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TAXONOMY OF *FUSARIUM* GENUS, A CONTINUOUS FIGHT BETWEEN LUMPERS AND SPLITTERS

ABSTRACT: The genus *Fusarium* comprises a high number of fungal species that can be plant-pathogenic, causing diseases in several agriculturally important crops including cereals, and also can be harmful for humans and animals since many of them are toxigenic.

The identification of mycotoxigenic *Fusarium* species still remains a most critical issue, given that the number of species recognized in the genus has been constantly changing in the last century in accordance with the different taxonomic systems. Together with the morphological identification, current criteria for *Fusarium* species identification are also based on biological and phylogenetic species recognition. However these criteria rarely agree to each other. Therefore, it is still a charming scientific challenge to ascertain the taxonomic status of *Fusarium* species, which in the years have been continuously „splitted" and „lumpered" by scientists. The major cases of the taxonomic debates amongst the *Fusarium* community will be here discussed.

KEY WORDS: *Gibberella*, morhology, biological identification, phylogeny.

INTRODUCTION

The genus *Fusarium* comprises a high number of fungal species that can be plant-pathogenic, causing diseases in several agriculturally important crops, including cereals, and can also be harmful for humans and animals. Many of them produce a wide range of biologically active secondary metabolites (e. g. mycotoxins) with extraordinary chemical diversity. The biological activity of *Fusarium* mycotoxins can be detrimental to plants, and it is associated with cancer and other diseases in humans and domesticated animals. The combined effect of *Fusarium* species infecting several crops and producing mycotoxins in the field is the contamination of cereal grains and other plant-based foods. With many pathogenic and opportunistic species of the genus colonizing plants as a part of the complex of *Fusarium* species, it provides an interesting example of biodiversity, as well as the consequences of different environmental conditions that exist in the various agro-ecosystems in which crops are

cultivated. These conditions can also influence the fungal-plant interactions of the single species and their capability to produce mycotoxins. Moreover, the ability of various *Fusarium* species within the complexes to produce different classes of secondary metabolites combined with their ability to coexist in the same host or/and occur in quick succession have allowed these complexes to become „invincible armadas" against many plants.[1] Plant infections by *Fusarium* can occur at all developmental stages, from germinating seeds to mature vegetative tissues, depending on the host plant and *Fusarium* species involved. Therefore, since most *Fusarium* species have specific mycotoxin profiles, early and accurate identification of the *Fusarium* species occurring in the plants, at every step of their growth, is critical to predict the potential toxicological risk to which plants are exposed and to prevent toxins entering the food chain. However, the unambiguous identification of mycotoxigenic *Fusarium* species still remains a most critical issue, given that the number of species (which stands now over 80)[2] recognised in the genus was constantly changing during the last century in accordance with the different taxonomic systems. Furthermore, this genus is provided of few morphological characters useful for species discrimination based only on traditional technique, although, fortunately, some of the most important toxigenic and pathogenic *Fusarium* species can be diagnosed, with some experience, by using only their morphological traits. Considering that the current criteria for *Fusarium* species identification (e. g. morphological (MSR),[3, 4] biological (BSR)[5] and phylogenetic species recognition (PSR)[6]) rarely concur, and that, out of 101 most economically important plants, 81 have at least one plant associated with *Fusarium* disease,[2] along with the fact that each *Fusarium* species keeps its own toxicological profile,[7] it is a challenge to ascertain the taxonomic status of *Fusarium* species on their phenotypical characteristics (including pathogenicity and toxigenicity) alone (www.apsnet.org/online/common/search.asp).

41.1.1. Classification and morphology of *Fusarium*

The genus *Fusarium* belongs to the *Ascomycota* phylum, *Ascomycetes* class, *Hypocreales* order,[8] while the teleomorphs of *Fusarium* species are mostly classified in the genus *Gibberella*, and for a smaller number of species, *Hemanectria* and *Albonectria* genera. For a complete review of the main taxonomic systems that have contributed to the defining of the modern taxonomy of *Fusarium*, see the excellent work of Leslie and Summerell,[2] which contains an updated description, not only morphological, of 70 species within the genus.

The main approach for the *Fusarium* classification is still morphology, and the primary trait for species to be placed in *Fusarium* genus is the occurrence of the asexual spores, the distinctive banana-shaped macroconidia, firstly diagnosed by Link.[9] *Fusarium* species produce three types of spores: macroconidia, microconidia and chlamydospores.[2] Septated macroconidia can be produced on monophialides and polyphialides in the aerial mycelium, but also on short monophialides in specialized structures called sporodochia.[10] A mono-

phialide is a conidiation cell with a unique pore from which the endoconidia are released; a polyphialide can possess several such openings. Microconidia can vary in shape and size, and are produced in the aerial mycelium in clumps or chains, both on monophialides and polyphialides. Finally, chlamydospores are resistance structures with thickened walls and high lipid content; in the case of their presence, they can form in the middle of the hyphae or at their termini. The different shape of macroconidia remains the most important feature for distinguishing the species. Moreover, other traits, such as the presence/absence of microconidia and their shape, the presence/absence of chlamydospores, and the characteristics of the micro- and macro-conidiogenous cells, contribute to distinguishing species in *Fusarium*. In order to identify the species, all taxonomists suggest the use of strain cultures derived from single--spore isolation, and growing the strains on special media under standard incubation conditions. All taxonomic systems developed so far are based on a seminal work by Wollenweber and Reinking[11] with various modifications.[4, 12] This publication organized the genus in 16 Sections, including 65 species, 55 varieties and 22 forms. The main discriminating criteria among the Sections were based on morphology; in particular, on the presence and shape of microconidia, on the presence and position of chlamydospores in the hyphae, on the shape of macroconidia and their basal cells. The taxonomic system described by Gerlach and Nirenberg[3] kept the number of the Sections as Wollenweber and Reinking, while Nelson et al.[4] proposed a simpler classification method that divided the genus in 12 Sections. Although the main taxonomic systems have organized the Sections with species sharing common morphological characteristics, thus, supposedly genetically related, not all researchers accept this Section concept since some of the used morphological characteristics are now considered of poor reliability from an evolutionary point of view, according to the recent molecular investigations. On the other hand, the classification of *Fusarium* species has still a number of open question marks that need to be solved and should require the use of all species recognition methods in an integrated approach.

The *G. fujikuroi* species complex. *Gibberella fujikuroi* (Sawada) Ito in Ito e K. Kimura has long been considered the teleomorph of several *Fusarium* species, morphologically placed by Nelson et al. in the *Liseola* section.[4] Within this complex, Nelson et al.[4] comprised 4 anamorphic species, including the maize pathogens, *F. moniliforme*, *F. proliferatum*, and *F. subglutinans*, and a minor species, *F. anthophilum*. On the other hand, Gerlach and Nirenberg[3] identified 10 species in Section *Liseola* and adopted the name of *F. verticillioides* (Sacc.) Nirenberg (teleomorph *Gibberella moniliformis*), instead of *F. moniliforme*, as generally accepted by the research community of *Fusarium*.[13] As for many other fungal phyla, taxonomic results based on MSR, BSR and PSR (see Introduction) have recently started being compared also for *G. fujikuroi* species complex. According to BSR, many reports have now clarified that *G. fujikuroi* species complex includes at least 11 different biological species or Mating Populations (MPs): MP-A (*G. moniliformis*, anamorph *F. verticillioides*), MP-B (*G. sacchari* anamorph *F. sacchari*), MP-C (*G. fujikuroi* anamorph *F. fujikuroi*), MP-D (*G. intermedia* anamorph *F. proliferatum*), MP-E

(*G. subglutinans* anamorph *F. subglutinans*), MP-F (*G. thapsina* anamorph *F. thapsinum*), MP-G (*G. nygamai* anamorph *F. nygamai*), MP-H (*G. circinata* anamorph *F. circinatum*), MP-I (*G. konza* anamorph *F. konzum*), MP-J (*G. gaditjirrii* anamorph *F. gaditjirrii*), and MP-K (*G. xylarioides* anamorph *F. xylarioides*).[2] The results of sexual crosses, integrated with morphological observations and molecular data by using RAPD, AFLP, RFLP and DNA sequencing,[6, 14—17] have shown that the results of the three classification methods (biological, morphological, phylogenetic) are largely congruent. However, phylogenetic analyses carried out by O'Donnell et al.[14] using several genes, among which b-tubulin and calmodulin, revealed 46 species in the *G. fujikuroi* complex, of which 23 are new to science. Among the 46 species, the 11 species identified by using biological species concept have been reported identical to the phylogenetic species, which indicates that phylogenetic approach can provide the same information as biological approach and that other MPs still need to be identified.

The *F. graminearum* species complex. The recent re-classification of *F. graminearum* (teleomorph, *G. zeae*), a worldwide pathogen of wheat and maize, is controversial. *Fusarium graminearum* produces several mycotoxins, mainly trichothecenes, which are tricyclic sesquiterpenes, that have been strongly associated with chronic and fatal toxicoses of humans and animals, and zearalenones, which have estrogenic activity.[7] *Fusarium graminearum* produces multiple trichothecene analogues, in particular deoxynivalenol (DON) and nivalenol (NIV), and their acetylated derivatives, 3-acetil-DON (3A-DON) and 15-acetil-DON (15A-DON). Within this species, strains differ in their trichothecene production profiles; some strain produce DON, some produce NIV, and others produce DON and NIV. Such chemotype diversity within *F. graminearum* is a result of loss of gene function. DNA sequence-based phylogenetic analysis of *F. graminearum* field isolates from six continents delineated eight phylogenetically distinct lineages that were considered biogeographically structured.[18] Among the lineages, lineage 7 was considered as the most geographically widespread, predominating on wheat and maize in North and South America, and in Europe, producing primarily DON.[18] The lineages were considered genetically isolated because each was reciprocally monophyletic within genealogies when the six nuclear genes were analyzed both individually and together. Further studies based on DNA sequence polymorphisms from eleven nuclear genes and three intergenic regions led O'Donnell et al.[19] to describe nine lineages within *F. graminearum* clade and to elevate these lineages to the rank of species. Finally, Starkey et al.[20] described two novel species within the *F. graminearum* species complex based on phylogenetic analyses of multi-locus DNA sequence data of 13 genes. However, not all *Fusarium* researchers agree with the division of *F. graminearum* into multiple species. Some authorities considered the lineages to be subspecies rather than species.[2] This opinion was supported by the finding that in general, isolates from partially inter-fertile phylogenetic species tend to have AFLP band identities in the range of 40—65%, and that *F. asiaticum* and *F. graminearum* have AFLP band identities of 50%. Moreover, only three of the nine lineages show conidial morphology traits useful for differentiating them, and there is no correlation between

lineages and specific mycotoxin profile.[18, 19, 21] Finally, sequencing a portion of *tri101* gene of 400 strains of the *F. graminearum* species complex, Leslie et al.[22] generated both a phylogenetic tree and a genetic network that led the authors to a conclusion that „there is only a single species within *F. graminearum/G. zeae*". Due to these apparently contradictory data, additional studies are necessary to determine whether the different lineages of *F. graminearum* represent distinct phylogenetic species or subspecies lineages. Such studies should also provide insight into the practical implications dividing *F. graminearum* into multiple species with respect to disease management, quarantine regulations and plant breeding strategies, and to understand the ecology, epidemiology, and population dynamics of *F. graminearum* species complex.

The third case, *Fusarium oxysporum*: a species complex? *Fusarium oxysporum* is a plant pathogen causing a wide range of plant diseases mainly related to vascular wilts. However, within the species, many populations isolated mainly from soil have been shown as non pathogenic and they are used as bio-control agents against several diseases also caused by *Fusarium* species.[23] Morphologically, these strains cannot be differentiated from pathogenic strains, although a wide genetic diversity of the population originating from soils has been reported.[23] On the other hand, the majority of the isolates causing vascular wilts are specific for a certain host plant. From taxonomic point of view, these strains have differentiated from each other on the basis of pathogenicity as *formae speciales*. Therefore, the identification of these strains traditionally involves tests of pathogenicity with the appropriate hosts, which are time consuming and can require several months for some *formae speciales*. Moreover, since pathogenicity is not an ancestral character, taxonomic distinctions of strains based only on this are not reliable from an evolutionary point of view, and *formae speciales* should not be considered monophyletic in origin. On the other hand, the basis for *formae speciales* names need not be grounded in traits that are monophyletic in origin [24,25] in order to avoid mistakes in breeding for resistance, and to set up inappropriate quarantine measures.[2]

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ТАКСОНОМИЈА РОДА *FUSARIUM*, СТАЛНА БОРБА МЕЂУ ТАКСОНОМИСТИМА

Антонио Н. Морети

Резиме

Род *Fusarium* обухвата велик број гљивичних врста које могу бити патогене за биљке и које изазивају обољења код неколико важних пољопривредних култура, укључујући и житарице; а како је већина њих токсигена, могу исто тако бити штетне за људе и животиње.

Идентификација микотоксигених *Fusarium* врста је још увек проблематична, с обзиром на то да се број познатих врста овог рода стално мењао у току прошлог века, а у складу са различитим таксономским системима. Уз морфолошку идентификацију, тренутни критеријуми идентификације *Fusarium* врста се базирају на идентификацији биолошких и филогенетичких врста. Међутим, ови критеријуми се ретко међусобно подударају. Из тог разлога је утврђивање таксономског статуса *Fusarium* врсте прави изазов, јер их научници све до данас разврставају по различитим системима који или истичу сличности или истичу разлике између врста. У раду ће бити размотрене неке од најважнијих научних дебата у вези са *Fusarium* заједницом.

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DETECTION METHODS FOR MYCOTOXINS IN CEREAL GRAINS AND CEREAL PRODUCTS

ABSTRACT: Analytical methods for mycotoxins in cereals and cereal-based products require three major steps, including extraction, clean-up (to eliminate interferences from the extract and concentrate the analyte), and detection/determination of the toxin (by using suitable analytical instruments/technologies). Clean-up is essential for the analysis of mycotoxins at trace levels, and involves the use of solid phase extraction and multifunctional (e.g. MycoSep®) or immunoaffinity columns. Different chromatographic methods are commonly used for quantitative determination of mycotoxins, including gas-chromatography (GC) coupled with electron capture, flame ionization or mass spectrometry (MS) detectors (mainly for type-A trichothecenes), and high-performance liquid chromatography (HPLC) coupled with ultraviolet, diode array, fluorescence or MS detectors. The choice of method depends on the matrix and the mycotoxin to be analyzed. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is spreading rapidly as a promising technique for simultaneous screening, identification and quantitative determination of a large number of mycotoxins. In addition, commercial immunometric assays, such as enzyme-linked immunosorbent assays (ELISA), are frequently used for screening purposes as well. Recently, a variety of emerging methods have been proposed for the analysis of mycotoxins in cereals based on novel technologies, including immunochromatography (i.e. lateral flow devices), fluorescence polarization immunoassays (FPIA), infrared spectroscopy (FT-NIR), molecularly imprinted polymers (MIPs) and optical biosensors.

KEYWORDS: cereals, mycotoxins, rapid methods, clean-up, GC, HPLC, immunoassays, LC-MS/MS, molecularly imprinted polymers

INTRODUCTION

Major mycotoxins that can occur in cereal grains and cereal-based products are *Fusarium* mycotoxins, deoxynivalenol (occurring mainly in wheat, maize, barley, oats, rye), T-2 and HT-2 toxins (oats, wheat, barley), zearalenone (maize, wheat) and fumonisins (maize), and *Aspergillus* or *Penicillium* mycotoxins, aflatoxins (maize) and ochratoxin A (maize, wheat, barley, rye). Human or animal exposure to these natural contaminants can lead to acute or chronic diseases, and in some cases death (R i c h a r d, 2007). According to

the results of risk assessment studies (SCOOP projects), cereals and cereal-based products are the main source of mycotoxin intakes by the EU population (http://ec.europa.eu/food/food/chemicalsafety/contamin ants/index_en.htm). In order to protect human health from exposure to these mycotoxins through the consumption of cereal-based foods, the European Commission has recently established regulatory limits for deoxynivalenol (DON), zearalenone (ZEA), fumonisins (sum of FB_1 and FB_2), aflatoxins (AFB_1 and sum of AFB_1, AFB_2, AFG_1 and AFG_2) and ochratoxin A (OTA) in raw cereals and derived products intended for human consumption, while permissible levels of T-2 and HT-2 toxins in cereals are under discussion (European Commission 2006, 2007).

Analytical methods for rapid, sensitive, and accurate determination of these mycotoxins in unprocessed cereals and cereal-based products are highly needed in order to properly assess both the relevant risk of exposure and the relevant toxicological risk for humans and animals, as well as to ensure that regulatory levels fixed by the EU or other international organisations are met. Analytical methods for mycotoxins in cereals and cereal-based products generally require toxin extraction from the matrix with an adequate extraction solvent, a clean-up step intended to eliminate interference from the extract and, finally, detection/determination of the toxin by suitable analytical instruments/technologies. Chromatographic methods commonly used for quantitative determination of mycotoxins in cereals include high-performance liquid chromatography (HPLC) coupled with ultraviolet (UV), diode array (DAD), fluorescence (FD) or mass spectrometry (MS) detectors, and gas-chromatography (GC) coupled with electron capture (ECD), flame ionization (FID) or MS detectors. In addition, commercial immunometric assays, such as enzyme-linked immunosorbent assays (ELISA) or membrane-based immunoassays, are frequently used for screening purposes. Recently, a variety of rapid methods that are emerging have been proposed for mycotoxin analyses. They are based on novel technologies, including immunochromatography, fluorescence polarization, infrared spectroscopy, molecularly imprinted polymers, and biosensors (S h e p h a r d, 2008). This review deals with the current analytical methodologies, traditional and emerging, available for the detection of main mycotoxins occurring in cereals and cereal-based products.

Sample preparation (extraction and clean-up)

Mycotoxins are commonly extracted from ground cereals by shaking or blending with mixtures of water or other polar solvents, such as methanol or acetonitrile. Purification of the extract is an essential step in the analysis of mycotoxins, especially when chromatographic techniques are used for their determination at trace levels. Solid phase extraction (SPE), multifunctional clean--up columns, e.g. MycoSep®, and immunoaffinity columns (IACs) are frequently used to clean-up the extracts of raw cereals, as well as cereal-processed products. MycoSep® columns are one of the most commonly used and commercially available columns for removing analytical interferences from raw extracts in one quick step (10—30 sec). The MycoSep® column contain-

ing various adsorbents, such as charcoal, Celite and ion-exchange resins, is pushed into a test tube (containing the extract) forcing the extract to filter upwards through the packing adsorbent material. The interferences adhere to the adsorbents in the column and the purified extract passes through a frit to the surface of the column. These columns are often used for the simultaneous and rapid clean-up of type A- and type B-trichothecenes, as well as AFs, OTA, ZEA and FBs. Immunoaffinity columns are based on monoclonal or polyclonal antibodies, and are commonly used for mycotoxin analyses. The specificity of antibodies provides cleaner extracts, with respect to other methods of purification, and good precision, accuracy and sensitivity of analytical methods that use this clean-up procedure. IACs are commercially available for AFs, OTA, FBs, ZEA, DON, T-2 and HT-2 toxins, and have been used to simultaneously detect the presence of these toxins by HPLC with good accuracy and precision (P a s c a l e and V i s c o n t i, 2008).

Recently, in the area of mycotoxin analysis, there has been an increasing interest in the potential use of molecularly imprinted polymers (MIPs) as adsorbents for SPE due to their low costs, easy preparation, high chemical stability and long shelf-life. MIPs are cross-linked polymers that are thermally, photochemically or electrochemically synthesized by the reaction of a monomer and a cross-linker in the presence of an analyte, e.g. mycotoxin, used as a template. After polymerization, the analyte is removed leaving specific recognition sites inside the polymer. MIPs provide biomimetic recognition elements capable of selective binding/rebinding to the analyte with efficiencies comparable to those of antibody-antigen interactions. The synthesis of MIPs with high affinity for DON, ZEA and OTA was already reported (P a s c a l e et al., 2008a). These polymers have been used as a stationary phase in chromatographic applications, or for the preparation of SPE columns that are to be used in sample clean-up, although, in a few cases, non-imprinted polymers, i.e. polymers synthesized without a mycotoxin template, performed similarly to molecularly imprinted polymers. Recently, itaconic acid has been identified by molecular modeling and computational design as a functional monomer with high affinity towards DON. Itaconic acid polymers, synthesized without the template (i.e. DON), were successfully used as adsorbents for SPE clean-up and pre-concentration of DON from pasta extracts prior to the HPLC analysis (P a s c a l e et al., 2008a).

Traditional technologies for detecting/quantifying mycotoxins

Gas chromatographic methods based on FID, ECD and MS detection are the most widely used methods for quantitative simultaneous determination of trichothecenes (mainly type A) in cereals and cereal-based products (K r s k a et al., 2001). These methods require a preliminary clean-up of extracts, generally by MycoSep® columns, and pre-column derivatization of the purified extract with specific reagents. Mass spectrometry (MS), or tandem mass spectrometry (MS/MS), offers an advantage in confirming the identity of chromatographic peak. The main problems associated to GC analysis include increa-

sed trichothecene responses (up to 120%), non-linearity of calibration curves, drifting responses, carry-over or memory effects from previous samples, and high variation in terms of reproducibility and repeatability (Petterson and Langseth, 2002).

HPLC coupled with UV, diode array (DAD) or fluorescence detector (FD) is currently the most widely used technique for the analysis of major mycotoxins occurring in cereals. AFs, OTA, FBs and ZEA are routinely analyzed by HPLC-FD, and DON by HPLC-UV (DAD) with good accuracy and precision. HPLC-FD is highly sensitive, selective and repeatable, so specific labeling reagents have been developed for the derivatization of non-fluorescent mycotoxins to form fluorescent derivatives. Either pre-column derivatization with trifluoroacetic acid (TFA), or post-column derivatization with Kobra Cell® (i.e. electrochemical bromination cell) or photochemical reactor (i.e. UV irradiation), can be used to enhance fluorescence of aflatoxins B_1 and G_1 (Papadopoulou-Bouraoui et al., 2002), whereas pre-column derivatization with OPA reagent is required for the detection of fumonisins B_1, B_2 and B_3, after purification of the extracts with immunoaffinity columns, solid phase extraction or MycoSep® columns. Recently, 1-anthroylnitrile (1-AN), 2-naphthoyl chloride (2-NC), and pyrene-1-carbonyl cyanide (PCC) have been used as fluorescent labeling reagents for T-2 and HT-2 detection by HPLC-FD (Visconti et al., 2005; Lippolis et al., 2008). The derivatization reaction was used to develop a sensitive, reproducible and accurate method for the simultaneous determination of T-2 and HT-2 after IAC clean-up in naturally contaminated cereal grains (wheat, maize, oats and barley). Several HPLC methods for identifying various mycotoxins in a number of cereals and cereal--based products have been validated by collaborative studies, and their performance characteristics, such as accuracy, repeatability, reproducibility, detection and quantification limits were established. These methods have been adopted as official or standard methods by the AOAC International or the European Standardization Committee (CEN). In particular, methods for measuring aflatoxins in maize (AOAC Official Method 991.31 and 2005.08), ochratoxin A in barley (2000.03), aflatoxin B_1 in baby food (2000.16), fumonisins B_1 and B_2 in maize flour and cornflakes (2001.04) that use IACs clean-up and HPLC-FD are approved as official methods by AOAC International (http://www.aoac.org). In addition, HPLC/IAC methods have been validated for the measurement of DON in cereals and cereal products, and ZEA in barley, maize, wheat flour, polenta and maize-based baby food (MacDonald et al., 2005a; MacDonald et al., 2005b).

Liquid chromatography coupled with mass spectrometry (LC-MS) has been used for many years, mainly as a technique for mycotoxin confirmation. At the present time, LC-MS and LC-MS/MS are the most promising techniques for the simultaneous screening, identifying and measuring of a large number of mycotoxins. Advances and recent trends in mycotoxin detection by LC-MS have been recently reviewed (Sforza et al., 2006; Songsermsakul and Razzazi-Fazeli, 2008). The following mycotoxins were examined: patulin, aflatoxins, ochratoxin A, zearalenone and its metabolites, trichothecenes and fumonisins. LC-MS/MS and Atmospheric Pressure Chemi-

cal Ionization (APCI) or Electro-Spray Ionization (ESI) interface was used for the simultaneous determination of the major type A- and type B-trichothecenes and ZEA in cereals and cereal-based products at trace levels (Berthiller et al., 2005). LC-APCI-MS/MS method using reversed phase SPE Oasis® HLB columns for extract clean-up has been recently developed for the simultaneous determination of NIV, DON, T-2 and HT-2 in cereals and cereal-based products (Lattanzio et al., 2008). The method was applied to a large number of cereals (wheat, barley and maize) and cereal-based foods (infant semolina, infant biscuits, bacon biscuits, cocoa wafers and coconut snacks) with detection limits in the μg/kg range. LC-ESI-MS/MS method has been recently developed for the simultaneous determination of major *Fusarium* toxins (DON, T-2, HT-2, FB_1, FB_2, ZEA) together with aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2) and OTA in maize, based on the use of new multi-toxin immunoaffinity columns, i.e. Mycodin1™ (Lattanzio et al., 2007). HPLC-MS/MS is also proved to be a powerful technique for the determination of masked mycotoxins, for example deoxynivalenol-glucosides, in wheat (Berthiller et al., 2007). Masked mycotoxins are mycotoxins conjugated to more polar substances, e.g. glucose, not detected by routine analytical methods, even though they can release their toxic precursors after hydrolysis. Accuracy, precision, and sensitivity of LC-MS methods may vary depending on the mycotoxin, matrix and instrument with the sensitivity of the method depending on the ionization technique used. Quantitative measurement of mycotoxins by LC-MS is often unsatisfactory due to matrix effects and ion suppression. Purification of extracts by MycoSep® or IACs is generally needed prior to MS detection (Lattanzio et al., 2009).

Immunological assays, such us ELISA, have become very popular in mycotoxin screening since the late 1970s (Pestka et al., 1995). In general, ELISA does not require clean-up procedures, and the extract containing the mycotoxin is analyzed directly. Even though they often lack accuracy at very low concentrations and are limited in the range of matrices examined, immunoassays provide fast, inexpensive screening assays. However, matrix interference or the presence of structurally related mycotoxins can interfere with the binding of conjugate and antibody, leading to mistakes in quantitative measurements of mycotoxins. ELISA kits should be used routinely only for the analysis of matrices that are extensively tested. Confirmatory analyses by more robust methods, e.g. HPLC with IAC clean-up or LC-MS, are required for the contamination levels that approach the legal limit. Several ELISA kits that use monoclonal or polyclonal antibodies against mycotoxins have been commercially developed for qualitative, semi-quantitative or quantitative analyses of the main known mycotoxins in cereal-based matrices. Some ELISAs have been validated by collaborative studies and adopted by the AOAC International as official methods for determination of aflatoxin B_1/total aflatoxins and ZEA in food and feed matrices (AOAC Official Methods No. 989.06, No. 990.32, No. 990.34, No. 991.45, No. 993.16 and No. 994.01). Nevertheless, the use of ELISAs to detect mycotoxins at contamination levels approaching the legal limits is inappropriate since these assays were validated at levels much higher than the legal limits. Direct ELISA has been validated for measu-

ring total fumonisins (i.e. sum of FB_1, FB_2 and FB_3) in maize at levels > 1.0 mg/kg (AOAC Official Methods No. 2001.06) with good precision.

Emerging technologies for mycotoxin analysis

Over the past years, rapid immunoassay-based tests have increasingly been used for the analyses of mycotoxins in cereals and cereal-based foods (Zheng et al., 2006; Goryacheva et al., 2007). Lateral flow devices (LFDs), also called immunochromatographic strip tests, are rapid immunoassays based on the interaction between specific antibodies, immobilized on a membrane strip, and antibody-coated dyed receptors, e.g. latex or colloidal gold, that react with analyte to form an analyte-receptor complex. LFDs have been developed for the most prevalent mycotoxins in cereals and are commercially available for the determination of AFs and FBs in maize, DON in wheat, and OTA, ZEA, T-2 and HT-2 in cereal grains. Portable photometric strip readers allow quantitative or semi-quantitative analysis (Krska and Molinelli, 2009).

Fluorescence polarization immunoassay (FPIA) is a simple technique that measures interactions between a fluorescently labeled antigen and a specific antibody in solution. FPIAs are developed for rapid determination of AFs, ZEA, FBs and DON, although low accuracy and sensitivity were observed when these assays were used with cereal samples (Nasir and Jolley, 2003; Maragos and Plattner, 2002; Chun et al., 2009). Recently, FPIA has been optimized for rapid determination of DON in durum and common wheat, semolina and pasta (Lippolis et al., 2006; Pascale et al., 2008b). The assay showed better accuracy and precision, with respect to a widely used HPLC-IAC method (MacDonald et al., 2005a), in the range of 100—2.000 μg/kg.

Fourier transform near-infrared (FT-NIR) spectroscopy and principal component analysis (PCA) have been recently used for the determination of DON content in durum and common wheat. A semi-quantitative model was developed to discriminate between blank and naturally contaminated wheat samples at 300 μg/kg (De Girolamo et al., 2009). A modified approach to evaluate DON content in scab-damaged wheat by using diffuse reflectance UV-vis spectroscopy was reported (Siuda et al., 2008). The applicability of NIR reflectance spectroscopy, combined with multivariate statistical methods, was evaluated for its ability to predict the incidence of fungal infection in maize and fumonisin B_1 content by analyzing 280 naturally and artificially contaminated maize samples, although the limit of detection of the method was not reported (Berardo et al., 2005). The advantages of these methods compared to other methods are the ease of operations, rapidity of analysis and non-destruction of samples.

Immunochemical biosensors that use surface plasmon resonance (SPR) or screen-printed carbon electrodes have been described for the detection of mycotoxins in cereals. Competitive SPR-based immunoassays have been recently described for the determination of DON in wheat, with or without

extract clean-up, showing a good correlation between DON concentration measured with biosensor and GC-MS, or HPLC-UV and HPLC-MS/MS as reference methods (P r i e t o - S i m o n et al., 2007). Competitive electrochemical ELISAs based on disposable screen-printed carbon electrodes have been developed for quantitative determination of ochratoxin A in wheat. The results from screen-printed carbon electrodes and HPLC/IAC clean-up methods for naturally contaminated wheat samples showed good correlation (A l a r c ò n et al., 2006).

CONCLUSION

Several analytical methods for the determination of major mycotoxins occurring in cereals and cereal-based products have been developed and continuously improved. Advantages and disadvantages of traditional and emerging methods are reported in Table 1. Among the traditional methods, immunoaffinity/MycoSep® column clean-up coupled with HPLC is the most frequently used technique for the measurement of main mycotoxins occurring in cereals and cereal-based products, although LC-MS/MS seems to be the most promising technique to be used in the future for multi-mycotoxins analysis. Various immunological methods, ELISA and other rapid antibody-based tests, are generally used for screening purposes, although these methods often require confirmatory analyses with more robust methods. Finally, few emerging technologies, which are often combined with immunochemical assays, have been proposed for rapid analysis of mycotoxins in cereals. Among these, FP immunoassay has the potential for reliable high-throughput analysis of DON in wheat and wheat-based products.

Tab. 1 — Advantages and disadvantages of traditional and emerging methods for mycotoxin analysis

Method	Advantages	Disadvantages
GC	Simultaneous analysis of mycotoxins, good sensitivity, may be automated (autosampler), provides confirmation (MS detector).	Expensive equipment, specialist expertise required, derivatization required, matrix interference problems, non-linear calibration curve, drifting response, carry-over effects from previous sample, variation in reproducibility and repeatability.
HPLC	Good sensitivity, good selectivity, good repeatability, may be automated (autosampler), short analysis times, official methods available.	Expensive equipment, specialist expertise required, may require derivatization.
LC/MS	Simultaneous analysis of mycotoxins, good sensitivity (LC/MS/MS), provides confirmation, no derivatization required.	Very expensive, specialist expertise required, sensitivity relies on ionization technique, matrix assisted calibration curve (for quantitative analysis), lack of internal standards.

ELISA	Simple sample preparation, inexpensive equipment, high sensitivity, simultaneous analysis of multiple samples, suitable for screening, limited use of organic solvents.	Cross-reactivity with related mycotoxins, matrix interference problems, possible false positive/negative results, confirmatory LC analysis required.
LFD	Rapid, no clean-up, no expensive equipment, easy to use, no specific training required.	Semi-quantitative (visual assessment), cross-reactivity with related mycotoxins, validation required for additional matrices.
FPIA	Rapid, no clean-up required, validated for DON in wheat.	Inconsistent with ELISA or HPLC analyses (except for DON), poor sensitivity in some cases, cross-reactivity with related mycotoxins, matrix interference problems.
IR spectroscopy	Rapid, non-destructive measurement, no extraction or clean-up, easy operation.	Expensive equipment, calibration model must be validated, knowledge of statistical methods, poor sensitivity.
Biosensors	Rapid, no clean-up procedure.	Cross-reactivity with related mycotoxins, extract clean-up needed to improve sensitivity, variation in reproducibility and repeatability.
MIP	Low cost, stable, reusable.	Poor selectivity.

GC = Gas Chromatography; HPLC = High Performance Liquid Chromatography; LC/MS = Liquid Chromatography/Mass Spectrometry; ELISA = Enzyme-Linked Immunosorbent Assay; LFD = Lateral Flow Device; FPIA = Fluorescence Polarization Immunoassay; IR = Infrared; MIP = Molecularly Imprinted Polymer.

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МЕТОДЕ ДЕТЕКЦИЈЕ МИКОТОКСИНА У ЖИТАРИЦАМА И ПРОИЗВОДИМА ОД ЖИТА

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Резиме

Методе анализе микотоксина у житарицама и производима од жита се састоје из три поступка: екстракције, пречишћавања (како би се уклониле интерференције екстракта и концентрисао аналит) и детекције/одређивања токсина (коришћењем одговарајућих аналитичких инструмената/технологије за анализу). Пречишћавање је кључни поступак у анализи микотоксина при његовим концентрацијама у траговима који обухвата примену екстракције чврстом фазом и мултифункционалне (нпр. MycoSep®) или имуноафинитетне колоне. Различите хроматографске методе се користе за квантитативно одређивање микотоксина, укључујући гасну хроматографију (GC) уз коришћење детектора са захватом електрона, пламено јонизационе детекторе или масену спектрометрију (углавном за трихотецене типа A) као и течну хроматографију високог учинка (HPLC) уз коришћење UV, DAJD, флуоресцентних и MS детектора. Избор методе зависи од матрикса и микотоксина који се анализира. Течна хроматографија у пару са масеном спектрометријом (LC-MS/MS) је техника која највише обећава у случају симултаног скенирања, идентификације и квантитативног одређивања великог броја микотоксина. Осим тога, комерцијална имунометријска испитивања, као што је ензимски имуносорпциони тест (ELISA), такође се често користе у сврхе скенирања. У последње време велик број метода које се базирају на новим технологијама могу се користити за анализу микотоксина, а међу њима су имунохроматографија (нпр. латерални проточни уређаји), имуно тестови са поларизацијом флуоресцентне емисије (FPIA), инфрацрвена спектроскопија (FT-NIR), полимери са молекуларним отиском (MIPs) и оптички биосензори.

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DIFFERENT MYCOTOXIN INACTIVATION APPLICATIONS AND THEIR INACTIVATION MECHANISMS

ABSTRACT: Control of mycotoxins is the need of the hour, since their occurrence in foods and feeds is continuously posing threats to both health and economics all over the world.

Besides the post-harvest preventive measures, it is important that suitable detoxification methods must be developed for inactivating or removing mycotoxins from the contaminated commodities, as the toxins are also produced by *Aspergillus flavus* and *A. parasiticus* even during pre-harvest stages of crop production.

Several physical and chemical detoxification methods developed so far have been critically discussed in different reviews for their advantages and limitations based on certain adopted strategies and specific criteria.

Understanding of mechanisms of mycotoxins detoxification by physical, chemical and microbiological methods will enable establishment of combined treatment procedures to effectively decontaminate, contaminated foods and feeds. Such treatment methods are expected to be cost effective and minimally deleterious to food constituents

KEY WORDS: mycotoxins, food, feed, methods, inactivation, detoxification

INTRODUCTION

Mycotoxins are toxic mould metabolites produced by toxigenic strains of different mould species. They have an important role in the occurrence of some human diseases such as liver cancer, chronic hepatitis and cirrhosis. When animals eat foodstuffs containing aflatoxin B1, these toxins are metabolized and excreted as aflatoxin M1 in their milk. Aflatoxin M1 is resistant to thermal inactivation and is not destroyed completely by pasteurization, autoclaving or other food processing procedures. The control of aflatoxins is the need of the hour, since their occurrence in foods and feeds is continuously posing threats to both health and economics all over the world. Since aflatoxin contamination is unavoidable, numerous strategies for its detoxification have been proposed. These include physical methods of separation, thermal inactivation, irradiation, solvent extraction, adsorption from solution, microbial inactivation and fermentation. Chemical methods of detoxification are also practiced

as a major strategy for effective detoxification. Besides the post-harvest preventive measures, suitable detoxification methods are developed for inactivating or removing aflatoxins from the contaminated commodities, since toxins are also produced by *Aspergillus flavus* and *A. parasiticus* even during the pre-harvest stages of crop production. Understanding of mechanisms of aflatoxin detoxification by physical, chemical and microbiological methods will enable the establishment of combined treatment procedures to effectively decontaminate contaminated foods and feeds. Such treatment methods are expected to be cost effective and minimally deleterious to food constituents.

Inactivation strategies

Several physical and chemical detoxification methods developed so far have been critically discussed in different reviews for their advantages and limitations based on certain adopted strategies and specific criteria. Detoxification by microbiological means is also reviewed with respect to the status on potential microorganisms and their enzymes that can degrade aflatoxins to less toxic or innocuous end products. Understanding the mechanisms of aflatoxin detoxification by physical, chemical and microbiological methods will enable the establishment of combined treatment procedures to effectively decontaminate contaminated foods and feeds. Such treatment methods are expected to be cost effective and minimally deleterious to food constituents (B a s a p p a and S h a n t a, 1996).

Application of ammonia

Ammoniation of corn, peanuts, cottonseed and meals for the alteration of toxic and carcinogenic effects of mycotoxin contamination has been the subject of intense research effort by scientists in various government agencies and universities worldwide. Engineers have devised workable systems of treatment of whole seeds, kernels or meals; chemists have identified and characterized the products formed from the reaction of aflatoxin B1 with ammonia, with and without meal matrix; biochemists have studied the biological effects of these compounds in model systems; and nutritionists have studied animal responses to rations containing ammoniated and non-ammoniated components. The results of aflatoxin/ammonia decontamination research demonstrate the efficiency and safety of ammoniation as a practical solution to aflatoxin detoxification in foods and animal feeds (P a r k, 1993).

Corn is, all over the world, frequently contaminated with the fungus *Fusarium moniliforme* that produces toxic fumonisins. However, ammonia detoxifies effectively aflatoxins in corn and cottonseed. Since corn can be contaminated by both fumonisins and aflatoxins, application of ammoniation of corn cultured with, or naturally contaminated by *F. moniliforme*, showed that Fumonisin B1 levels in the culture material and in naturally contaminated corn were reduced by 30 and about 45%, respectively, by the treatment. Despite the

apparent reduction in fumonisin content, the toxicity of the culture material in rats was not altered by ammoniation. Reduced weight gains, elevated serum enzyme levels and histopathological lesions, typical of *F. moniliforme* toxicity, occurred in rats fed with either ammoniated or non-ammoniated culture material. Atmospheric ammoniation of corn does not appear to be an effective method for the detoxification of *F. moniliforme*-contaminated corn (N o r r e d et al., 1991).

Although there was no significant change in dietary intake, body weight gain, and feed conversion ratio in chickens fed with ammonia treated aflatoxin contaminated maize, these parameters were suppressed in birds fed with aflatoxin-containing diet. These data suggest that replacement of aflatoxin-containing maize with ammoniated grains can significantly suppress aflatoxicosis, leading to an improvement in production parameters in broilers (A l l a m e h et al., 2005).

Rice, a wide spread cereal used for human and animal nutrition, is susceptible to aflatoxin contamination in the field and during storage. Therefore, the goal of the research was the evaluation of the efficacy and permanence of the ammoniation process through high pressure/high temperature (HP/HT) and atmospheric pressure/moderate temperature (AP/MT) conditions applied to rice samples artificially contaminated with aflatoxin B1. For this purpose, a 2(k) design was drawn up with temperature, rice moisture and the process time as its variables. Under both sets of conditions, aflatoxin B1 concentration was reduced in a range of 90—100%. In conclusion, the process efficacy and permanence were achieved through the use of high temperature and long process time for both sets of conditions (HP/HT and AP/MT), respectively (T r u j i l l o and Y e p e z, 2003).

Chlorine dioxide

The efficacy of chlorine dioxide (ClO_2) in the detoxification of trichothecene mycotoxins verrucarin A and roridin A, was evaluated. In the first experiment, verrucarin A (1, 5 or 10 mu g) and roridin A (5 or 10 mu g) were each inoculated onto square-inch sections of glass, paper, and cloth, and exposed to 1000 ppm of ClO_2 for either 24 or 72 h at room temperature. In the second experiment, verrucarin A and roridin A (1 or 2 ppm in water) were treated with 200, 500 or 1000 ppm ClO_2 for up to 116 h at room temperature. The results of the first experiment showed that ClO_2 treatment had no detectable effect on either toxin. In the second experiment, both toxins were completely inactivated at all tested concentrations in less than 2 h after treatment with 1000 ppm ClO_2. For verrucarin A, the effect was seen at 500 ppm level, but this effect was not as strong as that observed at 1000 ppm level. Roridin A toxicity was decreased after treatment with 200 and 500 ppm ClO_2, but this was not significant until the 24-hour exposure time was reached. These data show that ClO_2 (in solution) can be effective for detoxification of roridin A or verrucarin A at selected concentrations and exposure times (W i l s o n et al., 2005).

Citric acid

Chemical inactivation of aflatoxin B1(AFB1) and aflatoxin B2(AFB2) in maize grain by means of 1 N aqueous citric acid was confirmed by the AFLATEST™ immunoaffinity column method, high performance liquid chromatography (HPLC), and the Ames test (*Salmonella*-microsomal screening system). The AFLATEST™ assay showed that aflatoxins in the maize grain, with an initial concentration of 29 ng/g, were completely degraded, and 96.7% degradation occurred in maize contaminated with 93 ng/g when treated with the aqueous citric acid. Aflatoxin fluorescence strength of acidified samples was much weaker than the untreated samples, when observed in HPLC chromatograms (M e n d e z et al., 2005).

Sulfhydryl compounds

Most food toxicants have specific groups responsible for their deleterious effects. Modifying such sites with specific a-acids, peptides, and proteins lessens their toxicity. Sulfhydryl (thiol) compounds, such as cysteine, N-acetylcysteine, reduced glutathione, and mercaptopropionylglycine interact with disulfide bonds of plant protease inhibitors and lectins via sulfhydryl-disulfide interchange and oxidation-reduction reactions. Such interactions with inhibitors from soybeans and lectins from lima beans facilitate heat inactivation of the potentially toxic compounds, resulting in beneficial nutritional effects. Related transformations of protease inhibitors in soy flour are also beneficial. Since thiols are potent nucleophiles, they have a strong affinity for unsaturated electrophilic centers of several dietary toxicants, including aflatoxins, sesquiterpene lactones, such as elephantropin and parthenin, urethane, carbonyl compounds, quinones, and halogen compounds. Such interactions may be used *in vitro* to lower the toxic potential of the diet, and *in vivo* for prophylactic and therapeutic effects against oxidative damage. A number of examples are cited to illustrate the concepts and mechanisms of using sulfur amino acids to reduce the antinutritional and toxic manifestations of food ingredients (F r i e d m a n, 1994).

Feed additives

The possible protective effect of four feed additives against the toxic effects of T-2 toxin in growing broiler chickens was investigated in randomized trial consisting of six dietary treatments (control with no T-2 toxin or feed additive added, 2 ppm T-2 toxin alone, 2 ppm T-2 toxin plus 2.0 g/kg Mycofix, 2 ppm T-2 toxin plus 2.0 g/kg Mycosorb, 2 ppm T-2 toxin plus 2.5 g/kg MycoAd, and 2 ppm T-2 toxin plus 3.0 g/kg Zeolex). When no feed additive was included, 2 ppm dietary T-2 toxin significantly decreased BW and increased feed: gain ratio. When 2.0 g/kg Mycofix were added to the diet, the feed additive protected against the adverse effects of T-2 toxin on BW, BW gain, and feed: gain ratio. However, no protection against the adverse effects of T-2

toxin on final BW and BW gain were obtained by the supplementation of any of the other 3 feed additives. The results of trial indicate that the only feed additive capable of counteracting the adverse effects on performance caused by the dietary administration of 2 ppm T-2 toxin was the additive based on the enzymatic inactivation of the 12,13-epoxide ring of the trichothecenes (Mycofix). This study also confirms the previous reports showing that aluminosilicates are not effective against trichothecene mycotoxins (D i a z et al., 2005).

Aqueous extract of ajowan seeds was found to contain an aflatoxin inactivation factor (IF). Approximately 80% reduction in total aflatoxin content over the controls was observed. This observed phenomenon of reduction in total toxin was referred to as toxin inactivation. It was discovered that the temperature influenced the rate of toxin inactivation. At 45°C, toxin inactivation was rapid during the initial 5 hours, after which it decreased. The IF was found to retain considerable activity even after boiling and autoclaving, indicating partial heat stability. Toxin decontamination in spiked corn samples could be achieved using IF. This study emphasizes the potential of ajowan IF in aflatoxin removal from contaminated food commodities. However, the biological toxicity, if any, of the IF inactivated aflatoxins needs to be confirmed (H a j a r e et al., 2005).

Biological detoxification

Some toxin-producing fungi are able to degrade or transform their own products under suitable conditions. Pure cultures of bacteria and fungi which detoxify mycotoxins were isolated from complex microbial populations by screening and enrichment culture techniques. Genes responsible for some of the detoxification activities were cloned and expressed in heterologous hosts. The detoxification of aflatoxins, cercosporin, fumonisins, fusaric acid, ochratoxin A, oxalic acid, patulin, trichothecenes, and zearalenone by pure cultures were also reported (K a r l o v s k y, 1999).

Extrusion process

Cottonseed is an economical source of protein and is commonly used in balancing livestock rations. However, its use is typically limited by protein, fat, gossypol, and aflatoxin contents. The extrusion temperature study showed that aflatoxin levels were reduced by an additional 33% when the cottonseed was extruded at 160°C as compared to 104°C. Furthermore, the multiple-pass extrusion study indicated that aflatoxin levels were reduced by an additional 55% when the cottonseed was extruded four times as compared to one time. Total estimated reductions of 55% (three stages of processing at 104°C), 50% (two stages of processing at 132°C), and 47% (one stage of processing at 160°C) were obtained from the combined equations. If the extreme conditions (four stages of processing at 160°C) of the evaluation studies are applied to

the combined temperature and processing equation, the resulting aflatoxin reduction would be 76% (Michael et al., 2002).

Traditional nixtamalization and an extrusion method for making dough (masa) for corn tortillas, which requires the use of lime and hydrogen peroxide, were evaluated for the detoxification of aflatoxins. The traditional nixtamalization process reduced levels of aflatoxin B1 (AFB (1)) by 94%, aflatoxin M1 (AFM (1)) by 90% and aflatoxin B1-8,9-dihydrodiol (AFB (1)-dihydrodiol) by 93%. The extrusion process reduced levels of AFB(1) by 46%, AFM(1) by 20% and AFB(1)-dihydrodiol by 53%. Extrusion treatments with 0,0.3 and 0.5% lime reduced AFB (1) levels by 46,74 and 85%, respectively. The inactivation of AFB (1), AFM (1), and AFB (1)-dihydrodiol in the extrusion process using lime together with hydrogen peroxide showed higher elimination of AFB(1) than the treatments with lime or hydrogen peroxide alone. The extrusion process with 0.3% lime and 1.5% hydrogen peroxide was the most effective process of aflatoxin detoxification in corn tortillas, but the high level of those reagents negatively affected the taste and aroma of the corn tortilla as compared with tortillas elaborated by the traditional nixtamalization process (Elias et al., 2002).

Samples of corn flour experimentally contaminated with aflatoxin B1 (AFB1) (50 ppb) and deoxynivalenol (DON) (5 ppm) were extruded. The effects of three extrusion variables (flour moisture, extrusion temperature and sodium metabisulphite addition) were analyzed according to a two-level factorial design. The process was effective for the reduction of DON content (higher than 95%) under all the conditions assessed, but was only partially successful (10—25%) for the decontamination of AFB1.

The results show that extrusion cooking is effective for the inactivation of DON, but is of a limited value for AFB1, even when metabisulphite is added. More severe extrusion conditions are needed for the detoxification of AFB1. As DON contamination occurs mainly in the field, prior to harvesting, and that of AFB1 is normally produced during grain storage, maize is often contaminated with DON and not with AFB1. Under these conditions, the described extrusion process can be used for the detoxification of DON. The addition of sodium metabisulphite did not significantly affect the inactivation of AFB1. Extrusion cooking is therefore an appropriate treatment for vomitoxin-contaminated maize in the countries where, due to the prevailing conditions, these are the only toxins present (Cazzaniga et al., 2001).

Miscellaneous

Aflatoxins are also sensitive to UV light and gamma radiation. Exposure of artificially contaminated milk to UV light inactivated 3.6—100% of AFM1, depending on the exposure time. In the case of dried figs artificially contaminated with AFB1, the toxin level was reduced by 45.7%. Toxicity of a peanut meal contaminated with AFB1 was reduced by 75 and 100% after the irradiation by gamma rays at dose of 1 and 10 kg, respectively.

Solar energy is also widely used in the decrease of the amount of aflatoxins from 30—80% in peanut cakes, flakes peanut oil, and olive oils in different parts of the world. High hydrostatic pressure application is another method of inactivating mycotoxins present in food, but the pressure exciting 500 MPa has detrimental effects on the food itself.

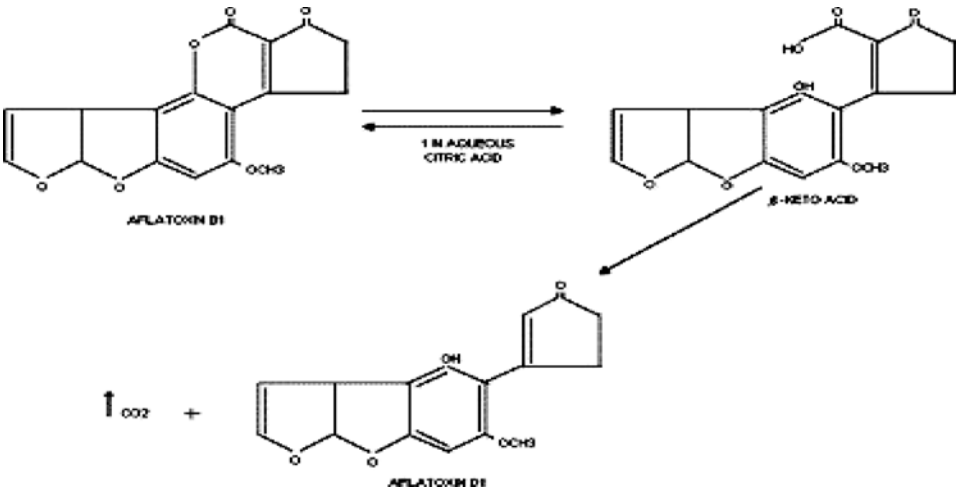

Fig. 1 — Proposed mechanism for the acidification of aflatoxin B_1 to produce aflatoxin D_1

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СТРАТЕГИЈЕ КОНТРОЛЕ И МЕХАНИЗМИ ИНАКТИВАЦИЈЕ МИКОТОКСИНА

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Резиме

Контрола микотоксина је ургентна потреба данашњице јер њихово присуство у храни и сточној храни угрожава здравље и економију у светским размерама.

Поред примене послежетвених превентивних мера, неопходно је развијати и одговарајуће методе детоксификације које служе за инактивацију и уклањање микотоксина из контаминиране хране, с обзиром на чињеницу да се токсини стално производе, чак и у току преджетвених фаза у производњи усева, нарочито од стране родова *Aspergillus flavus* и *A. parasiticus*.

Неки, данас познати, методи физичке и хемијске детоксификације критички су разматрани у више прегледних радова, са становишта њихових предности и ограничења а на основу одређених усвојених стратегија и неколико специфичних критеријума.

Разумевање механизама детоксификације микотоксина физичким, хемијским и микробиолошким методима ће омогућити успостављање процедура комбинованих третмана који могу ефикасно да деконтаминирају угрожену храну и сточну храну. Очекује се да ови третмани буду економични и да минимално утичу на састојке хране.

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COMPARISON OF ENZYME LINKED IMMUNOASSAY AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR DETERMINATION OF FUMONISIN IN DRIED FIGS

ABSTRACT: The occurrence of fumonisin in dried figs was investigated by Enzyme Linked Immunoassay (ELISA) and High Performance Liquid Chromatography (HPLC). Total fumonisins (FB_1, FB_2, FB_3) in dried figs were determined using ELISA, whereas only fumonisin B_1 (FB_1) was determined by HPLC. In the period 2003—2004, one hundred and fifty five dried fig samples were taken during their drying in 7 different districts in the Aegean Region. Among a total of 115 samples, the incidence of total fumonisin in the dried figs was 82% within the range of 0.16 — 108.34 μg/g when determined by ELISA. In comparison, FB_1 was detected in 86 samples (74.8%) within the range between 0.046 and 3.649 μg/g by HPLC.

Correlation between ELISA and HPLC methods was observed for all samples. However, no correlation between methods was recorded for the samples with less than 1 μg/g Fumonisin B_1 level (obtained by HPLC). Although there was a correlation between methods for all the samples, fumonisin levels obtained by ELISA were much higher than those obtained by HPLC. False positive results were obtained by ELISA in 11 out of 115 dried fig samples.

The results indicated that ELISA can be used as a screening method for determining the occurence of fumonisin in dried figs.

KEYWORDS: dried figs, enzyme linked immunoassay, fumonisin, high performance liquid chromatography

INTRODUCTION

Fumonisins are group of toxic and carcinogenic mycotoxins produced by *Fusarium verticilloides* and *Fusarium proliferatum*, fungi that commonly contaminate maize (Jackson and Jablonski, 2004; Scaff and Scussel, 2004). Among the fumonisin derivatives, FB_1 is the most common one and constitutes about 70—80% of the total fumonisin content of *F. verticilloides*

cultures and naturally contaminated foods. Fumonisin B_2 accounts for 15—25% of the total fumonisin, while fumonisin B_3 accounts for 3—8% (Rheeder et al., 2002).

Fumonisins induce several diseases in animals, such as equine leukoencephalomalacia in horses, porcine pulmonary oedema. A relationship between consumption of fumonisin containing maize and incidence of esophageal cancer in humans in certain areas of the world has been determined (Nelson et al., 1992; Castellá et al., 1999; Scaff and Scussel, 2004). According to the animal studies and epidemiological studies on humans, fumonisins have been classified as a possible human carcinogen (Group 2B) by IARC (IARC,1993).

Fumonisins can be determined by thin-layer chromatography (TLC) and liquid chromatography (LC), mass spectroscopy (MS), gas chromatography, immunochemical methods and capillary zone electrophoresis (CZE), but they are mainly determined by liquid chromatographic techniques. Enzyme Linked Immunosorbent Assay (ELISA) method is a useful tool for rapid detection of mycotoxins.

Dried fig is one of the most widely produced fruits in the world. It is an important agricultural product following raisin and dried apricot among Turkish dried fruit exports. Turkey is the first among the dried fig exporting countries with approximately 52.600 tons of dried figs in 2005, which is equivalent to 52% of the world's dried fig exports (FAO, 2007). Dried figs are a high risk commodity in terms of mycotoxins. The occurrence of aflatoxins (Iamanaka et al., 2007), ochratoxin A (Karbancıoglu-Güler and Heperkan, 2008), as well as fumonisin B_1 (Karbancıoglu-Güler and Heperkan, 2009) in dried figs were previously reported.

The purpose of this study was to compare the detection of fumonisin with Enzyme Linked Immunoassay (ELISA) and high performance liquid chromatography (HPLC) methods and to investigate the presence of fumonisin in dried figs. The fumonisin incidence and levels were determined in the Turkish dried figs, collected during the drying stage in seven different districts located in the Aegean Region in the period of two years.

MATERIAL AND METHODS

Samples

A total of 115 samples of dried figs were collected from the orchards in the Aegean Region during drying stage in 2003 and 2004 harvests. The samples were stored in polyethylene bags at –18°C until analysis. They were thawed and brought to room temperature before sample preparation. Approximately 250 g of each sample were minced with meat mincer, and then blended with 5 parts dried figs and 4 parts tap water (w/w). Representative sub-samples (45 g) were used in the analysis.

Extraction and cleanup

Methods of Romer Labs. (Anonymous, 2004), AOAC 995.15 (AOAC, 2000) and TS EN 13585 (TSE, 2002) developed for corn and corn products were modified for the extraction and determination of fumonisin in dried figs. A sample of dried fig slurry (45g ± 0,2 g) was blended in Waring Blender with 100 ml methanol: water (3:1; v/v) for 5 min. The pH of the mixture was adjusted to 6—9 with 0,1M NaOH after filtration through Whatman No.4 filter paper. A 10 ml aliquot was diluted with 10 ml methanol: water (3:1; v/v) mixture. 20 ml of diluted extract was cleaned up with conditioned MultiSep 211 Fum columns (Romer Labs, Union, MO) with a flow rate of < 2 ml/sn. The column was conditioned by the successive passage of 5 ml of methanol and 5 ml of methanol/water (3:1). In order to eliminate the impurities, the column was washed with 8 ml of methanol/water (3:1) and 3 ml methanol. Fumonisin was eluted from the column with methanol: acetic acid (99:1; v/v, 10 mL). The eluate was evaporated to dryness under a gentle stream of nitrogen at 60°C.

ELISA

The residue was dissolved in 1 ml of methanol:water (3:1, v/v), and 200 μl of diluted extract was applied to the ELISA plate (Romer Lab. AgraQuant® 1—5) in order to determine the total fumonisin content. Each sample and standards were applied in duplicate. Enzyme immunosorbent assay (ELISA) was performed according to the manufacturer's instructions. The intensity of the resulting yellow colour was measured in ELISA plate reader and evaluated according to the RIDAWIN program. The limit of detection was 0.1 mg/g.

HPLC

The residue was dissolved in 200 μl of methanol:water (3:1, v/v) for HPLC analysis. A 25 μl aliquot of this solution was derivatized with 225 μl of orthophthaldialdehyde (OPA) solution. Fumonisin-OPA derivatives were analyzed using a reversed-phase HPLC/fluorescence detection system within 1—2 minutes. Sample volumes of 20 μl were injected manually. The liquid chromatograph was an Agilent Technologies 1100 system equipped with a fluorescence detector set at an excitation wavelength of 335 nm and emission wavelength of 440 nm, quaternary pump, a vacuum degasser, the Rheodyne injector with a 20 μl loop. Data were processed by Chemstation 3D software. The separation was achieved at room temperature on ODS-Hypersil C18 reversed phase column (Supelco 250 mm x 4.6 mm, 5 μm particle size) with 1 ml/min flow rate. Methanol-0.1M $NaH_2PO_4 \cdot H_2O$ (77:23; v/v) solution adjusted to pH 3.35 with orthophosphoric acid was used as a mobile phase. The limit of detection was 0.1 μg/g.

RESULTS AND DISCUSSION

ELISA and High Performance Liquid Chromatography (HPLC) methods for the determination of fumonisins in dried figs were compared. Fumonisin analyses have been carried out in this study on naturally dried figs collected from orchards before any treatment. The distribution of fumonisin in dried figs is given in Table 1. The results obtained during the two years indicate that fumonisin presence is a potential risk in dried figs. Among a total of 115 dried fig samples, the incidence of total fumonisin in dried figs was 82% within the range of 0.16 — 108.34 μg/g, as it was determined by ELISA. The highest incidences of fumonisin positive samples were obtained within the range of 1.0—5.0 μg/g. 16.5% of the dried fig samples had fumonisin level exceeding 20 μg/g. In comparison, HPLC detected FB_1 in 86 samples (74.8%) within the range of 0.046 and 3.649 μg/g. FB_1 contamination was most frequently detected within the range of 0.046—0.100 μg/g. The levels of fumonisin determined by ELISA were higher than those determined by HPLC.

Tab. 1 — Frequency of fumonisin content according to the ELISA and HPLC methods in dried figs

Fumonisin levels (μg/g)	ELISA Total fumonisin		HPLC FB_1 levels	
	Number of samples	Frequency (%)	Number of samples	Frequency (%)
< 0.046[1]			29	25.2
< 0.1[2]	21	18.3	36	31.3
0.1—0.5	3	2.6	35	30.4
0.5—1.0	5	4.3	4	3.5
1.0—2.0	8	7.0	6	5.2
2.0—3.0	9	7.8	2	1.7
3.0—4.0	6	5.2	1	0.9
4.0—5.0	8	7.0	—	—
5.0—10.0	27	23.5	—	—
10.0—20.0	13	11.3	—	—
> 20	19	16.5	—	—

1: Limit of detection for HPLC
2: Limit of detection for ELISA

The levels of fumonisin determined by HPLC and ELISA were plotted in Figure 1 and the correlation between the two methods was investigated. Correlation between ELISA and HPLC methods was observed for all samples (R = 0.932). However, correlation coefficient between the methods was determined as 0.573 for the samples with less than 1 μg/g Fumonisin B_1 level (obtained by HPLC). Correlation coefficient was calculated as 0.905 for the samples containing above 1 μg/g fumonisin B_1. Although there was a correlation between the methods for samples above 1 μg/g, fumonisin levels obtained by ELISA were much higher than those obtained by HPLC. The ELISA results for some samples were up to 400-fold higher than HPLC results. Overestimation of the fumonisin content in ELISA has been also reported (P e t s k a

et al., 1994; O n o et al., 2000). ELISA was used to determine total fumonisin (FB_1, FB_2, FB_3) level in dried figs, whereas HPLC was used to determine only fumonisin B_1 (FB_1). Since Rheeder et al. (2000) had previously reported that FB_1 constitutes about 70—80% of the total fumonisin content of F. verticilloides cultures, the differences between methods may not be associated with FB_2 and FB_3 levels in dried figs.

Although solid phase extraction cartridge was used for cleaning in the ELISA, overestimated fumonisin levels in dried figs may be related to the high concentration of methanol in dissolving solvent. Hence, it was reported that solvent and matrix effect could be decreased by diluting the extract with phosphate buffer or distilled water. O n o et al. (2000) showed that extract dilution decreased the ELISA/HPLC ratio and reduced the matrix effect. Moreover, false positive results were obtained by ELISA in 11 out of 115 dried fig samples, and false negative results in 3 out of 115 samples. False positive and false negative results in ELISA were also reported previously (P e t s k a et al., 1994; O n o et al., 2000). O n o et al. (2000) reported that false negative results might be related to lower detection limit of HPLC compared to ELISA.

The results presented here showed that ELISA can be used as a screening method for determining the occurence of fumonisins in dried figs. The ELISA results should be confirmed by chromatographic methods, such as HPLC, to eliminate the false positive and false negative results.

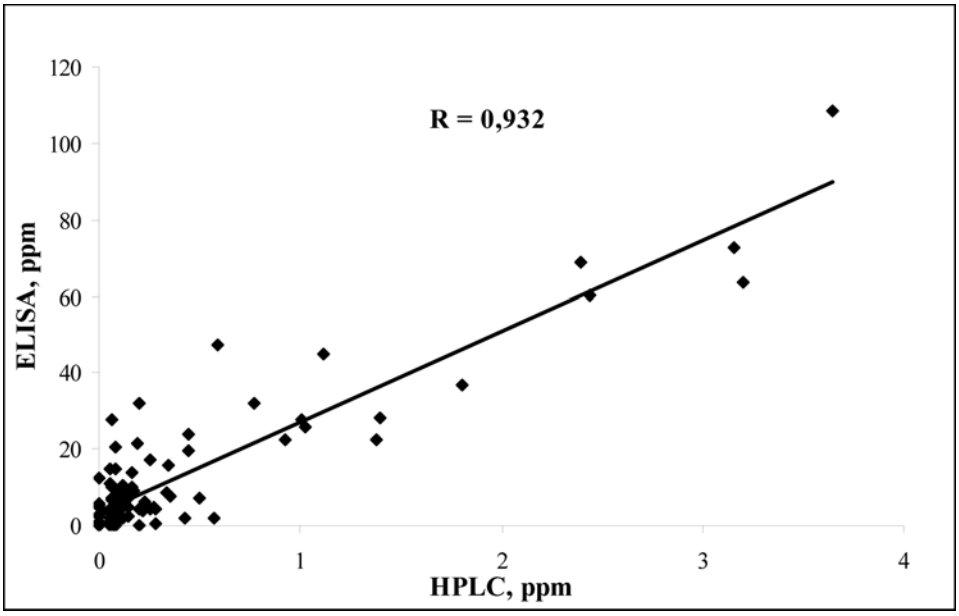

Fig. 1 — Correlation between ELISA and HPLC methods for the analysis of fumonisin in dried figs

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ПОРЕЂЕЊЕ ЕНЗИМСКОГ ИМУНОТЕСТА И ТЕЧНЕ ХРОМАТОГРАФИЈЕ ВИСОКЕ ПЕРФОРМАНСЕ ЗА ОДРЕЂИВАЊЕ ФУМОНИЗИНА У СУВИМ СМОКВАМА

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Резиме

Присуство фумонизина у сувим смоквама испитивано је помоћу ензимског имуносорпционог теста (ELISA) и високо перформансне течне хроматографије (HPLC). Применом теста ELISA утврђено је присуство свих фумонизина (FB1, FB2, FB3) у сувим смоквама, док је HPLC регистровао присуство само фумонизина B1 (FB1). У периоду између 2003. и 2004. године 155 узорака сувих смокава из 7 различитих области Егејског региона је узето током сушења. Од укупно 115 узорака, применом теста ELISA, присуство фумонизина је утврђено у 82% узорака, са концентрацијама у опсегу 0.16—108.34 μg/g. Применом HPLC метода, FB1 је регистрован код 86 узорака (74.8%), у концентрацијама од 0.046 до 3.649 μg/g.

Корелација између ELISA и HPLC метода утврђена је код свих узорака. Међутим, корелација између ове две методе није утврђена код узорака који су садржали мање од 1 μg/g фумонизина B1 (измереним помоћу HPLC). Иако је корелација између ова два метода утврђена код свих испитиваних узорака, показано је да се помоћу теста ELISA региструју много више концентрације фумонизина него применом HPLC метода. Применом ELISE, лажни позитивни резултати су добијени код 11 од укупно 115 узорака сувих смокава.

Резултати показују да се ELISA може користити као метод провере присуства фумонисина код сувих смокава.

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IDENTIFICATION OF MYCOBIOTA IN SERBIAN SLAUGHTERHOUSES

ABSTRACT: Mould growth within slaughterhouses is not acceptable and is considered as both economic and aesthetic problem. Aim of this paper was to determine filamentous moulds in two slaughterhouses by investigating air, water, environment area and equipment. There were a total of 100 samples collected. According to Samson (2004), 6 genera were determined among which *Penicillium* and *Aspergillus* were the most frequent. Three *Penicillium* species were identified; the most frequent were *P. brevicompactum* and *P. solitum.* The presence of *P. verrucosum* was not established. The presence of mycotoxigenic moulds, such as *P. brevicompactum*, indicates possible contamination of environmental area with mycophenolic acid (MPA). Results of investigation indicated that more comprehensive survey should be made in order to get a better insight of mycobiota in slaughterhouses in Serbia.

KEY WORDS: moulds, slaughterhouses, contamination

INTRODUCTION

Fungi are ubiquitously distributed and they contaminate meat and meat products which can further lead to meat spoilage. Contaminated meat also presents a health risk due to production of mycotoxins. The main sources of moulds contaminating carcasses are air, water, walls and floors of slaughterhouses (Refai et al., 1993). Refai et al. (1993) and Mansour (1986) reported air as a source of *Penicillium* and *Aspergillus*. Mansour et al. (1986) and Refai et al. (1993) reported that *Aspergillus, Penicillium, Cladosporium* and *Mucor* were frequently isolated from the floors and walls of slaughterhouses. Aim of this paper was to determine the occurrence of filamentous moulds within two slaughterhouses by investigating air, water, environment area and equipment in order to get a better insight of mycobiota in slaughterhouses in Serbia.

MATERIAL AND METHODS

The investigation was carried out in two beef slaughterhouses in central Serbia, in mid-autumn. A total of 100 samples were taken, fifty samples from each slaughterhouse. Ten samples of air, water, floors, walls and equipment were analyzed mycologically on the plates of Dichloran-Rosebengal-Chloramphenicol-agar medium. In order to investigate the presence of spores in air, exposed plate method was used, with each plate being exposed to the air of the slaughterhouse for 1 minute. For the water-borne moulds, 1 ml of water from water line within slaughterhouse was spread on each plate. Floor and wall area of 100 cm^2 each was swabbed using template. All plates were incubated at the temperature of 25°C for 7 days. Fungi were identified from macro- and microscopic features according to the criteria of Samson (2004) and Pitt (1988).

RESULTS AND DISCUSSION

The obtained data from the slaughterhouses were joined. The results of this study are presented in Table 1 and Figures 1 and 2. A total of six genera and eleven species of moulds have been isolated from environmental swabs. As it can be seen from the table, the most frequently isolated genera were *Penicillium* spp. and *Aspergillus* spp. Three *Penicillium* species were identified; the most frequent were *P. brevicompactum* and *P. solitum.* In this study we did not determine the production of mycotoxins. However, there were a couple of them reported to be toxigenic (Ostrý, 2001). The presence of mycotoxigenic moulds, such as *P. brevicompactum*, indicates a possibility of contamination of environmental area by mycophenolic acid (MPA). Presence of *P. verrucosum* was not established. There were no moulds in the water from the slaughterhouse pipelines which collides with the findings of Ismail (1995). Regarding the equipment swabs, *P. chrysogenum*, *P. brevicompactum* and *Cladosporidium* spp. were isolated in 2, 7 and 91% of the cases, respectively. However, members of the genus *Penicillium* and *Aspergillus* are reported to produce the widest range of mycotoxins, among which are ochratoxin A, aflatoxins, patulin, citrinin, citreoviridin, griseofulvin, rubratoxin, and penicillic, cyclopiazonic, secalonic, or mycophenolic acids. Regarding *Cladosporium* spp. and *Mucor* spp., no report on their ability for mycotoxin production in meat has been published so far (Ostry and Ruprich, 2001).

Tab. 1 — Species of moulds isolated from environmental area

Species	Incidence percentage			
	Air	Water	Floor	Walls
Penicillium brevicompactum	19	0	8	2
Penicillium solitum	34	0	28	
Penicillium chrysogenum	5	0	7	3
Aspergillus flavus	17	0	18	
Aspergillus clavatus	0	0	2	

Aspergillus terreus	2	0	8	32
Cladosporium sphaerospermum	8	0	5	20
Cladosporium cladosporioides	5	0	2	18
Cladosporium herbarum	2	0	4	25
Scopulariopsis brevicaulis	0	0	2	
Alternaria alternata	2	0	7	
Mucor racemosus	6	0	9	

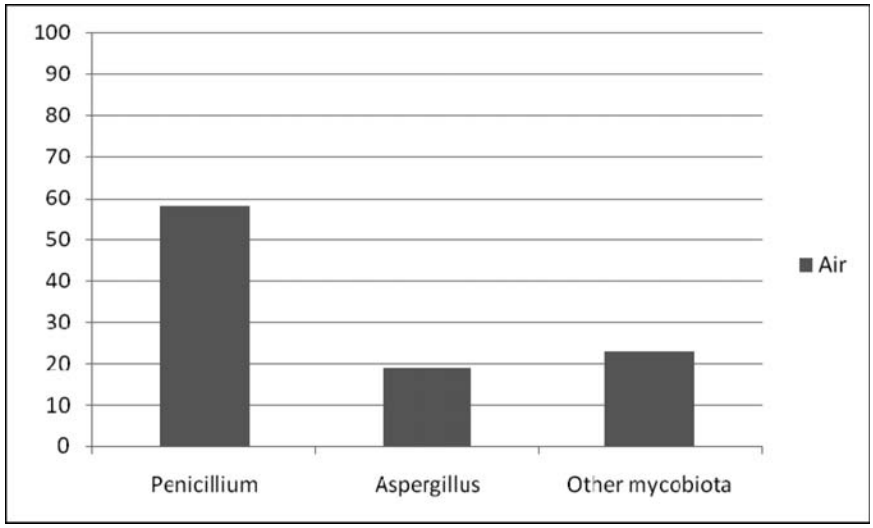

Fig. 1 — Share of *Penicillium* spp. and *Aspergillus* spp. in mycopopulation isolated from air

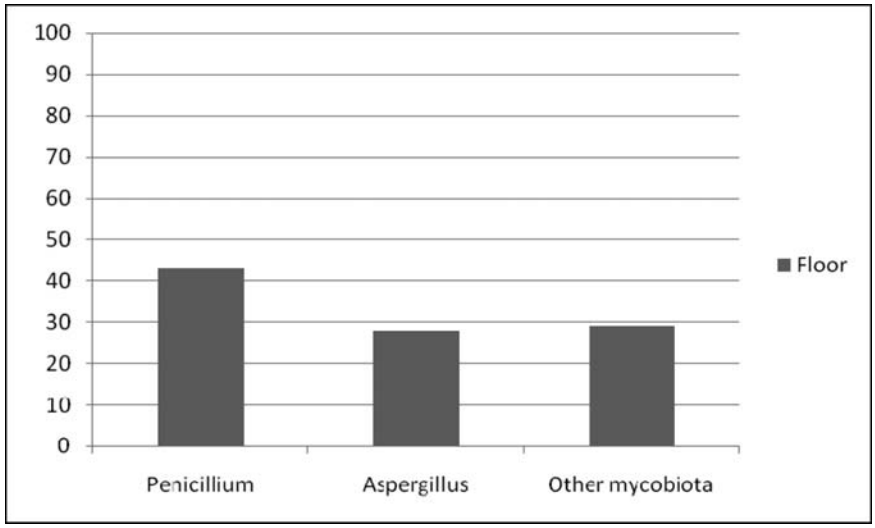

Fig. 2 — Share of *Penicillium* spp. and *Aspergillus* spp. in mycopopulation isolated from floor

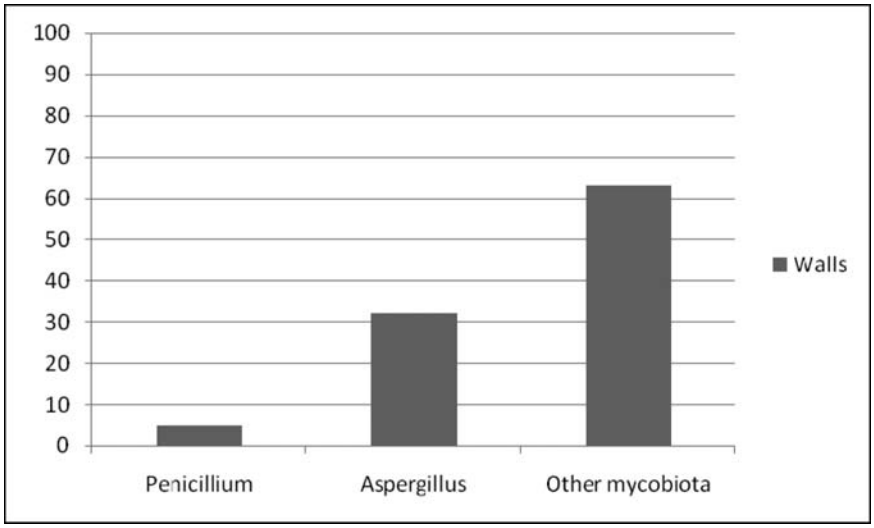

Fig. 3 — Share of *Penicillium* spp. and *Aspergillus* spp. in mycopopulation isolated from walls

CONCLUSION

The spores of moulds are always present in the environment enabling their survival even in extreme conditions. Therefore, it is practically impossible to eliminate them from food. The most frequent genera were *Penicillium* spp. and *Aspergillus* spp. There were no moulds in the water from the slaughterhouse pipelines. In general, the conditions during the slaughtering (relative humidity, air circulation) are suitable for cross-contamination and development of microscopic filamentous fungi on beef carcasses. Therefore, the prevention of mould development in the meat of slaughtered animals, as well as in the manufacturing rooms, stores and shops is of great importance in order to avoid the risk of mycotoxin production.

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ИДЕНТИФИКАЦИЈА МИКОБИОТА У КЛАНИЧНИМ ОБЈЕКТИМА У СРБИЈИ

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Резиме

Појава плесни у кланичним објектима није пожељна и сматра се економским и естетским проблемом. У циљу детерминације микобиота у два кланична објекта, ваздух, вода, радне површине и опрема испитани су на присуство филаментозних плесни. Испитано је укупно 100 узорака. Идентификацијом по Самсону утврђено је присуство 6 родова, а најчешћи су били *Penicillium* и *Aspergillus*. Идентификоване су 3 врсте из рода *Penicillium*, а у највећем броју узорака *P. brevicompactum* и *P. solitum*. Није утврђено присуство *P. verrucosum*. Присуство токсигених плесни као што је *P. brevicompactum* указује на могућност контаминације радних површина микофенолном киселином (MPA). Резултати истраживања указују на потребу интензивнијег испитивања и идентификације плесни у кланичној индустрији Србије.

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ANALYSIS OF OCHRATOXIN A IN PIG TISSUES USING HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC) AND LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC/MS/MS) AS CONFIRMATIVE METHODS

ABSTRACT: Two different analytical methods for the determination and confirmation of ochratoxin A (OTA) in blood serum, kidney and liver of pigs have been compared. Sample clean-up was based on liquid-liquid phase extraction. The detection of OTA was accomplished with high-performance liquid chromatography (HPLC) combined either with fluorescence detection (FL) or electro spray ionization (ESI+) tandem mass spectrometry (MS-MS). Comparative method evaluation was based on the investigation of 82 samples of blood serum, kidney and liver originating from different regions of Serbia. The analytical results are discussed in view of the respective method validation data and the corresponding experimental protocols. In general, analytical data obtained with LC-MS-MS detection offered comparably good results in the sub-ppb concentration level indicating that the liquid chromatography electro spray tandem mass spectrometric (LC-MS/MS) method was more specific and sensitive for the analysis and confirmation of ochratoxin A in pig tissues than high pressure liquid chromatography (HPLC) method after methylation of OTA.

KEYWORDS: ochratoxin A, pig, tissues, HPLC-FL, LC-MS/MS

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin produced by several *Aspergillus* and *Penicillium* species on different agricultural commodities [1—3]. It is a potent nephrotoxin and hepatotoxin with teratogenic, mutagenic and immunosuppres-

sive effects. In humans, the consumption of OTA-contaminated food has been related to the occurrence of Balkan endemic nephropathy [4], a disease characterized by severe kidney damage. In 1993, the International Agency for Research on Cancer (IARC) classified OTA as possibly carcinogenic for humans (group 2B) [5]. OTA occurs in a variety of food commodities of which cereals and cereal products, coffee, beer and wine are the most important sources of intake. Carry-over from contaminated feed-stuff has resulted in the detection of OTA in porcine tissue (liver, kidneys) and pig blood [6] while the mycotoxin is rapidly metabolized in ruminants [3]. Dietary exposure results in detectable levels in human serum and reaches significantly high levels in patients showing symptoms of ochratoxicosis [4]. Detection and determination of OTA is performed by immunochemical methods like enzyme linked immunosorbent assay (ELISA) [7] and chromatographic procedures, such as thin layer chromatography with densitometry detection [8], and especially by high performance liquid chromatography with fluorescence (LC-FLD) [9, 10, 11], mass spectrometry (LC-MS) [12] or tandem mass spectrometry (LC-MS-MS) detection [13]. Residue analysis should be simple and rapid to prevent the distribution of harmful products, but also economic enough to allow the development of programs for monitoring OTA over a wide number of food samples. Also, analytical methods for determination of mycotoxins must be fully validated according to the European specification [14] if they are to be used for the implementation of legislation and for monitoring and risk assessment studies. However, only a few of the methods found in the literature comply with these premises.

The purpose of this work was to develop a confirmative method which is sensitive, reliable, cost effective, rapid and adaptable to a routine work on the surveillance of ochratoxins A in pig tissues.

MATERIALS AND METHODS

Chemicals and reagents

OTA crystalline material was purchased from Sigma (St. Louis, MO, USA). Stock concentrated solution was prepared in toluene-acetic acid (99:1 v/v) at final concentration of 1 mg/ml, kept in security conditions at –20°C, and wrapped in aluminium foil due to the fact that OTA gradually breaks down under UV light. The OTA working solution was prepared by diluting the stock solution with toluene-acetic-acid (99:1 v/v) to č10 μg/ml. The actual concentration of OTA was calculated using UV spectrophotometer set at 333 nm (e 5.550). After suitable dilutions in water-methanol-acetic acid (50:49:1 v/v/v), the working solution was used to prepare the external calibration curve. A working standard OTA for HPLC was prepared just before starting the injection of a series of samples. Other reagents were HPLC grade. All other chemicals were reagent grade or chemically pure.

Sample collection

During a six month period (September 2006/February 2007), samples of blood, kidney and liver from each animal were collected from healthy slaughtered pigs (n = 90) originating from three different regions of Serbia where there is a significant swine industry. Slaughtered pigs were randomly sampled in the slaughterhouse during meat inspection. Serum samples were collected from each studied farm, and liver and kidneys were taken from the corresponding animals. About 50 ml blood/pig was sampled when slaughtered pigs were bled by jugular puncture. Blood samples remained at room temperature for 24 h to allow clotting to occur, and were then centrifuged at 3.000 x *g* for 20 minutes. Serum was decanted and stored at –20°C prior to analysis. About 100 g of liver and whole kidney were sampled from each pig. The whole sample was homogenized and stored at –20°C before analysis. No preservatives were added.

Extraction and clean-up for ochratoxin analyses from serum

Serum (0.8 ml) was extracted according to Curtui and Gareis [15] with 15% trichloroacetic acid (0.2 ml) and dichloromethane (1 ml) by vigorous vortexing for 30 s in a 2 ml safe-lock polypropylene conical-bottom centrifuge tube. The mixture was allowed to stand for 24 h at room temperature, and then centrifuged at 14.000 x g for 10 minutes. The lower dichloromethane phase was carefully withdrawn by a Pasteur pipette and transferred to a 1.5 ml safe--lock polypropylene conical bottom centrifuge tube. The acidic phase and the compact precipitate layer formed between the two phases were re-extracted with dichloromethane (0.5 ml) for 30 s on a vortex mixer and then centrifuged for 5 min at 14.000 x g. The pooled dichloromethane extract was evaporated to dryness at 40°C under a gentle nitrogen flow. The remaining residue was dissolved in methanol (80 ml) and transferred to a 300 μl HPLC vial.

Extraction and clean-up for ochratoxin analyses from kidney and liver

Kidney and liver analyses were performed by the method of Matrella et al. [16], which briefly includes a double extraction with acidic ethyl acetate. The organic phase was removed and extracted with 0.5M $NaHCO_3$, pH 8.4. The aqueous extract was acidified to pH-2.5 with 7M H_3PO_4. OTA was finally back extracted into ethyl acetate (3 ml). The organic phase was evaporated to dryness under N_2 steam, reconstituted in 150 μl mobile phases and a 20 μl aliquot injected.

Chromatographic conditions (HPLC)

An aliquot of 20 μl for serum samples and 50 μl for kidneys and liver samples were injected onto a Waters Symmetry Shield RP (Reversed phase)

18, high pressure liquid chromatography column (length and inner diameter 150 x 4,6 mm, particle size 5 μm) on a Waters Alliance HPLC system. The column was eluted with 4% acetic acid and acetonitrile (32:68 v/v) at 25°C and a flow rate of 1 ml/min. The measurements were performed by fluorescence detection at wavelengths of 334 nm (excitation) and 460 nm (emission) gains 10. The standards were injected with a volume of 10 μl, while the samples were injected with 20 μl. For more accuracy, 40 μl was re-injected in the case of the samples with an amount of OTA near the detection limit.

Confirmation procedures

Extraction and clean-up for ochratoxin analyses from serum, liver and kidney samples were performed by the methods described above [15, 16]. Sample extracts were evaporated to dryness under a gentle stream of nitrogen and stored at –18°C prior to analysis.

HPLC-FL after methylation of OTA

The methyl esters of ochratoxin A were prepared by adding 50 ml of methanol containing 14% of boron trifluoride (Sigma) to the evaporated sample extracts, and by keeping them at 80°C for 15 minutes [6]. Sample extracts were then evaporated to dryness under a gentle stream of nitrogen and stored at –18°C prior to analysis. Under the chromatographic conditions described above, the retention time of the methyl esters of OTA was in the range of 3.455 minutes.

Quantitation

Quantitation for the HPLC-FL method was performed using an external calibration curve from OTA and quantified using the standard addition method with calibration points set at 0.05, 0.1, 0.5, 1 and 3 ng/g.

2.4.2. Confirmation of OTA by liquid chromatography tandem mass spectrometric method (LC-MS/MS)

A Quattro II (Micro mass, UK) triple-quadrupole tandem mass spectrometer operated in the ESI+ mode using multiple reactions monitoring (MRM), and an atmospheric pressure chemical ionization (APCI) source was used. Data acquisition and processing, and instrument control were performed with Microsoft Windows based Mass Lynx software (Micro mass) on a Pentium Pro (Digital Equipment) computer. The following operating parameters were used: The electro spray capillary was set at 3.2 kV and the cone at 30 V. The ion source temperature was set at 115°C, and the flow rates for nitrogen bath and spray were 700 l/h and 55 l/h, respectively. The collision energy was 18

eV. The transition reactions monitored by LC-MS/MS were: precursor ions (*m/z*) 404.1 $[M+H]^+$, product ions (*m/z*) 358.5, and 239.4 of ochratoxin A.

Quantitation

External standard method. Calibration curves were constructed from the responses of each ion monitored plus the total ion current for OTA.

Linearity, detection limit, relative standard deviation and recovery

Fifteen blank samples were spiked with OTA standards at five different levels (0.05, 0.1, 0.5, 1 and 3 ng OTA/g). From the obtained responses (area of the mycotoxin divided by the area of the standard) the correlation coefficient (R) and limit of detection (LOD) were calculated. This LOD was verified by the signal-to-noise (S/N) ratio approach, measuring the chromatographic response of the compound and the chromatographic noise, which should be more than 3 for each compound. The coefficient of variation (C.V.) was calculated according to the five samples spiked at the same concentration level (the highest spiking level), which was performed under the same conditions. The overall recoveries of toxins were expressed as the mean recovery of three standards (n = 3) on two different days. The term recovery in this paper means the amount of substance obtained in the last quantification step (after the extraction) in relation to the amount of substance added to the material before the extraction, and is expressed as a percentage.

Statistical analyses

The arithmetic mean, standard deviation, the coefficient of variation (C.V. %), and the variance were calculated. The *t* test, double-sided, paired data, was used to determine the significance between the treatments.

RESULTS AND DISCUSSION

The results from these LC/MS/MS quantitation methods were compared with those from the conventional HPLC-Fl method and are summarized in Table 1 and 2, and Figure 1 and 2.

Method performance by HPLC-FL

The limit of detection (LOD) (S/N, 3:1) and the limit of quantification (LOQ) (S/N, 7:1) were estimated at 0.05, 0.1, 0.5, 1 and 3 ng OTA/ml(g), and were 0.1 and 0.2 ng/ml for serum samples, while the recovery was 86.8%

(C.V. = 9.6%). The limit of detection (LOD) (S/N, 3:1) and the limit of quantification (LOQ) (S/N, 7:1) for kidney and liver samples were 0.14 and 0.25 ng/g with a 71% (C.V. = 12%) mean recovery from artificially contaminated samples (n = 5).

Method performance by LC-MS/MS

The LOD and LOQ for serum were 0.20 and 0.40 ng/ml, while for kidney and liver were 0.25 and 0.50, respectively. Analyzed according to the proposed method, the recoveries obtained for kidney and liver samples, spiked at five levels (n = 5), ranged from 77 to 89% (C.V. = 13.9%).

Tab. 1 — Validation data according to the methods of HPLC-FD and LC-MS/MS

n = 5 (0.05, 0.1, 0.5, 1, and 3 ppb)	Separation/detection					
	HPLC-FL			LC-MS/MS		
	Serum	Kidney	Liver	Serum	Kidney	Liver
Limit of detection (ppb)	0.1	0.14	0.14	0.20	0.25	0.25
Limit of quantification (ppb)	0.20	0.25	0.25	0.40	0.50	0.50
Mean recovery (%)	86.8	71		92	77—89	
C.V. (%)	9.6	12		6.70	13.9	

Application to the real sample

The proposed method of extraction, followed by HPLC-FL and LC-MS/MS, was applied to 82 samples of serum, liver and kidney (Table 2). Figures 1 and 2 show the HPLC-FL, before and after methylation procedure, and LC--MS/MS chromatograms obtained by the proposed extraction procedure for positive samples. For tissue samples, the obtained results are shown in Table 2. The presence of OTA was detected by HPLC-FL in 82 out of 270 (30%) samples, whereas LC-MC/MS confirmed this in 78 samples. In 2.2% of the kidney samples, OTA levels were considerably higher and greatly exceeded the permissible levels of these toxins established in Serbia, including those proposed (10 ng/g) by the JECFA (2001) [17]. The presence of OTA was not detected by HPLC-FL, after methylation of OTA, in a positive sample which contained low levels of OTA (Figure 2 B1 and C1). The presence of problems due to the coextractive substances in the matrix was also observed by Wood et al. (1995) [23], thus, a confirmation of the positive analysis was necessary. This problem was resolved with confirmation procedures achieved by LC-MS/MS.

Tab. 2 — Incidence of ochratoxin A in different samples analyzed by LC-MS/MS method

Samples	No. of samples	No. of samples with OTA level			max. value of OTA (ng/g)
		< LOD	LOD-LOQ	> LOQ	
Serum	28	—	7	21	220.8
Liver	24	2	3	19	14.5
Kidney	30	2	6	22	52.5
Total	82	4	16	62	14.5—220.8

LOD and LOQ (See Materials and Methods)

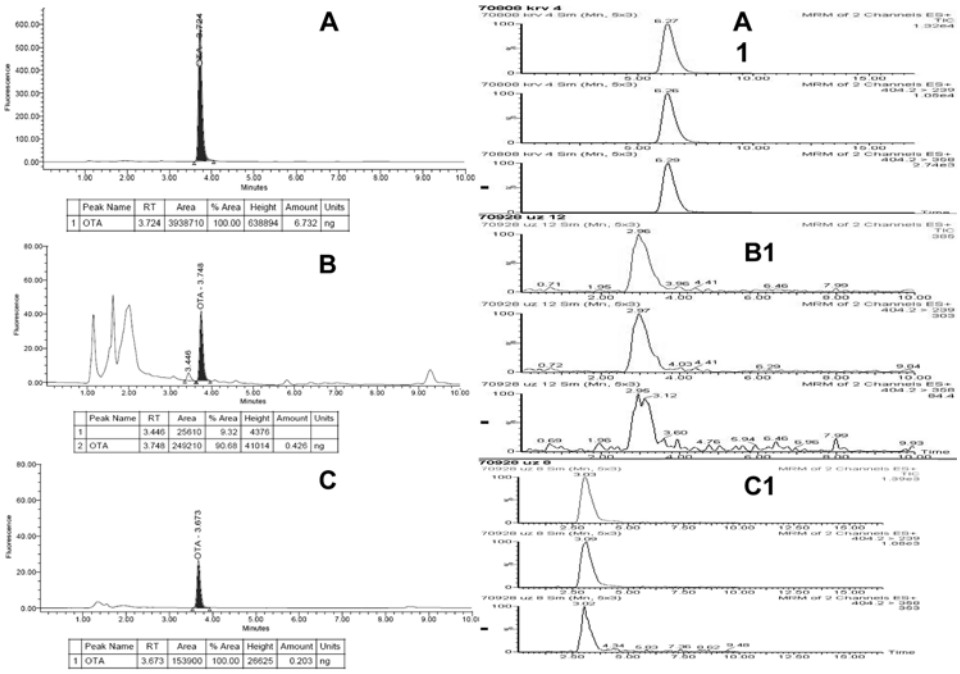

Fig. 1 — Chromatograms (HPLC-FL A, B,s C and LC-MS/MS A1, B1, C1) of a positive sample of serum, liver and kidney samples containing 220.8 ng/ml, 14.5 and 52.5 ng/g of OTA, respectively

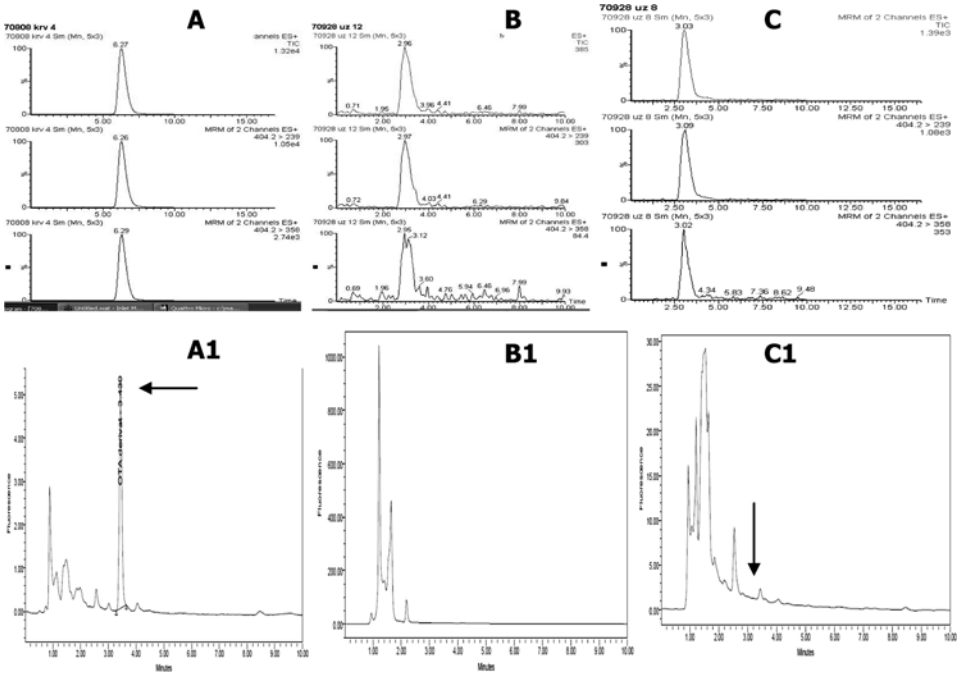

Fig. 2 — LC-MS/MS (A, B, C) and HPLC-FL (A1, B1, C1 with methylation) Chromatograms of a positive sample of serum, liver and kidney containing 220.8 ng/ml, 14.5 and 17 ng/g of OTA, respectively

The main differences in the performance of the detection techniques can be divided into three aspects: (i) sensitivity, (ii) unambiguous identification of the analytes, and (iii) necessary selectivity of sample clean-up to obtain non--biased results. The excellent detection limits are certainly an advantage of HPLC-FL. On the other hand, the LOD for MS/MS detection is 0.2 ng/ml and 0.25 ng/g, which is comparable with an earlier LC-MS/MS investigation of OTA in samples of serum, liver and kidney. Triple-quadrupole analyzers display high sensitivity when working in multiple reaction monitoring (MRM) mode, and are, thus, best suited to obtain the strict MRLs regulated for various toxic compounds in different food matrices. However, the MS method could be made more sensitive by using more samples for the liquid-liquid or SPE procedure. Higher concentration of matrix constituents should not pose any problems since no interfering peaks were observed in the LC-MS-MS chromatograms (Figures 1 and 2). Furthermore, MS sensitivity achieved in this and the previous study [19] can be considered sufficient to measure OTA concentrations in contaminated samples which could pose a threat to human health, taking WHO guidelines into account [20].

Considering the unambiguous analyte identification, the fluorescence properties of OTA may not provide a sufficient degree of certainty. To counteract this disadvantage, confirmation procedures are mainly performed by derivatization of OTA into its methyl ester using BF3/MeOH and the subsequent de-

termination of this compound. This method, however, poses two problems. First, the purity of the reagent employed is frequently uncertain so it itself could give rise to interferences [21]. Secondly, this method is not efficient for the confirmation of low levels of OTA [22, 23]. In the HPLC-FL chromatograms (Figure 2 B1) such signals are completely absent. Confirmation through the hydrolysis of OTA into OT¬ by the enzyme carboxypeptidase A is also employed in some studies and we have already used it for the confirmation of OTA in human plasma samples [24]. This procedure is no longer used in our laboratory because the identification of OTa is problematic due to its appearance at low retention time along with other substances in the sample. With MS/MS as a detection system, no further confirmation step is necessary, and this enables the avoidance of any error-prone derivatization steps and any further chromatographic analyses. In conclusion, the proposed method is an inexpensive, rapid, straightforward clean-up procedure for determining OTA in tissues, since around 10 samples can be processed per day.

CONCLUSION

A relatively high number of publications on the analyses of toxic substances in food samples by LC coupled with MS/MS shows that this technique has become a powerful tool in the quality control of food products and the safeguarding of human health. This paper has demonstrated excellent sensitivity and specificity of the LC-MS/MS method in the quantitative determination and confirmation of OTA in the samples of pig tissues at low- and sub-ppb levels. Despite lower sensitivity of the LC-MS/MS technique, this methodology has several advantages in terms of sample preparation, easy automatization and unambiguous analyte identification without any further time--consuming and error-prone confirmation steps. From this point of view, it seems that sample preparation and chromatographic separation may be simplified or even eliminated to achieve the highest possible sample throughput as, for example, in drug screening, pharmacokinetics and other areas of life sciences. The evaluation of the method with naturally contaminated samples demonstrated that the accuracy of the results is comparable to the most reliable HPLC-FL procedures, and that MS sensitivity is sufficiently high, taking into account the proposed OTA tolerance levels within the European Union. Since the detection of OTA in tissues of Serbian slaughtered swine that we analyzed serve as an end-point indicator of the widespread occurrence of OTA, it is important to determine which foods would be contaminated with this potent natural toxin. With the present analytical procedure, it should be possible to carry out surveillance on grains, animal feeds, meat, meat products and other foodstuffs to determine contamination levels of this toxin. As these techniques improve and become standardized, less controversial and more scientific evaluation of the human risks from exposure to mycotoxins will be possible [25].

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АНАЛИЗА ПРИСУСТВА РЕЗИДУА ОХРАТОКСИНА А У ТКИВИМА СВИЊА УПОТРЕБОМ ВИСОКОЕФИКАСНЕ ТЕЧНЕ ХРОМАТОГРАФИЈЕ (HPLC) И ТЕЧНЕ ХРОМАТОГРАФИЈЕ СА МАСЕНОМ СПЕКТРОМЕТРИЈОМ (LC/MS/MS) КАО КОНФИРМАТИВНИХ ТЕХНИКА

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Резиме

У овом раду приказани су резултати поређења две аналитичке технике за одређивање присуства и конфирмацију резидуа охратоксина А (ОТА) у крвној плазми, јетри и бубрезима свиња. Пречишћавање екстракта извршено је по методи за течно-течну екстракцију, а за детекцију ОТА коришћена је високоефикасна течна хроматографија са флуоресцентним детектором (HPLC-FL) и течна хроматографија са масеном спектрометријом (LC-MS/MS). Поређење метода извршено је на основу анализе 82 узорка крвне плазме, јетре и бубрега свиња, пореклом са различитих локалитета Србије. Добијени резултати су тумачени сходно методологији предвиђеној за валидацију, а на основу одговарајућег лабораторијског протокола. На основу добијених резултата може се закључити да су употребом течне хроматографије са масеном спектрометријом (LC-MS/MS) детектоване врло ниске концентрације ОТА (sub ppb), што указује да је LC-MS/MS техника много специфичнија и сензитивнија за одређивање присуства и конфирмацију ОТА у ткивима свиња, у односу на уобичајени дериватизациони поступак употребом HPLC-FL технике.

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PRESENCE OF MOULDS AND AFLATOXIN M1 IN MILK

ABSTRACT: Aflatoxin M1 (AFM1) appears in milk or dairy products as a direct result of the cattle's ingestion of feed contaminated with aflatoxin B1 (AFB1).

This study comprises mycological and mycotoxicological investigations of 23 milk samples (raw, infant food, pasteurized, whey and yoghurt).

The mycological testing showed dominant presence of genus *Geotrichum*. *G. candidum* was found in 9 samples, with the highest contamination in the raw milk samples.

The contamination level of AM1 is defined by using direct competitive enzyme-linked immunosorbent assay (ELISA). AFM1 was found in 9 samples. AFM1 levels were lower than the recommended limits.

However, as AFM1 is considered a probable human carcinogen (2B type), it is necessary to achieve a low level of AFM1 in milk.

Therefore, cows' feed samples from various cowsheds are supposed to be evaluated routinely for aflatoxin, and kept away from fungal contamination as much as possible.

KEYWORDS: milk, moulds, aflatoxin M1, enzyme immunoassay (ELISA)

INTRODUCTION

Aflatoxins are extremely toxic metabolites produced by the common fungi *Aspergillus flavus, A. parasiticus* and *A. nomius* (B a s k a y a et al., 2006). The most important are aflatoxin B_1(AB_1), G(AG_1) and their dihydro derivatives AB_2 and AG_2. Given that they are some of the most powerful mutagens and carcinogens, and on the basis of all-round scientific knowledge, in 1987 IARC classified them into Group 1 of human carcinogens.

Aflatoxin M1 appears in milk and milk products as a direct result of the intake of aflatoxin B1 contaminated feed by dairy cows (Van Egmond, 1989). AB1 may occur in various agricultural products that are used as feedstuff ingredients. The amount excreted as AM1, as a percentage of AB1 in feed, is usually 1—3%, but the values as high as 6% were reported by Pitet, 1998. Rothschild (1992) classified L.J. Rothschild , IARC classes AFB1 as class 1 human carcinogen. *Food Chem.* 34 (1992), pp. 62—66. AFM1 as 2B (probable carcinogen) human carcinogens. Lafont, Siriwardana and Lafont (1989) have also observed that AFM1 has high genotoxic activity, although AFM1 has been found to be about 10 times less carcinogenic than AFB1. In 2002, IARC classified AM1 into Group 1.

The risk posed by aflatoxins has been faced differently in different countries. In Europe, the maximum tolerated levels of AFM1 in milk and dairy products were regulated firstly by Regulation CE 2174/2003 (Off. J. Eur. Communities, 2003) that modified Regulation CE 466/2001 (Off. J. Eur. Communities, 2001), and then by Regulation 1881/2006 (Off. J. Eur. Communities, 2006). In accordance with these norms, the product to be screened is milk, in which AFM1 concentration must not exceed 0.05 mg/kg, while dairy products must be obtained using milk conforming to the above AFM1 limits.

In Serbia, maximum tolerated level of AM1 is 0.5 mg/kg (Official Gazette of SRY, No. 11/92).

This study was carried out to evaluate the presence of moulds and the prevalence of milk contamination with AM1.

MATERIALS AND METHODS

Mycological research

The mycological research encompassed determination of the total number of moulds in 1 ml of the tested samples and their identification. Twenty three different samples were tested (raw milk, infant food, whey, yoghurt and pasteurized milk). Seven samples of raw milk were taken from PKB, veterinary station, while the other samples were taken from the market.

Determination of the total number of moulds in 1 ml of milk was conducted in duplicate, according to the standard laboratory procedure.

Two types of culture media were used: Sabouraud-maltose agar (SMA) with the addition of antibiotics (1 ml chloramphenicol per 100ml of medium), and maltose yeast extract agar with 50% of glucose. The isolated species were identified on the basis of investigation of the macromorphological properties of colonies and micromorphological properties of conidial and other structures, and according to the key described by Samson and van Reenen-Hoekstra, 1988.

Mycotoxicological research

The quantitative analyses of AFM1 were performed with the enzyme immunoassay: Tecna — aflatoxin M1. The test kit is sufficient for 96 determinations (including the calibration curve).

The basis of the test is the antigen-antibody reaction. The wells in the microtiter strips are coated with specific antibodies reactive to AFM1. By adding AFM1 standards, or the sample solutions, the antibody binding sites are occupied proportionally to the AFM1 concentration. Any remaining free binding sites are occupied in the next stage by enzyme labeled toxin (enzyme conjugate). Any unbound enzyme conjugates are then removed in a washing step. Enzyme substrate (urea peroxide) and chromogen (tetramethylbenzidine) are added to the wells and incubated. Bound enzyme conjugate converts the colourless chromogen into a blue product. The addition of the stop reagent leads to a colour change from blue to yellow. The measurement is made photometrically at 450 nm (optional reference wavelength 600 nm). The absorption is inversely proportional to the AFM1 concentration in the sample.

RESULTS AND DISCUSSION

Mycological results

Eleven samples were without growth, while raw milk (samples 2 and 5) and infant food (sample 9) had the highest contamination level ($4 \cdot 10^3$/ml). Whey samples showed growth from $1.2 \cdot 10^2$/ml to $2 \cdot 10^2$/ml (Table 1). Comparison of the results pointed out that there were no drastic differences between the numbers of moulds grown on the two culture mediums.

According to the mycological research, genus *Geotrichum* was dominant in 9 out of 23 samples. The remaining moulds were from genus *Aspergillus*. *G. candidum* was isolated in raw milk (samples 1, 2, 3, 5 and 7), infant food (9 and 10), pasteurized milk (17) and yoghurt (sample 22). The level of contamination with *G. candidum* was the smallest in the samples 3, 7, 10, 17 and 22, while the samples 2, 5 and 9 had the highest level of contamination ($4 \cdot 10^3$/ml).

Aspergillus spp. were dominant in whey samples (14, 15 and 16), with the level of presence ranging from 1.2/ml — $2 \cdot 10^2$/ml.

The results are similar to the results of Torkar et al. (2007), who reported that the genera most frequently isolated from raw milk belonged to genera *Geotrichum* (51.5% of strains) and *Aspergillus* (33.8% of strains).

The presence of toxic metabolite AM1

Out of a total of 23 samples of various milk (raw, infant food, pasteurized, whey and yoghurt), 9 samples contained AFM1 in the quantities ranging from 0.007 to 0.250 μg/kg . AFM1 was not detected in any of the 6 samples

of pasteurized milk. The presence of AFM1 was very low in infant food (sample 9) and yoghurt (sample 20). All detected concentrations were practically below the maximum tolerated levels of AM1 — 0.5 μg/kg (Official Gazette of SRY, No. 11/92). Only three samples (raw milk) showed concentrations higher than the maximum tolerance limit of 0.05 μg/kg accepted by the EU regulations (samples of raw milk — 1, 2 and 5).

The percentages of absorbance obtained by the competitive enzyme immunoassay with the calibration curve (Table 1) allow the calculation of AFM1 concentration in mg/kg in the positive samples, for each type of milk.

Tab. 1 — Concentration of AM1 and total number of moulds in 1ml of milk

Sample	Aflatoxin M1 ($\mu g \cdot kg^{-1}$)	Total No. (moulds/ml)
Raw milk 1	0.250	$3 \cdot 10^3$
Raw milk 2	0.120	$4 \cdot 10^3$
Raw milk 3	0	20
Raw milk 4	0	0
Raw milk 5	0.200	$4 \cdot 10^3$
Raw milk 6	0	0
Raw milk 7	0.02	30
Infant food 8	0	0
Infant food 9	0.02	$4 \cdot 10^3$
Infant food 10	0	20
Pasteurized milk 11	0	0
pasteurized milk 12	0	0
pasteurized milk 13	0	0
Whey 14	0.042	$2 \cdot 10^2$
Whey 15	0.020	$2 \cdot 10^2$
Whey 16	0.008	$1.2 \cdot 10^2$
pasteurized milk 17	0	15
pasteurized milk 18	0	0
pasteurized milk 19	0	0
Yoghurt 20	0.007	0
Yoghurt 21	0	0
Yoghurt 22	0	20
Yoghurt 23	0	0

Considering the evaluation of AM1 by ELISA, in Italy, AFM1 was found in 16.6% of the cheeses tested. 31.3% of these were sheep-goat cheeses, 27.2% were cow cheeses, 16.7% were goat cheeses, and 12.8% sheep cheeses with no significant differences ($p > 0.05$). All the samples of buffalo milk cheese were consistently negative. Overall, the AFM1 values ranged from 50 to 250 ppt. Specifically, the concentrations of AFM1 in goat cheese ranged from 90 to 250 ppt, in cow cheese from 50 to 160 ppt, in sheep cheese from 50 to 215, and in sheep-goat cheese from 55 to 140 ppt (Montagna et al., 2008).

In Argentina, of a total of 77 various types of milk samples, with screening ELISA method, only 18 samples (approximately 23 %) were found to be contaminated with AFM1 at 0.010—0.030 μg/kg. All concentrations were below the maximum tolerated levels of AFM1 in liquid milk and powdered milk (Lopez et al., 2002).

In Iran, aflatoxin M1 was found in 100% of the examined milk samples. 390 samples (62.5%) had contamination less than 45 ng/l of AFM1, 123 samples (19.7%) contained 45—50 ng/l, 94 samples (15.1%) contained 50—80 ng/l, and the remaining 2.7% of the samples contained more than 80 ng/l of AFM1. In general, regardless of the 19.7% of the samples that were in borderline limit (45—50 ng/l), the amount of AFM1 in 17.8% of the samples was higher than the maximum tolerance limit (50 ng/l) accepted by the European Union (Alborzi et al., 2005).

CONCLUSION

In conclusion, considering that for various reasons many regions are obliged to feed dairy animals on stored forage or industrially produced pellets, it is important to reduce the occurrence of toxins (AFB1) in feedstuff and take prophylactic measures to prevent the factors enhancing toxin production. These factors include environmental temperature, humidity, and moisture content of the feed, as well as pH and mechanical damage to the grain affecting mould production.

Moreover, since AFM1 is well known to be mutagenic and carcinogenic, international regulations ensuring a minimal presence of this aflatoxin in cheeses are needed. In fact, by having a common European norm concerning the AFM1 threshold limits for dairy products, it will be possible to guarantee the distribution of safer, healthier foods, particularly in the light of the current norms on internal controls and HACCP (Off. J. Eur. Communities, 2004).

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УТВРЂИВАЊЕ ПРИСУСТВА ПЛЕСНИ И АФЛАТОКСИНА М1 У МЛЕКУ

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Резиме

Рад обухвата миколошку и микотоксиколошку анализу 23 узорка млека (свеже кравље млеко, дечја храна, пастеризовано млеко, сурутка и јогурт). Микролошка испитивања обухватала су одређивање укупног броја плесни у 1 ml узорка на две подлоге SMA и MA. Детерминација плесни извршена је на основу микроморфолошких и макроморфолошких особина на основу одговарајућих кључева. Микотоксиколошка испитивања обухватала су утврђивање присуства AM1 коришћењем ELISA теста фирме Тецна.

Детерминацијом плесни утврђена је доминантност врсте *Geotrichum candidum*. Микотоксиколошка испитивања потврдила су здравствену исправност свих узорака према нашем Правилнику.

Неопходно је обезбедити мониторинг сточне хране на присуство AB1, свежег млека на AM1, као и одговарајуће агротехничке мере.

Пројектовано повећање температуре на глобалном нивоу и учесталост и трајање топлотних интервала могу повећати ризик контаминације афлатоксинима и у умереним климатским регионима.

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OCHRATOXIN A *„IN VITRO"* BIOSYNTHESIS BY THE *ASPERGILLUS OCHRACEUS* E'G ISOLATE

ABSTRACT: This paper deals with the biosynthetic capacity for ochratoxin A (OTA) production by *Aspergillus ochraceus* E'G isolate derived from *A. ochraceus* CBS 108.08 strain, during 2007. Preliminary analysis of fungal potential for the production of OTA were performed according to the modified method of Filtenborg et al. (1983). Toxin production was tested in the following liquid media: (i) glucose-peptone-yeast extract broth (GPY — pH 5.6), (ii) potato-dextrose broth (PDB — pH 6.9), (iii) yeast extract-sucrose broth (YES — pH 6.5), and (iv) YES broth supplemented with 0.23 mg/l $ZnSO_4$ x 5 H_2O (YES^{Zn} — pH 6.5) after stationary and submerged cultivation. Dynamics of OTA biosynthesis was tested after the cultivation of *A. ochraceus* E'G on natural solid substrates, such as wet sterilized rice, corn and wheat grain. Cultivations were performed during different time periods (ranging from four days to few weeks) at different temperatures (ranging from 21°C to 30°C).

The presence of OTA was determined as follows: (i) in liquid media according to the method of Balzer et al. (1978) modified by Bočarov-Stančić et al. (2003), and (ii) in the solid substrates according to the Serbian official methods for sampling and analyzing of fodder (Official Gazette of SFRY, No. 15/87).

After the cultivation of *A. ochraceus* E'G isolate in liquid media, the highest yield of OTA (6.4 mg/l) was obtained after submerged cultivation in PDB (4 days, 128 rpm, 21—23°C). In the case of cultivation on solid substrates, the highest amount of OTA (800.0 mg/kg of dry matter) was recorded after several week long cultivation on wheat grain at 30 ± 1°C.

KEY WORDS: *Aspergillus ochraceus*, ochratoxin A, *in vitro* biosynthesis

INTRODUCTION

Ochratoxin A (OTA) is a potent kidney toxin which causes birth defects in test animals (rats, hamsters and mice). In terms of livestock and human health this mycotoxin is particularly important for the poultry and swine, because these monogastric animals lack the ability to degrade OTA rapidly, as compared to ruminants.

Ochratoxin A is the contaminant of a variety of plants and animal products. Although this mycotoxin is a world wide problem, its impact is the greatest in temperate regions where great amount of world's grain is produced and stored. Because of that, OTA has become a major concern to livestock producers, especially in Europe and North America. Once induced into feed of monogastric animals, it contaminates eggs, organs, fat, muscle tissue and blood (Abramson, 2008). The main sources of ochratoxin A in human diet are undoubtedly cereal products and swine products, although other commodities, such as coffee, wine, beer, cheese, poultry products, pepper etc. may also contain traces of this toxin (Gatti et al., 2003; Tjamos et al., 2004; Mahdavi et al., 2007). Medina et al. (2004) even hypothesized that bee pollen may constitute an important risk factor concerning the presence of OTA in the diet of consumers of that nutritious food.

A number of authors reported about the importance of OTA as one of the basic contaminants of fodder and feed components in the Republic of Serbia. During 1993—2003 period, Jakić-Dimić et al. (2003) analyzed 220 samples of fodder types for different growth categories of cattle and found that OTA and zearalenone were the most frequent contaminants. In 2004, in the samples of stored barley, taken from Kragujevac locality and intended for livestock nutrition, Škrinjar et al. (2007) recorded the presence of ochratoxin A in all samples, in the concentrations ranging from 0.54 to 7.3 μg/kg. According to Jurić et al. (2005), OTA occurrence is a serious problem in Serbia. In a 3-year period these authors analyzed 229 samples of feed components and found this mycotoxin in 50% of maize samples, and even 100% of sunflower pellets; the highest concentrations of OTA were 0.5—1.0 mg/kg.

Ochratoxin A can be produced by the number of fungal species belonging to the genera *Aspergillus* and *Penicillium*, but principal producers of this mycotoxin are *A. ochraceus* in warm regions of the world, and *P. aurantiogriseum* in temperate climates (Frisvald and Samson, 1991). As a rule, *Aspergillus* species grow in conditions of low water activity (min. 16%) and high temperatures, unlike typical field mycobiota, such as *Fusarium* species. Under laboratory conditions, they can grow in the range of 4.5—8.0 pH value, although the optimal pH for their growth is 5.5—7.5. Since the *Aspergillus* species, as well as other fungal species, are strictly aerobic, they generally require minimum 1—2% of oxygen for growth (Sinovec et al., 2006).

Accordingly, the aim of this paper was to examine the kinetics of OTA biosynthesis under laboratory conditions, and to complete the optimization of conditions for the OTA production by the newly isolated culture of *A. ochraceus* designated E'G.

MATERIAL AND METHODS

Microorganisms. *Aspergillus ochraceus* Wilhelm isolate E'G, derived in 2007 from CBS 108.08 strain, and CBS 108.08, known as ochratoxin A (OTA) producer, were investigated. The E'G isolate was obtained from the CBS 108.08 colony that showed segregation during 10-day growth on yeast extract (2%) sucrose (15%) agar (2%) with the addition of 0.23 mg/l $ZnSO_4$ x 5 H_2O (YESA^{Zn}, pH 6.5) (M ü c h l e n c o e r t, 2004) at 27 ± 1°C. The obtained isolate E' morphologically differed from the parent one: smaller conidial heads were observed (average diameter 173 μm compared with 244 μm of the original strain CBS 108.08). Also, the colour of the colony grown on potato-dextrose agar (PDA) was not typically ocher but rather green. After sowing of E' isolate on PDA plates and 10-day cultivation at 27 ± 1°C, the observed characteristics remained unchanged. After one year storage on PDA slants at 4—6°C, the E' isolate did not show any growth during the subcultivation on PDA plates at 27 ± 1°C, so the nutrient broth was used for enrichment and revivification of the E' culture. This procedure resulted in consistently olive green colony colour and smaller conidial heads during the cultivation on PDA at 27 ± 1°C, as well as at 30 ± 1°C. The obtained isolate was designated as E'G.

Preliminary analyses of fungal potential to produce OTA were performed according to the rapid screening method of F i l t e n b o r g et al. (1983) modified by B o č a r o v - S t a n č i ć et al. (in press) on the following media: YESA (2% yeast extract, 15% sucrose and 2% agar, pH 6.5), YESA^{Zn} (2% yeast extract, 15% sucrose, 0.23 mg/l $ZnSO_4$ x 5 H_2O, and 2% agar, pH 6.5), PPSA (2% peptone-1, 15% sucrose and 2% agar, pH 6.5), PPSA^{Zn} (2% peptone-1, 15% sucrose, 0.23 mg/l $ZnSO_4$ x 5 H_2O and 2% agar, pH 6.5) and PDA (potato-dextrose agar, pH 6.9).

Inoculations of liquid and natural solid fermentation media for testing kinetics of OTA biosynthesis were performed by 5 peaces (5 x 5 mm) of fungal material originating from Petri dish sowed with tested culture, and subcultivated for 7 days on potato-dextrose agar (PDA) at 27 ± 1°C.

Cultivation conditions. Toxin production was tested in the following types of liquid media: (i) broth with 5% glucose, 0.1% peptone-1 and 0.1% yeast extract, pH 5.6 (GPY), (ii) potato dextrose broth (PDB: 200 g/l of boiled sliced potato + 20 g/l dextrose, pH 6.9), (iii) broth with 2% yeast extract and 15% sucrose, pH 6.5 (YES), and (iv) broth with 2% yeast extract and 15% sucrose supplemented with 0.23 mg/l $ZnSO_4$ x 5 H_2O, pH 6.5 (YES^{Zn}). Inoculated Erlenmayer flasks (500 ml) containing 100 ml of each medium were cultivated for 4 days on rotary shaker (128 rpm) at room temperature (21—23°C), and 10 days stationary at 27 ± 1°C in chamber. pH value was measured after the cultivation of the isolate.

Inoculated Roux bottles containing 50 g of sterilized cereal kernels, such as rice, corn, and wheat, wetted with 50 ml of sterile water, were cultivated at 30 ± 1°C in chamber for four weeks, or at room temperature (21—23°C) for 3 weeks. In the case of corn and wheat substrates, the samples for analysis were taken weekly during the cultivation period. The moisture content of the tested

natural substrates was determined after drying at 105°C until constant weight was not achieved.

All cultivations were performed in two replications.

Analyses of OTA. After the cultivation on rotary shaker, liquid cultures were filtered. Crude OTA extracts were obtained by the use of acetonitrile/water (90 + 10 v/v), according to Balzer et al. (1978) method modified by Bočarov-Stančić et al. (2003). The modification consisted of adding the culture filtrate up to 20% of anh. Na_2SO_4, as well as 20% of silica gel for column chromatography (Kieselgel 60 extra pure — MERCK) before toxin extraction. The rest of the analysis was done according to the original Balzer et al. (1978) method.

The samples obtained after the cultivation on cereal grains were dried during 24 h or more at 60°C until constant weight was not obtained. After the pulverization of dried samples, OTA was analyzed according to the Serbian official methods for sampling and analyzing of fodder (Official Gazette of SFRY, No. 15/87). After the cultivation on natural substrates, the yield of OTA was expressed as number of mg per kg of dry matter of the sample.

All analyses were done in three replications.

RESULTS AND DISCUSSION

Results of the present investigation are shown in Tables 1—3.

Tab. 1 — Screening of ochratoxin A production by *Aspergillus ochraceus* CBS 108.08 and E'G isolates

Sample designation	*Temper. (°C)*	*Days*	*Medium*				
			PPSA^{Zn}	PPSA	PDA	YESA	YESA^{Zn}
CBS 108.08	27 ± 1	10	+	—	+++	++++	++++
E'G	21—24	7	n.a.	n.a.	+	++++	++++
		10	n.a.	n.a.	+	++++	++++
		14	n.a.	n.a.	+	++++	++++
	27 ± 1	10	+	—	++	n.a.	n.a.

Legend: n.a. — not analyzed; — no biosynthesis; + low intensity of biosynthesis, ++ moderate intensity, +++ high intensity, ++++ very high intensity

The ability of *A. ochraceus* isolate E'G to produce ochratoxin A was tested simultaneously after 10-day cultivation at 27 ± 1°C in chamber, and after 14-day cultivation at room temperature (21—24°C). Isolate E'G showed almost the same toxicological profile as the parent strain CBS.108.08, with the exception of cultivation on PDA plates where it produced lower quantities of OTA than CBS.108.08 did (Table 1).

During the cultivation of E'G isolate at room temperature (21—24°C) the best results were achieved on YESA and YESA^{Zn} plates. Under the same temperature conditions it was observed that biosynthesis of the tested mycotoxin did not change over time.

Tab. 2 — Biosynthesis of ochratoxin A by *A. ochraceus* E'G in liquid media

Temper. (°C)	*Cultiv. conditions*	*Days*	*pH*	*Medium*	*Yield (mg/l)*
21—23	128 rpm	4	5.12	GPY	n.d.
27 ± 1	stat.	10	4.75		n.d.
21—23	128 rpm	4	6.50	PDB	6.40
27 ± 1	stat.	10	5.80		0.80
21—23	128 rpm	4	5.15	YES	0.08
27 ± 1	stat.	10	5.04		0.04
21—23	128 rpm	4	5.28	YES^{Zn}	0.06
27 ± 1	stat.	10	5.01		n.d.

Legend: n.d. — not detected (< 0.004 mg/l)

The production of ochratoxin A in liquid media was tested simultaneously after stationary and submerged cultivation in: (i) GPY — pH 5.6, (ii) PDB — pH 6.9, (iii) YES — pH 6.5, and (iv) YES^{Zn} — pH 6.5. The initial pH of the tested media was adjusted to these values, having in mind the ascertainment of Mühlencoert (2004) that ochratoxin A biosynthesis can occur between 5.5 and 8.0 pH. At the end of the cultivation period the decrease of initial pH was recorded in all cases, although the most outstanding change occurred after 10-day stationary cultivation in chamber (27 ± 1°C) (Table 2).

During the testing of OTA production by *A. ochraceus* isolates, Mühlencoert (2004) used different liquid media, including YES broth (initial pH 6.5) in shaken incubation flasks (120 rpm) at 25°C in the dark. This author observed that after onset of OTA biosynthesis (after 72 h) there was a steady increase of toxin concentration, which was accompanied, like in our experiments, by a drop of pH.

During the presented experiments in different liquid media, OTA production was not at all recorded in GPY broth, while in YES broth and YES supplemented with Zn only small amounts of the toxin were found mostly after submerged cultivation (0.08 mg/l and 0.06 mg/l, respectively) (Table 2). The highest amounts of ochratoxin A were observed after the cultivation of E'G isolate in potato-dextrose broth. Although the temperature applied during the submerged cultivation in PDB was lower (21—23°C) than during the stationary one (27 ± 1°C) the obtained yield of OTA was much higher in the first case (6.40 mg/l compared to 0.80 mg/l) (Table 2). The obtained results pointed out that aeration influenced the toxin biosynthesis much more than the temperature during the cultivation of *A. ochraceus* E'G in liquid media did.

Much higher amounts of ochratoxin A, between 500 and 300 mg/l, were obtained by fungal isolates representing different sections of the *Aspergillus* genus (Varga et al., 2002). These authors have found the maximum of OTA biosynthesis after 7—10 days of incubation at 30°C in YES liquid medium.

Tab. 3 — Influence of type of the media, temperature and duration of cultivation on ochratoxin A production by *A. ochraceus* E'G

Temper. (°C)	*Days*	*Moist. (%)*	*Medium*	*Yield (mg/kg)*
21—23	21	63.6	rice	96.0
30 ± 1	7	—	corn	n.d.
		—	wheat	20.0
	14	37.8	corn	80.0
		48.8	wheat	240.0
	21	33.0	corn	80.0
		46.6	wheat	800.0
	28	32.0	corn	80.0
		43.0	wheat	800.0

Legend: n.d. — not detected (< 0.004 mg/kg)

The dynamics of OTA production by the isolate *A. ochraceus* E'G was tested during cultivation on three different types of wet cereal kernels (Table 3). The moisture content of corn and wheat grains decreased during prolonged cultivation from 37.8% for corn and 48.8% for wheat, respectively after 14-day cultivation to 32.0% for corn and 43.0% for wheat at the cultivation end (28 days).

Accumulation of the ochratoxin A was much higher in solid substrates then in the liquid media (Table 2 and 3). After three weeks of cultivation of E'G isolate, the similar concentrations of ochratoxin A were recorded on corn kernels and rice grain (80.0 mg/kg and 96.0 mg/kg, respectively) while the highest amounts were found on wheat grain (800.0 mg/kg). Thus, it was observed that the type of cereal grain affected the biosynthetic potential of the fungus, while the cultivation temperature was not so important factor for the toxin production.

Contrary to our findings, the investigations of Sanchis et al. (2006) on OTA production patterns on different substrates, such as YES, barley grains etc. did not show a significant influence of these substrates. On the other hand, these authors found that abiotic factors, such as temperature and water activity, affected significantly toxin biosynthesis. Maximal ochratoxin A accumulation was detected at 25°C and 0.98 a_w on all substrates tested.

The beginning of ochratoxin A production was observed seven days after the cultivation on wheat grain (20.0 mg/kg), and in the case of corn kernel, it was after the prolonged cultivation of *A. ochraceus* E'G for 14 days (80.0 mg/kg). Similar results were obtained by Häggblom (1982) who tested the dynamics of OTA biosynthesis on barley grain by *A. ochraceus* and *Penicillium viridicatum.* This author detected toxin 4 to 6 days after inoculation at 25°C, and observed its maximal accumulation after 28 days (from 7 to 46 mg/kg).

Contrary to wheat substrate, where yield of OTA was increasing until the 21st day of cultivation, the corn substrate did not show any changes in the quantity of the produced toxin from the 14th day of cultivation until the end of the same process (Table 3).

CONCLUSION

Preliminary analysis of *A. ochraceus* E'G potential for the production of ochratoxin A showed that isolate E'G had similar toxicological profile as its parent strain CBS.108.08.

At the end of the cultivation period, in different liquid media, the decrease of initial pH was recorded in all cases, although the most outstanding change occurred after the 10-day stationary cultivation in chamber (27 ± 1°C).

Aeration influenced the toxin biosynthesis much more than the temperature during the cultivation of *A. ochraceus* E'G in liquid media.

The highest yield of OTA (6.4 mg/l) was obtained after submerged cultivation in potato-dextrose broth.

Type of cereal grain used for OTA production showed a significant influence on the dynamic of the process and toxin yields.

After three weeks of cultivation of *A. ochraceus* E'G the similar concentrations of ochratoxin A were recorded on corn kernels and rice grain (80.0 mg/kg and 96.0 mg/kg, respectively) while the highest amounts were found on wheat grain (800.0 mg/kg).

Contrary to wheat substrate where the yield of OTA was increasing until the 21st day of cultivation, the corn substrate did not show any changes in the quantity of the produced toxin from the 14th day of cultivation until the end of the same process.

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IN VITRO БИОСИНТЕЗА ОХРАТОКСИНА А КОД ИЗОЛАТА *ASPERGILLUS OCHRACEUS* E'G

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Резиме

Испитивањем је био обухваћен изолат *Aspergillus ochraceus* E'G изведен из соја *A. ochraceus* CBS 108.08. Прелиминарне анализе присуства охратоксина А (ОТА) су извршене према модификованој методи Filtenborg-a и сар. (1983) на агаризобаним подлогама. Производња токсина је тестирана у следећим течним подлогама: 1) глукоза-пептон-екстракт квасца у бујону (GPY — pH 5,6), 2) кромпир — декстрозном бујону (PDB — pH 6,9), 3) екстракт квасца — сахарозном бујону (YES — pH 6,5) и 4) екстракт квасца-сахарозном бујону са додатком 0,23 mg/l $ZnSO_4$ x 5 H_2O (YES^{Zn} — pH 6,5) у условима стационарне и субмерзне култивације. Динамика биосинтезе охратоксина А праћена је након гајења на природним чврстим супстратима (зрно пиринча, кукуруза и пшенице) током вишенедељне култивације.

Охратоксин А је изолован из течних подлога за култивацију и продукцију применом методе Балзера и сар. (1978) модификоване према Бочаров-Станчић и сар. (2003), док је квантитација ОТА у природним чврстим супстратима извршена према Правилнику о методама узимања узорака и методама физичких, хемијских и микробиолошких анализа сточне хране („Сл. лист СФРЈ", бр. 15/87).

При гајењу изолата *A. ochraceus* E'G у течним подлогама највећи принос ОТА је добијен при коришћењу PDB (6,4 mg/l) и то у условима субмерзне култивације (4 дана, 128 о/мин, 21—23°C). У случају култивације на зрну житарица највећу количину ОТА је изолат *A. ochraceus* E'G биосинтетисао после вишенедељне култивације на зрну пшенице и 30 ± 1°C (800,0 mg/kg).

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MYCOTOXINS AS A RISK IN THE GRAIN FOOD

ABSTRACT: Mycotoxins are toxic secondary metabolites of fungi that contaminate a large variety of foods and have toxic effects on humans. The best protection against mycotoxins is to monitor their presence in food. This paper shows the screening results of mycotoxins present in 76 samples of different groups of grain foods. Samples of grain food were analyzed for contamination with aflatoxins, ochratoxin A, zearalenone, fumonisins and deoxynivalenol. Analysis were conducted using competitive enzyme-linked immunosorbent assay (ELISA). None of the samples was contaminated with aflatoxins. The most predominant mycotoxin was ochratoxin A with the mean level of 4.84 ± 4.49 ppb in 19.7% of the examined samples. Zearalenone, fumonisins, and deoxynivalenol were found in 9.21, 14.5 and 3.9% of the samples, respectively. Mycotoxin content in the investigated samples was compared with the regulations of Serbia and those of the European Union.

KEYWORDS: ELISA, grain food, monitoring, mycotoxins

INTRODUCTION

Mycotoxins are natural food and feed contaminants, mainly produced by moulds of genera *Aspergillus*, *Penicillium* and *Fusarium*. The number of mycotoxins known to exert toxic effect on human and animal health is constantly increasing, as well as the legislative provisions made to control their presence in food and feed (Zinedine et al., 2006). Currently, more than 400 mycotoxins are identified in the world. Considering their heat stability, these substances constitute a potential risk for human and animal health. The chemical and biological properties of mycotoxins and their toxic effects are extremely variable. These effects are carcinogecity, genotoxicity, teratogenicity, nephrotoxicity, hepatotoxicity and immunotoxicity. Mycotoxins are not only dangerous for the health of consumers, but they also deteriorate the marketable quality of the contaminated products, causing heavy economic losses (Zinedine, 2008).

Aflatoxins (AFs) B_1, B_2, G_1 and G_2 are produced by various strains of *Aspergillus*, mainly by *A. flavus*, *A. parasiticus*, *A. nomius* and *A. tamarii*. They have immunotoxic, mutagenic and carcinogenic effects (M o s s, 1988). Aflatoxins are often present in cereals (maize, sorghum, rice, wheat), oilseeds, spices and nuts. Aflatoxins were classified as group 1 carcinogen by the International Agency for Research on Cancer (IARC). Aflatoxin B_1 (Fig. 1, A) is the most frequent and the most toxic one (IARC, 1993).

Ochratoxin A (OTA, Fig. 1, B) is a secondary fungi metabolite mainly produced by *Penicillium verrucosum*, *Aspergillus ochraceus* and *Aspergillus carbonarius* (E s k o l a et al., 2001). These fungi are natural opportunist biodeterioration agents of carbohydrate-rich agricultural commodities. Therefore, this mycotoxin can occur in a large variety of commodities, such as cereals, dried fruits, coffee, beer, wine, and because of carry-over effect, in milk, blood, liver, kidney, and poultry meat from animals fed with contaminated feed (M a n t l e, 2002).

Zearalenone (ZEA, Fig. 1, C) is an estrogenic mycotoxin produced by several species of the fungal genera *Fusarium graminearum* and *Fusarium culmorum*. It widely exists in maize, barley, wheat, oats, sorghum and sesame seeds, as well as in hay and corn silage, which are prime ingredients in many food products for humans and animals (CAST, 2003). ZEA is classified by IARC under group 3 carcinogen (IARC, 1999).

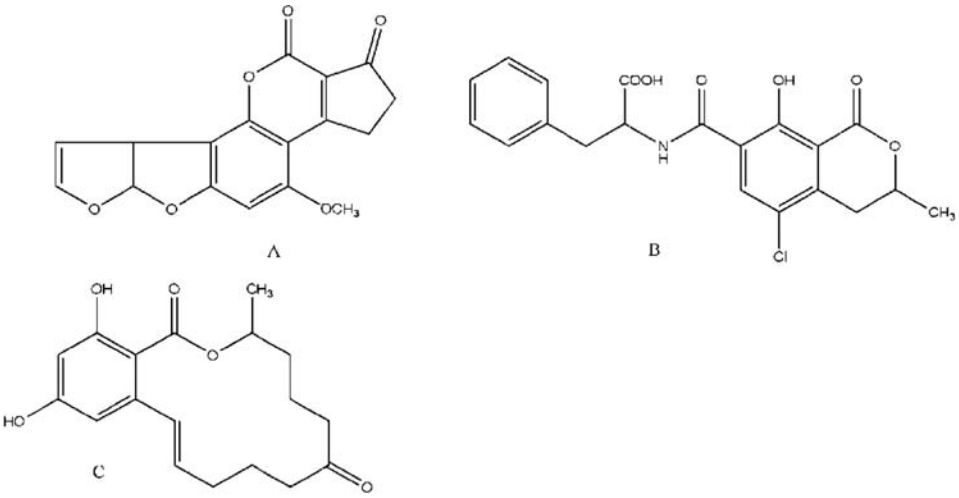
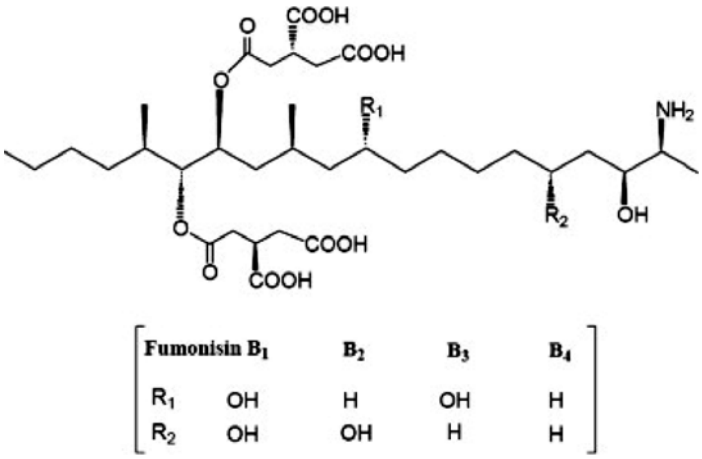


Fig. 1 — Structures of some mycotoxins: A — aflatoxin B_1, B — ochratoxin A, C — zearalenone

Fumonisins (Figure 2) are a group of mycotoxins produced by several agriculturally important fungi, including *Fusarium verticillioides*, which is a common fungal contaminant of corn and maize-derived products worldwide (W a n g, 2006). Fumonisins are classified into four main groups, the A, B, C and P-series. The B-series fumonisins are the most abundant analogs produced by the wild-type strains, with fumonosin B_1 (FB_1) accountig for approximately

70% of the total content (N e l s o n, 1993). FB_1 is also believed to be the most toxic constituent (M a r a s a s, 2001).



Fumonisin B_1		B_2	B_3	B_4
R_1	OH	H	OH	H
R_2	OH	OH	H	H

Fig. 2 — Chemical structure of the B-series fumonisins

Deoxynivalenol (DON) is a member of the trichothecene family of mycotoxins. The occurrence of deoxynivalenol is associated primarily with *Fusarium graminearum* and *Fusarium culmorum*, both of which are important plant pathogens commonly found in cereals and other crops (JECFA, 2001). DON is commonly detected in cereals and grains, particularly in wheat, barley, maize and their by-products. Although DON is among the least toxic of the trichothecenes, it is the most frequently detected one throughout the world, and its occurrence is considered to be an indicator of the possible presence of other, more toxic trichothecenes (L o m b a e r t, 2002).

The best protection against mycotoxins is to monitor their presence in food. Many countries have enacted regulations stipulating maximum permissible amounts of mycotoxins in food. Allowed limits of mycotoxins in food in Serbia (Pravilnik, 5/92) and the European Union (E C, No 1881/2006) are set by regulations. Maximum allowed limits for aflatoxins in Serbia (3 ppb) and EU (4 ppb) are similar. The limit for ochratoxin A in EU (3 ppb) is lower than that in Serbia (10 ppb). In the case of zearalenone, there is a large difference between the regulations, since the allowed limits in Serbia are much lower. The maximum allowed amounts for fumonisins and deoxynivalenol are not set by the Serbian regulation.

According to the Food and Agriculture Organization of the United Nations (FAO) over 25% of the agricultural commodities worldwide are significantly contaminated by mycotoxins. The best protection against mycotoxins is to monitor their presence in food.

The aim of this paper is to monitor the presence of five mycotoxins, aflatoxins, ochratoxin A, zearalenone, fumonisins and deoxynivalenol in the grain food originating from Serbia.

MATERIALS AND METHODS

Mycotoxin content was determined in 76 samples of different grain food. The selected commodity groups were: 7 samples of muesli, 4 samples of baby food, 17 samples of maize and its derived products (flour, corn flakes, pop corn, grits), 21 samples of grain and its derived products (oat, bran, wheat, barley, rye, flour), 11 samples of soybean and its derived products (milk, tofu, paté, soybean flakes), 2 samples of buckwheat, 5 samples of bread, and 9 samples of confectionary products. Samples were collected from local supermarkets in Novi Sad, Serbia.

The samples were analyzed by the ELISA method. The Neogen Veratox® AFs, OTA, ZEA, FUM and DON test kits were used for the analyses. Free mycotoxins in the samples and controls are allowed to compete with enzyme--labelled mycotoxins (conjugates) for the antibody binding sites. After a wash step, substrate was added, which reacted with the bound conjugate to produce blue colour. More blue colour meant less mycotoxin. The test was read in a microwell reader (Thermolabsystem, Thermo, Finland) to yield optical densities. The optical densities of the controls formed the standard curve, and the sample optical densities were plotted against the curve to calculate the exact concentration of mycotoxin. According to the manufacturer's description (Veratox®, Neogen) the detection limits for AFs, OTA, ZEA, FUM and DON were 1, 2, 25, 50 and 250 (ppb), respectively.

RESULTS AND DISCUSSION

The mycotoxin concentrations in grain food are shown in Table 1. None of the samples were contaminated with aflatoxins (AFs). The most predominant mycotoxin was ochratoxin A with the mean level of 4.84±4.49 ppb in 19.7% of the examined samples. Fumonisins (FUM), zearalenone (ZEA), and deoxynivalenol (DON) were also present in the examined grain food in 14.5, 9.21 and 3.9% of the samples, respectively. Between LOD and LOQ, 50.0, 31.6 and 17.1% of the invastigated samples contained DON, ZEA and OTA, respectively.

Tab. 1 — Mycotoxin concentrations in grain food

Mycotoxins	Percentage of contamination	Range of contamination (ppb)	Mean (SD)	Percentage of contamination between LOD&LOQ	Range of contamination (ppb)
Aflatoxins	0	1.0—8.0	0	0	0.5—1
Ochratoxin A	19.7	2.0—25.0	4.84 ± 4.49	17.1	1.0—2.0
Zearalenone	9.21	25—500	38.4 ± 13.2	31.6	10.0—25
Fumonisins	14.5	50—600	282 ± 246	0	50.0
Deoxynivalenol	3.9	250—2000	920 ± 930	50	100—250

SD: standard deviation

Table 2 presents the contamination frequencies and averages of the examined mycotoxins in various analyzed commodities. The results for muesli showed contamination with OTA and ZEA. The content of OTA in these samples was in accordance with the EC (3 ppb) regulation. The content of ZEA found in 1 sample of muesli was 10.6 ppb, which was also in accordance with the EC regulations (50 ppb). The baby food was contaminated with OTA. OTA was found in all examined samples of baby food, and in all samples, the content of OTA was much greater than the maximum allowed level set by the EC regulation (0.5 ppb). FUM, ZEA and OTA were found in 17 samples of maize and maize products with contamination frequency of 52.9, 11.8 and 5.88%, respectively. The concentrations of these mycotoxins are in accordance with the European regulations. Two samples of grain products had the content of OTA that was higher than the maximum level allowed by the European regulation (3 ppb). DON was found in 1 sample of grain, and this level is lower than the European level (750 ppb). OTA, ZEA and FUM were found in soybean products. The contents of ZEA and FUM in soybean products were in accordance with the EC regulations, but the contents of OTA in 2 samples were higher than the maximum level allowed (3 ppb). One sample of buckwheat had the content of OTA and DON that was much greater than allowed by the EC regulations. Bread samples contained OTA, ZEA and FUM in concentrations which were in accordance with the EC regulations. The confectionary products were contaminated with OTA and FUM, and the content of OTA was not in accordance with the EC regulations. 10 out of 76 samples contained the level of mycotoxins that was much higher than the maximum limits allowed by the European regulative.

Tab. 2 — Contamination frequency (CF), interval (CI), and mean (CM ± SD) of various analyzed commodities

Commodity groups (SN)		OTA	ZEA	FUM	DON
Muesli (7)	CF	28.6	14.3	0	0
	CI	2.01	10.6	0	0
	CM	2.01	10.6	0	0
Baby food (4)	CF	100	0	0	0
	CI	2.28—3.67	0	0	0
	CM	2.83 ± 0.60	0	0	0
Maize (17)	CF	5.88	11.8	52.9	0
	CI	2.71	35.5—53.3	58.2—600	0
	CM	2.71	44.4 ± 12.6	328.9 ± 249.1	0
Grain (21)	CF	9.52	0	0	4.76
	CI	3.66—13.5	0	0	390
	CM	8.6 ± 7.0	0	0	390
Soybean (11)	CF	18.2	36.4	9.09	0
	CI	3.72—4.88	25.1—60.1	56.4	0
	CM	4.3 ± 0.82	38.2 ± 15.4	56.4	0
Buckwheat (2)	CF	50.0	0	0	50.0
	CI	15.9	0	0	2000
	CM	15.9	0	0	2000

Bread (5)	CF	20.0	20.0	0	20.0
	CI	1.81	27.7	0	380
	CM	1.81	27.7	0	380
Confectionary products (9)	CF	11.1	0	11.1	0
	CI	3.18	0	82.5	0
	CM	3.18	0	82.5	0

SN: sample number, SD: standard deviation, CF (%), CI (ppb), CM (ppb)

The results of this test have shown that out of a total of seventy six samples, the presence of mycotoxins was detected in twenty six of them. Some of those 26 samples contained more than one mycotoxin. Table 3 shows the number of the contaminated samples with different number of mycotoxins. As it can be seen, most of the commodity groups were contaminated with one mycotoxin. Two different mycotoxins contaminated one sample of soybean, buckweat, and bread, and three samples of maize. Only one sample of soybean was contaminated with three different mycotoxins.

Tab. 3 — Number of the contaminated samples with different number of mycotoxins

Commodity groups	Number of mycotoxins			
	0	1	2	3
Muesli (7)	5	2	/	/
Baby food (4)	/	4	/	/
Maize (17)	8	6	3	/
Grain (21)	18	3	/	/
Soybean (11)	7	2	1	1
Buckwheat (2)	1	/	1	/
Bread (5)	4	/	1	/
Confectionary products (9)	7	2	/	/

From the obtained results it can be concluded that the regulatives in Serbia should be changed and corrected in accordance with the widely accepted regulatives within the European Union.

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МИКОТОКСИНИ КАО РИЗИК У ПРЕХРАМБЕНИМ ПРОИЗВОДИМА НА БАЗИ ЖИТА

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Резиме

У овом раду приказани су резултати скрининга микотоксина у прехрамбеним производима на бази жита. Узорци су анализирани директном ензимском имуноафинитетном методом ELISA, употребом Neogen Veratax® тестова. У 76 узорака на бази жита одређен је садржај афлатоксина, охратоксина А, зеараленона, фумонизина и деоксиниваленола. Циљ рада је био да се одреди концентрација наведених микотоксина и да се њихова количина упореди са Правилницима Србије и Европске Уније, као и да се укаже на ризик присуства микотоксина у овој врсти прехрамбених производа. Микотоксини су детектовани у 34.2% узорака, а у 13.2% количина микотоксина је била већа од максимално дозвољене прописане правилником Европске Уније. Ни у једном од анализираних узорака нису пронађени афлатоксини. Охратоксин А је пронађен у највећем броју узорака (19.7%), затим фумонизин (14.5%), зеараленон (9.21%) и деоксиниваленол (3.9%).

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SCREENING OF MYCOTOXINS IN ANIMAL FEED FROM THE REGION OF VOJVODINA

ABSTRACT: This paper shows the results of screening of mycotoxins in animal feed originating from the region of Vojvodina. Permanent screening is needed on all levels of production and storage, as well as the use of known methods to reduce mould contamination or toxin content in feedstuffs and feed. A total of 56 representative samples were collected from feed companies from the region of Vojvodina. Samples were collected during February 2009. The collected samples included 41 samples of feedstuffs (soybean, soybean meal, soybean grits, soybean cake, maize, sunflower meal, barley, wheat feed flour, rapeseed meal, dehydrated sugar beet pulps, alfalfa meal, yeast, dried whey, fish meal, meat-bone meal) and 15 samples of complete feedingstuffs. The amounts of aflatoxins, ochratoxin A, zearalenone, fumonisin and deoxynivalenol were determined. Screening method for the analysis was done using Neogen Veratox® testing kits. The test itself is a competitive direct enzyme-linked immunosorbent assay (CD-ELISA). Mycotoxins were present in 71.4% of the samples, but the values determined were below the maximum allowed limits for both Serbian and EC reference values. Zearalenone was found with the highest incidence (57.1% of samples), followed by ochratoxin A (37.5%), fumonisin (33.9%), deoxynivalenol (14.3%) and aflatoxins (3.6%).

KEYWORDS: animal feed, ELISA, mycotoxins, screening

INTRODUCTION

Mycotoxins are secondary metabolites produced by filamentous fungi that cause a toxic response (mycotoxicosis) when ingested by higher animals. Many fungi of the genera *Fusarium*, *Alternaria, Penicillium, Aspergillus*, *Cladosporium* and others are well known as producers of a great number of various toxic metabolites. The produced mycotoxins are thermo resistant and lose none of their toxicity during thermal processing (Stojanović et al., 2005).

Moulds that produce toxins may contaminate human foods and animal feeds through fungal growth prior to and during harvest, or during (improper) storage (Bhatnagar et al., 2004). Plants may be contaminated by mycotoxins in two ways: fungi growing as pathogens on plants, or growing saprophytically on stored plants. However, not all fungal growth results in mycotoxin formation, and the detection of fungi does not necessarily imply the presence of mycotoxins (Binder et al., 2007). The formation of mycotoxins is affected by biological, physical and chemical factors (D'Mello and Macdonald, 1997). The same toxin may be formed by a variety of species of fungi, but not necessarily by all the strains of the same species. Similarly, in certain instances, the same species of fungi may produce several forms of mycotoxins.

For practical consideration, in the feed manufacturing process, aflatoxins, trichothecenes, zearalenone, ochratoxins, and fumonisins are of particular interest (Table 1), though the extent of harm each toxin (group) can cause is highly species-dependent (Binder, 2007). Mycotoxins, when present in the diet, cause acute and/or chronic adverse health effects in animals and humans, depending upon the level consumed (Thieu et al., 2008).

Tab. 1 — Overview of the most relevant mycotoxins in animal production (Binder, 2007)

Major classes of mycotoxins	Most relevant representatives in grains and feed	Examples of mycotoxin-producing fungi	Effects observed in animals
Aflatoxins	Aflatoxin B1, B2, G1, G2	*Aspergillus flavus, Aspergillus parasiticus*	Liver disease (hepatotoxic, hepatocarcinogen), carcinogenic and teratogenic effects
Trichothecenes	Deoxynivalenol, T-2 toxin	*Fusarium graminearum, Fusarium sporotrichioides, Fusarium poae, Fusarium equiseti*	Immunologic effects, hematological changes, digestive disorders (emesis, diarrhea, reduced feed intake) dermatitis, oral lesions, hemorrhages of intestinal tissues, edema
Zearalenone	Zearalenone	*Fusarium graminearum*	Estrogenic effects (edema of vulva, enlargement of uterus), atrophy of ovaries and testicles, abortion
Ochratoxins	Ochratoxin A	*Aspergillus ochraceus, Penicillium verrucosum, Penicillium viridicatum*	Nephrotoxicity, porcine nephropathy, mild liver damage, immune suppression
Fumonisins	Fumonisin B1, B2, B3	*Fusarium verticillioides* (syn., *moniliforme), Fusarium proliferatum*	Pulmonary edema, leukoencephalomalacia, nephrotoxicity, hepatotoxicity

Mycotoxin-producing mould species are extremely common and can grow on a wide range of substrates under a wide range of environmental conditions. For agricultural commodities, the severity of crop contamination tends to vary

from year to year, based on climate and other environmental factors. Mycotoxins occur, with varying severity, in agricultural products all around the world. The estimate usually given is that one quarter of the world's crops are contaminated to some extent with mycotoxins (Fink-Gremmels, 1999; Mannon and Johnson, 1985).

Mycotoxins can enter the food chain in field, during storage, or at later points. Mycotoxin problems are exacerbated whenever shipping, handling, and storage practices are conducive to mould growth. Animal feeds are an essential part in the farm animal to human food chain; therefore, infectious and non-infectious hazards present in animal feeds pose a threat to human health. Mycotoxin contamination of feeds results in economic loss and transmission of toxins into the food chain.

Since it is normally impracticable to prevent the formation of mycotoxins, the food industry has established internal monitoring methods. Similarly, government regulatory agencies survey the occurrence of mycotoxins in foods and feeds and establish regulatory limits. Maximum tolerated levels of mycotoxins in animal feed have been established in many countries. Allowed limits for mycotoxins in feed on the territory of the European Union are regulated by the regulations of the European Union (EC 32/2002, EC 100/2003, EC 576/2006). Guidelines for establishing these limits are based on epidemiological data and extrapolations from animal models, taking into account the inherent uncertainties associated with both types of analysis. Estimations of an appropriate safe dose are usually stated as a tolerable daily intake (Kuiper-Goodman, 1998; Kuiper-Goodman, 1994; Smith et al., 1995). Countries that are members of the European Union have harmonized their regulations while other countries, like Serbia, have their own regulations. Allowed limits for mycotoxins in animal feed in Serbia are determined by official regulations of Serbia (Official Gazette of SFRY, 2/90, 27/90). The main differences between the EU and Serbian regulations for the feedstuffs and feedingstuffs are as follows: different categories of feedingstuffs; different values for allowed limits; in the EU, complete and complementary feedingstuffs categories are separated as opposed to the Serbian regulations, and in Serbia, the maximum allowed limits for FUM have not been determined. In Serbia, monitoring of mycotoxins is not obligatory at present, but the approval of a new law has been awaited, which will be in accordance with the EU law. By the new law, monitoring will be compulsory.

The aim of our work was to screen the presence of mycotoxins in animal feed originating from the region of Vojvodina. Permanent screening is needed on all levels of production and storage, as well as the use of known methods to reduce mould contamination or toxin content in feedstuffs.

MATERIAL AND METHODS

A total of 56 representative samples (1—2 kg per sample) were collected from the feed companies in Vojvodina. Samples were collected during February 2009. The collected samples included 41 samples of feedstuffs (soybean,

soybean meal, soybean grits, soybean cake, maize, sunflower meal, barley, wheat feed flour, rapeseed meal, dehydrated sugar beet pulps, alfalfa meal, yeast, dried whey, fish meal, meat-bone meal) and 15 samples of complete feedingstuffs.

The amounts of aflatoxins (AFS), ochratoxin A (OTA), zearalenone (ZEA), fumonisin (FUM) and deoxynivalenol (DON) were determined. Screening method for the analysis was done using Neogen Veratox® testing kits with limits of detection of 1 μg/kg (ppb) for ochratoxin A, 2 μg/kg (ppb) for aflatoxins, 10 μg/kg (ppb) for zearalenone, 50 μg/kg (ppb) for fumonisin and 0.1 mg/kg (ppm) for DON.

The test itself is a competitive direct enzyme-linked immunosorbent assay (CD-ELISA). Free mycotoxins in the samples and controls are allowed to compete with enzyme-labelled mycotoxins (conjugates) for the antibody binding sites. After a wash step, substrate is added, which reacts with the bound conjugate to produce blue colour. More blue colour means less mycotoxin. The test is read in a microwell reader (Thermolabsystem, Thermo, Finland) to yield optical densities. The optical densities of the controls form the standard curve, and the sample optical densities are plotted against the curve to calculate the exact concentration of mycotoxin.

RESULTS AND DISCUSSION

A total of 56 samples of feedstuffs and complete feedingstuffs were analyzed. Mycotoxins were found in 71.4% of the samples, but the values determined were below the maximum allowed limits according to both Serbian and EC reference values. ZEA was found with the highest incidence (57.1% of samples), followed by OTA (37.5%), FUM (33.9%), DON (14.3%) and AFS (3.6%). Incidence rate of aflatoxins was very low (3.6%) which was expected since aflatoxins are rarely found in Serbia (Jajic et al., 2008). The obtained results were compared with available literature. No results were found regarding the presence of FUM in feedstuffs and complete feedingstuffs in Serbia.

Tab. 2 — Occurrence of mycotoxins in maize samples

No. of samples	Feedstuff	AFS (ppb)	OTA (ppb)	ZEA (ppb)	FUM (ppb)	DON (ppm)
4	Maize	—[a]	—	—	438	—
		—	—	—	350	0.46
		—	—	—	543	—
		—	—	—	—	—

[a] toxin was not detected

The results of screening of mycotoxins in the maize samples are given in Table 2. FUM and DON were found in 75 and 25% of the samples, respectively, but none of the samples was contaminated with AFS, OTA and ZEA.

Jajić et al. (2008) analyzed DON in maize samples collected in 2004 (10 samples) and 2005 (66 samples). The number of positive samples for

DON in maize was 50% in 2004 and 43.9% in 2005. Mycotoxicological analyses of maize in the previous investigations showed significant contamination with AFS, OTA and ZEA. In 2000, Jajić et al. (2001) analyzed 38 samples of maize and found AFS in 73.6%, OTA in 78.9% and ZEA in 86.8% of the analyzed samples. Two years later, in the study conducted on the samples collected from the region of Vojvodina, Mašić et al. (2003) analyzed 53 samples of maize and found lower incidence of mycotoxins; AFS were found in 16.9%, OTA in 24.5% and ZEA in 35.8% of the analyzed samples. This shows that AFS, OTA and ZEA concentrations in maize may vary considerably, probably as a result of different drying techniques and weather conditions.

Tab. 3 — Occurrence of mycotoxins in soybean, soybean meal, soybean grits and soybean cake samples

No. of samples	Feedstuffs	AFS (ppb)	OTA (ppb)	ZEA (ppb)	FUM (ppb)	DON (ppm)
5	Soybean	—[a]	—	—	—	—
		—	—	—	—	—
		—	—	—	—	—
		—	2.63	36.5	—	—
		—	—	26.9	—	—
7	Soybean meal	—	5.12	55.3	—	—
		—	3.97	56.3	—	—
		—	3.52	61.0	—	—
		5.53	3.75	56.9	97.4	0.25
		5.20	2.61	69.5	—	—
		—	—	74.3	—	—
		—	4.87	61.8	—	—
2	Soybean grits	—	3.32	48.9	—	—
		—	—	50.2	—	—
1	Soybean cake	—	—	—	—	—

[a] toxin was not detected

The results of screening of mycotoxins in soybean, soybean meal, soybean grits and soybean cake samples are given in Table 3. 3 out of 5 analyzed samples of soybean did not contain mycotoxins. OTA was detected in one and ZEA in two samples. AFS, FUM and DON were not present in any soybean sample, which is in accordance with the previous results of Jakić et al. (2005) who investigated 63 samples of soybean collected in the period from 1999 to 2004, and their results showed that 0% of soybean samples were contaminated with aflatoxins. In soybean meal samples, ZEA was found with the highest incidence (100% of samples), followed by OTA (85.7%), AFS (28.6%), FUM (14.3%) and DON (14.3%). Jajić et al. (2001) analyzed 10 samples of soybean meal collected in 2001 and, according to their results, 100, 90 and 100% of the samples were contaminated with AFS, OTA and ZEA, respectively. This is in contrast to the data reported by Mašić et al. (2003) who investigated 43 samples of soybean meal from the region of Vojvodina in 2002, and found that 2.3, 6.9 and 4.6% of the samples were contaminated with AFS,

OTA and ZEA, respectively. Jajić et al. (2008) analyzed DON in soybean and soybean meal samples collected in 2004 (13 samples) and 2005 (11 samples). The number of positive samples for DON was 7.7% in 2004 and 9.1% in 2005, which is in accordance with our results.

Tab. 4 — Occurrence of mycotoxins in sunflower meal samples

No. of samples	Feedstuff	AFS (ppb)	OTA (ppb)	ZEA (ppb)	FUM (ppb)	DON (ppm)
7	Sunflower meal	—[a]	2.58	48.4	168	0.27
		—	—	41.3	—	—
		—	2.24	35.0	—	0.33
		—	3.82	32.8	—	—
		—	—	38.9	—	0.33
		—	2.62	44.2	—	—
		—	2.37	38.8	67.5	0.28

[a] toxin was not detected

The results of screening of mycotoxins in sunflower meal samples are given in Table 4. Of the 7 analyzed samples, ZEA was found with the highest incidence (100% of samples), followed by OTA (71.4%), DON (57.1%) and FUM (28.6%). None of the samples was contaminated with AFS. In the period between 1999 and 2001, Jajić et al. (2001) investigated sunflower meal (21 samples) for the presence of ZEA. The percentage of samples contaminated with ZEA during a 3-year period was 100%, which is in accordance with our results. Jajić et al. (2008) analyzed DON in sunflower and sunflower meal samples collected in 2004 (9 samples) and 2005 (10 samples). The number of positive samples for DON was 44.4% in 2004 and 50% in 2005. Our results show that the percentage of samples contaminated with DON was somewhat higher and was 57.1%. The incidence of OTA in sunflower meal was registered in 71.4% of the analyzed samples. Previous analyses (Jajić et al., 2001) showed that in years 1999, 2000 and 2001, the percentage of samples contaminated with OTA was higher (100, 92.3 and 100%, respectively). Analyses of sunflower meal showed that no samples were contaminated with aflatoxins, which is in accordance with the results reported by Mašić et al. (2003) who analyzed 19 samples of sunflower meal in 2002 from the region of Vojvodina.

Tab. 5 — Occurrence of mycotoxins in fish and meat-bone meal samples

No. of samples	Feedstuffs	AFS (ppb)	OTA (ppb)	ZEA (ppb)	FUM (ppb)	DON (ppm)
5	Fish meal	—[a]	6.83	—	—	—
		—	—	—	—	—
		—	—	—	—	—
		—	—	—	—	—
		—	—	—	—	—
2	Meat — bone meal	—	—	—	—	—
		—	—	—	—	—

[a] toxin was not detected

The results of screening of mycotoxins in fish and meat-bone meal samples are given in Table 5. A total of 5 samples of fish meal were analyzed. Only one sample was positive for the presence of OTA and other mycotoxins were not detected. In 2 samples of meat-bone meal mycotoxins were not detected.

Tab. 6 — Occurrence of mycotoxins in various feedstuffs samples

No. of samples	Feedstuffs	AFS (ppb)	OTA (ppb)	ZEA (ppb)	FUM (ppb)	DON (ppm)
1	Barley	—[a]	—	—	—	—
1	Wheat feed flour	—	4.79	35.7	—	0.60
1	Rapeseed meal	—	—	—	—	—
1	Dehydrated sugar beet pulps	—	—	—	—	—
2	Alfalfa meal	— —	9.48 3.49	177 159	— —	0.28 —
1	Yeast	—	2.26	31.8	—	—
1	Dried whey	—	—	—	—	—

[a] toxin was not detected

The results of screening of mycotoxins in various feedstuffs samples are given in Table 6. One sample of barley, wheat feed flour, rapeseed meal, dehydrated sugar beet pulps, yeast, dried whey and two samples of alfalfa meal were analyzed. Mycotoxins were not detected in barley, rapeseed meal, dehydrated sugar beet pulps and dried whey. Analyses showed that wheat feed flour, alfalfa meal and yeast were contaminated with OTA and ZEA, while DON was found in wheat feed flour and in one sample of alfalfa meal. None of the samples was contaminated with AFS and FUM. The number of analyzed samples was small and cannot be interpreted as the actual situation in the field conditions.

Tab. 7 — Occurrence of mycotoxins in complete feedingstuffs samples

No. of samples	Complete feedingstuffs	AFS (ppb)	OTA (ppb)	ZEA (ppb)	FUM (ppb)	DON (ppm)
15	Complete mash for piglets — pre-starter	—[a]	—	—	—	—
	Complete mash for piglets from 1 to 15 kg — starter	—	—	—	270	—
	Complete mash for piglets from 1 to 15 kg — starter	—	—	37.7	439	—
	Complete mash for piglets from 15 to 25 kg — starter	—	—	27.2	232	—
	Complete mash for pigs growth and fattening from 25 to 60 kg	—	—	—	555	—
	Complete mash for gilts	—	—	—	291	—
	Complete mash for gestating sows	—	—	—	288	—
	Complete mash for lactating sows and boars	—	—	37.8	479	—

Complete mash for dairy cows over 20 l milk per day	—	—	52.6	335	—
Complete mash for cattle fattening from 250 to 350 kg	—	—	40.2	415	—
Complete mash for cattle fattening from 250 to 350 kg	—	5.24	129	500	—
Complete mash for broilers I	—	8.89	36.7	430	—
Complete mash for broilers I	—	—	52.0	286	—
Complete mash for layers	—	2.66	58.4	270	—
Complete mash for trout	—	—	33.6	—	—

[a] toxin was not detected

The results of screening of mycotoxins in complete feedingstuffs samples are given in Table 7. A total of 15 samples of complete feedingstuffs were analyzed. FUM was found with the highest incidence (86.6% of samples), followed by ZEA (66.6%) and OTA (20%). None of the samples was contaminated with AFS and DON.

The difference in contamination level in our samples and samples analyzed in previous years could be attributed partly to agricultural factors and partly to variations in the susceptibility to different *Fusarium, Aspergillus* and *Penicillium* species in interaction with climatic factors.

CONCLUSION

Although this screening showed that 71.4% of the samples were contaminated with mycotoxins, concentrations were lower than the maximum level adopted by Serbian and European Commissions' regulations. Aflatoxins were found with the lowest incidence (3.6%) followed by deoxynivalenol (14.3%), fumonisin (33.9%), ochratoxin A (37.5%) and zearalenone (57.1%). Since all mycotoxins were found in the analyzed samples, it can be concluded that the monitoring is necessary. Given the vast diversity of commodities that may be infected by fungi, it is important to acknowledge the fact that the presence of specific fungi does not necessarily mean that a fungal toxin is present. It is, therefore, pertinent to analyse the presence of mycotoxins in all cases as far as possible. The results will help ensure better quality assurance in the feed, as well as develop the tools for management decision on the fate of feeds that do not meet the required standards. Based on the given results for the presence of fumonisins in feed, inclusion in the National regulation should be considered. Also, there is a need for harmonization of the National regulations with those of EU.

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СКРИНИНГ МИКОТОКСИНА У ХРАНИ ЗА ЖИВОТИЊЕ СА ПОДРУЧЈА ВОЈВОДИНЕ

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Резиме

Рад приказује резултате скрининга микотоксина у храни за животиње са подручја Војводине. Перманентан скрининг је потребан на свим нивоима производње и складиштења, као и коришћење познатих метода за смањење контаминације плеснима или токсинима у хранивима и храни за животиње. Прикупљено је укупно 56 репрезентативних узорака из фабрика за прозводњу хране за животиње на подручју Војводине. Узорци су прикупљени током фебруара 2009. Прикупљени узорци су обухватали 41 узорак хранива (соја, сојина сачма, сојин гриз, сојина погача, кукуруз, сунцокретова сачма, јечам, пшенично сточно брашно, сачма уљане репице, суви резанац шећерне репе, брашно од луцерке, сточни квасац, сурутка у праху, рибље брашно, месно коштано брашно) и 15 узорака потпуних смеша. Одређена је количина афлатоксина, охратоксина А, зеараленона, фумонизина и деоксиниваленола. Скрининг метода за анализу је изведена помоћу Neogen Veratox® тестова. Овај метод подразумева директну компетитивну ензимску имуноафинитетну методу (CD-ELISA). Микотоксини су детектовани у 71,4% узорака, али су утврђене вредности испод максималне дозвољене границе прописане правилником Србије и ЕУ. Зеараленон је пронађен у највећем броју узорака (57,1% узорака), затим охратоксин А (37,5%), фумонизин (33,9%), деоксиниваленол (14.3%) и афлатоксини (3.6%).

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YEAST BIODIVERSITY IN SLOVENIAN WINE REGIONS: CASE AMINO ACIDS IN SPONTANEOUS AND INDUCED FERMENTATIONS OF MALVASIA

ABSTRACT: Microbial biodiversity can also be reflected in final product composition. The work described in this paper investigates the differences in the amino acid composition of 14 Malvasia musts/wines fermented with local and commercial starter yeasts, comparing all to the spontaneous fermentations of must of the same origin. We tried to ascertain whether the changes were dependent upon different initiations of fermentations. A comparative study of free and total amino acid evolution was prepared. The total concentration of 15 amino acids studied was 1975 mg/l, and the concentration of fraee amino acids was 1061 mg/l. Spontaneous and induced fermentations showed different fermentation rates. Three to nine days were needed to reduce sugar by 50%. Although the proline is regarded as non-assailable amino acid, decreases in concentration were observed. Lysine was the only amino acid where the concentration increased. The minimal uptakes of amino acids occurred during spontaneous fermentations, whereas the maximal uptakes were observed in the fermentations inoculated with local starters.

KEYWORDS: fermentation, biodiversity, yeasts, must, wine, amino acids, proline, Malvasia

INTRODUCTION

The fermentation of grape juice into wine is a complex biochemical process involving interactions between yeasts, bacteria, fungi and their viruses. During natural fermentation many different *Saccharomyces cerevisiae* strains undergo sequential substitutions (Fleet and Heard, 1993). The use of inoculum dramatically changes the microbiology of the wine fermentation process (Raspor et al., 2002). Over the past years, there has been an increasing use of locally selected yeasts for controlled must fermentation in the countries with a winemaking tradition (Raspor et al., 2006). Though commercial yeasts exist to accomplish must fermentation, the use of local yeasts is believed to be

much more effective (Perez-Coello et al., 1999). Local yeasts are presumed to be more competitive because of their acclimation to the environmental conditions (Zagorc et al., 2001). Therefore, they would be able to dominate the fermentation successfully and become the most important biological agents responsible for the vinification (Povhe Jemec et al., 2001). Selection of the appropriate local yeasts assures the maintenance of the typical sensory properties of the wines produced in any given region (Regodon et al., 1997; Povhe Jemec and Raspor, 2005).

The nitrogenous compounds utilized by *Saccharomyces cerevisiae* include different substances, such as ammonia, urea, amino acids, small peptides, and purine and pyrimidine bases (Cooper, 1982). Major nitrogen species in average grape juice are proline, arginine, alanine, glutamate, glutamine, serine and threonine. Ammonium ion levels may also be high, depending on the variety and time of harvest (Henschke and Jiranek, 1993).

The composition of amino acids is of great importance in wine production, since they have a direct influence upon the aromatic composition of wines. Amino acids in wines have a variety of origins. Those indigenous to grape can be completely metabolized by yeasts during the growth phase, others can be excreted by live yeasts or released by proteolysis during the autolysis of dead yeasts, while some are produced by enzymatic degradation of grape proteins (Lehtonen, 1996).

The uptake and metabolism of nitrogen compounds by *S. cereviae* depend not only on the strain and physiological conditions, but also on the physical and chemical properties of the must and technological procedure (Ancin et al., 1996). A key step for the control of any metabolite utilization is the transport of the compound into the cell. Most nitrogen containing compounds are transported via active mechanisms since the cellular concentrations of each of these components would need to be higher than outside the cell. The amino acids active transport in yeast is typically coupled with the movement of ions (Cooper, 1982).

In typical grape juice fermentation, nitrogen-containing compounds present in low concentrations are taken up very quickly, within the decrease of the first two Brix units. Degradation of compounds as nitrogen sources occurs after the biosynthetic pools of amino acids are filled. The metabolism of nitrogen containing compound yields in end products is of great importance in determining wine quality. Nitrogen containing compounds in the grape must can be: (i) utilized as that compound directly in biosynthesis, (ii) converted into a related compound and utilized in biosynthesis, or (iii) degraded releasing nitrogen either as a free ammonium ion or as bound nitrogen. Amino acids are deaminated catabolically in order to release their nitrogen components, leaving behind carbon skeletons that will generally represent a waste product from the yeast's viewpoint. Deamination of amino acids can result in the formation of a-keto acids or higher (fusel) alcohols (Henschke and Jiranek, 1993).

The work described in this paper investigates the differences in the amino acid composition of Malvasia musts/wines fermented with local and commercial starter yeasts, comparing all to the spontaneous fermentations of must of the same origin. Many recent studies were focused on the development of free

amino acids during fermentations, so we concentrated our study on the total amino acid content. We studied the evolution of amino acids in 14 microvinifications to ascertain whether the changes were dependent upon the different initiations of the fermentations. For better understanding of the results obtained, we prepared a comparative study of free and total amino acid contents in a selected fermentation.

MATERIALS AND METHODS

Cultivar. The vine of Malvasia (*Vitis vinifera* L. cv. "Malvasia") can be found in several varieties in Mediterranean wine regions. Our study was focused on the "Istrian Malvasia" variety that grows in the coastal regions of Slovenia, Croatia and northern Italy (C o s m o and P o l s i n e l l i, 1964).

Must. The grapes of Malvasia were harvested in the vintage year of 1997, in the coastal region of Slovenia. The grape berries were ripe, undamaged and no *Botrytis cinerea* was present. The grape berries were crushed into 240 l of must and poured into a 500 l (PVC) container. The must was clarified by sedimentation at 13°C for 18 hours and no sulfur was added. The initial pH value of the must was 3.28 and the concentration of sugars was 227 gl^{-1} (113 mg/l glucose and 114 mg/l fructose).

Yeast starters. The strains belonged to the species *Saccharomyces cerevisiae* and they all possessed killer activity. All yeast strains were stored in the ZIM Culture Collection at the Biotechnical Faculty in Ljubljana.

a) Commercial strains: (B) ZIM 1749, (C) ZIM 1750, (D) ZIM 1751, (E) ZIM 1752.

b) Local strains: (F) ZIM 1640, (G) ZIM 1645, (H) ZIM 1709, (I) ZIM 1667.

Inoculum. Single colony cultures were cultivated in YM medium (0.3% yeast extract, 0.3% malt extract, 1% glucose) until they reached the late exponential phase. The strains were harvested by centrifugation and prepared in aliquots for final concentration in must 10^6 strains/ml. Commercial starters were prepared as suggested by their producer and added in the same concentration to the must.

Fermentation. Fourteen fermentations were performed in glass bottles containing 9 l of Malvasia must. Four commercial yeast starters (B, C, D and E) and four local yeast strains (F, G, H and I) were applied as starters in eight microvinifications. Six fermentations (A0, A1, A2, A3, A4 and A5) of Malvasia must were allowed to ferment spontaneously. The temperature of the cellar was monitored every morning and ranged from 12 to 13°C. Fermentations were followed for 24 days.

Sampling. The samples of Malvasia must/wine were taken from the center of the bottle four times during the fermentation processes. The results presented in this study refer to the following sampling points: (i) must before the inoculation, (ii) at č25% consumed sugar, (iii) at č75% consumed sugar, and (iv) wine at the end of fermentation, when the sugar content was constant. Sampling points refer to the following sampling times (i) after overnight cold settling, (ii) 2^{nd}, 3^{rd}, 5^{th} or 6^{th} day, (iii) 7^{th}, 9^{th} or 11^{th}, and (iv) 24^{th} day of fer-

mentation. Part of the sample was centrifuged (4000g, 15 min) and stored frozen for chemical analyses.

Yeast population monitoring. Part of the sample was immediately plated on YEPD agar plates in different dilution rates to determine the survival of the inoculated strain. The survival of the inoculated strain was determined by killer activity (S t u m m et al., 1997) and karyotype pattern analysis (R a s p o r et al., 2000) of the isolated yeast strains from the fermentations.

Determination of compounds

a) A modified method of C o h e n (1993) was used to detect free amino acids (AaF) and amino acids that compose proteinaceous components (AaP) in wine. The method was based on derivatization with aminoquinolyl-N-hydroxysuccinimidyl carbamate. The compound reacted with amino acids to form stable urea derivatives, which are amenable to analysis by HPLC. Analyses were performed with a Hewlett-Packard 1100 liquid chromatograph using Waters chromatography column Nova Pack C18; length 150 mm, diameter 4 mm, particle size 4 mm (Waters chromatography division, USA). Fluorescence of derivatives was detected by excitation at 250 nm and emission at 395 nm. The samples for total amino acid (AaP) analyses were previously degassed in an ultrasonic bath and filtrated, and the a-amino butyric acid as an internal standard was added. The samples were vaporized and hydrolyzed with 6 M HCl. Aliquots were buffered with borate buffer (pH = 9.3). Each sample was analyzed twice and the mean value was considered for the study.

b) Total nitrogen was determined with the Kjeldahl method (OIV, 1993). The samples were mineralized with H_2SO_4, $CuSO_4$ as a catalyst and K_2SO_4 to raise the boiling point. Distillation of the total nitrogen was performed with Tecator automatic steam equipment (Tecator AB, Sweden).

c) Gas-chromatographic analyses were carried out using a Hewlett-Packard 5890 gas chromatograph. Ethanol, methanol, higher alcohols and ethyl acetate were determined by the direct injection of 1 ml of wine to which 2-methyl-2-propanol was added as an internal standard. A HP-INNOWAX column (60 m x 0.25 mm with 0.25 μm film thickness) was used. The following temperature program was used: 35°C/10 min, then 5°C/min to 100°C and finally, 40°C/min to 200°C. Injector and detector temperatures were 200°C and 250°C, respectively. Hydrogen N-48 at 1 ml/min served as a carrier gas.

d) Sugar (fructose and glucose) was determined using a Hewlett-Packard 1100 liquid chromatograph with Bio-Rad HPX-87H column, and by detector based on refraction index differences. The mobile phase used was 0.007 M H_2SO_4 (OIV, 1993).

RESULTS AND DISCUSSION

Yeast population. During the fermentation of Malvasia the yeast population was monitored by checking the killer activity and by karyotyping. For this purpose, 2856 yeast strains were isolated and tested for their killer activity, and 1405 of them were characterized by karyotyping. In all induced fer-

mentations (commercial starter yeast B-E and local strains F-G) inoculated strains dominated over the indigenous yeast population of Malvasia must. All isolates that originated from the last sampling points of inoculated fermentations possessed identical karyotype patterns, whereas the strains isolated from the spontaneous fermentations (A0-A5) showed higher chromosomal polymorphism. All isolates from the inoculated fermentations (B-I) showed very strong killer activity, so we concluded that the inoculated killer strains were able to suppress the indigenous yeast population of Malvasia must effectively (Zagorc et al., 20001).

Composition of Malvasia must. Surveys of amino acids in grape juices from different vine growing regions have revealed immense variations in both the present amino acid, and the concentrations in which they appear (Henschke and Jiranek, 1993). Actual amount of N required to complete the fermentation is dependent on individual yeast strains present in the must, initial glucose concentration, and presence or absence of oxygen.

After cold settling, the Malvasia must was analyzed for its total nitrogen content using Kjeldahl method. The concentration determined was 811 mg N/l. HPLC determined 146 mg N/l free amino acids, and 169 mg N/l that originated from the amino acids from proteinaceous compounds of the Malvasia must.

Free and total amino acids. The total concentration of 15 amino acids studied was 1975 mg/l, including the amino acids released from proteinaceous substances (AaP) and free amino acids (AaF). The concentration of free amino acids was 1061 mg/l and they represented 53.7% of the AaF+AaP content (Table 1). The content of free amino acids of Malvasia must was poorer in comparison to Malvar must (Dizy and Polo, 1996), which contained 1625 mg/l of the same 15 free amino acids studied.

Tab. 1 — A comparative study of changes in free (AaF) and total (AaF+AaP) amino acid concentrations (mg/l) during the induced fermentation of Malvasia must/wine, inoculated with local yeast strain I

	AaF (mg/l)				AaF + AaP (mg/l)			
	Must	~25%	~75%	Wine	**Must**	~25%	~75%	Wine
Alanine	**123**	13.1	6.6	3.13	**137**	20.0	20.6	11.8
Arginine	**324**	1.41	0.79	0.53	**377**	12.6	10.7	6.46
Asparagine	**3.7**	*	*	0.62	**55.0**	26.5	16.2	16.2
Glutamine	**23.3**	2.53	*	3.29	**154**	31.5	26.1	19.1
Glycine	**4.0**	0.80	0.31	0.76	**81.3**	34.3	26.8	16.7
Histidine	**121**	0.84	*	0.85	**115**	14.3	14.6	6.13
Isoleucine	**8.4**	*	*	0.38	**40.5**	12.1	8.22	5.79
Leucine	**9.5**	*	*	0.49	**83.5**	24.3	15.5	10.1
Lysine	**12.6**	1.14	0.61	1.21	**10.4**	9.17	3.92	8.68
Methionine	**6.7**	*	*	0.48	**20.4**	2.45	4.42	3.95
Proline	**272**	114.6	86.3	88.1	**500**	241	266	231
Serine	**54.8**	2.07	0.94	0.61	**138**	33.6	27.5	12.0
Threonine	**58.7**	18.6	5.41	*	**175**	35.6	30.5	12.8
Tyrosine	**19.9**	0.85	0.54	0.78	**25.1**	3.91	2.84	6.71
Valine	**19.5**	0.52	0.31	0.67	**62.8**	16.4	13.1	8.93
Total	**1061**	156	102	102	**1975**	518	490	408

* < 0.3 mg/l

The most abundant amino acids in the must of Malvasia were proline, glutamine, and threonine, when only AaP was considered. In the AaP fraction, these three amino acids represented 52%. In the AaF fraction, the order of amino acids was different: arginine, proline and alanine, and they represented 68% of the amount of free amino acids. Comparing the amounts of amino acids in AaF and AaP fractions of the Malvasia must, it was observed that the amino acids appearing mainly in the proteinaceous form were asparagine, glutamine, glycine, isoleucine and leucine. On the contrary, alanine, arginine, histidine, lysine and tyrosine were present almost exclusively as free amino acids.

During the induced fermentation I, inoculated with the local strain I (ZIM 1667), the changes in AaF and AaF + AaP concentrations were analyzed four times (Table 1). The fermentation I was chosen for the comparative study, because the wine I was evaluated as the best one in the sensory analyses (results not shown). The rapid decrease of AaF took place in the first phase, when 85% of free amino acids were consumed. All free amino acids, with the exception of proline, threonine and alanine, were almost totally accumulated by yeasts during this phase. The AaF uptakes or releases were stabilized between the second and the third phase of fermentation. Untill the end of the fermentation process I, 68% of proline in free form was accumulated in yeasts, whereas threonine and alanine were slowly depleted from the must/wine towards the end of the fermentation. The final concentration of unconsumed free amino acids was 100 mg/l, with proline having the major part.

Peptides and proteins may also provide a source of N depending on the extent of hydrolysis to amino acids, since some non-*Saccharomyces* species, isolated from the fermenting must, posses significant acid protease activity (Lagace and Bisson, 1990; Spayd and Andersen-Bagge, 1996). This study showed that in the first phase of Malvasia fermentation, 552 mg/l of amino acids were depleted from the proteinaceous compounds. From the second to the third sampling point, the change in the AaP concentration was not significant, but from the third sampling point till the end of the fermentation it decreased by 80 mg/l. The final concentration of AaP in the wine was 300 mg/l. During the fermentation of wine I, the uptake of amino acids that originated from AaP reached 608 mg/l.

The results presented in Table 2 show the depletion of amino acids during the fermentation course in one group of fermentations (spontaneous and inoculated with local or commercial strains). The calculated S. E. M. (standard errors of the mean) was mostly around ± 10% of the mean value. There are few exceptions marked in Table 2. We could find no reasonable explanation for these deviations, as they occurred only in some amino acids and without any predictable cause. The uptake of amino acids in the form of AaF + AaP was higher during the fermentations induced with local strains, where it reached 1609 mg/l. In the case of commercial starter yeasts, the uptake of AaF + AaP was 1484 mg/l, and in spontaneous fermentations it was 1425 mg/l.

Tab. 2 — Mean values* of amino acid concentration (mg/l ± S.E.M.) during the *spont*aneous and induced (*comm*ercial and *local* starter yeasts) fermentations of Malvasia. Dotted squares: exceptions with higher S.E.M.; shading: the mean values that deviate from the other fermentation types (* Number of samples: spontaneous n = 6; local n = 4; commercial n = 4)

	Must	~25% fermented sugar			~75% fermented sugar			Wine		
		spont	comm	local	spont	comm	local	spont	comm	local
Alanine	137	23.4±1.9	30.4±6.1	19.5±0.6	61.7±26	13.3±1.2	13.4±2.4	34.7±12	20.0±3.2	15.4±2.3
Arginine	377	12.9±4.7	28.8±14.6	7.9±1.65	9.4±1.0	6.3±0.4	5.3±2.2	13.0±1.4	10.3±1.5	8.0±1.0
Asparagine	55.0	35.8±3.3	31.4±1.61	29.4±1.6	17.9±1.7	20.8±2.0	17.9±0.6	16.8±3.1	28.1±3.8	22.0±2.9
Glutamine	154	38.6±1.9	32.3±1.6	33.8±1.9	12.5±1.3	17.9±1.7	21.6±1.6	35.3±3.2	40.8±4.2	25.5±3.1
Glycine	81.3	26.9±1.1	35.1±3.9	27.6±2.5	21.9±1.9	15.7±1.7	17.9±3.0	46.7±3.5	39.6±4.9	17.1±1.8
Histidine	115	10.4±0.5	15.1±2.4	10.6±1.3	23.2±1.6	5.9±0.7	8.5±2.0	20.8±4.3	10.7±1.3	6.1±0.6
Isoleucine	40.5	10.7±0.6	12.8±1.5	10.4±0.7	10.2±0.6	6.8±0.7	6.5±0.6	12.1±0.4	12.1±2.2	7.9±1.1
Leucine	83.5	16.6±0.9	18.9±1.8	17.9±2.3	19.2±1.3	12.4±1.6	11.6±1.3	23.0±1.8	18.0±3.2	13.8±1.6
Lysine	10.4	15.6±1.7	13.2±1.1	11.8±1.0	3.8±1.0	9.4±0.6	7.1±1.0	13.0±0.7	16.8±2.5	12.3±1.6
Methionine	20.4	0.7±0.2	4.6±2.7	1.8±0.3	5.1±0.6	2.7±0.5	3.4±0.5	9.3±0.8	8.9±1.7	5.4±1.0
Proline	500	309±28	261±16	218±14	239±19	216±24	225±14	253±8.6	218±4.2	186±15
Serine	138	29.4±3.9	32.8±3.43	27.0±2.50	26.9±2.3	16.8±1.7	18.3±3.1	23.5±1.6	17.6±2.0	15.1±1.8
Threonine	175	19.3±1.0	34.9±7.7	23.4±4.2	30.0±3.4	14.3±1.5	18.4±4.1	26.8±2.7	17.5±1.9	15.3±1.6
Tyrosine	25.1	4.7±1.2	4.1±1.4	2.7±0.6	7.5±1.5	2.9±0.6	2.3±0.5	9.6±1.9	15.5±2.3	5.5±1.3
Valine	62.8	16.0±1.0	18.3±1.7	15.2±0.7	13.6±1.0	10.1±0.8	10.3±1.0	16.7±1.3	17.2±2.5	11.3±1.5
TOTAL	1975	618±39	574±52	457±28	502±45	372±29	387±33	550±12	491±38	366±22

According to the results (Tables 1 and 2), 4 groups of concentration curves resulting from uptakes and releases of amino acids can be distinguished. The first group consists of amino acids that appeared in AaF form and were completely consumed: alanine, arginine, histidine and tyrosine. The second group consists of amino acids which, besides the AaF form, partially accumulated the AaP form: asparagine, glutamine, glycine, isoleucine, leucine, methionine, serine and valine. Proline had a third pattern of depletion from the medium, although it is regarded as non-assailable. Lysine represents the forth one, since its concentration increased during the fermentation processes.

Studying the minimal and maximal changes in the concentration of AaF + AaP in the fermentation processes of induced and spontaneous fermentations of Malvasia (Table 3), some interesting differences can be detected. Among the 15 studied amino acids, the minimal uptake (light shading) in the most cases occurred during the spontaneous fermentations.

Tab. 3 — Minimal and maximal differences in the concentration of AaF + AaP (mg/l) in the must (C_m) and wines (C_w) of induced and spontaneous fermentations of Malvasia. The percentages present the accumulated part of the amino acid.

	C_m — C_w (m/l)						%					
	Spontaneous		Local		Commercial		Spontaneous		Local		Commercial	
	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	max
Alanine	47	122	115	125	112	125	33.4	89.1	84.9	91.2	81.7	91.2
Arginine	360	368	366	370	364	371	95.5	97.6	97.1	98.1	96.6	98.4
Asparagine	29	49	25	39	20	38	53.5	89.1	45.5	70.9	36.4	69.1
Glutamine	110	129	120	135	108	126	71.4	83.8	77.9	87.7	70.1	81.8
Glycine	24	48	59	67	34	54	29.5	59.0	72.6	82.4	41.8	66.4

Histidine	78	104	107	110	102	108	67.8	90.4	93.0	95.6	88.7	93.9
Isoleucine	27	30	29	35	24	34	66.7	74.1	71.6	86.4	59.3	83.9
Leucine	54	65	66	73	60	74	64.7	77.8	79.0	87.4	71.9	88.6
Lysine	–5	–0.4	1.7	–6.3	0.6	–11	*	*	16.3	*	5.8	*
Methionine	9	13	12	16	8	16	44.1	63.7	58.8	78.4	39.2	78.4
Proline	230	287	269	336	272	290	46.0	57.4	53.8	67.2	54.4	58.0
Serine	110	118	118	126	116	126	79.7	85.5	85.5	91.3	84.1	91.3
Threonine	141	156	155	162	154	163	80.6	89.1	88.6	92.6	88.0	93.1
Tyrosine	10	20	16	22	6	16	39.8	79.7	63.7	87.6	23.9	63.7
Valine	40	50	47	54	41	52	63.7	79.6	74.8	86.0	65.3	82.8

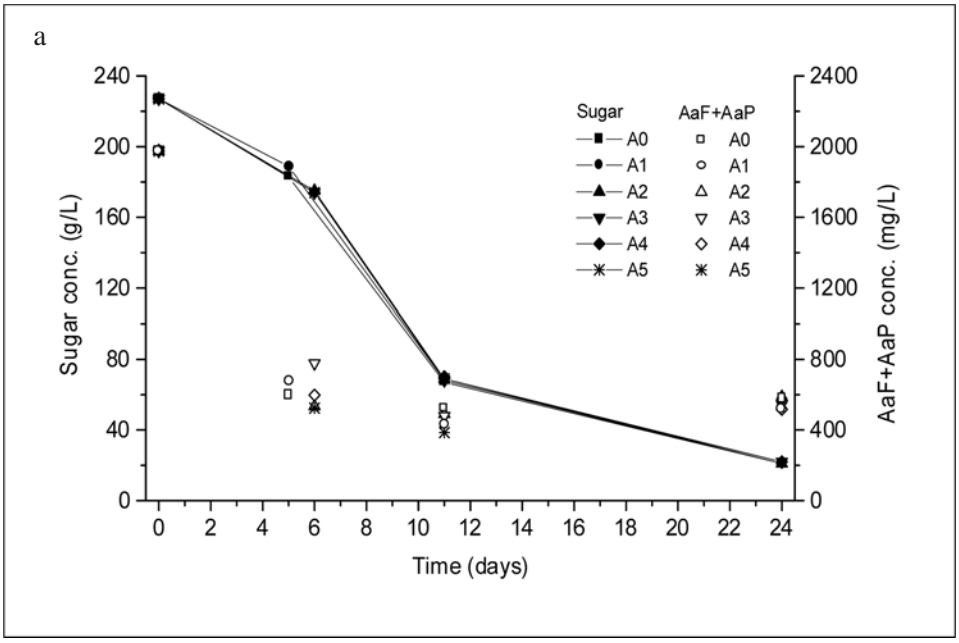
* Increased during fermentation

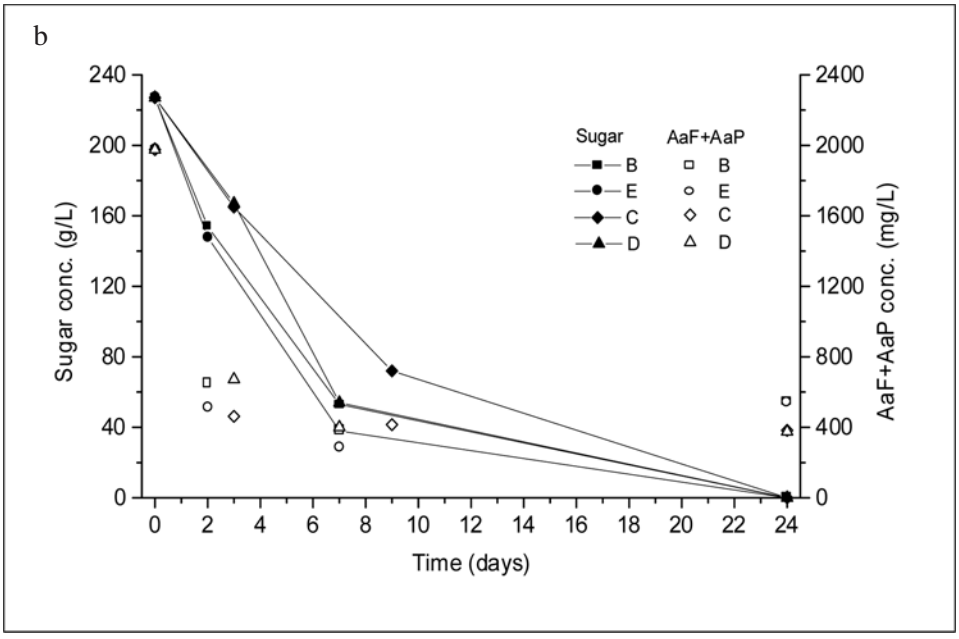
One of the explanations for this phenomenon could be a lower number of fermenting cells. The maximal uptake (dark shading) was in most cases seen in fermentations inoculated with local starters. This observation could support the hypothesis that local strains are more adapted to the fermentation process of a local substrate. According to the results, the maximal uptakes of amino acids were usually higher than 80%, only in the case of proline they were lower (67%). The minimal uptakes were at their lowest value, at around 30% in the case of asparagine, glycine, alanine, methionine and tyrosine, and around 60% in the case of leucine, isoleucine, valine and hystidine. The difference between minimal and maximal uptakes of arginine was not significant; the values were about 95%. An increase of amino acid could only be observed in the case of lysine.

Proline and lysine. The total concentration of lysine increased by 0.4 to 11 mg/l during the fermentations. In the Malvasia must, the initial concentration of lysine was 10.4 mg/l, which means that the final concentrations of lysine in the wines were, in some cases, two times higher than the initial. Our result coincides with those found in Garnacha fermentations (Goni and Ancin-Azpilicueta, 1999a, b), where lysine was found in low concentration, and its total concentration increased during fermentations, too. In chemically defined grape juice Jiranek et al. (1990) could observe only higher uptakes of lysine.

Although the proline is regarded as non-assailable amino acid, we noticed decreases in the concentration. Half of proline in both AaF and AaP form was already accumulated in the first phase of fermentation. During the induced fermentations, when local and commercial strains inoculated, the total amount of proline was reduced by 62 and 56%, respectively. During the spontaneous fermentations, the total amount of proline was reduced by 49%. According to the literature, the accumulation of proline could occur because of oxygen presence in the early phase of fermentation, since the mechanism of proline uptake is known. Under the typical anaerobic conditions of wine fermentations, proline is not utilized by yeast, but under aerobic conditions *Saccharomyces cerevisiae* can use proline as N source (Ingledew et al., 1987). On the contrary, Dizy and Polo (1996) showed in their study that during the fermentation of Malvar must, the concentration of proline increased, which could be also a consequence of arginine metabolism.

Fermentation rates. Goni and Ancin-Azpilicueta (1999a) observed that the fermentation rates of Garnacha musts were similar for both the


a
Sugar conc. (g/L)
AaF+AaP conc. (mg/L)
Time (days)
Sugar
AaF+AaP
A0
A1
A2
A3
A4
A5


b
Sugar conc. (g/L)
AaF+AaP conc. (mg/L)
Time (days)
Sugar
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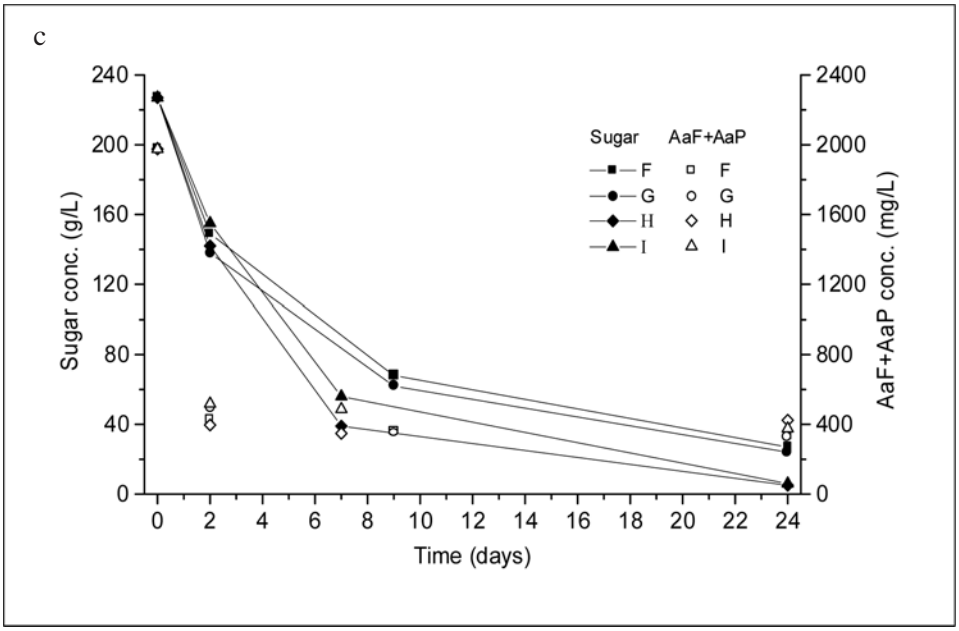

Fig. 1 — The fermentation rates of spontaneous (a), and induced fermentations inoculated with commercial (b), and local strains (c) with the total amino acid concentrations represented individually for each of 14 fermentations.

inoculated and spontaneous fermentations, which was not the case in our study. The fermentations induced with starter strains, or those allowed to ferment spontaneously, showed different fermentation rates. It is evident from Figure 1a that spontaneous fermentations needed 9 days to ferment 50% of sugar. Four to six days were needed for the same reduction in the fermentations induced by commercial strains (Figure 1b).

The fermentations induced by the local strains were even faster, 3 to 5 days were needed to ferment half of the sugar (Figure 1c). All available sugar was not fermented completely in some fermentations. In spontaneous fermentations, about 20 g/l of sugar were left, as well as in the fermentations induced with the local strains, F and G. However, in the fermentations induced by local strains, H and I, the concentration of residual sugar was below 5 g/l. The commercial starter strains possessed good fermentation characteristics, since all available sugar was consumed. The evolution of the total amino acids, AaF + AaP, for each of the 14 fermentation processes individually (Figure 1 a, b, c) support the observations previously described in the results in Table 3. It is evident that the local strains also showed an individual tendency toward higher amino acid uptake.

Metabolites of fermentation process. Ethanol, methanol and some higher alcohols were analyzed to study the impact of different starter strains on the evolution of metabolites of Malvasia fermentation processes. The analyses, where the minimal and maximal concentrations are marked by shading, are summarized in Table 4.

Tab. 4 — The final characteristics of Malvasia wines obtained in spontaneous (A0-A5) and induced (B-E: commercial, F-I: local strains) fermentations

Fermentor mg/l	Acetal-dehyde	Etilacetate	Methanol	n-Propanol	i-Butanol	i-Amyl-alcohol	Ethanol vol %
A0	18.2	31.5	50	16.7	31.2	195	12.7
A1	16.4	30.1	50	17.4	37.9	200	12.5
A2	18.9	31.7	50	17.8	37.6	200	12.4
A3	16.5	31.6	55	17.7	35.0	185	12.3
A4	14.7	29.1	50	15.8	34.1	179	12.7
A5	19.4	30.3	55	18.2	35.8	197	12.5
B	23.7	38.2	50	23.2	43.3	270	13.8
C	15.8	29.9	50	27.8	23.8	219	13.6
D	34.7	32.0	60	28.4	38.9	207	13.8
E	22.4	29.0	60	30.9	26.2	249	13.8
F	24.5	39.9	55	19.6	54.3	270	12.1
G	17.9	27.8	50	22.3	54.8	284	12.0
H	37.1	28.7	50	19.7	31.2	216	13.6
I	23.6	35.8	60	19.4	78.6	376	13.5

The amounts of ethanol produced in the fermentations of Malvasia coincide with the amount of fermented sugar (Figure 1). In all spontaneous (A0-A1) and two induced fermentations (F and G), the concentration of ethanol was lower, and so was the sugar consumption. Commercial strains (B-D) and two local strains (H and I) showed high tolerance to ethanol, since its final concentration reached 13.8 vol%. The methanol produced does not threaten the wine quality. In a similar study of Lema et al. (1996), where some components of Albariño wine aroma were monitored, significant differences were not observed. During the fermentation of Malvasia, more i-amyl alcohol and i-butanol, and less etylacetate were obtained.

The principal higher alcohols produced by yeast are n-propanol, isobutanol, isoamyl alcohol and aromatic alcohols. Although they exhibit harsh, unpleasant aroma, at the concentrations generally found in wine, below 300 mg/l, they are usually considered desirable (Rapp and Versini, 1991). Fermentation studies using dual-labeled amino acids and sugars have demonstrated that no simple relationships exist between the concentration of the parent amino acid and the corresponding alcohol. Nevertheless, availability and timing of amino acid uptake will influence higher alcohol formation and affect wine flavour. It is generally believed that esters contribute the most to the desirable aspects of the fermentation bouquet of wine, and account much for the greater aroma intensity of wines derived from must of higher nitrogen content (Chen, 1976; Henschke and Jiranek, 1993).

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БИОЛОШКА РАЗНОВРСНОСТ КВАСАЦА У СЛОВЕНАЧКИМ ВИНАРСКИМ ПОДРУЧЈИМА: СЛУЧАЈ АМИНОКИСЕЛИНА КОД СПОНТАНИХ И ИНДУКОВАНИХ ФЕРМЕНТАЦИЈА МАЛВАЗИЈЕ

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Резиме

Микробиолошка разноврсност може да се одрази и на састав крајњег производа. У раду су испитиване разлике у аминокиселинском саставу 14 малвазијских сировина ферментисаних помоћу локалних и комерцијалних стартер-култура квасаца, а поређење је извршено у односу на спонтане ферментације шире пореклом из истог подручја.

Покушали смо да утврдимо да ли су разлике зависиле од различитих почетака ферментација. Припремили смо компаративну студију развоја слободних и укупних аминокиселина.

Укупна концентрација 15 аминокиселина које су проучаване била је 1975 mg/l, док је концентрација слободних аминокиселина била 1061 mg/l. Спонтане и индуковане ферментације су показале различите брзине ферментација. Три до девет дана је било потребно како би се садржај шећера смањио за 50%. Иако се пролин сматра аминокиселином која није подложна деловању квасаца, примећено је смањивање његове концентрације. Лизин је била једина аминокиселина чија се концентрација повећала. Утрошак аминокиселина је био минималан током спонтаних ферментација, док је, у случају ферментација инокулисаних са локалним стартер-културама, утрошак био максималан.

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MEDICINAL MUSHROOM *GANODERMA LUCIDUM* IN THE PRODUCTION OF SPECIAL BEER TYPES

ABSTRACT: Mushrooms like *Ganoderma lucidum* have been used for thousands of years as a traditional medicine in the Far East. *Ganoderma* received wide popularity as an eating mushroom with high nutritive value, but even more as medical fungi. It has been used for the treatment of various diseases: hepatitis, hypertension, insomnia, and even cancer. Due to its extraordinary action, it is often called "Elixir of life", "Food of gods" and "Mushroom of universe". The intracellular and extracellular polysaccharides (β-glucane) inhibit the growth of several types of cancer. Mushroom produces triterpenes of which especially ganoderic acid showed cytotoxicity on primary tumor liver cells, inhibition of histamine release, hepatoprotective effect, stimulation of the immune system functions, inhibition of the aggregation of blood plates, etc. On the other hand, beer as a purely natural beverage obtained in the process of fermentation, contains a number of ingredients which are important for human organism, and in moderate usage has favourable reaction on the general health condition of the body. As such, beer is a very good basis for the development of a number of new products with defined pharmacodynamics influence.

In this work, we have investigated the possibilities of using extracts of mushroom *Ganoderma lucidum* in the production of special beer types. The composition of mushroom, properties of the most important active ingredients, extraction procedures, and sensory characteristics of the beers on the basis of such extracts were determined. The most important parameters of quality and possibility of adjustments using extracts of different medicinal herbs were investigated.

INTRODUCTION

Mushrooms like *Ganoderma lucidum* (Fr.) Karst (*Ganodermataceae*) has been a focus of public and health interest in recent years. It has been used in traditional Chinese medicine for centuries, and it is well known as *Ganoderma* in China, Reishi in Japan, and Young Zhi in Korea. During a long history period, it was regarded as a „Herb of Deathlessness" or „Miraculous King of the Herbs". Over the past decade, *Ganoderma* was extensively researched in medi-

cal area. It contains numerous bioactive natural components, polysaccharides, ganoderic acids, ergosterols, proteins, unsaturated fatty acids, vitamins and minerals, with properties conducive to normalizing and balancing the body. They can enhance health and help in relief of a multitude of diseases (Zhou et al., 2007). Numerous studies have proved that anti cancer properties of *Ganoderma* come from polysaccharides, mainly from b-glucans. Polysaccharides are extracted with hot water, salt solutions, alkali solutions and dimethyl sulfoxide solution. Extractions are preformed from mycelia to dry fruit body of fungi. Among those, neutral polysaccharides (β-1→3, β-1→6 homo D-glucan), acidic glucan and polyglycan are bioactive. Glucan consisting of (1→3)-, (1→4)-, and (1→6)-β-D linkages has been characterized with novel antitumor activity against oncogenesis and tumor metastasis (Yuen and Gohel, 2005). The cancer cell cytotoxicity can be explained as the direct killing of cells or the inhibition of cell proliferation. Using the tetrazolium (MTT) method, inhibition of proliferation has been shown in various cancer cell lines, including murine lymphocytic leukemia L1210 and Lewis lung carcinoma (LLC), human hepatoma PLC/PRF/5 and KB, human breast cancer MDA-MB-123, human prostate cancer PC-3, human breast cancer MCF-7, human cervix uteri tumor HeLa, and low-grade bladder cancer MTC-11 (Yuen and Gohel, 2005).

The other main group of *Ganoderma* components are triterpenoids. Their pharmacological effects are known as antioxidative, immune-modulating and antitumor. Major triterpenoides isolated from *Ganoderma* mushroom are different types of ganoderic acids. There are A, B and C ganoderic acids. Now, there are more than 130 oxygenated triterpenes (mostly lanostane-type triterpenes) that have been isolated from the fruiting bodies, spores, mycelia and culture media. They are divided into C30, C27 and C24 compounds according to the number of carbon atoms and based on the structure and the functional groups (Huie et al., 2004; Gao et al., 2005; Luo and Lin, 2002). It has been demonstrated that ganoderic acids -R, -T, -U, -V, -W, -X, -Y , and -Z; lucidimol-A and -B; Ganodermanondiol; ganoderiol F; and Ganodermanontriol exert cytotoxic-based carcinostatic effects on cancer cells, and many of them also possess anti-angiogenesis activity (Silva et al., 2003; Min et al., 2000). When we consider proteins and peptides isolated from *Ganoderma*, several active proteins and bioactive peptides have been isolated. Protein LZ-8 isolated from *Ganoderma* has shown to be mitogenic toward mouse splenocytes *in vitro* and immune-modulating *in vivo* by reducing antigen-induced antibody formation and by completely preventing the incidence of autoimmune diabetes in non-obese diabetic mice (Zhou et al., 2007). Immune-modulating activities of phytochemicals (Ganopoly) from *G. lucidum* affect the body's immune system through the following pathways: (1) By activating macrophage, Ganopoly facilitates theT-lymphocytes transferring to cytotoxic T cells, enhances the number and activity of the B-lymphocytes and the natural killer cells. (2) Ganopoly can activate the reticuloendothelial system and the complement system; induce the various immune factors, such as INF, TNF and so on. (3) It may have influence on the "Nerve Endocrine Immune System." (4) Ganopoly can facilitate RNA, DNA and protein synthesis in cells, and enhance the contents of the cGMP and cAMP in cells as well (Habijani et al.,

2001). Apart from the previously mentioned pharmacological functions, people in China use *Ganoderma* in the treatment of fatigue, coughing, asthma, insomnia, indigestion, hypertension, high cholesterol and neurosis; it could also reduce the side effects and pain during chemotherapy and radiotherapy for cancer patients (Z h o u et al., 2007).

In recent years, usage of natural substances, such as herbs and medicinal mushrooms, has significantly increased. Beer, as a fully natural product containing numerous health promoting ingredients, can serve as a very good basis for developing a wide variety of products with specific pharmacodynamic activity. The purpose of this study was to evaluate the potential effects of *Ganoderma lucidum* extract for the production of beer for special health use.

MATERIAL AND METHODS

Raw materials

Room temperature extraction of ethanol-soluble bioactive compounds from dried mushroom *Ganoderma lucidum*

Tissue of mushroom *Ganoderma lucidum* was cut into pieces and mixed with alcoholic solution 70% vol. of ethanol. Extraction was performed by daily mixing at a magnetic stirrer for 10 minutes and then leaving the solution to stand in a dry and dark place at room temperature. Period of the extraction was 21 days. After the extraction, the solution was filtered and concentrated in vacuum to 1/5 of the initial content.

The prepared extract was added aseptically to commercially produced bottled pills, taking into account the recommended daily doses and sensory acceptability. After injection the bottles were immediately closed and matured at 5°C for one day.

Analysis

Ganoderma extract, starting beer, and beers enriched with extract were analyzed by LC/MS and ^{1}H-NMR methods. LC/MS analysis was performed on an Agilent MSD TOF coupled to an Agilent 1200 series HPLC, using Zorbax Eclipse XDB-C18 column (RR, 30 x 2.1 mm i.d., 3.5 mm). Mobile A phase was 0.2 % formic acid in water, and mobile phase B was acetonitrile. The injection volume was 5 μl, and elution was performed at 0.7 ml/min with gradient program (0—1.5 min 5% B, 1.5—10 min 5—95% B, 10—15 min 95%B, 15—16 min). Mass spectra were acquired using an Agilent ESI-MSD TOF. Capillary voltage 4000 V, Fragmentor voltage 140 V, Nebulizer pressure 45 psig, drying gas 12 l/min, gas temperature 350°C, mass range (100—1500) m/z; negative and positive ionization mode. The processing of data was done with the software Molecular Feature Extractor.

The ^{1}H-NMR (200 MHz) spectra were recorded on a Varian Gemini 2000 spectrometer in $CDCl_3$. Chemical shifts are given on the δ scale relative to TMS as internal standard.

Sensorial evaluation

The consumers' acceptance test was carried out by 100 untrained consumers with the following average profile: 95% were 20—25 years of age, 29.5% of female gender, 72.4% with the consumption frequency of one or more beers per week, and 27.6% were beer non-drinkers. The beer samples were evaluated using a 5 point scale. Consumers did not have any formal training or experience in the description of beer flavour. An average value of each sensorial attribute and given consumers profile was calculated and expressed as "radial" diagrams.

RESULTS AND DISCUSSION

Relative contents of active constituents in *Ganoderma lucidum* extract are shown in Table 1.

Tab. 1 — Relative content of constituents of *Ganoderma lucidum* extract

Constituents	RT/MS	Bruto molecular formulae	RRI*
Ganoderic acid E, d	4.575	$C_{37}H_{36}O_2$; $C_{30}H_{40}O_7$	512.2
Ganoderic acid C 6	4.595	$C_{30}H_{42}O_8$	530.2
Ganoderic acid G	4.721	$C_{30}H_{44}O_8$	532.3
Ganoderic acid B, A	4.821	$C_{30}H_{44}O_7$	516.3
Ganoderic acid C 2, C	4.951	$C_{30}H_{46}O_7$	518.3
Ganoderic acid C, D, J	5.209	$C_{30}H_{42}O_7$	514.2
Ganoderic acid B, A	5.521	$C_{30}H_{44}O_7$	516.3
Ganoderic acid H	5.549	$C_{32}H_{44}O_9$	572.2
Ganoderic acid E,d	6.004	$C_{30}H_{40}O_7$	512.2
Ganoderic acid F	6.163	$C_{32}H_{42}O_9$	570.2
Ganoderic acid C, D, J	6.180	$C_{30}H_{42}O_7$	514.2
Ganodermanontriol, lucidumol A	7.389	$C_{30}H_{48}O_4$	472.3

* RRI-relative retention index

The presence of ganoderic acids A, B and Y was identified in the investigating extract. The isolated terpenoids have biological effect on cholesterol synthesis in human hepatic cell line *in vitro*. Ganoderic acids A and B inhibit hypoglycemic effects in several test systems and ameliorate the symptoms of diabetes. Ganoderic acids A and C inhibit farnesyl protein transferase, which catalyzes posttranslational farnesylation of Ras oncoprotein and is essential for the cell-transforming activity of Ras. Isolated ganoderic acids F contribute to atherosclerosis protection by the inhibition of angiotensin converting enzyme or platelet aggregation. Ganoderiol F, lucidimol A and ganodermanontriol identified from the extract have cytotoxic effect on LLC and Meth-A cancer cells. Ganodermanontriol founded in the extract inhibit HIV-1 protease (Z h o u et al., 2007).This means that the usage of *Ganoderma lucidum* extract could potentially have positive effect on individuals with poor health.

The results of the sensory acceptance test of the samples compared with the starting beer are given in Figures 1 to 4.

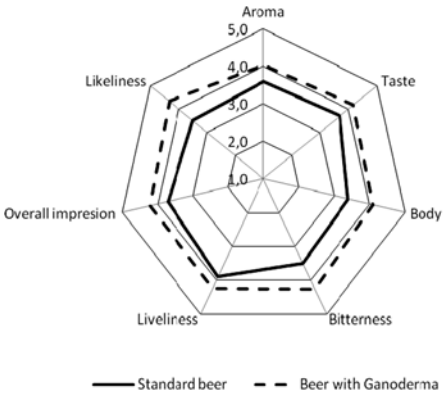

Fig. 1 — Sensory evaluation of standard beer samples and beer with *Ganoderma* by male beer drinkers

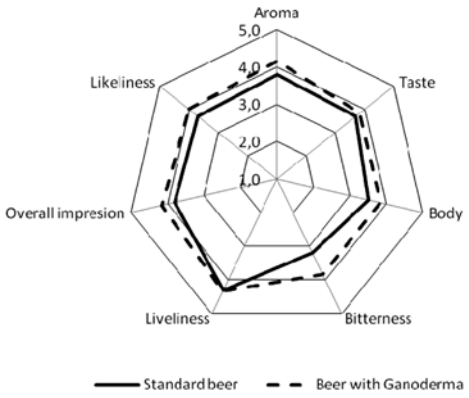

Fig. 2 — Sensory evaluation of standard beer samples and beer with *Ganoderma* by male beer abstinents

The consumers' acceptance test of male beer drinkers has shown that beer enriched with *Ganoderma* extract is absolutely compatible with standard beer. It is shown that in all analyzed parameters the enriched beer was even superior. The consumers have pointed out that fullness and bitterness were enhanced and enjoyable (Figure 1). The beer abstinents had almost similar opinions as the beer consumers, and the only exception was the freshness which was ranked as that of the standard beer (Figure 2). Both male beer drinkers and the abstinents have agreed that the obtained beer had full taste and pleasant bitterness. They have emphasized that bitterness was enhanced and the beer was strong.

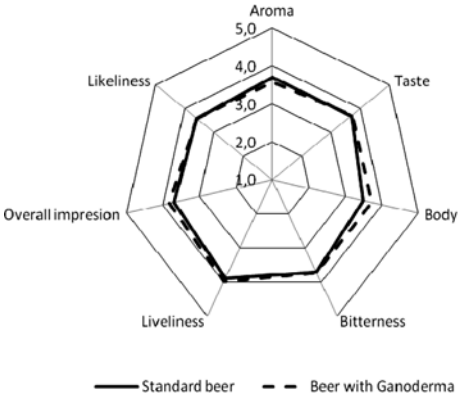

Fig. 3 — Sensory evaluation of standard beer samples and beer with *Ganoderma* by female beer drinkers

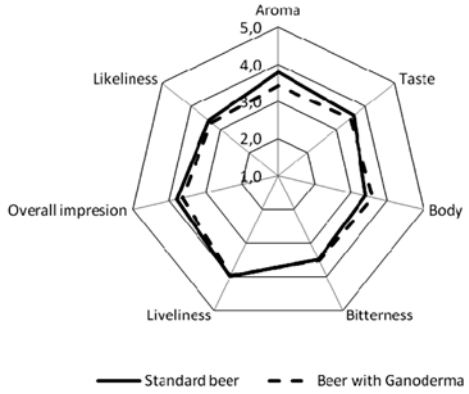

Fig. 4 — Sensory evaluation of standard beer samples and beer with *Ganoderma* by female beer abstinents

On the other hand, female beer consumers evaluated the starting standard beer with slightly higher grades than mail drinkers, and found no big difference between the standard beer and beer enriched with *Ganoderma* (Figure

3). According to their opinion, the beer with *Ganoderma* was almost similar to the standard beer with slightly enhanced fullness and overall impression. As it could be expected, the female beer abstinents graded the starting standard beer a bit worse than the beer drinkers, but also gave priority to the enriched beer. The only flaw they found was related to aroma, which was considered as rather poor (Figure 4). There was immense difference between male and female testers. Both male beer consumers and the abstinents prefered full taste and strong bitterness, while female consumers normally preferred beer with lighter taste and aroma.

CONCLUSION

The beer supplemented with *Ganoderma lucidum* extract as a natural source of nutritional supplements is very pleasant and even acceptable to beer abstinents. Male beer drinkers and abstinents conciliate in that the obtained beer is even superior in comparison to the starting standard beer in all tested sensory parameters. The obtained results indicate that standard beer can be successfully enriched with *Ganoderma* extracts as a source of natural nutritional supplements. Developed beer is superior not only in pharmacodynamic properties, but also in sensorial impression. Considering the above mentioned, it is obvious that the combination of beer and medicinal mushrooms and herbal extracts in recommended daily doses can give products with satisfactory sensorial properties. Numerous experiments showed different possibilities of using extracts derived from *Ganoderma lucidum* mushroom in various disease treatments, by improving immune system function which results in better general condition of organism. Such special beer base products should have predictable pharmacodynamic properties and can potentially be recognised as „traditional herbal medicinal products". Their base is beer, fully natural product with tradition longer than 6000 years, with evident positive effect on the overall health condition and with the presence of alcohol as the only limiting factor. By eliminating alcohol, using alcohol-free beer as a base product and/or implying to adequate „suggested use", this problem may be avoided.

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МЕДИЦИНСКА ГЉИВА *GANODERMA LUCIDUM* У ПРОИЗВОДЊИ СПЕЦИЈАЛНИХ ПИВА

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Резиме

Гљиве попут *Ganoderma lucidum* коришћене су хиљадама година у традиционалној народној медицини Далеког истока. Своју велику популарност *Ganoderma* је стекла као јестива гљива са високом нутритивном вредношћу, али још више као медицинска гљива. Коришћена је за лечење разних болести, између осталих и хепатитиса, хипертензије, инсомније, па и карцинома. Управо због свог изванредног деловања називана је „Еликсир живота", „Храна богова", „Гљива универзума". Њени интрацелуларни и екстрацелуларни полисахариди (β-глукани) доказано инхибирају раст неколико врста карцинома, а тритерпени које продукује, посебно ганодеринска киселина, показују цитотоксичност на примарне туморне ћелије јетре, инхибицију ослобађања хистамина, хепатопротективни ефекат, стимулацију функције имуног система, инхибицију агрегације крвних плочица итд. Са друге стране, пиво као потпуно природно пиће добијено ферментацијом, садржи многобројне састојке од значаја за организам и у умереној количини показује повољно деловање на опште здравствено стање организма. Као такво, пиво је веома добра подлога за развој низа нових производа дефинисаног фармакодинамичког деловања.

У раду је испитана могућност коришћења екстраката гљиве *Ganoderma lucidum* у производњи специјалних пива. Дати су састав гљиве, својства најважнијих активних састојака, поступци екстракције и сензорне карактеристике пива добијених на бази датих екстраката. Испитани су најважнији параметри квалитета и могућност кориговања употребом екстраката различитог лековитог биља.

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MAGNETICALLY ALTERED ETHANOL FERMENTATION CAPACITY OF *SACCHAROMYCES CEREVISIAE*

ABSTRACT: We studied the effect of static magnetic fields on ethanol production by yeast *Saccharomyces cerevisiae* 424A (LNH-ST) using sugar cane molasses during the fermentation in an enclosed bioreactor. Two static NdFeB magnets were attached to a cylindrical tube reactor with their opposite poles (north to south), creating 150 mT magnetic field inside the reactor. Comparable differences emerged between the results of these two experimental conditions. We found ethanol productivity to be 15% higher in the samples exposed to 150 mT magnetic field.

KEYWORDS: ethanol production, magnetic fields, *Saccharomyces cerevisiae*

INTRODUCTION

Process of fermentation of sugar into ethanol is one of the earliest biological reactions empirically undertaken by man. The significance of this process is even greater today than it has ever been, since humanity is constantly reaching for innovative uses of its major product, not only as the agent responsible for the rising of bread dough, or basis of alcohol industry, but also as sustainable biological fuel (Pimentel and Patzek, 2005). However, yeasts' ability to produce ethanol is regulated and constrained by their inability to survive high concentrations of this alcohol due to its toxic effects (Lau and Dale, 2008). Even the most tolerant yeast strains can not survive ethanol concentrations above 15% (Morais et al., 1996.). Therefore, during the last few decades, the subject of extensive investigations has been creating the yeast strains which are able to survive higher concentrations of ethanol, but which maintain their fermentative abilities. Some of those experiments involved genetic manipulations, even though the use of modified strains was abandoned since scientists had recognized possible collateral damage caused by unexpected effects on yeasts' metabolism, in case of accidental release into the en-

vironment. After failing to solve this problem by means and methods available to molecular biology, some efforts were employed in finding the solution at the level of quantum biology, utilizing electro-magnetic and static magnetic fields (M a n o l i u et al., 2005). Susceptibility of various microorganisms to electric and magnetic fields was thoroughly studied both *in vivo* and *in vitro* conditions (G a l o n j a and C o g h i l l, 1999).

MATERIAL AND METHODS

Saccharomyces cerevisiae culture was purchased from Albright laboratory, Abergavenny, and prepared for the fermentation. Normally, 1% toluene, 4% ethanol and 0.075% triton X-100 are added for the purpose of permeabilization of the cells. To avoid these permeabilizing supplements, we substituted them with 150 mT magnetic field which was capable of removing positively charged calcium ions and loosening membrane architecture. Subsequently, extra calcium enters the cell from the environment and stimulates cell metabolism. ATP, NAD^+, magnesium and inorganic phosphate were added in order to initiate the ethanol fermentation. The initial sugar concentration was 200 g/l. The pH of the culture medium was kept between 5 and 6.

Fig. 1 — BioFlo III fermentor, New Brunswick, with pH and temperature probes

Fermentation system consisted of one fermentation vessel (7.5 litre BioFlo III fermentor, New Brunswick, Figure 1) containing free cell yeast culture and fermentation medium, two permanent magnets attached to the reactor diametrically opposed, temperature probes and pH sensors. We opted for BioFlo III rather than BioFloo 310, because of greater feasibility in attaching the static magnets of our choice.

We monitored biomass growth of yeast culture by means of optical density correlated to dry cell mass, during sixteen hours of exposure in the reactor. Levels of sugar remained, and the concentrations of ethanol produced were measured every four hours.

RESULTS

At the end of a 16 hour experimental period, cell density and ethanol concentration values in magnetically treated samples and samples that were not exposed to 150 mT magnetic field showed significant differences.

Fermentation aided by static magnetic fields resulted in cell density of about 5.5 g/l with maximum ethanol concentration of 44 g/l. Average productivity was 2.75 g/l per hour, with 71.1 % of utilization of sugars. It was

not a linear process, however. Initial state being zero, suggests that ethanol production sped up four hours after the fermentation process started. At that checkpoint time, it was 6 g/l. The measurement taken after eight hours showed massive increase in ethanol production (19 g/l). After twelve hours of fermentation, we measured 39 g/l of ethanol in the fermentation medium (Chart 1).

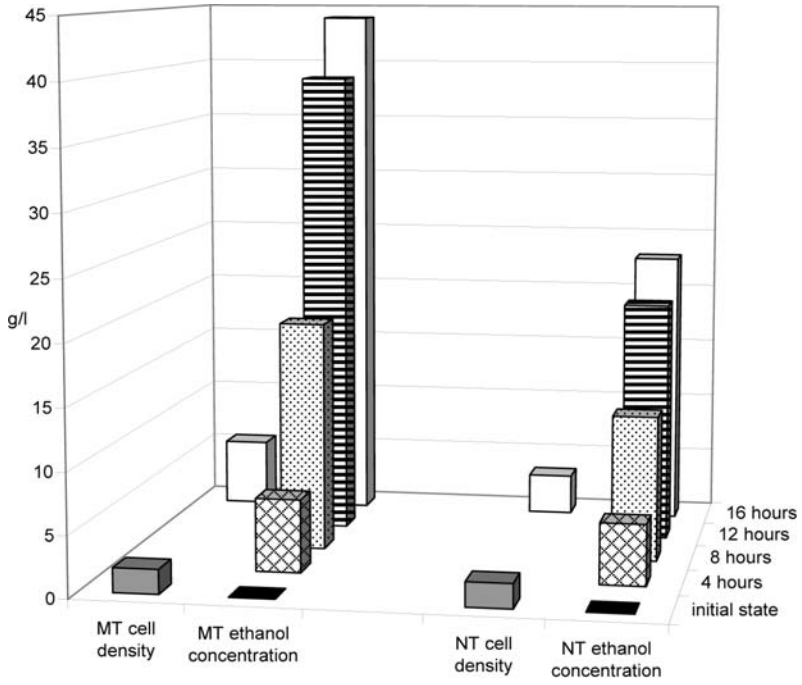

Chart 1 — Changes in cell density and ethanol concentration in magnetically treated samples (MT) and not treated samples (NT), during 16 hours of fermentation

Final cell densities in non-exposed samples were about 3.3 g/l. Ethanol production was not a strictly linear process, measuring 5, 12, 20 and 23 g/l after four, eight, twelve and sixteen hours of fermentation, respectively (Chart 1).

CONCLUSION

Magnetic fields do not only posses the capability of permeabilizing the cells and increasing their metabolic levels, but seem to neutralize bio-feedback mechanism of ethanol saturation, which normally leads to stopping the fermentation process. Some experiments suggest that yeast cells immobilized on Ca-alginate beads retain their ability to produce ethanol during four days (P e r e z et al., 2007). Although these results are encouraging, more investigation needs to be done into the optimum magnetic and/or electro-magnetic fields, comparison between free cell media and immobilized media performances, as well as many other parameters that come into focus as new results emerge.

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МАГНЕТСКИ ИЗМЕЊЕНА ЕТАНОЛ-ФЕРМЕНТАЦИОНА СПОСОБНОСТ *SACCHAROMYCES CEREVISIAE*

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Резиме

Алкохолну ферментацију шећера до етанола човек користи емпиријски од својих најранијих дана. Значај овог процеса данас је још већи, с обзиром на изналажење нових употребних могућности етанола, који у систему базираном на одрживом развоју има велику перспективу као биолошко гориво (Pimentel и Patzek, 2005). Ограничавајући механизам повратне спреге у овом процесу је неспособност квасаца да се одрже у медијуму који садржи високе концентрације етанола. Након покушаја да се методама генетичког инжењерства превазиђе овај проблем, решење би могла понудити квантна биологија, употребом електро-магнетских или статичних магнетских поља (Manoliu et al., 2005, Perez et al., 2007). Ова поља повећавају пермеабилност ћелија квасаца и интензивирају метаболичке процесе, притом повећавајући толерантност квасаца према вишим концентрацијама етанола. Ми смо користили статична магнетска поља 150 mT у

ферментационом систему BioFlo III fermentor, New Brunswick. Добијени резултати указују на значајно повећање ферментативне продуктивности квасаца, као и на повећање њихове толерантности према вишим концентрацијама етанола. Након шеснаесточасовне ферментације, при којој је коришћен слободноћелијски раствор квасца *Saccharomyces cerevisiae*, културе које су биле изложене магнетском пољу 150 mT продуковале су укупно 44 g/l етанола. Продуктивност култура које нису биле експониране овим пољима била је много мања и износила је укупно 23 g/l етанола.