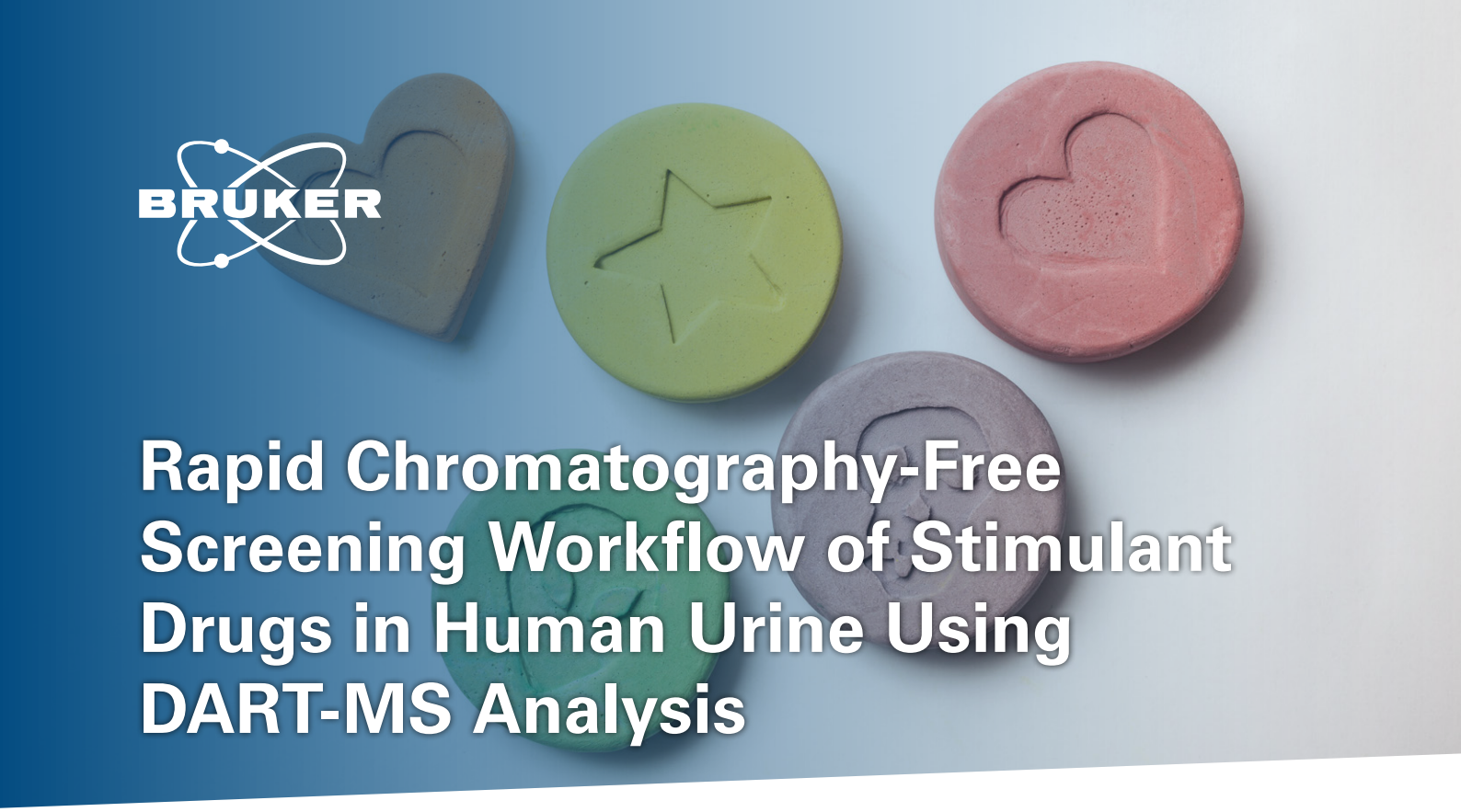

Rapid Chromatography-Free Screening Workflow of Stimulant Drugs in Human Urine Using DART-MS Analysis

Abstract

Immunoassays (IA) are most commonly used as a test method in initial Urine Drug Screening (UDS) tests for drugs of abuse in the field of forensic toxicology. The rapid generation of results and ease of adaptability to automation are the main reasons to use IA. However, IA results are considered to be presumptive in their accuracy due to the high frequency of false positives atributed to cross-reactivities with other ubiquitous co-analytes. Due to the number of potential interferents, a positive IA result must be confirmed by another analytical approach, typically a chromatography-based method. LC-MS and GC-MS are most commonly used as confirmatory assays due to their high degree of sensitivity, specificity, and accuracy. While chromatography-based approaches are well established and commonly achieve sub-ng/mL detection limits, they often are limited in throughput with time-consuming chromatography steps and rely on costly columns and mobile phases. In this work, we report the development of a chromatography-free method on the EVOQ® DART-TQ+ mass spectrometer employing a fully integrated direct analysis in real time (DART) source that is shown to accurately identify and measure four illicit phenylethylamine drugs--amphetamine, methamphetamine, 3,4-Methylenedioxyamphetamine (MDA), 3,4-Methylenedioxy methamphetamine (MDMA) along with phenylcyclohexyl piperidine (PCP). The detection of these five common illicit compounds commonly suffers from interferences in immunoassay-based urine screens. The optimized DART-MS based workflow achieves a throughput rate of 96 samples in less than 45 minutes that is roughly equivalent to IA. This chromatography-free workflow meets the low limits of detection and low %RSD for high repeatability in urine matrices and avoids interference from matrix or co-analytes.

Keywords:
Chromatography-free; DART; TQ; forensics; stimulants; quantitation; urine drug screen

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Overview of a toxicology workflow

Introduction

Simulants are commonly used, both for legitimate medical purposes and for illicit or non-prescribed use. There is a growing interest in the swift and effective measurement of these substances. Current LC-MS/MS and GC-MS/MS methodologies for quantifying simulants, are time-consuming and costly. An alternative approach, utilizing a chromatography-free workflow offers a simple, rapid and straightforward screen and quantification of these substances.

Phenylethylamines are a class of synthetic substances which act as central nervous system simulants that induce the effects of euphoria, increased energy, distortion of time, and enhanced enjoyment of tactile experiences[1]. PCP, a dissociative anesthetic, is often analyzed in an amphetamine drug panel. These compounds are classified as Schedule I substances under the Controlled Substances Act, and the related illicit drugs amphetamine, methamphetamine, MDA, and MDMA are commonly monitored in the field of toxicology (DEA) typically within the context of urine testing[2]. Traditional Urine Drug Monitoring (UDM) is comprised of two types of tests: presumptive Urine Drug Screening (UDS) by immunoassay followed by a confirmatory test using a spectrometric analytical technique such as LC-MS or GC-MS[3].

A limitation of testing for these small analyte compounds arises from their simple structure which leads to significant cross-reactivities with other analytes when using antibody-based immunoassays[1,3]. Cross-reactivity occurs with structurally related sympathomimetics commonly used as anti-hypertensive, anti-diabetic, antihistamine, antibiotic, and psychiatric drugs and is well documented, often leading to false positive test results within traditional UDS testing[4]. It has been shown that false positive results occur in as many as 15% of samples, resulting in unnecessary and expensive confirmatory testing[5].

Compared to presumptive and subjective IA techniques, MS-based techniques are capable of identification and quantitation of trace-level analytes with a high degree of specificity and accuracy. Tandem-MS provides enhanced levels of specificity and structural information about analytes of interest. Conventionally, MS and MS/MS approaches are typically preceded by a chromatographic separation to further improve the performance and detection of analytes in complex mixtures[6].

While chromatography improves specificity and sensitivity, analysis often takes between 10 and 30 minutes per sample which leads to severe bottlenecks in analytical workflows[7, 8]. Now, with the availability of ambient ionization techniques such as DART, the chromatography separation prior to the MS analysis is no longer necessary. On the EVOQ® DART-TQ+, DART-MS generates a signal that includes MS/MS data specific to the illicit compound. Because desorption conditions can be altered to favor lower boiling point and higher boiling point substances, DART is effective in separating compounds simply by changing parameters that control desorption and ionization. The resultant specific analysis method produces selective time versus intensity data that can be easily integrated to determine the amount of illicit compound present in a sample. Thus, DART-MS offers a rapid chromatography-free alternative with higher selectivity and specificity that significantly reduces high false-positive screening results in IA urine drug screens.

In this work, we perform a liquid-liquid extraction on urine samples containing a mixture of stimulants. Samples were processed using a ToxBox® custom drug panel (PinPoint® Testing) and analyzed using a chromatography-free workflow. All five compounds were analyzed with satisfying linearity and repeatability, confirming the efficacy of DART-MS as a urine drug screen.

Methods

Samples
A mixture of amphetamine, methamphetamine, methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA), and phenylcyclohexyl piperidine (PCP) was prepared in an aqueous solution. The mixture was analyzed in both neat and drug-free urine matrix.

Sample preparation
A concentration range of 125 – 10,000 ng/mL was used for amphetamine and methamphetamine. A range of 125 – 5,000 ng/mL was used for MDA and MDMA and a range of 6.25 – 250 ng/mL was used for PCP. All analytes and their corresponding deuterium-labeled equivalents were purchased from Millipore-Sigma. 500 µL of certified drug-free urine and 300 µL DI water were added to each well in a 96 deep-well ToxBox® customized Stimulants Validation plate from PinPoint Testing. The ToxBox custom drug panel contains reagent solutions A-C, a preloaded 96 well plate with selected analytes for an 8-point triplicate calibration curve, triplicate QC samples, along with sample and calibration blanks. The entire plate was then agitated for 10 minutes at 500 rpm on a horizontal plate shaker after which 600 µL of PinPoint Solution B was added to each well and aspirated 10X to mix. Samples were allowed to separate for 10 min. Next, the aqueous layer (800 µL) was removed from each well and discarded. The remaining organic layer was evaporated under nitrogen at 60 psi for 40 min followed by reconstitution in 50 µL of PinPoint Solution C. Reconstituted samples were agitated at 500 rpm on a horizontal plate shaker after which a 1 µL aliquot from each well was transferred onto a Bruker DART QuickStrip HTS-96 screen and allowed to dry under nitrogen gas at 40°C for 15 min. Table 4 displays the QC 1 and QC 2 concentrations present for the 5 compounds in this stimulants panel.

Instrumentation and software
DART-MS analysis was performed using an EVOQ® DART-TQ$^+$ (Bruker Daltonics) triple quadrupole mass spectrometer with a fully integrated DART source. Samples were vaporized using pulse sampling with a duration of 4 s at 250°C in positive ion mode. Pulse sampling saves a significant amount of He to minimize usage only during data acquisition. Each run used a 25 ms scan time per MRM transition that resulted in a cycle time of 27 seconds sample to sample. The cone temperature was set to 250°C with a pressure of 20 psi, and CID gas was set at 1.25 mTorr. Table 1 below contains the MRM transitions and collision energies for each analyte. tqControl software was utilized for method development, data acquisition, data processing, and data review.

Compound Transitions
For all five analytes and internal standards, the MRM transitions are shown in Table 1, as well as the optimized collision energies and scan times used.

Table 1. EVOQ® DART-TQ$^+$ MS method compound MRM transitions.

Analyte	MRM Transition (*m/z*)	CE (V)	Scan Time (msec)	Q1 Res	Q3 Res
Amphetamine	136 –> 91	5	25	0.7	0.7
Methamphetamine	150 –> 91	21	25	0.7	0.7
MDA	180 –> 135	18	25	0.7	0.7
MDMA	194 –> 163	9	25	0.7	0.7
PCP	244 –> 86	12	25	0.7	0.7
D5-amphetamine	141 –> 93	10	25	0.7	0.7
D5-methamphetamine	155 –> 92	10	25	0.7	0.7
D5-MDA	185 –> 110	18	25	0.7	0.7
D5-PCP	249 –> 86	15	25	0.7	0.7

DART and MS Parameters

Table 2. EVOO® DART-TQ+ MS method DART parameters and MS parameters used to analyze the five analytes.

DART Parameter	Value
Gas flow temperature	250°C
Grid Voltage	50 V
Scanning time	5 mm/sec
Ionization gas	He
Polarity	Positive
Array	96-well plate

MS Parameter	Value
Cone temperature	250°C
Cone gas pressure	20 psi
CID cell pressure	1.25 mTorr
Collision gas	Ar
Detector voltage	1.36 kV
Polarity	Positive

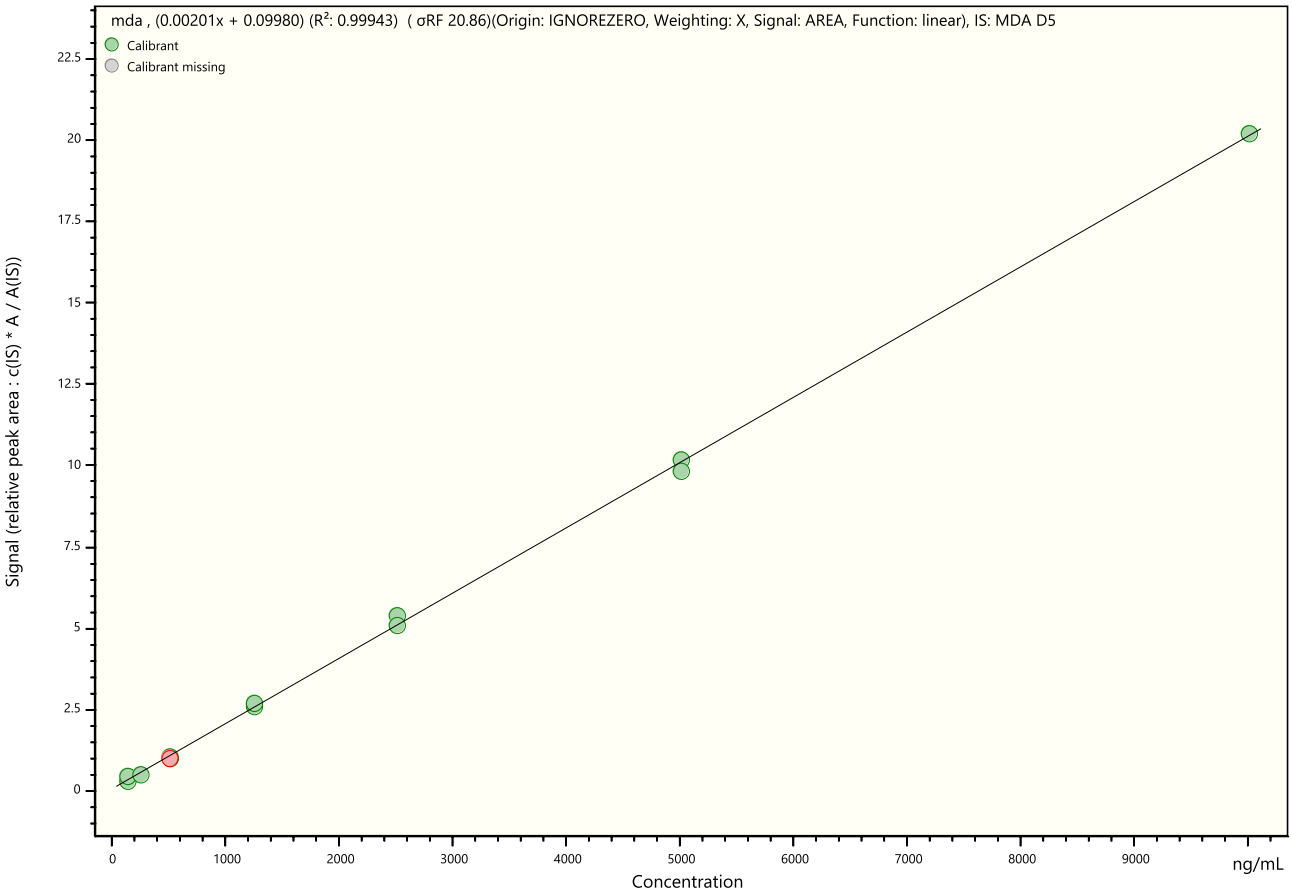
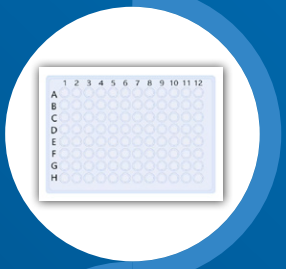


Figure 1. Shown is an example of the calibration curve that was generated for MDA, where a linear R^2 correlation value > 0.999 was realized. This shows the ability of DART-MS to accurately detect MDA across a wide concentration range with high confidence.

Chromatography-Free Screening Workflow

Sample Preparation

1. Load samples
2. Enzyme hydrolysis
3. Dilute
4. Mix/Agitate
5. Quench enzyme



Liquid Liquid Extraction

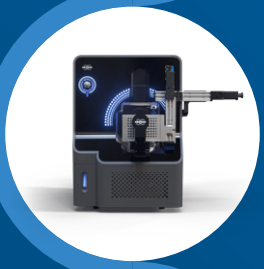
1. Add extraction solution
2. Mix/Agitate
3. Rest
4. Remove bottom aqueous layer
5. Dry down
6. Reconstitute

Load QuickStrip HTS Plate

1. Spot 8x12 format
2. Dry down

DART-MS Acquisition

1. Analysis of 96 samples in under 45 minutes
2. 27 seconds sample to sample



Data Review

1. Review calibration curve linearity and residual plot regression analysis
2. Review precision and accuracy
3. Identify screen hits

Report

1. Export to LIMS
2. Or printout

Results

DART-MS analysis of the stimulants panel resulted in good linear correlation with respect to the analyzed QC samples. They are completely within the performance criteria required for confident, reliable screening. Table 3 shows that four of the five analytes had $R^2 > 0.99$ while the fifth was $R^2 = 0.981$. The cutoff of 125 ng/mL was easily met for all compounds. Both accuracy and repeatability were well within the typical limits demonstrating the excellent performance of the method. Additionally, the Lower Level of Quantitation (LLOQ) ranged from 4.2 to 54.2 ng/mL for the five analytes, indicating that this quick chromatography-free workflow is sufficient in detecting these compounds in the urine matrix at levels at or below the common cutoffs within urine matrix[8]. The performance of this simple, fast chromatography-free quantitative screening workflow is significantly better than commonly used UDS assays and eliminates the high rate of false positives associated with IA UDS assays.

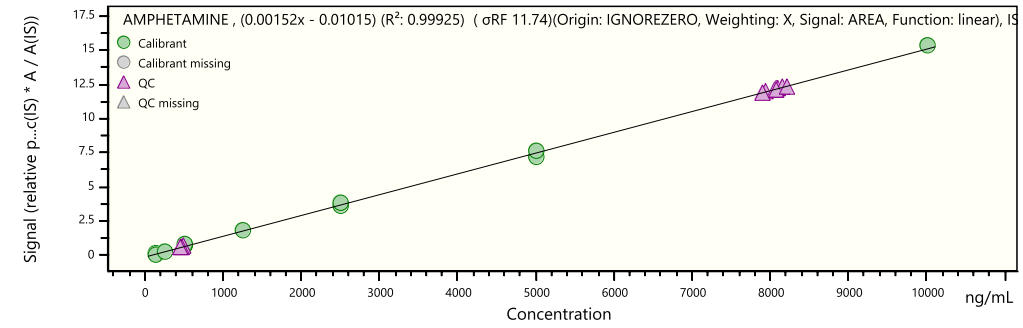
Table 3. Results of the analyzed compounds showing the linearity, limit of detection, lower limit of quantitation, accuracy of the two levels of Quality Controls for each analyte, and repeatability across the calibration range.

Analyte	Range (ng/mL)	R^2	Cutoff (ng/mL)	Slope	LOD (ng/mL)	LLOQ (ng/mL)	Accuracy (QC 1)	Accuracy (QC 2)	Repeatability (%RSD)
Amphetamine	125-10,000	0.999	125	0.002	14.4	47.9	96%	103%	5%
Methamphetamine	125-10,000	0.999	125	0.006	16.3	54.2	105%	97%	3%
MDA	125-5,000	0.999	125	0.002	11.7	39.1	106%	110%	3%
MDMA	125-5,000	0.981	125	0.060	6.18	20.6	104%	96%	12%
PCP	6.25-250	0.998	6.25	0.020	1.25	4.16	96%	92%	7%

Table 4. QC 1 and QC 2 concentrations for the five analytes.

Analyte	QC 1 ng/mL	QC 1 ng/mL
Amphetamine	375	7500
Methamphetamine	375	7500
MDA	375	750
MDMA	375	750
PCP	18.75	375

Amphetamine calibration curve and residual plot



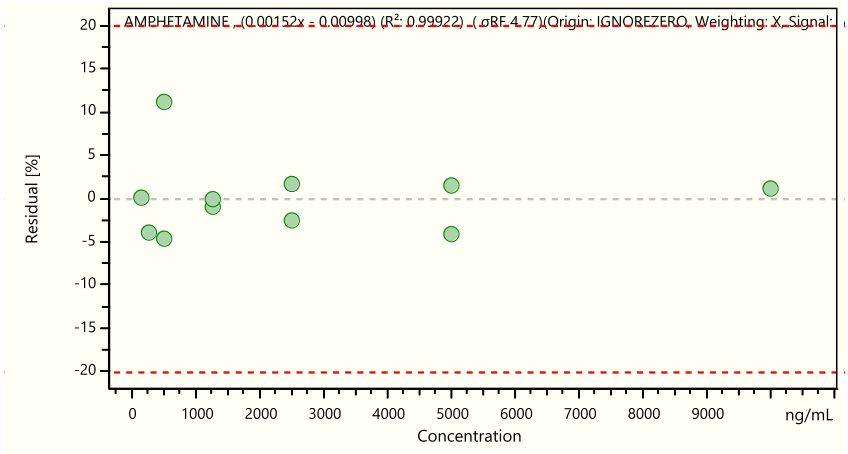


Figure 2. shows an example of the calibration curve that was generated for Amphetamine, where a linear R^2 correlation value > 0.999 was realized. Again, this shows the ability of DART-MS to accurately detect Amphetamine across a wide concentration range with high confidence. The residual plot illustrates the high quality of the assay regression fit for amphetamine with all points well under the ±20% limit of outliers.

Amphetamine

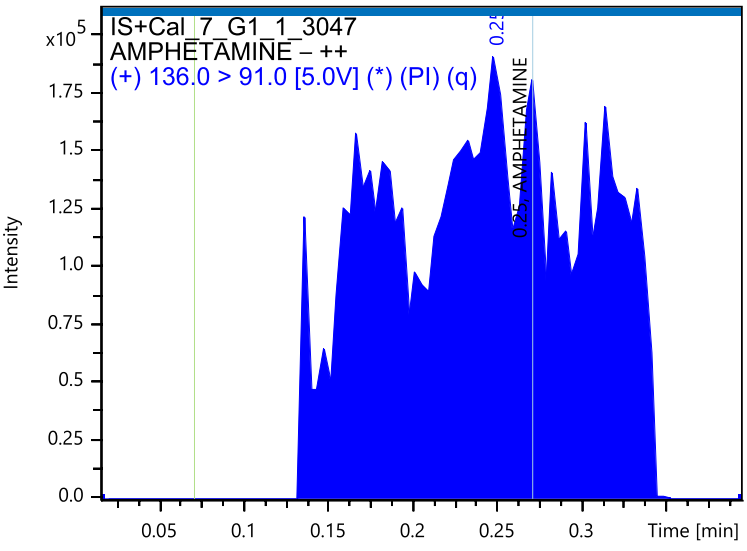


Figure 3. Amphetamine DART-MS raw 'unsmoothed' data signal.

Conclusions

This work demonstrates the utility of a simple chromatography-free screening in urine workflow for rapid quantitative drug screening for urine as a viable alternative to current IA UDS assays. The chromatography-free workflow is faster, more accurate, and quantitative. In less than 45 min, a plate of 96 samples can be fully analyzed. In addition, the chromatography-free workflow has the benefit of minimizing false positives typically associated with immunoassay screening and thus avoiding adding non-valued work to yield higher lab productivity. This high-performance workflow also minimizes the need for expensive and time-consuming chromatography based confirmatory tests.

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