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Development of HPLC-Based QuEChERS Method for High Recovery of Fungicides from Vegetable Samples

Abdul Majid Shad[1], Zeeshan Ali[1], Dilshad Hussain[2], Muhammad Sajid[1]*

[1]*Institute of Chemical Sciences, Bahauddin Zakariya University, Multan, Pakistan*
[2]*HEJ Research Institute of Chemistry, International Centre for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan*

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ABSTRACT

Vegetables are the most essential part of our diet. Several fungicides are used in controlling pathogens in vegetable plants. The control of phytopathogens with fungicides may be associated with many health hazards. Therefore, the present study was conducted to develop a simple high-performance liquid chromatography (HPLC)-based Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method for the recovery of fungicides (azoxystrobin and thiophenate methyl) from vegetables. Two types of vegetables; spinach and fenugreek used for this study were crushed, homogenized, and treated with acetonitrile in a centrifuge tube. Sodium sulphate and sodium chloride were added to maintain the pH of the sample solution and acetonitrile was added to remove the matrix by a clean-out method using dispersive solid-phase extraction (dSPE). Finally, HPLC analysis was carried out to determine the test fungicides in the two vegetable samples. The results indicated that recoveries of the test fungicides were in the range of 0.63 to 107.68% at spiking levels and different factors through percentage RSD between 3.6 and 4.7%. The limit of detection (LOD) and limit of quantification (LOQ) of the two fungicides were in the range of 0.0021 to 0.00181 mg/kg and 0.0706 to 0.00606 mg/kg, respectively. In the QuEChERS method, the different concentrations of the fungicides in the spinach vegetable were obtained, but in the fenugreek vegetable, these pesticides were absent. The findings of this study have shown that the proposed method is fast, easy, and efficient for fungicide analysis with high recovery, low limit of detection, and limit of quantification.

Keywords: Fenugreek, Fungicides, HPLC, QuEChERS, Recovery, Spinach, Vegetables.

Introduction

Azoxystrobin is a wide range pesticide that is used as a fungicide for many diseases of crops with different trade names including Amistar, Abound, Quadris, Bankit, and Heritage. It works by constraining mycelial growth,[1] spore germination, and mitochondrial respiration. Although, Azoxystrobin shows fewer toxic effects for bees, birds, mammals, and other terrestrial organisms,[2] it has been found to strongly work against various diseases of numerous ornamental and edible crops. A recent study illustrates that azoxystrobin degradation product; R234886 can percolate via loamy soils for a long period following application of the pesticide. This causes a potential threat to vulnerable aquatic environments and drinking water resources.[3] Some diseases controlled or prevented by this pesticide are late blight, rice blast, septoria, rusts, apple scab, powdery, and downy mildew.[4] In testing persons given azoxystrobin at a dose level of up to 117.1 mg/kg body weight per day for nearly two years, there was no numerical proliferation in the number of animals with tumours; benign, malignant, single or multiple, or metastatic tumours.[5] Azoxystrobin also remained low sub chronic in dermal testing of twenty-one days.[5] In the study, gross swelling of the bile duct convoyed by histological alteration was also observed. The pesticide had no developmental harm in the rabbit or rat by dose level of up to and level displayed to be maternally toxic.[5]

*Corresponding author. E mail: dr.msajid@bzu.edu.pk
Tel: +923040801998

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Azoxystrobin showed a weak response of clastogenic effect *in vitro* in mammalian cells at cytotoxic dosages and in *in vivo* studies, it did not induce genotoxicity.[7] Azoxystrobin is highly vulnerable to photolysis and is a non-volatile compound. It does not leach down in soil water, as it is not present in groundwater or drinking water. Because it does not leach, it is extremely unlikely to end up in water reservoirs unless it is sprayed directly or by accident. During laboratory studies, the chemical degrades in flooded anaerobic soils with a half-life of around seven weeks. In plants, it is metabolized having a complex with more than fifteen identified metabolites. These metabolites exist in very low amounts, usually considerably fewer than 5% of the Total Recoverable Residue (TRR). Thiophenate methyl is a class of fungicide that is used for different crops and vegetables to kill different germs causing different diseases. It is a colourless crystal,[8] with a melting point of 342°F.[9] The fungicide is frugally resolvable in many organic solvents at 25°C like acetone, methanol, ethyl acetate, dichloromethane, n-octanol, xylene, and n-hexane. It can be partially dissolved in a variety of organic solvents. [10] Thiophenate methyl is strongly soluble in methanol, acetone, and chloroform.[11] Its solubility in water is 26.6 mg/L at 20°C.[12] When excited to disintegrate, it releases exact poisonous gases and oxides of nitrogen and sulphur. It is cleared from the body in 24 hours, then goes into muscles for another 24 hours before being mostly eliminated in 96 h.[13]

Determination and quantification of pesticides in crops are usually done by the QuChERS method. The process of the QuEChERS method has several steps.[14,15] This comprises sample research and abstraction step. In this step, samples are mixed regularly; then, different acidic solutions and buffers are used to enhance the efficiency of extraction.[16] This is the clean-out step of extraction of a sample using the solid-phase extraction method.[17]

The present research was conducted to develop HPLC-based QuEChERS method for the determination of azoxystrobin and

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thiophenate methyl in two vegetables (spinach and fenugreek [Methi]).

Materials and Methods

Sources of chemical materials
Sodium sulphate (99-100%), azoxystrobin (95%), thiophenate methyl (70%), acetonitrile (99.9%), sodium chloride (99.9%), magnesium sulphate (98%), and charcoal (95%) were purchased from Sigma Aldrich and MERK. All the chemicals were of analytical grade.

Sources of vegetables
Different type of two vegetables; spinach (*Capsella bursa-pastoris (L.) Medik.; Brassicaceae;* Voucher no MG005997) and fenugreek Voucher no PIIUM 0226-1 were bought from the native supermarket, Muzaffar Garh, Pakistan in January 2020. These vegetables were washed thoroughly with water and dried in sunlight.

Sample preparation
Samples of the spinach and fenugreek were kept in sunlight for six days for water evaporation. These vegetables were dried, crushed, and the powder was filtered through 25 meshes (0.4500 mm or 0.0177 inch), and placed in a distinct envelope. The powdered vegetables were each weighed (1 g) and placed into a 15 mL centrifuge tube and saturated with 4 mL of deionized water for 2 min. Various working solutions of 7 ml were prepared, agitated, and vortexed strongly for 3 min. Then, analytical grade Acetonitrile (7 mL) was added into each centrifuge tube and further agitated for 3 min[18]. Each tube was filled with 1 g sodium chloride and 4 g anhydrous Na_2SO_4 and shaken for 3 minutes. The preparations were centrifuged in a TGL-16 high-speed centrifugal device at 4000 rpm for 5 min. The supernatant was discarded and 0.05 g activated carbon and 0.150 g Na_2SO_4 were added. This was followed by another round of centrifugation at 4000 rpm for 7 min. Acetonitrile organic layer was strained by Whatman filter paper and HPLC analysis was carried out. Dried vegetable 1g (spinach or fenugreek) was placed in a 15 mL tube; 4 mL of deionized water were added and saturated for 2 min. Then, 10 mL of acetonitrile were added and agitated strongly for 3 min through vortex mixing. Anhydrous Na_2SO_4 (4g) and sodium chloride (1g) were added to each centrifuge tube and agitated strongly for 3 min. Another round of centrifugation for 5 min at 4000 rpm was performed and the upper organic acetonitrile layer was removed and placed into a separate tube. Then, 0.05 g activated charcoal and 0.15 g Na_2SO_4 were added. This was followed by agitation and centrifugation at 4000 rpm. Finally, HPLC analysis was carried out. [19]

HPLC analysis
Series 1500 Lab Alliance C18 Agilent 5 HPLC was used for the analysis of fungicide residues in test samples. Reverse phase C18 column of length and width, 150 and 4.6 mm respectively, was used for the analysis. The mobile phase of the sample was a combination of the organic phase (acetonitrile) and water at a ratio of 80:20. The flow rate was set at 1 mL/min. UV -visible Lab Lines sensor Model No. 8266-9868 was used as a detector in the HPLC, which was fixed at 245 nm. Relative standard deviation RSD, limit of detection LOD, limit of quantification LOQ and percentage recovery of the two fungicides was calculated through HPLC.

Statistical analysis
The comparison of two different fungicides gave different values for Relative standard deviation RSD, limit of detection LOD, limit of quantification LOQ. RSD of azoxystrobin was 3.6%, LOD 0.00211, and LOQ was 0.0706. correlation coefficient (R^2) of azoxystrobin and thiophenate methyl were obtained with a value of 0.9990 and 0.9998 respectively Same experiment was repeated for the thiophenate methyl and values were 4.7%, 0.00181, and 0.00606 respectively. Fenugreek vegetable showed maximum recovery for both fungicides as compare to spinach given in table 2.

Results and Discussion

Spinach and fenugreek two different types of two vegetables were studied by the HPLC-based QuEChERS method. Firstly, blank solutions of both vegetables were analyzed by the HPLC with different concentrations of standard solutions of the fungicides (Figure 1). The concentration of Thiophenate and Azoxystrobin in the blank solutions of spinach was 8.84 mg/Kg and 6.96 mg/Kg, respectively. On the other hand, both pesticides were absent in the blank solution of fenugreek (Methi). The results showed that different fungicides are assimilated in vegetables or accumulated in the soils. The results of the solutions of the various concentrations (10, 20, 30, 40, and 50 mg/L) of azoxystrobin and thiophenate methyl analyzed by HPLC are highlighted in Figure 2. Peaks under the area of the two fungicides were determined and calibration curves for both fungicide standards were plotted (Figure 3). A straight-line graph for azoxystrobin and thiophenate methyl were obtained with a correlation coefficient (R^2) of 0.9990 and 0.9998 respectively. After a separate analysis of each standard solution of both pesticides, a mixture of each concentration was analyzed to estimate the effect of one pesticide on the other. The values of Relative Standard Deviation RSD, Limit of Detection LOD, and Limit of Quantification LOQ obtained for azoxystrobin were 3.6%, 0.00211, and 0.0706, respectively. Similarly, the RSD, LOD, and LOQ for the thiophenate methyl were 4.7%, 0.00181, and 0.00606 respectively. The detailed information is given in Table 1.

QuChERS method validation
The removal of fungicides and method authentication is based on the QuChERS method which is a very simple and easy method to determine the fungicide residue in vegetables without internal standards. Different concentrations such as 50, 75, and 100 mg/L of both pesticides were spiked in the vegetable samples. Acetonitrile was used as a solvent for the extraction of the fungicides, while water was used for the soaking of the samples.

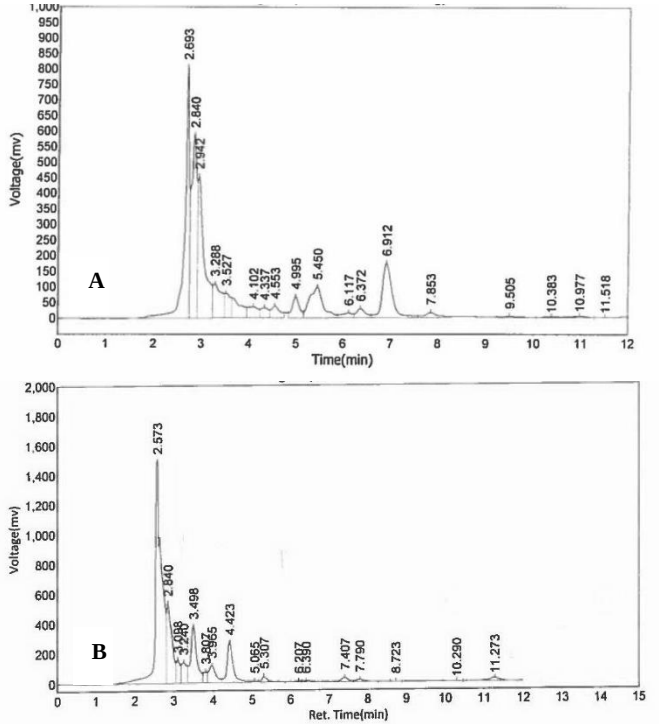

Figure 1: HPLC chromatograms of blank solutions of spinach (A) and fenugreek (B).

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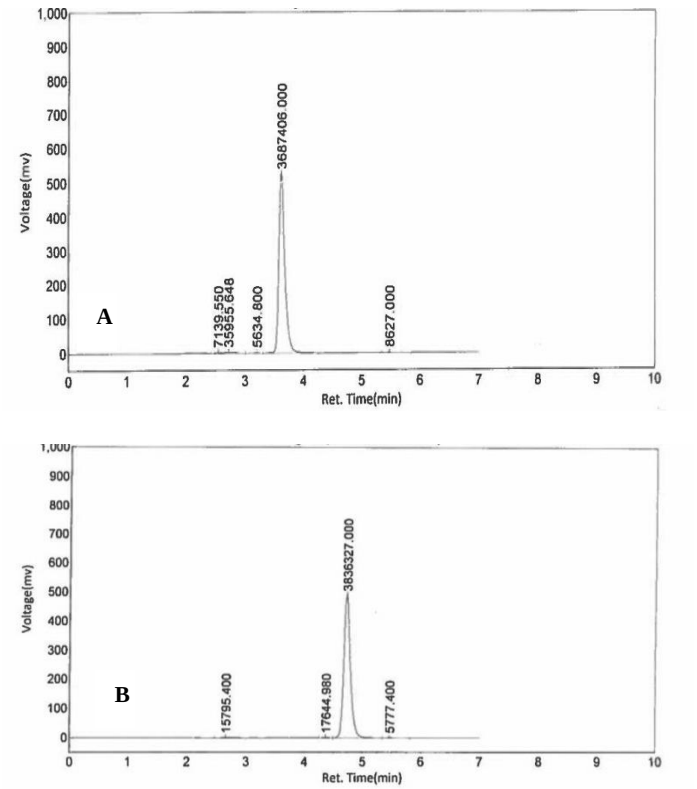

Figure 2: HPLC chromatograms of standard solutions of thiophenate methyl (A) and Azoxystrobin (B).

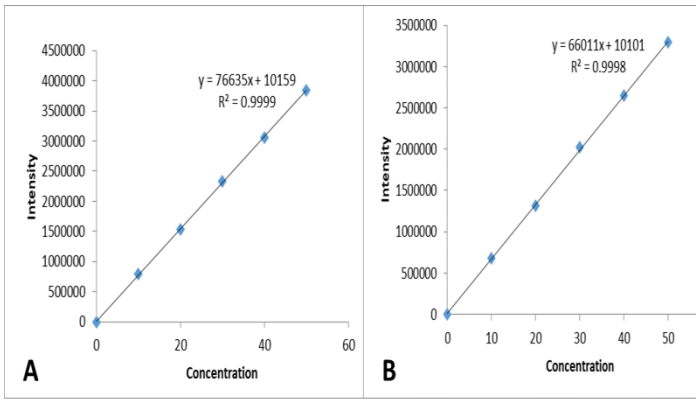

Figure 3: Calibration curves of thiophenate methyl (A) and azoxystrobin (B).

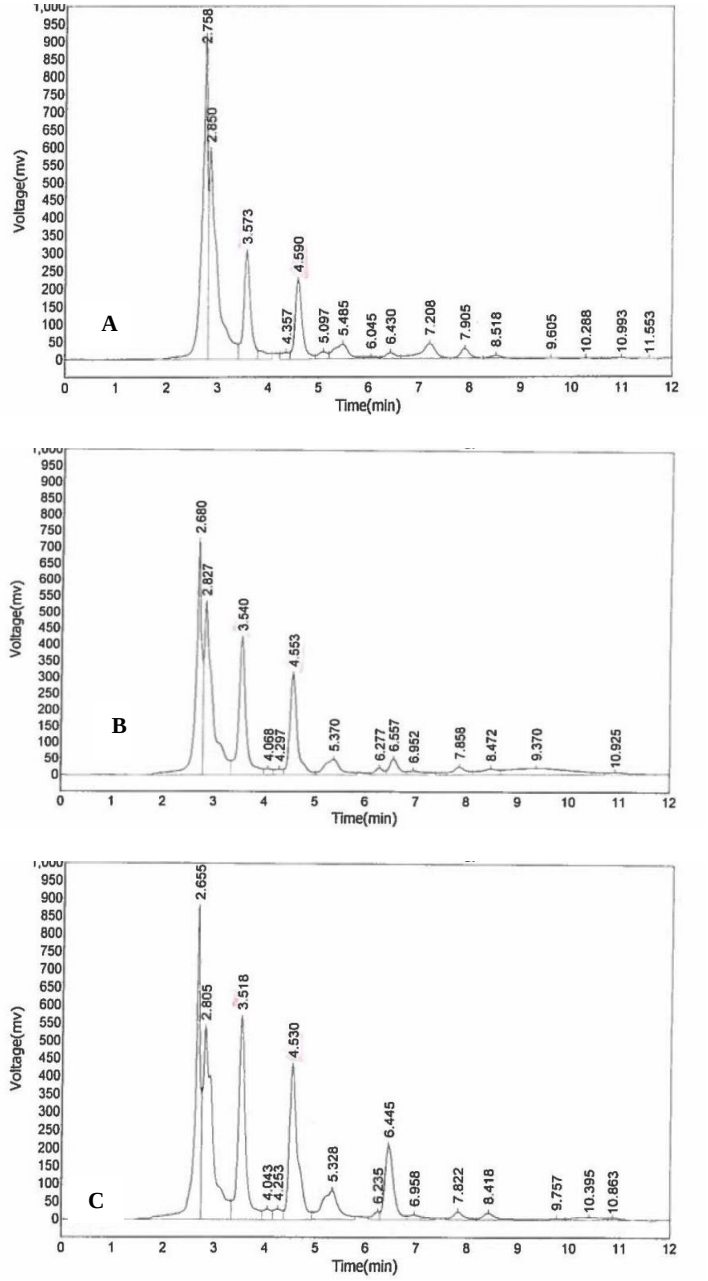

Figure 4: Chromatograms of azoxystrobin and thiophenate methyl in spinach at 50 mgL^{-1} (A); 75 mgL^{-1} (B); and 100 mgL^{-1} (C).

Table 1: Correlation coefficient (R^2), RSD, LOD and LOQ of test fungicides

Compound	R^2	RSD	Intercept of Y	Slop	LOD	LOQ
Azoxystrobin	0.9990	3.6 %	10101	66011	0.00211	0.0706
Thiophenate Methyl	0.9998	4.7 %	10159	76635	0.00181	0.00606

Chromatograms showing the retention times and peak areas for thiophenate methyl and azoxystrobin at 50, 75, and 100 mg/L spiking concentrations in spinach and fenugreek are shown in Figures 4 and 5, respectively. The extraction efficiency of the method for both pesticides is given in Table 2. Recovery (%) of both pesticides ranges from 63.98 to 107.68% at different spiked concentrations. Percentage recoveries of azoxystrobin in spinach and fenugreek samples were 65.38 and 87.56, respectively in the 50 mgL^{-1} spiking solution. Meanwhile, the percentage recoveries of thiophenate methyl in fenugreek and spinach were 107.68 and 73.36, respectively which shows positive results. Percentage recoveries of thiophenate methyl and azoxystrobin in spinach were 73.82 and 63.98, while in the fenugreek, values of 77.32 and 92.42 were obtained in the 75 mgL^{-1} spiking solution which was higher in values. Recovery % of azoxystrobin in spinach (75) and fenugreek (65.42), as well as that of thiophenate methyl in fenugreek (74.74) and spinach (72.45), showed the best results by spiking different concentrations of fungicides in the different vegetables. These results indicate that excellent recoveries were obtained for both pesticides in the vegetable samples using the HPLC-based QuChERS method.

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Table 2: Percentage recovery of test fungicides in vegetables at 50, 75, and 100 mgL^{-1} spiking levels

Pesticide	Vegetable	Peak area	Rt (min)	Conc. after Extraction (mg/kg)	Recovery %
Azoxystrobin	Spinach	2168622	4.590	32.69	65.38
	Fenugreek	2900330.500	4.423	43.78	87.56
Thiophenate Methyl	spinach	2821241.250	3.573	36.68	73.36
	Fenugreek	4136603.750	3.498	53.84	107.68
Azoxystrobin	Spinach	3178465.750	4.553	47.99	63.98
	Fenugreek	3838471	4.432	57.99	77.32
Thiophenate methyl	Spinach	4254090.500	3.540	55.37	73.82
	Fenugreek	5323110	3.507	69.32	92.42
Azoxystrobin	Spinach	4961264	4.530	75.00	75
	Fenugreek	4329061.500	4.457	65.42	65.42
Thiophenate methyl	Spinach	5562824.500	3.518	72.45	72.45
	Fenugreek	5738472	3.532	74.74	74.74

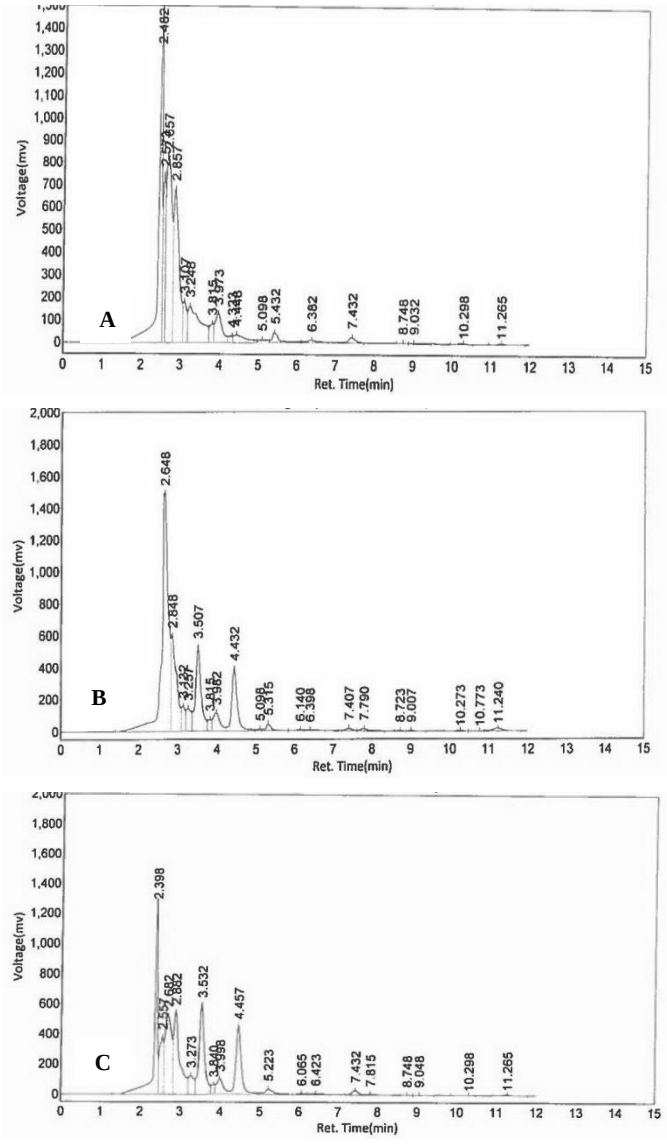

Figure 5: Chromatograms of azoxystrobin and thiophenate methyl in fenugreek at 50 mgL^{-1} (A); 75 mgL^{-1} (B); and 100 mgL^{-1} (C).

Conclusion

HPLC-based QuEChERS method for the determination of azoxystrobin and thiophenate methyl has been developed in two vegetables (spinach and fenugreek [Methi]). The findings of this study revealed that the developed scheme is an extremely quick, susceptible, and easy process for extraction of fungicide residues as compare to previously reported methods. Pesticides such as azoxystrobin and thiophenate methyl were extracted from the spinach and fenugreek samples. This extraction of pesticides by QuChERS method proved that developed scheme is simply precise, extra well-organized, cost effective, green and time saving. Also, the method is very sensitive in the extraction of the two pesticides in the different vegetables up to the level of mg/L.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

References

1. Reuveni M and Sheglov D. Effects of azoxystrobin, difenoconazole, polyoxin B (polar) and trifloxystrobin on germination and growth of Alternaria alternata and decay in red delicious apple fruit. Crop Protection. 2002; 21(10):951-955.
2. Bartlett DW, Clough JM, Godwin JR, Hall AA, Hamer M, Parr-Dobrzanski B. The strobilurin fungicides. Pest Manag Sci. 2002; 58(7):649-662.
3. Jørgensen LF, Kjær J, Olsen P, Rosenbom AE. Leaching of azoxystrobin and its degradation product R234886 from Danish agricultural field sites. Chemosphere. 2012; 88(5):554-562.
4. Špalková M, Beňová K, Falis M, Žďárský M, Renčko A. Study of interactions of selected pesticides, cadmium and low doses of ionizing radiation by means of a biotest on *Artemia franciscana*. Anim Protec Welfare. 2012; 3(10):167.
5. Kay P, Armstrong A, McDonald A, Parsons D, Best J, Peakall J, Walker A, Foulger M, Gledhill S, Tillotson M. A pilot study of the efficacy of residuum lodges for managing

sediment delivery to impoundment reservoirs. Water Environ J. 2009; 23(1):52-62.
6. Zafar MI, Belgers JD, Van Wijngaarden RP, Matser A, Van den Brink PJ. Ecological impacts of time-variable exposure regimes to the fungicide azoxystrobin on freshwater communities in outdoor microcosms. Ecotoxicology. 2012; 21(4):1024-1038.
7. Knight AW, Little S, Houck K, Dix D, Judson R, Richard A, McCarroll N, Akerman G, Yang C, Birrell L, Walmsley RM. Evaluation of high-throughput genotoxicity assays used in profiling the US EPA ToxCast™ chemicals. Regu Tox Pharm. 2009; 55(2):188-199.
8. Yang Y, Kung SC, Taggart DK, Xiang C, Yang F, Brown MA, Kruse TJ, Hemminger JC, Penner RM. Synthesis of PbTe nanowire arrays using lithographically patterned nanowire electrodeposition. Nano Lett. 2008; 8(8):2447-251.
9. Ray DE, Hayes WJ, Laws ER. Handbook of pesticide toxicology. Classes of Pesticides. 1991; 2:585.
10. O'Neil MJ, Smith A, Heckelman PE. The merck index: An encyclopedia of chemicals. Drugs Biol. 2006; 18(8):13.
11. Li J, Hu J, Sheng G, Zhao G, Huang Q. Effect of pH, ionic strength, foreign ions and temperature on the adsorption of Cu (II) from aqueous solution to GMZ bentonite. J Coll & Surf A: Physicochem Asp. 2009; 349(1-3):195-201.
12. González-Curbelo MÁ, Lehotay SJ, Hernández-Borges J, Rodríguez-Delgado MÁ. Use of ammonium formate in QuEChERS for high-throughput analysis of pesticides in food by fast, low-pressure gas chromatography and liquid chromatography tandem mass spectrometry. J Chromatogr. 2014; 1358(9):75-84.
13. Restrepo AR, Ortiz AF, Ossa DE, Mesa GA. QuEChERS GC–MS validation and monitoring of pesticide residues in different foods in the tomato classification group. Food Chem. 2014; 158(9):153-161.
14. Melo A, Mansilha C, Pinho O, Ferreira IM. Analysis of pesticides in tomato combining QuEChERS and dispersive liquid–liquid microextraction followed by high-performance liquid chromatography. Food Anal Meth. 2013; 6(2):559-568.
15. Ferreira I, Fernandes JO, Cunha SC. Optimization and validation of a method based in a QuEChERS procedure and gas chromatography–mass spectrometry for the determination of multi-mycotoxins in popcorn. Food Contr. 2012; 27(1):188-193.
16. Xu R, Wu J, Liu Y, Zhao R, Chen B, Yang M. and Chen J. Analysis of pesticide residues using the Quick Easy Cheap Effective Rugged and Safe (QuEChERS) pesticide multiresidue method in traditional Chinese medicine by gas chromatography with electron capture detection. Chemosphere. 2011; 84(7):908-912.
17. Stubbings G, Bigwood T. The development and validation of a multiclass liquid chromatography tandem mass spectrometry (LC–MS/MS) procedure for the determination of veterinary drug residues in animal tissue using a QuEChERS (QUick, Easy, CHeap, Effective, Rugged and Safe) approach. Anal Chimica Acta. 2009; 637(1-2):68-78.
18. Manousi N, Zachariadis GA, Deliyanni EA, Samanidou VF. Applications of metal-organic frameworks in food sample preparation. Molecules. 2018; 23(11):2896.
19. Dušek M, Jandovská V, Olšovská J. Analysis of multiresidue pesticides in dried hops by LC–MS/MS using QuEChERS extraction together with dSPE clean-up. J Inst Brewing. 2018; 124(3):222-229.