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When you have eliminated all which is impossible,

then whatever remains, however improbable, must be the truth.

Sir Arthur Conan Doyle, 1859-1930

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Table of Contents

1 Abstract

Systematic Toxicological Analysis is an ongoing challenge in forensic as well as in clinical toxicology. The development of new methods based on LC-MS has been in the focus of researchers during the last decade. In order to establish a routine toxicological screening, all factors influencing the procedure need to be identified. Beginning from the ionization process and acquisition settings to the reference library - multiple factors have to be evaluated. Moreover, reproducibility and transferability of spectra is another issue, yet without satisfying solution.

Introduction: For the development of a screening procedure a Linear Ion Trap was used in combination with HPLC and the ToxID library. Special emphasis was put on all parameters influencing a screening, including instrument features as well as on issues evolving from the library search. The aim was to identify the main factors that have to be considered when establishing LC-MS for Systematic Toxicological Analysis based on the used linear Ion Trap.

Material and Methods: The LTQ Velos (Thermo Fisher Scientific) was the MS used for this work, in combination with the RS 300 Ultimate HPLC (Dionex). A testmix with 13 toxicologically relevant substances was used for individual experiments. Deuterated standards were used to detect changes in the retention times of the chromatographic system. Single substances or a mix of compounds were either directly infused or injected via on-line HPLC. Pretests for limit of detection tests were performed for the compounds of the testmix and two real samples were analyzed with the newly established method. Screening results were evaluated manually and with ToxID - a library search tool provided by Thermo Fisher Scientific. Additionally the screening software SmileMS (GeneBio) was used.

Results and Discussion: The key-parameters for the creation of library spectra and consequently for practical screening are discussed, as well as the proper ionization settings for different applications. Several methods for multi-target- and general unknown screening were established and can be used for future routine analysis. Moreover, pitfalls for screening and library spectra creation were identified. For the methods, the number of scan events, dynamic exclusion settings, activation parameters and the importance of preselecting the targets are discussed. The Tox_Library and the search tools “ToxID” and “SmileMS” were evaluated and spectra were added for MS^2 and MS^3.

2 Introduction

"General unknown screening" - the analytical process that takes place when no prior information about possible toxic agents is available - is an ongoing challenge of forensic and clinical toxicology. This challenge has been addressed by a well-planned, systematic analytical approach - systematic toxicological analysis (STA). In the field of forensic toxicology, STA is the application of an appropriate analytical strategy for the identification of potentially toxic compounds and their metabolites in biological samples. The process of STA includes sampling, sample preparation (isolation), differentiation, detection, as well as identification. Due to the serious legal consequences of forensic cases, particular emphasis must be placed on the quality and reliability of analytical results.

(http://www.tiaft.org/node/53 on September 13th, 2011)

This definition given by the TIAFT (The International Association of Forensic Toxicologists) outlines the problems evolving from screening biological samples for toxic compounds. Toxins from different kinds of origin can harm the human body - including inorganic, organic, volatile and fairly stable substances, thus presenting toxicologists with a broad range of difficulties. Finding appropriate methods for identification is a demanding task. Each substance has multiple ways of entering the body, different metabolism, kinetic and elimination. By systematically using all established methods for sampling, sample preparation, differentiation, detection and identification the likelihood to find as many substances as possible is increased.

A method routinely used for toxicological analysis is Mass Spectrometry (MS). This very sensitive method detects ionized molecules. When additional fragmentation is applied, the path of dissociation for each substance is similarly unique to a fingerprint. Coupled with distinct sample separation techniques such as High Performance Liquid Chromatography (HPLC) or Gas Chromatography (GC), MS is one of the most frequently used techniques in forensic toxicology (Gergov, Weinmann et al. 2004; Mueller, Duretz et al. 2011).

Per definition, a “General Unknown Screening” (GUS) would include the search for ALL possible substances. However some compounds are not detectable by MS; others could be detected but are not found due to other reasons, such as the lack of the corresponding reference. Therefore Systematic Toxicological Analysis is performed. The systematic step-by-step approach is needed to find a large number of substances with different physical/chemical properties. GC-MS, HPLC with Diode Array Detection (DAD)

and immunological screening methods are combined to minimize the chance of false negative or positive samples.

Great handicaps for analysis are the different kinds of organic matrices. Neat spiked solutions as well as nearly matrix-free samples, such as tablets, are relatively simply analyzed and results generally are satisfactory (Oberacher, Pavlic et al. 2009; Pavlic, Schubert et al. 2010). However, the effect of matrix components on the analysis is unpredictable. Depending very much on the sample preparation process, some unknown biological compounds may not be removed sufficiently and thus interfere with the ionization of the analytes. In LC-MS, the signal can be suppressed or enhanced, thus possibly leading to false results, analytes at low concentrations could be missed. Moreover, additives or solvent residues can interfere.

The aim of this work was to evaluate the features and limits of toxicological MS analysis using a certain linear ion trap, the LTQ Velos (Thermo Fisher Scientific). Thus, the ideal settings for specific analytical questions were determined, while also considering HPLC influence. This work purely focuses on the possibilities for qualitative screening with the LTQ Velos mass spectrometer. Identification is the prerequisite for quantitative determination. Although it can be important to know the exact amount of an identified toxin (e.g. for determination of the cause of death), the methodical and instrumental requirements for quantitative analysis would differ greatly and were not addressed in this work.

2.1 Background

A number of different methods are commonly used for Systematic Toxicological Analysis. GC-MS is currently the “gold-standard” for most analytical questions; other methods commonly used are LC with DAD and immunological screening procedures. Although neither method is sufficient by itself, they complement each other well for a profound toxicological screening.

GC-MS utilizes very large existing libraries (e.g. “NIST MS 11”, currently 243,893 spectra of 212,961 compounds, on September 8th, 2011) and well established reliable methods. GC offers a high resolution power. Electron Impact (EI) ionization is highly reproducible through all instruments. EI triggers dissociation during ionization, no further collision step is required. EI is classified as “hard ionization method”, thus generating a higher amount of fragmentation, hence yielding more information. MS detectors work only under high vacuum, making GC a suitable separation technique. However, this advantage is also the method’s major drawback. Chromatography is performed in the gas phase, thus excluding non volatile or thermo labile compounds for direct analysis. Although derivatisation enlarges the amount of substances detectable, it makes time consuming sample preparation necessary. Although GC-MS is the gold-standard for STA,

development of routine LC-MS methods is progressing very fast, continuously gaining significance.

DAD as detector for LC is much less specific than MS. Identification can only be done by Retention Time (RT) and a corresponding reference UV/VIS spectrum. Moreover, the appearance of an optical spectrum can be strongly influenced by a number of parameters, such as the solvent, the pH and possible coeluents. Optical spectra yield much less information (e.g. structural) and are only available, when the molecule contains a chromophore. However, there are fewer problems with transferability of spectra, large libraries exist and liquid samples do not have to be transferred into gas phase/vacuum. Also, the technical complexity is much less than for mass detectors, with therefore increased cost effectiveness.

Immunological screening systems range from the quick and easy to use “on-site tests” to complex methods which require laboratory facilities and trained personnel. Usually predefined compounds are detected via reaction with specific antibodies. Unfortunately, this reaction is seldom absolutely specific. Depending on the method, false positive results due to cross-reactions can easily occur and tests are only available for a relatively small number of defined substances. The use of this method for quick testing especially outside laboratories is undisputed, as it can also be performed by untrained staff. However, no “STA” can be performed as these methods are always targeted on a limited number of substances. Furthermore, each substance or substance class requires a unique immunological method.

Next to these commonly used methods of GC-MS, LC-DAD and Immunological screening, LC-MS as method for GUS has been developed and routinely used since more than a decade. Coupling LC to MS was made possible through the development of new interfaces and Atmospheric Pressure Ionization (API) methods such as Electrospray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI). The need for these developments evolved from the problematic transfer of liquid into gas phase, especially at high liquid flow rates. At present, the combination of Liquid Chromatography and Mass Spectrometry is a widely used technique in many different fields of science. From big and very complex bio-molecules analyzed in proteomics to small-molecules analysis in toxicology, all fields are represented. A broad range of adapted instruments and methods are available for different applications. LC-MS is already being routinely used in clinical and forensic laboratories; however method development is still an ongoing process.

2.2 Approaches to Toxicological Screening

2.2.1 General Principles

The great range of available Mass Spectrometers makes many different screening approaches possible. Their common aim is to detect and identify relevant ions out of the vast amount of endogenous and exogenous components. Both clinical and forensic toxicologists have a high need for profound screening methods; the substances screened for are often similar. Methods should be validated, according to current guidelines (e.g. the International Conference on Harmonisation (ICH) or European Medicines Agency (EMA)), as the results have to be very reliable because of their serious medical or legal consequences.

Depending on the many different objects of screening, different principles have been observed. The problems of screening in matrix require more sophisticated methods than the analysis of simple tablet or powder samples. Post mortem brain samples have to be prepared in other ways than urine. Methods in clinical toxicology have to be very fast whereas forensic toxicology methods have to be very reliable. Living patients can offer additional information, whilst there might be no a priori information about the cause of death in a forensic case. In general, STA can be needed in clinical as well as forensic toxicology. The sample matrix can vary, as can the concentrations and specific requirements, but the general methods are the same.

Some common principles apply for screening on many different types of instruments. Methods can be classified as 'targeted' or 'non-targeted'. Targeted screening implies the search for predefined substances. This pre-selection is usually correlating with a MS library. Hence, the more entries there are in the library, the more profound the search. A very high number of toxins are known substances, e.g. prescription drugs, which can be easily added to a library by infusing neat standard solutions. The structures of new 'designer drugs' or many metabolites on the other hand, are often unknown and therefore not included in libraries. This is the main disadvantage of any targeted screening – no entry in the library means wrong results. The quality of the search is therefore highly dependent upon the quality of the library, the search algorithm and additional exclusion criteria. The quality of the reference spectra is another factor influencing search results. A number of methods have been developed for only a limited number or certain classes of substances (Nielsen, Johansen et al. 2010). The risk of false negative and positive hits is low; however it increases with the number of entries in the library. This cannot be compared to STA procedures and libraries with e.g. over 1500 entries where the chance of similar spectra is much higher. To eliminate false positives, search criteria have to be very strict, concerning deviation of RT and recorded mass spectrum. At the same time the search criteria must not be a reason for possible false negative results. Methods operating with larger libraries have been published, but only investigated urine or non-biological matrices (Pavlic, Schubert et al. 2010; Mueller, Duretz et al. 2011).

Targeted screening can either be performed in full scan mode with DDA or as SIM (Selected Ion Monitoring) or SRM/MRM (Selected Reaction Monitoring, Multiple Reaction Monitoring). Full scan spectra a usually yield the most information, SIM the least. SIM only monitors a single mass, without any fragments. This can be used for quantification of a known component or for screening with high resolution instruments, such as Time-of-Flight (TOF) detectors. SRM/MRM monitors the transition of a precursor ion into one or more specified product ions. Additional data is hereby obtained from the relative mass intensities and the retention time in the chromatogram. A full scan can be used for the initial survey scan and the following data-dependent product ion scan. This spectrum shows all produced product ions, including their intensities. The most commonly used method for fragmentation with LC-MS-MS instruments is Collision Induced Dissociation (CID). It can be performed in a quadrupole or an ion trap collision cell. However the spectra produced with either method are difficult to compare, as will be explained below (2.2.2).

The European Union Commission (Communites 2002) states that, for comparison to validated reference spectra, at least 4 ions should be present with at least 10% abundance of base peak for full scan spectra. For SIM/SRM/MRM experiments, the commission assigns Identification Points (IP), depending on the used method and the number of given ions. For example, LC-MS^2 with one precursor and two product ions would gain 4 IP (1 + 1.5 + 1.5 = 4); Minimum number of required IP is 4. Relative abundances also have to be taken into consideration, given limits have to be met. In general, full scan spectra or Enhanced Product Ion (EPI) spectra provide more reliable identification. High resolution data is awarded one additional IP for each ion (e.g. 2 instead of 1 for matching precursor).

Another option is performing a non-targeted GUS via intensities. (Wissenbach, Meyer et al. 2011) Hereby, no substances are predefined; the selection of precursor ions is based solely on their abundance. From one full scan, e.g. the 5 most abundant ions are selected for fragmentation. This minimizes the problem of pre-selection but becomes very difficult if real samples containing matrix are analyzed. Even the most thorough sample clean up cannot exclude all matrix in advance, especially without also excluding possible analytes. Besides, a toxin could be present at very low concentrations and would therefore not be selected for fragmentation. Matrix components with very high abundance can conceal other components. Known interferences might be put on a “reject” list and excluded from analysis beforehand, but there is still a chance for relevant components having identical m/z. Very strict “dynamic exclusion” settings can here be of considerable use. Success is also strongly dependent upon the separation power of the chromatography and the shape of the peaks.

A possible approach could be the combination of targeted and non-targeted screening. Ions on a list should be recorded, even when present at low concentrations. Additionally, high abundant ions not included on the list should be chosen too. Wissenbach et al. (Wissenbach, Meyer et al. 2011) uses multiple metabolites for the identification of one single compound. Of the many metabolites present in urine derived from one drug, not all might be identified. However, a sufficient number will be.

This is a rather robust method and additionally confirms body passage. Unfortunately, the method was exclusively designed for 45 antidepressants; already over 500 identified metabolite spectra were included in the search. For STA and a great range of possible analytes, the limits of this method are soon reached.

For targeted and non-targeted screening alike the selection of the highest screening order applied is a delicate task. It is possible to produce solely MS^2 or MS^3 spectra for comparison with a library, or a combination of both. Spectra in MS^2 often yield only little information (e.g. one single fragment), or two different structures may even show a very similar fragmentation pattern (e.g. derivates). Two ions with identical m/z could be present, resulting in a mixed spectrum. Here, MS^3 can be an advantage. On the other hand does applying MS^2 and MS^3 to every precursor ion result in fewer full scan to full scan cycles over time. This might even double the full scan to full scan cycle time, depending on the exact setup of the method. In general, MS^2 is mostly sufficient, in some cases even the highest order possible, however MS^3 can be an enhancement in information. A pragmatic approach might be the application of MS^3 only when necessary, e.g. through a predefined list, if the used software permits this sort of strategy.

For all strategies, ionization of substances in positive or negative mode has to be considered. The majority of toxicological relevant substances contains nitrogen and/or is basic, therefore ionizes in positive mode. Molecules with acidic functional groups generally also ionize well in negative mode. Newer MS instruments can rapidly switch between positive and negative scan mode, allowing detection of basic and acidic compounds in one run. The number of scans per cycle appointed to negative or positive mode should be chosen accordingly. Some strategies however, focus purely on basic compounds (Köhler, Grobosch et al. 2011). Using two separate ways of sample preparation is another possible approach (Stimpfl 2011). The extraction is specific for acidic/neutral and basic compounds, followed by two separate analytical runs.

2.2.2 Instrumental Strategies for Mass Spectrometry in Toxicology

Today's possibilities have strongly advanced since the first LC-MS attempts. Early strategies involved simple single MS or triple quadrupole instruments. Today, a Full Scan or MRM followed by a Data Dependent Acquisition (DDA) triggered full Product Ion (PI) scan, is the state of the art (Moffat, Osselton et al. 2004). Full scan mode leaves room for retrospective, untargeted, manual review whereas MRM is strictly confined to preselected targets. DDA can be either targeted or, based on intensities, untargeted. Even a mix of both is possible if MS^2 and MS^3 experiments can be combined.

Different strategies for MS are applied in Toxicology, with different aim and emphasis. In a quick overview, five different instrumental strategies will be introduced: The early stages of LC-MS screening were performed with 'in-source' CID on single-stage MS. Extensive research was put into development of reproducible spectra. Triple quadrupoles (QqQ), which are clearly the method of choice for quantification where

also assessed for qualitative screening. More profound information was gained when hybrid instruments such as quadrupoles with Linear Ion Traps (QqLIT) were used. Simultaneously, genuine ion traps were developed. High resolution Instruments, such as TOF detectors and especially the QqTOF hybrids are the most sophisticated instruments used for screening today.

For all LC-MS methods, two main sources of ionization are commonly used. The instrumentation requires API methods; ESI and APCI are equally popular. APCI can be used with a higher liquid flow and is less susceptible to ion suppression effects (Mueller, Duretz et al. 2011); On the other hand in-source fragments can be produced accidentally which might possibly conceal the molecular ion (Wissenbach, Meyer et al. 2011). Molecules can be fragmented after ionization. This takes place either right away at the source while entering by collision with the orifice ("in-source CID") or in a "collision cell" after the first Q-stage. However, in-source CID shows major drawbacks and is only necessary if no such cell is present (e.g. with single MS instruments). As collision cell, the second of three quadrupoles, Qq-hybrid instruments or simple ion traps can be used. The cell is filled with gas, e.g. nitrogen, helium or argon. Depending on the gas pressure, the amount of ions in the cell and the applied collision energy, the fragmentation can be regulated.

In source CID, mostly used with single MS, is strongly dependent upon the design of the instrument, the shape of the orifice and the placement in the source. Fragmentation can be influenced by variation of the cone voltage only. Sufficient reproducible spectra are only obtained with instruments of the same "family" from one manufacturer (Bristow, Nichols et al. 2002). Especially between instruments of different manufacturers the spectra do not only vary in the abundance of ions but also in spectral content. Two main strategies for increasing the reproducibility where assessed by Bristow et al., a tune compound protocol at low and medium Collision Energy (CE) and a $[M+H]^+$ ion attenuation protocol. The tune compounds where used to establish ideal conditions at two different collision energies. As this approach did not sufficiently work, the $[M+H]^+$ ion attenuation protocol was developed, whereby the extent of fragmentation is chosen so that the molecular ion is reduced to 50% of its original abundance. Both methods were supposed to adapt one instrument to the settings of another. It was found that neither approach would be sufficiently transferable to all other instruments.

Venisse et al. (Venisse, Marquet et al. 2003) states that only with in-source CID a complete GUS is possible because no ions are primarily excluded. They created their own library with reconstructed spectra from two different collision energies (±20V and ±80V) for routine application. A big emphasis for single-MS is on the chromatography because the noise reduces sensitivity greatly. Venisse et al. used enhanced data processing (for chromatography and spectra) to improve the signal/noise ratio. A 50 min gradient was used to reduce co-elution. Today, other methods are available to avoid pre-selection of ions. However it should considered that one can only identify what is already known, if no reference is available, no identification is possible. Moreover, the runtime of 50 min is another major drawback, as the time saving

advantage in comparison to GC is lost. Today, in-source CID is obsolete for single stage MS. However for single TOF instruments, in-source CID can still be a useful additive. The abundance of fragments and even the content of the spectrum have less relevance because the exact mass of all fragments is measured.

Nowadays, the strategies to systematic toxicological analysis mostly include Tandem-MS or high resolution detection. QqQ, QqLIT, simple traps, LIT or TOF detectors are available from different manufactures, with many available features.

QqQ instruments have a very high selectivity and also sensitivity, ideal for working with preselected ions. Their use for quantification is undisputed; however the possibility of screening is limited, due to the low sensitivity in this mode. To perform a SRM followed by an EPI scan needed for a comprehensive toxicological screening, two injections of the sample are necessary. Although 4 IPs would be sufficient for identification (one precursor ion and two product ions, 1+1.5+1.5=4) it may be doubted whether this really is a sensible STA screening. Limited speed and capacity might be another drawback for the application of this method in analytical toxicology. QqQ can be used for additional confirmation of substances identified in screening, and especially for their quantification.

QqLinear Ion Trap (QqLIT) instruments can be utilized in QqQ mode or with the third quadrupole working as a LIT. This allows collecting ions prior to detection in order to receive a full product ion scan after dissociation. Firstly, a MRM experiment is run to screen for a number of possible, preselected precursor ions and their most significant, also preselected, product ion. Secondly, after CID takes place in the second quadrupole, a full scan of the fragments is triggered via DDA. In general, one normalized CE is applied, regulated through the gas pressure and the applied voltage. All ions present in the quadrupole are activated and can possibly fragment. The pseudo-molecular ion is activated and dissociates into fragments which themselves are activated again. This process is repeated continuously, as long as the activation is performed, and fragmentation is therefore a cascading process. This is the major difference to 3D Ion Traps, as explained below.

A number of recently published strategies use the “QTrap” (ABSciex), a QqLIT (Mueller, Weinmann et al. 2005; Dresen, Ferreiros et al. 2010). To enhance the simple MRM/EPI scan methods, Dresen et al. introduced the scheduled MRM (sMRM) scan, which only screens for certain analytes within a specified timeframe, therefore allowing more analytes to be included in the search and minimizing interferences. They also performed semiquantitative analysis with the hybrid LIT; using deuterated standards to minimize the effects of fill time and matrix. However they cannot quantify as reliably as QqQ instruments, because the trap fill time greatly influences the measurement. Detection in traps is not performed continuously but rather dependent upon the amount of ions present in the trap. This is important for having a stable signal if only few ions are present and to avoid overloading the trap if a great amount is available.

Genuine **Ion Traps** can have a linear or 3D construction, where CID and detection scans are performed in the same space. Hence, the detection has to be performed in a gas

filled cell, therefore reducing sensitivity in detection. Prior to CID, ions of a selected m/z are isolated in the trap and activation is directed specifically. This increases the signal/noise ratio and, furthermore, it is the reason why spectra produced in quadrupole collision cells cannot be compared to spectra from an ion trap. No further cascading fragmentation process takes place. CID in traps often produces very few significant fragments, one of them typically being the “waterloss peak”. The waterloss fragment occurs when the molecular ion is activated and dissociates into $[M - H_2O]^+$ which is generally very stable and does not fragment further. New technical developments such as “Wideband”, “Stepped Collision Energy” (Thermo Fisher Scientific) or “Collision Energy Spread” (AB Sciex) can spread the activating energy over a broad range, thus fragmenting the waterloss peak and therefore yielding more information. These features will be discussed in the Material and Methods section (3.2.1).

Mueller et al. and Sturm et al. (Mueller, Weinmann et al. 2005; Sturm, Hammann et al. 2010) developed multi-target screening methods on LXQ and LCQ ion traps from Thermo Fisher Scientific. The general method is created with the Xcalibur software for both Instruments and is also comparable with the general method creation for the LTQ Velos.

The LTQ Velos used for this work is a further development of the basic ion traps. It is a LIT with one high and one low pressure cell, realizing a tandem-in-time rather than the tandem-in-space concept of QqLITs. The survey scan, the CID (with helium gas) and the product ion scan (after DDA with a certain threshold), are performed in two separate regions. In the low pressure region, detection is much more sensitive than in common traps, as no collision gas is present. No compromise has to be made and very fast switching from positive to negative mode in one cycle can make the information acquisition even more thorough. No GUS procedure performed on a LTQ Velos has yet been published. When connected to an “Orbitrap” (Thermo Fisher Scientific), the Velos can even produce additional high resolution information. Produced data would repeatedly be verified. The RT, molecular mass, MS^2 and/or MS^3 spectrum and exact mass measurement mutually confirm results.

TOF instruments combine accurate mass measurement with RT for identification. Unless in-source CID is applied, no deliberate fragmentation takes place. Although very much information can be gained through high resolution data, some issues cannot be resolved. One exact mass applies to all molecules of equal chemical formula. This includes structural isomers as well as random matches. However, data can also be reviewed retrospectively, because without pre-selection of ions, all peaks above a certain threshold are recorded.

Nielsen et al. (Nielsen, Johansen et al. 2010) developed a method for 52 substances in hair. They state that the selectivity of the method is equal to methods monitoring ion transitions and their relative ratios with MRM. As mentioned above, all single MS methods are very susceptible to interferences with matrix components or solvent residues. A big emphasis has to be put on the chromatography, which takes only 17

minutes for the described method. They were even able to partly quantify in the same run as the screening. This is actually not recommended by the TIAFT as they suggest that "Quantitation should ideally be performed on an aliquot of the sample other than that used for screening and/or qualitative analysis." (http://www.tiaft.org/node/82, 11.9.2011). Libraries for high resolution data can even be created from theoretical masses, possibly supplemented with spectral data.

High Resolution Hybrid Instruments (e.g. QqTOF or LIT-Orbitrap) have the big advantage of measuring the accurate mass in combination with fragmentation data. Both MRM and full scan screening are possible. Spectra created in a non-targeted screening (e.g. via intensities) can lead to identification of primarily unknown compounds, e.g. metabolites.

Broecker et al. (Broecker, Herre et al. 2011) developed a method for LC-QTOF-MS with DDA for more than 2500 substances. Spectra were taken after CID at 10, 20 and 40eV. The reconstructed spectra were checked for plausibility and adjusted according to their calculated exact mass. Again, retrospective analysis can be an advantage.

3 Material and Methods

3.1 Chemicals and Reagents

3.1.1 Internal Standard Solution

Three deuterated standards (morphine-d_3, benzoylecgonine-d_3 (BZE), and methadone-d_3) were purchased from Sigma-Aldrich Co. (USA), and solutions with 12 neat standards in three different concentrations (1 and 10mg/ml) in methanol were available from previous extractions. Diluted stock solutions of 10 µg/ml in 50% MeOH - acetonitrile (ACN) (1+1) with 0,1% glacial acetic acid were used to create library spectra.

For ensuring the reproducibility of the HPLC System a mix of the three deuterated standards was prepared ("Internal Standard Mix"). The substances were chosen to cover the standard 10 minute gradient run. Morphine-d_3 elutes at 0,84, BZE-d_3 at 4,07 and methadone-d_3 at 6,78 minutes, respectively.

The internal standards mix was used to evaluate retention times. Therefore, two different approaches were examined. Firstly, three MS^2 scans on the precursor masses were run permanently (Fig.1). Secondly, a DDA method was applied which only triggers a MS^2 scan if one of the three precursor masses appears with an intensity of over 100 counts in the full scan (Fig.2). The peaks were well visible in both Total Ion Chromatograms (TIC), though the first approach showed a better peak performance, allowing easier determination of the exact retention time.

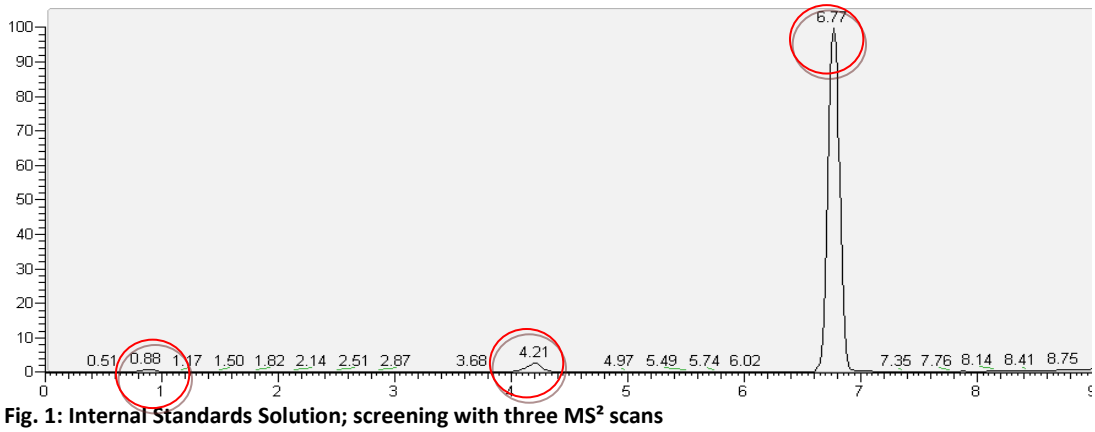

Fig. 1: Internal Standards Solution; screening with three MS^2 scans

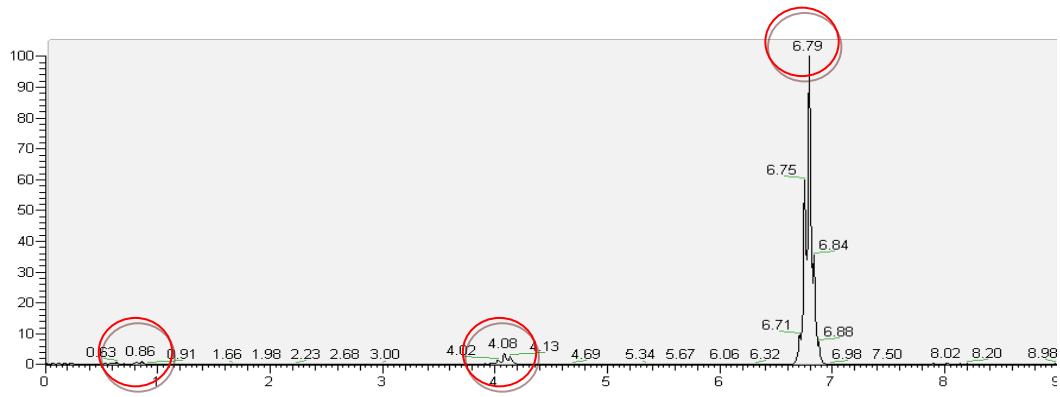

Fig. 2: Internal Standards Solution; screening with DDA

3.1.2 Testmix

A mixture of 13 substances, (from the 12 standard solutions plus one deuterated standard) as listed below (recommended in Toxichem Krimtech 2011; 78(1)-65), was prepared. These substances have different physical and chemical characteristics, chosen to cover different analytical problems. They have basic and/or acidic functional groups - to include positive and negative ionization, and different polarities to cover the whole chromatographic range. BZE-d_3 was used to differentiate between a product of degradation (of cocaine) and the added standard. All substances, their concentrations, precursor ions and the established RTs are listed in Tab. 1. THC refers to the delta-9-tetrahydrocannabinol and THCA to the 11-nor-9-Carboxy-THC. The Testmix was used to evaluate the prospective methods for screening and corresponding settings.

Substance	Concentration µg/ml	Relative RT (min) (12 min run)	m/z +H
Amphetamine	50	1,75	136
Benzoylecgonine-d_3	50	4,04	293,3
Cocaine	50	5,56	304,1
Codeine	50	1,89	300
Diazepam	50	6,32	285
Doxepine	50	6,35	280
Ibuprofen	500	6,49	207
Methadone	50	6,75	310
Metoprolol	50	5,16	286
Morphine	50	0,84	286
Phenobarbital	500	4,44	231 (-H)
THC	5	7,56	315
THCA	5	7,05	343 (-H)

Tab. 1: Testmix components used for the Experiments

3.2 Apparatus and Software

3.2.1 Mass Spectrometry

For the MS analysis a LIT (LTQ Velos, by Thermo Fisher Scientific, USA) was used. The novelty of this instrument in comparison to other, commonly used LITs are its two separated trap regions (Fig. 3). Inside the high-pressure cell the CID takes place, while detection happens in the low-pressure cell. The high vacuum makes the detection more precise. The LTQ Velos therefore realizes a tandem-in-time concept rather than a tandem-in-space concept applied by common hybrid systems.

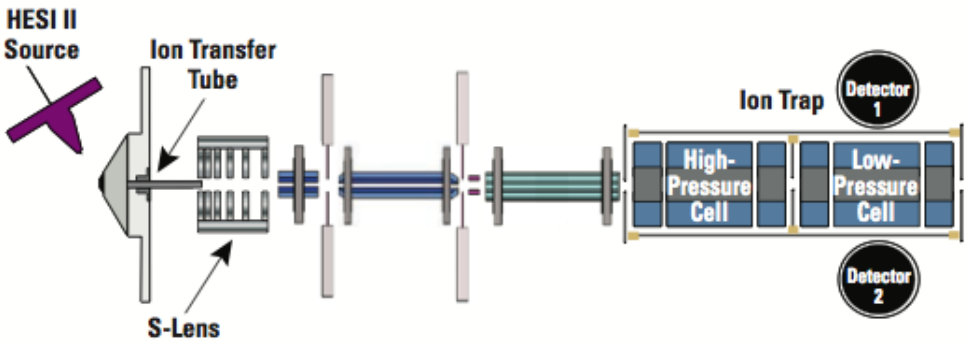

Fig. 3: LTQ Velos, Thermo Fisher Scientific

For ionization two different ESI source settings and one heated ESI (HESI) where applied. When combined with HPLC, ESI source settings had to be adapted to fully evaporate the solvent flow rate of 0,3 ml/min (Tab. 2).

Additional heating of the gas (HESI) implies less thermic stress on the molecules because the MS entrance capillary temperature can be set much lower. This approach was consequently applied.

Setting	Direct Inf.	HPLC	HESI
Sheath Gas Flow rate (arb)	8	35	25
Aux Gas Flow rate (arb)	0	10	5
Sweep gas Flow rate (arb)	0	0	0
Spray Voltage (kV)(+/-)	5	3	3/2,5
Source Heater Temp. (°C)	0	0	300
Capillary Temp (°C)	275	350	275

Tab. 2: ESI-Source Settings; for direct infusion (5-15µl/min), with HPLC and HESI (300µl/min)

Special features are the “Normalized” and “Stepped” Collision Energy (NCE, SCE) as well as the activation of Wideband. NCE allows a comparison of spectra taken on different instruments. The amount of collision energy (CE) applied varies according to the size of the molecules. Big molecules need more energy for the same degree of fragmentation than small molecules (Fig. 4). Thus the energy is not specified as eV but rather as percentage.

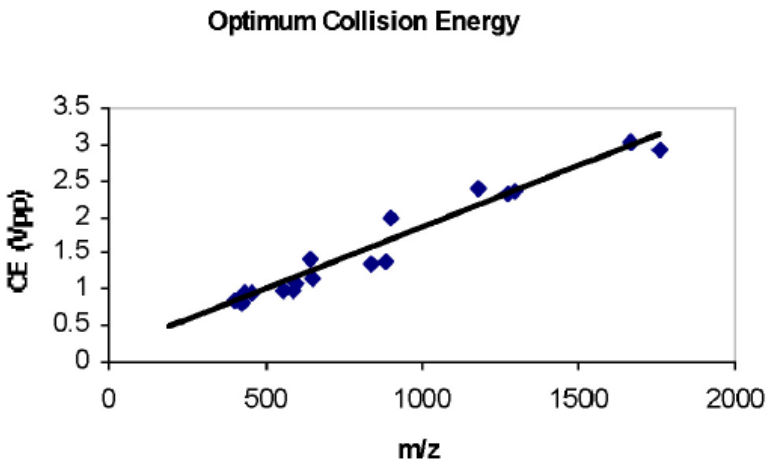

Fig. 4: Optimum CE for differently sized molecules (Thermo Fisher Scientific)

SCE means the application of more than one CE. This ensures complete fragmentation of molecular ions. No additional spectra are created and superimposed upon one another but the original is complemented by additional information. Steps can vary in size and number. For example, 20% SCE with 3 steps would result in the addition of -10, 0 and +10% to the NCE base value. At 40% NCE with SCE enabled, the precursor ions are fragmented with 30, 40 and 50% NCE, respectively.

Activation of the Wideband feature can advance the content of the spectra. Instead of generating only one significant fragment through the dissociation of water, the activation is performed over a larger range, after the isolation is performed on a rather small window. Hence, the waterloss peak is also activated and further fragmentation leads to more information in the spectrum (Fig. 5)

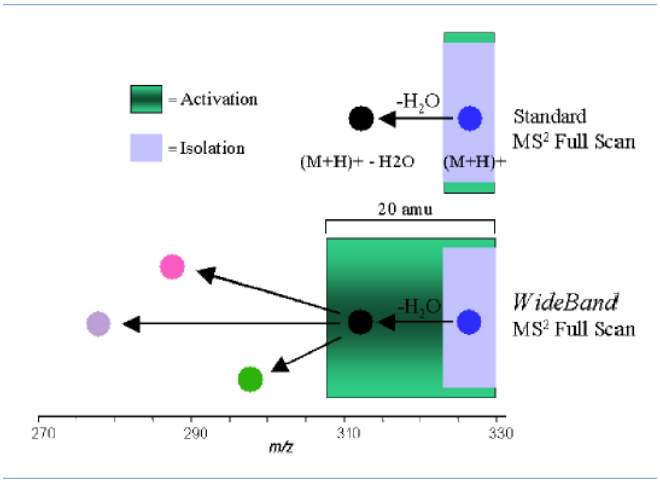

Fig. 5: Wideband Activation (Thermo Fisher Scientific)

Calibration and Tuning were performed according to the recommendations of the manufacturer of the instrument, using a mixture of 50% MeOH - ACN (1+1) with 0,1% glacial acetic acid; Calibration components were: caffeine, MRFA (L-methionyl-arginyl-

phenylalanin-alanine acetat *H_20, MW: 523,7), Ultramark 1621 (a mixture of fluorinated phosphazines) and n-butylamine.

Calibration was performed with each of the three prospective source settings, depending on the requirements of the proximate experiments. Tuning was usually performed immediately after calibration with the internal standard mix. Tuning was performed according the system (small molecules) which was subsequently used. Calibration is a source dependent procedure while tuning is dependent upon the analytes.

Calibration was continuously verified and performed weekly.

3.2.2 Software

Xcalibur (Version: 2.1) from Thermo Fisher Scientific was used to operate the LTQ Velos and for collection of data in the raw files. Chromeleon 6.8 (Dionex) was used for the HPLC. Both ToxID (Version: 2.1.1, Thermo Fisher Scientific) and SmileMS (Version: 1.2.3, Genebio) were used for data processing.

The library included (Tox_Library) was initially used and complemented with new spectra and additional information. Originally, 329 MS^2 spectra are included. No MS^3 spectra and no information about the chemical formula, structure, CAS number or synonyms are provided and had to be supplemented.

3.2.3 Xcalibur Methods

3.2.3.1 Methods for Direct Infusion

Neat standard solutions, the internal standards mix and the Testmix were directly infused for the creation of library spectra, as well as examining the effects of different parameters (such as NCE, SCE, Wideband activation, activation time, influence of solvent composition…).

3.2.3.1.1 Single Substance Methods

All methods were specifically programmed with the Xcalibur Software and adapted for each purpose. A runtime of typically five minutes guaranteed a stable ion spray and reproducible data. The number of scans per cycle was chosen individually for each experiment and either data dependent or for a preselected precursor ion. The following parameters were tested to enhance the information content of spectra: activation of wideband, stepped collision energy and different NCEs, as well as additional microscans and activation time (Tab. 3).

Tested Feature	Tested Settings	Optimum Setting
Normalized Collision Energy	20, 35, 40, 60 and 200%	40%
Wideband	Off/On	On
Additional Microscans	1/2/3/4	Dep. on MS Order
Stepped Coll. E	Off/On	On, with 20%, 3steps
Activation Time	10,30, 50 and 100 ms	30 ms

Tab. 3: Tested scan features

The infusion was performed at 5µl/min (with a Hamilton Syringe, 500µl) with the ESI source settings described above.

The default method, developed with the optimum setting of instrument features and parameters, used for library spectra creation consists of a full scan and specific MS^2 on the known precursor ion with 40% NCE, 20% Stepped CE (3 steps) and 30ms activation time. The full scan was performed to confirm the presence of the precursor ion and its overall abundance.

A selection of all used methods is listed in the appendix (App. 13) and discussed in the results (4.2).

3.2.3.1.2 Substance Mix Methods

Four different methods were developed to test initial settings of the LTQ Velos on the Testmix, without HPLC. All methods had the same sequential setup. After a full scan in the positive mode, DDA triggers MS^2 scans on the most intense ions from a precursor mass list (called "parent mass list" in Xcalibur). If no ion from that list is detected, no scan will be triggered. Afterwards a full scan in negative mode followed by DDA MS^2 scans is performed. The list includes m/z of ions within a RT frame to select for fragmentation. Additionally, a specific NCE can be assigned to each m/z and ions can be selected for MS^3. It should be mentioned that only ions included in the list can be selected, unless the option "most intense if no parent mass found" is applied; hence the methods are targeted. There are always more scans in positive mode than in negative because about 80% of all drugs are basic, hence ionize in positive mode (addition of H^+).

Methods in detail:

Method A (Fig. 6) is built up of 14 scans. One full scan in positive mode is followed by 10 positive DDA scans (MS^2).The 12th scan is a full scan in negative mode, followed by two negative DDA scans.

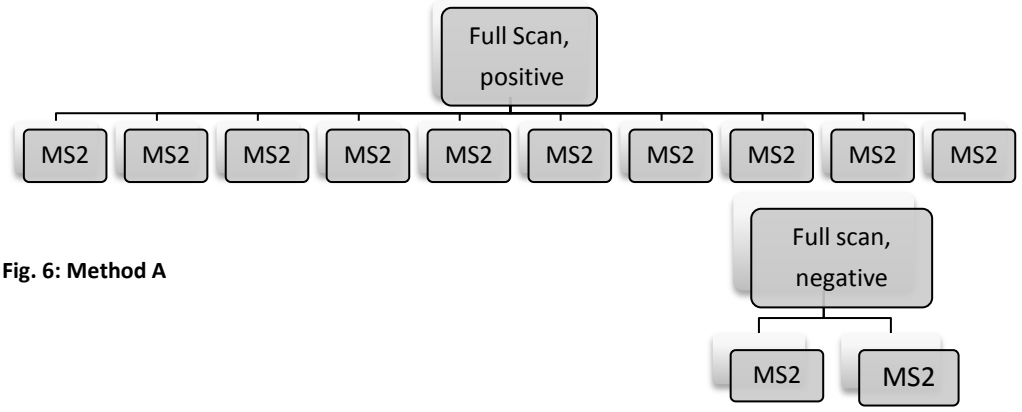

Fig. 6: Method A

Method B (Fig.7) is built up of six Scans in total. One full positive is followed by three positive DDA scans. The 5th is a full scan in negative and the 6th a negative DDA scan.

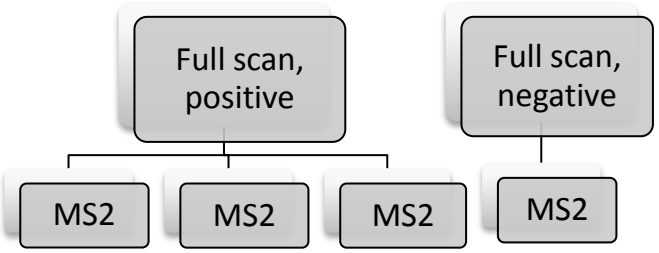

Fig. 7: Method B

Method C1 and C2 (Fig.8) are based on the method recommended by Thermo Fisher for STA and slightly adapted by one additional negative scan. They consist of 9 scan events in total with one full positive, five DDA positive, one full negative and two DDA negative scans. This method was also amplified by a special parent list. The so-called “128-500” parent mass list contains all m/z from 128 - 500 (including all mass from the library), hence simulating a real GUS.

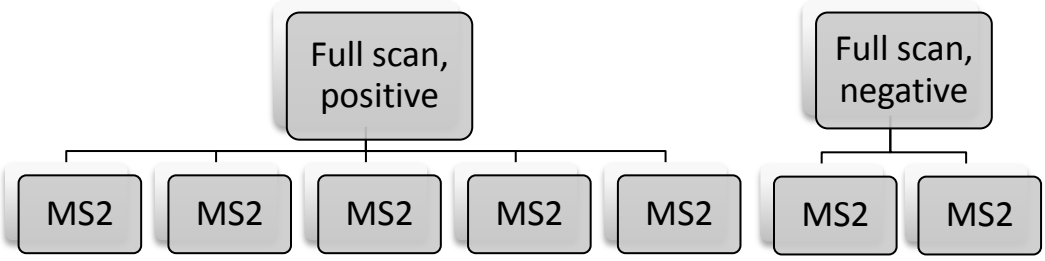

Fig. 8: Method C1 and C2; default for most experiments

The difference between C1 and C2 is the application of Dynamic Exclusion (DE) in C2, as stated in Tab. 4.

Repeat count	2
Repeat duration	10,0 sec
Exclusion list size	25
Exclusion duration	60 sec

Tab. 4: DE settings for method C2, direct infusion

The repeat count specifies the number of scans performed on a certain m/z, needed in a certain time frame (repeat duration) to trigger dynamic exclusion. The specific m/z will then be put on the exclusion list for a certain amount of time (exclusion duration). The exclusion list can be limited to a certain number of substances. If this number is exceeded, the earliest entry is removed to make room for new precursor masses.

3.2.3.2 HPLC – MS Methods

As detection of ions shows little success without prior separation through chromatography, specific methods for HPLC-MS screening were developed. Both the Testmix and the internal standards mix were used, as well as single substances for specific questions. Considering the approaches of Thermo Fisher for ToxID (Rezai, Kozak et al. 2007) for a default method for STA and Mueller et.al (Mueller, Duretz et al. 2011) concerning MS^2 and MS^3 combined screening, Method C1 and C2 were applied and developed further. Additionally, the "nth Order Double Play" approach was tested. Special methods were created to determine the RT of the internal standards (3.1.1).

The samples were injected at a mobile phase flow of 0,3ml/min with 2µl injection volume. The solutions and the HPLC gradient system were prepared and run as described below.

The *"nth Order Double Play"* method (Fig. 9) is another approach to screening with Xcalibur. This method facilitates a much higher scan rate. However, only either positive or negative mode is available within one run. The original method consists of only two scans within one segment. The first is a regular full scan while the second is a DDA scan

which can be repeated for up to 10 times. As these 10 scans are referred to as one scan, they cannot be modified individually.

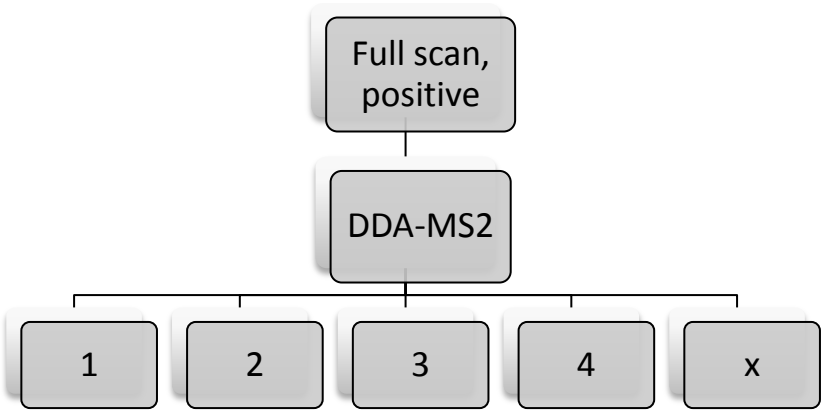

Fig. 9: The "nth Order Double Play" approach

Moreover, three different approaches to screen samples with additional MS^3 are available within Xcalibur. One was also applied by Mueller et al. (Mueller, Duretz et al. 2011) and another by Wissenbach et al. (Wissenbach, Meyer et al. 2011).

First, the concept of scans (Methods A, B and C) can be adapted to accommodate MS^3 for each MS^2 scan (Fig 10).

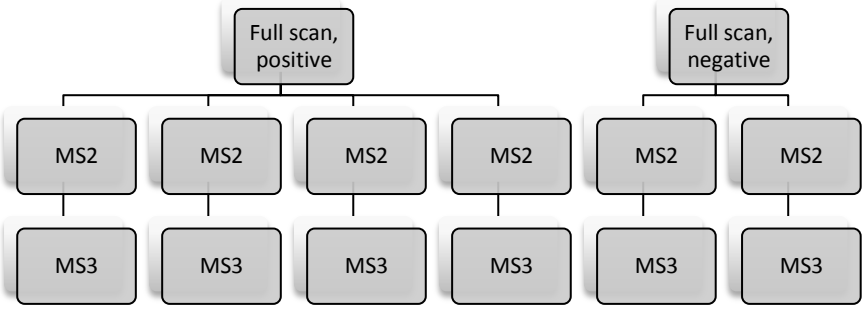

Fig. 10: Method A, adapted for MS^2+MS^3

There are two ways of determining which ion from the MS^2 scan is selected for MS^3.

- The “most intense” ion
- The “most intense ion from the list”

To choose the “most intense ion” means to collect MS^3 data for every MS^2 scan, whereas the “most intense ion from the list” option only optionally triggers a scan.

The list had to be expanded by the MS^3 precursor ions, hence a combined MS^2 and MS^3 parent mass list was used.

These two approaches could be taken further to accommodate MS^3, MS^4 or even higher order scans.

As a third approach, a certain fragment (produced in the MS^2 scan) was assigned to the substances on the parent mass list, and individual collision energy. Xcalibur then triggers the consecutive MS^3 scan automatically, if the parent mass is chosen for MS^2. This is possible up to MS^3 only. Individual treatment for certain substances was therefore possible, but MS^2 data was no longer available for selected ions.

ToxID allows a search for MS^2, MS^3 and combined MS^2&MS^3 spectra, respectively. Unfortunately, ToxID was only able to identify spectra produced with the "most intense ion from the list" approach.

3.2.4 Chromatography

For the High Performance Liquid Chromatography (HPLC) an Ultimate 3000 RS from Dionex was used, run by the Chromeleon (6.8) Software. The separation column was a Hypersil Gold PFP 50 x 2,1 mm, packed with 3 µm Material (Thermo Fisher Scientific). Column oven temperature was set to 35°C.

A linear binary gradient was used for chromatography: Mobile phase A was water with ammonium formiate (10 mMol, pH 3,3) and 0,1% formic acid; mobile phase B was ACN with 0,1% formic acid. The chromatography was run over 15 minutes. Beginning with an isocratic phase for 0,5 min (5% B), then raising the amount of B to 95% over 5 minutes, followed by 3 minutes isocratic elution at 95% B. One minute with 100% B preceded the 5 minutes equilibration phase (Fig. 11). The flow was set to 0,3ml/min unless otherwise stated. Injection volume was 2 µl, column oven temperature 35°C.

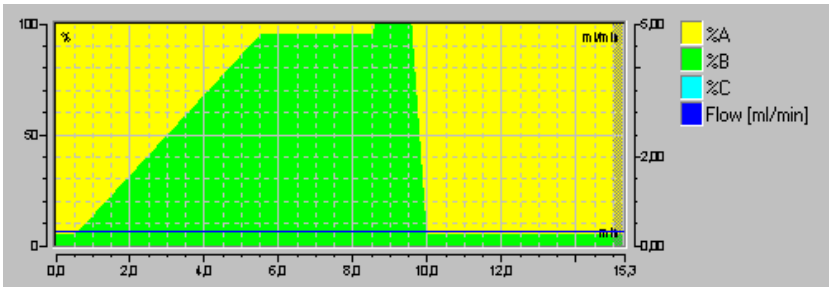

Fig. 11: Gradient elution

3.2.4.1 Retention Time Measurement/Stability

Stability of the retention of substances over time was tested at mobile phase flow of 0,3ml/min. Tab. 5 shows a selection of representative RT on two different days. An extended list of RTs of the Testmix over the whole period of work is given in the appendix (App. 14).

Datum/min	21.7.11	21.7.11	27.7.11	27.7.11	SD
Morphine-d_3	0,84	0,83	0,84	0,88	0,02
BZE-d_3	4,07	4,08	4,07	4,01	0,03
Methadone-d_3	6,78	6,78	6,74	6,8	0,02

Tab. 5: Representative selection of RTs of deuterated standards

For testing the stability of RT for all used components, the flow was set to 0,2, 0,4, and 0,5ml/min additionally to the default flow of 0,3ml/min. The linear gradient was also changed from 5 min to 10 and 15 min. In order to predict the occurring shifts of RTs an Excel calculation table (trend) was used. The internal standard solution was run with the altered settings (e.g. 0,4 instead of 0,3ml/min). The RTs for the testmix were then calculated, based on the change in RT of the three standards. This is necessary, because the scan as well as the identification of components is dependent upon the correct RT (Fig. 12).

	A	B	C	D	E	F
1		standard RT	measured RT		standard RT	calculated RT
2	MorphinD3	0,8	0,72	Morphine	0,8	0,71
3	BZED3	4,05	3,36	Amphetamine	1,75	1,49
4	MethadonD3	6,78	5,63	Codeine	1,9	1,61
5				BZE-D3	4,05	3,38
6				Phenobarbital	4,45	3,71
7				Metoprolol	5,16	4,29
8				Cocaine	5,57	4,63
9				Doxepin	6,34	5,26
10				Diazepam	6,38	5,29
11				Methadone	6,78	5,62
12				THCA	7,03	5,82
13				THC	7,58	6,28

Fig. 12: Calculation of RT after shifts; Excel

During the experiments at 0,2ml/min, shifting of some peaks, especially at early RT was observed. Since this may have been due to a problem with the mixing chamber, a “Step-gradient” was run to estimate the delay and accuracy of the mixing of the solvents.

3.2.4.2 Step-gradient elution

To test the formation of the gradient solvent A was Methanol, solvent B Methanol with 0,1% Acetone. The fraction of Solvent B was raised by 5% every 5 minutes. Detection via UV at 280nm and 245nm. Flow rates of 0,2, 0,3, 0,4 and 0,5ml/min were run. The mixing chamber contains a volume of 350 µl.

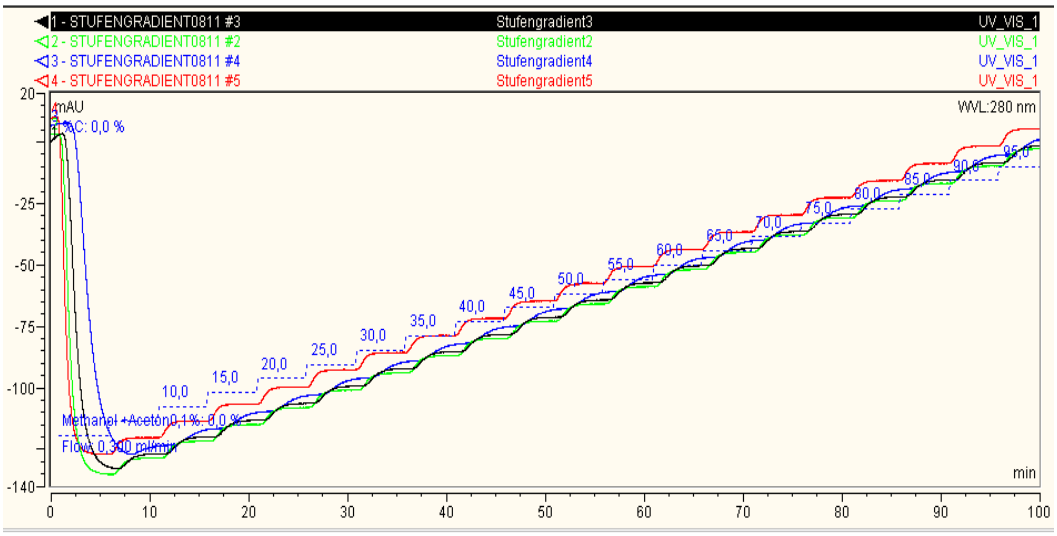

Fig. 13: Step-gradient; red: 0,5; green: 0,4; black: 0,3; blue: 0,2 ml/min

3.2.5 Limit of Detection – Pretests

In order to support future evaluations of the limit of detection, pretests were run at different dilutions. The components of the Testmix were originally present at 5µg/ml, 50µg/ml and 500µg/ml respectively, as mentioned above (3.1.2). The solution was diluted 1:10, 1:100 and 1:1000 with 50% MeOH - ACN (1+1) with 0,1% glacial acetic acid. Tab.6 shows the concentrations of the dilutions.

Concentration/Dilution	1:10	1:100	1:1000
5µg/ml	0,5µg/ml	0,05µg/ml	0,005µg/ml
50µg/ml	5µg/ml	0,5µg/ml	0,05µg/ml
500mg/ml	50µg/ml	5µg/ml	0,5µg/ml

Tab. 6: Concentration of components present in the Testmix and after dilution

The applied method was Method C (4.2.2.3) with standard HPLC conditions. Threshold was set to 100 counts, dynamic exclusion was disabled and Automatic Gain Control (AGC) was used to predict ion injection time.

As the injection volume was 2µl, the following concentrations were effectively applied:

Orig. Concentration/2µl of each Dilution	2µl at 1:10	2µl at 1:100	2 µl at 1:1000
5µg/ml	1ng	0,1ng	0,01ng
50µg/ml	10ng	1ng	0,1ng
500mg/ml	100ng	10ng	1ng

Tab. 7: Concentration injected via HPLC (2µl)

4 Results and Discussion

4.1 Introduction

Developing an LC-MS procedure for STA on the LTQ Velos MS requires the setup of relevant features. These, depending on the requirements indicated by sample origin and desired search outcome, include the instrument parameters, the setup of the method, the search criteria and the used libraries. Three different settings for the ESI Source were tested. Moreover, the influence of activation parameters on the information content and reproducibility of spectra was evaluated, for MS^2 and MS^3. Also, two ways to program a method for GUS were applied and examined. The used software (Xcalibur) was checked for applicability and practicability for the used methods. The screening software (ToxID) was primarily applied and eventually replaced by a product from a different manufacturer (SmileMS, Genebio). It had more features within a simpler setup, more sophisticated search functions and a considerably better interface.

Calibration was performed with the mentioned mix (3.2.1) and tested for stability over the whole working period of six months. It should be performed weekly and it should be followed by tuning with representative components – in this case with the 3 deuterated standards. Tuning should always be performed in analogy to the procedure applied, (e.g. high molecular compounds for proteomics and small molecules for toxicology). While calibration is a general procedure, tuning is specific.

Starting with the very basics of the instrument, the ionization and calibration procedures, already some influential parameters had to be considered.

4.1.1 MS-Source Settings

Three different MS-source conditions were tested, two with unheated ESI source and one with heated ESI (HESI).

APCI is said to be less prone to ion suppression effects and can handle higher solvent flow rates than ESI (Mueller, Duretz et al. 2011). However, APCI is less sensitive and tends to form artifacts in-source during ionization. As we were working with low solvent flow rates (max. 0,5ml/min) and did not observe relevant ion suppression effects during this work, ESI was considered adequate.

The solvent flow is one issue when considering source settings. Besides of the gas flow, the temperature in the source has to be high enough to evaporate all of the solvent.

However it should not compromise the stability of the analytes. The initial settings (see 3.2, ESI - needle temp. 275 ° C) were only used for direct infusion with a Hamilton syringe, at 5-15µl/min. To cope with the much higher flow from the HPLC (0,3ml/min), these settings had to be adapted. With an ESI needle temperature as high as 350°C, destruction of analytes during ionization is possible, however this was not observed with our Testmix. A much more gentle approach with the heated ESI source should protect thermolabile molecules. Heating the sheath gas, that flows alongside the capillary to assist evaporation, had less impact on the molecules than heating the needle itself. Therefore, HESI was considered the standard ionization method for future methods (Tab. 8).

The spray voltage of the needle was recommended by the manufacturer with 3 kV (positive mode) and 2,5 kV (negative mode) for HESI. Applying 5 kV (pos) and 3 kV (neg) instead had little impact on ionization intensity in positive mode, but much more in negative: The spray current (µA) rose to a high level of up to 30 µA (default value: < 5 µA), which resulted in difficulties with calibration. The calibration components caffeine and n-butylamine could not be correctly identified because they disappeared among this noise. The following spray conditions were ultimately taken as standard for future applications, for a solvent flow rate of approx. 300 µl/min. For direct infusion (5-15 µl/min), the source heater temperature could be turned off.

Sheath Gas Flow rate (arb)	**25**
Aux Gas Flow rate (arb)	**5**
Sweep gas Flow rate (arb)	**0**
Spray Voltage (kV) +/-	**3/2,5**
Source Heater Temp. (°C)	**300**
Capillary Temp (°C)	**275**

Tab. 8: Default HESI source settings

4.1.2 Scan Features

Producing spectra for a spectral library is a delicate task. Their quality and reproducibility influences every future search result. Ion suppressing or enhancing effects can influence the content or intensity of mass spectra. These effects especially may vary in different matrices. Moreover, biological samples are not homogenous and these effects might not be reproducible. To reduce this risk, spectra should be recorded under conditions very similar to future sample processing. The full scans in every cycle are controlled by the AGC. It ensures the trap is always filled with a sufficient number of ions and avoids overloading. AGC cannot be set manually; therefore it is not considered an “influenceable parameter”. Full scans can only be enhanced by applying additional microscans. However the default value is one, which is sufficient. Fragmentation for the DDA screening is initialized through activation in the trap and is therefore influenced by all activation parameters. The following parameters were tested (see also Tab.3; 3.2.3.1.1): NCE including stepped CE, wideband, additional microscans and activation

time. Generally applied values, taken from relevant literature (e.g. (Mueller, Duretz et al. 2011), are given in Tab.9:

Tested Features	Generally applied Values
Normalized Collision Energy	30-45 %
Stepped Collision Energy	± 5-10 %
Wideband	On
Additional Microscans	1 for full scan, 2-4 for higher order
Activation Time	10-50 ms

Tab. 9: Activation parameters: Generally applied values, taken from relevant literature

Relevant changes in spectra could be obtained by variation of NCE and activation time or by applying Wideband, each with different impact. Wideband generally enhances the spectrum content greatly by producing more information than just the waterloss peak.

NCE and activation time do not influence spectral content, but rather alter intensity ratios. The generally applied NCE can vary between instruments and is usually between 30 - 50%. It should be high enough to fragment a precursor ion completely. The performed experiments showed that for small molecules in the Velos-Trap it should be about 40%, with SCE enabled at 20% with 3 steps. This implies activation at 30, 40 and 50% respectively, covering the whole range from low to high CE in one spectrum. Spectra generated with this CE showed no or little remains of the molecular ion. This reduces variability due to CE.

Activation time also influences the amount of fragmentation of the precursor ion. As shown in the Morphine-d_3 spectra given in the appendix (App. 3), the molecular ion is still present after fragmentation at 10 ms. In general, spectra taken at 10 ms show more variation in intensities and are therefore slightly less stable. At 30 ms the molecular ion is usually fully dissociated, spectra are found to be stable and achieve high search index values. However, activation time of 50 ms (App. 4) and higher does not enhance the spectrum any further and is otherwise rather time consuming. This was also evaluated for MS^3 experiments, where it showed considerably more impact than with MS^2. For methadone and cocaine, the same response to activation time as for MS^2 was observed. The intensities of the precursor ion varied at 10 ms and were stable from 30 ms on. For the components morphine, doxepine and diazepam the MS^3 spectra taken at 10 ms were significantly unstable. They became reproducible at 30 and 50 ms; however they show differences between 30 and 50 ms (e.g. additional peak at m/z 91,1). Both spectra could however, be identified on the basis of the library spectrum taken at 30 ms. MS^3 spectra taken across the peak at 10, 30 and 50ms are given In the appendix (App.6, 7, 8). Depending on the weight that is put on the intensity relations for comparison with libraries, the impact of activation time can vary.

The number of microscans should not change the spectrum content but can improve statistical certainty of the results. Microscans can be set for full scans and for each order separately via the initial scan features or, alternatively, additional microscans can be applied for a certain method. However, additional microscans (as well as longer activation time) significantly extend the total scantime, which led from a low number of approx. 1500 scans/12 min run (30 ms, 4 microscans for MS^2) up to approx. 5000 scans/12 min run (10 ms and 1 microscan for MS^2). Microscans should be chosen by the principle "as much as necessary, as few as possible". Therefore, one microscan can be considered enough for all full scans and 2 - 3 for MS^2 and MS^3 respectively.

In summary, besides wideband, which changes the content, the greatest impact on spectra comes from the activation time. It can alter the degree to which the molecular ion is fragmented and is followed by the amount of CE applied. The least influence comes from the number of additional microscans. Tested and optimized features and settings are listed in the table below (Tab. 10).

Tested Features	Tested Settings	Optimum Setting
Normalized Collision Energy	20,35, 40, 60 and 200 %	40 %
Wideband	Off/On	On
Additional Microscans	1/2/3/4	Dep. on MS Order
Stepped Collision Energy	Off/On with 20 %, 3steps	On/w 20%, 3 steps
Activation Time	10, 30, 50 and 100 ms	30 ms

Tab. 10: Tested and optimized scan features

The total scantime (resulting from AGC, activation time and microscans) and thus the number of scans/run have significant impact on the efficiency of a screening result. To increase the number of scans/run while at the same time having reliable identification with 30 ms activation time and at least 2-3 microscans for higher order spectra, each sample could be analyzed in two separate runs, one in positive and one in negative mode. Separate sample workup as described by Stimpfl et.al (Stimpfl 2011) provides a good basis for both ionization modes. Additionally, this can help to confirm results as a number of ions can be detected in both modes, which would also lead to a higher IP score. On the other hand, the LTQ Velos is a very fast and sensitive instrument, producing stable scans in positive and negative mode within one run. This is not possible in other instruments (e.g. QqTOF) due to their slower work process and inability to switch quickly between negative and positive. However, specific preparation can also enhance the results, as it would result in a cleaner sample and therefore less interference. Moreover, low dose analytes will then more likely be detected.

Considering all of the above mentioned factors, activation parameters for library spectra creation from neat standard solutions on the LTQ Velos should be set as

follows: activation time 30ms; NCE: 40%±10% (SCE 20%, 3 steps); microscans according to the MS order. Wideband should always be activated. All spectra taken on the instrument under these conditions with different DDA methods were sufficiently reproducible and identifiable.

The two spectra of metoprolol given in the appendix (App. 1) show the very high resemblance of spectra taken within one run, making the comparison with library spectra possible if they are taken under corresponding conditions.

4.1.3. Method Setup

The setup of a MS-method includes the type, amount and order of included scan events, the DDA criteria and parent mass list features. The single substance methods were used to test individual behavior or to include substances into the library. The influence of the amount of scan events was evaluated with four different direct infusion methods, A, B, C1 and C2. A selection of all applied methods is given in the appendix (App. 13), including internal standard mix, Testmix, single substance and MS^3 methods. The full 15 minute HPLC run was not always applied – direct infusion methods where usually run between 3-5 minutes. This ensured stable spray conditions and a sufficient number of reproducible spectra. Methods could be divided into segments, which limit certain setups to a specified timeframe. Each segment can accommodate an individual amount of scans. Especially direct infusion methods were usually constructed for detecting a certain, known m/z. Therefore, no full scan and preceding DDA scans where used but rather simple MS^2/MS^3.

The number of DDA positive and negative scans after each full scan was varied as required.

The mass range for the detection was set to a default value of 128-827.4, unless otherwise stated. This includes all masses on the parent mass list. For MS^2/MS^3 experiments the lower value equals the lower cut-off of the Ion Trap instrument, which is about 1/3 of the precursor mass.

NCE, SCE, activation time and Wideband were varied as required, as well as the parent mass list and the DE settings.

4.2. Direct Infusion Methods

4.2.1. Specific Methods

Details for internal standard and single substance methods are given in the appendix (App.13). Various methods were used to test single features or to screen for individual components. They were applied to estimate RT for the internal standard mix to include spectra into the library, for limit of detection pretesting and for MS^3 experiments. For example, "*BZED3_1*" was run for five minutes, with four scan events. One full scan was followed by three MS^2 scans (directed to the m/z of benzoylecgonine-d_3; 293,3). The scan range was set from 85 to 350. The NCE was set to three different values at 30, 40, and 50 %. Activation time was 30 ms, Wideband was enabled as well as SCE. This method was used to evaluate the changes in a spectrum at three different CEs if SCE is enabled.

4.2.2. Testmix Methods

The under Material and Methods (3.2.3.1.2) described substance mix methods (Method A, B, C1 and C2) were used to test fundamental principles. The indicated full scan to full scan cycle time is only approximate, calculated from the average amount of scans/minute. In general, full MS scans take less time (about 0,09 - 0,1 sec) than MS^2 scans (about 0,6 - 0,8 sec).

4.2.2.1 Method A (Fig. 6) is built up of 14 scans, which is a high but still realistic number of scan events for one cycle. Method A was chosen to show the effect of a rather long cycle time in comparison to method B, which has a very low number of scans. The average full scan to full scan cycle time is approx. (14 scans x 0,25 seconds) 3,5 seconds. With one full positive, 10 DDA scans, followed by one full negative and two DDA scans. The larger fraction for positive ions is due to the natural distribution of basic and acidic drugs, putting more emphasis on the basic compounds. Of the 13 substances included in the Testmix screened with method A, 8 were found manually. Two of the three negatives were detected. The third one (phenobarbital, m/z 231), which had the highest abundance, was not detected, because it was not included in the negative list. This had not much influence on the results, as dynamic exclusion was disabled. Would m/z 321 have been included, one of the other two (m/z 205 Ibuprofen; m/z 343 THCA) would not have been detected instead. Amphetamine (m/z 136) and THC (m/z 315) were never selected for fragmentation, due to 1) their low concentration and 2) their hence low abundant signals. With DE enabled, they could have been detected. The low number of negative scans does make sense when considering the natural basic/acidic distribution of substances, but at the same time it might be a big drawback for coeluting compounds.

Consequently, 14 scans are an inefficiently high number, which is not justified for simple MS^2 screening, where half the number of scans would be sufficient. On the other hand, if half of the scan events are appointed to MS^3, 14 scans in total are an adequate number. Four MS^2 scans and four consecutive MS^3 scans would be available for each positive full scan, and two plus two in negative mode (Fig. 10).

ToxID identified only 7 substances - caused by discordances with the parent mass list and library, as explained later on (4.4).

4.2.2.2 Method B (Fig. 7) was chosen to show the effect of very few scan events per cycle. It is built up of six scans in total and the approximate full scan to full scan cycle time (6 x 0,25 sec) is 1,5 seconds. The full positive scan is followed by three and the full negative by one DDA scans. Naturally, only the four most abundant signals (three in positive mode, one in negative mode) were taken for fragmentation. Therefore, only four compounds were identified manually. Four and three compounds resp. could be identified by ToxID, one of them being metoprolol as it appeared in one scan as the most abundant ion in one single scan.

This method could only be applied with very strict DE settings, and via HPLC. Having a full scan every 1,5 seconds can be an advantage if the peak width is very narrow, but compounds with low intensity will hardly be chosen for fragmentation. Noise and especially isotopes of higher abundant molecules are often selected instead. For example, one isotope of methadone showed the third highest abundance (m/z 310 - methadone and m/z 311) in most full scans. The isotopes should be excluded from screening, e.g. by wider exclusion mass range for DE (m/z 2 instead of 1), but this might at the same time exclude other analytes. This phenomenon did not occur in all experiments with precedent HPLC.

4.2.2.3 Method C1 and C2 (Fig.8) were directly modified based on the method recommended by Thermo Fisher Scientific (Rezai, Kozak et al. 2007). Rezai et al. suggests the application of eight scan events in total. An additional DDA negative scan was included as the original method contained only one. However, three compounds of the Testmix ionized in negative mode. Therefore, especially without HPLC or DE, more than one scan was needed. In general, two DDA scans can be a great advantage, as interferences are always worse for negative ionization. The increase in time consumption is not relevant, especially if the DDA scans are confined to a parent mass list.

Therefore, method C was built up of nine scan events, where the full positive scan is followed by five DDA scans, and the full negative scan by two DDA scans.

In method C2, DE was enabled. This enhanced the method significantly and nine instead of just four analytes could be identified. The following parameters (Tab. 11) were primarily applied:

Repeat count	2
Repeat duration	10,0 sec
Exclusion list size	25
Exclusion duration	60 sec

Tab. 11: DE settings for method C2

The repeat count was set to two to guarantee doubtless results. The repeat duration was of little importance since the mix was directly and continuously infused for five minutes (5 µl/min), the exclusion list size was sufficient because there were only 13 neat standards included. The exclusion duration was chosen because the substances should be continuously rescanned within the five minutes. This does not represent realistic conditions considering combination with HPLC, but that was not the aim of this experiment.

THC, phenobarbital, BZE-d_3 and ibuprofen were not found due to problems with the library (4.4). It is very likely they would have been detected with method C2, if properly included. That would make C2 the only method suitable for screening with direct infusion. However, even for neat standard solutions or non organic samples (e.g. compounds in tablets) HPLC would be a great advantage. Direct infusion can only be a tool for certain analytical questions but not for profound STA. However, most general principles concerning DDA, threshold, parent mass list, etc apply to methods with and without LC in a similar way.

Methods C1 and C2 were consequently used as standard screening methods for most of the hyphenated LC-MS methods. Further consideration had to be put to all questions resulting from the chromatography. At the early stages, the parent mass list was adapted to compensate for problems evolving from HPLC, such as the RT dependent parent mass list. Different DDA and DE approaches were tested and the importance of the threshold was evaluated.

4.2.2.4 "nth Order Double Play" (Fig. 9) Xcalibur offers an additional approach to GUS with the this method, besides the introduced C1/C2 method. Full scan to full scan cycle times could be decreased significantly (average 0,15 sec/scan). This method only works in either positive or negative mode and consists of only two scan events. The first is a full scan and the second is a data dependent scan, which can be set for up to 10 ions. These are then selected as usual depending on the parent mass list or the relative intensities. This method is the fastest method available in Xcalibur. The highest number of scans/run was achieved (5300/12min), with a full scan to full scan cycle time of only 1,48 sec. The regulation options are more limited than with other method setups. As the method is considered to be built up of only two scan events (the second of which is repeatedly performed), the DDA scan features can only be set once. Therefore, NCE, parent mass or reject lists, activation time or additional microscans cannot be adapted

individually. This is of little relevance as all DDA scans usually have a common setup. However, no MS^3 scans can be added and the restriction to a single ionization mode can be a time consuming drawback.

4.3. Special Features

4.3.1 Parent Mass List/Precursor Mass List

For the pre-selection of ions in multi-target screening, a precursor mass list had to be written (syn: parent mass list in Xcalibur). Two general approaches to parent mass list setup were tested. First, the list from the ToxID software was used, containing all precursor masses of approximately 350 small molecules (drugs and pharmaceuticals) in the library. The list usually links substances to certain RTs, meaning that a certain m/z will only be selected if it occurs within the right timeframe. This can also be called a scheduled scan. For the direct infusion and early HPLC experiments the RT frame was irrelevant, and was therefore set from 0-12 minutes. Second, a list including all masses from m/z 128 to 500 was created to simulate a GUS without pre-selection. In the library 128 is the lowest m/z value and only 4 substances have a higher mass than 500. Masses above this value have low significance for small molecules analysis and have therefore been neglected. If a library is big enough to actually have a corresponding spectrum to each of these masses, the focus has to be put on the chromatography and limiting timeframes. A list containing all precursor masses from 128-500 produces a lot of MS^2 spectra from noise which do not lead to identification. The rejection of certain masses within a certain timeframe (e.g. a known contaminant or matrix component) is also available through a reject mass list. Unlike DE it does not bear such a high risk of excluding relevant ions, because the lists should be composed mutually. Ions are not excluded anywhere during the whole run but within a small timeframe.

4.3.2 Data dependent acquisition

Problems with the DDA settings include the number of scan events and the triggering criteria. Masses can be chosen from a list or via the intensities, without the restrictions of a list.

The number of scans per cycle should be chosen considering to the speed of the LC and MS. Too many scans in one cycle can make the overall cycle time to long. While fragmenting noise, upcoming relevant precursor masses might be missed. Short cycle times decrease that risk, but even with very sensible exclusion settings, substances with low intensity may not be detected. However, with only three DDA scan events, only the most abundant masses are taken into consideration. Very short cycles might be an advantage for UHPLC or other very fast LC methods. On the other hand are 14 scans also realistic because the cycle time is still acceptable, especially with fast MS. The importance of positive and negative scans has to be considered similarly to the methods

without LC. The majority of toxicologically relevant small molecules ionizes only or primarily in the positive mode. The scans should be assigned accordingly.

Techniques for DDA regulate whether a subsequent MS^2/MS^3 scan is triggered from a full scan. Predefined criteria direct this work process towards interpretable results. The acquisition process is restrained by a certain threshold and can be enhanced via DE.

In general, scans are triggered by intensities. Any mass has to exceed a given threshold. Furthermore, it has to be either the full scan's most abundant mass, or the most abundant mass from a specified list. An addition to the parent mass list approach is the "most intense if no parent mass found" option. This would detect substances not included on the list if their abundance is high enough. However the spectra would have to be analyzed manually. This can of course be of advantage if reference spectra are included at a later date, making retrospective analysis possible. Compounds not included in the list and only present at low concentrations will still escape the detection. The general aim should therefore be a very large and well established library, combined with the "most intense if no parent mass found" function. If MS^3 scans are also used for identification, the option can be disabled. In this case, the analytes are identified with MS^2, and MS^3 is used to confirm the results. However, if identification via MS^2 is not possible, the MS^3 spectrum will not be included in the library either. For this process, valuable time is wasted. Besides, not every ion produces detectable fragments in MS^3.

With a high-quality library, the pre-selective approach should be preferred, as all taken spectra can be processed and identified. When only small libraries are available, screening via intensities would be performed, as most relevant ions should be present in sufficient concentrations after proper sample workup, and spectra can be manually analyzed by trained personnel. The retrospective analysis might be a good approach for further development of libraries but it is probably no option for clinical toxicology, where the parent mass list approach is preferred.

4.3.3 Threshold

The LTQ Velos can produce spectra even with a very low number of ions, allowing to set a threshold as low as a hundred counts. When setting the threshold, it should be considered that analytes can be present in very low and very high concentrations, besides the strongly interfering matrix. The abundance of the pseudo-molecular ion will not only depend upon it's actual concentration in the sample, but also very much upon the efficiency of the ionization. This process is individual. Two components can be present at the same concentration in the body but only one might be detected. A higher threshold can of course exclude important analytes but can also prevent the method from fragmenting noise. Samples will always contain interfering ions with the same precursor mass as relevant analytes, especially under the use of large libraries. However, the evaluation of the actual threshold should be performed with real matrix-containing samples, and might have to be adapted for each matrix specifically.

4.3.4 Dynamic Exclusion

DE is a very helpful tool to make the most of all methods. It is needed to find components with very low abundance, as the selection of ions for fragmentation is always based on intensities - if possibly confined to a list. Applying DE to any method is a very delicate task; it can enhance any method or completely annihilate it. If a certain m/z is excluded to soon, a substance cannot be identified correctly. This happens if matrix components have the same m/z as an analyte. Moreover if the peak width is rather large and ions are selected for fragmentation at an early stage with low abundance of the ion, unstable spectra may result, which do not allow identification. This can be avoided by setting a sensible threshold.

Four DE parameters can be adjusted; hereby the aim of any specific method has to be considered. Changes in repeat count, repeat duration, exclusion list size and exclusion duration have varying impact on the screening. The exclusion list size is the only parameter with no influence on the screening. It should be large enough to guarantee the full length of exclusion time for any analyte. The factors with direct influence are the average peak width, the amount of scans needed for a reliable result and the signal/noise ratio.

Interferences are a big challenge for screening but should mostly be addressed by means of sample preparation. In neat standard solutions, noise should not be a problem. With biological matrix the interferences can be significant. The benefit of DE for neat solutions and clean samples is rather low. For running the 13 components of the Testmix via HPLC, DE was often obstructive and therefore disabled for all experiments, except those chosen to investigate DE. For example, after exclusion of a certain ion prior to elution of an analyte with the same m/z, the spectrum of the analyte was not recorded. Furthermore, to see how often one analyte is chosen by DDA across the peak, the work process had to run unhindered.

The average peak time in the described system is approximately 20 seconds. For components at very low concentrations such as THC peak time is only about 10 seconds. Considering the overall scan cycle time, only one or two scans are made of such a substance. Identification from one spectrum is possible, but not as reliable as e.g. from 4 scans. If only one scan is performed of a certain m/z, a second run should be performed for confirmation. Excluding a component after two scans can be necessary if the number of compounds in a sample is very high, but it is risky. In some cases, two scans were not sufficient for proper confirmation. Especially if the first scan is performed immediately after the peak begins to rise, the abundance of the compound might not be high enough for a reproducible spectrum. That would leave only one scan for comparison before exclusion. If any interfering ion has the same m/z as an analyte, this particular m/z could be excluded before the peak elutes. The peak can therefore not be assigned correctly; analytes might even be missed completely. The number of repeat counts should therefore be evaluated and chosen according to the matrix and peak width. Tab. 12 shows how many full cycles fit into one peak. The peak width is assumed with 20 and 10 seconds, respectively.

Cycle time/ peak width	20 sec	10 sec
Method A **3,5 s**	5,7	2,85
Method B **1,5 s**	13,3	6,67
Method C **2,25 s**	8,8	4,44
Nth Order double play **1,48 s**	13,5	6,75

Tab. 12: Number of full scan to full scan cycles across one peak

Two scans before exclusion are the minimum; however, a higher number would be favorable. Moreover, if much interference occurs, the repeat count has to be chosen lower than for clean samples (e.g. two vs. four). In general, interference in negative mode is generally more severe and disturbing. The relevant ions often "disappear" among the noise. Hence, the ion is visible in the full scan, but will not be taken for fragmentation within two scans (Method C, five DDA positive scans, two DDA negative scans) because it is not among the most abundant (provided no parent mass list is used). For the 4 most intense ions to be excluded, four full scan to full scan cycles have to be completed (DE as shown above for method C2; 2 repeat counts). 14 seconds (3,5 sec x 4 cycles) can already be too long for smaller peaks, especially considering bad efficiency of ionization in negative mode (e.g. ibuprofen). Therefore, a parent mass list is a great advantage as only relevant ions are chosen for fragmentation. The risk for 5 relevant ions to coelute is very small. In such cases, the chromatography has to be improved.

Wissenbach et al. (Wissenbach, Meyer et al. 2011) show a sensitive approach to DE with 2 repeat counts within 15 seconds and exclusion duration of 15 seconds. They state their average peak width of approximately 30 seconds, which allowed for any ion to be analyzed at least twice per peak. For the default method C on the LTQ Velos the DE settings should therefore be as given in Tab. 13 (average peak width 20 seconds).

Repeat count	2
Repeat duration	10,0 sec
Exclusion list size	Not relevant
Exclusion duration	10 sec

Tab. 13: DE settings for method C2

A simpler approach is the application of DE only to eliminate known disturbing components, such as endogenous cholesterol. A high number of repeat counts (e.g. 8 counts within 30 sec) excludes only very prominent ions or wide peaks. The exclusion duration can be over a minute, possibly two or three. Of course this would exclude possible coeluting analytes with the same m/z.

The more promising alternative is the use of the "reject list". Again, if the matrix includes known interferences, they can be excluded from screening. Moreover this exclusion can be limited to a certain timeframe.

Additionally, an early expiration option can be chosen. If a certain mass occurs too often during it's exclusion duration, it is included again before the time ends.

4.3.5 Additional MS^3 Scans/Higher Order Scans

It was also investigated if additional MS^3 scans could enhance the screening quality. When a spectrum contains only one significant product ion, even with Wideband activated, the information is low. Here, a MS^3 spectrum may enhance the results. This might also help to differentiate between two co-eluting substances. Three general method setups are shown under 3.2.3.2.

In method A half of the positive scan events were transformed into MS^3 scans. Thus, in positive mode the full scan is followed by four DDA MS^2 scans and four consecutive MS^3 scans to confirm the obtained information. This can be an advantage for very simple spectra (e.g. one single product ion peak). Furthermore, it can aid to distinguish between two analytes, if a mixed spectrum from 2 precursor ions of the same mass is obtained.

There are two ways of determining which ion from the MS^2 scan is selected for the subsequent MS^3 scan. Similar to MS^2 screening, the "most intense" ion or the "most intense ion from the list" can be selected.

The "most intense ion" to collects MS^3 data for every MS^2 scan, from the most intense of the original MS^2 fragments. This is time consuming, especially for ions not producing an MS^3 spectrum. Due to variation in intensities of the fragments (especially if two fragments are nearly equally abundant), the wrong precursor might be chosen for MS^3, making an identification impossible, maybe even leading to a false negative result. This phenomenon was observed, for morphine-d_3. At the rise of the peak the abundance of the ion was very low and the spectrum randomly varied in intensity ratios. The produced spectrum was not identified. However, a second spectrum was produced later on the same peak. This spectrum was more stable, the overall abundance higher and the ratios were therefore correctly correlating with the library spectrum.

The parent mass list had to be expanded by the MS^3 precursor ions; hence a combined MS^2 and MS^3 parent mass list was used. Substances not producing an MS^3 spectrum can be neglected. Unfortunately, all masses included for MS^3 scans are also available parent masses for MS^2 scans, as they are combined in one list. This makes the selection less precise, as noise might be chosen for fragmentation because an MS^3 ion has the same m/z. Improvement could come from scheduled scanning.

A third approach is to make changes in the parent mass list, instead of in the method setup. Hereby, all scan events are designated MS^2 scans. However, on the global parent mass list, the m/z of MS^2 fragments are assigned to their specific precursor. Xcalibur allows this up to MS^3 only. Treatment with individual collision energy for certain substances is also possible, but MS^2 data is lost for the selected precursors. If a certain ion is included into the list twice (e.g. morphine-d_3 with 289,0 and 289,1), MS^2 and MS^3

data are available but the handling of the list becomes more laborious - one of the major drawbacks of Xcalibur.

A schematic image of the Parent mass list is given in Tab. 14.

Mass	Retention time	MS^2 precursor	CE	Name
289	0,5-1,5min	144	40%	Morphine-d_3
289,1	0,5-1,5min			Morphine-d_3

Tab. 14: Global parent mass list in Xcalibur, schematic image

Unfortunately, the search interface ToxID was only able to identify spectra taken with designated MS^2 and MS^3 scan events (e.g. method A), with the "most intense ion from the list" approach. In general, ToxID allows a search for MS^2, MS^3 or combined MS^2&MS^3 spectra, respectively, if taken with the correct method. The search interface SmileMS on the other hand performs the library search with one of the three above mentioned approaches.

Apart from the above mentioned methods, an approach where the MS^3 scan is only triggered when needed, without losing MS^2 information and without generating a great amount of extra work would be desirable.

4.4 The Spectral Library

The quality of the spectral library is of utmost importance for the quality of the screening. Problems with transferring libraries between different laboratories or instruments have been the major drawback for LC-MS. Would the ionization process be as stable as with EI in GC-MS, LC-MS might already be the new gold standard for forensic toxicology. Features such as NCE or Wideband have significantly enhanced the reproducibility, although not to the same level as EI. The largest libraries available for LC-MS include up to 1500 substances, the largest library for GC-MS contains over 200.000 entries –including metabolites and adducts.1500 substances certainly cover most incidents and serve most needs; however, especially in forensics the unexpected and even very unlikely always has to be considered.

Spectra can differ to a variable degree when taken under alternating conditions, other instruments or matrices. Next to spectrum creation, the library management and search process is equally important. Before the development of "Stepped Collision Energy" (Thermo Fisher Scientific) or "Collision Energy Spread" (AB Sciex) a common approach was the inclusion of more than one spectrum per substance at different collision energies. These spectra were either saved separately or reconstructed to one spectrum (Sauvage, Saint-Marcoux et al. 2006). It has to be underlined that spectra produced in an Ion Trap cannot be compared to those recorded in a quadrupole collision cell due to the different ways of fragmentation. Libraries for high resolution single MS experiments

can even consist of theoretical data only as they represent the exact masses. Only data about RT or fragmentation has to be determined experimentally.

Thermo provides a 329 component library with the "ToxID" software. However, not all of the settings for recording the spectra for "Tox_Library" are stated. Upon inquiry, the settings were provided. Following conditions had been applied:

- Solution concentration: 10 mg/L
- Solvent mixture: 70:30 of H_2O with 10mM ammonium formiate and 0.1% formic acid; and ACN with 0.1% formic acid
- Flow rate: 10 µL/min;

Information provided in the library is rather poor. No synonyms, CAS-number, structure or molecular formula and no information about the instrument or the solvent for recording the spectrum are given. Upon inquiry, spectrum creation at different instruments (LCQ, LXQ and LTQ) was disclosed and Thermo stated that due to the different geometry of the traps, the NCE needed can differ from default (35%). Therefore, the LTQ Velos might need slightly more NCE (e.g. 40%). This was ultimately adopted for the default method. When comparing the different NCE, especially when SCE is enabled, the differences between spectra do not significantly influence the search results, although individual spectra may vary more. It can be assumed that the spectra might not be comparable between traps of different manufactures.

Much more discrepancy originates from the HPLC method. When developing a new method, all RT included in the library have to be checked and validated as the search is strongly dependent upon RT. RT are strongly dependent upon the used column, solvent, and other factors. This issue will be more closely discussed under 4.6 HPLC.

The solvent mixture is of relevance as the spectra vary between neutral (e.g. methanol) or acidic solvents (with glacial acid, formic acid), and between sources and instruments. Spectra were well comparable after adaption of activation settings. In general, spectra produced by direct infusion always bear the potential risk of varying from the ones taken via HPLC. An example for this phenomenon is given below (Fig. 14). The upper spectrum of THC was taken in methanolic solution (direct infusion) while the lower spectrum was from the library. This may be due to the missing H^+ ions needed for ionization. However, the abundance of these spectra is equal (9,99 E2 = $9{,}99*10^2$).

Spectra to be included in the library should therefore be taken under conditions as close to the actual method as possible.

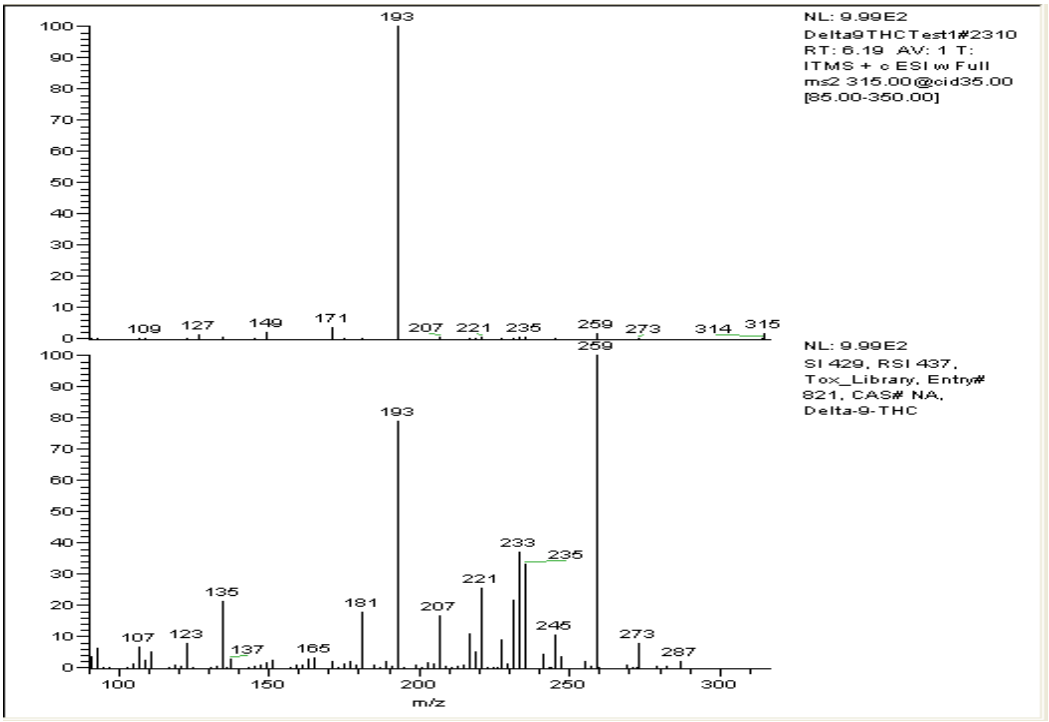

Fig. 14: THC spectrum in methanolic solution and Tox_Library spectrum

4.5 Screening Software: SmileMS vs. ToxID

Two available screening software packages were assessed. SmileMS (GeneBio) and ToxID (Thermo Fisher) were both used to process collected data and search the available library (Tox_library, Thermo Fisher). Besides the issues mentioned above, the two software packages were evaluated for their practicability and applicability.

In ToxID, all spectra are included in an excel sheet. The parent mass, one product ion mass, intensity threshold, Search Index (SI) and Reverse Search Index (RSI) have to be entered individually. While this enables unique criteria for each substance, the handling of the excel sheet is strenuous. There is no automatic update option for new library entries and no feature to easily manage and change data sets. Upon inquiry no easier solution to manage data and no convenient interface to run screenings could be provided. Additional search criteria can only be applied for each search request specifically. In the ToxID "setup" page the search can be limited with regard to m/z and RT. The software then produces a report showing all identified substances. For each substance, all scans which match a library entry are listed on a chromatogram. However, only the scan with the highest abundance is shown in the "Long Report" and can be checked manually. Beyond that, no further intervention or review of the results is possible. The program Xcalibur contains a library manager which allows manual analysis of spectra.

SmileMS allows the user to review the data and adapt exclusion parameters before creation of the report. All matching spectra are available for manual check. Moreover, SmileMS has a “library manager”, which can import any number of libraries from different providers. It saves all associated spectra under the name of the corresponding compound. MS^2 scans as well as MS^3 lead to the same entry. Apart from these more sophisticated features, the visual appearance is more appealing and the handling more user-friendly.

4.6 HPLC

The optimization of the conditions for the liquid chromatography was not the aim of this work. To develop a full LC-MS screening method however, chromatography is of great importance. The type of column, the solvent composition, the runtime, flow rate and gradient etc. have to be considered. Stability, robustness and other factors have to be tested. The establishment of a stable system would have gone beyond the scope of this work. However, some thoughts into fundamental issues concerning chromatography are compiled here. As the identification of substances always depends upon RT, shifts can be a problem. A possible system to predict the shift of analytes with an Excel calculation was developed (3.2.4.1).

For the 13 substances of the Testmix a shift in RT simulated by a flow of 0,4 ml/min instead of 0,3 ml/min was well predicted (Tab.15; Fig. 15) linear behavior was assumed.

D	E	F
	standard RT	calculated RT
Morphine	0,8	0,71
Amphetamine	1,75	1,49
Codeine	1,9	1,61
BZE-D3	4,05	3,38
Phenobarbital	4,45	3,71
Metoprolol	5,16	4,29
Cocaine	5,57	4,63
Doxepin	6,34	5,26
Diazepam	6,38	5,29
Methadone	6,78	5,62
THCA	7,03	5,82
THC	7,58	6,28

	0,4 µl/min
Morphine	0,72
Amphetamine	1,42
Codeine	1,85
BZE-D3	3,37
Phenobarbitone	3,58
Metoprolol	4,22
Cocaine	4,56
Doxepin	5,21
Diazepam	5,27
Methadone	5,6
THCA	5,96
THC	6,43

Tab. 15: Original RT (0,3 ml/min); Calculated RT (0,4 ml/min); Measured RT (0,4 ml/min)

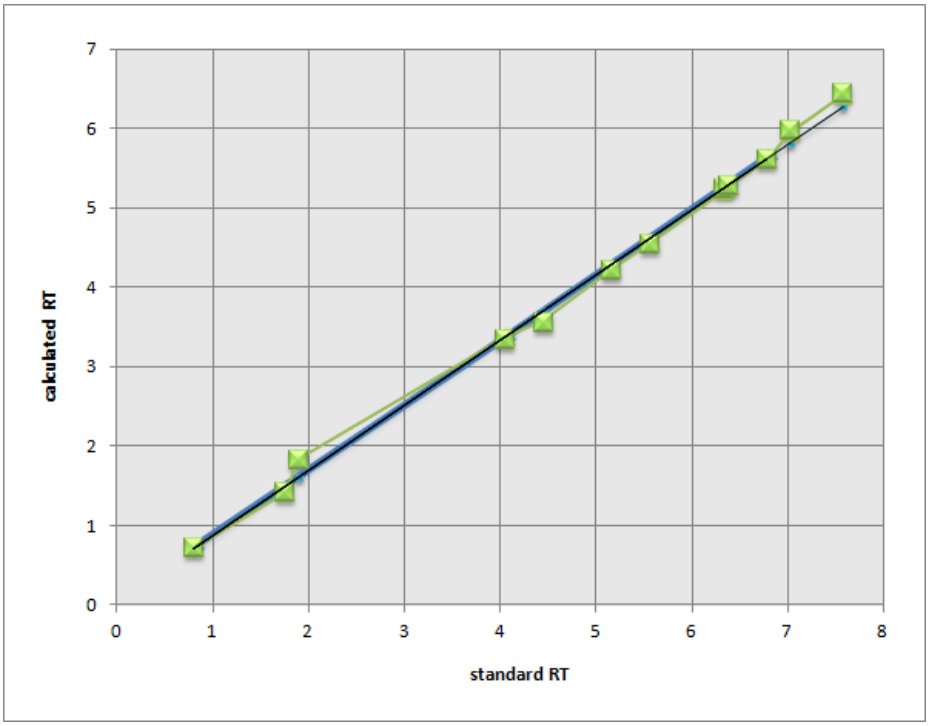

Fig. 15: Linearity of RT shifts; blue: calculated trend; green: measured values

4.7 Limit of Detection - Pretests

In order to support future limit of detection evaluations, pretests were run at different dilutions. The components of the Testmix were originally present at different concentration levels: 5µg/ml, 50µg/ml and 500µg/ml, respectively, as mentioned under Material and Methods (3.2.5). The Solution was diluted 1:10, 1:100 and 1:1000.

The applied method was method C. Threshold was set to 100 counts, and DE was disabled. All components could be identified up to a dilution - factor of 1:100. The search index and reverse search index (criteria for the matching with the library spectra) did not decrease with the lower concentration, although less scans were performed for each substance and therefore less information was available to confirm identification. Amphetamine was the substance with the lowest counts (222), followed by THC (418 counts). The threshold of 100 counts is presumably too low for matrix-containing samples. One component had an intensity over 1000 (ibuprofen 1717 counts) and all others were far over 10.000 counts.

At 1:1000 only seven components were identified, with phenobarbital and morphine at 2761 and 3631 counts, respectively. All other identified components showed over 10.000 counts. The TIC (Fig. 16) does not show the significant peaks anymore, only methadone still had a very good signal/noise ratio. The substances were still identified because they were included in the parent mass list.

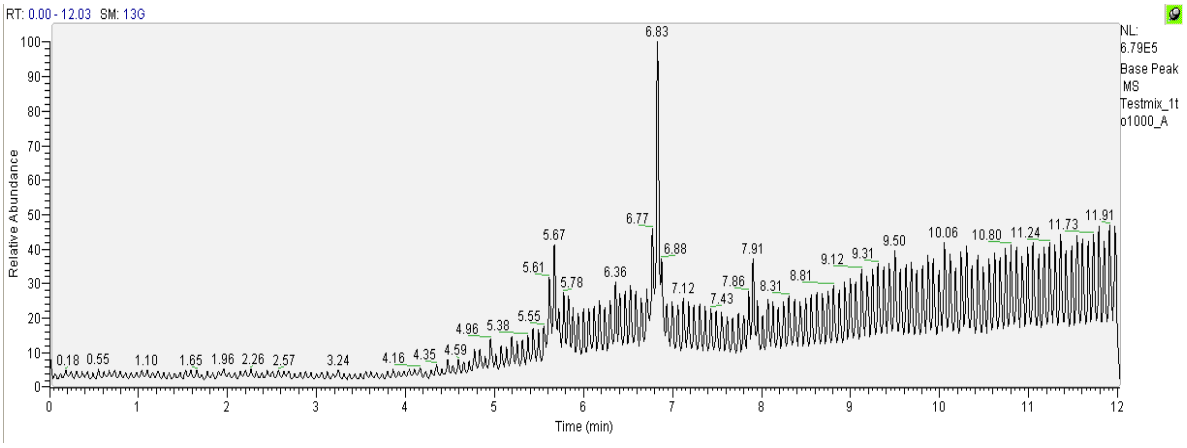

Fig. 16: TIC of the 1:1000 dilution

Substance	Orig. conc	1:10	1:100	1:1000
Amphetamine	0,05mg/ml	●	●	-
Benzoylecgonine-d_3	0,05mg/ml	●	●	●
Cocaine	0,05mg/ml	●	●	●
Codeine	0,05mg/ml	●	●	-
Diazepam	0,05mg/ml	●	●	●
Doxepine	0,05mg/ml	●	●	●
Ibuprofen	0,5mg/ml	●	●	-
Methadone	0,05mg/ml	●	●	●
Metoprolol	0,05mg/ml	●	●	●/-
Morphine	0,05mg/ml	●	●	●
Phenobarbital	0,5mg/ml	●	●	●
THC	0,005mg/ml	●	●	-
THCCOOH	0,005mg/ml	●	●	-

Tab. 16: LOD of Testmix components; original concentration in the Testmix, and detection via ToxID in dilutions:

Without the list, the identification would probably not have worked at the lowest concentration, similar to matrix-containing samples. The negative scan events show the need for a list as well. The molecular ion of phenobarbital (m/z 231) elutes around 4.4

minutes but it is selected for fragmentation during the whole twelve minute run. Although m/z 231 disappears among the noise before and after the peak, it is still selected because the ions included in the list are preferred.

The limit of detection does not correlate directly with the concentration of the analyte, as the ionization behavior has great impact. As an example, ibuprofen was present in higher concentrations than methadone but was not detected in the 1:1000 dilution.

4.8 Real Samples

4.8.1 Powder

A brown powder which was initially sold as heroine was analyzed by HPLC-DAD. Strong evidence was found for alprazolam, paracetamol, caffeine, and brucine. The sample was then analyzed by LC-MS (Method C) and additionally by GC-MS and LC-QqTOF-MS.

Since the library did not include the very uncommon component brucine (Fig. 17) initially, the search was not limited by the parent mass list but rather with the 128-500 approach. After analyzing a brucine/quinine standard (1mg/ml in MeOH) and adding MS^2 and MS^3 spectra to the library, the spectra were manually compared and the results confirmed (App. 9).

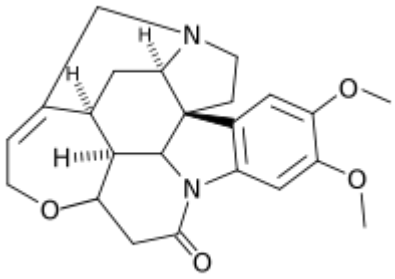

Fig. 17: Brucine

Knowing the molecular weight of brucine, MS^2 was performed on the specific mass of the standard and the sample in the same way (App. 10), leading to semiquantitative results. Brucine was found at approx. 100mg/g (UV/VIS: 104mg/g), an unexpected result for a heroine sample.

Additionally to brucine, alprazolam, paracetamol and caffeine, phenolphtalein was detected in LC-MS and GC-MS. Domperidone was only detected in LC-MS. Traces of strychnine were only found in GC-MS. High resolution data confirmed these results.

4.8.2 Urine

The urine of a patient with suspected ecstasy intoxication was diluted 1:1000 and analyzed with Method C (no DE, parent mass list from 128-500, RT frame from 0-12). Based on the results of HPLC-DAD and comparison with the library the unknown compound was identifies as the designer drug 4-Methylethcathinone (4-MEC; Fig.18)

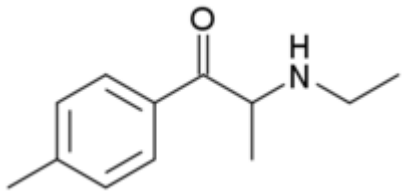

Fig. 18: 4-Methylethcathinone

Following components were identified via LC-MS: amiodarone, fentanyl, furosemide, lidocaine, midazolam and nicotine. An additional, high abundant peak was found at 1.4 minutes. As the substance was not included in the Tox_Library, a reference substance (indicated by the prior HPLC-DAD analysis) was used for identification. The data given in the appendix (App. 11) shows the total ion chromatogram of the urine as well as the full MS of the unknown peak (App. 12), showing a high abundant peak at 192 [$M+H^+$]. The chemical formula of 4-MEC is $C_{12}H_{17}NO$ with a nominal mass of 191.

For analysis by GC-MS, the sample was extracted (liquid-liquid) with Dichlormethan, pH 8 and additionally acetylated or silylated. The component was confirmed as 4-MEC.

5 Abbreviations

ACN	Acetonitrile
AGC	Automatic Gain Control
API	Atmospheric Pressure Ionization
APCI	Atmospheric Pressure Chemical Ionization
BZE	Benzoylecgonine
CE	Collision Energy
CID	Collision Induced Dissociation
DAD	Diode Array Detection
DDA	Data dependent Acquisition
DE	Dynamic Exclusion
EMA	European Medicines Agency
EPI	Enhanced Product Ion
ESI	Electrospray Ionization
GUS	General Unknown Screening
HPLC	High Performance Liquid Chromatography
ICH	International Conference on Harmonisation
IP	Identification Points
LIT	Linear Ion Trap
MeOH	Methanol
MRM	Multiple Reaction Monitoring
MRFA	L-methionyl-arginyl-phenylalanin-alanin acetate * H_2O
MS	Mass Spectrometry; Mass Spectrum
NCE	Normalized Collision Energy
PI	Product Ion
PFP	Perfluorophenyl

QqLIT	Quadrupole- Linear Ion Trap hybrid
QqQ	Quadrupole, here: Triple Quadrupole
RT	Retention Time
SCE	Stepped Collision Energy
SIM	Selected Ion Monitoring
SRM	Selected Reaction Monitoring
STA	Systematic Toxicological Analysis
TIAFT	The International Association of Forensic Toxicologists
TIC	Total Ion Current/Total Ion Chromatogram
THC	Delta-9-Tetrahydrocannabinol
THCA	11-nor-9-Carboxy-THC
TOF	Time of Flight detector

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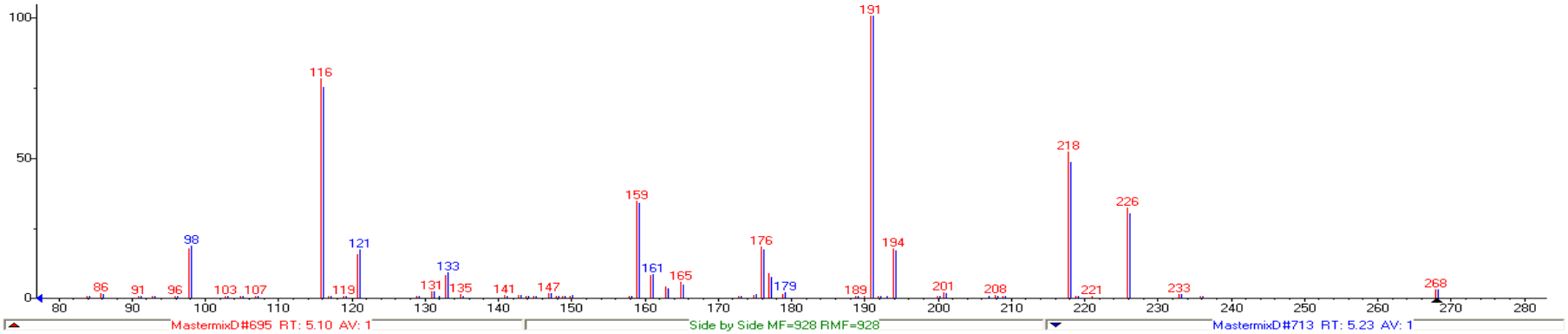
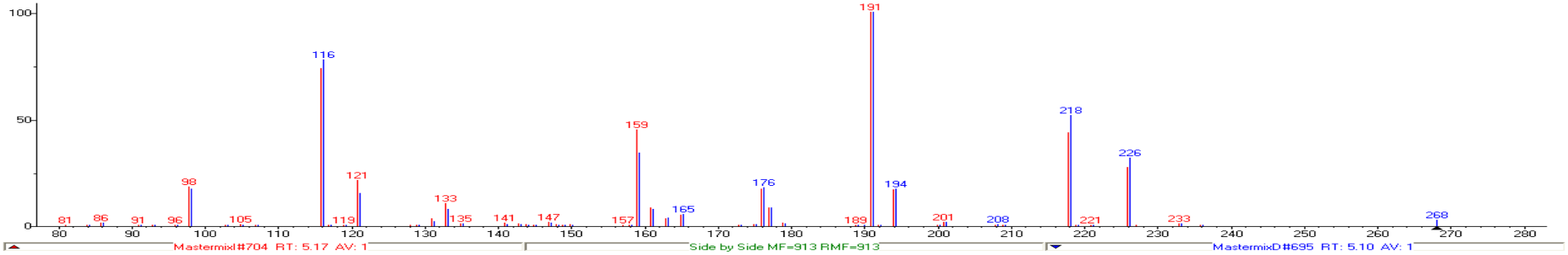
7 Tables and Figures

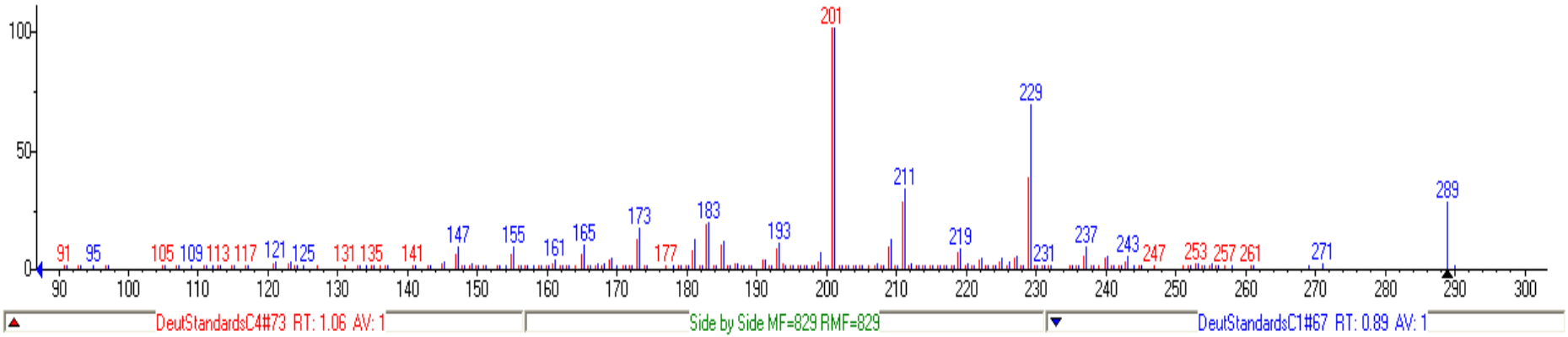
8. Appendix



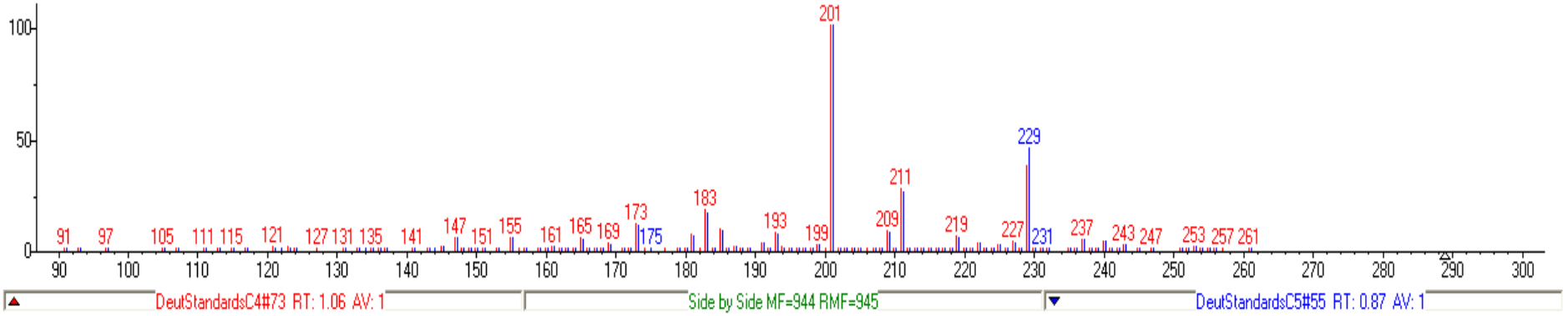
App. 1: Two Metoprolol Spectra (red and blue) taken in one run, across the peak, with the default method (40% ± 10%NCE; 30 ms)



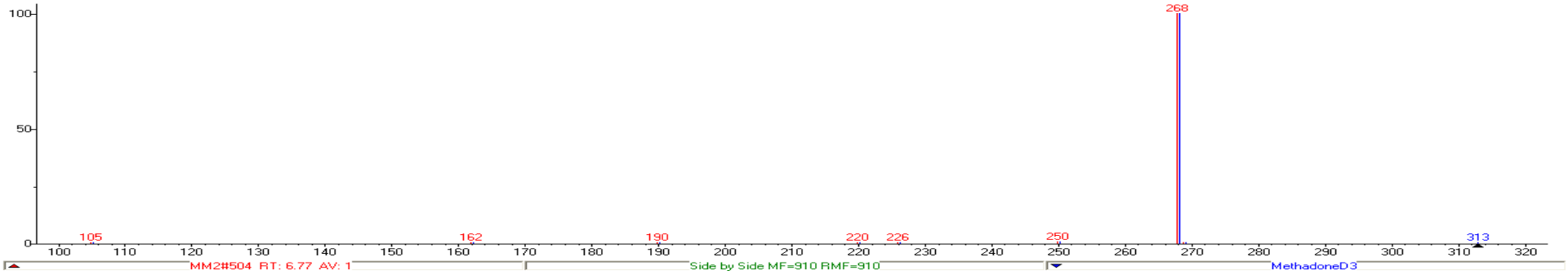
App. 2: Metoprolol spectra taken at NCE 35% (blue) vs. 40% ± 10% (default, red)



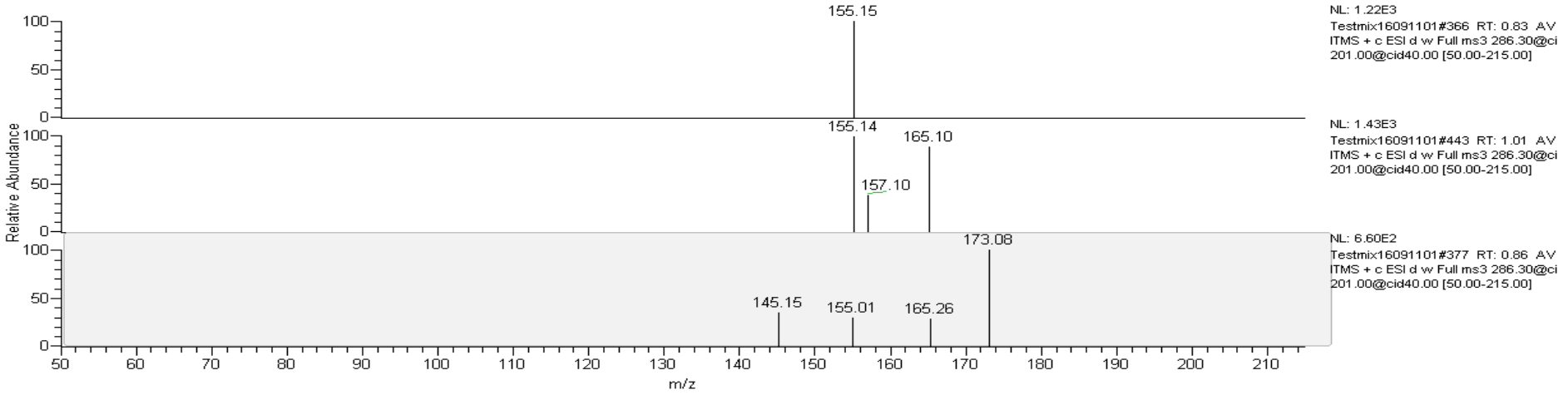
App. 3: Morphine-d_3 taken at 10 ms activation time (blue) and 30 ms activation time (default, red)



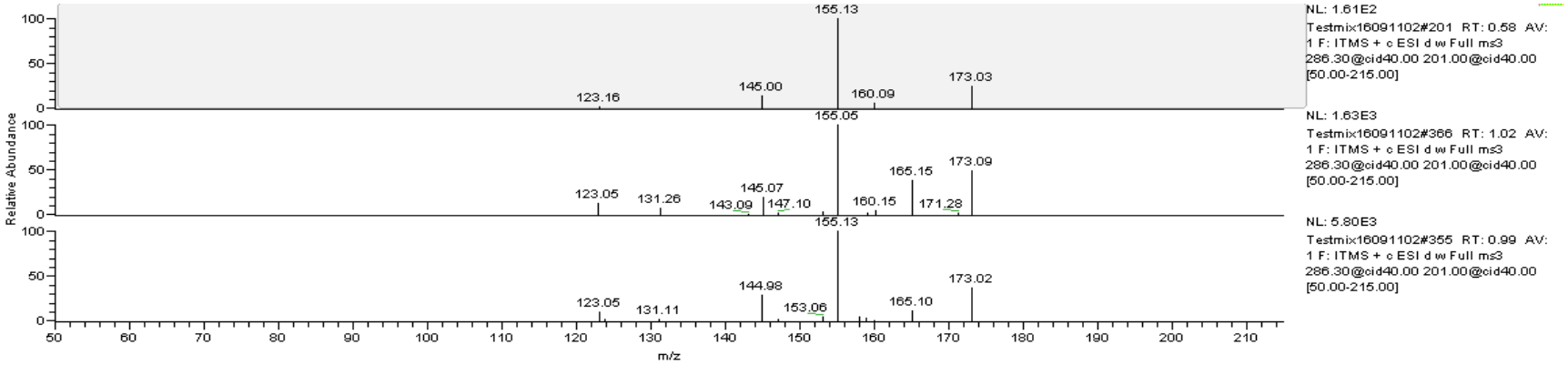
App. 4: Morphine-d_3; taken at 50 ms activation time (blue) and 30 ms activation time (default, red)



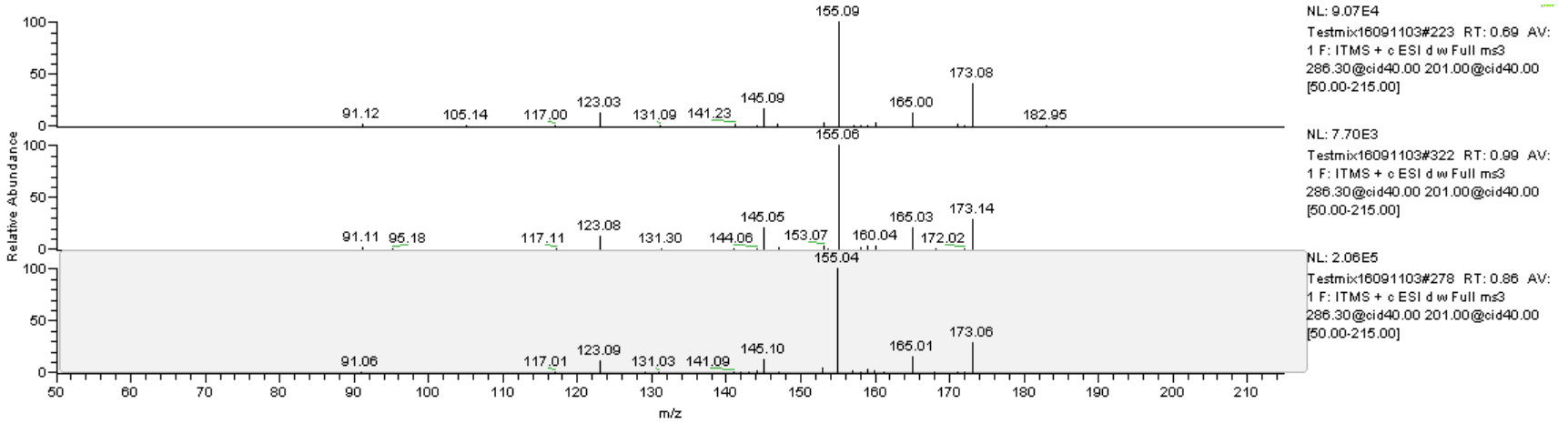
App. 5: Methadone-d_3 spectra taken at NCE 35% (blue) and 40% (red)



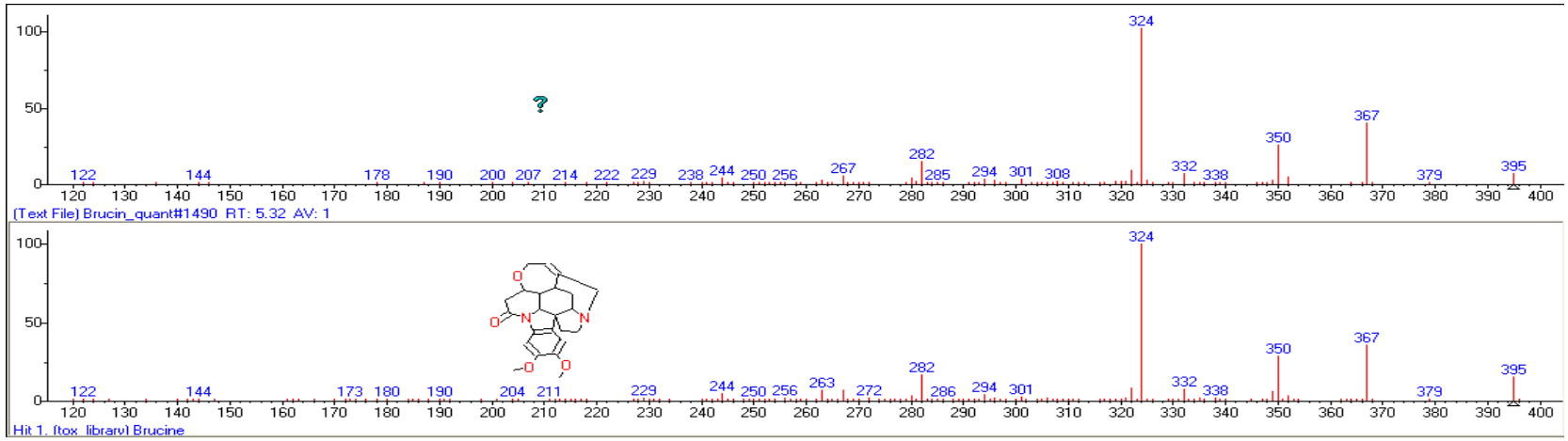
App. 6 :Morphine-d_3, three MS^3 spectra taken with 10 ms activation time across the peak (RT 0,84 min)



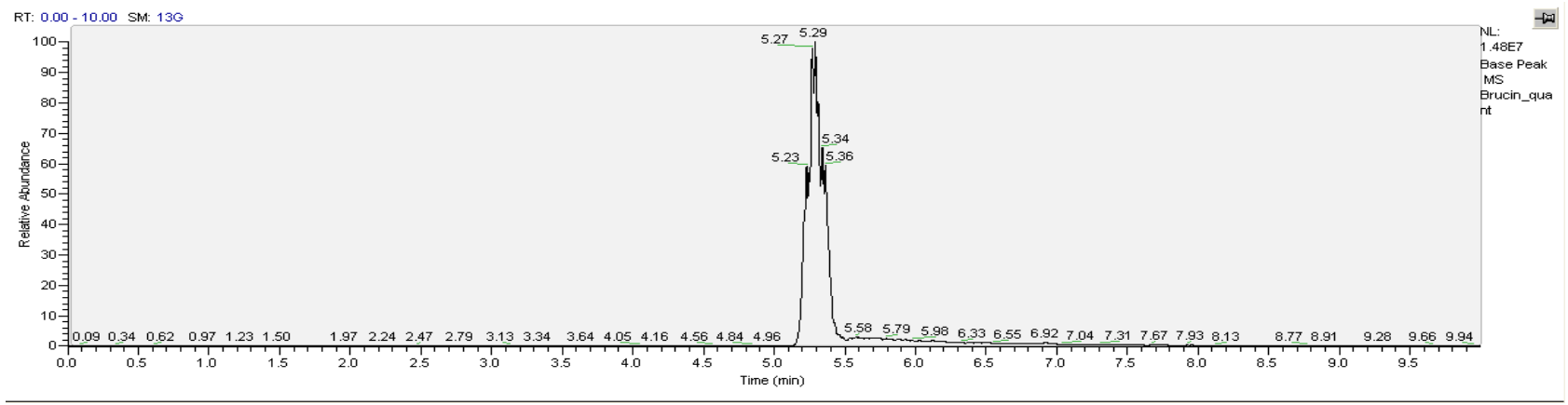
App. 7: Morphine-d_3, three MS^3 spectra taken with 30 ms activation time across the peak (RT 0,84 min)



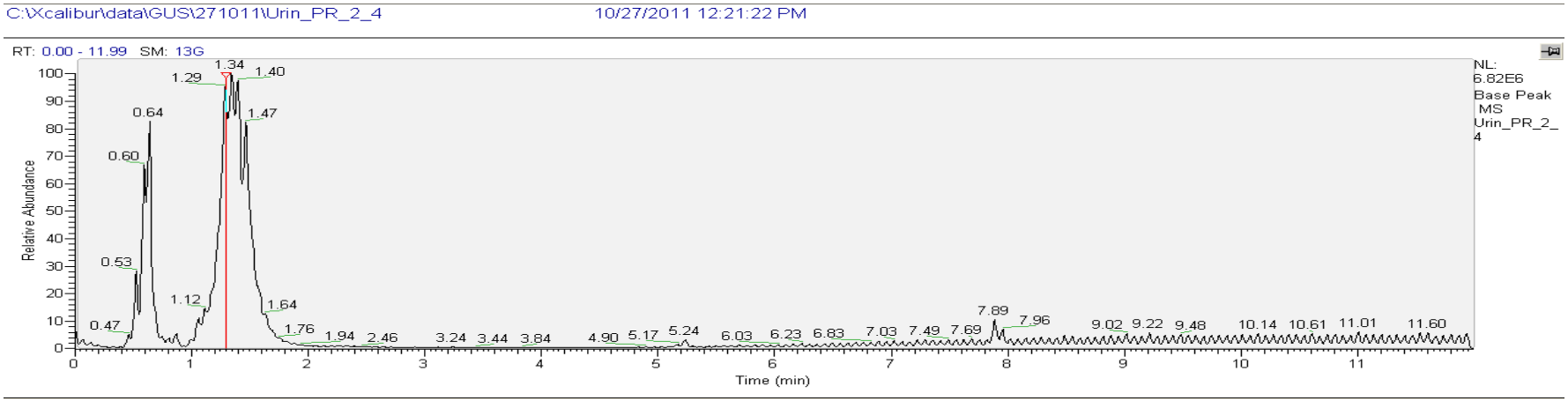
App. 8: Morphine-d_3, three MS^3 spectra taken with 50 ms activation time across the peak (RT 0,84 min)



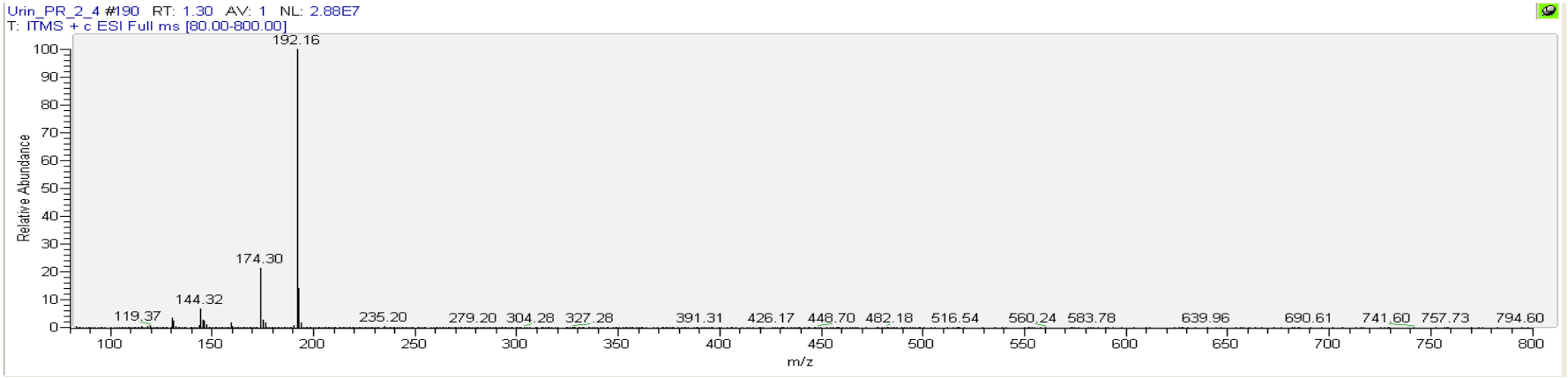
App. 9: Spectrum of brucine in powder sample (4.8.1) and Tox_Library spectrum



App. 10: Total ion chromatogram of brucine (MW: 394,47)



App. 11: TIC of the urine sample containing 4-MEC



App. 12: Full mass spectrum of the peak at 1,3 min

Name	HPLC precedent	runtime (min)	Segments	scans per segment	Type (DDA, MS2)	Fullscan included	DDA Scans	Range	NCE (%)	Acttime	Wideband	Parent Mass List	Step	Dynamic Exclusion
Internal Standard methods														
3standards	y	12	1	3	MS2	n		75-350	40	30	+			
3standardsB	y	12	1	3	MS2	n		75-350	40	50	+			
3standardsC	y	12	1	4	MS2	y		75-350	40	30	+			
Testmix methods														
GUS_DD012a	n	3	1	14	DDA	y	+10-2	default	35	30	+	012		
GUS_DD	y	12	1	9	DDA	y	+5-2	default	35	30	+	default		
GUS-DD012b	n	3	1	6	DDA	y	+3-1	default	35	30	+	012		
GUS-_DD_012step	y	12	1	9	DDA	y	+5-2	default	35	30	+	012	y	
GUS_DD_012stepDE	y	12	1	9	DDA	y	+5-2	default	35	30	+	012	y	5/10/50/30
GUS_DD_01240step	y	12	1	9	DDA	y	+5-2	default	40	30	+	012	y	
GUS_DD_01245	y	12	1	9	DDA	y	+5-2	default	45	30	+	012	n	
GUS_DD_01245step	y	12	1	9	DDA	y	+5-2	default	45	30	+	012	y	
GUS_DD_02040step	y	20	1	9	DDA	y	+5-2	default	40	30	+	020	y	
GUS_DD_RT	y	12	1	9	DDA	y	+5-2	default	40	30	+	default	y	
GUS_DD_RTDE	y	12	1	9	DDA	y	+5-2	default	40	30	+	MS^3	y	2/5/50/10
GUS_DD_testlist012	y	12	1	9	DDA	y	+5-2	default	35	30	+	012	n	
GUS_DD_testlist012Her1	n	5	1	9	DDA	y	+5-2	default	35	30	+	012	y	2/15/50/30
GUS_DD_testlist0120500	y	12	1	9	DDA	y	+5-2	default	40	30	+	012/128-500	y	
GUS_DD_testlist0120500A	n	3	1	9	DDA	y	+5-2	default	35	30	+	012/128-500	y	2/15/50/60

GUS_DD_testlist0120500AHer2	n	5	1	9	DDA	y	+5-2	default	35	30	+	012/128-500	y	2/15/50/30
GUS_DD_testlist0120500DE	y	12	1	9	DDA	y	+5-2	default	40	30	+	012/128-500	y	2/10/50/15
Master_RT	y	12	4	3/2/2/5	MS2	n		70-350	40	10	+		y	
Master_RT_DDA	y	12	4	3/4/3/4	MS2	y		75-350	40	10	+	exclparent	y	
Methanol	n	10	1	1		y	+	300-550						
Single Substance Methods														
BZED3	n	5	1	1	MS2	n		80-300	35	10	+		n	
BZED3_1	n	5	1	4	MS2	n		85-350	30/40/50	30	+		y	
Delta9THC	n	5	1	3	DDA/MS2	y	+1/MS2	85-350	40/35	30/10	+		y	
Delta9THC2	n	5	1	4	MS2	y		85-350	30/40/50	30	+		y	
Diazepam	n	5	1	1	MS2	n		75-300	35	10	+		n	
Diazepam_1	n	5	1	4	MS2	y		75-350	30/40/50	30	+		y	
Ibuprofen	n	12	1	2	MS2	y		55-250	35	10	+		y	
Ibuprofenb	n	5	1	4	MS2	y		55-250	35	10	+		n	
MetDia	n	5	1	3	MS2	y		70-300	35	10	+		n	
MetDiaB	n	3	2	3/3	MS2/DDA	y	MS2+/+2	70-300	40	10	+		y	
MetDiaC	n	2	1	3	DDA	y	+2	70-300	35	10	+	exclParent	n	
MethadonD3	n	5	1	1	MS2	n		85-350	35	10	+		n	
Metoprolol	n	5	2	1/1	MS2	n		70-300	35/40	10	+		n	
MetorprololDIL	n	5	1	1	MS2	n		70-300	35	10	+		n	
Metorprolol_1	n	5	1	4	MS2	y		70-350	30/40/50	30	+		y	
Morphin	n	5	1	4	MS2	y		75-350	30/40/50	30	+		y	
MorphinD3	n	5	1	3	MS2	y		75-350	40	10/30	+		y	
Phenobarbital	n	5	1	4	MS2/3	y		50-300	40(20/40)	30/10	+		y	
MS3 Methods														**DDA**
GUS_MS2_3	y	12	1	14	MS2/3	y	+4/4-2/2	default	40	30	-	default	y	MI

GUS_MS2_3w	y	12	1	14	MS2/3	y	+4/4-2/2	default	40	30	+	default	y	MI
GUS_MS2_3w_A	y	12	1	14	MS2/3	y	+4/4-2/2	default	40	30	+	default	y	MI - fL
GUS_MS3	y	12	1	9	MS3 (2)	y		default	40	30	+	MS3 in List	y	

App. 13: Methods used for different experiments

Step: always 20%, 3steps;
Range: default = 128-827.4
DDA Scantype: +/-: full pos, full neg scan, numbers give the amount of according DDA scans.
Parent list features:
default list: list with all parent masses, including correct RT, seperate pos/neg
128-500: For simulation of GUS, all masses from 128-500 included (small molecules range)
012: referring to RT frame, set from 0-12 minutes, therefore no scheduled scans.
exclparent: exclusive parent list, only specified precursor masses included
DDA: triggered by: *MI*: Most Intense*, MI - fL*: Most Intense from List

21.07.2011						
Testmix	A	B	C	D	E	F
Morphine	0,84	0,83	0,84	0,84	0,84	0,84
Amphetamine	1,75	1,75	1,745	1,76	1,75	1,76
Codeine	1,89	1,94	1,88	1,92	1,88	1,92
BZE-D3	4,04	4,04	4,1	4,08	4,02	4,04
Phenobarbital	4,44	4,46	4,45	4,515	4,43	4,45
Metoprolol	5,16	5,17	5,16	5,175	5,15	5,17
Cocaine	5,56	5,57	5,575	5,585	5,59	5,57
Doxepin	6,35	6,32	6,335	6,365	6,35	6,32
Diazepam	6,35	6,32	6,385	6,39	6,39	6,4
Methadone	6,75	6,76	6,7	6,765	6,74	6,75

THCA	7,05	7,02	7,03	7,04	7,04	7,05
THC	7,56	7,57	7,56	7,56	7,55	7,75
02.09.2011					**16.09.2011**	
Testmix	G	H	I	J	K	L
Morphine	0,82	0,87	0,85	0,86	0,84	0,79
Amphetamine	1,82	-	1,72	1,72	1,75	1,74
Codeine	2,06	-	1,87	1,96	1,93	1,82
BZE-D3	4,25	4,05	4,11	4,01	4,09	4,05
Phenobarbital	4,48	4,38	4,39	4,36	-	-
Metoprolol	5,18	5,19	5,22	5,15	5,22	5,16
Cocaine	5,66	5,66	5,62	5,6	5,67	5,62
Doxepin	6,36	6,35	6,35	6,31	6,36	6,34
Diazepam	6,37	6,35	6,39	6,39	6,39	6,35
Methadone	6,84	6,82	6,79	6,77	6,78	6,77
THCA	7,04	-	7	7,02	-	-
THC	7,59	-	7,55	7,55	7,54	7,54
Ibuprofen	6,53	-	6,51	6,48	-	-

App. 14: RT on three different days for all Testmix components

Zusammenfassung

Die Entwicklung neuer Methoden für die Systematisch-Toxikologische Analyse (STA) ist ein fortwährender Prozess, sowohl in forensischer als auch in klinischer Toxikologie. Die Entwicklung neuer Methoden, basierend auf LC-MS, ist bereits seit mehr als einem Jahrzehnt im Fokus der Forschung. Um Routinemethoden zu etablieren, müssen alle Faktoren, die ein Screening beeinflussen, bekannt sein. Angefangen bei dem Ionisierungsprozess über das Erfassen eines Spektrums bis hin zu der verwendeten Referenz-Bibliothek müssen viele Faktoren evaluiert werden. Des Weiteren ist die Reproduzierbarkeit und Übertragbarkeit der Spektren ein Thema, für das bisher kein völlig befriedigendes Ergebnis gefunden wurde.

Einleitung: Für die Entwicklung einer Routine Screening Methode wurde eine lineare Ionenfalle, in Kombination mit HPLC und der „Tox_Library“ verwendet. Hauptaugenmerk wurde auf alle Parameter, die ein Screening beeinflussen, gelegt. Dazu gehören sowohl die instrumentellen Parameter als auch die Punkte, die mit der Spektrenbibliothek in Zusammenhang stehen. Das Ziel dieser Arbeit war, jene Faktoren auf dem verwendeten Gerät zu identifizieren, die beachtet werden müssen, wenn eine LC-MS Methode für die Systematisch-Toxikologische Analyse etabliert werden soll.

Material und Methoden: Das Massenspektrometer “LTQ Velos” (Thermo Fisher Scientific) wurde zusammen mit einer RS 300 Ultimate HPLC (Dionex) für diese Arbeit verwendet. Ein Testmix aus 13 toxikologisch relevanten Substanzen wurde für individuelle Experimente herangezogen. Deuterierte Standards wurden verwendet, um Veränderungen im chromatographischen System zu detektieren. Einzelne Substanzen oder Substanzmischungen wurden entweder direkt infundiert oder via on-line HPLC eingebracht. Vorversuche für Limit of Detection Tests wurden durchgeführt und, um die erstellte Methode zu testen, wurden zwei reale Proben analysiert. Die verwendete Software für das Sammeln und Verarbeiten der Daten stammt von Thermo Fisher Scientific. Das Such-Interface “ToxID” mit der “Tox_Library” sowie der Bibliotheksmanager “SmileMS” von Genebio wurden auf ihre Anwendbarkeit hin überprüft.

Ergebnisse und Diskussion: Die Schlüsselparameter für die Erstellung von Bibliotheks-Spektren und ihre praktische Anwendung werden diskutiert sowie die optimalen Einstellungen für die Ionisation bei unterschiedlichen Anwendungen. Eine Auswahl an möglichen Methoden für das multi-target bzw. “General Unknown” Screening werden beschrieben und stehen für zukünftige Routinemethoden zu Verfügung. Des Weiteren werden Probleme beim Screening und beim Erstellen von Spektren für eine Bibliothek aufgezeigt: die Anzahl an Scan Events, Daten abhängige Acquisition, Dynamic Exclusion, Aktivierungsparameter und die Relevanz der Preselektion von Targets. Die Tox_Library und die Suchprogramme “ToxID” und “SmileMS” wurde evaluiert und Spektren für MS^2 und MS^3 aufgenommen.

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