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INTERLABORATORY COMPARISON FOR DETERMINATION OF OCHRATOXIN A BY ELISA IN MAIZE (Running title: DETERMINATION OF OCHRATOXIN A IN MAIZE)

ABSTRACT: Participation in interlaboratory comparison and proficiency testing schemes is important for laboratories to control the work quality. In this study, a sample of naturally contaminated maize was analyzed for the content of ochratoxin A (OTA) in three laboratories in Serbia. Participating laboratories used enzymatic immunoaffinity method (ELISA) for the determination of OTA and selection of the ELISA kit was free. Between-laboratory precision was acceptable as evidenced by Cochran's C test. Moreover, z-scores for all three laboratories were $z < \pm 2$, which is considered acceptable. Used OTA confirmation methods were thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC), with fluorescence detector. The results of different methods were comparable.

KEY WORDS: confirmation methods, ELISA, interlaboratory comparison, maize, ochratoxin A

INTRODUCTION

The primary aim of proficiency testing (PT) was to provide quality assurance for laboratories and demonstration of competence to an accreditation body by comparing their results with similar laboratories (ISO/IEC 17043, 2010; Santovac et al., 2010). An improvement and maintenance of quality in the laboratory can also be achieved by regular participation in interlabora-

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tory comparisons (ILC) (EA-4/18 TA, 2010). They are also useful tools for demonstrating the competence of laboratories, similar to PT in accreditation procedures. Both PT and ILC should be carefully and competently planned, prepared, carried out, interpreted and documented (ISO/IEC 17043, 2010).

It is also important that the cost-effective aspects and fitness-for-purpose of the use of PT were taken into account. Thus, in some cases it is useful to participate in ILC. ILC definition**:** "Organization, performance and evaluation of test on the same or similar test items by two or more laboratories in accordance with pre-determined conditions. Note – In some circumstances, one of the laboratories involved in the intercomparison may be the laboratory, which provided the assigned value for the test item" (ISO/IEC 17043, 2010). ILC can be designed for purposes other than PT: a) the validation of methods (for determining performance characteristics such as reproducibility, comparability, confidence intervals under comparable conditions, limiting values or robustness, measurement uncertainty etc.; b) the characterization of reference materials (to assign the certified value and estimate uncertainty of this value); c) self-assessment of a laboratory's performance in a test. ILC is useful and cost-effective external quality control in the following cases: due to changes of personnel; for the test methods to another matrix; for the extension of the scope of accreditation; for documented in-house methods; if laboratory use some procedural steps deviating from the standard methods; if the results of the PTs are unsatisfactory and corrective actions are necessary; if assistance in detecting systematic errors in the laboratory is required; and if the laboratory has no other means to provide evidence of its technical competence and quality of measurement (ILAC-G22, 2004).

Test materials used in ILC should be of appropriate quality. Sample must be carefully selected and prepared. It is very important that all laboratories get a homogeneous and stable test sample (ISO Guide 35, 2006).

If the laboratory did not have satisfactory results in the PT or in case of critical results, it should check and improve its work and implement any necessary corrective actions. The accreditation procedures defined for such cases should be followed (ILAC-G22, 2004; ISO/IEC 17043, 2010).

Ochratoxin A (OTA) is a mycotoxin produced by *Aspergillus* spp. and *Penicillium* spp.; it can be found in cereal grains and other food. OTA is possibly carcinogenic to humans (Group 2B) (WHO, 1997), and therefore, its confident and accurate determination and detection is important. This paper shows an example of organization of an ILC for mycotoxin determination and the obtained results were discussed.

MATERIAL AND METHODS

In this study, organizer-lab used maize sample, which was previously proved to contain a significant amount of *Penicillium* molds and ochratoxin A. The 1 kg sample was roughly grinded and homogenized and divided into parts.

An interlaboratory comparison involved three participating laboratories, codes labeled as Lh0, Lh1 and Lh2. The organizer-lab delivered 150 g of maize

sample to the participating laboratories. Participating laboratories applied enzymatic immunoaffinity method (ELISA) for the determination of OTA and ELISA kits from different producers were used: R-Biopharm AG, Romer Labs® and Tecna S.r.l.

For a thin-layer chromatographic method of maize analysis, extraction was done with acetonitrile–water. Sodium bicarbonate was added to separate the acidic OTA. After 1 mol/ dm^3 hydrochloric acid addition and chloroform extraction, reconstituted sample was spotted on TLC plate next to the standard, and then it was examined under ultraviolet light (B a l z e r et al., 1978).

The same sample was analyzed by the HPLC method after extraction with chloroform and 0.1 mol/dm phosphoric acid, filtration, evaporating and degreasing (S o l f r i z z o et al., 1998). The equipment consisted of an LC system – BioRad 2800 with Supelcosil™ LC-18-DB column (250 x 4.6 mm id, particle size 5 μm) with a fluorescence detector Hewlett Packard 1046A. Wavelength of excitation radiation was 330 nm and emission 460 nm. A mobile phase consisted of a mixture of acetonitrile–water–acetic acid (50:50:1), at a flow–rate of 1 ml/min. Chromatographic data were collected and processed using ValueChrom® Chromatography Software (Bio-Rad, USA). Calibration curve was constructed on the basis of the area under the chromatographic peak using five OTA working standard solutions. The linearity of the method was assessed by the standard, ranging from 0.3 to 3.0 μg/ml (Fig. 1B). Recovery of the method was determined using blank maize sample spiked with 1000 μg/kg.

RESULTS AND DISCUSSION

OTA is a mycotoxin undesirable in cereals. Nowadays, most laboratories apply ELISA method for the determination of OTA. Although this method has a number of advantages, it is not considered as standard method. In some cases, false positive results are possible and this is why, standard methods for confirmation are advisable (A n k l a m et al., 2002).

Lab-organizer prepared maize sample naturally contaminated with OTA for this study. In this sample, OTA was determined and confirmed by two standard methods before interlaboratory comparison.

Although TLC used in this study is a standard method (B a l z e r et al., 1978), fluorescence of OTA spots on thin layer plates was assessed visually, and thus, only semi quantitative results were obtained. After comparing the intensity of sample spots with a series of standard solution spots and taking into account the sample dilution, the obtained OTA content in the sample was 900 μg/kg.

Since the sample preparation for the applied HPLC method included liquid-liquid extraction instead of solid phase extraction, the recovery study using spiked maize sample was carried out (Fig. 1C). Recovery achieved by this method was very poor, only 42%, which was not enough for quantitative standard method, according to the regulations (EC, 2006). However, having

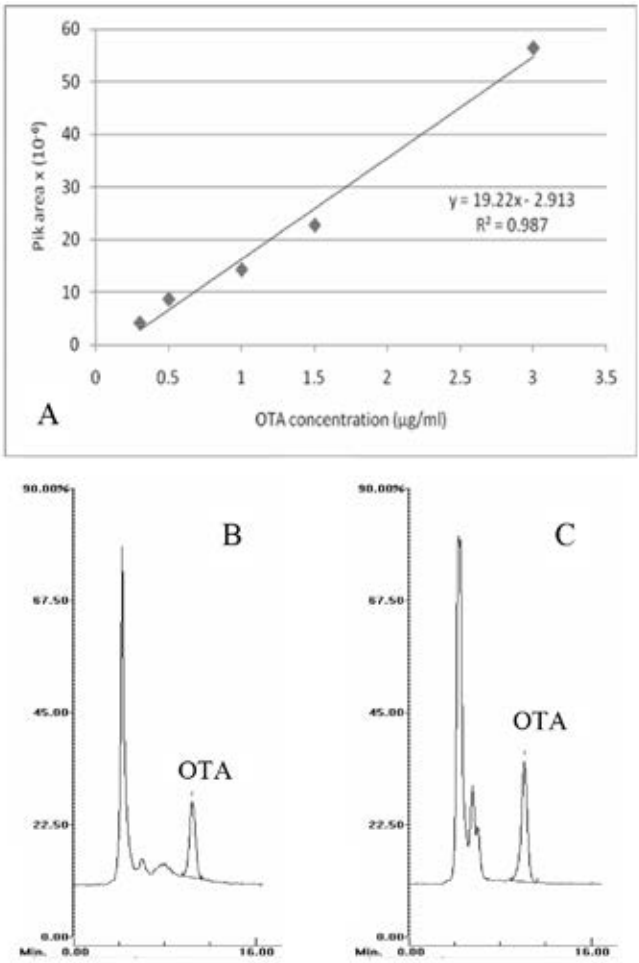

Fig. 1 – A Calibration curve for OTA determination by HPLC method;
B Naturally contaminated maize sample; C Blank maize sample spiked with 1000 µg/kg.

in mind this recovery, OTA content determined by HPLC in the maize sample (Fig. 1B) was 660 µg/kg.

Qualitative OTA confirmation included yellow florescence of OTA spots, i.e. blue fluorescence of OTA spots after treatment with ammonia vapors. Other qualitative evidence was retention time of OTA peak in the maize sample on HPLC chromatogram, which matched the peak of OTA standard. After this semi quantitative and qualitative confirmation of OTA in the maize sample, it was chosen for interlaboratory study. Laboratories that were participating in this interlaboratory comparison submitted the test results to the organizer-lab in predefined time. Since the number of tests in all series was the same, the estimation of inconsistent variance values was performed using

the Cochran´s C test (ISO 5725-2, 2002; Atanasijević et al., 1994). Furthermore, since the calculated Cochran´s coefficient was lower than critical value for comparing more than two series (0.55 < 1.44; Hadživuković, 1973), all variances were equal. Subsequently, the results were evaluated by calculating the deviation of the results, obtained in each particular laboratory, from the prescribed value. The prescribed value was determined based on a consensus-value of participating laboratories. The results were classified according to the recommendations of international norms (ISO/IEC 17043, 2010; ISO 13528, 2005) and are expressed as z–scores (Table 1). The expanded measurement uncertainty (k = 2) calculated from the standard deviation of bias based on proficiency testing was 19 µg/kg. The maize sample used in this study could then be used for interlaboratory internal review since it received consensual value and measurement uncertainty in described intralaboratory check (ISO Guide 35, 2006).

Figure 2 shows the comparison between ELISA and standard methods. It can be concluded that ELISA tests gave somewhat better results in OTA determination in comparison to TLC and HPLC.

Tab. 1 – Results of interlaboratory comparison and z–score of participating laboratories

Laboratory	Lh0	Lh1	Lh2
Results ± SD (µg/kg)	1073 ± 256	1039 ± 289	1082 ± 56
Attributed consensual value	1065 ± 23		
CV (%)	23.9	27.8	5.18
X max (µg/kg)	1250	1370	1089
X min (µg/kg)	780	837	1023
N	3	3	3
z–score	**+0.35**	**-1.13**	**+0.74**

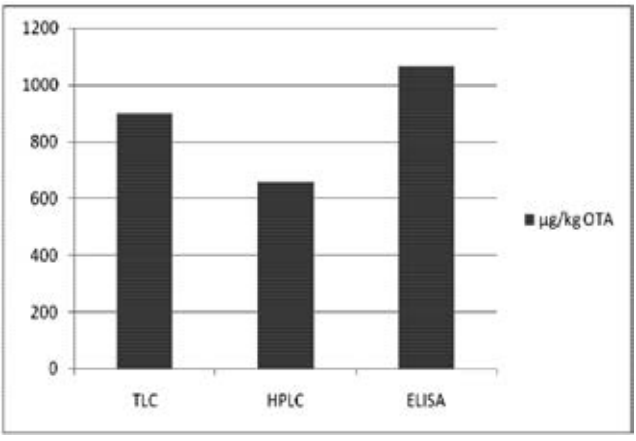

Fig. 2 – Content of OTA in naturally contaminated maize sample: interlaboratory ELISA result and standard methods results

Maximum permitted amount of OTA in animal feed was in the range of 0.1 to 1 mg/kg, depending on the types and categories of animals (Sl. Glasnik RS, 2010). These values were far outside the range of calibration ELISA and required multiple dilution of the sample. Our paper shows the possibility of using ELISA method in the case of highly contaminated samples at concentrations relevant to poultry feeding.

CONCLUSION

Although PT schemes organized by accredited providers are required for accredited laboratories, in some cases, when PT is not available, it is very useful for laboratories to participate in ILC. It is cheaper, faster and easier way to control the laboratory quality. In the comparison described in this paper, laboratories seized the opportunity to check their methods for determination of higher OTA concentration. In this way, the robustness of the methods was verified. By processing the results of tests for OTA content, and analysis of z–values for all three laboratories, it was concluded that $z <\pm 2$ was the acceptable result.

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МЕЂУЛАБОРАТОРИЈСКО ПОРЕЂЕЊЕ РЕЗУЛТАТА ОДРЕЂИВАЊА ОХРАТОКСИНА А ELISA МЕТОДОМ У КУКУРУЗУ

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Резиме

Учешће у међулабораторијским поређењима и шемама за испитивање оспособљености је важно за контролу квалитета рада лабораторије. У овом раду су дати резултати одређивања охратоксина А (ОТА) у природно контаминираном узорку кукуруза од стране три лабораторије у Србији. Лабораторије учеснице су за одређивање ОТА користиле ензимску имуноафинитетну методу (ELISA), а избор ELISA кита је био слободан. Међулабораторијска прецизност је била задовољавајућа, што је доказано Кохрановим критеријумом. Такође, анализом z-вредности је код све три лабораторије добијено $z < \pm 2$, што представља прихватљиве резултате. У истом узорку је ОТА одређен и стандардним методама – танкослојном (TLC) и течном хроматографијом под високим притиском (HPLC) са флуоресцентним детектором. Резултати добијени ELISA, TLC и HPLC методама су били упоредиви.

КЉУЧНЕ РЕЧИ: међулабораторијско поређење, ELISA, охратоксин А, кукуруз, стандардне методе

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