

# High chromatography reproducibility enables large panel MRM assays for pesticides in fruit and vegetables

## Characterizing the performance and robustness of the ExionLC 2.0 system

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The wide use of pesticides in agriculture to minimize crop loss by protecting against pests and the growth of unwanted plants has led to strong regulations that minimize hazards to human and animal health. Approved pesticides have maximum residue limits in food, requiring simultaneous identification and quantification of large panels of pesticide residues. LC-MS/MS with multiple reaction monitoring (MRM) provides high sensitivity and selectivity but, when performing a method with hundreds of compounds, data quality is extremely important and helps to ensure that every compound is effectively quantified. The Scheduled MRM Pro algorithm intelligently monitors the MRM transitions for a compound only during its elution time. This decreases the number of concurrent MRMs monitored at any point in time, allowing both the cycle time and dwell time to remain optimal. First, the elution times for each compound are determined, then a final optimized method is built using the Scheduled MRM Pro algorithm.

LC-MS/MS solutions must be robust and sensitive to meet the needs of food testing labs. When using the Scheduled MRM algorithm, it is critical to have stable retention times for the acquisition strategy to work effectively. An important determinant of success in achieving selectivity, reproducibility and robustness is having a high-quality up-front sample separation that provides stable retention.

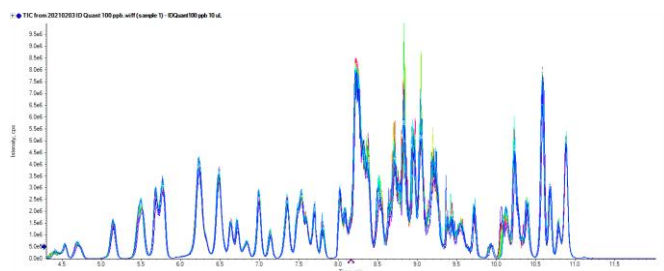
In this work, the flow rate precision and retention time reproducibility of the SCIEX ExionLC 2.0 system were investigated. The Scheduled MRM algorithm was used to acquire 428 MRM transitions, to accurately quantify pesticides



and confirm their identity based on the characteristic ratio of quantifier and qualifier transitions and a retention time match to reference standards. Owing to the inclusion of several pesticides in the panel that ionize preferentially in negative mode, a polarity switching method was also implemented.

### Key features of the ExionLC 2.0 system

- High-pressure, dual, serial piston pump rated to 860 bar (12,500 psi) at flow rates of 0.001 to 2 mL/min for maximum flexibility
- Precise and stable solvent flow delivering less than 0.1% coefficient of variation (CV) retention time variation allows the use of Scheduled MRM algorithm to monitor a large panel of compounds while maintaining quality of the data and confidence in identifications
- Accurate and precise quantification results with linear coefficient of determination performance ( $r^2$ ) > 0.99 and precision <10% coefficient of variation
- SecurityLink UHPLC finger-tight fittings and fixed tubing lengths aid in simplifying the LC system and column connections, providing consistent performance with torque limiting technology that prevents column damage or overtightening



**Figure 1. LC flow and gradient stability.** Overlaid total ion chromatograms (TICs) from 25 repeat injections of 10 µL of the same 100 ppb pesticide standard mixture.

## Methods

**Material and solutions:** All experiments were performed utilizing the iDQuant standards kit for pesticide analysis (SCIEX P/N 4465661) of certified reference material (ISP Guide 34, IOS/IEC 17025 and ISO 9001:2008) which includes 206 pesticides. The kit contains 10 mixes of about 20 pesticides each at initial concentrations of 100 µg/mL in 100% acetonitrile.

The kit was first used to optimize MRM conditions and adjust the retention times of existing methods to update the Scheduled MRM Pro algorithm for best performance.

Fruit and vegetable samples were extracted using an roQ QuEChERS extraction kit procedure [EN 15562 Kit, Phenomenex (P/N KS0-8909).

**Sample preparation:** Mix J of the SCIEX iDQuant kit for pesticide analysis, containing 20 compounds, was spiked into arugula sample and Mix C into red grape and used to verify method performance for identification and confirmation. Matrix calibrators of concentrations 1000, 500, 200, 100, 50 and 20 ng/mL were prepared.

Arugula and red grapes were frozen at -20°C, then thawed to room temperature prior to weighing and extraction. The following steps were performed, in the order listed:

- Preparation of 10 g of homogenized sample
- Addition of 10 mL acetonitrile and extraction by vigorous shaking for 1 min
- Addition of Phenomenex roQ QuEChERS kit buffer-salt mix (KS0-8909) and immediate vigorous shaking for 1 min using a vortex mixer
- Centrifugation for 10 min at 9000 rpm
- Transfer of a 1 mL aliquot of the sample extract into a tube containing Phenomenex roQ dSPE kit (KS0-8916, 8913, 8914 or 8915 depending on sample type)
- Cleanup by vigorous shaking for 30 sec and centrifugation at 9000 rpm
- Transfer of 500 µL of the cleaned sample extract into an autosampler vial
- 2x dilution with water prior to filtering through VWR 0.45 µm polypropylene filter and transfer to autosampler vial for LC-MS/MS analysis

**Chromatography:** LC separation was achieved using the SCIEX ExionLC 2.0 system and a Phenomenex Synergi 2.5 µm, 2.0 x 50 mm Fusion-RP column (P/N 00B-4423-B0). A 20 minute gradient of water and methanol with ammonium formate buffer

was used, with a flow rate of 0.4 mL/min and column temperature of 40°C. The SCIEX ExionLC 2.0 system autosampler was used with a configuration consisting of a 250 µL syringe, 250 µL buffer tubing, 100 µL sample loop and 15 µL needle tubing. In order to optimize sample consumption and minimize the injection cycle time, the injection mode used was the µL pick-up plus mode, and the injection volume was set to 2.0 µL. The syringe speed was set to low and speed factor to 1. Advanced rinse mode was used with 1000 µL acetonitrile/methanol/isopropanol (2:2:1 v/v/v) with 0.1% formic acid wash solvent and 1500 µL water/methanol (9:1 v/v) containing 5mM ammonium formate transport solvent.

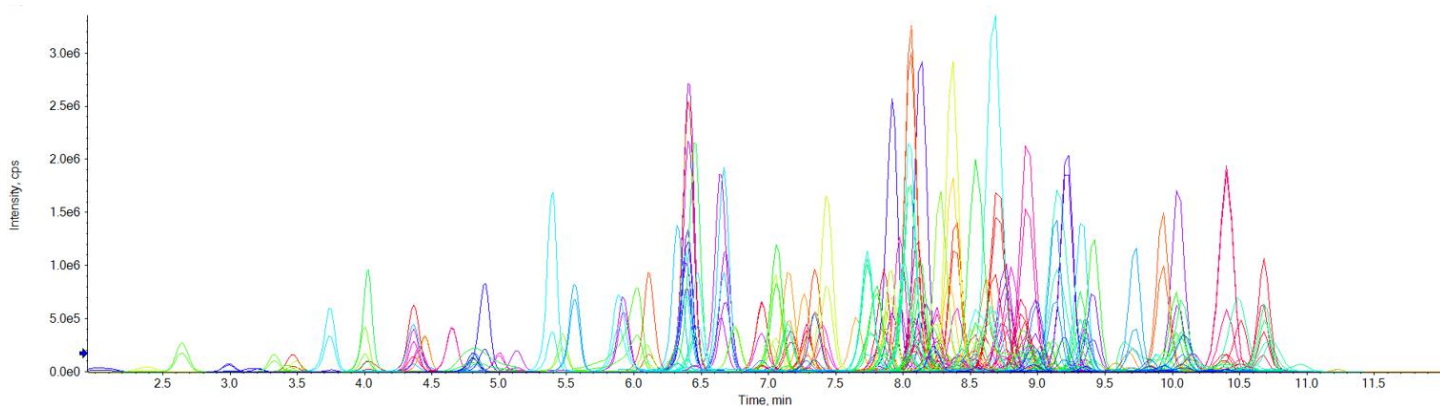
**Mass spectrometry conditions:** The SCIEX Triple Quad 3500 system was operated with the Turbo V ion source using an electrospray ionization (ESI) probe. 428 MRM transitions were monitored (194 compounds in positive mode (388 transitions) and 20 compounds in negative mode (40 transitions) using the Scheduled MRM Pro algorithm with a detection window of 40 sec and with polarity switching to allow analysis of all compounds in a single method.

Data acquisition was performed using Analyst software 1.7.2 with Components for the ExionLC 2.0 system. It is worth noting that the ExionLC 2.0 system is also fully supported for instruments in which data acquisition is performed using SCIEX OS software 2.1.5 and later.

**Data processing:** Data processing of mass spectrometry acquired data was performed using SCIEX OS software 2.1.5 in which calibration curves, precision and accuracy statistics were generated.

## High quality chromatography

428 MRM transitions were monitored using the Scheduled MRM Pro algorithm which allowed the quantification and identification of 200 pesticides in a single LC-MS/MS run. An example chromatogram of a matrix-matched standard at 20 ng/mL is shown in Figure 2. Very good separation was achieved, with narrow peak widths and very good peak symmetry, which is important when performing quantification. Peak symmetry was measured using the asymmetry factor—the distance from the center line of the peak to the back slope, divided by the distance from the center of the peak to the front slope, at 10% of the maximum peak—which is typically around 1.0. The example chromatograms shown in Figure 3 further highlight the narrow and symmetrical peak widths achieved with the LC system and selected chromatographic phase. For the data shown in Figure 3, the average asymmetry factor across the concentration range



**Figure 2. Extracted ion chromatogram showing the pesticide elution profile.** Approximately 200 pesticides detected using 400 MRM transitions with Scheduled MRM Pro algorithm.

was 1.1 for emamectin, 1.1 for metaflumizone, 0.9 for acetamiprid and 0.9 for Fenuron.

## Retention time reproducibility

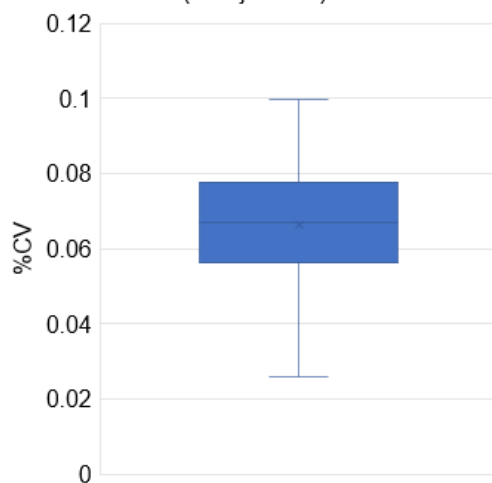
Very good retention time stability is critical when using narrow retention time windows to ensure the peaks remain within the detection windows. Stability of the LC pump over time is critical to ensure consistent retention times are delivered across large sample batches. This reduces the time spent readjusting methods to accommodate retention time drifts and minimizes any lost data due to peaks shifting out of target windows. Figure 1 highlights the excellent LC pump stability with overlaid chromatograms from 25 consecutive injections.

As shown in Figure 3, the retention time precision of each of the analytes across a range of retention times for these injections is less than 0.1% CV, with a mean of 0.066 % CV for the 150 compounds. For most compounds tested, the maximum retention time difference over 50 injections was <1.5 seconds.

Narrow retention time windows of 40 seconds were used in order to time-schedule such a large number of compounds in a single 20 minute acquisition method. Figure 4 illustrates the retention window width as centered around the detected peak.

When retention time stability is very good, more MRM transitions can be scheduled per run. In this method, two MRM transitions per compound were monitored in order to enable ion ratios to be computed between the quantifier and qualifier transitions. This is also highlighted in Figure 4, showing the ion ratio acceptance criteria lines that are automatically computed and plotted in Analytics in SCIEX OS software.

**% CV Retention Times for 150 compounds (50 injections)**

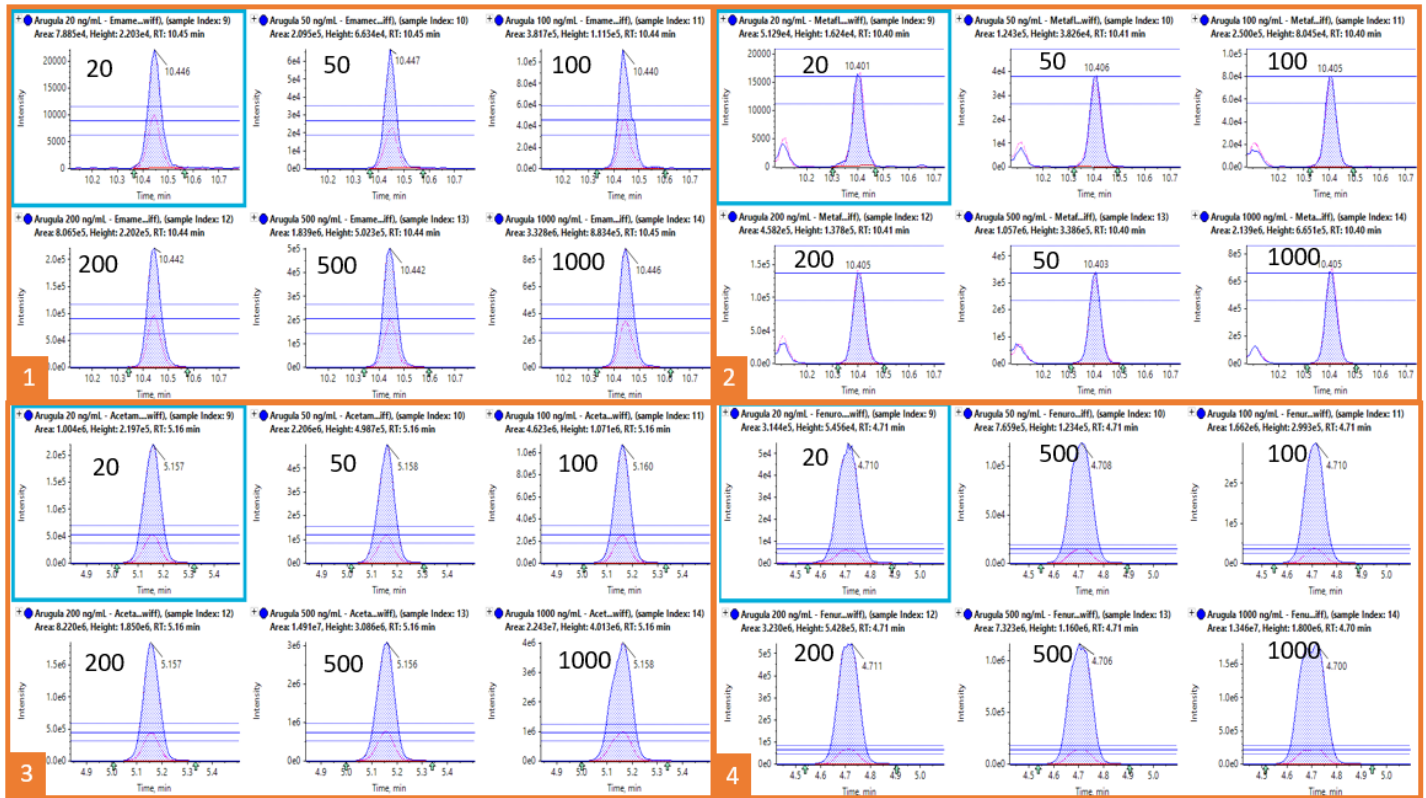


**Figure 3. Retention time precision.** Box and whisker plot showing the spread of the retention time %CV for 150 compounds from 50 injections of the 100 ppb pesticide standard mixture.

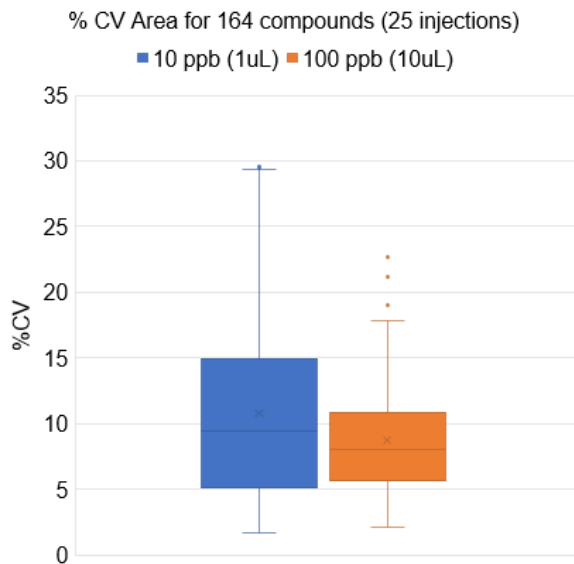
## Quantification performance

Solvent standards were injected at a concentration ranging from 20 to 1000 ng/mL. Example calibration lines are shown in Figure 5. Linear regression with 1/x weighting was used. The linear coefficient of determination ( $r^2$ ) was typically higher than 0.99.

The area reproducibility was also computed from 25 replicate injections of 1  $\mu$ L, then 25 replicate injections of 10  $\mu$ L, using the 100 ppb standard. Typical variance results are shown using selected compounds, without the use of internal standard, in Figure 6.



**Figure 4. Quantifier and qualifier MRM transitions of selected pesticides.** Four pesticides are shown here: (1) emamectin, (2) metaflumizone, (3) acetamiprid, (4) fenuron. The concentration range was from 20 to 1000 ng/mL. The MRM ratio of 30% is automatically displayed in the SCIEX OS software during peak review in Analytics to help confirm compound identification.



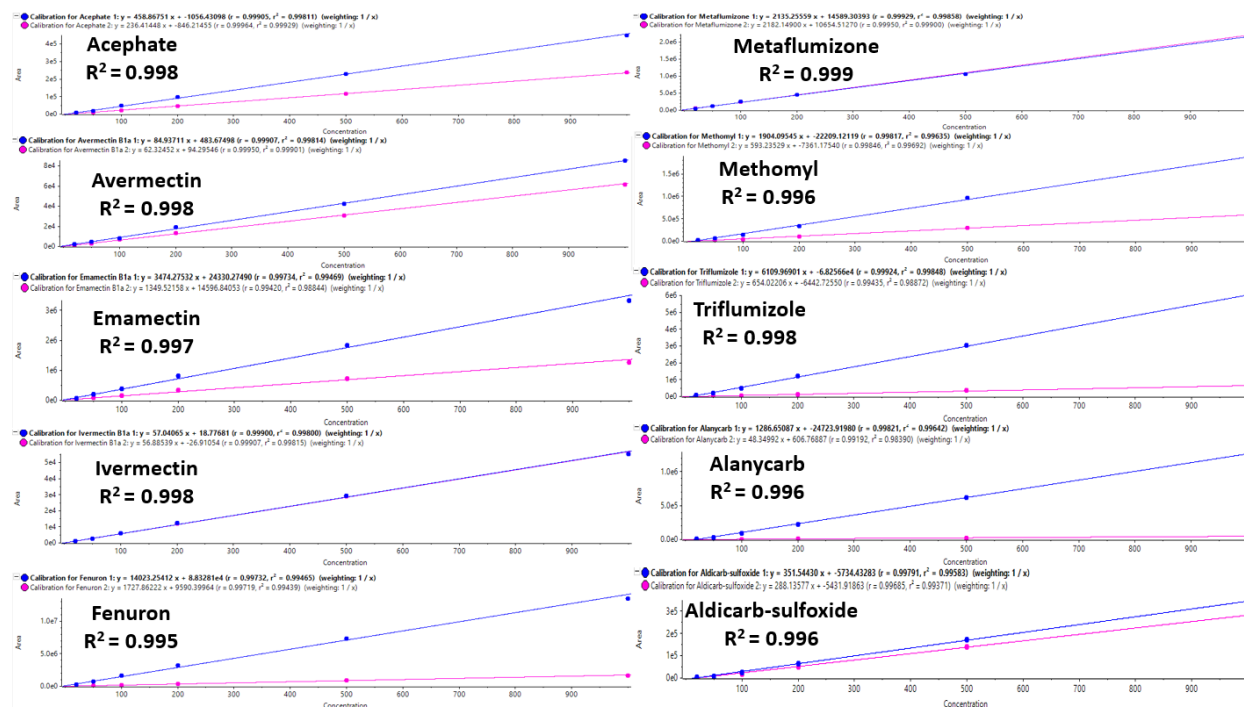
**Figure 6. Peak area precision.** Box and whisker plot showing the range of the peak area %CV for 164 compounds from 25 injections of the 100 ppb pesticide standard mixture, using either a 1  $\mu$ L injection (blue) or a 10  $\mu$ L injection (orange).

## Compound identification

Compound identification is typically performed by retention time matching and calculating the ratio between quantifier and qualifier MRM transitions. The ion ratio of a detected pesticide in unknown samples is compared to the ratio observed in standard samples and tolerance levels are applied to affirm the positive detection of that target pesticide. These tolerance levels are defined by several guidelines and can be user-defined in the SCIEX OS software.

MRM ratios were automatically calculated in SCIEX OS software. The ratio of quantifier and qualifier peak areas in the unknown samples is automatically compared to the average ratio of all included standard samples for compound identification. Tolerance levels are displayed in the peak review window in Analytics. Here, a generic tolerance of 30% was used, following SANCO/12682/2019 guidelines.<sup>1</sup>





**Figure 5. Concentration curves for selected pesticides.** Using the developed assay, concentration curves were generated on the pesticide mixture in solvent over a concentration from 20 ng/mL to 1000 ng/mL. Excellent linearity was observed with  $r^2$  values better than 0.995.

**Table 1. Pesticide identification.** Pesticides were identified in spiked food samples based on retention time (RT) matching, with a tolerance of 0.2 min, and MRM ratio comparison for qualitative method validation.

Pesticide in grape	RT (min)	RT error (%)	MRM ratio	% Ratio error
Benalaxyl	9.21	0.06	0.93	3.44
Benzoximate	9.41	0.02	0.42	2.71
Bifenazate	8.52	0.06	1.93	7.60
Cyazofamid	8.80	0.07	0.17	1.46
Fenamidon	8.10	0.26	0.69	2.85
Fenazaquin	10.67	0.18	0.25	1.45
Fenhexamid	8.52	0.18	0.57	2.61
Fenoxycarb	8.88	0.06	1.23	-1.50
Flufenacet	8.70	0.08	1.17	7.82
Furathiocarb	9.92	0.28	0.66	1.90
Indoxacarb	9.68	0.12	0.19	-0.28
Mepronil	8.36	0.19	1.53	10.42
Piperonyl-butoxide	10.02	0.28	0.49	-0.52
Quinoxifen	10.06	0.29	1.02	4.53
Thiobencarb, Benthocarb	9.33	0.36	0.25	-0.47
Zoxamide	9.13	0.22	0.49	0.48

## Verification of qualitative performance

Mix J of the SCIEX iDQuant kit for pesticide analysis containing 20 compounds was spiked into the arugula sample and Mix C was spiked into the red grape sample at 10 µg/kg.

As well as MRM ratio comparisons, compounds are also identified based on retention time matching. That also makes it critical to have good retention time stability, which is achieved through a combination of good sample clean up, chromatographic conditions and gradient pump stability. The results of identification based on retention time matching and MRM ratio comparison are summarized in Table 1. All 20 pesticides were confidently identified in all spiked samples. The average retention time error ranged from 0.017 to 0.355% and the average MRM ratio error from -1.50 to 10.42%.

## Conclusions

The SCIEX ExionLC 2.0 system has been successfully used in an LC-MS/MS method for the analysis of over 200 pesticides in food samples, after a QuEChERS based sample clean-up.

The key performance attributes of the SCIEX ExionLC 2.0 system, namely the flow rate precision and resulting retention time reproducibility, has enabled the use of the Scheduled MRM Pro algorithm workflows in the simultaneous identification and quantification of a large panel of pesticide residues. This has been achieved without any compromise in data quality by allowing both the cycle time and dwell time to remain optimal.

The method provided sufficient speed and sensitivity to quantify all of the pesticides (~200) with good linearity from 20 to 1000 ng/mL, with linear coefficient of determination ( $r^2$ ) performance typically above 0.99 and coefficient of variation typically well below 10%.

Qualitative method performance was verified by 20 compounds, into 2 different matrices at a concentration of 10 µg/kg. All compounds were confidently identified in all samples. This was, in part, due to the excellent pump stability afforded by the ExionLC 2.0 system, which was demonstrated by the retention time precision of each of the analytes across a range of retention times being less than 0.1% CV. Retention time errors observed were well below the ±0.1 min tolerance.

## References

1. SANCO/12682/2019 guidelines. Analytical quality control and method validation procedures for pesticide residues analysis in food and feed.

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