Research Article

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On the inter-instrument and inter-laboratory transferability of a tandem mass spectral reference library: 1. Results of an Austrian multicenter study

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The inter-instrument and inter-laboratory transferability of a tandem mass spectral reference library originally built on a quadrupole-quadrupole-time-of-flight instrument was examined. The library consisted of 3759 MS/MS spectra collected from 402 reference compounds applying several different collision-energy values for fragmentation. In the course of the multicenter study, 22 test compounds were sent to three different laboratories, where 418 tandem mass spectra were acquired using four different instruments from two manufacturers. The study covered the following types of tandem mass spectrometers: quadrupole-quadrupole-time-of-flight, quadrupole-quadrupole-linear ion trap, quadrupole-quadrupole-quadrupole, and linear ion trap-Fourier transform ion cyclotron resonance mass spectrometer. In each participating laboratory, optimized instrumental parameters were gathered solely from routinely applied workflows. No standardization procedure was applied to increase the inter-instrument comparability of MS/MS spectra. The acquired tandem mass spectra were matched against the established reference library using a sophisticated matching algorithm, which is presented in detail in a companion paper. Correct answers, meaning that the correct compound was retrieved as top hit, were obtained in 98.1% of cases. For the remaining 1.9% of spectra, the correct compound was matched at second rank. The observed high percentage of correct assignments clearly suggests that the developed mass spectral library search approach is to a large extent platform independent. **Copyright © 2009 John Wiley & Sons, Ltd.**

Keywords: tandem mass spectrometry; library; transferability

Introduction

The term *product ion tandem mass spectrometry* (MS/MS) summarizes mass spectrometric methods concerned with the selection of a particular ion (= precursor ion) and its dissociation to generate characteristic secondary fragment ions.[1–3] MS/MS-techniques are widely used for the fragmentation of ions gathered from 'soft' atmospheric pressure ionization (API) methods. In the majority of cases, collision-induced dissociation (CID) experiments are used to obtain structural information from a precursor ion. The combination of MS/MS-techniques with computational data interpretation routines represents a valuable tool for the characterization and identification of biopolymers such as peptides,[4,5] proteins,[6,7] oligosaccharides,[8] and oligonucleotides.[9–11] Biopolymers consist of a limited number of building blocks and the bonds that are preferably broken during CID are well known. Almost the same fragment ions are obtained from a certain precursor ion irrespective of the instrumental platform used for the MS/MS-experiment.[12,13] To a large extent fragment ion mass spectra are predictable and can be used for database search as well as for de novo derivation of a biopolymer's sequence.[14–16]

Characterization of the structure of small molecules represents another important field of application for MS/MS, which is extensively used for target-specific analysis and to a lower extent for general unknown screening procedures employing searchable mass spectral libraries.[17,18] In contrast to biopolymer MS/MS, the outcome of CID of a small molecule is difficult to predict. It is well known that the observed intensity of a potential fragment ion is controlled by kinetic (e.g. type and density of the collision gas and center-of-mass energy) and instrumental parameters (e.g. specifics of the applied ionization technique, efficiency of fragment ion collection, and mass discrimination effects of the detector).[19] The kinetics of a single ion-molecule-reaction is an intrinsic, generic, and thus transferable property of the investigated system. It is important to note, however, that a given set of experimental conditions may only be applicable to a limited

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range of compounds due to variations in collisional stabilities. Therefore, the collection of spectra acquired at low, medium, and high collision-energy settings has been proposed for library creation. The impact of instrument-specific parameters on CID of a small molecule is difficult to control and predict making the creation of a platform-independent mass spectral library a challenging task. A number of different types of instruments from diverse manufacturers have MS/MS-capabilities and can be classified either as 'tandem-in-space'- or as 'tandem-in-time' instruments.[20] There have been attempts to produce libraries with spectra recorded on a number of mass spectrometric platforms, which have been reviewed.[21] The use of tune compounds has been proposed for the 'normalization' of experimental conditions, which could help to reduce the inter-instrument variability of MS/MS spectra and to increase the success rate of automated library search procedures.[19,21–24] Nevertheless, still the inter-instrument differences in the relative intensities of ions present in MS/MS spectra are significant when strictly measured and statistically evaluated.[23,25–28] Thus, the common doctrine is that "MS/MS libraries are mostly in-house libraries successfully running only on a single apparatus or apparatus type".[29] The results of a recently published study, however, suggested that single MS/MS libraries might be sufficiently efficient for identification of unknowns and suitable for use with different tandem mass spectrometers.[30] In that particular study, 3126 MS/MS spectra, either taken from four large collections or from the literature, were used to construct a library. By performing a number of library searches with a subcollection of the library defined as 'unknown' spectra, it was shown that correct answers were obtained as the first rank in 60% of the search results.

In the present report, the examination of the inter-instrument and inter-laboratory transferability of a tandem mass spectral reference library originally built on a quadrupole-quadrupole-time-of-flight (QqTOF) instrument is presented.[31] In the course of a multicenter study, 418 tandem mass spectra of 22 compounds were collected in three different laboratories using four different instruments from two manufacturers and matched against the established reference library with the same search algorithm. The extent of platform independence of the established mass spectrometric library in combination with the developed search strategy was evaluated and discussed. As far as we know, there is no other study on the transferability of a tandem mass spectral reference library available that approximates the presented work in comprehensiveness and completeness.

Experimental Section

Reference library

The reference library was recently developed in the reference laboratory on a QqTOF instrument (Qstar XL, Applied Biosystems, Foster City, CA) and contained 3759 MS/MS spectra of 402 compounds.[31] For each reference compound, product-ion spectra were typically acquired at ten different collision-energy (CE) values between 5 and 50 eV. Because of possible saturation effects and to avoid false positive matching of the precursor ion with product ions originating from alternative compounds, all signals within a $\pm$4.0 amu window around the *m/z* of the precursor ion were deleted from the reference spectra obtained. To increase specificity further, reference spectra were filtered. Only those signals with a relative intensity above 0.01%, and which were observed at least in two spectra collected at different collision energies were regarded as suitable for identification. The remaining mass peaks were deleted from the reference spectra.

Test compounds

The sample set for checking the inter-instrument and inter-laboratory transferability of the library search approach consisted of 22 compounds (Table 1), of which 19 compounds were randomly selected from the collection of drug standards of the reference laboratory. Only the legal status of a compound was considered as sort of selection criterion. The ergot alkaloids (dihydroergotamine, ergotamine, and methysergide) were added to the sample set due to a pronounced scientific interest of one laboratory in mycotoxins.[32] To verify that the set of test compounds was suited to qualify the performance of the mass spectral library, the library was surveyed for entries that were likely to interfere with at least one test compound during library search (Table 1). All reference compounds with a molecular mass deviation of less than $\pm$1.0 amu from one of the test compounds' masses were taken into account (Table 1). Furthermore, the database entries were surveyed for compounds that show some structural similarity to one of the test compounds (Table 1). All but five belonged to groups of pharmaceuticals that were represented by several members in the library. Sulfamethoxazole and sulfamoxole, for example, are two of nine structurally closely related sulfanilamides that are part of the spectral library. A total of 118 compounds (nearly 30% of all database entries) were identified as highly probable cause for misleading search results. Before shipping to participating laboratories, the chemical identity of the samples was checked in the reference laboratory by GC/MS as described previously.[31]

Instrumentation and data acquisition

Three different laboratories participated in the multicenter study (Table 2). Tandem mass spectra were acquired using four different instruments from two manufacturers. The study covered the following types of tandem mass spectrometers: QqTOF, quadrupole-quadrupole-linear ion trap (QqLIT), quadrupole-quadrupole-quadrupole (QqQ), and linear ion trap-Fourier transform ion cyclotron resonance (LIT-FTICR) mass spectrometer. The QqLIT was operated in two different scanning modes: in 'product ion scan' (pi) and in 'enhanced product ion scan' (epi) mode. In both operational modes, precursor ions were selected in the first quadrupole and fragmented in the collision cell (= second quadrupole). The third quadrupole was either operated as quadrupole (pi) or as linear ion trap (LIT, epi) and was used to scan the fragment ions. On the LIT-FTICR instrument, product ions were generated in the LIT and were either analyzed at low resolution in the LIT or at high resolution in the Fourier transform ion cyclotron resonance (FTICR) mass spectrometer. In each participating laboratory, optimized instrumental parameters were gathered from routinely applied workflows. The experimental parameters are summarized in Table 2. Test samples were weighed and dissolved in 0.1% aqueous acetic acid solution containing 50% (v/v) acetonitrile before analysis. Depending on the performance of the different instruments as well as on the compound-specific ionization efficiencies, the concentrations of the sample solutions varied from 0.02 to 10 µg/ml. Samples were directly infused into the mass spectrometer. On each single instrumental platform, tandem mass spectra specific for a certain compound were acquired at three different collision-energy values. Additionally, on the QqLIT in epi mode a single spectrum

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Table 1. List of compounds with corresponding precursor ion masses as well as a summary of putative interfering reference compounds

Compound	Theoretical mass (amu) of $[M + H]^+$	Reference compounds within ±1.0 u	Similar or structurally related compounds
Amiloride	230.0551	Clonidine; propazine; sebuthylazine; terbuthylazine	–
Buphenin	300.1958	Chlordiazepoxide; metoclopramide; hydrocodone; codeine; clobazam; temazepam; carazolol	*Phenylethylamines*: hexoprenaline; mescaline; methoxamine; octopamine; oxyfedrine; suloctidil; verapamil
Cinchocaine	344.2332	Tetrahydrocannabinolic acid	–
Cyclizine	267.1855	Atenolol; desipramine; sulfamoxole; sulfisoxazole	*Piperazines*: cetirizine; cinnarizine; hydroxyzine; meclizine
Desipramine	267.1855	Atenolol; cyclizine; sulfamoxole; sulfisoxazole	*Dibenzazepines*: carbamazepine; clomipramine; imipramine; lofepramine; opipramol; trimipramine
Dihydroergotamine	584.2867	–	*Ergot alkaloids*: ergotamine; methysergide
Dosulepin	296.1467	Diclofenac; mebendazole; dibenzepin	–
Dyxirazine	428.2366	–	–
Ergotamine	582.2710	–	*Ergot alkaloids*: dihydroergotamine; methysergide
Ethambutol	205.1910	Bufotenin; dexpanthenol	–
Etilefrine	182.1175	Oxilofrine	*Ethanolamines*: epinephrine; octopamine; oxilofrine; phenylephrine; terbutaline
Etofylline	225.0982	Ethamivan; nifenalol; mesoranil	*Xanthines*: caffeine; pentifylline; theobromine; theophylline
Mefruside	383.0496	–	*Sulfonamides*: bumetanide; chlortalidone; indapamide; probenecid; *sulfanilamides*; sulthiame
Methysergide	354.2176	Butizide; acenocoumarol; yohimbine; vincamine	*Ergot alkaloids*: dihydroergotamine; ergotamine
Metoclopramide	300.1400	Carazolol; chlordiazepoxide; hydrocodone; codeine; buphenin; clobazam; temazepam	*Benzamides*: moclobemide; sulpiride
Phenazone	189.1022	–	*Pyrazolones*: aminopyrine; ketophenylbutazone; oxyphenbutazone; phenylbutazone
Phentermine	150.1277	Cathinone; metamphetamine	*Amphetamines*: 2,5-dimethoxy-4-bromoamphetamine; 2,5-dimethoxy-4-methylamphetamine; 3,4-methylenedioxyamphetamine; amphetamine; metamphetamine; *N*-methyl-3,4-methylenedioxyamphetamine
Phenytoin	253.0971	Oxcarbazepine; cimetidine; tizanidine; sulfamethoxazole	*Hydantoins*: mephenytoin
Sulfamethoxazole	254.0593	Oxcarbazepine; phenytoin; cimetidine; tizanidine; triamterene; tolpropamine	*Sulfanilamides*: sulfadiazine; sulfaguanidine; sulfameter; sulfamethoxypyridazine; sulfametrole; sulfamoxole; sulfaphenazole; sulfathiazole; sulfisoxazole
Sulfamoxole	268.0750	Atenolol; cyclizine; desipramine; sulfisoxazole; metoprolol	*Sulfanilamides*: sulfadiazine; sulfaguanidine; sulfameter; sulfamethoxazole; sulfamethoxypyridazine; sulfametrole; sulfaphenazole; sulfathiazole; sulfisoxazole
Sulthiame	291.0467	Norcocaine; benzoylecgonine; atropine; catechin; trimethoprim	*Sulfonamides*: bumetanide; chlortalidone; indapamide; mefruside; probenecid
Tetracycline	445.1605	Doxycycline	*Tetracyclines:* chlortetracycline; doxycycline; oxytetracycline

under 'collision-energy spread' conditions was measured. A total number of 418 fragment ion mass spectra were collected.

Data handling

Acquired mass spectra were centroided and exported as txt-files. Each txt-file contained information about the precursor ion mass and a list of the observed fragment ions (mass-to-charge ratios (m/z) and the corresponding relative intensities). The files were sent to the reference laboratory and are available for review from the authors upon request. All spectra were matched against the established reference library.[31] The principles of the applied library search procedure are described in the companion paper.

Results and Discussion

Characterization of the applied instrumental platforms

A total of 418 tandem mass spectra corresponding to 22 test compounds were collected in three different laboratories on four

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Table 2. Comparison of experimental settings used on different instrumental platforms for acquiring tandem mass spectra

	Reference laboratory	Laboratory 1	Laboratory 2	Laboratory 3	
Instrument	QqTOF	QqLIT	QqTOF	QqQ	LIT-FTICR
Company	Applied Biosystems	Applied Biosystems	Applied Biosystems	Thermo Fisher Scientific	Thermo Fisher Scientific
Trade name	QSTAR XL	QTrap 4000	QSTAR Pulsar i	TSQ Quantum Ultra	LTQ-FT Ultra
Alternative scan modi		Product ion scan (pi) or enhanced product ion scan (epi) with dynamic fill time option			LIT or LIT-FTICR
Ion source	TurboIonSpray	TurboIonSpray	Nanospray	H-ESI source	H-ESI source
Ion source parameters	Positive mode voltage: 4.0 kV nebulizer gas: 1–3 units turbo gas: 40 units source temperature: 200 °C	Positive mode voltage: 4.0 kV nebulizer gas: 30 psi turbo gas: 50 psi source temperature: 150 °C	Positive mode voltage: 850 V	Positive mode voltage: 4.5 kV sheath and sweep gas vaporization temperature: 50 °C ion transfer tube temperature: 200 °C	Positive mode voltage: 4.5 kV sheath and sweep gas vaporization temperature: 50 °C ion transfer tube temperature: 200 °C
Flow rate	3 µl/ min	10 µl/ min	<100 nl/ min	10 µl/ min	10 µl/ min
Sample concentration (µg/ml)	0.1–10	Pi mode: 0.2 epi mode: 0.02	0.1–1	10	10
Calibration	External with caffeine and reserpine	External with a mixture of polypropylenglycols	External using the peptide ALILTLVS	External using caffeine, MRFA and Ultramark	External using caffeine, MRFA and Ultramark
Collision gas	N_2 (5 arbitrary units)	N_2 (status 'high')	N_2 (4 arbitrary units)	Ar (1.5 mTorr)	He
Precursor ion isolation	Unit resolution	Unit resolution	Low resolution	Unit resolution	Width: 1.0 amu
Scan	50–700 amu accumulation time: 1.0 s	50–700 amu scan speed: 1000 amu/s	50–700 amu accumulation time: 1.0 s	50–700 amu scan speed: 325 amu/s	50–700 amu automatic gain control average of 3 microscans
Acquisition time	1.0 min	1.0 min	1.0 min	1.0 min	1.0 min
Software	Analyst QS 1.0 SP8	Analyst 1.4.1	Analyst QS 1.1	Xcalibur 2.0	Xcalibur 2.0

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different instruments (QqQ, QqLIT, QqTOF, and LIT-FTICR). QqQ, QqLIT, and QqTOF were classified as 'tandem-in-space' instruments and LIT-FTICR as 'tandem-in-time' instrument. The QqLIT was operated in two different scanning modes: 'pi' and 'epi' mode. On the LIT-FTICR instrument, product ions were either analyzed at low resolution in the LIT or at high resolution in the FTICR. Depending on the type of mass analyzer and the physical characteristics of each instrument used, mass accuracy was restricted. To estimate the performance of the instruments, the relative differences between measured and theoretical precursor ion masses were determined for all spectra per instrument and statistically evaluated. The results were reported as average measurement error ± standard deviation (QqQ: −440 ppm ± 176 ppm; LIT: −30 ppm ± 139 ppm; QqLIT: 70 ppm ± 102 ppm; QqTOF: −10.8 ppm ± 32.7 ppm; LIT-FTICR: 0.00 ppm ± 0.44 ppm). The standard deviation indicates the inherent mass accuracy of a certain instrument type and enables a ranking of the mass spectrometers as follows: QqQ < LIT < QqLIT < QqTOF < LIT-FTICR. The magnitude of the average mass measurement error can indicate the presence of some kind of systematic error arising from improper calibration. For all instrumental platforms but the QqQ, the calculated standard deviations were larger than the mean mass deviations. Obviously, all instruments but the QqQ were properly calibrated. Besides the precursor ion masses, the fragment ion masses were also affected by the improper calibration of the QqQ. All erroneous masses were corrected prior to further data processing.

Compound-specific mass spectra acquired on the different instrumental platforms were compared to estimate the extent of inter-instrument differences in fragmentation behavior. It is important to note in this context that no standardization procedure was applied to increase the comparability of MS/MS spectra. In each participating laboratory, optimized instrumental parameters were solely gathered from routinely applied workflows. Thus, certain fragment ions were detected on all experimental platforms, whereas others were preferentially produced by a certain type of instrument. Typically, more extended fragmentation was observed on 'tandem-in-space' instruments than on LIT/LIT-FTICR (an example is depicted in Fig. 1). Moreover, 'tandem-in-space' experiments favored the production of low-*m/z* fragment ions that were very weak or absent in the LIT (Fig. 1). Comparison of 'tandem-in-space' spectra clearly showed that differences regarding the observed fragmentation pathways were minor (Fig. 1(a)–(e)). Relative signal intensities, however, varied significantly. Particularly, the applied collision-energy settings were found to have a major impact on relative signal intensities. In this respect, 'tandem-in-time' spectra were found to be less affected than 'tandem-in-space' spectra.

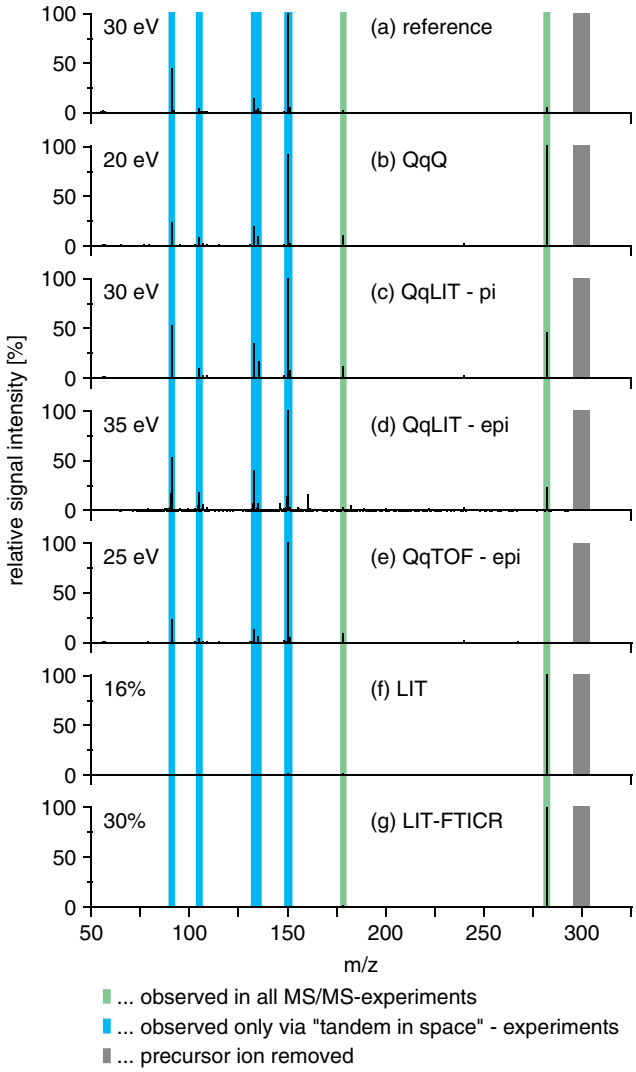

Figure 1. Comparison of buphenine-specific tandem mass spectra collected on different instrumental platforms.

Visual check of the collected fragment ion mass spectra

Before matching the collected sample spectra to the library, each spectrum was compared visually with the corresponding reference spectra to uncover noticeable discrepancies. In Fig. 2 putative mefruside spectra collected on the QqLIT in epi mode are depicted. Visual inspection revealed that the spectra collected at 20 eV (Fig. 2(c)) and under 'collision-energy spread' conditions (Fig. 2(e)) do not have any fragment ion in common with mefruside-specific reference spectra (Fig. 2(a)). Thus, both sample spectra did not retrieve mefruside as match. The origin of these two spectra was unclear. Most probably, the spectra got mixed up either during the extraction process or during the transfer as txt-files to the reference laboratory. Furthermore, abnormalities were also detected in all desipramine-specific spectra collected on the QqLIT in epi mode. The visual inspection of the spectra revealed that besides desipramine-specific ions a large number of cyclizine-specific ions were present (Fig. 3(c)). Desipramine and cyclizine have identical empirical formulas and therefore identical monoisotopic masses (Table 1) but different compound-specific fragment ions (Fig. 3(a) and (d)). Hence, if both the compounds are present in one sample solution due to contamination or due to carry-over effects, fragment ions corresponding to both species will appear in the fragment ion mass spectrum. For all affected spectra, cyclizine was obtained as top hit. After 'purification' of the sample spectra[31] simply by eliminating all cyclizine-specific ions, desipramine was retrieved as the best matching compound (Fig. 3(b)).

Evaluation of the library search results obtained from the Austrian multicenter study

Library search was performed against a 402-compound library using a sophisticated matching algorithm. The principles of the algorithm are described in the companion paper. The library

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Table 3. Summary of fragment ions observed (a) for phentermine and cathinone in selected reference spectra as well as (b) for phentermine on different instrumental platforms

(a)

Reference compound	*m/z* of precursor ion	*m/z* of compound-specific fragment ions							
Phentermine	150.13	133.10	–	–	105.07	91.05	–	–	65.04
Cathinone	150.09	133.06	132.08	117.06	105.07	91.05	90.05	77.04	65.04

(b)

Instrument – CE	*m/z* of precursor ion	*m/z* of fragment ions observed in sample spectra (relative intensity > cut-off)							
QqQ – 6 eV	150.12	133.12	–	–	–	91.09	–	–	–
QqQ – 12 eV	150.12	133.12	–	–	105.09	91.09	–	–	–
QqQ – 18 eV	150.12	–	–	–	–	91.09	–	–	–
QqLIT – epi – 10 eV	150.16	133.12	–	–	105.04	91.04	–	–	–
QqLIT – epi – 20 eV	150.16	–	–	–	105.04	91.04	90.08	–	–
QqLIT – epi – 30 eV	150.16	–	–	–	105.04	91.04	90.08	–	–
QqLIT – epi – 10, 20, 30 eV	150.16	133.12	–	–	105.04	91.04	90.00	–	–
LIT – 15%	150.11	133.11	–	–	–	–	–	–	–
LIT – 17%	150.11	133.08	–	–	–	–	–	–	–
LIT – 20%	150.11	133.08	–	–	–	–	–	–	–

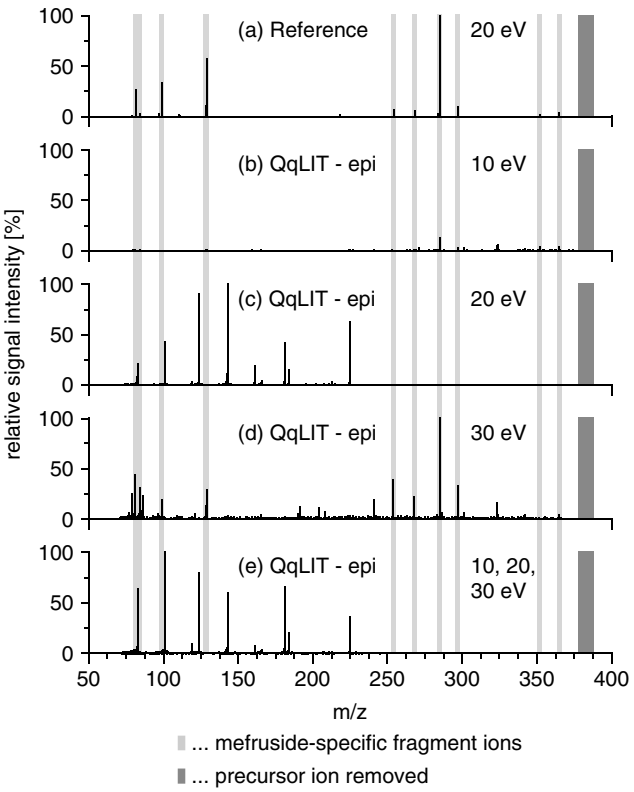

Figure 2. Visual inspection of putative mefruside-specific spectra collected at different collision-energy settings on the QqLIT in epi mode.

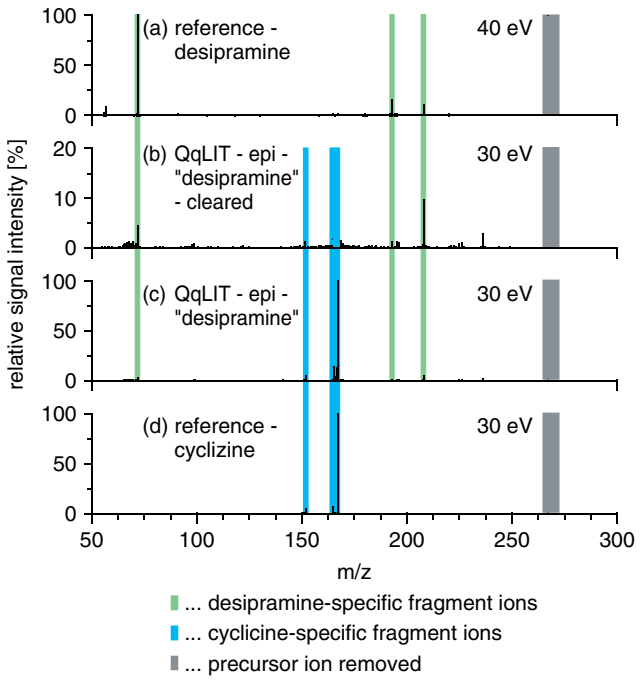

Figure 3. Visual inspection of a putative desipramine-specific spectrum for the presence of cyclizine-specific fragment ions.

search results are summarized in Fig. 4. The applied collision-energy settings are specified by the numbers given in each box. Different background colors are used to designate the obtained library search results. Here, any library search resulting in the test compound as top hit was called a *correct answer*. Of the initially collected set of 418 spectra, the above-mentioned questionable spectra (two mefruside- and four desipramine-specific spectra) were excluded from matching. Among the remaining 412 fragment ion mass spectra, an unexpected high percentage of incorrect assignments was obtained for phentermine-specific sample spectra. For nine spectra acquired on low-resolution instruments, cathinone was obtained as top match. Phentermine and cathinone have different empirical formulas, but identical nominal precursor ion masses (Table 3(a)). The two compounds are structurally related. In MS/MS experiments loss of NH_3 is observed for both compounds (nominal fragment ion mass: 133; Table 3(a)).

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instrument	scan mode	amiloride	buphenin	cinchocaine	cyclizine	desipramine	dihydroergotamine	dixyracine	dosulepin	ergotamine	ethambutol	etilefrine	etofylline	mefruside	metoclopramide	methysergide	phenazone	phentermine	phenytoin	sulfamethoxazol	sulfamoxole	sulthiame	tetracycline	correct assignments
QqQ	-	10 eV	10 eV	10 eV	5 eV	10 eV	15 eV	20 eV	15 eV	20 eV	8 eV	5 eV	10 eV	12 eV	10 eV	20 eV	20 eV	6 eV	5 eV	10 eV	10 eV	10 eV	10 eV	
		20 eV	20 eV	20 eV	12 eV	20 eV	30 eV	30 eV	25 eV	30 eV	16 eV	15 eV	20 eV	18 eV	20 eV	25 eV	30 eV	*12 eV*	15 eV	20 eV	20 eV	16 eV	20 eV	99%
		30 eV	30 eV	30 eV	20 eV	30 eV	40 eV	40 eV	35 eV	40 eV	24 eV	25 eV	30 eV	24 eV	30 eV	30 eV	40 eV	~~18 eV~~	25 eV	25 eV	30 eV	22 eV	30 eV	
QqLIT	pi	20 eV	20 eV	20 eV	20 eV	10 eV	20 eV	20 eV	20 eV	20 eV	10 eV	10 eV	10 eV	10 eV	10 eV	20 eV	20 eV	10 eV	10 eV	10 eV	~~10 eV~~	10 eV	10 eV	
		30 eV	30 eV	30 eV	30 eV	20 eV	30 eV	30 eV	30 eV	30 eV	20 eV	20 eV	20 eV	20 eV	20 eV	30 eV	30 eV	20 eV	20 eV	20 eV	20 eV	20 eV	20 eV	99%
		40 eV	40 eV	40 eV	60 eV	30 eV	40 eV	40 eV	40 eV	40 eV	30 eV	30 eV	30 eV	30 eV	30 eV	40 eV	40 eV	30 eV	30 eV	30 eV	30 eV	30 eV	30 eV	
	epi	~~10 eV~~	25 eV	20 eV	10 eV	10 eV	20 eV	20 eV	20 eV	30 eV	10 eV	*10 eV*	10 eV	10 eV	20 eV	20 eV	20 eV	*10 eV*	10 eV	~~10 eV~~	10 eV	10 eV	10 eV	
		20 eV	35 eV	30 eV	20 eV	20 eV	35 eV	30 eV	30 eV	45 eV	20 eV	20 eV	20 eV	20 eV	30 eV	35 eV	30 eV	~~20 eV~~	20 eV	20 eV	20 eV	20 eV	20 eV	
		30 eV	45 eV	40 eV	30 eV	30 eV	50 eV	40 eV	40 eV	60 eV	30 eV	30 eV	30 eV	30 eV	65 eV	50 eV	40 eV	~~30 eV~~	30 eV	30 eV	30 eV	30 eV	30 eV	95%
		10, 20,	25, 35,	20, 30,	10, 20,	10, 20,	20, 35,	20, 30,	20, 30,	30, 45,	10, 20,	10, 20,	10, 20,	10, 20,	10, 20,	20, 35,	20, 30,	*10, 20,*	10, 20,	10, 20,	10, 20,	10, 20,	10, 20,	
		30 eV	45 eV	40 eV	30 eV	30 eV	50 eV	40 eV	40 eV	60 eV	30 eV	30 eV	30 eV	30 eV	30 eV	50 eV	40 eV	*30 eV*	30 eV	30 eV	30 eV	30 eV	30 eV	
QqTOF		25 eV	20 eV	20 eV	10 eV	15 eV	20 eV	20 eV	20 eV	20 eV	10 eV	~~10 eV~~	20 eV	20 eV	20 eV	20 eV	25 eV	15 eV	20 eV	20 eV	20 eV	20 eV	30 eV	
		35 eV	25 eV	30 eV	15 eV	20 eV	30 eV	30 eV	30 eV	30 eV	20 eV	~~20 eV~~	30 eV	30 eV	30 eV	30 eV	30 eV	20 eV	25 eV	25 eV	25 eV	25 eV	35 eV	97%
		45 eV	30 eV	35 eV	20 eV	25 eV	40 eV	40 eV	40 eV	40 eV	30 eV	30 eV	40 eV	40 eV	40 eV	40 eV	35 eV	25 eV	30 eV	30 eV	35 eV	35 eV	40 eV	
LIT-FTICR	LIT	20%	12%	14%	12%	15%	12%	13%	15%	13%	14%	12%	15%	12%	13%	14%	25%	*15%*	15%	15%	15%	10%	10%	
		25%	16%	18%	15%	20%	15%	17%	20%	15%	18%	16%	23%	14%	17%	18%	30%	*17%*	18%	20%	20%	14%	13%	100%
		30%	20%	22%	18%	25%	18%	21%	25%	18%	22%	20%	30%	18%	21%	22%	35%	*20%*	21%	25%	25%	18%	16%	
	FTICR	25%	25%	20%	20%	20%	15%	20%	25%	14%	25%	25%	25%	14%	25%	25%	35%	15%	25%	25%	25%	15%	10%	
		30%	30%	25%	25%	25%	20%	25%	30%	19%	30%	30%	30%	19%	30%	30%	40%	17%	30%	30%	30%	20%	15%	100%
		35%	35%	30%	30%	30%	25%	30%	35%	24%	35%	35%	35%	24%	35%	35%	45%	20%	35%	35%	35%	25%	20%	

10 eV ... correct match ~~10 eV~~ ... incorrect match 10 eV ... wrong substance

10 eV ... correct match after the elimination of artifacts from reference spectra

Figure 4. Results of the Austrian multicenter study. Four different instruments were used to collect MS/MS spectra. The applied scan modes and collision-energy settings are given.

Moreover, the two compounds have several fragment ions in common (Table 3(a); 105.07, 91.05, and 65.04 amu). Cathinone, however, exhibited several fragment ions that were unique for this compound and can be used to unequivocally distinguish cathinone from phentermine (Table 3(a); 132.08, 117.06, 90.05, and 77.04 amu). The mismatched sample spectra contained one to four different fragment ions (Table 3(b)). Within three mass spectra acquired on the QqLIT in epi-mode one of the three possible fragment ions that represented unique identifiers for cathinone (Table 3(b); 90.08 amu) was observed giving rise to the appearance of cathinone as best matching compound. Although none of the cathinone-unique signals were observed in one of the remaining spectra listed in Table 3(b), all of them were preferentially matched to cathinone. In these cases, an assignable cause for the prevalence of cathinone was missing. A visual inspection of the cathinone-specific reference spectra (Fig. 5) revealed that artifacts with *m/z* values close to 105.07 and 133.06 amu were present and were found to represent the most probable source of overestimation. The mass differences between a true fragment ion and its neighboring artifact were so small that both ions matched to the corresponding signal in a sample spectrum, which increased the total number of 'matching fragments' as well as the corresponding 'match probability' values artificially. For low-resolution mass spectra where a *m/z* slot of 0.1 amu was used for signal assignment, cathinone was retrieved as top match instead of phentermine. We recognized that the artifacts arose from improper centroiding and bypassed the already installed filtering steps. To eliminate the artificially produced fragments from reference spectra, filtering was extended. The newly developed filtering step included scanning each reference spectrum for pairs of fragment ions whose *m/z*-difference was less than 0.05 amu and subsequent elimination of the fragment ion exhibiting lower signal intensity. Owing to filtering, six phentermine spectra and one etilefrine spectrum changed their status from incorrectly to correctly assigned (Fig. 4). The number of incorrect matches decreased from the initially 3.6% down to only 1.9%. Correct answers were

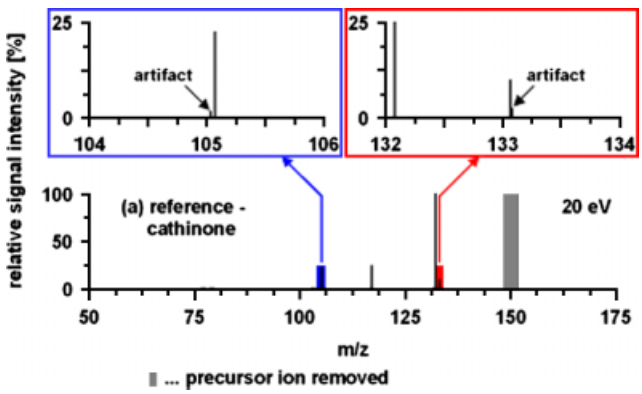

Figure 5. Identification of 'artifacts' within a cathinone-specific reference spectrum.

obtained in 98.1% of search results. For all eight spectra that were classified as incorrect, the correct compound matched at second rank. The lowest percentage of correct results (95.0%) was obtained for the QqLIT operated in epi mode. Nevertheless, we believe that the QqLIT operated in epi mode is as suitable for the collection of fragment ion spectra as all other examined platforms. Probably, the very low sample concentrations (0.02 μg/ml) were responsible for the somewhat reduced comparability of tandem mass spectra. Hundred percent correct search results were achieved with spectra acquired on the LIT-FTICR instrument.

Impact of the comprehensiveness of the mass spectral library on the search efficiency

It is well known that the observed intensity of a certain fragment ion is controlled by kinetic parameters (e.g. type and density of the collision gas and center-of-mass energy).[19] At certain experimental conditions, usually only a subset of all possible fragmentation pathways is observed. Thus, for the creation of a

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comprehensive MS/MS-spectral library, the collection of spectra acquired at several different collision-energy settings has been recommended. The use of three different collision-energy levels for acquiring compound-specific reference spectra is common.[27,33,34] We have recently proposed the collection of reference spectra acquired at ten different collision-energy values.[31] To determine the influence of the number of compound-specific reference spectra stored in a library on search efficiency, the sample spectra collection was matched to the following three databases: the entire library, one subset of the entire library consisting of spectra collected at three different collision energies (20, 35, and 50 eV), and another subset of the entire library consisting of spectra collected at a single collision energy of a mean level (35 eV). The performance of the library search decreased with decreasing comprehensiveness of the mass spectral library searched. For the entire library, for the library consisting of three compound-specific spectra, and for the library consisting of one spectrum, 98.1, 97.3, and 91.9% of matches, respectively, yielded correct results. The decline of the search efficiency, however, was rather moderate, which clearly suggests that the developed library search procedure has reached a high degree of development. Thus, even suboptimal collections of reference spectra can be successfully applied for unequivocal compound identification.

Conclusions

The tandem mass spectral reference library tested for its inter-instrument and its inter-laboratory transferability consisted of 3759 spectra collected from 402 reference compounds on a QqTOF instrument. In the course of a multicenter study, 418 tandem mass spectra of 22 compounds were acquired in three different laboratories using four different instruments from two manufacturers and matched against the library using a sophisticated peak matching algorithm. Thus, a comprehensive study on the inter-instrument and the inter-laboratory transferability of a tandem mass spectral reference library has been conducted. To point out that the results were authentic, problems were discussed that may occur in the course of a multicenter study and maybe also in laboratories during the use of such a gadget. Only a small number of spectra, however, were sorted out. Two out of 418 (0.5%) were of unknown origin and four others (1.0%) contained fragment ion masses of two species. All other spectra were useful for statistical evaluation of the performance of the library search approach. The overall high percentage of correct search results (98.1%) can be cited as evidence for the platform independence of the presented tandem mass spectral library search approach. The following factors are obligatory to obtain such an encouraging result: (1) For the library creation, a tandem mass spectrometric platform is necessary that enables the accurate and reproducible measurement of fragment ion masses. (2) Compound-specific reference spectra need to be collected at several different collision energies. (3) Reference spectra must be filtered before storage in the library to eliminate unspecific signals. (4) The instrument that is used to collect sample spectra must be properly calibrated. (5) The search algorithm must exhibit a high tolerance toward changes within the intensity distribution among different fragmentation pathways (for details on this topic please refer to the companion paper). Future work will show if the ongoing increase of the number of database entries will have a major impact on the efficiency and transferability of the mass spectral library.

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