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DETERMINATION OF CHLORPYRIFOS IN APPLE FROM THE RESEN REGION

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Abstract: *The aim of this research is to determine the presence of chlorpyrifos in two varieties of apples: Golden Delicious and Idared in two different locations (Evla and Krveni) from the Resen region in the country. Chlorpyrifos is organophosphate pesticide (insecticide) used to protect apples from insects which can cause significant damage in apple production. The apples are analyzed in four development phases and in each phase the presence of chlorpyrifos is determined. Chlorpiryfos analysis of the apples is performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) followed by an extraction/separation cleansing with acetonitrile and the dispersive SPE - QuEChERS - method. The results are compared with the maximum residue levels (MRL) prescribed by the legislation of the Republic of Macedonia. The conclusion is that chlorpyrifos is variously represented in most phases, regardless of the phase and location where the apple is grown. At certain phase the concentration exceedes MRL, however during the harvest, apples are safe to be consumed. Higher presence of chlorpyrifos is found in Idared apples at Kriveni location as compared to Evla. The presence of chlorpyrifos in Golden Delicious is almost equal in both locations. From the statistical analysis of the data it is noticeable that the use of chlorpyrifos does not depend on the (type) variety of apple but rather on the location where the apple is grown.*

Keywords: chlorpyrifos, pesticides, apples, Golden Delicious, Idaret

1. Introduction

The apple is the most important deciduous fruit consumed during a year. Its fruits can be frozen or used for various types of products (juice concentrate, apple acid and etc.) [1]. In the Republic of Macedonia, the most common fruits are: apples with about 62%, 13% plums, cherries 7%, peaches 7%, table grapes 7% and other types of fruit (pears, apricots, almonds, walnuts, etc.) with about 4% [2]. Domestic consumption of apples is 12 kg per citizen [3]. It is estimated that about 25% of the total production of apples is delivered to domestic market while the remaining 75% is processed or sold on foreign markets [2]. Fruit production occupies about 2.75% of the arable agricultural land, about 14 000 ha with 9 000 000 trees concentrated in regions at an altitude of 300-800 m (including the Resen region). In Macedonia, the total number of trees apple is 4 082 328, fruitful trees 3 811 548 with total production of 136 931 t or 36 kg per tree. In Resen, the number of fruit trees is 2 458 300, out of which fruitful trees are 2 256 800 while the apple production is of 90 450 tons of apples or 40 kg of apples per tree [3]. This shows that apple is mostly cultivated in Resen. It is said that apple is a measure for the development of fruit growing in every country, since increasing the number of apple trees it increases the intensity of fruit growing production, globally and conversely [4]. In

Resen the following varieties of apples are being grown: Idared, Golden Delicious, Mutcu, Red Delicious, Jonagold, Granny Smith, Fuji, Gala and Rubistar [5].

Idared is a variety of apple grown in Moscow, Idaho, by Lejf Verner. It is a diploid and secondary lush variety, blooming early. The fruit is coarse to very coarse, with a round shape. The epidermis is firm, elastic and smooth. The main color is green to red. The inner part of the fruit is white, mixed with chippings of green color, firmly, crispy, with good structure, juicy and sweet. It is harvested from late September to early November [6].

Golden Delicious is diploid, secondary lush variety of apples. Its blooming lasts long and late, being harvested very late. The fruits are medium large, round, have greenish - yellow surface mulch, which turns to golden yellow color at stage of maturity. It is harvested in the second half of September and can be stored until the end of May [6].

Producers of apples in Resen are facing with different kinds of pests in their fruit tree plantations that cause major economic damage. The most important apples pests are: codling moth *(Cydiapomonella),* apple green aphid *(Aphis pomi)* and phytophagous spiders, European red mite *(Panonychus ulmi)* and red spider mite *(Tetranychus urticae)* [4]. To protect apples from various diseases pesticides are used. Pesticides are chemical compounds with active substance affecting the suppression of the pest and cause of the disease. Poulsen *et. al*., in 2009 pointed out that pesticides are used in the production of fruit, vegetables and cereals [7]. Residuals of pesticides (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 [8]. 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 [9].

Chlorpyrifos (O, O - diethyl O - 3, 5, 6-trichloro – 2 - pyridyl phosphorothioate) is organophosphate pesticide and is used as an insecticide in order to increase the yield of agricultural products. Chlorpyrifos is mostly used in the following crops: cotton, corn, almonds, vegetables and fruits, especially oranges and apples [10]. Chlorpyrifos is moderately toxic to humans. Poisoning by chlorpyrifos can have a major impact on the central nervous system, cardiovascular system and respiratory system [11]. It can cause irritation to skin and eyes. Symptoms of acute and chronic exposure include: numbness, tingling, incoordination, headache, dizziness, nausea, abdominal cramps, sweating, blurred vision, difficulty in breathing or respiratory depression, and slow heartbeat. Very high doses may result in unconsciousness, incontinence, and convulsion fatality.

Integral protection of apples in recent years has gained in importance because there are major advantages. It involves using natural enemies to eliminate use of selective insecticides, which preserve the environment and the production of food safety [4, 12].

Student t-test is the most often used parametric test of significance testing a statistical significant difference between two arithmetic averages. The interpretation of the obtained values of the t-test is based on Student t-distribution with a number of degrees of freedom and table of critical values of t-distribution. If statistical t-value < t- critical value (SS and 0.05), the null hypothesis (Ho) is accepted and rejecting the alternative hypothesis (HA), of p = 0.05; P = 0.95 (95%).

The samples are statistically significantly different if the obtained p (probability) value is less than the p - critical value. If the obtained p-value is greater than or equal to the limit p-value, it says that the samples do not differ significantly. In the first case, the null hypothesis is rejected, while the second is accepted [21, 22].
We made Student t-test for the following hypothesis: does the presence of chlorpyrifos in both varieties of apples taken from the Kriveni and Evla are significantly different?
The aim of our research is to analyze this pesticide in two varieties of apples, in two different locations.

2. Matherials and methods

For the analysis two varieties of apples were taken: Golden Delicious and Idared. Samples were collected from two different locations (Evla and Kriveni) 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. Fresh apples with peel and mesocarp were analyzed. The sampling dates for analysis are the following: 31. 05. 2016, 09. 07. 2016, 21. 08. 2016, 25. 09. 2016 for each phase.
LC-MS/MS has become the most frequently used analytical method for quantification of polar pesticides in food [13-17]. Chlorpyrifos analysis was performed with modern methods LC-MS/MS following acetonitrile extraction/partitioning and clean-up by dispersive SPE - QuEChERS-method with MKS EN 15662: 2011 method. 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 [18].

The QuEChERS method uses acetonitrile, with the application of adequate combination of salts, dinatrium hidrogen citrata sesquihydrate, water-free magnesium sulfate and sodium chloride 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 [19, 20]. The homogenized sample of apple with weight of 10 g is transferred to a 50 ml centrifuge tube. 10 ml of acetonitrile is added and e.g. 100 µl of the internal standard solution, shaking vigorously for 1 min. This is the first extraction step. After extraction, in a vortex mixer for 1 minute, 4 g of magnesium sulfate anhydrous ($MgSO_4$), 1 g of sodium chloride (NaCl), 1 g of trisodium citrate dehydrate and 0.5 g of disodium hydrogencitrate sesquihydrate 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 extract was transferred into a clean-up tube containing 900 mg of MgSO4 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 autosampler vials to be used for the multi-residue determination. The analyses were carried out with a liquid chromatograph (LC) (Agilent 6420) with triple quadrupole mass spectrometry.

3. Results and discussion

The results are presented in graphs and diagrams. In order to see if the insecticide chlorpyrifos in the analyzed fruit is within the limits, the obtained values are compared with the Maximum Residue Levels (MRL) according to the legislation of the Republic of Macedonia [9].

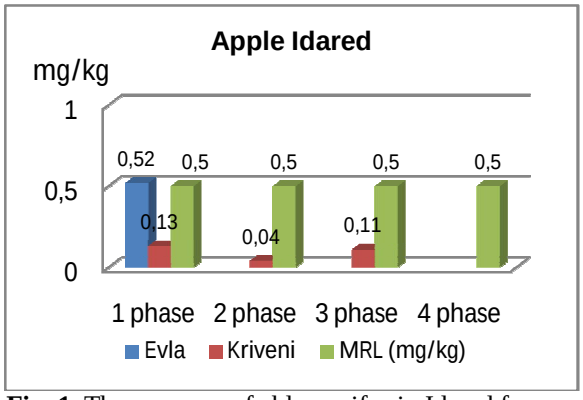

Fig. 1. The presence of chlorpyrifos in Idared from Evla and Kriveni and compared with the MRL

Fig. 1 represents the content of chlorpyrifos in Idared from two different locations. It is noticeable that chlorpyrifos at Evla location is present only in the first phase. In this phase, its concentration is 0.52 mg / kg and it is slightly above the allowed values. In the other phases chlorpyrifos is not present or is below the limit of detection. At the location Kriveni chlorpyrifos is more present than Evla and it is present in all three phases at a concentration that is acceptable according to the legislation of the Republic of Macedonia. It is seen that during the harvest time, apples do not contain residues of chlorpyrifos.

The presence of chlorpyrifos in Golden Delicious in two different locations is presented in Fig. 2.

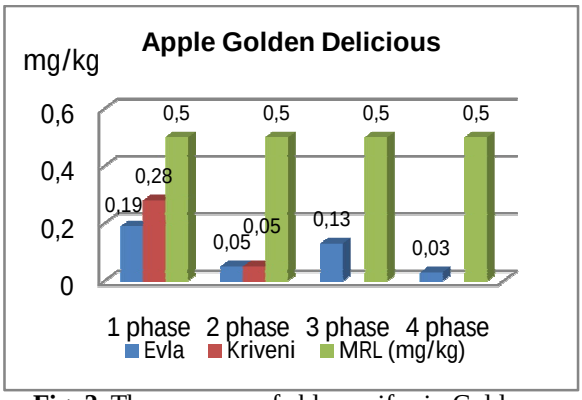

Fig. 2. The presence of chlorpyrifos in Golden Delicious from Evla and Kriveni and compared with the MRL

In Golden Delicious of Evla the chlorpyrifos is present in all four phases with the highest concentration of 0.19 mg / kg in the first phase and the lowest concentration of 0.03 mg / kg in the fourth phase, which is approximately 17 times less than the maximum allowed. At the location Kriveni it is present in the first two phases. The concentration of chlorpyrifos is reduced from 0.28 mg / kg in the first phase and finds with 0.05 mg / kg in the second phase which is 10 times smaller than the maximum residue levels. In the third and fourth phase the chlorpyrifos is not present in the analyzed samples.

We made a comparison of the presence of chlorpyrifos in both varieties of apples at both locations (Figs. 3 and 4).

From Fig. 3 it may be noticed that in the location Evla this pesticide is present in Golden Delicious in all phases and in Idared only in the first phase.

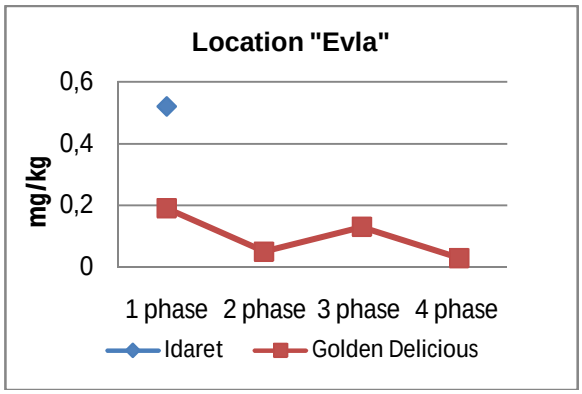

Fig. 3. The presence of chlorpyrifos in Idared and Golden Delicious from location Evla

Fig. 4 shows that the chlorpyrifos is more present in Golden Deliciuous than in Idared.

From the diagrams can be noticed that chlorpyrifos in Idared is more present at Kriveni. The presence of chlorpyrifos in Golden Delicious is almost equal in both locations because it is present in all the phases.

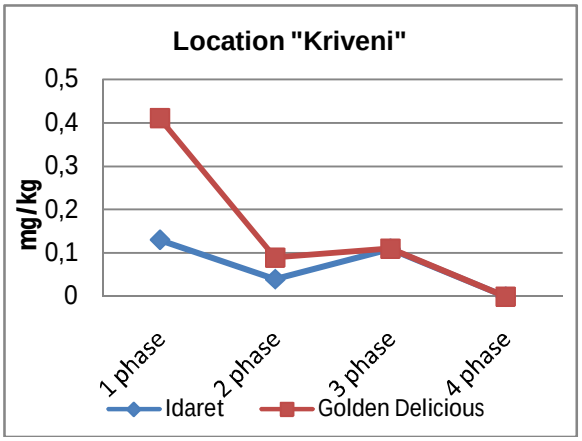

Fig. 4. The presence of chlorpyrifos in Idaret and Golden Delicious from location Kriveni

The obtained statistical values are presented in Table 1.

Table1.
Representation of the parameters from Student's t-test

Statistical parameters	**Idared**		**Golden Delicious**	
	Evla	**Kriveni**	**Evla**	**Kriveni**
$\bar{x}$	0.13	0.07	0.1	0.0825
t-test	0.534169		0.385922	
t-critical value	3.182446			
p-value	0.630237		0.725295	
p - critical value	0.025			

Table 1 presents the mean of value (x) on the concentration of chlorpyrifos shown in mg / kg in the two varieties of apples from both locations.

The value of t-test for Idared in both locations is 0.534169 and it is lower than the critical t-value 3.182446.

The value of t-test for Golden Delicious from both locations is 0.385922 and is less than the critical value of t-test.

That means that null hypotheses can be accepted and that there is a significant difference in the presence of chlorpyrifos in apples from both locations.

In both varieties of apples p-values are greater than the critical p-value. That means that null hypothesis can be accepted but the difference between the samples is not statistically significant.

4. Conclusions

Analyses were made on two varieties of apples: Golden Delicious and Idared from two different locations Kriveni and Evla in the Resen region. Chlorpyrifos is detected by LC - MS / MS and QuEChERS extraction is applied with the standard method MKS EN 15662: 2011.

Analyses show that at Evla chlorpyrifos is represented in the Idared variety only in the first phase while in the other phases it is absent. In Golden Delicious at location Evla chlorpyrifos is represented in all four phases but with approximately 17 times lower concentration than the maximum allowed in the fourth phase. At the location Kriveni chlorpyrifos in Golden Delicious is present in both phases with allowed concentration, while in Idared is present in all three phases. Although chlorpyrifos is available in two varieties of apples they are safe for consumption. Statistical analysis of data on apple from Kriveni and Evla showed that the use of chlorpyrifos depends on the variety of apple, but not on the location where it is grown. Further investigation will be made on other varieties of apples as well as in other locations.

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