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# C O N T E N T

# APPLICATION OF MICROBIAL KINETICS TO MODELING THE COMPOSTING PROCESS

**ORIGINAL SCIENTIFIC PAPER**

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**ABSTRACT**
In this study, the laboratory and numerical simulation of composting process was performed. Kinetic model based on microbial kinetics and reactor model (mass balances, heat balance, stoichiometry) were developed for composting process. Kinetic model considered two microbial populations that metabolized composting material which was split into two different fractions according to its degradability (easily degradable and hardly degradable fractions). Numerical simulations were performed in numerical software package POLYMATH. The experiment was conducted in a specially designed laboratory reactor under controlled conditions. Developed model was verified by comparing model performance to experimental data for substrate degradation, oxygen and carbon dioxide concentrations, temperature and moisture content. Model simulations provided results that fitted satisfactorily the experimental data. Some deviations were obtained during the degradation of hardly-degradable fraction. Maximum and mean differences between experiment and model are: 2.03% and 1.04% for organic matter conversion, 1.00% and 0.45% for oxygen concentration, 1.07% and 0.39% for carbon dioxide concentration, 10.44 °C and 5.46 °C for temperature, 4.57% and 2.03% for moisture content. Determined optimum value for the initial moisture content is 65%. Sensitivity analysis showed that the maximum conversion of organic matter is the most sensitive objective function, and revealed the most and the least influencing kinetic parameters.
**Keywords:** reactor model, kinetic model, composting, Monod kinetics, simulation, sensitivity analysis.

## INTRODUCTION

Many factors affect the composting process, but the most significant factors are content (availability) of oxygen and water. Temperature is also very a significant factor, however it is a result of microbial activity. Other important factors that may limit the process are pH and composition of substrate. The self-heating of organic matter during the process is a result of microbial respiration. Temperature increase affects the microbial population by changing mesophilic and thermophilic organisms, which is directly related to the rate of organic matter decomposition.

Mathematical models for the composting process improve the prediction of the process and optimize its performance. Kinetics in these models is often an empirical approximation and thus there is a lack of uniformity among current models[1]. Only a few composting models are based on microbial kinetics. Some models consider only one substrate and only one microbial population[2,3], while the other models consider several substrates and several microbial populations[4,5]. Taking into account the advantages of these models, there is a need to develop a new model that can help to improve prediction and optimization of the process performance.

The main objective of this study is to develop the mathematical model (kinetic and reactor) for the process of composting of the mixture of poultry manure and wheat straw, on the basis of existing mathematical models based on microbial kinetics. After developing such a model, it will be applied to experimental data. Comparison of simulation and experimental results will show the efficiency of the model for a further analysis, design and optimization of process.

## MATERIALS AND METHODS

### *Mathematical model*

The growth rate of microbial population is described by[2]:

$$\frac{dm_{x,i}}{dt} = \mu_i \cdot m_{x,i} - k_{d,i} \cdot m_{x,i} \quad (1)$$

where $m_{x,i}$ - mass of microbial population $i$ (kg), $\mu_i$ - specific growth rate of microbial population $i$ ($h^{-1}$), $k_{d,1}$ - specific death rate of microbial population i ($h^{-1}$), $t$ - time (h), $i$ – index for different fraction of substrate (1 = easily degradable fraction, 2 = hardly degradable fraction). The degradation rate of substrate $i$ is given by[2]:

$$\frac{dm_{S,i}}{dt} = -\frac{1}{Y_{X_i/S_i}} \cdot \left(\frac{dm_{x,i}}{dt}\right) + \beta_i \cdot m_{x,i} \quad (2)$$

where $Y_{X_i/S_i}$ - yield coefficient, cells produced / fraction consumed (kg $kg^{-1}$), $\beta_i$ -microbial maintenance coefficient of microbial population $i$ (kg $kg^{-1}h^{-1}$).

The specific growth of microbial population $i$ can be calculated as follows:

$$\mu_i = \mu_{\max,i} \cdot \left(\frac{m_{OM,i}}{K_{S,i} + m_{OM,i}}\right) \cdot k_{O_2} \cdot k_T \cdot k_{H_2O} \quad (3)$$

where $\mu_{\max,i}$ - maximum specific growth of microbial population $i$ ($h^{-1}$), $K_{S,i}$ - saturation constant of microbial population $i$ (kg $kg^{-1}$), $m_{OM,i}$ – organic matter content in fraction $i$ (-), $k_{O_2}$ - correction factor for oxygen (-), $k_T$ - correction factor for temperature (-), $k_{H_2O}$ - correction factor for moisture content.

The microbial maintenance coefficient of microbial population $i$ can be written as[2]:

$$\beta_i = \beta_{\max,i} \cdot \left(\frac{m_{OM,i}}{K_{S,i} + m_{OM,i}}\right) \cdot k_{O_2} \cdot k_T \cdot k_{H_2O} \quad (4)$$

where $\beta_{max,i}$ - maximum microbial maintenance coefficient of microbial population $i$ (kg $kg^{-1}$).

The mass fraction of organic matter content $i$ (wt%) is calculated as follows:

$$w_i = \frac{m_{S,i}}{m_{S,1} + m_{S,2} + m_{IM}} \cdot 100 \quad (5)$$

where $m_{IM}$ - mass of inorganic matter (kg).

Correction factor for oxygen is described by the following equation[6]:

$$k_{O_2} = \frac{c_{O_2}}{k_{O_2(0)} \cdot (K_{O_2} + c_{O_2})} \quad (6)$$

where $k_{O_2(0)}$ - correction factor for oxygen concentration in atmospheric air (20.95 vol %), $K_{O_2}$ - half velocity constant for oxygen (vol %), $\phi_{O_2}$ - volume fraction of oxygen in exhaust air (vol %).

The volume fraction of oxygen in exhaust air (vol %) can be calculated as follows[6]:

$$\phi_{O_2} = \frac{m_{O_2}}{\rho_{O_2} \cdot V} \cdot 100 \quad (7)$$

where $V$ - volume of composting mixture ($m^3$), $m_{O_2}$ – mass of oxygen (kg), $\rho_{O_2}$ - oxygen density (kg $m^{-3}$). The volume of mixture is given by:

$$V = 0.85 \cdot V_R \cdot \varepsilon \quad (8)$$

where is $V_R$ - reactor volume ($m^3$), $\varepsilon$ - porosity (-).

The oxygen density is calculated by the following equation (based on data[7]):

$$\rho_{O_2} = 1.4012 - 0.0041 \cdot T \quad (9)$$

The equation (9) is valid in the range between 0-70 °C.

Correction factor for temperature is described by the following equation[6]:

$$k_T = \frac{T \cdot (80 - T)}{1600} \qquad 0 < T < 80°C$$
$$k_T = \frac{T \cdot (60 - T)}{20 \cdot (80 - T)} \qquad 60°C < T < 80°C \quad (10)$$

Correction factor for moisture content is described by the following equation[8]:

$$k_{H_2O} = \frac{1}{e^{(-17.684 \cdot w_w + 7.0622)} + 1} \quad (11)$$

where $w_w$ - mass fraction of water in the mixture (-).
The mass fraction of water in the mixture (wt %) is calculated as follows:

$$w_w = \frac{m_w}{m_{S,1} + m_{S,2} + m_{IM} + m_w} \cdot 100 \quad (12)$$

where $m_w$ – mass of water in composting mixture (kg).
Mass balance of oxygen is derived as follows:

$$\frac{dm_{O_2}}{dt} = -Y_{O_2/S} \cdot \left( \frac{dm_{S,1}}{dt} + \frac{dm_{S,2}}{dt} \right) + \frac{q_{air}}{V} \cdot \left( m_{O_2,in} - m_{O_2,out} \right) \quad (13)$$

where $Y_{O_2/S}$ - oxygen yield coefficient, $O_2$ consumed / substrate consumed (kg kg$^{-1}$), $q_{air}$ - air flow rate (m$^3$ h$^{-1}$), $m_{O_2,in}$ - inlet oxygen mass (kg), $m_{O_2,out}$ - outlet oxygen mass (kg).
Mass balance of carbon dioxide is derived as follows:

$$\frac{dm_{CO_2}}{dt} = Y_{CO_2/S} \cdot \left( \frac{dm_{S,1}}{dt} + \frac{dm_{S,2}}{dt} \right) + \frac{q_{air}}{V} \cdot \left( m_{CO_2,in} - m_{CO_2,out} \right) \quad (14)$$

where $Y_{CO_2/S}$ -carbon dioxide yield coefficient, $CO_2$ produced / substrate consumed (kg kg$^{-1}$), $m_{CO_2,in}$ - inlet carbon dioxide (kg), $m_{CO_2,out}$ - outlet carbon dioxide mass (kg).
The volume fraction of carbon dioxide in exhaust air (vol %) can be calculated as follows:

$$\phi_{CO_2} = \frac{m_{CO_2}}{\rho_{CO_2} \cdot V} \cdot 100 \quad (15)$$

The carbon dioxide density is calculated by the following equation (based on data[7]):

$$\rho_{CO_2} = 1.9376 - 0.0057 \cdot T \quad (16)$$

Mass balance of water is derived as follows:

$$\frac{dm_w}{dt} = -Y_{W/S} \cdot \left( \frac{dm_{S,1}}{dt} + \frac{dm_{S,2}}{dt} \right) - q_{air} \cdot \rho_a \cdot \left( r_{air,in} - r_{air,out} \right) \quad (17)$$

where $Y_{W/S}$ -water yield coefficient, $H_2O$ produced / substrate consumed (kg kg$^{-1}$), $\rho_a$ - density of dry air (kg m$^{-3}$), $r_{air,in}$ -humidity ratio of inlet air (kg kg$^{-1}$), $r_{air,out}$ - humidity ratio of outlet air (kg kg$^{-1}$).
The density of dry air is calculated by the following equation (based on data[7]):

$$\rho_a = 1.271 - 0.0035 \cdot T \quad (18)$$

The humidity ratios of inlet and outlet air are calculated by the following equation (based on data[7]):

$$r_{air} = 0.1158 - 0.0072 \cdot T + 0.0001 \cdot T^2 \quad (19)$$

The equations (18-19) are valid in the range between 20-70°C.
Heat balance is derived as follows:

$$\frac{dT}{dt} = \frac{H_{R,1} \cdot \frac{dm_{S,1}}{dt} + H_{R,1} \cdot \frac{dm_{S,2}}{dt} - q_{air} \cdot \rho_a \cdot (h_{in} - h_{out}) - \dot{Q}}{c_{P,w} \cdot m_w + c_{P,IM} \cdot m_{IM} + c_{P,S1} \cdot m_{S,1} + c_{P,S2} m_{S,2}} \quad (20)$$

where $T$ – temperature of composting mixture (°C), $H_R$ – heat of reaction, heat produced / substrate consumed (J kg$^{-1}$), $h_{in}$ - enthalpy of inlet air (J kg$^{-1}$), $h_{out}$ - enthalpy of outlet air (J kg$^{-1}$), $\dot{Q}$ -heat loss by conduction through the reactor wall (J h$^{-1}$), $c_{P,w}$ - specific heat capacity of water (J kg$^{-1}$ °C$^{-1}$), $c_{P,IM}$ - specific heat capacity of inorganic matter (J kg$^{-1}$ °C$^{-1}$), $c_{P,S}$ - specific heat capacity of substrate (J kg$^{-1}$ °C$^{-1}$).

The enthalpies of inlet and outlet air are calculated by the following equation (based on data[7]):

$$h = 17844 + 1007.2 \cdot T \quad (21)$$

The equation (21) is valid in the range between 20-70 °C.

The heat loss by conduction through the reactor wall is given as:

$$\dot{Q} = U \cdot A \cdot (T - T_a) \quad (22)$$

where $U$ - overall heat transfer coefficient (J $h^{-1}m^{-2}°C^{-1}$), $A$ – heat transfer area ($m^2$), $T_a$ – ambient temperature.

The specific heat capacities are calculated by the following equation[13]:

$$c_p = 1.48 - 0.64 \cdot w_{IM} + 4.18 \cdot w_w \quad (23)$$

where $w_{IM}$ – inorganic matter content (-), $w_w$ - dry-basis moisture content (-).

With the assumption about known initial elementary composition of the substrate, the degradation of organic part of the substrate is presented by the equation[8]:

$$C_aH_bO_cN_d + \left(\frac{4a+b-2c-3d}{4}\right)O_2 \rightarrow aCO_2 + \frac{b-3d}{2}H_2O + dNH_3 \quad (24)$$

where $a$, $b$, $c$ and $d$ are indices which describe the molar fraction of carbon, hydrogen, oxygen and nitrogen, respectively. The values in the equation (24) are calculated using the known molecular formula of the substrate.

Physical, thermodynamic and stoichiometric constants/parameters were measured from the experiment, calculated from literature data and/or taken original or adjusted data from available literature[2,6,8-13]: $m_{X,1(0)}$ = 0.01 kg, $m_{X,2(0)}$ = 0.0006 kg, $\mu_{max,1}$ = 0.260 $h^{-1}$, $\mu_{max,2}$ = 0.13 $h^{-1}$, $k_{d,1}$ = 0.03 $h^{-1}$, $k_{d,2}$ = 0.05 $h^{-1}$, $\beta_{max,1}$ = 0.48 kg $kg^{-1}h^{-1}$, $\beta_{max,2}$ = 0.38 kg $kg^{-1}h^{-1}$, $Y_{X_1/S_1}$ = 0.35 kg $kg^{-1}h^{-1}$, $Y_{X_2/S_2}$ = 0.35 $kg_{X_2}$ $kg_{S_2}^{-1}$, $K_{S,1}$ = 0.5 kg $kg^{-1}$, $K_{S,2}$ = 0.5 kg $kg^{-1}$, $k_{O_2(0)}$ = 0.96189, $K_{O_2}$ = 0.75 %, $Y_{O_2/S_1}$ = 1.228 kg $kg^{-1}$, $Y_{O_2/S_2}$ = 1.296 kg $kg^{-1}$, $Y_{O_2/S_1}$ = 1.743 kg $kg^{-1}$, $Y_{O_2/S_2}$ = 1.793 kg $kg^{-1}$, $Y_{H_2O/S_1}$ = 0.400 kg $kg^{-1}$, $Y_{H_2O/S_2}$ = 0.495 kg $kg^{-1}$, $H_{R,1}$ = 15244 J $kg^{-1}$, $H_{R,2}$ = 16722 J $kg^{-1}$, $V_R$ = 0.032 $m^3$, $\phi$ = 0.85, $\varepsilon$ = 0.4, $q_{air}$ = 0.18 $m^3h^{-1}$, $T_a$ = 21.4°C, $c_{P,w}$ = 4200 J $kg^{-1}$ $°C^{-1}$, $c_{P,IM}$ = 840 J $kg^{-1}$ $°C^{-1}$, $c_{P,S1}$ = 1340 J $kg^{-1}$ $°C^{-1}$, $c_{P,S2}$ = 1403 J $kg^{-1}$ $°C^{-1}$, $UA$ = 4546.8 J $h^{-1}$ $°C^{-1}$.

The mathematical model consists of eight ordinary differential equations of the first order and corresponding equations. The Runge-Kutta-Fehlberg method was applied in order to obtain a numerical solution of the model. The model was implemented in the numerical software package POLYMATH 6.0[14].

### *Experimental materials and experimental methods*

Poultry manure and wheat straw were used as experimental materials and and were collected in polyethylene bags from farms near Gračanica in the Tuzla Canton of Bosnia and Herzegovina. Moisture content, organic matter content (dry basis), pH and electrical conductivity for poultry manure are 72.59%, 78.07%, 8.17 and 3.34 dS $m^{-1}$, respectively. Moisture content, organic matter content, pH and electrical conductivity for wheat straw are 10.87%, 87.91%, 7.18 and 1.91 dS $m^{-1}$, respectively. Moisture content, organic matter content, pH and electrical conductivity for composting mixture (poultry 83%, straw 27%, on dry basis) are 69.11%, 80.22%, 7.40 and 3.10 dS $m^{-1}$, respectively.

The experiment was conducted using a composting reactor made of polyethylene, insulated with a layer of polyethylene foam. Other details about the composting reactor can be found in literature[15]. The reactor was aerated using an air compressor with air flow rate 0.9 L $min^{-1}$ $kg^{-1}$ (measured by air flow meter). Temperature was measured at 15-min intervals using the thermocouple type T and the acquisition module.

Mixing of composting mixtures was performed several times per day. The samples were taken from the top, middle and bottom of the mixture in order to obtain representative samples. The moisture content and the organic matter content of the sample were analyzed by standard methods[16].

The following equation[17] was used to calculate the organic matter conversion, $X_{OM}$ (%):

$$X_{OM} = \frac{\{[w_{OM,m} - w_{OM,p}] \cdot 100\}}{w_{OM,m} \cdot [100 - w_{Om,p}]} \cdot 100 \qquad (25)$$

where $w_{OM,m}$ – mass fraction of organic matter content at the beginning of the process (mass %) and $w_{OM,p}$ – mass fraction of organic matter content at each sampling (mass %). Oxygen and carbon dioxide concentration were determined by an Orsat analyzer.

## RESULTS AND DISCUSSION

The agreement between the model and experimental data is shown in Figure 1. Maximum and mean differences between experiment and model are: 2.03% and 1.04% for organic matter conversion, 1.00% and 0.45% for oxygen concentration, 1.07% and 0.39% for carbon dioxide concentration, 10.44 °C and 5.46 °C for temperature, 4.57% and 2.03% for moisture content.

Comparison of experimental and simulated data for the organic matter content showed very good agreement (Figure 1a). Some small deviations were noticed between the third and the ninth day. Observed deviations can be explained by transition between the first and the second phase of the process where the most of the easily degradable fraction was decomposed due to a high process rate, while a small part of the hardly degradable fraction was decomposed due to a low process rate. Oxygen concentrations obtained by the model and experiment showed excellent agreement during the whole process. Some small deviations between the model and experimental data were probably caused by decomposition of the hardly degradable fraction, by excessive aeration, and by the fact that the oxygen concentration was measured in exhaust air.

Carbon dioxide concentration increased along with microbial activity. Therefore, the deviations that occurred between the 72$^{nd}$ and 120$^{th}$ hour can be explained by decreased microbial activity during this time interval. The calculated results of the maximum temperature during the process were 1.6 °C higher than the experimental results. The deviations between the model and experimental results occurred mostly during the cooling phase of the process. After reaching a thermophilic peak, cooling of the substrate started and the simulation results showed faster cooling in comparison to the experimental data. Explanation for these phenomena lies in the fact that each of the reactions, which normally occur during the biodegradation process, were not taken into account during the modelling. Comparison of model and experimental results for the moisture content showed that the model generally follows the profile of moisture content during the experiment. The deviations that occurred were most likely a result of material mixing. The simulation and experimental results of the final moisture content were 55.89% and 59.43%, respectively. The reason why the experimental results were higher than the simulation ones is in the fact that some amount

of water was condensed on the inside of the reactor lid and returned to composting mass. The evolution of different microbial populations considered in the model is shown in Figure 2. The profile of microbial populations was similar to the temperature profile. However, the microbial population 1 reached its peak sooner than the microbial population 2. The microbial population 1 was growing until the substrate 1 became a limiting factor for the process. At the same time, the microbial population 2 was growing at a slower rate. The exponential growth began after the most of easily degradable fraction had been degraded.

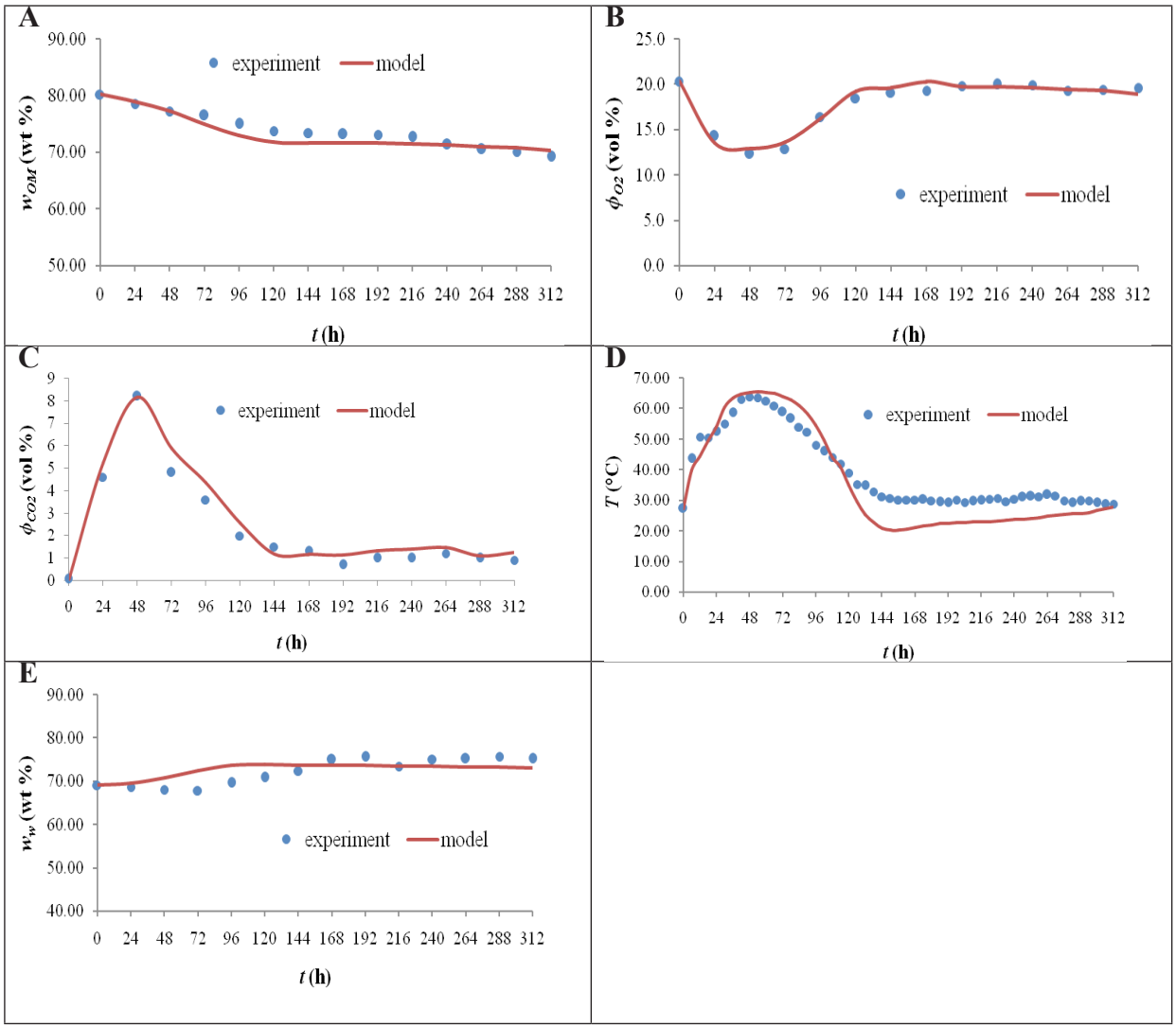


Figure 1. Comparison of experimental data and model for main dymamic state variables: a) $w_{OM}$, b) $\phi_{O2}$, c) $\phi_{CO2}$, d) T, e) $w_w$.

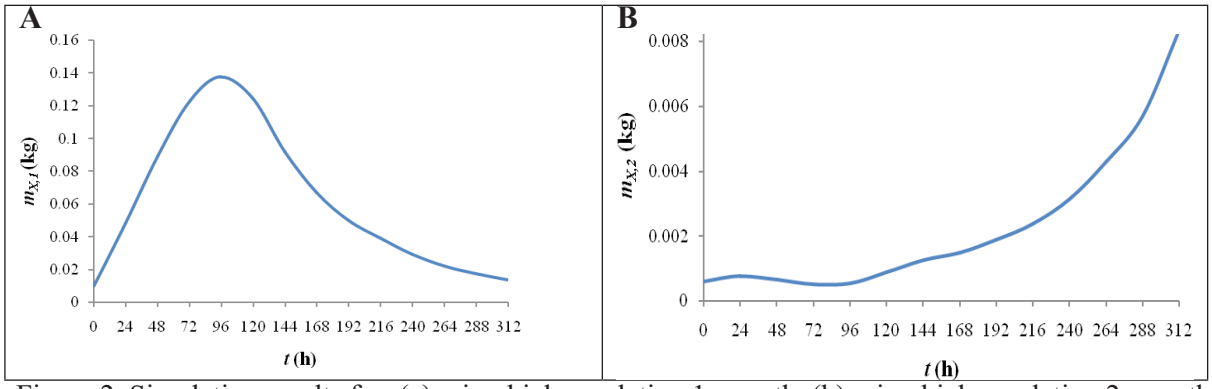


Figure 2. Simulation results for: (a) microbial population 1 growth, (b) microbial population 2 growth.

The profiles of easily degradable fraction and hardly degradable fraction are shown in Figure 3. The organic matter loss in the easily degradable fraction was significant in the first five days of the process and then it remained constant until the end of the experiment. Faster degradation of hardly degradable fraction was observed after the tenth day of the process. In the last phases of the composting process, stability of the mixture was influenced by degradation of the hardly degradable fraction.

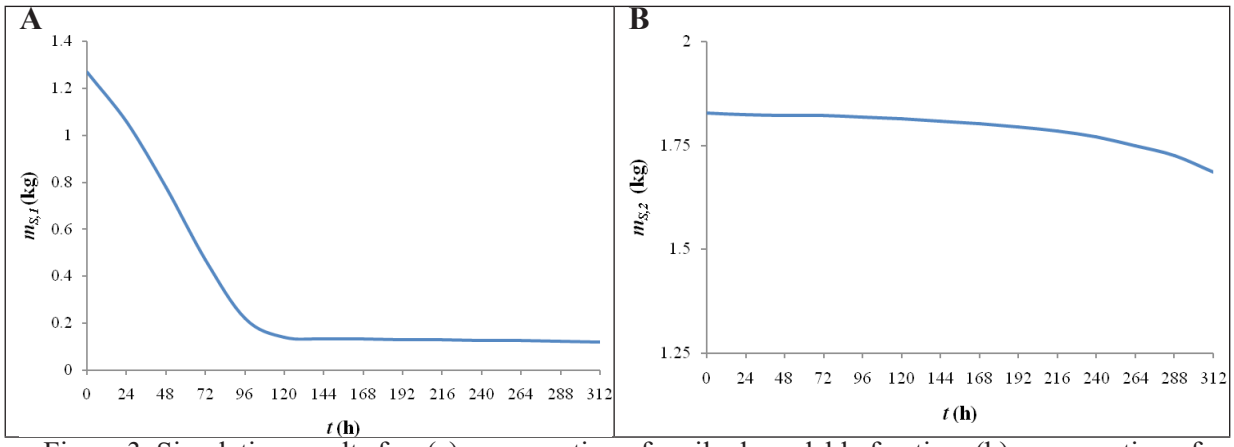


Figure 3. Simulation results for: (a) consumption of easily degradable fraction, (b) consumption of hardly degradable fraction.

Different simulations were performed with the model in order to study the effects of initial moisture content on organic matter conversion and carbon dioxide concentration (Figure 4). Seven moisture contents (45%, 50%, 55%, 60%, 65%, 70%, 75%) were tested. Simulations showed that initial moisture contents of 45% was limiting for the microbial activity. The highest values of organic matter conversion and carbon dioxide concentration were achieved with the initial moisture contents of 65%. Therefore, the optimum value of initial moisture content was 65%. Obtained value corresponds to the literature data for the same and different substrates[18-21].

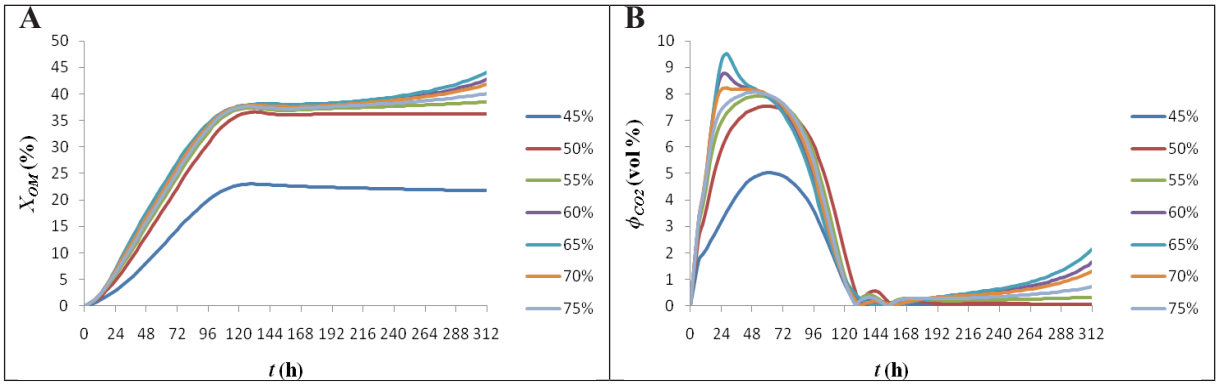


Figure 4. Simulations at different moisture contents: (a) influence on organic matter conversion, (b) influence on volume fraction of carbon dioxide in exhaust air.

For the purpose of evaluating the relative importance of kinetic parameters, the sensitivity analysis was performed. The parameter values were varied individually (-75%, -50%, -25%, +25%, +50%, and +75%) in each simulation run and 72 simulations were performed over a 13-day simulation period. The objective functions were maximum carbon dioxide concentration, maximum organic matter conversion and maximum substrate temperature. The effect of each parameter on the objective functions is shown in Figure 5. On the basis of sensitivity analysis it is found that the maximum organic matter conversion is the most sensitive objective function among the three selected, while the maximum substrate temperature is the least sensitive objective function. Among twelve examined parameters, $\mu_{max,1}$ is the most influencing parameter and $m_{X,1}$ is the least influencing parameter.

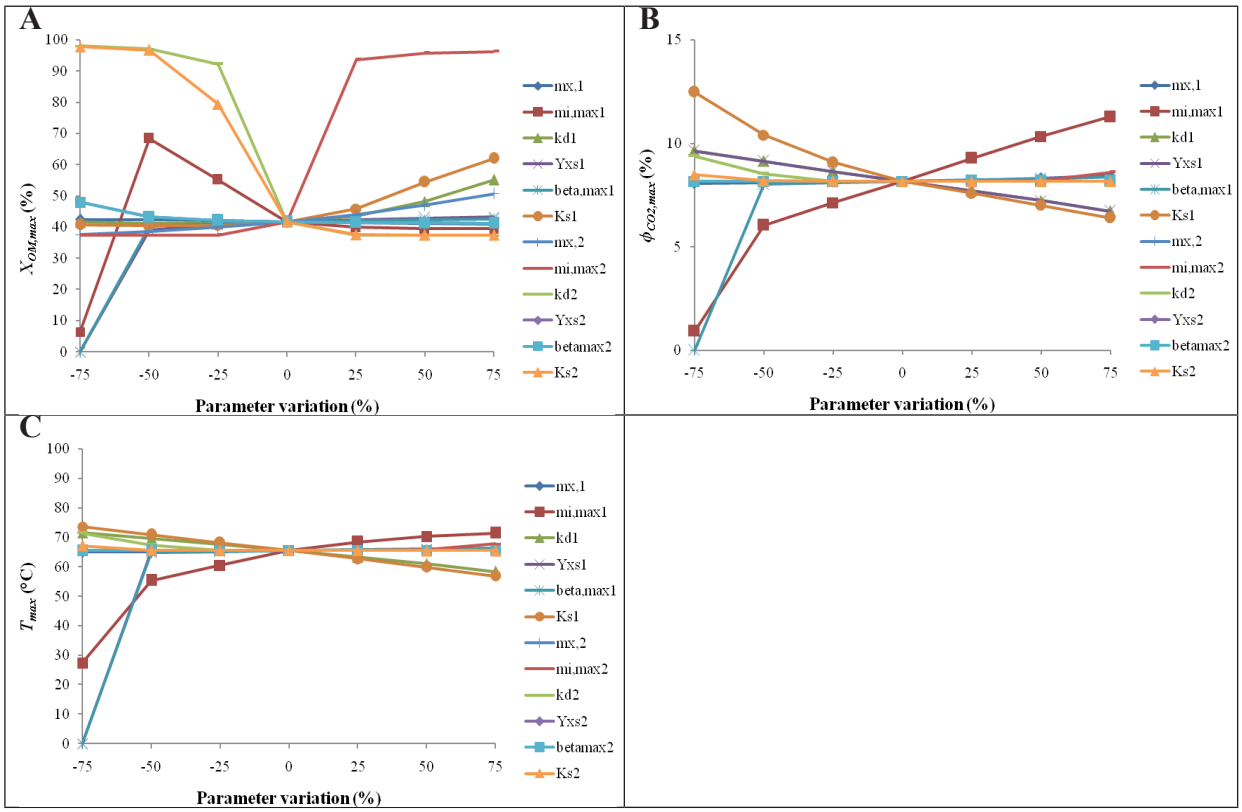


Figure 5. Effect of parameter variation on: (a) maximum organic matter conversion, (b) maximum volume fraction of carbon dioxide in exhaust air, (c) maximum temperature.

## CONCLUSIONS

Kinetic model based on microbial kinetics and reactor model were developed for the composting process of the mixture of poultry manure and wheat straw. Comparison of simulation results and experimental data for five dynamic state variables demonstrated that the model has very good predictions of the composting process. Maximum and mean differences between experiment and model are: 2.03% and 1.04 % for organic matter conversion, 1.00% and 0.45% for oxygen concentration, 1.07% and 0.39% for carbon dioxide concentration, 10.44 °C and 5.46 °C for temperature, 4.57% and 2.03% for moisture content. According to simulation results, the optimum value for initial moisture content is 65%. Sensitivity analysis showed that the maximum conversion of organic matter is the most sensitive objective function. Among twelve examined parameters, $\mu_{\max,1}$ is the most influencing parameter and $m_{X,1}$ is the least influencing parameter. Future work is directed to model modification in accordance with the results of sensitivity analysis.

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# CITOGENOTOXIC AND MICROBIOLOGICAL ASSESSMENT OF WATER QUALITY OF LAKE SNIJEŽNICA

**PROFESSIONAL PAPER**

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**ABSTRACT**
Lake Sniježnica is located in the north-eastern part of Bosnia and Herzegovina. It is formed in the 80's by building artificial dams on the River Brzava. Due to the fact that water is one of the most common components that have been violated in nature, it raises the question how to analyze and determine whether this kind of pollution is harmful to living organisms. The aim of this study was to make the genotoxic and microbiological assessment of water quality in Lake Sniježnica. Samples of water were taken from five different locations. The genotoxic and cytotoxic analyses were performed by *Allium* test. Tap water was used as the negative control, while the positive control contained different concentrations of ethylene di amino tetra (EDTA) acetic acid (1 mM, 2 mM, 4 mM, 6 mM and 8 mM). The length of *Allium cepa* roots, mitotic index and the number of chromosomal aberrations between samples taken from different locations, as well as results between control samples and negative control were compared. The largest increase in the average length was recorded from the roots grown in water samples taken from the location 4, and the lowest from the location 3. The highest values of the mitotic index and the highest number of chromosomal aberrations were observed in the roots grown in the water samples taken from the location 5, and the lowest from the location 1. The most common form of chromosomal aberrations was chromosomal breaks, and the aberrations observed in the lowest numbers were micronucleuses. By microbiological assessment of water quality, we found the presence of *E. coli* on the location 5, while on the other four locations, the bacteria of the genus *Klebsiella sp., Enterobacter sp. and Citrobacter sp.* were found. Based on our results, the water of lake Sniježnica can be classified in the second category water.
**Keywords:** Lake Sniježnica, Allium cepa's roots, mitotic index, chromosomal aberrations, microbiological assessment.

## INTRODUCTION

Hydro accumulation and the dam Sniježnica are located in the north-eastern part of Bosnia and Herzegovina, Tuzla Canton, township Teočak. They are located in the north-east part of Majevica Mountain, on the river Rastošnica, about 1 km upstream from its delta in the river Janja. Sniježnica is an artificial lake, formed in the early eighties (1982) for the needs of the mine and power plant Ugljevik, on the little river Brzava. The surface of the lake is about 1.7 km$^2$ (170 hectares), with the length of about 5 km and maximum depth of 49 meters. Hydrographically, the lake belongs to the Black Sea basin which consists of the river Rastošnica and the river Brzava with its tributaries[1]. There are no adequate data on the quantity and quality of waste water that is currently being discharged into surface waters in the basin of the lake, nor the information about which the biggest polluters of the lake are. From the current knowledge we can conclude that the most important polluters are agriculture and sewage from households located near the lake.

Genotoxicity is the ability of different substances or agents to cause changes in the genetic material, which in most cases has a significant impact on the entire organism[2]. Bioassays based on the use of the plant material are sensitive and can give a warning on the water status as a living environment. The root of plants is the most suitable material in biological tests, because it is first to be exposed to chemical changes in water and soil[3]. *Allium* test is one of the oldest assays applied for the bio toxic analysis. It is a suitable biological indicator system for determining the toxic and genotoxic effects of certain agents, whereby the cytotoxicity assay monitors root growth in length and the genotoxicity test

registers the value of the mitotic index and all changes related to chromosomes and the mitotic spindle during mitotic division[4]. Furthermore, highly clarified cytogenetic characteristics of *Allium cepa* and a small number of chromosomes in the diploid chromosome set (2n = 16) are some features that give this test a great advantage among other genotoxicity assays[5]. One of the criteria for the assessment of water quality and determination of its microbiological parameters is determining its faecal contamination. The aim of this study was to make the genotoxic and microbiological assessment of the water quality from Lake Sniježnica, which included analysing the samples taken from five different locations (north, east, west, south and centre of the lake).

## MATERIAL AND METHODS

Cytogenotoxic and microbiological assessment of water quality from the lake Sniježnica included five samples of water taken from five different locations: north (location 1), east (location 2), west (location 3), south (location 4) and centre of the lake (location 5). Samples were taken on the depth of 1m and 5m from the shore in sterile containers, and then transported to the laboratory in a portable fridge The results of *Allium* anaphase-telophase test were obtained by identifying and analyzing the mitotic activities (mitotic index) of meristematic root cells. The frequency of the individual phases of mitosis was calculated by analyzing microscope slides made from ten samples of the control and treated samples. Mitotic index is shown as a percentage of mitotic cells (cells that were in the certain phase of mitosis) in relation to the total number of the analyzed cells. *Allium* bulbs were prepared so that for 24 hours they were put in tap water to encourage the growth of roots, and then immersed in the sample water from different locations, where the roots grew taking additional 48 hours. The water samples were divided into five groups according to the location. In each group, 15 *Allium cepa* bulbs were set for the analysis, one bulb for the negative (tap water) and one for the positive control (EDTA). The daily growth of the roots was measured for the analysis of cytotoxic effect. The first measurement was performed after 24 hours, the second measurement after 48 hours and the third measurement after 72 hours. For making temporary microscopic slides and for determination of microscopic parameters, 10 bulbs from each group and bulbs from the positive control were used, while bulbs from the negative control were not used because there was no increase in their growth. The treated roots were washed after 72 hours in distilled water, cut into segments of 1-2 cm length from the top, fixed in glacial acetic acid (45%) for 30 min at room temperature, and then washed twice in ethanol (70%) 5 min. The samples were then transferred to 70% ethanol, sealed by an expandable film and stored at + 4°C until use. Just before slide preparation, the roots were hydrolyzed in1MHCl, 3 min at room temperature and then stained with a 2% solution of aceto-orcein 30-60 min. From each group of samples, ten slides were prepared, observed and analysed by optical microscope (for determination of mitotic index, and index of each phases and chromosomal aberrations). The root samples (1-2 mm of length) were crushed into small pieces and then transferred from the paint on the slide with the addition of 45% acetic acid. The slides were sealed using heated paraffin[5]. The value of the mitotic index is calculated by the proportion of the sum of all cells in the cell division and the total number of the observed cells, multiplied by 100.[6]

$$M\ (\%) = \Sigma\ (P+M+A+T)/\Sigma\ (P+M+A+T+I) \times 100$$

P-prophase, M-metaphase, A-anaphase,
T - telophase, I – interphase[6]

In the further cytogenetic analysis, the possible changes in the structure and kinetics of chromosomes were observed, i.e., irregularities at the level of cells and chromosomes during all phases of the cell cycle.

Microbiological analysis of water included methods for determination of the total number

of aerobic mesophilic and psychrophilic bacteria (BAS EN ISO 6222:2003; BAS EN ISO 6222:2003)[7], determination of total coliform bacteria and determination of the presence of coliform bacteria of fecal origin (BAS EN ISO 9308-2:2012).[8]
For statistical analysis of the results, we used T-test and ANOVA (analysis of variance).

## RESULTS

The results of cytotoxic effect of the water from the lake on the *Allium cepa* root system show that the mean values of the root length ranged from 0.40 cm (on the location 3), to 0.64 cm (on the location 4). The mean value of the root length for the control sample was 0.68 cm (Table 1).

Table 1. The mean values of the root length and values of the mitotic index

| | The mean value of the root length (cm) | Mitotic index (%) |
|---|---|---|
| Location 1 | 0.60 | 9.70 |
| Location 2 | 0.62 | 11.55 |
| Location 3 | 0.40 | 9.90 |
| Location 4 | 0.64 | 10.65 |
| Location 5 | 0.48 | 11.85 |
| Control sample | 0.68 | 12.85 |

Mitotic index values ranged from 9.70% (on the location 1) to 11.85% (on the location 5, Table 1). Micronucleus, anaphase bridges, chromosome breakage and lagging chromosomes in anaphase were found in root cells of *Allium cepa* in a form of chromosomal aberrations (Figure 1-4). The most common form of chromosomal aberrations was chromosomal breaks, while micronucleus was aberration observed in the lowest numbers. The largest number of abnormal cells was determined on the location 5, a total of 20 (1%), of which 9 cells with anaphase bridges, 7 cells with chromosomal breakages and 3 cells with lagging chromosomes (Table 2).

Table 2. Identified chromosomal aberrations in the total number of observed cells

| Treatment | Chromosomal aberrations | | | | | |
|---|---|---|---|---|---|---|
| | Metaphase spot (micronucleus) | Anaphase bridge | Chromosomal breaks | The lagging chromosomes | ∑ | % |
| Location 1 | 0 | 0 | 2 | 3 | **5** | 0.25 |
| Location 2 | 0 | 2 | 2 | 5 | **9** | 0.45 |
| Location 3 | 0 | 1 | 10 | 2 | **13** | 0.65 |
| Location 4 | 0 | 3 | 6 | 3 | **12** | 0.60 |
| Location 5 | 1 | 9 | 7 | 3 | **20** | 1.00 |
| Control sample | 0 | 0 | 0 | 0 | **0** | 0 |
| ∑ | **1** | **15** | **27** | **16** | = **59** | = **0.59** |

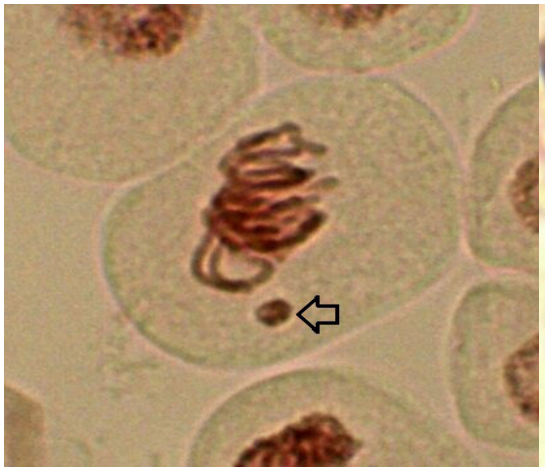

Figure 1. Cell with micronucleus

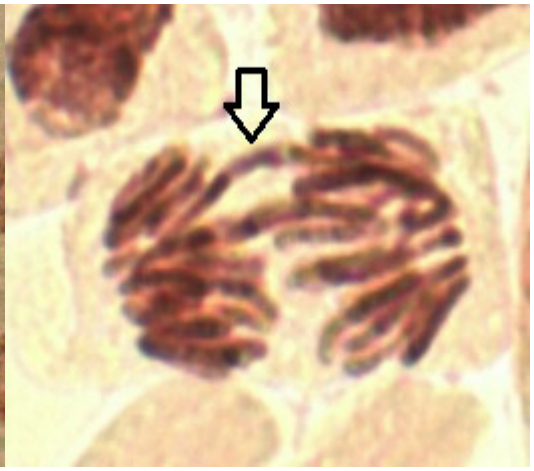

Figure 2. Cell with anaphase bridge

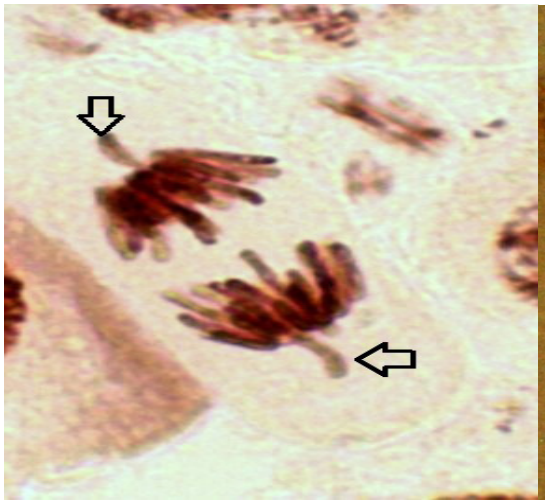

Figure 3. Cell with chromosomal breaks

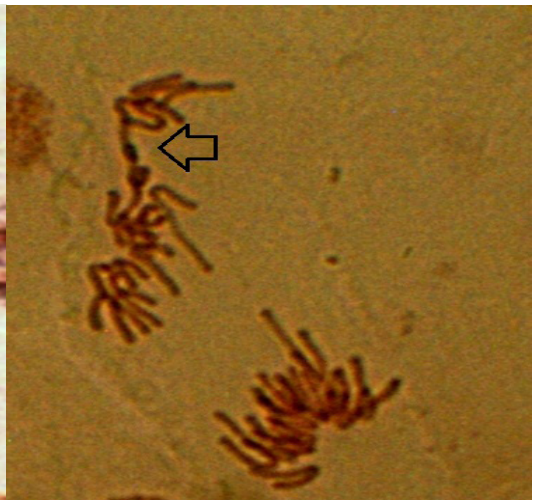

Figure 4. Cell with anaphase lagging chromosome

With analysis of variance (ANOVA) mitotic index showed statistically significant differences between the control group and cells treated water sample from the location 1 (F = 158.76 > 18.51). Statistical analysis also showed statistically significant differences between the control group and cells treated water samples from the location 2, (F = 19.88> 18.51), location 3, (F = 120.03> 18.51); and cells treated water sample from location 4 (F = 56.94> 18.51). Among the control group and cells treated water samples from the location 5, there is no statistically significant difference (F = 11.74 <18.51).

Total number of aerobic mesophilic bacteria psychrophilic bacteria in the water of Lake Sniježnica ranged from 4, 1 x $10^1$/1 ml on the location 4 to 1. 32 x $10^3$/1 ml on the location 5. MPN values on the locations 2 and 3 were the lowest (3 800/100 ml), while the locations 4, 5 and 1 had the highest values of MPN (24 000/100 ml). The indicators of faecal contaminations were isolated and identified in all tested samples.

Table 3. Total number of aerobic, mesophilic and psychrophilic bacteria (CFU/ml) and the most probable number of coliform bacteria in 100 ml sample

| | Microbiological indicator | | | |
|---|---|---|---|---|
| | Total number of aerobic bacteria (37 ºC) | Total number of psychrophilic bacteria (22 ºC) | The most probable number of coliform bacteria (MPN) | Identified bacteria |
| Location 1. | 292 | 305 | 24000 | ***Klebsiella sp.*** |
| Location 2. | 138 | 165 | 3800 | ***Enterobacter sp.*** |
| Location 3. | 46 | 980 | 3800 | ***Citrobacter sp.*** |
| Location 4. | 41 | 275 | 24000 | ***Citrobacter sp.*** |
| Location 5. | 960 | 1320 | 24000 | ***E. coli*** |

## DISCUSSION

Results of this study present the influence of water from the lake Sniježnica on mitotic activity and chromosomal behaviour of *Allium cepa* meristematic cells. Roots grown in water samples taken from the lake showed minor deviations in growth, then roots grown in control water samples. Analysed water samples had a negative effect on the growth and mitotic activity of the roots. With all tested samples of water, except for the control, chromosomal aberrations were observed whereby the rate of genetic damage does not exceed the tolerance of 3 to 5%, or more precisely the number of chromosomal aberrations in the total sample is only 0.59%. Comparing with other studies, number of chromosomal aberrations, in the water analysis of the Lake Vidara and Lukavac, was slightly higher, but the percentage also does not exceed acceptable 2% in either study.[9, 10]

The results indicate that there is a certain degree of genotoxic effects of lake water, which is manifested by changes in the mitotic phase. The control sample did not show evidence of aberrations, while the percentage of aberration in the tested water was 0.59%. The most common form of chromosomal aberrations was chromosomal breaks, while metaphase lagging chromosomes were observed in the fewest number.

With microbiological assessment of the water quality, we determined the presence of bacteria of the genus *Klebsiella sp*. at the location 1, the presence of bacteria of the genus *Enterobacter sp.* on the location 2, the presence of bacteria of the genus *Citrobacter sp.* at the location 3 and 4. Eikman test confirmed the presence of *E. coli* at the location 5. According to results of microbiological assessment, water from the lake Snježnica at the location 1, 4 and 5 falls into the third class of water, while the locations 2 and 3 shows quality of the second class of water[11,12]. Comparing our results with the results of the study on water quality of Modrac lake[13], we can conclude that this lake has a higher degree of pollution then Sniježnica lake while lake Bistarac and pit Mine Lake are in the same category.[10] The highest number of bacteria was determined at the location 5, which can be explained by the fact that this site is under the greatest human impact, since in this part of the lake a greater number of fishermen's houses were placed. Location 1 and 4 are also under strong anthropogenic influence; at the location 1 there is local beach, and at the location 4 there is estuary of the river Rastošnica which brings additional pollution and sewage from surrounding villages.

There are no significant industrial polluters near the lake Snježnica. However significant impact on pollution certainly has the sewer systems and waste water systems of surrounding villages, and rinsing farmland treated with pesticides by rain. Since the frequency of cytogenetic changes depends on the degree of harmful agents, there is a need for permanent monitoring of water from the lake, which would include genotoxic analysis of aquatic organisms. Therefore, this work should certainly encourage further microbiological, genocitotoxic and physic-chemical studies of Lake Sniježnica.

## CONCLUSION

Regarding the cytotoxic effect, the water from Lake Sniježnica has the inhibitory effect on the growth of *Allium cepa* roots. According to the results, *Allium cepa* roots grown in the water samples taken from the lake had a smaller increase in growth than the roots grown in control samples. A significant reduction in mitotic index of meristematic cells grown in the analyzed water was also observed.

According to the results, there is a degree of genotoxic effects of the lake water, which is manifested by changes in the mitotic stages. There was no evidence of chromosomal aberrations in the control samples, while the tested samples showed 0,59% of the aberrations. The highest numbers of aberrations were observed at the tested samples from the location 5. However, this result is within allowed limits and does not exceed the normal. On the horizontal profile, the water from Lake Sniježnica belongs to the second and third category of water. Indicators of faecal contamination were isolated from all five locations. These results are compatible with the fact that there are no significant pollutants close to the lake. However, the significant causes of pollution are the surrounding villages through sewer systems and waste water systems, and agricultural areas treated with pesticides located near the lake.

Considering the fact that the level of cytogenetic changes depends on the toxic pollution and that there is an increasing uncontrolled human impact on the lake water, there is a need for further analysis which would include genotoxic testing of aquatic organisms. Therefore, this work should certainly encourage further microbiological, genocytotoxic and physic-chemical studies of Lake Sniježnica.

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# LANDFILL LEACHATE QUALITY EVALUATION THROUGH THE LANDFILL POLLUTION INDEX

**ORIGINAL SCIENTIFIC PAPER**

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**ABSTRACT**

Recent regulations in the field of solid waste management require treatments before the disposal of municipal waste on landfills. Chemical and physical parameters of the landfill leachate can be used for evaluation of the landfill age as the main parameter for the stage of degradation inside the landfill body. In order to estimate the impact of leachate pollution into the environment, different pollution indexes can be calculated. One of common indexes is the Leachate Pollution Index (LPI) which has been developed for comparison of leachate pollution potentials of various landfill sites, in relation with the content of a particular pollutant. This calculation includes the concentration and significance of each pollutant and can be used as the main criterion for ranking of the environmental impact and for the design of leachate treatment processes. LPI has been calculated for the ″Bikarac″ landfill based on the following parameters: suspended matter, COD, BOD, ammonia nitrogen, zinc, copper, nickel, mercury, arsenic phenolic compounds, chlorides and pH value.

**Keywords:** Leachate, landfill, leachate pollution index (LPI)

## INTRODUCTION

Landfills represent a significant threat to groundwater quality, human health and ecosystems due to permanent liquid and gas emissions[1]. These emissions depend on solid waste composition, weather conditions, climate,natural water streams near the disposing area, moisture content, particle size and compaction of the waste[2-4]. In the underdeveloped and developing countries the uncontrolled landfills in nature pose aserious problem due to the disposal of municipal and hazardous waste on environmentally sensitive areas, close to aquifers and natural water streams[2]. This waste is disposed without any pre-treatment and processing, causing permanent hazardous pollution due to leaching of contaminants from the mixed waste which are distributed by groundwater flows, surface water runoff and biological decomposition. Rainfall is the main contributor to generation of leachates the composition of which varies widely and depends on the waste type and age. The leachate is usually characterized by three major groups of contaminants:

- Organic matters - acids, alcohols, aldehydes, other components determined by COD (chemical oxygen demand) and BOD(biological oxygen demand), DOC, volatile fatty acids (VFA), fulvic-like and humic-like compounds (HMW)
- Inorganic matters - sulphate, chloride, ammonium, calcium, magnesium, sodium, potassium, hydrogen carbonate, iron and manganese, heavy metals like lead, nickel, copper, cadmium, chromium and zinc
- Xenobiotic organic compounds - aromatic hydrocarbons, phenols, chlorinated aliphatichydrocarbons, pesticides, PCBs, Dioxins, PAHs, etc.

All these components are formed from the waste due to successive biological, chemical and physical processes. Landfill is considered to be a bio-

reactor where the activity of microorganisms' is responsible for degradation and production of intermediate organic compounds. Three basic phases of decomposition can be described in domestic landfills. In the first stage, the initial aerobic phase, consumption of oxygen and water infiltration inducesthe phase of acetogenic fermentation by producing leachate with high contents of BOD, COD and ammonia nitrogen. The methanogenic phase of decomposition starts and consumes organic compounds resulting from the acetogenic process to produce biogas. The stabilisation of the landfill takes place over time and the landfill age can be evaluated by determination of main chemical and physical parameters (Table 1)[6]:

Table 1. Physico-chemical parameters of landfill leachate necessary for evaluation of the landfill age.

| Leachate type | Young | Intermediate | Stabilised |
|---|---|---|---|
| Landfill age | <5 years | 5-10 years | >10 years |
| pH | <6.5 | 7 | >7.5 |
| COD mg/l | >20000 | 3000-15000 | <2000 |
| BOD/COD | >0.3 | 0.1-0.3 | <0.1 |
| TOC/COD | 0.3 | - | 0.4 |
| Organic matter | 70-90% VFA | 20-30% VFA | HMW |
| Metals mg/l | 2000 | <2000 | <2000 |
| Nitrogen mg/l | 100-2000 | 100-2000 | 100-2000 |

Note: TOC-total organic carbon, mg/l.

The determination of the accurate chemical composition and particle size of leachate, landfill age and discharge limits is necessary for selection of treatment before disposal into the environment in order to minimize emissions and maximize the economic feasibility of the process. Among all processes, the most commonly used are: aerobic biological treatments such us aerated lagoons, SBR- sequenced batch reactors, FBR - fluidized bed reactors, anaerobic biological treatments, membrane techniques, physico-chemical processes and electrochemical processes[6,7].

One of the most important operational tasks on each landfill is appropriate prevention of contamination of underlying soils and groundwater aquifers by the leachate generated from the landfills. This is prevented by installation of different depths of geo-membrane layers and water-resistant natural or synthetic materials, enabling collection of all generated leachate at different depths inside the landfill body. Containing a number of chemical compounds, the characteristics of municipal landfill leachate vary with landfill volume and age. Beside all mentioned factors of leachate composition, the pre-treatment of the solid waste as well as the content of recyclable materials affects the leachate content. Some authors have tried to evaluate the leachate pollution potential as the main parameter for remediation activities on a particular landfill. Kummar and Alapat (2003, 2005) have developed a technique to quantify the leachate contamination potential of different landfills on a comparative scale in terms of the leachate pollution index (LPI)[2,8-10]. Their study included 80 panellists from environmental science and engineering authorities, considering 50 commonly reported leachate parameters depending on their significance. Outof all considered pollutants, the most significant18 have been selected and included in the LPI analysis. These parameters are listed in Table 2, together with

weight factors or coefficients which indicate the importance of the individual pollutant.

For each parameter the ″sub index curve″ of pollutants has been developed,where abscissa means the level of strength or concentration of a particular variable and ordinate means the level of leachate pollution expressed as the sub index score. These graphs can be found elsewhere[2,8,9]. The sub index values or ″p″ value is evaluated from the intersection of concentration of leachate pollution with the sub index curve of specific pollutants. The sub index score can achieve values in the range ″5″ to ″100″. Minimum values of ″5″ are used to ensure that LPI values do not result in zero even if some pollutants do not show any pollution. The LPI can be calculated from equations:

$$\mathrm{LPI} = \sum_{i=1}^{n} w_i \, p_i \qquad (1)$$

$$\sum_{i=1}^{n} w_i \, p_i = 1 \qquad (2)$$

where: LPI - the weighted additive leachate pollution index; $w_i$- the weight for the $i^{th}$ pollutant variable; $p_i$- the sub index score of the $i^{th}$ pollutant variable; $n$- number of variables.

Table 2. Pollutant parameters included in LPI analysis.

| No. | Pollutant | Significance | Pollutant weight factor |
|---|---|---|---|
| 1. | pH | 3.509 | 0.055 |
| 2. | Total dissolved solids | 3.196 | 0.050 |
| 3. | $BOD_5$ | 3.902 | 0.061 |
| 4. | COD | 3.963 | 0.062 |
| 5. | Total nitrogen | 3.367 | 0.053 |
| 6. | Ammonia nitrogen | 3.250 | 0.051 |
| 7. | Total iron | 2.830 | 0.045 |
| 8. | Copper | 3.170 | 0.050 |
| 9. | Nickel | 3.321 | 0.052 |
| 10. | Zinc | 3.585 | 0.056 |
| 11. | Lead | 4.019 | 0.063 |
| 12. | Total chromium | 4.057 | 0.064 |
| 13. | Mercury | 3.923 | 0.062 |
| 14. | Arsenic | 3.885 | 0.061 |
| 15. | Phenolic compounds | 3.627 | 0.057 |
| 16. | Chlorides | 3.078 | 0.048 |
| 17. | Cyanide | 3.694 | 0.058 |
| 18. | Total coliform bacteria | 3.289 | 0.052 |
| | TOTAL | 63.165 | 1.000 |

When the data for all the pollutant variables from Table 2 are not available, the LPI can be calculated usingthe datafor available pollutants, according to the following equation:

$$LPI = \frac{\sum_{i=1}^{m} w_i \, p_i}{\sum_{i=1}^{m} w_i} \qquad (3)$$

where*m* is number of pollutant parameters for which data are available ($m$<18 and $\sum w_i$<1)[2,8]. In this paper we have performed the LPI analysis of the leachate sampled from the Bikaraclandfill, located in the Šibenik-Knin County, Croatia.

## MATERIAL AND METHODES

The leachate used in this study has been collected from the basin for collection of landfill leachate of the Bikarac landfill which is part of the Centre for Waste Management in the Šibenik-Knin County, Croatia. A sample of 1.5 l has been sampled in August 2013 and analysed in accredited laboratories of the Šibenik-Knin County and Split-Dalmatia County[11].The following parameters have been determined: pH, sampling temperature,electrical conductivity, suspended matter, ammonia nitrogen, nitrate nitrogen, total organic carbon, total phenols, COD, $BOD_5$, chlorides, sulphates, arsenic, cadmium, mercury, nickel, copper, zinc and lead. Results of analyses are shown in Table 3 and compared with maximumpermissible values for discharge in natural waters and sewage system according to Croatian laws (reported from NarodneNovine 80/13)[12].

Table 3. Results for physical and chemical parameters in the leachate from the Bikarac landfill compared with maximumpermissible values for discharge into natural waters and sewage system.

| Pollutant | Unit of measure | Result | Natural surface waters | Sewage system |
|---|---|---|---|---|
| pH | pH unit | 7.9 | 6.0-9.0 | 6.5-9.5 |
| Sampling temperature | °C | 24.6 | | |
| Suspended matter | mg/l | 110.1 | 25 | (a) |
| Electrical conductivity | µS/cm | 4800.00 | - | - |
| Ammonia nitrogen | mg/l | 50.6 | 5 | - |
| Nitrate nitrogen | mg/l | 0.4 | 1 | 10 |
| TOC | mg/l | 310 | 30 | - |
| Phenols | mg/l | < 1 | 0.1 | 10 |
| COD | mg $O_2$/l | 960 | 100 | 700 |
| $BOD_5$ | mg $O_2$/l | 391 | 20 | 250 |
| Chlorides | mg/l | 428 | - | 1000 (b) |
| Sulphates | mg/l | 482 | - | 200 (b) |
| Arsenic | mg/l | 0.02165 | 0.1 | 9.1 |
| Cadmium* | mg/l | <0.0003 | 0.1 | 0,1 |
| Mercury* | mg/l | <0.0003 | 0.01 | 0.01 |
| Nickel* | mg/l | 0.058 | 0.5 | 0.5 |
| Copper | mg/l | 0.0145 | 0.5 | 0.5 |
| Zinc | mg/l | 0.033 | 2 | 2 |
| Lead* | mg/l | <0.001 | 0.5 | 0.5 |

NOTE: (a) - depending on wastewater treatment facility

(b) - depending on construction materials of sewage system

*- pollutants which must not be discharged in groundwater.

## RESULTS AND DISCUSSION

The results for physical and chemical parameters in the leachate from the Bikarac landfill (Table 3) indicates that the content of suspended matter, ammonia nitrogen, TOC, COD and $BOD_5$ exceed maximal allowed (permissible) values for discharge into sewage waters or natural waters and that their treatment is necessary. The ratio $BOD_5/COD = 0.407$ indicates biodegradability of these waters, suggesting aerobic or anaerobic treatment as a possible method. According to physico-chemical parameters of landfill leachate for evaluation of the landfill age given in Table 1, the landfill leachate sampled from the Bikarac landfill can be characterized as of intermediate to stabilized age. However, for precise evaluation, it is necessary to perform the analysis depending on the season, weather conditions and different sampling sites.The measured number of pollutants in Table 3 is fewer than 18, which is recommended for calculation of the leachate pollution index. Thus, the LPI has been calculated on the basis of available data for 10 pollutants, using Equation (3). The calculated LPI value for leachate of the Bikarac landfill as well as the evaluated value of the sub index score $p_i$ and value $w_i$ from Table 2 are shown in Table 4.

According to reference[2,8], the calculated value of LPI = 8.526 is marked as low, and indicates low leaching pollution potential of the Bikaracland-fill. It means that the leachate generated from this site is relatively stabilized. However, as values of suspended matter, ammonia nitrogen, TOC, COD and $BOD_5$ exceed maximal allowed (permissible) values for discharge into sewage waters or natural waters, the leachate should be treated before allowing discharging.

Table 4.The leachate pollution index (LPI) for the Bikarac landfill site.

| Pollutant | Measured value | Individual pollution rating, e.g. „sub index score“ $p_i$ | Weights (from Table 3) $w_i$ | Overall pollution rating $p_i w_i$ |
|---|---|---|---|---|
| pH | 7.9 | 5 | 0.055 | 0.275 |
| Total dissolved solids | 110.1 | 5 | 0.05 | 0.25 |
| $BOD_5$ | 960 | 28 | 0.061 | 1.708 |
| COD | 391 | 11 | 0.062 | 0.682 |
| Total nitrogen | - | - | - | - |
| Ammonia nitrogen | 50.6 | 7 | 0.051 | 0.357 |
| Total iron | - | - | - | - |
| Copper | 0.0145 | 5 | 0.05 | 0.25 |
| Nickel | 0.058 | 5 | 0.052 | 0.26 |
| Zinc | 0.033 | 5 | 0.056 | 0.28 |
| Lead | BDL | - | - | - |
| Total chromium | - | - | - | - |
| Mercury | BDL | - | - | - |
| Arsenic | 0.02165 | 5 | 0.061 | 0.305 |
| Phenolic compounds | BDL | - | - | - |
| Chlorides | 428 | 6 | 0.048 | 0.288 |
| Cyanide | - | - | - | - |
| Total coliform bacteria | - | - | - | - |
| TOTAL | | | $\Sigma$=0.546 | $\Sigma$=4.655 |
| **LPI = 8.526** | | | | |

Note: data for suspended solids have been used instead of TDS. BDL-below detection limit.
All values are in mg/l, except pH and total coliform bacteria (cfu/ml).

## CONCLUSIONS

The physico-chemical parameters of the landfill leachate sampled from the Bikarac landfill have characterized this site as of intermediate to stabilized age. The $BOD_5$/COD ratioequals 0.407 indicating the leachate biodegradability, whichsuggests the implementation of biological wastewater treatments. The LPI was calculated on the basis of available data for 10 pollutants. The calculated value of LPI=8.526 indicates that the leachate generated from these sites is relatively stabilized. The LPI can be used as a simple tool for evaluation of the leachate pollution threat to the environment and human health.

**Acknowledgement**

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# EXTRACTION OF CURCUMINOIDS FROM TURMERIC (*Curcuma longa* L.) WITH SUBCRITICAL WATER

**PRELIMINARY COMMUNICATION**

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**ABSTRACT**

In this study the isolation of curcuminoids from turmeric (*Curcuma longa* L.) was performed using subcritical water extraction. The effect of extraction parameters, such as temperature and time, on the extraction yield of curcuminoids was observed. The obtained extracts were analyzed by HPLC for the content of curcumin, demethoxycurcumin and bis-demethoxycurcumin and the antioxidant activities were determined using DPPH radical-scavenging spectrophotometric methods.

Optimum extraction conditions where the highest extraction yield was obtained were pressure of 30 bar, temperature of 150 °C and time 60 min, however at this temperature, the degradation of curcuminoids was also observed with the prolonged extraction time. The highest amount of total curcuminoidswas extracted at temperature of 100 °C, 30 bar and 120 min (3 mg/g material). The curcuma extracts showed potent DPPH radical-scavenging activities in range between 1.36 to 21.62 %.

**Key words:** Curcuminoids, Subcritical water, Extraction, HPLC, DPPH

## INTRODUCTION

*Curcuma longa* L., also known as turmeric, is grown in warm, rainy regions of the world such as China, India, Indonesia, Jamaica and Peru. The rhizome of turmeric is an important source of a yellow natural pigment, which in the past was used as a spice, a colouring agent in the food industry, for household medicine and as insect repellent. The yellow colour, which is characteristic for turmeric rhizome, is due to the presence of 3-5% of curcuminoids. Curcuminoids are practically insoluble in water at ambient conditions; therefore, water cannot be used as an extraction solvent for these compounds[1].

On the other hand, when heating water above its boiling point and applying just enough pressure to maintain it in liquid state, its properties change significantly. At these conditions, the polarity decreases drastically, which enhances the solubility of many less-polar organic compounds, such as the curcuminoids. Nevertheless, when high temperatures are applied the possibility of hydrothermal degradation reactions of the organic compounds exists, which consequently lowers the quality of obtained extract. Extraction parameters should therefore be studied in detail and optimized[2-5].

## MATERIALS AND METHODS

Subcritical water extraction

For extraction of curcuminoidswith subcritical water a 60 mL cylindrical stainless steel high-temperature high-pressure autoclave was used. The temperature was regulatedusing an electric heater and the medium was stirred using a magnetic stirrer. The pressure inside the autoclave was established by filling itwith nitrogen gas. The applied extraction pressure for all extractions was 30 bar.

The extractions were carried out at three different temperatures, namely 100 °C, 150 °C and200 °C for 5 min to 120 min at a material to water ratio of 1/20 g/mL.After extraction, the suspension was filtered and the obtained liquid extract was filtered again through a 22 μm filter head. The filtered extract was then evaporated until dryness and the obtained solid extracts were kept in sealed beakers at -20 °C until further analysis[2].

HPLC analysis

The extracts were analyzed by the HPLC methodthat has been previously described by our research group[1].

The Agilent 1100 HPLC system consisted of a binary pump, column heater, autosampler and variable wavelenght detector (VWD). The separation was achieved on a chromatographic column Agilent Eclipse XDB-C18 (150 mm × 4.6 mm; 5 μm particle size). The mobile phases were 2% acetic acid in water (elution A) and 2% acetic acid in acetonitrile (elution B). The solvent gradient was as follows: 0–3 min, 10% B; 8 min, 20% B; 13 min, 25% B; 18 min, 35% B; 28–33 min, 55% B and then held for 3 min before returning to initial conditions. The solvent flow rate was 1.0 mL/min and the column temperature was 30 °C. The volume of injection was 10 μLand peaks were monitored at 420 nm. Quantification of single curcuminoids was done using calibration curves obtained from curcuminoid standards. All measurements were performed in triplicate and averages were calculated.

DPPH radical-scavenging activity

The antioxidant activities of curcuma extracts were measured spectrophotometrically using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method. Extract solutions were prepared by dissolving approximately 10 mg of extract in 10mL of methanol. 3 mL of DPPH solution was added into a dark flask containing 77 μL of extract solution.The mixed solutions were kept in the dark for 15 min at room temperature and the absorbance was measured using a UV-VIS spectrophotometer at 515 nm[1].The radical-scavenging activities of curcuma extracts were calculated using Eq.(1).

$$\% \text{ inhibition} = \frac{A_B - A_A}{A_B} \times 100 \qquad (1)$$

where $A_B$ is absorbance of the blank ($t$= 0 min) and $A_A$ is absorbance of the extract solution after 15 min of incubation.

## RESULTS AND DISCUSSION

Figure 1 represents the extraction yield of turmeric obtained with subcritical water extraction at 100 °C, 150 °C and 200 °C. It can be observed that at 100 °C the extraction yield increases with increasing time. After 60 min of extraction the yield equals to 26.2% and after this point the yield does not increase with increasing extraction time any more. At 150 °C the overall yield of curcuma is much higher compared to 100 °C. The maximum yield is obtained after 60 min of extraction (61.7%). The yield however is not highly dependent on extraction time. Although at 200 °C the highest yield of extraction is already observed after 10 min equalling to roughly 65.3%, the yield after this time begins to decrease with time and reaches a value of 36.15% after 120

min. The high temperature probably degrades the compounds in curcuma extracts into volatile compounds (volatile organic acids or solid components which are not soluble in water) which can be removed in the separation step thus lowering the extraction yield.

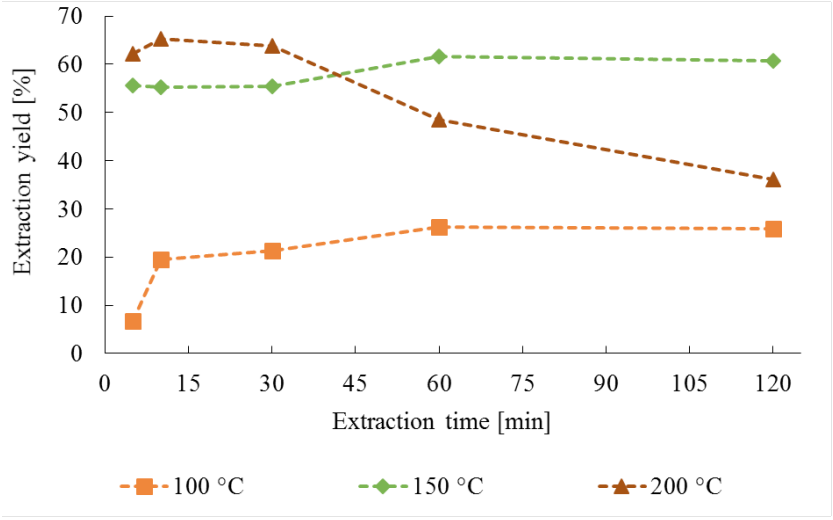


Figure 1. Extraction yield of curcuma obtained with subcritical water extraction at different temperatures.

Figure 2 depicts the total curcuminoids content in curcuma extracts obtained with subcritical water extraction. It can be observed that the curcuminoid content is highly dependent on temperature, namely at the lowest observed temperature (100 °C) the curcuminoids content increases with increasing extraction time while at 150 °C the content already drastically decreases to a zero value after 30 min of extraction. At 200 °C practically no curcuminoids are present in the extracts. This indicates that the curcuminoids are highly thermally labile compounds and are quickly degraded into other compounds. The compounds may represent hydroxycinnamic acids (ferulic acid and *p*-coumaric acid) or even benzoic acid derivatives (vanillin and *p*-hydroxybenzaldehyde)[6]. These compounds do, however, exhibit higher antioxidant activities, which is discussed in the following chapter.

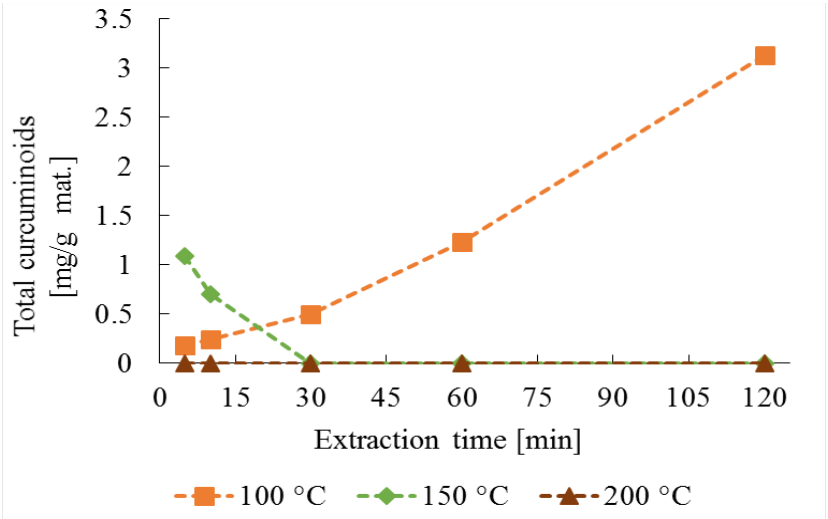


Figure 2. Total curcuminoids content in curcuma extracts obtained with subcritical water extraction.

**Table 1** presents the antioxidant activities of curcuma extracts obtained at the three different temperatures. From the table it can be observed that the antioxidant activity of curcuma extracts at 100 °C is not affected by extraction time and remains more or less constant throughout the whole extraction time period. At 150 °C, however, the antioxidant activity increases with increasing extraction time. At 200 °C the rate of increase of antioxidant activity is even higher compared to the rate at 150 °C. This indicates that indeed the degradation of curcuminoids results into compounds which exhibit higher antioxidant activities.

Table 1. Antioxidant activities of curcuma extracts.

| Extraction time | % inhibition | | |
|---|---|---|---|
| [min] | 100 °C | 150 °C | 200 °C |
| 5 | 2.95 | 1.36 | 4.02 |
| 10 | 3.05 | 1.63 | 3.76 |
| 30 | 3.15 | 2.75 | 12.18 |
| 60 | 2.90 | 4.40 | 17.27 |
| 120 | 3.11 | 4.55 | 21.62 |

## CONCLUSION

In this study, subcritical water is proposed as an extraction medium for the isolation of bioactive curcuminoids from curcuma. It was observed that in the range from 100 to 200°C extraction yield increases with increasing temperature, however at the highest temperature the yield starts to decrease with increasing extraction time.This indicates, that at 200°C the extracted compounds probably start to degrade with prolonged extraction time to volatile or solid compounds not soluble in water at atmospheric conditions, so they are removed in the separation step.

Furthermore it was observed, that curcuminoids are highly thermally labile compounds. The highest amount of curcuminoids was extracted at the lowest temperature 100 °C, while at temperatures of 150 and 200 °C all curcuminoids were degraded to hydroxycinnamic acids as *p*-coumaric and ferulic acids. These compounds did, however, exhibit much higher antioxidant activities.

It should be emphasized, that previous investigations[4] showed huge improvements in the extraction yields of curcuminoids when the pH was adjusted to a very low value. Under very acidic conditions, curcumin is probably protonated and exhibits greatly increased water solubility[4]. Future research will therefore be focused on the influence of the process conditions as temperature, pH and extraction time on the degradation mechanisms of curcuminoids in subcritical water.

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# MONOSACCHARIDES DETERMINATION IN *Lepidium meyenii* capsules BY GC-MS

**ORIGINAL SCIENTIFIC PAPER**

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**ABSTRACT**
A GC-MS method for separation and quantification of monosaccharides fructose and glucose in commercially available *Lepidium meyenii* (maca) samples was developed. The method with a formation of trimethylsilyl derivatives using N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) as a derivatization reagent enabled good separation and quantitative determination of fructose and glucose. Quantitative analysis of monosaccharides in the maca sample was carried out after solid-liquid extraction using water or methanol as an extraction solvent (0.5 g of sample/10 mL of solvent) at various temperatures (40 °C, 50 °C, 70 °C and 100 °C) performed at two different time intervals (1h and 3h). The contents of fructose and glucose were determined from calibration curves using phenyl-β-D-glucopyranoside as an internal standard. The total contents of investigated monosaccharides (free and bounded) were determined after the acid hydrolysis of the samples using an aqueous solution of HCl (concentrations 0.1 M, 0.5 M and 1 M; 0.5 g of sample/10 mL of solvent) at two temperature settings (50°C and 70°C) and two different time intervals (1h and 3 h). The highest concentrations of investigated compounds were confirmed in the maca sample hydrolyzed with 0.5 M HCl and heated for 3 hours at 70°C. The average concentrations of fructose and glucose were 224 mg/g and 303 mg/g of dry weight sample, respectively. It was also detected that the maca samples contained larger quantities, of sucrose, but it was not quantitatively determined.
**Keywords:** maca, monosaccharides, gas chromatography, mass spectrometry

## INTRODUCTION

Maca (*Lepidium meyenii*), a Peruvian plant of the Brassicaceae family, has been used in food and traditional medicine for a long time.[1,2] Recently, it has been widely used as a food supplement in the form of dry powdered roots, mostly, because of its potential positive effects on physical and sexual activity.[3] Maca, in addition, showed anti-proliferative functions and can slow down the prostate weight increase induced by testosterone treatment.[4] Maca roots contain several secondary metabolites including fatty acids esters, phytosterols, alkaloids and alkamides (macamides).[5] It has also been proved that maca roots contain between 59% and 76% of carbohydrates.[6] The most useful solvents for extraction of carbohydrates from maca samples are water, methanol and ethanol.[7,8] From the scientific literature it is obvious that the most common used techniques for the determination of monosaccharides are high-performance liquid chromatography (HPLC) with evaporative light-scattering detector (ELSD)[9] or refractive index detector (RI)[10], gas chromatography (GC)[11,12], or capillary electrophoresis[13]. Detection by UV-VIS, as commonly used in HPLC, is not possible in sugar analysis, as they are not absorbing components. Sample preparation for GC analysis involves the derivatization of sugars with special reagents to transform them into more volatile compounds.[14,15]. Because of the longer sample preparation process, using GC-MS in quantification of monosaccharides is relatively limited for routine analysis, but in comparison with other methods mentioned, GC-MS offers several advantages, including high-resolution separation, sensitive detection, unambiguous identification and quantitation of all sugar isomers.

The aim of this study was to determine the content of the two selected monosaccharides (glucose and fructose) in maca capsules, available in the Slovenian market. Selected monosaccharides were

identified and quantified by gas chromatography coupled to mass spectrometry (GC-MS). For the determination of free monosaccharides, extractions with methanol and water were used, while for the determination of total (free and bounded) monosaccharides, samples were hydrolyzed with aqueous hydrochloric acid (0.1 M, 0.5 M and 1 M). The optimal extraction and hydrolysis conditions were determined.

## MATERIALS AND METHODS

### *Chemicals*

All reagents and solvents used were of analytical grade. The standard compounds: D-glucose (99%), as well as solvents: tetrahydrofuran (99.5%) and pyridine (99.9%) were supplied from Merck (Germany). Derivatization reagent, N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and D-fructose (99%) were supplied from Sigma (USA). Phenyl-β-D-glucopyranoside (97%) was supplied from Aldrich (USA). Methanol (99.8%) was by ChemLab (Belgium). Toluene (99.5%) and HCl (36.5%) were supplied from Carlo Erba (Italy).

### *Preparation of standard solutions for the calibration curves*

Standard stock solutions of fructose, glucose and phenyl-β-D-glucopyranoside (used as internal standard- ISTD) were prepared by accurately weighing 10 mg each of them into 10 ml volumetric flasks, and dissolving in methanol. Six calibration solutions in the concentration range 10-400 mg $L^{-1}$ were prepared by combining different volumes of the fructose and glucose standard stock solutions (10, 20, 30, 100, 200 and 400 µL) with 50 µL of ISTD in the conical, glass flasks. The solvent was evaporated in vacuum oven at 40 °C and pressure 2 mbar. The absolutely dry residues were derivatized according to the procedure described below.

### *Derivatization procedure*

Trimethylsilyl (TMS) derivatives of the compounds were prepared by adding 100 µL of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), 50 µL of pyridine (a basic catalyst for the reaction) and by heating for 90 minutes at 70-80 °C. After derivatization was finished, the samples were quantitatively transferred to the 1 ml flasks and made up to the mark with the toluene. Individual isomers were identified by comparing their spectral properties with those reported in the Willey and NIST mass spectra libraries or in literature. The quantities of glucose and fructose in the samples were determined from the corresponding calibration curves using ISTD and by summing the peak areas of individual isomers. The curves were constructed by linear regression of the peak area ratio of individual sugar to the ISTD (y) versus the concentration in mg $L^{-1}$ (x).

### *Preparation of maca samples*

Commercially available maca capsules produced by PlantBIO were used for analysis. For each analysis homogenized sample made from the contents of twenty maca capsules was used for the further work. Each analysis was performed in duplicates. The aim of the experimental work was to evaluate the effect of temperature, time and the solvent on the content of free monosaccharides in the final extracts. As the extraction solvents methanol and water were used (0.5 g sample/10 ml solvent). Extractions were carried out in glass flasks equipped with a condenser in water bath by heating at 40 °C and 70 °C for methanol, and at 50 °C and 100 °C for deionized water at two different time intervals (1h and 3h). After the extractions were finished, the extracts were filtered through PTFE filter (I.D. 0.45-µm). 50 µL of the extract was spiked with 50 µL of ISTD (1000 mg $L^{-1}$), solvent was evaporated and the sample was further prepared by the procedure described in the chapter 1.3 and analyzed by GC-MS.

Additionally, quantification of the total (free and bounded form) monosaccharides contents in the samples was performed. For this reason, glycoside bounded monosaccharides (di-, oligo- and polysaccharides) were determined after the cleavage of the bonds by acid hydrolysis. For

this purpose, 0.5 g of dry homogenized maca sample was weighed in the round-bottomed flask, 10 mL of an aqueous solution of HCl (0.1, 0.5 and 1 M) was added and the sample was heated in a water bath (at 50 °C and 70 °C, for 1h and 3h). After the extraction was finished, the extract was filtered through the PTFE filter. 50 µl of the extract was spiked with 50 µL ISTD, the solvent was evaporated and the sample was derivatized and analyzed by GC-MS.

### *Instrumentation and GC–MS conditions*

The analysis was performed on a Varian 3900 gas chromatograph (GC), coupled to Varian Saturn 2100T ion trap mass spectrometer (MS). GC separation was performed using a Varian nonpolar capillary column VF-5ms CP8944 (30 m×0.25 mm, with the 0.25 µm film thickness). 1 µL of the sample was injected in split mode (split ratio 1:10). Carrier gas was He (5.0 UHP) at 1.0 mL $min^{-1}$ flow rate. The temperature program of the column was as follows: 1 min at 40 °C, 10 °C $min^{-1}$ to 300 °C, and finally, fixed for 5 min at 300 °C. The total run time was 32 min. The injection-port and transfer-line were set to 250 °C and 170 °C, respectively. The mass spectrometer recorded the entire spectrum (SCAN mode) in range from 50 to 650 *m/z*, using electronic ionization energy at 70 eV.

### *Validation of developed GC-MS method*

The method was validated for linearity, limit of detection (LOD) and limit of quantitation (LOQ). For linearity determination, all calibration curves were constructed using the internal standard method. The curves were fitted to linear least-squares regression. The limit of detection (LOD) was calculated using the equation $(3.3 + s_y)/b_1$ and the limit of quantitation (LOQ) was calculated from the equation $(10 + s_y)/b_1$ (where $s_y$ is standard deviation of linear regression and $b_1$ is slope of the calibration line).

## RESULTS AND DISCUSSIONS

Individual isomers of glucose and fructose (Figure 1) were identified by comparing their spectral properties with those reported in the Willey and NIST mass spectra libraries or in the literature.[16,17,18]

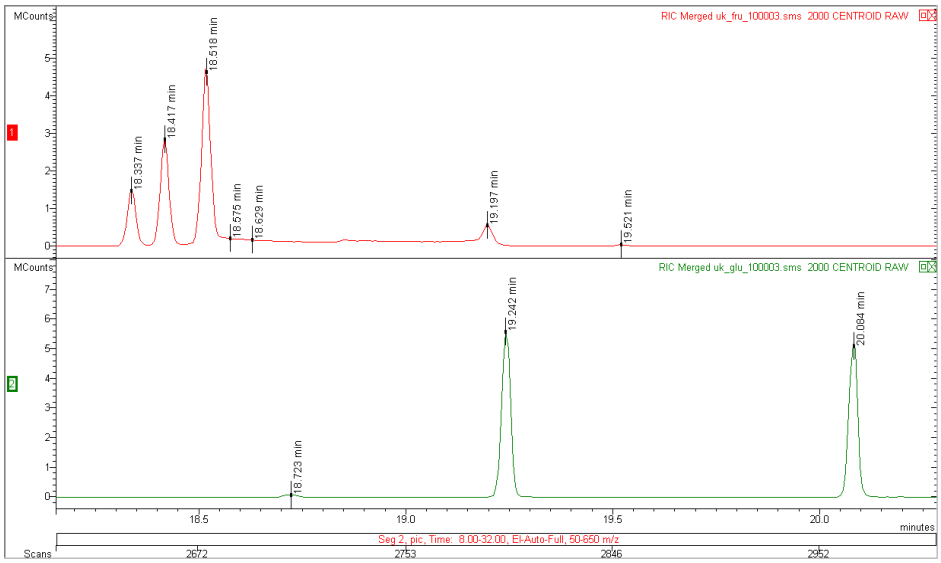


Figure 1. Chromatogram of fructose and glucose with its isomer derivatives.

Linear regression analysis proved that the responses for the both investigated monosaccharides were linear over the tested concentration range (10−400 mg $L^{-1}$), with correlation coefficients ($r^2$) above 0.999. The validation parameters are shown in Table 1.

Table 1. Validation parameters for investigated monosaccharides

| Monosaccharide | Linear correlation | $r^2$ | LOD (ppm) | LOQ (ppm) |
|---|---|---|---|---|
| Fructose | Y= 0.02x + 0.0756 | 0.9995 | 3.85 | 10.39 |
| Glucose | Y= 0.0232x + 0.0952 | 0.9992 | 4.50 | 10.14 |

By the extraction with methanol and deionized water, the aim was to determine the contents of free monosaccharides in maca samples, as well as to determine the optimal extraction conditions which will enable the maximum yields of the investigated compounds in the final extracts. The results are shown in Table 2.

Table 2. The contents of free monosaccharides in the maca samples obtained after different extraction conditions

| Solvent | **Methanol** | | | | **Water** | | | |
|---|---|---|---|---|---|---|---|---|
| Time | **1 h** | | **3 h** | | **1 h** | | **3 h** | |
| Temperature | 40 °C | 70 °C | 40 °C | 70 °C | 50 °C | 100 °C | 50 °C | 100 °C |
| *w*(fructose) [mg $g^{-1}$] | 21 | 30 | 25 | 32 | 34 | 31 | **33** | 33 |
| *w*(glucose) [mg $g^{-1}$] | 14 | 14 | 20 | 14 | 39 | 23 | **45** | 25 |

The results indicated that the extraction yields for free monosaccharides were higher when water was used as an extraction solvent in comparison with methanol, in all tested conditions. Longer extraction time, performed at the same temperature, did not significantly affect the concentrations of free monosaccharides in the final extracts. After the extraction using methanol, by heating the sample at the temperature of 70 °C, slightly higher concentrations of fructose were obtained. After the extraction with water and heating the samples at 100 °C, lower concentrations of both monosaccharides, particularly glucose, were obtained. It is known that the compounds like glucose and fructose are sensitive to higher temperatures, and they could be decomposed or transformed into other compounds[19], and the same fact can also be concluded from these results. The study showed that the most optimal conditions for the extraction of free monosaccharides in the maca samples are: extraction with water by heating the samples for 3h at 50 °C.

The additional aim of the study was to optimize the conditions for the extraction of total monosaccharides contents (free and bounded) from the samples. A reliable determination could be achieved after the cleavage of glycosidic bonds using the classical acid hydrolysis preformed under different conditions (different acid molarity, different temperatures and different time of hydrolysis). The results are shown in Table 3.

Table 3. The total contents of monosaccharides (free and bonded) performed under different hydrolysis conditions.

| Time of extraction | **1 h** | | **3 h** | |
|---|---|---|---|---|
| Temperature | **50 °C** | **70 °C** | **50 °C** | **70 °C** |
| *c*(HCl) | 0.1 M | | | |
| *w*(fructose) [mg $g^{-1}$] | 105 | 102 | 128 | 133 |
| *w*(glucose) [mg $g^{-1}$] | 110 | 116 | 142 | 147 |
| *c*(HCl) | 0.5 M | | | |
| *w*(fructose) [mg $g^{-1}$] | 98 | 105 | 120 | **141** |
| *w*(glucose) [mg $g^{-1}$] | 113 | 171 | 135 | **303** |
| *c*(HCl) | 1 M | | | |
| *w*(fructose) [mg $g^{-1}$] | 88 | 91 | 99 | 135 |
| *w*(glucose) [mg $g^{-1}$] | 129 | 139 | 157 | 241 |

The highest extraction yields for both of investigated monosaccharides were obtained after the hydrolysis using 0.5 M HCl, performed at 70 °C for 3 h. Amount of extracted glucose after hydrolysis at 70 °C during 3h with 0.1M, 0.5M and 1M HCl were 147, 303 and 241 mg/g respectively. It seems that using such conditions molar concentration of HCl had significantly affect the amount of extracted glucose. Some examples of GC chromatograms of the maca extracts obtained after hydrolysis (under different conditions) are shown in Figure 2.

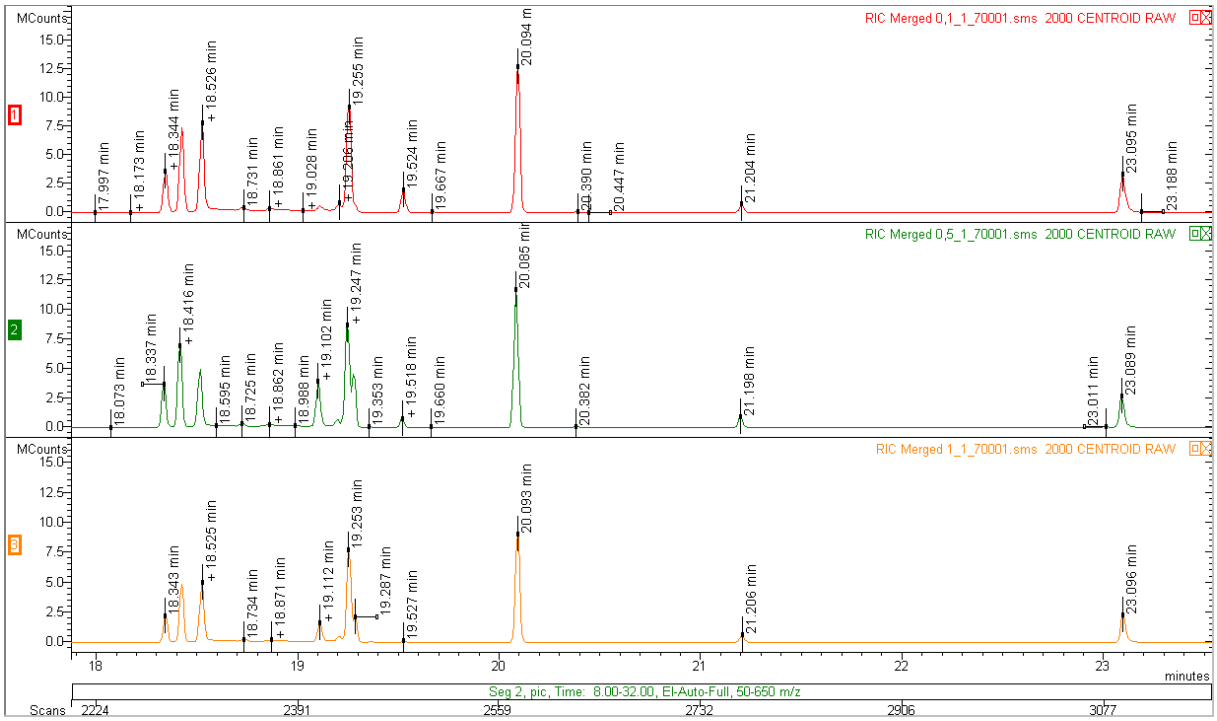


Figure 2. Examples of the chromatograms of maca extract after hydrolysis.

## CONCLUSION

The developed GC-MS method allows determination of selected monosaccharides, fructose and glucose, in the maca samples. The extraction procedure was optimized according to the extraction yields from the aspect of extraction solvent, temperature and extraction time.

The highest concentrations of free fructose and glucose were determined in the maca sample extracted with deionized water by heating for 3h at 50 °C. The average contents of free fructose and glucose in sample were 33 mg $g^{-1}$ and 45 mg $g^{-1}$ of dry weight, respectively. The total contents of monosaccharides (free and bounded) were determined after acid hydrolysis using HCl at different molar concentrations, different temperatures and different extraction times. The highest extraction yields for both of investigated monosaccharides were obtained after the hydrolysis using 0.5 M HCl, performed at 70 °C for 3 h. The average content of fructose in the samples was 224 mg $g^{-1}$ and the average content of glucose was 303 mg $g^{-1}$ of dry weight.

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# THE EFFECT OF THE EXTRACTION TECHNIQUES ON THE YIELD, KINETICS AND TOTAL PHENOLIC AND FLAVONOIDS CONTENT OF AQUEOUS-METHANOLIC EXTRACTS FROM NETTLE roots (*Urtica dioica* L.)

**ORIGINAL SCIENTIFIC PAPER**

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**ABSTRACT**
The goal of this study was to define the optimal extraction technique for obtaining the maximum yields of the extractive matter, maximal total phenolic and total flavonoids content of nettle roots (*Urticae radix*) as well as to determine the parameters in the extraction kinetics equations. Five extraction techniques: maceration, reflux, Soxhlet, Tillepape and ultrasonic extraction, were used to obtained the extractive matter from nettle roots. The total phenolic and total flavonoid contents were determined according to the Folin-Ciocalteu method and by the complexation reaction with $AlCl_3$, respectively. Two kinetics models - model Ponomarev and a non-stationary diffusion model through the plant material were successfully used for modeling extraction process of extractive matter. The extract obtained by Soxhlet extraction contained the highest total extractive matter yield (16.22 g/100g of dry plant material), as well as total phenolic (497.25 mg GAE/g dry extract) and total flavonoids (11.42 mg RE/g dry extract). The use of ultrasound reduced the extraction time, as well as total phenolic and total flavonoids content. The results indicate that aqueous-methanolic extracts from the nettle root are natural products with potential application in the food and pharmaceutical industry.

**Key words:** extraction techniques; extraction kinetics; nettle root; total phenolic; total flavonoids.

## INTRODUCTION

Many plant species, either medicinal or spicy have diverse applications. Plant raw material could be used as a drug, food, spice *etc*. simultaneously, so it is hard to claim what use is the most important one. Medicinal, aromatic and spice plants are, on the other hand, industrial raw material and significant export product as well. Plant material has mostly been used in the medicinal, food, pharmaceutical and cosmetic industry. They have also been used in technological procedures for milk and meat processing, in the patisserie and bakery industry and in households[1].

Nettle (*Urtica dioica* L., *Urticaceae*) is a perennial herbaceous plant, native to Europe, Asia, North Africa and North America used in traditional medicine as a medicinal plant for centuries. According to the most recent performed studies it is effective in the daily diet as well as in phytotherapy. Nettle herb, root and leaves are most frequently used and studied plant parts. The herb is used as diuretic as well as in treatment of rheumatic condition and urinary tract, the root in treatment of benign prostate hyperplasia, and fresh freeze-dried leaves for treatment of allergies[2,3].

Nettle contains carotenoids, vitamins C, $B_2$, $B_5$, iron, calcium, magnesium, phosphorus, silicylic acid, formic acid and histamine. On the other hand, the presence of organic acids, amines (acetylcholine, betaine, histamine, serotonin), flavonoids, carotenoids, β-sitosterol, tannins, coumarins (scopoletin), glucokikin, vitamins

C and K have been demonstrated in nettle leaves[4]. Steroids, terpenoids, phenylpropanoids, coumarins[5], polysaharides[6] and lectins[7] have been isolated from the netlle root. This plant part also contains β-sitosterol that regulates normal prostate function, so it is frequently used in tea mixtures for treatment of the enlarged prostate disease and urinary tract inflammations. Extracts from the nettle root are used in the treatment of benign prostate hyperplasia[8,9].

The main goal of this work was to define the optimal extraction technique for obtaining the maximum yields of the total extractive matter, as well as the maximum total phenolic and flavonoids content of aqueous-methanolic extracts from the nettle root.

## MATERIALS AND METHODS

### *Plant material*

Nettle root (*Urticae radix*) was purchased from Institute for Medicinal Plant Research "Dr. Josif Pančić", Belgrade, Serbia. The moisture content, determined by drying at 105 °C to a constant weight, was 8.78%. The plant material was milled in a laboratory disintegrator (laboratory electric mill "BRAUN AROMATIC KSM2"), just before extraction.

### *Reagents*

All solvents and reagents used in this investigation were of analytical grade. Methanol (Centrochem, Stara Pazova, Serbia), Folin-Ciocalteu's reagent, gallic acid, rutin, aluminum (III) chloride hexahydrate and potassium acetate (Sigma Chem. Company, St.Louis, USA).

### *Total extractive matter content in the plant material*

The measured quantity (10 g) of plant material was placed in the Soxhlet extraction apparatus and 200 ml of 50% V/V methanol was added to the receiving flask. The extraction was performed at boiling temperature for 360 minutes. At the end of the extraction, the solvent was evaporated on the rotary vacuum evaporator at 40 °C. The obtained extract was dried in the vacuum dryer at 40 °C till constant mass and the content of total extractive matter, TEM (dry extract), in the initial plant material ($q_0$) was calculated on the basis of dry residue content (16.90 g/100g dry plant material).

### *Maceration and reflux extraction*

Plant material (1.5 g) was extracted with 50% V/V methanol with solvomodule (ratio of plant material : solvent) 1:20 m/V at 25 °C (maceration), 50°C and boiling point temperature (reflux extraction). Extraction kinetics of TEM was monitored at specified periods of time (5-120 minutes).

### *Soxhlet and Tillepape extraction*

The measured quantity (10 g) of plant material and 50% V/V methanol (200 ml) were put into the Soxhlet and Tillepape apparatus and extractions were performed for 240 minutes, with solvomodules 1:20 m/V. Extraction kinetics of TEM was monitored at specified time periods (15-240 minutes).

### *Ultrasonic extraction*

Plant material (1.5 g) was extracted with 50% V/V methanol with solvomodule 1:20 m/V in the presence of low-frequency ultrasound. Extraction was performed for 60 minutes using an ultrasonic bath (Sonic, Niš, Serbia; internal dimensions: 30´15´20 cm; total nominal power: 3´50 W; and frequency: 40 kHz) at 25 °C. Extraction kinetics of TEM was monitored at specified time periods (5-60 minutes).

Dry extracts from each of the extraction technique used were obtained using the same procedure as described in the section: *Total extractive matter content in the plant material* and stored at +4 °C for subsequent analysis.

### *Extraction kinetics*

Two kinetical models were used for modeling the extraction kinetics of TEM from the nettle

root: the model of Ponomarev[10] (Model A) and a non-stationary diffusion model through the plant material[11] (Model B). This models were used in our early investigation[12,13,14].

Model A: $$\frac{q_0 - q_i}{q_0} = b + k \cdot t \quad (1)$$

Model B: $$\frac{q_i}{q_0} = (1-b) \cdot e^{-kt}; \ \ln\frac{q_i}{q_0} = \ln(1-b) - k \cdot t \quad (2)$$

where: $q_0$ is the content of TEM in the initial plant material (g/100 g dry plant material); $q_i$ - content of TEM in the plant material after the period $t$ (g/100 g of dry plant material); $b$ - coefficient of the fast extraction period, $k$ - coefficient of the slow extraction period ($min^{-1}$); $t$ - extraction time (min)[12].

***Total phenolic content***

The total phenolic content was determined according to the Folin-Ciocalteu method (using gallic acid as a standard)[15], by previously described procedure[16]. The reaction mixture was prepared by mixing 1 ml of the methanolic solution of the extract, 9 ml of distilled water, 1 ml of Folin-Ciocalteu's reagent and 10 ml of 7% sodium carbonate. After the 90 minutes incubation at room temperature, the absorbance was determined spectrophotometrically at 765 nm. The total phenolic content was expressed as milligram GAE (galic acid equivalent) per gram dry extract[16].

***Total flavonoids content***

The total flavonoids content was determined according to the aluminum chloride colorimetric method[17] (using rutin as a standard), by previously described procedure[16]. Methanolic solution of the extract (2 ml) was mixed with 0.1 ml of 10% aluminium chloride hexahydrate, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. After the 40 minutes incubation at the room temperature, the absorbance of the reaction mixture was determined spectrophotometrically at 415 nm. Rutin was chosen as a standard and the total flavonoid content was expressed as milligram RE (rutin equivalent) per gram of dry extracts [16]. All experiments were carried out in three replications.

## RESULTS AND DISCUSSION

Figure 1 shows the effect of temperature (25 °C, 50 °C and boiling point) and extraction time on the yield of TEM from the nettle root. The yield of TEM was increased with the increase in temperature. The highest TEM yield (14.23 g/100 g dry plant material) was obtained on boiling point temperature for 120 minutes; therefore, the boiling point was accepted as the optimal extraction temperature. The yield of TEM of 13.05 and 13.50 g/100 g dry plant material was achieved on the extraction temperature of 25 and 50 °C, respectively. These results are probably conditioned by character of components which are present in the investigated plant material, *i.e.* by their highest solubility in 50% methanol V/V on higher temperature.

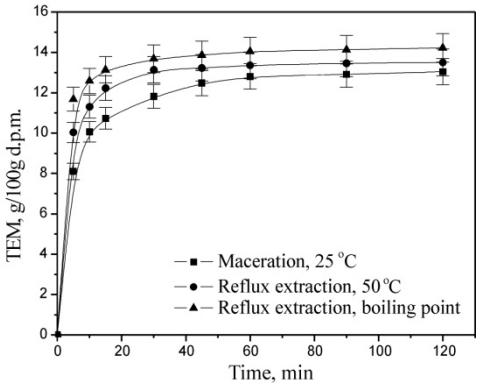


Figure 1. The variation of TEM yield from nettle root at various temperatures during maceration and reflux extraction

Figure 1 shows that there are two periods of the TEM yield increasing: a period of fast extraction and a period of slow extraction. The period of fast extraction is 30 minutes at 50 °C at the boiling temperature, or 45 minutes at 25 °C. The highest yield of TEM was realized in the fast extraction period (about 81% of the initial content in plant

material) at boiling temperature of the solvent. The yields are significantly less increased in the period of slow extraction. The dependence of TEM yield from the nettle root on Tillepape, Soxhlet and ultrasonic extraction time is shown in Figure 2. The figure shows that higher yields of TEM were obtained by circulation extraction techniques compared to reflux extraction at the boiling temperature. The yield of TEM obtained by Soxhlet extraction was 16.22 g/100 g of dry plant material while yield obtained by Tillepape extraction was 14.69 g/100 g of dry plant material for a period of 240 minutes, which is higher for about 12% and 3%, respectively, compared to the yield obtained by reflux extraction at the boiling temperature for extraction period of 120 minutes.

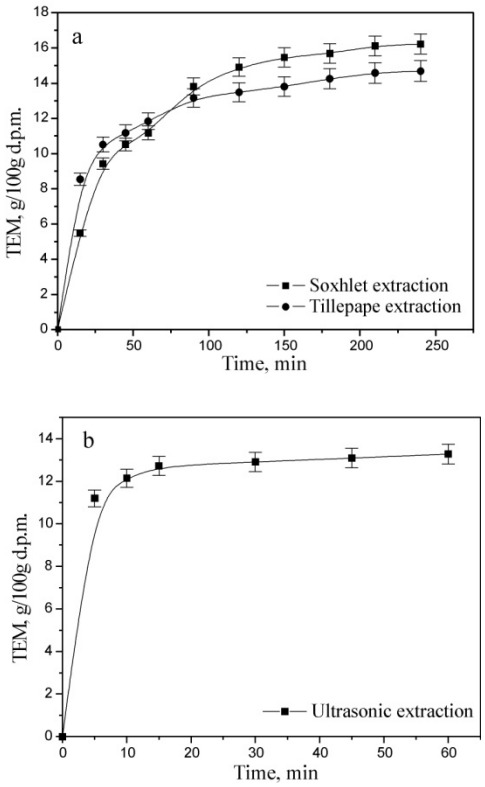


Figure 2. The variation of TEM yield during the Soxhlet, Tillepape (a)

and ultrasonic extraction (b)

The yield of TEM obtained by 60 minutes of ultrasonic extraction at room temperature was 13.28 g/100 g of dry plant material, which is 1.7% more than the yield obtained by classic maceration, for a period of 120 minutes, under the same other operating conditions. The effect of ultrasound has a positive effect on the rate of TEM extraction from the nettle root. This can be attributed to the rapid destruction of the cell walls by ultrasound, particle size reduction as well as better mass transfer of TEM from plant cells [18-21]. The most likely mechanism of ultrasound action is mass transfer intensification and easier penetration of the solvent into the cells of the plant material. Normal diffusion mechanism through cell walls is represented in the classic maceration, so this process requires a much longer extraction time[20].

### *Extraction kinetics*

Figures 3 and 4 show the kinetics of TEM from the nettle root by different extraction techniques by kinetics models A and B, respectively.

Extraction kinetics curves (Figure 3) are characteristic for extraction from cellular material with two period of extraction[10,12,14,22]. In the first period, fast extraction takes place *i.e.* washing extractive matter by solvent from the surface of destroyed cells. In the second period, slow molecular diffusion of extractive matter from inside of undestroyed cells takes place (slow extraction). The highest extraction level of TEM was obtained by Soxhlet extraction for a period of 240 minutes (Figure 3b).

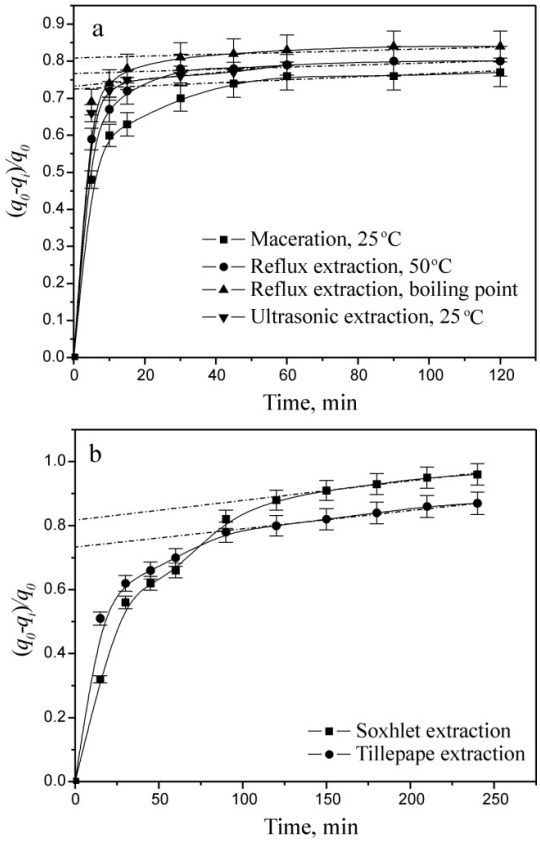


Figure 3. Extraction kinetics of nettle root TEM (Model A) (a - maceration, reflux extraction and ultrasonic extraction, b - Soxhlet and Tillepape extraction)

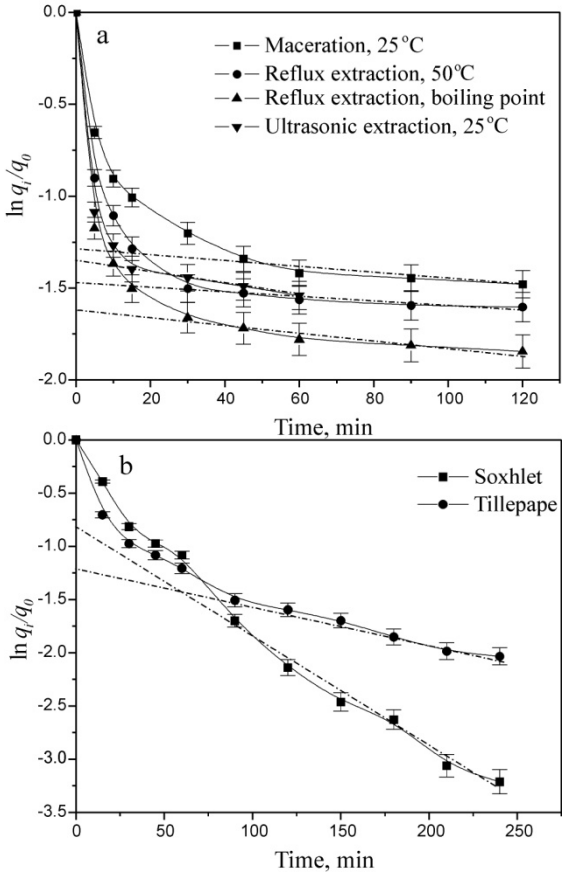


Figure 4. Extraction kinetics of nettle root TEM (Model B) (a - maceration, reflux extraction and ultrasonic extraction, b - Soxhlet and Tillepape extraction)

The values of coefficients *b* and *k*, fast extraction time (FET), extraction level of TEM in the period of fast extraction (EL, %) in the kinetics equations, by using different extraction techniques are presented in the Table 1.

Table 1. The fast extraction time (FET), the extraction level (EL) in the period of fast extraction and the values of *b* and *k* coefficients in the extraction kinetics

| Extracion technique | FET, min | EL, % | Model A | | Model B | |
|---|---|---|---|---|---|---|
| | | | *b* | $k\times10^4$, $min^{-1}$ | *b* | $k\times10^3$, $min^{-1}$ |
| Maceration | 45 | 74.0 | 0.73 | 3.28 | 0.73 | 1.63 |
| Reflux extraction, 50 °C | 30 | 78.0 | 0.77 | 2.59 | 0.77 | 1.14 |
| Reflux extraction, boiling point | 30 | 81.0 | 0.81 | 3.33 | 0.80 | 1.94 |
| Ultrasonic extraction | 15 | 75.0 | 0.74 | 8.67 | 0.74 | 3.76 |
| Tillepape extraction | 90 | 78.0 | 0.73 | 0.62 | 0.69 | 9.16 |
| Soxhlet extraction | 120 | 88.0 | 0.81 | 6.67 | 0.65 | 3.18 |

In the period of fast extraction, from 74 to 88% of TEM was extracted by rinsing and dissolving extractive matters from the surface of destroyed plant material cells (Table 4). These results show that the disintegration of nettle root used for the investigation is relatively high, and that a high level of destruction of cells increases the surface area from which the TEM is washed down and quickly dissolved during the fast period, thus providing a high degree of their extraction in this period.

The highest extraction level, calculated on the base of the maximum content extracted with 50% v/v methanol ($q_0$), in the fast extraction period was obtained by Soxhlet extraction (88.0%) for a period of 120 minutes. Extraction level of 75.0% for a period fast extraction of 15 minutes was obtained by ultrasonic extraction. This extraction technique achieves a slightly higher yield of TEM in three times a shorter period of fast extraction than the yield obtained by maceration (45 minutes) under the same operating conditions. This is probably due to easier solvent penetration into particles of plant material, mass transfer rate increasing as well as plant cells destruction under the influence of ultrasound. The effects of other factors cannot be seen easily. They are probably combined with the impact of extraction system mixing by ultrasound.

Modeling of the kinetics of TEM extraction from the nettle root was in accordance with extraction kinetics of bioactive substances from sage [23], St. John's worth[24], dill[13] and nettle leaves[14]. The coefficient $k$ values in kinetics extraction equations in a model of non-stationary diffusion (Model B) are higher than those of coefficient $k$ in model Ponomarev (Model A) kinetics extraction equations. Coefficients $b$ in kinetics equations of TEM extraction, by Ponomarev and non-stationary diffusion model, are slightly different except in case of Soxhlet extraction (Table 1). The obtained results suggested that both kinetics models can be used for modeling of TEM extraction from the nettle root by 50% methanol as solvent.

***Total phenolic and total flavonoids content in extracts from nettle root***

Total phenolic and total flavonoids content in aqueous-methanolic extracts from the nettle root obtained by different extraction techniques are presented in Table 2.

Table 2. Total phenolic and total flavonoids content in extracts from nettle roots obtained by different extraction techniques

| Extraction technique | Total phenolic, mg GAE/g d.e. | Total flavonoids, mg RE/g d.e. |
|---|---|---|
| Soxhlet extraction | 497.25±14.92 | 11.42±0.571 |
| Tillepape extraction | 474.05±14.22 | 11.07±0.55 |
| Reflux extraction, boiling point | 365.01±10.95 | 1.26±0.06 |
| Maceration | 313.97±9.42 | 1.12±0.06 |
| Ultrasonic extraction | 309.33±9.28 | 0.98±0.05 |

d.e. – dry extract

Total phenolic and total flavonoids content is higher in the extracts obtained by circulation extraction techniques compared to those obtained by maceration and ultrasonic extraction. The highest total phenolic and total flavonoids content was determined in the extract obtained by Soxhlet extraction. This is probably due to the solvent circulation flow until the complete depletion of plant material. Total phenolic and total flavonoids content in the extract obtained by ultrasonic extraction is lower for 37.8% and 92%, respectively in comparison to the extract obtained by Soxhlet extraction. This phenonomenon could be explained by degradation of phenolic and

flavonoid compounds under the influence of ultrasound[25]. Comparing to the results obtained for aqueous-ethanolic extracts from nettle leaves in the earlier study[14] it has been found that the total phenolic and total flavonoids content determined in the present study is lower. Otles and Yalcin[26] (2012) found lower total phenolic content in 80% methanolic extract from nettle root originated from Turkey comparing to our research results. Total phenolic content expressed by galic acid equivalents (GAE) can be divided into three ranges[27]: extracts with low (10 mg GAE/g of dry plant material), intermediate (10-20 mg GAE/g of dry plant material) and high (more than 40 mg GAE/g of dry plant material) total phenolic content. On the basis of the results obtained (Table 2) it could be concluded that aqueous-methanolic extracts from the nettle root possess high phenolic content but much lower total flavonoids content.

## CONCLUSIONS

The yield and extraction kinetics of the total extractive matter (TEM), as well as total phenolic and flavonoids content from the nettle root depend on the extraction technique applied. The highest TEM content, total phenolic and flavonoids content was obtained by Soxhlet extraction, during 240 minutes. The use of ultrasound reduced the extraction time, as well as total phenolic and total flavonoids content. It has been found that two models, model Ponomariev (model A) and non-stationary diffusion model (model B) could be applied for following extraction kinetics. Equation parameters for the extraction kinetics models applied to the TEM extraction by maceration, reflux, Tilepape, Soxhlet and ultrasonic extraction were determined. On the basis of the results obtained, it could be concluded that aqueous-methanolic extracts from the nettle root represent a natural source of phenols and flavonoids.

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# BIOMASS WASTE – A SOURCE OF RAW MATERIALS

**ORIGINAL SCIENTIFIC PAPER**

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**ABSTRACT**
Biomass represents an immense and renewable source for the production of bio-fuels and valuable chemicals. Using liquefaction reaction, lignocellulosic components are depolymerised to low molecular mass compounds with high reactivity, high hydroxyl group content and can be used in many useful applications. Liquefied biomass was used as a feedstock in polymer chemistry, such as synthesis of polyesters, polyurethane foams and adhesives. Herein the optimized procedure for rapid preparation of nanocrystalline cellulose is presented by liquefaction of its amorphous part of cellulose, lignin and hemicelluloses in ethylene glycol under acidic catalysis. A special attention was given to the utilization of the liquefied lignocellulosic materials as a new energy source with high heating value. The utilization of liquefied lignocellulosic materials can at least partially reduce the crude oil consumption, thus increasing the use of the renewable resources in large extent.
**Keywords:** Biomass liquefaction, Polyester, Adhesives, Biomass fuel, Nanocellulose

## INTRODUCTION

Biomass represents an immense and renewable source for the production of bio-fuels and valuable chemicals[1,2,3]. Agricultural crop residues, such as straw, corn stover and wood and wood wastes such as leftovers from timber cutting, broken furniture, sawdust, residues from paper mills etc. contain appreciable quantities of cellulose, hemicelluloses and lignin. New applications and methods for thermochemical conversion of biomass wastes into several new products have been developed by many research groups worldwide.

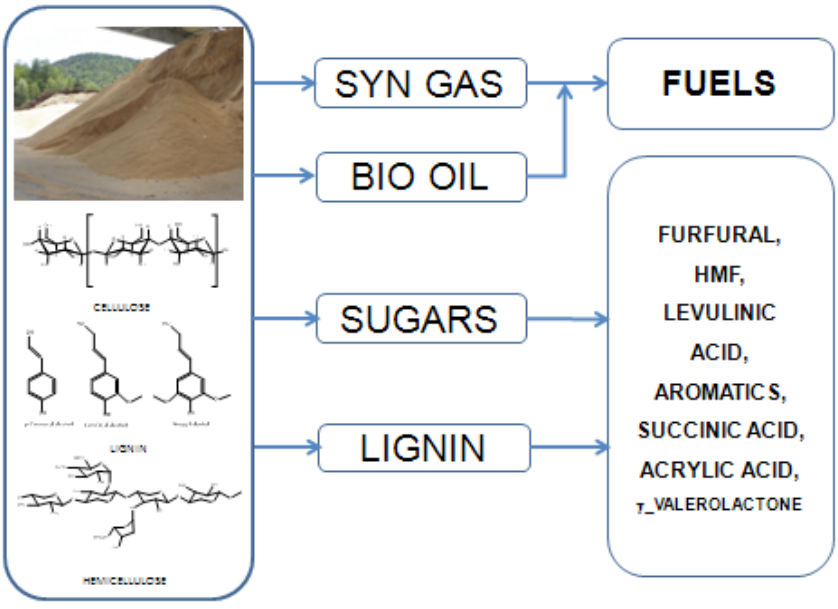


Figure 1. Chemical conversion of lignocellulosic biomass

One of the effective methods of chemical conversion of biomass into feedstock for polymers and fine chemicals is the liquefaction process with glycols and mild acid catalysis[4]. During liquefaction, lignocellulosic components are depolymerised to low molecular mass compounds with high reactivity, high hydroxyl group content and can be used in many useful applications.

Liquefied biomass has been used as a feedstock in polymer chemistry, such as synthesis of polyesters[5], polyurethane foams and adhesives[6]. Polyester polyols were prepared by using adipic acid and/or phthalic acid anhydride in a high temperature polycondensation - esterification reaction. The products were reacted with isocyanates to give a series of polyurethane foams that were comparable with commercial foams.

Cellulose represents the most abundant renewable polymer that is biodegradable and non-toxic. Nanocrystalline cellulose (NCC) has been realized as a new class of nanomaterials and has been the subject of a wide array of research efforts as reinforcing agent in nanocomposites[7]. The NCC has high strength due to its dense and ordered crystalline structure. The NCC is produced from cellulose by isolation of its crystalline regions. Typical procedure currently employed for the production of NCC consists of subjecting the pure cellulosic material to strong acid hydrolysis under strictly controlled conditions of temperature, agitation, and time[8,9]. The concentration of sulfuric acid for hydrolysis reaction is usually 65 wt%, however, the temperature can range from room temperature up to 70 °C and the corresponding hydrolysis time varies from 30 min to overnight, depending on the temperature.

Authors wish to present an overview of their research on liquefaction of biomass using glycol and acid catalyst. In this paper, the optimized method for the liquefaction of cellulosic biomass is described. The same procedure has been modified for rapid preparation of nanocrystalline cellulose by liquefaction of its amorphous part of cellulose, lignin and hemicelluloses in ethylene glycol under acidic catalysis[10].

## MATERIALS AND METHODS

### *Materials*

Lignocellulosic biomass samples were the spruce wood sawdust (provided by GGP Postojna, Slovenia), eucalyptus wood (*Eucalyptus Globulus*, from Agronelli Agroindustria, Uberaba, Brasil), Chinese silver grass (Miscanthus Sinensis, from the experimental plantation of Petrović U., Ljubljana, Slovenia). The reagents were supplied by Sigma-Aldrich (>98%, GC, Reagent Plus).

### *Biomass Liquefaction*

The liquefaction of wood was carried out in a 1000mL three-neck glass reactor, equipped with the mechanical stirrer and condenser. The reactor was charged with 100 g of dry biomass and 300 g of glycol (3:1). 9 g of p-toluenesulfonic acid or methane sulphonic acid was added. The proportion of the constituents in the reaction mixture was chosen after the initial trials[11]. The mixture was heated for 3 hours at 180 °C while being constantly stirred. A sample was withdrawn from the reaction system periodically and immersed in cold water to quench the reaction.

### *Polyester synthesis*

The liquefied wood was introduced into the four-necked 1000 $cm^3$ glass reactor, equipped with a water condenser and mechanical stirrer. The reactor was placed in an electric jacket heater. Adipic acid and/or phthalic acid anhydride were added when the liquefied wood reached 180 °C. Dibutyl tin oxide (0.2% w/w) was added as the esterification/transesterification catalyst. The mixture was heated gradually up to 200 °C, under stirring and was held at this temperature. Water was continuously distilled from the reaction system. A slight stream of nitrogen was introduced into the

reactor for easier transport of water vapour into a condenser. A sample was withdrawn periodically from the reaction system and its acid value was determined. The total reaction time was between 160 minutes and 180 minutes. After completion of the reaction, when the acid value was reduced to less than 30 mgKOH/g, the reaction mixture was cooled to ambient temperature.

***Nanocrystalline cellulose***

Lignocellulosic biomass was dispersed in glycol and methane sulfonic acid was used as a catalyst. The equipment used was the same configuration as for the liquefaction of biomass. The reaction was carried out at 150 °C for 180 minutes. The NCC was isolated as a residue, rinsed with 1,4-dioxane and centrifuged.

***Characterization***

The extent of liquefaction was evaluated by determining the residue after the washing out the sample with dioxane and water (4:1 v/v). The conversion yield was calculated as the weight percentage based on the starting wood material.

Size exclusion chromatography was performed on chromatographic system HP-Agilent, equipped with a UV detector, set at 280 nm. Analyses were carried out at 40 °C using 0.01 M DMAc/LiBr as the eluent at flow rate 0.7 ml/min.

Particle board mechanical properties were measured acccording to the appropriate European standards (EN). These concern thickness (EN 324), density (EN 323), swelling in thickness after immersion in water (EN 317), bending strength and modulus of elasticity (EN 310), internal bond strength (EN 319), surface soundness (EN 311) and the total formaldehyde content was determined using the Perforator extraction method, according to the European standard EN 120.

The NCC samples were characterized by wide-angle X-ray diffraction using a XPert PRO MPD diffractometer from PANalytical with a Cu anode as the X-ray source, at wavelength Cu Kα1: 1.5406 Å. The crystallinity index was calculated according to the Segal method[12] and the crystallite sizes were estimated by using well known Scherrer equation.

The average hydrodynamic diameter of the species in the aqueous NCC suspensions was determined by dynamic light scattering at 25 °C, using a Malvern Zetasizer Nano-ZS, (Malvern Instruments Ltd.).

The microtopographies of NCC samples were observed by SEM. The SEM micrographs were taken on a Zeiss Supra 35 VP scanning electron microscope. The same suspensions were applied to a glass substrate from which the acetone quickly evaporated. The dried glass support with its NCC particles on the surface was then coated with gold and used for SEM observations.

## RESULTS AND DISCUSSION

***Adhesives***

One of the main practical values of our research is the utilization of the liquefied lignocellulosic materials in adhesives for the wood particle boards, veneer boards and plywood boards.

We have proven that such adhesives emit less formaldehyde and products have the same or even better mechanical and physical properties. The results are presented in Table 1.

Table 1. Properties of laboratory prepared particle boards with regard to the pressing conditions (Meldur H-97 resin precursor and liquefied spruce wood 50/50).

| | | Pressing temperature | |
|---|---|---|---|
| | **Required values** | 180 °C | 160 °C |
| Board thickness EN 324 (mm) | **16.0 ± 0.3** | 16.1 | 16.1 |
| Density EN 323 (g/cm$^3$) | **-** | 0.68 | 0.60 |
| Internal bond strength EN 319 (N/mm$^2$) | **Above 0.35** | 0.40 | 0.39 |
| Surface soundness EN 311 (N/mm$^2$) | **Above 0.8** | 1.0 | 1.1 |
| Bending strength EN 310 (N/mm$^2$) | **Above 13** | 15.9 | 13.6 |
| Swelling in Thickness EN 317 (%) | **Below 15** | 14.6 | 15.8 |
| Formaldehyde release EN 120 (mg/100g board) | **Below 8** | 5.7 | 5.4 |

***Saturated polyesters and polyurethane foams***

Liquefied wood was used as a component in polyester synthesis due to the large number of hydroxyl groups that are available in the liquefied wood. The liquefied wood was used as a substitute for part of the polyhydroxy alcohols that are standard raw material in polyester formulation. Different polyesters were prepared using adipic acid and/or phthalic acid anhydride. The polyesters were prepared under the standard high temperature polycondensation conditions, confirming the use of the liquefied wood as a raw material in polyester synthesis. The hydroxyl value was reduced from 1043 mgKOH/g to values between 400 and 800 mgKOH/g. The average molar mass was icreased from 20000 Dalton to more than 40000 Dalton but viscosity was reduced from 118 Pa.s to 2 Pa.s. As a result of the utilization of liquefied wood in polyester-polyol synthesis, 22% to 23% of the polyhydric alcohols were replaced with wood.

These polyesters were used in polyurethane foam processing, as a polyol that reacts with polymeric diisocyanate[13]. Standard silicone surfactants and amine additives were used in all formulations. The mechanical testing proved that such polyurethane foams have compressive strength at 10% strain more than 72 kPa, tensile strength more than 127 kPa and thermal conductivity less than 0.036 $Wm^{-1}K^{-1}$. Such values meet the criteria for the isolation purposes.

***Liquefied biomass as a fuel***

The innovative biofuel obtained through solvolysis of spruce wood with glycols and p-toluene sulfonic acid catalyst was tested in an experimental turbine engine[14]. Results indicated that variations of primary air temperature, viscosity and combustion parameters influence the emission of CO, THC and $NO_x$. It was found that NOx emissions were lower than those when standard diesel fuel was used. All emissions and combustion efficiency can be optimized by selecting the proper combustion parameters, viscosity of fuel and the primary air temperature.

***Nanocrystalline cellulose***

Method for preparation of NCC by acid hydrolysis in ethylene glycol is a model procedure for NCC isolation from different natural cellulosic sources such as biomass with high yields and products with high crystallinity index. The yields depend on the percentage of cellulose present in the starting material. The results are summarized in Table 2, where yields, crystallinity index and crystal dimensions of nanocrystalline cellulose from different sources are presented.

| Biomass | NCC recovery (%) | Crystallinity index ($C\ I)_r$ | Average NCC crystal length (nm) | Average NCC crystal width (nm) |
|---|---|---|---|---|
| **Cotton linter** | 74.5 | 89% | 242 | 12.7 |
| **Spruce wood** | 61.5 | 68% | 235 | 6.3 |
| **Chinese silver grass** | 55.6 | 80% | 203 | 6.8 |
| **Eucalyptus wood** | 63.0 | 79% | 273 | 7.3 |

The product was a NCC suspension in 1,4-dioxane. The yields were lower when using biomass since they depended on the initial cellulose content in the particular raw material. The NCC was characterized by SEM microscopy, X-ray diffraction and $^{1}H$ and $^{13}C$ NMR spectroscopy. The average particle size was between 200 nm and 300 nm, with diameter from 5 nm to 20 nm. The method was also tested in the pilot plant reactor. The main advantage of this method is that NCC is in a form of a suspension in organic solvent, suitable for further derivatization and functionalization.

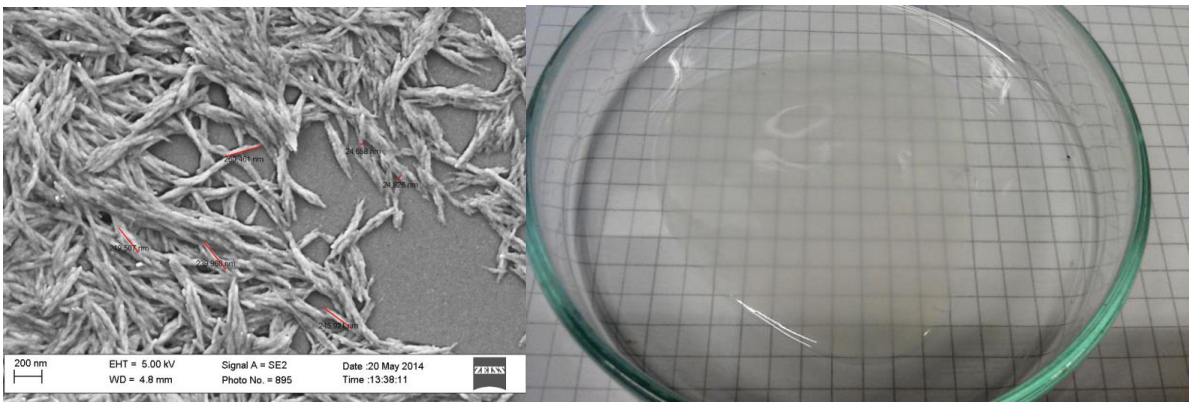


Figure 2. SEM micrograph of nanocrystalline cellulose (left) and suspension of nanocrystalline celulose in 1,4-Dioxane (right).

## CONCLUSIONS

Lignocellulosic biomass is a natural source of many valuable chemicals. The initial step needed is its depolymerisation and derivatization. The liquefaction reaction in glycols with mild acid catalysis was used for the liquefaction of different types of biomass. Adhesives, saturated polyesters and polyurethane foams were synthesized by using liquefied biomass as a feedstock. The properties of these products were within the range of commercially available materials. The same reaction was used for the isolation of nanocrystalline cellulose. The yields, crystallinity index and the dimensions of the nanocrystals were similar to those published in literature. A special attention was given to the utilization of the liquefied lignocellulosic materials as a new energy source with high heating value. Most of liquefied products have a heating value higher than 22 KJ/kg, that is in the range of pure ethanol and higher than brown coal. Initial tests have indicated that these products could also be used as a motor fuel. Since the production of such liquid fuel utilizes a huge variety of lignocellulosic wastes and takes place under very mild reaction conditions, an overall energy output is high. The utilization of liquefied lignocellulosic materials can at least partially reduce the crude oil consumption, thus increasing the use of the renewable resources in large extent.

**Acknowledgments**

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# INFLUENCE OF LIGNITE ON PHYSICO-CHEMICAL PROPERTIES OF THE SOIL AND ITS POTENTIAL USE AS A SOIL SUBSTRATE

**ORIGINAL SCIENTIFIC PAPER**

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**ABSTRACT**
Microstructure of lignite coal and its chemical properties, the ability to be linked to different organic and inorganic compounds in ionic and molecular form by physical and chemical forces of different strengths, makes lignite coal a very good substrate. Lignite is not fertilizer per se, but he may improve soil conditions as well as support plant nutrition, mainly due to high content of humic substances, especially humic acids. Since the input of main plant nutrients are mediated by humic substances, the growth of plants is directly or indirectly under the influence of these components. Humic substances effect stimulating plant growth by increasing intake of major plant nutrients, N, P and K. In addition, when appropriate humic substances are present in soil, they reduce the demand for NPK (nitrogen-phosphorous-kalium) fertilizers.In addition, when appropriate humic substances are present in soil, they reduce demand for the NPK fertilizer. In this study, the lignite mine "Šikulje" and mine "Kreka" of lignite ore was used as substrate in soil. Lignite used in experiments was shredder to granulation size of 0-5 mm and dried in an oven at 105°C. Investigations were conducted by growing strawberries at eight different combinations of soil in greenhouse experiment with the addition of certain amounts of lignite, manure and/or mineral fertilizer NPK 7:15:30, including the control plot of soil without any additive. It has been found that the addition of lignite in soil improve physico-chemical properties of soil (electrical conductivity, pH value, content of humic acids) increasing plant yield.

**Keywords**: soil, coal, humic acid, plant yield, fertilizer,strawberry.

## INTRODUCTION

Most of organic matter in coals is lignine, humic acid and humin, which holds all the physical and chemical properties of coal. In lignite humic acids prevail, substantially in the form of their salts, humates. Humic acids are high molecular organic acids with a relative molecular weight of 1000-30000 and molecule sizes 6-8 nm.[1] The structure of humic acid, as shown in Figure 1., contains three phenolic OH groups, quinone structures, nitrogen and oxygen as bridge units and COOH groups variously distributed on aromatic rings.[2]

Lignine and humic acids are typical colloids, so a substantial water content, swelling and distinct autooxidative properties of younger coals may be attributed to these contents. Humic acids are associated with the mineral fraction of soil, forming colloidal complexes humus-clay and humus-sludge. These units increase cohesive forces that cause attraction of clay components and very fine soil particles. Humate compounds may incorporate metals in chelate complexes, allowing their bioavailability.[3]

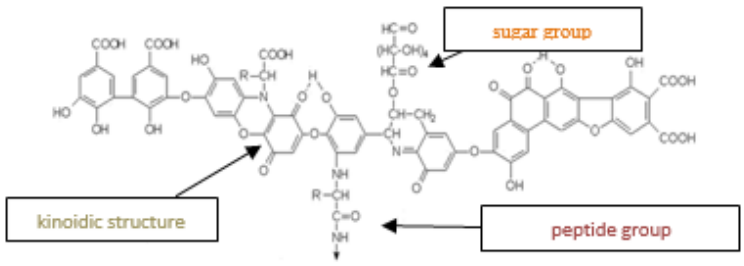


Figure 1. Model of humic acid structure[4]

Many of these humus components are heterogeneous, relatively large stable organic complexes[5]. Oxidation of coal proceeds in forming of humic acids, which penetrate into plant cells with polyphenol and kinoid groups, stimulating DNA-RNA system synthesis. In this way, humic acids increase the energy of plant organism.[6] Exchangeable cations affect the structure of soil, water and air regime, biological activity, the reaction of soil and formation of various types of soil. Cations linked to the colloidal particle of soil are protected from rinsing and they are available to plants. Cations that bind electrostatically can be easily exchanged with other cations from soil solution and thus be available to plants.[7] Exchangeable cations, with the other criteria of soil fertility may be an indicator of the soil quality and productivity.[8] Differences in cation exchange capacity, can be attributed to different contents of organic substances.[9] Early studies have shown that the addition of humic substances to soil changes its pH value. Soil being neutralized and many trace elements, which were in alkaline or acidic conditions attached to the ground and inaccessible, became available to plant roots. Since the input of main plant nutrients is mediated by humic substances, the growth of plants is directly and indirectly affected by humic substances. Molecules of humic acids influence rapid development of root system, formation of specific enzymes that increase the resistance of plants to negative stress factors (drought, frost), improve the assimilation of nitrogen (or inhibit forming of nitrates) and at the same time establish the synthesis of chlorophyll, sugar, vitamins, essential amino acids, fats, etc. Soil pH and availability of nutrients are the most important soil properties which are primarily determined by the type and amount of clay minerals and organic matter. Organic matter affects many chemical, biological and physical properties of soil.[10] Functioning of organic matter in soil depends on its quantity, macro-structure, and the ratio between organic matter and soil aggregates.[11] Fractions of organic matter affect biological, physical and chemical activities of soils.[12] Previous studies have shown a positive effect of lignite on the yield of different plants when it was applied with the appropriate amount of inorganic fertilizers, even in high quality soil.[13] It was shown that lignite contributed to the controlled intake of plant nutrients, most likely by forming organic-mineral complexes. In appropriate conditions, humic acids form a layer which protects the plant cells from the most negative impacts during plant growth and development, so that cells energy can mainly be used for its development. Coaly material called "biochar" showed a great efficiency during fertilization improving soil fertility and productivity. These "biochars" retain and make available to plants much more mineral nutrients than manure, compost and other fertilizers. The advantage of coal application is in the fact that it binds $CO_2$ in soil and thus reduces its emission into the atmosphere, preventing global warming of the planet.

Humic acid as one of the most important components of humic substances help in transferring micronutrients from soil to plants, improves water retention, increase seed germination and encourage the development of soil microbiological population.[14]

Complexing ability is partly attributed to the humic acid carboxyl and phenolic groups and in rare cases can be included amino groups.[15] Humic acids can act as electron shuttles and mediate biogeochemical cycles, thereby influencing the transformation of nutrients and environmental pollutants.[16] Research lignite of Tuzla basin, which are performed with the aim of exploring the possibilities of its application in agriculture, have shown that this lignite, thanks to the favorable physico-chemical properties, can be considered as a potential raw material for the production of humic fertilizers.[17]

## MATERIAL AND METHODS

Present study used lignite from ore mine ″Šikulje″ and lignite mine ″Kreka″, which was taken from the roof seam dug, fragmented and shredded to the granulation size of 0-15 mm. Lignite is further seeded in laboratory on granulation size 0 - 5 mm, and dried in oven at 105 °C. Experiments were performed by growing crop plants, strawberries (*Fragaria Vesca*, R., Frigo type seedlings material, Italy, EU, virus free). Investigations were carried out by growing this plant culture on eight various combinations of soil, lignite, manure and fertilizer NPK 7:15:30, including the control point, i.e. soil without any additive. Experiments were carried out with two repetitions. Investigations were conducted by growing strawberries at eight different combinations of soil in greenhouse experiment with the addition of certain amounts of lignite, manure and/or mineral fertilizer NPK 7:15:30, including the control plot of soil without any additive. The quantity of applied lignite, NPK fertilizer and manure in plots have been calculated on the basis of their recommended optimal amount for strawberry cultivation, according to the literature data.[18,19] Plots are presented in Table 1.[17]

Table 1. Lignite, NPK fertilizers and manure applied in soils of eight plots during cultivation of strawberries (Fragaria Vesca, R.)

| Plots number | Lignite (grounded) | NPK fertilizer 7:15:30 | Manure |
|---|---|---|---|
| **1 (control)** | - | - | - |
| **2** | 60 t/ha (10.2 kg) | 700 kg/ha (119 g) | - |
| **3** | 90 t/ha (15.3 kg) | 700 kg/ha (119 g) | - |
| **4** | 60 t/ha (10.2 kg) | - | 50 t/ha (6.8 kg) |
| **5** | 90 t/ha (15.3 kg) | - | 50 t/ha (6.8 kg) |
| **6** | 60 t/ha (10.2 kg) | - | - |
| **7** | - | - | 50 t/ha (6.8 kg) |
| **8** | - | 700 kg/ha (119 g) | - |

Results of the determination of elements in soil are classified in three cycles (I, II and III) which include the next periods of soil analysis:
I: before planting strawberries (control plot)
II: vegetation period (at rest seedlings) and
III: the period of the fruit forming (berry picking).

The study was conducted following the next methods of analysis:
1. Electrical conductivity of soil (according to standard EN 0000: 2003).
2. pH of soil in $H_2O$ (active acidity) and KCl (substitution acidity), electrochemicaly on pH-

meter with ion selective electrode in a suspension of soil.
3. The content of humic acid (total humic acid, free humic acid, easily soluble humate) in soil and lignite (*Shaminade* method with calcium acetate).
4. Cation exchange capacity of soil, CEC method with ammonium acetate.

Goal of this work was to determine the effect of humic acid on some physico-chemical characteristics of the soil and the plants yield.

## RESULTS AND DISCUSSION

### *Humic fractions in researched soils*

Figure 2. presents the results of determining the content of total humic acid (THA) as a mass percentage share in soils in three cycles of studies.

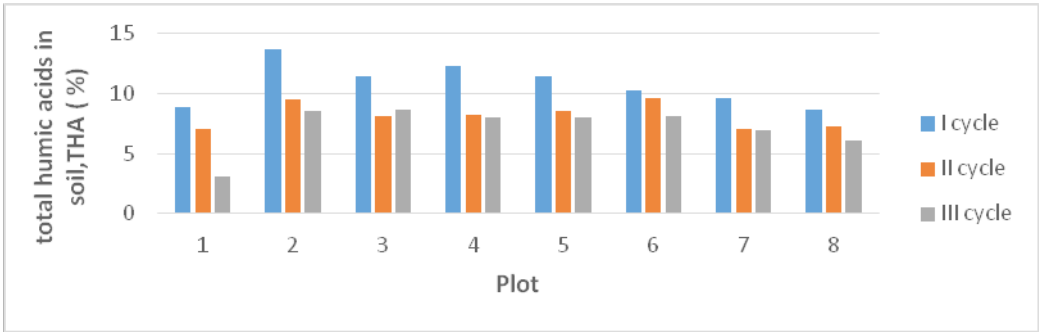


Figure 2. Content of total humic acids in soil

At the beginning of investigation, THA content in soils of certain plots decreases in the following order: 2> 4> 5> 3> 6> 7> 8>1. The results indicate a correlation between the content of total humic acids and the amount of applied lignite. In the second and third cycle of studies in all plots content of THA was reduced comparing to the initial phase. However, in the third cycle, the THA contents remained highest on plots where lignite was applied, alone or in various combinations with NPK and manure (3> 2> 6> 5> 4). The minimum content of THA in all three cycles were observed in soil sample from a control plot 1 (3.13%) where any additive was not applied. The results of ANOVA analysis show that there is a significant statistical difference on the level of significance of 0.05 between the amount of used coal and the total amount of humic acid (p=5.75E-4). Figure 3. presents the results of determination of free humic acids (FHA) as a weight percentage concentration in soils during three cycles of studies.

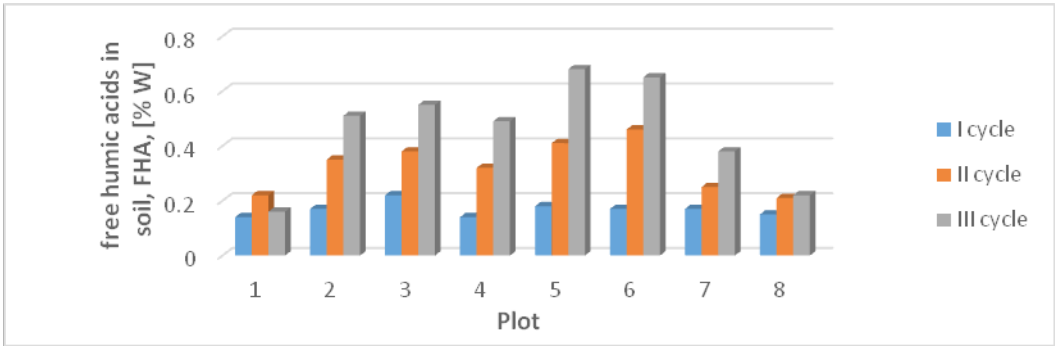


Figure 3.The content of free humic acid

The content of free humic acid (FHA) in the soil gradually increased in the second and third cycle in comparison with the beginning of the experiment, especially in plots treated with lignite. This can be only explained by the process of coal degradation and subsequent oxidation in soil. The highest content of FHA in the third cycle was in the plot 5 (0.68%) at which lignite

and manure were applied, then the plot 6 (0.65%) to which only lignite was applied and plot 3 (0.55%), where lignite and NPK fertilizer were applied.

The change of the FHA content in all plots was negatively correlated with the THA change (except in plot 1), i.e. decreased content of THA in soils increased the content of FHA. Decreased amount of FHA in the third cycle in the control plot is the result of a small amount of THA in the original soil. The results of ANOVA analysis show that there is a significant statistical difference on the level of significance of 0.05 between the amount of used coal and the free humic acid (p=5.12E-4). Figure 4. presents the results of determination of the content of easy soluble humate (ESH) as a weight percentage concentration in soils during three cycles of studies.

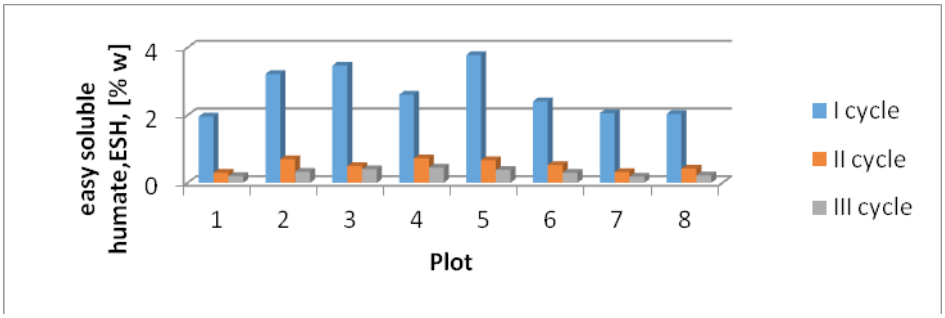


Figure 4. The content of easily soluble humates, ESH

At the begining of the study, the content of ESH in some plots decreased in the following order: 5> 3> 2> 4> 6> 7> 8>1. The highest content of ESH was measured in soils treated with large quantities of lignite (90 t/ha): 5 (3.78%) and 3 (3.47%) and the lowest in control plot. As might be expected, the mass of applied lignite directly determines the resulting amounts of humic fractions. In the treated soil samples in second and third cycle of studies, there was first a sudden, and then a gradual reduction in the content of ESH in comparison with their content in the first cycle.

The change of ESH in all three cycles was positively correlated with the change of THA in all plots and negatively correlated with the content of FHA. Humates play an important role in the growth and development of plants. Easy soluble humates are more available to plants, and the percentage of their contents in soils showed the highest raduction in plots 5 and 3 on which higher quantity of lignite was applied. However, ESH content in soils remained the highest in these plots after ripening of strawberries, i.e. applied lignite remained in soil even after the biological maturation of cultivated strawberries. The results of ANOVA analysis show that there is a significant statistical difference on the level of significance of 0.05 between the amount of used coal and the easily soluble humates (p=2,04E-10).

***The effect of lignite on soil pH***

Figure 5. presents the results of pH determination in soils and applied substrates, lignite and manure. Figure 6. presents the results of pH determinations of soils in certain plots.

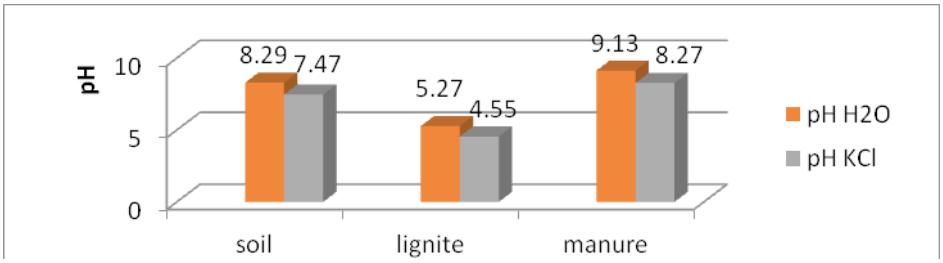


Figure 5. pH values in soils and applied substrates

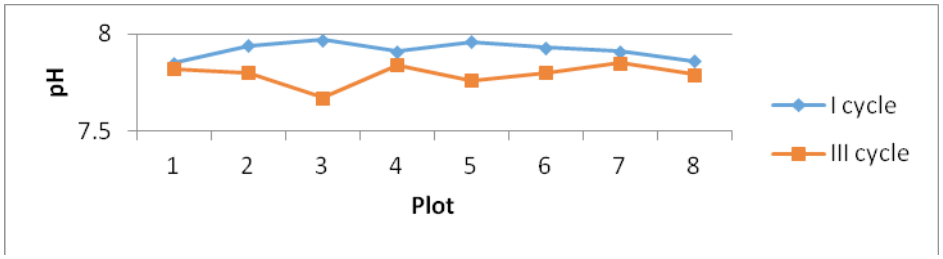


Figure 6. pH valueS of soil in the I and III cycle of determination.

At the end of one-year experiment, pH values of soils decreased, although it was still above 7 in all plots, i.e. ground remained nearly neutral. Comparing soil pH at the beginning and at the end of experiment, it is obvious that pH values were more decreased in plots 2, 3, 4, 5 and 6, where lignite was added (Δ pH = 0.13 to 0.3). It was expected because humus in lignite is acid (pH = 5.27). The changes of pH values were negligible in the control plot and in plots treated with manure or NPK, which ranged from 0.03 (plot 1) to 0.07 (plot 8). The results of ANOVA analysis show that there is a significant statistical difference on the level of significance of 0.05 between the amount of total humic acid and soil pH values (p=2.12E-4).

***The effect of lignite on soil electrical conductivity (EC)***

Value of soil electrical conductivity shows the ability of water in soil to carry electrical charge. This value is a good indicator of the quantity of available nutrients that plants may absorb. All macro- and micro-nutrients which are important for plants are in the form of cations or anions. Electrical conductivity and the amount of nutrients that plants can translocate depend on dissolved ions in soil water.

Figure 7. shows the results of electrical conductivity determination at the beginning and the end of experiment.

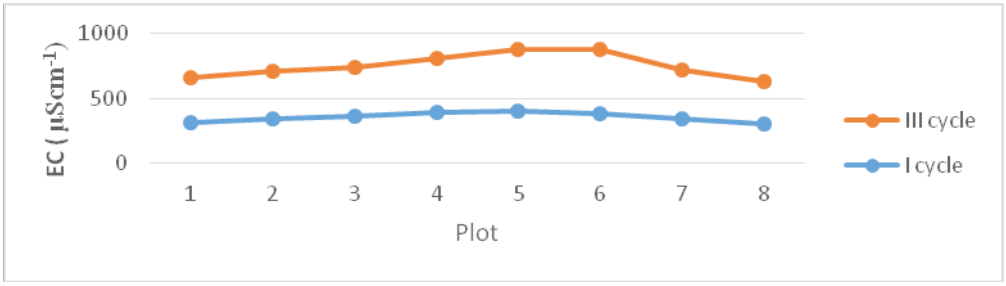


Figure 7. EC value in the I and III cycle of determination

It can be seen from the results that EC values at the end of experiment increased. The maximum EC value was observed in the soil sample from plot 6 (496 $\mu Scm^{-1}$), i.e. in plot where only lignite (60 $m^3$/ha) was added. Also, a high EC value was recorded in plots 5, 4 and 3. Contribution of applied manure may be observed, which together with coal in plots 4 and 5 influenced the increase of these values. Most likely the reason is an increased content of ions, i.e. nutrients which originate from lignite and manure humus and organic substances. The results of this study are in accordance with literature data which show that the optimal EC values in soil are in range between 200 $\mu Scm^{-1}$ and 1200 $\mu Scm^{-1}$.[14]

The results of ANOVA analysis show that there is a significant statistical difference on the level of significance of 0.05 between the amount of total humic acid and soil EC values (p=3.05E-11).

**Effect of lignite on cation exchange capacity, CEC**

Figure 8. shows the results of determination of exchangeable cations (Ca, Mg, K, Na) in soils at the end of the experiment where the results of plot 1 can be used as a control point.

Figure 9. shows the results of determination of soil cation exchange capacity (CEC).

Mg content in soil (mg/kg) increased with increasing amounts of the applied lignite, in plot 3 (425.53) to which lignite (90t/ha) with NPK was applied, and plot 4 (423.33) at which the lignite (60t/ha) with manure was applied. Also, the high concentration of exchangeable Mg (415.8) was determined in plot 7, where only manure was added. Organic substances from lignite and manure in soil obviously affected Mg adsorption. The lowest contents of exchangeable magnesium were observed in plots without added substrates and no significant effects of organic matter (plots 1 and 8).

On contrary, the content of exchangeable potassium decreased with increasing amounts of applied lignite and the lowest content of K was in plots where lignite and manure were applied, with the greatest amount of organic matter and humic fractions.

Most likely, there was a large K translocation from soils in strawberries especially in plots with larger quantities of lignite or manure, because of strawberry necessities for this essential element.

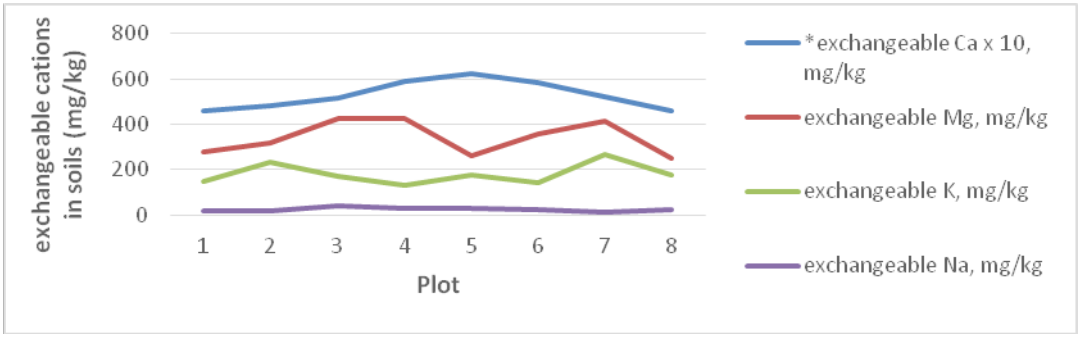


Figure 8.The content of exchangeable elements in soils (Ca * concentration x10 mg/kg)

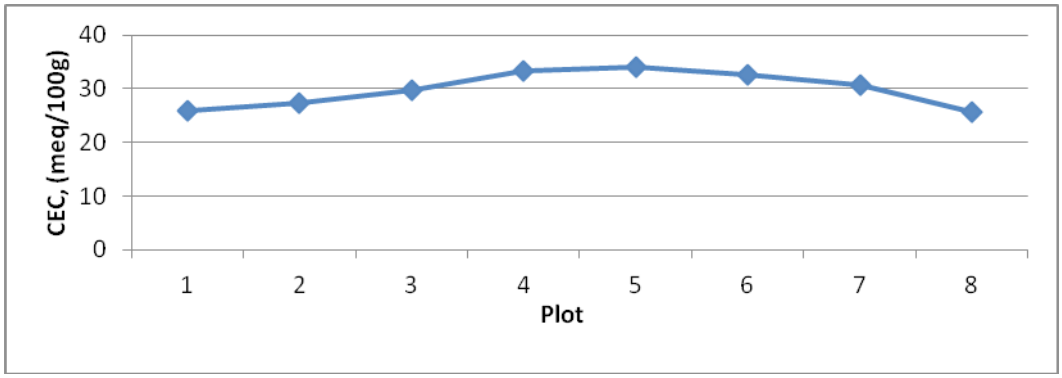


Figure 9. Cation exchange capacity (CEC) of soils in studied plots

The higher value of cation exchange capacity means that the soil has a higher capacity to retain cations. The results in Fig 9. show that the addition of lignite have positive effect on soil increasing CEC values. Maximum values of CEC (meq/100g) were recorded in plots with larger applied lignite: plot 5 (34.03), 4 (33.06), and 6 (32.61). Contribution of manure substrate to the higher CEC values was also evident. Comparing these values in plot 6 (32.61), only lignite added, with plot 7 (30.79), only manure added, a higher contribution of coal to CEC value is observed. The composition and morphological structure allows to lignite humic acids to retain nutrients in soil and react as a ″sponge″. The results of ANOVA analysis show that there is a significant statistical difference on the level of significance of 0.05 between the amount of total humic acid and soil EC values (p=3.05E-11).

***Effect of lignite to fruit yield***

The results of different content of applied lignite to yield fruit strawberries are shown in Figure 10. The value of fruits yield in some plots decreased in following order: 3>5>2>4>7>6> 8>1. The results clearly show the increased yield of strawberries in all plots treated with lignite compared to the control plot 1 or plots 7 and 8, which were treated with manure or mineral NPK fertilizer.

The higher yields of strawberries were in plots 3 and 5 to which a greater amount of lignite (90 t/ha) was applied. Fruit yields in plots treated with combination of coal and mineral fertilizers (2 and 3) were higher than in plots 4 and 5 treated with combination of coal and organic fertilizer (manure). Comparing fruit yields in plot 4 (lignite and manure) and 6 (only lignite), it can be concluded that the impact of manure is limited and that the fruit yield depends on the amount of applied lignite. The results of ANOVA analysis show that there is a significant statistical difference on the level of significance of 0.05 between the amount of total humic acid and fruit yield (p=5.35E-11).

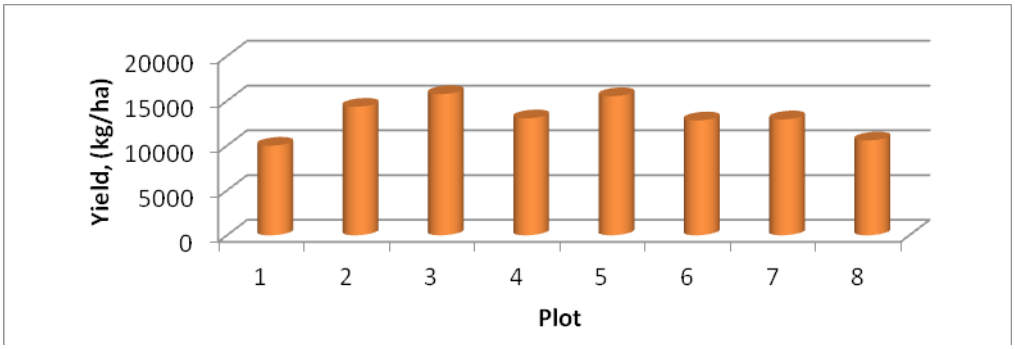


Figure 10. Strawberry fruit yield in the studied plots, kg/ha

## CONCLUSION

The use of small fraction of lignite as a substrate in soil, alone or in various combinations with manure and NPK fertilizer, show that lignite contributes to increasing content of total and free humic acids in soil. Lignite also contributes to increasing content of easy soluble humates that bind nutrients from soil insoluble complexes making them available to plants. Although humus in lignite is acid, soils treated with lignite are nearly neutral. The addition of lignite in soils also has a positive effect on EC and CEC contributing to these increased values. Humic fractions from lignite have a direct positive impact on strawberry yields.

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# THE TEMPERATURE PROFILE DURING COOKIE BAKING AS A FUNCTION OF SUGAR GRANULATION

**ORIGINAL SCIENTIFIC PAPER**

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**ABSTRACT**

The aim of this paper was to determine the effect of sugar granulation on temperature profile of cookies. Each formulation of cookies differ with respect to used sugar granulation (>1000 **µm,** 700-800 **µm and** <50µm) and baking temperature (180 °C, 205 °C i 230 °C). The temperature was measured with thermocouples type T. The texture of cookies was analysed using the texture analyser, and the colour was determined using a Chroma Meter (Konica Minolta Chroma Meter, CR-400). The changes in water content of cookies were also measured.

Results of monitoring the temperature inside the cookies during baking showed that the cookies with smaller sugar granulation in formulation achieved the temperature of water evaporation faster. Results of texture analysis showed that granulation of sugar do not significantly affect the parameters of hardness and fracturability. According to results of the colour determination, cookies with the addition of sugar granulation >1000 **µm** had lowest and cookies with the addition of powdered sugar (<50 **µm**) had the largest total colour change.

**Keywords:** cookies, sugar granulation, temperature profile, texture, colour

## INTRODUCTION

By definition, the biscuits and cookies related products, are products of certain nutritional and sensory properties, derived from grain mill products, fats, sugar, starch and other raw materials and additives, technological mixing, beatings, shaping, baking and other proceedings. Cookies, one of the most popular parts of the baking industry, are frequently consumed due to their nutritive value and long shelf life[1,2,3,4,5]. Baking is a complex process inducing physical, chemical and biochemical changes in the cereal matrix such as crust formation and colour changes[6]. During baking simultaneous heat and mass transfer occur due to elevated temperatures. Heat is transferred from the hot air to the product surface by convection and throughout the product by conduction while the moisture evaporates from the product. These processes influence on the qualitative parameters of cookies: colour formation[7,8] as well as textural properties – hardness and fracturability.

Product appearance plays a vital role in making a purchase decision. Among other, the colour is a significant factor that influences purchase decisions of bakery products. Browning at the surface of product is generally quantified by CIE $L*a*b*$ scale using either a colorimeter or computer vision based image analysis systems[9,10,11,12]. Browning is the final step of both the Maillard reaction and caramelization, one of the end-points of the baking process and the final result of sugar degradation during baking. The objective of this study was to investigate the influence of the sugar granulation on temperature profiles and quality

parameters (water content, colour formation and textural properties: hardness and fracturability) after cookie baking process.

## MATERIALS AND METHODS

### *Sample preparation*

Cookie dough was prepared from commercial cookie flour (Tena T550, Djakovo, Croatia), shortening (Zvijezda d. d., Zagreb, Croatia), sugar (different granulation: particle size >1000 μm, 700-800 μm and < 50 μm), kitchen salt (sodium chloride) and sodium bicarbonate from a local market, glucose and water. The cookie doughs were prepared by weighing the appropriate amount of each constituent in accordance with AACC Method 10-50D[13] and mixing the ingredients using an electronic mixer (Gorenje MMC800W, Slovenia) with a flat beater. First sugar, salt, sodium bicarbonate and shortening were mixed at low speed for 3 min (scrape down after each minute), then distilled water glucose solution was added and mixed at low speed for 1 min, scraped down and mixed for 1 min at medium speed. Finally, the flour was added and mixed at low speed for 2 min, scraped down after each 30 s. After mixing cookie dough rested in a refrigerator (8 °C) for 30 min. Dough was flattened and rolled by a rolling pin to the desired thickness (7 mm). At the end of the sample preparation process cookie dough was cut with a cookie cutter (60 mm inside diameter) and shaped cookies were placed at a baking surface (cold baking pane covered with baking paper). Dough leftover was discarded.

### *Cookie baking and temperature measurement*

Baking process was conducted in a convection-oven (Wiesheu Minimat Zibo, Wiesheu GmbH, Germany). A 36 gauge copper–constantan, type T thermocouple (diameter 0.0254 cm, Cole-Parmer, International, U.S.A.) was used to measured cookie temperature during baking and also to record the temperature profile inside the cookies during baking. The temperature of the hot air inside the oven was maintained at 180, 205 and 230 °C with the precision of ±1 °C.

Baking and cooling process was conducted on all samples (5 replications of each type) during 10 minutes of baking and 30 minutes of cooling.

The thermocouples were placed in the cookie dough by threading the wire through the cookie dough cylinder on central positions inside the dough cylinder. Position of the thermocouple was observed at the end of each baking process to ensure the final placement of the probe. During baking, samples were extracted from the oven every minute and used for further analysis. One cookie dough cylinder was baked with inserted thermocouple for monitoring the temperature profile during baking which was repeated 10 times (10 replications) and the collected data was analysed in accordance with statistic rule 3σ.

Thermocouples sampling rate was 1 second, and values were acquired with data acquisition software and hardware, PicoLog Recorder and PicoLog Player, connected to a PC.

### *Moisture determination*

Moisture analysis was preformed according to AACC Approved Methods[14] before, during (every minute during 10 minutes of baking) and after baking (after complete cooling at room temperature).

### *Qualitative parameters analysis*

Cookies colour measurement was performed by Minolta Chroma Meter (Konica Minolta, CR–400, Japan). Data was stored in CIE $L^*a^*b^*$ colour model and colour changes during baking process (10 minutes) were evaluated. The total colour difference ($\Delta E$), in CIE $L^*a^*b^*$ colour model was calculated by the equation[15,16]

$$\Delta E = \sqrt{\left(L_0^* - L^*\right)^2 + \left(a_0^* - a^*\right)^2 + \left(b_0^* - b^*\right)^2}$$

where $L_0^*$, $a_0^*$ and $b_0^*$ indicate colour parameters of raw cookies (dough), parameter $L^*$ refers to the lightness of the samples, and ranges from black ($L$=0) to white ($L$=100), a negative value of parameter $a^*$ indicates green and a positive one indicates red–purple colour, and positive value of parameter $b^*$ indicates yellow while blue indicates negative value. The Minolta CR-400 Chroma Meter D65 calibration plate was used for calibration. All measurements were repeated in five replicates.

Textural analysis was performed by TA.XT2i SMS Stable Micro Systems Texture Analyzer (Stable Microsystems Ltd., Surrey, England). Six cookies were evaluated using the three-point break technique. Quantified parameters were hardness (g) - peak fracturability force and fracturability (mm) - mean distance at break. Measurement was performed with the test speed of 3 mm/s and the distance between the two bottoms supports was adjusted to 50 mm. Textural analysis test was conducted three hours after baking.

### *Statistical data analysis*

Obtained experimental values were analysed by analysis of variance (ANOVA) and Fisher's least significant difference test (LSD), with significance defined at $p < 0.05$. Statistical analysis was carried out with Statistica ver. 12.0 Stat Soft Inc. Tulsa, OK, USA.

## RESULTS AND DISCUSSION

The Figures 1, 2 and 3 shows temperature profiles inside the cookies during the baking process (10 min) at 180, 205 and 230 °C, and during cooling to room temperature (30 min). Results are shown with respect to the granulation of sugar that is used in formulations. From the figures it can be seen that there was the same trend of rising temperatures during baking and fall of temperature during cooling cookies for all formulations. During the first 300 seconds of baking temperature in the center of the cookies rise to the evaporating temperature (100.2 to 102 °C) which remains constant during the process of evaporation of water. Thereafter again temperature rise as cookies continue to baked whereby a maximum temperature is in the range of 105 – 118 °C depending on the baking temperature and granulation of sugar. Evaporation temperature of water that is recorded in samples of cookies is higher in relation to the evaporation temperature of distilled water due to dissolved solids that are found in the free water with regard to formulation[17].

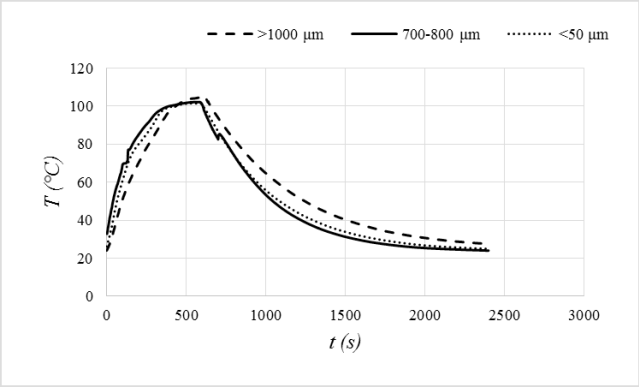


Figure 1. The temperature profile within the cookies during baking at 180 °C and cooling at room temperature regarding the sugar particle size

Based on statistical analysis and conducted Fisher's LSD test ($p < 0.5$) the obtained value of the temperature in the middle of the cookies differ significantly for cookies with size of sugar crystals over 1000 μm from the other two cookies (granulation sugar with crystal size 700-800 μm, and the powdered sugar with a particle size up to 50 μm) which do not differ significantly as shown in Figure 4. This difference is attributable to the fact that a larger sugar crystals dissolve more slowly and thus gradually creating spaces (cavities) that cause resistance to heat transfer. The cavities influence the slower achievement of the temperature of evaporation in relation to other formulation of cookies with smaller sugar granulation.

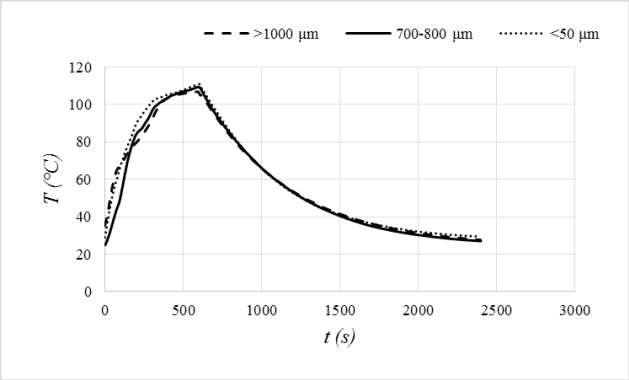


Figure 2. The temperature profile within the cookies during baking at 205 °C and cooling at room temperature regarding the sugar particle size

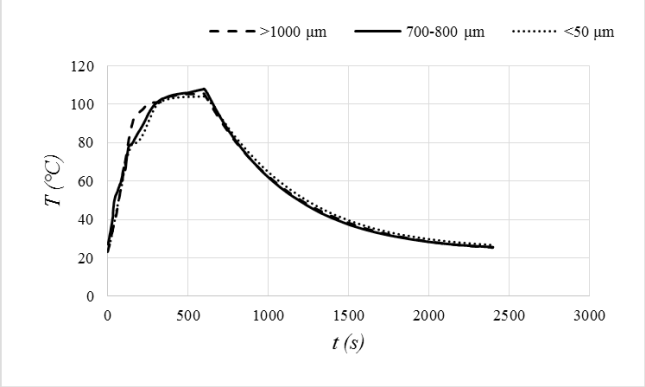


Figure 3. The temperature profile within the cookies during baking at 230 °C and cooling at room temperature regarding the sugar particle size

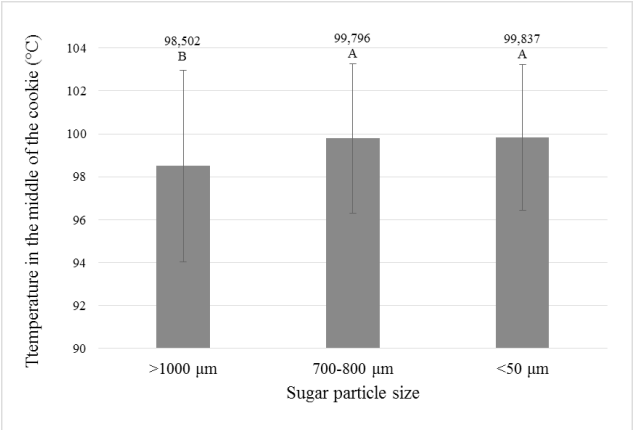


Figure 4. Temperature in the middle of the cookies from 5th to 7th minutes of baking depending on the sugar particle size regardless of the cooking temperature (the data are presented as mean ± standard deviation; the values marked with the same letter are not significantly different ($p < 0.5$) according to Fisher's least significant difference (LSD) test)

The statistical analysis and Fisher's LSD test ($p < 0.5$) showed that the obtained value of the temperature in the middle of the cookies is significantly different for cookies baked at 180 °C, regardless of the sugar granulation, compared to those baked at higher temperatures (205 and 230 °C) (Figure 5).

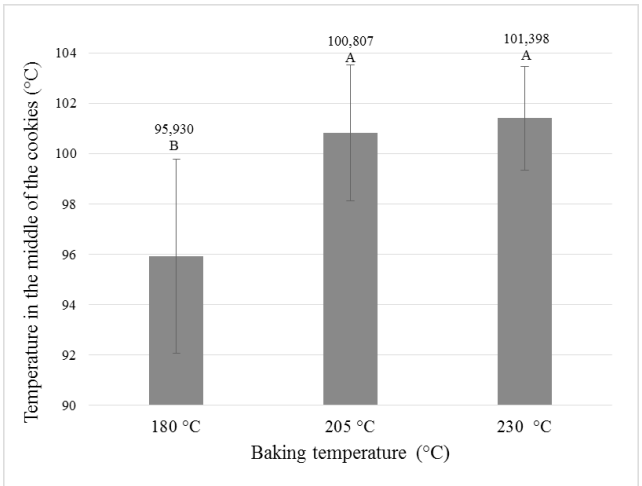


Figure 5. The results of measuring the temperature in the middle of the cookies from 5th to 7th minutes, depending on the given baking temperature regardless of sugar particle size (the data are presented as mean ± standard deviation; the values marked with the same letter are not significantly different ($p < 0.5$) according to Fisher's least significant difference (LSD) test)

The figures 6, 7 and 8 shows the results of water content in cookies depending on the sugar granulation and baking temperatures. It can be observed that intense water loss over time occurred between 5$^{th}$ and 7$^{th}$ minute followed by a period of establishing a uniform rate of water loss from the samples.

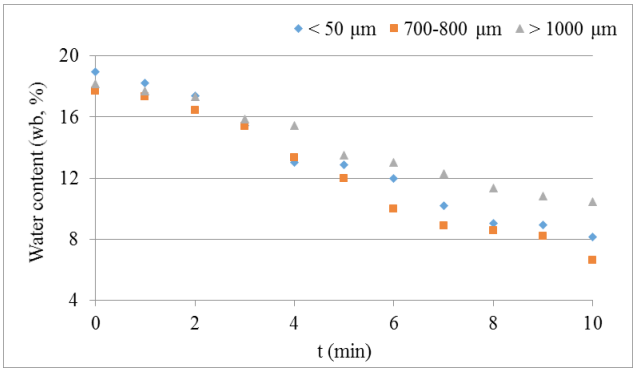


Figure 6. The water content of cookies with different sugar granulation versus time baked at 180 °C

Based on statistical analysis and conducted Fisher's LSD test ($p$ <0.5), it is evident (Figure 9) that the values obtained for water content significantly differ for cookie formulations with sugar granulation over 1000 µm in relation to cookies with sugar granulation of 700-800 µm and powdered sugar (particle size up to 50 µm) regardless of the baking temperature.

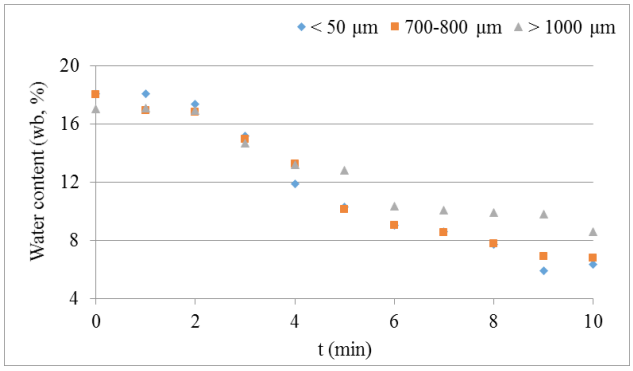


Figure 7. The water content of cookies with different sugar granulation versus time baked at 205 °C

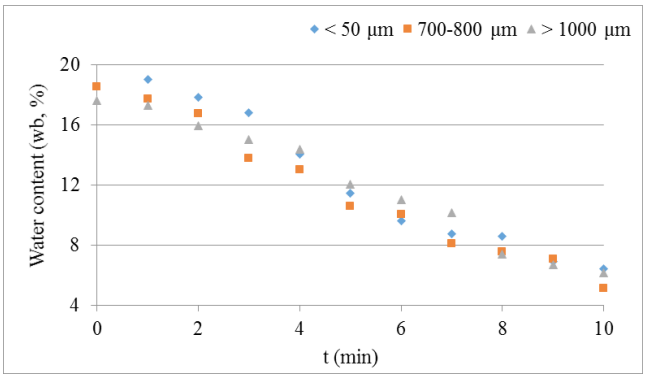


Figure 8. The water content of cookies with different sugar granulation versus time baked at 230 °C

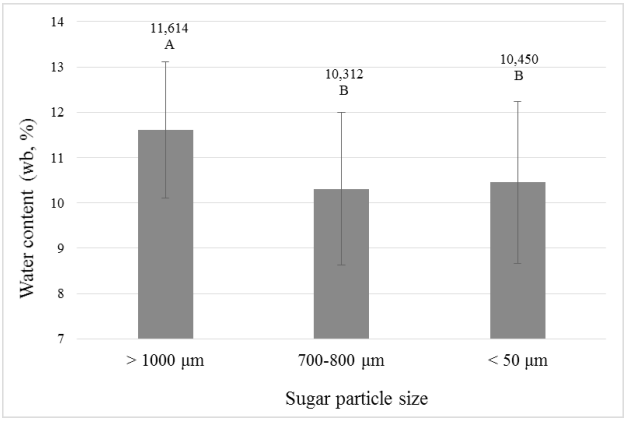


Figure 9. The water content of cookies from 5th to 7th minute of baking depending on the sugar particle size regardless of the cooking temperature (the data are presented as mean ± standard deviation; the values marked with the same letter are not significantly different ($p < 0.5$) according to Fisher's least significant difference (LSD) test).

From figure 10, based on statistical analysis and conducted Fisher's LSD test ($p < 0.5$), it is evident that the values obtained for water content are significantly different for cookies baked at a temperature of 180 °C compared to the cookies baked at temperatures of 205 and 230 °C, regardless of the granulation of sugar.

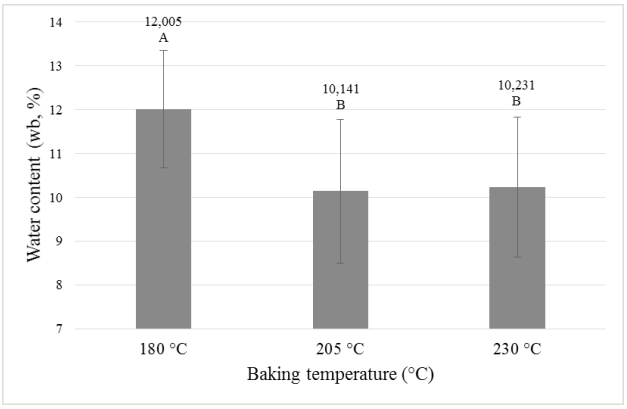


Figure 10. The water content of cookies from 5th to 7th minute of baking depending on the baking temperature regardless of the sugar particle size (the data are presented as mean ± standard deviation; the values marked with the same letter are not significantly different ($p < 0.5$) according to Fisher's least significant difference (LSD) test)

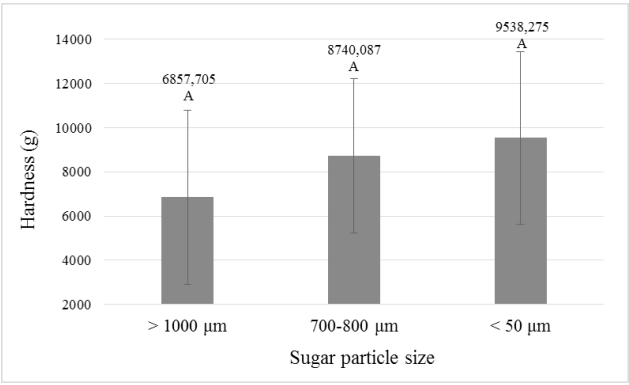


Figure 11. Cookie hardness depending on the sugar granulation regardless of the baking temperature (the data are presented as mean ± standard deviation; the values marked with the same letter are not significantly different ($p < 0.5$) according to Fisher's least significant difference (LSD) test)

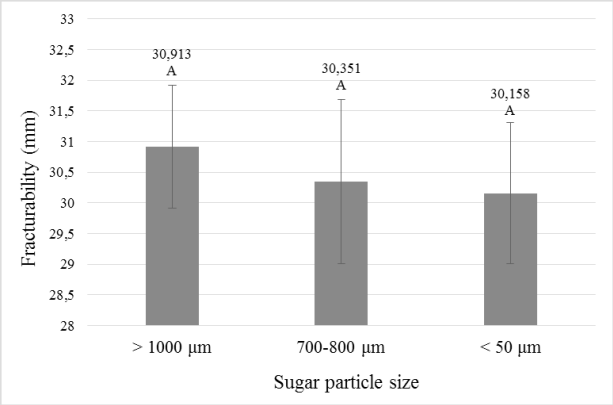


Figure 12. Cookie fracturability depending on the sugar granulation regardless of the baking temperature (the data are presented as mean ± standard deviation; the values marked with the same letter are not significantly different ($p < 0.5$) according to Fisher's least significant difference (LSD) test)

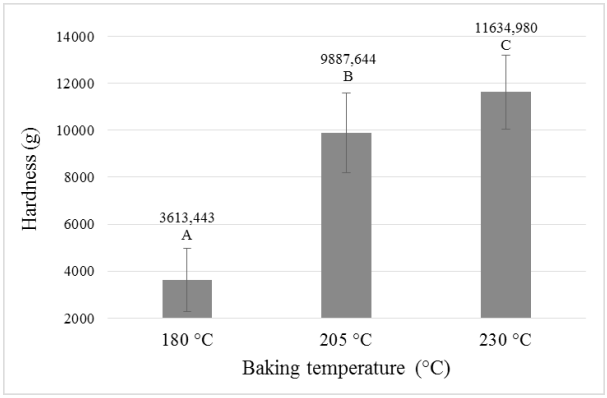


Figure 13. Cookie hardness depending on the baking temperatures regardless of the sugar granulation (the data are presented as mean ± standard deviation; the values marked with the same letter are not significantly different ($p < 0.5$) according to Fisher's least significant difference (LSD) test)

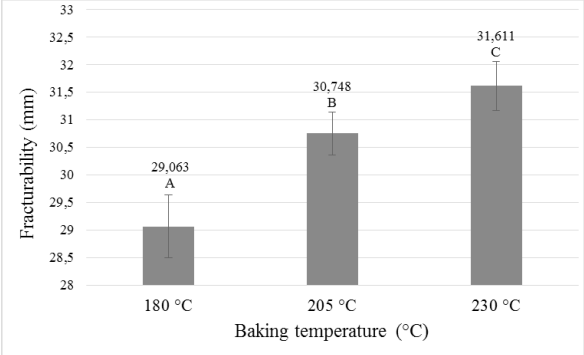


Figure 14. Cookie fracturability depending on the baking temperature regardless of the sugar granulation (the data are presented as mean ± standard deviation; the values marked with the same letter are not significantly different ($p < 0.5$) according to Fisher's least significant difference (LSD) test)

Figures 11 and 12 shows the results of determination of changes of hardness and fracturability of cookies considering the granulation of sugar and cooking temperature. There was no statistically significant difference in the hardness and fracturability of the studied cookie samples due to the sugar granulation according to Fisher's LSD test ($p$ <0.5) but it is evident that samples with highest sugar particle size had the lowest hardness. It can be explained by the fact that the large sugar particles needs more time to melt and during that time encourages spreading of cookies. During baking more of the sugar dissolves, which causes the dough to soften and spread.

In addition, according to the data shown in Figures 13 and 14, it is evident that there is a statistically significant difference ($p$ <0.5) in cookie hardness and fracturability due to the applied baking temperature.

Figure 15 shows the results of measurements of total colour change after 10 minutes of baking considering the granulation of sugar that was used in formulations. The results showed that increasing the baking temperature, increases the value of the overall color change for all the samples. The values of total color change in the 10$^{th}$ minute of baking considering sugar granulation, were in the range: from 14.05 to 24.29 for samples with powdered sugar, from 18.95 to 21.37 for samples of cookies with sugar granulation of 700-800 μm and from 9.56 to 20.83 in samples with sugar granulation larger than 1000 μm.

Samples with the largest sugar particle size (<1000 μm) showed the lowest colour change at all three baking temperatures that also can be explained with slow dissolving of large sugar particles which can led to delaying the browning caused by the caramelization and the Maillard reactions.

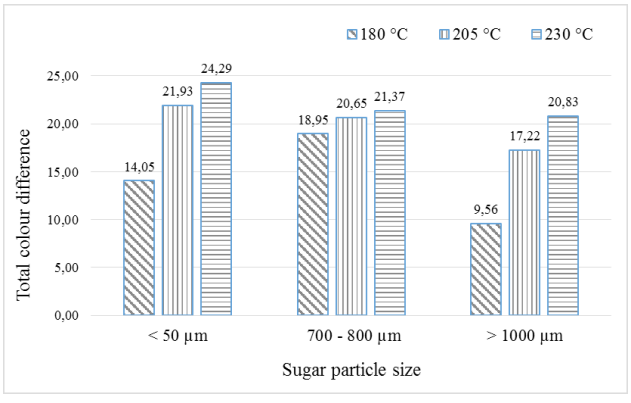


Figure 15. Total colour change of cookies after 10 minutes of baking at different temperatures

## CONCLUSIONS

Sugar particle size significantly affects cookie quality parameters. When cookies containing powdered sugar and sugar granulation of 700-800 μm in their formulations are baked, they achieve temperature at which the water evaporation occurs (cca 100 °C) between the 5$^{th}$ and 7$^{th}$ minute of baking but the cookies containing sugar granulation greater than 1000 μm needs to bake longer to achieve evaporation temperature. Baking temperature significantly affects the fracturability and hardness of cookies in a way that increasing the baking temperature increases the fracturability and hardness of cookies. Textural analyses of cookies showed that granulation of sugar does not significantly affect hardness and fracturability but still, samples with the highest sugar particle size had the lowest hardness due to more time needed for sugar melt which causes the dough to soften and spread.

Slow dissolving of large sugar particles also influence the total colour change of cookies during baking delaying the browning caused by the caramelization and the Maillard reactions.

Using the powdered sugar in cookie formulation caused the highest colour change.

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# THE MODEL OF UTILISING THE MARKET RESEARCH AND CRM POTENTIAL IN THE FUNCTION OF PRODUCTION MANAGEMENT IN THE BAKING INDUSTRY

**ORIGINAL SCIENTIFIC PAPER**

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**ABSTRACT**

In the 20th century, when the industrial age was reaching its peak, production of many products was based on little or no knowledge about consumer needs. Product volume, type and quality in such production conditions were primarily defined by production potential. As a result, product surpluses, which could not or would not be absorbed by the market, were often generated. Such surpluses in the baking industry are quite common in the Republic of Croatia even today. The growth of competition and the growth of product transparency in the market have led to a global decrease in the margin. This has resulted in the necessity of finding the ways of learning about consumer needs in order to optimise production and reduce product surpluses, thus making production more economically feasible. The potentials offered by the concept of market research and the concept of customer relationship management play a great role in this process. Being an integral part of marketing, these two concepts should be integrated as a model into a management concept for the bakery production to make production of bakery products more economically feasible, i.e. to ensure survival of large non-flexible production systems in the baking industry in particular. Namely, small production systems in the baking industry are very flexible, whereas large systems are facing greater challenges in their efforts to adapt to modern ways of doing business. Therefore, the key element of survival of large systems in modern production conditions is to find a solution to the problem of management information that will ensure production optimisation.

**Keywords:** Market research, marketing, relationship marketing, CRM, model

## INTRODUCTION

In terms of economic processes, the modern world is characterised by an overall increase of competition caused, on the one hand, by the growing number of businesses, and on the other, by the growing transparency of businesses, as well as the mobility and knowledgeability of contemporary consumers. The result of these trends is a worldwide decline in margins in the developed economies which in turn decreases revenues and increases the sensitivity of companies to possible adverse developments in the business, i.e. increases the risk of operating losses and the risk of business failure. Therefore, modern businesses are increasingly focusing on the rational decision-making which is within the scope of company management, as well as marketing which, in the present-day conditions, has transformed from a business function into a management philosophy which focuses on meeting the needs of consumers. Increased competition in the market has caused the need to produce only those products for which there is a demand which is a conse-

quence of the actual consumer needs. Marketing is the business philosophy that unlike production and sales philosophy does not push the product, and does not try to persuade the customer to buy a product that he/she does not need, but rather investigates consumer needs and, based on the perceived needs, initiates the rational decision-making process concerning the production program, the selling price, as well as the method of distribution and promotion.

When it comes to the baking industry, the problem is further complicated by the decline in consumption of bakery products in developed countries worldwide. In fact, the consumption of bakery products in the developed world has been falling due to the knowledge about human health, as well as the increasing volume and availability of other food products. Therefore, the management of baking industry companies should as soon as possible abandon the production concept as the main business model and apply the marketing concept, especially, if possible, the relationship marketing that promotes an individualized approach to the consumer, with an aim to meet the demands of the modern market. Taking into account its size, it is important for a baking industry companyto find the optimum balance between the traditional methods of market research and continuous market research through interaction with consumers using the principles of relationship marketing by applying customer relationship management (CRM).

## MATERIALS AND METHODS

At a time when the global decline in margins in developed markets impels businesses to become more rational in their operations, baking industry companies are also required to successfully apply marketing, especially relationship marketing. A particular challenge is the problem of choosing the optimal way of acquiring knowledge about the needs of consumers, i.e. finding an optimal balance between market research on the one hand and the application of customer relationship management as an alternative source of information about consumer needs on the other. In addition to providing high-quality interaction of businesses with the market, the information about consumer needs will also allow for the optimization of the production program in the baking industry by decreasing production surpluses, thus enabling production optimization in the baking industry. Based on the above arguments, the following hypothesis can be proposed:

*An appropriate system for market research and customer relationship management in the function of production management in the baking industry is possible and desirable.*

The research goals are the following:

1. Explore and outline the situation in the baking industry today
2. Explore the consumption of bread and bakery products in Croatia for determination of the product optimisation needs
3. Outline the potentials offered by the concept of market research and the concept of customer relationship management for optimising the production in the baking industry
4. Develop an appropriate conceptual descriptive model consisting of the two described concepts to make production of bakery products more economically feasible.

Overall, a deductive method of investigation was used as the initial hypothesis was explored in a mental experiment. The main method utilized to accomplish the research goal is the modelling method. In order to correctly define the analogies in the process of modelling between the original and the model we will apply the systematic approach. Other scientific research methods have also been used in addition to the above-mentioned.

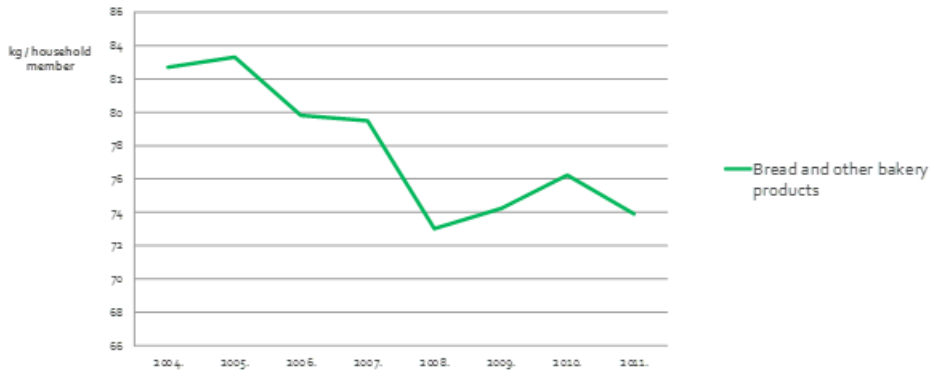
## RESULTS AND DISCUSSION

### *Analysis of the consumption of bread and other bakery products in the Republic of Croatia*

Until the mid-twentieth century, the Republic of Croatia, as a predominantly agricultural area in which bread represented a very important food product, was falling behind in terms of economic performance in comparison to the developed industry-oriented economies mainly from Western Europe. Until the end of the 20th century, industrialization was implemented in Croatia, which due to the then present socialist framework was significantly lagging behind the industrial potential of the developed parts of Europe. Nevertheless, industrialization has led to urbanization, lifestyle changes, and a relative increase in the purchasing power of consumers, consequently changing the eating habits of the Croatian population. Almost until the end of the 20th century bread and bakery products were produced predominantly in large-scale establishments, in large quantities and with a small range of products. The awareness and the needs of the population were such that everything that was produced was absorbed. Such business conditions were favourable for the production concept in which technocracy-oriented managers developed production programs with very small variations in the assortment.

The end of the 1980s was marked by a rapid growth of awareness of the population in this area and a desire to adopt shopping and business habits of the Western world resulting in significant economic and social changes. The re-emergence of capitalism brought about mass appearance of small flexible producerswho emerged under the influence of entrepreneurial aspirations of the population on the one hand, and the influence of changed and differentiated needs of the population on the other. The changed and differentiated consumer habits required producers to put greater emphasis on marketing and meeting consumer needs. This affected the bakery products in that a large number of small flexible privately-owned bakeries offering a large range of different products were established. However, it should be noted that the margins in that period were high enough to compensate for the lack of cost-effectiveness in the production of bakery enterprises.

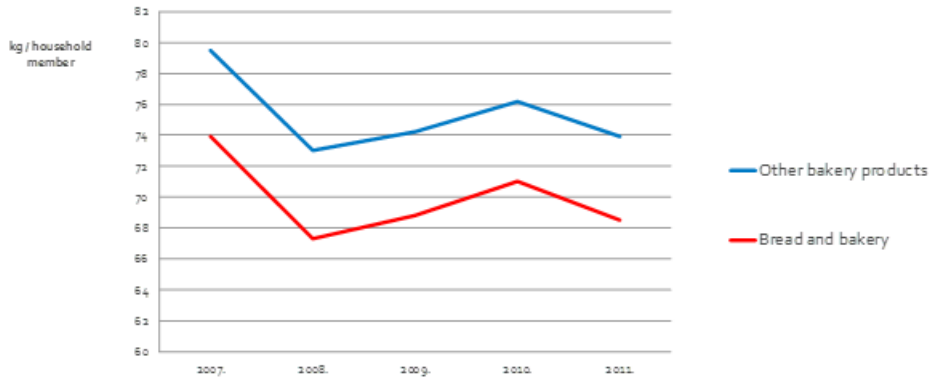
The situation has significantly changed at the beginning of the 21st century. The advent of the Internet and information and communication technologies led to globalization, high level of transparency, further increase in the level of awareness of the population, as well as to the influx of cheap Asian goods to Western markets. This in turn led to a global decrease in margins and the need for rationalisation of business operations. Although the baking industry in the Republic of Croatia was not directly affected by globalization because the bakery products are still mainly locally produced, imports of other products that replace bakery products, as well as nutrition trends promoting minimal consumption or a complete elimination of bakery products from the diet of the population have contributed to the decline in margins in the baking industry. The survival of the baking industry in the Republic of Croatia in present-day business conditions requires rapid adaptation, rationalization of production, and, above all, a marketing orientation, which means producing products that people actually need. The general trends in bread consumption in the Republic of Croatia in the new millennium are shown in Figure 1.




Source: Central Bureau of Statistics, www.dzs.hr.Accessed October 1, 2015.[1]

Figure 1. Quantities of food consumed in households in Croatia 2004-2011

Figure 2 shows that in the new millennium the level of bakery product consumption exceeded the level of bread consumption.




Source: Central Bureau of Statistics, www.dzs.hr. Accessed October 1, 2015.[1]

Figure 2. Bread consumption in comparison to the consumption of other bakery products in Croatia, 2007-2011

Based on the above and thesecondary sources, the following can be concluded concerning the baking industry in Croatia:

- still functioningwithin the Production/ Product Concept and Selling Concept
- The growth of competition and the growth of product transparency in the market have led to a global decrease of margins
- product surpluses in the baking industry are quite common in the Republic of Croatia even today
- small production systems are more flexible than large systems
- bread and bakery products consumption is decreasing.

### *Marketing definition and evolution*

Although a number of definitions have been used in modern science to describe marketing, most of them concur with the definition provided by the American Marketing Association. The definition has changed over time to become what it is today: ″Marketing is the activity, set of institutions, and processes for creating, communicating, delivering, and exchanging offerings that have value for customers, clients, marketers, and society at large.″[2].The preferred definition of marketing in the business practice is the one provided by the CIM (The Chartered Institute of Marketing) which reads follows: ″The management process responsible for identifying, anticipating and satisfying customer requirements profitably.″[3].It is important to note that in the 1980s marketing was considered to be a business function. Today, marketing is recognized as a management philosophy that enables the survival of business organizations by satisfying customer needs.

Given the trends resulting from ICT evolution, it is necessary to look at marketing from a different standpoint. Marketing can be defined as an information process whose primary task is to:

- provide information to business organizations about customer needs,
- provide information to customers about products which can satisfy their needs.

Marketing as an information process involves communication in two directions: from a customer to a business organization and vice versa. A business organization is at its centre, using the marketing research process to collect data about the market, customer needs and requirements, as well as customer behaviour. The data collected are processed by business organization information system and the information and knowledge extracted are used for business decision-making. The information and knowledge are the bases for market segmentation and customer profiling, as well as the development and implementation of marketing plans. A marketing plan contains, as a minimum, the following information about the marketing mix: the product production plan, the product price, the methods of product distribution and product promotion. Product promotion is an extension of the information process which involves the dissemination of information about products to customers.

Outside the framework of the marketing theory, marketing is usually perceived as and equated with the product promotion process and understood as a selling concept. On the contrary, marketing is focused on marketing research, especially on customer needs identification. Fundamental marketing concepts claim that it is not necessary to promote a product that completely satisfies customer needs. Therefore marketing is a philosophy according to which a customer pulls a product from a business organization, while in a selling concept a business organization pushes a product to the customers regardless of whether they need it or not. Thus, in the marketing concept collecting information about customer needs is a primary process, while in the selling concept the main process is the dissemination of information through aggressive promotion. It follows that marketing is a ‘win-win’ concept, while the selling concept is a ‘win’ concept, because in the latter only merchandisers’ needs have to be satisfied. Hence, one can argue that the simplest way is to consider marketing as a business philosophy that should be primarily used by management, and whose task is to satisfy consumers’ needs. To achieve this, marketing focuses on consumers. Whereas the selling concept is focused on the buyer and finding the ways to persuade him/her to buy a product, the marketing concept explores consumers’ needs and tends to satisfy these needs through identification of a suitable product, its price, distribution method and method of presenting the product and providing information about it. Consequently, the selling concept is aggressive in its approach, whereas the marketing concept tends to communicate with buyers and receive from them information about their needs, and provide information about the product. Figure 3 shows the development phases in the business philosophy


Source: Meler, M. (2005): Osnove marketinga, Osijek, Croatia: Ekonomski fakultet u Osijeku, p. 12.[4]

Figure 3. Evolutionary processes that resulted in marketing

Marketing is not a homogenous business philosophy. It has evolved through several phases. Meler, who analyzed the evolution of marketing through the decades, identified the following phases in its evolution[5]:

1950-1960 – mass marketing

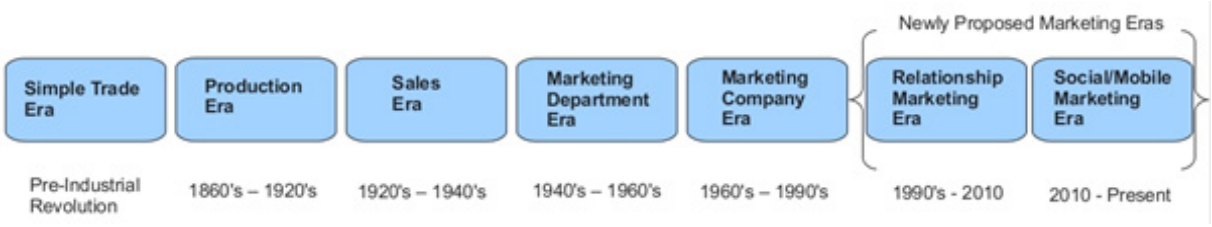
1970s – market segmentation

1980s – niche marketing

1990s – micro-marketing.

Meler[4] used the level of market segmentation as the basis for delineating different phases in the evolution of marketing. The last phase, i.e. micro-marketing has evolved from one-to-one marketing to relationship marketing. While all the phases prior to relationship marketing support asynchronous one-way communication between a business entity and a customer, relationship marketing involves synchronous bidirectional communication. Asynchronous one-way communication means that the stage during which information about custumers' needs is received is clearly separated from the stage in which information about products is conveyed through promotional activities. Synchronous bidirectional communication means that there is continuous parallel transfer of information about customers' needs and products (continuous marketing research and promotion). Modern ICTs enable synchronous bidirectional communication which is the reason why relationship marketing and its application concept, i.e. Customer Relationship Management (CRM), emerged in the 1990s.

Steven With recognized the five phases in marketing evolution (see Figure 4), but argued that there were two more: relationship marketing which emerged in the 90s and Social/Mobile marketing which developed within the Web 2.0 concept– social web concept.




Source: With, S.: *The Evolution of Marketing*, Available at http://dstevenwhite.com/2010/06/18/the-evolution-of-marketing/.Accessed February 4, 2015.[6]

Figure 4. Evolutionary processes in marketing

### *Marketing research*

According to Božić, the purpose of market research is to collect data and information necessary for planning, organizing and controlling of business processes[7]. Based on reliable information obtained from market research, the management of a business entity makes key decisions related to the business. Market research is the first of five stages of the marketing process, as shown in Figure 5.

Market research is of key importance when introducing a new product or production program because it allows a business entity to obtain information on the needs and desires of potential consumers. As observed earlier, ″the planning period and projection represents the basis of every initial stage of production development. Moderate evolution of a production program is a guaranteeof business stability. The best policy for a long–term production development program is one that allows for new or renewed products to appear beforehand. In order to define a long–term production development program plan, one needs to carry out many complex activities generally known as marketing research product development.″[8]


Source: http://www.teaminternetmarketing.com/the-tim-internet-marketing-process/,accessed April 4, 2015.[9]

Figure 5. Marketing process stages

It is important to bear in mind that the original application of marketing was mainly associated with the initial market research as the basis for identifying customer needs. However, it was soon realised that a product has its lifetime and that it should be adjusted over time to meet the evolving needs of the consumer. This is why, in addition to the initial market research, periodic market surveys are conducted to measure customer satisfaction with the product and analyze marketing performance. Studies and analyses to be carried out for the purpose of introduction, development or evolution of a product are the following[8].

- Research and market analysis with the intention of affirming a general tendency of demands, wishes and market needs for a product's business system in an upcoming longer period, through ascertaining trends of important facts such as: product quality, product technical characteristics, product functionality, product aesthetics, product price, evaluation of market needs for particular products, evaluation of competitive market product share, market purchasing power and mode of selling (cash, credit);
- Research and concurrent analysis with emphasis on the evaluation of advantages or

disadvantages of the concurrent program and business policy by comparison with one's own program through: technical estimation and commercial characteristics of individual products by comparison with one's own relative products, cost evaluation of particular products (if possible), quality evaluation by individual products by comparison with one's own, trend evaluation for future development (what, when, how), evaluation of concurrent ability for development (ideas, staff, finance, etc.);

- Research and analysis of own existent production program with the intention of establishing which products can have further production, which production should be stopped, and which products could stay in further production when some characteristics or parameters could be improved. Such matters can be researched through: quality evaluation,technical characteristics, functionality and aesthetics of each product, relative evaluation cost and selling price of each product and spare parts, evaluation of historical market attachment to each product from its own program, evaluation of change upon eventual reconstruction of each product and cost size.

Market research includes the following stages[7]:

1. Identifying the problem and research objectives
2. Collection and evaluation of secondary data
3. Designing of primary research
4. Collection of primary data
5. Processing, analysis and interpretation of data collected
6. Drawing up of the research report.

The primary data are collected as part of the market research by means of observation and surveys. Observation can be conducted by people; however, recently electronic devices have been increasingly used to record data about consumer behaviour. Examples include POS (Point of Sale) devices that enable the implementation of the so-called loyalty programmes under which, by identifying a buyer's card, his/her buying habits are monitored. However, traditional primary methods of market research prefer research carried out by means of surveys or in-depth interviews with consumers, by direct interviews, telephone interviews or interviews using information and communication systems. The key features of the traditional methods of market research are as follows:

- Discontinuity - carried out periodically; it often involvesagencies specializing in market research;
- Limited to a single sample - the research focuses on the sample of consumers, rather than the total basic set of consumers in a potential market (overall or total market is divided into the market of absolute non-consumers and the market of potential consumers. The potential market is made up of the market of relative non-consumers and the market of consumers. The market of consumers itself is made up of the consumers of the products of a particular business entity versus the consumers of its competitors' products);
- Focus on averages – rather than seeking to identify the needs of each individual consumer, it is focused on the common needs (averages) of a particular market segment or specific niches.
- Focus on a market segment (niche) – rather than researching the entire market, they are predominantly focused on potential or actual consumers.
- Static – theresults of the research are accepted as accurate for a longer period of time ignoring the rapid changes in the modern dynamic market.

### *Fundamentals of the CRM*

We live in an age when marketing orientation and information superiority are the key factors of competitiveness. Kotler and Caslione[10] make the following point: ″The most important thing you have to remember is that in the age of turbulence the greatest change will occur with your

customers. For this reason you have to change too. If you know where your customers are headed, you must be ready to adjust your offer. It is not enough to cut your costs. You have to adapt your product lines and service packages.″ Adapting products and services to meet the needs of consumers in the dynamic business environment prevailing today in the market is only possible if the market research is carried out continuously. This continuous and permanent market research, aimed at establishing and developing long-term relationships with consumers, is the very focus of the latest major development of marketing, and that is relationship marketing. Not only is the relationship marketing focused on continuous and permanent relationship with the consumers, but the monitoring and determining consumer behaviour is investigated down to a minute detail. Thus, relationship marketing does not involve identification of common characteristics of a market segment or niche, but is focused on each individual customer, his/her needs and habits, aimed at establishing and developing relationships with them. CRM is an application of relationship marketing. Furthermore,CRM represents a complex business philosophy which integrates a marketing concept, management and information technology. It is impossible to organize a CRM system without information technology support, but technology is also, owing to its information and communication potentials, capable of ensuring information superiority. The importance of information superiority as the basis for competitiveness has received special attention over the past decade, which has seen the evolution of Customer Relationship Management in response to the need of retaining market positions. Panijan[11] states: ″Modern trends have shifted the focus of attention from studying the macro aspects and general rules of behaviour of consumers as a collective body to research directed to individual user, to his particular needs and behaviour. This has set up the prerequisites for the development of a new managerial discipline – customer relationship management.″ In line with the business philosophy of CRM, the retaining of market positions can be achieved through continuous care for the customer, who, under pragmatic conditions, is transformed and evolves into a client.

The key is how and where CRM works, and the way in which it is used to establish and develop a relationship with consumers. In pragmatic conditions it is very often an upgrade to the existing enterprise resources planning (ERP) system. In terms of its structure, a CRM system is composed of the following three components[12]:

- collaborative CRM,
- operational CRM and
- analytical CRM.

While collaborative CRM is used at the point of contact, it is supported by operational CRM, the back-office of collaborative CRM. The task of the analytical CRM is to analyse data collected for the purpose of making management decisions. There is a wide range of CRM systems, but the most common are software applications used at retail points (e.g. loyalty systems), and various types of contact centres. Regardless of the specific type and mode of action of a particular CRM system, their common characteristic is a systematic, permanent and continuous monitoring of consumer behaviour for the purpose of determining their needs, preferences and desires, and ultimately using the collected information to define a consumer behaviour model which will serve as the basis for optimization of the production program. This means that unlike market research CRM works as follows:

- continuously - every interaction with the consumer at point of contact is recorded;
- a basic set is used to monitor the behaviour of all persons with whom contact was made, rather than just using a sample;
- it is focused on an individual, seeking to identify the needs of each individual consumer**;**
- it is focused on the entire market; the aim is to collect as much information as possible about

as many consumers as possible (a ″friendly″ relationship to the Big data concept);

- it is dynamic; customer behaviour is constantly monitored to detect deviations in their behaviour.
-

## CONCLUSIONS

Given the dynamism and globalization of markets in the developed countries, the time has come for Croatian bakery companies to identify production concepts, as especially large systems in the baking industry worldwide have already done, and adapt to the current environment and ensure their survival by applying the marketing concept. Marketing concept is based on the customer satisfaction: ″You do not sell what you can make, you make what you can sell.″ In order to systematically apply the marketing concept to the operation of baking industry businesses, it is necessary first of all to solve the problem of the collection of data concerning the needs of consumers and their behaviour. It is necessary to properly conduct market research, regardless of whether it is traditional market research or continuous market research provided by CRM systems. Figure 6 gives an explanation as to the application of a particular type of market research.

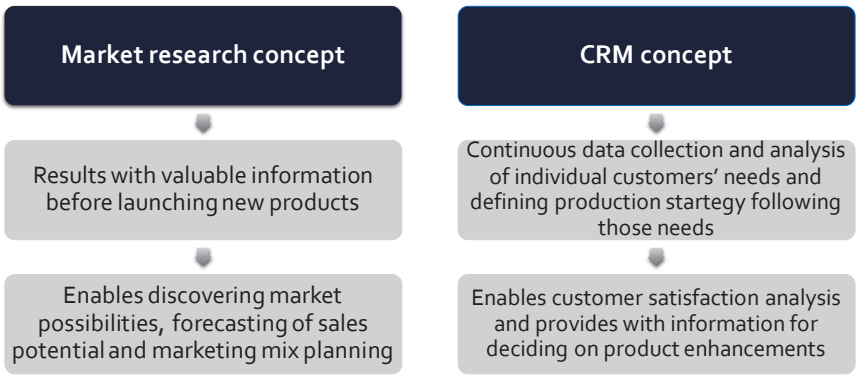



Figure 6. Choice of optimal market research system

At the point of contact, all available information and communication technologies as well as concepts built on it can be used for the collection of data on the needs and desires of consumers and their behaviour. They are as follows:

- Contact centres
- Geolocation systems
- Loyalty schemes
- Biometric systems
- Social networks.

Based on the above the following conclusions can be made:

- An appropriate system for market research and customer relationship management in the function of production management in the baking industry can help in solving the problem of product optimisation.
- Proper information about customers' needs can help in deciding about the production volume and product quality features on time and in different stages of product lifecycle.
- Learning about customers' needs is achieved with market research activities before launching new products and with customer relationship management activities in later stages of product lifecycle.
- This integrated concept (model) of mar-

ket research and the concept of customer relationship management can help in finding optimum ways of learning about consumer needs in order to optimise production of bread and bakery products and reducing product surpluses.

- It can help in making production of bakery products more economically feasible and it is also the key element of survival of large systems in modern production conditions.

Suggestions for future research are as follows:

- The issue of managing customer satisfaction in the baking industry when online sales increase
- The problem of customer satisfaction tracking in small systems.

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# THE EFFECT OF OAT β-GLUCAN ADDITION ON COOKING LOSS, COLOUR AND TEXTURAL ATTRIBUTES OF CHICKEN SURIMI GELS AFTER FROZEN STORAGE

**ORIGINAL SCIENTIFIC PAPER**

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**ABSTRACT**

Cooking loss, texture profile analysis (TPA) and instrumental colour parameters of chicken surimi gels mixed with oat β-glucans ($w$ = 0 - 6%), after frozen storage were investigated. Chicken surimi gels were prepared from broiler meat, mixed with oat β-glucans ($w$ = 0 - 6%), quickly frozen and stored for 12 weeks on -30 °C. Instrumental colour measurements ($L^*$, $a^*$, and $b^*$ values) were taken using a Hunter-Lab Mini ScanXE. The Hunter $L^*$, $a^*$, and $b^*$ values respectively correspond the lightness, greenness ($-a^*$) or redness ($a^*$), and blueness ($-b^*$) or yellowness ($b^*$). Texture profile analysis (TPA) tests were performed using a TA.XT2i Texture Analyzer equipped with an aluminium cylindrical probe P/75. The following parameters were quantified: hardness, springiness, cohesiveness and chewiness. Instrumental colour parameters ($L^*$, $a^*$ and $b^*$) and whiteness ($L^* - 3b^*$) of chicken surimi gels were significantly ($p < 0.05$) affected by addition of oat β-glucans. Hardness and chewiness also increased significantly ($p < 0.05$) and cooking loss decreased significantly ($p < 0.05$) by addition of oat β-glucans. Cohesiveness and springiness of chicken surimi gels were not significantly ($p > 0.05$) affected by addition of oat β-glucans. Increase in colour and textural attributes ($L^*$, whiteness, hardness, gumminess and chewiness) and decrease in cooking loss of chicken surimi gels indicate possible interactions between chicken myofibrillar proteins and oat β-glucans.

**Keywords:** Chicken Surimi, Cooking Loss, Texture, Instrumental Colour, β-Glucans, Frozen Storage

## INTRODUCTION

Chicken myofibrillar protein concentrate, produced with modified technology used for fish surimi[1,2] are characterized by very good technological properties, such as high water holding capacity and high ability to form strong gels after being heated. The most frequently used food preservation technique for this kind of food product is freezing and frozen storage. To protect myofibrillar proteins from denaturation during frozen storage and maintain its possible high processability, some cryoprotectants (i. e. disaccharides, polysaccharides, polyalcohol's, organic acids and polyphosphates) are generally added[3,4]

Colour and texture are the major factors responsible for the final acceptance of surimi-based products by consumers. To better suit the textural preferences of consumers, ingredients must be added to surimi that modify the textural and water mobility properties of the surimi[5]. In a composite food such as surimi the additives can modify the texture. Protein additives, such as egg white, are used to increase gel strength and to give a whiter and glossier appearance to the gel [6]. The final surimi-based product can assume almost any desired texture through its gel forming capacity.

β-glucans are composed of glucose molecules, which are linked with β-(1,3), (1,4) and (1,6) glycosidic bonds. (1,3), (1,4)-β-D-glucans are commonly isolated from wheat, barley and oats. Although found in all grains, their concentration

is highest in oats (4.6 - 4.9%) and barley (1.8 to 6%). β-glucans from various sources are used in the food industry as a thickening agent, dietary fibers, emulsifiers, etc.[7]. Studies have shown that the addition of β-glucans to meat batter increases the denaturation enthalpy of myofibrillar proteins, which suggests that β-glucans interact with meat proteins and stabilize them[8].

The objective of this study was to determine influence of oat β-glucans after frozen storage on chicken surimi cooking loss, instrumental colour ante textural attributes.

## MATERIAL AND METHODS

### *Sample preparation*

Chicken surimi samples were prepared in the laboratory from broiler meat (mainly lat. *Pectoralis major M. and Pectoralis minor M.*) using the modified procedure of Yang and Froning (1992)[9] since washing and leaching was performed with distilled water, instead of with tap water. β-glucans (Sigma-Aldrich, Taufkirchen, Germany) (isolated from oat) were added to samples in mass fractions of 2, 4 and 6%. Mass fractions were determined as percent of total mass. The pH level was measured in a homogenate of the sample with distilled water (1:10, p/v) with pH/Ion 510 – Bench pH/Ion/mV Meter (Eutech Instruments Pte Ltd/ Oakton Instruments, USA). Water activity ($a_w$) was determined using a Rotronic Hygrolab 3 (Rotronic AG, Bassersdorf, Switzerland) at a room temperature (20 ± 2 ºC). The FoodScan Meat Analyser was used to determine moisture, total protein share, total fat share and collagen content according to the AOAC 2007. 04[10].

### *Textural analysis (TPA) and cooking loss*

Samples of chicken surimi were placed into plastic test tubes with an inside diameter of 10 mm. After defrosting, test tubes with their content were heated for 25 min in a water bath at 80 °C. Test tubes with produced gels were cooled in ice water until the temperature of approx. 20 °C was obtained inside the sample. After that, they were stored at 4 - 6 °C until the next day. Cooking loss was calculated as a weight difference of the sample prior to the cooking and after the removal of the cooked gel from the test tubes. Cooking loss was expressed as a percent of the fresh sample weight. Texture profile analysis (TPA) tests were performed using a TA.XT2i SMS Stable Micro Systems Texture Analyzer (Stable Microsystems Ltd., Surrey, England) equipped with a cylindrical probe P/75. This involved cutting samples into 1.5 cm thick slices, compressed twice to 60% of their thickness. Force-time curves were recorded at across-head speed of 5 mm/s and the recording speed was also 5 mm/s. The following parameters were quantified[11]: hardness (g), maximum force required to compress the sample, springiness (ratio), the ability of the sample to recover its original form after the deforming force was removed, cohesiveness, the extent to which the sample could be deformed prior to rupture (ratio) and chewiness (g), the work required to masticate the sample before swallowing, which is calculated hardness cohesiveness springiness, was measured.

### *Determination of colour*

Colour measurements ($L^*$, $a^*$, and $b^*$ values) were taken using a Hunter-Lab Mini ScanXE (A60-1010-615 Model Colorimeter, Hunter-Lab, Reston, VA, USA). The instrument was standardized each time with a white and black ceramic plate ($L^*0 = 93.01$, $a^*0 = -1.11$, and $b^*0 = 1.30$). The Hunter $L^*$, $a^*$, and $b^*$ values correspond to lightness, greenness ($-a^*$) or redness ($+a^*$), and blueness ($-b^*$) or yellowness ($+b^*$), respectively.

The whiteness (W) was calculated: $L^* - 3\,b^*$. The colour measurements were performed on chicken surimi at a room temperature (20 ± 2 ºC).

***Statistical analysis***

Three determinations for basic chemical composition, cooking loss, pH, $a_w$, seven for TPA and colour parameters were measured from each sample. Experimental data were analyzed by the analysis of variance (ANOVA) and Fisher's least significant difference (LSD), with significance defined at $p < 0.05$. Statistical analysis was carried out with Statistica ver. 8.0 StatSoft Inc. Tulsa,OK. USA.

## RESULTS AND DISCUSSION

The mean basic chemical composition, pH and $a_w$ values of chicken surimi samples before mixing with β-glucans are presented in Table 1. The mass fraction of water, protein and fat in chicken surimi were similar to the results reported by Stangierski and Kijowski[12] for myofibril concentrate prepared from mechanically recovered poultry.

Table 1. Basic chemical composition, $a_w$ i pH of chicken surimi samples

| Water *w* (%) | Proteins *w* (%) | Fat *w*(%) | Collagen *w* (%) | pH | $a_w$ |
|---|---|---|---|---|---|
| 84.75 ± 0.28 | 13.06 ± 0.58 | 0.73 ± 0.07 | 0.79 ± 001 | 6.95 ± 0.04 | 0.98 ± 0.01 |

Values are means ± Standard deviation of triplicate

The cooking loss of chicken surimi mixed with different mass fracion of oat β-glucans after 12 weeks of frozen storage are presented in Fig 1. The additon of oat β-glucans ($w = 2$ - 6%) caused a significant reduction ($p < 0.05$) of cooking loss in the obtained gels. The lowest cooking loss showed the sample of chicken surimi mixed with 6% of oats β-glucan. Stangierski and Kijowski[13] reported similar results were by for mechanically recovered washed and frozen stored poultry meat with the addition of Cremodan and Pork Stock.

Instrumental colour parameters of chicken surimi with addion of oats β-glucans are presented in Table 2. Generally, the demand is higher for surimi gels with high lightness ($L^*$), low yellowness ($+b^*$) and high whiteness (W). Heat-induced chicken surimi gels exhibited a higher $L^*$ (76.31 – 79.71) as the mass fraction of oats β-glucans increases ($w = 2$ - 6%).

Table 2. Instrumental colour parameters of chicken surimi mixed with different mass fracion of oat β-glucans after 12 weeks of frozen storage.

| *w* (%) | L* | a* | b* | W |
|---|---|---|---|---|
| 0 | 76.31b ± 1.13 | 2.39a ± 0.86 | 18.75a ± 0.51 | 20.06b ± 0.27 |
| 2 | 78.06b ± 1.12 | 1.63b ± 0.63 | 17.78b ± 0.54 | 24.74b ± 0.14 |
| 4 | 78.81ab ± 1.23 | 1.57b ± 0.63 | 17.56b ± 0.53 | 23.53b ± 0,33 |
| 6 | 79.71a ± 1.06 | 1.52b ± 0.78 | 17.39b ± 0.82 | 24.34a ± 0.62 |

Values are means ±SD of seven measurements. Values in the same row with different letters (a-b) are significantly different (p < 0.05)

Chicken surimi gels redness (+*a**) decreased significantly ($p$ <0.05) with the addition of oats β-glucans ($w$ = 2 - 6%). Yellowness (+*b**) de



creased with the addition of oats β-glucans ($w$ = 2 - 6%) from 18.75 to 17.39.

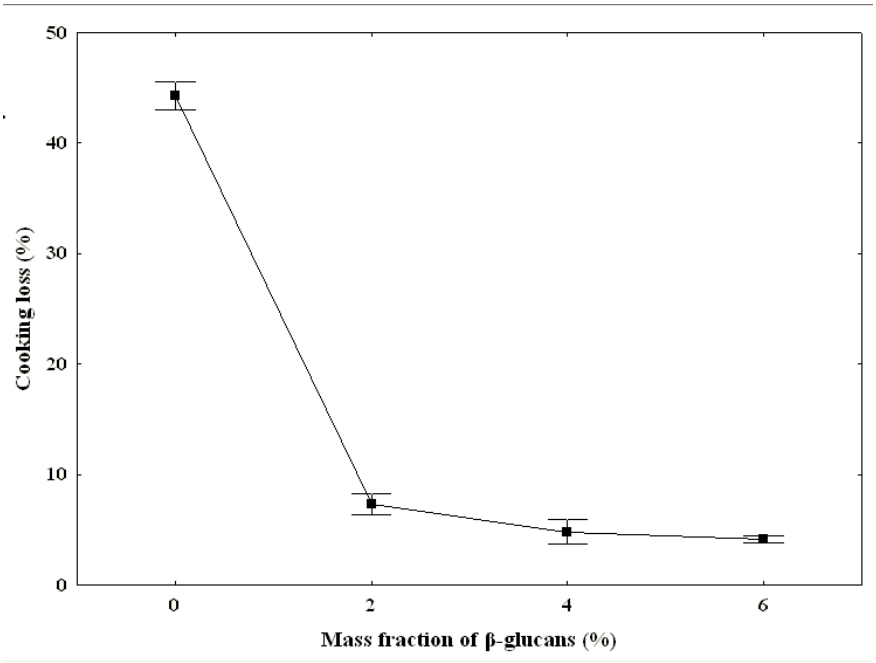


Figure 1. The Cooking loss chicken surimi samples actin mixed with different mass fracion of oat β-glucans after 12 weeks of frozen storage.

Since the most important quality parameter in surimi is whiteness, and in order to better predict the behaviour of additives, whiteness was calculated W = $L$* - 3$b$*. The whiteness of chicken surimi samples varied from 20.06 to 24.74. Similar increase in lightness and whiteness for the heat induced fish surimi gels mixed with potato starch, egg white and oat bran reported Tabilo-Muniza-

ga and Barbosa-Canovas[14] and Alakhrash et al [15] . The addition of β-glucans significantly increased whiteness ($p < 0.05$) of chicken surimi samples.

Table 3. Texture profile of chicken surimi mixed with different mass fracions of oat β-glucans after 12 weeks of frozen storage.

| *w* (%) | Hardness (g) | Springiness | Cohesiveness | Chewiness (g) |
|---|---|---|---|---|
| 0 | 1000.04b ± 74.31 | 0.75a ± 0.06 | 0.40b ± 0.04 | 299.44b ± 22.41 |
| 2 | 1013.16b ± 52.49 | 0.75a ± 0.04 | 0.43b ± 0.03 | 327.99b ± 17.59 |
| 4 | 1148.89b ± 41.2 | 0.77a ± 0.11 | 0.44b ± 0.04 | 353.72b ± 12.57 |
| 6 | 1306.87a ± 32.98 | 0.79a ± 0.06 | 0.46a ± 0.03 | 474.92a ± 29.05 |

Values are means ±SD of seven measurements. Values in the same row with different letters (a-b) are significantly different ($p < 0.05$)

Texture profile analysis parametrs of chicken surimi mixed with different mass fracion of oat β-glucans after 12 weeks of frozen storage are presented in Table 3. Four parametrs were obtained: hardness, springness, cohesivness and chewiness.

The hardness of chicken surimi sampels incresed significantly ($p < 0.05$) from 1000.04 to 1306.87 g with the increase of mass fraction of oat β-glucans ($w$ = 0 - 6%). The springiness of chicken surimi samples were in range from 0.75 to 0.79 and did not vary significantly ($p > 0.05$) with the increase of β-glucans mass fraction. Chicken surimi samples cohesiveness was not significantly ($p > 0.05$) affected with the increase of β-glucans mass fraction (Table 3). The main differences in TPA among different mass fraction of β-glucans added were obtained in hardness and chewiness.

## CONCLUSIONS

The results of this study showed statistically significant ($p < 0.05$) reduction of cooking loss, increase of lightness ($L^*$) and whiteness (W), decrease of greenness ($+a$) and yellowness ($+b$), increase of some TPA parameters (hardness, chewiness) of chicken surimi samples after frozen storage with the increase of the mass fraction oat β-glucans. This can indicate possible interaction of oat β-glucans with the chicken myofibrillar proteins and its stabilisation.

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# FACULTY OF TECHNOLOGY

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