ISSN 1313 - 8820 (print)
ISSN 1314 - 412X (online)
Volume 10, Number 1
March 2018

AGRICULTURAL SCIENCE AND TECHNOLOGY

2018

An International Journal Published by Faculty of Agriculture,
Trakia University, Stara Zagora, Bulgaria

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The journal is accepted to be indexed with the support of a project № BG051PO001-3.3.05-0001 "Science and business" financed by Operational Programme "Human Resources Development" of EU. The title has been suggested to be included in SCOPUS (Elsevier) and Electronic Journals Submission Form (Thomson Reuters).

This issue is printed with the financial support by Contract No. DNP 06-41/20.12.2017, financed from Fund 'Scientific Research' grant Bulgarian scientific periodicals.

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AGRICULTURAL SCIENCE AND TECHNOLOGY, VOL. 10, No 1, pp 63 - 66, 2018
DOI: 10.15547/ast.2018.01.015

Product Quality and Safety

Residue analysis of difenoconazole in apple fruits grown in Republic of Macedonia

V. Jankuloska[1]*, I. Karov[2], G. Pavlovska[1]

[1]Faculty of Technology and Technical Sciences - Veles, "St. Kliment Ohridski" University - Bitola, Dimitar Vlahov 57, 1400 Veles, Republic of Macedonia
[2]Faculty of Agriculture, University Goce Delchev, 2400 Shtip, Republic of Macedonia

(Manuscript received 18 July 2017; accepted for publication 11 November 2017)

Abstract. *The purpose of this research was to determine the residue analysis of difenoconazole in apple fruits from Resen region, Republic of Macedonia. Analyzed were two varieties of apples, Golden Delicious and Idared. Samples were taken from two different locations in Resen in the year 2016. Difenoconazole analysis was performed with liquid chromatography-tandem mass spectrometry (LC-MS/MS) after a previous extraction of residue by applying the QuEChERS method. The obtained concentrations of residues of difenoconazole are compared with the maximum residue limit regulated by the Macedonian legislation. Analyses showed that the concentration of difenoconazole in both varieties of apples from two different locations is in the range of 0.01 to 0.41 mg/kg. In certain development phase of the apple fruits the concentration reaches 80 times lower concentration than the maximum limit. The data show that apple fruits can be safely consumed according to the recommended maximum residue limit (MRL) for difenoconazole in apples (0.8 mg/kg). Statistical processing of the data suggests that there is no significant difference between samples but presence of difenoconazole in apple fruits from both locations is with significant difference.*

Keywords: Golden delicious, Idared, pesticides, difenoconazole, liquid chromatography-tandem mass spectrometry

Introduction

Fruits are one of the supplementary sources of carbohydrates, vitamins, minerals, antioxidants and other biologically active components (Lozowicka et al., 2013). Apple fruits are the most important deciduous fruit consumed during a year in fresh form and various types of many products (Jankuloska et al., 2017). Domestic consumption of apple fruits is 12 kg per citizen per year (State statistical office, 2015). Depending on weather conditions in the year, apple fruits are treated at least 10 to 15 times with pesticides. Pesticides are chemical substances which are commonly used in modern agriculture practices to protect the crops from different pests and diseases (Guler et al., 2010; Lozowicka et al., 2013). For treating the crops permitted active substances are used (Official Gazette of the Republic of Macedonia, 2013). Unfortunately, not all farmers follow legal practices with pesticides during production (Lozowicka et al., 2013).

Extensive use of pesticides in modern agriculture to combat plant pests has begun to receive more attention because pesticide residues in food commodities may be hazardous to human health (Mansour, 2004). As a result of treatment with pesticides, apple fruits can be a potential source of toxic substances such as pesticides.

Residues of pesticides have to be monitored and controlled. Maximum residues limits of certain substances (pesticides, mycotoxins, heavy metals and other contaminants) in the Republic of Macedonia are presented in the Regulation on general requirements for food safety (Official Gazette of the Republic of Macedonia, 2013). A maximum residue limit is only accepted if it is guaranteed that the concentration does not have any harmful effects on human health according to the latest scientific findings available. If these maximum residue limits are complied with, then the products are considered "safe" within the meaning of consumer health protection (Lukassowitz, 2008).

Difenoconazole (Cis, Trans-3-chloro-4-[methyl-2(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl] phenyl 4-chlorophenyl ether) is a broad-spectrum fungicide being used for pathogen control in many fruits, vegetables, cereals and other field crops (NRS, 2006). Difenoconazole acts by inhibition of demethylation during ergosterol synthesis (Nasr et al., 2007). Difenoconazole is a fungicide that inhibits sterol demethylation and is widely used against *Ascomycetes*, *Basidiomycetes* and *Deuteromycetes* families (Filimon et al., 2015). It acts as a seed treatment, foliar spray and systemic fungicide. It penetrates through the surface of the infected plant and is translocated to all parts of the plant. It has a curative effect and a preventative effect (Ellen et al., 1997).

The liquid chromatography-tandem mass spectrometry (LC-MS/MS) is one of the most powerful techniques for the analysis of pesticides in a variety of complex matrices. In particular, it has been shown to be very sensitive on determination of pesticide residues in fruits and fruit juices (Grujic et al., 2005; Sagratini et al., 2007; Radisic et al., 2009).

The use of the multiresidue method of analysis is generally preferred for reducing the analysis time and cost, especially when the pesticide application history is not known (Radisic et al., 2009). The Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method, is a simple sample preparation methodology for pesticide multi-residue analysis that was first reported in 2003 (Anastassiades et al., 2003; Sinha et al., 2012). The QuEChERS technique was used for isolation of pesticides from the samples (Sinha et al., 2012). The QuEChERS method uses acetonitrile, with the application of adequate combination of salts, dinatrium hydrogen citrate sesquihydrate, water-free magnesium sulfate and sodium chloride

* e-mail: vezirka.jankuloska@gmail.com

with the purification procedure by primary-secondary amine (PSA) with the addition of water-free magnesium sulfate which results in a better separation of phases without dilution (Anastassiades et al., 2003; Vukovic et al., 2012).

Student's t-test deals with the problems associated with inference based on “small” samples. This data can be used to determinate if the averages of two samples are significantly different (Newbold et al., 2007; Levine et al., 2008).

Fruit consumption has increased worldwide because people have become more health conscious. Although the analysis of pesticide in food samples has been documented (Shermerhorn et al., 2005; Marin et al., 2012; Abdallah et al., 2014) there is little information regarding the analysis of difenoconazole in fresh apple but there are literary data about difenoconazole analyzed by liquid chromatography with electrospray ionization in tomatoes and its concentration is 0.01 mg/kg (Sannino et al., 2004).

The aim of our research was to determine the amount of difenoconazole in two varieties of apple fruits from different locations. From the obtained results it will be determined whether the concentrations of pesticide residue in both varieties of apples from different locations in Resen region are in accordance with Macedonian regulations and whether Resen apples are safe for consumption.

Material and methods

Samples for analysis (apple fruits) are taken from two locations (Evla and Kriveni villages) of Resen region in 2016. Apple varieties Golden Delicious and Idared were analyzed. Sampling from the locations was performed at random from the orchards. Samples were collected in four developmental phases of apples:

- I - phase, apple at the size of hazelnuts;
- II – phase apple at the size of a walnut;
- III - phase, early ripening of apple;
- IV – phase, during the apple harvest.

The mass of samples for analysis was 1 kg (for sampling). Fresh apples with peel and mesocapr without treatment (e.g. washing with water) were analyzed. Analyses were performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) after a previous extraction residue by applying the QuEChERS method.

The homogenized sample of apple fruits with weight of 10 g is transferred at 50 ml centrifuge tube. Add 10 ml of acetonitrile and e.g. 100 µl of the internal standard solution. Shake vigorously for 1 min. This is the first extraction step. The use of acetonitrile in QuEChERS had many advantages, including its ability to separate from water upon the addition of salt the need to employ a nonpolar solvent and good compatibility with LC applications (Sinha et al., 2012). After extraction on a vortex mixer for 1 minute, 4 g of magnesium sulfate anhydrous (MgSO4), 1 g of sodium chloride (NaCl), 1 g of trisodium citrate dehydrate ($C_6H_5Na_3O_7$. 2 H_2O) and 0.5 g of disodium hydrogen citrate sesquihydrate ($C_6H_8Na_2O_8$) were added and the mixture was shaken for 1 min and centrifuged for 5 minutes at 3000 rpm. This is the second extraction with phase separation. After centrifugation, 1 ml of the extracts was transferred into a clean-up tube containing 900 mg of $MgSO_4$ and 150 mg of primary-secondary amine (PSA). Shake for 30 sec. Centrifuge for 5 minutes at 4500 rpm. The cleaned and acidified extracts are transferred into auto sampler vials to be used for the multi-residue determination.

Analyses were carried out with a liquid chromatograph (Agilent 6420) with triple quadrupole mass spectrometry (LC-MS/MS). The statistical processing of date was made Student's t-test which is the most often used parametric test of significance testing a statistical significant difference between two arithmetic averages. The statistical difference in the samples is determined by the p-value.

Results and discussion

The results of determination the difenoconazole in apple fruits from different location are shown on Figure 1.

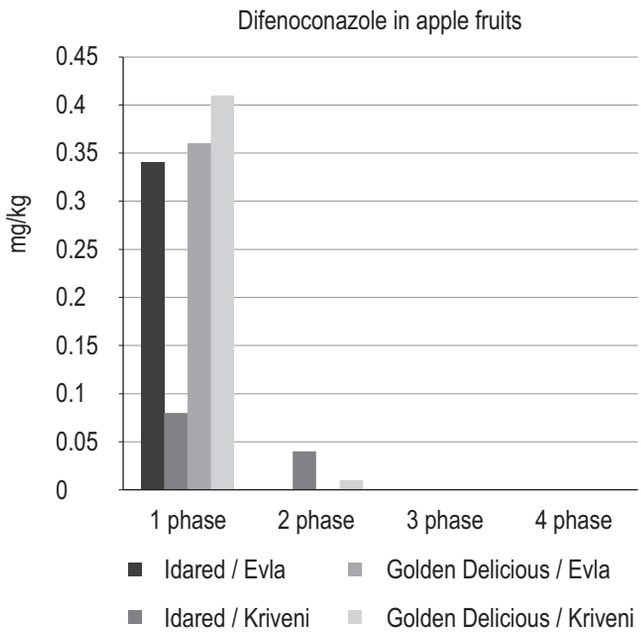

Figure 1. Difenoconazole in apple fruits from different location

Difenoconazole is the most present in the first phase (apple at the size of hazelnuts) and second phase (apple at the size of a walnut). While in the stages before maturing and during the harvest of apples it is not detected or is found at a concentration below the level of detection. It may be noted that in the first phase difenoconazole is detected in Golden Delicious and Idared from both locations. While in the second phase it is detected only in apples from Kriveni (in both varieties of apples) and not in apples from Evla (Figure 1).

In order to see if the difenoconazole in the analysed fruit is within the limits, the obtained values are compared with the maximum residue limits (MRL) according to the legislation of the Republic of Macedonia (Figures 2, 3, 4 and 5). The maximum residue limit (MRL) of difenoconazole in apple fruits is 0.8 mg/kg.

From Figure 2 it can be seen that the concentration of difenoconazole in Idared from Evla is 0.34 mg/kg and it is 2.5 times smaller than the maximum residue limit (0.8 mg/kg).

Difenoconazole in Idared from Kriveni in the first phase is 0.08 mg/kg and is 10 times lower than the limit and in the second phase it is 0.04 mg/kg and 20 times lower than the limit. In the third and fourth phase it was not detected (Figure 3).

In Golden Delicious from Evla the concentration of difenoconazole is 2.2 times lower than the maximum residue limit (Figure 4).

In Golden Delicious form Kriveni difenoconazole is represented in the first phase with concentration 1.95 times lower than the limit

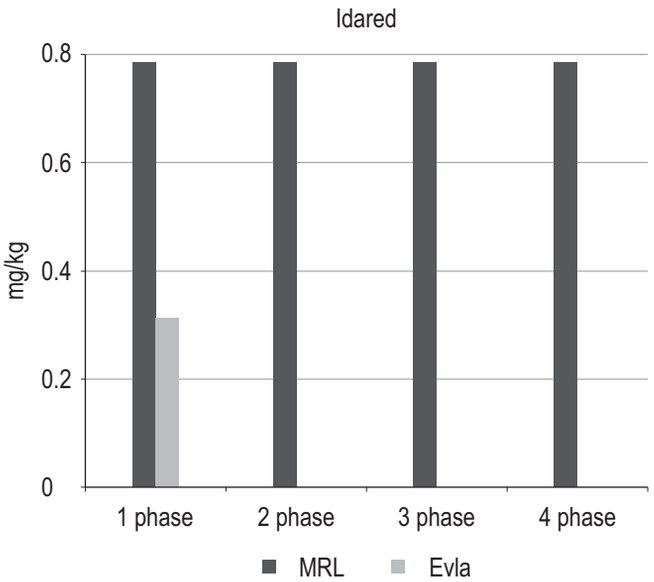

Figure 2. Difenoconazole in Idared from location Evla and maximum residue limits (MRL)

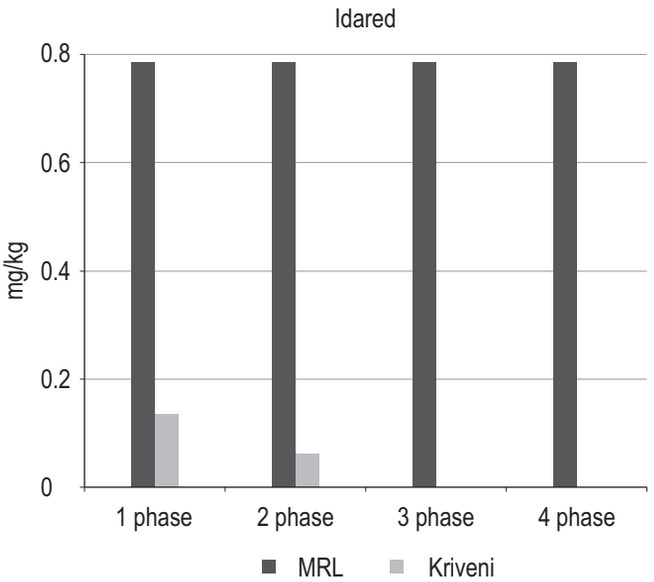

Figure 3. Difenoconazole in Idared from location Kriveni and maximum residue limits (MRL)

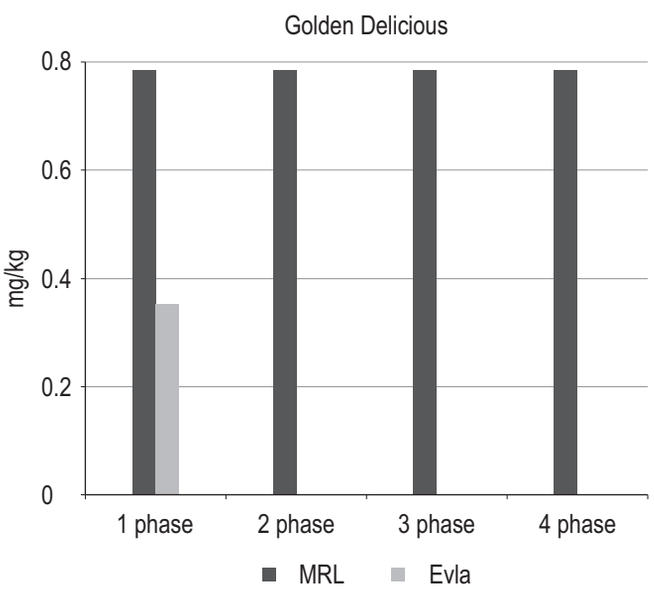

Figure 4. Difenoconazole in Golden Delicious from Evla and maximum residue limits (MRL)

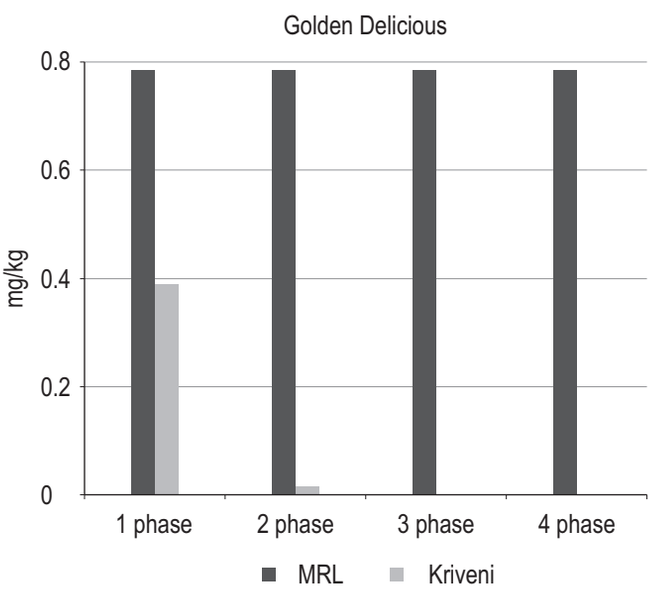

Figure 5. Difenoconazole in Golden Delicious from Kriveni and maximum residue limits (MRL)

and in the second phase it is present with the lowest concentration of 0.01 mg/kg and 80 times lower than the limit. In the phase before and during the harvest of apples it was not detected (Figure 5).

The mean difenoconazole concentration values from Idared is 0.085 mg/kg for Evla village and 0.03 mg/kg for Kriveni village. The mean value from Golden delicious is 0.09 mg/kg for Evla village and 0.105 mg/kg for Kriveni village.

The obtained t-test value for Idared from both locations is 0.483536 while for Golden Delicious from both locations it is 0.296689. Because of the values which are less than the critical t-value, that means that there is a significant difference in the presence of difenoconazole in apple fruits from both locations. The calculated p-value for Idared is 0.630237 and 0.725295 for Golden Delicious. In both varieties of apples p-values are greater than the critical p-value. That means the difference between the samples is not statistically significant.

Conclusion

After the analysis of two varieties of apple fruits (Golden Delicious and Idared) from Resen region in the Republic of Macedonia, it can be concluded that the concentration of difenoconazole is in the range from 0.01 to 0.41 mg/kg. Difenoconazole is most present in the phases of apple in the size of hazelnuts and apple in the size of a walnut while in the other two developmental phases (phases of apple before ripening and during harvesting) it is not detected or it is at a concentration which is below the level of detection (0.01 mg/kg). Difenoconazole is detected in the first phase in two varieties of apple fruits from both locations (Evla and Kriveni), while in the second phase it is detected in Golden delicious and Idared from the location of Kriveni and not in apple fruits from location Evla. From the statistical calculations and analysis above, we can make the conclusion that the difference

between the samples from both locations is not statistically significant. The presence of difenoconazole in apple fruits from both locations is with significant difference. The data show that apple fruits could be safely consumed according to the recommended maximum residue limit (MRL) for difenoconazole in apple fruits (0.8 mg/kg).

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Mauff G, Pulverer G, Operkuch W, Hummel K and Hidden C, 1995. C3-variants and diverse phenotypes of unconverted and converted C3. In: Provides of the Biological Fluids (ed. H. Peters), vol. 22, 143-165, Pergamon Press. Oxford, UK.

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Volume 10, Number 1
March 2018

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