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Original Paper

Determination of insecticides in honey samples collected from Gümüşhane-Turkiye; Box-Behnken design evaluation of experimental parameters

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ABSTRACT

This study deals with the investigation of cyfluthrin, cypermethrin, deltamethrin, and malathion residues in local honey samples from Gümüşhane, Turkey. The determination was performed with GC/MS-MS method with HP-5MS column under certain conditions: 120 °C oven temperature, 250 °C injection temperature, 121.9 kPa pressure and 1.2-1.8 mL/min flow rates. The samples were picked from eighteen stations of Gümüşhane. Standard addition method was employed in chromatographic determination. No pesticide detected in samples of fifteen stations, nevertheless, subjected pesticides were determined in samples collected from other three stations. The residue levels varied from 0.18 mg/kg to 9.50 mg/kg at 1.5 mL/min flow rate. The results were also evaluated with Box-Behnken Design (BBD) optimization. Multivariate experimental design (flow rate and station, pesticide type) was employed for constructing quadratic models. Regression analysis showed that the experimental results and the predictive values yielded by model are quite close to each other with determination coefficient (R^2) of 0.985.

I. INTRODUCTION

Pesticides are widely used around the world to eliminate harmful organisms. They are utilized for destroying and/or preventing insects, rodents, weeds, microorganisms *etc*. Many chemicals such as organochlorines, organonitrogens, organophosphates and pyrethroid class have been employed for this purpose. It has been known that pesticides have mutagenic and carcinogenic effects besides their toxic properties [1]. It is known that pyrethroids have been widely used in agricultural purposes due to having high efficiency and low residue besides their lower toxicity and biodegradability in plants [2]. The mobilization of pesticides in environmental systems must be monitored. When used, it can be drifted by wind or migrate beyond soil sheets and leak into surface waters or accumulate in another organism. Because pesticides are stable chemicals and have long-term effects on the organism [3], consequently, it may join the food chain and be consumed by humans.

As you know, honey is a natural product fabricated by honey bees from nectar of flowering plants [4]. It is widely used for nourishment (as direct food or a component of manufactured foods) and medicinal purposes [5]. Besides its health-promoting and immunity-enhancing properties, it has antibacterial, antifungal, antimicrobial, antioxidant, antiviral and antimutagenic effects [6, 7]. Jakkielska, et al. defined honey as a bioindicator of environmental pollution [6]. The determination and extraction of pesticides from honey is significant in the context of food safety, as it enables the detection of harmful residues in food matrices. Therefore, analyses of many pollutants such as arsenic, organonitrogens organochlorines, polyaromatic hydrocarbons, tetracyclines *etc.* were

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studied by many researchers using various analytical and instrumental method [4, 6, 8-11]. El-Osmani et al., optimized solid phase extraction (SPE) method using modified styrene-based polymer for sensitive detection of organonitrogen and organochlorine pesticides They determined fifteen pesticides by GC-MS following by solid phase extraction at optimized flow rate, pH, and elution speed, conditions [4]. To investigate pesticide exposure in honey bees, Ostiguv et al. analyzed pesticide residues in samples collected from stationary apiaries in six U.S. states. As result, they detected 79 different pesticides and declared that detected pesticides were more than fungicides or herbicides [12].

A popular technique for modeling and optimizing process variables that reduces the number of trials in an economical and timely way is the Box-Bhenken design (BBD) [13]. Box-Bhenken design, a surface response methodology, is an approach utilized to evaluate the effects of three or more three-level factors on the response by minimizing the number of experimental runs. Using Design Expert (DOE) version 11.0.5.0 software (stat-Ease Inc., USA), BBD was used in this study to examine the impact of process variables and the relationship between these variables on the answer. In recent years, this method has been increasingly employed to detect components in complex matrix structures, such as honey, and to optimize these processes. Through this approach, researchers can obtain more data in less time and with reduced experimental work compared to traditional methods [14]. In this context, there is a paucity of studies in literature, particularly regarding the optimization of pesticide extraction from honey. While most of these studies have focused on enhancing extraction efficiency, they have generally concentrated on parameters such as solvent quantity, pesticide type, and extraction duration [13, 15, 16]. Although many acaricides (*i.e.* amitraz, cymiazole, flumethrin, imidacloprid and fipronil) prevail in honey due to protect the hive from *Varroa jacobsoni* and *Ascosphaera apis*, other pesticides used in agriculture also exist in honey [17]. It is known that the most used pesticides in Gümüşhane region are cyfluthrin, cypermethrin, deltamethrin and malathion. Therefore, in this study, the determination of those pesticides in honey samples collected from the assigned locations of Gümüşhane was studied to introduce the pollution around the city. For this purpose, after samples picked from eighteen stations (shown in Figure 1) were extracted, extracts were passed through florisil cartridge, then the extracts were analyzed with GC/MS-MS using certain analysis conditions at different flow rates. Also, Box–Behnken Model was employed to design the experiments. The amount of pesticide was employed as a response, while the process variables were stations, flow rate, and pesticide kind. The novelty of this study lies in its analysis of different stations and flow velocities using an experimental design method, which has not been previously reported in the literature.

Figure 1. Sample collection points

II. EXPERIMENTAL METHOD

2.1. Materials and Preparation Techniques

Cyfluthrin, cypermethrin, deltamethrin and malathion (all are Supelco) were purchased from Sigma-Alrich, while methanol was provided from Merck. Honey samples were collected from various points in Gümüşhane (Figure 1). The samples picked were kept in capped vessels at room temperature until treatment.

A SHIMADZU GC-MS TQ8040 system equipped with a triple quadrupole mass spectrometry detector and an electron ionization (EI) source was used in the experiments. The capillary column (HP-5MS) is 30 m x 25 mm x 0.25 mm dimension. Helium with 99.9% purity was used as carrier gas at different flow rates. Oven and injection temperatures are 120 ℃ and 250 ℃, respectively. The pressure is 121.9 kPa, total flow 19.5 mL/min and purge flow 3.0 mL/ min. The accuracy of the applied method was controlled by using reference material. As the equipment includes the SPME unit (Smart SPME Arrow), further solid phase extraction pretreatment was not required in this study.

2.2. Chromatographic analysis

2 grams of honey samples were dissolved in 50 mL methanol: distilled water (1:1) mixture on a magnetic stirrer for 1 h at room temperature. The sample solutions were filtered through Whatman No. 1 filter paper. The standard addition method was used to determine very low concentrations in a complex matrix. Five samples for each station were prepared for chromatographic analysis. 1 mL of filtered sample (in vial) was spiked with volume of pesticide standard solution to last concentration will be between 0.1 and 0.9 mg/L. Then, the last volume was completed to 1.5 mL with methanol. All tests are performed in triplicate.

III. RESULTS AND DISCUSSIONS

The regression equations, determination coefficients, LOD and LOQ values were given in Table 1. As understood from the table, the lowest LOD and LOQ values were obtained for Cyfluthrin. The capillary column HP-5MS, 5% diphenyl/95% dimethyl siloxane crosslinked polymer, is between moderately polar and non-polar character (Figure 2). The pyrethroid pesticides have halogen groups on the tail of the aliphatic chain. Cypermethrin and cyfluthrin contain chloro-groups, while deltamethrin has bromo- groups. Additionally, cyfluthrin has fluoro groups on one of its aromatic rings.

According to Briggs et al., the pyrethroids are highly lipophilic compound [18]. Although cypermethrin and deltamethrin are classified as non-polar pesticides [19], a slight difference in polarity between these molecules is expected due to the presence of halogens with differing electronegativity. Cyfluthrin might have a higher polarity than the rest. Malathion should also possess a slight polar character.

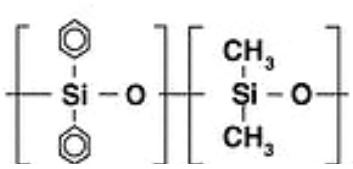
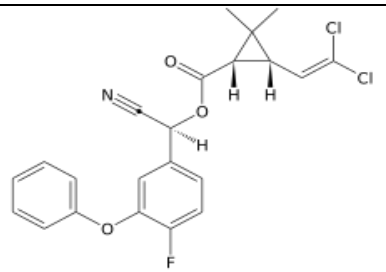
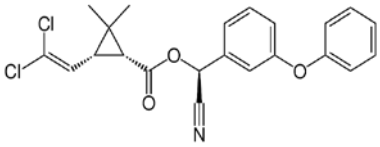
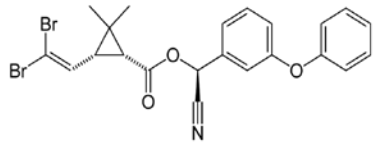
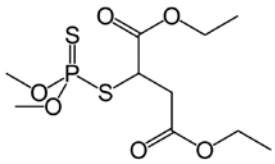


Figure 2. The chemical structure of HP-5MS column

Table 1. Regression equations, determination coefficients, LOD and LOQ values

Analyte	Chemical Structure	Regression Equation	R^2	LOD (µg/L)	LOQ (µg/L)
Cyfluthrin		y=406386x + 2775	0.999	21	70
Cypermethrin		y=498583x + 3175	0.999	29	98
Deltamethrin		y=83706x - 173	0.999	55	182
Malathion		y=2000000x + 16091	0.998	24	79

The pesticide retention on column material diphenyl, dimethyl siloxane polymer can be explained by the formation of the π-π interaction and interaction via slightly polar structures. Considering the earliest obtained peak belongs to malathion (8.53), it is said that malathion has the lowest affinity to material of stationary phase, followed by cyfluthrin (11.9), cypermethrin (12.18), and deltamethrin (13.9), respectively. So, deltamethrin must be the molecule whose affinity to column material is the highest. The existence of aromatic rings in pyrethroid pesticides also supports the contribution of π-π interactions. The lowest detection limit of cyfluthrin is most probably sourced from SPE stage.

No pesticide detected in samples except collected from Stations 1, 6, and 18. Furthermore, cyfluthrin was detected in any sample of each station. The detected pesticides levels obtained at three different flow rates were introduced in Table 2.

The Acceptable Daily Intake (ADI) for cypermethrin, deltamethrin, and malathion are reported by Lu et al. as 0.05, 0.01, and 0.02 mg/kg per day, respectively [20]. According to EPA reports, the oral LD50 values of cypermethrin for male and female rats are 187-326 mg/kg and 150-500 mg/kg, respectively [21]. Similarly, it is reported that this value for deltamethrin varies between 30 and over 5000 mg/kg for rats according to by which vehicle it is taken with aqueous or an oily way [22].

Malathion is also reported as being a low-toxicity pesticide considering the LD_{50} values varying between 5400 mg/kg and 5700 mg/kg up to generation of rats. [23]. Considering the limitations mentioned, it is said that the obtained data indicates a minimal risk associated with exposure via oral consumption of honey collected from Stations 1, 6, and 18. But even so, they are legible so that the detected pesticide residues in samples are rather

below the limited values. Nahhal et al. inspected insecticide residues in 2020 honey samples picked from 27 countries all over the world. As result of their research, they declared that fifty-two different insecticides were encountered in any country, even at least one pesticide. They also reported that the most encountered class of pesticide is organophosphorus insecticides, followed by pyrethroids as cypermethrin and permethrin [24].

Table 2. The pesticides levels obtained at three different flow rates

Station	Flow rate mL/min	Cypermethrin mg/kg	Deltamethrin mg/kg	Malathion mg/kg
	1.2	0.33 ± 0.05	2.80 ± 0.65	0.46 ± 0.06
1	**1.5**	0.51 ± 0.03	3.95 ± 0.60	0.65 ± 0.08
	1.8	0.09 ± 0.01	nd	nd
	1.2	0.55 ± 0.01	3.37 ± 0.85	0.46 ± 0.05
6	**1.5**	0.32 ± 0.02	9.50 ± 0.20	nd
	1.8	0.54 ± 0.05	0.25 ± 0.08	0.18 ± 0.02
	1.2	0.28 ± 0.06	0.61 ± 0.02	0.18 ± 0.05
18	**1.5**	0.20 ± 0.07	3.12 ± 0.20	0.28 ± 0.02
	1.8	0.20 ± 0.03	nd	nd

3.1. Modeling the determination of pesticide amount in honey using Box-Behnken design

Table 3 displays the experimental design matrix for coded and real process variables as well as response. There are a total of 17 runs in the experimental design, which includes three independent process variables with three levels and five central points, as shown in Table 3. The location (A) (Stations 1, 6, 18), pesticide type (B) (deltamethrin, cypermethrin, and malathion), and flow rate (C) (1.2, 1.5, and 1.8 mL/min) are the independent process factors.

Table 3. Independent process variables (coded and actual) and experimental design matrix for determination of pesticide amount.

Coded Factors				**Actual Factors**			**Response (Amount of Pesticide mg/kg)**	
Run	**A**	**B**	**C**	**Station**	**Pesticide**	**Flow rate (mL/min)**	**Actual Value**	**Predicted Value**
1	-1	-1	0	18	Deltamethrin	1.5	3.12	3.1100
2	1	-1	0	1	Deltamethrin	1.5	3.95	3.7300
3	-1	1	0	18	Malathion	1.5	0.28	0.4956
4	1	1	0	1	Malathion	1.5	0.65	0.6619
5	-1	0	-1	18	Cypermethrin	1.2	0.28	0.3388
6	1	0	-1	1	Cypermethrin	1.2	0.33	0.5875
7	-1	0	1	18	Cypermethrin	1.8	0.20	0
8	1	0	1	1	Cypermethrin	1.8	0.54	0.4813
9	0	-1	-1	6	Deltamethrin	1.2	3.37	3.3200
10	0	1	-1	6	Malathion	1.2	0.46	0.1856
11	0	-1	1	6	Deltamethrin	1.8	2.50	2.7700
12	0	1	1	6	Malathion	1.8	0.18	0.2269
13	0	0	0	6	Cypermethrin	1.5	0.32	0.3240
14	0	0	0	6	Cypermethrin	1.5	0.35	0.3240
15	0	0	0	6	Cypermethrin	1.5	0.32	0.3240
16	0	0	0	6	Cypermethrin	1.5	0.29	0.3240
17	0	0	0	6	Cypermethrin	1.5	0.34	0.3240

Experiments were carried out in triplicate in accordance with the BBD experimental design matrix, and the pesticide quantity was ascertained by utilizing the mean experimental data. From the results in Table 3, the amount of pesticide varies between 3.95 and 0.18 mg/kg depending on the experimental conditions. Based on the results, the highest pesticide rate was found at Station1. It was determined that the pesticide with the highest concentration at this station was deltamethrin and its amount was 3.95 mg/kg. The lowest pesticide amount was determined at Station 6. It was observed that the pesticide with the lowest concentration at this station was malathion and its amount was 0.18 mg/kg. Furthermore, in addition to these findings, the highest pesticide concentration (9.5 mg/kg) was observed at Station 6 at a flow rate of 1.5 mL/min in preliminary experiments conducted outside this experimental design. In this study, four mathematical models—quadratic, cubic, two-factor interactions (2FI), and linear—were assessed for their suitability in describing the pesticide amount.

Additionally, the experimental data were subjected to two separate tests: the model summary statistics and the sequential model sum of squares (SMSS). The examination of the data, which are shown in Table 4, showed that the quadratic model for pesticide quantity had a reduced standard deviation and a higher determination coefficient (R^2) value. When compared to other models, the model showed statistical significance overall.

Table 4. Regression statics for pesticide amount

Source	Standard Deviation	R2	R2adj	R2pre	Press	
AC/CS-PVA:3.0						
Linear	0.8843	0.6202	0.5326	0.2944	18.89	
Interactive (2FI)	1.00	0.6263	0.4020	-0.5487	41.46	
Quadratic	0.2376	0.9852	0.9663	0.7650	6.29	Suggested
Cubic	0.0230	0.9997	0.9907		Not defined	Aliased

These findings imply that the connection between the response variables and the independent factors is well described by the quadratic model. The amount of pesticides have the R^2 of 0.985 and a standard deviation of 0.238. With the pesticide amount model accounting for 98.5% of the total variance, the models were statistically significant, as evidenced by the high R^2 value. The high values of the adjusted and predicted R^2 values, which show a respectable degree of proximity to one another, further support the validity of the model. The discrepancy between the adjusted R^2 and the predicted R^2 value should not be greater than 0.2, according to a well-established criterion [25]. As evident from Table 4, the quadratic model most effectively satisfies this condition. Although the R^2adj and R^2 values were approximately equivalent, the cubic model was determined to be aliased. This determination was based on the press and R^2 values, which were deemed unsuitable for selecting this model. Figure 3 shows the actual and predicted value of the response given in Table 3. The actual and predicted values of the response for pesticide amount was very close to each other. This result supported a strong relationship between the actual and predicted value of the responses and strengthens the accuracy of the determined model.

To determine the best independent factors for the pesticide quantity and the most effective parameters among the process variables, analysis of variance (ANOVA) was used. Thus, evaluation of the regression model's applicability at a 95% confidence level was performed. Also, it is understood from the ANOVA whether the means of the experimental results differed significantly from one another.

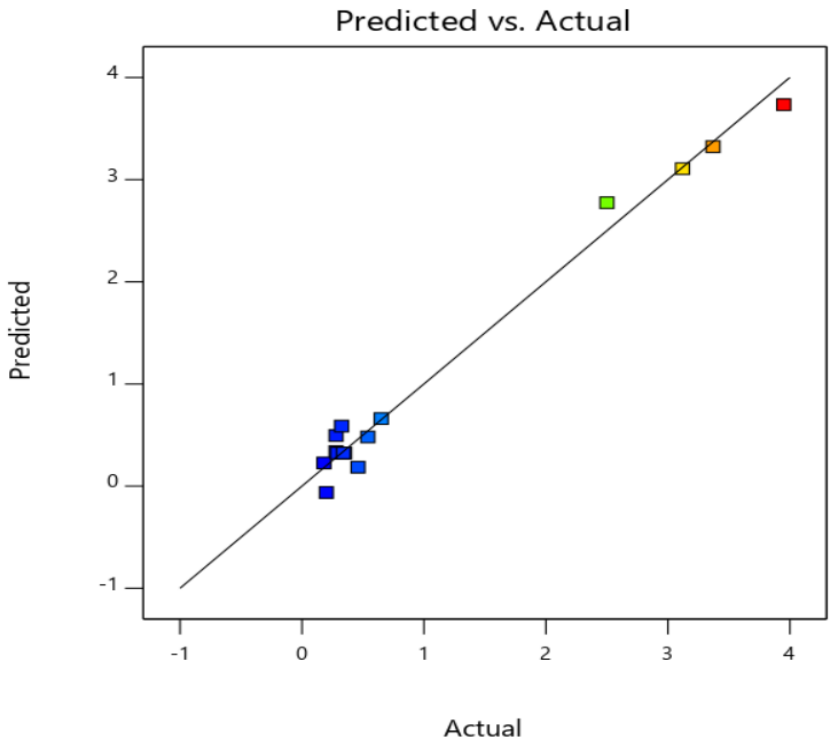


Figure 3. Actual and predicted value of pesticide amount

To enhance the reliability of the experimental results, the model for pesticide quantification was refined based on F-value, p-value, and R-squared, as evaluated through ANOVA. A p-value below 0.05 indicates statistical significance for the model, dependent, and independent variables. Additionally, the F-value and R-square values must be sufficiently high for the model to be deemed statistically significant. Table 5 provided an overview of the pesticide amount ANOVA results. As shown in Table 5, A, B, and C correspond to the type of pesticide, the station, and the flow rate, respectively. The model terms AB, AC, and BC represent the interaction between two independent variables. For example, the interaction between the type of pesticide and the station is indicated by the term AB. The quadratic impacts of the independent variables are represented by the additional model terms A^2, B^2, and C^2. As understood from the inspection of Table 5, the quadratic model has the lowest p-value (<0.0001), demonstrating the model's significance. Furthermore, the model's F-value was determined to be 51.92. The quadratic model demonstrated a strong correlation between the independent variables and response, as evidenced by the comparatively high F-value and very low p-value in the pesticide quantity model.

In other respects, the model-independent parameter exhibiting the lowest p-value (<0.05) and highest F-value was determined to be the most effective parameter for the response. According to first-order independent variables (A, B, C), Table 5 indicated that the p-values of the pesticide type demonstrated lowest value. Consequently, the pesticide type was identified as the most influential parameter for pesticide amount. The station had the highest F-value after the pesticide type for amount of pesticide, suggesting that the station exerted a greater influence on the pesticide amount compared to the flow rate. Moreover, considering all the variables, it can be clearly seen from Table 3, the model parameters B, B^2, A, A^2, (<0.0001, <0.0001, 0.0504) had significant p-value (<0.05). If the F-values are analyzed for significant model parameters, it can be said that the most effective parameters are B> B^2>

A> A^2> C^2>C>BC>AB (286.33> 164.22> 5.56> 2.76> 2.42> 2.28>1.54>0.9373) for pesticide amount respectively.

Table 5. ANOVA analysis for pesticide amount.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	26.37	9	2.93	51.92	< 0.0001	significant
A-station	0.3140	1	0.3140	5.56	0.0504	
B-Pesticide type	16.16	1	16.16	286.33	< 0.0001	
C-Flow rate	0.1288	1	0.1288	2.28	0.1747	
AB	0.0529	1	0.0529	0.9373	0.3652	
AC	0.0218	1	0.0218	0.3855	0.5543	
BC	0.0870	1	0.0870	1.54	0.2543	
A^2	0.1558	1	0.1558	2.76	0.1405	
B^2	9.27	1	9.27	164.22	< 0.0001	
C^2	0.1366	1	0.1366	2.42	0.1637	
Residual	0.3951	7	0.0564			
Pure Error	0.0021	4	0.0005			
Lack of Fit	0.4006	3	0.1335			
Cor Total	26.77	16				

3.2. Response surface analysis

In the current study, 3D surfaces plots produced by a response surface model created with Design Expert software were used to investigate the impacts of independent factors on response. Figure 4 shows the 3D surface graphs for pesticide quantity that were produced using the quadratic model. Three response 3D surfaces were produced since the regression model included three independent variables and one constant factor at the center levels. Figure 4 (a), (b) and (c) represent 3D response surface plots for the combined effect of station-pesticide type, flow rate-station, and pesticide type-flow rate independent variables on pesticide amount, respectively. From the graphs, it is clearly seen that the pesticide amount varied according to the station and pesticide type, and the amount of pesticide does not significantly alter as the experimental flow rate is changed. The pesticide concentration was increased from 0.28 to 0.65 mg/kg, respectively, when the station changes from Station 18 to 1 at constant flow rate (1.5 mL/min) and pesticide type (Malathion). Thus, the 3-fold increase in the amount of pesticides indicates that there can be significant differences in the amount of pesticides, even between different stations in the same region.

The Malathion and Deltamethrin concentrations were found to be 0.28 and 3.12 mg/kg, respectively, for Station 18 and at constant flow rate (1.5 mL/min) was kept constant, and approximately 1188.57% increase in the amount of pesticide was observed. This situation may be associated with the use of agricultural chemicals with different pesticide contents and concentrations in the region. The pesticide concentrations of the samples picked from Station 18 were 0.28 and 0.2 mg/kg at flow rate of 1.2 and 1.8 mL/min, respectively, whereas the Station 18 and pesticide type (cypermethrin) were constant, and no significant change was observed in the amount of pesticide.

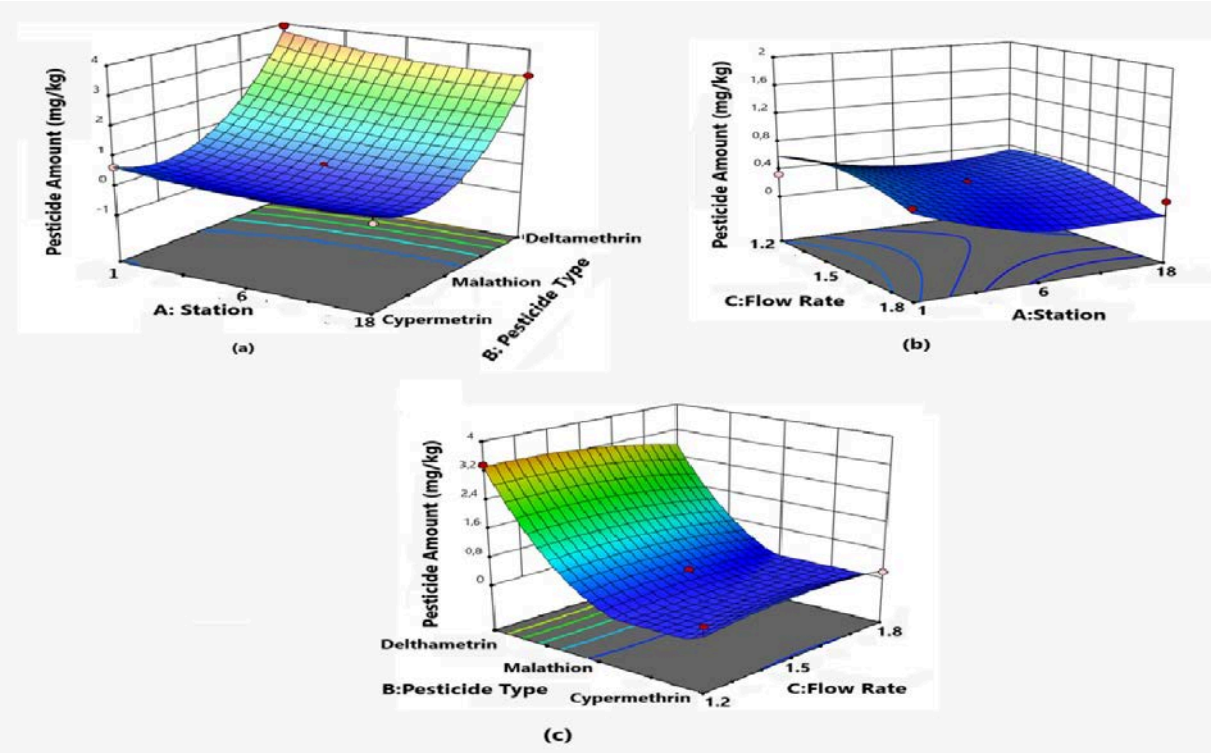


Figure 4. 3D response surface plots for pesticide amount presenting the effect of (a) station and pesticide type (b) flow rate and station (c) pesticide type and flow rate

IV. CONCLUSIONS

It is possible to encounter pesticide residues in honey due to extensive flight times and distance, as well as contact with several plants; even the producer makes tiny distinctions. That's why it is assumed that honey is an excellent indicator for environmental pollution. The investigation of cyfluthrin, cypermethrin, deltamethrin, and malathion residues in local honey samples collected from 18 stations was performed with GC-MS/MS. Cypermethrin, deltamethrin, and malathion were detected in the samples from three stations. The residue levels varied from 0.18 mg/kg to 9.50 mg/kg at 1.5 mL/min flow rate. Although the determined values are rather below the limits of LD_{50} for rats via oral intake, these pesticides were detected in the samples of some stations. 3D response surface plots showed that the station and pesticide type are decisive for the pesticide amount, and that changes in the experimental flow rate had little effect on the amount of pesticide. The results are significant in terms of enabling that further precautions should be taken and that additional monitoring studies should be carried out.

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Authorship Contribution Statement

Erol Erçağ: Supervision, Conceptualization, Methodology, Writing – original draft, review & editing. Berrin

Saygı: Data curation, Writing – original draft, review & editing. Murat Şahin: Investigation. Jülide Hızal: Data curation.

Declaration of Competing Interest:

The authors declare that there is no financial or personal conflicting interest that could influence the work reported in this paper.

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