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REVIEW OF OCCURRENCE OF MYCOTOXINS IN SERBIAN FOOD ITEMS IN THE PERIOD FROM 2005 TO 2022

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Abstract: This paper aimed to review the publications on mycotoxins' presence in cereals and foodstuffs originated from the Serbian market covering the period from 2005 to 2022. The review covers all the important steps in mycotoxins analysis including sampling, sample preparation, instrumental analysis, and concentration ranges in which the mycotoxins were found. Also, the results were interpreted from the European Union regulation point of view. The review emphasizes the importance of multi-mycotoxins analysis for determining the simultaneous presence of mycotoxins that can negatively affect the Serbian human population. The most frequently used instrumental technique in the mycotoxin analysis of Serbian products was the Enzyme-Linked Immunosorbent Assay followed by the Ultra-High Performance Liquid Chromatography coupled with triple quadrupole mass spectrometry. Most of the studies undertaken in Serbia until now investigated a few groups of matrices such as wheat, maize, milk, and dairy products. Only a few studies involved specific matrices such as nuts, dried fruits, biscuits, cookies, and spices. The review showed that contamination of milk and dairy products with aflatoxin M1 (AFM1), occurred at the very beginning of 2013, was the major health issue related to the population health. The contamination of milk and dairy products with the AFM1 was a consequence of maize contamination with aflatoxins which occurred in the year 2012, characterized by drought conditions. The studies dealing with the analysis of masked and emerging mycotoxins are rare and more attention should be paid to monitoring the presence of these types of mycotoxins in foodstuffs from Serbia.

Key words: *aflatoxins, trichothecenes, food analysis, instrumental techniques, Serbia*

INTRODUCTIONS

Mycotoxins, as natural secondary metabolites produced by toxigenic fungi, primarily, *Aspergillus*, *Fusarium*, and *Penicillium*, pose a serious threat to food and feed safety (Bryla, Ksieniewicz-Woźniak, Waśkiewicz, Szymczyk & Jędrzejczak, 2018). Although more than 400 mycotoxins are known, the most commonly analyzed mycotoxins are: aflatoxins (AFs), ochratoxins (OTs), zearalenone (ZEA), trichothecenes type A (HT-2, T-2) and type B deoxynivalenol (DON), fumonisins (FMs), ergot alkaloids, (Brodal, Hofgaard, Eriksen, Bernhoft & Sundheim, 2016). Mycotoxins have harmful effects on human and animal health

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causing various acute or chronic problems such as carcinogenicity, mutagenicity, teratogenicity, and hepatotoxicity (Khaneghah, Moosavi, Oliveira, Vanin & Sant'Ana, 2020). The toxicity of the commonly analyzed mycotoxins classifies into several groups: 1, 2A, 2B, 3, 4, and 5 (Ostry, Malir, Toman & Grosse, 2017). For example, aflatoxin B1 (AFB1) is classified as Group 1 (i.e. carcinogenic to humans) by the International Agency for Research on Cancer (IARC, 1993). OTA and FMs belong to Group 2B, considered possibly carcinogenic for humans. Furthermore, DON, ZEA, T-2, and HT-2 toxins belong to Group 3, not defined carcinogenic to humans due to a lack of evidence (IARC, 2012; Ostry et al., 2017). In addition, a significant threat to human health represents additive or synergistic effects caused by the simultaneous presence of mycotoxins in the tested samples (Lee & Ryu, 2017; Frisvad et al., 2019).

Contamination of food and feed with mycotoxins is a global concern because 25-50% of the world's harvest can be contaminated with them, which represents serious economic losses worldwide (Marin, Ramos, Cano-Sancho & Sanchis, 2013). Mycotoxins contaminate vast range of commodities such as corn, wheat, barley, oats, rice, figs, nuts, milk, and cheese (Juraschek, Kappenberg & Amelung, 2022). Contamination of crops can occur before and during the harvest, storage, and processing, which negatively affects the quality of food and feed (Sforza, Dall'Asta & Marchelli, 2006). It should be noted that in addition to the division of mycotoxins based on toxicity, they can also be sorted by geographical occurrence, which is directly related to the type of contaminated crops and climatic conditions (Juraschek et al., 2022). In this context, mycotoxins that mainly characterize tropical and subtropical regions such as Asia, Africa, and parts of Europe are AFs and FMs (Smith, Madec, Coton & Hymery, 2016), while toxins from the group of trichothecenes (DON, NIV, T-2, and HT-2 toxin and ZEA) can be found in northern regions of Europe, America, and parts of Asia with humid and cold conditions (Schuhmacher-Wolz, Heine & Schneider, 2010; EFSA Panel on Contaminants in the Food Chain, 2013; Smith et al., 2016). Contamination of food and feed with mycotoxins is a global problem because every year many crops around the world become contaminated due to extreme climatic conditions, which prevents their further processing, and leads to serious economic losses (Luo, Du, Kebede, Liu & Xing, 2021). To protect the health of consumers and raise awareness of the prevention and control of mycotoxins, the European Commission has adopted comprehensive and detailed regulations on the content of mycotoxins in foodstuffs (EC, 2006b). Continuous monitoring of mycotoxins in food and feed is crucial for reducing potential problems related to food distribution and consumption, as well as consumer confidence (Agriopoulou, Stamatelopoulou & Varzakas, 2020). To provide databases that would enable a comprehensive assessment of mycotoxin exposure, comparable data obtained for the same type of crop are needed, as well as representative samples adequately prepared and analyzed by reliable instrumental methods. However, the existing data do not allow the above mentioned, as data were obtained mainly on a limited number of unrepresentative samples, while in some cases mycotoxins are analysed using uncertified laboratory reference procedures (IARC, 2015; Misihairabgwi, Ezekiel, Sulyok, Shephard & Krska, 2019). With this in mind, many laboratories and researchers have focused their attention on standard analytical procedures, established by various regulatory authorities (Vargas Medina, Bassolli Borsatto, Maciel & Lanças, 2021). Food matrices are complex matrices containing numerous interfering compounds. Thus, it is necessary to develop rapid analytical methods for performing complex mycotoxin analysis to achieve high sample throughput. Considering the different chemical and physical properties of mycotoxins and the complex composition of the analyzed matrices, very sensitive and selective techniques are necessary to enable the simultaneous detection of mycotoxins. In this context, multi-analyte methods based on high-performance liquid chromatography (HPLC) coupled with (tandem-) mass spectrometry (MS/MS) that offer higher selectivity and allow multi-toxin determination without dedicated purification of the samples are an absolute trend in mycotoxin analysis (Sulyok, Krska & Schuhmacher, 2010). Earlier, Sulyok, Berthiller, Krska & Schuhmacher (2006) reported that using the latest generation of mass spectrometers allows quantitative determination of mycotoxins in crude food extracts, provides sufficient characterization of the extraction efficiency and mat-

rix effects for all tested analyte/matrix combinations. Likewise, the researchers indicated that from their point of view and many years of analytical experience, two analytical steps are crucial in determining mycotoxins in foods: sample preparation and instrumental analysis (Vargas et al., 2021). Although the review published by Udovicki, Audenaert, De Saeger and Rajkovic (2018) dealt with the mycotoxins incidence in Serbian food products, mainly in cereals and dairy products in the period from 2004 to 2016, this study includes the new data published after 2016. The mentioned review (Udovicki et al., 2018) gave insight into mycotoxin presence in different matrices separately (mycotoxin by mycotoxin).

In this review paper, the matrices were evaluated in terms of simultaneous mycotoxins presence. Accordingly, the multi-mycotoxin contamination of particular matrices can be easily observed. Also, this review provided insight into sampling protocols, frequently used sample preparation and instrumental analysis procedures. Thus, this review brings an overview of the most commonly used and most effective analytical methods for the determination of mycotoxins in food. Additionally, the review will discuss the occurrence of mycotoxins in food products in Serbia and compare their content with those available in the literature.

SAMPLING PROTOCOL AND MYCOTOXIN ANALYSIS

Many countries around the world have established effective food control systems to ensure food safety and protect consumer health (FAO, 2007). However, the prevention and control of mycotoxins in the food chain is a major challenge faced by analysts and reference laboratories due to the complexity of food matrices and the heterogeneous distribution of fungi in them. This indicates the need for efficient and reliable sampling protocols and analytical methods that would allow rapid detection and continuous control of mycotoxins in samples of interest (Maestroni & Cannavan, 2011). Representative sampling is considered to be the most important part of an analytical protocol that should provide a reliable sample with satisfactory information such as the purpose of the study, the nature of the food matrix tested, and the nature of the toxin (Iqbal, 2021). Food shipments should be tested to determine if they "fit for purpose". This suggests that the sampling process should provide the answers to the following questions "why, where, and when" to take samples and analyze them. In addition, it should be borne in mind the aforementioned heterogeneous distribution of mycotoxins in samples and the fact that approximately 0.1% of the sample might be contaminated at high toxin levels, while the other part of the sample might be free of mycotoxins (Miraglia, De Santis, Minardi, Debegnach & Brera, 2005). Miraglia et al. (2005) pointed out that if the subject of analysis is a highly contaminated part of the sample or a part with no mycotoxin presence, this may lead to inaccurate analytical results. Accordingly, collecting as many incremental samples as possible in bulk amounts to obtain a representative sample for analysis of the whole lot is highly relevant **(**EC, 2006a). European Commission (EC, 2006a) gives the procedures to fix general criteria with which the sampling method and method of analysis should comply. The regulation covers detailed instruction concerning the sampling protocols for different matrices such as cereals and cereal products, dried fruit, groundnuts, nuts, spices, milk, dairy products, coffee, coffee products, fruit juices, and baby food. Additionally, EC (2006a) gives performance criteria related to the method recovery, precision, and measurement uncertainty.

It is worth noting that extraction is a challenging step in the analysis of mycotoxins as chemically different compounds need to be isolated, indicating that the extraction method is a compromise of achievable recoveries. Furthermore, sample preparation for mycotoxin analysis should be based on simplified procedures using environmentally friendly solvents (Somsubsin, Seebunrueng, Boonchiangma & Srijaranai, 2018). Today, conventional liquid-liquid extraction (LLE) is used to extract mycotoxins from liquid samples, while solid-phase extraction (SPE) is most commonly employed for solid samples (Liu et al., 2014). As it is known, mixtures of methanol-water or acetonitrile-water in different ratios are most often used for the extraction of mycotoxins (Zhang, Dou, Zhang, Logrieco & Yang, 2018). Adding water to the organic solvents increases the polarity and allows easier penetration of the organic solvents into the food matrix (Iqbal, 2021). Moreover, the extraction effi-

ciency will be enhanced by adding an acidic buffer to the extraction mixture, which would help to break the strong bonds between the analyte and the food components (protein and sugar) (Whitaker & Wiser, 1969). One of the frequently used approaches in multi-mycotoxin analysis is the use of the so-called crude or diluted sample extract analysis without clean-up (Sulyok, Berthiller, Krska & Schuhmacher, 2006; Herebian, Zühlke, Lamshöft & Spiteller, 2009; Martos, Thompson & Diaz, 2010; Škrbić, Malachova, Živančev, Veprikova & Hajslová, 2011; Škrbić, Živančev, Đurišić-Mladenović & Godula, 2012). This approach has its advantages and disadvantages. The extensive matrix effects and instrument contamination are major disadvantages of this approach (Garrido Frenich, Vidal Martínez, Romero-González & Aguilera-Luiz, 2009). However, the advantage of injecting the crude extract is reflected in the possibility of including a large number of chemically diverse mycotoxins in a single analysis (Sulyok et al., 2006). Additionally, Sulyok, Krska & Schuhmacher (2007a) and Sulyok, Krska & Schuhmacher (2007b) pointed out that methods based on the crude extract allow easy introduction of new analytes and matrices, assuming that previously optimized extraction and chromatographic conditions are applicable. In recent decades, researchers have focused on the removal of unwanted compounds and remaining interferences from extracts of interest, and the clean-up step of the obtained extract was considered important during mycotoxin extraction. Data from the available literature indicate that the sample clean-up procedures are mainly based on different forms of solid-phase extraction (SPE) and the so-called QuEChERS extraction (**Qu**ick, **E**asy, **Ch**eap, **E**ffective, **R**ugged, **S**afe). Alshannaq and Yu (2017) reported that immune affinity columns (IAC) and columns intended for solid-phase extraction are the most commonly used to clean up the extracts for mycotoxin due to their high sensitivity and selectivity. In recent years, advanced extraction methods have been used to extract mycotoxins from samples of interest, such as accelerated solvent extraction (ASE), supercritical liquid extraction (SFE), and microwave-assisted extraction (MAE) (Wang, Kong & Yang, 2016). Although these methods offer better extraction efficiency with lower solvent consumption due to some difficulties (e.g. in optimization and routine use, as well as the need to invest in special equipment), these advanced extraction methods have not gained significant application in multi-mycotoxin methods.

Determination of mycotoxins in food is based on various techniques that offer qualitative and quantitative analysis. The expansion of the development of analytical methods for the determination of mycotoxins began in the 1960s, and initially, the procedures were based on thin-layer chromatography (TLC) (Kralj Cigić & Prosen, 2009). Very quickly, the TLC technique was completely replaced by other techniques, among which the enzyme immunesorbent assay (ELISA) was probably the most widely used (Meneely, Ricci, Van Egmond & Elliott, 2011). ELISA offers some advantages over TLC, such as high-speed analysis, ease of use, low cost, portability, and ease of handling. The disadvantages of this technique are unreliable results, i.e. false positive or false negative results due to its cross-reactivity with structurally similar toxins or matrix compounds. Besides the mentioned techniques, chromatographic techniques have a significant role in determining mycotoxins i.e. HPLC combined with fluorescence (FLD), ultraviolet-visible (UV-Vis), and other detectors. Although HPLC in combination with the mentioned detectors has found application in the determination of mycotoxins, the absence of structural information, the inability to separate co-eluting compounds, and the need to determine multiple compounds in a single run, indicated the need for more sophisticated detection techniques (Rahmani, Jinap & Soleimany, 2009). Thus, HPLC/UHPLC in combination with MS analyzers is considered a reference technique for the determination of mycotoxins, taking into account the following: a) the possibility of performing multi-toxin analyzes regardless of their chemical structure and b) the determination of low concentration levels due to high sensitivity and excellent selectivity of MS analyzers (Pereira, Fernandes & Cunha, 2014). MS analyzers enable successful quantification of mycotoxins in matrices of interest, because, apart from the high selectivity that contributes to the identification of analytes based on molecular mass, additional structure-specific information can be obtained e.g. through specific fragmentation reactions (De Berardis et al., 2018). LC-MS/MS systems are highly desirable in multi-

mycotoxin analysis due to good features such as high sample throughput, high detection sensitivity (ng/g levels), and satisfactory analysis efficiency (Iqbal, 2021). The importance of the LC-MS/MS systems has been confirmed by numerous studies in which they have been used to determine the content of mycotoxins in different foods and concentration ranges (Perrone, Ferrara, Medina, Pascale & Magan, 2020; Leite, Freitas, Silva, Barbosa & Ramos, 2021). In the last years, UHPLC has been extremely useful in multi-mycotoxin analysis because it offers higher separation power compared to conventional HPLC due to using packing material having particles less than 2.5 mm; e.g. Beltran, Ibañez, Sancho and Hernández (2009) reported that thanks to UHPLC, 11 mycotoxins were efficiently separated in only 4 min. A new relevant trend in the multi-mycotoxin analysis is the use of LC-HRMS (High-Resolution Mass Spectrometry) systems for targeted, post-targeted, and non-targeted analysis that can be performed in a single run (Izzo et al., 2020; Narváez, Rodríguez-Carrasco, Castaldo, Izzo & Ritieni, 2020). Apart from the targeted HRMS analysis of mycotoxins in food, these analyzers are a suitable tool for performing retrospective data analysis of non-target compounds (Vargas Medina et al., 2021). Recently published studies reporting targeted HRMS analysis of mycotoxins in food have also been followed by retrospective analysis of unknown coexisting compounds (Castaldo et al., 2019; Izzo et al., 2020; Narváez et al., 2020).

OCCURRENCE OF MYCOTOXINS IN FOODSTUFFS FROM SERBIA

The studies dealing with mycotoxins determination in foodstuffs from Serbia were mainly directed to the analysis of cereals (Table 1). Table 1 presents mycotoxin results published in studies conducted from 2005 to 2022. The most commonly analyzed cereals are wheat and maize (Škrbić et al., 2011; Kos, Mastilović, Janić Hajnal & Šarić, 2013; Jajić, Krstović, Kos & Abramović, 2014). In addition to the cereals analysis, powdered red paprika, white, and black pepper (Škrbić, Koprivica & Godula, 2013) were also analyzed as well as different types of nuts (walnut, hazelnut, almond, and peanut) (Škrbić, Živančev & Godula, 2014). As a consequence of the outbreak of milk contamination by AFM1 that occurred in Serbia at the very end of February 2013 a few studies were reported dealing with the determination of AFM1 in milk and dairy products (cheese) (Kos, Lević, Đuragić, Kokić & Miladinović, 2014; Škrbić, Živančev, Antić & Godula, 2014a; Tomašević et al., 2015). Additionally, the presence of mycotoxins was also investigated in biscuits, cookies, dried fruit, and fruit jams (Škrbić, Antić & Cvejanov, 2017). The mentioned mycotoxins studies included aflatoxins (AFB1, AFB2, AFG1, AFG2, and AFM1), fumonisins (FB1 and FB2), ZEA DON, OTA, HT-2, and T-2 toxins. Among the selected mycotoxins the most frequently investigated are AFs.

For the sample preparation/analysis, the most often used technique was ELISA (Kos et al., 2013; Kos et al., 2014a; Kos et al 2014b; Tomašević et al., 2015). The method is normally conducted by following the manufacturer's procedure which is specific for each type of matrix and kit producer. Also, the mass of the sample should be in accordance with the manufacturer's procedure as well as each step involved in the analysis. The frequently used sample preparation method for mycotoxins determination in different foodstuffs (wheat, maize, different types of pepper, nuts) was crude extract analysis followed by UHPLC-MS/MS quantification (Škrbić et al., 2014b; Škrbić et al., 2017; Kos et al., 2020). The method is based on the extraction of mycotoxins with a combination of solvents such as acetonitrile/water (80:20, v:v) or acetonitrile/water/acetic acid (79:19:1, v:v:v) and direct analysis of crude extracts (usually after a 4-fold dilution with the mobile phase). Also, when the fatty samples were analyzed (Škrbić et al., 2014b; Škrbić et al., 2017) after the solid-liquid extraction, an additional defatting step was introduced into the sample preparation that involved the use of hexane to remove fat from the sample extracts. Furthermore, when the hard types of cheese were analyzed on AFM1 content, an additional clean-up step with SPE was introduced (Škrbić, Antić & Živančev, 2015).

Regarding the instrumental analysis, ELISA was the technique that is the most often used for quantitative analysis of mycotoxins (Kos et al., 2013; Kos et al., 2014a; Kos et al., 2014b; Tomašević et al., 2015; Kos et al., 2017; Torović, Popov, Živković-Baloš & Jakšić,

2021). Very often an additional confirmation step was introduced to confirm the concentrations determined by ELISA (Udovicki et al., 2021; Torović et al., 2021). UHPLC-MS/MS was the second technique of choice for mycotoxins determination in food matrices (Škrbić et al., 2011; Škrbić et al, 2012; Škrbić et al., 2013; Kos et al., 2020). Also, in a few studies (Jajić et al., 2014; Janić Hajnal et al., 2017), the quantification of mycotoxins was carried out by LC- Diode-Array Detection (DAD) or FLD detectors.

In the study undertaken by Udovicki et al., (2021), AFB1 was investigated in peanut-based products (n = 94), maize milling products and maize-based products (n = 117), mixed composition products (n = 41), tree nuts (n = 125), rice (n = 41), millet (n = 14), dried figs (n = 25) and dried ground red paprika (n = 6) (Table 1). Total AFB1 occurrence across all categories of analyzed products was 29.2%. 16 samples (4 peanut-based products, 7 maize milling and maize-based products, 3 tree nuts, and 2 millet) out of 463 were contaminated with AFB1 above the EU Maximum Residue Limit (MRL) defined for foodstuffs. Muesli with cornflakes was the matrix with the highest percentages of positive samples (70%), while the highest mean values were found in peanut butter (7.86 μg/kg), and maize flour (4.67 μg/kg).

The study published by Kos et al. (2020) reports the results of mycotoxins presence (AFs including AFM1), OTA, FMs (FB1, FB2, FB3, FB4, FA1, FA2), ZEA, DON among others) in 204 systematically taken maize harvested in Northern Serbia during the period from 2012 to 2015. The study included maize samples produced during the seasons of extreme drought (2012), and extreme precipitation (2014) as well as the seasons with hot and dry conditions (2013 and 2015). The results show that the extreme drought in 2012 had a great influence on the production of AFs. Namely, except for AFG1 and AFG2, AFs were quantified in more than 50% of analyzed samples collected in 2012. AFB1 was quantified in an extremely high concentration of 205 μg/kg (mean value of 44±49 μg/kg). It is interesting to note that AFM1 was detected in 57% of analyzed maize samples in 2012 with a concentration range of 0.5–7 μg/kg, although AFM1 is usually formed in the liver of humans and lactating animals that have been fed with AFB1 contaminated food. Also, in 2012 the simultaneous occurrence of all five mycotoxins (i.e. AFs) was observed in 8% of analyzed samples. Opposite to the previously discussed weather conditions, in 2013, the absence of prolonged and extreme drought conditions during the maize growing season conditioned in much lower contamination of maize samples with AFs (<35%). In 2014, none of the analyzed maize samples had detectable levels of AFs. The mentioned year was characterized by extremely rainy conditions during the growing season of maize. A similar trend of occurrence was observed for OTA (25% in 2012; 2% in 2013; and 18% in 2015), while FMs, DON, and ZEA dominated in 2014, which was characterized by extremely rainy and humid conditions.

Regarding the presence of AFM1 in milk and dairy products after the aflatoxins crisis which was happened in 2013 numerous studies were published (Kos et al., 2014b; Škrbić et al., 2014; Škrbić et al., 2015; Tomašević et al., 2015; Torović et al., 2021) dealing with the determination of AFM1 in these products. As already mentioned prolonged drought during the spring and summer of 2012 affected the contamination of maize by AFs and consequently the contamination of milk and dairy products. To protect the struggle of the Serbian dairy industry, the authority changed the MRL for AFM1 from 0.05 to 0.5 μg/kg. In the following years, the MRL for AFM1 was changed several times (from 0.25 μg/kg to 0.5 μg/kg). The comprehensive study published by Kos et al., (2014b) investigated the AFM1 content in 175 milk samples (cow's, goat's, donkey's, and breast milk) and 1 infant formula sample. AFM1 was quantified in 98.7% of analyzed cow's milk samples, while 86.0% of cow's milk samples had a concentration greater than the MRL of 0.05 μg/kg defined by EU Regulation. Another comprehensive investigation of the AFM1presence in 1438 samples (678 samples of raw milk, 438 thermally processed milk and 322 dairy products (white cheeses, hard cheeses, yoghurts, ice creams and baby formulas)) of milk and dairy products was carried out by Tomašević et al. (2015) in the period 2013-2014. Results indicated that AFM1 content exceeded the EU MRL in 56.3% of raw milk, 32.6% of heat-treated milk, and 37.8% of milk product samples. It is interesting to note that the highest mean concen-

trations of AFM1 were quantified in the milk powders (mean value of 0.847 μg/kg). Another very important study was carried out by Milićević et al. (2019) where 20235 samples of milk were analyzed during the four years of investigation (2015-2018).

The main aim of the study was to evaluate the occurrence of AFM1 associated with climate change by using chemometrics and multivariate techniques. The results showed that among the selected environmental variables (temperature, relative humidity, drought, and stress condition), temperature and moisture significantly influenced AFM1 production.

In the absence of the EU regulation, the market placement of cheese is subject only to national regulations. However, cheese and other dairy products are not included in the Serbian regulation. The studies dealing with the determination of AFM1 in milk products are also conducted after the outbreak with mycotoxins that happened in 2013 (Tomašević et al., 2015; Škrbić et al., 2015; Torović et al., 2021). In the study published by Škrbić et al., (2015) the presence of AFM1 was investigated in commercial and homemade white and hard cheeses. In 17.3% of analyzed commercial and 40% of hard cheese samples, the concentrations of AFM1 were above the maximum allowed level of 0.25 μg/kg sets by the national regulations of Austria, Switzerland, and France for AFM1 in cheese.

Although the AFM1 was determined in homemade cheese samples (in 10 out of 21 analyzed samples), all the quantified concentrations were lower than 0.250 μg/kg. Cheese contamination with the AFM1 was also investigated in the period 2019-2020, where 60 domestic and imported cheese samples were analyzed (Torović et al., 2021). In 75% of soft and semi-hard and 43% of hard cheeses samples, the concentrations of AFM1 were higher than 0.025 (Maximum Detection Level, MDL) μg/kg. In total, 3.3% of domestic hard cheese samples exceeded the established limit of 0.250 μg/kg (Torović et al., 2021).

The multi-mycotoxins methods were not very often used in mycotoxins analysis of foodstuffs in Serbia (Škrbić et al., 2012; Škrbić et al., 2014b; Škrbić et al., 2017; Hajnal et al., 2020; Kos et al., 2020). In 2013, a multi-mycotoxins method based on crude extract analysis was used for mycotoxins analysis in various types of nuts (walnut, hazelnut, peanut, almond). Weak contamination of nuts samples was observed. Out of 17 analyzed samples, only two samples showed detectable levels of ZEA in concentrations of 1.20 and 3.48 μg/kg (Škrbić et al., 2014b). The second study dealing with the mycotoxins (AFs, OTA, ZEA, DON, HT-2, and T-2) analysis was carried out on cereal-based products (biscuits (n=39), cookies (34)), dried fruits (14), and fruit jams (10) (Škrbić et al., 2017). Biscuit samples were found to be contaminated with ZEA (mean value of 2.64 μg/kg), OTA (4.10 μg/kg), T-2 (8.13 μg/kg), AFB1 (1.32 μg/kg), while fruit jams were found to be contaminated with OTA (mean value of 17.7 μg/kg), T-2 4.37 (μg/kg), AFB1 (2.0 μg/kg) and AFB2 (1.15 μg/kg) mycotoxins, respectively. The majority of analyzed samples complied with the EU MRL regulation except for eight biscuits and three fig jam samples where contents of OTA were higher than the existing OTA limits. Figure 1 presents the overview of the mycotoxins which were analyzed in cereals and foodstuffs produced in Serbia based on the data presented in Table 1. Although T-2 and HT-2 toxins are not regulated mycotoxins by the EU and Serbian regulations, as a consequence of their frequent determination in cereals and foodstuffs originnating from the Serbian market, T-2 and HT-2 toxins are included in this review paper.

Although this review deals with the overview of regulated mycotoxins presence in cereals and foodstuffs produced in Serbia, it is interesting to mention the study published by Janić Hajnal et al., (2020) which dealt with the non-regulated mycotoxin and other fungal metabolites analysis in maize (n=204) harvested in Serbia in the period 2012–2015. The mentioned study covers the periods of extreme drought (2012), extreme precipitation and flood (2014), and moderate drought conditions (2013 and 2015). In each of the analyzed samples between 13 to 55 non-regulated fungal metabolites were detected with a total of 109 different non-regulated fungal metabolites found. The highest frequency of occurrence was observed for moniliformin and beauvericin.

Table 1.
An overview of regulated mycotoxins presence in analyzed cereals and foodstuffs produced in Serbia

Mycotoxins analyzed	**Matrix**	**Sample prep/ Instrumental analysis**	**Number of samples analyzed/Year**	**Concentration range/ mean concentration, µg/kg**	**Reference**
FB1, DON ZEA, T-2,	Winter wheat	ELISA	103/**2005 and 2007**	2005 FB1: 750-5400 (2079.45) DON: 52-3306 (605.52) ZEA: 10-143 (19.74) T-2: 60-495 (171.52) 2007 FB1: 750-4900 (918.76) DON: 50-1090 (282.84) ZEA: 16-201 (29.01) T-2: 86-200 (86.75)	Stanković et al. (2012)
DON, ZEA, HT2	Wheat	Crude extract analysis /UHPLC-MS/MS	54/**2007**	DON: 41-309/33 HT-2: 128-129/9	Škrbić et al., (2011)
AFB1, AFB2, AFG1, AFG2, OTA	Red pepper powder, white and black pepper	crude extract analysis /UHPLC-MS/MS	13 red pepper, 2 black pepper, 2 white pepper , 17/**2010**	n.d.	Škrbić et al., (2013)
DON	Wheat and barley	SPE/LC-DAD	128 wheat, 11 barley/**2010**	Wheat: 64 – 4808/546 Barley: 118 – 355/306	Jajić et al., (2014)
AFB1, AFB2, AFG1, AFG2	Maize	ELISA	380/**2009-2012**	2009: n.d. 2010: n.d. 2011: n.d. 2012: 1.01 - 86.1/36.3	Kos et al., (2013)
AFB1, AFB2, AFG1, AFG2, FB1, FB2, DON, ZEA, HT-2 toxin, T-2 toxin, OTA	Wheat flour	Crude extract analysis /UHPLC-MS/MS	15/**2011**	DON: 17.5-976/325 ZEA: 1.9-21.1/4.6 T-2: 9.8-26.9/4.1	Škrbić et al., (2012)
FMs, DON, ZEA, HT-2,T-2	Maize	ELISA	90/**2012**	FMs: 500.4-5800/1730 DON: 600.0-700.0/650 ZEA: n.d. HT-2: < LOD (0.0002) - 1.44/ 0.30 T-2: 25.09-209.0/50.93	Kos et al. 2014a
AFM1	Milk	SPE-UHPLC-MS/MS	50/**2013**		Škrbić et al., (2014a)
AFB1, AFB2, AFG1, AFG2, FB1, FB2, ZEA, HT-2 toxin, T-2 toxin, OTA	Nuts (walnut, hazelnut, almond, and peanut)	crude extract analysis /UHPLC-MS/MS	17/**2013**	ZEA (walnut): 1.20 - 3.48	Škrbić et al., (2014b)
AFM1	Cow's milk,	ELISA	150 cow's milk,	Cow: 0.01-1.2 /0.144	Kos et al.,

	goat's milk, donkey's milk, breasts milk 1 infant formula		10 goat's milk, 5 donkey's milk, 10 breasts milk, 1 infant formula, 176/**2013**	Goat: 0.008-0.240/0.080 Donkey: 0.005-0.035/0.020 Breasts milk: 0.001-0.022/0.010 Infant formula: 0.02/0.02	(2014b)
AFM1	Raw milk, heat-treated milk, and milk product	ELISA	678 raw milk, 438 heat-treated milk, 322 milk products, 1438/**2013-2014**	*Raw milk: 0.282 (mean) Heat-treated milk: 0.090 Milk products: 0.268	Tomašević et al. (2015)
AFM1	Milk, infant formulae	IAC -HPLC-FLD	80 milk, 21 infant formulae/**2013 and 2014**	Milk (2013): 0.024-0.319 /0.134 Milk (2014): <LOD-0.104, 0.026 Infant formulae (2013): in only one sample, 0.020	Torović (2015)
AFB1, AFB2, AFG1, AFG2, DON, ZEA, HT-2 toxin, T-2 toxin, OTA	Biscuits, cookies, dried fruits, and fruit jams	Crude extract analysis /UHPLC-MS/MS	39 biscuits, 34 cookies, 14 dried fruits, 10 fruit jams/**2014**	Biscuits: AFB1: 0.91 - 1.92/1.32; ZEA: 1.05-8.86/2.64; T-2: 2.90-26.7/8.13; OTA: 1.75–11.6/ 4.10 Cookies: OTA: 18.3 Dried fruits: n.d. Fruit jams: AFB1: 0.93–4.17/2.00; AFB2: 1.15; T-2: 3.82–4.92/4.37; OTA: 12.0-25.0/17.7	Škrbić et al., (2017)
AFM1	White and hard type of cheese	Crude extract for white cheese, SPE for hard cheese - UHPLC-MS/MS	23 commercial white cheese, 21 homemade white cheese, 10 hard cheese/**2014**	White cheese: 0.13 - 0.55/0.11 Hard cheese: 0.08 – 2.23/0.64 Household white cheese: 0.13 - 0.22/0.08	Škrbić et al., (2015)
AFM1	Milk	ELISA/LC-MS/MS	20235/**2015-2018**	2015: 0.005-1.26 2016: 0.005-1.10 2017:0.005-1.09 2018:0.005-1.09	Milićević et al., (2019)
AFB1, AFB2, AFG1, AFG2	Maize	SPE /HPLC-FLD	180/**2015**	AFB1: 1.3–88.8/11.4 AFB2: 0.60–2.8/1.3 AFG1: 1.8–28.5/8.6 AFG2: 2.1–7.5/3.8	Janić Hajnal et al., (2017)
DON	Maize	ELISA	1800/**2013-2015**	2013: 260.1-1388/642.3 2014: 260.4-9050/3063.3 2015: 252.3-6280/921.1	Kos et al., (2017)
AFB1, AFB2, AFG1, AFG2, AFM1, FMs, DON, ZEA, OTA	Maize	Crude extract/ LC-MS/MS	204/**2012-2015**	2012 AFB1: 0.6-205/44 AFB2: 0.7-22/5 AFG1: 0.4–141/10 AFG2: 2–73/16 AFM1: 0.5–7/2 DON:10-1855/128 FB1:211- 13396/4121	Kos et al., (2020)

				FB2: 72-3118/828 ZEA: 5-6726 OTA: 2-318/53 2013 AFB1: 0.5-48/8 AFB2: 0.7-2/1 AFG1:3/- AFG2: n.d. AFM1: 0.6 DON: 21-436/120 FB1: 88-16187/4690 FB2: 20-3811/967 ZEA: 1-97/17 OTA: 1/- 2014 AFB1: n.d./- AFB2: n.d./- AFG1: n.d./- AFG2: n.d./- AFM1: n.d./- DON: 428-16350/3522 FB1: 193-27103/5846 FB2: 160-4651/1463 ZEA: 15-2596/598 OTA: n.d./- 2015 AFB1: 0.4-41/8 AFB2: 0.8-2/2 AFG1: 0.3-1/0.8 AFG2: n.d./- AFM1: 0.5-0.7/0.6 DON: 22-460/84 FB1: 192-4253/1905 FB2: 46-1019/431 ZEA: 1-58/14 OTA: 0.5-27/6	
AFB1, AFB2, AFG1, AFG2	Maize kernels	ELISA / HPLC-FLD	700 for AFs determination, 32 for AFB1 determination) /**2012-2013**	AFs: <1->50 AFB1: 9.50-183/-	Kos et al., 2018
AFM1	Soft cheese, semi-hard/hard cheese	ELISA / HPLC-FLD	36 (domestic origin), 24 imported from EU/**2019- 2020**	*Soft cheese: 0.026-0.237/0.061 Semi-hard/hard: 0.030-0.591/0.075	Torović et al., (2021)
AFB1	Peanut-based products, maize milling products and maize-based products, tree nuts, rice, millet, mixed composition	Immunoaffinity Columns/ ELISA-LC-MS/MS	94 peanut-based products, 117 maize milling products and maize-based products, 125 tree Nuts, 41 rice, 14 millet , 41mixed composition products, 25 dried figs,	*Total peanuts and peanut based products: 0.25-13.10 Total maize and maize based products: 0.28–28.15 Total mixed (muesli, flips, etc.) composition products: 0.56-1.55 Total nuts: 0.21-3.36 Dried figs: 1.01–1.51 Rice: 0.17–1.60 Milet: 1.03–2.27 Dried ground	Udovicki et al. (2021)

	products, dried figs, and dried ground red paprika.	6 dried ground red paprika, 463/**2017-2019**	Red paprika: 0.5–2.88

*n.d. mycotoxins were not detected; *the data are provided for the whole studied period*

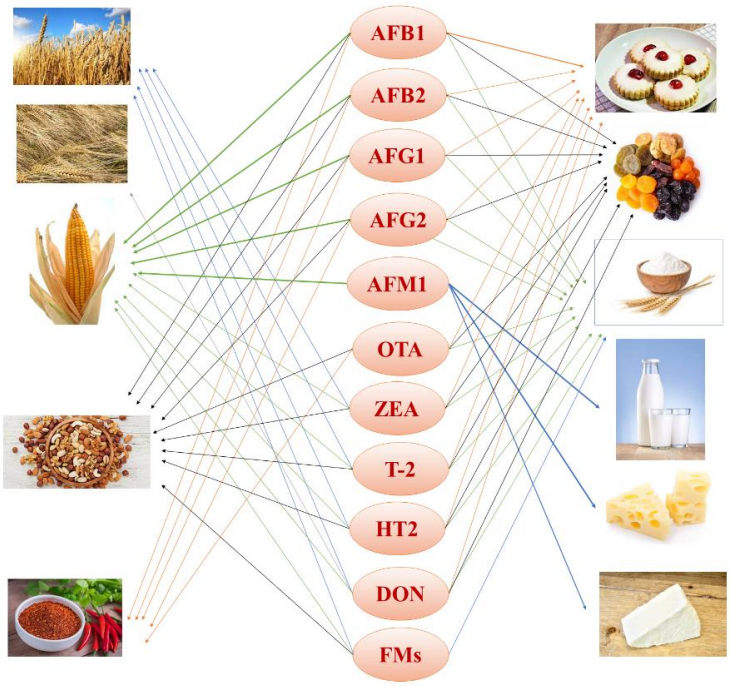

Figure 1. Overview of matrices originated from Serbia in which the mycotoxins were investigated based on the data provided in Table 1

DISTRIBUTION OF MYCOTOXINS WORLDWIDE

Mycotoxins' presence in feed, animal-derived food products, cereals, cereal-based products, and other foodstuffs is a topic that attracts considerable attention worldwide over the years. Numerous review studies provide overviews of thousands of investigations on the presence of regulated and non-regulated mycotoxins in foodstuffs (Tolosa, Rodríguez-Carrasco, Ruiz & Vila-Donat, 2021; Leite et al., 2021). Also, several international organizations such as World Health Organization (WHO), the European Food Safety Authority (EFSA), and Food and Agriculture Organization (FAO) provided databases regarding the occurrence of mycotoxins. These data are valued in risk assessment studies with the main goal of evaluating the risk to human and animal health when mycotoxins contamination is under consideration (Perrone et al., 2020).

The distribution and co-occurrence of mycotoxins varied among the region where the investigations were carried out (climate-related abiotic conditions) as well as among the type of matrices (crops or foodstuffs) that were considered. In Europe, the contamination of maize, nuts, spices, and dried fruit with AFs is rare to occasional. On the other hand, the contamination of wheat, barley, maize, coffee, wine, and beer with OTA is frequent as well as the contamination of wheat, maize, and barley with DON and T-2/HT-2 toxins. Furthermore, frequent contamination of maize samples with FBs and ZEA was observed in Europe also (Perrone et al., 2020). For example, DON and ZEA were the most prevalent mycotoxins in the maize chain (grain-maize and whole maize plants) according to the review article dealt with the description and evaluation of studies on the presence of regulated, masked, and emerging mycotoxins in maize food (Leite et al., 2021), while AFB1 was the major toxin of

dairy feed followed by DON and ZEA. Regarding the presence of mycotoxins in raw feed material AFs, FMs, OTA, trichothecenes, and ZEA were the most prevalent ones, while AFM1 and OTA were the most prevalent mycotoxins in milk and dairy products and meat by-products, respectively (Tolosa et al., 2021).

When it comes to contamination of cereal and cereal-derived food products, AFs represent a significant problem in Africa and Asia with a frequency of occurrence up to 63%, while a low frequency of occurrence (15%) was observed in South America. As was already mentioned, DON very often contaminates wheat samples with a frequency of occurrence of 50% and 76% in Asia and Africa, respectively. FMs follow a similar trend as DON in Asia and Africa (frequency of occurrence of 62% for both mycotoxins). Interestingly, the high frequency of occurrence of FMs was observed in North and South America (up to 95%). Regarding the ZEA low frequency of occurrence was reported for Asia (15%), while the incidence of ZEA in Africa is high (59%). OTA was found with a frequency of occurrence of 42% in North America (Lee & Ryu, 2017).

FUTURE CHALLENGES

Although more than 400 mycotoxins are known to date, the most commonly monitored and analyzed are regulated mycotoxins such as AFs, FMs, DON, ZEA and OTA (Agriopoulou et al., 2020). Together with these mycotoxins in food and feed can occur so-called "masked", "modified" and "emerging" mycotoxins, which are less known from a scientific point of view (Streit et al., 2013). Masked mycotoxins are plant metabolites of mycotoxins or “biologically-modified” mycotoxins (Rychlik et al., 2014). Data from the literature indicate that deoxynivalenol-3-glucoside (DON-3-G) and zearalenone-sulphate (ZEA-S) are the most common masked mycotoxins in food and feed (Scarpino, Vanara, Sulyok, Krska & Blandino, 2021). Among the group of emerging mycotoxins, aflatoxin precursors, ergot alkaloids, enniatins (ENN), beauvericin (BEA) and moniliformin (MON) are the most frequently reviewed and described (Agriopoulou et al., 2020). To date, there are no regulations for emerging mycotoxins and risk assessment studies are still ongoing because available data on the toxicological effects of these compounds and their synergistic or additive toxic effects are limited. Since little is known about the occurrence and the fate of masked, modified, and emerging mycotoxins in food and feed, the trend to investigate these forms of compounds have become the focus of researchers in recent years (Schollenberger, Müller, Rüfle, Suchy & Drochner, 2008; Scarpino, Vanara, Reyneri & Blandino, 2020). Lu et al. (2020) reported that modified mycotoxins, together with other toxins, were detected in foodstuffs, animal feeds, and herbal medicines and widespread in Europe, North America, and Asia. Taking into account their prevalence and scarce data that indicate the occurrence, properties, and health hazards caused by their presence in food, processing steps, ongoing investigations and monitoring seem to be necessary. Detection of masked mycotoxins and determination of their content in matrices of interest remains a major analytical challenge. The reason is the unavailability of analytical and isotope-labeled standards that are necessary for the accurate determination of mycotoxins. Although LC-MS/MS is a widely used technique for the simultaneous determination of masked mycotoxins, adequate sample preparation is still necessary to reduce the interference of the remaining coeluting matrix constituents. As known, the transfer of modified mycotoxins usually occurs during food processing as well as from raw materials to the final products. Furthermore, during food processing, modified mycotoxins can be degraded or lost, but also partially converted into their parent forms by external processing. However, it should be emphasized that pharmacokinetic information for metabolites of modified mycotoxins remains unknown. Thus, a reliable biomarker of exposure is required to adequately assess the intake of modified mycotoxins (Lu et al., 2020). Considering the limited data on the presence of masked and emerging mycotoxins in Serbian market baskets, further research should be focused on the analysis of these contaminants. However, the study published by Jajić et al. (2019) offers rare data on emerging mycotoxins (MON, ENs, BEA and FUS) belonging to the *Fusarium* mycotoxin group and analyzed in Serbian maize samples (n = 190) harvested in the period from 2016 to 2018. Among the analyzed emerging mycotoxins, MON, BEA, and FUS were quantified with a frequency of occurrence higher than 20%. The mean values ranged from 72.40 to 12272.00

μg/kg, 1.96–34.79 μg/kg, and 34.40–920.10 μg/kg for FUS, BEA, and MON, respectively.

Besides the above-mentioned challenge related to modified mycotoxins, another currently extremely unexplored topic is the occurrence of mycotoxins in soil and the environment (Juraschek et al., 2022). Literature data indicated that only a few studies were carried out to explore the environmental fate of mycotoxins and their occurrence and behavior in soils (Accinelli, Abbas, Zablotowicz & Wilkinson, 2008; Hartmann et al., 2008a; Muñoz et al., 2015 and 2017). The dominant sources of mycotoxins in the soil are plant residues that remain in the field after harvest and mycotoxins introduced through the application of manure. In this way, the soil becomes a potential reservoir of mycotoxins that can cause contamination of plants and animals due to contact with the soil and watercourses into which mycotoxins arrive due to soil leaching and erosion (Hartmann et al., 2008b). All this indicates the need to include soils and agro-environmental samples in the ecological assessment of mycotoxins.

Furthermore, it should be observed that climate change and the warming of the climate system will have a significant influence on the life cycles of toxigenic fungi. This means that the interactions between the particular plant host and toxigenic fungi will be changed. This will result in changing the resistance of some plant hosts to fungal infections for which the plant host had shown different levels of resistance in the past. In addition, the same plant host will be attacked by different toxigenic fungi resulting in multi-mycotoxin contamination resulting in changes in traditional host-pathogen interactions (Perrone et al., 2020).

CONCLUSIONS

To demonstrate acceptable accuracy of mycotoxins quantification in food matrices, appropriate sample selection and validated sample preparation procedures are very important. In most cases, a clean-up step needs to be involved in the sample preparation method for removing the matrix interferences which can lead to false-positive results. Finally, the selection of convenient instrumentation is probably the crucial step for obtaining adequate detection limits and reliability in mycotoxins quantification. All the mentioned steps need to be validated for obtaining results that are accurate, precise, reliable, and reproducible. The instrumental techniques based on mass spectrometry may satisfy all the mentioned requirements to fit the purpose of mycotoxins quantification in all types of foodstuffs, especially for multiple mycotoxins methods as well as quantification of masked or modified mycotoxins. ELISA has long been the technique of choice for mycotoxin quantification due to the inability of scientific centers and laboratories to purchase modern equipment based on mass spectrometry. Recently, this negative experience was changed, and most of the published papers used sophisticated equipment mainly based on mass spectrometry. According to the review articles dealing with the mycotoxin's determination in food matrices originating from Serbia, it can be concluded that the years 2012 and 2013 were the most demanding periods where the Serbian human population was at the highest risk regarding the AFB1 and AFM1 contamination. Additionally, the study undertaken in the period from 2015-2018 revealed the high frequency of exceedance of EU MRL level for AFM1. As a consequence, systematic monitoring of AFM1 presence in milk and dairy products should be enforced as there is evidence that the risk to the Serbian human population was not limited to the period when the AFM1 crisis happened (2013). Since environmental conditions in Serbia can be favourable for the occurrence of mycotoxigenic fungi, future monitoring of "traditional" but also "emerging" toxins is essential for producing safe food and feed. Additionally, the data reviewed in this study may be used for the evaluation of year-to-year mycotoxins' presence in some of the most important cereals and foodstuffs. The review article provides evidence that the co-occurrence of mycotoxins in feed raw materials and food tends to be the rule rather than the exception. As a consequence, it can be concluded that the multi-matrix methods are favorable in mycotoxins analysis.

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OSVRT NA PRISUSTVO MIKOTOKSINA U HRANI SA SRPSKOG TRŽIŠTA U PERIODU OD 2005. DO 2022. GODINE

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Sažetak: Pregledni rad ima za cilj da prikaže publikacije koje su se bavile analizom prisustva mikotoksina u žitaricama i hrani proizvedenoj u Srbiji u periodu od 2005. godine do danas. U preglednom radu su obrađeni najvažniji koraci u analizi mikotoksina kao što su uzorkovanje, priprema uzoraka, instrumentalna analiza i raspodela koncentracija kvantifikovanih mikotoksina u matriksima od interesa. Takođe, poseban osvrt dat je na poređenje određenih koncentracija mikotoksina sa regulativom Evropske unije koja definiše maksimalno dozvoljene sadržaje u hrani i hrani za životinje. Pregledni rad ukazuje na važnost primene multi-mikotoskin metoda u analizi mikotoksina pri čemu se dobijaju podaci o istovremenom prisustvu većeg broja mikotoksina u ispitivanim uzorcima. Time se dobija realnija slika o mogućim štetnim uticajima višekomponentne kontaminacije uzoraka po zdravlje humane populacije u Srbiji. Najčešće korišćena tehnika u analizi mikotoksina do sada je bila ELISA, a sledeća u nizu je ultrapritisna tečna hromatografija sa trostrukim kvadruponim masenim analizatorom. Najveći broj istraživanja sprovedenih u Srbiji u periodu od 2005. godine do danas bio je usmeren na analizu mikotoksina u kukuruzu, pšenici, mleku i mlečnim proizvodima. Retke su studije koje su se bavile analizom specifičnih matriksa kao što su orašasti plodovi, suvo voće, biskviti, začini. Pregledom dostupne literature ustanovljeno je da je kontaminacija mleka i mlečnih proizvoda aflatoksinom M1 (AFM1), koja se desila početkom 2013. godine, bila jedna od najvećih pretnji po zdravlje stanovništa u Srbiji kada se uzme u obzir prisustvo regulisanih mikotoksina u hrani. Kontaminacija mleka i mlečnih proizvoda AFM1 bila je posledica kontaminacije hrane za životinje aflatoksinom B1, koja se desila 2012. godine, usled nepovoljnih klimatskih uslova. Nadalje, dostupna literatura ukazala je da ne postoji veliki broj studija koje su se pored prisustva regulisanih mikotoksina, bavile i utvrđivanjem prisustva „maskiranih“ i „novootkrivenih“ mikotoskina u hrani sa srpskog tržišta.

Ključne reči: *aflatoksini, trihoteceni, hrana, instrumentalne tehnike, Srbija*

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