ORIGINAL ARTICLE

Evaluation of Tetracycline Antibiotic Residue in Honey Samples Using ELISA and HPLC

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ABSTRACT: Honey is used worldwide due to its medicinal and nutritional properties. Antibiotics are used to treat diseases such as American foulbrood and European foulbrood or as a drug for preventing disease in the beehives. Antibiotic residues should be carefully monitored because they can have adverse effects on the general health of human. In this study, the amount of tetracycline residue was measured in honey samples. A total of 80 honey samples were collected from different regions of Qazvin province, Iran. The methods used included enzyme-linked immunosorbent (ELISA) assay and high-performance liquid chromatography (HPLC). ELISA method showed that the maximum and minimum levels of tetracycline residue were 40 ppb and 1.26 ppb, respectively. The areas with values above the kit's LOD include Takestan (14.28%), Abeyek (4.76%), and Alamot-e-gharbi (4.54%), respectively. In the Alamot-e-sharghi samples, the antibiotic values above the kit's LOD were not found. Samples with values above the kit's LOD in ELISA method were measured using HPLC method. According to ELISA results, of the 80 honey samples, 4 samples (5%) had antibiotic more than the highest LOD of the kit. These 4 samples were tested using HPLC method. The results of HPLC showed that out of 4 honey samples, one sample was more than 40 ppb, but 3 samples were less than 40 ppb. There is a significant difference between ELISA and HPLC ($p < 0.05$). If the antibiotic residue levels of tetracycline are too high in food, it can cause serious harm to the health of consumers, therefore, monitoring of antibiotics residue in food is very necessary.

INTRODUCTION

Honey has medicinal and nutritional properties and is therefore widely used by consumers [1]. In human history for a long time, honey was the only sweetener and important source of carbohydrate until industrial sugar took its place [2]. Honey is rich in nutrients and used worldwide, however, due to honey is produced in a natural

environment it maybe contaminates by various natural substances [3]. Annual production of honey is approximately 1.4 million tons [4]. It has been forecasted that the World Honey Market will reach 2.4 million tons by 2022 and most of this growth is thought to be owing to the fact that honey is a natural product and there are no obesity problems related to honey consumption that are found in other sources of sugar [5]. Nowadays, honey is produced in areas infected with different materials. For example, environmental pollutants, including bacteria, heavy metals, radioactive substances and pesticides [4]. Veterinary drugs are widely applied to control or prevent diseases. However, the use of inappropriate methods can lead to the possible presence of drugs in foods of animal origin and also have detrimental effects on human health [6]. Antibiotics are compounds used to prevent or treat illness in humans, plants, and animals [7]. It is appraised that 100-200 thousand tons of antibiotic materials are produced worldwide, and according to the WHO, roughly half of the antibiotics produced in non-humane programs are consumed [8]. Tetracyclines are a group of antibiotics that act against gram-negative and gram-positive bacteria [9]. There are currently more than 20 types of tetracycline antibiotics, among them, tetracycline, oxytetracycline, doxycycline, and chlortetracycline are the most applied in veterinary drugs [10]. Tetracycline is used because of its good stability, high antimicrobial ability, and low cost for the prevention and treatment of disease [11]. The European Union has set a maximum residue limit (MRL) of antibiotic tetracycline in food of animal origin to be 100 μg kg^{-1} that is equivalent to 140μg l^{-1} [9]. If antibiotic residues of tetracycline are exceeded in food, they will cause serious damage, especially in infants and children younger than 12 years of age [12]. Due to having allergen and carcinogen factors, antibiotic residues in foods can endanger public health, so they must be carefully controlled. Antibiotic residues can also lead to bacterial resistance [7]. Nowadays, various methods are used to identify and determine drug residues in food products [13]. Among different methods,

enzyme-linked immunosorbent (ELISA) is widely used due to its high ability to analyze test results. Additionally, in many articles ELISA method has been used to detect tetracycline [14]. High performance liquid chromatography (HPLC) is one of the strongest devices which are capable of isolating, diagnosing, and measuring drug residues in foods. Its application in the analysis of the remaining materials in food products is on the rise [15].

In this study, Tetracycline was measured in honey samples. Samples were collected from different regions of Qazvin province. The first method used to measure the antibiotic residue was ELISA. Then samples with values above the kit's Limit of detection (LOD) in ELISA method were measured using HPLC.

MATERIALS AND METHODS

Sampling

80 honey samples were collected from different regions of Qazvin province (Takestan, Abeyek, Alamot-e-sharghi and Alamot-e-gharbi) in 2019. Samples were transferred to Food Safety Laboratory of Qazvin University of Medical Sciences under appropriate conditions and kept at 4°C until the start of the experiments.

Analysis of tetracycline residue

ELISA assay

The antibiotic residue of tetracycline was analyzed using ELISA method. In order to determine tetracycline, ELISA method according to the constructor's instructions [RIDASCREEN tetracycline ELISA kit (R 1501), Europeroxima, Netherlands] was accomplished. One gram of honey sample was mixed with 1.5 ml of methanol (80%). Then, to remove impurities, the solution was centrifuged for 5 minutes at 2,000g.

According to the kit instruction, first, into each well in duplicate were added 50 μl of antibiotic standards and tetracycline extracts. Then, 50μl of conjugate (tetracycline-HRP) was added to all wells, except H$_1$ and H$_2$. In the next step, 100 μl of substrate solution was added into each well.

At the last step, at the presence of desired antibiotics, the addition of the stop reagent (which contains 1 M sulphuric acid) led to a color change from blue to yellow. The limit of detection (LOD) and limit of quantification (LOQ), respectively were 0.02 ng g^{-1} and 0.05 ng g^{-1}. The measurement was made by spectrophotometry at 450 nm (Multiskan plus MK2 spectrophotometer from Elvetec Service, France) [4].

HPLC analysis of honey samples

Sample preparation

The samples with values above the kit's LOD in ELISA method were measured using HPLC. 10 g of honey samples was transferred to the 50 ml Falcon tube. 20 ml PBS was added with pH 7.2. The samples were then divided into small pieces and homogenized using Ultra Turrax T25. The extraction method was followed by adding 20 ml PBS, vortexing for 2 minutes, and sonicating for 15 minutes. The tubes were centrifuged for 10 minutes with at 1400 g. Then, supernatants were transmissive to fresh tubes, and 3 ml of tri-chloroacetic acid (15%) to each tube was added. After vortexing, samples were centrifuged for 10 minutes at 1400 g, and then, supernatant was transferred to a solid phase extraction (SPE).

Solid phase extraction (SPE)

To perform SPE, before extraction with water and methanol, cartridge C18 (3 ml, 5 mg, J.T. Baker, Netherlands) was distilled. Samples of chemical extraction were transmitted via the cartridge. The columns were laundered with distilled water then, dried with N2 stream. Eventually, the bound compound to the cartridge was washed with methanol. The washed samples under a gentle N2 stream were dried and dissolved in the mobile phase for analysis of HPLC.

HPLC

According to the method described above, the level of Tetracycline in the extracted samples was determined using the HPLC method. The chromatography system consisted of dual pumps (HPLC wellchrom pump, K-1001, KNAVER Germany) and automatic sampling (Autosampler Triathlon type 900, Germany). A total volume of 20 µl of the extracted sample was injected into ODS C18 (250 _ 4.60 mm column, 5 mm, Phenomenex). The mobile phase consists of a mixture of water–methanol (60:40, V / V) at a flow rate of 1.0 ml min^{-1}. Tetracycline was determined at the wavelength of 276 nm using a UV detector (RF-10AXL KNAUER, Germany). The levels of Tetracycline were determined using an external standard by measuring the areas under the peaks and comparing them with the peaks produced by Tetracycline in HPLC. The LOD for tetracycline was found by measuring the signal-to-noise ratio at 3 and 2.5ng g^{-1}. In order to obtain a calibration curve for tetracycline, different standardized concentrations of 0 to 100 ng / ml (r2 = 0.9992) were used. For each sample Mean recovery and RSD were acquired with three times spiking of 5ng g^{-1} [7]. In Figure 1, the chromatogram of mixed standard solution of tetracycline (100 ppb) has been shown.
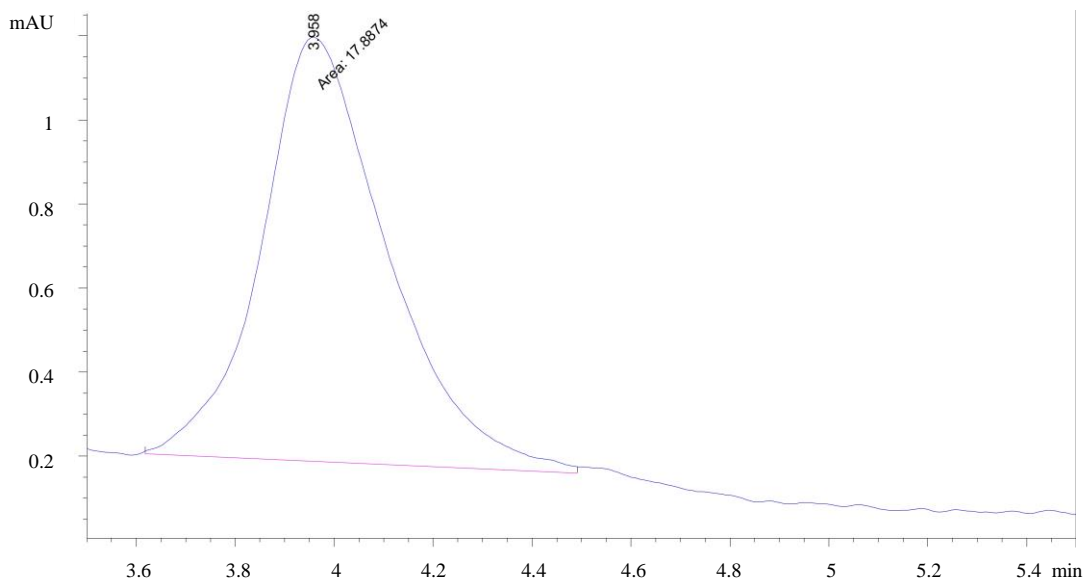
Figure 1. Chromatogram of tetracycline mixed standard solution **(100 ppb)**

Statistical analysis

The data are expressed as mean ± SD, minimum and maximum. In this study, the analysis of variance (ANOVA) was used to determine the statistical significance for mean of tetracycline residue in different regions of Qazvin province. Mann-Whitney test was used to analyze the difference between ELISA method and HPLC. Statistical analyses were performed using the statistical package SPSS for Windows, PC software, version 11.5.1 (SPSS Inc., Chicago, IL, USA). The statistical significance was defined as $p < .05$.

RESULTS AND DISCUSSION

The results of ELISA showed that out of 80 honey samples, 71 samples had normal level of antibiotic, 4 samples had values above the kit's LOD and 5 samples contained the antibiotic levels less than the minimum detectable amount of kit. The highest and the lowest antibiotic residues of tetracycline detected by ELISA were 40 ppb and 1.26 ppb, respectively. According to Table 1, 14.28% of Takestan samples, 4.76% of Abeyek samples, 4.54% of Alamot-e-gharbi samples had values above the kit's, while the antibiotic values above the kit's LOD were not found in Alamot-e-sharghi samples. The results of HPLC showed that out of 4 honey samples, one sample was more than 40 ppb, but 3 samples were less than 40 ppb. Figures 2–5 illustrates the standard curve of different concentrations of tetracycline. Box plot for the antibiotic residue of tetracycline in different regions presented in Figure 6. The plot includes five values: the minimum value, the 25th percentile (Q_1), the median (The thick line within the box), the 75th percentile (Q_3), and the maximum value. Moreover, Q3-Q1 is interquartile range (IQR), maximum value defined as Q3+1.5IQR and if a value is bigger than maximum value, it is considered outlier (as an instant sample No. 19 and 31, which tetracycline level was 18.23 and 40 ppb, was considered an outlier). According to Table 2, there is a significant difference between ELISA and HPLC ($p < 0.05$).

Table 1. Results of analysis of honey samples using ELISA method

	Number of samples	Mean	Std. Deviation	Min (ppb)	Max (ppb)	Concentration standard (ppb)	Samples containing tetracycline with values above the kit's LOD (%)
Takestan[a]	14	15.9493	11.16455	6.63	40.00	100	**14.28**
Abeyek[b]	21	7.4700	8.40906	1.26	40.00	100	**4.76**
Alamot-e-sharghi[b]	23	9.4730	9.96586	2.19	38.80	100	**0**
Alamot-e-gharbi[b]	22	8.6832	9.00409	1.26	40.00	100	**4.54**
Total	80	9.8634	9.80518	11.34	158.8	100	**5.0**

a-b: different superscripts within a column indicate significant differences ($p < 0.05$).
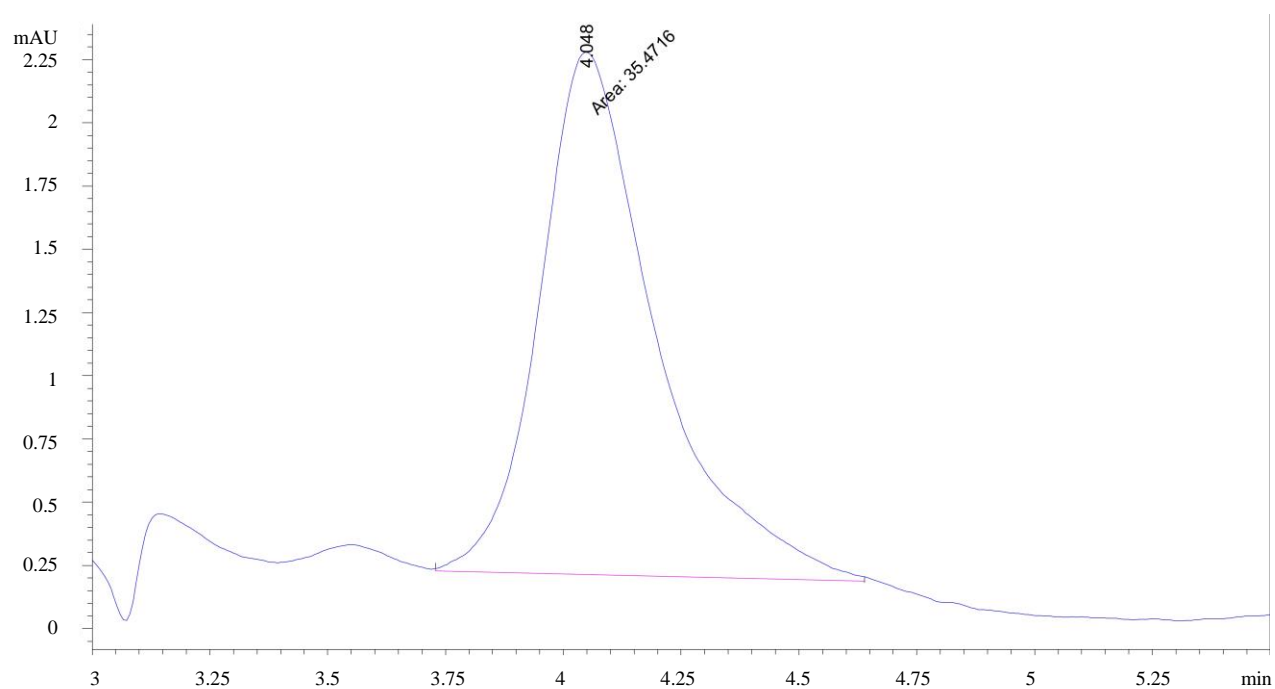

Figure 2. The chromatogram peak of sample No 1 - the calculated concentration is **52.58 ppb**
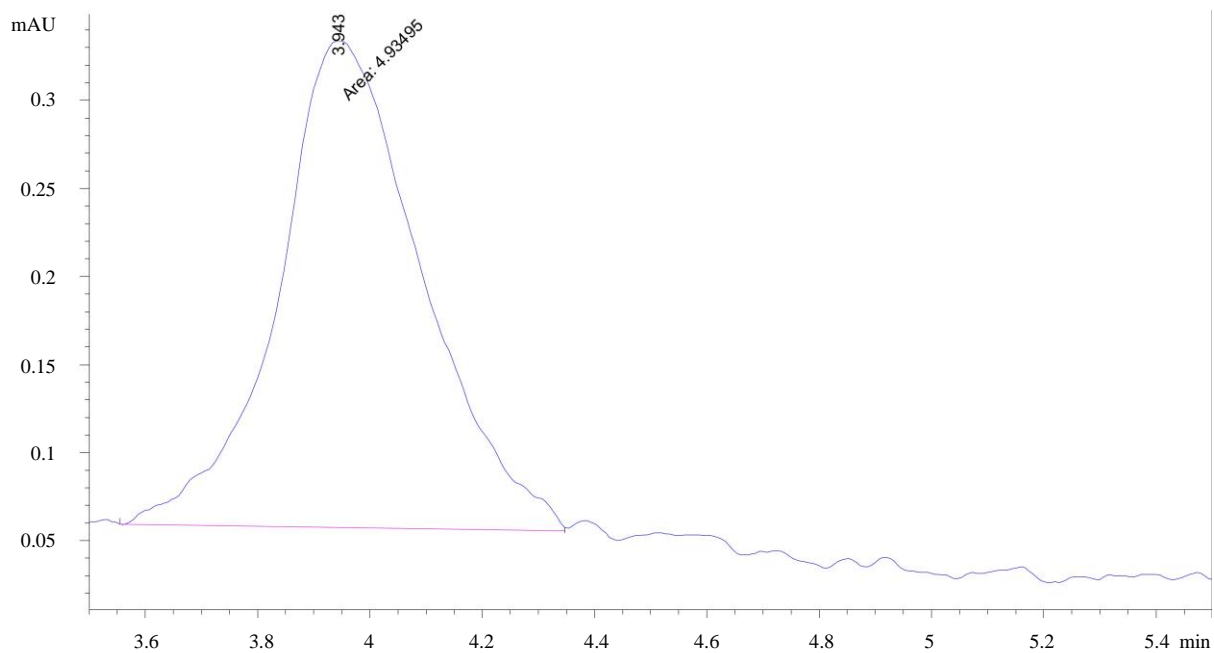
Figure 3. The chromatogram peak of sample No 2 - the calculated concentration is **14.49 ppb**
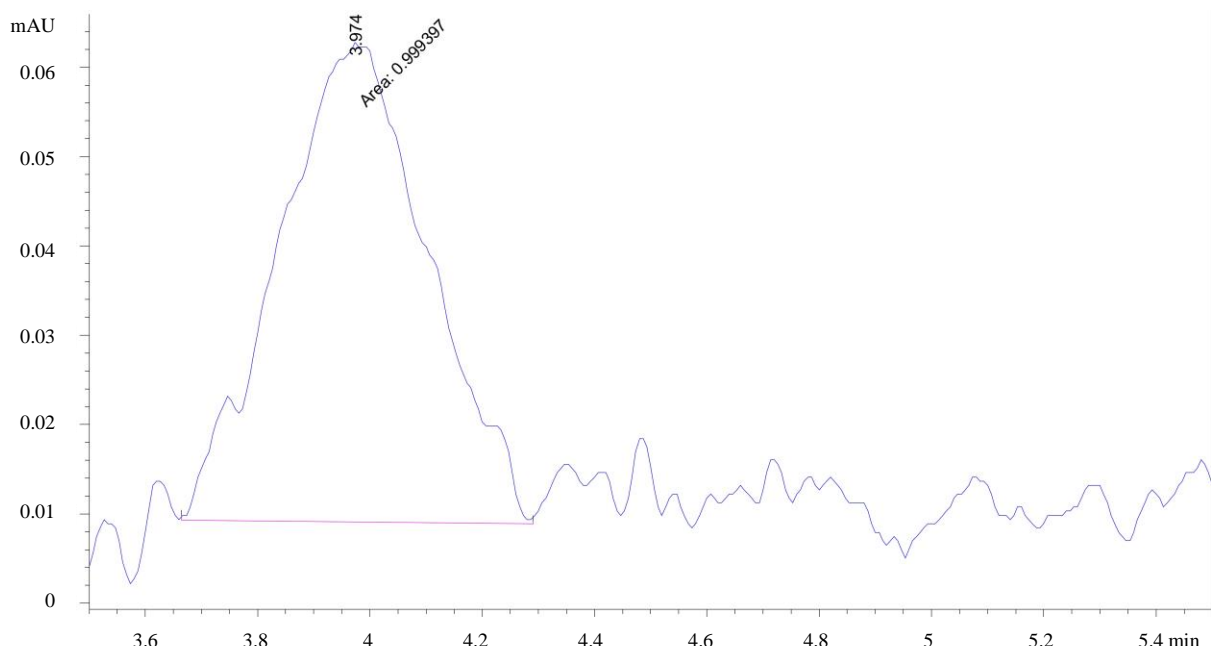

Figure 4. The chromatogram peak of sample No 3 - the calculated concentration is **2.92 ppb**
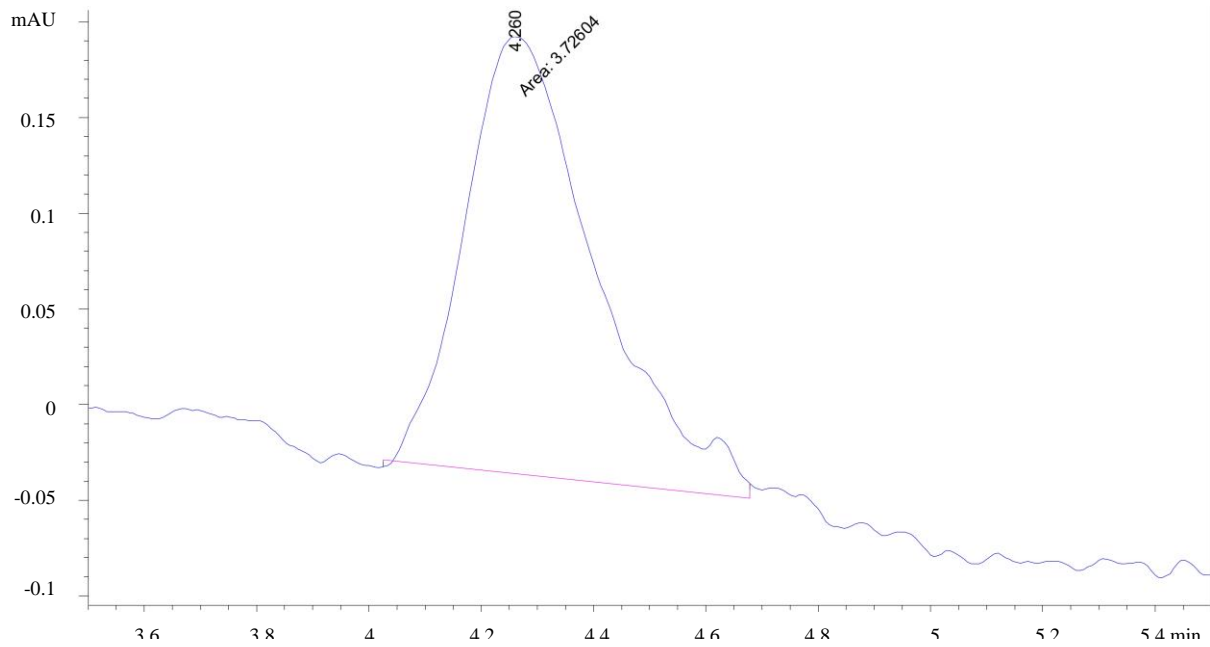
Figure 5. The chromatogram peak of sample No 4 - the calculated concentration is **10.93 ppb**
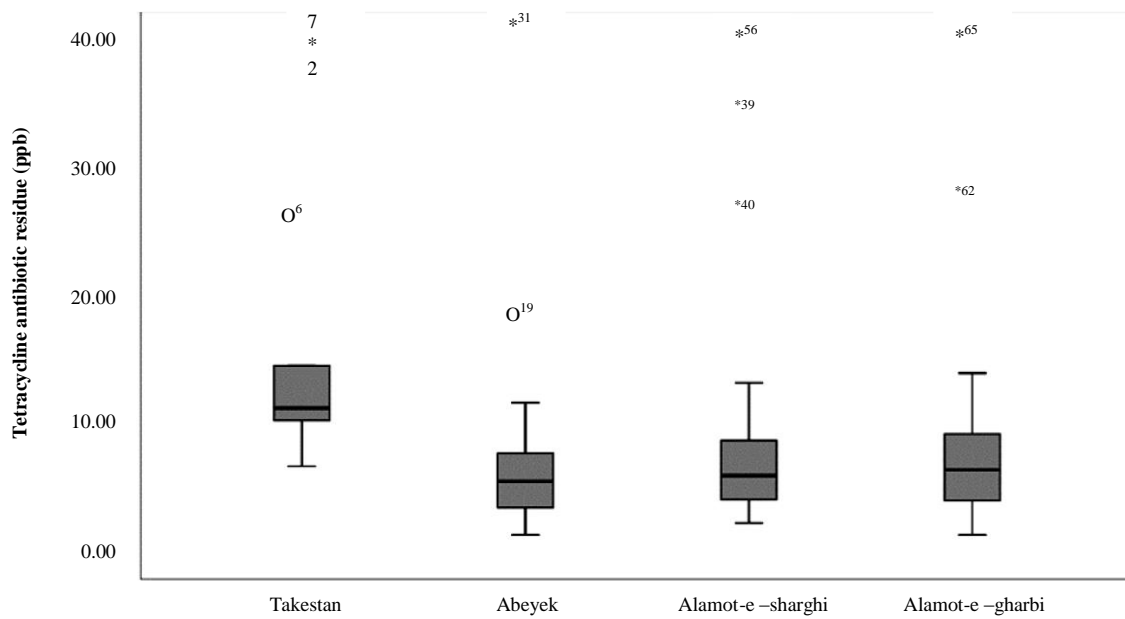

Figure 6. Box plot for Tetracycline antibiotic residue in different regions (* Extreme outlier ° Outlier)

Table 2. Comparison of the results obtained by ELISA and HPLC.

	Mean	Standard Deviation	Minimum	Maximum
ELISA[a]	40.00	.00	40.00	**40.00**
HPLC[b]	20.23	22.10	2.92	**52.58**

a-b: different superscripts within a column indicate significant differences ($p < 0.05$).

The widespread use of antibiotics by beekeepers to cope with diseases in beehives may result antibiotic residues in honey, causing many concerns among consumers. In this study, the antibiotic residue of tetracycline in honey samples collected from different areas of Qazvin province was tested using ELISA and HPLC methods. There are many studies on antibiotic residues in, honey; some of these studies are listed below:

A research on 145 honey samples in Ardebil province was conducted to determine the amount of oxytetracycline antibiotic using FPT, ELISA, and HPLC methods. The results showed that of the145 honey samples, 34 samples contained oxytracycline antibiotic residue. The minimum and the maximum residue levels of oxytracycline were 5.32 and 369.1 ng g^{-1}, respectively. HPLC analysis confirmed ELISA results, although the level of oxytetracycline detected by HPLC method was remarkably lower than ELISA [7]. An experiment was conducted in Qazvin province on 135 samples of honey using ELISA to identify the antibiotic residues, including penicillin, enrofloxacin, chloramphenicol, tylosin, tetracycline, sulfonamide, and gentamicin. The results showed that the highest and the lowest rates of contamination were related to enrofloxacin (10.8 ± 1.6), and then, penicillin (4.4 ± 2.9) and chloramphenicol (0.1 ± 0.1). The highest antibiotic residues (71.85%) were found in the honey samples collected in the autumn season [4]. A study in Turkey was conducted on 50 honey samples prepared from several fragrances from different regions using a Liquid Chromatograph-Mass Spectrometer (LC-MS) system to determine the amount of sulfonamide antibiotic residue (sulfadiazine, sulfatizole, sulfamazazine, sulfadimotoxin) and oxytetracycline. According to the results, the residues were not detected in tested samples. This indicates that either their concentrations were lower for detection by the system used or the samples lacked antibiotic residues [16]. Another study was conducted in Greece on 251 honey samples to measure the amount of tetracycline antibiotic residue using HPLC. According to reports by research, 34 samples had oxytetracycline, 27 samples had doxycycline, and one

sample had chloratetracycline. 29% of honey samples contained tetracycline antibiotic residue. In 20.3% of them, more than one tetracycline derivative was observed. Most of the samples were 0.018 - 0.055 mg kg^{-1} and some had more than 0.100 mg kg^{-1} tetracycline residue [17]. A research was performed on 16 honey samples collected from supermarkets and 5 honey samples collected from beekeeping to measure 17 types of antibiotics (macrolides, tetracyclines, quinolones and sulfanamides) by Ultra performance liquid chromatography tandem-mass spectrometry (UPLC-MS/MS) in Spain. The results indicated that one supermarket honey sample contained 8.6 μg kg^{-1} erythromycin while another sample contained sarafloxacin. In samples collected from beekeeping, different types of antibiotics were found, among them the concentrations of sarafloxacin and tylosin were 14.6 μg kg^{-1} and 3.2 μg kg^{-1}, respectively. Sulfadimidine and sulfachlorpyridazine were also found in honey samples collected from beekeeping [18]. Another study in Spain was conducted on 567 honey samples with the aim of determining tetracycline, sulfanamide and chloramphenicol by Liquid chromatography-fluorescence detection (LC-FD) method. According to the results of the study, 24 honey samples contained tetracycline residue, and the amount of this residue was in the range of 15 to 920 μg kg^{-1}. 68 samples had sulfanamide antibiotics. Chloramphenicol was not detected in any of the samples [19]. A research in Germany was performed on 47 imported honey samples and 30 domesticly produced honey samples with the purpose of determining antibiotic residues. The results of the study showed that 22 samples (approximately 50%) of honey imported from Argentina, China, and Canada had antibiotic residues, mainly sulfamethoxazole, and only one sample of domesticly produced honey contained antibiotic residues [20].

In another study, 66 honey samples were collected from different provinces of Italy and experiments were performed to evaluate 6 types of antibiotics. According to the results of the study, 40 samples had antibiotic. 38 honey samples contained antibiotic tylosin, 36 samples had

tetracycline, 27 samples contained quinolone, 21 samples contained thiamphenicol, 19 samples contained cephalosporin, and in one of the honey samples, streptomycin was found [21]. A total of 27 types of veterinary drugs in sulfanamide, nitromidazole and quinolone groups were determined by LC-MS / MS in Italian honey samples. According to the results obtained from 74 honey samples, the level of sulfonamides was confirmed in 9 samples (12%) [22].

The present study showed that there is a significant difference between ELISA and HPLC. According to ELISA results, of the 80 honey samples of collected from different regions of Qazvin province 88.8% of samples had antibiotic at normal level, 5.0% had values above the kit's LOD, and the antibiotic level of 6.3% of samples was less than the minimum detectable amount of kit. The areas with values above the kit's LOD include Takestan (14.28%), Abeyek (4.76%), and Alamot-e-gharbi (4.54%), respectively. In the Alamot-e-sharghi samples, the antibiotic values above the kit's LOD were not found. The level of tetracycline detected by HPLC method was remarkably lower than ELISA, so that in HPLC method, of the 4 honey samples, 1 sample contained the antibiotic level more than the level detected by ELISA method. From the present study, it can be concluded that tetracycline antibiotics are widely used by beekeepers in beehives for prevention and treatment of diseases. Honey containing more antibiotics than MRL has harmful effects on the consumers' health. Additionally, the antibiotic residues in honey cause change in its organoleptic properties.

CONCLUSIONS

Currently, the presence of antibiotics in honey has created a very serious problem for honey trade. Considering that honey is an important food for human health, there are many concerns over the antibiotic residues in honey. Therefore, it is very necessary to control these residues. Among methods used to control the drug residues in honey we can refer to improving the level of knowledge of beekeepers about the correct use of drugs, prescribing the drug under the supervision of the veterinarian, and the use of drug in accordance with manufacturer's instruction.

Conflict of interest

The authors declared no conflict of interest.

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