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EXAMINATION OF AFLATOXINS B_1 AND G_1 IN FEED

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Aflatoxins B_1 (AFB_1) and G_1 (AFG_1) are micotoxins derived from molds *Aspergillus flavus* and *Aspergillus parasiticus* present in animal food stored in an environment with relative humidity of 85%, moisture content of the surface about 30%, temperature of 25°C and a suitable substrate. Tests for AFB_1 and AFG_1 are made in 60 feed samples (corn, concentrate and silage) from 20 locations. Two methods have been used, Charm II 6600 Luminometer where aflatoxins fluorescent strongly in ultraviolet light of 365 nm, B_1 generates a blue fluorescent and G_1 green fluorescent, and with VICAM where AFB_1 are extracted in portions with acetonitrile/methanol, and application of HPLC. In corn C(AFB_1)/ppb with Charm II 6600 Luminometer was 3.5±2.0647; in concentrate 3.25±1,8883, and in silage 6.45 ±2.0124; for C(AFG_1)/ppb was: 3.2±1.9358, 2.9±1.9324, and 5.7±2.22663, respectively. In the same samples C(AFB_1)/ppb determinate with VICAM are: 4.49±2.3548, 4.93±2.7941 and 7.73±1.766054. The results obtained by HPLC are with two decimal places and for a unit higher. Aflatoxins present in the feed used by the Pelagonian region vary within the limits of normal (0–10 ppb) except silage which has a higher value due to its greater exposure to mold. Determining the concentration of AFB_1 and AFG_1 in animal feed is of great importance, the feed with aflatoxins results in high toxicity, carcinogenicity and mutagenicity and affects the health of livestock and their application in nutrition and health of people.

Key words: aflatoxins; aflatoxin B_1; aflatoxin G_1; animal feed

ИСПИТУВАЊЕ НА АФЛАТОКСИНИТЕ B_1 И G_1 ВО ДОБИТОЧНА ХРАНА

Афлатоксините B_1 (AFB_1) и G_1 (AFG_1) се микотоксини добиени од мувлите *Aspergillus flavus* и *Aspergillus parasiticus* присутни во добиточна храна чувана во средина со релативна влажност од 85%, содржина на влага на подлогата околу 30%, температура од 25 °C и погоден супстрат. Испитувања за AFB_1 и AFG_1 се направени на 60 примероци добиточна храна (пченка, концентрат и силажа) од 20 локации. Користени се две методи: Charm II 6600 Luminometer, каде афлатоксините се силно флуоресцентни во ултравиолетовата светлина од 365 nm, B_1 создава сина флуоресценција, а G_1 зелена флуоресценција, и со VICAM, каде AFB_1 се екстрахира во порции со ацетонитрил/метанол и со примена на HPLC. Според методата со Charm II 6600 Luminometer концентрацијата на AFB_1/ppb во пченката изнесува 3,5±2,0647, во концентратот 3,25±1,8883, во силажата 6,45±2,0124, а концентрацијата на AFG_1/ppb е 3,2±1,9358, 2,9±1,9324, и 5,7 ± 2,22663, соодветно. Во истите примероци AFB_1/ppb определуван со VICAM е: 4,49±2,3548, 4,93±2,7941 и 7,73±1,766054, соодветно. Резултатите добиени со HPLC се со две децимали и за единица повисоки. Афлатоксините присутни во добиточната храна која се користи во пелагонискиот регион се движат во границите на нормала (0–10 ppb), со исклучок на силажата кај која имаат повисока вредност, што е резултат на нејзината поголема подложност на мувли. Определувањето на концентрацијата на AFB_1 и AFG_1 во добиточната храна е од големо значење, бидејќи храната со афлатоксини е со висока токсичност, канцерогеност и мутагеност и влијае врз здравјето на добитокот, а преку исхрана и врз здравјето на луѓето.

Клучни зборови: афлатоксини; афлатоксин B_1; афлатоксин G_1; добиточна храна

INTRODUCTION

Aflatoxins (AF) are toxic by-products of mold growth on certain agricultural commodities. Since their discovery in the early 1960's, aflatoxins have been shown to be carcinogenic to "laboratory test animals". In 1969, the Food and Drug Administration (FDA, 2009), set an action

level for AF at 20 ppb for all foods, including animal feeds, based on FDA's analytical capability and the agency's aim of limiting aflatoxin exposure to the lowest possible level. In 1990, FDA issued guidelines that AF in peanut products intended for use as feed ingredient are no more toxic to these same subgroups of animals than aflatoxin is in corn (Gary et al., 2009).

Most European countries have given notice that 50% of import peanuts contain AF. Because of toxic effects of AF their quantities are regulated by rules that permit the maximum allowable concentrations. Maximum allowed concentrations of aflatoxins B_1 and G_1 (AFB_1 and AFG_1) under Regulation quality and food safety no. 118/2005, allowed amounts of AF in feed. Amounts of AF permitted in feed: rye, oats, wheat, corn, barley, are 5 μg kg^{-1} (Pešić-Mikulec D., 2006).

Aflatoxins are toxic secondary metabolites of many saprofite molds which the organisms of animals and people get in food infected with spores or fragments of mycelium. Aflatoxins are secreted by molds of the species *Aspergillus flavus* and *Aspergillus parasiticus*. From the molds *Aspergillus flavus*, pale yellow crystalline substance with a strong fluorescence, aflatoksin B_1 (AFB_1) blue and aflatoksin G_1 (AFG_1) green fluorescence (Yuan I., Naoki H., 2004).

The fungus *Aspergillus flavus* is quite common in nature, but its population increases during hot dry weather. Aflatoxin contamination is greater in corn that has been produced under stresssed conditions. By Tvrtković (2006) they are developed in relative humidity of 85%, moisture content of the surface about 30%, temperature of 25°C and a suitable substrate. Thus, drought, heat, insect, nematode and fertilizer stress are all conductive to high levels of AF. Seed companies are in the process of developing corn hybrids with some level of resistance to the fungus or that have less tendency to accumulate the toxin. Although these hybrids will tend to have lower levels of AF than others grown under the same conditions, complete resistance is unlikely. Management practices such as irrigation, good insect control and timely fertilization may reduce stress to the corn plant and thus lower AF levels (Udom I. E., et al., 2012).

Samples of two types of cattle feed concentration compounded at the feed mill section of the National Veterinary Research Institute (NVRI), Vom, Nigeria, were evaluated for incidence of toxigenic *Aspergillus* section *Flavi*. Aflatoxins in the feed were quantified by thin-layer chromatography with fluorescent detection in order to determine the risk of AF to the cattle. Aflatoxins B_1 (AFB_1), B_2 and G_1 were detected in the samples at varying concentrations. About 92% of the concentrate feed samples for dairy cattle had AFB_1 concentrations exceeding the stipulated 5 μg kg^{-1} maximum limit set by the European Union (EU) for dairy cattle. AFB_1 concentrations in all samples of the maintenance feed concentrate were within the EU maximum acceptable limits of 20 μg kg^{-1}. This study has shown that the risk of aflatoxicosis is high in dairy cattle due to the high levels of AFB_1 in the feed concentrates. This may affect the milk products obtained from the cattle due to biotransformation of AFB_1 into AFM_1 (Odoemelam S. A. et al., 2009).

In cattle, chronic ingestion of aflatoxin in sillage causes various adverse effects such as increased susceptibility to disease, loss of reproductive performance, and in case of dairy cattle a decrease in yield and quality of milk production. Aflatoxins, particularly AFB_1, have been described both acute and chronic (Meggs, 2009). In June 2004, in Kenya there was an outbreak of acute aflatoxicosis and high levels of AFB_1 in stored corn at high humidity conditions were found (Lewis, 2005). Aflatoxins B_1 have been found in different countries as a contaminant in feed of dairy, cotton-seed, barley, soy-bran, pellet wheat, peanut shells, corn silage and sorghum silage (Decastelli et al., 2007). In the case of AFB_1, its presence in the food of dairy cattle leads to the emergence of AFM_1 in milk and dairy products (Lopez A., 2008).

As corn silage consists of grinding and storing the whole corn plant, it includes not just grain but a high percentage of stalks and stover and represents a new important bulky feed source for dairy and beef cattle. Nutritionally, corn silage, for example, has a balance between the energy density of the grain and fibber and digestibility of the green plant that makes it suitable for feeding ruminants in the phases of maximum nutritional needs (Molina et al., 2004).

A method for the determination of aflatoxins B_1, B_2, G_1 and G_2 in maize silage using high-performance liquid chromagraphy with fluorescence detection has been developed and validated, Method using HPLC, Uganda standard (2009). Recoveries of aflatoxins B_1, B_2, G_1 and G_2 spiked over the 0.25 to 5 μg kg^{-1} range averaged 74–94%.

The results of laboratory scale and farm scale ensiling experiments indicated that aflatoxins could increase when silage is exposed to air during conservation or during the feed-out phase (Cavallarin L et al., 2011).

Aflatoxin B_1 (AFB_1) is bis-furanocoumarin derivative usually dominant in cereals and food products (Aggarwal M., 2009). After entering the body it can be metabolized in the liver reactive epoxide intermediate or hydrolyzed and get less harmful aflatoxins M_1 and M_2. Aflatoxin G_1 (AFG_1) commonly encountered in contaminated foods such as peanuts and their products, coffee, cotton, etc. with toxic effect similar as AFB_1, but one degree lower (Huang B., et al., 2010) has similar structure as AFB_1 with difference that instead cyclopentenone contains pyran ring (Cismileanu G. et al., 2008), occurs during the synthesis of nucleic RNA or synthesis of proteins.

Growth and development of molds in food animals are related to utilization of nutrients in foods that reduce the nutritional value and change the organoleptic properties. The natural yellow color of corn can be changed to white, pink, red or green depending on the type of mold that grow on it and that is why the food has a suffocating odor (Hocking A. D. et al., 2006). The development of molds in the utilization of nutrients reduces the content of dry matter and the loss can be up to 50%. Grains contaminated with molds and stored 30 days at humidity of 18% lost 40% dry matter, and for 60 days 8% dry matter (Dimić D. J. et al, 2009).

The presence and development of molds in foods reduce fat and carbohydrates and thus reduce the metabolic energy for 5%, even up to 25%. Food for the young and high production animals that utilize high energy rations which significantly increases the cost of food. On the other hand, the formulation of meals based on table values without prior knowledge of changes in nutritional values may lead to malnutrition and poor production result (Meggs W., 2009).

The use of inhibitors, adsorbens, acids and other chemical compounds could reduce the amount of molds, but also will not affect mycotoxins already produced, even to remove molds. In recent times the applications of different biological adsorbens that competatively tie mycotoxins prevent their harmful effect. However, the best way of preventing the occurrence of mycotoxins is the use of quality, microbiological and chemical raw materials (Sumić Z. et al., 2007).

MATERIAL AND METHODS

For this examination for testing samples of animal feed (corn, concentrate and silage) were taken from 20 locations with farms of different size of droves from 5 to 100 heads of cattle in the Pelagonian region. The food was used for feeding the cattle during the winter period in 2011. The examination of the concentration of aflatoxins B_1 and G_1 was carried out with two different devices, by two different methods. One was fluorometric, Luminometer was also used by Leszczynska J., (2007), and the other thin-layer chromatography, HPLC was used by Yuan I., (2004). Although the methods differ in their performance, the results were not very different.

Determination of aflatoxins with Charm II 6600 Luminometer

The appliance Charm II Luminometer is a significant and substantial progress in sanitary microbiology and toxicology. The Charm II aflatoxin test is fast (about 15 minutes) to test and find if aflatoxins are present within the limits of 5–40 ppb. The test uses the same Charm II equipment used for testing antimicrobial drugs (medication). The limit of the linear calibration of Charm is usually 10^4 to 10^5, greater than the limit of detection. The detection limit of elements is from 5 to 40 ppb. The precision expressed as relative standard deviation is typically 1% and the concentrations are lower 100 times greater than the limit of detection.

Preparation of solutions: 80% methanol (80 ml 100% methanol is added to 20 ml distilled water), basic standard solution (mixture) of aflatoxins B and G – 100 mg/l, standard (mixture) of aflatoxins B, G – 1 mg l^{-1}. One ml of basic solution is taken and is added to the pumpkin 100 ml with 80% methanol. The standard is kept in a fridge at 2 to 6 ^oC for 48 hours. Reconstructed standard can be packed up to 2 months at a temperature of 15 ^oC or lower temperature. The working standard solution of aflatoxins B, G is prepared from 50 ml pumpkin by appropriate dilution of the transitional solution. The working standards are with the following concentrations: working standard solution of aflatoxin B_1/μl kg^{-1} (2, 4, 6, 8, and 10); working standard solution of aflatoxin G_1/μl kg^{-1} (1, 3, 5, 7, and 9).

Preparation of samples for analysis: off samples that are tested (corn concentrate sided) are

measured in a 50 g bag to grind in a blender. The bag is added to methanol (the digester) to mix 30 seconds, wait 10 seconds and then 2 ml extract is transferred into the test tube to centrifuge 3 minutes, and then the extract is used for testing.

Test procedure: use laboratory tweezers to put the table in the empty test tubes and add 5 ml of distilled water. Mix with vortex (10 cycles) with 3700 rotates per minute (rpm). Add 100 l of the sample extract and it matches again 10 cycles with 3700 rpm, then it is incubated at 35 ^{o}C of a time of 2 min. Then we added a purple tablet in the test tube and immediately mixed it with vortex of 3700 rpm, after being incubated at 35 ^{o}C during 2 min, in the centrifuge 5 minutes and carefully decanted of the liquid. We added 100 l of distilled water and mixed the sediment of vortex of 3700 rpm 10 cycles. We added 3 ml scintillating liquid in the test tube then closed and mixed it in vortex. The prepared sample is placed in the appliance of analyses and after 1 minute concentrations of aflatoksin B and aflatoksin G are seen.

Determination of aflatoksins with Vicam

Analytical laboratories use one of several procedures such as a thin-layer chromatography, to determine aflatoxin levels. These procedures are highly accurate and quantitative. The laboratory should grind the entire sample of corn together before taking subsamples for analysis. Technology allows the aparatures Vicam down with a limit of detection to 0.10 ppb, with an accuracy of 0 to 20 ppb, and the precision expressed as a relative standard deviation is usually around 1% and lower concentrations 100 times greater than the limit of detection.

The method for determination of aflatoxin B_1 in feed: take 50 g of the sample and add 10 ml NaCl, mix in a blender jar. Add 200 ml methanol (HPLC grade)/water; the extraction solution of 80/20 is ground 1 min at high speed, and filtered through a filter. 10 ml of the filtered extract is taken in a 10 ml clean beaker and add 40 ml water, mix throughly. Then the mixture is again filtered through a 1.5 min in a clean glass microfibre filter (Whatman) or a syringe barel of 10 ml. Take 10 ml of filtrate and pass through the AflaTest affinity column flow 1–2 drops/second. Through the column to pass 2×10 ml water. After the second washed place clean under outlet of the column, add 1 ml methanol and clean the syringe with the flow of 1–2 drops/second. Then add 1 ml methanol and 1 ml Aflatest Developer Solution in a cuvette, well mixed on Vortex and placed column in a fluorometer (Vicam, USA) in which the reading of the result is in 1 minute.

The results were processed with statistical methods variaton in Microsoft Office Excel, average and standard deviation (SD) and verified the accuracy of the method.

RESULTS AND DISCUSSION

The experimental values for the presence of aflatoxins B_1 and G_1 confirm the validation of the appliance Charm II Luminometer for repeatability, accuracy and precision of the method.

1. *Calibration curve for determining the concentration of AFB$_1$*

Linearity of the method is tested with a standard formula in the areas of concentration of 2 to 10 μg kg^{-1}. Based on the measured values the calibration curve is shown in Figure 1. Calibration graph for determining the concentration of aflatoxin B_1 confirms the assumption of linearity in the range 2 to10 μg kg^{-1} and coefficient of dependence of 0.99997.

$$y = 619.65x + 165.4n = 10$$

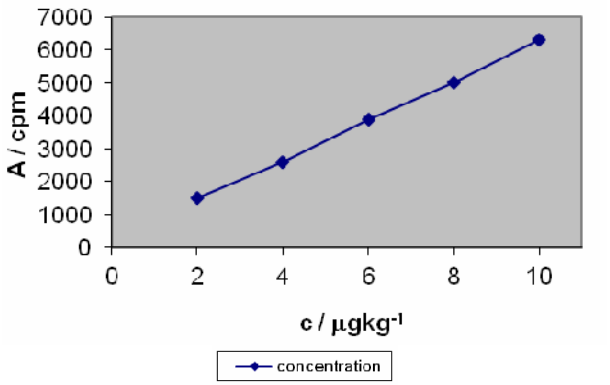

Fig. 1. Calibration diagram concentration of AFB$_1$/μg kg^{-1}

2. *Calibration curve for determining the concentration of AFG$_1$*

Calibration curve for determining the concentration of aflatoxin G_1 is shown in Figure 2. The values are obtained with the regression analysis using the method of least squares which confirms the linearity of the method in the area of 1 to 9 μg kg^{-1}.

$$y = 515.26x + 114.26n = 10$$

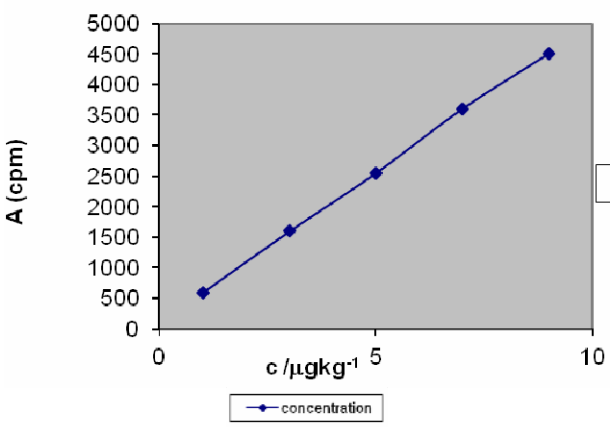

Fig. 2. Calibration diagram concentration of AFG_1/µg kg^{-1}

Experimental values for aflatoxins B_1 and G_1 in animal feed

The results of Table 1 show determination on Charm II Luminometer, that have high values of aflatoxins B_1 and G_1 only in silage but not higher than the permissible limit for their presence. From Figures 3 and 4 it can be seen that the aflatoxin G_1 has more deviations from the mean in all three types of feed.

From the obtained values it can be concluded that the presence of AFB_1 and AFG_1 is within the limits allowed (0–10 ppb). The value of AFB_1 in the large silage is greater than in corn and concentrate, which confirms the fact that silage is a easier subject to the occurrence of molds.

Table 1

Concentration of aflatoxins B_1 and G_1 in livestock feed determinant of Charm II Luminometer

Locations	Aflatoxin B_1/ppb in different type livestock feed			Aflatoxin G_1 /ppb in different type livestock feed		
	Corn	Concentrate	Silage	Corn	Concentrate	Silage
1	9	5	6	8	6	7
2	4	5	8	3	6	8
3	4	6	7	3	5	7
4	6	1	11	5	2	6
5	3	1	5	1	2	4
6	5	3	7	6	2	5
7	1	3	7	1	2	7
8	3	1	8	4	1	7
9	3	2	4	2	1	5
10	1	3	4	2	2	5
11	3	1	3	2	2	1
12	5	5	8	6	5	8
13	1	3	6	1	2	5
14	2	7	7	2	1	8
15	4	3	9	4	5	9
16	4	3	7	3	2	6
17	5	4	7	4	3	8
18	1	2	3	1	2	2
19	1	1	7	2	1	3
20	5	6	5	4	7	3
$\bar{x}$	3,5	3,25	6,45	3,2	2,95	5,7
SD	2,064742	1,88833	2,01246118	1,935812	1,932411	2,226633

Research was done on behalf of the Veterinary School in Trinidad, where 40 samples of feed from 6 areas were examined AFB_1 was detected in 20% of the samples at the minimum limit of detection ranged from 0.5 ppb, 10 ppb, 20 ppb, examination by Offiah N & Adesiyun (2007). In Por-

tugal, Martins, H. M. et al. (2007) did testing for the presence of AFB_1 in feed for 10 years (1995–2004) where the boundaries from 1 to 5.0 ppb were 312 positive samples, from 5.1 to 10 ppb were 37 positive samples, from (10–20 ppb) there were 18 positive samples, and from 20.1 to 80 ppb were only 7 positive samples.

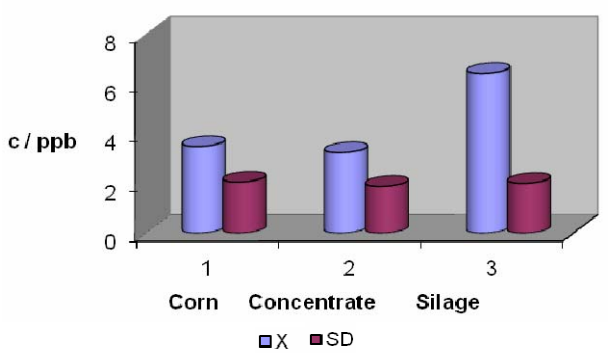

Fig. 3. Graphic presence of average and standard deviation on aflatoxin B_1 in the livestock feed of Charm II Luminometer

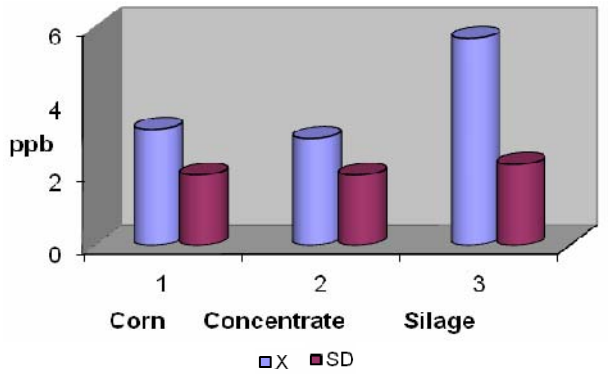

Fig. 4. Graphic presence of average and standard deviation on aflatoxins G_1 in the livestock feed of Charm II Luminometer

Research in Nigeria from Alonso, V. A., et al. (2012) for aflatoxin B_1 in feed arose within (30.22 – 210.20 ppb) detection of minimal (<0.05 ppb). In China examination of AFG_1 was made and of 73 positive tested samples 14 had concentrations of AFG_1 within the limits of 0.03 to 14.5. In Great Britain a survey of 120 samples of feed was made, from which 98 were positive with the presence of AFG_1 and concentration of 0–20 ppb.

Examining AFB_1 with the thin-layer chromatography the VICAM appliance showed similar values as obtained from Charm II Luminometer for corn and concentrate but as for the silage value it is little higher. Thus the AFB_1 presence in silage confirms deviations from the mean, which is the lowest among the AFB_1 (silage heighten precise of this method).

Table 2

Concentration of aflatoxin B_1 in the livestock feed determinant of Vicam

Locations	Aflatoxin B_1/ ppb in different type livestock feed		
	Corn	Concentrate	Silage
1	10	6.9	8.6
2	5.1	7.5	9
3	5.1	7.6	9
4	7.5	14	8.9
5	4.2	2.8	6.1
6	7.3	3.5	8.2
7	1.3	3.3	8.7
8	4.5	1.8	9.6
9	3	2.7	7.6
10	2.1	4.4	5.7
11	3.5	2.6	4.2
12	6.7	5.1	9
13	1.8	4.5	7.2
14	2.1	3.7	8.9
15	4.5	5.1	10.1
16	4.2	3.7	7.3
17	6.6	5.2	8.1
18	1.8	3.5	3.7
19	2.2	2.8	8.4
20	6.3	7.9	5.7
$\bar{x}$	4.49	4.93	7.3
SD	2.354816	2.794186	1.766054

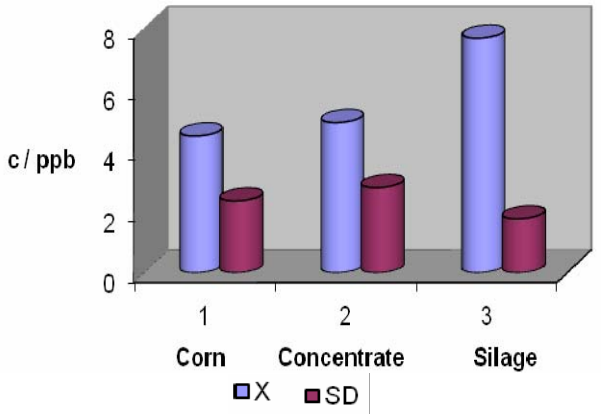

Fig.4. Graphic presence of average and standard deviations on aflatoxins B_1 in livestock feed of Vicam

In Pakistan testing was performed for the presence of AFB in wheat from 1995 to 2000 with 250 samples where 16 examined were positive and the minimum detection (13 ppb) in February and April, while most were (39 ppb) in March (published by Bhatti et al., 2001). The examination of AFB_1 in feed in Nigeria compared with many countries during 2008 showed very high values amounted to 30.22–210.20 ppb, in Ghana 20–355 ppb, in Botswana 0.3–23 ppb, in North Africa 0–25 ppb, in Senegal 20–200 ppb, in India 917–2110 ppb, in Bangladesh 65 ppb, in Turkey 0.126–5 ppb and in Argentina 20–200 ppb (presented by Odoemelam, et al., 2009).

CONCLUSIONS

Particular attention has been paid to the concentration of aflatoxins B_1 and G_1 present in animal foods used in the Pelagonia region, which varies in limits of the FDA.

The paper covers the methods for determination of aflatoxins B_1 and G_1 in the feed to Charm II 6600 Luminometer and Vicam, which can be used for the routine analysis in the laboratory. Linearity of methods is evaluated through calibration diagrams and calculations with statistical parameters which proves that the methods are accurate, precise and repeatable.

The compared results of two different methods applied in determining contamination with aflatoxin B_1 in the feed from the Pelagonian region concluded that HPLC is a more sophisticated method and Vicam gives the most accurate results.

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