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Scientific paper

EXAMINATION OF AFLATOXINS IN MILK AND ACCURACY OF THE METHOD

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Abstract

In this study, we determined the accuracy Vicam method for the determination of aflatoxin B_1 in feed and aflatoxin M_1 in milk and dairy products by applying the method of standard addition. Analytical yield in all causes ranged from 93 to 95%, indicating a high accuracy of the method. The determination was made on aflatoxin B_1 in (n = 20) samples of corn and adequately in milk and dairy products taken from animals that were fed the same corn. The samples are taken from small farms Pelagonia region. The results were in the normal range for corn and matches the reference value given approval for accreditation of laboratories in our country. For milk that was not sufficiently investigated field we get results that are in accordance with the Rules of the European Union, the aflatoxin M_1 presence to 0.05 ppb.

We conclude that the determination of aflatoxin B_1 important, their presence in animal feed in order to avoid the presence of aflatoxin M_1 in milk and dairy products, and it certainly depends on the season when the use of corn in the diet, time, storage temperature, etc.

***Key words**: mycotoxins, aflatoxin B1, aflatoxin M1, Vicam method.*

INTRODUCTION

Aflatoxin M_1 is hidroksilisated metabolite of aflatoxin B_1 (4-Hydroxyaflatoxin B) under the action of enzymes from the liver. Once set out in the external environment via urine and milk, can be found in dairy products come from animals that consumed contaminated food [2]. Aflatoxin B_1 (AFB_1) and M_1(AFM_1) are acute and chronic toxic. AFB_1 is one of the most hepatocancerogene substances, while AFM_1 is cytotoxic and its toxicity is similar to one degree

weaker than that of AFB_1. AFM_1can cause damage to genetic mutations in DNA, chromosomal anomalies and cell transformation [1].

Maximum allowable amount of AFM_1 is 0,05 g / kg (0,5 ppb) in raw milk and milk intended for dairy products, or 0,025 g / kg if intended for feeding young children. AFM_1 can be detected in milk from 12 to 24 hours after intake of AFB_1 in the body of the animal [3].

If the AFM_1 is detected in milk, then the dairy cows should be given clean does contaminated maize containing bentonite or diet hemisorbent. If the level of AFM_1 is above 0,5 ppb, then it is useless, and if the 0,5 ppb or the milk is accepted, but in any case grain foods should be tested to determine the extent and presence of aflatoxins [4].

European Commission seeks measures to limit the presence of AFM_1 and by the EU is not allowed milk with AFM_1 can be used for human consumption [1].

AFM_1 during the heat treatment during pasteurization and direct heating of the milk of fire for a period of 3-4 hours is stable and no significant change in the amount of its quantity, it occurs in cold or frozen storage where storage of frozen milk contaminated and dairy products for several months given change in the amount of AFM_1. The production of yogurt and kefir, also did not significantly reduce the presence of AFM_1 in products [6].

Inactivation of AFM_1 in milk involves the application of chemical and physical treatments. Chemicals that are applied to the decomposition of AFM_1 are sulphites, bisulphite and hydrogen peroxide (H_2O_2). When natural raw milk contaminated with AFM_1 were treated with 0,4% potassium bisulphite at 25^0C for 5 hours, the concentration decreases by 45%. Larger amount of bisulphite is less effective. In the presence of 1% hydrogen peroxide at 30^0C for 30 minutes will significantly reduce the AFM_1, and the addition of H_2O_2 in a concentration of 0,05 to 0,1% in the presence of laktoperoksidaza amount of AFM_1 is reduced by 50%. Physical processes that are used to reduce the AFM_1 in milk include adsorption and radiation. 5% bentonite in milk adsorb 89% of the presence of AFM_1. In a study of the effects of UV hydrogen peroxide, the concentration of radiation with or without AFM_1 was reduced from 3,6 to 100% of the length of time that the milk was exposed to radiation, the volume of processed milk, and the presence of hydrogen peroxide.

To be able to control AFM_1 is necessary to reduce the presence of AFB_1 in the diet of animals with various preventive measures [3]. Maize and products are based on corn, are among the most contaminated food products where the risk factor of having AFB_1 contamination of corn is equated with risk factors of existence AFM_1 contamination in milk and dairy products. During the production of corn, the production of aflatoxins commonly occurs during harvest, fertilization, irrigation and storage. At harvest grain moisture is quite low and it is exposed to contamination by mold species Aspergillus. The occurrence of mechanical damage and changes in temperature are factors that need to be controlled [1].

For determination of fat in milk can apply all common methods for determination of fat in animal products, but as the most practical method proved Gerber. This method is based on the dissolution of all components of milk in sulfuric acid, but not fat are separated on the surface.

Separation of fat is facilitated by the addition of amyl alcohol, which reduces surface tension, and accelerates the spin [15]. The technology with Vicamfor determining the presence of aflatoxins, allows determining the presence of aflatoxin in milk without the use of toxic solvents. Afla M used in laboratories where lead quality control of milk because it is very quick and easy method. During the analysis included all parameters that directly or indirectly affect the quantitative and qualitative properties of raw milk obtained after milking.

According to the certificate of accreditation of laboratories in our country there are reference values only for aflatoxin in products of plant origin and that the HPLC method (grain, grain products, dried figs, raisins, walnuts, almonds, pistachios, peanuts and nuts) and in milk and dairy products still has not sufficiently investigated. Considering the fact that micotoxsicology control of cereals and fodder is very topical issue worldwide, and milk and dairy products are received by an entity that feeds them, expect their evidence to get real, reliable and accurate results for presence [4]. Method called Vicam, for determining aflatoxins in animal feed, raw milk, commercial milk and dairy products apply to all monitoring these toxins in some milk and dairy products.

MATERIALS AND METHODS

Verification method for the determination of aflatoxins in milk, method Vicam was used the method of standard addition. Samples of raw milk, yogurt and cheese were taken at random from the market and added as a standard addition. Were examined and 20 samples of corn and raw milk from individual farms from agricultural regions of Pelagonia.

The first determined fat content in milk and with acidbutirometric, Gerber method after which is important data for evaluating quality of milk. This method is based on the dissolution of all ingredients in milk with sulfuric acid, except fat are separated on the surface. Separation of fat by adding amyl alcohol ρ_{20}=0,810-0,813 (g/cm^3), T=128-130 °C, which reduces surface tension and accelerates with the centrifugation. For this purpose use graduated butirometer (which show the percentage of fat) and appropriate centrifuge [15].

Procedure: In butirometer, Gerber with special pipette placed 10cm^3 solution of H_2SO_4 Gerber. Then carefully of walls with a special pipette add 11 cm^3 milk (brought to 20°S ± 2°S) and 1 cm^{3}amil alcohol. When it should be careful not to wet the throat butirometer with milk, is closed with a rubber stopper and shake a few times when it is proteins dissolved and lactose become caramel,the mixture gets brown color. Because mixture wasstrongly heated rolls in cloth while still warm butirometer placed in Gerber centrifugal 5 min. (800-1200 rpm / minutes).

Rubber shutter of butirometer should be inside the bearing centrifuge after spinning sets shutter down and leave 3-5 minutes in a water bath at 65-70 ^{o}C. At that temperature is seen in the table % butirometer it brings the border layer to 0 or portion scale and read the lower meniscus detached fat.

Determination of aflatoxinsof apparatusVicam

Detection of the presence and content of AB_1 and AM_1 was derived flourometric, using Vicamfluorometer method and determination of (AflaB TM andAfla M TM test) [14].

Technology of Vicam equipment enables determination of the presence of aflatoxin M in milk; test Afla M, without the use of toxic solvents such as chloroform and methyl chloride. The method is characterized by repeatability of results, where the limit of detection up to 0,10 ppb with an accuracy of the apparatus of the 0-2,0 ppb. Precision expressed by relative standard deviation is about 1% and lower concentrations 100 times greater than the limit of detection.

Calibration settings are made with AflaTest-M and calibration standards: green (-0,10), red (2,20) and yellow (1,10 ± 0,2).

Analytical yield R (recovery of 80% total aflatoxin M is present between 0,010-3 ppb.

1. *Method for determination of aflatoxin M in milk and dairy products:*

Taken 50 ml of milk for examination centrifuged at 1540 rpm during 15 minutes that separates the outer adipose layer and further defining use lower fat free. Then 50 ml of milk completely passed through the column M with 1-2 drops / second until the start of the syringe to air out (total time of flow should not exceed 20 minutes). Through the column passes 2 x 10 ml water, 1,25 ml acetonitrle: methanol (3:2) with a flow through column 1 drop / 2-3 seconds. Through the column passes 1,25 ml water with 1 drop / 2-3 seconds and collected in clean cuvette (V = 2,5 ml). Mix on Vortex and read Vicam (HPLC).

2. *Method for determination of aflatoxin B_1 in feed :*

take 50 gr of the sample and add 10 ml NaCl, mix in a blender jar. Added 200 ml metanol (HPLC grade) / water, extraction solution 80/20 is grinding 1 min at high speed, filtered through a filter. 10 ml of filtered extract is taken in 10 ml clean beaker and add 40 ml water , mix throughly. Then the mixture is again filtered through a 1,5 min in clear glass microfibre filter (Whatman) or syringe barel of 10 ml. Taken 10 ml of filtrate and passing through AflaTest affinity column flow 1- 2 drops / second. Through the column to pass 2 x 10 ml water. After the second washed place clean under outlet of the column and add 1 ml metanol clean the syringe with the flow 1-2 drops / second. Then in cuvette add 1 ml metanol and add 1 ml Aflatest Developer Solution, well mixed on Vortex and placed column in fluorometer (Vicam, USA) in which the reading of the result is in 1 minutes.

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

R (%) = weight of the resulting component / weight of starting component x 100

RESULTS AND DISCUSSION

1. Determination of fat by method of Gerber

In all samples of milk is determined fat content of which varies within the limits of 2.8 to 3.2% fat, which confirms the correctness of milk for its use.

2. To determine the accuracy and precision of Vicam method

For determination of aflatoxin B_1 and aflatoxin M_1 in various types of milk and dairy products, were applied the method of standard addition. As a standard accessory is used standard that uses a mixture of aflatoxin B_1 in corn and M_1 in raw milk, the obtained values are shown in Table 1.

Table 1. Presence of aflatoxins B_1 in corn and M_1 in raw milk by the method of standard addition

Aflatoksins	Certain amount wthout % standard addition (μ g/kg)	+ 3 μ g/kg			+ 5 μ g/kg			+ 7 μ g/kg		
		Obtained value	Calculated value	R (%)	Obtained value	Calculated value	R (%)	Obtained value	Calculated value	R (%)
B_1	2,11	4,6	6,81	94	6,76	7,11	96,6	9,11	9,11	98
M_1	1,21	3,7	4,21	93	5,79	6,21	95	8,21	8,21	96

The values of analytical yield, R, ranging within the limits of94-98% for aflatoxins B_1 and for aflatoksin M_1 by 93-96%, confirming the accuracy of quantity of the method. The presence of aflatoksin B_1 in corn and aflatoxin M_1 in milk is shown Fig.1, by the method of standard addition.

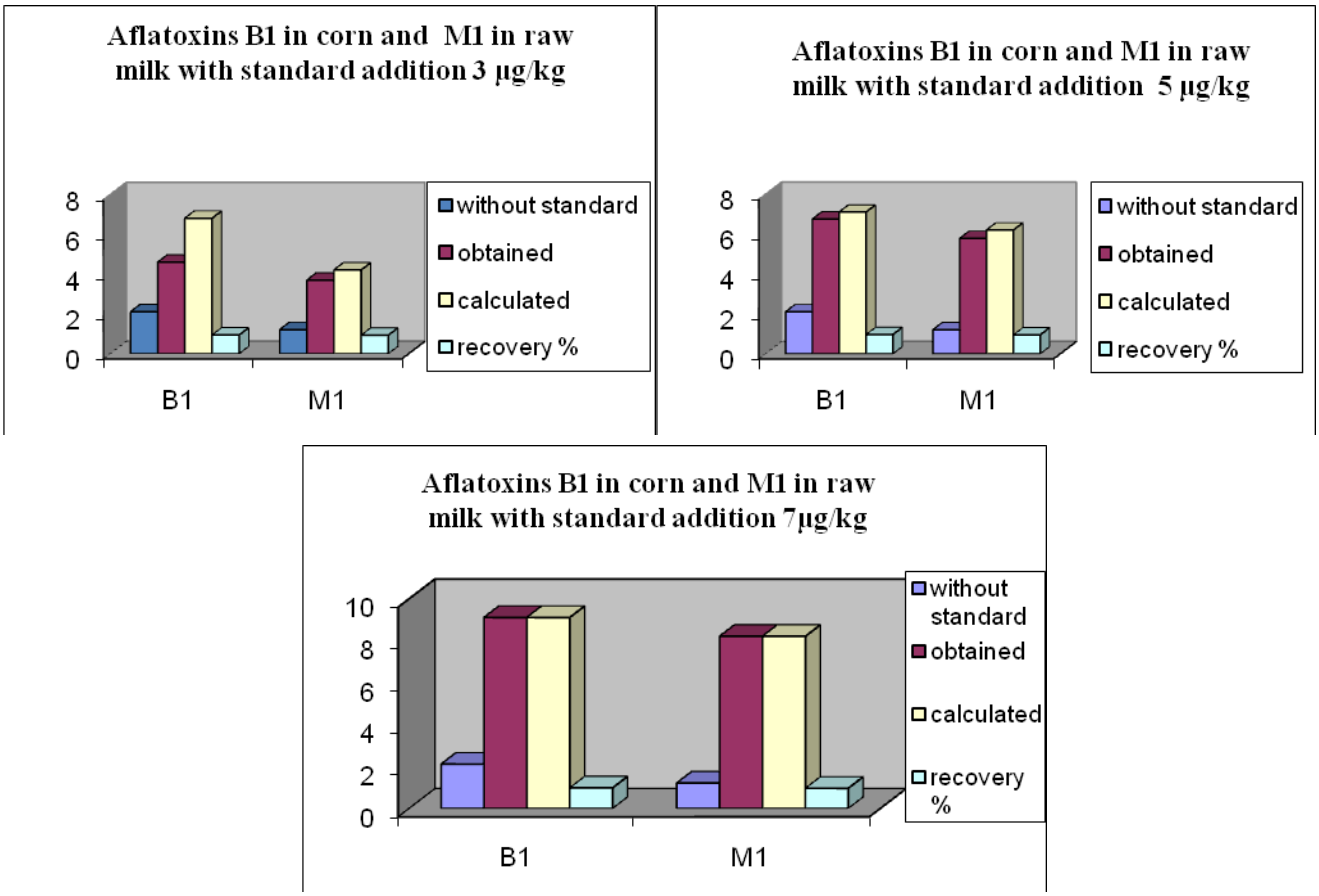

Figure 1. Graphic presentation of aflatoxins B_1 in corn andM_1in raw milk by the method of standard addition

Done is an examination of samples of yogurt-milk product made from milk of cattle which had previously been fed with contaminated corn, corn that found the presence of aflatoxin B_1. As a standard accessory is taken standard containing a mixture of aflatoxin B_1 in corn and M_1yogurt.

The values of analytical yield in Table 2 are moving limits: aflatoxin B_1 by 96-98% and aflatoxin M_1 from 93-99%.These values confirm the accurate quantitative method. The presence of aflatoksin B_1 in corn and M_1 yogurt method of standard addition is shown in Figure 2.

Table 2.Values of aflatoxins B_1 in corn and M_1 in yogurt by the method of standard addition

Afla toxins	Certain amount without % standard addition (μ g/kg)	+ 4 μ g/kg			+ 5 μ g/kg			+ 6 μ g/kg		
		Obtained value	Calculated value	R (%)	Obtained value	Calculated value	R (%)	Obtained value	Calculated value	R (%)
B_1	2,13	5,98	6,13	97	6,98	7,13	98	5,91	6,13	96
M_1	1,92	5,76	5,92	97	6,41	6,92	93	7,89	7,92	99

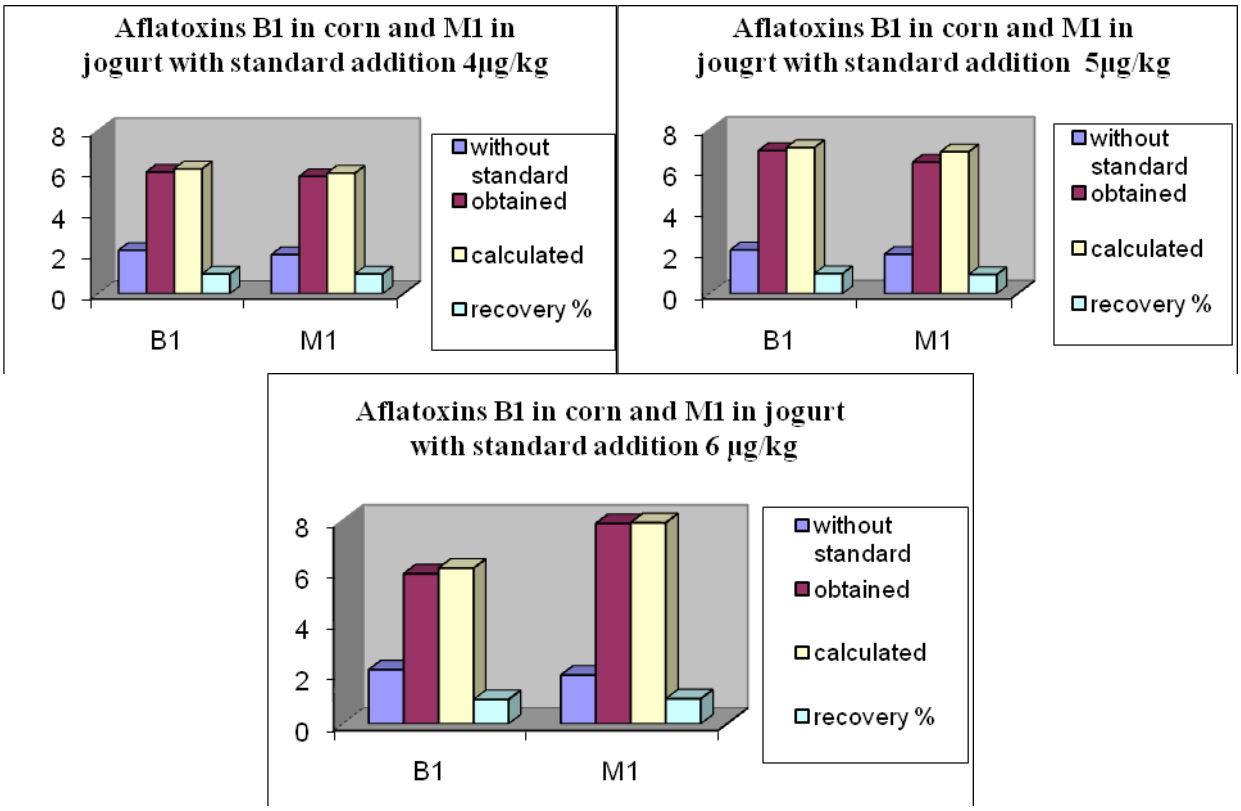

Figure 2. Graphic presentation of aflatoxins B_1 in corn andM$_1$in yogurt by the method of standard addition

Examination is done and in cheese made from milk of cattle which fed on contaminated food, corn, which was detected the presence of aflatoxin B_1. As a standard accessory is taken standard containing a mixture of aflatoxin B_1 in corn and M_1 in cheese?

Table 3.Values of aflatoxins B_1 in corn and M_1 in cheese by the method of standard addition

Afla toksins	Certain amount wthout % standard addition (μ g/kg)	+ 4 μ g/kg			+ 6 μ g/kg			+ 8 μ g/kg		
		Obtained value	Calculated value	R (%)	Obtained value	Calculated value	R (%)	Obtained value	Calculated value	R (%)
B_1	2,58	6,18	6,58	94	7,99	8,58	95	10,1	10,58	96
M_1	2,12	5,89	6,12	98	7,98	8,12	98	10,0	10,12	99

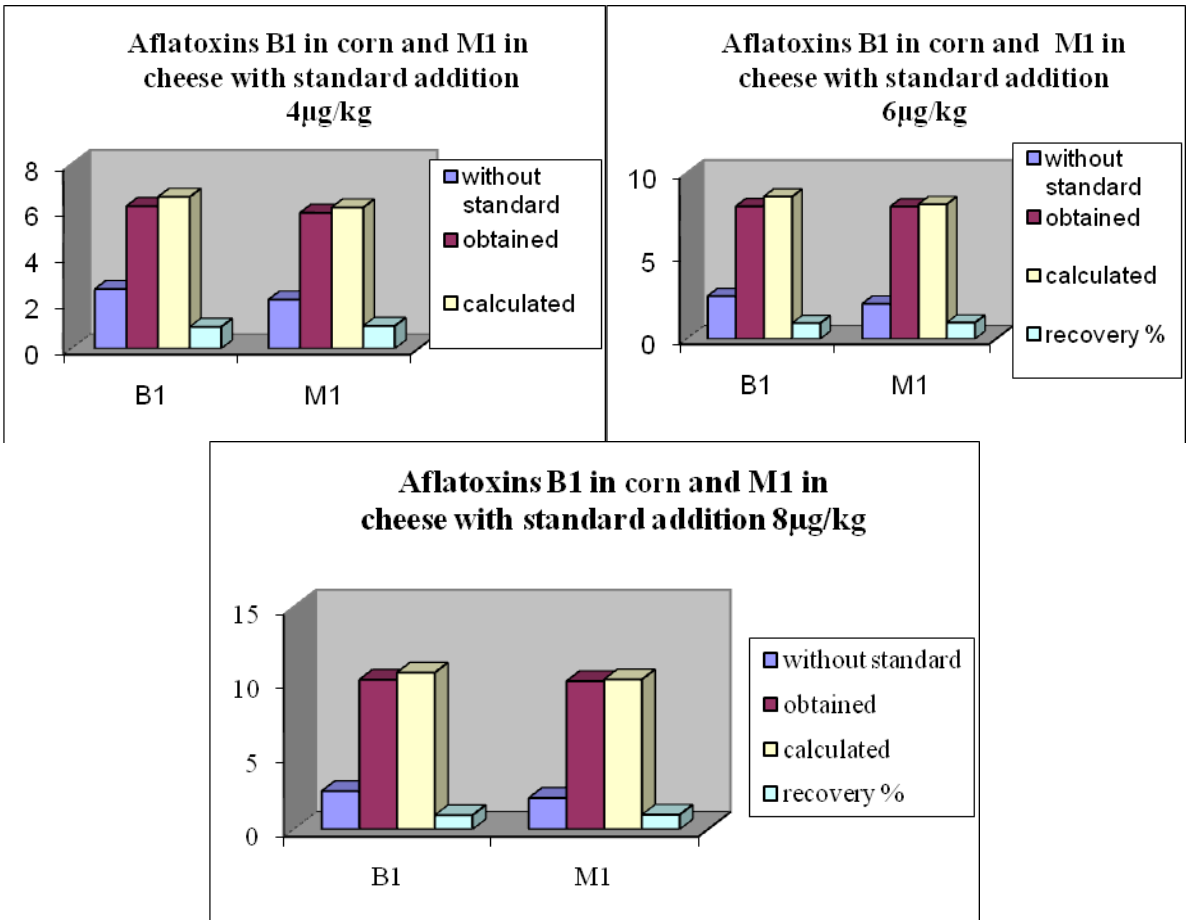

Figure 3. Graphic presentation of aflatoxins B_1 in corn andM_1in cheese by the method of standard addition

The values of analytical yield range within the aflatoksin B_1 by 94-96% and aflatoksin M from 98 to 99% that confirmed the same for accurate quantitative method. The presence of aflatoksin B_1 in corn and cheese M_1 method of standard addition is shown in Figure 3.

Determination of aflatoxin B_1 in corn and M_1 in milk Pelagonia region

Investigated the presence of aflatoxin in 20 samples of corn and appropriate in 20 milk samples from cattle that are fed with that food, Table 4.

From the results can be seen that the presence of aflatoxin $B_1$is in the limits of 4,49 ± 2,3548, and until it is observed the presence of aflatoxin in milk (0,02549 ± 0,071635).

Table 4. Values of aflatoxins B_1 in corn and M_1 in milk from Pelagonia region

Location	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
AFB_1(ppb)in corn	10	5,1	5,1	7,5	4,2	7,3	1,3	4,5	3,0	2,1	3,5	6,7	1,8	2,1	4,5	4,2	6,6	1,8	2,2	6,3
AFM_1(ppb) in milk	0	0,1	0	0	0	0	0	0	0,3	0	0	0	0	0	0	0	0	0,1	0	0

The following table statistically calculated values for the presence of aflatoxins B_1 and M_1 in tested samples taken from Pelagonia region.

Table 5. Statistical values of aflatoxins B_1 in corn and M_1 in milk from Pelagonia region

AFB_1	$\bar{x}$ = 4,49	SD = 2,354867
AFM_1	$\bar{x}$ = 0,025	SD = 0,071635

During the test the accuracy of the method for determining the vice aflatoxins in milk and dairy products, the method of standard addition is taken into account parameters that directly affect the milk. First is some fat that is a factor of quality of milk and affecting other components of milk such as calcium, phosphorus, proteins, peptides, amino acids and others. This kind of examination is important to introduce our population with their presence, which directly or indirectly affect health [10].

The method of Vicamdetermination of aflatoxins B_1, and M_1, a quantitative and repeatable values that speak to the accuracy of the method and can be applied to monitoring of aflatoxins in animal feed and in all milk and dairy products [5].

In Taiwan made tests for the presence of aflatoxin M_1 in milk taken from a commercial milk network throughout 18 hours, where the measurements after one hour the concentration amounted aflatoksins M_1 (75 ppb), amounted to 6 hours (243 ppb), and na18 hours amounted to (532 ppb). This indicates that the cattle from which samples are taken of milk was fed with food contaminated with aflatoxinB1 [8].

Also speaking tests made in Iran contamination of aflatoxins in milk and dairy products marketed in the trade network where the 47 examined samples of raw milk, 41 were positive concentration of aflatoxins M_1 (98,9 ppb), in 40 samples of yogurt from which 22 samples were positive with a concentration (20, 6 ppb), in 35 samples cream of which 35 were positive which amounted concentration (20,3 ppb) and in 45 samples of cheese examined 38 were positive with a concentration of (23,2 ppb) [9].

A team of scientists from Turkey investigated the presence of aflatoksins M_1 in milk and dairy products marketed in the trade network from 7 different companies throughout the year and in the first season (March, April, May), the minimum concentrations of aflatoksins M_1 amounted (0,193 ppb), and maximum (0,535 ppb), the second season (June, July, August) minimum (0,000 ppb), maximum (0,324 ppb), the third season (September, October, November) was the minimum (0,262 ppb), most (0,705 ppb), and the fourth season (December, January, February) was the minimum (0,350 ppb), and maximum (0,372 ppb) [10].

The study made in Turkey in the UHT milk showed that 47% of samples exceeded the allowed limit of the EU. The average value found was 0,108 g/l (Unusan, 2006). We must also take into account the data taken from the EU before introduction the regulation that sets 0,05 g/l as a limit in 2003 [12].

In the north ofItaly in the period of 2003 surprisingly high concentration of aflatoxin B_1 infood for feeding milk cows were found resulted in higher concentration ofaflatoxin M_1 in milk (EFSA, 2004) [14]. As a result of the situation in the country,strategy of surveillance and control was erected which is already near timeframe and so far has given towards the good results reduction of the percentageof contaminated samples of food and milk (Decastelliet al., 2006) [11].

From 11,831 processed samples from the territory of the European Union, 280 were from individual small farms, the concentration of aflatoxin M_1 in all the samples greater than 0,05 g/l detected in only 0,06% of samples, which indicates that the incidence of this mycotoxin are extremely low (EFSA, 2004) [13]. Countries in the region that are not members of the EU, which implemented some kind of monitoring, have higher values of concentration aflatoxin M_1 in milk and milk products then the member countries. In the study taken in Albania on samples of milk from individual farms, 42% of samples had lower value ten 0,05 g/l, while 58% of samples had higher concentration of aflatoxin M_1 [7].

The study of Mary et al. (2011) been done examining the degree of contamination with aflatoxins in animal feed in Vojvodina, during all seasons over a year and that samples taken from 4 different farms (n = 35), and determining the presence and quantity of aflatoksin B_1 (AB1), where the most common were the genus Asspergilus(12) species despite ohratoksin A (O TA) and zearalenon (ZEA) in these foods [11].

Prevention of contamination of molds, both during fattening, and the period of storage of feed is of utmost importance not to appear aflatoxinM_1 in milk, but the method for decontamination and detoxification have very little [14].

CONCLUSIONS

For assessment the quality of milk is important to determine the amount of fat in the milk, it is made fat in each sample before examination to determine the levels of aflatoxins M_1. Determination concentration of each sample of milk and dairy products by theGerbermethod before determine the amount of aflatoxins M_1, which varies within the limits of normal 2,9 to 3,2 %. From results we conclude that all milk samples satisfy the % representation in fat, that is good quality milk.

Examination the accuracy of the method Vicam for determination of aflatoxin M_1 in milk andin dairy products by the method of standard addition to certifying its validation and can be used in laboratory for routine analyzes.

In determining the presence and detection of aflatoxin M_1 in milk and dairy products in samples from Pelagonia obtained values ranging within the limits.

Determination of aflatoxin B_1 in animal feed is of great importance for the health of the animals from which milk is obtained and the people who are fed with milk and dairy products. Of course if you reduce the amount of B_1 will be reduced and the amount of M_1 in milk. Must not allow the presence of aflatoxins B_1, and that means you need to reduce the use of chemical plant protection and to increase the yield of cereals which could lead to an increase in the presence of toxic substances, aflatoxins. Therefore it is necessary occasionally making monitoring and determination of aflatoxins in feed, milk and dairy products for the prevention and protection of human and animal health.

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Naučni rad

ISPITIVANJA AFLATOXINA U MLEKA I TAČNOST METODA

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Izvod

U ovom radu utvrdili smo tačnosti Vicam metoda za određivanje aflatoksina B_1 u hrani i aflatoksina M_1 u mleku i mlečnim proizvodima primenom metode standardnih dodatka. Analitički prinos u svim uzrocima kretao se od 93 do 95%, to ukazuje na visoku tačnost metode. Određivanje je napravljeno na aflatoksin B_1 u 20 uzorka kukuruza i adekvatno u mleku i mlečnim proizvodima uzetih iz grla koji su hranjeni sa istim kukuruzom. Uzroci su uzeti iz male farme Pelagonia region. Rezultati su bili u granicama za normalni kukuruz i poklapa se sa referentnim vrednostima date Saglasnost za akreditaciju laboratorija u našoj zemlji, a za mleko nije dovoljno istraživana oblas tdobili smo rezultate koje su u skladusa Pravilnika Evropske Unije, prisutnost na aflatoxina M_1 do 0,05 ppb.

Zaključujemo da je važno određivanje aflatoksina B_1, njihovo prisustvo u hrani za životinje da bi se izbeglo prisustvo aflatoksina M_1u mleku i mlečnim proizvodima, a to svakako zavisi od sezone kada se kukuruz koristi u ishrani, od vremena, temperature skladištenja, itd.

***Ključne reči**: mikotoksini, aflatoksin B_1, aflatoksin M_1, Vicam metod.*