



EVALUATION OF ELISA TESTS AS SCREENING METHODS FOR DETERMINATION OF ANTIBIOTICS AND SULFONAMIDES IN HONEY

Sandra M. Jakšić*, Željko A. Mihaljev, Brankica D. Kartalović, Jelena B. Babić, Suzana L. Vidaković, Milica M. Živkov Baloš

Scientific Veterinary Institute „Novi Sad“, 21000 Novi Sad, Rumenački put 20, Serbia

*Corresponding author:

Phone: +381214895363

Fax: +38121518544

E-mail address: sandra@niv.ns.ac.rs

ABSTRACT: The health safety of honey includes the correctness in terms of the presence of different contaminants, which also implies the antibiotic residue. The presence of antibiotics in honey is prohibited, and methods of food analysis are prescribed in order to reliably determine antibiotics in food. In this paper the application of ELISA tests for determination of selected antibiotics and sulfonamides in honey is shown. The possibility of using four ELISA tests for the analysis of tetracyclines, streptomycines, chloramphenicol and sulfonamides was examined. Each test was evaluated after application on honey samples spiked with standard solution of a particular analyte. Samples were prepared according to the instructions of the ELISA test manufacturer referring to honey. Results of investigation of all ELISA tests, except for sulphonamides, have shown satisfactory accuracy (73–111%) and precision (14–16%). Recovery for sulfametyoxypyridazin was low (40%), and for low tetracycline concentration was somewhat higher than acceptable (139%). The detection limits were in accordance with the limits given by the ELISA kit manufacturer and are also satisfactory in relation to the requirements of the legislation (0.075–3 µg/kg). The test kits can be used to screen the presence of tetracycline, chloramphenicol and streptomycin in the honey, taking into account the obtained validation parameters.

Key words: honey, tetracyclines, streptomycines, chloramphenicol, sulphonamides, ELISA

INTRODUCTION

Honey has an important role in human nutrition. In addition to the quality of honey (Prica et al., 2014), its safety for the presence of unwanted and toxic substances is very important (Al-Waili et al., 2012). Moderate continental climate including floral and plant richness make perfect conditions for beekeeping in Serbia (Babić, 2014; Vidaković et al., 2017). The use of antibiotics in beekeeping for combating bacterial diseases is prohibited and accordingly there is no prescribed maximum permissible concentration of them in ho-

ney in Serbia (Pravilnik, 2002). Legislation does not allow the presence of antibiotics in honey in EU (EC 1990, 2009, 2010). Treatments with antibiotics are not allowed in the EU, while other countries (e.g. USA, Canada, Argentina) allow the use of antibiotics to keep the disease in control (Reybroeck et al., 2012). However, antibiotics and chemotherapeutics could be used in the EU in apiculture based on the 'cascade' system as described in Directive 2001/82/EC, and amended by Directive 2004/28/EC (EC, 2001; 2004). Antibiotic

residues can originate mostly from the environment and improper beekeeping practices from treatments against the brood diseases American Foul Brood or European Foul Brood (Bogdanov, 2006). There are several international reports of antibiotic residues in honey samples from different countries (Al-Waili et al., 2012), and from Serbia (Živov-Baloš et al., 2017), although taking honey from treated hives is forbidden (Babić et al, 2017).

The ways of controlling antibiotics presence in EU are also prescribed (EC, 2002, 2003). Requirements for methods of antibiotic examination in honey refer to the sensitivity, specificity and qualitative confirmation. In particular, very important is to achieve as lower detection limit (LOD) as possible, prescribed as the Minimum Required Performance Limit (MRPL).

According to EU regulations, only chloramphenicol has a MRPL in honey of 0.3 µg/kg (EC, 2003). Because of that, some countries, like Switzerland, UK and Belgium, have established action limits for antibiotics in honey, which generally lay between 10 and 50 µg/kg for each antibiotic group. Action limits are the level of antibiotics in honey beyond which the sample is deemed non-compliant (Jonson and Jardon, 2010).

Different methods for antibiotics determination can be used (Bargańska et al., 2011). Often it is difficult to choose the appropriate method according to regulations as well as the need or possibilities of the laboratory.

Analytical challenges include difficulties in detecting low-level antibiotic contamination in complex honey matrices. Therefore, appropriate sample preparation and pre-concentration methods are needed to isolate these analytes from honey samples.

In routine residue determination, the vast majority of samples usually are estimated by "screening" methods (microbiological and immune-enzyme tests). According to our previous results, the modified method 4 plates can be used as a first step in screening procedures, especially in regular monitoring of the presence of antibiotic residues in honey (Apić et al., 2015).

However, this method is time-consuming and lack of specificity. Immunochemical methods such as enzyme-linked immunosorbent assays (ELISA) can be used for antibiotics determination as an alternative screening method (Pastor-Navaro et al., 2007; Jakšić et al., 2018). ELISA tests are fast, cheap and simple methods for determination of different antibiotics. Those methods are based on specific antigen-antibody reaction.

A number of such tests are available in the market and some of them are applicable to honey samples. Nonetheless, ELISA tests are considered as a screening method and confirmation of positive samples and determination of the amount of residues present in foodstuffs should be carried out with more sensitive methods such as chromatographic methods. These confirmative methods require sophisticated equipment, and can be used to test routine samples for the presence of all groups of antibiotics (Petrović et al, 2005; Jakšić et al, 2018).

The aim of the study presented in this paper was to investigate possibility of application ELISA tests for determination of tetracycline, chloramphenicol, sulphamide and streptomycin in honey.

MATERIAL AND METHODS

For investigation of ELISA method, ELISA kits produced by R-Biopharm (Darmstadt, Germany) were used (Table 1). Sample preparation for tetracycline and chloramphenicol determination included only extraction step.

For determination of sulphonamides and streptomycin, clean-up step of crude honey extract was needed, by using solid phase extraction method. For that purpose, C18 columns (Rida® C18, Art No. R2002, R-Biopharm, Germany) were used according to proposed procedure of ELISA manufacturer for honey samples.

The basis of all applied ELISA tests is the antigen-antibody reaction. Antibodies against analyte of interest are placed in microtiter wells. Free molecules of analyte and enzyme conjugate compete for the antibiotic antibody sites. Test principle is competitive enzyme immunoassay.

Table 1.

Characteristics of ELISA tests used in evaluation (R-Biopharm, Germany)

| ELISA test | Specificity and cross-reactivities | Sample preparation | LOD (µg/kg) |
|--|--|---|-------------|
| Ridascreen® Tetracycline Art. No. R3505 | Tetracycline 100% Chlortetracycline 70% Rolitetracycline 34% Demeclocycline 26% Oxytetracycline 13% Minocycline 3% Doxycycline 2% | Extraction with phosphate buffer solution | 4 |
| Ridascreen® Chloramphenicol Art. No. R1505 | Chloramphenicol 100% Chloramphenikol baza 12% Tiamphenikol <0,1% | Extraction with etyl-acetate | 0.025 |
| Ridascreen® Sulfonamide Art. No. R3004 | Sulfamethoxy-pyridazine 100% Sulfapyridine >100% Sulfamethoxydiazine 75% Sulfametoxazole 58% Sulfadimethoxin 41% Sulfaquinoxaline 34% Sulfachloropyridazine, sulfamerazine, sulfadiazine, sulfamethizole, sulfadoxine, sulfachloropyrazine, sulfaguanidine <20% Sulfaphenazole, sulfamethazine, sulfisoxazole, sulfanilamide, sulfacetamide <2% | Extraction with sodium-acetate buffer and Rida® C18 column purification (Art. No. R2002) | 2 |
| Ridascreen® Streptomycin Art. No. R3103 | Streptomycin 100% Dihydrostreptomycin 149% | Extraction with buffer made from heptan-sulfonic acid (sodium salt) and trisodiumphosphate and Rida® C18 column purification (Art. No. R2002) | 5 |

After washing step and substrate addition wells are incubated. Chromogen is converted into blue product and addition of the stop reagent change colour to yellow. Photometric measurements of ELISA plates were performed using Thermo Scientific Multiskan FC ELISA reader (Shanghai, China) at 450 nm. Special software Rida®Soft Win (Z9999, R-Biopharm, Germany) was applied for data evaluation and calculation of toxin concentration.

ELISA methods were tested using blank honey sample, which has been spiked with concentration levels of antibiotic that are important for evaluating of honey safety according to regulations about methods requirements. Blank honey sample was confirmed to be free from antibiotics by using microbiological method "Modified method 4 plates" (Heitzman, 1994). For

sample spiking, following standards of antibiotics were used: tetracycline hydrochloride (Dr Ehrenstofer, 17396150), Rida®Sulfonamide Sulfamethoxy-pyridazin-Spiking solution (R3099, R-Biopharm, Germany), Rida®Streptomycin Spiking solution (R3199, R-Biopharm, Germany), and Rida®Chloramphenicol spiking solution (R1599, R-Biopharm, Germany).

The analytical quality of the ELISA methods was assured by the use of spiked blank honey samples. To this end, 1 g of sample was spiked with appropriate volume of tetracycline hydrochloride standard solution which contain 1 µg/ml. For chloramphenicol ELISA testing, honey sample was spiked by using standard solution of chloramphenicol containing concentration of 50 ng/ml. 2 g of sample was spiked with 40 i.e. 80 µl of standard solution for ex-

pected contamination of sample to be 100 and 200 ng/kg, respectively. Recovery experiment for sulfonamides was done using blank honey sample (3 g) spiked with 300 µl standard spiking solution containing 0.1 µg/ml sulfametoksy-pyridazine. For streptomycine test evaluation, honey sample (3 g) was spiked using spiking solution with concentration 1 µg streptomycine/ml, in the level of 40 µg per kg of honey.

RESULTS AND DISCUSSION

ELISA test for tetracycline

The LOD was determined by the series of 6 repeated measurements of blank honey sample and calculated as the sum of the average value of the blind probe and 3 standard deviations. Quantification limit (LOQ) was determined as the sum of the average value and 10 standard deviations. Obtained LOD was 2 µg/kg, and LOQ was 5 µg/kg.

Obtained results of spiked samples determination (Table 2) showed high recovery for low concentration of tetracycline, however, average recovery was 125%. In that case, recalculation of results is mandatory. Average relative standard deviation calculated under repeatability was 16%.

ELISA test for chloramphenicol

For determination of chloramphenicol, honey sample was extracted with ethyl-acetate and extract was used for ELISA testing without additional clean-up. Negative blank honey sample matrix gave matrix effect and sample was positive. That is in accordance with manufacturer warning.

They recommend to avoid these effects by using solid phase extraction columns for sample extract clean-up. Since this way of sample preparation is more expensive and slower, for this investigation, we applied manufacturer recommendation to set LOQ as the "cut of" value for spiked samples.

The efficiency obtained by calculating the impact of the matrix was 88%, while relative standard deviation calculated under repeatability conditions was 13%. Taking into account the requirements of the EU

directive for the performance of the chloramphenicol method of determination (0.3 µg/kg), this method can be used as a screening test without using the column, because in this way a LOD is 0.075 µg/kg.

Since the different types of honey gave a positive reaction in ELISA microtiter plate, it is necessary to examine in more detail the influence of the matrix on the chloramphenicol determination.

ELISA test for sulfonamide

The LOD was determined as in tetracycline ELISA. Obtained LOD was 3 µg/kg, and LOQ was 8 µg/kg. In the determination of sulfamethoxy-pyridazine in spiked honey samples, low efficiency of the method was obtained (Table 2).

Expected concentration in sample was 10 µg/kg, and result for recovery was 40%. The critical phase of this analysis is the purification of the sample on the C18 columns, and in further studies it is necessary to optimize this phase of the analysis.

ELISA test for streptomycin

The LOD and LOQ were determined as in previous ELISAs. Obtained LOD was 2 µg/kg, and LOQ was 5 µg/kg. Obtained results (Table 2) showed recovery of 73%. Relative standard deviation calculated under repeatability was 14%.

The technical lack of ELISA tests for the determination of sulfonamide and streptomycin is the necessity to use solid-phase extraction for the preparation of honey samples (C18 columns).

Namely, according to the manufacturer's instructions, columns with 100 mg adsorbents are used. An aqueous solution of honey (in the case of sulfonamide 3 g in 6 ml) is applied to the columns, but it very difficultly passes through the column.

The vacuum on the automatic manifold is not sufficient to extract the entire sample extract and rinse solution, but it is necessary to manually pass one by one sample. Consequence is that this determination phase lasts longer than predicted by the ELISA kit maker.

Table 2.

Efficacy (recovery) and repeatability of tetracycline, chloramphenicol, sulfametoxypridazin and streptomycin determination by ELISA tests obtained by spiking experiments

| Analyte | Target concentration (µg/kg) | Recovery (%) | RSD (%) | n |
|---------------------|---------------------------------|-----------------|------------|---|
| Tetracycline | 10 | 139 | 18 | 3 |
| | 50 | 111 | 14 | 2 |
| Chloramphenicol | 0.1 | 115 | 3 | 3 |
| | 0.2 | 62 | 24 | 2 |
| Sulfametoxypridazin | 10 | 40 | 34 | 2 |
| Streptomycin | 40 | 73 | 14 | 4 |

RSD- relative standard deviation
n- number of measurements

As can be seen, efficacy varies from 40 to 139% depending on the concentration and the type of the analyte. Low efficiency of 40% can be interpreted by the great influence of the density of the honey solution on the purification of SPE columns.

On the other hand, a high efficiency of 139% can be a consequence of the matrix effect on the competitive immuno-chemical method itself.

In the literature there are studies on the development of ELISA methods for the determination of antibiotics (Burkin et al., 2018), as well as studies on their application on real samples (Shen and Jiang, 2005; Mahmoudi et al, 2014), but there are no data on similar evaluations. Certainly, the European requirements for screening methods are stricter (EC, 2002), and these methods should be examined in order to determine detection capability, as well as check them by using reference materials based on honey matrices and participation in proficiency tests.

CONCLUSION

Although, the honey matrix is complicated, the evaluated ELISA methods can detect tetracyclines successfully without sample pre-treatment. ELISA test for chloramphenicol was not specific enough by using only ethyl-acetate extraction without sample extract clean-up.

Despite the difficulty in purifying the extract of honey samples, the method of determining streptomycin gave satisfactory efficiency and a low detection limit. Also, all those methods provide limit of detection low enough according to the requirements

of the legislation. For sulphonamides, further optimization is needed, as already mentioned.

It can be concluded that the ELISA technique is fast, sensitive, economical, and can be successfully used for routine screening antibiotic residues in the honey, except for the determination of sulfametoxypridazine residues in honey.

Certainly, the determination of antibiotics in honey samples that have been shown to be positive after testing by ELISA tests should be carried out with more specific and accurate modern confirmatory methods by using instrumental technics.

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ОЦЕНА ЕЛИСА ТЕСТОВА КАО СКРИНИНГ МЕТОДЕ ЗА ОДРЕЂИВАЊЕ АНТИБИОТИКА И СУЛФОНАМИДА У МЕДУ

Сандра М. Јакшић*, Жељко А. Михаљев, Бранкица Д. Карталовић, Јелена Б. Бабић,
Сузана Л. Видаковић, Милица М. Живков Балаш

Научни институт за ветеринарство „Нови Сад“, 21000 Нови Сад, Руменачки пут 20,
Србија

Сажетак: Здравствена исправност меда обухвата исправност у погледу присуства различитих контаминаната који подразумевају и резидуе антибиотика. Присуство антибиотика у меду је забрањено, а прописане су и методе анализе хране у циљу поузданог одређивања антибиотика у храни. У овом раду је приказана примена ЕЛИСА тестова за одређивање одабраних антибиотика и сулфонамида у меду. Испитана је могућност примене четири ЕЛИСА теста, за анализу тетрациклина, стрептомицина, хлорамфеникола као и сулфонамида. Сваки тест је испитан на узорцима меда обогаћеним стандардним раствором одређеног анализата. Узорци су припремани према упутству произвођача кита. Резултати испитивања свих примењених ЕЛИСА тестова, осим за сулфонамиде, показали су задовољавајућу тачност (73–111%) и прецизност (14–16%). Ефикасност одређивања сулфаметоксипиридазина била је ниска (40%), а при одређивању ниске концентрације тетрациклина била је нешто виша од прихватљиве (139%). Границе детекције су у складу са границама датим од стране произвођача ЕЛИСА тестова и такође су задовољавајуће у односу на захтеве законске регулативе (0,075–3 µg/kg). Испитани китови се могу користити за скрининг присуства тетрациклина, хлорамфеникола и стрептомицина у меду, узимајући у обзир добијене валидационе параметре.

Кључне речи: мед, тетрациклин, стрептомицин, хлорамфеникол, сулфонамиди, ЕЛИСА

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