

# Performance Evaluation of Three LC/MS Methods Implemented on an Ion Trap Mass Spectrometer for Drug Testing in Urine

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## Overview

**Purpose:** Compare identification performance in screening using MS<sup>2</sup>, both MS<sup>2</sup> and MS<sup>3</sup>, and MS<sup>3</sup> spectrum.

**Methods:** Liquid chromatography (LC) with electrospray ionization (ESI) implemented on the Thermo Scientific LXQ ion trap mass spectrometer was used for the screening of human urine samples prepared by solid-phase extraction (SPE) against libraries of MS<sup>2</sup> and MS<sup>3</sup> spectra. Data was processed and reported with Thermo Scientific ToxID automated screening software.

**Results:** Methods collecting MS<sup>2</sup> and MS<sup>3</sup> or just MS<sup>3</sup> spectra for compound identification provide strong confirmation and are less affected by interferences. The sensitivity of identification for some compounds is negatively affected by longer data acquisition cycle in methods collecting MS<sup>2</sup> or MS<sup>2</sup> and MS<sup>3</sup> spectra.

## Introduction

A screening procedure identifying compounds based on MS<sup>2</sup> spectrum is commonly employed by forensic toxicology laboratories to analyze for drugs of abuse and other compounds in urine and other matrices. Screening applications have to analyze a broad range of compounds in the shortest possible time. This demand leads to a non-specific sample preparation procedure and samples containing endogenous matrix compounds analyzed with compromised chromatographic separation. Consequently, a false negative might be reported due to combined MS<sup>2</sup> spectra. Compound identification based on MS<sup>2</sup> and MS<sup>3</sup> spectra or based just on MS<sup>3</sup> spectra could eliminate false positives seen in MS<sup>2</sup> based identification. We investigated advantages and limitations of MS<sup>2</sup>/MS<sup>3</sup> and MS<sup>3</sup> workflows and compared them to commonly used MS<sup>2</sup> based workflow.

## Methods

**Sample Preparation:** SPE was performed using 200 mg mixed mode Thermo Scientific HyperSep Verify-CX C18 solid phase extraction cartridges. One mL of urine was spiked with 10, 100 and 1000 ng of analytes of interest, as well as 100 ng of three deuterated standards. Basic, acidic and neutral fractions were collected, combined, evaporated to dryness, reconstituted in 100 µL and injected onto the LCMS.

**LC Conditions:** A 13-minute LC method was used. Gradients from 5%-95% B were employed, with flow rates of 200 µL/minute.

**Solvent A:** Water, 10 mM Ammonium Formate w/0.1% Formic Acid

**Solvent B:** Acetonitrile, 0.1% Formic Acid

**Column:** Thermo Scientific Hypersil PFP, 50x2.1mm, 5 µm

FIGURE 1. Compounds identification with MS<sup>2</sup> spectrum – data acquisition method scan events diagram

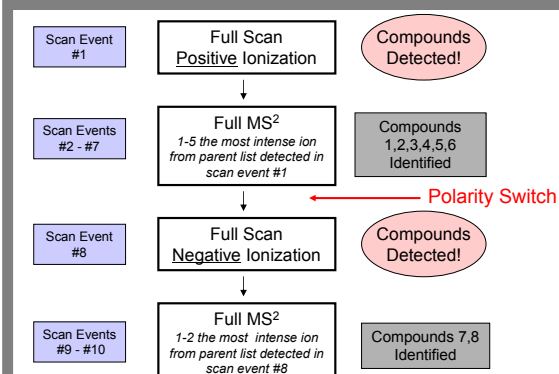


FIGURE 2. Compounds identification with MS<sup>2</sup> and MS<sup>3</sup> spectra – data acquisition method scan events diagram

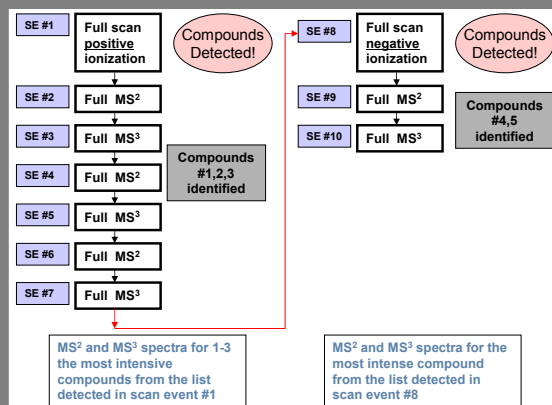
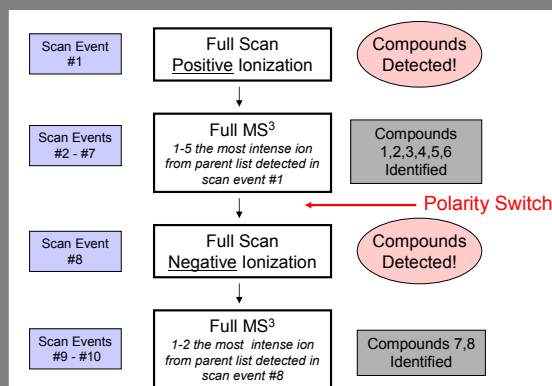


FIGURE 3. Compounds identification with MS<sup>3</sup> spectrum – data acquisition method scan events diagram

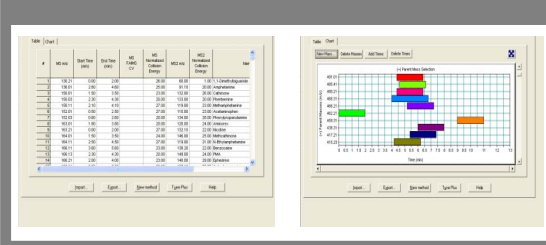


**MS Conditions:** The SPE prepared samples were analyzed on an LXQ™ ion trap mass spectrometer with an ESI source, in a scan-dependent polarity-switching methods. (Figures 1 – 3)

**Scan events cycle time:** In all three mass spectrometry methods maximum injection time for full scan event was set to 50 ms and maximum injection time for full MS<sup>2</sup> and full MS<sup>3</sup> experiments was set to 100 ms. As automated gain control (AGC) was implemented, the cycle time depended on concentration of ionized compounds in the sample. It was in range of 0.07-0.09 min for method identifying compounds with MS<sup>2</sup> spectra and of 0.12-0.16 min in method identifying compounds with MS<sup>2</sup>/MS<sup>3</sup> and MS<sup>3</sup> spectra.

**Spectra libraries:** MS<sup>2</sup> spectra library and MS<sup>3</sup> spectra library were created for 300 compounds. All spectra were collected with optimized collision energy for maximum signal intensity and spectra specificity.

FIGURE 4. Parent mass list table



The parent mass table (Figure 4) allows the instrument to only search for masses within retention time windows specific to each compound. This greatly conserves instrument resources by preventing searches for all masses throughout the entire run.

Figures 5 – 7 show examples of data collected in all three methods.

FIGURE 5. Compounds identification with MS<sup>2</sup> spectrum 100 ng/mL Chlorprothixene in urine

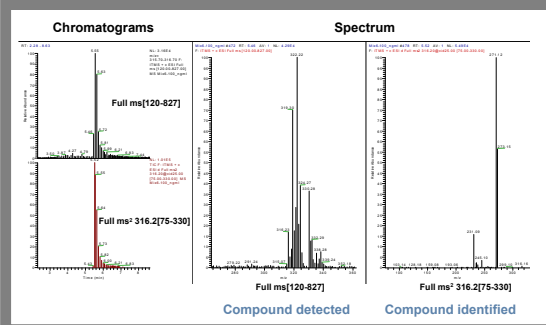


FIGURE 6. Compounds identification with MS<sup>2</sup> and MS<sup>3</sup> spectra 100 ng/mL Chlorprothixene in urine

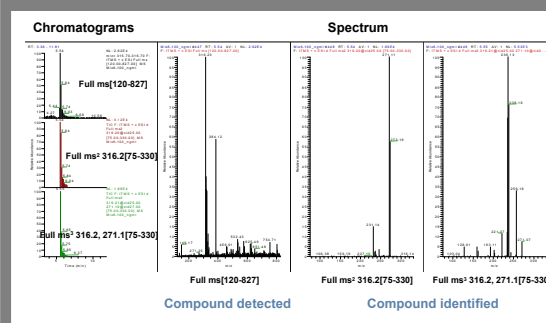
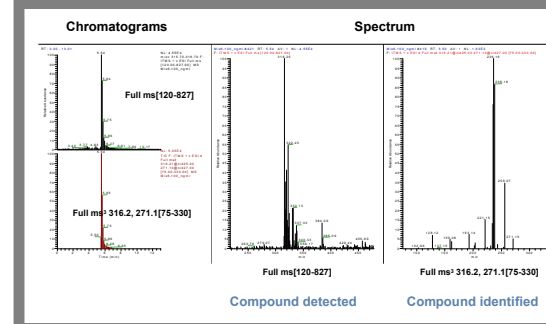


FIGURE 7. Compounds identification with MS<sup>3</sup> spectrum 100 ng/mL Chlorprothixene in urine



## Method Validation

We wanted to answer the following questions in the method validation process:

- How number of compounds in MS<sup>2</sup>/MS<sup>3</sup> and MS<sup>3</sup> methods will affect identification limit
- Will longer cycle time in MS<sup>2</sup>/MS<sup>3</sup> and MS<sup>3</sup> methods effect identification limit

To answer these questions:

- We compared identification limits for a selected group of compounds in MS<sup>2</sup>/MS<sup>3</sup> and MS<sup>3</sup> methods targeting different numbers of compounds to those obtained in MS<sup>2</sup> method targeting list of 300 compounds.

- Identification limits were obtained by spiking urine with 10 randomly selected compounds to concentrations of 10, 100, 500 and 1000 ng/mL. The samples were processed with SPE procedure and were analyzed with all three methods.
- Data was automatically processed and reported with ToxID™ software. The hit was reported if SI and RSI indexes calculated with NIST software had values of at least 600 and 700 for SI and RSI respectively.

Method validation results are presented in Figures 8 – 10.

FIGURE 8. Limits of detection for a selected group of analytes in three screening methods: MS<sup>2</sup> method targeting 300 compounds and MS<sup>2</sup>/MS<sup>3</sup> and MS<sup>3</sup> methods targeting 54 compounds

Compound	MS <sup>2</sup> identification	MS <sup>2</sup> /MS <sup>3</sup> identification	MS <sup>3</sup> identification
7-aminoflurazepam	10 ng/mL	10 ng/mL	10 ng/mL
BDB	10 ng/mL	10 ng/mL	10 ng/mL
Benzocaine	500 ng/mL	500 ng/mL	1000 ng/mL
Benzoylcegonine	100 ng/mL	100 ng/mL	10 ng/mL
Alprazolam	10 ng/mL	10 ng/mL	10 ng/mL
Clozapine N-Oxide	100 ng/mL	10 ng/mL	10 ng/mL
Chlorpromazine	10 ng/mL	10 ng/mL	10 ng/mL
Alprazolam	10 ng/mL	10 ng/mL	10 ng/mL
Chlorprothixene	10 ng/mL	10 ng/mL	10 ng/mL
3-Hydroxystanzolol	500 ng/mL	500 ng/mL	500 ng/mL

FIGURE 9. Limits of detection for selected group of compounds in three screening methods: MS<sup>2</sup> method targeting 300 compounds and MS<sup>2</sup>/MS<sup>3</sup> and MS<sup>3</sup> methods targeting 113 compounds

Compound	MS <sup>2</sup> identification	MS <sup>2</sup> /MS <sup>3</sup> identification	MS <sup>3</sup> identification
Cinnarizine	10 ng/mL	10 ng/mL	10 ng/mL
Cisapride	100 ng/mL	10 ng/mL	10 ng/mL
Citalopram	10 ng/mL	10 ng/mL	10 ng/mL
Fluoxetine	10 ng/mL	10 ng/mL	10 ng/mL
Flurazepam	10 ng/mL	10 ng/mL	10 ng/mL
Delta-9-THC	100 ng/mL	100 ng/mL	100 ng/mL
Desmethyldoxepin	10 ng/mL	10 ng/mL	10 ng/mL
Diazepam	10 ng/mL	10 ng/mL	10 ng/mL
Enalapril	10 ng/mL	10 ng/mL	10 ng/mL
EMDP	10 ng/mL	10 ng/mL	10 ng/mL

FIGURE 10. Limits of detection for selected group of compounds in three screening methods: MS<sup>2</sup> method targeting 300 compounds and MS<sup>2</sup>/MS<sup>3</sup> and MS<sup>3</sup> methods targeting 245 compounds

Compound	MS <sup>2</sup> identification	MS <sup>2</sup> /MS <sup>3</sup> identification	MS <sup>3</sup> identification
Hydroxyzine	100 ng/mL	10 ng/mL	10 ng/mL
Ketamine	10 ng/mL	100 ng/mL	100 ng/mL
Ibogaine	100 ng/mL	100 ng/mL	100 ng/mL
Labeltolol	100 ng/mL	500 ng/mL	500 ng/mL
Ketoprofen	1000 ng/mL	500 ng/mL	1000 ng/mL
MDA	10 ng/mL	10 ng/mL	10 ng/mL
Malathion	100 ng/mL	100 ng/mL	100 ng/mL
MDMA	10 ng/mL	100 ng/mL	10 ng/mL
Mirtazapine	10 ng/mL	100 ng/mL	10 ng/mL
Nalorphine	10 ng/mL	500 ng/mL	100 ng/mL

## Conclusions

- All 245 compounds screened with MS<sup>2</sup>/MS<sup>3</sup> and MS<sup>3</sup> methods in spiked urine samples were identified.
- The probability of non-specific endogenous peak ion selection for spectra acquisition increases as the number of targeted compounds in screening method increases.
- Identification limits in methods with longer scan events cycle (MS<sup>2</sup>/MS<sup>3</sup> and MS<sup>3</sup> methods) are affected more by collecting spectra for endogenous compounds than methods with shorter scan events cycle (MS<sup>2</sup> method).
- Methods collecting MS<sup>2</sup> and MS<sup>3</sup> or just MS<sup>3</sup> spectra provide stronger hit confirmation. They are less affected by interference but may result in higher detection limits for some compounds compared to method using just MS<sup>2</sup> spectra.

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