Pesticide Residues in Tobacco

Using the Agilent 6470 Triple Quadrupole LC/MS/MS System

Application Note

Food Testing and Agriculture

Authors

Prasanth Joseph and Dr. Samir Vyas
Agilent Technologies, Inc.
Bangalore, India

Abstract

A sensitive and specific MRM method was developed for 40 pesticides in tobacco samples using the Agilent 6470 triple quadrupole LC/MS/MS system. The Agilent pesticide test mix and pesticide database were used for easy method development. To minimize the matrix effect, an Agilent QuEChERS kit was used for extraction of pesticides, followed by dilution before injection. Matrix-matched linearity ranging from 0.05 to 100 ng/mL was generated by post spiking the required concentration of pesticides into the diluted matrix extract. The method was validated with excellent reproducibility at the limits of quantification (LOQ), accuracy, and precision in daily and day-to-day samples with acceptable recovery. The developed method provided high throughput with minimal sample preparation, and can be adopted for routine quality control of tobacco samples.

Agilent Technologies

Introduction

Tobacco is one of the world's leading high-value crops. It is a plant prone to many diseases. Pesticides are widely used in the cultivation of tobacco. Pesticides applied during cultivation can remain in the tobacco leaves at harvest, and even during postharvest processing treatments, and can appear in the finished products. "Up to 16 applications of pesticides are recommended during the three-month growing period of the tobacco plant"[1]. There is a global concern about pesticide residues that accumulate in the body through the consumption and use of various tobacco products.

Multipesticide residue analysis in tobacco is a challenge in both sample preparation and instrumental detection. The QuEChERS sample preparation technique has been accepted worldwide for multipesticide residue analysis. Multiple reaction monitoring (MRM) based LC/MS/MS methods have been used increasingly in detection and quantification of multiple pesticide residues in food and agricultural products. MRM uses a combination of a specific precursor mass and a unique product ion, which is generally an unambiguous and sensitive method to selectively monitor and quantify compounds of interest in complex mixtures and matrices. SANTE regulations specify that a minimum of two product ions are required for the identification of pesticides. The ion ratio should be within 30 % of the average of calibration standards from the same sequence[2].

Dynamic MRM (dMRM) creates more powerful quantitative methods by grouping MRMs in a retention time window instead of time segments. Compound-specific MRMs and corresponding retention times can easily be imported into dMRM methods, which can quantify up to 4,000 compounds in a single run[3].

Because of the analytical sensitivity of the Agilent 6470 triple quadrupole LC/MS/MS system, sample extracts can be diluted to reduce or remove matrix effects. Dilution or removal of matrix effects is an attractive feature for many routine testing labs because the requirement for cleanup can be avoided. To prepare recovery samples, a blank tobacco sample was spiked with the pesticide mix standard, and extracted using the QuEChERS method. Tobacco samples processed by QuEChERS are complex, containing various matrix components. An aliquot of the extract was diluted 10-fold, followed by filtration, and the sample was ready for LC/MS/MS analysis. By diluting the extracted sample, the quantity of matrix injected into the LC/MS system was limited. The result was less matrix interference, increased robustness of the analytical method, and minimized instrument contamination.

Experimental

Chemicals and reagents

MS grade acetonitrile was purchased from Fluka. Ammonium formate (AR grade) and formic acid (MS grade) were from Sigma-Aldrich. The Milli-Q ultrapure water system was used as a source of water for the preparation of the mobile phase and needle wash. Pesticide reference standards were bought from LGC Standards. Agilent Bond Elut QuEChERS extraction salts with tubes (p/n 5982-5650) was used for the extraction of pesticides from tobacco samples.

Standard solutions

A standard mix stock solution of 1 mg/L was prepared by mixing individual stock solutions of 100 mg/L of pesticide standards. Stock solutions were available in acetonitrile. Further dilutions were prepared by mixing the stock mix solution with water/acetonitrile (80:20 v/v) as diluent. All working standard solutions were stored at 4 °C

Sample preparation

Approximately 1.0 g of tobacco was accurately weighed in a 50-mL polypropylene tube. Ten milliliters of water were added to the tobacco sample, and kept for 30 minutes. Ten milliliters of acetonitrile were added as an extraction solvent. The mixture was thoroughly shaken for 1 minute, and stored for 10 minutes at –18 °C. Agilent Bond Elut QuEChERS extraction salts with tubes (p/n 5982–5650) were then added to the mixture. The tube was closed tightly and shaken for 1 minute. Samples were centrifuged for 5 minutes at 5,000 rpm. One milliliter of the clear supernatant was diluted 10 times using the diluent. The diluted extract was filtered through a 0.45-µm PTFE syringe filter.

Instrumentation

This study was performed on an Agilent 1290 Infinity II coupled to an Agilent 6470 Triple Quadrupole LC/MS/MS with an Agilent Jet Stream source operated in positive ionization mode. Gradient elution was used on an Agilent Rapid Resolution High Definition (RRHD) Eclipse Plus C18 (2.1 × 100 mm, 1.8 µm) stationary phase for separation of targeted analytes. Table 1 shows further parameters.

Agilent MassHunter acquisition software version B.08.00 was used for acquisition. MassHunter qualitative software version B.07.00 was used for data processing, and MassHunter quantitative software version B.07.01 was used for quantification of the pesticides in real samples and recovery samples. MRM transitions were imported from the tMRM database. LC/MS acquisition of 100 ng/mL standard was performed. Based on the generated MRM chromatogram, the method was converted to a dMRM method. All further samples were acquired using dMRM. For each analyte, an extra fragment ion was defined as a confirmatory ion, and the method complied with the EU directive criteria of four identification points for the reporting of results. Limit of detection (LOD), limit of quantification (LOQ), accuracy, reproducibility, linear range, and recovery were determined as part of the method validation.

Results and Discussion

The LOQ of the standards was calculated using the formula:

LOQ = (t × RSD × concentration)/100

(t for 99 % confidence level, n–1 degrees of freedom) by considering the %RSD of nine replicate injections of 0.05 ng/mL. For all tested compounds, the LOQ calculated was ≈ 0.03 ng/mL. Noise was calculated using the auto RMS algorithm available in the MassHunter qualitative software. All samples were effectively diluted by 100 times (1 g sample in 10 mL acetonitrile followed by 10 times dilution), and the method detection limit (MDL) was determined to be 10 ng/g. For most of the pesticides, lower LOQs and MDLs may be achieved when considering the minimum signal-to-noise ratio (S/N) of 10:1 required to calculate the LOQ.

Linearity range

Three-order linearity from 0.05 to 100 ng/mL was established with eight concentration levels in both solvent and matrix-matched standards. Concentration levels required for matrix-matched calibration curves were prepared by post spiking the diluted extracted matrix. For example, a matrix-matched concentration level of 0.05 ng/mL was prepared by diluting 100 µL of 5 ng/mL to 900 µL of extracted tobacco matrix, followed by 10-fold dilution. Six replicate injections at each level were used to plot the calibration curve. The linearity of all pesticides in the study had a regression coefficient (R^2) >0.9950.

Table 1. Instrument conditions.

Parameter	Value		
Mobile phase	A) 5 mM ammonium formate + 0.01 % formic acid in water B) 5 mM ammonium formate + 0.01 % FA in water:acetonitrile (5:95)		
Gradient elution program	Sl. no.	Time (min)	% B
	1	0	5
	2	1	5
	3	4	50
	4	9	95
	5	12	5
	Post run	2	5
Flow rate	0.4 mL/min		
Injection volume	2 µL		
Source parameters			
Sheath gas	12 L/min		
Heated gas	6 L/ min		
Nebulizer	35 psi		
Sheath gas temperature	400 °C		
Heated gas	325 °C		
Capillary voltage	4,000 V		

Table 3. S/N of some of the pesticides at 10 ng/mL.

Sl. no.	Pesticide ID	S/N at 10 ng/mL	Sl. no.	Pesticide ID	S/N at 10 ng/mL
1	Methomyl	1,175	16	Phosphamidon	11,610
2	Tricyclazole	1,877	17	Pirimiphos-methyl	1,828
3	Carbendazim (Azole)	2,367	18	Fenazaquin	1,842
4	Fenobucarb (Baycarb)	2,076	19	Tebuconazole	1,323
5	Propoxur	1,175	20	Triazophos	5,204
6	Carbofuran	14,099	21	Benalaxyl	39,126
7	Methabenzthiazuron	14,099	22	Bitertanol	1,269
8	Acetamiprid	10,127	23	Propiconazole	2,972
9	Monocrotophos (Azodrin)	7,968	24	Thiodicarb	11,214
10	Pirimicarb	12,357	25	Flufenacet (Fluthiamide)	24,140
11	Imidacloprid	3,431	26	Profenofos	3,168
12	Penconazole	4,612	27	Dimethomorph	6,029
13	Metolachlor	2,744	28	Azoxystrobin	3,849
14	Isoprothiolane	4,909	29	Diazinon	428
15	Thiamethoxam	3,766	30	Buprofezin	579

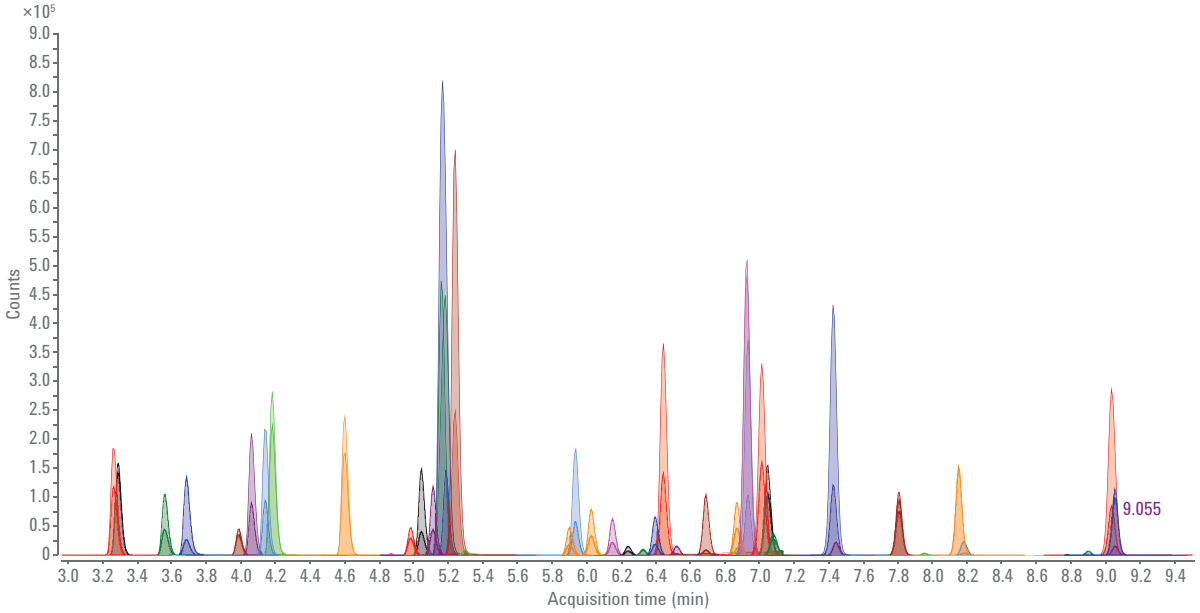

Figure 1. Representative chromatogram of 100 ng/mL pesticide standard mix.

Recovery studies

Recovery studies were conducted at three different fortification levels: at 10, 100, and at 200 ng/g. To prepare the recovery samples, blank tobacco samples were prespiked with pesticide working solutions. For example, the recovery sample at 10 ng/g was prepared by prespiking 100 µL of 100 ng/mL pesticide working standard into 1 g of blank tobacco sample. The prespiked samples were extracted with 10 mL of acetonitrile, followed by QuEChERS extraction, and diluted 10 times. Recovery samples prepared in this way were compared against the matrix-matched calibration graphs. Matrix-matched calibration curves from 0.05–100 ng/mL were prepared by post spiking pesticide standards in the diluted matrix (Figure 3). As per SANTE 11945/20152, residue data adjustment is not required when the mean recovery ranges between 70 and 120 %. All 40 pesticides in this study had a recovery percentage between 75 and 120 %, and recovery correction was not required.

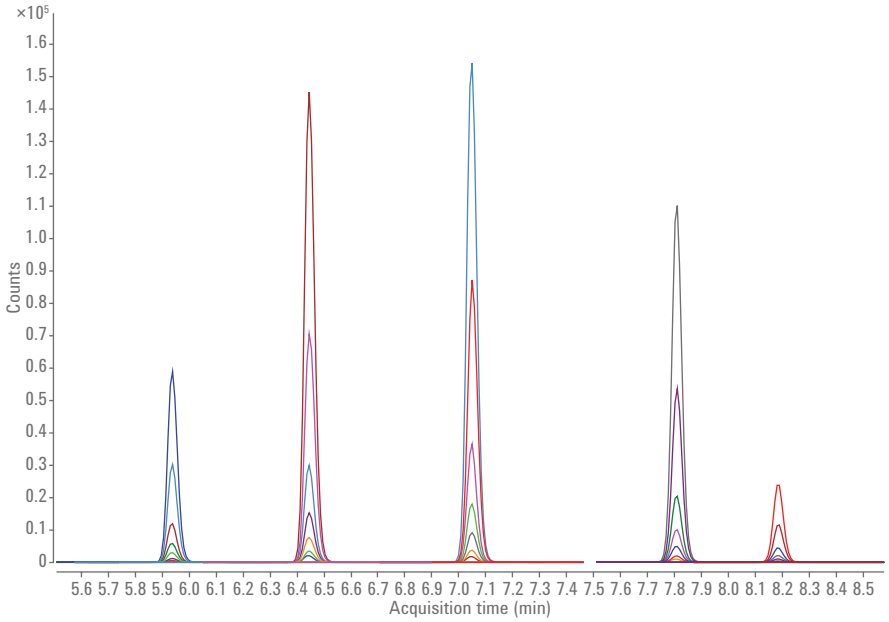

Figure 2. Overlaid chromatograms of selected pesticides from 0.05 ng/mL to 100 ng/mL

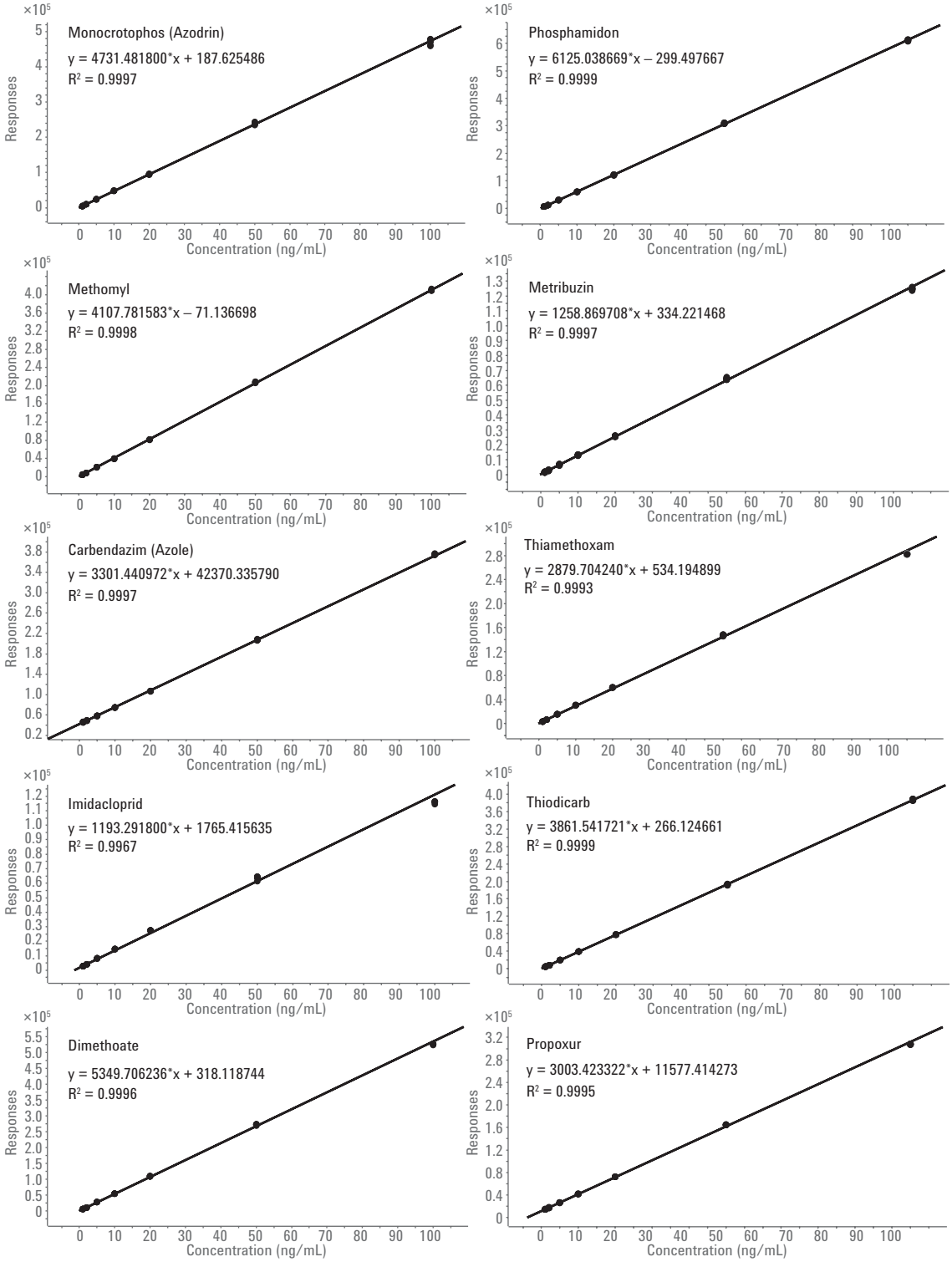

Figure 3. Matrix-matched calibration graphs of representative pesticides in this study.

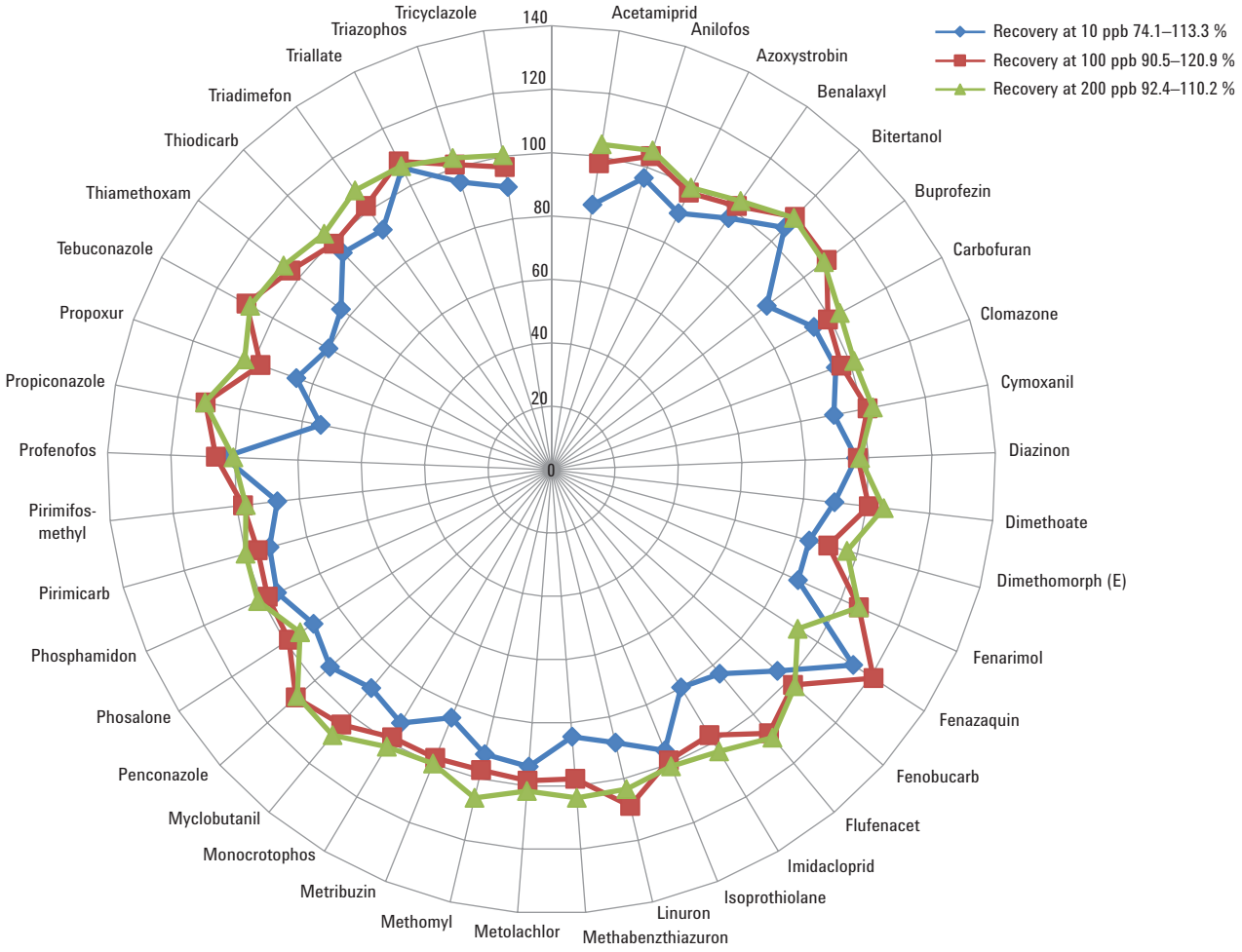

Figure 4. Radar plot of recovery of various pesticides in the study at 10, 100, and 200 ng/g spiking levels.

Repeatability

Six replicate injections of matrix-matched standard spiked at 10 ng/mL (0.1 ng/mL spiked at 1 mL of the diluted tobacco extract) concentration level were performed on a daily basis. The percentage relative standard deviation (%RSD) was calculated for the area. All 40 pesticides in this study showed excellent reproducibility, with RSD <5 %.

Sample analysis

To study the suitability of the developed method for the routine analysis of tobacco samples, five commercially available branded tobacco samples were collected from various locations of southern India, and analyzed in triplicate. Concentration calculated by quantitative software was multiplied by the dilution factor of 100 to achieve the original concentration of pesticides in the analyzed tobacco samples. Most of the analyzed pesticides had less concentration than the LOQ level in all five tobacco samples. The concentration of carbendazim was found in ppm levels in two of the samples. Azoxystrobin was detected at 0.9 ppm in sample 5. Concentrations of imidacloprid, pendimethalin, triadimefon, and tebuconazole were detected at less than 200 ng/g in many samples.

The ion ratio of the MRM transitions helps in the identification and elimination of false positives. The major challenge for this study was to obtain blank tobacco matrices. Several samples were tested, and the sample that had a lower concentration of pesticides was chosen for the spike recovery study. Analytes that were detected in the blank matrix were excluded from this study. In the case of low concentration pesticides, the peak area from nonfortified matrix extracts were subtracted from the matrix-matched standards to evaluate the corrected recovery percentage.

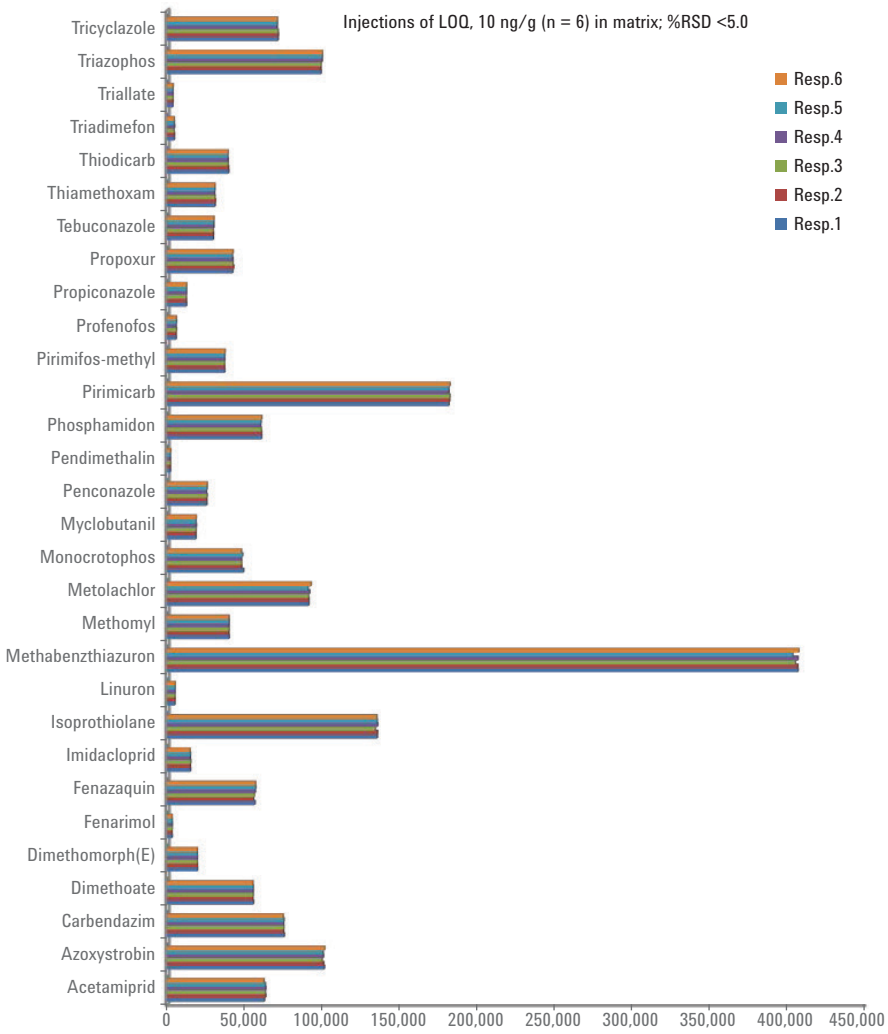

Figure 5. Bar diagram plotting response of six replicate injections, showing excellent reproducibility for area response.

Table 4. Pesticides quantified in samples (above 10 ng/g).

Pesticides detected above 10 ng/g	Concentration in sample 1 (ppb)	Concentration in sample 2 (ppb)	Concentration in sample 3 (ppb)	Concentration in sample 4 (ppb)	Concentration in sample 5 (ppb)
Carbendazim		1,097.7	4,680.7		385
Imidacloprid		270.7	348.0		121.7
Pendimethalin	130.9	205.7	252.0		
Triadimefon	201.1			39.2	
Tebuconazole		329.5	129.5		199.2
Azoxystrobin	63.2	306.6	315.4		903.6
Methomyl	19.1				
Tricyclazole			11.3		
Propoxur					22.4
Acetamiprid		89.7	28.1		39.3
Thiamethoxam					28.5
Dimethomorph	52.3			32.7	

Conclusion

The developed method allows the user to both screen and quantify 40 pesticides in tobacco samples using the Agilent 6470 triple quadrupole LC/MS/MS system. This is a fast method in terms of short run time and relatively simple sample preparation. The dynamic MRM method is efficient in reducing cycle time and enhancing the number of data points across the chromatographic peak. The developed and validated method is sensitive, linear within the specified concentration range, and reliable and reproducible for the routine analysis of pesticides in a difficult matrix such as tobacco. Dilution of samples reduces the matrix effect, resulting in lower contamination of the analytical column and the LC/MS/MS instrument. The method was successfully tested with spiked quality control samples and with real samples. Excellent recoveries, between 75 and 120 %, were obtained at a concentration level of 10 ng/g. The developed method can be adopted in routine quality control of tobacco samples.

References

1. Geiss, O.; *et al.* Tobacco, Cigarettes and Cigarette Smoke, an Overview; http://publications.jrc.ec.europa.eu/repository/bitstream/111111111/8885/1/7472%20
2. Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed. https://ec.europa.eu/food/sites/food/files/plant/docs/pesticides_mrl_guidelines_wrkdoc_11945.pdf
3. Agilent Technologies, publication nnumber 5991-4541EN, **2014**. https://www.youngin.com/upload/equipment/2014/10/1/iyqqciyumm_20141001175905118.pdf
4. Shadiah, S.; Baz. Qualitative and Quantitative analysis of some imported cigarette samples locally used in Saudi Arabia by Scanning Electron Microscope and Energy Dispersive Spectroscopy, *Journal of American Science* **2015**, *11(5)*.
5. Commission Directive 2000/42/EC of 22 June 2000 amending the Annexes to Council Directives 86/362/EEC.86/363/EEC and 90/ 642/EEC on the fixing of maximum levels for pesticide residues in and on cereals, foodstuffs of animal origin and certain products of plant origin, including fruit and vegetables respectively (Text with EEA relevance). Official Journal L 158, 30/06/2000, pp. 0051–0075.
6. Fei Yang; *et al.* Analysis of 118 Pesticides in Tobacco after Extraction With the Modified QuEChERS Method by LC–MS-MS' *Journal of Chromatographic Science* **2013**, 1–5. doi:10.1093/chromsci/bmt112
7. Lehotay, S. J.; Mastovska, K.; Lightfield, A. R. Use of buffering and other means to improve results of problematic pesticides in a fast and easy method for residue analysis of fruits and vegetables; *Journal of AOAC International* **2005**, *88*, 615–629.
8. Kwon, H.; Lehotay, S., Geis-Asteggiante, L. Variability of matrix effects in liquid and gas chromatography–mass spectrometry analysis of pesticide residues after QuEChERS sample preparation of different food crops. *Journal of Chromatography A* **2012**, *1270*, 235–245.
9. Zweigenbaum, J.; *et al.* Multi-Residue Pesticide Analysis with Dynamic Multiple Reaction Monitoring and Triple Quadrupole LC/MS/MS - Fast and Effective Method Development Using an Application Kit and a Pesticides Compound Parameter Database, *Agilent Technologies Application Note*, publication number 5990-4253EN, **2009**.
10. Xianyu Chen, V.; Zhai, A. Analysis of Multipesticide Residues in Tobacco – The Agilent Triple Quadrupole GC/MS/MS Analyzer for Pesticides in Tobacco, *Agilent Technologies Application Note*, publication number 5991-5763EN, **2015**.
11. Stone P.; *et al.* New Dynamic MRM Mode Improves Data Quality and Triple Quad Quantification in Complex Analyses, *Agilent Technologies Technical Overview*, publication number 5990-3595EN, **2009**.

www.agilent.com/chem

Published in the USA, September 1, 2017
5991-8327EN

Agilent Technologies