UNIVERSITY „Ss. CYRIL AND METHODIUS" IN SKOPJE
FACULTY OF VETERINARY MEDICINE – SKOPJE

BOOK OF PROCEEDINGS

DAYS OF VETERINARY MEDICINE 2012

3rd International Scientific Meeting

Republic of Macedonia
2-4 September 2012

EXECUTIVE COMMITTEES OF DAYS OF VETERINARY MEDICINE 2012

Organizing Committee

Prof. Dr. Dine Mitrov, Prof. Dr. Velimir Stojkovski, Prof. Dr. Zehra Hajrulai-Musliu, Prof. Dr. Slavco Mrenoski, Prof. Dr. Vlatko Ilieski, Prof. Dr. Blagica Sekovska, Prof. Dr. Plamen Trojacanec, Prof. Dr. Igor Ulcar, Prof. Dr. Pavle Sekulovski, Prof. Dr. Toni Dovenski, Asst. Prof. Dr. Jovana Stefanovska, Asst. Prof. Dr. Lazo Pendovski, Asst. m-r Dean Jankuloski, Asst. m-r Ljupco Mickov, Asst. m-r Irena Celeska

International Scientific Committee

Prof. Dr. Marjan Kosec
University of Ljubljana, Slovenia
Prof. Dr. Jelka Zabavnik-Piano
University of Ljubljana, Slovenia
Prof. Dr. Dinko Dinev
Trakia University of Stara Zagora, Bulgaria
Prof. Dr. Aleksandar Pavlov
Trakia University of Stara Zagora, Bulgaria
Prof. Dr. Tomislav Dobranic
University of Zagreb, Croatia
Prof.Dr. Alen Slavica
University of Zagreb, Croatia
Prof. Dr. Andrej Kirbis
University of Ljubljana, Slovenia
Prof. Dr. Geert Opsomer
University of Gent, Belgium
Prof. Dr. Robert Farkas
University of Budapest, Hungary
Prof. Dr. Almedina Zuko
University of Saraevo, Bosnia and Herzegovina
Prof. Dr. Mehmed Muminovic
University of Saraevo, Bosnia and Herzegovina
Prof. Dr. Danijela Kirovski
University of Belgrade, Serbia
Prof. Dr. Miodrag Lazarevic
University of Belgrade, Serbia
Prof. Dr. Ivanco Naletoski
Joint FAO/IAEA Division, Vienna, Austria
Prof. Dr. Giovanni M. Lacalandra
University of Bari, Italy
Prof. Dr. Kiro R. Petrovski
University of Adelaide, Australia
Prof. Dr. Mustafa Atasever
Istanbul University, Turkey
Prof. Dr. Halil Gunes
Istanbul University, Turkey

Secretariat

Asst. Prof. Dr. Florina Popovska-Percinik, D-r Elizabeta Dimitrievska-Stojkovik Asst. m-r Aleksandar Dodovski, Asst. m-r Iskra Cvetkovik, Asst. m-r Ksenija Ilievska, Asst. m-r Kirili Krstevski, Asst. m-r Igor Dzadzovski, Asst. m-r Nikola Adamov, Asst. m-r Igor Esmerov, Asst. m-r Katerina Blagoevska, Asst. m-r Branko Atanasov, m-r Biljana Stojanovska – Dimzoska, Asst. Sandra Kostova, Ljupco Angelovski, Mirko Prodanov, Marija Ratkova, Sinisa Acevski, Branko Angelovski

Topics of the Days of Veterinary Medicine 2012

Animal Health
Food Safety and Veterinary Public Health
Animal Welfare and Genetics
Animal Reproduction

Editors:

Prof. Dr. Dine Mitrov
Assist. Prof. Dr. Lazo Pendovski

Published by:

Faculty for veterinary medicine – Skopje, Lazar Pop Trajkov 5/7, 1000 Skopje
Tel: ++389 2 3420 700 Fax: ++ 389 2 3114 619
www. fvm.ukim.edu.mk

CIP - Каталогизација во публикација
Национална и универзитетска библиотека "Св. Климент Охридски", Скопје
636.09(062)
DAYS of veterinary medicine 2012 : book of proceedings : 3rd International scientific meeting, 2-4 September, 2012 Republic of Macedonia / [editors Dine Mitrov, Lazo Pendovski]. - Skopje : Faculty of Veterinary medicine, 2012. - 308 стр. : граф. прикази ; 21 см
Текст на мак. и англ. јазик. - Библиографија кон трудовите
ISBN 978-9989-774-23-2
1. Mitrov, Dine [главен уредник] 2. Pendovski, Lazo [уредник]
а) Ветеринарна медицина - Собири
COBISS.MK-ID 91886090

UDC: 636.2.09:616.63-097

DETERMINATION OF ZERANOL RESIDUES LEVELS IN BOVINE URINE WITH ELISA METHOD

Hajrulai-Musliu Zehra[1], Uzunov Risto[1], Dimitrieska-Stojkovik Elizabeta[1], Stojanovska-Dimzoska Biljana[1], Sekulovski Pavle[1], Stojkovski Velimir[2], Todorovic Aleksandra[1]

[1]Food Institute, [2]Institute for Biomedicine and Reproduction, Faculty of Veterinary Medicine, University Ss. "Cyril and Methodius", Skopje, Macedonia

*Corresponding author: zhajrulai@fvm.ukim.edu.mk

ABSTRACT

Zeranol is a synthetic derivative of zearalenone which has been used as an anabolic substance in sheep and cattle to increase growth at food producing animals. The usage of zeranol is prohibited in most countries of the European Union and in Macedonia. In the illegal use of zeranol it is difficult to determine its presence because the amount of zeranol given and period is not known. A high affinity polyclonal antibody-based enzyme linked immunosorbent assay (ELISA) was developed for the quantification of zeranol in bovine urine. In the improved ELISA, the linear response range was between 0.025 and 3 ng/ml , and the detection limit was 0.22 ng/ml for the assay. The overall recoveries and the coefficients of variation (CVs) were in the range of 87.9%~92.3% and 2.4%~5.6%, respectively. Thirty-six bovine urine samples spiked with zeranol (ranging from 0.2 to 10 ng/ml) were detected by the ELISA, and good correlations was obtained (R^2=0.9929). We conclude that this improved ELISA is suitable tool for a mass zeranol screening method for zeranol in bovine urine. A total of 87 bovine urine samples were screened for the presence of zeranol as part of national monitoring residues where these documented cases presented zeranol concentrations were much lower than the MRPL set by the according the guidance letter from Community Reference Laboratories' (7 December 2007).

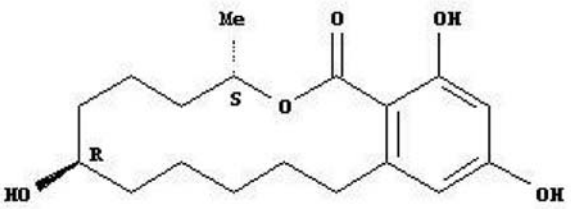
Key words: Rezorcylic acid, urine, ELISA, zeranol

INTRODUCTION

Zeranol is a non-steroidal oestrogenic growth promoter that increases the live weight gain in food animals. Fig 1. It is a semi-synthetic product derived from the naturally occurring mycotoxin zearalenone. Its administration has been banned within the European Union (EU) (Council Directive 96/22/EEC) and Member States are required to monitor food-producing animals for possible abuse (Council Directive 96/23-EC). Zearalenone is also known as the fusarium spp. toxin (F2-toxin) and is commonly found in animal feed. Zeranol and zearalenone are known to give identical metabolites, including zeranol itself, can also occur naturally in bovine urine after metabolism of Fusarium spp. toxin. The traditional method for the analysis of zeranol and other rezorcylic acid in the present is gas chromatography (GC) with liquid chromatography (Tobioka and Kawashima 1978, 1981) and mass spectrometry (Bagnati et al., 1990; Sawaya etal., 1998; Talat et al., 1999; Leslie et al., 2003). Large-scale surveillance programsrequire a rapid analysis of zeranol therefore an enzymelinked immunosorbent assay (ELISA) appeared suitable. Such assays have been developed in our laboratories since 2006.

Finally, the optimized ELISA was applied to determine zeranol in bovine urine samples. The use of zeranol in food producing animals is prohibited in most countries of the EU and in Macedonia. The aim of the present study was to determine the levels zeranol in cattle urine of various sex and age using validated ELISA methods, to get an insight into the residual levels that might indicate to the illegal use of zeranol on farm animals in this region.

Zeranol [CASRN 26538-44-3]



MATERIALS AND METHODS

A total of 87 bovine urine samples were screened for the presence of zeranol as part of national monitoring residue plan. The samples were collected within period of 12 months as they were delivered by the authorised veterinary inspectors. Samples were kept frozen at -20 ºC until analysis. A I' screen zeranol kit for ELISA was provided by Tecna (R&Diagnostics- Biotechnology, Italy). Each kit contained a microtiter plate with 96 wells coated with antibodies to rabbit IgG, zeranol standard solutions (0; 0,025; 0, 1; 0, 3; 1 and 3 ng/mL), enzyme-conjugate zeranol, anti-zeranol antibody, substrate/chromogen solution, stop reagent, conjugate and antibody dilution buffer, and washing buffer. The extraction and clean-up procedures were those described by the ELISA kit manufacturer (R&Diagnostics- Biotechnology, Italy). Urine samples (0, 5 ml) were diluted with 2.5 ml of sodium acetate buffer 50 mM pH 4.8, then was added 10 µl of β glucoronidase aril-sulfatase of Helix pomatia, the pH was controlled and in case was adjusted it at

4.8-5. The entire supernatant was allowed to reach room temperature (20-25°C) an overnight and then underwent clean-up with RIDA C18 column. Subsequently, 1 ml of the elute was pippeted into a glass tube and evaporated at 50/60ºC under a stream of nitrogen, and the residue was redisolved in 0.5 ml of kit dilution buffer. Fifty microliters of standards and control were pippeted into the standard/ sample wells in duplicate and 50 microliters of conjugate was added. The microtiter plate was covered with adhesive film, gently tapped from side to side, and incubated for 90 min at room temperature. The plate was inverted and the liquid was tapped out. The microtiter plate was washed 4 times with working wash solution diluted diluent over a 10-15 minute period. After the final wash, it was tapped onto a tissue paper. Immediately after washing, 100 µl of of developing solution was pippeted into each well. The microtiter plate was gently tapped and incubated for 15 minutes at room temperature in the dark. The color reaction was stopped by addition of 50µl of stop solution per well. A color change of blue to yellow was evident, and the optical density was measured at 450 nm within 10 minutes. Data were analyzed using a special software RIDAWIN ELISA (R-Biopharm, Darmstadt, Germany). The mean absorbance values obtained for the standards and the samples divided by the absorbance value of the first standard (zero standard) and multiplied by 100 was the % absorbance. The zero standard was thus made equal to 100 % and the absorbance values were quoted in percentages. The method recovery was evaluated by fortifying negative urine samples with zeranol standards (1, 2 and 3 µg/kg).

Method validation.

The limit of detection (LOD) was obtained by spiking with 0, 5 from MRPL which is 2 ppb according the guidance letter from Community Reference Laboratories' (7 December 2007). The method recovery was determined at three level by spiking urine samples with 0,5; 1 and 1,5 times the MRPL level. For determination of repeatability, the same steps were repeated on two occasions in the same analytical conditions. Detection capabilities (CCβ) was evaluated by analyzing 20 spiked samples at 0,5 MRPL level. A typical ELISA standard curve is presented in Figure 1. Final zeranol concentrations in urine were calculated by taking the average recoveries into account.

RESULTS AND DISCUSSION

Validation results of quantitative ELISA methods include determination of the recovery, repeatability and detection capability (CCβ) of the test methods.

The estimated LOD for urine samples for zeranol was 0,22 ng/ml. The CCβ for urine sample for zeranol was 1,21 ng/ml The results of method recovery (n=18) and repeatability (n=54) are presented in Table 1.

Table 1. *Recovery and repeatability of zeranol*

Validation parameter	No. of replicates	Spiked concentration µg/kg	Determinated concentration µg/kg	Mean recovery %	Coefficient of variation %
	6	1	0.923	92.3	2,4
	6	2	1.759	87.9	4,7
Recovery	6	3	2.689	89.6	5,6
	18	1	0.901	90.0	4,9
	18	2	1.782	89.1	6,8
Repeatability	18	3	2.801	93.3	8,6

Validation of the method used in zeranol determination resulted in the mean recovery of 87.9%-92,3% and repeatability of 89.1%-93,3% with coefficient of variation (CV) of 2,4%-5,6% and 4,9%-8,6% respectively.

As can be seen in Fig.1, the zeranol calibration curve was found to be virtually linear in the 0.025 to 3 ng/ml.

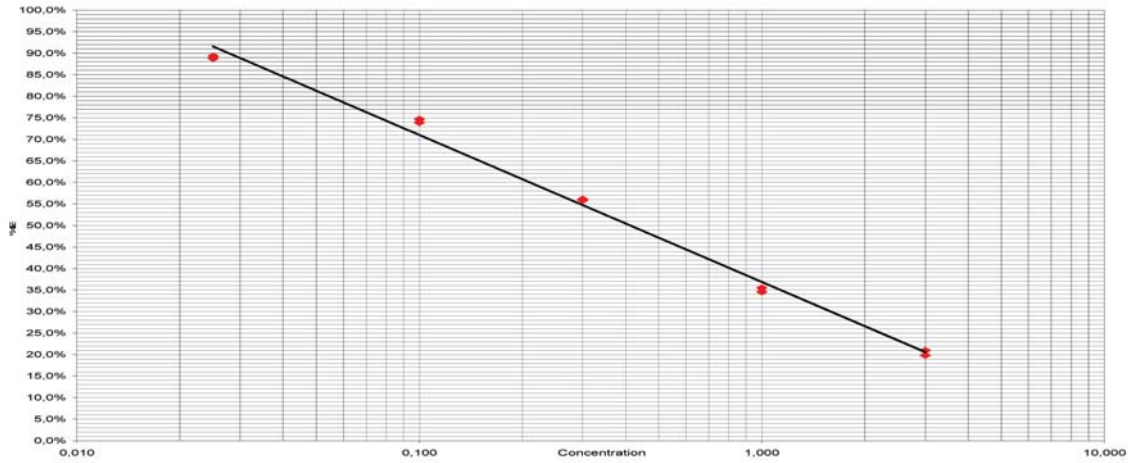


Figure 1. *Linearity of calibration curve for zeranol standards*

In Fig.2 the correlation between the absorbance ratio and zeranol concentration was evaluated over the range 0 – 3 ng/ml, R^2=0.9929.

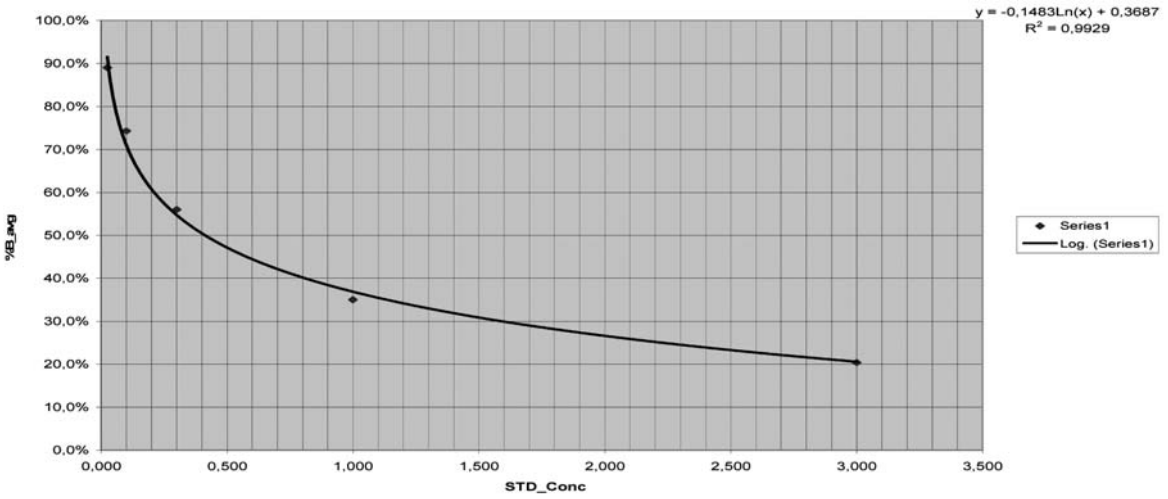


Figure 2. *Calibration curve for zeranol standards (0.025-3 ng/ml)*

Zeranol and its metabolites act as estrogen receptor agonists and exert typical estrogenic effects on animals (Lamming 1987, Le Guevel and Pakdel 2001, Leffers et al. 2001, Nagel et al. 1998, Nikaido et al. 2005). The presence of mycotoxins and serum levels of zearalanone were associated with early thelarche and mastopathy in Hungarian girls (Szuets et al. 1997). Furthermore, the presence of mycotoxins was strongly correlated with precocious puberty, and exposure to the mycoestrogenic zearalenone is thought to trigger central precocious puberty in young girls (Massart et al. 2008). Natural estrogen is a known cause of human breast and uterine cancer and increased exposure to zeranol may similarly increase risk from the existing burden of the natural compound. Most, but not all, of the short-term assays to assess mutagenicity of zeranol and some metabolites (zearalanone and taleranol) were negative (Metzler and Pfeiffer 2001). Because of the potential toxicity of these compounds, whether from natural or synthetic sources, it is important to prevent human exposure. Identifying zeranol in biospecimens is not sufficient to prove exposure to the synthetic hormone, as ingestion of *Fusarium* contaminated corn can produce similar results. However, laboratory methods exist to distinguish between metabolites resulting from exposure to zeranol and those resulting from exposure to the *Fusarium* mycotoxin. Elevated levels of zeranol or its metabolites could indicate that measures aimed at keeping the synthetic hormone out of the food supply are not adequate or that efforts to prevent *Fusarium* contamination of the food supply are not adequate. Zearalanone is stored in adipose tissue (Pillay et al. 2002). In one study (Nagel et al. 1998), an oral dose of zearalanone had a half-life of 22 hours in human blood. Both the presence of mycotoxins and serum levels of zearalanone in the range of 18.9-103µg/L were associated with early thelarche and mastopathy in Hungarian girls (Szuets et al. 1997). Since zeranol use has been banned in the European Union, the interest in detecting illegal zeranol use has resulted in the development of GC-MS (Blokland et al. 2006) and immunoassay (Tuomola et al. 2002) techniques that can be used to distinguish between exposure to zeranol and exposure to *Fusarium* toxins in biological specimens. LC/MS methods exist to detect zeranol in sub-ppb quantities in animal urine (Launay et al. 2004, Schmidt et al. 2008, Rúbies et al. 2007). In the illegal use of zeranol it is difficult to determine its presence because the amount of zeranol given and period is not known. In the improved ELISA, the linear response range was between 0.025 and 3 ng/ml , and the detection limit was 0.22 ng/ml for the assay. The overall recoveries and the coefficients of variation (CVs) were in the range of 87,9%~92,3% and 2.4%~5.6%, respectively. The levels of zeranol in bovine urine, which require due measures to be taken for suspect abuse are defined according the Council Directive 1996/22/EC. The borderline urine level of zeranol demanding due measures has been set at 1,21 ng/ml to obviate the possibility of a great number of false-positive results. In comparison with physiological values reported in the literature, the results obtained in the present study and data on study animals indicated that illegal use ofzeranol residues could not be suspected in none of the studied animals. As data on urine zeranol concentrations have not yet been precisely determined, are quite inadequate for different animal species and categories, and depend on numerous factors, additional studies are definitely necessary. On the other hand, because there is possibility to occur high rates of false positive zeranol samples in urine, confirmation of the ELISA test should always be carried out by chromatographic methods coupled to spectrometric methods.

REFERENCES

1. Council directive 96/22/EC of 29 April 1996 concerning the prohibition on the use in stock framing of certain substances having hormonal or thyrostatic action and of beta-agonists and repealing Directives 81/602/EEC, 88/146/EEC and 88/299/EEC
2. 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/EECand 86/469/EEC and Decisions 89/187EEC and 91/664/EEC
3. Ch.L. Xu, Ch.F. Peng, L. Liu, L. Wang, Z.Y. Jin and X.G. Chu (2006) Development of an enzyme-linked immunosorbent assay for the determination of hexoestrol. Journal of Animal and Feed Sciences, 15, 2006, 159–171
4. Bagnati R., Castelli M.G., Airoldi L., Oriundi M.P., Ubaldi A., Fanelli R., 1990. Analysis of diethylstilbestrol, dienes-

trol and hexestrol in biological samples by immunoaffinity extraction and gas chromatography-negative-ion chemical ionization mass spectrometry. J. Chromatogr. 527, 267-278
5. Sawaya W.N., Lone K.P., Hasain A., Dashti B., Al-Zenki S., 1998. Screening for estrogenic steroids in sheep and chicken by the application of enzyme- linked immunosorbent assay and a comparison with analysis by gas chromatography–mass spectrometry. J. Agr. Food Chem. 63, 563-569
6. Talat S., Esmael N., Nisar A., 1999. Assessment of the levels of anabolic compoumds in Kiwait meat industry: optimization of a multiresidue method and the results of a priliminery survey. J. Food Control 10, 169-174
7. Leslie C.D., MacNeil J.D., Reid J.A., Fesser A.C.E., 2003. Validation of screening method for residues of diethylstilbestrol, dienestrol, hexestrol, and zeranol in bovine urine using immunoaffinity chromatography and gas chromatography/mass spectrometry. J. AOAC Int. 86, 631-639
8. Lamming GE. 1987. Scientific report on anabolic agents in animal production. Veterinary Record. 121:389–392.
9. Le Guevel R., and Pakdel F. 2001. Assessment of oestrogenic potency of chemicals used as growth promoter by in-vitro methods. Hum Reprod. 16:1030–1036.
10. Leffers H., Naesby M., Vendelbo B., Skakkebae NE., Jorgensen A. 2001. Oestrogenic potencies of Zeranol, oestradiol, diethylstilboestrol, Bisphenol-A and genistein: implications for exposure assessment of potential endocrine disrupters. Hum Reprod. 16:1037–1045.
11. Nagel SC, vom Saal FS, Welshons WV. 1998. The effective free fraction of estradiol and xenoestrogens in human serum measured by whole cell uptake assays: physiology of delivery modifies estrogenic activity. Proc Soc Exp Biol Med. Mar;217(3):300-9.
12. Nikaido Y, Danbara N, Tsujita-Kyutoku M, Yuri T, Uehara N, Tsubura A. 2005. Effects of prepubertal exposure to xenoestrogen on development of estrogen target organs in female CD-1 mice. In Vivo. May-Jun;19(3):487-94.
13. Szuets P., Mesterhazy A., Falkay GY., Bartok T. 1997. Early telarche symptoms in children and their relations to zearalenone contamination in foodstuffs. Cereal Research Communications 25(3I):429-436.
14. Massart F, Meucci V, Saggese G, Soldani G. 2008. High growth rate of girls with precocious puberty exposed to estrogenic mycotoxins. J Pediatr. May;152(5):690-5.
15. Metzler M. and Pfeiffer E. 2001. Genotoxic potential of xenobiotic growth promoters and their metabolites. Acta Pathologica, Microbiologica et Immunologica Scandinavica 109:89-95.
16. Pillay D., Chuturgoon AA., Nevines E., Manickum T., Deppe W., Dutton MF. 2002. The quantitative analysis of zearalenone and its derivatives in plasma of patients with breast and cervical cancer. Clin Chem Lab Med. Sep;40(9):946-51.
17. Schmidt K., Stachel C., Gowik P. 2008. Development and in-house validation of an LC-MS/MS method for the determination of stilbenes and resorcylic acid lactones in bovine urine. Anal Bioanal Chem. Jun;391(4):1199-210.
18. Launay FM., Young PB., Sterk S., Blokland M., Kennedy D. 2004. Confirmatory assay for zeranol, taleranol and the Fusarium spp. toxins in bovine urine using liquid chromatography-tandem mass spectrometry. Food Addit Contam. 21:52–62.

ОПРЕДЕЛУВАЊЕ НА РЕЗИДУАЛНИ НИВОА НА ЗЕРАНОЛ ВО УРИНА СО ELISA МЕТОД

Хајрулаи-Муслиу Зехра[1]*, Узунов Ристо[1], Димитриеска-Стојковиќ Елизабета[1], Стојановска-Димзоска Билјана[1], Секуловски Павле[1], Стојковски Велимир[2], Тодоровиќ Александра[1]

[1]Институт за храна, [2]Институт за биомедицина и репродукција, Факултет за ветеринарна медицина, Универзитет "Св. Кирил и Методиј", Скопје, Македонија

*Автор за коресподенција: zhajrulai@fvm.ukim.edu.mk

АПСТРАКТ

Зеранолот е синтетички производ на зеараленонот кој се користи како анаболичка супстанца кај овците и говедата за зголемување на растот на животните кои се одгледуваат за производство на храна. Користењето на зеранолот е забрането во сите земји на Европската Унија и во Македонија. При нелегалната употреба на зеранол, утврдувањето на неговото присуство претставува потешкотија, бидејќи не се познати количината на аплицираниот зеранол и периодот на апликација. Беше разработен високо афинитетен ензимски имуносорбентен метод (ELISA) на основа на поликлонални антитела, за квантификација на зеранол во урина од говеда. Кај подобрената ELISA линеарниот одговор беше во опсегот помеѓу 0,025 и 3 ng/mL, со лимит на детекција на методот од 0,22 ng/mL. Вкупниот аналитички принос и коефициент на варијанца (CV) беше во опсегот од 97,9-92,3 % и 2,4-5,6 %, соодветно. 36 примероци од говедска урина спикувани со зеранол (од 0,2 до 10 ng/mL) беа детектирани со ELISA и постигната е добра корелација (R^2=0,9929). Може да се заклучи дека оваа подобрена ELISA претставува соодветна алатка како масовен метод за скрининг на зеранол во говедска урина. Вкупно 87 примероци од говедска урина, како дел од националниот мониторинг на резидуи, анализирани се за утврдување на присуство на зеранол. Во овие документирани случаи утврденото присуство на зеранол беше значително пониско отколку MRPL определен со циркуларното упатство на Референтните Лаборатории на ЕУ (7 декември 2007).

Клучни зборови: резорцинска киселина, урина, ELISA, зеранол.