Application Note

Food Safety

Intelligent Reflex Targeted Reinjection Workflow for Pesticide Screening in Food Matrices

Using the Agilent Revident LC/Q-TOF

Authors

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Abstract

This application note details a methodology for screening of pesticides in complex food matrices using the Agilent Revident quadrupole time-of-flight LC/MS (LC/Q-TOF) with Agilent MassHunter Quantitative Analysis software, version 12.1. Intelligent reflex automated targeted reinjection was used for the acquisition of compounds that were screened as present or questionable. This method describes all steps including sample preparation, untargeted screening, automated targeted reinjection, and reporting for routine pesticides screening.

Introduction

Robust and reliable screening methods are a requirement for pesticide analysis in food matrices and a continuous point of challenge with the growing number of matrix samples, varying detection limits, and a continuously expanding list of emerging contaminants.[1] Data-independent acquisition (DIA) methods allow users to simultaneously screen for targets of interest while also evaluating valuable information on unknown compounds. With this simultaneous acquisition, past data files can be evaluated for contaminants of future interest retroactively.

The Revident LC/Q-TOF (Figure 1) facilitates DIA and enhances confidence in identification through extended dynamic range, stable accurate mass measurements, isotopic fidelity, and enhanced acquisition speeds. Different from its predecessors, the Revident LC/Q-TOF boasts a new detector, producing superior mass accuracies. Further, the temperature-controlled flight tube enables long-time mass stability, the proven ion optics assures robustness, the pump-based calibrant delivery is improved, and the VacShield technology provides seamless and fast capillary maintenance.

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

Although complex, DIA analysis can be simplified with curated libraries and simple software workflows. The LC Screener Tool in MassHunter Quantitative Analysis 12.1 provides a streamlined screening overview with clear "present," "questionable," and "not detected" designations. The curated libraries from the Agilent ChemVista library manager software allow for comparison to selected fragment ions, increasing confidence in identification. Despite these benefits, DIA workflows could lead to compounds with ambiguous results as a product of the indiscriminate fragmentation. The common workflow for these compounds would be manual data analysis by the scientist and reinjection of the sample in a targeted MS/MS mode of acquisition.

New to the Revident LC/Q-TOF, this manual interpretation and reinjection can now be automated using the intelligent reflex workflow. DIA data files are automatically processed with the LC Screener Tool and new injections are appended to the worklist for targeted reinjection of compounds that are found to either be present, questionable, or both. This process speeds up the screening workflow by reducing the amount of time required by the analyst to achieve confident identifications.

Herein, this workflow has been used for pesticide screening of three different food matrices using intelligent reflex targeted reinjection screening to evaluate present pesticides in real-world samples by liquid chromatography mass spectrometry.

Experimental

Sample preparation

All foods for the experiment were sourced from local stores and were not labeled as organic. Broccoli, strawberry, and celery were prepared following the QuEChERS (EN) standard protocol. The food samples were chopped and frozen at –80 °C, then homogenized to a fine material. The homogenized samples (10 g) were weighed and extracted with acetonitrile (10 mL) using the Agilent Bond Elut QuEChERS EN extraction kit (part number 5982-5650CH). Extracts were prepared further with dispersive solid phase extraction (dSPE): broccoli and celery were prepared using the Agilent Bond Elut QuEChERS Pigmented Fruit and Vegetables dSPE kit (part number 5982-5256), and strawberry using the Agilent Bond Elut QuEChERS General Fruits and Vegetables dSPE kit (part number 5982-5056).[2] The resulting matrices were stored at –20 °C in scintillation vials.

The Agilent LC/MS Pesticide Comprehensive mix (part number 5190-0551), which contains over 200 compounds, was used as the standard mixture for calibration curve preparation. The standard was spiked into the prepared matrices at eight points between 1 and 100 ppb, along with four deuterated standards spiked in at 50 ppb.

Data acquisition

The samples were analyzed using C18 reversed-phase chromatography with a UHPLC guard column (part numbers 959759-302 and 821725-901, respectively) on an Agilent 1290 Infinity II LC with the Revident LC/Q-TOF. Data were collected in positive mode using the untargeted All Ions acquisition mode at three different collision energy settings: 0, 20, and 40 V (Tables 1 and 2). Two reference ions were used during acquisition to ensure consistent mass accuracy, *m/z* 121.0509 and 922.0098. Calibration was completed before the worklist was started; the instrument ran for over two days without stopping for additional calibration.

Table 1. LC parameters for the Agilent 1290 Infinity II LC.

Parameter	Value
Analytical Column	Agilent ZORBAX RRHD Eclipse Plus C18, 3.0 × 150 mm, 1.8 µm (p/n 959759-302)
Guard Column	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 mm, 1.8 µm, UHPLC guard column (p/n 821725-901)
Column Temperature	45 °C
Injection Volume	4 µL
Sampler Temperature	4 °C
Needle Wash	Standard wash, 10 s, MeOH:IPA (50:50)
Mobile Phase	A) Water + 4.5 mM ammonium formate + 0.5 mM ammonium fluoride + 0.1% formic acid B) MeOH + 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 2 0.5 2 1.0 50 4.0 65 16.0 100 18.0 100 18.1 2 20.0 2
Post-Time	4 min

Table 2. MS parameters for the Agilent Revident LC/Q-TOF.

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	2,500 V
Nozzle Voltage	200 V
Reference Mass	*m/z* 121.0509 and 922.0098 (positive)
All Ions Acquisition	MS only with 0, 20, and 40 V
All Ions Acquisition Rate	6 Hz
All Ions MS Range	*m/z* 50 to 1,000
Targeted MS and MS/MS Range	*m/z* 100 to 1,000 and *m/z* 40 to 600, respectively
Targeted MS and MS/MS Acquisition Rate	6 and 36 Hz
Targeted Isolation Width	Narrow (*m/z* 1.3)
Targeted Collision Energy	30 V

The 24-minute chromatographic method allowed for sufficient separation of analytes from the standard mixture over the full course of acquisition. The standard mixture of compounds included six isomeric pairs, of which most were separated chromatographically but two required an additional data analysis method curation to screen simultaneously.

Screening parameters

Target and suspect screening data analysis was performed with MassHunter Quantitative Analysis 12.1, applying the embedded LC Screener Tool. The LC Screener Tool summarizes subsets of information based on the presence, absence, or possible detection as decided by modifiable screening settings based on attributes of data such as mass accuracy, signal-to-noise ratio (S/N), mass match score, coelution of specified fragment ions, and retention time (Table 3).

Table 3. LC Screener Tool outlier settings.

LC Screener Outlier Parameters	Values
Retention Time Window	10%
Minimum S/N	3
Coelution Score Limit	80
Mass Accuracy Limit	5 ppm
Mass Match Score Minimum	65
Number of Verified Ions Minimum	2

Screening parameters were set to the requirements of SANTE guidelines.[3] This requires the mass accuracy to be ≤ 5 ppm for two ions, including at least one fragment ion, S/N ≥ 3, and full coelution of the precursor and product ions in the extracted ion chromatogram. The coelution of fragments can easily be assessed by MassHunter Quantitative Analysis 12.1; the evaluation of the coelution is scored by comparing the retention time, peak width, and peak symmetry of the quantifier and imported fragment qualifier ions. Based on this comparison, a score out of 100 was designated for each fragment ion, describing the coelution and the likelihood that the fragment is associated with the targeted quantifier ion. Furthermore, a mass match score of 65 was required, coelution score was set to 80 of 100, and retention time window to ± 0.4 minutes. Based on the evaluation of these parameters, a green (identified), orange (questionable), or red (not detected) designation was assigned.

Intelligent reflex workflow

The intelligent reflex workflow used was Target Screening Confirmation. The All Ions acquisition results are automatically analyzed and used to create the targeted list for the targeted MS/MS acquisition. The decision state for the initial screening was "green and orange" (present and questionable). Two MassHunter Quantitative Analysis 12.1 methods were loaded with the screening parameters described above. The resulting report template combines All Ions screening and targeted MS/MS results. Each tested sample of broccoli, strawberry, and celery was spiked with internal standards (50 ppb) and evaluated with the intelligent reflex workflow. The initial screening results were used to create a targeted MS/MS acquisition method automatically, and the reinjection was appended to the worklist for targeted evaluation of green and orange analytes.

Results and discussion

Data-independent acquisition—All Ions

Untargeted data acquisition requires precise mass accuracy measurements to confidently inform the identification of features. The use of reference ions during acquisition, which are added at a specific flow volume and rate as a feature of the new calibrant delivery system on the Revident Q-TOF LC/MS, makes for an even more stable accurate mass measurement. Independent of matrix or spiked pesticide concentration, mass accuracy was maintained over all experimental measurements made. Of the 200 compounds measured, the average mass accuracies of 100% of analytes were below ± 5 ppm in all detected calibration levels and matrices. Further, the average mass accuracy of each compound over all calibration levels was below ± 2 ppm for 98% of measurements. As shown in Figure 2, the mass accuracies of 170 compounds detected at all concentrations across all matrices were plotted for concentrations of 2.5, 10, 50, and 100 ppb, showing nearly all measurements within ± 2 ppm over 9,000 data points.

The mass match score is a weighted aggregate of mass accuracy, isotope distribution, and isotope spacing. A value out of 100 is used to define how well these parameters match the theoretical isotope measurements for the experimental target. The mass match score represents assurance of isotopic fidelity, which, while important for known targets with defined retention times and available fragment spectra, can be even more valuable to the evaluation of suspects that may not have additional identification criteria. At the 10 ppb calibration level, over 95% of evaluated compounds scored above 80 for mass match scores in all of the complex matrices.

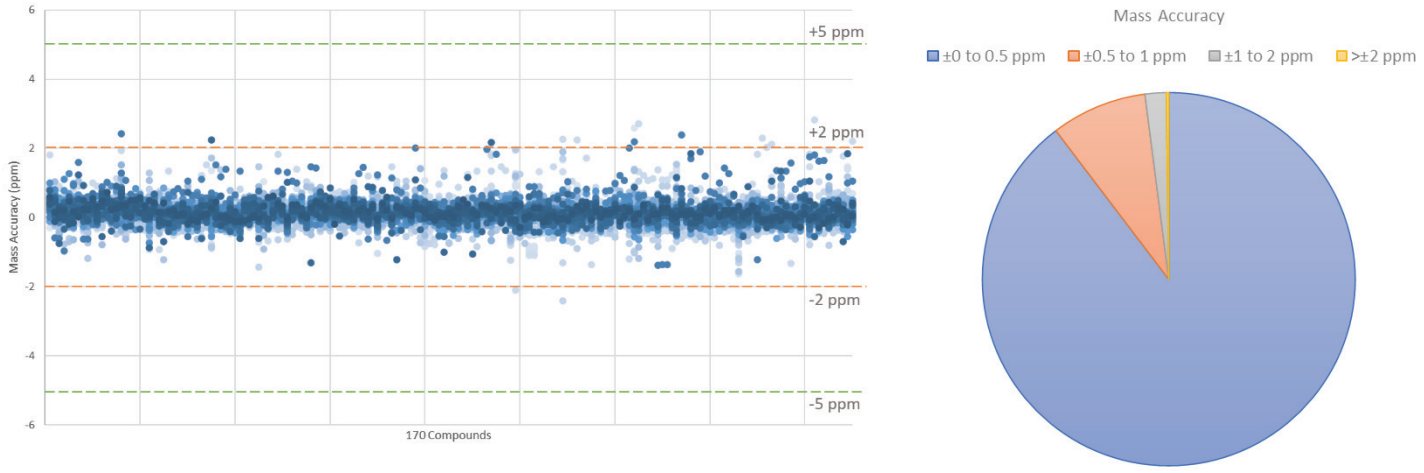


Figure 2. Mass accuracy plot over 9,000 data points including four different calibrant levels and all three food matrices.

Results can be easily viewed using the batch-at-a-glance interface, where all quantifier and qualifier values are visible, including calibration curves, coelution of fragment ions and ratios, and peak results (Figure 3).

Matrix interferences can also be quickly evaluated and corrected using a qualifier/quantifier swap within the MassHunter Quantitative Analysis method editor. This quantifier/qualifier swap was required in the case of methoxyfenozide in strawberry matrix, while it was not needed for broccoli or celery extract (Figure 4).

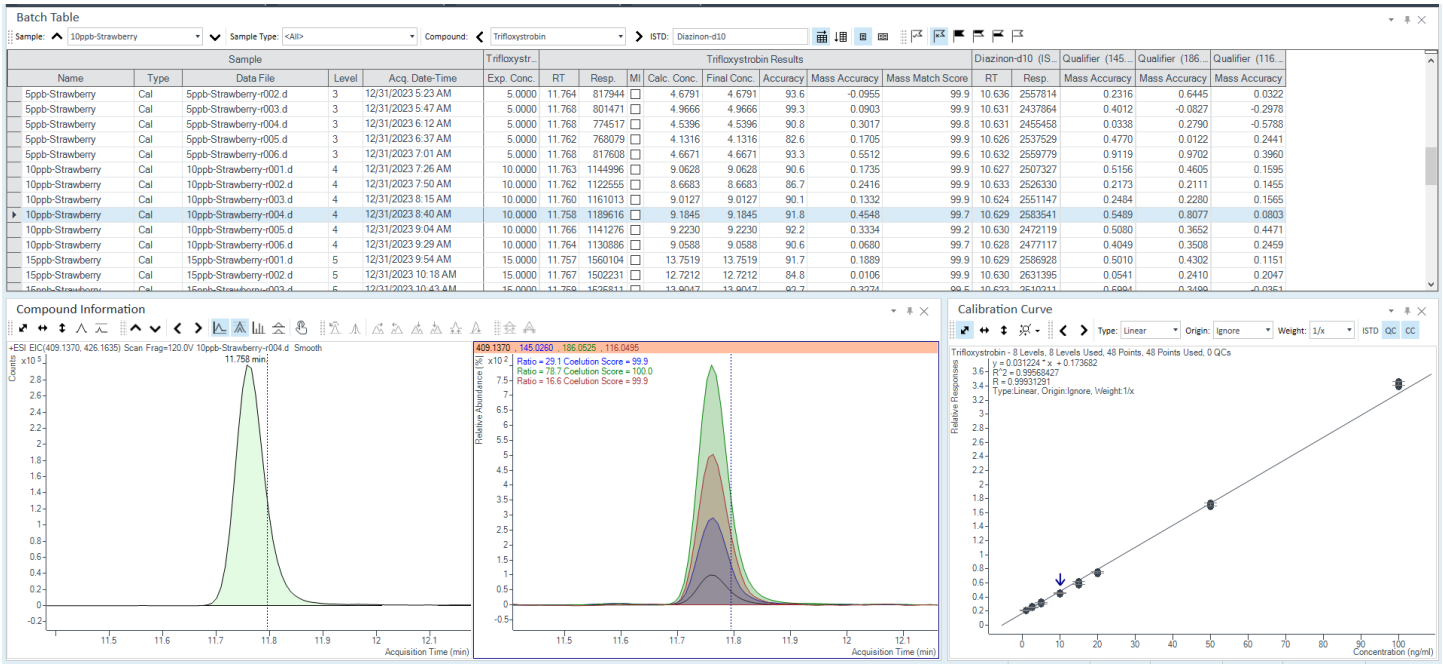


Figure 3. Batch-at-a-glance interface for trifloxystrobin in strawberry at the 10 ppb calibration level. Data files and results are displayed in the top table, and quantifier, qualifiers, and calibration curves are displayed in the bottom left to right, respectively.

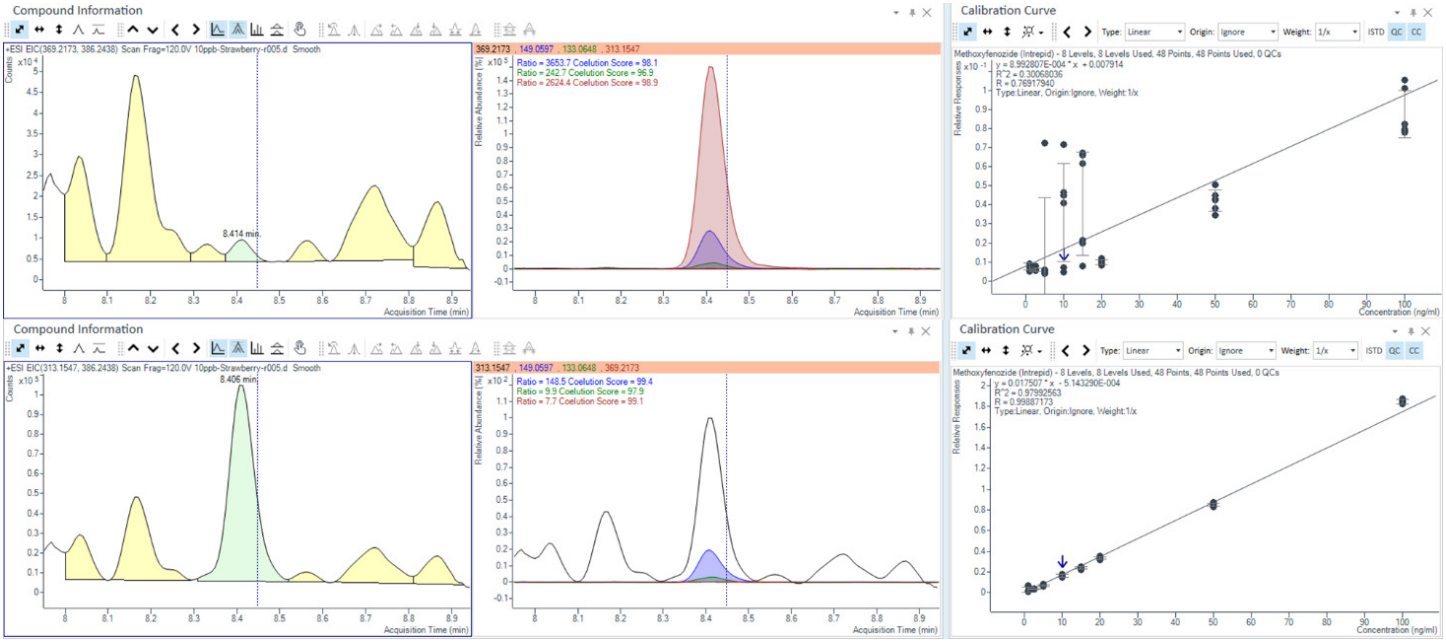


Figure 4. Qualifier and quantifier swap for methoxyfenozide at 10 ppb in strawberry to manage matrix interference.

A summary of this data can be viewed with the LC Screener Tool—a program embedded into MassHunter Quantitative Analysis. Here, all the detailed data can be set with qualifying parameters and reported in an easy-to-view table that works in combination with the batch-at-a-glance interface, making large screening experiments easier to review.

LC Screener Tool

Analysis of the untargeted data was carried out using detailed libraries containing MS/MS spectra and curated retention times evaluated with the chromatography. The spectral libraries were built within ChemVista—the new library management software from Agilent. Here, compounds can be sorted, assigned retention times with specific chromatography, and built to even more specificity, with the ability to import open-source spectral libraries. With this, the MassHunter Quantitative Analysis methods were curated to contain sensitive and specific fragment ions, further increasing confidence in identification.

Screening parameters were set to SANTE guidelines (as previously described in Table 3). Therefore, if two or more qualifying parameters were out of bounds, a red (not detected) designation was assigned; if one parameter fell out, an orange (questionable) designation was assigned; and if all parameters were met, a green (identified) designation was assigned. Although curated to specific guidelines here, screening parameters can be set on a per-group or a per-analyte basis with modifiable settings. Further, defining compounds as suspects or targets can simplify screening parameters and be used to sort upon evaluation of the data.

Untargeted acquisition via All Ions is advantageous to the workflow as evaluation of targets, suspects, and unidentified constituents occur simultaneously. Through the addition of collision energies, fragmentation information is acquired for everything in the sample, enabling confident identification of suspects and targets, as well as the retrospective investigation of data upon emergence of a new compound of interest.

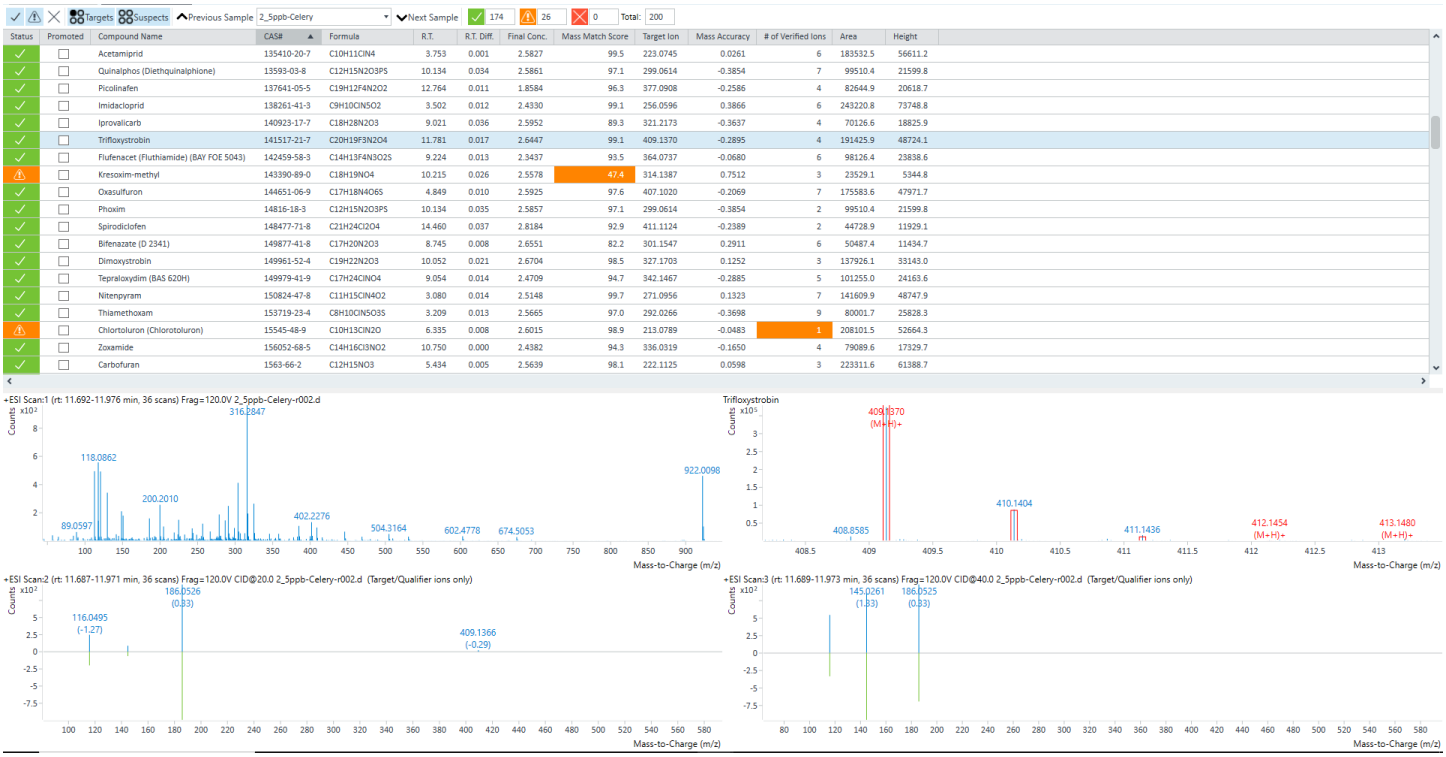


Figure 5. LC Screener Tool interface displaying results for celery at the 2.5 ppb level with detailed spectra of trifloxystrobin. Compound counter for sample in the top right with summary counts for green, orange, and red compound designations. Compound results are promotable from this interface.

The matrix spiked samples at three different calibration levels were quickly evaluated using the screening parameters in Table 3. The data can be evaluated clearly for interference and effective fragmentation by the separate fragment spectra for each collision energy as well as the molecular ion overlaid with theoretical isotope boxes (Figure 5). Screening results showed the majority of compounds were confidently detected at all concentration levels in each matrix tested. At lower concentration levels, more compounds were questionably detected, and at all levels shown, no compounds were undetected (Table 4).

Table 4. Overall LC Screener Tool results for three different calibration levels in matrices. Identified (green), questionable (orange), and not detected (red).

Matrix/ Concentration	Broccoli			Celery			Strawberry		
5 ppb	183	17	0	185	15	0	181	19	0
10 ppb	190	10	0	195	5	0	192	8	0
100 ppb	198	2	0	198	2	0	198	2	0

Despite the advantages of All Ions, this nonspecific fragmentation method can result in questionable identification due to interference with matrix or other compounds of interest, quickly summarized by the LC Screener Tool. Manual workflows would often require the re-evaluation of these questionable or identified designations.

Here, the Revident LC/Q-TOF's new intelligent reflex workflows, specifically the Target Screening Confirmation workflow, was used to automate this targeted review and reinjection process. This reduces the need for manual intervention and instrument idle time between completion of the All Ions analysis and initiation of the targeted analysis. Further, the combined report template details and compares the direct analyte results from the two injections for a quick and informative summarization of the experimental results.

Targeted reinjection

To engage the intelligent reflex workflow, two acquisition methods were created: (1) untargeted and (2) targeted. Further, two quantitative analysis methods were created with loaded screening parameters: (1) untargeted and (2) targeted, along with a report template for combined reporting of the untargeted and targeted screening results. In Agilent MassHunter Acquisition software, version 12.0, these methods and the report template were loaded into the intelligent reflex DA panel with the TS Confirmation workflow selected and screening status selected for compounds designated as green and orange upon initial All Ions data acquisition (Figure 6).

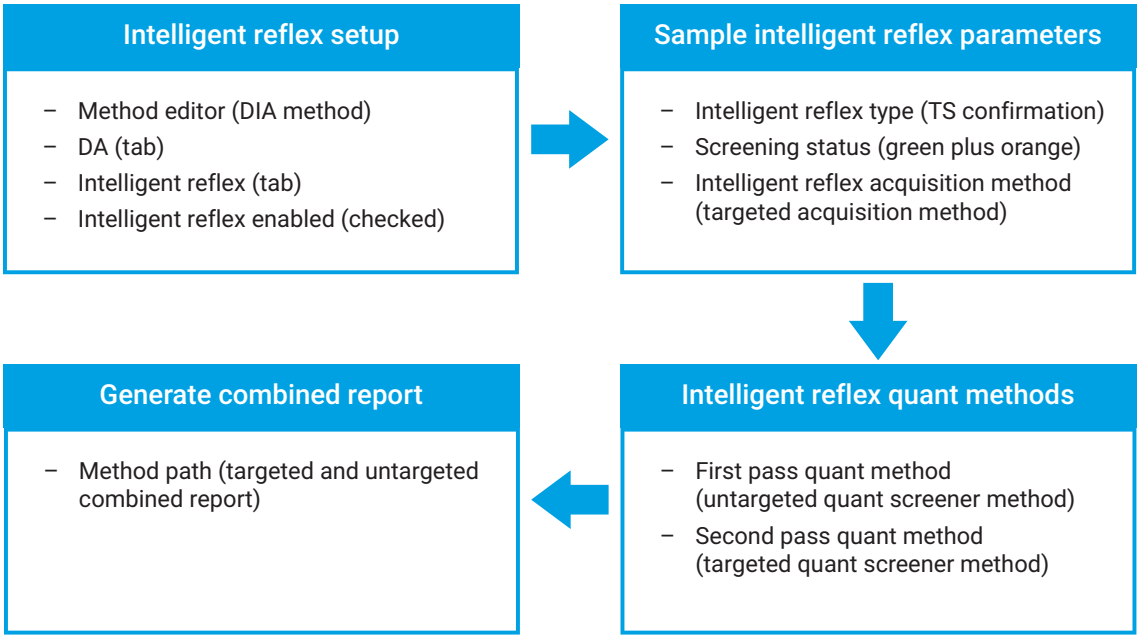


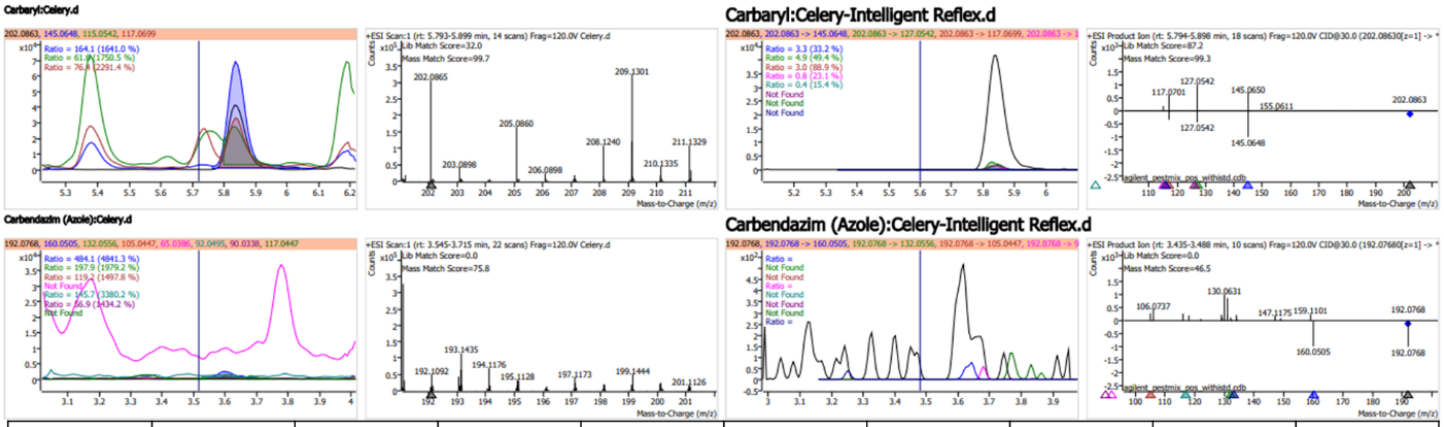
Figure 6. Intelligent reflex acquisition setup in the Agilent MassHunter Acquisition 12.0 Method Editor.

Through this workflow, samples were evaluated first by an All Ions, untargeted method, and data analysis was automated using the screening restrictions set in the LC Screener Tool for the untargeted MassHunter Quantitative Analysis 12.1 method. Upon analysis, MassHunter Acquisition 12.0 began the targeted method, selecting only those compounds that were designated orange, green, or green and orange, as specified by the user. The targeted reinjection was appended to the running worklist along with a QC and blank injection (Figure 7). The originally created worklist was completed, followed immediately by the targeted reinjection without manual input by the scientist.

The results of the automated targeted reinjections were automatically analyzed using the targeted MassHunter Quantitative Analysis 12.1 method and the results were combined into a single report format (Figure 8).

	Status	Sample Name	Data File	Sample Type	Intelligent Reflex Type	Intelligent Reflex Acq. Method	First Pass Quant	Second Pass Quant	Screening Status
2	Completed	Blank	ACN Blank-r001.d	Blank	No Intelligent Reflex Workflow	Pesticide-Targeted-1700-Long-ξ	D:\Projects\OPC	D:\Projects\OPC_N	
3	Completed	Blank	ACN Blank-r002.d	Blank	No Intelligent Reflex Workflow	Pesticide-Targeted-1700-Long-ξ	D:\Projects\OPC	D:\Projects\OPC_N	
4	Completed	QC 15 ppb	QC-15ppb.d	QC					
5	Completed	Blank ACN ISTD	Blank ACN ISTD-01.d	Blank	No Intelligent Reflex Workflow	Pesticide-Targeted-1700-Long-ξ	D:\Projects\OPC	D:\Projects\OPC_N	
6	Completed	Broccoli	Broccoli .d	Sample	TS Confirmation	Pesticide-Targeted-1700-Long-ξ	D:\Projects\OPC	D:\Projects\OPC_N	Green plus Orange
7	Completed	Celery	Celery.d	Sample	TS Confirmation	Pesticide-Targeted-1700-Long-ξ	D:\Projects\OPC	D:\Projects\OPC_N	Green plus Orange
8	Completed	Strawberry	Strawberry.d	Sample	TS Confirmation	Pesticide-Targeted-1700-Long-ξ	D:\Projects\OPC	D:\Projects\OPC_N	Green plus Orange
9	Completed Intelligent Reflex	Broccoli -Intelligent Reflex-Blank	Broccoli -Intelligent Reflex-Blank.d	Blank	No Intelligent Reflex Workflow			D:\Projects\OPC_N	
10	Completed Intelligent Reflex	Broccoli -Intelligent Reflex	Broccoli -Intelligent Reflex.d	Sample	No Intelligent Reflex Workflow			D:\Projects\OPC_N	
11	Completed Intelligent Reflex	Celery-Intelligent Reflex-Blank	Celery-Intelligent Reflex-Blank.d	Blank	No Intelligent Reflex Workflow			D:\Projects\OPC_N	
12	Completed Intelligent Reflex	Celery-Intelligent Reflex	Celery-Intelligent Reflex.d	Sample	No Intelligent Reflex Workflow			D:\Projects\OPC_N	
13	Completed Intelligent Reflex	Strawberry-Intelligent Reflex-Blank	Strawberry-Intelligent Reflex-Blank.d	Blank	No Intelligent Reflex Workflow			D:\Projects\OPC_N	
14	Completed Intelligent Reflex	Strawberry-Intelligent Reflex	Strawberry-Intelligent Reflex.d	Sample	No Intelligent Reflex Workflow			D:\Projects\OPC_N	

Figure 7. Intelligent reflex worklist modifications as a result of automated data analysis by targeted screening confirmation workflow.



Compound	Status	RT (min)	MZ	Mass Accuracy	Mass Match Score	Final Conc	Status	RT (min)	Library Match Score
Carbaryl	Green	5.834	202.0865	1.0039	99.7	22.1575	Green	5.841	87.2
Carbendazim (Azole)	Orange	3.650	209.1035	1.0225	75.8	0.000	Red	3.450	0.0

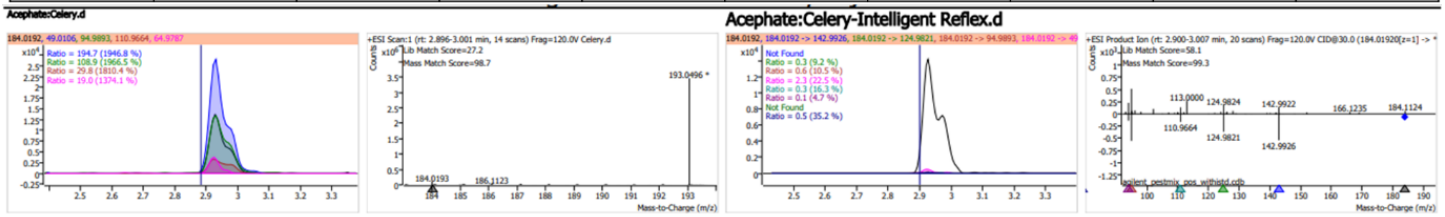
Figure 8. Intelligent reflex report showing the combined spectra results (top) of the untargeted (left) and targeted (right) results along with mass match scores and library match scores. The values for both acquisition results are displayed (bottom) in a table format.

The targeted MS/MS acquisition method allows unambiguous identification of the targeted analyte by selecting only the precursor ion for fragmentation, avoiding the risk of interference from the matrix. For instance, in the case of broccoli, a first pass with All Ions for acephate was designated green with a mass match score of 97.6 and library match score of 4.0. However, a second pass with targeted MS/MS maintained a good mass match score of 93.9 but a library score of 0.0, as selective fragmentation of precursor ions did not yield any product ions specific to acephate, leading to this compound to be downgraded to orange. On the other hand, for acephate in celery, an initial green status from All Ions screening was confirmed by presence of characteristic fragments and a library score match of 58.1, which increased from 27.2 (Figure 9).

The screening and reinjection results were consistent with reported usage of acephate on the food matrices, broccoli and celery. Samples available in the USDA Pesticides Data Program showed 0% hits for acephate in broccoli, while 31.5% of tested celery samples had detectable levels of acephate.[4]

The production of targeted data using reflexive logic from the automatic data analysis and targeted reinjection of compounds within the resulting parameters specified by the user reduced manual interference, instrument idle time, and data analysis workload.

Compound	Status	RT (min)	MZ	Mass Accuracy	Mass Match Score	Final Conc	Status	RT (min)	Library Match Score
Acephate	Green	2.928	184.0193	0.6843	98.7	7.4075	Green	2.927	58.1



Acephate	Green	2.814	184.0194	1.4676	97.6	3.6199	Orange	2.850	0.0

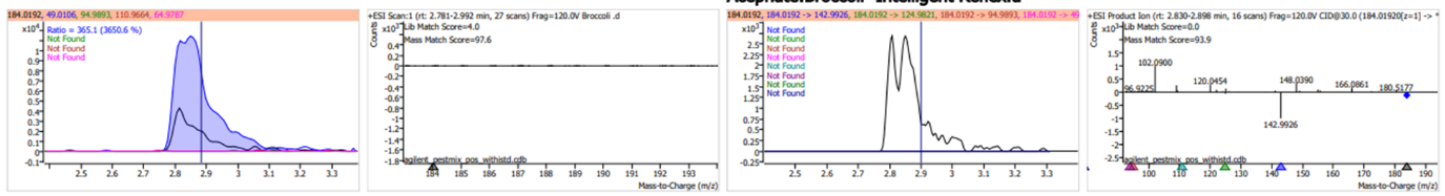


Figure 9. Intelligent reflex report for acephate in celery (top) and broccoli (bottom).

Conclusion

This application note demonstrates a DIA methodology for the screening of pesticides in complex matrix with the Agilent Revident Q-TOF LC/MS. The data analysis was supported by the LC Screener Tool in Agilent MassHunter Quantitative Analysis 12.1, which provided a color-coded summary of the presence and absence of targets and suspects in a sample. Further, the intelligent reflex workflow for automated targeted reinjection of compounds screened using All Ions acquisition was demonstrated along with some specific examples of increasing identification confidence through the workflow. The Revident Q-TOF LC/MS is a flexible instrument supported by great mass accuracy and isotopic fidelity, and it can effectively detect compounds of interest at low concentrations in complex matrix.

References

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