

*Original Scientific Article***DETERMINATION OF AFLATOXINS AND OCHRATOXIN A IN ANIMAL LIVER USING HPLC-FD METHOD WITH IMMUNOAFFINITY COLUMN CLEAN-UP**

Biljana Stojanovska-Dimzoska, Elizabeta Dimitrieska-Stojkovic, Zehra Hajrulai-Musliu, Risto Uzunov, Aleksandra Angeleska, Dean Jankuloski, Katerina Blagoevska

*Food Institute, Faculty of Veterinary Medicine Skopje,  
Lazar Pop Trajkov 5-7, 1000 Skopje, R. North Macedonia*

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**ABSTRACT**

Analytical methods based on immunoaffinity column clean-up and quantitative determination with liquid chromatography-fluorescence detection were used to determine aflatoxins and ochratoxin A in liver samples. The validation of the procedures was performed. The linearity of the methods was checked, and a good coefficient of correlation was found for all aflatoxins and OTA as well. The LOD and LOQ were acceptable: 0.003 µg/kg and 0.009 µg/kg for AFB<sub>1</sub>; 0.001 µg/kg and 0.005 µg/kg for AFB<sub>2</sub>; 0.006 µg/kg and 0.020 µg/kg for AFG<sub>1</sub>; 0.007 µg/kg and 0.022 µg/kg for AFG<sub>2</sub>; 0.08 µg/kg and 0.27 µg/kg for OTA. The results for the repeatability estimated by the relative standard deviation (RSD<sub>r</sub>) were satisfactory and the obtained values were in the acceptable range (1.97–14.41% for all aflatoxins and 3.76–8.31% for OTA) at three proposed concentration levels. RSD<sub>r</sub> values showed acceptable correlation between two analysts for all four aflatoxins and OTA. The RSD<sub>r</sub> values were as followed: 2.37% and 5.60% for AFB<sub>1</sub>; 6.71% and 8.78% for AFB<sub>2</sub>; 4.40% and 7.00% for AFG<sub>1</sub> and 10.30% and 13.91% for AFG<sub>2</sub> (for the first and second analyst, respectively). The RSD<sub>r</sub> values for OTA were 4.91% and 3.15% (1 µg/kg); 3.76% and 4.12% (5 µg/kg) and 8.31% and 8.21% (10 µg/kg). The mean recovery for total aflatoxins and OTA were 78.10% and 93.34%, respectively. All validation parameters were in accordance to European legislation. They indicate that the proposed analytical procedures are suitable and they could be methods of choice for the determination of aflatoxins and OTA in liver samples.

**Key words:** aflatoxins, ochratoxin A, HPLC-FD, liver samples, validation

**INTRODUCTION**

Mycotoxins are secondary metabolites produced by various genera of molds (*Aspergillus*, *Penicillium*, *Fusarium*). Cereals contaminated with mycotoxins, when are ingested by animals, can cause a toxic response (mycotoxicosis) resulting with carcinogenic, estrogenic, teratogenic or immunosuppressive effects (1). Consumption of a mycotoxin-contaminated diet may have direct consequences on animal health and economic

impact as well. This includes reduced weight gain and reproductive capacities, feed refusal and poor feed conversion, less meat and egg production, increased disease incidence (due to immune-suppression) and lower productivity (1-2).

Due to modern laboratory methods and a growing interest in this field of research, more than 300 mycotoxins have been differentiated so far. Among them, aflatoxins, especially aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) exhibits the highest toxicity and carcinogenicity and it can be found as a residue in animal liver after ingestion of contaminated feed (3). International Agency for Research on Cancer (IARC) made classification of naturally occurring AFB<sub>1</sub> as carcinogenic to humans (Group 1). Ochratoxin A (OTA) contamination is also a significant public food safety concern due to accumulation of its residues in animal tissues, predominant in kidney, but also in liver, muscle and fat. OTA is involved in the etiology of Balkan Endemic Nephropathy

*Corresponding author:* Dr. Biljana Stojanovska-Dimzoska, PhD

*E-mail address:* bsdimzoska@fvm.ukim.edu.mk

*Present address:* Food Institute, Faculty of Veterinary Medicine Skopje, Lazar Pop Trajkov 5-7, 1000 Skopje, R. North Macedonia

*Phone:* ++389 2 3240745

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(BEN), a human disease characterized by progressive renal fibrosis and by tumors of the urinary tract. IARC made classification of OTA as possibly carcinogenic to humans (Group 2B). Mycotoxin content in food and feed is regulated by legislation worldwide (Commission Regulation 466/2001, 2174/2003, 1881/2006 for food and Commission Directive 2002/32 and 2003/100 for feed). The Commission of European Communities has not set maximum residue limits (MRL) for mycotoxins in animal tissues, but it recommends that OTA levels should be reduced to below 5 ng/kg of body weight per day. Several countries have enforced their own regulation for animal origin foodstuff. They vary in the range of 1-10 µg/kg for pork and bovine meat (4).

Different analytical methods are used for aflatoxins and OTA analysis which are mainly based on liquid chromatography with fluorescence detection and previous clean-up step. Most of them are greatly improved with the commercial availability of immunoaffinity columns (IAC), which are simple and rapid to use, also provide good recovery values. Several articles report the use of these methods for aflatoxins (5, 6, 7) and OTA (8, 9, 10) in food and feed. To the best of our knowledge, very few reports give methods for determination of aflatoxins (11, 12) and OTA (13, 14, 15) in food of animal origin. Having in mind the significant health risks in human and animals associated with the presence of aflatoxins and OTA in food and feed, it is important to establish an analytical method for the determination of aflatoxins and OTA in food of animal origin.

The purpose of this work was to provide and validate a precise and reliable analytical methods for determination of aflatoxins and OTA in animal liver using simple immunoaffinity column clean-up and liquid chromatography with fluorescence detection. Postcolumn bromination (Kobra®cell) was used in order to determine aflatoxins. The validation procedure was performed according to European Decision 2002/657/EC (16) and Regulation 401/2006/EC amending mycotoxins (17). Also, the aim of this study was to carry out an overview for presence of aflatoxins and OTA in animal liver samples taken from slaughterhouses in Republic of North Macedonia over the period of six years (2015-2020). To the best of our knowledge, such investigation has not been done so far in our country. This research would provide a screening of the natural occurrence of aflatoxins and OTA in animal tissues, having in mind its potential health hazard for human consumption.

## MATERIAL AND METHODS

### *Apparatus*

HPLC analysis for aflatoxins were performed with a Perkin Elmer (PE) chromatographic system equipped with binary pump (PE LC-250), manual injector (PE Rheodyne 7125) and fluorescence detector (PE LC-240). Aflatoxins were separated on RP C18 column (250 mm x 4.6 mm, 5 µm). The mobile phase consisted mixture of water:acetonitrile:methanol (600:350:50, V/V/V) with addition of 119 mg KBr and 350 ml 4N HNO<sub>3</sub>. The flow rate was 1 ml/min and the injection volume was 100 µl. The detection was carried out at  $\lambda_{\text{ex}} = 360$  nm and  $\lambda_{\text{em}} = 440$  nm.

HPLC system (Waters Alliance) was used for determination of OTA (Waters separation module e2695 and Waters fluorescence detector 2475). An analytical column RP C18 (150 mm x 4.6 mm, 5 µm) was used with mobile phase consisting of acetonitrile:water:acetic acid mixture (99:99:2, V/V/V). Flow rate was 1 ml/min. The detection was carried out at  $\lambda_{\text{ex}} = 333$  nm and  $\lambda_{\text{em}} = 460$  nm.

### *Reagents and standard solutions*

HPLC reagents (methanol, acetonitrile, water) and chemicals (benzene, KBr, NaCl, dichloromethane, ethyl acetate, citric acid, diatomaceous earth, anhydrous Na<sub>2</sub>SO<sub>4</sub>, 65% HNO<sub>3</sub>, 85% phosphoric acid, glacial acetic acid, 2M NaCl, 0.5 M H<sub>3</sub>PO<sub>4</sub>, phosphorus buffer 7.0 or PBS buffer) were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (USA). For clean-up step immunoaffinity columns Aflaprep and Ochraprep (R-Biopharm Rhône, Glasgow, Scotland) were used. As a standard, aflatoxins mix (AFB<sub>1</sub> 982 ng/ml, AFB<sub>2</sub> 284 ng/ml, AFG<sub>1</sub> 1034 ng/ml, AFG<sub>2</sub> 333 ng/ml) (Supelco, Sigma-Aldrich, USA) was used and OTA standard with concentration of 50 µg/ml (Supelco, Sigma-Aldrich, USA) was used too.

Aflatoxins mix stock solution (AFB<sub>1</sub> 100 ng/ml, AFB<sub>2</sub> 28.4 ng/ml, AFG<sub>1</sub> 103.4 ng/ml, AFG<sub>2</sub> 33.3 ng/ml) was prepared from the aflatoxins mix standard, dissolving aliquot in a volumetric amber flask of 10 ml. Seven working standard solution (for AFB<sub>1</sub> in a range 0.25-15.00 ng/ml; for AFB<sub>2</sub> in a range 0.071-4.260 ng/ml; for AFG<sub>1</sub> in a range 0.258-15.510 ng/ml and for AFG<sub>2</sub> in a range 0.083-4.990 ng/ml;) were prepared from the stock solution in volumetric amber flasks of 5 ml. All working standards were kept in a refrigerator at 2-8 °C.

Aliquot of OTA standard (50 µg/ml) was used to prepare an OTA stock solution with concentration

of 5.0 µg/ml using salinized glass vial in order to keep the mycotoxin in the solution. It was used for preparation of intermediate solutions with concentration of 1000 ng/ml and 100 ng/ml. Seven working solution in range 0.10–20 ng/ml were obtained from the intermediate solution at 100 ng/ml. All working standards were dissolved in filtrated mobile phase and were kept in a refrigerator at 2-8 °C.

### *Samples*

During validation procedure, samples of animal liver were used, purchased in the local market, intended for human consumption. For validation purpose, samples were spiked with AFB<sub>1</sub> working standard solution prior to the extraction, at three concentration levels (0.10, 0.25 and 0.50 µg/kg). For OTA spiking, also three concentration levels were analyzed (1.0, 5.0 and 10.0 µg/kg). Those portions of standards were applied to a measured liver sample and they were kept for approximately 30 min. to addition of the extraction solvent.

For screening purpose, animal liver samples were taken from slaughterhouses or brought by food inspectors from all over the country. They were stored in specimen containers at -18 °C until analysis.

### *Analytical procedure*

The extraction and purification of aflatoxins from liver samples was done according to our reference (12) and modified AOAC procedure (18). HPLC-FD detection was performed according to ISO 16050:2003 standard (19). Homogenized liver sample (50.0 g) was mixed well with 5 ml of 20% citric acid and 10 g diatomaceous earth. The mixture was extracted with 100 ml dichloromethane on shaker for 30 min. Then, the mixture was filtered and dried by addition of 5 g anhydrous Na<sub>2</sub>SO<sub>4</sub> and filtered again. Twenty (20) ml of the filtrate was evaporated until dryness at 60 °C. Dry residue was dissolved in 5 ml of methanol and the solution was mixed with 25 ml of phosphorus buffer (pH 7.0). The solution was mixed well and filtered again through microfiber filter. The entire sample was applied onto the immunoaffinity column. Ten (10) ml of water was used for the washing step. Aflatoxins were eluted with 1 ml of methanol. The elution step was repeated one more time with 1 ml of water. Then, 100 µl of methanol-water solution was used for further analysis.

The process for extraction and purification of OTA in animal tissues was done according to modified method (14). Volume of 10 ml extraction

solvent (dichloromethane:ethyl acetate (1:3)) and 10 ml 0.5 M H<sub>3</sub>PO<sub>4</sub> in 2M NaCl was added to 25 g tissue sample and blended or homogenized in a baker for a few minutes. Then the samples were put for 30 min on a horizontal shaker. The mixture was filtered using filter paper. An aliquot of the filtrate (10 ml) was evaporated to dryness. Dry residue was dissolved in 2 ml methanol and 30 ml PBS buffer. Then the solution was filtered again using microfiber filter. Twenty (20) ml of the filtrate passed through the immunoaffinity column. Twenty (20) ml of water was use for washing step. OTA was eluted with 4 ml of methanol in a vial. The solution was evaporated under of stream of nitrogen until dryness. The dry residue was re-dissolved in 1 ml of mobile phase for further chromatographic analysis.

### *Validation procedure*

The validation procedure was achieved in agreement to general Decision 2002/657/EC (16) and Regulation 401/2006/EC (17) which is related to mycotoxins. Seven working standard solutions were used for the linearity test in the following range: for AFB<sub>1</sub> (0.25-15.00 ng/ml), for AFB<sub>2</sub> (0.071-4.260 ng/ml), for AFG<sub>1</sub> (0.258-15.510 ng/ml), for AFG<sub>2</sub> (0.083-4.990 ng/ml) and OTA (0.1–20.0 ng/ml).

The limit of detection (LOD) was calculated as 3.3xSD/slope, where the slope was determined from the calibration curve. Standard deviation (SD) was based on measurement of analytical background response of 10 blank samples. The limit of quantification (LOQ) was calculated as 10xSD/slope in the same way as LOD calculation.

Because the certified reference material was not available, recovery was determined as a part of trueness of the method. Method for standard addition at the following fortified concentration levels was applied: for AFB<sub>1</sub> (0.10, 0.25 and 0.50 µg/kg), for AFB<sub>2</sub> (0.028, 0.071 and 0.142 µg/kg), for AFG<sub>1</sub> (0.103, 0.258 and 0.517 µg/kg), for AFG<sub>2</sub> (0.033, 0.083 and 0.166 µg/kg) and for OTA (1.0, 5.0 and 10.0 µg/kg), using liver sample which was free from aflatoxins and OTA (previous determined with HPLC-FD). The analysis was performed with six replicates at each level of fortification.

Repeatability was predicted through the standard deviation and relative standard deviation, using the data of the recovery measurements. Within-laboratory reproducibility of the method was determined in the same fortified concentration levels with six replicates at each level. Those steps were repeated in different days, using the same methods and the same apparatus. Two different analysts performed the analysis.

The expanded measurement uncertainty  $U$  at the proposed concentration level (0.50  $\mu\text{g}/\text{kg}$  and 5.0  $\mu\text{g}/\text{kg}$  for AFB<sub>1</sub> and OTA, respectively) was calculated from the within-laboratory reproducibility and other factors: standard solution, pipettes that we used and balance as well, using coverage factor 2 (95% confidence level). All of those factors were adjusted as a percentage according to NIST Uncertainty Guideline (20).

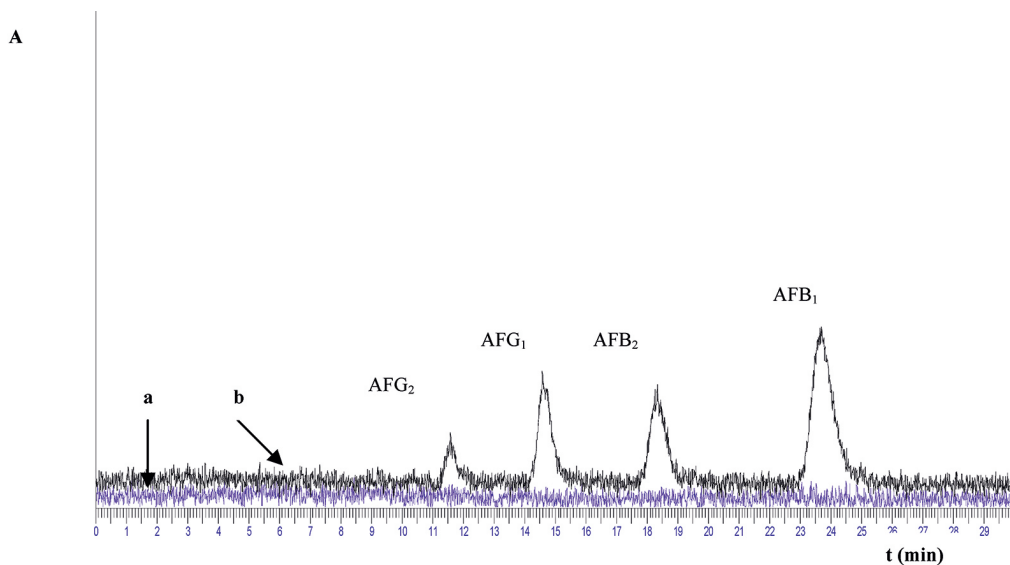
## RESULTS

Seven-point calibration curves were linear in the suggested concentration range for all four aflatoxins and OTA as well, with satisfactory coefficient of correlation ( $R^2 > 0.998$ ). The methods

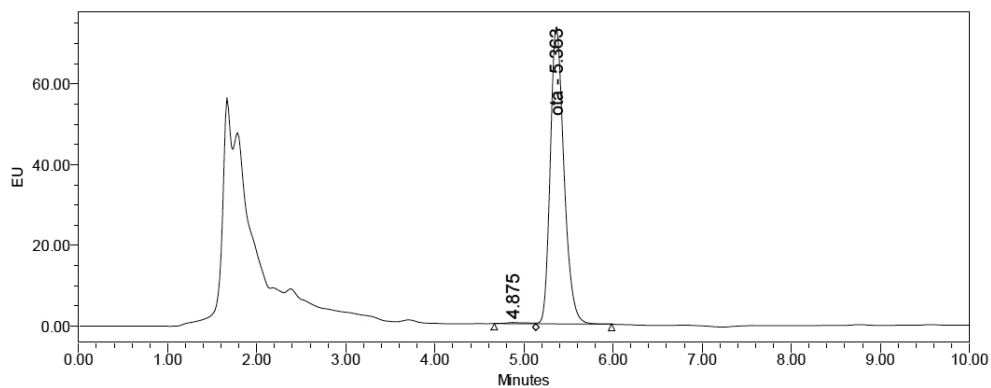
were appropriate over the tested concentration range, having in mind that there is no requirement for the maximum permitted level of aflatoxins and OTA in liver within European legislation.

The chromatograms obtained when IAC clean-up procedure was performed, were clean, with flat baseline, no matrix effects and practically no impurity and unidentified peaks, as can be seen in Fig. 1 and Fig. 2.

The limit of detection was acceptable (for AFB<sub>1</sub> it was 0.003  $\mu\text{g}/\text{kg}$ ; for AFB<sub>2</sub> 0.001  $\mu\text{g}/\text{kg}$ , for AFG<sub>1</sub> 0.006  $\mu\text{g}/\text{kg}$  and for AFG<sub>2</sub> 0.007  $\mu\text{g}/\text{kg}$ ). Limit of quantification was also satisfactory (for AFB<sub>1</sub> it was 0.009  $\mu\text{g}/\text{kg}$ ; for AFB<sub>2</sub> 0.005  $\mu\text{g}/\text{kg}$ , for AFG<sub>1</sub> 0.02  $\mu\text{g}/\text{kg}$  and for AFG<sub>2</sub> 0.022  $\mu\text{g}/\text{kg}$ ). LOD and LOQ values for OTA were found 0.08  $\mu\text{g}/\text{kg}$  and 0.27  $\mu\text{g}/\text{kg}$ , respectively.



**Figure 1.** Chromatograms of (a) blank liver sample and (b) fortified liver sample with concentration of AFB<sub>1</sub> 0.50  $\mu\text{g}/\text{kg}$ ; AFB<sub>2</sub> 0.142  $\mu\text{g}/\text{kg}$ ; AFG<sub>1</sub> 0.517  $\mu\text{g}/\text{kg}$  and AFG<sub>2</sub> 0.166  $\mu\text{g}/\text{kg}$



**Figure 2.** Chromatogram of fortified liver sample with concentration of OTA 5.0  $\mu\text{g}/\text{kg}$

The results for the repeatability estimated by the relative standard deviation ( $RSD_r$ ) are presented in Table 1 at three proposed concentration levels for all four aflatoxins. Accuracy and precision results

for OTA are presented in Table 2. The results for the recovery are given in the same Table 1 and Table 2 for aflatoxins and OTA, respectively.

**Table 1.** Repeatability, estimated by relative standard deviation ( $RSD_r$ ) for aflatoxins

	Spiked concentration level ( $\mu\text{g/kg}$ )	Determined concentration level ( $\mu\text{g/kg}$ )	$SD_r^*$	$RSD_r$ (%)	Recovery (%)	Mean recovery (%)	Mean recovery (%) for total aflatoxins
AFB <sub>1</sub>	0.100	0.085	0.008	9.967	84.500	89.630	78.100%
	0.250	0.253	0.005	1.976	101.200		
	0.500	0.416	0.010	2.376	83.200		
AFB <sub>2</sub>	0.028	0.025	0.003	11.054	88.730	90.130	
	0.071	0.067	0.004	5.328	94.360		
	0.142	0.124	0.008	6.716	87.320		
AFG <sub>1</sub>	0.103	0.077	0.007	8.867	74.270	85.030	
	0.258	0.236	0.025	10.803	91.290		
	0.517	0.463	0.020	4.408	89.550		
AFG <sub>2</sub>	0.033	0.011	0.002	14.415	33.930	47.610	
	0.083	0.040	0.004	10.430	48.070		
	0.166	0.101	0.010	10.300	60.840		

$n^*=6$

**Table 2.** Repeatability, estimated by relative standard deviation ( $RSD_r$ ) for OTA

	Spiked concentration level ( $\mu\text{g/kg}$ )	Determined concentration level ( $\mu\text{g/kg}$ )	$SD_r^*$	$RSD_r$ (%)	Recovery (%)
OTA	1.0	0.81	0.04	4.91	80.83
	5.0	5.18	0.20	3.76	103.63
	10.0	9.56	0.79	8.31	95.57

$n^* = 6$

**Table 3.** Within-laboratory reproducibility, estimated by standard deviation ( $SD_R$ ) and relative standard deviation ( $RSD_R$ ) for aflatoxins

		I analyst (day 1)	II analyst (day 2)
Spiked concentration level ( $\mu\text{g/kg}$ )	AFB <sub>1</sub>	0.500	0.500
	AFB <sub>2</sub>	0.142	0.142
	AFG <sub>1</sub>	0.517	0.517
	AFG <sub>2</sub>	0.166	0.166
Determined concentration level ( $\mu\text{g/kg}$ )	AFB <sub>1</sub>	0.416	0.430
	AFB <sub>2</sub>	0.124	0.125
	AFG <sub>1</sub>	0.463	0.465
	AFG <sub>2</sub>	0.101	0.108
$SD_R^*$ $n^*=6$	AFB <sub>1</sub>	0.010	0.024
	AFB <sub>2</sub>	0.008	0.011
	AFG <sub>1</sub>	0.020	0.033
	AFG <sub>2</sub>	0.010	0.015
$RSD_R$ (%)	AFB <sub>1</sub>	2.376	5.609
	AFB <sub>2</sub>	6.716	8.781
	AFG <sub>1</sub>	4.408	7.067
	AFG <sub>2</sub>	10.300	13.910
Recovery (%)	AFB <sub>1</sub>	83.200	86.000
	AFB <sub>2</sub>	87.320	88.020
	AFG <sub>1</sub>	89.550	89.940
	AFG <sub>2</sub>	60.840	65.060

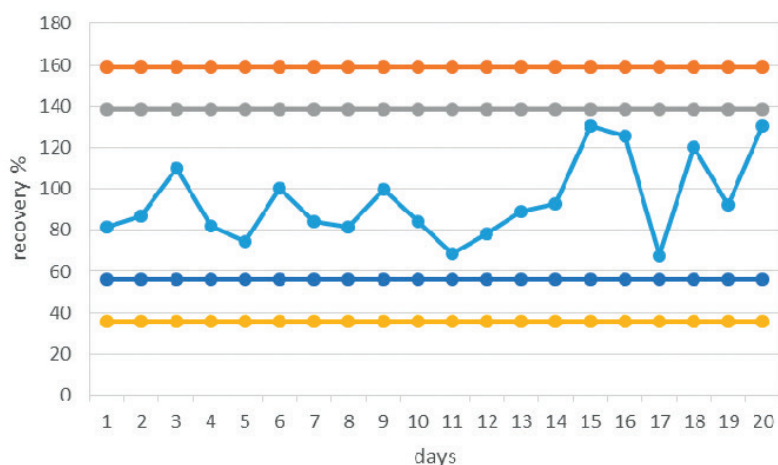
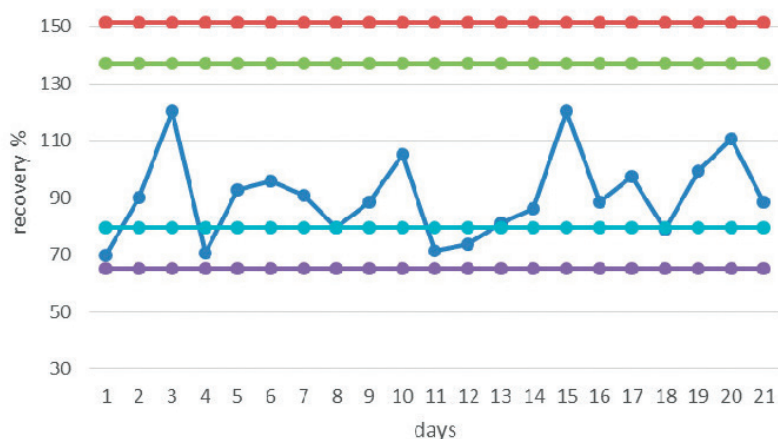
**Table 4.** Within-laboratory reproducibility, estimated by standard deviation ( $SD_R$ ) and relative standard deviation ( $RSD_R$ ) for OTA

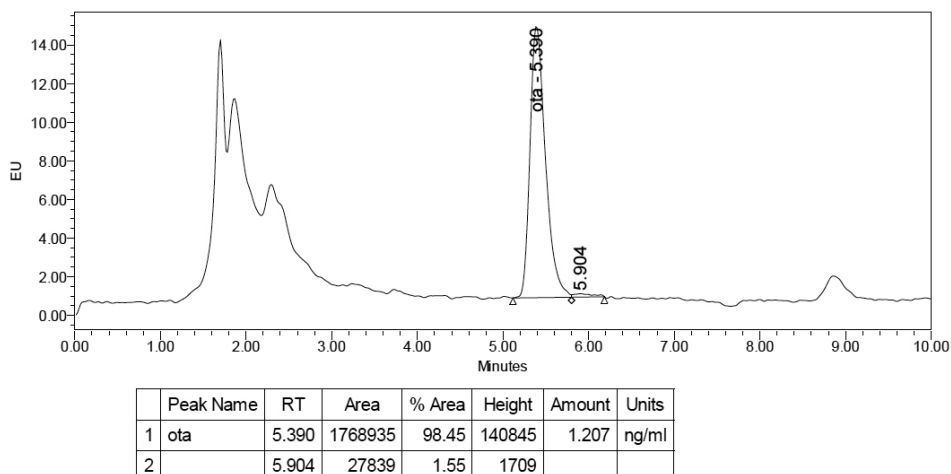
	I analyst (day 1)			II analyst (day 2)		
	1.0 $\mu\text{g}/\text{kg}$	5.0 $\mu\text{g}/\text{kg}$	10.0 $\mu\text{g}/\text{kg}$	1.0 $\mu\text{g}/\text{kg}$	5.0 $\mu\text{g}/\text{kg}$	10.0 $\mu\text{g}/\text{kg}$
<b>Mean concentration</b>	0.81	5.18	9.56	0.78	4.98	9.34
<b>STD (<math>n^* = 6</math>)</b>	0.04	0.20	0.79	0.03	0.31	1.04
<b><math>RSD_R</math> (%)</b>	4.91	3.76	8.31	3.15	4.12	8.21
<b>Recovery%</b>	80.93	103.63	95.57	78.00	99.60	93.40

In Table 3 the results for the within-laboratory reproducibility expressed with the standard deviation ( $SD_R$ ) and the relative standard deviation ( $RSD_R$ ) are given.  $RSD_R$  values results show adequate correlation in all four aflatoxins. For  $AFB_1$   $RSD_R$  values were 2.37% (for the first analyst) and 5.60% (for the second), for  $AFB_2$  values were 6.71% and 8.78%, for  $AFG_1$  values were 4.4% and 7.0% and for  $AFG_2$  values were 10.30% and 13.91%, respectively.

Reproducibility results for two days for OTA are demonstrated in Table 4. Within-laboratory reproducibility for OTA also show good correlation between two days (4.91% and 3.15% for the concentration level of 1.0  $\mu\text{g}/\text{kg}$ ; 3.76% and 4.12% for the concentration level of 5.0  $\mu\text{g}/\text{kg}$  and 8.31% and 8.21% for the concentration level of 10.0  $\mu\text{g}/\text{kg}$ ).

The stability of the method was approved following the recovery over a period of one year. The values obtained for recoveries are presented in Fig. 3 and Fig. 4.

**Figure 3.** Shewhart control chart for  $AFB_1$  (spiking level 0.50  $\mu\text{g}/\text{kg}$ )**Figure 4.** Shewhart control chart for OTA (spiking level 5  $\mu\text{g}/\text{kg}$ )



**Figure 5.** Chromatogram of liver sample with concentration of OTA 0.86  $\mu\text{g}/\text{kg}$  (dilution factor is 1.4)

The measurement uncertainties determined at the proposed concentration level (0.50  $\mu\text{g}/\text{kg}$  for  $\text{AFB}_1$  and 5.0  $\mu\text{g}/\text{kg}$  for OTA) were 15.50% and 11.26 % for  $\text{AFB}_1$  and OTA, respectively.

The monitoring of presence of aflatoxins and OTA in animal liver samples was conducted over a period of six years (2015-2020). In thirty seven (37) samples out of 297 OTA was present in concentration range of 0.130-0.922  $\mu\text{g}/\text{kg}$ . Most of the samples were with OTA concentration below LOD. No one sample was found for presence of aflatoxins. Chromatogram of liver sample with concentration of OTA 0.86  $\mu\text{g}/\text{kg}$  is presented on Fig. 5.

## DISCUSSION

Linearity of the methods with high coefficients of correlation demonstrated reliable results. As can be seen in Fig. 1, Fig. 2 and Fig. 5 there is no potential risk of interfering compounds around selected mycotoxins. This is due to the IAC selectivity. The columns which are considered to be state-of-the-art in the field of analytical chemistry, contained a gel suspension of monoclonal antibody specific to the toxin of interest. Spiked blank liver sample showed good peaks separation, specific for preferred mycotoxin (Fig. 1). Hence, taking into account the characteristics of IAC, the selectivity and specificity were recognized to be satisfactory for bought methods. We compared some studies (14, 15, 21, 22) with the methods we worked on, and our study showed efficiency and appropriateness for detection of the very low levels for determined mycotoxins (low LOD and LOQ values).

All results obtained from the validation procedure were suitable for evaluation according to the criteria (16, 17). Our results for repeatability, determined by  $\text{RSD}_r$  (Table 1 and Table 2) were in the acceptable range (1.97–14.45% for all aflatoxins and 3.76–8.31% for OTA) according to the benchmark (mean value for  $\text{RSD}_r$  shall not exceed 20%). Recovery values as a part of trueness, were satisfactory and in accordance to European legislation (2002/657/EC) and performance criteria set up in Commission Regulation 401/2006. They were in range of 47.61-90.13% as a recovery for individually aflatoxins, or as mean recovery for total aflatoxins (78.10%). For OTA, recovery values were in the range of 80.83–103.63% for three concentration levels of determination. As reported by regulation (16), data corrected with the recovery are only acceptable when they fall within the range -50% to +20% of measured value. Within-laboratory reproducibility, estimated by standard deviation ( $\text{SD}_R$ ) and relative standard deviation ( $\text{RSD}_R$ ) given in Table 3 and Table 4 were considered acceptable.

The stability of the method, which was tested through the recovery, showed satisfactory results. The values for recoveries were found to be stable over the tested period, having in mind the Shewhart control chart (Fig. 3 and Fig. 4). These limits of the chart, reflect that the process operate as consistently as possible without fundamental changes.

The results obtained from the conducted monitoring for presence of aflatoxins and OTA in animal liver samples, showed presence of OTA in 12.45% of samples (37 out of 297). OTA was present in very low concentration range (0.130-0.922  $\mu\text{g}/\text{kg}$ ). The greater number of the samples (87.55%) were

with OTA concentration below LOD (0.08 µg/kg), although some studies (14, 15, 22, 23) showed higher value of OTA in pig tissues (more than 15.0 µg/kg). We didn't find any sample with presence of aflatoxins. Although there isn't official MRL's for mycotoxins in animal tissues, the attention should be paid and regular monitoring for presence of mycotoxins should be organized.

## CONCLUSION

It was confirmed, through the validation procedures, that the proposed methods are rapid, reliable, providing satisfactory recoveries (mean value for total aflatoxins was 78.10% and mean value for OTA was 93.34%), with acceptable precision values in the range of 1.97–14.41% for all four aflatoxins and 3.76–8.31% for OTA, at three proposed concentration levels. The methods also showed high peak selectivity and low values for LOD and LOQ. All the validation parameters are in accordance to the requirements of European regulation and all of them indicate that those methods are suitable for the determination of aflatoxins and OTA in animal liver. The methods were found to be applicable to determine significantly low concentration of proposed mycotoxins. The overall overview in a period of six years, showed no significant contamination in liver samples in Republic of North Macedonia. However, regular aflatoxins and OTA monitoring plan in animal liver is highly recommended.

## CONFLICT OF INTEREST

The authors declared that they have no potential conflict of interest with respect to the authorship and/or publication of this article.

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## AUTHORS' CONTRIBUTION

BSD conducted the validation, analyses, interpretation of the results and she wrote the manuscript. EDS and ZHM supervised the laboratory work and they corrected the manuscript. RU and KB supervised the conduction of validation and participated in the

interpretation of results. AA conducted the laboratory work. DJ organized the laboratory work and corrected the manuscript.

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