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PECULIARITIES OF SAMPLE PREPARATION FOR THE DETERMINATION OF CERTAIN MYCOTOXINS IN GRAIN PRODUCTS AND FRUITS BY IMMUNOBIOSENSOR ANALYSIS

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ABSTRACT

Our current report focuses on effective ways of sample preparation for detection of mycotoxins aflatoxin B1 and T2 in grain products (wheat, maize, sunflower and oats) as well as patulin in fruits (plum, apple and grape) by the surface plasmon resonance (SPR) immunobiosensor. An effective method for carrying out analysis of mycotoxins by an SPR Immunobiosensor was assessed, and the results obtained were compared with those determined in a traditional ELISA method. The analysis with the immunobiosensor is capable to be carried out in a direct format on the basis of mycotoxin-specific antibodies immobilized on the sensor surface, but its sensitivity was found to be lower than that obtained in a competitive format on the basis of an immobilized mycotoxin conjugate. Due to this characteristic difference in analytical sensitivity, we recommend the former format for screening for the presence of mycotoxins in environmental samples. In turn, the latter format is proposed for verification of the obtained results of analysis. The competitive analysis by the immunobiosensor has demonstrated sensitivity in the range of the traditional ELISA method. Organic solvent extraction of the three mycotoxins, aflatoxin B1, T2 and patulin from produce samples was carried out with acetonitrile and methanol, and the former solvent was concluded that preferable. Particular attention was paid to the problem of matrix components detrimentally affecting extractability and the immunochemical reaction: difficulties due to the presence of

small quantities of vegetable oil in grain products needed to be overcome. In cases of samples of substantial vegetable oil content, acetone as an extraction solvent is recommended, the use of which did not appear to affect immunoanalysis results. It was demonstrated that the analysis of patulin in fruits depends on pH of the obtained samples, especially if mycotoxin content is determined in an unripe stage. In such cases, pH determination and neutralization by a special buffer prior to immunoanalytical measurements are recommended

KEYWORDS: Surface plasmon resonance, immunobiosensor, ELISA, mycotoxins, sample preparation.

1. INTRODUCTION

Biosensors technologies currently find expanding new aspects for practical application.[1-5] To promote this process fundamental investigations are needed to be accelerated in numerous important directions, which concerns first of all not only technical approaches of transducer fabrication, namely their technical construction and the creation of the selective sensitive layers, but also the analytical efficacy of the biosensors, which depends both on the main algorithm of its fulfilment in general and on the analytical methods used for the preparation of the samples to be analysed. There is a wide range of sample preparation approaches available, and their applicability highly depends on type of substances to be analysed. This process characterises some specific features in the case of mycotoxins, too. Sample preparation for mycotoxins analysis is a procedure of utmost importance, because in most cases the source material cannot be used directly. Preliminary purification and concentration of the substances analysed are also needed occasionally in mycotoxin analysis. It is noted that such procedures are quite important, because they significantly affect assay performance. These methods have specific characteristic features depending on the analystes and matrices surveyed, and methods used. These approaches widely differ from each other, and are typically complicated, in particular, in the case of traditional analytical methods. The process of toxin extraction is characterised by the several features: (a) the use of non-miscible solvent with the sample, (b) the difficulty of separation of polar and ionic components of the aqueous solutions, and (c) the use of large amounts of organic solvents. Solid-phase extraction variants also exist, and although these methods strongly reduce the use of organic solvents, their process is time consuming and expensive, and requires particular attention in application.[6] For this reason solvent extraction often remains in use as a simple process. However, in that cases problems occur since mycotoxins are predominantly hydrophobic

substances with a very low level solubility in water –approximately 10-20 mg/L. Aflatoxins are well soluble in solvents of moderate polarity, for example, in methanol, chloroform or dimethyl sulfoxide,[7] and approximately the same is typical for most others mycotoxins. Taking this into consideration, investigators try to use a wide range of extraction solvents: acetonitrile, methanol, chloroform, toluene and others. The task is to seek an optimum between maximised mycotoxin extraction level on the on hand and, minimised negative effects of the solvent one immunocomplex formation by the selective antibodies used for detection, on the other hand. Both problems are genuine and require solution. If the samples contain certain levels of fat or substances with extra alkaline or acidic properties, additional difficulties have to be faced during sample preparation for analysis of mycotoxins by the immunoanalytical methods including approaches based on biosensor technology. Considering this problem, we try to compare the efficiency of the extraction of mycotoxin T2 and aflatoxin B1 from grain products and patulin from several fruits using acetonitrile and methanol as most used solvents (mentioned above). Moreover, the effect of the pH and fat content in the samples is estimated by additional approaches.

2. MATERIAL AND METHODS

The selected sample of shredded wheat, maize, sunflower grain or oats weighing 2 g was placed in a 50 ml flask and was soaked in 80% aqueous methanol or acetonitrile at a ratio of 1:3 (weight: volume). Extraction was performed for 12 hours. Possibilities to shorten the sample preparation period and the overall time of mycotoxins analysis was investigated in certain commodities by assessing the effect of shortened extraction times on the extraction efficiency (see below). After extraction the sample was centrifuged for 15 min at 500×g. The supernatant was taken for further analysis. Before immunoanalysis the sample was diluted with 5 mM phosphate buffer setting the concentration of the extraction solvent in the diluted extract to 20-40%. In special experiments it was demonstrated that the final level of acetonitrile and methanol may not exceed 40% and 20%, respectively. Further increases in their concentration in the sample analysed lead to reduced intensitiesy of the immunochemical reaction, asdemonstrated in Fig. 2.1. Analysis by the ELISA method was performed in a competitive format, when a mycotoxin–gelatine conjugate was immobilized on the wells of the micro plates and competed with free mycotoxins for the specific rabbit antibodies.[8,9] The resulting immunocomplex was found using secondary anti-rabbit antibodies labelled with horseradish peroxidase ("Sigma", USA).

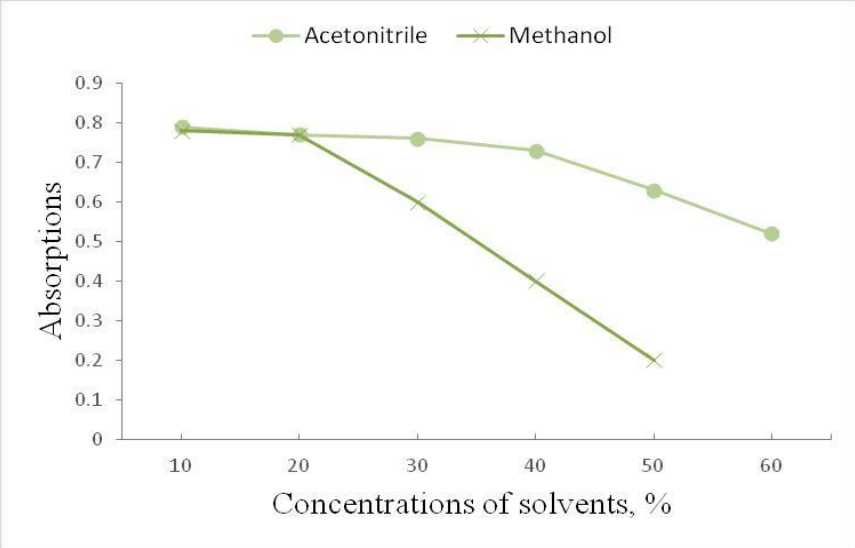

Fig. 2.1: Intensity between extinction of the enzymatic reaction at a wavelength of 492 nm and concentration of the organic solvents used for sample preparation pror to the ELISA method.

Solvents acetonitrile and methanol and mycotoxin aflatoxin B1 were used in the model experiment.

In parallel to the ELISA method, an immunobiosensor based on surface plasmon resonance (SPR) was also used for comparative purposes.[10] The biosensors were devised both in direct and competitive formats. In the former one the specific antibodies immobilised on the transducer surface interact with the appropriate analyte mycotoxin. The latter one is devised when the free analyte and its derivative conjugated to bovine serum albumin and immobilised on the transducer surface compete with each other for the specific antibodies in solution.

In model experiments matrices were spiked with the mycotoxins used. Thus, the solution of each mycotoxin was prepared with the application of an appropriate solvent and was then added to the grain/fruit matrix prior to the process of extraction at a final concentration of 50.0ng/ml. In case of the experiments with fruits, apple, plum and grape were chosen as models and all procedures with the extraction of mycotoxins were the same as described above.

To check the effect of the presence of low quantities of fat in the extracted materials sunflower grain was used as a model for aflatoxin B1 extraction with or without subsequent application of acetone to remove fatty components.[6] In this case the addition of acetone was tested as a model to eliminate this factor. The extraction procedure was carried out similarly

as described above, but with the addition of 0.1% of sunflower oil and then acetone at up to 5% ratio to the total volume.

3. RESULTS AND DISCUSSION

3.1. Comparison of the sensitivity of two types of protocol for the determination of mycotoxins by the immune biosensor and traditional ELISA method.

Mycotoxins are low molecular weight substances and they have very restricted field for the interaction with the specific antibodies. In this case the competitive way of analysis is suitable for their determination by the traditional ELISA-method.[9] The calibration curves of the determination of T2 mycotoxin, aflatoxin B1 and patulin in model solutions are shown in Fig. 3.1.1. According to the presented data the limit of detection (LOD) is about 1 ng/ml and a linear plot is in the range from 1 to 500ng/ml. The values of these indicators are retained for all investigated mycotoxins with minor deviations for some of them.

The immunobiosensor based on SPR allows direct determination of concentration of the mycotoxins in the solution. The calibration curves for all above-mentioned mycotoxins in this case are presented in Fig. 3.1.2. According to the data obtained the LOD for all of mycotoxins tested is about 50ng/ml and a linear plot lies in the range of 10-500 ng/ml.

The established level of the sensitivity may be enough for screening purposes of the dispersion of mycotoxins in the environment, since the analytical procedure is very simple. But for verification of the preliminary obtained results the sensitivity is needed to be increased by one order of magnitude as a minimum. It was found that this can be achieved by the immunobiosensor devised in the competitive format (Fig. 3.1.3). In this case the LOD of mycotoxin determination is about 1 ng/ml, i.e. practically the same as in the ELISA method as demonstrated above. Such sensitivity is sufficient for the verification of results obtained in the screening tests. The method of analysis is not particularly complicated but introduces an additional component in form of special conjugate of mycotoxin with protein.

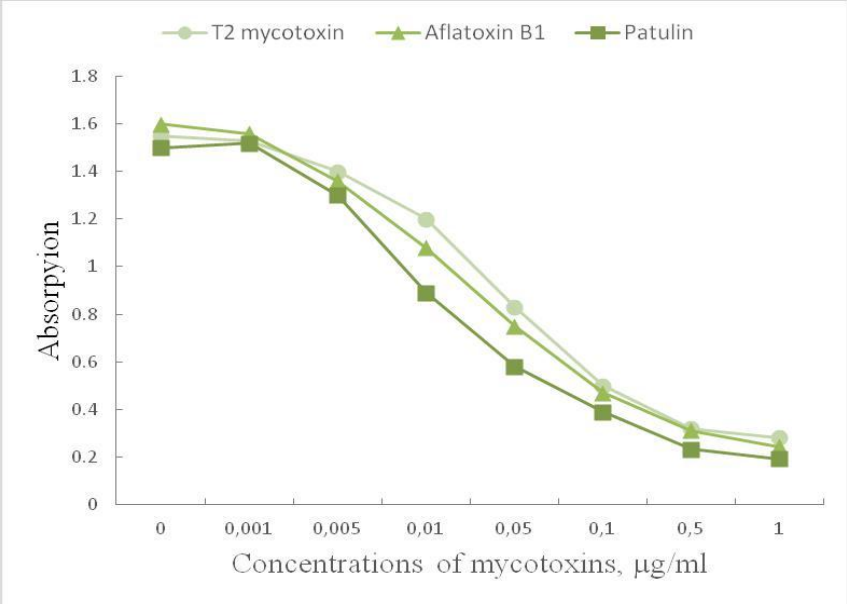

Fig. 3.1.1: Calibration curves between the absorbance at 492 nm and concentration of mycotoxins by an ELISA method in model solutions of T2 mycotoxin, aflatoxin B1 and patulin.

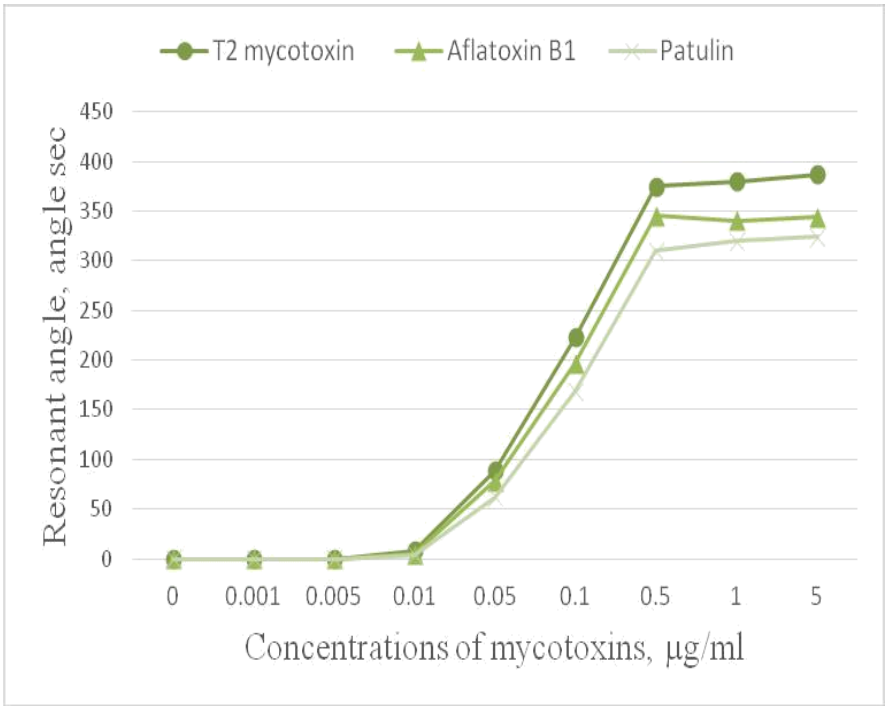

Fig. 3.1.2: Calibration curves between the resonance angle and concentration of mycotoxins using the immunobiosensor in the direct format in model solutions of T2 mycotoxin, aflatoxin B1 and patulin.

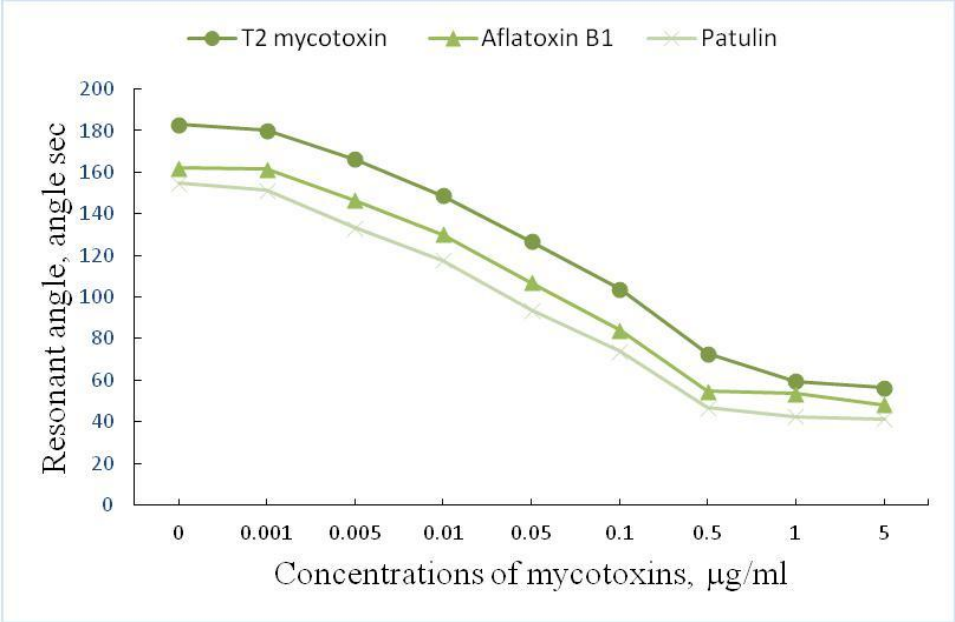

Fig. 3.1.3: Calibration curves between the resonance angle and concentration of mycotoxins using the immunobiosensor in the competitive format in model solutions of T2 mycotoxin, aflatoxin B1 and patulin.

3.2. Efficiency of different approaches for the extraction of mycotoxins from grain products.

It is necessary to mention that according to both analytical approaches, namely, the ELISA and the immunobiosensor methods, at the extraction of the T2 mycotoxin from shredded wheat it is possible to reveal about 40% when using methanol and approximately 60% in case of application of acetonitrile from the theoretical calculated level of their presence in the sample to be analyzed.

Similar data were obtained for application these procedures for such grain products as maize and oat. Unfortunately, from last products T2 mycotoxin was extracted in above 85% and 75% from its total content in samples with acetonitrile and methanol, respectively. The level of the extraction of aflatoxin B1 by both solvents was somewhat higher and achieved up to 90% and 80% for acetonitrile and methanol, respectively (Fig. 3.2.1). The observed difference in this case is related to the different solubility of T2 mycotoxin and aflatoxin B1.

At the same time, the level of extraction of both above-mentioned mycotoxins is substantially lower when shredded sunflower grain was used in the experiments. One of reasons of this effect, according to our opinion, is the presence of some quantity of the vegetable oil components in the matrix. To test this hypothesis we added sunflower oil into the extracted

grain, maize and oat samples in the range of 0.1%. The level of registered aflatoxin B1 and, especially, T2 mycotoxin was decreased (Table 3.2.1). It confirms that the presence of oil in the analysed products may affect the result of the analysis. It has to be underlined that the presence of sunflower oil in the control sample affected the result of analysis the most in the case when acetonitrile was used as a solvent in comparison to methanol, and this difference may exceed 10-15%. The effect is possibly due to additional dissolving of fat and entering it in the test sample, and then impacting the effectiveness of the interaction between the antigen and the antibodies. To prevent this effect, we tried to use the treatment of sample after the extraction process by acetone about 5% of the total volume of the sample. The results of these investigations indicated that the preliminary treatment of the analysed sample by acetone increases the determined level of aflatoxin B1 and T2 mycotoxin to that seen before oil addition. The effect may result both from the prevention of the effect of the oil content on the immunochemical reaction and from increasing efficacy of mycotoxin extraction. For either reasons, such additional treatment of the extraction samples with acetone is recommended, especially, if their oil content is noteworthy.

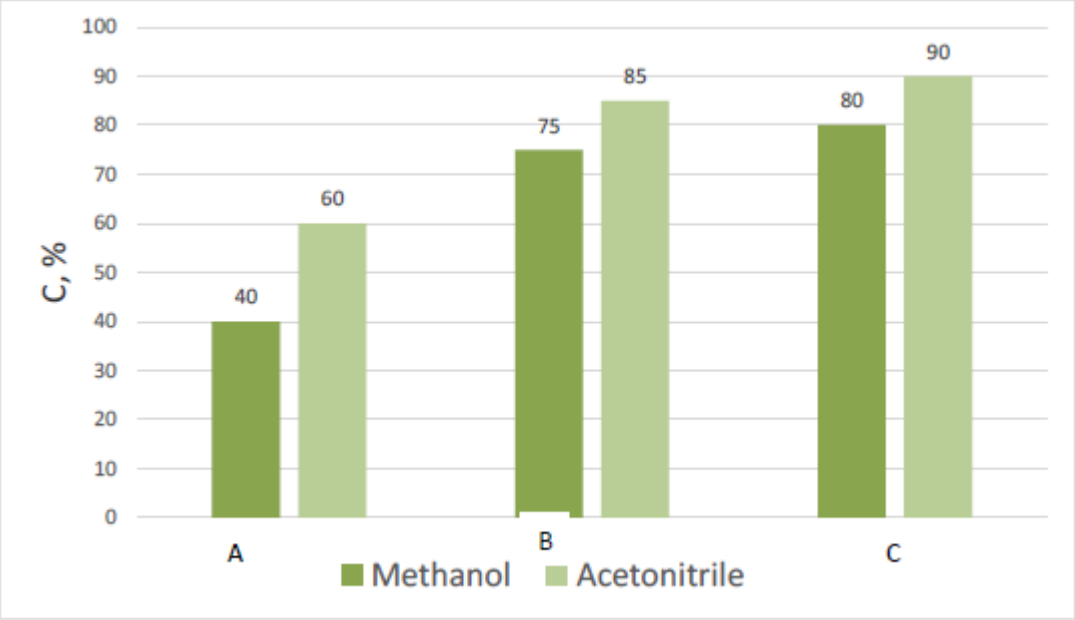

Fig. 3.2.1. Efficiency of the extraction T-2 mycotoxin from the shredded wheat with the help of methanol (dark), acetonitrile (lighter) in comparison with theoretically calculated level.

Finally, we have also analysed how the duration of the extraction procedure affects the efficiency of the extraction of mycotoxins. The duration of the extraction procedure was set

between 1 h and 12 h. The obtained results are presented in Fig. 3.2.2. As shown, the extraction of T2 mycotoxin from shredded wheat shows a saturation pattern Maximal extraction levels is reached practically in 12 h, and represent good recoveries relative to the theoretically calculated level (up to 90 and 80% for acetonitrile and methanol, respectively). As for initial extraction efficacy, approximately 50% of the content of this mycotoxin is extracted within the first 1 h treatment. Simlar results were obtained in the extraction of aflatoxin B1, as well as for the use of other types of grain products, in particular, maize and oat. Therefore, in screening observations it is possible to use short time (up to 1 h) procedures of mycotoxin extraction from commodity samples. Nonetheless, validation of the results such screening tests by precise mycotoxin content determination based on exhaustive extraction require 10-12 h long extraction processes.

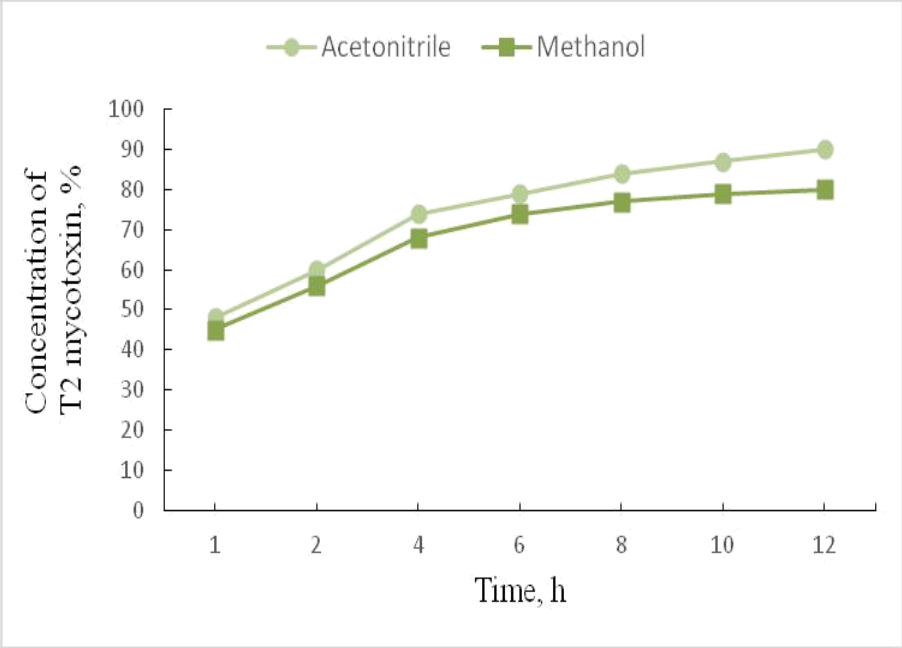

Fig. 3.2.2. Dependence of the level of T2 mycotoxin detected on the duration of its extraction from shredded wheat by acetonitrile and methanol.

Table 3.2.1. Varies of the efficiency of extraction of aflatoxin B1 and T2 mycotoxin by acetonitrile from maize and oats at the influence of the sunflower oil in comparison with the theoretical calculated level, %.

		Samples			
No.	**Mycotoxins**	**maize**		**oats**	
		without oil	**with oil**	**without oil**	**with oil**
1.	Aflatoxin B1	84±1,8	75±3,5	91±1,5	73±3,3
2.	T2 mycotoxin	78±1,6	62±4,1	86±1,7	75±2,2

3.3. Efficiency of different approaches for the extraction of mycotoxins from fruits

Extraction efficacies were tested in fruit matrices as well, using fruits as apple, grape and plums, and mycotoxin patulin as an analyte. In these experiments, the extraction process was the same as described above for grain products. At first it was revealed that the level of the deviation of the determined actual patulin content from the theoretically calculated level shows a strong variation for individual groups of the same fruits. Especially noteworthy variation within the same fruit species was seen between ripe and immature groups. The detected level of variation relative to the theoretically calculated level renged between 10% and 40%, in with worse recoveries for immature fruits that for ripe fruits in all cases. Deviations up to 40% were far not typical of most mature fruits. In view of this, it was suggested that this effect may be due to different acididity levels in these groups. Indeed, by checking the pH level it was confirmed that in homogenates of individual samples the pH range was 6.9-5.5. Moreover, the lower the pH was recorded, the greater the deviation was detected in the content of patulin in the sample with respect to the originally theoretically specified its level (Table. 3.3.1). Obviously, some acidification of the analyzed sample adversely affects on the antigen reaction with the antibodies. It is known that neutral pH is optimal for such interaction, whereas its decrease leads to decrease in the antibody-antigen complexation.[11]

There is yet another visible effect, namely, a somewhat greater variation in the pH value during the extraction of patulin with acetonitrile, and the discrepancy in the revealed amount of this mycotoxin in compared to the theoretical level. Simlar effects were seen for methanol as an extraction solvent as well. Apparently this is to some extent a reflection of their level of hydrophobicity.

Table 3.3.1: Level of pH and relative (in %) level of decreasing patulin revealed content in fruit extracts certain obtained with acetonitrile and methanol.

N, item	Products	pH of extract obtained by:		Deviation (%) of patulin content on the theoretical calculated level.	
		acetonitrile	methanol	acetonitrile	methanol
1.	Mature plums	6.8	6.9	10,0	9.0
2.	Immature plums	6.4	6.1	14,0	17,0
3.	Mature apple	6.6	6.8	12,2	10.1
4.	Immature apple	5.7	6.0	40,0	36,2
5.	Mature grapes	6.6	6.8	16,3	12,2
6.	Immature grapes	5.5	5.7	38,8	36,4

4. CONCLUSION

It has to be underlined that the SPR-based immunobiosensor may provide high level of sensitivity for the analysis of mycotoxins. In the regime of the competitive format of analysis its LOD is even on the similar level (up to 1 ng/ml) as it was demonstrated by the traditional ELISA method. Moreover, this immunobiosensor has several advantages compared to traditional immunoanalysis, as its performance is simpler, does not demand additional expensive conjugates, and the measurement can be completed in 40 min or even much more rapidly, if the transducer surface together with the samples will be preliminarily prepared. This strongly contrasts with the fact that completion of the ELISA method requires 6-8 hours. The direct format of the immunobiosensor is less sensitive than the competitive one, thus, this simple to carry out immunobiosensor format is recommended for screening purposes. The competitive immunobiosensor format is capable to be used for validation purposes i.e. verification of the results of the preliminary sceening tests. Therefore, for the screening tests, the time of mycotoxin extraction from the investigated commodities may be shortened to 1 h. In case of the verification analyses, exhaustive extraction procedures are required, lasting 10-12 h. A number of factors, potentially affecting extraction efficacy of mycotoxins by acetonitrile and methanol, are needed to be considered during sample preparation. Our results indicate that acetonitrile is more suitable for organic solvent extraction than methanol for two reasons. On the one hand, it allows higher levels of extraction of mycotoxins e.g. aflatoxin B1 and mycotoxin T2 from grain products. And on the other hand, it affects the immunochemical reaction less, as antibodies used for immunoanalysis of mycotoxins may tolerate acetonitrile at up to 40% in the extract. In contrast, the corresponding levels of methanol should not exceedmore 20%, which only but it may also affect the extraction of mycotoxins. To prevent non-desirable effects by the presence certain oil substances in grain products on the analysis of mycotoxins, acetone was shown to be useful for an effective pre-treatment, which removes these substances and allows obtaining the real information about the content of mycotoxins in grain products such as sunflower.

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