADRINE                               PROBABILITY =   2.16 %
AMITRIPTYLINE                              PROBABILITY =   2.16 %


RF-VALUE :  64
RFC-VALUE :   6.5744680851064E+001
CANDIDATES ON IDENTIFICATION WITH PROBABILITY :
LIGNOCAINE                                 PROBABILITY =  35.12 %
YOHIMBINE                                  PROBABILITY =  19.32 %
CAFFEINE                                   PROBABILITY =  19.32 %
CLEMIZOLE                                  PROBABILITY =   9.16 %
PRAMOXINE                                  PROBABILITY =   4.01 %
NIKETHAMIDE                                PROBABILITY =   1.53 %
NICOTINYL ALCOHOL                          PROBABILITY =   1.53 %
IPRONIAZID                                 PROBABILITY =   1.53 %
NIALAMIDE (D)                              PROBABILITY =   0.23 %
DEXTROMORAMIDE                             PROBABILITY =   0.23 %
PAPAVERINE                                 PROBABILITY =   0.10 %
LYSERGIDE                                  PROBABILITY =   0.09 %
MEPIVACAINE                                PROBABILITY =   0.09 %
GUANETHIDINE                               PROBABILITY =   0.09 %
NAPHAZOLINE                                PROBABILITY =   0.09 %
PROTRIPTYLINE                              PROBABILITY =   0.09 %
AMETAZOLE                                  PROBABILITY =   0.09 %
CYCLOPENTAMINE                             PROBABILITY =   0.09 %
ATROPINE                                   PROBABILITY =   0.09 %
STRYCHNINE                                 PROBABILITY =   0.09 %
DESIPRAMINE                                PROBABILITY =   0.09 %
ANTAZOLINE                                 PROBABILITY =   0.09 %
NORTRIPTYLINE                              PROBABILITY =   0.09 %
METHYLAMPHETAMINE                          PROBABILITY =   0.09 %
EPHEDRINE                                  PROBABILITY =   0.09 %
CHLORPHENIRAMINE                           PROBABILITY =   0.09 %
HYDROMORPHONE                              PROBABILITY =   0.09 %
CARBINOXAMINE                              PROBABILITY =   0.09 %
AMPHETAMINE                                PROBABILITY =   0.09 %
PROTHIPENDYL                               PROBABILITY =   0.09 %
PHENYLPROPANOLAMINE                        PROBABILITY =   0.09 %
METHAPYRILENE                              PROBABILITY =   0.09 %
ETHOHEPTAZINE                              PROBABILITY =   0.09 %
PROMAZINE                                  PROBABILITY =   0.09 %
PHENIRAMINE                                PROBABILITY =   0.09 %
METHADONE                                  PROBABILITY =   0.09 %
MEPYRAMINE                                 PROBABILITY =   0.09 %
TRIPELENNAMINE                             PROBABILITY =   0.09 %
PROCYCLIDINE                               PROBABILITY =   0.09 %
MORPHINE                                   PROBABILITY =   0.09 %
THIORIDAZINE                               PROBABILITY =   0.09 %
IMIPRAMINE                                 PROBABILITY =   0.09 %
DIPHENYLPYRALINE                           PROBABILITY =   0.09 %
CARBETAPENTANE                             PROBABILITY =   0.09 %
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Figure 2
Computer printout of the possible candidates matching the R_f^c-values of 25.32 and 65.74, respectively, as found for the two unknown spots in thin-layer chromatogram on silica gel with methanol as solvent. Probabilities are expressed at the 95% confidence level.

```
COMPOUND :  1

SYSTEM :         1
RF-VALUES :     26
RFC-VALUES :    25.32
GASCHROMATOGRAPHYVALUE :  1795
CANDIDATES ON IDENTIFICATION WITH PROBABILITY :
PETHIDINE                                  PROBABILITY =  96.05 %

COMPOUND :  2

SYSTEM :         1
RF-VALUES :     64
RFC-VALUES :    65.74
GASCHROMATOGRAPHYVALUE :  2656
CANDIDATES ON IDENTIFICATION WITH PROBABILITY :
CLEMIZOLE                                  PROBABILITY =  99.83 %
```

Figure 3
Computer printout of the possible candidates matching both the retention index values and the R_f^c-values as found in Figs 1 and 2 for one configuration, at the 95% probability level.

```
COMPOUND :  1

SYSTEM :        1
RF-VALUES :    26
RFC-VALUES :   25.32
GASCHROMATOGRAPHYVALUE :  2656
CANDIDATES ON IDENTIFICATION WITH PROBABILITY :
DIAMORPHINE                                   PROBABILITY = 99.56 %

COMPOUND :  2

SYSTEM :        1
RF-VALUES :    64
RFC-VALUES :   65.74
GASCHROMATOGRAPHYVALUE :  1795
CANDIDATES ON IDENTIFICATION WITH PROBABILITY :
CAFFEINE                                      PROBABILITY = 99.18 %

TOTAL NUMBER OF CONFIGURATIONS :        2
```

Figure 4
As in Fig. 3, but for the other configuration.

methylsilicone phase comparable to SE-30 or OV-1, but not exactly the same. Thus, there may be differences between brands of SE-30/OV-1 capillary columns; moreover, there may be differences between a packed SE-30/OV-1 column and its capillary counterpart. This is illustrated in Tables 3–6 [18]. Table 3 shows data on barbiturates on packed columns and on three different types of capillary columns. It can, however, be concluded that there is fairly good agreement between packed and capillary data, in particular those on fused silica columns.

Table 3
Comparison of retention indices of barbiturates on CP-SIL 5 capillary columns and SE-30 or OV-1 packed columns

Compound	CP-Sil 5 columns*			Packed columns [15]
	Glass WB†	FS NB‡	Fs WB§	
Allobarbital	1577	1590	1588	1605
Amobarbital	1696	1712	1716	1720
Aprobarbital	1592	1618	1614	1620
Hexobarbital	1841	1865	1868	1855
Pentobarbital	1721	1738	1730	1745
Phenobarbital	1938	1943	1965	1960
Secobarbital	1768	1784	1783	1790

* Chrompack, Middelburg, The Netherlands [18].
† Glass wide-bore.
‡ Fused silica narrow-bore.
§ Fused silica wide-bore.

Table 4 depicts acceptable agreement between narrow-bore fused silica columns and packed columns, the deviations being within ± 50 RI-units [15]. However, the values on wide-bore fused silica columns are higher by some 60 RI-units. A similar tendency is seen for antidepressants in Table 5, but Table 6 shows quite different patterns. Column instability, especially above 250°C, has also been observed, resulting in irreproducible retention times over a longer period of time [19].

A second problem in CGLC is associated with the load capacity of the system. In STA the concentration of the drugs to be encountered is usually unknown — indeed, it may well vary by three to four orders of magnitude. In a recent study [20] the author's group found that retention indices of drugs in CGLC were not constant over such a concentration range. Retention indices tended to increase with concentration, some-

Table 4
Comparison of retention indices of some pesticides on CP-SIL 5 capillary columns and SE-30 or OV-1 packed columns

Compound	CP-Sil 5 columns*			Packed columns [15]
	Glass WB	FS NB	FS WB	
Aldrin	n.d.†	1950	2008	1945
Dieldrin	n.d.	2156	2214	2110
Endrin	n.d.	2194	2258	2180

* Chrompack, Middelburg, The Netherlands [18].
† Not determined.

Table 5
Comparison of retention indices of antidepressants on CP-SIL 5 capillary columns and SE-30 or OV-1 packed columns

Compound	CP-Sil 5 columns*			Packed columns [15]
	Glass WB	FS NB	FS WB	
Amitriptyline	2195	2197	2234	2205
Clomipramine	2419	2420	2460	2415
Desipramine	2241	2244	2286	2250

* Chrompack, Middelburg, The Netherlands [18].

Table 6
Comparison of retention indices of miscellaneous drugs on CP-SIL 5 capillary columns and SE-30 or OV-1 packed columns

Compound	CP-Sil 5 columns*			Packed columns [15]
	Glass WB	FS NB	FS WB	
Antazoline	2295	2299		2350
Caffeine	1780	1796		1810
Codeine	2376	2384		2385
Gallamine		2603		2700
Hexachlorophene			2860	2795
Hydroxyzine	2867	2890	2934	2850
Isoniazide		1447	1447	1630
Isopropamide		1998	2037	2060
Methadone	2150	2150	2182	2150
Methaqualone	2142	2150	2181	2115
Naphazoline	1993	1996	2044	2065
Nicotinyl alcohol	1092	1100		1150
Phenazone			1875	1830
Phencyclidine			1932	1904
Strychnine	3115	3163	>3200	3115
Theophylline	1947	1932		2105
Yohimbine	3168	3210		3290

* Chrompack, Middelburg, The Netherlands [18].

times by as much as 50–100 RI-units. Moreover, peak-splitting was observed at higher concentrations. This was found not only for drugs (Fig. 5), but also for reference alkanes (Fig. 6).

It will be clear that the above phenomena make CGLC rather unsuitable for STA, at least for the time being. Unless a standardized phase system becomes available it would be useless to start building a databank for CGLC. Furthermore, technological developments are required to solve the problem of the concentration dependence of RI-values.

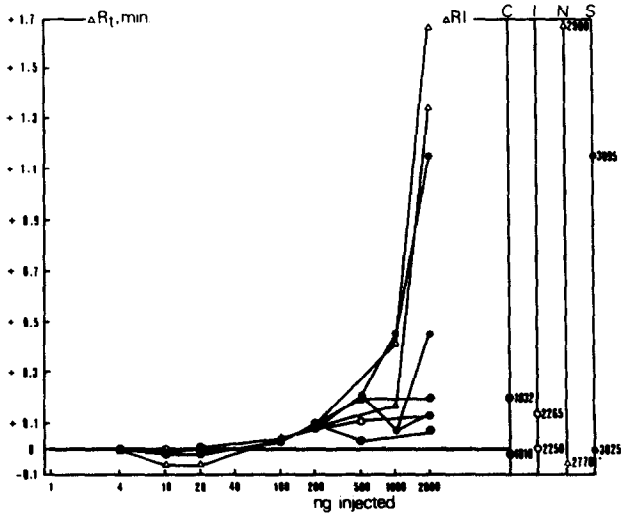

Figure 5
Concentration-dependent behaviour of drugs on a capillary fused silica column coated with CP Sil 5. Sample code: ● = caffeine; ○ = imipramine; △ = nitrazepam; * = strychnine. Solutions in ethyl acetate; injection volume 2 μl; splitless injection; further details in [20]. Left: retention time differences in minutes with lowest concentration taken as arbitrary zero point. Right: retention index differences between highest and lowest values observed.

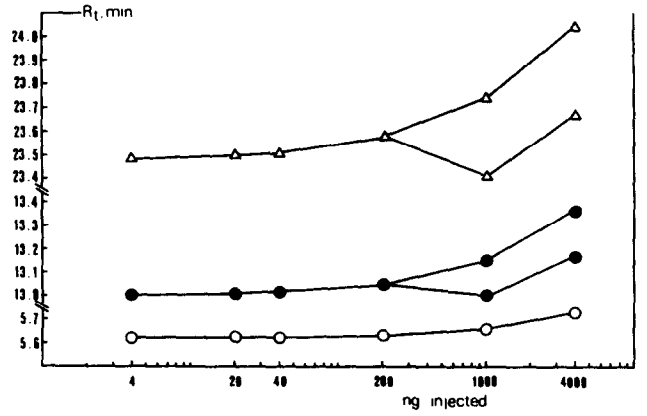

Figure 6
Concentration-dependent behaviour of alkanes on a capillary fused silica column coated with CP Sil 5. Sample code: ○ = C_{12}; ● = C_{22}; △ = C_{32}. Further details in [20].

High performance thin layer chromatography (HPTLC)

This technique can be considered as a sophisticated form of TLC in which the sorbent layer and the development procedure are thoroughly standardized and optimized. This can result in increased separation power, shorter development times and enhanced reproducibility [21]. Although these parameters are of paramount importance for STA, the technique has not been generally accepted in this area. This may be due to the fact that HPTLC requires special instrumentation for sample application and development, as well as some skill from the operator. On the other hand, the load capacity of HPTLC is usually less than in classical TLC, so that 'dirty' extracts, which are not uncommon in toxicological analysis, can overload the plates or block the sample applicator. It would therefore seem that, similar to CGLC, the fundamental advantages of HPTLC are overshadowed by some practical aspects characteristic for toxicological analysis as such. It should be noted, however, that shorter development times in classical TLC can be obtained simply by using a shorter development distance (7–8 cm) than the classical but arbitrary 10 cm. This may lead to a gain in development time of 30–50%, without essentially affecting the R_f-values, resolution or reproducibility. Moreover, with shorter development distances, better sensitivity can be obtained [22, 23].

High-performance liquid chromatography (HPLC)

At first sight, HPLC would seem to have much to offer to STA, in view of its high separation power, good reproducibility and general applicability, including thermolabile and relatively non-volatile compounds. Furthermore, in HPLC as in TLC separations can be influenced and optimized by manipulating both the mobile and the stationary phases. The reversed-phase mode (RP-HPLC) also allows aqueous samples to be analysed directly. This explains the enormous increase in the application of HPLC, especially for quantitative purposes. However, although some HPLC systems have been recommended for screening purposes [24, 25], the use of HPLC for STA is still quite limited. Various factors are responsible for this situation, the following being the most important:

(a) Up till now, it has been virtually impossible to obtain adequately standardized stationary phase materials. Similar types of packing materials from different manufacturers usually differ markedly in performance and batch-to-batch differences within the same brand are also quite common [26].

(b) An adequate, universal detection system for HPLC does not exist. Though UV-detection appears to be the best choice, there is uncertainty as to which wavelength(s) should be used.

(c) A generally acceptable method to express retention in a standardized form does not yet exist. Relative retention times for reference drugs have serious drawbacks, especially in view of (a), since the use of a new batch of packing material may already cause considerable changes in relative retention times or even changes in elution order [26]. Obviously, alkanes cannot be used to calculate RI-values because they do not exhibit UV-absorption.

As a result, research on the evaluation of HPLC systems to find those most suitable for STA has been delayed. Nevertheless, various activities are underway in all the three areas outlined above, in order to find solutions to these problems. The biggest bottleneck is the availability of packing materials of constant quality and reproducibility. Once they become available, progress can also be made in the other areas, especially in (c).

As for the detection problem, the new generation of multichannel photodiode array

detectors may represent a significant improvement. These detectors can monitor the complete absorption spectrum over a range of 200–800 nm. Strictly speaking, the spectrum is not scanned; the photodiodes acquire the spectrum within a few milliseconds and this can be repeated at rapid intervals. Since this provides a tremendous amount of data if the complete chromatogram is monitored, the data are first captured and transferred to disc or tape, and then recalled and inspected after the run. These detectors have already demonstrated their value in screening for metabolites [27] and are presently being evaluated for STA. Although they have several drawbacks, including their price, these detectors do have a unique feature in that they provide both a chromatographic parameter and a UV-spectrum in one and the same run.

Gas chromatography–mass spectrometry (GC–MS)

Although the coupling of a mass spectrometer (MS) to a gas chromatograph does not yield a new chromatographic technique as such, special mention of GC–MS seems warranted here. Undoubtedly, MS offers unique possibilities for substance identification and indeed, many analysts tend to believe that MS is the ultimate infallible test. It should be stressed that this is not the case. Several studies indicate that up to 50% of the identifications by GC–MS may be incorrect, generating false positive as well as false negative results [28, 29]. These results were obtained with drugs commonly encountered in STA.

The main reason for this deplorable situation appears to be the fact that the MS databanks contain spectra of pure substances, taken under experimental conditions which are usually not specified. However, in the daily practice of GC–MS in STA, the eluting peaks are quite often contaminated by either endogenous or exogenous compounds, so that a mixed mass spectrum is obtained. Furthermore, it is well known that mass spectra are dependent on the experimental conditions as well as on the instrument used. Other factors involved are that even the larger MS databanks do not contain spectra on many toxicologically relevant substances and/or they are out of date in recording spectra of the newer drugs. False positive findings are easily explained if it is realized that the computer search system always tries to come up with a match and that the matching factors given are extremely difficult to interpret.

Thus, it appears that this is yet another area where a potentially very powerful technique is available, but where it remains to be seen how it can be best employed under the conditions inherently characteristic of toxicological screening. Although HPLC–MS is still in its infancy, it is to be expected that once it becomes established, similar problems to those in GC–MS may well be encountered too.

Conclusions

Currently TLC and GLC are the work-horses for screening in toxicological analysis. This will remain the case for the years to come because of the expertise and experience that has been accumulated with these techniques. It is suggested, though, that a set of recommended systems be adopted for general use in STA and that a large databank be set up for these recommended systems. Such a databank should be generally accessible, contain data on as many toxicologically relevant drugs as possible and should be kept up to date. A prerequisite for such a bank is the continued availability of TLC and GLC materials of constant quality.

CGLC should be investigated further, especially with regard to designing suitable

stationary phases of constant quality that are closely comparable to the SE-30/OV-1 phases in GLC, since this will allow one databank to be used both for CGLC and GLC. If this turns out to be impossible, it will mean that a separate databank will have to be established for CGLC. In addition, methods to ensure that retention times become concentration-independent over a large range will be required.

The role of HPTLC in STA seems to be rather limited, as the advantages of the technique are relatively small in view of the disadvantages, bearing in mind the possibilities of classical TLC. HPLC could become an important technique in the near future, if well defined, constant quality column materials became available, and which are not immediately superseded by newer developments. Then the systems most suited to STA need to be established, including the detection mode, followed by establishing a databank, as described above.

So far, the subject of sensitivity has not been addressed. Suffice it to say that in STA one is always looking for increased sensitivity in order not to overlook toxicants present at low levels. With the advent of newer drugs with extremely high potency, this trend will definitely continue, and will increase the problems in STA in two respects: first, the lower the concentration of a drug, the greater the risk of losses in the analytical procedure; second, the trace levels need to be found and identified against a large background of endogenous material, or other contaminants picked up in the detection process. An example is given in Fig. 7, in which a post mortem blood sample was analysed for the presence of unknown poisons [30].

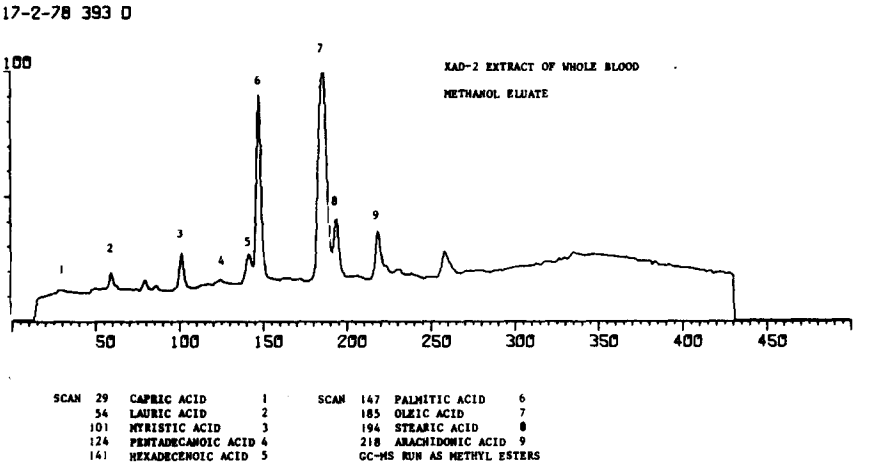

Figure 7
GC–MS analysis in screening for the presence of unknown substances in post-mortem blood.

Thus, it can be concluded that much remains to be done in STA. Potentially powerful chromatographic techniques are on hand, but a concerted research effort is needed to establish how they can be adapted to meet the requirements dictated by the special character of qualitative toxicological analysis.

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