Application Note

Food

Simultaneous Screening and Quantification of Pesticide Residues in Red Cabbage

All Ions LC/Q-TOF MS/MS approach for the EUPT pesticide screening proficiency test

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Abstract

This application note introduces a UHPLC-Q-TOF/MS method to simultaneously qualitatively screen and quantitatively determine 415 pesticide residues in red cabbage. The cabbage samples were extracted using a conventional SPE approach according to the current China GB method 20769-2008. The obtained extract was filtered through a 0.22 µm membrane. This was followed by separation with an Agilent 1290 Infinity II LC and detection with an Agilent 6545 LC/Q-TOF MS/MS under Agilent All Ions MS/MS scanning mode. A total of 415 pesticides in red cabbage were evaluated with matrix-matched standards within the concentration range of 1 to 200 µg/kg. The screening detection limit (SDL) and limit of quantification (LOQ) for the majority of pesticides were less than 5 µg/kg and 10 µg/kg, respectively, which was further confirmed by spiking the corresponding concentration into the matrix for determination. The linear correlation coefficients (R^2) in the concentration range were all greater than 0.99 for all pesticides. At spiking levels of 1 × LOQ, 2 × LOQ, and 10 × LOQ, the recoveries for 413 out of 415 pesticides ranged from 70 to 118.8%. Only two

pesticides had recoveries within 65 to 70% at the 1× LOQ level. The relative standard deviations were below 20% for all pesticides. This method was used to conduct the unknown pesticide residue screening and quantitative measurement when participating the EU pesticide screening proficiency test program in 2019. All officially spiked pesticides were correctly identified and accurately quantified. The results demonstrate that the method is very reliable, capable of screening unknown pesticides, and capable of quantitating pesticides with simultaneous data acquisition in both accurate mass and accurate MS/MS fragment levels. This greatly improves the screening throughput of pesticide residues in red cabbage while meeting the screening criteria. This method can be expanded to other vegetable and fruit matrices for pesticide residue screening.

Introduction

Ultrahigh performance liquid chromatography (UHPLC) combined with high resolution mass spectrometry (HRMS) has been widely used in high-throughput screening of pesticide residues in food due to its advantages in high mass accuracy and high mass resolution, and less dependence upon chemical standards.[1,2] According to the EU SANTE guidelines on pesticide residue screening, to identify pesticides using high resolution mass spectrometry, an analysis must show that certain criteria are met. Two ions must have accurate *m/z* within ±5 ppm of the theoretical values. In addition to retention time consistency for the two ions, one ion must be the molecular adduct ion, and the other should be one of the MS/MS fragment ions.[3] High-resolution mass spectrometry approaches for food safety typically involve two kinds of acquisition workflows: data-dependent analysis and data-independent analysis. The data-dependent MS/MS acquisition mode allows for the acquisition of both the precursor ion and the corresponding MS/MS fragment spectra simultaneously, based on the automatic selection of the precursor ions according to their abundance.[4] However, the duty cycle of the mass analyzer may limit highly sensitive detection in complex samples with many potential analytes.

In recent years, data-independent acquisition mode (such as with the Agilent All Ions MS/MS targeted screening workflow for the Q-TOF mass analyzer) has been widely used.[5] Accurate *m/z* values for the molecular adduct ion and fragment ion can be simultaneously obtained through single data acquisition and a reliable data mining approach. By combining with chemical standards, the screening result can be further quantitated. The All Ions MS/MS acquisition function of the 6545 LC/Q-TOF ramps the collision energy of the collision cell at the front end of the TOF flight tube to simultaneously obtain both molecular adduct ions and their fragments. Based on our recent report,[6] this application note describes an approach in detail using the All Ions MS/MS scanning mode for high-throughput screening and accurate quantitation of 415 pesticide residues in red cabbage.

Experimental

Materials and methods

All pesticide standards were purchased from Dr. Ehrenstorfer GmbH (Germany) with purity ≥95%; formic acid and ammonium acetate were of LC/MS purity. Acetonitrile and toluene were of chromatographic purity, obtained from Fisher (US). Acetic acid, sodium chloride, anhydrous sodium sulfate and anhydrous magnesium sulfate were of analytical purity, obtained from Beijing chemical company (China). Carbon/NH_2 SPE cartridges (500 mg/500 mg) were obtained from Agilent Technologies (US).

Sample preparation

The sample extraction and clean-up procedure used is similar to China GB method 20769-2008.[7]

1. Weigh out 10 g of red cabbage sample and transfer it into an 80 mL centrifuge tube.
2. Transfer 40 mL of 1% acetic acid acetonitrile to the tube.
3. Homogenize the mixture at a speed of 13,500 rotations per minute for one minute.
4. Add 1 g of sodium chloride and 4 g of anhydrous magnesium sulfate to the mixture. Shake this mixture for 10 minutes sequentially, followed by centrifugation at 4,200 rotations per minute for five minutes.
5. Evaporate and concentrate the resultant supernatant (20 mL) to about 2 mL in a parallel concentrator at 37 °C and 150 rotations per minute before purification.
6. For purification, transfer anhydrous sodium sulfate into the carbon/NH_2 cartridge with 2 cm height before fixing on the automatic solid-phase extractor.
7. Set the extractor parameters at 4 mL acetonitrile: toluene mixing solvent (3:1, V:V) to elute the SPE column. Discard the effluent.
8. Transfer the concentrated sample into the carbon/NH_2 column, wash the sample container three times with 2 mL acetonitrile and toluene (3:1, V:V), and transfer the washing liquid into the carbon/NH_2 column.
9. Use 25 mL acetonitrile and toluene (3:1, V:V) for elution, and collect the eluent into the test tube.
10. Using the parallel concentrator, evaporate and concentrate the eluent to about 0.5 mL at 37 °C, centrifuging at 150 rotations per minute. Blow under nitrogen until completely dry.

11. Dissolve the residue in a 1 mL acetonitrile/water solution (3:2, V:V), mixing thoroughly. Filter with a 0.22 µm membrane for UHPLC-Q-TOF/MS analysis.

Table 1. LC separation conditions

Parameter	Value
LC	Agilent 1290 Infinity II LC
Column	Agilent ZORBAX SB-C18 (100 mm × 2. 1 mm, 3.5 µm, (p/n 861775-902)
Mobile Phase	A: 0.1% formic acid/5 mM ammonium acetate; B: acetonitrile
Column Temperature	40 °C
Injection Volume	5 µL
Flow Rate	0.4 mL/min
Gradient Profile	Time (min) %B 0 to 3 1 to 30 3 to 6 30 to 40 6 to 9 40 9 to 15 40 to 60 15 to 19 60 to 90 19 to 23 90
Post Time	4 min

Table 2. MS/MS conditions

Parameter	Value
MS	Agilent 6545 LC/Q-TOF with Agilent Dual Jet Stream ESI
Polarity	Positive ionization
Drying Gas Temperature	325 °C
Drying Gas Flow Rate	12 L/min
Nebulizer Gas Pressure	35 psi
Sheath Gas Temperature	375 °C
Sheath Gas Flow Rate	11 L/min
Capillary Voltage	4,000 V
MS Scan Range	*m/z* 50 to 1,000
Scan Mode	Agilent All Ions MS/MS screening workflow
CE Value	0, 15 V, 35 V
Reference Ions	*m/z* 121.0509/922.0098

Qualitative screening method evaluation

For screening detection level (SDL) measurement, the SANTE/12682/2019 guidelines for pesticide screening were followed.[3] The red cabbage samples were spiked with a series of concentrations for each pesticide, with 20 replicates at concentration levels of 1, 2, 5, 10, 20, and 50 ug/kg. The resulting samples were extracted, cleaned up and analyzed using the method described in the experimental section. The criteria for positive identification include agreement with the database with respect to retention time for reference compounds (±0.35 minutes) and accurate mass for the precursor ion and at least one fragment ion (with mass accuracy within ±5 ppm). For pesticides with *m/z* less than 200, up to 1 mDa mass deviation is allowed. For the unknown sample from EUPT, analysis was performed following the procedure described above, with the exception of spiking pesticide compounds into the red cabbage. Information regarding the 415 pesticides (including chemical names, CAS numbers, retention time, and both the accurate *m/z* of the quantitative and qualitative ions) can be found in reference.[6]

Quantitative method evaluation

Six blank red cabbage samples were initially subjected to sample preparation following the method described previously. The resulting blank sample matrix residue was added to a series of standard pesticide mixture solutions, with a final concentration of each pesticide ranging from 5.0 to 100 µg/kg. The resultant samples were filtered through a membrane and subjected to LC-Q-TOF/MS analysis. A matrix-matched standard calibration curve was then applied for quantitative analysis to avoid quantitation bias.

The calculated limit of quantitation (LOQ) was determined to be the level at which S/N = 10, based on the lowest matrix-matched calibration level for each compound. For convenience of method evaluation, if the calculated LOQ was less than 1 µg/kg, the LOQ was rounded up to 1 µg/kg. Similarly, if it was between 1 and 2 µg/kg, the LOQ was rounded up to 2 µg/kg. If the calculated LOQ was between 2 and 5 µg/kg, the LOQ was rounded up to 5 µg/kg. If the calculated LOQ was between 5 and 10 µg/kg, the LOQ was rounded up to 10 µg/kg. The rounded-up LOQ level was further validated by analyzing the spiking samples at the corresponding concentration level to ensure S/N ≥10. Only the rounded-up LOQ levels which passed validation could be then set as the LOQs of the method.

The accuracy and precision of the method were evaluated via a spiking recovery test at three concentration levels in the red cabbage: the LOQ, 2 × LOQ, and 10 × LOQ with six replicates at each spiking level. The spiked samples were then subjected to the same sample preparation procedure and analyzed using the UHPLC-Q-TOF/MS.

Results and discussion

UHPLC-Q-TOF/MS method optimization

Initially, a PCDL containing over 800 pesticides was customized in the lab following the Agilent PCDL creation guidelines. A UHPLC-Q-TOF/MS method under TOF scan mode developed previously[4] was applied for separation of the selected 415 pesticide compounds, with a slight modification in the elution profile to ensure all pesticides could be distributed relatively evenly within the elution time window to minimize the interference among the pesticides themselves. The parameters for the ionization source of the mass spectrometer were then optimized so that an overall acceptable response for all selected pesticides could be

obtained. The optimized parameters for separation and detection are shown in Tables 1 and 2 of the experimental section.

All Ions MS/MS parameter optimization

For the Q-TOF mass analyzer, All Ions MS/MS mode can be applied to conduct data-independent acquisition.[8] As multiple energy channel acquisition is required to generate All Ions MS/MS spectra, it is necessary to optimize the collision energies and acquisition rate to ensure sufficient qualitative ions can be generated for each compound, and that enough data points for each compound can be used for accurate quantitation. Initially, a group of 215 compounds in solvent were evaluated in terms of the response sensitivity under 2 and 3 collision energy channels for fragmentation at a fixed acquisition rate of two spectra per second. As shown in Figure 1A, though application of the two channels (0 to 15 V) could identify 197 out of 215 compounds, there were still 18 compounds which could not be sensitively and reliably identified. In comparison, when applying three energy channels sequentially, it was observed that all three energy channels (0, 15, and 30 V and 0, 15, and 35 V) could improve the number of identified pesticides. Of these, 0, 15, and 35 V can identify the highest number of pesticides (209 out of 215 compounds). Hence, collision energy channels of 0, 15, and 35 V were selected for sequential data acquisition optimization under All Ions MS/MS mode.

Acquisition rate can affect the number of data points of a chromatographic peak, and a sufficient number of data points for one chromatographic peak is required for quantitation accuracy. The higher the acquisition rate is, the higher the number of data points are. However, the acquisition rate can also affect the accumulation number of ion pulses during each individual data point acquisition. As shown in Figure 1B, with the increase in acquisition rate from 2 to 4 spectra per second, the number of compounds meeting the qualification criteria increased from 209 to 215 compounds. When acquisition rate is equal to or greater than 5 spectra per second, the sensitivity decreases slightly. Only 4 out of 215 compounds did not meet the qualitative criteria as strictly, as the sensitivity for their fragment ions is not high enough for accurate identification. As the condition of 4 spectra per second can provide enough data points for accurate quantitation, it was selected as the optimal acquisition rate.

Under the optimized collision energies and acquisition rate, the UHPLC-Q-TOF/MS method was applied to evaluate a total of 415 compounds spiked in red cabbage. Figure 2 shows the typical overlapped extracted ions chromatograms for all 415 pesticide compounds. Retention time, quantitative and qualitative ions and their relative mass accuracies for each compound can be found in reference.[6]

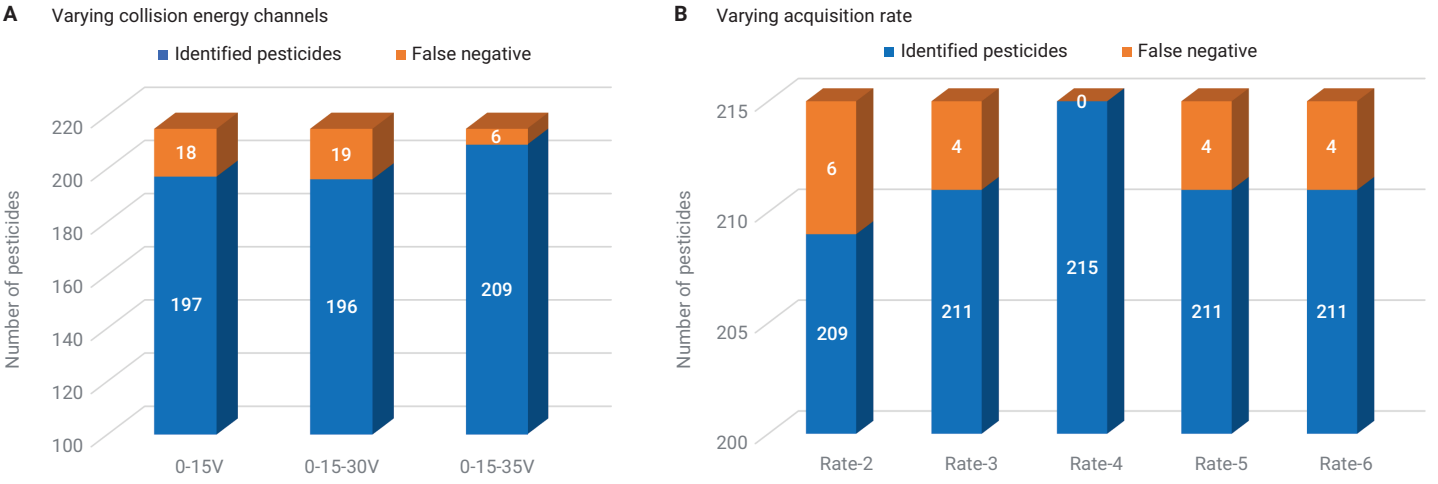


Figure 1. (A) The number of correctly identified and false-negative pesticides under different collision energies and (B) acquisition rates using the All Ions MS/MS mode. Note that the concentration of each pesticide in the solvent was fixed at 100 µg/L.

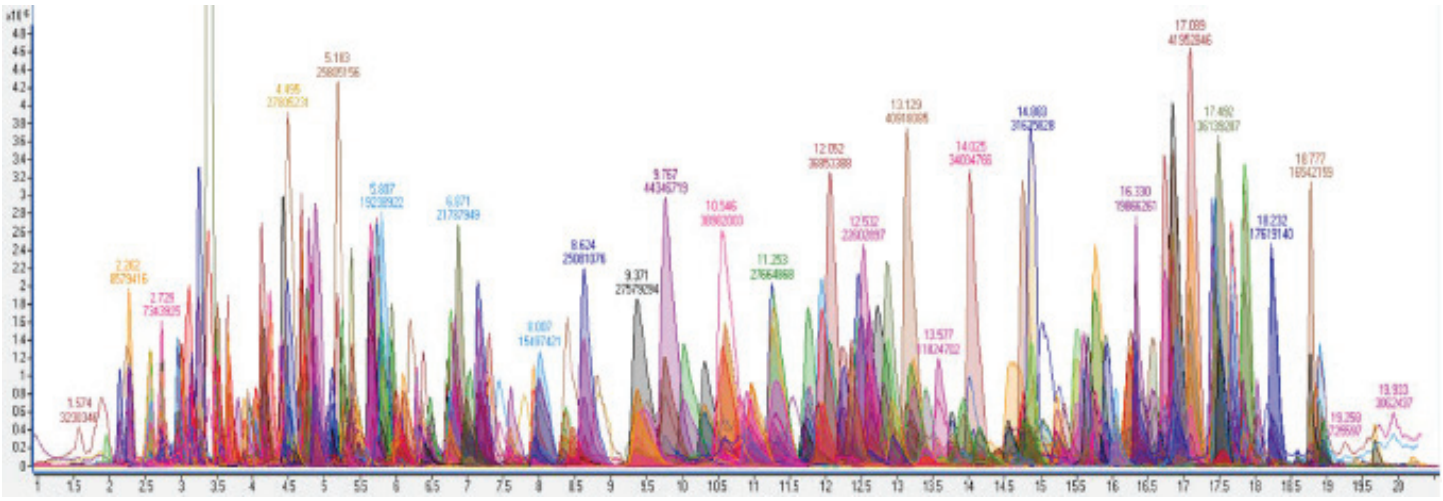


Figure 2. Typical overlapped extracted ion chromatograms for the pesticides under the experimental condition.

Method performance evaluation

Determination of the screening detection level (SDL)

Following the SANTE/12682/2019 guidelines, the spiked red cabbage samples were prepared and analyzed at a series of concentrations for each pesticide, with 20 replicates at each concentration level. The SDL for each pesticide was then obtained based on the criteria listed in the experimental section. As shown in Figure 3A, the majority of the open blue circles (411 total) which represent the SDL of each pesticide, are less than or equal to 5 µg/kg. Only 3 pesticides (overlapping with orange triangles – fluorochloridone, isoprocarb, and terbufos-oxon) show SDLs at 10 µg/kg. One pesticide, terbucarb, shows an SDL at 20 µg/kg.

Matrix-matched standard calibration for linearity evaluation

The matrix-matched calibration standard solutions were prepared using blank red cabbage matrix extract, with a final concentration of each pesticide ranging from 5.0 to 100 µg/kg. The resultant samples were filtered through a membrane and subjected to LC-Q-TOF/MS analysis, as described in the experimental section. A matrix-matched standard calibration curve was then established. The linear relationship of 415 pesticides were very good, with the linear regression correlation coefficients (R^2) all greater than 0.990 (Figure 3B). Of these, 368 pesticides had a linear range from 5 to 100 µg/kg, accounting for 88.7% of pesticides.

Determination of limit of quantitation (LOQ)

Following the procedure for determination of LOQ in the experimental section, the LOQ of the method for each pesticide was obtained. As shown in Figure 3A, up to 413 of 415 pesticides have LOQ values at or below 10 µg/kg. Only diazinon and terbucarb exhibit LOQs at 20 µg/kg. It is worth pointing out that both the SDL and LOQ for some pesticides are the same (terbucarb, fluorochloridone, isoprocarb and terbufos-oxon). This is mainly because here, SDL is not determined by the conventional method (normally set as the level which can achieve a signal-to-noise ratio of three), but rather, determined based on the frequency for positive screening on a set of 20 spiked samples.

Accuracy and precision

The accuracy and precision of the method were evaluated via a spiking recovery test at 3 concentration levels in red cabbage: LOQ, 2 × LOQ, and 10 × LOQ. As shown in Figure 3C (with the exception of ethirimol and chlormeq, which show recovery values of 65.7 and 68.3% at the spiking level of LOQ, respectively) all other recovery values at the 3 spiking levels for these 415 pesticides are within the range of 70 to 120%, with relative standard deviation (RSD) below 20% (Figure 3D). This demonstrates that the method is both highly accurate and reliable.

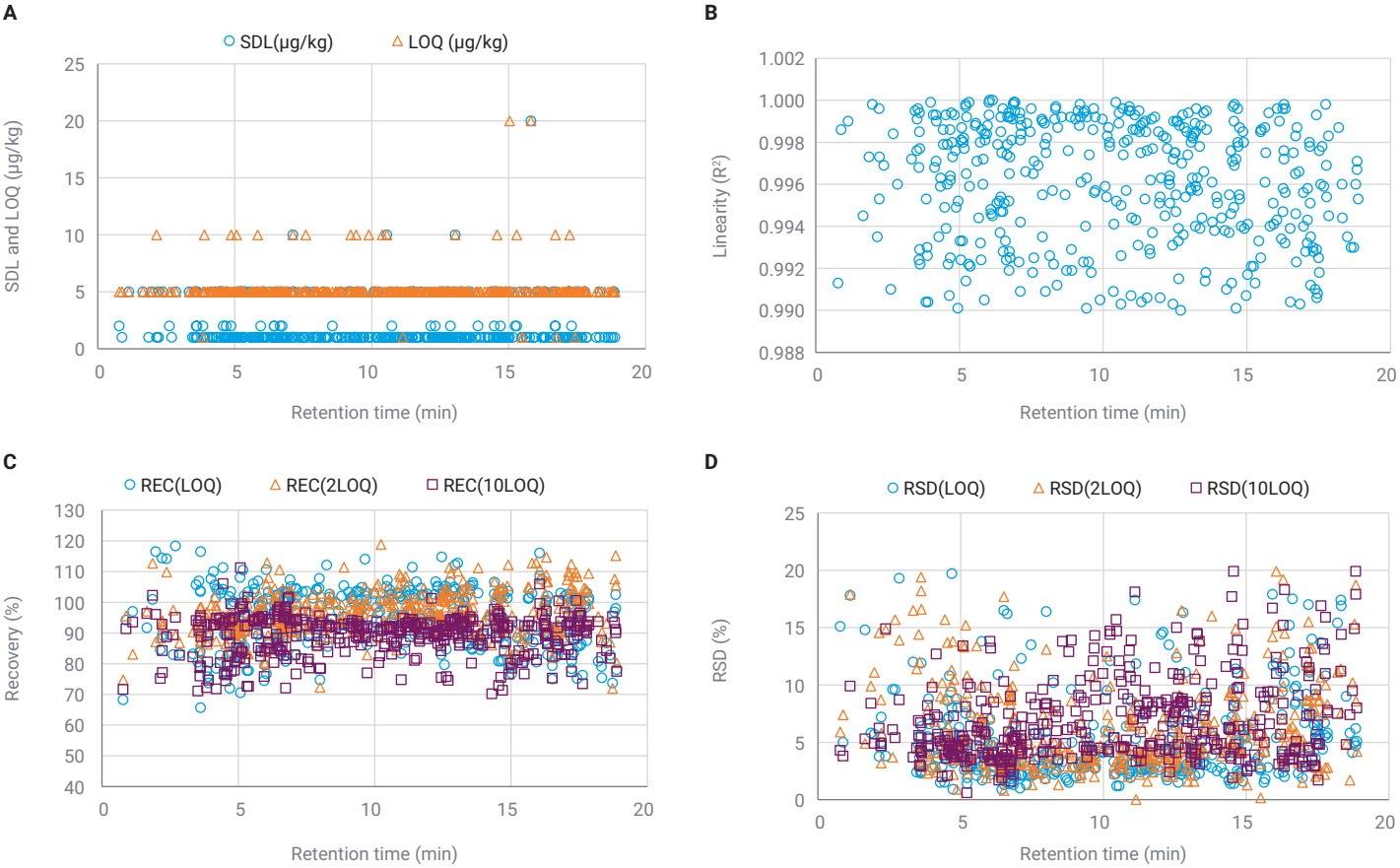
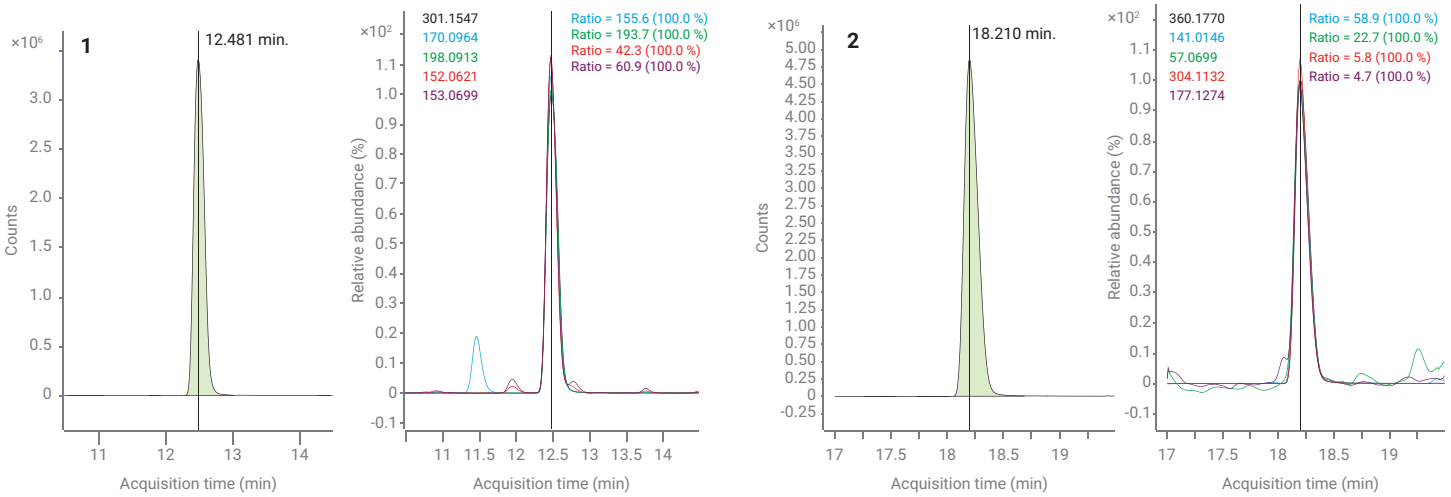


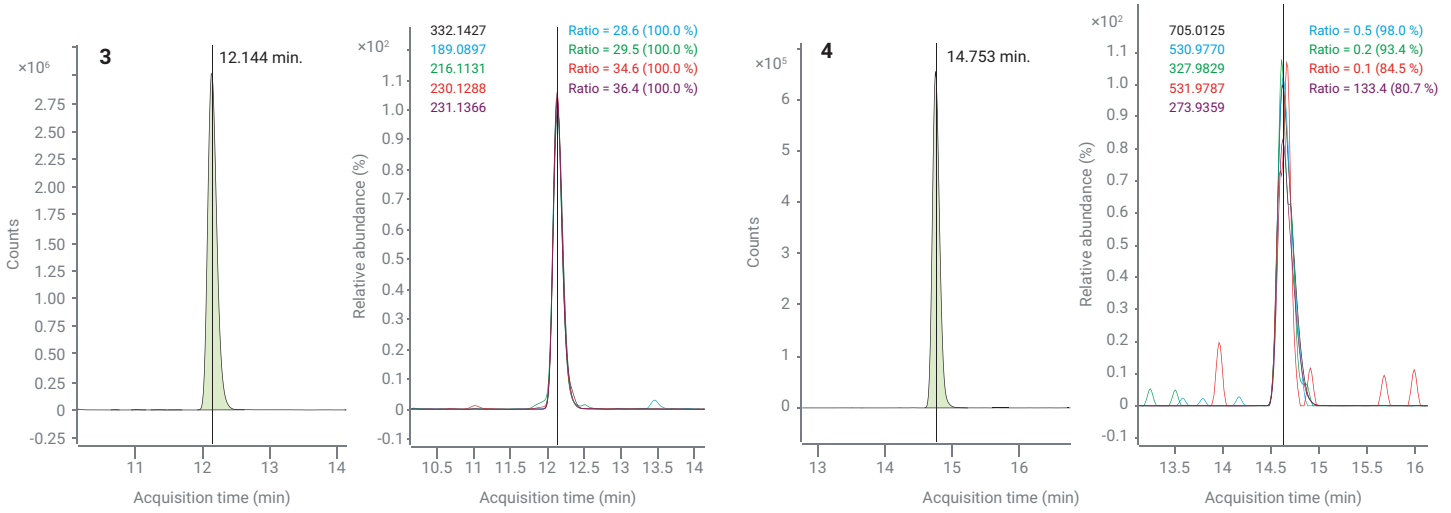
Figure 3. Method performance evaluation in terms of (A) screening detection limit (SDL) and limit of quantitation (LOQ), (B) linearity, (C) method accuracy (recovery), and (D) method precision (RSD).

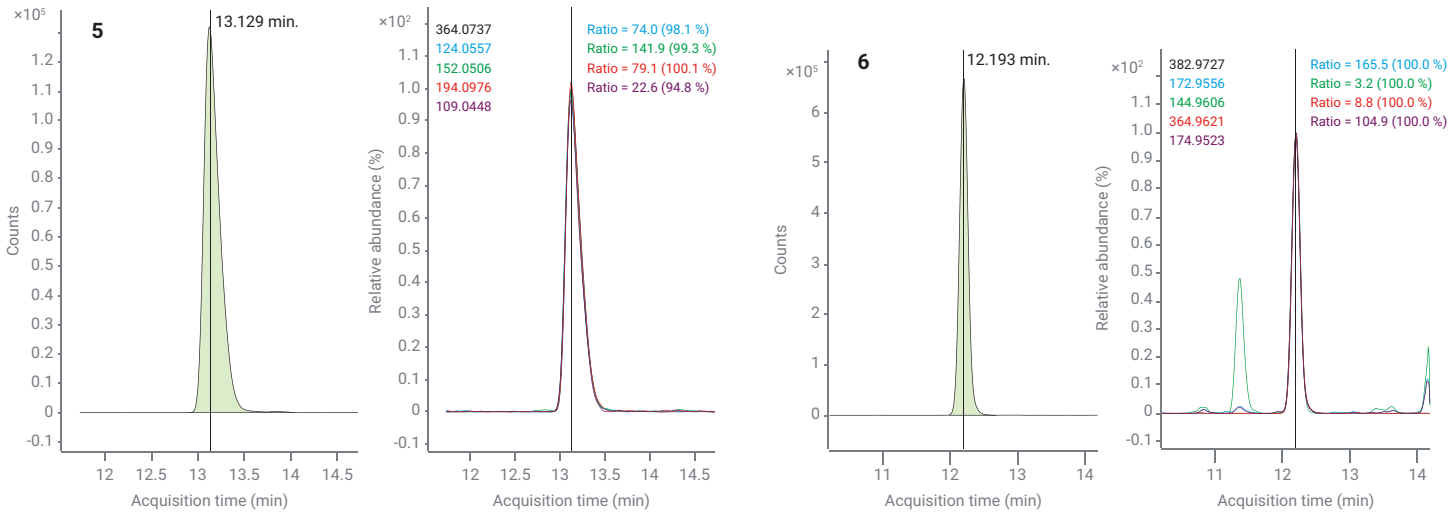
Method validation by determination of EU proficiency test samples

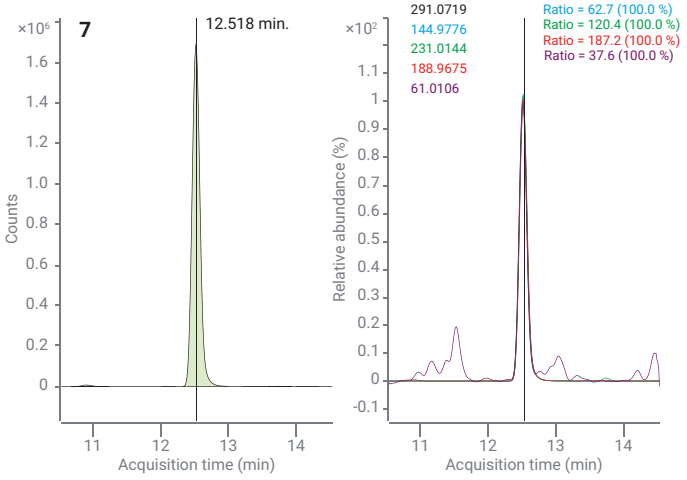
The established qualitative screening and quantitative determination method was applied to the analysis of both unknown and quantitative screenings of red cabbage samples provided by the EU pesticide screening proficiency test program. For the unknown pesticide screening sample (not specified in the scope of the pesticides) 16 pesticides were correctly screened out using the in-house PCDL (includes over 800 pesticides) and all pesticides are within the list of 415. The result is completely consistent with the naturally officially incurred pesticides, and no false positive/ false negative results were present. For all 16 identified pesticide compounds (other than the precursor ions) at least 3 fragment ions exist for each pesticide which can be used to support accurate identification. All mass accuracies of these precursor ions and fragment ions are consistent with those present in the reference spectra of the PCDL, with mass accuracy no greater than 5 ppm. In addition, the retention time for all identified pesticide compounds are within 0.2 minutes of the reference time in the PCDL. The extracted ion chromatograms and their qualitative data are shown in Figure 4. Table 3 also lists these compounds with retention time and the two ions for qualification.

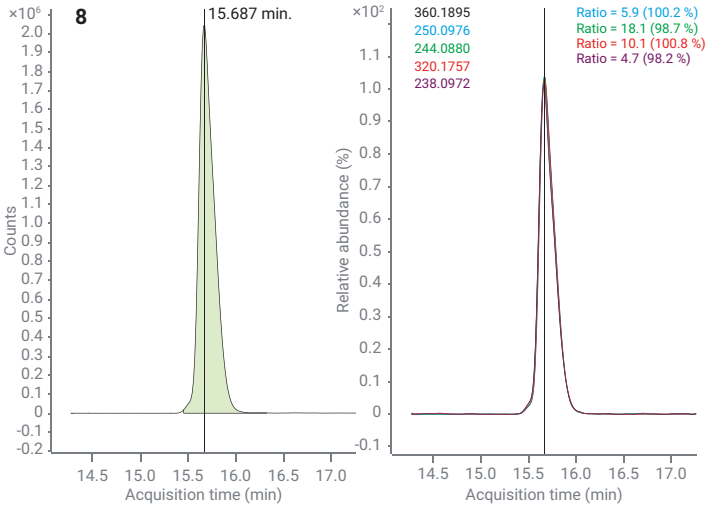
For the quantitative samples with incurred pesticides (a specified subset of 209 of the 415 described in this application), all 21 spiked pesticide compounds were correctly identified, including omethoate. The identified pesticides were then quantitated using the standard compounds. Omethoate was excluded from quantitative evaluation due to low robustness among registered laboratories. The other 20 pesticides identified (Table 4) showed that measured values are in excellent agreement with the reference values, as the absolute value of Z-scores is not greater than 1.1. The successful identification of unknown pesticides, and the accurate measurement of pesticides within a specific list, rendered the lab with performance category A (“good”, the highest level) for pesticide screening.

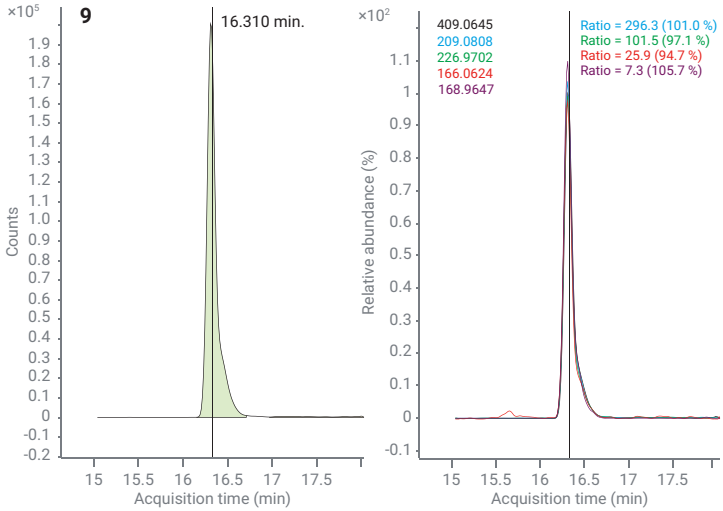

1
12.481 min.
Counts
Acquisition time (min)
301.1547
170.0964
198.0913
152.0621
153.0699
Ratio = 155.6 (100.0 %)
Ratio = 193.7 (100.0 %)
Ratio = 42.3 (100.0 %)
Ratio = 60.9 (100.0 %)
Relative abundance (%)
2
18.210 min.
360.1770
141.0146
57.0699
304.1132
177.1274
Ratio = 58.9 (100.0 %)
Ratio = 22.7 (100.0 %)
Ratio = 5.8 (100.0 %)
Ratio = 4.7 (100.0 %)

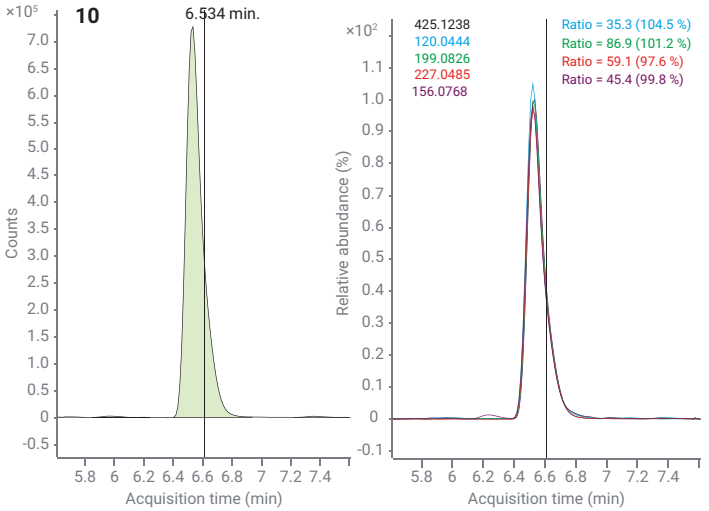

3
12.144 min.
Counts
Acquisition time (min)
332.1427
189.0897
216.1131
230.1288
231.1366
Ratio = 28.6 (100.0 %)
Ratio = 29.5 (100.0 %)
Ratio = 34.6 (100.0 %)
Ratio = 36.4 (100.0 %)
Relative abundance (%)
4
14.753 min.
705.0125
530.9770
327.9829
531.9787
273.9359
Ratio = 0.5 (98.0 %)
Ratio = 0.2 (93.4 %)
Ratio = 0.1 (84.5 %)
Ratio = 133.4 (80.7 %)

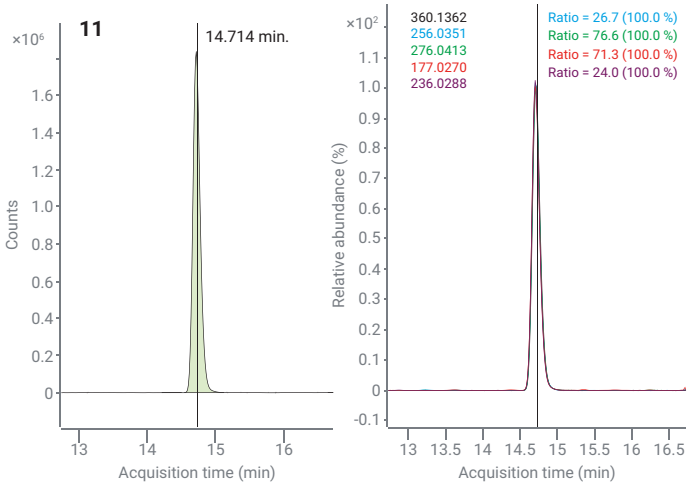

5
13.129 min.
Counts
Acquisition time (min)
364.0737
124.0557
152.0506
194.0976
109.0448
Ratio = 74.0 (98.1 %)
Ratio = 141.9 (99.3 %)
Ratio = 79.1 (100.1 %)
Ratio = 22.6 (94.8 %)
Relative abundance (%)
6
12.193 min.
382.9727
172.9556
144.9606
364.9621
174.9523
Ratio = 165.5 (100.0 %)
Ratio = 3.2 (100.0 %)
Ratio = 8.8 (100.0 %)
Ratio = 104.9 (100.0 %)

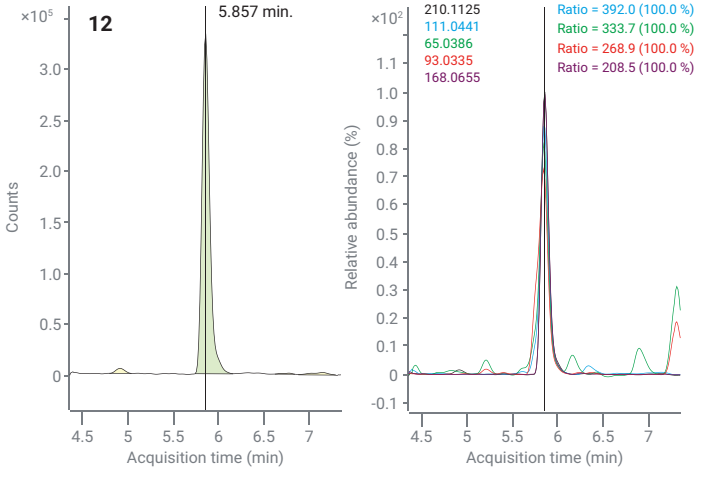

7
12.518 min.
Counts
Acquisition time (min)
291.0719
144.9776
231.0144
188.9675
61.0106
Ratio = 62.7 (100.0 %)
Ratio = 120.4 (100.0 %)
Ratio = 187.2 (100.0 %)
Ratio = 37.6 (100.0 %)
Relative abundance (%)


8
15.687 min.
Counts
Acquisition time (min)
360.1895
250.0976
244.0880
320.1757
238.0972
Ratio = 5.9 (100.2 %)
Ratio = 18.1 (98.7 %)
Ratio = 10.1 (100.8 %)
Ratio = 4.7 (98.2 %)
Relative abundance (%)


9
16.310 min.
Counts
Acquisition time (min)
409.0645
209.0808
226.9702
166.0624
168.9647
Ratio = 296.3 (101.0 %)
Ratio = 101.5 (97.1 %)
Ratio = 25.9 (94.7 %)
Ratio = 7.3 (105.7 %)
Relative abundance (%)


10
6.534 min.
Counts
Acquisition time (min)
425.1238
120.0444
199.0826
227.0485
156.0768
Ratio = 35.3 (104.5 %)
Ratio = 86.9 (101.2 %)
Ratio = 59.1 (97.6 %)
Ratio = 45.4 (99.8 %)
Relative abundance (%)


11
14.714 min.
Counts
Acquisition time (min)
360.1362
256.0351
276.0413
177.0270
236.0288
Ratio = 26.7 (100.0 %)
Ratio = 76.6 (100.0 %)
Ratio = 71.3 (100.0 %)
Ratio = 24.0 (100.0 %)
Relative abundance (%)


12
5.857 min.
Counts
Acquisition time (min)
210.1125
111.0441
65.0386
93.0335
168.0655
Ratio = 392.0 (100.0 %)
Ratio = 333.7 (100.0 %)
Ratio = 268.9 (100.0 %)
Ratio = 208.5 (100.0 %)
Relative abundance (%)

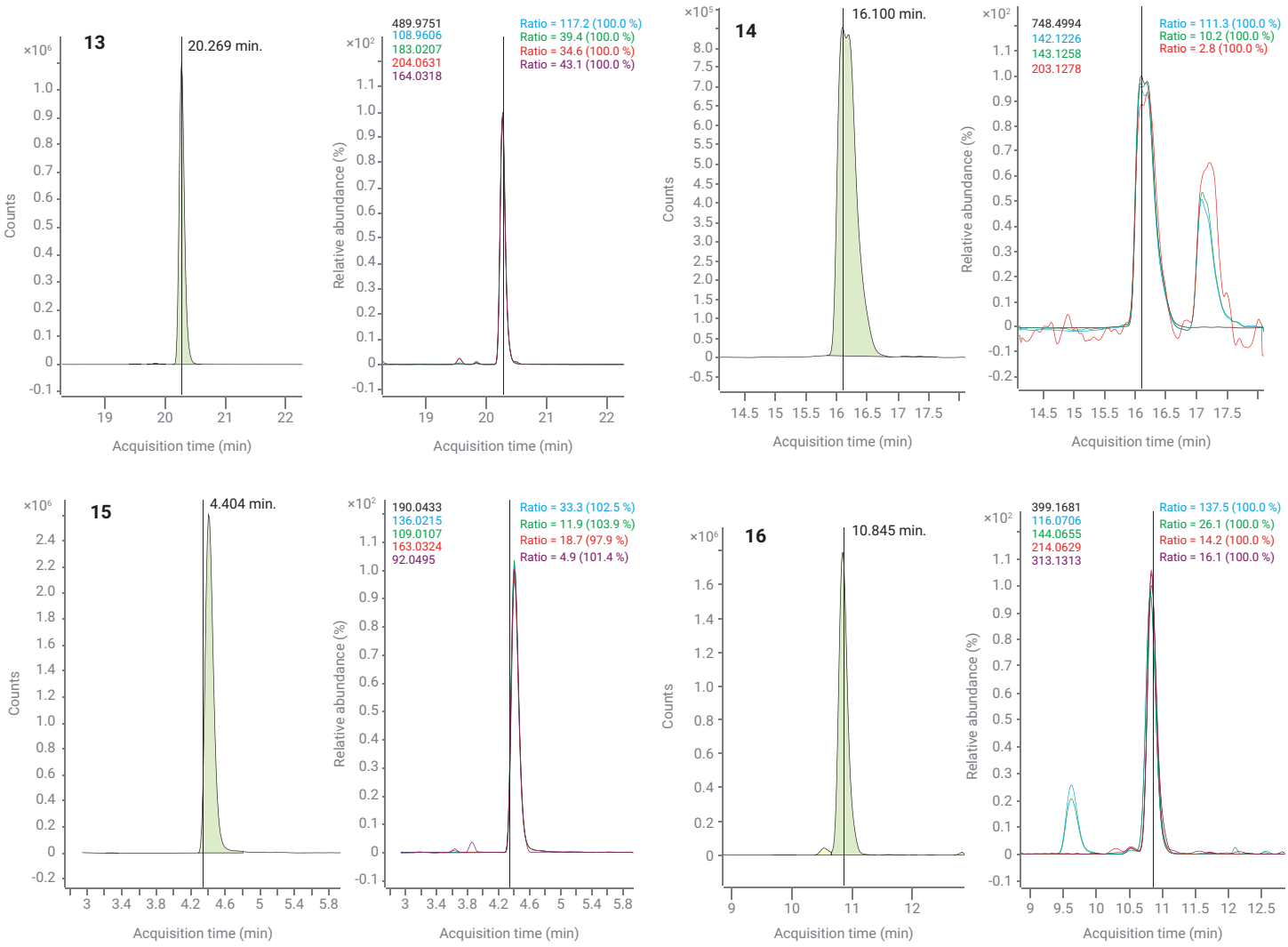


Figure 4. The extracted ion chromatograms for both quantitative and qualitative ions of each identified pesticide compound in the unknown red cabbage sample provided by the EU proficiency test on pesticide residue program. The identification number of each pesticide is listed in Table 3. For each pesticide ID, the left graph shows the quantitative ion chromatogram, and the right graph shows the overlapping of both the extracted quantitative and the qualitative ion chromatograms.

Table 3. The identified pesticide compounds in the unknown red cabbage sample, each listed with one precursor ion and one major fragment ion (higher intensity) for qualification (the leftmost is the precursor ion).

No.	Identified Pesticides	RT (min)	Major Qualitative Ions	No.	Identified Pesticides	RT (min)	Major Qualitative Ions
1	Bifenazate	12.48	301.1547, 198.0913	9	Metrafenone	16.31	409.0645, 209.0808
2	Etoxazole	18.21	360.1770, 141.0146	10	Orthosulfamuron	6.54	425.1238, 120.0444
3	Fenpyrazamine	12.14	332.1427, 189.0897	11	Penthiopyrad	14.71	360.1362, 256.0351
4	Flubendiamide	14.75	705.0125, 530.9770	12	Propoxur	5.86	210.1125, 111.0441
5	Flufenacet	13.13	364.0737, 124.0557	13	Pyridalyl	20.27	489.9751, 108.9606
6	Fluopicolide	12.19	382.9727, 172.9556	14	Spinetoram	16.10	748.4994, 142.1226
7	Isoprothiolane	12.52	291.0719, 144.9776	15	Tricyclazole	4.40	190.0433, 136.0215
8	Isopyrazam	15.69	360.1895, 250.0976	16	Valifenalate	10.85	399.1681, 116.0706

Table 4. Measured values and Z-scores for the identified pesticides in red cabbage.*

No.	Compound	Result (mg/kg)	Z-Score	No.	Compound	Result (mg/kg)	Z-Score
1	Acetamiprid	0.1510	−0.5	11	Metaflumizone	0.1527	−1.1
2	Chlorantraniliprole	0.1160	−0.6	12	Propamocarb	0.1671	−0.1
3	Chlorpropham**	0.0676	−0.8	13	Propyzamide	0.0715	−0.6
4	Chlorpyrifos	0.0406	−0.8	14	Pyraclostrobin	0.0668	−0.5
5	Clothianidin	0.0481	−0.3	15	Teflubenzuron	0.0941	−0.6
6	Diazinon	0.0597	−0.6	16	Trifloxystrobin	0.1863	−0.2
7	Difenoconazole	0.1282	−0.9	17	Triflumuron	0.4360	−0.3
8	Dimethoate	0.0980	−0.6	18	Penthiopyrad	0.1936	−0.1
9	Fenamidone	0.5334	−0.1	19	Spinetoram	0.0531	−0.2
10	Fluxapyroxad	0.4264	−0.2	20	Tritosulfuron	0.0538	−0.7

* The Z-score refers to the EUPT website (https://www.eurl-pesticides.eu/), omethoate was excluded from evaluation by EUPT due to low robustness, ** was reported using the other method.[9]

Conclusion

This application note describes a method for the simultaneous screening and quantification of 415 pesticide residues in red cabbage using the Agilent 6545 LC-Q/TOF. The method developed for red cabbage matrix has sensitive and reliable screening capacity, with a majority of SDLs at or below 5 ug/kg. The method also has accurate and robust quantitation capacity with majority of LOQs at or below 10 µg/kg and RSD below 20%. This method can also be expanded to include many other food matrixes of plant origin for qualitative and quantitative pesticide residue screening.

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