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Comparison of two analytical methods (ELISA and LC-MS/MS) for determination of aflatoxin B_1 in corn and aflatoxin M_1 in milk

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Abstract

The aim of this paper is to assess the closeness of agreement between results of ELISA and LC-MS/MS methods for determination of aflatoxin B_1 in corn and aflatoxin M_1 in milk. Samples of corn (n=100) and milk (n=250) were simultaneously analyzed using ELISA and LC-MS/MS methods, after the severe drought that affected Serbia in summer 2012 resulting in occurrence of aflatoxin B1 in corn and aflatoxin M_1 in milk. Regression analysis showed higher level of agreement between aflatoxin B_1 samples (R2=0.994), compared to aflatoxin M_1 samples (r^2=0.920). However, both techniques were satisfactory in meeting the requirements for official control purposes.

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Keywords: aflatoxin B1; aflatoxin M1; corn; milk; ELISA; LC-MS/MS

1. Introduction

Aflatoxins (Af) are secondary metabolites of molds belonging to the *Aspergillus* genera[1]. Climate conditions are the primary factor determining the development of the mold on the fields, and consequent occurrence of these

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compounds in crops. Dry and warm climate facilitates the process of fungal growth and aflatoxins production. Aflatoxin B_1 (AfB_1) is the most significant mycotoxin, being both the most abundant and related to the severe health implications. AfB_1 is highly carcinogenic and mutagenic, while its hepatocarcinogenic metabolite - aflatoxin M_1 (AfM_1) is obtained by hydroxylation of AfB_1 in liver, and is excreted through milk of the animals that consumed feed contaminated with AfB_1. According to Creppy[2], carcinogenicity of AfM_1 is only 2-10% of the AfB_1. Nevertheless, International agency for research and cancer (IARC), included both compounds into the group I of human carcinogens[3].

Having in mind the ubiquity and health hazards of both AfB_1 and AfM_1, almost every country in the world established tolerance limits for both toxins[4], which imposed the need for development and validation of numerous analytical methods utilizing a number of techniques. Due to its simplicity, high-throughoutput capability and very low limits of quantification, ELISA has become the screening technique of choice for determination of mycotoxins in general, especially in situations where large number of samples is to be analyzed in a short period of time[5]. However, one of the major drawbacks of immunoassays is the lack of information on the structure of the analyte. This restricts the technique to screening purposes only. On the other hand, liquid chromatography coupled to triple-quadrupole mass spectrometry operating in modes capable of monitoring both molecular ions and their fragments, which are formed after collision with molecules of noble gases such as Ar_2 (LC-MS/MS in MRM or SIR mode), is the confirmatory technique providing structural information of the analyte and its unequivocal identification. The drawbacks of LC-MS/MS technique are high costs of analysis and limited availability of laboratories having the technical and human resources to perform such measuring.

A severe draught in summer of 2012 affected the countries of the southeastern Europe and Balkan Peninsula including Serbia[6], resulting in high levels of AfB_1 recorded in corn, especially in Serbia which is the major corn producer in the region[7]. Due to the severity of the corn contamination, elevated concentrations of AfM_1 were found in milk countrywide. A very high number of corn and milk was analyzed for the presence of AfB_1 and AfM_1 in order to assess the intensity and distribution of these compounds throughout the country and to undertake necessary steps in order to control the crisis adequately. Having the access to a significantly large number of contaminated corn and milk samples, we decided to compare closeness of agreement between results of AfB_1 and AfM_1 determination in corn and milk respectively, using ELISA and LC-MS/MS analytical techniques.

2. Materials and methods

Corn and milk were sampled between November 2012 and March 2013. Corn samples were taken from siloes, feed-production facilities and dairy farms. Milk samples were taken at major and minor dairies and from the retail. Samples were taken for the purposes of official controls by the veterinary inspectors as well as for the self-monitoring purposes of the feed and dairy producers. Total of 680 corn samples and 6,625 milk samples were taken during the sampling period and analyzed using ELISA technique. Of that number, 100 corn samples and 250 milk samples were randomly chosen to be simultaneously analyzed using LC-MS/MS technique for the purposes of comparing the analytical techniques performances.

2.1. ELISA

Determination of AfB_1 in corn was conducted using "Celer AFLA B_1" ELISA kit (Tecna S.r.l., Italy). Sample preparation was carried-out according to the instructions from the manufacturer. Optical density was measured using ELISA-reader Thermo Scientific (Waltham, MA, SAD), model 364, at wavelenght of 450 nm. Ascent softwate (v. 1.0) was used for data aquisition and processing. Detection limit of the method was 1 μg/kg, specificity was 100%, 5%, 19% and 1% for AfB_1, AfB_2, AfG_1 and AfG_2 respectively. Relative standard deviation of reproducibility was 3%. Recovery was 91%.

Determination of AfM_1 in milk was conducted using "Aflatoxin M_1" ELISA kit (Tecna S.r.l., Italy). Sample preparation was carried-out according to the instructions from the manufacturer. Optical density was measured using ELISA-reader Thermo Scientific (Waltham, MA, SAD), model 364, at wavelenght of 450 nm. Ascent softwate (v. 1.0) was used for data aquisition and processing. Detection limit of the method was 0.005 μg/kg, specificity was 100%, and 16% for the AfM_1 and AfM_2 respectively. Relative standard deviation of reproducibility was 6%.

Recovery was 110%.

2.2. LC-MS/MS

LC-MS/MS analysis of AfB_1 was carried out according to the method published by Sulyok, Krska and Schuhmacher[8]. The instrument was Waters Acquity UPLC system (Waters, Milford, MA, USA) coupled by TQD mass spectrometer (Waters Micromass, Manchester, UK). Purospher Star (Merck, Darmstadt, Germany) RP-18 column (50x2.1 mm, 2 μm particle size) was used for the separation of AfB_1. Mobile phase was 0.1% acetic acid and methanol (60:40). Isocratic flow was maintained at 0.25 mL/min.

The instrument operated in positive elctrospray ionisation mode and multiple reaction monitoring (MRM) mode of the quadrupoles. Three product ions were monitored (313.1>285.2 Da; 313.1>270.1 Da and 313.1> 241.1 Da). Quantification ion was 285.2 Da. MassLynx 4.1 software was employed for data aquisition and processing. Detection limit of the method was 0.5 μg/kg, Relative standard deviation of reproducibility was 4%. Recovery was 86-92%.

LC-MS/MS analysis of AfM_1 was carried out according to the method published by Sørensen i Elbæk[9]. The same instrument and column was used. Mobile phase was 0.1% acetic acid and methanol (35:65). Isocratic flow was maintained at 0.3 mL/min. Two product ions were monitored (329>273 Da and 329>259.1 Da). Quantification ion was 273 Da. MassLynx 4.1 software was employed for data aquisition and processing. Detection limit of the method was 0.02 μg/kg, Relative standard deviation of reproducibility was 5.4%. Recovery was 65-81%.

Linear regression analysis was performed using JMP v.10 software.

3. Results and discussion

Figure 1 shows regression correlation curve for the results of AfB_1 (a) and AfM_1 (b) concentrations in μg/kg for 100 corn samples and 250 milk samples analyzed by ELISA and LC-MS/MS method.

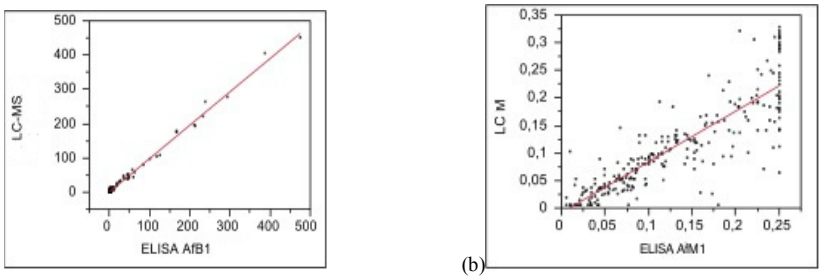

Fig. 1. Correlation curves for AfB_1 in corn and AfM_1 in milk analyzed using ELISA and LC-MS/MS analytical techniques.

Equations of the regression curves and coeffitients of determination are as follows:

AfB_1: $y = 0.9749x + 0.214$ ($r^2 = 0.994$)
AfM_1: $y = 0.9201x - 0.0082$ ($r^2 = 0.920$)

From the graphs and the equations, it can be seen that the higher closeness of agreement between results from ELISA and LC-MS/MS are obtained in the case of AfB_1 comparing to AfM_1. This can be explained considering that AfB_1 concentrations are 200 – 1000 times lower than measured AfM_1 concentrations and that the higher degree of scattering is expected in such low concentration range. From the other hand, number of analyzed samples of milk is 2.5 times higher, which also to some extent, contributes to lower coefficient of determination for AfM_1.

From the dispersion pattern in the graph showing AfM_1, it can be concluded that the deviations between ELISA and LC-MS/MS methods are higher as the measured values are higher which would imply the amount of heteroscedasticity, that can be confirmed using analysis of residuals or employing e.g. Park test. However, this is not

the consequence of invalidated regression analysis or the deficiencies in used data. Closer look at the data suggests that ELISA generally underestimates the concentration values of AfM_1 when these values approach to 0.2 μg/kg and higher. This anomaly can be explained by the fact that much of the analysed milk samples have AfM_1 concentrations that exceed the limit of ELISA linearity range (up to 0.25 μg/kg) which can also be observed in the form of vertical dots forming a straight line at the far end of the graph. In these cases, results obtained using LC-MS/MS technique are more accurate due to much wider dymanic range of the instrument.

4. Conclusions

On the basis of presented results, it can be concluded that the closeness of agreement between concentration values of AfB_1 in corn and AfM_1 in milk using ELISA and LC-MS/MS is high, and that both techniques can be used for control or regulatory purposes, having in mind their inherent advantages and limitations.

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References

1. Milicevic D, Nedeljkovic-Trailovic J, Masic Z. Mikotoksini u lancu ishrane – analiza rizika i znacaj za javno zdravstvo. *Tehn mesa* 2014;**55(1)**:22-38.
2. Creppy EE. Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicology Letters* 2002;**127**:19-28.
3. IARC, 2002. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. IARC Monogr. Eval .Carcinog. Risk Hum, Lyon, France, pp. 1–556.
4. FAO, 2004. Worldwide regulations for mycotoxins in food and feed in 2003. Rome, FAO.
5. Stroka J, Anklam E. New strategies for the screening and determination of aflatoxins and the detection of aflatoxin-producing moulds in food and feed. *TrAC* 2002;**21(2)**:90–5.
6. RHMZ, 2013. Republicki hidrometeoroloski Zavod, 2012. Sezonski bilten za 2012. godinu. Nacionalni centar za klimatske promene. available at <www.hidmet.gov.rs>.
7. FAO. 1996. Basic facts on the world cereal situation. Food Outlook, 5/6. Rome.
8. Sulyok M, Berthiller F, Krska R, Schuhmacher R. Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. *Rapid Communications in Mass Spectrometry* 2006;**20(18)**:2649–59.
9. Sørensen LK, Elbæk TH. Determination of mycotoxins in bovine milk by liquid chromatography tandem mass spectrometry. *J Chrom* B 2005;**820**:183–96.