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DETECTION OF RESIDUES OF ANTIMICROBIAL COMPOUNDS IN EGGS BY THE RAPID SCREENING METHODS

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ABSTRACT

Eggs belong to the most frequently consumed products of animal origin worldwide, and therefore the safety of eggs is a substantiated issue. Conventional poultry rearing involves the use of antimicrobials added to their feed or potable water particularly for disease treatment, however, in some countries also for the prevention of diseases, promotion of growth and better utilisation of the feed. Thus, effective control of residues of such substances in eggs is very important for the protection of the public health. The aim of this study was to detect the potential presence of antimicrobial residues in fresh hen eggs using commercially available rapid screening methods (Premi®Test and EXP Ampulle test) and compare the results of both of these tests. We examined 22 samples randomly selected from among 66 samples purchased in 11 European countries. We respected the procedures as supplied by the manufacturers of the tests together with their respective test kits. The examination of eggs by the Premi®Test did not detect the presence of antimicrobial residues in the samples, while the EXP Ampulle test provided 8 positive and 6 dubious results. Our results allowed us to conclude that the EXP Ampulle appears to be more sensitive and allows one to carry out more effective control of the presence of antimicrobial residues in hen eggs intended for human consumption.

Key words: antibiotics; detection; eggs; residues

INTRODUCTION

The essential role of egg production is to ensure the reproduction of the respective species. The development of the progeny takes place outside of the maternal body, therefore eggs must contain all of the important nutrients necessary for the embryonal development. Eggs contain high levels of full-value proteins that are a rich source of essential amino acids. Other important components are fats, vitamins and minerals. Eggs are an inevitable product for human nutrition. Besides their direct consumption, eggs are used also as a raw material in many branches of the food industry but also in other industries. They are important

also for their use in human and veterinary medicine, e.g. in vaccine production or as an insemination diluent [7].

In poultry production, antibiotics and antiparasitics are used to prevent and treat infectious diseases. Although in some countries antimicrobials are still used as growth promoter, in the European Union the use of antibiotics for this purpose was banned in 2006 in all species of food producing animals due to the increasing risk of the development of antimicrobial resistance [9]. The exception are coccidiostats that are administered preventively to poultry as feed supplements intended for killing or inhibition of the growth of pathogenic protozoa. Other antimicrobials most frequently used in poultry include polypeptides, tetracyclines, penicillins and sulphonamides, either separately or in combination with trimethoprim and chinolones [8].

In order to ensure good functioning of the internal market involving food of animal origin and to protect the public health, the EU accepted Commission Regulation (EU) No. 37/2010 as of 22 December 2009 on pharmacologically active substances and their classification regarding the maximum residue limits (MRL) in foodstuffs of animal origin. These MRL, accepted in the respective food matrix of the respective animal species, guarantee its safety to consumers [10]. The presence and the level of residues in products of animal origin are affected by a number of factors. The most important include: the dose of the medicine and the way and length of its administration, combination of medicines, physico-chemical properties and metabolism, withdrawal period, contamination of feed and water, age and physical condition of the animal and the composition of the food matrix itself [1, 2].

Egg consists of the egg white and the egg yolk. After administration of antimicrobials to layer hens, their residues occur in one or both egg components. Absorption of drugs in the intestine and their transport by the blood circulation to the left ovary, the protein-producing part of the oviduct (magnum) and uterus results in the deposition of antimicrobial residues in the egg yolk and thick and thin egg white during egg formation. The amount of accumulated residues depends first of all on the physico-chemical properties of the drug, its polarity, ability to bind to plasma proteins and the duration of relevant phases of formation of individual egg components [6].

The presence of residues in eggs for consumption is subject to compulsory testing. The EU has established a unified effective system for the determination of the presence of antimicrobial residues in products of animal origin in agreement with the Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC [14]. This effective system of residue monitoring consist of two linked-up steps, the screening and confirmation. The Directive 96/23/EC stipulates that the screening of residues can be carried out only by those analytical methods for which it can be demonstrated in a documented traceable manner that they are validated, and at the level of interest (concentration of the analyzed substance in the sample important for determination of its compliance with legislative regulations – MRL) have a false compliant rate of < 5 % (β-error). In the case of a suspected non-compliant result, this result shall be confirmed by a confirmatory method.

The screening methods that comply with the Directive 96/23/EC include also Premi®Test and EXP Ampulle test. Both these tests are broad-spectrum qualitative commercial testing systems combining the principle of agar diffusion and indicator colour change as a result of the active metabolism of the test strain *Bacillus stearothermophilus* var. *calidolactis* with the absence of an inhibitor. If the sample contains substances inhibiting the growth of the test strain, the purple colour of the indicator remains unchanged.

As the Premi®Test is currently the official method for laboratory diagnosis approved by the relevant authority of the Slovak Republic for the detection of antibiotic residues in products of animal origin including eggs [11], and EXP Ampulle test is one of the most recent available commercial tests, also recommended for detection of antibiotic residues in products of animal origin including eggs, the aim of our study was to detect the potential presence of antimicrobial residues in fresh hen eggs available on the market of 11 European countries by both above mentioned commercial rapid screening methods and compare the results of both tests.

MATERIALS AND METHODS

In the period of 2017—2018, we tested 22 samples randomly selected from among 66 samples of eggs purchased in chain stores in 11 European countries (Tab. 1) using Premi®Test (R-Biopharm AG, Germany) and EXP Ampulle

test (Packhaus Rockmann GmbH, Germany). In the case of a positive result, we tested separately the egg yolk and the egg white by the test that provided the initial positive result. Both tests are based on the inhibition of growth of the test strain *Bacillus (Goebacillus) stearothermophilus*, susceptible to a broad spectrum of antimicrobial substances used in veterinary medicine (β-lactams, cephalosporins, tetracyclines, macrolides, sulphonamides, and aminoglycosides) at their MRL level. The preparation and analysis of the samples complied with the procedures recommended by the manufacturers of Premi®test and EXP Ampulle test.

Table 1. Labeling of the samples and country of their origin/purchase

Sample No.	Country of origin
1, 2	Italy
3, 4	Spain
5, 6	France
7, 8	Rumania
9, 10	Bulgaria
11, 12	Ukraine
13, 14	Hungary
15, 16	Czechia
17, 18	Austria
19, 20	Poland
21, 22	Slovakia

Preparation of samples

The contents of the eggs used for the analysis of residues (whole egg content; egg yolk; egg white) were transferred to a sample container and homogenised thoroughly.

Procedure

Premi®Test: using a micropipette of a defined volume we transferred 100 µl of the homogenised sample to a Premi®Test ampoule (containing an agar medium, pH indicator and spores of *Bacillus stearothermophilus* and covered the ampoule with an adhesive foil supplied by the test manufacturer. The ampoule was then labelled and inserted into a thermoblock (Acublock Digital Dry Bath D 1200, Labnet, USA) where it was first pre-incubated at 80 °C for 10 min and then incubated for approximately 3—3.5 hours at 64 ± 0.5 °C. The incubation was terminated when the colour of the agar medium in the negative control sample turned from purple to yellow.

EXP Ampulle: 10 ml of sterile demineralised water was added to 10 ml of the homogenized egg contents. After thorough mixing, the diluted sample was warmed up for 3 min in a water bath at 100 °C with occasional mixing by a glass rod to prevent coagulation. Then, using a micropipette of a defined volume, 100 µl of the diluted homogenised sample were transferred to an ampoule supplied with the EXP Ampulle kit, covered the ampoule with a provided adhesive foil and inserted it into a thermoblock where it was incubated at 65 °C ± 1 °C for approximately 3 hours (2 hours and 30 min — 3 hours and 15 min), until the agar medium of the negative control samples turned from purple to yellow.

Evaluation of results

The colour of the agar medium in the sample ampoule was compared with that of the negative control. The yellow colour of the agar medium indicated the absence of antibiotics, therefore a negative result. The purple colour of the agar medium indicated the presence of antibiotics and the sample was evaluated as positive. The yellow-purple colour of the agar medium is indicative of the presence of antibiotics at the level equal to the detectability of the test. Such results are considered as dubious, because any colour differing from the yellow colour of the agar medium of the negative control indicates the presence of a residue. In the case of a dubious result, the analysis should be repeated again.

RESULTS AND DISCUSSION

The results of both tests were evaluated by comparing the colour of the agar medium with the colour chart which is part of the commercial test kit (Fig. 1 and 2). On the basis of this comparison we could determine whether the relevant sample was positive, negative or dubious.

The samples for the EXP Ampulle test were prepared according to the manufacturer's instructions. However, during the primary pre-incubation of the samples at 100 °C for 3 min, the samples coagulated despite their previous dilution with a sterile demineralized water (1 : 1 v/v). Because the pre-incubation of eggs is important for the inactivation of the enzyme lysozyme present in the egg white, which has an antimicrobial effect and thus can lead to false positive

Fig. 1. Evaluation of the change of colour of the agar medium bythe Premi®test

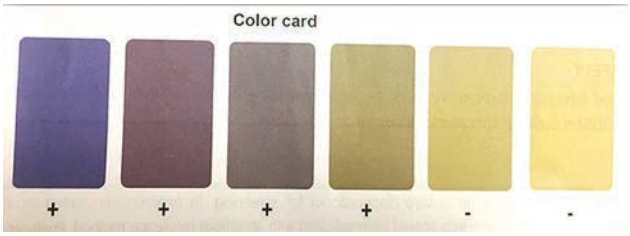


Fig. 2. Evaluation of the change of colour of the agar medium by the EXP Ampulle test

Table 2. Results of the examination of the whole egg content obtained by the Premi®testom and EXP Ampulle tests

Test	Sample																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Premi®test	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
EXP Ampulle	+	–	–	–	+	–	–	–	+	–	+	+	–	+	–	–	–	–	+	+	–	–

+ — positive sample; ± — dubious sample; – — negative sample

Table 3. Results of the examination of individual components of the positive eggs by the EXP Ampulle test

	Sample							
	1	5	9	11	12	14	19	20
Egg yolk	–	–	±	±	±	±	±	±
Egg white	+	+	+	+	+	+	+	+

+ — positive sample; ± — dubious sample

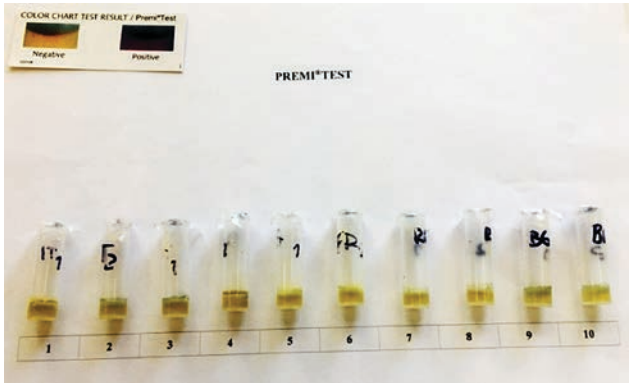
results, we respected the requirement of sample dilution but pre-incubated the samples using the thermal regimen of the Premi®test (pre-incubation at 80 °C for 10 min).

The testing of all 22 samples (whole egg content) by the Premi®test provided no positive results while with the EXP Ampulle test 8 samples tested positively. The results of the determination of residues of antimicrobial compounds in whole egg content obtained by Premi®test and EXP Ampulle test are presented in Table 2 and Figures 3a, 3b, 4a, 4b.

Eight positive results (samples 1, 5, 9, 11, 12, 14, 19, 20) obtained by the EXP Ampulle test when testing the whole egg content inspired us to test the individual components of the eggs, i.e. the egg yolk and the egg white separately. When examining the egg yolks, 6 samples tested dubious, but all 8 egg whites tested positive. The results of the determination of antimicrobial residues in egg whites and egg yolks of the positive samples by EXP Ampulle test are presented in Table 3 and Figures 5a, 5b and 5c.

The national plans of monitoring the residues of antimicrobial compounds in the food of animal origin are implemented by the EU member states. The residue control plan is aimed at surveying and revealing the reasons for residue hazards in foods of animal origin on farms, slaughterhouses, dairies, fish processing plants, and egg collecting and packing stations. The plan of residue control must agree with respect to the extent and frequency of sampling with the requirements set by the Directive 96/23/EC and the national legislative regulations.

The levels and frequencies of sample collection for the monitoring of some compounds and their residues in eggs are stipulated in the Annex of the Commission Decision 97/747/EC, which supplements the levels and frequencies



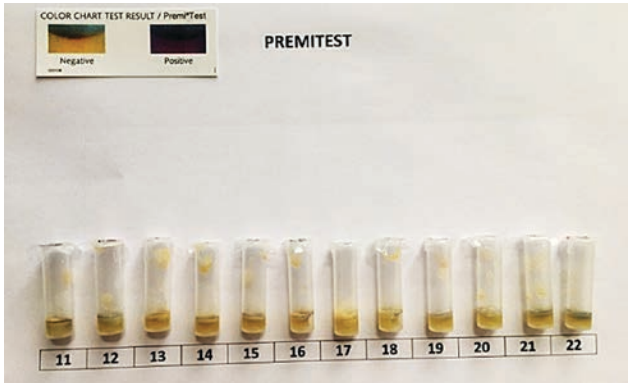
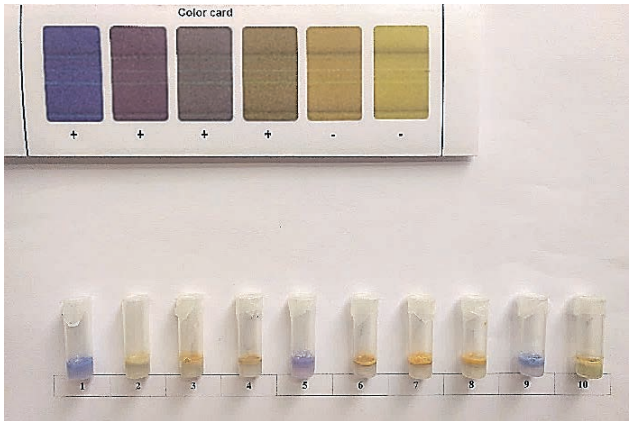


Fig.3a (left) and 3b (right). Determination of the antimicrobial residues in whole egg contents by the Premi®test



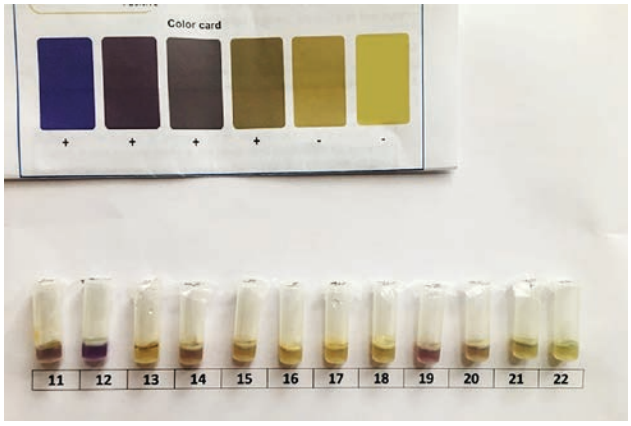


Fig. 4a (left) and 4b (right). Determination of the antimicrobial residues in whole egg contents bythe EXP Ampulle test

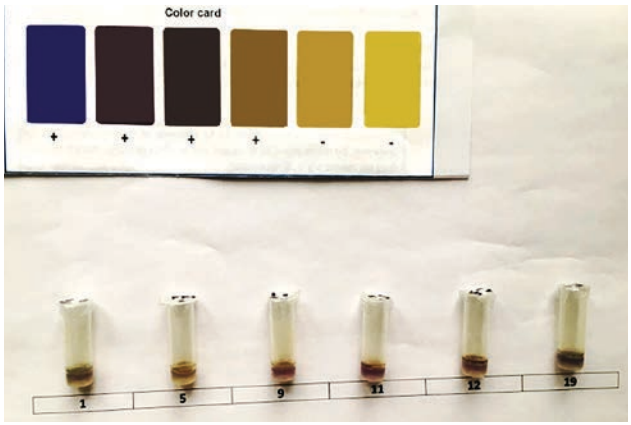


Fig 5a. Results of the examination of egg whites of the positive eggs (1, 5, 9, 11, 12, 19) usingthe EXP Ampulle test

Fig. 5b. Results of the examination of egg yolks of the positive eggs (1, 5, 9, 11, 12, 19) using the EXP Ampulle test

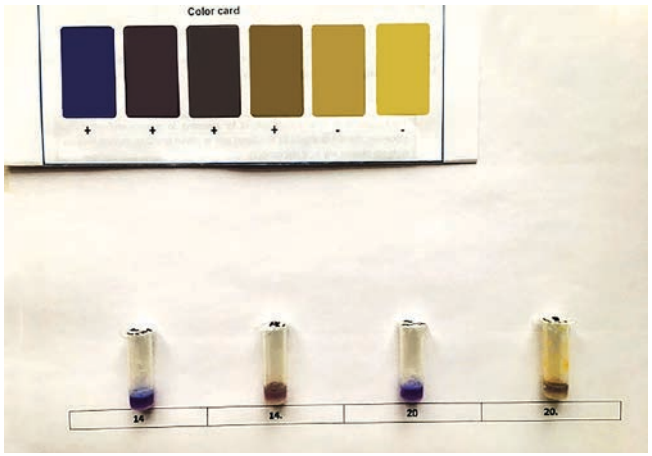


Fig 5c. Results of the examination of the egg white and egg yolks of the positive eggs (14/white/, 14/yolk/, 20/white/, 20/yolk) usingthe EXP Ampulle test

of sampling described in the Annex IV of the Directive 96/23/EC [13]. In the case of hen eggs, each official sample must be collected by the authorised veterinary administration body in a manner that allows one to identify retroactively the farm from which the eggs originated. The samples can be collected either at the farm level or grading or packing stations. The size of the sample is 12 or more eggs, according to the analytical methods used. The number of samples collected per year must be equal to at least one sample per 1,000 tonnes of annual production of eggs intended for consumption; the minimum being 200 samples.

Each member state of the EU can decide about the structure of sample collection depending on the structure of the egg industry, particularly with regard to its integration level. At least 30 % of the samples must be collected from egg grading and packing stations that represent the most significant proportion of eggs supplied for human consumption. From the point of view of the monitored compounds, 70 % of the samples must be tested for at least one compound from group A 6 (unauthorised substances), B 1 (antibacterial compounds /beta-lactams, tetracyclines, macrolides, aminoglycosides/including sulphonamides and chinolones) and B 2, letter b) (anticoccidials including nitroimidazoles) listed in the Annex II of the Directive 96/23/EC, and 30 % of samples must be collected according to the situation in the respective country, however, this must include some analysis of compounds from group B 3, letter a) (organochlorine compounds including chlorinated biphenols).

In the case of eggs from other poultry species, the proportions of samples from these species are determined individually by each member state according to the level of production and the detected problems. The eggs from other poultry species must be included in the plan of sample collection in the form of supplementary samples to samples of hen eggs.

The detection of antibacterial compounds by many screening methods based on microbiological analyses involves the testing of samples or sample extracts by means of the inhibition of growth of the test bacterial strains. The inhibition of the growth of the respective bacterial strain after some period of incubation indicates the presence of an antibacterial compound in the sample. Some EU member states use microbiological methods as a part of their specific control programmes intended for the control of antimicrobial residues. In some cases, the results of such tests are sufficient to decide about the presence of residues in the samples and no additional physical or chemical analysis are required for confirmation or identification of the relevant compounds. In the cases that require additional confirmatory tests, the positive result of the screening test is confirmed by immunochemical or physico-chemical tests capable of identifying the relevant compounds and determining whether its concentration is lower or higher than the respective MRL [12].

The three latest reports on the results of the monitoring of the residues of veterinary medicines and other substances in live animals and animal products in the years 2014,

2015 and 2016, published by the European Food Safety Authority (EFSA), presented the following results for eggs: in 2014 the examination of 4,674 samples of eggs for the presence of antimicrobials showed that 4 (0.09 %) of the samples were positive for doxycycline, enrofloxacin, flumechin and sulfadiazine, and the examination of 4,367 samples of eggs for the residues of coccidiostats revealed that 18 (0.41 %) of the samples were positive for diclazuril, lasalocid, monensin, narasin, nicarbazin, robenidin, salinomycin and toltrazuril sulfone [3]; in the 2015 examination of 4,454 samples of eggs for the presence of antimicrobials showed that 7 (0.16 %) of the samples were positive for doxycycline, enrofloxacin, sulfadimetoxine in combination with trimethoprim and sulfadiazine, while of 4,823 samples of eggs examined for anticoccidials and coccidiostat residues 23 (0.54 %) of the samples were positive for diclazuril, lasalocid, maduramycin, monensin, narazin, nicarbazin, robenidin, salinomycin and toltrazuril sulfone [4]; the examination of 4,476 samples of eggs for antimicrobial residues in 2016 revealed positivity of 8 (0.18 %) samples for doxycycline, enrofloxacin, flumechin and sulfadiazine, and tests for anticoccidial and coccidiostat residues conducted in the same year on 3,933 egg samples detected positivity of 32 (0.81 %) samples for lasalocid, monensin, narasin, salinomycin, diclazuril, dinitrokarbanyl, toltrazuril, toltrazuril sulfone and robenidin [5].

The results above clearly indicate that the monitoring and control of residues are essential to the protection of the public health and to validation the safety of products of animal origin. The results presented in our study clearly indicated the presence of antimicrobial residues in fresh hen eggs intended for human consumption that were randomly selected for screening tests. Hen eggs are an important food matrix and their safety in the first link of the food chain, i. e. by egg producers, should be guaranteed.

CONCLUSIONS

The aim of this study was to use and compare the current methods available for screening of residues (Premi®Test and EXP Ampulle tests) for the determination of the potential presence of antimicrobial residues in fresh hen eggs available on the market in 11 European countries. These two commercial tests were developed for the detection of a broad spectrum of antimicrobial residues in various food matrices including eggs. The higher number of positive and dubious results detected by EXP Ampulle test indicates the relevance of the residue control and use of microbiological methods for simple, rapid and economic detection of potentially non-compliant results. However, the confirmation of this potentially non-compliant results and necessity of taking relevant corrective measures requires identification and quantification of the respective antimicrobial compound.

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REFERENCES

1. **Aerts, M. M. L., Hogenboom, A. C., Brinkman, U. T., 1995:** Analytical strategies for the screening of veterinary drugs and their residues in edible products. *J. Chromatogr. B. Biomed. Sci. Appl.*, 667, 1—40.
2. **Beyene, T., 2016:** Veterinary drug residues in food-animal products: its risk factors and potential effects on public health. *J. Vet. Sci. Technol.*, 285, 1—7.
3. **EFSA, 2016:** *Report for 2014 on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products*. https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/sp.efsa.2016.EN-923.
4. **EFSA, 2017:** *Report for 2015 on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products*. https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/sp.efsa.2017.EN-1150.
5. **EFSA, 2018:** *Report for 2016 on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products*. https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/sp.efsa.2018.EN-1358.
6. **Hester, P. Y., 2017:** *Egg Innovations and Strategies for Improvements*, 1st edn., Academic Press, London, UK, 646 pp.
7. **Jurajda, V., 2002:** *Diseases of Poultry and Birds. Viral Infections* (In Czech). Veterinary and Pharmaceutical University in Brno, Brno, Czech Republic, 19—46.
8. **Méheust, D., Chevance, A., Moulin, G., 2015:** Suivi des ventes de médicaments vétérinaires contenant des antibiotiques en France en 2015 — Rapport annuel. *Fougères: Anses*, 2016, 1—106. https://hal.archives-ouvertes.fr/hal-01398388.

9. **Regulation (EC) No. 1831/2003** of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. *Official Journal of the European Union*, L268, 29—43.
10. **Commission Regulation (EU) No. 37/2010** of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. *Official Journal of the European Union*, L15, 1—72.
11. **R-26:** *Determination of residues of inhibitory substances in meat by the PREMI® TEST method. Official method of laboratory diagnostics*. http://www.svssr.sk/dokumenty/zakladne_info/R_26.pdf
12. **European Commission, 2009:** *Commission Staff working document on the implementation of national residue monitoring plans in the Member States in 2009*. https://ec.europa.eu/food/sites/food/files/safety/docs/cs_vet-med-residues_workdoc_2009 _en.pdf.
13. **Commission Decision No. 97/747/EC** of 27 October 1997 fixing the levels and frequencies of sampling provided for by Council Directive 96/23/EC for the monitoring of certain substances and residues thereof in certain animal products. *Official Journal of the European Union*, L303, 76—79.
14. **Council Directive No. 96/23/EC** of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC. *Official Journal of the European Union*, L125, 71—93.

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