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ANALYSIS OF TRIFLOXYSTROBIN IN GOLDEN DELICIOUS AND IDARET BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (LC/MS/MS)

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Abstract

Apple is the most common crop cultivated in Resen region, Republic of Macedonia. In this region, apple powdery mildew caused by *Podosphaera leucotricha* (Ellis & Everh.) E. S. Salmon, and apple scab (caused by *Venturia inaequalis* (Cooke) G. Wint) are important pests in apple production. Trifloxystrobin is fungicide which acts against these pests and improves the yield of agricultural crops.

We analyzed the presence of trifloxystrobin in two varieties of apples: Golden Delicious and Idaret from two different locations: Evla and Kriveni in Resen region, Macedonia. Fungicide was analyzed in four apple development phases, or from apple of the size a hazelnut, to the phase of harvest. Trifloxystrobin is analyzed by liquid chromatography-tandem mass spectrometry (LC-MS / MS) followed by an extraction/separation treatment with acetonitrile and the dispersive SPE - QuEChERS - method. Obtained values are compared with the maximum residues levels (MRL) according to the legislation of the Republic of Macedonia. We made a statistical processing of the data with Student's t-test.

The analysis revealed that trifloxystrobin is present in Golden Delicious on both locations with a concentration within the range of 0.04 to 0.14 mg/kg, but mainly on Kriveni location. In the Idaret from Kriveni, it is detected only in early phase of apple maturing, while in Evla there is none or is below the detection limit. The value of t-test for Golden Delicious and Idaret from both locations are 1,946173039 and 1. They are lower than the critical t-value. That means that there is a significant difference in the presence of trifloxystrobin in apples from Kriveni and Evla.

After comparing the obtained values with the MRL it can be concluded that apples from the two locations are safe for consumption.

***Key words**: Trifloxystrobin, Fungicides, Apples, Golden Delicious, Idaret.*

1. Introduction

Fruits and vegetables are important components of human diet as they are rich in proteins, carbohydrates, minerals, vitamins and fibers. The benefits of fruits and vegetables are often to their high antioxidant content. Research supports a role of secondary plant metabolites particularly polyphenols in the prevention of degenerative diseases e.g. cardiovascular diseases and cancer. Apple fruit are an important source of secondary plant metabolites and one of the major phenol sources being consumed during the whole year [1]. Its fruits can be frozen or used for various types of products (fresh fruits, juice concentrate, apple acid and etc.) [2].

Apple is the best known and most important fruit growing species from temperate climate. The importance becomes from the: culture age, area and obtained production, food, dieted value, and therapeutic value of fruit, yield and suitability to the various culture technologies of varieties [3].

Apple is the most common crop cultivated in Resen region, Republic of Macedonia. In Resen, the number of fruit trees is 2,458,300, out of which fruitful trees are 2.256,800 while apple production is 90,450 tons of apples or 40 kg of apples per tree [4]. In Resen are grown the following varieties of apples: Idaret, Golden Delicious, Mutcu, Red Delicious, Jonagold, Granny Smith, Fuji, Gala and Rubistar [5].

In this region, apple powdery mildew (caused by *Podosphaera leucotricha* (Ellis & Everh.) E. S. Salmon and apple scab (caused by *Venturia inaequalis* (Cooke) G.

Wint, are important diseases in apple production. Apple scab is the most economically important disease in apple (*Malus 9* x *domestica* Borkh) worldwide. *Podosphaera leucotricha* (Ellis & Everh.) E. S. Salmon is the causal agent of apple powdery mildew, one of commercially the most important fungal diseases in apple. Its occurrence is rather regular and, in susceptible cultivars (Jonatan, Idared) it: causes leaf necrosis and drying, decelerates growth and reduces the formation of buds. Apple powdery mildew is spread in all apple-growing regions throughout the world [6, 7].

Crop losses result directly from fruit infection and indirectly from repeated defoliation, which can reduce tree growth and yield [8, 9, and 10]. Economic damage includes: yield loss, deteriorated fruit quality, reduced ability to bear fruit over the following season and increased susceptibility to frost.

Successful apples growing is not possible without intensive application of chemical fungicides. In order to keep trees scab-free during the growing season, more than 20 treatments may be needed [11, 12]. Chemical control of pests and diseases represents a considerable part of biotechnological resources necessary to protect apple orchards and maintain profitable apple production [13].

This major disease is managed in apple orchards by integrating several practices, but fungicidal control is the most widely practice used [8, 9, and 10]. Such a large number of treatments with different or the same fungicides often results in toxic effects in soil and the environment in general, and the negative impact occurs especially as a residual effect on the fruit, and consequently on other processed food products consumed by people.-

"Pesticide residues" are residues, including active substances, metabolites and/or breakdown products or products of the reaction of the active substances that were used at the time or before, in products for plant protection [14].

Pesticide residues must be monitored and controlled. The European Commission has adopted a list of maximum residue levels (MRL) of pesticides used in food production and animal feed regulated by Regulation EC 396/2005 [15]. Maximum residues levels of certain substances (pesticides, mycotoxins, heavy metals and other contaminants) in the Republic of Macedonia are given in the Regulation on general requirements for food safety [14].

Trifloxystrobin (methyl (E) -methoxyimino -{(E)-α-{1-(ααα-trifluoro-m-tolyl) ethylideneaminooxy]-o-tolyl} acetate), a strobilurin is a fungicide [16] with outstanding biological activity [17], and has been widely applied for efficient control of plant fungal diseases since the 1990s [18]. It is the first strobilurin compound with an oximether side chain, which exhibits broad-spectrum fungicidal bio-efficacy against a variety of crop pests [19, 20]. Strobilurins have a wide spectrum of activity against fungal species, and are registered for use in a number of cultures [21]. They can be also used to protect cereals against fungal diseases such as: powdery mildew, stem rust, leaf rust, and black stem rust, and to protect apple and pear orchards against scab and mildew [22].

The fungicides thus have a wide range of applications and are commonly used in agriculture; it is, therefore, necessary to determine levels of trifloxystrobin residues in plant material [22].

Detection and quantification of pesticide residues in food, particularly fruits and vegetables, is of growing concern for producers, consumers, and governments [23]. Therefore, contamination of vegetables and fruit in general are ones of the most important aspects of the food quality assurance.

The main objective of our study was to investigate the presence of the residues of pesticides (trifloxystrobin) on apples.

2. Materials and Methods

For the analysis were taken two varieties of apples: Golden Delicious and Idaret from two different locations: Evla and Kriveni from Resen. Samples are taken 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.

Samples were collected for the period from May to September 2016. 1 kg of sample (from both varieties of apples from different locations, separately) are prepared for analysis by liquid chromatography (LC).

LC-MS/MS has become the most frequently used analytical method for quantification of polar pesticides in food [24 -28]. Trixloxystrobin is analyzed with liquid chromatography - tandem mass spectrometry (LC-MS/MS). The extraction/separation and purification was done with acetonitrile and dispersive SPE - QuEChERS - method. The sample analysis is performed with standard methods MKS EN 15662: 2011 [29]. This method is accredited in a flexible range of the Institute for Accreditation of the Republic of Macedonia (IRAM) for MKS EN ISO/IEC 17025 : 2006 under number LT - 036 [30].

QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method, which minimizes the number of sample-preparation steps, has been developed for the extraction of pesticides in fruits and vegetables. QuEChERS is a novel sample preparation methodology for pesticide multiresidue analysis introduced by

Anastassiades *et al.*, in 2003 [31]. The QuEChERS method has several advantages over traditional sample preparation methods used in pesticide residue analysis: high recoveries are achieved for wide polarity and volatility ranges of pesticides; high accuracy; high sample throughput; low amounts of solvents, glassware, and bench space are required; use of no chlorinated solvents; low expense; and the only devices needed are a chopper, balance, and centrifuge. The main disadvantage of the QuEChERS method compared to other common methods is that higher LOQs are obtained because of a lower enrichment factor. Also, acetonitrile has a large vaporization expansion volume, which tends to limit the GC injection volume [23].

Numerical statistical analyses of data are made with applying Student t-test. Student's t-test deals with the problems associated with inference based on "small" samples. Two sets of data can be used to determine if the averages of your two samples are significantly different. The p-value (probability) is a measure of how likely you are to get this spot data if no real difference exists [32 - 34].

3. Results and Discussion

3.1 Determination of trifloxystrobin in apples

The results are presented with graphs and diagrams. In order to see if trifloxystrobin in analyzed fruit is within the limits, the obtained values are compared with the Maximum Residue Levels (MRL) according the legislation of the Republic of Macedonia [14]. In the Figure 1 is shown the amount of trifloxystrobin in Golden Delicious from both locations.

The analysis has shown us that trifloxystrobin in Golden Delicious form Kriveni is present mostly in the first phase with a concentration of 0.14 mg/kg. After third phase its concentration decreases and reaches the 0.05 mg/kg which is about 14 times lower than the maximum allowed concentration.

In Evla location, trifloxystrobin is present only in the third and fourth phase and the concentration is in the range of 0.06 to 0.04 mg/kg. The concentration of trifloxystrobin in the last phase of Evla is 17.5 times lower than the maximum allowed concentration (0.7 mg/kg) provided by the legislation of the Republic Macedonia.

The amount of trifloxystrobin in apple Idaret from Evla and Kriveni is shown in Figure 2.

The analysis has shown that trifloxystrobin was not detected in apples Idaret (Figure 2) from Evla location at any phase or it is at a concentration below the detection limit. In samples taken from Kriveni it's detected only in the third phase with concentration of 0.04 mg/kg which is 17.5 times lower than the maximum allowed concentration.

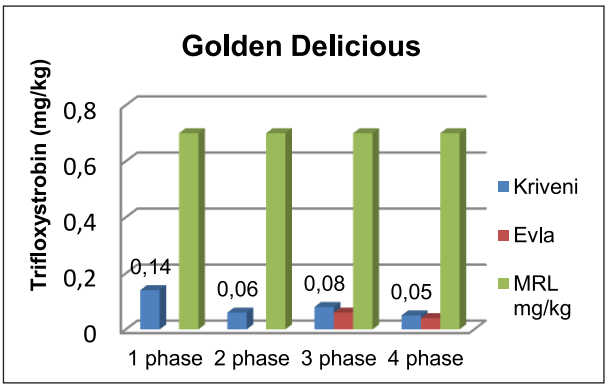

Figure 1. Amount of trifloxystrobin in Golden Delicious at Kriveni and Evla and comparison with MRL

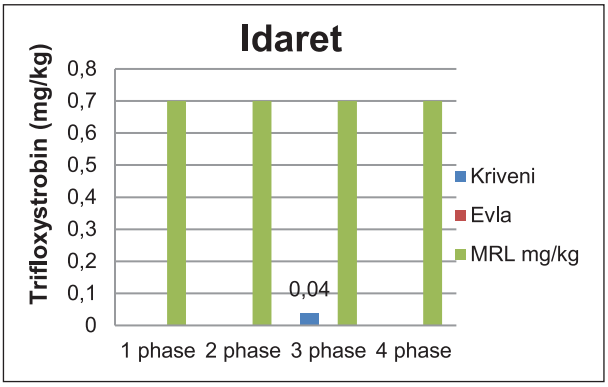

Figure 2. Amount of trifloxystrobin in Idaret from Kriveni and Evla and comparison with MRL

Figure 3. The presence of trifloxystrobin in Idaret and Golden Delicious from location Evla

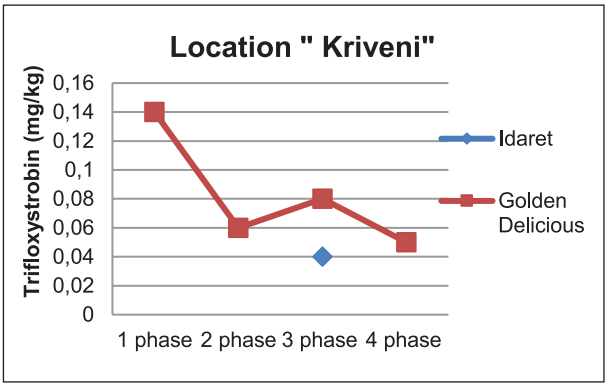

Figure 4. The presence of trifloxystrobin in Idaret and Golden Delicious from location Kriveni

We made a comparison of the presence of trifloxystrobin in apples at both locations (Figure 3, and Figure 4).

In the Figure 3 is shown that trifloxystrobin in Golden Delicious form Evla is present at third and fourth phases with different concentration and it's not present in variety Idaret.

In the Figure 4 is shown that trifloxystrobin in Golden Delicious from Kriveni is present at all development phases while in variety Idaret is present only in the third phase.

From the comparison made in locations, it can be concluded that trifloxystrobin is more present in the variety Golden Delicious than in variety Idaret. Trifloxystrobin is more present in apple from Kriveni location.

3.2 Results of the statistical data processing

We used the t-test for two groups to formalize this calculation.

Table 1. Representation of the parameters from Student's t-test

Statistical parameters	Golden Delicious		Idaret	
	Kriveni	Evla	Kriveni	Evla
mean	0.0825	0.025	0.01	0
t-test	1.946173039		1	
t-critical	3.182446			
p-value (probability) (statistical significantly, p < 0.025)	0.146822133		0.391002219	

The statistical parameters (mean, t-test and p-value) are presented in Table 1. From Table 1 can be shown that the average value (mean) of the amount of trifloxystrobin (mg/kg) is different in all samples.

The value of t-test for Golden Delicious from both locations is 1.946173039 and it is lower than the critical t-value 3.182446.

The value of t-test for Idaret from both locations is 1 and is less than the critical value of t-test.

The two values of t-test is lower than critical value, which means that there is a significant difference in the presence of trifloxystrobin in apples from both locations.

Obtained p-value is greater than the limit p-value and it says that the samples don't differ significantly.

4. Conclusions

- It can be concluded that trifloxystrobin in Golden Delicious variety is present in both locations, but more at Kriveni location. Throughout phases, the concentration decreases and reaches 0.05 mg/kg which is about 14 times lower than the MRL. In the apples from Evla, concentration reaches 0.04 mg/kg which is 17.5 times smaller than the MRL.

- Trifloxystrobin in Idaret from Kriveni is detected only in the third phase at a concentration of 0.04 mg/kg and this concentration is 17.5 times smaller than the MRL. In Idaret from Evla location it's not detected or it is at in concentration below the detection limit.

- From the comparison made in both locations, can be concluded that trifloxystrobin is more present in the Golden Delicious variety of Kriveni location than in the Idaret variety. Trifloxystrobin is more present in apple from Kriveni location.

- The value of t-test for Golden Delicious and Idaret from both locations are 1.946173039 and 1. They are lower than the critical t-value. That means that there is a significant difference in the presence of trifloxystrobin in apples from Kriveni and Evla.

- From processing of statistical data can be concluded that there is a difference in the presence of trifloxystrobin in both varieties of apples, but the difference between the samples is not statistically significant.

- Although trifloxystrobin is detected in certain phases and locations, its concentration is within the allowable concentrations.

- After comparing the obtained values with the MRL it can be concluded that apples from the two locations are safe for consumption.

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