New Acquisition and Processing Tools for Targeted and Unknown Screening Approaches in Forensic Toxicology

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Overview

Purpose: To present the benefits of the variable data-independent acquisition (vDIA) approach to acquire specific and sensitive MS^2 data for compounds at low concentration level in complex matrices. The benefits of different software processing tools will also be discussed for both targeted and unknown approaches.

Methods: Chromatographic separation was achieved on a Thermo Scientific™ Accucore™ Phenyl-Hexyl column using gradient elution. Mass spectrometric analysis was performed on a Thermo Scientific™ Q Exactive™ Plus MS using electrospray ionization. Full scan data in m/z range 100-800 amu followed by higher energy collisional dissociation (HCD) were collected in positive and negative switching ionization modes using vDIA and data dependent experiment (DDE) approaches. Resolution was set to 70,000 and 17,500 (FWHM @ m/z 200) for full scans and MS^2 scans respectively.

Results: Specificity as well as sensitivity obtained using DDE and vDIA approaches were compared and discussed. TraceFinder was evaluated for targeted screening approach. mzCloud and Mass Frontier will be discussed for unknown compound elucidation.

Introduction

Forensic Toxicology laboratories require sensitive and selective analytical methods to properly identify a broad range of analytes in complex matrices. The existing methods need to be constantly updated as new compounds such as designer drugs are available every day on the market. Liquid chromatography coupled to mass spectrometry has been widely used in this area for years. So far, the preferred technology has been the triple quadrupole for its selectivity in selected reaction monitoring (SRM) mode. But this approach suffers from the limitation of being able to perform only targeted analysis and not unknown screening. For this reason the use of high resolution accurate mass (HRAM) instruments has gained popularity, offering the possibility to perform screening in full scan mode to identify new substances. The two main technologies used for screening purposes are the time-of-flight (ToF) and the Orbitrap. We will focus here on Thermo Scientific™ Orbitrap™ technology that provides superior resolution compared to the ToF systems and very good mass accuracy without the need for an internal mass calibration.

Untargeted screening approaches need data acquisition methods that gather as much MS and MS/MS information from a sample as possible, regardless of the nature of the sample or the primary analytic purpose. To date, combinations of full-scan measurements and wide-range fragmentation techniques like all-ion fragmentation (AIF) are commonly used methods in this approach. These scan modes fragment all ions in a single fragmentation event without precursor ion isolation and detect all fragment ions in a single mixed spectrum. As a result, they suffer from limitations in sensitivity, selectivity and dynamic range compared to data-dependent acquisition methods where detected precursors are isolated with narrow isolation windows prior to fragmentation and detection. A new HRAM scan mode, termed variable data-independent acquisition (vDIA) will be described for both targeted and unknown screening approaches and compared to standard data-dependent acquisition (DDA) methods.

Thermo Scientific™ TraceFinder™ software identifies and confirms substances using the exact mass of the analyte, the isotopic pattern, the fragment ions and the retention times. Identification of unknown compounds can be performed using mzCloud, a unique HRAM MS^n spectral database. Structures can be confirmed using Thermo Scientific™ Mass Frontier™ software that is capable to automatically generate possible fragments at an expert level.

Methods

Sample Preparation

Plasma samples were extracted by protein precipitation. 300 µL of acetonitrile were added to 100 µL of plasma, vortex-mixed and centrifuged. The supernatant was collected, evaporated to dryness and the reconstituted with 70% mobile phase A and 30% of mobile phase B (see below).

Liquid Chromatography

The UHPLC system was a Thermo Scientific™ UltiMate™ 3000 RS Dual Pump. Chromatographic separation was achieved using an Accucore Phenyl-Hexyl (100 x 2.1 mm , 2.6 µ) column. The gradient elution was performed with a mobile phase A (water containing 2 mM Ammonium formate and 0.1% formic acid) and a mobile phase B (MeOH/ACN 50/50 containing 0.1% formic acid, 1 % water and 2mM Ammonium formate) at a flow rate of 500 µl/min. The LC conditions are reported in Table 1.

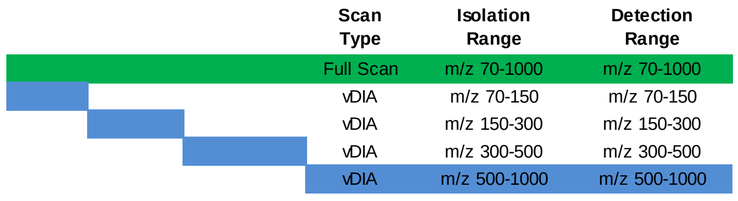
TABLE 1: LC conditions

	Retention [min]	Flow [ml/min]	%B	%C	%D	Curve
1	0.000	0.500	1.0	0.0	0.0	
2	0.000	0.500	1.0	0.0	0.0	
3	1.000	0.500	1.0	0.0	0.0	
4	10.000	0.500	99.0	0.0	0.0	
5	11.500	0.500	99.0	0.0	0.0	
6	11.700	0.500	1.0	0.0	0.0	
7	17.000	0.500	1.0	0.0	0.0	

Mass Spectrometry

Data were acquired using a Q Exactive Plus high resolution instrument. Two methods were used and compared. For the first one, called DDE, full scan data in the m/z range 100-800 amu followed by HCD fragmentation were collected in positive and negative switching ionization modes. Each full scan experiment was followed by 8 high-resolution MS^2 scans in positive mode and 3 high-resolution MS^2 scans in negative mode. Precursor selection was performed in the data-dependent operation mode where the most intense ions of the previous scan were selected for fragmentation. The second approach was vDIA. In this case, vDIA uses 4 isolation windows ranging from 70 to 1000 and covering the entire mass range of the preceding full scan. Figure 1 diagrams a representative vDIA method set-up. Resolution for both vDIA and DDE approaches was set to 70,000 FWHM (at m/z 200) for each full scan mode and 17,500 FWHM (at m/z 200) for MS^2 scan acquisition.

FIGURE 1. Example of vDIA set-up with 4 scan windows, covering a mass range from m/z 70 to 1000



Data processing

Data processing was performed using TraceFinder 4.0 processing software. For generation of extracted ion chromatograms, an extraction window of 3 ppm was used. Targeted screening analysis was performed using a database containing retention time and fragment ions. mzCloud and Mass Frontier were used for unknown screening capabilities.

Results

Targeted screening - Comparison between the DDE and vDIA approaches

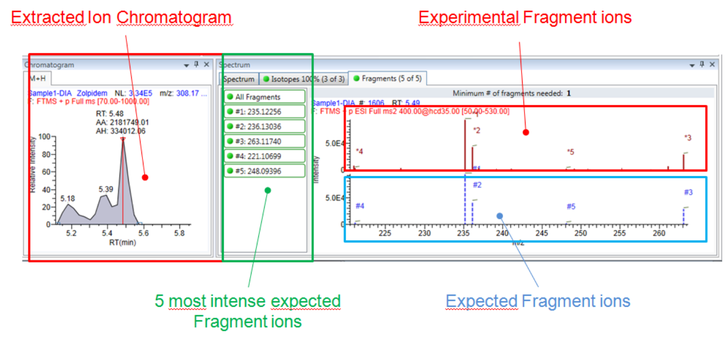
Three plasma samples were spiked with 53 molecules and analysed using the two acquisition methods (DDE and vDIA). Identification of substances was confirmed using the exact mass of the parent ion, its retention time and isotopic distribution and the exact mass of the fragment ions. Table 2 summarizes the number of analytes identified and confirmed for each sample using the DDE and the vDIA approaches. As reported in this table, we have been able to identify 42 analytes in the DDE mode versus 53 in the vDIA. The vDIA mode led to an increase of the identification rate by 26%. The extra molecules identified with vDIA are the ones giving a pretty low signal (intensities below 10e6 cps). For this reason, the ions are not selected by the acquisition software to trigger MS^2 data in the DDE approach.

TABLE 2: Number of analytes identified in 3 spiked plasma samples using DDE and vDIA workflows

Sample #	Identified compounds	
	DDE	vDIA
Sample 1	14	17
Sample 2	15	19
Sample 3	13	17
TOTAL	**42**	**53**

Figure 2 shows an example of an analyte (Zolpidem) that has been identified in sample 1 using the vDIA approach only. As reported in this figure, the full scan spectrum shows potential isobaric interferences in the chromatogram at a retention time very close to the one of Zolpidem. The intensity of the parent ion is pretty low ($3.10e^5$) but all expected fragment ions have been identified in the MS^2 spectrum. The retention time has also been used as a confirmation criteria.

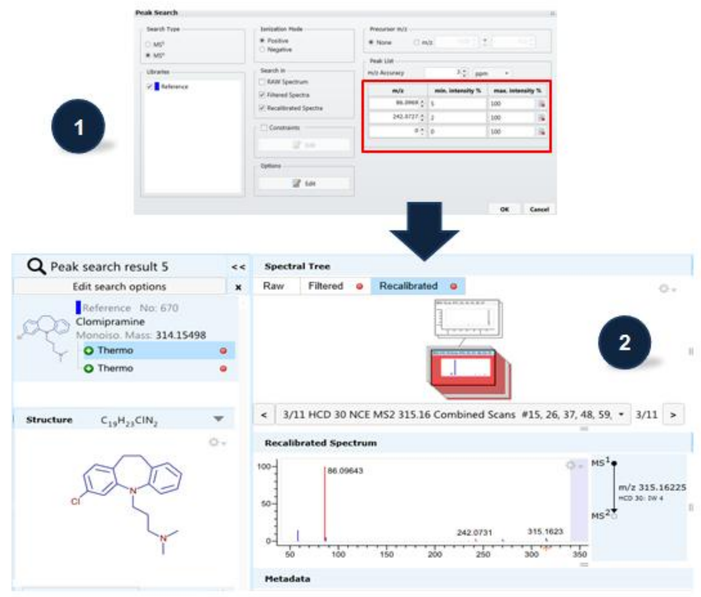
FIGURE 2: XIC and MS^2 spectrum of an analyte (Zolpidem) that has been identified using the vDIA approach only



Unknown screening approach

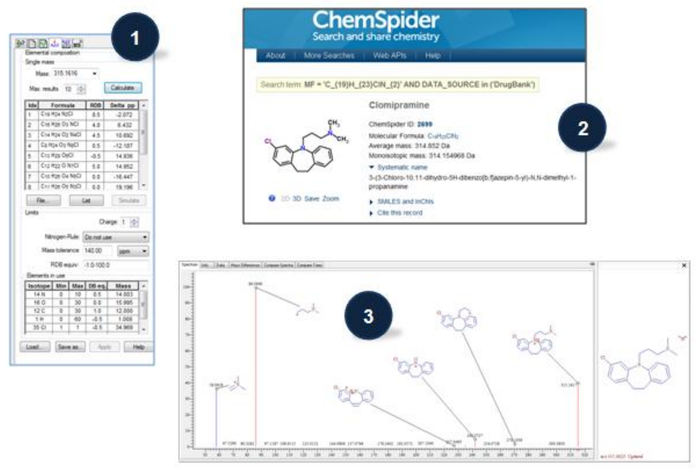
The second approach, called unknown screening, requires to identify drugs that are not available in databases and spectral libraries such as new designer drugs. Identification can be performed using mzCloud and Mass Frontier software. mzCloud is a modern MS^n spectral database searchable from spectra, structures, fragment and precursor ions. It currently contains more than 4722 compounds and 1,215,000 spectra. All spectral peaks are structurally annotated and have been acquired at a resolution of 140.000 (FWHM @ m/z 200). Figure 3 shows an example of searching using fragment ions. The 2 main fragment ions from an MS^2 spectrum are reported into the peak search window and searched against the library. One single hit is provided. It corresponds to Clomipramine.

FIGURE 3: Library searching from a fragment peak list using mzCloud



Mass Frontier™ software is another powerful tool for structural elucidation. It is able to automatically generate possible fragments at an expert level, including complete fragmentation and rearrangement mechanisms, starting from a user-supplied chemical structure. The knowledge base used to predict fragmentation reactions consists of 24 ionization, fragmentation and rearrangement rules along with more than 100,000 published fragmentation mechanisms. Figure 4 presents the workflow used to identify unknown analytes from an exact mass. The first step consists in assigning a formula to an exact mass; in this example the isotopic distribution is characteristic from an analyte containing a chloride atom. Only one formula within the set mass accuracy (3 ppm) is provided by the software : $C_{19}H_{24}N_2Cl$. This formula is then searched against a specific database called Drugbank. The only hit provided is Clomipramine. You can then confirm this result using Mass Frontier software: from the clomipramine structure, Mass Frontier software is going to generate all possible fragment ions and assign these fragments to the one obtained in the experimental MS^2 spectrum. As reported in this figure, all fragments have been assigned to a structure. So, the presence of Clomipramine in the sample is confirmed.

FIGURE 4: Workflow for identification of unknown compounds and confirmation using Mass Frontier software



Conclusion

- In comparison to the DDE approach, vDIA increases the number of analytes that can be identified for targeted screening.
- mzCloud contains more than 1,215,000 spectra and allows spectral database search starting from spectra, structures, fragment and precursor ions.
- Mass Frontier software generates possible fragments at an expert level and can be used to confirm identification of unknown analytes by identifying fragment ions in an MS^2 spectrum.

For forensic use only

vDIA in not available in the United States of America

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