

Poster Reprint

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Automated Fast Screening Workflow for Novel Psychoactive Substances by High Resolution Mass Spectrometry

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Data Independent Acquisition for Routine Metabolomic Profiling

The emergence of novel psychoactive substances (NPS) presents an analytical challenge due to compound diversity, low prevalence, and turnaround time requirements. Traditional broad screening workflows often require extensive data review before determination of a confirmatory analysis. Utilizing reflexive injection logic that reduces the need for initial review reduces workload and turnaround time. Herein, an automated two-step workflow integrating initial fast screening with a secondary longer chromatographic reinjection that includes fragment analysis, triggered upon initial detection of targeted features, is described. This approach enabled by a high-resolution mass spectrometry platform optimized for small molecule analysis provides good mass accuracy, isotopic fidelity and extended dynamic range necessary for untargeted NPS screening. The LC/Q-TOF is an ideal platform for routine untargeted screening. Acquisition with All Ions methodology includes fragment ions that assist in confident identification of analytes of interest.



Figure 1. Revident LC/Q-TOF with 1290 Infinity II LC.

Performance elements of the Revident LC/Q-TOF are a new detector, with better mass accuracies over extended concentration range, and an increased dynamic range compared to previous instrument generations. In combination with the temperature-controlled flight tube, contributing to mass stability, the overall mass accuracy has improved. These factors make the Revident LC/Q-TOF suitable for routine screening as demonstrated herein.

Data Acquisition with Revident LC/Q-TOF

A panel of 23 NPS compounds was spiked into whole blood and processed using protein precipitation and filtration with Agilent Captiva EMR-Lipid cartridges. Samples were crashed with cold ACN:MeOH, vortexed, and centrifuged. The supernatant was loaded onto filtration cartridges, then rinsed with an ACN:H₂O mixture to enhance analyte recovery. The eluate was dried under nitrogen at 45°C and reconstituted in 50:50 MeOH:H₂O. Analysis was performed on a LC/Q-TOF using two chromatographic methods, a short Phenyl-Hexyl MSOnly screen and a longer C18 All Ions screen, to evaluate sensitivity and screening capabilities of the workflow. Data was analyzed using MassHunter Quantitative Analysis 12.1 and the LC Screener Tool.

Table 1. LC Conditions with 1290 Infinity II LC.

LC Conditions				
Column	Agilent Poroshell 120 Phenyl-Hexyl, 2.1 x 50 mm, 1.9 μm (pn 699675-912) and Agilent Poroshell 120 EC-C18, 2.1 x 100 mm, 1.9 μm (pn 695675-902)			
Column temp	55 °C			
Injection	2 μL	Autosampler	5 °C	
Mobile phase	A = H2O + 0.1% formic acid B = MeOH + 0.1% formic acid			
Flow rate	0.800 mL/min	0.500 mL/min		
Gradient program	Short		Long	
	Time	%B	Time	%B
	0.0	5	0.0	5
	0.15	8	0.50	8
	0.60	11	1.20	11
	1.00	25	2.00	25
	1.50	45	6.00	45
	2.00	70	7.50	70
	3.00	98	8.50	98
	3.50	98	9.50	98
			10.00	5

Table 2. Source parameters with Revident LC/Q-TOF.

Parameters	Value
Ion mode	AJS, positive
Gas temperature	250 °C
Drying gas flow	11 L/min
Nebulizer gas	40 psi
Sheath gas temperature	350 °C
Sheath gas flow	12 L/min
Capillary voltage	2500 V
Nozzle voltage	0 V

Automated Reinjection Workflow from MS Only Fast Screening to Extended All Ions Fragmentation Analysis

The Revident LC/Q-TOF combined with intelligent reflex automated injection logic works to address the evaluation of NPS compounds in complex matrix samples without extensive analyst review and unnecessary long chromatographic injections for samples without any hits.

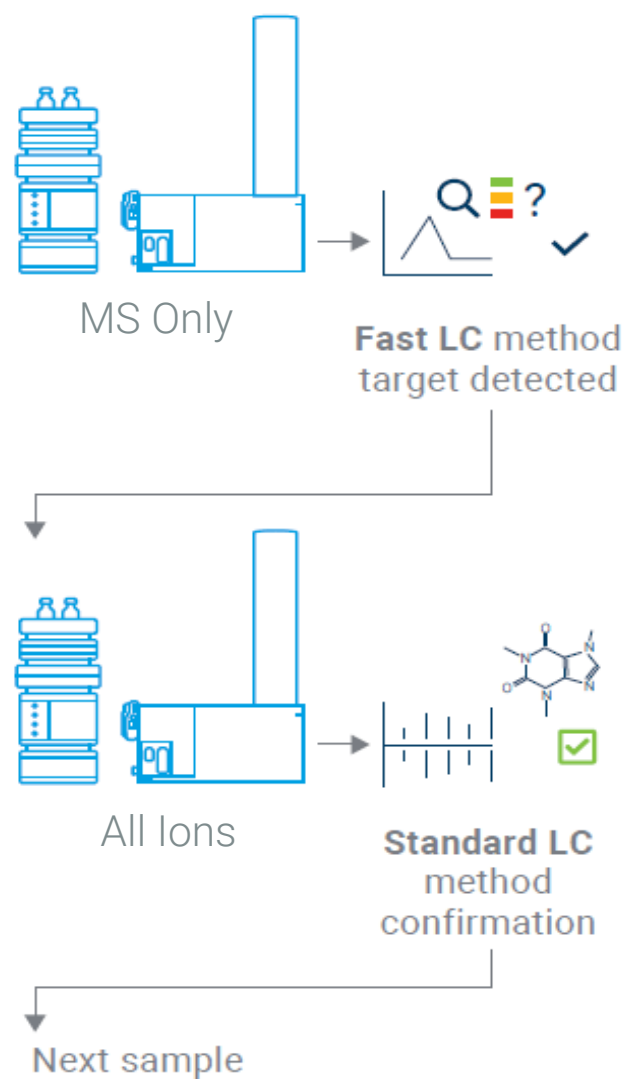


Figure 2. Combination workflow first-pass short MSOnly and second-pass long All Ions. First-pass hits determined by LC Screener outlier analysis automatically and reinjected upon Green or Orange hit.¹

Fast Screening with LC Screener Tool in MassHunter Quantitative Analysis

The data from both methods were analyzed using MassHunter Quantitative Analysis 12.1 with the LC Screener Tool and a spectral library with retention times organized with ChemVista, was used to build the methods. The screening methodology reports the confident presence, questionable presence, or absence of compounds. The report highlights the compound status with green, orange, or red flags. A compound labeled as present fulfills all qualifier settings, a questionable compound has an outlier that falls outside of the settings, and absent compounds fall outside of two or more outlier restrictions. Outliers include retention time, minimum signal to noise, fragment coelution, mass accuracy, mass match score and number of verified ions (Table 3).

Table 3. LC Screener outlier parameters. (MSOnly required 1 verified ion and no coelution requirement)*

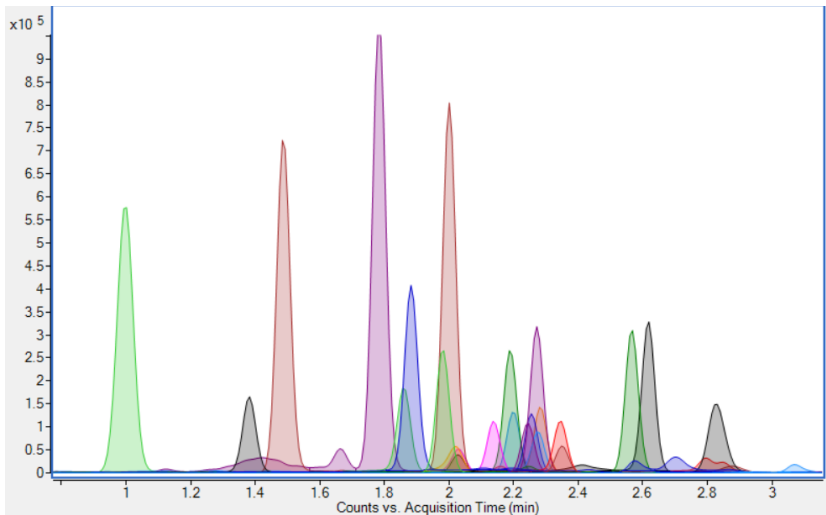
LC Screener Outlier Parameters	Values
Retention Time Window	10%
Minimum S/N	3
Coelution Score Limit	0 & 70*
Mass Accuracy Limit	5 ppm
Mass Match Score Minimum	75
Number of Verified Ions Minimum	1 & 2*

To enable the workflow two different Quant methods were established. Both required LC Screener parameters. In the methods, calibration curves were included for quantitation calculations, although this is not required for the workflow.

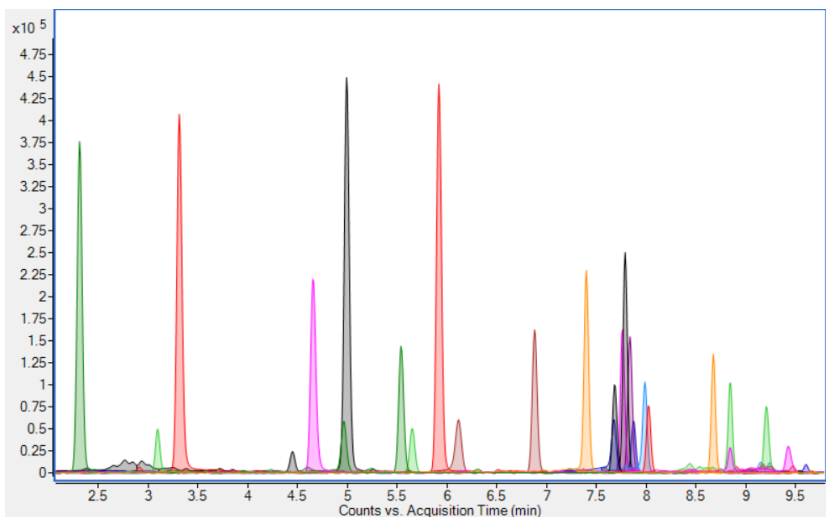
Additional Chromatographic Separation, Linearity, and Fragment Analysis with Second-Pass Method

The combination of All Ions fragmentation and selective chromatography allows for more confident identification of hits.

First-Pass / Shorter / MSOnly



Second-Pass / Longer / All Ions



- NPS Compounds**
- 4-OH Xylazine
 - Xylazine
 - Metonitazene
 - Adinazolam
 - Medetomidine
 - Brorphine
 - Pyrazolam
 - Nifoxipam
 - ortho-Methylfentanyl
 - Bromazepam
 - Clonazolam
 - Flualprazolam
 - Bromazolam
 - Phenazolam
 - Flubromazepam
 - Etizolam
 - Desalkylgidazepam
 - MDMB-INACA
 - MDMB-4en-PINACA
 - Ethylbromazolam
 - Rilmazolam
 - 5F-MDMB-PINACA(5F-ADB)
 - HHC

Figure 3. First (top) and second (bottom) pass chromatograms with EICs of all 23 targets at 10 ppb reportable level. All NPS compounds listed (right).

Most targets were detectable at a reportable level of 1 ppb. This level was consistent between the shorter and longer chromatographic methods. Across all compounds method performance was robust, retention time RSDs below 1% for all measurements and over 90% of area RSDs under 10%. Linearity was excellent, with R^2 values >0.99 for all detected concentration levels (Figure 4).

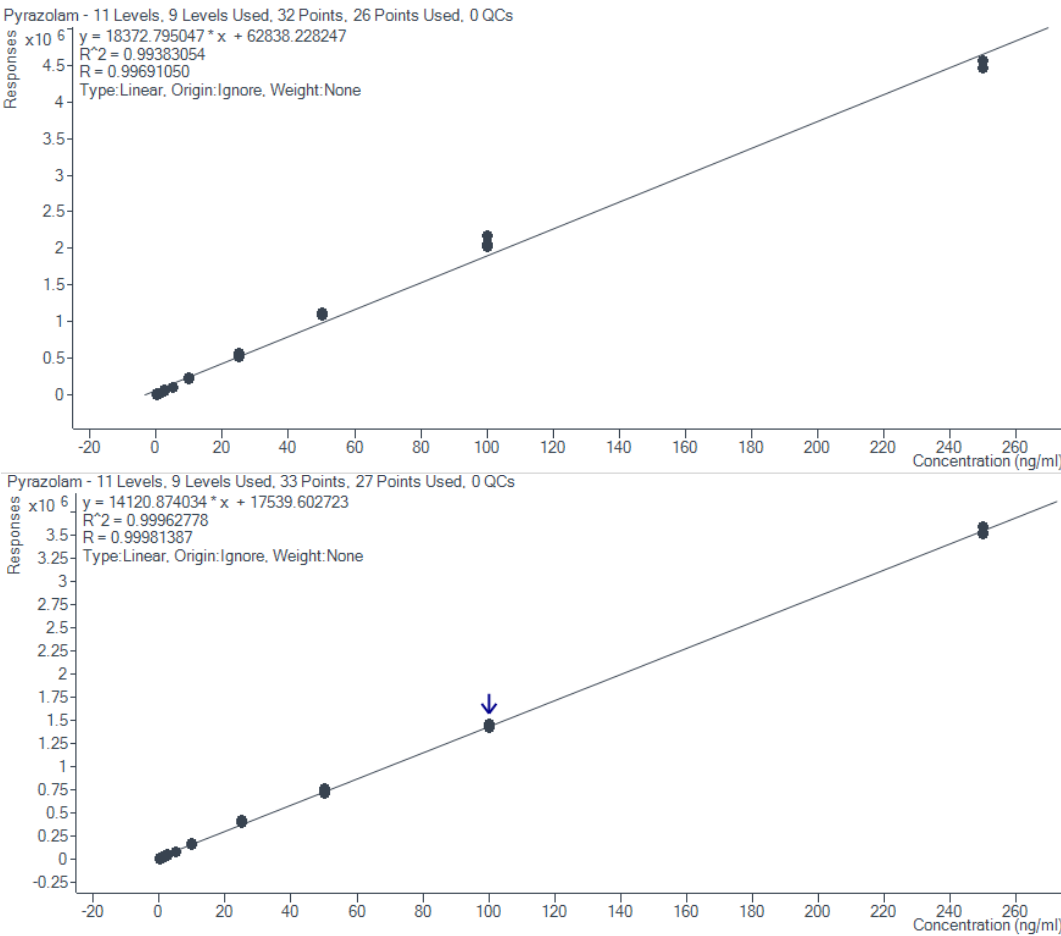


Figure 4. Example concentration curves of Pyrazolam, first (top) and second (bottom) pass from 0.5 to 250 ppb.

Mass accuracy averaged within ± 1 ppm for all compounds and mass match score, a combined score of mass accuracy, isotopic spacing and isotopic abundance, was over 90/100 for the majority of compounds, supporting identification.

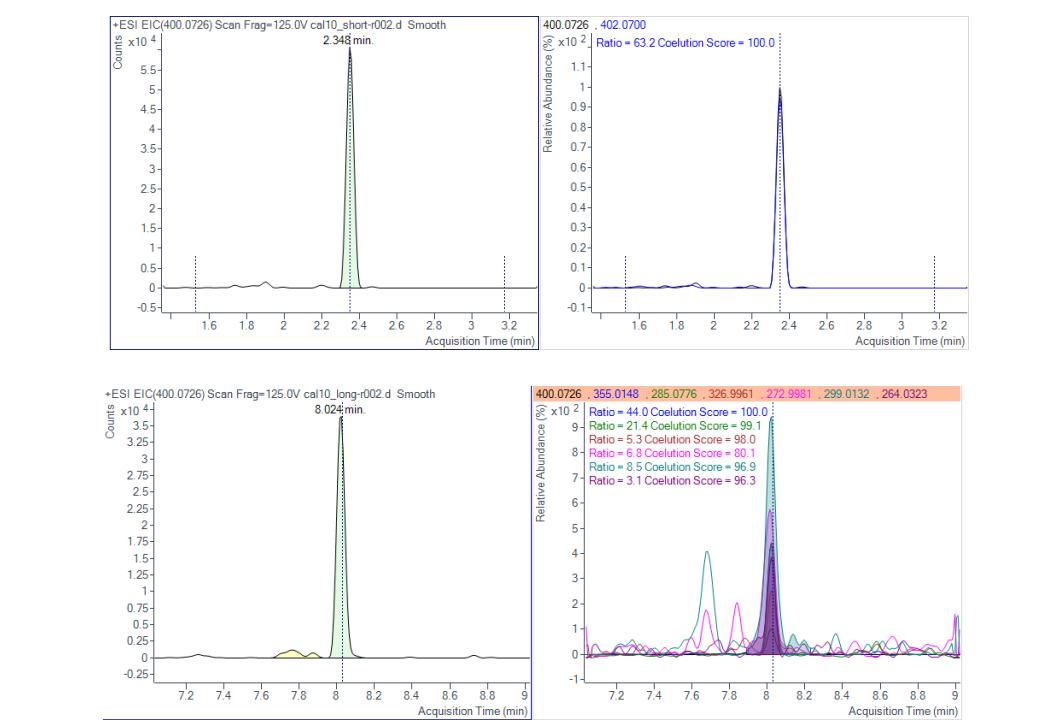


Figure 5. EIC results for Rilmazolam at 10 ppb in blood with (top) first-pass including isotopic overlay and second (bottom) with fragment coelution.

<https://www.agilent.com/en/promotions/asms>

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Automated Reinjection Workflow with LC Screener Tool

Based on the results of the first-pass the worklist is appended with additional injections using a different chromatographic method and column. A blank is injected before the first reinjection for equilibration and QCs at a chosen interval. As shown in figure 6, the appended injections are highlighted for visibility and clearly marked as the result of an intelligent reflex decision.

104	✓	Completed	P1-F4	asm26_dhugp_shortcolumn_msonly.m	sample1_short.d	Sample	As Method	TS Confirmation	D:\Projects\Cate	D:\Projects\Cate\Q	Green plus Orange
105	✓	Completed	P1-F5	asm26_dhugp_shortcolumn_msonly.m	sample2_short.d	Sample	As Method	TS Confirmation	D:\Projects\Cate	D:\Projects\Cate\Q	Green plus Orange
106	✓	Completed	P1-F6	asm26_dhugp_shortcolumn_msonly.m	sample3_short.d	Sample	As Method	TS Confirmation	D:\Projects\Cate	D:\Projects\Cate\Q	Green plus Orange
107	✓	Completed	P1-F7	asm26_dhugp_shortcolumn_msonly.m	sample4_short.d	Sample	As Method	TS Confirmation	D:\Projects\Cate	D:\Projects\Cate\Q	Green plus Orange
108	✓	Completed	P1-F8	asm26_dhugp_shortcolumn_msonly.m	sample5_short.d	Sample	As Method	TS Confirmation	D:\Projects\Cate	D:\Projects\Cate\Q	Green plus Orange
109	✓	Completed	P1-F9	asm26_dhugp_shortcolumn_msonly.m	sample6_short.d	Sample	As Method	TS Confirmation	D:\Projects\Cate	D:\Projects\Cate\Q	Green plus Orange
110	✓	Completed	Intelligent Reflex	P1-D8	asm26_dhugp_column_allions.m	sample1_short-Intelligent Reflex-Blank	Blank	As Method	No Intelligent Ref	D:\Projects\Cate\Q	
111	✓	Completed	Intelligent Reflex	P1-F4	asm26_dhugp_column_allions.m	sample2_short-Intelligent Reflex.d	Sample	As Method	No Intelligent Ref	D:\Projects\Cate\Q	
112	✓	Completed	Intelligent Reflex	P1-F4	asm26_dhugp_column_allions.m	sample3_short-Intelligent Reflex-Blank	Blank	As Method	No Intelligent Ref	D:\Projects\Cate\Q	
113	✓	Completed	Intelligent Reflex	P1-F5	asm26_dhugp_column_allions.m	sample4_short-Intelligent Reflex.d	Sample	As Method	No Intelligent Ref	D:\Projects\Cate\Q	

Figure 6. Worklist results for reinjection of samples carried out with intelligent reflex. Decision is made upon presence, absence, or questionable status.

The results of the reinjection can be reviewed by a detailed or summary-based report or within the Quant LC Screener tool page. (Figure 7)

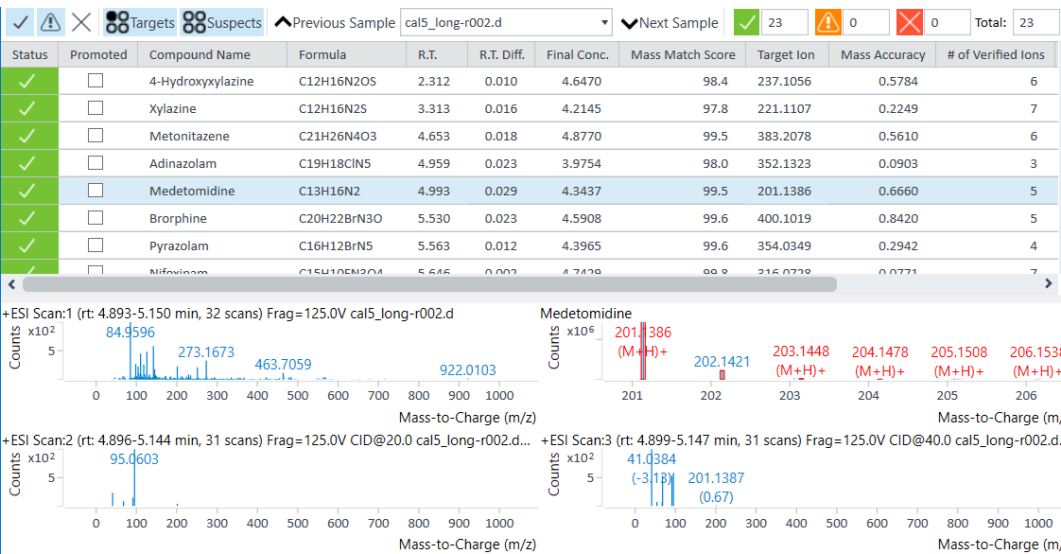


Figure 7. LC Screener tool results for All Ions second-pass, 5 ppb concentration level in blood.

Conclusions

Workflow for Automated Screening of NPS compounds in Whole Blood

- Easily executable automated workflow for the fast screening of hits followed by required complementary method reinjection
- 23 NPS compounds detected at low levels, with good linearity, using MSONly and All Ions acquisition
- Example of use for LC Screener tool in Quant with automated intelligent reflex workflow

References

1 Simmermaker, C et al. Intelligent Relfex Targeted Reinjection Workflow for Pesticide Screening in Food Matrices. Agilent App. Note 5994-7312EN. 2024.