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ANTIOXIDANT POTENTIAL OF *Clinopodium menthifolium, Satureja montana* AND *Salvia Sclarea* (Lamiaceae) EXTRACTS

ABSTRACT: Plants which belong to Lamiaceae family are good potential sources of natural antioxidants useful for preventing oxidative stress-related diseases. The food industry is becoming increasingly interested in aromatic herbs, including plants from Lamiaceae family, because of their anti-inflammatory properties and antioxidant activities, due to growing consumer demands for healthy foods of natural origin. In the present investigation, the comparative antioxidant potential of aqueous and acetone extracts of three Lamiaceae species are described: *Clinopodium menthifolium* (Host), *Satureja montana* L., and *Salvia sclarea* L., using three methods: 2,2–azinobis (3–ethyl–benzothiazoline–6–sulfonic acid) radical (ABTS) scavenging, 1,1–Diphenyl–2–picrylhydrazyl radical (DPPH) scavenging, and ferric reducing antioxidant power (FRAP) assay and their correlations with total phenolic and flavonoid contents. Antioxidant capacity showed a positive relationship comparing three above mentioned tests. Antioxidant capacity detected by antioxidant ABTS, DPPH, and FRAP assays was positively correlated with total phenolics content. Aqueous extract of *C. menthifolium* showed greater antioxidant potential.

KEYWORDS: ABTS assay, *Clinopodium menthifolium* (Host), DPPH assay, FRAP value, *Salvia sclarea* L., *Satureja montana* L.

INTRODUCTION

Plants or medicinal herbs may contain a wide variety of molecules which are rich in antioxidant activity (Cai et al., 2004). The concept of antioxidant capacity describes the ability of redox molecules to scavenge free radicals (Floegel et al., 2011), such as reactive oxygen species (ROS): superoxide anions ($O_2^{-\bullet}$), and hydroxyl ($^{\bullet}OH$) or hydroperoxyl ($HO_2^{\bullet}$) radicals. The defensive ef-

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fects of a natural antioxidant are mostly related to three major groups: vitamins (ascorbic acid), phenolic compounds (e.g. phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), and carotenoids (Thaiponga et al., 2006; Halliwell, 1996).

The plants chosen for the present study are flowering plants belonging to the family Lamiaceae, also called the mint family (Raja, 2012). The Lamiaceae family consists of approximately 200 genera and between 3,200 and 6,500 species with a cosmopolitan distribution, particularly well represented in tropical areas such as the Mediterranean region (Derakhshani et al., 2012; Dorman et al., 2004). The aerial parts of most aromatic plants belonging to family Lamiaceae are added to foods for their organoleptic properties (Dorman et al., 2004). Some of Lamiaceae species are used in folk phytomedicine (Matkowski et al., 2008). Capecka et al. (2005) reported that fresh and dried herbs of three Lamiaceae species (lemon balm, oregano, and peppermint) are rich sources of antioxidants, particularly from the group of phenolic compounds. Lamiaceae plants are good potential sources of natural antioxidants useful for either prevention or treatment of oxidative stress-related diseases (Firuzi et al., 2010). Because of their medicinal and anti-inflammatory properties or antioxidant activities, the food industry is becoming increasingly interested in aromatic herbs due to growing consumer demands for healthy foods of natural origin (Hossain et al., 2010).

One antioxidant assay cannot evaluate all types of antioxidants present in plant extracts (Ajaib et al., 2013). Therefore, several different assays have been frequently used to evaluate antioxidant capacities in plants including 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and the oxygen radical absorption capacity (ORAC) (Thaiponga et al., 2006). In the present investigation, the comparative antioxidant potential of aqueous and acetone extracts of three Lamiaceae species are presented: *Clinopodium menthifolium* (Host), *Satureja montana* L., and *Salvia sclarea* L., using three methods: 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) radical (ABTS) scavenging, 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) scavenging, and ferric reducing antioxidant power (FRAP) assay and their correlations with total phenolic and flavonoid contents.

MATERIALS AND METHODS

Plant materials

The wild, aromatic plants, *Clinopodium menthifolium* (Host) and *Satureja montana* L. were collected at locality near the Adriatic coast in Montenegro in May–June 2012. The aerial parts of the flowering plant *Salvia sclarea* L. were collected in the south of Serbia, in July 2012. Voucher specimens of collected plants were numbered 2-1543, 2-1544 and 2-1545 (*C. menthifolium*, *S.*

montana, and *S. sclarea*, respectively) deposited in the Herbarium of The Department of Biology and Ecology, Faculty of Science, University of Novi Sad. The geographical locations of the sampling areas are given in Table 1.

Table 1. Geographical location of the sample areas

Sample	Longitude	Latitude	Altitude (m)
Clinopodium menthifolium	19.00.30,10 E	42.09.52,19 N	31
Satureja montana	19.20.02,17 E	42.32.23,21 N	123
Salvia sclarea	21.53.09,23 E	42.22.40,44 N	494

Extractions

The air-dried plant materials were ground into powder. The powdered materials (0.2 g) were extracted with 70% acetone and distilled water (10 mL) by maceration for 24 h in both solvents, respectively. After 24 h, the extracts were filtered through filter paper and kept at 4 °C until application.

Determination of total phenolic and flavonoid contents

The total phenolic content of *C. menthifolium*, *S. montana*, and *S. sclarea* extracts were determined according to the Folin-Ciocalteu method (Hagerman et al., 2000). The 0.02 mL of extract, 3.36 mL of deionized water, 0.4 mL of 20% sodium carbonate, and 0.2 mL of 33% Folin-Ciocalteu reagent were mixed using a Vortex. The solution was incubated at room temperature for 30 min. The absorbance of the reaction mixture was measured at 720 nm using a spectrophotometer (Thermo Scientific Evolution 220). The results are expressed as mg gallic acid equivalents/g dry weight (mg GA equivalents g^{-1} d.w.).

The total flavonoids were estimated according to the method described by Marckam (1989). Extract (0.4 mL) was mixed with 1 mL of deionized water and 2.5 mL of 2% aluminum chloride hexahydrate solution. After incubation at room temperature for 15 min, the absorbance of the reaction mixture was measured at 430 nm. The data are expressed as mg rutin equivalents/g dry weight (mg rutin equivalents g^{-1} d.w.).

Antioxidant activity determinations

For ABTS assay, the procedure followed the method of Re et al. (1999) with some modifications. The stock solutions included 7.4 mM $ABTS^{\cdot+}$ solution and 2.6 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities for 12 h in the dark. The 0.1 mL of extract was mixed with 2 mL of the $ABTS^{\cdot+}$ solution. Af-

ter incubation at room temperature for 2 h in a dark condition, the absorbance of the reaction mixture was measured at 734 nm. Results are expressed in mg trolox equivalents/g dry weight (mg TE g^{-1} d.w.).

The DPPH assay was done according to the method of Lee et al. (1998). The DPPH solution was prepared by dissolving DPPH with 70% acetone. The 0.01 mL was mixed with 3 mL DPPH solution using a Vortex. The solution was incubated at room temperature for 30 min. The absorbance of the reaction mixture was measured at 517 nm. Results are expressed in mg trolox equivalents/g dry weight (mg TE g^{-1} d.w.).

The FRAP assay was done according to Benzie and Strain (1999) with some modifications. The stock solutions included 300mM acetate buffer (pH 3.6), 10mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3x6H_2O$ solution. The fresh working solution was prepared by mixing acetate buffer, TPTZ solution, and $FeCl_3x6H_2O$ solution in the ratio 10 : 1 : 1. The 0.05 mL of extract was mixed with 3 mL of the FRAP solution. The absorbance of the reaction mixture was measured at 593 nm. Results are expressed in mg trolox equivalents/g dry weight (mg TE g^{-1} d.w.).

Statistical analysis

Each antioxidant activity assay was done three times from the same extract in order to determine their reproducibility. Analysis of variance was used to test any difference in antioxidant activities resulting from these methods. Correlations among data obtained were analyzed using STATISTICA for Windows version 11.0.

RESULTS AND DISCUSSION

Very important plant constituents, plant phenolics, are aromatic metabolites that possess one or more phenolic hydroxyl groups. They appear to serve a variety of essential physiological functions like screening high levels of visible and UV light (Smirnof, 2005) and defend plants from infection and injury (Ćetković et al., 2007). Also, phenolics have an important role in general protection against oxidative stress (Smirnof, 2005) acting as hydrogen-donating antioxidants with reactive oxygen and reactive nitrogen species (Pereira et al., 2009), inactivating free radicals or preventing decomposition of hydroperoxides into free radicals (Ćetković et al., 2007). Thus, the phenolic content of plants is correlated with their antioxidant activity (Ajaib et al., 2013).

The content of total phenolics and total flavonoids in acetone extracts of three Lamiaceae species *C. menthifolium*, *S. montana*, and *S. sclarea* are given in Table 2. The total phenolic content of the acetone extracts was 61.58 ± 2.26 mg gallic acid equivalents/g dry weight for *C. menthifolium*, 53.97 ± 3.90 mg

gallic acid equivalents/g dry weight for *S. montana* and 21.20 ± 0.49 mg gallic acid equivalents/g dry weight for *S. sclarea*. The amount of total phenolic content was significantly different among *C. menthifolium*, *S. montana,* and *S. sclarea* acetone extracts, and the minimum amount was observed in the *S. sclarea* extract. The total flavonoid content was 7.25 ± 0.35 mg rutin equivalents/g dry weight for *C. menthifolium*, 3.50 ± 0.09 mg rutin equivalents/g dry weight for *S. montana*, 5.29 ± 0.11 mg rutin equivalents/g dry weight for *S. sclarea*. The total phenolic and flavonoid content depends on applied extraction means. Ćetković et al. (2007) found that the quantity of phenolic compounds was significantly higher in ethyl acetate and n-butanol *S. montana* extracts than in other investigated extracts. Furthermore, Gülçin et al. (2004) found that acetone extract of *S. sclarea* had higher total phenolic compounds than chloroform extract.

Table 2. Total phenolic and flavonoid contents of *C. menthifolium*, *S. montana* and *S. sclarea* acetone extracts

Plants	Total phenolics[a]	Total flavonoids[b]
Clinopodium menthifolium	61.58 ± 2.26	7.25 ± 0.35
Satureja montana	53.97 ± 3.90	3.50 ± 0.09
Salvia sclarea	21.20 ± 0.49	5.29 ± 0.11

The data are mean values ± standard error. [a]Total phenolics content expressed in mg gallic acid equivalents/g dry weight. [b]Total flavonoids content expressed in mg rutin equivalents/g dry weight.

In the present study, the antioxidant activity of *C. menthifolium*, *S. montana,* and *S. sclarea* aqueous and acetone extracts was examined using DPPH, ABTS and FRAP tests. The DPPH test is based on the reduction of the purple $DPPH^{\cdot}$ radical to DPPH-H, whereas colour changes from purple to yellow. The $DPPH^{\cdot}$ radical is widely used because of the ease of the reaction (Baba and Malik 2015) and rapid way to measure antioxidant activity (Mensor et al., 2001). The second one is $ABTS^{\cdot+}$ radical which is a relatively stable radical and often preferred in the assessment of radical scavenging activity (Koleva et al., 2002). The ABTS test is based on the generation of a blue/green $ABTS^{\cdot+}$ radical that can be reduced by antioxidants. The FRAP assay is based on the ability of antioxidants to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}).

Table 3. Antioxidant activity of *C. menthifolium*, *S. montana* and *S. sclarea* aqueous and acetone extracts

Extracts	*Clinopodium menthifolium*	*Satureja montana*	*Salvia sclarea*
ABTS			
70 % acetone	126.1 ± 1.55	122.2 ± 0.75	28.05 ± 2.85
distilled water	219.4 ± 2.10	91.10 ± 8.10	14.90 ± 2.70
DPPH			
70 % acetone	40.35 ± 1.30	37.05 ± 1.40	19.75 ± 5.50
distilled water	76.30 ± 4.50	49.30 ± 1.90	09.90 ± 2.80
FRAP			
70 % acetone	31.64 ± 3.60	52.05 ± 0.75	10.30 ± 1.05
distilled water	82.13 ± 1.80	48.55 ± 3.07	11.38 ± 2.06
The data are mean values ± standard error. The results are expressed in mg trolox equivalents/g dry weight.			

The antioxidant activity of aqueous and acetone extracts of *C. menthifolium*, *S. montana*, and *S. sclarea* is presented in Table 3. The highest antioxidant capacity showed *C. menthifolium* aqueous extract (for DPPH assay: 76.30 ± 4.50 mg trolox equivalents/g dry weight; for ABTS assay: 219.4 ± 2.10 mg trolox equivalents/g dry weight, and for FRAP assay: 82.13 ± 1.80 mg trolox equivalents/g dry weight, respectively). The lowest antioxidant capacity showed *S. sclarea* aqueous extract (for DPPH assay: 9.90 ± 2.80 mg trolox equivalents/g dry weight; for ABTS assay: 14.90 ± 2.70 mg trolox equivalents/g dry weight, and for FRAP assay: 11.38 ± 2.06 mg trolox equivalents/g dry weight, respectively) and *S. sclarea* acetone extract (for DPPH assay: 19.75 ± 5.50 mg trolox equivalents/g dry weight; for ABTS assay: 28.05 ± 2.85 mg trolox equivalents/g dry weight, and for FRAP assay: 10.30 ± 1.05 mg trolox equivalents/g dry weight, respectively). It was noticed that plant extracts had different antioxidant activity through DPPH, ABTS and FRAP assays. The highest antioxidant effect showed *C. menthifolium* aqueous extract. By examining the antioxidant potential of aqueous and acetone extracts of *C. menthifolium* through all tests, higher antioxidant activity showed more polar extracts. These findings are in agreement with earlier studies which reported that more polar partitions had higher antioxidant activity in contrast to the less polar having lower antioxidant activity (Mensor et al., 2001). Miliauskas et al. (2004) found that ethyl acetate and acetone extracts of seven investigated plants were less effective $DPPH^{\cdot}$ radical scavengers compared to methanolic extract.

In the case of plant *S. montana* the degree of neutralization $ABTS^{\cdot+}$ and $DPPH^{\cdot}$ radicals depends on the applied extraction means. The higher effect in the neutralization of $ABTS^{\cdot+}$ radicals showed acetone extract, while aqueous extract was better in the neutralization of $DPPH^{\cdot}$ radicals. Koleva et al. (2002) suggested that the activity of plant extracts is influenced by the origin of the plant sample but not by the polarity.

Table 4. Correlation between antioxidant capacities measured by DPPH, ABTS and FRAP assay and total phenolic and flavonoids content.

Correlated parameters	DPPH	ABTS	FRAP	Phenolics	Flavonoids
DPPH	1	0.943*	0.948*	0.987*	0.494
ABTS	0.943*	1	0.934*	0.963*	0.603
FRAP	0.948*	0.934*	1	0.950*	0.306
Phenolics	0.987*	0.963*	0.950*	1	0.493
Flavonoids	0.494	0.603	0.306	0.493	1
*Correlation is significant, p<0,050					

It is shown in Table 4 that statistical analysis of the results established a significant positive correlation of antioxidant capacity between the antioxidant assays. The correlations ranged between 0.93 and 0.95. High correlation between antioxidant assays was found in other plants. Thaipong et al. (2006) found high correlation between DPPH, ABTS, FRAP, and ORAC in guava. Dudonné et al. (2009) reported a significant correlation between DPPH, ABTS, and FRAP assays in the 30 plant extracts. Furthermore, antioxidant capacity was strongly positively correlated with total phenolics content (for DPPH: $\rho = 0.987$; $p < 0.05$; for ABTS: $\rho = 0.963$; $p < 0.05$; for FRAP: $\rho = 0.950$; $p < 0.05$, respectively). In this study, no statistically significant correlation was observed between antioxidant activity and total flavonoids content. Poor correlation between the content of flavonoids with antioxidant activity points to the fact that extracts contain another class of compounds as dominant antioxidants.

CONCLUSIONS

By examining the antioxidant potential of aqueous and acetone extracts of *S. montana*, *C. menthifolium*, and *S. sclarea* by applying the above mentioned tests, it can be concluded that more polar extracts usually show greater antioxidant potential (aqueous extract of *C. menthifolium* showed higher antioxidant activity than acetone extract in all tests, aqueous extract of *S. montana* is of greater capacity of the neutralization of $DPPH^{\cdot}$ radicals than acetone extract). The highest antioxidant effect showed *C. menthifolium* aqueous extract. These two facts can be explained by the higher amount of polar phenolic components that contribute to the antioxidant activity such as phenolic acids, tannins, and different content of these components in the plants extracts.

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АНТИОКСИДАНТНИ ПОТЕНЦИЈАЛ ЕКСТРАКАТА БИЉАКА *Clinopodium menthifolium, Satureja montana* И *Salvia sclarea* (Lamiaceae)

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РЕЗИМЕ: Биљке фамилије Lamiaceae су добри извори природних антиоксиданаса који су корисни за спречавање болести повезаних са оксидативним стресом. Прехрамбена индустрија је све више заинтересована за ароматичне биљке, укључујући биљке из породице Lamiaceae, због њихових антиинфламаторних својстава и антиоксидативних активности. У овом раду испитан је антиоксидативни потенцијал водених и ацетонских екстраката три биљке фамилије Lamiaceae: *Clinopodium menthifolium* (Host), *Satureja montana* L. и *Salvia sclarea* L. помоћу три методе: ABTS, DPPH и FRAP, као и корелација добијених резултата са садржајем укупних фенола и флавоноида. Највећу активност уклањања

радикала показао је водени екстракт биљке *C. menthifolium*. Утврђена је позитивна корелација са садржајем укупних фенола.

КЉУЧНЕ РЕЧИ: ABTS тест, *Clinopodium menthifolium* (Host), DPPH тест, FRAP вредност, *Salvia sclarea* L., *Satureja montana* L.

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NON-NATIVE AND INVASIVE TREE SPECIES – THEIR IMPACT ON BIODIVERSITY LOSS

ABSTRACT: The paper gives an overview of non-native and invasive tree species, and offers the basic data about their current presence in European forests. It deals with definitions and classifications and explains how these species can affect new environment with the emphasis on local biodiversity loss. The process of invasion is also explained, emphasizing that there is a lag phase in which a species does not reveal its invasiveness, which can last even a few centuries (for certain species). That is why it is important to monitor non-native species and prevent their further establishment. Invasive species are less likely to develop in habitats with intense inter-species competition over a long period of time, in undisturbed areas with high biodiversity, but also in areas with the lack of nutrients, light and water or in areas where these resources are being heavily exploited by local plant communities.

KEYWORDS: biodiversity, invasive species, non-native species

INTRODUCTION

Non-native plants species are being introduced into new areas for multiple purposes, but some of them become invasive and therefore represent a serious threat to local biodiversity (Campagnaro et al., 2017). Features that contribute to a species turning into invasive are mainly morpho-anatomic and physiological features: fast growing, early flowering, high quality seed, sexual polymorphism, autogamy, allogamy, etc. (Tucović et al., 2000). This paper analyzes some of the most important topics related to this subject, with the aim to provide appropriate definitions and classification of non-native and invasive species, description of stages of invasion process, analysis of impact that invasive species have over the environment and factors that can contribute to reducing invasiveness.

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Forests cover 33% of Europe's total land area and that is equal to 215 million hectares. In Europe, coniferous forests occupy 45%, broadleaves forests 36%, and mixed-species forests 19% of total forest area (FAO, 2015).

According to the data provided by Forest Europe (2015) approximately 9 million hectares (4%) of forests in Europe are dominated by non-native tree species. There are assumptions that approximately 160 non-native tree species are grown in European forests, while 29 of them are listed as invasive in at least one country. The Features that contribute a species turning se are: *Robinia pseudoaccacia* L., *Ailathus altissima* (Mill.) Sw., *Acer negundo* L., *Eucalyptus* sp., *Fraxinus pennsylvanica* Marshall, *Prunus serotina* Ehrh., *Pseudotsuga menziesii* (Mirb.) Franco, etc. (Wohlgemuth, 2015).

In order to keep track and control of non-native invasive species in Europe many programs have been established and the most comprehensive one is DAISE (Deriving Alien Invasive Species Inventories for Europe). The main objectives of DAISE (2009) are: creating an inventory of invasive species which threaten terrestrial and aquatic ecosystems; structuring the inventory as a base for prevention and control of biological invasions; summarizing ecological, economic and health risk and impact of dominant invasive plants and distributing the results obtained.

Non-native and invasive species: definitions and classification

Non-native species are the ones with the natural occurrence outside the observed area (Jovanović, 2007). According to the USDA (2003), non-native species are those that have been "introduced from another place to exist among types of organisms with which they were not previously found". Other definition states that a non-native plant belongs to "plant taxa in a given area whose presence is due to intentional or unintentional human involvement" (Pyšek et al., 2004). The synonyms for non-native species are: "alien", "non-indigenous" or "exotic" species (Protopopova et al., 2006), and all these terms can be found in the scientific literature.

Based on the time of their introduction, non-native trees in Europe are classified as (Mosyakin and Yavorska, 2003):

- archaeophytes – introduced in pre-Columbian era, and
- neophytes – introduced after 1492.

Most of the trees one can encounter in Europe nowadays belong to the category of neophytes (e.g. *Thuja occidentalis* L., *Quercus rubra* L., *Juglans nigra* L., *Pseudotsuga menziesii* (Mirb.) Franco, etc.).

Based on the reports, 1/3 of introductions of non-native species in Europe was intentional, while 2/3 happened unintentionally (Wohlgemuth, 2015). Reasons for introduction of non-native species were numerous: food source (e.g. *Amygdalus communis* L.), wind and erosion control (e.g. *Robinia pseudo-*

acacia L.), ornamental properties (e.g. *Salix matsudana* Koidz., *Paulownia tomentosa* (Thunb.) Steud.), etc. In addition to that, non-native species can provide habitat, shelter and food for native species (Schlaepfer et al., 2011) or can be introduced because of high timber productivity (Branco et al., 2015).

The most comprehensive classification of non-native species was proposed by Pyšek et al. (2004) and is presented in Figure 1.

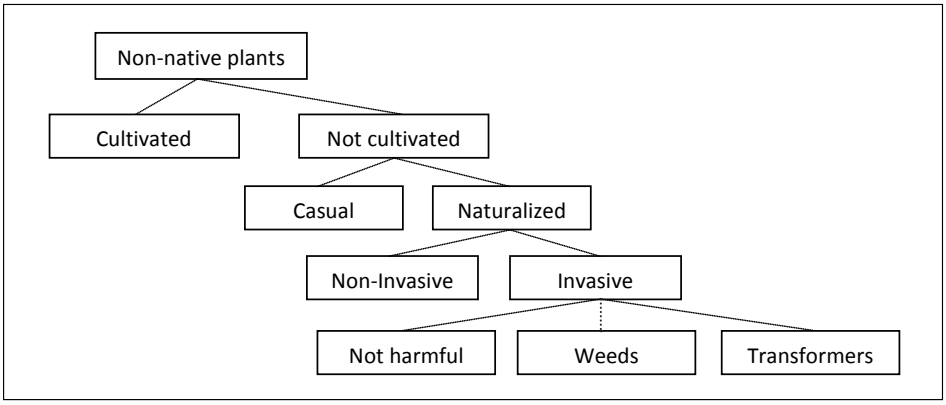

Figure 1. Classification of non-native plants (Pyšek et al., 2004)

Pyšek et al. (2004) defined each of the categories as described in the following text. *Cultivated plants* – aside from crops, fruit trees and ornamental plants, this term involves forest tree plantations such as hybrid poplars. *Casual non-native plants* are the ones which are able to grow or even reproduce outside the cultivation, but eventually die out. One example of casual non-native tree is *Pinus strobus* L. in several European countries. *Naturalized plants* are the ones whose entire life cycle can be established outside the natural distribution range and without further human intervention (e.g. *Chamaecyparis lawsoniana* (Murr.) Parl., *Abies grandis* (Douglas ex D. Don) Lindl.). *Transformers* are highly competitive plants with a broad ecological niche and capable for severe modifications within ecosystems, while *weeds* are similar to them but with a smaller transforming capacity. Both transformers and weeds can be either native or non-native species.

According to the Convention on Biological Diversity – CBD (UN, 1992; Secretariat of the CBD, 2014) invasive species are defined as the ones whose introduction and consequent spread cause harm to the environment, economy or human health. Invasive species can also be defined as a species "that has characteristics that allow it to become both quickly established and abundant in new areas" (USDA, 2003).

Even though there is a well-known estimation by Williamson and Fitter (1996) stating that only 1% of non-native species eventually become invasive, the non-native species are more often seen as a treat for the local biodiversity

because of their invasiveness potential (Mack et al., 2000; Muñoz-Vallés and Cambrollé, 2015). Figure 2 presents the possible interactions of both native and non-native species with the new environment and determines corresponding invasiveness status.

		Initially transported into the area by humans	
		NO	*YES*
Spreads out in new habitats and has negative impact on species already present	*NO*	native	non-invasive non-native
	YES	invasive native	invasive non-native

Figure 2. Classification of native and non-native species and their invasiveness (Alpert et al., 2000)

Stages of the invasion process

The invasion process consists of two main phases (Müller-Schärer et al., 2004): (1) initial introduction and establishment, and (2) spread within a new habitat. Most of the invasive plants make the initial introduction spreading as seeds, and the phase of the establishment means successful survival and reproduction until establishing a self-sustaining population. The phase of spread in the new habitat consists of three sub-phases: (a) *lag phase* during which the species are present with the low density and are hardly noticeable, (b) *phase of explosive growth* when the populations start to increase rapidly, and (c) *phase of reaching carrying capacity* when the populations stop with the further spread. The described phases are illustrated in Figure 3.

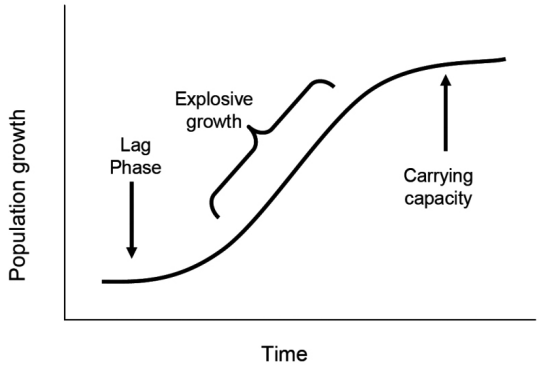

Figure 3. The stages of the invasion process (ISSG, 2005)

It is important to emphasize that the duration of the lag phase is different, for some species it can last a few months and for others – even decades or centuries. Therefore, it is important to prevent a non-native species from establishing, because low abundance of a species at the moment does not imply that it will not become invasive over time.

Impact of non-native species on the environment

Table 1 presents an overview of impacts of non-native species on biodiversity, ecosystem services, human well-being, and economy.

Table 1. Impact of non-native species (NNS) on the environment (European Environmental Agency, 2012)

Impact	Description
Impact of invasive NNS on biodiversity	Competition with local species
	Causing harm to local species
	Hybridization with native species
	Modifying ecosystem structure and local habitats
Impact of invasive NNS on ecosystem services	Interfering provisioning services
	Interfering regulating services
	Interfering cultural services
Impact of invasive NNS on human well-being	Impacting human health
Impact of invasive NNS on economy	Damaging infrastructure
	Damaging agriculture
	Damaging natural landscapes

Briefly, it can be said that introduction and establishment of non-native species affect the local biodiversity in many different ways. These species can quickly become competitive and therefore might suppress native flora, causing the loss of local habitats and modification of ecosystem structure. Invasive non-native species affect all three categories of ecosystem services defined by Millennium Ecosystem Assessment (2003) and these are: provisioning, regulating, and cultural services. Some of the invasive non-native species can cause allergic reactions, and therefore that is one of their common threats for human health. Economic consequences are related to damages non-native species cause to infrastructure, agricultural production, and natural landscapes.

Factors that reduce invasiveness potential of species

Figure 4 shows the factors that reduce the invasiveness potential of the invasive species; they are related to evolutionary history, disturbance, community structure and stress, and have a strong interaction with each other.

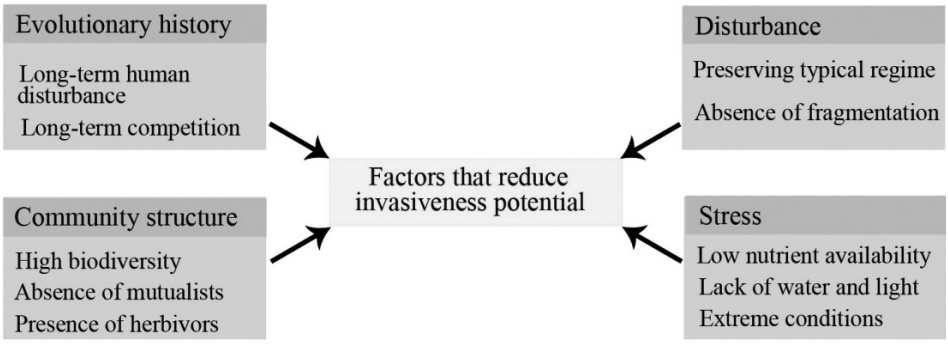

Figure 4. Factors that reduce species' invasiveness potential (Alpert et al., 2000, modified)

Evaluation history affects the invasiveness success in a following way: if a habitat had an intense competition over evolutionary time, it is likely that native species that remained have a high competition ability and are capable for outcompeting potential invasiveness. Community structure is also important for reducing invasiveness, e.g. more diverse communities exploit resources more completely and therefore diminish their availability to the invasive species. When it comes to disturbance, it is important to emphasize that invasion can take place in both disturbed and undisturbed habitats (Wiser et al., 1998), but if the disturbance is present it will usually increase invasiveness. Stress is a factor that does not favor both invasive and local species and the main limiting factors are: lack of nutrients, water and light, and extreme conditions that might occur.

CONCLUSION

Non-native tree species can be introduced to the new environment as: food source, material for applying bioengineering measures (erosion control, wind control, phytoremediation, etc.), forestry practice (when introduced species are characterized by rapid growth), but also for their ornamental features (decorative leaves, flowers, fruits, extended flowering phase, etc.). Some of non-native species can become invasive in the new environment, suppressing local flora and colonizing different types of habitats.

In Europe, 4% of forests are dominated by non-native species, and there are 29 of them registered as being invasive in at least one country. Among invasive species, category known as "transformers" is the most dangerous threat to local biodiversity because these species have a potential to make severe modification within an ecosystem. Tree species such as: *Robinia pseudoaccacia* L., *Ailathus altissima* (Mill.) Sw., *Acer negundo* L., *Fraxinus pennsylvanica* Marshall belong to transformers category and they are present in almost entire Europe, including Serbia (Lazarević et al., 2012). The general recommendation for future landscape measures would be to give a priority to native species and if the non-native species are being introduced than sterile clone trees would be preferred, in order to exclude further spread of species by seed dispersal.

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АЛОХТОНЕ И ИНВАЗИВНЕ ВРСТЕ ДРВЕЋА И ЊИХОВ УТИЦАЈ НА ГУБИТАК БИОДИВЕРЗИТЕТА

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РЕЗИМЕ: У раду је приказан преглед у вези са алохтоним и инвазивним врстама дрвећа, као и основни подаци о њиховој застуљености у шумама у Европи. Рад обезбеђује приказ дефиниција и класификација и објашњава на који начин ове врсте угрожавају животну средину, посебно локални биодиверзитет. Између осталог, описан је и процес освајања станишта и наглашено је да постоји фаза мировања у којој врсте не откривају свој инвазивни карактер, а која, у изузетним случајевима, може да траје и неколико векова. Због тога је важно да се врши непрекидно осматрање алохтоних врста и спречи њихово даље ширење. Инвазивне врсте теже могу да освоје станишта која имају развијене односе компетиције између врста током дугог временског периода, затим очувана станишта са израженом биолошком разноликошћу, као и просторе на којима постоји недостатак хранљивих материја, воде и светлости или просторе на којима ове ресурсе скоро у потпуности искоришћавају локалне биљне заједнице.

КЉУЧНЕ РЕЧИ: алохтоне врсте, инвазивне врсте, биодиверзитет

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AFLATOXIN M1 IN SERBIA: A SYSTEMATIC REVIEW OF OCCURRENCE AND EXPOSURE ASSESSMENT – AN UPDATE

ABSTRACT: Milk is a highly nutritious diet for all age groups including YOPI (young, old, pregnant and immune suppressed) patients, because it contains numerous important nutrients such as proteins, vitamins, and minerals. On the other hand, contamination of milk is considered as one of the major public health problems, which mainly arises due to aflatoxin M1 (AFM1) contamination, recently reported in our region. Hence, the main objectives of this study were to evaluate the prevalence and possible trends of AFM1 contamination of milk and milk products reported between 2007 and 2016 in Serbia, and to compare collected results with similar research in neighboring countries since aflatoxin crisis has broken in order to identify the predisposing factors for AFM1 contamination. In addition, this paper gives an evaluation of potential public health risk due to consumption of AFM1 contaminated milk.

KEYWORD: aflatoxin M1, risk assessment, public health

INTRODUCTION

Aflatoxins (AFs) are secondary metabolites produced by different filamentous fungi (mainly *Aspergillus* species) and they are known to represent serious hazard to the health of consumers due to their mutagenic, teratogenic, carcinogenic, and immunosuppressive effects (Milicevic et al., 2010). Aflatoxin M1 is the most significant aflatoxin in milk and dairy products. This compound is the hydroxylated form of the aflatoxin B1 (AFB1) and it is usually present in milk and urine of mammals after they have fed on contaminated food or feed (Flor-Flores et al., 2015). The amount of AFM1 excreted in milk as a percentage of AFB1 is in average 2.5%. This can vary from animal to animal and from season to season. The AFM1 is detected in milk 12–24 h af-

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ter the first AFB1 ingestion, reaching a high level after a few days. If there is no further intake of AFB1 through feed, the AFM_1 concentration in milk decreases to an undetectable level after 72 h (Battacone et al., 2012).

Incidences of AFM1 in milk products have been reported globally (Iqbal et al., 2015) with particular attention paid to Balkan Peninsula because of the "aflatoxin crisis" (Torović, 2015; Polovinski Horvatovic et al., 2016; Milicevic et al., 2017). Currently, available researches demonstrate that AFM1 can also be present in a wide range of milk-derived or products containing milk, such as cheese, yogurt, cream, and chocolate (WHO, 2010; Škrbić et al., 2014), because of the stability of aflatoxins during processes involved in preparation of these commodities (Iqbal et al., 2015), and most commonly at higher concentrations than in milk (Mashak et al., 2016). Aflatoxins are one of the major etiological factors in the development of hepatocellular carcinoma (IARC, 2012), and more recently associations between childhood aflatoxin exposure and growth stunting have been reported (Gong et al., 2004). It has been calculated that about 27% of the hepatocellular carcinoma cases reported in Southeast Asia is aflatoxin induced (Liu and Wu, 2010). AFs also cause serious genetic damages including gene mutation, teratogenic effects, causing congenital malformation and, consequently affecting normal growth in children. Aflatoxin M1 is about eight-times less toxic than AFB1 but the metabolite carcinogenic and animal experiments show that it is a potent carcinogen (Hsieh et al., 1984). However, because of the toxic and carcinogenic effects of AFM1, IARC of WHO reconsidered its carcinogenic categorization and changed it from Group 2B to Group 1 (IARC, 2002).

For this reason, and taking into account the significance of milk and milk products in human diet (especially for children), many preventive measures have been proposed in order to stop AFB1 contamination of feeds for dairy cattle, especially lactating cows. Moreover, the maximum allowed levels of AFM_1 are strictly regulated worldwide (van Egmond 2004). Food and Drug Administration from USA limits the concentration of AFM1 in milk and processed milk products at 0.5μg/kg (FDA, 2005). However, European Community Legislation is even more restrictive and does not allow AFM1 levels in milk and infant formula above 0.050 and 0.025 μg/kg, respectively (EC, 2001, 2006) (Table 1). In early 2013, extensive controls of milk samples were carried out following an incidence of unusually elevated concentrations of AFM1 in milk from dairy farms in Serbia. The aim of this paper is to systematically review research studies conducted on AFM1 contamination of milk and dairy products between seasons of 2007 and 2016, and to investigate its seasonal variation during this period. In addition, the authors have compared the obtained results with international standards in relation to permissible limits, assessed dietary exposure to AFM1 and made risk characterization of these mycotoxins regarding human health due to milk consumption in Serbia, and finally extrapolated the obtained results to concentration of AFB1 in the feeds. Practical strategies to prevent AFs contamination during food chain are also given.

Table 1. Regulation on AFM1 in milk and milk products in different countries.

Country	Foodstuffs	MRL (μg/kg)
EU. Islamic Republic.	Raw milk. heat-treated milk and milk for the manufacturer of milk-based products	0.050
USA. CAC	milk	0.50
Serbia	Milk and milk products	0.50[1]
	Raw milk. heat-treated milk and milk for the manufacturer of milk-based products	0.05[2]
		0.50[3]
		0.05[4]
		0.25[5]

[1] *Official Bulletin of the SRJ* (5/1992); [2] *Official Bulletin of the Republic of Serbia* (28/11); [3] *Official Bulletin of the Republic of Serbia* (20/13); [4] *Official Bulletin of the Republic of Serbia* (29/14); [5] *Official Bulletin of the Republic of Serbia* (84/15). CAC-Codex Alimentarius Commission

Data sources and searches

A systematic literature review was conducted on the occurrence of AFM1 in milk and dairy products in Serbia and neighboring countries where aflatoxin crisis occurred. Studies were conducted by searching relevant literature databases including Kobson and national databases published in Serbia since 2007. Several criteria were used to select eligible studies: authors, study period, year of publication, and country/location of the study, number of samples, study design, used methodology, and associated risk factors.

Occurrence of AFM1 in milk

In many scientific researches has been revealed that animals and humans share risk of exposure to biological and chemical toxic agents. The occurrence of AFM1 in milk and dairy products in Europe has been mainly reported in Turkey, France, Italy, Spain, Greece, and recently in Croatia and Serbia. According to the EFSA report, the levels and incidence of AFM1 in milk and dairy products in Europe is less than in the countries from other regions, which may be the result of strict regulations on these mycotoxins in feed and milk products, correlated with good agriculture and storage practices. Incidence of AFs in Europe did not pose a public health concern. Regarding previous studies in Serbia, only a few papers suggested that the incidence of AF species or the presence of AFs in food and feed was at a very low level. Basically, food originating from tropical and subtropical regions has been susceptible to aflatoxin contamination.

The first report on the incidence of the AFs in our region came from Slovenia in 2011, and then from the rest of the Balkan countries. In Serbia, during 2012/2013 the incidence of aflatoxin in corn, and consequently in milk, had the characteristics of an epidemic. Several studies have been conducted since 2012.

Nowadays, the incidence and levels of AF contamination in maize and milk are most commonly investigated in various institutions in Serbia. But, none of work is done on the identification of fungal species and the relationships or interactions among them, as well as their impacts on mycotoxin contamination. This could be explained by the fact that the identification of fungal species is often complicated due to processes such as gene mapping requiring expensive equipment.

In this study, milk and milk products were investigated for the presence of AFM1. The frequency distribution of AFM1 concentrations during the period of investigation and regulations on AFM1 in milk and milk products in different countries, and particularly in Serbia since the aflatoxin crisis, are presented in Tables 2 and 3. The results are presented chronologically per year for each author. Collected results showed that the elevated incidence of AFM1 in raw milk started in 2013 (Kos et al., 2014; Škrbić et al., 2014; Stefanovic et al., 2015), maintaining a high level through all season to 2016 (up to 95.4%), followed by high incidence of raw milk samples that exceeded the EU MRL value (0.05 μg/kg) (Milicevic et al., 2017/a). The results of the last study indicate that the incidence of AFM1 in raw milk (80.7%) was smaller compared to heat processed milk (90.6%), but in raw milk (31%) a higher percentage of analyzed samples exceeded the maximum limit for AFM1 permitted by the EU (0.05 μg/kg).

Also, results by month showed that the highest mean concentrations of AFM1 was measured in December 2015 (0.137±0.18 μg/kg), while the highest concentrations of AFM1 was measured in October of the same year (1.26 μg/kg). According to the results obtained (Table 2), a significant seasonal difference in the level of AFM1 in milk samples was observed during four seasons of same year. Regarding regional and seasonal variability, the raw milk samples during cold and wet season showed higher risk for AFM1 contamination, which indicated that seasonal factors should be considered for the control of aflatoxins in raw milk. In this study, research was particularly focused on the risk assessment of the AFM1, not only on the occurrence of AFM1.

The occurrence and/or mean level of AFM_1 contamination in research conducted by Milicevic *et* al., (2017) is close to that obtained by Polovinski-Horvatovic et al. (2009/a,b; 2016), Kos et al. (2014), Spiric et al. (2015), Skrbic et al. (2014), Torovic et al. (2015), Tomašević et al. (2015), and Miocinovic et al. (2017). The occurrence of AFM1 in raw milk was varying with seasons. The percentage of raw milk samples collected from 2013–2016 which exceeded the EU legal limit were 73.3% in 2013, 39.4% in 2014, 28.6% in 2015 and 13.9% in 2016, with mean levels of contamination during period of investigation 34.4% (Jaksic et al., 2017).

Similarly to the results of that investigation, the percentage of positive samples in many of the studies conducted in neighboring countries was high: Slovenia (Jakovac-Strajn et al., 2012), Croatia (Bilandzic et al., 2014a, 2015),

Bosnia and Herzegovina (Tankovic, 2015), and Kosovo (Rama et al., 2015). On the other hand, with the exception of a few cases (Bilandzic et al., 2014/b; Rama et al., 2015), the observed concentrations of AFM1 were relatively low, as well as the percentage of samples with levels of AFM1 above the legal limit (0.05 μg/kg). Some of the previous researches from this region did not indicate the possibility of the high presence of AFM1 in milk (Polovinski-Horvatovic et al., 2009/a,b; Bilandzic et al., 2010; Rama et al., 2016; Dimitrieska-Stojkovic et al., 2016). Before 2008, *Aspergillus* spp. in grain occurred rarely under Serbian climate condition, but the very high temperatures and extreme drought since 2010 caused *A. flavus* to occur in epidemic proportions (Lević et al., 2013). Serbia has a largely agrarian economy, and thus aflatoxins contamination of agricultural products has had a strong negative impact on Serbian trade, especially with the EU and neighboring countries markets.

However, the variations of AFM1 levels in milk and dairy products in studies mentioned above could be particularly attributed to different methods used for detection of the toxin, geographical conditions, climate and seasonal variations, differences in feeding systems, feed storage practices, and farm management practices (Iqbal et al., 2013). In the light of Serbian legislation, it could be concluded that the safety of milk in Serbia has been improved in recent years. The number of samples which exceed MRL has been decreasing in recent years. Although the improvement of dairy products safety was evident in 2016 when compared to 2013 and 2014, the cause of high concentrations of aflatoxins in raw milk has not been resolved yet (Miocinovic et al., 2017). According to the EU regulation, a large percentage of raw milk (31%) was still contaminated with levels above the maximum permitted level in the EU (EC, 2006). Elevated levels of AFM1 in raw cow milk samples from different regions of Serbia clearly indicated the use of contaminated feedstuff in some farms during the period of samples collecting.

Climate changes resulted in specific extreme conditions during 2012 to 2016 production years in Serbia, which have not occurred previously. According to a report by the Republic Hydrometeorological Service of Serbia (2016), the 2015 production year was the third hottest year in the period from 1951 to the present. Prolonged periods of extremely high air temperatures during summer (daily temperatures around 40 °C), as well as precipitation deficit, resulted in severe and extreme droughts in many regions of Serbia. In addition, regarding the agro-meteorological conditions 2016/2017 production year was unfavorable to many agricultural crops. Besides the extreme climate condition (late spring frosts, snowfall in April, droughts and heat waves in the summer), the quality and quantity of yields of certain agricultural crops, during the time of very important vegetative and generative processes in agricultural crops, were also affected by insufficient application of appropriate agro-technical measures. Furthermore, AFB1 contamination is prevalent in warm and humid climates, particularly in regions which are extremely humid during the rainy season, but also in temperate climates following severe drought, like it was in Serbia. Therefore, a seasonal trend in milk contamination could be expected.

Table 2. Incidence and levels of AFM1 contamination in milk and milk products reported between 2007–2017 in Serbia.

Sample	Year of sampling	N	Incidence n (%)	Mean (μg/L±/SD)	Range (μg/L)	>MRL n (%)			Ref.
						SRB[1-5]	EU[a,b]	USA[c]	
Pasteurized	2008.	34	7 (20.5)	14	10 - 30	0	0	0	Polovinski-Horvatović et al., 2009a
UHT		31	11(35.5)			0	0	0	
Raw cow's milk	2007-2008	23	16 (70)	n.r.	10 – 150	0	7 (30.4%)	0	Polovinski-Horvatović et al., 2009b
Pasteurized		35	9 (13.43)	n.r.	10 - 30	0	0	0	
UHT		32	11 (16.42)	n.r.	10 - 50	0	0	0	
Various milk	2008.	23	9 (39)	30 ± 0.06	7 - 250	0	3 (13)	0	Janković et al., 2009
Raw cow's milk	2013	40	38 (95)	190 ± 0.20	5 - 900	5 (12.5%)	30 (75)	5 (12.5%)	Kos et al., 2014
Pasteurized		35	35 (100)	220 ± 0.23	60 - 1200	6 (17.4)	35 (100)	6 (17.4)	
UHT		69	69 (100)	190 ± 0.09	20 - 410	0	63 (91.3)	0	
Organic		6	6 (100)	30 ± 002	10 - 80	0	1 (16.7)	0	
Goat		10	8 (80)	80 ± 0.09	8 - 240	0	4 (40.0)	0	
Donkey	2013	5	3 (60.0)	20 ± 0.02	5 - 35	0	0	0	
Breast milk		10	6 (60.0)	10 ± 0.0006	6 - 22	0	0	0	
Raw cow's milk	2013	8	8 (100)	482 ± 0.44	10 - 1400	3 (37.5)	6 (75)	3 (37.5)	Škrbić et al., 2014
Pasteurized and sterilized milk		42	32 (76.2)	300 ± 0.21	10 - 800	10 (24)	32 (76)	4 (9.5)	
White cheese	2013	44	19 (43.2)	n.r.	130 - 550		24 (13)		Škrbić et al., 2015
Hard cheese		10	10 (100)	n.r.	80 - 2230				
Raw cow's milk	2013	2045	934 (45.67)	n.r.	5 - 1250	266 (13)	1329 (65)	266 (13)	Spirić et al., 2015

Sample	Year of sampling	N	Incidence n (%)	Mean (µg/L±/SD)	Range (µg/L)	>MRL n (%)			Ref.
						SRB[1-5]	EU[a,b]	USA[c]	
Pasteurized and sterilized milk	2013-2014	20 60	20 (100) 54 (90)	133 ±0.086 26	24 - 320 3-104	0 0	17 (85) 6 (10)	0 0	Torović, 2015
Raw cow's milk	2013-2014	678	540 (79.6)	282 ± 0.358	26 - 1000		382 (56)	165 (24.6)	Tomasevic et al., 2015
Heat treated milk		438	317 (72)	90 ± 0.145			143 (32.7)	12 (2.7)	
Milk products - yoghurt. cheese (white. hard)		322	184 (57)	268 ± 0.952			121 (37.8)	23 (7.1)	
Raw cow's milk	2015	42 38	32 (84)	7.94 ± 9.36 230 ± 257	4.50 - 39.8 6-864	0 13 (34)	0 24 (63)	0 0	Polovinski-Horvatović et al., 2016
Raw milk	2015	1207	503 (41.7)	37±0.041	5-263		353 (29.2)		Miocinovic et al., 2017
Dairy products*		997	236 (23.7)	19±0.024	5-320		42 (4.21)		
Raw milk	2013-2016	75	55 (73.3)		LOD->0.8		55 (73.3)	8 (10.6)	Jaksic et al., 2017
		66	26 (39.4)		LOD-0.8		26 (39.4)	1 (1.5)	
		178	51 (28.6)		LOD->0.8		51 (28.6)	2 (1.1)	
		108	15 (13.9)		LOD->0.8		15 (13.9)	1 (0.9)	
Raw milk	2015-2016	5054	4078 (80.7)	71±0.13	5-1260	450 (9)	1557 (30.1)	159 (3.1)	Milicevic et al., 2017a
Heat-treated milk		1233	1117 (90.6)	35±0.029	5-280	14 (1.1)	214 (17.3)	0	
Dairy products*		501	61 (12)	21±0.017	5-147	1 (0.01)	3 (0.6)	0	Milicevic et al., 2017b

N – total number of analysed samples; n – number of samples; MRL (µg/kg) [1] *Official Bulletin of the SRJ* (5/1992); [2] *Official Bulletin of the Republic of Serbia* (28/11); [3] *Official Bulletin of the Republic of Serbia* (20/13); [4] *Official Bulletin of the Republic of Serbia* (29/14); [5] *Official Bulletin of the Republic of Serbia* (84/15). [a] – EU Regulation 466/2001, [b] – EC, 2006 (0.05 µg/kg and 0.025 µg/kg), [c] – FDA, 2005 (0.5 µg/kg), *Dairy products- infant formulae, milk powder, dairy drink.

Table 3. Results of studies on AFM1 contamination in milk in neighboring region

µSample	Country Year of sampling	N	Incidence n (%)	Mean (µg/L±/SD)	Range (µg/L)	>MRL[a,b] n (%)	Ref.
Dietary cow milk	Greek 2009-2010	196	91 (46.5)	10.0	8.6–11.4	2 (1.0)	Tsakiris et al., 2013
Raw cow's milk	Slovenia 2012	240	152 (63)	n.r.	5-2833	19 (8.0)	Jakovac-Strajn et al. 2012
Raw cow's milk	Croatia, 2013	337	278 (82.5)	16.35±14.14	2.69-162.3	13 (3.8)	Bilandzić et al., 2014a
Raw cow's milk							Bilandzić et al., 2014b
UHT milk	Croatia, 2013	3736	1722 (46.1)	46.6±75.9	3.25-1135.0	1039 (27.8)	
Raw cow's milk	Croatia, 2013-2014	706	279 (39.5)	25.7 ±18.5	3.98-183.5	68 (9.6)	Bilandzić et al., 2015
		3543	253 (7.1)	10.56±12.67	0.11- 764.4	72 (2.0)	
Raw cow's milk	Croatia, 2014	386					Bilandzić et al., 2016a
Raw cow's milk			n.r.	5.65±7.95	1.30-123.8	2 (0.52)	
UHT milk	BIH 2014.	285	n.r.	6.22± 6.36	1.39-60.0	2 (0.7)	
Raw cow's milk	Croatia, 2015	165	n.r.	5.64±3.92	2.45-21.4	0	Bilandzić et al., 2016b
Raw milk	Kosovo	548	n.r.	4.14±1.13	2.02-10.6	0	Rama et al., 2016
UHT milk	2009-2010	826	23 (2.8%)	5.19±5.19	5.2-26.6	0	
Pasteurized milk	Kosovo	69	2 (2.6%)	5.10±0.64	7.2-9.9	0	Rama et al., 2015
UHT milk	2013	84	70 (83.3)	27.8±27.0	5.16-110.93	18 (21)	
		94	74 (78.7)	17.9±14.0	5.02-62.26	4 (4.2)	
Raw milk	Macedonia 2013	3635	349 (9.6)	14.3±18.6	<6.6-408.4	105(2.9)	Dimitrieska-Stojkovic et al., 2016

Legend: BIH – Bosnia and Herzegovina; n.r. – Not reported; [a] – EU Regulation 466/2001, [b] – EC, 2006 (MRL 0.05 µg/kg).

Estimated daily intake of AFM1 by human consumers

The intake of aflatoxins by food, moreover the regular uptake of minor amounts, poses serious health risks for consumers that should be avoided. The carcinogenic potential of AFM1 in humans was widely assessed and discussed before, whereas numerous hepatotoxicity and histopathological signs were demonstrated in *Sub-Saharan Africa.* Taking into account that AFM1 is relatively stable to drying and thermal processing (decomposition temperatures of aflatoxins are between 237 ºC and 306 ºC), ionizing radiation, and addition of enzymes and food additives (Iha et al., 2013; Mashak et al., 2016), raw milk contamination by AFM1 means that this mycotoxin is likely to be found in the final product.

Risk assessment data of mycotoxin contamination of food related to human health in Serbia are still limited mainly due to scientific, political, sociological and economic reasons. Based on the data described before and despite a lack of data concerning milk consumption by different age groups in Serbia, it seems that the average estimated daily intake (EDI) reported by some authors (Skrbic et al., 2014; Torovic et al., 2015; Milicevic et al., 2017/a,b) since the outbreak of aflatoxin crisis was higher when compared with the studies which reported on European diet (0.11 μg/kg bw/day), and particularly regarding infants which represent a risk group because of *a higher milk intake*.

In a study conducted by Skrbic (2014), EDI for AFM1 reported during February, April and May was 1.42; 0.77 and 0.5 μg/kg bw/day. The AFM1 daily intake levels through milk consumption by infants and adults reported in another study in Serbia (Kos et al., 2014) were ~6 and ~0.5 μg/kg bw/day, respectively, indicating a significant difference in the EDI values for AFM1 during different months of a year which is in accordance with a recent study conducted by Milicevic (2017/a,b). The highest EDI values for AFM1 in raw milk in this study were calculated for infants (1–4 years old) (2.257 and 2.206 μg/kg bw/day for males and females, respectively). The EDI values for AFM1 were found to decrease with increasing age; thus, the lowest values were recorded for adult females (age 16–25 years; 0.144 μg/kg bw/day) and males (age >25 years; 0.168 μg/kg bw/day). Considering mean values of AFM1 in heat-treated cow's milk during different seasons of investigation period, in this study in the period from September 2015 to June 2016 EDI was higher than 0.2 μg/kg bw/day, the amount recommended by Kuiper-Goodman (1990). The EDI values of AFM1were affected by the changes of seasons in a similar manner as the concentration of AFM1 in milk i.e. maximum in autumn and followed by winter, spring, and summer. However, in another study conducted in Serbia Torovic 2015), EDI was approximately two-fold lower in August 2013 (0.30 μg/kg bw/day), followed by a further drop until April 2014 (0.08 μg/kg bw/day) and December 2014 (0.03 μg/kg bw/day). The mean level of exposure in 2014 was 0.06 μg/kg bw/day.

Therefore, the comparison of the present results with the ones at the international level on the basis of the mean concentrations of AFM1 in milk and the milk consumption in the GEMS/Food regional diets (JECFA, 2002), as well as the assumption that 1% of the population carries the hepatitis B virus, indicate that there is a potential risk for liver cancer in Serbian consumers due to

the consumption of milk. Moreover, for this type of carcinogen it is generally felt that there is no threshold dose below which no tumor formation would occur. In other words, only a zero level of exposure would be harmless. Therefore, more attention needs to be paid to milk safety in Serbia in order to minimize the health hazards. In addition, the authors indicated the necessity of total diet study in order to evaluate co-occurring risk assessments, using probabilistic estimation, especially for children.

Extrapolated intake of AFB1 in feed

Based on data for the determined concentrations of AFM1 in milk samples, concentration of AFB1 in the feeds consumed by the producing cows could be estimated (Walte et al., 2016). A recently conducted study (Milicevic et al., 2017a) on the large number of analyzed milk samples provides reliable data on the occurrence of AFB1 in corn in the range from 2 to 125 µg/kg (Figure 1). It could be concluded than during the period of investigation a large percentage of contaminated corn was used in feeds intended for dairy cows feeding set by the Serbian regulations (2014). The same back calculation of the average values of AFM1 obtained from analysis of different types of milk samples collected from February to June 2013 revealed that average concentration of AFB1 in feeds consumed by the producing cows was 18.75 µg/kg, which is 3.8 times higher than the maximum allowed level in feeds for dairy cattle set by the European Directives (Directive 2002/32/EC (EC, 2002) and amending Directive 2003/100/EC (EC, 2003) to be 5 µg/kg (Skrbic et al., 2014). However, it is important to emphasize that carry-over rates range from 1 to 6% (reported level for high yielding cows), depending on factors such as the genetics of the animals, seasonal variation, the milking process and the environmental conditions (Iqbal et al., 2015).

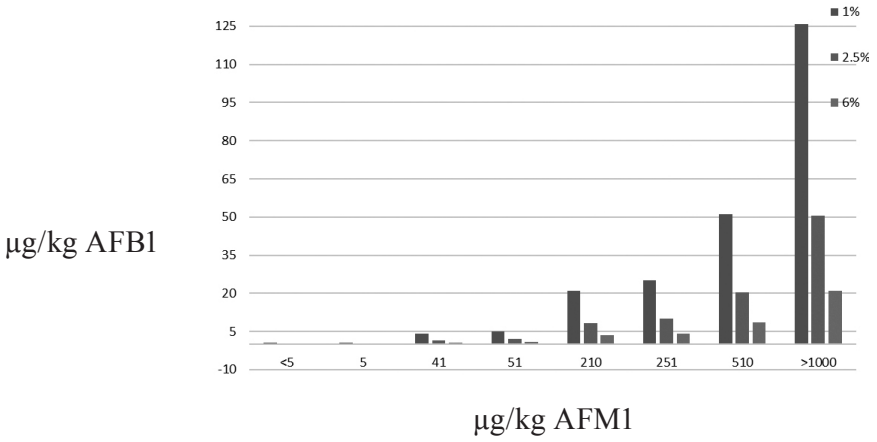

Figure 1. Estimation of the carry-over rate of AFB1 to AFM1

CONCLUSION

AF contamination of milk and milk products has been a serious problem in the last few years in Serbia. The results of this study revealed a relatively high occurrence of AFM1 contamination in raw cow milk. Moreover, a seasonal trend of AFM1 contamination with higher occurrence and levels of the toxin during cold seasons was recorded. Elevated temperatures and extreme weather events, such as droughts and floods that have recently occurred in Serbia, have a direct and indirect impact on the dairy industry. In fact, the presence of mycotoxin producing fungi on crops is extremely dependent on environmental conditions. It is expected that aflatoxigenic species will become more prevalent in such climate conditions, particularly under inadequate storage conditions. As a consequence, the contamination risk for maize-derived products and for milk will be higher than in the past. More precise risk estimates must be made at the national or local level, based on contamination levels and food consumption, particularly in vulnerable groups or regions (carriers of hepatitis B). Thus, the most effective way of controlling AFM1 is to monitor feed for AFB_1. In an integrated food safety control system such as Hazard Analysis and Critical Control Points (HACCP), assigning food safety responsibility to food business operators and finally, fast and accurate identification of potential hazard from food by public health laboratories, ensure not only better safety and quality of products, but the possibility to adopt timely preventive measures to avoid the spread of hazard in case of an outbreak.

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АФЛАТОКСИН М1 У СРБИЈИ: СИСТЕМАТИЧАН ПРЕГЛЕД ЗАСТУПЉЕНОСТИ И ПРОЦЕНА ИЗЛОЖЕНОСТИ – НОВИ ПОДАЦИ

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РЕЗИМЕ: Млеко представља високовредну намирницу животињског порекла. Међутим, у њему врло често могу бити присутни биолошки и хемијски хазарди који су опасни по здравље људи. Као један од најзначајнијих хазарда у последње време сматра се афлатоксин М1 (АФМ1). Услед климатских промена и последично високих температура, незабележених у историји хидрометеорологије у Србији, афлатоксин Б1 и његов хидроксилисани метаболит афлатоксин М1 који се излучује млеком, последњих година представљају једну од највећих опасности за јавно здравље. Стога је циљ овог рада био да се анализирају резултати истраживања контаминације крављег млека и производа од млека АФМ1 у периоду од 2007 до 2016. године, с посебним осврtом на период од 2013. године до данас. Подаци су прикупљени из релевантних база података, публикованих у међународним и домаћим часописима. На основу прикупљених података, може се закључити да је инциденца АФМ1 у млеку и производима од млека доста висока, те је самим тим и изложеност становништва Србије АФМ1 путем млека током периода испитивања била већа у односу на становништво Европске уније (0,11 μg/kg bw/day). Свеукупно сагледавајући добијене резултате, може се закључити да је током периода испитивања, кукуруз контаминиран АФМ1 коришћен за исхрану млечних крава. Из тог разлога мониторинг хране за животиње представља прву карику у ланцу безбедности хране, а тиме и унапређења јавног здравља.

КЉУЧНЕ РЕЧИ: афлатоксин М1, процена ризика, јавно здравље

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DETECTION OF MYCOTOXINS TROUGH DIFFERENT ANALYTICAL METHODS

ABSTRACT: Mycotoxins are secondary metabolites produced by fungi which can affect a variety of feedstuffs. These compounds elicit toxicological effects which represent risk for both humans and animals. Their toxicity occurs at very low concentrations, therefore there is a need for sensitive and reliable methods for their detection. This review aims to evaluate classical and emerging methods for the analysis of mycotoxins concerning their advantages and disadvantages. Currently, several sensitive methods based on chromatographic or immunochemical technique are commercially available. Especially widely are used different chromatographic methods for quantitative determination of mycotoxins, including gas-chromatography (GC) and high-performance liquid chromatography (HPLC) coupled with ultraviolet, fluorescence or MS detectors. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is used as a promising technique for screening, identification and quantitative determination of a large number of mycotoxins. Immunometric assays, such as enzyme-linked immunosorbent assays (ELISA), are frequently used for screening purposes. On the other hand, a variety of emerging methods have been proposed. They are based on novel technologies, including immunochromatography (i.e. lateral flow devices), fluorescence polarization immunoassays (FPIA), infrared spectroscopy (FT-NIR), molecularly imprinted polymers (MIPs), and optical biosensors. In addition, during the last years, the highlight was put on nanoscale materials included in biosensors, which are some of the smart devices used for determination of mycotoxins.

KEYWORDS: biosensors, ELISA, FT-NIR, GC, HPLC, LC-MS/MS, molecularly imprinted polymers

INTRODUCTION

Among the naturally occurring toxic compounds there are mycotoxins. The impact of mycotoxins on health depends on the amount of the mycotoxin consumed, the toxicity of the compound, acute or chronic exposure, the body weight of the individual, the presence of other mycotoxins, and other dietary effects (Kuiper-Goodman, 1991).

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Most cereal grains, oil seeds, tree nuts, and dehydrated fruits are susceptible to fungus contamination and mycotoxin formation. They are produced under appropriate environmental conditions by fungi species. The most important species are *Aspergillus niger, Aspergillus parasiticus, Aspergillus flavus, Penicillium, Fusarium, Alternaria,* etc. (Zinedine et al., 2007; Oancea and Stoia, 2008; Turner et al., 2009). The mycotoxins produced by those molds are shown in Table 1.

Table 1. The most important moulds and produced mycotoxins

Mould species	Mycotoxins produced
Aspergillus parasiticus	Aflatoxins B_1, B_2, G_1, G_2
Aspergillus flavus	Aflatoxins B_1, B_2
Fusarium sporotrichioides	T-2 toxin
Fusarium graminearum	Deoxynivalenol, Zearalenone
Fusarium moniliforme (F. verticillioides)	Fumonisin B_1
Penicillium verrucosum, Aspergillus ochraceus, Aspergillus carbonarius	Ochratoxin A

As can be seen in Table 1, the *Aspergillus* species produce aflatoxins which are highly toxic compounds and can cause chronic toxicity in humans and animals and they are also associated with mycotoxicosis. These molds can grow on peanuts, corn, cotton seeds, nuts, copra, cereals, oilseeds such as sunflower and soybeans, unrefined vegetable oils, spices (paprika and chili pepper), dried fruits (dried figs and raisins), coffee, cocoa, and feed. *Fusarium* species produce Deoxynivalenol, T-2 toxin, and Fumonisin. These toxins occur worldwide and are frequently found in maize. Ochratoxin A is produced by *Penicillium*. The cereal grains are considered to be the main human dietary source of Ochratoxin A. However, Cicoňová et al. (2010) suggested that pork products may also be a significant source of this toxin.

Because mycotoxins are toxic to humans and animals, there is a tremendous need for analytical methods for their measurement. Different analytical methods that have different sensitivity and accuracy, which could be used for different purposes, have been developed. Commonly used methods for mycotoxin analysis are thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) with ultraviolet (UV) or fluorescence detection (FD), and enzyme immunoassays (EIAs). Recently, liquid chromatography–mass spectrometry (LC-MS) and gas chromatography–mass spectrometry (GC-MS) techniques have become accessible for the qualitative and quantitative determination of mycotoxins.

This article reviews different analytical methods for detection of mycotoxins occurring in feedstuffs.

Sample preparation

Mycotoxins are commonly extracted from ground cereals by shaking or blending with mixtures of water or other polar solvents, such as methanol or acetonitrile. Purification of the extract is an essential step in the analysis of mycotoxins, especially when chromatographic techniques are used for their determination at trace levels. Solid phase extraction (SPE), multifunctional clean-up columns, e.g. MycoSep®, and immunoaffinity columns (IACs) are frequently used to clean up the extracts of raw cereals, as well as cereal-processed products. MycoSep® columns are among the most commonly used and commercially available columns for removing analytical interferences from raw extracts in one quick step (10—30 sec). The MycoSep® column, containing various adsorbents, such as charcoal, Celite and ion-exchange resins, is pushed into a test tube (containing the extract) forcing the extract to filter upwards through the packing adsorbent material. The interferences adhere to the adsorbents in the column and the purified extract passes through a frit to the surface of the column. These columns are often used for the simultaneous and rapid clean-up of type A- and type B-trichothecenes, as well as AFs, OTA, ZEA, and FBs. Immunoaffinity columns are based on monoclonal or polyclonal antibodies, and are commonly used for mycotoxin analyses. IACs are commercially available for AFs, OTA, FBs, ZEA, DON, T-2, and HT-2 toxins, and have been used to simultaneously detect the presence of these toxins by HPLC with good accuracy and precision (Pascale and Visconti, 2008).

METHODS FOR DETECTION

Nowadays, there are several recommended methods for detection of mycotoxins in food and feedstuffs.

Thin-layer chromatography (TLC)

This method is one of the most used for determination of mycotoxins. TLC can be used to help determine the number of components in a mixture, the identity of compounds, and the purity of a compound. It is important to note that it is cheap, simple and suitable for rapid screening, but the lack of automation caused TLC to be replaced by other techniques (Roseanu et al., 2010). In sample preparation, the procedures for extraction and clean-up of given matrices depend on the physico-chemical properties of the mycotoxins. To visualize the mycotoxin spots on thin-layer plates, two kinds of techniques have been most frequently applied − examination under UV light of long or short wavelength for naturally fluorescent mycotoxins like aflatoxins, citrinin, ochratoxin A, and spraying the plates with a chemical reagent that reacts with the mycotoxins to produce a colored or a fluorescent product.

Gas chromatography (GC)

Gas chromatographic methods based on FID, ECD, and MS detection are the most widely used methods for quantitative simultaneous determination of trichothecenes (mainly type A) in cereals and cereal-based products (Krska et al., 2001). These methods require a preliminary clean-up of extracts, generally by MycoSep® columns, and pre-column derivatization of the purified extract with specific reagents. Of the published GC methods prevail those developed for trichothecenes and other *Fusarium* toxins. Onji *et al.* (1998) reported an analysis of native compounds (zearalenone, DON, T-2 toxin, and others) using cool on-column injection. There are other reports on direct analysis (Wilkes and Sutherland, 1998), although the adsorption of more polar mycotoxins to column has been observed. An obvious advantage is the combination with MS detection, as the identity of compounds can be confirmed, which is especially important in the analysis of metabolites. Russo et al. (2007) mentioned that direct analysis of T-2 toxin using a GC-GC tandem system for sequential extraction and separation could be also accomplished.

Liquid chromatography (LC)

LC can be classified into 3 parts: column chromatography, mini-column chromatography, and HPLC. Column chromatography is used for clean-up. Many factors like particle size, particle size distribution and surface area, packing density, pH, and many other factors affect its performance. Therefore, columns have been replaced by commercial prepacked cartridges. Mini-columns were used for screening of different mycotoxins (usually aflatoxins, ochratoxin A, and ZEA). LC is currently the dominate type of chromatography and is even replacing GC in its more traditional applications of mycotoxin analysis. Advantages of LC compared to GC are as follows: LC can be applied to the separation of any compound that is soluble in a liquid phase; LC is more useful in the separation of biological compounds, synthetic or natural polymers; liquid mobile phase allows LC to be used at lower temperatures than those required by GC; LC is better suited than GC for separating compounds that may be thermally labile. Liquid chromatography coupled with mass spectrometry (LC-MS) has been used for many years, mainly as a technique for mycotoxin confirmation.

At present, LC-MS and LC-MS/MS are the most promising techniques for the simultaneous screening, identifying, and measuring of a large number of mycotoxins. In this regard, Vucović et al. (2016) showed that by the validation of the LC-MS/MS using Vicam AOZTM immunoaffinity column clean-up for the extraction of AFB1, AFB2, ATG1, and AFG2 can be successfully performed. They concluded that the method provides a very high sensitivity, good reproducibility, appropriate linearity and can be ap-

plied with a high reliability to the analysis of the AFs content in real maize samples. LC-MS/MS is also proven to be a powerful technique for the determination of masked mycotoxins, for example deoxynivalenol-glucosides, in wheat (Berthiller et al., 2005).

HPLC is the most popular method for the analysis of mycotoxins in foods and feeds. Actually, it is a quantitative technique that is suited for online clean-up of sample extract and could be combined with different detectors. This technique is widely accepted as an official method for the determination of mycotoxins. It is applied in conjunction with UV, fluorescence, amperometric or spectrofluorimetric detection. A number of mycotoxins already have natural fluorescence (ochratoxin, citrinin) and thus can be detected directly by HPLC-fluorescence detection (HPLC-FD) (Toskani et al., 2007).

HPLC–MS/MS is also used for determining fumonisins. A procedure was developed for the determination of fumonisins B1, B2, and B3 in herbal tea and medicinal plants. Sample preparation was carried out with the aid of immunoaffinity columns. Separation was performed on an XBridge column; methanol and formic acid were used as a mobile phase. The limit of detection was 0.025 or 0.5 μg/kg for fumonisins B1 and B3 or B2, respectively (Vucović et al., 2010).

Enzyme-linked immunosorbent assay (*ELISA)*

The ELISA is another technique for mycotoxin detection. It is based on the specific antigen-antibody reaction and can be direct or indirect competitive. In direct technique, an extract of the sample is added to the solution and the dissolution of the mycotoxin covalently linked to enzyme is observed. Indirect competitive uses a second antibody directed to the constant region of the first antibody. The assay is mostly performed in a 96-well plate, allowing simultaneous analysis of up to 45 samples in duplicate. Incubation times are 0.5–2 h and the developed color is usually measured spectrophotometrically (Schneider et al., 2004). In general, ELISA does not require clean-up procedures, and the extract containing the mycotoxin is analyzed directly. Even though they often lack accuracy at very low concentrations and are limited in the range of matrices examined, immunoassays provide fast, inexpensive screening assays. However, matrix interference or the presence of structurally related mycotoxins can interfere with the binding of conjugate and antibody, leading to mistakes in quantitative measurements of mycotoxins. The results of aflatoxin determination in red-scaled, red and black pepper determined by ELISA showed a good correlation with HPLC, since ELISA can be used in the routine screening of aflatoxin contamination in spices in terms of simplicity, rapidity, reliability, and cost-effectiveness (Colak et al., 2006). Aflatoxin M1 in milk has been determined by ELISA by some researchers (Decastelli et al., 2007).

Other immunoassay techniques are based on novel technologies, including immunochromatography (i.e. lateral flow devices), fluorescence polarization immunoassays (FPIA), infrared spectroscopy (FT-NIR), molecularly imprinted polymers (MIPs), and optical biosensors.

Fluorescence polarization immunoassay (FPIA)

This is a means of identifying and quantifying the amount of an antigen in a specimen, in which a fluorescently labeled antibody mixed with a sample thought to contain the antigen is exposed to polarized light. Bound fluorescently labeled antibody reacts to polarized light in a characteristic fashion not demonstrated by unbound antibody, allowing for identification and measurement of the ligand. FPIAs are developed for rapid determination of aflatoxins, zearalenone, fumonisins, and DON, although low accuracy and sensitivity were observed when these assays were used with cereal samples (Chun et al., 2009). Recently, FPIA has been optimized for rapid determination of DON in durum and common wheat, semolina and pasta (Lippolis et al., 2006). The assay showed better accuracy and precision when compared to a widely used HPLC-IAC method (MacDonald et al., 2005) in the range of 100–2.000 mg/kg.

Infrared spectroscopy (FT-NIR)

Infrared (IR) spectroscopic methods are among the most promising strategies for determining mycotoxin contamination in agricultural commodities or processed food products. IR-based methods are rapid and nondestructive techniques that require minimal technical training and sample preparation. Analysis is usually not labor-extensive, and large quantities of chemicals are not required in comparison to the existing sophisticated chromatographic techniques usually requiring advanced technical competence. These intrinsic qualities of IR-based methods render them an attractive option for high-throughput analysis of foodstuffs on site (McMullin et al., 2015).

Biosensors

Bioassay by biosensor is designed as an inhibition assay. A fixed concentration of mycotoxin-specific antibody is mixed with a sample containing an unknown amount of mycotoxin in these methods. The antibody and mycotoxin form a complex. Then the sample is passed over a sensor surface to which mycotoxin has been immobilized. Non-complexed antibodies are measured as they bind to the mycotoxin on the sensor surface. The responses generated over a range of standard mycotoxin concentrations are used to create a calibration curve and table. Finally, unknown samples are determined by referring to the

calibration curve. Actually, the biosensors have emerged as a rapid, sensitive, practical, and convenient method for mycotoxin analysis. They consist of a recognition element, commonly of biological origin, that produces a quantifiable response in a signal transduction element when in contact with the target analyte (Figure 1) (Sertova, 2015).

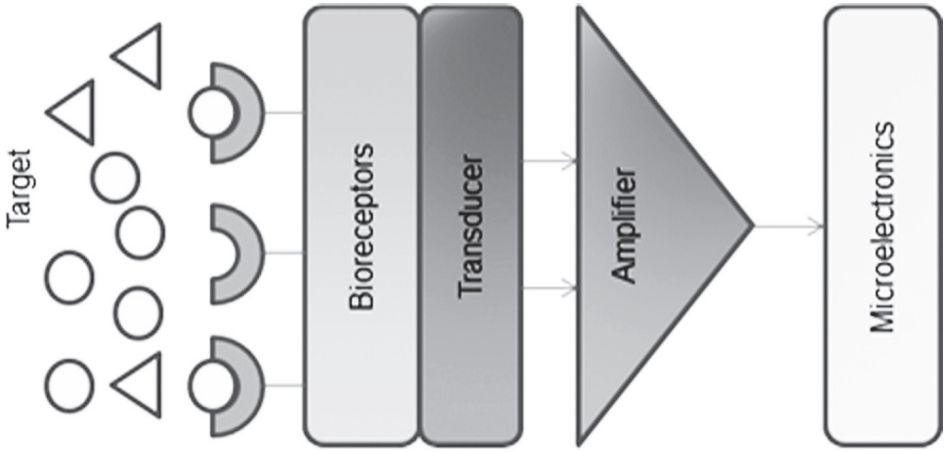

Figure 1. Schematic representation of biosensor

Most signal transduction mechanisms are optical (colorimetric, fluorescence, enhanced chemiluminescence), electrochemical or surface plasmon resonance (Wang and Wang, 2008; Yuan et al., 2009). Tissue biosensors, optical immunosensors, enzyme sensors, electrochemical sensors, quartz crystal, array and plasmon resonance biosensors have been applied to detect ochratoxin, aflatoxins, fumonisin, and deoxynivalenol in different commodities. For example, zearalenone and its derivatives were detected in milk products with a yeast whole-cell bioluminescent sensor (genetically modified *Saccharomyces cerevisiae*), allowing detection at nanomolar concentrations (Valimaa et al., 2010). Compared to other traditional analytical techniques, biosensors offer the possibility to monitor a large number of samples thus being a very convenient tool, that can also be automated, for screening toxins in routine analysis. The main limitation is regeneration of the receptor surface. The improvement of their specificity, sensitivity, reproducibility and stability are important requirements for future applications on a large scale. In addition, during the last years, the nanoscale materials have been included in biosensors, which are some of the smart devices used for detection of mycotoxins. The nanotechnology is playing an increasingly important role in the development of biosensors. The sensitivity and performance of biosensors could be improved by using nanomaterials for their construction. Nowadays, there are different effective systems working for detection of mycotoxins. One of them,

based on the properties of nanomaterials, is included in the nanobiosensor devices. The nanosensor's structure includes materials at nano level, and the most popular are nanoparticles. Nanoparticle is defined as a small object that behaves as a whole unit in terms of its transport and properties and has one dimension which is 100 nm or less in size (Sertova, 2015). The most important fact in this case is that the biosensors are binding the specific analyte of interest to the sensor for the measurement with minimum of interference from other components in complex mixture.

Molecularly imprinted polymers (MIPs)

Recently, in the area of mycotoxin analysis, there has been an increasing interest in the potential use of MIPs as adsorbents for SPE due to their low costs, easy preparation, high chemical stability, and long shelf life. MIPs are cross-linked polymers that are thermally, photochemically or electrochemically synthesized by the reaction of a monomer and a cross-linker in the presence of an analyte, e.g. mycotoxin, used as a template (Pascale et al., 2008). For mycotoxins applications, MIPs have mainly been used as clean-up media for sample extraction and pretreatment and also as sensing receptors (Urraca et al., 2006). MIPs are synthetic receptors with high-affinity sites that can selectively recognize a target analyte, based on its shape, size, or functional group distribution. These receptors are promising due to their easy preparation, thermal stability, chemical inertness, and long shelf life at room temperature and humidity. From the point of view of analytical chemistry, this protocol is very promising for applications in the extraction and analysis of ochratoxins. Recent investigations have led to the synthesis of new MIPs for a wider range of mycotoxins. Currently, MIPs are not selective enough in the aqueous environment to compete with natural antibodies (Cozzini et al., 2008), and better shape selectivity must be achieved in future development. In addition, mycotoxins are costly for large-scale preparation of MIPs. Molecular-imprinting polymerisation has been the focus of intense research interest in recent years and been developed for the preparation of selective separation materials and as sensing layers in sensor devices (Piletsky et al., 2006).

Comparing the abovementioned methods used for the analysis of mycotoxins, some pros and cons could be shown (Table 2).

Table 2. Pros and cons of methods for mycotoxin analysis

Method	Pros	Cons
TLC	Less equipment are used, simple and sensitive method	The sample stays in the open, it may be affected by humidity and temperature
GC	Good sensitivity, simultaneous analysis of mycotoxins, may be automated	Specialist expertise required, expensive equipment, variation in reproducibility and repeatability
LC/MS	No derivatization required, simultaneous analysis of mycotoxins, provides confirmation	Specialist expertise, very expensive
HPLC	Good selectivity, good sensitivity, automated by autosampler, short time analyses, good repeatability	Expensive equipment, may require derivatization, specialist expertise required
ELISA	Suitable for screening, limited use of organic solvents, inexpensive equipment, high sensitivity, simple sample preparation	Possible false positive/negative results, cross-reactivity with related mycotoxins
FTIR	No extraction or clean-up, easy operation, rapid measurement	Poor sensitivity, expensive equipment
Biosensors	No clean-up procedure, rapid	Extract clean-up to improve sensitivity, cross-reactivity with related mycotoxins
MIP	Reusable, low cost	Poor selectivity

CONCLUSION

Several methods are now available in mycotoxin detection. There is an ongoing development toward quick methods providing rapid yes/no decision or semi quantitative results. Easy-to-use methods are in general expensive or show a lack of sensitivity. The main disadvantage of the conventional electrode-based immunosensor is the difficulty of regeneration of immunorecognition phase. Nowadays, the researchers have developed various electrochemical immunosensors for the detection of mycotoxins in different types of samples. The advantages of electrochemical immunosensors are portability and low cost. The nanotechnology has also contributed to the construction of biosensors, which enable the analysis of very small quantities of different residue including mycotoxins. Notwithstanding all this, new methods are required for detection for mycotoxins.

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ДЕТЕКЦИЈА МИКОТОКСИНА ПУТЕМ РАЗЛИЧИТИХ АНАЛИТИЧКИХ МЕТОДА

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РЕЗИМЕ: Микотоксини су секундарни продукти метаболизма гљива који могу да утичу на различите намирнице. Ова једињења изазивају токсиколошке ефекте који представљају ризик и за људе и за животиње. Њихова токсичност

јавља се у веома ниским концентрацијама, због чега постоји потреба за осетљивим и поузданим методама за њихово откривање. Циљ овог рада је евалуација класичних и нових метода за анализу микотоксина узевши у обзир њихове предности и недостатке. Тренутно је комерцијално доступно неколико осетљивих метода заснованих на хроматографским или имунохемијским техникама. Посебно се широко користе различите хроматографске методе за квантитативно одређивање микотоксина, укључујући гасну хроматографију (GC) и течну хроматографију високих перформанси (HPLC) заједно са ултраљубичастим, флуоресцентним или MS детекторима. Течна хроматографија са тандемском масеном спектрометријом (LC-MS/MS) користи се као обећавајућа техника за скрининг, идентификацију и квантитативно одређивање великог броја микотоксина. Имунолошки тестови, као што су ензимски везани имуносорбентни тестови (ELISA), често се користе за потребе скрининга. С друге стране, предложене су различите нове методе. Оне се базирају на новим технологијама, укључујући имунохроматографију (тј. уређаје за бочни проток), флуороимунолошко поларизацијско одређивање (FPIA), инфрацрвену спектроскопију (FT-NIR), молекуларно утиснуте полимере (MIPs) и оптичке биосензоре. Осим тога, током последњих година нагласак је стављен на наноматеријале који су инкорпорирани у биосензоре и служе као паметни уређаји за одређивање микотоксина.

КЉУЧНЕ РЕЧИ: биосензори, ELISA, FT-NIR, GC, HPLC, LC-MS/MS, молекуларно утиснути полимери

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ALLELOPATHIC ACTIVITY OF *Myriophyllum spicatum* L. ON NATURAL PHYTOPLANKTON ASSEMBLAGES

ABSTRACT: Widespread eutrophication of the water bodies and consequential occurrence of toxic algal blooms is one of the most serious environmental problems. Considering that aquatic macrophytes and microalgae compete for nutrients and light, allelopathic inhibition of algal growth is considered to be an effective macrophyte competitive strategy against algae that can bloom and thus significantly decrease an amount of light that reaches macrophytes. Three different concentrations of *Myriophyllum spicatum* ethanolic extract were tested for their inhibitory allelopathic activity on natural phytoplankton assemblages. After applying the extract, the average biomass of 3 replicates was measured during the experimental time. All the three concentrations of the *M. spicatum* extracts showed inhibitory effect to a certain extent. The maximal inhibitory effect was achieved with the 5g/50 ml concentration of extract at first sampling time. The inhibitory effect of extracts is evident within all recorded algal phyla. Phylum Cyanobacteria is found to be the most sensitive to applied extracts compared with Chlorophyta and Bacillariophyta.

KEYWORDS: allelopathy, antialgal activity, extract, *Myriophyllum spicatum* L., natural phytoplankton assemblages

INTRODUCTION

Living in the same water habitat with limited amount of resources, photoautotrophic organisms, such as plants and microalgae compete for nutrients and especially for light. Therefore, light is the most important factor which determines the type and intensity of interaction between submerged plants (and macrophytes in general), epiphyton and phytoplankton. This happens because those epiphytic and especially blooming planktonic algae consequently decrease the amount of light that reaches the plants (Hilt, 2006).

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During their long-time evolution, plants, as well as algae have developed numerous competitive strategies where allelopathy takes an important place.

Allelopathy includes the release of organic compounds by plants or bacterial species that affects other plants or bacterial species, which is regarded as a form of interference competition (Jiang et al., 2014). The chemical compounds produced in this biological phenomenon are named allelochemicals.

The production and excretion of allelochemicals provide to aquatic macrophytes an effective defense strategy against other photosynthetic organisms competing for light, e.g. other macrophytes, algae, and cyanobacteria (Gross et al., 1996).

The numerous allelochemicals with algicidal and algistatic activity produced by plants have been discovered until now, but only a few of them are structurally elucidated. The reported allelochemicals with the antialgal activity in literature include mainly polyphenols, fatty acids, terpenoids alkaloids, and polyethers (Meng et al., 2015).

Myriophylum spicatum (L.) (family Haloragaceae), known as Eurasian milfoil, is a submerged species, native to Europe, Asia and northern Africa. Milfoil is a very invasive aquatic plant which propagated fast in eastern USA and Canada after its introduction from Europe at the end of the last century (Chambers et al., 1993). Beside that, it may displace the native vegetation, and milfoil-dominated lakes usually have low phytoplankton densities (Gross et al., 1996).

M. spicatum (L.) contains up to 30% polyphenols based on dry weight in apical meristems and exhibits a strong inhibitory action against various cyanobacteria and algae, which is mainly based on the polyphenol tellimagrandin II (Bauer et al., 2009). Further, gallic and ellagic acid, which caused algal inhibition, are allelochemicals found in crude milfoil extracts, too (Gross et al., 1996). There are several modes of inhibition for some allelochemicals: linkage with extracellular algal proteins (e.g. alkaline phosphatase) which makes them inactive (Gross et al., 1996), as well as inhibition of photosystem II (Leu at al., 2002).

The basic idea in this experiment was to study allelopathic activity of the different concentrations of the *Myriophyllum spicatum* extracts on the structure and dynamics of natural phytoplankton assemblages in *ex situ* conditions. The results would potentially provide insight into the patterns of using this plant allelochemicals for algal bloom control, given that control and elimination of harmful algal blooms became crucial in the management and mitigation of aquatic ecosystems (Zhang et al., 2014).

MATERIALS AND METHODS

Sampling

The natural phytoplankton community was collected from the Sava Lake on 20 May 2016 by a plankton net (net frame 25 cm, mesh size 22 μm). Thereafter, the fresh *M. spicatum* macrophytes were collected from the same lake.

Phytoplankton samples preparation

In sterile conditions (laboratory), 50 ml of the collected phytoplankton samples were added in each of 12 pre-autoclaved 250 ml flasks filled with 150 ml of BG-11 medium (Rippka et al., 1979). These flasks were sealed with a sterile gauze tampons coated with aluminum foil and cultured for 16 hours in an incubator using a light intensity of 400 lux, at 20±3 ˚C and photoperiod 15 L : 9 D, for proper acclimation.

Macrophyte extract preparation

Representative plants with fresh shoots were chosen and rinsed carefully with tap-water to remove all impurities, epiphytic algae and other organic materials. The chosen plant material was dried at 65 ˚C during 24 hours and powdered with lab ceramic mortar and pestle. One gram, 5 g and 25 g of powdered plant material were added to three different flasks (250 ml) containing 50 ml of 40% ethanol. The flasks were covered to prevent evaporation and vibrated for 13 hours at room temperature. After 13 h, each flask solvent was filtered with a vacuum pump and 1.2 μm cellulose filter to remove insoluble residue, giving three different concentration ethanol extracts of *M. spicatum*: A (1 g/50 ml), B (5 g/50 ml) and C (25 g/50 ml).

Plant extracts adding and experimental sampling

One milliliter of each extract of certain concentration was added to 3 experimental flasks which resulted in 4 series marked as: A, B, C, and K named after the concentrations of the added plant extract (1; 5 and 25 g/50 ml, respectively). The fourth, control series (K) was prepared only with 1 ml of ethanol (Figure 1). After the extract adding, the experimental flasks were returned to the incubator where the experiment took place.

During the experiment, 15 ml of microalgae suspension was taken four times from each flask: before extract application (0 state), 4 h (state I), 8 h (state II), and 24 h after adding extracts. It is noteworthy that the plant extracts

were added only once, at the beginning of the experiment. Collected sub-samples were taken in sterile conditions, fixed with Lugol's solution and kept in the dark at a room temperature.

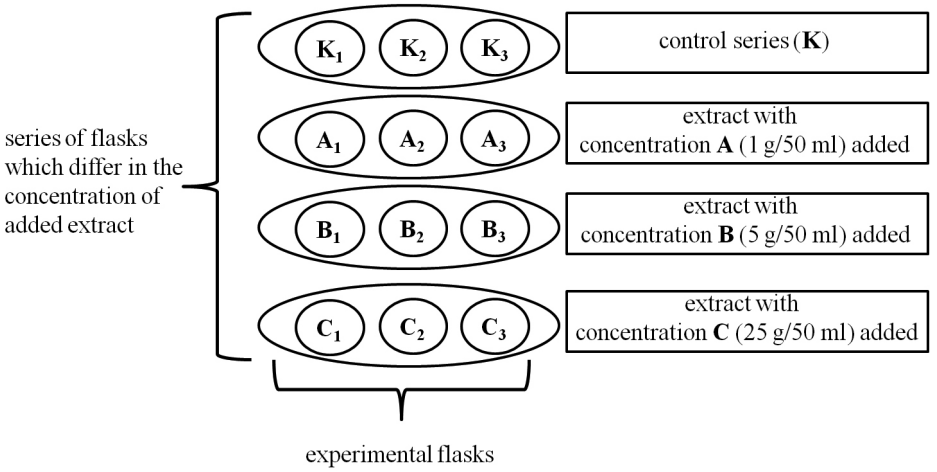

Figure 1. Experimental design

Qualitative analysis of taken sub-samples

Detailed analysis of phytoplankton population structure was made by Carl Zeiss AxioImager M.1 microscope equipped with digital camera AxioCam MRc5 and AxioVision 4.8 software. For the identification of the particular taxa, standard taxonomic literature was used (Hofman et al., 2013; Huber-Pestalozzi et al., 1983; Starmach, 1974, 1983, 1985; Popovský and Pfiester, 1990; Ettl, 1978; Komárek, 2013; Komárek and Anagnostidis, 1998, 2005).

Quantitative analysis of taken sub-samples

For quantitative analysis of phytoplankton, the Utermöhl's method (Utermöhl, 1958) was applied using an inverted-microscope Leica DMIL. For counting individuals were used 10 ml volume Hydro-Bios plankton chambers.

The phytoplankton biomass was estimated from the approximate geometric volume of each taxon (Hillebrand et al., 1999) and expressed in microgram per liter (μg/l).

Statistical analysis

CANOCO for Windows, version 5.0 (ter Braak and Smilauer, 2012) was used for statistical analysis of experimental results where particular phylum biomass was set as response variable, while extract concentrations and experimental sampling time were set as explanatory variables.

RESULTS AND DISCUSSION

A total of 67 algal species classified in 8 phyla (Cyanobacteria, Bacillariophyta, Chlorophyta, Chrysophyta, Cryptophyta, Dinophyta, Euglenophyta, and Xanthophyta) were detected by qualitative analysis of sub-samples. More than a half recorded species were members of Chlorophyta phylum, while some of the phyla (Xanthophyta, Cryptophyta, Euglenophyta) were represented by very few species, as well as individuals.

The results of quantitative analysis of the sub-samples were expressed by the average value of the total series (K, A, B, C) biomass (from 3 flasks that belong to the same series in the particular sampling time), and their dynamics are shown in Figure 2.

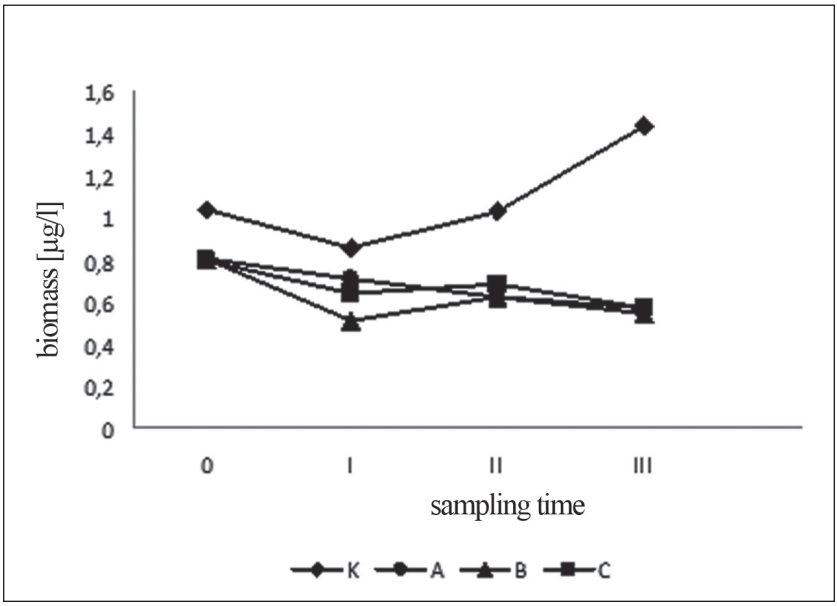

Figure 2. The effect of three different concentrations (A, B, C) of the *M. spicatum* extracts on the total biomass of the particular series during the experimental time

Figure 2 shows that, according to the expectations, the total biomass in the control series increased during the experimental time (with a slight exception of the first sampling time), while all the three concentrations of the *M. spicatum* extracts inhibited the algal growth and caused biomass decrease to a certain extent.

The maximal growth inhibition after the extract adding was recorded in most cases for the first sampling time and concentration B of the extract: 25 g of powdered plant material in 50 ml of 40% ethanol in the first sampling time showed the most powerful inhibitory effect although that was expected for concentration C. The explanation of this phenomenon is that the extraction efficiency has its own maximum which depends on the amount of powdered plant, as well as on the volume of solvent (ethanol). The extract with concentration B was obviously closer to reach that maximum compared to the extract with concentration C which most likely exceeded the maximal amount of powdered plant material for the ethanol volume, so it decreased its inhibitory activity.

After reaching the greatest rate of algal growth inhibition, most likely due to the decomposition of the active allelochemicals, it comes to the gradual recovery of the survived algal populations, so there is a slight increase in biomass noted.

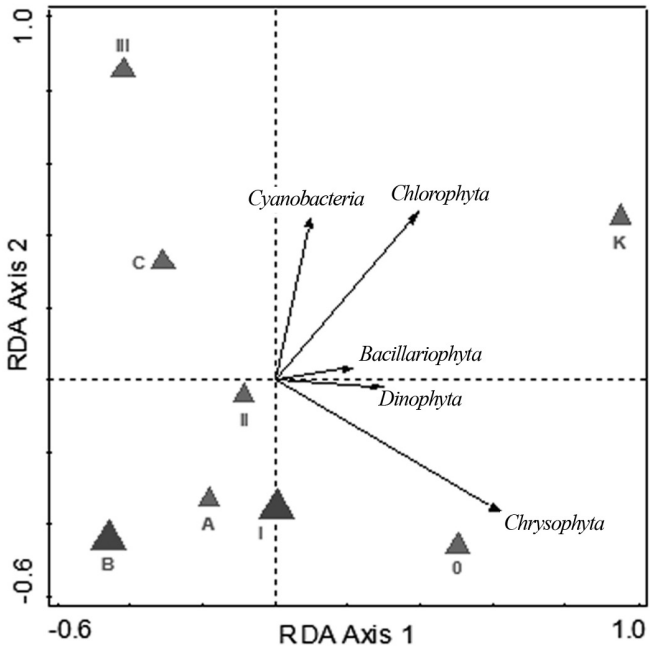

Figure 3. Redundancy analysis of the experiment results (different extract concentrations – K, A, B, C; different sampling time – 0, I, II, III)

Redundancy analysis showed that there are statistically significant differences between the control and the treatments. The first two axes taken together display 24.3% of total variation, and Figure 3 confirms that the extract with concentration B achieved the maximal inhibitory effect on total biomass at the first sampling time. Speaking in terms of different sensitivity of algal phyla, Figure 3 also indicates that Cyanobacteria is the group most sensitive to the extract B compared with Chlorophyta and Bacillariophyta, which is in accordance with the results of Planas et al. (1981), Gross et al. (1996), Körner and Niklisch (2002), Hilt and Gross (2008), and Švanys et al. (2013).

Numerous studies (Gross et al., 1996; Nakai et al., 1999; Körner and Niklisch, 2002; Mulderij et al., 2003; Hilt, 2006; Eigemann, 2013) also pointed that there are differences in sensitivity to allelochemicals not only in particular phyla, but also in different genera, as well as species in the phylum. Although most studies show that Cyanobacteria have the highest sensitivity to the allelochemicals extracted from *M. spicatum*, some species, such as *Anabaena flos-aquae*, could be characterized as exceptions (Körner and Niklisch, 2002). Differences in susceptibility among cyanobacterial species were also established by Nakai et al. (1999), but the reasons for it still remained unknown (Eigemann, 2013).

CONCLUSION

The analysis of the obtained results leads to the conclusion that different concentrations of *Myriophylum spicatum* extracts show inhibitory allelopathic effects on algal growth. The rate of inhibitory activity is correlated with the extract concentration. Because of the reduced extraction efficiency during the preparation of the extract of concentration C, the extract of concentration B at the first sampling time showed the strongest inhibitory effect on the total algal biomass, which is confirmed by the redundancy analysis of the results of the experiment.

The inhibitory effect of different concentration extracts is evident within all recorded algal phyla, where Cyanobacteria reached the maximum of susceptibility to applied extract, compared with Chlorophyta and Bacillariophyta.

Considering the trend of the worldwide and comprehensive eutrophication of water bodies accompanied by frequent and harmful algal blooms, as well as the advantage of potential allelopathic control of this natural phenomenon, the obtained results give the opportunity for a more detailed and specific research that will have an effective and sustainable management and recovery of water bodies as an ultimate goal.

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АЛЕЛОПАТСКА АКТИВНОСТ ЕКСТРАКТА *Myriophyllum spicatum* L. НА ПРИРОДНУ ЗАЈЕДНИЦУ ФИТОПЛАНКТОНА

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РЕЗИМЕ: Изражена еутрофикација водних тела која за последицу има појаву честог и интензивног цветања алги које продукују токсине представља један од највећих проблема у управљању и одржавању водених екосистема. Насељавајући иста станишта, акватичне макрофите и алге ступају у најразличитије видове компетитивних односа за нутријенте и нарочито светлост, при чему алелопатија представља врло ефектну компетитивну стратегију против алги које цветају и на тај

начин смањују количину светлости која до макрофита доспева. У овом истраживању испитиван је инхибиторни алелопатски утицај три различите концентрације етанолског екстракта *Myriophyllum spicatum* на природне фитопланктонске заједнице. Након додавања екстракта, у одређеним временским интервалима израчунавана је просечна вредност биомасе фитопланктона од три реплике. Све три концентрације екстракта *Myriophyllum spicatum* показале су у одређеном степену инхибиторни ефекат на раст алги. Најјачи инхибиторни ефекат на заједницу алги имао је екстракт концентрације 5 g/50 ml у првом времену узорковања. Инхибиторни ефекат је, такође, запажен у оквиру свих група алги. Највећа осетљивост на додате екстракте уочена је код раздела Cyanobacteria у поређењу са осетљивошћу раздела Chlorophyta и Bacillariophyta.

КЉУЧНЕ РЕЧИ: алелопатија, антиалгална активност, екстракт, *Myriophyllum spicatum* L., природна фитопланктонска заједница

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EFFECT OF WATER ACTIVITY ON THE RADIAL GROWTH OF FUNGI ISOLATED FROM DRY-CURED SHEEP HAM, *IN VITRO* (SERBIA)

ABSTRACT: In the Western Balkans, traditional dry-cured sheep ham called Pastrma or Stelja is produced. Dry-cured sheep ham from Sjenica is produced in a very complex manner, and a prerequisite for its production is the sanitary safety of raw materials in accordance with veterinary and sanitary regulations. Isolation and preliminary identification of fungi from sheep ham were carried out in this study, as well as *in vitro* testing of the effects of water activity (a_w) on the growth of isolated fungi. Fungi were isolated from 9 samples of dry-cured sheep meat taken from three households in the area of Sjenica in two productive years (2015 and 2016). Species of genus *Penicillium* were isolated as dominant in all the investigated samples. Water activity was tested on MY50GF agar from the series of malt extract yeast extract glucose fructose agar. Water activity was set to values of 0.87, 0.89, and 0.97. The results of the research showed that the growth of fungal colonies is under the direct influence of water activity. Fungi grew fastest at water activity of 0.97, and the highest growth of all tested species was recorded after 3 (*A. niger*), 7 (*P. patulum*), and 10 days (*A. nidulans*).

KEYWORDS: sheep ham, fungi, water activity, growth

INTRODUCTION

Dry-cured sheep meat – Pastrma or Stelja (in Turkish pastyrma or bastyrma) is a characteristic product of the Western Balkans (Stamenković and

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Dević, 2006). It is produced in the traditional way from whole carcasses of animals aged 1–6 years, using the meat of fattened male castrates and barren sheep (Stamenković and Dević, 2006).

Sjenica sheep Pastrma (Serbia) is prepared by cutting the whole carcass from sternum to pelvis, removing the head, spinal cord and internal organs. Only kidneys with surrounding fat are left on the carcass while leg muscles are removed for ham production. Such boneless meat salted and dry-cured is called Stelja (Stojković et al., 2015). Air-drying and ripening take place during the winter period of 4 to 6 months, whereby meat develops its characteristic microbiota that defines its organoleptic properties and quality.

Complex production method and the fermentation of the product influence the richness of microbiota which develops in dry-cured ham from Sjenica. It is mostly composed of lactic acid bacteria and coagulase-negative staphylococci, but some fungi can be found as a part of the microbiota as well. Composition of microbial population depends on microorganisms found in the meat or in the environment during the production process to the product itself (Žugić-Petrović et al., 2016). Dried sheep meat is characterized by low water activity (a_w) and high concentrations of salt, which are good conditions for the development of xerophilic fungi (Sonjak et al., 2011).

Fungi play an important role in the production of dried meat products; their growth on the surface is often desirable so they are often added as a starter in the production of some meat products (Canel et al., 2013). Fungi directly affect the product's quality, and can be responsible for the formation of specific taste and aroma of dried meat, due to their lipolytic and proteolytic activity (Ludemann et al., 2004; Scolari et al., 2003). Proteolytic changes occurring in dry-cured sheep ham can lead to an increase in free amino acids, which serve as precursors of volatile compounds (Martin et al., 2004). Fungi form a barrier on the surface of smoked meat which prevents the penetration of light and oxygen into deeper layers of the product thus making it more stable (Sonjak et al., 2011).

Diversity of fungi species growing on the surface of Stelja depends on the quality of raw materials and hygienic quality of the production environment. Dry-cured meat products are characterized by long and complicated production process with each stage of the production process requiring specific physico-chemical conditions that may have a positive impact on the growth of certain fungi. Some conditions may determine and accelerate the development of the dominant fungi species (Scolari et al., 2003).

The aim of this research was to isolate and preliminary identificate the fungi from dry-cured sheep meat, as well as to investigate the impact of water activity on their growth. Fungi were isolated from the samples of dry-cured sheep meat – Sjenica sheep ham (Western Serbia), produced with the traditional method at three different households, under identical microclimate conditions and from the meat of Pramenka – autochthonous sheep species from Sjenica and Pešter.

MATERIALS AND METHODS

Samples of sheep ham

Nine samples of sheep ham were taken from three producers (A, B, and V) in the territory of Sjenica (Western Serbia) during two production years (2015 and 2016). All samples were produced in the traditional manner without the addition of starters and under the identical microclimate conditions from the meat of Pramenka – autochthonous sheep species from Sjenica and Pešter.

In the first production year (2015) 3 samples were taken from the producer A. Six samples of sheep ham in total were taken in the second production year (2016), 3 from each household of B and V. Sampling of dry-cured sheep meat was conducted according to the regulations of general and specific food hygiene requirements at any stage of production, processing and transport (*Official Gazette RS*, 72/2010).

Isolation and identification of fungi

Isolation of fungi from the surface of the sheep ham was conducted using dichloran 18% glycerol agar (DG18 agar) (Merck, Darmstadt). Surface of the tested sheep ham was leaned against DG18 agar surface and kept for 30 seconds in order to transmit fungal spores from the sheep ham to the surface of the substrate. Then the substrate was incubated at 25 °C for 5 days. After that, the colonies for which, based on the macromorphological properties, it was assumed to belong to genera *Penicillium*, *Aspergillus*, and *Eurotium*, were subcultured onto the Czapek Yeast Extract Agar (CYA) (Merck, Darmstadt). Inoculated mediums were incubated for 7 days at 25 °C. Isolates for which it was presumed to belong to the genus *Mucor* were grown on Malt Extract Agar (MEA) (Merck, Darmstadt), for 7 days at 25 °C. Obtained pure cultures of fungi were identified according to the keys for determination (colony diameter, color and texture, microscopic characteristics – hyphae and conidiophore appearance, size and shape of vesicles, metulae, phialides, and conidia) described in literature by Klich (2002), Samson et al. (2004), Samson and Frisvard (2004), and Pitt and Hocking (2009). Isolated and identified fungal cultures were kept on Sabouraud Maltose Agar (SMA) (Torlak, Beograd, Serbia) at 4 °C as part of the collection of the Laboratory for Food Microbiology at the Faculty of Technology, University of Novi Sad, Serbia.

Investigation of the effect of water activity on the growth of fungi

The effect of different water activity values (0.87 a_w, 0.89 a_w, and 0.97 a_w) on the fungal growth was tested for the following fungal cultures: *Penicillium corylophilum, Penicillium carneum, Penicillium patulum, Aspergillus nidulans,*

Aspergillus niger, Eurotium herbariorum and *Mucor racemosus* isolated from dry-cured sheep ham from Sjenica (Western Serbia).

Substrates with different a_w values were prepared using Malt extract yeast extract glucose 50% agar (MY50G) substrates (Pitt and Hocking, 1985). The substrates with different a_w values were prepared by adding different concentration of glucose to the basic medium MY50G (20% for 0.87 a_w, 50% for 0.89 a_w and 60% for 0.97 a_w) (Beuchat and Hocking, 1990). Water activity values of prepared substrates were tested by using Meter group INC 40515 device, in three repetitions. Media are poured into Petri dishes (ø90 mm TER) and centrally inoculated with mature spores of seven-day fungi cultures (incubated at 25 °C, on SMA).

Colony growth was monitored during 15 days at 25 °C on third, fifth, seventh, tenth, twelfth and fifteenth day when the diameter of the colonies was measured using a ruler in three repetitions.

RESULTS AND DISSCUSION

Species of genus *Penicillium* were isolated as dominant in all the investigated samples in two production years (2015 and 2016) (Table 1).

Table 1. Isolated species of fungi from the surface of sheep ham

Mould species	A (2015)	B (2016)	V (2016)
	Frequency of isolation (%)		
Penicillium carneum	18	0	33
Penicillium caseifulvum	6	0	13
Penicillium confertum	2	30	7
Penicillium corylophilum	0	6	0
Penicillium crustosum	0	0	2
Penicillium polonicum	8	40	25
Penicillium rugulosum	30	0	0
Penicillium solitum	30	0	16
Aspergillus nidulans	0	2	0
Aspergillus niger	2	0	0
Aspergillus penicillioides	2	0	2
Eurotium chevalieri	0	0	2
Eurotium herbariorum	2	4	0
Mucor racemosus	0	13	0
Mucor plumbeus	0	5	0

Three producers in two production years A (2015); B (2016); V (2016).

Macromorphological characteristics of some of the isolated types of fungi are shown in Figure 1 and 2. *M. racemosus* formed wavy, white colonies on the SMA. Colonies of *E. chevalieri* were yellow-ocher with an elevated center in the middle and irregular edges. *A. niger* formed a compact white mycelium with a thick layer of dark brown to black conical heads. Colonies of *P. corylophilum* had dark-green fasciculus texture (conidiophore bundles) with pronounced coremias in the central part and with the presence of a colorless exudate. *P. carneum* formed fluffy, pistachio green colonies with concentric circles and radial folds on CYA. Colonies of *P. polonicum* were fluffy, blue-green in color, with a reddish-brown pigment in the base.

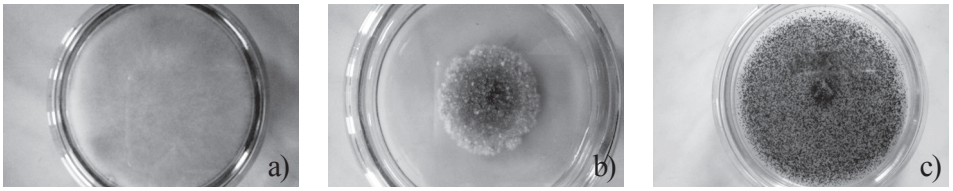


Figure 1. M. racemosus on SMA (a), *E. chevalieri* on CYA (b), *A. niger* on CYA (c)

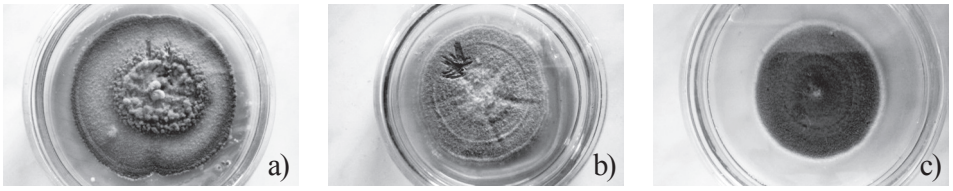


Figure 2. P. corylophilum on CYA (a), *P. carneum* on CYA (b), *P. polonicum* on CYA (c)

The obtained results are consistent with studies of other authors. Sonjak et al. (2011) in their work highlighted the importance of *Penicillium* genus as the largest part of the surface microbiota in all the studied dry meat products. *Penicillium* species have also been confirmed in a large percentage (88.3%) in the Norwegian smoked meat products, where *Penicillium nalgiovense* was the dominant species (Asefa et al., 2009). Toledano et al. (2011) proved the good potential of *P. nalgiovense* as a starter culture which was isolated from the ham.

Microbiota isolated from San Daniele dry ham was largely comprised of 2 genus *Penicillium* spp. and *Aspergillus* spp., which were found during the ripening process (Comi et al., 2013).

Among the 65 analyzed dry hams, *Aspergillus* and *Penicillium* were the dominant genera (Rojas et al., 1991). *Aspergillus* spp. primarily consisted of *Aspergillus glaucus, Aspergillus fumigatus, Aspergillus niger*, and *Aspergillus flavus* (Rojas et al., 1991). *Aspergillus* species were present in all samples of Sjenica dry-cured sheep ham. *E. herbariorum* and *M. racemosus* were isolated to a lesser extent. In the research of fungi from Istria ham, Comi et al. (2004) identified five genera, where *Eurotium* spp., *Aspergillus* spp. and *Penicillium*

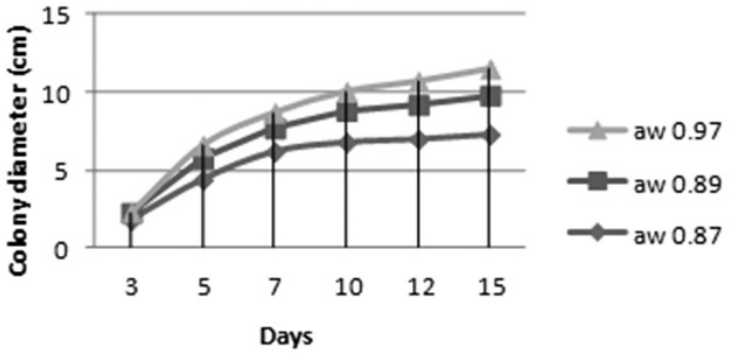
spp. were most frequently isolated from ham samples. *Eurotium* strains isolated from Nebrodi hams*, E. herbariorum, Eurotium rubrum* and *Eurotium cristatum* have been detected (Berni et al., 2012).

Growth of fungi is influenced by a variety of environmental or intrinsic factors, such as the composition of the product, pH, and temperature. Water availability is probably the single most important environmental factor affecting germination, growth, and establishment on nutrient-rich substrates of fungi (Dantigny et al., 2005).

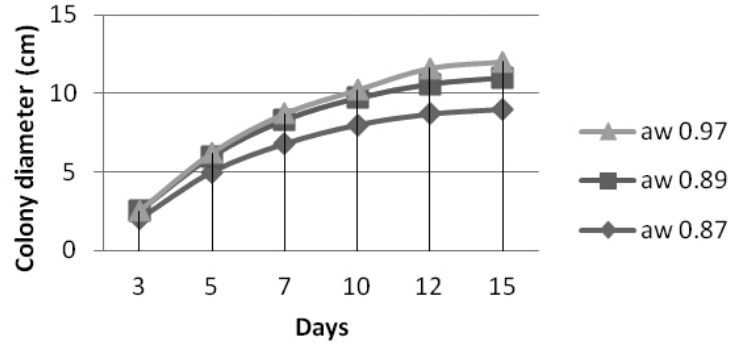
Temperature and a_w are the most important factors that determine the ability of fungi to grow in meat products (Dantigny et al., 2005).

The effect of a_w to isolated strains of fungi *P. corylophilum, P. carneum, P. patulum, A. nidulans, A. niger, E. herbariorum* and *M. racemosus* are shown in Figure 3.

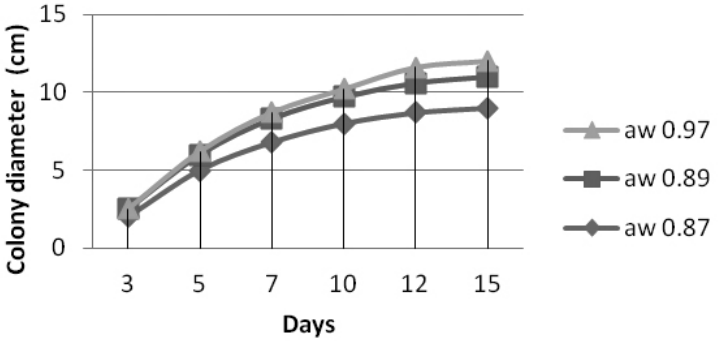
Penicillium corylophilum



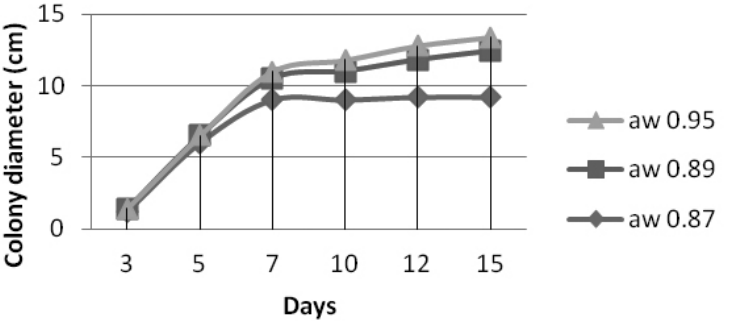
Penicillium carneum



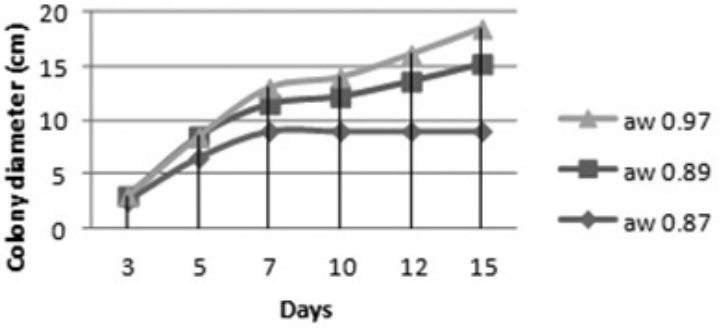
Penicillium patulum



Aspergillus nidulans



Aspergillus niger



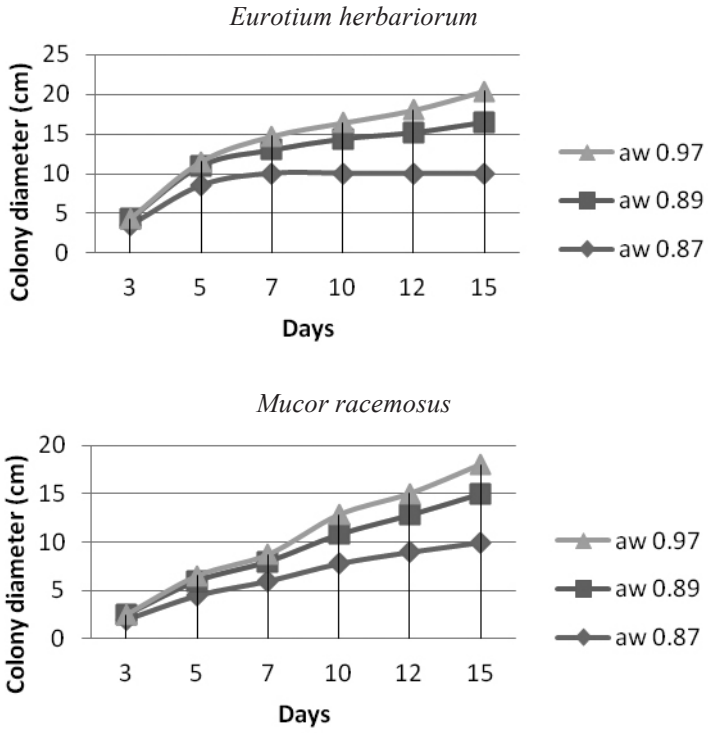


Figure 3. Colony diameters of tested fungi on medium with different a_w values

Table 2. Mean value and SD of colony diameters on medium with different a_w values after 15 days of growth

Fungi	a_w 0.97%	a_w 0.89	a_w 0.87
P. corylophilum	5.58±2.10	1.43±0.71	1.04±0.60
P. carneum	6.58±2.67	1.43±0.57	0.51±0.41
P. patulum	7.26±3.22	1.66±1.17	0.55±0.46
A. nidulans	7.26±3.22	1.66±1.7	0.55±0.46
A. niger	7.5±2.64	3.16±2.0	1.51±1.32
E. herbariorum	8.66±2.6	3.73±2.0	1.81±1.44
M. racemosus	6.55±2.98	2.63±1.63	1.40±1.16

The growth of fungi isolated from the dry-cured sheep meat was under the direct influence of water activity (Table 2). All isolates grew well at the water activity of 0.97. The maximum growth of *P. patulum, A. niger*, and *E. herbariorum* was reached in 7 days, while the isolates of *P. corylophilum, P. carneum*, and *M. racemosus* reached their maximum after 15 days.

Growth reduction of the isolated fungi at lower a_w values was more noticeable (Table 2). Thus, in the majority of fungal isolates germination of spores was completely inhibited the first 5 (*A. nidulans, P. patulum*) and 3 days (*P. corylophilum, P. carneum, A. niger* and *M. racemosus*) at the value of a of 0.87 (Figure 3).

The effect of 0.89 a_w influenced the growth of the isolates less when compared to the growth at 0.87 a_w. At 0.89 a_w in the first few days of growth the diameter of the colonies was observed to be 0.2 (*P. patulum*) to 1.5 cm (*E. herbariorum*). After 15 days of incubation colony diameter of *P. corylophilum, P. carneum*, and *P. patulum* isolates varied from 2 (*P. carneum*) to 3.2 cm (*P. patulum*) (Figure 3).

The results show that the growth of *E. herbariorum* was least affected by a_w value. The size of colonies in all tested a_w had the same value after 12 and 15 days of incubation (Figure 3).

Gibson et al. (1994) studied the effect of a_w on the growth of fungi by using ten different water activities (a_w) between 0.995 and 0.810, adjusted with equal mixtures of glucose and fructose at 30 °C to *Aspergillus* genus *flavi (A. flavus, A. oryzae, A. parasiticus* and *A. nomius*). In their paper they predicted the growth of colonies and proved that the colony diameter for each different a_w value changed about 3mm on the average. The diameters of the colonies were between 7.8 (10^{th} day) to 10 cm (15^{th} day). Gibson et al. (1994) pointed out that the minimum water activity for the growth of *A. flavus* ranged from 0.81 to 0.95.

CONCLUSION

Dominant species of fungi were isolated and identified by using samples of dry-cured sheep ham from Western Balkans. They include strains from the genera *Penicillium, Aspergillus*, *Eurotium,* and *Mucor* (*P. corylophilum, P. carneum, P. patulum, A.s nidulans, A. niger, E. herbariorum*, and *M. racemosus*). The growth of fungi isolated from the dry-cured sheep meat was under the direct influence of water activity as one of the most important intrinsic growth factors. In the tested range of 0.87 to 0.97 a_w, the growth of the tested fungi and colony diameter decreased as a_w decreased.

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УТИЦАЈ АКТИВНОСТИ ВОДЕ НА РАДИЈАЛНИ РАСТ ПЛЕСНИ ИЗОЛОВАНИХ ИЗ ОВЧИЈЕ СТЕЉЕ, *IN VITRO* (СРБИЈА)

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РЕЗИМЕ: На западном Балкану производи се традиционално сушено овчије месо која се назива пастрма или стеља. Овчија стеља са подручја Сјенице (Западна Србија) производи се на веома сложен начин, а предуслов за производњу меса је хигијенска сигурност сировина која испуњава ветеринарске и санитарне услове производње. У раду је вршена изолација и прелиминарна категоризација, као и испитивање *in vitro* ефеката активности воде (a_w) на раст плесни из сувог меса овчијег трупа (овчија стеља). За потребе истраживања коришћено је девет узорака овчијег сувог меса узетих из три домаћинства у две производне године (2015, 2016) с подручја Сјенице. Изоловано је и идентификовано седам различитих врста плесни и то: *Penicillium corylophilum, Penicillium carneum, Penicillium patulum, Aspergillus nidulans*, *Aspergillus niger*, *Eurotium herbariorum* и *Mucor racemosus*. Активност воде истраживана је на MY50GF агру из серије подлога Малт екстракт квасац екстракт глукоза фруктоза агара. Активност воде је подешена на вредности од 0,87; 0,89 и 0,97. Резултати истраживања су показали да је раст колонија под директним утицајем активности воде. Плесни су најбрже расле при активности воде од 0,97 a_w, при чему је код свих испитиваних врста највећи пораст забележен између 3 (*A. Niger*), 7 (*P. patulum*) и 10 дана (*A. nidulans*).

КЉУЧНЕ РЕЧИ: овчија стеља, плесни, активност воде, брзина раста

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ENVIRONMENTAL ANALYSIS OF CONSERVATIONALLY SIGNIFICANT *Eumerus* AND *Platycheirus* SPECIES (Diptera: Syrphidae) IN SERBIA

ABSTRACT: Due to raising environmental pressures, the number of species exposed to risk of extinction is also increasing. One of the first steps in species preservation is their legal protection. However, it is impossible to protect all species, therefore, conservation priorities are to be established. The aim of this study was to analyze the environmental niches of species from two genera: *Eumerus* Meigen, 1822 and *Platycheirus* le Peletier et Serville, 1828 recognized as important for conservation in Serbia (strictly protected and protected according to national legislation or ones to be suggested for future protection). For species of genera *Eumerus* and *Platycheirus*, distributional patterns in relation to altitude, annual precipitation and annual mean temperature were established. In order to compare environmental niches of these species, Principal Component Analysis (PCA) was carried out, which indicated partial overlap of the environmental niches of these two genera, but *Platycheirus* species seemed to be better adapted to harsher conditions. Species richness maps indicated that for *Eumerus* the most species-rich areas were Bačka and mountains of Eastern Serbia, while Dinaric mountains in Western Serbia were rich in species of both genera. Protecting habitats with different combination of climatic conditions will contribute to protection and conservation of species with different environmental preferences.

KEYWORDS: conservation, environmental niches, hoverflies, PCA, protected species

INTRODUCTION

Global biodiversity is constantly being lost as a result of different changes in the environment (Thomas et al., 2004). Due to these changes, it is anticipated

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that the extinction risk for many species in the future will increase (Bellard et al., 2012). Traits making species particularly prone to environmental perturbations are narrow range, limited dispersal capacity, low reproductive power, and high level of specialisation towards the particular habitat type. Species with restricted range, such as endemic species, possess most of these traits, which makes them especially sensitive (Wulf et al., 2013).

Hoverflies are a Dipteran family, with approximately 6,000 species described worldwide, of which 1,200 occur in Europe (Pape et al., 2011). In Serbia, around 400 species have been registered so far, making this region one of the most species-rich in terms of hoverfly diversity (Vujić, pers. comm.). Syrphids are important not only because of their pollinator role (Petanidou et al., 2011), but also because they have the potential to be used as bioindicators, reflecting the potential environmental perturbations through the changes in their presence and/or abundance (Rotheray and Gilbert, 2011).

One of the first steps in species preservation is their legal protection. However, it is impossible to protect all species, therefore, conservation priorities are to be established. In Serbia, according to the Code on the declaration and protection of strictly protected and protected wild species of plants, animals and fungi, 33 species of hoverflies are declared strictly protected, while 44 species are defined as protected. In addition, Vujić et al. (2016) designated 102 endemic or endangered species for potential future protection using expert-generated, criteria-driven approach.

This paper focuses on target species (strictly protected, protected or the ones suggested for future protection) from two genera: *Eumerus* Meigen, 1822 and *Platycheirus* le Peletier et Serville, 1828. Genus *Eumerus* is one of the largest phytophagous genera in the Palearctic region, with 140 species (Peck, 1988). Within this genus there are 9 protected species or the ones designated for potential protection in Serbia. On the other hand, genus *Platycheirus* is also one of the most numerous genera, with more than 50 species present in Europe, but with zoophagous type of larval development (Speight, 2015). In Serbia, 11 species have been marked as significant or endangered and designated for potential protection. Both species groups have an important role in ecosystems. Association of phytophagous hoverfly species with bulbous plants supports the pollination of these species. It has been observed that Syrphids predominantly pollinate those plant species on which they grow (Van Eck, 2016). On the other hand, species with zoophagous larvae can have significant contribution to biological control of pests (White et al., 1995).

If species protection is going to have a practical application, not just the theoretical one, it is important to know biology and ecology of these species in order to conduct appropriate conservation measures for their preservation, if needed. General aims of this study were to (I) analyze and compare the environmental niches of *Eumerus* and *Platycheirus* species designated as important for conservation in Serbia, and (II) to establish regions in Serbia with the highest diversity of these species. Specific aim was to inspect the differences

in environmental niches of the species with the different type of larval development, considering that *Platycheirus* species have larvae that are zoophagous, contrary to *Eumerus* with phytophagous larval type.

MATERIAL AND METHODS

Selection of species was based on national legislative (where protected and strictly protected species were selected) and a recent study of Vujić et al. (2016) (concerning species that are going to be proposed for future protection). For selected species of genera *Eumerus* and *Platycheirus* analyses of species relation to altitude, annual precipitation and annual mean temperature were conducted. In order to analyze environmental niches of important species from these two genera, PCA analysis was conducted to reduce the number of used bioclimatic variables (details on used variables are available at http://www.worldclim.org/bioclim) into a smaller number of principal components (PC axes) that account for most of the variance. PCA was carried out applying a normal varimax rotation of factor loadings. Principal components with eigenvalue greater than one were retained as predictor variables. Variables with a factor loading greater than 0.7 were interpreted as meaningfully correlated with PC axes. A scatter plot of PCA score values was used to graphically display the position of the analyzed species in environmental space. All analyses were conducted using Statistica (StatSoft, Inc. v. 13.2).

To display the distribution of important *Eumerus* and *Platycheirus* species, as well as to make species richness maps, software package ArcGIS (ArcGIS 10, ESRI) was used.

RESULTS AND DISCUSSION

Results of analysis in relation to altitude for *Eumerus* species showed that *Eumerus grandis* Meigen, 1822 is adapted to the largest altitudinal range (300–1,500 m), followed by *Eumerus clavatus* Becker, 1923 (150–800 m). Three species, *E. banaticus* in litt, *E. basalis* Loew, 1848, and *E. panonicus* Ricarte, Vujić et Radenković, 2016 can only be found in the lowland regions (0–100 m). When it comes to *Platycheirus* species, *Platycheirus nielseni* (Dusek et Laska), 1976 has the largest altitudinal range (700–2,000 m). Most of the species can be found at altitudes over 1,000 m (Figure 1 A,B).

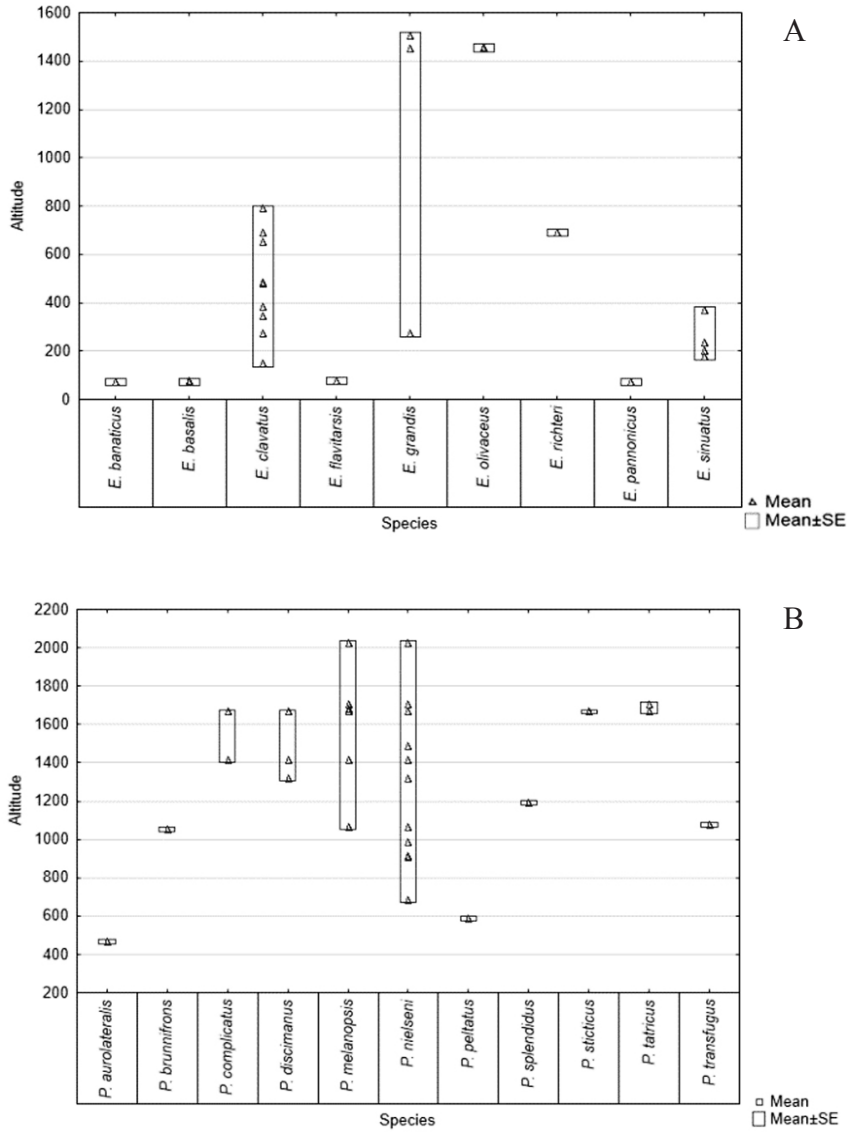

Figure 1. Eumerus (A) and *Platycheirus* (B) species in relation to altitude

Considering annual precipitation, most of the species of genus *Eumerus* prefer more arid habitats (600–800 mm). In contrast, most of the *Platycheirus* species prefer humid areas (800–1,000 mm) (Figure 2 A,B). Again, *Eumerus grandis* and *Platycheirus nielseni* showed the largest variation regarding this parameter.

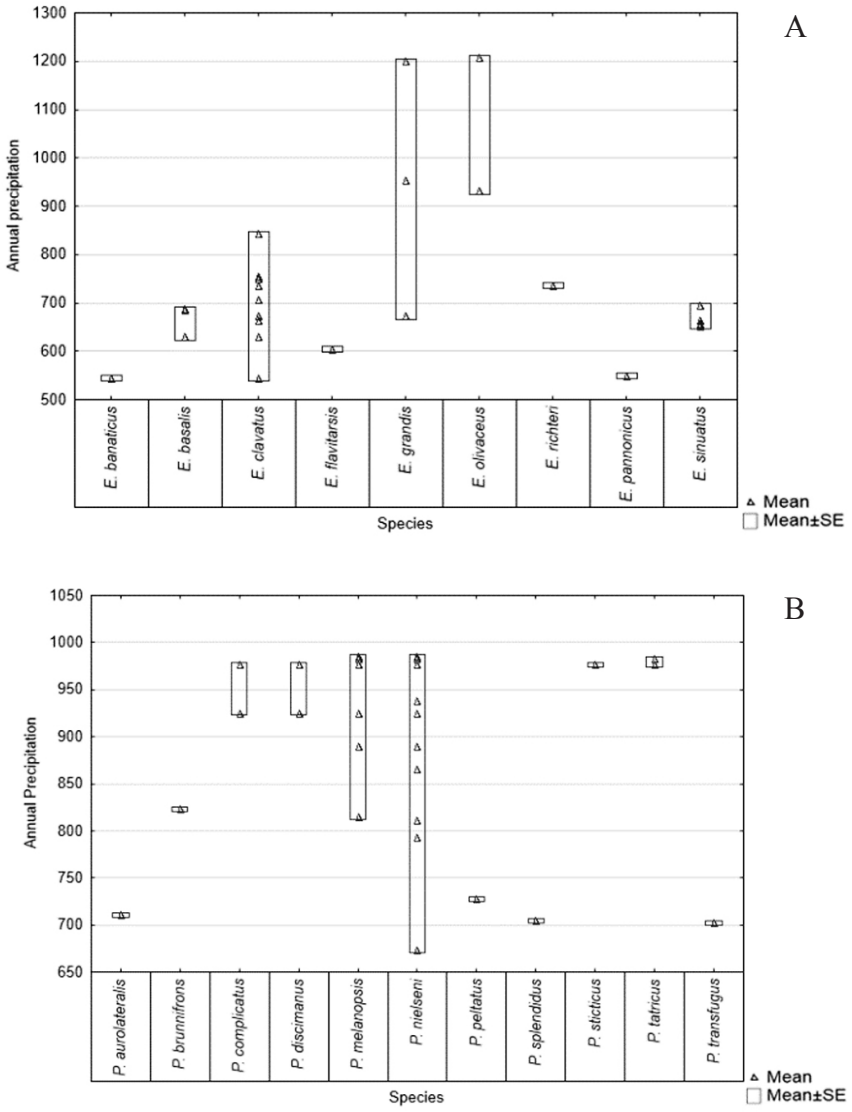

Figure 2. Eumerus (A) and *Platycheirus* (B) species in relation to annual precipitation

Environmental analysis of species in relation to annual mean temperature showed that most *Eumerus* species prefer areas with annual mean temperature above 7 °C, with *Eumerus grandis* being the most tolerant species to variations (Figure 3A). However, the majority of *Platycheirus* species can tolerate temperatures between 2.5 and 7 °C, and can be found in colder areas (Figure 3B). In case of variation of annual mean temperature, *Platycheirus nielseni* again showed highest tolerance.

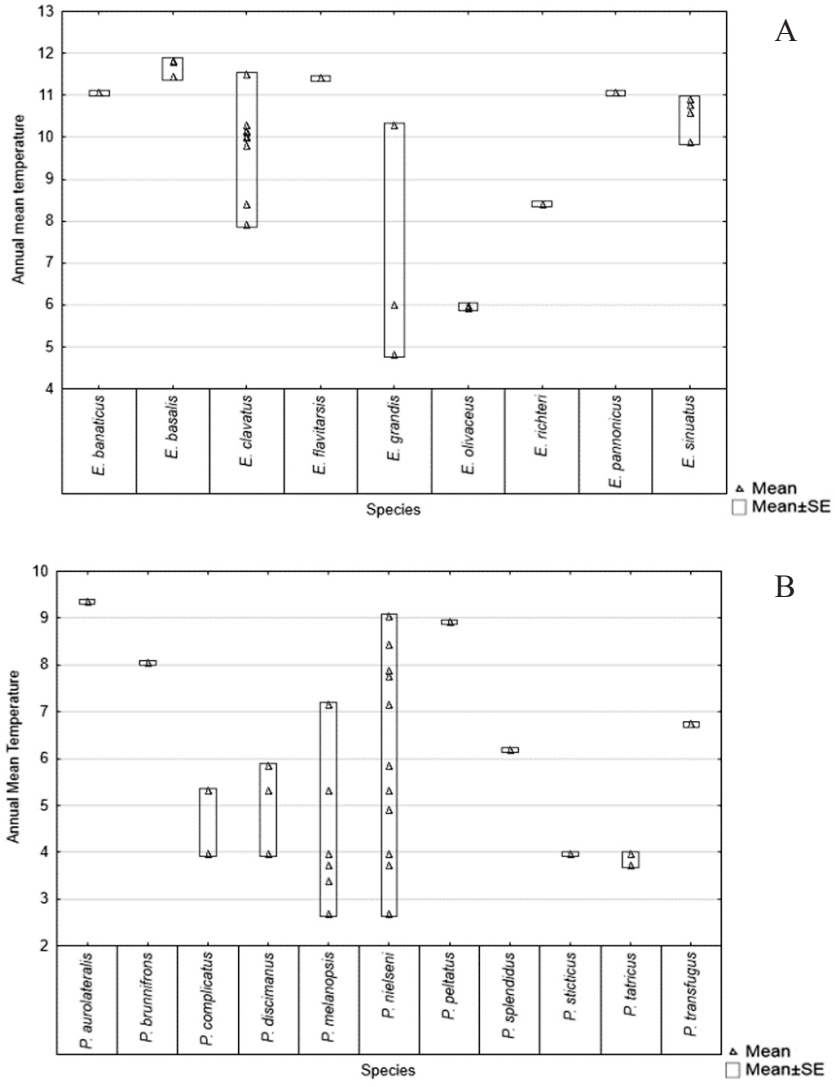

Figure 3. Eumerus (A) and *Platycheirus* (B) species in relation to annual mean temperature

Results of PCA analysis and significant variables identified by it were in accordance with known biology of the species of both genera. First PC axis explained 72% and second 18% of variability. All temperature variables from PC1 axis were positively correlated, while most of precipitation variables were negatively correlated. Also, temperature variables from second PC were positively correlated, while precipitation variable showed negative correlation (Table 1).

Table 1. Principal component analysis of bioclimatic variables for *Eumerus* and *Platycheirus* species. Factor loading values higher than ±0.7 are marked in bold.

Factor Loadings		
Factors	PC1	PC2
BIO1	**-0.98452***	-0.057236
BIO2	**0.96528***	0.127866
BIO3	0.68197	0.664892
BIO4	0.03391	**0.927682***
BIO5	**0.98904***	-0.020128
BIO6	**0.96065***	0.192223
BIO7	**0.96132***	0.077164
BIO8	**0.92003***	0.331038
BIO9	**0.86414***	-0.422901
BIO10	-0.12109	**0.912293***
BIO11	**0.97344***	0.108101
BIO12	**0.94504***	0.182095
BIO13	**-0.95362***	-0.157847
BIO14	**-0.83644***	-0.337325
BIO15	**-0.91988***	-0.262268
BIO16	**0.82189***	0.086556
BIO17	**-0.89204***	-0.264611
BIO18	**-0.90863***	-0.286745
BIO19	-0.44757	**-0.867150***
Variability (%)	**72.56**	**18.34**

Scatter plot used to display the position of analysed species in environmental space showed that environmental niches of genera *Eumerus* and *Platycheirus* overlap to some extent, but there are some significant differences (Figure 4).

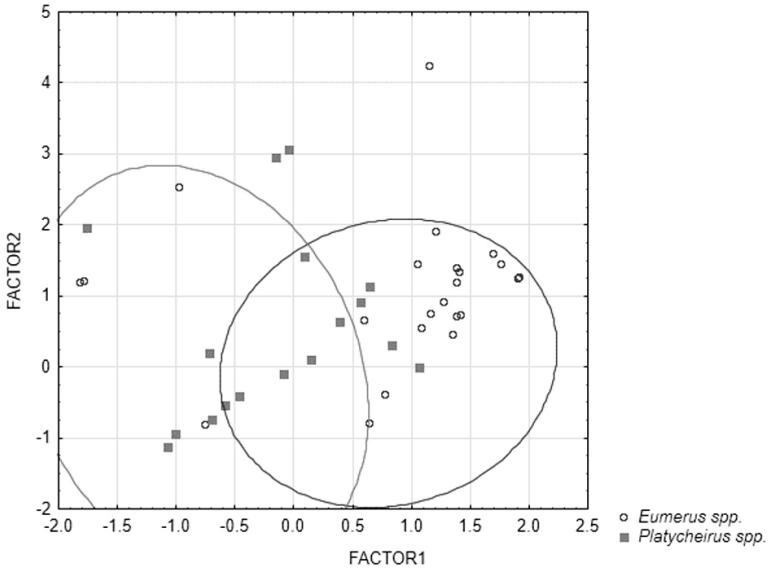

Figure 4. Environmental niche comparison among analyzed *Eumerus* and *Platycheirus* species

According to the PCA analysis, most of *Eumerus* species prefer higher temperatures and lower levels of precipitation. Figures 2A and 3A confirm this notion. Indeed, most of these species are thermophilous and can be found in grasslands and at the borders of woods and bushes (Van Veen, 2004), in which they seek warm and sunny places to rest (Speight, 2015). Species from genus *Eumerus* are widely distributed in Palaearctic, Afrotropical, Oriental and Australian region (Stackelberg, 1961). In Europe, *Eumerus* species are predominantly distributed in Mediterranean region (Ricarte et al., 2008) indicating the preference of these species towards warmer and more arid conditions. Contrastingly, *Platycheirus* species can tolerate lower temperatures and more precipitation. Many of them stay active during rainy and cold weather and observations suggest that lower temperatures are optimal for this genus (Van Veen, 2004). Most of the species are distributed in Nearctic Region (Vockeroth, 1990), while European ones are mostly distributed in the northern parts of the continent, or on high mountains of central and southern part of Europe (Speight, 2015). Flight period for analysed species of this genus starts from the beginning of April and lasts until October (Speight, 2015), which confirms that *Platycheirus* species can tolerate lower temperatures. Overall, *Platycheirus* species seem to be adapted to harsher conditions. This fact could also be connected with different types of larval development of species from these two genera. Larvae of the genus *Eumerus* are phytophagous, developing in the bulbs of plants, thus strictly connecting the development of these species with the period of development of their host plant, which is again dependant on temperature. On the other hand, *Platyche-*

irus species have zoophagous (aphidophagous) larvae and are not conditioned by the time of appearance of the host plants and therefore are indirectly less dependent on weather conditions, which may vary from season to season.

Analyses of environmental niches have been proved useful in many other cases. Except for revealing the environmental preferences of species, this type of analysis was used for delimiting species borders among closely related species. In the genus *Merodon* (Meigen, 1803), PCA analysis of climatic profiles of species contributed in discovering several cryptic species among them (Ačanski, 2017), while for the genus *Chrysotoxum* (Meigen, 1803) this analysis, in combination with geometric morphometry and genetic analyses, helped identify differences between two morphotypes (Nedeljković et al., 2015). It is worth mentioning that the analysed species of *Merodon* (with phytophagous larvae) and *Chrysotoxum* (having aphidophagous larvae) did not show constant pattern regarding climatic preferences, thus indicating that other biological and ecological traits of species, except the type of larval development, are connected with climatic preferences.

Figure 5 shows the distribution of analyzed species of two genera in Serbia. Species richness maps created in order to determine the areas with the highest number of species showed that for *Eumerus* most species rich areas were Bačka (western part of Vojvodina), mountains of Eastern Serbia, and Dinaric mountains in Western Serbia (Figure 6A). The last one coincides with the area richest in *Platycheirus* species (Figure 6B).

Figure 5. Distribution of conservationally significant *Eumerus* and *Platycheirus* species in Serbia.

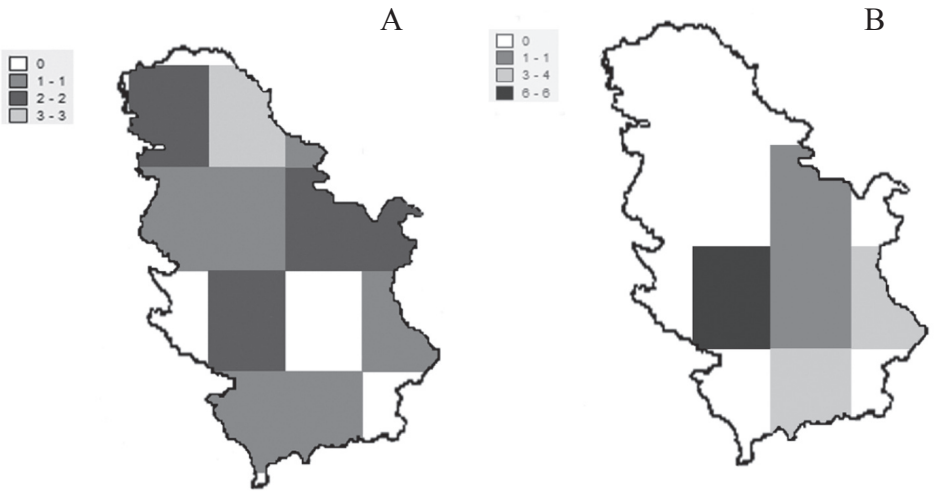

Figure 6. Species richness map of analyzed (A) *Eumerus* and (B) *Platycheirus* species. Darker areas indicate higher species richness

CONCLUSION

Our results confirm that conservationally significant *Eumerus* and *Platycheirus* species have different ecological preferences. Additionally, they are distributed in different regions across Serbia. Therefore, it is important to preserve and protect different types of habitats, which have different combinations of suitable climatic conditions. Protection of these habitats will contribute to species protection and conservation.

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АНАЛИЗА ФАКТОРА ЖИВОТНЕ СРЕДИНЕ *Eumerus* И *Platycheirus* ВРСТА (Diptera: Syrphidae) ЗНАЧАЈНИХ ЗА КОНЗЕРВАЦИЈУ У СРБИЈИ

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РЕЗИМЕ: Услед растућих притисака средине, број врста изложених ризику од изумирања је такође у порасту. Један од првих корака у очувању врста јесте њихова легална заштита. Међутим, немогуће је заштитити све врсте, стога је неопходно установити конзервационе приоритете. Циљ овог рада је анализа климатских профила врста из два рода: *Eumerus* Meigen, 1822 и *Platycheirus* le Peletier et Serville, 1828 које су препознате као значајне за конзервацију у Србији (заштићене и строго заштићене врсте на основу националног законодавства или врсте које ће бити предложене за заштиту у будућности). За врсте родова *Eumerus* и *Platycheirus* установљени су дистрибуциони обрасци у односу на надморску висину, годишњу преципитацију и просечну годишњу температуру. У циљу поређења климатских ниша ових врста, спроведена је анализа главних компоненти (PCA), која је указала на делимично преклапање срединских ниша врста ова два рода, док су врсте рода *Platycheirus* изгледа боље прилагођене на оштрије услове средине. Мапе богатства врста показале су да су подручја најбогатија врстама рода *Eumerus* Бачка и планине источне Србије, док су динарске планине у западној Србији подручја најбогатија врстама оба рода. Заштита станишта која имају различиту комбинацију климатских услова допринеће заштити и очувању врста са различитим климатским преференцама.

КЉУЧНЕ РЕЧИ: конзервација, срединске нише, осолике муве, PCA, заштићене врсте

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DETERMINATION OF INORGANIC ANIONS IN HERBAL TEA INFUSIONS USING ION CHROMATOGRAPHY

ABSTRACT: The ionic content was examined in nine aqueous tea extracts in which time of boiling, acidification of the medium using lemon juice and way of preparation were observed as factors. Ion chromatography was used for determination of inorganic anion content, and data were processed using CANOCO program for multivariate analysis. The variations in ionic content were observed among different tea samples. The highest concentrations of chloride, nitrate, phosphate, and sulphate ions were found in nettle, while the highest concentrations of fluorides were detected in elderflower tea infusion. The effect of boiling time (5, 10, and 20 min), acidification of the medium and different preparation procedure (boiling and cooling at room temperature) were statistically presented using principal component analysis. The examined factors did not have a significant effect on the ionic concentration in tea infusions.

KEYWORDS: herbal tea, inorganic anions, ion chromatography, principal component analysis, tea infusion

INTRODUCTION

Nowadays, traditional methods of treatment that replace synthetic pharmaceuticals are increasingly used. One of such traditional treatment methods includes phytotherapy, which involves the use of herbal teas, more precisely,

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herbal tea infusions (Pohl et al., 2016; Pytlakowska et al., 2012; Martín-Domingoa et al., 2017). Herbal tea infusion represents a combination of boiling water and different parts of a plant, such as leaves, flowers, berries, seeds, roots, separately or combined (Altintig et al., 2013; Emekli et al., 2009; Kara, 2009; Pohl et al., 2016). These infusions are often consumed as an alternative or non-conventional medicaments for their physical or medicinal effects, especially for their stimulant, relaxant or sedative properties, helping thus in the treatment of different diseases (Altintig et al., 2013; Atoui et al., 2005; Kara, 2009; Malinowska et al., 2008; Martín-Domingoa et al., 2017; Pohl et al., 2016; Pytlakowska et al., 2012). It is known that herbal teas can positively influence human health due to the presence of different useful compounds such as amino acids, polysaccharides, vitamins, antioxidants, minerals and ions (Das et al., 2017; Pohl et al., 2016). The concentrations of these compounds in plant depend on many factors like plant species, the origin of the plant, climatic conditions, the characteristics of the substratum on which certain plant grows, the capability of plant to accumulate certain substances, and industrial processes in tea production and preparation (Martín-Domingoa et al., 2017; Pohl et al., 2016; Pytlakowska et al., 2012). Inorganic anions frequently released into water solution during tea preparation include fluorides, chlorides, nitrates, sulphates, and phosphates. Michalski (2006) and Mincă et al. (2013) recorded that inorganic anions and carboxylic acid contribute to the acidity of the medium influencing the flavor and taste of tea itself. For most of them, important biological role, as well as beneficial effects on the function of the human organism has been recorded. Chlorides are the basic constituent ions of extracellular fluid responsible for the proper muscle functioning (Mincă et al., 2013). Phosphates are responsible for the growth and tissue regeneration, and also for proper cell metabolism functioning (Mincă et al., 2013), while sulphates are the main source of sulfur that is necessary for the synthesis of amino acids cysteine and methionine (Balcerzak and Janiszewska, 2015). Fluorides, regarded as the most prominent anions, are very beneficial in small concentrations, enabling proper bones and teeth functioning (Chan et al., 2013; Das et al., 2017; Giljanović et al., 2012). However, many substances that have the positive effect on human health can be harmful if used in higher concentrations than allowed: for example, fluorides in higher concentrations can cause dental and skeletal fluorosis (Emekli et al., 2009; Koblar et al., 2012; Chan et al., 2013; Das et al., 2017; Giljanović et al., 2012) and nitrates and nitrites paralysis of vasomotor center (Mincă et al., 2013).

The aim of this paper was to analyze the inorganic anions in herbal tea infusions commonly consumed in this geographical area using the ion chromatography. The effect of different extraction time at the same temperature, as well as the way of the tea preparation were also taken into consideration, as in Popović et al. (2017) where the influence of these factors on metals release was observed.

MATERIALS AND METHODS

Analytical procedure

The content of inorganic anions was determined by ion chromatography using ICS 2020i Dionex ion chromatographic system consisting of an isocratic eluent delivery pump with the flow of 0.8 mL/min, sample injection port, guard and separation column (AG22 and AS22 Dionix IonPac), suppressor (ASRS 300 Dionix), and conductivity detector. As an eluent, a mixture containing 3.2 mM Na_2CO_3 and 1.0 mM $NaHCO_3$ was used. Calibration solutions of analyzed inorganic anions were prepared by diluting the stock standard solution (1000 mg/L fluorides (F^-), chlorides (Cl^-), nitrates (NO_3^-), phosphates (PO_4^{3-}), and sulphates (SO_4^{2-})) to the required concentration. Ultra-pure water with the electrical conductivity of <0.05 μS/cm was used during the experiment. The solution was injected into the chromatographic column after filtration through 0.45 μm membrane filter.

Sample preparation

As described in Popović et al. (2017), eight different commercial herbal tea samples and one green tea sample of the same brand were used for the analyses: hawthorn (*Crataegus monogyna* Jacq.), St John's wort (*Hypericum perforatum* L.), nettle (*Urtica dioica* L.), elderflower (*Sambucus nigra* L.), green tea (*Camellia sinensis* (L.) Kuntze), bearberry (*Arctostaphylos uva-ursi* (L.) Spreng.), thyme (*Thymus serpyllum* L.), yarrow (*Achillea millefolium* L.), and mint tea (*Mentha piperita* L.). Some of them were associated to "leaf" or "flower" teas depending on which part of the plant was mostly used for the preparation of tea when packed in tea bags. Bags were opened and content was mixed and homogenized prior to drying at 105 °C to constant weight. After homogenization of each sample, five different sets of aqueous extractions were prepared as described in Popović et al. (2017). Each set was prepared by weighing approximately 2 g of each tea sample and soaking it in 100 mL of boiling ultra-pure water: the first set was boiled for five minutes, the second for ten and the third for twenty minutes; for the fourth set, boiling water was added to the tea sample, then it was covered and left at room temperature for five minutes; the fifth set included the addition of lemon juice (5 mL), then it was covered and left for five minutes at room temperature. Conductivity and pH value were also measured in each tea sample water extract using WTW LF 191 conductivity meter and pH meter WTW inoLab pH 730. For each set of samples, a blank was prepared following the same procedure.

Limits of detection (LOD) and limits of quantification (LOQ) of the instrument were calculated as three times and ten times of the residual standard deviation of the low concentration standard (Table 1).

Table 1. Limit of detection and limit of quantification for all anions

Anion	F^-	Cl^-	NO_3^-	PO_4^{3-}	SO_4^{2-}
LOD, mg/L	0.29	0.18	0.30	0.51	0.29
LOQ, mg/L	0.99	0.60	1.00	1.70	0.99

Data analysis

Statistical analysis was done using CANOCO for Windows, Version 5.0 (Ter Braak and Smilauer 2012). Two principal component analyses (PCA) were performed. The first PCA was used to evaluate the relationship between anion concentration released in water after boiling for 5, 10, and 20 min with following supplementary variables included: tea samples, time of boiling, part of the plant included in the making of the tea, pH, and conductivity. The second PCA was used to demonstrate the relationship between the same supplementary variables and anions released in water solution from samples that were boiled for 5 min, that were covered and left at room temperature for 5 min, and samples that were treated with lemon juice covered and left at room temperature for 5 min.

RESULTS AND DISCUSSION

The average contents of the determined anions in the examined tea samples are given in Table 2. The highest variation among different tea samples is observed in nitrate ion values. According to the results, in most tea samples phosphates had the highest concentrations, while the concentrations of other anions were significantly lower. The highest values of phosphates, as well as nitrates, were found in nettle and elder-flower water extracts.

Few data exist for nitrates, chlorides, phosphates, and sulphates. The analysis of these anions was done in few tea samples, mainly in green and mint tea. According to Ozcan et al. (2007) the accumulation of nitrates is possible in leaves, as well as in roots of vegetables, and the concentration itself depends on the plant type, fertilization form, and amount and harvest time, used together with organic fertilizer or industrial fertilizer. So, the concentration of nitrates can be very variable in different plants, but also in the same plant of different geographical origin. Michalski (2006) in his study reported higher

Table 2. The average concentrations of the determined anions in tea infusions

Type of tea	Concentration (mg/g)±SD				
	Fluorides	Chlorides	Nitrates	Phosphates	Sulphates
Hawthorn	2.13±0.08	0.20±0.02	0.66±0.06	3.83±0.10	1.61±0.07
Yarrow	0.39±0.02	2.56±0.01	0.36±0.08	3.12±0.05	1.51±0.03
St John's wort	0.79±0.03	0.99±0.04	1.04±0.06	3.58±0.01	1.38±0.02
Elderflower	2.37±0.10	0.40±0.07	7.34±0.03	6.60±0.04	2.56±0.04
Nettle	0.97±0.05	2.84±0.01	8.27±0.02	7.00±0.03	4.70±0.02
Thyme	0.51±0.04	1.93±0.05	1.29±0.07	3.01±0.07	2.01±0.02
Mint tea	1.49±0.02	1.52±0.01	1.25±0.08	4.58±0.02	2.97±0.08
Bearberry	0.97±0.09	0.16±0.20	0.22±0.07	1.86±0.05	0.73±0.08
Green tea	1.47±0.07	0.37±0.02	0.18±0.02	1.92±0.04	1.28±0.03

nitrates content than in this research and lower content was reported in the study done by Mincă et al. (2013), Balcerzak et al. (2015), and Ozcan et al. (2007), with similar values of this anion for green tea. Considering chlorides, we obtained lower values in green tea than Mincă et al. (2013) and Balcerzak et al. (2015), and also in mint tea reported by Michalski (2006). For phosphates, Balcerzak et al. (2015) reported similar results for green tea, while the content of sulphates was slightly higher. The concentrations of phosphates and sulphates were higher for mint tea (Michalski 2006) and for green tea in study by Mincă et al. (2013). When speaking about fluorides, the data are much more diverse compared to other mentioned anions. Lower concentration of fluorides than in this study was reported by Chan et al. (2013), Emekli et al. (2009), Karak and Bhagat (2010), Kjellevold et al. (2006), Michalski (2006), and Yuwono (2005). According to Das et al. (2017) the content of fluorides in some herbal teas was lower, but similar results for green tea were documented. Similar values of fluorides in tea infusions were also reported by Chan et al. (2013) (0–30 mg/L). On the other hand, Michalski (2006) and Mincă et al. (2013) reported higher values of this anion in their examined tea infusions. According to Malinowska et al. (2008) and Yi and Cao (2008) the content of fluorides can be used as a tool for the estimation of the tea quality. Furthermore, the content of fluorides in green tea, for example, depends on the age of plant burgeon, where young burgeons (with two leaves) release significantly lower concentration of fluorides, compared to older ones where the fluoride concentration can be 2–4 times higher, and according to Chan et al. (2013) even to 10 times higher. Fluoride concentration can also be dependent on the land type, for example,

higher concentrations of fluorides in plants are observed when the plant is growing on acidified substratum (Emekli et al. 2009; Chan et al. 2013).

Figure 1 shows the relationship between ion concentrations in infusions, tea samples, and time of tea boiling. PCA explained a total of 81.2% of variations in our data (PCA axis 1 and PCA axis 2 together explained 73.7% of the variation). It can be seen that the highest content of nitrates and sulphates are in nettle and elderflower extracts, while chlorides have high concentrations in nettle, but also in thyme and yarrow. The highest concentrations of fluorides and phosphates were found in elderflower tea infusion. Supplementary variables that refer to 5, 10, and 20 min of boiling are placed near the center of the ordination diagram. As it was the case with extracted metals from the same tea samples (Popović et al. 2017), their position indicates that significant differences were not observed in the concentrations of ions from the tea samples with changes in boiling time. Nominal variables "flower" and "leaf" were also placed in the center of the ordination diagram, meaning that ions were equally extracted from these plant parts. Conductivity vector and pH are oriented toward the right side of the ordination diagram, and their highest values were recorded in nettle tea infusion.

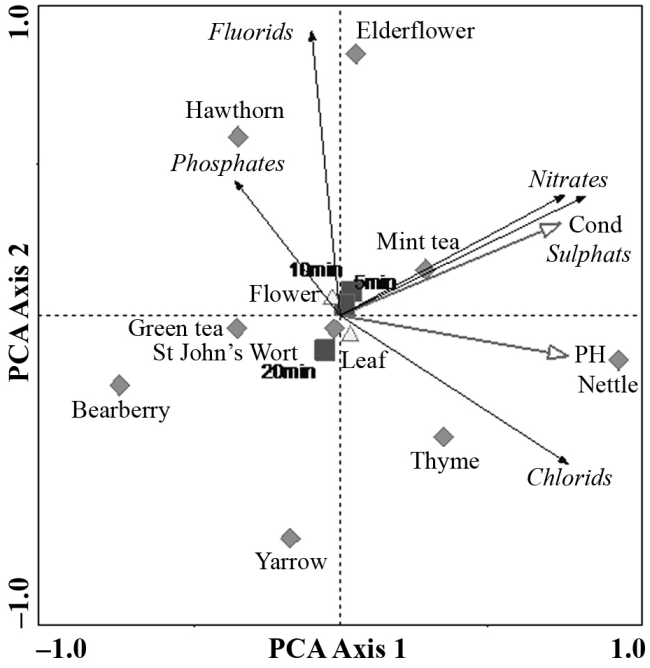

Figure 1. Principal component analysis (PCA) of ions from water extract after boiling for 5, 10, and 20 min. The supplementary variables include tea as blue diamonds (hawthorn, St John's wort, nettle, elderflower, green tea, bearberry, thyme, yarrow, and mint tea); red squares refer to boiling time (5, 10, and 20 min); and white triangles represent the part of the plant from which the tea is primarily produced (leaves, flowers)

Fluorides are the most examined ions when speaking about tea infusions and time as a factor. Giljanović et al. (2012) examined how brewing time affected fluoride concentration for tested tea samples, and concluded that in most cases fluoride concentration reached maximum in 10 to 20 min, which especially referred to mint tea. However, different tea samples acted completely different: the increase in fluoride concentrations with time was observed in mint and pomegranate samples, while in green tea a positive correlation was documented (Giljanović et al., 2012). Similar findings were given by Yuwono et al. (2005), where the increase was observed only in green and black tea samples. Emekli et al. (2009) demonstrated that there was no significant difference in fluoride content in different herbal teas if time of boiling was higher than five minutes. According to Chan et al. (2013) the highest concentration of anions was released after two minutes of boiling, and the prolonged time of boiling did not influence the changes in the concentration of anions – the concentration did not increase with time. On the other side, Chan et al. (2013), Karakand Bhagat (2010), and Malinowska et al. (2008) documented the increase in fluoride content with time of boiling. Giljanović et al. (2012) also recorded that the content of released fluorides in the tea water solution depended on the way of tea packing procedure, and also highlighted that the extraction was better from teas that consisted of smaller parts of plants, than from those consisting of larger parts. Kjellevold et al. (2006) pointed out that in some cases tea plant acted as an adsorption agent when concentrations of fluorides were higher in tea infusions, and emphasized the fact that the concentration of total fluorides in tea leaf did not affect its adsorption capacity. In this study, higher concentrations of fluorides were obtained when compared to a large number of papers dealing with this topic. It is possible that in this case some form of balance between the release and absorption exists, which is responsible for similar results obtained after 5, 10, and 20 min of tea boiling.

Since no major differences were observed after 5, 10, and 20 min of tea boiling, we decided to analyze samples obtained after 5 minutes of tea preparation (tea samples boiled for five minutes, tea samples in which boiling water was added, then covered and left at room temperature for five minutes; tea samples in which lemon juice (5 mL) was added, after which they were covered and left for five minutes at room temperature). According to Das et al. (2017) and Yuwono et al. (2005) five minutes represent the ideal length for the tea preparation since during five minutes the best taste and low levels of tannins are guaranteed.

The analysis of differently prepared tea extracts for 5 minutes time showed similar results (Figure 2). PCA explained a total of 91.3% of variations in our data (PCA axis 1 and PCA axis 2 together explained 74.0% of the variation). Unlike metals (Popović et al. 2017) ions behaved differently when these three different ways of preparation for 5 minutes were observed. All supplementary variables referring to 5 minutes tea preparation are placed in the center of the ordination diagram, showing that different ways of preparation do not influence the ion concentrations in tea infusions. So, unlike metals, higher concen-

trations of ions have not been observed in acidified tea infusions. The relationship between ion concentrations in infusions and tea samples is exactly the same as in the previous figure, leading to the conclusion that ion release after all five different ways of preparation is the same, or that a different way of preparation available in this study does not influence the changes in the ion concentrations in tea infusion.

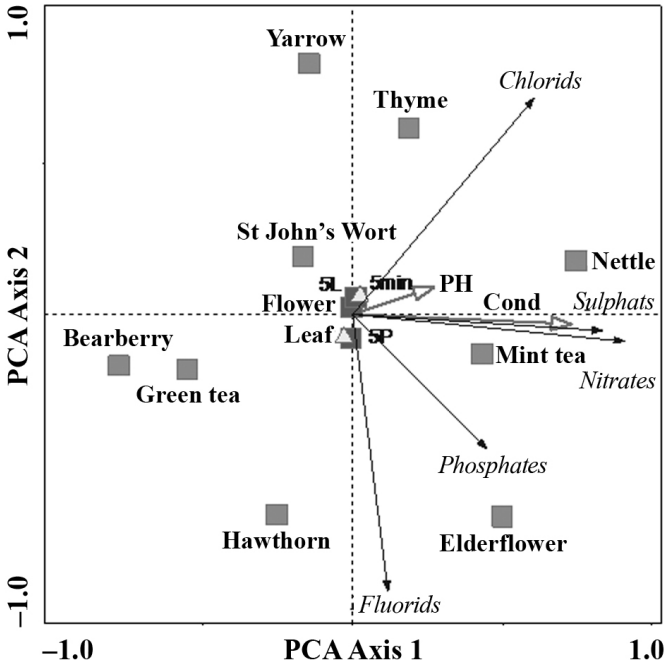

Figure 2. Principal component analysis (PCA) of ions from water extract after boiling for 5 min (5 min), after samples were covered and left at room temperature for 5 min (5P), and after samples were covered and left at room temperature for 5 min with added lemon juice (5L). The supplementary variables include tea as blue diamonds (hawthorn, St John's wort, nettle, elderflower, green tea, bearberry, thyme, yarrow, and mint tea); red squares refer to boiling time; and white triangles represent the part of the plant from which the tea is primarily produced (leaves, flowers)

In the majority of tea samples, pH value showed slightly lower values – the samples were slightly acidic. The same finding was observed by Mincă et al. (2013) where infusions of green, white, and black tea were examined. The values of pH ranged from 4.71 to 5.91 in all tea samples except in nettle tea infusion, where pH of 7.74 was measured. The conductivity ranged from 474 μS/cm (bearberry) to 1995 μS/cm (nettle).

Acidification of samples with lemon juice does not significantly influence the release of anions in tea infusions. However, in nettle infusions some differences not seen in the ordination diagram were observed in acidified

samples. More precisely, nettle infusion is the only infusion having pH values above 7. After acidification, a slight increase in chlorides and nitrates was observed, while sulphate content was decreased. On the other hand, the content of phosphates was significantly higher in acidified sample. This suggested that pH had an indirect impact on the anion concentration in samples.

It is worth mentioning that the results of the anion analysis were hard to compare with the results from other studies. It is probably due to the different methods used, the origin of herbs itself, different examined herbal teas, type of the land on which the herb is grown and its acidity, or content of fluorides in land.

CONCLUSION

Results indicated that all investigated anions showed variations in their concentrations among different tea samples. In almost all tea samples phosphate ion had the highest concentrations and its highest release was observed in elderflower and nettle tea infusions. Beside phosphate content, nettle tea infusion also had the highest chloride, nitrate, and sulphate content. According to the performed principal component analysis, no significant difference in ion concentration after boiling for 5, 10, and 20 minutes, as well as after acidification of the medium was observed. In the aqueous solution of herbal tea, pH of the solution did not have an effect on ion concentration, but slight variations were observed in nettle, where the highest pH of the tea infusion was measured. The only difference was found in nettle infusions, where acidification showed higher ion concentrations, except for fluoride ions. A presumption could be made that there is some indirect influence on the concentration change rather than on pH value. The obtained results can contribute to the general knowledge of inorganic anions in herbal tea infusions that can be beneficial in human diet, as well as to better understanding of the effect of different factors on their release.

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ОДРЕЂИВАЊЕ НЕОРГАНСКИХ ЈОНА У РАСТВОРУ БИЉНИХ ЧАЈЕВА ЈОНСКОМ ХРОМАТОГРАФИЈОМ

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САЖЕТАК: Јонски садржај испитиван је код девет водених раствора чаја, при чему је на екстракцију јона посматран утицај дужине кувања, киселости средине (која је постигнута додатком лимуновог сока) и начина припреме. Јонска хроматографија коришћена је за одређивање садржаја неорганских анјона, а подаци су обрађени помоћу *Canoco* програма за мултиваријациону анализу. Јонски садржај варира код различитих узорака чаја. Највеће концентрације хлоридних, нитратних, фосфатних и сулфатних јона одређене су у коприви, док су највеће концентрације флуорида одређене у инфузији кантариона. Утицај времена кључања (5, 10 и 20 мин.), киселост медијума и различити начини припреме (кључање и хлађење на собној температури) статистички су приказани помоћу анализе главних компоненти. Испитивани фактори нису имали значајан утицај на јонску концентрацију у чајним инфузијама.

КЉУЧНЕ РЕЧИ: анализа главних компоненти, биљни чајеви, чајне инфузије, јонска хроматографија, неорганski анјони

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EVALUATING NATURAL MONUMENTS MANAGEMENT OBJECTIVES USING *SMART* AND *SMARTER* METHODS

ABSTRACT: This research presents the process of assessment of management objectives for natural monuments by applying two multi-criteria methods: SMART and SMARTER. For SMART application three decision makers performed the required assessments, while SMARTER results were gained by using the ranking of objectives. Namely, in both cases, the bases of the assessments were the management guidelines defined by the IUCN organization. In SMARTER analysis the ranking of objectives defined by the IUCN was the only input data, while SMART application included decision makers who assigned the points to each objective respecting the IUCN ranking. The obtained results represent the weights (cardinal values) of management objectives for natural monuments, which can be a convenient basis for further assessment and evaluation of management plans for this category of protected areas.

KEYWORDS: natural monuments, management objectives, SMART, SMARTER

INTRODUCTION

Natural monuments are usually smaller protected areas with well preserved natural features. In addition, natural monuments can be individual trees with distinguished botanical values (Vujić, 2008). There are three parks in Novi Sad: Dunavski, Futoški, and Kamenički park (Lakićević et al., 2017), and six trees have been declared natural monuments. Among the trees, the most famous example is a tree of *Celtis australis* L. in Modena Street in the city center.

IUCN (International Union for Conservation of Nature) organization defined management objectives for all categories of protected areas. The headquarters of the organization is in Gland, Switzerland, but there is also a regi-

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onal office for Eastern Europe in Belgrade, in the building of the Institute for Nature Conservation of Serbia. IUCN published many guidelines dealing with management of protected areas (e.g. IUCN, 2008, 2013). This paper analyses the natural monuments management objectives by introducing two multi-criteria analysis methods SMART (Edwards and Barron, 1994) and its version SMARTER (Edwards and Barron, 1994). Namely, the objectives proposed by the IUCN were processed within two multi-criteria methods, in order to obtain cardinal values representing the importance of objectives. Those results can serve as a support when analyzing and selecting management plans developed for the natural monuments. Even though the importance of management objectives can vary depending on a specific case study area, the results provide a general outline for their assessment.

When applying SMART method, there were three decision makers who assessed the set of objectives by assigning certain number of points to each of the objective, and based on those values it was possible to estimate the importance of management objectives.

Application of SMARTER method, in this research, did not require the assessments of the decision makers. As a matter of fact, the ranking of objectives proposed by the IUCN was the only input data for calculating the importance of management objectives.

At the end of the research, results of both methods were compared and the differences were discussed. The paper explains the process of assessing the importance of management objectives using two prominent multi-criteria methods. The demonstrated procedure can be repeated for many different assignments in the subject area.

MATERIALS AND METHODS

The objectives analyzed in the research are presented in Table 1. Their importance is defined by the IUCN, using values from 1 to 3, where: "1" represents primary objective, "2" secondary objective, and "3" potential objective, and there are also some in between values.

Table 1. Natural monuments management objectives (IUCN, 2013)

Label	Objective	Importance
O_1	Scientific research	3
O_2	Preservation of habitats, species and genetic diversity	1–2
O_3	Protection of specific natural/cultural features	1
O_4	Recreation and tourism	1–2
O_5	Education	2
O_6	Maintenance of cultural /traditional landscape features	2

The criteria in the Table 1 were processed within SMART and SMARTER methods, and for the SMART assessments there were three decision makers with the background in forestry and nature protection. The explanation of the methods is provided in the text that follows.

SMART (Simple Multi-Attribute Ranking Technique) is based on assigning numerical values to decision elements (objectives, alternatives) in accordance to their importance. The process usually starts by giving 10 points to the least important element, and the rest of points are being given accordingly (Kangas et al., 2015). The calculation of final weights is performed as:

$$a_j = \frac{p_j}{\Sigma_{i=1}^{m} p_i} \quad (1)$$

where a_j is the weight of the j^{th} element, p_j is number of points assigned to the j^{th} element, and m is the number of elements.

SMARTER (Simple Multi-Attribute Rating Technique Exploiting Ranks) is a version of SMART that is primarily based on the ranking of elements. Within this method there are different techniques for calculating final weight and in this research we used two of them − Rank order centroid (ROC) and Rank reciprocal rule (RR).

Rank order centroid – ROC (Edwards and Barron, 1994) provides the weights following the rule:

$$a_j = (1/m)\sum_{i=j}^{m} 1/i \quad (2)$$

where a_j is the weight of the j^{th} element, i is a rank of the j^{th} element, and m is the number of elements.

Rank reciprocal rule – RR (Stillwell et al., 1981) establishes final weights by dividing reciprocal of rank of the j^{th} element with the sum of the reciprocals for all elements:

$$a_j = \frac{1/\eta}{\Sigma_i 1/n} \quad (3)$$

Both SMART and SMARTER methods have been reported as suitable in the area of natural resources management (Gärtner et al. 2008).

RESULTS AND DISSCUSSION

Table 2 presents the evaluations of importance of six management objectives for natural monuments following the SMART procedure. There were three decision makers (DMs) included in the research, and they have assigned points to each objective respecting their ranking defined by the IUCN. That means that the ranking of objectives was considered as predefined and therefore is the same for all the decision makers included in the research, but relative importance of objectives differs according to decision makers' individual assessments.

Table 2. SMART assessment of management objectives

Objectives	SMART points		
	DM_1	DM_2	DM_3
O_3	40	60	30
O_2	25	40	20
O_4	25	40	20
O_5	15	25	12
O_6	15	25	12
O_1	10	10	10

Based on the values provided in the Table 2 it was possible to calculate the weights of objectives for all decision makers and the results are presented in Table 3.

Table 3. SMART weights of management objectives

Objectives	SMART weights		
	DM_1	DM_2	DM_3
O_3	0.308	0.577	0.288
O_2	0.192	0.385	0.192
O_4	0.192	0.385	0.192
O_5	0.115	0.240	0.115
O_6	0.115	0.240	0.115
O_1	0.077	0.096	0.096

The same decision problem was processed within SMARTER method and the results for two techniques applied are shown in Table 4. For estimating

the weights of objectives only input data were the importance ranks of objectives defined by the IUCN.

Table 4. SMARTER weights of management objectives

Objectives	SMARTER weights	
	ROC technique	RR technique
O_3	0.408	0.408
O_2	0.200	0.170
O_4	0.200	0.170
O_5	0.082	0.092
O_6	0.082	0.092
O_1	0.028	0.068

In order to compare the results gathered by applying SMART and SMARTER method, the arithmetic mean of weights was calculated (Table 5). The arithmetic means of SMART results for three decision makers are presented in the first column, while the second column represents the arithmetic means of weights obtained by using ROC and RR techniques.

Table 5. SMART and SMARTER weights of management objectives (average values)

Objectives	SMART weights	SMARTER weights
O_3	0.391	0.408
O_2	0.256	0.185
O_4	0.256	0.185
O_5	0.157	0.087
O_6	0.157	0.087
O_1	0.090	0.048

Analysis of values presented in Table 5 shows that the application of both methods provided similar results. The main difference between two methods applied is in the type of input data; SMARTER requires only defining the rank of elements, while SMART input data are points assigned to each element which discovers the relative ratio between set of elements.

The interpretation of the results gained in this research (Table 5) shows that the relative importance of the most important objective – *protection of specific natural/cultural features* – is equal to 0.391 according to SMART

method and slightly higher, 0.408, according to SMARTER method. On the other side, the relative importance of the least important objective – *scientific research* – derived by SMART and SMARTER is equal to 0.090 and 0.048, respectively. The importance of all objectives takes U shape more sharply if the method SMARTER is used instead of SMART. Enhanced boundary values obtained by the SMARTER method indicate that this method could be more useful than SMART when a sharp distinction between objectives is required.

CONCLUSION

Management of protected areas requires evaluating the set of different objectives and that task can be performed by applying multi-criteria methodologies. This paper presents the results of evaluation of the natural monuments managing objectives using SMART and SMARTER methods. The results gained can be useful for defining and evaluating the management plans for natural monuments. In this research, management objectives were processed using two multi-criteria methods, but these methods can be applied for the assessment of the standard decision making hierarchy – including the set of alternatives i.e. management plans.

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ВРЕДНОВАЊЕ ЦИЉЕВА УПРАВЉАЊА СПОМЕНИЦИМА ПРИРОДЕ ПРИМЕНОМ *SMART* И *SMARTER* МЕТОДА

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РЕЗИМЕ: Рад приказује поступак вредновања циљева управљања споменицима природе применом два метода вишекритеријумске анализе – *SMART* и *SMARTER*. За примену метода *SMART* укључена су три доносиоца одлука, док су *SMARTER* резултати добијени коришћењем дефинисаног рангирања циљева. Наиме, у оба случаја основа за вредовања биле су смернице за управљање заштићеним подручјима које је дефинисала Међународна унија за заштиту природе – *IUCN*. За *SMARTER* анализу рангирање циљева, како је дефинисала ова организација, био је једини улазни податак, док је примена метода *SMART* укључила доносиоце одлука који су доделили поене сваком од циљева поштујући *IUCN* рангирање. Добијени резултати представљају тзв. тежине (кардиналне вредности) циљева управљања споменицима природе, што може да буде пригодна основа приликом будућих вредновања управљачких планова овим заштићеним природним добрима.

КЉУЧНЕ РЕЧИ: споменици природе, циљеви управљања, *SMART, SMARTER*

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Editor-in-Chief

IVANA MAKSIMOVIĆ

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1.3. Template for the authors can be found at: http://maticasrpska.org.rs/wordpress/assets/INSTRUCTION-TO-ATHORS.pdf

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6.1. SI units of measurement (Système international d'unités) should be used, but other officially accepted units are acceptable when necessary.

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