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Flower honey as a potential bio-indicator of environmental pollution by pesticides in the Varaždin County

Cvjetni med kao potencijalni bio-indikator onečišćenja okoliša pesticidima u Varaždinskoj županiji

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Summary

Aim: In this research samples of floral honey were used as bio-indicators of environmental pollution by pesticides.

Material and methods: A total of 40 (4x10) samples from bee farms were collected at 4 different locations (sub-regions) including 16 municipalities in the Varaždin County, as well as 20 (2x10) samples from two locations – bee-farms registered as "ecological producers" in the neighborhood County, both located far away from conventional agricultural production. Analyses were carried out using the gas chromatography joint system with mass spectrometry (GCMS) and high-performance liquid chromatography (HPLC).

Results: Identification and quantification of 78 pesticide active ingredients were performed which are part of a total of approximately 400 pesticides that are usually analyzed in fruits and vegetables whereby the laboratory had available analytical equipment that also determined the number of detected pesticides.

Due to identified and quantified active ingredients of pesticides, the results of analyses might indicate a possible cause-effect relationship with conventional agricultural activities in the Varaždin County. It was evident from the obtained results on the shares of identified and quantified pesticide residues, and with respect to admissibility, that the share of permitted pesticides in the samples of floral honey accounted for 55.2%, while the share of illegal pesticides accounted for 44.8%. Analyzing the number of detected insecticides and fungicides, on a total of all 4 locations (sub-regions), it was found that floral honey contained 13 different types of insecticides and 3 types of fungicides.

Conclusion: The results and statistical data analysis showed that floral honey is a good bio-indicator of environmental pollution by pesticides, therefore this study can serve as a basis for scientists and experts engaged in the research of harmful effects of pesticides on the environment and human health.

Key words: honey, pollution, pesticides, environment

Sažetak

Cilj: U ovom istraživanju uzorci cvjetnoga meda korišteni su kao bio-pokazatelji onečišćenja okoliša pesticidima.

Materijal i metode: Ukupno je prikupljeno 40 (4x10) uzoraka sa 4 različite prostorne lokacije (subregije), u ukupno 16 općina, od proizvođača meda u Varaždinskoj županiji, kao i 20 (2x10) uzoraka s dvije različite lokacije proizvođača u susjednoj županiji, registriranih kao "ekoloških proizvođači", a udaljenih od poljoprivrednih površina pod konvencionalnom proizvodnjom. Analize su provedene korištenjem vezanog sustava plinske kromatografije i spektrometrije masa (GCMS) i tekućinskom kromatografijom visoke djelotvornosti (HPLC).

Rezultati: Identificirano je i kvantificirano 78 aktivnih sastojaka pesticide, koji su dio od ukupno cca 400 pesticida koji se analiziraju u voću i povrću, a za koje je laboratorij raspolagao analitičkom opremom koja i dijelom određuje i vrstu i broj pesticida koje se detektiraju. Rezultati analiza ukazali su na mogući uzročno-

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posljedični odnos između konvencionalnih poljoprivrednih aktivnosti u Varaždinskoj županiji s identificiranim i kvantificiranim aktivnim sastojcima pesticida. Iz rezultata dobivenih na udjelima identificiranih i kvantificiranih ostataka pesticida, s obzirom na dopustivost, vidljivo je da je udio dopuštenih pesticida u uzorcima cvjetnoga meda iznosio 55,2%, dok je udio ilegalnih pesticida iznosio 44,8%. Analizirajući broj otkrivenih insekticida i fungicida, ukupno na sve 4 lokacije utvrđeno je da cvjetni med sadrži 13 različitih vrsta insekticida i 3 vrste fungicida.

Zaključak: Rezultati i statistička analiza podataka pokazuju da je cvjetni med dobar bio-indikator onečišćenja okoliša pesticidima, te stoga ova studija može poslužiti kao osnova za znanstvenike i stručnjake koji se bave istraživanjem štetnih učinaka pesticida na okoliš i ljudsko zdravlje.

Ključne riječi: med, onečišćenje, pesticidi, okoliš

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Introduction

People are able to adapt the environment to their needs. During such adjustments they use different tools that enable them to fight disease or pests. Through the discovery and development of pesticides, the efficiency of the race with pests, which compete with humans for food, significantly increased. The use of pesticides commonly requires treatment of large soil surfaces by using concentrations that are able to kill certain plants or animal species. The methods and frequency of daily use of pesticides have led to the inability to control pesticides from circulating in the environment. Slow degradation of pesticides, as well as an excessive and inappropriate use, can lead to environmental pollution of water, soil, air, and indirectly of people.[1,2,3]

Pesticides, which are used in agriculture, are usually in the form of a liquid or an emulsion that is sprayed onto plants or soil. Sometimes pesticides in the form of granules are dispersed onto soil prior to planting, or a pre-treated seed is used[3]. All factors of application of pesticides, their formulation, target pests, spray techniques, and weather conditions affect the quantity that will enter the environment.

The amount of pesticides that comes in direct contact or which is consumed by a pest is very small compared to the amount that is applied[1]. In most researches, the portion of applied pesticides that reaches the targeted pest is less than 0.3%, therefore it might be presumed that 99.7% of it ends up in the environment.[4]

Their chemical properties, such as their ability to bind to the soil particles, vapor pressure, solubility in water, and resistance to degradation leads to the fact that there is no segment of our environment where pesticide residues cannot be found[5]. Besides the soil[6-8], underground water,[9] surface water,[10] ground water,[11] air,[12] and harbor sediments,[13] pesticide residues can be found in all animals and even in infant hair, cord blood and meconium of human fetuses.[14]

We can say that every organism and its home form a mirror pair whereby one cannot exist without the other. The mirror image of living beings and their biotope allows us to use certain organisms as biological indicators of environmental pollution.[15] Therefore, in this paper floral honey as a bio-indicator of pollution by pesticides was used.[15]

Honey is a natural and healthy product consumed by people all around the world. It is used as a natural sweetener in food, in cosmetics, but also in the pharmaceutical industry in the production of various food supplements and medicines.[16] It is produced by the bees (*Apis mellifiera*) from the nectar of honey plants or plant secretions and excretions of insects (Honeydew), which the bees collect, add their own excrement, extract the excess water, and deposit in the honeycomb cells to mature. Protection of plants and products used in agriculture do not only affect the bees, but also their products, especially honey.[15] The absence of light and relatively stable temperature in the beehive, combined with absorbent surface (waxes) and a high concentration of sugar in honey, create a stable environment for organic compounds.[15] In many scientific papers, honey is used as a bioindicator of air pollution[16,17] and environmental pollution,[18] by metals,[19,20] pesticides and other pollutants[21-26.]

Materials and Methods

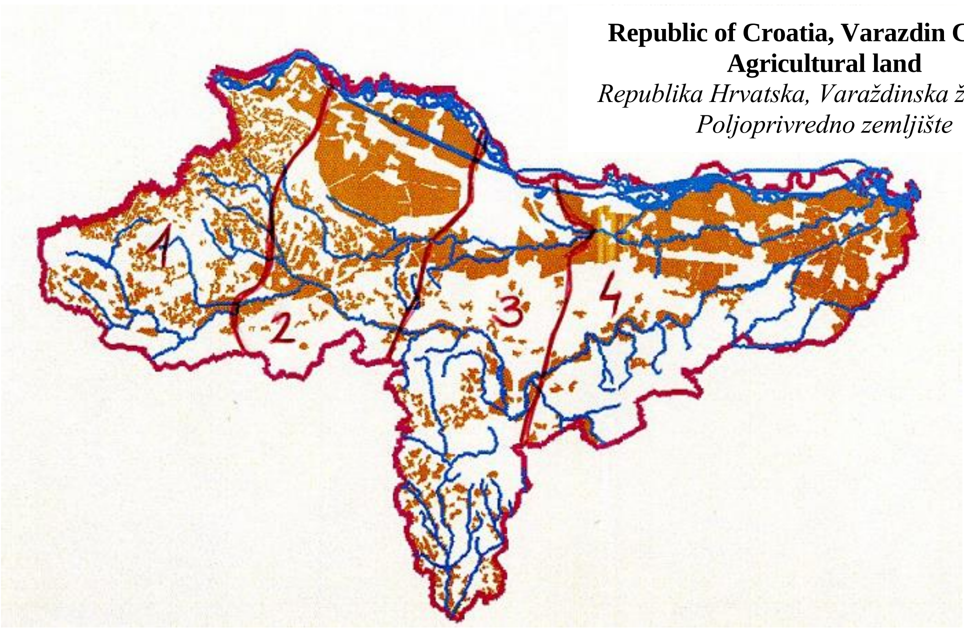
For the purpose of this study, samples were collected from domestic honey producers from stationary beehives in glass jars with screw caps to avoid any contamination. A total of 40 (4x10) samples of floral honey were collected at 4 different locations (sub-regions) – bee farms in the Varaždin County, as well as 20 (2x10) samples from two locations – bee-farms registered as "ecological producers" in the neighborhood County, located far away from conventional agricultural production, given to the fact that in the Varaždin County there are no "eco-honey producers" away from agricultural areas. Other group samples were taken from eco-honey producers from organic production in the area of the Lonja Field and Bosiljevo. Until the beginning of the analysis, samples were kept in the dark at +10°C, as described in scientific literature.[18,23] Before the analysis, it was confirmed that all samples corresponded to floral honey. Samples of honey and bees were prepared according to the modified method EN 15662: 2008, "Foods of plant origin. Determination of pesticide residues using GC-MS and/or LC-MS/MS after extraction by acetonitrile and purification with dispersive SPE". "Quechers method" (e*ng. Quick, Easy, Cheap, Rugged, Safe, Quechers*). The Quechers method was introduced in the world of analytics by Anastassiades[27] in 2003., and the group of Italian authors.[28,29] The method was originally used for the extraction of pesticide residues from fruit and vegetables,[30] and its simplicity and flexibility intrigued scientists who began to use it for the preparation of other types of samples.[28-30]

After standard sample preparation, it was proceeded with the identification and quantification of pesticide residues using a joint system of gas chromatography and mass spectrometry (GC-MS), and the chromatographic technique of high-performance liquid chromatography (HPLC).[28] Mass fraction of pesticide residues was determined by GCMS-QP2010 plus device equipped with PTV (Pressure temperature volume) autoinjector model AOC-20i. Restek RTX-capillary column OPPesticides (30m x 0.25 mm i.d.) and 0.25 film thickness with the helium flow (He 6.0) of 1.99 ml/min were used for separation. The determination of the type and quantity of residues was carried out using the selective ion monitoring (SIM) mode analysis, which is based on one main ion and two confirmatory ions and the retention time. Concentration was calculated automatically using GCMS-solution program based on the ratio of the peak area of analyte divided by the peak area of standard.

The Shimadzu liquid chromatograph was used to determine the amount of residues of pesticide Imidacloprid, which belongs to the class of neo-nicotinoids. A liquid chromatograph with UV-Vis detector was used, and the recording was made at a wavelength of 280 nm. C18 column was used for separation, and acetonitrile was the mobile phase: water in a 60:40 ratio. Concentration was calculated automatically using the LC-solution program based on the ratio of the peak area of analyte divided by the peak area of standard.

Results

A total of 40 samples of floral honey taken at 4 locations (sub-regions) (including 16 municipalities) in Varaždin County and from 2 locations in the neighborhood county with 20 control samples of floral honey being analyzed (Map 1).



Map 1 Four locations (sub-regions) in Varazdin County where sampling was performed
Source: author based upon regional program of Varaždin County, 2006
Mapa 1. Četiri lokacije (sub-regije) u Varaždinskoj županiji gdje su prikupljeni uzorci
Izvor: Autorove oznake na regionalnom programu Varaždinske županije, 2006.

Each location (sub-region) in Varaždin County included several municipalities: location 1 (Bednja, Lepoglava, Klenovnik, Donja Voća, Cestica); location 2 (Ivanec, Maruševec, Vidovec, Vinica, Petrijanec, Sračinec) location 3 (Novi Marof, Sveti Ilija, Turčin, Beretinec, Varaždin) and location 4 (Ljubešćica, Varaždinske toplice, Jalžabet, Trnovec, Martijanec, Ludbreg).

One sample consisted of 10 g of honey. Samples of honey were stored at +10°C in the dark until analysis.

The determination of types and amounts of pesticide residues was conducted, which included 78 active ingredients of pesticides, and the results of obtained concentrations are given in Table 1.

Table 1 Mass fractions of pesticide residues in floral honey
Tablica 1. Razlomak mase ostataka pesticida u cvjetnom medu

Analyte	MAQ values mg/kg *MAQ vrijednosti*	Floral Honey *Cvjetni med* N (40) Range mg/kg* *Omjer*
Aldrin	-	n.d.
Alpha-HCH	-	n.d.
Azinphos-ethyl	-	n.d.
Azinphos-methyl	-	n.d.
Azoxystrobin	-	n.d.
Beta-HCH	-	n.d.
Bioallethrin[D]	≤0.01	0.022-0.348(4)
Boscalid	-	n.d.
Bromophos-ethyl	-	n.d.
Carbaryl[N]	≤0.01	n.d.-0.0046(2)
Chlorfevinphos	-	n.d.
Chloropropham	-	n.d.
Chlorpyrifos-methyl	-	n.d.
Chorpyrifos	-	n.d.
Cis Chlordane	-	n.d.
Cyfluthrin-beta 1	-	n.d.
Cyfluthrin-beta 2	-	n.d.
Cyfluthrin-beta 3	-	n.d.
Cypermethrin 1	-	n.d.
Cypermethrin 2	-	n.d.
Cypermethrin 3	-	n.d.
DDD-4,4,	-	n.d.
DDE	-	n.d.
DDT-4,4	-	n.d.
Diazinon[N]	≤0.01	0.0026-0.179(8)
Dichlorvos	-	n.d.
Dieldrin	-	n.d.
Dimethoate	-	n.d.
Disulfoton sulfone		n.d.
Disulfoton[N]	≤0.01	0.171(1)
Disulfoton-sulfoxide[N]	≤0.01	0.0031-0.078(2)
Endosulfan alfa	-	n.d.
Endosulfan beta	-	n.d.
Endosulfan sulfate	-	n.d.
Endrin	-	n.d.
Ethion[N]	≤0.01	n.d.
Fenamiphos	-	n.d.
Fenchlorphos	-	n.d.
Fenitrothion	-	n.d.
Fenpropathrin	-	n.d.
Fensulfothion	-	n.d.
Fenthion[N]	≤0.01	0.0023(1)
Fludioxonil[D]	≤0.01	0.0087(1)
Heptachlor	-	n.d.
Heptachlor epoxide	-	n.d.
Imazalil[D]	≤0.05	0.023-1.688(16)
Imidacloprid[D]	≤0.05	0.329(1)
Lindane	-	n.d.
Malaoxon[D]	≤0.02	0.001-0.003(2)
Malathion[D]	≤0.02	0.001-0.021(7)
Metacriphos[N]	≤0.01	0.001-0.012(3)
Methamidophos	-	n.d.

Methidathion	-	n.d.
Methyl parathion	-	n.d.
Mevinphos^{N}	≤0.01	0.006(1)
Myclobutanil		n.d.
Paraoxon^{N}	≤0.01	0.004-0.892(2)
Parathion ethyl^{N}	≤0.01	0.0268(1)
Permethrin cis		n.d.
Permethrin trans		n.d.
Phorate^{N}	≤0.01	n.d.
Phosalone	-	n.d.
Phosphamidon	-	n.d.
Pirimentalin		n.d.
Pirimicarb		n.d.
Pirimiphos methyl		n.d.
Procimidon^{N}	≤0.01	0.004-0.009(2)
Pyrazophos		n.d.
Quinalphos	-	n.d.
Resmethrin		n.d.
Tetradifon		n.d.
Tetramethrin^{D}		n.d.
Tolclophos		n.d.
Trans-Chlordane		n.d.
Trifloxystrobin^{D}		n.d.
Vinclozolin		n.d.

Legend / *Legenda:*

- * number of samples in which analyte was identified / *broj uzoraka u kojima je identificiran analit*

- n.d. – not detectible with the applied method / *nije moguće ustanoviti primijenjenom metodom*

- (Analyte)^{D} – permitted use of active substance of pesticide in the Republic of Croatia / *dopuštena upotreba aktivne supstance pesticida u Republici Hrvatskoj*

- (Analyte)^{N} – prohibited use of active substance of pesticide in the Republic of Croatia */ zabranjena upotreba aktivne supstance pesticida u Republici Hrvatskoj*

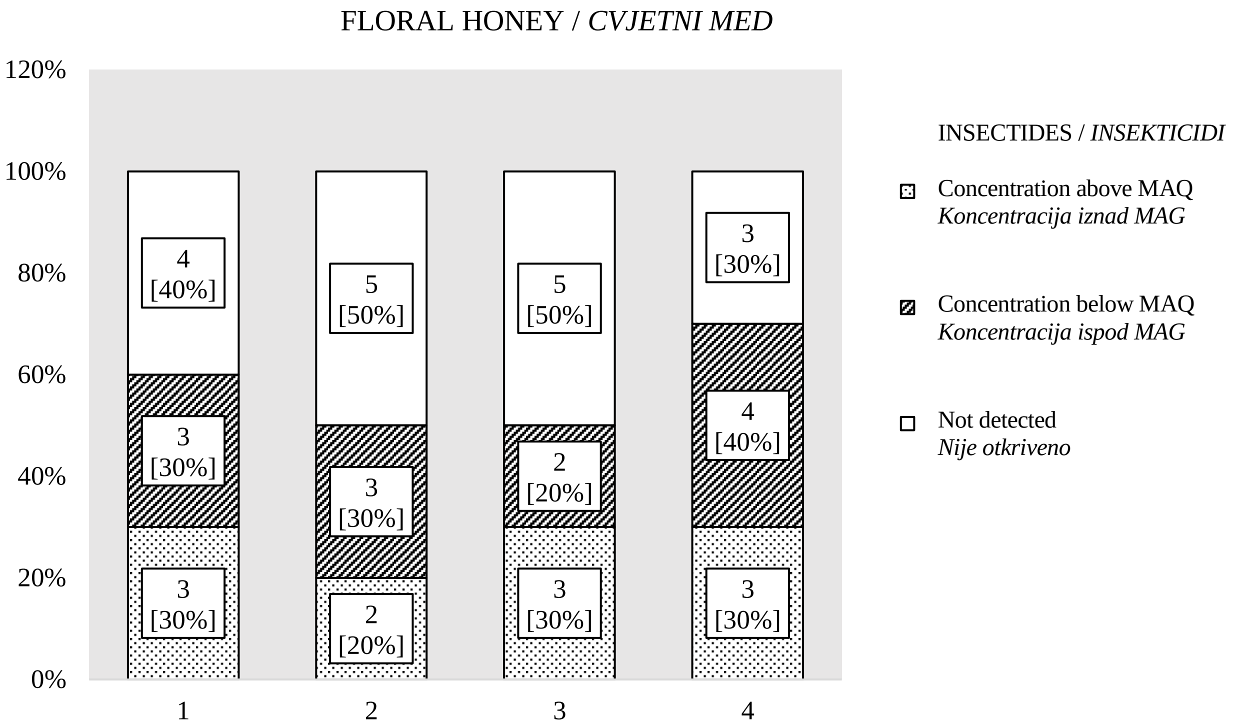
In this research, a total of 13 different types of insecticides and 3 types of fungicides were identified in 40 samples of floral honey. The share of identified and quantified permitted pesticides accounted for 55.2%, while the share of illegal pesticides accounted for 44.2%. The highest number of samples (n = 16) contained a fungicide Imazalil, whose concentrations ranged from 0.0233 to 1.688 mg/kg. From a total of 40 samples, in eight samples of floral honey insecticide Diazinon, whose use is prohibited, was found in concentrations ranging from 0.0026 to 0.179 mg/kg. Seven samples contained the insecticide Malathion in concentrations from 0.001 to 0.021 mg/kg and in two samples Malathion metabolite Malaoxon was identified and quantified in concentrations of from 0.001 to 0.003 mg/kg. Insecticide Bioallethrin was identified and quantified in four samples in concentrations from 0.022 to 0.348 mg/kg. In three samples of floral honey, residues of prohibited insecticide Metacrifos were identified and quantified in concentrations from 0.001 to 0.012 mg/kg. In two different samples residues of pesticides Disulfoton sulfoxide, Carbaryl, Paraoxon and Procymidone were found. The concentrations of Disulfoton sulfoxide residues ranged from 0.0031 to 0.078 mg/kg, of Carbaryl n.d.-0.0046 mg/kg, Paraoxon 0.004 to 0.892 mg/kg and of Procymidone from 0.004 to 0.009 mg/kg. Single samples of floral honey contained Disulfoton in concentration of 0.171 mg/kg, Fenthion in concentration of 0.0023 mg/kg, Fludioxonil in concentration of 0.087 mg/kg, Mevinphos in concentration of 0.006 mg/kg, Ethyl Parathion in concentration of 0.0268 mg/kg and Imadacloprid in concentration of 0.329 mg/kg.

Tables 2 and 3, as well as Pictures 1 and 2, show the results of the statistical analysis of the concentration difference of insecticides in floral honey considering the sampling location. Obtained statistical differences might be connected with differences in special distance of farm from agricultural fields and with differences in individual practice of pesticide usage among locations (sub-regions), but further investigation should be conducted before further conclusions.

Table 2 Differences in insecticide concentrations in floral honey regarding location distribution
Tablica 2. Razlike kod koncentracija insekticida u cvjetnom medu sukladno lokaciji distribucije

			Insecticides: concentration *Koncentracija insekticida*			Total *Sveukupno*
			Not detected *Nije otkriveno*	Concentration below MAQ *Koncentracija ispod MAG*	Concentration above MAQ *Koncentracija iznad MAG*	
Location *Lokacija*	1	N	4	3	3	10
		%	40%	30%	30%	100%
	2	N	5	3	2	10
		%	50%	30%	20%	100%
	3	N	5	2	3	10
		%	50%	20%	30%	100%
	4	N	3	4	3	10
		%	30%	40%	30%	100%
Total *Sveukupno*		N	17	12	11	40
		%	42.5%	30%	-	100%

	Value / *Vrijednost*	df	*P*
χ^2 test	1.586	6	0.954
Total *Sveukupno*	40		

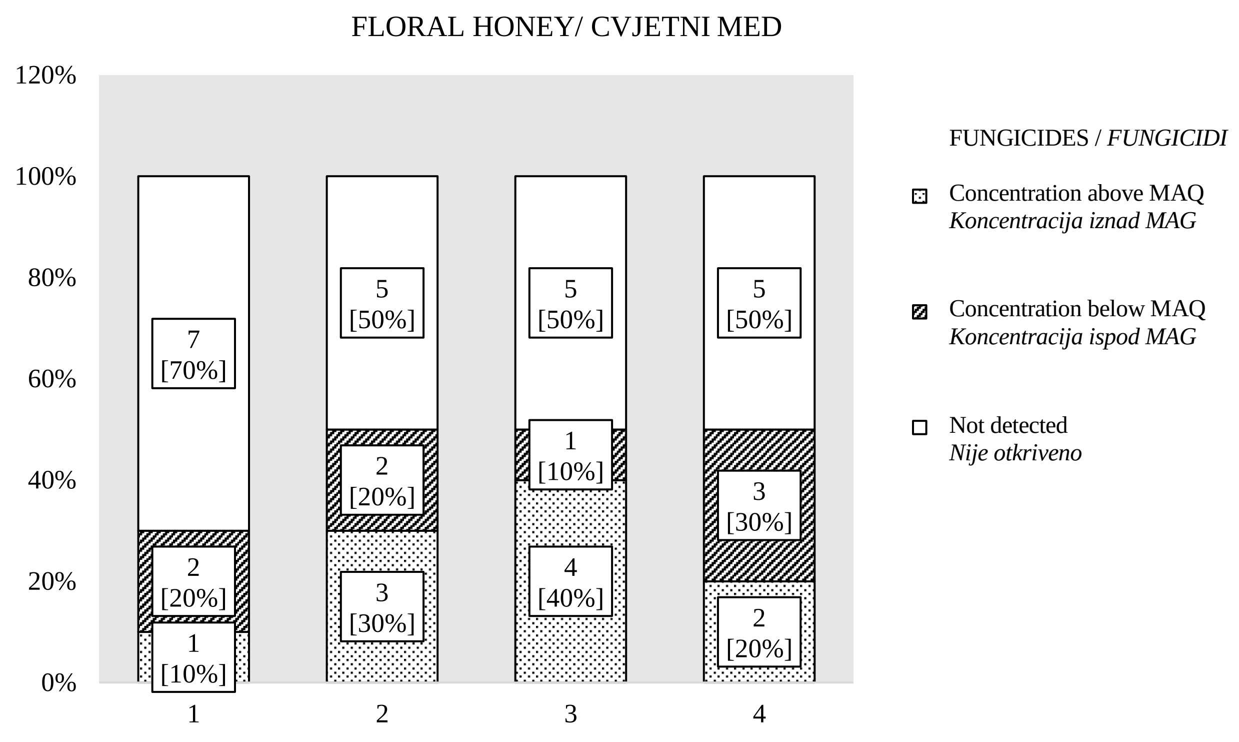


Picture 1 Differences in insecticide concentrations in floral honey regarding location distribution
Slika 1. Razlike kod koncentracija insekticida u cvjetnom medu sukladno lokaciji distribucije

Table 3 Differences in fungicide concentrations in floral honey regarding location distribution
Tablica 3. Razlike kod koncentracija fungicida u cvjetnom medu sukladno lokaciji distribucije

			Fungicides: concentration *Koncentracija fungicida*			Total *Sveukupno*
			Not detected *Nije otkriveno*	Concentration below MAQ *Koncentracija ispod MAG*	Concentration above MAQ *Koncentracija iznad MAG*	
Location *Lokacija*	1	N	7	2	1	10
		%	70%	20%	10%	100%
	2	N	5	2	3	10
		%	50%	20%	30%	100%
	3	N	5	1	4	10
		%	50%	10%	40%	100%
	4	N	5	3	2	10
		%	50%	30%	20%	100%
Total *Sveukupno*		N	22	8	10	40
		%	55%	20%	25%	100%

	Value/ *Vrijednost*	df	*P*
χ^2 test	3.545	6	0.738
Total *Sveukupno*	40		



Picture 2 Differences in fungicide concentrations in floral honey regarding location distribution
Slika 2. Razlike kod koncentracija fungicida u cvjetnom medu sukladno lokaciji distribucije

Discussion

In this paper, floral honey samples were used as bio-indicators of environmental pollution by pesticides. The obtained concentrations of pesticide residues ranged from very low to above maximum permissible concentrations prescribed by the European Commission Directive 396/2005.[31] Flower honey, by its pollen characteristics, is multifloral honey, because it contains more than one type of pollen grains of different plants, depending on the geographic and climate region, such as grass grains (*Poaceae*), legumes (*Fabaceae*), buckthorn (*Rhamnaceae*), labrid (*Lamiceae*), daisy (*Asteraceae*), amorpha (*Amorpha fructicosa*), sweet chestnut (*Castanea sativa*), willow (*Salix sp.*), rose (*Roasaceae*), cabbages (*Brassicaceae*), and other cultivated plants.

According to research conducted on pollen analysis in the Varaždin County, it was established that floral honey may contain a greater proportion of pollen grains of cultivated plants such as plum (*Prunus sp.*), rapeseed (*Brassica napus L.*), and pollen grains of maize (*Zea mays L.*).[32] An increased proportion of plum pollen grains was as high as 36%, while the proportion of pollen grains of seed in the same sample of honey amounted to 21%.[32] An increased proportion of pollen grains of cultivated plants in the flower honey also explains the remains of active ingredients of pesticides used in agriculture that were found.

In most of the specimens, fungicide Imazalil was identified and quantified, which indicates that this fungicide is mostly used in agriculture during the activity of bees collecting flower pollen. Research on 80 active ingredients of pesticides in honeybees and pollen, which was conducted in France, confirmed the occurrence of Imazalil in honey. The amount of 80 active substances of pollutants was determined in order to demonstrate the presence of pesticides and antibiotics in the environment, as a potential factor affecting the mortality of bees. In the conducted research, in 4% of the analyzed samples of honey, Imazalil was identified and quantified[22]. In previously conducted research, it was noticed that Imazalil was the most commonly detected fungicide identified and quantified in other foods[30].

The residues of the active substance Diazinon were identified and quantified in ten samples of floral honey. Diazinon is an organophosphorus, non-systematical insecticide used to control harmful insects of grapes and apples. Research conducted in Poland on 45 honey samples from different botanical origin, collected from the Polish beekeepers throughout 2010, supported the obtained results. In their research, the scientists described that the presence of some pesticide residues was confirmed in as many as 29% of the analyzed samples.[21] Of the 30 analyzed residues of active ingredients of pesticides, the most commonly found were Prophenophos, Diazinon, and Dimoksistrobin.[21] In seven samples of floral honey, Malathion metabolite Malaoxon was identified and quantified, while in two samples Malathion was identified and quantified.[21] Malathion is an organophosphorus insecticide used in fruit and vegetable growing and gardening to control insects. In 2003 and 2004, research was conducted in Brazil on honey samples. The scientists used a simple and quick multiresidue method to determine 48 selected pesticides in honey samples collected from local honey producers in Brazil.[28,33] Quantities of identified and quantified Malathion in this study ranged from 0.243 mg/kg for 2003 and 0.209 mg/kg for 2004.[28] Since other pesticides were found at low concentrations in this study, relatively high concentrations of Malathion may be associated with environmental pollution as a result of treatments for the tiger mosquito, as described in some other scientific papers.[34] Allethrin was quantified in 4 samples of floral honey. Allethrin is a pyrethroid, or a synthetic form of the active substance, which is found naturally in plants *Chrysantemum*. It is used in households as a repellent for various insects (Raid, Neopitroid), but is also used for a wider application in combating mosquitoes through activities of pest control, like warm fogging. Its high solubility affects bioaccumulation and persistence in beeswax, and thus in honey. Identified traces of prohibited pesticide Metacrifos, in three floral honey samples, indicate contamination of the environment by pesticides and by pesticide residues circulation, even long after the cessation of use.

Other identified and quantified pesticides were found in a small number of samples, but their presence indicates the prevalence of different types of organic pollution circulating through the environment.

The fact that in the control samples of floral honey sampled from eco-beekeepers from the areas of Bosiljevo and the Lonja Field, which are far from farms, no pesticide residues were identified and quantified speaks in favor of how agricultural activities affect the environment pollution by pesticides. These results confirm the causal relationship of agriculture activities with identified and quantified active ingredients of pesticides.

Conclusion

The results of this study suggest a causal relationship of agricultural activities with identified and quantified residues of pesticides and indicate an increased environmental pollution with pesticides. Following the conducted statistical analysis, regarding the amount of insecticides and fungicides analyzed in individual samples of floral honey, it was observed that there was no significant difference in the levels of identified and quantified insecticides and fungicides in different locations, and that the distribution was uniform. Results of this study indicate the need for further research of honey, in a larger number of samples, to obtain a global picture of pollution.

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