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Napredne spregnute tehnike u analizi ksenobiotika

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Izvod:
IZ
Prisustvo organskih zagađujućih supstanci (farmaceutski aktivnih komponenata i prirodnih toksina-mikotoksina) u uzorcima životne sredine i namirnicama je u porastu kao posledica novih industrijskih procesa i ostalih antropogenih aktivnosti, kao i klimatskih promena. Takođe veliku pažnju javnosti privlače i neorganske zagađujuće supstance kao što su teški elementi. S obzirom da zagađujuće supstance imaju negativan uticaj na životnu sredinu i zdravlje ljudi, u svetu se preduzimaju mere u cilju smanjenja stepena izloženosti toksičnim jedinjenjma i posledicama izlaganja. Trenutno, jedan od najvećih izazova, jeste procena rizika povezana sa velikim brojem zagađujućih supstanci u tragovima ili u tzv. ultratragovima, uključujući "novo" otkrivena zagađujuća jedinjenja, a jedan od osnovnih trendova je razvoj i primena brzih i efikasnih metoda za njihovu analizu u ispitivanim uzorcima na bazi naprednih hromatografskih i spektrometrijskih tehnika.
Tehnike bazirane na tečnoj hromatografiji sa različitim masenim analizatorima za kvantifikaciju organskih zagađujućih supstanci kao i metode zasnovane na atomskoj apsorpcionoj spektrometriji za određivanje ultratragova neorganskih zagađujućih supstanci postale su referentne na međunarodnom nivou. Ovakve napredne spregnute tehnike postale su važne za identifikaciju, kvantifikaciju i praćenje različitih zagađujućih supstanci u uzorcima životne sredine i namirnicama i proceni njihovog štetnog uticaja na zdravlje čoveka. S obzirom da su u literaturi retka istraživanja koja se bave razvojem i primenom metoda zasnovanih na naprednim hromatografskim i spektrometrijskim tehnikama i određivanju organskih i neorganskih zagađujućih supstanci u matriksima životne sredine i namirnicama sa prostora zapadnog Balkana, a uzimajući u obzir njihovu važnost, specifični ciljevi disertacije su:

- unutrašnja („*in-house*") provera kvaliteta i pouzdanosti postojeće „multi-rezidualne" metode zasnovane na UHPLC-QqLIT-MS/MS za analizu 81 farmaceutski aktivne komponente (PhAC) u otpadnoj, površinskoj, podzemnoj i pijaćoj vodi i po prvi put dobijanje sveobuhvatnih rezultata njihovog prisustva u različitim tipovima vode sa područja Srbije;
- unutrašnja („*in-house*") provera kvaliteta i pouzdanosti postojeće „multi-toksin" metode za analizu 8 *Fusarium* mikotoksina u uzorcima ozime pšenice različitih sorti zasnovane na HPLC-QqQ-MS/MS radi određivanja regionalnih razlika između žitnih regiona kao i otpornosti ispitivanih sorti pšenice na kontaminaciju *Fusarium* toksinima;
- modifikacija postojeće „multi-toksin" metode zasnovane na UHPLC-QqQ-MS/MS za analizu 11 osnovnih mikotoksina u uzorcima brašna i njena unutrašnja („*in-house*") provera kvaliteta i pouzdanosti, kao i provera kroz interlaboratorijsko poređenje, radi dobijanja podataka za procenu štetnog uticaja ispitivanih mikotoksina na zdravlje populacije;
- razvoj „multi-toksin" (višekomponentne) i „multi-matriks" (za više matriksa) metode bazirane na UHPLC-QqQ-MS/MS za analizu 10 mikotoksina u različitim vrstama koštuničavog voća čija provera kvaliteta je zasnovana na intralaboratorijskoj proveri tačnosti i preciznosti dobijenih rezultata;
- primena postojeće analitičke procedure zasnovane na naprednoj tehnici pripreme (mikrotalasnoj digestiji) različitih uzoraka biljnog i životinjskog porekla i provera kvaliteta metode identifikacije i kvantifikacije zasnovane na atomskom apsorpcionom spektrometru sa grafitnom kivetom (GFAAS) radi dobijanja sveobuhvatnih rezultata o prisustvu teških elemenata (arsena, olova i kadmijuma) radi procene izloženosti stanovništva Srbije toksičnim neorganskim elementima.

Postignuti rezultati predstavljaju jedinstvene rezultate za područje Srbije dobijene primenom naprednih spregnutih tehnika koje imaju značajnu ulogu u praćenju prisustva većeg broja organskih i neorganskih zagađujućih supstanci u izabranim uzorcima životne sredine i namirnica, (regulisanih postojećim zakonodavstvom) radi procene stepena zagađenosti ili u slučaju jedinjenja koja nisu regulisana zakonodavstvom radi sticanja novih saznanja o njihovom prisustvu i proceni mogućeg negativnog uticaja na životnu sredinu i zdravlje populacije.
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Abstract:
AB
The presence of organic pollutants in environmental samples and food (pharmaceutically active components and natural toxins-mycotoxins) is increased as a result of new industrial processes and other anthropogenic activities, as well as climate change. Similarly heavy elements as inorganic pollutants have attracted worldwide attention. Since, these pollutants have negative impact on environment and human health, extremely efforts are undertaken in the world to reduce the level of exposure to these pollutants and consequences of the exposure. Currently, one of the highest challenges is to assess the risk associated with a large number of pollutants in trace or ultra trace levels, including "new" (emerging) discovered pollutants, and one of the main trends is development and implementation of fast and efficient methods for their analysis on the basis of advanced chromatographic and spectrometric techniques. Therefore, coupled techniques have become important for the identification, quantification and monitoring of various pollutants in environmental samples and food and assessment of their hazard impact on human health. Since, there are scarce data about the development and application of advanced methods based on chromatographic and spectrometric techniques for determination of organic and inorganic pollutants in environmental matrices and food from the Western Balkan, and taking into account their importance, specific objectives of the dissertation were:

- internal ("in-house") quality control of the existing "multi-residual" method based on UHPLC-QqLIT-MS/MS for analysis of 81 pharmaceutically active components (PhAC) in wastewater, surface, underground and drinking water due to obtaining for the first time comprehensive results of their presence in different types of water from Serbia;
- internal ("in-house") quality control of the existing "multi-toxin" method for the analysis of 8 *Fusarium* mycotoxins in samples of different winter wheat cultivars based on HPLC-QqQ-MS/MS to determine the differences among wheat-growing regions as well as the resistance of the analysed wheat cultivars towards *Fusarium* toxins;
- modification of existing "multi-toxin" method based on UHPLC-QqQ-MS/MS for analysis of 11 principal mycotoxins in samples of flour and its internal ("in-house") quality control as well as verification through the interlaboratory comparison, in order to obtain data for assessing the hazard effect of these mycotoxins on the health of the population;
- the development of "multi-toxin" and "multi-matrix" method based on UHPLC-QqQ-MS/MS for the analysis of 10 mycotoxins in various types of nuts based on intra-laboratory verification of the accuracy and precision of the obtained results;
- application of analytical procedure based on advanced preparation technique (microwave digestion) and atomic absorption spectrometer with a graphite furnace (GFAAS) and its verification in order to obtain comprehensive results on the presence of heavy elements (arsenic, lead and cadmium) in different samples of plant and animal origin to assess the exposure of the Serbian population to toxic inorganic elements.

The obtained results are unique for the Serbia. They are obtained by applying advanced coupled techniques that have a significant role in monitoring the presence of a numerous organic and inorganic pollutants in analyzed samples of the environment and food. The presented results contribute to the assessment of pollution degree and in the case of new (emerging) not regulated polutant they might give new information about the possible negative impact on the environment and health of the population.	
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Disertacija predstavlja deo rezultata postignutih na projektu br. 172050 finansiranog od strane Ministarstva prosvete, nauke i tehnološkog razvoja Republike Srbija, u periodu 2011-2014., pod rukovodstvom prof. dr Biljane Škrbić.

Ova doktorska disertacija urađena je u Centru izvrstnosti za bezbednost hrane i nove rizike na Tehnološkom fakultetu u Novom Sadu i u Katalonskom Institutu za ispitivanje voda, Đirona, Španija, pod mentorstvom dr Biljane Škrbić, redovnog profesora, kojoj dugujem najveću zahvalnost ne samo na nesebičnom angažovanju tokom celokupnog rada na disertaciji, već od početka mog profesionalnog razvoja. Duboku zahvalnost dugujem prof. Škrbić na ukazanom poverenju i pruženoj šansi da budem deo njenog istraživačkog tima u momenutu kada je nosila ogromnu odgovornost za realizaciju FP7 međunarodnog projekta kojim je rukovodila. Od prvog dana kada me je izabrala za saradnika i uvela u svet nauke, pokazala mi je da visoko postavljene ciljeve je moguće ostvariti samo uz ogromnu želju, odricanje, veliku posvećenost i rad. Njena predanost i posvećenost istraživačkom radu su mi bile smernice na putu kojim sam stigla do odbrane doktorske disertacije. Profesorka, hvala Vam na svemu što ste me naučili, čast mi je, zadovoljstvo i privilegija što sam deo Vašeg tima!

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Isto tako, zahvaljujem se dr Jeleni Cvejanov, vanrednom profesoru, na nesebičnoj podršci i korisnim sugestijama u svakodnevnom radu.

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U Novom Sadu, *Jelena Živančev*

08.07.2014.

SADRŽAJ

LISTA SKRAĆENICA

3-ADON	3 acetil deoksinivalenol
ADON	Acetil deoksinivalenol
AFB1	Aflatoksin B1
AFB2	Aflatoksin B2
AFG1	Aflatoksin G1
AFG2	Aflatoksin G2
ALT	Alternariol
AME	Alternariol metil eter
AOH	Alternuen
APCI	Hemijska jonizacija
APPI	Fotojonizacija
As	Arsen
ASE	Ubrzana ekstrakcija pod pritiskom
a_w	Aktivnost vode
BEA	Beauvericin
BEN	Balkanska endemska nefropatija
CIT	Citrinin
CRM	Sertifikovani referentni materijal
Cu	Bakar
Da	Dalton
DART	Direktna analiza u realnom vremenu
DAS	Diacetoksiskirpenol
DC	Jednosmerna struja
DON	Deoksinivalenol
DON-3-Glc	Deoksinivalenol-3-glukozid
d-SPE	Disperzivna ekstrakcija na čvrstoj fazi
EI	Elektronska jonizacija
ELISA	Imunoadsorpcioni enzimski test
ENN	Eniatin
ENN B	Eniatin B
EPI	"*Enhanced product ion*"

ERG	Ergot alkaloid
ESI	Elektrosprej jonizacija
FAAS	Plamena atomska apsorpciona spektrometrija
FB1	Fumonizin B1
FB2	Fumonizin B2
FB3	Fumonizin B3
Fe	Gvožđe
FHB	„*Fusarium head blight*“
FID	Plameno jonizacioni detektor
FLD	Fluorescentni detektor
FT-ICR	Analizator sa jon-ciklotronskom rezolucijom i Furijerovom transformacijom
FUS-X	Fuzarenon X
FHWM	Širina pika na polovini njegove visine
GC	Gasna hromatografija
GFAAS	Atomska apsorpciona spektrometrija sa grafitnom kivetom
H-ESI	Grejana elektrosprej jonizacija
Hg	Živa
HPLC	Visoko pritisna tečna hromatografija
HRMS	Visokorezolucioni maseni spektrometar
HT-2	HT-2 toksin
IA	Imunoafinitetne kolone
IARC	Međunarodna agencija za istraživanje raka
ICP-MS	Induktivno-kuplovana (spregnuta) plazma sa masenom spektrometrijom
ICP-OES	Optička emisiona spektrometrija sa induktivno spregnutom plazmom
IT	Analizator na principu jonske zamke
LC/MS	Tečna hromatografija sa masenom spektrometrijom
LOD	Granica detekcije
LOQ	Granica kvantifikacije
MALDI	Jonizacija potpomognuta laserskom desorpciom iz matriksa
MON	Moniliformin
MRM	Mod za praćenje višestruke fragmentacije
MS/MS	Tandemska masena spektrometrija

n.a.	Nije određivano
Ni	Nikal
NIV	Nivalenol
NSAID	Nesteroidni anti-inflamatorni lekovi
OTA	Ohratoksin A
PAT	Patulin
Pb	Olovo
PhAC	Farmaceutski aktivne komponente
PSA	„*Primary secondary amine*“ sorbent
PT	Interlaboratorijsko poređenje
QIT	Kvadrupol “*ion trap*”
QqLIT	Hibridni trostruki (tripl) kvadrupol “*ion trap*”
QqQ	Trostruki (tripl) kvadrupol
QqTOF	Hibridni analizator na bazi vremena preleta
QuEChERS	Brza, laka, jeftina, efikasna, robustna i sigurna priprema uzorka
R^2	Koeficijent determinacije
RF	Rediofrekfentni napon
RP	Reversna tečna hromatografija
RSD	Relativna standardna devijacija
RT	Retenciono vreme
SCF	Naučni odbor za hranu
S/N	Odnos signala i šuma
SD	Standardna devijacija
SIM	Mod za praćenje pojedinačnog jona
SPE	Ekstrakcija na čvrstoj fazi
SRM	Mod za praćenje izabranog fragmenta
SSE	Suzbijanje ili povećanje signala analita
STE	Sterigmatocistein
T-2	T-2 toksin
TDI	Tolerantni dnevni unos
TISP	„*Turbo ion spray*“ jonski izvor
TLC	Tankoslojna hromatografija
tm	Telesna masa
TOF	Analizator na bazi vremena preleta

UHPLC	Ultra pritisna tečna hromatografija
UV	UV detektor
Zn	Cink
ZON	Zearalenon
α-ZOL	α-zearalenol

1. UVOD

Istraživanja koja se bave zagađenjem životne sredine kao posledice antropogenih aktivnosti dobila su veliki značaj i dospela u žižu interesovanja naučne i šire javnosti od pojave prvih dokaza o rizicima i štetnom uticaju po zdravlje ljudi, životinja i same okoline. Tek sa razvojem novih naprednih analitičkih metoda i procedura, veći deo društva je upoznat sa određenim ekološkim problemima i rizikom po ljudsko zdravlje. U laboratorijama za identifikaciju i kvantifikaciju organskih zagađujućih supstanci iz različitih uzoraka životne sredine uobičajene su metode koje se baziraju na hromatografskim tehnikama, kao što su tečna (HPLC) i gasna (GC) hromatografija, dok se za neorganske zagađujuće supstance koriste atomske spektrometrijske tehnike. Sa pooštravanjem kriterijuma definisanih regulativama Evropske komisije u odnosu na pouzdanost primenjenih metoda, pred laboratorije se postavljaju sve strožiji analitički zahtevi, te razvoj novih instrumenata za detekciju zagađujućih supstanci ide u pravcu povećanja njihove osetljivosti, selektivnosti i opsega primene, kako bi se u jednoj analizi dobio što veći broj pouzdanih podataka o prisustvu različitih vrsta zagađujućih supstanci prisutnih u tragovima. Tako na primer, u poslednjih dvadeset godina, uočljiv je trend snižavanja eksperimentalno postignutih instrumentalnih granica detekcije koje su ranije bile na nivou nanograma, a danas su na nivou pikograma pri čemu je značajan doprinos ovom trendu dao razvoj tečne hromatografije sa masenom spektrometrijom. U poslednjih nekoliko godina, tečni hromatografski sistemi sa jednim masenim spektometrijskim analizatorom (LC/MS) zamenjeni su novim, naprednim spregnutim sistemima kao što je hromatografski sistem sa trostrukim (tripl) kvadrupolnim (LC-QqQ-MS/MS) ili sa hibridnim trostrukim (tripl) kvadrupolnim linearnim „ion trap“ masenim analizatorom (LC-QqLIT-MS/MS). Takođe, razvoj tečne hromatografije i to UHPLC („*ultra high performance liquid chromatography*“) povezane sa tripl kvadrupolnim masenim analizatorima, čine ovu tehniku veoma značajnom i primenljivom na različitim poljima istraživanja. UHPLC-QqQ/QqLIT-MS/MS ima široku primenu, zbog potvrđene selektivnosti, osetljivosti i mogućnosti identifikacije i kvantifikacije različitih organskih zagađujućih supstanci „multi-rezidualnim/multi-toksin“ metodama u koncentracionim nivoima ispod 1 ppb primenom jednostavnih i brzih metoda pripreme veoma složenih matriksa. Usavršavanjem postojećih (elektrosprej-ESI, izvor sa hemijskom jonizacijom-APCI) i razvojem novih jonizacionih izvora kao što je grejani elektrosprej (HESI) i fotojonizacioni (APPI) izvor, učinili su ovu tehniku primenjivu kako za polarne, tako i za nepolarne organske supstance. Sa razvojem UHPLC kao nove tehnologije koja omogućava rad na 1000 bara (dok HPLC radi na 400 bara), moguće je koristiti kolone prečnika 1mm sa punjenjem čestica od 1-2 μm kao i znatno veće protoke mobilne faze u poređenju sa HPLC. Navedene karakteristike UHPLC-QqQ/QqLIT-MS/MS čine ovu tehniku superiornom u odnosu na druge jer je vreme jednog analitičkog ciklusa za „multi-rezidualne/multi-toksin“ metode do 10 minuta ili kraće, što je značajano zbog brzine dobijanja pouzdanih rezultata velikog broja uzoraka, uštede korišćenih rastvarača i zaštite životne sredine.

Nadalje, veliku pažnju javnosti privlače neorganske zagađujuće supstance kao što su teški elementi, kao i analitičke metode i tehnike namenjene za njihovo određivanje. Jedna od tehnika koja se primenjuje za određivanje elemenata u tragovima je atomska apsorpciona spektrometrija sa grafitnom kivetom (GFAAS) koja ima značajnu ulogu na polju analize neorganskih zagađujućih supstanci.

1.1. Cilj rada

Napredne analitičke metode imaju značajnu ulogu u analizi vrlo kompleksnih uzoraka i potvrdi njihovog stepena zagađenja. Pri tome, prednost imaju brze, jednostavne, ekonomski isplative metode pripreme, koje omogućavaju dobijanje, u kratkom vremenskom roku, pouzdanih rezultata za veći broj uzoraka koji sadrže hemijski različite zagađujuće supstance, i time istovremeno pružaju mogućnost brze procene izloženosti populacije kako organskim tako i neorganskim zagađujućim supstancama. Ove metode pripreme u kombinaciji sa naprednim analitičkim tehnikama za identifikaciju i kvantifikaciju zagađujućih supstanci primenjuju se kako u zaštiti životne sredine, tako i u prehrambenoj i farmaceutskoj industriji i biomedicini. Istraživanja zasnovana na naprednim spregnutim tehnikama u razvijenim zemljama Evrope usaglašena sa Regulativom Evropske unije (96/23/EC)[1] zahtevaju da organske zagađujuće supstance budu potvrđene sa tri identifikaciona jona; tj. jednim prekursor jonom i dva produkt jona, ukazujući na potrebu razvoja i primene „multi-rezidualnih/multi-toksin“ metoda i određivanje organskih zagađujućih supstanci sa UHPLC-QqQ/QqLIT-MS/MS. Pored toga, postoje mnoge oblasti nauke i industrije gde je potrebno odrediti prisustvo neorganskih zagađujućih supstanci u veoma niskim koncentracionim nivoima, s obzirom da ove supstance mogu da imaju negativan uticaj na zdravlje ljudi i životnu sredinu, te potreba da se razviju i primene analitičke tehnike zasnovane na atomskim spektrometrijskim tehnikama su od vitalnog značaja.

Osobine i izvori ispitivanih zagađujućih supstanci u izabranim matriksima predstavljeni su u poglavlju OPŠTI DEO. Napredne spregnute tehnike, koje se najčešće koriste, a koje su primenjene u ovom radu, predstavljene su takođe u ovom poglavlju, kao i izabrani radovi u kojima se koriste ove tehnike.

U EKSPERIMENTALNOM DELU je opisano uzimanje uzoraka, kao i metode pripreme i analize uzoraka primenjene u Centru izvrsnosti za bezbednost hrane i procenu rizika, Tehnološkog fakulteta Novi Sad i Katalonskog Instituta za ispitivanje voda, Đirona, Španija.

Tehnike bazirane na tečnoj hromatografiji sa različitim masenim analizatorima za kvantifikaciju organskih zagađujućih supstanci kao i metode zasnovane na atomskoj

[1] Council Directive 96/23/EC, OJ L 221, 17.8.2002, str. 16-17 (u daljem tekstu: Regulativa Evropske unije (EU, 2002)[1])

apsorpcionoj spektrometriji za određivanje ultratragova neorganskih zagađujućih supstanci postale su referentne na međunarodnom nivou. Ovakve napredne spregnute tehnike postale su važne za identifikaciju, kvantifikaciju i praćenje različitih zagađujućih supstanci u uzorcima životne sredine i namirnicama i proceni njihovog štetnog uticaja na zdravlje čoveka. S obzirom da su u literaturi retka istraživanja koja se bave razvojem i primenom metoda zasnovanih na naprednim hromatografskim i spektrometrijskim tehnikama i određivanjem organskih i neorganskih zagađujućih supstanci u matriksima životne sredine i namirnicama sa prostora zapadnog Balkana, a uzimajući u obzir njihovu važnost, specifični ciljevi disertacije su:

- unutrašnja („*in-house*“) provera kvaliteta i pouzdanosti postojeće „multi-rezidualne“ metode zasnovane na UHPLC-QqLIT-MS/MS za analizu 81-e farmaceutski aktivne komponente (PhAC) u otpadnoj, površinskoj, podzemnoj i pijaćoj vodi i po prvi put dobijanje sveobuhvatnih rezultata njihovog prisustva u različitim tipovima vode sa područja Srbije;

- unutrašnja („*in-house*“) provera kvaliteta i pouzdanosti postojeće „multi-toksin“ metode za analizu 8 *Fusarium* mikotoksina u uzorcima ozime pšenice različitih sorti zasnovane na HPLC-QqQ-MS/MS radi određivanja regionalnih razlika između žitnih regiona kao i otpornosti ispitivanih sorti pšenice na kontaminaciju *Fusarium* toksinima;

- modifikacija postojeće „multi-toksin“ metode zasnovane na UHPLC-QqQ-MS/MS za analizu 11 osnovnih („*principal*“) mikotoksina u uzorcima brašna i njena unutrašnja („*in-house*“) provera kvaliteta i pouzdanosti, kao i provera kroz interlaboratorijsko poređenje, radi dobijanja podataka za procenu štetnog uticaja ispitivanih mikotoksina na zdravlje populacije;

- razvoj „multi-toksin“ (višekomponentne) i „multi-matriks“ (za više matriksa) metode bazirane na UHPLC-QqQ-MS/MS za analizu 10 mikotoksina u različitim vrstama koštuničavog voća čija provera kvaliteta je zasnovana na intralaboratorijskoj proveri tačnosti i preciznosti dobijenih rezultata;

- primena postojeće analitičke procedure zasnovane na naprednoj tehnici pripreme (mikrotalasnoj digestiji) različitih uzoraka biljnog i životinjskog porekla i provera kvaliteta metode identifikacije i kvantifikacije zasnovane na atomskom apsorpcionom spektrometru sa grafitnom kivetom (GFAAS) radi dobijanja sveobuhvatnih rezultata o prisustvu teških elemenata (arsena, olova i kadmijuma) radi procene izloženosti stanovništva Srbije toksičnim neorganskim elementima.

Rezultati su predstavljeni i prodiskutovani u poglavlju REZULTATI I DISKUSIJA, a zaključci su izneti u petom poglavlju - ZAKLJUČAK. Postignuti rezultati predstavljaju jedinstvene rezultate za područje Srbije dobijene primenom naprednih spregnutih tehnika koje imaju značajnu ulogu u praćenju prisustva većeg broja organskih i neorganskih zagađujućih supstanci u izabranim uzorcima životne sredine i namirnica, (regulisanih postojećim zakonodavstvom) radi procene stepena zagađenosti ili u slučaju jedinjenja koja nisu

regulisana zakonodavstvom radi sticanja novih saznanja o njihovom prisustvu i mogućem negativnom uticaju na životnu sredinu i zdravlje populacije.

Rezultati prikazani u radu su međunarodno prihvaćeni i obajavljeni u vodećim međunarodnim časopisima, koji su sastavni deo disertacije (Prilog I).

2. OPŠTI DEO

2.1. Razvoj naprednih metoda analize ksenobiotika

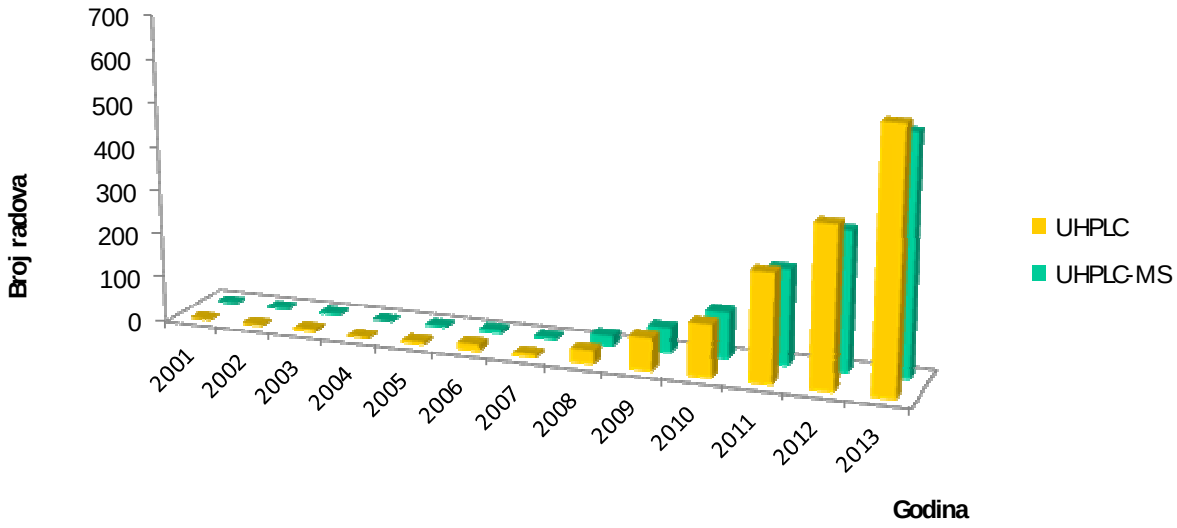
Postoji više od sto hiljada ksenobiotika[2] na tržištu Evropske unije. Procenjuje se da 70.000 ksenobiotika može imati hazardno delovanje na čoveka i/ili ekosistem. Hemijski, ksenobiotici se mogu podeliti na: organske i neorganske zagađujuće supstance (Ivančev-Tumbas, 2009). Organski ksenobiotici su mnogo brojniji u odnosu na neorganske. Organske ksenobiotike čine: pesticidi, prirodni toksini (mikotosini), lekovi u humanoj medicini i veterini, kozmetička sredstva i sredstva za ličnu higijenu (deterdženti), boje, lakovi i drugo. Najznačajnija grupa neorganskih ksenobiotika su teški elementi. Prisustvo organskih zagađujućih supstanci u uzorcima iz životne sredine i namirnicama je posledica novih industrijskih procesa i ostalih antropogenih aktivnosti, kao i klimatskih promena. S obzirom da organske zagađujuće supstance imaju negativan uticaj na životnu sredinu i na zdravlje ljudi, u svetu se preduzimaju mere u cilju smanjenja stepena izloženosti toksičnim supstancama i posledicama izlaganja. Trenutno, jedan od najvećih izazova, jeste procena rizika povezana sa velikim brojem različitih organskih zagađujućih supstanci u tragovima ili čak i u tzv. ultratragovima, a jedan od glavnih trendova je razvoj brzih i efikasnih procedura za njihovu analizu na bazi naprednih hromatografskih i maseno spektrometrijskih tehnika.

Gasna hromatografija (GC) u kombinaciji sa masenom spektrometrijom (MS) uspešno se primenjuje samo na ograničen broj nepolarnih i lako isparljivih organskih jedinjenja. Pri gasno hromatografskoj analizi polarnih organskih supstanci potrebno je znatno više vremena, zbog koraka derivatizacije koji je neophodan posle koraka prečišćavanja ekstrakta uzorka. Uloga derivatizacije je da prevede polarna organska jedinjenja u manje polarne isparljivije i termički stabilnije analite čime se omogućava GC/MS analiza odnosno poboljšano razdvajanje, detekcija i kvantifikacija, ali koja je često praćena neadekvatnom ponovljivošću. Nedvosmislena identifikacija sa GC/MS nije moguća za veliki broj supstanci, uglavnom zbog toga što analizirana jedinjenja nisu isparljiva na ulazu u gasni hromatograf. Ovo se može izbeći samo primenom naprednih instrumentalnih tehnika kao što je tečna hromatografija (LC) u kombinaciji sa masenom spektrometrijom (MS). Tečni hromatograf omogućava razdvajanje složene smese jedinjenja sa širokim spektrom hemijske polarnosti i različitim molekulskim masama, a maseni spektrometar njihovu detekciju zbog visoke osetljivosti i selektivnosti (Boyd i sar., 2008). Visoko pritisna tečna hromatografija (HPLC) ili u novije vreme razvijena ultra pritisna tečna hromatografija (UHPLC) u kombinaciji sa masenim spektrometrom (MS), zbog visoke selektivnosti i osetljivosti, postala je prioritetna tehnika za „multi-rezidualne“ (višekomponentne ili, u slučaju prirodnih toksina, „multi-toksin“) metode (Malik i sar., 2010).

[2] Reč vodi poreklo iz grčkih reči *xenos*-stran i *bios*-život.

Prvi komercijalni UHPLC sistemi na tržištu su se pojavili 2004. godine sa Agilent RRHT kolonom (1,8 µm veličina čestica) i Waters Acquity BEH kolonom (1,7 µm). Uspešnost ove tehnologije pripisana je prednostima punjenja kolona sa veličinom čestica ispod 2 µm. U cilju adekvatnog korišćenja ovih kolona, neophodno je bilo razviti i dizajnirati pumpe koje će omogućiti veći protok mobilne faze na pritiscima do 1000 bara i iznad, obezbeđujući na taj način visoku moć hromatografskog razdvajanja ispitivanih analita. Na ovaj način, mnogo brže hromatografsko razdvajanje velikog broja komponenata postiže se u odnosu na konvencionalnu visoko pritisnu tečnu hromatografiju. Kratko vreme analize koje omogućava UHPLC je veoma važno zbog analize većeg broja uzoraka u kratkom vremenskom roku. Sa druge strane, uzani hromatografski pikovi su rezultat visoke rezolucije korišćenih kolona koje omogućavaju smanjenje broja ko-eluirajućih pikova koji predstavljaju interferencije za ciljane analite. Rezultat primene ovih kolona je smanjenje uticaja matriksa tokom analize. Efikasnost hromatografskog razdvajanja značajno zavisi od izbora mobilne i stacionarne faze (Snyder i sar., 1997). Širok dijapazon stacionarnih faza sa različitim punjenjima, koja se koriste u svrhe razdvajanja u tečnoj hromatografiji, dostupan je na tržištu. Najčešće korišćeni mehanizam zadržavanja ispitivanih analita podrazumeva hromatografiju gde se primenjuje nepolarna stacionarna faza i polarna mobilna faza, i ovaj tip hromatografije se zove hromatografija sa obrnutim fazama ili tzv. reversna tečna (RP) hromatografija. RP hromatografija je uglavnom zasnovana na hidrofobnim interakcijama između analita i stacionarne faze, i omogućava zadržavanje šireg spektra hemijski različitih jedinjenja od interesa (Snyder i sar., 1997). Izbor mobilne faze koja se koristi za hromatografsko razdvajanje jedinjenja od interesa zavisi od osobina ispitivanog analita (kao npr. rastvorljivosti), postizanja željenog retencionog vremena (RT) i selektivnosti analita (Snyder i sar., 1997). U LC/MS analizama, potrebno je razmotriti sastav mobilne faze zavisno od MS analize; jer jedan od zahteva je da korišćena mobilna faza treba da je isparljiva i stoga kompatibilna sa procesom jonizacije (Boyd i sar., 2008). Slično ekstrakciji, smesa vode i organskih rastvarača, najčešće acetonitrila i metanola se koristi za hromatografsko razdvajanje ispitivanih analita primenom „multi-rezidualnih/multi-toksin“ metoda. Fizičko-hemijske osobine acetonitrila i metanola su različite i stoga utiču na različita RT jedinjenja od interesa, kao i na selektivnost u hromatografskom razdvajanju (Snyder i sar., 1997). Dodatno, modifikatori se dodaju u mobilne faze u cilju poboljšanja hromatografskog i maseno spektrometrijskog „ponašanja“ ispitivanog analita (Boyd i sar., 2008). Organske kiseline (najčešće sirćetna i mravlja) i njihove soli uobičajeno se koriste kao modifikatori u LC/MS analizama, i često se dodaju u eluente korišćene u „multi-rezidualnim/multi-toksin“ metodama (Sulyok i sar., 2006; Desmarchelier i sar., 2010; Jin i sar., 2010; Petrović i sar., 2014).

Napredak i primena ove tehnike od momenta razvoja do danas je u stalnom porastu, što je prikazano na slici 2.1. Uporedo sa razvojem UHPLC, usavršavanje masenih analizatora i njihova primena na polju analize organskih zagađujućih supstanci u kojim je potrebno odrediti koncentracije u nivoima od µg/kg i niže takođe je u stalnom porastu (slika 2.1).



***Slika 2.1**. Rastući broj publikovanih radova u kojima se primenjuju metode zasnovane na UHPLC ili UHPLC-MS u periodu od 2001-2013. Izvor ovih podataka je elektonska baza SCIENCE DIRECT (podaci iz avgusta 2013. god.)*

Masena spektrometrija (MS) postala je popularna tehnika, jer MS analizatori su vrlo osetljivi i imaju široku primenu u "otkrivanju" jedinjenja koja su prisutna u vrlo niskim koncentracionim nivoima u složenim matriksima (Boyd i sar., 2008). Ovi analizatori su visoko selektivni pri identifikaciji analita što je zasnovano na molekulskoj masi, a dodatne strukturne informacije mogu se dobiti preko specifičnih fragmentacionih reakcija (Boyd i sar., 2008) tj. modom za praćenje izabranog fragmenta („*selected reaction monitoring",* SRM)[3] ili modom za praćenje višestruke fragmentacije („*multiple reaction monitoring"*, MRM)[3]. Dakle, snaga masene spektrometrije leži u činjenici da su maseni spektri različitih jedinjenja dovoljno specifični da omogućavaju njihovu identifikaciju sa velikim stepenom poverenja, ako ne i sa potpunom sigurnošću.

[3] Mod za praćenje izabranog fragmenta („*selected reaction monitoring",* SRM) i mod za praćenje višestruke fragmentacije („*multiple reaction monitoring"* MRM) su dva pojma (sinonima) koji označavaju isti način snimanja masenog spektra pomoću trostrukih (tripl) kvadrupolnih masenih analizatora, pri čemu je SRM pojam uveden od strane kompanije Thermo Fisher Scientific, USA, a MRM od strane kompanije Waters, Milford, USA.

Veza između tečnog hromatografa i MS analizatora izvedena je preko jonizacionog izvora čija svrha je da ukloni višak mobilne faze isparavanjem na određenoj temperaturi i jonizuje jedinjenja od intersa, koja se nakon toga prenose u sekciju masenog analizatora koji radi pod vakuumom (Boyd i sar., 2008). U masenom analizatoru, joni ispitivanih analita razdvajaju se prema odnosu mase i naelektrisanja (m/z) koji je u većini slučajeva 1, na osnovu čega se jednostavno može reći prema masi (Boyd i sar., 2008). Ovo se postiže delovanjem magnetnog ili električnog polja u samom masenom analizatoru. Postoji veliki broj komercijalno dostupnih masenih analizatora, kao što su na primer trostruki (tripl) kvadrupol (QqQ), magnetski sektorski, sa jon-ciklotronskom rezolucijom i Furijerovom transformacijom (FT-ICR), analizator na principu jonske zamke („*ion trap*“ -IT), analizator na bazi vremena preleta (“*time of flight*” -TOF), detektor baziran na Orbitrap tehnologiji (Sulyok i sar., 2006; Tanaka i sar., 2006; Herebian i sar., 2009; Martos i sar., 2010), hibridni trostruki (tripl) kvadrupol „*ion trap*“ (QqLIT) (Petrović i sar., 2014). Maseni analizatori su različiti po konfiguraciji i dizajnu, opsegu masa koje detektuju, rezoluciji[4], tačnosti merenja mase[5], i

[4] Sposobnost masenog spektrometra da razdvoji dve mase definiše se kao rezolucija (R). Najčešća matematička definicija rezolucije data je po sledećoj jednačini:

$$R = \frac{m}{\Delta m}$$

gde *m* predstavlja odnos *m/z* (mase i naelektrisanja) za dati pik, dok se *Δm (razlika dve vrednosti m/z)* razlikuje u zavisnosti od primenjene metodologije izračunavanja rezolucije. Dakle, rezolucija se izračunava primenom prethodno navedene jednačine s tom razlikom da se vrednost *Δm* određuje na različite načine:

1. Rezolucija se može definisati preko procentualne veličine tzv. „doline“ između dva susedna pika, tj. procentualnim stepenom radvojenosti dva pika. Obično se pri definisanju zadovoljavajuće rezolucije na ovaj način koristi stepen razdvajanja od 10%. Ređi su slučajevi kada se kao zadovoljavajući stepen razdvojenosti uzima *Δm* dolina od 5% ili 50%. Važno je da pri određivanju rezolucije na ovaj način visine i širine pikova budu približno iste.

Obično se kod magnetskih sektorskih instrumenata rezolucija predstavlja sa procentualnim stepenom razdvajanja između dva pika od 10%. Sve ostale opšteprihvaćene definicije rezolucije odnose se na izračunavanje rezolucije posmatranjem samo jednog pika. Tako da se pri poređenju rezolucija iz različitih izvora mora voditi računa o načinu na koji je rezolucija predstavljena, odnosno izračunata u svakom pojedinačnom slučaju.

2. Drugi način definisanja rezolucije, koji je direktno (bez potrebe za preračunavanjem) uporediv sa prethodno opisanim načinom predstavljanja, jeste način izražavanja rezolucije gde se za *Δm* uzima širina pika na 5% od njegove visine.

3. Najviše korišćeni metod za izračunavanje rezolucije kod instrumenata tipa kvadrupola, FT-ICR, masenog spektrometra na bazi Orbitrap tehnologije i TOF, jeste da se za vrednost *Δm* uzima širina pika na polovini njegove visine („*full width at half maximum*“ - FWHM).

[5] Tačnost merenja mase, u kombinaciji sa odgovarajućom rezolucijom, utiče na pouzdanost identifikacije jedinjenja primenom MS smanjujući broj mogućih molekulskih formula za dati maseni spektar. Tačnost određivanja mase se obično izražava kao greška (izražena u ppm) u određivanju odnosa *m/z* u odnosu na tačno izračunatu masu :

$$\Delta m / z = \frac{m_{sr} - m_e}{m_e} \cdot 10^6$$

stoga se koriste za različite analitičke svrhe (Boyd i sar., 2008). U tabeli 2.1 prikazane su uporedne karakteristike najčešće korišćenih masenih analizatora.

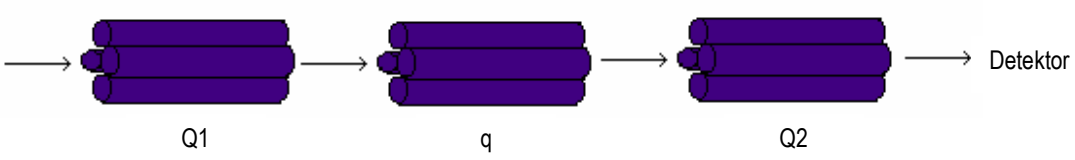
Tabela 2.1. *Uporedne karakteristike najčešće korišćenih masenih analizatora*

Maseni spetrometar	m/z opseg	Tačnost merenja mase	Masena rezolucija
Magnetni sektorski	10,000	1-2 ppm	100,000
MS (jednostruki kvadrupol)	10,000	100,000 ppm	4,000
MS/MS (QqQ)	10,000	100,000 ppm	5,000
QqLIT	1,000	100,000 ppm	7,000
Linearni IT	10,000	50-200 ppm	1,000
TOF	100	5 ppm	15,000
FT-ICR	>5,000	> 1 ppm	500,000
Orbitrap	>5,000	5 ppm 1-2 ppm	200,000

Trostruki (tripl) kvadrupol. Kvadrupol kao analizator koristi se još od polovine prošlog veka. Danas, iako je još uobičajeno korišćenje uređaja sa jednim kvadrupolom u rutinskim analizama (više u kombinaciji sa GC nego sa LC), mogućnost korišćenja tripl kvadrupola (QqQ) za analizu različitih jedinjenja daje bolju osetljivost, jednostavnost rada i mogućnost određivanja ciljanih komponenata u različitim uzorcima zbog njegove selektivnosti. Kvadrupoli rade kao maseni filteri i baziraju se na prenosu jona. Kvadrupol maseni analizator sastoji se od četiri hiperbolične ili cilindrične šipke postavljene paralelno u radijalnom nizu, koji rade pod delovanjem radiofrekventnog (RF) napona. Polje generisano između šipki kvadrupola, kombinacijom jednosmerne struje (DC) i RF napona, uzrokuje da joni koji prolaze kroz kvadrupol imaju složene putanje. Primenom odgovarajućeg napona, joni koji dospevaju u kvadrupol mogu se filtrirati duž centralne ose između šipki: joni koji imaju stabilnu putanju prolaze kroz kvadrupol i stižu na detektor dajući maseni spektar. QqQ je maseni spektrometar koji se sastoji od dva kvadrupola i kolizione ćelije; u prvom kvadrupolu (Q1) prekursor jon (roditeljski jon) ispitivanog analita se selektuje, zatim se fragmentiše (cepa) u kolizionoj ćeliji (q) usled sudara sa molekulima argona, a nastali specifični joni („produkt“ joni–ćerke joni) selektuju se u drugom kvadrupolu (Q2) i prate se specifičnim tranzicionim reakcijama to jest tzv. MRM mod-om (Boyd i sar., 2008) ili SRM mod-om (Škrbić i sar., 2012, 2013a, 2014). Dakle, u MS/MS analizi, pouzdanost u identifikaciji ispitivanog analita potvrđuju specifični „produkt“ joni koji su nastali od prekursor jona (Boyd i sar., 2008). Jedan od najosetljivijih i selektivnih QqQ, koji je komercijalno dostupan je TSQ Vantage (Thermo Fisher Scientific, USA) koji radi pri niskoj rezoluciji tj. jediničnoj. Kvadrupol može biti povezan sa

gde m_e predstavlja tačnu masu molekula izračunatu sabiranjem masa svakog pojedinačnog atoma u molekulu, m_{sr} predstavlja masu izmerenu primenom odgovarajućeg masenog spektrometra.

drugim analizatorima kao što je TOF, IT ili drugim kvadrupolom. Specifične konfiguracije QqQ mogu se povezati sa GC ili LC. Osim, MRM (tj. SRM) moda, QqQ ima višestruke funkcije skeniranja kao što su mod za praćenje pojedinačnog jona („*single ion monitoring*“, SIM)[6] „*neutral loss*“[7], prekursor mod („*precursor mod*“)[8] i mod za kompletno skeniranje („*full scan*“)[9], dok najveći značaj ima SRM mod koji povećava selektivnost i čini ovaj analizator vrlo superiornim za svrhu kvantifikacije jedinjenja od interesa. QqQ je tehnika koja ima veliki značaj u analizi različitih jedinjenja u uzorcima životne sredine i namirnicama zbog osetljivosti, jednostavnosti i robustnosti. Ograničenje „multi-rezidualnih/multi-toksin“ LC/MS-MS metoda sa QqQ je nemogućnost ovog analizatora da obezbedi podatke za neciljane komponente koje mogu biti prisutne u uzorku. Na slici 2.2 data je shema QqQ.



Slika 2.2. *Shema QqQ*

Jonska zamka. Između 1950. i 1960. godine, maseni analizator nazvan jonska zamka razvijen je od strane fizičara Wolfgang Paula i Hans Georg Dehmelta. Najprostije rečeno, jonska zamka je uređaj u kojem dolazi do zarobljavanja jona delovanjem elektromagnetnog polja. IT sadrži prstenastu elektrodu („*ring electrode*“) i dve konveksne „*end-cap*“ elektrode (slika 2.3), između kojih se uspostavlja trodimenzionalno elektromagnetno polje u kome joni u širokom opsegu m/z vrednosti prate stabilnu, kompleksnu putanju odn. zarobljeni su. U ovom analizatoru svi eksperimenti izvode se na istom mestu, ali u različito vreme. IT može da radi u različitim modovima: u „*full scan*“ modu sa visokom efikasnošću svi joni zarobljeni su u prostoru između elektroda, u SIM modu dolazi do destabilizacije neželjenih jona i povećanja osetljivosti selektovanih jona koji se koriste za MS^n eksperimente. Ovaj poslednji mod dozvoljava višestruko fragmentisanje određenog jona i omogućava tumačenje strukture

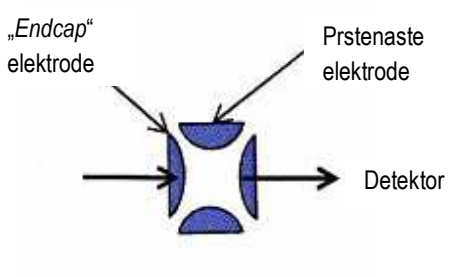
[6] Kada instrument radi u mod za praćenje pojedinačnog jona („*single ion monitoring*“, SIM), kvadrupol omogućava prolaz samo jonu sa definisanom vrednošću m/z.

[7] „*Neutral loss*“ mod: u prvom kvadrupolu dolazi do razdvajanja jona na osnovu odnosa m/z, nakon čega joni odlaze u kolizionu ćeliju gde se vrši fragmentacija. Treći kvadrupol razdvaja „produkt“ jone na osnovu njihovog odnosa m/z.

[8] Prekursor („*parent* (*precursor*)“) mod: u prvom kvadrupolu parametri su postavljeni tako da se omogući prolaz svim jonima različitog odnosa m/z. U kolizionoj ćeliji dolazi do fragmentacije svih „parent“ jona, nakon čega, „produkt“ joni dolaze u treći kvadrupol gde samo selektovani „produkt“ joni (zadatih m/z vrednosti) prolaze do detektora.

[9] U modu za kompletno skeniranje („*full scan*“), kvadrupol skenira sve mase u opsegu između dve zadate mase i rezultujući maseni spektar se sastoji od intenziteta svih m/z jona koji se nalaze u navedenom opsegu masa (tj. m/z).

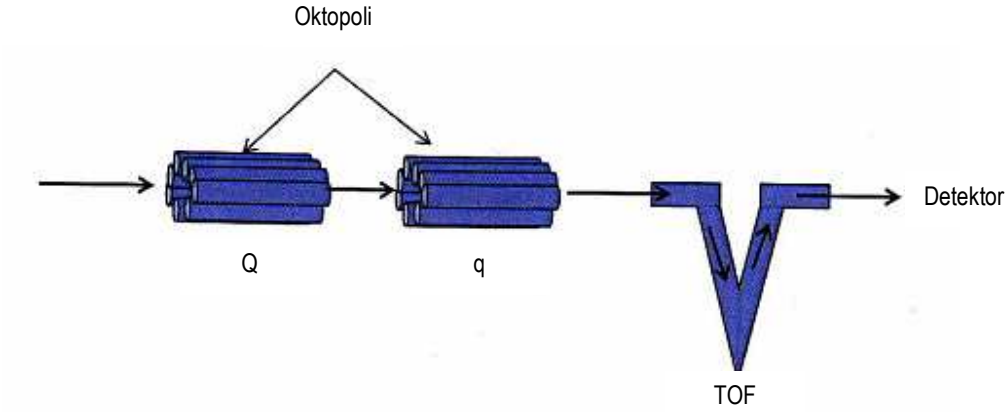
nepoznatih jedinjenja. Međutim, analizator na bazi jonske zamke ima nisku rezoluciju (jediničnu) i mogućnost nastajanja neželjenih jon-molekulskih reakcija unutar analizatora. U cilju smanjenja pojave jon-molekulskih reakcija neophodno je uvođenje helijuma kao „*damper*" gasa, koji ima funkciju da putem kolizija "hladi" jone (smanjuje im kinetičku energiju) i fokusira ih u centru zamke. Glavne prednosti ovog masenog spektrometra su što je jednostavan za rukovanje i održavanje, zatim može biti povezan sa drugim analizatorima kao što je kvadrupol, a kada je reč o separacionim tehnikama može biti i u kombinaciji sa GC i sa LC.



Slika 2.3. *Shema jonske zamke*

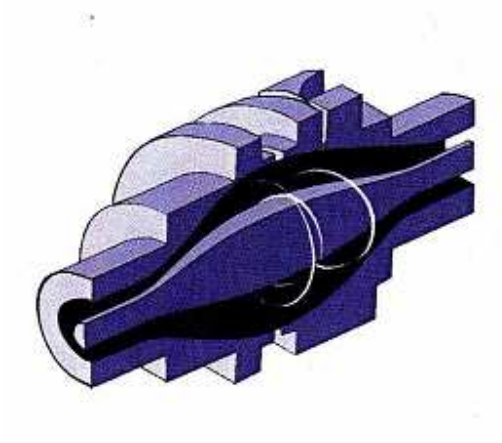
Hibridni kvadrupol TOF. Hibridni analizator TOF (QqTOF) povezuje odličnu selektivnost kvadrupola i srednju do visoke (*med-to-high*) rezoluciju TOF, te se zbog ovakve strukture (slika 2.4) i visoke osetljivosti, koristi za analizu velikih molekula kao što su proteini. Osnovni princip rada instrumenta je merenje vremena preleta ubrzanih jona od jonskog izvora do detektora. Kretanje jona od izvora do detektora dešava se usled primene potencijala i zavisi od odnosa mase i naelektrisanja (m/z). Ovaj maseni analizator može biti povezan sa GC ili LC, kapilarnom elektroforezom, ili pulsnim jonizacijonim izvorom za uvođenje uzoraka kao što je MALDI[10]. QqTOF omogućava dobijanje precizne mase identifikovanih „produkt" jona, jer ima mogućnost određivanja tačne mase i predstavlja važnu tehniku na polju analize degradacionih proizvoda (Jones i sar., 2003; Martin i sar., 2004; Guo i sar., 2008).

[10] Jonizacija potpomognuta laserskom desorpcijom iz matriksa („*matrix-assisted laser desorption ionization*", MALDI) je meka jonizaciona tehnika u masenoj spektrometriji, koja omogućava jonizaciju biomolekula (biopolimera kao što su proteini, peptidi, šećeri) i velikih organskih molekula (kao što su polimeri) koji imaju osobinu da su „krhki" i da se fragmentišu pri jonizaciji usled primene drugih konvencionalnih jonizacionih tehnika.



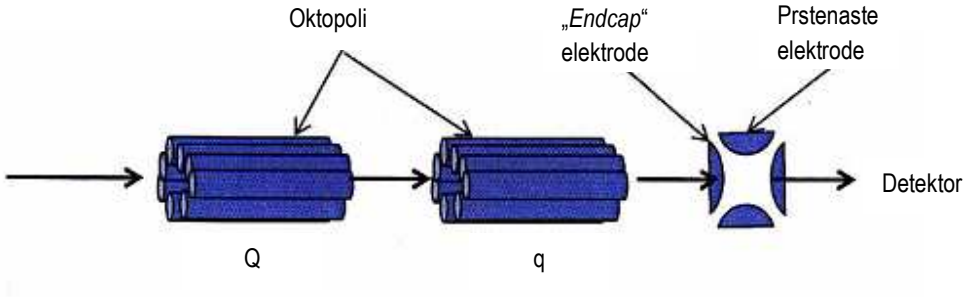
Slika 2.4. *Shema QqTOF*

Orbitrap. Orbitrap maseni analizator patentiran je od strane Makareva (2000) i radi na istom principu kao maseni spektrometar sa jon-ciklotronskom rezolucijom i Furijerovom transformacijom (FT-ICR): joni su zarobljeni u električnom polju između vretenaste unutrašnje elektrode i para elektroda u obliku zvona (koji okružuju vretenastu elektrodu), i u ravnoteži su delovanjem centrifugalnih sila (slika 2.5). Kružno kretanje jona oko centralne elektrode je u funkciji električnog polja, i takođe njihovog oscilatornog kretanja zbog odnosa mase i naelektrisanja (m/z). Orbitrap kao i TOF imaju mogućnost i kvalitativnog i kvantitativnog određivanja. Oba tipa analizatora imaju osobinu da obezbede odgovarajuću selektivnost pri detekciji analita kao potvrdu njegovog identiteta tj. primenu srednje ili visoke rezolucije (≥ 10^4 mereno kao širina pika na polovini visine, FWHM), kao i detekciju analita pri visokoj masenoj tačnosti (krajnje od 2 do 5 ppm) i preciznosti (Boyd i sar., 2008; Malik i sar., 2010). Ovi instrumenti imaju veliku spektralnu brzinu koja omogućava snimanje praktično neograničenog broja komponenata.



Slika 2.5. *Shema orbitrapa*

Hibridni trostruki (tripl) kvadrupol linerani „ion trap“. Osetljivost i selektivnost kvadrupola (Q) i visoka efikasnost jonske zamke (IT) čine hibridni tripl kvadrupol linearni „ion trap“ (QqLIT ili QTrap) jednim od najinteresantnijih masenih analizatora za analizu bioloških i uzoraka životne sredine. Ovaj maseni analizator najčešće je povezan sa LC sistemom. U IT joni su zarobljeni u polju kvadrupola što je suprotno od Q kvadrupola kroz koji joni prolaze. Ovo omogućava izvođenje MS^3 eksperimenata, što je veoma korisno za strukturno tumačenje identifikacije različitih komponenata tokom degradacije. Tipično, QqLIT se sastoji od kvadrupola (Q), kolizione ćelije (q) i linearne jonske zamke (LIT) koju čine dve hiperbolične „*endcap*“ elektrode i prstenasta elektroda (slika 2.6).



Slika 2.6. *Shema hibridnog tripl kvadrupolnog linearnog „ion trap“*

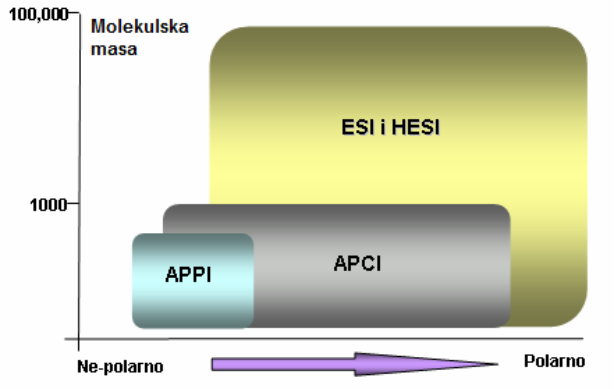
Primenom ovog masenog analizatora moguće je izvršiti skeniranje u oba analizatora, SIM u kvadrupolu i skeniranje u LIT koje se naziva „*enhanced product ion*“ (EPI) i skeniranje kao što je „*neutral loss*“, SRM i MS^3. QqLIT je maseni spektrometar koji je po principu rada u tesnoj vezi sa QqQ. QqQ i QqLIT popularno nazvani MS/MS instrumenti stekli su široku primenu zbog pouzdane primenjivosti u kvantitativnoj analizi.

Pri LC/MS analizi, najčešće korišćeni izvori za jonizaciju analita na atmosferskom pritisku (API) su: elektrosprej jonizacioni izvor (ESI) uključujući grejani elektrosprej izvor (H-ESI), izvor sa hemijskom jonizacijom (APCI) i fotojonizacioni izvor (APPI). Jonizacioni izvori sadrže optiku za usmeravanje putanje kretanja jona molekula ispitivanih analita od izvora sa atmosferskog pritiska do sekcije masenog analizatora koji radi pod vakuumom (Boyd i sar., 2008). Međutim, fizičko-hemijski procesi koji dovode do nastajanja jona molekula različiti su između ESI/H-ESI, APCI i APPI.

Elektrosprej jonizacija. ESI prevodi jone molekula koji se nalaze u rastvoru (mobilnoj fazi) u jone u gasovitoj fazi, kada se jako električno polje primeni na mobilnu fazu koja prolazi kroz usku kapilaru. Pod dejstvom električnog polja mobilna faza koja napušta kapilaru prelazi u aerosol sastavljen od visoko naelektrisanih kapljica. Pomoću struje azota i blagim zagrevanjem, rastvarač isparava, a kapljice se smanjuju i u određenom trenutku, kada površinski napon ne može da izdrži nagomilano naelektrisanje, dolazi do eksplozije kapljica. Ovaj proces se ponavlja i kao rezultat toga stvaraju se joni analita, oslobođeni od rastvarača, koji kroz jonsku kapilaru stižu u maseni analizator. Dve gasne struje potpomažu ESI jonizaciju: „*sheath*“ potpomaže raspršivanje mobilne faze, dok „*auxiliary*“ potpomaže de-

solvataciju. HESI prevodi jone iz rastvora u jone u gasovitoj fazi pomoću ESI u kombinaciji sa zagrejanim „*auxiliary*“ gasom.

Hemijska jonizacija. Dok se kod ESI, molekuli u uzorku jonizuju pre uklanjanja rastvarača, u APCI se jonizacija dešava u gasovitoj fazi. Mobilna faza prolazi kroz silikatnu kapilaru koja je zagrejana na 400-600^{o}C. Gas koji doprinosi zagrevanju i raspršivanju mobilne faze u fini aerosol je obično azot. Smesa isparenog rastvarača i analita kreće se prema regionu visokog napona gde se koronarnim pražnjenjem kroz seriju hemijskih reakcija između mobilne faze u parnom stanju i azota formiraju „reagens“ joni. Ovi „reagens“ joni u sudaru sa neutralnim molekulima koji se nalaze u gasovitom stanju izazivaju njihovu jonizaciju.
Fotojonizacija. Tokom fotojonozacije (APPI) molekul se prevodi u jonsko stanje nakon kontakta (sudara) sa fotonom (hu) koji dolazi od svetlosnog izvora. Svetlosni izvor koristi „krypton“ lampu za emisiju fotona energije 10 i 10,6 eV. Molekuli sa jonizacionim potencijalom manjim od ovih vrednosti se jonizuju i prelaze u M^+ ili $(M + H)^+$ jonizacioni oblik, dok se molekuli sa većim jonizacionim potencijalom ne jonizuju.
Opsezi primene pomenutih jonizacionih tehnika ilustrovani su na slici 2.7.



Slika 2.7. *Opsezi primenjivosti jonizacionih izvora pri atmosferskom pritisku*

Treba reći da različite klase zagađujućih jedinjenja mogu biti prisutne u uzorcima te se oni često moraju analizirati više puta zbog potrebe određivanja svake klase prisutnih organskih jedinjenja. Ovo je podstaklo istraživače da razviju tzv. „multi-rezidualne/multi-toksin“ metode koje omogućavaju istovremenu identifikaciju većeg broja hemijski različitih jedinjenja, omogućavajući time analizu većeg broja uzoraka, čineći tako metodu ekonomski opravdanom.

Sa razvojem savremenih analitičkih instrumenata, razvijene su „multi-rezidualne/multi-toksin“ metode koje za cilj imaju istovremenu detekciju većeg broja hemijski različitih jedinjenja i postale su vrlo važne u istraživanjima vezanim za zaštitu životne sredine i bezbednosti namirnica, a to se proteže na farmaceutski aktivne komponenate (PhAC), ostatke pesticida, prirodne toksine (mikotoksine), itd. (Malik i sar., 2010; Škrbić i sar., 2011a, 2012, 2014;

Petrović i sar., 2014). Takođe, poseban doprinos „multi-rezidualnim/multi-toksin“ metodama dale su savremene LC/MS tehnike koje omogućavaju istovremenu jonizaciju značajnog broja analita u u pozitivnom ili negativnom modu u okviru jednog hromatografskog ciklusa, čime se eliminiše problem ko-eluiranja dva analita sa suprotnim MS jonizacionim potrebama. Dakle, jonizacija analita u pozitivnom ili negativnom modu u okviru jednog hromatografskog ciklusa omogućava veću fleksibilnost prilikom optimizacije LC/MS uslova za veći broj analita od interesa.

Uobičajna analitička procedura za određivanje ispitivanih analita primenom LC/MS tehnike obuhvata sledeće korake: uzimanje uzoraka, homogenizaciju uzorka, ekstrakciju analita, prečišćavanje ekstrakta, razdvajanje analita tečnom hromatografijom i na kraju detekciju i kvantifikaciju analita masenim spektrometrom (Berthiller i sar., 2007). Primenom ovih metoda brzo se mogu dobiti informacije o većem broju jedinjenja prisutnih u uzorku. Dodatno, primenom ovih metoda postiže se analiza većeg broja uzoraka u kratkom vremenskom periodu, primenom jedne, a ne više pojedinačnih metoda pripreme. Uobičajena priprema uzorka podrazumeva korak tečne ekstrakcije analita pomoću polarnog rastvarača i korak prečišćavanja ekstrakta baziran na ekstrakciji na čvrstoj fazi („*solid phase extraction*“-SPE) (Lattanzio i sar., 2007; Monbaliu i sar., 2009; Zachriasova i sar., 2010, Gros i sar., 2012; Petrović i sar., 2014). Ovoj korak predstavlja izazov pri optimizaciji „multi-rezidualnih“ metoda za PhAC, jer je potrebna istovremena ekstrakcija ovih analita iz kompleksnih matriksa (npr. otpadne vode) koji imaju različitu polarnost. Ukoliko sve ciljane komponente ne mogu biti ekstrahovane u jednom koraku, one se mogu klasifikovati u dve ili više SPE grupa, u skladu sa njihovim fizičko-hemijskim osobinama. Međutim, jedan od novih trendova u pripremi uzoraka je pokušaj smanjenja predtretmana ekstrakta uzorka, to jest analiza ekstrakta uzorka sa minimalnim ili bez koraka prečišćavanja (Sulyok i sar., 2006) tj. korišćenje tzv. sirovog ekstrakta („*crude extract*“) najčešće u slučaju mikotoksina. Ono što predstavlja izazove u kvantitativnom „multi-rezidualnom/multi-toksin“ određivanju jeste podešavanje uslova svakog analitičkog koraka za sve analite od interesa koji mogu pokazivati veoma različite hemijske osobine. Ipak, optimalni uslovi ne mogu biti postignuti za sve analite i često neki od njih se izostavlja zbog neadekvatnog koraka ekstrakcije, lošeg hromatografskog razdvajanja ili neodgovarajuće jonizacije u izvoru MS (Rundberget i Wilkins 2002; Sulyok i sar., 2006) i krajnja „multi-rezidualna/multi-toksin“ metoda predstavlja kompromis između postignutih performansi razvijene metode (Sulyok i sar., 2006; Gros i sar., 2012; Petrović i sar., 2014).

U idealnom slučaju, „multi-rezidualne/multi-toksin“ metode za određivanje organskih zagađujućih supstanci u tragovima u različitim matriksima, treba da ispune nekoliko kriterijuma:

a) priprema uzorka i koncentrisanje trebalo bi da se izvedu u jednom koraku, iako analiti imaju različite fizičko-hemijske osobine;

b) granica detekcije („*limit of detection*“-LOD) i kvantifikacije („*limit of quantification*“-LOQ) trebalo bi da su dovoljno niske za svaki analit od interesa; i

c) jednostavna primena za različite matrikse (npr. voda za piće, otpadna voda, površinska voda).

Međutim, istovremena analiza jedinjenja sa sasvim različitim fizičko-hemijskim osobinama primenom „multi-rezidualnih/multi-toksin“ metoda nameće kompromis između postignutih performansi razvijene metode. Na primer, Castiglioni i sar. (2005) dobili su „recovery“[11] vrednosti u opsegu od 65-131% za 30 analiziranih PhAC, osim za amoksicilin koji je imao veoma nizak „recovery“ samo 36%. Potrebno je reći, da nizak „recovery“ u ovim metodama ne može se smatrati nedostatkom, ukoliko drugi parametri kao što je LOD ili standardna devijacija (SD) imaju nisku vrednost (Petrović i sar., 2014).

Takođe, pri razvoju „multi-rezidualnih/multi-toksin“ metoda, potrebno je ispuniti kriterijum za identifikaciju i potvrđivanje („*confirmation*“) analiziranih jedinjenja definisan u Regulativi Evropske unije (EC, 2002)[1]. Naime, za potvrdu prisustva analizirane komponente od interesa u ispitivanim uzorcima sa LC/MS, u SRM/MRM modu je da analizirano jedinjenje bude potvrđeno sa tri identifikaciona jona (1 prekursor jon i 2 produkt jona) (Regulativa Evropske unije (EC, 2002)[1]). Drugi kriterijum koji se koristi je relativni odnos dobijenih produkt jona i RT. Potrebno je reći da u slučaju slabe fragmentacije nekih jedinjenja (kao npr. anti-inflamatora kao što su ibuprofen i ketoprofen i lipid regulatora gemfibrozila) zbog čega je nemoguće ili teško dobiti 2 karakteristična „produkt“ jona, njihova potvrda se dobija na osnovu RT jedinjenja od interesa u uzorku i standardnom rastvoru (Gros i sar., 2007). Da bi se ova potvrda smatrala dovoljno tačnom, odstupanje RT analiziranog jedinjenja u uzorku u odnosu na RT analiziranog jedinjenja u standardnom rastvoru ne sme da prelazi 2,5%.

Pri analizi zagađujućih supstanci u uzorcima iz životne sredine i namirnicama sa LC/MS, jonizacioni izvori su osetljivi na uticaj komponenti iz matriksa, naročito u slučaju složenih matriksa (npr. otpadna voda), čak i kada se koristi SRM/MRM mod jer se mogu dobiti „lažno-negativni“ (zbog suzbijanja signala („*ion suppression*“)) ili „lažno-pozitivni“ (zbog povećanja signala („*ion enhancement*“)) rezultati jedinjenja od interesa. U cilju smanjenja problema netačne kvantifikacije, nekoliko pristupa je usvojeno u analitičkoj praksi. Najčešće korišćeni pristupi kojima se kompenzuje uticaj matriksa su: korišćenje spoljašnje kalibracije pripremljene u nekontaminiranom uzorku („*matrix match calibration*“, u daljem tekstu matriks standard kalibracija), metoda standardnog dodatka i metoda unutrašnjeg standarda (sa strukturno sličnim neoznačenim ili izotopski označenim standardima). Uticaj matriksa na suzbijanje ili na povećanje signala analita („*signal suppression and enhancment*“ - SSE) se izračunava kao:

$$SSE\ (\%) = 100 \times nagib_{\text{matriks standard kalibracije}} / nagib_{\text{kalibracije sa čistim standardima}}$$

U odsustvu matriks efekta, nagibi za obe kalibracione krive međusobno su u okviru eksperimentalne greške odstupanja. Manji nagib dobijen za matriks standard kalibraciju

[11] Efikasnost izdvajanja ciljanog analita primenjenom metodom pripreme ili eng. „recovery“ se određuje u svim analitičkim procedurama koje se zasnivaju na ekstrakciji analita u tragovima. „Recovery“ metode se određuje na tri načina: „spajkovanjem“ (dodatkom, obogaćivanjem), analizom sertifikovanog referentnog materijala (CRM) i kroz interlaboratorijsko poređenje (PT). Obogaćen („spajkovan“) uzorak se priprema dodavanjem poznate zapremine standardnog rastvora ispitivanog analita u ispitivani uzorak (Thompson i sar., 1999). „Recovery“ se izražava u %, i predstavlja odnos izdvojene količine analita i stvarne količine analita prisutne u uzorku. Najefikasniji način određivanja „recovery“ metode je kroz PT, a najjednostavniji i najekonomičniji je obogaćivanjem („spajkovanjem“).

(SSE<100%) ukazuje na suzbijanje signala analita, dok veći nagib (SSE>100%) ukazuje na povećanje signala.

2.2. Farmaceutski aktivne komponenate

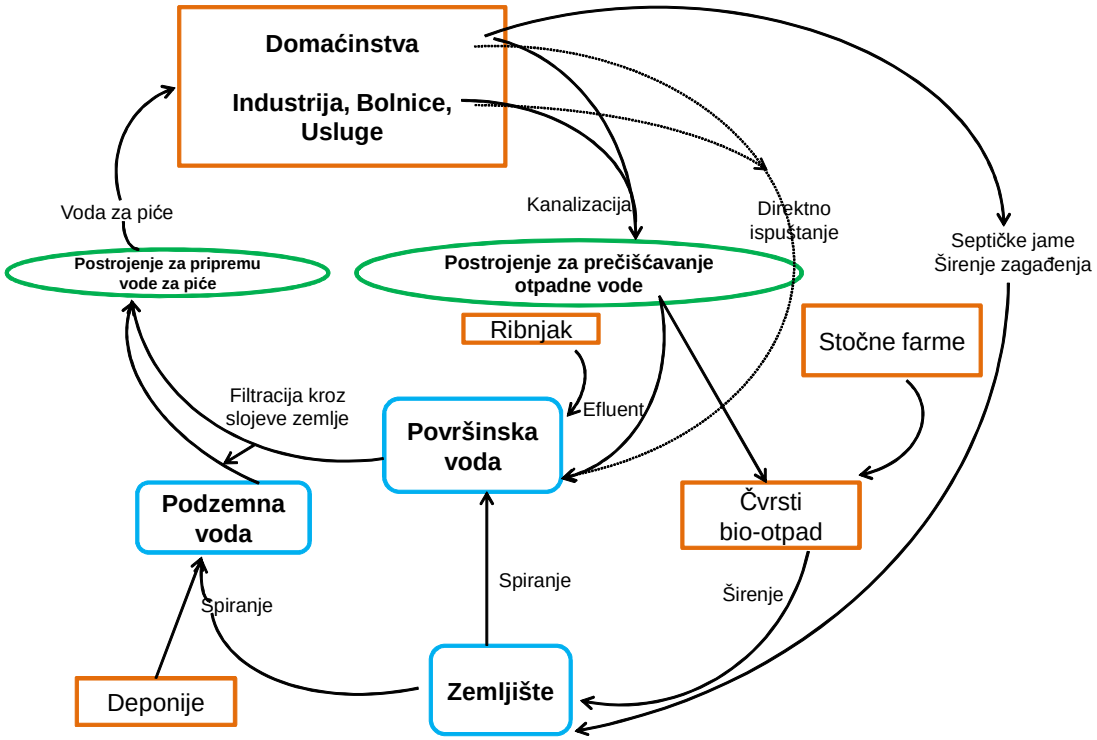
2.2.1. Osobine i rasprostranjenost

Farmaceutski aktivne komponente (PhAC) su velika i raznovrsna grupa jedinjenja koja se uobičajno koriste u humanoj i veterinarskoj medicini za sprečavanje i lečenje bolesti i poboljšanje zdrastvenog stanja ljudi i životinja. PhAC i njihovi metaboliti klasifikovani su u 24 terapeutske klase (analgetici/anti-inflamatori, regulatori nivoa holesterola (statini), lekovi koji deluju na centralni nervni sistem (tj. antiepileptici, antidepresivi, benzodijazepini, antipsihotici, itd.), histamini H1 i H2, β-blokatori, diuretici, antidijabetici, antihipertenzivi, antitrombotici, lekovi za hiperplaziju prostate, lekovi za lečenje astme, antikoagulanti, kontrastna sredstva u radiologiji (X-zraci), antihelmentici, sintetički glukokortikoidi, lekovi za sedaciju i relaksaciju mišića, lekovi za smirenje, antibiotici, blokatori kalcijumovih kanala, itd.), među kojima su 4 dominantne na osnovu do sada objavljenih relevantnih studija. Oko 40% studija odnosi se na istraživanja nesteroidnih anti-inflamatornih lekova („*nonsteroidal anti-inflammatory drugs*"-NSAID), dok preostale tri grupe su lekovi koji deluju na centralni nervni sistem, antibiotici i lipidni regulatori sa procentom istraživanja između 25-30%. Kada je reč o NSAID glavni predstavnici ove grupe su ibuprofen i diklofenak, dok je gemfibrozil najviše proučavani lipidni regulator. Iz grupe lekova koji deluju na centralni nervni sistem, karbamazepin (antiepileptik) je najčešće ciljano ispitivana komponenta u većini literaturno dostupnih studija (Reinstorf i sar., 2008). Antibiotici su terapeutska grupa koju karakteriše veliki izbor PhAC. Činjenica je da mnogi antibiotici koji se koriste u humanoj i veterinarskoj medicini imaju sličan mehanizam delovanja kao i najšire korišćeni antihelmintici odnosno ivermetcin (Sanderson i sar., 2007), zatim diklofenak iz grupe NSAID (Fent i sar., 2006), itd.

Upotreba i potrošnja PhAC je u stalnom porastu zbog otkrića novih komponenata, porasta broja stanovnika kao i promene starosne strukture u populacijama širom sveta (Daughton, 2003). Kao što je prethodno pomenuto potrošnja PhAC se sve više povećava, i godišnja potrošnja u Evropi za najčešće korišćene PhAC je nekoliko stotina tona (Fent i sar., 2006). Na primer, kao NSAID aspirin (acetilsalicilna kiselina), paracetamol, ibuprofen, diklofenak proizvedeni su u sledećim količinama 836, 622, 345 i 86 tona, redom, u Nemačkoj u 2001. godini, dok metformin (antidijabetik) i karbamazepin (antiepileptik) proizvedeni su u količini od 517 i 88 tona, redom (Fent i sar., 2006). Štaviše, potrošnja antibiotika dostiže i do nekoliko hiljada tona godišnje zbog njihove široke upotrebe u poljoprivredi, ribnjacima i humanoj medicini, tako da je više od 13000 tona antibiotika potrošeno (65% za ljudsku upotrebu) u Evropskoj uniji i Švajcarskoj u 1999. godini (Kemper, 2008), pri čemu je Francuska bila pozicionirana kao najveći potrošač u Evropi. Ibuprofen, kao NSAID je jedan od najčešće prepisivanih lekova u Evropi i ranije je bilo poznato da je to jedan od najčešće identifikovanih anti-inflamatora u površinskim vodama. Godišnja potrošnja ibuprofena bila je 166 t/godini u Francuskoj sa 55,5 miliona stanovnika u 1998; 128 t/godini u Nemačkoj sa 82,4

miliona stanovnika u 2011; 276 t/godini u Španiji sa 43,2 miliona u 2003; i 180 t/godini u Kanadi sa 30 miliona stanovnika u 2001 (Verlicchi i sar., 2010). U Srbiji u 2010. godini, 28 tona ibuprofena je potrošeno za 7,5 miliona stanovnika (Radonjić i Šipetić, 2010), što ibuprofen čini najčešće korišćenim lekom. Potrošnja karbamazepina u Srbiji (280 mg/godini/stanovniku) (Radonjić i Šipetić, 2010) niža je u poređenju sa razvijenim zemljama kao što su Nemačka (1010,9 mg/godini/stanovniku); Švajcarska (875,5 mg/godini/stanovniku); Francuska (554,3 mg/godini/stanovniku); Švedska (820,2 mg/godini/stanovniku) i Španija (438,0 mg/godini/stanovniku) (Ortiz de Gracía, 2013). U Evropskoj uniji oko 3000 PhAC jedinjenja koja pripadaju različitim terapeutskim grupama odobreno je za upotrebu u humanoj medicini (Howard i Muir, 2011; Mompelat i sar., 2009).

Tačna procena uticaja PhAC na životnu sredinu je „težak“ zadatak jer postoji veoma veliki broj njihovih mogućih izvora u životnu sredinu bez dokaza o kvantitativnim podacima u vezi sa raspodelom PhAC iz svih izvora emisije (slika 2.8). PhAC jedinjenja u vodene sisteme dospevaju u osnovnom obliku, kao metaboliti ili konjugovane forme i to usled neodgovarajućeg odlaganja tj. direktnog ispuštanja u kanalizacijske odvode, ili izlučivanjem putem urina i fecesa s obzirom da se PhAC ne metabolišu potpuno u organizmu čoveka. Ipak treba reći da ljudi i životinje predstavljaju glavni izvor zagađenja vode za piće, odnosno i drugih vodenih resursa (površinske i podzemne vode), jer se PhAC kvalitativno, kvantitativno, prostorno i vremenski raspodeljuju u različitim pravcima u zavisnosti od toga da li se pacijenti nalaze u privatnim domaćinstvima, bolnicama, školama ili drugim mestima. Ranije, prepisani lekovi za lečenje težih oblika bolesti isključivo su korišćeni za pacijente u bolnici, a ne u domaćinstvu. To je bio slučaj sa antineoplasticima (tzv. citostaticima) koji se koriste u hemoterapiji kancera i isključivo su prepisivani i davani (intravenski ili oralno) pacijentima u bolnici. Dakle, ova vrsta lekova ispuštana je u bolnički efluent gde su utvrđene koncentracije bile u rasponu od 5-50 µg/l (Kümmerer, 2001), ali danas 75% ovih lekova primenjuje se u ambulantnim odeljenjima sa velikom tendencijom za oralnu primenu kod kuće (Johnson i sar., 2008; Kümmerer i Schuster, 2008).



Slika 2.8. *Poreklo i putevi kretanja PhAC (Petrović i sar., 2003)*

Nakon unošenja PhAC u organizam čoveka i metaboličkih procesa, oni se izlučuju putem urina i fecesa u osnovnom obliku ili obliku metabolita, i kanalizacionom mrežom i gradskom otpadnom vodom stižu do postrojenja za prečišćavanje otpadnih voda, dok se u seoskim domaćinstvima direktno ispuštaju u septičke jame (Carrara i sar., 2008). Septički sistemi nisu napravljeni da uklanjaju visoko polarne mikropolutante kao što su PhAC, ali zavisno od prirode PhAC i postrojenja za prečišćavanje otpadnih voda, stepen njihovog uklanjanja pokriva ceo opseg između 0 i 100% (Rađenović i sar., 2007; Drewes, 2007). Na primer, u tabeli 2.2 data su PhAC jedinjenja sa procentom njihovog uklanjanja nakon prolaska kroz postrojenja za primarno (mehaničko) i sekundarno (biološko) prečišćavanje (Collado i sar., 2014).

***Tabela 2.2**. Procenat uklanjanja određenih PhAC jedinjenja nakon njihovog prolaska kroz postrojenja za primarno (mehaničko) i sekundarno (biološko) prečišćavanje (Collado i sar., 2014).*

PhAC	
Nadalol (β-blokator) Eritromicin, ofloksacin (antibiotici) Karbamazepin (antiepileptik) Irbesartan, lozartan (antihipertenziv) Hidrohlorotijazid (diuretik) Diklofenak, kodein (anti-inflamatori)	0-35% (neznatno uklanjanje)
Fenazon (anti-inflamator) Sulfametaksazol (antibiotik) Trazodon (antidepresiv) Metaprolol (β-blokator)	35-70% (umereno uklanjanje)
Ranitidin (histamine H2) Atenolol (β-blokator) Gemfibrozil (regulator nivoa holesterola) Furosemid (diuretik) Valsartan (antihipertenziv) Acetaminofen, ibuprofen (anti-inflamatori)	> 70% (dobro uklanjanje)

Među PhAC koje prolaze kroz postrojenje za preradu otpadnih voda je i karbamazepin koji se zavisno od vrste postrojenja za prečišćavanje uklanja od 0-20% (Rađenović i sar., 2007; Pérez i Barceló, 2007; Collado i sar., 2014), a zatim se zajedno sa drugim neuklonjenim PhAC putem prečišćene vode ispušta u površinske vodene sisteme (reke, jezera, potoke). Štaviše, kanalizacioni odvodi mogu da ispuste neobrađenu otpadnu vodu sa PhAC u životnu sredinu u slučaju vremenskih nepogoda i jakih kiša (Tamtam i sar., 2008). Dodatno, u seoskim područjima gde su domaćinstva međusobno udaljena, otpadna voda iz septičkih jama usled padavina se takođe izliva noseći sa sobom i PhAC koje tada dospevaju direktno u podzemnu vodu (Carrara i sar., 2008). Sa druge strane, direktno ispuštanje veterinarskih lekova u životnu sredinu događa se usled njihove primene u ribnjacima (odnosno pri uzgoju ribe), ali i na indirektan način lečenjem stoke i uglavnom preko spiranja i curenja vode kroz poljoprivredna polja na koja je nanešeno stajsko (prirodno) đubrivo, ali i od stočnog otpada (Boxall i sar., 2004; Sarmah i sar., 2006; Sanderson i sar., 2007; Boxall, 2008; Khan i sar., 2008; Kemper, 2008). Podzemna i površinska voda su fizički usko povezane, pa samim tim one se mogu kontaminirati međusobno (Jux i sar., 2002). Krajnji korak uklanjanja PhAC iz sirove vode pre njene distribucije kao vode za piće je prolazak kroz postrojenje za prečišćavanje.

Osim, akcidentnih situacija kada PhAC iz proizvodnje dospeju u životnu sredinu (Reddersen i sar., 2002), takođe nepravilno odlaganje neiskorišćenih ili lekova sa isteklim rokom trajanja koji se direktno bacaju u toaletne odvode ili završavaju na deponijama mogu se smatrati drugim značajnim lokalnim mestima potencijalnog zagađenja. Da bi se sprečilo nekontrolisano odlaganje PhAC u životnu sredinu, prema Direktivama Evropske

unije, 2001/83/EC[12] i 2004/27/EC[13], države članice su u obavezi da obezbede odgovarajući sistem i mesto za medicinske proizvode koji su neiskorišćeni ili su sa isteklim rokom trajanja.

Koncentracija pojedinačnih komponenata u površinskoj vodi je u rasponu od nekoliko desetina do nekoliko stotina ng/l, iako su koncentracije u nivou od µg/l takođe određivane za neke komponente (Kolpin i sar., 2002). Ove koncentracije smatraju se suviše niskim da bi predstavljale akutnu opasnost po zdravlje ljudi[14], međutim još uvek nije poznato da li su drugi „neciljani" organizmi kao što su vodeni osetljivi na delovanje pojedinačnih PhAC ili njihovih kombinacija. Do danas, samo u malom broju slučajeva PhAC su identifikovani u tragovima u vodi za piće (Stolker i sar., 2004; Hummel i sar., 2006; Mompelat i sar., 2009). Prisustvo PhAC u vodama u životnoj sredini, a posebno u vodi za piće i sirovoj neprečišćenoj vodi za njenu proizvodnju je pitanje od izuzetne važnosti za zdrastvenu bezbednost ljudi (Gros i sar., 2012). Stoga je neophodno pratiti i identifikovati prisustvo svih PhAC u vodenim sistemima životne sredine, jer to je značajan preduslov za adekvatnu procenu rizika. Takođe, treba reći da su koncentracije PhAC u vodenim sistemima promenjive i zavise od nekoliko parametara kao što su geografski položaj, efikasnost prečišćavanja otpadnih voda, blizine postrojenja za preradu otpadnih voda i meteoroloških uslova (uglavnom padavina).

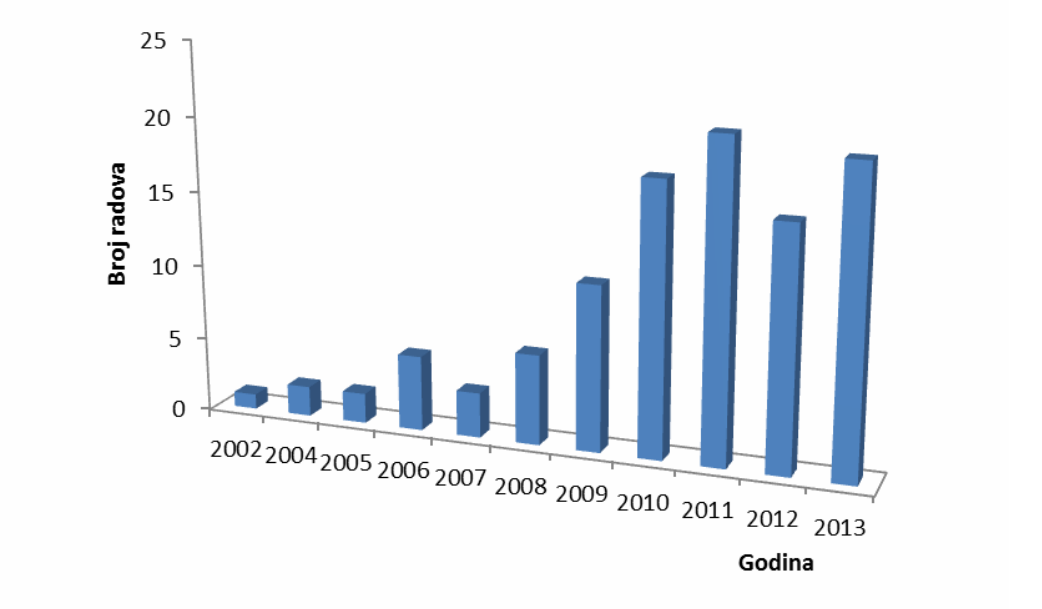
Veoma malo se zna o dugoročnim efektima PhAC i njihovom „ponašanju" u vodenoj sredini, posebno podzemnoj vodi (López-Serna i sar., 2010) u koje dospevaju usled prodiranja površinskih voda i curenja sa deponija. Takođe, nepoznato je da li kombinacije PhAC koje imaju zajednički mehanizam delovanja ispoljavaju toksični sinergetski efekat (Hernando i sar., 2004). Prisustvo PhAC u životnoj sredini dovodi do brojnih mogućih rizika vezanih za njihov biološki potencijal na floru, faunu i ljude. PhAC se smatraju potencijalno štetnim jedinjenjima, jer su neki od njih sveprisutni u životnoj sredini, postojani i biološki aktivne komponente sa poznatim negativnim uticajem na endokrini sistem (Kasprzyk-Hordern i sar., 2008). Na primer, hormoni kao estrogen poznati su po negativnom uticaju na endokrini sistem jer mogu da prouzrokuju negativne uticaje na reproduktivni razvoj (Lai i sar., 2002; Ingerslev i sar., 2003; Robinson i sar., 2007), kao i feminizaciju muških riba kada su prisutni samo u nivou od ng/l. Dodatno, zbog stalnog uvođenja PhAC u životnu sredinu oni se smatraju pseudo-perzistentnim jedinjenjima, a usled sinergetskih efekta kroz kombinovana i paralelna delovanja, čak i jedinjenja sa vrlo malom postojanošću mogu prouzrokovati neželjene efekte u životnoj sredini (Daughton i Ternes, 1999; Fent i sar., 2006). Za PhAC kao što su eritromicin, ciklofosfamidom, naproksen, sulfametaksazol, sulfasalazin poznato je da su postojani više od godinu dana u vodenim sistemima u životnoj sredini (Zuccato i sar., 2000). Nadalje, procena rizika obično se vrši za pojedinačne PhAC (tj. aktive supstance), dok farmaceutska jedinjenja se obično detektuju u smesama sa drugim antropogenim kontaminentima (Kolpin i sar., 2002).

[12] Directive 2001/83/EC, OJ L 311, 28.11.2001., str. 67-128 (u daljem tekstu: Direktiva Evropske komisije (EC, 2001)[12])

[13] Directive 2004/27/EC, OJ L 136, 30.4.2004., str. 34-57 (u daljem tekstu: Direktiva Evropske komisije (EC, 2004)[13])

[14] Na primer, ukoliko je u vodi prisutan acetaminofen sa koncentracijom od 10 ng/l to bi značilo da bi bilo potrebno popiti 100 miliona litara vode, da bi se unela količina ovog jedinjenja koja se nalazi u jednoj piluli.

Krajem prošlog i početkom ovog veka, prisustvo PhAC kao novih zagađujućih jedinjenja u životnoj sredini, pre svega u vodi privlači sve veću pažnju u širim naučnim krugovima (Gros i sar., 2006; Pérez i Barceló, 2007; López-Roldán i sar., 2010; Ferreira da Silva i sar., 2011; López-Serna i sar., 2013). Međutim, još uvek postoji ograničeno znanje o koncentraciji, „sudbini“ i uticajima PhAC na životnu sredinu, a oni još uvek nisu obuhvaćeni ni jednom regulativom za životnu sredinu. U skorije vreme, prvi put evropska komisija je uvrstila tri PhAC – inflamatorni lek za bolove (diklofenak) i dva hormona (17 alfa etinil estradiol i 17 beta estradiol) na listu „posmatranja“ („*Watch list*“) novootkrivenih polutanata koji bi mogli biti dodati na prioritetnu listu hemikalija za koje se zna da predstavljaju rizik za bezbednost površinskih voda. Suprotno razvijenim zemljama koje imaju značajnu količinu podataka o PhAC, podaci o pojavi i prisustvu ovih jedinjenja u zemljama u razvoju su retki i nesistematični. Iako se efekti PhAC ispituju kroz bezbednosne i toksikološke studije, potencijalni uticaj PhAC na životnu sredinu od proizvodnje do upotrebe je manje poznat i postao je značajna tema istraživačkog interesovanja (Ferreira da Silva i sar., 2011). Na ovo ukazuje i broj naučnih radova u periodu od 2002-2013 za površinsku vodu (slika 2.9), jer su podaci o koncentraciji i „sudbini“ PhAC u životnoj sredini predmet istraživanja brojnih studija u poslednjoj deceniji ovog veka (Terzić i sar., 2008; Grujić i sar., 2009; López-Roland i sar., 2010; Vazquez-Roig i sar., 2010; Ferreira da Silva i sar., 2011; Jelić i sar., 2011; Valcárcel i sar., 2011; López-Serna i sar., 2012; Petrović i sar., 2014).



***Slika 2.9**. Ilustracija porasta broja radova u međunarodnim časopisima iz elektronske baze SCIENCE DIRECT u kojima se pojavljuju reči „pharmaceutical active compounds and river water“ (podaci iz avgusta 2013. god.)*

Literaturni podaci ukazuju da je oko 160 PhAC namenjenih za upotrebu u ljudskoj i veterinarskoj medicini do sada ispitivano u različitim matriksima životne sredine. PhAC su određivane paralelno sa razvojem analitičkih tehnika za njihovu identifikaciju i kvantifikaciju u

vodenim sistemima. Njihova „sudbina“ u životnoj sredini i uklanjanje tokom procesa prečišćavanja otpadnih voda ili tokom prerade sirove vode namenjene za proizvodnju vode za piće je takođe predmet istraživanja (Mompelat i sar., 2009). Izbor PhAC i teškoće iznalaženja modela zagađenih vodenih sistema sa PhAC povezani su sa zdrastvenom praksom svake zemlje i prepisivanim receptima (Zuccato i sar., 2006; Goossens i sar., 2005). Primer za to je vankomicin jedan od najprepisivanijih antibiotika u Sjedinjenim Američkim Državama (USA) koji stoga može biti i predmet istraživanja u USA, međutim isti antibiotik ne spada u najprepisivanije u evropskim zemljama (Kümmerer, 2001). Dodatno, izbor PhAC koji će biti predmet istraživanja zasniva se osim na razmatranju njegove potrošnje, i na toksičnom uticaju na vodenu floru (Fent, 2008; Nentwig, 2008), faunu i/ili ljude kao i na njegovoj postojanosti u životnoj sredini.

2.2.2. Metode određivanja farmaceutski aktivnih komponenata

Za analizu PhAC prisutnih u životnoj sredini važno je razviti brze, relevantne i pouzdane analitičke metode koje će omogućiti određivanje velikog broja PhAC u vodenim sistemima u niskim koncentracionim nivoima. Danas, veliki broj analitičkih metoda je razvijen za određivanje PhAC u površinskoj i otpadnoj vodi (Bueno i sar., 2007; Kasprzyk-Hordern i sar., 2007; Fatta i sar., 2007; Gros i sar., 2009; Ferrer i sar., 2010; Lopez-Serna i sar., 2010; Gracia-Lor i sar., 2011). Sa druge strane, u literaturi je dostupan znatno manji broj metoda za određivanje PhAC u morskoj vodi i vodi za piće, zbog analitičkih poteškoća usled njihove kvantifikacije u izuzetno niskim nivoima u kojima su prisutni u ovim tipovima vode. Primeri analitičkih metoda koje se koriste za analizu PhAC u vodenim sistemima prikazani su u tabeli 2.3.

Tabela 2.3. *Pregled primene LC/MS metoda u analizi PhAC*

Broj ispitivanih PhAC	Broj terapeutskih grupa	Vrsta uzorka	Priprema uzoraka	LC-MS	Literatura
6	-	Otpadna i površinska voda	SPE (Lic EN kolone)	LC-ESI-MS	Farré i sar. (2001)
23	10	Površinska voda	SPE (Oasis MCX, LiCrolutEN kolone, C18)	LC-„*turbo ion spray*“-QqQ-MS/MS	Calamari i sar. (2003)
22	13	Površinska voda	SPE (Oasis MCX)	LC-„*turbo ion spray*“-QqQ-MS/MS	Zuccato i sar. (2005)
7	1	Otpadna voda i čvrsti bio-otpad	ASE	LC-ESI-QqQ-MS/MS	Miao i sar. (2005)
15	7	Rečna voda	SPE (Oasis HLB)	GC-EI-MS	Moldovan (2006)
28	6	Rečna voda	SPE (Oasis HLB)	LC-ESI-QqQ-MS/MS	Gros i sar. (2007)

Nastavak tabele 2.3

Broj ispitivanih PhAC	Broj terapeutskih grupa	Vrsta uzorka	Priprema uzoraka	LC-MS	Literatura
44	10	Otpadna voda (gradska i industrijska)	SPE (Oasis HLB)	LC-ESI-QqQ-MS/MS	Terzić i sar. (2008)
38	13	Površinska voda	SPE (Oasis MCX)	UHPLC-ESI-QqQ-MS/MS	Kasprzyk-Hordern i sar. (2008)
73	16	Površinska i otpadna voda	SPE (Oasis HLB)	LC-ESI-QqLIT-MS/MS	Gros i sar. (2009)
76	9	Otpadna voda	SPE (Oasis HLB)	LC-ESI-QqQ-MS/MS	Shao i sar. (2009)
28	6	Rečna voda	SPE	LC-ESI-QqQ-MS/MS	López-Roldán i sar. (2010)
10	1 (estrogeni)	Rečna voda	„*On-line*“ SPE	LC-ESI-QqQ-MS/MS	
24	3	Rečna voda i voda za piće	SPE (Oasis HLB)	LC-„*turbo ion spray*“-QIT-MS/MS	Valcárcel i sar. (2011)
6	1	Zemljište	Sokslet ekstrakcija potpomognuta mikrotalasima	LC-ESI-QqQ-MS/MS	Tamtam i sar. (2011)
20	10	Rečna voda i voda za piće	SPE (Oasis HLB)	UHPLC-ESI-QqQ-MS/MS	Mompelat i sar. (2011)
74	16	Podzemna, rečna i otpadna voda	SPE (Oasis HLB)	UHPLC-ESI-QqQ-MS/MS	López-Serna i sar. (2011)
43	10	Otpadna voda i mulj	SPE (Oasis HLB) ASE	HPLC-„*turbo ion spray*“-QqLIT-MS/MS	Jelić i sar. (2011)
43	9	Površinska voda, zemljište i sediment	SPE (Oasis HLB) ASE	HPLC-„*turbo ion spray*“-QqLIT-MS/MS	Ferreira da Silva i sar. (2011)
81	19	Površinska, otpadna i voda za piće	SPE (Oasis HLB)	UHPLC-ESI-QqLIT-MS/MS	Gros i sar. (2012)
77	10	Površinska voda	„*On-line*“ TFE kolone (TurboFlow™)	UHPLC-ESI-QqQ-MS/MS	López-Serna i sar. (2012)
95	10	Podzemna voda	„*On-line*“ SPE	LC-ESI-QqQ-MS/MS	López-Serna i sar. (2013)

Nastavak tabele 2.3

Broj ispitivanih PhAC	Broj terapeutskih grupa	Vrsta uzorka	Priprema uzoraka	LC-MS	Literatura
81	19	Otpadna, površinska, podzemna i voda za piće	SPE	UHPLC-ESI-QqLIT-MS/MS	Petrović i sar. (2014)

SPE – ekstrakcija na čvrstoj fazi, „*On-line*“ SPE – „*on-line*“ ekstrakcija na čvrstoj fazi, ASE („*accelerated solvent extraction*“) – ubrzana ekstrakcija pod pritiskom, EI - elektronska jonizacija, ESI – elektrosprej jonizacija, LC – tečna hromatografija, MS – masena spektrometrija, QqQ – tripl kvadrupol, QIT - kvadrupol „ion trap“, QqLIT – tripl kvadrupol linearni „ion trap“, GC – gasna hromatografija, UHPLC – ultra pritisna tečna hromatografija, „*turbo ion spray*“- „*turbo ion spray*“ jonski izvor

2.2.2.1. Metode pripreme uzoraka u analizi farmaceutski aktivnih komponenata

Priprema uzoraka za analizu PhAC je u saglasnosti sa novim pristupima u preparativnim tehnikama koji se oslanjaju na princip „zelene hemije“ („*green chemistry*“), u cilju smanjenja potrošnje organskih rastvarača i primene manje manipulativnih koraka tokom pripreme uzorka („*on-line*“ ekstrakcija na čvrtoj fazi) (Busetti i sar., 2008; Petrović i sar., 2010). Međutim, SPE još uvek ostaje najčešće i najšire korišćena preparativna tehnika za istovremenu ekstrakciju i koncentrisanje organskih supstanci iz vodenih uzoraka (Gros i sar., 2006; Gómez i sar., 2006; Zhang i Zhou, 2007; Lee i sar., 2007a, 2007b; Xiao i sar., 2008; Grujić i sar., 2009; Petrović i sar., 2014). Među SPE kolonama koje su punjene različitim sorbentima (nepolarnim, jono-izmenjivačkim, polimernim), Oasis® HLB (Waters, Manchester, UK) polimerne kolone najčešći su izbor za ekstrakciju polarnih i nepolarnih PhAC komponenti (Hao i sar., 2007). Ove kolone obezbeđuju vezivanje ciljanih analita na obrnutim fazama jer su punjeni monomerima N-vinilpirolidina i lipofilnog divinilbenzena. Tokom SPE procedure parametri koji utiču na efikasnost izolovanja ciljanih komponenata su pH vode, vreme sušenja punjenja u koloni kao i vrsta rastvarača koji se koristi za eluiranje ciljanih analita. Dodatno, stepen razblaženja uzoraka vode i zapremina dejonizovane vode koja se koristi kao rastvarač u koraku čišćenja kolone takođe su važni za smanjenje uticaja matriksa pri analizi različitih uzoraka (na primer, morske vode, otpadne vode, itd.). pH vrednost vodenih uzoraka ima važnu ulogu u efikasnosti SPE ekstrakcije. Dokazano je da pH 2 obezbeđuje najveći „recovery“ za kisele ciljane analite pri korišćenju Oasis HLB kolona (Kasprzyk-Hordern i sar., 2008). Ovo se objašnjava vezom između pH vrednosti uzorka vode i slabo kiselih PhAC jedinjenja. Pre početka eluiranja ciljanih komponenata, punjenje u koloni se suši u struji azota radi uklanjanja zaostale vode iz prethodnog koraka čišćenja jer ima značajan uticaj na efikasnost ekstrakcije. Wu i sar. (2010) su utvrdili da je optimalno vreme sušenja bilo 10 minuta za ciljane analite, jer su se „recovery“ vrednosti za diklofenak, gemfibrozil i ibuprofen povećavale tokom perioda sušenja punjenja kolone od 5 do 10 minuta, a kada je vreme sušenja produženo od 10 do 20 minuta „recovery“ vrednosti su se smanjivale. „Recovery“ vrednost naproksena je imala sličan trend ponašanja kao prethodno spomenute „recovery“ vrednosti navedenih lekova u periodu sušenja od 5-10 minuta,

međutim u slučaju naproksena sa povećanjem vremena sušenja od 10-20 minuta i efikasnost („recovery“) bila je veća, ali optimalno vreme sušenja za sve analite je bilo 10 minuta. Efikasnost („recovery“) SPE metode za ciljane analite zavisi i od polarnosti rastvarača korišćenog za eluiranje i njegove pH vrednosti. U literaturno dostupnim studijama utvrđeno je da metanol omogućava postizanje zadovoljavajućeg „recovery“ za ciljane PhAC komponente (Petrović i sar., 2005; Gros i sar., 2006). Ovo može biti objašnjeno činjenicom da se visoko polarne komponente mogu lako eluirati sa rastvaračem relativno visoke polarnosti kao što je metanol.

2.2.2.2. Instrumentalne metode analize farmaceutski aktivnih komponenata

Trenutna istraživanja vezana za razvijene analitičke metode za određivanje PhAC u uzorcima životne sredine usmerena su na poboljšanje metodologije sa ciljem povećanja broja analiziranih uzoraka u kratkom vremenskom periodu, smanjenje predtretmana uzorka, kao i smanjenje potrošnje rastvarača i povećanje ukupne efikasnosti metode u odnosu na selektivnost i osetljivost. Napredne LC-MS/MS tehnike kao što su QqQ ili IT za kvantitativno određivanje PhAC, idu u pravcu poboljšanja osetljivosti, omogućavajući njihovu detekciju u koncentracionim nivoima ispod ppt, i takođe istovremenu identifikaciju velikog broja komponenata bez gubitka osetljivosti. Sa druge strane, kompleksni matriksi iz životne sredine često zahtevaju primenu visokorezolucionih tehnika, kao što je Orbitrap i TOF ili hibridne masene spektrometre kao što je QqTOF i QqLIT, u cilju dobijanja dodatnih strukturnih informacija za nedvosmislenu identifikaciju PhAC i potvrdu pozitivnih uzoraka. Ove instrumente karakteriše velika brzina skeniranja, visoka tačnost pri određivanju mase ispitivanih analita (QqTOF) i povećanje osetljivosti.

2.3. Mikotoksini

2.3.1. Osobine i rasprostranjenost

Mikotoksini su sekundarni toksični metaboliti proizvedeni od saprofitnih plesni roda *Aspergillus, Penicillium* i *Fusarium* koje rastu na različitim usevima i namirnicama uključujući i hranu za životinje. Ova jedinjenja predstavljaju potencijalnu opasnost po zdravlje ljudi i životinja kroz unos namirnica proizvedenih od kontaminiranih useva. Ranija istraživanja izvedena tokom 1960. godine su ukazala da su mikotoksini odgovorni za mnoge bolesti. Broj poznatih mikotoksina sa toksičnim dejstvom na zdravlje ljudi i životinja konstantno se povećava kao i zakonske odredbe kojima se kontroliše njihovo prisustvo u hrani i hrani za životinje.

Naziv mikotoksin potiče od kombinacije grčke reči za plesan „*mykes*“ i latinske reči „*toxicum*“ što znači otrov. Dodatno, pojam mikotoksina uobičajno se vezuje za toksične hemijske

proizvode relativno malih molekulskih masa (~ 700 Da[15]) koji su nastali kao sekundarni metaboliti nekoliko plesni koje se lako „kolonizuju“ kako na žitaricama u polju tako i na žitaricama posle žetve. Procenjeno je da je 25% žitarica od ukupno proizvedenih u svetu i 20% od proizvedenih u Evropskoj uniji kontaminirano mikotoksinima (Zöllner i Mayer-Helm, 2006).

Mikotosini se uobičajeno unose u organizam čoveka putem hrane zagađene njima, ali i udisanjem toksičnih spora kao i direktnim kontaktom preko kože. Trenutno više od 400 mikotoksina je identifikovano u svetu. S obzirom da su mikotoksini termički stabilna jedinjenja, predstavljaju konstantan rizik po zdravlje ljudi i životinja. Većina mikotoksina su hemijski stabilna jedinjenja koja imaju sposobnost da prežive proces skladištenja i prerade namirnica, čak i visoke temperature pri njihovoj termičkoj obradi. Tako su na primer, nesmetano prisutni u hlebu nakon procesa pečenja ili u žitnim pahuljicama za doručak nakon procesa prerade. Ovo ukazuje na činjenicu da je izuzetno važno izbeći uslove koji dovode do pojave i prisustva mikotoksina u namirnicama, što nije uvek i slučaj u praksi. Mikotoksine je izuzetno teško ukloniti iz namirnica i najbolji način kontrole njihovog prisustva je prevencija. Hemijske i biološke osobine mikotoksina su različite i njihovi toksični uticaji su veoma promenjivi, kao što su: kancerogeni, genotoksični, teratogeni, nefrotoksični, hepatotoksični i imunotoksični. Mikotoksini nisu samo „opasni“ po zdravlje ljudi, već dovode do značajnih ekonomskih gubitaka, u slučaju velikog stepena zagađenja različitih namirnica. Generalno, usevi koji se čuvaju više od nekoliko dana postaju potencijalne „mete“ za razvoj plesni i pojavu mikotoksina. Pojava mikotoksina karakteristična je i za umereno tople i tropske regione sveta, zavisno od vrste plesni karakterističnih za to područje. Namirnice koje su najčešće kontaminirane mikotoksinima su žitarice, koštuničavo i sušeno voće, kafa, kakao, začini, seme uljarica, sušeni grašak, pasulj, a od voća posebno jabuke. Takođe, mikotoksini mogu biti prisutni u pivu i vinu kao posledica upotrebe kontaminiranog ječma, drugih žitarica, grožđa i njihovih proizvoda. Dodatno, mikotoksini se mogu uneti u lanac ishrane za ljude preko mesa ili proizvoda životinjskog porekla kao što su jaja, mleko i sir što je posledica hranjenja životinja kontaminiranom hranom. Najčešće su ispitivani mikotoksini čiji je sadržaj u hrani i hrani za životinje definisan postojećim regulativama, a to su: aflatoksini, ohratoksin (OTA), trihoteceni, zearalenon (ZON) i fumonizini.

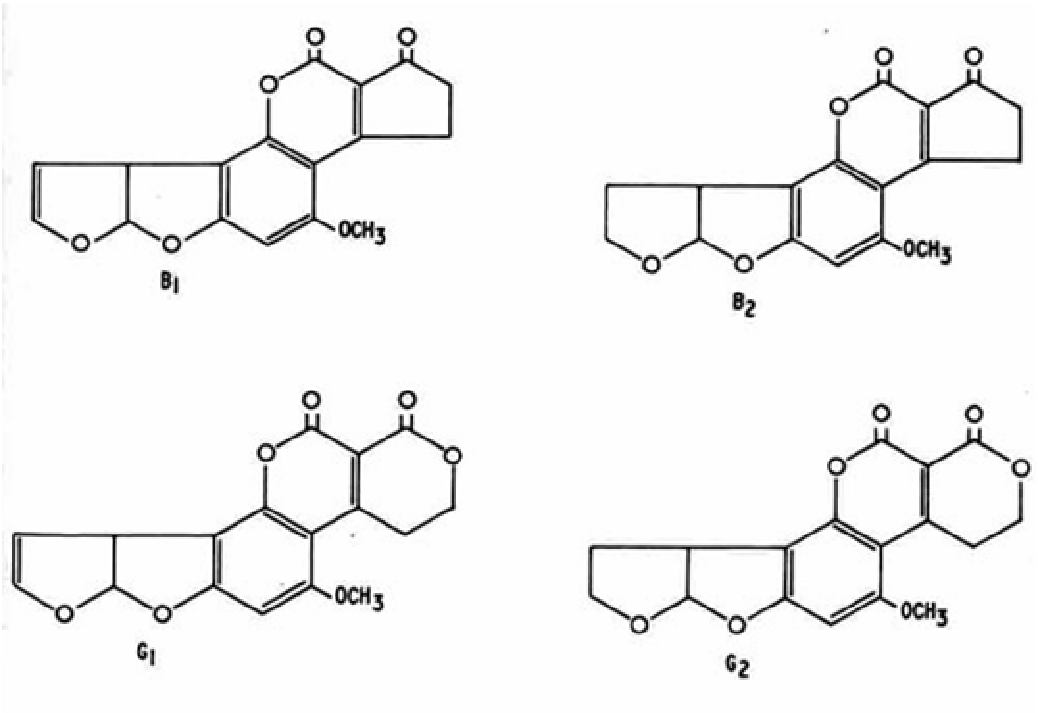
Aflatoksini

Aflatoksini su široko rasprostranjeni toksini proizvedeni od rodova *Aspergillus flavus, Aspergillus parasiticus* i *Aspergillus nomius* (Kurtzman i sar.,1987). *A. flavus* proizvodi samo B aflatoksine, dok druge dve vrste proizvode i B i G aflatoksine (Creppy, 2002). Aflatoksini predstavljaju veliki problem u Evropi i Svetu, zbog njihovih štetnih karcenogenih, mutagenih, teratogenih i imunosupresivnih uticaja na zdravlje ljudi i životinja (Eaton i Gallagher, 1994). Hepatokancerogena svojstva aflatoksina B1 (AFB1) dokazana su na sisarima; Internacionalna agencija za istraživanje raka klasifikovala je ovaj toksin kao karcinogen iz

[15] 1Da = 1/12 mase atoma ugljenikovog izotopa ^{12}C, gde se za masu ugljenikog izotopa ^{12}C po dogovoru uzima tačno 12 masenih jedinica; Da = AMU (engl. Atomic Mass Unit) =Th (Thompson).

grupe 1[16] (International Agency for Research on Cancer, IARC, 1993). U Keniji 2004. i 2005. godine, usled zagađenja kukuruza aflatoksinima zabeležena su 123 smrtna slučaja. Epidemiološke studije iz ovog slučaja ukazale su na vezu između kontaminacije kukuruza i lokalnih metoda žetve, pripreme i skladištenja kukuruza. U navedenom periodu u Keniji, prisustvo aflatoksina utvrđeno u kukuruzu bilo je do 1000 µg/kg (Centers for Disease Control, 2004).

Na slici 2.10 prikazane su strukturne formule aflatoksina B1, B2, G1 i G2.



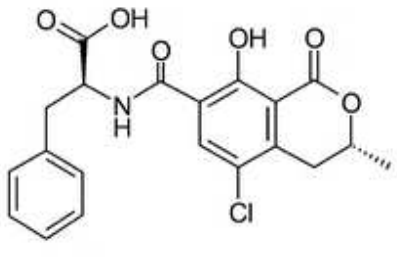
Slika 2.10. *Aflatoksini B1, B2, G1 i G2*

Ohratoksin (OTA)

OTA je sekundarni metabolit nekoliko vrsta plesni rodova *Aspergillus* i *Penicillium* (slika 2.11). Ove plesni su široko rasprostranjene, pošto se obe vrste mogu razvijati pri različitim klimatskim uslovima (Pohland, 1982; Harwig i sar., 1983; Ramos i sar., 1998; Frisvad i Lund, 1993; Lee i Magan, 2000). OTA toksin je prvi put otkriven 1965. godine kao

[16] Međunarodna agencija za istraživanje raka („International Agency for Research on Cancer", IARC) je međuvladina agencija formirana kao deo Svetske zdravstvene organizacije Ujedinjenih nacija koja vrši kategorizaciju različitih supstanci, smesa ili uslova izloženosti na osnovu njihove kancerogenosti, pa Grupa 1 podrazumeva supstance, smese i uslove izloženosti za koje postoji dovoljno dokaza o njihovoj kancerogenosti za ljude ili je njihova kancerogenost dokazana eksperimentalno na životinjama.

metabolit plesni koji je pokazao toksično ponašanje kod životinja (Van der Merwe i sar., 1965).



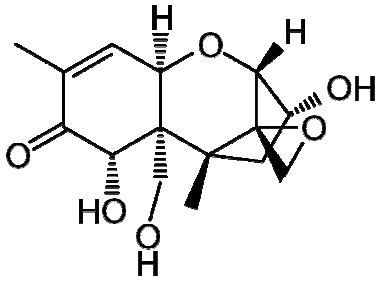
Slika 2.11. *Struktura ohratoksina A*

Uobičajeno, žitarice i druge namirnice bogate skrobom, zatim kafa, začini i sušeno voće mogu biti zagađeni OTA toksinom (International Agency for Research on Cancer, IARC, 1993; Studer-Rohr i sar., 1995). Prisustvo ovog toksina ne prelazi nekoliko ppb. Krajem prošlog i početkom ovog veka, prisustvo OTA toksina identifikovano je u krvi ljudi i životinja, kao i u mesu, vinu i pivu (Gharbi i sar., 1993; Jørgensen, 1998; Brera i sar., 2002). OTA je „stabilan" toksin koji se unosom preko hrane u telo životinja nagomilava u krvotoku, jetri i mišićnom tkivu, što dalje dovodi do unosa ovog mikotoksina u organizam čoveka. Literaturni podaci ukazuju na imuno – supresivnu prirodu ovog toksina (Turner i sar., 2009), zatim teratogene, mutagene i karcinogene osobine (International Agency for Research on Cancer, IARC, 1993; Turner i sar., 2009) kao i njegov negativan uticaj na plodnost (Biró i sar., 2003). Prisustvo OTA je povezano i sa balkanskom endemskom nefropatijom (BEN), hroničnom bolesti bubrega u regionu jugoistočne Evrope (Mantle, 2002; Petzinger i Weidenbach, 2002). Internacionalna agencija za istraživanje kancera (International Agency for Research on Cancer, ICRA, 1993) klasifikovala je OTA toksin kao moguće kancerogeni toksin (grupa 2B). Zbog ovih pronalazaka mnoge zemlje definisale su maksimalno dozvoljenu vrednost OTA toksina u hrani, između 1 i 10 ppb zavisno od vrste namirnice.

Trihoteceni

Trihoteceni su porodica mikotoksina koju čine više od 60 metabolita proizvedenih od brojnih plesni rodova *Fusarium*, *Myrothecium*, *Phomopsis*, *Stachybotrys*, *Trichoderma* i *Trichothecium.* Deoksinivalenol (DON) ili vomitoksin je toksin koji pripada trihotecenima tipa B (slika 2.12). Kada je reč o toksičnosti DON izaziva dva toksična efekta: smanjenje unošenja hrane (anoreksiju) i povraćanje. Iako je manje toksičan od mnogih drugih trihotecena, DON je najprisutniji u pšenici, ječmu, kukuruzu, raži, suncokretovom semenu i smesama žitarica koje se koriste u ishrani životinja. Toksini T-2 i HT-2 pripadaju trihotecenima tipa A i proizvedeni su od *Fusarium sporotrichioides, Fusarium poae, Fusarium equiseti* i *Fusarium acuminatum* (Creppy, 2002). Ova dva toksina prisutna su u žitaricama kao što su pšenica, kukuruz, ovas, ječam, zatim u pirinču, soji i takođe u proizvodima na bazi žitarica. Zagađenje hrane ovim

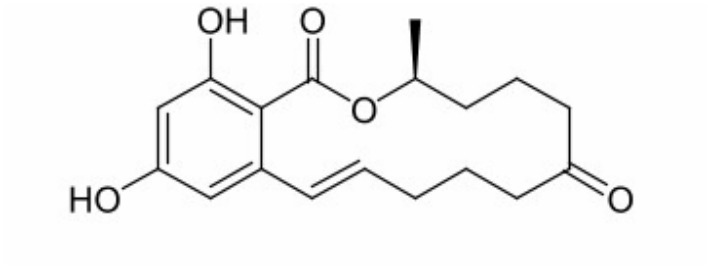
toksinima kod ljudi izaziva mučninu, povraćanje, razdražljivost ždrela, bolove u stomaku i nadimanje, dijareju, vrtoglavicu, krvarenje iz nosa i usta (Bennett i Klich, 2003).



Slika 2.12. *Struktura deoksinivalenola*

Zearalenon (ZON)

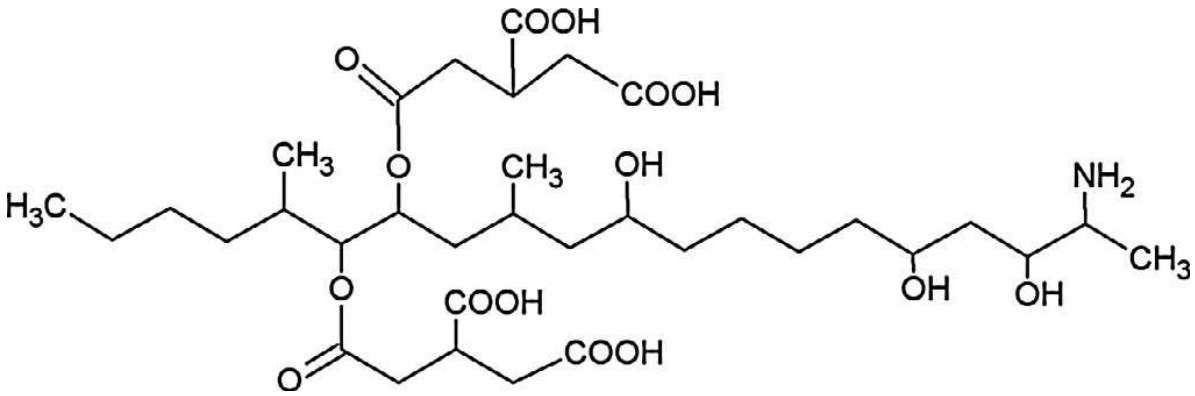
ZON, ranije poznat kao F-2 toksin, proizvode različite *Fusarium* plesni i karakterističan je za zemlje sa umereno toplom i toplom klimom (slika 2.13; Zinedine i sar., 2007). Plesni koje proizvode ZON mogu se naći na kukuruzu i u manjoj meri ječmu, ovsu, pšenici, prosu i pirinču. ZON ima relativno malu akutnu toksičnost, što je dokazano na miševima i pacovima nakon oralne upotrebe. Prethodne studije dokazale su da ZON stimuliše rast ćelija kancera dojke (Ahamed i sar., 2001; Yu i sar., 2005).



Slika 2.13. *Struktura zearalenona*

Fumonizini

Fumonizini su grupa mikotoksina koji proizvode *Fusarium* plesni rodova *Fusarium verticillioides* i *Fusarium proliferatum* (Marin i sar., 2013). Iz ove grupe mikotoksina najčešće su ispitivani fumonizin B1 (FB1), B2 (FB2) i B3 (FB3), ali najveću pažnju privlači FB1 (slika 2.14).



Slika 2.14. *Struktura fumonizina B1*

Kukuruz, posebno onaj koji se uzgaja u toplim regionima najčešće je zagađen fumonizinima. *Fusarium verticillioides* i *Fusarium proliferatum* rastu u područjima sa širokom opsegom temperatura i pri relativno visokoj aktivnosti vode[17] (a_w>0,9). Zagađenje kukuruza fumonizinima moguće je pre i tokom rane faze skladištenja. Osim u ekstremnim uslovima skladištenja, koncentracija fumonizina se ne povećava u kukuruzu tokom skladištenja. Fumonizini su prilično termički stabilni i sadržaj toksina može se smanjiti samo tokom termičkih procesa obrade u kojima temperatura prelazi 150^{o}C. Stoga, tokom procesa fermentacije degradacija fumonizina je neznatna (EFSA, 2005).

2.3.1.1. Uticaj klimatskih promena na razvoj mikotoksina na prostoru Evrope

Poznato je da klimatske promene utiču na pojavu mikotoksina u poljoprivrednim kulturama (Paterson i Lima, 2011). Toplije vreme, period visokih temperatura, kao i veća količina padavina, a i suše utiču na razvoj mikotoksina zavisno od regiona u kojem se istraživanja vrše.

Tako, „Međuvladin panel za klimatske promene“ (IPCC, 2007) predviđa uvećanje regionalnih razlika u prirodnim resursima na prostoru Evrope. Na primer, očekuje se da visoke temperature i suše smanje dostupnost vode i proizvodnju useva na jugu Evrope. Povećanje temperature za 3^{o}C uticaće na razvoj mnogih biljnih bolesti i dovešće do velikih promena unutar mnogih biljnih zajednica (Pritchard, 2011). Primer za ovo je uočena pojava OTA toksina na vinovoj lozi (Paterson i Lima, 2010) i porast njegovog sadržaja u vinima u Ujedinjenom Kraljevstvu (UK) (Paterson i Kozakiewicz, 2001). Očekuje se da će klimatske promene u UK dovesti do smanjenja padavina tokom proleća i povećanja temperature tokom letnjeg i zimskog perioda u narednih 50 godina (Hulme i sar., 2002; Miraglia i sar., 2009). Prognoza klimatskih uslova u UK tokom 21. veka nagoveštava blage/vlažne zime, topla/suva leta i pojavu ekstremnih vremenskih uslova. Klimatske promene u UK mogu dovesti i do

[17] Indikator prisustva vode za mikrobiološka ispitivanja; većina svežih namirnica ima a_w vrednost <0,99; većina bakterija koje izazivaju kvarenje ne rastu pri a_w<0,91, dok plesni uzročnici kvarenja rastu i pri vrednostima <0,80.

uzgajanja novih useva koji nisu karakteristični za UK, ali se može očekivati i njihovo zagađenje mikotoksinima. Kao posledica povećanja temperature u UK predviđa se pojava aflatoksina, dok će pojava fumonizina biti karakteristična za submediteranske zemlje (na primer, severni Portugal). Prisustvo aflatoksina već je zabeleženo u severenoj Italiji (Giorni i sar., 2008).

U nekim oblastima pogoršaće se kvalitet zemljišta, a u drugim će se pojaviti klizišta i erozije zbog padavina (Miraglia i sar., 2009). Povećanje temperature i izmenjen režim padavina uticaće na gubitak minerala prisutnih u zemljištu usled ispiranja i erozije. U južnoj Evropi doći će do smanjenja prinosa useva zasejanih tokom proleća kao što su kukuruz, suncokret i soja i predviđa se da će pogodni regioni za njihovo gajenje postati severnije oblasti gde se očekuje porast proizvodnje kukuruza od 30-50%.

U južnoj i jugo-istočnoj Evropi (Portugal, južna Francuska, Italija, Slovenija, Srbija, Grčka, Malta, Kipar, Bugarska i južna Rumunija) povećanje temperature od 4-5^{o}C uticaće na smanjenje dostupnosti vode posebno u letnjim mesecima. Povećanje jakih padavina uticaće na eroziju i gubitak organske materije iz zemljišta (EC, 2007). Takođe će se zahtevati posebne mere pravilnog sušenja useva radi smanjenja stepena njihove zagađenosti mikotoksinima tokom perioda skladištenja.

U oblasti centralne Evrope koja obuhvata Poljsku, Češku Republiku, Slovačku, Mađarsku, Severnu Rumuniju, južnu i istočnu Nemačku, istočnu Austriju, očekuje se da će doći do povećanja temperature od 3-4^{o}C što će povećati količinu padavina tokom zime, a smanjiti tokom leta, sa povećanim rizikom od poplava. Ovo će dalje dovesti do erozije zemljišta, gubitka organske materije zemljišta, migracije štetočina i bolesti, sušom tokom leta i visokim temperaturama što će sve neminovno uticati na poljoprivredu. Sve ovo dovešće do pojave mikotoksina, gde oni nisu bili prisutni pre, kao što je pojava aflatoksina na grožđu 2004. godine u Mađarskoj (Varga i sar., 2007).

U Norveškoj, Švedskoj, Finskoj i Baltičkim zemljama (severna Evropa) povećanje temperature u opsegu od 3,5-4^{o}C i godišnje povećanje padavina do 40% uticaće na pojavu poplava. Ukupan rezultat klimatskih promena biće „produžena vegetaciona sezona i duži period bez mraza“ što će dovesti do povećanja prinosa useva od 10-30% sa porastom temperature od 1-3^{o}C i mogućnosti gajenja novih kultura. Dakle, u narednom periodu očekuje se da će klimatske promene uticati na povećan razvoj plesni te time i češću pojavu mikotoksina u usevima čak i u predelima gde je njihovo prisustvo do sada bilo retko.

2.3.2. Metode određivanja mikotoksina

S obzirom da je većina mikotoksina toksična u vrlo niskim koncentracijama (na primer, 1 ng/kg telasne mase po danu za aflatoksine (Scientific Committee on Food, SCF, 1994)) veoma su važni podaci o njihovom prisustvu u različitim namirnicama radi procene izloženosti i rizika po zdravlje. Radi određivanja njihovog prisustva u namirnicama neophodne su potvrđene i pouzdane anlitičke metode. Različite fizičko-hemijske osobine mikotoksina i

njihovi različiti koncentracioni opsezi u uzorcima u kojima su prisutni, predstavljaju pravi izazov za istraživače, te je do nedavno većina instrumentalnih metoda bila usmerena na određivanje pojedinačnih „ciljanih“ mikotoksina (Zöllner i Mayer-Helm, 2006; Krska i sar., 2008; Köppen i sar., 2010).

Plesni imaju tendenciju da se razviju na pojedinačnim delovima namirnica i nisu homogeno rasprostranjene po namirnicama tokom skladištenja. Stoga je važno da razvijeni protokol uzimanja uzorka obezbedi reprezentativnost uzorka u odnosu na celokupnu seriju uzoraka koji su predmet ispitivanja. Poznato je da nehomogenizovani i pojedinačni uzorci daju nerealnu i lošu procenu sadržaja mikotoksina, zbog čega se do 90% greške pri određivanju sadržaja mikotoksina pripisuje neadekvatnom načinu uzimanja uzoraka. Kako su mikotoksini nehomogeno raspoređeni u žitaricama i drugim uzorcima, uzimanje uzorka koji će omogućiti dobijanje pouzdanog rezultata tokom analize je „ozbiljan“ zadatak (Lauren i sar., 2006).

U tabeli 2.4 je dat pregled metoda za analizu mikotoksina u različitim uzorcima useva i namirnicama koje su zasnovane na naprednim tehnikama detekcije, prvenstveno na primeni LC/MS. Zbog velikih razlika među toksinima i vrstama uzoraka koji se analiziraju, a u kojima mikotoksini mogu biti prisutni, broj razvijenih i validovanih metoda je značajan u literaturi. Pri tome, „multi-toksin“ pristup postaje sve neophodniji za analizu mikotoksina kao i metode namenjene analizi šireg spektra fizičko-hemijski različitih toksina. U literaturi su objavljene „multi-toksin“ metode za hranu i hranu za životinje koje obuhvataju od 4 do 106 hemijski različitih jedinjenja (najčešće poreklom od nekoliko plesni) (tabela 2.4). Treba istaći da su metode koje obuhvataju veliki broj hemijski različitih jedinjenja uglavnom tzv. „skrining“ metode koje daju više uvid u kvalitativnu identifikaciju nego u pouzdano kvantitativno određivanje.

Tabela 2.4. *Metode analize mikotoksina u hrani i hrani za životinje bazirane na tečnoj hromatografiji i masenoj spektrometriji*

Broj ispitivanih analita	Vrsta analita	Vrsta uzorka	Korak pripreme i/ili prečišćavanja *„clen-up“*	LC/MS	Izvor
4	NIV, DON, FUS-X, 3-ADON	Kukuruz	SPE (Carbograph-4)	LC-TISP/APCI-MS/MS	Laganà i sar. (2003)
13	Trihteceni, ZON, aflatoksini	Kukuruz, pšenica, kukuruzne pahuljice, biskvit	SPE (Multisep 226)	HPLC-APCI-TOF-MS	Tanaka i sar. (2006)
39	Trihteceni, ZON+derivati, fumonizini, ENN, ERG, OTA, aflatoksin, MON	Pšenica, kukuruz, pirinač, ječam	Sirovi ekstrakt	HPLC-ESI-QIT-MS/MS	Sulyok i sar. (2006); Sulyok i sar. (2007a)

Nastavak tabele 2.4

Broj ispitivanih analita	Vrsta analita	Vrsta uzorka	Korak pripreme i/ili prečišćavanja „*clen-up*“	LC/MS	Izvor
11	Aflatoksini, OTA, fumonizini, trihoteceni	Kukuruz	SPE (IA, Myco-6-in-1)	HPLC-ESI-QIT-MS/MS	Lattanzio i sar. (2007)
17	aflatoksini, OTA, fumonizini, trihoteceni, CIT, STE	Kikiriki buter, kukuruz	SPE (Mycosep 226)	UHPLC-ESI-QqQ-MS/MS	Ren i sar. (2007)
11	Aflatoksini, OTA, DON, HT-2, T-2, fumonizini, ZON	Kukuruz, pšenična pasta, dečija nrana na bazi žitarica	Sirovi ekstrakt	UHPLC-ESI-QqQ-MS/MS	Beltrán i sar. (2009)
12	Aflatoksini, OTA, DON, NIV, T2, HT-2, ZON, fumonizini	Kukuruz, orah, biskvit, žitarice	Sirovi ekstrakt	HPLC-ESI-QqQ-MS/MS	Garrido Frenich i sar. (2009)
6	Aflatoksini	Jaja, mleko, meso i proizvodi od mesa	SPE	HPLC-FLD/UV	Herzallah (2009)
32	Trihoteceni, ZON, Þ-ZOL, fumonizini, aflatoksini, ERG, PAT, CIT, OTA, MON, ALT, AOH, AME, ENN B, BEA, „*gibberellic*“ kiselina	pšenica, kukuruz	Sirovi ekstrakt	HPLC-ESI-QqQ-MS/MS „*MicroLC*“-ESI-Orbitrap-MS	Herebian i sar. (2009)
11	Trihoteceni, ZON, fumonizini	Žitarice, proizvodi na bazi žitarica	QuEChERS ili sirovi ekstrakt	UHPLC-ESI-TOF-MS UHPLC-APCI-Orbitrap-MS	Zachariasova i sar. (2010)
106	Metaboliti različitih rodova plesni	Hrana kontaminirana plesnima	Sirovi ekstrakt	UHPLC-ESI-QqQ-MS/MS	Sulyok i sar. (2010)

Nastavak tabele 2.4

Broj ispitivanih analita	Vrsta analita	Vrsta uzorka	Korak pripreme i/ili prečišćavanja „*clen-up*“	LC/MS	Izvor
9	OTA, aflatosini, ZON, DON, T-2, HT-2	Žitarice	Sirovi ekstrakt	HPLC-APPI-QIT-MS/MS	Capriotti i sar. (2010)
6	OTA, aflatoksini, ZON	Začinska paprka	IA (R-Biopharm Rhône LTD)	HPLC-FLD	Santos i sar. (2010)
11	DON, 3-AcDON, NIV, DON, FUS-X, DAS, ZON, STE, ALT, AOH, AME	Pšenica, kukuruz, proso	Direktna jonizacija iz ekstrakta žitarica	HPLC-DART-Orbitrap	Vaclavik i sar. (2010)
6	Aflatoksini, OTA	Dečija hrana i mleko	SPE (IA, Aflaochra HPLC™, Vicam)	UHPLC-HESI-QqQ-MS/MS	Beltrán i sar. (2011)
10	aflatoksini, ENN, CIT, OTA	Jaja	QuEChERS	UHPLC-ESI-QqQ-MS/MS	Garrido Frenich i sar. (2011)
8	HT-2, T-2, DON, DON-3-Glc, ADON, FUS-X NIV, ZON	Pšenica	Sirovi ekstrakt	UHPLC-APCI-QqQ-MS/MS	Škrbić i sar. (2011a)
10	Aflatoksini, OTA, DON, fumonizini, T-2, HT-2	Žitarice i proizvodi na bazi žitarica	Sirovi ekstrakt	UHPLC-APCI-QqQ-MS/MS	Queslati i sar. (2012)
11	aflatoksini, ZON, OTA, DON, fumonizini, T-2, HT-2	Pšenično brašno	Sirovi ekstrakt	UHPLC-HESI-QqQ-MS/MS	Škrbić i sar. (2012)
5	Aflatoksini i OTA	Začinska paprika i biber	Sirovi ekstrakt	UHPLC-HESI-QqQ-MS/MS	Škrbić i sar. (2013a)
10	Aflatoksini, ZON, OTA, fumonizini, T-2, HT-2	Orah, badem, kikiriki i lešnik	Sirovi ekstrakt	UHPLC-HESI-QqQ-MS/MS	Škrbić i sar. (2014)

ADON – acetil deoksinivalenol, 3-ADON – 3 acetil deoksinivalenol, ALT - alternariol, AME – alternariol metil eter, AOH – alternuen, APCI – hemijska jonizacija, APPI - fotojonizacija, BEA – beauvericin, CIT – citrinin, DAS – diacetoksiskirpenol, DART – direktna analiza u realnom vremenu, DON-deoksinivalenol,

DON-3-Glc – deoksinivalenol-3-glukozid, FLD – fluorescentni detektor, FUS-X – fuzarenon X, ENN – eniatin, ENN B – eniantin B, ERG – ergot alkaloid, ESI – elektrosprej jonizacija, HESI - grejana elektrosprej jonizacija, HPLC – visoko pritisna tečna hromatografija, HT-2 – HT-2 toksin, IA – imunoafinitetne kolone, LC – tečna hromatografija, MON – moniliformin, MS – masena spektrometrija, MS/MS – tandemska masena spektrometrija, NIV-nivalenol, OTA – ohratoksin, PAT – patulin, QIT – kvadrupol ion trap, QuEChERS - brza, laka, jeftina, efikasna, robustna i sigurna priprema uzorka, QqQ – tripl kvadrupol, STE – sterigmatocistein, SPE – ekstrakcija na čvrstoj fazi, T-2 – T-2 toksin, TISP – „turbo ion spray" jonski izvor, TOF – „analajzer na bazi vremena preleta", UHPLC – ultra pritisna tečna hromatografija, UV – UV detektor, ZON – zearalenon, α-ZOL – α-zearalenol

LC/MS „multi-toksin" metode uglavnom su razvijene za ciljane tzv. target analite odnosno za identifikaciju i kvantifikaciju mikotoksina od interesa. Većina „multi-toksin" metoda namenjena je za određivanje mikotoksina u poljoprivrednim proizvodima, gde se oni najčešće javljaju, a to su žitarice, kukuruz, pirinač, soja, orasi, začini, sušeno voće, sveže povrće, hrana za životinje, i itd. (Sulyok i sar., 2006; Boonzaaijer i sar., 2008; Spanjer i sar., 2008; Garrido Frenich i sar., 2009; Monbaliu i sar., 2009; Desmarchelier i sar., 2010, Škrbić i sar., 2011a, 2012, 2013a, 2014). „Multi-toksin" metode obuhvataju one toksine za koje su definisane maksimalno dozvoljene količine ili se planira njihovo određivanje Regulativama Evropske unije. Iako, se neki toksini mogu odrediti samo kvalitativno, metode koje se primenjuju za njihovu identifikaciju imaju važnu ulogu zbog dobijanja informacija o kvalitetu proizvoda u vrlo kratkom vremenskom roku, kao i kada je potrebno identifikovati faktore koji dovode do zagađenja hrane i hrane za životinje, odnosno odrediti poreklo toksina. Primenjivost ovih metoda moguća je kroz tzv. "skrining" mikotoksina u namirnicama kao što su hleb, sir, orasi, džem i vino (Sulyok i sar., 2010).

2.3.2.1. Metode pripreme uzoraka u analizi mikotoksina

Priprema uzoraka pre LC/MS analize, odnosno korak ekstrakcije predstavlja veliki izazov u „multi-toksin" analizi zbog hemijske različitosti analita koje treba ekstrahovati iz uzorka (Lattanzio i sar., 2007) i dobijanja adekvatne vrednosti „recovery" za sve mikotoksine od interesa (Sulyok i sar., 2006). Na osnovu „multi-toksin" metoda prikazanih u tabeli 2.4 jasno se uočava da apsolutni trend u pripremi uzoraka pripada sirovom ili razblaženom ekstraktu (Sulyok i sar., 2006, 2010; Beltrán i sar., 2009; Garrido Frenich i sar., 2009; Herebian i sar., 2009; Škrbić i sar., 2011a; Queslati i sar., 2012; Škrbić i sar., 2012, 2013a, 2014); ali suprotno PhAC jedinjenjima za čije izolovanje iz uzoraka se najviše primenjuje SPE, u slučaju mikotoksina pored sirovog ekstrakta koriste se i drugi načini pripreme uzoraka. Prednost pripreme sirovog ekstrakta je mogućnost ekstrakcije više mikotoksina u jednom koraku, jednostavnost analitičkog postupka, vremenska i ekonomska opravdanost. U slučaju sirovog ekstrakta, korak odmašćivanja sa heksanom izvodi se istovremeno sa ekstrakcijom uzorka u cilju smanjenja količine lipida u krajnjem ekstraktu uzorka. Ovo je jedini korak prečišćavanja ekstrakta uzorka, nakon čega se ekstrakt može upariti, a potom rekonstituisati u rastvaraču koji je kompatibilan sa korišćenim LC mobilnim fazama. Tako, na primer, ekstrakti žitarica ili hrane za životinje odmah se injektiraju i analiziraju LC/MS posle ekstrakcije, ekstrakcije i odmašćivanja ili nakon koncentrisanja i rekonstituisanja (Garrido Frenich i sar., 2009; Herebian i sar., 2009; Capriotti i sar., 2010; Škrbić i sar., 2011, 2012, 2013a, 2014).

Nedostatak ovog načina ekstrakcije je izolovanje i „neciljanih“, odnosno neželjenih jedinjenja iz uzorka koje ometaju pouzdanu identifikaciju, prouzrokujući intenzivan uticaj matriksa i kontaminaciju instrumenta (Garrido Frenich i sar., 2009). Uticaj matriksa na signal ispitivanih analita može se smanjiti razblaživanjem krajnjeg ekstrakta uzorka vodom pre početka instrumentalne analize (Herebian i sar., 2009; Škrbić i sar., 2011, 2012, 2013a). Dodatno, matriks efekat može se kompenzovati korišćenjem matriks standard kalibracija (Sulyok i sar., 2006).

Nekoliko autora ukazalo je da je potreban visok procenat metanola ili acetonitrila (>75%) u ekstrakcionoj smesi za ekstrakciju većine mikotosina, kao što su aflatoksini, ZON, OTA, trihoteceni (Sulyok i sar., 2006; Lattanzio i sar., 2007; Ren i sar., 2007; Spanjer i sar., 2008; Martos i sar., 2010), dok se efikasna ekstrakcija fumonizina postiže povećanjem udela vode i/ili smanjenjem pH vrednosti ekstrakcione smese (Škrbić i sar., 2014). U poslednjoj deceniji istraživanja većina razvijenih „multi-toksin“ metoda za istovremenu ekstrakciju hemijski različitih mikotoksina koristi smesu acetonitrila/vode (Sulyok i sar., 2006; Beltrán i sar., 2009; Garrido Frenich i sar., 2009; Škrbić i sar., 2011a, 2012, 2013a). Najbolji rezultati dobijeni su za 34 hemijski različita mikotoksina iz pšenice sa visokim procentom acetonitrila (Sulyok i sar., 2006), jer su „recovery“ vrednosti bile između 87 i 111% sa smesom acetonitrila/vode (75/25, v/v) sa izuzetkom za PAT i fumonizin (čiji „recovery“ je od 17-35%), dok su sa smesom metanola/vode (50/50, v/v) dobijene različite „recovery“ vrednosti od 27 do 111% („recovery“ vrednosti za PAT i ZON-4-glukozidaze bile su izvan pomenutog opsega).

„Multi-toksin“ metoda ima i svojih nedostataka, koji se pre svega odnose na pomeranje granice kvantifikacije (tj. mogu biti neprihvatljive u odnosu na maksimalno dozvoljene vrednosti definisane postojećim regulativama) usled izostavljanja koraka prečišćavanja ekstrakta uzorka (Sulyok i sar., 2006). Takođe, jedan od nedostataka je „zaprljanje“ LC/MS instrumenta. Na primer, postepeno povećanje signala MS zbog „zaprljanja“ instrumenta uočili su Sulyok i sar. (2007b) usled uzastopnog injektiranja razblaženog ekstrakta žitarica. Sa druge strane, Herebian i sar. (2009) dokazali su da uprkos uočenom pogoršanju izgleda hromatograma, preko 200 injekata neprečišćenih ekstrakata žitarica nije dovelo do pada osetljivosti instrumenta. Neki istraživači su da bi izbegli varijacije MS signala prouzrokovane kontaminacijom usled „prenosa“ uzoraka, uveli korak ispiranja instrumenta odgovarajućim rastvaračom između svakog analitičkog ciklusa (Rundberget i Wilkins, 2002; Rasmussen i sar., 2010).

Analiza neprečišćenog ekstrakta uzorka je praktičan pristup pri određivanju većeg broja hemijski različitih mikotoksina u okviru samo jednog analitičkog ciklusa (Sulyok i sar., 2006). Dodatno, pomenuti pristup omogućava lako uvođenje novih analita i uzoraka, pod predpostavkom da su prethodno optimizovana ekstrakcija i hromatografski uslovi primenjivi (Sulyok i sar., 2007a; 2007b). Na primer, Sulyok i sar. (2006; 2007a; 2010) postepeno su proširivali svoj metod sa prvobitnih 39 na 106 mikotoksina u uzorcima žitarica, a potom su razvijeni metod primenili na druge namirnice kao što su med, džem i vino. Dodatno, „skrining“ nepoznatih metabolita jedino je moguć analizom neprečišćenog ekstrakta uzoraka, jer u suprotnom usled prečišćavanja može doći do gubitka jedinjenja od potencijalnog interesa (Herebian i sar., 2009).

Jednostavnost, brzina analize i količina informacija dobijena iz samo jednog analitičkog ciklusa često se navode kao važni ciljevi „multi-rezidualnih“ metoda, a to je moguće samo direktnom analizom sirovog ekstrakta ispitivanog uzorka (Mol i sar., 2008; Garrido Frenich i sar., 2009; Martos i sar., 2010; Škrbić i sar., 2011, 2012, 2013a, 2014) primenom novo razvijenih spomenutih spregnutih tehnika.

Sa druge strane, neki istraživači u cilju izbegavanja neželjenog uticaja matriksa i „neciljanih“ komponenata primenjuju korak prečišćavanja („*clean-up*“) ekstrakta uzorka (Laganà i sar., 2003; Tanaka i sar., 2006; Lattanzio i sar., 2007; Ren i sar., 2007; Zachariasova i sar., 2010; Santos i sar., 2010; Beltrán i sar., 2011; Garrido Frenich i sar., 2011). Procedure pripreme koje obuhvataju korak prečišćavanja ekstrakta uzorka podrazumevaju različite tipove SPE i brzu, jednostavnu, jeftinu, efikasnu, robustnu i sigurnu tzv. QuEChERS („*QuEChERS*“ je skraćenica od „quick, easy, cheap, effective, rugged and safe“) ekstrakciju.

Uobičajeno korišćene SPE kolone su sa obrnutim i normalnim fazama i jono izmenjivačke, a u poslednje vreme veliku primenu našle su i Multisep® i Mycosep® (Römer Labs, Tulln, Austria) kolone kao i specifične imunoafinitetne kolone (IA) (Tanaka i sar., 2006; Ren i sar., 2007; Lattanzio i sar., 2007). Imunoafinitetne kolone zasnovane su na imunosorbentima koji sadrže imobilisana antitela koja vezuju specifične analite čime se obezbeđuje vrlo selektivan postupak izolovanja jedinjenja od interesa iz ekstrakta uzorka.

Multisep® i Mycosep® (Römer Labs, Tulln, Austria) SPE kolone namenjene su za prečišćavanje ekstrakata uzoraka hrane i hrane za životinje za analizu selektovanih grupa mikotoksina i njihova primenjivost u „multi-toksin“ analizi je dokazana. Multisep 226® i Mycosep 226® Aflazon+ kolone korišćene su u određivanju 13 i 17 mikotoksina u proizvodima na bazi žitarica i u kikiriki buteru (Tanaka i sar., 2006; Ren i sar., 2007). Prema proizvođačkim specifikacijama, kolone koje sadrže sorbent baziran na siliki imaju i polarnu i nepolarnu funkcionalnost, odnosno sposobnost da vežu (uklone) interferirajuće komponente iz uzorka, dok selektovani mikotoksini kao što su aflatoksini, ZON, PAT i trihoteceni nesmetano prolaze kroz kolonu. Pri korišćenju ovih kolona, deo ekstrakta uzorka u 85% acetonitrilu nanosi se na kolonu čime se postiže adsorpcija ometajućih jedinjenja iz uzorka i direktno vrši eluiranje aflatoksina, ZON i trihotecena (Tanaka i sar., 2006; Ren i sar., 2007). Tanaka i sar. (2006) i Ren i sar. (2007) pokazali su da u obe metode primena pomenutih kolona omogućava „recovery“ vrednosti ≥70,6% za sve mikotoksine od interesa.

Lattanzio i sar. (2007) razvili su „multi-toksin“ metod za određivanje 11 mikotoksina (aflatoksini, OTA, fumonizini, DON, ZON, T-2 i HT-2 toksin) iz kukuruza, nakon primene multifunkcionalne IA Myco6in1® (Vicam/Waters corp., Manchester, UK) kolone koja sadrže antitela aflatoksina, OTA, fumonizina, DON, ZON, T-2 i HT-2 toksin. U ovom istraživanju, Lattanzio i sar. (2007) primenili su duplu ekstrakciju odnosno, prvi korak ekstrakcije bio je sa rastvorom fosfatnog pufera (PBS), a drugi metanol/voda (80/20, v/v), nakon čeka je primenjen korak prečišćavanja. Analiti su u smesi PBS (rastvor fosfatnog pufera):metanola naneti na kolonu, a potom eluirani sa antitela kolone čistim metanolom. Lattanzio i sar. (2007) ukazali su da uneta zapremina i udeo metanola u IA kolonu mogu imati negativan uticaj na uspešno izolovanje DON. Poznato je da su antitela u IA sorbentima osetljiva na organske

rastvarače i to ukazuje na činjenicu da metanol prisutan u prvom koraku nanošenja ekstrakta uzorka na kolonu delimično denaturiše antitela DON, što dovodi do nezadovoljavajućeg vezivanja pomenutog analita (Lattanzio i sar., 2007). Dobijene „recovery" vrednosti za ispitivane analite nakon optimizovane ekstrakcije i prečišćavanja bile su u opsegu od 79 do 104%.

U poslednjih nekoliko godina, nova preparativna tehnika tzv. QuEChERS metod primenjuje se u „multi-toksin" metodama (Zachariasova i sar., 2010; Garrido Frenich i sar., 2011), iako je prvobitno razvijena za istovremenu ekstrakciju pesticida u širokom opsegu polarnosti (polarni i nepolarni pesticidi) iz uzoraka voća i povrća (Anastassiades i sar., 2003). QuEChERS metoda bazira se na ekstrakciji (obično sa acetonitrilom) analita i istovremenom razdvajanju organske i vodene faze, dodatkom soli i puferovanih jedinjenja (npr. citrata) koji podstiču prelazak analita u organsku fazu koja se potom prečišćava disperzivnom ekstrakcijom na čvrstoj fazi (d-SPE) primenom izabranog PSA sorbenta („*primary secondary amine*"-PSA) (Lehotay i sar., 2010). Razdvajanje pomenutih faza i deljenje analita između njih (tj. postizanje zadovoljavajućih „recovery" vrednosti) optimizuje se sa vrstom i količinom dodate soli (Anastassiades i sar., 2003).

U „multi-toksin" metodama, koriste se modifikovane QuEChERS procedure koje obuhvataju ekstrakciju analita i korak razdvajanja organske i vodene faze, ali ne obuhvataju korak prečišćavanja sa d-SPE. Dakle, mikotoksini se iz uzoraka ekstrahuju sa acetonitrilom koji je zakišeljen da bi se omogućila ekstrakcija fumonizina (Zachariasova i sar., 2010). Razdvajanje organske i vodene faze ekstrakta uzorka i prelazak mikotoksina u organsku fazu vrši se dodatkom 4 g magnezijum sulfata ($MgSO_4$) ili 4 g $MgSO_4$ i 1 g natrijum hlorida (NaCl) (Vaclavik i sar., 2010; Zachariasova i sar., 2010), što je u skladu sa originalnom QuEChERS procedurom optimizovanom za pesticide (Anastassiades i sar., 2003).

2.3.2.2. Instrumentalne metode analize mikotoksina

Savremena analiza mikotoksina u velikoj meri se oslanja na primenu U/HPLC-MS/MS. Prethodno uobičajeno korišćene metode za kvantitativno određivanje mikotoksina su: tankoslojna hromatografija (TLC), HPLC u kombinaciji sa UV, fluorescentnim (FLD) ili MS i GC u kombinaciji sa elektron apsorbujućim, plameno jonizacionim (FID) ili MS detektorom. Zbog slabe isparljivosti mikotoksina, njihovo određivanje GC/MS (Krska i sar., 2001; Melchert i Pabel, 2004; Schollenberger i sar., 2008) moguće je samo nakon odgovarajućeg koraka derivatizacije. Dodatno, imunoadsorpcioni enzimski test (ELISA) se koristi u svrhe brze „skrining" analize mikotoksina sa nedostatkom dobijanja „lažno"-pozitivnih rezultata i ponekad, neprihvatljive tačnosti, stoga zahteva dodatnu analizu potvrđivanja („*confirmation*") drugom tehnikom (Sforza i sar., 2006; Ren i sar., 2007). U novije vreme prema srpskom standardu SRPS EN 15891 (en) (Službeni glasnik RS, br. 56/2012) određivanje sadržaja DON u žitaricama i proizvodima od žitarica se bazira na HPLC/UV tehnici, a određivanje sadržaja AFB1 i ukupnog sadržaja AFB1, AFB2, AFG1 i AFG2 u žitaricama, jezgrastom voću i njihovim proizvodima na HPLC/FLD tehnici (SRPS EN ISO 16059 (en), Službeni glasnik RS,

br. 56/2012); pri čemu, ove tehnike zahtevaju specifičnu pripremu uzoraka tj. dobijeni ekstrakti se prečišćavaju skupim imunoafinitetnim kolonama koje su selektivne samo za pomenute mikotoksine i ne omogućavaju izolovanje drugih mikotosina od interesa.

Da bi se obezbedilo poštovanje važećih evropskih i nacionalnih regulativa neophodno je razviti pouzdane i tačne „multi-toksin“ analitičke metode koje će omogućiti nedvosmislenu identifikaciju i preciznu kvantifikaciju mikotoksina u veoma niskim koncentracionim nivoima. U poslednjih nekoliko godina, U/HPLC u kombinaciji sa masenim analizatorom koji je baziran na kvadrupolu tj. QqQ postala je univerzalna i prioritetna tehnika u analizi mikotoksina (Laganà i sar., 2003; Ren i sar., 2007; Beltrán i sar., 2009; Herebian i sar., 2009; Beltrán i sar., 2011; Garrido Frenich i sar., 2011; Škrbić i sar., 2011a; Oueslati i sar., 2012; Škrbić i sar., 2012, 2013a, 2014). Tri najčešće korišćena API jonizaciona izvora u „multi-toksin“ analizama su: ESI uključujući H-ESI (Škrbić i sar., 2011a, 2012, 2013a, 2014), APCI i APPI (Sulyok i sar., 2006; Tanaka i sar., 2006; Capriotti i sar., 2010). Takođe, treba reći da su to tzv. „meke“ jonizacione tehnike.

Danas, pored najčešće korišćenog masenog analizatora QqQ u analizi mikotoksina se koristi i kvadrupl „ion trap“ (QIT) (Sulyok i sar., 2006; Lattanzio i sar., 2007; Capriotti i sar., 2010). U literaturi su dostupne metode za analizu mikotoksina koje se baziraju samo na jednom masenom visokorezolucionom analizatoru (HRMS) (Tanaka i sar., 2006; Herebian i sar., 2009; Zachariasova i sar., 2010). Za detekciju jedinjenja od interesa MS detektorima najvažnije je postići odgovarajuće uslove za efikasnu jonizaciju analita, radi nastajanja jona koji mogu da proizvedu dovoljno jake signale za uspešnu kvantifikaciju. Dodatno, negativan efekat na signal jedinjenja od interesa mogu da imaju i ostala jedinjenja ispitivanog uzorka tzv. matriksa koji treba ispitati i kompenzovati u svakom pojedinačnom slučaju.

2.4. Teški elementi

2.4.1. Osobine i rasprostranjenost

U protekle dve decenije pojam teški elementi („*heavy elements*“) naišao je na široku upotrebu. Često se koristi kao grupno ime za metale i metaloide i povezano je sa zagađenjem zemljišta, vode, biljaka, a samim tim i lanca ishrane. Međutim, u dostupnoj relevantnoj literaturi ne postoji jasna definicija ovog pojma od strane autorizovanih tela kao što je IUPAC, što je dovelo do konfuzije u pogledu značenja tog pojma. Preko 60 godina ovaj pojam se koristi za različite grupe elemenata, često slične, ali ne u potpunosti identične, bilo na osnovu njihove gustine (pri čemu su predložene različite granične vrednosti gustine za podelu elemenata na lake i teške), atomske mase, atomskog broja i drugih hemijskih osobina (na primer, reaktivnosti u odnosu na ditizone) ili toksičnosti (Duffus, 2002). Međutim, pojam teški metali ili teški elementi kada se pominju i metaloidi kao što je arsen (As) čine grupu neorganskih polutanata sa toksičnim efektima po zdravlje ljudi.

Teški elementi mogu se klasifikovati kao toksični (arsen, olovo, kadmijum, živa), potencijalno esencijalni (vanadijium, kobalt) i esencijalni (gvožđe, bakar, cink, mangan, itd.) (Zhu i sar., 2011). Toksični elementi su veoma štetni čak i u niskim koncentracijama kada se unose u organizam čoveka u dužem vremenskom periodu (Unak i sar., 2007). Esencijalni elementi mogu takođe biti toksični kada se unose u organizam u prekomernim dozama (Škrbić i sar., 2006; Gopalani i sar., 2007).

Visoke koncentracije teških elemenata u urbanim sredinama su posledica širokog spektra ljudskih aktivnosti kao i prirodnih geohemijskih procesa. Izvori teških elemenata u životnoj sredini su posledica antropogenih aktivnosti: industrijske kao što su topionice, fabrički kompleksi, obojena metalurgija, olovni benzin, itd. (Pisani i sar., 2008; Wang i sar., 2010). Kroz vlažnu i suvu atmosfersku depoziciju teški elementi prouzrokuju zagađenje svih delova životne sredine tj. zemljišta, vode i vegetacije. Zagađenje zemljišta teškim elementima koje se koristi u poljoprivredne svrhe je posledica atmosferskih padavina, primene pesticida, veštačkog đubriva i navodnjavanja vodom lošeg kvaliteta (Škrbić i Đurišić-Mladenović, 2013). Ekološka važnost metala u zemljištu usko je povezana sa zdravljem ljudi usled značajnog potencijala transfera (Morton-Bermea i sar., 2009), naročito kada je u pitanju zemljište urbanih sredina, gde je izloženost ljudi teškim elementima povećana, bilo putem udisanja, unosa preko digestivnog trakta ili kontakta preko kože (Wong i sar., 2006). Sa druge strane, voda i hrana su glavni izvor esencijalnih elemenata, ali takođe su i medijum kroz koji su ljudi izloženi različitim toksičnim elementima (Škrbić i sar., 2013b). Indrektne posledice kontaminacije životne sredine podrazumevaju migraciju metala putem gradske otpadne vode do vodenih sistema i organizama koji žive u njima, utičući na kvalitet vodenih organizama kroz bioakumulaciju[18] i biomagnifikaciju[19], potencijalno prouzrokujući kontaminaciju lanca ishrane. Lanac ishrane identifikuje se kao glavni izvor izloženosti ljudi toksičnim elementima u poređenju sa drugim načinima izlaganja kao što su inhalacija i kontakt preko kože (Qian i sar., 2010). Prisustvo metala u hrani zavisi od više faktora, kao što su: karakteristike zemljišta, genotip biljaka, korišćenih đubriva, primenjenih pesticida, kontaminacije usled procesa proizvodnje ili kontaminacije kao posledica korišćenja metalne opreme tokom proizvodnog procesa (Škrbić i sar., 2013b). Stoga, neophodno je proceniti nivo teških elemenata u uzorcima životne sredine i hrani u cilju ispitivanja stepena kontaminacije i štetnosti po zdravlje ljudi.

2.4.2. Metode pripreme uzoraka i instrumentalne metode analize teških elemenata

U cilju određivanja sadržaja teških elemenata u uzorcima životne sredine i hrane, neophodno je adekvatno pripremiti uzorke, odnosno ukloniti organsku materiju i dobiti kiseli rastvor sa neorganskim analitom. Priprema uzoraka vrši se razaranjem (digestijom), odnosno vlažnim i suvim putem. Ova dva načina pripreme koriste se za digestiju različitih vrsta uzoraka.

[18] Bioakumulacija – nakupljanje, koncentrisanje toksičnih jedinjenja u organizmima; javlja se kada je brzina unosa ovih supstanci u organizam veća od brzine procesa njihove razgradnje i/ili izlučivanja.

[19] Biomagnifikacija – povećanje koncentracije toksičnih jedinjenja u višim karikama lanca ishrane.

Digestija suvim putem je jednostavan postupak koji podrazumeva žarenje uzoraka na 500-600^{o}C u dužem vremenskom periodu; ne zahteva intenzivan laboratorijski rad i skupe hemikalije, a može se koristiti za analizu većeg broja uzoraka. Nedostaci ovog načina pripreme su dugo vreme pripreme od 12-24 h, i pri tome gubitak lako isparljivih elemenata kao što su: bakar (Cu), gvožđe (Fe), živa (Hg), nikal (Ni), cink (Zn), arsen (As).

Drugi način pripreme uzoraka je digestija vlažnim putem koja omogućava uklanjanje organske materije i istovremeno prevođenje metala od interesa u rastvor za analizu. Za pripremu uzoraka digestijom vlažnim putem, uzorak se odmerava u normalni sud u koji se dodaje jaka kiselina (azotna, perhlorna, sumporna) i/ili oksidaciono sredstvo (vodonik peroksid), a zatim se vrši zagrevanje (Todorović i sar., 1997). Vlažna digestija vrši se na dva načina: zagrevanjem u otvorenim posudama (klasična digestija) ili u zatvorenom sistemu, kao što je sistem za mikrotalasnu digestiju. Prednosti klasične digestije vlažnim putem su manji gubitak isparljivih elemenata zbog primene nižih temperatura u odnosu na digestiju suvim putem kao i kraći vremenski period pripreme; nedostaci ovog postupka su intenzivan laboratorijski rad, specijalne posude za pripremu uzoraka ako se koristi perhlorna kiselina zbog njene štetnosti po okolinu kao i priprema manjeg broja uzoraka po radnom ciklusu.

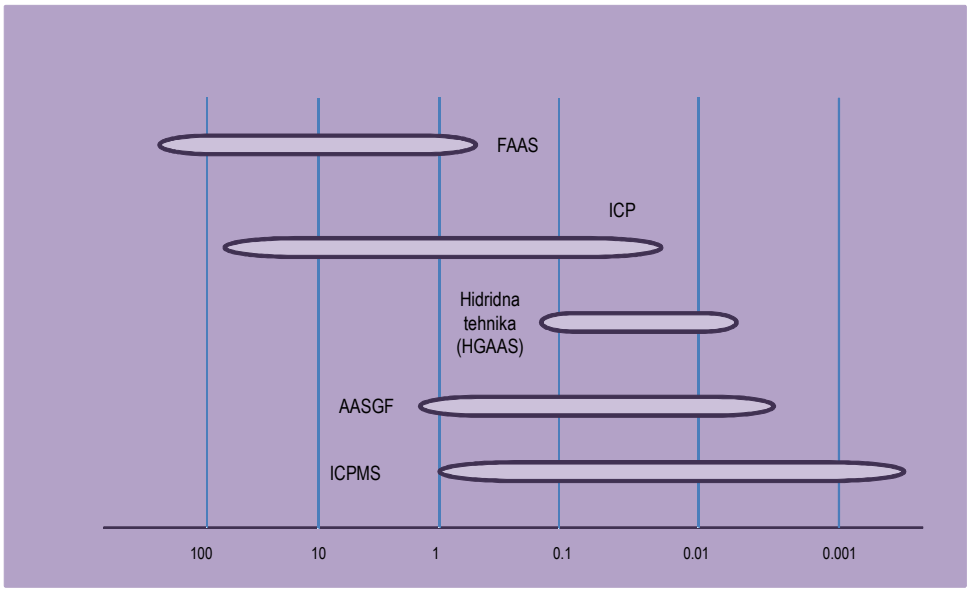
Poslednjih godina, mikrotalasna digestija koristi se za pripremu različitih vrsta uzoraka kao efikasna alternativa klasičnoj digestiji vlažnim putem. U mikrotalasnom sistemu, uzorak je u direktnom kontaktu sa kiselinom i/ili oksidacionim sredstvom u zatvorenoj posudi i digestija se vrši pod kontrolisanim uslovima visoke temperature i pritiska. Prednosti primene mikrotalasnih sistema u pripremi uzoraka su: smanjena mogućnost kontaminacije uzorka tokom digestije, minimalan gubitak lako isparljivih elemenata, smanjena potrošnja kiseline tokom pripreme uzorka i znatno kraće vreme digestije u poređenju sa tradicionalnim metodama. Efikasnost mikrotalasne digestije zavisi od više faktora kao što su temperaturni program, radni pritisak, zapremine i vrste korišćene kiseline za pripremu uzoraka.

Nakon digestije dobijeni rastvor može se analizirati u odnosu na sadržaj teških elemenata različitim tehnikama kao što su: plamena atomska apsorpciona spektrometrija (FAAS), atomska apsorpciona spektrometrija sa grafitnom kivetom (GFAAS), optička emisiona spektrometrija sa induktivno spregnutom plazmom (ICP-OES) i induktivno-kuplovana (spregnuta) plazma sa masenom spektrometrijom (ICP-MS). Ove instrumentalne tehnike dele se prema načinu dobijanja spektra na: optičke apsorpcione i optičke emisione metode. Optičke apsorpcione metode zasnivaju se na merenju smanjenja intenziteta zračenja usled apsorpcije pri prolasku kroz ispitivanu supstancu. Optičke emisione metode zasnivaju se na ispitivanju svetlosti koju emituje analizirana supstanca. Poređenje instrumentalnih tehnika za određivanje teških elemenata prikazano je u tabeli 2.5, dok je opseg granica detekcije za ove tehnike prikazan na slici 2.15 (Atomic Spectroscopy, A Guide to Selecting the Appropriate Technique and System, Perkin Elmer, 2009; www.perkinelmer.com/atomicspectroscopy).

Tabela 2.5. *Poređenje instrumentalnih tehnika za određivanje teških elemenata*

	FAAS	GFAAS	ICP-OES	ICP-MS
Broj elemenata				
jedan	■			
nekoliko		■		
mnogo			■	■
Nivo određivanja				
ppb	■		■	
> ppb		■	■	■
> ppb-ppm				■
> ppt				■
Broj uzoraka[a]				
malo	■	■		
mnogo			■	■
Zapremina				
> 5 ml	■	■	■	■
< 1-2 ml		■		

[a]Broj uzoraka predstavljen je relativno između tehnika koje se opisuju: s obzirom da su ICP multielementarne tehnike i omogućavaju istovremeno analizu većeg broja elemenata od interesa po uzorku, a time i značajno kraće vreme analize za veći broj uzoraka, AAS tehnike zbog pojedinačne analize svakog elemeta od interesa u svakom uzorku zahtevaju duže vreme analize.



Slika 2.15. *Opseg granica detekcije (µg/l ili ppb) za različite instrumentalne tehnike*

Plamena FAAS je manje osetljiva tehnika od GFAAS, jer osetljivost FAAS je u opsegu koncentracionih nivoa od ppm, dok GFAAS je znatno osetljivija tehnika namenjena detekciji

tragova elemenata od interesa odnosno za koncentracione nivoe od nekoliko ppb. Kada govorimo o zapremini uzorka koja se koristi za analizu, razlika između ove dve tehnike je značajna, jer FAAS zahteva zapreminu u ml, dok za GFAAS reč je o µl. Dodatno, FAAS je jeftinija tehnika i zahteva znatno niži nivo veštine i obučenosti analitičara u poređenju sa GFAAS. Vreme trajanja analize sa FAAS je kraće u poređenju sa GFAAS. Bez obzira na vrstu tehnike, i FAAS i GFAAS zahtevaju korišćenje šuplje katodne lampe za svaki analit od interesa.

U novije vreme na polju analize teških elemenata sve veću pažnju privlače ICP-OES i ICP-MS. Obe tehnike omogućavaju brzu i multielementarnu analizu odnosno istovremenu detekciju većeg broja elemenata od interesa. Za razliku od ICP-OES čija je granica detekcije u zavisnosti od ispitivanog matriksa i elemenata od interesa u opsegu od ppm-ppb, ICP-MS ima veću osetljivost, sa mogućnošću detekcije koncentracionih nivoa i ispod ppt nivoa. Veštine i obučenost analitičara za rad sa ICP-OES i ICP-MS su na višem nivou nego za FAAS i GFAAS, dok među ove četiri tehnike ICP-MS zahteva najveći stepen znanja i veštine od analitičara koji radi sa ovim instrumentom. Dodatno, ICP-MS je najskuplja tehnika od ovih instrumentalnih tehnika kao i troškovi održavanja (Lewen, 2011).

3. EKSPERIMENTALNI DEO

3.1. Uzorkovanje

Uzorci vode za analizu farmaceutski aktivnih komponenata

Za određivanje sadržaja PhAC u različitim tipovima vode, uzorci su uzeti sa više različitih lokacija na teritoriji Srbije. Uzorkovanje je izvršeno u proleće 2012. god. i obuhvaćeno je 25 lokacija (slika 3.1; Petrović i sar., 2014).

Uzeti su uzorci neprečišćene otpadne industrijske vode, gradske otpadne vode, podzemne, površinske i vode za piće. Opis uzetih uzoraka vode prikazan je u tabeli 3.1. Svi uzorci pripremljeni su kao kompozitni mešanjem više pojedinačno uzetih uzoraka. Uzorkovanje je izvršeno od strane javnog vodoprivrednog preduzaća „Vode Vojvodine“. Ručno uzorkovanje izvedeno je od strane inspektora koji su ovlašćeni za zvaničnu kontrolu vode. Za svaki tip vode, pet pojedinačnih uzoraka jednake zapremine (500 ml za površinsku, podzemnu i vodu za piće i 200 ml za otpadnu vodu) uzeto je diskontinualno (sa prekidima) u konstantnom vremenskom intervalu od 2 h i izmešano.

Uzorci vode stavljeni su u plastične boce od 500 ml, prethodno isprane sa ultra čistom vodom. Uzeti uzorci, od mesta uzorkovanja do laboratorije čuvani su na 4^{o}C i pripremljeni za analizu u roku od 48 h.

Slika 3.1. *Mapa Srbije sa lokacijama gde je izvršeno uzorkovanje: Novi Sad, Zrenjanin, Vrbas i Obrenovac (detaljan opis uzoraka dat je u tabeli 3.1.)*

Table 3.1. *Opis uzetih uzoraka vode sa različitih lokacija u Srbiji, u kojima je analizirano prisustvo PhAC komponenti*

Broj uzorka	Lokacija	Tip vode	Opis
1	Obrenovac	Otpadna voda	Neprečišćena otpadna voda iz farmaceutske industrije posle proizvodnje biljnog sirupa
2	Obrenovac	Otpadna voda	Neprečišćena otpadna voda uzeta tokom pranja opreme iz farmaceutske industrije
3	Novi Sad	Otpadna voda	Neprečišćena otpadna voda koja se direktno uliva u Dunav, Šangaj
4	Novi Sad	Površinska	Kanal, prigradsko naselje Adice
5	Novi Sad	Površinska	Kanal Dunav–Tisa–Dunav blizu Rafinerije nafte
6	Novi Sad	Površinska	Kanal Dunav–Tisa–Dunav dalje od Rafinerije nafte
7	Zrenjanin	Površinska	Reka Begej
8	Zrenjanin	Površinska	Malo jezero, Peskara
9	Novi Sad	Površinska	Reka Dunav, plaža Štrand uzvodno od centra grada
10	Novi Sad	Površinska	Reka Dunav, plaža Oficirac, nizvodno od centra grada
11	Novi Sad	Površinska	Kanal Bukovac
12	Bečej	Površinska	Reka Tisa
13	Vrbas	Površinska	Kanal-Dunav–Tisa-Dunav-I
14	Vrbas	Površinska	Kanal-Dunav–Tisa-Dunav-II
15	Novi Sad	Podzemna	Bunar na privatnom posedu, prigradsko naselje Adice-I
16	Novi Sad	Podzemna	Bunar na privatnom posedu, prigradsko naselje Adice-II
17	Novi Sad	Podzemna	Javna česma-Spens
18	Novi Sad	Podzemna	Javna česma, Limanski park
19	Novi Sad	Podzemna	Javna česma, Jodna Banja
20	Vrbas	Podzemna	Javna česma u parku
21	Novi Sad	Voda za piće	Česmenska voda
22	Novi Sad	Voda za piće	Sirova neprečišćena voda iz vodovoda
23	Novi Sad	Voda za piće	Hlorisana voda iz vodovoda
24	Zrenjanin	Voda za piće	Sirova neprečišćena voda iz vodovoda
25	Zrenjanin	Voda za piće	Hlorisana voda iz vodovoda

Uzorci za analizu 8-11 mikotoksina

Prisustvo mikotoksina analizirano je u sledećim uzorcima: ozimoj pšenici, pšeničnom brašnu i koštuničavom voću.

Za analizu *Fusarium* mikotoksina, uzimanje uzoraka ozime pšenice zasejane u oktobru 2006. god. i požnjevene tokom jula 2007. god. izvršeno je u okviru godišnjeg monitoringa Instituta za kukuruz, Zemun Polje. Uzorci pšenice različitih sorti uzeti su odmah nakon žetve iz 10 različitih žitnih regiona Srbije. Uzorci su pripadali sledećim sortama: Evropa 90, Renesansa, Pobeda, Kraljevica i Nora. Ukupno 54 reprezentativna uzorka ozime pšenice analizirana su na prisustvo *Fusarium* mikotoksina. U tabeli 3.2 prikazana je shema uzorkovanja sa brojem uzoraka različitih sorti ozime pšenice iz izabranih regiona. Ručno uzorkovanje izvedeno je žitnom sondom od strane inspektora koji su ovlašćeni za zvaničnu kontrolu žitarica. Zbog neujednačene distribucije mikotoksina u žitaricama, uzorkovanje je izvršeno prema zahtevima Regulative Evropske unije (401/2006/EC)[20]. Određeni broj pojedinačnih uzoraka (tabela 3.2) od oko 200 g uzeto je nasumično za svaku sortu i namešano (kombinovano) u kompozitne uzorke sorti od 3-9 kg za svaki region.

Tabela 3.2. *Broj uzoraka različitih sorti ozime pšenice uzete iz 10 regiona Srbije koji su namešani u cilju dobijanja kompozitnih uzoraka sorti i dalje anlizirani na prisustvo mikotoksina*

Region	Broj pojedinačnih (kompozitnih) uzoraka po sorti					Broj kompozitnih uzoraka po regionu [a]
	Evropa 90	Renesansa	Pobeda	Kraljevica	Nora	
Srem	51 (2)	45 (2)	50 (2)			6
Zapadna Bačka	59 (2)	50 (2)	52 (2)			6
Južna Bačka	77 (2)	54 (2)	64 (2)			6
Severna Bačka	64 (2)	65 (2)	71 (2)			6
Južni Banat	64 (2)	68 (2)	66 (2)			6
Srednji Banat	54 (2)	49 (2)	47 (2)			6
Severni Banat	75 (2)	61 (2)	68 (2)			6
Beograd	35 (2)	35 (2)	39 (2)			6
Zaječar				52 (2)	59 (2)	4

[20] Commission Regulation 401/2006/EC, OJ L 70, 9.3.2006., str. 15-33 (u daljem tekstu: Regulativa Evropske unije (EC, 2006)[20])

Nastavak tabele 3.2

Region	Broj pojedinačnih (kompozitnih) uzoraka po sorti					Broj kompozitnih uzoraka po regionu [a]
	Evropa 90	Renesansa	Pobeda	Kraljevica	Nora	
Toplica				25 (1)	24 (1)	2
UKUPNO						54

[a]Kompozitni uzorak svake sorte analiziran je i dobijeni rezultati korišćeni su za procenu regionalnih razlika u odnosu na prisustvo mikotoksina.

Petnaest uzoraka brašna kupljeno je u prodavnicama u Novom Sadu, tokom januara 2011. god. (Škrbić i sar., 2012) za analizu 11 osnovnih mikotoksina čiji sadržaj je definisan postojećim regulativama kao i oni za koje je utvrđeno da mogu biti prisutni u ovom tipu uzoraka. Pet različitih najzastupljenijih brendova izabrano je na osnovu njihovog udela u ukupnoj proizvodnji brašna u Srbiji. Pakovanja pšeničnog brašna korišćena za analizu su od 1000 g.

Sedamnaest uzoraka različitih vrsta koštuničavog voća (orah, lešnik, badem i kikiriki) uzeto je u februaru 2013. god. u Novom Sadu (Škrbić i sar., 2014) za analizu deset mikotoksina čiji sadržaj je definisan postojećim regulativama kao i oni za koje postoje indikacije da mogu biti prisutni u ovim vrstama uzoraka. Prema poreklu uzorci su razvrstani na „domaće“ (8 uzoraka oraha i 2 uzorka lešnika uzeti su sa privatnih poseda) i „komercijalne“ (1 uzorak oraha, 1 uzorak lešnika, 3 uzorka kikirikija i 2 uzorka badema kupljeni su u prodavnicama u Novom Sadu). Komercijalna pakovanja uzoraka su od 75 do 500 g.

Svi uzorci dopremljeni su na Tehnološki fakultet, Univerziteta u Novom Sadu i čuvani su na niskoj temperaturi i tamnom mestu. Neposredno pre analize uzorci su samleveni, izmešani i analizirani na prisustvo mikotoksina od interesa.

Uzorci za analizu teških elemanata u namirnicama potrošačke korpe Srbije

U januaru 2012. godine i martu 2013. godine kupljene su namirnice koje ulaze u sastav potrošačke korpe Srbije na osnovu podataka Statističkog zavoda Srbije (Statistički zavod, Republike Srbije, 2011). Namirnice su kupljene u različitim prodavnicama sa područja Novog Sada. Analizirano je ukupno 114 kompozitnih uzoraka. Svaki pojedinačni analizirani uzorak je predstavljao kompozitni, koji je dobijen namešavanjem tri uzorka istog brenda kupljenog u različitim prodavnicama. Za svaku ispitivanu namirnicu analizirana su tri kompozitna uzorka tri različita brenda. U okviru ovog istraživanja (Škrbić i sar., 2013b) analizirane su sledeće prehrambene grupe: povrće, voće, ulja i masti, slatkiši, mleko/mlečni proizvodi i jaja, meso i proizvodi na bazi mesa, riba, pšenica i proizvodi na bazi pšenice i ostali proizvodi. U tabeli 3.3 prikazane su analizirane grupe namirnica iz potrošačke korpe Srbije (Statistički zavod, Republike Srbije, 2011) i potrošnja svake od analiziranih namirnica u g/danu.

Tabela 3.3. *Namirnice obuhvaćene potrošačkom korpom Srbije*

Prehrambena grupa (udeo u ukupnoj potrošačkoj korpi Srbije, %)	Namirnice svrstane u prehrambene grupe		Ukupan % ispitivanih namirnica u datoj prehrambenoj grupi
		Potrošnja (g/dan)	
Voće (8,6%)	Jabuka	91,4	95,9
	Pomorandža	14,0	
	Banana	16,1	
	Orasi	3,2	
	Suve šljive	1,1	
Povrće (25,5%)	Salata	18,3	66,3
	Kupus	47,3	
	Pasulj	16,1	
	Šargarepa	17,2	
	Krompir	139,8	
	Luk	30,1	
	Šampinjoni	5,4	
Ulja i masti (3,0%)	Ulje	32,3	83,3
	Margarin	5,4	
Slatkiši (1,2%)	Keks	9,1	75,8
	Čokolada	2,2	
	Bomboni	2,2	
Mleko/mlečni proizvodi i jaja (22%)	Mleko	145,2	87,5
	Jogurt	69,9	
	Meki sir	32,3	
	Tvrdi sir	3,2	
	Jaja	43,0	
Meso i proizvodi na bazi mesa (9,9%)	Juneće meso	7,5	92,2
	Svinjsko meso	43,0	
	Pileće meso	48,4	
	Slanina	5,4	
	Kobasica	10,8	
	Viršla	4,8	
	Salama	15,1	
	Pašteta	4,3	
Pšenica i proizvodi na bazi pšenice (25,4%)	Beli hleb	266,7	88,5
	Drugi tipovi hleba	17,8	
	Pasta	10,8	
	Pšenično brašno	48,4	
Riba (0,9%)	Morska riba oslić	11,8	100
	Riba u konzervi (sardine u ulju)	2,2	
Drugi proizvodi (3,7%)	Začinska paprika	1,6	72,4
	Šećer	37,6	

Sve namirnice pre početka pripreme obrađene su kao što se to čini u domaćinstvu, tj. meso je očišćeno od kosti i kože, krompir i korenasto povrće očišćeni su od kore, itd. Pre analize, svaki uzorak je samleven i homogenizovan korišćenjem laboratorijskog mlina (A11 Basic, IKA, Nemačka). Do početka analize, uzorci su čuvani u njihovoj originalnoj ambalaži na 4^{o}C.

3.2. Hemikalije

U tabeli 3.4 prikazani su korišćeni standardni rastvori PhAC komponenti visokog stepena čistoće (>90%) .

Tabela 3.4. *PhAC komponente klasifikovane po terapeutskim grupama i izotopski označeni unutrašnji standardi korišćeni za njihovu kvantifikaciju (označeni deuterisani standardi)*

Terapeutska grupa	PhAC	Broj	CAS broj	Izotopski označeni unutrašnji standard
Analgetici/anti-inflamatori (14)	Ketoprofan	1	22071-15-4	Ibuprofen-d_3
	Naproksen	2	22204-53-1	
	Ibuprofen	3	15687-27-1	
	Diklofenak	4	15307-79-6	
	Idometacin	5	53-86-1	Idometacin-d_4
	Acetaminofen	6	103-90-2	Acetaminofen-d_4
	Salicilna kiselina		69-72-7	
	Fenazon	8	60-80-0	Fenazon-d_3
	Propifenazon	9	479-92-5	
	Piroksikam	10	36322-90-4	Meloxicam-d_3
	Tenoksikam	11	59804-37-4	
	Meloksikam	12	71125-39-8	
	Oksikodon	13	124-90-3	Karbamazepin-d_{10}
	Kodein	14	76-57-3	
Regulatori nivoa holesterola (statini) (5)	Bezafibrat	15	41859-67-0	Bezafibrat-d_6
	Gemfibrozil	16	25812-30-0	Gemfibrozil-d_6
	Pravastatin	17	81131-70-6	
	Fluvastatin	18	93957-54-1	
	Atorvastatin	19	134523-03-8	

Nastavak tabele 3.4

Terapeutska grupa	PhAC	Broj	CAS broj	Izotopski označeni unutrašnji standard
Lekovi koji deluju na centralni nervni sistem (15)	Karbamazepin 2-Hidroksikarbamazepin[a] 10,11-epoksiarbamazepin[a] Akridon[a] Olanzapin	20 21 22 23 24	298-46-4 68011-66-5 36507-30-9 578-95-0 132539-06-1	Karbamazepin-d_{10}
	Sertralin	25	79559-97-0	Fluoksetin-d_5
	Citalopram	26	59729-32-7	Citalopram-d_4
	Venlafaksin	27	99300-78-4	Venlafaksin-d_6
	Trazodon Fluoksetin Norfluoksetin[a] Paroksetin	28 29 30 31	25332-39-2 56293-78-7 83891-03-6 110429-35-1	Fluoksetin-d5
	Diazepam Lorazepam Alprazolam	32 33 34	439-14-5 846-49-1 28981-97-7	Diazepam-d5
Histamin H1 i H2 (5)	Loratadin Desloratadin[a] Ranitidin Famotidin Cimetidin	35 36 37 38 39	79794 100643-71-8 66357-59-3 76824-35-6 51481-61-9	Cimetidin-d_3
β-blokatori (6)	Atenolol Sotalol Propranolol Metoprolol Nadolol Karazolol	40 41 42 43 44 45	29122-68-7 959-24-0 318-98-9 51384-51-1 42200-33-9 57775-29-8	Atenolol-d_7
Diuretik (3)	Hidrohlorotijazid	46	58-93-5	Hidrohlorotijazid-d_2
	Furosemid Torasemid	47 48	54-31-9 56211-40-6	Furosemid-d_5
Antidijabetici (1)	Glibenclamid	49	10238-21-8	Gilburid-d_5
Antihipertenzivi (4)	Amlodipin Lozartan	50 51	111470-99-6 124750-99-8	Amlodipin-d_4
	Irbesartan Valsartan	52 53	137862-53-4 138402-11-6	Valsartan-d_8
Antitrombotici (1)	Klopidogrel	54	135046-48-9	Gilburid-d_3
Lekovi za hiperplaziju prostate (1)	Tamsulosin	55	106463-17-6	Sulfametaksazol-d_4
Lekovi za lečenje astme (1)	Salbutamol	56	51022-70-9	Atenolol-d_7
Antikoagulant (1)	Varfarin	57	81-81-2	Varfarin-d_5

Nastavak tabele 3.4

Terapeutska grupa	PhAC	Broj	CAS broj	Izotopski označeni unutrašnji standard
Kontrastno sredstvo u radiologiji (X-zraci) (1)	Jopromid	58	73334-07-3	Sulfametaksazol-d_4
Antihelmentici (3)	Albendazol	59	54965-21-8	Ronidazol-d_3
	Tiabendazol	60	148-79-8	
	Levamizol	61	16595-80-5	
Sintetički glukokortikoid (1)	Deksametazon	62	50-02-2	Deksametazon-d_4
Lek za sedaciju i relaksaciju mišića (1)	Ksilazin	63	7361-61-7	Ksilazin-d_6
Sredstvo za smirenje (2)	Azaperon	64	1649-18-9	Azaperon-d_4
	Azaperol[a]	65	2804-05-9	
Antibiotici (13)	Eritromicin	66	59319-72-1	Eritromicin-N,N$^{13}C_2$
	Azitromicin	67	83905-01-5	Azitromicin-d_3
	Klaritromicin	68	81103-11-9	
	Tetraciklin	69	64-75-5	Sulfametaksazol-d_4
	Sulfametaksazol	70	723-46-6	
	Trimetoprim	71	738-70-5	
	Ofloksacin	72	82419-36-1	Ofloxacin-d_3
	Ciprofloksacin	73	85721-33-1	
	Metronidazol	74	443-48-1	Ronidazol-d_3
	Metronidazol-OH[a]	75	4812-40-2	
	Dimetridazol	76	551-92-8	
	Ronidazol	77	7681-76-7	
	Cefaleksin	78	15686-71-2	
Blokatori kalcijumovih kanala (3)	Diltiazem	79	42399-41-7	Karbamazepin-d_{10}
	Verapamil	80	152-11-4	Verapamil-d_6
	Norverapamil[a]	81	67812-42-4	

[a]Metaboliti

Pojedinačni standardni rastvori PhAC komponenti kao i izotopski označeni unutrašnji standardi rastvoreni su u metanolu. Posle pripreme standardi su čuvani na -20°C. Standardni rastvori antibiotika pripremani su mesečno zbog njihove ograničene stabilnosti, dok standardni rastvori ostalih PhAC pripremani su na tri meseca. Smesa svih PhAC pripremljena je kroz odgovarajuća razblaženja pojedinačnih standardnih rastvora u metanolu-vodi (10:90, v/v). Izotopski označeni unutrašnji standardi, korišćeni za kvantifikaciju PhAC komponenti metodom unutrašnjeg standarda, pripremljeni su u metanolu, a zatim razblaženi u smesi metanola-vode (10:90, v/v).

Pojedinačni standardni rastvori mikotoksina AFB1 (2 µg/ml), AFB2 (0,5 µg/ml), AFG1 (2 µg/ml), AFG2 (0,5 µg/ml), OTA (1000 µg/ml), HT-2 toksin (100 µg/ml), T-2 toksin (100 µg/ml), DON (100 µg/ml), DON-3-Glc (100 µg/ml), NIV (100 µg/ml), FUS-X (100 µg/ml), ADON (100 µg/ml), ZON (100 µg/ml), FB1 i FB2 (50 µg/ml) korišćeni za analizu kupljeni su od Supelco Co. (Bellefonte, PA) i Sigma-Aldrich (Nemačka). Standardni rastvori mikotoksina rastvoreni

su u acetonitrilu i čuvani na - 20^{o}C u tamnim vijalima. Radni standardni rastvori pripremljeni su od prethodno pomenutih standardnih rastvora u acetonitrilu i njihova razblaženja korišćena su za pripremu kalibracionih standarda spoljašnje matriks standard kalibracije.

Za analizu PhAC i mikotoksina korišćeni su: metanol, acetonitril, amonijum acetat (svi LC-MS kvaliteta) kupljeni od J.T. Baker (Deventer, Holandija); amonijum formijat i amonijak kupljeni od Sigma-Aldrich (Steinham, Nemačka); glacijalna sirćetna kiselina (p.a. kvaliteta) kupljena od LTG Promochem (Wesel, Nemačka); mravlja kiselina (98%) kupljena od Merka (Darmstadt, Nemačka); heksan (HPLC kvaliteta, ≥98.5%) kupljen od Sigma-Aldrich (Hamburg, Germany). Stepen čistoće azota koji je korišćen za uparavanje ekstrakta uzoraka vode za analizu PhAC je 99,9990% (Abello Linde S.A, Španija). Ultra-čista voda (stepena čistoće I, električne otpornosti 18,2 MΩ/cm) dobijena je sistemom za prečišćavanje Milli-Q (Millipore, Molsheim, Francuska).

Sve hemikalije korišćene za određivanje sadržaja teških elemenata u ispitivanim uzorcima su visokog stepena čistoće. Koncentrovana 69% azotna kiselina (ccHNO_3) (za analizu metala u tragovima), 30% vodonik peroksid (H_2O_2) i standardni rastvori As, Cd i Pb (1000 µg/ml) kupljeni su od J.T.Baker. Pre početka analize teških elemenata, plastično i stakleno posuđe prvo je potopljeno u 20% hlorovodoničnu kiselinu (preko noći), zatim u 20% azotnu kiselinu (preko noći), i na kraju isprano ultra čistom vodom. Ultra čista voda (stepena čistoće I, električne otpornosti 18,2 MΩ/cm, Simplicity, Millipore, Francuska) korišćena je za pripremu standardnih rastvora i razblaženja rastvora uzoraka nakon digestije.

Radni standardni rastvori od 1 µg/ml za svaki element pripremljeni su u 3% azotnoj kiselini. Kalibracione krive ispitivanih elemenata dobijene su korišćenjem rastvora („*bulk*“) dobijenog mešanjem radnih standardnih rastvora u odgovarajućem razblaženju. Primenjena „*Automix*“ opcija, atomskog apsorpciong spektrometra sa grafitnom kivetom (GFAAS) omogućila je automatsku pripremu kalibracionih standarda.

3.3. Metode pripreme uzoraka

Priprema uzoraka vode za analizu farmaceutski aktivnih komponenata

Određivanje 81 PhAC u različitim tipovima uzoraka vode koji su uzeti sa 25 različitih lokacija u Srbiji bazira se na ekstrakciji na čvrstoj fazi. PhAC komponente koje pripadaju različitim terapeutskim klasama određene su u dobijenim ekstraktima radi utvrđivanja prisustva i raspodele ovih komponenata u otpadnoj, površinskoj, podzemnoj i pijaćoj vodi.

Uzorci vode i to: 500 ml površinske, podzemne i vode za piće i 200 ml neprečišćene otpadne vode pripremljeni su ekstrakcijom na čvrstoj fazi sa Oasis HLB kolonama (masa sloja 60 mg, zapremine 3 ml) (Waters, Milford, MA, U.S.A.) (Gros i sar., 2009). Ciljane PhAC komponente izolovane su iz uzoraka vode ekstrakcijom na čvrstoj fazi (SPE) korišćenjem Backer vakuum

sistema (J.T. Baker, Deventer, Holandija). SPE kolone kondicionirane su sa 5 ml metanola, zatim sa 5 ml vode (HPLC kvaliteta) sa protokom od 2 ml/min. Uzorci vode propušteni su kroz najlonski filter - 0,45 µm (Whatman, U.K.). Nadalje, uzorci su propušteni sa protokom 5 ml/min i koncentrovani na SPE kolonama, koje su potom isprane sa 5 ml vode (HPLC kvaliteta), osušene pod vakuumom 15-20 min da se ukloni ostatak vode, a zatim su PhAC komponente eluirane sa 2x4 ml metanola. Dobijeni ekstrakti upareni su u struji azota, a zatim rekonstituisani sa 1 ml smese metanola-vode (10:90, v/v). Pre instrumentalne analize, u dobijene ekstrakte uzoraka dodato je 10 µl smese izotopski označenih unutrašnjih standarda čija je krajnja koncentracija u ekstraktu uzoraka bila 10 ng/ml.

Priprema uzoraka za analizu 8-11 mikotoksina

Određivanje mikotoksina u zrnu pšenice izvedeno je primenom jednostavne i brze metode pripreme tj. sa jednostepenom ekstrakcijom za istovremeno (simultano) izdvajanje mikotoksina različitih fizičko-hemijskih osobina i dobijanje neprečišćenog tzv. sirovog ekstrakta za analizu *Fusarium* mikotoksina u uzorcima ozime pšenice radi određivanja razlika među žitnim regionima Srbije (iz kojih su uzorci pokupljeni) kao i otpornosti sorti pšenice na kontaminaciju *Fusarium* toksinima (Škrbić i sar., 2011a). Određivanje 11 osnovnih („*principal*“) mikotoksina u pšeničnom brašnu zasnovano je na prethodno pomenutoj metodi koja je modifikovana u odnosu na količinu uzorka, zapreminu rastvarača za ekstrakciju i razblaženje ekstrakta uzorka (Škrbić i sar., 2012); dok za određivanje 10 mikotoksina od interesa u koštuničavom voću razvijena je „multi-matriks“ metoda koja se bazira na jednostepenoj kiseloj ekstrakciji i obuhvata korak odmašćivanja dobijenog sirovog ekstrakta sa heksanom u cilju uklanjanja lipida koji potiču iz ispitivanih uzoraka.

Priprema uzoraka ozime pšenice i pšeničnog brašna vršena je jednostepenom ekstrakcijom u cilju postizanja brze kvantitativne analize osam *Fusarium* i 11 osnovnih („*principal*“) mikotoksina. Ekstrakcija 12,5 g homogenizovanog samlevenog uzorka ozime pšenice izvršena je sa 50 ml smese acetonitrila/vode (84:16, v/v) automatskom mućkalicom (IKA Laboratortechnik, Nemačka) u trajanju od 1 h; dok je pet grama uzorka pšeničnog brašna ekstrahovano sa 20 ml smese acetonitrila/vode (84:16, v/v) automatskom mućkalicom (Promax 2020, Heidolph Instruments, Germany) u istom vremenskom intervalu. Nakon završene ekstrakcije, sirovi ekstrakti ozime pšenice i pšeničnog brašna su profiltrirani. 4 ml profiltriranog ekstrakta ozime pšenice upareno je do suva u struji azota, a zatim rastvoreno u 1 ml smese vode/metanola (1:1, v/v); dok je 1 ml sirovog filtriranog ekstrakta pšeničnog brašna razblaženo sa 3 ml mobilne faze korišćene za hromatografsko razdvajanje (95% A (voda/sirćetna kiselina (99:1, v/v)) i 5% B (metanol/sirćetna kiselina (99:1, v/v); oba eluenta sadržala su 5 mM amonijum acetata)). Pre početka analize (tj. kvantitativnog određivanja), sirovi ekstrakti uzorka ozime pšenice i pšeničnog brašna propušteni su kroz najlonski filter - 0,2 µm.

Za razliku od prethodne modifikovane metode tzv. sirovog ekstrakta korišćenog za pšenično brašno (Škrbić i sar., 2011b, 2012) i papriku (Škrbić i sar., 2013a), za pripremu koštuničavog voća razvijena je kisela ekstrakcija koja obuhvata i korak odmašćivanja dobijenog sirovog

ekstrakta sa heksanom. Naime, kako su prethodne studije (Škrbić i sar., 2011b, 2012) za analizirane mikotoksine iz pšeničnog brašna pokazale da ekstrakcija rastvaračima bez kiseline ne omogućava dobijanje prihvatljivih „recovery" vrednosti za FB1 i FB2 (>60%), sirćetna kiselina dodata je u smesu rastvarača za ekstrakciju (79:20:1, v/v/v, acetonitril/voda/sirćetna kiselina) (Sulyok i sar., 2006, 2007a, 2010; Abia i sar., 2013; Varga i sar., 2013; Škrbić i sar., 2014), da bi se omogućilo efikasno izolovanje ovih toksina. Zatim, odmašćivanje dobijenog sirovog ekstrakta sa heksanom je uvedeno u proceduru pripreme uzoraka u cilju uklanjanja lipida, jer njihovo prisustvo može imati negativan uticaj na signal mikotoksina od interesa tokom analize sa UHPLC-HESI-QqQ-MS/MS. Ovako dobijeni sirovi ekstrakti uzoraka korišćeni su za dalju analizu bez koraka prečišćavanja.

Uzorci su pripremljeni na sledeći način: 10 g homogenizovanog uzorka ekstrahovano je sa 40 ml acetonitrila/vode/sirćetne kiseline (79:20:1, v/v/v) u trajanju od 1 h automatskom mućkalicom (Promax 2020, Heidolph Instruments, Nemačka). Nakon ekstrakcije, suspenzija je propuštena kroz filter papir (Whatman No. 4). 20 ml profiltriranog sirovog ekstrakta preneto je u plastičnu kivetu (za centrifugu), a zatim je dodato 20 ml heksana i mešano 2 min u cilju uklanjanja lipida. Smesa je centrifugirana sa 5000 o/min u trajanju od 5 min. Nakon razdvajanja faza, heksan je uklonjen. Pre početka analize, acetonitrilni ekstrakt propušten je kroz najlonski filter - 0,2 μm.

Priprema uzoraka za analizu teških elemenata u namirnicama potrošačke korpe Srbije

Uzorci su prepremljeni sistemom za mikrotalasnu digestiju (Ethos One, Milestone, Italija). Pre početka digestije, oko 0,5 g svakog homogenizovanog kompozitnog uzorka odmereno je u teflonsku kivetu u koju je dodata smesa 7 ml ccHNO_3 (69%) i 1 ml H_2O_2 (30%). Temperaturni program korišćen za digestiju uzoraka podešen je prema uputstvu proizvođača. Nakon završetka digestije i hlađenja, rastvor uzoraka („*digest*") razblažen je ultra čistom vodom do konačne zapremine od 25 ml u normalnom sudu, a potom prenet u polipropilenski sud. Za svaki ispitivani uzorak tri alikovota su pripremljena i svaki je analiziran u triplikatu.

3.4. Instrumentalna analiza

Instrumentalna analiza farmaceutski aktivnih komponenata

Instrumentalna analiza PhAC komponenata od interesa u ekstraktima uzoraka vode izvršena je visoko-pritisnim tečnim hromatografom sa hibridnim trostrukim (tripl) kvadrupolom „ion trap" masenim spektrometrom (UHPLC–ESI-QqLIT–MS/MS) (UHPLC, Acquity Ultra-Performance™, Waters, Milford, MA, USA; QqLIT–MS/MS, 5500 QTRAP, Applied Biosystems, Foster City, CA,USA) (Gros i sar., 2012).

Hromatografsko razdvajanje PhAC komponenata analiziranih u pozitivnom modu izvršeno je sa T_3 (Acquity UHPLC HSS) kolonom (dužine 50 mm, unutrašnjeg prečnika 2,1 mm (i.d.), sa punjenjem čestica prečnika 1,8 μm), dok je za PhAC komponenate analizirane u negativnom

modu korišćena T4 kolona (Acquity UPLC BEH C18) (dužine 50 mm, unutrašnjeg prečnika 2,1 mm (i.d.), sa punjenjem čestica prečnika 1,7 μm), obe od Waters korporacije. Temperatura kolona podešena je na 30^{o}C, a autosamplera na 4^{o}C. Za analizu PhAC komponenti u pozitivnom modu, korišćena je mobilna faza A - metanol i mobilna faza B - voda (HPLC kvaliteta) sa 10 mM amonijum formijata/mravlje kiseline (pH=3,2) sa protokom od 0,5 ml/min. Korišćen je sledeći gradijentni program: početni uslovi 5% A; 0-4,5 min, 5-95% A; 4,5-4,6 min, 100% A; 4,6-6 min, 100% A; od 6-6,1 min vraćanje na početne uslove; od 6,1 do 6,7 min, kondicioniranje kolone pri početnim uslovima. Za analizu PhAC komponenti u negativnom modu, korišćena je mobilna faza A - acetonitril i mobilna faza B - voda sa amonijum acetatom/amonijakom (pH=8), sa protokom 0,6 ml/min. Injektirana zapremina kalibracionih standarda i uzoraka je 5 μl u oba moda. Korišćeni gradijentni program za negativni mod je: 0-1,5 min, 0-60% A (početni uslovi); 1,5-2 min, 100% A; 2-3 min, 100% A; zatim se 3-3,2 min vraća se na početne uslove; od 3,2 do 3,7 min, kondicioniranje kolone pri početnim uslovima.

PhAC komponente od interesa analizirane su u MRM modu (tabela 3.5). Optimizovani parametri ESI jonskog izvora primenjeni za analizu PhAC komponenata u pozitivnom modu su: „*curtain*" gas – 30 V; temperatura jonskog izvora – 650^{o}C; voltaža jonskog spreja – 5500 V; gasovi jonskog izvora G1 i G2 – 60 i 50 V, redom. Za komponente analizirane u negativnom modu, ovi parametri su: „*curtain*" gas – 30 V; temperatura jonskog izvora – 650^{o}C; voltaža jonskog spreja – 3500 V; gasovi jonskog izvora G1 i G2 – 60 i 70 V, redom. Azot je korišćen kao kolizioni gas. Svi podaci dobijeni su i obrađeni korišćenjem Analyst 1.5.1 programa.

Tabela 3.5. *Parametri UHPLC–ESI-QqLIT–MS/MS za PhAC komponente pod optimizovanim uslovima Acquity Ultra-Performance™ – 5500 QTRAP*

PhAC komponente	Prekursor jon (m/z)	Q1[a]	Q3[b]
PhAC komponente analizirane u pozitivnom modu			
Metronidazol-OH	187 $[M+H]^+$	126	123
Sotalol	273 [M+H]+	255	133
Salbutamol	240 [M+H]+	148	122
Atenolol-d_7 (IS)	274 [M+H]+	145	-
Ronidazol	201 [M+H]+	140	-
Atenolol	267 [M+H]+	145	190
Ronidazol-d_3	204 [M+H]+	143	-
Metronidazol	172 [M+H]+	128	82
Ranitidin	315 [M+H]+	176	130
Famotidin	338 [M+H]+	189	259
Cimetidin-d_3 (IS)	256 [M+H]+	95	-
Cimetidin	253 [M+H]+	159	95
Jopromid	792 [M+H]+	573	300

Nastavak tabele 3.5

PhAC komponente	Prekursor jon (m/z)	Q1[a]	Q3[b]
PhAC komponente analizirane u pozitivnom modu			
Codeine	300 [M+H]+	152	115
Oksikodon	316 [M+H]+	298	241
Levamizol	205 [M+H]+	178	91
Dimetridazol	142 [M+H]+	96	95
Trimetoprim	291 [M+H]+	230	261
Cefaleksin	348 [M+H]+	158	106
Nadolol	310 [M+H]+	254	201
Olanzapin	313 [M+H]+	256	198
Ofloksacin-d_3 (IS)	365 [M+H]+	321	-
Ofloksacin	362 [M+H]+	318	261
Sulfametaksazol-d_4 (IS)	258 [M+H]+	160	-
Sulfametaksazol	254 [M+H]+	92	156
Tetraciklin	445 [M+H]+	410	154
Ciprofloksacin	332 [M+H]+	288	245
Fenazon-d_3 (IS)	192 [M+H]+	59	-
Fenazon	189 [M+H]+	77	56
Sulfadoksine-d_3 (surogat[c])	314 [M+H]+	156	-
Ksilazin-d_6 (IS)	227 [M+H]+	90	-
Ksilazin	221 [M+H]+	90	77
Metoprolol	268 [M+H]+	133	121
Azaperol	330 [M+H]+	121	78
Tiabendazol	202 [M+H]+	175	131
Tamsulosin	409 [M+H]+	228	200
Azaperon-d_4 (IS)	332 [M+H]+	127	-
Azaperon	328 [M+H]+	123	95
Sulfadimetoksine-d_6 (surogat[c])	317 [M+H]+	162	-
Karazolol	299 [M+H]+	116	222
Trazodon	372 [M+H]+	176	148
Azitromicin-d_3 (IS)	752 [M+H]+	594	-
Venlafaksin-d_6 (IS)	284 [M+H]+	64	-
Venlafaksin	278 [M+H]+	58	260
Azitromicin	749 [M+H]+	592	116
Propranolol	260 [M+H]+	116	183
10,11- epoksi CBZ	253 [M+H]+	180	236
2-hidroksi CBZ	253 [M+H]+	210	208
Citalopram-d_4 (IS)	329 [M+H]+	113	-
Citalopram	325 [M+H]+	109	262
Norfluoksetin	296 [M+H]+	134	-
Akridon	196 [M+H]+	166	167

Nastavak tabele 3.5

PhAC komponente	Prekursor jon (m/z)	Q1[a]	Q3[b]
PhAC komponente analizirane u pozitivnom modu			
Akridon	196 [M+H]+	166	167
Verapamil-d_6 (IS)	461 [M+H]+	165	-
Norverapamil	441 [M+H]+	165	150
Verapamil	455 [M+H]+	165	77
Diltiazem	415 [M+H]+	178	109
Karbamazepin-d_{10} (IS)	247 [M+H]+	204	0
Desloratadin	311 [M+H]+	259	258
Karbamazepin	237 [M+H]+	194	193
Propifenazon	231 [M+H]+	189	56
Amlodipin-d_4 (IS)	413 [M+H]+	238	-
Paroksetin	330 [M+H]+	192	123
Eritromicin	734 [M+H]+	576	158
Eritromicin-N,N$^{13}C_2$ (IS)	736 [M+H]+	578	-
Lorazepam	321 [M+H]+	275	303
Alprazolam	309 [M+H]+	281	205
Fluoksetin-d_5 (IS)	315 [M+H]+	44	-
Fluoksetin	310 [M+H]+	44	148
Amlodipin	409 [M+H]+	238	294
Sertralin	307 [M+H]+	159	276
Albendazol	266 [M+H]+	234	191
Klaritromicin	748 [M+H]+	158	590
Diazepam-d_5 (IS)	290 [M+H]+	198	-
Diazepam	285 [M+H]+	193	154
Varfarin-d_5 (IS)	314 [M+H]+	163	-
Varfarin	309 [M+H]+	163	251
Glibenklamid	494 [M+H]+	369	169
Glibenklamid-d_3 (IS)	497 [M+H]+	372	-
Klopidogrel	322 [M+H]+	212	184
Loratadin	383 [M+H]+	337	267
PhAC komponente	Prekursor jon (m/z)	Q1[a]	Q3[b]
PhAC komponente analizirane u negativnom modu			
Acetaminofen-d_4 (IS)	154 [M-H]-	111	-
Acetaminofen	150 [M-H]-	107	-
Salicilna kiselina	137 [M-H]-	93	66
Hidrohlorotijazid-d_2 (IS)	298 [M-H]-	270	-
Hidrohlorotijazid	296 [M-H]-	269	205
Tenoksikam	336 [M-H]-	152	272
Piroksikam	330 [M-H]-	146	266
Valsartan	434 [M-H]-	179	350
Valsartan-d8 (IS)	442 [M-H]-	179	-

Nastavak tabele 3.5

PhAC komponente	Prekursor jon (m/z)	Q1[a]	Q3[b]
PhAC komponente analizirane u negativnom modu			
Naproksen	229 [M-H]-	170	185
	232 [M-H]-	171	-
Furosemid-d_5 (IS)	334 [M-H]-	290	-
Furosemid	329 [M-H]-	285	205
Ketoprofan-d_3 (surogat[c])	256 [M-H]-	212	-
Pravastatin	423 [M-H]-	321	303
Ketoprofan	253 [M-H]-	209	-
Meloksikam-d_3 (IS)	353 [M-H]-	289	-
Meloksikam	350 [M-H]-	146	286
Bezafibrat-d_6 (IS)	366 [M-H]-	280	-
Bezafibrat	360 [M-H]-	274	154
Torasemid	347 [M-H]-	262	196
Lozartan	421 [M-H]-	127	179
Ibuprofen-d_3 (IS)	208 [M-H]-	164	-
Ibuprofen	205 [M-H]-	161	-
Diclofenak	294 [M-H]-	250	214
Indometacin-d4 (IS)	360 [M-H]-	316	-
Indometacin	356 [M-H]-	312	297
Irbesartan	427 [M-H]-	193	399
Deksametazon	451 [M-H]-	361	307
Deksametazon -d_4 (IS)	395 [M-H]-	363	-
Gemfibrozil-d_6 (IS)	255 [M-H]-	121	-
Gemfibrozil	249 [M-H]-	121	127
Fluvastatin	410 [M-H]-	210	348
Atorvastatin	557 [M-H]-	278	397

[a]Jon korišćen za kvantifikaciju („*quantifier*").
[b] Jon korišćen za identifikaciju („*qualifier*").
[c]Surogat standardi su analiti koji se dodaju u uzorak u poznatoj koncentraciji u cilju određivanja efikasnosti ekstrakcije.

Instrumentalna analiza mikotoksina

Analiza *Fusarium* mikotoksina u dobijenim sirovim ekstraktima izvršena je sa visoko-pritisnim tečnim hromatografom sa trostrukim (tripl) kvadrupolnim masenim spektrometrom (HPLC-APCI-QqQ-MS/MS, Vantage, Thermo Fisher Scientific, US). Hromatografsko razdvajanje analita uzorka izvršeno je na koloni sa obrnutim fazama (dužine 150 mm, unutrašnjeg prečnika 3 mm, sa punjenjm čestica prečnika 4 μm, Synergi Hydro RP, Phenomenex, USA). Temperatura kolone podešena je na 40^{o}C. Za analizu *Fusarium* mikotoksina korišćena je voda sa 10 mM amonijum acetata - mobilna faza A i metanol – mobilna faza B, čiji protok je podešen na 0,5 ml/min. Zapremina uzorka koja se unosi u tečni hromatograf je 30 μl. Identifikacija i kvantifikacija mikotoksina izvršena je sa tripl-kvadrupolnim spektrometrom (QqQ) (Hajšlová i sar., 2007). Parametri APCI izvora u oba

moda (pozitivnom i negativnom) su sledeći: temperatura kapilare – 150^{o}C, temperatura isparivača („*vaporizer*“) – 450^{o}C, protok azota kao „*sheath*“ gasa – 1,2 l/min, protok azota kao „*auxiliary*“ gasa – 3 l/min, napon izvora – 6 kV. Gas u kolizionoj ćeliji je helijum, a režim skeniranja je SRM mod.

Za hromatografsko razdvajanje 11 osnovnih („*principal*“) mikotoksina u sirovom ekstraktu pšeničnog brašna i 10 mikotoksina u ekstraktima koštuničavog voća korišćen je visoko pritisni tečni hromatograf (UHPLC, Accela, Thermo Fisher Scientific, USA). Korišćena je Hypersil GOLD™ kolona, dužine 50 mm, unutrašnjeg prečnika 2,1 mm (i.d.), sa punjenjem čestica prečnika 1,9 µm (Thermo Fisher Scientific, USA). Temperatura kolone je podešena na 30^{o}C, a protok mobilne faze na 0,5 ml/min. Zapremina ekstrakta uzorka koja se unosi u tečni hromatograf je 10 µl. Mobilna faza A je voda/sirćetna kiselina (99:1, v/v), a B metanol/sirćetna kiselina (99:1, v/v). Oba eluenta sadržala su 5 mM amonijum acetata. Razdvajanje mikotoksina izvršeno je korišćenjem gradijentnog programa prikazanog u tabeli 3.6.

Tabela 3.6. *Gradijentni program korišćen za analizu mikotoksina u uzorcima brašna i koštuničavog voća*

Vreme (min)	Mobilna faza A (%)	Mobilna faza B (%)	Protok (ml/min)
0,00	95	5	0,5
0,50	95	5	0,5
3,90	5	95	0,5
6,00	5	95	0,5
6,20	95	5	0,5
8,00	95	5	0,5

Za identifikaciju i kvantifikaciju mikotoksina od interesa u uzorcima brašna i koštuničavog voća korišćen je tripl kvadrupol maseni spektrometar (QqQ) TSQ Vantage (Thermo Fisher Scientific, USA) sa grejanim elektrosprej jonizacijonim izvorom (HESI-II, Thermo Scientific, USA). Parametri jonskog izvora su sledeći: napon spreja – 3,4 kV, temperatura isparivača („*vaporizer*“) - 350°C, pritisak „*sheath*” gasa - 40 arbitarnih jedinica, pritisak „*auxiliary*” gasa - 10 arbitarnih jedinica, temperatura kapilare - 270°C.

Parametri MS/MS optimizovani su direktnom infuzijom standardnog rastvora (5 µg/ml) svakog mikotoksina (osim u slučaju AFB1 i AFG1 (2 µg/ml) i AFB2 i AFG2 (0,5 µg/ml)) rastvorenog u prvom gradijentnom stepenu mobilne faze (95% A i 5% B) sa protokom od 10 µl/min. Identifikacija ispitivanih analita izvedena je u „*full scan*“ modu u cilju utvrđivanja prekursor jona. Uslovi cepanja (fragmentacije) prekursor jona odnosno koliziona energija i voltaža „S-lens“ optike optimizovana je za svaku pojedinačnu komponentu tj. tranziciju. Optimizovani parametri UHPLC-HESI-QqQ-MS/MS (Accela-TSQ Vantage) za razdvajanje i identifikaciju ispitivanih mikotoksina prikazani su u tabeli 3.7.

Table 3.7. *Parametri UHPLC-HESI-QqQ-MS/MS za mikotoksine pod optimizovanim uslovima Accela - TSQ Vantage sistema*

Mikotosini	t_R [a], min	„Dwell" vreme [b], s	Prekursor jon, m/z	Produkt joni [c], m/z	CID [d], eV
AFB1	3,17	0,1	313,1 $[M+H]^+$	285,1/241,1	33/22
AFB2	3,07	0,1	315,1 $[M+H]^+$	287,2/259,2	28/25
AFG1	2,97	0,1	329,1 $[M+H]^+$	200,2/215,1	26/40
AFG2	2,87	0,1	331,0 $[M+H]^+$	245,0/189,1	40/28
OTA	3,91	0,1	404,1 $[M+H]^+$	239,0/221,0	35/23
DON	1,82	0,1	295,0 [M-H]-	265,1/205,2	-18/-24
HT-2	3,57	0,1	447,2 $[M+Na]^+$	345,5/285,5	19/20
T-2	3,74	0,1	489,0 $[M+Na]^+$	245,1/327,1	26/23
ZON	3,88	0,1	317,1 $[M-H]^-$	175,1/131,1	-26/-32
FB1	3,70	0,1	722,6 $[M+H]^+$	334,3/352,4	38/34
FB2	3,98	0,1	706,5 $[M+H]^+$	336,3/318,3	36/38

[a] Retenciono vreme.
[b] Zadati opseg masa i brzina skeniranja hromatograma određuju vreme praćenja određene mase tj. „*Dwell*" vreme.
[c] Jon korišćen za kvantifikaciju („*quantifier*") /jon korišćen za identifikaciju („*qualifier*" jon).
[d] Optimizovana koliziona energija za dobijeni „*quantifier*"/„*qualifier*" jon pri cepanju prekursor jona.

U cilju pouzdane kvantifikacije mikotoksina korišćen je mod za praćenje specifičnih tranzicionih reakcija koje se dešavaju u QqQ analizatoru tj. SRM mod, gde primenom odgovarajuće kolizione energije za svaki prekursor jon (mikotosina) nastaju njegove tranzicije tj. produkt joni (ili ćerke joni). Dva produkt jona su određena za svaki analit i jedan je korišćen za kvantifikaciju („*quantifier*"), a drugi za identifikaciju („*qualifier*"). Masena rezolucija za prvi i treći kvadrupol podešena je na 0,7 Da, a širina prozora („*scan width*") na 0,5 m/z. Kontrola instrumenta i obrada podataka urađeni su sa programom Xcalibur 2.1.0 (Thermo Fisher Scientific, USA).

Instrumentalna analiza teških elemenata

Koncentracije As, Cd i Pb u ispitivanim uzorcima određene su atomskim apsorpcionom spektrometrom sa grafitnom kivetom (GFAAS, Varian AA240/GTA120) uz korekciju pozadinske apsorpcije korišćenjem deuterijumske lampe. Operativni uslovi GFAAS korišćeni pri analizi As, Cd i Pb dati su u tabeli 3.8. Kontrola instrumenta vršena je programom SpectrAA.

Tabela 3.8 *Parametri analize za određivanje As, Cd i Pb sa GFAAS u namirnicama potrošačke korpe Srbije*

	As			Cd			Pb		
Talasna dužina, nm	193,7			228,8			283,3		
Uzorak (µl)	15			15			20		
Modifikator (µl)	10			5			-		
Faze	Piroliza	Atomizacija	Čišćenje	Piroliza	Atomizacija	Čišćenje	Piroliza	Atomizacija	Čišćenje
Temperatura (°C)	1400	2600	2600	400	1800	1800	400	2100	2100
Vreme zagrevanja (s)	5	0,6		5	0,8		5	0,9	
Zadržavanje (s)	3	2	2	5	2	2	3	2	2
Beleženje signala	-	On		-	On		-	On	-
Protok gasa argona (ml/min)	300	0	300	300	0	300	300	0	300

Korišćeni modifikator je 0,1% paladijum nitrat.
Temperatura tokom koraka sušenja je od 85-120°C tokom 55 s.

3.5. Parametri unutrašnje („in-house“) kontrole kvaliteta i pouzdanosti primenjenih i razvijenih metoda

Provera kvaliteta i pouzdanosti primenjenih i razvijenih metoda za PhAC komponente, mikotoksine i teške elemente izvršena je korišćenjem sledećih parametara: koeficijenata determinacije (R^2), granica detekcije/kvantifikacije (LOD/LOQ), efikasnosti („recovery“) i preciznosti (izražene kao % relativne standardne devijacije, %RSD).

Kvantifikacija PhAC komponenti izvršena je metodom unutrašnjeg standarda. Osam standardnih rastvora različitih koncentracija korišćeno je za kalibraciju za svaki analit od interesa. Svaki standardni rastvor analiziran je po tri puta pri konstrukciji kalibracione krive. Kalibracione krive su linearne u celom kalibracionom rasponu od 0,1, do 50 ili 100 µg/l zavisno od PhAC komponenata i očekivanih koncentracija u analiziranim uzorcima vode na osnovu dostupnih literaturnih podataka (Gros i sar., 2012).

Kvantifikacija mikotoksina u sirovim ekstraktima ozime pšenice, pšeničnog brašna i koštuničavog voća izvršena je metodom spoljašnjeg standarda tj. matriks standard kalibracijom. Standardni rastvori za matriks kalibracije pripremljeni su u nekontaminiranim (”*blank*”) sirovim ekstraktima ozime pšenice, pšeničnog brašna i koštuničavog voća. Od šest do jedanaest standardnih rastvora različitih koncentracija korišćeno je za kalibraciju za svaki mikotoksin od interesa (zavisno od vrste analiziranih uzoraka tj. maksimalno dozvoljene vrednosti analita od interesa definisane postojećim regulativama). Svaki standardni rastvor analiziran je po tri puta pri konstrukciji kalibracionih krivih. Kalibracione krive za sve *Fusarium* mikotoksine su linearne u radnom opsegu od 5 µg/kg do 10000 µg/kg; dok su kalibracione krive za 11 osnovnih („*principal*“) mikotoksina linearne u radnom opsegu od 1 do 1760 µg/kg. Za kvantifikaciju 10 mikotoksina u koštuničavom voću korišćeni su standardni rastvori od 1 do 80 µg/kg.

Kvantifikacija teških elemenata određena je na osnovu kalibracionih krivih dobijenih snimanjem apsorbanci kalibracionih standarda pripremljenih sa istom kiselinom, koja je korišćena za razaranje uzoraka.

LOD i LOQ vrednosti za PhAC i mikotoksine od interesa određene su na osnovu odnosa signala (ispitivanog analita) i šuma (S/N) bazne linije; LOD za svaki ispitivani analit je vrednost koncentracije za koju je S/N = 3, odnosno za LOQ, S/N = 10. Vrednosti LOD, odnosno LOQ za teške elemente određene su na osnovu vrednosti dobijenih analizom slepe probe u pet ponavljanja. LOD analiziranih elemenata predstavlja srednju vrednost signala slepe probe uvećane za trostruku vrednost standardne devijacije signala slepe probe, dok za LOQ srednja vrednost signala slepe probe uvećana je za desetorostruku vrednost standardne devijacije signala slepe probe.

Provera efikasnosti („recovery”) analitičkih metoda određena je analizom obogaćenih („spajkovanih”) uzoraka. Obogaćen („spajkovan“) uzorak se priprema dodavanjem poznate zapremine standardnog rastvora ispitivanog analita u ispitivani uzorak. Korišćeni nivoi

obogaćivanja („spajkovanja“) u skladu su sa maksimalno dozvoljenim količinama za analite od interesa definisane postojećim regulativama; za analite čiji sadržaj nije definisan postojećim regulativama nivoi obogaćivanja („spajkovanja“) birani su proizvoljno ili na osnovu literaturno dostupnih podataka.

Uzorci vode (n=3) obogaćeni („spajkovani“) su sa smesom PhAC komponenti koja je sadržala i unutrašnje standarde: 100 ng/l za otpadnu vodu i 50 ng/l za površinsku vodu i vodu za piće.

Provera efikasnosti metode za analizu *Fusarium* mikotoksina određena je obogaćivanjem („spajkovanjem“) nekontaminiranog („*blank*“) uzorka ozime pšenice sa sledećim nivoima obogaćivanja („spajkovanja“): 1250 µg/kg za DON, DON-3-Glc, Fus-X i ADON, 100 µg/kg za ZON, 200 µg/kg za NIV, HT-2 i T-2 toksin. Efikasnost analitičke metode za 11 osnovnih („*principal*“) mikotoksina određena je kroz ”*in-house recovery*” nekontaminiranog pšeničnog brašna obogaćenog (”spajkovanog”) u sledećim nivoima: 2 µg/kg za svaki aflatoksin, 3 µg/kg za OTA, 350 µg/kg za DON, 100 µg/kg za HT-2 i T-2 toksin, 35 µg/kg za ZON i 300 µg/kg za fumonizine. Dodatno, efikasnost metode proverena je i analizom uzoraka kukuruza dobijenih za interlaboratorijsko poređenje („proficiency test“, PT) u cilju određivanja do 11 mikotoksina u kukuruzu LC-MS/(MS). Interlaboratorijsko poređenje organizovano je od strane CNR-ISPA, Institute of Sciences of Food Production, u okviru MoniQA projekta u 2011. godini koji je podržan od šestog okvirnog programa Evropske komisije (FP6; www.moniqa.org/mycotoxins). Za proveru efikasnosti metode za analizu 10 mikotoksina u koštuničavom voću dva nivoa obogaćivanja (”spajkovanja”) su korišćena. Naime, nekontaminirani (”*blank)*” uzorci koštuničavog voća spajkovani su sa 4 µg/kg za svaki aflatoksin, što odgovara maksimalno dozvoljenoj količini afaltoksina koja je definisana Regulativom Evropske unije (165/2010/EU)[21]. Za mikotoksine (OTA, HT-2, T-2, ZON, FB1 i FB2), čiji sadržaj nije definisan postojećim regulativama, proizvoljan nivo od 40 µg/kg izabran je za obogaćivanje („spajkovanje“). Obogaćeni („spajkovani“) uzorci (ozime pšenice, pšeničnog brašna i koštuničavog voća) ostavljeni su preko noći na sobnoj temperaturi radi uklanjanja (isparenja) rastvarača i uspostavljanja ravnoteže između dodatih jedinjenja i matriksa i analizarani su u duplikatu ili triplikatu.

Efikasnost („*in-house recovery*“) metode za analizu teških elemenata proverena je obogaćivanjem („spajkovanjem“) ispitivanih uzoraka u nivou maksimalno dozvoljenih vrednosti za analizirane teške elemente u skladu sa Regulativom Evropske unije (1881/2006/EC)[22] i/ili važeće regulative u Srbiji (Pravilnik)[23]. Obogaćeni („spajkovani“) uzorci

[21] Commission Regulation 165/2010/EU, OJ L 50, 27.2.2010., str. 11 (u daljem tekstu: Regulativa Evropske unije (EU, 2010)[21])

[22] Commission Regulation 1881/2006/EC, OJ L 364, 20.12.2006., str. 15-20 (u daljem tekstu: Regulativa Evropske unije (EC, 2006)[22])

[23] Službeni glasnik RS, br. 28/11, (u daljem tekstu: Važeća regulativa u Srbiji (Pravilnik)[23])

analizirani su kroz pet ponavljanja. Dodatno, efikasnost metode proverena je analizom sertifikovanog materijala (CRM) i kroz interlaboratorijsko poređenje (PT).

Preciznost primenjenih metoda izražena je kao relativna standardna devijacija (RSD, %), tri ili pet vrednosti dobijenih pri analizi obogaćenih („spajkovanih“) uzoraka. Svaki uzorak analiziran je u duplikatu ili triplikatu i rezultat je predstavljen kao srednja vrednost. Slepa proba je analizirana u cilju provere prisustva mogućih nečistoća prisutnih u korišćenom posuđu i hemikalijama.

4. REZULTATI I DISKUSIJA

4.1. Prisustvo farmaceutski aktivnih komponenata u vodi

S obzirom na činjenicu da su farmaceutski aktivne komponente (PhAC) nova klasa jedinjenja, maksimalno dozvoljene vrednosti za ove komponente nisu definisane postojećim regulativama, kao ni kriterijumi (na primer, LOD, LOQ, „recovery" vrednosti) koje primenjena metoda treba da zadovolji.

LOD, LOQ, "recovery" vrednosti i preciznost (RSD, %) „multi-rezidualne" metode analize za PhAC komponente od interesa određene u okviru disertacije kroz unutrašnju („*in-house*") proveru kvaliteta i pouzdanosti primenjene metode prikazane su u tabeli 4.1 (Petrović i sar., 2014). Kalibracione krive su linearne sa koeficijentom determinacije R^2>0,9900. „Recovery" vrednosti dobijene za većinu PhAC komponenata od interesa su veće od 50%. Međutim, za neke komponente kao što su hidrohlorotijazid, salbutamol, losartan, tiabendazol, metronidazol i hidroksimetronidazol dobijene su niže „recovery" vrednosti. Za druge komponente, kao što su cimetidin, ranitidin, dimetridazol, ronidazol, fluvastatin i atorvastatin dobijene su takođe niske „recovery" vrednosti, ali samo u nekim matriksima kao što je voda za piće. Ovo se može objasniti činjenicom da primenjeni eksperimentalni uslovi nisu odgovarajući za ove specifične komponente. Ovo je i jedan od nedostataka „multi-rezidualnih" metoda, kod kojih nema optimalnih uslova za sve ciljane analite, i stoga, „recovery" vrednosti predstavljaju kompromis između primenjenih analitičkih uslova i broja analita. Međutim, niske „recovery" vrednosti za pomenute PhAC komponente ne mogu se smatrati nedostatkom za njihovo određivanje u uzorcima vode, jer su granice detekcije i kvantifikacije primenjene „multi-rezidualne" metode prilično niske (tabela 4.1). LOD je određen u rasponu od 0,01-20 ng/l za sve analizirane tipove vode, dok je LOQ 0,1-50 ng/l. Vrednosti LOD/LOQ za većinu PhAC u vodi za piće (tabela 4.1) su ispod 10 ng/l, što predstavlja ciljanu („target") vrednost definisanu za genotoksične supstance i steroidne hormone u vodi za piće na osnovu nedavno uvedenog pristupa na bazi toksikološkog praga („Treshold of Toxicological Concern", TTC) u Holandiji (Mons i sar., 2013). Na osnovu TTC, za sve druge organske supstance ciljana vrednost u pijaćoj vodi je 100 ng/l (Mons i sar., 2013).

Tabela 4.1. *Parametri kvaliteta i pouzdanosti UHPLC-ESI-QqLIT-MS/MS metode primenjene za određivanje 81 PhAC komponente*

PhAC komponente	LOD (LOQ) (ng/l)			%Recovery (%RSD) (n=3)		
	VP[a]	PV[b]	OV[c]	VP[a]	PV[b]	OV[c]
PhAC komponente analizirane u pozitivnom modu						
Metronidazol-OH	n.m. (n.m.)[d]	9,3 (30,0)	14 (48,0)	n.m.	40 (15,0)	43 (8,2)
Sotalol	n.m. (n.m.)	1,1 (3,5)	9,0 (10,0)	n.m.	101 (5,9)	83 (8,8)
Salbutamol	n.m. (n.m.)	0,1 (0,2)	0.9 (3,0)	n.m.	30 (9,3)	30 (4,4)
Ronidazol	0,5 (1,8)	2,5 (8,3)	15 (51,0)	30 (6,4)	90 (6,0)	108 (10,1)
Atenolol	0,1 (0,1)	0,3 (1,0)	9 (12,9)	57 (9,7)	57 (13,5)	61 (3,3)
Metronidazol	n.m. (n.m.)	2,7 (8,5)	26 (44,0)	n.c.	30 (11,5)	109 (4,7)
Ranitidin	0,1 (0,2)	0,1 (0,2)	0,4 (1,4)	45 (3,1)	40 (7,4)	92 (12,7)
Famotidin	0,1 (0,3)	0,2 (0,7)	1,4 (4,8)	50 (12,4)	95 (14,9)	87 (6,8)
Cimetidin	0,1 (0,3)	0,5 (1,0)	3,0 (10,0)	24 (16,5)	83 (11,1)	76 (9,7)
Jopromid	0,3 (0,9)	1,4 (4,7)	6,8 (22,8)	157 (16,2)	158 (22,7)	130 (10,8)
Kodein	0,1 (0,2)	1,0 (3,4)	8,8 (28,0)	82 (8,5)	88 (4,2)	100 (10,9)
Oksikodon	0,2 (0,6)	1,9 (6,3)	17,1 (50,0)	75 (7,6)	73 (5,8)	112 (5,8)
Levamizol	0,01 (0,01)	0,2 (0,3)	0,4 (1,2)	50 (5,9)	82 (11,8)	74 (8,3)
Dimetridazol	1,0 (2,8)	4,4 (15,0)	15 (50,0)	30 (8,9)	65 (15,1)	109 (11,1)
Trimetoprim	0,1 (0,3)	0,6 (2,0)	2,4 (8,1)	56 (12,9)	53 (1,2)	67 (7,1)
Cefaleksin	n.m. (n.m.)	1,1 (3,8)	5,0 (16,6)	n.m. (n.m.)	31 (9,2)	70 (8,6)
Nadolol	0,01 (0,1)	0,1 (0,2)	0,7 (2,4)	40 (2,0)	60 (3,9)	56 (4,4)
Olanzapin	n.m. (n.m.)	0,5 (1,8)	0,5 (1,8)	n.m. (n.m.)	66 (8,5)	66 (9,9)
Ofloksacin	n.m. (n.m.)	0,6 (2,1)	0,6 (1,8)	n.m. (n.m.)	67 (12,2)	116 (15,9)
Sulfametaksazol	0,1 (0,3)	2,0 (6,5)	5,5 (18,0)	83 (19,7)	78 (1,0)	81 (11,3)
Tetraciklin	7,2 (20,0)	12,0 (41,0)	7,0 (23,0)	128 (4,7)	112 (13,1)	127 (6,3)
Ciprofloksacin	n.m. (n.m.)	5,5 (18,3)	7,0 (23,0)	n.m. (n.m.)	113 (9,1)	140 (21,2)
Fenazon	0,1 (0,5)	0,7 (2,3)	2,0 (6,5)	83 (10,8)	98 (1,43)	91 (6,0)
Sulfadoksin-d_3 (surogat)	-	-	-	78 (4,6)	82 (7,8)	93 (6,8)
Ksilazin	0,4 (1,4)	1,6 (5,3)	4,0 (13,0)	90 (0,3)	93 (1,7)	102 (8,5)
Metoprolol	0,3 (0,9)	1,3 (4,2)	8,7 (28,0)	53 (1,5)	77 (3,5)	70 (2,0)
Azaperol	0,1 (0,4)	0,3 (1,0)	0,5 (1,7)	66 (6,2)	80 (4,6)	66 (10,7)
Tiabendazol	0,01 (0,03)	0,03 (0,1)	0,2 (0,6)	20 (5,0)	40 (5,6)	22 (7,1)
Tamsulosin	0,1 (0,4)	0,3 (0,9)	1,2 (4,0)	71 (5,1)	73 (1,0)	117 (6,5)
Azaperon	0,2 (0,4)	0,5 (1,0)	0,9 (1,7)	52 (17,6)	100 (4,4)	92 (14,5)
Sulfadimetoksine-d_6 (surogat)	-	-	-	63 (2,9)	70 (9,0)	74 (1,5)
Karazolol	0,2 (0,7)	0,3 (0,9)	0,6 (2,1)	50 (11,1)	90 (3,7)	98 (16,6)
Trazodon	0,1 (0,4)	0,2 (0,8)	2,1 (7,1)	46 (4,3)	41 (6,6)	76 (12,4)
Venlafaksin	0,1 (0,3)	0,2 (0,7)	0,6 (2,1)	88 (2,9)	107 (3,1)	92 (2,4)
Azitromicin	0,4 (1,4)	0,14 (0,5)	0,4 (1,2)	144 (10,7)	128 (20,0)	111 (11,6)
Propranolol	0,4 (1,4)	1,5 (4,9)	3,5 (13,3)	80 (10,8)	120 (5,7)	88 (1,0)
10,11- epoksi CBZ	4,7 (13,0)	15,2 (50,7)	19,0 (50,0)	127 (7,6)	82 (6,9)	66 (4,5)
2-hidroksi CBZ	1,6 (5,2)	4,2 (13,9)	8,0 (25,0)	91 (7,8)	86 (6,3)	142 (13,9)
Citalopram	0,4 (1,2)	0,5 (2,7)	1,0 (3,3)	80 (1,3)	96 (2,1)	109 (5,4)
Norfluoksetin	0,3 (0,9)	1,3 (4,5)	2,6 (8,6)	82 (16,4)	122 (2,6)	113 (14,6)

Nastavak tabele 4.1

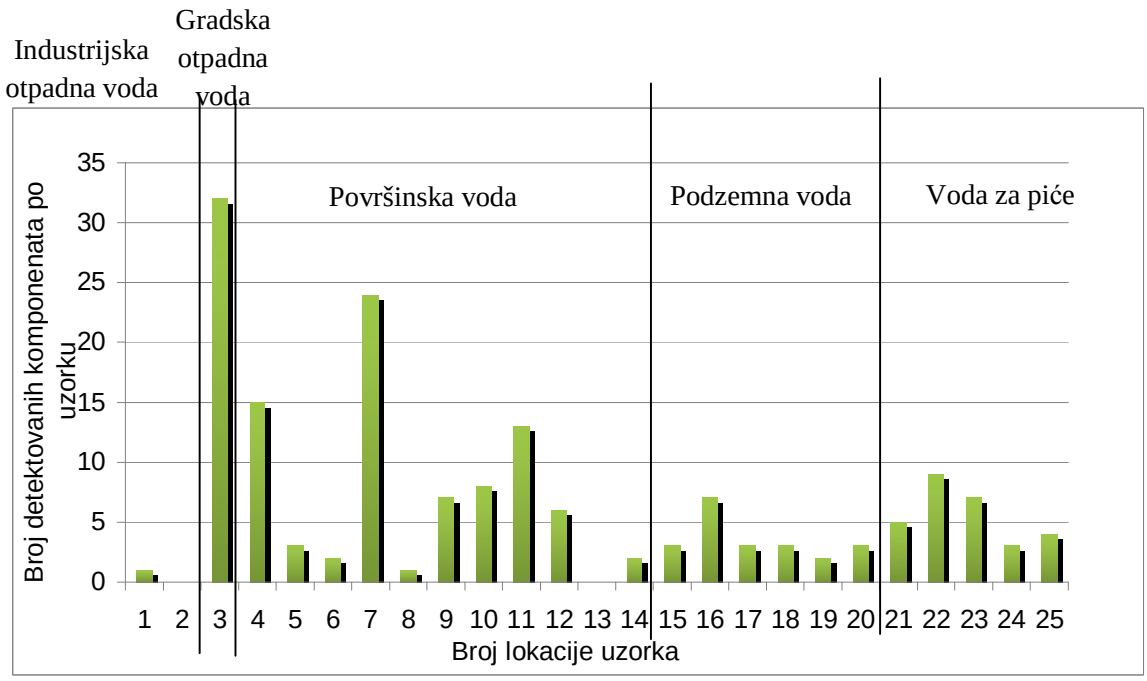
PhAC komponente	LOD (LOQ) (ng/l)			%Recovery (%RSD) (n=3)		
	VP[a]	PV[b]	OV[c]	VP[a]	PV[b]	OV[c]
PhAC komponente analizirane u pozitivnom modu						
Akridon	0,04 (0,1)	0,6 (2,0)	2,6 (8,8)	84 (4,7)	83 (0,4)	84 (10,6)
Norverapamil	0,1 (0,4)	0,4 (1,3)	8,4 (28,0)	88 (9,9)	96 (1,9)	83 (11,1)
Verapamil	0,3 (1,1)	1,2 (5,8)	3,9 (13,0)	72 (7,4)	64 (12,5)	103(10,1)
Diltiazem	0,2 (0,5)	0,5 (1,7)	0,7 (2,4)	115 (2,9)	95 (4,9)	140 (4,1)
Desloratadin	0,2 (0,6)	0,9 (3,0)	2,3 (7,6)	41 (23,3)	82 (1,4)	146 (10,0)
Karbamazepin	0,2 (0,6)	0,7 (2,4)	2,4 (8,0)	108 (4,6)	108 (3,3)	124 (6,5)
Propifenazon	0,2 (0,5)	1,6 (5,2)	1,3 (4,5)	61 (1,5)	77 (2,1)	116 (5,8)
Paroksetin	1,8 (6,02)	4,24 (14,2)	7,4 (24,0)	67 (13,3)	122 (2,6)	145 (13,2)
Eritromicin	0,3 (0,9)	1,5 (5,1)	1,1 (3,5)	30 (15,6)	52 (20,7)	137 (18,0)
Lorazepam	1,4 (4,5)	5,3 (17,7)	13,0 (42,0)	93 (1,7)	99 (5,8)	107 (4,6)
Alprazolam	0,1 (0,2)	0,5 (1,5)	1,3 (4,4)	50 (3,2)	63 (2,2)	61 (3,5)
Fluoksetin	1,0 (2,0)	2,7 (9,0)	2,0 (6,5)	62 (19,1)	93 (2,7)	89 (13,9)
Amlodipin	n.m. (n.m.)	6,6 (20,9)	9,9 (33,1)	n.m. (n.m.)	69 (17,6)	75 (16,3)
Sertralin	2,0 (6,7)	9,7 (32,0)	12,0 (40,0)	130 (21,5)	96 (2,1)	109 (5,4)
Albendazol	n.m. (n.m.)	1,1 (3,6)	3,2 (10,5)	n.m. (n.m.)	115 (7,9)	130 (6,1)
Klaritromicin	0,4 (1,3)	0,6 (1,9)	1,3 (4,3)	137 (9,4)	116 (7,8)	106 (12,5)
Diazepam	0,2 (0,4)	1,1 (3,5)	1,1 (3,7)	97 (1,9)	98 (6,0)	101 (2,5)
Varfarin	0,2 (0,5)	0,5 (1,7)	2,1 (6,8)	94 (4,7)	100 (1,5)	103 (7,5)
Glibenklamid	-	-	-	88 (7,2)	101 (1,9)	88 (10,1)
Klopidogrel	0,1 (0,3)	0,7 (2,3)	0,3 (1,1)	97 (3,8)	104 (2,6)	112 (16,3)
Loratadin	3,1 (10,0)	1,1 (3,7)	3,2 (10,0)	96 (17,2)	80 (7,3)	130 (8,0)
PhAC komponente	LOD (LOQ) (ng/l)			%Recovery (%RSD) (n=3)		
	VP[a]	PV[b]	OV[c]	VP[a]	PV[b]	OV[c]
PhAC komponente analizirane u negativnom modu						
Acetaminofen	0,8 (1,0)	9,2 (20,0)	6,0 (20,0)	92 (0,3)	91 (0,3)	139 (7,3)
Salicilna kiselina	0,1 (0,2)	0,8 (2,6)	4,2 (13,0)	137 (43,6)	146 (3,4)	137 (6,0)
Hidrohlorotijazid	0,1 (0,3)	0,5 (1,7)	1,0 (2,6)	30 (5,4)	33 (8,9)	55 (20,0)
Tenoksikam	0,2 (0,8)	0,5 (1,8)	1,8 (6,0)	75 (5,7)	68 (6,6)	65 (5,5)
Piroksikam	0,2 (0,6)	1,1 (2,1)	2,0 (6,5)	79 (3,4)	56 (2,5)	76 (3,2)
Valsartan	0,5 (1,5)	6,7 (21,9)	4,0 (13,0)	98 (15,0)	60 (17,0)	100 (19,7)
Naproksen	0,4 (1,3)	1,3 (4,4)	3,5 (11,5)	88 (9,1)	136 (1,2)	104 (14,6)
Furosemid	0,7 (2,4)	4,7 (15,6)	8,9 (29,0)	60 (7,4)	91 (5,1)	70 (7,9)
Ketoprofan-d_3 (surogat)	-	-	-	106 (2,0)	111 (4,2)	105 (7,5)
Pravastatin	0,5 (1,8)	2,7 (9,1)	4,2 (14,1)	58 (6,1)	93 (11,0)	70 (9,5)
Ketoprofan	4,0 (13,0)	9,0 (30,1)	9,0 (30,0)	122 (21,2)	127 (1,2)	108 (14,6)
Meloksikam	0,1 (0,2)	0,2 (0,6)	0,5 (1,5)	82 (4,7)	102 (4,9)	107 (9,8)
Bezafibrat	0,1 (0,3)	0,3 (1,0)	1,0 ()3,3	96 (4,8)	107 (2,4)	96 (8,6)
Torasemid	0,1 (0,2)	0,5 (1,7)	1,1 (3,5)	91 (2,3)	97 (5,9)	103 (8,8)
Lozartan	0,9 (3,0)	3,5 (11,7)	4,1 (13,6)	32 (10,5)	41 (2,9)	40 (14,6)
Ibuprofen	0,5 (1,8)	3,2 (10,5)	1,1 (3,8)	105 (8,6)	86 (27,8)	174 (3,5)
Diklofenak	0,3 (1,0)	4,1 (13,5)	5,2 (17,1)	50 (3,0)	116 (10,0)	57 (4,2)
Indometacin	1,3 (4,4)	1,7 (5,5)	4,9 (16,4)	88 (3,0)	94 (9,7)	79 (12,9)

Nastavak tabele 4.1

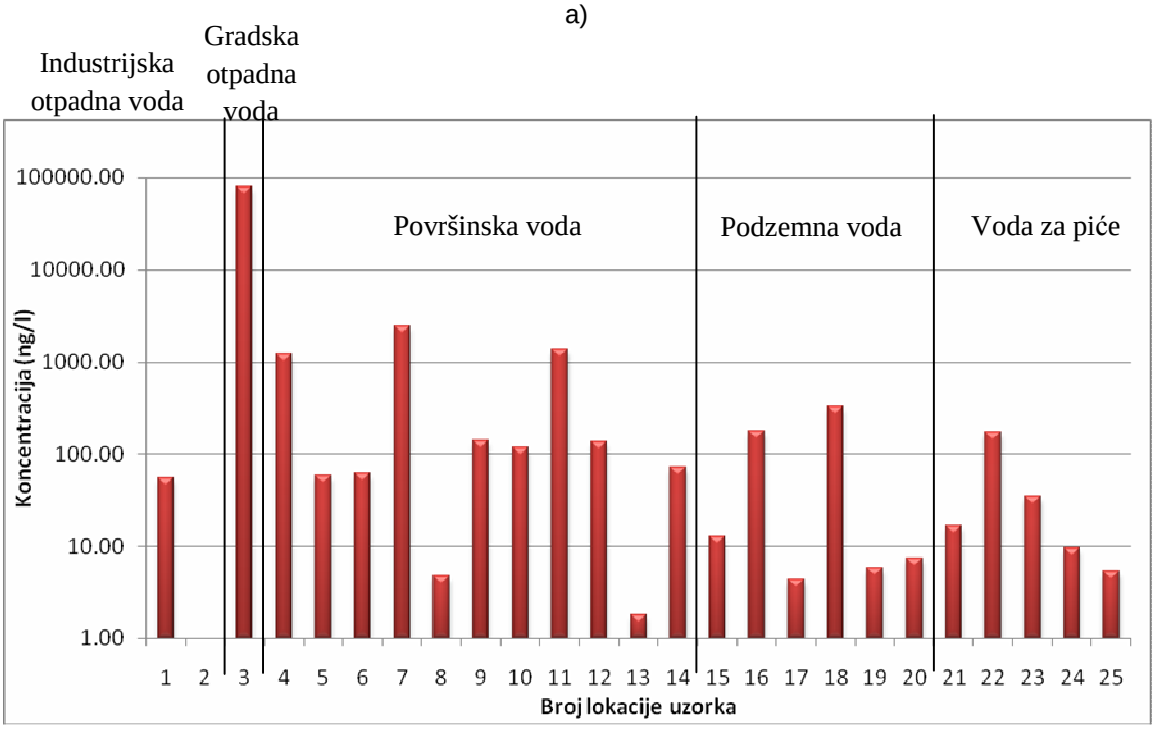
PhAC komponente	LOD (LOQ) (ng/l)			%Recovery (%RSD) (n=3)		
	VP[a]	PV[b]	OV[c]	VP[a]	PV[b]	OV[c]
PhAC komponente analizirane u negativnom modu						
Irbesartan	0,1 (0,3)	0,3 (1,1)	1,2 (3,9)	61 (11,7)	62 (2,7)	58 (9,5)
Deksametazon	0,1 (0,4)	0,4 (1,2)	0,9 (2,8)	84 (5,1)	88 (14,9)	83(7,6)
Gemfibrozil	0,04 (0,1)	0,6 (1,9)	0,4 (1,3)	90 (4,2)	42 (22,5)	99 (1,0)
Fluvastatin	0,1 (0,4)	0,6 (2,1)	2,6 (8,6)	32 (11,0)	117 (14,0)	97 (11,7)
Atorvastatin	0,03 (0,08)	0,1 (0,25)	0,2 (0,9)	12 (9,4)	89 (2,2)	56 (10,6)

[a] VP-voda za piće; [b] PV-površinska voda; [c] OV- otpadna voda; [d] nije mereno

„Multi-rezidualna“ metoda (Gros i sar., 2012) primenjena je za određivanje 81 PhAC komponente (najčešće korišćenih) u uzorcima vode koji su sakupljeni sa različitih lokacija u Srbiji. Na osnovu dobijenih rezultata utvrđeno je da većina ispitanih uzoraka sadrži neke od PhAC komponenti od interesa. Od 81 ispitane PhAC komponente 47 je detektovano u ispitivanim uzorcima vode iz sledećih terapeutskih grupa: analgetici/anti-inflamatori, regulatori nivoa holesterola (statini), antipsihotici, histamin H1 i H2, β – blokatori, diuretici, antihipertenzivi, lekovi za lečenje astme, kontrastna sredstva u radiologiji (x-zraci), antihelmintici, antibiotici i blokatori kalcijumovih kanala (tabela 4.2). Na slici 4.1a prikazana je raspodela identifikovanih PhAC komponenti u ispitivanim uzorcima vode; na osnovu čega je uočljivo da najveći broj komponenata je određen u uzorku neprečišćene gradske otpadne vode koja se direktno uliva u Dunav (uzorak br. 3). Najveći zbirni (kumulativni) nivo određen je u uzoraku br. 3 (79292,10 ng/l) zatim uzoraku br. 7 (2433,60 ng/l, reka Begej), 11 (1386,40 ng/l, kanal Bukovac) i 4 (1209,80 ng/l, kanal, prigradsko naselje Adice) (slika 4.3b). Najniži zbirni (kumulativni) nivo (1,80 ng/l) određen je za uzorak br. 13 (kanal-Dunav-Tisa-Dunav-I, slika 4.1b).



a)



b)

Slika 4.1. *Raspodela identifikovanih komponenata u ispitivanim uzorcima (broj lokacije uzoraka je dat u tabeli 3.1): a) Broj detektovanih komponenata po uzorku, b) Ukupni (kumulativni) nivoi detektovanih komponenata po uzorku*

Rezultati dobijeni za učestalost prisustva i koncentracioni opseg za PhAC komponente u ispitivanim uzorcima vode prikazani su u tabeli 4.2. Koncentracioni nivoi PhAC komponenti su u opsegu od nekoliko ng/l do više od 1 µg/l (za neke od detektovanih komponenata kao što su ibuprofen, diklofenak, kodein, valsartan, acetaminofen, 2-hidroksikrabamazepin i 10,11-epoksikrabamazepin). Učestalost prisustva PhAC komponenata detektovanih u ispitivanim uzorcima u grupi analgetika/anti-inflamatora smanjuje se u sledećem nizu: salicilna kiselina (41,67%) > ibuprofen (16,67%) > propifenazone = naproksen (13,89%) > diklofenak (11,11%) > meloksikam = ketoprofan = fenazone (8,33%) > kodeine (5,56%) > indometacin = acetaminofen (2,78%). Iz ove terapeutske grupe, minimalan broj PhAC komponenata je nađen u vodi za piće (samo ketoprofan i salicilna kiselina). Kao što se vidi iz tabele 4.2, u neprečišćenoj gradskoj otpadnoj vodi koja se direktno uliva u Dunav (uzorak br. 3) određene su visoke koncentracije četiri PhAC komponente: 1,0 µg/l za kodein, 1,3 µg/l za diklofenak, 15,7 µg/l za acetaminofen i 20,13 µg/l za ibuprofen. Takođe, u regionu zapadnog Balkana, Terzić i sar. (2008) utvrdili su za diklofenak maksimalnu koncentraciju od 4,2 µg/l, dok je najveći nivo ibuprofena u otpadnoj vodi bio 11,9 µg/l.

Tabela 4.2. *Učestalost prisustva i koncentracioni opseg[a] dobijen za PhAC komponente koje pripadaju različitim terapeutskim klasama u analiziranim uzorcima vode (prikazani rezultati korigovani su za „recovery" vrednosti)*

PhAC komponente	Ukupna učestalost prisustva	Industrijska otpadna voda (n=2) [b]	Gradska otpadna voda (n=1) [b]	Površinska voda (n=11)		Podzema voda (n=6)		Voda za piće (n=5)	
	%	ng/l	ng/l	%	ng/l	%	ng/l	%	ng/l
Analgetici/anti-inflamatori									
Ketoprofan	8,33	<LOQ	247	9,09	45		<LOQ	20	16
Naproksen	13,89	<LOQ	208	27,27	<LOQ-74,2	16,67	27,6		<LOQ
Ibuprofen	16,67	<LOQ	20130	18,18	<LOQ-346	16,67	92		<LOQ
Idometacin	2,78	<LOQ	<LOQ	9,09	19,5		<LOQ		<LOQ
Acetaminofen	2,78	<LOQ	15719		<LOQ		<LOQ		<LOQ
Salicilna kiselina	41,67	54,5	204	9,09	2,7	83,33	<LOQ-2,5	100	<LOQ-1,4
Diclofenak	11,11	<LOQ	1338	27,27	<LOQ-324		<LOQ		<LOQ0
Fenazon	8,33	<LOQ	13,5	9,09	125	16,67	23,4		<LOQ
Propifenazon	13,89	<LOQ	99,5	9,09	5,8	50	<LOQ-24,8		<LOQ
Meloksikam	8,33	<LOQ	5,0	18,18	<LOQ-1,8		<LOQ		<LOQ
Kodeine	5,56	<LOQ	1017	9,09	7,3		<LOQ		<LOQ
Regulatori nivoa holesterola (statini)									
Bezafibrat	5,56	<LOQ	<LOQ	18,18	<LOQ-1,6		<LOQ		<LOQ
Atorvastatin	5,56	<LOQ	40,5	9,09	1,5		<LOQ		<LOQ
Lekovi koji deluju na centralni nervni sistem									
Karbamazepin	36,11	<LOQ	303	72,73	<LOQ-35,5	16,67	3,4	60	<LOQ-8,7
2-Hidroksikarbamazepin	5,56	<LOQ	15939	9,09	160		<LOQ		<LOQ
10,11-Epoksikarbamazepin	13,89	<LOQ	16208	27,27	<LOQ-932		<LOQ	20	128
Venlafaksin	13,89	<LOQ	154	27,27	<LOQ-5,3		<LOQ		<LOQ
Lorazepam	5,56	<LOQ	184	9,09	30,1		<LOQ		<LOQ

Nastavka tabele 4.2

PhAC komponente	Ukupna učestalost prisustva	Industrijska otpadna voda (n=2) [b]	Gradska otpadna voda (n=1) [b]	Površinska voda (n=11)		Podzema voda (n=6)		Voda za piće (n=5)	
	%	ng/l	ng/l	%	ng/l	%	ng/l	%	ng/l
Histamin H1 i H2									
Ranitidin	8,33	<LOQ	<LOQ	27,27	<LOQ-54,5		<LOQ		<LOQ
Farmotidin	2,78	<LOQ	301		<LOQ		<LOQ		<LOQ
β-blokatori									
Atenolol	8,33	<LOQ	670	18,18	<LOQ-50,6		<LOQ		<LOQ
Sotalol	5,56	<LOQ	91,3		<LOQ		<LOQ	20	0,4
Propranolol	30,56	<LOQ	78,5	9,09	10,4	66,67	<LOQ-4,5	100	<LOQ-4,3
Metoprolol	16,67	<LOQ	584	27,27	<LOQ-26,3		<LOQ	40	<LOQ-3,5
Karazolol	5,56	<LOQ	<LOQ		<LOQ	16,67	3,3		<LOQ
Diuretik									
Hidrohlorotijazid (HCTZ)	27,28	<LOQ	1070	54,55	<LOQ-164		<LOQ	20	24
Furosemid	11,11	<LOQ	362	27,27	<LOQ-101		<LOQ		<LOQ
Antihipertenzivi									
Lozartan	11,11	<LOQ	229	27,27	<LOQ-154		<LOQ		<LOQ
Irbesartan	30,56	<LOQ	11,6	63,64	<LOQ-15,3		<LOQ	60	<LOQ-2,2
Valsartan	5,56	<LOQ	1086	9,09	89,6		<LOQ		<LOQ
Lekovi za lečenje astme									
Salbutamol	5,56	<LOQ	<LOQ		<LOQ		<LOQ	40	<LOQ-5,4
Kontrastno sredstvo u radiologiji (X-zraci)									
Jopromid	19,44	<LOQ	804	63,64	<LOQ-75,2		<LOQ	20	6,8

Nastavka tabele 4.2

PhAC komponente	Ukupna učestalost prisustva	Industrijska otpadna voda (n=2) [b]	Gradska otpadna voda (n=1) [b]	Površinska voda (n=11)		Podzema voda (n=6)		Voda za piće (n=5)	
	%	ng/l	ng/l	%	ng/l	%	ng/l	%	ng/l
Antihelmentici									
Albendanzol	13,89	<LOQ	<LOQ		<LOQ	33,33	<LOQ-1,9	60	<LOQ-2,8
Levamizol	2,78	<LOQ	<LOQ	9,09	1,5		<LOQ		<LOQ
Antibiotici									
Eritromicin	2,78	<LOQ	<LOQ	9,09	292		<LOQ		<LOQ
Klaritromicin	5,56	<LOQ	<LOQ	18,18	<LOQ-616		<LOQ		<LOQ
Ofloksacin	2,78	<LOQ	220		<LOQ		<LOQ		<LOQ
Ciprofloksacin	8,33	<LOQ	278	18,18	<LOQ-28,2		<LOQ		<LOQ
Sulfametaksazol	2,78	<LOQ	432		<LOQ		<LOQ		<LOQ
Trimetoprim	5,56	<LOQ	259	9,09	8,1		<LOQ		<LOQ
Cefaleksin	5,56	<LOQ	803	9,09	283		<LOQ		<LOQ
Blokatori kalcijumovih kanala									
Diltiazem	8,33	<LOQ	217	18,18	<LOQ-6,9		<LOQ		<LOQ

[a] Vrednost umesto opsega znači da postoji samo jedan pozitivan uzorak.
[b] Učestalost prisustva nije data jer su analizirana samo dva uzorka industrijske otpadne vode i jedan uzorak gradske otpadne vode.

Potrebno je reći da je među PhAC komponentama u grupi analgetika/anti-inflamatora, najveća koncentracija određena za ibuprofen u površinskoj (reka Begej, uzorak br. 7) i podzemnoj (bunar na privatnom posedu, prigradsko naselje Adice, Novi Sad, uzorak br. 16) vodi (tabela 4.2): 346 ng/l (18.18%) i 92 ng/l (16.67%), redom.

Maksimalna koncentracija ibuprofena određena u površinskoj vodi koja je uzeta iz reke Begej (346 ng/l, uzorak br. 7) odgovara koncentraciji (277 ng/l) određenoj u rečnoj vodi u Španiji (Ferreira da Silva i sar., 2011). U većini Evropskih studija, ibuprofen je detektovan u površinskim vodama (Vanderford i sar., 2003; Roberts i Thomas, 2006), dok je u Sjedinjenim Američkim Državama najveća određena koncentracija ovog anti-inflamatora bila 200 ng/l (Kolpin i sar., 2002). Veoma visoka potrošnja ibuprofena povezana je sa njegovom najčešćom detekcijom od svih PhAC komponentama, ukazujući i na visoke koncentracije od 17 µg/l (Valcárcel i sar., 2011) i 1,4 µg/l (Martinez Bueno i sar., 2010) ovog leka određene u rečnoj vodi u regionu Madrida. Takođe, u istraživanjima Farré i sar. (2001) određene su visoke koncentracije ibuprofena (2,7 µg/l) u rekama Katalonije (severno-istočna Španija).

Sa druge strane, u terapeutskoj grupi regulatora nivoa holesterola (statini) samo bezafibrat i atorvastatin detektovani su u ispitivanim uzorcima sa istom frekvencijom prisustva od 5,56% (tabela 4.2). Maksimalna koncentracija atorvastatina je 40,5 ng/l određena u neprečišćenoj gradskoj otpadnoj vodi koja se direktno uliva u Dunav (uzorak br. 3), dok maksimalna koncentracija bezafibrata od 1,6 ng/l određena je u jednom uzorku površinske vode (Dunav, uzvodno od centra grada, plaža Štrand, uzorak br. 9). Suprotno ovome, Valcárcel i sar. (2011) takođe su pronašli bezafibrat u rečnoj vodi u Španiji, ali u značajno većoj koncentraciji (vrednost medijane je 682 ng/l).

Najdominantnija PhAC komponenta iz terapeutske grupe lekova koji deluju na centralni nervni sistem je karbamazepin sa ukupnom učestalošću prisustva od 36,11% u analiziranim uzorcima i maksimalnom koncentracijom od 303 ng/l u otpadnoj vodi (neprečišćena gradska otpadna voda koja se direktno uliva u Dunav, uzorak br. 3). Poznato je, da je karbamazepin jedan od najčešće pronađenih PhAC komponenti u vodenoj sredini, iako se iz organizma čoveka izlučuje u osnovnom obliku (netransformisan u toku metabolizma) u samo nekoliko procenata (Ternes, 1998). Učestalost prisutva karbamazepina u uzorcima površinske vode je 72,73%, a maksimalna koncentracija od 35,5 ng/l određena je u kanalu Dunav – Tisa – Dunav (uzorak br. 14). Prethodna istraživanja sprovedena u Srbiji (Grujić i sar., 2009) otkrila su da je 80% analiziranih uzoraka vode sadržalo karbamazepin, i koncentracioni opseg je bio od 6–130 ng/l. Takođe, koncentracije ovog antiepileptika detektovane u površinskim i podzemnim vodama Srbije slične su koncentracijama utvrđenim u Švajcarskoj (30–250 ng/l) (Öllers i sar., 2001), Španiji (9–37 ng/l) (Pedrouzo i sar., 2007), Južnoj Koreji (4,5–61 ng/l) (Kim i sar., 2007), Italiji (34,2 ng/l) (Zuccato i sar., 2005). Nadalje, koncentracija krabamazepina u podzemnoj vodi je 3,4 ng/l (javna česma-Vrbas, uzorak br. 20) i vodi za piće 8,7 ng/l (uzorak br. 25). U Nemačkoj, Heberer i sar. (2001) pronašli su karbamazepin u koncentraciji od 20 ng/l u vodi za piće koja je uzeta iz napuštenog bunara udaljenog 100

m od jezera Wannsee gde je izmerena koncentracija ovog antiepileptika bila 135 ng/l. U regionu Mediterana, Rabiet i sar. (2006) analizirali su sedam uzoraka vode za piće (bunarske) i samo u dva uzorka (dva bunara) određen je karbamazepin sa koncentracijama od 13,9 i 43,2 ng/l. Ovaj antiepileptik je rezistentan u životnoj sredini, i njegova koncentracija u podzemnim vodama je do 900 ng/l (Sacher i sar., 2001) što istvoremeno objašnjava činjenicu da je karbamazepin prisutan u velikom broju uzoraka podzemne vode, a takođe, je određen i u vodi za piće sa koncentracijom od 30 ng/l (Ternes, 2001). Pored ovoga, treba imati na umu, da lečenje epilepsije traje obično ceo život za razliku od povremenog uzimanja antibiotika, analgetika/antipiretika i to je razlog stalnog unosa ovog leka u prirodni vodeni sistem. Značajno visoke koncentracije za dva metabolita karbamazepina iz grupe lekova koji deluju na centralni nervni sistem određene su u gradskoj otpadnoj vodi (uzorak br. 3). Najveća koncentracija određena je za 10,11–epoksikarbamazepin metabolit karbamazepina u neprečišćenoj gradskoj otpadnoj vodi koja se direktno uliva u Dunav (uzorak br. 3) (16,2 µg/l), površinskoj vodi uzetoj iz reke Begej (0,93 µg/l, uzorak br. 7) i uzorku vode za piće (0,13 µg/l, uzorak br. 22). Za drugi metabolit, 2-hidroksikarbamazepin, utvrđena je koncentracija od 15,9 µg/l u istom uzorku (uzorak br. 3). Nivoi određenih metabolita karbamazepina su veći u poređenju sa nivoima objavljenim u drugim studijama, ali sa sličnim odnosom osnovne komponente (karbamazepin)/metabolita. Na primer, López-Serna i sar., (2012) detektovali su 10,11–epoksikarbamazepin (sa maksimalnom koncentracijom od 1600 ng/l) u slivu reke Erbo u koncentraciji koja je za jedan red veličine veća od koncentracije karbamazepina.

Dodatno, iz terapeutske grupe lekova koji deluju na centralni nervni sistem, slične maksimalne koncentracije izmerene su za venlafaksin (154 ng/l) i lorazepam (184 ng/l) u neprečišćenoj gradskoj otpadnoj vodi koja se direktno uliva u Dunav (uzorak br. 3) (tabela 4.2).

Analiza PhAC komponenti iz teraputske grupe antihistaminika potvrdila je prisustvo ranitidina u uzorku površinske vode tj. reke Begej (uzorak br. 7) sa maksimalnom koncentracijom od 54,5 ng/l i famotidina u otpadnoj vodi (neprečišćena gradska otpadna voda koja se direktno uliva u Dunav, uzorak br. 3) sa nivoom koji je pet puta veći u odnosu na pomenuti antihistaminik. Nešto niža vrednost antihistaminika ranitidina određena je u reci Lambro u Italiji (38,5 ng/l, Zuccato i sar., 2005). Preostale tri PhAC komponente iz grupe antihistaminika, loratadin, desloratadin i cimetidin nisu detektovane ni u jednom od ispitivanih uzoraka.

Među šest analiziranih β–blokatora, najzastupljeniji je propranolol koji je određen u svim tipovima ispitivanih uzoraka vode (ukupna učestalost je 30,56%). Potrebno je reći da samo jedan β–blokator, nadalol nije detektovan. Koncentracije atenolola (670,3 ng/l, uzorak br. 3) i metaprolola (573,9 ng/l, uzorak br. 3) su značajno veće u odnosu na druge lekove iz ove terapeutske grupe u neprečišćenoj gradskoj otpadnoj vodi. U istom uzorku otpadne vode, propranolol i sotalol određeni su u koncentracijama od 78,5 ng/l i 91,3 ng/l, redom. Nivoi β–blokatora određeni u otpadnoj vode su značajno niži u odnosu na koncentracije β–blokatora određene u uzorcima otpadne vode u ranijim istraživanjima u

regionu zapadnog Balkana (Terzić i sar., 2008). Naime, srednje vrednosti β–blokatora u uzorcima otpadne vode koje su analizirali Terzić i sar. (2008) bile su: 1,88 µg/l za atenolol, 0,95 µg/l za metaprolol, 0,22 µg/l za sotalol i 0,13 µg/l za propranolol. Različite studije pokazale su da iz terapeutske grupe kardiovaskularnih lekova tj. β–blokatora, atenolol (Garner i sar., 1994) i propranolol (Alder i sar., 2010) su najčešće određene PhAC komponente u površinskim vodama. Međutim, ove dve PhAC komponente određene su sa niskom pojavom učestalosti u površinskim vodama Srbije (18,18% i 9,09%, redom, tabela 4.12) i nižim nivoima (50,6 ng/l i 10,4 ng/l, redom) u poređenju sa onim određenim u površinskim vodama južnog Velsa (Velika Britanija) (Kasprzyk-Hordern i sar., 2008), u severo-istočnoj Španiji (Gros i sar., 2007), u Italiji (Zuccato i sar., 2000; Calamari i sar., 2003) i u regionu Madrida (Španija, Martinez Bueno i sar., 2010; Valcárcel i sar., 2011).

Iz grupe diuretika, najzastupljenija PhAC komponenta je hidrohlorotijazid određen u 27,28% ispitanih uzoraka i maksimalna koncentracija ovog diuretika je 1070 ng/l u uzorku neprečišćene gradske otpadne vode koja se direktno uliva u Dunav (uzorak br. 3). Drugi srodan diuretik furosemid je 2,5 puta manje prisutan u ispitivanim uzorcima u poređenju sa hidrohlorotijazidom sa maksimalnom koncentracijom od 362 ng/l u uzorku br. 3. U slučaju uzoraka površinske vode iz grupe diuretika određeni su hidrohlorotijazid u reci Begej (uzorak br. 7) sa koncentracijom od 164 ng/l i furosemid u kanalu prigradskog naselja Adice, Novi Sad (uzorak br. 4) sa koncentracijom od 101 ng/l. Suprotno ovome, u regionu Madrida, Španija, Valcárcel i sar. (2011) odredili su visoke sadržaje hidrohlorotijazida sa maksimalnom koncentracijom od 18 µg/l u površinskoj vodi, dok je drugi diuretik furosemid takođe određen u povišenim koncentracijama i njegova maksimalana vrednost je 3,2 µg/l. Takođe, mora se naglasiti da koncentracija hidrohlorotijazida određena u površinskoj vodi u Srbiji (tj. ovom istraživanju) je slična koncentraciji ovog diuretika određenog u reci Erbo u Španiji (Ferreira da Silva i sar., 2011).

PhAC komponente koje pripadaju grupi antihipertenzivnih lekova određeni su u otpadnoj, površinskoj i vodi za piće. Maksimalne određene koncentracije su za valsartan (1,1 µg/l) i losartan (228,6 ng/l) u neprečišćenoj gradskoj otpadnoj vodi koja se direktno uliva u Dunav (uzorak br. 3), dok je znatno niža koncentracija detektovana za irbesartan (11,6 ng/l) u istom uzorku. Takođe, maksimalne koncentracije irbesartana, valsartana i losartana određene u uzorcima površinske vode su 15,3 ng/l (reka Begej, uzorak br. 7), 89,6 ng/l (reka Begej, uzorak br. 7) i 154,1 ng/l (kanal, Bukovac, uzorak br. 11), redom.

Salbutamol je iz grupe lekova za lečenje astme i određen je samo u uzorku vode za piće (uzorak br. 24) sa maksimalnim nivom od 5,4 ng/l. Sa druge strane, López-Serna i sar. (2010) nisu detektovali salbutamol u ispitivanim uzorcima vode za piće u regionu Barselone, Španija, dok u Italiji, Zuccato i sar. (2005) odredili su salbutamol u uzorcima uzetim iz reka Lambro (2,5 ng/l) i Po (1,7 ng/l).

Nadalje, jopromid, iz terapeutske grupe kontrastna sredstva u radiologiji (x-zraci), određen je u gradskoj otpadnoj, površinskoj i vode za piće sa maksimalnom koncentracijom od 804, 75,2 i 6,8 ng/l, redom, što je značajno niže u poređenju sa nivom od 6263 ng/l koji je određen u otpadnoj vodi u Španiji (Gros i sar., 2012). Druge studije pokazale su da visoke

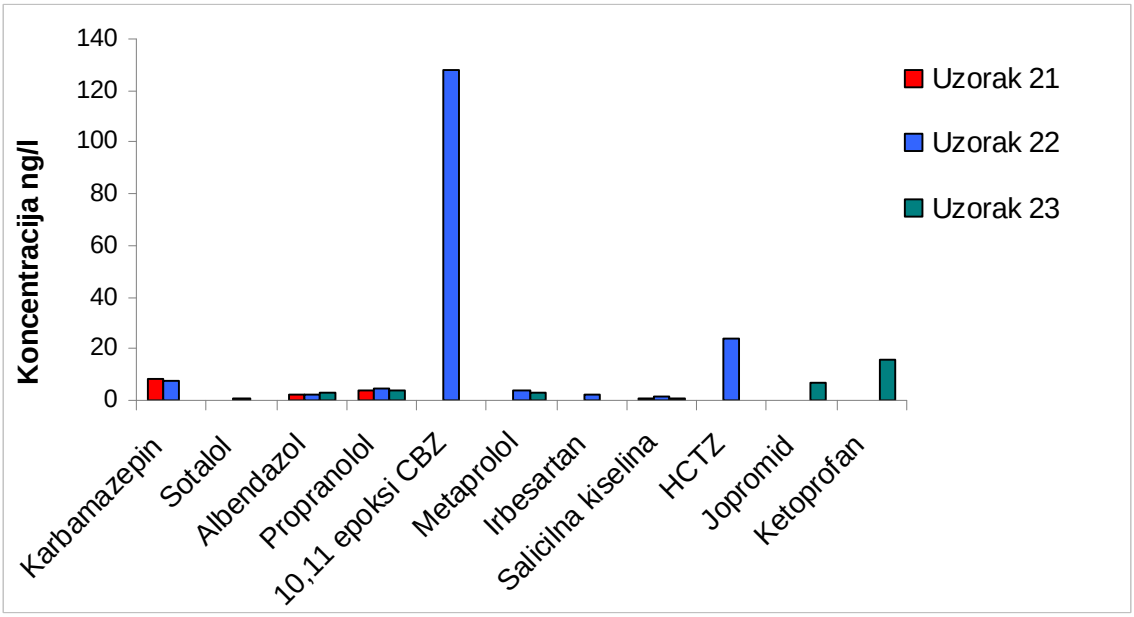
koncentracije ove komponente mogu biti određene, posebno u otpadnim vodama iz bolnica (Gros i sar., 2012). Generalno, koncentracija jopromida se značajno povećava u bolničkom efluentu tokom radne nedelje, jer se radiološki pregledi obavljaju u bolnicama pretežno od ponedeljka do petka (Ternes i Hirsch, 2000).

Kada se govori o antibioticima, najčešće određena PhAC komponenta je ciprofloksacin (u 8,33% uzoraka), dok ukupna učestalost za druge antibiotike kao što su klaritromicin, trimetoprim i cefaleksin je 5,56%. Među analiziranim antibioticima u svim ispitivanim uzorcima najmanje su zastupljeni sa 2,78% eritromicin, ofloksacin i sulfametoksazol.

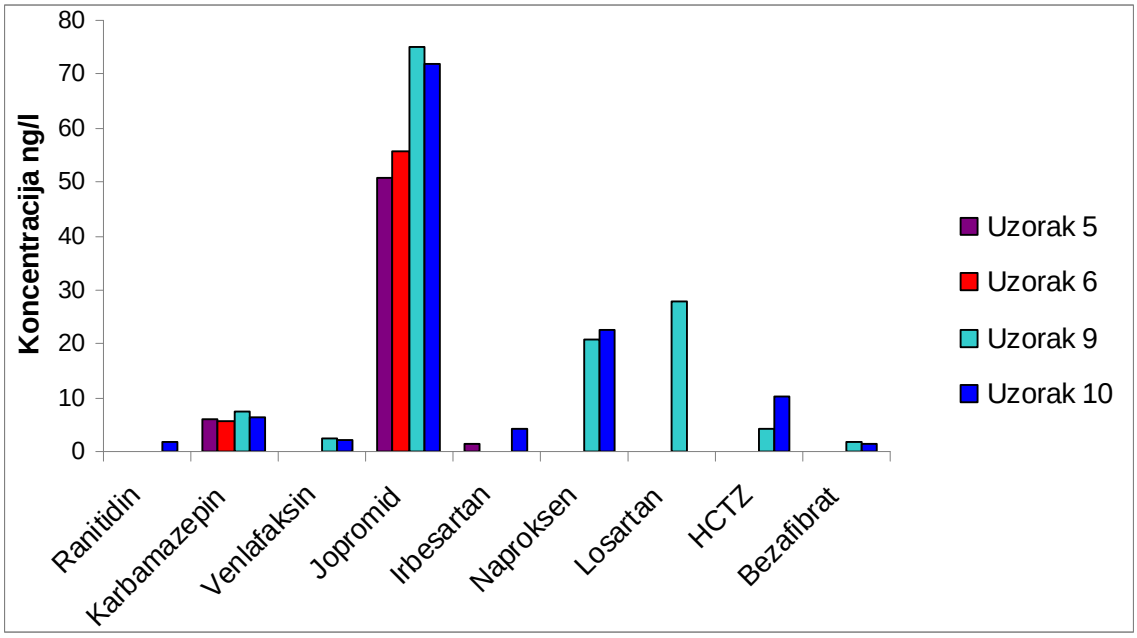
Vrlo visoka koncentracija klaritromicina (616 ng/l) određena je u površinskoj vodi (površinska voda kanala u prigradskom naselju Adice, Novi Sad, uzorak br. 4). Eritromicin je određen u znatno manjoj koncentraciji (292 ng/l, površinska voda kanala u selu Bukovac, uzorak br. 11) u odnosu na klaritromicin u istom tipu vode. Ovo se može objasniti činjenicom da eritromicin prelazi u eritromicin-H_2O u vodenim sistemima, i do ovog zaključka u svojim istraživanjima došli su Hirsch i sar. (1999). Sa druge strane, znatno manje koncentracije klaritromicina do 20,3 ng/l određene su u uzorcima površinske vode reka Lambro i Po u Italiji (Zuccato i sar., 2005). U grupi antibiotika, maksimalna koncentracija cefaleksina od 283 ng/l određena je u površinskoj vodi reke Begej (uzorak br. 7). Osim ovih PhAC komponenti iz grupe antibiotika, ofloksacin, ciprofloksacin, sulfametoksazol, trimetoprim i cefaleksin određeni su u neprečišćenoj gradskoj otpadnoj vodi koja se direktno uliva u Dunav (uzorak br. 3) sa koncentracionim rasponom od 220-803 ng/l.

Iz terapeutske grupe, blokatora kalcijumovih kanala, određen je samo diltiazem u površinskoj (18,18%) i otpadnoj gradskoj vodi sa maksimalnim koncentracijama 6,9 ng/l (površinska voda kanala u selu Bukovac, uzorak br. 11) i 217 ng/l (neprečišćenoj gradskoj otpadnoj vodi koja se direktno uliva u Dunav, uzorak br. 3), redom.

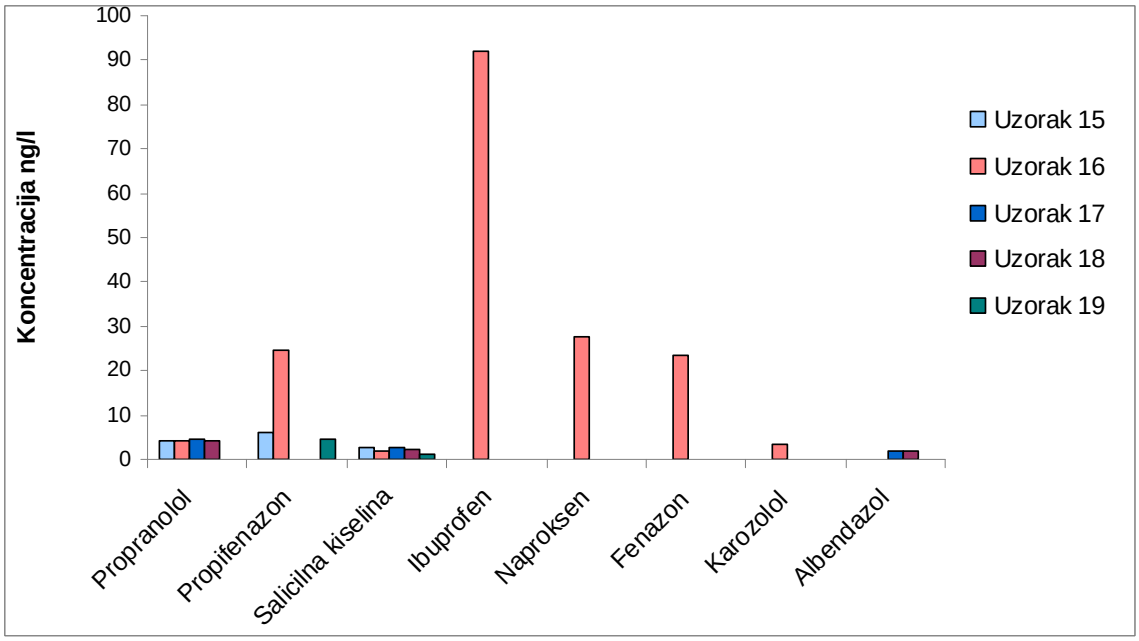
Da bi se utvrdilo da li postoji sličnost među raspodelom PhAC komponenti u uzorcima različitih tipova vode koji su uzeti na bliskim lokacijama u Novom Sadu, na slici 4.2 prikazane su određene koncentracije analiziranih komponenti u uzorcima vode za piće (slika 4.2a), u uzorcima površinske vode - reka Dunav i kanal Dunav-Tisa-Dunav (slika 4.2b), u uzorcima podzemne vode (slika 4.2c), u uzorku površinske vode – kanal Adice (slika 4.2d), i uzorku neprečišćene gradske otpadne vode (slika 4.2e).


Koncentracija ng/l
140
120
100
80
60
40
20
0
Uzorak 21
Uzorak 22
Uzorak 23
Karbamazepin
Sotalol
Albendazol
Propranolol
10,11 epoksi CBZ
Metaprolol
Irbesartan
Salicilna kiselina
HCTZ
Jopromid
Ketoprofan

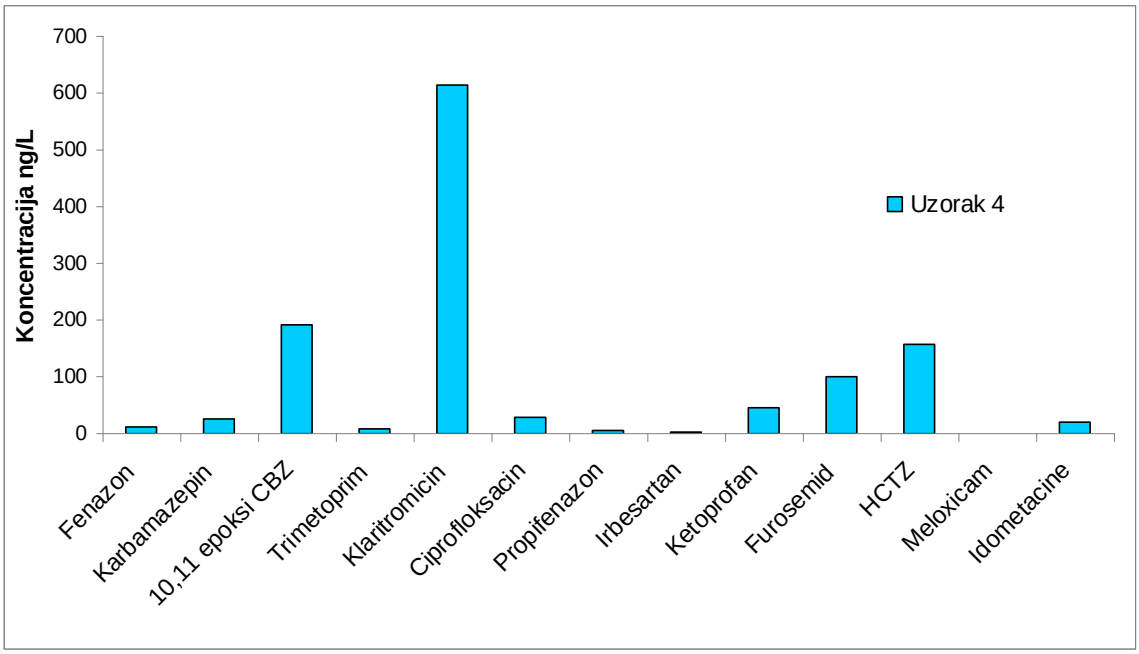
a)


Koncentracija ng/l
80
70
60
50
40
30
20
10
0
Uzorak 5
Uzorak 6
Uzorak 9
Uzorak 10
Ranitidin
Karbamazepin
Venlafaksin
Jopromid
Irbesartan
Naproksen
Losartan
HCTZ
Bezafibrat

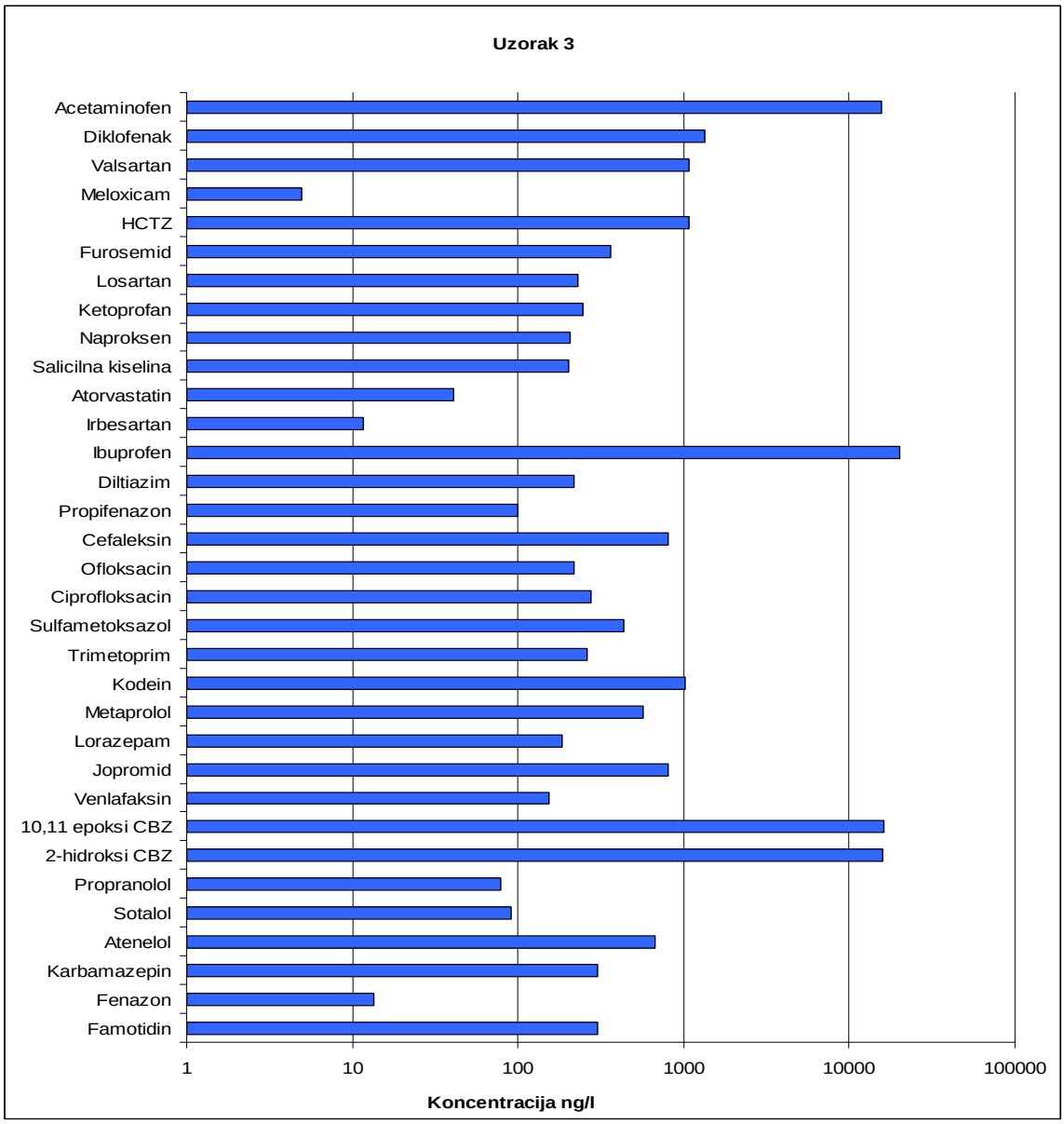
b)


Koncentracija ng/l
100
90
80
70
60
50
40
30
20
10
0
Uzorak 15
Uzorak 16
Uzorak 17
Uzorak 18
Uzorak 19
Propranolol
Propifenazon
Salicilna kiselina
Ibuprofen
Naproksen
Fenazon
Karozolol
Albendazol

c)


Koncentracija ng/L
700
600
500
400
300
200
100
0
Uzorak 4
Fenazon
Karbamazepin
10,11 epoksi CBZ
Trimetoprim
Klaritromicin
Ciprofloksacin
Propifenazon
Irbesartan
Ketoprofan
Furosemid
HCTZ
Meloxicam
Idometacine

d)



e)

Slika 4.2. *Prisustvo PhAC komponenti u uzorcima različitih tipova vode koji su uzeti na području Novog Sada: a) voda za piće, b) reka Dunav (uzorci br. 9 i 10) i kanal Dunav-Tisa-Dunav (uzorci br. 5 i 6) c) podzemna voda (Adice), d) kanal Adice (uzorak br. 4) i e) otpadna gradska voda (uzorak br. 3)*

Na slici 4.2a vidi se da su slične koncentracije albendazola, propranolola i salicilne kiseline određene u svim tipovima vode za piće (česmenska (uzorak br. 21), neprečišćena sirova (uzorak br.22) i hlorisana voda (uzorak br. 23) iz gradskog vodovoda), što ukazuje da

prečišćavanje i distribucija vode iz gradskog vodovoda nisu imali uticaj na ove tri PhAC komponente. Značajno visoka koncentracija 10,11-epoksikarbamazepin (10,11 epoksi CBZ) i hidrohlorotijazida (HCTZ) određena je u sirovoj neprečišćenoj vodi iz gradskog vodovoda (uzorak br. 22), za razliku od hlorisane (uzorak 23) i česmenske vode (uzorak 21) gde ove PhAC komponente nisu određene što ukazuje da je primenjeni tretman prečišćavanja uticao na njihovo uklanjanje. Nadalje, jasno se vidi da (slika 4.2b) prisustvo PhAC komponenti je slično u oba uzorka Dunavske vode uzete uzvodno sa plaže Štrand (uzorak br. 9) i sa plaže Oficirac (uzorak br. 10) nizvodno od prethodne. Jedina značajna razlika utvrđena je za losartan detektovan u uzorku br. 9. Koncentracije jopromida i karbamazepina u uzorcima (br. 5 i 6) uzetih iz kanala Dunav-Tisa-Dunav blizu gradskog područja Novog Sada su slične sa koncentracijama u uzorcima reke Dunav, kao i irbersartana koji je određen u tragovima. Može se primetiti da prisustvo PhAC komponenti u vodi reke Dunav i vodi za piće je različito, iako je poznato da se izdan vode za snabdevanje gradskog vodovoda uglavnom snabdeva vodom iz Dunava. Prisustvo jopromida i ketoprofana određeno je samo u uzorku hlorisane vode (uzorak 23) uzete iz gradskog vodovoda (slika 4.2a), dok ove komponente nisu određene u česmenskoj vodi (uzetoj u našoj Laboratoriji). U slučaju uzoraka podzemne vode, sa slike 4.2c vidi se sličnost između uzoraka 17 i 18, jer je gotovo isti sadržaj propranola, salicilne kiseline i albendazola određen u oba uzorka. Ova dva uzorka uzeta su iz dve javne česme, međusobno udaljene 1 km, i očigledno je da se prisustvo istih PhAC komponenti može objasniti istim poreklom vode tj. da ista podzema voda je izvor za ove dve česme. Sa druge strane, jasne razlike su uočljive između PhAC komponenti koje su prisutne u uzorcima podzemne vode (br. 15 i 16) uzete iz privatnih bunara koji su međusobno udaljeni ~500 m, posebno, ako se uzme u obzir prisustvo ibuprofena, naproksena, fenazona, propifenazona i karozolola. Interesantno je reći da se manja kompanija za proizvodnju kozmetičkih preparata nalazi u neposrednoj blizini kanala Adice, gde je uzet uzorak br. 4. Međutim, ne može se primetiti da postoji sličnost u prisustvu PhAC komponenti između uzorka podzemne vode (uzorak br. 16) i uzorka uzetog iz pomenutog kanala (uzorak br. 4, kanal Adice, slika 4.2d). PhAC profil uzorka br. 3 (slika 4.2d) specifičan je u odnosu na druge profile uzoraka sa 33 detektovane komponente, jer je to uzorak neprečišćene gradske otpadne vode u kome su najveće koncentracije određene za: ibuprofen, 10,11 epoxy CBZ, 2-hidroksi CBZ i acetominofen. Na osnovu prikazanih PhAC profila može se zaključiti da HTCZ i karbamazepin su dve najčešće prisutne komponente u svim ispitanim tipovima vode sa područja Novog Sada.

4.2. Prisustvo *Fusarium mikotoksina u zrnu pšenice*

Podaci o prisustvu mikotoksina u žitaricama i proizvodima na bazi žitarica u Srbiji su veoma retki i nesistematični. Kada je reč o postojećim podacima vezanim za prisustvo mikotoksina u žitaricama i proizvodima na bazi žitarica, može se govoriti samo o kvantitativnom određivanju jednog mikotoksina kao što je DON (Jajić i sar., 2008) ili kvalitativnom određivanju grupe srodnih mikotoksina kao što su AFB1, AFG1, AFB2 i AFG2 (Kos i sar., 2013). U Srbiji prve podatke o istovremenom (simultanom) prisustvu 8 *Fusarium* mikotoksina u zrnu pšenice i prisustvu 11 osnovnih („*principal*“) mikotoksina u pšeničnom brašnu objavili su Škrbić i sar. (2011a, 2012, redom); naime, u Centru izvrsnosti za bezbednost hrane i nove rizike osnovanom u okviru FP7 međunarodnog projekta CEFSER na Tehnološkom fakultetu Novi Sad, primenjena je „multi-toksin“ metoda za kvantitativno određivanje 11 najčešće prisutnih mikotoksina u žitaricama i proizvodima na bazi žitarica.

Kroz unutrašnju („*in-house*“) proveru kvaliteta i pouzdanosti „multi-toksin“ metode za određivanje *Fusarium* mikotosina u uzorcima ozime pšenice dobijeni su prihvatljivi parametri (Škrbić i sar., 2011a). Kalibracione krive za sve analite su linearne u rasponu od 5 do 10000 µg/kg sa R^2 od 0,9991-0,9999. Rezultati dobijeni za *Fusarium* mikotosine tokom unutrašnje („*in-house*“) provere „multi-toksin“ metode za LOD, LOQ, efikasnost („recovery“) i preciznost (%RSD) primenjene metode dati su u tabeli 4.3. Vrednosti „recovery“ dobijene za DON, ZON, T-2 i HT-2 za primenjenu „multi-toksin“ metodu analize su u rasponu koji je definisan Regulativom Evropske unije (EC, 2006)[20], a visoke „recovery“ vrednosti (iznad 80%) dobijene su za DON-3-Glc, FUS-X i ADON. „Recovery“ vrednost za NIV nije definisana Regulativom Evropske unije (EC, 2006)[20]. Relativna standardna devijacija za primenjenu metodu je oko 4% što ukazuje na prihvatljivu preciznost ovo metode za analizu mikotoksina od interesa.

Tabela 4.3. *Parametri kvaliteta i pouzdanosti HPLC-APCI-QqQ-MS/MS metode primenjene za analizu Fusarium mikotoksina u uzorcima ozime pšenice*

	NIV	DON	DON-3-Glc	FUS-X	ADON	HT-2	T-2	ZON
LOD (µg/kg)	5	0,3	1	1	5	5	0,3	1
LOQ (µg/kg)	10	1	5	5	10	10	1	5
„Recovery“ (%)	43	87	85	85	80	86	83	69
RSD (%)	3,5	3,6	3,2	1,4	1,5	3,9	3,9	2,5

„Multi-toksin“ metoda primenjena je za simultano određivanje prisustva *Fusarium* mikotosina u 54 uzorka pet sorti (Evropa 90, Renesansa, Pobeda, Kraljivica i Nora) ozime pšenice iz 10 izabranih žitnih regiona Srbije. Dobijeni rezultati prikazani su u tabelama 4.4-4.6 (Škrbić i sar., 2011a). Najzastupljeniji mikotoksin u ispitivanim uzorcima je DON, zatim DON-3-Glc i HT-2 toksin, dok ADON, FUS-X, NIV i ZON nisu identifikovani ni u jednom od analiziranih uzoraka. Kao što se vidi iz tabele 4.4, DON, DON-3-Glc i HT-2 toksin

pronađeni su u 28%, 13% i 6% od ukupnog broja analiziranih uzoraka, redom, a njihove srednje vrednosti su: 33 µg/kg za DON, 9 µg/kg za HT-2 i 5 µg/kg za DON-3-Glc.

Tabela 4.4. *Učestalost prisustva i parametri deskriptivne statistike za analizirane Fusarium mikotoksine u 54 uzorka ozime pšenice iz 10 izabranih regiona Srbije za žetvu 2007*

Mikotoksin	Broj pozitivnih uzoraka (učestalost prisustva, %)	Srednja vrednost[a] (µg/kg)	Medijana (µg/kg)	Koncentracioni opseg[b] (µg/kg)	Maksimalno dozvoljena vrednost (µg/kg) (EC, 2007)[24]
NIV	0	< LOD	< LOD	< LOD	n.d.[c]
DON	15 (27,78)	33	< LOD	41-309	1250
DON-3-Glc	7 (12,96)	5	< LOD	17-83	n.d.
FUS-X	0	< LOD	< LOD	< LOD	n.d.
ADONs	0	< LOD	< LOD	< LOD	n.d.
HT-2	3 (5,56)	9	< LOD	128-129	n.d.
T-2	0	< LOD	< LOD	< LOD	n.d.
ZON	0	< LOD	< LOD	< LOD	100

[a] Ako je sadržaj nekog mikotoksina ispod LOD primenjene metode u svim ispitivanim uzorcima,za njegovu srednju vrednost uzima se ispod LOD; u suprotnom (kada je deo rezultata pozitivan i iznad LOD a deo ispod LOD), za izračunavanje srednje vrednosti ispitivanih mikotoksina, za količine ispod LOD korišćeno je LOD/2.
[b] U pozitivnim uzorcima (tj. prirodno kontaminiranim uzorcima)
[c] Nije definisano

U svim analiziranim uzorcima ozime pšenice, nivo DON je ispod maksimalno dozvoljene vrednosti od 1250 µg/kg za neprerađene žitarice osim durum pšenice, definisane Regulativom Evropske unije (1126/2007/EC)[24] ili od 1000 µg/kg za proizvode namenjene za ljudsku ishranu preporučene od strane US Food and Drug Administration (Murphy i sar., 2006). S druge strane, ZON nije određen ni u jednom uzorku, te su svi uzorci u skladu sa Regulativom Evropske unije (EC, 2007)[24]. Maksimalno dozvoljene vrednosti za druge mikotoksine koji su određeni u uzorcima ozime pšenice (DON-3-Glc i HT-2 toksin) još uvek nisu definisane postojećim regulativama.

U tabeli 4.5 vidi se da učestalost prisustva i nivoi *Fusarium* mikotosina variraju u uzorcima pšenice iz različitih žitnih regiona Srbije. U slučaju DON, maksimalna vrednost određena u Južnoj Bačkoj (309 µg/kg) je pet puta veća od vrednosti određene u Srednjem Banatu

[24] Commission Regulation 1126/2007/EC, OJ L 225, 29.9.2007., str. 16-17 (u daljem tekst: Regulativa Evropske unije (EC, 2007)[24])

(58 µg/kg). Dakle, poredeći žitne regione u Srbiji, prosečna vrednost DON može se predstaviti sledećim opadajućim redom: Južna Bačka > Severna Bačka > Beograd = Severni Banat > Srednji Banat. Neznatna razlika primećena je u pojavi DON-3-Glc kroz ispitane regione u poređenju sa DON: Južna Bačka ≈ Srem > Severni Banat > Srednji Banat. Sa druge strane, zastupljenost HT-2 toksina je najmanja. On je određen u samo tri regiona (tabela 4.5) sa skoro istom koncentracijom. Nadalje, koncentracija *Fusarium* mikotosina u uzorcima ozime pšenice uzete u Toplici i Zaječaru je ispod LOD primenjene metode. Ova pojava može se objasniti činjenicom da su oba regiona na jugu Srbije, suprotno od pomenutih regiona (severni deo) gde je pojava mikotoksina određena. Uočene razlike između severnih i južnih regiona u istoj žetvenoj godini posledica su različitih klimatskih uslova, a samim tim i perioda žetve.

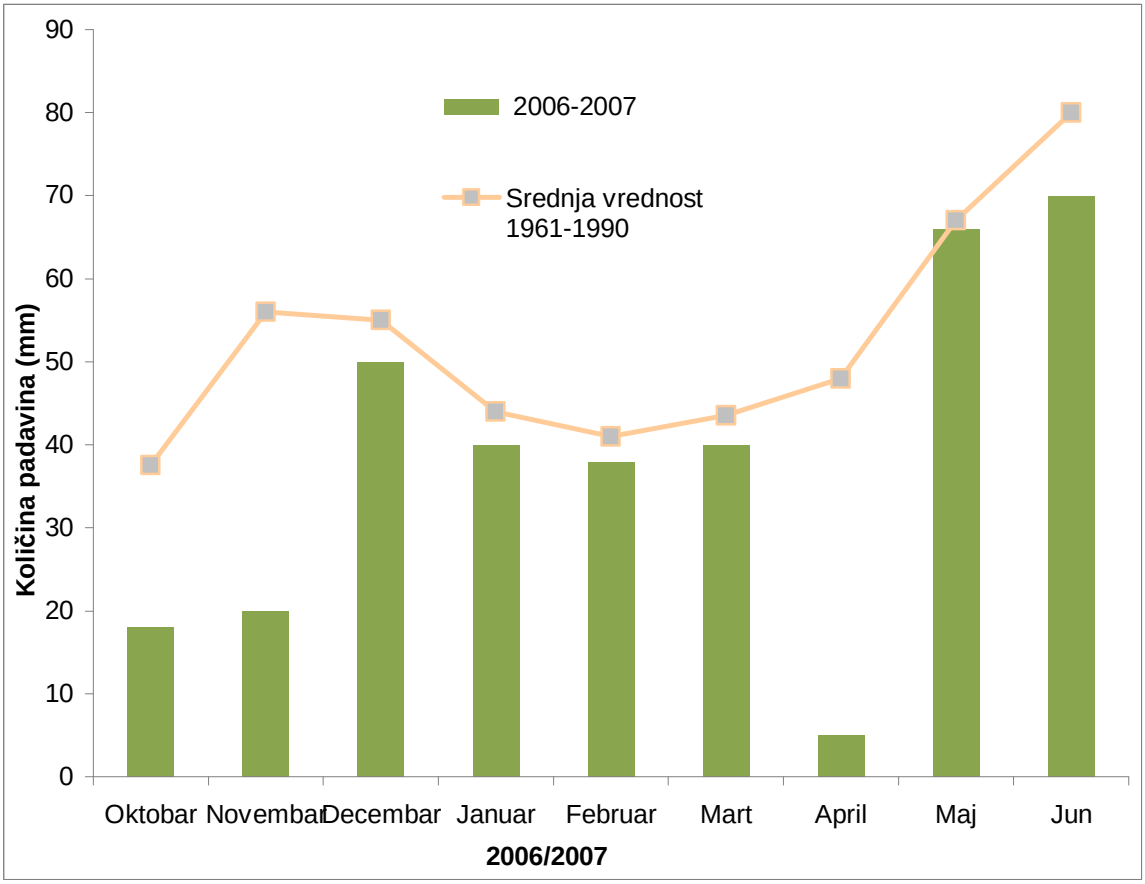
Tabela 4.5. *Srednja vrednost i koncentracioni opseg određenih Fusarium mikotoksina u ispitivanim uzorcima različitih sorti pšenice iz 10 izabranih žitnih regiona Srbije za žetvu 2007*

Region	Mikotoksin					
	DON		DON-3-Glc		HT-2	
	Srednja vrednost[a]	Koncentarcioni opseg[b] (µg/kg)	Srednja vrednost	Koncentracioni opseg (µg/kg)	Srednja vrednost	Koncentracioni opseg (µg/kg)
Južna Bačka	260	211-309	44	41-46	< LOD	< LOD
Zapadna Bačka	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Severna Bačka	108	54-269	< LOD	< LOD	44	< LOD
Južni Banat	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Severni Banat	89	57-111	24	21-30	< LOD	< LOD
Srednji Banat	54	49-58	9	< LOD	< LOD	<LOD
Srem	93	41-145	42	< LOD	66	< LOD
Beograd	89	49-164	< LOD	< LOD	34	<LOD
Zaječar	< LOD	< LOD	< LOD	<LOD	< LOD	<LOD
Toplica	< LOD	<LOD	<LOD	< LOD	< LOD	< LOD

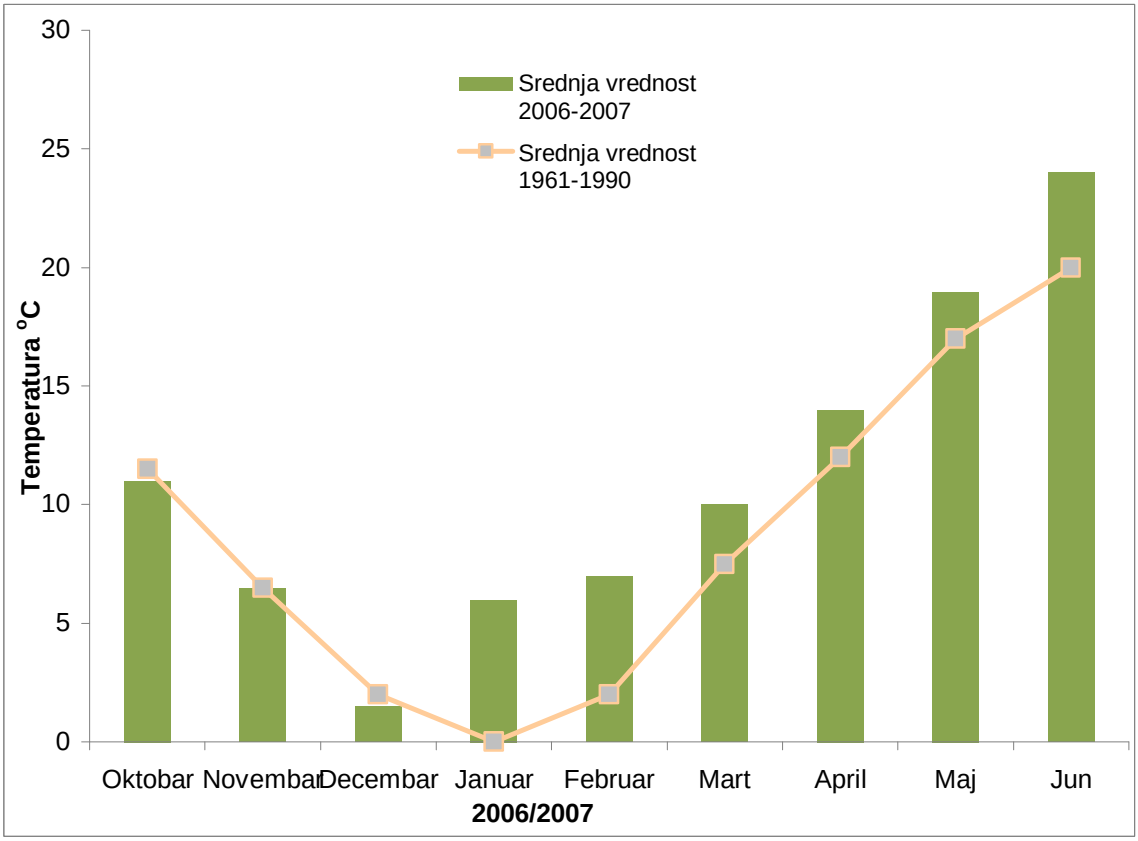
[a] Ako je sadržaj nekog mikotoksina ispod LOD primenjene metode u svim ispitivanim uzorcima, njegova srednja vrednost prikazana je ispod LOD; u suprotnom (kada je deo rezultata pozitivan i iznad LOD, a deo ispod LOD) za izračunavanje srednje vrednosti mikotoksina u svim uzorcima, za količine koje su ispod LOD korišćeno je LOD/2.

[b] U pozitivnim uzorcima

Da bi se razumeo uticaj klimatskih faktora na pojavu *Fusarium* mikotoksina u ispitivanim uzorcima, treba reći da je Srbija smeštena u kontinentalnom klimatskom pojasu i objasniti vremenske uslove u periodu gajenja ispitivane pšenice. Na slici 4.3 prikazano je poređenje količine padavina i srednje vrednosti temperature u Srbiji u periodu od oktobra 2006. do jula 2007. godine sa srednjim vrednostima za višegodišnji period od 1961-1990. godine (podaci dobijeni od Republičkog hidrometeorološkog zavoda Srbije, 2007). Ovaj period definiše poslednje uobičajene vremenske uslove u Srbiji, odnosno aritmetičku sredinu klimatskih elemenata dobijenih za tri uzastopne decenije. Klimatski faktori u periodu 2006/2007. godine su značajni tokom ovog istraživanja, jer ispitivani uzorci pšenice zasejani su tokom oktobra 2006. godine, a njihova žetva bila je tokom jula 2007. godine. Sa slike 4.3b vidi se da su srednje vrednosti temperature tokom 2006/2007. godine slične ili neznatno više u poređenju sa višegodišnjim periodom od 1961-1990. godine, dok je količina padavina manja (slika 4.3a). Ovakvi uslovi ne mogu se smatrati pogodnim za razvoj mikotoksina, jer pojava *Fusarium head blight* (FHB) je usko povezana sa vlažnim vremenskim uslovima, odnosno period vlažnog vremena značajniji je za FHB od količine padavina (JECFA, 2001).



a)



b)

Slika 4.3. *Poređenje mesečnih količina padavina (a) i srednjih vrednosti temperature vazduha (b) tokom 2006/2007. godine sa srednjim vrednostima za višegodišnji period (1961-1990) u Srbiji*

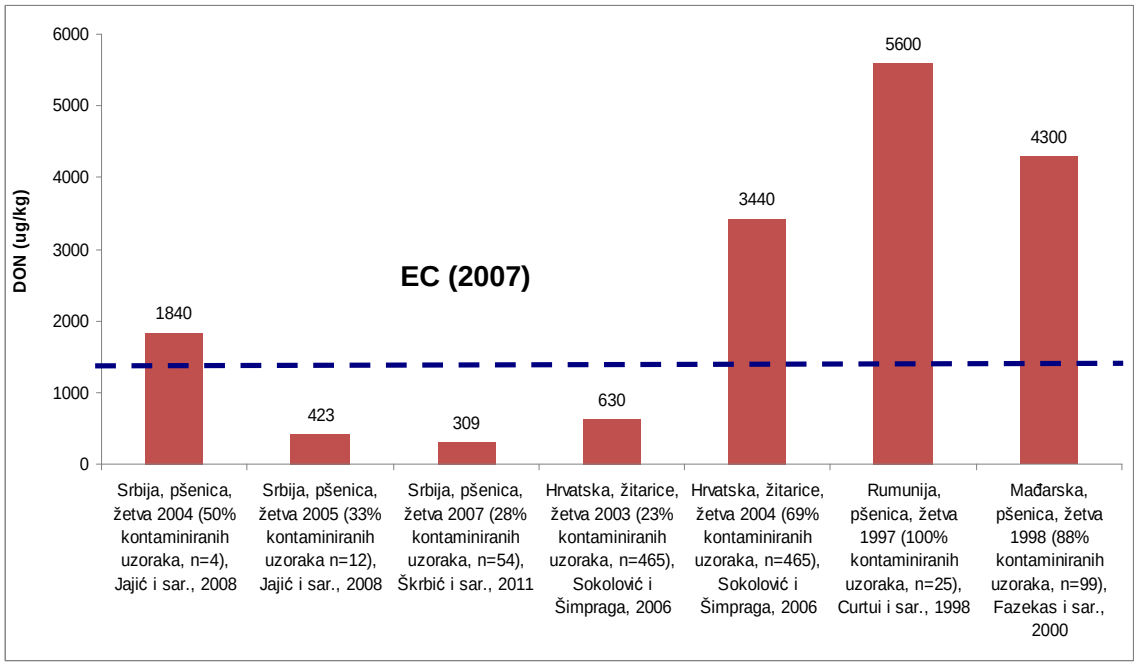
Kao što je prethodno pomenuto *Fusarium* mikotosini nisu identifikovani u uzorcima pšenice koja je uzeta u južnim srpskim regionima Toplici i Zaječaru koje karakteriše specifična mikroklima sa veoma malom količinom padavina. U Zaječaru tokom juna i jula 2007. godine količina padavina bila je 30 mm i 10 mm, redom, što je znatno manje od srednje vrednosti količine padavina u Srbiji za ovaj period (~80 mm i ~60 mm) i srednje vrednosti količine padavina za Južnu Bačku (75 mm i 40 mm). Dakle, sušni vremenski uslovi u regionu Toplice i Zaječara nisu pogodovali razvoju plesni. Međutim, potrebno je naglasiti da razvoj FHB zavisi takođe i od otpornosti ispitivanih sorti (Hajšlová i sar., 2007; Muthomi i sar., 2008). Iz tabele 4.6 vidi se da prosečna koncentracija DON u uzorcima pšenice analiziranih sorti u ovoj studiji ima sledeći trend: Renesansa > Evropa 90 > Pobeda, dok u uzorcima sorti Kraljevice i Nore sadržaj ovog mikotoksina je ispod LOD primenjene metode. Dakle, razlike između sorti uzgajanih u geografski bliskim (severnim) regionima (Evropa 90, Renesansa i Pobeda) ukazuju na različitu osetljivost ispitivanih sorti pšenice ka DON, dok razlike uočene između sorti u južnim (Kraljevice i Nore) i severnim regionima se mogu pripisati različitim klimatskim uslovima tokom rasta pšenice.

***Tabela 4.6**. Koncentracija Fusarium mikotoksina određena u uzorcima pšenice različitih sorti za žetvu 2007. godine u Srbiji*

Sorte ozime pšenice	DON			DON-3-Glc			HT-2		
	Broj kontaminiranih uzoraka (ukupan broj uzoraka)	Opseg (μg/kg)	Prosečna koncentracija (μg/kg)	Broj kontaminiranih uzoraka (ukupan broj uzoraka)	Opseg (μg/kg)	Prosečna koncentracija (μg/kg)	Broj kontaminiranih uzoraka (ukupan broj uzoraka)	Opseg (μg/kg)	Prosečna koncentracija (μg/kg)
EVROPA 90	7 (16)	< LOD-269	119	4 (16)	< LOD-46	27	1 (16)	< LOD-128	128
RENESANSA	6 (16)	< LOD-309	137	3 (16)	< LOD-83	51	2 (16)	< LOD-129	129
POBEDA	2 (16)	< LOD-54	52	0 (16)	< LOD	< LOD	0 (16)	< LOD	< LOD

Podaci o *Fusarium* mikotoksinima u žitaricama koje se gaje u Srbiji veoma se retki, i postoji samo jedna literaturno dostupna studija koja se bavi prisustvom jednog *Fusarium* mikotoksina – DON-a u malom broju uzoraka žitarica u Srbiji (Jajić i sar., 2008). Rezultati dobijeni u ovom istraživanju (Škrbić i sar., 2011a) upoređeni su sa postojećom Regulativom Evropske unije i sa literaturno dostupnim podacima za susedne zemlje Hrvatsku, Bugarsku, Rumuniju i Mađarsku, uzimajući u obzir su da gajeni usevi, agrotehničke mere i klimatski uslovi slični u ovom delu jugoistočne Evrope.

Na slici 4.4. prikazano je poređenje rezultata dobijenih u ovoj studiji sa literaturno dostupnim rezultatima za Srbiju i susedne zemlje prvenstveno za pojavu DON, jer je ovo najčešće analizirani *Fusarium* mikotoksin u žitaricama. Dodatno, na slici 4.4 u cilju što jasnijeg ilustrativnog predstavljanja, dobijeni rezultati upoređeni su sa maksimalno dozvoljenom količinom DON koja je definisana Regulativom Evropske unije (EC, 2007)[24] za neprerađene žitarice osim durum pšenice.



***Slika 4.4**. Poređenje maksimalnih koncentracija DON određenih u žitaricama u Srbiji i susednim zemljama sa maksimalno dozvoljenom količinom DON (1250 µg/kg) definisanom Regulativom Evropske unije (EC, 2007)[24] za neprerađene žitarice osim za durum pšenicu. Procenat učestalosti i broj analiziranih uzoraka u citiranim studijama prikazani su u zagradama.*

U prethodnoj studiji sprovedenoj u Srbiji, Jajić i sar. (2008) ispitali su prisutvo DON u jaroj pšenici (zasejana u maju, požnjevena u oktobru) u dve uzastopne godine 2004/2005 i

procenat pozitivnih uzoraka bio je 50% (2004) i 33% (2005). Razlike između rezultata Jajića i sar. (2008) i rezultata dobijenih u ovoj studiji su posledica vremenskih uslova tokom 2004. i 2005. godine, koje su bile znatno vlažnije u poređenju sa 2006/2007.godinom. Naime, Jajić i sar. (2008) navode da je 2004. godina bila nešto „vlažnija" u odnosu na višegodišnji prosek koji su koristili za poređenje (1971-2000), dok su 2005. godinu okarakterisali kao „ekstremno vlažnu", tj. tokom maja i juna 2004/2005. godine kiše su bile česta pojava, što je pogodovalo razvoju plesni, a negativno je uticalo na razvoj jare pšenice. Tokom ova dva meseca količina padavina bila je 100-125% veća od prosečne vrednosti za višegodišnji period (1971-2000) koji su Jajić i sar. (2008) koristili za poređenje. Nadalje, rezultati ovih autora ukazuju na veći stepen kontaminacije ispitivanih uzoraka pšenice kao i na određenu veću koncentraciju DON tokom „vlažnije" 2004. godine, nego tokom „ekstremno vlažne" 2005. godine. Takođe, potrebno je naglasiti da autori u svom istraživanju ukazuju na činjenicu da veći stepen kontaminacije uzoraka pšenice za žetvu 2004. godine je i posledica neadekvatnog skladištenja uzoraka u ambarima, što je pogodovalo razvoju plesni (jer su uzorci analizirani nakon jedne godine skladištenja). Takođe, Jajić i sar. (2008) su naveli da je broj analiziranih uzoraka bio prilično mali (samo 4 za žetvu 2004; dok je za žetvu 2005 analizirano 12 uzoraka), što ne predstavlja realnu situaciju, već samo preliminarnu procenu sadržaja DON u uzorcima jare pšenice za žetvu 2004. i 2005. godine u Srbiji. Kada je reč o našim susedima, na prostoru zapadne Rumunije, Curtui i sar. (1998) ispitivali su prisustvo mikotoksina u uzorcima pšenice roda 1997. godine i odredili su visok stepen kontaminacije analiziranih uzoraka sa DON i ADON. Klimatski uslovi koji su preovlađivali u letnjim mesecima u zapadnom delu Rumunije, pogodovali su obilnim padavinama pre žetve, što je dovelo do kontaminacije uzoraka *Fusarium* toksinima, i 100% kontaminacije analiziranih uzoraka pšenice sa DON kao i maksimalne koncentracije od 5600 μg/kg (Curtui i sar., 1998; slika 4.2).

Fazekas i sar. (2000) analizirali su 99 uzoraka pšenice roda 1998. godine na prisustvo DON u Mađarskoj, i odredili njegovo prisustvo u 88% uzoraka sa maksimalnom koncentracijom od 4300 μg/kg. Ovako visok procenat pozitivnih uzoraka pšenice u odnosu na DON kao i njegova visoka koncentracija (maksimalno određena vrednost je 3,44 puta veća od maksimalno dozvoljene vrednosti – 1250 μg/kg definisane Regulativom Evropske unije (EC, 2007)[24]) posledica su izuzetno kišnog leta, jer vlažan period pogoduje nastajanju DON-a.

Sokolović i Šimpraga (2006) su analizirali pojavu DON u žitaricama u Hrvatskoj u periodu između 2001. i 2004. godine. Njihovi rezultati pokazali su da od 465 uzoraka uzetih tokom četvorogodišnjeg perioda, 41% ispitanih uzoraka je bio pozitivan na analizirani mikotoksin. Nivo DON u uzorcima pšenice iz 2004. godine u Hrvatskoj bio je veći od maksimalno dozvoljene količine (1250 μg/kg) definisane Regulativom Evropske unije (EC, 2007)[24], jer su 2003 i 2004. godina bile ekstremno tople, sa temperaturama iznad proseka i sa čestim kišama tokom proleća, jeseni i zime, pa su ih autori okarakterisali kao „ekstremno vlažne godine" koje su doprinele pojavi DON (slika 4.2). Dodatno, prema istraživanjima ovih autora, visok procenat pozitivnih uzoraka žitarica sa T-2 toksinom određen je tokom 2004. godine, tj., oko 59% ispitivanih uzoraka bilo je pozitivno, dok je najveća određena

koncentracija bila 520 μg/kg. Nivo T-2 mikotoksina bio je ipod granice detekcije (LOD) u istraživanju koja su bila predmet ove disertacije (Škrbić i sar., 2011a).

Istraživanja sprovedena u Bugarskoj (Manova i Mladenova, 2009) pokazala su da je nivo ZON bio 10 puta manji u odnosu na maksimalno dozvoljenu vrednost od 100 μg/kg definisanu Evropskom Regulativom (EC, 2007)[24] za neprerađene žitarice. U pozitivnim uzorcima nivo ispitivanog toksina je bio do 10 μg/kg, dok u našim istraživanjima ZON nije određen ni u jednom od uzoraka pšenice ispitivanih u okviru disertacije (Škrbić i sar., 2011a).

Neophodno je takođe naglasiti da osim pomenutih susednih zemalja, za ostale zemlje iz okruženja kao što su Makedonija, Bosna i Hercegovina, Crna Gora i Albanija ne postoje relevantni podaci dostupni u literaturi.

Dakle, može se zaključiti da je ovo prvo istraživanje urađeno u cilju istovremenog određivanja osam *Fusarium* mikotoksina u Srbiji, i jedinstveno u regionu zapadnog Balkana. Dobijeni podaci ukazuju da ispitivani uzorci pšenice različitih sorti imaju različitu otpornost na kontaminaciju *Fusarium toksinima*, i stoga je neophodno vršiti sveobuhvatan, dugoročni monitoring iz kojeg će biti moguće dobiti što jasniju sliku o rasprostranjenosti ispitivanih toksina.

4.3. Prisustvo 11 osnovnih („principal“) mikotoksina u pšeničnom brašnu i procena izloženosti

Tokom unutrašnje („*in-house*“) provere kvaliteta i pouzdanosti modifikovane „multi-toksin“ metode za 11 osnovnih („*principal*“) mikotoksina u pšeničnom brašnu dobijeni su zadovoljavajući parametri (Škrbić i sar., 2012). Kalibracione krive su linearne u rasponu od 1 do 1760 μg/kg sa R^2 od 0,9913-0,9990 što ukazaje na dobru linearnost. U svim slučajevima, LOD su manji od maksimalno dozvoljenih vrednosti za mikotoksine definisane Regulativom Evropske unije (EC, 2006)[22], ukazujući na pouzdanost primenjene metode za određivanje mikotoksina prisutnih u tragovima. Prema unutrašnjoj (”*in-house*”) proceduri ”recovery” vrednosti za primenjenu „multi-toksin“ metodu su u rasponu koji je definisan zahtevima Regulative Evropske unije (EU, 2006)[20] osim za FB1 i FB2 čije ”recovery” vrednosti su < 60% (tabela 4.7).

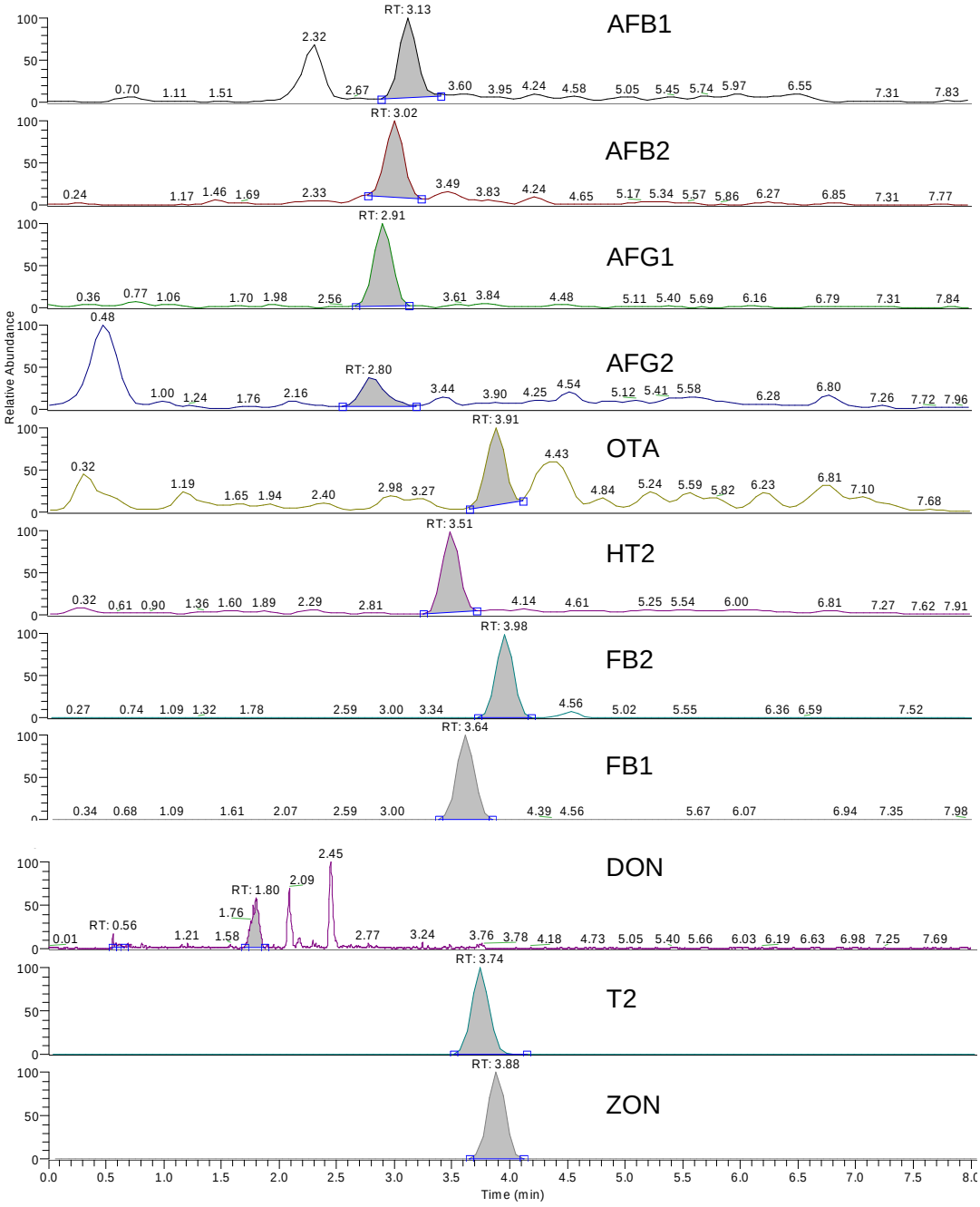
Table 4.7. *Parametri kvaliteta i pouzdanosti UHPLC/HESIQqQ-MS/MS metode primenjene za analizu 11 osnovnih ("principal") mikotoksina u sirovom ekstraktu pšeničnog brašna*

	AFB1	AFB2	AFG1	AFG2	DON	ZON	HT-2	T-2	OTA	FB1	FB2
LOD (µg/kg)	0,7	0,2	0,5	0,9	0,3	0,4	0,9	1,4	2,1	0,05	0,01
LOQ (µg/kg)	2,3	0,7	1,7	3,0	1,0	1,3	3,0	4,7	7,0	0,2	0,03
"Recovery"[a] (%)	121	110	112	113	72	74	130	107	87	<60	<60
"Recovery"[b] (%)	115	100	114	115	n.d.[c]	82	79	136	80	<60	<60

[a] "Recovery" vrednosti dobijene su "in-house" procedurom tj. obogaćivanjem ("spakovanjem") nekontaminiranog uzorka pšeničnog brašna sa mikotoksinima kao što je opisano u poglavlju 3.2.2.
[b] "Recovery" vrednosti dobijene "PT" procedurom opisanom od strane organizatora interlaboratorijskog poređenja organizovanog u 2011. god. od CNR-ISPA, Institute of Sciences of Food Production, Bari, Italija, u okviru MoniQA projekta (www.moniqa.org/mycotoxins): nekontaminirani ("*blank*") uzorak kukuruznog brašna obogaćen ("spajkovan") je smesom mikotoksina (obezbeđenom od PT organizatora) i ostavljen preko noći na sobnoj temperaturi kao što je prethodno opisano.
[c] n.d.: nisu određeni

Dodatno, efikasnost ("recovery") metode proverena je i u interlaboratorijskom poređenju („proficiency test"; PT) analizom uzoraka kukuruza dobijenih u cilju provere efikasnosti određivanja 11 mikotoksina u kukuruzu primenom LC-MS/(MS) tehnike, organizovanom od CNR-ISPA, Institute of Sciences of Food Production, u okviru MoniQA projekta u 2011. godini, koji je finansiran u šestom okvirnom programu Evropske komisije (FP6; www.moniqa.org/mycotoxins). Prema protokolu obogaćivanja („spajkovanja") koji je dobijen od PT organizatora, 20 g „*blank*" kukuruznog brašna obogaćeno („spajkovano") je sa 600 µl smese svih mikotoksina (tj. za PT učesnike sa nepoznatom koncentracijom) i ostavljeno preko noći na sobnoj temperaturi. Sve materijale korišćene u PT poređenju obezbedili su organizatori. Dobijene PT „recovery" vrednosti prikazane su u tabeli 4.7. „Recovery" vrednosti dobijene za mikotoksine kroz unutrašnju („*in-house)*" kontrolu kvaliteta i interlaboratorijsko poređenje su slične (tabela 4.7), osim za HT-2 i T-2 toksine. Dobijene „recovery" vrednosti za FB1 i FB2 su manje od 60% za sirovi ekstrakt kukuruznog brašna (tj. PT materijal) ukazujući na neefikasnu ekstrakciju ovih toksina. Do istih zaključaka došli su Sulyok i sar. (2006) ispitujući prisustvo mikotoksina u sirovom ekstraktu pšenice i kukuruza. Razlike između „recovery" vrednosti koje su dobijene za HT-2 i T-2 toksine sa „*in-house*" i „PT" procedurom su posledica različitog uticaja matriksa (pšeničnog i kukuruznog brašna) tokom procesa jonizacije. Slika 4.5 prikazuje

hromatogram svih analiziranih mikotoksina za najniži kalibracioni nivo u sirovom ekstraktu pšeničnog brašna.



Slika 4.5. *Hromatogrami svih analiziranih mikotoksina za najniži kalibracioni nivo u sirovom ekstraktu pšeničnog brašna.*

„Multi-toksin“ metoda za određivanje 11 osnovnih („*principal*“) mikotoksina primenjena je za analizu uzoraka pšeničnog brašna i dobijeni rezultati prikazani su u tabeli 4.8

(Škrbić i sar., 2012). Dobijeni rezultati određenih mikotoksina korigovani su za „in-house" „recovery" (tabela 4.7). Uzorci u kojima je koncentracija mikotoksina između granice detekcije i kvantifikacije smatrani su „pozitivnim" i njihovi nivoi uključeni su u statističku analizu. Za slučajeve kada je sadržaj mikotoksina u svim ispitivanim uzorcima ispod LOD, njegova srednja vrednost je <LOD; u suprotnom (kada je deo rezultata pozitivan i iznad LOD, a deo ispod LOD), za izračunavanje srednje vrednosti mikotoksina u svim uzorcima, za koncentracije koje su <LOD uzima se LOD/2. Najzastupljeniji mikotoksin u ispitivanim uzorcima je DON koga sledi ZON i T-2 toksin, dok AFB1, AFB2, AFG1, AFG2, OTA, HT-2, FB1 i FB2 nisu određeni ni u jednom od ispitivanih uzoraka. Procenat kontaminacije ispitivanih uzoraka sa DON, ZON i T-2 toksinom je 86,7%, 33,3% i 26,7%, redom. Srednja vrednost i medijana dobijeni za DON su 325 μg/kg i 292 μg/kg, redom. Samo jedan od ispitivanih uzoraka sa koncentracijom DON od 976 μg/kg je iznad maksimalno dozvoljene koncentracije od 750 μg/kg koja je definisana Regulativom Evropske unije (EC, 2006)[22] u žitaricama (uključujući brašno) koje su namenjene za direktnu ljudsku upotrebu, uključujući i brašno. Srednja vrednost dobijena za ZON i T-2 toksin u analiziranim uzorcima je 4,6 i 4,1 μg/kg, redom, dok je njihova medijana <LOD. Maksimalna koncentracija ZON (21,1 μg/kg) određena u uzorcima brašna je ispod maksimalno dozvoljene koncentracije (75 μg/kg) definisane Regulativom Evropske unije (EC, 2006)[22] za žitarice (uključujući brašno) namenjene za direktnu ljudsku upotrebu. Maksimalno određena koncentracija T-2 toksin u svim ispitanim uzorcima je 26,9 μg/kg. Za T-2 toksin maksimalno dozvoljena koncentracija nije definisana Regulativom Evropske unije (EC, 2006)[22].

Tabela 4.8. *Učestalost i nivo analiziranih mikotoksina u pšeničnom brašnu u Srbiji*

Mikotoksini	Broj pozitivnih uzoraka (učestalost, %)	Srednja vrednost[a] (μg/kg)	Medijana (μg/kg)	Koncentracioni opseg[b] (μg/kg)
AFB1	0	< LOD	< LOD	
AFB2	0	< LOD	< LOD	
AFG1	0	< LOD	< LOD	
AFG2	0	< LOD	< LOD	
DON	13(86,7)	325	292	17,5-976
ZON	5 (33,3)	4,6	< LOD	1,9-21,1
FB1	0	< LOD	< LOD	
FB2	0	< LOD	< LOD	
T-2	4 (26,7)	4,1	< LOD	9,8-26,9
HT-2	0	< LOD	< LOD	
OTA	0	< LOD	< LOD	

[a] Ako je sadržaj nekog mikotoksina ispod LOD primenjene metode u svim ispitivanim uzorcima, njegova srednja vrednost prikazana je ispod LOD; u suprotnom (kada je deo rezultata pozitivan i iznad LOD, a deo ispod LOD) za izračunavanje srednje vrednosti mikotoksina u svim uzorcima , za količine koje su ispod LOD korišćeno je LOD/2.
[b] U pozitivnim uzorcima.

Prisustvo DON, ZON i T-2 toksina u komercijalnom pšeničnom brašnu je tema mnogih studija, međutim, nijedna od studija nije do sada izvedena u Srbiji ili zemljama iz okruženja, jedino su 11 osnovnih („*principal*“) mikotoksini bili predmet istraživanja izvedenih u okviru ove disertacije. Relevantni literaturni podaci ukazuju da je DON najčešće identifikovan mikotoksin u brašnu, ali procenat uzoraka u kojima je nivo DON jednak ili veći od 750 μg/kg (maksimalno dozvoljena koncentracija za brašno koje se koristi kao sirovina za prehrambene proizvode) retko prelazi 6-7% (Schothorst i van Egmond, 2004). Prema studiji izvedenoj u jugozapadnoj Nemačkoj 1998. godine (Schollenberger i sar.,1999), procenat učestalosti DON u 134 uzorka belog brašna je bio 78% sa maksimalnom koncentracijom od 624 μg/kg, dok je u slučaju 77 uzoraka integralnog brašna 66% uzoraka bilo pozitivno na ovaj toksin sa maksimalno određenom koncentracijom od 1670 μg/kg. Isto tako, Schollenberger i sar. (2002) ponovo su analizirali prisustvo DON u uzorcima pšeničnog brašna (n=60) iz jugozapadne Nemačke. Procenat zastupljenosti DON u analiziranim uzorcima bio je oko 100%. Prema relevantnim podacima iz literature, određeni nivoi ZON u uzorcima brašna retko su prevazilazili maksimalno dozvoljenu vrednost definisanu Regulativom Evropske uniji (EC, 2006)[22]. Pérez-Torrado i sar. (2010) analizirali su 21 uzorak brašna (kukuruzno, pšenično, pirinčano i ražano brašno i njihove mešavine proizvedene u različitim zemljama) i odredili su, da je sadržaj ZON u ispitivanim uzorcima bio ispod 75 μg/kg što predstavlja maksimalno dozvoljenu vrednost ovog toksina u žitaricama namenjenim za direktnu ljudsku upotrebu u Evropi (Regulativa Evropske unije (EC, 2006)[22]. U Južnoj Koreji, Kassim i sar. (2011) ispitivali su prisustvo T-2 toksina u 15 uzoraka pšenice/pšeničnog brašna i odredili su njegovo prisustvo u 3 uzorka sa visokom koncentracijom od 431 μg/kg, dok je koncentracija HT-2 toksina bila u rasponu od 30,8–355,3 μg/kg. Autori su ovako visok nivo T-2 i HT-2 toksina objasnili kao posledicu neadekvatnog uzimanja uzoraka, pakovanja i skladištenja.

Može se zaključiti da se razlike između rezultata koji su dobijeni u našem istraživanju i drugim studijama mogu pripisati, između ostalog, različitom poreklu žitarica. Međutim, potrebno je naglasiti da je poređenje dobijenih rezultata među zemljama teško zbog različitih analitičkih procedura, klimatskih uslova, skladištenja, vrste i broja ispitivanih proizvoda. Na primer, treba istaći da metode pripreme i određivanja mikotoksina u prethodno pomenutim studijama se baziraju na: ekstrakciji na čvrstoj fazi, dok je identifikacija određena na GC/MS nakon derivatizacije (Schollenberger i sar., 2002); ekstrakciji pod pritiskom, dok je identifikacija određena na HPLC/MS (Pérez-Torrado i sar., 2010); ekstrakciji metanolom/vodom i prešišćavanju dobijenog ekstrakta sa imunoafinitetnim kolonama, dok je identifikacija određena na HPLC/FD nakon derivatizacije (Kassim i sar., 2011). Međutim potrebno je ipak istaći dominantno prisustvo DON u pšeničnom brašnu dobijenom od pšenice zasejane u različitim zemljama uključujući i Srbiju.

U Srbiji se pšenica uglavnom koristi za dobijanje brašna od kojeg se proizvode najrazličitije vrste hleba, kao i razni drugi pekarski proizvodi i testenine. U tom kontekstu, dobijeni

rezultati na prisustvo mikotoksina u uzorcima brašna su upotrebljeni za procenu[25] moguće izloženosti stanovništva mikotoksinima kroz potrošnju brašna i proizvoda na bazi brašna. Suprotno razvijenim zemljama (Thuvander i sar., 2001; Schothorst van Egmond, 2004; Leblanc i sar., 2005a; Boon i sar., 2009) u Srbiji nema podataka u vezi sa procenom unosa mikotoksina kroz potrošnju brašna i proizvoda na bazi brašna. Srednje vrednosti DON, ZON i T-2 toksina (tabela 4.8) određene u analiziranim uzorcima brašna korišćene za procenu izloženosti populacije Srbije analiziranim mikotoksinima primenom metodologije koja omogućava poređenje dobijenih vrednosti sa vrednostima tolerantnih dnevnih unosa (TDI), koje su dobijene na bazi dugoročne izloženosti (FAO/WHO 1997; Thuvander i sar., 2001) i posmatranja potencijalno negativnog uticaja na zdravlje čoveka. U tabeli 4.9 prikazane su vrednosti procenjenog dnevnog unosa DON, ZON i T-2 toksina za prosečnog odraslog potrošača i decu u Srbiji kroz prosečnu potrošnju proizvoda na bazi pšenice (prema potrošačkoj korpi Srbije, Statistički zavod Republike Srbije, 2011). Dnevni unos svakog mikotoksina predstavljen je kao procenat postojećeg tolerantnog dnevnog unosa predloženog od strane Naučnog odbora za hranu (Scientific Committee on Food, SCF, 2000, 2001, 2002) Evropske unije koji iznosi: 1 µg/kg tm/danu za DON, 0,2 µg/kg tm/danu za ZON i 0,06 µg/kg tm/danu za T-2 toksin.

Kao što se vidi iz tabele 4.9, dnevni unos ZON i T-2 toksina kroz potrošnju proizvoda na bazi pšenice, za prosečnu odraslu osobu u Srbiji je ispod postojećih TDI. Međutim, procenjeni dnevni unos DON je iznad postojećeg TDI (tabela 4.9). Ovo ukazuje da pojedinci čija ishrana je bazirana na većoj potrošnji proizvoda na bazi žitarica od prosečne

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[25] Procena dnevne izloženosti stanovništva u Srbiji kroz potrošnju pšeničnog brašna dobijena je kombinovanjem prosečne dnevne potrošnje brašna sa prosečnom koncentracijom mikotoksina koja je određena u ispitivanim uzorcima, na sledeći način:

$$\text{Procena dnevnog unosa mikotoksina (µg/kg tm/dan)} = \frac{[tok\,\text{sin}] * [potrošnja\ brašna]}{[telesna\ masa]}$$

gde [toksin] prosečna koncentracija mikotoksina u (µg/kg) određena u pšeničnom brašnu korigovana za „recovery" (tabela 4.5), [potrošnja brašna] je količina pšeničnog brašna (kg) koju dnevno konzumira prosečan potrošač u Srbiji, [telesna masa] je telesna masa prosečnog potrošača (kg) (tm je skraćenica za telesnu masu). Količina pšeničnog brašna korišćena za procenu dnevnog unosa mikotoksina dobijena je kao ukupna količina brašna koja se koristi za prizvodnju prosečne dnevne porcije hleba, paste, lisnatog testa i keksa prema potrošačkoj korpi Srbije (Statistički zavod Republike Srbije, 2011). Na bazi dokumentovanih proizvođačkih specifikacija proizvoda, dnevno konzumirania ukupna količina brašna korišćena za proizvodnju hleba, paste, lisnatog testa i keksa je 192,9 g, 11,6 g, 16,7 g i 4,8 g, redom. U obzir je uzeta i prosečna dnevna količina brašna od 50,0 g koju potroši prosečan potrošač prema potrošačkoj korpi Srbije (Statistički zavod Republike Srbije, 2011). Na ovaj način, procenjena ukupna prosečna potrošnja pšeničnog brašna je 267,7 g/danu za prosečnog odraslog potrošača. Dakle, iako mikotoksini nisu analizirani u proizodima na bazi pšenice, osnovna pretpostavka je da su ove namirnice prozvedene iz pšeničnog brašna i imaju prosečnu koncentraciju mikotoksina koja je određena u brašnu.

Prosečna telesna masa (tm) za odrasle osobe je 60 kg, a za decu 25 kg.

Procenjeni dnevni unos mikotoksina (u µg/kg tm/danu) je upoređen sa postojećim TDI (µg/kg tm/danu) i procenat TDI za proizvode na bazi pšenice izračunat je na sledeći način:

%TDI = [Procenjeni unos mikotoksina]/[TDI]∗100

potrošnje (u skladu sa potrošačkom korpom Srbije), mogu uneti veću količinu DON tj. sadržaj koji je iznad postojeće toksikološke vrednosti.

Ovo može biti slučaj sa specifičnim potrošačkim grupama, kao što su vegani/makrobiotičari zbog veće potrošnje proizvoda na bazi pšenice u odnosu na prosečnu potrošnju koja je korišćena za procenu u ovoj studiji. Takođe, dokazano je da izloženost dece mikotoksinima kroz potrošnju proizvoda na bazi pšenice je veća nego za odrasle osobe, s obzirom na znatno manju telasnu masu (Boon i sar., 2009). S obzirom da u Srbiji nema podataka o prosečnom dnevnom unosu namirnica na bazi pšenice za decu, a da bi se procenila njihova izloženost mikotosinima, u obzir je uzeta polovina od prosečne potrošnje odraslih osoba (na primer, potrošnja pšeničnog brašna za decu je 133,9 g). Dakle, za decu sa prosečnom telasnom masom od 25 kg (Castillo i sar., 2008) stepen izloženosti DON ne može se zanemariti, jer je dnevni unos 1,7 μg/kg tm/danu što predstavlja 174% od postojeće TDI vrednosti (tabela 4.9). Međutim, postoji nekoliko literaturno dostupnih studija koje su dokazale smanjenje nivoa DON (od 24 do 71%) u hlebu i drugim pekarskim proizvodima u poređenju sa pšeničnim brašnom koje je korišćeno za njihovu poroizvodnju tokom procesa fermentacije i/ili termičke obrade (Samar i sar., 2001; Bullerman i Bianchini, 2007; Kushiro, 2008; Pacin i sar., 2010). Sa druge strane, Berthiller i sar. (2009) utvrdili su povećanje koncentracije DON u nekim proizvodima na bazi žitarica (pšeničnim pahuljicama i krofnama) tokom procesa proizvodnje i ovu pojavu objasnili kao posledicu prisustva pratioca DON ili tzv. „maskiranog" toksina kao što je DON-3-Glc. Stoga promene koncentracije mikotoksina tokom fermentacije i pečenja je značajno odrediti radi dobijanja što realnije slike o prisustvu mikotoksina u proizvodima na bazi žitarica i procene njihovog dnevnog unosa. Potrebno je naglasiti da dobijene vrednosti za dnevni unos toksina treba „smatrati" približne, ali i prve ove vrste koje se odnose na procenu dnevne izloženosti stanovnišva u Srbiji kroz unos proizvoda na bazi pšenice. Pouzdanija procena izloženosti zahteva određivanje sadržaja mikotoksina i u drugim namirnicama iz potrošačke korpe Srbije osim proizvoda na bazi pšenice.

Tabela 4.9. *Procena i poređenje dnevnog unosa DON, ZON i T-2 toksina (μg/kg tm/dan) kroz prosečnu potrošnju pšeničnog brašna i proizvoda na bazi pšenice na osnovu potrošačke korpe Srbije, u odnosu na postojeće TDI vrednosti predložene od Scientific Committee on Food Evropske unije (SCF, 2000, 2001, 2002)*

Mikotoksini	Srednja vrednost[a] (μg/kg)	Unos[b] (μg/kg tm/danu)		(%) od TDI[c]	
		odrasli	deca[d]	odrasli	deca
DON	325	1,5	1,7	145	174
ZON	4,6	0,02	0,02	10,2	12,2
T-2	4,1	0,02	0,02	30,7	36,9

[a] Srednja vrednost koncentracije mikotoksina (Tabela 4.8).
[b] Telesna masa odraslih osoba korišćena za izračunavanje dnevnog unosa je 60 kg, a za decu 25 kg (Castillo i sar.,2008).
[c] Postojeće TDI vrednosti predložene od SCF (2000, 2001, 2002).
[d] Kako u Srbiji nema zvaničnih podataka o potrošnji namirnica za decu, polovina dnevnog unosa proizvoda na bazi pšenice za odrasle osobe je korišćena za svrhu procene dnevne izloženosti.

U analiziranim uzorcima pšeničnog brašna uzetog sa tržišta Srbije (u okviru ove disertacije) koncentracije AFB1, AFB2, AFG1, AFG2, OTA i HT-2 toksina su ispod LOD primenjene metode, i njihovi dnevni unosi ne mogu se smatrati nulom, već je realnije da je to vrednost u rasponu od nule do vrednosti izračunate za polovinu LOD primenjene metode (Bakker i sar., 2003; Škrbić, 2008). U ovakvim slučajevima, tzv. "*middle bound scenario*" može biti usvojen za procenu dnevnog unosa mikotoksina tj. za uzorke gde je sardžaj mikotoksina <LOD primenjene metode, koncentracija mikotoksina korišćena za procenu dnevnog unosa je jednaka polovini LOD (Leblanc i sar., 2005a; Boon i sar., 2009).

Poređenje procenjenih dnevnih unosa u različitim studijama je ograničeno zbog velikih razlika u načinu uzorkovanja, primenjenim analitičkim metodama, broju uzoraka, metodologiji primenjenoj za izračunavanje dnevne izloženosti. Stoga su vrednosti za procenu dnevne izloženosti „orijentacione" i treba ih pažljivo koristiti. Slično proceni koja je dobijena u ovoj disertaciji, prethodne studije koje su se bavile procenom dnevnog unosa mikotoksina potvrdile su činjenicu da je populacija najviše izložena DON kroz unos proizvoda na bazi pšenice i drugih žitarica (Schothorst i van Egmond, 2004; Leblanc i sar., 2005a; Boon i sar., 2009). U Holandiji su Boon i sar. (2009) sproveli istraživanje za procenu rizika dnevne izloženosti dece i utvrdili da dnevni unos DON kroz proizvode na bazi žitarica je 0,3 μg/kg tm/danu za decu uzrasta od 2 do 6 godina, što je oko 5,5 puta manje od vrednosti dobijene za srpsku decu (tabela 4.9). Nadalje, Schothorst i van Egmond (2004) objavili su rezultate za DON, NIV, T-2 i HT-2 toksin kao i procenu dnevnog unosa ovih toksina kroz ispitivane namirnice u okviru SCOOP (Scientific Cooperation on Questions relating to Food), projekta za 2001. god. za zadatak 3.2.10. pod nazivom „Prikupljanje podataka za *Fusarium* toksine u hrani i procena dnevnog unosa ovih toksina kroz ispitane namirnice za stanovnike država članica Evropske unije" ("Collection of occurence data for *Fusarium* toxins in food and assessment of dietary intake by the population of EU Member States"). Oni su dokazali da od *Fusarim* toksina, najzastupljeniji mikotoksin bio je DON, stoga procenjeni unos ovog toksina kroz potrošnju pšenice, pšeničnog brašna i hleba bio je oko 90%. U ovom istraživanju, iako je prosečan unos DON za odrasle osobe ispod TDI vrednosti, za decu je bio vrlo blizu postojeće vrednosti TDI. Tokom istraživanja u okviru SCOOP projekta, ukupni unos HT-2 i T-2 toksina u većini slučajeva je bio iznad postojeće TDI vrednosti. U Francuskoj su Leblanc i sar. (2005a) sproveli istraživanje i došli do saznanja da je francuska populacija najviše izložena mikotoksinima kroz unos proizvoda na bazi žitarica. Ovi autori su utvrdili da je najveći unos DON u odnosu na postojeće toksikološke vrednosti kroz proizvode na bazi žitarica (90%) posebno hleba i dvopeka (45-70%) (Leblanc i sar., 2005a). U Francuskoj, prosečan unos DON za odrasle kroz potrošnju hleba i dvopeka je 188 ng/kg tm/danu, dok je veći unos ovog toksina procenjen za vegetarijansku populaciju, sa prosečnim unosom između 320 i 410 ng/kg tm/danu (Leblanc i sar., 2005a). Procenat populacije za koju procenjeni unos DON prevazilazi postojeću toksikološku vrednost je 0,4% za odrasle, 4% za decu i 4-5% za vegetarijance (Leblanc i sar., 2005a).

4.4. Prisustvo 10 mikotoksina u koštuničavom voću

Vrednosti „recovery“ i preciznosti (RSD, %) određene za razvijenu i kroz unutrašnju („*in-house*“) proceduru kontrole kvaliteta i pouzdanosti proverenu „multi-toksin“ metodu za orah, lešnik, badem i kikiriki prikazane su u tabeli 4.10 (Škrbić i sar., 2014). Rezultati su predstavljeni kao srednja vrednost tri ponavljanja. U ovoj studiji, performanse metode određene su za svaki mikotoksin obogaćivanjem („spajkovanjem“) realnih uzoraka, i upoređene sa zahtevima Regulative Evropske unije (EU, 2006)[20].

„Recovery“ vrednosti analiziranih mikotoksina za sve ispitivane matrikse najpre su određene u odnosu na spoljašnu matriks standard kalibraciju pripremljenu u sirovom nekontaminiranom ekstraktu oraha. Orah je izabran jer je najčešće konzumiran među stanovništvom Srbije, bilo da je proizveden u zemlji ili uvezen iz drugih zemalja. Dodatno, orah je jedina vrsta koštuničavog voća uvrštena u potrošačku korpu Srbije sa mesečnom potrošnjom od 0,1 kg po osobi (Statistički zavod Republike Srbije, 2011). Na ovaj način, korišćenjem orah matriks standard kalibracija za analizu mikotoksina iz ekstrakata badema, lešnika i kikirikija, ispitane su „multi-matriks“ osobine primenjene metode. U slučaju kvantifikacije mikotoksina u odnosu na orah matriks standard kalibracije zadovoljavajuće „recovery“ vrednosti (>60% i ≤130%) dobijene su za većinu mikotoksina za analizirane matrikse, osim za izuzetke objašnjene u tekstu koji sledi (tabela 4.10). „Recovery“ vrednosti za AFB1, AFB2, AFG1 i AFG2 za sve ispitivane matrikse u odnosu na orah matriks standard kalibracije su od 79-120%, što je u skladu sa Regulativom Evropske unije (EC, 2006)[20]. Kao što se vidi iz tabele 4.10 u svim ispitivanim matriksima, „recovery“ vrednosti za FB2 su prihvatljive, sa rasponom od 77-91%, osim u slučaju oraha za koji je dobijen „recovery“ od 140%, ali RSD vrednost je ukazala na njegovu zadovoljavajuću preciznost. Stoga, primenjena metoda sa orah matriks standard kalibracijom može se smatrati „multi-matriks“ metodom za analizu AFB1, AFB2, AFG1, AFG2 i FB2 u različitim vrstama koštuničavog voća.

Izuzeci koji nisu imali prihvatljive „recovery“ vrednosti u odnosu na orah matriks standard kalibracije su sledeći: „recovery“ vrednosti za OTA toksin u lešniku, kikirikiju i bademu jer su >130%; „recovery“ vrednosti za ZON i T-2 toksin u bademu su takođe >130%. Za pomenute izuzetke odgovarajuće matriks kalibracije su pripremljene u cilju provere, da li su postojale nejednakosti među matriksima, koje su uticale na dobijanje višeg ili nižeg signala određivanih mikotoksina. Stoga, da bi se ispitalo da li matriks oraha utiče na jonizaciju OTA toksina na različit način od matriksa lešnika, pojačavajući njegov signal i dajući „recovery“ od 161%, lešnik matriks standard kalibracija je pripremljena u cilju smanjenja uticaja ispitivanog matriksa. U slučaju OTA toksina, potpuna kompenzacija uticaja matriksa postignuta je u odnosu na odgovarajuće matriks standard kalibracije, tj. sirovi ekstrakti („*blank*“) svakog matriksa obogaćeni (spajkovani) su radnim standardnim rastvorom ovog toksina. Primenom orah matriks standard kalibracija dobijene „recovery“ vrednosti za T-2 toksin i ZON u svakom ispitivanom matriksu su do 118% i 126%, redom, što je u skladu sa zahtevima Regulative Evropske unije (EC, 2006)[20], osim za “recovery” vrednosti

za ove toksine u bademu (tabela 4.10). "Recovery" vrednosti za badem se prilično razlikuju od vrednosti dobijenih za druge ispitivane matrikse (orah, lešnik, kikiriki), ukazujući na različit uticaj ovog matriksa na proces jonizacije ovih toksina i njihov MS signal. Dakle, badem matriks standard kalibracije za T-2 toksin i ZON su primenjene i dobijene „recovery" vrednosti su zadovoljavajuće (tabela 4.10). „Recovery" vrednost FB1 za lešnik nije uzeta u obzir, jer lešnik matriks standard kalibracija nije dobijena sa prihvatljivim R^2 (R^2<0,99), dok "recovery" vrednost FB1 za badem je <60% ukazujući na činjenicu da proces ekstrakcije nije omogućio efikasnu izolaciju ovog toksina iz matriksa u acetonitrilni rastvor.

Za HT-2 toksin, orah i lešnik matriks standard kalibracione krive nisu dobijene sa zadovoljavajućim R^2 (R^2<0,99), i prihvatljive "recovery" vrednosti za ovaj toksin dobijene su samo u odnosu na badem i kikiriki kalibracione krive (71% i 102%, redom). Dakle, razlike u sastavu matriksa izazivaju različite smetnje (tj. interferencije) signala mikotoksina od interesa, i utiču na kalibraciju i krajnju kvantifikaciju toksina u sirovim ekstraktima koji su dobijeni od različitih vrsta koštuničavog voća. Različite vrste koštuničavog voća koje su ispitivane imaju različit sadržaj glavnih komponenata matriksa kao što su proteini ili lipidi (na primer, sadržaj protina u orahu je 13%, u bademu 20%, u lešniku je 10% i u kikirikiju 23%; dok sadržaj lipida u analiziranim uzorcima opada u sledećem nizu: lešnik (63%) ~ orah (60%) > badem = kikiriki (47%)). Stoga, za pouzdanu kvantifikaciju mikotoksina korišćene su odgovarajuće matriks standard kalibracije u cilju kompenzacije uticaja matriksa i poboljšanja analitičkih parametara, kada „recovery" vrednosti dobijene u odnosu na orah matriks kalibracione krive nisu bile zadovoljavajuće. Treba imati na umu da je analiza sirovog ekstrakta sa UHPLC-QqQ-MS/MS brza procedura, tako da priprema i injektiranje odgovarajućih matriks standard kalibracija za određene serije uzoraka ne utiču na povećanje ukupnog vremena trajanja eksperimenta.

Nadalje, za sve ispitane mikotoksine, dobra ponovljivost dobijena je za svaki matriks, jer RSD vrednost za svaki analit je manja od 20% (tabela 4.10). Relativna standardna devijacija za primenjenu metodu je oko 11-12% za sve analizirane tipove koštuničavog voća, ukazujući na prihvatljivu preciznost primenjenog metoda za mikotoksine od interesa.

Tabela 4.10. *„Recovery" vrednost (%) i preciznost (RSD, %) primenjene metode za mikotoksine od interesa u uzorcima oraha, lešnika, kikirikija i badema (u zagradama su „recovery" i RSD vrednosti dobijene za odgovarajuće matriks standard kalibracije); van zagrade su „recovery" i RSD vrednosti dobijene u odnosu na orah matriks standard kalibracije*

Mikotoksin	Orah		Lešnik		Kikiriki		Badem	
	Recovery[a] (%)	RSD[a] (%)	Recovery[a] (%)	RSD [a] (%)	Recovery[a] (%)	RSD[a] (%)	Recovery[a] (%)	RSD[a] (%)
AFB1	99	6,22	88	4,40	102	7,00	112	5,45
AFB2	110	8,25	113	6,78	111	7,28	103	8,00
AFG1	101	6,12	79	6,36	79	5,88	96	7,19

Nastavak tabele 4.10

Mikotoksin	Orah		Lešnik		Kikiriki		Badem	
	Recovery[a] (%)	RSD [a] (%)	Recovery[a] (%)	RSD [a] (%)	Recovery[a] (%)	RSD[a] (%)	Recovery[a] (%)	RSD[a] (%)
AFG2	100	6,64	120	8,95	79	7,40	104	6,50
ZON	123	3,29	126	2,88	120	3,77	161 (94)	3,28 (8,00)
OTA	115	2,98	161 (114)	5,57 (3,80)	154 (114)	9,32 (5,24)	182 (120)	7,45 (1,16)
T-2	96	9,67	115	6,14	88	5.44	158 (118)	6,00 (5,64)
HT-2	-[b]	-[b]	- (-)[b]	- (-)[b]	(71)	(8,74)	(102)	(8,21)
FB1	96	11,91	- (-)[b]	-	<60 (98)	(10,78)	<60 (<60)	-
FB2	140	10,61	77	10,83	87	10,60	91	11,87

[a] Rezultati dobijeni na osnovu tri obogaćena („spajkovana“) uzoraka (n=3).

[b] „Recovery“ vrednosti nisu određene jer linearnost matriks standard kalibracija u sirovom ekstraktu oraha i lešnika nije dobijena sa prihvatljivim R^2 (R^2<0,99) (vidi tabelu 4.11).

Linearnost dobijena za matriks standard kalibracione krive (tabela 4.11) procenjena je na osnovu dobijenih R^2. Odlična linearnost (R^2≥0,9903) dobijena je za sve kalibracione krive (tj. za sve analite) pripremljene u matriksu oraha, osim za HT-2 toksin. Kao što se vidi iz tabele 4.11, orah i lešnik matriks standard kalibracije za HT-2 toksin nisu dobijene sa prihvatljivim R^2. Takođe, R^2 je neprihvatljiv za lešnik matriks standard kalibraciju za FB1. Dakle, može se pretpostaviti, da komponente iz sirovog ekstrakta oraha i lešnika ometaju signale ovih toksina tokom analize. U ovim slučajevima neophodno je uvesti korak prečišćavanja („*clean-up*“) sirovog ekstrakta oraha i lešnika u cilju uklanjanja matriks interferencija i dobijanja odgovarajućih matriks standard kalibracionih krivih sa prihvatljivim R^2. Dodatno, kao što je prikazano u tabeli 4.11, R^2 je prihvatljiv za mikotoksine (ZON, OTA, HT-2, T-2, FB1), tj. matriks standard kalibracione krive pripremljene u sirovom ekstraktu lešnika, kikirikija i badema, osim za kikiriki matriks standard kalibraciju za FB1 (0,9689).

U tabeli 4.11 prikazane su LOD i LOQ vrednosti za mikotoksine od interesa za ispitivane matrikse. LOD vrednosti za mikotoksine od interesa u sirovom ekstraktu oraha su između 0,02 i 2,00 µg/kg, dok vrednosti LOQ su u rasponu od 0,07 do 6,66 µg/kg. Procenjene vrednosti LOD (LOQ) za badem su 0,05 µg/kg (0,15 µg/kg) za T-2, 0,13 µg/kg (0,42 µg/kg) za OTA, 0,62 µg/kg (2,05 µg/kg) za FB1, 0,63 µg/kg (2,10 µg/kg) za HT-2 i 0,06 µg/kg (0,21 µg/kg) za ZON. Potrebno je naglasiti da su vrednosti LOD/LOQ za OTA toksin vrlo niske i slične među ispitivanim vrstama koštuničavog voća (tabela 4.11). U slučaju aflatoksina, LOD vrednosti su manje od maksimalno dozvoljenih vrednosti za ove toksine definisane Regulativom Evropske unije (EC, 2010)[21], što ukazuje na pouzdanost primenjenog metoda za određivanje ovih kontaminenata u tragovima.

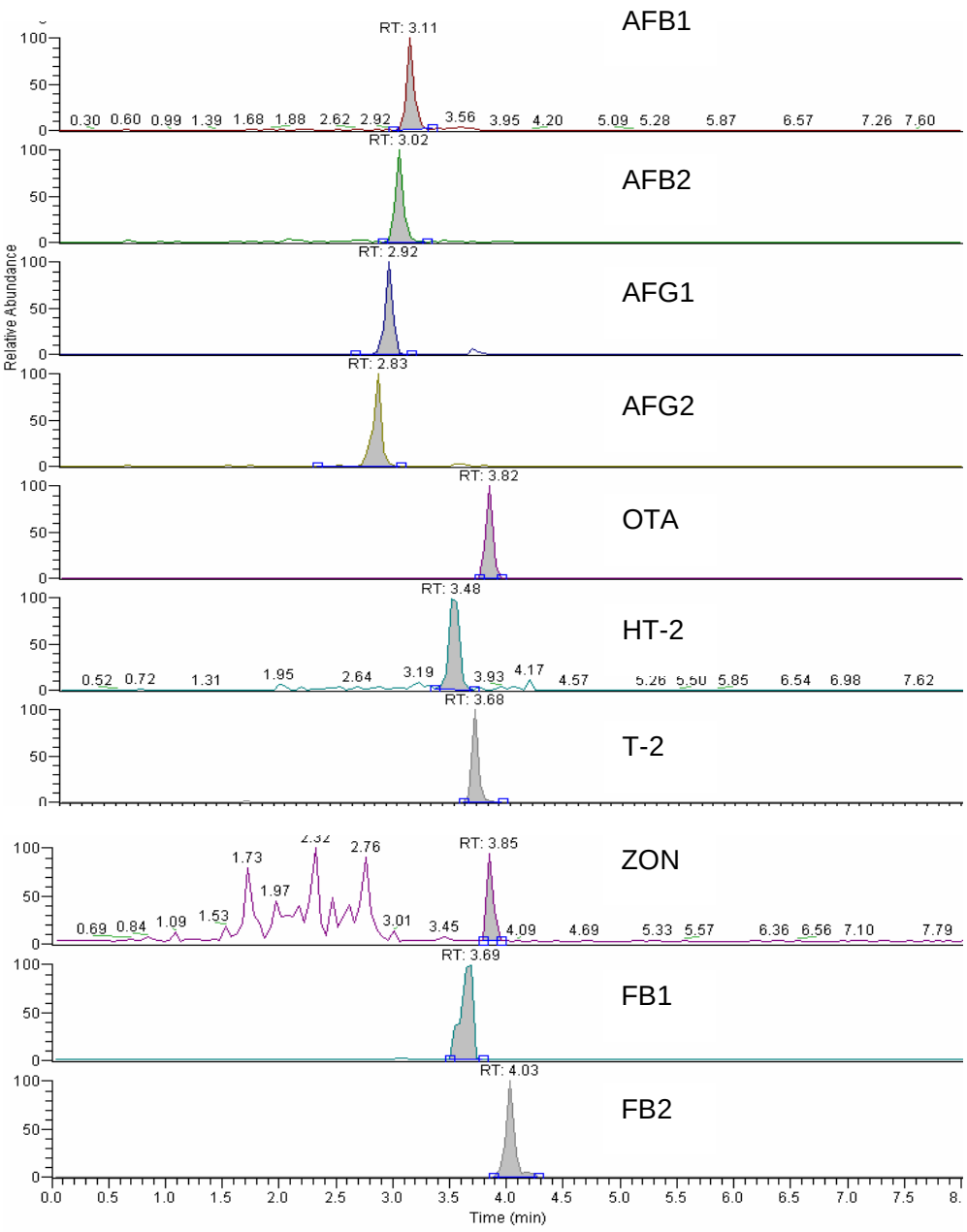
Tabela 4.11. *Karakteristike primenjene "multi-toksin" metode za analizu 10 mikotoksina u različitim tipovima koštuničavog voća.*

Mikotoksini	Orah			Lešnik[a]			Kikiriki[a]			Badem[a]		
	R^2	LOD, µg/kg	LOQ, µg/kg	R^2	LOD, µg/kg	LOQ, µg/kg	R^2	LOD, µg/kg	LOQ, µg/kg	R^2	LOD, µg/kg	LOQ, µg/kg
AFB1	0,9966	1,50	5,00									
AFB2	0,9903	1,20	4,00									
AFG1	0,9907	0,03	0,10									
AFG2	0,9935	0,55	183									
ZON	0,9949	0,06	0,21							0,9951	0,06	0,21
OTA	0,9929	0,02	0,07	0,9960	0,01	0,04	0,9967	0,10	0,33	0,9935	0,13	0,42
T-2	0,9924	2,00	6,66							0,9948	0,05	0,15
HT-2	-[b]	-[b]	-[b]	-[b]	-[b]	-[b]	0,9931	0,89	2,96	0,9959	0,63	2,10
FB1	0,9946	0,24	0,80	-[b]	-[b]	-[b]	0,9689	0,89	2,96	0,9912	0,62	2,05
FB-2	0,9916	0,05	0,17									

[a] Za matriks standard kalibracione krive korišćen je matriks oraha; kada "recovery" vrednosti dobijene za mikotoksine u odnosu na orah matriks standad kalibracije u drugim matriksima nisu bile prihvatljive (<60% ili >130, tabela 4.8), pripremljene su kalibracione krive u odgovarajućim matriksima za svaki mikotoksin.

[b] Lineranost kalibracionih krivih u matriksu oraha i lešnika nije dobijena sa prihvatljivim R^2, (R^2<0,99)

Slika 4.6 prikazuje hromatogram svih analiziranih mikotoksina za najniži kalibracioni nivo u sirovom ekstraktu oraha.



Slika 4.6. *Hromatogrami svih analiziranih mikotoksina za najniži kalibracioni nivo u sirovom ekstraktu oraha.*

U tabeli 4.12 dat je pregled preparativnih i analitičkih metoda uključujući i „recovery“ vrednosti za mikotoksine dobijene u različitim vrstama koštuničavog voća u literaturno dostupnim studijama uzimajući u obzir i rezultate dobijene u našem istraživanju. Kao što se vidi iz tabele 4.12 u većini studija korak prečišćavanja je uključen u metodu pripreme uzoraka, dok ekstrakcija zasnovana na sirovom ekstraktu uzorka je retko korišćena (Abia i sar., 2013; Varga i sar., 2013). Obe metode pripreme uzoraka (sa ili bez koraka prečišćavanja ekstrakta) daju sličan raspon „recovery“ vrednosti, osim u istraživanjima Varga i sar. (2013) gde je za analizirane mikotoksine dobijen „recovery“ raspon od 31-127%. Ovi autori, niske „recovery“ vrednosti dobijene za fumonizin (31-41%) u svim analiziranim matriksima, objašnjavaju činjenicom da „multi-toksin“ metode uključuju veliki broj hemijski različitih analita i „recovery“ vrednosti predstavljaju kompromis između primenjenih analitičkih uslova i broja analiziranih analita. Međutim, suštinska razlika između sirovog i prečišćenog ekstrakta uzorka je u broju određivanih toksina, jer analiza sirovog ekstrakta uzorka omogućava identifikaciju i kvantifikaciju znatno većeg broja mikotoksina u poređenju sa prečišćenim ekstraktom, što ima i vremensku i ekonomsku opravdanost.

Tabela 4.12. *Raspon „recovery“ vrednosti određen za mikotoksine u različitim vrstama koštuničavog voća u literaturno dostupnim studijama*

Izvor	Mikotoksini	Uzorci	Metod pripreme	Analitički metod	“Recovery”, (%)[a]
Set i Erkmen, (2010)	AFB1, AFB2, AFG1, AFG2	Pistaći	Imunoafinitetne kolone	HPLC-FLD	61-89,3%
Huang i sar., (2010)	AFB1, AFB2, AFG1, AFG2	Kikiriki i puter od kikirikija	Kolone pripremljenje u laboratoriji	UHPLC-MS/MS	80,1-86,8%
Luttfullah i Hussain, (2011)	AFB1, AFB2, AFG1, AFG2	Sušeno voće, badem, orah, kikiriki, pistaći	Imunoafinitetne kolone	HPLC-FLD	83,5-92,5%
Rubert i sar., (2011)	AFB1, AFB2, AFG1, AFG2 i OTA	Zemljani badem (“Tiger-nuts”)	Disperziona ekstrakcija na čvrstoj fazi (d-SPE)	LC-MS/MS	71-83%
Baquião i sar., (2012)	AFB1, AFB2, AFG1, AFG2	Brazilski orah	SPE (C18)	HPLC-FLD	80,44-84,26%
Gracía-Cela i sar., (2013)	AFB1, AFB2, AFG1, AFG2 i OTA	Pistaći	Imunoafinitetne kolone	HPLC-FLD	71-122%
			ELISA		72,8-107,9

Nastavak tabele 4.12

Izvor	Mikotoksini	Uzorci	Metod pripreme	Analitički metod	"Recovery", (%)[a]
Abia i sar., (2013)	AFB1, AFB2, AFG1, AFG2, OTA, ZON, T-2, HT-2, FB1 i FB2	Kikiriki i proizvodi na bazi kikirikija	Sirovi ekstrakt	LC-MS/MS	73-86%
Varga i sar., (2013)	AFB1, AFB2, AFG1, AFG2, OTA, ZON, T-2, HT-2, FB1 i FB2	Badem, lešnik, kikiriki i pistaći	Sirovi ekstrakt	UHPLC-MS/MS	31-127%
Škrbić i sar., (2014)	AFB1, AFB2, AFG1, AFG2, OTA, ZON, T-2, HT-2, FB1 i FB2	Orah, lešnik, kikiriki, badem	Sirovi ekstrakt	UHPLC-MS/MS	71-140%

[a]Vrednosti su date kao u citiranim studijama.

Nakon provere kvaliteta i pouzdanosti, „multi-toksin" metoda je primenjena za simultano određivanje mikotoksina od interesa u uzorcima oraha, lešnika, badema i kikirikija. Kvantifikacija analiziranih uzoraka urađena je u odnosu na odgovarajuće matriks standard kalibracione krive dobijene sa prihvatljivim R^2. Dobijeni rezultati ukazuju na slabu kontaminaciju ispitivanih uzoraka (samo dva pozitivna od 17 uzoraka). Koncentracije mikotoksina u većini ispitivanih uzoraka koštuničavog voća su ispod granica detekcije primenjene metode analize. U dva uzorka oraha (domaća, čuvana bez ljuske) ZON je određen u nivoima od 1,20 i 3,48 µg/kg. Dakle, svi ispitani uzorci u skladu su sa Regulativom Evropske unije (EC, 2010)[21], kao i postojećom regulativom u Srbiji (Pravilnik)[23] koja definiše iste maksimalno dozvoljene vrednosti za aflatoksine kao i pomenuta evropska Regulativa.

Koncentracije određivanih mikotoksina u koštuničavom voću u ovom istraživanju znatno su manje u poređenju sa koncentracijama mikotoksina koje su određene za koštuničavo voće u literaturno dostupnim studijama. U Maroku, Zinedine i Mañes (2009) utvrdili su da uzorci oraha i pistaća su kontaminirani sa AFB1 u rasponu od 0,56 do 2500 µg/kg i od 0,04 do 1430 µg/kg, redom. Takođe, u Maroku prosečna koncentracija OTA toksina u pozitivnim uzorcima oraha bila je 0,11 µg/kg (Zinedine i Mañes, 2009). Literaturno dostupni podaci iz Pakistna (Luttfullah i Hussain, 2011), ukazali su da samo dva od deset ispitivanih uzorka oraha (u ljusci) su pozitivna na aflatoksine u nivoima od 7,8 i 13,5 µg/kg i iznad su maksimalno dozvoljene vrednosti za aflatoksine definisane Regulativom Evropske unije (EC, 2010)[21].

Pored mikotoksina (aflatoksina) za koje su maksimalno dozvoljene vrednosti definisane Regulativom Evropske unije (EC, 2010)[21], postoje dokazi o prisustvu OTA toksina i

fumonizina u kikirikiju (Abia i sar., 2013). Abia i sar. (2013) utvrdili su da 40% od 15 analiziranih uzoraka kikirikija je pozitivno na OTA toksin, 73% na FB1 i 33% na FB2, dok su maksimalno određene koncentracije ovih mikotoksina u ispitivanim uzorcima 17 µg/kg, 6 µg/kg i 3 µg/kg, za FB1, FB2 i OTA toksin, redom. Dakle, može se zaključiti da je prisustvo mikotoksina u različitim vrstama koštuničavog voća različito u zavisnosti od geografskog područja i klimatskih uslova.

4.5. Prisustvo teških elemenata u namirnicama potrošačke korpe Srbije i procena izloženosti

U tabeli 4.13 prikazani su rezultati unutrašnje („*in-house*“) kontrole kvaliteta i pouzdanosti metode GFAAS za određivanje sadržaja As, Cd i Pb u ispitivanim uzorcima nakon digestije mikrotalasnim sistemom (Škrbić i sar., 2013b).

Tabela 4.13. *Parametri kontrole kvaliteta i pouzdanosti GFAAS metode primenjene za određivanje sadržaja As, Cd i Pb u namirnicama prema potrošačkoj korpi Srbije*

Uzorak	Elementi	„Recovery“ „in-house“[a] (%)	RSD[a] (%)
Krompir	As	74	20
	Cd	120	7
	Pb	108	8
Šampinjoni	As	74	20
	Cd	86	5
	Pb	132	0.6
Jabuka	As	104	4
	Cd	74	7
	Pb	77	8
Ulje	As	78	5
	Cd	112	2
	Pb	111	7
Keks	As	83	2
	Cd	94	5
	Pb	104	2
Mleko	As	66	15
	Cd	92	2
	Pb	84	8

Nastavak tabele 4.13

Uzorak	Elementi	„Recovery“ „in-house“[a] (%)	RSD[a] (%)
Hleb	As	117	12
	Cd	88	9
	Pb	88	8
Meso	As	60	15
	Cd	73	3
	Pb	89	6
Šećer	As	95	4
	Cd	120	8
	Pb	108	2
Orah	As	71	5
	Cd	80	7
	Pb	120	10
Jaje	As	70.5	5
	Cd	107	13
	Pb	87	6
Začinska paprika	As	60	2
	Cd	99	7
	Pb	80	3

[a] Rezultati dobijeni na osnovu pet obogaćenih („spajkovanih“) uzoraka (n=5)

R^2 su veći od 0,9950 što ukazuje na dobru linearnost kalibracionih krive određivanih elemenata. Dobijene vrednosti LOD (LOQ) za As, Cd i Pb su 0,03 mg/kg (0,07 mg/kg), 0,0003 mg/kg (0,0003 mg/kg) i 0,003 mg/kg (0,003 mg/kg), redom. U slučaju, Cd i Pb, LOD i LOQ vrednosti su u skladu sa kriterijumima za performanse primenjene metode za određivanje sadržaja ovih elemenata, definisane Regulativom Evropske unije (EC, 2011)[26]: tj. ako je maksimalno dozvoljena vrednost ispod 0,1 mg/kg, LOD je manji ili jednak jednoj petini (LOQ je manji ili jednak dve petine) maksimalno dozvoljene vrednosti za ove elemente Regulativom Evropske unije (EC, 2006)[22]; u slučaju kada je maksimalno dozvoljena vrednost veća ili jednaka 0,1 mg/kg, LOD je manji ili jednak jednoj desetini (LOQ je manji ili jednak jednoj petini) maksimalno dozvoljene vrednosti za ove elemente (EC, 2006)[22]. Efikasnost („recovery“) metode određena je obogaćivanjem („spajkovanjem“) uzoraka različitih namirnica (krompir, šampinjoni, jabuke, ulje, keks, mleko, meki sir, meso, hleb, orasi, jaja, šećer i začinska paprika) u nivoima maksimalno dozvoljenih vrednosti definisanih Regulativom Evropske unije (EC, 2006)[22] ili postojećom regulativom u Srbiji (Pravilnik)[23]. Opseg „recovery“ vrednosti je od 60-130% zavisno od vrste uzorka (tabela 4.13). Preciznost merenja analiziranih elemenata izražena je kao relativna standardna

[26] Commission Regulation 836/2011/EC, OJ L215, 20.8.2011, str. 13-15 (u daljem tekstu: Regulativa Evropske unije (EC, 2011)[26])

devijacija (RSD) pet obogaćenih („spajkovanih“) uzoraka, sa rasponom od 1-20% (tabela 4.13); vrednosti RSD dobijene za Cd i Pb u skladu su sa kriterijumima koji su definisani Regulativom Evropske unije (EC, 2011)[26], tj. sa HORRAT vrednosti.

Efikasnost metode („recovery“) proverena je i analizom sertifikovanog referentnog materijala (CRM) GBW 10011. „Recovery“ vrednosti (i %RSD) dobijene za As, Cd i Pb analizom CRM su sledeće: 113% (12%), 92% (8%) i 96% (10%), redom. Dodatno, efikasnost metode proverena je i kroz učešće u interlaboratorijskom poređenju (PT) koje je organizovano od strane FAPAS (tokom jula-avgusta 2012. godine) tj. kroz određivanje sadržaja Pb u začinskoj paprici. Dobijena efikasnost metode (i %RSD) kroz PT-test je 80% (4%). Dakle, svi podaci provere kvaliteta ukazuju na pouzdanost primenjene metode za određivanje tragova As, Cd i Pb u ispitivanim uzorcima.

GFAAS metoda za As, Cd i Pb primenjena je za određivanje sadržaja ovih elemenata u uzorcima namirnica potrošačke korpe Srbije. Koncentracije As, Cd i Pb dobijene za ispitivane uzorke namirnica prikazane su u tabeli 4.14 i rezultati su predstavljeni kao srednja vrednost tri analizirana uzorka svake namirnice. Uzorci u kojima je sadržaj elemenata između LOD i LOQ vrednosti primenjene metode analize, smatraju se „pozitivnim“ i njihovi sadržaji uključeni su u statističku analizu. Ako je sadržaj nekog elementa ispod LOD primenjene metode u svim ispitanim uzorcima, njegova srednja vrednost prikazana je <LOD; u suprotnom (kada je deo rezultata pozitivan i iznad LOD, a deo ispod LOD), za izračunavanje srednje vrednosti elemenata u svim uzorcima, za količine <LOD korišćeno je LOD/2 (Škrbić i Čupić, 2005).

Tabela 4.14. *Sadržaj i dnevni unos As, Cd i Pb iz različitih tipova namirnica koje čine potrošačku korpu Srbije*

Element	Namirnica	Srednja vrednost[a] (mg/kg)	Dnevni unos (µg/dan)	Maksimalno dozvoljena vrednost (mg/kg), Važeća regulativa u Srbiji (Pravilnik)[23]	Maksimalno dozvoljena vrednost (mg/kg), Regulativa Evropske unije (EC, 2006)[22]	EU[b] (mg/kg)	Prva francuska studija za ukupnu potrošačku korpu (Leblanc i sar., 2005b) (mg/kg)
As	Krompir	<0,03	2,097	0,3		0,002	
	Luk	<0,03	0,452	0,3			
	Šampinjoni	<0,03	0,0012	0,3		0,09	
	Zelena salata	<0,03	0,275	0,3			
	Kupus	<0,03	0,710	0,3			
	Pasulj	<0,03	0,242	0,3			
	Šargarepa	<0,03	0,258	0,3			

Nastavak tabele 4.14

Element	Namirnica	Srednja vrednost[a] (mg/kg)	Dnevni unos (µg/dan)	Maksimalno dozvoljena vrednost (mg/kg), Važeća regulativa u Srbiji (Pravilnik)[23]	Maksimalno dozvoljena vrednost (mg/kg), Regulativa Evropske unije (EC, 2006)[22]	EU[b] (mg/kg)	Prva francuska studija za ukupnu potrošačku korpu (Leblanc i sar., 2005b) (mg/kg)
As	Jabuka	<0,03	1,371	0,3		0.006-0.014	
	Pomorandža	<0,03	0,210	0,3			
	Banana	<0,03	0,242	0,3			
	Orasi	<0,03	0,048			0,006-0,029	0,169
	Suve šljive	<0,03	0.017	0.5			
	Ulje	0,03	1,244	0,1		0,003-0,005	0,045
	Margarin	0,03	0,210	0,1			0,060
	Keks	<0,03	0,137	0,5		<0,1	0,003
	Čokolada	<0,03	0,033	0,5		0.0128	0.007
	Bomboni	<0,03	0,033	0,005			
	Mleko	<0,03	2,178	0,1		<0,005-0,003	0,003
	Jogurt	<0,03	1,049	0,1		0,002	
	Tvrdi sir	<0,03	0,048	0,1			
	Meki sir	<0,03	0,485	0,1		0,004	0,003
	Jaja	<0,03	0,645	0,1		0,0009-0,005	0,008
	Beli hleb	<0,03	4,001	0,5		0,005	0,04
	Druge vrste hleba	<0,03	0,258	0,5			
	Pasta	<0,03	0,162	0,5		0,018	0,003
	Pšenično brašno	<0,03	0,726	0,5			
	Morska riba, oslić	<0,03	0,177	4		9,7	
	Riba u konzervi	0,43	1,577				
	Juneće meso	<0,03	0,113	0,1		0,0053	
	Svinjsko meso	<0,03	0,645	0,1		0,01	
	Pileće meso	<0,03	0,726	0,1			

Nastavak tabele 4.14

Element	Namirnica	Srednja vrednost[a] (mg/kg)	Dnevni unos (µg/dan)	Maksimalno dozvoljena vrednost (mg/kg), Važeća regulativa u Srbiji (Pravilnik)[23]	Maksimalno dozvoljena vrednost (mg/kg), Regulativa Evropske unije (EC, 2006)[22]	EU[b] (mg/kg)	Prva francuska studija za ukupnu potrošačku korpu (Leblanc i sar., 2005b) (mg/kg)
As	Slanina	<0,03	0,081				
	Kobasica	0,04	0,485				
	Viršla	<0,03	0,072				
	Salama	<0,03	0,227				
	Pašteta	<0,03	0,065				
	Začinska paprika	<0,03	0,024	5,0			
	Šećer	<0,03	0,564	1,0		0,005	0,018
	Ukupan dnevni unos		21,89				
Cd	Krompir	0,009	1,049	0,1	0,05	0,010-0,0686	
	Luk	0,003	0,075	0,1	0,05	0,002-0,1288	
	Šampinjoni	0,005	0,0004	0,2	0,2	0,016-0,081	
	Zelena salata	<0,0003	0,003	0,2	0,2	0,013-0,1514	
	Kupus	<0,0003	0,007	0,2	0,2	0,005-0,0862	
	Pasulj	<0,0003	0,002				
	Šargarepa	<0,0003	0,003	0,1	0,05	0,031-0,12	
	Jabuka	0,001	0,128	0,05	0,05	0,0029-0,2025	
	Pomorandža	<0,0003	0,002	0,05	0,05		
	Banana	<0,0003	0,002	0,05	0,05		
	Orasi	<0,0003	0,0005			0,0588-0,198	0,0187
	Suve šljive	0,003	0,004	0,3			
	Ulje	0,001	0,029	-	-	0,006	0,0004
	Margarin	<0,0003	0,0008	-			0.0004
	Keks	0,013	0,126	0,05	0,1		0,0004
	Čokolada	0,034	0,080	0,2			0,0004

Nastavak tabele 4.14

Element	Namirnica	Srednja vrednost[a] (mg/kg)	Dnevni unos (µg/dan)	Maksimalno dozvoljena vrednost (mg/kg), Važeća regulativa u Srbiji (Pravilnik)[23]	Maksimalno dozvoljena vrednost (mg/kg), Regulativa Evropske unije (EC, 2006)[22]	EU[b] (mg/kg)	Prva francuska studija za ukupnu potrošačku korpu (Leblanc i sar., 2005b) (mg/kg)
Cd	Bomboni	0,028	0.066	-			
	Mleko	0,001	0,160	0,01	-	0,0002-0,006	0,0004
	Jogurt	<0,0003	0,010	0,02			
	Tvrdi sir	<0,0003	0,005	0,1		0,003	0,0004
	Meki sir	0,0008	0,003	0,1			
	Jaja	<0,0003	0,006	0,05		0,0006-0,005	0,0004
	Beli hleb	0,021	6,187	0,05		0,0284-0,04	0,0048
	Druge vrste hleba	0,006	0,114	0,05			
	Pasta	0,008	0,099	0,05		0,05	0,0022
	Pšenično brašno	0,010	0,552	0,1		0,03-0,05	
	Morska riba, oslić	0,003	0,048	0,05	0,05	0,005	
	Riba u konzervi	0,029	0,087				
	Juneće meso	<0,0003	0,001	0,05	0,05	0,004	
	Svinjsko meso	<0,0003	0,006	0,05	0,05		
	Pileće meso	<0,0003	0,007	0,05	0,05	0,013	
	Slanina	0,003	0,022				
	Kobasica	0.033	0.488				
	Viršla	0,003	0,019				
	Salama	<0,0003	0,002				
	Pašteta	0,007	0,041			0,10-0,12	
	Začinska paprika	0,118	0,191			0,070-0,112	
	Šećer	0,060	1,880			0,004-0,005	0,0004
	Ukupan dnevni unos		11,51				

Nastavak tabele 4.14

Element	Namirnica	Srednja vrednost[a] (mg/kg)	Dnevni unos (µg/dan)	Maksimalno dozvoljena vrednost (mg/kg), Važeća regulativa u Srbiji (Pravilnik)[23]	Maksimalno dozvoljena vrednost (mg/kg), Regulativa Evropske unije (EC, 2006)[22]	EU[b] (mg/kg)	Prva francuska studija za ukupnu potrošačku korpu (Leblanc i sar., 2005b) (mg/kg)
Pb	Krompir	<0,003	0,210	0,1	0,1	0,003-0,34	
	Luk	<0,003	0,045	0,1	0,1		
	Šampinjoni	0,006	0,0003	0,3	0,3	0,16-0,226	
	Zelena salata	0,080	1,356	0,3	0,3	0,018	
	Kupus	0,050	2,190	0,3	0,3	0,05	
	Pasulj	0,060	0,895		0,1		
	Šargarepa	0,060	0,956	0,1	0,1	0,011	
	Jabuka	0,013	1,545	0,1	0,1		
	Pomorandža	0,085	1,545	0,1	0,1		
	Banana	0,060	1,254	0,1	0,1		
	Orasi	0,010	0,0003			0,01-0,12	0,022
	Suve šljive	0,263	0,376	3,0			
	Ulje	0,023	0,669	0,1	0,1	0,005-0,089	0,005
	Margarin	0,014	0,07	0,1			0,004
	Keks	0,010	0,087	0,4	0,2		0,008
	Čokolada	0,143	0,303	1,0		0,031	0,028
	Bomboni	0,323	0,683	0,5			
	Mleko	0,011	1,902	0,02	0,02	0,004-0,05	0,003
	Jogurt	0,030	2,495	0,4		0,0024-0,02	
	Tvrdi sir	<0,003	0,048	1,0		0,031-0,058	0,016
	Meki sir	0,045	0,172	1,0		0,006	
	Jaja	<0,003	0,065	0,25		<0,001-0,021	0,011
	Beli hleb	0,133	40,298	0,4		<0,040-0,025	0,026
	Druge vrste hleba	0,150	2,933	0,4			
	Pasta	0,010	0,123	0,4			0,012

Nastavak tabele 4.14

Element	Namirnica	Srednja vrednost[a] (mg/kg)	Dnevni unos (µg/dan)	Maksimalno dozvoljena vrednost (mg/kg), Važeća regulativa u Srbiji (Pravilnik)[23]	Maksimalno dozvoljena vrednost (mg/kg), Regulativa Evropske unije (EC, 2006)[22]	EU[b] (mg/kg)	Prva francuska studija za ukupnu potrošačku korpu (Leblanc i sar., 2005b) (mg/kg)
Pb	Pšenično brašno	0,071	3,906	0,4			
	Morska riba, oslić	<0,003	0,018	0,3	0,3		
	Riba u konzervi	0,011	0,027				
	Juneće meso	0,038	0,320	0,1	0,1		
	Svinjsko meso	0,020	0,968	0,1	0,1	0,05	
	Pileće meso	0,098	5,329	0,1	0,1		
	Slanina	0,030	0,182				
	Kobasica	0,04	0,720				
	Viršla	<0,003	0,007				
	Salama	0,010	0,180				
	Pašteta	0,040	0,205				
	Začinska paprika	0,080	0,160				
	Šećer	<0,003	0,056	1,0		0,0054-0,039	0,036
	Ukupan dnevni unos		72,30				

[a] Ako je sadržaj nekog elementa ispod LOD primenjene metode u svim ispitanim uzorcima, njegova srednja vrednost prikazana je <LOD; u suprotnom (kada je deo rezultata pozitivan i iznad LOD, a deo ispod LOD), za izračunavanje srednje vrednosti elemenata u svim uzorcima, za količine <LOD korišćeno je LOD/2.

[b] Koncentracioni raspon ili srednja vrednost koncentracije teških elemenata određena u analiziranim namirnicama u zemalja članicama Evropske unije (SCOOP, 2004).

Kao što se vidi iz tabele 4.14 As je određen samo u četiri namirnice: ribi u konzervi (0,43 mg/kg), kobasicama (0,04 mg/kg), ulju (0,03 mg/kg) i margarinu (0,03 mg/kg).

Sadržaj As u margarinu i ulju je u nivou LOD primenjene metode analize, što je skoro tri puta manje od masimalno dozvoljene vrednosti za As u ovim proizvodima što je definisano postojećom regulativom u Srbiji (Pravilnik)[23]. U drugim uzorcima analiziranih namirnica sadržaj As je <LOD.

Kvantifikovani sadržaj Cd u analiziranim namirnicama je ispod maksimalno dozvoljene vrednosti koja je definisana Regulativom Evropske unije (EC, 2006)[22] ili postojećom regulativom u Srbiji (Pravilnik)[23] za ovaj element. Namirnice u kojima je određen Cd, mogu se prikazati prema opadajućem sadržaju ovog elementa: začinska paprika > čokolada > bomboni = riba u konzervi > beli hleb > šećer > kobasice > keks > krompir, zatim, pasta, pašteta, drugi tipovi hleba, šampinjoni, luk, suve šljive, slanina, viršla, oslić, dok je najniži sadržaj Cd u ulju, mleku, jabukama, pšeničnom brašnu i tvrdom siru.

Takođe, sadržaj Pb u uzorcima ispitivanih namirnica je ispod maksimalno dozvoljenih vrednosti koje su definisane Regulativom Evropske unije (EC, 2006)[22] ili postojećom regulativom u Srbiji (Pravilnik)[22]. Za većinu namirnica sadržaj Pb je jednak ili manji od 0,08 mg/kg, osim u slučaju hleba, čokolade, suvih šljiva i bombona, gde je sardžaj ovog elementa od 0,133 do 0,323 mg/kg.

Sadržaj Cd za krompir, šampinjone, margarin, meki sir, oslić, paštetu, pileće meso, pšenično brašno, pastu, jaja, zelenu salatu, kupus, šargarepu i juneće meso je sličan ili manji od (minimalne) vrednosti Cd određenog u namirnicama koje su analizirane u zemalja članicama Evropske unije, dok je suprotan slučaj za ulje, keks, čokoladu, mleko, šećer i začinsku papriku (SCOOP, 2004; Leblanc i sar., 2005b). Koncentracija Cd određena u hlebu odgovara sadržaju ovog elementa u SCOOP izveštaju (2004), dok je ova vrednost skoro četiri puta veća od vrednosti određene u hlebu u Francuskoj (Leblanc i sar., 2005b). Dodatno, vrednosti Pb određene u povrću i voću (krompir, šampinjoni, jabuke), keksu i mekom siru iz potrošačke korpe Srbije manje su od vrednosti za iste proizvode koji su analizirani u zemalja članicama Evropske unije (tabela 4.14), dok su za ulje, margarin, čokoladu, mleko, hleb, svinjsko meso, pastu, tvrdi sir, jogurt, šargarepu, kupus, zelenu salatu vrednosti Pb slične ili manje od vrednosti dobijenih u SCOOP izveštaju (2004) ili prvoj francuskoj studiji za ukupnu potrošačku korpu (Leblanc i sar., 2005b). Sadržaj As (0,03 mg/kg) određen u uzorcima ulja koji su uzeti sa srpskog tržišta je sličan koncentracijama određenim tokom prve francuske studije za ukupnu potrošačku korpu (Leblanc i sar., 2005b), ali mora se pomenuti da je ova vrednost 10 puta veća od vrednosti koja je objavljena u SCOOP izveštaju (2004). Interesantno je primetiti (tabela 4.14) da su maksimalne vrednosti određene za neke namirnice u zemljama članicama Evropske unije veće od maksimalno dozvoljenih vrednosti; na primer, koncentracije Cd u uzorcima luka i jabuka su 4 puta veće od dozvoljenih vrednosti, dok vrednosti Pb u krompiru i mleku su 3,5 puta iznad dozvoljene vrednosti. Slično ovome, Millour i sar. (2011) utvrdili su sadržaj Pb za neke od ispitivanih uzoraka mleka (3/38) iznad vrednosti dozvoljene za Pb od 0,020 mg/kg; u delimično obranom mleku sadržaj Pb je 2 puta iznad maksimalno dozvoljene vrednosti, dok u druga dva slučaja, neobranom i obranom mleku sadržaj ovog elementa je neznatno iznad maksimalno dozvoljene vrednosti. Dodatno, Millour i sar. (2011) utvrdili su da je od analiziranog povrća, spanać imao najveći sadržaj Cd (n=16; srednja vrednost je 0,073 mg/kg), ali određene vrednosti su bile ispod maksimalno dozvoljene vrednosti.

Nadalje, srednje vrednosti As, Cd i Pb (date u tabeli 4.14.) korišćene su za procenu dnevne izloženosti[27] stanovništva Srbije kroz potrošnju analiziranih namirnica, tj. za izračunavanje dnevnog unosa elemenata od interesa. Dnevni unos elemenata kroz potrošnju namirnica zavisi od određene koncentracije elemenata u analiziranim namirnicama i količine njihove potrošnje. U tabeli 4.14 prikazani su procenjeni dnevni unosi As, Cd i Pb za prosečnog odraslog potrošača u Srbiji kroz unos namirnica iz potrošačke korpe Srbije (Statistički zavod Republike Srbije, 2011). Kao što se vidi iz tabele 4.14 dnevni unos As i Cd kroz unos namirnica od intresa je ispod postojećih toksikoloških vrednosti (210 μg/dan (EFSA, 2009) i 58 μg/dan (JECFA, 2011), redom), dok se u slučaju Pb procenjeni unos ne može zanemariti, jer je skoro dva puta veći od postojeće toksikološke vrednosti za bubrežnu funkciju (44 μg/dan) (EFSA, 2010). Sa druge strane, unos Pb manji je od toksikološke vrednosti za kardiovaskularnu funkciju (105 μg/dan) (EFSA, 2010).

Procenjeni unos As za prosečnog odraslog potrošača Srbije kroz ispitivane namirnice je 21,89 μg/dan, dok je unos Cd skoro dva puta manji od unosa As (11,51 μg/dan, tabela 4.14). Potrebno je reći da ispitivane namirnice daju mali doprinos dnevnom unosu As i Cd u odnosu na postojeće toksikološke vrednosti. Suprotno unosu As i Cd, veći unos Pb (72,30 μg/dan, tabela 4.14) procenjen je za namirnice potrošačke korpe Srbije. Najveći doprinos unosu sva tri elementa daje hleb, zbog njegove visoke potrošnje. Dodatno, visokom unosu Pb značajno doprinosi visoka koncentracija ovog elementa određena u hlebu.

Potrebno je napomenuti da su dostupna relevantna istraživanja koja su se bavila određivanjem sadržaja teških elemenata u različitim namirnicama u cilju pronalaženja glavnih izvora emisije ovih elemenata i procenom njihovog dnevnog unosa kako za celokupnu populaciju, tako i za visoko rizične grupe stanovništva (Llobet i sar., 2003; SCOOP, 2004; Leblanc i sar., 2005b; Rubio i sar., 2005; Rubio i sar., 2006; Becker i sar., 2011; Martorell i sar., 2011; Hernández-Martínez i Navarro-Blasco, 2012; Domingo i sar., 2012; Arnich i sar., 2012). Stoga su procenjeni dnevni unosi za As, Cd i Pb (Škrbić i sar., 2013b) kroz analizirane namirnice upoređeni sa literaturno dostupnim

[27] Procena dnevne izloženosti stanovništva u Srbiji kroz potrošnju ispitivanih namirnica zasnovana je na prosečnoj dnevnoj potrošnji namirnica i srednjoj vrednosti sadržaja As, Cd i Pb koje su određene u analiziranim uzorcima:

Procena dnevnog unosa elemenata (μg/danu) = [element] • [prosečna dnevna potrošnja namirnica]

gde je [element] koncentracija ispitivanih elemenata (mg/kg) u ipitivanim namirnicama korigovana za vrednosti „recovery" (Tabela 4.13), [prosečna dnevna potrošnja namirnica] je količina namirnica koju prosečan potrošač Srbije konzumira dnevno.

Količina namirnica korišćena za proračun dnevnog unosa dobijena je na osnovu potrošačke korpe Srbije (Statistički zavod Republike Srbije, 2011) (Tabela 3.7).

podacima (Llobet i sar., 2003; SCOOP, 2004; Leblanc i sar., 2005b; Rubio i sar., 2005; Rubio i sar., 2006; Becker i sar., 2011; Martorell i sar., 2011; Domingo i sar., 2012; Arnich i sar., 2012) (tabela 4.15), u cilju dobijanja prve uporedne procene dnevne izloženosti stanovništva Srbije sa stanovnicima drugih zemalja. Pomenute studije ukazale su na značajne razlike u dnevnom unosu As (Llobet i sar., 2003; SCOOP, 2004; Leblanc i sar., 2005b; Martorell i sar., 2011; Domingo i sar., 2012; Arnich i sar., 2012; tabela 4.15). Prema istraživanjima sprovedenim u Kataloniji, zemljama članicama Evropske unije, prvoj i drugoj studiji za ukupnu potrošačku korpu u Francuskoj, utvrđeno je da su riba i drugi morski plodovi glavni izvor As u ishrani odraslih osoba. Naime, tokom prve francuske studije za ukupnu potrošačku korpu, Leblanc i sar. (2005b) utvrdili su da riba, drugi morski plodovi i voće najviše doprinose izloženosti stanovništva As (49-50%, 8-13% i 15-17%, redom), za razliku od ostalih proizvoda, čiji doprinos je zanatno manji sa udelom od 5% u ukupnoj izloženosti ovom elementu. Nadalje, tokom druge francuske studije za ukupnu potrošačku korpu, procenjeni dnevni unos As kroz potrošnju ribe bio je manji (oko 30%) (Arnich i sar., 2012). Interesantno je naglasiti da potrošačka korpa Srbije obuhvata samo jednu vrstu morske ribe (oslić) i jednu vrstu morske ribe u konzervi sa veoma malom zastupljenošću i potrošnjom među stanovništvom (poglavlje 3.3; tabela 3.7). Doprinos analizirane ribe procenjenom unosu As za prosečnog potrošača Srbije je veoma nizak (8%).

Tabela 4.15. *Poređenje rezultata dobijenih u različitim studijama za procenjeni dnevni unos As, Cd i Pb u organizam čoveka*

Izvor	Dnevni unos µg/dan			Lokacija
	As	Cd	Pb	
Llobet i sar.,(2003)	223,59	15,73	28,37	Španija (Katalonija)
SCOOP, (2004)	125	14,4	42	Zemlje članice Evropske Unije
Leblanc et al., (2005b)	62	2,7	18,4	Francuska
Rubio i sar., (2005)	-	-	72,8	Španija (Kanarska ostrva)
Rubio i sar., (2006)	-	11,165	-	Španija (Kanarska ostrva
Becker i sar., (2011)	n.d.[a]	10,0	7,0	Švedska

Nastavak tabele 4.15

Izvor	Dnevni unos µg/dan			Lokacija
	As	Cd	Pb	
Martorell i sar., (2011)	328	19,5	101	Španija (Katalonija)
Domingo i sar., (2012)	199	49,5	19,8	Španija (Katalonija)
Arnich i sar., (2012)	54,60	10,99	14,07	Francuska
Škrbić i sar., (2013b)	21,89	11,51	72,30	Srbija

[a] Nije određivano

Kao što se vidi iz tabele 4.15, dnevni unos Cd dobijen za prosečnog potrošača u Srbiji u skladu je sa dnevnim unosima ovog elementa koji su određeni u istraživanjima Llobet i sar. (2003), SCOOP (2004), Rubio i sar. (2006), Becker i sar. (2011) i Arnich i sar. (2012). Procenjeni dnevni unos Cd određen za prosečnog potrošača u Srbiji je 4 puta manji od dnevnog unosa ovog elementa za odraslu populaciju Katalonije (2012), dok je najniža dnevna izloženost za Cd određena za francusku odraslu populaciju tokom prve studije za ukupnu potrošačku korpu (2005) (tabela 4.15). Prema rezultatima dobijenim u istraživanju Becker i sar. (2011) za potrošačku korpu Švedske, glavni izvori Cd bili su proizvodi na bazi žitarica (48%), krompir (19%) i povrće (11%). Slično ovome, tokom prve francuske studije za ukupnu potrošačku korpu (2005) najveći unos Cd u odnosu na postojeće toksikološke vrednosti procenjen je za povrće tj. doprinos unosu Cd u organizam čoveka za povrće i skrobno povrće bio je 21-23 i 21-27%, redom. Nadalje, u istraživanjima Arnich i sar. (2012) tokom druge francuske studije za ukupnu potrošačku korpu, glavni izvori Cd za stanovništvo Francuske bili su hleb i brašneni proizvodi (22% i 13%, redom) i krompir i proizvodi na bazi krompira (12% i 14%, redom). Takođe, u SCOOP studiji (2004) određeno je da su voće, povrće, žitarice, meso i riba glavni izvori Cd u ishrani stanovništva u zemljama članicama Evropske unije. Mora se naglasiti da iako je dnevni unos Cd znatno ispod postojećih toksikoloških vrednosti, žitarice i povrće čine 2/3 od ukupnog dnevnog unosa ovog elementa za stanovništvo u zemljama članicama Evropske unije. Suprotno ovome, u SCOOP studiji utvrđeno je da prehrambene grupe kao što su mlečni proizvodi, ulja i masti, jaja i pića vrlo malo doprinose dnevnom unosu Cd.

Najveći dnevni unos Pb procenjen je za stanovništvo Katalonije (Martorell i sar., 2011; tabela 4.15). Tokom ovog istraživanja, Martorell i sar. (2011) ukazali su na povećanje dnevnog unosa Pb kroz namirnice u periodu od 2000-2008, što je suprotno značajnom smanjenju nivoa Pb u atmosferi. Suprotno ovome, rezultati istraživanja Becker i sar. (2011) ukazuju na smanjenje dnevnog unosa Pb kroz namirnice potrošačke korpe za 2,5 puta tj. od 17 µg/dan za istraživanja tokom 1987. godinu do 7 µg/dan za istraživanja sprovedena

tokom 1999. godine. Dodatno, autori potkrepljuju svoja istraživanja činjenicom da je smanjeni nivo Pb u namirnicama rezultat korišćenja bezolovnog benzina kao i drugih preduzetih mera u cilju smanjenja emisije Pb u Švedskoj.

U skladu sa ovim treba pomenuti da je u Srbiji upotreba olovnog benzina skoro zabranjena (2010. godine). Dnevni unos Pb za prosečnog odraslog potrošača Srbije je oko 1,5 puta manji od dnevnog unosa ovog elementa za odrasle potrošače Katalonije (Martorell i sar., 2011). Međutim, dnevni unos Pb određen za stanovništvo Srbije sličan je procenjenom unosu ovog elementa za stanovništvo Kanarskih ostrva (Rubio i sar., 2005), dok je 10 i 4 puta veći od unosa procenjenih za stanovništvo Švedske (Becker i sar., 2011) i Francuske (Leblanc i sar., 2005b), redom (tabela 4.15). Glavni izvor Pb u ishrani stanovništva Srbije je hleb što je u skladu i sa drugim istraživanjima. Međutim, postoji više faktora koji utiču na procenu dnevnog unosa ispitivanih elemenata u različitim istraživanjima. Najvažniji faktor koji ima uticaj na unos elemenata je izbor prehrambenih grupa čije namirnice će biti analizirane. Drugi značajni faktori su razlike u načinu uzorkovanja, primenjenoj analitičkoj metodi, broju uzoraka, metodologiji korišćenoj za izračunavanje dnevnog unosa. Takođe, prisustvo (ili odsustvo) određene namirnice sa visokom (ili niskom) koncentracijom ispitivanih elemenata i koja se konzumira u većoj (ili manjoj) količini može imati značajan uticaj na procenu unosa analiziranih elemenata za prehrambenu grupu kojoj namirnica pripada.

5. ZAKLJUČAK

Na osnovu postavljenih ciljeva u disertaciji, sprovedenih istraživanja i rezultata predstavljenih u prethodnom poglavlju, zaključci do koji se došlo su:

- Brza i jednostavna „multi-rezidualna“ metoda bazirana na primeni napredne spregnute tehnike tj. ultra-pritisne tečne hromatografije sa hibridnim trostrukim (tripl) kvadrupolnim „ion trap“ masenim analizatorom (UHPLC–QqLIT–MS/MS) uspešno je proverena kroz unutrašnju (“*in-house*”) proceduru kontrole kvaliteta i pouzdanosti i primenjena za određivanje 81 farmaceutski aktivne komponente (PhAC) u različitim tipovima vode. Postignute su visoke vrednosti koeficijenata determinacije iznad 0,99 za sve ispitivane komponente. Granice detekcije su u rasponu od 0,01-26 ng/l. Efikasnost metode tj. „recovery“ vrednosti bile su veće od 50% za većinu PhAC od interesa. Samo, za neke komponente dobijene su niže „recovery“ vrednosti, i to u pojedinim matriksima kao što je voda za piće. Ovo se može objasniti činjenicom da primenjeni eksperimentalni uslovi nisu odgovarajući za sve komponente od interesa što predstavlja i jedan od nedostataka multi-rezidualnih metoda, jer se ne mogu postići optimalni uslovi za sve ciljane analite, te se stoga, male „recovery“ vrednosti za pojedine komponente prihvataju kao kompromis između primenjenih analitičkih uslova i broja analita. Dakle, male „recovery“ vrednosti za pojedine PhAC komponente ne mogu se smatrati nedostatkom za njihovo određivanje u ispitivanim uzorcima, naročito zbog toga što su postignute granice detekcije i kvantifikacije primenjene metode prilično niske. Četrdest sedam od ukupno 81 ispitivane komponente određeno je u ispitivanim uzorcima površinske, podzemne, pijaće i otpadne vode. Ispitivane farmaceutski aktivne komponente određene su u ispitanim uzorcima vode u koncentracionom rasponu od nekoliko ng/l do više od 1 µg/l. Veće koncentracije ibuprofena, diklofenaka, kodeina, valsartana, acetaminofena, 2-hidroksikarbamazepina i 10,11-epoksikarbamazepina određene su u gradskoj otpadnoj vodi. Dobijeni rezultati upoređeni su sa relevantnim podacima u dostupnoj literaturi, na osnovu čega se može zaključiti da su određene koncentracije za većinu ispitivanih komponenata u različitim tipovima vode u opsegu vrednosti karaktrističnih za analizirane tipove vode, osim u slučaju metabolita karbamazepina tj. 2-hidroksikarbamazepina i 10,11-epoksikarbamazepina čije visoke koncentracije su određene u otpadnoj vodi sa područja Novog Sada. Dobijeni rezultati predstavljaju prve podatke do sada objavljene o sadržaju 81 farmaceutski aktivne komponente u različitim tipovima vode sa područja Srbije.

- „Multi-toksin“ metoda bazirana na naprednoj spregnutoj tehnici tj. ultra-pritisnoj tečnoj hromatografiji sa trostrukim (tripl) kvadrupolnim masenim analizatorom

(UHPLC–QqQ–MS/MS) razvijena je za određivanje prirodnih mikotoksina u sirovim ekstraktima različitih uzoraka. Razvijena metoda proverena je kroz unutrašnju („*in-house)*“ proceduru za proveru kvaliteta i pouzdanosti njene primenjivosti, kao i kroz interlaboratorijsko poređenje. Parametri provere bili su prihvatljivi (R^2>0,99; LOD je u opsegu od 0,01-5 µg/kg; recovery je u opsegu od 69-130% za većinu mikotoksina od interesa u uzorcima pšenice i brašna) i u skladu sa zahtevima relevantne Evropske Regulative za većinu mikotoksina od interesa. U slučaju fumonizina metoda ekstrakcije je bila modifikovana zakišeljavanjem ekstrakcione smese pri čemu je dobijena zadovoljavajuća efikasnost metode (77-140%); u suprotnom fumonizini se ne mogu izdvojiti. S obzirom da pri analizi sirovog ekstrakta radi identifikacije i kvantifikacije ispitivanih mikotoksina, a usled uticaja komponenti iz matriksa može doći do „lažno-pozitivnih“ ili „lažno-negativnih“ rezultata, korišćena je kalibracija primenom tzv. matriks standarda. Modifikacija metode ekstrakcije je uvedena za uzorke sa većim udelom masnoća, tj. uveden je korak odmašćivanja odgovarajućih sirovih ekstrakata sa heksanom. Efikasnost modifikovane metode je od 71-140%. Na taj način, primenom odgovarajućih modifikacija u zavisnosti od vrste uzorka i ciljanih mikotoksina pokazano je da razvijena multi-toksin metoda je primenjiva na različite matrikse dokazujući istovremeno i njenu multi-matriks primenu. Razvijena metoda primenjena je na različite uzorke sa srpskog tržišta, omogućavajući po prvi put u Srbiji pouzdano, ponovljivo i selektivno određivanje toksina od interesa prisutnih u tragovima u analiziranim uzorcima. Prisustvo mikotoksina od interesa u većini ispitivanih uzoraka je ispod određenih granica detekcije, dok su pozitivni uzorci ispod maksimalno dozvoljenih vrednosti definisanih postojećom evropskom ili srpskom regulativom.

- Metoda korišćena za određivanje sadržaja teških elemenata primenom atomskog apsorpcionog spektrometra sa grafitnom kivetom proverena je kroz unutrašnju („*in-house*“) kontrolu kvaliteta i puzdanosti, interlaboratorijsko poređenje i analizu sertifikovanog referentnog materijala i dobijeni parametri provere potvrdili su njenu primenjivost. Opseg koncentracija u ispitivanim uzorcima je: <LOD-0,43 mg/kg za arsen, <LOD-0,118 mg/kg za kadmijum i <LOD-0,150 mg/kg za olovo. Rezultati analize sadržaja arsena, olova i kadmijuma u različitim uzorcima sa srpskog tržišta, poslužili su za procenu izloženosti srpske populacije toksičnim teškim elementima. Dobijeni rezultati ukazali su na moguć povećan procenjeni unos olova (72,30 µg/dan) u organizam čoveka, što je posledica doskorašnje upotrebe olovnog benzina u Srbiji, čija upotreba je zabranjena 2010. god. Procenjeni unos aresena (21,89 µg/dan) i kadmijuma (11,51 µg/dan) dobijen u ovoj disertaciji niži je ili sličan u poređenju sa unosom ovih elemenata u dostupnim evropskim studijama. Dobijeni rezultati predstavljaju prve do sada objavljene podatke koji se odnose na procenu izloženosti srpske populacije unosom arsena, kadmijuma i olova kroz konzumirane namirnice zastupljene u prosečnoj potrošačkoj korpi Srbije.

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7. PRILOG

Radovi koji su objavljeni na osnovu rezultata istraživanja u okviru rada na doktorskj disertaciji

M21 – Rad u vrhunskom međunarodnom časopisu

1. Petrović, M., Škrbić, B., Živančev, J., Ferrando-Climent, L., Barcelo, D. Determination of 81 pharmaceutical drugs by high performance liquid chromatography coupled to mass spectrometry with hybrid triple quadrupole–linear ion trap in different types of water in Serbia. Science of the Total Environment 468–469 (2014) 415–428. (IF=3,258)

2. Škrbić, B., Živančev, J., Godula, M. Multimycotoxin analysis of crude extracts of nuts with ultra-high performance liquid chromatography/tandem mass spectrometry. Journal of food composition and analysis 34 (2014) 171-177. (IF=2,088)

3. Škrbić, B., Živančev, J., Mrmoš, N. Concentrations of arsenic, cadmium and lead in selected foodstuffs from Serbian market basket: Estimated intake by the population from the Serbia. Food and Chemical Toxicology 58 (2013) 440–448. (IF=3,010)

4. Škrbić, B., Živančev, J., Đurišić-Mladenović N., Godula, M. Principal mycotoxins in wheat flour from the Serbian market: Levels and assessment of the exposure by wheat-based products. Food Control 25 (2012) 389-396. (IF=2,738)

5. Škrbić, B., Malachova, A., Živančev, J., Veprikova, Z., Hajšlová, J. Fusarium mycotoxins in wheat samples harvested in Serbia: A preliminary survey. Food Control 22 (2011) 1261-1267. (IF=2,738)

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Determination of 81 pharmaceutical drugs by high performance liquid chromatography coupled to mass spectrometry with hybrid triple quadrupole–linear ion trap in different types of water in Serbia

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HIGHLIGHTS

- Comprehensive study of 81 pharmaceuticals in different water types from Serbia was conducted.
- Forty seven compounds of 81 drugs were found in analyzed water samples.
- A widespread occurrence of drugs in waters was proven from ng L^{-1} to more than 1 μg L^{-1}.
- Low levels of pharmaceuticals were found even in underground and drinking waters.
- The highest concentrations of pharmaceuticals were found in municipal waste water samples.

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ABSTRACT

The aim of the work was to study the occurrence of pharmaceuticals in waste, surface, underground, and drinking water samples collected in Serbia. A multi-residue method for the analysis of 81 pharmaceutical drugs from different therapeutic classes in the various types of water was applied. Twenty-five composite water samples were prepared using solid-phase extraction and the presence of 81 pharmaceutical compounds in the extracts was analyzed by ultra-high performance liquid chromatography coupled to mass spectrometry with hybrid triple quadrupole–linear ion trap (UPLC–QqLIT–MS/MS). Forty seven compounds of 81 drugs were found in four different types of analyzed water. The highest concentrations of ibuprofen of 20.1 μg L^{-1}, 10,11-epoxycarbamazepine of 16.2 μg L^{-1}, 2-hydroxycarbamazepine of 15.9 μg L^{-1} and acetaminophen of 15.7 μg L^{-1} were found in municipal waste water sample. Results revealed the presence of salicylic acid in 41.67% of water samples, carbamazepine in 36.11%, propranolol and irbesartan in 30.56%. The obtained results were discussed in relation to the relevant data available in literature. This is the first attempt to assess the occurrence of these 81 pharmaceutical residues in water samples in Serbia.

1. Introduction

In recent years, pharmaceuticals, an important group of emerging contaminants in the environment, have attracted worldwide attention (Gros et al., 2006; Pérez and Barceló, 2007; López-Roldán et al., 2010; Ferreira da Silva et al., 2011; López-Serna et al., 2013). The usage and consumption are increasing consistently due to the discoveries of new drugs, the expanding population, and the inverting age structure in the general population (Daughton, 2003). For instance, in the European Union (EU) around 3000 different pharmaceutically active compounds (PhACs) are approved for use in human medicine. Although the effects of the pharmaceuticals are investigated through safety and toxicology studies, the potential environmental impacts of their production and use are less understood and have recently become a topic of research interest (Ferreira da Silva et al., 2011).

Pharmaceuticals, usually used in human and veterinary medicine, can enter the aquatic environment as parent compounds, metabolites or conjugates of both. Sometimes, these compounds are not managed properly and are discharged directly into drains, while at other times they are not completely metabolized by the human body and are excreted via urine and feces. Thus, the occurrence of pharmaceuticals in the environment leads to various unanswered questions with regard to their biological potency in flora, fauna, and humans. Generally, very little is known about the long-term effect and behavior of pharmaceutical

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residues in the aquatic environment, and in groundwater in particular (López-Serna et al., 2010). Also, it is unknown if the combination of drugs that share a common mechanism of action exhibits toxic synergistic effect (Hernando et al., 2004).

Additionally, one of main concerns is contamination of groundwater through surface water filtration and landfill leakage. The presence of pharmaceuticals in environmental waters, especially in drinking water and raw waters used for its production should be considered as important issue in terms of human health safety (Gros et al., 2012). Therefore, it is necessary to identify and monitor the occurrence of pharmaceuticals in various environmental waters.

Although, PhACs are new contaminants, the information concerning their concentration and environmental fate has been reported in the last decade in a number of studies (Terzić et al., 2008; Grujić et al., 2009; López-Roldán et al., 2010; Vazquez-Roig et al., 2010; Ferreira da Silva et al., 2011; Jelić et al., 2011; Valcárcel et al., 2011; López-Serna et al., 2012). The concentrations of individual compounds in surface waters are typically in the range of several tens to hundreds of ng L^{-1}, although concentrations at the μg L^{-1}level are also reported for some compounds and specific sites (Kolpin et al., 2002). These concentrations are generally considered too low to pose an acute risk for humans; however, it is still unknown whether other receptors in non-target organisms, like aquatic organisms, are sensitive to individual pharmaceutical residues, or their mixtures. To date, only in a few cases, pharmaceuticals have been detected at trace levels in drinking water (Stolker et al., 2004; Hummel et al., 2006; Mompelat et al., 2009). Nevertheless, there is still limited knowledge on concentration, fate and effect of drugs in the environment and they have not yet been included in any environmental regulation.

Furthermore, environmental risk assessment is often carried out for individual pharmaceutical compound (active ingredients), while pharmaceutical compounds are typically detected in mixtures with other anthropogenic contaminants (Kolpin et al., 2002). Monitoring of wide-range pharmaceuticals in surface and ground waters is as a prerequisite for proper risk assessment.

In the light of these concerns, the aim of the present work was to study the occurrence and distribution of pharmaceuticals in waste, surface, underground, and drinking water collected in the northern part of Serbia. Therefore, 81 pharmaceuticals belonging to different therapeutic groups (i.e. analgesics and anti-inflammatory drugs, antidiabetic drugs, psychiatric drugs, histamine H1 and H2 receptor antagonists, antihypertensives drugs, antibiotics, β-blockers, diuretics, lipid regulator and cholesterol lowering statin drugs, anti-histamines, antiplatelet drugs, prostatic drugs, anticoagulant drugs, X-ray contrast agents, antihelmintics, synthetic glucocorticoid, sedation and muscle relaxation, tranquilizer, calcium channel blockers) were monitored in different type of Serbian water. This is the first comprehensive study on the simultaneous occurrence of pharmaceuticals in different types of water from Serbia, and this information is necessary and of high priority in order to assess the risk of exposure to these compounds.

2. Materials and methods

2.1. Pharmaceuticals selected

Target compounds analyzed in this study belong to different medical classes and they were selected taking into account different selection criteria. First of all, there is a need for comprehensive study of as wide as possible range of PhAC in the Serbian waters, particularly in those from the northern part of Serbia, which is dominated almost entirely by the drainage basin of the river Danube and could therefore reflect water contamination by drugs throughout Eastern Europe. Hence, this study aims to complement the scarce studies (Moldovan, 2006; Grujić et al., 2009) that report results on PhAC from this region. Within the groups of compounds, analyzed in this study, some were selected because of their high consumption and ubiquity in the aquatic environment (e.g. ibuprofen is one of the most frequently prescribed pharmaceuticals in Europe previously reported as being one of the most common drug in surface water (Farré et al., 2001; Vanderford et al., 2003; Roberts and Thomas, 2006; Martinez Bueno et al., 2010; Ferreira da Silva et al., 2011; Valcárcel et al., 2011)) while some were targeted due to their known persistency (carbamazepine, β-blockers, diclofenac, etc.). In order to get as much as possible information on simultaneous occurrence of pharmaceuticals in different types of water in Serbia the most comprehensive method found in literature is applied. This method allows determination of 81 compounds that were included in this report (Table 1).

2.2. Chemicals and reagents

All pharmaceutical standards (see Table 1) were of high purity grade (>90%).

The solvents, HPLC grade methanol, acetonitrile, water (Lichrosolv) and formic acid 98% were provided by Merck (Darmstadt, Germany). Nitrogen for drying 99.9990% of purity was from Abello Linde, S.A. (Spain). A Milli-Q-Advantage system from Millipore Iberica, S.A. (Spain) was used to obtain HPLC-grade water. Ammonium formiate, ammonium acetate, and ammonia were from Sigma-Aldrich (Steinheim, Germany).

The cartridges used for solid phase extraction were Oasis HLB (60 mg, 3 mL) from Waters Corporation (Milford, MA, USA).

The individual standard solutions as well as isotopically labeled internal standard solutions were prepared on a weight basis in methanol. After preparation, standards were stored at −20 °C. Fresh stock solutions of antibiotics were prepared monthly due to their limited stability while stock solutions for the rest of substances were renewed every 3 months. A mixture of all pharmaceuticals was prepared through appropriate dilution of individual stock solutions in methanol–water (10:90, v/v) and was renewed before each analytical run. A separate mixture of isotopically labeled internal standards, used for internal standard quantification, was prepared in methanol and further diluted in methanol–water (10:90, v/v) mixture.

2.3. Sampling sites and sample collections

Sampling was performed in spring 2012 and included 25 locations in Serbia (i.e. northern part, Fig. 1). The majority of samples from Serbia were untreated industrial and municipal waste water, surface, underground, and drinking water. The list of samples analyzed and description is given in Table 2. All the analyzed samples were composite ones prepared by mixing of grab samples. Appropriate sampling was ensured by public water company "Vode Vojvodine". Manual sampling was performed by trained inspectors, which are authorized for official control of contaminants in these types of samples. Briefly, for each type of water, five grab samples of equal volume (500 mL for surface, underground and drinking water and 200 mL for effluent) were taken discontinuously at constant intervals over a period of 2 h and blended.

Water samples were collected in 500 mL amber PET bottles, previously rinsed with ultrapure water. Once prepared collected samples were kept at 4 °C until arrival to the laboratory and processed within 48 h.

2.4. Sample preparation

Procedures for preparation of water samples for instrumental analysis were described in detail previously (Gros et al., 2009).

Target compounds were extracted from water samples by solid phase extraction (SPE) (Oasis HLB cartridges, 3 cm^3, 60 mg; Waters Corporation, Milford, MA) using a Baker vacuum system (J.T. Baker, Deventer, The Netherlands). Briefly, 60 mg SPE cartridges were conditioned with 5 mL of methanol followed by 5 mL of HPLC—grade water at a flow rate of 2 mL min^{-1}. Water samples previously passed through 0.45 μm nylon membrane filters (Whatman, UK) were preconcentrated

Table 1
Target compounds organized in their therapeutical groups and the isotopically labeled internal standards assigned for their quantification.

Therapeutic groups	Compounds	Number	CAS number	Corresponding internal standard
Analgesics/anti-inflammatories (14)	Ketoprofen	1	22071-15-4	Ibuprofen-d_3
	Naproxen	2	22204-53-1	
	Ibuprofen	3	15687-27-1	
	Diclofenac	4	15307-79-6	
	Indomethacine	5	53-86-1	Indomethacine-d_4
			103-90-2	Acetaminophen-d_4
	Acetaminophen	6	69-72-7	
	Salicylic acid			
	Phenazone	8	60-80-0	Phenazone-d_3
	Propyphenazone	9	479-92-5	
	Piroxicam	10	36322-90-4	Meloxicam-d_3
	Tenoxicam	11	59804-37-4	
	Meloxicam	12	71125-39-8	
	Oxycodone	13	124-90-3	Carbamazepine-d_{10}
	Codeine	14	76-57-3	
Lipid regulators and cholesterol lowering statin drugs (5)	Bezafibrate	15	41859-67-0	Bezafibrate-d_6
	Gemfibrozil	16	25812-30-0	Gemfibrozil-d_6
	Pravastatin	17	81131-70-6	
	Fluvastatin	18	93957-54-1	
	Atorvastatin	19	134523-03-8	
Psychiatric drugs (15)	Carbamazepine	20	298-46-4	Carbamazepine-d_{10}
	2-Hydroxycarbamazepine[a]	21	68011-66-5	
	10,11-Epoxycarbamazepine[a]	22	36507-30-9	
	Acridone[a]	23	578-95-0	
	Olanzapine	24	132539-06-1	
	Sertraline	25	79559-97-0	Fluoxetine-d_5
	Citalopram	26	59729-32-7	Citalopram-d_4
	Venlafaxine	27	99300-78-4	Venlafaxin-d_6
	Trazodone	28	25332-39-2	Fluoxetine-d5
	Fluoxetine	29	56293-78-7	
	Norfluoxetine[a]	30	83891-03-6	
	Paroxetine	31	110429-35-1	
	Diazepam	32	439-14-5	Diazepam-d5
	Lorazepam	33	846-49-1	
	Alprazolam	34	28981-97-7	
Histamine H1 and H2 receptor antagonists (5)	Loratadine	35	79794	Cimetidine-d_3
	Desloratadine[a]	36	100643-71-8	
	Ranitidine	37	66357-59-3	
	Famotidine	38	76824-35-6	
	Cimetidine	39	51481-61-9	
β-Blocking agents (6)	Atenolol	40	29122-68-7	Atenolol-d_7
	Sotalol	41	959-24-0	
	Propranolol	42	318-98-9	
	Metoprolol	43	51384-51-1	
	Nadolol	44	42200-33-9	
	Carazolol	45	57775-29-8	
Diuretic (3)	Hydrochlorothiazide	46	58-93-5	Hydrochlorothiazide-d_2
	Furosemide	47	54-31-9	Furosemide-d_5
	Torasemide	48	56211-40-6	
Antidiabetic (1)	Glibenclamide	49	10238-21-8	Glyburide-d_5
Antihypertensives (4)	Amlodipine	50	111470-99-6	Amlodipine-d_4
	Losartan	51	124750-99-8	
	Irbesartan	52	137862-53-4	Valsartan-d_8
	Valsartan	53	138402-11-6	
Antiplatelet agent (1)	Clopidogrel	54	135046-48-9	Glyburide-d_3
Prostatic hyperplasia (1)	Tamsulosin	55	106463-17-6	Sulfamethoxazole-d_4
To treat asthma (1)	Salbutamol	56	51022-70-9	Atenolol-d_7
Anticoagulant (1)	Warfarin	57	81-81-2	Warfarin-d_5
X-ray contrast agents (1)	Iopromide	58	73334-07-3	Sulfamethoxazole-d_4
Antihelmintics (3)	Albendazole	59	54965-21-8	Ronidazole-d_3
	Thiabendazole	60	148-79-8	
	Levamisole	61	16595-80-5	
Synthetic glucocorticoid (1)	Dexamethasone	62	50-02-2	Dexamethasone-d_4
Sedation and muscle relaxation (1)	Xylazine	63	7361-61-7	Xylazine-d_6
Tranquilizer (2)	Azaperone	64	1649-18-9	Azaperone-d_4
	Azaperol[a]	65	2804-05-9	
Antibiotics (13)	Erythromycin	66	59319-72-1	Erythromycin-N,N$^{13}C_2$
	Azithromycin	67	83905-01-5	Azithromycin-d_3
	Clarithromycin	68	81103-11-9	
	Tetracyclin	69	64-75-5	Sulfamethoxazole-d_4
	Sulfamethoxazole	70	723-46-6	

(continued on next page)

Table 1 (*continued*)

Therapeutic groups	Compounds	Number	CAS number	Corresponding internal standard
	Trimethoprim	71	738-70-5	
	Ofloxacin	72	82419-36-1	Ofloxacin-d_3
	Ciprofloxacin	73	85721-33-1	
Antibiotics (13)	Metronidazole	74	443-48-1	
	Metronidazole-OH[a]	75	4812-40-2	Ronidazole-d_3
	Dimetridazole	76	551-92-8	
	Ronidazole	77	7681-76-7	
	Cefalexin	78	15686-71-2	
Calcium channel blockers (3)	Diltiazem	79	42399-41-7	Carbamazepine-d_{10}
	Verapamil	80	152-11-4	Verapamil-d_6
	Norverapamil[a]	81	67812-42-4	

[a] Metabolites.

on Oasis HLB cartridges, which were further rinsed with 5 mL of HPLC-grade water, dried under vacuum for 15–20 min, to remove excess of water, and eluted with 2 × 4 mL of methanol. The extracts were then evaporated under a gentle nitrogen stream, reconstituted with 1 ml of methanol–water (10:90, v/v). In all cases, 500 mL of surface and drinking waters, and 200 mL of effluent waste waters were loaded onto the cartridges at a flow rate of approximately 5 mL min^{-1}. Prior to instrumental analysis, the extracts were fortified with 10 μL of a mixture of internal standards to a final concentration of 10 ng mL^{-1}, since the quantitation based on internal standard calibration method was applied. Addition of internal standards in the final extract compensates possible matrix effect (ion suppression or enhancement), but does not compensate any of the possible procedure losses (extraction, evaporation).

2.5. Instrumental analysis

Instrumental analysis of all samples was done by high performance liquid chromatography coupled to mass spectrometry with hybrid triple quadrupole–linear ion trap (UPLC–QqLIT–MS/MS) using a method previously developed by Gros et al. (2012).

Briefly, UPLC analysis was performed using Waters Acquity Ultra-Performance™ (Milford, MA, USA) equipped with autosampler and connected in series to a 5500 QTRAP mass spectrometer equipped with a turbo ion spray source (Applied Biosystems–Sciex, Foster City, CA, USA). Chromatographic separations were achieved with an Acquity UPLC HSS T_3 column (50 mm × 2.1 mm i.d., particle size 1.8 μm) for the compounds analyzed under positive electrospray ionization (PI), and an Acquity UPLC BEH C_{18} T_4 column (50 mm × 2.1 mm i.d., particle size 1.7 μm) for the compounds analyzed under negative electrospray ionization (NI), both purchased from Waters Corporation. The optimum temperature for columns was adjusted on 30 °C, while for autosampler temperature was set at 4 °C. For the analysis in PI mode, eluent A was methanol and eluent B was HPLC-water with 10 mM ammonium formiate/formic acid (pH = 3.2) at a flow rate 0.5 mL/min. The gradient program started with: initial conditions 5% A; 0–4.5 min, 5–95% A; 4.5–4.6 min, 100% A; 4.6–6 min, 100% A; from 6 to 6.1 return to initial conditions; and 6.1 to 6.7 min, equilibration of the column. The analysis in NI mode was performed using acetonitrile (A) and 5 mM ammonium acetate/ammonia (pH = 8) (B) at a flow rate of 0.6 mL/min. The injection volume was 5 μL for both modes. The gradient program started with: 0–1.5 min, 0–60% A; 1.5–2 min, 100% A; 2–3 min, 100% A; 3.2 return to initial conditions; and 3.2 to 3.7 min, equilibration of the column. The sample injection volume was set at 5 μL in chromatographic method.

The target compounds were analyzed in multiple reaction monitoring (MRM) mode, monitoring two transitions between the precursor ion and the most abundant fragment ions for each compound.

Settings for source-dependent parameters were determined by Flow Injection Analysis and are as follows: for compounds analyzed under PI, curtain gas (CUR), 30 V; nitrogen collision gas (CAD) medium; source temperature (TEM) was 650 °C; ion spray voltage was 5500 V; and ion source gasses GS1 and GS2 were set at 60 and 50 V, respectively. For compounds analyzed under NI, such parameters were: curtain gas (CUR), 30 V; nitrogen collision gas (CAD) medium; source temperature (TEM) was 650 °C; ion spray voltage was −3500 V; and ion source gasses GS1and GS2 were set at 60 and 70 V.

All data were acquired and processed using Analyst 1.5.1 software.

Further information on the methodology and its performances can be found elsewhere (Gros et al., 2012).

2.6. Quality control

Extraction recoveries for target compounds were determined for different matrices by spiking samples (n = 3) at 50 ng L^{-1} for drinking (DW) and surface (SW) water and 100 ng L^{-1} for effluent wastewater (WWE) (see Table 3). For each type of water samples, recoveries were determined by comparing the concentrations obtained after the whole SPE procedure, calculated by internal standard calibration, with the initial spiking levels. As both surface and wastewaters spiked already contained target compounds, blanks (non-spiked samples) were analyzed in order to determine their concentrations, which were afterwards subtracted to the spiked waters. Recoveries achieved for majority of target compounds were higher than 50% (Table 3). However, some substances such as hydrochlorothiazide, salbutamol, losartan, thiabendazole, metronidazole and hydroxy-metronidazole showed much lower recovery (Table 3). Moreover, other substances, such as cimetidine, ranitidine, dimetridazole, ronidazole, fluvastatin and atorvastatin yielded also low recovery but only in certain matrices (especially in drinking waters, Table 3). This could be mainly attributed to the fact that experimental conditions chosen are not the most appropriate for those specific compounds. In fact, this is one of the limitations of multi-residue methodologies, where not the best conditions for all target analytes are achieved and therefore, a compromise on the final analytical conditions has to be reached. Nevertheless, the low recovery was not considered as an obstacle for its determination in environmental waters, as its sensitivity was fairly good (see Table 3 for LODs and LOQs).

Precision of the method was determined by calculating the relative standard deviation (%RSD) of the triplicate spiked samples.

Quantification of target analytes, based on peak area, was performed by the internal standard approach, and the results were corrected for the recovery.

Calibration curves were generated using linear regression analysis and showed good fits ($r^2 > 0.9900$) over the established concentration points ranging from 0.1, 0.5, 1, 5, 10, 20, to 50 or 100 μg L^{-1} depending of the compound. Limit of detection (LOD) and limit of quantification (LOQ) were determined for DW, SW and WWE as the minimum

Fig. 1. Map of Serbia and sampling locations: Novi Sad, Zrenjanin, Bečej, Vrbas and Obrenovac (detailed description of samples is in Table 2).

detectable amount of analyte with a signal-to-noise ratio of 3 and 10, respectively (Table 3).

3. Results and discussion

Developed analytical method was applied in a survey on residues of 81 most frequently used pharmaceuticals, which were detected in different type of water collected in Serbia.

The study has shown that majority of the investigated samples contained drug residues. In the total, 47 out of 81 investigated drugs were detected from the following therapeutic groups: analgesics/anti-inflammatories, lipid regulators and cholesterol lowering statin drugs, psychiatric drugs, histamine H1 and H2 receptor antagonists, β-blocking agents, diuretic, antihypertensives, to treat asthma, X-ray contrast agents, antihelmintics, antibiotics and calcium channel blockers (Table 4). Fig. 2a shows distribution of occurrence of the detected components in the analyzed samples; it could be easily seen that the highest number of the compounds was detected in sample of untreated municipal waste water discharged directly into the Danube River without any treatment (sample 3), in which the highest

Table 2
List of sampling sites and types of samples analyzed.

Sample mark	Locations	Type of water	Description
1	Obrenovac	Industrial waste water	Waste water (without treatment) from pharmaceutical industry after production of plant syrup.
2	Obrenovac	Industrial waste water	Waste water during the washing of equipment from pharmaceutical industry.
3	Novi Sad	Municipal waste water	Sewerage on the out flow to the Danube, Šangaj
4	Novi Sad	Surface	Canal water from suburb settlement, Adice.
5	Novi Sad	Surface	Canal Danube–Tisa–Danube near oil refinery
6	Novi Sad	Surface	Canal Danube–Tisa–Danube farther from the oil refinery
7	Zrenjanin	Surface	River water, Begej River
8	Zrenjanin	Surface	Little lake, Peskara
9	Novi Sad	Surface	Danube River beach upstream from the city center, Štrand.
10	Novi Sad	Surface	Danube River beach downstream from the city center on the opposite bank from the centre, Oficirac.
11	Novi Sad	Surface	Canal water from village Bukovac
12	Bečej	Surface	River water, Tisa River
13	Vrbas	Surface	Canal-Danube–Tisa–Danube-I
14	Vrbas	Surface	Canal-Danube–Tisa–Danube-II
15	Novi Sad	Underground	Private well ("fountain") water from suburb settlement, Adice-I
16	Novi Sad	Underground	Private well ("fountain") water from suburb settlement, Adice-II
17	Novi Sad	Underground	Public well ("fountain") water near city centre-Spens
18	Novi Sad	Underground	Public well ("fountain") water in Limanski park
19	Novi Sad	Underground	Public well ("fountain") water from park, Jodna Banja
20	Vrbas	Underground	Public well ("fountain") water in park
21	Novi Sad	Drinking water	Tap water
22	Novi Sad	Drinking water	Raw untreated water in public company for city water supply.
23	Novi Sad	Drinking water	Chlorinated water in public company for city water supply.
24	Zrenjanin	Drinking water	Raw untreated water in public company for city water supply.
25	Zrenjanin	Drinking water	Chlorinated water in public company for city water supply.

cumulative level of target compounds was obtained (79,292 ng L^{-1}) followed by samples 7 (2434 ng L^{-1}, Begej River), 11 (1386 ng L^{-1}, canal water from village Bukovac) and 4 (1210 ng L^{-1}, canal water from suburb settlement Adice) (Fig. 2b). The lowest cumulative level of analyzed compounds of 1.8 ng L^{-1} was found for sample 13 (canal-Danube–Tisa–Danube-I, Fig. 2b).

Chromatograms of a standard solution (10 ng mL^{-1}) versus a real waste water sample 3, for some of the detected compounds are given in the Supplementary data 1.

In Table 4, frequency of occurrence and concentration ranges obtained for target compounds in investigated samples of water are presented. Levels of target compounds were in the ng L^{-1} range but concentrations of some of them exceed level of 1 μg L^{-1} (ibuprofen, diclofenac, codeine, valsartan, acetaminophen, 2-hydroxycarbamazepine and 10,11-epoxycarbamazepine). According to the frequency of occurrence of target compounds found in this study the analyzed analgesics/anti-inflammatories could be order as follows: salicylic acid (41.67%) > ibuprofen (16.67%) > propyphenazone = naproxen (13.89%) > diclofenac (11.11%) > meloxicam = ketoprofan = phenazone (8.33%) > codeine (5.56%) > idomethacine = acetaminophen (2.78%). From this group analgesics/anti-inflammatories, minimum number of components was detected in drinking water (only ketoprofen and salicylic acid). As shown in Table 4, four drugs were detected in municipal waste water (city sewerage on the out flow to the Danube River, sample no. 3) with fairly high concentrations: codeine of 1.0 μg L^{-1}, diclofenac of 1.3 μg L^{-1}, acetaminophen of 15.7 μg L^{-1} and ibuprofen of 20.1 μg L^{-1}. Similarly, Terzić et al. (2008) detected in Western Balkan region maximum concentration of 4.2 μg L^{-1} for diclofenac, while the highest level of ibuprofen was 11.9 μg L^{-1} in municipal waste water. As can be seen from Table 4, maximum level found for analgesics/anti-inflammatories in surface (Begej River, sample no. 7) and underground (private well from suburb settlement Adice, Novi Sad, sample no. 16) water was for ibuprofen: 346 ng L^{-1} (18.18%) and 92 ng L^{-1} (16.67%), respectively. The maximum level of ibuprofen detected in surface water taken from Begej River (346 ng L^{-1}, sample no. 7) was similar to the level (277 ng L^{-1}) reported for river water in Spain (Ferreira da Silva et al., 2011). In most European studies, ibuprofen has been detected in surface water (Vanderford et al., 2003; Roberts and Thomas, 2006), and even in the USA the highest concentration obtained by Kolpin et al. (2002) was 200 ng L^{-1}. Ibuprofen, an NSAID (nonsteroidal anti-inflammatory drug), is one of the most frequently prescribed pharmaceuticals in Europe and has been previously reported as being one of the most common drug residues in surface water. The annual consumption of ibuprofen was equal to 166 t $year^{-1}$ in France with population of 55.5 million in 1998; 128 t $year^{-1}$ in Germany with population of 82.4 million in 2001; 276 t $year^{-1}$ in Spain with population of 43.2 million in 2003; and 180 t $year^{-1}$ in Canada with population of 30 million in 2001 (Verlicchi et al., 2010). In 2010, 28 tones of ibuprofen were used in Serbia populated with 7.5 millions of inhabitants (Radonjić and Šipetić, 2010), making ibuprofen the most consumed pharmaceutical. Very high consumption of ibuprofen means that this drug has been the most widely detected: high levels of 17 μg L^{-1} (Valcárcel et al., 2011) and 1.4 μg L^{-1} (Martinez Bueno et al., 2010) were found in river waters of the Madrid Region (Spain). Also, in the study conducted by Farré et al. (2001) considerably high concentrations of ibuprofen (2.7 μg L^{-1}) were reported in surface water of the principal rivers of Catalonia (north-east Spain).

On the other hand, within therapeutic group lipid regulators and cholesterol lowering statin drugs only bezafibrate and atorvastatin were detected with same total frequency of 5.56%. Maximum level of atorvastatin was 40.5 ng L^{-1} in municipal waste water (city sewerage on the out flow to the Danube River, sample no. 3), while the highest level found for bezafibrate was 1.6 ng L^{-1} in one sample of surface water (the Danube River beach upstream from the city center of Novi Sad, sample no. 9). Valcárcel et al. (2011) also detected bezafibrate in river water in Spain, but at far higher concentration (median value was 682 ng L^{-1}).

The most detected component from group of psychiatric drugs was carbamazepine with a total occurrence frequency of 36.11% and the highest concentration level of 303 ng L^{-1} in municipal waste water (the Novi Sad City sewerage on the out flow to the Danube River, sample no. 3). It is well known that carbamazepine is one of the most frequently detected drugs in water environment (found in sewage treatment plant effluents, surface waters, groundwater and occasionally in drinking water all over the world), although it is excreted unmetabolized in only few percent (Ternes, 1998). Consumption of carbamazepine in Serbia (280.0 mg $year^{-1}$ $inhabitant^{-1}$) (Radonjić and Šipetić, 2010) is lower than the estimated consumption in the developed countries

Table 3
UPLC–MS/MS parameters of target compounds under optimized conditions on Acquity Ultra-Performance™—5500 QTRAP. Parameters indicating the performance of the analytical method: limit of detection (LOD), limit of quantification (LOQ) and recoveries obtained for target compounds in all matrices studied.

Compounds	Precursor ion (m/z)	Q1[a]	Q3[b]	LOD (LOQ) (ng L^{-1})			%Recoveries (%RSD) (n = 3)		
				DW	SW	WWE	DW	SW	WWE
Compounds analyzed under PI mode									
Metronidazole-OH	187 $[M + H]^+$	126	123	n.c. (n.c.)	9.3 (30)	14 (48.0)	n.c. (n.c.)	40 (±15.0)	43 (±8.2)
Sotalol	273 [M + H]+	255	133	n.c. (n.c.)	1.1 (3.5)	9.0 (10.0)	n.c. (n.c.)	101 (±5.9)	83 (±8.8)
Salbutamol	240 $[M + H]^+$	148	122	n.c. (n.c.)	0.1 (0.2)	0.9 (3.0)	n.c. (n.c.)	30 (±9.3)	30 (±4.4)
Atenolol-d_7 (IS)	274 $[M + H]^+$	145	–						
Ronidazole	201 $[M + H]^+$	140	–	0.5 (1.8)	2.5 (8.3)	15 (51.0)	30 (±6.4)	90 (±6.0)	108 (±10.1)
Atenolol	267 $[M + H]^+$	145	190	0.1 (0.1)	0.3 (1.0)	9 (12.9)	57 (±9.7)	57 (±13.5)	61 (±3.3)
Ronidazole-d_3	204 $[M + H]^+$	143	–						
Metronidazole	172 $[M + H]^+$	128	82	n.c. (n.c.)	2.7 (8.5)	26 (44.0)	n.c.	30 (±11.5)	109 (±4.7)
Ranitidine	315 $[M + H]^+$	176	130	0.1 (0.2)	0.1 (0.2)	0.4 (1.4)	45 (±3.1)	40 (±7.4)	92 (±12.7)
Famotidine	338 $[M + H]^+$	189	259	0.1 (0.3)	0.2 (0.7)	1.4 (4.8)	50 (±12.4)	95 (±14.9)	87 (±6.8)
Cimetidine-d_3 (IS)	256 $[M + H]^+$	95	–						
Cimetidine	253 $[M + H]^+$	159	95	0.1 (0.3)	0.5 (1.0)	3.0 (10.0)	24 (±16.5)	83 (±11.1)	76 (±9.7)
Iopromide	792 $[M + H]^+$	573	300	0.3 (0.9)	1.4 (4.7)	6.8 (22.8)	157 (±16.2)	158 (±22.7)	130 (±10.8)
Codeine	300 $[M + H]^+$	152	115	0.1 (0.2)	1.0 (3.4)	8.8 (28.0)	82 (±8.5)	88 (±4.2)	100 (±10.9)
Oxycodone	316 $[M + H]^+$	298	241	0.2 (0.6)	1.9 (6.3)	17.1 (50.0)	75 (±7.6)	73 (±5.8)	112 (±5.8)
Levamisole	205 $[M + H]^+$	178	91	0.01 (0.01)	0.2 (0.3)	0.4 (1.2)	50 (±5.9)	82 (±11.8)	74 (±8.3)
Dimetridazole	142 $[M + H]^+$	96	95	1.0 (2.8)	4.4 (15.0)	15 (50)	30 (±8.9)	65 (±15.1)	109(±11.1)
Trimethoprim	291 $[M + H]^+$	230	261	0.1 (0.3)	0.6 (2.0)	2.4 (8.1)	56 (±12.9)	53 (±1.2)	67 (±7.1)
Cefalexin	348 $[M + H]^+$	158	106	n.c. (n.c.)	1.1 (3.8)	5.0 (16.6)	n.c. (n.c.)	31 (±9.2)	70 (±8.6)
Nadolol	310 $[M + H]^+$	254	201	0.01 (0.1)	0.1 (0.2)	0.7 (2.4)	40 (±2.0)	60 (±3.9)	56 (±4.4)
Olanzapine	313 $[M + H]^+$	256	198	n.c. (n.c.)	0.5 (1.8)	0.5 (1.8)	n.c. (n.c.)	66 (±8.5)	66 (±9.9)
Ofloxacin-d_3 (IS)	365 $[M + H]^+$	321	–	–	–	–			
Ofloxacin	362 $[M + H]^+$	318	261	n.c. (n.c.)	0.6 (2.1)	0.6 (1.8)	n.c. (n.c.)	67 (±12.2)	116 (±15.9)
Sulfamethoxazole-d_4 (IS)	258 $[M + H]^+$	160	–	–	–	–			
Sulfamethoxazole	254 $[M + H]^+$	92	156	0.1 (0.3)	2.0 (6.5)	5.5 (18.0)	83 (±19.7)	78 (±1.0)	81 (±11.3)
Tetracycline	445 $[M + H]^+$	410	154	7.2 (20.0)	12.0 (41.0)	7.0 (23.0)	128 (±4.7)	112 (±13.1)	127 (±6.3)
Ciprofloxacin	332 $[M + H]^+$	288	245	n.c. (n.c.)	5.5 (18.3)	7.0 (23.0)	n.c. (n.c.)	113 (±9.1)	140 (±21.2)
Phenazone-d_3 (IS)	192 $[M + H]^+$	59	–	–	–	–			
Phenazone	189 $[M + H]^+$	77	56	0.1 (0.5)	0.7 (2.3)	2.0 (6.5)	83 (±10.8)	98 (±1.43)	91 (±6.0)
Sulfadoxine-d_3 (surrogate)	314 $[M + H]^+$	156	–	–	–	–	78 (±4.6)	82 (±7.8)	93 (±6.8)
Xylazine-d_6 (IS)	227 $[M + H]^+$	90	–	–	–	–			
Xylazine	221 $[M + H]^+$	90	77	0.4 (1.4)	1.6 (5.3)	4.0 (13.0)	90 (±0.3)	93 (±1.7)	102 (±8.5)
Metoprolol	268 $[M + H]^+$	133	121	0.3 (0.9)	1.3 (4.2)	8.7 (28.0)			
Azaperol	330 $[M + H]^+$	121	78	0.1 (0.4)	0.3 (1.0)	0.5 (1.7)	66 (±6.2)	80 (±4.6)	66 (±10.7)
Thiabendazole	202 $[M + H]^+$	175	131	0.01 (0.03)	0.03 (0.1)	0.2 (0.6)	20 (±5.0)	40 (±5.6)	22 (±7.1)
Tamsulosin	409 $[M + H]^+$	228	200	0.1 (0.4)	0.3 (0.9)	1.2 (4.0)	71 (±5.1)	73 (±1.0)	117 (±6.5)
Azaperone-d_4 (IS)	332 $[M + H]^+$	127	–	–	–	–			
Azaperone	328 $[M + H]^+$	123	95	0.2 (0.4)	0.5 (1.0)	0.9 (1.7)	52 (±17.6)	100 (±4.4)	92 (±14.5)
Sulfadimethoxine-d_6 (surrogate)	317 $[M + H]^+$	162	–	–	–	–	63 (±2.9)	70 (±9.0)	74 (±1.5)
Carazolol	299 $[M + H]^+$	116	222	0.2 (0.7)	0.3 (0.9)	0.6 (2.1)	50 (±11.1)	90 (±3.7)	98 (±16.6)
Trazodone	372 $[M + H]^+$	176	148	0.1 (0.4)	0.2 (0.8)	2.1 (7.1)	46 (±4.3)	41 (±6.6)	76 (±12.4)
Azithromycin-d_3 (IS)	752 $[M + H]^+$	594	–	–	–	–			
Venlafaxine-d_6 (IS)	284 $[M + H]^+$	64	–	–	–	–			
Venlafaxine	278 $[M + H]^+$	58	260	0.1 (0.3)	0.2 (0.7)	0.6 (2.1)	88 (±2.9)	107 (±3.1)	92(±2.4)
Azithromycin	749 $[M + H]^+$	592	116	0.4 (1.4)	0.14 (0.5)	0.4 (1.2)	144 (±10.7)	128 (±20.0)	111 (±11.6)
Propranolol	260 $[M + H]^+$	116	183	0.4 (1.4)	1.5 (4.9)	3.5 (13.3)	80 (±10.8)	120 (±5.7)	88 (±1.0)
10,11-EpoxyCBZ	253 $[M + H]^+$	180	236	4.7 (13.0)	15.2 (50.7)	19.0 (50.0)	127 (±7.6)	82 (±6.9)	66 (±4.5)
2-HydroxyCBZ	253 $[M + H]^+$	210	208	1.6 (5.2)	4.2 (13.9)	8.0 (25.0)	91 (±7.8)	86 (±6.3)	142 (±13.9)
Citalopram-d_4 (IS)	329 $[M + H]^+$	113	–	–	–	–			
Citalopram	325 $[M + H]^+$	109	262	0.4 (1.2)	0.5 (2.7)	1.0 (3.3)	80 (±1.3)	96 (±2.1)	109 (±5.4)
Norfluoxetine	296 $[M + H]^+$	134	–	0.3 (0.9)	1.3 (4.5)	2.6 (8.6)	82 (±16.4)	122 (±2.6)	113 (±14.6)
Acridone	196 $[M + H]^+$	166	167	0.04 (0.1)	0.6 (2.0)	2.6 (8.8)	84 (±4.7)	83 (±0.4)	84 (±10.6)
Verapamil-d_6 (IS)	461 $[M + H]^+$	165	–	–	–	–			
Norverapamil	441 $[M + H]^+$	165	150	0.1 (0.4)	0.4 (1.3)	8.4 (28.0)	88 (±9.9)	96 (±1.9)	83 (±11.1)
Verapamil	455 $[M + H]^+$	165	77	0.3 (1.1)	1.2 (5.8)	3.9 (13.0)	72 (±7.4)	64 (±12.5)	103(±10.1)
Diltiazem	415 $[M + H]^+$	178	109	0.2 (0.5)	0.5 (1.7)	0.7 (2.4)	115 (±2.9)	95 (±4.9)	140 (±4.1)
Carbamazepine-d_{10} (IS)	247 $[M + H]^+$	204	0	–	–	–			
Desloratadine	311 $[M + H]^+$	259	258	0.2 (0.6)	0.9 (3.0)	2.3 (7.6)	41 (±23.3)	82 (±1.4)	146 (±10.0)
Carbamazepine	237 $[M + H]^+$	194	193	0.2 (0.6)	0.7 (2.4)	2.4 (8.0)	108 (±4.6)	108 (±3.3)	124 (±6.5)
Propyphenazone	231 $[M + H]^+$	189	56	0.2 (0.5)	1.6 (5.2)	1.3 (4.5)	61 (±1.5)	77 (±2.1)	116 (±5.8)
Amlodipine-d_4 (IS)	413 $[M + H]^+$	238	–	–	–	–			
Paroxetine	330 $[M + H]^+$	192	123	1.8 (6.02)	4.24 (14.2)	7.4 (24.0)	67 (±13.3)	122 (±2.6)	145 (±13.2)
Erythromycin	734 $[M + H]^+$	576	158	0.3 (0.9)	1.5 (5.1)	1.1 (3.5)	30 (±15.6)	52 (±20.7)	137 (±18.0)
Erythromycin-N,N$^{13}C_2$ (IS)	736 $[M + H]^+$	578	–	–	–	–			
Lorazepam	321 $[M + H]^+$	275	303	1.4 (4.5)	5.3 (17.7)	13.0 (42.0)	93 (±1.7)	99 (±5.8)	107 (±4.6)
Alprazolam	309 $[M + H]^+$	281	205	0.1 (0.2)	0.5 (1.5)	1.3 (4.4)	50 (±3.2)	63 (±2.2)	61 (±3.5)
Fluoxetine-d_5 (IS)	315 $[M + H]^+$	44	–	–	–	–			
Fluoxetine	310 $[M + H]^+$	44	148	1.0 (2.0)	2.7 (9.0)	2.0 (6.5)	62 (±19.1)	93 (±2.7)	89 (±13.9)
Amlodipine	409 $[M + H]^+$	238	294	n.c. (n.c.)	6.6 (20.9)	9.9 (33.1)	n.c. (n.c.)	69 (±17.6)	75 (±16.3)
Sertraline	307 $[M + H]^+$	159	276	2.0 (6.7)	9.7 (32.0)	12.0 (40.0)	130 (±21.5)	96 (±2.1)	109 (±5.4)
Albendazole	266 $[M + H]^+$	234	191	n.c. (n.c.)	1.1 (3.6)	3.2 (10.5)	n.c. (n.c.)	115 (±7.9)	130 (±6.1)

(continued on next page)

Table 3 (*continued*)

Compounds	Precursor ion (m/z)	Q1[a]	Q3[b]	LOD (LOQ) (ng L^{-1})			%Recoveries (%RSD) (n = 3)		
				DW	SW	WWE	DW	SW	WWE
Clarithromycin	748 $[M + H]^+$	158	590	0.4 (1.3)	0.6 (1.9)	1.3 (4.3)	137 (±9.4)	116 (±7.8)	106 (±12.5)
Diazepam-d_5 (IS)	290 $[M + H]^+$	198	–	–	–	–			
Diazepam	285 $[M + H]^+$	193	154	0.2 (0.4)	1.1 (3.5)	1.1 (3.7)	97 (±1.9)	98 (±6.0)	101 (±2.5)
Warfarin-d_5 (IS)	314 $[M + H]^+$	163	–	–	–	–			
Warfarin	309 $[M + H]^+$	163	251	0.2 (0.5)	0.5 (1.7)	2.1 (6.8)	94 (±4.7)	100 (±1.5)	103 (±7.5)
Glibenclamide	494 $[M + H]^+$	369	169	0.5 (1.7)	3.7 (12.5)	1.7 (5.7)	88 (±7.2)	101 (±1.9)	88 (±10.1)
Glibenclamide-d_3 (IS)	497 $[M + H]^+$	372	–	–	–	–			
Clopidogrel	322 $[M + H]^+$	212	184	0.1 (0.3)	0.7 (2.3)	0.3 (1.1)	97 (±3.8)	104 (±2.6)	112 (±16.3)
Loratadine	383 $[M + H]^+$	337	267	3.1 (10.0)	1.1 (3.7)	3.2 (10.0)	96 (±17.2)	80 (±7.3)	130 (±8.0)
Compounds	Precursor ion (m/z)	Q1[a]	Q3[b]	LOD (LOQ) (ng L^{-1})			%Recoveries (%RSD) (n = 3)		
				DW	RW	WWE	DW	RW	WWE
Compounds analyzed under NI mode									
Acetaminophen-d_4 (IS)	154 $[M - H]^-$	111	–	–	–	–			
Acetaminophen	150 $[M - H]^-$	107	–	0.8 (1.0)	9.2 (20.0)	6.0 (20.0)	92 (±0.3)	91 (±0.3)	139 (±7.3)
Salicylic acid	137 $[M - H]^-$	93	66	0.1 (0.2)	0.8 (2.6)	4.2 (13.0)	137 (±43.6)	146 (±3.4)	137 (±6.0)
Hydrochlorothiazide-d_2 (IS)	298 $[M - H]^-$	270	–	–	–	–			
Hydrochlorothiazide	296 $[M - H]^-$	269	205	0.1 (0.3)	0.5 (1.7)	1.0 (2.6)	30 (±5.4)	33 (±8.9)	55 (±20.0)
Tenoxicam	336 $[M - H]^-$	152	272	0.2 (0.8)	0.5 (1.8)	1.8 (6.0)	75 (±5.7)	68 (±6.6)	65 (±5.5)
Piroxicam	330 $[M - H]^-$	146	266	0.2 (0.6)	1.1 (2.1)	2.0 (6.5)	79 (±3.4)	56 (±2.5)	76 (±3.2)
Valsartan	434 $[M - H]^-$	179	350	0.5 (1.5)	6.7 (21.9)	4.0 (13.0)	98 (±15.0)	60 (±17.0)	100 (±19.7)
Valsartan-d_8 (IS)	442 $[M - H]^-$	179	–	–	–	–			
Naproxen	229 $[M - H]^-$	170	185	0.4 (1.3)	1.3 (4.4)	3.5 (11.5)	88 (±9.1)	136 (±1.2)	104 (±14.6)
	232 $[M - H]^-$	171	–						
Furosemide-d_5 (IS)	334 $[M - H]^-$	290	–	–	–	–			
Furosemide	329 $[M - H]-$	285	205	0.7 (2.4)	4.7 (15.6)	8.9 (29.0)	60 (±7.4)	91 (±5.1)	70 (±7.9)
Ketoprofen-d_3 (surrogate)	256 $[M - H]^-$	212	–	–	–	–	106 (±2.0)	111 (±4.2)	105 (±7.5)
Pravastatin	423 $[M - H]^-$	321	303	0.5 (1.8)	2.7 (1.9)	4.2 (14.1)	58 (±6.1)	93 (±11.0)	70 (±9.5)
Ketoprofen	253 $[M - H]^-$	209	–	4.0 (13.0)	9.0 (30.1)	9.0 (30.0)	122 (±21.2)	127 (±1.2)	108 (±14.6)
Meloxicam-d_3 (IS)	353 $[M - H]^-$	289	–	–	–	–			
Meloxicam	350 $[M - H]^-$	146	286	0.1 (0.2)	0.2 (0.6)	0.5 (1.5)	82 (±4.7)	102 (±4.9)	107 (±9.8)
Bezafibrate-d_6 (IS)	366 $[M - H]^-$	280	–	–	–	–			
Bezafibrate	360 $[M - H]^-$	274	154	0.1 (0.3)	0.3 (1.0)	1.0 (3.3)	96 (±4.8)	107 (±2.4)	96 (±8.6)
Torasemide	347 $[M - H]^-$	262	196	0.1 (0.2)	0.5 (1.7)	1.1 (3.5)	91 (±2.3)	97 (±5.9)	103 (±8.8)
Losartan	421 $[M - H]^-$	127	179	0.9 (3.0)	3.5 (11.7)	4.1 (13.6)	32 (±10.5)	41 (±2.9)	40 (±14.6)
Ibuprofen-d_3 (IS)	208 $[M - H]^-$	164	–	–	–	–			
Ibuprofen	205 $[M - H]^-$	161	–	0.5 (1.8)	3.2 (10.5)	1.1 (3.8)	105 (±8.6)	86 (±27.8)	174 (3.5)
Diclofenac	294 $[M - H]^-$	250	214	0.3 (1.0)	4.1 (13.5)	5.2 (17.1)	50 (±3.0)	116 (±10.0)	57 (±4.2)
Indomethacine-d_4 (IS)	360 $[M - H]^-$	316	–	–	–	–			
Indomethacine	356 $[M - H]^-$	312	297	1.3 (4.4)	1.7 (5.5)	4.9 (16.4)	88 (±3.0)	94 (±9.7)	79 (±12.9)
Irbesartan	427 $[M - H]^-$	193	399	0.1 (0.3)	0.3 (1.1)	1.2 (3.9)	61 (±11.7)	62 (±2.7)	58 (±9.5)
Dexamethasone	451 $[M - H]^-$	361	307	0.1 (0.4)	0.4 (1.2)	0.9 (2.8)	84 (±5.1)	88 (±14.9)	83(±7.6)
Dexamethasone-d_4 (IS)	395 $[M - H]^-$	363	–	–	–	–			
Gemfibrozil-d_6 (IS)	255 $[M - H]^-$	121	–	–	–	–			
Gemfibrozil	249 $[M - H]^-$	121	127	0.04 (0.1)	0.6 (1.9)	0.4 (1.3)	90 (±4.2)	42 (±22.5)	99 (±1.0)
Fluvastatin	410 $[M - H]^-$	210	348	0.1 (0.4)	0.6 (2.1)	2.6 (8.6)	32 (±11.0)	117 (±14.0)	97 (±11.7)
Atorvastatin	557 $[M - H]^-$	278	397	0.03 (0.08)	0.1 (0.25)	0.2 (0.9)	12 (±9.4)	89 (±2.2)	56 (±10.6)

n.c.—not calculated.
[a] Quantification.
[b] Confirmation.

such as Germany (1010.9 mg $year^{-1}$ $inhabitant^{-1}$); Switzerland (857.5 mg $year^{-1}$ $inhabitant^{-1}$); France (554.3 mg $year^{-1}$ $inhabitant^{-1}$); Sweden (820.2 mg $year^{-1}$ $inhabitant^{-1}$) and Spain (438.0 mg $year^{-1}$ $inhabitant^{-1}$) (Ortiz de García et al., 2013). The level of carbamazepine found in surface water of canal Danube–Tisa–Danube (sample no. 14) was up to 35.5 ng L^{-1} with frequency occurrence of 72.73%. Previous study from Serbia published by Grujić et al. (2009) revealed that in 80% of analyzed water samples, residue of carbamazepine was found, in the concentration range 6–130 ng L^{-1}. Levels of this antiepileptic detected in surface and ground waters in Serbia were similar to those reported for surface waters in Switzerland (30–250 ng L^{-1}) (Öllers et al., 2001); Spain (9–37 ng L^{-1}) (Pedrouzo et al., 2007); South Korea (4.5–61 ng L^{-1}) (Kim et al., 2007); and Italy (34.2 ng L^{-1}) (Zuccato et al., 2005). Carbamazepine was also detected in underground and drinking water where measured concentrations were 3.4 ng L^{-1} (public well-Vrbas, sample no. 20) and 8.7 ng L^{-1} (sample no. 25), respectively. Heberer et al. (2001) detected carbamazepine at 20 ng L^{-1} in an abandoned drinking water well located 100 m away from a lake where carbamazepine was measured at 135 ng L^{-1}. Rabiet et al. (2006) investigated seven drinking water wells in the Mediterranean region and carbamazepine was detected in two wells at concentrations of 43.2 and 13.9 ng L^{-1}. Carbamazepine is very resistant in the environment, as it passes through natural bank filtration and is detected in ground waters at concentrations up to 900 ng L^{-1} (Sacher et al., 2001). This also explains why carbamazepine has been detected in a number of ground water samples and was also found with a concentration of 30 ng L^{-1} in drinking water (Ternes, 2001). Also, it should be taken into account that epilepsy treatment usually lasts for life, as opposed to occasional consumption of antibiotics and analgesics/antipyretics, so there is constant input of this drug into natural waters. Additionally, carbamazepine has an extremely low removal rate (lower than 7%) in sewage treatment plants (STP), and it is an excellent tracer substance for pharmaceutical agents in the environment (Moldovan, 2006).

Remarkably high values were found in municipal waste water for two metabolites of carbamazepine from group of psychiatric drugs. The highest

Table 4
Frequency of occurrence and concentration ranges[a] for pharmaceutical compounds belonging to different therapeutic classes detected in the analyzed water samples (presented results are corrected for recovery).

Compounds	Total frequency of occurrence	Industrial waste water (n = 2) [b]	Municipal waste water (n = 1) [b]	Surface water (n = 11)		Underground water (n = 6)		Drinking water (n = 5)	
	%	ng L^{-1}	ng L^{-1}	%	ng L^{-1}	%	ng L^{-1}	%	ng L^{-1}
Analgesics/anti-inflammatories									
Ketoprofen	8.33		247	9.09	45			20	16
Naproxen	13.89		208	27.27	<LOQ—74.2	16.67	27.6		
Ibuprofen	16.67		20130	18.18	<LOQ—346	16.67	92		
Indomethacine	2.78			9.09	19.5				
Acetaminophen	2.78		15719						
Salicylic acid	41.67	54.5	204	9.09	2.7	83.33	<LOQ—2.5	100	<LOQ—1.4
Diclofenac	11.11		1338	27.27	<LOQ—324				
Phenazone	8.33		13.5	9.09	12.5	16.67	23.4		
Propyphenazone	13.89		99.5	9.09	5.8	50	<LOQ—24.8		
Meloxicam	8.33		5.0	18.18	<LOQ—1.8				
Codeine	5.56		1017	9.09	7.3				
Lipid regulators and cholesterol lowering statin drugs									
Bezafibrate	5.56			18.18	<LOQ—1.6				
Atorvastatin	5.56		40.5	9.09	1.5				
Psychiatric drugs									
Carbamazepine	36.11		303	72.73	<LOQ—35.5	16.67	3.4	60	<LOQ—8.7
2-Hydroxycarbamazepine (2-hydroxy CBZ)	5.56		15939	9.09	160				
10,11-Epoxycarbamazepine (10,11 epoxy CBZ)	13.89		16208	27.27	<LOQ—932			20	128
Venlafaxine	13.89		154	27.27	<LOQ—5.3				
Lorazepam	5.56		184	9.09	30.1				
Histamine H1 and H2 receptor antagonists									
Ranitidine	8.33			27.27	<LOQ—54.4				
Famotidine	2.78		301						
β-Blocking agents									
Atenolol	8.33		670	18.18	<LOQ—50.6				
Sotalol	5.56		91.3					20	0.4
Propranolol	30.56		78.5	9.09	10.4	66.67	<LOQ—4.5	100	<LOQ—4.3
Metoprolol	16.67		574	27.27	<LOQ—26.3			40	<LOQ—3.5
Carazolol	5.56					16.67	3.3		
Diuretic									
Hydrochlorothiazide (HCTZ)	27.28		1070	54.55	<LOQ—164			20	24
Furosemide	11.11		362	27.27	<LOQ—101				
Antihypertensives									
Losartan	11.11		229	27.27	<LOQ—154				
Irbesartan	30.56		11.6	63.64	<LOQ—15.3			60	<LOQ—2.2
Valsartan	5.56		1086	9.09	89.6				
To treat asthma									
Salbutamol	5.56							40	<LOQ—5.4
X-ray contrast agents									
Iopromide	19.44		804	63.64	<LOQ—75.2			20	6.8
Antihelmintics									
Albendazole	13.89					33.33	<LOQ—1.9	60	<LOQ—2.8
Levamisole	2.78			9.09	1.5				
Antibiotics									
Erythromycin	2.78			9.09	292				
Clarithromycin	5.56			18.18	<LOQ—616				
Ofloxacin	2.78		220						
Ciprofloxacin	8.33		278	18.18	<LOQ—28.2				
Sulfamethoxazole	2.78		432						
Trimethoprim	5.56		259	9.09	8.1				
Cefalexin	5.56		803	9.09	283				
Calcium channel blockers									
Diltiazem	8.33		217	18.18	<LOQ—6.9				

[a] Single number instead of range means that there was only single positive sample.
[b] Frequencies are not reported since only two samples of industrial waste water and one sample of municipal waste water were analyzed.

concentrations were found for 10,11-epoxycarbamazepine, one of the human metabolites of carbamazepine, in municipal waste water sample (the Novi Sad City sewerage on the out flow to the Danube River, sample no. 3) (16.2 μg L^{-1}), surface water sample taken from Begej River (0. 93 μg L^{-1}, sample no. 7) and drinking water sample (0.13 μg L^{-1}, sample no. 22). Other metabolite, 2-hydroxycarbamazepine, was found

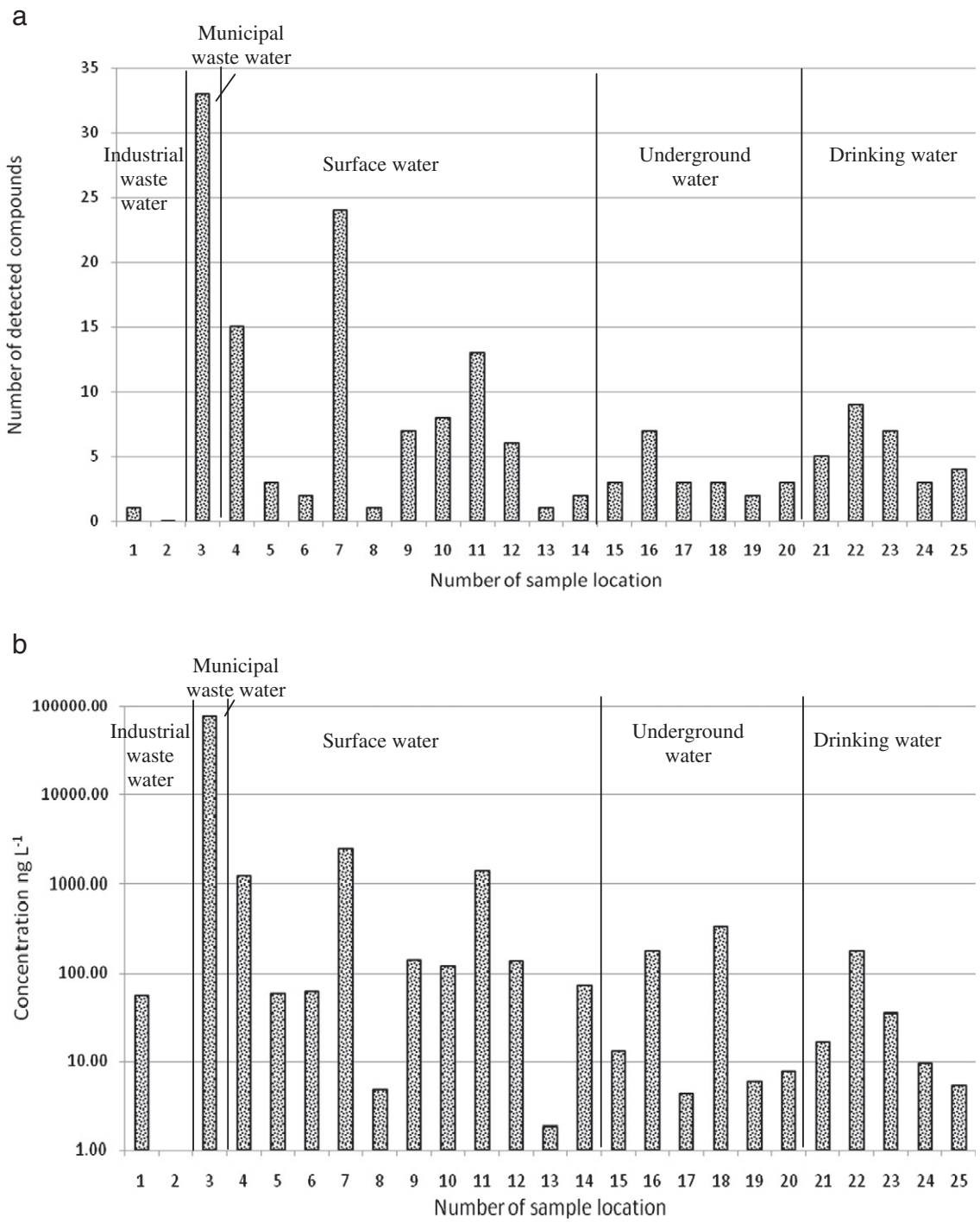

Fig. 2. Occurrence of detected components in the analyzed samples: a) Number of detected compounds per sampling site, b) cumulative levels of detected compounds per sampling site.

in concentration of 15.9 μg L^{-1} in municipal waste water sample (the Novi Sad City sewerage on the out flow to the Danube River, sample no. 3). The levels of detected metabolites of carbamazepine are higher than those reported in other studies, but with similar ratio of parent compound/metabolite. For example, López-Serna et al. (2012) detected 10,11-epoxycarbamazepine (maximum concentration of 1600 ng L^{-1}) in the Ebro river basin at concentrations more than one order of magnitude higher than that of parent carbamazepine. In addition, from this therapeutic group, similar maximum levels were measured for venlafaxine (154 ng L^{-1}) and lorazepam (184 ng L^{-1}) in municipal waste water (city sewerage on the out flow to the Danube River, sample no. 3).

Analyses of histamine H1 and H2 receptor antagonists confirmed the presence of ranitidine in surface water of Begej River (sample no. 7) with maximum concentration of 54.4 ng L^{-1} and famotidine which was detected five times higher than ranitidine in municipal waste water (city sewerage on the out flow to the Danube River in the Novi Sad municipality, sample no. 3). According to the study performed in Italy, Zuccato et al. (2005) detected ranitidine at concentration of 38.5 ng L^{-1} in the River Lambro. The other three selected representatives of this group: loratadine, desloratadine and cimetidine were not detected in any of analyzed samples.

Among six different β-blocking agent drugs monitored, the most frequent was propranolol, which was detected in all types of investigated

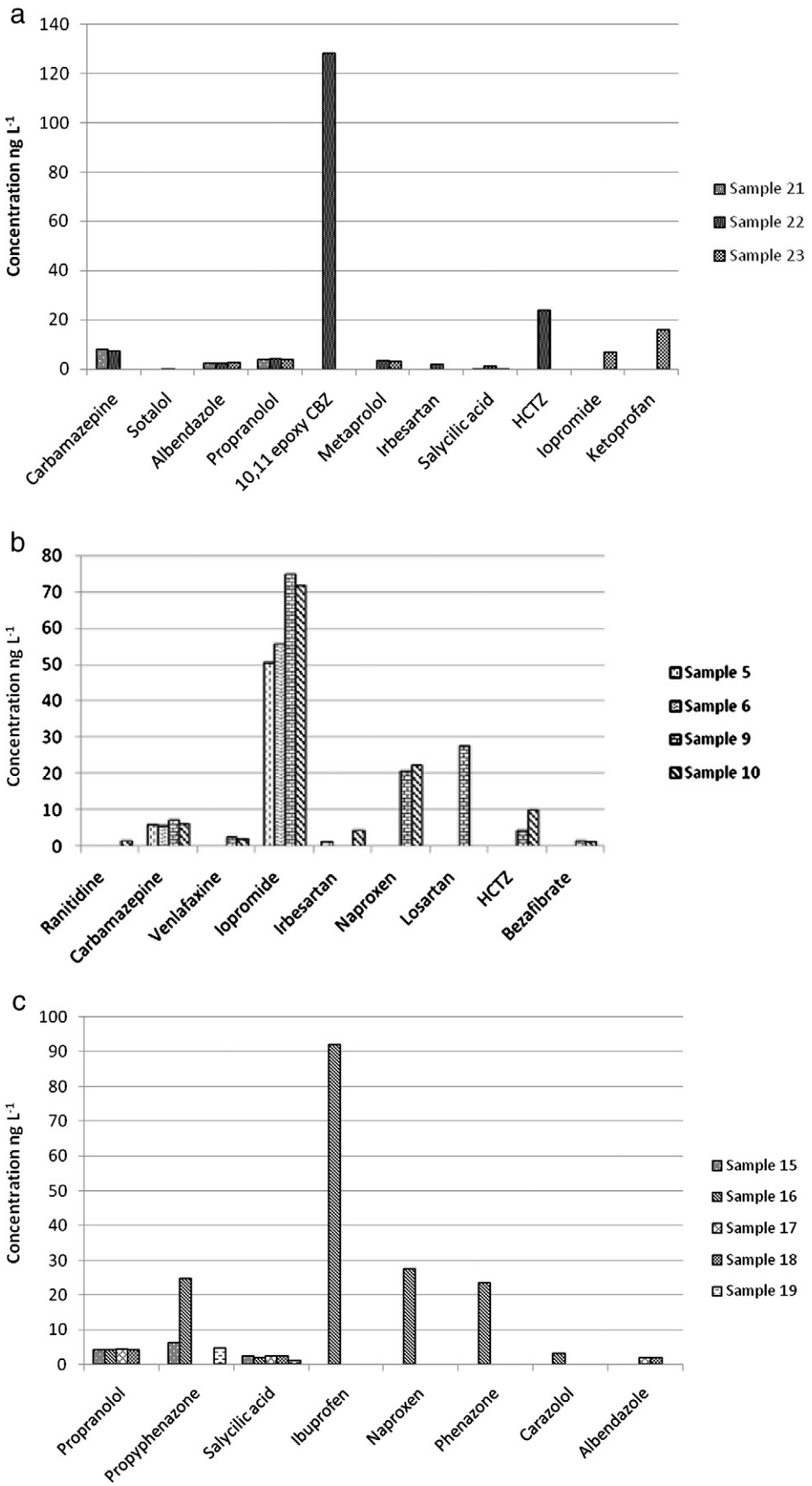

Fig. 3. Co-occurrence of pharmaceuticals in different types of water collected within Novi Sad city area: a) Drinking water in Novi Sad, b) the Danube River (samples 9 and 10) and canal (samples 5 and 6) water in Novi Sad, c) underground water in Novi Sad, d) canal water—Adice (sample 4) and e) municipal waste water in Novi Sad (sample 3).

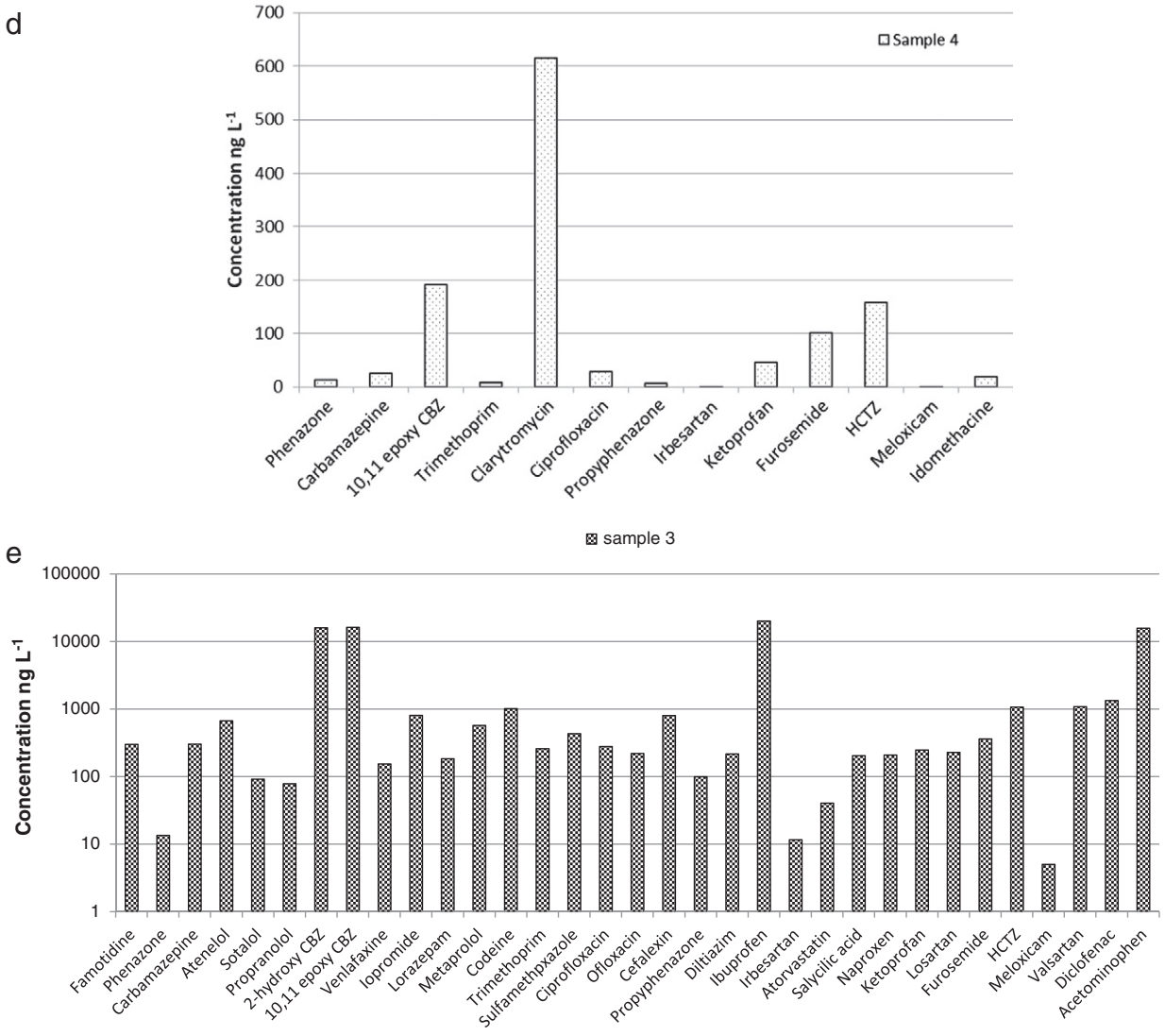

Fig. 3 (*continued*).

samples (total occurrence frequency was 30.56%). Only compound of β-blocking agents which was not detected in any water samples was nadolol. The maximum levels among analyzed β-blockers were found for atenolol (670 ng L^{-1}) and metoprolol (574 ng L^{-1}) in sample of municipal waste water (sample no. 3), being significantly higher in comparison with other agents from this group. In the same sample of municipal waste water, propranolol and sotalol were detected in concentrations of 78.5 ng L^{-1} and 91.3 ng L^{-1}, respectively. The levels of detected β-blockers appeared to be significantly lower than those reported in earlier study for Western Balkan region (Terzić et al., 2008). Namely, Terzić et al. (2008) found the average concentrations of β-blockers in samples of municipal waste water as follows 1.88 μg L^{-1}, 0.95 μg L^{-1}, 0.22 μg L^{-1} and 0.13 μg L^{-1} for atenolol, metoprolol, sotalol and propranolol, respectively. Various studies have shown that among the group of cardiovascular drugs, β-blockers such as atenolol (Garner et al., 1994) and propranolol (Alder et al., 2010) belong to the most ubiquitous group of pharmaceuticals in surface waters. However, these two agents were found in low frequency in surface waters analyzed here (18.18% and 9.09%, respectively, Table 4) with levels (50.6 ng L^{-1} and 10.4 ng L^{-1}, respectively) lower than those found for surface water in South Wales (UK) (Kasprzyk-Hordern et al., 2008), in north-east Spain (Gros et al., 2007), in Italy (Zuccato et al., 2000; Calamari et al., 2003), and in the Madrid Region (Spain, Martinez Bueno et al., 2010; Valcárcel et al., 2011).

The most prominent diuretic drug was hydrochlorothiazide, which was detected in 27.28% of the samples and reached maximum level of 1070 ng L^{-1} in municipal waste water (city sewerage on the out flow to the Danube River, sample no. 3). The other related drug, diuretic furosemide was 2.5 times less frequent than hydrochlorothiazide with maximum level of 362 ng L^{-1} in the same sample. Diuretics detected in samples of surface water were hydrochlorothiazide in Begej River (sample no. 7) at concentration level of 164 ng L^{-1}, and furosemide in canal water from suburb settlement in the Novi Sad municipality (sample no. 4) in concentration of 101 ng L^{-1}. In region of Madrid, Spain, Valcárcel et al. (2011) found high quantities of hydrochlorothiazide with maximum level of 18 μg L^{-1} in surface water, while other diuretic, furosemide was found at elevated quantities with maximum level of 3.2 μg L^{-1}. Additionally, it should be noted that level of hydrochlorothiazide found in surface water in Serbia was very similar to the level of this diuretic detected in water of Erbo River in Spain reported by Ferreira da Silva et al. (2011).

The drugs belonging to the class of antihypertensives were identified in municipal waste, surface and drinking water. The most abundant ones were valsartan and losartan in sample of municipal waste water

(city sewerage on the out flow to the Danube River, sample no. 3) with maximum levels of 1.1 μg L^{-1} and 229 ng L^{-1}, respectively, while significantly lower concentration was found for irbesartan (11.6 ng L^{-1}) in the same sample. The maximum level of irbesartan, valsartan and losartan detected in samples of surface water was 15.3 ng L^{-1} (Begej River, sample no. 7), 89.6 ng L^{-1} (Begej River, sample no. 7) and 154 ng L^{-1} (canal water from Bukovac village, sample no. 11), respectively.

In this study, salbutamol was only detected in drinking water (sample no. 24) at the maximum concentration of 5.4 ng L^{-1}. López-Serna et al. (2010) did not detect salbutamol in investigated samples of drinking water in region of Barcelona, Spain, while Zuccato et al. (2005) found salbutamol in Lambro River and Po River at concentrations of 2.5 and 1.7 ng L^{-1}, respectively.

Furthermore, X-ray contrast agent iopromide was detected in municipal waste, surface and drinking water with maximum levels of 804, 75.2 and 6.8 ng L^{-1}, respectively, which is significantly lower than the level of 6263 ng L^{-1} reported for wastewater in Spain (Gros et al., 2012). Other studies have shown that this compound could be found at high concentrations, especially in hospital waste waters (Gros et al., 2012). Generally, the loads of the X-ray contrast media are significantly increased on weekdays, because X-ray examinations are performed in hospitals and radiological practices predominately from Monday to Friday (Ternes and Hirsch, 2000). Ternes and Hirsch (2000) stated that compared to the other drug residues, the iodinated X-ray contrast media exhibited generally higher maximum levels in STP effluents.

The most commonly detected drug from group of antibiotics was ciprofloxacin (in 8.33% of water samples), while total frequency of occurrence of clarithromycin, trimethoprim and cefalexin was 5.56%. In this group, the lowest frequency of occurrence was 2.78% for erythromycin, ofloxacin and sulfamethoxazole for all investigated samples.

Very high concentration of clarithromycin (up to 616 ng L^{-1}) was observed in surface water (canal water from suburb settlement, Adice, Novi Sad, sample no. 4). Erythromycin was found at lower level (292 ng L^{-1}, canal water from Bukovac village, sample no. 11) than clarithromycin in the same type of water. This might be caused by the conversion of erythromycin to erythromycin-H_2O in the aquatic environment as it has been previously reported (Hirsch et al., 1999). On the other hand, much lower concentrations of clarithromycin up to 20.3 ng L^{-1} were detected in Lambro River and Po River (Zuccato et al., 2005). In this group, cefalexin was detected with maximum concentration of 283 ng L^{-1} in surface water of Begej River (sample no. 7). Apart from these drugs from group of antibiotic, ofloxacin, ciprofloxacin, sulfamethoxazole, trimethoprim and cefalexin were found at concentrations ranging from 220 to 803 ng L^{-1} in municipal waste water (city sewerage on the out flow to the Danube River, sample no. 3).

From therapeutic group, calcium channel blocker only detected drug was diltiazem in surface (18.18%) and municipal waste water with maximum concentrations of 6.9 ng L^{-1} (canal water from Bukovac village, sample no. 11) and 217 ng L^{-1} (sewerage on the out flow to the Danube River, sample no. 3), respectively.

In order to investigate if there are similarities among the PhAC patterns of different types of waters collected from the close locations, the bar plots representing the concentrations of the detected compounds in drinking water samples (Fig. 3a), the Danube River and canal water samples (Fig. 3b), underground water samples (Fig. 3c), canal water—Adice (Fig. 3d) and municipal waste water sample (Fig. 3e) taken from the Novi Sad City area are presented in Fig. 3.

Fig. 3a shows that albendazole, propranolol and salicylic acid were found in similar levels in all the types of drinking water either raw, treated (chlorinated) or tap drinking water, indicating that treatment and distribution of water in the city water supply system did not affect the presence of the three pharmaceutical compounds. Significantly higher levels of 10,11 epoxycarbamazepine (10,11 epoxy CBZ) and of hydrochlorothiazide (HCTZ) were found in sample 22 (raw untreated water in public company for city water supply) while these compounds were found neither in chlorinated water (sample 23) nor in tap water (sample 21) suggesting that applied treatment influence their removal. As can be observed from Fig. 3b occurrence of drugs was very similar in two samples of the Danube River water taken upstream and downstream from the city center (sample 9 and sample 10, respectively). The only significant difference was found for losartan detected in the sample (9) taken upstream from the city center. In water sample from canal Danube–Tisa–Danube from the city area (samples 5 and 6) iopromide and carbamazepine were found in levels similar to the Danube River water as well as traces of irbesartan. The observed PhAC pattern of the river water was quite different than the pattern found in drinking water, even though it is known that the aquifer of the water supply source is predominantly fed from the Danube River. The presence of iopromide and ketoprofan only in sample of chlorinated water taken in the water supply company (Fig. 3a), without detecting these compounds in tap water (taken at our Lab), implied their sporadic and low occurrence probably as a result of the treatment processes. Concerning the underground water samples, similarity between samples 17 and 18 was observed, since almost the same contents of propranolol, salicylic acid and albendazole were found in both samples. The public fountains from which these two samples were taken are located about 1 km from each other; obviously, their similar PhAC patterns resulted from the same origin, i.e. the same underground water feeds these two public fountains. However, clear difference of the PhAC patterns might be seen between the samples of underground waters 15 and 16 taken at the private wells at close locations (~500 m from each other), particularly concerning the presence of ibuprofen, naproxen, phenazone, propyphenazone and carazolol. It is interesting to note that the small private company for production of cosmetic is located in the very vicinity of the canal where sample 4 was taken. Nevertheless, the similarity between the PhAC pattern of sample of the underground water (sample 16) and the nearby canal water—sample 4 (canal water—Adice, Fig. 3d) could not be seen. The PhAC pattern of sample 3 (Fig. 3e) was specific with over 30 (exactly 33) compounds detected; as it was the sample of the municipal waste water discharged without any treatment, it reflected drugs most often used by the citizens of the Novi Sad and some of their metabolites: ibuprofen, 10,11 epoxy CBZ, 2-hydroxy CBZ and acetaminophen. Comparison of all presented PhAC patterns revealed that HTCZ and carbamazepine were two compounds most often found in almost all types of water samples taken in the Novi Sad City area.

4. Conclusions

Pharmaceuticals belonging to different therapeutic groups and having different physicochemical properties were analyzed in different types of waters collected from 25 locations in the northern part of Serbia. The pharmaceuticals were selected based on their very frequent usage and therefore ubiquitous presence in different types of waters.

A UPLC–QqLIT–MS/MS method was applied to determine simultaneously 81 pharmaceuticals from different therapeutic classes in extracts of surface, underground, drinking and waste waters (industrial and municipal) obtained by solid phase extraction. Forty seven of 81 pharmaceuticals were found in investigated samples of water collected from Serbia. The highest concentrations of pharmaceuticals were found in sample of municipal waste water, while the lowest occurrence of pharmaceuticals were in waters intended for drinking water supply (untreated and treated), in which the highest concentration was found for carbamazepine metabolite (10,11-epoxycarbamazepine), the predominant compound in all types of water within the group of psychiatric drugs. A widespread occurrence of pharmaceuticals in the analyzed waters was proven, with general levels, when detected, from ng L^{-1} to more than 1 μg L^{-1} as found for some of drugs such as ibuprofen, diclofenac, codeine, valsartan, acetaminophen, 2-hydroxycarbamazepine and 10,11-epoxycarbamazepine in sample of municipal waste water. This is the first attempt to assess the occurrence of these 81 pharmaceutical residues in water samples in Serbia.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2013.08.079.

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Original Research Article

Multimycotoxin analysis of crude extracts of nuts with ultra-high performance liquid chromatography/tandem mass spectrometry

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ABSTRACT

A reliable, fast and simple method using ultra-high performance liquid chromatography with heated electrospray ionization triple quadrupole mass spectrometry (UHPLC/HESI-MS/MS) was developed for the simultaneous determination of aflatoxins B1, G1, B2 and G2, ochratoxin A (OTA), zearalenone (ZEA), HT-2 toxin, T-2 toxin and fumonisins B1 and B2 in crude extracts of various types of nuts. The procedure is based on the simultaneous extraction of selected mycotoxins with a mixture of acetonitrile/water/acetic acid (79:20:1, v/v/v) and defatting the obtained extract with hexane in order to remove the lipids. The validation data indicated that the analysis of different types of crude extracts of nuts is feasible and sensitive enough for determination of the majority of the studied mycotoxins. The recoveries of various nuts matrices ranged between 71.25% and 140.11% with relative standard deviation lower than 12%. The satisfactory recoveries were obtained for the most of mycotoxins using walnut matrix-matched calibration curves indicating the multi-matrix feasibility of the method. The applicability of the method was successfully demonstrated on 17 samples of nuts collected in a region of northern Serbian province of Vojvodina. Total frequency of the occurrence of the selected mycotoxins was 12%.

1. Introduction

Food contamination by mycotoxins is a continuous concern in food safety. The number of mycotoxins known to exert a toxic effect on human and animal health is constantly increasing, and more and more legislative provisions are taken to control their presence in food and feed (Zinedine and Mañes, 2009). These toxins occur naturally in plant products such as cereals, nuts and dried fruit and in their by-products as well (Bennett and Klich, 2003; Miraglia and Brera, 2002).

Nuts are among the most nutritious human foodstuffs because of their high content of proteins, carbohydrates, unsaturated lipids, vitamins and essential minerals (USDA, 2010). Nuts are commonly consumed by all age groups and across social strata in both developed and developing countries. Per capita consumption is expected to increase in the world wide with continuous promotion of their properties as healthy food. However, nuts have low water activity (a_w) so fungi are the major microbiological contaminants. Some of these molds are mycotoxigenic, thus high levels of mycotoxins have frequently been reported in nuts from the orchards and from the market (Bayman et al., 2002; Fernane et al., 2010). It is well known that nuts are among the commodities with the highest risk of contamination by aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2). Reports on mycotoxin contamination of nuts have mainly focused on *Aspergillus* and *Penicillium* mycotoxins, such aflatoxins and OTA (Huang et al., 2010; Luttfullah and Hussain, 2011; Rubert et al., 2011) with scanty records on some other fungal metabolites including *Fusarium* mycotoxins like fumonisins B1 (FB1) and B2 (FB2), ZEA, HT-2, T-2, etc. (Abia et al., 2013; Varga et al., 2013).

Currently, only aflatoxins have been included in the European regulation for nuts, Regulation EC 165/2010 amending the Commission Regulation No. 1881/2006, which established the maximum levels (ML) for aflatoxins as follows: 10 μg/kg for

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aflatoxins (AFB1, AFG1, AFB2, AFG2) and 5 μg/kg for AFB1 for hazelnut; 4 μg/kg for aflatoxins and 2 μg/kg for AFB1 for walnut/peanut; 10 μg/kg for aflatoxins and 8 μg/kg for AFB1 for almond. Nevertheless, more information is needed on other mycotoxins as well, such as fumonisins, HT-2, T-2, ZEA, OTA, etc.

Hence the implementation of a reliable, rapid, cost-effective analytical strategy providing comprehensive data is an important task. The crucial condition for obtaining good recoveries is an efficient isolation of analytes from plant matrix. It should be noted that the physicochemical properties of mycotoxins vary widely, thus the choice of an efficient extraction procedure enabling high recoveries for all the target analytes is not an easy task (Zachariasova et al., 2010). Many laboratories routinely use preparatory methods based on extraction/cleanup/pre-concentration steps for only one or a small group of similar mycotoxins. For example, many authors have used methods employing clean-up for analysis of aflatoxins and OTA in nuts (Set and Erkmen, 2010; Huang et al., 2010; Luttfullah and Hussain, 2011; Rubert et al., 2011; Baquião et al., 2012). Although these methods are well established, and in some cases interlaboratory validated, the current trend is to introduce simple (one-step), broad-scope procedures which, thanks to the use of modern separation/detection instrumental technologies, allow accurate determination of as many as possible major mycotoxins even at low levels in crude extracts, and do not require a labor/cost-demanding clean-up step (Škrbić et al., 2011a, 2011b; Škrbić et al., 2012; Škrbić et al., 2013).

The Serbian population consumes nuts, mostly walnuts directly or as ingredients in cookies and other confectionary products. The Serbian regulation (28/2011) sets mycotoxins maximum levels at the same levels as the EC regulation (165/2010). However, until now there has been no information about the safety of nuts consumed by the Serbian population. Consequently, it is important to study the presence of mycotoxins, since there is a lack of information in the literature about their occurrence in these products.

Thus, the aim of this study was: (i) to develop a simple and simultaneous method for efficient extraction of regulated and non-regulated mycotoxins from nuts; (ii) to validate UHPLC/HESI-MS/MS multi-mycotoxin method using the obtained crude extracts from nuts; and (iii) to apply the method on samples collected within Novi Sad, the capitol of the northern Serbian province of Vojvodina.

As the crude extract method followed by UHPLC–HESI-MS/MS is a technique that has been frequently used in the literature as a routine analytical technique for mycotoxins in cereals, our intention was also to extend the scope of the previously developed method (Škrbić et al., 2011a, 2011b, 2012, 2013) and prove the availability of crude extract use for mycotoxin analysis of nuts matrices.

2. Materials and methods

2.1. Reagents and chemicals

Individual standard stock solutions of AFB1 (2 μg/mL), AFB2 (0.5 μg/mL), AFG1 (2 μg/mL), and AFG2 (0.5 μg/mL), OTA (10 μg/mL), HT-2 toxin (100 μg/mL), T-2 toxin (100 μg/mL), ZEA (100 μg/mL), FB1 (50 μg/mL) and FB2 (50 μg/mL) were purchased from Supelco Co. (Bellefonte, PA, USA). All standards dissolved in acetonitrile were stored at −20 °C in amber glass vials, and brought to room temperature before use. Composite working standard solutions were prepared by diluting the above-mentioned stock solutions in acetonitrile and they were added in appropriate dilution to the extract of the uncontaminated sample to prepare matrix-matched calibration standards in concentration ranges that include the maximum allowable concentrations and also the expected range of mycotoxin occurrence (in accordance to the available literature data). Ultra-pure water was produced by Milli-Q purification system (Millipore, Molsheim, France). Methanol, acetonitrile and ammonium acetate (all LC–MS grade) were supplied from J.T. Baker (Deventer, The Netherlands), glacial acetic acid (p.a.) was obtained from LTG Promochem (Wesel, Germany). Hexane (HPLC grade, ≥98.5%) was supplied from Sigma–Aldrich (Hamburg, Germany).

2.2. Collection of samples

Seventeen samples of different nuts were collected within Novi Sad, the capitol of the northern Serbian province of Vojvodina, in February 2013. Samples could be classified according to the origin as "domestic" (8 walnut (*Juglans*) and 2 hazelnut (*Corylus avellana*) samples were taken from private resources) and "commercial" (1 walnut, 1 hazelnut, 3 peanut (*Arachis hypogaea*) and 2 almond (*Prunus dulcis*) samples were selected randomly from different supermarkets within Novi Sad). The commercial packs of selected samples weighed from 75 to 500 g. Before analysis, each sample was ground and homogenized using a laboratory mill (A11 Basic, IKA, Germany). The samples were kept at 4 °C until analysis.

2.3. Sample preparation

Previously developed methods for the so-called crude extract of wheat flour (Škrbić et al., 2011b, 2012) and paprika (Škrbić et al., 2013) were slightly modified. Namely, since the previous studies of mycotoxin analysis in wheat flour (Škrbić et al., 2011b, 2012) showed that non-acidified extraction solvent could not recover FB1 and FB2 in a satisfactory manner (above 60%), acetic acid was added to the mixture of solvents for extraction (79:20:1, v/v/v, acetonitrile/water/acetic acid) (Sulyok et al., 2006, 2007, 2010; Abia et al., 2013; Varga et al., 2013), in order to enable the isolation of these toxins. Then, defatting of the obtained crude extracts with hexane was introduced into the sample preparation procedure in order to remove the lipids that might interfere with the mycotoxin analysis by UHPLC–HESI-MS/MS. Such prepared crude extracts of the samples were used for further analysis without any purification step.

Briefly, the samples were prepared as follows: 10 g of homogenized samples (walnuts, hazelnuts, peanuts or almonds) were extracted by shaking with 40 mL of acetonitrile/water/acetic acid mixture (79:20:1, v/v/v) for an hour using an automatic shaker (Promax 2020, Heidolph Instruments, Germany). After extraction, the suspensions were filtered through Whatman filter paper No. 4, and an aliquot (20 mL) of filtered crude extracts was transferred into a plastic flask. Then, 20 mL of hexane was added to the filtered crude extract (20 mL) and the content was thoroughly mixed for 2 min in order to remove the lipids. The mixture was centrifuged at 5000 rpm for 5 min. After separation of the two phases, hexane was eliminated. Before injection into the UHPLC/HESI-MS/MS, the crude extract in acetonitrile was passed through a 0.2 μm nylon syringe filter.

2.4. Instrumental conditions

Separation and detection were performed as described in previous studies (Škrbić et al., 2011b, 2012, 2013). The steps could be summarized as follows: ultra-high performance liquid chromatography (UHPLC) performed by Accela™ (Thermo Fisher Scientific, San Jose, United States) was used for separation of sample components. Hypersil GOLD™, 50 mm × 2.1 mm i.d., 1.9 μm column (Thermo Fisher Scientific) was used with a flow rate of 0.5 mL/min, and the column temperature was maintained at 25 °C. The injection volume was 10 μL. The mobile phase consisted

Table 1
UHPLC/HESI-MS/MS parameters of mycotoxins under optimized conditions on Accela-TSQ Vantage.[a]

Mycotoxins	t_R[b], min	Precursor ion, *m/z*	Product ions[c], *m/z*	CID[d], eV
AFB1	3.17	313.1 $[M+H]^+$	285.1/241.1	33/22
AFB2	3.07	315.1 $[M+H]^+$	287.2/259.2	28/25
AFG1	2.97	329.1 $[M+H]^+$	200.2/215.1	26/40
AFG2	2.87	331.0 $[M+H]^+$	245.0/189.1	40/28
OTA	3.91	404.1 $[M+H]^+$	239.0/221.0	35/23
HT-2	3.57	447.2 $[M+Na]^+$	345.5/285.5	19/20
T-2	3.74	489.0 $[M+Na]^+$	245.1/327.1	26/23
ZEA	3.88	317.1 $[M-H]^-$	175.1/131.1	−26/−32
FB1	3.70	722.6 $[M+H]^+$	334.3/352.4	38/34
FB2	3.98	706.5 $[M+H]^+$	336.3/318.3	36/38

[a] Dwell time for all compounds was set to be 0.1 s.
[b] Retention time.
[c] Numerical values are given in the order quantifier/qualifier ion.
[d] Collision-induced dissociation energy for quantifier/qualifier ion.

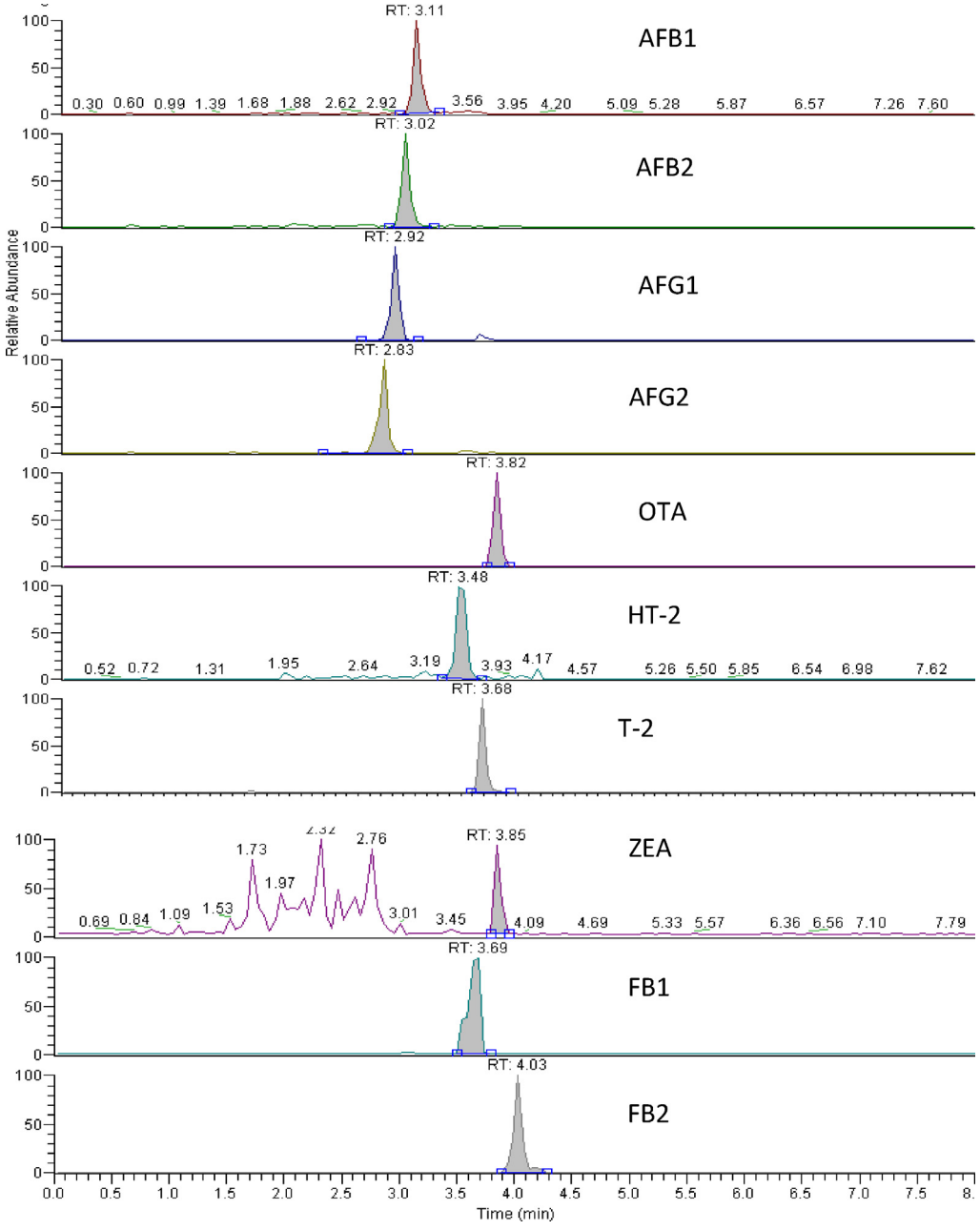

Fig. 1. Chromatograms of the mixture of all analyzed mycotoxins spiked in crude extract of walnut at the lowest calibration levels.

of eluent A containing water/acetic acid (99:1, v/v), and eluent B consisting of methanol/acetic acid (99:1, v/v). Both eluents contained 5 mM ammonium acetate. The gradient program started with 95% A and 5% B and was kept for 0.5 min, afterwards a linear gradient was applied, reaching 95% B after 3.04 min (holding time 2.1 min) and then switched back (6.20 min) to 95% A (holding time 1.80 min), which was maintained until the end of the run at 8 min.

For detection of analytes, a triple quadrupole mass spectrometer (MS/MS) TSQ Vantage equipped with heated-electrospray ionization probe HESI-II (Thermo Fisher Scientific), was used. Parameters of the ion source were as follows: spray voltage: 3.4 kV, vaporizer temperature: 350 °C, sheath gas pressure: 40 arbitrary units, auxiliary gas pressure: 10 arbitrary units, and capillary temperature: 270 °C.

Details on MS acquisition and optimization were given in previous studies (Škrbić et al., 2011b, 2012, 2013). UHPLC/HESI-MS/MS parameters of mycotoxins separation and identification under optimized conditions on the Accela-TSQ Vantage system are shown in Table 1. Fragmentation reactions were carried out in selected reaction monitoring mode (SRM) by choosing the optimum voltage of collision energies for each compound. Two product ions were measured for all compounds: one was used as the quantifier ion and the other was used as the qualifier ion. In SRM mode, a mass resolution of 0.2 Da full width at half maximum (FWHM) was set on the first (Q1) and to 0.7 Da FWHM at the third (Q3) quadrupoles and a scan width of 0.5 m/z were used. Chromatograms of the mixture of all analyzed mycotoxins spiked in crude extract of walnut at the lowest calibration levels are presented in Fig. 1. Instrument control and data collection were handled by computer equipped with Xcalibur 2.1.0 (Thermo Fisher Scientific).

2.5. Validation of the method

The developed method was validated by "in-house" quality control procedure. Parameters taken into account were: instrumental linearity, limits of detection (LOD) and quantification (LOQ), recovery and precision (expressed as relative standard deviation, RSD, in %).

Mycotoxins were quantified by external matrix-matched calibration procedure. Calibration solutions for matrix-matched calibration curves were prepared in uncontaminated nuts extract. To assess linearity, matrix-matched calibration curves ranging from 1 to 80 μg/kg for AFG1, ZEA, OTA and FB2; from 2 to 80 μg/kg for AFG2; from 4 to 80 μg/kg for AFB2; from 5 to 8 μg/kg for AFB1; T-2 from 7 to 80 μg/kg; from 3 to 80 μg/kg for HT-2 and FB1 were constructed.

LODs and LOQs were estimated by analyzing matrix matched standards at the lowest calibration level, and they were determined as the lowest concentration of the analytes that produce chromatographic peak at S/N of 3 and 10, respectively (Table 3).

Validation of the analytical method was carried out by determination of ("in-house") mycotoxin recoveries from spiked blank samples. Two levels of fortification were chosen. Namely, the uncontaminated nut samples were fortified with 4 μg/kg of each aflatoxins, corresponding to the order of magnitude (scale) of the maximum allowable contents of aflatoxins set by the European Commission Regulations (EC, 165/2010). For all the other investigated analytes (OTA, HT-2, T-2, ZEA and fumonisins), which are not regulated by the existing regulations for nuts, the arbitrary level of 40 μg/kg was chosen for the spiking. Spiked samples were left overnight at room temperature to allow solvent evaporation and equilibration between analytes and matrix. The recoveries of the method were determined as average values of the mycotoxin contents in three fortified samples of each investigated matrix; and each of them was analyzed three times. The repeatability of the method was determined as relative standard deviation (RSD, in %) of the mycotoxin content in three fortified samples of each investigated matrix.

All samples were analyzed in triplicates. Blank samples were included in every batch of samples to check for possible contamination.

3. Results and discussion

3.1. Results with nuts-based calibration curves

Recovery and repeatability values of multi-mycotoxin method obtained for walnut, hazelnut, peanut and almond are presented in Table 2. The results are presented as average values of three replicates. In this study, method performance was evaluated for each mycotoxin by spiking the real samples, and the performance parameters were compared with the ones established in relevant European regulation (EC, 401/2006).

The mycotoxin recoveries were first determined using the walnut-based calibration curves. This matrix was chosen knowing that walnut is the most widely consumed nut by the Serbian population, either domestically produced or imported. Moreover, walnut is the only nut type included in the Serbian market basket with monthly consumption rate of 0.1 kg per person (Statistical Office of the Republic of Serbia, 2011). Thus, the multi-matrix feature of the method was tested using the walnut calibration standards for mycotoxins analysis in the other investigated types of nuts. Table 2 demonstrates the applicability of the walnut

Table 2
Accuracy (%) and precision (RSD, %) of determination of selected mycotoxins (AFB1, AFB2, AFG1, AFG2, ZEA, OTA, T-2, HT-2, FB1, FB2) in walnut, hazelnut, peanut and almond (numbers in parenthesis are recovery and RSD values obtained for corresponding matrix-matched calibration standards); otherwise the numbers are recoveries and RSD determined using the walnut matrix-matched calibration standards.

Compound	Walnut		Hazelnut		Peanut		Almond	
	Recovery[a] (%)	RSD[a] (%)	Recovery[a] (%)	RSD[a] (%)	Recovery[a] (%)	RSD[a] (%)	Recovery[a] (%)	RSD[a] (%)
AFB1	99.12	6.22	88.46	4.40	102.05	7.00	112.35	5.45
AFB2	109.91	8.25	113.29	6.78	110.98	7.28	103.06	8.00
AFG1	101.29	6.12	78.81	6.36	78.85	5.88	95.91	7.19
AFG2	99.83	6.64	120.01	8.95	79.01	7.40	104.19	6.50
ZEA	123.12	3.29	126.60	2.88	120.56	3.77	160.65 (94.49)	3.28 (8.00)
OTA	115.63	2.98	160.96 (114.41)	5.57 (3.80)	153.59 (114.04)	9.32 (5.24)	181.84 (120.01)	7.45 (1.16)
T-2	96.44	9.67	114.93	6.14	88.16	5.44	157.71 (118.21)	6.00 (5.64)
HT-2	–[b]	–[b]	– (–)[b]	– (–)[b]	(71.25)	(8.74)	(102.13)	(8.21)
FB1	95.96	11.91	– (–)[b]	– (–)[b]	<60.00 (98.49)	(10.78)	<60.00 (<60.00)	–
FB2	140.11	10.61	76.86	10.83	87.45	10.60	91.00	11.87

[a] On the base of the results obtained by analysis of the three spiked samples ($n = 3$).
[b] Recoveries were not determined since the linearity of calibration curves in the walnut and hazelnut matrices were not approved with acceptable R^2 ($R^2 < 0.99$) (see Table 3).

calibration curves for majority of the studied mycotoxins and the investigated nuts matrices because the satisfactory recovery values (above 60.00% and equal or lower than 130.00%) were obtained, except in cases explained hereafter. Recovery values for aflatoxins for all nut samples in the case of walnut-based calibration were within the range of 78.81–120.01%, being in general in compliance with the Commission Regulation (EC, 401/2006). According to Table 2 the recovery values of FB2 were satisfactory, being from 76.89% to 91.00%, except in the case of walnut, for which recovery of 140.11% was obtained, but the RSD value indicated its satisfactory repeatability. Thus, the applied method with walnut-based calibration might be regarded as multi-matrix method for aflatoxins and FB2 analysis in various nut varieties.

Exceptions of the satisfactory recoveries, when walnut based calibration curves were used, are as follows: recoveries for OTA in hazelnut, peanut and almond were all higher than 130.00%; ZEA and T-2 recoveries from almond were also higher than 130.00%. For the listed exceptions the corresponding matrix-matched standards were prepared in order to check if disparity among the matrices caused too high or too low recoveries of particular mycotoxins. Thus, to investigate if walnut matrix influences ionization of OTA in a different way than hazelnut matrix enhancing the toxin signal and resulting in the recovery of 160.96%, the hazelnut-matched calibration standards of OTA were prepared. In the case of OTA toxin, the full compensation of matrix effects was achieved using the corresponding matrix-matched calibration curves, i.e. blank extract of each selected matrix spiked with the working standard. Furthermore, using walnut matrix matched calibration curves, the recoveries obtained for T-2 and ZEA in each selected matrix were up to 118.21% and 126.60%, respectively, being in general in compliance with the EC Regulation (EC, 401/2006), except for recovery values of these toxin in almond (Table 2). The obtained recoveries for almond matrix were quite different from the ones determined for other types of nuts, implying different influence this matrix had on the ionization process of these toxins and the MS signals. Hence, almond matched calibration standards for T-2 and ZEA were used and the obtained recoveries were in the range of the acceptable values (Table 2). Recovery of FB1 from hazelnut was not considered because hazelnut matched calibration curve was not approved with the acceptable squared correlation coefficient ($R^2 < 0.99$), while the recovery of FB1 for almond was lower than 60.00%, indicating that the extraction procedure did not enable efficient transfer of this toxin from sample into the acetonitrile solution. Since the walnut- and hazelnut-matched calibration curves for HT-2 were not obtained with satisfactory R^2 ($R^2 < 0.99$), the recoveries of HT-2 were considered only against the almond- and peanut-calibration curves, and their recovery values were acceptable (71.25% and 102.13%, respectively).

Thus, differences in matrix (background) composition could cause different interferences of the mycotoxin signals, influencing the calibration and final quantification of mycotoxins in extracts obtained from different types of nuts. The nut varieties studied in this work have different contents of main constituents like proteins or lipids (for instance, the content of proteins in walnut is 13%, in almond, 20%, in hazelnut, 10% and in peanut, 23%; while the content of lipids in analyzed nuts could be ordered as follows: hazelnut (63%) ~ walnut (60%) > almond = peanut (47%)). Thus, the use of corresponding matrix-matched standards calibration is required in order to compensate matrix effects and improve the analytical parameters when recoveries obtained against walnut matrix matched calibration curves were unacceptable. Bearing in the mind that the UHPLC–MS/MS analysis of crude extract is a fast procedure, the preparation and injection of corresponding matrix-matched standards did not increase the analysis time per batch considerably.

For all mycotoxins considered, good repeatability was obtained for each matrix, since the RSD for each compound was below 20% (Table 2). Relative standard deviations were about 11–12% for all four types of nuts, showing the acceptable precision of the applied procedure for selected mycotoxins.

The linearity of the calibration graphs obtained for the matrix-matched standards (presented in Table 3) was evaluated by calculating the squared correlation coefficient (R^2). For walnut matrix, excellent linearity ($R^2 \geq 0.9903$) was obtained for all the calibration curves (i.e. for all analytes), except of HT-2. As shown in Table 3, walnut- and hazelnut-matrix matched calibration curves for HT-2 were obtained with unsatisfactory R^2. In a similar manner, R^2 was unacceptable for hazelnut matrix matched calibration for FB1. Hence, it could be assumed that matrix compounds from walnut and hazelnut interfered with the signals of these toxins. Thus, to get rid of the matrix interferences, it seems that clean-up of the walnut and hazelnut crude extracts is necessary in order to obtain the corresponding matrix matched calibration curves with satisfactory squared correlation coefficients (R^2). Additionally, it is shown that R^2 obtained for some mycotoxins in samples of hazelnut, peanut and almond were satisfactory, except for peanut where linearity was worse for FB1 (0.9689).

LODs and LOQs are shown in Table 3 for analyzed mycotoxins in the studied matrices. LODs in walnut matrix were found to be between 0.02 and 2.00 μg/kg, while LOQs ranged between 0.07 and 6.66 μg/kg. Estimated values for almond of LODs (LOQs) were 0.05 μg/kg (0.15 μg/kg) for T-2, 0.13 μg/kg (0.42 μg/kg) for OTA, 0.62 μg/kg (2.05 μg/kg) for FB1, 0.63 μg/kg (2.10 μg/kg) for HT-2, 0.06 μg/kg (0.21 μg/kg) for ZEA. Generally, it could be seen from Table 3 that the estimated LODs/LOQs for OTA were very low and similar among investigated types of nuts.

Table 3
Method performance characteristics for analysis of regulated and non-regulated mycotoxins in nuts samples.

Compound	Walnut			Hazelnut[a]			Peanut[a]			Almond[a]		
	R^2	LOD, μg/kg	LOQ, μg/kg	R^2	LOD, μg/kg	LOQ, μg/kg	R^2	LOD, μg/kg	LOQ, μg/kg	R^2	LOD, μg/kg	LOQ, μg/kg
AFB1	0.9966	1.50	5.00									
AFB2	0.9903	1.20	4.00									
AFG1	0.9907	0.03	0.10									
AFG2	0.9935	0.55	1.83									
ZEA	0.9949	0.06	0.21							0.9951	0.06	0.21
OTA	0.9929	0.02	0.07	0.9960	0.01	0.04	0.9967	0.10	0.33	0.9935	0.13	0.42
T-2	0.9924	2.00	6.66							0.9948	0.05	0.15
HT-2	–[b]	–[b]	–[b]	–[b]	–[b]	–[b]	0.9931	0.89	2.96	0.9959	0.63	2.10
FB1	0.9946	0.24	0.80	–[b]	–[b]	–[b]	0.9689	0.89	2.96	0.9912	0.62	2.05
FB2	0.9916	0.05	0.17									

[a] Walnut was chosen as a matrix for the method validation and instrument calibration; when recoveries obtained against walnut matrix matched calibration curves were unacceptable (<60% or >130, see Table 2), the instrument was calibrated against the particular matrix matched standards of that mycotoxin.
[b] The linearity of calibration curves in the walnut and hazelnut matrices were not approved with acceptable R^2, ($R^2 < 0.99$).

Table 4
Recovery ranges for selected mycotoxins determined in this study and reported in some previous studies in nuts matrices.

References	Mycotoxins	Samples	Method preparation	Analytical method	Recovery range (%)[a]
Set and Erkmen (2010)	Aflatoxins (B1, B2, G1, G2)	Pistachio nut	Immunoaffinity column	HPLC-FLD	61–89.3%
Huang et al. (2010)	Aflatoxins (B1, B2, G1, G2)	Peanut, musty peanut, peanut butter	Home-made mixed cartridge	UHPLC–MS/MS	80.1–86.8%
Luttfullah and Hussain (2011)	Aflatoxins (B1, B2, G1, G2)	Dried fruits and nuts (almond, walnut, peanut, pistachio)	Immunoaffinity column	HPLC-FLD	83.5–92.5%
Rubert et al. (2011)	Aflatoxins (B1, B2, G1, G2) and OTA	Tiger-nuts and their beverages	Matrix solid phase dispersion	LC–MS/MS	71–83%
Baquião et al. (2012)	Aflatoxins (B1, B2, G1, G2)	Brazil nuts	SPE (C18)	HPLC-FLD	80.44–84.26%
García-Cela et al. (2013)	Aflatoxins (B1, B2, G1, G2) and OTA	Pistachio	Immunoaffinity column ELISA	HPLC-FLD	71–122% 72.8–107.9%
Abia et al. (2013)	Aflatoxins (B1, B2, G1, G2), OTA, ZEA, fumonisins (B1 and B2)	Peanut and its by-products	Crude extract	LC–MS/MS	73–86%
Varga et al. (2013)	Aflatoxins (B1, B2, G1, G2), OTA, ZEA, T-2, HT-2, fumonisions (B1 and B2)	Almond, hazelnut, peanut, pistachios	Crude extract	UHPLC–MS/MS	31–127%
This study	Aflatoxins (B1, B2, G1, G2), OTA, ZEA, T-2, HT-2, fumonisins (B1 and B2)	Walnut, hazelnut, peanut, almond	Crude extract	UHPLC–MS/MS	71.25–140.11%

[a] Values are presented as given originally in the cited source.

In the cases of aflatoxins, LODs were lower than the maximum residue limits established by European Union (EC, 165/2010), indicating the suitability of the proposed method for the determination of trace concentration of these compounds.

The overview of the sample preparation and analytical methods applied together with the obtained recoveries for the selected mycotoxins in various nuts reported in previous studies are given in Table 4 including the summarized results obtained in this study. It could be seen that in most studies a purification step was included in the applied sample preparation methods, while crude extract analysis was rarely used (Abia et al., 2013; Varga et al., 2013). In general, both types of sample preparation methods (with or without purification of the extracts) gave similar ranges of recoveries except in the case of the analyzed mycotoxins with reported recoveries of 31–127% (Varga et al., 2013). Low recoveries obtained for fumonisins (31–41%) in all studied matrices (Varga et al., 2013) could be expected when a multi-target method covering a huge number of chemically diverse analytes is used; therefore a compromise on the final analytical conditions has to be reached. However, the real difference in these two types of applied methods is in the number of analytes: crude extract analysis enables identification and quantification of more mycotoxins than in the case of the purified extracts. Moreover, the sample preparation of crude extract is cheaper and faster.

3.2. Mycotoxins analysis in nuts samples

The method was applied to the simultaneous determination of the studied mycotoxins in walnut, hazelnut, almond and peanut samples collected within Novi Sad, the capitol of the northern Serbian province of Vojvodina, in February 2013. The quantification of the nut samples was performed against the corresponding matrix-matched calibration curves approved with acceptable R^2. Results obtained in this study showed a weak contamination of nuts (only two positive samples out of 17). In most of the investigated samples of walnut, peanut, hazelnut and almond, levels of mycotoxins were below the determined LODs. In two samples of walnut (domestic, stored without shell) ZEA was detected at levels of 1.20 and 3.48 μg/kg. In two samples of almond (commercial, crude) the levels of AFB2 were below LOQ. Thus, all the analyzed nuts samples were in compliance with the relevant EC regulations (EC, 165/2010), as well as with the Serbian Regulation (28/2011) that defines the same maximum allowable concentration of aflatoxins as EU regulations.

Zinedine and Mañes (2009) showed that in Morocco, walnut and pistachio were contaminated with AFB1 levels ranging from 0.56 to 2500 μg/kg and from 0.04 to 1430 μg/kg, respectively. Also, in Morocco the average value of OTA in positive samples of walnut was 0.11 μg/kg (Zinedine and Mañes, 2009). Literature available data on the occurrence of aflatoxins in walnut from Pakistan indicated that two investigated walnuts samples had contamination levels of 7.8 and 13.5 μg/kg, respectively, and they were above the suggested limit (Luttfullah and Hussain, 2011).

Besides mycotoxins subjected to the EU regulation, there is also an evidence of the occurrence of OTA and fumonisins in groundnuts (Abia et al., 2013). For instance, Abia et al. (2013) found that 40% of the 15 samples were naturally contaminated with OTA, 73% were contaminated with FB1 and 33% with FB2, while the highest amounts found in these samples were 17 μg/kg, 6 μg/kg and 3 μg/kg, for FB1, FB2 and OTA toxin, respectively.

4. Conclusion

The multimycotoxin and multimatrix method developed for simultaneous determination of 10 regulated and non-regulated mycotoxins in nuts according to the requirements of the relevant EU regulations has demonstrated the applicability and effectiveness of crude extract. The obtained recoveries of the developed method were satisfactory between 71.25% and 140.11% in all matrix tested with RSD values lower than 12%. However, the same analytical approach could not be satisfactorily applied for obtaining linear matrix matched calibration curves for FB1 (for hazelnut) or HT-2 (for walnut and hazelnut), due to marked matrix interferences and their impact on the MS response. Total frequency of the occurrence of the selected mycotoxins in the analyzed types of nuts was 12%.

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Concentrations of arsenic, cadmium and lead in selected foodstuffs from Serbian market basket: Estimated intake by the population from the Serbia

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ABSTRACT

In this study arsenic (As), cadmium (Cd) and lead (Pb) were determined in 114 samples of various food items collected at supermarkets located in Novi Sad, the capitol of the northern Serbian province of Vojvodina in January 2012 and March 2013. The considered items represented the most consumed foodstuffs according to the "national market basket". The highest concentrations were obtained for Pb in candy (0.323 mg kg^{-1}), for Cd in paprika (0.118 mg kg^{-1}) and for As in canned fish (0.43 mg kg^{-1}). The results were compared with the relevant data on the occurrence of these toxic elements available in literature for other European countries. Human health risk assessment through dietary exposure was evaluated for Serbian adult consumers. The estimated intakes were compared with available toxicological references to assess the risk of As, Cd and Pb intake through consumption of analysed food items. The highest intake were estimated for Pb being 72.30 μg day^{-1} for adult population, while intakes of As and Cd were significantly lower (21.89 μg day^{-1} and 11.51 μg day^{-1}, respectively).

1. Introduction

The presence of heavy elements in the environment and food chain represents a serious problem, which is recognised in most of the countries around the world. To ensure food safety, the European Commission Regulation (EC, 1881/2006) has established maximum levels for some contaminants, including also heavy elements like Pb and Cd in human foods whereas for As the limit levels have not yet been proposed.

Similarly, the latest Official Bulletin of the Republic of Serbia No 28/11 (Serbian regulation, 2011) has established the maximum levels for Pb and Cd in foodstuffs in line with the EC regulation, but it regulates wider spectrum of food commodities and it also set the maximum levels of As.

Most of the elements including As, Cd and Pb are extremely toxic because of their solubility in water. Even low concentrations of As, Cd and Pb have damaging effects to humans and animals because there is no good mechanism for their elimination from the body. Food and water are the main sources of essential elements, but these are also the media through which humans are exposed to various toxic elements. The presence of these elements in food depends on several factors. They might come from: the soil, environment, genotype of the plant, fertilizers and/or metal-containing pesticides, introduced during the production process or by contamination from the metal processing equipment. Hence, the accumulation of these toxic elements in the food was of increasing concern due to food safety issues and potential health risk. Thus, it is imperative that the presence of heavy elements in foodstuffs is controlled in accordance to the defined maximum residue levels set by the EU/national authorities, or in the absence of such limits to perform constant monitoring and comparison with the available data in literature. The monitoring of the heavy element levels in food samples as well as the estimation of these food contaminants intake are essential for risk evaluation and investigation of possible contamination that would represent a health hazard.

Concerning the health risks derived from the intakes of the toxic elements As, Cd and Pb, the results derived from this study were compared with the available toxicological values for these elements. On the basis of data related to lung cancer in humans, the European Food Safety Authority (EFSA) concluded that a range of 0.3–8 μg kg^{-1} bw day^{-1} should be used as a single reference point for characterising risk associated with inorganic arsenic (EFSA, 2009b). In 2010, Joint FAO/WHO Expert Committee on Food Additives (JECFA) withdrew the provisional tolerable weekly intake (PTWI) of 15 μg kg^{-1} bw $week^{-1}$ that had been defined in 1989 (JECFA, 2011a). According to new modelling approaches of EFSA, based on increased incidence of lung cancer in human, the reference point would be 3 μg kg^{-1} bw day^{-1} (i.e. 210 μg day^{-1} for adult of 70 kg body weight).

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Regarding Cd, the CONTAM Panel (EFSA, 2009a) established a tolerable weekly intake of 2.5 μg kg^{-1} (i.e. 25 μg day^{-1} for adult of 70 kg body weight), which replaced the previous PTWI of 7 μg kg^{-1}. Afterwards, in 2010, JECFA set a provisional tolerable monthly intake (PTMI) of 25 μg kg^{-1} bw $month^{-1}$ (i.e. 58 μg day^{-1} for adult of 70 kg body weight) (JECFA, 2011b).

Regarding Pb, the previously established PTWI of 25 μg kg^{-1} bw $week^{-1}$ set by JECFA in 1986. Recently, EFSA (EFSA, 2010) and JECFA (JECFA, 2011b) both acknowledged that this PTWI was not sufficiently protective but that they were unable to establish a new health-based guidance value, since a no-observed adverse effect level could not be identified on the basis of current data. However, EFSA identified three reference dietary intake values, two for adults and one for children/pregnant women and women of childbearing age. They were respectively 0.63 μg kg^{-1} bw day^{-1} (i.e. 44 μg day^{-1} for adult of 70 kg body weight) for nephrotoxic effects, 1.5 μg kg^{-1} bw day^{-1} (i.e. 105 μg day^{-1} for adult of 70 kg body weight) for cardiovascular effects, and 0.5 μg kg^{-1} bw day^{-1} for neuro-developmental effects (EFSA, 2010). A number of serious health problems can arise as a result of excessive uptake of heavy elements through the diet. Considering pathways such as intake, inhalation, and dermal contact, it is well established that more than 90–95% of the total daily exposure to elements comes from the diet (Bocio et al., 2005; Martí-Cid et al., 2009). Arsenic has been the only human carcinogen with registered evidence of carcinogenic risk by both inhalation and ingestion and it has been connected with certain types of cancer, including lung, liver, skin and bladder cancer in humans (IARC, 2004; Kapaj et al., 2006). It is also directly toxic and is accumulative. Cadmium is classified as "carcinogenic to humans" (group 1) by the International Agency for Research on Cancer (IARC, 1993) and ranked in category 2 by the European Union (OJEC, 2004). Cadmium is a highly toxic metal with a natural occurrence in soil, but it is also spread in the environment due to human activities (Zhu et al., 2011). Lead similarly to cadmium, has no beneficial role in human metabolism, producing progressive toxicity (Zhu et al., 2011). Lead creates health disorders such as sleeplessness, tiredness, hear and weight loss. The International Agency for Research on Cancer classified inorganic lead as probably carcinogenic to humans (Group 2) in 2006 (IARC, 2006).

Therefore, estimation of food intakes is necessary for risk evaluation, and possibly to determine relationships between adverse effects observed in humans and exposure to particular substances. Such exposure evaluations are also useful when making decision on the regulation of chemical contaminants and the safety of food products. Dietary exposure to elements such as As, Cd and Pb has been associated with toxic and adverse health effects (FAO/WHO, 2007). Dietary intakes of heavy elements of high public concern need to be monitored on a regular basis and rapidly updated to identify recent dietary intakes of heavy elements in different countries.

The World Health Organization (WHO), through its Global Environment Monitoring System—Food Contamination Monitoring and Assessment Programme (GEMS/Food), is encouraging countries to undertake total diet studies as the most cost-effective method for assessing dietary exposure to chemical contaminants in the diet (FAO/WHO, 2007). Representative datasets on consumption of foods are combined with data on concentration in foods of the compounds of interest, to derive the average dietary exposure.

Concerning the toxic elements occurrence in Serbian food and the corresponding dietary intake by Serbian population there is scarce data available in the literature (Škrbić and Čupić, 2005; Škrbić and Gyura, 2007) contrary to numerous studies from European countries (Llobet et al., 2003; SCOOP, 2004; Leblanc et al., 2005; Rubio et al., 2005, 2006; Becker et al., 2011; Martorell et al., 2011; Domingo et al., 2012; Arnich et al., 2012). In fact, to the best of our knowledge there is no comprehensive study on the levels of heavy elements in foods frequently consumed in Serbia. Thus, the main focus of this study was investigation on the presence of three toxic heavy elements Pb, Cd and As in major food items from the Serbian market basket to provide information for both producers and consumers.

Through comparison of the obtained results with the national and EC regulation, as well as to the relevant information from other countries, the aim was also to estimate the potential health risk of general population through the daily consumption of the studied foodstuffs from the Serbian market basket.

2. Materials and methods

2.1. Sample collection

In this study, the following food groups were included: fruit, vegetables, oils and fats, sweets, milk/dairy products and eggs, meat and meat – based products, fish, wheat and wheat products and other products. Table 1 presents the percentage of these groups in the total Serbian market basket according to the Statistical Office of the Republic of Serbia (2011), as well as food items included in the food groups with the percentage of their consumption rates in relation to the food group consumption.

In January 2012 and March 2013, selected foodstuff samples were collected randomly from different supermarkets within Novi Sad, the capitol of the Vojvodina Province, where the biggest producers of food in Serbia are located. A total of 114 composite samples were analysed. Within each food commodity, 3 composite samples of different brands were prepared by mixing in each of them 3 individuals samples of one brand collected from different markets. The food items in each food group were treated as they would be in a general household, e.g. meat was freed from skin and bones, potatoes and root vegetables were peeled. Before analysis, each sample was ground and homogenised using laboratory mill (A11 Basic, IKA, Germany). The samples were stored in their original packs at 4°C until analysis was carried out.

2.2. Reagents and solutions

All chemical were of analytical reagent grade. Ultra-pure deionized water type Milli-Q (Simplicity, Millipore, France) with a specific resistivity of 18.2 MΩ cm^{-1} was used for preparation of standards and sample solutions. Concentrated 69% nitric acid (ccHNO_3) ("for trace metals analysis" grade) and 30% hydrogen peroxide (H_2O_2) were purchased from J.T. Baker. All the plastic and glassware were cleaned by soaking in a 20% hydrochloric solution overnight then in 20% nitric acid overnight and finally rinsed with Milli-Q water. The As, Cd and Pb stock standard solutions (1000 μg mL^{-1}) were supplied by J.T. Baker. The working standard solutions of 1 μg mL^{-1} for each element were obtained by diluting stock solutions in 3% nitric acid. The calibration curve was prepared using the so-called bulk solution prepared by mixing the standard solutions and the subsequent dilution. Automix option of the GFAAS was applied enabling automatic preparation of the calibration standards.

2.3. Microwave digestion

Microwave (Ethos One, Milestone, Italy) with segmented rotor of high pressure (HPR-1000/10S) and internal temperature sensor was used for digestion of the samples.

About 0.5 g of previously homogenised composite samples was weight inside high-pressure Teflon (TFM) vessels and 7 mL of ccHNO_3 (69%) and 1 mL of H_2O_2 (30%) were added. The operational conditions and the heating program used were carried out according to the conditions recommended by the manufacturer.

After cooling, digests were diluted with Milli-Q water to 25 mL in glass flask and finally, transferred to previously acid-cleaned and labelled polypropylene vessel for further analysis. From each kind of food samples three aliquots were digested and each sample solution was then analysed in triplicates.

2.4. Instrumentation

A Varian AA240/GTA120 model atomic absorption spectrometer (AAS) with deuterium background correction, equipped with a graphite furnace (GF) for electrothermal atomization and an automatic sampler was used in this study. The assembly was operated from an interfaced computer running SpectrAA software. Varian hollow cathode lamps were used as line sources for all analytes. The operating conditions for GFAAS are given in Table 2. Argon was used as the inert gas.

Table 1
Foods included in market basket purchased in Novi Sad, Vojvodina Province 2011 and 2013.

Food group (percentage of the total Serbian market basket)	Food items included in the food group		Total % of chosen food items in the food group
		Consumption (g/day)	
Fruit (8.6%)	Apple	91.4	95.9
	Orange	14.0	
	Banana	16.1	
	Walnut	3.2	
	Prunes	1.1	
Vegetables (25.5%)	Lettuce	18.3	66.3
	Cabbage	47.3	
	Bean	16.1	
	Carrot	17.2	
	Potato	139.8	
	Onion	30.1	
	Mushrooms	5.4	
Oil and fats (3.0%)	Oil	32.3	83.3
	Margarine	5.4	
Sweets (1.2%)	Cookies	9.1	75.8
	Chocolate	2.2	
	Candy	2.2	
Milk and dairy products, and eggs (22%)	Milk	145.2	87.5
	Yogurt	69.9	
	Soft cheese	32.3	
	Hard cheese	3.2	
	Egg	43.0	
Meat and meat-based products (9.9%)	Beef	7.5	92.2
	Pork	43.0	
	Chicken	48.4	
	Bacon	5.4	
	Sausage	10.8	
	Hot dog	4.8	
	Salami	15.1	
	Pate	4.3	
Wheat and wheat-based products (25.4%)	White bread	266.7	88.5
	Other types of bread	17.8	
	Pasta	10.8	
	Wheat flour	48.4	
Fish (0.9%)	Sea fish, hake	11.8	100
	Canned fish (sardines in oil)	2.2	
Other products (3.7%)	Paprika-dried spice	1.6	72.4
	Sugar	37.6	

2.5. Quality assurance

Analytical method used was accredited according to ISO 17025. Thus, appropriate quality assurance procedures and precautions were carried out to ensure the reliability of the results. The developed method was validated by: (a) in-house quality control procedure, (b) certified reference material (CRM), and (c) through participation in proficiency testing (PT) scheme. Parameters taken into account were: instrumental linearity, limits of detection (LOD) and quantification (LOQ), recovery and precision (expressed as relative standard deviation, RSD).

Summary of validation data of GFAAS method for analysis of As, Cd and Pb in samples digested by microwave are given in Table 3.

Calibration curves were obtained with acified aqueous element standards by external calibration procedure except in the case of meat samples, when standard addition method was applied. The correlation coefficients obtained for calibration curves were all greater than 0.9950. LOD and LOQ were calculated as the mean signal of five blanks plus three or ten times, respectively, the standard deviation. The LODs (LOQs) obtained for As, Cd and Pb were 0.03 mg kg^{-1} (0.07 mg kg^{-1}), 0.0003 mg kg^{-1} (0.0003 mg kg^{-1}) and 0.003 mg kg^{-1} (0.003 mg kg^{-1}), respectively. In the case of Cd and Pb, LODs and LOQs were in line with performance criteria for methods of analysis for these elements established by European Union (EC, 836/2011): LODs were less than one tenth (LOQs were less than one fifth) of the respective maximum level sets by Regulation (EC, 1881/2006). The exception was accepted for milk according EC (836/2011) that defines LOD (LOQ) which should be less than one fifth (two fifth) of the maximum level in the case when the maximum level for Pb is less than 0.1 mg kg^{-1}. Validation of the method accuracy was carried out by determination of the element recoveries from different foodstuffs (potato, mushrooms, apple, oil, cookies, milk, soft cheese, meat, bread, walnut, egg, sugar and paprika) samples spiked at maximum residual levels set by EC or Serbian regulation. For this purpose, each type of samples was spiked at five replicates. The recoveries ranged from 60% to 132% (Table 3). Repeatability expressed as relative standard deviation of 5 spiked samples, ranged from 1% to 20% (Table 3); RSD obtained for Cd and Pb are in accordance to the criteria established by EU (836/2011) with corresponding HORRAT values less than 2. All samples were analysed in triplicate ($n = 3$). Blank samples were included in every batch of samples to check for possible contamination.

The CRM, wheat GBW 10011, was also used to check the bias of the method. The recoveries (and %RSD) of As, Cd and Pb regarding CRM were as follows: 113% (12%), 92% (8%) and 96% (10%), respectively. Additionally, the accuracy of the method was checked by involvement in PT for determination of Pb in chilli powder organised by FAPAS in July–August 2012. The recovery (and %RSD) obtained in PT test was 80% (4%). Thus, all validation data indicated the suitability of the proposed method for determination of trace concentration of As, Cd and Pb.

2.6. Intake calculation

Calculation of the exposure of the Serbian population through consumption of investigated foodstuffs was based on the average daily portion of the selected foodstuffs and the average element concentrations found in this study, corrected for the recovery values.

Following equation was used for estimation of the element intake:

$$\text{Estimate of element intake } (\mu g/\text{day}) = [\text{element}] \cdot [\text{foodstuffs consumption}]$$

where [element] is the concentration of element in (mg kg^{-1}) detected in foodstuffs adjusted for recovery (Table 3), [foodstuffs consumption] is the amount of selected foodstuffs (kg) consumed per person per day.

For calculations, when the concentration of an element was under the respective LOD, that value was assumed to be equal to one half of the LOD (1/2 LOD).

The amounts of investigated foodstuffs used for calculation of the intakes were obtained according to the Serbian market basket (Statistical Office of the Republic of Serbia, 2011) and they are presented in Table 1.

3. Results and discussion

3.1. Concentration As, Cd and Pb in selected foodstuffs

The concentrations of As, Cd and Pb determined in selected foodstuff samples are presented in Table 4. Results are expressed

Table 2
Operating parameters for determination of As, Cd and Pb by GFAAS in selected food samples.

Wavelength (nm)	As 193.7			Cd 228.8			Pb 283.3		
Parameter step	Pyrolysis[a]	Atomization	Cleaning	Pyrolysis[a]	Atomization	Cleaning	Pyrolysis[a]	Atomization	Cleaning
Temperature (°C)	1400	2600	2600	400	1800	1800	400	2100	2100
Ramp time (s)	5	0.6		5	0.8		5	0.9	
Hold time (s)	3	2	2	5	2	2	3	2	2
Read	–	On		–	On		–	On	–
Gas flow (ml min^{1})	300	0	300	300	0	300	300	0	300
Sample volume(μl)[b]	15			15			20		
Modifier (μl)[b]	10			5			–		

Matrix modifier was 0.1% palladium nitrate.
[a] Drying step was in the range 85–120 °C in 55 s.
[b] Injected before pyrolysis.

Table 3
Summary of validation data of GFAAS method for As, Cd and Pb analysis.

Samples	Elements	Recovery "in-house"[a] (%)	RSD[a] (%)
Potato	As	74	20
	Cd	120	7
	Pb	108	8
Mushrooms	As	74	20
	Cd	86	5
	Pb	132	0.6
Apple	As	104	4
	Cd	74	7
	Pb	77	8
Oil	As	78	5
	Cd	112	2
	Pb	111	7
Cookies	As	83	2
	Cd	94	5
	Pb	104	2
Milk	As	66	15
	Cd	92	2
	Pb	84	8
Bread	As	117	12
	Cd	88	9
	Pb	88	8
Meat	As	60	15
	Cd	73	3
	Pb	89	6
Sugar	As	95	4
	Cd	120	8
	Pb	108	2
Walnut	As	71	5
	Cd	80	7
	Pb	120	10
Egg	As	70.5	5
	Cd	107	13
	Pb	87	6
Paprika	As	60	2
	Cd	99	7
	Pb	80	3

[a] On the base of the results obtained by analysis of the five spiked samples ($n = 5$).

as the average of three samples analysed. Samples with the element concentrations between LOD and LOQ were considered to be positive and their levels were included in the statistical analysis. If element was below LOD in all of the samples, its average value was reported as "<LOD"; otherwise for calculation of the averages, quantities below LOD were considered as LOD/2 (Škrbić and Čupić, 2005).

In present study, As was detected in only four analysed food items: canned fish (0.43 mg kg^{-1}), sausage (0.04 mg kg^{-1}), oil (0.03 mg kg^{-1}) and margarine (0.03 mg kg^{-1}).

Arsenic was found in margarine and oil products at the level of LOD, which was almost 3 times lower than the maximum residue level of 0.1 mg kg^{-1} set by Serbian regulation. In other food items it was below the limit of detection.

The Cd levels quantified in analysed foodstuffs were lower than maximum corresponding levels set by EC (1881/2006) or Serbian regulations (No. 28/11). According Cd contents found in this study the analysed food items could be order as follows: paprika > chocolate > candy = canned fish > white bread > sugar > sausage > cookies > potatoes, followed by pasta, pate, other types of bread, mushrooms, onion, prunes, bacon, hot dog, hake whereas the lowest content was found in oil, milk, apple, wheat flour and hard cheese samples.

The Pb levels in all investigated foodstuffs were found to be lower than respective maximum levels set by EC (1881/2006) or Serbian regulations (No. 28/11). In majority of samples Pb concentrations were very low being equal or lower than 0.08 mg kg^{-1}, except in the case of bread, chocolate, prunes and candy for which the highest concentrations from 0.133 to 0.323 mg kg^{-1} were found.

The results were compared with the European data (Table 4) obtained within the reports on tasks for scientific cooperation - SCOOP 3.2.11 that is the keystone of European Union (EU) risk assessment of food and feed safety in Member States (MSs) and also with the first French total diet study by Leblanc et al. (2005). The Cd values found for potato, mushrooms, margarine, soft cheese, hake, pate, chicken, wheat flour, pasta, egg, lettuce, cabbage, carrot and beef were similar or lower of the (minimum) values reported for Cd contents in corresponding foodstuffs from MSs, while opposite was found for oil, cookies, chocolate, milk, sugar and paprika (SCOOP, 2004; Leblanc et al., 2005). Value for the presence of Cd in bread from this study corresponded to those found in SCOOP report (2004), whereas it was almost 4 times higher than the value reported by Leblanc et al. (2005). Moreover, Pb contents in vegetables and fruits (potato, mushrooms, apple), cookies and in soft cheese obtained in this investigation were lower than those given in Table 4 for MSs, while in oil, margarine, chocolate, milk, bread, pork, pasta, hard cheese, yoghurt, carrot, cabbage, lettuce similar or higher values were found here (SCOOP, 2004; Leblanc et al., 2005). Concerning the presence of As detected in the Serbian oil samples, it was found to be similar to the level obtained by Leblanc et al. (2005) and it was about 10 times higher than the corresponding levels found in MSs (SCOOP, 2004).

It is worth to note that maximum values of the ranges reported for some foodstuffs from MSs (Table 4) were found to be higher than the relevant maximum allowable concentration (MAC); for instance, Cd levels in some samples of onion and apple were higher 4 times than the limit value, while Pb content in potato and milk were up to 3.5 times higher than respective limit. Similarly, Millour et al. (2011) also found some samples of milk (3/38) which exceeded the maximum Pb level of 0.020 mg kg^{-1}; in case of semi-skimmed milk detected level was almost 2 times higher than MAC, while in the other two cases, for whole and skimmed milk it was slightly above the MAC. Additionally, Millour et al. (2011) revealed of in analysed vegetables, spinach ($n = 16$) was by far the most contaminated by Cd (mean 0.073 mg kg^{-1}) although it was below MAC.

3.2. Intake of heavy elements by consumers through market basket

Intake of elements through food consumption is dependent on element concentrations in food and amount of food consumed. The average levels of As, Cd and Pb (presented in Table 4) were used in the intake calculation, since this methodology is internationally recognised to provide satisfactory estimates of long-term exposure, suitable for comparison with the respective toxicological values. Table 4 shows the estimates of As, Cd and Pb intakes for the general Serbian adults through consumption of the foodstuffs from the market basket. As can be seen from Table 4, the intakes of As and Cd through consumption of the investigated foodstuffs were estimated to be generally below the respective toxicological values (210 μg day^{-1} and 58 μg day^{-1}, respectively), while in the case of Pb the estimated intake cannot be ignored because it is almost two times higher than the reference toxicological value for nephrotoxic effects (44 μg day^{-1}). On the other hand, the estimated intake of Pb was lower then reference dietary intake values for cardiovascular effects (105 μg day^{-1}).

For adults the estimated intake of As through the studied foodstuffs was 21.89 μg day^{-1}, and almost two times lower intake of Cd (11.51 μg day^{-1}, Table 4) estimated through the selected foodstuffs. The studied food commodities gave little contribution to

Table 4
The content and intakes of As, Cd and Pb by different types of foodstuffs from the Serbian market basket.

Analyte	Type	Average value[a] (mg kg^{-1})	Daily intake (μg day^{-1})	MAC[b], Serbian regulation (No. 28/11) (mg kg^{-1})	MAC[b], EU (1881/2006) (mg kg^{-1})	EU, MSs[c] (mg kg^{-1})	First French total diet study (Leblanc et al., 2005) (mg kg^{-1})
As	Potato	<0.03	2.097	0.3		0.002	
	Onion	<0.03	0.452	0.3			
	Mushrooms	<0.03	0.0012	0.3		0.09	
	Lettuce	<0.03	0.275	0.3			
	Cabbage	<0.03	0.710	0.3			
	Bean	<0.03	0.242	0.3			
	Carrot	<0.03	0.258	0.3			
	Apple	<0.03	1.371	0.3		0.006–0.014	
	Orange	<0.03	0.210	0.3			
	Banana	<0.03	0.242	0.3			
	Walnut	<0.03	0.048			0.006–0.029	0.169
	Prunes	<0.03	0.017	0.5			
	Oil	0.03	1.244	0.1		0.003–0.005	0.045
	Margarine	0.03	0.210	0.1			0.060
	Cookies	<0.03	0.137	0.5		<0.1	0.003
	Chocolate	<0.03	0.033	0.5		0.0128	0.007
	Candy	<0.03	0.033	0.005			
	Milk	<0.03	2.178	0.1		<0.005–0.003	0.003
	Yogurt	<0.03	1.049	0.1		0.002	
	Hard cheese	<0.03	0.048	0.1			
	Soft cheese	<0.03	0.485	0.1		0.004	0.003
	Egg	<0.03	0.645	0.1		0.0009–0.005	0.008
	White bread	<0.03	4.001	0.5		0.005	0.04
	Other types of bread	<0.03	0.258	0.5			
	Pasta	<0.03	0.162	0.5		0.018	0.003
	Wheat flour	<0.03	0.726	0.5			
	Sea fish, hake	<0.03	0.177	4		9.7	
	Canned fish	0.43	1.577				
	Beef	<0.03	0.113	0.1		0.0053	
	Pork	<0.03	0.645	0.1		0.01	
	Chicken	<0.03	0.726	0.1			
	Bacon	<0.03	0.081				
	Sausage	0.04	0.485				
	Hot dog	<0.03	0.072				
	Salami	<0.03	0.227				
	Pate	<0.03	0.065				
	Paprika	<0.03	0.024	5.0			
	Sugar	<0.03	0.564	1.0		0.005	0.018
	Intake by total food considered		21.89				
Cd	Potato	0.009	1.049	0.1	0.05	0.010–0.0686	
	Onion	0.003	0.075	0.1	0.05	0.002–0.1288	
	Mushrooms	0.005	0.0004	0.2	0.2	0.016–0.081	
	Lettuce	<0.0003	0.003	0.2	0.2	0.013–0.1514	
	Cabbage	<0.0003	0.007	0.2	0.2	0.005–0.0862	
	Bean	<0.0003	0.002				
	Carrot	<0.0003	0.003	0.1	0.05	0.031–0.12	
	Apple	0.001	0.128	0.05	0.05	0.0029–0.2025	
	Orange	<0.0003	0.002	0.05	0.05		
	Banana	<0.0003	0.002	0.05	0.05		
	Walnut	<0.0003	0.0005			0.0588–0.198	0.0187
	Prunes	0.003	0.004	0.3			
	Oil	0.001	0.029			0.006	0.0004

	Margarine	<0.0003	0.0008				0.0004
	Cookies	0.013	0.126	0.05	0.1		0.0004
	Chocolate	0.034	0.080	0.2			0.0004
	Candy	0.028	0.066				
	Milk	0.001	0.160	0.01		0.0002–0.006	0.0004
	Yogurt	<0.0003	0.010	0.02			
	Soft cheese	<0.0003	0.005	0.1		0.003	0.0004
	Hard cheese	0.0008	0.003	0.1			
	Egg	<0.0003	0.006	0.05		0.0006–0.005	0.0004
	White bread	0.021	6.187	0.05		0.0284–0.04	0.0048
	Other types of bread	0.006	0.114	0.05			
	Pasta	0.008	0.099	0.05		0.05	0.0022
	Wheat flour	0.010	0.552	0.1		0.03–0.05	
	Sea fish, hake	0.003	0.048	0.05	0.05	0.005	
	Canned fish	0.029	0.087				
	Beef	<0.0003	0.001	0.05	0.05	0.004	
	Pork	<0.0003	0.006	0.05	0.05		
	Chicken	<0.0003	0.007	0.05	0.05	0.013	
	Bacon	0.003	0.022				
	Sausage	0.033	0.488				
	Hot dog	0.003	0.019				
	Salami	<0.0003	0.002				
	Pate	0.007	0.041			0.10–0.12	
	Paprika	0.118	0.191			0.070–0.112	
	Sugar	0.060	1.880			0.004–0.005	0.0004
	Intake by total food considered		11.51				
Pb	Potato	<0.003	0.210	0.1	0.1	0.003–0.34	
	Onion	<0.003	0.045	0.1	0.1		
	Mushrooms	0.006	0.0003	0.3	0.3	0.16–0.226	
	Lettuce	0.080	1.356	0.3	0.3	0.018	
	Cabbage	0.050	2.190	0.3	0.3	0.05	
	Bean	0.060	0.895		0.1		
	Carrot	0.060	0.956	0.1	0.1	0.011	
	Apple	0.013	1.545	0.1	0.1		
	Orange	0.085	1.545	0.1	0.1		
	Banana	0.060	1.254	0.1	0.1		
	Walnut	0.010	0.0003			0.01–0.12	0.022
	Prunes	0.263	0.376	3.0			
	Oil	0.023	0.669	0.1	0.1	0.005–0.089	0.005
	Margarine	0.014	0.07	0.1			0.004
	Cookies	0.010	0.087	0.4	0.2		0.008
	Chocolate	0.143	0.303	1.0		0.031	0.028
	Candy	0.323	0.683	0.5			
	Milk	0.011	1.902	0.02	0.02	0.004–0.05	0.003
	Yogurt	0.030	2.495	0.4		0.0024–0.02	
	Soft cheese	<0.003	0.048	1.0		0.031–0.058	0.016
	Hard cheese	0.045	0.172	1.0		0.006	
	Egg	<0.003	0.065	0.25		<0.001–0.021	0.011
	White bread	0.133	40.298	0.4		<0.040–0.025	0.026
	Other types of bread	0.150	2.933	0.4			
	Pasta	0.010	0.123	0.4			0.012
	Wheat flour	0.071	3.906	0.4			
	Sea fish, hake	<0.003	0.018	0.3	0.3		
	Canned fish	0.011	0.027				
	Beef	0.038	0.320	0.1	0.1		
	Pork	0.020	0.968	0.1	0.1	0.05	
	Chicken	0.098	5.329	0.1	0.1		
	Bacon	0.030	0.182				

(*continued on next page*)

Table 4 (*continued*)

Analyte	Type	Average value[a] (mg kg^{-1})	Daily intake (μg day^{-1})	MAC[b], Serbian regulation (No. 28/11) (mg kg^{-1})	MAC[b], EU (1881/2006) (mg kg^{-1})	EU, MSs[c] (mg kg^{-1})	First French total diet study (Leblanc et al., 2005) (mg kg^{-1})
	Sausage	0.04	0.720				
	Hot dog	<0.003	0.007				
	Salami	0.010	0.180				
	Pate	0.040	0.205				
	Paprika	0.080	0.160			0.0054–0.039	0.036
	Sugar	<0.003	0.056	1.0			
	Intake by total food considered		72.30				

[a] If the element content was below LOD in all the samples, its average value is reported as "<LOD"; otherwise for calculation of averages, quantities below LOD were considered as LOD/2.
[b] Maximum allowable concentration.
[c] Concentration range or mean levels of heavy elements in particular food item from Member States (SCOOP, 2004).

As and Cd intake levels, when compared to the respective toxicological values. However, higher total intake was estimated for Pb (72.30 μg day^{-1}, Table 4). The highest contribution to the total intakes of all three elements gave the bread due to its highest consumption rate. Moreover, in the case of Pb the significantly higher intake of bread was also a consequence of the markedly higher concentration found.

There are several studies reporting heavy element contents in different foodstuffs in order to provide the identification of the major dietary sources of the elements and the estimation of the intakes by both the whole population and by any high-risk subgroups (Llobet et al., 2003; SCOOP, 2004; Leblanc et al., 2005;Rubio et al., 2005, 2006; Becker et al., 2011; Martorell et al., 2011; Hernández-Martínez and Navarro-Blasco, 2012; Domingo et al., 2012; Arnich et al., 2012).

The estimated daily intakes of food groups investigated in this study were compared with available data in literature (Llobet et al., 2003; SCOOP, 2004; Leblanc et al., 2005; Rubio et al., 2005, 2006; Becker et al., 2011; Martorell et al., 2011; Domingo et al., 2012; Arnich et al., 2012) (Table 5), in order to get the first comparative assessment of Serbian dietary exposure intake. The mentioned studies revealed significant differences in daily intake of As for adults (Llobet et al., 2003; SCOOP, 2004; Leblanc et al., 2005; Martorell et al., 2011; Domingo et al., 2012; Arnich et al., 2012; Table 5). According to total food study from Catalonia, MSs and the first and the second French total diet study, fish and other seafood were the main source of As in the diet of the mean adult. Namely, during the first French total diet study, Leblanc et al. (2005) reported that fish, shellfish and fruits contributed mostly to the population exposure for As (49–50%, 8–13% and 15–17%, respectively) while other foodstuffs contributed less than 5% of the total food exposure. During the second French total diet study, contribution of fish to the estimated daily intake of As was lower being about 30% (Arnich et al., 2012). Serbian market basket included one sort of see fish (hake) and canned see fish with very low consumption rate (Table 1). The contribution of analysed fish to the estimated intake of As was very low 8%.

As can be seen from Table 5, similar daily intake of Cd was estimated for adult population by several authors (Llobet et al., 2003; SCOOP, 2004; Rubio et al., 2006; Becker et al., 2011; Arnich et al., 2012) as well as for adult population in this study.

The Cd daily intake for Serbian adult population was almost 4 times lower than estimated intake for adult population from Catalonia (2012), while the lowest dietary exposure was estimated for French adults population during the first total diet study (2005) (Table 5). According to the Swedish market basket study published by Becker et al. (2011), main sources of Cd were cereals products (48%), potatoes (19%), and vegetables (11%). In French study (2005) the highest intake relative to the existing toxicological references was estimated for Cd in vegetables; vegetables and starchy vegetables were foodstuffs contributing most (21–23 and 21–27%, respectively) to the exposure of the populations. Arnich et al. (2012) reported in the second French total diet study that the main contributors to cadmium exposure in adult populations are bread and dried bread products (22% and 13%, respectively) and potatoes and potato products (12% and 14%, respectively). SCOOP study (2004) also revealed that fruit, vegetables, cereals, meat and fish were the main sources of Cd in the diet of people from MSs, as they are highly consumed such staple foods. Even if the level of cadmium is generally low, cereals and vegetables contributed approximately 2/3 of the total Cd intake for population from MSs. In MSs the food groups which gave little contribution to Cd intake levels were: dairy products, fats and oils, eggs and beverages. Similarly, this study (Table 4) also showed that cereal based products like bread followed by potato, gave the highest contribution to the total estimated intake of Cd, because of their high consumption rates.

Table 5
Comparison of daily intakes of As, Cd and Pb for adults obtained from different studies.

Comparison of different study	Daily intake for adults (μg day^{-1})		
	As	Cd	Pb
Common foods consumed by the population of Catalonia (Llobet et al., 2003)	223.59	15.73	28.37
EU, MSs (SCOOP, 2004)	125	14.4	42
First French total diet study (Leblanc et al., 2005)	62	2.7	18.4
Common foods consumed by the population of Canary Islands (Rubio et al., 2005)	–	–	72.8
Common foods consumed by the population of Canary Islands (Rubio et al., 2006)	–	11.165	–
Swedish market basket diets (Becker et al., 2011)	n.d.[a]	10.0	7.0
Total food study from Catalonia (Martorell et al., 2011)	328	19.5	101
Duplicate diet study from Catalonia (Domingo et al., 2012)	199	49.5	19.8
Second French total diet study (Arnich et al., 2012)	54.60	10.99	14.07
This study	21.89	11.51	72.30

[a] Not determined.

Generally, the highest daily intake of Pb was estimated for population from Catalonia (Martorell et al., 2011; Table 5). Additionally, Martorell et al. (2011) found the continued increase of Pb intake in the period from 2000 to 2008, in contrast to the significant decrease of the atmospheric levels of Pb. Contrary to this founding, Becker et al. (2011) reported that Pb exposure decreased by about 2.5 times from 17 μg/day in the 1987 market basket study to 7 μg/day of the PTDI in the 1999 market basket study. Authors concluded that decrease in Pb concentrations in foods were probably a result of the elimination of Pb from petrol and other measures taken to reduce Pb emissions in Sweden.

In light of this conclusion it is worth to note that, leaded gasoline has been banned recently (2010) in Serbia. Still, daily intake of Pb for Serbian adult population was almost 1.5 times lower than daily intake of this element for adults in Catalonia (Martorell et al., 2011). Nevertheless, it was very similar to the estimated intake for population of Canary Islands reported by Rubio et al. (2005), and 10 and 4 times higher than intakes calculated in Sweden (Becker et al., 2011) and France (Leblanc et al., 2005), respectively (Table 5). This study showed that bread contributed in the highest rate to the estimated intake of Pb similarly as it was found in most of the other intake studies. However, several factors have an impact on the intake estimation in different study. The most important are different choices of analysed food groups. Other factors are differences in the sampling strategies, applied analytical methods, number of samples, calculation methodology. Also, the presence (or absence) of a particular food with a very high (or very low) occurrence level which is much (or little) consumed may thus have a great impact on the intake from some food group. Moreover, there are evidence derived from animal studies that the administration of Fe and Se interact with Cd (Groten and van Bladeren, 1994; Škrbić et al., 2009) efficiently counteract the toxicity of Cd. Also, Pb uptake ranged from 3% to 7% of the total ingested when it was taken with a meal or with leafy vegetables, which had taken up lead from its root (Heard et al., 1983). James et al. (1985) reported a similar drop in Pb uptake (4%) when subjects balanced meals.

4. Conclusion

This study presents the first comprehensive insight into the concentrations of three toxic elements in foodstuffs produced or commerzialed in Serbia contributing to the national market basket in the largest shares. The study highlights the fact that levels of As, Cd and Pb in investigated foodstuffs collected from the supermarkets in Novi Sad were in compliance with the current Serbian and EC legislation. It also emphasises that there was no concern about intake of As and Cd through different foodstuffs by adult consumers. However, it appeared that particular attention should be paid to the exposure of Pb since the estimated intake of Pb was almost two times higher than the reference value for nephrotoxic effects. Comparison with available diet intake studies from Europe revealed that the seen differences could be mainly attributed to the variations in the element concentrations found in the analysed foods, as well as to the differences in the dietary habits of the studied populations. This is the first attempt in Serbia to contribute to the global food monitoring and assessment programme by assessing the national dietary exposure to the investigated heavy elements.

5. Conflict of Interest

The authors declare that there are no conflicts of interest.

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Principal mycotoxins in wheat flour from the Serbian market: Levels and assessment of the exposure by wheat-based products

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ABSTRACT

The presence of eleven principal mycotoxins from the wheat flour bought in supermarkets in Novi Sad, the capitol of the northern Serbian province of Vojvodina, was determined. The samples were prepared by simple one-step method and analyzed by ultra-high performance liquid chromatography with heated-electrospray ionization triple quadrupole mass spectrometry (UHPLC/HESI-MS/MS). Deoxynivalenol (DON) was the predominant mycotoxin for all analyzed samples followed by zearalenone (ZON) and T-2 toxin, with frequency of occurrence: 88.7%, 33.3% and 26.7%, respectively. Aflatoxins (AFs), ochratoxin A (OTA), HT-2 toxin, fumonisins B1 (FB1) as well as B2 (FB2) were below the limit of detection. All the samples complied with current European/Serbian legislation, except one sample that exceeded the DON maximum level of 750 μg/kg. In addition, mycotoxin intakes through consumption of wheat-based products were estimated for average adult consumers based on Serbian market basket and then compared with the tolerable daily intake (TDI) proposed by Scientific Committee on Food of the European Union. The calculated intakes of ZON and T-2 were lower than the respective TDIs. However, intakes of DON were assessed to be close to the level of TDI for adults. This is the first study on the intake assessment for mycotoxins present in the wheat flour through the consumption of wheat-based products on the Serbian market.

1. Introduction

Cereals and cereal-based products are the main food crops worldwide. However, cereals like many other crops are susceptible to fungal attack either in the field or during subsequent processing (drying) and storage. A great variety of the fungi can produce mycotoxins, low molecular mass metabolites. In fact, it was reported that approximately 25% of cereals produced in the world are contaminated by mycotoxins (Devegowda, Raju, & Swang, 1998). Mycotoxin contamination of cereals causes adverse effects on consumers of all age groups since cereal-based products are notably consumed in various diets worldwide. Taking into account that the occurrence of mycotoxins in cereals is a problem difficult to avoid and that they could be only partly removed by industrial processing, monitoring of the production chain is essential to assess the risks to which consumers may be exposed.

Currently, more than 400 mycotoxins are identified in the world, but the most important groups of mycotoxins that occur quite often in food are: aflatoxins (AFs: AFB1, AFG1, AFB2, AFG2) produced by *Aspergillus* species, ochratoxin A (OTA) produced by both *Aspergillus* and *Penicillium* species, trichothecenes (type A: HT-2 and T-2 toxin, and type B: deoxynivalenol (DON)), zearalenone (ZON), and fumonisins B1 and B2 (FBs: FB1 and FB2) produced by *Fusarium* species (Salem & Ahmad, 2010). These groups of mycotoxins are of major health concern for humans and animals, because of the adverse effect including carcinogenicity. To protect consumer's health, the commission of the European Communities established the maximum level for most of these (principal) mycotoxins in cereals (EC, 2006b; EC, 2007): 4 μg/kg for total AFs (AFB1, AFG1, AFB2, AFG2), and 2 μg/kg for AFB1 for all cereals and all products derived from cereals; 3 μg/kg for OTA for all products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption; 750 μg/kg for DON and 75 μg/kg for ZON in cereals intended for direct human consumption; and 1000 μg/kg for fumonisins (FBs) in maize intended for direct human consumption. It should be noted that regulations regarding HT-2 and T-2 toxins in cereals have not yet been established due to scarce data on the occurrence of these toxins; nevertheless, they are in the preparation.

Estimates of food contaminants intake are essential for risk evaluation and are also important to determine the relationships

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between adverse effects observed in humans and exposure to particular substances. Such exposure evaluations are also useful when making decision on the regulation of chemical contaminants and the safety of food products. Since toxicity of mycotoxins has not been evaluated for all the toxins found in food, the total impact of these naturally occurring contaminants on human health cannot be assessed. Even for the most well-documented toxins, the tolerable daily or weekly intakes (TDI, TWI) are established/proposed by relevant authorities. FAO/WHO Joint Expert Committee on Food Additives (JECFA) and EU's Scientific Committee on Food (SCF) have remained temporary tolerable daily intake (t-TDI), provisional tolerable weekly intake (PTWI) or provisional maximum tolerable daily intake (PMTDI) for several well known mycotoxins due to insufficient toxicological information (Leblanc, Tard, Volatier, & Verger, 2005).

In recent years, principal mycotoxins were subject of evaluation of SCF (1994, 1998, 1999, 2000, 2001, 2002 and 2003). Namely, several trichothecenes were evaluated and for four of them toxicological reference values were allocated. Thus, for DON (a type B trichothecene) a PMTDI of 1 μg/kg body weight (bw), and for T-2 and HT-2 toxins (type A trichothecenes) a group PMTDI of 0.06 μg/kg bw (separately or in combination) were allocated, respectively. For the other *Fusarium* toxins studied, the SCF established a temporary tolerable daily intake (t-TDI) for ZON of 0.2 μg/kg bw based on short-term study in pigs. For FBs separately or in combination, in 2001 the JECFA established a PMTDI of 2 μg/kg bw based on short- and long-term studies of renal toxicity in rodents. The SCF established a temporary tolerable daily intake of 2 μg/kg bw for FB1 based on chronic studies in the rat, while the established OTA tolerable daily intake of 5 ng/kg bw is calculated from the nephrocarcinogenic effects observed in the rat. Concerning AFs, SCF concluded that even very low levels of exposure to AFs, as little as 1 ng/kg bw per day or even less, could contribute to a risk of liver cancer, recommending that levels should be reduced as low as reasonably achievable.

Among cereals, wheat is the most used crop for food by the Serbian population: FAO Food Balance Sheet (FAO, 2007) showed an average of 508500 tones of wheat used in food supply in 2006 and 2007 with per capita supply of 51.7 kg/year: most of the wheat for food production is of national origin. Thus, wheat-based products play an essential role in the Serbian diet and constitute one of its most important characteristic features. Namely, in Serbia, human consumption of wheat-based products is greater than of products made from other cereals. The most important wheat-based products are wheat flour, bread, pasta, pastry and cookies, which average consumption figures in 2011 (Statistical Office of the Republic of Serbia, 2011) are: 50.0, 275.5, 11.1, 16.7 and 9.5 g per person per day, respectively. These foodstuffs represent approximately 26% of Serbian market basket (Statistical Office of the Republic of Serbia, 2011). Thus, the main objective of this study was to: a) identify and quantify the occurrence of 11 principal mycotoxins in wheat flour collected from the Serbian markets in order to b) estimate the exposure to these toxins through consumption of wheat-based products (flour, bread, pasta, pastry and cookies) by the Serbian population. Mycotoxins included in the investigation were those of major health concern for human and/or their occurrence in cereals has been subjected to the EU regulations. It should be noted that the recent Serbian regulation (2011) has established the maximum level for AFs, OTA, DON, ZON, FB1 and FB2 in line with the relevant EU regulations (EC, 2006b; EC, 2007). The determined levels were compared with results of available studies, while the consumer exposure estimates were compared with appropriate tolerable intakes to assess the safety of wheat-based products consumed by the Serbian population. This is the first report on the simultaneous occurrence of these mycotoxins in wheat flour from the Serbian market, and this information is necessary and of high priority in order to protect the consumer's health from the risk of exposure to these toxins.

2. Materials and methods

2.1. Reagents and chemicals

Individual standard stock solutions of AFB1 (2 μg/ml), AFB2 (0.5 μg/ml), AFG1 (2 μg/ml), AFG2 (0.5 μg/ml), OTA (1000 μg/ml), HT-2 toxin (100 μg/ml), T-2 toxin (100 μg/ml), DON (100 μg/ml), ZON (100 μg/ml), FB1 (50 μg/ml) and FB2 (50 μg/ml) were purchased from Supelco Co. (Bellefonte, PA). All standards dissolved in acetonitrile were stored at −20 °C in amber glass vials, and brought to room temperature before use. Composite working standard solutions were prepared by diluting the above-mentioned stock solutions in acetonitrile and they were added in appropriate dilution to the extract of the uncontaminated sample to prepare matrix-matched calibration standards in concentration ranges that cover the maximum allowable concentrations and also the expected range of mycotoxin occurrence (in accordance to the available literature data). Ultra-pure water was produced by Milli-Q purification system (Millipore, Molsheim, France). Methanol, acetonitrile and ammonium acetate (all LC-MS grade) were supplied from J.T. Baker (Deventer, The Netherlands), glacial acetic acid (p.a.) was obtained from LTG Promochem (Wesel, Germany).

2.2. Collection of samples

In Serbia, the major food crops are cultivated mainly in relatively small area of the northern province of Vojvodina, which is the main growing region accounting for about 62% of the national wheat production. In January 2011, fifteen flour samples were collected randomly from different supermarkets within Novi Sad, the capitol of the Vojvodina Province, where the biggest Serbian producers of flour in Serbia are located. Five different brand names were selected in accordance with their rate in total Serbian flour production to have a market-representative sampling. Packs of wheat flour were 1000 g. Flour samples analyzed in this study belonged to type 400 (T 400; n (number of samples) = 5) and type 500 (T 500; n = 10). Ash content for T400 flour is up 450 mg/100 g (dry matter (d.m.)), while for T500 it is 460–550 mg/100 g d.m. (Serbian regulation, 1995; Serbian regulation, 1988). The samples were stored in their original packs at 4–5 °C until analysis was carried out.

2.3. Sample preparation

Many routine laboratories use preparatory methods based on extraction/clean-up/pre-concentration steps regarding only single or small group of similar mycotoxins (Manova & Mladenova, 2009; Sokolović & Šimpraga, 2006). Although these methods are well established, and in some cases interlaboratory validated, the current trend is to introduce simple (one-step), broad scope procedures which allow, thanks to use of modern separation/detection instrumental technologies, accurate determination of as many as possible major mycotoxins even at low levels in crude extracts, not applying labor/cost demanding clean-up step (Škrbić, Malachova, Živančev, Veprikova, & Hajšlová, 2011). Thus, simple sample preparation technique with only one-step of extraction was chosen to be used in this work in order to allow fast analysis of eleven mycotoxins (AFB1, AFG1, AFB2, AFG2, OTA, ZON, DON, T-2, HT-2, FB1, and FB2).

Method used to prepare the crude extracts of the wheat flours was previously described by Škrbić et al. (2011) for preparation of wheat extracts for *Fusarium* mycotoxins analysis by high

performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). Only slight modifications were made with respect to amount of sample, volume of solvent used for extraction and dilution of the extract. Briefly, 5 g of homogenized wheat flour samples were extracted by shaking with 20 ml of acetonitrile/water mixture (84:16,v/v) for an hour using an automatic shaker (Promax 2020, Heidolph Instruments, Germany). After, the suspensions were filtered and an aliquot (1 ml) of filtered crude extracts were transferred into glass vials and diluted with 3 ml of mobile phase used for liquid chromatographic analytical separation (95% A and 5% B; explanation of the composition of the eluent A and B is given in the subsequent section). Thus, the final extracts contained 0.0625 g sample per ml. Before injection into the liquid chromatograph system, the extracts were passed through the 0.2 μm nylon syringe filter.

2.4. Instrumental conditions

Ultra-high performance liquid chromatography (UHPLC) performed by Accela™ (Thermo Fisher Scientific, USA) was used for separation of sample components. Hypersil GOLD™, 50 mm × 2.1 mm i.d., 1.9 μm column (Thermo Fisher Scientific, USA) was used with a flow rate of 0.5 ml/min, and the column temperature was maintained at 30 °C. The injection volume was 10 μl. The mobile phase consisted of eluent A containing water/acetic acid (99:1, v/v), and eluent B consisting of methanol/acetic acid (99:1, v/v). Both eluents contained 5 mM ammonium acetate. The gradient program started with 95% A and 5% B and was kept until 0.5 min, afterwards a linear gradient was applied, reaching 95% B after 3.04 min (holding time 2.1 min) and then switch back (6.20 min) to 95% A (holding time 1.80 min), which was maintained till the end of the run at 8 min.

For analytes detection, triple quadrupole mass spectrometer (MS/MS) TSQ Vantage (Thermo Fisher Scientific, USA) equipped with heated-electrospray ionization probe (HESI-II, Thermo Scientific, USA) was used. Heated-electrospray ionization (HESI) transforms ions in solution into ions in the gas phase by using electrospray ionization (ESI) in combination with heated auxiliary gas (Huls, Zuiderent, & Ghosh, 2007). Parameters of the ion source were as follows: spray voltage – 3.4 kV, vaporizer temperature – 350 °C, sheath gas pressure – 40 arbitrary units, auxiliary gas pressure – 10 arbitrary units, and capillary temperature – 270 °C.

Acquisition parameters of the mass spectrometer were optimized by direct continuous pump infusion of 5 μg/ml standard solutions of each individual analyte standards (except for AFB1 and AFG1 (2 μg/ml) and AFB2 and AFG2 (0.5 μg/ml)) dissolved in initial mobile phase into the mass spectrometer using a syringe pump at a flow rate of 10 μl/min. Data acquisition was performed initially in full scan to determine an abundant precursor ion. Next, the MS/MS fragmentation conditions were investigated and collision energies and S-lens voltages were optimized for each individual compound and/or transition. UHPLC/HESI-MS/MS parameters of mycotoxins separation and identification under optimized conditions on the Accela-TSQ Vantage system are shown in Table 1. Fragmentation reactions were done in selected reaction monitoring mode (SRM) by choosing the optimum voltage of collision energies for each compound. Two product ions were measured for all compounds: one was used as the quantifier ion and the other was used as the qualifier ion. In SRM mode, a mass resolution of 0.7 Da full width at half maximum (FWHM) was set on the first (Q1) and the third (Q3) quadrupole and a scan width of 0.5 m/z were used. Chromatograms of the mixture of all analyzed mycotoxins spiked in crude extract of wheat flour at the lowest calibration levels are presented in Fig. 1. Instrument control and data collection were handled by computer equipped with Xcalibur 2.1.0 (Thermo Fisher Scientific, USA).

Table 1
UHPLC/HESI-MS/MS parameters of mycotoxins under optimized conditions on Accela-TSQ Vantage.

Mycotoxins	t_R,[a] min	Dwell time, s	Precursor ion, m/z	Product ions,[b] m/z	CID,[c] eV
AFB1	3.17	0.1	313.1 $[M + H]^+$	285.1/241.1	33/22
AFB2	3.07	0.1	315.1 $[M + H]^+$	287.2/259.2	28/25
AFG1	2.97	0.1	329.1 $[M + H]^+$	200.2/215.1	26/40
AFG2	2.87	0.1	331.0 $[M + H]^+$	245.0/189.1	40/28
OTA	3.91	0.1	404.1 $[M + H]^+$	239.0/221.0	35/23
DON	1.82	0.1	295.0 $[M - H]^-$	265.1/205.2	−18/−24
HT-2	3.57	0.1	447.2 $[M + Na]^+$	345.5/285.5	19/20
T-2	3.74	0.1	489.0 $[M + Na]^+$	245.1/327.1	26/23
ZON	3.88	0.1	317.1 $[M - H]^-$	175.1/131.1	−26/−32
FB1	3.70	0.1	722.6 $[M + H]^+$	334.3/352.4	38/34
FB2	3.98	0.1	706.5 $[M + H]^+$	336.3/318.3	36/38

[a] Retention time.
[b] Numerical values are given in the order quantifier/qualifier ion.
[c] Collision-induced dissociation energy for quantifier/qualifier ion.

2.5. Quality control

The developed method was validated by in-house quality control procedure. Parameters taking into account were: instrumental linearity, limits of detection (LOD) and quantification (LOQ), and recovery.

Mycotoxins were quantified by external matrix-matched calibration procedure. Calibration solutions for matrix-matched calibration curves were prepared in uncontaminated flour extract. Calibration curves for all mycotoxins in the wheat flour were linear within the working range from 1 μg/kg to 1760 μg/kg. Squared correlation coefficients (R^2) showed good linearity in the range of 0.9913–0.9990.

The detection and quantification limits for all mycotoxins were assessed at a signal to noise ratio of 3:1 and 10:1, respectively (Table 2). In all the cases, LODs were lower than the maximum residue limits established by European Union (EC, 2007), indicating the suitability of the proposed method for the determination of trace concentration of these compounds.

Validation of the analytical method was carried out by determination of recoveries ("in-house") of uncontaminated wheat flour sample spiked at level of 2 μg/kg for each of AFs, 3 μg/kg for OTA, 350 μg/kg for DON, 100 μg/kg for HT-2 and T-2, 35 μg/kg for ZON and 300 μg/kg for FBs. Recovery experiments were performed in duplicate. Spiked samples were left overnight at room temperature to allow solvent evaporation and equilibration between analytes and matrix. The "in-house" recoveries were in the range defined by EU requirement (EC, 2006a), except for FBs, which recoveries were <60% (Table 2).

Additionally, the accuracy of the method was checked by analysis of a maize sample provided during the proficiency test (PT) for simultaneous determination of up to 11 mycotoxins in maize by LC–MS (MS) organized by CNR-ISPA, Institute of Sciences of Food Production, within MoniQA project in 2011, funded by the European Commission Sixth Framework Programme (FP6; www.moniqa.org/mycotoxins). According to the spiking protocol described by the PT organizers, 20 g of blank maize flour samples was spiked with 600 μl of a mixture of mycotoxins (with unknown concentrations for the PT participants) and left overnight at room temperature. All materials used in PT were provided by the organizers. The obtained "PT" recoveries are summarized in Table 2. As could be seen, similar recoveries were obtained for "in-house" and "PT" results (Table 2), except for HT-2 and T-2. Again, recoveries for FB1 and FB2 were lower than 60%, proving low extraction capacity of the chosen method with respect to these two toxins. Thus, results reported for FBs hereafter should be taken with caution, since extraction of these

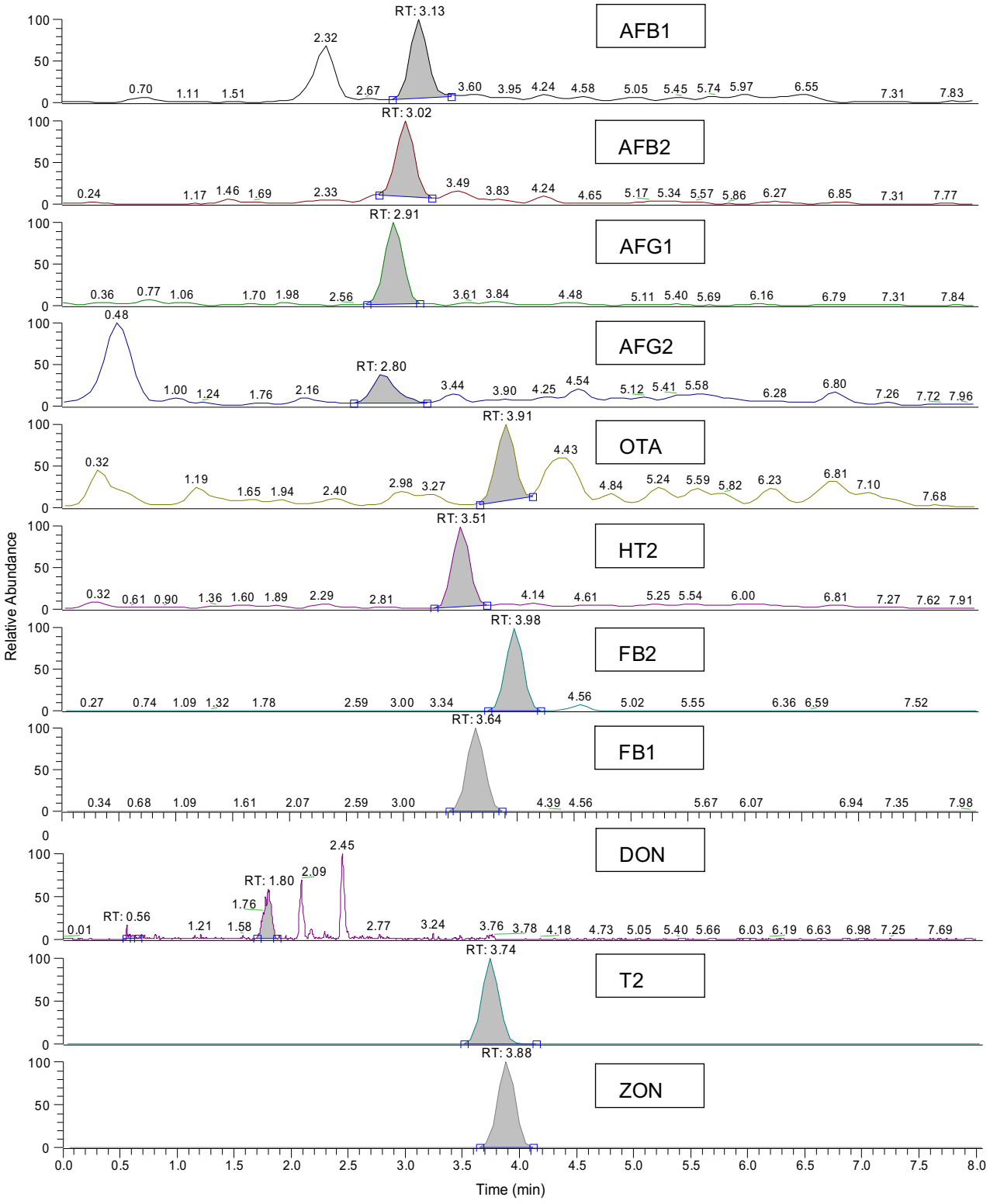

Fig. 1. Chromatograms of the mixture of all analyzed mycotoxins spiked in crude extract of wheat flour at the lowest calibration levels.

toxins was not satisfactory, indicating a need for more specific preparatory step. Similarly, Sulyok, Berthiller, Krska, and Schuhmacher (2006) investigated 39 mycotoxins in crude extracts of wheat and maize obtained by extraction with acetonitrile/water/acetic acid (79:20:1, v/v/v) and found also low (unsatisfactory) recoveries of fumonisins (<60%). Differences between HT-2 and T-2 recoveries obtained by "in-house" and "PT" spiking protocol could be ascribed to differences in matrix effect that wheat and maize flour introduced into the MS/MS ionization source.

All samples were analyzed in duplicate. Blank samples were included in every batch of samples to check for possible contamination.

Table 2
Summary of validation data of UHPLC/HESI-MS/MS method.

	AFB1	AFB2	AFG1	AFG2	DON	ZON	HT-2	T-2	OTA	FB1	FB2
LOD (μg/kg)	0.7	0.2	0.5	0.9	0.3	0.4	0.9	1.4	2.1	0.05	0.01
LOQ (μg/kg)	2.3	0.7	1.7	3.0	1.0	1.3	3.0	4.7	7.0	0.2	0.03
Recovery "in-house"[a] (%)	121	110	112	113	72	74	130	107	87	<60	<60
Recovery "PT"[b] (%)	115	100	114	115	n.d.[c]	82	79	136	80	<60	<60

[a] Recovery values obtained by "in-house" spiking procedure using domestic uncontaminated wheat flour samples spiked with mycotoxins as described and left overnight at room temperature.
[b] Recovery values obtained by "PT" spiking protocol described by the organizers of the proficiency test PT organized in 2011 by CNR-ISPA, Institute of Sciences of Food Production, Bari, Italy, within FP6 MoniQA project (www.moniqa.org/mycotoxins): blank maize flour sample provided by PT organizers was spiked with mycotoxin solution (also provided as the PT organizers) and left overnight at room temperature as described.
[c] n.d.: not determined.

2.6. Intake calculation

Calculation of the exposure of Serbian population by wheat flour consumption was done by combining data on the average daily consumption of the wheat flour with the average mycotoxin concentrations found here, as follows:

$$\text{Estimate of mycotoxin intake } (\mu g/kg\ bw/day) = \frac{[\text{toxin}]*[\text{wheat flour consumption}]}{[\text{bw}]}$$

where [toxin] is the concentration of mycotoxin in (μg/kg) detected in wheat flour adjusted for recovery (Table 2), [wheat flour consumption] is the amount of wheat flour (kg) consumed per person per day, and [bw] is the body weight (kg).

Wheat flour consumption amount used for calculation of the intakes was obtained as a sum of average daily per capita consumption figure for flour and the amounts of wheat flour used for production of average daily portion of bread, pasta, pastries and cookies according to the Serbian market basket (Statistical Office of the Republic of Serbia, 2011). Thus, on the basis of documented product recipe specifications, the approximate content of flour needed for the production of corresponding amounts of bread, pasta, pastry, and cookies daily consumed was calculated (192.9 g, 11.6 g, 16.7 g and 4.8 g, respectively) and added to the average daily per capita consumption of flour estimated for Serbian market basket to be 50.0 g (Statistical Office of the Republic of Serbia, 2011). In this way, total individual average consumption of wheat flour was assumed to be 267.7 g/day for adults. Similar approach of the intake estimation has been applied previously for mixed food categories and assessment of dietary intakes of polychlorinated biphenyls since the share of foodstuffs like bread and pastries could be estimated from the categories of which they consist (Bakker, Baars, Baumann, Boon, & Hoogerbrugge, 2003; Škrbić, 2008). Hence, even though mycotoxins were not analyzed in the wheat-based products, the basic assumption was that these food categories were produced mainly from wheat flour, having the average level of mycotoxins as determined.

In this estimation, we have ignored the amount of imported wheat and wheat-based products as negligible in relation to the quantities of national origin. The average body weight (bw) of 60 kg for adults and 25 kg for children was used for calculating daily intakes per kg bw.

The estimated mycotoxin intakes (in μg/kg bw/day) were than compared with the relevant tolerable daily intake (TDI, μg/kg bw/day) and percentage of TDIs from consumption of wheat-based products was calculated as follows:

$$\%TDI = [\text{Estimate of mycotoxin intake}]/[TDI] * 100$$

3. Results and discussion

3.1. Occurrence of mycotoxins in wheat flours

The data on occurrence of mycotoxins analyzed in the wheat flour is given in Table 3. The presented results were corrected for "in-house" recovery (Table 2). Samples with a toxin concentration between LOD and LOQ were considered to be positive and their levels were included in the statistical analysis. If mycotoxin was below LOD in any of the samples, its average value is reported as "<LOD"; otherwise for calculation of averages, quantities below LOD were considered as LOD/2. The most prevalent mycotoxin was DON followed by ZON and T-2, while AFs, OTA, HT-2, FB1, as well as FB2 were not detected in any of the samples. DON, ZON and T-2 were detected in 86.7%, 33.3% and 26.7% of the total number of samples, respectively. The average and median values obtained for DON were 325 and 292 μg/kg, respectively. Only one sample with concentration of 976 μg/kg exceeded the limit of 750 μg/kg set by EC (2007) for allowed presence of DON in cereals (including cereal flour) intended for direct human consumption. The average value obtained for ZON and T-2 in analyzed samples were 4.6 and 4.1 μg/kg, respectively, while their median values were below LODs. The maximum level for ZON (21.1 μg/kg) detected in flour was below the limits (75 μg/kg) for cereals (including cereal flour) for direct human consumption established by EC regulation (EC, 2007). Since the limit for T-2 has not been regulated yet, maximum level found in this study (26.9 μg/kg) could not be compared.

The incidence of DON, ZON and T-2 in commercial wheat flours has been reported in several studies, none of these referred to Serbia or its neighboring countries; only the mycotoxins in cereal grains have been subject of the regional investigations. In general,

Table 3
Frequency of occurrence and levels of mycotoxins analyzed in wheat flour from Serbia.

Mycotoxins	No. of positive samples (frequency of occurrence, %)	Average value[a] (μg/kg)	Median (μg/kg)	Concentration range[b] (μg/kg)
AFB1	0	<LOD	<LOD	
AFB2	0	<LOD	<LOD	
AFG1	0	<LOD	<LOD	
AFG2	0	<LOD	<LOD	
DON	13 (86.7)	325	292	17.5–976
ZON	5 (33.3)	4.6	<LOD	1.9–21.1
FB1	0	<LOD	<LOD	
FB2	0	<LOD	<LOD	
T-2	4 (26.7)	4.1	<LOD	9.8–26.9
HT-2	0	<LOD	<LOD	
OTA	0	<LOD	<LOD	

[a] In calculating the average values of mycotoxin concentrations half of the limit of detection (LOD) values were taken for samples with concentration below LOD, except in the case when the frequency was 0%.
[b] In positive samples.

DON is frequently found in cereal flours but the percentage of samples with DON level of 750 μg/kg (limit proposed for flour used as raw material in food products) or higher rarely exceeds 6–7% (Schothorst & van Egmond, 2004). According to a study carried out in southwest Germany (Schollenberger, Suchy, Jara, Drochener, & Müller, 1999) the incidence of DON in 1998 for white flour ($n = 134$) and whole grain flour ($n = 77$) were 78% and 66%, with maximum contents of 624 μg/kg and 1670 μg/kg, respectively. Likewise, an investigation on the occurrence of DON in 1999 from southwest Germany reviewed by Schollenberger, Jara, Suchy, Drochner, and Müller (2002) showed again that there were samples of wheat flour ($n = 60$) that exceeded the DON maximum permitted level of 750 μg/kg established in the EU and that the frequency of occurrence of DON in wheat flour was very high with almost 100%. Concerning the ZON occurrence, it has been shown that its levels rarely have exceeded the maximum allowed concentration. A survey done by Pérez-Torrado, Blesa, Moltó, and Font (2010) revealed that twenty one samples of cereal flour (such as maize, wheat, rice, ray and its mixtures) produced in different countries, contained ZON below the maximum limit of 75 μg/kg established for cereals intended for direct human consumption in Europe (EC, 2007). Kassim et al. (2011) investigated 15 wheat/wheat powder samples for the presence of T-2 in South Korea, and found this mycotoxin in 3 samples with high level of 431 μg/kg, while the concentration of HT-2 were in the range 30.8–355.3 μg/kg. The authors claimed that high level of the toxin may be attributed to the conditions of the sampling sources, packaging and storage facilities.

Differences between these data and the results of the present study may be attributed, among others, to a different origin of cereals; however, comparison among the countries is difficult due to different analytical procedures, climatic and storage conditions, information provided on kind and number of cereal-based products studied. Nevertheless, prevalent occurrence of DON in wheat flour obtained from grains cultivated in different countries, including Serbia, is evident.

3.2. Consumer exposure estimation

Serbia is an agricultural country and grain production, especially wheat, is an important economic factor. Wheat is utilized mainly as flour for the production of a large variety of breads, as well as for the manufacture of a wide variety of bakery products and pasta.

In this context, estimation of the possible exposure to mycotoxins by wheat-based products consumption is very important parameters of the risk assessment. Contrary to the developed nations (Boon et al., 2009; Leblanc et al., 2005; Schothorst van Egmond, 2004; Thuvander et al., 2001) to date no survey exists in Serbia concerning the intakes of mycotoxins.

The average levels of DON, ZON and T-2 were used in the intake calculation, since this methodology is internationally recognized to provide satisfactory estimates of long-term exposure, suitable for comparison with the respective TDI values, which are also estimates based on long-term exposure (FAO/WHO 1997; Thuvander et al., 2001). Table 4 shows the estimates of DON, ZON and T-2 intakes for the general Serbian adults and also children through consumption of wheat-based products. The intake of each mycotoxin is expressed as a percentage to the TDI proposed by the SCF of the European Union to be 1 μg/kg bw/day for DON, 0.2 μg/kg bw/day for ZON and 0.06 μg/kg bw/day for T-2. As can be seen from Table 4, the intakes of ZON and T-2 by adults through wheat-based products consumption generally seemed to be below the respective TDIs. However, intakes of DON were estimated to be above the level of TDI (Table 4), implying further that individuals with a wheat-based products consumption higher than the average represented by the Serbian market basket might ingest DON at levels that exceed the reference toxicological values. This could be the case with specific population groups, like vegans/macrobiotics, due to their higher consumption rates of the wheat-based products than used for estimation in this study. Furthermore, it is shown that exposure of children to mycotoxins by consuming wheat-based products may also be higher than the respective intakes of adults, since children consume more food compared to adults when expressed per kg body weight (Boon, Bakker, van Klaveren, & van Rossum, 2009). For instance, in absence of the food consumption figures for children in Serbia, if a half of the adult daily portion (i.e. 133.9 g of wheat flour) was concerned as a children' daily portion and body weight of 25 kg is assumed as the average for the children (Castillo et al., 2008), the calculated DON intake is 1.7 μg/kg bw/day, representing 174% of the TDI (Table 4). However, there are few studies published by now reporting reduction of DON (24–71%) in bread and other bakery products in comparison to the wheat flour used for their making due to fermentation losses and/or thermal decomposition (Bullerman & Bianchini, 2007; Kushiro, 2008; Pacin, Ciancio Bovier, Cano, Taglieri, & Hernandez Pezzani, 2010; Samar, Neira, Resnik, & Pacin, 2001). On the other hand, increase of DON concentrations in some products (cereal flakes, donuts) has been also reported and explained by its release from "masked" forms (e.g. DON-glycosides) during processing (Berthiller, Schumacher, Adam, & Krska, 2009). Thus, consideration of the changes in mycotoxin levels due to fermentation and a baking would give a more realistic figure for mycotoxin presence in the wheat-based products ("as consumed") and the corresponding intakes. Anyway, the calculated values should be treated as approximate values and the first of such kind assessed for the mycotoxin exposure of the Serbian population through wheat-based products. To make more precise and reliable exposure estimates than those presented here, large-scale study and determination of mycotoxin levels in foodstuffs ("as consumed") other than wheat-based products is needed.

Concerning AFs, OTA and HT-2 that were below the limit of detection (<LOD) in analyzed wheat flour samples from the Serbian

Table 4
Estimated DON, ZON and T-2 daily intakes (μg/kg bw/day) through consumption of the wheat flour and wheat-based products according to the Serbian market basket relative to the respective tolerable daily intakes (TDI) proposed by the Scientific Committee on Food of European Union (SCF, 2000, 2001, 2002).

	Average[a] (μg/kg)	Intake[b] (μg/kg bw/day)		(%) of TDI[c]	
		Adults	Children[d]	Adults	Children
DON	325	1.5	1.7	145	174
ZON	4.6	0.02	0.02	10.2	12.2
T-2	4.1	0.02	0.02	30.7	36.9

[a] Average of the mycotoxin levels (Table 3).
[b] A body weight (bw) of 60 kg was assumed for adults intake, while 25 kg was used for children body weight (Castillo et al., 2008).
[c] Respective TDI proposed by SCF (2000, 2001, 2002).
[d] Since, there is a lack of food consumption figures for children in Serbia, half of the adult daily portion of wheat – based products has been assumed to be the daily portion for children.

market, it must not be assumed that their intakes would be zero, but, more realistically, they could be expected to be in the range from zero to the values calculated for the respective LODs (Bakker et al., 2003; Škrbić, 2008). In such cases, the so-called "middle bound scenario" might be adopted for the mycotoxin intake assessment: for "below the limit of detection" samples in which toxins were below the limit of detection, it might be assumed to contain concentrations equal to half of LOD (Boon et al., 2009; Leblanc et al., 2005).

Even though comparison of intakes estimated in different studies is of limited value as there are great differences in the sampling strategies, applied analytical methods, number of samples, calculation methodology, it should be used only approximately and with great caution. Similarly to the estimates obtained here, previous studies on mycotoxin intakes also proved that the highest exposure of population could be expected for DON and through consumption of wheat- and other cereal-derived products (Boon et al., 2009; Leblanc et al., 2005; Schothorst & van Egmond, 2004). Comprehensive study on risk assessment of dietary exposure to contaminants in young children in the Netherlands (Boon et al., 2009) reported that wheat-based products contributed mostly to the DON long-term dietary exposure of children aged between 2 and 6 years; the mean DON long-term exposure through the wheat-based products of Dutch children was estimated to be 0.3 μg/kg bw/day, which is about 2 times lower than the value calculated for children in this study. Schothorst and van Egmond (2004) reported results on the DON, NIV, T-2 and HT-2 occurrence and intakes assessed in 2001 for SCOOP (Scientific Cooperation on Questions relating to Food), task 3.2.10. "Collection of occurrence data for *Fusarium* toxins in food and assessment of dietary intake by the population of EU Member States". By far, most of the occurrence data were obtained for DON in wheat. Thus, estimated intakes of DON by consumption of wheat, wheat flour or bread were up to about 90%. Even though mean intakes of DON were below TDI, the mean intakes for young children were sometimes (very) close to TDI. According to the total intake of the sum of HT-2 and T-2 it was in most of the cases above TDI. Leblanc et al. (2005) reported results on the assessment of dietary exposure of the French population to the principal mycotoxins in the French diet ("as consumed"), showing that cereal – derived products is a food group that contributed mostly to the population exposure. The highest intake relative to the existing toxicological references was estimated for DON; the foodstuffs contributing mostly (90%) to this exposure were cereal-based products, particularly bread/rusk group (45–70%). The mean estimated intake of DON for adults by consumption of bread/rusk was 188 ng/kg bw per day. The higher intakes were assessed for vegetarian population, with the average DON intake between 320 and 410 ng/kg bw per day. The proportion of individuals whose theoretical intake exceeded the TDI for DON was estimated to be 0.4% among adults, 4% among children and 4–5% among vegetarians (Leblanc et al., 2005).

4. Conclusion

The study highlights the fact that the levels of eleven principal mycotoxins in the wheat flour collected from Serbian local markets were satisfactory with regard to the current EU legislation. It also emphasizes that there was no concern about intake of the mycotoxins through wheat-based products by mean adult consumer in the area surveyed. However, it appeared that particular attention should be paid to the exposure of specific population groups and individuals with consumption rates higher than the average included in the Serbian market basket, who may ingest certain mycotoxins, particularly DON, at levels that exceed the reference toxicological values. Children are especially vulnerable group due to their higher food consumption level per kg body weight. Therefore, results implied that constant monitoring throughout the cereals production chain is necessary in order to minimize health risks related to the intake of mycotoxins present in food.

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Fusarium mycotoxins in wheat samples harvested in Serbia: A preliminary survey

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ABSTRACT

The objective of this study was to determine the occurrence of trichothecenes of both the A-type and B-type, masked mycotoxin derived from DON - deoxynivalenol-3-glucoside (DON-3-Glc), 3- and 15-acetyldeoxynivalenol (ADONs), fusarenon-X (FUS-X) and nivalenol (NIV)) as well as zearalenone (ZON) in winter wheat. Total of 54 samples were collected during the harvest of 2007 representing the most important Serbian wheat-growing regions. The samples were prepared by one-step simple method and analyzed by high performance liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). The obtained recoveries proved that the used method could be successfully applied for multi-component analysis of *Fusarium* mycotoxins. DON, DON-3-Glc and HT-2 contents were detected approximately in 28%, 13% and 6% of the total number of samples, respectively. The amount of these toxins ranged from 17 μg/kg for DON-3-Glc to 309 μg/kg for DON. ADONs, FUS-X, NIV, T-2 toxin as well as ZON were below the limit of detection. Different susceptibility of wheat cultivars towards detected mycotoxins was observed. The results were compared to the EC Regulative and with available the literature data concerning the neighboring countries. This is first report on the simultaneous presence of 8 Fusarium mycotoxins in the wheat cultivated in the Balkan Countries region.

1. Introduction

Mycotoxins are secondary metabolites generated by several species of fungi, e.g. *Fusarium*, *Aspergillus, Alternaria*, *Penicillium, Claviceps,* etc (Herebian, Zühlke, Lamshöft, & Spiteller, 2009) that widely contaminate plant origin products such as crops, foods and feeds. More than 200 kinds of mycotoxins are known to toxicologists, however approximately 30 of these mycotoxins are considered relevant for agriculture and food. Fungi with its mycotoxins infect agricultural crops both in the field and in storage (Abramson, Hulasare, York, White, & Jayas, 2005). Due to their various toxic effects and good thermal stability, the presence of mycotoxins in food and feed is potentially hazardous to the health of both humans and animals (Royer, Hump, & Guy, 2004). Usually, exposure is through consumption of contaminated food, which causes diseases known as mycotoxicosis (Prandini, Sigolo, Filippi, Battilani, & Piva, 2009). The occurrence, amount and kind of mycotoxin may depend on the environment, species of the fungus present, severity of infection and the cultivar or kind of crop (Doohan, Brennan, & Cooke, 2003; Muthomi, Ndung'u, Gathumbi, Mutitu, & Wagacha, 2008). A weather condition during plant growth, in particular at flowering period, is a key factor influencing on the mycotoxin formation and production (Manova & Mladenova, 2009). The most favorable conditions for infection are prolonged periods (48–72 h) of high humidity and warm temperatures (25–30 °C) (Muthomi et al., 2008). Although the moisture content and temperature are the most critical factors, several other factors such as - mechanical injury, insect damage, mineral nutrition of the plant, chemical treatment, rapidity of drying, leakage in storage and hot spots can affect mould growth (Llorens, Mateo, Hinojo, Valle-Algarra, & Jimenez, 2004; Mateo, Mateo, & Jimenez, 2002). The occurrence, frequency and implications of mycotoxins entering the food chain through cereal grain have gained global attention, especially in the last decade with frequent outbreaks of Fusarium head blight (FHB) in many cereal-growing regions (Schaafsma & Hooker, 2007). Surveys have revealed that *Fusarium* spp. are ubiquitous fungi occurring on a broad variety of hosts including barley, maize, millet, oat, rice, rye, and wheat (Krska, Baumgartner, & Josephs, 2001). Species of *Fusarium* range from those, which grow saprophytically on dead plant materials to those, which are fairly specific plant pathogens. These plant pathogens grow parasitically on living plants and damage the mature plant tissue. Infection of the grains takes place in the preharvest period, whereby climatic conditions during growth and in particular during flowering show major impact on the mycotoxin contents in final products (Biselli & Hummert, 2005). A variety of *Fusarium* moulds produce a number

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of different toxins of which mainly trichothecenes (deoxynivalenol (DON), masked mycotoxin derived from DON - deoxynivalenol-3-glucoside (DON-3-Glc), 3- and 15-acetyldeoxynivalenol (ADONs), fusarenon-X (FUS-X) and nivalenol (NIV)), fumonisins and zearalenone (ZON) have been analyzed in cerales. There are also other *Fusarium* mycotoxins grouped into the emerging *Fusarium* mycotoxins such as enniatins, fusaproliferin, beauvericin and moniliformin, which have been recently reviewed and described (Jestoi, 2008). Toxic effects of *Fusarium* mycotoxins largely differ within the groups of organisms (Hajšlová et al., 2007). In experimental animals, type A trichothecenes, such as HT-2 and T-2 toxin were shown significantly more toxic than type B trichothecenes, e.g. DON, NIV, FUS-X, ADONs (Weidenbörner, 2001). Although DON is the least toxic compound of the trichothecene family, it is the most widely presented. European study (EC, 2003) on occurrence of *Fusarium* toxins revealed that 57% of the wheat samples (11022 samples) from 11 countries (from Europe, South America and Asia) were positive for DON. The main effect of DON is an inhibition of protein and DNA synthesis. It affects food intake, body weight gain and immunological functions at high doses (Royer et al., 2004).

The Commission of the European Communities (EC, 2007) established the maximum level for several mycotoxins. Considering DON maximum levels, they are regulated in the following way: 1250 μg/kg of unprocessed cereals other than durum wheat; 1750 μg/kg of unprocessed durum wheat, oats and maize; and 750 μg/kg of cereal flours. Moreover, US Food and Drug Administration (FDA) has advised that products with DON levels above 1000 μg/kg are not acceptable for human consumption (Murphy, Hendrich, Landgren, & Bryant, 2006). Currently, maximum level for ZON is regulated in unprocessed cereals other than maize to be 100 μg/kg and in cereals intended for direct human consumption of 75 μg/kg. It should be noted that regulations regarding HT-2 and T-2 toxins in cereals have not yet been established due to scarce data on the occurrence of these toxins, nevertheless, they are in the preparation (EC, 2007).

The data on *Fusarium* mycotoxins in cereals grown in Serbia have not been studied systematically and there is only one report published for the Serbian cereals regarding one of the *Fusarium* mycotoxins-DON (Jajić, Jurić, & Abramović, 2008). Thus, the present study was undertaken with the aim to provide a preliminary survey on simultaneous occurrence of the trichothecenes of both the A-type (HT-2 and T-2) and the B-type (DON, masked mycotoxin derived from DON, i.e. DON-3-Glc, ADONs, FUS-X and NIV) as well as of ZON in winter wheat harvested in 2007, from different growing regions of Serbia and to determine their co-occurrence. The results were compared to the existing European regulation and with the available literature date for the neighboring countries like Croatia, Bulgaria, Romania and Hungary, knowing that the choice of crops, the use of agrotechnical measures, and food production techniques as well as the climatic conditions are similar in this part of Southeast Europe. Since, none of these previous works dealt with cooccurence of 8 *Fusarium* toxins in wheat, this study could be regarded as the first one reporting their levels in wheat cultivated in the Western Balkan region.

2. Material and methods

2.1. Samples

Serbia is an agricultural country and grain production, especially wheat and maize, is an important economic factor. Cereals represent a staple food for population in Serbia, having high social, economic and nutritional relevance. Moreover, cereals contribute approximately to 20% of the agricultural output. Furthermore, in Serbia, the average annual consumption of wheat per capita has been reported to be around 150 kg. On average, Serbia consumes 1.5 million tons of cereals each year (Statistical Office of the Republic of Serbia, 2007).

Grain samples were collected in the frame of the yearly monitoring of winter wheat sowed in October 2006 and harvested during July 2007 in Serbia. For the purpose of this study, 10 administrative wheat-growing regions were selected, and grain samples of different cultivars were collected immediately after grain harvesting. Five cultivars were included: Evropa 90, Renesansa, Pobeda, Kraljevica and Nora. The sampling scheme with the number of samples of different winter wheat cultivars collected per each region is given in Table 1.

In total 54 representative samples of winter wheat were analyzed. Due to irregular mycotoxin distribution among the crops and kernels, a proper sampling was ensured according to EU requirements (EC, 2006). Manual sampling was performed by trained inspectors with grain probes, which are authorized for official control of contaminants. A certain number of individual samples (see Table 1) of about 200 g were taken randomly for each cultivar and were combined in composite cultivar samples of 3–9 kg weight representative for particular region. Each sample was transported immediately to the Faculty of Technology, University of Novi Sad, and was stored at low temperature in a dark place. All samples were grounded, mixed and a portion was taken for analysis of contaminants.

2.2. Reagents and chemicals

Mycotoxin standards – DON, NIV, Fus-X, ADONs, HT-2, T-2 and ZON - were purchased from Sigma–Aldrich (Germany) and Biopure (Austria). Certified reference materials (CRM), DON in wheat flour (<0.05 mg/kg, BCR 396, Belgium) and DON in naturally contaminated wheat (0.7 ± 0.1 mg/kg, R-Biopharm, Rhone, UK) were used for the quality assurance in the mycotoxin analysis. Both analytical methods described below were accredited (ISO 19010) and as a part of external quality control, the trueness of the generated data was demonstrated through participation in Food Analysis Performance Assessment Scheme (FAPAS) organized by Central Science Laboratory (York, UK).

2.3. Preparation of samples

Many routine laboratories use preparatory methods based on extraction/cleanup/pre-concentration steps regarding only single or

Table 1
Number of samples of different winter wheat cultivars collected from 10 Serbian regions mixed to obtain the composite cultivar samples that were further analyzed to assess the mycotoxins occurrence.

Region	No. of individual (composite) samples per cultivar					No. of composite samples per region [a]
	Evropa 90	Renesansa	Pobeda	Kraljevica	Nora	
Srem	51 (2)	45 (2)	50 (2)			6
West Bačka	59 (2)	50 (2)	52 (2)			6
South Bačka	77 (2)	54 (2)	64 (2)			6
North Bačka	64 (2)	65 (2)	71 (2)			6
South Banat	64 (2)	68 (2)	66 (2)			6
Middle Banat	54 (2)	49 (2)	47 (2)			6
North Banat	75 (2)	61 (2)	68 (2)			6
Belgrade	35 (2)	35 (2)	39 (2)			6
Zaječar				52 (2)	59 (2)	4
Toplica				25 (1)	24 (1)	2
TOTAL						54

[a] One composite sample of each cultivar was analyzed and the obtained results were used to assess the regional differences in mycotoxins occurence in Serbia.

small group of similar mycotoxins (Curtui, Usleber, Dietrich, Lepschy, & Märtlbauer, 1998; Manova & Mladenova, 2009; Sokolović & Šimpraga, 2006). Although these methods are well established, and in some cases interlaboratory validated, the current trend is to introduce simple (one step-), broad scope procedures which allow, thanks to use of modern separation/detection instrumental technologies, accurate determination of as many as possible major mycotoxins even at low levels in crude extracts, not applying labor/cost demanding clean-up step (Zachariasova et al., 2010).

Thus, in this paper simple sample preparation tecnique with only one step of extraction was chosen to be used in order to allow fast analysis of 8 *Fusarium* mycotoxins. Namely, 12.5 g of homogenized ground samples of winter wheat were extracted by shaking with 50 mL of acetonitrile-water mixture (84:16, v/v) for an hour using an automatic shaker (IKA Laboratortechnik, Germany). The crude extracts were filtered. Four mL of filtered extracts were evaporated under vacuum to dryness, dissolved in 1 ml water-methanol mixture (1:1, v/v) and passed through a 0.2 μm microfilter (Alltech, USA) prior to further analysis.

2.4. Analytical method

In this work, LC–MS/MS was used for mycotoxins analysis. Chromatographic separation of the sample components was carried out on a reverse phase column with polar endcapping (Synergi Hydro RP, 150 mm × 3 mm × 4 μm, Phenomenex, USA) at 40 °C in a gradient of mobile phase A (10 mM ammonium acetate in distilled water) and mobile phase B (methanol). The flow rate was set to 0.5 ml/min and the injection volume was 30 μl. The identification and quantification were performed using a tandem mass spectrometry with the following MS/MS parameters previously described by Hajšlová et al., (2007): ion source type – APCI in both negative and positive ion modes, capillary temperature – 150 °C, vaporizer temperature – 450 °C, nitrogen sheath gas flow – 1.2 l/min, nitrogen auxiliary gas flow – 3 l/min, source voltage – 6 kV, collision gas – helium, scan type – selected reaction monitoring.

2.5. Quality assurance/Quality control

Calibration curves for all analytes were linear within the working range from 5 μg/kg to 10000 μg/kg. Squared correlation coefficients (R^2) were in the range of 0.9991–0.9999 for 11 point calibration curves. The limits of detection (LOD), limit of quantification (LOQ) and recoveries that were obtained within the validation process are reported in Table 2. The results showed recoveries achieved for DON, HT-2, T-2, and ZON using the simple preparation technique and reliable, selective and sensitive detection method LC-MS/MS (VANTAGE, Thermo Fisher Scientific, US), were in the range defined by EU requirements (EC, 2006). Moreover, the high recoveries (above 80%) were gained for DON-3Glc, Fus-X, ADONs. However, the recovery for NIV (43%) was lower, being less than the lowest level of 50% requested by EC (2006) for other mycotoxins, implying that further modification of the preparatory method is needed for accurate determination of the NIV occurrence in wheat. Thus, in this study, the presence of NIV concentrations was only indicative. All the presented results were not corrected for recovery.

Table 2
Summary of validation data of LC/MS–MS method.

	NIV	DON	DON-3Glc	Fus-X	ADONs	HT-2	T-2	ZON
LOD (μg/kg)	5	0.3	1	1	5	5	0.3	1
LOQ (μg/kg)	10	1	5	5	10	10	1	5
Recovery (%)	43	87	85	85	80	86	83	69

Table 3
Frequency of occurrence and descriptive statistic parameters for mycotoxins analyzed in winter wheat cultivars in 10 selected Serbian regions from the 2007 harvest of the total number of 54 samples.

Mycotoxin	No. of positive samples (frequency of occurrence, %)	Average value[a] (μg/kg)	Median (μg/kg)	Concentration range[b] (μg/kg)
NIV	0	< LOD	< LOD	< LOD
DON	15 (27.78)	33	< LOD	41–309
DON- 3 - Glc	7 (12.96)	5	< LOD	17–83
FUS-X	0	< LOD	< LOD	< LOD
ADONs	0	< LOD	< LOD	< LOD
HT-2	3 (5.56)	9	< LOD	128–129
T-2	0	< LOD	< LOD	< LOD
ZON	0	< LOD	< LOD	< LOD

[a] Half of the limit of detection (LOD) values were taken in calculating the average values of mycotoxin concentrations that were below LOD, except in the case of the frequency of occurrence of 0%.
[b] In positive samples.

3. Results and discussion

The occurrence of *Fusarium* mycotoxins in winter wheat was determined in 54 samples of 5 cultivars: Evropa 90, Renesansa, Pobeda, Kraljevica and Nora, from 10 selected Serbian wheat-growing regions, and the results are given in Tables 3–6. The most prevalent was DON followed by DON-3-Glc and HT-2. ADONs, FUS-X, NIV as well as ZON were not detected in any of the samples. As can be seen, DON, DON-3-Glc and HT-2 were detected in 28%, 13% and 6% of the total number of samples, respectively (Table 3). Considering the average value for all samples, the highest one was found for DON (33 μg/kg), followed by HT-2 (9 μg/kg) and DON-3-Glc (5 μg/kg).

The overview of the contamination patterns in the winter wheat samples is summarized in Table 4. In all samples with co-occurrence of DON-3-Glc and DON, the DON/DON-3-Glc ratio was in a range 2–7. Concerning HT-2, it was found co-occurrently with DON only in 2 samples: in one of them, HT-2 was more abundant than DON (about 3 times) while in the other it was present in slightly lower level than the latter.

In all investigated samples of winter wheat, the level of DON was below the maximum one established by EC (2007) to be 1250 μg/kg for unprocessed cereals other than durum wheat or 1000 μg/kg for products intended for human consumption as advised by FDA (Murphy et al., 2006). As ZON was not detected in any of the samples, all the samples were in compliance with EC regulation (EC, 2007). The maximum levels for the other mycotoxins that were detected in winter wheat samples (DON-3-Glc and HT-2) have not been regulated by the existing regulation.

It could be noted that the incidence and levels of mycotoxins varied in samples from different wheat-growing regions (Table 5). With respect to DON, maximum value found in South Bačka (309 μg/kg), was five folds of the one found in Middle Banat (58 μg/kg). Comparing the wheat-growing regions in Serbia, average DON values could be ordered as follows: South Bačka>North Bačka> Belgrade = North Banat > Middle Banat. All these regions are located in northern part of Serbia, i.e. in the Vojvodina Province, except the

Table 4
Frequency of co-occurrence of *Fusarium* mycotoxins determined in 54 winter wheat samples analyzed in this study.

Mycotoxin	Positive samples (%)
DON	6 (11.11)
DON+ DON-3-Glc	7 (12.96)
DON+HT-2	2 (3.70)
HT-2	1 (1.85)

Table 5
Average values and concentration range of mycotoxin contents in investigated samples of all studied cultivars from 10 selected wheat-growing regions in Serbia from the 2007 harvest.

Region	Mycotoxins (μg/kg)					
	DON		DON-3-Glc		HT-2	
	Average value[a]	Concentration range[b] (μg/kg)	Average value	Concentration range (μg/kg)	Average value	Concentration range (μg/kg)
South Bačka	260	211–309	44	41–46	< LOD	< LOD
West Bačka	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
North Bačka	108	54–269	< LOD	< LOD	44	< LOD
South Banat	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
North Banat	89	57–111	24	21–30	< LOD	< LOD
Middle Banat	54	49–58	9	< LOD	< LOD	<LOD
Srem	93	41–145	42	< LOD	66	< LOD
Belgrade	89	49–164	< LOD	< LOD	34	<LOD
Zaječar	< LOD	< LOD	< LOD	<LOD	< LOD	<LOD
Toplica	< LOD	<LOD	<LOD	< LOD	< LOD	< LOD

[a] In calculating the average values of mycotoxin concentrations for each region, for the content below LOD, half of the limit of detection (LOD) value was taken.
[b] In positive samples.

Belgrade region adjacent to them. A slightly different distribution of DON-3-Glc was found throughout the regions investigated in this study: South Bačka ≈ Srem > North Banat > Middle Banat. HT-2 toxin had the lowest frequency and it was found in only three regions (Table 5) with almost a constant concentration. The concentration of *Fusarium* mycotoxins in winter wheat from Toplica and Zaječar was under LOD. These two regions are situated on the south of Serbia, contrary to the other regions where occurrence of toxins were detected. The changes observed between northern and southern regions within the same year, are attributed primarily due to differences in climate conditions and consequently in period of collection.

In general, Serbia is located in the continental climate belt. Fig. 1 shows the amount of rainfall and average temperature in Serbia in the period October 2006–July 2007 compared to the average values for the long term period of 1961–1990 (obtained from the Republic Hydrometeorogical Service of Serbia, 2007), which defines the latest normals for Serbia, i.e. arithmetic means of a climatological elements computed over three consecutive decades in Serbia. The period 2006/2007 was considered since the investigated winter wheat samples were sowed during October 2006, while their harvesting were during July 2007. The average temperatures in the 2006/2007 period were similar or slightly higher than the ones of the period 1961–1990 (Fig. 1b), while the amount of rainfalls were lower (Fig. 1a). For instance, in June 2007 average temperature was about 25% higher than the one for the long-term average (1961–1990), whereas the amount of rainfall was less for 10% than for the 1961–1990 average. Such conditions could not be regarded as favorable for the development of mycotoxins, as the incidence of Fusarium head blight (FHB) is strongly associated with humid conditions.

Furthermore, the southern Serbian regions like Toplica and Zaječar where mycotoxins were not detected in wheat samples are characterized with specific microclimate with very low amount of rainfalls. For instance, in June and July 2007, the rainfall amounts in Zaječar were about 30 mm and 10 mm, respectively, that were several times lower than the Serbian average (~80 mm and ~60 mm) and the average of South Bačka station located on the north of Serbia (75 mm and 40 mm). Obviously, arid conditions in Toplica and Zaječar regions did not favor the growth of moulds. However, it should be noted that the development of FHB depends also on the resistance of the particular cultivar (Hajšlová et al., 2007; Muthomi et al., 2008). The average results of DON contamination in winter wheat cultivars analyzed in this study (Table 6) ordered cultivars in the following way: Renesansa > Evropa 90 > Pobeda, while in Kraljevica and Nora cultivars the content was below LOD. This implies different susceptibility of cultivars towards DON, although the found differences were also a consequence of different climate conditions during cultivation, since Kraljevica and Nora cultivars were collected in two southern Serbian regions.

The comparison of the results with the ones reported in the literature for Serbia and neighboring countries was made preferentially regarding the DON occurrence (Fig. 2), since it has been the most frequently analyzed *Fusarium* mycotoxins. In order to illustrate more clearly the reported results in accordance to the EC regulation (EC, 2007) maximum allowed content of DON in unprocessed cereals other than durum wheat is also presented in Fig. 2.

In previous study concerning DON occurrence in spring wheat (planted in May, harvested in October) in Serbia, in two consecutive years, 2004 and 2005, reported by Jajić et al (2008), incidence rates of DON for these years were 50% and 33%, respectively. Difference between the results of Jajić et al (2008) and those obtained in this study could be attributed to the fact that 2004 and 2005 were more humid than 2006/2007. Namely, Jajić et al (2008) reported that the year 2004 was somewhat "more humid" than the long-term average they used for comparison (1971–2000), while they classified 2005 as "highly humid". In these years, rains were frequent in

Table 6
Mycotoxin concentrations detected in different cultivars in Serbia from the 2007 harvest (only mycotoxins detected of the samples are presented).

Winter wheat cultivar	DON			DON- 3 -Glc			HT-2		
	No. of positive samples (total number of samples)	range (μg/kg)	Average of positive samples	No. of positive samples (total number of samples)	range (μg/kg)	Average of positive samples	No. of positive samples (total number of samples)	range (μg/kg)	Average of positive samples
EVROPA 90	7 (16)	< LOD-269	**119**	4(16)	< LOD-46	**27**	1 (16)	< LOD-128	**128**
RENESANSA	6 (16)	< LOD-309	**137**	3 (16)	< LOD-83	**51**	2 (16)	< LOD-129	**129**
POBEDA	2 (16)	< LOD-54	**52**	0 (16)	< LOD	**< LOD**	0 (16)	< LOD	**< LOD**

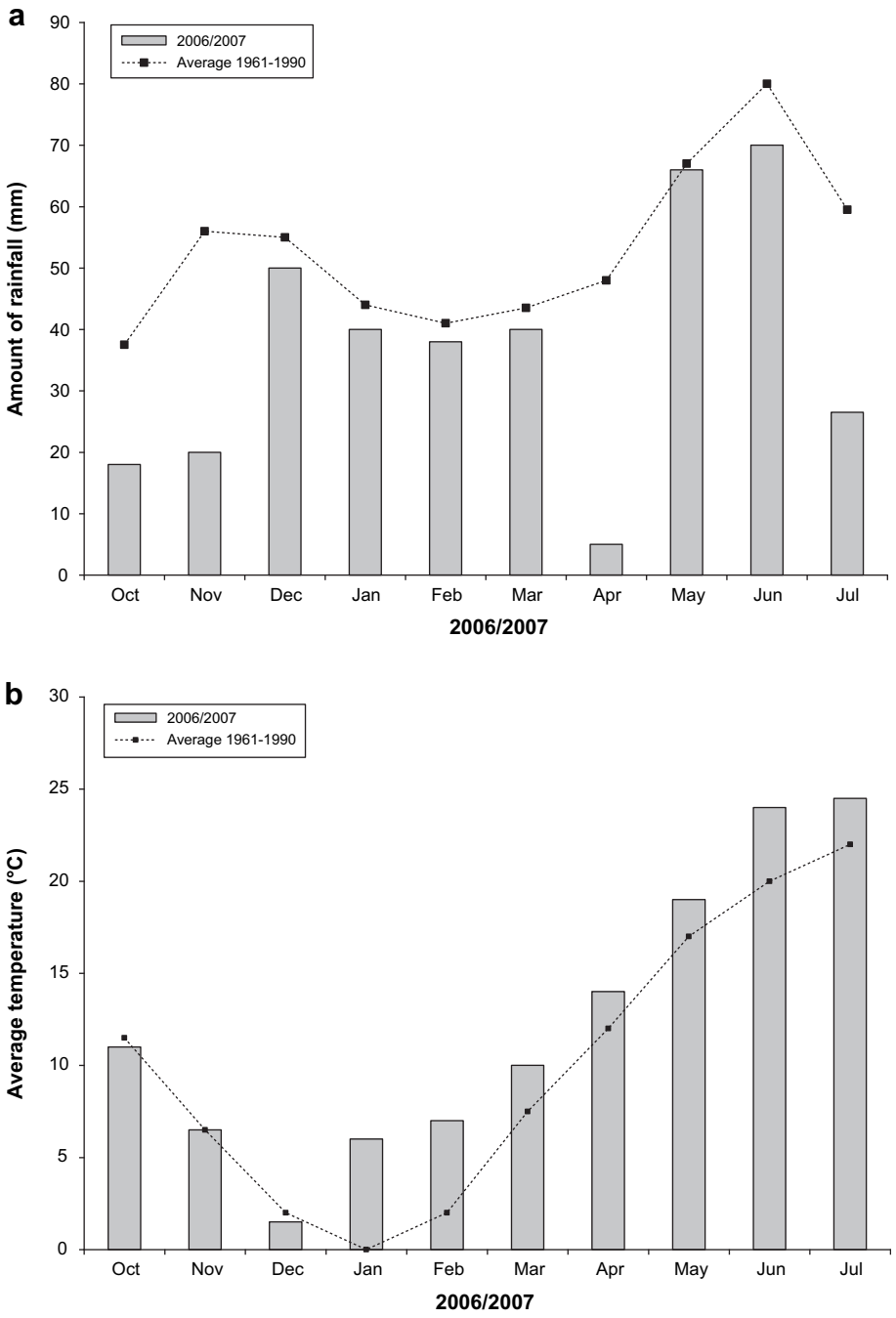

Fig. 1. Comparison of monthly amounts of rainfall (a) and average air temperatures (b) for Serbia in 2006/2007 with average values in the long-term period (1961-1990).

the period May–June, the critical period for development of fungi in spring wheat. During these two months, amounts of rainfall were about 100–125% higher than the average value for the 1971–2000, Jajić et al (2008) used for comparison. Still, they found the higher incidence rate and the maximum value of DON in the wheat samples cultivated during "more humid" 2004, not during the "highly humid" 2005. They speculated that higher contamination of the 2004 harvest was most likely because the samples were analyzed one year after storage in barns, which enabled further mycotoxin production. Furthermore, Jajić et al (2008) emphasized that number of the analyzed samples was rather small (from 2004 it was only four; while from 2005 it was twelve samples), and it could not be interpreted as the actual situation, but only as a preliminary evaluation of DON contents in spring wheat cultivated in Serbia during 2004 and 2005.

An investigation on the occurrence of mycotoxins in wheat harvested in 1997 from western Romania reviewed by Curtui et al (1998) showed that the highest contamination rates and levels were found for DON and its acetylated analogues (ADONs). Climatic conditions prevailing in the summer months in the western part of Romania, characterized by heavy rainfall before harvest, and are known to favor high infection rates with *Fusarium*, explained 100% frequency of DON contamination in analyzed wheat samples, with maximum concentration of 5600 μg/kg (Fig. 2). Furthermore, by investigating 99 feeding wheat samples from 1998 harvest for the presence of DON in Hungaria, Fazekas, Hajdu, Tar, and Tanyi (2000)

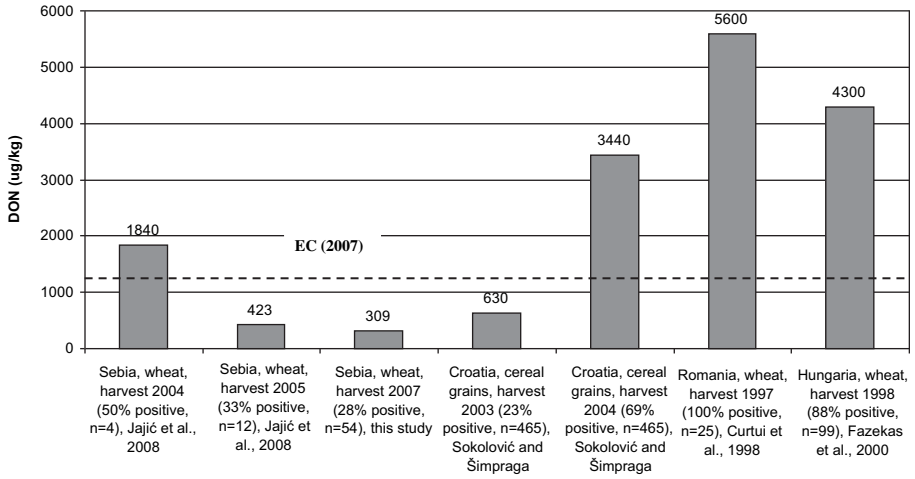

Fig. 2. Maximum concentrations of DON detected in cereal grain in Serbia and neighboring countries compared to the EC maximum limit set for 1250 μg/kg of unprocessed cereals other than durum wheat. Frequency percentage and total number of samples analyzed in the cited studies are given in parentheses.

found that this mycotoxin was present in 88% of samples with the highest concentration of 4300 μg/kg. The authors related such high incidence of occurrence and concentration of DON in wheat samples with the rainy summer weather. Sokolović and Šimpraga (2006) analyzed DON in cereal grains in Croatia in the period between 2001 and 2004, and their results indicated that from total of 465 samples collected during the four year period there was about 41% of positive samples. Wheat harvested in Croatia in 2004 (Sokolović & Šimpraga, 2006), contained levels of DON higher than the EC limit of 1250 μg/kg (Fig. 2), since the years 2003 and 2004 were extremely warm, high above the average and with frequent rains in spring, fall and winter, classifying them as "highly humid". Additionally, according to the report of Sokolović and Šimpraga in 2004 the highest incidence rate of about 59% was found for T-2 toxin with maximum concentration of 520 μg/kg, while the levels of the same mycotoxin were below the LOD in the current study. A survey done by Manova and Mladenova (2009) revealed that samples of wheat grains produced in Bulgaria, contained ZON at levels about 10 times lower than the maximum limit of 100 μg/kg established for unprocessed cereals in Europe (EC, 2007) since in positive samples the concentrations were found to be up to 10 μg/kg, while in the current survey, ZON was not detected in any of the investigated wheat samples. As for other neighboring countries such as Macedonia, Bosnia and Herzegovina, Montenegro, Albania, there are no relevant literature data for mycotoxins level in the cultivated crops, further comparison of data was not feasible.

As this is the first report on simultaneous occurrence of 8 *Fusarium* mycotoxins from Serbia, and even wider in the Western Balkan countries region and the obtained data suggested different resistance of the wheat cultivars investigated in this study with respect to *Fusarium* infection, it is necessary to take a more comprehensive, long-term monitoring program to draw a general conclusion on this issue.

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