## K38 Method Validation Using Multiple Compound Mixtures for Screening/Confirmation of 800+ Novel Psychoactive Substances (NPS) by Liquid Chromatography/Triple Quadrupole/Mass Spectrometry (LC/QqQ/MS)

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After attending this presentation, attendees will be aware of the ability to use mixtures, rather than running individual compounds, to validate a screening/conformational method for detecting NPS in biological fluids using LC/QqQ/MS. The goal of this presentation is to include more than 800 NPS in the final validated method for screening. The 800 NPS that will be included in this method will cover a wide variety of drug classes with a focus on synthetic cannabinoids due to their current importance in many cases entering toxicology laboratories.

This presentation will impact the forensic science community by introducing the development of a validated, comprehensive screening method for NPS, which would help with the screening of potential drug abusers. This proposed method will help with the detection of NPS so that a higher number can be detected, thus decreasing the number of false negatives. The goals of this presentation are to be able to use one comprehensive method to screen for the majority of NPS, which may not be detected through current screening methods, and to present an alternative to current screening methods, as this method is capable of detecting a higher number of compounds.

This presentation is intended to demonstrate the use of standard mixtures rather than individual compounds to validate a screening/confirmatory method for detecting NPS in biological fluids using LC/QqQ/MS). The ultimate goal is to include more than 800 NPS in a final validated method. The NPS that are included in the method cover a wide variety of NPS drug classes and metabolites, with a focus on synthetic stimulants and cannabinoids, due to their current importance in forensic toxicology casework. NPS have become a major issue in toxicology laboratories because of their potentially high potency, their ability to remain undetected by many current screening methods, and their rapid development in attempts to avoid current scheduling laws. The proposed method is anticipated to allow the comprehensive detection of the majority of NPS in a single run, with concomitant reduction in the number of false negatives.

An Agilent® 1290 Infinity High-Performance Liquid Chromatography (HPLC) system and Agilent® 6460 QqQ/MS with Jet Stream Technology Electrospray Ion Source (ESI) was used for this research. A total of 826 compounds to be included in the final method were analyzed using both Flow Injection Analysis (FIA) and Agilent® Optimizer software for optimizing fragment transitions in order to create a triggered Multiple Reaction Monitoring (tMRM) method. Once the tMRM method was created, all compounds were run through an Agilent® ZORBAX® Rapid Resolution HD Eclipse Plus C18 column (3.0mm x 100mm; 1.8µm particle size) in order to obtain retention times. Retention times were collected with a gradient of 95% A (5mM ammonium formate in HPLC water with 0.1% formic acid) and 5% B (methanol with 0.1% formic acid) from 0min-1min, increasing to 95% B over 1min-9.5min, then 98% B for the remainder of the run. All retention times were used to create the final method for validation.

In order to fully validate the method, calibration curves must be created for each drug standard. Since completing individual calibration curves for each of the 826 NPS included would be extremely time-consuming and

inefficient, an approach using a series of standard calibration mixes was explored. In order to validate the proposed method for 826 compounds, mixes were created to evaluate multiple compounds at a time. Mixes contained 29 to 37 different compounds, leading to 25 total mixes encompassing all 826 NPS. Criteria for individual mixes included the presence of unique transitions for each component, no co-eluting compounds, and a minimum of 0.2min between compound peaks in the same mixture. Seven different calibration levels were chosen for method validation as follows: 1ng/mL, 2ng/mL, 5ng/mL, 10ng/mL, 20ng/mL, 50ng/mL, and 100ng/mL. All calibrators also incorporated an internal standard "supermix" composed of 22 deuterated standards representing multiple drug classes. Calibrations were performed with both methanol-based and spiked matrix (urine) mixtures for method optimization. For urine, calibrations were completed using a "dilute and shoot" approach, with a 1:5 dilution injected directly into the instrument.

To date, individual calibration curves have been created for eight different NPS mixtures representing 240 of the 826 proposed compounds for the final validated method. The majority of compounds exceeded the required $R^2$ value for validation using six replicates at each level. LC chromatograms were analyzed using the Find-by-MRM software algorithm, which identifies each compound in the mixture based on its targeted transitions. This approach was capable of identifying all compounds in each mixture. The results of these experiments clearly demonstrate the value of using standard mixes for method validation in comprehensive toxicological analysis. Work is continuing to create calibration curves for the remaining compounds, with the goal of using mixtures containing a maximum number of compounds in order to limit the number of mixtures needed for full validation.

**LC/QqQ/MS, Method Validation, Novel Psychoactive Substances**