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Ksenija D. NEŠIĆ[1], Boris P. PISINOV[1], Sandra M. JAKŠIĆ[2], Aleksandra M. TASIĆ[1], Božidar M. SAVIĆ[1], Nikola J. PAVLOVIĆ[1]*

[1] Institute of Veterinary Medicine of Serbia
Autoput 3, Belgrade 11070, Republic of Serbia
[2] Scientific Veterinary Institute "Novi Sad"
Rumenački put 20, Novi Sad 21000, Republic of Serbia

COMPARISON OF *ELISA* AND *HPLC* METHODS FOR THE DETECTION OF MYCOTOXINS BY ANALYSING PROFICIENCY TEST RESULTS

ABSTRACT: Different analytical techniques for the detection of mycotoxins have been developed in order to control the levels of mycotoxins in food and feed. Conventional analytical methods for mycotoxin determination are involving techniques such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC). Also, rapid methods for mycotoxin analysis have become increasingly important. Enzyme-linked immuno-sorbent assay (ELISA) is one of the most common rapid methods for determination of these natural contaminants. The aim of this study was to provide a comparison between two different methods of analysis (HPLC and ELISA) for the detection of different mycotoxins using data that originate from commercial proficiency tests. Based on the statistical evaluation of the results for both methods, in three proficiency tests for various mycotoxins (aflatoxins, ochratoxin and zearalenone), it could be concluded that both techniques can equally be used, although ELISA is considered to be the screening one.

KEYWORDS: ELISA, HPLC, food and feed, mycotoxins

INTRODUCTION

Mycotoxins are a major analytical challenge due to the range of chemical compounds that they represent and the vast array of feed matrices in which they are found. Analysis is essential for determining the extent of mycotoxin contamination, for risk analysis, confirming the diagnosis of a mycotoxicosis and for monitoring mycotoxin mitigation strategies (Nesic *et al*. 2013). In order to control the levels of these natural contaminants in food and feed, different analytical tools for their detection have been developed. The most common

* Corresponding author. E-mail: ksenija_n@yahoo.com

are chromatographic techniques, but also rapid immunochemical methods have become increasingly important.

Chromatographic techniques are based on the physical interaction between a mobile phase and a stationary phase. The components to be separated are distributed between these two phases. The mobile phase is usually a fluid that penetrates through or along the stationary bed (liquid or solid). Liquid, gas and supercritical fluids are currently used as mobile phase and chromatographic techniques derive their names from the nature of the mobile phase: liquid chromatography, gas chromatography and supercritical fluid chromatography, respectively. In practice, the sample to be analyzed is dissolved in the mobile phase and applied as a spot on the stationary phase. The analyte or sample is carried along by the mobile phase and partitions between the solid and liquid stationary phase are called the sorbent. The various constituents in the analytes travel at different speeds resulting in differential partitioning of the constituents between the mobile and the stationary phases. The most commonly used chromatography techniques for analysis of mycotoxins are thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC). Although many of the chromatographic techniques are very sensitive, they require trained skilled technician, cumbersome pretreatment of sample and expensive apparatus/equipment (Wacoo *et al.* 2014).

High-performance liquid chromatography (HPLC) is the most popular chromatographic technique for separation and determination of organic compounds. It is basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster. All chromatographic separations, including HPLC, operate under the same basic principle – separation of a sample into its constituent parts because of the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation. HPLC instrumentation includes a pump, an injector, a column, a detector and an integrator or an acquisition and a display system. The heart of the system is the column where separation occurs (Brown and DeAntonois, 1997).

High-performance liquid chromatography provides fast and accurate mycotoxin detection results within a short time. A sensitivity of detection as low as 0.1 ng/kg has been reported. However, the disadvantage of using HPLC for this purpose is the requirement of rigorous sample purification using immunoaffinity columns. In addition, HPLC requires tedious pre- and post column derivatization processes to improve the detection limits. Therefore, to overcome the challenges associated with derivatization processes in mycotoxin analysis, a modification of the HPLC method, whereby the HPLC is coupled to mass spectroscopy, has been made and is currently employed in the determination of mycotoxins (Wacoo *et al.* 2014). Since the mass spectrometer requires neither use of UV fluorescence nor the absorbance of an analyte, the need for chemical derivatization of compounds is eliminated. The HPLC-MS/MS uses small amounts of sample to generate structural information and exhibits low detection limits (Rahmani *et al*. 2009). However, HPLC-MS/MS is bulky and very expensive equipment which

can only be operated by trained and skilled personnel. Besides, this also limits its use to laboratory environment only and not field conditions.

Immunochemical detection methods vary from simple immunoassay to highly sophisticated immunosensors, but they all rely on the specificity of binding between antibodies and antigens. The immunochemical reaction, i.e, the binding of antibody and antigen, in an assay is not visible and therefore several means to detect the reaction product, the immune complex, have been developed based on signal-generating components and appropriate measuring devices. The various immunoassays are named based on the signal-generating component or tracer: Radioimmunoassay (RIA), Enzyme-Linked Immunosorbent assay (ELISA), Chemiluminenscent Immunoassay (CL-IA), Fluorescent Immunoassay (FIA), Lateral Flow Immunoassay (LF-IA) and immunosensors (Meulenberg, 2012). The popularity of the antibody-antigen based techniques, since their development in the late 1970s, is due to their high level of specificity and sensitivity even in the presence of contaminating materials. Besides, immunochemical methods do not require skilled and highly trained personnel to troubleshoot in case of any problems during separation; they are less labor intensive and consume less time, in which respects they are preferable to both chromatographic and spectrophotometric techniques.

ELISA test kits are well favored as high through-put assays with low sample volume requirements and often less sample clean-up procedures compared to conventional methods such as TLC and HPLC. They are rapid, simple, specific, sensitive and have become the most common quick methods for the detection of mycotoxins in foods and feeds. However, although the antibodies have the advantage of high specificity and sensitivity, because the target compounds are mycotoxins but not the antigens, compounds with similar chemical groups would also interact with the antibodies. This so-called matrix effect or matrix interference commonly occurs in ELISA methods, which can give rise to underestimates or overestimates in mycotoxin concentrations in commodity samples. Additionally, insufficient validation in ELISA methods causes the methods to be limited in the range of matrices examined. Therefore, an extensive study on the accuracy and precision of the ELISA method over a wide range of commodities is still needed and a full validation for an ELISA method is essential and critical (Mohamadi *et al*. 2012). The conventional wisdom is that ELISA kits should be used routinely only for the analysis of matrices that are extensively tested. Confirmatory analyses by more robust methods, e.g. HPLC with IAC clean-up or LC-MS, are required for the contamination levels that approach the legal limit (Pascale, 2009).

When monitoring for the presence of mycotoxins either in raw and derived agricultural products, there is a large choice of methods. Depending on the purpose, either rapid detection or validation according to the regulations, one can use quantitative and qualitative methods. Among the available conventional methods, HPLC has been traditionally applied, and among the immunochemical methods, ELISA has also been used. Having all that on our mind, the aim of this paper is to provide a comparison between two most used methods for the detection of different mycotoxins (HPLC and ELISA) using data that originate from commercial proficiency tests.

MATERIAL AND METHODS

The data were obtained from the official reports of three commercial international proficiency tests (PT):

- Aflatoxin PT in 2015. The sample was naturally contaminated corn. The material was sent to 222 laboratories and the results returned from 160 laboratory (72%). The following methods were reported to be used: ELISA in 93 labs, HPLC with a variety of detection systems (FLD, MS, MS/MS) in 58 labs, fluorimeter in 1 lab, LFD (Lateral Flow Device – strips for rapid determination) in 4 laboratories and 4 laboratories did not declare the method they used.
- Zearalenone PT in 2014. The test material was naturally contaminated wheat. Sample was sent to 170 laboratories, while 124 of them (73%) sent the feedback. Among them ELISA method was used in 75 laboratories, HPLC with a variety of detection systems (FLD, MS, MS/MS) in 45 laboratories, LFD (Lateral Flow Device – strips for rapid determination) in 2 laboratories, TLC in 1 laboratory and 1 laboratory did not express the method.
- Ochratoxin PT in 2013. Sample was naturally contaminated wheat. The material was sent to 98 laboratories and the results were reported by 78 laboratories (80%), where: 30 laboratories used ELISA method and 48 laboratories used HPLC with a variety of detection systems (FLD, MS, MS/MS).

In order to compare the most used methods (HPLC and ELISA) for statistical analysis of data in all three proficiency tests, for calculations and graphs, MS Excel 2002 was applied (Schmuller, 2009), as well as Grubbs' test on-line calculator on the GraphPad portal www.graphpad.com/quickcalcs/grubbs2/ (Motulsky, 1999). For the calculation of robust parameters of data distribution free Excel add-in Robust Statistics Toolkit was used, which can be downloaded from the portal of Royal Society of Chemistry, United Kingdom, Analytical Methods Committee (AMC, 2001). Also, GraphPad Prism v4.0 for Mann-Whitney U test, which is considered a non-parametric t-test, was applied. In the analyzed PT schemes target standard deviation SD_P is calculated, depending on the target concentration, by the first of three forms of modified Horwitz equation (Thompson, 2000; FAPAS, 2016):

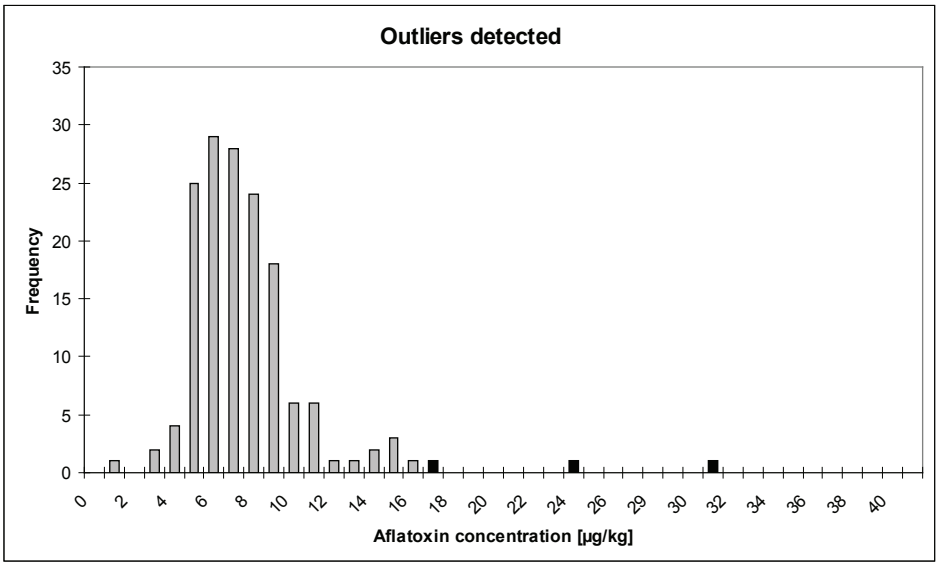
$SD_p = 0{,}22$ x assigned value If assigned value < 120 μg/kg (ppb)

RESULTS AND DISCUSSION

Aflatoxin

According to the report of the PT provider, the assigned value for total aflatoxins in the proficiency test 2015 was 6.4 μg/kg (ppb). The assigned value was calculated as the robust mean by Huber's H15 method. Target standard

deviation SD_p calculated by modified Horwitz equation was SD_p = 1.4 ppb. This means that the target accuracy was (CV%) 22%. Satisfactory results (z <2) were achieved by 78.5% of laboratories that used ELISA method and 82.8% who used HPLC.

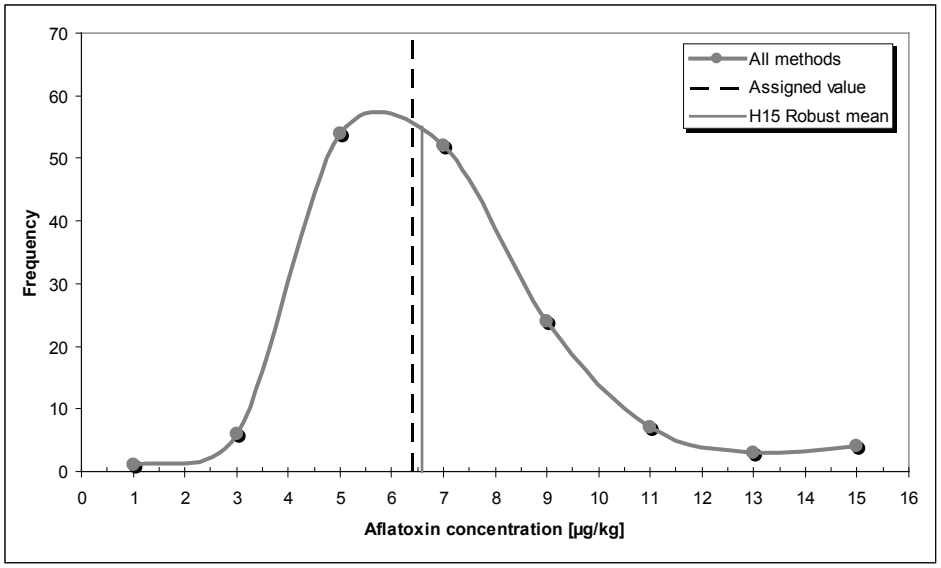


Graph 1. Outliers in the overall aflatoxin PT results

First of all, in analysis of data available in the PT report, results in non-numerical form (given as e.g. "< 2 ppb") were removed. Outliers were excluded using Grubbs' test on-line calculator. On the Graph 1, with the data sorted into the class intervals of 1 ppb, outliers are colored in black. From a total of 160 results 9 of them were excluded from further analysis.

For the rest of results it was calculated: Mean value = 6.89 ppb (higher than assigned value 6.4 ppb); SD = 2.41 ppb (higher than SD_p = 1.40 ppb); CV% = 34.9% (the accuracy of all methods, the real inter-laboratory reproducibility); Median = 6.50 ppb; MAD_e = 2.05 ppb (Median of Absolute Deviations); Huber's H15 Robust Mean = 6.59 ppb; Huber's H15 Robust SD = 2.03 ppb; Trimmed Mean 25% = 6.61 ppb; Modus = 5.92 ppb, calculated over the data sorted into class intervals width of 2 ppb.

The asymmetry of data distribution – Skewness = 1.103 (where 0 means there is no asymmetry, while a positive number means that there is a right-sided asymmetry distribution). Asymmetry is visible on the chart with the data sorted into class intervals width of 2 ppb, but we estimate that it is not too big and in with further calculations it can be considered that the data have a normal distribution.

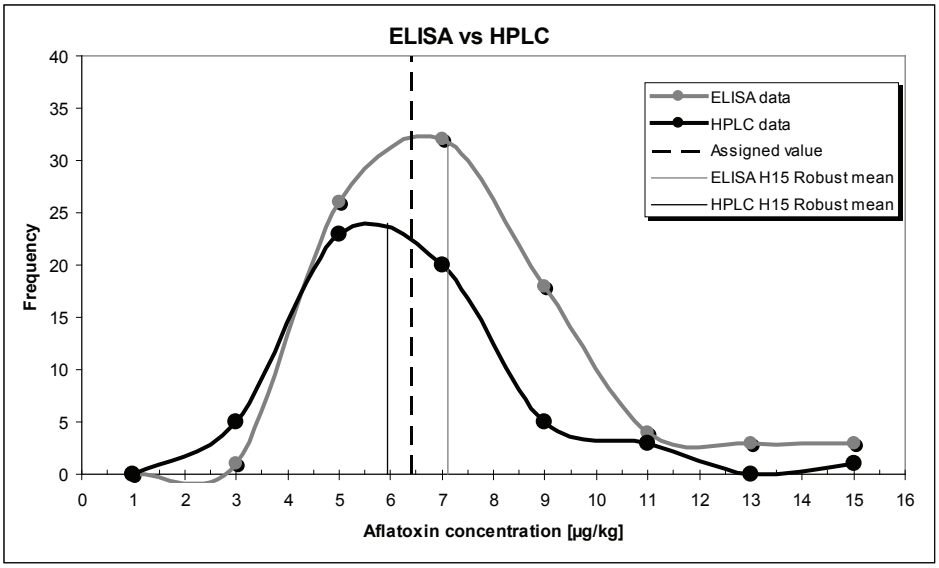


Graph 2. Frequency distribution of aflatoxin PT participants results

For each method following parameters have been separately calculated and showed in Table 1:

Table 1. Statistical parameters for ELISA and HPLC aflatoxin detection

	ELISA	HPLC
Mean [ppb]	7.47	6.23
SD [ppb]	2.38	2.22
CV%	31.8%	35.6%
Median [ppb]	7.10	6.03
MAD_e [ppb]	1.85	1.97
H15 Robust Mean [ppb]	7.11	5.94
H15 Robust SD [ppb]	1.93	1.78
Trimmed Mean 25% [ppb]	7.15	5.97
Modus [ppb]	6.60	5.71
n	87	57
Skewness	1.297 right-skewed	1.267 right-skewed



Graph 3. Visual comparation of ELISA and HPLC frequency distributions of aflatoxin results

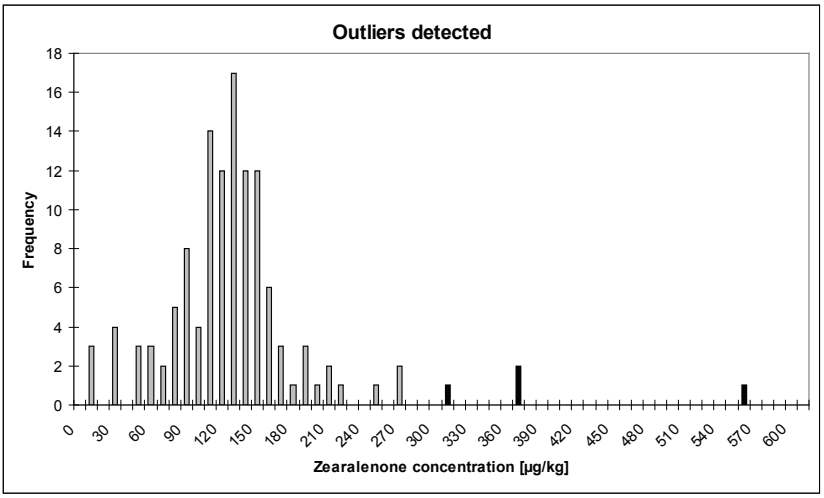
F test Two-Sample for Variances showed that there was no statistically significant difference in the variation coefficient (interlaboratory reproducibility – precision) between the ELISA and HPLC methods ($p = 0.29$).

Non-parametric (robust) Mann-Whitney U-test and parametric t-test two-tailed, equal variances, showed that there was a statistically highly significant difference between median and mean values of these two methods ($p = 0.004$ and $p = 0.002$), but as they are on opposite sides almost equally distant from the assigned value, we believe that there is no difference in accuracy between them.

Zearalenone

The PT assigned value for zearalenone was 119 µg/kg (ppb). The assigned value was calculated as the robust mean by Huber's H15 method. Calculated target standard deviation was $SD_p = 26$ ppb. This means that the target accuracy was (CV%) 22%. According to the analysis done by PT provider, satisfactory results ($z < 2$) were shown by 64.0% of the laboratory that used ELISA method and 97.8% which used HPLC.

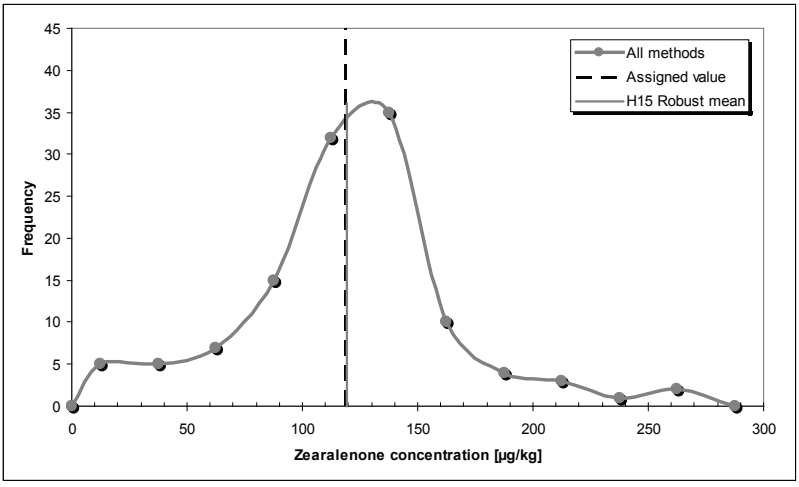
At the beginning, from the reported quantifications all non-numerical results in the form "<2 ppb" were removed. Removing outliers was done by Grubbs' test on-line calculator. On the Graph 4 data are sorted into class intervals width of 10 ppb and outliers are colored in black. Totally 5 of 124 results were removed.



Graph 4. Outliers in the overall zearalenone PT results

Other data were used to calculate: Mean = 118.4 ppb (assigned value 119 ppb); SD = 47.5 ppb (higher than SD_p = 26 ppb); CV% = 40.1% (the accuracy of all methods, the real inter-laboratory reproducibility); Median = 123 ppb; MAD_e = 32.5 ppb (Median of Absolute Deviations); Huber's H15 Robust Mean = 119.5 ppb; Huber's H15 Robust SD = 35.0 ppb; Trimmed Mean 25% = 118.8 ppb; Modus = 127.7 ppb, calculated over the data sorted into the class interval width of 25 ppb.

Asymmetry of data distribution – Skewness = 0.185 (mild right-sided asymmetry), which means that it can be considered that the data have a normal distribution.

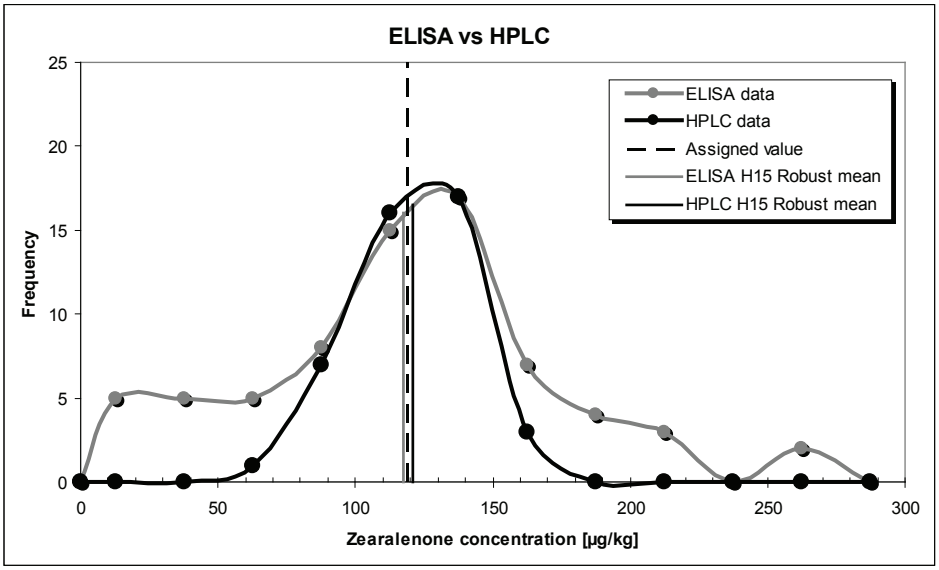


Graph 5. Frequency distribution of zearalenone PT participants results

The following parameters were calculated for each method separately and showed in Table 2:

Table 2. Statistical parameters for ELISA and HPLC zearalenone detection

	ELISA	HPLC
Mean [ppb]	116.6	119.9
SD [ppb]	56.6	21.5
CV%	48.6%	17.9%
Median [ppb]	117.0	124.6
MAD_e [ppb]	49.8	22.4
H15 Robust Mean [ppb]	117.4	120.9
H15 Robust SD [ppb]	49.5	20.2
Trimmed Mean 25% [ppb]	116.4	120.5
Modus [ppb]	129.2	126.7
n	71	44
Skewness	0.124 right-skewed	-0.234 left-skewed



Graph 6. Visual comparation of ELISA and HPLC frequency distributions of zearalenone results

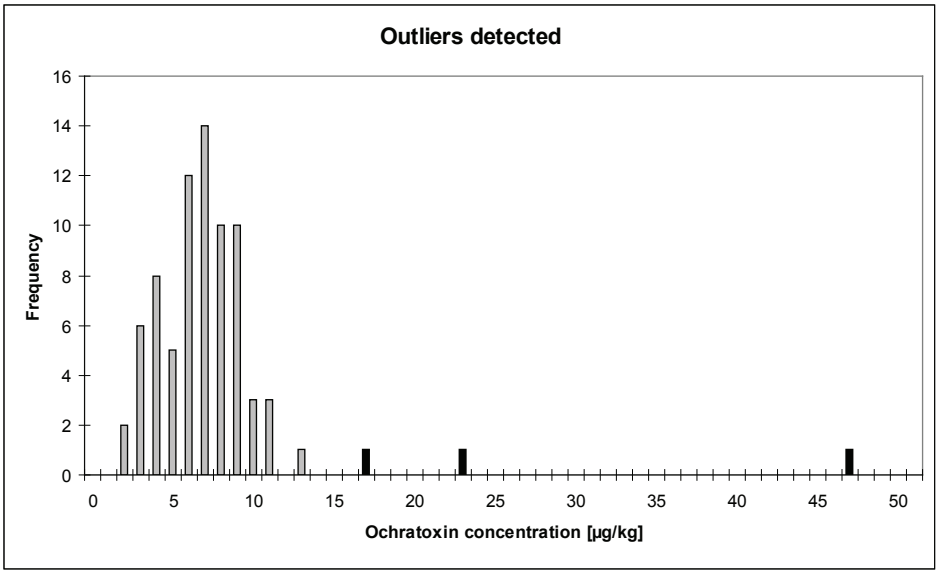
F test Two-Sample for Variances showed that there was statistically significant difference in the variation of results (in interlaboratory reproducibility – precision) between the ELISA and HPLC methods ($p = 3.4 \cdot 10^{-10}$) and that in this case, as it could be seen in the Graph 6, an ELISA method showed lower precision.

Non-parametric (robust) Mann-Whitney U-test and parametric t-test two-tailed, unequal variances, showed that there was no statistically significant difference between median and mean values of two methods ($p = 0.97$ and $p = 0.65$), which means that there was no statistically significant difference in accuracy between these two methods.

Ochratoxin

The assigned value for ochratoxin in the proficiency test 2013 was 6.8 μg/kg (ppb). It was calculated as the robust mean by Huber's H15 method and target standard deviation SD_p was $SD_p = 1.5$ ppb. This means that the target accuracy was (CV%) 22%. According to the report of the PT provider, 63.3% of the laboratory that had used ELISA demonstrated satisfactory results ($z < 2$), as well as 79.2% who had used HPLC.

Analyzing the data available in the PT report, all results in non-numerical form (given as e.g."< 2 ppb") were removed. Outliers were excluded using Grubbs' test on-line calculator. On the Graph 7, with the data sorted into the class intervals of 1 ppb, outliers are colored in black. From a total of 78 results 4 of them were removed.

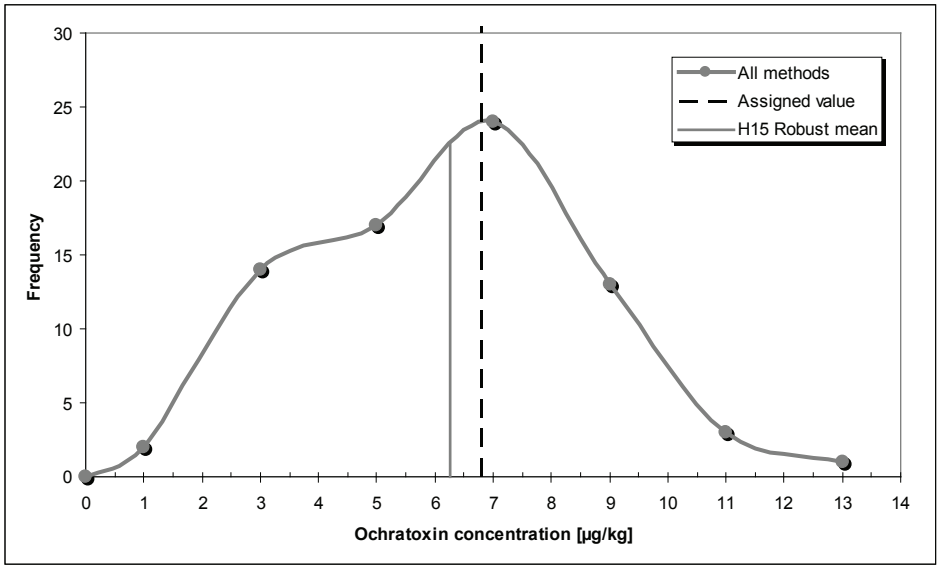


Graph 7. Outliers in the overall ochratoxin PT results

The rest of the laboratory results were used to calculate: Mean = 6.22 ppb (assigned value 6.8 ppb); SD = 2.33 ppb (higher than $SD_p = 1.5$ ppb); CV% = 37.4% (the accuracy of all methods, the real inter-laboratory reproducibility); Median

= 6.35 ppb; MAD_e = 2.21 ppb (Median of Absolute Deviations); Huber's H15 Robust Mean = 6.26 ppb; Huber's H15 Robust SD = 2.43 ppb; Trimmed Mean 25% = 6.23 ppb; Modus = 6.78 ppb, calculated over the data sorted into the class interval width of 2 ppb.

Asymmetry of data distribution – Skewness = 0.07 (almost no asymmetry).

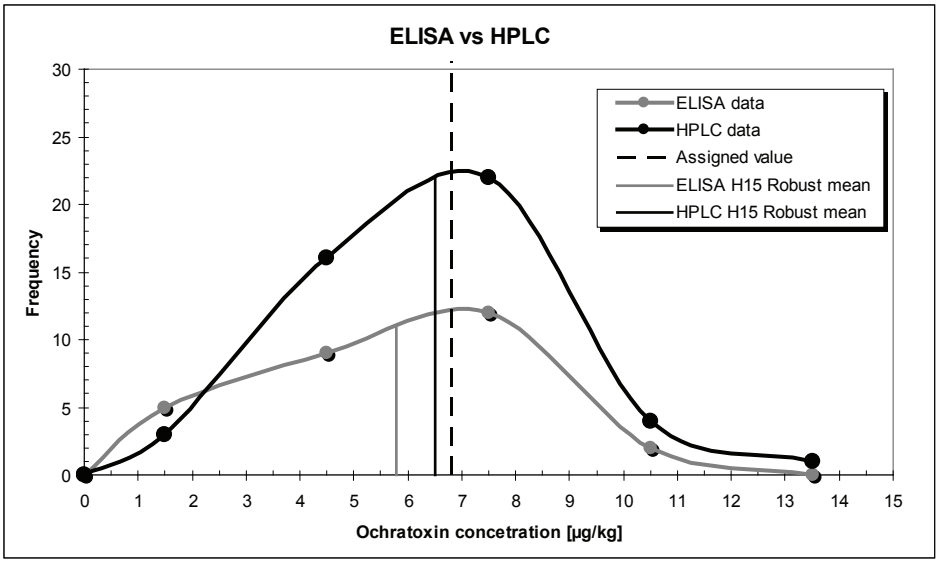


Graph 8. Frequency distribution of ochratoxin PT participants results

Statistical parameters showed in Table 3 have been separately calculated for each method:

Table 3. Statistical parameters for ELISA and HPLC ochratoxin detection

	ELISA	HPLC
Mean [ppb]	5.80	6.47
SD [ppb]	2.53	2.18
CV%	43.7%	33.7%
Median [ppb]	5.85	6.44
MAD_e [ppb]	3.31	1.85
H15 Robust Mean [ppb]	5.78	6.51
H15 Robust SD [ppb]	3.00	2.04
Trimmed Mean 25% [ppb]	5.75	6.48
Modus [ppb]	6.69	6.75
n	28	46



Graph 9. Visual comparation of ELISA and HPLC frequency distributions of ochratoxin results

F test Two-Sample for Variances showed that there was no statistically significant difference in the variation of results (in interlaboratory reproducibility – precision) between the ELISA and HPLC methods ($p = 0.18$).

Non-parametric (robust) Mann-Whitney U-test and parametric t-test two-tailed, equal variances showed that there was no statistically significant difference between median and mean values of two methods ($p = 0.27$ and $p = 0.23$), which means that there was no statistically significant difference in accuracy between these two methods.

In all proficiency tests the estimated standard deviation of PT (SD_P), an important parameter for the calculation of z-score and ranking of laboratories, was always much smaller than the actual variability of the reported results. It is now clear that the SD_P was calculated only on the basis of the assigned value and that it was an estimation of the provider as variability of results should be, not what they were actually. Therefore, we express certain doubts into the usefulness of such a parameter that is not based on reality.

Taking into account all results mentioned above and in spite of some literature data (Pascale, 2009; Mohamadi *et al*. 2012), we abandoned the strict statement that one method is more useful than the other. Although such comparison of methods in this case is rather crude, because of different detection systems in HPLC and various kits for ELISA method that were used, we noted well performance of both techniques and we consider information from proficiency tests extremely beneficial for the analysis. It appeared that the use of ELISA method is entirely appropriate for the determination of mycotoxins,

especially in animal feed where the legal limits are higher. Due to its simplicity and accessibility, analysts of different profiles and little training can apply it. This may also be the cause of less accurate determination of zearalenone in PT2014. In the Graph 6 it could be seen that there was a core of 64% of laboratories (which had satisfactory results of z-score <2) whose distribution curve resulted in a sharp peak and almost exactly matched the results of the HPLC method. It seems like good proof of usability of ELISA method also for zearalenone detection, but that reliable results could be achieved with good training and enough experience.

In laboratory practice, HPLC is the "number one" technique for the measurement of main mycotoxins occurring in cereals and cereal-based products and LC-MS/MS is the most promising technique to be used in the future for multi-mycotoxins analysis. Also, the common perception is that various immunological methods, ELISA and other rapid antibody-based tests should generally be used for screening purposes only and that these methods often require confirmatory analyses with more robust methods. Some authors (Pascale, 2009; Mohamadi *et al.* 2012) emphasize that ELISA kits should be used routinely only for the analysis of matrices that are extensively tested and that confirmatory analyses by e.g. HPLC with IAC clean-up or LC-MS, are required for the contamination levels that approach the legal limit. Despite of general acceptance of this view, the results obtained by ELISA method in analyzed proficiency tests showed that it is very reliable and that the importance of this technique can not be neglected, even for forensic purposes.

CONCLUSION

Final reports of proficiency tests contain important information for the general scientific and professional community and upon our experience it would be good to have them more available. Based on the analysis of more than 360 laboratory results of the tests presented here, in which our laboratory took part, we came to conclusions useful not only for direct participants, but for the wider society. Considering that parts of these reports are useful as a scientific and technical literature, we analyzed them in order to check whether the HPLC method has such clear advantages in comparison to the ELISA. Finally, it was concluded that both techniques could be successfully used for mycotoxin control, especially of animal feed where legal limits are higher than in food.

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ПОРЕЂЕЊЕ *ELISA* И *HPLC* МЕТОДЕ ЗА ДЕТЕКЦИЈУ МИКОТОКСИНА АНАЛИЗОМ РЕЗУЛТАТА *PROFICIENCY* ТЕСТОВА

Ксенија Д. НЕШИЋ[1], Борис П. ПИСИНОВ[1], Сандра М. ЈАКШИЋ[2], Александра М. ТАСИЋ[1], Божидар М. САВИЋ[1], Никола Ј. ПАВЛОВИЋ[1]

[1] Научни институт за ветеринарство Србије,
Аутопут 3, Београд 11070, Република Србија
[2] Научни институт за ветеринарство „Нови Сад“,
Руменачки пут 20, Нови Сад 21000, Република Србија

РЕЗИМЕ: У циљу контроле присуства микотоксина и нивоа контаминације хране и хране за животиње развијене су различите аналитичке технике за детекцију ових природних контаминената. Конвенционалне аналитичке методе за утврђивање микотоксина су танкослојна хроматографија (ТЛЦ), течна хроматографија високих перформанси (ХПЛЦ) и гасна хроматографија (ГЦ). Такође, брзе

методе за микотоксиколошке анализе постају све важније, међу којима је ЕЛИСА (*Enzyme linked immuno-sorbent assay*) једна од најприменљивијих. Циљ овог рада био је да се две различите и најчешће коришћене лабораторијске методе за утврђивање различитих микотоксина (ХПЛЦ и ЕЛИСА) упореде анализом података који потичу из комерцијалних тестова оспособљености (*proficiency* тестова). На основу детаљне статистичке процене резултата добијених применом ових метода за квантификацију афлатоксина, охратоксина и зеараленона, у три комерцијална *proficiency* теста, може се закључити да обе технике могу равноправно да се користе с великом поузданошћу, иако се често наводи да је ЕЛИСА погодна само за почетну тријажу узорака.

KEYWORDS: ELISA, HPLC, food and feed, mycotoxins