Kafkas Univ Vet Fak Derg
22 (1): 1-5, 2016
DOI: 10.9775/kvfd.2015.13236

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Determination of Ochratoxin-A in Cattle Liver By HPLC-FD Method

Başak KÜÇÜKÇAKAN [1] Biljana STOJANOVSKA-DIMZOSKA [1] Zehra HAJRULAI-MUSLIU [1]
Elizabeta DIMITRIESKA-STOJKOVIC [1] Risto UZUNOV [1] Katerina DAVCHEVA [1]

[1] Food Institute, Faculty of Veterinary Medicine, Ss. Cyril and Methodius University, Lazar Pop Trajkov 5-7, 1000 Skopje, MACEDONIA

Article Code: KVFD-2015-13236 Received: 26.02.2015 Accepted: 09.09.2015 Published Online: 05.10.2015

Abstract

Ochratoxin-A (OTA) is a mycotoxin which is produced by several *Penicillium* and *Aspergillus* spp. OTA has carcinogenic, hepatogenic, nephrotoxic and immunosuppressive effects in human. The toxin accumulates in the animal tissues (kidney, liver and muscle) which could be potential harmful source for human health. Following results of previous investigations our aim was to validate HPLC-FD method for OTA determination in cattle liver which could be implemented in laboratory practise, complying with the European Union Commission Decision (2002/657/EC). Specificity, linearity of method, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision were evaluated. The linearity of the method was determined by preparation of eight standard solutions. The standard curve showed high correlation coefficient (r^2>0.9999). LOD and LOQ were determined as 0.088 ng/ml and 0.268 ng/ml, respectively. Accuracy and precision were established in three concentration levels: 1.0, 5.0 and 10.0 μg/kg. Recovery was in the range of 76.1 to 102.5% and repeatability (RSD_r%) was expressed through relative standard deviation which resulted in the range of 3.7 to 14.28%. Reproducibility results (RSD_R%) were found in the range of 0.16-11.8% for the first day and for the second day in the range of 2.15-17.88%. (Post-hock analysis of samples (n=15), only in one sample OTA was detected (0.2 μg/kg) as close to the LOQ value. As a result, we recommend this method for OTA detection and quantification in cattle liver, for concentrations which are lower than the maximum residue limits (MRL).

Keywords: *Validation, Ochratoxin-A, HPLC-FD, Cattle liver*

Sığır Karaciğerinde Okratoksin-A Varlığının HPLC-FD Metodu İle Belirlenmesi

Özet

Okratoksin- (OTA), *Penicillium* ve *Aspergillus* spp. tarafından üretilen bir mikotoksindir. OTA insanlar üzerinde karsinojenik, nefrotoksin ve immunosupresif etkilere sebep olmaktadır. Ayrıca toksin hayvan dokularında (böbrek, karaciğer, kas) birikerek, insan sağlığı için potansiyel tehlike kaynağı oluşturmaktadır. Bu çalışmadaki amaç, sığır karaciğerinde OTA varlığının HPLC-FD metodu ile araştırıldığı diğer çalışmaların sonuçlarını takiben, 2002/657/EC sayılı komisyon kararına uygun bir HPLC-FD metodu geliştirmektir. Bu amaç doğrultusunda; spesifite, doğrusallık, tespit limiti (LOD), tayin limiti, (LOQ), doğruluk ve kesinlik gibi parametreler değerlendirilmiştir. Doğrusallık; sekiz adet standart solüsyonun analizi ile belirlenmiştir. Korelasyon katsayısı (r^2)>0.9999, tespit limiti 0.088 ng/ml ve tayin limiti 0.268ng/ml değerinde bulunmuştur. Doğruluk ve kesinlik analizleri üç farklı konsantrasyon seviyesi (1.0, 5.0 and 10.0 μg/kg) denk alınarak uygulanmıştır. Geri kazanım, %76.1-102.5 arasında bulunmuştur. Tekrarlanabilirlik relatif standart sapma (RSD_r %) göre ifade edilerek; %3.7-14.28 arasında hesaplanmıştır. Tekrar üretilebilirlik sonuçları (RSD_R%); birinci gün %0.16- 11.8, ikinci gün %2.15-17.88 arasında bulunmuştur. Metot oluşturulduktan sonra, OTA varlığı 15 adet doku örneğinde araştırılmıştır. Sadece 1 örnekte tayin limitine yakın oranda (0.2 μg/kg) OTA tespit edilmiştir. Sonuç olarak, üzerinde çalıştığımız bu HPLC-FD metodunun sığır karaciğerinde maksimum kalıntı limitleri altındabulunan OTA miktarlarının belirlemesinde kullanılmasını önermekteyiz.

Anahtar sözcükler: *Validasyon, Okratoxin-A, HPLC-FD, Sığır karaciğeri*

INTRODUCTION

Ochratoxin - A (OTA) is a toxic metabolite which is produced by fungi *Aspergillus* and *Penicillium* spp. The molds grow on food products (such as; cereals, grains, coffee, beverages, cacao and spices) and produce the toxin under favorable conditions of temperature, moisture and humidity. For example, OTA originates from *Penicillium verrucosum* at 0.8a_w and below 30°C. Also, the toxin can be produced at lower temperatures (5°C) by some *Penicillium*

İletişim (Correspondence)
+389 23240760
zhajrulai@fvm.ukim.edu.mk

spp.[1-3]. OTA contamination is a significant public food safety concern due to accumulation of its residues in animal tissues. Animals, especially pigs, when consume OTA contaminated animal food, the toxin accumulates in kidney, liver, muscle and fat. The researches found out that in German markets, among 620 meat and meat products; 77% of blood sausages and 68% of liver-type sausages were OTA contaminated. Human are exposed to OTA when the toxin contaminated animal products (such as salami, dry-cured ham, sausages) are consumed [3,4]. It has been reported that OTA has carcinogenic, teratogenic, nephrotoxic, neurotic, immunosuppressive, genotoxic and mutagenic effects on experimental animals [5]. International Agency for Research on Cancer (IARC) classified OTA in group 2B (possibly cancerogenic for humans) [6].

Kidneys are the target organs of OTA and nephrotoxicity is the most pronounced toxicity. The toxic effects of OTA in kidneys are mostly affective in non-ruminants like, pigs, birds, rodents, dogs and young ruminants [7,8]. It was observed that in Bulgaria, in several farms, pigs were prone to have Mycotoxic Porcine Nephropathy Disease (MPN) due to consumption of OTA contaminated feed. Additionally, the research revealed that morphological changes and damages in kidney during porcine nephropathy were similar to human kidney disease; endemic nephropathy. Endemic nephropathy is a chronic tubulointerstitial disease with unknown aetiological agent, which is mostly seen in Europe and Balkan countries (Bulgaria, Romania, Serbia, Croatia, Bosnia, Herzegovina, Slovenia, and Macedonia). The disease is also called Balkan Endemic Nephropathy (BEN), due to its common incidence in these regions. It has been reported that in these countries at least 20.000 people have suffered from the disease [9].

The Commission of the European Communities has not set maximum residue limits (MRL) for OTA in animal products. Hence, several countries, including Macedonia, have enforced their own regulations for animal origin foodstuff. MRL for each country were given as following: Denmark (pig kidney) 10 µg/kg, Italy (pork meat and derived products) 1 µg/kg, Romania (pig kidney, liver and meat) 5 µg/kg, Slovenia (milk and meat) 5 µg/kg and Macedonia (bovine liver) 10 µg/kg [10,11]. Determination of public exposure to OTA was evaluated by both international and internal bodies in different countries. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a Provisional Tolerable Weekly Intake (PTWI) of 112 ng/kg body weight (16ng/kg body weight per day). The European Commission's Scientific Committee for Food (SCF) estimated tolerable daily intakes (TDI) of 1.2 to 14 ng/kg body weight [12]. In France, regulations have been in force for pork meat and derived products for OTA since 1978 and in Italy since 1999 [13,14].

OTA analytical methods are based on three steps of analyses; extraction, clean-up and detection of the toxin; respectively. Since now, in several scientific articles, different techniques and equipment have been used for OTA detection in animal tissues. Extraction of toxin from tissues usually has been done with different solvent mixtures such as; ethyl acetate-phosphoric acid, chloroform-phosphoric acid or dichloromethane-ethyl acetate-phosphoric acid. Solid phase extraction (SPE) with immunoaffinity columns has been employed for the clean-up step. Chemical solutions such as methanol and dichloromethane have been used for OTA elution from the SPE columns. Immunochemical and chromatographic methods have been developed for OTA detection and quantification. ELISA is usually applied as a screening method especially in the laboratories which are dealing with big number of samples. HPLC-FD is enough sensitive, precise and mostly used method for the detection and the quantification of the toxin and LC-MS/MS is usually used for confirmation of positive samples or multi-toxin analysis [13-16].

In this study we worked on validation of HPLC-FD method for OTA determination in cattle liver tissues which could be implemented in laboratory practise. The validation parameters were conducted complying with the European Commission Decision (2002/657/EC) [17].

MATERIAL and METHODS

Samples

The validation procedure was done with OTA free cattle liver samples (previously determined with HPLC-FD). Liver samples were analyzed before the validation procedure to control OTA occurrence according to the methodology from the study [18] which were brought by food inspectors from all over Macedonia. After the method validation, cattle liver samples (n=15) were analyzed according to the validated method. Before the analysis, all the samples were stored in specimen containers at -18°C.

HPLC-FD Equipment Condition

HPLC system (λ_{ex} = 333 nm and λ_{em} = 460 nm) and analytical column (RP C18150 mm 4.6 I.D.5 mm) was used. The mobile phase consisted isocratic mixture of water: acetonitrile: glacial acetic acid (99:99:2 v/v/v) at a flow rate 1.0 ml/min. The sample injection volume was 100 µl and run time was 10 min.

Reagents

OTA standard solution was obtained with the concentration level about 50 µg/ml in benzene: acetic acid (99:1). Aliquot of OTA standard were dissolved in benzene: acetic acid 99:1 (v/v) in amber volumetric flask of 10 ml, in order to obtain OTA stock solution at 5.0 µg/ml. The stock solution was kept in refrigerator at-18°C and used for preparation of 1.000 ng/ml OTA standard solution. Intermediate solution with concentration of 100 ng/ml was made from OTA standard solution at level of 1.000 ng/

ml. Calibration (working) OTA solutions with concentration levels 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 20.0 and 50.0 ng/ml were obtained from the intermediate solution at 100 ng/ml.

Sample Extraction and Clean-up

A 25 g of tissue sample was measured in glass beakers for extraction. 100 ml extraction solvent (dichloromethane: ethylacetate (1:3)) and 10 ml 0.5 M H_3PO_4 in 2M NaCl solution were added to the 25 g of tissue sample. The samples were blended for a few minutes using homogenizer and mixed for 30 min on a horizontal shaker. The mixture was filtered by using filter paper and an aliquot of the filtrate (10 ml) was evaporated under nitrogen evaporator until the liquid dried. The residue was dissolved in 2 ml methanol and in 30 ml PBS buffer and the new solution was filtered by using microfiber filter. 20 ml of the filtrate passed through the immunoaffinity column and the column was washed with 20 ml of distillate water. OTA was eluted with 4 ml of methanol in a glass tube and was evaporated under stream of nitrogen. The dry residue was redissolved in 1 ml of mobile phase in a glass vial. The samples were ready for HPLC-FD analyses [18].

Method Validation Parameters

Method validation was performed according to the European Union Commission Decision (2002/657/EC) [17]. Specificity, linearity of method, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision were evaluated as follows: Specificity measurement was done with one blank and one fortified tissue at concentration level of 5.0 µg/kg. Linearity of method was determined by preparation of eight standard solutions with concentration levels at 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 20.0 and 50.0 ng/ml. Also, regression analyses are done. The standard solutions were replicated six times in order to obtain calibration curve. Standard deviation of the response and slope was calculated in order to estimate LOD and LOQ according to the formula [19]. Standard deviation (SD) was obtained by analysing 20 tissue samples which were fortified at the lowest concentration of the calibration curve (0.1 ng/ml).

Accuracy was determined by analyzing fortified samples at three levels (1.0, 5.0 and 10.0 µg/kg) with 6 times replication for each concentration. Accuracy was expressed through recovery (%) levels and precision of the method was performed through repeatability (RSD_r%) and reproducibility (RSD_R%) measurements. The same three concentration levels with 6 replicates were also used for reproducibility. The measurements were done using same sample, same method, personal and equipment in two different days as it written in the EU Directive (2002/657/EC) [17]. F-test (two-sample for variances) was done for the three level of concentration (1.0, 5.0 and 10.0 µg/kg). Also, t-test (two-sample assuming unequal variances) was applied only for concentration of 5.0 µg/kg.

Determination of uncertainty was aimed to estimate the errors associated with the various stages of the analysis. Variance of 5.0 µg/kg concentration in two days was calculated in order to obtain uncertainty for reproducibility. Precision of method was evaluated according to measurements from eight standards (0.1, 0.5, 2.5, 5.0, 1.0, 10.0, 20.0, 50.0 ng/ml). Standard error (sy/x) of the calibration curve was calculated according to regression analysis. Later, combined uncertainty of the calibration curve was calculated.

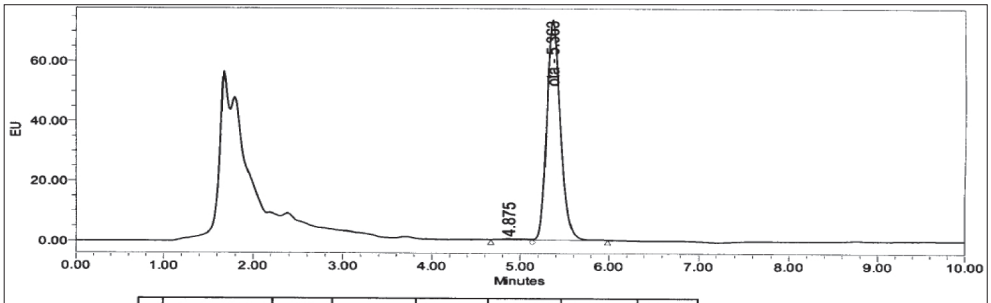
The uncertainty results of OTA Standard Solution 50 µg/ml, calibration curve, reproducibility; pipette (1000 µl) and balance were adjusted as a percentage according to the NIST Uncertainty Guideline [20]. In the respect of the guideline, expanded uncertainty was calculated with coverage factor of 2, at 95% confidence level. Maximum standard uncertainty was determined according to the 'Fitness-for-purpose' approach in the respect of European Commission Decision (401/2006) [21].

RESULTS

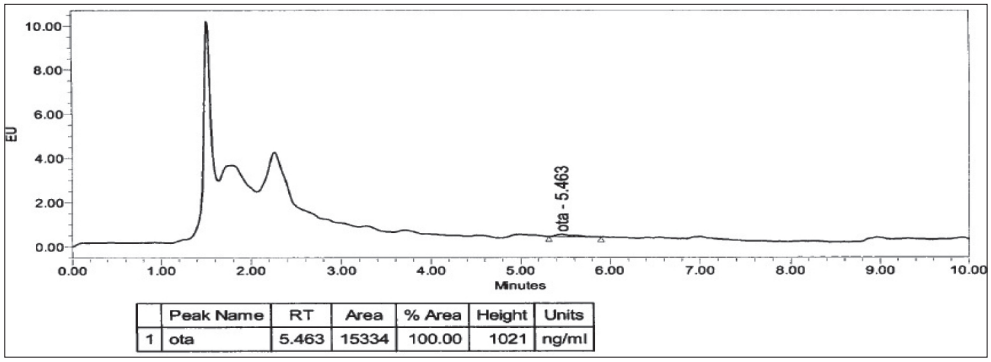
The method was found linear with coefficient correlation (r^2)>0.999 with the formula (y=1E+06x-152916). According to the regression analyses, F-value was significantly smaller, which indicated strong relationship between y and x values (P<0.05). The retention time for OTA was measured between 5-6 min in *Fig. 1*. Chromatography of blank sample was clean without any interferences at the retention time *(Fig. 2)*. LOD and LOQ values were found

Fig 1. HPLC-FD chromatogram of Ochratoxin -A (OTA) in cattle liver sample fortified at 5.0 µg/kg

Şekil 1. 5.0 µg/kg konsantrasyon seviyesinde Okratoxin-A (OTA) içeren sığır karaciğeri HPLC-FD kromotogramı



	Peak Name	RT	Area	% Area	Height	Amount	Units
1		4.875	45147	0.57	2459		
2	ota	5.363	7851054	99.43	735924	7.150	ng/ml



	Peak Name	RT	Area	% Area	Height	Units
1	ota	5.463	15334	100.00	1021	ng/ml

Fig 2. HPLC-FD chromatogram of Ochratoxin -A (OTA) in blank cattle liver sample

Şekil 2. Okratoxin -A (OTA) içermeyen sığır karaciğerinin HPLC-FD kromatogramı

0.088 ng/ml and 0.268 ng/ml, respectively. Accuracy and precision results for 1.0, 5.0 and 10.0 µg/kg concentration levels were presented in *Table 1*.

Reproducibility results for different two days were demonstrated in *Table 2*. F-test calculations were resulted as follows: For the concentration of 1.0 µg/kg; F=1.97 (F critical = 5.05 and P=0.23) and for the concentration of 10.0 µg/kg; F=2.63 (F critical = 5.05 and P=0.15). For the concentration of 5.0 µg/kg, F- test didn't provide equality but t-test did (t-stat = 0.063 and t-critical = 2.57).

Table1. *Determination of mean, standard deviation (SD), repeatability (RSD_r%) and recovery (%) values for 1.0, 5.0 and 10.0 µg/kg concentration levels*

Tablo 1. *1.0, 5.0 ve 10.0 µg/kg konsantrasyon seviyelerindeki ortalama, standart sapma (SD), tekrarlanabilirlik (RSD_r) ve geri kazanım (%) değerlerinin belirlemesi*

Parameter	Concentration		
	1.0 µg/kg	5.0 µg/kg	10.0 µg/kg
Mean	0.76	5.12	9.36
SD	0.04	0.19	1.34
RSDr%	4.85	3.70	14.28
Recovery (%)	76.1	102.5	93.6

Table 2. *Reproducibility results (RSD_R% values)*

Tablo 2. *Tekrar üretilebilirlik sonuçları (RSD_R% değerleri)*

Period	Concentration		
	1.0 µg/kg	5.0 µg/kg	10.0 µg/kg
1 Day	2.76	0.16	11.18
2 Day	2.15	5.48	17.88

Precision of the system was resulted as follows: Mean RSD% value was 0.62%. The uncertainty of predicted sample was estimated 0.11 µg/kg, (At the 95% confidence interval the predicted sample was calculated 11.3±0.26 µg/kg.). The uncertainty of reproducibility for fortified sample (concentration level 5 µg/kg) was 0.28 µg/kg (5±0.28 µg/kg) and for the standard solution was 0.407 µg/ml (50.46±0.407 µg/ml). Expanded uncertainty was resulted as 5.63±11.26% with coverage factor of 2 at the 95% confidence interval. Maximum standard uncertainty was 2.0 µg/kg. Only one of the analyzed samples (n=15) had OTA amount of 0.2 µg/kg which was close to the level of LOQ.

DISCUSSION

Linearity of method with the high coefficient determination showed reliable results. As can be seen from the *Fig. 2*, specificity was satisfactory. There were no potential interfering compounds around the OTA retention time. In several studies, OTA analyses in pig tissues with HPLC-FD method were successful enough to determine very low levels of LOD and LOQ [22-25]. Some of these studies were compared with our study and demonstrated in *Table 3*. Our study showed that the HPLC-FD method which we worked on was efficient and appropriate for detection of the toxin level at the low concentrations in cattle liver.

The uncertainty measurements for each variable were complying with the 'Fitness-for-purpose' approach. There

Table 3. *Demonstration of recovery (%), limit of detection (LOD) and limit of quantification (LOQ) levels from several studies (µg/kg)*

Tablo 3. *Diğer çalışmalarda elde edilen geri kazanım (%), tespit limiti (LOD) ve tayin limiti (LOQ) değerleri (µg/kg)*

Tissue	Recovery (%)	LOD	LOQ	References
Kidney	71±19	0.02	0.06	[18]
Meat	53±10	0.03	0.09	[18]
Kidney	86	0.05	0.16	[13]
All tissues	85±15	0.14	0.52	[22]
Kidney and liver	71	0.14	0.25	[25]
Liver	76.1±12, 102.5±12	0.088	0.26	Our Study

were not any higher uncertainty values than the maximum standard uncertainty (2 µg/kg). The results were equally suitable to be evaluated according to the performance criteria for OTA [21]. Our results for accuracy, repeatability, reproducibility-*(Table 1* and *Table 2)* were consistent with the values (for the concentrations between 1-10 µg/kg: Recovery %70-110, RSD_R 30%, RSD_r 20%) which were written in European Union Commission Decision (401/2006) [21].

Higher values of OTA in pig tissues were found in other studies. Especially, kidney was the most contaminated tissue with the levels of 15.0, 27.5, 52.5 and 23.8 µg/kg [18,22,23,25,26]. Beside kidney, in muscle 2.9 µg/kg, in spleen 0.5 µg/kg, in urinary bladder 11.5 µg/kg and in liver 5.3, 14.5 µg/kg were reported in different studies from several countries (Denmark, Italy, Serbia) [18,22,23,25].

As a result of this study we achieved to validate method for OTA detection and quantification in cattle liver according to European Union Commission Decision (2002/657/EC) [17]. The method was found applicable to analyze significantly lower concentrations than the maximum residue limits. Sample analyses from our study, did not show any significant contamination in liver samples. However, we recommend administration of regular OTA monitoring program in cattle liver samples in Republic of Macedonia.

REFERENCES

1. European Food Safety Authority (EFSA): Opinion of the scientific panel on contaminants in the food chain on a request from the commission related ochratoxin-A in food. *EFSA J,* 365, 1-56, 2006.

2. El-Ghreeb W, Darwish W, Tharvat E, El-Deskoy K, Hussein A: Aflatoxin and Ochratoxin-A residues in some meat additives. *Life Sci*, 10 (4): 3411-3414, 2013.

3. European Food Safety Authority (EFSA): Opinion of the scientific panel on contaminants in food chain on a request from the commission related to ochratoxin-A (OTA) as undesirable substance in animal feed. *EFSA J,* 101, 1-36, 2004.

4. Asta C, Galaverna G, Bertuzzi T, Moseriti A, Pietri A, Dossena A, Marchelli R: Occurrence of ochratoxin- A in raw ham muscle, salami and dry-cured ham from pigs fed with contaminated diet. *Food Chem,* 120, 978-983, 2010. DOI: 10.1016/j.foodchem.2009.11.036

5. The Joint FAO/WHO Expert Committee on food Additives (JECFA): Ochratoxin A.JECFA Food Additive Series 47, Geneva, 2001. http://www.inchem.org/documents/jecfa/jecmono/v47je04.htm, *Accessed:* 09.01.2015.

6. International Agency Research on Cancer (IARC): Agents classified by the IARC monographs volumes 1-111. 2014. http://monographs.iarc.fr/ENG/Classification/ClassificationsAlphaOrder.pdf, *Accessed:* 09.01.2015.

7. Mobasar M, Hummal J, Blank R, Sudekum K: Ochratoxin-A in ruminants - Review on its degradation by gut microbes and effects on animals. *Toxins*, 2, 809-839, 2010. DOI: 10.3390/toxins204809

8. Reddy L, Bhoola K: Ochratoxins-food contaminants impact on human health. *Toxins*, 2, 771-779, 2010. DOI: 10.3390/toxins2040771

9. Stoev D: Complex etiology, prophylaxis and hygiene control in mycotoxic nephropathies in farm animals and humans. *Int J Mol Sci*, 9, 578-605, 2008.

10. Daurte C, Lino M, Angelina P: Food safety implications of ochratoxin-A an animal-derived food products. *Vet J,* 192, 286-292, 2012. DOI: 10.1016/j.tvjl.2011.11.002

11. Scientific Cooperation (SCOOP) Task Reports: Task 3.2.7. Assessment of dietary intake of ochratoxin-A by the population of EU member states. 27, Italy, 2002. http://ec.europa.eu/food/fs/scoop/3.2.7_en.pdf, *Accessed:* 09.01.2015.

12. Codex Alimentarius Commission (CAC): Position paper on ochratoxin-A. Codex committee on food additives and contaminants 31th session, 1-9, The Netherlands, 1999.

13. Dragacci S, Grosso FR, Bire R, Fremy M, Coulon S: A French monitoring programme for determining ochratoxin-A occurrence in pig kidneys. *Nat Toxins,* 7, 167-173, 1999. DOI: 10.1002/(SICI)1522-7189(199907/08)7:4<167::AID-NT55>3.0.CO;2-Q

14. Matrella R, Monaci L, Milillo M, Tantillo G, Palmisano F: Ochratoxin-A determination in paired kidneys and muscle samples from swines slaughtered in southern Italy. *Food Control,* 17, 114-117, 2006. DOI: 10.1016/j.foodcont.2004.08.008

15. Guillamont ME, Lino CM, Baeta ML, Pena ASM, Silveira IN, Vinuesa J: A comparative study of extraction apparatus in HPLC analysis of ochratoxin-A in muscle. *Anal Bioanal Chem,* 383, 570-575, 2005. DOI: 10.1007/s00216-005-0051-4

16. Monaci L, Tantillo G, Palmisano F: Determination of ochratoxin-A in pig tissues by liquid-liquid extraction and clean-up and high-performance liquid chromatography. *Anal Bioanal Chem*, 378, 1777-1782, 2004. DOI: 10.1007/s00216-004-2497-1

17. Commission of the European Communities: Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC). *Off J Eur Union*, L 2218-2236, 2002.

18. Jorgensen K, Petersen A: Content of ochratoxin-A in paired kidney and meat samples from healthy Danish slaughter pigs, *Food Addit Contam*, 19, 562-567, 2002. DOI: 10.1080/02652030110113807

19. Singh R: HPLC method development and validation- on overview. *J Pharm Educ Res,* 4 (1): 26-33, 2013.

20. Taylor B, Kuyatt C: Guidelines for evaluating and expressing the uncertainty of NIST Measurement Results. **In,** NIST Editorial Review Boards. NIST Technical Note 1297, 1-20, US, 1994.

21. Commission of the European Communities: Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Off J Eur Union*, L 70, 12-34, 2006.

22. Ceci E, Bozzo G, Bonerba E, Pinto A, Tantillo MG: Ochratoxin-A detection by HPLC in target tissues of swine and cytological and histological analysis. *Food Chem,* 105, 364-368, 2007.

23. Milićević D, StefanovićS, Janković S, Radićević T: Risk analysis and exposure assessment of ochratoxin-A in Serbia. *Vet World,* 5, 412-416, 2012. DOI: 10.5455/vetworld.2012.412-416

24. Milićević D, Jurić V, Daković A, Jovanović M, Stefanović SM, Petrovic Z: Survey of slaughtered pigs for occurrence of ochratoxin-A and porcine nephropathy in Serbia. *Int J Mol Sci*, 9, 2169-2183,2008. DOI: 10.3390/ijms9112169

25. Milićević D, Jurić V, Stefanović SM, Vesković- Moracanin SV, Janković S: Analysis of ochratoxin-A in pig tissues using high pressure liquid chromatography (HPLC) and liquid chromatography tandem mass spectrometry as confirmative methods. *Proc Nat Sci Matica Srpska Novi Sad*, 117, 51-61, 2009. DOI: 10.2298/ZMSPN0917051M

26. Georgescu M, Constantin S, Dobrea M, Milca I, Georgescu D: Comparative assessment of ELISA and HPLC for ochratoxin-A detection in pork kidney samples. *Bulletin UASVM Anim Sci Biotechnol*, 70 (2): 223-232, 2013.