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Fully-automated systematic toxicological analysis of drugs, poisons, and metabolites in whole blood, urine, and plasma by gas chromatography–full scan mass spectrometry

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Abstract

The availability of automated, rapid and reliable methods for the systematic toxicological analysis (STA) of drugs and poisons in biosamples is of great importance in clinical and forensic toxicology laboratories. Gas chromatography–continuous scan mass spectrometry (GC–MS) possesses a high potential in STA because of its selectivity and identification power. However, in order to develop a fully automated STA method based on GC–MS two main obstacles have to be overcome: (a) sample preparation is rather sophisticated owing to the need to isolate analytes from the aqueous matrix and to allow a correct GC repartition of polar analytes; (b) the large amount of information collected within a single analysis makes it difficult to isolate relevant analytical information (mass spectra of analytes) from the chemical noise. Using a bench-top GC–MS system equipped with a laboratory robot for sample preparation (the Hewlett-Packard 7686 PrepStation) and an original method for mass spectral purification, a fully automated STA procedure was developed involving isolation of drugs from the sample (whole blood with minimal pretreatment, plasma, urine) by means of solid-phase extraction, derivatization (trimethylsilylation) of the acidic–neutral and of the basic extracts, GC–MS analysis, processing of data, and reporting of results. Each step of the procedure, and the method for data analysis in particular, can be easily integrated with other existing STA methods based on GC–MS.

Keywords: Systematic toxicological analysis; Screening; Drugs; Poisons

1. Introduction

The introduction of chromatographic techniques in analytical toxicology in the late 1950s represented the first key moment in the evolution of systematic toxicological analysis (STA). Since that time it became possible to detect the presence of an organic poison in a biological sample from its behavior in a given chromatographic system, providing that the same compound had been previously analyzed in that system and its retention behavior had been recorded. From the first approaches with paper and thin-layer chromatography we have witnessed within a few years a rapid development of different chromatographic techniques, such as gas chromatography with the introduction of capillary columns (GC), and high-performance liquid chromatography (HPLC), particularly with the introduction of the reversed-phase mode, that have found widespread application in STA. A great effort was also made towards the standardization of chromatographic data with the introduction of retention indexes [1] and with the construction of large databases [2,3] in order to make this information usable on an interlaboratory basis.

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The development of rapid-scanning (1 scan/s or higher) multichannel spectroscopic detectors able to record the whole spectrum of the column eluate can be considered as the second key moment in the evolution of STA. The introduction in the 1970s of mass spectrometers (MS) and UV diode array spectrophotometers (DAD) specifically designed as chromatographic detectors has transformed the two-dimensional world of chromatographers into a three-dimensional world, thus multiplying the amount of information obtainable from a single analysis.

Among the combined chromatographic and spectroscopic techniques, HPLC–DAD has been proposed as suitable for STA, its strong points being the possibility of direct analysis of compounds scattered on a wide range of polarity, stability, and molecular mass as well as the solvent compatibility of the sample (urine, serum) with the chromatographic system. These features have proved to be favourable towards the development of fast and fully automated methods of drug screening in biosamples [4,5].

Nevertheless, the separation power of capillary GC as well as the selectivity of detection of MS make GC–MS the technique of choice for STA [6]. The inherent simplicity of the information contained in a mass spectrum (representable as a vector) on one side and the availability of large databases of mass spectra of reference compounds [7,8] on the other allow a fast and reliable computer-aided identification of unknown compounds based on the method of fingerprint superimposition, the so-called library search. Moreover, by including in the procedure a single-step broad spectrum derivatization [9,10], and a reliable method for the automated detection and identification of unknowns in GC–MS data files [11,12], most of the weak points of the technique can be overcome.

As a consequence of the development of extraction methods for STA based on mixed-mode solid-phase extraction (SPE) columns [13] as well as of the recent introduction of instruments for the automated sample preparation allowing efficient evaporation/derivatization of the extracts, full automation of STA methods based on GC–MS analysis is at hand. Using a Hewlett-Packard bench top GC–MS instrument equipped with the HP 7686 PrepStation System, a fully automated procedure was developed involving isolation of drugs from the sample by means of SPE, derivatization of the acidic–neutral and of the basic extracts, GC–MS analysis, processing of data, and reporting of results. The procedure is suitable for the analysis of whole blood, urine and plasma samples.

2. Experimental

2.1. Chemicals

All solvents were of analytical reagent grade (Carlo Erba, Milan, Italy). N-Methyl, N-trimethylsilyl trifluoroacetamide (MSTFA) and trimethylchlorosilane (TMCS) were obtained from Sigma (Milan, Italy). DAU solid-phase 100-mg extraction cartridges (Part No. G1204-62097) for automated extraction where purchased from Hewlett-Packard (Milan, Italy), while Bond-Elut Certify columns (10-ml volume) for manual extraction were purchased from Varian (Milan, Italy). β-Glucuronidase from *Escherichia coli* (specific activity: at least 200 U/ml at 37°C with 4-nitrophenyl-β-D-glucuronide as substrate) was obtained from Boehringer Mannheim (Mannheim, Germany).

2.2. Biological samples

Samples of whole blood, plasma, and urine collected from suspectedly intoxicated patients as well as of blank urine and lyophilized plasma spiked with different drugs were used throughout the study.

In order to evaluate the performance of the method, blank urine (1 ml, $n=4$) spiked with 16 different drugs, namely the barbiturates amobarbital, butobarbital, butalbital, phenobarbital, pentobarbital and secobarbital, the benzodiazepines alprazolam, diazepam, nordazepam and oxazepam, the tricyclic antidepressants amitriptyline, desipramine, imipramine and nortriptyline, the drugs of abuse morphine and benzoylecgonine was prepared. With the exception of alprazolam (0.4 mg/l), all the drugs were present at concentrations of 0.2 mg/l.

2.3. Instrumentation

A Hewlett-Packard bench-top GC–MS system consisting of the following components was used:

HP 6890 gas chromatograph; HP 5973 mass selective detector; HP 5673 autosampler; HP 7886 PrepStation; HP Vectra, Series 4, 5/150 personal computer. Instrument control, data acquisition and data analysis were carried out using the HP Enhanced CHEMSTATION software G1701AA (Version A.03.00). Further processing of data was carried out using Microsoft EXCEL (Version 7.0). The system is able to carry out the analytical procedure described below, from SPE to reporting of results, in a fully automated way.

2.4. Analytical procedure

2.4.1. Sample pretreatment (manual)

The following pretreatment was adopted in the case of whole blood in order to avoid clogging of the SPE cartridge and of the PrepStation tubings: a 0.7-ml sample was placed in a glass tube containing 1.8 ml of 0.1 *M* phosphate buffer (pH 6.0), and the internal standards (heptabarbital, 2 mg/l and nalorphine, 1 mg/l) were added; after vortex mixing (10 s) the tube was placed for 15 min in a sonic bath and then centrifuged (1000 *g*, 5 min); a volume of 1.8 ml of the supernatant was then collected and transferred in a 2-ml vial. Urine or plasma samples (0.5–1 ml) were placed in glass tubes and, after the addition of the internal standards, diluted to 2 ml with phosphate buffer. After centrifugation (1000 *g*, 5 min) 1.8 ml of the diluted samples were transferred to 2-ml vials. In the case of urine, pretreatment may include a hydrolysis step in order to increase the detectability of drugs excreted mainly as conjugates with glucuronic acid. Enzymatic hydrolysis was carried out on the sample adjusted to pH 6–6.5 (with 1 *M* NaOH or 1 *M* HCl, if needed) by adding 50 μl of β-glucuronidase and heating at 45°C for 1 h.

2.4.2. Solid-phase extraction (automated)

The vial was placed in the tray of the autosampler/PrepStation and submitted to the extraction procedure (described in detail in Fig. 1, steps 1–12), according to the method proposed by Chen et al. [13] with minor modifications [14]. The procedure is based on the use of mixed-mode SPE columns. From each sample submitted to extraction an acidic–neutral (EAC) and a basic (EAL) extract were obtained.

2.4.3. Evaporation of the extracts and derivatization (automated)

Both the EAC and the EAL extracts were evaporated to dryness under nitrogen flow and derivatized with a mixture of MSTFA–toluene (1:4, v/v) containing trimethylchlorosylane (TMCS, 5%), prepared just before use and filtered over anhydrous sodium sulphate [9,10]. The derivatization procedure is described in detail in Fig. 1 (steps 13–28).

2.4.4. GC–MS analysis (automated)

GC separation of the EAC and EAL extracts was carried out using a HP-5 Ultra 2 5% phenyl, methyl silicone column (12 m×0.2 mm I.D., 0.33 μm film thickness). The GC settings were as follows: carrier gas, helium (column head pressure, 72 kPa, constant flow mode, 1.0 ml/min); injector, pulsed splitless (2.25 min) with a column head pressure of 172 kPa, at 250°C; oven, 100°C (2.25 min), first ramp at 40°C/min from 100°C to 180°C, second ramp at 10°C/min from 180 to 290°C, final isotherm at 290°C for 15 min; transfer line, 280°C. EI (70 eV) MS analysis was performed in scan mode (40–600 u, 2.64 scan/s), with a source temperature of 230°C.

2.4.5. Detection and identification of peaks (automated)

Raw GC–MS data files were processed using an original procedure for the automated purification of mass spectra from the total ion chromatogram [11,12]. The procedure is based on the subtraction of the total intensity of consecutive scans in the total ion chromatogram (TIC). Briefly, two transformed chromatograms are produced by subtracting from the total abundance of each scan that of the preceding scan (first transformed chromatogram) as well as that of the following scan (second transformed chromatogram). Each peak in the TIC corresponds to two peaks (one in the positive and one in the negative direction of the intensity scale) in each transformed chromatogram (Fig. 2). The two transformed chromatograms, that are specular and displaced of a scan, are then processed as follows: the program detects positive and negative peaks in the transformed chromatograms, and searches the corresponding background-subtracted mass spectra (apex-start and apex-end) against one or more reference libraries.

PREPSTATION METHOD

Current Method = STAB.TSP 07/30/97 11:20:22

```
[1]   Rinse_System with 4.000 mL of P04 buffer using Entire System flow path
[2]   Aspirate 1.750 mL from campione
[3]   SPE_Load 1.850 mL of aspirate sample with tampone P04 wash solvent
[4]   SPE_Condition dau with 2.000 mL of methanol
[5]   SPE_Condition dau with 2.000 mL of P04 buffer
[6]   SPE_Apply to <waste> with 3.900 mL of water using dau
[7]   SPE_Wash dau with 1.200 mL of 0.01 M acetic acid
[8]   Preheat at 75 deg C
[9]   Dispense 1.000 mL of methanol into <waste>
[10]  SPE_Dry dau for 5.00 min using wash_Elute flow path
[11]  SPE_Elute dau to EAC with 2.000 mL of clorof./acetone
[12]  SPE_Elute dau to EAL with 1.200 mL of methanol/NH3
[13]  Evaporate EAC at 75 deg C for 5.00 minutes
[14]  Evaporate EAC at 75 deg C for 3.00 minutes
[15]  Evaporate EAC at 90 deg C for 4.00 minutes
[16]  Dispense 1.000 mL of toluene into <waste>
[17]  Transfer 0.050 mL from mstfa to EAC
[18]  Mix EAC at Medium speed for 0.50 minutes
[19]  Heat EAC at 90 deg C for 14.00 minutes
[20]  Mix EAC at Medium speed for 0.50 minutes
[21]  Sample_Ready EAC
[22]  Evaporate EAL at 90 deg C for 5.00 minutes
[23]  Evaporate EAL at 90 deg C for 3.00 minutes
[24]  Evaporate EAL at 90 deg C for 4.00 minutes
[25]  Transfer 0.050 mL from mstfa to EAL
[26]  Mix EAL at Medium speed for 0.50 rninutes
[27]  Heat EAL at 90 deg C for 14.00 minutes
[28]  Mix EAL at Medium speed for 0.50 minutes
[29]  Sample_Ready EAL
[30]  Preheat off
[31]  Rinse_System with 4.000 mL of methanol using Entire System flow path
-- END --
```

Vial/Cartridge Information Table

Name	Type	Number of Uses
dau	cartridge	1
campione	sample	N/A
EAC	empty vial	1
EAL	empty vial	1
mstfa	reagent	25

Solvent Information

SPE SPE Module 2.5 mL syringe

Station	Port	Solvent	Size
SPE	1	methanol	250.0
SPE	2	water	250.0
SPE	3	P04 buffer	250.0
SPE	4	0.01 M acetic acid	50.0
SPE	5	clorof./acetone	50.0
SPE	6	methanol/NH3	50.0
SPE	8	toluene	250.0
SPE	-	Air	N/A

Fig. 1. Scheme of the automated method for sample preparation.

Purified mass spectra were submitted to search against the reference library using the Probability Based Matching algorithm (PBM) [15]. For each identified mass spectrum, PBM provides a match quality value, a value from 1 to 99 defined as the probability that the identified reference spectrum is contained in the unknown spectrum. The reference library consisted of a modified version of the Pfleger, Maurer, Weber Library [8] to which about 200 mass spectra of compounds of toxicological interest and of their trimethylsilyl (TMS) derivatives obtained with the previously described instrumentation were added.

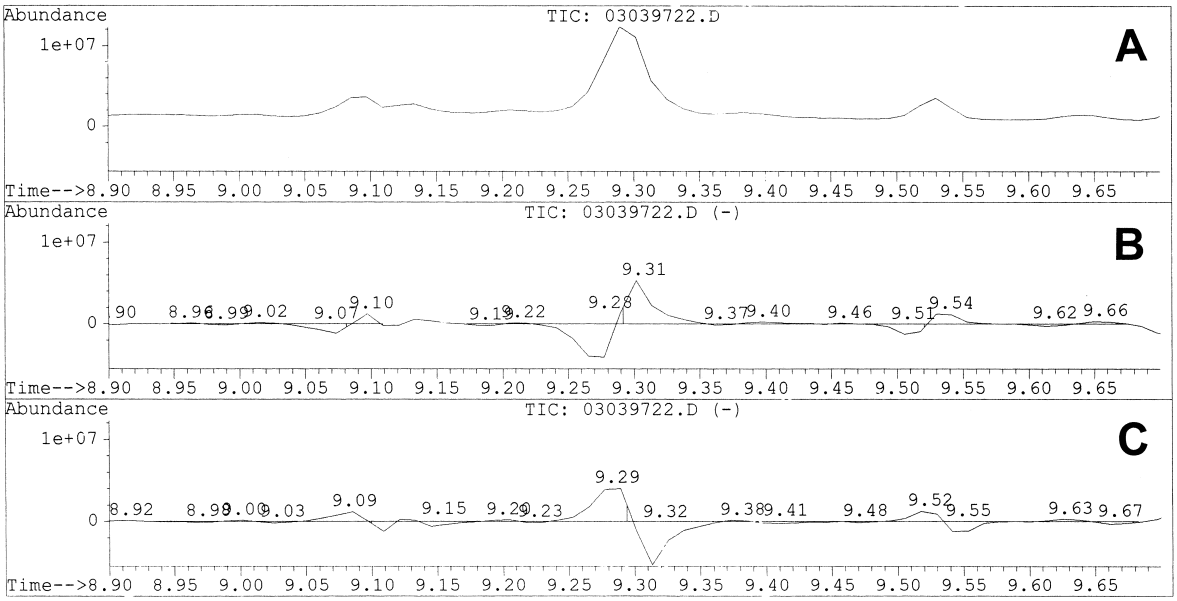


Fig. 2. Example showing the transformation of the total ion chromatogram (A) into two transformed chromatograms (B and C).

2.4.6. Reporting of results (automated)

Mass spectra tentatively identified by library search with match quality values lower than a given threshold value (usually in the range 40–70) or whose estimated retention index [12] differed from the calculated retention index of the reference for more than a given value (usually in the range ±50–200) were automatically discarded in order to reduce the number of false positives. The search report consists of a summary report in which tentatively identified compounds are listed in decreasing order of match quality (see some examples in Figs. 3 and 4), and of a detailed report in which together with the name of the compound and the match quality value, the following data are reported: retention time, reference number in the searched library, CAS number, number of the scans that were subtracted to obtain the purified mass spectrum (see the example in Fig. 8). To execute the above described procedure for data treatment and reporting of results a macro was prepared using the macro language of the HP ENHANCED CHEMSTATION software (based on the Qual_rpt.mac macro). The raw search report produced by the macro was then submitted to further automated elaboration using Microsoft EXCEL in order to improve the readability of the report itself.

2.4.7. Evaluation of results

A macro was created for the rapid retrieval of tentatively identified mass spectra using the purification method described above. Together with the purified mass spectrum, the macro displays on the screen the mass chromatogram of the base mass peak (time window of ±0.5 min around the retention time of the tentatively identified compound). Mass spectra were evaluated using the following criteria: (a) toxicological relevance (e.g. usual interferences from blood were not considered); (b) pK_a (e.g. basic compounds identified in the acidic extract were not considered); (c) fragmentation patterns (e.g. compounds whose tentatively identified mass spectra did not contain one or more significant mass peaks of the reference were not considered).

2.5. Optimisation of the PBM search parameters

The PBM search algorithm is based on reverse search, that is, it looks for the presence of the mass peaks of each reference mass spectrum in the unknown mass spectrum. This feature is quite important for identifying unknown mass spectra in mixtures, which is a frequent occurrence in mass spectra obtained from STA of biosamples. The

search performance of PBM can be customized by varying the settings of different parameters: $U+A$, Tilting, Minimum Estimated Purity (MEP), and Flag Threshold (FT) [15]. A detailed explanation of the PBM search parameters is beyond the scope of the present article; they are only briefly described here in order to understand their effect on search performance.

$U+A$ (default, 2): to each mass peak of each reference mass spectrum and of the unknown mass spectrum an $U+A$ value is assigned where U represent the uniqueness of the mass peak (e.g. the uniqueness of m/z 243 is higher than that of m/z 43). As the probability of the presence of a signal at a certain m/z value increases rapidly at the decrease of abundance, U has to be corrected by a value (A) representing the relative abundance (the lower the relative abundance of the mass peak, the lower A is). The $U+A$ parameter in the PBM search is used as a prefilter search: when the $U+A$ parameter is set to 1, the most significant peaks in the unknown spectrum are selected among those with the highest $U+A$ value or with the highest $U+A$ value minus 1. The reference mass spectra that will be considered in the search will be those whose most significant peak (i.e. that with the highest $U+A$) is included among the most significant peaks of the unknown. If the $U+A$ parameter is increased, the number of most significant peaks in the unknown is increased and a higher number of reference spectra will be considered in the search. At the maximum value of the $U+A$ parameter (9) the prefilter is off and the entire reference library is searched.

Tilting (default, on): when set to on the search attempts to improve the matching among reference and unknown spectrum by scaling the reference to fit the unknown spectrum.

MEP (default, 50): this parameter, operating after the prefilter but before the actual search comparison occurs, requires that the base peak in the reference spectrum is present in the unknown spectrum at a minimum specified relative abundance (%). High MEP values provide good performance when relatively pure mass spectra are searched, while low values allow identification of spectra in mixtures.

FT (default, 3): when a significant mass peak in the reference spectrum is not present in the unknown it is flagged and if the maximum number of flagged ions is reached the reference spectrum is discarded. The FT parameter prevents a certain ion mass from being inappropriately flagged if its relative abundance is low. For example, a value of 3 means that any ion with relative abundance less than 3% will not be flagged as a mismatch.

With the only exception of Tilting, that in general provides best search performance when set to on, $U+A$, MEP and FT may significantly influence search performance and require careful optimisation, particularly when heavily contaminated mass spectra are submitted to library search.

To this purpose, a ‘dirty’ chromatogram (Fig. 5) was obtained by submitting a large volume of urine (5 ml) spiked with seven barbiturates (amobarbital, butalbital, butobarbital, heptabarbital, secobarbital, pentobarbital and phenobarbital) and five benzodiazepines (alprazolam, desalkylflurazepam, diazepam, nordazepam and oxazepam) each at 0.2 mg/l, to manual extraction with Bond-Elut Certify columns (10-ml volume) using a previously described procedure [14] quite similar to the automated procedure proposed here. Then, the obtained GC–MS data files were submitted to automated search by varying the settings of MEP (0, 30, 50%), FT (0, 3, 10%), and $U+A$ (1, 2, 3). The following parameters were evaluated: time required for processing, number of true positives, minimum match quality value, number of false positives.

3. Results and discussion

The potential of mixed-mode SPE columns in the application to STA of biosamples has been discussed elsewhere [13,14]. Both Bond-Elut Certify and WWM-DAU columns provide clean extracts and high versatility in the recovery of compounds scattered on a wide range of polarity, molecular mass and pK_a values [16].

Derivatization of the extract prior to GC analysis is mandatory if polar drugs and metabolites have to be included in the screening. Among the different derivatization procedures proposed for GC–MS analysis, trimethylsilylation under the described conditions has the following advantages: (a) it is highly versatile, allowing the derivatization of different functional groups such as hydroxy, carboxy, amidic,

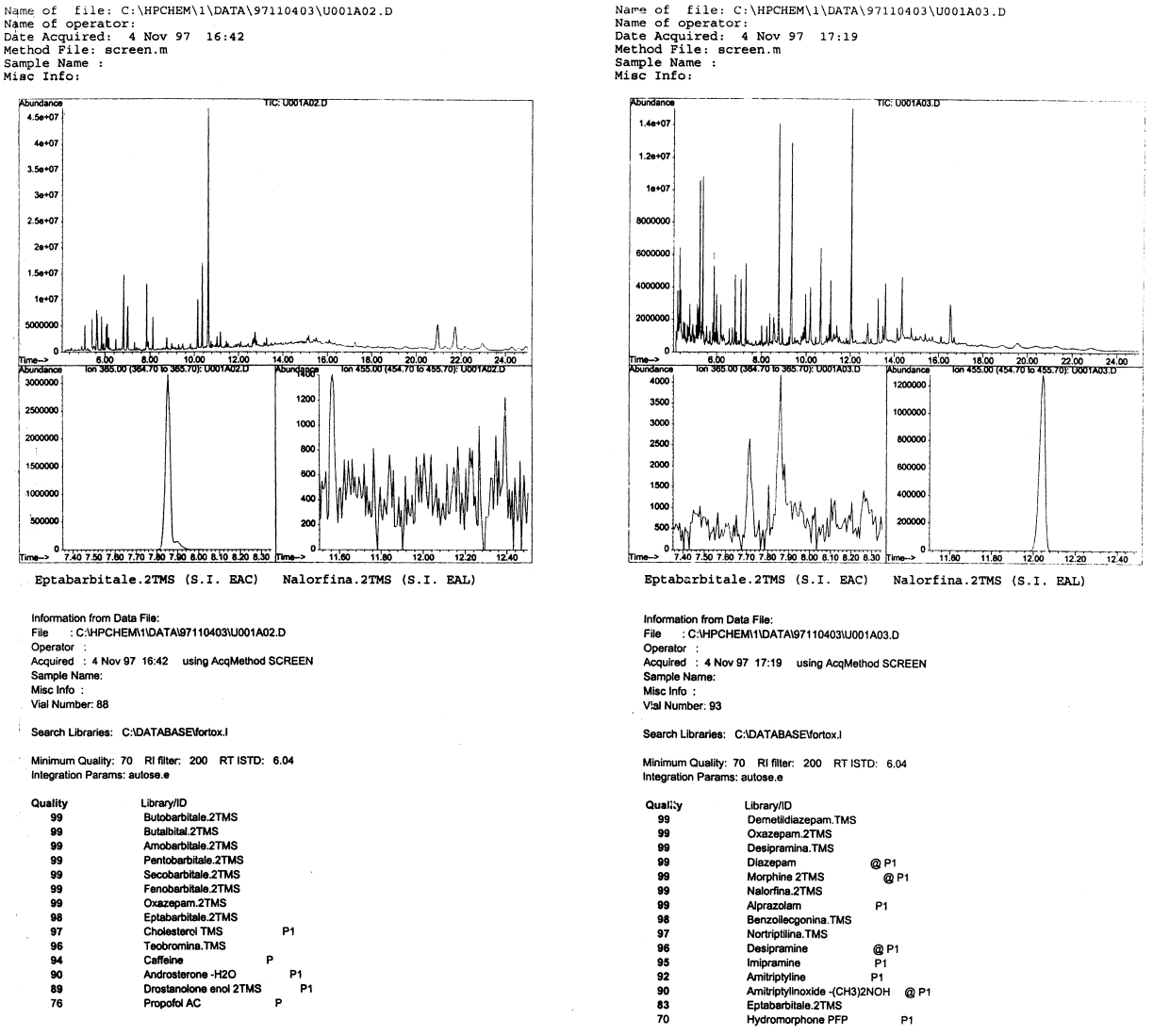


Fig. 3. Search reports obtained for the analysis of the acidic–neutral and of the basic extract of a 1-ml urine sample spiked with sixteen different acidic, neutral and basic drugs.

and, to some extent, aminic ones [9,10]; (b) the reaction occurs under mild conditions and evaporation of the excess reactant is not required prior to injection; (c) it gives a considerable gain in molecular mass of derivatizable compounds, thus increasing, in many cases, the selectivity of mass spectral information; (d) it is effective in improving the repartition of highly concentrated endogenous interferences (such as fatty acids and cholesterol) in biological extracts, thus enhancing the overall chromatographic resolution and, consequently, the detectability of analytes. An important limitation in the use of trimethylsilylation as a general purpose derivatization procedure for STA is the scarcity of TMS derivatives in commercial libraries of mass spectra. This limitation was partly overcome by adding mass spectra of TMS derivatives of many drugs and toxins to the commercial library adopted in our study, the Pfleger, Maurer, Weber Library of Mass Spectral and GC Data of Drugs, Poisons,

Pesticides, Pollutants and their Metabolites [8]. However, the new edition of the library, that should be published at the beginning of 1998, will provide a practical complete coverage of trimethylsilylated derivatives [H.H. Maurer, personal communication].

The adoption of the Hewlett-Packard Prepstation system allowed an easy and complete automation of the sample preparation procedure. Beside automating the SPE step, the PrepStation provided efficient solvent evaporation and heating of vials for derivatization purposes. Furthermore, the PrepStation can be coupled to Hewlett-Packard bench top GC–MS instruments, as in the system used in the present study, thus allowing full automation of the procedure from sample preparation until the printout of the search report. Although probably slower than a skilled operator (it works on one sample at a time), the PrepStation allows overlap of different steps of the procedure (e.g. while one extract is analyzed by GC–MS the following sample is submitted to extraction) and the overall time required for the completion of one screening is less than 2 h. Although this time may be thought too long for the needs of clinical toxicology laboratories, it should be considered for the notable amount of information that is collected within a single analysis.

Fig. 3 shows the report for the automated analysis of a urine sample spiked with 16 different acidic, neutral and basic drugs. The search report contains the TIC, the mass chromatograms of the two internal standards (m/z 365 for heptabarbital bisTMS, and m/z 455 for nalorphine TMS), and the list, in decreasing order of match quality, of the compounds identified by automated search. A detailed report is

Table 1
Identification and reproducibility data for the tested compounds spiked into urine

Compound	Spike level (mg/l)	Derivative	Match quality value[a]	Base mass peak (m/z)	t_R (min)	Acidic–neutral extract[b]	Basic extract[b]	C.V.%[c]
Barbiturates								
Amobarbital	0.2	bisTMS	97±1	355	5.87	P		16.9
Butalbital	0.2	bisTMS	99±0	353	5.67	P		16.5
Butobarbital	0.2	bisTMS	99±0	341	5.44	P		17.4
Phenobarbital	0.2	bisTMS	98±1	146	7.02	P		17.4
Pentobarbital	0.2	bisTMS	99±0	285	5.86	P		15.5
Secobarbital	0.2	bisTMS	99±0	297	6.12	P		14.6
Benzodiazepines								
Alprazolam	0.4		99±0	279	14.35		P	9.6
Diazepam	0.2		99±0	283	10.66		P	2.7
Nordazepam	0.2	TMS	99±0	341	9.35		P	3.7
Oxazepam	0.2	bisTMS	99±0	429	10.16	P	P	8.2[d]
Tricyclic antidepressants								
Amitriptyline	0.2		65±18	58	8.71		P	4.3
Desipramine	0.2		95±1	195	9.09		P	7.8
Desipramine	0.2	TMS	99±0	208	10.20		P	6.9
Imipramine	0.2		95±1	234	8.94		P	5.2
Nortriptyline	0.2	TMS	94±3	116	9.98		P	9.2
Drugs of abuse								
Benzoylecgonine	0.2	TMS	98±1	182	9.34		P	18.2
Morphine	0.2	bisTMS	99±0	429	11.11		P	4.1

[a] Mean±S.D. (n=4).
[b] P=present in the extract.
[c] Calculated for the ratio peak area of the analyte/peak area of internal standard.
[d] For the peak in the acidic–neutral extract.

also produced in which all the data for each tentatively identified mass spectrum are provided (see an example in Fig. 8).

Identification and reproducibility data for the 16 tested compounds are reported in Table 1. All the compounds added were identified: 15 out of the 16 compounds with a match quality value higher than 90, and in one case only (amitriptyline) the match quality was lower (65). Owing to the sample preparation adopted the method provides multiple identification for some compounds, for example, oxazepam, whose bisTMS derivative was detected in both the acidic–neutral and in the basic extract, and of desipramine which was identified in the basic extract in both the derivatized and nonderivatized form. It is well known that neutral compounds such as benzodiazepines may be found in both the acidic–neutral and in the basic extracts obtained from mixed-mode SPE columns [14], while quantitative trimethylsilylation of secondary aminic groups such as that of desipramine was never obtained with the described derivatisation procedure [10]. These features, although possibly limiting the use of the procedure in such cases for semi-quantitative purposes, have undoubtedly positive implications on the possibility of identifying unknown drugs, as the probability of identifying a compound split into two or more chromatographic peaks (and mass spectra) increases. Although the proposed method is intended mainly for qualitative purposes, the data shown in Table 2 indicate that the ratio between peak area of the analytes and peak area of internal standard is fairly

Table 2
Optimisation of the search parameters of PBM (see text for details)

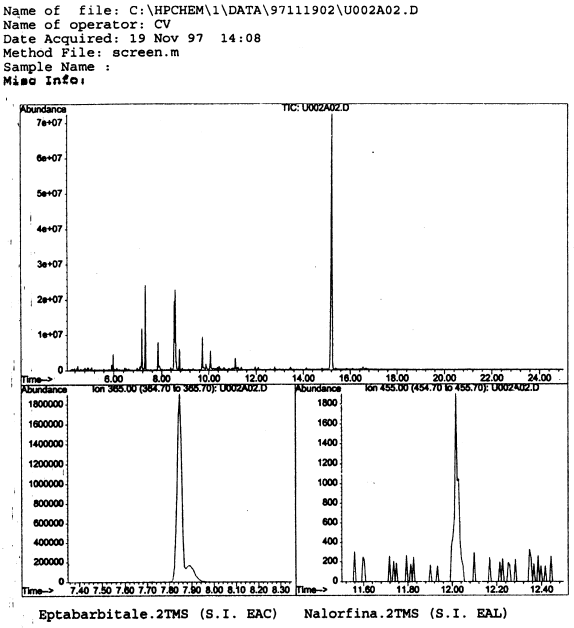
Purity	Flag	$U+A$	Processing time (min)	True positives			False positives		
				No.	Minimum quality	Maximum quality	No.[a]	Minimum quality	Maximum quality
0	0	1	6.28	12	70	99	13	76	97
0	0	2	7.55	12	70	99	13	78	97
0	0	3	9.88	12	70	99	13	78	97
0	3	1	6.88	12	70	99	3	90	93
0	3	2	8.85	12	70	99	4	90	95
0	3	3	10.98	12	70	99	4	90	91
0	10	1	6.87	12	70	99	0		
0	10	2	9.35	12	70	99	0		
0	10	3	11.70	12	70	99	0		
30	0	1	4.72	12	45	99	3	49	93
30	0	2	5.35	12	45	99	4	55	92
30	0	3	8.40	12	45	99	5	49	92
30	3	1	5.30	12	70	99	1		93
30	3	2	6.18	12	70	99	0		
30	3	3	6.78	12	70	99	0		
30	10	1	5.65	12	70	99	0		
30	10	2	6.47	12	70	99	0		
30	10	3	6.95	12	70	99	0		
50	0	1	4.45	12	9	99	59	9	93
50	0	2	4.92	12	9	99	89	9	92
50	0	3	5.48	12	9	99	99	9	92
50	3	1	5.18	12	38	99	11	38	93
50	3	2	5.75	12	38	99	13	38	59
50	3	3	6.20	12	38	99	14	38	59
50	10	1	5.40	12	38	99	11	38	59
50	10	2	5.90	12	38	99	11	38	59
50	10	3	6.57	12	38	99	11	38	59

[a] Using the minimum match quality of true positives as threshold.

reproducible, thus allowing also semi-quantitation, which is of course extremely important in emergency toxicology.

Fig. 4 shows the search report obtained for the analysis of a sample of cadaveric blood obtained from an autopsy case of suspected acute narcotism. Automated STA allowed identification of several benzodiazepines, together with the opiates morphine and codeine, cotinine and a few endogenous compounds.

An important aspect of the described procedure is the original method for peak detection and mass spectral purification adopted. Basically, the method consists of subtracting from each other different scan selected along the profile of each raw peak in the TIC. Therefore, when a chromatographic peak is produced by the coelution of different compounds, the method is able in most cases to obtain one or more purified mass spectra of each component. Compared to other methods for automated detection and identification of mass spectra, this approach has the substantial advantages of not requiring a priori criteria for identification (unlike methods based on selected ion retrieval) and of being of quite simple application (it does not require sophisticated programming) [11].

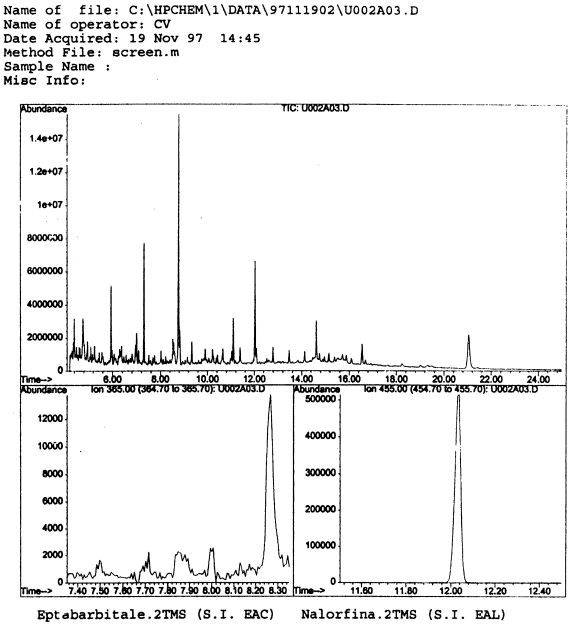


Information from Data File:
File : C:\HPCHEM\1\DATA\97111902\U002A02.D
Operator : CV
Acquired : 19 Nov 97 14:08 using AcqMethod SCREEN
Sample Name: xxx
Misc Info :
Vial Number: 87

Search Libraries: C:\DATABASE\fortox.l

Minimum Quality: 70 RI filter: 200 RT ISTD: 6.04
Integration Params: autose.e

Quality	Library/ID	
99	Eptabarbitale.2TMS	
99	Clordemetildiazepam.TMS	
99	Demetildiazepam.TMS	
99	Lorazepam.2TMS	
95	Cholesterol TMS	P1
87	Glucose 5TMS	P1
80	Cholesterol -H2O	P1



Information from Data File:
File : C:\HPCHEM\1\DATA\97111902\U002A03.D
Operator : CV
Acquired : 19 Nov 97 14:45 using AcqMethod SCREEN
Sample Name: xxx
Misc Info :
Vial Number: 92

Search Libraries: C:\DATABASE\fortox.l

Minimum Quality: 70 RI filter: 200 RT ISTD: 6.04
Integration Params: autose.e

Quality	Library/ID	
99	Demetildiazepam.TMS	
99	Clordemetildiazepam.TMS	
99	Morphine 2TMS	@ P1
99	Nalorfina.2TMS	
97	Cholesterol TMS	P1
90	Codeine TMS	P1
87	Nicotine-M (cotinine)	@ P
87	Diazepam	@ P1
72	Oxazepam isomer-1 2ME	P1

Fig. 4. Search reports obtained for the analysis of a cadaveric blood sample obtained from an autopsy case of suspected acute narcotism.

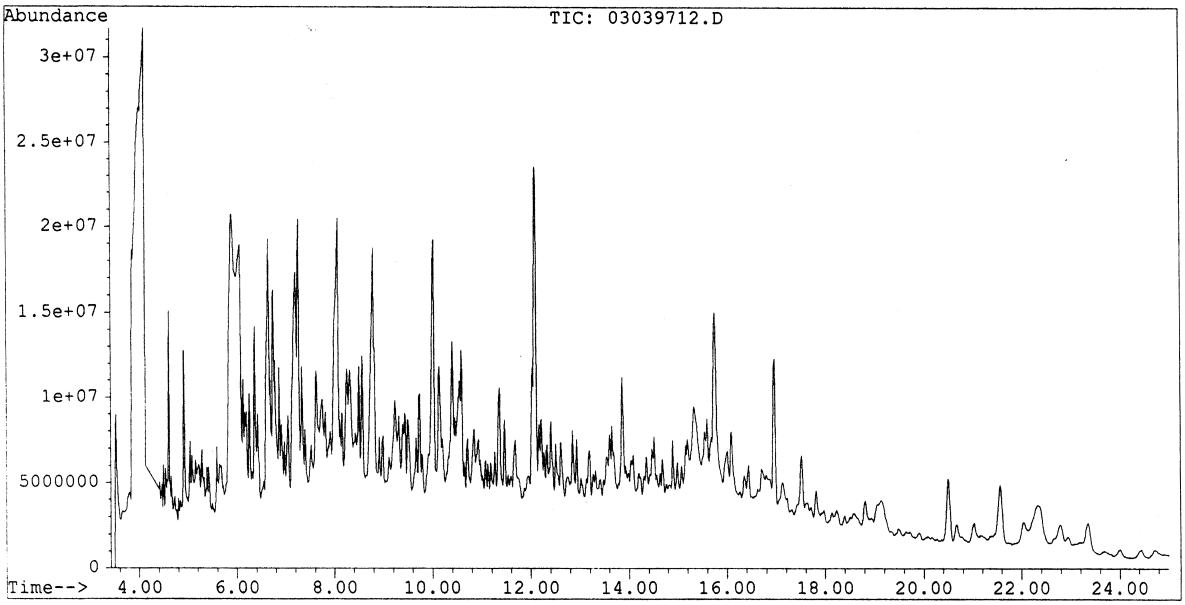


Fig. 5. Total ion chromatogram obtained for the analysis of the acidic–neutral extract from a 5-ml urine sample spiked with seven barbiturates and five benzodiazepines at the 0.2 mg/l level.

Fig. 5 shows the 'dirty' chromatogram used for the optimization of the PBM search parameters. It was obtained for the GC–MS analysis of the derivatized acidic–neutral extract of a urine sample (5 ml) spiked with different barbiturates and benzodiazepines at the usual cut-off values of many immunochemical screening methods (0.2 mg/l). The results of the optimization of PBM search parameters are reported in Table 2. Although in all cases all the 12 added compounds were identified, important differences were observed both in the minimum match quality of true positives and also in the number of false positives. Of the three parameters considered, $U+A$ appeared to be the less important as it had no effect on search performance. When increased from 1 to 3, the minimum match quality of true positives and the number of false negatives were not affected, while processing time was substantially increased ($U+A$ values higher than 3 gave further increase in processing time but no improvement in performance). The combined effect of MEP and FT (with $U+A$ set at default value of 2) on search performance is illustrated in Fig. 6. Higher minimum match quality values for true positives and lower number of false positives, but also higher processing times were obtained by decreasing MEP and by increasing FT. The combination of 30% MEP and 3% FT gave the best compromise in terms of minimum match quality for true positives (70), number of false positives using the minimum match quality as threshold (0), and processing time (6.18 min).

The extreme efficiency of the PBM search algorithm, when carefully optimized, in identifying mass spectra in mixtures is well known [15]. However, PBM is not a method for mass spectral purification. This is clearly demonstrated by the example illustrated in Fig. 7, showing the identification of the bisTMS derivative of secobarbital (Sec) in the TIC of Fig. 5. The mass spectrum A, showing good similarity with the reference spectrum of Sec (B), was obtained using the proposed method for mass spectral purification (match quality value, 87). On the other hand, the scan at which Sec maximized (C), although identified with a high match quality value (91), looks quite different from the reference mass spectrum owing to the coelution of Sec with caffeine (MEP was set to 0% in order to allow identification under heavy contamination of mass spectra). Moreover, the proposed method provides a

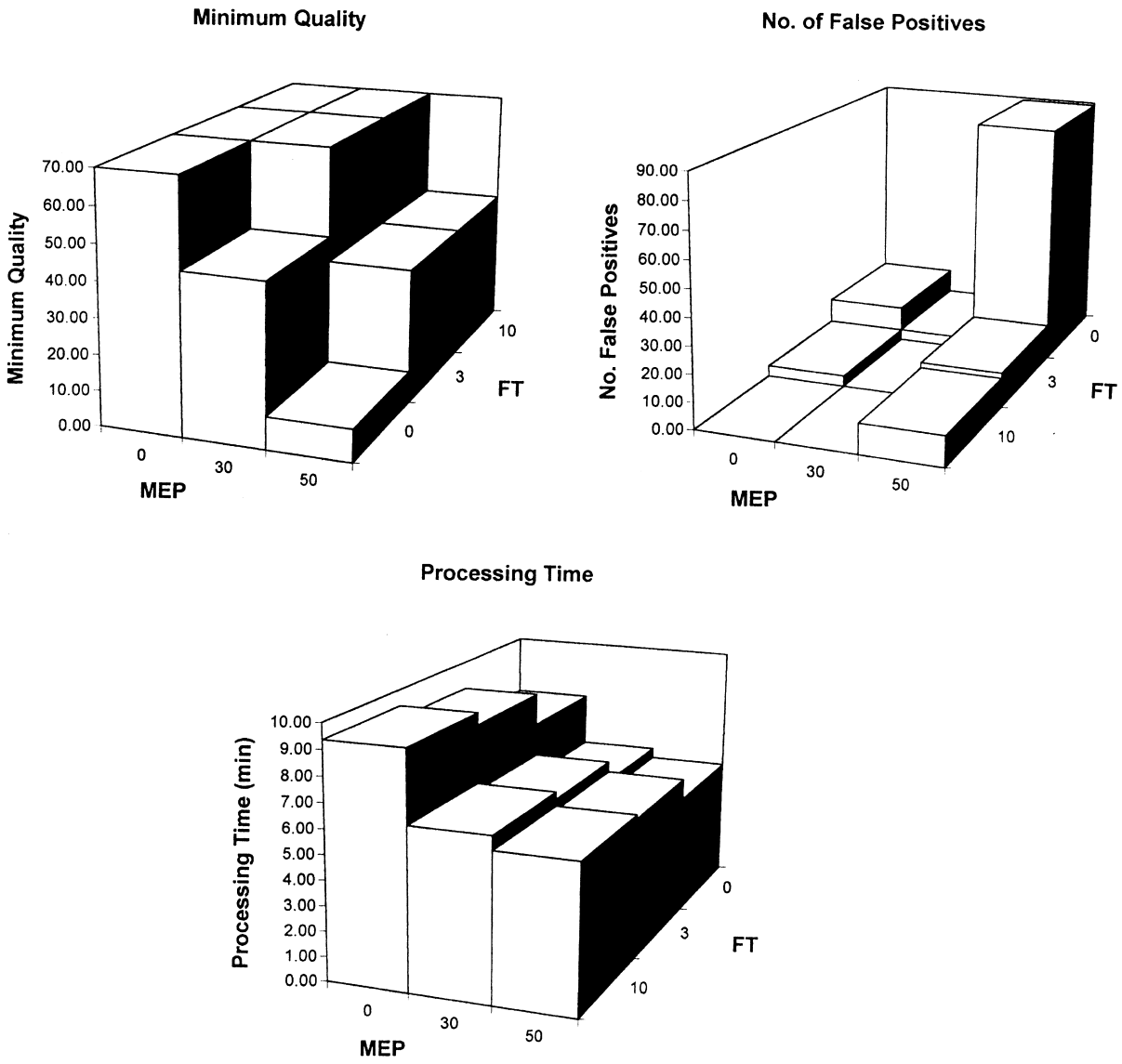


Fig. 6. Effect of the PBM search parameters minimum estimated purity (MEP) and flag threshold (FT) on the minimum match quality of true positives, on the number of false positives, and on processing time of GC–MS data.

tool for selecting mass spectra to be library searched, thus reducing to reasonable limits the time required for processing a GC–MS data file.

Among the features that a method for the automated identification of unknown mass spectra in GC–MS data files must possess is not only the ability to identify drugs present in the sample (true positives) but also of minimizing the number of false positives. To this purpose, the proposed program for automated search makes use of two filters [12]. The first filter is a match quality threshold: compounds identified with match quality lower than the defined threshold are discarded. The choice of the match quality threshold is a compromise among the two opposed demands of minimizing at the same time false positives and false negatives. According to our

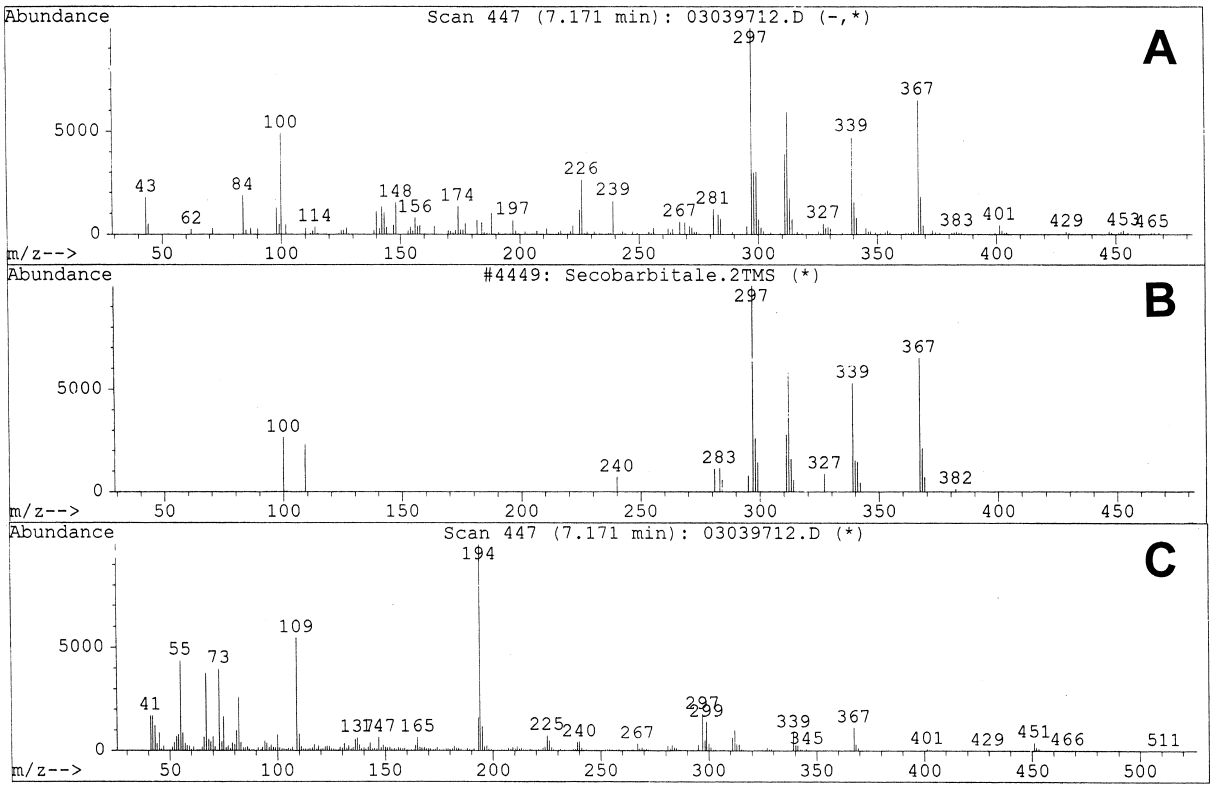


Fig. 7. Purification of the mass spectrum of secobarbital bisTMS (Sec) from GC–MS data of Fig. 5. (A) purified mass spectrum of Sec with the proposed method; (B) reference mass spectrum of Sec; (C) mass spectrum corresponding to the scan at which Sec maximized in the chromatogram.

experience, a threshold of 50 is the best compromise as the risk of discarding poorly identified true positives is minimum, while the number of false positives is dramatically reduced. A second important filter is based on retention data. The program for automated search estimates the retention index (RI) of each integrated peak in the chromatogram and when this differs from that of the identified reference compound for more than a given value, the identified compound is discarded. Taking into account the error in the estimate of retention index and the variability of the chromatographic system, a window of ±200 around the reference RI allows to minimize the number of false positives with no loss of true positives.

When both filters are omitted the automated search for a GC–MS data file such as that shown in Fig. 3 gives as a result a list of 4000–6000 identified compounds. When only one filter (match quality threshold or retention index filter) is adopted the number of identified compounds is reduced to a few hundreds. The reports shown in Fig. 3 were obtained with the application of both filters (match quality threshold, 70; RI filter: ±200 around the RI of the reference mass spectrum): together with true positives, endogenous compounds (e.g. cholesterol TMS) and impurities coming from the materials used (e.g. ionol), only three false positives were included in the search report for the acidic–neutral extract, and one false positive was present in the report for the basic extract. A rapid check of these mass spectra allowed easy rejection of these identifications. To this purpose, a macro was created to retrieve purified mass spectra tentatively identified by the method: using the data listed in the detailed search report (Fig. 8A), the mass spectrum as well as the ion chromatogram

A

	RT	Quality	Ref#	CAS#	Subtraction		Library/ID	
	10.81	96	4390	000000-00-0	747	743	Demetildiazepam.TMS	
	10.81	93	4390	000000-00-0	747	0	Demetildiazepam.TMS	
	10.85	80	4390	000000-00-0	750	0	Demetildiazepam.TMS	
	11.64	99	4442	000000-00-0	815	813	Oxazepam.2TMS	
	12.24	80	481	000439-14-5	864	861	Diazepam	@ P1
→	12.29	94	481	000439-14-5	868	870	Diazepam	@ P1
	17.49	94	3209	001856-05-9	1294	1291	Cholesterol TMS	P1
	17.52	92	3209	001856-05-9	1296	0	Cholesterol TMS	P1
	17.54	99	3209	001856-05-9	1298	0	Cholesterol TMS	P1

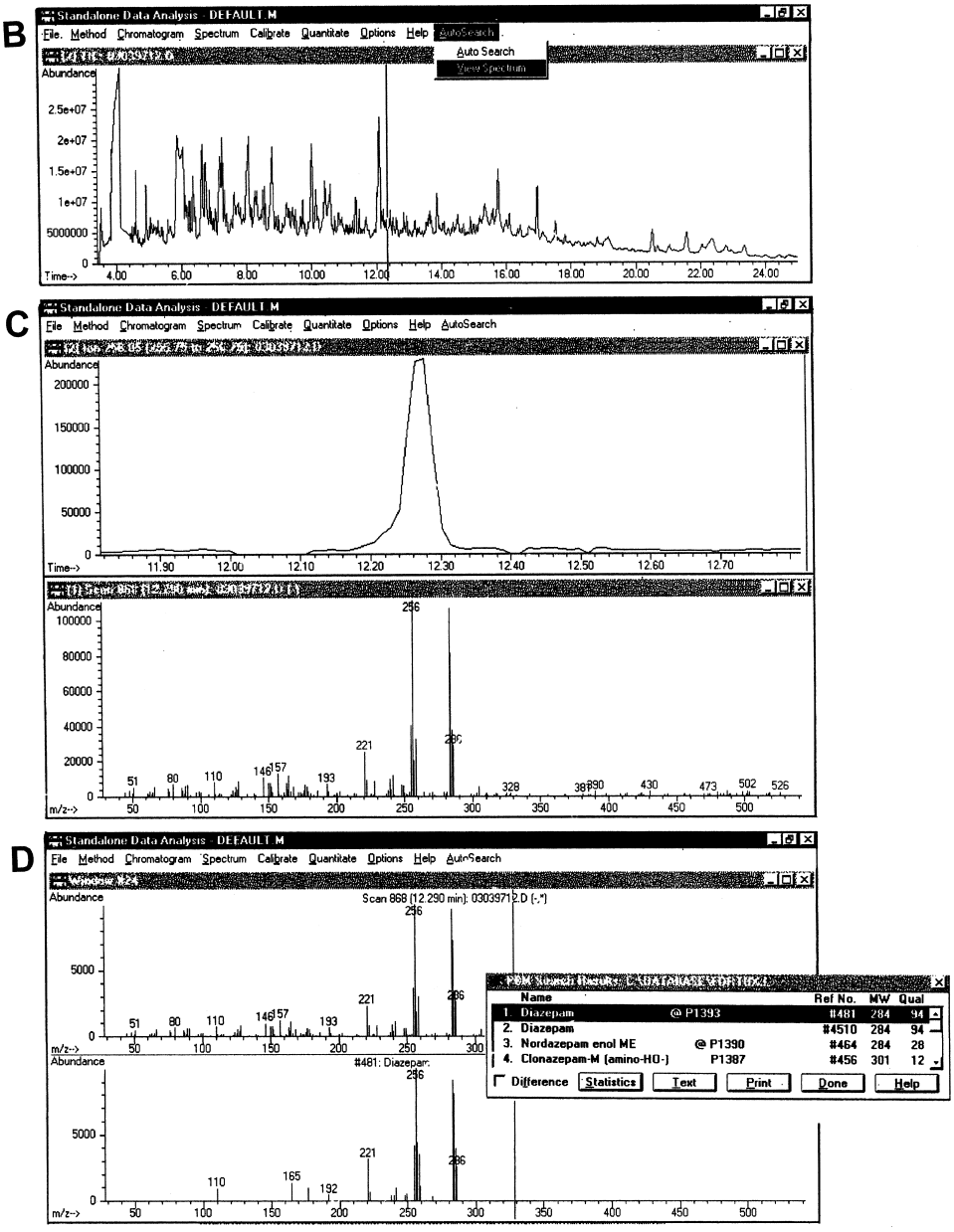


Fig. 8. Retrieval and evaluation of the mass spectrum identified as diazepam from GC–MS data of Fig. 5. (A) Part of the detailed search report (the arrow indicates the retrieved mass spectrum); (B) and (C) retrieval of the mass spectrum and of the ion chromatogram of the base mass peak; (D) execution of library search.

of the base peak in the mass spectrum are displayed on the screen (Fig. 8B and C) for further evaluation or for library search (Fig. 8D).

4. Conclusions

Although more investigations are required in order to evaluate in detail the performance of the described method, the results here presented confirm the high potential of GC–MS in the screening of biological samples for drugs, poisons and metabolites. The combination of a versatile SPE, a broad spectrum derivatization, a reliable method for mass spectral purification, a careful optimization of the PBM library search algorithm, and the effective full automation of the procedure provide an extremely powerful tool for the detection and unequivocal identification of unknown exogenous substances at therapeutic levels.

In the authors' opinion, the large amount and high quality of the information obtained with the application of the proposed procedure largely counterbalances both the high cost of the apparatus and the relatively low sample throughput ($\sim 0.5\ h^{-1}$).

Notwithstanding the excellent performance exhibited, it should not be forgotten that this GC–MS screening procedure, like all methods of identification based on fingerprint superimposition, suffers at present the insurmountable limitation of being able to identify only unknown compounds whose reference data (i.e. mass spectrum) are available in the searched database.

References

[1] E. Kovats, Helv. Chim. Acta 27 (1958) 1915.
[2] R.A. de Zeeuw, J.P. Franke, H.H. Maurer, K. Pfleger (Editors), Gas Chromatographic Retention Indices of Toxicologically Relevant Substances and their Metabolites (Report of the DFG commission for clinical toxicological analysis, special issue of the TIAFT bulletin), VCH Publishers, Weinheim, New York, Basel, 3rd ed., 1992.
[3] D.W. Hill, A.J. Kind, J. Anal. Toxicol. 18 (1994) 233.
[4] S.R. Binder, M. Regalia, M. Biaggi-McEachern, M. Mazhar, J. Chromatogr. 473 (1989) 325.
[5] K.S. Kalasinisky, T. Schaefer, S.R. Binder, J. Anal. Toxicol. 19 (1995) 412.
[6] H.H. Maurer, J. Chromatogr. 580 (1992) 3.
[7] NIST/EPA/MSDC Mass Spectral Database, v. 3.0, National Institute for Standards and Technology, Gaithersburg, 1992.
[8] K. Pfleger, H.H. Maurer, A. Weber. Mass Spectral and GC Data of Drugs, Poisons, Pesticides, Pollutants and their Metabolites. VCH, Weinheim, 2nd Edition, 1992.
[9] A. Groppi, A. Polettini, C. Stramesi, M. Montagna, in: P. Sandra, G. Devos (Editors), Proc. XVIII International Symposium on Capillary Chromatography, Riva del Garda, May 20–24, 1996, Vol. II, p. 922.
[10] A. Groppi, G. Marrubini, A. Polettini, M. Montagna, in: W. Bernhard (Editor), Proc. 34th T.I.A.F.T. Meeting, Interlaken, August 11–15, 1996, Abstract book, p. 85, in press.
[11] A. Polettini, J. Anal. Toxicol. 20 (1996) 579.
[12] A. Polettini, C. Stramesi, G. Marrubini, A. Groppi, M. Montagna, in: W. Bernhard (Editor), Proc. 34th T.I.A.F.T. Meeting, Interlaken, August 11–15, 1996, Abstract book, p. 15, in press.
[13] X.H. Chen, J.P. Franke, J. Wijsbeek, R.A. de Zeeuw, J. Anal. Toxicol. 16 (1992) 351.
[14] A. Polettini, A. Groppi, C. Stramesi, M. Montagna, in: A.V. Kovatsis, H. Tsoukali-Papadopoulou (Editors), Proc. 33rd T.I.A.F.T. Meeting, Thessaloniki, August 27–31, 1995, p. 413.
[15] D.B. Stauffer, F.W. McLafferty, E.D. Ellis, D.W. Peterson, Anal. Chem. 57 (1985) 1056.
[16] X.H. Chen, J.P. Franke, J.W. Wijsbeek, R.A. de Zeeuw, J. Chromatogr. 617 (1993) 147.