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Application News

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Liquid Chromatography Mass Spectrometry

Applying 'MRM Spectrum Mode' and Library Searching for Enhanced Reporting Confidence in Routine Pesticide Residue Analysis

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Abstract

To help reduce the incidence of false positive and false negative reporting in pesticide residue monitoring routine multiple-reaction monitoring (MRM) methods have been enhanced to monitor a higher number of fragment ion transitions to increase specificity and reporting confidence. In this workflow, typically 6-10 fragment ion transitions were monitored for each target pesticide as opposed to a conventional approach using 2-3 fragment ions. By acquiring a high number of fragment ion transitions, each target pesticide had a corresponding fragmentation spectra which could be used in routine library searching and compound verification using reference library match scores. This 'MRM Spectrum Mode' was applied to quantify and identify 193 pesticides using 1,291 MRM transitions without compromising limits of detection, linearity or repeatability.

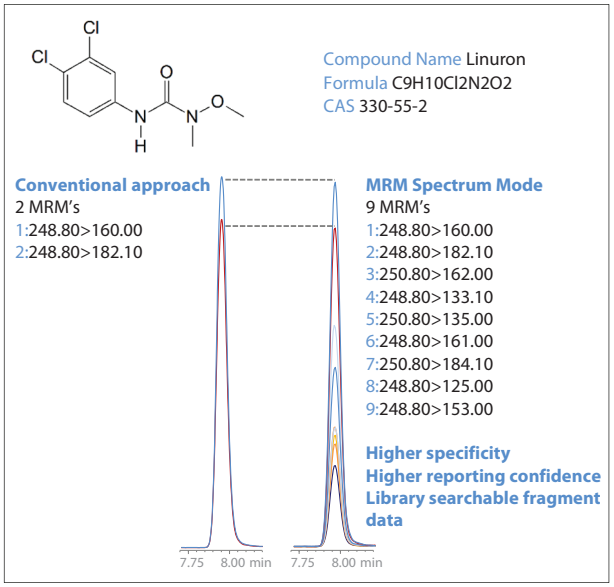

Fig. 1 Using a higher number of fragment ions in MRM data acquisition increases the specificity of detection and reduces false negative and false positive reporting. In the case of linuron, 9 precursor-fragment ion transitions were used to increase confidence in assay specificity. There is no compromise in data quality between methods despite a higher number of fragment ions monitored. Signal intensity, linearity, reproducibility are in good agreement between both methods.

Introduction

Multiple Reaction Monitoring (MRM) based LC-MS/MS techniques are widely used on triple quadrupole platforms for targeted quantitation as a result of high selectivity, sensitivity and robustness. In a regulated environment such as food safety there is a growing need to enhance the capability in routine monitoring programs by increasing the number of pesticides measured in a single analysis and at the same time delivering the highest confidence in compound identification to reduce false detect reporting. For pesticide analysis in the EU, identification criteria in SANTE/11945/2015 requires the retention time and the ion ratio from at least 2 MRM transitions to be within acceptable tolerance limits.[*1] However, even applying this criteria it is well reported that false positives can occur in certain pesticide/commodity combinations.[*2-*4]

To reduce false negative and false positive reporting a higher number of MRM transitions were used for each target pesticide to increase the level of confidence in assay specificity. The number of fragment ion transitions monitored for each target pesticide was dependent upon the chemical structure with typically between 6-10 fragment ions for each compound. MRM Spectrum mode combines conventional MRM quantitation with the generation of a high quality MRM product ion spectrum which can be used in routine library searching and compound verification and identification.

In this application paper we present the development of a method for 193 pesticides, with 1,291 MRM transitions, and a 15 minute cycle time. In order to acquire this number of MRM transitions using a short run time a 3 msec dwell time was applied to each MRM transition and a 5 msec polarity switch was used. On average 7 MRM transitions were applied to each compound. The method was quickly set up using the Shimadzu Pesticide Method Package, a data base with more than 750 pesticides and over 6,000 MRM transitions designed to accelerate method set-up and help compound verification. MRM Spectrum mode was also compared to a conventional pesticide monitoring method with 2 MRMs per compound (386 MRMs in total) in order to assess the effect on data quality when adding additional MRM transitions to the method. Several different food commodities were analysed with varying complexity (turmeric, plum, peppermint, parsnip, cherry, lime, pumpkin, tomato, potato). Data was processed using LabSolutions Insight software which provides automated library searching of target MRM spectrum.

Experimental

Pesticide spiked samples, extracted using established QuEChERS based methods, were provided by Scientific Analysis Laboratories, UK. In order to test the performance of the MRM Spectrum Mode database and library searching a number of matrices were tested including turmeric, plum, peppermint, parsnip, cherry, lime, pumpkin, tomato and potato. Final extracts were prepared in acetonitrile without any dilution and directly injected into the LC-MS/MS. A water co-injection method, performed automatically in the auto-sampler, was used to improve early eluting peak shapes in addition to a sub 2 micron particle size column to improve peak capacity (Table 1) .

Calibration curves were prepared in the range 0.01 to 0.2 mg/kg. Repeatability of the method was tested using avocado matrix at 0.1 mg/kg. In the final method samples were analysed in ESI +/- using a polarity switching time of 5 msec.

On average 7 MRM transitions were applied to each compound, with more than 10 MRM transitions applied to 34 compounds. All MRM transitions were acquired throughout the MRM window without the need for triggering thresholds. The method includes a total of 1,291 MRM transitions for 193 pesticides in a run time of only 15 minutes. A dwell time of 3 msecs was applied to every MRM transition. In order to evaluate the data quality from the MRM Spectrum Mode method, the same method was set up with 2 MRMs applied to each compound (386 MRMs in total) using the same acquisition method (Table 2).

LabSolutions software was used to automatically optimize the fragmentation for all pesticides and generate a MRM Spectrum mode method. The MRM Spectrum Mode method for library searching and compound verification could be simply and quickly set up using the Shimadzu pesticide database. This database contains more than 6,000 MRM transitions for over 750 pesticides.

LabSolutions Insight v3.0 software was used to review quantitative data and MRM Spectrum mode library searching with advanced filtering tools to review by exception and to reduce false detect reporting.

Table 1 LC acquisition parameters

Liquid chromatography		
UHPLC	Nexera LC system	
Analytical column	HSS T3 (100 × 2.1, 1.7 µm)	
Column temperature	40 °C	
Flow rate	0.4 mL/minute	
Solvent A	5 mmol/L ammonium formate and 0.004 % formic acid	
Solvent B	5 mmol/L ammonium formate and 0.004 % formic acid in methanol	
Binary Gradient	**Time (mins)**	**%B**
	1.50	35
	11.50	100
	13.00	100
	13.01	3
	15.00	Stop
Injection volume	0.1 µL (plus 30 µL water)	

Table 2 MS/MS methods used to acquire data in MRM Spectrum Mode and a conventional MRM method with 2 MRM transitions per compound. As part of the comparative study, the same LC conditions were used for both methods.

LC-MS/MS Mass spectrometry	MRM Spectrum Mode: generating library searchable spectra	2 MRM method
Target number of compounds	193	193
Total number of MRM transitions	1,291 transitions (1,229 in ESI+ and 62 in ESI-)	386 (374 in ESI+ and 12 in ESI-)
Pause time/dwell time	1 msec./3 msec.	1 msec./3 msec.
Ionisation mode	ESI +/-	ESI +/-
Polarity switching time	5 msec	5 msec
Interface temperature	350 °C	350 °C
Heat bl°Ck temperature	300 °C	300 °C
Desolvation line temperature	150 °C	150 °C
Nebulising gas	3 L/min	3 L/min
Heating gas	10 L/min	10 L/min
Drying gas	10 L/min	10 L/min

Results and Discussion

In developing monitoring programs for chemical contamination methods are designed to determine a list of known analytes with a focus on delivering a rapid, cost-effective analysis that generates no false-negative or false-positive results. Guidelines for compound identification have been published by the EU in directive SANTE/11945/2015 . This identification criteria requires at least two MRM transitions with an ion ratio and retention time within defined tolerance limits.

To help reduce false detect reporting in pesticide monitoring programs, a MRM method was developed with a higher number of MRM transitions for each target pesticide to increase the level of confidence in assay specificity. By combining multiple MRM transitions for a compound into a product ion spectrum, pesticide identification can be verified and confirmed against a MS/MS reference spectral library. Using MRM Spectrum mode can help markedly reduce false detect reporting without affecting the data quality for optimized quantitation or identification.

Fig. 2, shows the MRM chromatogram for all 193 pesticides spiked at 0.010 mg/kg measured with MRM Spectrum mode. Using this mode 1,291 MRM transitions were measured for 193 pesticides. Despite the high data density acquired with MRM Spectrum Mode (for example, 151 MRM transitions were registered in the same time window during the analysis, see Fig. 3) sensitivity was not affected by the high data acquisition rate.

Method performance

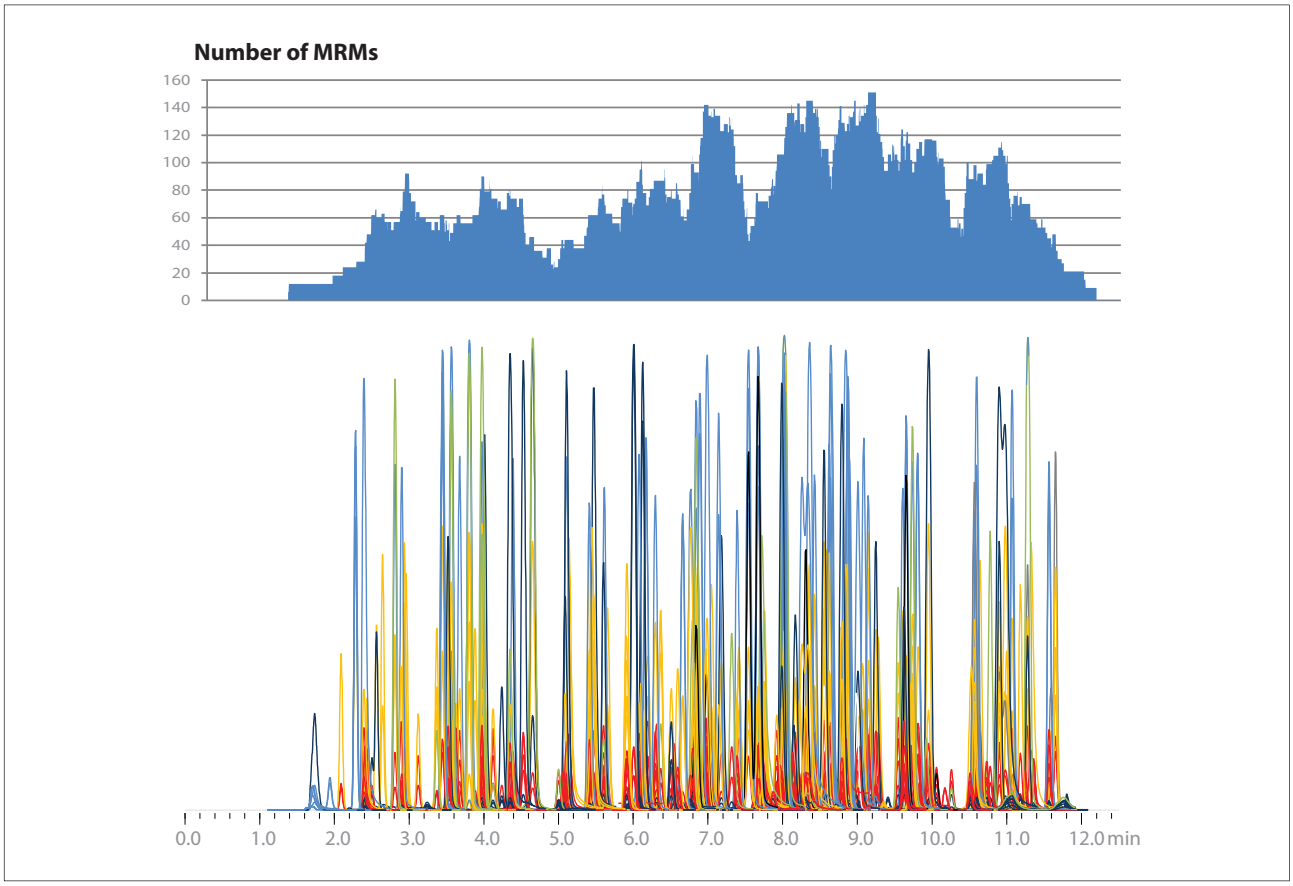

Fig. 2 Histogram showing the number of MRM transitions monitored at each time point and chromatogram showing all 193 target compounds. The highest number of overlapping MRM's acquired was 151. Even at such a high data sampling rate the response was in agreement with a conventional 2 MRM method with peak area variation less than 5.2% (n=5). This data is displayed below in more detail, Fig. 3.

Table 3 Between 8.80 mins and 9.30 mins151 MRM transitions in both positive and negative ion were monitored. Peak area repeatability for the 22 compounds eluting in this time period is shown below.

	Ret. Time	# MRMs	Polarity	Peak Area %RSD (n=5)
Dichlofluanid	8.80	6	ESI+	2.2
Dichlofluanid 2	8.80	6	ESI+	3.4
Dichlofluanid 1	8.80	5	ESI+	2.6
Fluoxastrobin	8.82	12	ESI+	2.0
Fenhexamid	8.83	11	ESI+	2.2
Iprovalicarb	8.88	6	ESI+	2.3
Spirotetramat	8.89	6	ESI+	2.6
Azinphos-ethyl	8.90	5	ESI+	3.1
Chromafenozide	8.91	5	ESI+	3.2
Triticonazole	8.93	5	ESI+	2.1
Cyazofamid	9.01	5	ESI+	2.1
Prothioconazole desthio	9.07	10	ESI+	1.9
Diflubenzuron	9.09	4	ESI+	2.0
Pyrifenox	9.11	8	ESI+	2.0
Dodemorph	9.17	6	ESI+	2.1
Fenoxycarb	9.17	6	ESI+	2.0
Rotenone	9.17	6	ESI+	2.4
Fipronil	9.20	10	ESI-	5.2
Bixafen	9.25	8	ESI-	2.8
Tebufenozide	9.27	6	ESI+	3.9
Bensulide	9.27	6	ESI+	2.6
Neburon	9.30	9	ESI+	1.7
		Total MRM's 151		Average 2.6 %RSD

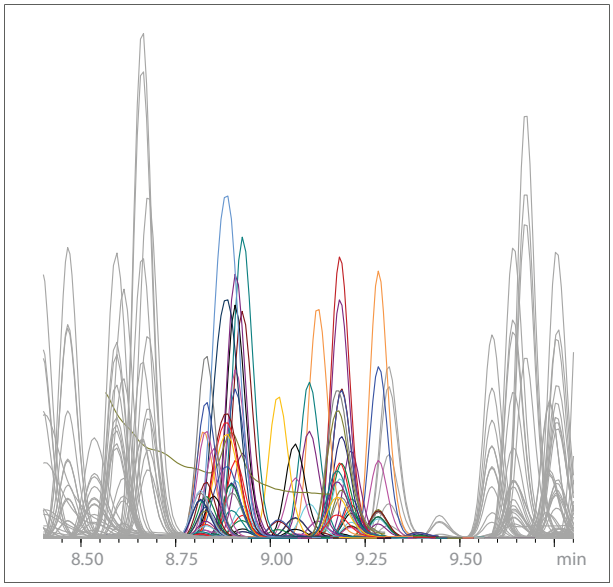

Fig. 3 Between 8.80 mins and 9.30 mins151 MRM transitions in both positive and negative ion were monitored. During this time period 22 target pesticides eluted with a peak area variation less than 5.2 % RSD. Data was acquired in an avocado sample matrix at a concentration of 0.1 mg/kg.

Method performance

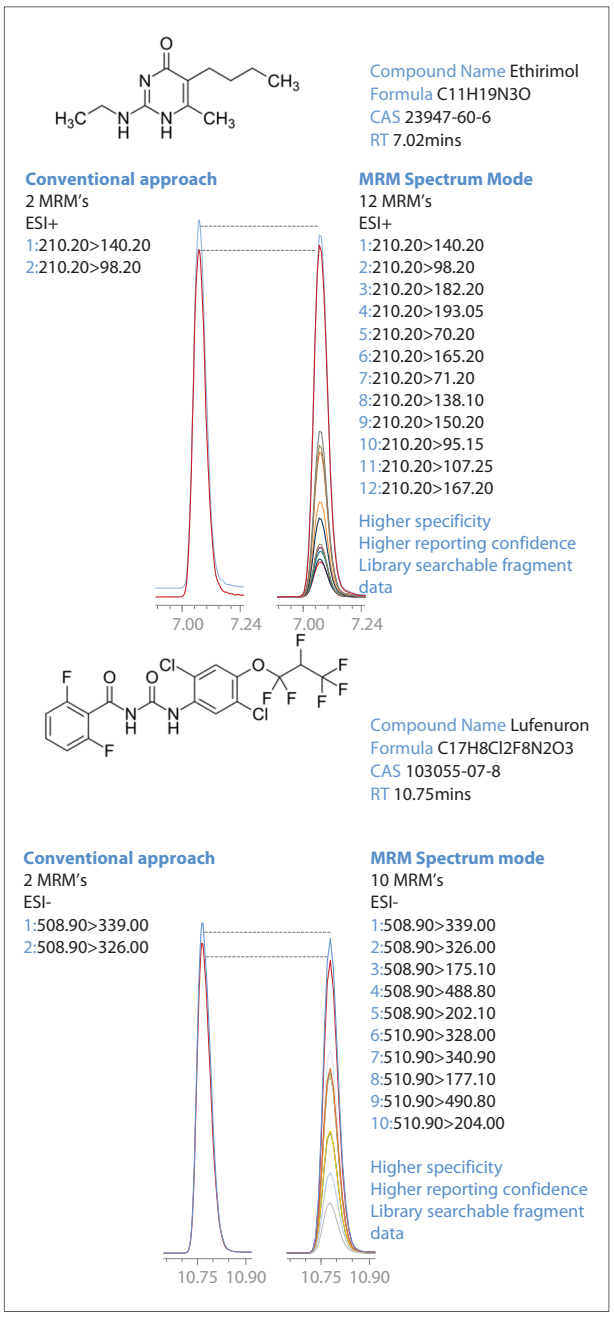

Fig. 4 MRM chromatograms for ethirimol (positive ion) and lufenuron (negative ion) acquired using a conventional 2 fragment ion MRM method and compared to a method with a higher number of precursor-fragment ions to increase confidence in assay specificity and reporting.

Despite acquiring a higher number of MRM transitions the library searchable MRM approach (acquiring 1,291 transitions in a single method) results in the same signal intensity compared to a conventional 2 fragment ion MRM method (acquiring 386 MRM transitions in a single method). The repeatability for each MRM method was evaluated by repeatedly injecting (n=5) an avocado extract corresponding to a concentration of 0.1 mg/kg. In each MRM method the %RSD was less than 3.5% for both compounds.

To minimize the possibility of false positive and false negative reporting LC-MS/MS methods were developed with a high number of MRM transitions for each pesticide. The performance of this approach was compared with a conventional MRM method monitoring 2 transitions for each pesticide.

In Fig. 4, the MRM chromatograms for 2 compounds, ethirimol and lufenuron, are shown for the same sample extract acquired using different MRM methods (the sample is avocado spiked at 0.1 mg/kg). The MRM chromatograms show un-smoothed data and are scaled to the same signal intensity for each compound. Ethirimol and lufenuron elute at 7.02 and 10.75 mins corresponding to time windows of high data density with more than one hundred MRM transations monitored in the same time segment. However, regardless of the high number of fragment ions monitored, the absolute signal intensity for both approach's is near identical in positive and negative ion mode.

Fig. 5 shows the correlation between the peak areas for all pesticides measured using 2 different MRM methods. The linear regression curve shows a good agreement between the peak areas measured for all pesticides spiked into sample matrix with a slope value near unity and an intercept near zero.

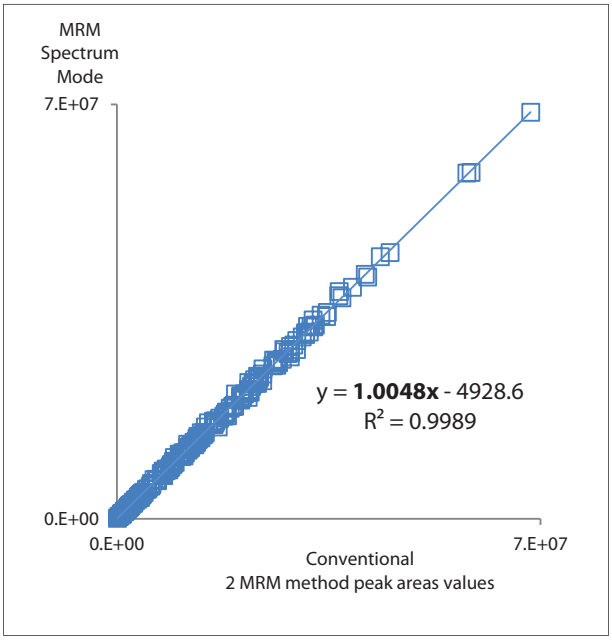

Fig. 5 Absolute peak area response for all 193 pesticides acquired using a conventional MRM method with 386 transitions compared to a MRM method with 1,291 transitions designed for library searchable verification. Both approaches result in near identical peak areas regardless of the number of fragment ions used to verify and identify each pesticide.

Spectrum based identification

In this study, the number of qualifier fragment ion transitions was increased for each pesticide and the combined transitions were used to create a MRM product ion spectrum. This product ion spectrum derived from MRM acquisitions was used in conventional library matching routines comparing against a reference spectrum to generate a similarity score.

In Fig. 6, demeton-S-methyl sulphone was to highlight library matching in different matrices including cumin, potato, mucuna pruriens powder, tomato, black pepper, peppermint tea and turmeric. Even in the presence of complex spice matrices the library matching approach identified demeton-S-methyl sulphone with a high similarity score and a high degree of confidence for data reporting.

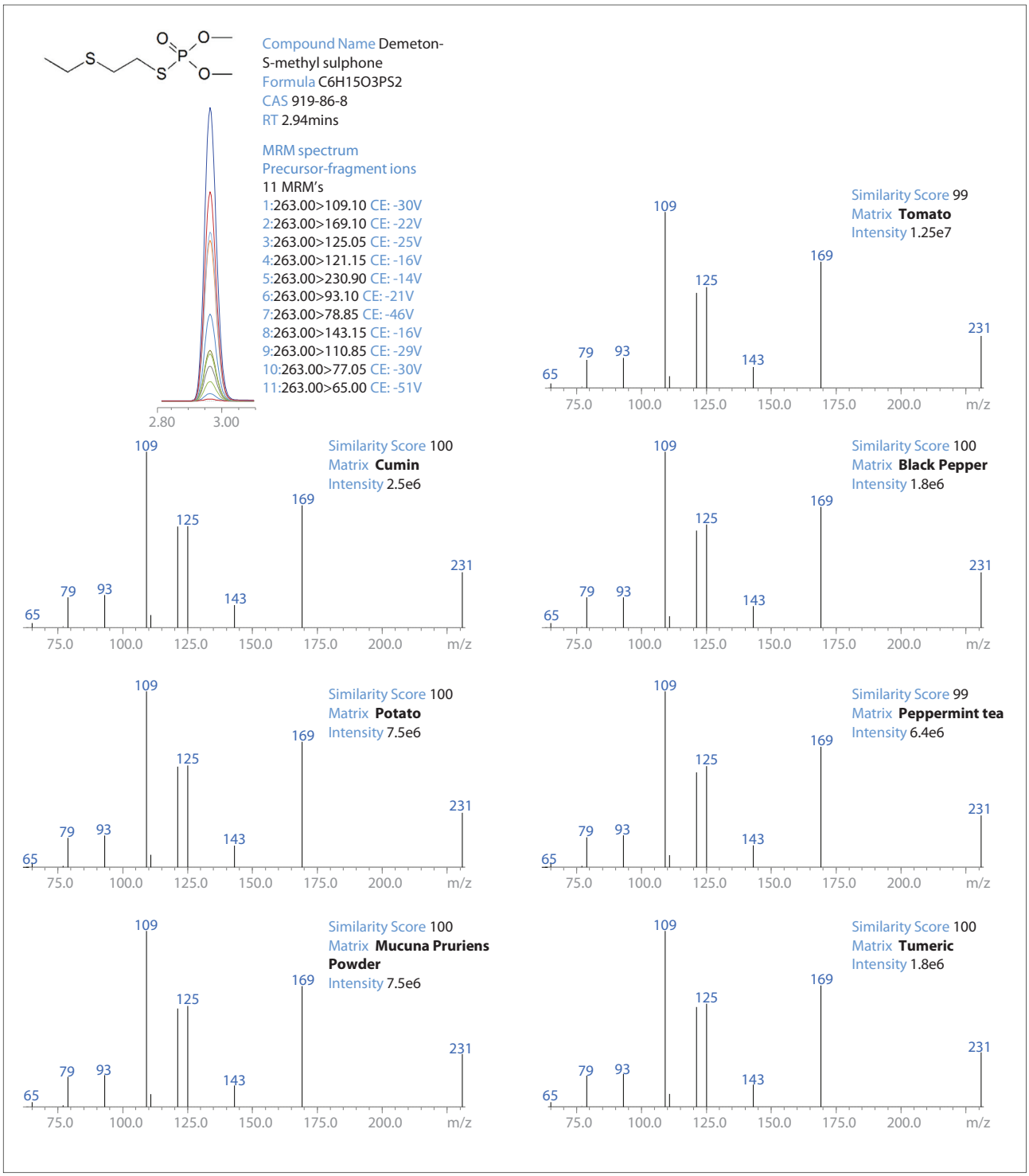

Fig. 6 MRM spectrum identification in different matrices for demeton-S-methyl sulphone

Spectrum based identification

To increase the confidence in reporting results the number of qualifier transitions was increased for each pesticide and the combined MRM transitions were used to create a product ion spectrum. This MRM product ion spectrum can then be automatically compared against a reference spectrum to generate a product ion spectrum match score using conventional library matching.

Fig. 7 highlights the advantage of using a library searchable fragment ion spectrum in identifying and quantifying desmedipham and phenmedipham. Both desmedipham and phenmedipham share several common fragment ions and have similar retention times. Using MRM Spectrum Mode and comparing to a library searchable spectra, both desmedipham and phenmedipham are positively identified (fragment ions at *m/z* 154 and 182 are absent in product ion spectrum for phenmedipham).

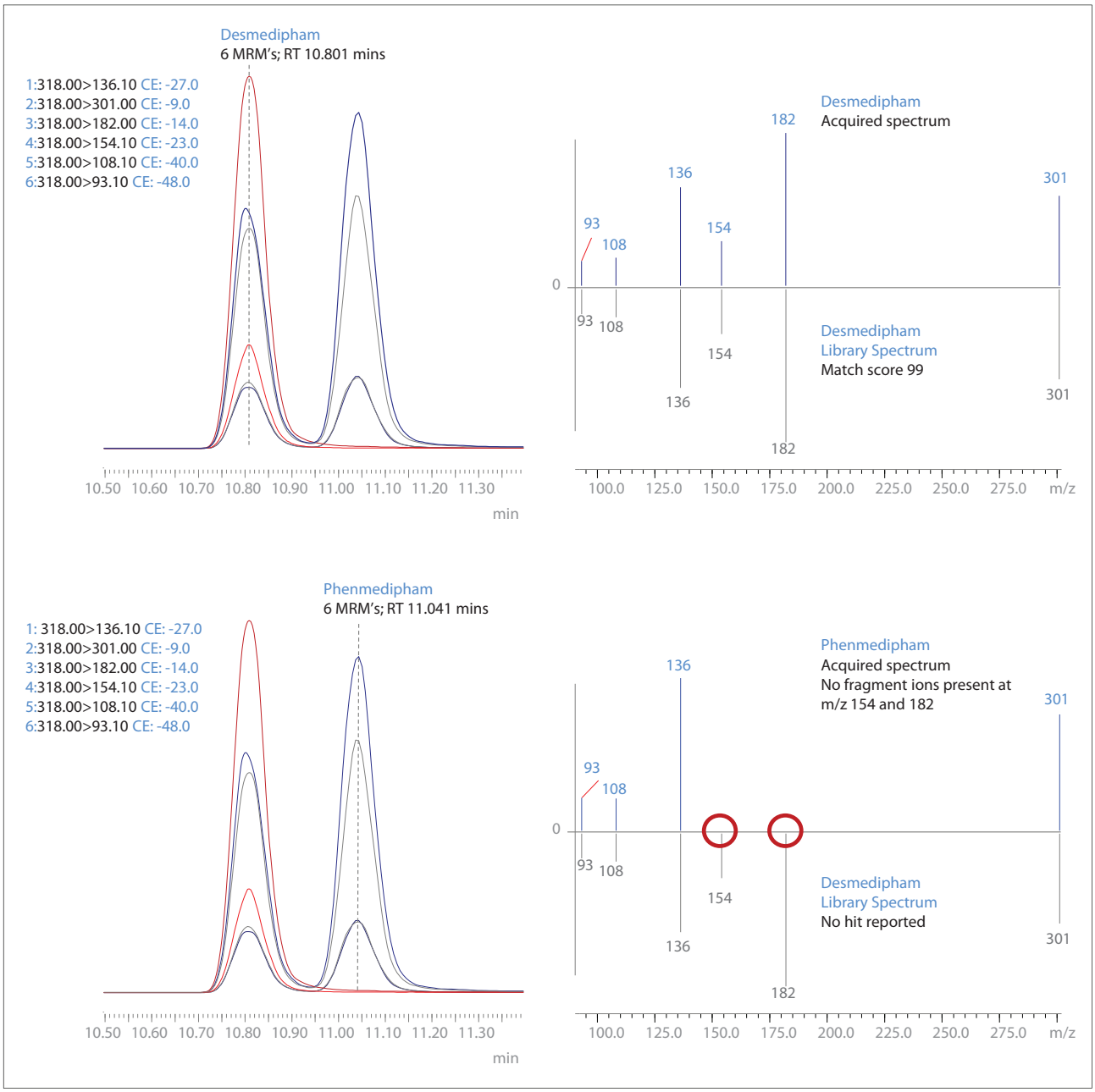

Fig. 7 MRM chromatogram for desmedipham and phenmedipham spiked into a cumin extract at 0.1 mg/kg. As phenmedipham shares common transitions and elutes at a similar retention time as desmedipham the MRM spectrum can be used to distinguish between both pesticides to avoid false positive reporting.

Quantitation

As one example, carbendazim was spiked into a matrix at three different concentration levels. In Fig. 8, all MRM transitions were detected even at the reporting level of 0.010mg/kg with a signal to noise for all fragment ion transitions greater than 9. The response was linear for all transitions throughout the calibration range (0.010-0.200mg/kg) as shown Fig. 9.

The limit on the number of MRM transations used to generate a product ion spectrum is dependent on the chemical structure of the pesticide molecule. In the case of carbendazim, several bonds could be broken using collision energies between 10-60V resulting in a product ion spectrum of 12 fragment ions. The product ion spectrum can then be used for library search and analyte confirmation as shown in Fig. 10. For each calibration level ranging from 0.010-0.200mg/kg the library similarity score was greater than 99 confidently confirming the target analyte. The advantage of this technique is that library searchable product ion spectrum data is used in target compound identification without compromising sensitivity, accuracy and robustness in quantitative data reporting.

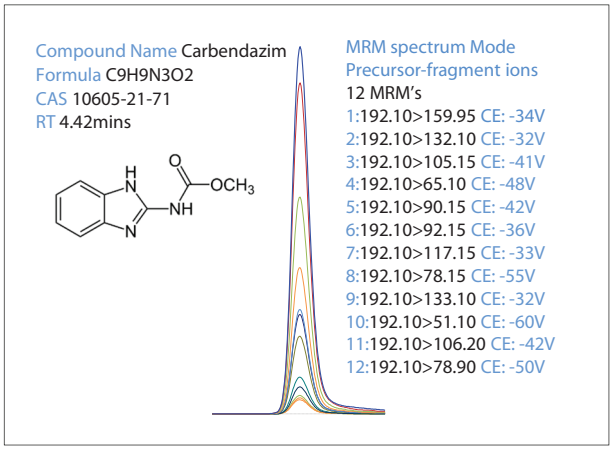

Fig. 8 By applying a range of collision energies to carbendazim 12 precursor-fragment ions are generated. MRM 192.10>159.95 was used in generating sensitive and robust quantitation whilst the product ion spectrum using all 12 fragment ions was used in confirming peak identification.

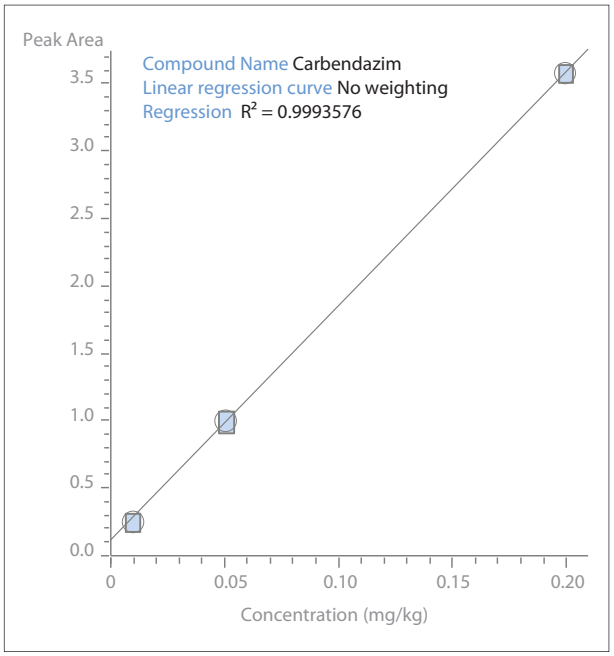

Fig. 9 Calibration curve for carbendazim using the optimized quantitation ion transition (MRM 192.10>159.95). The response was linear for all calibration and QC samples. All 12 fragment ions were above a signal to noise ratio of 10 even at the reporting level of 0.010mg/kg.

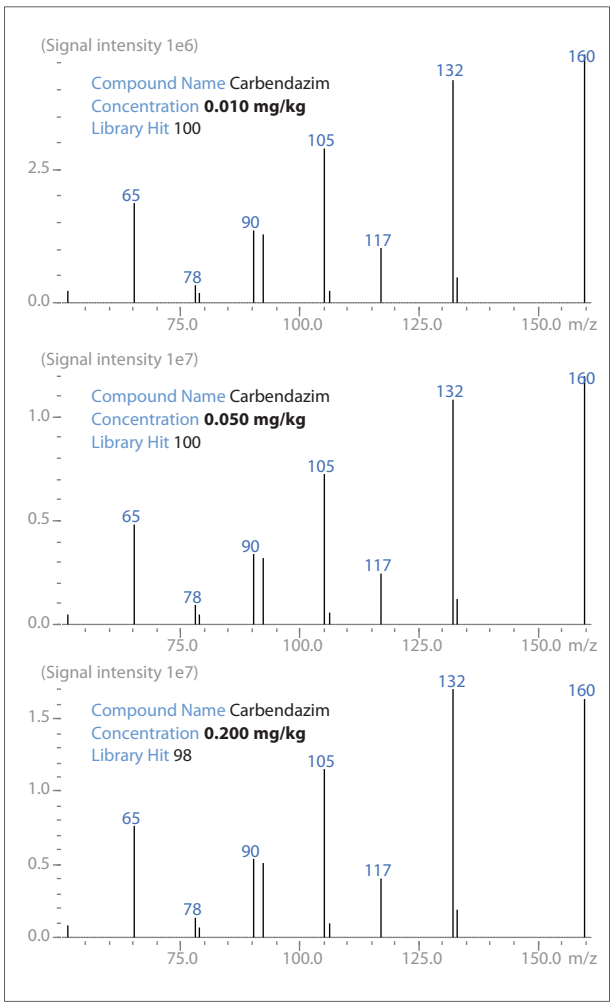

Fig. 10 MRM Product ion spectrum data for carbendazim in 3 calibration levels (0.010-0.200mg/kg) spiked into a food matrix was compared with an authentic library spectrum of carbendazim. In all library searches the similarity score was greater than 99 indicating a very high confidence in compound verification and reporting.

Data Reporting

Automated reference library matching and quantitation results can be simply viewed using LabSolutions Insight software (Fig 11).

LabSolutions Insight software helps to review by exception and to reduce false positive reporting by verifying compound identification using library matching scores and retention time variation from a calibration standard.

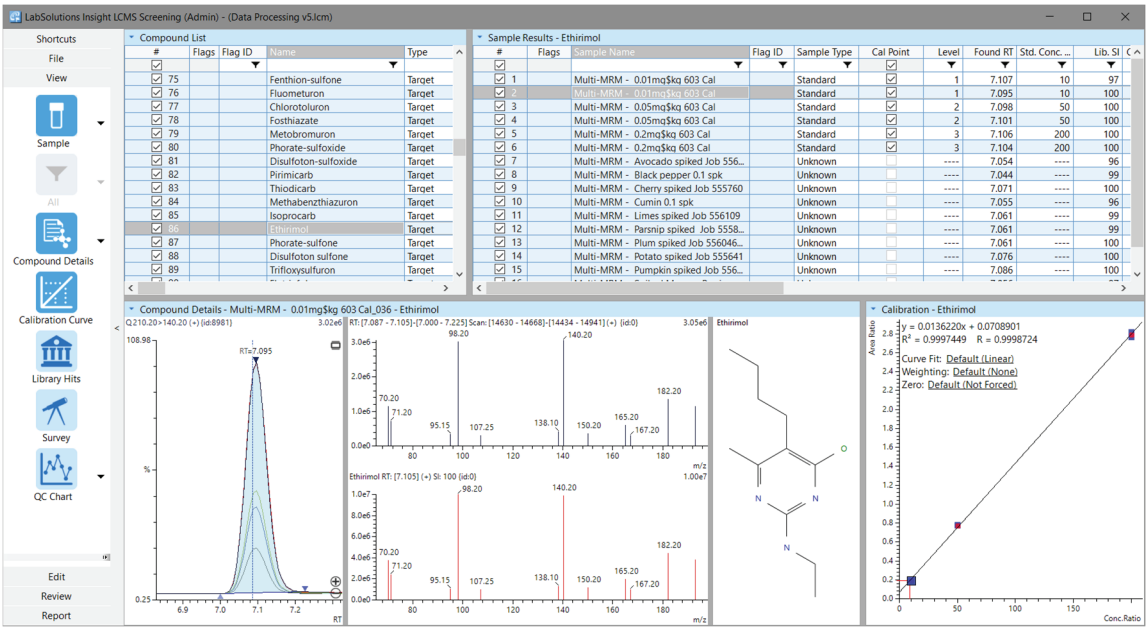

Fig. 11 LabSolutions Insight software helps to review quantitative and reference library matching results quickly and easily. Flexible filtering and sorting tools can be used to help reduce reporting false detects, especially in high throughput laboratories by filtering results based upon a similarity score with a reference library product ion spectrum.

Conclusions

False positive results are a major issue for all pesticide residue monitoring laboratories. EU regulations require that retention time and the ion ratio between 2 MRM transitions are within a set threshold. However, even applying this criteria false positives may occur for certain pesticide/commodity combinations.

In this application paper, we have applied MRM Spectrum Mode to identify and quantify 193 target pesticides in a number of different sample matrices. The library score is used as an additional identification criterion in order to improve identification confidence.

Acquisition of the MRM Spectrum mode method (1,291 MRM transitions) did not compromise data quality when compared to a conventional 2 MRM per compound method (386 MRM transitions) with consistent signal response and repeatability in both methods. The MRM product ion spectrums were demonstrated to be consistent across the linear range and between different matrices. The method acquired data in both positive and negative ion modes with a polarity switching time of 5 msec enabling fast cycle times and a high data collection rate.

All 1,291 MRM transitions were acquired throughout the MRM window. No 'triggering' of MRM transitions was necessary due to the short dwell times that were applied using the LCMS-8060. Therefore, MRM transitions can be swapped between qualifier and qualifier if needed and the peak shape of the additional MRM transitions can be assessed.

References

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