Metabolomics and Lipidomics in Health and Disease

Simultaneous Quantitation and Untargeted Discovery (SQUAD) workflow using multiple fragmentation techniques for quantitation and characterization of trace level nitrosamine in biological matrices

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Abstract

Purpose: Develop and optimize a SQUAD method for detecting and quantifying nitrosamines in biological matrices

Methods: Serial dilution of Nitrosamine standards were subjected to Simultaneous Quantitation and Discovery (SQUAD) analysis using Thermo Scientific™ Vanquish™ Horizon UHPLC system and the Thermo Scientific™ Orbitrap IQ-X™ Tribrid™ mass spectrometer . Nitrosamine spiked matrices of human pooled urine and human Serum were subjected to SQUAD analysis.

Results: The limit of detection range of NDMA and other nitrosamines is 1 ppb. The limit of quantification is 2 ppb.

Introduction

N-Nitrosamines are a class of carcinogenic chemical compounds that pose threat to humans exogenously through matrices like drinking water, food, cosmetics, and endogenously by nitrosation in gastrointestinal tract, nitrate to nitrite conversion, etc. An accurate, reliable analytical method is critical for detection and quantification of these trace-level, polar and low molecular weight compounds to understand the association between their exposure and related diseases.

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An optimized SQUAD workflow is presented that discovers unknown compounds in the matrix by using high resolution orbitrap and a sensitive linear ion trap detector that quantifies the low intensity nitrosamines in a single run. The workflow uses multiple fragmentation types – HCD, CID and UVPD and MSn to help characterize isomeric structures.

Materials and methods

Sample Preparation

Dilution series (100 fg/mL - 20 ng/mL) of EPA 521 Nitrosamine Mix (Sigma - Aldrich) standards were prepared in methanol. A commercially available Human Pooled Urine (Gender unspecified) from BioIVT was diluted with methanol in the ratio of 1:1, vortexed and spiked with nitrosamine standards (20 ng/mL to 1pg/mL) and 20 ng/mL pooled internal standards.

Human serum (BioIVT) was also diluted with methanol in the ratio 1:1, vortexed, spiked with EPA 521 nitrosamine standards mixture (20ng/mL – 1pg/mL) and 20 ng/mL pooled internal standards. The spiked mixture is vortexed again and centrifuged. The top clear extract was collected for SQUAD analysis. Water and Methanol with 0.1% formic acid (Fisher Scientific) were used as chromatography solvents.

Test Method(s)

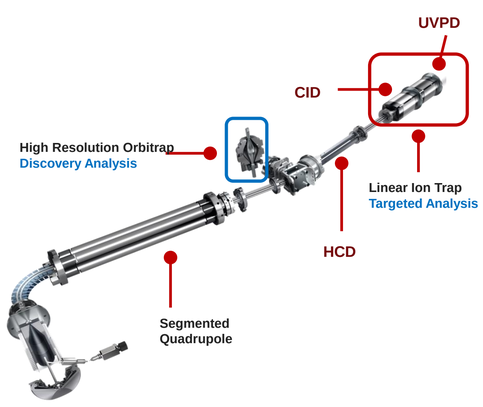
ACQUITY UPLC BEH Phenyl column (100 x 2.1mm, 1.7um). connected to Vanquish Horizon UHPLC system and Orbitrap IQ-X Tribrid MS was used to conduct analytical experiments. Thermo Scientific™ EASY-Spray™ C18 HPLC Column connected to Thermo Scientific™ Vanquish™ Neo UHPLC system and Orbitrap IQ-X Tribrid MS were used to conduct nano flow experiments.

SQUAD experiments using UVPD, HCD, and CID fragmentation techniques were performed on dilution series (100 fg/mL to 25 ng/mL) of EPA 521 Nitrosamine Mix (Sigma-Aldrich). Human pooled Urine and human serum spiked extracts with EPA521 Nitrosamine standards were subjected to SQUAD analysis

Data Analysis

Spectra were evaluated for nitrosamines using Thermo Scientific™ FreeStyle™ software and annotated using Thermo Scientific™ Mass Frontier™ 8.0 software. Untargeted data analysis was performed using Compound Discoverer 3.4 software. Calibration curves were generated using Thermo Scientific™ TraceFinder™ 5.2 software.

Figure 1. Orbitrap IQ-X Tribrid mass spectrometer Instrument View.



The Orbitrap IQ-X Tribrid mass spectrometer has three mass analyzers. Quadrupole, Orbitrap and an ion trap. Ions are isolated in quadrupole by default unless the user makes setting to use ion trap. High resolution Orbitrap mass analyzer detects ions with high mass accuracy. Ion trap isolates ions, acts as a trap for fragmentation and detects ions as well.

Having both Orbitrap and ion trap mass analyzers on the same instrument provides great flexibility for performing parallel analysis and employing multiple activation types. In the parallel analysis SQUAD experiment, a full scan is performed in high resolution Orbitrap followed by a data dependent analysis for detecting unknown compounds in the drug matrix and targeted quantitation of the known nitrosamines is performed in sensitive ion trap in a single run.

Figure 2. SQUAD method editor set up

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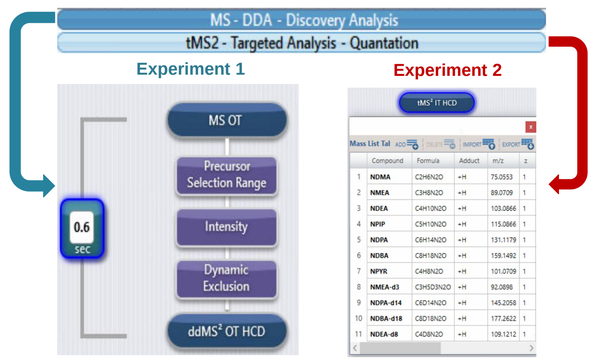
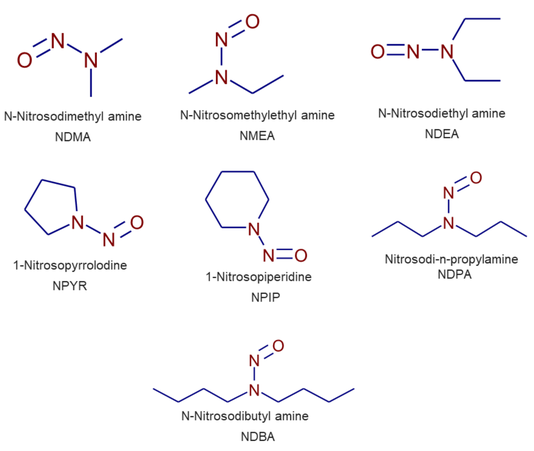


Experiment 1 is Discovery analysis and is performed in the Orbitrap detector. The method consists of a full scan and untargeted data dependent MS^2 scan.
MS^2 data is analyzed using compound discoverer to find library matched compounds with high confidence

Experiment 2 is Targeted Quantitative analysis. Multiple targeted scan experiments are added in the same method. The method scans targets compounds of interest with known retention time in the sample. Targeted analysis is performed in the ion trap detector.

Figure 3. Chemical structures of Nitrosamine compounds present in EPA 521 Nitrosamine mix



Nitrosamines are a class of nitroso compounds with a functional group N-N=O and carcinogenic in nature. They form as impurities in the drug manufacturing process due to the reaction of amines present in raw materials with an acid.

Results

All the seven nitrosamine compounds were detected and quantified in the matrix at picogram level through targeted analysis in the ion trap in analytical flow and nano flow chromatography analyses. Unknown compounds were also identified and characterized through discovery analysis in the Orbitrap .

Figure . XIC's (0.5 ppb) of MS2 ions of spiked nitrosamines detected and quantified in spiked pooled human serum extract.

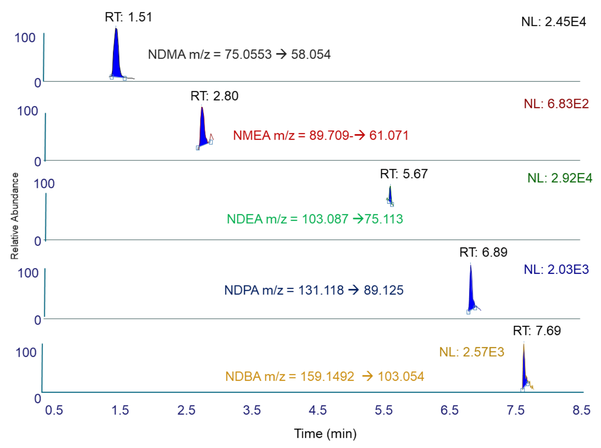


Figure 5a-5b. Linear charts of NDMA (m/z = 75.0553) in standards mixture and spiked Urine

Limit of Quantitation is 0.5 ppb

Fig 5a. Spiked Human Pooled Urine extract

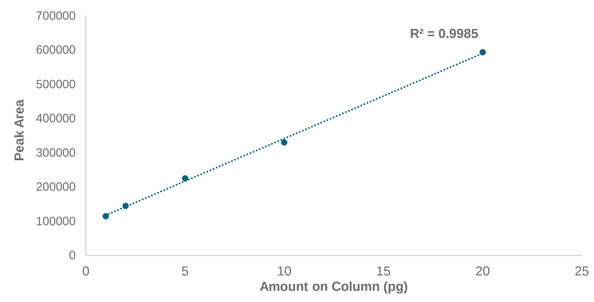


Fig 5b. EPA 521 Nitrosamine Mix - NDMA (75.0553)

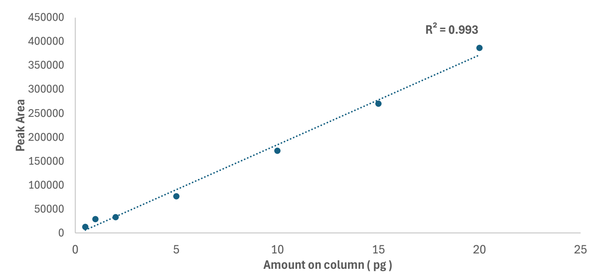
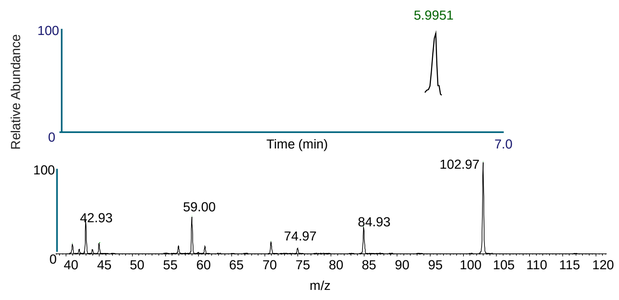


Table 1. LOD and LOQ values for all seven Nitrosamines in human pooled urine extract

	LOD ppb	LOD Amount on Column (pg)	LOQ ppb	Amount on Column (pg)
NDMA	0.5	0.5	1	1
NMEA	0.2	0.2	0.5	0.5
NDEA	0.2	0.2	0.5	0.5
NPYR	2	2	5	5
NPIP	0.5	0.5	2	2
NIPA	0.05	0.05	0.2	0.2
NDBA	0.5	0.5	0.5	0.5

Figure 6. Spiked human pooled serum extract XIC of m/z 75.113 ion from MS^2 spectra of N- Nitrosodiethyl amine (NDEA m/z 103.087) - LOQ 5ppt Nano flow LC



By employing nano flow chromatography low levels of quantitation was achieved. Human pooled serum extract was spiked with EPA 521 nitrosamine mix subjected to nano flow chromatography analysis low level of quantitation was achieved.

Figure 7. Human pooled urine extract spiked Nitrosamine Mix – Discovery Analysis – $FTMS^2$ spectrum of 2-Methylparaben(C_8H_8O3) m/z = 152.0473

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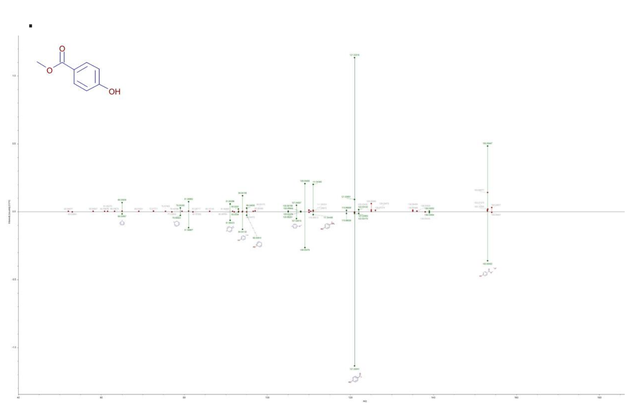

Discovery analysis in high resolution Orbitrap identified unknowns successfully untargeted Orbitrap scans collected HRAM MS2 data on Menadione during the experiment.

Conclusions

- Detection limits as low ppb levels (0.2 ppb –0.5 ppb) and quantitation limits (0.2 ppb- 5 ppb) in presence of the matrix were achieved for all seven nitrosamines by applying parallel ion trap analysis
- SQUAD analysis was run on Orbitrap IQ-X Tribrid MS and achieved low ppb level quantitation in ion trap and identified unknown compounds through discovery analysis in Orbitrap.
- Employing nano LC helped achieve lower limit of quantitation (5 ppt)

Trademarks/licensing

Conflict of Interest

The authors SY, BB, RD, BA, SB and VZ are employees of Thermo Fisher Scientific whose instrumentation and software were used to acquire and process the data.