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Detection of tetracycline antibiotics in honey using high-performance liquid chromatography

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ABSTRACT

It is hard to overestimate the value of honey since the constituent substances in it are of great importance in the food industry and medicine. The quality of honey is established by the legislation of the Russian Federation (GOST). But the levels of these standards are regulated by outdated methods of analysis, which can not give reliable results. The detection of antibiotic residues in honey is a central issue in the quality and safety control of this product. Accumulation of drugs in honey used to treat bee colonies can cause allergies and dysbiosis in people who have eaten such honey, as well as develop antibiotic resistance in microorganisms. At present, one of the promising directions in the field of detecting medicines in honey is the use of high-performance liquid chromatography (HPLC). It helps selectively and accurately detect antibiotic substances in honey bee productA sample preparation algorithm was developed, and the conditions for chromatographing combined indication of tetracyclines in honey at an acceptable concentration according to the MRL (0.01 mg/kg) with Agilent 1260 Infiniti liquid chromatograph equipped with a column thermostat, a gradient pump, and a UV detector were selected. Under these conditions, the antibiotic retention time was determined: 4.069 minutes for oxytetracycline, 4.331 minutes for tetracycline hydrochloride, 4.642 minutes for chlortetracycline. The developed HPLC method for the simultaneous determination of tetracycline antibiotics in honey was tested on honey bee products from the regions of the Republics of Tatarstan and Bashkortostan.

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INTRODUCTION

There are many obstacles to the delivery of Russian honey abroad. The international requirements have increased for honey-import countries concerning the safety of this product – the presence of antibiotic residues used by beekeepers while treating bees (Oka *et al.*, 1989; Brabander *et al.*, 2009). Antibiotics are also added to honey in order to avoid fermentation and to preserve the marketable state of it. These tricks lead to the transformation of a useful product into a dangerous one. Long-term use of such honey leads to dysbacteriosis and weakens the immune system. Even daily minimum concen-

trations of antibiotics in honey bee products kill sensitive to them bacteria in the human body. Antibiotics are stored in commercial honey for more than three years.

The World Health Organization's (WHO) assessment of the situation with antibiotics is interpreted as follows: "Antibiotics have revolutionized the treatment of infectious diseases in humans" (Viñas *et al.*, 2004). However, their widespread and incorrect use has led to the emergence and development of antibiotic resistance. Currently, this problem is urgent for public health: every year only in the European Union countries, over 25 thousand people die from infections caused by resistant bacteria (Cristina *et al.*, 2012; Zai *et al.*, 2013; Singh *et al.*, 2015). Therefore, there is a need for a large number of studies to detect antibiotic residues in honey and control their permissible limits in honey bee products.

In Russia, the analysis of honey for the maintenance of antibiotics is practically not conducted due to the fact that there are no perfect analysis methods (Udalova *et al.*, 2015).

A number of methods for detecting tetracycline residues in honey have been developed, for example, enzyme immunoassay and high-performance liquid chromatography (HPLC) with a mass spectrometric detector.

Due to the fact that honey contains a large number of components, including substances with pronounced antimicrobial properties, data of enzyme immunoassay are assessed as indicative.

Indirect factors that also give false-positive results are fibreboards used for making hives which produce chemical substances at high temperature, acidity, and humidity that are identical in composition to antibiotics.

One of the promising methods for detecting drugs is a high-performance liquid chromatography with a mass spectrometric detector. It helps selectively and accurately detect toxic substances in honey (Singh *et al.*, 2015). A significant drawback with this method that limit the widespread use of it is the application of expensive, technically sophisticated equipment that needs high-quality reagents and consumables.

The goal of our experiment was to develop a reproducible, precision method for isolating and concentrating tetracycline antibiotics (tetracycline hydrochloride, oxytetracycline, and chlortetracycline) in honey and to quantify them using reversed-phase high-performance chromatography.

In Russia, there is no mandatory control over the amount of antibiotics in honey bee products (Carrasco-Pancorbo *et al.*, 2008; Mahmoudi *et al.*, 2014; Rama *et al.*, 2015). In this connection, we also set a goal to test the method developed by us for the simultaneous detection of tetracycline antibiotics by HPLC method on honey samples received from the regions of the Republics of Tatarstan (RT) and Bashkortostan (RB).

MATERIALS AND METHODS

The studies were conducted with Agilent 1260 Infiniti liquid chromatograph. The separation was carried out on a column (250:4 mm) Reprosil ODS – AC 18 (5 μm) in the gradient elution mode of the mobile phase. The concentration and purification of the extracts were carried out by the SPE method to eliminate effectively the interfering influence of the components of the matrix without using a large number of organic solvents. Standard solutions of tetracycline antibiotics within MRL – 0.01 mg/kg were added to control honey.

Experimental honey samples: 15 test portions from the regions of the Republic of Tatarstan and 15 test portions from the Republic of Bashkortostan.

RESULTS AND DISCUSSION

The method for detecting tetracycline antibiotics (tetracycline hydrochloride, oxytetracycline, chlortetracycline) is as follows. A 2g honey sample was placed in a polypropylene centrifugation tube and dissolved in 10 ml of Mcllvaine buffer, mixed with a shaker for 20 minutes. Then the tube was placed in a centrifuge for 10 min at 3000 rpm. The solution was passed through a membrane filter with a pore diameter of 0.45 microns. The filtrate was cleaned with an Oasis HLB cartridge 3 cm^3x60 mg at a speed of 2 drops per second. Before it, the cartridge was condensed with 3 ml of methanol and 3 ml of water. The sample was washed with 15 ml of water, eluted with 6 ml of methanol. The collected eluate was evaporated to dryness in a nitrogen current and redissolved in 5 ml of a 0.1% formic acid aqueous solution and passed through a membrane filter. The final volume was used for HPLC analysis.

In the course of the work, an optimal mobile phase was selected based on acetonitrile/methanol/aqueous solution of KH_2PO_4 (0.05M, pH=6.0) (15:45:40 v/v/v) with 0.1M salt solution of disodium ethylenediaminetetraacetic acid (EDTA) in the aqueous part of the mobile phase to increase the efficiency of the separation of the chromatogram peaks in the samples and to increase the sensitivity of the analysis.

Table 1: The data of chromatogram while detecting oxytetracycline, tetracycline hydrochloride and chlortetracycline in honey with artificial primer at a dose of 0.01 mg/kg at a wavelength of 254 nm

The number of peak	Void time (min)	Peak type	Breadth (min)	Area, [mAU*s]	Area, %	The name of an antibiotic
1	4.069	VV	0.1483	214.69307	14.8401	oxytetracycline
2	4.331	VB	0.1534	149.34583	10.3231	tetracycline hydrochloride
3	4.642		0.6087	104.27613	7.2078	chlortetracycline

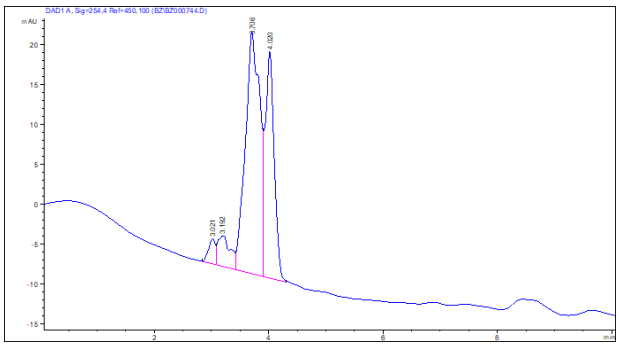

Figure 1: Chromatogram of the honey sample produced in the RT. Column ReprosilODS– AC 18 250*4 mm, column temperature 25^{0}C, flow rate 0.5 ml/min, eluent– acetonitrile, methanol, aqueous solution of KH_2PO_4 (0.05 M, pH = 6.0), detection at 254 nm

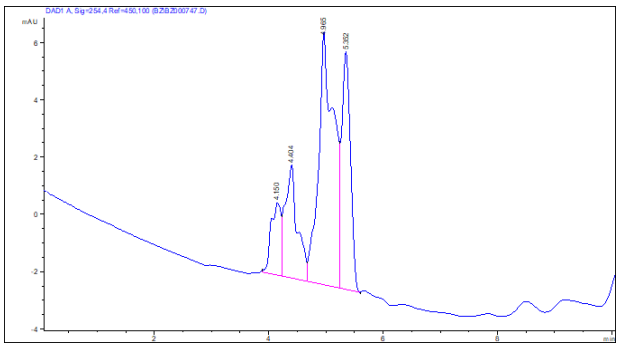

Figure 2: Chromatogram of the honey sample produced in the RB. Column ReprosilODS– AC 18 250*4 mm, column temperature 25^{0}C, flow rate 0.5 ml/min, eluent– acetonitrile, methanol, aqueous solution of KH_2PO_4 (0.05 M, pH = 6.0), detection at 254 nm

When using this mode of chromatography, no skewness of peaks was recorded.

The Table 1 shows the data of chromatogram – void time, type, breadth, and area (in absolute and percentage terms) of the peaks of detected antibiotics (oxytetracycline, tetracycline hydrochloride, chlortetracycline).

The lower detection limit of tetracyclines in honey is 0.01 mg/kg. The recovery ratio is 80.3% for tetracycline hydrochloride, 82% – for oxytetracycline, and 81.5% – for chlortetracycline.

Figures 1 and 2 show chromatograms of honey samples from the RT and the RB regions that were tested for the tetracycline residues by HPLC method with Agilent 1260 Infiniti chromatograph with a UV detector.

In the range of detection of tetracycline antibiotics in the honey from the RT by HPLC method Figure 1, specific peaks were not recorded while meeting control conditions.

In the range of detection of tetracycline antibiotics in the honey from the RB by the HPLC method Figure 2, specific peaks were not recorded while meeting control conditions.

The chromatograms show peaks of mobile phase composition and organic compound residues in honey. The void time of peaks do not coincide with the void time of antibiotics, that proves their absence in the studied honey samples.

According to the results of the research, drugs were not detected in the honey samples from the Republics of Tatarstan and Bashkortostan. Chromatograms 1-2 did not reveal specific peaks for tetracycline antibiotics, that proves the absence of antibiotics in the studied honey samples.

CONCLUSIONS

The sample preparation scheme has been developed for the simultaneous detection of tetracycline antibiotics in honey followed by HPLC indication, including liquid and solid-phase extraction. The method of simultaneous analysis of tetracycline antibiotics in honey allows us to detect them at the level of MRL (0.01 mg/kg) according to Sanitary Regulations and Norms 2.3.2.1078-01. The developed HPLC method

for the simultaneous determination of tetracycline antibiotics in honey was tested on honey bee products from the regions of the Republics of Tatarstan and Bashkortostan. The results obtained to exclude the possibility of honey contamination with antimicrobial agents. However, to obtain pure honey, it is necessary to monitor the state of the environment and exclude the use of highly toxic chemical and drugs.

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