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The Faculty of Technology Dean's Address at the 55th Anniversary Ceremony

"Dear friends, dear honourable former and current professors, students and dear employees, this is the jubilee ceremony.

In retrospect, the year 1959 was the time when the National Assembly of the People's Republic of Bosnia and Herzegovina adopted the law establishing the Faculty of Technology, which was at the time a part of the University of Sarajevo. Since that time, thousands of students studied here, thousand of lectures were held and thousands of final theses, master and doctoral dissertations were defended. All those who worked at the Faculty at any time became an inextricable part of it enabling it to grow and evolve into the respectable institution.

Apart from the Register Commission, special credit for the establishment of the Faculty of Technology goes to Mr. Nikola Andrić and engineer Halid Čokić, the president and the vice-president of Tuzla district respectively; to distinguished republican officials Mr Pašaga Mandžić and Academician Edhem Čamo PhD, the provost of University of Sarajevo, as well as to the town of Tuzla.

On November 13, 1959, in this very amphitheatre, 120 freshmen from almost all regions of former Yugoslavia entered for the first time. Fifty years seems to be a long period of time, a human lifetime. After the years have passed and when things have settled down, and each of us has chosen their path a long time ago. It has been a period during which the Faculty has experienced its successful and less successful times, during which a great number of employees including professors, associate professors, administrative assistants, and auxiliary staff have worked.

Let me say something about us professors and students, about our mission and prospects for the good of our town and country, which we frequently represent. When we are young we are usually surrounded by people we trust by birth, our family. However, later on some of us learn to have faith in God or in people, whereas others learn to trust in both God and people. After some time we begin to have doubts in people while at the same time faith in ourselves grow stronger. It is the way we function – it is the way the world functions.

Everyone looked after us during the first years of our existence. These were the years of triumphant and rapid success, and then we were left to develop on our own. I believe we managed it well and thereby matured. Perhaps, it might have gone unnoticed how much we contributed to the economic development of contemporary Yugoslavia, especially Bosnia and Herzegovina and our region. However, our numerous research studies, surveys and projects, whose authors were the professors of the Faculty of Technology, bear witness to our contribution. In addition, this helped in the establishment of, the technological development of and the modernization of factories, such as ***Solana*** *Tuzla,* ***Sisecam SODA, Koksara, Cementara***[1] *. These are the companies which operate successfully even today.*

1 Solan – a salt processing plant; Sisecam SODA – a plant for ammonia soda production; Koksara – a coke plant; Cementara – a factory for cement production.

Unfortunately, some are closed or no longer exist but they used to be the Chemical industry giants of contemporary Yugoslavia: ***Hlor – alkaline complex*** *well known as* ***HAK I and II****, and a factory for detergents DITA. Moreover, the wartime and post - war period strongly affected the Faculty of Technology and the need for chemical engineers and technologists, and environmental protection engineers, since the Chemical industry was now practically destroyed, and once was a reason for opening the Faculty of Technology. Yet, today 20 years after the war, we have managed to reemerge and be recognized again, and emphasise the importance and need for chemical, environmental protection and food engineers who are a priority and are responsible for the development of a country.*

Today at the time of the fifty-fifth anniversary, it has to be admitted, without false modesty, that a lot was achieved in the period between 2010 and 2014. During 2010 and 2011 we carefully monitored what was happening in the economy and the labour market, we visited the business community to who we contribute by developing human resources. Therefore, in the academic year 2011/2012 we introduced changes to the curriculum and academic programmes of the first and second cycle of study. We changed the concept and also developed the curriculum which includes general and joint academic programmes that are offered at all Departments in the first and second year of studies allowing our students to choose specialized areas of study, that interests them most, after they have completed the second year of study. In addition to the current academic programmes of the first cycle of study at the Chemical Engineering and Technology Department, a Chemical and Materials Engineering programme has been introduced. A Food Safety and Quality programme has been introduced at the Department of Food Technology as well. Thereby, we have greatly harmonized our curriculum with curricula of related faculties in Europe. We believe it is significant and of great importance that after so many years, the English Language has been introduced as a compulsory subject in all Departments. These changes provide great opportunities for better mobility of our students. As for the second cycle of study, we have also introduced changes to the curriculum and offered new attractive academic programmes. I would single out the Food Safety and Quality Management programme which has attracted interest. In this academic year 2014/2015, we have introduced the Nutrition programme into the curriculum of the second cycle of study, which is the first one in Bosnia and Herzegovina.

In the period between 2010 and 2014 we introduced the third cycle or doctoral studies: Chemical Engineering, Food Engineering and Environmental Protection Engineering. Unfortunately, in the academic year 2013/2014 an insufficient number of students prevented us from starting the teaching programme. However, we feel certain, according to interest shown in the meantime, that in the academic year 2014/2015 we will have an adequate number of students. I would also like to emphasise that we have the Environmental Protection Engineering programme which is the only specialized programme in this field of study in the region, and about which we regularly receive enquires about the admissions and when admissions would be announced.

In coordination with the Department of Chemistry at the Faculty of Science, we developed the doctoral studies of Applied Chemistry. We are the first ones that have organized a joint degree at University. From this point of view, we have to admit that it was not easy

and we had to invest a lot of effort to coordinate ideas and justify the need for the degree. The first applicants are expected this year.

I will take a brief look at the scientific research and activities conducted in the previous period. At the beginning of 2011, we initiated accreditation of the Laboratory for Wastewater Analysis in accordance with ISO 17025. This was not easy to manage and we faced numerous administrative and financial obstacles. However, we were persistent and brought it to a conclusion.

Since 2010, the Faculty as an equal partner has taken part in implementing many international projects: Tempus projects, International project funded by the Royal Norwegian Ministry of Foreign Affairs – HERDA in cooperation with the University of Trondheim, EUREKA projects and a project funded by the Japan International Cooperation Agency. Thus, a total of 5 international projects were implemented during those 4 years. The Faculty greatly benefited from those projects. A computer room was equipped, laboratory equipment valued at 250 000KM was purchased, and the Technology Transfer Centre was opened.

These were not the only activities. The laboratory was renovated within the project supported by the Federal Ministry of Education and Science which also funded purchase of the most recent literature.

In the following period, we intend to implement international projects. The Faculty is a partner in the IPA project which is to be implemented until 2016. Also, the project ECO – INNOVATIVE SOLUTIONS in HORIZON 2020 is currently under preparation. It is hoped that higher education and scientific research (especially at University) will have a more significant place in the process of allocating budget funds since it is required for development of society as a whole.

We particularly take pride in our professional journal ***Tehnologica Acta****, which has been issued in English since the end of 2010. The journal is also indexed, and the two issues from 2014 will be promoted today.*

In this period we organised two significant international conferences: ***Environmental Potential, Sustainable Development and Food Production*** *and* ***With Food to Health****. In addition, during those four years the Faculty initiated and was one of the organisers of numerous professional conferences on a range of topical issues.*

Finally, we graduated from our schools as well as abroad, we learned from the best professors and maintain contact with them. We raise standards all the time. We strive to teach and shape our students to become stronger, more enduring and better individuals than we are. We teach them not to create because they wish to or because they have to, rather we teach them to create because they both wish and have to since progress of a society and the quality of life depend on educated citizens!

We are descendants of those who absorbed colours of their time and wove them into us: Professors Tihomil Marković, Irena Ranogajac, Leopold Fridman, Stjepan Ivić, Zdenka Dušek, Sadik Latifagić, Slavoljub Perdija, Mahmud Ahmetbašić, Nihada Latifagić, Ranko Babić, Oleg Ščedrov, Branko Popović, Hajrudin Simičić, and many others.

We continued to strengthen our capacities and develop ourselves on a legacy of firm foundations supported by old and powerful educational institutions of former Yugoslavia.

It is certain that both teaching programmes and scientific research should be further developed. The organisational structure of the Faculty has already been adjusted to meet the requirements of new times by introducing a new organisational scheme of the Faculty. All these changes are taking place at the time of reconstruction of the chemical and chemical related industries, changes of market structure and transition to market economy based on a European model. In addition to typical development of technologies which were used in big plants of the Chemical Industry, for which this region was recognized, today we witness a growing tendency for development of small and medium plants for manufacturing special materials and products designed for environmental protection. A prerequisite for adopting and following such development is well organized and qualified research specialists and professors capable of swiftly recognizing and responding to changes with modification of existing curricula. In addition to those changes in the economy, which are going at their particular pace, thorough changes at University are still to come. The University is expected to give different, better but more specific support for scientific research. Consequently, the Faculty, with better equipment and appropriate rooms, continuous work and university staff development, will certainly encourage young people but also stabilize its position in the process of education of chemical, food and environment protection engineers.

Where do we find our strength?

The students of the Faculty are our strength. They encourage us to believe and hope; being "hooked" to their youth to create, improve and plan.

The most important fact is that our diplomas are recognized all over the world, which is regularly confirmed by our students who graduated from this University but who, to our deep regret, have decided to pursue their future abroad, outside Bosnia and Herzegovina.

In the end, I would like to thank he team that harmoniously worked together in the previous period. I would also like to thank the Dean of Academic Affairs, Professor Husejin Keran for his patience and engagement in considering all our requests. I would like to thank the Dean of Scientific Research Professor Zoran Iličković for his ideas but also for his readiness to realize our ideas; to Professor Vahida Selimbašić Head of the Environment Protection Engineering Department, Professor Gordan Avdić Head of the Engineering and Technology Department, Professor Milica Vilušić Head of the Food Technology Department and Docent Sabina Begić Head of the Agronomy Department, the youngest department which has just been opened; I would like to thank Nidret Ibrić PhD for making our web site alive and maintaining it, but also for giving it a new design; and I would like to thank to my secretary Ms Nermina Jahić as nothing would have been possible without her support.

Thank you all for coming here today. I hope you will feel well and enjoy today's ceremony."

C O N T E N T

GC-MS DETERMINATION OF GLUCOSE AND MANNITOL AFTER OXIMATION AND TRIMETHYLSILYLATION IN OLIVE LEAVES EXTRACTS

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT
The determinations of carbohydrates in *Olea europaea* L. have been mostly limited to olive-fruits, although olive leaves can also contain significant amounts of sugars. In this study an analytical method for the simultaneous determination of D-glucose and D-mannitol in wild olive leaves, obtained from Slovenian Karst region, was developed, optimised and validated.
Olive leaves were extracted by conventional solid – liquid extractions with water, water/acetone and water/ethanol mixtures at various sample-to-solvent ratios (1:10, 1:20, 1:50 gmL^{-1}). The aim of the research was also to establish how the extractions carried out under different conditions affect the concentrations of the sugars in the final extracts. Sugars were quantified as oxime-trimethylsilyl (o-TMS) derivatives by gas chromatography and mass spectrometry (GC-MS), although direct silylation was also used. The reactions of the oxime- and the TMS- derivatives formation were optimized separately. Optimized oximation reaction was carried out with the addition of $NH_2OH \cdot HCl$ dissolved in pyridine (2%) and by heating at 80 °C for 45 min. The quantitative conversion of carbohydrates to the TMS derivatives was achieved by adding MSTFA and heating for 60 min at 80 °C. Chromatograms with one peak for mannitol, and not more than two peaks for glucose were obtained as a final result in all GC-MS analyses. Method was validated where linearity, precision as repeatability, limit of quantitation (LOQ) and limit of detection (LOD) were determined. The linearity of the method was tested within the concentration range 20-200 mg L^{-1}. Method is linear with the correlation coefficients (r^2) in the range of 0.993 to 0.999. The LOD's for glucose and mannitol were 0.35 mg L^{-1} and 0.25 mg L^{-1}, respectively. The LOQ's for both compounds were 1 mg L^{-1}. It was proved that the method is repeatable (precise) (RSD<4%), and accurate. The average contents of glucose in olive leaves ranged from 31 to 154 mg, whilst contents of mannitol ranged from 89 up to 218 mg per g of dry weight extract.
Keywords: glucose, mannitol, gas chromatography, mass spectrometry

INTRODUCTION

D-glucose, also called dextrose or blood sugar, is the most widely occurring of all monosaccharides in nature and has the most important function within the human metabolism. It is well known that in nearly all living organisms, D-glucose serves as a source of energy for biochemical reactions. Glucose is present in different fruits, rice, wheat, soybeans, and many other plants. D-mannitol is a sugar alcohol (polyol), which can be mostly found in marine algae, yeast, lichens, different fruits and vegetables like pineapples, asparagus, sweet potatoes, carrots, onions, mushrooms etc. Mannitol is assumed to have several beneficial effects and is commonly used as an artificial sweetener in the food industry. Both of the mentioned sugars are applicable in food products, in pharmaceuticals, chemicals, and as a medicine.[1-4] Many articles with different separation techniques have been published on the chromatographic analysis of monosaccharides but the usual methods are the reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detection,[5-7] or gas chromatography coupled to mass spectrometry (GC-MS).[6,8-17] Identification using only an UV detector is very difficult due to the similarities in molecular structures and due to the lack of light-absorbing chromophores in carbohydrates' structures. Therefore it seems that MS is much better choice for unambiguous confirmation of the compounds. Direct GC is one of the less suitable methods for the separation of monosaccharides because of their high polarity and low volatility. In order to increase the volatility and enhance the

sensitivity, derivatisation must be conducted before the GC determination of these compounds.[8-10, 13, 14, 16] In addition tautomers of reducing sugars can cause further significant problems during conventional GC analysis. Namely, in solutions the open-chain (acyclic) form of reducing sugar glucose exists in equilibrium with several cyclic isomers (either α- or β-cyclic-furanose, and cyclic-pyranose), each containing a ring of carbons, closed by one oxygen atom. In aqueous solutions however, more than 99% of the existing glucose molecules exist as pyranose. The open-chain form is limited to about 0.25% and furanose exists in negligible amounts. Since carbohydrates can be present in solutions in various forms, multiple peaks may occur in the GC chromatograms as a result of sample preparation. Multiple peaks may confound both qualitative identification and quantitative determination of a single analyte. A lot of different more or less complicated and time-consuming procedures for derivatisation before GC analysis has been published. In the analytical methods that include silylation before GC, reagents such as hexamethyldisilazane (HMDS) or N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) have been repeatedly proven to be the best choice for sugars' trimethylsilyl (TMS) derivatives preparation, although N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) has some advantages over BSTFA and HMDS.[15,18,19] In the literature there is only limited information about carbohydrate composition of *Olea europaea* L. *Olea europea* L. is one of the more important oleaginous crops of the Mediterranean basin. Most of the determinations have been limited to olive-fruits [20-24] but on the other hand, tissues, such as olive-leaves, can also contain extensive sugar quantities. Undoubtedly, it is very important to also determine enumerated compounds in olive leaves because they can serve as excellent and rich source of sugars, and are now utilised in the food industry, pharmacy, chemistry, and medicine. Therefore the aim of this study was twofold: **(I)** to optimise and validate relatively simple, rapid and certain preparation method, which will include appropriate derivatization, followed by GC-MS analysis for the simultaneous separation and determination of D-glucose and D-mannitol in different wild olive leaves' samples obtained from Slovenian Karst region and **(II)** to establish how the conventional solid – liquid extractions performed under different conditions (using different extraction solvents, using various sample-to-solvent ratios, performed at different temperatures) affect the sugars' concentrations in the final extracts.

MATERIALS AND METHODS

Chemicals and reagents

All the reagents and solvents used were of analytical grade. Dry pyridine (PYR), methanol (MeOH), acetone, ethanol, toluene and hydroxylamine hydrochloride ($NH_2OH \cdot HCl$) were purchased from Merck (Germany), *N*-O-bis-trimethylsilyl trifluoroacetamide (BSTFA) and *N*-O-bis-trimethylsilyl trifluoroacetamide with 1% of trimethylchlorosilane (BSTFA + 1% TMCS) were from Fluka Chemie (Switzerland), *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA), D-glucose (99.5%), D-mannitol (98%), and phenyl-β-D-glucopyranoside (99%) were supplied by Sigma-Aldrich (Germany).

Preparation of calibration solutions and calibration curves with trimethylsilyl (TMS) and oxime-trimethylsilyl (o-TMS) derivatives

Preliminary studies were carried outin order to analyze sugars as volatile derivatives by GC-MS method. Glucose and mannitol standards were prepared and analysed as their TMS derivatives, according to literature data: **(I)** without prior formation of the oxime derivatives with direct silylation (single-step reaction) and **(II)** with prior formation of oxime derivatives before silylation (two-step reaction). Both steps were optimized separately. To optimize the silylation process, the reaction was carried outat various time intervals (10 min, 1 h, 1.5 h, 2 h, 3 h) and at different temperatures (room temperature, 70-80°C, 100-110°C), using various silylating reagents (MSTFA, BSTFA and BSTFA+1%TMCS). In all cases pyridine was used as the catalyst. Silylating reagents were added in the same quantities, 200 µL for each sample. For the optimization of the oxime derivatives formation procedure, the reaction was also perform edunder different conditions. Hydroxyl amine hydrochloride dissolved in pyridine ($NH_2OH \cdot HCl$/PYR, 2-2.5%) was used as a reagent. The samples were heated forvarious lengths of time (30-120 min) at different temperatures (room temperature, 70-110 °C).

(I) Optimized prepration of TMS derivatives without priorformation of the oxime derivatives(directsilylation; single-step reaction)

Standard stock solutions of glucose and mannitol were prepared by weighing 10 mg of each pure standard into separate 10 mL glass flasks and dissolving in a solvent mixture of methanol and water (MeOH-H_2O, v/v=1/1). Then after wards the flasks were placed in an ultrasonic bath for at least 15 min. Calibration solutions were prepared by combining different aliquots (20–200 µL) of glucose and mannitol stock solutions into separate conical glass flasks. These solutions were evaporated to absolute dryness, using a rotary evaporator. Then 100 µL of pyridine and 200 µL MSTFA were added, and derivatisation (silylation) was carried out by heating for 50–60 min at 70–80°C on a sand bath. Prior to GC-MS analysis the solutions were diluted with toluene to the same final volume of 1 mL and 1 µL of the final solutions were injected into the GC-MS system in triplicate; also three replicates of separate analyses were performed. Curves were constructed by linear regression of the peak-areas of individual sugars (y), versus the concentration (x) in mg L^{-1}.

(II) Optimised preparation of o-TMS derivatives (with prior formation of oxime derivatives; two-step reaction)

Standard stock solutions were prepared as follows: 10 mg of glucose, 10 mg of mannitol, and 5 mg of phenyl-β-D-glucopyranoside (internal standard-ISTD) were weighed with analytical precision into three different 10 mL glass flasks. Standard compounds were dissolved in 10 mL of $NH_2OH \cdot HCl$/PYR (2%). Calibration solutions were prepared by combining different aliquots (20–200 µL) of glucose and (20–200 µL) of mannitol stock solutions with 200 µL of ISTD stock solution into separate conical glass flasks. Solutions were heated on a sand bath at 70–80°C for 45 min then cooled to room temperature. Then 200 µL of MSTFA were added, and samples were heated for 50–60 min at 70–80°C. Prior to analysis the solutions were diluted with toluene to 1 mL and 1 µL of these solutions were injected into GC-MS system in triplicate; also three replicates of separate analyses

were performed. Curves were constructed by linear regression of the peak-area ratio (y) of individual sugar to the ISTD versus the concentration (x) in mg L^{-1}.

Olive leaves extracts preparation

The olive leaves extracts, used in this work, were prepared by the extraction of dried (60°C) and ground olive leaves (particle size fraction < 1 mm), collected within the Karst area (Slovenia). The corresponding amount of ISTD was added, prior to the extractions. Extractions were done in duplicate with water and mixtures of water/ethanol or water/acetone (v/v = 1:1) at various sample-to-solvent ratios (Table 1) in flasks, equipped with a condenser. The temperature was maintained by a water bath at 80°C (for the water) and at 60°C (for the mixtures of water/organic solvents). The extraction time was 2 hours. After extraction, the olive leaves' particles were removed by filtration. The extracts were evaporated to dryness at a reduced pressure at 40°C and stored in glass bottles within a refrigerator until chromatographic analysis. Approximately 20 mg of each dry sample was weighed into a 10 mL glass flask and dissolved in 10 mL $NH_2OH{\cdot}HCl$/PYR. An aliquot of 100 µL from each sample was transferred into conical glass flasks and additional 200 µL of the $NH_2OH{\cdot}HCl$/PYR were added. Samples were heated on the sand bath at 70–80°C for 45 min, then cooled to room temperature. The samples were trimethyl silylated by adding 200 µL of MSTFA and heating at 70–80°C for 60 min. Prior to analysis the solutions were diluted with toluene to 1 mL and 1 µL analysed by GC-MS in triplicate; three replicate analyses were also performed. The quantities of glucose and mannitol from the extracts were determined from corresponding calibration curves, using ISTD. The investigated compounds were confirmed using standard compounds, by comparing their retention times, mass spectral properties and by the study of fragmentation patterns of o-TMS or TMS derivatives. All other compounds were identified by comparing their spectral properties with those, reported in the Willey and NIST mass spectra libraries or in literature.

Validation parameters

The method was validated for linearity, precision as repeatability, accuracy, limit of detection (LOD), and limit of quantitation (LOQ).

Instrumentation and GC-MS Conditions

A gas chromatograph Varian 3900 coupled with ion trap mass spectrometer Saturn 2100T was used to analyze the TMS and o-TMS derivatives of carbohydrates. Chromatographic separation was performed on a VF-5MS capillary column (30 m x 0.25 mm i.d., 0.25 µm thick). Helium was used as the carrier gas with a constant flow of 1 mL min^{-1}. The temperature program was as follows: initial 60°C (2 min), 10°C min^{-1} to 270°C (3 min), 5°C min^{-1} to 300°C (2 min) (total elution time: 34 min). Injector temperature was set at 250°C. Samples were injected in split less mode. The injection volume was 1 µL. The transfer line temperature was held at 200°C. MS was operated in the electron positive-mode ionisation (EI), with electron energy at 70 eV. Source temperature was 235°C. The MS data were obtained in full scan mode (mass range 50-650 *m/z*).

RESULTS AND DISCUSSION

Optimization of TMS and o-TMS derivatives' preparation and identification of the compounds

Untreated sugars are unsuitable for GC-MS analysis, because of their polarity and low volatility. One of the more widely used methods for decreasing the polarity and increasing the volatility is silylation. To optimize the silylation reaction, the different silylation reagents, reaction times, and temperatures were tested. Based on literature data, HMDS, BSTFA or BSTFA + 1% TMCS have been repeatedly proven to be the best choice for sugars' trimethylsilylil (TMS) derivatives preparation[6, 8, 11, 12, 17], but our results did not confirm this fact. BSTFA silylating potential is similar to that of MSTFA and both reagents should react similarly as donors for TMS groups. In our experiments using BSTFA or BSTFA + 1% TMCS in pyridine the derivatising yield was lower compared to MSTFA and in some cases a precipitate was formed in the reaction vial. After silylation using BSTFA and BSTFA + 1% TMCS, with or without prior oximation, at least five peaks appeared in the chromatograms for glucose-TMS derivative (Fig. 1c). These draw backs were not removed either by changing the temperature or reaction time. Also the peak-area ratios were continuously changing. In our experiments much better results were obtained using known powerful silylation reagent MSTFA (Figs. 1a, 1b, 1d). Only MSTFA provided complete silylation and without (Fig. 1a) or with prior oximation (Figs. 1b, 2) gave as expected one sharp peak for mannitol and no more than two peaks for glucose in all chromatograms. After direct silylation of the glucose (single-step reaction), using MSTFA, the two peaks in the chromatograms, were actually α- and β-glucopyranose TMS isomers. In this case peak-area ratios for both isomers were constant throughout the whole concentration range, and were between 0.8 and 1. The silylation reaction for mannitol was completed in 30 min at room temperature, but this was not the case for glucose. Glucose was completely silylated after 60 min by heating at 70–80°C. Heating at 80°C for over 60 min did not change the results for mannitol, but on the other hand, longer heating had the effect on glucose. Namely, after heating for longer time intervals (over 70 min) at 70°C, 80°C or 100°C degradation of the compound occurred, as on the chromatograms for glucose, again multiple peaks appeared. For further analyses 80 °C was set as the maximum silylating temperature. Finally, the reaction time was also optimised from 30 to 120 min and a maximum derivatisation yield was obtained after 50–60 min for both compounds. The formation of the oxime derivatives just before silylation was also tested. Oximation reaction did not affect the sugar alcohol mannitol, but it was essential for reducing sugar glucose, because in this way aldehyde group was converted into oxime, which locked the reducing sugar into open-chain configuration, thus eliminated the anomericcentre and its ability to form furanoic and pyranoic ring structures. Hydroxylamine hydrochloride diluted in pyridine (2%) was used as a suitable reagent and several reaction times and temperatures were tested. The results confirmed that for optimal oxime formation, reaction for both compounds was completed in 45 min by heating at 70–80°C. Gentle heating accelerated the reaction but the maximum temperature was again 70–80°C. Higher temperatures (100°C) caused evaporation of the pyridine and smeared sample all over the flask. It was also important that for optimal oximation always more than 200 μL of the solution was heated, otherwise smaller volumes led to incomplete reaction and unrepeatable results. Optimal conditions

for oxime derivatives' preparation were therefore heating of a minimum 200 µL of the solution for 45 min at 70–80°C. After two-step reaction generally for glucose acyclic oxime -TMS *syn*- and *anti*-derivatives were formed (Figs. 1b, 1d).[8] The peak-area ratios for both isomers were stable (ratio was approx. 4) and independent of its analysed amounts.

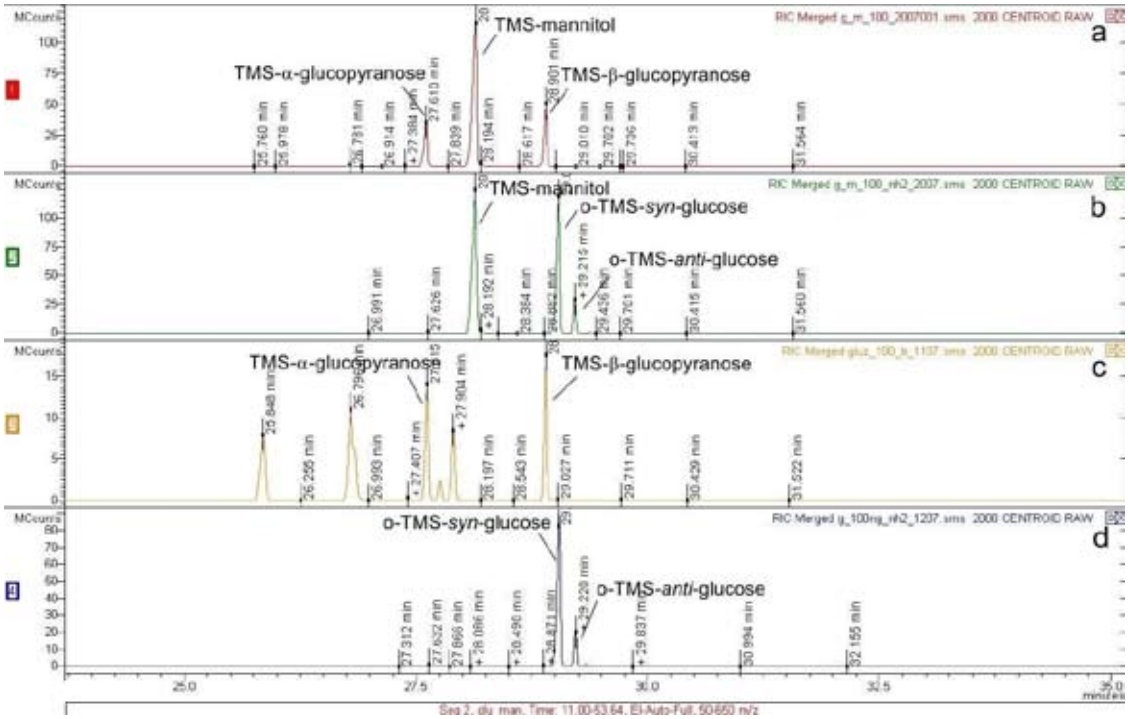

Figure 1.Total ion chromatograms (TIC) of: (a) directly silylated glucose and mannitol with MSTFA (single-step reaction); (b) silylated glucose and mannitol with MSTFA with prior oximation (two-step reaction); (c) directly silylated glucose with BSTFA (single-step reaction); (d) silylated glucose with MSTFA with prior oximation (two-step reaction)

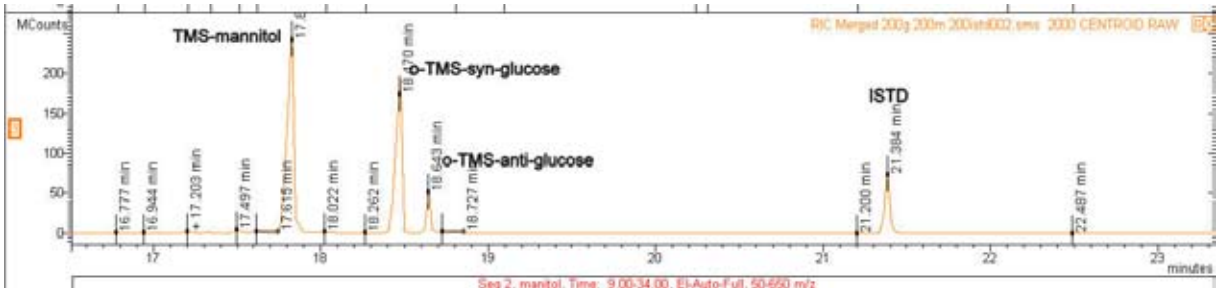

Figure 2.Total ion chromatogram (TIC) of glucose and mannitol obtained after prior oximation followed by silylation (two-step reaction), using MSTFA as silylation agent and phenyl-β-D-glucopyranoside as ISTD

Validation parameters

The responses of both investigated compounds were linear over the tested concentration range from 20−200 mg L^{-1}. The correlation coefficients (r^2) were between 0.993 and 0.999. The LOD's forglucose and mannitol were 0.35 mg L^{-1} and 0.25 mg L^{-1}, respectively. The LOQ's for both compounds were 1 mg L^{-1}. It was proven that this method is repeatable (precise), and accurate (RSD<3,9%).

Confirmation of TMS and o-TMS derivatives

Mono-saccharides sometimes can not be successfully separated using HPLC-UV system due to the similarity in skeletons of these compounds. Especially when two compounds with very close retention times and identical UV spectra appear in a chromatogram, it is necessary to use more sophisticated detectors that can yield much more structural information without requiring the isolation of the compounds. MS is often used to overcome these drawbacks. We have repeatedly proved that GC-MS can also serve as a suitable alternative way of determination, chiefly in case of isomers present in complex natural matrices, such as plant extracts.[25,26] Although mannitol and both isomers of glucose had very similar structures, they were well distinguished by order of elution during gas chromatography (Figs. 1,2), and by fragmentation patterns in their mass spectra's (Fig.3). In order to unambiguously identify the isomers of glucose, retention times (*tr*) should be regularly checked and compared to the *tr´*s of the standard compounds, obtained under the same chromatographic conditions. Actually, β-TMS-glucopyranose was retained longer then α-TMS-glucopyranose and an o-TMS-*anti*-isomer was always retained longer than o-TMS-*syn*-isomerafter GC separation. Fragmentation patterns of o-TMS-*syn*-glucose and o-TMS-*anti*-glucose were studied and were almost the same as they contained the same fragment ions (Figs. 3a, 3b), only their intensities were different. Molecular ion can be observed at *m/z* 628 for both compounds, but its intensity is very low. The loss of a methyl group from the molecular ion results in a fragment ion *m/z* 613, whilst the next loss of the TMSOH group provides the *m/z* 523 ion. However, the most intense fragment is formed at *m/z* 319-320 upon the cleavage of C3-C4 bond within the glucose molecule. Other fragments at *m/z* 422, 217, 147, 103 and 73 are also observed in the EI spectra of o-TMS-glucose. In contrast to glucose, mannitol did not form isomers in chromatograms. In the EI^+ mass spectra of the 6TMS mannitol molecular ion [$M^{+\cdot}$ = 614] is very low (intensity < 1%) (Fig. 3c). Important fragment ion signals are found at *m/z* 599 (loss of CH_3 group from the molecular ion), *m/z* 525 (further elimination of TMS group) and *m/z* 419 (further elimination of TMSOH and CH_3 groups). The characteristic sugar fragments at *m/z* 217 (TMSO-CH=CH-CH-OTMS) and at *m/z* 320, are also present. The ion at *m/z* 345 is characteristic for hexitols like mannitol and can be described as the loss of one TMS group together with one CH_3 group and two TMSOH molecules from the molecular ion.(Fig. 3c).

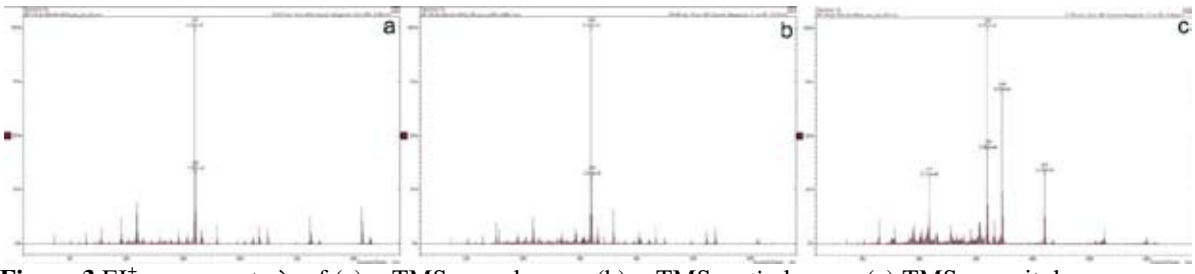

Figure 3.EI^+ mass spectra`s of (a) o-TMS-*syn*-glucose; (b) o-TMS-*anti*-glucose; (c) TMS-mannitol.

The sugar concentrations in the real samples were determined over two-step derivatization procedure, included oximation, as well as trimethylsilylation from the corresponding calibration curves, using phenyl-β-D-glucopyranoside as an ISTD. Figure 4 presents a typical TIC chromatogram of the investigated compounds, present in olive leaves' extract. The average contents of glucose and

mannitol in seven different olive leaves' extracts were determined and expressed in mg per g of dry weight extract (Table 1). The contents of D-glucose ranged from 31 to 154 mg, whilst contents of D-mannitol ranged from 89 up to 218 mg per g of dry weight extract. The lowest concentration of D-glucose was determined in the sample, where water-acetone mixture with sample-to-solvent ratio1:20 [g mL^{-1}] was used for extraction. The lowest amount of D-mannitol was found in the sample, where water-acetone mixture with sample-to-solvent ratio 1:10 [g mL^{-1}] was used. In the sample, prepared using only water as an extraction solvent with sample-to-solvent ratio 1:20 [g mL^{-1}], and heating at 80°C for 2 hours both compounds were presented in the highest amounts. On the basis of this study it can be concluded that the optimum solvent for the extraction of different mono-saccharides from olive leaves is water with a sample-to-solvent ratio 1:20 [g mL^{-1}], while the addition of organic solvents (acetone and ethanol) to the water resulted in lower sugar contents in the final extracts. It can also be assumed that sugar contents also depend on the extraction temperature, as the samples with the addition of organic solvents were extracted at slightly lower temperature (60°C).

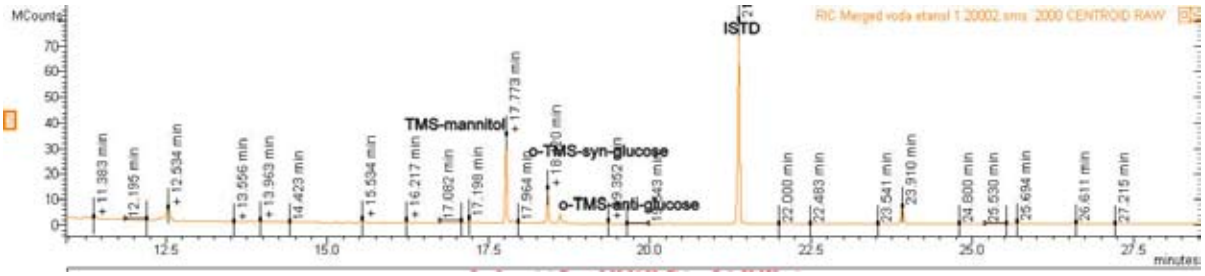

Figure 4. Typical TIC chromatogram of silylated investigated compounds present in olive leaves' extract, prepared using water-ethanol mixture as an extraction solvent with sample-to-solvent ratio 1 : 20 [g mL^{-1}].

Table 1. Concentrations of glucose and mannitol determined from the corresponding calibration curves using internal standard method

Extraction solvent	Sample to solvent ratio [g mL^{-1}]	Concentration of glucose [mg g^{-1} of dry weight extract]	Concentration of mannitol [mg g^{-1} of dry weight extract]
water	1:10	134.3	176.6
water	1:20	**154.4**	**217.9**
water	1:50	102.7	213.7
water-acetone*	1:10	47.57	**88.7**
water-acetone*	1:20	**30.9**	102.5
water-ethanol*	1:10	102.1	142.9
water-ethanol*	1:20	85.37	184.1

* volume to volume ratio 1:1 was used for extraction

CONCLUSION

The main difficulties in monosaccharide analysis arise from the existence of their numerous structurally – related stereoisomers with close chemical and physical properties, and their occurrence as mixture in complex natural matrices. Our goal was to determine different types of sugars by GC-MS simultaneously over one single run with easier sample preparation that would provide optimal results and a minimal number of peaks in chromatograms for the investigated compounds. The described method with the previous formation of oxime - (o) and

later trimethylsilyl - (TMS) derivatives or with direct silylation provides a good separation and quantitative determination of thermally stable and volatile o-TMS or TMS derivatives of glucose and TMS derivatives of mannitol in different olive leaves extracts. The method is sensitive, quantitative, and suitable for simultaneously tracking the levels of structurally diverse monosaccharide species, as well as their numerous isomers even if they are present in the trace amounts. Never the less in all samples relatively high concentrations of sugars were determined and therefore this study also suggests that olive leaf can serve as an important source of naturally derived sugars which can be utilized in food industry, chemistry, and pharmacy.

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LISTERIA MONOCYTOGENES IN THE SURFACE WATERS OF THE NORTH-EAST BOSNIA

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT
L. monocytogenes is a facultative intracellular pathogen which inhabits a wide range of natural habitats. It causes listeriosis, a serious human and animal infection. Listeriosis is placed among diseases with a very frequent fatal outcome which amounts to 30%. Listeriosis is primarily a zoonosis which is responsible for various diseases that affect both domestic and wild animals in natural conditions. Considering the fact that *L. monocytogenes* is a ubiquitous microorganism and that it is isolated from almost every environment and all food categories, it becomes clear that listeria is not only spread as zoonosis, but that there are numerous sources of its origin. The aim of this research was to confirm the presence of the mentioned bacterium in the water environment of the north-east Bosnia. The bacteria from the genus *Listeria* was present in 49.07% of 110 analyzed samples (54/110). Three species were identified: *L. monocytogenes*, *L. innocua* and *L. seeligeri*, with the following distribution by species: *L. monocytogenes* 1.81% (2/110), *L. innocua* 43.63% (48/110) and *L. seeligeri* 3.63% (4/110). The conducted research confirmed the presence of the bacteria in analyzed river and lake waters at the territory of the north-east Bosnia, which indicates the need for a serious approach in prevention of this phenomenon. One of the first steps is the introduction of obligatory legal measures of water control and the engagement of all relevant institutions in order to solve this problem.
Key words: *L. monocytogenes*, listeriosis, water environment

INTRODUCTION

L. monocytogenes has been recognized as an important human and animal pathogen, and in the last two decades it has become the subject of interest in medical, veterinary and food microbiology[1]. Listeriosis is a serious human and animal infection, mostly caused by consuming the food contaminated by *L. monocytogenes* bacterium, and the outcome of the listeria infection is manifested in series of different clinical conditions. Listeria infection in humans appears sporadically, but numerous outbreaks do also occur. Out of thirteen known bacteria serotypes, the serotypes 4b, 1/2a and 1/2b are the causes of diseases in most cases[2,3]. Considering that it is a ubiquitous microorganism, *L. monocytogenes* is isolated from many sources including soil, plants, rotting vegetation, waste waters, rivers, sea backwaters, and from almost all groceries and food categories, milk and dairy products, meat and meat products, fruits and vegetables, sea food[4].

Listeriosis is primarily a zoonosis, and it causes various diseases of domestic animals which represent a great economic problem. Sheep and cattle are domestic animals that are mostly affected by this bacterium[5]. It has been isolated from 37 mammalian species[6]. This bacterium possesses a high resistance to external conditions, which enables it to survive. *L. Monocytogenes* grows at temperature from 1-45°C and is resistant to negative effects of freezing, drying and heat. Tolerance of the bacteria to high concentrations of NaCl (up to 12%), reproduction within the wide range of pH (4.3-9.6) and at a refrigerator temperature (+4°C) additionally enable the survival of listeria during food processing which represents a great problem in food industries and a potential danger for human health[7].

Domestic animals – ruminants – maintain the listeria circulation by fecal-oral contamination of soil and water. Inappropriately prepared silage represents the greatest risk for animal contamination.

The conducted research indicates the connection between the faecal excreta microorganism and its presence in in adequately prepared feed for dairy animals. *L. monocytogenes* can be transmitted from fodder and animal feed into digestive tract of animals by ingestion, and in this way, the animal carriers of bacteria become the source of meat and dairy products contamination[8].

Considering the fact that listeria is naturally the part of human and animal digestive tract, the presence of *L. monocytogenes* in rivers, lakes and channels is the result of their contamination by human and animal faecal excreta. The research indicates that 5-10% of human population carry *L. monocytogenes* in their intestinal tracts without any visible indication of a disease (asymptomatic carriers)[6]. Therefore, the contamination of environment is possible by saliva and excreta of healthy animals and humans, not only the sick ones. Faecal excreta contaminate the environment – soil, water, plants, fruits, vegetables, and so on, which represents a great danger for humans.

In the research conducted at the territory of Tuzla, all samples of the Jala River and its tributaries were positive for *Listeria spp*., where the presence of *L. monocytogenes* amounted to 60%. In the same research, the presence of *L. monocytogenes* in samples of water from Lake Modrac was 13.33%[9]. According to the data from professional literature, besides the previous research, there are no other published works about the presence of *L. monocytogenes* in the water environment of Bosnia and Herzegovina. Concerning the fact that *L. monocytogenes* is not enough studied at the territory of Bosnia and Herzegovina, the attention in this work was paid to the presence of this pathogen in the water environment.

MATERIAL AND METHODS

The microbiological examination was conducted in the period from June 2012 until February 2013, in the microbiology laboratory at the Faculty of Mathematics and Natural Sciences in Tuzla. The examination included 110 samples of water collected at 11 localities at the territory of the north-east Bosnia. Analyzed samples were taken from the following rivers: Spreča, Drina, Drinjača, Sava, Gradašnica, Tinja and Turija, including the lakes: Hazna, Vidara, Modrac and Bistarac. Ten samples of water were collected at every of the mentioned localities. At the Spreča River, samples were collected at length span 2-6 km down stream, and at the following regions: Caparde, Osmaci, Šeher, Kalesija, Miljanovci, Jeginov Lug, Rainci Donji, Vukovije Donje, Dubrave Donje and Živinice. At the region of the Drina River samples were taken at length span of 3-10 km downstream and at the following places: Zvornik, Karakaj, Tršić, Tabanci, Kozluk, Roćević, Šepak, Pilica, Glavičice and Janja. The sampling of water at the Drinjača included the following places: Brateljevići, Kladanj, Starić, Ravne, Prijevor, Turalići, Tišća, Dragosavci, Konjević Polje and Milići. The samples were collected at the length span of 2-5 km downstream. The sampling at the Sava River was conducted at the following places: Domaljevac, Mahala, Orašje, Vidovice, Vučilovac, Gorice, Grbavica, Brčko, Gredice and Brezovo Polje. These samples were also collected down stream at the length of 5-10 km. The samples for analysis at the Gradašnica River were collected 1-2 km down stream at the following places: Bagdale, Bukva, Centar, Svirac, Varoš, Škorići, Požarike, Sibovac, Ledenice Gornje and Srednja Slatina. The water samples at the river Tinja were taken for microbiological analysis at length span of 2-4 km downstream at the following places:

Avdibašići, Donja Dragunja, Osoje, Previle, Tinja, Podpeć, Bjelave, Duboki Potok, Srebrenik and Špionica. Ten samples of the Turija River were collected at the following places: Pribitkovići, Borovac, Seona, Lozna, Savičići, Orahovica, Mujagići, Turija, Babice and Prokosovići. The sampling was conducted at the distance range of 2-3 km downstream. The water samples for microbiological analysis were collected at every lakeside and from the middle of the mentioned lakes. The samples were placed in 1000 ml sterile bottles, appropriately marked and transported in a portable fridge to the microbiological laboratory where they were subjected to further analysis. In order to isolate and identify the bacteria of *Listeria* genus, we applied the Horizontal method for the detection and enumeration of *L. monocytogenes* – the International standard ISO 11290-1:1996/Amd.1:2004 (E)[10]. A hundred millilitres was measured of every sample and filtered in a vacuum filtration pump. After that process, the filters were aseptically transmitted into 90 ml ½ Fraser broth using sterile tweezers and incubated at 30°C/24 h. Twenty four hours later, each sample that changed the colour of Fraser broth from yellow to black (which indicates the positive reaction to esculin) was transferred into test tubes with 10 ml of concentrated Fraser broth (0.1 ml of inoculum). The incubation of the inoculated sample lasted for 48 h/35°C. After the incubation, the content of esculin+ in a test tube was heated by a sterile esa on the both Oxford and Aloe agars, and incubated at the temperature of 35-37°C/48 h. After the process of incubation was completed, we watched the grown colonies. The characteristic bacteria colonies from the genus *Listeria* on the Oxford agar were small (1-2 mm), grayish-black with indented middle part and brownish-black halo around colony. The colonies of *L. monocytogenes* on the Aloe agar were bluish-green with transparent whitish blur surrounding the colonies. The characteristic listeria colonies were streaked from the Oxford agar to Tryptone soya agar containing 0.6 per cent of the yeast extract (TSYEA – Triptone soya yeast extract agar) and incubated at 37°C/18-24 h. Aiming for identification from pure TSYEA cultures, the following tests were conducted: catalase test, Gramm stain procedure, haemolysis and degradation of carbohydrates. In order to identify the bacteria, we used API – Microgene *Listeria* ID System. The following biochemical reactions were tested: esculin hydrolysis, mannitol fermentation, xylose, arabitol, ribose, rhamnose, trehalose, tagatose, glucose-I-phosphates, methyl-D-glucose and methyl-D-mannose. Microgene *Listeria* ID System includes the haemolysis test which was additionally confirmed in this manner.

RESULTS AND DISCUSSION

All the isolated species had a specific image of the Gramm positive rods of a palisade arrangement with the length of about 0.5-2 µm and the width of 0.4-0.5 µm. The nonsporogenus bacilli that were arranged separately, by two or scattered in the shape of letters V or Y were also visible by the preparation. The macroscopic aspect of the colonies from the genus *Listeria* which were grown on the Oxford agar had a specific form. After the incubation was completed, the grayish-black colonies grew to 1-2 mm, with indented middle part and a brownish-black halo surrounding the colony. The listeria cultures had an unpleasant smell that reminded of a rancid butter. On the Aloe agar, *L. monocytogenes* formed bluish-green colonies with transparent whitish blur around the colony. The remaining isolated species from the genus *Listeria* also formed bluish-green colonies but without the blur surrounding colonies. On blood agar, *L. Monocytogenes* formed

small, round, smooth, raised and opaque colonies, around which was formed a tight zone of β haemolysis. *L. seeligeri,* which was also isolated in work, led to the lysing of blood cells in the sample around and under the heated colonies forming the zone of haemolysis. On the other hand, the heated colonies of *L. innocua* did not indicate the formation of haemolysis zone and the heated plates of blood agar remained unchanged. All the isolated species from the genus *Listeria* produced the enzyme catalase. The biochemical characteristics of the bacteria were confirmed by the tests of Microgene *Listeria* ID System. The positive reaction of *L. monocytogenes* to esculin, arabitol, rhamnose, trehalose, methyl-D-glucose, methyl-D-mannose and blood cells haemolysis was confirmed, and the negative reaction to mannitol, xylose, ribose, tagatose and glucose-I-phosphate.

The microbiological analysis of the water environment on 11 localities at the territory of the north-east Bosnia, confirmed the bacteria presence of the *Listeria* genus in in 49.07% of samples out of 110 analyzed water samples (54/110). Three species were identified: *L. monocytogenes, L. innocua* and *L. seeligeri* with the following distribution by species: *L. monocytogenes* was found in 1.81% of samples (2/110), *L. innocua* in 43.63% of samples (48/110) and the presenceof *L. seeligeri* amounted to 3.63% (4/110). The total results overview of the microbiological sample analysis conducted at the water environment of the north-east Bosnia are presented in Table 1. The results of water analysis in the rivers and lakes at the territory of the north-east Bosnia indicate that the presence of *L. monocytogenes* amounts to 1.81% (2/110). *L. monocytogenes* was found in the region of the Drinjača River where it was identified in two samples of water. It is most likely that the waste waters containing human and animal faecal excreta caused the contamination. Forty eight samples of 110 analyzed water samples tested positive for *L. innocua* (43.63%). The first recorded case of a fatal bacteriemia caused by this particular bacterium confirms that *L. innocua*is not as harmless as it seems[11]. The presence of other species from the genus *Listeria*, except *L. monocytogenes*, can indicate the possible contamination with *L. monocytogenes* because of the similar conditions needed for growth and reproduction.

All samples of the Jala River and its tributaries were positive for *Listeria spp*., where the presence of *L. monocytogenes* amounted to 60%. The same research confirmed the presence of *L. monocytogenes* in Lake Modrac (13.33%)[9]. The microbiological water analysis of Lake Modrac in this research confirmed the presence of *L. innocua*in 40% of the samples. *L. monocytogenes* was not found in the mentioned lake. The research conducted in the north-west England did not show the presence of *L. monocytogenes*. *Listeria spp*. was isolated in eight samples (27%), out of 30 samples. Six of them were identified as *L. seeligeri*, one was *L. innocua* and the last one was *L. welshimeri*[12]. The water analysis of the rivers and lakes in Greece confirmed the lower level of presence of *L. monocytogenes* which amounted to 4%[13]. In the South Nation River in Canada (Ontario), 10% of samples were positive for *L. monocytogenes*[14]. *Listeria spp*. were also present in the great percentage of treated water (84.4%) and crude mud (89.2%) in six French city facilities for water treatment and one for compost[15]. Research conducted in California confirmed the presence of *L. monocytogenes* in 62% of water samples collected at the rivers and coastal sea[13]. The results of the previously conducted research do not mach with this one because they indicate the higher level of contamination of water with *L. monocytogenes*.

The results showed that not many samples were positive for the pathogenic bacteria *L. monocytogenes*. The most probable

explanation for this phenomenon is the relatively small number of samples in analyzed waters of rivers and lakes. Concerning the fact that there are no facilities for treating waste waters and that the state of sewage system is quite poor, the presence of this pathogenic bacterium in mentioned localities is unquestionable. A lot of the villages and cities that are close to the tested areas do not have suitable sewage networks, which creates a great possibility for their contamination from that source and spreading downstream. There are many agricultural areas at riversides which presents a multiple risk for listeriosis contamination since these waters are used for cattle feeding and watering agricultural crops. The lake waters are used for fishing, swimming and water sports and they are often the source for contamination of wild animals, which also represents a risk and the potential source of a disease. Therefore, the presence of this pathogen in waters represents a great danger for human and animal health. While there are many facilities for the treatment of waste waters in developed countries, waste waters in Bosnia and Herzegovina are let into rivers and lakes without being previously processed. When it comes to wastewater drainage and treatment in our country, the situation is quite alarming. The construction of suitable sewage networks and facilities for wastewater refinement are the first steps in prevention of listeria expansion and contamination.

Although *L. monocytogenes*is present in small percentage, this research confirmed the presence of the bacteria from the genus *Listeria* in tested river and lake waters, which calls for a serious approach to the prevention of this phenomenon and the legal obligatory control of this pathogen would be one of the first steps in solving this problem.

The control of water quality in Bosnia and Herzegovina is not paid enough attention to, which represents a serious epidemiological risk especially during the summer months. The increased water consumption due to the higher standards of living, urbanization and industrialization led to the growing amounts of waste waters that are let into natural water environment, which quite disturbs the natural balance and causes its contamination. By the pollution of surface waters, amounts of pure ground waters, which are mostly used for drinking, are also reduced.

Water is an irreplaceable resource which is a main prerequisite for the survival of all living beings and therefore, it should be protected from destruction and pollution. In order to protect present and future generations, it is necessary to protect the water environment from pollution which calls for the responsibility of every country and its population, and the engagement of all relevant institutions in solving this problem.

The conducted research can be regarded as the foundation for the further and more extensive researches concerning the fact that it is the original work of this domain in our country.

Table 1. The total results overview of the microbiological sample analysis conducted at the water environment of the north-east Bosnia

Locality	Number of samples	Number of isolated *Listeria spp.* (%)	*Listeria monocytogenes* (%)	Number of isolates *Listeria innocua*(%)	*Listeria seeligeri (%)*
1. Spreča	10	4 (40%)	-	1 (10%)	3 (30%)

2. Drinjača	10	5 (50%)	2 (20%)	3 (30%)	-
3.Drina	10	5 (50%)	-	5 (50%)	-
4.Sava	10	5 (50%)	-	5 (50%)	-
5.Hazna	10	2 (20%)	-	1 (10%)	1 (10%)
6.Vidara	10	0 (0%)	-	-	-
7.Gradašnica	10	9 (90%)	-	9 (90%)	-
8.Tinja	10	10 (100%)	-	10 (100%)	-
9.Turija	10	10 (100%)	-	10 (100%)	-
10.Modrac	10	4 (40%)	-	4 (40%)	-
11.Bistarac	10	0 (0%)	-	-	-
Total	110	54 (49.07%)	2 (1.81 %)	48 (43.63 %)	4 (3.63%)

CONCLUSION

The presence of the bacteria from the genus *Listeria* was confirmed in 54 samples out of 110 analyzed water samples collected at the rivers and lakes in the north-east Bosnia (49.07%). Within this genus three species were identified: *L. monocytogenes*, *L. innocua* and *L. seeligeri*. *L. monocytogenes* was present in 1.81% samples (2/110), *L. innocua* in 43.63% (48/110) and *L. seeligeri* in 3.63% (4/110). *L. monocytogenes* was found at the territory of the Drinjača River, where the incidence of *L. monocytogenes* was accomplished with 1.81% (2/110). *L. seeligeri* was found at the territory of the Spreča Riverand Lake Hazna. *L. innocua* was isolated at the following rivers: Spreča, Drinjača, Drina, Sava, Gradašnica, Tinja and Turija, and in the lakes: Hazna and Modrac. The presence of other species from the genus *Listeria*, besides *L. monocytogenes*, may refer to the contamination with *L. monocytogenes* since they need same conditions for growth and reproduction. Concerning the fact that there are no facilities for treating waste waters and that the state of sewage networks is quite poor, the presence of this pathogenic bacterium in the mentioned localities is unquestionable. The low level of *L. monocytogenes* isolation in this research refers to the fact that the small number of isolated bacteria is the result of not enough water samples of rivers and lakes. It has to be noted that there is an objective danger for human and animal health because of the presence of the mentioned pathogen in water. It is evident that the bacteria of *Listeria* genus are present in our water environment and this data should be the reason for introduction of obligatory legal measures and a serious approach to the prevention of this phenomenon. One of the priorities is the legal obligatory water control to this pathogen.

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PROPERTIES OF EXTRUDED CORN PRODUCTS WITH ADDITION OF RYE FLOUR

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT
The aim of this research was to examine the potentiality of enrichment of extruded corn products by addition of rye, which is arich source of dietary fibres and bioactive components. Corn grits with particle size ≤500 µm and grits with particle size >500 µm were used. Rye flour was added in 20% (d. m.). Extrudates were produced in the laboratory single screw extruder at 135/170/170 °C. Rye addition caused a decrease of expansion ratio, lightness and fracturability and an increase of bulk density, colour saturation and hardness of extrudates. When grits of larger particle size were used, lightness of samples increased after extrusion, while it decreased for smaller particle size. Starch damage largely increased after extrusion, as well as the absorption index and solubility, while viscosity decreased. Rye addition decreased starch damage.
Keywords: rye, extrusion, physical properties, starch damage

INTRODUCTION

Extrusion is widely used in the food industry. Among others, snack products and modified flours for the bakery and confectionery industry are commonly produced by this process. Basic materials for extrusion are starch and/or protein rich raw materials, such as corn grits.
Since consumers are increasingly aware of proper diet importance and corn based snacks are considered as an "empty calorie source", attempts are being made to make these snacks healthier. Jozinović et al. investigated properties of corn extrudates enriched with buckwheat and chestnut flour[1], Anton et al. fortified snacks with common bean[2], Lazou and Krokida added lentil to corn snacks[3,4] and Obradović et al. gave an overview of improvement of nutritional and functional properties of extruded products[5].
Whole grain rye flour is a rich source of fibres, with 4.7% soluble dietary fibre, 11.7% insoluble dietary fibre, 1.5% water soluble pentosan, 7.9% total pentosan and 2.4% ß-glucan (based on dry matter)[6]. Although it is health-beneficial, due to a specific polyphenolic and small peptides composition, it has a specific bitter flavour and is often mixed with other cereal flours[6].
The aim of this paper was to research the potentiality of production of extruded corn products fortified with rye flour.

MATERIALS AND METHODS

Corn grits with granulation ≤500 µm (*Resli*) and >500 µm (*Specijal*) were kindly supplied by ŽitoJSC. Osijek, Croatia. Rye was purchased at Family farm Jazbec, Ivanovac, Croatia and milled at a laboratory mill IKA MF10 (IKA Werke GmbH, Staufen, Germany) to produce wholegrain flour.
Corn grits and rye flour were mixed in a ratio of 80:20 and moisture of mixtures was set to 15%. Prepared flour mixtures were kept overnight at 4°C to even moisture through the samples and extruded in the laboratory single screw extruder Brabender 19/20 DN (Brabender GmbH & Co, Duisburg, Germany) with screw configuration 4:1, 4 mm die and temperature regime 135/170/170 °C. Extrudates were air-dried overnight and analysed.

Expansion ratio was determined according to Brnčićet al.[7], *bulk density* according to Alvarez-Martinez et al.[8], and *texture properties* and *colour* according to Jozinović et al.[1].

Starch damage was determined according to AACC 76-31.01 method[9], with Megazyme kit.

*Water absorption index (WAI) and water solubility index (WSI)*were determined according to Anderson et al.[10].

Pasting properties were determined according to Jozinović et al.[1], using Brabender micro viscoanalyser 803202, Brabender GmbH & Co, Duisburg, Germany.

Experimental data were analysed by the analysis of variance (ANOVA) and Fisher's least significant difference (LSD) with significance defined at $P < 0.05$ using software Statistica 12.

RESULTS AND DISCUSSION

Figure 1 represents the expansion ratio of extruded samples. Expansion was better when larger corn grits were used (>500 µm), but it was significantly reduced by rye addition, regardless of corn grits granulation. The same trend was observed by Anton et al. after pea flour addition[2]and Pastor-Cavada et al. after leguminous flour addition due to an increased fibre content[11].

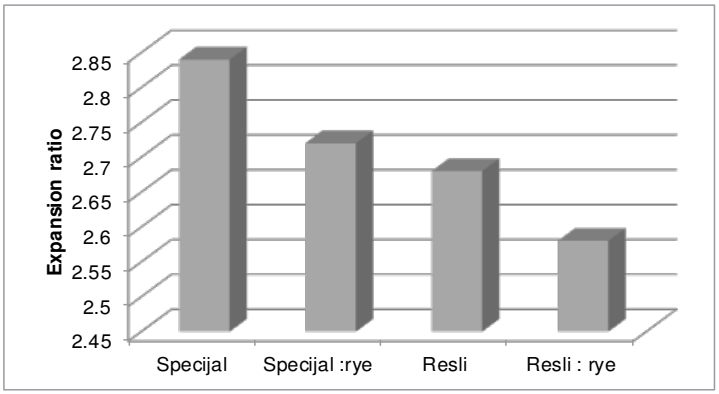

Figure 1. Expansion ratio of extrudates produced from corn grits with particle sizes >500 µm (*Specijal*) and ≤500 µm (*Resli*) with and without addition of wholerye flour in 20% (d. m.)

Bulk density (Fig. 2) increased as the expansion ratio decreased. The same results were reported by Stojceska et al. after addition of brewer's spent grain[12], Lazou and Krokida after lentil flour addition[3,4] and Yagci and Gogus after defatted hazelnut flour addition[13].

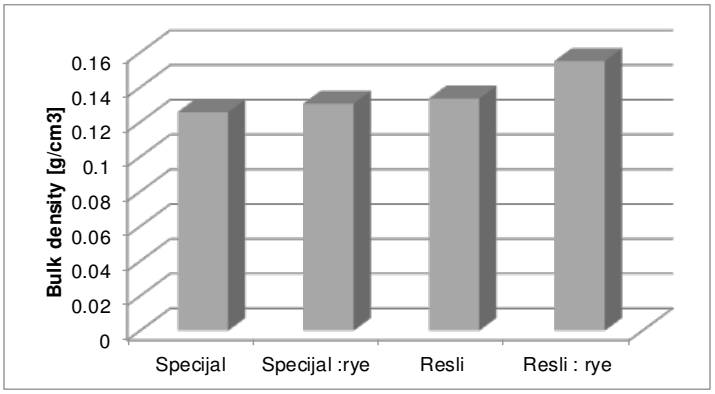

Figure 2. Bulk density of extrudates produced from corn grits with particle sizes >500 µm (*Specijal*) and ≤500 µm (*Resli*) with and without addition of wholerye flour in 20% (d. m.)

Rye flour addition increased hardness and decreased fracturability of extrudates (Fig. 3 and 4).

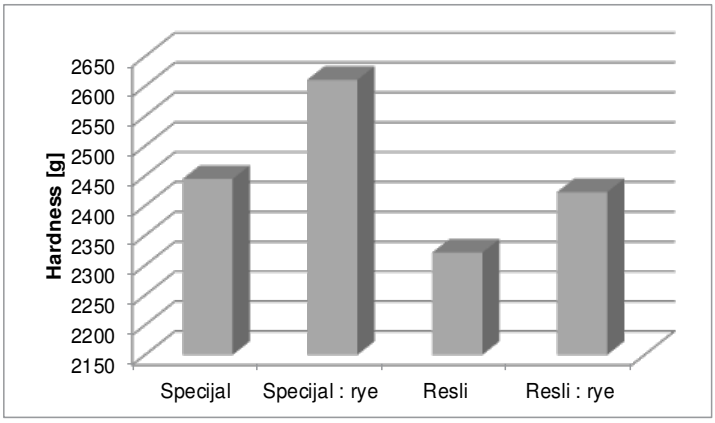

Figure 3. Hardness of extrudates produced from corn grits with particle sizes >500 µm (*Specijal*) and ≤500 µm (*Resli*) with and without addition of wholerye flour in 20% (d. m.)

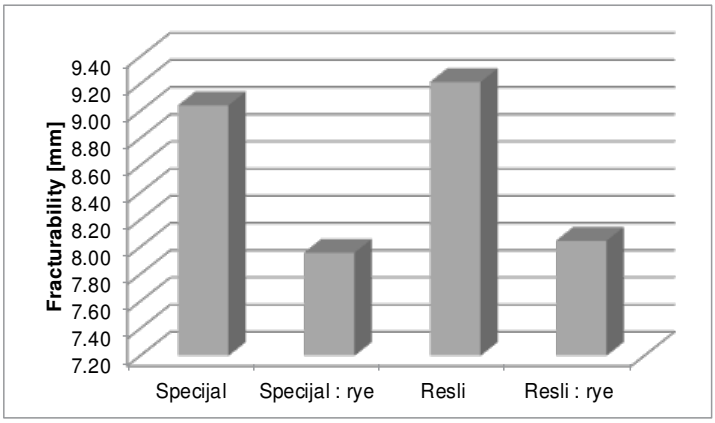

Figure 4. Fracturability of extrudates produced from corn grits with particle sizes >500 µm (*Specijal*) and ≤500 µm (*Resli*) with and without addition of wholerye flour in 20% (d. m.)

Our previous research[1] also showed an increase of hardness and a decrease of fracturability after addition of buckwheat and chestnut flour. Although the texture properties are mainly influenced by sample moisture[14,15], other parameters, such as temperature profile, screw configuration and rate, also have influence[3,4,16,17].

Colour parameters of both extruded and non-extruded samples are shown in Table 1. Rye addition to corn grits caused a decrease of lightness (L*), yellowness (b*) and increaseda green component of colour (a*). Extrusion additionally decreased the lightness of samples, except for the sample *Specijal* (>500 μm) without addition of rye flour. Colour saturation (C*) and hue did not show a clear trend, since they were influenced both by particle size and rye flour addition. In addition, during extrusion Maillard products are formed, which also cause the colour change[18-21].

Table 1. Colour properties of extruded and non-extruded corn grits with particle sizes >500 μm (*Specijal*) and ≤500 μm (*Resli*) with and without addition of wholerye flour in 20% (d. m.)

Sample	Non-extruded				
	L	**a**	**b**	**C**	**h°**
Specijal	82.32±0.04^{b}	0.25±0.02^{d}	44.70±0.17^{d}	44.70±0.17^{d}	89.68±0.02^{a}
Specijal:rye	77.16±0.02^{a}	-0.66±0.05^{c}	30.18±0.21^{b}	30.19±0.21^{b}	91.24±0.08^{b}
Resli	87.37±0.02^{d}	-2.15±0.04^{b}	35.42±0.03^{c}	35.48±0.02^{c}	93.48±0.07^{c}
Resli:rye	83.87±0.02^{c}	-3.08±0.02^{a}	28.52±0.12^{a}	28.69±0.12^{a}	96.17±0.04^{d}
	Extruded				
	L	**a**	**b**	**C**	**h°**
Specijal	85.51±0.01^{d}	-4.02±0.06^{a}	38.97±0.06^{d}	39.18±0.06^{d}	95.89±0.08^{d}
Specijal:rye	76.66±0.06^{a}	0.05±0.01^{c}	31.17±0.02^{b}	31.17±0.02^{b}	89.90±0.02^{b}
Resli	84.86±0.03^{c}	-2.63±0.03^{b}	32.62±0.02^{c}	32.73±0.03^{c}	94.61±0.06^{c}
Resli:rye	76.86±0.03^{b}	0.51±0.03^{d}	27.73±0.02^{a}	27.74±0.02^{a}	88.94±0.07^{a}

Samples with different superscripts in the same column are statistically different (p<0.0

Extrusion caused increased starch damage (Fig. 5) due to high pressure and high temperature in the extruder. However, rye flour addition reduced starch damage.

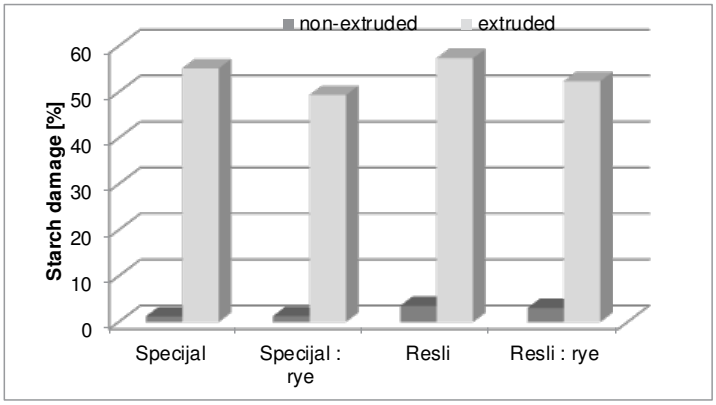

Figure 5. Starch damage of extrudates produced from corn grits with particle sizes >500 µm (*Specijal*) and ≤500 µm (*Resli*) with and without addition of wholerye flour in 20% (d. m.)

Water absorption index (WAI) was not influenced by rye flour addition (Fig. 6).

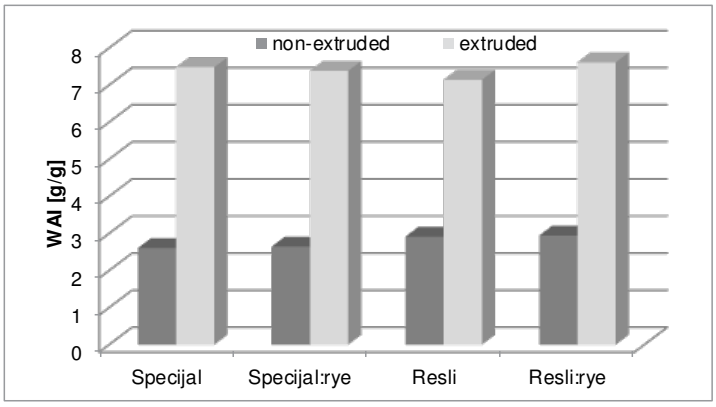

Figure 6. Water absorption index (WAI) of extrudates produced from corn grits with particle sizes >500 µm (*Specijal*) and ≤500 µm (*Resli*) with and without addition of wholerye flour in 20% (d. m.)

However, it increased after extrusion, mainly due to significant starch damage. WAI increase after extrusion was observed in other researches as well[1,22]. Water solubility index (WSI) increased after addition of rye flour to corn grits prior to the extrusion (Fig. 7). Extrusion further increased WSI, which can also partly be explained by starch damage and a larger number of smaller molecules[23]. Yagci and Gogus (2008) also reported increased WSI after the addition of defatted hazelnut flour[13].

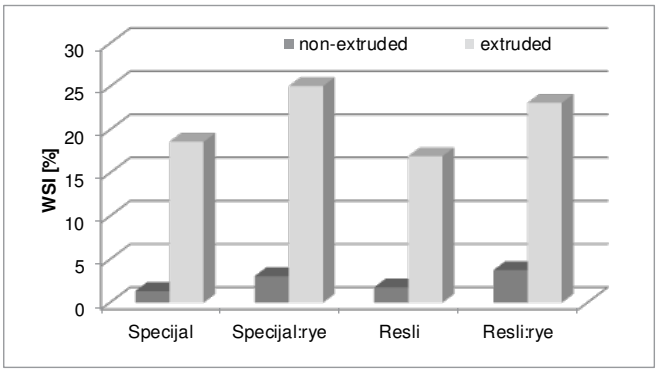

Figure 7. Water solubility index (WSI) of extrudates produced from corn grits with particle sizes >500 µm (*Specijal*) and ≤500 µm (*Resli*) with and without addition of wholerye flour in 20% (d. m.)

Pasting properties of the extruded and non-extruded samples are shown in Table 2. Rye addition to corn grits caused a decrease of peak viscosity both for extruded and non-extruded samples and the extruded samples had the lower values of peak viscosity compared to the non-extruded counterparts.

Table 2. Pasting properties of extruded and non-extruded corn grits with particle sizes >500 μm (*Specijal*) and ≤500 μm (*Resli*) with and without addition of wholerye flour in 20% (d. m.)

	Peak viscosity [BU]	Viscosity at 92 °C [BU]	After mixing at 92 °C [BU]	Viscosity at 50 °C [BU]	After mixing at 50 °C [BU]	Breakdown [BU]
			Non-extruded			
Specijal	608± 7.0^{c}	27.5±8.5^{b}	607.5±23.5^{c}	1009±18.0^{d}	985±4.0^{d}	3.5±3.5^{a}
Specijal:rye	456±24.0^{b}	25±1.0^{b}	457±23.0^{b}	940.5±1.5^{c}	917.5±3.5^{c}	0±0.0^{a}
Resli	903.5±5.5^{D}	898.5±0.5^{C}	775.2±2.5^{C}	1311.2±1.5^{C}	1347±17.0^{C}	126±5.0^{B}
Resli:rye	804±4.0^{C}	636.5±18.5^{B}	736.5±4.5^{B}	1236.5±17.5^{B}	1258±14.0^{B}	66.5±2.5^{A}
			Extruded			
Specijal	81.5±16.5^{a}	0±0.0^{a}	0±0.0^{a}	85.5±18.5^{b}	92.5±21.5^{b}	81.5±16.5^{b}
Specijal:rye	89±8.0^{a}	0±0.0^{a}	0±0.0^{a}	17.5±8.5^{a}	26±8.0^{a}	89±8.0^{b}
Resli	64±16.0^{A}	0±0.0^{A}	0±0.0^{A}	19.5±13.5^{A}	23.5±12.5^{A}	64±16.0^{A}
Resli:rye	152.5±14.5^{B}	0±0.0^{A}	0±0.0^{A}	11±11.0^{A}	13±13.0^{A}	152.5±14.5^{B}

Samples with different superscripts in the same column are statistically different ($p<0.05$). Statistical analysis was performed separately for Specijal and Resli.

The breakdown values are indicators of paste stability during shearing at high temperatures. According to these, the most stable was non-extruded *Specijal*: rye mixture (0.00 BU), followed by non-extruded *Specijal* (3.5 BU), extruded *Resli* (64.0 BU), non-extruded *Resli*:rye (66.5 BU), extruded *Specijal* (81.5 BU), extruded *Specijal*: rye (89.0 BU), non-extruded *Resli* (126.0 BU), and least stable was extruded *Resli*: rye mixture (152.5 BU).

The set back values are indicative of starch retrogradation during cooling. The results obtained in this research show that the non-extruded samples have the higher tendency towards retrogradation than the extruded samples.

CONCLUSION

Expansion ratio decreased and hardness and bulk density of extrudates increased after rye flour addition. These are not desirable characteristics and additional research is needed to solve the expansion problem. The colour of extrudates was noticeably changed after rye flour addition, but this is not necessarily a disadvantage.

Starch damage was large after extrusion and thus modified flour could not be used as single flour in the bakery industry. However, WAI and WSI, as well as pasting properties show it could have the application in the bakery and other industries as partial supplementation for flour.

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PHYSICO-CHEMICAL AND SENSORIAL CHARACTERIZATION OF DISTILLATES PRODUCED FROM FRESH AND DRIED FIG (*Ficus carica* L.)

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT
The aim of this study was to characterize distillates produced from fresh and dried figs (*Ficus carica* L.) in the Istria region of Croatia. Four autochthonous fig varieties were used for production: Bjelica, Bružetka Bijela, Crnica and Zimica. Fresh and dried fruits were crushed, ensiled, and subjected to alcoholic fermentation. After completion of the fermentation, fig pomaces were distilled, and obtained distillates were re-distilled. Produced distillates were subjected to standard physico-chemical analyses, and GC-FID analysis of major volatile constituents. Distillates were also evaluated sensorially by the 20-positive point method. In most cases, fresh fig distillates were characterized by higher methanol content (5035.15 to 10977.21 mg/L a.a.), while dried fig distillates exhibited composition with higher amounts of total acidity (3700 to 13440 mg/L p.A.), acetaldehyde (78.23 to 245.72 mg/L p.A.), ethyl acetate (1043.24 to 7343.29 mg/L p.A.) and total higher alcohols (1909.35 to 3381.52 mg/L p.A.). Sensory evaluation showed that investigated distillates also differed in organoleptic attributes.
Keywords: fresh fig (*Ficus carica L.*), dried fig, distillate, physico-chemical parameters, volatile constituents, sensory evaluation

INTRODUCTION

The cultivated fig, *Ficus carica* LINN, is a member of the Moraceae (mulberry family). Nutritional values of figs were recognized as far back as the times of ancient Egyptians who attributed it with medicinal effects, and Cleopatra arguably owed her beauty to her favourite fruit - fig. Early Olympic athletes were given figs as a training food and figs were given as laurels to the winners of the first Olympics as a "medal"[1]. According to Roman belief, Romulus and Remus were born underneath a fig-tree. The fig enjoys the reputation of an ecological product because it cannot grow in a contaminated environment. It is healthy food, as well as medicine and a treat. Fig fruits contain considerable amounts of sugars, minerals and polyphenols[2]. To a large extent figs are eaten fresh, but they may be dried. Dried figs contain more calcium and magnesium than the comparable quantity of milk. On some family farms, fresh fruits are also used for preparing cakes, bakery products, jams, syrups, liqueurs. The fig contains a high rate of sugar and 73.97% of this sugar is glucose and fructose (invert sugar)[3]. The alcohol yield from ripe figs is about the same as that from plums, *viz.* 30 to 33 litres of 54 per cent alcohol from 100 kilos of dried figs, one hectare of fig trees producing 800 to 900 litres of 90 percent alcohol[4].
The tradition of fig cultivation in the Balkans goes a long way back in the history. The fig tree was imported into the Adriatic growing region in the age of

the Illyrians and Greek colonization. In west Balkan countries the fig cultivation is a supplementary activity with good market prospects that preserves the traditional cultural heritage and typical Mediterranean landscape. There are 19 registered fig varieties in Croatia, but there are more than 300 known worldwide. The interest for fig cultivation in Croatian region Istria is growing, due to the wish to achieve the diversity of agricultural activities. Quality of fig brandy is influenced by many factors, namely, climate and soil characteristics, and technological characteristics of manufacturing process. Also fig variety had a significant influence on quality of brandy. Furthermore, composition of volatile aroma compounds determining the sensory quality of distillates, and is essential for consumers' perception[5]. Aroma profile of wine and all distilled alcoholic beverages consists of the same major groups of volatile compounds (alcohols, esters, acids, aldehydes, acetals) and their proportions are quite different owing to the different origins of raw material and different processing techniques[6].

In spite of its importance as an arid, semiarid and sub humid species, a little effort has been made in the study of chemical composition and sensory properties of distillates produced from autochthonous fig varieties in Istria. The aim of this investigation was to characterize distillates produced from fresh and dried figs (*Ficus carica* L.) offour autochthonous fig varieties: Bjelica, Bružetka Bijela, Crnica and Zimicawere used. Bružetka Bijela, Zimnica and Bjelica are white figs varieties, while Crnica is dark fig.

MATERIALS AND METHODS

Reagents and standards

Dichloromethane, p.a., ethanol, 96% p.a., methanol, p.a., sodium sulfate, p.a. and acetic acid, p.a., were supplied by Kemika (Zagreb, Croatia). Pure deionized water was obtained from an Elix 3 purification system (Millipore, USA). Acetaldehyde, p.a., ethyl acetate, p.a., 1-propanol, p.a., 2-methyl-1-propanol (isobutanol), p.a., and isomers 2-methyl-1-butanol and 3-methyl-1-butanol (isoamyl alcohol), p.a. were purchased from Fluka (Buchs, Switzerland). Chemical standards were purchased from Merck (Darmstadt, Germany) and Fluka.

Table 1. Sugar content in fig mash

Fig varieties	Sugar content (g/L)
Zimnicafresh	248.0
Zimnica dried	229.0
Crnica fresh	259.0
Crnica dried	276.0
Bjelica fresh	242.0
Bjelica dried	229.0
BružetkaBijelafresh	236.0
BružetkaBijela dried	248.0

Plant material

Fig plants are part of a pilot fig orchard on a location of Kazela, close to the touristic town of Medulin in the region of Istria, Croatia. The orchard was planted by SKINK Company Rovinj in spring 1999[7]. Four autochthonous fig varieties were used for the investigation: Bjelica, Bružetka Bijela, Crnica and Zimica. Fruits were harvested at full ripeness in 2008 and were used in fresh and dried state. Drying of whole fig samples was performed in a pilot plant cabinet dryer (Rasadnik "Skink" Rovinj, Croatia), drying capacity 150 kg of fresh material, heater power 2 x 4 kW, consisting of 17 trays, each weighing 2.5 kg. The dryer was equipped with temperature and airflow velocity controllers. Drying temperature was 60 °C. The drying process started when the drying conditions had been achieved. Fig samples were placed onto trays in the cabinet dryer and measurements started from this point. Sample weight loss was recorded every ten minutes during drying using a digital balance. Drying lasted until a moisture content of about 25% (wet base) was achieved.
Fresh and dried figs were crushed, ensiled, and subjected to alcoholic fermentation.

Fermentation procedure

Production of distillates from fresh and dried fruit contains five steps: harvest, preparing and drying fruit in a case of production of distillates from dried fig; crushing and pressing fruit; alcoholic fermentation of the mark; immediate distillation of fermented mark. The figs were squashed by an electric squasher. For distillates production, 10 kg of fresh and 5 kg of dried figs of each variety were used. The sugar content in fig mash was measured according to Lane-Eynon method[8].The results for sugar content are shown in Table 1.
Fermentation of the fig mash was carried out in appropriate vessels, with adding hot water at 52^{o}C, (ratio figs mash: water was 1:3; m/v).The mash was inoculated with a commercial yeast strain (*Saccharomyces ellipsoideus*, Ceppo 20 Castelli) up to a concentration of approximately10^{6} cells/ml. Also the matched nutrient, mineral, vitamin mix was added, to ensure that the yeast has all of its nutrition requirements considered, even when used in nutrient deficient mash. Temperature was regulated at 24^{o}C and fermentation of the mash was carried out over the period of 25 days. After completion of the fermentation, fig pomaces were distilled, and obtained distillates were re-distilled.

Distillation procedure

The first distillation of the fermented fig pomaces was carried out in a traditional alembic still[9], producing a distillate with an alcohol content between 18.5% and 22.8% (v/v). Re-distillation of weak distillates was done in the same apparatus. The first distillation was carried out with the maximum alcohol obtained, with separation of 1% first-made distillate.
The second distillation took place in an alembic still, yielding a final alcohol content of 37.5–45.4% (v/v). During the second distillation, the same amount (1%) of first-made distillate was separated. To prevent aroma loss, the

distillates were kept at 5°C until analysis.The final spirits were stored in 1 l glass flasks and after six months distillates were analyzed.

Analysis of the distillates

Produced fig distillates were subjected to standard physico-chemical analyses, and GC-FID analysis of major volatile constituents, prescribed by the Ordinance on the Performance of Analytical Methods of Analysis of Spirit Drinks and Alcoholic Beverages[10].The alcohol content was determined by electronic densimetry applying a hydrostatic balance (Gibertini, Milano,Italy)(Official Gazette 138/05, method Ic). Reducing sugar was determined according to Elmaci and Altug[11]. Total acid was determined by volumetric titration with 0.1 N NaOH and expressed as acetic acid[7]. Total dry extract was determined gravimetrically[7]. Analyses were done in duplicate.

Gas chromatography

Gas chromatographic analyses were performed on a Varian 3350 gas chromatograph (Varianinc., SAD), equipped with a split/splitless injector and a flame ionization detector (FID).Separations of major volatile compounds in the samples of fig distillates (methanol, ethyl acetate, 1-propanol, isobutanol and isoamyl alcohol) were done using a 30m × 0.25 mm I.D.× 0.25 μmdf capillary column Rtx-WAX (Restek, USA) with the following parameters: aninitial oven temperature was 40°C, raised after 4 min at 5°C/min to 90°C, then it was programmed at 15°C/min to 235°C and then kept for 10 min. The injector and detector temperatures were 160 and 240°C, respectively. The carrier gas was helium at a velocity of 1.1 ml/min. Two microlitres of the double diluted sample were injected and the injection mode was split with the split ratio 1:20 and by the standard addition analysis. Calibration graphs used for the calculations and quantifications were prepared by GC analysis of dichloromethane solutions containing known amounts of the standards and of the internal standard (1-pentanol). Calibration curves (relative peak area versus concentration ratio of volatile compound/internal standard) and all quantifications were performed by the internal standard method using Varian Star 4.51 software.GC-FID analysis of major volatile constituents fig distillates (acetaldehide, methanol, etil-acetate, 1-propanol, 2-butanol, isobutanol, isoamyl alcohol) were done according to Lukićet al[12]. Analyses were done in duplicate.

Sensory testing

Sensory testing of fig distillate samples was performed by six trained tasters, highly experienced in fig marc distillates hedonic and wine descriptive sensory testing. Tasters were seated in separate purpose-made booths, and the environment was free of interference in terms of noise, visual stimulation and ambient odour. The positive point methodwas used. The model was based on the evaluation of four basic properties (colour, clearness, odour and taste), which were marked with scores from 1 to 20 [1, not satisfactory (strong defect); 20, excellent (the standard of quality)].

RESULTS AND DISCUSSION

Fruit quality is based on its aroma composition.As previously reported[13,14] the ripening period had very important role in volatile composition of figs. Aroma profile of *Ficus carica* fruits include a lot of volatiles that contribute to the pleasant note and taste of the fruit at maturity. The differences in volatile comosition occur also due to the variety of fig[15]. The production of fruit distillates in the region of Istria in Croatia has a long tradition, and today it is an inseparable part of local customs and gastronomy. It is mostly related to small family farms, where it is carried out using traditional copper alembics[16].

In this study, volatile compounds of distillates produced from fresh and dried fig varieties wereidentified and quantified and are reported in Table 2.Alcohol content ranged from 37.5% for fig varieties Zimnica dried and Bijelicadried to 45.4% for Crnica dried.

Based on the ^{o}Brix on fig mash, the theoretical alcohol production as expected, in consideration of the final reducing sugar content was zero. The all fig distillates produced comply with thequality standard requirements prescribed by Croatian Rules[17] (minimal alcohol content in fruit distillates is 37.5%).As regards the macro constituents in Table 2, we observed among the alcohols that the methanol content never exceeds the legal limit of 1500 g/hL[17]. In most cases, the fresh fig distillates were characterized by higher methanol content then the distillates produced from dried figs. The highest content of methanol, 10977.21 mg/L p.A., was determined in distillates of fresh fig variety Bružetka Bijela. During fig processing and fermentation, methanol is formed by the hydrolytic demethoxylation of esterified methoxyl groups of the pectin polymer by pectic enzymes[18]. Excessive amounts of methanol can be harmful to humans[16] so fig distillate produced from fresh variety Bružetka Bijela, which exhibited exceptionally high methanol content, indicating that seriousproblems may arise in the production of distillates fromthis variety.

Table 2. Chemical composition of distillates produced from fresh and dried fig

Varieties	Zimnica fresh	Zimnica dried	Crnica fresh	Crnica dried	Bjelica fresh	Bjelica dried	Bružetka Bijelafresh	BružetkaBijela dried
Alcohol (vol%)	40.6	37.5	42.3	45.4	37.7	37.5	38.3	41.2
Total extract (g/L)	0.07	0.17	0.1	0.16	0.13	0.11	0.23	0.09
Reducing sugar (g/L)	-	-	-	-	-	-	-	-
Total acids (mg/L p.A.[1])	2000.0	13440.0	1930.0	5340.0	4830.0	3700.0	3440.0	4360.0
Ethyl-acetate (mg/L p.A.)	116.75	7343.29	553.77	1132.79	668.76	1043.24	610.72	1183.73
Acetaldehide (mg/L p.A.)	75.69	245.72	178.90	218.43	77.48	116.89	71.68	78.23
Methanol (mg/L p.A.)	5540.45	5570.94	5030.51	1400.76	6100.16	4300.73	10970.72	4290.26
1-Propanol (mg/L p.A.)	181.13	498.59	288.93	371.86	169.93	468.64	205.87	384.39
Isobutanol (mg/L p.A.)	757.03	872.33	835.81	577.24	268.98	703.75	375.96	519.01
1-Butanol (mg/L p.A.)	4.27	5.97	6.11	3.66	2.87	5.62	4.47	4.89
Isoamil alcohol (mg/L p.A.)	1089.91	2004.6	1018.57	956.59	424.54	1771.49	492.57	1295.09
Higher alcohols[2] (mg/L p.A.)	2032.34	3381.52	2149.42	1909.35	866.31	2949.50	1078.87	2203.38
Total volatile compounds[3](mg/L p.A.)	4220.48	24410.05	4810.21	8600.06	6440.25	7800.96	5200.13	7820.53

[1]pure alcohol, absolute ethanol
[2]the sum of 1-propanole, isobutanole, 1-butanol and isoamil alcohol (mg/L p.A.)
[3]the sum of total acids, ethyl-acetate, acetaldehide and total volatile compounds (g/hLp.A.)

In almost all cases the dried fig distillates exhibited composition with higher amounts of total acidity, 3440 mg/L p.A. to 13440 mg/L p.A. for Bjelica fresh and Zimica dried respectively. Distillates from dried fig variety Zimnica had attracted more attention related to content of total acids (13440 mg/L p.A.), ethyl-acetate (7343.29 mg/L p.A.) and total volatile compounds (24410.05 mg/L p.A.) compared to the contents in other investigated varieties. Also, the content of acetaldehyde, ethyl acetate and total higher alcohols is highest in dried fig distillates. The content of acetaldehyde ranges from 71.68 mg/L p.A. for Bružetka Bijela fresh to 245.72 mg/L p.A. for Zimnica dried. The lowest determined concentration of ethyl acetate (668.76 mg/L p.A.) was for Bjelica fresh and the highest (7343.29 mg/L p.A.) for Zimnica dried. The content of total higher alcohol is within the boundaries from 866.31 mg/L p.A. for Bjelica fresh to 3381.52 mg/L p.A. for Zimnica dried distillates. Themost represented higher alcohol is isoamil alcohol, then isobutanol and 1-propanol, while the content of 1-butanol is the lowest. The minimum content of total volatile compounds (200 g/hLp.A.)[17] was reached in all variants.

Numerous volatile esters contribute to fruity and flowery odours, while higher levels of ethyl acetate may cause undesirable smells[19]. Acetic and other fatty acids are responsible for sharp smells described as sour, rancid,fatty and cheesy, which are generally considered negative, while the main aldehyde, acetaldehyde, can contribute positively or negatively, depending on its concentration[20].

The results of the sensory testing of distillate samples using the positive point method are reported in Table 3.The produced fig distillates can be described as colourless and clear, having odour and taste characteristic of this sort of alcoholic beverages. In Table 3 the average organoleptic rating for the fig distillates is shown. Zimnica dried and Bjelica fresh distillates showed the highest organoleptic rating (18.0), while organoleptic rating for Bjelica dried was the lowest (12.16). Zimnica and Bjelica

are aromatic varieties of fig, and the applied traditional technological process of production has an influence on sensory characteristics, too.

Table 3. Organoleptic ratings of fig distillates

Varieties	**Organoleptic Rating($\overline{X}$)**
Zimnicafresh	16.58
Zimnica dried	18.00
Crnica fresh	15.66
Crnica dried	12.66
Bjelica fresh	18.00
Bjelica dried	12.16
BružetkaBijelafresh	15.83
BružetkaBijela dried	12.25

Sometimes even a detailed knowledge of volatile aroma compound composition[5] is insufficient topredict the aroma of a whole distillate's system as perceived by a trained sensory testing panel. But obtained results about the volatile compounds represent a valuable contribution to the understanding of the aroma of fig distillates.

CONCLUSION

Distillates produced from autochthonous fig varieties in Istria Region have good chemical and sensoryproperties. The raw material (fresh or dried figs, and fig variety) had significant influence on the composition of the distillate.Fresh fig distillates were characterized by higher methanol content then distillates produced from dried figs.The content of acetaldehyde, ethyl acetate, higher alcohols and total volatile compoundswere highest in dried fig distillates. Different volatile compound groups have contribution to occurrence ofcertain specific odours in the aroma and flavour of the fig distillates.

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MYCOTOXINS AND FUNGI OCCURRENCE IN WHEAT FROM EASTERN CROATIA STORED UNDER DIFFERENT CONDITIONS

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT
In this study, 25 wheat samples (harvest 2009) were collected from storage tanks of the local producers from five eastern Croatian Counties and analyzed on mycotoxins occurrence. The samples were stored in diverse ways: in concrete silos, metal silos or foot warehouse equipped with or without temperature sensors, and with or without cooling or ventilation systems. Wheat quality parameters such as moisture, protein and starch content, sedimentation value, wet gluten content, gluten index and falling number were also measured.
Seven fungi species were detected (*Asperigillus*, *Alternaria*, *Cladosporium*, *Fusarium*, *Penicillium*, *Rhizopus* and *Cephalosporum*) at low contamination levels ($1.0 \cdot 10^2$fungi/g - $1.7 \cdot 10^4$fungi/g). *Fusarium* was present in all samples. Four mycotoxins were found (aflatoxin, ochratoxin, deoxynivalenol, zearalenone and T-2/HT-2) reaching concentrations that do not exceed the maximum permissible level of mycotoxins in cereals for unprocessed grains (total aflatoxin, 2.5 µg/kg; DON, 50-850 µg/kg; zearalenone, 0.2-89.8 µg/kg, and T-2/HT2, 0.5 µg/kg).
Key words: mycotoxins, fungi, silo, Croatia

INTRODUCTION

Mycotoxins are secondary metabolites generated by several fungi species contaminating various agricultural commodities in the field or after the harvest[1-4]. Mycotoxigenic fungi belong mainly to *Fusarium, Alternaria, Aspergillus* and *Penicillium*genera. *Fusarium* and *Alternaria* usually represent a high mycotoxicological risk in the preharvested or freshly harvested plant, whereas *Aspergillus* and *Penicillium* represent a higher risk for products during storage or used in food and feed processing[5]. More than 400 mycotoxins have been identified so far, but the most important ones concerning human and animal health that occur quite often in food and feed are aflatoxins, ochratoxin A, trichothecenes (deoxynivalenol, nivalenol), zearalenone and fumonisins[6,7]. Conditions favouring the appearance of mycotoxins in cereals, before and after the harvest, concern mostly the temperatures (range from 20 to 25°C) and air humidity (around 80%)[8].

Storage systems, such as silos and warehouses, are often not flexible in terms of volume. They are rigid systems, not always of a proper size for the cereal quantity to be stored with the frequent presence of certain headspaces as well as environmental conditions optimal for fungal growth and mycotoxin production [9].The occurrence, concentration and type of mycotoxin are mostly influenced by environmental and storage conditions, fungi species, infection severity and a cultivar or type of the crop[2,10-12].

Previous studies reported the occurrence of mycotoxins in wheat in Croatia showing especially their common presence in samples from the eastern part of Croatia and their appearance correlation with climatic conditions[8,13,14].

This study investigated the occurrence of fungi and mycotoxins in wheat grains harvested in the area of eastern Croatia during 2009, concerning the storage conditions. Quality control parameters of the wheat samples were also determined.

MATERIALS AND METHODS

Wheat samples

Wheat samples were collected from 25 storage tanks located in five Counties in eastern Croatia: Vukovar-Syrmia County (VSC), Osijek-Baranja County (OBC), Brod-Posavina County (BPC), Požega—Slavonia County (PSC) and Virovitica-Podravina County (VPC) after the harvest 2009. The samples were collected in October and November. Sampling and sampling preparation were conducted in accordance with the standard sampling procedure of Croatian Regulations[15].

Storage conditions

Wheat grain samples were collected from the local producers' storage tanks of different storage conditions (Table 1): a) eight "big" concrete silos with a capacity from 44.000 to 80.000 tons; b) two "small" concrete silos with acapacity from 1.000 to 13.000 tons; c) fourteen metal silos with a capacity from 1.600 to 12.000 t; d) one foot warehouse. The measured temperature inside the silos equipped with temperature sensors ranged from 17 to 22°C.

Microbiological analysis and mycotoxins determination

Microbiological analysis was performed according to HRN ISO 7954:2002. Concentrations of five mycotoxins (aflatoxin, ochratoxin, deoxynivalenol, zearalenone and T-2/HT-2) were measured in all the wheat samples using immuno assay *CD-ELISA Veratox test kits* (Neogen Europe Ltd.) and Stat Fax Microplate Reader (Neogen Europe Ltd.).

Wheat quality parameters measurements

The following wheat quality parameters were determined: moisture content (ICC 110/1), protein content (ICC 303), starch content (122/1), Zeleny sedimentation value (ICC No 115/1), wet gluten content and gluten index (ICC No 155) and falling number (ICC 107/1).

RESULTS AND DISCUSSION

Based on the results of wheat studies performed in Croatia during past decades, it can be noticed that *Fusarium* fungi and mycotoxins produced by them are the most common contaminants of storage cereals grown in Croatia[8]. Biosynthesis of mycotoxins in cereals is dominantly influenced by climate conditions, mechanical injury, insect damage, mineral plant nutrition, rapidity of plant drying, storage conditions, chemical treatment, etc. [16-19] and due to the continental climate and its location eastern Croatia has optimal conditions for fungal development. Therefore, special care should be taken to ensure good storage conditions. In this research fourteen metal silos, one metal warehouse and ten concrete silos were used for wheat storage. In addition, some of them were equipped with the cooling system Granifrigor™ (FrigorTec, Germany). Storage conditions (silo type and capacity, presence of ventilation and cooling system, temperature sensors), detected fungi species and a total number of fungi in wheat samples taken for each silo are presented in Table 1.

Table 1. Storage conditions and detected fungi species in wheat samples from different silos in the area of eastern Croatia after the harvest 2009

No. Silo*	Capacity [t]	Temp. sensor	Ventilation system	Granifrigor™	MC	PC	ST	SV	FN	WG	GI**	Number of fungi / g	Fungi species***
Vukovar-Syrmia County													
1 (M)	10 000	Yes	Yes	No	12.5	12.0	68.7	33	360	21.8	99.5	$1.1 \cdot 10^2$	*ASP, ALT, FUS, RHZ*
2 (C)	78 000	Yes	Yes	No	12.3	10.8	68.6	25	370	20.4	99.5	$1.0 \cdot 10^2$	*ASP, CLD, FUS, PEN*
3 (M)	9 000	Yes	Yes	No	12.4	11.5	69.0	30	370	23.1	98.5	$4.5 \cdot 10^2$	*ASP, FUS, PEN, RHZ*
4 (M)	2 100	No	Yes	No	13.1	11.8	68.14	33	260	21.6	99.0	$1.1 \cdot 10^2$	*ASP, ALT, CLD, FUS, RHZ*
5 (M)	6 300	Yes	No	Yes	11.4	11.5	68.93	29	240	24.0	97.0	$1.5 \cdot 10^4$	*ASP, CLD, FUS, MUC, PEN, RHZ*
6 (M)	Foot wearhouse	-	-	-	12.1	13.0	67.03	40	310	31.3	84.7	$1.6 \cdot 10^4$	*ALT, FUS, MUC, RHZ*
7 (C)	13 000	No	No	No	12.5	11.8	68.75	33	320	23.4	99.0	$1.3 \cdot 10^3$	*ASP, ALT, FUS, PEN, RHZ*
8 (C)	1 000	No	Yes		11.5	12.3	68.75	35	360	27.6	97.1	$4.4 \cdot 10^2$	*ASP, CLD, FUS*
9 (M)	1 600	No	Yes	Yes	12.8	12.0	68.95	33	310	25.0	97.5	$5.9 \cdot 10^2$	*ASP, ALT, FUS, RHZ*
10 (M)	5 400	Yes	Yes	Yes	12.3	12.0	68.9	33	340	26.5	85.0	$1.8 \cdot 10^3$	*ASP, CLD, FUS, RHZ*
11 (C)	55 000	Yes	Yes	Yes	12.9	12.1	68.55	33	300	22.5	98.4	$1.6 \cdot 10^4$	*ASP, CLD, FUS, PEN*
Osijek-Baranja County													
12 (C)	44 000	Yes	Yes	Yes	12.5	11.5	68.93	28	235	20.5	99.0	$2.9 \cdot 10^2$	*ASP, CPH, FUS, RHZ*
13 (C)	80 000	Yes	Yes	Yes	12.1	11.8	69.1	32	310	23.4	98.0	$1.6 \cdot 10^4$	*ASP, CLD, FUS, PEN*
14 (M)	7 500	Yes	Yes	No	13.1	11.5	68.85	28	315	20.0	99.5	$1.5 \cdot 10^4$	*ASP, CLD, FUS, PEN*
15 (C)	72 000	Yes	Yes	No	11.8	11.7	69.1	30	170	25.2	92.2	$2.9 \cdot 10^2$	*ASP, ALT, FUS*
16 (C)	68 000	Yes	No	Yes	12.4	11.6	68.65	29	305	22.2	97.5	$9.2 \cdot 10^2$	*ASP, ALT, CLD, FUS*
17 (M)	3 400	No	No	No	12.8	12.5	68.8	34	225	24.4	98.4	$1.3 \cdot 10^3$	*ASP, ALT, CLD, FUS*
18 (M)	12 000	Yes	Yes	No	12.5	12.2	69.0	34	210	26.8	94.6	$1.2 \cdot 10^3$	*ASP, ALT, CLD, FUS, PEN*
19 (C)	47 500	Yes	Yes	No	11.8	11.7	68.45	30	270	20.4	98.8	$1.3 \cdot 10^3$	*ASP, CLD, FUS*
Brod-Posavina County													
20 (C)	55 000	Yes	No	No	12.0	12.0	68.75	32	220	22.5	98.5	$1.4 \cdot 10^3$	*ASP, FUS, RHZ*
21 (M)	3 000	No	Yes	No	12.3	13.6	67.8	41	100	31.4	94.7	$1.6 \cdot 10^3$	*CLD, FUS*
22 (M)	11 000	No	No	Yes	12.4	11.8	68.9	31	275	23.2	98.1	$1.4 \cdot 10^3$	*ASP, CLD, FUS, PEN*
Požega—Slavonia County													
23 (M)	3 000	Yes	Yes	No	12.3	11.1	68.2	27	85	19.7	99.5	$2.8 \cdot 10^3$	*ASP, FUS, RHZ*
24 (M)	5 000	Yes	Yes	No	12.6	11.8	67.85	32	120	21.6	99.2	$1.7 \cdot 10^4$	*ASP, CLD, FUS*
Virovitica-Podravina County													
25 (M)	10 000	Yes	Yes	Ne	11.1	11.0	69.0	27	250	21.4	99.5	$9.3 \cdot 10^2$	*ASP, CPH, FUS, PEN, RHZ*

**M- metal silo; C - concrete silo;*

***MC – moisture content, %; PC – protein content, %; ST – starch, %; SV – sedimentation value, mL; FN – falling number, sec; WG – wet gluten, %; GI – gluten index, %*

****ASP - Aspergillus* sp.*; ALT - Alternaria*sp.*; CLD - Cladosporium* sp., *CPH - Cephalosporum*sp., *FUS - Fusarium*sp.*; MUC- Mucor* sp.; *PEN - Penicillium*sp.*; RHZ - Rhizopus*sp.

The results show that the wheat stored in different silo types in eastern Croatia was contaminated by fungi in the concentration which ranged from $1.0 \cdot 10^2$ fungi/g to $1.7 \cdot 10^4$ fungi/g. Detected fungi belonged to species *Asperigillus*, *Alternaria*, *Cladosporium*, *Fusarium*, *Penicillium*, *Rhizopus* and *Cephalosporum*, however only *Fusarium* was detected in all the samples. 92% of samples were contaminated by *Aspergillus* sp., while *Penicillium* sp. was found in 40% of samples. Mycotoxins, a number of samples contaminated by mycotoxins, measured mycotoxin concentration and maximum permissible level are presented in Table 2.

Table 2. Mycotoxins detected in wheat samples from different silos in the area of eastern Croatia after the harvest 2009

Mycotoxin	ML* (µg/kg)	Number of contaminated samples	Concentration [µg/kg]	County	Type of silo
Total aflatoxins	4.0	1/25	2.5	VSC	Concrete
Ochratoxin	3.0	0/25	0	-	-
Deoxynivalenol		4/25	50	VSC	Concrete
			50	VSC	Metal
			850	OBC	Metal
			90	BPC	Metal
Zearalenone	750	9/25	14.4	VSC	Metal
			17	VSC	Metal
			43.5	VSC	Concrete
			19.4	OBC	Concrete
			89.8	OBC	Metal
			25.1	OBC	Concrete
			7.7	OBC	Concrete
			1	OBC	Metal
			0.2	VPC	Metal
T-2/HT2	-	1/25	0.5	BPC	Metal

** Maximum level (ML)in accordance toCroatian legislation and Commission Regulation (EC) No 1881/2006*

According to the obtained results of detected fungi number and species (Table 1 and 2) and bylinear regression analysis, no relationship was observed between the amount of mycotoxins and mold number/g was found. Moreover, it was shown that silo types do not have influence on the fungi presence and the mycotoxins occurrence in wheat samples harvested in 2009. In the research by Gregori et al.[19] it was reported that mycotoxins contamination is in agreement with fungal behavior; even if the level detected in the silo bags at the beginning of the storage period differed between the two seasons, no significant variation occurred during cereal storage.

The results presented in Table 2 show that 52% of wheat samples were contaminated with one or two types of mycotoxins. One wheat sample contained Aflatoxin produced by *Aspergillus* sp. in concentration of 2.05 µg/kg, while all other detected mycotoxins originated from *Fusarium* sp.

As it can be observed from Table 2, the concentration of 2.05 µg/kg of total aflatoxin was measured in one sample taken from the concrete silo (Vukovar-Syrmia County), while in other contaminated samples mostly mycotoxins produced by *Fusarium* sp. were present. DON was detected at concentrations from 50 to 850 µg/kg in 4 samples which presents 16% of total contamination, while zearalenone was found in 9 samples (36% of total contamination) in concentrations from 0.2 to 89.75 µg/kg. One sample contained T-2/HT-

2 toxin at a concentration of 0.5µg/kg. Two samples contained both DON and ZEA. No ochratoxin was found. According to Croatian Legislation[15] and Commission Regulation[20] the wheat samples were suitable for human consumption while none of the samples exceeded the maximum permissible level of mycotoxins in cereals for unprocessed grains.

Since the contamination of cereals by moulds and mycotoxins cause a significant hazard to the food chain via losses in dry matter, quality, and nutritional value[9], wheat quality parameters such as moisture, protein and starch content, wet gluten content, gluten index, sedimentation and falling number were analyzed and the obtained results are presented in Fig. 1-3.

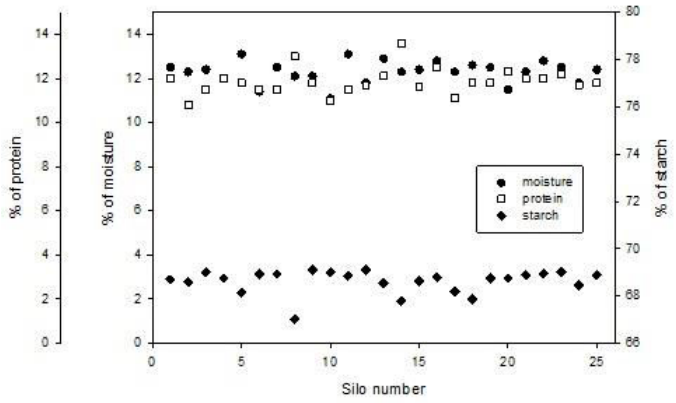

Figure 1. Moisture, protein and starch content [%] in wheat samples

Moisture, protein and starch content in wheat samples taken from silos in the area of eastern Croatia are shown in Fig. 1, the data of sedimentation volume and falling number are presented in Fig. 2 while wet gluten content and gluten index are presented in Fig. 3. According to the obtained results it can be observed that all wheat samples had moisture content between 11.1 to 13.1% which was in accordance with Croatian legislation[15]. The analyzed wheat samples are classified in the first category (the best quality) – the wheat with reduced moisture content, since this amount of moisture prevents or significantly slow biochemical changes inside of the kernel[21,22]. It can be also observed that reduced moisture content in collected wheat samples contributed to the sporadic fungi and mycotoxins occurrence.

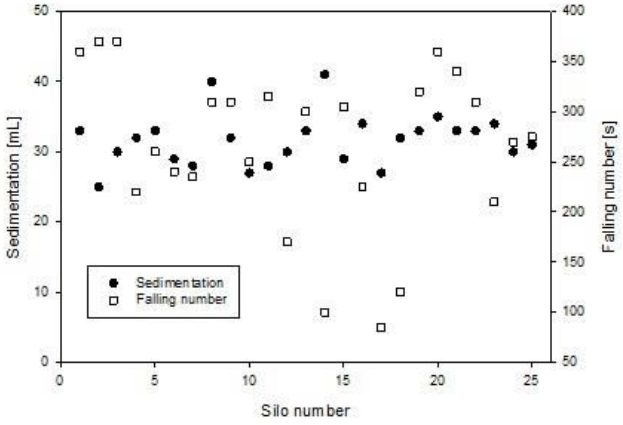

Figure 2. Sedimentation volume [mL] and falling number [s] of analyzed wheat samples

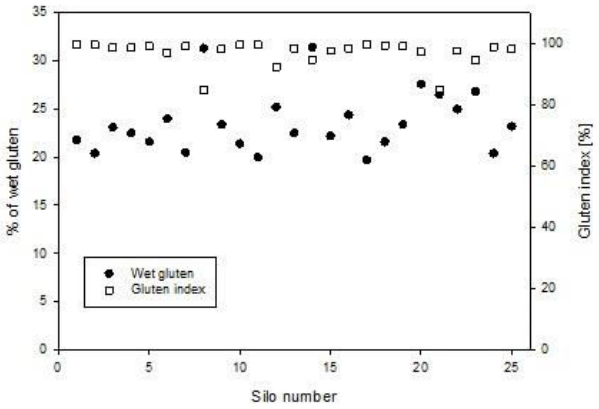

Figure 3. Wet gluten content and gluten index [%] in wheat sample*s*

Since the humidity and temperature, during both wheat growth and storage, are the critical control points regarding fungi formation and, thus, mycotoxins production, low contamination levels of fungi ($1.0 \cdot 10^2$ fungi/g to $1.7 \cdot 10^4$ fungi/g) and mycotoxins (2.5 µg/kg of total aflatoxin, 50 to 850 µg/kg of DON, 0.2 to 89.8 µg/kg of zearalenone and 0.5 µg/kg of T-2/HT2) observed in this study are probably the result of weather conditions during the harvest 2009 in the investigated part of Croatia, e.g. warm and dry weather. According to weather data obtained from the Metrological and Hydrological Service of Croatia, the period from June to August 2009 was warm with the air temperatures above average, while the rainfall was far less than usual and, although the harvest started at the end of June, when the alternation of sunny and rainy days was noted, the stronger impact of weather conditions on fungi formation and mycotoxins occurrence was not observed. The relationship between mycotoxin occurrence and weather conditions were also emphasized by Sokolović and Šiəmpraga[14]. They analyzed mycotoxins occurrence in cereal grains in Croatia between 2001 and 2004 and reported DON presence in 41% of total 465 analyzed samples collected during the four year period. The authors also reported that the highest average concentrations of DON were noted during harvest 2004 (over EC limit of 1250 mg/kg) which was associated to "highly humid" weather conditions, i.e. extremely warm

weather with frequent rains in spring, fall and winter during 2003 and 2004. During the four-year investigation period, the highest incidence rate of mycotoxin T-2 (59% of total 465 analyzed samples) with maximum concentration of 520 mg/kg was determined in grains harvested in 2004, which was also related to weather conditions during wheat growth and storage in 2004.

Wheat can be also classified according to grain properties for specific applications. Considering the protein content and sedimentation value, wheat is usually classified into three grades of quality. Protein content in analyzed wheat samples was between 10.8 to 13.6% (dry matter). Two samples had protein content higher than 13%, 22 samples from 11.5 to 13.6%, and three samples from 10.8 to 11.5%. Protein content is strongly influenced by environment and is negatively correlated with grain yield[8].

Sedimentation is a parameter that indicates the quality of the protein, as well as quantity and quality of gluten[23,24]. In all the analyzed wheat samples, sedimentation values were in the range between 25 and 41 mL. Owing to the protein content and sedimentation value, the wheat samples taken from two metal silos (6 and 21) were of the first class quality which is characterized by 13% / DM protein and sedimentation more than 40 mL. 17 samples were classified as the second class quality (>11.5% / DM protein and sedimentation between 30 and 40 mL) and 6 wheat samples were classified as the third class quality (> 11.5% / DM protein and sedimentation between 18 and 30 mL).

Gelation ability of starch is a very important indicator of wheat quality. It depends on the presence of amylolitic enzymes and starch nature. One of the methods that indicate α-amylase presence and activity in wheat is the falling number method. As the falling number decreases the amylase activity increases. Optimum amylase activity in unsprouted grains is reached between 200 and 300 sec.

In ten analyzed wheat samples, the measured falling number was between 200 and 300 sec, in 11 samples it was longer than 300 sec while in 3 samples it was below 150 sec.

Wet gluten in wheat flour is viscoelastic substance made of gliadin and glutenin and usually highly correlates with protein content[25]. Wet gluten content, as visible from Fig. 3, in all analyzed samples ranged from 19.7 to 31.4%.

The gluten index is a measure of gluten characteristics, which indicates whether gluten is weak, normal or strong. The values of gluten index ranged from 84.7 to 99.5% which indicates strong and high-quality gluten.

Starch is a plant polysaccharide that forms the largest part of the endosperm of wheat. Starch content in the analyzed samples ranged from 67.03 to 69.1% (dry matter) which were inversely proportional to the protein content, as expected.

Regarding the wheat technological quality, in four samples the quality was reduced due to the high amilolytic activity as a result of sprouting grain before the storage.

CONCLUSIONS

The purpose of this study was to determine the influence of storage conditions on the occurrence of fungi and mycotoxins in wheat grains harvested in 2009 in the area of eastern Croatia. Twenty five wheat samples, stored under different conditions, were taken from fourteen metal, ten concrete silos and one metal warehouse. The results indicated the presence of *Fusarium* sp. in all samples, while *Asperigillus* sp. was detected in 92%,

Alternaria sp. in 36%, *Cladosporium* sp. in 60%, *Cephalosporum* sp. in 8%, *Penicillium* sp. in 36%, and *Rhizopus* sp. in 48% of analyzed samples. The presence of the following mycotoxins was determined: aflatoxin (in 1 of 25 samples), deoxynivalenol (in 4 of 25 samples), zearalenone (in 9 of 25 samples) and T-2/HT-2 (in 1 of 25 samples), but their concentrations were in compliance with criteria set by Croatian regulation. Comparing the obtained results of the fungi and mycotoxin analysis with the silo type, no relationships were determined.

Analyzing the effects of moisture, protein, starch content, sedimentation value, wet gluten content, gluten index and falling number onto wheat quality, it was observed that four samples had too high or too low falling number which indicated poor technological quality of wheat. All other analyzed wheat quality parameters were incompliance with criteria set by Croatian regulation and no relationships between values of wheat quality parameters and occurrence of fungi/mycotoxins were not observed.

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