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## Combined use of ESI–QqTOF-MS and ESI–QqTOF-MS/MS with mass-spectral library search for qualitative analysis of drugs

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**Abstract** The potential of the combined use of ESI–QqTOF-MS and ESI–QqTOF-MS/MS with mass-spectral library search for the identification of therapeutic and illicit drugs has been evaluated. Reserpine was used for standardizing experimental conditions and for characterization of the performance of the applied mass spectrometric system. Experiments revealed that because of the mass accuracy, the stability of calibration, and the reproducibility of fragmentation, the QqTOF mass spectrometer is an appropriate platform for establishment of a tandem-mass-spectral library. Three-hundred and nineteen substances were used as reference samples to build the spectral library. For each reference compound, product-ion spectra were acquired at ten different collision-energy values between 5 eV and 50 eV. For identification of unknown compounds, a library search algorithm was developed. The closeness of matching between a measured product-ion spectrum and a spectrum stored in the library was characterized by a value called “match probability”, which took into account the number of matched fragment ions, the number of fragment ions observed in the two spectra, and the sum of the intensity differences calculated for matching fragments. A large value for the match probability indicated a close match between the measured and the reference spectrum. A unique feature of the library search algorithm—an implemented spectral purification option—enables characterization of multi-contributor fragment-ion spectra. With the aid of this software feature, substances comprising only 1.0% of the total amount of binary mixtures were unequivocally assigned, in addition

to the isobaric main contributors. The spectral library was successfully applied to the characterization of 39 forensic casework samples.

**Keywords** Mass-spectral library · Electrospray ionization mass spectrometry · Time-of-flight mass spectrometry · “General unknown screening”

### Introduction

Gas chromatography (GC) hyphenated with electron impact ionization mass spectrometry (EI-MS) is the “golden standard” for general screening of samples in environmental and forensic toxicology [1]. In recent decades very large libraries of standardized spectra have been created for GC–MS techniques, enabling simultaneous screening for thousands of relevant toxicants. Despite its proven record of success, GC–MS has the disadvantage of poor detectability of polar, thermally labile, and high-mass molecules [2, 3]. Complementary ionization techniques have therefore been developed. Among these, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the interfaces most commonly used for analysis of toxicants [4–8].

ESI and APCI are soft ionization techniques. Usually, only molecular ions are formed. By accurate mass measurement the elemental formula of a molecule can be determined [9]. Time-of-flight (TOF)-MS is currently the most cost-effective technique for performing accurate mass analysis on a routine basis. In addition to high mass accuracy, the benefits of TOF-MS include high resolving power (typically, resolution of approximately 20,000 can be obtained), wide mass range, and rapid mass spectral acquisition with high full-scan sensitivity [10]. To some extent, accurate monoisotopic masses enable detection and identification of a priori unknown substances, as has been shown, for example, for pesticides [11, 12] and for drugs [13–17] and their metabolites [18, 19]. Monoisotopic masses calculated from empirical formulas have been used to build target-specific databases. Because of the inability of

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MS to distinguish between isobaric substances, pure formula databases were extended by incorporating chromatographic libraries to ensure unequivocal identification of unknown compounds. Use of retention times as compound-specific markers makes the approach rather inflexible and susceptible to changes of experimental conditions. Any modification of the chromatographic conditions may induce shifts of substance-specific retention times, complicating unequivocal identification.

Collision-induced dissociation (CID) [20, 21] can be used to obtain structure-related information about analytes. Diagnostic fragment ions are either formed unspecifically in the ion-transfer region (in-source CID) or selectively in the collision cell of an instrument dedicated to tandem MS (MS/MS). Both techniques have been used to establish mass-spectral libraries for screening purposes. The implementation of in-source CID libraries was hampered by the poor reproducibility of the mass spectra. For ESI and APCI, the energy characteristics of ion production and activation are much less well defined than for EI (a detailed discussion is available elsewhere [22]). The ions must cross a high-pressure region, where their internal energy can be modified, before they enter the mass-analyzer system. Consequently, in-source CID spectra may differ substantially, depending on the experimental conditions used (pressure, acceleration potentials, nature of the solution and the gas phases), which makes them difficult to compare [23, 24]. Nevertheless, transferable in-source CID libraries have been established by standardization of fragmentation with appropriate tuning compounds and by the application of a set of different collision energies for CID [24–26]. In comparison with in-source CID spectra, the reproducibility of MS/MS spectra is typically better, because only two experimental factors, the collision energy (CE) and the collision-gas pressure, can affect the appearance of a fragment-ion spectrum [25, 27, 28]. To increase the tolerance toward variability of these instrumental settings, application of a set of different collision energies for collection of reference spectra has been proposed [27, 29]. Established mass-spectral libraries are dedicated to the identification of drugs, pesticides, or explosives [30]. More or less all of these have been built by use of quadrupole, triple quadrupole, or ion-trap mass spectrometers [31–38]. A database was recently established by use of a quadrupole–quadrupole–TOF (QqTOF) mass spectrometer [39] and successfully applied to qualitative and quantitative analysis of toxicants in blood samples.

Valuable methods for identification of unknown compounds have been based either on accurate measurement of molecular mass with high-resolution mass spectrometers or on mass-spectral libraries usually established by use of low-resolution instruments. Our goal was to integrate these two approaches on an ESI–QqTOF mass spectrometer (QSTAR XL; Applied Biosystems, Foster City, CA, USA). Because of the accuracy of precursor-ion and fragment-ion mass measurements, the combined use of ESI–QqTOF-MS and ESI–QqTOF-MS/MS with mass-spectral library search should be a powerful tool for identification of unknown compounds. To prove this theory, a database containing 319 drugs and xenobiotics of forensic interest was

established. For each reference sample, product-ion spectra were collected at ten different CE values between 5 eV and 50 eV. Because of the lack of any kind of database feature within the operating software of the mass spectrometer, a library search algorithm was developed. A unique purity-control option was implemented in the software to enable identification of multiple contributors simultaneously present within a product-ion mass spectrum. This screening method was successfully applied to the characterization of forensic casework samples. Active agents and extenders in 37 seized drug samples and two autopsy samples were unequivocally identified.

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## Experimental

### Chemicals

Acetonitrile (HPLC gradient-grade), acetic acid (p.a.), and water (HPLC gradient-grade) were obtained from Fluka (Buchs, Switzerland). Drug standards from the laboratory's collection were used and were of the highest available purity.

### ESI–QqTOF-MS and ESI–QqTOF-MS/MS

ESI–MS was performed with a Qstar XL mass spectrometer (Applied Biosystems). A modified TurboIonSpray source (MicroTurboIonSpray) was used. The modifications included replacement of the PEEK tubing transfer line and of the stainless steel sprayer capillary by fused silica capillary tubing (transfer line 375  $\mu\text{m}$  o.d., 20  $\mu\text{m}$  i.d.; sprayer capillary 90  $\mu\text{m}$  o.d., 20  $\mu\text{m}$  i.d.; Polymicro Technologies, Phoenix, AZ, USA). Mass calibration and optimization of instrument settings were performed in the positive-ion mode by infusion, at a flow rate of 2.0  $\mu\text{L min}^{-1}$ , of a mixture of 1.0  $\text{mg L}^{-1}$  caffeine and 1.0  $\text{mg L}^{-1}$  reserpine dissolved in 0.05% aqueous acetic acid solution containing 50% (v/v) acetonitrile. The spray voltage was 4.0 kV. Gas flows of 1–3 arbitrary units (nebulizer gas) and 40 arbitrary units (turbo gas) were used. The temperature of ion-source gas 2 was adjusted to 200 °C. The accumulation time was 1.0 s and four time bins were summed. Mass spectra were collected in the range between 50 u and 700 u. For MS/MS, the Q1 resolution was set to unit resolution. The collision gas ( $\text{N}_2$ ) flow was set to 5 arbitrary units. CE values between 5 eV and 50 eV were used to generate product-ion mass spectra. Total-ion chromatograms and mass spectra were recorded on a personal computer by use of Analyst QS software (1.0, service pack 8, Applied Biosystems).

### Mass-spectral library of drugs

For collection of product-ion mass spectra characteristic of a drug, a solution of the drug in 0.05% aqueous acetic acid solution containing 50% (v/v) acetonitrile was prepared and infused directly into the mass spectrometer at a flow rate of

2.0  $\mu\text{L min}^{-1}$ . Depending on the ionization efficiency of the compound, solutions with concentrations in the range 0.10 to 10.0  $\text{mg L}^{-1}$  were used. Product-ion mass spectra were measured at ten different CE values. Starting at 5 eV, the CE was increased in steps of 5 eV to 50 eV. For each CE level, mass spectra collected over a period of at least 1.0 min were averaged.

### Computer-aided data interpretation

All calculations were performed on a personal computer under Windows XP operating system (1.7 GHz Pentium, 1.0 GB RAM). Measured MS/MS spectra were exported from the Analyst QS software (Applied Biosystems) as text files. Extracted files were processed. Data treatment included the deletion of any signal detected within a range of  $\pm 2$  u around the mass-to-charge ratio ( $m/z$ ) of the precursor ion. From files which served as reference spectra stored in the library, all signals were deleted which were not found in at least two spectra collected at different collision energies or which had relative signal intensities smaller than 0.01%. Automated library search was performed with a program written in ActivePerl 5.6.1 (Active State Corporation, Vancouver, BC, Canada).

### Characterization of real world drug samples

The sample set consisted of 37 drug samples seized by the Austrian police and of two samples collected during autopsies. The samples (100 ng–1.0 mg) were separately dissolved in 1.0 mL acetonitrile. Aliquots (250  $\mu\text{L}$ ) of the stock solutions were mixed with an equal volume of 0.1% aqueous acetic acid solution and analyzed by direct infusion into the mass spectrometer at a flow rate of 2.0  $\mu\text{L min}^{-1}$ . Mass spectra collected over a period of at least 1.0 min were averaged. Product-ion mass spectra were acquired at least at three different CE values. Results were checked by screening for common drugs by GC–MS using an HP6890 GC device with an HP5973 mass-selective detector (Agilent Technologies, Palo Alto, CA, USA) and an HP MS ChemStation with G1034C software version D01.00 including a mass-spectral library (Pfleger–Maurer–Weber, 3rd edn). The capillary column (DB-XLB, 30  $\text{m} \times 0.25$  mm i.d.  $\times 0.25$   $\mu\text{m}$  film thickness) was obtained from J&W Scientific (Folsom, CA, USA). Helium was used as carrier gas at a flow rate of 1.0  $\text{mL min}^{-1}$ . The injection volume was 1  $\mu\text{L}$  (splitless) and the injection port temperature was 250  $^{\circ}\text{C}$ . The column temperature was maintained constant at 50  $^{\circ}\text{C}$  for 1.0 min then programmed at 25  $^{\circ}\text{min}^{-1}$  to 150  $^{\circ}\text{C}$  and then at 10  $^{\circ}\text{min}^{-1}$  to 320  $^{\circ}\text{C}$  which was held for 8.0 min. The MS conditions were: full-scan mode,  $m/z$  50–800 u; EI mode, ionization energy, 70 eV; capillary direct interface temperature 310  $^{\circ}\text{C}$ .

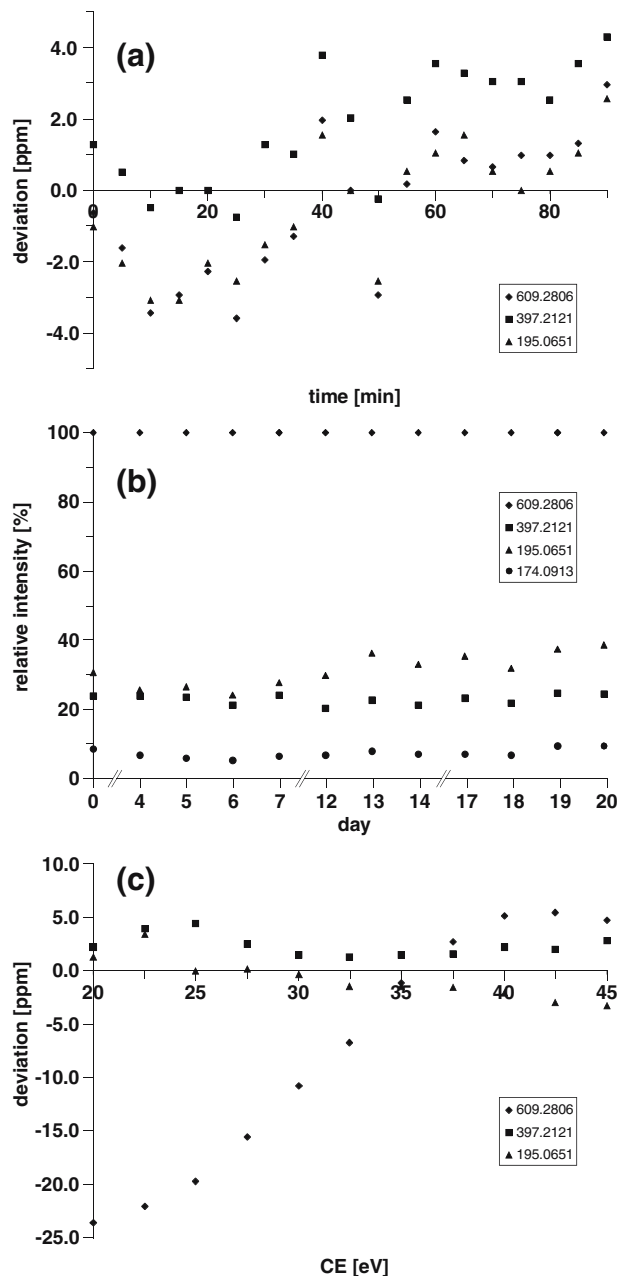
## Results and discussion

### Stability of mass spectrometric conditions

To identify compounds by library searching, the product-ion spectra obtained from a sample must be reliably assigned to the corresponding reference spectra stored in the database, even if a long period of time has elapsed between collection of the reference spectra and the acquisition of the sample spectra. The reproducibility of fragment ion formation and the accuracy and reproducibility of measurement of the fragment ion masses are the two factors with the greatest impact on the similarity between a sample spectrum and the corresponding reference spectrum.

The performance of the QqTOF instrument used were evaluated in a set of experiments in which reserpine was used as test compound. To check the stability of calibration over a time period reasonable for a single analysis, production spectra of reserpine were collected over a period of 90 min. Recalibration of the instrument was accomplished immediately before beginning of the experiment. For calibration, the  $m/z$  values of the precursor ion (609.2806 u) and the two most intense product ions of reserpine (195.0651 and 397.2121 u) were used. The CE was set to 35 eV. To simplify data treatment, 60 mass spectra at a time were averaged to produce a single spectrum. Accordingly, each data point reflected the mean mass deviation over one minute of analysis (Fig. 1a). Average mass deviations of  $-0.5$  ppm (609.2806 u), 1.8 ppm (397.2121 u), and  $-0.5$  ppm (195.0651 u), respectively, were measured. The observed maximum deviations were  $-3.6$  ppm and 4.3 ppm. Obviously, fragment ion masses can be reproducibly measured by use of the QqTOF mass spectrometer with accuracy surpassing that achievable with other types of instrument (quadrupole, triple quadrupole, and ion-trap mass spectrometers) commonly used as platforms for mass-spectral library approaches [40]. In the context of matching a sample spectrum with a reference spectrum, greater accuracy of fragment ion mass measurement reduces the possibility of false-positive assignment and increases the significance of a true positive match; overall this enhances the accuracy of compound identification by library search. For this reason, use of a high-resolution QqTOF may be favored over use of low-resolution instruments for building a library of mass spectra.

The repeatability of MS/MS fragmentation depends mainly on the reproducibility of the CE settings and on the stability of the collision gas flow. Fragmentation investigated on triple quadrupole instruments was shown to be reproducible, which enabled the identification of compounds even years after the creation of the spectral library [25]. To evaluate the reproducibility of fragment-ion formation on the QqTOF instrument, product-ion spectra of reserpine (CE 35 eV, collision gas flow 5 units) were collected on twelve different days over a period of twenty days (Fig. 1b). In each spectrum the highest signal intensity was observed for the precursor ion (609.2806 u). Relative



**Fig. 1** Evaluation of the stability of mass spectrometric settings in MS/MS mode: (a) stability of calibration over time; (b) stability of relative intensities over time; (c) effect of applied CE on the stability of the calibration. Sample, 1.0 mg L<sup>-1</sup> solution of reserpine in 0.05% aqueous acetic acid containing 50% (v/v) acetonitrile. The CE was set to 35 eV in (a) and (b)

intensities (mean±SD) of 31.5±4.8%, 22.9±1.5%, and 7.3±1.3% were obtained for the three most intense product ions (195.0651 u, 397.2121 u, and 174.0913 u, respectively). For all three product ions the relative standard deviations were smaller than 18% and, therefore, clearly within the tolerances of ±20% up to ±50% permitted by EU guidelines for LC-MS/MS [41]. The observed reproducibility of fragmentation clearly indicates that the QqTOF mass spectrometer is an appropriate platform for creation of a mass-spectral library.

## The database strategy

Mass spectrometer settings were tuned in full-scan mode by direct infusion, at a flow rate of 2.0 μL min<sup>-1</sup>, of a mixture of 1.0 ng μL<sup>-1</sup> caffeine and 1.0 ng μL<sup>-1</sup> reserpine dissolved in 0.05% aqueous acetic acid solution containing 50% (v/v) acetonitrile. The settings of the ion optics were optimized to ensure maximum ion transmission combined with a minimum amount of in-source fragmentation. Although the QqTOF instrument can, in principal, achieve a resolution exceeding 20,000 (FWHM) for the [M+H]<sup>+</sup> ion of reserpine, the resolution was set to approximately 16,000 to increase signal intensities in both MS and MS/MS modes. Tuning was rechecked at the beginning of every working day.

To build the spectral library 319 substances were used as reference samples. At the current stage of development the database mainly consists of drugs for therapeutic purposes and illicit substances. All investigated compounds are of forensic or toxicological interest, because they can cause severe or even fatal intoxication. The examined collection was classified according to the physiological effects of the agents. A few typical representatives of each group are listed in Table 1. A complete summary of the reference substances is available as a Table in the [Electronic Supplementary Material](#). To confirm the identity of the reference compounds, all samples were checked by GC-MS. For ESI-MS experiments, samples were dissolved in 0.05% aqueous acetic acid solution containing 50% (v/v) acetonitrile. Because of the acidic conditions, only minor alkali adduct formation was observed. Depending on substance-specific mass spectrometric detectability, solutions of concentration in the range 0.10 to 10.0 mg L<sup>-1</sup> were used as reference samples.

As the QqTOF mass spectrometer used is operated such that parent ion selection in Q, parent ion dissociation in q, and product ion mass analysis in TOF occur sequentially in space as ions traverse the instrument, the MS-MS process is tandem-in-space. Hence, in comparison with tandem-in-time instruments (ion-trap mass spectrometers) variation of the CE in QqTOF-MS/MS should have a significant effect on the appearance of fragment-ion mass spectra [27, 42]. To increase the tolerance of the library toward the applied CE, product-ion spectra of reference compounds were acquired at ten different CE values between 5 eV and 50 eV. As expected, the applied CE affected the number of fragments detected and the measured relative signal intensities. Spectra indicative of low, medium, and high levels of fragmentation were observed. Only a slight effect on measured *m/z* values was observed. The deviations of the *m/z* values of the two most intense product ions of reserpine (195.0651 u and 397.2121 u) from the theoretical values were within ±5.5 ppm, irrespective of the CE used (Fig. 1c). Larger errors were observed for the precursor ion (609.2806 u), most probably because of detector saturation caused by high signal intensities at low CE values [10].

Because of saturation effects and to avoid false positive matches of the precursor ion with product ions from alternative compounds, all signals within a 4.0-u window

**Table 1** Summary of major substance groups in the spectral library, including a few examples

Groups	N	Examples
Sympathetic acting drugs	33	Adrenalin, celiprolol, prazosine, propranolol
Antibiotics/antiseptics	32	Chloroquine, metronidazole, rifampicin, tetracycline
CNS affecting drugs	27	Carbamazepine, clozapine, haloperidol, thioridazine
Sleeping drugs	23	Chlordiazepoxide, diazepam, flunitrazepam, methaqualone
Psychostimulants	23	Amphetamine, cocaine, MDMA, phentermine
Opiates/opioids	20	Buprenorphine, codeine, methadone, morphine
Antidepressants	19	Amitriptyline, citalopram, doxepin, maprotiline
Antihistamines	18	Chlorpheniramine, diphenhydramine, meclizine, pheniramine
Analgesics	17	Diclofenac, naproxen, acetaminophen, phenacetin
(Local) anaesthetics	12	Lidocaine, etomidate, ketamine, procaine
Vascular dilators	11	Cinnarizine, diltiazem, dipyridamole, verapamil
Diuretics	9	Amiloride, chlorthalidone, hydrochlorothiazide, triamterene
Parasympathetic acting drugs	9	Atropine, pirenzepine, pyridostigmine, scopolamine
Bronchial/antitussive drugs	9	Emetine, eprazinone, diprophyllyne, theophylline
Oral antidiabetics	6	Glibenclamide, gliclazide, gliquidone, tolbutamide
Others	51	Dihydroergotamine, mescaline, phencyclidine, reserpine, THC

Compounds are classified in accordance with their physiological effects.

around the  $m/z$  of the precursor ion were deleted from the reference spectra obtained. To increase specificity further, all signals found in a reference spectrum that could not positively contribute to precursor identification were eliminated. Only those signals with a relative intensity above 0.01%, and which were observed twice or more often within a collection of substance-specific product-ion spectra were regarded as suitable for identification. The remaining species were deleted from the reference spectra. As a result of these data-processing steps some spectra collected at low CE values no longer contained product ion signals. Deletion of these blanks reduced the number of stored reference spectra from 3210 to 3023. For detection of possible duplicates, all reference spectra stored in the database were matched with each other.

#### Development of the database search algorithm

For routine use of a spectral database an efficient algorithm for similarity search is obligatory. As the software package provided with the mass spectrometer used (Analyst QS 1.0, service pack 8, Applied Biosystems) did not include any kind of library-management function, we developed such a software feature. The principle steps of the algorithm are outlined in Fig. 2. The measured product-ion spectrum of an unknown substance was the input. To avoid false positive matches of the precursor ion with product ions of other substances, all signals within a 4.0 u window around the  $m/z$  of the precursor ion are deleted from the input spectrum (Fig. 2, step 1). The treated spectrum is compared with all the mass spectra stored in the library (Fig. 2, step 3). Comparison of the input spectrum with a single reference spectrum yields the “number of matching fragments” ( $mf$ ). For a match, the difference between the

$m/z$  values must be smaller than 0.01 u. Next, the match probability ( $mp$ ) is calculated by use of the equation:

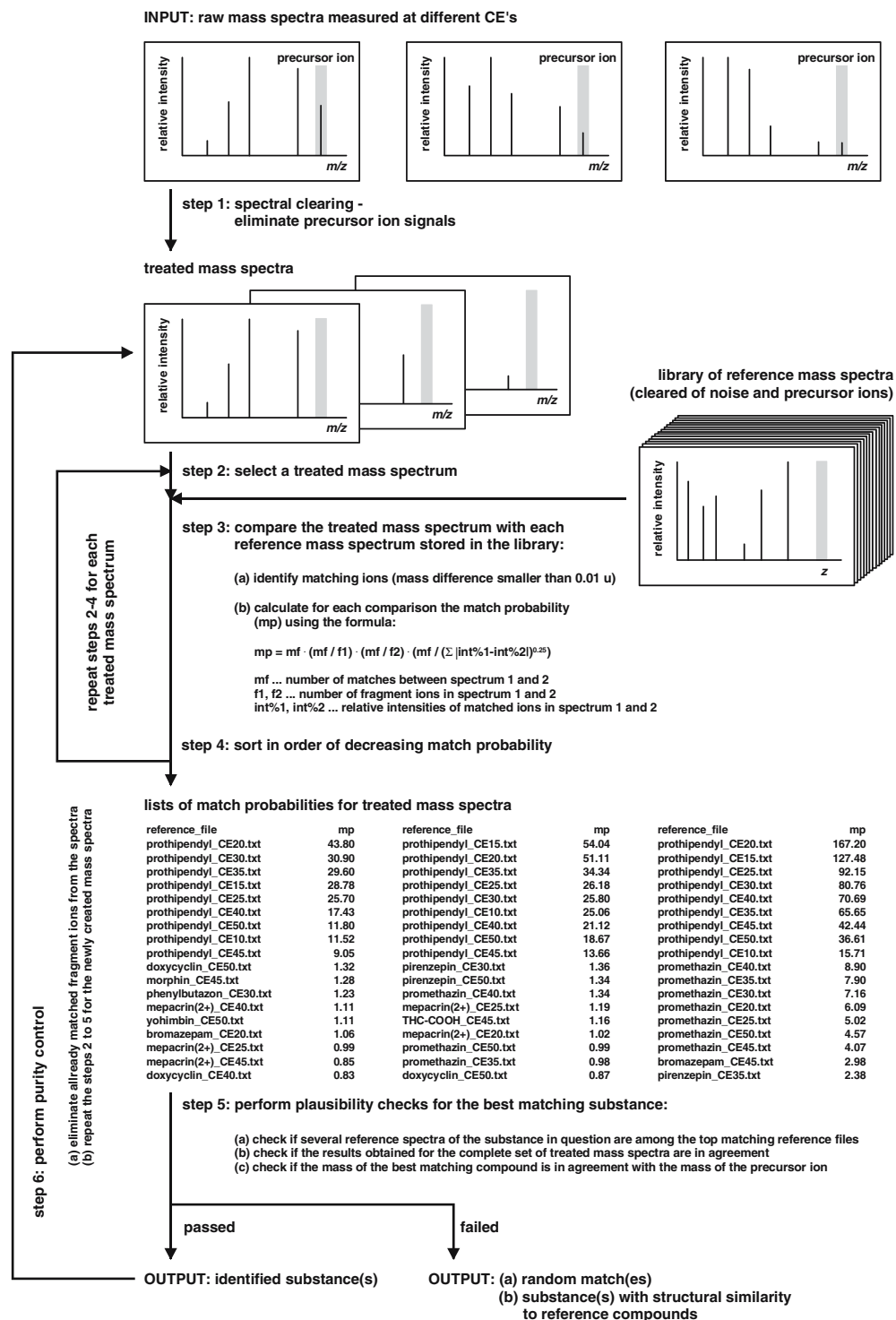
$$mp = mf \times (mf/f1) \times (mf/f2) \times \left( mf / \left( \sum |int\%1 - int\%2| \right)^{0.25} \right)$$

where  $f1$  and  $f2$  are the number of fragments in the input and in the reference, respectively. Accordingly,  $mf/f1$  and  $mf/f2$  reflect the coverage of the input spectrum by the reference spectrum and vice versa.  $\sum |int\%1 - int\%2|$  represents the sum of the intensity differences observed for matching fragments. As the relative intensities can vary to some extent, less weight was assigned to the peak intensities by use of the exponent 0.25 [43]. It is obvious that  $mp$  becomes larger with increasing correlation of two spectra.

The output of the similarity search algorithm is a list which is sorted in order of decreasing  $mp$  (Fig. 2, step 4). The substance with the highest  $mp$  is regarded as representing the unknown compound. To avoid a false positive assignment, several reference spectra corresponding to the best matching substance should be among the top matching reference files (Fig. 2, step 5a). If possible, the identity of the unknown compound should be confirmed by results obtained from product-ion spectra collected at several different CE (Fig. 2, step 5b). To achieve final confirmation, the monoisotopic mass of the best matching substance must be in accordance with the measured monoisotopic mass of the precursor ion (Fig. 2, step 5c).

Instead of being the final criterion, the conformity of the monoisotopic masses could have been used as selection criterion before performing the library searching. Such an

**Fig. 2** Outline of the steps involved in the identification of compounds by automated spectral library search



approach would help to reduce computing time and power, because only a limited number of reference spectra must then be matched to the spectra of the sample. On the other hand such a strategy would disregard any information about the quality of the obtained “best match” in comparison with improper matches, or about compounds with some structural similarity to the unknown substance.

### Characterization of spectral mixtures

The database currently contains 25 pairs of isobaric substances and even more compounds with nearly identical molecular masses that can barely be resolved from each other with a quadrupole filter used for isolation of precursor ions. Because of the occurrence of spectral interference two or more species can simultaneously contribute to a product-ion spectrum. Depending on the

concentration ratios, the ionization efficiencies, and the fragmentation behavior of the components, all or, more likely, only a limited number of species may be recognized within a multi-contributor mass spectrum via a single-stage library search. Consequently, toxicologically important compounds, and thus either illicit drugs or, even worse, drugs responsible for severe intoxication, may be overlooked. For unequivocal detection of multiple contributors within a product-ion spectrum, a purity-control option was added to the search algorithm (Fig. 2, step 6): All product ions that have been assigned to the best matching compound of the previous stage of library search are subtracted from the measured product-ion spectrum. The library search procedure is then repeated with the purified spectrum to assign an additional contributor. Examples demonstrating the value of the purity-control feature are given in the following paragraphs.

Bromazepam and clonazepam are benzodiazepine tranquilizers with sedative and relaxing effects. Benzodiazepines are widely used to treat sleep disorders and anxiety states and are, therefore, often observed in routine forensic casework. The calculated  $m/z$  values of  $[M+H]^+$  for bromazepam and clonazepam are 316.0079 u and 316.0483 u, respectively. In MS mode, the resolving power of the TOF analyzer enabled resolution of the two species (Fig. 3a). Because of the lower resolving power of the quadrupole filter both species were simultaneously fragmented in MS/MS mode and so the product-ion spectrum obtained represented a mixture of two compound-specific spectra (Fig. 3b). Using our library search algorithm clonazepam was identified as the primary hit. By use of the two subsequent steps—spectral purification and repeated library search—the second contributor was identified as bromazepam. For visual inspection of the results obtained, the best matching reference spectra were aligned with the input spectrum in a reflected arrangement along the abscissa (Fig. 3b,c).

Another example of spectral interference is shown in Fig. 3d. The first isotope peak of 3,4-methylenedioxy-*N*-methylamphetamine (MDMA, 195.1146 u) and the mono-isotopic peak of caffeine (195.0876 u) have almost identical  $m/z$  values. MDMA is better known as ecstasy, a designer drug that mainly causes mild euphoria, increased sociability, and increased blood pressure, pulse, and body temperature. Ecstasy is commonly sold in colored tablets that can contain several other compounds besides MDMA. Caffeine is very often added as extender. Analysis of seized ecstasy tablets, most often meaning analysis of a mixture of MDMA and caffeine, is therefore a common task in forensic laboratories. The mass spectrum obtained from analysis of a mixture containing 1.0 mg L<sup>-1</sup> MDMA and 0.10 mg L<sup>-1</sup> caffeine is depicted in Fig. 3d. MDMA was unequivocally identified by library search for the product-ion spectrum obtained from its monoisotopic signal (194.1175 u). Identification of caffeine in the presence of excess MDMA would have been a difficult task without computer-aided product-ion spectrum interpretation. In full MS mode the monoisotopic peak of caffeine and the first isotopic peak of MDMA overlapped. The presence of a

second species besides MDMA was indicated by slight peak broadening, which can easily be overlooked. The production spectrum obtained (Fig. 3e) was a mixture of the signals from MDMA and caffeine. MDMA was assigned as the best matching compound. In the purified mass spectrum, caffeine was unequivocally detected (Fig. 3f).

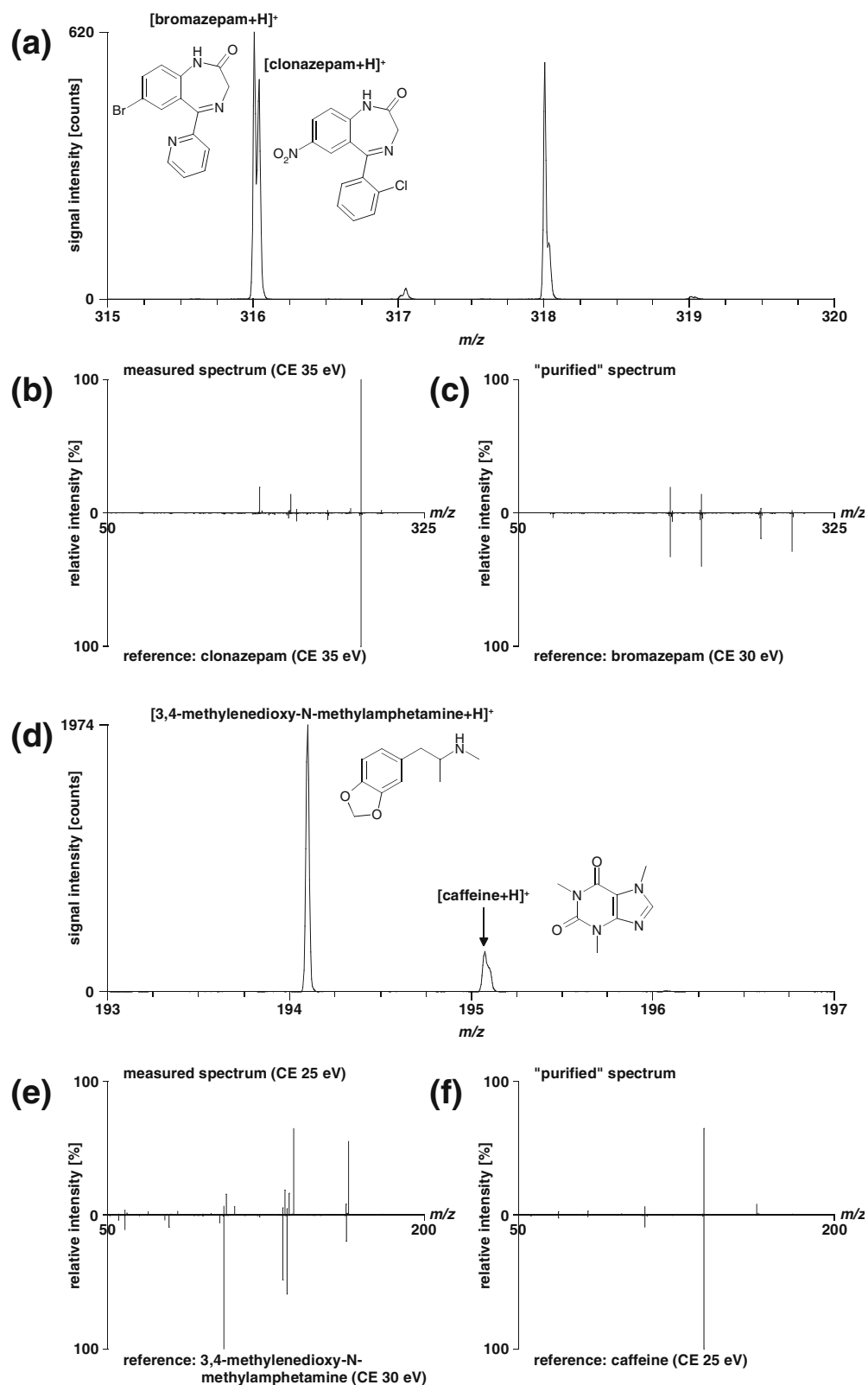
The simultaneous presence of two species with identical empirical formulas is the worst case of mass spectral interference. Neither the measured monoisotopic mass nor the observed isotopic peak pattern indicates the presence of a mixture. Discrimination of two isobaric compounds is only feasible with the aid of structure-specific information obtained from MS/MS experiments. To evaluate the capability of the MS/MS approach to differentiate two isobaric species the two substances isoaminile and xylometazoline (Fig. 4a and b) were used as test compounds. Isoaminile, an opioid, is manufactured synthetically and is used as an antitussive drug. Xylometazoline is a sympathomimetic substance which mainly causes a constriction of blood vessels. It is used for local treatment of rhinitis. Solutions of the pure compounds and mixtures of different ratios of isoaminile and xylometazoline (99:1, 90:10, 80:20 down to 20:80, 10:90, 1:99) were prepared and analyzed (Fig. 4). No evidence of a second contributor was found in the fragment-ion spectra of the pure compounds. Because of the high sensitivity of the mass spectrometric detection system used and the quality of the library search algorithm, both compounds were unequivocally detectable in all mixtures. “Raw” and subsequently “purified” product-ion spectra obtained from mixtures containing only 1.0% of the minor component are depicted in Fig. 4c–f. Although huge signal intensity differences were observed between the purified spectra and the matching reference spectra, and although several unspecific signals were present within the purified spectra (Fig. 4d and f), the search algorithm unequivocally assigned the correct compounds, which clearly proves the efficiency of the algorithm.

#### Concentration dependence of library-search results

To evaluate the concentration dependence of the library-search results, ten different solutions of reserpine with concentrations between 15.6 µg L<sup>-1</sup> and 8.0 mg L<sup>-1</sup> were analyzed by direct infusion at a flow rate of 2.0 µL min<sup>-1</sup>. In both full-scan MS and full-scan MS/MS modes (scan range for both 50–700 u) 15.6 µg L<sup>-1</sup> was the lowest detectable concentration of reserpine. Fragmentation was accomplished at a CE of 35 eV, and all scans acquired over a time period of 1.0 min were averaged. Before each measurement the system had been recalibrated with a 0.50 mg L<sup>-1</sup> solution of reserpine (CE 35 eV, 195.0651 u, 397.2121 u, 609.2806 u).

Within the linear dynamic range of the mass spectrometer the mass measurement error was typically below 6.2 ppm. Deviations up to 55.3 ppm were observed at concentrations equal to or greater than 2.0 mg L<sup>-1</sup>, because of detector saturation, and at concentrations near the detection limit, because of problems with the peak shape. Nevertheless,

**Fig. 3** Characterization of mixtures. Part 1 (a–c): two species with nearly identical  $m/z$  values. Part 2 (d–f): two species for which the first isotope peak of one species has almost the same  $m/z$  value as the monoisotopic peak of the second species. Samples: (a–c) mixture of bromazepam ( $1.0 \text{ mg L}^{-1}$ ) and clonazepam ( $1.0 \text{ mg L}^{-1}$ ) dissolved in 0.05% aqueous acetic acid containing 50% (v/v) acetonitrile; (d–f) mixture of 3,4-methylenedioxy-*N*-methylamphetamine (MDMA,  $1.0 \text{ mg L}^{-1}$ ) and caffeine ( $0.10 \text{ mg L}^{-1}$ ) dissolved in 0.05% aqueous acetic acid containing 50% (v/v) acetonitrile



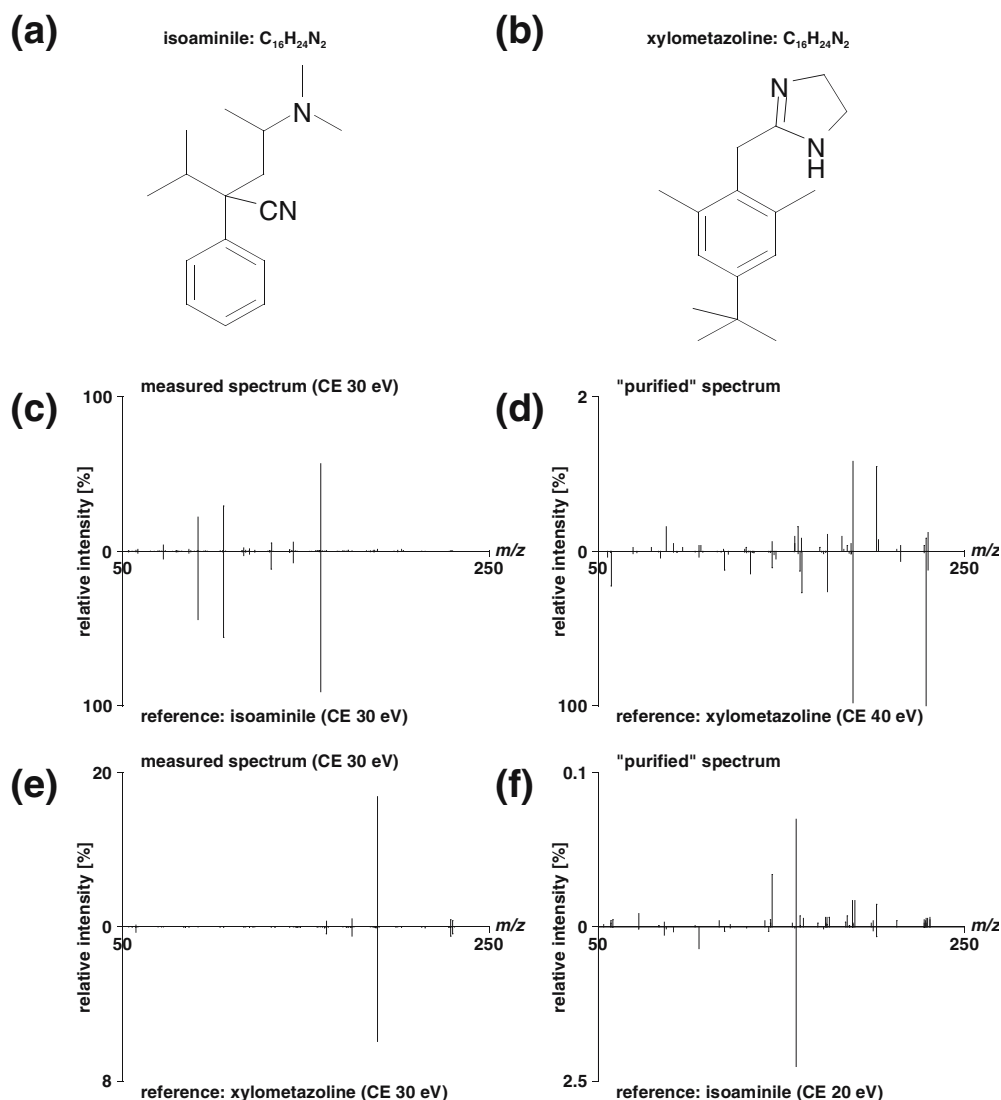
reserpine was always unequivocally assigned as the best matching compound, which suggests that the concentration of the analyzed compound has no decisive effect on the library-search result.

#### Application to real drug samples

The created database and the search algorithm developed were applied to the analysis of 39 samples commonly observed in routine forensic casework. All samples were



**Fig. 4** Characterization of mixtures. Part 3: two species with identical empirical formulas. Samples: mixtures of isoaminile and xylometazoline dissolved in 0.05% aqueous acetic acid containing 50% (v/v) acetonitrile; (c, d) 0.99 mg L<sup>-1</sup> isoaminile and 0.01 mg L<sup>-1</sup> xylometazoline; (e, f) 0.01 mg L<sup>-1</sup> isoaminile and 0.99 mg L<sup>-1</sup> xylometazoline



characterized both by GC-MS and by direct-infusion MS and subsequent MS/MS experiments (Table 2). Direct-infusion MS has the clear advantage that the time and effort required for sample preparation steps such as extraction and chromatography can be saved. Typically, characterization of a sample, including collection of spectra and the execution of multiple library search runs, was accomplished within a few minutes. Most of the total analysis time can be used for MS/MS experiments. Collected fragment-ion spectra may therefore be of better quality than those obtained, e.g., by information-dependent acquisition-mediated LC-MS screening [39]; this increases the probability of a positive match with a spectrum in a mass-spectral library. In the absence of a chromatographic separation step, however, matrix effects may become a concern. Thus, the applicability of the described approach to the analysis of complex biological samples, for example tissues, blood, urine, and feces, is restricted.

Two samples were collected during autopsies. One was a swab of turquoise residues found in the corners of the mouth of one patient (Table 2, sample 1); the other was apparent

drug residues in the stomach contents of another patient (Table 2, sample 2). It is important to note that in both cases macroscopic identification by classification of drug characteristics was impossible. The mass spectrometric information obtained enabled identification of prothipendyl (a central nervous system-active drug, mainly used for sedative purposes) in sample 1 and diclofenac (a nonsteroidal anti-inflammatory drug, often used as a painkiller) in sample 2 (Table 2).

The remaining 37 samples were a variety of seized illicit drug materials. Fourteen samples were suspected to be cocaine, eight to be heroine, ten to be amphetamines or designer drugs, and five samples were apparently hashish or marijuana. In many cases the samples were only partially soluble in acetonitrile, enabling separation of the active agents from inorganic additives and sugars.

Among the suspected cocaine samples one was found to be lidocaine, a substance often used as extender in cocaine preparations, because its local anesthetic effect mimics that of cocaine (Table 2, sample 3). The other samples were proven to be cocaine. Cocaine and its hydrolysis product

**Table 2** Summary of compounds identified in 39 real samples

Sample	Description	Compounds identified by ESI-MS and ESI-MS/MS	GC-MS
1	Turquoise residues from mouth	Prothipendyl	Prothipendyl
2	White mush from stomach	Diclofenac	Diclofenac
3	White powder	Lidocaine	Lidocaine
4	Two white pressed blocs	Cocaine, benzoylecgonine, truxillines	Cocaine, benzoylecgonine, methylecgonine, benzoic acid, cinnamoylcocaine
5	Crude white powder	Cocaine, benzoylecgonine, truxillines	Cocaine, benzoylecgonine, methylecgonine, benzoic acid, cinnamoylcocaine, tropacocaine, trimethoxycocaine
6	White powder	Cocaine, benzoylecgonine, truxillines	Cocaine, benzoylecgonine, methylecgonine, benzoic acid, cinnamoylcocaine, tropacocaine, trimethoxycocaine
7	White powder with lumps	Cocaine, benzoylecgonine, truxillines	Cocaine, benzoylecgonine, methylecgonine, benzoic acid, cinnamoylcocaine, tropacocaine, trimethoxycocaine
8	Beige powder with lumps	Cocaine, benzoylecgonine, truxillines	Cocaine, benzoylecgonine, methylecgonine, benzoic acid, cinnamoylcocaine, tropacocaine, trimethoxycocaine
9	Beige powder	Cocaine, benzoylecgonine, truxillines	Cocaine, benzoylecgonine, methylecgonine, benzoic acid, cinnamoylcocaine, tropacocaine, trimethoxycocaine, benzocain
10	White powder	Cocaine, benzoylecgonine, truxillines	Cocaine, benzoylecgonine, methylecgonine, benzoic acid, tropacocaine, trimethoxycocaine
11	White pressed round blocs	Cocaine, benzoylecgonine, truxillines, caffeine	Cocaine, benzoylecgonine, methylecgonine, benzoic acid, cinnamoylcocaine, tropacocaine, trimethoxycocaine, caffeine
12	Beige powder with lumps	Cocaine, benzoylecgonine, truxillines, phenacetin	Cocaine, benzoylecgonine, methylecgonine, benzoic acid, cinnamoylcocaine, tropacocaine, trimethoxycocaine, phenacetin
13	One small packet with white powder (body packer)	Cocaine, benzoylecgonine, truxillines, diltiazem	Cocaine, benzoylecgonine, methylecgonine, benzoic acid, cinnamoylcocaine, trimethoxycocaine, diltiazem
14	White powder	Cocaine, benzoylecgonine, truxillines, chlorprothixene, phenacetin	Cocaine, benzoylecgonine, methylecgonine, benzoic acid, cinnamoylcocaine, tropacocaine, trimethoxycocaine, phenacetin, chlorprothixene
15	White lumps with some powder	Cocaine, benzoylecgonine, truxillines, diltiazem, phenacetin, lidocaine	Cocaine, benzoylecgonine, methylecgonine, benzoic acid, cinnamoylcocaine, trimethoxycocaine, diltiazem, phenacetin, lidocaine, benzocaine, caffeine
16	Off-white powder	Cocaine, benzoylecgonine, truxillines, diltiazem, phenacetin, lidocaine	Cocaine, benzoylecgonine, methylecgonine, benzoic acid, cinnamoylcocaine, tropacocaine, trimethoxycocaine, benzocaine, diltiazem, phenacetin, lidocaine
17	Beige-brownish powder	Heroin, 6-acetylmorphine, acetylcodeine, papaverine, noscapine, caffeine, acetaminophen	Heroin, 6-acetylmorphine, acetylcodeine, papaverine, noscapine, meconin, hydrocotamine, caffeine, acetaminophen
18	Beige-brownish powder	Heroin, 6-acetylmorphine, acetylcodeine, papaverine, noscapine, caffeine, acetaminophen	Heroin, 6-acetylmorphine, acetylcodeine, papaverine, noscapine, meconin, hydrocotamine, caffeine, acetaminophen
19	Beige-brownish powder	Heroin, 6-acetylmorphine, acetylcodeine, papaverine, noscapine, caffeine, acetaminophen	Heroin, 6-acetylmorphine, acetylcodeine, papaverine, noscapine, meconin, hydrocotamine, caffeine, acetaminophen

Table 2 (continued)

Sample	Description	Compounds identified by ESI-MS and ESI-MS/MS	GC-MS
20	Beige-brownish powder	Heroin, 6-acetylmorphine, acetylcodeine, papaverine, noscapine, caffeine, acetaminophen	Heroin, 6-acetylmorphine, acetylcodeine, papaverine, noscapine, meconin, hydrocotamine, caffeine, acetaminophen
21	Brownish powder	Heroin, 6-acetylmorphine, acetylcodeine, papaverine, noscapine, caffeine	Heroin, 6-acetylmorphine, acetylcodeine, papaverine, noscapine, meconin, acetylthebaol, hydrocotamine, caffeine
22	Brownish powder	Heroin, 6-acetylmorphine, acetylcodeine, papaverine, noscapine, caffeine, acetaminophen, alprazolam, phenolphthalein	Heroin, 6-acetylmorphine, acetylcodeine, papaverine, noscapine, meconin, thebaol, hydrocotamine, caffeine, acetaminophen, alprazolam, phenolphthalein
23	Brownish powder	Heroin, 6-acetylmorphine, acetylcodeine, papaverine, noscapine, caffeine, acetaminophen, alprazolam, phenolphthalein	Heroin, 6-acetylmorphine, acetylcodeine, papaverine, noscapine, meconin, thebaol, hydrocotamine, caffeine, acetaminophen, alprazolam, phenolphthalein
24	Brownish powder	Heroin, 6-acetylmorphine, acetylcodeine, papaverine, noscapine, caffeine, acetaminophen	Heroin, 6-acetylmorphine, acetylcodeine, papaverine, noscapine, meconin, caffeine, acetaminophen
25	Yellow tablets "Tp" stamping, Ø: 8 mm, th: 3 mm, wt: 218 mg, no breakline	3,4-Methylenedioxyamphetamine	3,4-Methylenedioxyamphetamine
26	Yellow tablets "Tp" stamping, Ø: 8 mm, th: 3 mm, wt: 218 mg, no breakline	3,4-Methylenedioxyamphetamine	3,4-Methylenedioxyamphetamine
27	Yellow tablets "Tp" stamping, Ø: 8 mm, th: 3 mm, wt: 218 mg, no breakline	3,4-Methylenedioxyamphetamine	3,4-Methylenedioxyamphetamine
28	Yellow tablets "Tp" stamping, Ø: 8 mm, th: 3 mm, wt: 218 mg, no breakline	3,4-Methylenedioxyamphetamine	3,4-Methylenedioxyamphetamine
29	Yellow tablets "Tp" stamping, Ø: 8 mm, th: 3 mm, wt: 218 mg, no breakline	3,4-Methylenedioxyamphetamine	3,4-Methylenedioxyamphetamine
30	White powder	3,4-Methylenedioxy- <i>N</i> -methylamphetamine	3,4-Methylenedioxy- <i>N</i> -methylamphetamine
31	Beige tablets, "STARDUST" stamping, Ø: 8 mm, th: 3 mm, wt: 240 mg, 1 breakline	3,4-Methylenedioxy- <i>N</i> -methylamphetamine, 3,4-methylenedioxy- <i>N</i> -ethylamphetamine	3,4-Methylenedioxy- <i>N</i> -methylamphetamine, 3,4-methylenedioxy- <i>N</i> -ethylamphetamine
32	Yellowish powder	3,4-Methylenedioxy- <i>N</i> -methylamphetamine, amphetamine, caffeine	3,4-Methylenedioxy- <i>N</i> -methylamphetamine, amphetamine, caffeine
33	Yellowish powder	3,4-Methylenedioxy- <i>N</i> -methylamphetamine, amphetamine, caffeine	3,4-Methylenedioxy- <i>N</i> -methylamphetamine, amphetamine, caffeine
34	Yellowish powder	3,4-Methylenedioxy- <i>N</i> -methylamphetamine, amphetamine, caffeine	Amphetamine, caffeine
35	Hashish, 1 clump	$\Delta$ -9-Tetrahydrocannabinol	$\Delta$ -9-Tetrahydrocannabinol, cannabidiol, cannabidiol, cannabigerol, cannabidivariol
36	Hashish, 3 plates and 2 chunks	$\Delta$ -9-Tetrahydrocannabinol	$\Delta$ -9-Tetrahydrocannabinol, cannabidiol, cannabidiol, cannabigerol, cannabidivariol
37	Hashish, 32 plates	$\Delta$ -9-Tetrahydrocannabinol	$\Delta$ -9-Tetrahydrocannabinol, cannabidiol, cannabidiol, cannabigerol, cannabidivariol
38	Marihuana	$\Delta$ -9-Tetrahydrocannabinol	$\Delta$ -9-Tetrahydrocannabinol, cannabidiol, cannabidiol, cannabigerol, cannabidivariol
39	Marihuana	$\Delta$ -9-Tetrahydrocannabinol	$\Delta$ -9-Tetrahydrocannabinol, cannabidiol, cannabidiol, cannabigerol, cannabidivariol

benzoylecgonine were identified by use of both ESI-MS and GC-MS (Table 2, samples 4–16). The thermally labile truxillines were detected in their native form with the soft-ionization technique ESI whereas, as a result of thermal decomposition, the corresponding monomer cinnamoylcocaine was observed by use of GC-MS. Other compounds that are commonly part of cocaine preparations—methyl-ecgonine, tropacocaine, trimethoxycocaine and benzoic acid—were found by use of GC-MS only. It is possible that low ionization efficiencies combined with ion-suppression effects were responsible for the inability to detect these species by use of ESI-MS. In some samples extender substances, for example phenacetin, caffeine, diltiazem, or chlorprothixene, were identified by the library-search approach, and confirmed in all but one instance by GC-MS (Table 2, sample 15). Caffeine in sample 15 and benzocaine in samples 15 and 16 were detected solely by use of GC-MS. These species were probably not detectable by direct infusion ESI-MS because of the small amounts

present in the drug samples and because of ion-suppression effects.

Samples 17 to 24 (Table 2) were suspected heroin preparations. Heroin is produced semi-synthetically by acetylation of raw opium extracts. Thus, every heroin sample contains a variety of alkaloids from the opium poppy plant. Some were detected by ESI-MS. The full-scan mass spectrum obtained from analysis of sample 22 (Table 2) is depicted in Fig. 5a. Nine very intense peaks were detected. To identify these species, MS/MS experiments and subsequent library searches were performed. The acquired product-ion mass spectra and the best-matching library files are shown in Fig. 5b–i. Seven different compounds (heroin, 6-acetylmorphine, papaverine, noscapine, caffeine, acetaminophen, and alprazolam) were unequivocally identified. Codeine was assigned to the species with a measured  $m/z$  value of 342.1638 u by library search (Fig. 5h). Similar results were obtained with mass spectra acquired at two more CE (35 eV and 40 eV).

**Fig. 5** Application of the library search strategy to characterization of a seized drug sample. The sample was 50 ng of sample 22 dissolved in 0.05% aqueous acetic acid containing 50% (v/v) acetonitrile

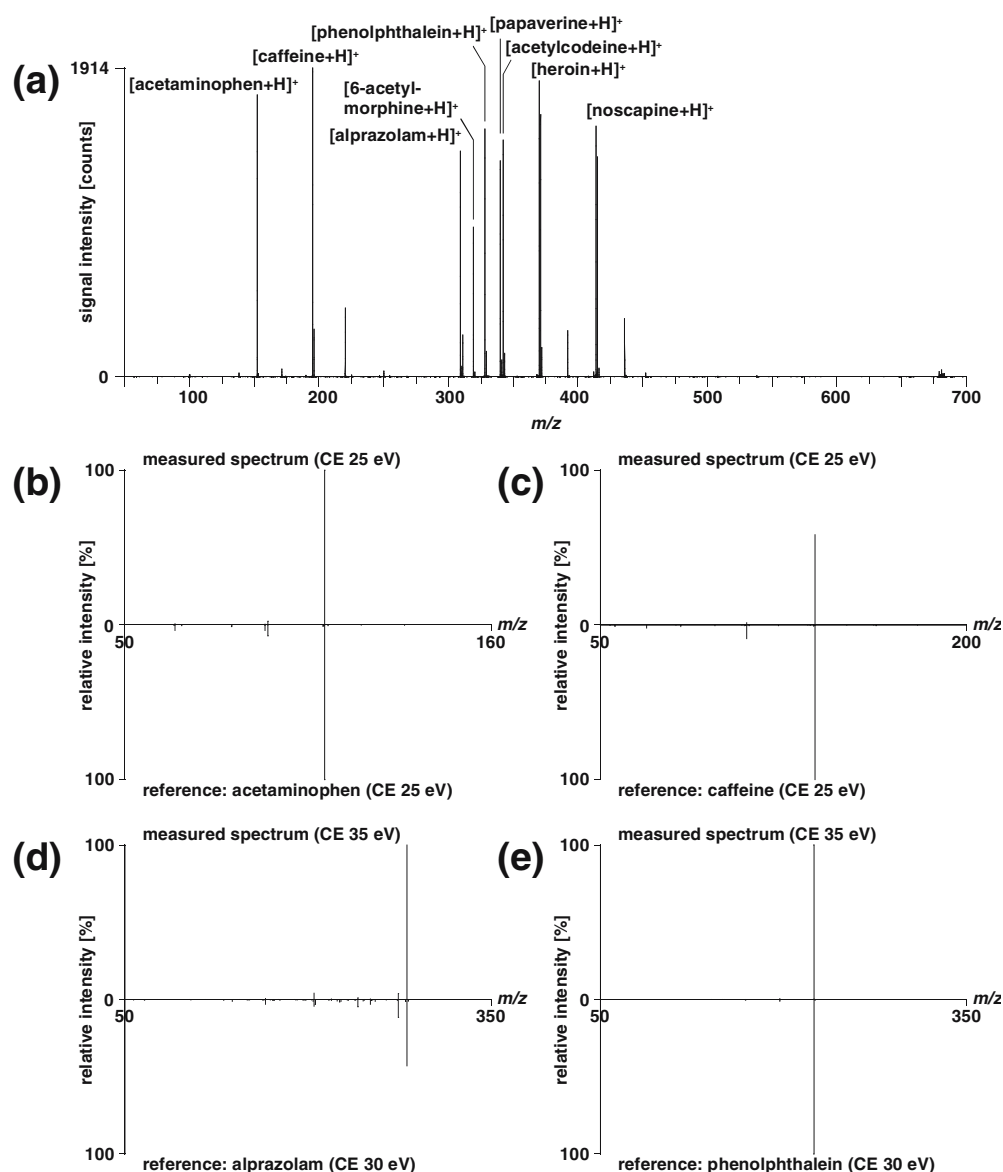
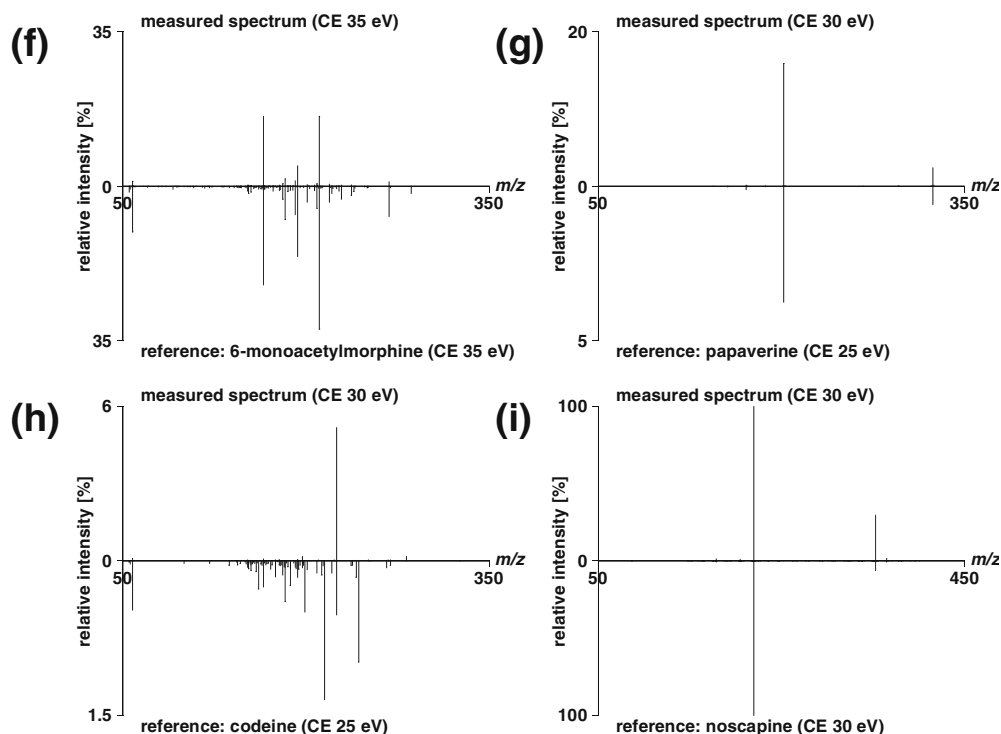


Fig. 5 (continued)



Nevertheless, as the measured monoisotopic mass did not agree with the theoretical  $m/z$  of [codeine+H]<sup>+</sup> (300.1594 u), the potential match “codeine” was ruled out by the final plausibility check implemented in the search algorithm. The calculated mass difference of 42.0042 u suggested addition of an acetyl group to codeine (theoretical mass difference: 42.0105 u). Hence, the unknown compound was tentatively identified as acetylcodeine. The presence of acetylcodeine in the drug sample was confirmed by GC–MS (Table 2, sample 22). This example clearly proves the applicability of the library search approach to the identification of derivatized compounds or metabolites.

Besides acetylcodeine, a second component (measured  $m/z$ : 319.0988 u) was unidentifiable in this specimen. A search within the library did not return any reasonable result. By GC–MS analysis, phenolphthalein was detected as impurity (Table 2, sample 22). As the theoretical  $m/z$  of [phenolphthalein+H]<sup>+</sup> (319.0964 u) correlated well with the measured mass, the unknown compound was tentatively identified as phenolphthalein. To prove the identity, phenolphthalein-specific product-ion spectra were added to the library. A search within the extended database enabled unequivocal identification of the unknown compound as phenolphthalein (Fig. 5e). This example proves the high flexibility of the proposed screening approach. Substance-specific data is obtainable from compounds, which are not part of an existing library. Hence, a subsequent extension of the library can lead to the identification of these unknowns. Hence, even uncommon or unexpected substances, which would have been ignored by any kind of targeted screening technique, are identifiable with the library-search approach.

Low and medium-abundance species observed in ESI–MS analysis of sample 22 (Fig. 5a) were product ions

produced by in-source fragmentation and alkali metal adducts.

In all suspected heroin samples (Table 2, samples 17–24) the opiates heroin, 6-acetylmorphine, acetylcodeine, papaverine, and noscapine, and the extender substances caffeine, acetaminophen, alprazolam, and phenolphthalein were found by ESI–MS and confirmed by GC–MS. Traces of meconine, (acetyl)thebaol, and hydrocotarnine were detected solely by GC–MS.

Samples 25 to 34 (Table 2) were seized designer drugs containing amphetamine derivatives. The results obtained by ESI–MS were, in all except one instance, confirmed by GC–MS. In sample 34 traces of MDMA were found by ESI–MS that were not detectable by GC–MS.

Hashish and marijuana preparations contain the cannabinoid  $\Delta$ -9-tetrahydrocannabinol (THC) as the main psychoactive component. This compound was unequivocally identified by ESI–MS in all the samples analyzed (Table 2, samples 35–39). Other cannabinoids produced by the cannabis hemp plant were detected by GC–MS only.

## Conclusions

ESI–QqTOF-MS and ESI–QqTOF-MS/MS combined with mass-spectral library search is a powerful tool for “general unknown screening”. The proven accuracy of the measurement of precursor ion masses and of the corresponding fragment-ion masses (mass measurement error is typically in the range of a few ppm) ensures a low probability of false positive matches. Hence, in comparison with the low-resolution instruments commonly used for mass-spectral

library search approaches, greater identification accuracy can be achieved by use of TOF instruments.

The established mass-spectral library contains production spectra of 319 reference compounds. For each compound reference spectra were collected at ten different CE values between 5 eV and 50 eV. A library search algorithm was developed that includes a unique purity control option. This option was found to be advantageous for the identification of several compounds in a single product-ion mass spectrum.

The library search approach was applied to 39 real samples. GC-MS was used to check the results obtained. Comparison of the results reveals that ESI-MS and GC-MS are both valuable tools for characterization of drugs. Nevertheless, none of the two techniques was able to detect and identify all the different compounds present in the samples. Depending on their chemical properties and their contents, several compounds were preferentially or solely detected by one of the two ionization techniques. Hence, the combined use of the two complementary techniques (GC-MS and ESI-MS) is the best approach for forensic "general unknown screening".

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