

Determination of Tetracycline and Oxytetracycline Residues in Honey by High Performance Liquid Chromatography

Shaif Mohammed Kasem Saleh¹, Amer Mohammed Mussaed¹ and Fadhil Mohammed Al-Hariri²

1. Department of Chemistry, Faculty of Science & Education, University of Aden, Aden, Yemen

2. National Quality Control Laboratory, Aden, Yemen

Abstract: Honey is being used as a pure natural and as an ingredient in many foods, pharmaceuticals and cosmetics than ever before, so honey testing has become essential to maintain its healthful characteristics and protect public health. Tetracyclines (TCs) are used for the treatment or prevention of American and European foul broad in bee colonies which are caused especially by two species of bacteria—*Paenibacillus* larvae and *Melissococcus pluton*. The present study aimed to determine the tetracycline residues in different types of local and imported honey. The reversed-phase high performance liquid chromatography (HPLC) with ultraviolet detection (UV) was used. Tetracycline and oxytetracycline were extracted from honey with 0.01 M sodium succinate buffer. Of the examined samples, 31.25% and 12.50% had these oxytetracycline and tetracycline residues, respectively. It was observed that all the samples studied have been reported residues of tetracycline and oxytetracycline more than international standards of maximum residue limit (MRLs).

Key words: Honey, antibiotic residues, tetracycline, oxytetracycline, HPLC, Yemen.

1. Introduction

Honey is used widely throughout the world. Honey is one of the best natural products in terms of biomedical value and its endless use, and is the greatest natural product as well as food. It contains many active compounds that carry out the functions of antioxidants along with a lot of material that earn human activity body and protect it from several diseases [1, 2]. Recently, the Yemeni markets have been invaded by many works, such as honey imported from Turkish, German and Kashmiri, etc., and due to the high price of Yemeni honey, these types have received great demand among consumer [3]. Studies show that in recent years, honey and other bee products face problems in the growing use of antibiotics and pesticides, which means that honey may become contaminated with residues of antibiotics,

pesticides and toxic substances, and thus affects the human health and environment. Thus, the need of a lot of research is prompted in order to detect antibiotic residues and pesticides in honey and control them within the limits of these pollutants through the development of legislative specifications [1, 2, 4-10].

Tetracyclines (TCs) are used for the treatment or prevention of American and European foul broad in bee colonies, which are caused especially by two species of bacteria—*Paenibacillus* larvae and *Melissococcus pluton* [6, 8-10]. The presence of tetracycline and its degradation products in honey may have harmful effects on consumers, such as possible allergic reactions, liver damage, yellowing of teeth and gastro-intestinal disturbance due to the selective pressure of antibiotics on the micro flora of human gut [9]. The European Union Regulation 396/2005 [11] has recommended zero level tolerance for oxytetracycline residues in honey. According to Regulation (EC) No. 470/2009 [12] and Regulation

Corresponding author: Shaif Mohammed Kasem Saleh, associate professor, research fields: environmental chemistry and pollution.

(EU) No. 37/2010 [13] in the European Union, no maximum residue limit (MRL) for tetracycline in honey has been set. This means that the presence of tetracycline residues in honey is not allowed. Despite this decision, some countries have established action limits or tolerated levels for tetracycline in honey. For instance, in Belgium, the action limit for the group of tetracycline has been fixed at 20 µg/kg. France applies a nonconformity limit for tetracycline in honey of 15 µg/kg, the reporting limit in Great Britain is 50 µg/kg, while the tolerance level in Switzerland is 20 µg/kg [10]. In Japan, based on microbiological research, a value of 0.1 mg/kg was introduced as the allowed residual quantity of tetracycline in honey [8]. Australia, India, America and the US Food and Drug Administration (USFDA) have set MRL for only oxytetracycline in honey at 300 ppb [4, 14, 15].

The present study aimed to determine tetracycline and oxytetracycline residues in different types of local and imported honey by high performance liquid chromatography (HPLC).

2. Materials and Methods

2.1 Honey Samples

In this study, there are total seven locally produced honey and nine imported honey were studied. Among seven locally produced honey, four fresh honey were collected from honey bee breeding location (*Acacia tortilis* and Sidr honey from Dowan in Hadramout; Sider honey from Jardan, Bayhan in Shabwa; Sider honey from Radfan), while the other three samples were collected from commercial markets of the city of Aden (Osaimi sider honey from Sana'a; Soctotra honey from Soctotra; Marai honey from Abyan).

Nine samples of imported honey included Kashmiri honey imported by Galap (India), pure bees honey from Germany, black forest honey from Germany, tropical plants honey from Thailand, nutrition honey from Australia, Kashmiri honey imported by Saudi Arabia (India), Kashmiri honey imported by Yemen (India), black bean honey from (Egypt) and Bashaori

honey from Pakistan, which are randomly from different commercial markets of the city of Aden.

2.2 Apparatus

The LC system consisted of Shimadzu VP series (Japan). This was equipped with binary LC-10AD pump, DGU-14A degasser, SLC-10A system controller, CTO-10AS column oven, SIL-10AF auto-injector and SPD-10A UV-VIS detector set at 360 nm wavelength. The separation was performed on a Nucleosil 100 RP-18, 5 µm column, 250 mm × 4.6 mm internal diameter (ID) with a guard column (4.6 mm × 7.5 mm ID) containing same packing material.

The optimal selected mobile phase was 10 mM oxalic acid, acetonitrile and methanol (16:3:2), and the symmetrical non-tailed peaks were obtained. The flow rate was 1 mL/min, the injection volume was 50 µL and the column temperature was 35 °C.

2.3 Reagents

Ultra pure water (LC grade) and the solvents methanol and acetonitrile (LC grade), ethanol absolute (reagent grade) were used. The extraction solution was a sodium succinate buffer. A 0.1 M succinic acid solution was prepared and adjusted to right pH with 5 M sodium hydroxide. The buffer solution is stable at 4 °C.

2.4 Calibration

Under the optimized experimental conditions, there were good linearities found between tetracyclines concentration and peak area responses ranged from 10-50 µg/mL with $r^2 > 0.999$. Method validation results were satisfied with linear regression equations, as shown in Table 1.

2.5 Analytical Procedure for Honey Samples

A total of 16 honey samples (weighing 30 g each) were obtained from local and imported honey. Honey samples were transferred to National Quality Control Laboratory, Aden, under appropriate conditions, and

Table 1 Validation results of oxytetracycline and tetracycline*.

Tetracyclines	Retention time (min)	Regression equation	Correlation coefficient (r^2)
Oxytetracycline	4.1	$y = 19,496.8 x + 29,605.8$	0.9997
Tetracycline	4.8	$y = 23,718.8 x + 33,353.9$	0.9993

* Based on three replicates of five concentration in the range of 10-50 µg/mL.

then analyzed immediately after preparations.

Tetracycline and oxytetracycline were detected according to Bonta et al. [6], which include sample extraction, clean-up procedure, analytical procedure and method validation.

The honey sample was weighted and dissolved in sodium succinate extraction solution. The solution was stirred and centrifuged at 2,500 rpm for 10 min. The solution was filtered through a 0.45 µm nylon chromatographic filter and then injected into the HPLC system. Calibration curves were performed for each tetracycline from standard chromatograms, by plotting concentration against peak area.

3. Results and Discussion

The results of tetracycline and oxytetracycline residues analysis of 16 samples of local and imported honey are shown in Table 2.

3.1 Imported Honey Samples

Oxytetracycline was detected in most of the imported honey samples. The concentration of oxytetracycline residues was found to be high in the black Forest honey with 13,800 µg/kg, followed by Kashmiri honey imported by Yemen (sample 3: 9,033 µg/kg), pure bees honey (sample 1: 3,610 µg/kg) and Kashmiri honey imported by Saudi Arabia (sample 4: 3,430 µg/kg). However, the oxytetracycline residues were higher than the MRL established by European Union Regulations [2, 11-13], and Australia, Indian, American and USFDA [4, 14, 15]. The rest of the samples did not show for oxytetracycline residues in honey or probably less than the limits of detection device. Tetracycline residues were detected less in the imported honey samples. The study showed the

presence of residues of the antibiotic tetracycline only in the Bashaori honey from Pakistan with 2,330 µg/kg. The tetracycline residues were higher than the MRL for international specifications [4, 14].

3.2 Locally Produced Honey

The concentration of oxytetracycline residues was found 7,140 µg/kg in the Sider honey of Jordan. Tetracycline residues were detected less in the honey imported samples. The study showed the presence of residues of the antibiotic tetracycline only in the Marai honey of Lowder with 2,850 µg/kg. However, the oxytetracycline and tetracycline residues were higher than the MRL for international specifications [4, 6, 11, 14, 15].

Tetracycline and oxytetracycline residue values of honey samples in the present study are considerable compared with other country honey samples. And the results of their comparison are shown in Tables 3 and 4.

As shown in Table 3, the range of oxytetracycline residue in contamination samples of current study was between 3,430 µg/kg and 13,800 µg/kg, lower than those obtained by Narin and Supaporn from Thailand (60,150-106,900 µg/kg) [8] and higher than the results of applied studies in Greece [5], Iran [1], Pakistan [16] and India [2, 7].

The tetracycline comparison results were shown in Table 4. It revealed that the range of tetracycline residue in contamination samples of the current study was 2,330-2,850 µg/kg, lower than those obtained by Narin and Supaporn from Thailand (7,180-14,060 µg/kg) [8] and more than that reported in Refs. [5, 10].

In general, of the examined samples, 31.25% and 12.50% had these oxytetracycline and tetracycline residues, respectively. It was observed that the

Determination of Tetracycline and Oxytetracycline Residues in Honey by High Performance Liquid Chromatography

Table 2 Results of tetracycline and oxytetracycline residues analysis from different honey samples.

No.	Honey types	Country (origin)	Average concentration ($\mu\text{g}/\text{kg}$) (mean \pm SD)	
			Tetracycline	Oxytetracycline
Honey imported samples				
1	Pure bees honey	Germany	ND	3,610 \pm 0.036
2	Black forest honey	Germany	ND	13,800 \pm 0.265
3	Kashmiri honey imported by Yemen	India	ND	9,033 \pm 0.058
4	Kashmiri honey imported by Saudi Arabia	India	ND	3,430 \pm 0.006
5	Nutrition honey	Australia	ND	ND
6	Kashmiri honey imported by Galap Co.	India	ND	ND
7	Tropical plants honey	Thailand	ND	ND
8	Black bean honey	Egypt	ND	ND
9	Bashaori honey	Pakistan	2,330 \pm 0.000	ND
Locally produced honey				
10	Acacia tortilihoney, Dowan	Hadramout, Yemen	ND	ND
11	Sidr honey (Zizyphusspina-christi), Dowan	Hadramout, Yemen	ND	ND
12	Sider honey (Zizyphusspina-christi), Jordan	Shabwa, Yemen	ND	7,140 \pm 0.000
13	Osaimi sider honey (Zizyphusspina-christi), Omran	Sana'a, Yemen	ND	ND
14	Soctotra honey	Soctotra, Yemen	ND	ND
15	Marai honey, Lowder	Abyan, Yemen	2,850 \pm 0.000	ND
16	Sider honey (Zizyphusspina-christi), Radfan	Lahij, Yemen	ND	ND
Least significant difference (LSD)			0.000	0.162

ND: not detected.

Table 3 Comparison of oxytetracycline residue in honey samples between the present study and other countries.

Country	Author and year	Analysis methods	Contamination percent	Range ($\mu\text{g}/\text{kg}$)
Greece	Saridaki-Papakonstadinou et al., 2006 [5]	HPLC	14.30%	23.00-335.00
India (imported)	Jonson and Jadon, 2010 [7]	HPLC-FLD	50.00%	27.10-250.40
Thailand	Narin and Supaporn, 2010 [8]	HPLC-FLD	66.66%	60,150-106,900
Iran	Mahmoudi et al., 2014 [1]	ELISA, HPLC	23.44%	2.10-120.60
Pakistan	Muhammad Zai et al., 2013 [16]	HPLC	7.00%	1.12-2.13
India (produced with country)	Chilumuru et al., 2015 [2]	HPLC-UV	100.00%	0.05-0.50
Yemen (imported)	Saleh et al., 2016 (current study)	HPLC-UV	44.44%	3,430-13,800
Yemen (produced with country)	Saleh et al., 2016 (current study)	HPLC-UV	14.29%	7,140

HPLC-FLD: high performance liquid chromatography with fluorescence detection; ELISA: enzyme-linked immuno-sorbent assay; HPLC-UV: HPLC with ultraviolet detector.

Table 4 Comparison of tetracycline residue in honey samples between the present study and other countries.

Country	Author and year	Analysis methods	Contamination percent	Range ($\mu\text{g}/\text{kg}$)
Greece	Saridaki-Papakonstadinou et al., 2006 [5]	HPLC	4.78%	18-57
Thailand	Narin and Supaporn, 2010 [8]	HPLC-FLD	33.33%	7,180-14,060
Romania	Monica et al., 2012 [10]	ELISA	50.00%	15.47- 60.67
Yemen (imported)	Saleh et al., 2016 (current study)	HPLC-UV	11.11%	2,330
Yemen (produced with country)	Saleh et al., 2016 (current study)	HPLC-UV	14.29%	2,850

tetracycline and oxytetracycline detected in the samples studied have been reported to be more than MRLs.

4. Conclusions

Result showed that of the examined samples, 31.25% and 12.50% had these oxytetracycline and tetracycline residues, respectively. Therefore, the honey samples did not have the desired conditions because of presence of oxytetracycline and tetracycline residues more than MRLs. Other studies are necessary to evaluate other drug residues in honey samples and the hazards of these residues in relation with daily intakes and other related factors.

References

- [1] Mahmoudi, R., Moosavy, M., Norian, R., Kazemi, S., Nadari, M., and Mardani, K. 2014. "Detection of Oxytetracycline Residues in Honey Samples Using ELISA and HPLC Methods." *Pharmaceutical Sciences* 19 (4): 145-50.
- [2] Chilumuru, R. M. R., Lakkineni, C. A. K., and Chandra, B. S. 2015. "Quantitative Analysis of Oxytetracycline Residues in Honey by High Performance Liquid Chromatography." *Int. Res. J. Biological Sci.* 4 (5): 59-65.
- [3] Khanbash, M. S., and Al-Kaff, H. A. 2007. "Using of Yemeni Honey in Dressing of Gluteal and Breast Abscesses." Honeybee Center, Hadhramout University, Yemen. Accessed July, 2014. <http://hust.edu.oak.arvixe.com/>.
- [4] Center for Food Safety (CFS). 2006. "Antibiotics Found in Honey." CFS, Hong Kong. Accessed January 5, 2013. <http://www.cfs.gov.hk/eindex.html>.
- [5] Saridaki-Papakonstadinou, M., Andredakis, S., Burriel, A., and Tsachev, I. 2006. "Determination of Tetracycline Residues in Greek Honey." *Trakia J. of Sci.* 4 (1): 33-6.
- [6] Bonta, V., Marghitas, L. A., Dezmirean, D., Moise, A., Bobis, O., and Maghear, O. 2007. "Optimization of HPLC Method for Quantifying Tetracycline Residue in Honey." *Bull. Uni. Agri. Sci. Veter. Med.* 63-64: 186-90.
- [7] Johnson, S., and Jadon, N. 2010. *Antibiotic Residues in Honey*. New Delhi, India: Center for Science and Environment.
- [8] Narin, T., and Supaporn, S. 2010. "HPLC-Fluorescence Detection Method for Quantitative Determination of Tetracycline Antibiotic Residues in Honey." *Naresuan Uni. Science J.* 6 (2): 147-55.
- [9] Thanasarakhan, W., Kruanetr, S., Deming, R. L., Liawruangrath, B., Wangkarn, S., and Liawruangrath, S. 2011. "Sequential Injection Spectrophotometric Determination of Tetracycline Antibiotics in Pharmaceutical Preparations and Their Residues in Honey and Milk Samples Using Yttrium (III) and Cationic Surfactant." *Talanta* 84 (5): 1401-9.
- [10] Monica, C. C., Gabriela-Alina, D., Mirel, G., and Delia, P. 2012. "Stability of Tetracycline Residues in Honey." *J. Serb. Chem. Soc.* 77 (7): 879-86.
- [11] European Union Regulation (EC) No. 396/2005—MRLs. 2013. "A Database of EU MRLs and Pesticide Authorization Status (Dir 91/414/EEC)." Accessed June 6, 2013. https://ec.europa.eu/food/safety_en.
- [12] Official Journal of the European Union L152. 2009. *Regulation (EC) No. 470/2009 of the European Parliament and of the Council*.
- [13] Official Journal of the European Union L15/1. 2010. *Regulation (EU) No. 37/2010*.
- [14] FAO/WHO. 2008. "Codex Alimentarius: Veterinary Drugs Residues in Food." Accessed July, 2014. <http://www.fao.org/fao-who-codexalimentarius/standards/veterinary-drugs-mrls/en/>.
- [15] CDC. 2000. "Interagency Task Force on Antimicrobial Resistance: A Public Health Action Plan to Combat Antimicrobial Resistance." The Centers for Disease Control and Prevention, the Food and Drug Administration, and the National Institutes of Health. Accessed July 17, 2014. http://www.cdc.gov/drugresistance/itfar/introduction_overview.html.
- [16] Zai, I. U. M., Rehman, K., Hussain, A., and Shafqatullah. 2013. "Detection and Quantification of Antibiotics Residues in Honey Samples by Chromatographic Techniques." *Middle-East Journal of Scientific Research* 14 (5): 683-7.