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Detection of Pesticide Residues in Olive Leaves From İzmir, Turkey

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Abstract

Turkey, especially the Aegean region, has very suitable lands for olive cultivation. In this work, the olive leaves extracts were treated with pesticide solution and also Bordeaux mixture. Olive varieties in their natural environment were collected after 1 year, and the amount of pesticides they contained was measured. As a result of the QuEChERS method using LC-MS/MS and GC-MS/MS techniques, no pesticide residues were detected in any samples. The pesticide residues determined in the samples used in this study do not exceed the maximum limits specified in the Turkish Food Codex and the European Commission. There are no quantifiable residues of abamectin, acetamiprid, azoxystrobin, difenoconazole, diflubenzuron, diflufenican, dimethoate, dodine, emamectin benzoate, indoxacarb, lufenuron, malathion, novaluron, phosmet, pyriproxyfen, spinosad, thiacloprid, and triflumuron were detected in any olive samples by using LC-MS/MS method. Also, alpha-cypermethrin, beta-cyfluthrin, cyflutrin, deltamethrin, and lambda-cyhalothrin were not detected in samples by GC-MS/MS method. Accordingly, they agree with the results found. The results obtained showed that the pesticide solution used protected the olives from pests and did not have a harmful effect on the olives.

Key words: Deltamethrin, GC-MS/MS, QuEChERS method, LC-MS/MS, Olive

1. Introduction

The olive tree (*Olea europaea* L.) has been cultivated in many parts of the world since ancient times. Today, the major agricultural area for the production of the plant is the Mediterranean region. The Mediterranean region is rich in phenols naturally derived from the olive plant. However, this region is also known for its abundant supply of olive resources, which contain nutritious, healthy, and antioxidant benefits (Benavente-García et al., 2000; Ferreira et al., 2007; Lockyer et al., 2016). In our country, the main agricultural area of olive trees is known as the Aegean region. Olive leaves have become popular recently due to their good source of bioactive compounds such as phenolics and flavonoids. They are discarded as a food by-product, and it is reported that the amount of olive leaves that accumulate annually from the industries may exceed 1 million tons (Lama-Muñoz et al., 2019). Olive leaf, which is one of the traditional herbal teas that Mediterranean people often use to treat many diseases such as colds, malaria, and tropical diseases, is also consumed as tea (El and Karakaya, 2009).

Moreover, olive leaves, which can be explained both as wastes from olive oil production and as medicinal and aromatic plants, include many phenolic compounds; oleuropeosides (oleuropein and verbascoside), flavonols, flavones, flavan-3-ols (catechin), substituted phenols, oleoside and secoiridoid glycoside (oleuricin A and oleuricine B) (Putnik et al., 2018; Giacometti et al., 2018; Wang et al., 2021).

Olive fruit is consumed as food after processing, and its oil is a valuable and important food source (Aparicio and Harwood, 2013). Olive varieties have a richness that varies according to the region. In this work, 7 different olive varieties were used. Arbequina olives origin from Spain. Their colours are black when ripe and have a small and spherical-symmetrical shape. The apex is rounded and sessile, its cross-section is rounded and slightly rests on the base. The nucleus is oval and symmetrical. Gemlik variety origins from Gemlik, Bursa. They are known as Trilye, Kaplık and Kıvırcık also. Their fruits are of medium size, close to round, and cylindrical. They are partially resistant to cold. Domat olives origin from Akhisar, Manisa. Their fruits are large and cylindrical. The fruit tip is flat or rounded. They generally produce green-filled olives. Çekişte olives origin from Ödemiş, Izmir. The fruit shape is large, oval. The fruit tip is slightly protruding. It is used as green table olives, crushed olives and oil. Uslu olives origin from Akhisar, Manisa. It grows vigorously under irrigated conditions. The fruit is oval in shape and medium in size, and the nipple is round in shape. The core is easily separated and used as black table olives. Ayvalık variety origins from Edremit, Balıkesir. It constitutes 25% of the tree that exists in the Aegean region. The yield is good and moderate. It is partially resistant to cold. Fruit size is medium and its shape is cylindrical, close to round. It is also known as Şakran, Midilli, and Edremit olives. Karamürsel olives origin from Kocaeli. Its fruit is very large and oval and matures early. The yield is medium. It is considered as a table food because it contains a low rate of bitterness. They are also known as water olive (Çetin et al., 2015).

The olive tree is affected by several diseases such as anthracnose (*Colletotrichum gloeosporioides* Penz.), olive canker, olive fly (*Bactrocera oleae*), olive wolf (*Coenorrhinus cribripennis*), olive moth (*Prays oleae*), Lepra fruit rot, *Parlatoria oleae* and *Aspidiotus nerii* (Uguz and Uysal, 2021). The most efficient way to protect fruits and vegetables from harmful organisms and diseases is the use of pesticides.

Especially when Bordeaux mixture is applied to fruit trees in autumn (when 75% or all of the leaves are shed) and in spring, it is an effective and economical agricultural pesticide against many potential diseases that may occur in the season. When this slurry is used in the appropriate dose and period, it protects against many fungal and bacterial diseases. It increases the resistance of our tree against adverse weather conditions and frost risk that may occur in winter. It helps to protect trees from frost in winter and increases their resistance to drought in summer. Among the main diseases in which it is used are branch cancer (*Pseudomonas savastanoi*) and ring spot diseases (*Spilocaea oleginea*) for olives (Turkmenoglu et al., 1974; Demircan and Yilmaz, 2005).

Deltamethrin is an α-cyano pyrethroid insecticide widely used in pest defense. Although initially thought to be of low toxicity, recent studies contain data on toxicity in a wide variety of animal species (Pham et al., 1984). However, its antioxidant effect has also been proven by studies. As it is effective against various harmful organisms in many vegetables and fruits, its effectiveness in olives is against *Bactrocera oleae*, *Prays oleae* and *Saissetia oleae* (Hasibur et al., 2006; Radovanovic et al., 2017).

The QuEChERS (quick, easy, cheap, effective, rugged, and safe) method is widely used as an extraction method in pesticide residue analysis for fruits and vegetables (Gonzalez-Curbelo et al., 2015; Acosta-Dacal et al., 2021). Some parameters, such as the polarity of the solvent and the volume of solvent used, were adjusted to enable the quantitative determination of target compounds. The method has been validated for linearity, accuracy, and precision. The validated method accurately quantifies 23 pesticides through two complementary analyses by LC-MS/MS and GC-MS/MS (Lopez et al., 2020). To the best of our knowledge, there is no study about the determination of pesticide residues on olive leaves collected from İzmir, Turkey. Therefore, the purpose of this study was to optimize the validated QuEChERS-based extraction method for the quantitative detection of pesticide residues in olive leaves, including many pesticides currently being investigated in food products, as well as other environmental pesticides.

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2. Material and Methods

2.1. Plant Materials

Dried 7 different olive leaves (*Olea europaea* L.) were collected from Dallık, Aktepe village, Beydag (Odemis, Izmir, Turkey) in June 2021. Leaves were stored in a dry and dark room until extraction procedures. Herbs were milled using a knife mill (Retsch GmbH SK 1; Germany). Afterward, extracts of the powdered plants were prepared using 96% ethanol at a concentration of 5 g/100 ml. Extraction was completed in a shaker incubator (Thermo Scientific Max Q 6000) at room temperature for 24 hours. The extracts obtained were filtered, and after the liquid filtrates obtained of the extract were concentrated and evaporated to dry at 40°C temperature with the help of a rotary evaporator under vacuum pressure to get the crude extracts. The yield of the extract was calculated as 6.4%.

2.2 Chemicals and reagents

QuEChERS kits were obtained from Chromascience. Ammonium formate (5 mM), formic acid (1%) and acetonitrile (100%) were obtained from Merck. Certified pesticide standards [Abamectin (HPC Standards, 90.20%), acetamiprid (HPC Standards, 99.90%), azoxystrobin (Toronto Research Chemicals, 98%), difenoconazole (HPC Standards, 98.61%), diflubenzuron (HPC Standards, 99.60%), diflufenican (HPC Standards, 99.90%), dimethoate (Sigma Aldrich, 99.40%), dodine (Sigma Aldrich, 90.00%), emamectin benzoate (HPC Standards, 98.51%), indoxacarb (HPC Standards, 99.30%), lufenuron (HPC Standards, 99.50%), malathion (HPC Standards, 98.20%), novaluron (HPC Standards, 99.60%), phosmet (HPC Standards, 98.40%), pyriproxyfen (Toronto Research Chemicals, 96%), spinosad (Chem Service, 94.20%), thiacloprid (HPC Standards, 99.90%), triflumuron (Sigma Aldrich, 99.90%), alpha-cypermethrin (HPC Standards, 98.10%), beta-cyfluthrin (HPC Standards, 98.60%), cyflutrin (HPC Standards, 98.60%), deltamethrin (HPC Standards, 99.70%), and lambda-cyhalothrin (HPC Standards, 99.64%),] were obtained from Skygen.

2.3. Selection of pesticides

Bordeaux mixture (Bordo mix or Bordeaux slurry) is a mixture of copper (II) sulfate and slaked lime used as a fungicide in vineyards. It is used against fungal diseases especially in the garden, nursery, and field plants, especially vineyard downy mildew plant disease caused by *Plasmopara viticola* fungus. It was invented in the Bordeaux region of France. All olive species were exposed to the Bordeaux mixture in February 2020. Moreover, deltamethrin was used as a pesticide in April 2020. This pesticide, which is frequently used in our country, was also applied to the plants in this study.

2.4. Preparation of standard solutions

The stock solutions of each pesticide standard were prepared by exactly weighed of each analyte in volumetric flasks and dissolving in acetone, ethanol, acetonitrile, methanol, and etc., according to the solubility of pesticides to reach 2000 ppm stock solutions. The standard mixture was prepared from the stock solution of each pesticide to reach 100 or 200 ppm standard mixture, and the standard solution was kept at -20 °C. Using the working standard solutions, 5 series of calibration standards were prepared with the range of 10-200 µg/L by serial dilution in acetonitrile.

2.5. Extraction procedure and analysis techniques

The QuEChERS method can be summarised as 15 g of sample was weighed into falcon tube than 15 ml acetonitrile (1% acetic acid) and QUECHERS extraction pouch (1.5 g sodium acetate + 6 g magnesium sulphate) added into the falcon. The mixture was shaken for 1 min by using a vortex. After this process, the mixture was immediately centrifuged at 500 rpm/min for 4 min at 4 ºC. Nearly 8 ml of supernatant (organic phase) was taken and cleaned using the QUECHERS clean-up kit (1200 mg magnesium sulphate + 400 mg PSA). The mixture was shaken for 1 min by using a vortex. Then, the mixture was immediately centrifuged at 500 rpm/min for 4 min at 4 ºC. The supernatant phase was filtered through a 0.20 µm filter and taken into a vial. After these procedures, the vials were ready for LC-MS/MS and GC-MS/MS routine pesticide analysis (Figure 1).

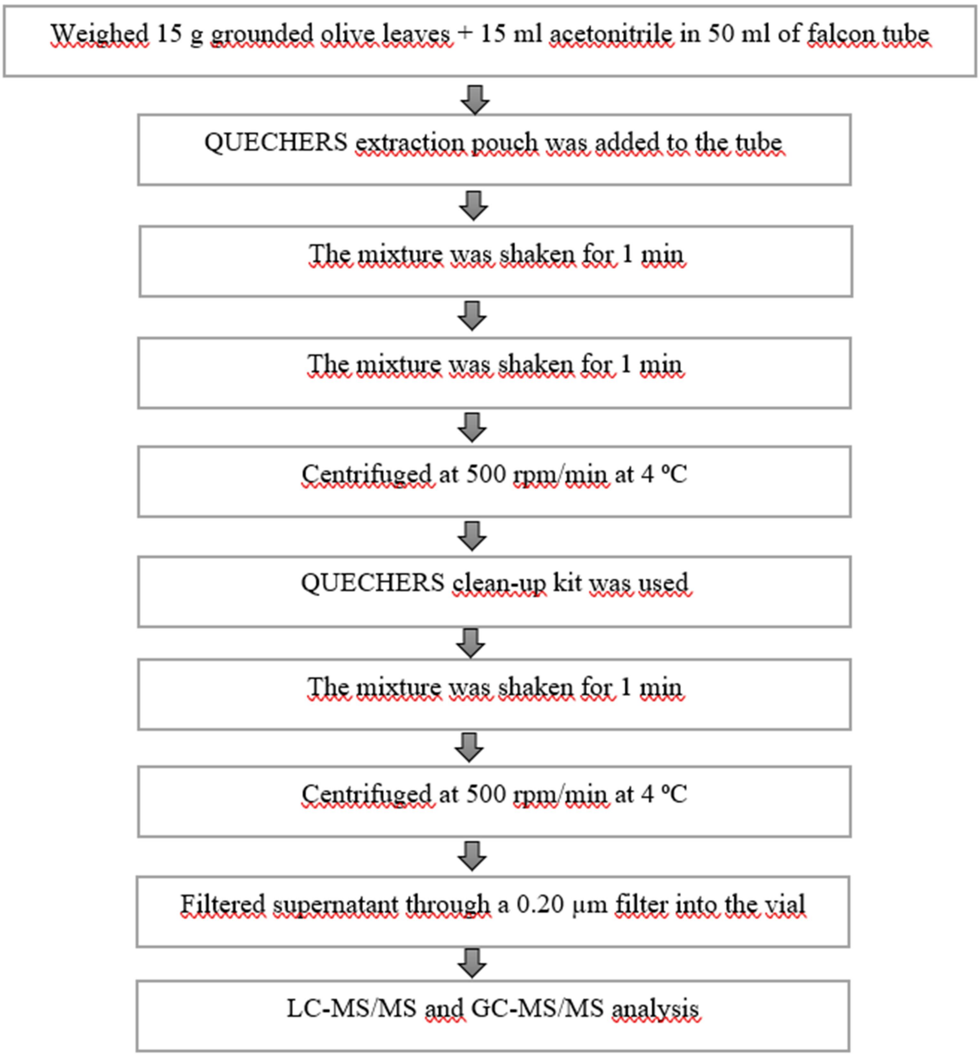


Figure 1. Flow chart of the analytical method applied for the pesticide determination of olive leaf extracts.

LC-MS/MS (8060 Shimadzu) analysis parameters were summarised as; 20 min analysis time, 0.7 ml/min. Total flow rate, 40 ºC column oven, 5 µL injection volume. 2 mobile phases were used. Mobile phase A consisted of ammonium formate (5 mM), formic acid 1%, and distilled water. Mobile phase B was consist of 100% acetonitrile. GC-MS/MS (PQ 8040 NX) analysis parameters were set as 16 min analysis time, 1.33 µL/min total flow rate, 120 ºC column temperature (T0: 120 to Te: 300 ºC), 2 µL injection volume, and helium carrier gas.

2.6. Method validation

The QuEChERS method was in-house validated according to SANTE/11813/2017 guideline (European Commission, 2017). Amplified calibration curves on empty olive leaves for quantification (1, 2, 5, 10, 50, 100, and 200 µg/kg). The calibration curves for pesticide standards were generated by plotting the relative responses to relative concentrations of pesticide standards. The pesticide standards were spiked into blank olive leaves matrices with four replicates to evaluate the recovery of the method. Relative standard deviation (RSD) values (0.94%) were measured for deltamethrin using signals supporting figure 1A, spiked into blank olive leaves matrices with five replicates to evaluate the recovery and precision of the method. Sensitivity studies were done to determine the limit of detection (LOD). For that reason, the GC-MS/MS column was analyzed using various concentrations of deltamethrin (linear range between 0.002 and 0.4 μM). Test results gave a response between the selected deltamethrin concentrations. The LOD value was calculated for GC-MS/MS as 0.54 μM. LOD studies were based on linear regression. The instrument response y is assumed to be linearly related to the standard concentration x for a limited concentration range for a linear calibration curve. This can be expressed in a model such as $y=a+bx$. This model is used to calculate sensitivity b and LOD. Therefore, LOD can be defined as $LOD=3Sa/b$; where Sa can be expressed as the standard deviation of the response and b as the slope of the calibration curve (Sahyar et al., 2019). The accuracy (97-101 %) and recovery (103.58%) values were also calculated.

3. Results and Discussion

In this study, common pesticides used in olive, trees, leaves, and related products to control and detect pesticide contamination were analyzed. There is no cross-contamination of these pesticides, and deltamethrin is also degraded from pesticide application to harvesting time.

3.1. GC-MS/MS analysis

Alpha-cypermethrin, beta-cyfluthrin, cyflutrin, deltamethrin, and lambda-cyhalothrin are the pesticides, which were analyzed by GC-MS/MS.

3.2. LC-MS/MS analysis

Abamectin, acetamiprid, azoxystrobin, difenoconazole, diflubenzuron, diflufenican, dimethoate, dodine, emamectin benzoate, indoxacarb, lufenuron, malathion, novaluron, phosmet, pyriproxyfen, spinosad, thiacloprid, and triflumuron are the pesticides, which were analyzed by LC-MS/MS.

3.3. Linearity

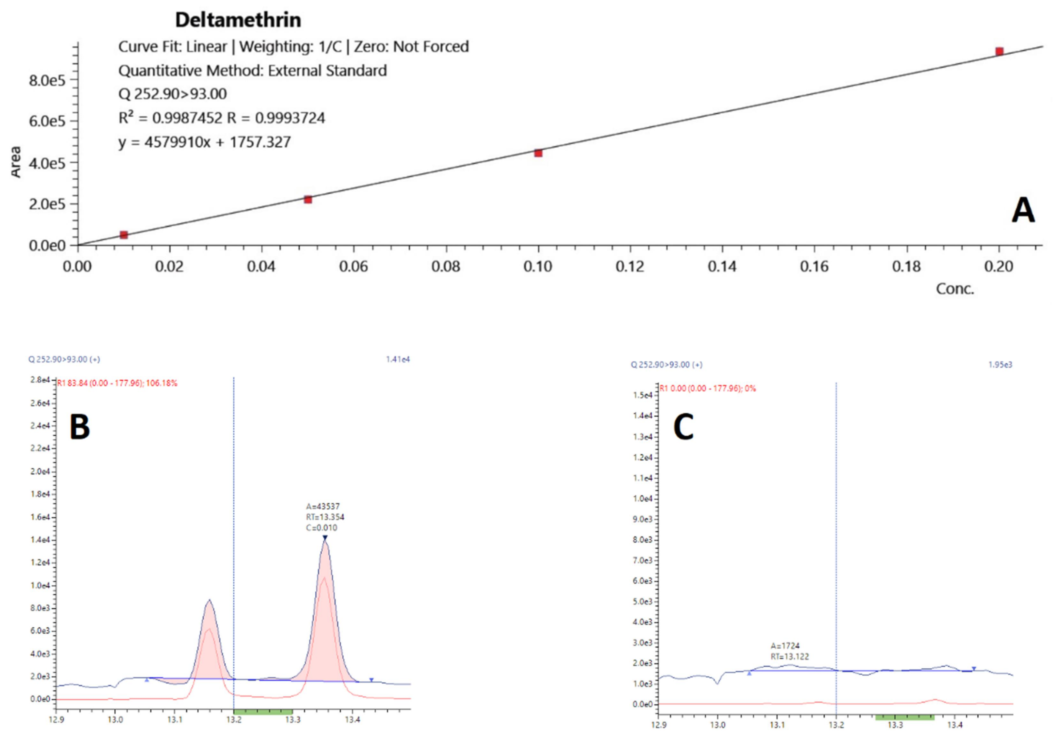
These pesticides are generally used in olive, trees, leaves, and related products. Compounds analyzed in olive samples with the category of use, legal status, analysis technique and values of maximum residue limit (MRL) were shown in Table 1. Analysis results are shown that our product is not included these pesticides.

Table 1. Compounds analyzed in samples with the category of use, legal status, analysis technique and mass spectrometric conditions.

N°	Compound	Category[a]	Legal status in EU	MRL µg kg^{-1}	Technique[d]
1	Abamectin	I, A, AH	Approved[b]	0.04[c]	LC
2	Acetamiprid	I	Approved	0.9	LC
3	Alpha-cypermethrin	I	Approved	0.05	GC
4	Azoxystrobin	F	Approved	0.01	LC
5	Beta-cyfluthrin	I	Not	0.02	GC
6	Cyflutrin	I	Not	0.02	GC
7	Deltamethrin	I, A	Approved	0.04	GC
8	Difenoconazole	F, MB, WP	Approved	0.6	LC
9	Diflubenzuron	I	Approved	0.01	LC
10	Diflufenican	H	Approved	0.02	LC
11	Dimethoate	I	Not	0.01	LC
12	Dodine	F	Approved	0.01	LC
13	Emamectin benzoate	I	Approved	0.01	LC
14	Indoxacarb	I	Approved	0.02	LC
15	Lambda-cyhalothrin	I	Approved	0.2	GC
16	Lufenuron	I	Not	0.01	LC
17	Malathion	I	Not	0.02	LC
18	Novaluron	I	Approved	0.01	LC

[a]A – acaricide, MB – microbiocide, AH – anthelminthic, F – fungicide, H – herbicide, I – insecticide, WP – wood preservative. [b]The legal status reflecting the EU Pesticide Database was considered (https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=activesubstance.selection&language=EN), which is valid for the entire EU. [c]Pesticide MRL (maximum residue limits) values according to European Commission. [d]GC: Gas chromatography, LC: liquid chromatography.

Deltamethrin standard calibration curve and sample chromatograms can be seen in Figure 2.



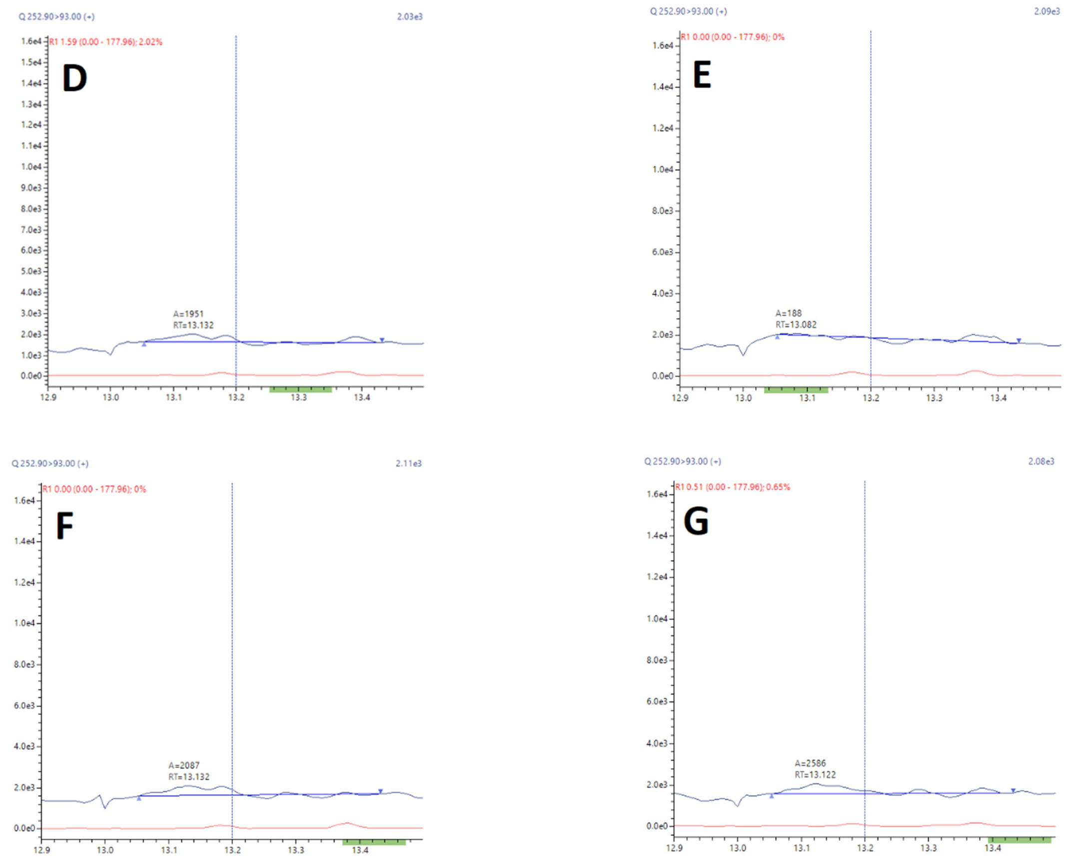


Figure 2. (A) Deltamethrin standard calibration curve, (B) 10 ppb standard chromatogram of deltamethrin and chromatograms of (C) Arbequina olives sample, (D) Domat olives sample, (E) Gemlik olives sample, (F) Karamürsel olives sample, (G) Çekişte olives sample, (H) Uslu olives sample, (I) Ayvalık olives sample.

4. Conclusion

The values of MRL given in the European Commission are shown in Table 1. Besides, according to Turkish Food Codex, MRL values of deltamethrin, thiacloprid, lambda-cyhalothrin, cyflutrin, dimethoate and alpha-cypermethrin for olives are 1 mg/kg, 4 mg/kg, 1 mg/kg, 0.02 mg/kg, 2 mg/kg and 0.05 mg/kg, respectively. And also the average MRL of abamectin, acetamiprid, azoxystrobin, indoxacarb and thiacloprid are 0.02 mg/kg, 0.3 mg/kg, 1 mg/kg, 0.3 mg/kg and 0.3 mg/kg, respectively for various vegetables (Turkish Food Codex, 2020). Accordingly, they are in agreement with the results found. Besides, in another reference, the tolerance limits of malathion, dodine, spinosad for vegetables are 3 mg/kg, 5 mg/kg and 0.02 mg/kg, respectively (PFA, 1954). When the EU MRL values are also examined, it is seen that they are within the given limit values (Table 1). As a result of this study, the fact that the olive cultivars in question do not contain any pesticide residues in their natural environment without being exposed to any pesticide cleaning process increases their importance. The pesticides used for the pests on the olive protected the olives from diseases, but fortunately, they did not leave any harmful effects on the olives.

With the application of the exposure determination method, none of the samples were found to exceed the toxicological reference value. Therefore, there is no harm in consuming olive leaves as food. The determination of deltamethrin and other pesticide residues in vegetables and fruits is important to confirm compliance with EU MRLs and determine the risk associated with their consumption as food.

When the literature studies are examined, it is seen that there are studies of pesticide residues in olive oil and different olive samples (Pena et al., 2006; Tognaccini et al., 2019; Barrek et al., 2003; Sarbishegi et al., 2018; Zuntar et al., 2019; Rebora et al., 2020). However, no pesticide analysis study was found with the olive leaves varieties used in this study, which increases the importance of the study.

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Conflicts of Interests

Authors declare that there is no conflict of interest.

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