Original Scientific Article UDC: 637.12.07:[636.09:615.33.074

SCREENING OF VETERINARY DRUG RESIDUES IN MILK FROM INDIVIDUAL FARMS IN MACEDONIA

Dimitrieska-Stojkovic Elizabeta, Hajrulai-Musliu Zehra, Stojanovska-Dimzoska Biljana, Sekulovski Pavle, Uzunov Risto

Institute for food, Faculty for veterinary medicine, "Sts Cyril and Methodius" University in Skopje, Republic of Macedonia

Corresponding author: edimitrieska@fvm.ukim.edu.mk

ABSTRACT

A total of 497 raw milk samples collected at individual farms and collection tanks for milk from eight regions from Macedonia were examined for chloramphenicol, sulfonamides, quinolones and tetracyclines from October 2008 until April 2011. Immunoassay methods were used for the determination of chloramphenicol, sulfonamides and quinolones, and high performance liquid chromatography with Diode Array detection was applied for screening of tetracyclines. The methods were validated according to the recommendations laid down by European Commission Decision 2002/657/EC. The obtained data confirmed that the methods were appropriate for detection of antibiotics determined, at the concentration level of interest. Measured range of concentrations (in μg/kg) was 13.5-147.9 for sulfonamides, 0.6-22.0 for quinolones and 17.4-149.1 for tetracyclines, with calculated mean values (in μg/kg) 24.7 for sulfonamides, 12.6 for qinolones and 41.9 for tetracyclines. None of the analyzed samples showed presence of chloramphenicol over the minimum required performance level value of the screening method. The calculated estimated daily intakes for the average daily consumption of 200 mL of milk for an adult in Macedonia, for the examined antimicrobials, obtained levels 2 to 100 times lower than the values of the acceptable daily intakes fixed by World Health Organization. This indicates that toxicological risk associated with the consumption of analyzed milk could not be considered as a public health issue with regards to these veterinary drugs.

Key words: *milk, veterinary drug residues, chlorampenicol, tetracyclines, sulfonamides quinolones, ELISA, HPLC-DAD, estimated daily intake*

INTRODUCTION

Antimicrobial agents are widely used at milk producing animals. Improper administration of the veterinary medicinal products (VMP) for disease therapy and growth promotion may result in presence of antimicrobial residues in milk and dairy products, and can contribute to the development of microbial drug resistance and the spread of resistant bacteria (1). Also, the residues of VMP present a potential risk to the consumer, particularly with the appearance of allergic reactions and interferences of intestinal micro-flora (2). From the technological prospective, residues of antimicrobial agents in milk can cause significant losses in fermented products, by inhibition of bacterial fermentation in the production processes of cheese or yoghourt (3).

Due to harmful effects of VMP residues, surveillance systems are enforced in the European Union related to the requirements laid down in the Council Directive 96/23/EC (4). According with these requirements, the Macedonian legislation was fully aligned with the EU legislation concerning residues of VMP in foodstuffs of animal origin in

regarding the Council regulation 37/2010/EU (5).

Therefore, accurate detection of low levels of antimicrobial drug residues in milk is of great importance for the diary industry and for farmers, with a purpose to ensure that the contaminated milk form individual cows are not consigned to the bulk tank (6). Consequently, it is necessary to monitor a large number of milk samples for the presence of the most important antimicrobial drug residues, by using rapid and reliable microbiological (7,8), immunological (9,10) or physico-chemical screening methods (11-13). All methods used for that purpose have to detect antibiotics at or below their permissible limits or MRLs and also have to be validated in accordance with the Council Directive 2002/657/EC (14).

Unlike the other countries from the region, the annual consumption of milk and diary products in Macedonia is somewhat lower, and for 2009 it was around 72 L per capita (15,16). In the last decades there were few studies about the screening of VMP residues in raw milk samples collected from Macedonia (17-19). In order to monitor veterinary drug contamination in milk samples from diary farms and individual production facilities, residual concentrations of chloramphenicol, sulfonamides, qinolones and tetracyclines were examined. Furthermore, an estimation of the dietary intake of veterinary drugs residues derived from milk consumption was performed.

MATERIALS AND METHODS

Sampling

A total of 497 raw milk samples were collected from October 2008 to April 2011 at individual small milk-producing facilities and from the collection tanks of milk routes in eight regions form Macedonia. Samples were stored at 4-8^{o}C if analyzed within 24 hours after sampling, or kept at less than -20^{o}C for four weeks.

Reagents and standard solutions

The Chloramphenicol ELISA kit (type AB630) and quinolones (enrofloxacin, ciprofloxacin, norfloxacin, danofloxacin, oxolinic acid, flumequine) ELISA kit (type AB630) were purchased by TECNA (Trieste, Italy). The Multi-sulfonamides (sulfadimidine, sulfamerazine, sulfachloropyridazine, sulfisoxazole, sulfadiazine, sulfachloropyridazine, sulfametoxazole) ELISA kit (type 5101SULMp) was supplied from Europroxima (Arnhem, The Netherlands). Ethyl acetate, citric acid, disodium hydrogen phosphate, zink sulphate heptahydrate, potassium hexacyanoferrate (II) and oxalic acid were with p.a. purity, supplied from Merck (Darmstadt, Germany). Sodium EDTA was supplied by Sigma (St. Louis, USA). Methanol, acetonitrile and water were with HPLC grade, and were supplied from Sigma (St. Louis, USA).

The certified pure neat standards of sulfadimidine, sulfisoxazole, oxytetracycline hydrochloride, tetracycline hydrochloride, chlorotetracycline hydrochloride, doxycycline hyclate, enrofloxacin and chloramphenicol were supplied from Sigma-Aldrich (St. Louis, USA).

Standard solutions and spiking of samples

Standard stock solutions with concentration of 1 mg/mL were prepared on monthly basis by dissolving the analytes in methanol. Intermediate working solutions were prepared prior to usage by diluting tetracycline's solutions in methanol, while sulfonamides, fluoroquinolones and chloramphenicol solutions were prepared in sample dilution buffers, provided in the EIA kits. These solutions were used for spiking blank milk samples at different levels. Following fortification, samples were allowed to equilibrate for 15 minutes before extraction.

Instruments

For sample preparation vortex model Relax Top by Heidolph (Schwabach, Germany), centrifuge model 2K15 by Sigma (St. Louis, USA), evaporator model DriBlock DB-3D by TECHNE (Staffordshire, UK) were used. The optical density at 450 nm for the EIA tests was measured by microplate reader BDSL Immunoscan (Labsystem, Switzerland). Liquid chromatographic separation of the tetracyclines in isocratic mode was performed on reverse-phase C_8 Ascentis column, supplied by Supelco (Belafonte, USA). Applied mobile phase was a mixture of 0,01 mol/dm^3 oxalic acid, methanol and acetnitrile in ratio 70:20:20 (V/V/V). Detection of tetracyclines was performed on Perkin Elmer LC 235C diode array instrument (Norwalk, USA) at 365 nm.

Sample preparation for the screening methods

Sample preparation, as well as preparation of all reagents for chloramphenicol, sulfonamides and quinolones, was according to the manufacturer's instructions. Milk samples for chloramphenicol and sulfonamides were prepared by defatting of samples by centrifugation at +4 °C and 3000 rpm, extraction with ethylacetate, and evaporation under the stream of nitrogen. The prepared samples were finally dissolved in sample dilution buffers, included in the EIA kits, and applied on the microtiter plates. For chloramphenicol the dilution factor was 0.1, while for sulfonamides it was 20. The milk samples for analysis of quinolones were deproteinized by adding Carrez I and Carrez II reagents, centrifuged and diluted with sample dilution buffer in ratio 1:9 (tenfold dilution).

Tetracyclines (oxytetracycline, tetracycline, chlorotetracycline and doxycycline) were determined by modified and optimized screening reverse-phase High-performance liquid chromatography/diode array detector (HPLC/DAD) method (20), with previous extraction with McIlvaine buffer and purification of the sample extracts on OASIS HLB solid-phase extraction cartridges (Waters, Milford, USA). The samples were finally dissolved in 0.01 mol/dm^3 oxalic acid buffer, filtered through 0.45 μm syringe filters and injected into the HLPC system. The dilution factor was 0.2.

Validation of the screening methods

Performance characteristics of EIA methods and the screening HPLC-DAD method were determined as prescribed for qualitative screening methods in Commission Decision 2002/657/EC (14). Also the limit of detection (LOD) and limit of quantification (LOQ) were obtained by adding 3 and 10 times the standard deviation of 20 blank samples to the mean blank value. Recovery was assessed by performing the experiments where fortified milk samples were analyzed in ten replicates, at the respective maximum residue level (MRL) or minimum required performance level (MRPL) values for the substances being analyzed. From the recovery experiments the method precision was obtained, as well. The detection capability (CCβ) for tetracyclines, quinolones and sulfonamides was determined by spiking of 20 blank samples at the one half of the MRL value set at 100 μg/kg (5), while for chloramphenicol the spiking level was below the established MRPL value of 0,3 μg/kg (21). The calculations were performed by the formula provided in the EU Commission Decision (14).

Calculation of the estimated daily intake

The estimated daily intake (EDI) was calculated by the equation given by Bilandžić et al. (22), whereas the data for the mean detected concentrations for the analyzed residues and average daily consumption based on 60 kg body weight were taken into account. According to the literature data, the average daily milk consumption for an adult in Macedonia was 200 mL (17,18).

RESULTS AND DISCUSSION

The objective of this study was to determine the residual levels of chloramphenicol as a prohibited substance, and tetracyclines, quinolones and sulfonamides throughout two and a half year of raw milk samples monitoring in Macedonia, to reveal if the maximum residue limits have been exceeded in terms of consumer health protection.

All VMP residues analyses were performed using in-house validated screening methods according to the criteria laid down in Commission Decision 2002/657/EC (14). Validation data corresponding to the screening method performance (LOD, LOQ, CCβ, recovery and precision) are presented in Table 1. For all screening methods the obtained CCβ values were less than the fixed MRLs or MRPL, with recoveries higher than 70 %. Moreover, the precision values were lower than the recommended maximal by the Horwitz equation (14). The validation data indicated that the applied methods were appropriate for the detection of residues of VMP measured.

Table 1. Method validation data for the screening methods of the antibiotics analyzed in milk samples

Analyte	LOD	LOQ	CCβ	Recovery	Precision
	(μg/kg)	(μg/kg)	(μg/kg)	%	%
Sulfonamides	13.5	45.0	64.5	97.5	4.5
Oxytetracycline	9.0	29.9	56.6	84.1	9.7
Tetracycline	13.8	46.1	53.8	80.4	10.5
Chlorotetracycline	16.7	55.7	58.4	99.1	7.1
Doxycycline	20.7	69.2	53.9	85.1	12.4
Quinolones (Enrofloxacine)	4.2	10.8	58.6	78.3	16.2
Chloramphenicol	<0.01	0.014	0.18	75.0	17.1

A total of 497 milk samples were subjected to the screening methods. The determined antimicrobial's concentrations and MRLs for tetracyclines, quinolones and sulfonamides, as well as the MRPL value for chloramphenicol are summarized in Table 2. All concentrations found for chloramphenicol were lower than the critical value established by Commission Decision 2003/181/EC (21). In the case of tetracyclines and sulfonamides a few samples were found exceeding the MRLs set by Commission Regulation (EU) 37/2010 (5). None of the samples examined contained residues of quinolones over the established MRL. In a previous study, residues of veterinary drugs were detected in total 4.3 % of milk samples collected in Macedonia during the 2010, using a screening diffusion test (Delvo test, DSM Food Specialities, Delft, The Netherlands) (19).

The frequencies of detection of the individual antimicrobial substances (expressed in %) are presented on Figure 1. From the presented data it can be clearly concluded that in the raw milk samples the most frequently detected were the residues of tetracyclines (48.9 %). The occurrence of the individual substances was 32.7 %, 13.1 %, 3.4 % and 2.5 % for oxytetracycline, tetracycline, chlortetracycline and doxycycline, respectively. Sulfonamides were detected in 18.4 % and quinolones in 6.8 % of the samples tested for residues of antimicrobial substances.

Table 2. Veterinary drug residues (range and mean) in milk samples collected in the period from October 2008 until April 2011 and critical values regulated by EU and Macedonian legislation

Analyte	n	Range	Mean	MRL
		(μg/kg)	(μg/kg)	(μg/kg)
Sulfonamides	497	13.5-147.9	4.71	100
Oxytetracycline	497	17.4-149.1	12.9	100
Tetracycline	497	19.7-80.5	4.6	100
Chlorotetracycline	497	13.6-63.9	0.9	100
Doxycycline	497	28.2-76.4	1.2	NE**
Total tetracyclines	497	13.6-149.1	19.6	100
Quinolones	497	0.6-22.0	12.5	100
Chloramphenicol*	497	0.002-0.074	0.019	0.3

* Chloramphenicol is not authorized for use in food producing animals in the European Union and in Macedonia (in MRL column the indicated value is MRPL)

**NE: not established; MRL value is not established for doxycycline, not authorized for milk producing animals in the European Union and in Macedonia

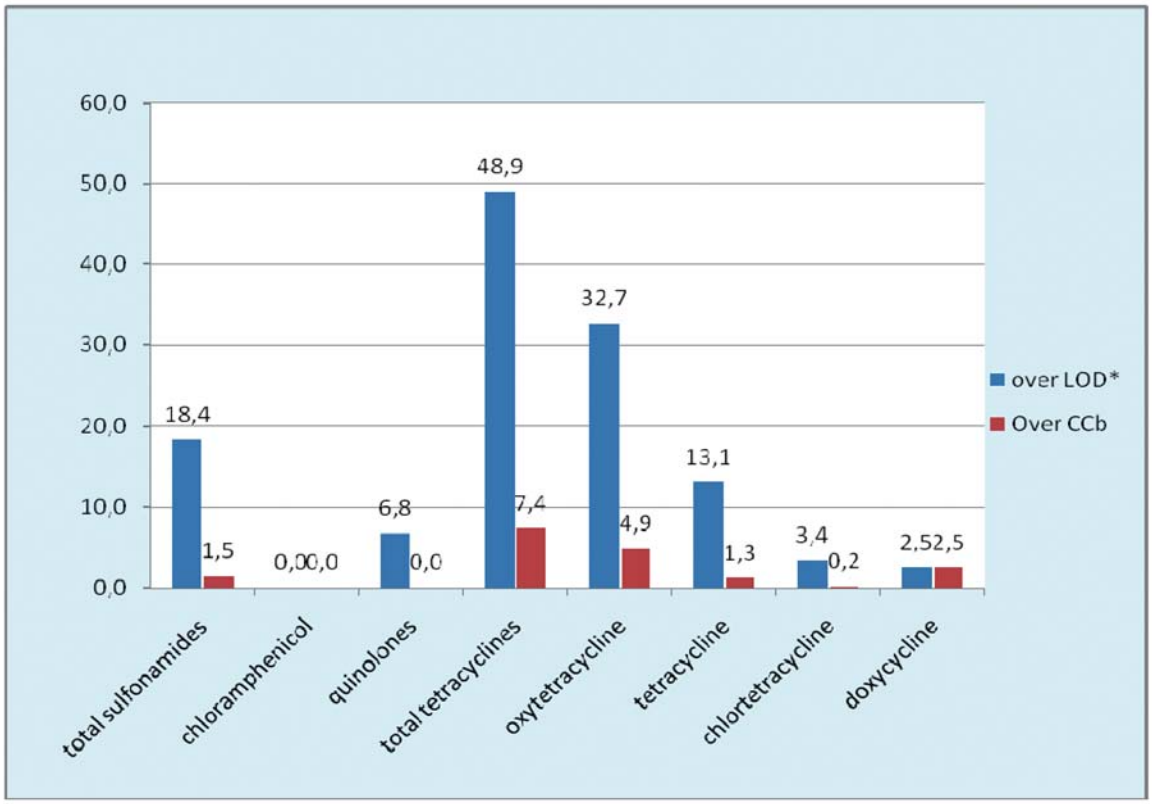

Figure 1. Occurrence of individual antimicrobials or groups of antimicrobials (in %) regarding the LOD and CCβ values

*for chloramphenicol LOD value indicates the MRPL value

Sulfonamides play important role as effective chemotherapeutics of bacterial and protozoan diseases and as growth promoters in veterinary medicine. The Committee for Veterinary Medicinal Products considers that the sum of all substances belonging to the sulfonamide group in raw milk should not exceed 100 µg/kg (23). During the past decades there have been reports on presence of sulfonamides in raw milk samples from Germany (24,25), whereas the prevalence for sulfonamides was 1,1 and 1,6 %. Other authors (26) have reported prevalence for total sulfonamides of 51,3 % and obtained concentration range from 1,9 to 180 µg/kg. In the present study, the mean concentration of determined sulfonamides was approximately 21 times lower than the established MRL. According to World Health Organization (WHO) (27) the acceptable daily intake (ADI) for sulfadimidine was established at 3 mg for 60 kg BW.

Tetracyclines are globally used as broad spectrum antibiotics in veterinary medicine against a wide range of Gram-positive and Gram-negative aerobics and anaerobic bacteria (28). Screening of locally produced milk samples in Czech Republic indicates a presence of tetracyclines in 50,6 % of the analyzed samples with concentrations under the MRL value (29). In the present study, the highest tetracycline level detected has exceeded the MRL value and it was 149.1 µg/kg. However, the mean tetracycline concentration was more than five times lower than the assigned MRL.

Quinolones are a group of relatively new antimicrobials synthesized from 3-quinolone carboxylic acid. They show excellent activity against both Gram-positive and Gram-negative organisms, as well anaerobes (30). Qunolones, especially enrofloxacine, are considered to be one of the most frequently applied MVP on the Belgian market. Despite this fact, during one investigation of the presence of these antimicrobials in 2008 by *E.coli* test in microplate format, no quinolone residues were detected (31). In this study, the mean quinolones concentration (expressed as enrofloxacin) showed levels more than eight times below the established MRLs.

Chloramphenicol is a broad spectrum antibiotic

active against both Gram-positive and Gram-negative bacteria and an effective therapeutic agent for the treatment of mastitis in cattle. Due to the potential risk to human health, the use of chloramphenicol is prohibited in food-producing animals in the European Union (5). The European Union introduced the concept of the minimum required performance limit (MRPL) of 0.3 µg/kg, the highest concentration level at which the screening and confirmatory method shall demonstrate satisfactory performances regarding the sensitivity, accuracy and precision (21).

In this investigation, the measured chloramphenicol mean concentration of 19 ng/kg was substantially lower than the MRPL value, and in practically it is the signal obtained from the blank. However, in the past decade there have been reports of incidences in other European countries for unauthorized presence of chloramphenicol: >0.2 µg/kg in 2000 and 0.5 µg/kg in 2001 in Slovenia, 0.72 µg/kg in 2006 in Poland, 0.3 µg/kg in 2002 in Lithuania, 0.14 µg/kg in 2004 in Estonia (32). The recent publications for presence of chloramphenicol residues found in 39 samples ranging from 0.3 to 1.27 mg/kg has been reported in milk and dairy products mainly from Eastern European countries (33).

For the purpose of evaluation of dietary exposure with veterinary drugs residues through the intake of raw milk controlled in the present study, the (EDIs) for consumers were estimated. Table 3 shows the EDIs of veterinary drug residues based on the concentrations found in the present work, calculated with a presumed average daily milk consumption for an adult of 200 mL (15,16). Residue values of all MRL drugs measured, ranged from 0.033 to 1.63 mg/kg BW/day, and were 2 to 100 times lower than the set values of ADIs (29, 34, 35). However, ADIs values have not been established for chloramphenicol.

Table 3. Estimation of daily intakes (EDIs) of veterinary drug residues through milk consumption based on the mean concentrations found in the period end of 2008 - beginning of 2011

Analyte	EDI	ADI
	µg/kg BW/day	µg/kg BW/day
Sulfonamides	0.177	50
Oxytetracycline	0.472	1
Tetracycline	0.169	1
Chlorotetracycline	0.033	1
Doxycycline	0.1	NE**
Total tetracyclines	1.63	3
Enrofloxacin	1.042	372
Chloramphenicol*	0.74*	NE**
Total value	3.624	428

*EDI for chloramphenicol was expressed as ng/kg BW/day

**NE: Not yet have been established

The highest individual calculation for EDI was obtained for oxytetracycline, which was approximately one half of the ADI value. The total EDI value obtained was 3.624 mg/kg BW/day, substantially lower than the total acceptable daily intake. Therefore, the toxicological risk associated with the consumption of analyzed milk could not be considered as a public health issue with regards to these veterinary antimicrobial substances.

CONCLUSION

The methods used for antibiotic determination in milk were validated according to Commission Decision 2002/657/EC and proved to be rapid, simple and reliable, exhibiting good accuracy and repeatability, with recovery higher than 70 %. In the two and a half year period of monitoring milk samples, the residues measured for quinolones were

far below the maximum residue limits (MRLs) set by the legislation. None of the samples exceeded the MRPL value for chloramphenicol. On the other hand, there have been a few samples containing amounts of sulfonamides and tetracyclines exceeding the established MRLs, that should be confirmed by application of confirmatory methods. The EDIs calculated showed that the contribution of milk to dietary intake of the investigated antibiotics were 2 to over 100 times below the ADIs proposed by EMEA and WHO. This indicates that the raw milk in Macedonia, in average, contains low levels of veterinary drugs, and therefore, it could be considered as safe for human consumption.

REFERENCES

1. Stolker, A.A.M., Zuidema, T., Nielen, M.W.F. (2007): Residue analysis of veterinary drugs and growth-promoting agents. *Trends in Analytical Chemistry* 26, 967-979.

2. Dewdeny, J. M., Maes, L., Raynaud, J.P., Blanc, F., Scheid, J.P., Jackson, T., Lens, S., Verschueren, C. (1991): Risk assessment of antibiotic residues of beta-lactams and macrolides in food-products with regard to their immunoallergic potential, *Food and Chemical Toxicology* 29, 477-483.

3. Molina, M.P., Althausb, R.L., Molinac, A., Fernándeza, N. (2003): Antimicrobial agent detection in ewe's milk by the microbial inhibitor test brilliant black reduction test – BRT AiM®, *International Diary Journal* 13, 821-826.

4. 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 OJ L 125, 23.5.1996, p. 10–32.

5. Commission Regulation (EU) 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, OJ L 15/1, 20.01.2010, p. 1–2 (ANNEX I).

6. Mitchell, J.M., Groffiths, M.W., Mcewen, S.A., McNab, W.B., Yee, A.J. (2002): Antimicrobial drug residues in milk and meat: Causes, concerns, prevalence, regulations, tests and test performance, *Journal of Food Protection* 61, 742-756.

7. Gaudin V., Maris P., Fuselier R., Ribouchon J.-L., Cadieu N., Rault A. (2004) Validation of a microbiological method: the STAR protocol, a five-plate test, for the screening of antibiotic residues in milk, *Food Addit. Contam.*, 21: 422–433.

8. Kress C., Seidler C., Kerp B., Schneider E., Usleber E. (2007) Experiences with an identification and quantification program for inhibitor-positive milk sample, Anal. Chim. Acta, 586: 275–279.

9. Pastor-Navaro N., Maquieira A., Puchades R. (2009) Immunoanalytical determination of tetracycline and sulfonamide residues in edible products: a review, Anal. Bioanal. Chem., 395: 907–920.

10. Ricci, F., Volpe, G., Micheli, L., Palleschi, G. (2007): A review on novel developments and applications of immunosenzors in food analysis, Anal. Chim. Acta, 605: 111–129.

11. Terzano C., Gamba V., Moretti S., Galarini R., Dusi G. (2008): Development and validation of a confirmatory method for the determination of sulfonamides in milk by HPLC with Diode Array detection, *Proceedings of the EuroResidue VI Conference*, 1: 223–227, Egmond aan Zee, The Netherlands 19–21 May.

12. Koesukwiwat, U., Jayanta, S., Leepipatpiboon, N. (2007): Solid-phase extraction for multiresidue determination of sulfonamides, tetracyclines and pyrimethamine in bovine`s milk, J. Chromatogr. A., 1149: 102–111.

13. Stoev, G., Michailova, Al. (2000): Quantitative determination of sulfonamide residues in foods of animal origin by high-performance liquid chromatography with fluorescence detection, *J. Chromatogr. A*, 871: 37–42.

14. Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results OJ L 221, 17.8.2002, p. 8–36.

15. Regional figures for milk/diary products consumption for 2009. (http://eastagri.org, accessed at 26.10.2011).

16. Krstevska, A., Nacka, M., Janeska, I., Georgiev, N. (2009): An assessment of the competitiveness of the diary food chain in the Republic of Macedonia. (http://www.euroqualityfiles.net/AgriPolicy/report%201.1/FYROM%20Agripolicy %20D2-1.pdf)

17. Dimitrieska-Stojkovic, E., Hajrulai-Musliu, Z., Stojanovska-Dimzoska, B., Sekulovski, P., Uzunov, R., Prodanov, R. (2010): Assesment of compliance of raw milk samples from Republic of Macedonia for residues of tetracyclines according to method performance requirements laid down in the Commission Decision 657/2002/EC, Book of abstracts of Days of veterinary medicine p. 64, 28-30 October, Ohrid, R. of Macedonia.

18. Dimitrieska-Stojkovic, E., Prodanov, R., Hajrulai-Musliu, Z., Dimzoska-Stojanovska, B., Sekulovski, P., Uzunov, R. (2011): Screening of raw milk samples from Republic of Macedonia for residues of sulfonamides, Proceedings of Days of veterinary medicine p. 106-107, 09-11 September, Ohrid, R. Macedonia.

19. Angelovski, Lj., Sekulovski, P., Jankulovski, D., Ratkova, M., Kostova, S., Prodanov M. (2011): Microbiological quality and occurrence of antibiotic residues in ewe milk, Proceedings of Days of veterinary medicine p. 101, 09-11 September, Ohrid, R. Macedonia.

20. Cinquina, A. L., Longo, F., Anastasi, G., Gianetti, L., Cozzaani, R. (2003): Validation of high-performance liquid chromatography method for the determination of oxytetracycline, tetracycline, chlorotetracycline and doxycycline in bovine milk and muscule, J. Cromatogr. A, 987: 227–223.

21. Commission Decision 2003/181/EC of 13 March 2003 amending Decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results OJ L 221, 17.8.2002, p. 8–36.

22. Bilandžić N., Solomun-Kolanovic B., Varenina I., Scortichini G., Annunziata L., Brstilo M., Rudan N. (2011) Veterinary drugs residues determination in raw milk in Croatia, Food Control, 22 (12) 1941-1948.

23. EMEA (1995a): Sulfonamides, summary report (2), Committee for veterinary medicinal products, EMEA. EMEA/MRL/026/95.

24. Suhren, G., Heeschen, W., Walte, H. G. (1996): Application of an integrated system for the detection of antimicrobials in car tanker milk, *Proceedings of the EuroResidue III Conference*, 2: 922–926, Veldhoven, The Netherlands 6–8 May.

25. Kress, C., Seidler, C., Kerp, B., Schneide,r E., Uslebe,r E. (2007): Experiences with an identification and quantification program for inhibitor-positive milk sample, Anal. Chim. Acta, 586: 275–279.

26. Tolentino, R.G., Perez, M.N., Gonzales, D.D., Vega, S.L., Flores, G.P. (2005): Determination of the presence of 10 antimicrobial residues in mexican pasteurized milk, Intercsiencia, 3: 291–294

27. JECFA (1995): Joint FAO/WHO expert Committee on food additives. WHO technical report series 851, forty second meeting of JECFA.

28. JECFA (1998): Joint FAO/WHO expert Committee on food additives. WHO technical report series 888, fifty meeting of JECFA.

29. Navratilova, P., Borkovcova, I., Dračkova, M., Janštova, B., Vorlova, L. (2009) Occurrence of tetracycline, chlortetracyclin, and oxytetracycline residues in raw cow's milk, *Chech J. Food Sci.*, 27: 379–385

30. Bogialli S., D`Ascenzo G., Di Corcia A., Lagana A., Nicolardi S. (2008) A simple and rapid assay based on hot water extraction and liquid chromatography–tandem mass spectrometry for monitoring quinolone residues in bovine milk, *Food Chemistry*, 108: 354-360.

31. Reybroeck W. (2010) Screening for residues of antibiotics and chemotherapeutics in milk

and honey, *PhD Thesis*, Faculty of Veterinary Medicine, University of Ghent, Belgium.

32. Dolajš, H., Doganoc, D.Z., Šinigoj-Gačnik K., Kirbiš A., Verkvenik-Flajs V. (2007): Residues of certain veterinary drugs in raw milk in Slovenia in the 2000-2002 period, International Journal of Environmental and Pollution 31, 155-166.

33. RASSF (2011): Rapid Alert system for Food and Feed (https://webgate.ec.europa.eu/rasff-window/portal/)

34. EMEA (1995b): Oxytetracycline, Tetracycline, Chlortetracycline, summary report (3) Committee for veterinary medicinal products, EMEA, EMEA/MRL/023/95.

35. EMEA (2002): Enrofloxacin, summary report (5) Committee for veterinary medicinal products, EMEA, EMEA/MRL820/02 final, January 2002.