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Review

Systematic toxicological analysis of drugs and poisons in biosamples by hyphenated chromatographic and spectroscopic techniques

Aldo Polettini*

Department of Legal Medicine and Public Health, University of Pavia, Via Forlanini 12, 27100 Pavia, Italy

Abstract

The introduction of hyphenated chromatographic-spectroscopic techniques represented a substantial step-forward for Systematic Toxicological Analysis (STA), increasing the amount and quality of information obtainable from the analysis of a biological sample, and enhancing the possibilities of identifying unknown drugs and poisons. STA methods based either on GC–MS or on HPLC–UV published in the last decade are reviewed in this paper. The different analytical phases, i.e. sample preparation (pretreatment, extraction, derivatisation), chromatographic separation and detection/identification are examined in detail in order to emphasise the complementarity of the two approaches. In addition, the first STA method based on HPLC–MS is illustrated and some applications of TLC–UV to drug screening are also described. Finally, an overview of semi- and fully-automated STA methods is given.

Keywords: Systematic toxicological analysis; Drug screening; Reviews; Detection, spectroscopic

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*Tel.: +39-0382-507-829; fax: +39-0382-528-025.
E-mail address: apole@unipv.it (A. Polettini)

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1. Introduction

If one thinks back to the times when the first approaches based on rudimental chromatographic techniques were applied in acute intoxication cases, it is striking how and how much Systematic Toxicological Analysis (STA), i.e. the screening for drugs and poisons, has advanced.

The diffusion of drug use, abuse and misuse has brought to an increasing awareness that drugs are not only harmful to life, but may also affect different aspects of human behaviour (e.g. the performance in work, in driving, in sport). As a consequence, new fields of application of STA have come up (e.g. workplace drugs testing, drugs and driving, antidoping control) [1–4]. That therapeutic more than toxic concentrations have to be searched for in such cases has put pressure on technological progress towards the increase of sensitivity of analytical techniques.

The widespread use of pesticides in agriculture and of drugs with therapeutic or growth promoting action in zootechnics has raised concern on the effects of the indirect exposure to residues of such substances present in food, and has raised the demand for screenings of xenobiotics in foodstuffs before marketing. The continuous input into the environment of new chemical substances exerting an actual or potential biological effect on the organism has directed the evolution of screening techniques towards higher separation and discrimination powers.

New applications of STA have originated as a consequence of the progresses of medicine, as is the case of transplantation surgery where the presence of drugs in the organ's donor has to be excluded [3].

Even the approach to the most traditional application of STA, i.e. the diagnosis of acute/lethal intoxication, has undergone significant changes. As properly suggested by de Zeeuw [5], STA is increasingly applied on a routine basis to all intoxication cases, regardless of the fact that the substance involved is known or strongly suspected. In fact, the possibility that other toxic compounds may have contributed to the observed biological effect cannot be excluded. For example, it is well known that 'pure' heroin intoxications are relatively rare, the neurodepressant action of heroin being frequently modified as a consequence of the concomitant intake of other substances (e.g. cocaine, alcohol, benzodiazepines, barbiturates, tricyclic antidepressants etc.) [6].

No doubt that such an evolution of STA has been made possible by a corresponding progress in separation and detection techniques. In particular, the introduction of rapid scanning multichannel spectroscopic detectors (UV spectrophotometers and mass spectrometers), able to record the whole spectrum of the column eluate, represented a key factor in the evolution of STA methods. The possibility to combine chromatographic and spectroscopic techniques added a third dimension to the world of chromatography and allowed to multiply the amount of analytical information obtainable within a single chromatographic run [7,8].

The aim of this paper is to review methods developed for the screening of drugs, poisons, and their metabolites in different biological samples (serum, whole blood, urine, tissues, hair) based on hyphenated chromatographic and spectroscopic techniques in the last ten-year period. Most of the STA methods available in the literature are based either on GC–MS [9–20] or on HPLC–UV [20–36], and on these combined techniques the paper is mainly focused. In addition, some applications based on TLC–UV [37–39] and the first application of LC–MS [40] to STA are described.

2. GC–MS

The development of an effective GC–MS interface posed minor technical problems as the removal of the gaseous mobile phase from the ion source of the mass spectrometer is relatively easy. Moreover, after

the introduction of capillary columns requiring much lower flow rates than packed columns, direct GC–MS interfacing was made possible. This opened-up the way to the introduction in the 1970s of low-priced bench-top GC–MS systems and, consequently, to the large diffusion of this technique in analytical toxicology labs.

It is universally recognised that GC–MS now represents the 'golden standard' for drug analysis [8], although the recent development of effective LC–MS interfaces will probably modify this situation in the future [41,42].

Together with these practical advantages, however, GC–MS has important features that make this technique well suited for the bioanalysis of drugs and particularly for STA [43]: (a) the higher separation power of capillary GC compared to HPLC; (b) the higher selectivity of detection of MS compared to UV spectroscopy; (c) the inherent simplicity of the information contained in a mass spectrum, allowing its easy handling and storage on electronic medium; (d) the availability of a well established and standardised ionisation technique (electron impact, EI) that allowed the construction of large databases of reference mass spectra; (e) the consequent possibility to develop a fast and reliable computer-aided identification of unknown compounds based on the method of fingerprint superimposition, the so-called library search.

On the other hand, GC has well known weak points as a separation technique compared to HPLC. First, it is unsuitable for the direct analysis of polar compounds, although this limitation can be partly overcome with the adoption of an adequate derivatisation method. Second, there is no compatibility between the chromatographic system and the aqueous biological matrix. As a consequence, sample preparation is necessarily more laborious and time-consuming than for HPLC analysis.

2.1. Sample preparation

2.1.1. Sample pretreatment

Owing to the characteristics of drug metabolites (e.g. conjugates with glucuronic acid) or to the nature of the matrix itself (e.g. tissues, hair) the sample cannot be always submitted directly to the extraction procedure and requires a pretreatment.

Urine samples are often submitted to acidic [14] or enzymatic [9] hydrolysis in order to obtain the cleavage of conjugates that are not amenable to GC analysis owing to their high polarity and molecular weight.

Whole blood needs to be diluted or deproteinised when submitted to solid-phase extraction (SPE) in order to avoid cartridge clogging [44]. Among the different methods tested by Chen et al. [45], sonication followed by dilution in 0.1 *M* phosphate buffer (pH 6) was found to provide the best performance in terms of analytes' recovery.

Acetylation carried out in the aqueous medium was proposed by Brooks and Smith for urine [10] and later adapted to plasma [11]. Such pretreatment, besides producing the acetyl derivatives of primary and secondary amino drugs that undergo a more selective fragmentation under EI than the corresponding underivatised drugs, prevented the loss of volatile basic drugs (e.g. amphetamine) during evaporation of the extraction solvent. The sample was added with $NaHCO_3$ followed by acetic anhydride and then allowed to stand for 10–15 min before extraction. The authors stressed that it is important not to mix again after the addition of acetic anhydride, so that $NaHCO_3$ settles at the bottom of the tube and acetic anhydride floats on the surface, thus avoiding the violent reaction between sodium bicarbonate and the acetic acid formed, and obtaining a controlled CO_2 development.

Biological tissues (e.g. liver) require homogenisation followed by protein precipitation, acid hydrolysis or enzymatic digestion, the latter method yielding higher recoveries and cleaner extracts [46].

Incubation of powdered hair in 0.1 *M* hydrochloric acid or in water (56°C/12 h) was proposed by Gaillard and Pepin [20] to bring in solution basic and acidic/neutral drugs, respectively, before solid-phase extraction (SPE).

2.1.2. Extraction

The great influence of biological matrices on recovery and detectability of analytes has been emphasised by Bogusz et al.[47], who evaluated the performance of different extraction methods based on liquid–liquid partition (LLE) as well as on SPE.

LLE with dichloromethane–acetone (2:1, v/v) was adopted by Brooks and Smith [10] to extract

acetylated and non acetylated basic, neutral and weakly acidic drugs from urine (see Section 2.1.1). After acetylation, the sample was diluted with water and a saturating amount of NaCl was added before extraction. Besides producing the 'salting-out' effect, NaCl saturation contributed to a very sharp interface between phases and to make the aqueous phase denser, thus facilitating the aspiration of the extract. Recoveries in the range 50–100% were obtained from urine spiked with 13 different analytes. Extraction of carboxylate-containing drugs was achieved by replacing the acetylation step with urine acidification at pH 2. The same authors developed a more laborious method to extract drugs from plasma [11].

Toxi-Lab (Irvine, CA) extraction tubes A and B, for alkaline and acidic extraction respectively, were used by Chia and Gere [17].

Following acidic hydrolysis and pH adjustment to 8–9, the extraction of basic and neutral drugs from urine with a dichloromethane–isopropanol–ethyl acetate (1:1:3, v/v/v) mixture was obtained by Maurer et al. [13]. The same authors used extractive methylation for the isolation of acidic drugs from urine [13,15]: after mixing with the phase-transfer reagent consisting of 0.02 *M* tetrahexylammonium (THA) hydrogensulfate in 1 *M* sodium phosphate buffer adjusted to pH 12 with sodium hydroxide, the sample was shaken with a solution of 1 mol/l methyl iodide in toluene for 30 min at 50°C. THA salts were then removed from the organic phase using a diol phase cartridge.

The use of SPE, although in the past it was preferred for the selective extraction of specific drugs or drug groups, is rapidly gaining popularity in drug screening procedures. SPE methods adopted in STA have been excellently reviewed by Franke and de Zeeuw [44] and therefore this subject will not be treated here. However, the method developed by Chen et al. [45] based on mixed-mode SPE cartridges needs to be mentioned as it proved to be excellent in terms of versatility and cleanness of extracts. The sample, adjusted to pH 6, is applied to a pre-conditioned phase containing both hydrophobic and cation exchange functional groups. Weakly acidic and neutral compounds are retained by hydrophobic groups, whereas protonated basic compounds are retained by cation exchange groups. After washing with deionised water and phase acidification with diluted acetic acid, differential elution of analytes is achieved with acetone–chloroform (1:1, v/v) mixture (acidic–neutral fraction) followed by ammoniated ethylacetate, or ammoniated dichloromethane–isopropanol (8:2, v/v) (basic fraction). For a thorough discussion on advantages and limits in the application of mixed-mode SPE to STA see Ref. [44]. Based on this extraction procedure a number of manual or automated STA methods have been later developed [9,19,48–50].

2.1.3. Derivatisation

As previously mentioned, the inability to provide efficient repartition of polar compounds represents a major drawback in the application of GC to STA. However, this limit can be partly overcome with the adoption of a suitable method of derivatisation of polar functional groups.

Acetylation with acetic anhydride–pyridine (3:2, v/v, 60°C/0.5 h) has been proposed by Maurer et al. [13] for the derivatisation of basic and neutral compounds, and the same authors have recently developed a procedure for acidic drugs based on extractive methylation (see details in Section 2.1.2) [13,15].

A method based on trifluoroacetylation with MBTFA for basic drugs and methylation of acidic drugs with ethereal diazomethane was adopted by Neill et al.[16].

Trimethylsilylation (TMS) is the most common derivatisation technique for GC–MS analysis [51]. The ability of silylating agents to react with a high number of functional groups (hydroxyl, carboxyl, amidic and amino groups) makes this derivatisation method very versatile and, therefore, suitable for application in STA [9]. On the other hand, the use of such derivatisation method for STA purposes is presently limited by the low number of reference mass spectra of TMS derivatives available in commercial libraries. However, the new edition of the Pfleger, Maurer, Weber library will contain a practically complete coverage of TMS derivatives [52].

Silyl derivatives are very moisture-sensitive and strictly anhydrous conditions have to be maintained in order to allow the reaction to occur or to avoid hydrolysis of derivatives. Highly efficient and reproducible derivatisation was obtained with a mix-

ture of MSTFA–toluene (1:4, v/v) containing 5% TMCS (75°C/0.5 h or 1 min exposure to microwaves, 2450 mHz, 750 W), prepared just before use and filtered over anhydrous sodium sulphate [53]. Together with versatility, further advantages of trimethylsilylation are: (a) evaporation of the excess reactant is not required prior to injection into the GC; (b) it provides a considerable gain in molecular mass of derivatisable compounds, thus increasing, in many cases, the specificity of mass spectral information; (c) it is effective in improving the repartition of highly concentrated endogenous interference compounds (such as fatty acids and cholesterol) in biological extracts, thus enhancing the overall chromatographic resolution and, consequently, the detectability of analytes [9,53,54].

TMS derivatives of drugs were obtained by Cardenas et al. [12] by eluting analytes previously retained on an Amberlite XAD-2 sorbent with a BSTFA–chloroform–acetone (5:4:1) mixture (see Section 6).

2.2. Chromatographic separation

Conditions for GC separation in STA methods are well established. Apolar phases, such as 5% phenyl, dimethylpolysiloxane [9,10,12,16–20] or dimethylpolysiloxane [15,55] are usually preferred. Moderate polarity stationary phases (e.g. 14% cyanopropyl, dimethylpolisiloxane) have been also proposed [17,56].

The choice of column dimensions (12–30 m length×0.2–0.25 mm I.D., 0.25–0.33 μm film thickness) and of the carrier gas (usually He) flow rate (0.9–2.0 ml/min) are obviously limited by the efficiency of the MS pumping system. Recently designed instruments allow to maintain a constant carrier gas flow rate at the increase of temperature during the run [9,20].

Sample injection is always carried out in splitless mode (1–2 μl; purge time 0.5–1 min) at temperatures in the range 200–280°C. Oven temperature programs often consist of a single ramp starting from 40–100°C (1–3 min initial isotherm) up to 280–310°C at a rate of 5–30°C/min [12,14–16,19,20,55]. A first ramp from 70–100°C (0.7–2.25 min) to 140–180°C at 20–40°C/min followed by a slower ramp to 290°C at 10°C/min have also been adopted [9,56]. A four-ramp temperature program was developed by Brooks and Smith [10]: 50°C (0.5 min) to 140°C at 30°C/min, 15°C/min to 210°C, 5°C/min to 300, and 2°C/min to 320°C.

The GC–MS direct interface is usually held at 260–300°C.

Great effort was devoted in the past to the construction of large databases of Kovats retention indices in order to improve the reliability of retention-based identification [57]. However, after the diffusion of GC–MS, retention data have obviously become of secondary importance with respect to spectral information. Nevertheless, retention data are very useful in order to prefilter reference mass spectra to be compared with the unknown, thus speeding up the library search routine. Retention data are also important in the case of compounds with poorly specific mass spectra, such as amino compounds with base peaks at m/z 44, 58, 72, or 86 and very low fragments in the high-mass range (e.g. some tricyclic antidepressants) and many barbiturates (with prominent ions at m/z 141 and 156).

It should be noted, however, that retention behaviour in capillary GC columns is concentration-dependent. Bogusz et al. [58] found that variations of over 100 retention-index units occurred in the more pronounced cases when the injected amount of compounds exceeded 400 ng. This may be a serious problem for STA applications where typically the injected amount of analytes in unknown. Modifications of retention behaviour due to matrix interference should be also taken into account.

2.3. Detection and identification

MS detection is obviously carried out using electron impact (70 eV) ionisation mode in order to obtain mass spectra comparable to those contained in commercially available reference libraries and, to this respect, it is important to note that all MS detectors used in STA methods are based on the quadrupole technology [9,10,12,14–20,55].

MS acquisition is usually started after a suitable time (2.5–4 min) in order to allow the removal of the injection solvent from the ion source and to avoid rapid oxidation of the filament.

The scan range is generally between 35–50 and 440–650 u, with scan speeds of 1–1.5 scan/s. Last

generation MS detectors allow considerably higher scan speeds (more than 2.5 scan/s) with a consistent increase in chromatographic resolution [9].

Different aspects have to be considered when selecting the scan range. On one hand, noisy background ions at low mass (e.g. nitrogen at m/z 28, and CO_2 at m/z 44) should be avoided, on the other the heaviest analytes that may be eluted from the column must be included. Often, m/z 50 is chosen as first mass owing to the practical reason that mass spectra of commercial libraries usually start from m/z 50. However, this may lead to low sensitivity for those amino compounds that exhibit a base mass peak at m/z 44 $[C_2H_6N]^+$ and very low abundances at higher masses (e.g. amphetamine, nortriptyline). A practical solution to this problem is to acquire standard reference spectra of such compounds starting from m/z 40–42 and adding the obtained spectra to the database used for comparison.

If derivatisation is performed prior to GC analysis, the mass gain of the derivatives (e.g. 42, 72, and 96 u in the case of acetyl, TMS, and TFA derivatives, respectively) has to be considered when selecting the scan range.

It is also important to note that, when quadrupole mass analysers are used, the scan width affects chromatographic resolution: a wider scan range results in lower scan speed and, consequently in lower resolution. As compounds are generally eluted from a capillary GC column within 4–6 s, a scan speed of about 1 scan/s should be sufficient to collect at least two mass spectra close to the peak apex.

Owing to the low selectivity of the MS detector operated in continuous scan mode, total ion chromatograms (TIC) obtained for the systematic toxicological analysis of biological samples are usually characterised by high chemical noise that may preclude the possibility to select mass spectra sufficiently clean for a positive identification by library search. However, based on the fact that the TIC can be decomposed into as many mass chromatograms as the number of masses in the scan range or into as many mass spectra as the number of scans, different methods have been proposed in order to enhance the selectivity of continuous scan MS detection, from simple background subtraction to sophisticated methods of spectral reconstruction or factor analysis [8,59–66]. Two mass spectral purification methods will be briefly described here for their effectiveness and relatively easy applicability within automated search routines. One is based on selected ion retrieval followed by search of the mass spectra of the peaks detected in the resulting ion chromatograms. Maurer proposed a ‘general unknown’ computer-monitoring program for extracting the signal of 48 selected fragment ions from the TIC [8]. A substantial increase in selectivity is obtained with this approach when the fragment selected is not present in the mass spectra of coeluting interferences. However, the major limit of this strategy is that the overall performance of the method depends on the fragments selected: if none of the fragments of the mass spectrum of the unknown analyte are included within the list of fragments selected, there is no chance to detect/identify it.

A simple method that is not based on a priori criteria for detection/identification is that proposed by Polettini [60], which consists of subtracting from each other different mass spectra selected along the elution profile of a chromatographic peak. Two transformed chromatograms, specular and displaced of one scan, are obtained by subtracting the total intensity of each scan of the TIC from that of the preceding and, respectively, of the following scan. Each chromatographic peak in the TIC corresponds to two peaks in each transformed chromatogram, one in the positive and one in the negative direction of the intensity scale. Detected positive and negative peaks in both transformed chromatograms are then background subtracted (apex-start, and apex-end) and searched against the reference library.

Methods for the evaluation of peak purity are available in the data analysis software of bench-top GC–MS systems, such as the Peak Purity algorithm of the Hewlett-Packard MS Chemstation. This is a stand-alone application that may be applied either on single chromatographic peaks or on the whole chromatogram, and that can be integrated in routine library search reports [67].

The notable elaboration speed reached by PCs makes it possible to search all the scans of a TIC (a 20 min TIC acquired at a 1 scan/s speed contains 1200 mass spectra), with or without subtraction of

adjacent scans, against the reference library within a few minutes. This search strategy, combined with good filters (e.g. based on match quality and/or retention time) able to reduce the number of false positive identifications [68], proved to be highly effective in the detection of unknowns even in extremely 'dirty' chromatograms [67].

Among the different available search algorithms, the Probability Based Matching developed by McLafferty [69] has rapidly become the standard for STA and other analytical applications. Being a reverse search algorithm it is able, to some extent, to identify mass spectra in mixtures. However the search parameters have to be carefully optimised in order to get the best search performance [9].

The database of reference mass spectra [52,70–74] against which unknown spectra are searched is obviously of utmost importance for the overall performance of an STA method. Owing to the practical impossibility to identify even only the prominent peaks of the chromatogram by manual interpretation of mass spectra, automated search routines have to be used. As a consequence, only those compounds whose reference spectrum is included in the searched library will have a chance of being identified.

Different aspects should be evaluated in the choice of the reference library (RL). Obviously, the higher the number of reference spectra, the higher the probability that an unknown spectrum will be identified. However, the size of the RL is not necessarily correlated with the number of *toxicologically relevant* mass spectra it contains. Furthermore, the larger the RL the slower the speed of the data processing step during which the library search routine may need to be repeated hundreds of times. Another important aspect is that the RL should contain not only spectra of parent drugs but also of metabolites and, if required, derivatives. Some RLs contain different spectra for the same compound collected with different instruments or by different laboratories in order to prevent possible errors. It is also good practice to construct a home-made RL with spectra produced with the same instrumentation used to obtain unknown spectra, although search algorithms are usually able to adjust for small instrument-dependent differences between unknown and reference spectrum. Some MS databases are also available on the internet [75–78].

3. HPLC–UV

High-performance liquid chromatography (HPLC) was introduced in the 1970s. However, this technique found initially limited application in STA owing to the low selectivity of UV single-wavelength detectors. Photodiode-array detectors (DAD) were developed at the beginning of the 1980s and their use in combination with HPLC for analytical toxicological purposes was proposed in 1984 [79]. Many HPLC–UV methods for the screening of drugs in biosamples have been published in the literature since then [20–36].

HPLC has interesting advantages over GC for the analysis of non-volatile organic compounds, and the reversed-phase (RP) mode is at present the most common separation method for such compounds. It is a highly versatile technique, allowing the analysis of compounds scattered over a wide range of polarity, molecular weight, and thermal stability. The compatibility of the mobile phase (at least in the RP mode) with the aqueous biological matrices makes sample preparation simple and fast and, consequently, easily automated [25].

On the other hand, resolution of HPLC is generally low compared to capillary GC, and owing to this reason the identification power of HPLC–DAD has been found to be slightly lower than the combination of GC and UV spectroscopy [31]. Also, UV spectroscopy is less selective that MS because of the weak relationship between chemical structure and UV spectral characteristics.

By converse, the relatively low selectivity of UV can be usefully exploited in STA for the detection of metabolites. In many cases, in fact, metabolic transformations do not affect the ultraviolet chromophores of the molecule. As a result, UV spectra of metabolites closely resemble that of their parent compound [22,23]. This is different from what usually happens in MS where biotransformations result in a shift of the m/z ratio of the molecular ion and of the ionic fragments of the metabolite. An even more important advantage of the low selectivity of UV spectroscopy

is that, for the same reasons mentioned above, compounds belonging to the same class often display similar absorbance patterns. Therefore, unknown compounds not previously characterised can be tentatively assigned to a certain class on the basis of UV spectral characteristics in order to assist with further analysis [32].

3.1. Sample preparation

As mentioned previously, HPLC analysis in the RP mode allows a simple and fast sample preparation. In principle, in fact, there is no need (i) to hydrolyse the sample as intact conjugates can be directly analysed [23], (ii) to derivatise polar groups of the analytes, and (iii) to isolate analytes from the aqueous matrix.

A very simple method for sample preparation is that proposed by Drummer et al.[33] for forensic toxicological purposes. Whole blood was deproteinised by shaking with an equal volume of acetonitrile. The sample was than allowed to stand for 10 min, centrifuged at approximately 10 000 *g* for 10 min, and a 20 μl aliquot was then injected into the HPLC.

Liquid–liquid extraction is by far the most common method for STA purposes. Bogusz and Wu [28] extracted autopsy blood adjusted at pH 4.6 with ethyl acetate.

Solvent extraction with a mixture of chloroform–2-propanol–*n*-heptane (60:14:26, v/v/v) from blood or plasma adjusted to pH 9.5 was proposed by Tracqui et al.[29], and the Toxi-Lab (Irvine, CA) extraction system for basic and neutral drugs (Toxi-Tube A) was adopted by Gaillard and Pepin [21]. The poor recoveries of acidic compounds like salicilates, barbiturates, and some NSAIDs obtained with such methods was not considered as a severe drawback as these drugs have large therapeutic doses resulting in high levels in biological samples. In such cases, low recovery of extraction should on the contrary be considered as an advantage as saturation of the detector with possible negative impact on identification is avoided [21].

Extraction with dichloromethane from strongly basified plasma (NaOH, 1 *M*) was adopted by Turcant et al. [22].

Lambert et al. [30] extracted basic compounds from blood, urine, stomach content and tissue homogenates with a mixture of *n*-hexane–ethyl acetate (7:3, v/v) after adjusting pH to 9.5.

Different preparation methods for serum, blood and liver homogenates including acidic hydrolysis, and various solvent extractions were investigated by Bogusz and Erkens [80] in order to evaluate their effect on chromatographic behaviour and on spectral characteristics of analytes. These authors observed that, whereas RI values were virtually unaffected, the detection of some analytes was impaired by matrix peaks occurring regularly in biological extracts.

Solid-phase extraction (SPE) for drug screening purposes by HPLC–UV has been also proposed [20,23,32,35,81]. SPE methods for STA have been recently reviewed by Franke and de Zeeuw [44] and this topic will be only outlined here.

The use of two coupled cartridges containing polymeric resins has been adopted by Binder et al. in their automated system [25]. This approach is based on sample preparation on-line with a coupled column chromatographic separation allowing a fully automated screening of drugs in urine, whereas serum, whole blood and biological tissues require a preliminary off-line isolation of the analytes from the matrix [81].

A twin C-18 SPE procedure was developed by Gaillard and Pepin [20] for the purification of acidic–neutral and basic drugs from hair incubation media.

Cation-exchange SPE was adopted by Logan et al.[32] in their screening for basic drugs.

Lai et al. [23] adapted a previously developed mixed-mode SPE method [45] (see Section 2.1.2) to their HPLC–UV screening procedure in urine. Instead of separating acidic–neutral from basic analytes by carrying out two separate elutions, a single elution with methanol containing 10% ammonia was adopted. This approach, although resulting in a notable speed-up of analysis (one chromatographic run instead of two per sample), had a negative impact on selectivity of the method. In order to overcome this drawback the composition of the mobile phase was modified by adding octyl sulfate as an anionic pair ion in order to obtain a better spread of analytes over the entire chromatogram and to separate basic compounds from the peaks in the early part of the chromatogram, probably due to extra drug metabolites extracted by SPE. The authors demon-

strated that their SPE–HPLC–UV method is suitable for the detection of intact glucuronides of different drugs. They compared extraction recoveries from urine for ten different drugs obtained by SPE and by solvent extraction with Toxi-Tube A (Toxi-Lab, Irvine, CA), and found that SPE performed better, particularly in the case of acidic (e.g. furosemide, naproxen), and amphoteric (e.g. morphine) polar compounds.

The medium in which the final extract is dissolved before HPLC analysis has to meet the opposite demands of solubilising hydrophobic analytes and of avoiding peak broadening during injection [22]. Generally, a 1:1, v/v mixture of water and organic modifier, either methanol [30] or acetonitrile [21,23], with [23,30] or without [21] the other constituents of the mobile phase, is chosen.

3.2. Chromatographic separation

RP-HPLC with acidic pH mobile phases and gradient elution is the most commonly adopted strategy in STA owing to the possibility of separating a huge number of compounds with different physico-chemical properties within a single run [20–24,27,28,31–33].

However, isocratic elution is generally considered to be preferable as (a) retention behaviour is more reproducible [36], (b) the background contributed by the mobile phase to the UV spectra does not change during chromatography [25] and (c) it is more efficient for quantitation [82]. Therefore, alternative approaches to gradient elution have been proposed, such as the use of different isocratic chromatographic systems in order to cover a broad range of drugs [36], or the use of coupled-column liquid chromatography (RP and silica column) [25]. Tracqui et al. [29] proposed isocratic elution with high percentage of organic modifier in the mobile phase (methanol–tetrahydrofuran–pH 2.6 phosphate buffer 65:5:30, v/v/v). The loss in selectivity due to the reduced separation power (35 pairs of drugs exhibiting similarity in both retention behaviour and UV spectra over a database of 311 compounds) was considered acceptable for the authors' purposes.

HPLC retention data are more difficult to standardise compared to GC data because of the lower intra- and interlaboratory reproducibility of the technique [28]. Many authors have, therefore, pointed out the need for calculating secondary parameters of retention, such as relative retention times or retention indexes using different scales: alkane-2-one, alkylarylketone, and 1-nitroalkane scales [26–28,82]. Further corrections using drugs mixtures in order to overcome the difference in chromatographic behaviour between drugs and scales of neutral homologues have been also proposed [83]. The technical progress in columns and pumps manufacturing as well as the control of environmental conditions (separation is often carried out at constant temperature, usually in the range 25–50°C) have contributed to reduce the problem and recently published methods generally exhibit excellent reproducibility of retention behaviour [21].

Deactivated silica is the standard stationary phase for STA applications, separation being achieved by exploiting hydrophobic interactions of the drugs with hydrocarbon chains. Octadecyl silica columns (100–300 mm length×2.1–4.6 mm I.D., 4–5 μm particle size) are commonly used, although octyl silica [32], 1,1-diysopropyloctyl silica [27], and a mixture of octadecyl and cyanopropyl silica [82] have been also proposed as stationary phases. Owing to the type of analysis and to the complexity of extracts, a guard column in front of the analytical column is a must.

Linear gradient elution is generally carried out starting from low percentage of organic modifier, usually acetonitrile [20,22–24,27,28,31–33]. Typical conditions are 0–15% acetonitrile in phosphate buffer (0.05 *M*, pH 3–3.8) with linear increase up to 50–80% acetonitrile within 15–30 min, 5 min isocratic, and return to initial conditions (3–5 min), with 25–45 min total run time including after-run equilibration. Flow rates are in the range 0.4–2 ml/min. A two-step gradient from 15% to 35% acetonitrile after 6.5 min and from 35% to 80% after 25 min (with 3 min hold) with linear increase of the flow rate from 1 to 1.5 ml/min during the run has been proposed by Gaillard and Pepin [21]. Two consecutive linear gradients (from 10% to 30% in 5 min acetonitrile and from 30% to 60% acetonitrile in 10 min) with an isocratic step in between (20 min) has been adopted by Drummer et al. [33].

When deactivated silica columns are used, ionic interactions of basic compounds with the remaining free silanol groups or with trace metals in the

stationary phase may occur, resulting in peak tailing. In order to suppress ionisation of silanol groups, the use of low pH mobile phases (in the range 2–4) is therefore usually proposed, together with the addition of a competing base (triethylamine). Peak tailing can be also considerably reduced by using octadecyl silica packing material with so-called end-capping characteristics, obtained by elimination of trace metals and by deactivation of the free silanols in the stationary phase.

Low pH mobile phases provide good separation of acidic compounds whose ionisation is suppressed in order to fully exploit hydrophobic interactions with the stationary phase.

The use of alumina based packing coated with butadiene has been proposed in alternative to deactivated silica [30]. This material has a simplified retention mechanism owing to the absence of free silanols. In addition, it is stable over a wider pH range (pH 2 to 12) compared to deactivated silica, thus allowing separation of basic compounds under alkaline conditions. However, acidic drugs (e.g. containing phenolic or carboxylic groups) are poorly retained at alkaline pH and a second chromatographic system has to be developed for these compounds [26].

3.3. Detection and identification

UV detection is usually carried out in the range 200–400 nm, with a resolution of 1–2 nm and a scan speed of ca. 1 scan/s. Some authors use narrower ranges: 210–350 nm [23], 225–350 nm [21]. Lower initial wavelengths - 190 nm [32] – or higher final wavelengths – 650 nm, [33] – have been proposed in some cases. The signal of one or more wavelengths in the range 200–254 nm is usually monitored during the chromatographic run.

Although some authors have emphasised the importance of chromatographic data for identification purposes in HPLC–UV analysis [28,82], the most common strategy is to use retention data as a prefilter of possible candidates: reference spectra included in a given time window around the absolute (RT) or relative (RRT) retention time of the unknown are submitted to the UV spectral comparison routine. Time windows of $\pm$0.04–0.06 RT or RRT have been used [21,22,33]. Tracqui et al. [29] adopted a time window of RT$\pm$0.5 min widened, for compounds eluting after 9 min, to RT$\pm$1.0 min. A match factor combining retention behaviour with UV spectral data was proposed by Harstra et al. [84,85].

Spectral comparison routines [24,86] are usually preceded by the evaluation of peak purity. This has been done manually (e.g. by comparing UV spectra recorded along the peak profile) or automatically using one of the available peak deconvolution routines [26].

Background subtraction is also a good practice in order to compensate for the shift caused by solvent gradient, especially at wavelengths lower than 220 nm [22].

Some UV spectra libraries are commercially available [25,74,87] or accessible through the internet [88]. An ultraviolet spectra library is also commercially available from Merck (Darmstadt, Germany), known as the Merck Tox Screening System (MTSS).

However, as previously stated, UV spectra are difficult to standardise owing to the possible effect of small changes in local conditions (mobile phase composition, temperature, gradient accuracy, etc.) on spectral shape. For these reason, many authors recommend the construction of in-house UV spectra databases. Indeed, almost all the HPLC–UV methods available in the literature use home-made libraries for spectral comparison [21,25,27,29]. Together with retention data and a UV spectrum of the analyte, some authors have proposed the storage in the library also of the urinary metabolic profiles as obtained from previous intoxication cases [23] or from excretions studies and of the UV spectra of metabolites [21] in order to give a further aid to identification.

Identification based on comparison of UV spectra may be problematic for those compounds having poor UV spectra such as barbiturates, lidocaine, phenytoine, zipeprol [21].

Criteria for positive identification on the basis of the matching of the reference UV spectrum with the unknown have been proposed in some cases [21,29,33,34]: match factors higher than 90–95% of the maximum match value provided by the library search algorithm are in general considered as possible correct identifications. Lower values may be accepted for compounds with poor UV spectra, with retention data gaining more significance in such cases [21].

4. HPLC–MS

After the development of effective and easy-to-handle interfaces, such as electrospray (ES) and atmospheric-pressure chemical ionisation (APCI), and the introduction on the market of relatively low-priced instruments, HPLC–MS has rapidly gained popularity among analytical toxicologists [41,42]. Indeed, HPLC–MS is a very promising hyphenated technique as it combines the high versatility of HPLC separation to the selectivity of detection of MS.

Nevertheless, a number of obstacles limit the application of HPLC–MS in STA. First of all, due to the different ionisation processes, the existing large databases of reference EI mass spectra [52,70–74] are useless in HPLC–MS. Particle beam mass spectra constitute an exception to this statement. However, this interface has found limited success owing to its low versatility in term of analytes' polarity. Moreover, though well established and widespread HPLC–MS ionisation techniques are now available, they have been developed only in the last few years, which means that large databases of ES and/or APCI mass spectra do not exist yet.

A further problem is that HPLC–MS ionisation techniques are relatively soft. Therefore, mass spectra generally consist almost only of the pseudomolecular ion and, consequently, give little structural information. Increased fragmentation by collision-induced dissociation (CID) can be obtained by increasing the repeller voltage in the ion source or by means of tandem mass spectrometric techniques. Recently, Marquet et al.[40] proposed an interesting approach to obtaining ES mass spectra with a fair number of diagnostic ions to be used in library search routines. After HPLC separation, carried out on a 150 mm×1 mm I.D. octadecyl silica column (5 μm particle size) by gradient elution from 5 to 95% acetonitrile in 5 m*M* ammonium formate (flow rate 40 μl/min), MS acquisition (100–1100 *u*) was carried out by alternating both positive and negative ionisation and low and high energy in the source. Alternating positive and negative ionisation allowed the authors to include in the screening strongly acidic and basic compounds that may not show up in the positive and negative ion mode, respectively. The low and high energy mass spectra (the former with a prominent pseudomolecular ion and little or no fragmentation, and the latter with extensive fragmentation) were summed and the resulting mass spectrum was stored in the database. The authors constructed a library containing more than 600 of such summed spectra. The power of the method in the identification of unknowns was demonstrated by comparing its performance with that of current GC–MS and HPLC–UV procedures.

5. TLC–UV

Although largely overcome in terms of selectivity and sensitivity by HPLC and GC, TLC is a highly versatile and low-cost technique and it is still very popular in analytical toxicology labs. Its usefulness in combination with UV detection for STA purposes has been demonstrated [37]. Ojanperä extracted basic and amphoteric drugs from urine or from enzyme-digested liver samples using an ion-pair extraction procedure [38,39]. Separation was carried out on silica gel plates using a mixture of toluene–acetone–94% ethanol–25% ammonia (45:45:7:3, v/v). A second TLC system (octadecyl silica plates eluted with methanol–water–37% hydrochloric acid, 50:50:1, v/v) having low correlation with the first one was later added in order to increase the identification power of the method [38]. Detection was carried out using a scanning densitometer in absorbance mode (220 nm) and spectra were recorded between 190 and 400 nm at 5 nm increments. A software was developed for comparing the obtained corrected R_F values (prefilter) and UV spectra with data contained in the reference database.

6. Automated STA methods

A fully automated HPLC–UV system for the screening of drugs in urine and serum was proposed by Binder et al. in 1989 [25] and is commercially available under the name of REMEDi HS (Bio-Rad, Hercules, CA). This system, that takes about half an hour for the processing of a sample, includes on-line SPE extraction (see Section 3.1), analysis by coupled-column liquid chromatography (see Section 3.2), UV scanning of the column eluate, peaks identifica-

tion based on retention behaviour and on library search of UV spectra, and printout of the final report (Fig. 1). Good performance in the identification of drugs in clinical and forensic intoxication cases has been reported [81,89–91]. However, low chromatographic and spectroscopic selectivity has been observed in the case of some drugs classes (e.g. benzodiazepines, barbiturates, NSAIDs). Recently, a REMEDi HS assay for benzodiazepines has been developed [92].

A fully automated STA GC–MS method was developed by Polettini et al.[9] using the Hewlett-Packard 7886 PrepStation coupled to a HP 5973 GC–MS system. This method involves extraction of urine or diluted and sonicated plasma/serum/whole blood by SPE using mixed-mode solid-phase cartridges. The acidic–neutral and basic extracts are then separately evaporated, derivatised (trimethylsilylation, see Section 2.1.3) and analysed by GC–MS in scan mode. After data processing consisting of mass spectral purification and library search (see Section 2.3), a final report is printed out with a list of tentatively identified compounds (Fig. 2). The whole procedure takes less than two hours for the completion of one screening. The method exhibited good performance in the identification of unknowns at therapeutic levels, although with limitations in the case of highly polar substances not amenable to GC analysis.

Another method based on the coupling of a benchtop HP GC–MS system with the HP PrepStation has been proposed by Gougnard et al. [19]. It consists of mixed-mode SPE (with combination of the acidic–neutral and of the basic extracts) and of direct GC–MS analysis of the reconstituted extract (no derivatisation applied). Review of GC–MS data was carried out manually.

Chen et al. [93] developed a semi-automated version of their sample preparation method based on mixed-mode SPE (see Section 2.1.2) [45] using the ASPEC automatic extraction system (Gilson Medical Electronics, Villiers le Bel, France). After SPE, the extracts had to be manually evaporated before reconstitution (no derivatisation applied) and GC analysis.

A system for automatic SPE and derivatisation has been proposed by Cardenas et al. [12]. The urine sample (1 ml) was passed through a Amberlite XAD-2 cartridge at a flow-rate of 0.4 ml/min. After rinsing with 1 ml of 0.1 *M* phosphate buffer, pH 7, the cartridge was eluted with a mixture of BSTFA–chlorofrom–acetone (5:4:1, v/v/v) at a flow-rate of 0.2 ml/min, thus obtaining the displacement and simultaneous derivatisation of analytes, and 1 μl of the collected fraction (100 μl) was injected into the GC–MS system.

Methods for the automated processing of GC–MS data able to select relevant spectra form a data file, perform library search, and produce a final report with a list of tentatively identified substances have been developed by different authors [18,60,94,95].

7. Conclusions

A good number of excellent methods for the STA of drugs and poisons in biosamples is currently available. Both GC–MS and HPLC–UV appear to be well suited to this purpose, although they should be considered complementary more than alternative techniques. A recent study, for example, demonstrated that an integrated approach based on GC–MS analysis of serum or whole blood and of HPLC–UV (REMEDi HS) analysis of urine is able to increase the number of positive identifications in clinical and forensic intoxication cases with respect to the application of the methods alone [96]. The improved performance appeared to be largely due to the complementary features of the techniques more than to the different types of samples analysed (serum/blood versus urine).

It is predictable that the next years will see a progressive increase in the application of HPLC–MS to STA, although this process will be probably slowed down by the present lack of comprehensive databases of LC–MS mass spectra produced with standardised ionisation conditions [41,42].

The diffusion of automated STA methods is to be hoped for as it would allow toxicologists to concentrate on the most crucial step of the STA procedure: data evaluation [9]. The availability of fast and reliable laboratory robots able to carry out even complex sample preparation procedures such as those required for GC, the good level of automation of chromatographic analysis and of data acquisition reached both by GC–MS and HPLC–UV systems, and the increasing capabilities of operative systems

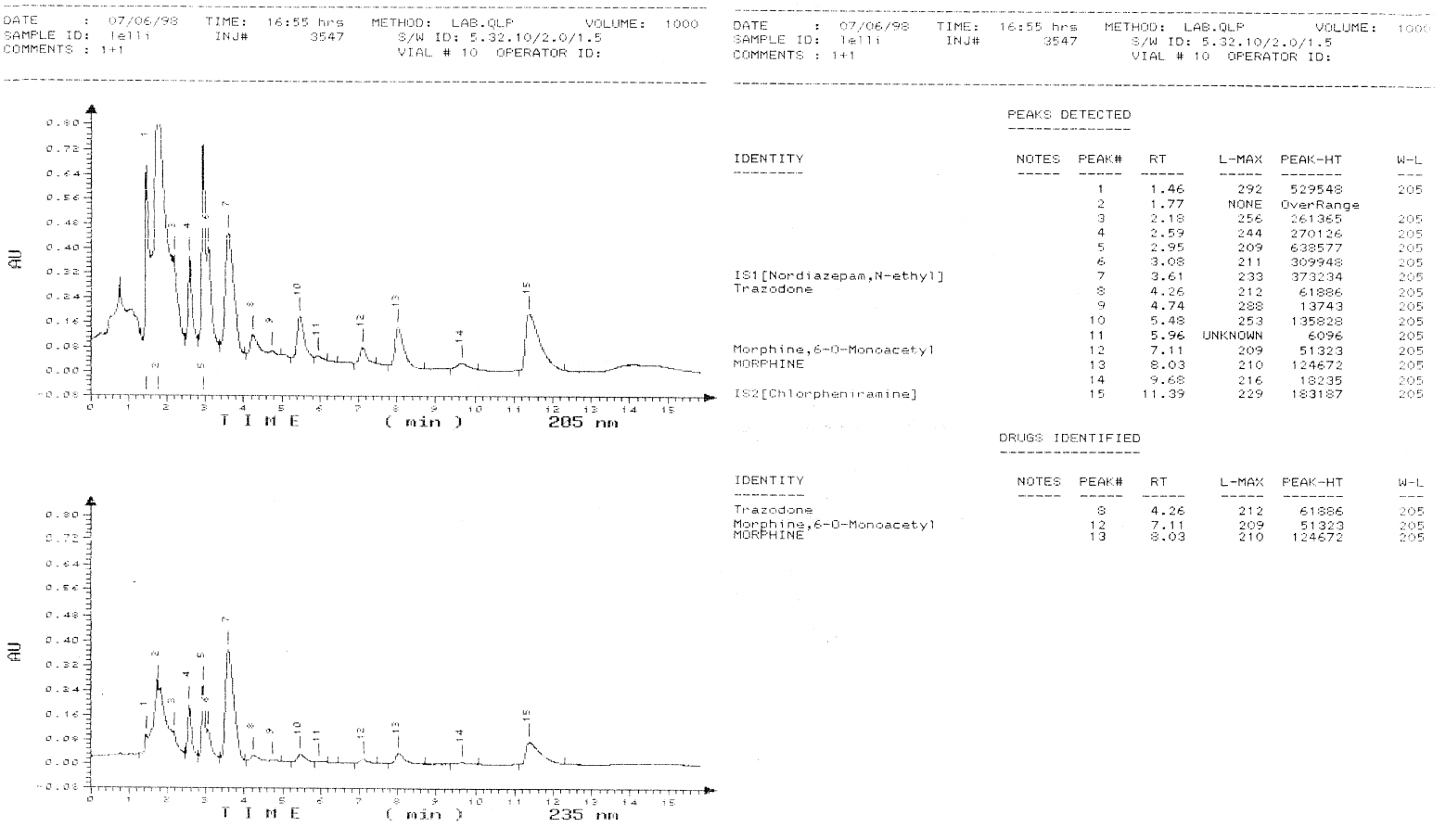
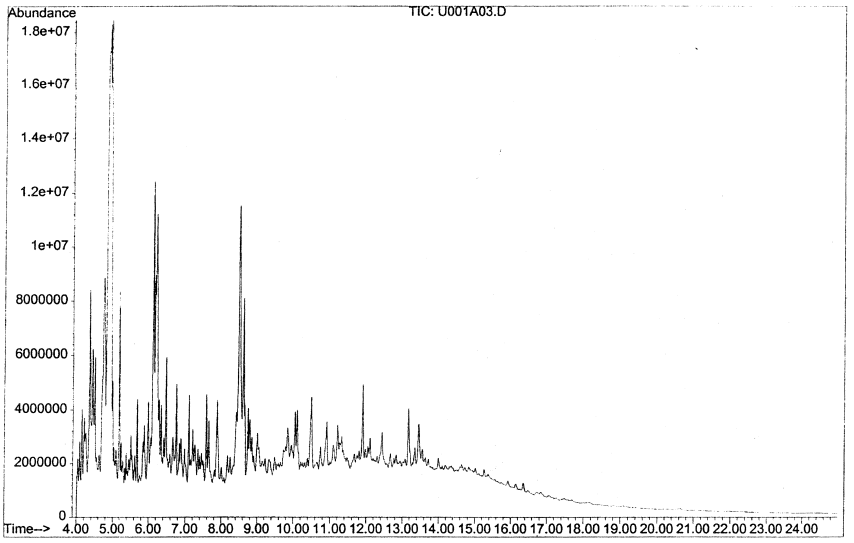


Fig. 1. Example of the search report produced by the REMEDi HS (Bio-Rad, Hercules, CA) [25].

```
File       : C:\HPCHEM\1\DATA\99030402\U001A03.D
Operator   : CV
Acquired   :  4 Mar 99  14:54     using AcqMethod SCREEN
Instrument :    GC/MS Ins
Sample Name:              eal
Misc Info  :
Vial Number: 96
```



Information from Data File:
File : C:\HPCHEM\1\DATA\99030402\U001A03.D
Operator : CV
Acquired : 4 Mar 99 14:54 using AcqMethod SCREEN
Sample Name eal
Misc Info :
Vial Number: 96

Search Libraries: C:\DATABASE\fortox.l

Minimum Quality: 50 RI filter: 200 RT ISTD: 6.07
Search Type: TC Integration Params: autosep.e

RT	Quality	Library/ID
5.14	59	MDA TMS
5.29	52	Reserpine-M ME @ P1
9.05	59	Dobutamine-M 2AC @ P1
9.27	***93***	***Nordazepam.TMS***
9.69	50	Amitriptyline-M (HO-) -H2O @ P1
9.72	53	Amitriptyline-M (HO-) @ P1
9.73	50	Chlorphenoxamine-M (HO-) P1
9.73	60	Doxepin-M (HO-dihydro-) AC P1
9.74	68	Amitriptyline-M (HO-) AC @ P1
9.74	***59***	***Amitriptyline P1***
9.83	59	Benzoctamine.TMS
10.96	***59***	***Nortriptyline.TMS***
11.15	***84***	***Chlordiazepoxide artifact (desoxo) P1***
11.42	53	Perhexiline-M (di-HO-) -H2O P1
11.94	99	Nalorphine.2TMS (ISTD)
12.39	***83***	***7-aminoflunitrazepam.TMS***
12.70	47	Aloe-emodin 2TMS P1
12.70	72	Naloxone TMS P1
13.09	92	Pholcodine-M 3TMS P1
15.06	91	Cholesterol TMS P1

Fig. 2. Example of the search report produced by the fully automated GC–MS method developed by A. Polettini et al. [9]. Compound names in italic are confirmed identifications.

in data processing will surely contribute to the diffusion of automation in STA. In particular, there is a demand for automated methods able to reliably and rapidly process the large amount of analytical information produced by hyphenated chromatographic and spectroscopic techniques: the manual processing of a chromatogram containing several dozens of peaks requires a considerable effort often not repaid with satisfactory results.

Nevertheless, it should be always kept in mind that approaches to identification of unknowns based on automated library search, although unavoidable, have a currently insurmountable limitation. None of the methods described in this review will be able to identify an unknown substance whose reference data are not included in the searched database. The currently available expert systems for the interpretation of analytical data [97,98] are far from being sufficiently reliable and are too sophisticated to be adopted in routine analysis. The identification of unknown substances based on the correlation between analytical information and structural features will be among the future challenges of STA.

8. List of abbreviations

APCI	atmospheric pressure chemical ionisation
BSTFA	*N,O*-bis(trimethylsilyl)trifluoroacetamide
CID	collision-induced dissociation
DAD	diode array detection
EI	electron impact ionisation
ES	electrospray ionisation
GC	gas chromatography
HPLC	high-performance liquid chromatography
LLE	liquid–liquid extraction
MBTFA	*N*-methyl-bis(trifluoroacetamide)
MS	mass spectrometry
MSTFA	*N*-methyl, *N*-trimethylsilyltrifluoroacetamide
NSAIDs	non-steroidal anti-inflammatory drugs
RP	reversed-phase
RT	absolute retention time
RRT	relative retention time
SPE	solid-phase extraction
STA	systematic toxicological analysis
TFA	trifluoroacetyl
THA	tetraethylammonium
TIC	total ion chromatogram
TLC	thin layer chromatography
TMCS	trimethylchlorosylane
TMS	trimethylsilyl
UV	ultraviolet spectroscopy

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