Poster Reprint

ASMS 2023
Poster number ThP 035

From data-independent acquisition (DIA) to targeted MS/MS: Automatic reinjection for additional confirmation in suspect screening

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Introduction

From DIA to targeted MS/MS via automatic reinjection

Multi-residue screening in food safety is challenged with an ever-growing list of regulated residues. As a result, high resolution instruments are becoming increasingly popular for initial screening. To comply with SANTE guidelines for the simultaneous identification and quantitation of compounds, overlapping molecular ions and a specified number of fragments are required over the chromatographic peak. DIA workflows fulfill these requirements but can yield questionable results. These require manual inspection and often reinjection, leading to delays in reporting results. Here we present the concept of an intelligent reflex, where after the initial sample run in DIA mode (All Ions) the data is processed, and automatically appended to the running worklist to confirm questionable or identified residues via targeted MS/MS.

Key performance elements of the new Revident LC/Q-TOF are a new detector, resulting in better mass accuracies over a broad range of abundances, as well as an increased dynamic range compared to previous instrument generations. The overall mass accuracy has significantly improved due to a new detector and the integration of the Thermo-controlled flight tube, which ensures long-term mass stability. In addition to maintaining isotopic fidelity and uncompromised resolution, the acquisition speeds of the instrument support the specificity of fragment ions in All Ions mode and enable subsequent targeted MS/MS analysis. As demonstrated in this study, the Revident LC/Q-TOF is highly suitable for routine screening in food safety applications.

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

Experimental

Sample Preparation and All Ions Acquisition for Complex Food Matrices

An organic broccoli matrix was prepared with QuEChERS for pigmented fruits and vegetables, after being homogenized. The supernatant was spiked with over 200 pesticides (PN 5190-0551) at 8 different concentrations from 0.625 ppb to 100 ppb and 4 heavy labeled standards at 50 ppb (n=6).[1] The samples were analyzed utilizing reverse phase chromatography on a 1290 Infinity II LC and All Ions non-targeted acquisition in both positive and negative mode. The collision energies (CE) used were 20 V and 40 V to fragment molecular ions. Two reference ions were used to ensure mass accuracy.

Table 1. LC method with Agilent 1290 Infinity II LC.

LC Conditions	
Analytical Column	Agilent ZORBAX Eclipse Plus C18, 3.0 x 150 mm 1.8 µm, (p/n 959759-302)
Guard Column	ZORBAX Eclipse Plus C18, 2.1 mm, 1.8 µm, UHPLC guard column (p/n 821725-901)
Column temperature	45 °C
Injection volume	3 µL
Autosampler temp	4 °C
Needle wash	Standard Wash, 10 sec, MeOH:IPA (50:50)
Mobile phase	A = Water + 4.5 mM ammonium formate + 0.5 mM ammonium fluoride + 0.1% formic acid B = ACN + 4.5 mM ammonium formate + 0.5 mM ammonium fluoride + 0.1% formic acid
Flow rate	0.45 mL/min
Gradient program	Time %B 0.00 2 0.50 2 1.00 50 4.00 65 16.00 100 18.00 100 18.10 2 20.00 2
Post Time	4 min

Table 2. Revident LC/Q-TOF acquisition parameters.

Parameter	Value
Sheath Gas Temperature	375 °C
Sheath Gas Flow	12 L/min
Gas Temperature	325 °C
Gas Flow	10 L/min
Nebulizer	35 psi
Capillary Voltage	2500 V
Nozzle Voltage	200 V
MS Mode	Positive and Negative
Acquisition	All Ions with 0, 20, and 40 CE experiments
MS Range	*m/z* 50 to 1,000
Reference Mass	*m/z* 121.0509 and 922.0098 (positive) *m/z* 119.0363 and 966.0007 (negative)

The chromatography and analysis allowed for the detection of 214 compounds, including the separation of 5 isomeric pairs in the standard mixture.

The Stepping Stones to Confident Screening: Mass Accuracy, Stable Area Counts and Fragments

The 200+ compounds were measured with good mass accuracies, most within ±2 ppm (Figure 2). The %RSD of the abundance were under 20% for > 99% of measurements made at the 5 ppb and 10 ppb levels for all target compounds. As detection limits were pushed the RSDs increased, but the system proved very sensitive even at low limits. The calibration curves showed good linearity with a $R^2 \geq 0.99$ for all target compounds from 0.625 ppb to 100 ppb.

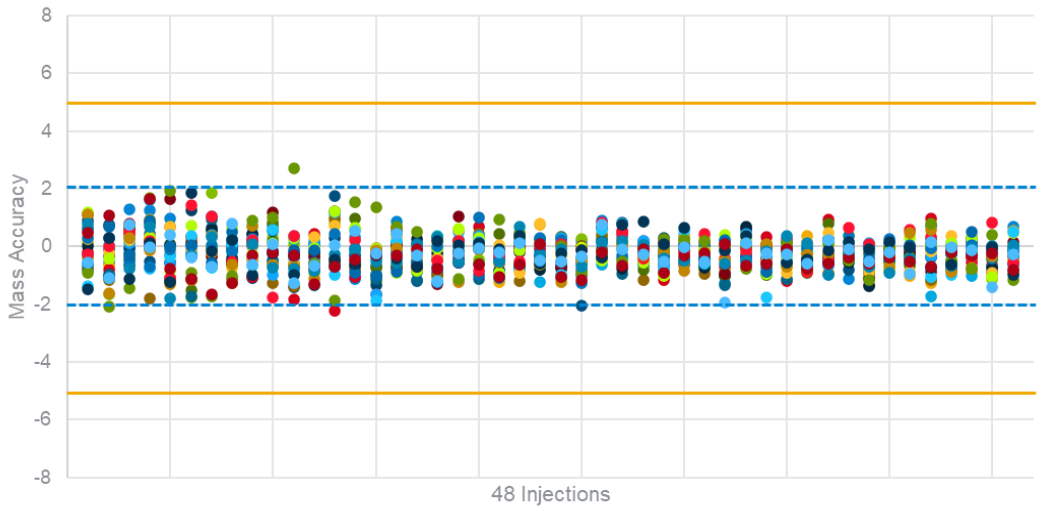


Figure 2. Mass accuracy stability of 25 different analytes (various colors) over the entire calibration range from 48 injections over 18 hours. (blue dotted line ±2 ppm and yellow solid ±5 ppm)

The All Ions data, analyzed in the LC/Q-TOF screener tool in MassHunter Quantitative Analysis 12.1 also provides fragment ions for confident identification scored for ratio and coelution to the molecular ion, allowing for the processing to distinguish matrix interferences from specific fragment ions (Figure 3). All the analytes have consistent abundance and mass accuracy over the full calibration range (Figure 2).

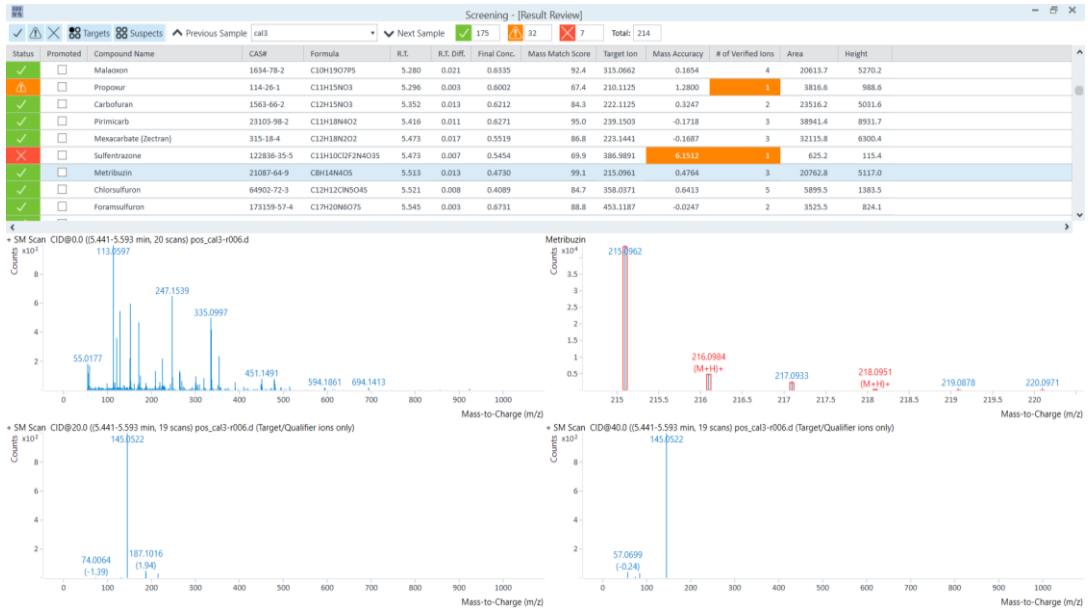


Figure 3. LC Screener user interface showing identified suspects (green) as well as questionable suspects (orange) that require further analysis.

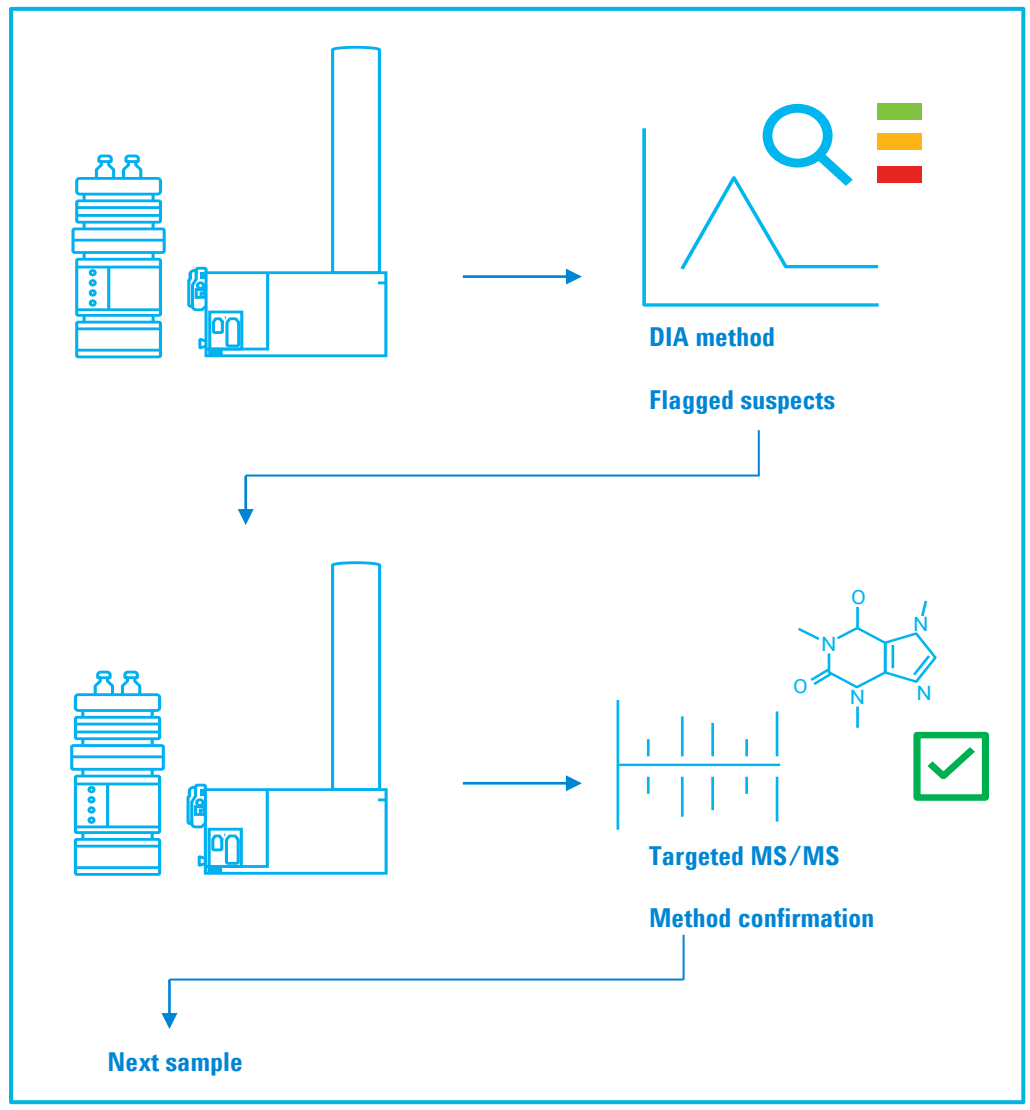


Figure 4. Targeted MS/MS Confirmation Intelligent Reflex workflow to automatically reinject a sample after suspects were found in a DIA analysis. The second run is then done using targeted MS/MS for maximum selectivity and orthogonal identification.

Screening criteria were set according to SANTE guidelines[2], including the requirement of mass accuracy ≤ 5 ppm, fragment coelution score above 80%, the signal-to-noise ≥ 3, and the 2 ions verified with mass accuracy.

After completion of the first run, all information of the detected suspects are automatically transferred to a second, targeted MS/MS method, where the information of RT and *m/z* are used to populate the acquisition table of compounds for precursor selection and fragmentation (Figure 4).

Using this novel automatic reinjection approach can dramatically increase the productivity of a lab, eliminating the time the instrument is normally idle after completing the DIA worklist and the analyst performs manual data inspection and manual set-up of the targeted MS/MS parameters.

As an example, a sample (10 ppb) “identified” 182 of 254 pesticides, with 22 being “questionable” and 50 “not identified”. Upon targeted MS/MS reinjection 18 of the 22 “questionable” compounds could be unambiguously “identified” using the LC Screener Tool. Driven by the spectral simplification displayed in Figure 6 and 7.

SureMass Algorithm for Maximum Sensitivity

All data was analyzed using SureMass, a new algorithm to detect features on profile mode peak detection. The advantage of this algorithm is the full MS peak is analyzed, not just the centroid, leading to extended quantitative accuracy while maintaining trace analysis capabilities (Figure 5).

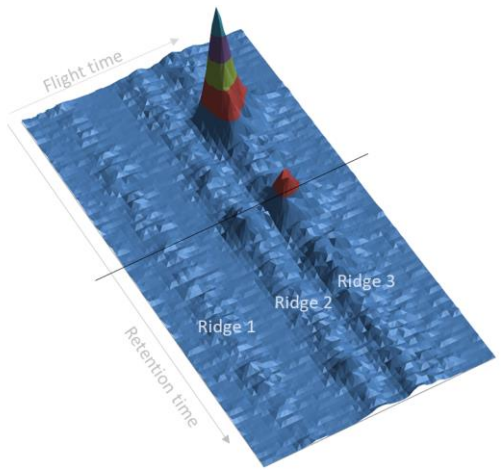


Figure 5. SureMass 3-dimensional signal processing of ridges and associated chemical features.

Targeted MS/MS for Highest Selectivity

Fragment spectra using All Ions can be very complex, with the possibility of peak interferences with matrix, as well as overlap of co-eluting residues with similar retention times and similar fragments. To overcome this ambiguity, targeted MS/MS with quadrupole isolation of the precursor prior fragmentation offers the highest confidence in identification (Figure 6).

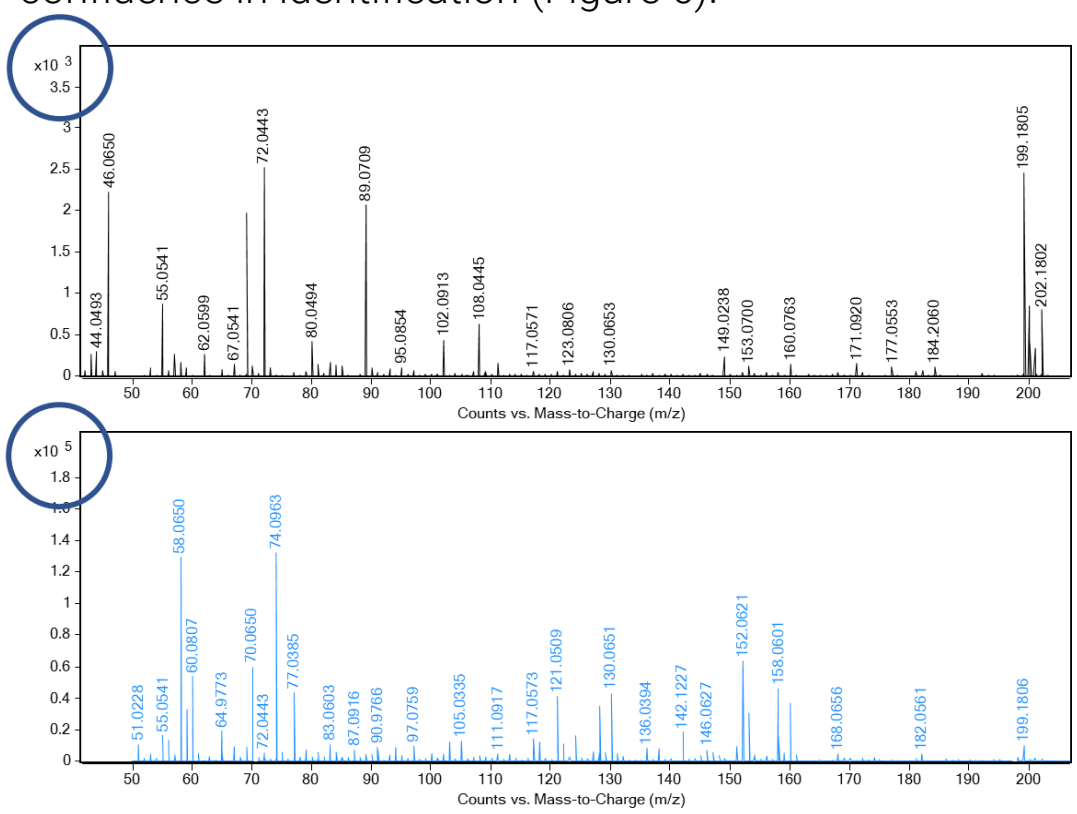
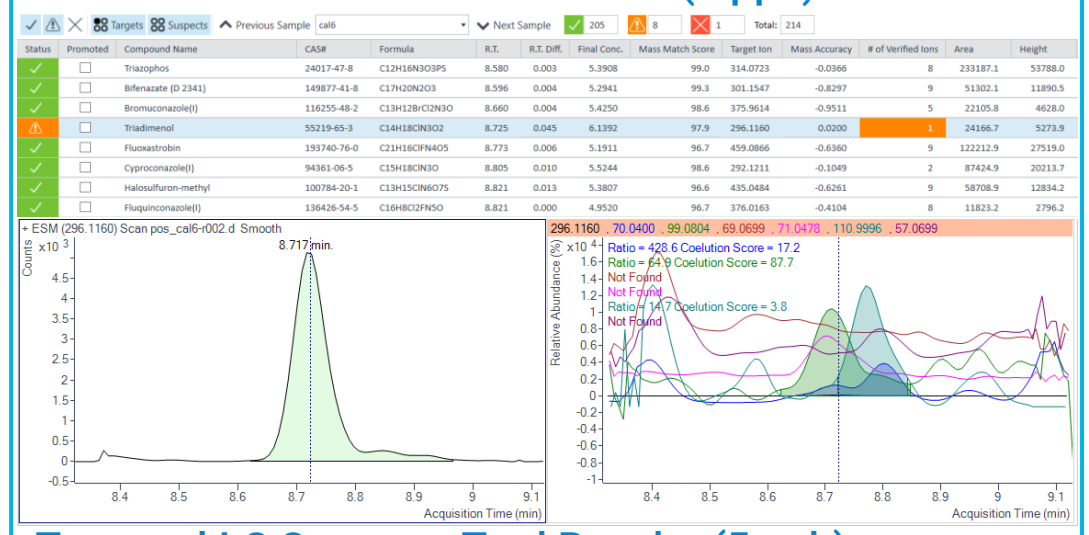


Figure 6. Fragment spectra using targeted MS/MS (top) and All Ions (bottom)

Using a DIA workflow, data would traditionally need to be manually analyzed and possibly reinjected. This workflow can be simplified with intelligent reflex automation to transform questionable screening into confident identification.

All Ions LC Screener Tool Results (5 ppb)



Targeted LC Screener Tool Results (5 ppb)

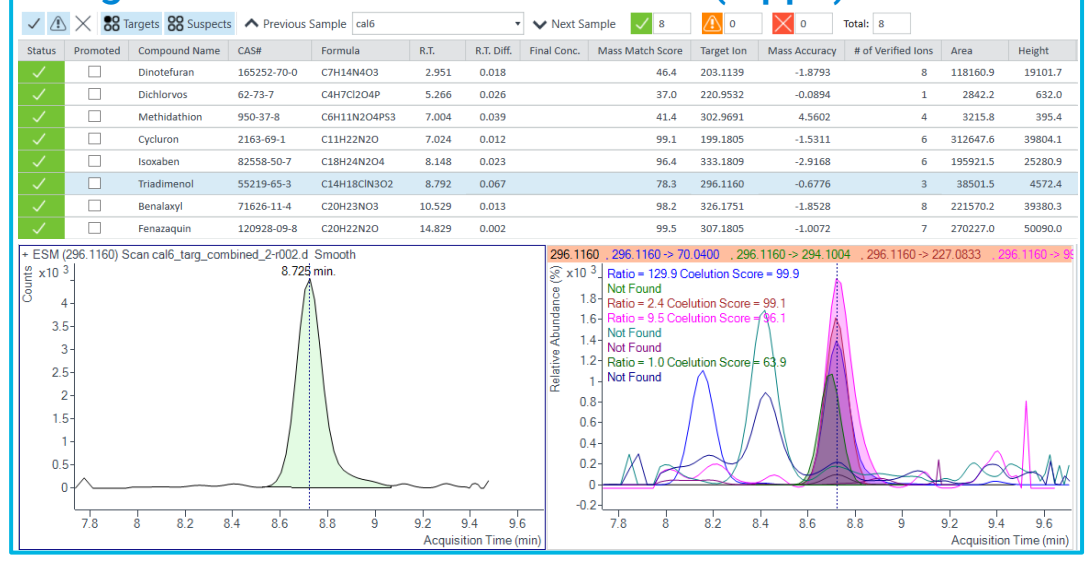


Figure 7. LC Screener and analyte results at 5 ppb with DIA acquisition (top) and targeted MS/MS acquisition (bottom).

Conclusions

Confident Pesticide Screening by combining automatically DIA with targeted MS/MS

Over 200 pesticides were screened using All Ions acquisition from 0.625 ppb to 100 ppb with:

- New Agilent Revident LC/Q-TOF
- Good Mass Accuracy, Low RSD's, Scored Coelution of Fragment Ions
- Sample Preparation and Detection in Complex Matrix
- Analysis with MassHunter Quant 12.1 allows combined reporting of All Ions and targeted MS/MS runs

References

[1]Yannell, K.; *et al.* Enhanced Food Safety Testing: A Pesticide Screening Methodology Using the Agilent 6546 LC/Q-TOF and MassHunter Quantitative Analysis Software 10.0 LC/Q-TOF Screener Tool, *Agilent Technologies Application Note,* 5994-0738EN, 2019.

[2] Analytical Quality Control and Method Validation Procedures for Pesticide Residues Analysis in Food and Feed SANTE 11312/2021.

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DE12059019

Published in USA, May 31,2023

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