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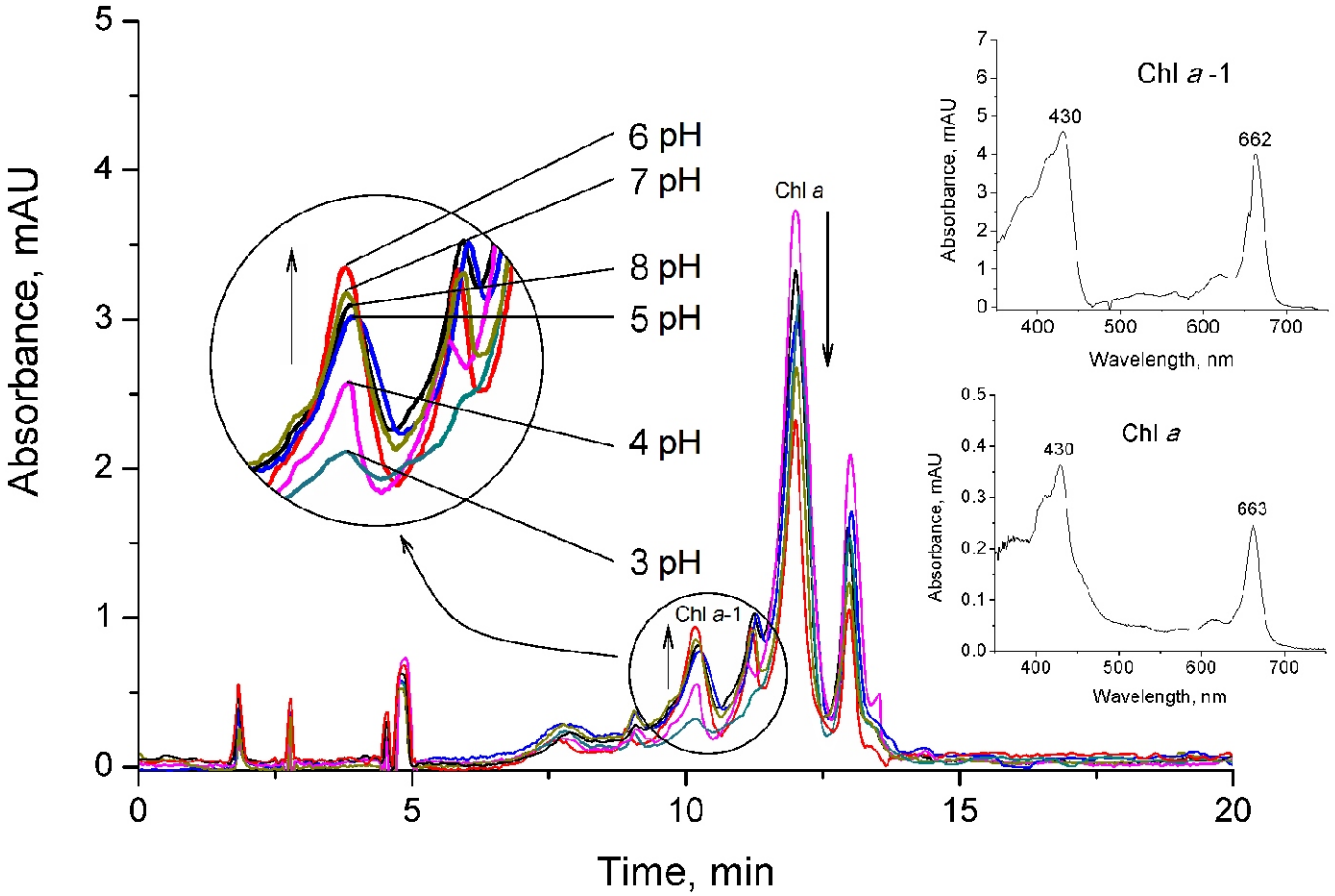

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Centrifugal separation of liquid carbon dioxide from natural gas

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Abstract

Natural gas has become a global commodity in the energy consumption. New technologies, like as gas-to-liquid conversion technology, contribute to this. But more than 16% of the currently known global gas reserves cannot be produced, because they are heavily contaminated by CO_2 and/or H_2S: (CO_2 > 10% and H_2S > 5%). The traditional technology of amine treatment is not capable to economically remove these contaminants. The objective of this article is to investigate the possibilities of centrifugal separation in a way to resolve the existing problem. After analyzing the existing situation, in the centrifugal separation of natural gas, some innovations in separators' design and theory are suggested. The aim of the presented theoretical considerations is that the complex theory of separation should be adjusted to the needs of engineers engaged in design, development and operation of these devices.

Keywords: gas, calorific value, liquid, separation, efficiency.

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Natural gas, used by consumers, is almost entirely composed of methane. Natural gas, extracted from gas deposits, composed primarily of methane, is by no means pure. Impurities, that can occur in natural gas, are [1–5]: particles of rock material; liquid (water and crude oil); gases (carbon dioxide, hydrogen sulfide, oxides of nitrogen, etc.).

Fields with a high purity of methane (CH_4) are commonly referred to as “sweet gas fields”. Fields that contain significant amount of hydrogen sulfide (H_2S) are called “sour” gas fields.

Fields that are contaminated with significant amounts of acidic gases, *e.g.*, carbon dioxide (CO_2) or hydrogen sulfide (H_2S), are called “acid” gas fields.

Removal of impurities: solid, liquid and gaseous, increases the possibility of gas exploitation at the gas fields, increases the heating value of gas, makes transport easier, protects the machine from corrosion, wear, etc. The cleaning process of natural gas can be divided into two technology related phases:

1. Primary cleaning of solid and liquid contaminants;
2. Secondary cleaning of gaseous impurities.

Particulate removal devices operate basically on the principle that a gas stream, containing particles, is passed through a region where the particles are acted on by external forces (gravitational or centrifugal) thereby separating them from the gas stream. This technology is well known and in this paper will not be represented.

When gas analyzed gas with high contents of gaseous pollutants (Tables 1 and 2), [6–9] the traditional technologies of treatments are not able to economically remove these pollutants. Finding the optimal solution, for cleaning such polluted gases, is important task of modern science.

Table 1. Acid natural gas volumetric composition [6]

Field	Gas							
	CH_4	C_2H_6	C_3H_8	C_4H_8	C_4H_{10}	C_nH_m	N_2	CO_2
Srbobran	75.15	1.95	0.50	0.12	0.25	0.16	11.90	10.00
Miloševo	28.76	0.65	0.41	0.19	–	–	6.94	63.05
Bečej 1	9.30	–	–	–	–	–	6.70	84.00
Bečej 2	65.74	0.09	–	–	–	–	2.32	31.50
Čantavir	76.41	2.51	0.43	0.05	–	–	7.69	12.80

Centrifugal separation is one of solutions for gas cleaning. There are three methods of gas separation by centrifugation [2–4]:

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Table 2. Biogas composition [6]

Biogas	CH_4 (%)	CO_2 (%)	O_2 (%)	N_2 (%)	H_2S (ppm)	Benzene (mg/m^3)	Toluene (mg/m^3)
Deposit I	47–57	37–41	<1	<1–17	36–115	0.6–2.3	1.7–5.1
Anaerobic digesters I	61–65	36–38	<1	<2	–	0.1–0.3	2.8–11.8
Biogas	55–58	37–38	<1	<1–2	32–169	0.7–1.3	0.2–0.7

1. Gas/gas separation in a rotating cylinder based on the difference in molecular weight of the gaseous components;
2. Gas centrifugation with wall condensation;
3. Centrifugal separation of condensed contaminant.

The particular interest in this paper is the condensed contaminant centrifugal separation (C3-sep). C3-Sep process has two steps [2–4]:

1. Cooling the gas to a temperature whereby the gaseous contaminant becomes liquid in the form of a mist of micron-sized droplets;
2. Separating the mist from the gas by the rotational particle separator (RPS).

The concept (C3-Sep) is particularly suited for the application of CO_2 and H_2S removal from natural gas. It has the potential to boost recoverable gas reserves by amounts which are energetically equivalent to multi-billions of barrels of oil.

C3-Separation splits the mixture into two phases: a liquid phase which is enriched in CO_2 and a gaseous phase that is enriched in methane CH_4. The liquid phase forms a mist of micron-sized particles. Centrifugal separation can rapidly remove the micron size particles using a rotating particle separator (RPS).

The core of the separator is the RPS element, *i.e.*, a rotating cylindrical body which consists of a multitude of axially oriented channels, Figure 1a and b [2]. The diameter of the channels is typically 1–2 mm, its length is 0.2–0.5 m. The element is about 0.4–1 m in diameter. After entering the channels of the rotating body, liquid mist particles, entrained in the gas, are rejected towards the collecting walls, Figure 1c. They then form a liquid film flowing downwards parallel with the gas.

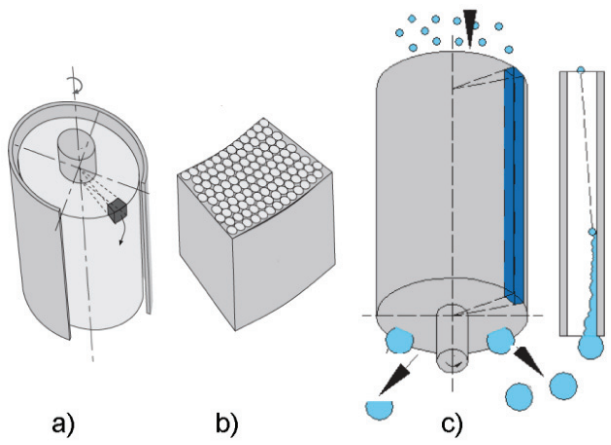

Figure 1. Rotational separator. a – Body, b – elements with different channel configurations and c – flow of liquid phase.

At the exit of the channels, the liquid film breaks up into droplets of 50–100 μm in diameter.

These droplets are rejected to the casing wall and subsequently leave the device via a liquid drain, Figure 2 [2].

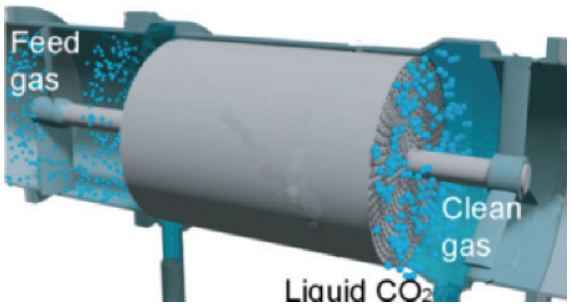

Figure 2. Rotation particle separator (RPS).

This technical solution, apart from the numerous advantages (such as its simple construction), has certain weaknesses:

- The separated liquid is accumulated, under the influence of the centrifugal force, on the tubes' wall. Flow, of liquid gas, goes through the axial canals whose axis is the same as the rotation axis. The only source of energy for liquid flow is the gas drag force at the contact of liquid-gas fraction.
- At the tube's exit the liquid suddenly turns (under the influence of the centrifugal force) in radius direction, which, in case of the sharp edges of the tube's ends, can lead to cavitation i.e. part of the liquid fraction, due to the local pressure drop, can evaporate.
- The separation process, of the liquid component from the gas component, is not equal for all tubes, since the influential centrifugal forces depend on the rotation radius, *i.e.*, on the position of the tube in the separator's construction.
- The drops of liquid, exiting the tubes that are closer to the axis of rotation, must cover a long way until they reach the inner wall of the separator body. Along that way they are exposed to the influence of pure gas stream. A significant problem, which accompanies such manner of discharge, the friction of liquid drops against the gas stream, results in micro-heating at the drop's surface, which can to spoil the quality of cleaning.

Analyzing the observed disadvantages, as well as numerous existing problems [2–4,10] the authors suggested a technical solution of the separator which, in their opinion could be a step forward in the technological process of cleaning the natural gas, biogas and a number of other gases.

NEW TECHNICAL SOLUTION

The basic idea of a new technical solution is that the primary (removal of fine solid particles and small particles of frosty water) and secondary (removal of pollutant, liquid phase which is enriched in CO_2 and H_2S) separation will be made in one step. The prototype of the centrifugal separator was based on existing technical solutions-disk stack centrifuges [11–13], with a number of innovations that are protected by patent applications [14,15]. One technical solution has been done and tested on tasks of solid–liquid mixture treatment. The results confirmed the initial assumptions in the design of the separator [16].

The prototype, Figure 3, consists the following sections: product feed device with vortex tube (I), rotor (II) with disk stack insert, pump (III) and centrifugal compressor (IV). Motor with frequent regulator, ancillaries system and measuring equipment are also involved.

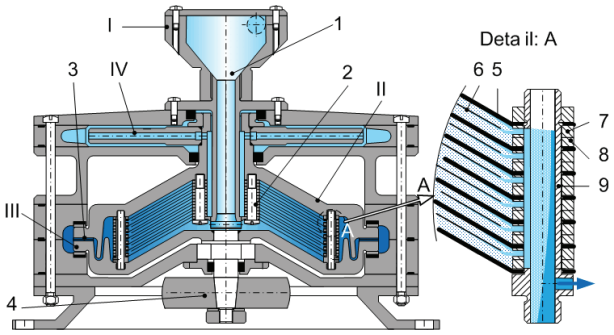

Figure 3. Centrifugal separator – prototype. 1. Vortex tube, 2. rotor; 3. pump; 4. compressor.

The lower half of the rotor, with disk stack insert (2), is connected to the motor via the belt drive (4). The disks split the working space into thinner segments in which the separation process is developing easier because the path of the heavier particles to the inner surface of disk is short.

Also, one of the observed disadvantages of the previous solutions [5] is the grip of the liquid fraction by the gas flow.

In new solution of separator such problem was removed by rings (7) (Detail A, Figure 3). The rings have the function of taking over the liquid that is drained through the inner surface of the disks (5) and liquid is directed towards the drainage tubes (9), *i.e*., to the rotor bottom.

Introducing the rings (8) will increase the gap between the discs (5). It is necessary, that the process of separation is done in optimal conditions, by imposing additional discs (6), smaller in diameter. The size of the gap (the thickness) is vital for the capacity and the quality of separation and it is theoretically defined, and experimentally verified. The goal of this innovation was made to further improve the efficiency of separation.

Why this innovation?

The technical solution presented in the paper [16] is designed for the separation of solid-liquid mixture. Discs have the radial edge. The relationship of acceleration is: $g/\omega^2 << 1$, so that the output path of solid particles is strictly radial. As the velocity (vertical component) in the free space of rotor is too small, the direction of the particles remains radial. This solution is sufficient for good separation of solid-liquid mixture.

If the same construction of discs is used for the separation of gas–liquid mixture, the layer of liquid, after impact with the disc edge, would be again dispersed into tiny droplets.

Micro-droplets would be drawn into the gas and re-introduced into the separation process, lowering the separation efficiency.

On the periphery edge, on the upper and lower half of the rotor, are the pumps channels (3) used for the drainage of the separation products (liquid). The rotor drainage is controlled by opening and closing the plugs, which are part of the pump construction.

The sealing of the periphery edge of the rotor (pump) is realized using mechanical seals. These seals are constructed in a way that at the same time they have the function of axial bearings. Such technical solution, in cases of wet friction of the sliding rings, reduces the friction resistance. This kind of solution enables achieving the great rotation speeds and the rotor stability is significantly improved. The liquid, a product of separation, is used as a lubrication fluid in the zone of sliding rings.

The lower half is connected to the upper half of the rotor by bolts. Upper half holds the impellers of centrifugal compressor.

In the field of centrifugal acceleration (Figures 3 and 4) [16–19] the liquid-gaseous mixture is separated down to its basic components.

The liquid particles and small particles of frosty water are immediately separated. Leaning, on the inner side of the rotor body, the particles move at the discharge opening of the rotor. The mixture (gas and fine liquid fraction of CO_2 and H_2S) gets caught into the gaps of the rotor insert, goes through the gaps and has been separated to the main components.

The liquid fraction gets caught on the insert rings and directed trough a channel and pipes (9) to the inner side of rotor body, *i.e*., to the pump.

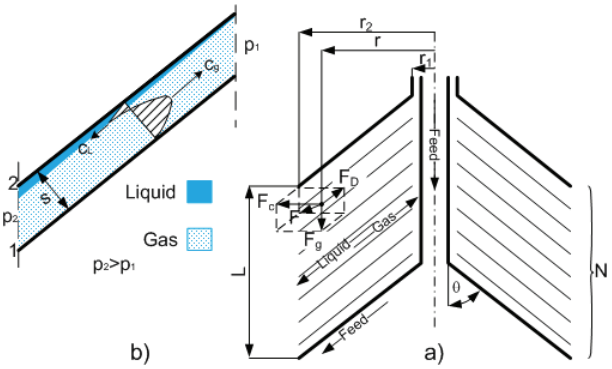

Figure 4. Forces acting on a particle due to centrifugal acceleration. F_D – Drag, F_g – gravity, F_c – centrifugal, F – total.

The easiest fraction (gas) gets caught at the place closest to the rotation axis and directed to the centrifugal compressor.

Innovations introduced in the separator construction were as following [14,15]:

- the periphery edge of the upper and lower half of rotor is constructed as an impeller of pump for liquid transport;
- the sealing of impellers is done by mechanical seals, which are, also, axial bearings for rotator;
- the specific construction of the insert allows the liquid drops to be caught after a short fall and directs the drops towards the bottom of the rotor, towards the discharge opening;
- by introducing the centrifugal compressor into the separator construction, suction of mixtures (liquid-gaseous) is facilitated and also the losses of pressure are compensated.

The advantages of solutions thus formed are as following:

- central charging and self-suction make separator independent of auxiliary equipment (feed pump);
- the vortex tubes (1) conducted the largest droplet separation. They are, sliding down the first disk, immediately directed to the drain hole in the body of the rotor.
- one engine controls the power of several sections, which on the other hand has an disadvantage that the conditions in the section cannot be independently changed;
- combining radial–axial bearings, the great rotation speeds can be achieved together with excellent rotor stability;
- by moving away from the rotation center, the liquid fraction is exposed to the greater centrifugal force (due to greater density), which results in better quality separation from the gas fraction.

Some of the weaknesses that should be mentioned are:

- the complex construction;
- heavy-duty, low temperature, leading to the frequent breakdowns;
- it is necessary to invest significant means into the technical solution in the prototype phase, so that all theoretical and constructive assumptions can be verified.

Perceived weaknesses in the theoretical considerations [16], while defining the separation quality and capacity, imposed the need for reconsidering the existing theoretical basis for defining the separation capacity and quality. The acquired results of the theoretical considerations are given in this paper.

THEORETICAL BASES

Many researchers [1–4,10,16,20,21] have dealt with this problem and the acquired results emphasize the following: the solid particle (droplet) size (d_s in m), settling velocity (c_g in m/s), length of settling road (L in m), settling time (t in s) and viscosity (η in Pas) of the feed fluid have a great influence to the separation feed flow rate and quality of separation.

The movement of the particles within the rotor and the separator's rotor insert is complex and consists of (Figure 4):

- vortex movement for rough separation of large drops in the pipe (1);
- radial movement under the influence of the centrifugal force in the insert of the rotator (2);
- axial movement vertically upwards, in the inner space of rotator;
- tangential movement of the liquid fraction – on disc surface and in the canals of the collection rings (7).

Separation of the mixture (in pre-separator, I) is theoretically and experimentally very well treated [3,4,16,17] and, in this paper, of particular interest is the movement of mist (gas–liquid CO_2) into the gaps of the rotor insert.

Moving practically (tg $\theta = g/\omega^2 r << 1$) radial in the gap between the disks, the liquid particle touches the inner disc side, Figure 4, and while sliding on it comes to the disk's exit edge (radius r_2). The layer thickness, at the contact point disk-mixture, is proportional $\sqrt{\nu}$ and inversely proportional $\sqrt{\omega_L}$. The minimal values of the boundary layer thickness are [22]:

$$\delta_{l,\min} = 3.71\sqrt{\frac{\nu}{\omega_L}}, \text{m} \tag{1}$$

for laminar flow, and:

$$\delta_{T,\min} = 0.525\left(\frac{\nu}{R^2\omega_L}\right)^{\frac{1}{5}}, \text{m} \tag{2}$$

for turbulent flow.

The gas, being the lighter fraction, fulfills the space bordered by the exterior disc surface (1), Figure 4, and a layer of liquid CO_2 which lies on the inner surface of the disc. In this gap the gas moves from the periphery to the rotation center at the speed of c_g. The amount of fluid (mixture of gas–liquid) which flows through the insert gap depends on the total flow q_v (m^3/s) through the gaps and the number of gaps ($z = N–1$) [17]:

$$q = \frac{q_v}{z}, \mathrm{m}^3/\mathrm{s} \tag{3}$$

There are two objectives of this theoretical consideration:

1. Definition of pressure drop in the inset (2) of rotor, that is of particular importance for separation efficiency and the size of the gap between the disks;

2. Defining the stability of liquid film flow, that is of particular importance for the quality of the final product.

During this complex movement, of gas–liquid mixture, the following resistances appear in:

- Liquid fraction friction against the inner disc surface (2);
- Gas fraction friction against the layer of liquid fraction;
- Gas friction against the exterior disc surface (1).

The consequence of friction are the losses (pressure drop) which are important for defining flow and power of the centrifugal separator. For all considerations [16,23] the common thing is that the friction coefficient (f), significantly depends on:

- Gap size ($s \le R_2$)
- Relative gap size, s / R_2 ;
- Relative roughness size, Δ / R_2
- Reynold's number:

$$\mathrm{Re}_\omega = \frac{q}{2\pi s \nu} = \frac{R_2^2 \omega}{\nu}$$

It is important to note that the angular velocity of liquid (ω_L) and gas (ω_G) are not the same to angular velocity of disc (ω_D), because the process of drive transfer, in rotating disc, is accompanied by slides. Many studies [17,22] confirm the position:

$$\begin{aligned} \omega_L &= K_L \omega_D \\ \omega_G &= K_G \omega_D \end{aligned} \quad (K_L \approx 0.5;\ K_G \approx 0.3) \tag{4}$$

It is customary that the coefficient of friction at the contact: the fluid–disc wall, is defined by [20,21]:

$$f_G = C_G \left[\frac{Rc_G}{\nu} \right]^{-n};\ f_L = C_L \left[\frac{Rc_L}{\nu} \right]^{-n} \tag{5}$$

where:

$C_G = C_L = 0.0791$, $n_G = n_L = 0.25$ – for turbulent flow;

$C_G = C_L = 16$, $n_G = n_L = 1$ for laminar flow.

A similar, but in our opinion, for these conditions acceptable, approach has Vasiljcev [22] that the friction coefficient is defined by the expression:

$$c_{fo} = A_\chi \left(\frac{s}{R_2}\right)^{m_i} \mathrm{Re}_\omega^{n_i} \tag{6}$$

The coefficient values: A_χ, m_i, n_i, depend on the fluid flow regime and are given in Table 3.

Table 3. The coefficient values: A_χ, m_i, n_i

Flow	m_i	n_i	A_χ
Laminar	–1	–1	$1-(1-\chi)^4$
Turbulent	–0.25	–0.25	$1-(1-\chi)^{4.75}$

The coefficient $\chi = (R_2 - r)/R_2$ defines how soaked is the disc surface (for: $r = r_2$, $\chi = 0$, *i.e.*, the disc is surrounded by gas; for $r = 0$, $\chi = 1$, *i.e.*, the disc is completely covered with liquid). Calculation of (c_{fo}) is complex, and a digram given in Figure 5 [22], is most frequently used.

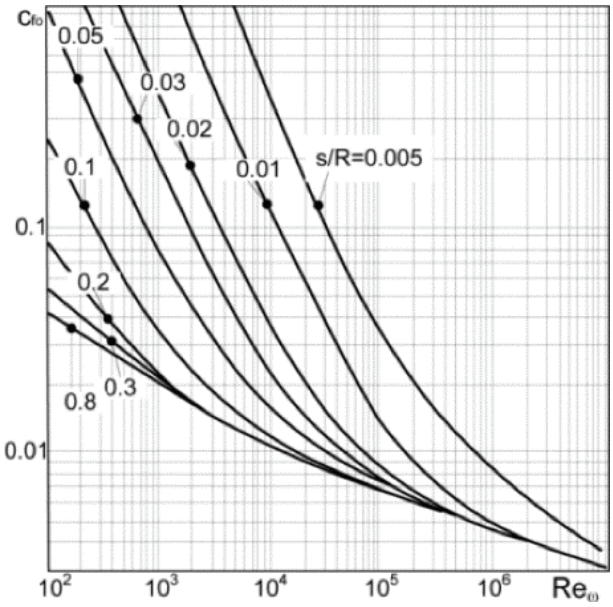

Figure 5. Friction coefficient c_{fo}.

The pressure drop, in insert of rotator, is defined by the formula (1 – gas; 2 – liquid CO_2):

$$\Delta p = f_{1,2} (K_{1,2} \omega_D)^2 R_2^2 \left[1 - (1-\chi)^2 \right] \frac{\rho_{1,2}}{2}, \mathrm{Pa} \tag{7}$$

According to this analysis (criteria – minimal pressure drop), the minimal gap value is defined by formula:

$$s_{\text{min.}} = (3.2-4.2)\sqrt{\frac{\nu}{\omega_L}}, \text{m} \quad (8)$$

The actual size of the gap is:

$$s = s_{\text{min.}} + 2\delta_{l(T)}, \text{m} \quad (9)$$

For r_2 = 0.1 m, ω_L = 150 s^{-1} and $\nu = 1\times10^{-6}$ m^2/s the optimal gap value is: 0.0007 m (0.7 mm). Gaps are commonly 2–3 mm.

The analysis of fluid behavior at the contact of gas-liquid CO_2 is important for two reasons:

- defining the drag force on the contact of liquids-gas boundary zone and its impact on the capacity and quality of separation;
- defining the impact of gas flow on the stability of liquid film and quality of separation.

The micro-mixture is formed on the contact layer with density of:

$$\rho_M = P_G\rho_G + P_L\rho_L, \text{ kg/m}^3 \quad (10)$$

Speed of movement in the boundary zone is [23]:

$$\bar{c} = \frac{P_G\rho_G c_G + P_L\rho_L c_L}{\rho_M}, \text{ m/s} \quad (11)$$

Mode of movement in this zone defines Reynold's number:

$$\text{Re}_\omega = \frac{\bar{c}s}{\nu} \quad (12)$$

for:

- Re < 50 motion is laminar, with a flat area on the liquid-gaseous contact;
- 50 <Re < 400 motion is laminar, with a wavy surface of liquid–gaseous contact;
- Re > 400 motion is turbulent.

Shear stress is:

$$\tau_{LG} = \frac{1}{2} f_{LG}\rho_G\bar{c}^2 \quad (13)$$

As the fluids (gas and liquid CO_2) move in opposite directions, the critical velocity, c_c, is defined by the expression [20]:

$$c_c = \sqrt{2(\frac{\sigma g}{\rho_L})^{0.5}\frac{\rho_L}{\rho_G}}, \text{ m/s} \quad (14)$$

Group of authors [20] proposed that the coefficient of friction (f_{LG}), in this zone, is defined by comparing the coefficient of friction of pure fluids in contact with the wall.

For ($c_G + c_L$) < c_c coefficient of friction is:

$$\frac{f_{LG}}{f_G} = 5 \quad (15)$$

For ($c_G + c_L$) ≥ c_c coefficient of friction is:

$$\frac{f_{LG}}{f_G} = 5 + 15(\frac{\delta}{D_2})^{0.5}\left[\frac{c_G + c_L}{c_c} - 1\right] \quad (16)$$

Also in use is the expression [21]:

$$f_{LG} = \frac{24}{\text{Re}_\omega}(1 + 0.15\text{Re}_\omega^{0.687}) + \frac{0.42}{1 + 4.25\times10^4\,\text{Re}_\omega^{-1.16}} \quad (17)$$

At low content of liquid and speeds that are less than the speed of sound, the liquid film is separated, from the disc, in compact layer length 2–3 mm. After that, the film breaks up into the droplets of 0.1–0.2 mm in diameter.

The aim of this part of the article is to find criteria which will define the stability of liquid film flow. From the theory of the liquid film flow [23–25] and analysis of the gas influence on the stability of the liquid film (Kelvin–Helmholty instability) the Weber's number required criteria:

$$We = \frac{\rho_G\bar{c}^2 d}{\sigma} \quad (18)$$

When the value of Weber's number is less than 5.5, the flow of the liquid film is stable and it is not breaking up into droplets. Time, until film reach the critical stage, is:

$$t_{kr} = 1.65\frac{\delta}{c_c}\sqrt{\frac{\rho_L}{\rho_G}}, \text{ s} \quad (19)$$

Critical areas are zones of maximum diameter of the disc (r_2), where the speed of the liquid fraction (c_L = = $r_2\omega$) has maximum or zone of minimum diameter of the disc (r_1), where the rate of gaseous fractions (c_G) has maximum. The influential value for gas and liquid carbon dioxide are shown in Table 4.

Table 4. Physical properties of liquid CO_2 and gas CH_4

Methane CH_4, gas	Carbon dioxide CO_2, liquid
Temperature, t = –70 °C	Temperature, t = –70 °C
Pressure, 5 bar	Pressure, 5 bar
Density, 4.9 kg/m^3	Density 1216.7 kg/m^3
Viscosity	Viscosity
Dynamic, 7.93×10^{-6} Pa s	Dynamic, 0.32×10^{-3} Pa s
Kinematic, 1.618×10^{-6} m^2/s	Kinematic, 0.226×10^{-6} m^2/s
	Surface tension, $\sigma = 0.001$ N/m

DISCUSSION

The possibility of the cleaning of the natural gas (about 16% of world gas reserves are in the category of non-balanced reserves due to a high content of carbon dioxide, CO_2, and hydrogen sulfide, H_2S) could activate

numerous gas deposits. The technological procedures, used for cleaning the natural gas, are numerous, but it has been proved that many of them are connected to the complex technology which requires expensive facilities and great operation expenses. The condensation of the polluting gases and their removal from the natural gas is one of the directions of further development in this field. The research is facing two directions:

- Finding a technologically, technically and economically feasible solution for the condensation of carbon dioxide.
- Finding a technically and economically feasible solution for the separation of liquid carbon dioxide (CO_2) from gas (CH_4).

Based on the analysis of the so far developed procedures, the cleaning of natural gas by separation, a bigger group of authors [3,4,14] as well as authors of this paper [16], observed numerous disadvantages of which we emphasize the following:

- separation in gas-gas separators is slow and economically unacceptable for the cleaning of natural gas;
- separation of the natural gas through condensation CO_2 on the rotor wall is also inefficient and unacceptable for working with natural gas;
- For now, the most suitable solution, the C3-sep procedure with RPS-separators for separating the liquid fraction is more and more accepted in the gas industry, but this solution also has certain disadvantages, some of which the authors have presented in this paper.

The authors have tried to remove the observed disadvantages suggesting a construction of a new separator [14,15], which in their opinion will not only remove the observed disadvantages, but will also enable a continual separation of the natural gas with a continual discharge of pure gas and products (impurities) of separation. While working on the development of a prototype of this separator, the authors face various theoretical and technical problems. Some of these problems the authors represented in their paper that analyze the separation of the liquid-liquid, liquid-solid mixture [16].

Why is it important to precisely define the friction coefficient in the rotor separator insert? First of all for the simple need of defining the pressure drop in the rotor separator insert. The construction of the insert is such that in a certain operation regime (gap size, angular speed) it can act as a disc pump or centrifugal seal, *i.e.*, complete termination or reverse gas movement can appear.

The thickness of the liquid layer, on the disc, and its speed can form a drag force which will draw a great amount of gas to the disc periphery. The consequence of such movement is the reduced capacity and lower quality of separation. This paper gives theoretical solutions of the observed problems.

Relying on established theoretical assumptions we, in this paper, show the process of defining the diameter (d_g) of liquid droplets, given in Table 5 and Figure 6.

Also we calculated the values of the Weber's number, which is important for the stability of the flow of liquid, given in Table 6. Both calculations were done for a prototype that was also used for the treatment of solid–liquid mixtures and its reconstruction is in progress.

Table 5. Definition of d_g for CH_4–liquid CO_2

ω / s^{-1}	R / m	p / bar	H / m	Re×10^{-6}	c_i / m s^{-1}	Ai / m^2	q_i×10^3 / m^3 s^{-1}	q_g×10^3 / m^3 s^{-1}	q_L×10^3 / m^3 s^{-1}	d_G / μm [16]
200	0.0078	5	0.05	0.7	28.1	28.2×10^6	0.7	0.6	0.13	14.8
400				1.5	39.7		1.1	0.9	0.18	13.1
600				2.2	50.6		1.4	1.1	0.23	11.8
800				3	62.8		1.7	1.4	0.28	10.9
100				3.7	75.6		2	1.7	0.28	10.3

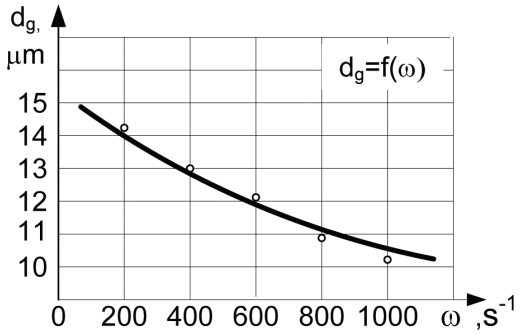

Figure 6. Dependence $d_g = f(\omega)$.

The fact is that each theoretical solution must be tried out on a prototype and the aim of the next paper will be the procedure and the results of research, i.e. confirmation or negation of certain theoretical assumptions.

CONCLUSION

By presenting the new technical solution of the centrifugal separator, the authors point to the expected

Table 6. Definition of Webers number

ω / s^{-1}	$q_T\times10^6$ / m s^{-1}	$c_T\times10^3$ / m s^{-1}	c_g / m s^{-1}	ρ_m / kg m^{-3}	$\bar{c}$ / m s^{-1}	f	$\tau_T\times10^{-6}$ / Pa	δ_T / μm	*We*
200	0.13	0.4	2.17	5.01	2.12	0.0083	0.8	100	0.157
400	0.18	0.6	3.26		3.19	0.0072	1.5	70	0.209
600	0.23	0.8	3.98		3.89	0.0066	5.5	58	0.230
800	0.28	1	5.07		4.89	0.0062	3.7	50	0.270
100	0.28	1	6.15		6.02	0.0060	3.7	45	0.310

improvements, both in the capacity and the quality of gas-liquid mixture separation. Innovations introduced in the separator construction are:

- the periphery edge of the upper and lower half of rotor is constructed as an impeller for the pump for liquid transport;
- the sealing of impellers is done by mechanical seals, which are, also, axial bearings for the rotator;
- the specific construction of the insert allows the liquid film to be caught immediately after a very short fall, directing liquid to the bottom of the lower half of rotor and discharging them immediately next to the rotor's wall against which they slide to the discharge opening;
- by introducing the centrifugal compressor into the separator construction, suction of mixtures (liquid––gaseous) is facilitated and also the losses of pressure are compensated.

The aim of this paper was to point out the certain advantages of the new solution, by presenting the new solution, but what is more important to form theoretical basis for the calculation and construction of the separator. This paper emphasizes the technological part: defining the pressure drop in the separator insert and criteria (critical velocity, c_c) for stability of liquid film flow. Of course, such theoretical considerations must be checked through laboratory and semi-industrial experiments, since the next paper will be dedicated to that task.

Nomenclature

A, χ, m_i, n_i - Coefficients
c, c_i c_m - Speed, m/s
f, f_o, - Coefficient of friction
f_{LG} - Coefficient of friction at the contact of gas–liquid
K, K_L, K_G - Coefficients
N - The number of gaps
q - Flow through the gap, m^3/s
q_v - The flow through the separator, m^3/s
R, R_2- Disk radius, m
Re, Re_ω- Reynolds number
P - Percentage, %
Δp - Pressure drop, Pa
s - The thickness of the gap, mm
We - Weber number
δ, δ_L, δ_T - The thickness of the layer of liquid, m
Δ - Absolute roughness, mm
ρ, ρ_i, ρ_m - Density kg/m^3
ν - Kinematic viscosity, m^2/s
ω_L, $\omega_{D\ LG}$ - The angular speed of the liquid, disc and gas, s^{-1}
σ - Surface tension, N/m
τ - The shear stress, Pa

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IZVOD

CENTRIFUGALNA SEPARACIJA TEČNOG UGLJEN-DIOKSIDA IZ PRIRODNOG GASA

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(Naučni rad)

Prirodni gas se sve više koristi u globalnoj energetskoj potrošnji pre svega zbog lakog transporta do potrošača kao i zbog relativno malog uticaja na životnu sredinu. Nove tehnologije, kao što je konverzija u tečnost, olakšavaju transport i primenu prirodnog gasa. Prirodni gas, dobijen sa eksploatacionog polja, prate brojni zagađivači (tečni, čvrsti i gasoviti) koje treba ukloniti kako bi tržišna vrednost gasa bila veća. Čvrsti i tečni zagađivači se relativno lako uklanjaju, ali više od 16% trenutno poznatih rezervi gasa ne može se koristiti zbog teškog uklanjanja gasovitih zagađivača (CO_2 i/ili H_2S: CO_2 > 10%, H_2S > 5%). Tradicionalna tehnologija, kao što je amino-tretman, se ne može ekonomično primeniti za uklanjanje ovako velikih količina zagađivača. U radu se istražuju mogućnosti primene centrifugalnih separatora za rešavanje ovog problema. Posle analize postojećeg stanja, u centrifugalnoj separaciji prirodnog gasa, neke inovacije u konstrukciji separatora su predložene. Inovacije se sastoje pre svega u mogućnostima, separatora da u jednoj celini, pogonjenoj jednim motorom, realizuje proces čišćenja gasa od: čvrstih, tečnih, a posle tretmana hlađenjem, i gasovitih zagađivača (CO_2 i/ili H_2S). Prednosti ovako koncipiranog tehničkog rešenja bi bile: centralno punjenje i samopražnjenje separatora; jedan motor pogoni čitavo postrojenje; kombinovano radijalno–aksijalno oslanjanje obezbeđuje stabilan rad separatora i pri velikim brzinama rotacije; moguće je postići velika centrifugalna ubrzanja što olakšava proces razdvajanja. Naglasak, u ovom radu, je dat na teorijska razmatranja procesa razdvajanja tečnog ugljen-dioksida (CO_2) od gasovitog metana (CH_4). Cilj predloženih teorijskih razmatranja je da se složena teorijska osnova prilagodi potrebama inženjera pri razvoju, konstruisanju i izradi ovih postrojenja. Kapacitet i kvalitet, separacije su parametri koji čine osnovu ekonomičnosti procesa šišćenja prirodnog gasa. Ovi parametri, uz stabilnost rotora separatora, su osnovni kriterijumi za ocenu kvaliteta konstrukcije jednog separatora. Naravno da se ovako formirane teorijske osnove moraju detaljno proveriti ispitivanjima na laboratorijskim, poluindustrijskim i industrijskim postrojenjima. Deo ovih ispitivanja je urađen [13,16] ali suštinska ispitivanja tek slede.

Ključne reči: Gas • Kalorična vrednost • Tečnost • Separacija • Efikasnost

Determination of kinetic parameters for complex transesterification reaction by standard optimisation methods

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Abstract

This paper represents a methodology for kinetic parameter estimation based on standard optimization methods. The parameter estimation procedure is applied to the example of modelling of non-catalytic transesterification reaction, based on laboratory experiments performed under elevated pressure. The kinetic model employed in this study consists of three consecutive and parallel reversible reactions of the second order, with six kinetic constants. The influence of the mass transfer effects was considered as well. The best results were obtained by Genetic Algorithm method. The application of this method resulted in kinetic parameters with improved accuracy in predicting concentrations of important reaction intermediates, *i.e.*, diglycerides and monoglycerides. Activation energies of kinetic parameters obtained by the Genetic Algorithm method are in line with theoretical values determined by molecular orbital calculations.

Keywords: optimisation technique, genetic algorithm, short-cut method, kinetic parameters, high pressure process.

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Research on chemical kinetics of complex reactions of industrial importance often requires the estimation of rate or equilibrium coefficients by nonlinear regression [1]. A variety of methods are applied to solve practical problems in parameter optimization, starting from traditional calculus methods and ranging to the so-called ''evolutionary algorithms'' [2]. These methodologies have been extensively applied during the past two decades to the modelling of various industrial processes [3].

The transesterification reaction represents challenge for many researchers, mainly due to its application for synthesis of renewable biofuels. This reaction can be performed without presence of homogeneous or heterogeneous catalyst, if reaction pressure and temperature are elevated above certain values. The subcritical transesterification is a promising method for a more environmentally friendly biodiesel production, as a result of its feedstock flexibility, production efficiency and environmentally friendly benefits. The investigation of subcritical and supercritical methanolysis of triglycerides and determination of kinetic parameters has been the objective of several papers published in the last ten years [4–9].

The methanolysis of triglycerides that forms fatty acid methyl esters (FAME), as end products, is the complex reaction which can be represented as parallel--consecutive kinetic model consisting of three second-order reversible reactions [4]. The composition of reaction mixture at the end of synthesis comprises mainly the products: methyl esters, the small amount of glycerol (up to 5 mass%), and the large excess of methanol. Monoglycerides and diglycerides are formed as intermediate products and could be present only in traces in a mixture upon reaction completion. However, those minute concentrations of reaction intermediates could impact on biodiesel quality and compliance with the current technical standards. Therefore, it is extremely important to be able to predict an optimum reaction time or a reactor space time, for this reaction [5,8].

This paper presents the determination of kinetic parameters of the non-catalytic biodiesel synthesis under elevated pressure and temperature. Three different numerical methods: Simulated annealing, the lsqcurvefit with Levenberg–Marquardt algorithm and Global optimization technique – Genetic Algorithm, have been used to calculate kinetic parameters. The influence of mass transfer was calculated, since it was found to influence strongly on the reaction rate in the initial phase of the reaction and consequently to the values of reaction kinetic parameters.

EXPERIMENTAL PROCEDURE

The high pressure batch reactor, volume of 2 dm^3, mechanically agitated (Ernst Haage, Germany), was used for the analysis of reaction rate of triglycerides methanolysis at 150 and 210 °C and 1.0 and 4.5 MPa.

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The specified amounts of methanol and sunflower oil corresponding to molar ratio 42 to 1 were used. Detailed description of the experimental procedure can be found in the literature [6,8]. The composition profile during reaction for both analysed conditions is shown in Table 1.

Mathematical modelling and numerical optimisation

Kinetic model description

Transesterification or FAME synthesis under sub-critical conditions is a catalyst-free chemical reaction between triglycerides (the major component in vegetable oils, animal fats, and used vegetable oils) with a low molecular weight alcohol, usually methanol, at a temperature and pressure under the critical point of triglyceride-alcohol mixture. The overall reaction occurs as a sequence of three steps, parallel with respect to alcohol and consecutive with respect to triglyceride. Triglyceride (TG) reacts with an alcohol (ROH) in the first reaction and forms diglyceride (DG) and fatty acid methyl ester (FAME). Monoglycerides (MG) and fatty acid methyl esters (FAME) are formed in the second reaction from diglyceride (DG) and methanol. The final products, appearing as products of the third reaction, are glycerol (GL) and again fatty acid methyl esters (FAME). The reaction scheme is shown below:

$$\mathrm{TG} + \mathrm{ROH} \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} \mathrm{DG} + \mathrm{FAME} \quad (1)$$

$$\mathrm{DG} + \mathrm{ROH} \underset{k_{22}}{\overset{k_{21}}{\rightleftharpoons}} \mathrm{MG} + \mathrm{FAME} \quad (2)$$

$$\mathrm{MG} + \mathrm{ROH} \underset{k_{32}}{\overset{k_{31}}{\rightleftharpoons}} \mathrm{GL} + \mathrm{FAME} \quad (3)$$

It is important to point out that the reversible reactions of DG, MG and GL with FAME control the maximum/equilibrium conversion of triglycerides. The reaction rate for each component in the system (constant volume batch system) can be represented by the following set of equations where each reaction step

Table 1. The reaction mixture composition profile during methanolysis of sunflower oil at 150 °C and 1.0 MPa, and at 210 °C and 4.5 MPa

Time, min	Concentration, kmol/dm^3					
	MeOH	TG	DG	MG	FAME	Glycerol
Experiment 1 (150 °C and 1.1 MPa)						
0	2.833	6.84E-02	0.00	0.00	0.00	0.00
256	2.807	4.75E-02	1.51E-02	3.71E-03	2.60E-02	8.18E-04
613	2.795	3.92E-02	1.98E-02	5.83E-03	3.83E-02	1.60E-03
1228	2.772	2.95E-02	2.83E-02	9.78E-03	6.07E-02	2.30E-03
1433	2.762	2.40E-02	3.13E-02	1.49E-02	7.08E-02	4.48E-03
1633	2.747	1.74E-02	2.02E-02	2.16E-02	8.57E-02	6.23E-03
1933	2.715	1.03E-02	9.10E-03	2.83E-02	1.18E-01	6.97E-03
2623	2.699	7.49E-03	7.87E-03	2.34E-02	1.34E-01	9.83E-03
3028	2.676	3.04E-03	6.56E-03	1.58E-02	1.57E-01	1.68E-02
9000	2.639	0.00	0.00	0.00	1.94E-01	6.06E-02
Experiment 2 (210 °C and 4.5 MPa)						
0	2.835	6.78E-02	0.00	0.00	0.00	0.00
130	2.806	3.56E-02	1.96E-02	1.92E-02	3.35E-02	0.00
160	2.755	3.42E-02	1.49E-02	2.54E-02	4.17E-02	0.00
190	2.735	2.92E-02	1.38E-02	2.83E-02	5.67E-02	0.00
220	2.715	2.20E-02	1.40E-02	2.80E-02	7.97E-02	4.37E-03
250	2.698	1.70E-02	7.89E-03	3.12E-02	1.05E-01	1.24E-02
280	2.675	1.29E-02	5.45E-03	2.78E-02	1.28E-01	2.23E-02
340	2.659	7.02E-03	5.90E-03	1.56E-02	1.58E-01	3.99E-02
400	2.648	3.65E-03	5.13E-03	7.92E-03	1.75E-01	5.17E-02
460	2.641	2.66E-03	5.04E-03	4.64E-03	1.79E-01	5.61E-02
520	2.637	1.49E-03	3.57E-03	2.48E-03	1.86E-01	6.09E-02
580	2.633	3.35E-04	4.96E-04	1.84E-03	1.95E-01	6.58E-02
640	2.632	2.49E-04	2.40E-04	1.44E-03	1.98E-01	6.65E-02
700	2.630	2.31E-04	2.90E-05	1.28E-03	2.00E-01	6.69E-02
760	2.630	0.00	0.00	7.61E-04	2.02E-01	6.77E-02

is assumed to be second order in both directions and therefore dependent on concentration of reacting components:

$$r_{TG} = \frac{dC_{TG}}{dt} = -k_{11}C_{TG}C_{ROH} + k_{12}C_{DG}C_{FAME} \quad (4)$$

$$r_{DG} = \frac{dC_{DG}}{dt} = k_{11}C_{TG}C_{ROH} - k_{12}C_{DG}C_{FAME} - \\ -k_{21}C_{DG}C_{ROH} + k_{22}C_{MG}C_{FAME} \quad (5)$$

$$r_{MG} = \frac{dC_{MG}}{dt} = k_{21}C_{DG}C_{ROH} - k_{22}C_{MG}C_{FAME} - \\ -k_{31}C_{MG}C_{ROH} + k_{32}C_{GL}C_{FAME} \quad (6)$$

$$r_{GL} = \frac{dC_{GL}}{dt} = k_{31}C_{MG}C_{ROH} - k_{32}C_{GL}C_{FAME} \quad (7)$$

$$r_{FAME} = \frac{dC_{FAME}}{dt} = -3\left(\frac{dC_{TG}}{dt}\right) - 2\left(\frac{dC_{DG}}{dt}\right) - \left(\frac{dC_{MG}}{dt}\right) \quad (8)$$

$$r_{ROH} = \frac{dC_{ROH}}{dt} = -\frac{dC_{FAME}}{dt} \quad (9)$$

If the reaction scheme is represented by the above equations the concentrations of reacting species are assumed to be numbers of moles divided by the overall reaction volume, regardless of the existence of multiple phases. Each reaction is characterized by its reaction rate constant (both forward and reverse reactions). The slowest forward reaction rate controls the overall reaction rate along with reaction equilibrium constant for each of the reversible reactions shown above [6].

Batch reaction system containing triglycerides and methanol, at investigated pressure and temperature, is characterised by the equilibrium between two liquid phases at the beginning of reaction. During methanolysis of triglycerides, the phase distribution is changing according to the actual composition of reaction mixture, temperature and pressure, for a given time of reaction. Distribution of methanol between the oil phase, the methyl esters phase and the glycerol rich phase strongly depends on operating conditions [8].

The rate of triglycerides methanolysis depends also on the phase equilibrium and on the methanol distribution in the oil-rich phase. Up to 150 °C and 1.1 MPa the oil is present only in one liquid phase together with a smaller amount of dissolved methanol [6,8]. The methanol to oil molar ratio is 1:1, three times less than required by stoichiometry, thereby causing very low reaction rate (complete conversion could be achieved after approximately 150 h). At 210 °C and 4.5 MPa the methanol to oil molar ratio in the oil phase is changing from 6:1 to 10:1 through the course of reaction, thus increasing the rate of reaction and resulting in complete conversion of triglycerides in approximately 10 h. The methyl esters being produced form the third liquid phase which contains almost all diglycerides, monoglycerides and glycerol, together with certain amount of methanol. This is also the period when probability for reverse reaction between glycerol and FAME is much higher than probability of reaction between glycerides (present in low concentrations) and methanol. At the end of reaction (at 210 °C and 4.5 MPa) one liquid phase contains methanol, FAME and glycerol, as well as certain small amount of monoglycerides and diglycerides [6,8].

The sigmoid shape of the conversion curves, during FAME synthesis, points out to a complex reaction mechanism. Initially slow reaction rate increases with conversion and in its final stage the FAME yield curve reaches a plateau. At the beginning of reaction, the interfacial area between the phases containing reactants is dependent on the agitation intensity and mass transfer controls the overall reaction rate ($k_{kinetic}$ >> >> $k_{mass\ transfer}$). This is more pronounced at conditions corresponding to low methanol solubility in the oil phase, *e.g.*, under lower pressure and temperature. Therefore, the kinetic constant for the forward reaction of triglycerides conversion could be corrected with following equation, introducing the mass transfer effects [6]:

$$k_{11}' = k_{mt} + k_{11}(C_{DG} + C_{MG})/C_{TGo} \quad (10)$$

where the k_{mt} represents the mass transfer controlled kinetic constant in the initial phase of the reaction. Increasing conversion followed by changing phase distribution, increasing concentrations of intermediates (mono and diglycerides) and the enhancement of interfacial area will result in the increase of overall kinetic constant [6]. Based on the calculation of mass transfer coefficient in mechanically stirred system the numerical value of k_{mt} for the first and second experiment was determined as 9.2×10^{-7} and 1.5×10^{-6} dm^3/(kmol min) respectively [6]. Intense agitation provides sufficiently high values of mass transfer coefficient to accommodate for methanol consumed by the reaction in oil phase, while the extent of reaction in methanol rich phase can be neglected due to very low concentrations of glycerides in that phase [6,8]. Hence, the calculation of apparent values for kinetic parameters using the overall reaction volume can be applied with the incorporation of mass transfer effects at the beginning of reaction.

Parameter determination by different optimization techniques

The proposed kinetic model for biodiesel synthesis is nonlinear implying that finding the best set of values for kinetic parameters requires the use of optimization techniques and/or their combinations. In general, the techniques used for parameters estimation can be

divided into two groups: the short cut methods and the global optimization techniques that do not require initial guesses [10–15].

Due to the fact that most of the methods for parameters estimation require good initial guess to find the optimal set of results, finding the initial values of unknown parameters are essential for successful parameters estimation. In this study model parameters, or reaction constants, were determined using Simulated Annealing (SA), lsqcurvefit (LM) and Genetic Algorithm (GA) methods (as defined functions in MATLAB). In order to make a comparison among these optimization methods the following objective function was defined for all of them:

$$\text{Objective functon} = \sum_{i=1}^{6}\sum_{j=1}^{n}\left(\frac{C_{i,j}^{\text{Exp}} - C_{i,j}^{\text{Model}}}{C_{i,\max}^{\text{Exp}}}\right)^2 \qquad (11)$$

where *i* refers to the component (TG, DG, MG, GL, FAME and ROH), and *j* refers to the experimental data for each component. $C_{i,\max}^{\text{Exp}}$ is the maximum concentration of component *i* within the experimental data set.

The default parameters were defined as the parameters predetermined by MATLAB. The only deviation from this was the applied algorithm for solving optimization by lsqcurvefit which was changed from trust-region-reflective (default) to Levenberg-Marquardt.

Simulated annealing (SA)

Simulated annealing is a probabilistic global search method [16,17]. SA is stochastic search techniques and it is used when the structure of a space is not well understood or is not smooth. In particular, these techniques are frequently used to solve combinatorial optimization problems, such as the travelling salesman problem. The goal is to find a point in the space at which a real valued energy function (or cost function) is minimized. Simulated annealing is a minimization technique which has given good results in avoiding local minima; it is based on the idea of taking a random walk through the space at successively lower temperatures, where the probability of taking a step is given by a Boltzmann distribution [16,17].

The lsqcurvefit with Levenberg–Marquardt algorithm (LM)

The lsqcurvefit solves nonlinear data-fitting problems. The lsqcurvefit requires a user-defined function to compute the vector-valued function *F(x, xdata)*. The size of the vector returned by the user-defined function must be the same as the size of *ydata* [18,19].

The Levenberg–Marquardt algorithm (LMA), also known as the damped least-squares (DLS) method, is an iterative technique which provides a numerical solution to the problem of minimizing a function, generally nonlinear, over a space of parameters of the function. These minimization problems arise especially in least squares curve fitting and nonlinear programming. It has become a standard technique for non-linear least-squares problems, widely adopted in a broad spectrum of disciplines [18,19].

The LMA interpolates between the Gauss–Newton algorithm (GNA) and the method of gradient descent. When the current solution is far from the correct one, the algorithm behaves like a steepest descent method (slow but certain convergence). When the current solution is close to the correct solution, it becomes a Gauss-Newton method [17,18]. The LMA is a very popular curve-fitting algorithm used in many software applications for solving generic curve-fitting problems. However, the LMA finds only a local minimum, not a global minimum [18,19].

The first initial guess required for these two techniques (LM and SA) was found by applying combination of two short cut methods. This approach combines differential method of analysis [20] and method for kinetics parameters estimation proposed by Glowinski and Stocki [21]. In order to reveal the dependency of the optimum solution to the initial guess, three guesses were considered for each experiment. The first guess is $0.1\times\mathbf{x}_0$, the second guess is $\mathbf{x}_0$, and the last one is $10\times\mathbf{x}_0$, where $\mathbf{x}_0$ is the vector containing the obtained values of reaction constants by linear technique.

Global optimization technique - Genetic Algorithm

Nowadays this technique is widely used. Commonly, genetic algorithm optimization technique is used to estimate initial values of parameters, because this approach does not require any initial assumption of parameters' value. The method compares experimental data of species' concentration with values predicted by the model. Due to the fact that concentration of species' values differ from each other by several orders of magnitude and sum of squares of deviation between experimental and modelled values are summarized in one objective function, the values should be brought to the same numeric interval. There are several techniques to achieve this: *i*) minimize square of relative error between experimental and model predicted values of concentration and *ii*) involve variances of experimental measurements. The drawback of using square of relative error is that error of each experimental measurement has significant impact on objective function. On the other side, using variance of experimental measurements are more convenient, since error of measurements is averaged which results in lower impact of individual measurement error on objective function. If values of variances of experimental values are not available, they can be optimized together with unknown rate reaction parameters. The drawback of this technique is that it increases number of parameters to be optimized [22–27].

The genetic algorithm technique makes use of the Darwinian survival of the fittest procedure. These are search procedures based on mechanics of natural genetics and natural selection [22]. Five GA operators are used in the DNA-GA to enhance the searching ability of the GA [23]. In particular, the "mutation step" helps in avoiding getting trapped in local minima during the search procedure [23]. The parameters were searched within non-negative numbers.

RESULTS AND DISCUSSION

The kinetic model, as defined by Eqs. (4)–(9), including modification of k_{11} rate constant as shown by Eq. (10), was applied to the experimental data of transesterification reaction performed under subcritical conditions (150 °C and 1.1 MPa and 210 °C and 4.5 MPa). Six kinetic parameters for forward and reverse reactions were obtained by different approaches using numerical methods.

The results obtained by SA method are presented in Table 2. The initial guess was obtained using linear technique. In order to reveal the dependency of the optimum solution on the initial guess, three guesses were considered for each experiment. The first guess is $0.1\times\mathbf{x}_0$, the second guess is x_0, and the last one is $10\times\mathbf{x}_0$. The optimum values of the kinetic constants depended significantly on the initial guess, and the second initial guess was found to result in the minimum error (Eq. (11)).

The results obtained by LM method are shown in Table 3. The same initial guesses as in the case of SA were used. The results have indicated that the optimum value is practically insensitive and independent on the initial guess, and therefore only one set of results is shown in Table 3. Also, error values are considerably lower than in the case of SA method. In order to check whether the results obtained by LM are a local optimum or the global one, the LM program was performed with various initial guesses. For generating logical and different initial guess values and to avoid trapping into a local minimum, the initial guesses were determined using GA with sufficient population size (the default population size was changed from 20 to 500). In this way the time required for running the GA program increased significanly, but it has been confirmed that correct initial guess values have been obtained. The results showed that the data presented in Table 3 are really the global optimum.

The kinetic parameters obtained by GA method are shown in Table 4. The error values are slightly higher than in the case of LM method. By comparing results shown in Tables 2–4, it can be observed that LM method results in lower minimum error than SA method and even lower than GA method.

Table 2. Kinetic constants (kmol/(dm^3 s)) obtained by SA method

Constant	Exp. 1		Exp. 1 with modified k_{11}		Exp. 2		Exp. 2 with modified k_{11}	
	Initial guess	Optimum	Initial guess	Optimum	Initial guess	Optimum	Initial guess	Optimum
k_{11}	7.58E-07	5.83E-06	7.58E-07	1.95E-05	3.70E-06	5.73E-05	3.70E-06	2.32E-04
k_{12}	0.00E+00	7.40E-06	0.00E+00	3.75E-05	0.00E+00	3.12E-03	0.00E+00	8.58E-06
k_{21}	2.20E-07	6.38E-06	2.20E-07	8.45E-06	7.83E-06	6.32E-05	7.83E-06	1.13E-04
k_{22}	0.00E+00	0.00E+00	0.00E+00	4.60E-06	0.00E+00	1.17E-04	0.00E+00	8.15E-04
k_{31}	2.15E-07	3.20E-05	2.15E-07	8.37E-05	6.18E-06	2.37E-03	6.18E-06	7.98E-04
k_{32}	0.00E+00	2.98E-05	0.00E+00	1.28E-04	0.00E+00	2.85E-03	0.00E+00	6.32E-05
Error		3.286		3.741		6.129		5.359
k_{11}	7.58E-06	1.67E-05	7.58E-06	2.80E-05	3.70E-05	3.70E-05	3.70E-05	2.63E-04
k_{12}	0.00E+00	4.45E-06	0.00E+00	1.58E-05	0.00E+00	0.00E+00	0.00E+00	3.62E-04
k_{21}	2.20E-06	5.03E-06	2.20E-06	1.25E-05	7.83E-05	7.83E-05	7.83E-05	1.30E-04
k_{22}	0.00E+00	0.00E+00	0.00E+00	2.13E-05	0.00E+00	0.00E+00	0.00E+00	7.35E-04
k_{31}	2.15E-06	4.82E-06	2.15E-06	5.20E-06	6.18E-05	6.18E-05	6.18E-05	9.30E-04
k_{32}	0.00E+00	0.00E+00	0.00E+00	1.78E-05	0.00E+00	0.00E+00	0.00E+00	1.20E-03
Error		2.678		2.656		1.552		4.999
k_{11}	7.58E-05	1.83E-05	7.58E-05	4.45E-05	3.70E-04	4.78E-05	3.70E-04	3.30E-04
k_{12}	0.00E+00	5.80E-04	0.00E+00	1.73E-04	0.00E+00	9.85E-04	0.00E+00	5.50E-04
k_{21}	2.20E-05	1.08E-05	2.20E-05	1.47E-05	7.83E-04	6.17E-04	7.83E-04	2.05E-04
k_{22}	0.00E+00	2.15E-04	0.00E+00	3.88E-04	0.00E+00	5.40E-03	0.00E+00	2.53E-03
k_{31}	2.15E-05	4.23E-05	2.15E-05	6.73E-05	6.18E-04	1.92E-03	6.18E-04	1.27E-03
k_{32}	0.00E+00	1.16E-03	0.00E+00	3.72E-04	0.00E+00	1.10E-02	0.00E+00	2.75E-03
Error		2.996		3.642		7.237		5.738

Table 3. Kinetic constants (kmol/(dm^3 s)) obtained by LM method

Constant	Exp. 1	Exp. 1 with modified k_{11}	Exp. 2	Exp. 2 with modified k_{11}
k_{11}	5.92E-06	2.77E-05	4.25E-05	2.30E-04
k_{12}	7.15E-05	3.92E-04	3.72E-19	1.57E-03
k_{21}	5.42E-06	5.80E-06	8.38E-05	1.43E-04
k_{22}	3.70E-20	3.70E-20	3.72E-19	2.12E-04
k_{31}	3.42E-06	3.52E-06	4.28E-05	4.68E-05
k_{32}	3.70E-20	3.70E-20	3.72E-19	3.70E-19
Error	0.582	0.683	1.167	0.892

Table 4. Kinetic constants (kmol/(dm^3 s)) obtained by GA method

Constant	Exp. 1	Exp. 1 with modified k_{11}	Exp. 2	Exp. 2 with modified k_{11}
k_{11}	5.03E-06	1.60E-05	4.25E-05	2.10E-04
k_{12}	3.82E-06	1.19E-05	1.40E-05	1.37E-04
k_{21}	5.93E-06	8.03E-06	8.53E-05	1.54E-04
k_{22}	1.04E-07	5.17E-06	1.39E-05	1.41E-04
k_{31}	3.60E-06	4.62E-06	4.35E-05	5.12E-05
k_{32}	5.43E-09	6.62E-07	1.78E-06	1.19E-06
Error	0.629	0.999	1.182	1.040

Model equations (4)–(9) were solved using kinetic parameters obtained by LM and GA methods. The resulting composition profiles for each component, at analysed temperatures and pressures, are shown in Figure 1 for LM method and Figure 2 for GA method.

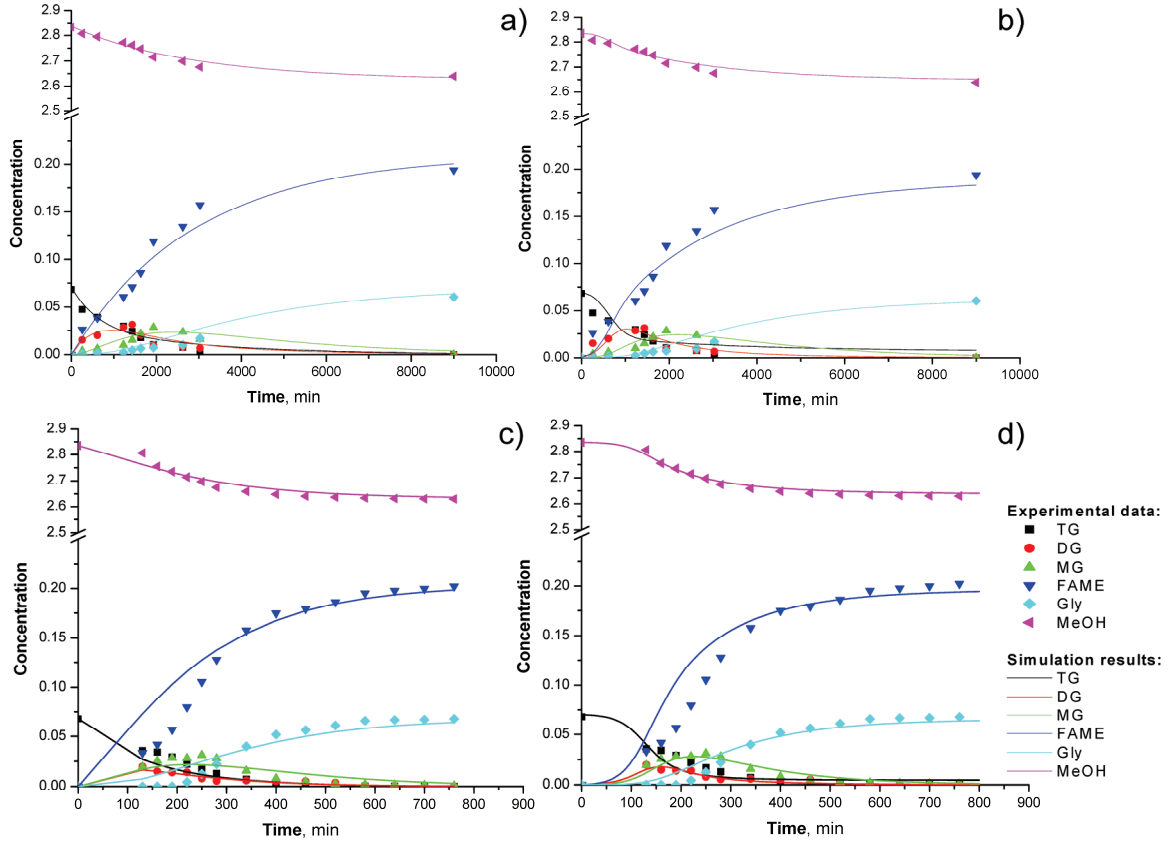

Figure 1. The comparison of model simulation results (kinetic constants obtained by LM method) and experimental results: a) Exp. 1; b) Exp. 1 with modified k_{11}; c) Exp. 2; d) Exp. 2 with modified k_{11}.

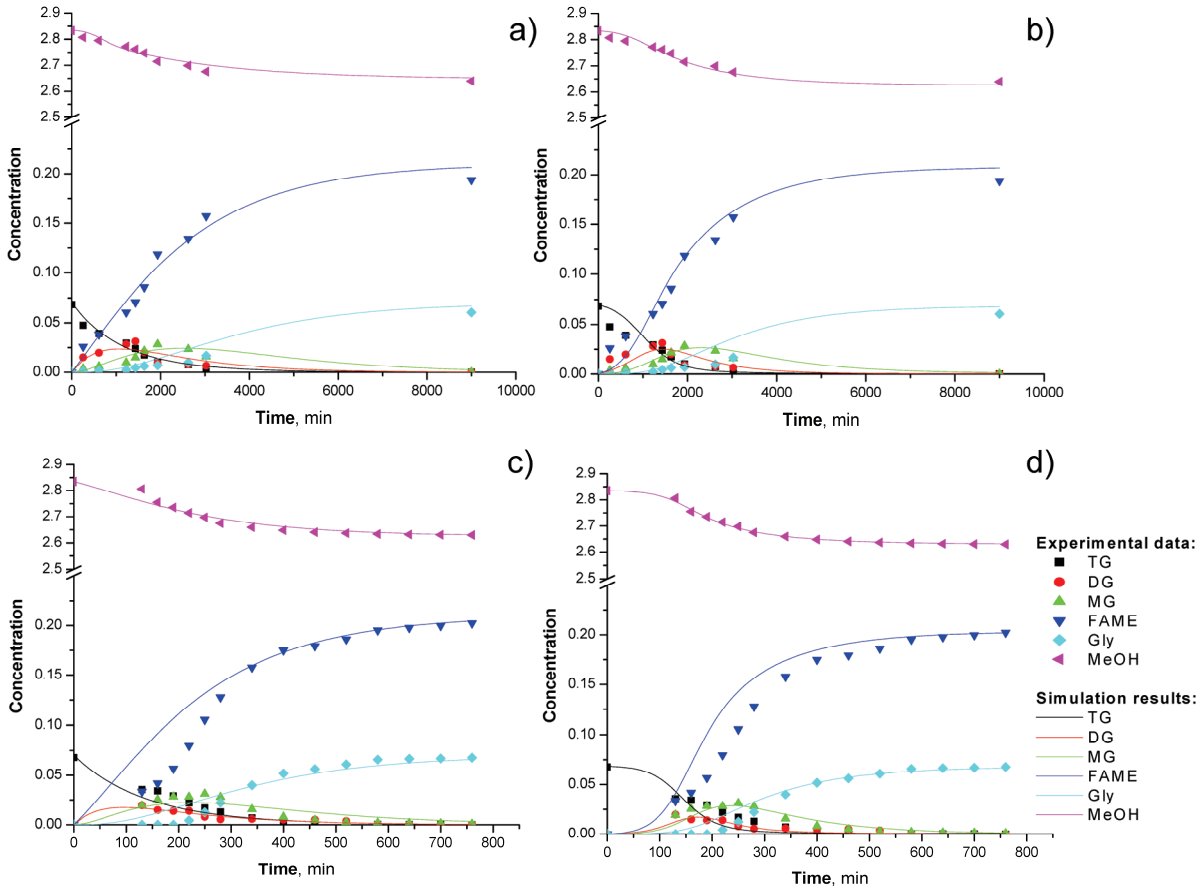

Figure 2. The comparison of model simulation results (kinetic constants obtained by GA method) and experimental results: a) Exp. 1; b) Exp. 1 with modified k_{11}; c) Exp. 2; d) Exp. 2 with modified k_{11}.

The obtained results indicate that kinetic parameters determined by LM and GA are in line with experimental data obtained at 150 °C and 1.1 MPa (Exp. 1) with (Figure 1a and b) or without (Figure 2a and b) mass transfer limitations included in the expression for kinetic constant k_{11}. However, kinetic parameters deviate considerably from the experimental results at 210 °C and 4.5 MPa, if the mass transfer limitations are not included in the kinetic constant k_{11} (Figures 1c and 2c). Introducing the mass transfer modification of k_{11} kinetic constant improves accuracy, but still doesn't lead to the excellent correlation of experimental data. This is especially the case for concentration profile of FAME. Judging by FAME concentration profile, it would seem that kinetic parameters obtained by numerical optimisation methods correlate experimental data slightly worse than the parameters obtained by simplified kinetic procedure (Figure 6b in reference [6]).

Error values were thus calculated (using Eq. (11)) for the procedure based only on triglycerides conversion and these were found to be 3.899 and 3.459 for the Exp. 1 and Exp. 2, respectively. Obviously these values are considerably higher than for LM and GA methods. Concentration profiles of intermediate species (diglycerides and monoglycerides) for these three methods are shown in Figure 3.

It is clear that kinetic parameters obtained by optimisation methods are superior in predicting concentration profiles of diglycerides and monoglycerides, and this is especially the case for GA method. The ability to predict accurately concentrations of intermediates could be extremely important for proper design of reactor system for FAME synthesis. This is in fact critical parameter for FAME biodiesel quality due to limitations imposed by pertaining technical standards.

As stated previously the error values of LM method are slightly better than for GA method. However, careful inspection of values for kinetic parameters k_{11}–k_{32} in Table 3 shows that kinetic constant for the first reverse reaction k_{12} is higher than forward reaction. However, this type of behaviour shouldn't be seen in this type of reactive system [28]. Also, values of k_{22} at 150 and 210 °C indicate that activation energy for k_{22} should be equal to 1026.2 kJ/mol which is hardly possible when compared to the values obtained in theoretical study using molecular orbital calculations [29,30].

Kinetic constants obtained by the GA method (Table 4) indicate that equilibrium constants are always above

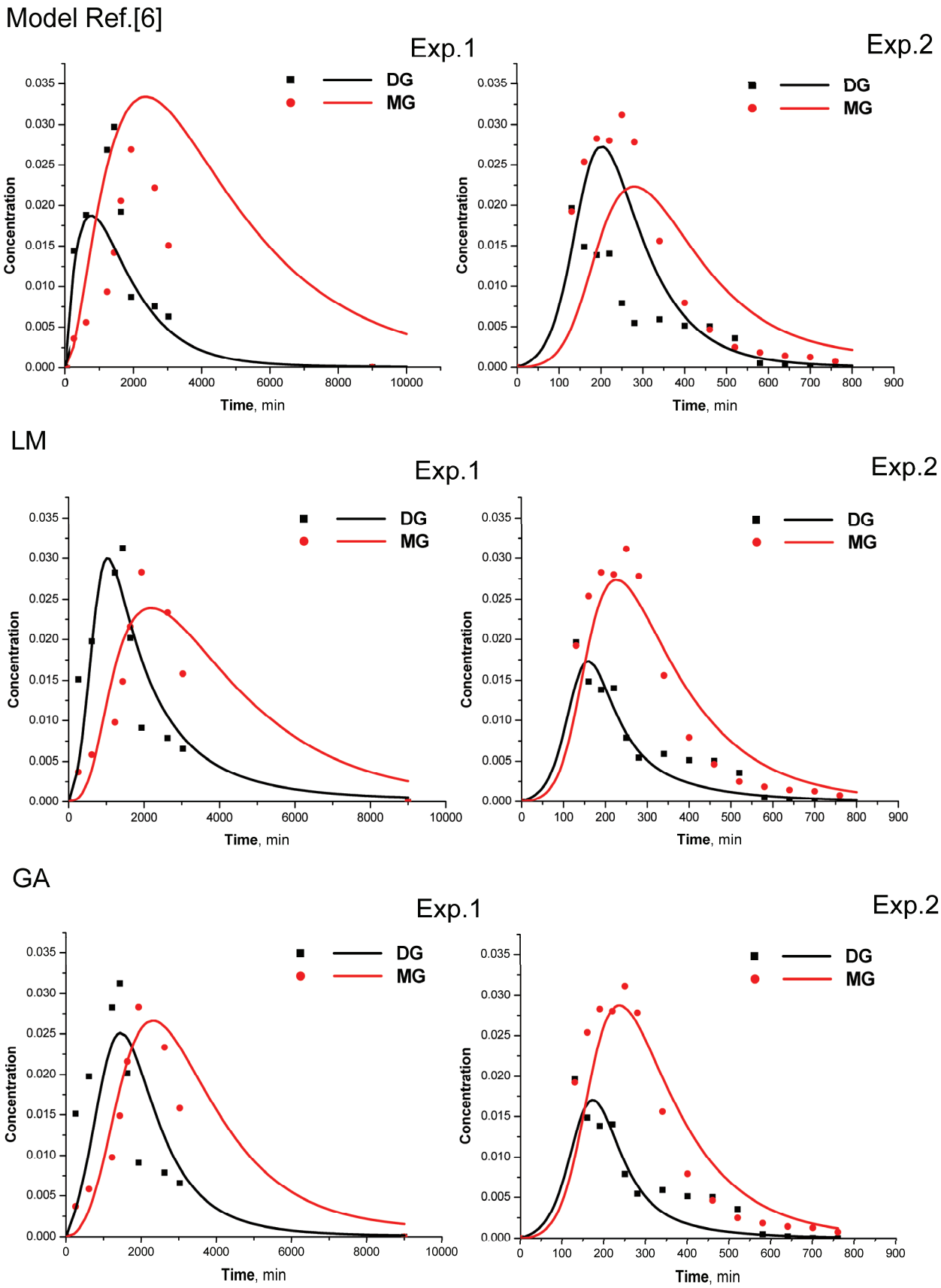

Figure 3. Comparison of the experimental and simulated data using kinetic constants obtained with the GA and LM optimisation methods and the simplified procedure [6].

1 and also that equilibrium constants decrease with increasing temperature, which is in accordance with theoretical calculations of phase and chemical equilibrium [28]. Furthermore, the values of activation energies for forward reactions (in k_{11}, k_{21} and k_{31}) are in line with the values obtained through theoretical calculations based on molecular orbital calculations for synthesis under acidic conditions [29–31]. Values for route (c) from [30] have been used to make comparison and this is shown in Table 5.

Table 5. The comparison of Ea (kJ/mol) for forward reactions based on molecular orbital calculations [30] and values obtained in this study by the GA method

Constant	*Ea* taken from [30]	*Ea* by GA method
k_{11}	79.4	72.8
k_{21}	71.1	83.5
k_{31}	83.6	68.0

CONCLUSION

The parameter estimation procedures were applied to the determination of kinetic parameters of non-catalytic fatty acids methyl esters (FAME) synthesis under elevated pressure. Experimental data were obtained using mechanically agitated batch reactor of constant volume at 150 °C and 1.1 MPa and 210 °C and 4.5 MPa. The kinetic model used in this study consisted of three consecutive and parallel reversible reactions of second order. The kinetic constants for forward and reverse reaction steps were obtained by three different types of numerical methods: Simulated Annealing (SA), lsqcurvefit with Levenberg–Marquardt algorithm (LM) and Genetic Algorithm (GA). The proposed kinetic model and kinetic parameters determined by the GA method, with the inclusion of mass transfer limitations, fitted well the experimental data. Predictions of triglycerides conversion and FAME yield were very good, as well as the predicted concentrations of intermediates (mono- and diglycerides). The activation energies of forward reactions are in line with values obtained by molecular orbital calculations.

Nomenclature

Symbols

- C concentration (kmol dm^{-3})
- E_A activation energy (kJ kmol^{-1})
- K equilibrium constant
- k kinetic constant of reaction (kmol dm^{-3} min^{-1})
- P pressure (MPa)
- T temperature (^{o}C)
- t time (minute or second)
- V volume (dm^3)

Abbreviations

- TG triglycerides
- DG diglycerides
- MG monoglycerides
- FAME fatty acid methyl esters
- MeOH methanol

Subscripts

- *n1* n = 1,2,3, index for the forward reaction
- *n2* n = 1,2,3, index for the reverse reaction

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IZVOD

ODREĐIVANJE KINETIČKIH PARAMETARA SLOŽENE REAKCIJE TRANSESTERIFIKACIJE PRIMENOM STANDARDNIH OPTIMIZACIONIH METODA

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(Naučni rad)

U ovom radu je predstavljena metodologija i rezultati određivanja kinetičkih parametara za složenu reakciju transesterifikacije korišćenjem standardnih optimizacionih metoda. Određivanje kinetičkih parametara različitim optimizacionim metodama izvedeno je za nekatalizovane reakcije transesterifikacije suncokretovog ulja sa metanolom. Reakcija je ispitivana eksperimentima u šaržnom reaktoru pod uslovima povišenog pritiska i temperature. Ova reakcija predstavlja jedan složen reakcioni sistem, kako sa aspekta kinetike hemijskih reakcija tako i sa aspekta ravnoteže faza i prenosa mase. Sistem je višekomponentni pri čemu se ukupni sastav znatno menja tokom reakcije, a u zavisnosti od temperature i pritiska, reakcioni sistem menja i broj i sastav faza u ravnoteži. Primenjeni kinetički model čini sistem od tri povratne uporedno-uzastopne reakcije drugogog reda. Pod uslovima pritiska i temperature ispitanim u ovom radu (150 °C i 1,1 MPa; 210 °C i 4,5 MPa), ovaj reakcioni sistem u početnoj fazi čine dve tečne faze u termodinamičkoj ravnoteži. Zbog ove činjenice u početnoj fazi reakcije u obzir je uzet i uticaj prenosa mase na kinetičke parametre. Tokom reakcije koju karakteriše nastajanje međuproizvoda, mono i diglicerida, dolazi do stvaranja mikroemulzije što je potvrđeno prethodnim istraživanjima. Stoga se ovakav sistem u naknadnoj fazi može smatrati uniformnim po celoj zapremini. Optimizacione metode primenjene u ovom radu zasnivaju se na primeni Levenberg–Marquardt logaritma, *Simulated Anneling* i genetskog algoritma. Najbolji rezultati su dobijeni primenom genetskog algoritma, odnosno kinetički parametri dobijeni tim proračunom pokazuju najmanje odstupanje predviđanja koncentracija svih reaktanata i proizvoda u odnosu na eksperimentalne podatke. Primenom optimizacionih metoda na ovako složen sistem moguće je dobiti bolje predviđanje koncentracija intermedijera u odnosu na druge metode koje se mogu naći u literaturi. Takođe, postoje razlike u kvalitetu rešenja dobijenim različitim optimizacionim metodama. Kinetički parametri dobijeni primenom Levenberg–Marquardt algoritma dobro numerički opisuju realni sistem ali vrednosti kinetičkih parametara ne odgovaraju teoretskim vrednostima koje se očekuju na osnovu mehanizma reakcije. Sa druge strane, vrednosti energija aktivacije ove složene reakcije koje su dobijene primenom genetskog algoritma, u veoma su dobrom slaganju sa vrednostima energija aktivacije koje su dobijene proračunom molekulskih orbitala.

Ključne reči: Optimizacione metode • Genetski algoritam • Kinetički parametri složene reakcije transesterifikacije • Matematičko modelovanje nekatalizovane transesterifikacije triglicerida

Separation of digoxin by liquid–liquid extraction from extracts of foxglove secondary glycosides

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Abstract

The present study deals with the extraction of digoxin (Dgx) from chloroform and trichloroethylene extracts of the secondary glycosides of fermented foxglove (*Digitalis lanata* Ehrh.) foliage by liquid–liquid extraction. The extraction degree (*ED*) of Dgx achieved by maceration and percolation using 10 vol.% aqueous ethanol solution was higher than 95%. Using trichlorethylene and chloroform, *ED* of Dgx of about 100 and 96%, respectively, from the liquid ethanolic extracts (macerate or percolate) were achieved by the four-cycle extraction. Fifteen separating funnels were employed for the liquid-liquid extraction. Three different four-component two-phase systems (ethanol:water–chloroform:ethyl acetate, ethanol:water–chloroform:trichloroethylene and ethanol:water–trichloroethylene:ethyl acetate) were tested as an extracting solvent to get the final product having more than 98% of Dgx. The initial amount of the chloroform or trichloroethylene extract in the light phase was varied between 5 and 25 g/L, while the volume ratio of light and heavy phases was in the range of 1:1 to 1:2. The best Dgx yield of 98% was achieved with the system ethanol:water–chloroform:trichloroethylene 35:15:20:30 at the volume ratio of the phases of 1:1.1 and at the initial amount of the extract of 15 g/L. Purity of the separated digoxin was 99.8%.

Keywords: digoxin, foxglove, *Digitalis lanata* Ehrh., liquid–liquid extraction, solid–liquid extraction.

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Bioactive products from plant materials, known as phytochemicals, are often used in manufacturing of phytopharmaceuticals and phytotherapy because of their specific characteristics [1]. Phytopreparations are increasingly being used for various treatments in both human and veterinary medicine, especially for treating the most serious diseases such as: heart disease, cancer, AIDS, herpes, leukemia and viral diseases [1–7]. These substances are rare, expensive and difficult to find on market.

Phytochemicals are obtained from plant materials by specific isolation and purification processes. The most important phases in their isolation from plant raw materials and further purification are solid-liquid extraction [8–14], crystallization [12] and liquid–liquid extraction [10–12]. Highly valuable bioactive substances are usually accompanied with various compounds of similar structure, and are difficult to be isolated in their pure crystalline forms as they crystallize isomorphically [12]. This is also the case with digoxin (Dgx), a highly valuable and efficient secondary cardiotonic glycoside isolated from the fermented foliage of foxglove (*Digitalis lanata* Ehrh.) [15]. This compound, which is irreplaceable drug for a heart disease (so-called elderly heart), has not been synthesized yet because of its complex structure. Besides cardiotoxic effects, Dgx has moderate diuretic effects, as well as anticancer and antiviral activities (herpes) [3–7].

Dgx is, as a rule, found in a mixture of secondary glycosides, such as digitoxin (Dx) and gitoxin (Gx), having very similar structure. One of the methods for isolation of Dgx from this mixture obtained by the extraction from the fermented foliage of foxglove [16–21], with a high degree of purity, is the liquid–liquid extraction [10,11,14]. Pekić [14] studied the batch liquid–liquid extraction of Dgx from the crystallizate of secondary glycosides isolated from the fermented foliage of foxglove.

The present study deals with the isolation of Dgx from chloroform and trichloroethylene extracts of the secondary glycosides of fermented foxglove (*Digitalis lanata* Ehrh.) foliage by liquid-liquid extraction using 15 separating funnel. In a previous procedure, the secondary glycosides were extracted from the fermented foxglove foliage by an aqueous ethanol solution (10 or 50 vol.%) using maceration or percolation. Then, the secondary glycosides were extracted from the aqueous ethanol extract by chloroform or trichloroethylene and were further purified. Finally, three different four-component two-phase systems (ethanol:water–chloroform:ethyl acetate, ethanol:water–chloroform:trichloroethylene and ethanol:water–trichloroethylene:ethyl

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acetate) were tested as an extracting solvent to get the final product having more than 98% of Dgx. The initial amount of the chloroform or trichloroethylene extract in the light phase was varied between 5 and 25 g/L, while the volume ratio of light and heavy phases was in the range of 1:1 to 1:2. The main goal was to define the operating conditions of Dgx isolation (type of extracting system, the initial concentration of the extract, volume ratio of the phases and number of separating stages). Ethyl acetate is used for the first time as a component of an extracting system for Dgx isolation from extracts of secondary glycosides of foxglove by liquid–liquid extraction.

EXPERIMENTAL

Materials

Plant material

Dried foliage of plantation-grown foxglove (*Digitalis lanata* Ehrh.) (Borča, Serbia) was used. Dark green leaves, 10–12 cm in length, about 3 cm wide, hairy on the surface, having characteristic odor, were stored in paper bags. The plant material contained 0.3 to 0.5% of lanatozide C. Identification and quality control of the foliage were done by the official methods [22]. The foliage was chopped in a hammer mill and then sieved. The fraction having the average particle size of 7.0 mm was employed.

Chemicals

Lanatoside C, Dx, Gx, Dgx, digoxigenin, aucubin, acetoside, sodium nitrite, sodium molybdate and sodium hydroxide were purchased from Merck. Chloroform, ethanol, methanol, methylene chloride, acetonitrile, ethyl acetate, anhydrous formic acid, glacial acetic acid, hydrochloric acid, sulfuric acid (98%), lead(II) acetate, ammonium hydroxide, methylene chloride, $CuSO_4$. $5H_2O$, $CoCl_2 \cdot 6H_2O$, $FeCl_3 \cdot 6H_2O$, hexamethylene tetramine, hydrazine sulfate potassium bromide (previously dried for 1 h at 250 °C), silica gel F_{254} and silica gel G were obtained from Fluka. All chemicals were pro analysis quality, unless otherwise indicated. Distilled water was also used.

Reagents

Basic lead(II) acetate solution. Concentrated ammonium hydroxide was added into a solution of lead(II) acetate (30%) until tests with 2–3 drops of this solution in the presence of phenolphthalein turned the color of white wine.

Xanthydrol reagent. Xanthydrol (10 mg) was dissolved in glacial acetic acid (50 mL) in a 100 mL flask, applying slight heating. A hydrochloric acid solution (1 mL, 25%) was added to the flask, which was then filled up with glacial acetic acid to the mark. A fresh solution of xanthydrol was prepared for each determination.

Plant material fermentation

Chopped foliage (5 g) was soaked with distilled water in a mass ratio of 1:2, well homogenized, put in plastic bags, sealed and left to ferment at 37 °C for 48 h [16]. The yields of Dx, Gx and Dgx were about 99–100%. The yields of Dx, Gx and Dgx were defined as the amount of glycoside formed divided by the theoretically amount of glycoside expected to be obtained from lanatozide A, B and C present in the plant material.

Extraction of secondary glycosides from fermented plant material

Secondary glycosides were extracted by maceration and percolation using 10 or 50 vol.% ethanol as an extracting solvent. The Dx, Gx and Dgx extraction degrees (EDs) were defined as the percentages of Dx, Gx and Dgx extracted from the fermented plant material, respectively.

Maceration and percolation

Maceration was carried out according to the modified procedure [16]. The fermented plant material (100 g) and the extracting solvent (10 or 50 vol.% ethanol, 1 L) were added to the extracting vessel equipped with a stirrer. The plant material was macerated at room temperature for 1 h. The liquid extract (macerate) was separated from the plant material by vacuum filtration. The extraction was repeated two more times in the same way. The macerates obtained were mixed in a separating funnel. The filtration cake of the exhausted plant material was washed 3 times with appropriate solvent (100 mL). Washing solutions were added to the total macerate in the separating funnel.

For percolation, a battery of 10 stainless steel percolators (inner diameter: 20 cm; height: 60 cm; empty volume: 18.84 L) having a ball valve at the bottom was employed. The appropriate volume of the extracting solvent was poured in the percolator, and the fermented plant material (particle size: 7.0 mm) was added to the plant layer height of 30 cm. Aqueous solutions of ethanol (10 or 50 vol.%) were used as extracting solvents. The percolation was performed at room temperature at a volumetric flow rate of percolate of 4.0 L/h using a constant volume of the extracting solvent (24 L). The percolate leaving a percolator was used as the extracting solvent in the next percolator. In this way, the percolate leaving the tenth percolator entered the first percolator. When the Dgx extraction degree for the percolate leaving a percolator was higher than 95%, the exhausted fermented foliage was replaced by a fresh plant material, and the process continued further. Samples of the liquid extract (percolate) were taken with the progress of percolation, and the content of Dgx was determined in each sample using the HPLC method.

Extraction of secondary glycosides using chloroform or trichlorethylene

The liquid extracts (macerate or percolate) obtained were treated by liquid-liquid extraction using chloroform or trichlorethylene as the extracting solvent to obtain the liquid fractions of secondary glycosides. The liquid-liquid extraction was performed four times with the extract-to-solvent volume ratio of 1×1:2 and 3×1:4 within 20 min in separating funnels. The extract-to--solvent ratios and the extraction time were previously defined by laboratory tests. The goal of this extraction was to extract more than 95% Dgx from aqueous––alcoholic extracts. The chloroform and trichlorethylene extracts were concentrated by vacuum evaporation to the 1/20 of their initial volume.

Treatment of concentrated extracts with MgO. MgO was added to the concentrated extract (MgO-to-the initial plant material mass ratio 1:10), mixed well and left to stand for 45 min, with occasional stirring. The resulting suspension was filtered under vacuum to separate the concentrate. The MgO cake was washed with the appropriate solvent (chloroform or trichloroethylene, 3×250 mL). The filtrates were added to the concentrate, which was then washed with distilled water (4 times, each with 2 L). The washed concentrate was evaporated under vacuum, and the solid product was further dried at 60 °C under vacuum to the moisture content up to 6%. The contents of Dx, Gx and Dgx in the obtained products were determined by the HPLC method [22].

Liquid–liquid extraction of highly-pure Dgx from purified extracts

Three four-component two-phase systems, namely a) ethanol:water–chloroform:ethyl acetate (EtOH:H_2O––$CHCl_3$:EtOAc), b) ethanol:water–chloroform:trichlorethylene (EtOH:H_2O–$CHCl_3$:TCE) and c) ethanol:water––trichlorethylene:ethyl acetate (EtOH:H_2O–TCE:EtOAc), were used to extract Dgx from the concentrated extracts by liquid–liquid extraction. These systems were at first equilibrated at the volume light (EtOH:H2O)-to-heavy (organic solvent mixture $CHCl_3$:EtOAc, $CHCl_3$:TCE or TCE:EtOAc) phase ratio of 1:1 at room temperature (20–25 °C) in fifteen separating funnels (10 L) and then used to separate Dgx from Dx and Gx. Volume fractions of two-component light and heavy phases were varied from 10:40 to 40:10 vol. to define the optimum composition of both phases. Different amounts (10 to 25 g) the chloroform or trichlorethylene extracts having different contents of Dgx, Dx and Gx were added to the light phase of the extracting system in the first separating funnel.

The light phase (3 L) was poured into 15 separating funnels (10 L). The volume ratio of equilibrated light and heavy phase was varied from 1:1 to 1:2. The heavy phase (3 L) was added to the first separating funnel, mixed (5 min) and left to separate. The heavy phase was transfered to the second sepratating funnel and the procedure (vigorous shaking followed by separation of the phases) was repeated until the last separating funnel. The contents of Dgx, Dx and Gx were determined in both the light and heavy phase after each separation step. For this, samples were taken from both phases of each separating funnel and evaporated to get more than 50 mg of dry residue for determining the secondary glycosides.

The light phases containing Dgx and traces of Dx ili Gx (less than 0.5%) were combined and evaporated to 1/20 of the initial volume under vacuum (100-200 mm Hg). The crystals of Dgx obtained from the concentrated solution of the light phase were separated by vacuum filtration, washed with a small portion of cold ethanol and then dried at 80 °C under vacuum. The ethanol filtrates from washings were collected and evaporated to recuperate the solvent.

The heavy phases containing Dx, Gx and traces of Dgx were also collected and evaporated to dry under vacuum (100–200 mmHg).

Determination of Dx, Gx and Dgx

The basic solution preparation

The fermented plant material (5 g) was transferred into a flask, methanol (50 mL, 50%) was added, and the suspension was shaken for 1 h. Then, while shaken, 5 mL of freshly prepared 30% solution of basic lead(II) acetate was gradually added into the suspension, the content was well shaken, left to rest for 5 min and then the excess of lead(II) acetate was precipitated by adding a sodium sulfate solution (5%). If the extract in the flask gave a positive reaction to Pb^{2+} with a 2% solution of potassium iodide (2–3 drops of clear extract mixed on a watch glass with 2–3 drops of 2% solution of potassium iodide; a yellow precipitate was formed in the presence of Pb^{2+}), the sodium sulfate solution was added until obtaining a negative reaction (no yellow precipitate). After the sedimentation of excess lead(II) acetate, the suspension was filtered through a quantitative filter paper set on a rapid filtration funnel. The filtrate was separated in a separating funnel, and the first turbid filtrate was returned for re-filtration until the filtrate remained totally clear. The precipitate on the filter paper was rinsed 3 times with a methanol solution (50 mL, 50%). The secondary glycosides and the accompanying extraction materials were re-extracted from the combined filtrate with chloroform (once 25 mL and 4 times with 12.5 mL). The collected chloroform re-extracts were passed through anhydrous sodium sulfate (sodium sulfate on the filter paper in the filtration funnel) into a round bottom flask (250 cm^3). Sodium sulfate was rinsed 3 times with 10 mL of chlo-

roform each. The chloroform re-extract, together with chloroform solutions obtained by rinsing, was evaporated to dry under vacuum at 60-70 °C to get a dry chloroform re-extract.

Determination of Dx, Gx and Dgx content

The dry chloroform re-extract (50 mg) was dissolved in 100 mL of methanol (basic solution), and the obtained solution was used to determine the contents of Dx, Gx and Dgx by the HPLC method [22].

Preparation of the standard solution of Dx,Gx and Dgx

Standards of Dx, Gx and Dgx (50 mg), previously dried to constant weight in a vacuum desiccator over phosphorus(V) oxide, was dissolved in methanol (100 mL).

HPLC Analysis of Dx, Gx and Dgx

Apparatus: Agilent 1100 Series. Column: length = 0.15 m, diameter = 3.9 mm, stationary phase: octadecylsilyl–silica gel for chromatography (5 μm). Mobile phase A: acetonitrile:water (10:90, *V*/*V*). Mobile phase B: acetonitrile:water (90:10, *V*/*V*).

Detection: 220 nm. Flow rate: 1.5 ml/min. Volume of injection: Inject in 10 mL basic solution and standard solution. Temperature: room. Retention times and concentrations of mobile phases A and B are given in Table 1.

Calculation (Ph. Eur., 7th ed., 2012, Method 2.2.29) [22]:

$$\%\ \text{Glycoside} = \frac{\text{Ppr} \times Wst \times K}{\text{Pst} \times \text{Wpr}} \cdot \frac{(100 - a_{\text{st}})}{(100 - a_{\text{pr}})}$$

where Ppr is the Dx, Gx or Dgx peak area of the investigated basic solution, Pst is the Dx, Gx or Dgx peak area of the standard solution, Wpr is the weight of the investigated substance (mg), *Wst* is the weight of the standard substance (mg), *K* is the Dx, Gx or Dgx content in the working standard (%), a_{pr} is the drying loss of the investigated substance (%) and a_{st} is drying loss of the standard substance (%).

RESULTS AND DISCUSSION

Extraction of Dgx by maceration and percolation from fermented plant material

The extraction degrees (*ED*) of Dx, Gx, Dgx and total glycoside (*TG*) achieved by maceration and percolation using 10 and 50 vol.% aqueous ethanol solutions were higher than 95% (Table 2). The 10 vol.% aqueous ethanol solution was recommended for extraction at higher scales although it yielded somewhat lower EDs (by 1–2%) than the more concentrated ethanol solution (50 vol.%). However, the use of a smaller amount of ethanol for the Dgx extraction would reduce the extraction costs. The percolation using 10 percolators was as efficient as the three-step maceration, so both techniques could be used for Dgx extraction from the fermented plant material.

Liquid–liquid extraction of secondary glycosides from ethanolic extracts

Chloroform or trichlorethylene was used to extract Dx, Gx and Dgx from the liquid ethanolic extracts (macerate or percolate) by the four-cycle liquid-liquid extraction (the extract-to-solvent volume ratio of 1×1:2 and 3×1:4; 20 min per a cycle). The values of EDs of Dx, Gx, Dgx and total glycosides (TGs) are presented in Table 3. The best *ED* of about 100 and 96% were achieved using trichlorethylene and chloroform, respectively by the four-cycle extraction. The dry chloroform and trichloroethylene extracts had different contents of Dx, Gx, Dgx and TGs (Table 4). The latter extract contained Dx, Gx, Dgx and TGs at higher levels than the former extract. Therefore, trichlorethylene

Table 1. Retention time and the concentration of mobile phases A and B

Time, min	Mobile phase A	Mobile phase B
0 → 5	78	22
5 → 15	78 → 30	22 → 70
15 → 16	30 → 78	70 → 22
16 → 30	78	22

Table 2. ED values (relative to the content in the fermented plant material, %) of Glycoside achieved by maceration and percolation using 10 and 50 vol.% aqueous ethanol solutions (fermented plant material-to-solvent mass ratio: 1:10; extraction time: 3×1 h; average particle size: 7 mm; room temperature)

Glycoside	Maceration		Percolation	
	10 vol.%.	50 vol.%	10 vol.%.	50 vol.%
Digitoxin	96.0	98.0	97.5	98.5
Gitoxin	95.0	96.0	97.0	97.5
Digoxin	97.0	98.0	98.5	99.5
Total glycosides	101.0	102.0	101.5	102.5

Table 3. Values of ED (relative to the content in the macerate or the percolate, %) of secondary glycosides achieved by chloroform and trichlorethylene from macerates and percolates obtained by the 10% vol. aqueous ethanol solution under optimal conditions

Type of extraction	Extraction cycle	Extracting agent							
		Chlorophorm				Trichlorethylene			
		Digitoxin	Gitoxin	Digoxin	Total glycosides	Digitoxin	Gitoxin	Digoxin	Total glycosides
Maceration	1	72	75	80	83	75	82	83	90
	2	86	84	86	88	92	89	87	92
	3	89	88	90	92	90	90	91	95
	4	93	93	94	96	94	95	95	100
Percolation	1	84	79	84	86	83	86	85	91
	2	87	86	88	90	88	90	90	93
	3	90	91	92	96	92	92	93	98
	4	95	96	96	99	100	100	100	102

Table 4. Contents of Dx, Gx, Dgx and TG in dry chloroform and trichlorethylene extracts (Relative to the mass of extract, %)

Glycoside	Extracting agent	
	Trichlorethylene	Chlorophorm
Digitoxin	35.2	12.3
Gitoxin	12.6	5.6
Digoxin	46.2	43.8
Total glycosides	81.8	80.0

was selected as extracting solvent to recover secondary glycosides from the liquid ethanolic extracts.

Separation of Dgx from extracts of secondary glycosides by liquid-liquid extraction

Tables 5–7 presents the results of separating Dx, Gx and Dgx from the trichlorethylene extract by three two-phase systems: EtOH:H_2O–$CHCl_3$: EtOAc, EtOH:H_2O–$CHCl_3$:TCE and EtOH:H_2O–TCE:EtOAc. The optimal systems ensuring the highest Dgx content in the light phase of 78, 76 and 85% were as follows: EtOH:H_2O–$CHCl_3$:EtOAc 25:25:30:20, EtOH:H_2O–$CHCl_3$:TCE 30:20:30:20 and EtOH:H_2O–TCE:EtOAc 35:15:20:30 *V*/*V*, respectively.

Table 5. Separation of Dx, Gx and Dgx by the two-phase system EtOH:H_2O–$CHCl_3$:EtOAc (concentration of trichlorethylene extract in the light phase: 15 g/l; light-to-heavy phase volume ratio: 1:1; volume of phases: 3 L; room temperature)

Volume ratio EtOH:H_2O:$CHCl_3$:EtOAc	Content of glycosides, relative to the content in the initial solution of the trichlorethylene extract from the light phase, %					
	Light phase (EtOH:H_2O)			Heavy phase ($CHCl_3$:EtOAc)		
	Digitoxin	Gitoxin	Digoxin	Digitoxin	Gitoxin	Digoxin
10:40:10:40	45.5	26.7	35.0	64.5	63.3	65.0
10:40:20:30	35.0	28.0	45,5	65.0	72.0	54.5
10:40:30:20	25,7	33.0	42.5	74.3	67.0	57.5
10:40:35:15	22.5	37.0	44.6	77.5	63.0	55.4
10:40:40:10	18.8	41.0	46.0	81.2	59.0	64.0
20:25:10:40	34,6	55.5	48.0	65.4	45.5	32.0
20:25:20:30	28.5	45.7	52.0	71,5	54.3	40.0
20:25:30:20	25.0	34.0	55.5	75,0	56.0	44.5
20:25:35:15	20.4	27.0	60.0	74.6	63.0	55.0
20:25:40:10	17.0	15.5	63.0	83.0	84.5	70.0
25:25:10:40	40.5	45.0	62.0	65.0	55.0	44.5
25:25:20:30	35.0	40.8	64.0	68.0	59.2	55.0
25:25:30:20	22.5	35.0	78.0	72.0	65.0	22,0
25:25:35:15	20.5	30.5	58.0	69.5	69.5	42,0
25:25:40:10	19.0	20.0	60.5	71.0	80.0	39.5

Table 5. Continued

Volume ratio EtOH:H_2O:$CHCl_3$:EtOAc	Content of glycosides, relative to the content in the initial solution of the trichlorethylene extract from the light phase, %					
	Light phase (EtOH:H_2O)			Heavy phase ($CHCl_3$:EtOAc)		
	Digitoxin	Gitoxin	Digoxin	Digitoxin	Gitoxin	Digoxin
30:20:10:40	35.0	30.0	52.5	65.0	70.0	47.5
30:20:20:30	24.0	27.0	48.5	76.0	73.0	51.5
30:20:30:15	15.5	20.0	45.5	84.5	80.0	55.5
30:20:40:10	12.0	17.0	40.5	88.0	83.0	59.5
35:15:10:40	25.0	25.5	55.0	75.0	74.5	45.0
35:15:20:30	21.0	21.0	50.0	79.0	79.0	50.0
35:15:30:20	18.0	16.0	45.5	82.0	84.0	54.5
35:15:35:15	16.5	13.0	42.0	83.5	87.0	48.0
35:15:40:10	15.0	11.5	40.0	85.0	88.5	60.0
40:10:10:40	20.0	20.5	38.0	80.0	79.5	62.0
40:10:20:30	16.0	15.5	35.5	84.0	84.5	64.5
40:10:30:20	14.0	14.0	32.5	86.0	86.0	68.5
40:10:35:15	12.0	12.5	31.0	88.0	87.5	69.0
40:10:40:10	10.0	11.0	30.0	90.0	89.0	70.0

Table 6. Separation of Dx, Gx and Dgx by the two-phase system EtOH:H_2O–TCE:EtOAc (concentration of trichlorethylene extract in the light phase: 15 g/l; light-to-heavy phase volume ratio: 1:1; volume of phases: 3 L; room temperature)

Volume ratio EtOH:H_2O:TCE:EtOAc	Content of glycosides, relative to the content in the initial solution of the trichlorethylene extract from the light phase, %					
	Light phase (EtOH:H_2O)			Heavy phase (TCE:EtOAc)		
	Digitoxin	Gitoxin	Digoxin	Digitoxin	Gitoxin	Digoxin
10:40:10:40	56.5	24.7	30.0	43.5	75.3	70.0
10:40:20:30	45.0	30.0	34.5	55.0	70.0	65.5
10:40:30:20	35.5	35.0	40.5	64.5	65.0	59.5
10:40:35:15	30.5	41.0	46.6	69.5	49.0	53.4
10:40:40:10	28.0	43.0	20.0	72.0	80.0	80.0
20:25:10:40	24.6	20.5	25.0	79.5	79.5	75.0
20:25:20:30	36.5	20.7	38.0	63.5	79.3	62.0
20:25:30:20	35.0	25.0	33.0	65,0	87.0	77.0
20:25:35:15	35.0	30.0	37.0	65.0	70.0	63.3
20:25:40:10	30.0	36.0	40.0	70.0	64.0	60.0
25:25:10:40	50.0	40.0	46.0	50.0	60.0	54.0
25:25:20:30	53.0	42.8	50.0	47.0	57.2	50.0
25:25:30:20	46.0	35.0	45.0	54.0	65.0	55.0
25:25:35:15	38.0	27.5	49.0	72.0	72.5	51.0
25:25:40:10	35.0	20.0	57.0	65.0	80.0	43.0
30:20:10:40	30.0	17.0	65.0	70.0	83.0	35.0
30:20:20:30	25.0	15.0	70.0	75.0	85.0	30.0
30:20:30:20	20.0	12.0	76.0	80.0	88.0	24.0
30:20:30:15	18.5	10.0	68.5	81.5	90.0	31.5
30:20:40:10	15.0	8.0	56.5	85.0	92.0	43.5
35:15:10:40	45.0	37.5	60.0	55.0	62.5	40.0
35:15:20:30	36.0	30.0	50.0	74.0	70.0	50.0
35:15:30:20	30.0	25.0	46.0	70.0	75.0	54.0
35:15:35:15	25.0	20.0	36.0	75.0	80.0	64.0
35:15:40:10	18.0	15.0	30.0	82.0	85.0	70.0

Table 6. Continued

Volume ratio EtOH:H_2O:TCE:EtOAc	Content of glycosides, relative to the content in the initial solution of the trichlorethylene extract from the light phase, %					
	Light phase (EtOH:H_2O)			Heavy phase (TCE:EtOAc)		
	Digitoxin	Gitoxin	Digoxin	Digitoxin	Gitoxin	Digoxin
40:10:10:40	30.0	28.5	48.0	70.0	71.5	62.0
40:10:20:30	22.0	25.0	38.5	78.0	75.0	61.5
40:10:30:20	18.0	20.0	36.5	82.0	80.0	63.5
40:10:35:15	14.0	16.5	33.0	86.0	83.5	67.0
40:10:40:10	12.0	14.0	31.0	88.0	86.0	69.0

Table 7. Separation of Dx, Gx and Dgx by the two-phase system EtOH:H_2O–$CHCl_3$:TCE (concentration of trichlorethylene extract in the light phase: 15 g/l; light-to-heavy phase volume ratio: 1:1; volume of phases: 3 L; room temperature)

Volume ratio EtOH:H_2O–$CHCl_3$:TCE	Content of glycosides, relative to the content in the initial solution of the trichlorethylene extract from the light phase, %					
	Light phase (EtOH:H_2O)			Heavy phase ($CHCl_3$:TCE)		
	Digitoxin	Gitoxin	Digoxin	Digitoxin	Gitoxin	Digoxin
10:40:10:40	36.5	6.7	35.0	65.5	43.3	65.0
10:40:20:30	35.0	60.0	54,5	65,0	40.0	45.5
10:40:30:20	30,5	58.0	48.5	69.5	42.0	41.5
10:40:35:15	26.5	51.0	46.6	73.5	49.0	26.5
10:40:40:10	25.0	42.0	50.0	75.0	58.0	50.0
20:25:10:40	44,6	65.5	60..0	55.4	35.5	40.0
20:25:20:30	38.5	55.7	65.0	61,5	47.3	35.0
20:25:30:20	30.0	43.0	70.0	70,0	67.0	30.0
20:25:35:15	26.5	35.0	67.0	73.5	65.0	43.0
20:25:40:10	21.0	24.5	65.0	79.0	75.5	35.0
25:25:10:40	50.5	57.0	73.0	49.5	43.0	27.0
25:25:20:30	43.0	52.8	78.0	57.0	47.2	22.0
25:25:30:20	30.5	43.0	82.0	69.5	67.0	28,0
25:25:35:15	26.5	37.5	69.0	73.5	62.5	31,0
25:25:40:10	22.0	27.0	66.5	78.0	73.0	33.5
30:20:10:40	40.0	42.0	80.0	60.0	58.0	20.0
30:20:20:30	44.0	37.0	85.5	66.0	23.0	14.5
30:20:30:20	48.0	44.0	89.0	52.0	56.0	11.0
30:20:35:15	26.5	32.0	58.5	73.5	68.0	41.5
30:20:40:10	25.0	29.0	52.5	75.0	71.0	47.5
35:15:10:40	35.0	33.5	60.0	65.0	66.5	40.0
35:15:20:30	30.0	20.0	85.0	70.0	80.0	15.0
35:15:30:20	25.0	22.0	50.5	75.0	78.0	49.5
35:15:35:15	20.0	16.0	48.0	80.0	84.0	88.0
35:15:40:10	18.0	15.0	45.0	82.0	85.0	55.0
40:10:10:40	30.0	28.5	48.0	70.0	71.5	62.0
40:10:20:30	22.0	25.0	38.5	78.0	75.0	61.5
40:10:30:20	18.0	20.0	36.5	82.0	80.0	63.5
40:10:35:15	14.0	16.5	33.0	86.0	83.5	67.0
40:10:40:10	12.0	14.0	31.0	88.0	86.0	69.0

Table 8 represents the EDs of Dx, Gx and Dgx for the optimum light and heavy phases obtained from chloroform and trichlorethylene extracts by liquid-liquid extraction using the three optimum two-phase systems. The highest ED of Dgx in the light phase achieved in the first ten seprataing funnels (Figures 1 and 2) was

in the range between 92.0 and 99.8%, while the contents of Dx and Gx were ignorable (0.0 to 0.5%). The best two-phase system was EtOH:H_2O–$CHCl_3$:TCE 35:15:20:30, which ensured the highest ED of Dgx of 99.5 and 99.8% in the case of the chlorophorm and trichlorethylene extracts, respectively. This system was accepted as the optimum one for separation of Dgx from the chloroform and trichlorethylene extracts.

Table 8. The ED values (extraction degree relative to the content in the initial solution, %) of Dx, Gx and Dgx for the optimum liquid-liquid extraction systems obtained from chloroform and trichlorethylene extracts; C – chlorophorm extract; TCE – trichlorethylene extract

Liquid–liquid extraction system, volume ratio	Phase											
	Light						Heavy					
	Dx		Gx		Dgx		Dx		Gx		Dgx	
	C	TCE	C	TCE	C	TCE	C	TCE	C	TCE	C	TCE
EtOH:H_2O-$CHCl_3$:EtOAc, 25:25:30:20	0.5	0.3	0.0	0.0	92.0	99.5	99.5	97.7	100.0	100.0	8.0	0.5
EtOH:H_2O-$CHCl_3$:EtOAc, 30:20:30:20	0.5	0.5	0.0	0.0	94.0	95.5	99.5	99.5	100.0	100.0	6.0	5.5
EtOH:H_2O-$CHCl_3$:TCE, 35:15:20:30	0.3	0.2	0.0	0.0	99.5	99.8	99.7	99.8	100.0	100.0	0.5	0.2

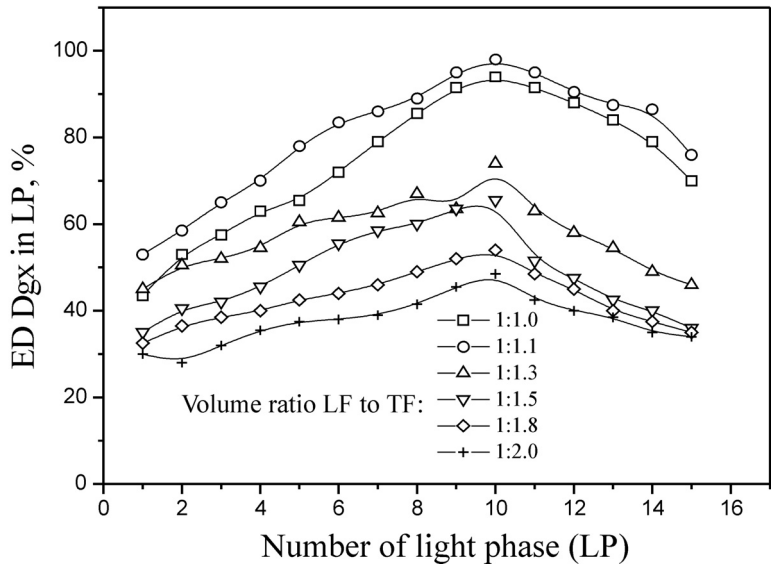

Figure 1. Distribution Dgx in the light phase of the system EtOH:H_2O–$CHCl_3$:TCE, 35:15:20:30 volume ratio, in the separating funnels at different volume ratios of the light and heavy phase (amount of the trichlorethylene extract in the first separating funnel: 15 g/L; volume of the phases: 3 L; room temperature; shaken of the phases in the separating funnels: 5 min).

Figures 1 and 2 shows the distribution of Dgx in the light phase of the optimum system (EtOH:H_2O–$CHCl_3$:TCE, 35:15:20:30 volume ratio) in the 15 separating funnels at different volume ratios of the phases (Figure 1) and at different amounts of trichlorethylene extract in the light phase in the first separating funnel (Figure 2). The ED of Dgx increased with increasing the number of separating funnels up to the tenth funnel and then decreased, independently of the volume ratio of the phases and the initial amount of the glycoside extract (Figures 1 and 2). The highest ED of Dgx was achieved at the volume ratio of the phases of 1:1 (98%, Figure 1) and the amount of the trichlorethylene extract of 15 g/L (99%, Figure 2). Therefore, the volume ratio of the phases of 1:1,1 and the amount of the trichlorethylene extract of 15 g/L were accepted as the optimum ones. The purity of the extracted Dgx product obtained from the concentrate of the combined light phases from the ten separating funnels. The yield of Dgx (with respect to its content in the trihlorethylene extract) was 89%.

CONCLUSION

The optimum operating conditions of the liquid–liquid extraction of Dgx using a four-component system EtOH:H_2O–$CHCl_3$:TCE, 35:15:20:30 volume ratio. Fifteen separating funnels were employed for the liquid–liquid extraction with 3 L of the light and heavy phase each. The volume ratio of the phases was 1.0:1.1. The initial amount of the chloroform or trichlorethylene extract in the light phase of the first separating was 15 g/L. Under these operating conditions, more than 98% Dgx of high purity (over 99%)

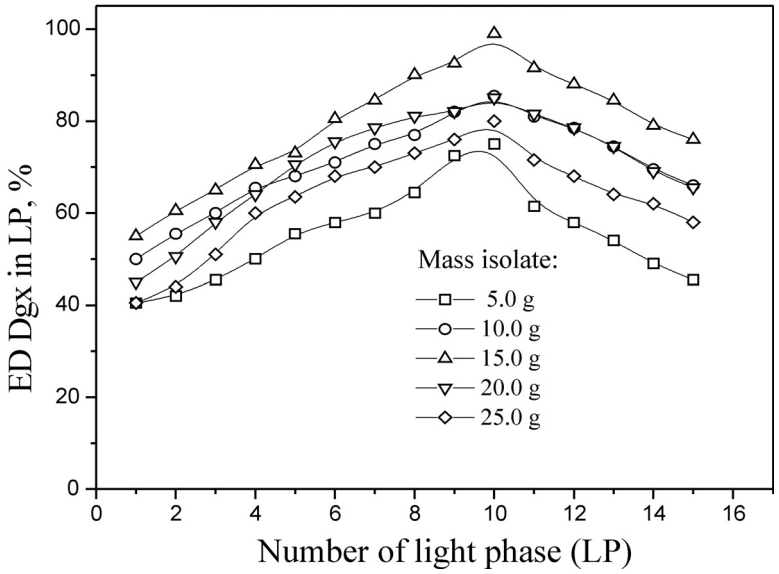

Figure 2. Distribution Dgx in the light phase of the system EtOH:H_2O–$CHCl_3$:TCE, 35:15:20:30 volume ratio, in the separating funnels at different amounts of trichlorethylene extract in the light phase in the first separating funnel (volume ratio of the light and heavy phase: 1:1.1; volume of the phases: 3 L; room temperature; shaken of the phases in the separating funnels: 5 min).

was obtained from chloroform and trichlorethylene extracts of secondary glycoside of foxglove foliage (*Digitalis lanata* Ehrh.).

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IZVOD

IZOLACIJA DIGOKSINA EKSTRAKCIJOM TEČNOST–TEČNOST IZ EKSTRAKATA SEKUNDARNIH GLIKOZIDA VUNASTOG DIGITALISA

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(Naučni rad)

Ispitan je uticaj operativnih uslova na efikasnost izdvajanja digoksina ekstrakcijom tečnost-tečnost u levcima za odvajanje iz suvih hloroformskih i trihloretilenskih izolata sekundarnih glikozida vunastog digitilisa. Kao ekstrakcioni rastvarači korišćena su tri četvorokomponentna dvofazna sistema: a) etanol:voda-hloroform:etilacetat, b) etanol:voda-trihloretilen:etilacetat i c) etanol:voda-trihloretilen:hloroform. Ispitivanja su uključila sledeće procesne uslove: sastav ekstrakcionog sistema, koncentracija rastvora suvih hloroformskih i trihloretilenskih ekstrakata sekundarnih glikozida u lakoj fazi prethodno uravnoteženoj teškom fazom u opsegu 5–25 g/L, zapreminski odnos lake i teške faze, broj uravnotežavanja faza i odnos zapremina lake i teške faze 1:1 do 1:2. Definisani su optimalni operativni uslovi za izdvajanje preko 98% digoksina u lakoj fazi, i to: koncentracija sekundarnih glikozida u lakoj fazi 15 g/L; sastav četvorokomponentni sistema etanol:voda–hloroform:trihloetilen, 35:15:20:30; i odnos zapremina lake i teške faze 1,0:1,1. Iz lake i teške faze su, koncentrovanjem i kristalizacijom, izdvajeni digoksin i smeša glikozida digitoksina i gitoksina. Čistoća izdvojenog digoksina je 99,8%.

Ključne reči: Digoksin • Vunasti digitalis • *Digitalis lanata* Ehrh. • Ekstrakcija tečno–tečno • Ekstrakcija čvrsto–tečno

Effect of segmental baffles on the shell-and-tube heat exchanger effectiveness

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Abstract

The results of the experimental investigations of fluid flow and heat transfer in laboratory experimental shell-and-tube heat exchanger are presented in this paper. Shell-and-tube heat exchanger is with one pass of warm water on the shell side and two passes of cold water in tube bundle. Shell-and-tube heat exchanger is with 24×2 tubes (U-tube) in triangle layout. During each experimental run, the pressure drops and the fluid temperatures on shell side, along the shell-and-tube heat exchanger (at positions defined in advance) have been measured. The special attention was given to the investigation of the segmental baffles number influence of the shell-and-tube heat exchanger effectiveness.

Keywords: shell and tube heat exchanger, experiment, effectiveness, baffles.

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Shell-and-tube heat exchangers (STHE) are one of the most often used apparatuses in chemical industry. One of STHE manufacturer's main goals is to improve their exploitation reliability and efficiency. Improving the STHE design is possible by two approaches: the experimental investigation, which is very expensive and long-lasting, because of shell side complex geometry, and by numerical investigations. The numerical simulations can be used to check the old and to develop new, more efficient STHE designs.

Although STHE's are not specially compact, their robustness and design make them well suited for high pressure operations, both in chemical and energy production plants. They are commonly used as oil coolers, in processes involving aggressive or dangerous fluids.

Baffles, placed on the shell side space, are providing the cross flow direction of shell side fluid and so the more intensive heat exchange between fluids could be realized. Besides, baffles are carriers of tube bundle, which helps them to decrease the deflection and vibrations in apparatuses [4,6].

On the shell side, there is not just one stream, beside a main cross-flow stream the four leakage or bypass streams exist, as a result of design-type-baffle to tubes, baffle to shell and tube bundle to shell gaps (tube-to-baffle hole leakage stream, bundle bypass stream, pass-partition bypass stream and baffle-to-shell leakage stream).

Basically, one can conclude that heat transfer between fluids in STHE's is highly influenced not only by thermal and flow quantities, such as inlet temperatures and velocities, but also with baffle cut size, baffle spacing, size of inlet and outlet zones and number of baffles [2–9].

To investigate the influence of mentioned parameters, thermal, flow and geometric, in other words, to find the „apparatus response" to thermal and fluid properties and shell side geometry, in steady regime, by experimental and numerical methods, it was necessary to concept one compact experimental STHE [8].

In this paper, the special attention was given to the experimental investigation of the segmental baffles number influence of the shell-and-tube heat exchanger effectiveness.

EXPERIMENTAL

The experimental STHE, type 1-2U, was projected and manufactured in cooperation with company MIN Inžinjering from Niš. The experimental installation shown in Figure 1 and schematic shown in Figure 2 was formed in the boiler house of the Mechanical Engineering Faculty in Niš.

Figure 1. Experimental installation.

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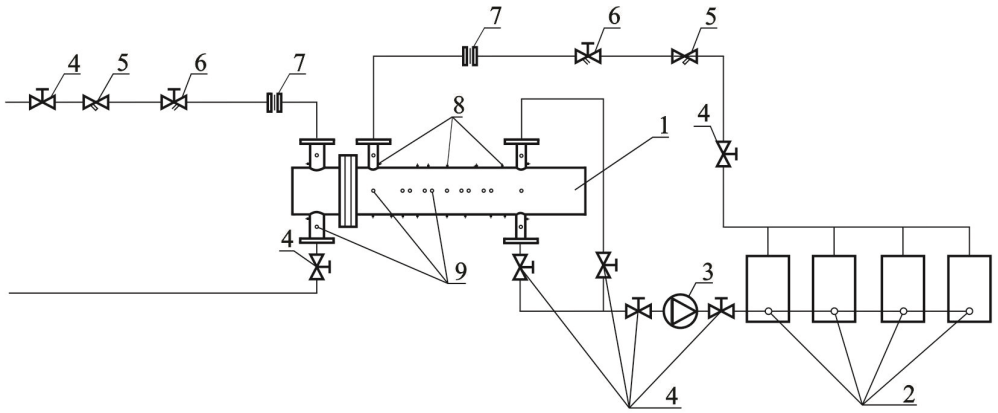

1. Heat exchanger, 2. Electrical boilers, 3. Pump, 4. Valve, 5. Impurity catchers, 6. TA STAD valve,
7. Measuring orifice, 8. Pressure measuring taps, 9. Openings for thermocouple carriers

Figure 2. Experimental installation (shematic).

The basic part of the installation is STHE (Figure 3). Tube bundle is made of copper U-pipes, ∅15/13mm, with rotated triangular tube layout and tube pitch of 21 mm. There are 48 tubes in the tube bundle. The STHE's active area for heat exchange is 1.9 m^2.

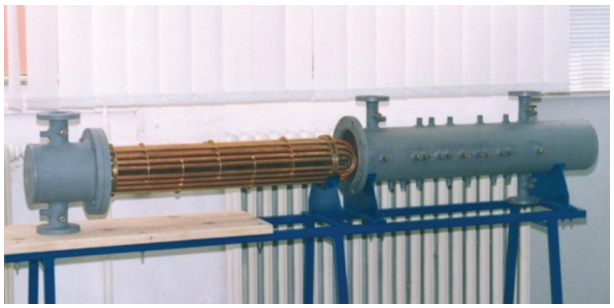

Figure 3. Experimental shell and tube heat exchanger.

The shell is made of carbon steel, ∅193.7/182.9 mm. The STHE's full length is 1217 mm. Three packages of segmental baffles with baffle cuts of 22, 27 and 32% are located in the shell. There are 5 segmental baffles in every package. Segmental baffles are made of copper.

Range of change of fluids operational and geometric parameters

The experimental investigation is done under summer ambient conditions in the period May-September. The heating fluid is warm water, which heated the cold water from the local water supply. In all experiments, the constant volume flow rate (V_h = 9 m^3/h), as well as inlet temperature (t_{1h} = 15 °C) of cold fluid, were maintained. Volume flow rate of heated fluid (V_t = 3, 4 and 5 m^3/h) and temperature (t_{1t} = 50 and 60 °C) were varied at the inlet. At the beginning the series of experiments without baffles (*Np* = 0) in the shell were done. After that, baffles (with baffle cut of *BC* = 22, 26 and 32%) were set into the shell (one *Np* = 1; two *Np* = 2; three *Np* = 3; four *Np* = 4 and five *Np* = 5 segmental baffles). Six experiments were done for every geometric configuration.

MEASURING RESULTS

Heating and heated fluid flow rate measurements were done with standard measuring diaphragms, as well as with pressure drop measurements on TA-STAD valves with CBI Acquisition system (computerized balancing instrument).

The pressure in the closed circulation heating fluid circle was measured with laboratory mechanical manometer. The manometer indicators were in the range 3––3.5 bar in all experiments.

The pressure drop in the STHE's tube bundle and on the measuring diaphragms was done with the hydrostatic manometer (U-pipe with mercury). In the measurements done by average tube bundle, the pressure drop was 3710 Pa.

Shell side pressure drops were measured by specially designed system consisting of taps set up on the shell, transparent plastic hoses, collector with 16 connections, valves, shanks, millimeter partitioned scale and hand pump. All taps from the STHE's shell are connected by transparent rubber hoses into a collector (totally 15 branches). One side of the collector is closed and the other side is connected to the compressed air source through a valve. The compressed air increases the pressure on the water column surfaces in the transparent plastic hoses. As this pressure is the same in all the hoses, it decreases the water column height in them. By this way the shell side pressure drop, as well as pressure drop along STHE's shell, i.e. pressure drop between two baffles and pressure drop in the baffle cut, was measured. Table 1 shows the total shell side pressure drop increase comparing with the case with no baffles.

Table 1. Total shell side pressure drop increase comparing with the case with no baffles

Volume flow rate, m^3/h	Baffles number with baffle cut 22%	Total shell side pressure drop increase comparing with the case with no baffles, %
3	1	6
	5	38
4	1	10
	5	49
5	1	16
	5	51

During each experiment, after accomplishing steady state heat exchange regime (which was accomplished after 25–30 min in experiments done), the fluid temperatures are measured with 16 previously calibrated chromel–allumel thermocouples of 0.2 mm diameter, using the Hewlett–Packard acquisition system. The cold sides of all thermocouples were immersed into insulated tank filled with 1:2 water and ice mixture. All thermocouples were set into so-called "movable platforms".

Apparatus' inlet and outlet, heating and heated fluid temperatures (five thermocouples), as the heating fluid temperatures in the central shell's plane and 5mm from the shell wall, on eleven formerly defined locations along the STHE, were measured. The heated fluid inlet temperatures in the beginning and outlet temperatures in the end of the experiment were checked with a mercury thermometer.

The precision of the TA CBI measuring system for the water flow measurement is 0.001 l/s. Accuracy varied in the range of 0.029–0.200%. For the measuring orifice accuracy was in the range 0.704–0.819%. The precision of the thermocouple measurements was 0.1 °C. Temperature measurement accuracy was in the range of 0.143–0.769%. Considering these accuracy levels, it is obvious that these measurements were in the laboratory class.

Measuring results are shown in the form of diagrams. The measuring place position is shown on the *x*-axis and temperature value on the *y*-axis. Figure 4 shows the heating fluid temperature change along the STHE's axis for $Np = 1$, $BC = 22\%$ and $t_{1t} = 50$ °C depending on the volume flow rate of heated fluid.

Figure 5 shows the heating fluid temperature change along the STHE's axis for $Np = 5$, $BC = 22\%$ and $t_{1t} = 50$ °C, depending on the volume flow rate of heated fluid.

Figure 6 shows the heating fluid temperature change along the STHE's axis for $Np = 3$, $V_t = 4$ m^3/h, $BC = 22\%$, depending on the inlet temperature of heated fluid.

Figure 7 shows the heating fluid temperature change along the STHE's axis for $V_t = 5$ m^3/h, $t_{1t} = 60$ °C, $BC = 22\%$, depending on the segmental baffles number.

Figure 8 shows the heating fluid temperature change along the STHE's axis for $Np = 5$, $V_t = 3$ m^3/h, $t_{1t} = 60$ °C, depending on the baffle cut.

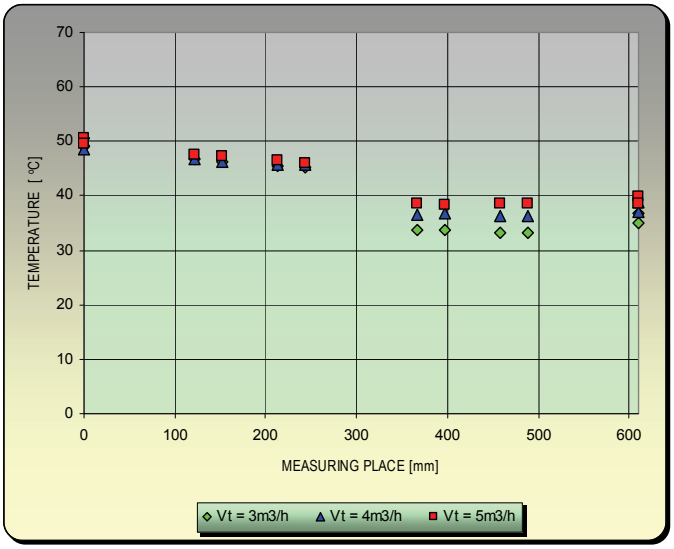

Figure 4. Heating fluid temperature change along the STHE's axis for Np = 1, BC = 22% and $t_{1t} = 50$ °C depending on the volume flow rate of heated fluid.

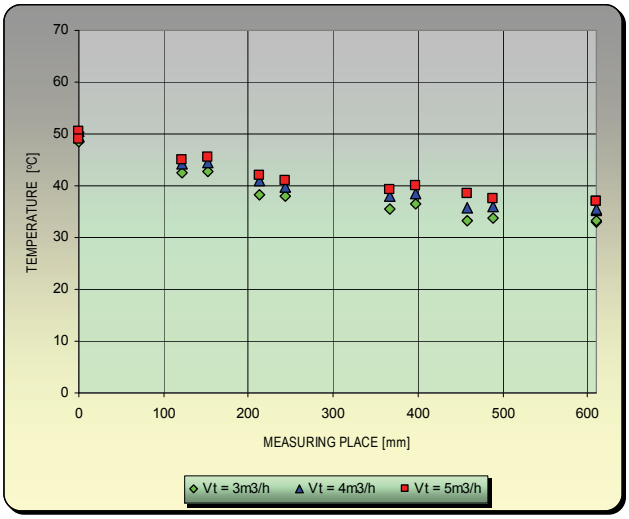

Figure 5. Heating fluid temperature change along the STHE's axis for Np = 5, BC = 22% and t_{1t} = 50 °C depending on the volume flow rate of heated fluid.

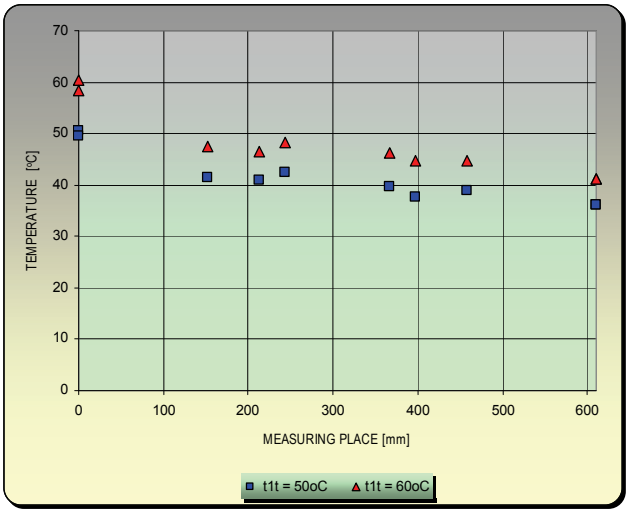

Figure 6. Heating fluid temperature change along the STHE's axis for Np = 3, V_t = 4 m^3/h and BC = 22% depending on the inlet temperature of heated fluid.

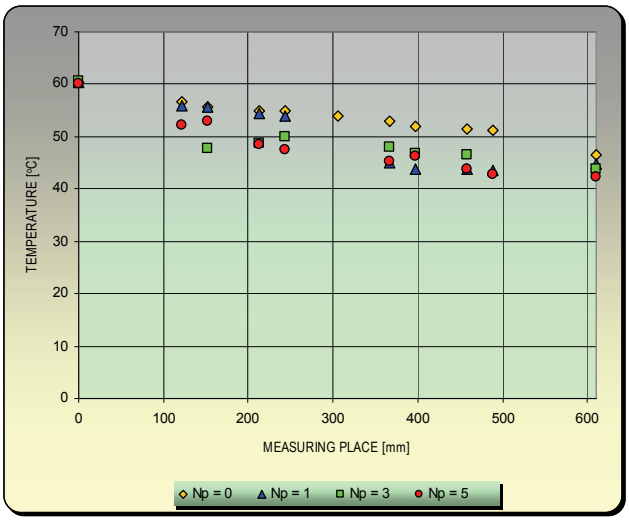

Figure 7. Heating fluid temperature change along the STHE's axis for V_t = 5 m^3/h, t_{1t} = 60 °C and BC = 22% depending on the segmental baffles number.

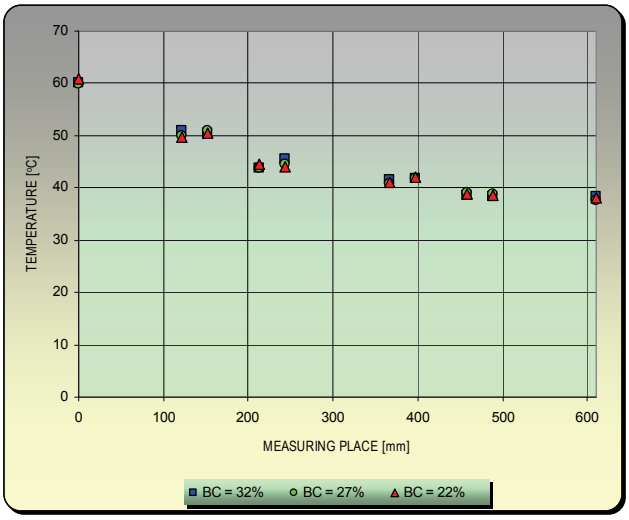

Figure 8. Heating fluid temperature change along the STHE's axis for Np = 5, V_t = 3 m^3/h, t_{1t} = 60 °C depending on the baffle cut.

Figure 9 shows the heat transfer rate depending on the segmental baffles number with baffle cut 22%.

In Table 2 the heat exchanger effectiveness is shown [1,9], the increase comparing with the case with no baffles.

CONCLUSION

The results of performed experiments show that STHE's heat exchange strongly depends on the shell side geometry (number of segmental baffles, baffle cut

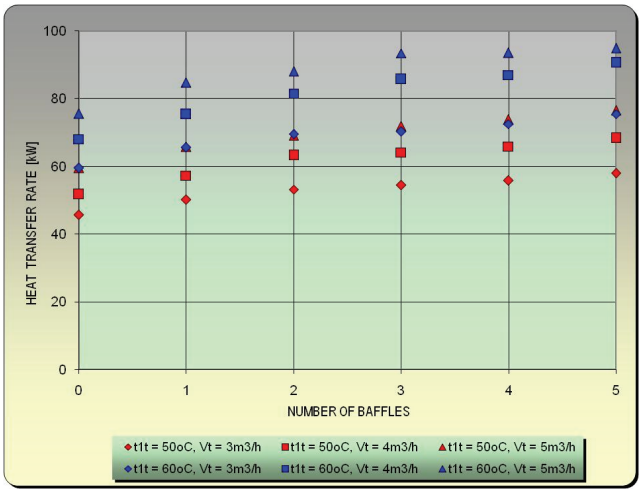

Figure 9. Heat transfer rate depending on the segmental baffles number with baffle cut 22%.

Table 2. Heat exchanger effectiveness increase comparing with the case with no baffles

Volume flow rate, m^3/h	Baffles number with baffle cut 22%	Heat exchanger effectiveness increase comparing with the case with no baffles for t_{1t} = 60 °C, %
3	1	11.1
	3	17.4
	5	25.0
4	1	9.1
	3	22.1
	5	29.2
5	1	13.6
	3	22.0
	5	28.5

size, baffle distance, the first and the last baffle position to inlet and outlet nozzle, size of the constructive clearances).

With the experimental results analysis the following can be concluded:

- the STHE design, including baffles, drastically changes the fluid flow characteristics;
- downstream fluid temperatures are significantly lower (up to 10 °C behind the first baffle), which is caused by intensive heat exchange in the cross flow ahead and before the baffle and by axial flow in the baffle cut. The influence of the baffle to shell leaking and cooling of the fluid through the shell wall should be carefully considered;
- increasing the heating fluid flow rate decreases temperature drops behind the baffle;
- increasing the heating fluid inlet temperature and/or flow rate maintains temperature change trend in the axis and near the shell wall;
- the most intensive heat exchange is in the STHE's inlet zone (up to the first baffle);
- heating the fluid temperature near the shell wall is lower, compared to the heating fluid temperature in the STHE's axis, up to the first baffle, and after the first baffle situation is reversed, which is consequence of the baffle to shell leaking, because it doesn't take place in the heat exchange;
- increase of segmental baffles number has higher influence to the STHE's effectiveness than the increase of the heating fluid flow rate;
- when segmental baffles are present in HE shell, the values of heat characteristics are increasing [9]. For example, for case of one segmental baffle with baffle cut of 22%, at t_{1t} = 60 °C and V_t = 3 m^3/h, the heat exchanger effectiveness was increased for 11.1% comparing to the case without baffles in a shell.

The results of this investigation have resulted in the series of improved STHE's manufactured in the company MIN Inžinjering from Niš, mostly as transformer oil cooler.

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IZVOD

UTICAJ SEGMENTNIH PREGRADA NA EFIKASNOST RAZMENJIVAČA TOPLOTE SA CEVNIM SNOPOM I OMOTAČEM

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(Naučni rad)

Zahtevi savremenog inženjerskog projektovanja toplotnih aparata, kao što su razmenjivači toplote sa cevnim snopom i omotačem, ogledaju se, pored poznavanja odziva aparata na promenu ulaznih veličina i geometrije aparata i u poznavanju polja temperatura, polja pritiska, polja brzina, polja turbulentnih karakteristika unutar samog aparata. Ovakva saznanja neophodna su pri optimizaciji kako konstrukcije aparata, tako i procesa u ovim aparatima i mogu u znatnoj meri uticati na pouzdanost, efikasnost i cenu aparata. U ovom radu posebna pažnja posvećena je istraživanju uticaja termo-strujnih veličina radnih fluida (protok i temperatura grejnog fluida na ulazu u aparat) i geometrije međucevnog prostora (broj segmentnih pregrada i veličina okna pregrade, a samim tim i rastojanje između pregrada, veličina ulazne i izlazne zone aparata, kao i broj poprečnih nastrujavanja na snop cevi) na intenzitet razmene toplote, odnosno na efikasnost razmenjivača toplote. Eksperimentalna istraživanja izvršena su na laboratorijskom razmenjivaču toplote sa cevnim snopom i omotačem na ispitnom štandu koji je formiran na Mašinskom fakultetu u Nišu. Eksperimentalni razmenjivač toplote je izrađen u saradnji sa firmom MIN Inženjering iz Niša. Nakon probnih eksperimenata, prema planu eksperimentalnih istraživanja, izvršene su pet serija eksperimenata. U svim eksperimentima održavan je konstantan protok grejanog fluida (9 m^3/h), kao i temperatura grejanog fluida (15 °C) na ulazu u snop cevi. Najpre je obavljena serija eksperimenta (šest eksperimenta) bez pregrada u omotaču razmenjivača toplote. Varirani su protok (3, 4 i 5 m^3/h) i temperatura (50 i 60 °C) grejnog fluida na ulazu u omotač. U drugoj seriji eksperimenata (30 eksperimenta) korišćene su segmentne pregrade sa veličinom okna od 22%. Varirani su protok (3, 4 i 5m^3/h) i temperatura (50 i 60 °C) grejnog fluida na ulazu u omotač i broj segmentnih pregrada (jedna, dve, tri, četri i pet pregrada). U trećoj i četvrtoj seriji eksperimenata (po šest eksperimenta) varirani su protok (3 i 5m^3/h) i broj segmentnih pregrada (jedna, tri i pet pregrada) za temperaturu grejnog fluida na ulazu u omotač od 60 °C. Rezultati eksperimenata prikazani su u vidu dijagrama i tabela. Analizom rezultata izvršenih eksperimentalnih istraživanja može se zaključiti sledeće:

- pregrada locirana u omotaču aparata u znatnoj meri utiče na karakter strujanja fluida u međucevnom prostoru;
- najintenzivnija razmena toplote je u ulaznoj zoni aparata (do prve pregrade);
- sa povećanjem početne temperature, kao i sa povećanjem protoka grejnog fluida zadržava se trend promene temperature fluida u omotaču;
- najizraženije promene toplotnih karakteristika aparata nastaju postavljanjem jedne segmentne pregrade u omotač aparata;
- veći uticaj na efikasnost aparata ima povećanje broja segmentnih pregrada u odnosu na povećanje protoka grejnog fluida;
- najintenzivnija razmena toplote u omotaču aparata ostvarena je sa segmentnim pregradama sa veličinom okna od 22%;
- broj segmentnih pregrada u omotaču aparata, u odnosu na ostale razmatrane geometrijske veličine, ima odlučujući uticaj na toplotne karakteristike aparata.

Ključne reči: Razmenjivač toplote sa cevnim snopom i omotačem • Eksperiment • Efikasnost • Pregrade

Porcelain veneers – preparation design: A retrospective review

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Abstract

This paper discusses the preparation of tooth design for porcelain veneers. It follows the literature more than three past decades. From the very begining, the porcelain veneers were placed to no/minimaly prepared tooth substance, showing different problems in clinical use. Later, the technique of etching the porcelain and controlling the reduction of tooth structure presented the great steps forward in porcelain veneers accepting. The special accent concerning the preparative design was placed on variations of incisal edge preparation - the problem, which is still present in current practice. Additionally, the paper emphasizes the extremely demanding protocols in making the porcelain veneers, as well as their expanded clinical indications.

Keywords: literature review, porcelain veneers, preparation design.

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REVIEW PAPER

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In the aesthetic dentistry, the porcelain veneers present the first class clinical conservative modalities. The current literature recognizes them as the state of the art of each auspicious dental practice. As being less invasive, for both hard and soft tissues and granting satisfactory aesthetic outcome, the rehabilitation procedure with porcelain veneers has been widely welcomed by the patients. In addition, the modern improvement of composite cements, adhesive systems and simplified cementation procedures also enable the promotion of this effective treatment approach among the dentists.

But, different literature data bring to the practitioners various dilemmas concerning tooth preparation design, as well as the clinical recommandation with expanded indications, opening the controversial sugestions.

The following literature review with retrospective glance will thoroughly highlight this topic.

HOW DID IT ALL BEGIN?

The use of porcelain veneers goes back in the late 1930s. The wish of a famous Hollywood actress to „urgently" alters the looks of her several teeth represents the true begining of these restorations; at least that is how the idea that some aesthetic problems could be solved in this non-agressive way was born. The procedure was performed by Charles Pincus, one of the pioneers in aesthetic dentistry, who applied thin veneers and provisionally fixed them by means of using prosthesis adhesive powder. Created as an emergency solution, porcelain veneers of Pincus's time were labelled as false front or Hollywood veneers. The most commonly created as thin porcelain veneers, they covered the irregularities of existing teeth: diastemas, rotations and malpositions, and established the desired shapes of the dental arch. Sometimes they were also placed on the teeth in the lateral regions in order to fill in too narrow or sunken cheeks of the actors. Due to the unresolved problem of their fixing and huge functional stresses to which they were exposed, these veneers had a short life spain in the mouth [1].

The inovation of acrylic resin and their fast development marked a second step in the application of aesthetic materials for the acrylic veneers fabrication. A number of physical performances of resins limit their clinical longevity in the mouth: high degree of polymerization shrinkage, poor edge adaptation, high coefficient of thermal expansion, risk of restoration edges recolouring, insufficient abrasion hardness, increased water absorption, resin softening and change of the basic colour. Nowdays, viewed from this time distance, it can be said that the application of acrylics as aesthetic materials meant for dental science and practice is a true driving force for the inovation of new and better materials [1–5]. Lately, indirect acrylic veneers were recommended as an alternative to direct composite veneers. Unfortunatelly, instead of the improved characteristic of these modality, two unsettled problems remained to discredit them as high quality restorations: low resistance to abrasion and separation from composite resin due to poor chemical bonding to cement [1,3,6,7].

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Through the next decades the great emphasis in dentistry has been placed on development of the microfine composite cements and reliable etching to dental enamel [8,9]. The resulting effects led to the introduction of composite veneers for masking different tooth discolorations. These restorations showed a short clinical lifetime of four years or less and various problems such as polymerisation shrinkage, staining, poor wear resistance and thermal dimensional changes [10].

The impossibility of achieving long term aesthetic results with use of composite veneers reactivated the interest of the profession, once again directed it towards porcelain as a chosen material for veneer fabrication. The idea of special preparation of porcelain veneers and their bonding to tooth enamel was first mentioned in prophy text in 1975 [11], but the technique of ethching the inner porcelain veneer surface with hydrofluoric acid was developed later, in 1981 [12]. By measuring the tensile bond strength of etched porcelain to composite cement, NYUCD researches concluded that sufficient retention was obtained [13,14].

The first indications for porcelain veneers were amelogenesis imperfecta, intrinstic staining and anatomically malformed teeth, while teeth in an edge-to--edge or cross-bite relationship were contraindicated. If the teeth being restored have old restorations class III, IV or V, these must be retreated; or if the teeth are incorrectly positioned, they need minor cosmetic countouring first. Early cases of porcelain veneers in 1982 were placed without removing tooth substance and the lingually inclined teeth were selected for that purpose. The literature of that time criticized the possible concequnces of increasing the tooth emergence profile and undesirable material bulk in such cases (periodontal problems and unnatural aesthetic appearance of the restorations). Therefore, the professional standpoint was planning the optimal tooth reduction, which simplifies the fabrication and placement of porcelain veneers [14,15].

The first step in preparation procedure was to make a lingual plaster index suggested to be obtained in a wax tray. The labial extension of the plaster was trimmed, and the incisal edge of tooth was reduced 1 mm, using the lingual plaster as a guide. If the incisal length of the anterior teeth needs a modification, the aesthetic planning is done on the stone models (study cast) with tin foil painted on the stone teeth and a lingual plaster index [15]. The controlled reduction of tooth labial surface is critical. It was recommended to be done into two planes, gingival and incisal, using cut horizontal grooves 0.5 mm depth marked with a lead pencil (to protect against over reduction). The two planes should merge smoothly into each other forming a gentle labial curve. The gingival portion was prepared with a diamond bur to create chemfer and was extended up to the free gingival margin. The same bur was used to prepare the rest of the labial surface. When the teeth possessed proximal contacts, enamel reduction was followed toward in the proximal embrasure without eliminating the contact points. To avoid the unsightly appearance of the juction of the proximal porcelain/tooth substance and gingival-proximal bulk, it was suggested to extend the preparation proximally into gingival area. The enamel reduction was 0.5 mm at incisal edge, and the edge was rounded. In addition, it was mentioned as modality, the incisal overlap with finishing line on inciso-lingual portion. It hides the incisal margin, makes the new one in porcelain more esthetic, provides the incisal edge reinforced and allows a positive seat for veneer. In those cases, the incisal edge should be reduced approximately 0.5 mm with 0.75 mm at mesio/disto incisal angles [14]. Also, some authors prefered local anesthesia during preparation procedure [15].

The article printed in the late eighties, stresses the main contraindications in use of porcelain veneers: teeth with poor quality enamel, rotated or overlapped teeth and broken down teeth which may not offer enough support for veneer. Also, there is opinion that if teeth are in linguoversion, retroinclined or are peg-shaped, reduction of enamel can usually be avoided, especially in the young patients. But, if there is need to mask out strong discoloration or prevent an overbulked restoration, better aesthetic appereance would be achived by reduction of enamel tissue at least for 0.5 mm. In addition, there are recommandations for preparation protocols. The first step is the establishment of a confluent finish line proximally and gingivally with round diamond bur which creates a positive chamfer. Cervically, the finish outline is in level with the contour of the free gingival margin in the most cases. Rarely, it could be positioned 0.5–1 mm subgingivally. The proximal finish line is extended into the embrasures, but usually short of the contact point. The second phase is the reduction of labial enamel by applying a series of 0.5 mm deep vertical tracer cuts close together, which in final provide tooth reduction of 0.5 mm labially. The most critical step is preparation of the incisal surface, which may considerable vary. The preferred way is the reduction of incisal edge by applying a bevel at the expanse of a labial surface and incisal edge to a depth of 0.5-1 mm. The other possible designs are: feathered incisal edge or window preparation. Also, the overlapped incisal edge preparation is useful in circumstances where it is necessary to change the tooth dimensions or to protect part of the palatal surface. The final step in the preparation procedure is achieving the smooth enamel surface and round off sharp angles with fine diamonds and flexible discs [10].

At the begining of nineties, over 68% of general dentists have placed at least one porcelain veneer in their practice. Instead of the fact, that laminates are believed to be the simplest aesthetic modality, they are very sofisticated and need special skill and accuracy by all dental team [16]. The clinical problems associated with veneers are poor marginal integrity [17], unaesthetic monocromatic color, unpredictability of cemetation [18–20], extensive placement time and unrealistic long term expectations by patients [19]. The dental profession stated that the definite tooth preparation must be done in enamel (ideally) with a chamfer linish line; only in some instances, the praparation includes a rounded incisal edge and terminates lingually with a heavy chamfer demarcation [10,21–23]. At that time the first special diamond set of instruments for depth cutting (LVS-1 or LVS-2) (Brasseler laminate veneer system set 4151, Brasseler, USA) were born. The remaining excess enamel was removed with two-grit diamond stone (LVS-3 or LVS-4), and the margins were polished with a 12-fluted finishing bur (brasseler H283K016). All other internal surfaces of preparation are left non polished intentionally to create the optimal bond to composite cement. The cervical margins are placed sligtly above the gingival level, and the application of the retraction cord is not always necessary. Additionally, it was highly recommended the use of magnification for checking and visualization of all the phases of the preparation procedure [23].

WHAT IS NEXT? (1990–2000)

During the last decade of the old millenium, literature′ s data announced the statistic details about the quantity of prepared tooth structure in porcelain veneers making: only 25% of the practitioners remove 0.75 mm of tooth tissue, while 65% of the therapists remove less, around 0.5 mm. Also, 84% of the practitioners create the cervical chamfer as demarcation, 22% provide complete coverage of the incisal edge while 78% of the clinicians offering complete coverage occasionaly [24].

Still, there are controversy as to whether or not tooth preparation is required on labial surface. Among the results of 26 literatute reports which have been published since 1991, majority of them, 22 studies favored some of the preparation modality [25]. Those days, the scientific community discussed the problem of three different incisal edge design variations: the „window“ or intra-oral preparation, the „overlapped“ and the „feathered“ incisal edge preparation. The window style of preparation was recommended as it can withstood the highest load until failure (dynamic stress analysis) and transmitted the least forces through the veneer (photo-elastic stress analysis) compared to the other two designs. It was stated that the porcelain is the weakest point in tooth-cement – veneer system, and if the selection of preparation is based on mechanical criteria, the window type of edge preparation can be one of the most optimal conversative choices [25,26].

Studies evaluating the marginal integrity of porcelain veneers depicted different, but considerable discrepancies which range from 60–292 μm. It would be ideally, to create the porcelain margin in enamel enabling the excellent veneers sealing. But, clinical situations such as root recession, caries, abrasion cavities, aesthetic demands, very often impose to the practioners to finish the porcelain margins on dentin or cementum, significantly increased the potential for microleakage. Also, there were professional attemps of using the denting bonding agents to pretreat the dentin surface and to promote wetting of the composite cement. Glass-ionomers as pretreatment modality did provide no resistance to microlaekage; therefore it seems that acceptable solution to reduce the microleakage is to seal all finished veneer margins with unfilled resin [27––33].

It was discussed the possibility how to restore worn mandibular anterior teeth. As complete crowns are the last option (weaken the teeth), the porcelain veneers could be used in cases, when the vertical dimension need to be increased. But, such a choice of restoration must be carefully checked, because the mandibular teeth have the important role in anterior guidance and they will be subjected to significant occlusal force in function. It was considered that patients with excessive vertical overlap and little horizontal overlap are not good candidates to carry the porcelain veneers. In addition, when restoring all anterior mandibular teeth, maxillary palatal surfaces could be restored with porcelain to minimize the possible wear effects (similar materials) [34].

Still, there is a promotion of incisal edge design without overlapping, instead of an existing opinion, which supports the overlapping way as standard procedure. There are results about the efficency of different preparation style of incisal edge: feathered edge, window preparation, incisal bevel and overlapped incisal edge in the 3-years follow up study. It was illustrated that wrap-over method is not optimally conservative and must be avoided in young patients. It appears, that the veneer should not be brought into contact with the opposite tooth, as porcelain is known to be very brittle material (etched porcelain with silane produced bond strength which surpasses the cohesive strength of porcelain). Under normal overbite a preparation modality without incisal overlapping will be preferable [35]. At the same time, the critics of the incisal edge window preparation design have started. It is „accused“ of leaving a weak enamel margins of

poorly supported enamel prisms, which may result in chipping during protrusion movement in future. Moreover, with window preparation, resin cement will be bonded to the longitudinal oriented enamel prisms. Such a situation produces a weaker bond between the enamel and porcelain veneers, and leads to the veneers' debonding (polymerization shrinkage of composite cement) [10,36,37].

During mid-nineties, the porcelain veneers are recognized as accepted method to restore malformed, malaligned, discolored and fractured teeth. They presented a good alternative to complete ceramic crowns, when combine with correct techique and careful application [38,39]. Literature data emphasized the necessity of the tooth preparation procedure for several reasons: shear bond strength of composite cement to etched enamel is increased, particulary if a coarse diamond bur is used, it is possible to provide the sufficient place to prevent overcontouring at the gingival margin and also is easier to control stress distribution in the veneer [15,40]. It was advocated to use the specially designed depth gauge burs for rational removing of the tooth substance to avoid the improvization. The „handfree" technique, which is very often emloyed by the practitioners, illustrated different drawbacks. The studies showed the significant reduce of tooth structure in cervical and proximal regions, in excess of 0.5 mm (till 1.2 mm), while the least reduction occured in the incisal third (0.2–0.4 mm). The consequence of such preparation technique is overcountered veneer in mid-incisal level and exposed dentin in cervical zone [41]. The other studies confirmed that the incisors enamel thickness in gingival third is 0.3–0.4 mm, so it seems that 0.5 mm reduction at this level would result in dentin exposure or possibly complete elamination of enamel (laterals) [42]. The use of hydrophilic dentin bonding system has demonstrated penetration of resin into dentinal tubules, therefore that procedure could be benefitial in decreasing the sensitivity and microleakage [43].

The interesting longitudinal studies which followed the efficiency and the other parameters relevant for the clinical performance of porcelain veneers were published during the late nineties. The results of 6.5 years long study, which evaluated the survival rate of 372 porcelain veneers fixed mostly (90%) on not prepared teeth, showed the high failure rates, 22–39%, with overall probability of a veneer surviving with no problems only in 50%. The main technical reasons contributed to the failures were the two clinical protocols: veneers were bonded to unprepared enamel and the veneers were sandblasted and silanated only, they were not etched with HF acid [44]. On the other hand, the retrospective report of 3500 placed porcelain veneers observed during the 15th period, showed approximately 7% of failure rate manifested as fracture, debonding or leakage. The fractures are described as static, cohesive or adhesive, leaving the leakage almost between the tooth and resin. The author promotes an enamel substrate as a critical element to a successful clinical outcome, as well as tooth preparation including an intraenamel preparation (whenever is possible). Also, the porcelain veneers were reffered as an enamel ceramic restorations. In the commentary, it was disscused the new trends in tooth preparation for veneers which are more aggressive than initially desrcibed, concluded that veneers were very often primarily adhered to a dentin substrate in clinical reality [45,46]. Another ten-year longitudinal study of 191 porcelain veneers presented the excellent results with survival probability of the veneers of 97% in five years and 91% in 10.5 years. Over the observation period only 4% of examined restorations failed. Veneers' failures like gingival recession, marginal discoloration debonding or porcelain fracture, were more likely when the restorations were bonded to dentin or when the patient suffered of CMD (clenching, griding) [47]. Also, in a 2.5 year interim evaluation of veneers durability, it was shown no difference between the techniques of preparation (without the reduction of incisal edge or incisal reduction with palatal bevel). The study recommended that incisal edge should be left unprepared if possible (aesthetic reasons). Additionally, preparation of incisal edge was considered to be unnecessary to assure or improve the veneers strength, therefore it would be avoided [48].

During this period, the profession states a problem concering the long clinical duration of porcelain veneers, turning the interest toward to the other structures essential for their functional quality. A 2-dimensional finite element analysis (FEA), known in mathematics, is used to show the stress distribution in the veneers and the fracture mode at maximum load. It was noticed that the different designs of cervical demarcation (feather edge, chemfer and shoulder) were of less importance than the masticatory loading condition. For the first time, it was mentioned, that the most significant factor for stress variation in porcelain veneers was the cement layer. In that sense, it was recommended to pay attention to moisture control and proper handling of composite luting, as procedures relevant for the veneers' success [49].

The research on crack propensity of porcelain veneers frequently occupied the scientists at the end of the 20th century. Incisal chipping and development of cracks that occur before and during the cementation, appear to be primarily, a consequence of therapeutic skills in handling and positioning of the veneers. However, considerong the polymerization cement shrinkage, as well as temperature variations (consuming of

food and beverages), there is considerable disagreement in the CTE of the teeth and porcelain, which in turn produces significant stress in the porcelain. Then, they began seriously thinking about a new design that includes a proximal surface of the teeth, "wrap around", the importance of location and configuration of the cervical demarcation and the relative thickness ratio of porcelain/cement layer. It was pointed out the importance of: 1) uniform tooth reduction (special attention is focused at facial axial level of the preparation which thickness is critical), 2) improved quality of tooth preparation (smooth contours, absence of undercuts), 3) the optimal CER/CPR relationship (above 3) and 4) the application of die spacers during laboratory procedures (to define the uniform cement layer); all of them presented the key elements of good clinical practice [50].

The most of the available literature data from the beginning of XXI century brought the recommendations regarding the preparation of teeth for porcelain veneers. Those studies supported removal of varying amounts of tooth structure, contrary to early concepts of no tooth preparation [15,51–53]. In particular, the literature data highlight the removal of aprismatic top surface of mature unprepared enamel, which offers a minor retention capacity and can jopardize the bond strength of the composite cement to tooth structure [54]. On the other side, the preparation must be maintained completely in enamel to achive the optimal bond of different supstrates [55]. If dentin is exposed, it must be protected for the period between preparation and cementation. It could be done by means of primers, hydrophilic reactive monomers in organic solvents, which seems not to decrease adhesion of veneers system, also making possible the further cementation [56,57]. The proposed alternative, is the application of denting bonding agent immediately after the preparation. This procedure may prevent the development of bacterial leakage and dentin sensitivity [58]. If temporary resin veneers must be created (aesthetic, phonetic reasons), it is indicated to use eugenol free provisional cement or to fixed it by a small area of etched enamel (Table 1) [53,59].

Again, there are polemics concerning the differences in the amount of tooth substance which must be removed during the preparation procedure. Free--hand technique tends to leave underprepared labial surface, with possible overcontouring of the finally restored tooth. The excessive bulk in the gingival portion of the restoration changes the emergence profile and could initiate gingival inflammation. Overcontouring in the incisal part of the restoration alters the protrusive relationship, promotes atypical incisal loading of the veneer, creates subsequent fracture and pro-

Table 1. Descriptive statistics of porcelain veneers clinical trials with reference to material brand and type of tooth design

Author	Number of veneers	Number of patients	Porcelain/adhesive system	Preparation design
Clyde and Gilmoure	200	Not specified	Chameleon (Terec)/duo-cure (Terec)	Feathered incisal edge In. Bevel Palatal overlap
Calamia	115	17	Chameleon/Comspan?+Ultrabond (Den-Mat)	No preparation Slight incisal overlap
Jordan *et al*.	80	12	Not specified/dual cure (not spec.)	Conventional (no incisal overlap)
Rucker *et al.*	44	16	Vitadur –N /Vita)/Heliolink + dual cement (Vivadent)	Incisal bevel Feathered incisal edge
Christensen and Christensen	163	45	Cerinate (Den-Mat) ultrabond (Den Mat)	Feathered incisal edge
Nordbq	135	41	Ceramco (Ceramco Inc)/Porcelite LC (Kerr)	Conventional (no incisal overlap)
Jäger *et al.*	80	25	Mirage/Mirage FLC +Mirage Bond (FA Mirage)	Palatal overlap
Strassler and Weiner	291	60	Cerinate (Den Mat)/Ultrabond (Den-Mat)	No preparation Conventional (no incisal overlap)
Walls	54	12	Fiber reinforced porcelain/Heliolink (Vivadent) + Gluma (Bayer)	Special preparation for worn teeth
Meijering	56	Not specified	Flexo-ceram (Elephant Ceramics)/not specified	Conventional (no incisal overlap) Palatal overlap
Peumans *et al*.	87	25	GC Cosmotech Porcelain/CG Cosmotech Bonding set (CG) + Scutabond 2 (3M)	Palatal overlap
Kihn *et al.*	59	12	Ceramco Colorlogic/Ceramco Colorogic Bonding System	Conventional (no incisal overlap) Palatal overlap

duces poor aesthetic outcome. Teeth prepared with a silicone key or depth gauge bur can be overprepared with exposed dentin, particulary in the cervical third of the preparation (enamel is very thin). Therefore, it was recommanded to use 0.4 mm depth gauge bur for limitted removal of tooth structure. In fact, the orientation grooves involve additional smoothing out of the grooves done by depth bur, so the tooth removal would be higher than 0.4 mm, approximatelly 0.5 mm. Also, there are situations where free-hand technique is the proper choice: severely discoloured teeth and non-carious tooth surface loss. In addition, the silicone index is more helpful than a depth bur when reducing the incisal edge and bevelling or overlapping the incisal/palatal surface [60].

The study of stresses within the porcelain veneers with different preparation design, using 2D finite element analysis, has shown unexpected interesting results. Incisal overlap preparation model was associated with less compressive stress within porcelain and composite, than the window preparation design. Also, the tensile stresses with labial and palatal loading were significantly greater for the chamfer and shoulder design (25 times) compared with knife-edge preparation design. The authors confirmed that porcelain physical properties, and the bond strength at the composite-tooth interface, as well as composite-porcelain interface, presented critical points for veneer restorations clinical success. Additionally, using the incisal overlap design, the porcelain veneers with knife-edge labial margins could better introduce occlusal stresses without fracture [61]. The other similar study stated that the lowest values for the loadability for the overlapped preparation is more than three times higher, if compared with the biting force for incisor (axial direction), therefore this design may be used in different clinical indications safely (to re-establish the proper anterior guidance) [62].

The actual scientific literature criticizes the professional attitude which does not offer a relevant information of the preparation design responsible for veneers longevity. Still, it remains controversial whether various tooth preparation design could influence the fracture strength of veneers or whether one tooth preparation modality is superior to another. Currently, a new design in veneers preparation technique, named „butt joint“ configuration is introduced. It is created by cutting the incisal surface (edge) 2 mm flat, without forming the palatal chamfer. The substitution of a palatal chamfer with a new design offers a several advantages: 1) provides an optimal ceramic/composite ratio at the palatal surface, 2) decreases the risk of postinsertion palatal cracks caused by shrinkage of composite cement (polymerization contaction, natural thermal changes in the mouth), 3) permittes the preservation of a peripherial enamel layer around all margins, which is essential for eliminating microleakage at the palatal/restoration contact and counteracting shear stresses, 4) allows for optimal characterization of incisal third of veners, 5) butt join preparation is easier, less time-consuming, easily reproduced on the model, 6) provides a significant support for ceramic layers, 7) the path of insertion could be buccal-palatal or incisal-cervical and 8) the risk of fracture for thin palatal edges of ceramic is controlled with butt-join design.

The treatment with bonded all-ceramic restorations, including the porcelain veneers, is based on adhesive properties of different materials and not classical micromechanical retention and resistance. If it so, the palatal chamfer is not only essential for providing retention to tooth structure for the ceramic veneers. Ceramic as a brittle material fails at a critical strain of 0.1%, and if the bond to tooth fails, the ceramic would be broken easilly [63–66].

„BACK TO THE FUTURE“ (2000–2012)

The first decade of the new millennium has brought the studies concerning tooth preparation design for porcelain veneers, mainly in terms of possible clinical failures over the longer lifetime. Interesting, but somewhat confusing, were the results of the study which emphasis the importance of the knife-edge cervical finish line combine with the “overlap” incisal edge design that produced the smallest tensile stresses in the porcelain and composite cement compared to the other recommended designs [61].

During this period the profession asked a real question that demanded an answer. In fact, it appears, that this question has always been controversial in some way. How thin veneers made of brittle ceramic materials survive in the conditions of the oral environment for a long time?

What is surely known is the information that porcelain veneers can clinically fail due to the development of flaws on the restoratons surface [50]. The surface imperfactions act as a potential source of deeper cracks initiation. The microcraks and the place of stress concentrations may be inherent to the porcelain or may occured during laboratorial and clinical procedures (PV manufacturing, pre-cementation treatment, cementation). The slow crak growth at the tips of surface flaws is obvious in the moist environment due to the hydrolysis of silicate bonds [67]. But, equally, the surface flaws may become expanded as the result of stresses induced by thermal variations of every day ingested food and drinks (10 extreme thermocycles would occur per day). A large flaw on the porcelain surface may turn into premature fracture, if the temperature differences are greater as well as the imposed tensile stresses [68].

According to some professional opinions there are generally two techniques for localization of the incisal finish line during the veneers preparation procedure [69]. The first way leaves the lingual surface unprepared, that is window or intraenamel preparation, obtained when the facial surface is finished at the incisal edge. The second technique terminates on the lingual tooth surface, while the incisal edge is reduced. Literature data showed the interesting connection between microleakage and different incisal edge preparation. The marginal fit and integrity, as well as resistance to microleakage, are important elements for clinical success of porcelain veneers. Marginal leakage involves percolation of fluids and invasion of different enzymes, acids and bacteria. The percolation is the result of the mismatch of CTE between tooth structure and restorative materials and curing shrinkage of luting cements [70]. The porcelain-composite bond could be compromised by hydroscopic expansion of the resin cement or by hydrolysis of the silane [71,72]. Studies, using radioactive isotopes ($CaCl_2$, pH 7), indicate that the incisal edge preparation type affects the microleakage features at the incisal finish line. The window veneers preparation has the greater preventive potential in decreasing the microleakage at the incisal margin than the overlapped modality. However, the cervical microleakages were of the similar degree in the two different tested incisal margins [69].

Two impressive publications, which came out in 2002/2003, dealt with the problems of minimal invasive modalities in aesthetic dentistry, with especially emphasis on porcelain veneers. Biomimetic approach in aesthetic reconstruction of the anterior teeth with bonded porcelain veneers is an extraordinary issue, which offers a new restorative solution that balanced the expanded indications for porcelain veneers and various clinical references. The great topic of this publication underlines the porcelain stiffnes and the biomechanical strength achieved through tooth/composite/porcelain bonding, as new powerful structure enabled as a whole entity to support different masticatory activities.

Generally speaking, the tactics of tooth preparation for the porcelain veneers depend on appropriate selection of the patient and a correct diagnostic stages. The first task during tooth preparation is the maximum preservation of remaining sound tooth structure, bearing in mind that porcelain veneers significantly differentiated from traditional cemented restorations. The preparation of sequential procedure starts with detailed case analysis and making the silicone index (template) over diagnostic wax-up. It is pointed out that horizontally sectioned silicon key is the most useful tool for enamel reduction. The next step is the preparation of the axial surface where tooth reduction is done trought three phases using the tapered, rounded-end diamand burs of differnt diameter. The smallest diameter bur is used first to cut the proximal reduction grooves. Afterwords it is suggested to place the deflection cord to improve visability of the paragingival margin, while in second phase the medium-diameter bur is used to create vertical facial grooves. The depth of each groove is individually controlled by silicon template. The third phase is gross preparation or axial reduction III, which is created with a larger bur to prevent the penetration into the grooves. The final effect of those phases is removing of 0.5–0.7 mm tooth tissue uniformly and producing enough space to ceramic at the proximal and axial levels. Going further, the incisal edge is reduced at least 1.5 mm and established the palatal finish line as the last step of tooth preparation. At the end, it is essential to realized the preparation without sharp angles and undercuts during the finishing procedure. In the cervical and proximal areas it is necessary to make a light, clinically accepted chamfer. The intrasulcular margins are recommended only when closing interdental black triangle (loss of papillae) or diastema to enabled the ceramist to produce a progressive emergence profile [73,74]. Additionally, the excessive interdental penetration must be avoided, except for the two particular cases such as wrapping of old class III restorations and reduction of diastema/“black triangles”. The introduction of sonic oscillating technique and instruments seem promising when used on large interdental contact surfaces, overlapping teeth (a more conservative proximal preparation as compared to burs) and also for cases of subgingival margins, when is possible to finish the margins without damaging the soft tissues.

Talking about the palatal extension of porcelain veneers, which is always critical, the authors stated that the extent of tooth substance loss have to be considered. The main rule to respect is to avoid the palatal extension of the preparation in the zone of palatal concavity. That part of the palatal surface is the place of the maximum tensile stresses generated during loading. Therefore, the use of a butt margin instead of a mini chemfer (finish line) could provide the restoration margin with a bulk of porcelain.

If it is case of maximum remaining tooth substance, it would be possible to create incisal overlaps with butt margin or mini chamfer, but is imperativ to avoid a long chamfer extended into palatal concavity. For moderate crown fracture that involved incisal one third, or severe wear, it is recommended to make butt margin which limits the extension of the ceramic and reduce the amount of stress at tooth/veneer interface [75]. In addition, a horizontal butt margin is correct choice for fractured teeth, especially from the aspect of future adhesive bonding, which would be obtained with ena-

mel prisms obliquely sectioned at angle greater than 50°. One of the interesting solution which could be useful in fractured teeth to avoid the palatal fossa, is to make a composite builtup „stress breaker," when is expected a stress redistribution into the more flexible material [76]. The clinical situations with severe crown fracture that involved incisal two thirds are less complex than moderate tooth fracture, because the palatal margins (butt margins or mini-chamfer) could be situated in the low tensile stress area of smooth cingulum. It must be noticed that evaluation of indication spectrum for pocelain veneers toward replacement of great quantity of tooth substance complicated their original preparation design, making it closer to partial crowns design.

Another fascinating book about porcelain laminate veneers presents a comprehensive theoretical and clinical issues, essential for understanding the procedures, practical guidelines and the doctrinal rules, important in veneers production. Also, the publication illustrates the differences in the veneers preparation design compared to protocols mentioned earlier [73–76], showing the authors distinct individual approach to this topic.

The fact, that enamel has different thickness at the gingival (0.3–0.5 mm), middle (0.6–1 mm) and incisal (1.0–2.1 mm) 1/3rds of the tooth labial surface, requires a special diamond instrument to facilitate the labial preparation. Diamond depth cutters have different cutting depths whitin themselves and the weels can cut trough the enamel until the shift is flush with the surface, creating the horizontal grooves. The technique will be sucessful only if the depth cutter bur is hold at three different angles, thus enablingthe preparation of the labial surface into different planes. But, the clinical practice confirms that is impossible to cutt the three grooves simultaneously (natural curve of the facial surface) especially in lower premolars or canines. It is therefore recommended to begin with the cervical and medial striations, afterwards adjusting the angle of the instrument and tracing the occlusal (incisal) groove, with the medial one as a guide [42]. The remaining tooth structure between the orientation grooves, is removed using the tapered round-end fissure diamond bur. The labial surface design has to reproduce its natural convexity, respecting, first of all, the biological principles. A minimum reduction thickness of 0.7 mm in junction of the middle and incisal thirds of the tooth is necessary to achive the optimal thickness and promising optical properties of the future porcelain veneers. Cutting the silicone index, that had been made before, into horizontal slides, it is possible to control the vertical levels of labial surface preparation.

A mini-chemfer 0.3 mm is preferred as finish line for all the gingival margins, practically done with the round end fissure diamond bur. The bur must be held parallel to the inclination of the cervical 1/3rd of the labial preparation, moving from the distal towards mesial interproximal surface. Additionally, it is important to remove serrated, overhanging enamel prisms, to achive a distinct finish line. The gingival extension should be placed in the enamel whenever is possible, supragingivally, providing the great benefits. Very often, if the cervical finish line is located subgingivally (tetracycline staining, „gummy smile", case of caries, old fillings), it will be created in dentin, with all the risk for adhesive bonding [77]. If deeper chamfer preparations need to be placed at the gingival margin, it should be recommended to use fine-grit round ended fissure diamond burs of a larger diameter.

The preparation design of the tooth proximal surfaces is planned in details, before the preparation of labial surface and positioning the gingival finish line. The basic principles are: to preserve the contact area (problems: the cases of natural diastema, fractured tooth angle, encompass a proximal composite fillings; margin should be extended further in a lingual direction) and to place the margins beyond the visible area (important for aesthetic appereance, especially if there are major differences in tooth and veneers shade) [78].

It is very interesting to pay attention on authors' instructions concerning the proximal tooth preparation. There is suggestion to follow the preparation procedure in two regions: the gingivoproximal area and direct proximal contact area, both necessary to be prepared differently. The simplest way to prepare the gingivoproximal margin (extends gingivally from the interdental contact zone or point) is to start preparation after the gingival prep is finished (labial finish line). The same, round end tapered fissure diamond bur is used, held at 60° following the gingival margin towards the palatal, from mesial and distal. Looking from mesiolabial aspect, the curve that looks like interproximal elbow, frequently positioned supragingival [79]. Direct proximal reduction (area which is located in the incisal 2/3rd of the proximal surface) is natural extension of the labial tooth reduction. Using the same bur, as used before, the gingivoproximal reduction is continued by uprghting the angle of the bur vertically into the proximal area, parallel to the long tooth axis and to the mid-line. Mostly, the proximal wall should end 0.25 mm labial to the contact area, following buccolingual tooth inclination. It is considered as some kind of interlock which can improve the mechanical stability and resistance of the cemented porcelain veneers. In some particular cases, the metal matrix band or oscillating instruments (one side is noncutting flat area) could be useful to protect the proximal surfaces of adjecent non prepared teeth during this phase.

The author underlines the two basic techniques for the placement of the incisal finish line. The first ends at the incisal edge and can be in a form of window (intraenamel preparation, without incisal reduction) or feathered incisal preparation (there is no prep of the lingual surface). The second technique looks for the incisal edge reduction, overlapping the incisal edge with porcelain, and finishing the preparation on the lingual/palatal surface.

However, it is still a great dilemma whether the incisal edge of the tooth should be included in the preparation design for porcelain veneers, or not.

The contemporary dental practice frequently favors the overlapped preparation modality as well as finishing the porcelain veneer on the palatal surface. In that case the incisal edge must be shortened 1 mm or 1.5–2 mm for canines and lower incisors with light palatal chamfer 0.5 mm wide, terminated 1 mm far away from the central contact points, not involving the palatal concavity. The same effect can be obtained with the flat shoulder finish line, butt joint, design.

Also, localization of opposing tooth should prevent any centric contacts at the border of the porcelain veneer and tooth structure. All sharp internal angles and corners of the preparation need to be rounded off, to reduce the restoration stresses during luting procedures and function, decreasing the number of weak points in the porcelain.

The most of the complex indications for porcelain veneers (diastema, atipical teeth, lower incisors, porcelain palatal laminates on canines, tooth discoloration) demand for the specificities in the preparation design, related to different amounts of tooth structure to be removed, the position of orientation grooves and the protection of exposed dentin [80].

The results of 7-year long-term survival of 110 porcelan veneers with [51] and without [71] incisal porcelain coverage, published in 2004, did not indicate the statistically significant differences between the different approach in preparation design [81].

Later on, in 2005, the retrospective evaluation result of the clinical performance of porcelain veneers placed in the anterior region over a 12-year period was published. Various features, like color match, porcelain surface, marginal discoloration, and marginal integrity of the porcelain veneers were clinically tested using modified CDA/Ryge criteria [82]. The obtained probability of survival rate of 182 veneers was 94.4%, while the clinical failure rate shows only 5.6%. The study stated that the porcelain veneers are associated with nearly the same risk of loss by fracture as metal-ceramic crowns and all anterior ceramic crowns [83–85]. In addition, it is important for successful clinical longevity of porcelain veneers to be carefuly bonded with a correct adhesive technique. Previosly, the proffesion has been informed that acid-etching the porcelain, as necessary procedure for adhesive cementation, would weakening the porcelain surface compared to alumina abrasion treatment. But, also, composite polymerisation shrinkage may help to strengthen the porcelain surface, providing the benefical compressive stress on the porcelain surface [86].

At the moment, when it appears that all the dilemmas related to the porcelain veneers profession have been discussed and clarified, the new (old) chapter is opened, which promotes minimally agressive modality in its true sense – no preparation porcelain veneers. Often, this professional thinking is referred to as „returning to the future“ (back to the future). The proponents of this direction consider expanded indications for application of the porcelain veneers do not fall within the slightly invasive procedure, and that there is nothing conservative about leaving only a quarter of the tooth unprepared. Also, it is emphasized that dental materials do not determine preparation for the treatment that has to be accomplished, but depending on the case (quality of dental tissues), it is necessary to define the use of dental materials. The consecutive development of aesthetic dentistry should reconsider aggressive media advertisements, and the procedures of accelerated „before and after“ images, that resulted mostly with miscommunication going between patients and therapists. All this, to some extent, helped to promote that no-prep (aration) veneers last five years, confirming electivity of aesthetic dentistry as a discipline (Figures 1 and 2) [87,88].

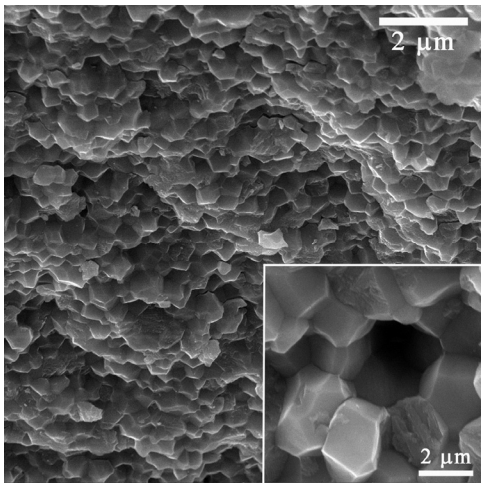

Figure 1. SEM Micrograph of high strength and zirconia ceramic material (Wieland, Germany) with uniform microstructure, used in porcelain veneer fabrication, unpublished authors date.

Porcelain veneers, designed for no prepared or minimally prepared tooth surfaces, are facets with the thickness of 0.3–0.5 mm, similar to thickness of the contact lens. Generally, minority of cases are consi-

dered as ideal for no-prep veneers: individuals with pleasing teeth arraignments as well as minor tooth demage and discolorations able to tolerate an increase in tooth bulk [89]. Practically, the clinical situations that can be regarded appropriate for minimally invasive porcelain veneers are much wider: changing natural tooth shades, masking tooth discolorations, overlaying existing composite restorations (III, IV or V class), closing diastemas, reshaping undersize teeth and peg-shaped incisors, restoring worn, chipped, and fractured teeth, changing the minor misalignments of anterior teeth and reparing existing porcelain restorations trough resurfacing [90–92].

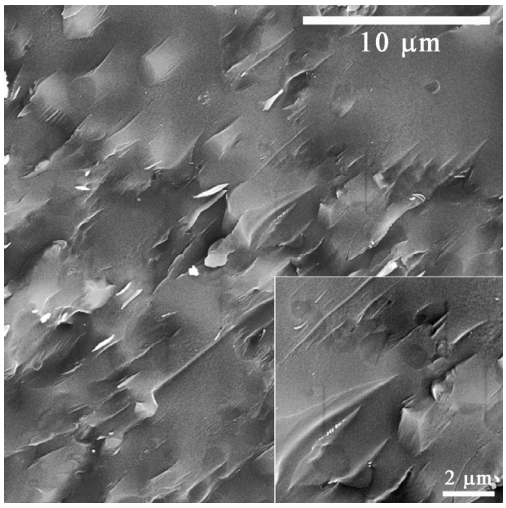

Figure 2. SEM Micrograph of aesthetic, high-strength translucent lithium disilicate ceramic (Ivoclar, Lichtenstein), often used in porcelain veneer fabrication, unpublished authors date.

This type of porcelain veneer offers several advantages for both the patient and therapist: lack of need for anesthesia, painless procedure, elimination of post-operative sensitivity, conservation of the tooth structure, no need for provisionals, longer-lasting restorations due to enamel bonding, higher level of acceptance by the patients, and the others. The great disadvantages of no-prep veneers are: bulky or over-countered appearance, opaque, monotone look with limited translucence, periodontal problems, inability to significant changes of tooth width, possible overcontouring of margins and inadvertent alteration of occlusion [91–93].

To optimize the outcome of no-prep porcelain veneers, it is necessary to perform a comprehensive aesthetic examination of the patients before selecting and planning the therapy. That include: patients expectations, midline position, lip fullness, incisal edge position, tooth shape, disered color change and occlusal schemes. Additionally, only such a detailed concept will facilitate the therapist decision if some degree of tooth preparation is necessary (slight modification of enamel 0.3–0.5 mm with untouched dentin) or not [94].

All the websites of commercial examples of no/minimal-preparation veneer products (Lumineers by cerinate, Vivaneers, DURAthin veneers, da Vinci Veneers, MAC Veneers, IPS e.max Press lithium disilicate veneers) have the same advertising claims, but there is very little evidence in the literature about these types of veneers. The exceptions are Lumineers by Cerinate, which have proved clinical successful longevity for peroid of 20 years (94% survival rate) [95]. Certainly, it is essential for unlocking the problems with no-prep technique, to make the proper selection of the patient/case (small teeth with space, with slight lingual alignment). A stronger emphasis on the minimal preparation veneers concept compared to no-preparation veneers is highly advised [96,97].

Still, there are actual polemics about the current trend toward ceramic veneers for everybody, which is negative phenomena that needs correction by the profession. Different modalities, that should be considered as porcelain veneers alternatives, which is overused in everyday practice, are well known: orthodontic therapy, bleaching/whitening teeth, periodontal plastic surgery, tooth recontouring, conservative resin-based composite restorations, all ceramic crowns, and the combination of the above therapies. Most of these mentioned procedures can preserve the tooth structure and grant the patient a pleasant appearance for lifetime [98].

Enclosing this literature review, it must be admitted that tooth preparation design, especially incisal edge design, continues to be one of the most contraversial aspects of porcelain veneers. For now, there are four types of preparation designs proposed as optimal: the window preparation (limited to the labial surface); the feather incisal edge preparation (extended to the incisal margin, but without definite demarcation line); shoulder finish line, butt joint design; and overlapped incisal edge preparation with a palatal chemfer. All the design possibilities have the advantages and weak points, but the very recent studies promote incisal butt joint design with an addition of palatal chamfer as most promising especially in worn tooth structure [99].

The therapist choice is in a large degree determined by the specificity of the clinical case and should be in advance detailed planned through the close communication with the patient and ceramist. Also, it should not be forgotten that the porcelain veneers are extremely demanding conservative modality. Therefore, the satisfying final expectations as well as their clinical longevity could be reached only with the great respect to the next stages in porcelain veneers realization (impression and cementation protocols).

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IZVOD

PORCELANSKE FASETE – PREPARATIVNI DIZAJN: REVIJALNI PREGLED

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Pregledni rad)

Rad diskutuje preparativni dizajn zuba u izradi porcelanskih faseta kroz literaturni revijalni osvrt duži od tri decenije. Upoznajući se sa porcelanskim fasetama, dentalna profesija nije prepoznavala na pravi način značaj preparacije zuba koji će nositi fasete, pa su fasete aplikovane na nepreparine površine zuba. Veliki korak napred u kliničkoj prihvatljivosti porcelanskih faseta donela je tehnika nagrizanja površine gleđi i kontrolisana redukcija ovog tkiva. Poseban akcenat u uspešnoj ekspolataciji faseta predstavlja iznalaženje odgovarajućeg dizajna incizalne ivice, kao i rešavanje problema fraktura tankih porcelanskih faseta. U vezi sa tim, rad diskutuje jedan od modaliteta u preparaciji incizalne ivice koji uključuje samo njeno skraćivanje do 2mm, but joint dizajn. Istraživanja pokazuju niz prednosti ovog dizajna u odnosu na palatinalni žleb: optimalan odnos keramike i cementa na palatinalnoj strani, smanjen rizik od postcemetirajućeg loma nastalog polimerizacionom kontrakcijom i prirodnim temperaturnim varijacijama u ustima i dr. Uz to, ovaj dizajn omogućava prezervaciju perifernog sloja gleđi, koji je kritičan u eliminaciji mikropukotine na palatinalnom spoju faseta i zuba, jasno se suprostavljajući silama smicanja. Skraćivanje incizalne ivice je jednostavnije, brže, a laboratorijski model je jasniji. Ravna površina ostavlja bolji oslonac keramičkoj faseti, pa su i rizici loma tankih palatinalnih ivica keramike kontrolisani ovim dizajnom. U novije vreme, prošireno indikativno polje u izradi faseta, koje je donelo brojne specifičnosti u preparativnoj tehnici i dizajnu zuba, biva kritikovano od „back to future“ promotera u struci, koji se zalažu za upotrebu tankih non-prep porcelanskih faseta. Uz to, revijalni pregled apostrofira značaj poštovanja veoma zahtevnih, različitih kliničkih faza u preparaciji zuba za prihvatanje sofiticiranog konzervativnog modaliteta u struci, kakve su porcelanske fasete.

Ključne reči: Literaturni pregled • Porcelanske fasete • Preparativni dizajn

Antioxidant capacity and contents of phenols, ascorbic acid, β-carotene and lycopene in lettuce

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Abstract

The antioxidant activity of three lettuce varieties (*Lactuca sativa* L.) Emerald, Vera and Neva, cultivated in two kinds of protected spaces, a glasshouse and a plastic greenhouse, under controlled conditions, was determined. The content of antioxidant compounds: total phenols, flavonoids, L-ascorbic acid, β-carotene and lycopene, were determined in ethanolic extracts of the lettuce with spectrophotometric methods. The largest content of total phenols (78.98±0.67 mg GAE/g of dry extract) was found in ethanolic extract of the lettuce variety Neva cultivated in a plastic greenhouse, whereas the largest content of flavonoids (35.45±0.95 mg RU/g of dry extract) was displayed in the lettuce Emerald cultivated in a glasshouse. It was observed that the lettuce cultivated in the glasshouse contained a somewhat higher content of L-ascorbic acid than the lettuce same variety from plastic greenhouse. The content of lycopene in the examined lettuce is negligible, and the content of β-carotene is low. On the other hand, the high content of phenolic components causes favourable antioxidant properties found in all varieties of examined lettuce.

Keywords: lettuce, antioxidant, phenolics, ascorbic acid, beta-carotene, lycopene.

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The formation of free radicals is related to the normal metabolism of aerobic cells. The consummation of oxygen typical during the growth of cells leads to the formation of a series of oxygenic free radicals. The interaction of these free radicals with the molecules of lipidic nature produces new radicals, hydroperoxides and different peroxides [1–3]. This group of radicals (superoxide, hydroxyl and lipid peroxides) can react with biological systems in a cytotoxic manner. It was shown that flavonoids and phenols display an important antioxidant activity towards these radicals, which is generally based on the redox properties of their phenolic hydroxyl groups [4–6]. The phenolic components inactivate the lipid free radicals and prevent the dissolution of hydroperoxides to the free radicals [7]. It is believed that free radicals and their uncontrolled products are one of the important causes of the development of some pathogenic processes such as prostate and colon cancers [8] and coronary heart diseases [9].

Over the last years the doubt regarding the toxicity of certain synthetic compounds used in nutrition has increased, and therefore the interest in natural products has also grown [10,11]. With this aim, researchers consider the possibilities of isolating bioactive compounds from natural products by extraction and purification. Antioxidant compounds can scavenge free radicals and therefore slow the processes of lipid peroxidation, which is one of the main reasons for deterioration of food products during processing and storage [12]. Thus, over the course of recent years, the interest in replacing the synthetic antioxidants with natural antioxidants, especially those of plant origin, has been growing [13].

Vegetables and fruits are rich sources of antioxidants such as vitamins A, C and E, carotenoids, polyphenolic compounds and flavonoids [14], which prevent the attack of free radicals decreasing the risk of chronic diseases. It is believed that the consumption of dietary antioxidants from natural sources serves as a good prevention of cardiovascular diseases especially atherosclerosis [15]. Antioxidant properties of different sorts of vegetables are being increasingly researched in fundamental science, as well as in food industry with the aim of determining the nutritive values of vegetables and protecting the human health.

Lettuce (*Lactuca sativa* L.) belongs to the family Asteraceae and it is the most popular leaf vegetable consumed in increasingly greater amounts because of its nutritive values [16], as well as the fact that it is used fresh so that all the ingredients remain intact. Lettuce is used almost throughout the year since there are a number of varieties which are successfully cultivated in early spring, during the summer and winter. In everyday nutrition lettuce is of great significance primarily for its content of biologically active substances, especially phenolic compounds, ascorbic acid, vitamins

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A and K, folates and carotenoids [17]. The nutritive content varies depending on the lettuce type [18,19].

The aim of this paper has been to determine the content of antioxidant components (total phenols, flavonoids, L-ascorbic acid, β-carotene and lycopene) and antioxidant activity of three lettuce varieties.

EXPERIMENTAL

Plant material

The research included three different varieties of lettuce (*Lactuca sativa* L.); two domestic varieties, Vera and Neva, the selection was made by the Institute for Vegetable Crops, Smederevska Palanka, and the variety Emerald, originating from the Netherlands. All lettuce varieties are head-forming types, type Butterhead, green-leafed, intended for cultivation during winter. The experiments were conducted in two kinds of protected spaces, in a glasshouse and a plastic greenhouse, on experimental lots of the Institute for Vegetable Crops in Smederevska Palanka, Serbia. In the phase of technological maturity of lettuce, laboratory samples were taken. For preparing ethanol extracts of lettuce the fresh lettuce was used.

Sample preparation

Green leaves of the lettuce (samples) (20.0 g) were being extracted by 96% ethanol (200.0mL) for 24 h in the process of cold maceration. The solutions have been filtrated after that time and ethanol was removed by a rotary evaporator (Devarot, Elektromedicina, Ljubljana, Slovenia) under a vacuum and was dried at 40 °C. The dried extracts were stored in glass bottles at 4 °C to prevent oxidative damage untill analysis.

Chemicals

All chemicals and reagents were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Alfa Aesar (Karlsruhe, Germany) and Aldrich Chemical Co. (Steinheim, Germany).

Spectrophotometric measurements

The spectrophotometric measurements were performed using an ultraviolet-visible spectrophotometer (model MA9523-SPEKOL 211, ISKRA, Horjul, Slovenia).

Total phenols content

Total phenols in the lettuce ethanolic extracts were estimated according to the Folin–Ciocalteu method [20]. The extract was diluted to the concentration of 1 mg/mL, and aliquots of 0.5 mL were mixed with 2.5 mL of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 mL of $NaHCO_3$ (7.5%). Aliquots were left for 15 min at 45 °C, and then the absorbance was measured at 765 nm with a spectrophotometer against a blank sample. Gallic acid (GA) was used to calculate the standard curve. The assays were carried out in triplicate; the results were the mean values ± standard deviations and expressed as mg of gallic acid equivalents per gram of dry extract (mg of GA/g).

Total flavonoids content

The aluminium chloride colorimetric method [21] was used to measure the flavonoids content of the lettuce extracts. Two percent aluminium chloride (0.5 mL) in methanol was mixed with the same volume of methanol solution of plant extract. After 1 hour-incubation at room temperature, the absorbance of the mixtures was measured at 415 nm using UV/Vis spectrophotometer. Rutin was used as standard for the calibration curve. Estimation of the total flavonoids was carried out in triplicate. The results were mean values ± standard deviations and expressed as rutin equivalents (mg of RU/g of dry extract).

Total antioxidant capacity

The total antioxidant capacity of the three varieties of lettuce extracts was evaluated by the phosphomolybdenum method [22]. The assay is based on the reduction of Mo(VI) to Mo(V) by antioxidant compounds and subsequent formation of a green phosphate/Mo(V) complex at acid pH. A total of 0.3 mL of sample extract was combined with 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95 °C for 90 min. After cooling to room temperature the absorbance of the solution was measured at 695 nm with a spectrophotometer against methanol as the blank. Ascorbic acid (AA) was used as the standard, and total antioxidant capacity was expressed as micrograms of AA per gram of dry extract (μg AA/g dry extract). The experiment was performed in triplicate and the average absorption ± standard deviation was used to express the total antioxidant capacity.

2,2-Diphenyl-1-picrylhydrazyl free radical scavenging activity

The capacity to scavenge the "stable" free radical DPPH was monitored according to the method of Takao *et al.* [23] adopted with suitable modifications from Kumarasamy *et al.* [24]. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (8 mg) was dissolved in methanol (100 mL) to obtain a concentration of 80 μg/mL. Serial dilutions were prepared with the stock solution (1mg/mL) of the extract. Various concentrations of lettuce extract (2 mL) were then mixed with 2 mL of methanolic solution containing DPPH and left for 30 min in the dark (until stable absorption values were obtained). The absorbance was measured at 517 nm. Ascorbic acid (AA), gallic acid (GA), and butylated hydroxytoluene (BHT)

were used as reference standards and were prepared by being dissolved in methanol to obtain the stock solution with the same concentration (1mg/mL). The control sample was prepared containing the same volume without test compounds or reference antioxidants. The 95% methanol was used as a blank. The 50% inhibition concentration (IC_{50}) value, defined as the concentration of the test material that leads to 50% reduction of the free radical concentration, was calculated as micrograms per millilitre through a sigmoidal dose-response curve.

Hydroxyl radical scavenging activity

The ability of examined lettuce to inhibit a non-site specific hydroxyl radical-mediated peroxidation was carried out according to the method described by Hinneburg *et al.* [25]. The reaction mixture contained 100 μL of extract dissolved in water, 500 μL of 5.6 mM 2-deoxy-D-ribose in KH_2PO_4–NaOH buffer (50 mM, pH 7.4), 200 μL of premixed 100 μM $FeCl_3$ and 10^4 mM EDTA (1:1 *V*/*V*) solution, 100 μL of 1.0mM H_2O_2 and 100 μL of 1.0 mM aqueous AA. Tubes were vortex-mixed and incubated at 50 °C for 30 min. Then, 1mL of 2.8% trichloroacetic acid and 1 mL of 1.0 % thiobarbituric acid were added to each tube. The samples were vortex-mixed and heated in a water bath at 50 °C for 30 min. The extent of oxidation of 2-deoxyribose was estimated from the absorbance of the solution at 532 nm. The percentage inhibition values were calculated from the absorbance of the control and of the sample, where the controls contained all the reaction reagents except the extract or positive control substance. The values are presented as the mean values of triplicate analyses.

Ascorbic acid content

Ascorbic acid was determined according to the method of Klein and Perry [26]. The dried ethanolic extract (100 mg) was extracted with 10 ml of 1% metaphosphoric acid for 45 min at room temperature and filtered through Whatman No. 4 filter paper. The filtrate (1 ml) was mixed with 9 ml of 2,6-dichlorophenolindophenol and the absorbance was measured within 30 min at 515 nm against a blank. The content of ascorbic acid was calculated on the basis of the calibration curve of standard l-ascorbic acid (0.020–0.12 mg/ml). The results were expressed as milligrams of ascorbic acid/100 g of fresh lettuce.

β-Carotene and lycopene content

β-Carotene and lycopene were determined according to the method of Nagata and Yamashita [27]. The dried ethanolic extract (100 mg) was vigorously shaken with 10 ml of acetone–hexane mixture (4:6) for 1 min and filtered through Whatman No. 4 filter paper. The absorbance of the filtrate was measured at 453, 505, 645 and 663 nm. Contents of β-carotene and lycopene were calculated according to the following equations:

$$\text{Lycopene (mg/100 ml)} = -0.0458A_{663} + 0{,}204A_{645} + 0.372A_{505} - 0.0806A_{453}$$

$$\beta\text{-Carotene (mg/100 ml)} = 0.216A_{663} - 1{,}22A_{645} - 0.304A_{505} + 0.452A_{453}$$

Statistical analysis

In order to determine the lowest possible significant differences among the researched parameters the one way ANOVA was applied.

RESULTS AND DISCUSSION

Table 1 shows the total phenols and flavonoids as well as the antioxidant activity of extracts of the examined lettuce varieties cultivated in the glasshouse and plastic greenhouse under controlled conditions.

Table 1. Total phenols, flavonoids, total antioxidant capacity and antioxidant activity of the lettuce extracts tested and standards

Sample plant	Total phenols mg GA/g	Flavonoids mg RU/g	Total antioxidant capacity μg AA/g	IC_{50} / μg ml^{-1}	
				DPPH scavenging activity	Hydroxyl radical scavenging activity
Emerald[a]	75.88±0.54	29.95±0.39	69.50±1.00	21.09±0.85	90.98±0.97
Emerald[b]	68.87±0.55	35.45±0.95	68.99±0.67	21.87±1.03	89.67±0.97
Vera[a]	70.56±0.35	34.65±0.89	70.55±1.02	25.45±1.05	93.87±0.95
Vera[b]	69.67±0.85	34.23±0.89	70.98±0.35	24.57±0.95	91.45±0.79
Neva[a]	78.98±0.67	28.09±0.85	70.15±0.54	24.65±0.89	87.56±1.05
Neva[b]	73.67±0.67	27.87±1.03	69.95±0.35	24.23±0.87	91.67±0.85
$LSD_{0.05}$	5.151	5.481	0.614	1.397	5.697
$LSD_{0.01}$	7.495	7.975	0.893	2.033	8.289
Gallic acid	–	–	–	3.79±0.69	59.14±1.10
Ascorbic acid	–	–	–	6.05±0.34	160.55±2.31
BHT	–	–	–	15.61±1.26	33.92±0.79

[a]Plastic greenhouse; [b]glasshouse

The table also displays IC_{50} (μg/ml) values of DPPH scavenging activity and hydroxyl radical scavenging activity of the examined lettuce extracts and standards in relation to which these determinations were performed.

The results show that lettuce extracts have high content of total phenols and flavonoids, which has been established by other authors in case of other lettuce varieties [28]. The highest content of total phenols is in the lettuce variety Neva cultivated in the plastic greenhouse (78.98±0.67 mg GA/g). If we compare the amounts of phenols in the plastic greenhouse lettuce, somewhat lower content of phenols that the Neva lettuce is in the variety named Emerald (75.88± ±0.54 mg GA/g), and the lowest is in the Vera lettuce (70.56±0.35 mg GA/g) [13]. The lettuce cultivated in the glasshouse has lower concentrations of total phenols, but the variety Neva has the highest content of total phenols in these conditions as well (73.67±0.67 mg GA/g), whereas other two varieties cultivated in the glasshouse display a similar amount of total phenols [17]. The total content of phenols in the research does not represent the significant level of differences among the researched varieties. Significant difference has been found for the place of growth – that is – for the phenol content is significantly higher in lettuces grown in plastic houses comparing to glass-house production.

The lettuce Emerald from the glasshouse contains the highest amount of flavonoids (35.45±0.95 mg RU/g), and Neva from the glasshouse contains the lowest (27.87±1.03 mg RU/g). It was established that there is a difference in the amount of flavonoids in these two varieties of lettuce if cultivated in different conditions, whereas in case of the lettuce Vera this difference is small. No significant difference of the flavonoid content has been found both among the varieties and among the place of growing (plastic- or glasshouse).

The presence of phenols and flavonoids points to the existence of an antioxidant activity of the biological system especially regarding their oxidation-reduction properties which play a significant role in the absorption and neutralization of free radicals, quenching of mono- and triplet-oxygen and dissolution of peroxides [29]. A significant property of flavonoids is their excellent radical scavenging ability, which enables therapeutic use [30]. The results have shown that ethanolic extracts of the examined lettuce varieties possess an antioxidant activity with total antioxidant capacity ranging from 70.98±0.35 μg AA/g in the lettuce Vera cultivated in the glasshouse to 68.99±0.67 μg AA/g in the lettuce Emerald from the glasshouse (Table 1). Antioxidant capacity of the lettuce extracts of different varieties cultivated in glasshouses and plastic greenhouses do not differ one from another to a great extent, which was expected since they belong to the same type of lettuce, while the research with different lettuce varieties showed great differences in anti-oxidative activity [17]. Differences among the varieties for the total oxidative capacity for the variety are at the significant level ($p \leq 0.01$), while the place of growth was without statistical significance.

IC_{50} values were determined for each lettuce extract (Table 1). The extract of the Emerald lettuce cultivated in the plastic greenhouse has better antioxidant properties (lower IC_{50} DPPH values) than the other lettuce varieties' extracts. For this parameter, a highly significant difference at the level of variety was found, which represents highly determinated genotype, while the place of growth was not significant.

The content of L-ascorbic acid, β-carotene and lycopene is presented graphically (Figure 1).

The highest content of l-ascorbic acid is found in the extract of the Neva lettuce cultivated in the glasshouse (10.9 mg/100 g fresh lettuce), and the lowest in the extract of the Emerald lettuce cultivated in the plastic greenhouse (7.3 mg/100 g fresh lettuce). It has been observed that the lettuce produced in the glasshouse contains a somewhat higher content of l-ascorbic acid. Accoriding to some researches [18,19], the quantity of L-ascorbic acid depends upon lettuce genotype, but it can be increased by increasing the light intensity. As the light intensity is greater in a glasshouse than in a plastic greenhouse, the lettuce cultivated in glasshouses has a somewhat higher content of L-ascorbic acid.

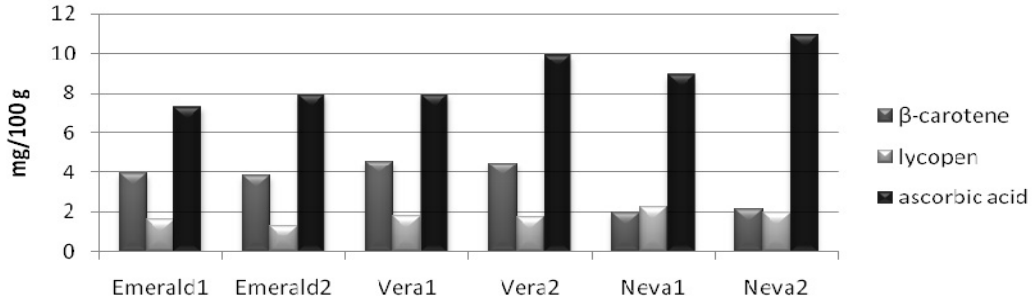

Figure 1. β-Carotene, lycopene and L-ascorbic acid concentrations (mg/100 g fresh lettuce) of three lettuce varieties; 1– plastic greenhouse and 2– glasshouse cultivated plants.

The extracts of examined lettuce contain a smaller amount of β-carotene; the greatest amount of it is in the Vera lettuce (4.35 mg/100 g fresh lettuce), and the smallest in the Neva lettuce (1.97 mg/100 g fresh lettuce). It has been determined that the lettuce extracts contain vestigial amounts of lycopene; from 1.32 mg/ /100 g fresh lettuce in the Emerald lettuce to 2.22 mg/ /100 g fresh lettuce of the Neva variety. The values determined in the stated lettuce (L-ascorbic acid, β-carotene and lycopene) are in accordance with the data obtained by other authors [17,31–33].

CONCLUSION

The presented research of the content of the antioxidant components (total phenols, flavonoids, L-ascorbic acid, β-carotene and lycopene) and the antioxidant activity of the three lettuce varieties confirmed the values of lettuce in everyday nutrition. On the basis of the obtained results it could be concluded that none of the three examined lettuce varieties (Emerald, Vera and Neva) could be singled out as the one containing all the antioxidant components in the highest amount. Thus the Neva lettuce has the highest content of total phenols, L-ascorbic acid and lycopene, whereas the Emerald lettuce contains the greatest amount of flavonoids, and the Vera lettuce has the highest content of β-carotene. All three varieties of the examined lettuce display a solid antioxidant activity and are rather rich in phenolic compounds. Since the cultivation conditions were the same for all the examined lettuce varieties, it can be concluded that the content of antioxidant components (total phenols, flavonoids, β-carotene and lycopene) depends on the lettuce genotype. However, the content of L-ascorbic acid also depends on the light, which proved to be correct in this research as well, so that the lettuce cultivated in a glasshouse has a somewhat greater content of l-ascorbic acid.

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IZVOD

ANTIOKSIDATIVNI KAPACITET I SADRŽAJ FENOLA, ASKORBINSKE KISELINE, β-KAROTENA I LIKOPENA U SALATI

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(Naučni rad)

Određena je antioksidativna aktivnost tri sorte salate (*Lactuca sativa* L.) Emerald, Vera i Neva gajene u dve vrste zaštićenog prostora stakleniku i plasteniku pri kontrolisanim uslovima. Sadržaji antioksidativnih komponenti: ukupni fenoli, flavonoidi, L-askorbinska kiselina, β-karoten i likopen, određeni su u etanolskim ekstraktima salata spektrofotometrijskim metodama. Dobijeno je da najveći sadržaj ukupnih fenola (78,98 ± 0,67 mg GAE/g suvog ekstrakta) ima etanolski ekstrakt salate sorte Neva gajene u plasteniku, dok salata Emerald gajena u stakleniku ima najveći sadržaj flavonoida (35,45±0,95 mg RU/g suvog ekstrakta). Zapaženo je da salate gajene u stakleniku imaju nešto veći sadržaj L-askorbinske kiseline od salate istih sorti iz plastenika. Sadržaj likopena u ispitivanim salatama je zanemarljiv, a β-karotena je nizak. S druge strane visok sadržaj fenolnih komponenti uzrokuju dobre antioksidativne osobine nađene u svim sortama ispitivanih salata.

Ključne reči: Salata • Antioksidans • Fenoli • Askorbinska kiselina • Beta-karoten • Likopen

Influence of fermentation conditions on production of plum (*Prunus domestica* L.) wine: A response surface methodology approach

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Abstract

Plum (*Prunus domestica* L.) is the most important and most commonly grown fruit species in Serbia, one of the leading plum-producing countries. It is mainly used for table consumption, drying and fruit brandy production. The use of plums for wine production is not sufficiently investigated. The aim of this study was to investigate the influence of temperature, pH and duration of fermentation on the plum wine composition and quality, and to optimize these factors by response surface methodology (RSM). Second order polynomial equations, which represent fitted models for investigated responses, are shown as adequate (R^2 > 0.90 and P < 0.05). The average values of ethanol and glycerol content in plum wine were 6% and 5 g/L, respectively, while high methanol concentrations (above 1000 mg/L) were recorded in all wine samples. This requires further investigation of possible procedures to reduce the methanol content in the wines, according to its toxic properties to human. The optimal conditions for plum wine production, obtained by the application of RSM, were 18.3 °C, pH 3.0 and 7 days fermentation time. Apart from the problem of very high methanol concentrations, the plum wine produced with the optimal conditions had good sensory properties and acceptability.

Keywords: plum, wine, optimisation, fermentation conditions, methanol.

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Wine is a product of alcoholic fermentation of grape or any other fruit juice with a good proportion of sugar. In general, the main raw material for the wine production is grape, but the suitability of different fruits for the wine-making has been investigated significantly in the previous decade. Highly acceptable fruit wines are obtained from apple [1,2], mango [3,4], banana [5], peach [6], raspberry [7], blackberry [8], etc.

A plum is a common name for a large number of species belonging to the genus Prunus, generally cultivated in the temperate zones with numerous varieties and hybrids that are suitable for many soils and regions [9]. China is the leading plum producer with approximately 42% share of the total world production in 2003, followed by Romania and the United States. Plum (*Prunus domestica* L.) is the most important and most commonly grown fruit species in Serbia. With the average production of 577000 tonnes the Serbia is one of the leading plum-producing countries [9,10]. Plum trees are precocious and well cropping, have small requirements for ecological conditions and orchard management practices and can be grown at higher altitudes. The fruits are used for table consumption, drying, freezing and processing. The largest amount of plum fruits produced in Serbia (more than 75%) is processed into brandy [10].

Plum contains 10–16% (w/v) of sugar and 5–14 g/kg of total acids. Glucose, fructose and sucrose are the principal sugars in ripened plum, while malic, citric, succinic, quinic and fumaric acids are dominant organic acids [10,11]. The share of malic acid is up to 70% of total organic acids in ripened plums [12]. Plum juice is also good source of vitamins A (345 IU) and C (10 mg/L), as well as of potassium (157 mg/L) [9]. High content of natural phenolic phytochemicals, such as flavonoids and phenolic acids, is reported in plums. These compounds are effective natural antioxidants in human diet which reduce the risk of cancer and other chronic diseases [13]. Plums demonstrated high scavenger activity against oxygen-derived free radicals, such as hydroxyl and peroxyl radicals, and that activity is especially emphasized [14].

Research on plums' composition, their volatiles content and antioxidant potential were reported by many authors [11,12,15–17]. Furthermore, in European plum-producing countries, the special attention is paid to the production of the plum brandy, as a distillate of plum fermented must [18,19]. On the other hand, there are very few published studies about the production of plum wine [20]. There is a lack of relevant data about fermentation conditions for plum wine production and a need for characterization of the obtained wine. Hence, these factors must be studied in more detail in order to develop new vinification technologies

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which would ensure production of wine with the best sensory characteristics. Response surface methodology (RSM) is a statistical method widely used for optimization of fermentation medium and conditions, because it can simultaneously consider several factors at many different levels and corresponding interactions among these factors, using a small number of observations [3,21,22]. The aim of this research was to investigate the influence of temperature, pH and duration of fermentation on the plum wine composition and quality, and to optimize these factors by use of RSM and central composite design (CCD).

MATERIALS AND METHODS

Plum pomace preparation and fermentation

Plum variety Čačanska lepotica resulted from the cross of Požegača and Wangenheims Fruhzwetsche in 1961. It was released in 1975 and patented in 1991 by the Fruit Research Institute, Čačak, Serbia. Today it is one of the most widely grown plum varieties in Serbia. The plums for this research were procured at commercial maturity in early September 2011 from the local market of Novi Sad, Serbia. Plums were halved and pits were carefully removed by hand after which plums were subjected to crushing. Obtained pomace was treated with $K_2S_2O_5$ (SO_2 level was set to 50 mg SO_2/kg pomace), to prevent contamination and oxidation processes, and with 0.02g/kg of commercial pectinase Lallzyme-oe (Lallemand S.A., St. Simon, France) for 3 h at 25 °C. The amount of pectinase was used according to the manufacturer's instructions. The plum juice sample was extracted by passing through cheesecloth and then subjected to analysis of total and reducing sugars, total acidity, pH and fermentable nitrogen.

The entire amount of pomace was divided into 5 L glass jars (3 kg of pomace in each) fitted with a fermentation bung for CO_2 release. The adjustment of pH to values 2.8, 3.0, 3.3, 3.6 and 3.8 was carried out by means of mixture solution of malic, citric and tartaric acid (1:1:0.5, respectively) and calcium carbonate. Alcoholic fermentation was conducted at desired temperatures (15–25 °C). All the runs were carried out according to the central composite design. Inoculation was performed with 0.25 g/kg of previously rehydrated commercial wine yeast *Saccharomyces cerevisiae* (Anchor WE372, South Africa). Wine was passed through the cheesecloth when the fermentation was finished. SO_2 level was adjusted to 50 mg/L and the wine was poured into 500 mL bottles, closed with screw caps and kept at 12–13 °C in the absence of light. After two months, during which clarification and stabilization processes took place, young plum wines were subjected to sensory analysis.

Analytical methods

Pomace samples taken for analysis were previously centrifuged (Tehtnica LC-321, Železniki, Slovenia) at 3500 rpm, 10 min and 20 °C. Total and reducing sugars, sucrose, total acidity and pH were determined using official methods [23]. The pH was measured directly in the pomace by the laboratory multi-parameter analyser Consort C860 (Consort, Turnhout, Belgium) with the glass electrode (SP10T). Fermentable nitrogen was determined using Formol titration [24]. Glycerol was estimated by the enzymatic method [25], using commercially available glycerol assay kit (Megazyme, Ireland).

Ethanol and methanol content in wine samples were determined by gas chromatography, using an HP 5890 Series II GC (Agilent Technologies Inc, Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and Carbowax 20 M column. Chromatography conditions were set according to the previously described procedure [3].

Experimental design

Optimization of conditions for plum wine production was carried out using RSM. The experimental design and statistical analysis were performed using Stat-Ease software (Design-Expert 7.0.0 Trial, Minneapolis, MN, USA). Experiments with three independent variables, fermentation temperature (X_1), fermentation time (X_2) and pH (X_3), were carried out by full factorial central composite experimental design (CCD) [26]. CCD was used to evaluate the combined effect of the three independent variables. A 2^3 factorial experiment with 6 axial points (α = 1.682) and six replicates at the centre points (n_0 = 6) leading to a total of 20 experiments. Response parameters were ethanol, methanol and glycerol content. The levels of independent variables and design matrix are shown in Tables 1 and 2, respectively. Mean values of triplicate determinations were analysed to fit the following second-order polynomial model (1) which is used to calculate predicted responses:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + \\ + b_{33}X_3^2 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 \quad (1)$$

where Y is the predicted response, X_1, X_2 and X_3 correspond to the independent variables, b_0 is intercept, b_1, b_2 and b_3 are linear effects, b_{11}, b_{22} and b_{33} are squared effects and b_{12}, b_{23} and b_{13} are interaction effects of the factors. The goodness of fitting and the

Table 1. Values of factors in central composite design (CCD)

Factor	Name	Low value	High value
X_1	Temperature, °C	15	25
X_2	Fermentation time, day	3	7
X_3	pH	3.0	3.6

Table 2. CCD matrix and responses

Temperature, °C	Fermentation time, day	pH	Ethanol content, vol.%	Methanol content, mg/L	Glycerol content, g/L
X_1	X_2	X_3	Y_1	Y_2	Y_3
11.59	5.00	3.30	1.11	387	1.3
15.00	3.00	3.60	0.64	787	2.53
15.00	3.00	3.00	0.4	580	2.1
15.00	7.00	3.60	4.35	1094	3.41
15.00	7.00	3.00	3.64	955	3.36
20.00	1.64	3.30	0.6	489	0.96
20.00	5.00	3.30	4.23	1207	4.25
20.00	5.00	3.30	3.97	1169	4.05
20.00	5.00	3.30	4.34	1222	4.4
20.00	5.00	3.80	4.42	1240	4.7
20.00	5.00	3.30	4.62	1199	4.3
20.00	5.00	3.30	4.41	1135	4.1
20.00	5.00	3.30	4.19	1188	4.39
20.00	5.00	2.80	3.72	1006	3.75
20.00	8.36	3.30	6.08	1204	4.95
25.00	3.00	3.60	3.74	1101	3.45
25.00	3.00	3.00	3.34	1028	3.4
25.00	7.00	3.00	5.9	1240	5.34
25.00	7.00	3.60	6.23	1265	5.72
28.41	5.00	3.30	5.96	1236	4.8

significances of all terms in the polynomial equations were determined through appropriate statistical methods (coefficient of determination (R^2), *F*-value at a probability (*P*) of 0.05).

Sensory analysis

Plum wine produced by the optimised conditions was subjected to sensory evaluation by the 20-point Bux-Baum method. A five-member panel evaluate following wine properties: colour (max. 2 points), clarity (max. 2 points), aroma (max. 4 points) and taste (max. 12 points). OIV Wine Descriptor Codes were used for the sensory description of the wines [27].

RESULTS AND DISCUSSION

Characteristics of plum pomace

The physicochemical characteristics of the base plum pomace were determined in order to evaluate a potential of plum, as a raw material, for fruit wine production. The total sugar concentration was 125 g/L, where the share of reducing sugars was 60% and the rest was mostly sucrose (35%). Total acidity was 7.1 g/L, expressed as malic acid, while an initial pH of plum pomace was 3.64. The content of fermentable nitrogen was 266 mg/L, which showed that additional nitrogen sources were unnecessary for normal fermentation process.

Statistical analysis

The most important parameters affecting the production of wine, in general, are temperature, pH and time of fermentation. In order to ensure the best quality characteristics of plum wine it is necessary to investigate and optimize fermentation parameters. The effects of these factors on ethanol, glycerol and methanol content in plum wine are shown in Table 2. Multiple regression analysis was performed to fit the

Table 3. Second-order polynomial models for investigated responses ($Y_{1–3}$); X_1: temperature (°C); X_2: fermentation time (day); X_3: pH; Y_1: ethanol content (vol.%); Y_2: methanol content (mg/L); Y_3: glycerol content (g/L); R^2: determination coefficient

Parameter	Equation	R^2
Ethanol	$Y_1 = -27.8149 + 0.9035X_1 + 1.8612X_2 + 7.6636X_3 - 0.0237X_1X_2 - 0.0183X_1X_3 + 0.0833X_2X_3 - 0.0114\,X_1^2 - 0.0884\,X_2^2 - 1.0631\,X_3^2$	0.987
Methanol	$Y_2 = -5550.7745 + 306.2226X_1 + 489.5587X_2 + 919.1365X_3 - 3.8250X_1X_2 - 20.6667X_1X_3 - 24.1667X_2X_3 - 4.5037\,X_1^2 - 25.0548\,X_2^2 - 27.3553\,X_3^2$	0.934
Glycerol	$Y_3 = -3.0893 + 0.6409X_1 + 0.9975X_2 - 3.3613X_3 + 0.0259X_1X_2 - 4.1667E\text{-}3X_1X_3 - 0.0104X_2X_3 - 0.0144\,X_1^2 - 0.0982\,X_2^2 + 0.6226\,X_3^2$	0.958

response functions (Y_{1-3}), and second order polynomial equations (Table 3) have been obtained. Regression coefficients (b_0,b_1,b_2,…,b_{13}) were used to generate response surface plots for investigated variables (Y_{1-3}). Response surface plots (Figures 1–3) are used to illustrate the effects of temperature, pH and fermentation time on the responses. Coefficient of determination (R^2) was used to evaluate the goodness of fitted models. The analysis of variance (ANOVA) is used to determine the adequacy and the significance of the quadratic models. The analyses were done by means of Fisher's *F*-test, and the results are shown in Table 4. The regression models were significant ($P < 0.05$) with a satisfactory value of determination coefficients ($R^2 > 0.90$), implying that at least 90% of the variability in the response could be explained by the second-order model equations.

Effects of fermentation temperature, time and pH

Optimal temperature and pH values for *Saccharomyces cerevisiae* activity are in the range of 25–30 °C and 4.5–6.5, respectively. However, wine fermentations are usually conducted at a relatively lower temperature (15–25 °C) and pH values (3.3–3.6), despite the risk of slower ethanol production, in order to

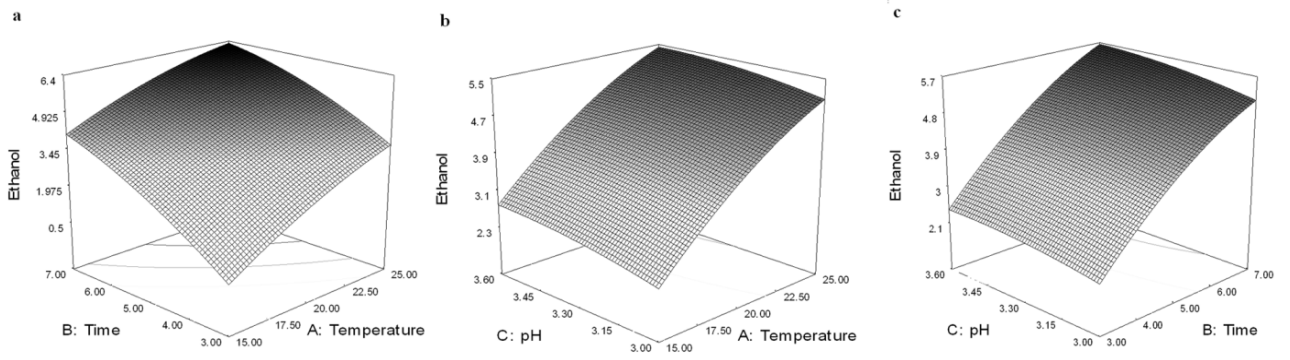

Figure 1. Response surface plots of the interaction of a) temperature– time (pH 3.3), b) temperature– pH (time = 5 days) and c) time–pH (temperature = 20 °C), and their influence on ethanol content.

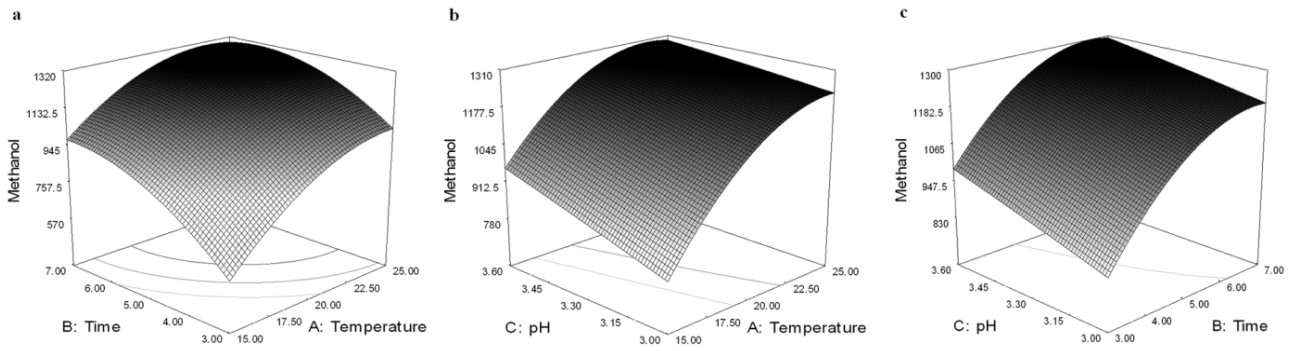

Figure 2. Response surface plots of the interaction of a) temperature–time (pH 3.3), b) temperature–pH (time = 5 days) and c) time–pH (temperature = 20 °C), and their influence on glycerol content.

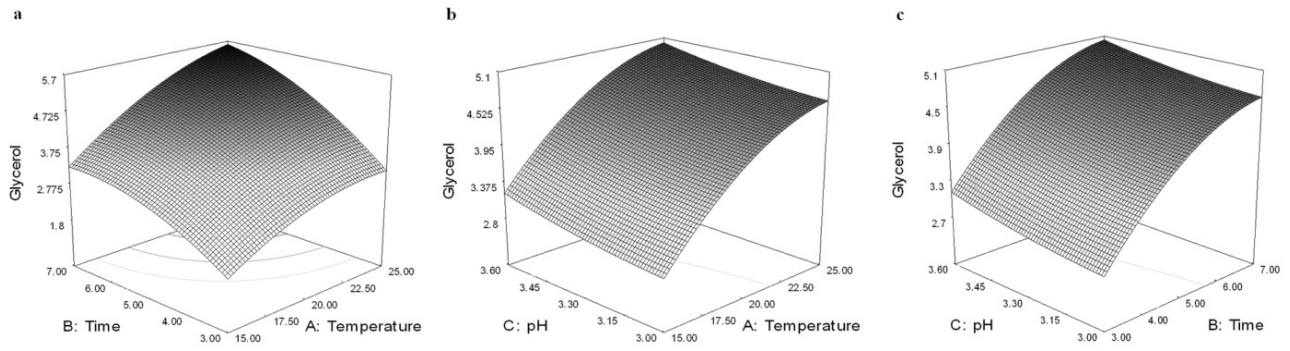

Figure 3. Response surface plots of the interaction of a) temperature–time (pH 3.3), b) temperature–pH (time = 5 days) and c) time–pH (temperature = 20 °C), and their influence on glycerol content.

*Table 4. Analysis of variance (ANOVA) for the experimental results; X_1: temperature (℃); X_2: fermentation time (day); X_3: pH; Y_1: ethanol content (vol.%); Y_2: methanol content (mg/L); Y_3: glycerol content (g/L); *significant at P < 0.05; **not significant*

Source	*F*-Value			*P*-Value		
	Y_1	Y_2	Y_3	Y_1	Y_2	Y_3
Model	152.02	15.76	25.67	< 0.0001*	< 0.0001*	< 0.0001*
X_1	548.74	57.63	90.62	< 0.0001*	< 0.0001*	< 0.0001*
X_2	734.62	42.06	100.59	< 0.0001*	< 0.0001*	< 0.0001*
X_3	13.32	5.77	3.71	0.0045*	0.0371*	0.0830**
X_1X_2	10.06	1.32	4.31	0.0100*	0.2780**	0.0645**
X_1X_3	0.13	0.86	2.5E-3	0.7211**	0.3744**	0.9610**
X_2X_3	0.45	0.19	2.5E-3	0.5195**	0.6729**	0.9610**
X_1^2	26.06	20.54	14.99	0.0005*	0.0011*	0.0031*
X_2^2	40.20	16.27	17.93	< 0.0001*	0.0024*	0.0017*
X_3^2	2.94	9.8E-3	0.36	0.1172**	0.9230**	0.5595**

decrease the risk of potential wine spoilage [28]. Hence, the influence of these levels of fermentation conditions on plum wine quality was investigated in this study.

Ethanol is the main product of the alcoholic fermentation of sugar in fruit juices and it contributes to the body and mouthfeel of a wine. As an organic solvent, ethanol helps to extract colour and phenolic compounds from the skins of fruit during fermentation. In addition to flavour, it also provides microbial stability to wine. We can notice (Figure 1) that the fermentations at 15 °C were not completed in the observed time (7 days), according to the ethanol content (3.64–4.35 vol.%) and the amount of available sugar in plum pomace (125 g/L). The maximum ethanol content (6.23%) was reached during fermentation at 25 °C and pH 3.6. Increase in temperature of fermentation has caused an intense increase in ethanol production, while the influence of pH was not so pronounced. Figure 1 shows the response surface plots for the effect of the independent variables on the ethanol content. The obtained model (Y_1), with a determination coefficient R^2 = 0.987 is proved as significant (P < 0.05), with only 1.13% of the total variations not explained by the model. The model terms X_1, X_2, X_3, X_1^2, X_2^2 and X_1X_2 are significant at 0.05 level (P < 0.05) while terms X_1X_3, X_2X_3 and X_3^2 do not have a significant effect on ethanol content (Table 4). Ethanol content was positively affected by the fermentation temperature, time and pH, and negatively by interactions between temperature and time, as well as by the quadratic terms of temperature and time of fermentation.

Methanol has no organoleptic impact on wine. It is not formed by alcoholic fermentation, but exclusively from enzymatic hydrolysis of the methoxyl groups of pectins during fermentation [29]. From Figure 2 it can be seen that most of the methanol is formed in the first 4 days of fermentation when the activity of pectin methylesterase is highest. Furthermore, exponential increase in methanol concentration is observed with the increase of fermentation temperature. Increase in pH values of plum pomace has caused a slight increase in its content. Hence, the maximum methanol content (1265 mg/L) is obtained when process parameters were 25 °C and pH 3.6, after 7 days of fermentation. Generally, the high methanol content was obtained in all wine samples. This is a problem because methanol is toxic to human through ingestion and inhalation. Its oxidation leads to production of formic aldehyde and formic acid, both toxic to the central nervous system [29]. Oral lethal dose of methanol for human ranges from 340 μg to 1 mg/kg of body weight [30]. A possible explanation for these results can be found in the fact that plums have high pectin content (2.0–3.5 mass%) and the high degree of esterification [31]. For instance, mango has 0.7–1 mass% of pectins [32] and its wine can contain up to 800 mg/L of methanol [33]. Wine from apples, another fruit that has high content of pectins – 0.7–0.84 mass% [34], can have high methanol concentrations (up to 700 mg/L) [35]. The addition of pectinase in pomace is also causing an increase in methanol level [33,36]. According to the Serbian regulations on alcoholic drinks quality, the maximal dose of methanol in fruit wines is 250 mg/L and in fruit brandies 10–14 g on the litre of absolute ethanol. From the regression model (Y_2) of methanol concentration, the value of determination coefficient (R^2 = 0.934) indicates that only 6.6% of the total variance could not be explained by the model. Among the model terms X_1, X_2, X_3, X_1^2, X_3^2 are significant with the probability of 95% (Table 4). The interactions between X_1, X_3 and X_3, as well as quadratic term X_3^2 however, did not have significant influence on methanol production. The influence of pH is less significant compared to the influence of temperature and time. Production of methanol was positively affected by the linear effects of temperature, time and pH, while quadratic terms of the first two factors had a negative influence.

Glycerol is a non-volatile compound, without aromatic properties, but which significantly contributes to wine quality by providing sweetness and fullness [29,37]. It can be noticed (Figure 3) that glycerol content increased with the increase of fermentation temperature and pH of pomace. The highest glycerol content (5.72 g/L) was obtained during fermentation at 25 °C and pH 3.6. Production of glycerol was more intensive during first 4 days of fermentation, especially at the higher temperatures (25 °C). As already mentioned, a temperature of 15 °C delayed the fermentation and left residual sugars, but according to the fact that glycerol production is connected with the first 50 g of sugar fermented [29], extension of fermentation would not increase significantly its concentration. The previous studies have also shown that an increase in temperature resulted in greater glycerol production [3,4,37]. It was reported that a pH increase from 2.8 to 3.7 has only a slight effect on glycerol yield [38]. The amount of this parameter in plum wine was lower than in wines produced from grapes (4–9 g/L), mango (5.5––8.4 g/L) and raspberry (5–10 g/L) [4,7,38]. The possible explanation may be found in the fact that higher concentrations of glycerol are obtained in mediums with higher content of glucose [39]. According to the determination coefficient of glycerol R^2 = 0.958 it can be concluded that only 4.2% of the total variance could not be explained by the model Y_3, which is proved to be significant (P < 0.05). The significance at 0.05 level (P < < 0.05) is associated with following model terms: X_1, X_2, X_1^2, X_2^2; on the other hand, terms X_3, X_1X_3, X_1X_3, X_2X_3 and X_3^2 are insignificant (P > 0.05). It can be seen that the glycerol content was positively affected by fermentation temperature and time. Furthermore, quadratic terms of temperature and time of fermentation negatively affected glycerol production.

Optimisation

The obtained response surfaces (Figures 1–3) were used as guidelines in the optimisation of investigated parameters for plum wine production. According to the general winemaking practices, it is expected that the optimisation should be aimed to maximise ethanol, glycerol and minimise methanol yield. Methanol is a limiting factor in this study because of the very high values of its content obtained in all wines. The optimisation was made by use of desirability function concept which combines multiple responses into one response by assigning a value from 0 (one or more characteristics are unacceptable) to 1 (all process characteristics are on target). After the transformation of estimated responses into individual desirability values (from 0 to 1), the overall desirability of the process is calculated as geometric mean of the individual desirability functions [21]. The final optimised fermentation conditions, obtained with RSM, were 18.3 °C, pH 3.0 and 7 days fermentation time, which should ensure the production of 4.72 vol.% of ethanol, 1122 mg/L of methanol and 4.23 g/L of glycerol. The predicted optimum was verified and the models were proven as adequate after a repeated experiment (triplicate set), with the optimal fermentation conditions, was done (4.95% of ethanol, 1087 mg/L of methanol and 4.10 g/L of glycerol were obtained). The obtained desirability function value was 0.558. This value is relatively low because of the strong limiting effect of the high methanol content. Reduction of the methanol content could lead to an increase of the overall desirability function value.

Sensory evaluation of the plum wine produced with optimized fermentation conditions showed good quality and overall acceptability, according to the assigned average values for colour (1.9), clarity (2.0), aroma (3.4) and taste (9.1), in total 16.4.

CONCLUSION

The results of this study are significant for improvement of plum wine production, primarily in terms of optimisation of fermentation conditions. From the results it can be seen that changes in ethanol, methanol and glycerol during fermentation are well described by the obtained second order equations, according to the high coefficients of determination (R^2 > 90%) and statistical significance (P < 0.05). It has been shown that fermentation at 15 °C could not be completed in the observed time (7 days). Considering the content of ethanol and glycerol, the average values of 6% and 5 g/L, respectively, were obtained. Generally, high methanol concentrations (above 1000 mg/L) were recorded in all wine samples. The optimal conditions for plum wine production were 18.3 °C, pH 3.0 and 7 days within which the production of 4.72% of ethanol, 1122 mg/L of methanol and 4.23 g/L of glycerol should be ensured. This was confirmed through the validation experiment. Apart from the problem of very high methanol concentrations, plum wine produced with the optimal conditions has good sensory properties and acceptability. Future studies will have to deal with the investigation of possible procedures to reduce the methanol content in plum wines. Furthermore, the suitability of other plum varieties for wine production should be checked.

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Ispitivanje uticaja uslova fermentacije na proizvodnju vina od šljiva (*Prunus domestica* L.) primenom metode odzivne površine

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(Naučni rad)

Šljiva (*Prunus domestica* L.) predstavlja najvažniju i najzastupljeniju vrstu voća u Srbiji koja je ujedno i jedan od najvećih proizvođača ovog voća u svetu. Njena upotreba je uglavnom vezana za potrošnju u svežem stanju, sušenje i proizvodnju voćne rakije šljivovice. Upotreba šljiva u proizvodnji vina nije uobičajena, ali ni dovoljno istražena. Cilj ovog rada bio je da se ispita uticaj temperature, pH i trajanja fermentacije na hemijski sastav i kvalitet vina od šljiva, kao i da se optimizuju pomenuti faktori procesa fermentacije upotrebom metode odzivnih površina (RSM). Pokazano je da dobijene jednačine drugog reda adekvatno predstavljaju fitovane modele za ispitivane parametre kvaliteta vina (R^2 > 0,90 i P < < 0,05). Prosečna vrednost za sadržaj etanola u proizvedenim vinima je bila 6 zapr.%, glicerola 5 g/l, dok je koncentracija metanola bila veoma visoka (iznad 1000 mg/l) u svim uzorcima. S obzirom na toksična svojstva metanola, ovakvi rezultati zahtevaju opsežna ispitivanja mogućih postupaka za smanjenje sadržaja ovog jedinjenja u vinu od šljiva. Optimalni uslovi fermentacije za proizvodnju vina šljive dobijeni su primenom metode funkcije poželjnosti, koja je bila usmerena na postizanje maksimalnog prinosa etanola i glicerola i minimalnog prinosa metanola. Primenom pomenute metode dobijene su sledeće optimalne vrednosti: 18,3 °C (temperatura ferementacije), pH 3,0 i 7 dana trajanja fermentacije. Ako se zanemari nedostatak usled visokih koncentracija metanola, vino od šljiva proizvedeno pri optimalnim uslovima imalo je dobre senzorne karakteristike i sveukupnu prihvatljivost.

Ključne reči: Šljiva • Vino • Optimizacija • Uslovi fermentacije • Metanol

Electrochemical characterization and determination of carbamazepine as pharmaceutical standard and tablet content on gold electrode

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Abstract

The anodic behaviour of carbamazepine (CBZ), an anticonvulsant drug, has been studied on gold electrode in 0.1 mol dm^{-3} phosphate buffer of pH 7.0 by using cyclic voltammetry. It has been found that the value of the oxidative current of pure CBZ at 0.90 V *vs*. SCE is a linear function of the concentration in a range from 1.0×10^{-7} to 1.0×10^{-4} mol dm^{-3}. The detection of CBZ in the concentration of 1.0×10^{-8} mol dm^{-3} is among the lowest that have been reported for this drug using voltammetric techniques. CBZ as a content of tablet Gal-epsine® has been quantitatively determined. It has also been demonstrated that the modification of gold electrode with bovine serum albumin (BSA) results in a decrease of the oxidative peak current due to the binding of the drug to BSA.

Keywords: carbamazepine, voltammetric determination, gold electrode.

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Carbamazepine (CBZ) is one of the safest and the most effective anticonvulsant drugs, which is used in the management of tonic-clonic, complex partial and mixed-type seizures for a long time [1,2] (Fig. 1). Currently, various mechanisms of its action are proposed. The anticonvulsant activity of CBZ principally involves limitation of seizure propagation by reduction of post-tetanic potentiation of synaptic transmission [3]. The drug has also demonstrated sedative, anticholinergic, antidepressant, muscle relaxant, antiarrhythmic, antidiuretic and neuromuscular transmission inhibitory actions [4].

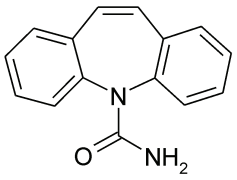

Figure 1. Chemical structure of carbamazepine (5H-dibenzo-[b,f]azepine-5-carboxamide).

Several analytical methods have been presented in the literature for the determination of CBZ and its impurities in the bulk drug and biological fluids. Fluorescence polarization immunoassay [5] and HPLC [6–8] are usually used for the routine determination of this and other anticonvulsant drugs. Conventional chromatographic techniques, such as gas chromatography [9,10], gas chromatography combined with mass spectrometry [11] and micellar electrokinetic capillary chromatography [12] have also been employed. Few recent studies have investigated the applicability of electrochemical methods for the characterization of CBZ. Its electrochemical oxidation at glassy carbon electrode (GCE) in phosphate buffer solution (pH 7.4) has been studied by Kalanur and Seetharamappa [13]. Two oxidation peaks have been observed, whereby no peak has been observed in the reverse scan suggesting that the oxidation process is an irreversible one. The probable reaction mechanism of oxidation of CBZ has been proposed based on the reported mechanism of oxidation of imipramine (3-(10,11-dihydro-5*H*-dibenzo-[*b,f*]azepin-5-yl)-*N*,*N*-dimethylpropan-1-amine), which has an almost similar structure and belongs to same class of tricyclic compounds. Taking into account that the voltammogram of imipramine closely resembled that of CBZ, the authors have proposed that the reaction mechanism includes the oxidation of CBZ to a radical, which dimerizes rapidly and subsequently undergoes oxidation to form a dimmer radical. Veiga *et al.* have applied a multiwalled carbon nanotubes film-coated GCE for the voltammetric determination of CBZ in phosphate buffer solution (pH 6.89) [14]. The electrochemical reduction of CBZ in acetonitrile and dimethylformamide using GCE and microelectrode has also been studied [15]. The reduction process is suggested to follow electrochemical–chemical mechanism involving a two electron transfer accompanied by a first order reaction. Pruneanu *et al.* have employed graphene––gold nanoparticle composite deposited on gold electrode to detect CBZ [16]. A two-wave oxidation peak has been observed accompanied by a small reduction

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peak, which have suggested an oxidation pathway with formation of dimmers similar to the one proposed in [13]. Lin *et al.* have used differential pulse voltametry to determine the serum level of CBZ in rabbits and reported a detection limit of 59.2×10^{-8} mol dm^{-3} [17]. Excellent enhancement effects on electrochemical response of CBZ have been achieved through the use of fullerene–C_{60} modified GCE [18]. Cyclic voltammogram has shown two oxidation and reduction peaks at fullerene–C_{60} modified GCE in phosphate buffer solution (pH 7.2). In this case, a detection limit of 16.2×10^{-9} mol dm^{-3} has been reported.

Different electrode materials have offered possibilities for improving the electrochemical behavior of CBZ, as well as for reaching lower limits of its detection. The aim of this paper is at first the investigation of the electrochemical behaviour of CBZ (pharmaceutical standard) on gold electrode and gold electrode modified with BSA using cyclic voltammetry. The electrochemical identification of its tablet content is presented in this regard. The proposed method is observed to be very sensitive and allows determination of CBZ in a wide concentration range in solid dosage forms.

EXPERIMENTAL

Materials

The CBZ standard (Sigma Aldrich) was used as a pure substance without further purification, dissolved (1 vol.% of methanol and 99 vol.% of phosphate buffer) and added into the electrolyte (0.1 mol dm^{-3} phosphate buffer, pH 7.0), so that the final range of the drug concentrations was 1.0×10^{-8}–1.0×10^{-4} mol dm^{-3}.

The commercial CBZ, as a content of Galepsin® tablets (Galenika a.d., Serbia), was dissolved in methanol and added into the electrolyte (0.1 mol dm^{-3} phosphate buffer, pH 7.0); the final range of concentrations was 1.0×10^{-6}–1.0×10^{-4} mol dm^{-3}. BSA (Sigma Aldrich) was used as a pure substance without further purification and modification. Phosphate buffer solution was prepared by standard procedure and pH has been measured by Mettler Toledo (FiveGo) pH Meter. Monobasic sodium phosphate and dibasic sodium phosphate were p.a. purity. Water was purified by Milli-Q system.

Apparatus and preparation of electrode surfaces

Standard equipment was used for the cyclic voltammetry measurements and the three electrode electrochemical cell was described in detail previously [19–21]. Polycrystalline gold (bare gold) served as the working electrode; a gold wire was used as the counter electrode and a saturated calomel electrode as the reference electrode. All the potentials are given *vs*. SCE. Prior to the addition of CBZ, the electrolyte was deoxygenated by purging with nitrogen. All the experiments were performed at room temperature.

BSA modified gold electrode was prepared by transferring a droplet of 2×10^{-6} cm^3 of BSA solution onto the surface of gold electrode and by air-drying overnight. The electrode was then soaked in sterile water for at least 4 h before being rinsed with water to remove any unadsorbed BSA [22].

Polycrystalline gold (surface area 0.500 cm^2), which served as the working electrode, was polished with diamond paste, cleaned with a mixture of 18 MΩ cm deionised water and sulfuric acid and further cleaned with 18 MΩ cm deionised water in an ultrasonic bath.

RESULTS AND DISCUSSION

Due to its low water solubility (~72×10^{-5} mol dm^{-3} at 25 °C), the electrochemical characterization of CBZ has been mostly performed with addition of organic solvents such as acetonitrile and dimethylformamide [14–17]. However, these solvents interact with the surface of gold electrode and easily adsorb on it [23], which makes them inconvenient for the purposes of the present research. In the initial experiment, we have employed bicarbonate solution as electrolyte, but the obtained cyclic voltammogram of CBZ has shown its low electrooxidation activity when compared with the ones obtained in phosphate buffer solution. Because of this, phosphate buffer has been further used for the electrochemical determination of CBZ. To overcome the problem of the low solubility of CBZ and excipients used in tablet (Galepsin®), methanol has been added to the electrolyte (0.1 and 1 vol.% for the determination of CBZ as pharmaceutical standard and tablet content, respectively). It has already been shown that methanol is not electroactive on gold electrode at the value of the applied sweep rate (50 mV s^{-1}) [24].

In the presence of the CBZ standard (at the concentration of 1.0×10^{-8} mol dm^{-3}), the reaction of the oxidation occurs in the area of the oxide formation with the apparent anodic activity from 0.90 to 1.10 V, as it is presented in Fig. 2. The small lowering of the first gold oxide peak at 0.75 V is also apparent only for the concentration of 1.0×10^{-8} mol dm^{-3}. In the reverse sweep, the reaction of the oxide reduction has been decreased in the presence of CBZ at 0.44 V, which may be attributed to the reduction of products formed in the described anodic reactions, as it is the case with macrolide antibiotics [19–21]. Furthermore, for the all of the investigated concentrations of CBZ from 1.0×10^{-7} to 1.0×10^{-4} mol dm^{-3}, in anodic direction, cyclic voltammograms exhibited the changed shape compared to the cyclic voltammograms in the presence of 1.0×10^{-8} mol dm^{-3} of CBZ, as well as in its absence. The increasing anodic current values from 0.30 to 1.10 V due to the increased CBZ concentrations lead to the

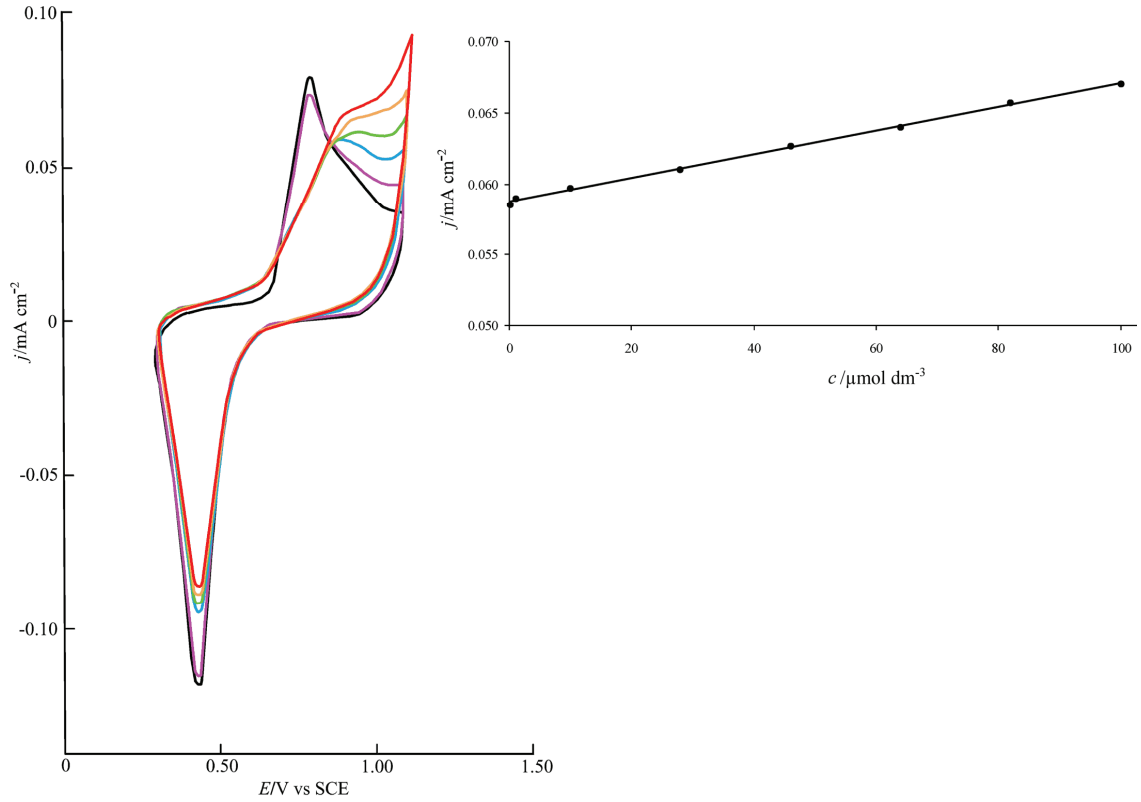

Figure 2. The cyclic voltammograms of bare gold electrode (black line) and in the presence of the carbamazepine (CBZ) standard in the concentration from 1.0×10^{-8} to 1.0×10^{-4} mol dm^{-3} (1.0×10^{-8} mol dm^{-3} pink line, 1.0×10^{-7} mol dm^{-3} blue line, 1.0×10^{-6} mol dm^{-3} green line, 1.0×10^{-5} mol dm^{-3} orange line, 1.0×10^{-4} mol dm^{-3} red line) in 0.1 mol dm^{-3} phosphate buffer (pH 7.0), sweep rate 50 mV s^{-1} (only the first sweep is recorded). Inset: The dependency of the value of the oxidative currents of CBZ at 0.90 V on the concentration in the range 1.0×10^{-7}–1.0×10^{-4} mol dm^{-3}.

formation of the current shoulder with the maximum value at 0.95 V. The first gold oxide peak at 0.75 V completely diminished as shown in Fig. 2. Because it has tendency to undergo poisoning after the first sweep, gold electrode has been polished between successive additions of suitable aliquots of the working solution of CBZ in phosphate buffer solution to obtain good reproducible results, improved sensitivity and resolution of voltammetric peaks. Comparing to GCE, gold electrode does not require anodic pretreatment for its activation and its polishing and cleaning between the consecutive concentrations of analyte is quite simple and cheap procedure [13]. Both electrodes exhibit abilities for efficient, fast simple and cheap voltammetric determination of CBZ. The value of the oxidative currents of pure CBZ at 0.90 V in 0.1 dm^{-3} phosphate buffer at the scan rate of 50 mV s^{-1} is a linear function of the concentration in a range of 1×10^{-7}–1×10^{-4} mol dm^{-3}. This linearity is presented by Eq. (1) and given in the left corner of Fig. 2:

$$j\ (\text{mA cm}^{-2}) = 0.0588 + 83.3c\ (\text{mol dm}^{-3}),\ R = 0.998 \qquad (1)$$

Thus, the proposed method, using cyclic voltammetry on gold electrode, enabled the detection of CBZ, even at the concentration of 1.0×10^{-8} mol dm^{-3}, which is among the lowest that have been reported for this drug using voltammetric techniques so far [1, 18].

The voltammetric characterization of CBZ, as a content of tablets (Galepsin®) in the concentration range from 1.0×10^{-6} to 1.0×10^{-4} mol dm^{-3}, is presented in Fig. 3. The cyclic voltammograms of Galepsin® tablets shows the same shape and the current maximum at 1.10 V, as it is observed for the standard. The negligible smaller anodic activity from 0.80 to 1.00 V may be attributed only to the presence of the excipients. The present excipients obviously do not affect the electrooxidation of CBZ and enable its quantitative determination as content of tablets (Galepsin®).

The value of the oxidative currents of CBZ (in Galepsin®) at 0.90 V *vs.* SCE in 0.1 mol dm^{-3} phosphate buffer solution at the scan rate of 50 mV s^{-1} is a linear function of the concentration in the range of 1.0×10^{-6}–1.0×10^{-4} mol dm^{-3}. This linearity is presented by Eq. (2) and given in the left corner of Fig. 3:

$$j\ (\text{mA cm}^{-2}) = 0.0506 + 118c\ (\text{mol dm}^{-3}),\ R = 0.995 \qquad (2)$$

Serum albumin is the most abundant protein in blood plasma and is responsible for the binding and transportation of various drugs. BSA is an ideal model protein for serum albumin due to its availability, stab-

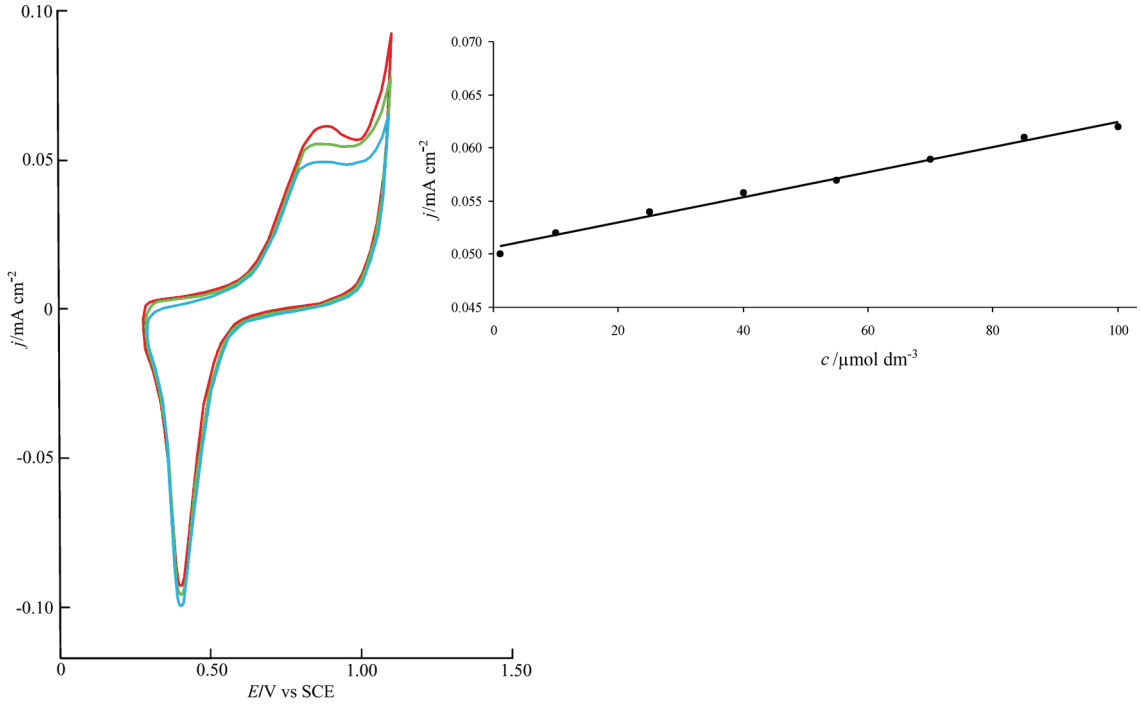

Figure 3. The cyclic voltammograms of carbamazepine (CBZ) as a content of tablets (Galepsin®) in the concentration from 1.0×10^{-6} to 1.0×10^{-4} mol dm^{-3} (1.0×10^{-6} mol dm^{-3} blue line, 1.0×10^{-5} mol dm^{-3} green line, 1.0×10^{-4} mol dm^{-3} red line) in 0.1 mol dm^{-3} phosphate buffer (pH 7.0), sweep rate 50 mV s^{-1} (only the first sweep is recorded). Inset: The dependency of the value of the oxidative currents of CBZ at 0.90 V on the concentration in the range 1.0×10^{-6}–1.0×10^{-4} mol dm^{-3}.

ility and incomparable binding property [25]. The electrochemical behaviour of various proteins at gold electrode surfaces has been widely investigated in recent years (*e.g.*, a gold electrode modified by gelatin [26], nafion-riboflavin [27], BSA [28]). To study the binding of CBZ to plasma proteins, its electrochemical behaviour on gold electrode modified with BSA, has been investigated, whereby a decrease of the oxidative peak currents as a result of the drug adsorption on the surface of the modified electrode has been expected. Figure 4 represents the cyclic voltammogram of gold electrode modified with BSA, as well as in the presence of the CBZ standard at the concentration of 1.0×10^{-4} mol dm^{-3} in 0.1 mol dm^{-3} phosphate buffer solution. This concentration of the drug has been chosen, because its blood plasma levels may range from 2.1×10^{-6} to 1.1×10^{-4} mol dm^{-3} [29].

Gold modified by BSA exhibited almost two times lower currents in the whole region of the applied potential, which results from the strong adsorption of BSA on gold. It can be seen that the oxidative peak current has been decreased after adding the CBZ standard at the concentration of 1.0×10^{-4} mol dm^{-3} due to the binding of the drug to BSA. An ultraviolet and fluorescence spectroscopic study has indicated that the interaction between CBZ and BSA in Tris-HCl buffer solution (pH 7.4) is mainly driven by the hydrophobic force; the apparent binding constant and the binding site values at 27 °C are 1.8×10^{4} and 0.97, respectively [30].

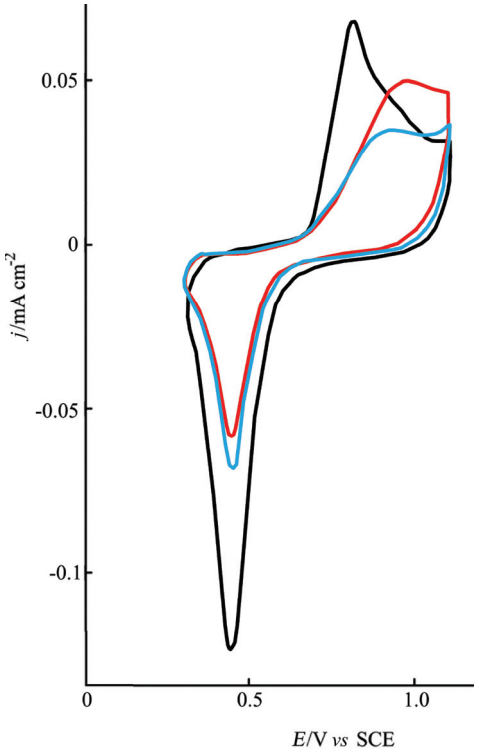

Figure 4. The cyclic voltammograms of bare gold electrode (black line), modified with bovine serum albumine (red line) and in the presence of the carbamazepine standard (blue line) at the concentration of 1.0×10^{-4} mol dm^{-3} in 0.1 mol dm^{-3} phosphate buffer (pH 7.0), sweep rate 50 mV s^{-1} (only the first sweep is recorded).

CONCLUSION

The proposed procedure enabled the detection of CBZ even at the concentration of 1.0×10^{-8} mol dm^{-3}, which is among the lowest that have been reported for this drug using voltammetric techniques. Thus, the anodic behaviour of CBZ on gold electrode in 0.1 mol dm^{-3} phosphate buffer of pH 7.0 has shown that the value of the oxidative currents at 0.90 V *vs*. SCE is a linear function of the concentration in a range from 1.0×10^{-7} to 1.0×10^{-4} mol dm^{-3}. The CBZ, as a content of tablet Galepsine®, has also been quantitatively determined in a range from 1.0×10^{-6} to 1.0×10^{-4} mol dm^{-3}.

The electrooxidative behaviour of CBZ on gold electrode modified with BSA indicates a strong interaction between the drug and BSA, which is mainly driven by the hydrofobic force.

The simple, fast and cheap voltammetric procedure using gold electrode for the CBZ determination can be further developed as an additional method offering useful combinations with HPLC and with already investigated electrodes, such as glassy carbon electrode.

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ELEKTROHEMIJSKO KARAKTERISANJE I ODREĐIVANJE KARBAMAZEPINA KAO FARMACEUTSKOG STANDARDA I SADRŽAJA TABLETE NA ELEKTRODI OD ZLATA

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(Naučni rad)

Anodno ponašanje jednog od najsigurnijih i najefikasnijih antikonvulzivnih lekova karbamazepina (CBZ) proučavano je po prvi put na elektrodi od zlata u 0.1 mol dm^{-3} fosfatnom puferu pH 7.0 pomoću ciklične voltametrije. Karbamazepin kao standard i kao sastojak komercijalnog oblika (tablete) Galepsin® podleže anodnoj oksidaciji. Utvrđeno je da je vrednost struja oksidacije čistog CBZ-a na 0.90 V linearna funkcija koncentracija u opsegu od 1.0×10^{-7} do 1.0×10^{-4} mol dm^{-3}. CBZ je detektovan i u koncentaraciji od 1.0×10^{-8} mol dm^{-3} koja je među najnižim koje su objavljene do sada za ovaj lek korišćenjem voltametrijskih tehnika. CBZ kao sadržaj tablete Galepsin® je kvantitativno određen, pri čemu je na 0.90 V takođe dobivena linearna zavisnost vrednosti struja oksidacije od ispitivanih koncentracija leka (1.0×10^{-6}–1.0×10^{-4} mol dm^{-3}). Pokazano je da prisutni ekscipijenti u tableti ne utiču na proces elektrooksidacije CBZ u fosfatnom puferu. Takođe je pokazano da modifikovanje elektrode od zlata albuminom goveđeg seruma (BSA) ima za posledicu smanjenje struja oksidativnog pika CBZ-a usled vezivanja leka za BSA. Kvantitativno određivanje CBZ i kao sadrzaja tablete Galepsin® na elektrodi od zlata cikličnom voltametrijom u fosfatnom puferu pH 7.0 je brza, jednostavna i efikasna metoda koja je korisna dopuna standardnoj metodi, tečnoj hromatografiji visokih performansi (HPLC).

Ključne reči: Karbamazepin • Voltametrijsko određivanje • Elektroda od zlata

PROIZVODNJA BIODIZELA IZ ULJA MIKROALGI

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Izvod

Jedan od obećavajućih izvora ulja za proizvodnju biodizela predstavljaju mikroalge čijim gajenjem se može proizvesti do 100 puta više biodizela po jedinici površine u odnosu na suncokret i uljanu repicu. Sadržaj ulja u mikroalgama može biti do 77% suve biomase, a produktivnost do 122 mg/l/d. U ovom radu je prikazan pregled dosadašnjih proučavanja mogućnosti korišćenja mikroalgi (tehnike izolovanja, gajenja i izdvajanja biomase, kao i načini konverzije ulja) za dobijanje biodizela. Prednosti upotrebe mikroalgi je povećana efikasnost proizvodnje, mogućnost gajenja u sredinama koje su neodgovarajuće za gajenje biljaka, pri čemu ne zahtevaju puno prostora za gajenje i nemaju negativan uticaj na svetske zalihe hrane i vode. Zbog trenutno većih proizvodnih troškova, mikroalge još uvek nisu održivi izbor za proizvodnju biodizela jer je cena biodizela iz mikroalgi veća od cene dizela.

Ključne reči: biodizel, mikrooalge, gajenje, ekstrakcija, *in situ* transesterifikacija.

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Glavni nedostatak upotrebe "sirovina prve generacije" (jestive uljarice, žitarice, itd.) za dobijanje biodizela je stalna dilema: hrana ili gorivo, odnosno rast cena hrane na bazi jestivih ulja zbog njihove povećane potrošnje u proizvodnji biogoriva [1]. Zbog toga, nova istraživanja se usmeravaju ka "sirovinama druge generacije", u koje spadaju nejestivi i lignocelulozni materijali, kao što su: ostaci pri preradi šećerne trske, drveta i useva, komunalni čvrsti otpad, itd. [2]. U skorije vreme, sve veći broj istraživača proučava upotrebu tzv. "sirovina treće generacije", u koje spadaju mikroorganizmi, kao sto su: kvasci, gljive i alge, čija se biomasa može koristiti kao sirovina za dobijanje biodizela.

Jedan od obećavajućih alternativnih izvora ulja koje se može upotrebiti kao sirovina za proizvodnju biodizela predstavljaju mikroalge. To su jednoćelijski ili kolonijalni fotosintetski organizmi, koji poslednjih godina imaju sve veću industrijsku primenu u proizvodnji hemikalija i nutritivnih suplemenata [3]. Značaj algi u prirodi je da, zahvaljujući procesu fotosinteze, učestvuju u obnavljanju i održavanju količine kiseonika u atmosferi. Procenjuje se da mikroalge proizvode oko polovinu ukupnog atmosferskog kiseonika. Do sada je opisano oko 35.000 vrsta algi, od ukupno 200.000––800.000 vrsta koliko se pretpostavlja da postoji u prirodi [4] Potencijalna industrijska primena algi proizilazi iz činjenice da je preko 15.000 komponenti (antioksidansi, masne kiseline, enzimi, peptidi, toksini i steroli) dobijeno iz algi. Osim toga, mikroalge mogu rasti veoma brzo i za 24 h (odnosno za 3,5 h u eksponencijalnoj fazi rasta) mogu udvostručiti svoju masu [5]. Jednostavna jednoćelijska struktura mikroalgi omogućava veliku brzinu fotosinteze, efikasno vezivanje ugljenika i brzu akumulaciju ulja u biomasi (do 77% suve biomase, odnosno produktivnost ulja pri fototrofnom gajenju mikroalgi do 122 mg/l dnevno [6]). Gajenjem algi može se postići veći energetski prinos po jedinici površine u odnosu na kopnene useve [7].

Mikroalge pružaju mogućnost proizvodnje do 100 puta više biodizela po jedinici površine gajenja u odnosu na suncokret i uljanu repicu [8]. Pored toga, prednosti upotrebe mikroalgi kao izvora za proizvodnju biogoriva su povećana efikasnost i smanjeni troškovi proizvodnje. Troškovi izdvajanja i transporta mikroalgi su niži u odnosu na troškove transporta ostalih sirovina za proizvodnju biodizela. Isto tako, mikroalge ne zahtevaju puno prostora za gajenje, pri čemu, za razliku od upotrebe žitarica, uljarica i drugih biljnih kultura, upotreba algi kao sirovine za dobijanje goriva nema negativan uticaj na svetske zalihe hrane i vode [9]. Mikroalge se mogu gajiti u različitim sredinama koje su nepogodne za ostale biljke, na primer u slatkoj, otpadnoj ili morskoj vodi, kao i na neobradivom zemljištu. Mogu se, takođe, gajiti na farmama ili u bioreaktorima.

Ograničenja rasta mikroalgi odnose se, uglavnom, na intenzitet svetlosti i temperaturu tokom gajenja. Solarna energija u tropskim i subtropskim oblastima pruža više energije za fotosintezu ali, s druge strane, dovodi do povećanja temperature koja može biti letalna za mikroalge [10]. U kontekstu proizvodnje biodizela, samo one mikroalge sa visokim sadržajem stearinske (C18:0) i oleinske (C18:1) kiseline su potencijalni izvori, jer lipidi iz ovakvih mikroorganizama oponašaju svojstva ulja veće vrednosti, pospešuju oksidativnu stabilnost i imaju veću mogućnost prilagođavanja u industrijskoj proizvodnji biodizela [11].

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Biodizel se iz mikroalgi, nakon gajenja i sakupljanja biomase, može dobiti na dva načina. Prvi način podrazumeva prethodnu ekstrakciju ulja iz mikroalgalne biomase uz pomoć rastvarača, praćenu reakcijom transesterifikacije i/ili esterifikacije odgovarajućim alkoholom u prisustvu katalizatora (kiselog, baznog ili enzima) ili u odsustvu katalizatora pod natkritičnim uslovima metanola. Ovaj način podrazumeva utrošak rastvarača i energije za ekstrakciju i transesterifikaciju, što utiče na povećanje cene sirovog biodizela, a doprinosi i zagađenju životne sredine. Drugi način je direktna transesterifikacija algalne biomase, tzv. *in situ* postupak, kojim se unapređuje proces proizvodnje biodizela iz mikroalgi u smislu smanjenja proizvodnih troškova [12].

Makroalge mogu, takođe, biti sirovina za proizvodnju biodizela [13], iako je sadržaj ulja u njima manji nego kod mikroalgi. Ispitivanja bazno katalizovane metanolize ulja dobijenih iz nekih vrsta makroalgi pokazala su da se veća količina ulja, a samim tim i biodizela, moze dobiti iz makroalgi roda *Oedigonium* spp., dok je količina sporednih proizvoda – glicerola, pigmenata i drugih sastojaka veća kod upotrebe ulja makroalgi roda *Spirogyra* spp. [13].

U ovom radu je prikazan pregled dosadašnjih proučavanja mogućnosti korišćenja mikroalgi za dobijanje biodizela. Prikazane su tehnike za izolovanje, gajenje i izdvajanje algi, kao i načini konverzije ulja algi u biodizel.

KULTURE MIKROALGI ZA DOBIJANJE ULJA

Izolovanje mikroalgi

Mikroalge koje se koriste za proizvodnju biodizela mogu se izolovati iz prirodnog staništa ili kupiti iz ogovarajućih kolekcija kultura, kao što su: UTEX (SAD), ANACC (Australija), CCAP (Velika Britanija), NIES (Japan), SAG (Nemačka), CPCC (Kanada), itd. Iako su vrste iz kolekcija kultura dobro proučene, usled kontinualnog presejavanja može doći do gubitka određenih karakteristika, pa se preporučuje izolovanje mikroalgi iz okoline [14].

Postupak izolovanja mikroalgi obuhvata izbor odgovarajućeg prirodnog staništa iz koga se izdvajaju, kao i obogaćivanje i prečišćavanje izolovane kulture. Kako naseljavaju veliki broj staništa, različite vrste mikroalgi se mogu izolovati iz različitih uzoraka vode i zemljišta. Obogaćivanje kulture podrazumeva obezbeđivanje pogodnih uslova koji omogućavaju rast i razmnožavanje odgovarajuće vrste uz, istovremeno, sprečavanje rasta ili uništavanje ostalih vrsta mikroorganizama [15]. Jedan od načina obogaćivanja mikroalgi je dodatak male količine uzorka u odgovarajuću hranljivu podlogu i inkubacija na odgovarajućoj temperaturi tokom 3–4 nedelje. Nakon toga, fotosintetske vrste se prebacuju na čvrste mineralne podloge, dok se za miksotrofne i heterotrofne vrste koriste supstrati sa organskim izvorom ugljenika. Prečišćavanje mikroalgi se vrši presejavanjem morfološki različitih izolata na nove odgovarajuće podloge sve dok se ne dobiju identične kolonije. Pre prenošenja na čvrste podloge, mikroalge koje se izoluju iz uzoraka vode mogu se izdvojiti filtracijom. Isto tako, izolovanje mikroalgi se može vršiti direktno upotrebom mikromanipulatora, pri čemu se individualne ćelije prenose na odgovarajuću čvrstu ili tečnu podlogu [16].

Podloga koja se koristi za rast mikroalgi mora da zadovolji određene uslove, odnosno da sadrži [17]:

- odgovarajuće vrste i količine soli,
- izvor ugljenika – kako oko 50% biomase čini ugljenik, dovoljno snabdevanje ugljenikom je od posebnog značaja za uspešno gajenje, pri čemu izvor ugljenika može biti neorganski (gasoviti CO_2 ili bikarbonati) i organski (šećeri ili acetati),
- odgovarajući i ekonomičan izvor azota – azot predstavlja važan element pri gajenju mikroalgi (čini do 10% suve biomase) jer može uticati na metaboličke puteve i promenu sastava ćelije algi, pri čemu se, zavisno od vrste koje se gaji i optimalne pH vrednosti, najčešće nitrati, amonijak ili urea koriste kao izvori azota,
- odgovarajuće koncentracije ostalih važnih elemenata (na primer, kalijum, magnezijum, natrijum, fosfor i sumpor),
- elemente u tragovima neophodne za rast koji se dodaju u veoma malim količinama i
- ukoliko je potrebno, organska jedinjenja ili supstance koje podstiču rast, kao što su vitamini, hormoni i sl.

Minimalne nutritivne potrebe mikroalgi mogu se izračunati iz približne molekularne formule za biomasu: $CO_{0,48}H_{1,83}N_{0,11}P_{0,01}$ [18]. Neki nutritijenti, kao što je fosfor, moraju se dodati u značajnom višku. Fosfati grade komplekse sa jonima metala, tako da ćelije algi ne mogu iskoristiti celokupnu količinu dodatog fosfora [18].

Kako uzorci iz kojih se vrši izolovanje mikroalgi sadrže i veliki broj drugih vrsta mikroorganizama (uglavnom protozoe i bakterije), dobijanje unialgalne kulture zahteva njihovo uklanjanje. Prečišćavanje kulture može se vršiti [16]:

- ispiranjem ćelija serijskim prenošenjem u novu sterilnu podlogu,
- UV zračenjem (ćelije algi su otpornije na UV zrake u odnosu na bakterije, tako da se izlaganjem UV zračenju, ispiranjem, razblaživanjem uzorka i prenošenjem na selektivnu čvrstu podlogu mogu dobiti čiste kulture mikroalgi) i
- dodatkom antibiotika (različiti antibiotici se mogu koristiti za elimisanje plesni i cianobakterija iz uzorka).

Primena genetičkog inženjerstva za poboljšanje proizvodnjih svojstava algi

Mnoge mikroalge nemaju sposobnost proizvodnje velikih količina ulja tokom eksponencijalne faze rasta. Primenom genetičkog inženjerstva može se uticati na promenu metaboličkih puteva sinteze određenih komponenti i poboljšati produktivnost i prinos mikroalgi [19]. Tako, genetički modifikovane mikroalge se mogu koristiti za sintezu rekombinantnih proteina i vakcina [20], proizvodnju biohidrogena [21] i bioremediaciju kontaminiranog zemljišta [22]. Do sada je genetički modifikovano više od 30 vrsta algi [23].

Ekspresijom gena moguće je uticati na sintezu ulja i razmnožavanje mikroalgi. Povećanje sinteze ulja može dovesti do smanjenja deobe ćelija. U ovom slučaju, ekspresija gena može, ipak biti poželjna ukoliko se može kontrolisati i aktivirati kada ćelije dostignu dovoljnu gustinu [24]. Inhibicija metabolizma ulja može, takođe, izazvati probleme sa razmnožavanjem i produktivnošću biomase jer se obezbeđivanje energije i prekursora za deobu ćelija zasniva na kataboličkim putevima [25]. Početak primene metaboličkog inženjerstva zasnivao se na konverziji acetil-koenzima A ((acetil--CoA) u malonil-CoA u prisustvu acetil-CoA karboksilaze, koja predstavlja prvi korak u biosintezi masnih kiselina. Međutim, nekoliko pokušaja povećanja sinteze acetil-CoA karboksilaze u cilju povećanja sadržaja ulja nije dalo željene rezultate [26]. Drugu mogućnost predstavlja blokiranje određenih metaboličkih puteva, što dovodi do akumuliranja supstanci bogatih energijom [25]. Komplementarna strategija za povećanje akumulacije ulja je smanjenje katabolizma ulja koje, osim povećanja skladištenja ulja, može imati štetan efekat na rast i razmnožavanje ćelija [25]. Osim primene genetičkog inženjerstva u cilju povećanja produkcije ulja, moguće je težiti i poboljšanju kvaliteta ulja, kako bi bilo pogodnije za proizvodnju biodizela.

U sastavu ulja nekih vrsta *Chlorophyceae*, najzastupljenije su palmitinska (C16:0) i oleinska (C18:1) kiselina, zatim polinezasićene masne kiseline linolna (C18:2) i linoleinska (C18:3), dok su polinezasićene masne kiseline sa brojem C atoma iznad 18 malo zastupljene [27]. Dužina lanca i stepen nezasićenosti masnih kiselina u ulju mikroalgi utiču na svojstva dobijenog biodizela, i to: jodni broj, oksidativnu stabilnost, cetanski broj i tačku začepljenja hladnog filtera [28]. Mali cetanski broj i veliki jodni broj biodizela povezuje se sa metil estrima polinezasićenih masnih kiselina (C18:2 i C18:3). Takođe, oksidativna stabilnost biodizela smanjuje se povećanjem sadržaja metil estara polinezasićenih masnih kiselina. Biodizel sa većim sadržajem metil estara masnih kiselina sa većim brojem C-atoma pokazuje nepovoljniju tačku začepljenja hladnog filtera, dok je biodizel sa visokim sadržajem oleinske kiseline pokazivao znatno bolja svojstva [29]. Metil estri ulja mikroalgi roda *Chlorella*, koji su sastavljeni uglavnom iz metil--oleata (65%) i metil-linoleata (18,5%), imaju zadovoljavajuća svojstva goriva: tačka začepljenja hladnog filtera: 13 °C, jodni broj 112,2 g I_2/100 g, kinematska viskoznost od 4,43 mm^2/s na 40 °C i oksidativna stabilnost oko 4,5 h [30].

GAJENJE MIKROALGI I IZDVAJANJE ULJA

Gajenje mikroalgi

Mikroalge se najčešće gaje kao fotoautotrofne kulture u kojima ćelije koriste svetlosnu energiju i CO_2 kao izvor ugljenika. Međutim, proizvodnja biomase fotoautotrofnih kultura je ograničena usled smanjenja dostupnosti svetlosti sa povećanjem broja ćelija. U suprotnom, niska koncentracija biomase kultura povećava troškove izdvajanja ćelija mikroalgi. Jedna od mogućih alternativa je upotreba heterotrofnih kultura koje, u odsustvu svetlosti, koriste organska jedinjenja (šećere i organske kiseline) kao izvor ugljenika. Za razliku od fotoautotrofnih, heterotrofne kulture se mogu gajiti u konvencionalnim mikrobnim bioreaktorima. Ovakav način gajenja ima određene prednosti, kao što su jednostavnija promena procesnih uslova i veći prinos biomase od 20 do 100 g/l [31]. Takođe, kontrolisanjem uslova rasta može se postići povećana proizvodnja željenog proizvoda, na primer, povećana akumulacija ulja [32]. Tako, u odnosu na autotrofni rast, heterotrofni rast vrste *Chlorella protothecoides* odlikuje se većom proizvodnjom biomase i četiri puta većem sadržaju ulja u ćelijama [33,34]. Xu i sar. [34] su ustanovili da je integracija heterotrofnog rasta mikroalge *C. protothecoides* i transesterifikacije, jeftin, izvodljiv i efikasan metod za dobijanje biodizela visokog kvaliteta iz mikroalgi. Međutim, heterotrofni uslovi nisu pogodni za sve vrste algi, a mogu izazvati i promene u sastavu ćelija [31]. Osim toga, pri proceni dugoročne održivosti ovakvog načina proizvodnje, moraju se uzeti u obzir i troškovi proizvodnje organskih supstrata [32]. Smanjenje troškova izdvajanja biomase može se postići i primenom miksotrofnih kultura koje, kao izvor ugljenika, istovremeno koriste CO_2 i organska jedinjenja [35]. Pojedini sistemi za proizvodnju biomase pomoću miksotrofnih kultura podrazumevaju pripremu početnog inokuluma pomoću organskih supstrata, kako bi se dobila veća koncentracija ćelija pre prenošenja u otvorene bazene. Glavna prednost ovih sistema je povećana proizvodnja biomase ali se, kao i kod heterotrofnih sistema, mora izvršiti procena ekonomske isplativosti upotrebe organskog izvora ugljenika [32].

Za gajenje mikroalgi mogu se koristiti tri tipa industrijskih reaktora: fotobioreaktori, otvoreni bazeni i hibridni sistemi. Otvoreni bazeni predstavljaju plitke bazene u kojima se dotok nutritijenata obezbeđuje proticanjem rastvora. Glavna prednost ovih sistema je

njihova jednostavnost koja uslovljava niske troškove proizvodnje biomase. Tehnička i biološka ograničenja ovih sistema dovela su do razvoja zatvorenih fotobioreaktora, koji predstavljaju zatvorene osvetljene sudove za kontrolisanu proizvodnju biomase. Iako zahtevaju veća ulaganja, fotobioreaktori imaju određene prednosti u odnosu na otvorene sisteme [36]:

- rizik od kontaminacije je minimalan, što omogućuje gajenje unialgalne kulture,
- pružaju bolju kontrolu procesnih uslova (pH vrednost, temperatura, intenzitet svetlosti, koncentracija CO_2 itd.),
- manji gubitak CO_2,
- sprečeno je isparavanje vode,
- omogućavaju dobijanje veće koncentracije ćelija i
- omogućavaju proizvodnju složenih biofarmaceutika.

Najčešće se koriste fotobioreaktori u vidu vertikalnih kolona, ravnih ploča ili cevni fotobioreaktori. U tabeli 1 naglašene su prednosti i nedostaci fotobioreaktora koji se koriste u proizvodnji mikroalgi.

Svetlost u fotobioreaktorima se obezbeđuje prirodnim ili veštačkim osvetljenjem koje može biti spoljašnje ili unutrašnje [37]. Prinos ulja u sistemima na otvorenom koji koriste dnevnu svetlost iznosi 100–130 m^3/ha [18]. S druge strane, prinos ulja pri upotrebi veštačkog osvetljenja može dostići do 172 m^3/ha usled stabilnosti i konstantne izloženosti osvetljenju [38]. Pri visokom intenzitetu svetlosti, maksimalna brzina nastajanja kiseonika tokom fotosinteze u cevnom fotobioreaktoru može dostići 10 g O_2/(m^3min). Nivo rastvorenog kiseonika koji je mnogo veći od vrednosti koja odgovara zasićenosti vazduhom inhibira fotosintezu. Takođe, visoke koncentracije kiseonika u kombinaciji sa jakim osvetljenjem dovode do fotooksidativnog oštećenja ćelija [39]. Povećan intenzitet svetlosti može dovesti do fotoinhibicije, pri čemu dolazi do smanjenja brzine rasta mikroalgi. Do fotoinhibicije dolazi pri povećanju intenziteta svetlosti malo iznad intenziteta koji obezbeđuje maksimalnu brzinu rasta [18]. U zavisnosti od intenziteta svetlosti i vremena izlaganja, fotoinhibicija može biti reverzibilna ili ireverzibilna [40].

Hibridni sistemi koji se koriste za kultivaciju mikroalgi su kombinacija otvorenih bazena i fotobioreaktora u kojima se proces proizvodnje biomase izvodi u dve faze. Prva faza se izvodi u fotobioreaktoru i obuhvata pripremu početnog inokuluma sa dobrim karakteristikama rasta i minimalnom kontaminacijom. U drugoj fazi, inokulum se prenosi u otvoreni bazen, kako bi se maksimizirao rast biomase i akumulacija ulja [32].

Sadržaj ulja u mikroalgama varira u zavisnosti od vrste i može se kretati od 5 do 77% u odnosu na suvu biomasu [18,41]. Tako, sadržaj ulja kod vrste *Chlorella* iznosi od 5 do 58% [42–49], kod vrste *Chlorococcum* od

Tabela 1. Prednosti i nedostaci bioreaktora koji se koriste za gajenje mikroalgi [33]
Table 1. Advantages and disadvantages of bioreactor types used for microalgae cultivation [33]

Tip bioreaktora	Prednosti	Nedostaci
Otvoreni bazeni	Relativno ekonomični	Loša kontrola uslova gajenja
	Lako čišćenje	Poteškoće u gajenju mikroalgi na duži vremenski period
	Pogodni za masovno gajenje mikroalgi	Niska produktivnost
		Visoki zahtevi za površinom
		Primena ograničena samo na nekoliko vrsta mikroalgi
		Podložni kontaminaciji
Vertikalne kolone	Visok stepen prenosa mase	Mala površina izložena svetlosti
	Dobro mešanje uz mali napon smicanja	Konstrukcija zahteva složene materijale
	Niska potrošnja energije	Sa povećanjem razmere smanjuje se površina izložena svetlosti
	Visok potencijal za povećanje razmere	
	Jednostavnija sterilizacija i održavanje temperature	
	Pogodnost za imobilizaciju mikroalgi	
	Smanjena fotoinhibicija i fotooksidacija	
Ravne ploče	Velika površina izložena svetlosti	Povećanje razmere zahteva izgradnju pregrada
	Pogodni za imobilizaciju mikroalgi	Određeni stepen rasta na zidovima reaktora
	Visoka produktivnost biomase	Mogućnost uticaja hidrodinamičkog pritiska na određene vrste mikroalgi
	Relativno jeftini	
	Lako čišćenje i održavanje temperature	
	Niska akumulacija nagrađenog kiseonika	
Cevni reaktori	Velika površina izložena svetlosti	Postojanje gradijenta ph rastvorenog kiseonika i co_2 duž reaktora
	Pogodni za gajenje kultura na otvorenom	
	Dobra produktivnost biomase	Stvaranje naslaga
	Relativno jeftini	Određeni stepen rasta na zidovima reaktora
		Visoki zahtevi za površinom

19 do 25% [45, 47], *Scenedesmus* od 1 do 29% [42,44––45,47,50], *Botryococcus* od 6 do 28% [46,51], *Desmodesmus* od 17 do 58% [10,45,48,52] i *Chlamydomonas* od 25 do 51% [42,52]. Osim razlike u sadržaju, uočena je razlika u sastavu ulja, tako da su neke vrste bogatije neutralnim lipidima od drugih [41].

Rast mikroalgi zavisi od nekoliko faktora koji uključuju abiotičke (svetlost, temperatura, koncentracija nutritijenata, kiseonik, CO_2, pH, salinitet i prisustvo toksičnih elemenata), biotičke (prisustvo patogena i konkurentnost u odnosu na druge vrste algi) i procesne (mešanje, stepen razblaženja, frekvencija uklanjanja biomase i dodatak bikarbonata) faktore [8]. Tokom početnih faza rasta nastaju velike količine polarnih lipida i polinezasićenih C16 i C18 masnih kiselina. Međutim, sa ulaskom u stacionarnu fazu sastav nastalih ulja se menja i sastoji se, uglavnom, od neutralnih zasićenih masnih kiselina dugih lanaca (18:0 i 16:0). Ipak promene u sastavu ulja zavise od vrste mikroalgi, pa plavo-zelene mikroalge pokazuju vrlo malu promenu u sastavu tokom ciklusa rasta. Sadržaj polinezasićenih C16 i C18 masnih kiselina, mono- i di-galaktozil-diglicerida, sfingolipida i fosfoglicerida u vrstama *Euglena gracils* i *Chlorella vulgaris* povećava se sa povećanjem količine svetlosti [14]. Morske vrste mikroalgi proizvode veću količinu fosfolipida u odnosu na količinu triacilglicerola (TAG) [53]. Fosfolipidi nisu pogodni za proizvodnju biodizela transesterifikacijom. S druge strane, slatkovodne vrste proizvode velike količine zasićenih neutralnih lipida, pa su mnogo pogodnije za proizvodnju biodizela [54]. Produktivnost biomase, sadržaj, produktivnost i načini ekstrakcije ulja, kao i uslovi gajenja pojedinih slatkovodnih mikroalgi prikazani su u tabeli 2.

Tabela 2. Produktivnost biomase, sadržaj ulja i produktivnost ulja pojedinih slatkovodnih mikroalgi
Table 2. Biomass productivity, oil content and oil productivity of some fresh water microalgae

Vrsta mikroalge	Podloga[a]	Tehnika				Produktivnost biomase, g/l/d	Sadržaj ulja, %	Produktivnost ulja, mg/l/d	Lit.
		Gajenje	Izdvajanje biomase	Razaranja ćelija	Ekstrakcije ulja				
Chlorococcum sp.	BG11	Fototrofno	Centrifugiranje i zamrzavanje	–	Metanol: hloroform 2:1	0,28	19,3	53,7	[47]
Chlorococcum sp.	BBM	Fototrofno	Centrifugiranje	–	Metanol: hloroform 2:1	0,05	11	5,71	[48]
Chlorococcum macrostigmatum	BG11	Fototrofno	Centrifugiranje	–	Hloroform: metanol 2:1	–	25,1	–	[45]
Chlorella sp.	BG11	Fototrofno	Centrifugiranje	–	Metanol: hloroform 2:1	0,23	18,7	42,1	[47]
Chlorella sp.	BG11	Fototrofno	Centrifugiranje	–	Metanol: hloroform 1:2	–	20	–	[45]
Chlorella sp.	BBM	Fototrofno	Centrifugiranje	–	Metanol: hloroform 2:1	0,04	46	19,64	[48]
Chlorella sorokiniana	BG11	Fototrofno	Centrifugiranje	–	Metanol: hloroform 2:1	0,23	19,3	44,7	[47]
Chlorella vulgaris	BG11	Fototrofno	Centrifugiranje	–	Metanol: hloroform 2:1	0,20	18,4	36,9	[47]
						0,17	19,2	32,6	
	BG11	Fototrofno	Centrifugiranje i zamrzavanje	–	Metanol: hloroform 1:1	0,07	5	3,5	[46]
				Autoklav		0,07	10	7	
				Perlice		0,07	8	5,6	
				Mikrotalasi		0,07	10	7	
				Ultrazvuk		0,07	6	4,2	
				Osmotski pritisak		0,07	8	5,6	
	BBM	Fototrofno	Centrifugiranje	–	Metanol: hloroform 1:2	0,56	20	112	[6]
	BBM	Fototrofno	Centrifugiranje	–	Metanol: hloroform 1:2	0,08	26	21	[42]
	–	Fototrofno	Centrifugiranje i zamrzavanje	Ultrazvuk	*n*-Heksan	0,18	5,1	7,4	[44]

Tabela 2. Nastavak
Table 2. Continued

Vrsta mikroalge	Podloga[a]	Tehnika				Produktivnost biomase, g/l/d	Sadržaj ulja, %	Produktivnost ulja, mg/l/d	Lit.
		Gajenje	Izdvajanje biomase	Razaranja ćelija	Ekstrakcije ulja				
Chlorella protothecoides	Hidrolizat jerusalimske artičoke	Heterotrofno	Centrifugiranje i liofilizacija	–	*n*-Heksan	3,4–4,1	43–46	1400–1700	[43]
	–	Heterotrofno	Centrifugiranje i zamrzavanje	–	*n*-Heksan	2,2–7,4	50,3–57,8	1210–3701	[49]
	–	Heterotrofno	Centrifugiranje i zamrzavanje	–	*n*-Heksan	0,62	55	343	[30]
Scenedesmus sp.	BG11	Fototrofno	Centrifugiranje	–	Metanol: hloroform 2:1	0,26	21,1	53,9	[47]
						0,21	19,6	40,8	
	BG11			–	Metanol: hloroform 1:1	0,07	1	0,7	[46]
				Autoklav		0,07	4	2,8	
				Perlice		0,07	8	5,6	
				Mikrotalasi		0,07	10	7	
				Ultrazvuk		0,07	6	4,2	
				Osmotski pritisak		0,07	5,8	4,1	
	BG11	Fototrofno	Centrifugiranje	–	Metanol: hloroform 1:2	–	20,7–25	–	[45]
Scenedesmus sp.	BBM	Fototrofno	Centrifugiranje	–	Metanol: hloroform 1:2	0,03–0,04	22–45	13,2–16,1	[48]
Scenedesmus quadricauda	BG11	Fototrofno	Centrifugiranje	–	Metanol: hloroform 2:1	0,19	18,4	35,1	[47]
Scenedesmus obliquus	–	Fototrofno	Centrifugiranje i zamrzavanje	Ultrazvuk	*n*-heksan	0,09	17,7	15,9	[44]
	N11	Fototrofno	Centrifugiranje		Metanol: hloroform 2:1	0,06	12,7	7,14	[50]
	N11+ glukoza	Miksotrofno				0,1–0,5	6,6–11,8	11,6–58,6	
	BBM	Fototrofno	Centrifugiranje		Metanol: hloroform 1:2	0,09	29	25	[42]
Botryococcus sp.	modifikovana Chu 13	Fototrofno	Centrifugiranje i zamrzavanje	Ultrazvuk	*n*-Heksan	0,06–0,22	5,7–25,8	3,5–46,9	[51]
	BG11	Fototrofno	Centrifugiranje i zamrzavanje	–	Metanol: hloroform 1:1	0,04	8	3,2	[46]
				Autoklav		0,04	11	4,4	
				Perlice		0,04	28	11,2	
				Mikrotalasi		0,04	28,5	11,4	
				Ultrazvuk		0,04	8,5	3,4	
				Osmotski pritisak		0,04	10	4	

Tabela 2. Nastavak
Table 2. Continued

Vrsta mikroalge	Podloga[a]	Tehnika				Produktivnost biomase, g/l/d	Sadržaj ulja, %	Produktivnost ulja, mg/l/d	Lit.
		Gajenje	Izdvajanje biomase	Razaranja ćelija	Ekstrakcije ulja				
Desmodesmus sp.	Modifikovana Bold 3N	Fototrofno	Centrifugiranje	Perlice	Metanol: hloroform 2:1	–	36–58	–	[10]
	BG11	Fototrofno	Centrifugiranje	–	Metanol: hloroform 1:2	–	21,7	–	[45]
	BBM	Fototrofno	Centrifugiranje i zamrzavanje	Ultrazvuk	Metanol: hloroform 1:2	0,093	19,7	18	[52]
Desmodesmus sp.	BBM	Fototrofno	Centrifugiranje	–	Metanol: hloroform 2:1	0,06	34	5,7	[48]
Desmodesmus elegans	BG11	Fototrofno	Centrifugiranje	–	Metanol: hloroform 1:2	–	16,9	–	[45]
Chlamydomonas spp.	BBM	Fototrofno	Centrifugiranje i zamrzavanje	Ultrazvuk	Metanol: hloroform 1:2	0,134	25,3	34	[52]
Chlamydomonas pitschmanii	BBM	Fototrofno	Centrifugiranje		Metanol: hloroform 1:2	0,05	51	25	[42]
Chlamydomonas mexicana	BBM	Fototrofno	Centrifugiranje		Metanol: hloroform 1:2	0,07	28	21	[42]
Neochloris oleabundans	–	Fototrofno	Centrifugiranje i zamrzavanje	Ultrazvuk	n–Heksan	0,09	29	26,1	[44]
	BBM	Fototrofno	Centrifugiranje	–	Metanol: hloroform 1:2	0,53	23	122	[6]
Monodus subterraneus	BG11	Fototrofno	Centrifugiranje		Metanol: hloroform 2:1	0,19	16,1	30,4	[47]

[a]Sastav podloga (g/dm^3):

BG11: $NaNO_3$ 1,5; $K_2HPO_4 \cdot 3H_2O$ 0,04; $MgSO_4 \cdot 7H_2O$ 0,075; $CaCl_2 \cdot 2H_2O$ 0,036; $C_6H_8O_7$ 0,006; Feri–amonijum citrat 0.006, $Na_2MgEDTA$ 0,001; Na_2CO_3 0,02; H_3BO_3, 0,003; $MnCl_2 \cdot 4H_2O$ 0,002; $ZnSO_4 \cdot 7H_2O$ 0,0002; $Na_2MoO_4 \cdot 2H_2O$ 0,0004; $CuSO_4 \cdot 5H_2O$ 0,00008; $Co(NO_3)_2 \cdot 6H_2O$ 0,00005

BBM: $NaNO_3$ 0,249; $CaCl_2 \cdot 2H_2O$ 0,0250; $MgSO_4 \cdot 7H_2O$ 0,075; K_2HPO_4 0,072; KH_2PO_4 0,175; NaCl 0,025; EDTA 0,16; KOH 0,077; $FeSO_4 \cdot 7H_2O$ 0,012; H_3BO_3 0,028; $ZnSO_4 \cdot 7H_2O$ 0,019; $MnCl_2 \cdot 4H_2O$ 0,004; MoO_3 0,002; $CuSO_4 \cdot 5H_2O$ 0,004; $Co(NO_3)_2 \cdot 6H_2O$ 0,001.

Modifikovana Chu 13: KNO_3 0,2; K_2HPO_4 0,04; $MgSO_4 \cdot 7H2O$ 0,1; $CaCl_2 \cdot 2H_2O$ 0,054; $FeC_6H_6O_7$ 0,01; $C_6H_8O_7$ 0,1; $NaHCO_3$ 0,036; H_3BO_3 0,003; $MnCl_2 \cdot 4H_2O$ 0,002; $ZnSO_4 \cdot 7H_2O$ 0,0002; $Na_2MoO_4 \cdot 2H_2O$ 0,0004; $CuSO_4 \cdot 5H_2O$ 0,00008; $Co(NO_3)_2 \cdot 6H_2O$ 0,00005

Modifikovana Bold 3N: $NaNO_3$ 0,374; $CaCl_2$ 0,019; $MgSO_4$ 0,036; K_2HPO_4 0,038; KH_2PO_4 0,088; NaCl 0,025; $FeCl_3 \cdot 6H_2O$ 0,002; $Na_2EDTA \cdot 2H_2O$ 0,005; $ZnSO_4 \cdot 7H_2O$ 0,00007; $CoSO_4 \cdot 7H_2O$ 0,00002; $MnSO_4 \cdot 4H_2O$ 0,0005; $Na_2MoO_4 \cdot 2H_2O$ $1{,}48 \times 10^{-8}$; Na_2SeO_3 $1{,}73 \times 10^{-7}$; $NiCl_2 \cdot 6H_2O$ $1{,}49 \times 10^{-8}$; tiamin–HCl 0,0001; biotin 2×10^{-6}, B12 1×10^{-6}

N11: KNO_3 1; $Na_2HPO_4 \cdot H_2O$ 0,083; KH_2PO_4 0,052; $MgSO_4 \cdot 7H_2O$ 0,050; $CaCl_2 \cdot 2H_2O$ 0,010; Fe–EDTA 0,01; $MnCl_2 \cdot 4H_2O$ 0,099; $NiSO_4 \cdot 6H_2O$ 0,024; $ZnSO_4 \cdot 7H_2O$ 0,063; $CuSO_4 \cdot 5H_2O$ 0,005; $CoSO_4 \cdot 7H_2O$ 0,003; NH_4VO_3 0,003; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0,002

Proučavanje uticaja temperature na rast pokazalo je da su mikroalge otpornije na niže temperature i da mogu preživeti temperature i do 18 °C niže od optimalnih, dok je povišenje temperature iznad optimalne za samo 2-4 °C letalno. Zbog toga, veoma je važno održavati temperaturu tokom gajenja mikroalgi na 20–26 °C [8]. Mešanje je jedan od veoma važnih procesnih faktora jer dovodi do ravnomerne distribucije ćelija, toplote i metabolita, uz istovremeno ubrzavanje prenosa mase gasova. Takođe, određeni stepen turbulencije je poželjan da bi se obezbedila cirkulacija mikroalgi iz mračnog u osvetljeni deo reaktora.

Promenom uslova gajenja mikroalgi moguće je uticati na produktivnost ulja. Smanjenje pH vrednosti usled povećanog sadržaja CO_2 može inhibirati rast dok aeracija sa većim sadržajem CO_2 dovodi do povećanja produktivnosti ulja kod *Nannochloropsis oculata, Scenedesmus obliquus* i *Chlorella kessleri* [55,56]. Neke vrste algi mogu povećati sadržaj ulja za 10 do 20% usled smanjenja koncentracije kiseonika [57]. Najefikasnija metoda za povećanje akumulacije ulja je ograničavanje sadržaja azota koja, osim povećane akumulacije, dovodi i do promene sastava ulja od slobodnih masnih kiselina (SMK) do TAG [58]. Razmnožavanje ćelija pri limitiranoj količini azota je sprečeno, ali se ugljenik još uvek koristi

i prevodi u TAG koji se skladište unutar ćelija [11]. Visoke koncentracije gvožđa povećavaju, takođe, akumulaciju ulja u *C. vulgaris*, što ukazuje na to da se određeni metabolički putevi mogu modifikovati izlaganjem visokim koncentracijama oligoelemenata u podlozi [59].

Izdvajanje biomase

Izdvajanje biomase zahteva jedan ili više koraka odvajanja tečne i čvrste faze. Biomasa se može izdvojiti centrifugiranjem, filtracijom ili taloženjem (tabela 2). U cilju lakšeg izdvajanja biomase, ovi procesi mogu biti praćeni flokulacijom. Izdvajnje biomase predstavlja poseban problem zbog male veličine ćelija (3–30 μm) i relativno malog sadržaja ćelija (< 0,5 kg suve biomase/m^3 pri komercijalnoj proizvodnji) mikroalgi [60]. Ćelije mikroalgi izdvojene tokom trajanja stacionarne faze imaju niži sadržaj polarnih lipida u odnosu na ćelije sakupljene tokom eksponencijalne faze [61].

Izbor metode za izdvajanje biomase zavisi od karakteristika mikroalgi (na primer, veličine i gustine ćelija), kao i od vrednosti proizvoda. Izdvajanje biomase se, najčešće, vrši u dva koraka. Prva faza se sastoji u odvajanju biomase iz suspenzije, pri čemu se vrši koncentrovanje 100–800 puta, kako bi se dobila koncentracija suve materije od 2–7% (sadržaj biomase zavisi od njene početne koncentracije i primenjenih metoda koje uključuju flokulaciju, flotaciju ili sedimentaciju). Druga faza podrazumeva koncentrisanje biomase centrifugiranjem, filtracijom ili agregacijom ultrazvukom i zahteva veću potrošnju energije [62].

Flokulacija predstavlja stvaranje agregata ćelija dodatkom flokulanata, čime se ubrzava proces odvajanja ćelija iz tečnosti. Pri agregaciji čestica uključene su dve vrste sila: na većim rastojanjima deluju elektrostatičke odbojne sile, dok na veoma malim rastojanjima deluju jače intermolekularne ili Van der Valsove sile. Ćelije mikroalgi nose negativno naelektrisanje koje sprečava agregaciju ćelija u rastvoru. Površinsko naelektrisanje može se neutralisati ili smanjiti dodatkom flokulanata, kao što su viševalentni katjoni i katjonski polimeri. Flokulanti trebaju biti jeftini, netoksični, efikasni u malim koncentracijama i bez uticaja na dalji tok procesa [63]. Najčešće se kao flokulanti koriste gvožđe(III)-hlorid, gvožđe(III)-sulfat i aluminijum(III)-sulfat. Dodatkom flokulanata koji sadrže dvovalentne i trovalentne katjone smanjuje se negativno naelektrisanje površine ćelija. Efikasnost elektrolita da izazove flokulaciju meri se kritičnom koncentracijom flokulanta ili koncentracijom neophodnom da izazove brzu flokulaciju [63]. Osim elektrolita, flokulaciju može izazvati promena pH uz upotrebu nejonskih polimera [64], bioflokulanata (na primer, hitozan) [65] ili ultrazvuka [66].

Flotacija predstavlja proces izdvajanja pri kome se mehurići gasa ili vazduha pripijaju uz čvrste čestice i nose ih na površinu tečnosti. Flotacija je pogodnija i efikasnija za izdvajanje mikroalgi od sedimentacije [67]. Takođe, za razliku od flokulacije, ne zahteva dodavanje hemijskih agenasa [68]. Međutim, postoji veoma malo dokaza o ekonomskoj i tehničkoj održivosti primene flotacije za izdvajanje biomase [62].

Izdvajanje biomase taložnim metodama zavisi od gustine i veličine ćelija mikroalgi, kao i od brzine taloženja [62]. Nedostatak ove metode je neefikasno taloženje mikroalgi male gustine [69], kao i ograničenost primene na ćelije mikroalge veće od 70 μm, kao što je *Spirulina* spp. [70].

Primena filter presa pod pritiskom ili vakuumom pogodna je za velike količine biomase, dok u određenim slučajevima može biti relativno spora. Dodatno, filtracija je pogodnija za veće mikroalge, kao što su *Coelastrum proboscideum* i *Spirulina platensis,* a ne može se koristiti za ćelije mikroalgi manjih dimenzija, npr. *Scenedesmus, Dunaliella* ili *Chlorella* [60]. U tom slučaju, kao moguće rešenje može se primeniti membranska mikrofiltracija ili ultra-filtracija [62].

Većina mikroalgi se može izdvojiti iz suspenzije centrifugiranjem. Izdvajanje centrifugiranjem predstavlja brz ali energetski zahtevan metod izdvajanja biomase, koji zavisi od taložnih karakteristika ćelija i vremena zadržavanja mulja u centrifugi. Nedostaci procesa ogledaju se u velikoj potrošnji energije i troškovima održavanja opreme [66]. Prednosti centrifugiranja su visoka efikasnost izdvajanja [71] i mogućnost upotrebe za velike količine suspenzije [62].

Jedna od mogućnosti gajenja mikroalgi koja dodatno olakšava proces izdvajanja ćelija je primena imobilizacionih tehnika sa ciljem ograničavanja kretanja ćelija algi. Najčešće se koristi metoda imobilizacije ćelija u gel, pri čemu se, zbog male toksičnosti i visoke transparentnosti, najčešće kao nosači koriste prirodni polisaharidi, kao što su agar i alginati [72]. Proces se sastoji u mešanju polimera sa ćelijama mikroalgi i stabilizaciji dodatkom dvovalentnih jona, kako bi se formirale kuglice. Kako su imobilizovane kuglice relativno velike u poređenju sa ćelijama mikroalgi, njihovo izdvajanje iz rastvora može se izvršiti filtracijom koja ne zahteva značajnu količinu energije. Primena imobilizacije u gajenju mikroalgi za proizvodnju biodizela pokazala se veoma pogodnom jer je proces energetski efikasan, a dobijeno ulje ima sličan profil masnih kiselina i ME kao i ulje uljarica [73].

Nakon izdvajanja, biomasa sadrži 5–15% suve materije i podložna je kvarenju, tako da je neophodno preraditi je neposredno nakon izdvajanja. Najčešće se za produžavanje stabilnosti biomase koriste metode sušenja: na suncu, pri niskom pritisku, zamrzavanjem, u fluidizovanom sloju ili sprej-sušenje [62]. Temperatura na kojoj se odvija sušenje može uticati na sastav i prinos ulja u biomasi. Na primer, pri sušenju biomase na 60 °C zadržava se visoka koncentracija TAG u mastima i

vrlo malo smanjuje prinos ulja, dok više temperature dovode do smanjenja i koncentracije TAG i prinosa ulja [58].

Razaranje ćelija

Proizvodnja biodizela u određenim slučajevima zahteva oslobađanje ulja iz ćelija mikroalgi. Ovaj postupak se mora obaviti na najekonomičniji i energetski najefikasniji način uz izbegavanje korišćenje velikih količina organskih rastvarača i maksimiziranje prinosa biodizela, a bez značajnijeg prisustva sporednih proizvoda. Ukupan prinos biodizela značajno zavisi od primenjene metode razaranja ćelija i uređaja koji se koristi [19]. Razaranje ćelija može se vršiti primenom različitih metoda, kao što su: u autoklavu, primena mikrotalasa i ultrazvuka ili dodatak 10% rastvora NaCl. Mikrotalasi visoke frekvencije mogu se koristiti kao efikasno sredstvo za razaranje ćelija uljarica [74], ali se mogu primeniti i za razaranje velikih količina mikroalgi [46]. Ultrazvuk izaziva razaranje ćelijskog zida i membrane usled kavitacije i često se primenjuje za razgradnju mikrobnih ćelija [75]. Metoda dezintegracije ćelija pomoću perlica izaziva direktna mehanička oštećenja ćelija usled obrtanja velikom brzinom u prisustvu finih perli [75].

Ekstrakcija ulja

Idealni metod za ekstrakciju ulja iz mikroalgi treba da bude specifičan za lipide, kako bi se minimizirala ekstrakcija ne-lipidnih supstanci ali, isto tako, selektivan za određene frakcije lipida, na primer neutralne lipide koji sadrže mono-, di- i tri nezasićene masne kiseline [76]. Kako uklanjanje vode zahteva značajna ulaganja, potrebno je da primenjeni metod ekstrakcije ulja bude efikasan kada se primenjuje direktno na vlažnu biomasu [19]. Primenom kisele i bazne hidrolize mogu se ekstrahovati ulja iz vlažne biomase bez upotrebe organskih rastvarača i uz smanjenje koncentracije hlorofila u dobijenim uljima [77].

Ekstrakcija ulja iz mikroalgi je otežana usled prisustva debelog ćelijskog zida. Zbog toga, za ekstrakciju ulja iz mikroalgi retko se koriste mehaničke prese koje se najčešće primenjuju za ekstrakciju ulja iz uljarica. Najčešće se proces ekstrakcije vrši primenom organskih rastvarača, koji su pogodni za primenu na suvoj biomasi, ili superkritičnih fluida (na primer, superkritični CO_2) pogodnih za primenu na vlažnoj algalnoj biomasi [78]. Nakon ekstrakcije ulja, smeša koja se sastoji od rastvarača, preostale vode, ulja i ostataka ćelija, podvrgava se razdvajanju tečno-čvrsto (na primer, filtracijom), kako bi se uklonili ostaci ćelija. Pri ekstrakciji organskim rastvaračima, nakon razdvajanja tečno-čvrsto, primenjuje se neka od metoda za razdvajanje tečno-tečno, kao što su destilacija ili vakuum uparavanje u cilju uklanjanja rastvarača i preostale vode [57]. Pri upotrebi smeša nepolarnih i polarnih rastvarača, voda se uklanja iz rastvarača i ulja dvofaznom separacijom i dekantovanjem. S druge strane, tokom ekstrakcije superkritičnim fluidima, smanjenje pritiska dovodi do isparavanja vode i rastvarača i taloženja lipida.

Pri izlaganju ćelija bikompatibilnom rastvaraču, molekuli rastvarača ulaze u ćeliju, izazivajući oštećenja membrane [79]. Da bi se postigla potpuna ekstrakcija, moraju se razoriti veze između lipida i ostalih nelipidnih komponenti, a istovremeno treba izbeći degradaciju lipida. Pri ulasku nepolarnog rastvarača (hloroform ili heksan) u ćeliju, dolazi do stvaranja van der Waalsovih sila između rastvarača i neutralnih lipida i građenja kompleksa rastvarač-lipidi. Usled koncentracionog gradijenta dolazi do izlaska kompleksa iz ćelije i ulaska čistog rastvarača. Na ovaj način, neutralni lipidi se ekstrahuju iz ćelije i ostaju rastvoreni u rastvaraču. Međutim, neki neutralni lipidi se nalaze u citoplazmi vezani za polarne lipide tako da van der Waalsove sile koje se formiraju nisu dovoljno jake da raskinu veze između nepolarnih i polarnih lipida. S druge strane, polarni rastvarači, kao što je metanol ili izopropanol, mogu da raskinu veze između lipida i proteina gradeći vodonične veze sa polarnim lipidima iz kompleksa. Pri upotrebi smeše polarnog i nepolarnog rastvarača, rastvarači prodiru u ćeliju i reaguju sa lipidnim kompleksom. Nepolarni rastvarač stvara van der Waalsove sile sa neutralnim lipidima iz kompleksa, dok polarni rastvarač gradi vodonične veze sa polarnim lipidima. Vodonične veze su dovoljno jake da poremete veze lipida i proteina, pri čemu se kompleks rastvarača i lipida može odvojiti od ćelijske membrane i izbaciti iz ćelije. Dodatak polarnog rastvarača nepolarnom ubrzava ekstrakciju neutralnih lipida koji su vezani za membranu što, međutim, neizostavno dovodi i do ekstrakcije polarnih lipida [57].

Najčešće se, kao rastvarač, koristi smeša hloroform/metanol (tabela 2) u zapreminskim odnosima 1/2 [10,47], 2/1 [6,42,45] ili 1/1 [46], pri čemu preostala voda predstavlja tercijalnu komponentu u smeši i omogućava potpunu ekstrakciju neutralnih i polarnih lipida. Primena smeše rastvarača ne zahteva potpuno sušenje biomase. Donju, organsku fazu čini hloroform i deo metanola, kao i rastvoreni neutralni i polarni lipidi, dok je gornja, vodena faza sastavljena od vode sa delom metanola i većeg dela nelipidnih komponenti (proteini i ugljeni hidrati) [80].

Iako je klasična Folch-ova ekstrakcija hloroformom pogodna za ekstrakciju ulja mnogih mikroalgi, manje toksični organski rastvarači se sve češće koriste. Efikasnost *n*-heksana pri ekstrakciji ulja iz mikroalgi je manja u poređenju sa hloroformom, ali je njegova toksičnost manja i ima veoma mali afininitet prema nelipidnim kontaminantima i visoku selektivnost prema frakcijama neutralnih lipida [19]. Primenom smeše hloroform/ /metanol postižu veći prinosi ekstrahovanog ulja algi (32 mas.%) u odnosu na *n*-heksan (22 %), a primenjeni

sistem ekstrakcije ne utiče na sastav MEMK [81]. Međutim, troškovi i zaštita životne sredine su problemi koji se moraju rešiti pre primene organskih rastvarača u procesu [82].

Smeša heksan/izopropanol (3/2, v/v) može se koristiti kao manje toksična zamena za smešu hloroform/ /metanol sa sličnim delovanjem [83]. Čisti alkoholi, kao što su butanol, izopropanol i etanol su jeftini, lako isparljivi i imaju jak afinitet prema kompleksu lipida koji je vezan za membranu ćelija. Međutim, njihova polarna priroda je nedostatak jer ograničava interakcije sa slobodnim globulama neutralnih lipida [57].

Upotreba superkritičnih fluida zasniva se na poboljšanju prenosa mase usled dobre rastvorljivosti, visoke difuzivnosti i niske viskoznosti. U poređenju sa konvencionalnim procesima, ekstrakcija superkritičnim fluidima daje veći prinos, olakšava recikliranje i obezbeđuje dobijanje proizvoda visoke čistoće [84]. Najčešće se koristi superkritični CO_2. Nizak kritični pritisak CO_2 (72,9 atm) uslovljava male troškove kompresije, dok niska kritična temperatura CO_2 (31,1 °C) omogućava uspešnu ekstrakciju termički osetljivih frakcija lipida bez degradacije [57]. Osim toga, prednosti upotrebe superkritičnog CO_2 ogledaju se u njegovoj niskoj toksičnosti i nezapaljivosti, kao i slaboj reaktivnosti [76].

TRANSESTERIFIKACIJA I ESTERIFIKACIJA ULJA IZ MIKROALGI

Reakcije tranesterifikacije i esterifikacije ulja se mogu katalizovati homogenim katalizatorima, heterogenim katalizatorima i enzimima, a mogu se izvoditi i u odsustvu katalizatora na povišenoj temperaturi i povišenom pritisku (tzv. superkritičnim uslovima).

Homogeno katalizovane reakcije

Homogeni bazni katalizatori (KOH i NaOH), koji se, inače, upotrebljavaju u cilju ubrzanja reakcije transesterifikacije različitih biljnih ulja, kao, na primer: suncokretovog [85–88], palminog [89,90] ili sojinog [91], ne mogu se primeniti u reakciji transesterifikacije lipida iz mikroalgi zbog prisustva velikih količina SMK. U reakciji SMK sa baznim katalizatorom stvaraju se sapuni [92], čime se smanjuje prinos biodizela i otežava izdvajanje glicerola [93].

Prommuak i sar. [94] su ispitivali bazno katalizovanu metanolizu ulja ekstrahovanog iz suvih mikroalgi *C. vulgaris*, u opsegu koncentracije KOH od 2–8% (računato na biomasu), pri odnosu metanol: biomasa (v/w) 8:1, 12:1 i 16:1, na temperaturi od 60 °C, u trajanju od 1, 2, 3 ili 4 h. Najveći prinos metil estara masnih kiselina (MEMK) postiže se pri sledećim uslovima: koncentracija KOH 6%, odnos metanol:biomasa 16:1 u toku 2 h reakcije. Povećanje koncentracije KOH do 6 % (molski odnos metanol:biomasa 16:1, vreme trajanja reakcije 4 h) dovodi do porasta prinosa biodizela. Ukoliko se količina katalizatora poveća na 8%, prinos biodizela se smanjuje, jer se deo katalizatora koristi za reakciju saponifikacije, pri čemu dolazi do nastajanja sapuna i vode, koji ometaju reakciju transesterifikacije.

U slučaju dobijanja biodizela iz mikroalgi, prihvatljivija je primena kiselih katalizatora, npr H_2SO_4, koji nisu osetljivi na prisustvo SMK, i koji istovremeno katalizuju esterifikaciju SMK i transesterifikaciju TAG [78]. Uprkos ovoj prednosti, kiselo katalizovana transesterifikacija ulja algi je do sada malo proučavana. Johnson i Wen [95] su, transesterifikacijom ulja prethodno ekstrahovanog iz mikroalgi *Schizochytrium limacinum* u prisustvu sumporne kiseline kao katalizatora, ostvarili prinos sirovog biodizela od 57% (računato na masu algi). Sadržaj MEMK u sirovom biodizelu, dobijenom nakon centrifugisanja i razdvajanja gornjeg – metilestarskog sloja od donjeg – glicerolno-metanolnog sloja, bio je približno 66%, dok su ostatak činili i monoacilgliceroli, diacilgliceroli, TAG i masne kiseline.

Ulja mikroalgi se mogu podvrgnuti tzv. kiselom predtretmanu koji uključuje esterifikaciju SMK u prisustvu kiselog katalizatora. Ovim predtretmanom se smanjuje sadržaj SMK do nivoa kada je moguća primena baznih katalizatora (dvostepeni proces). Glavni nedostatak ovog dvostepenog procesa je upotreba viška baznog katalizatora u cilju neutralizacije kiselog katalizatora, što doprinosi povećanju troškova proizvodnje biodizela [78]. Dvostepeni proces koji kombinuje kiselo-katalizovanu esterifikaciju i bazno-katalizovanu transesterifikaciju može se prihvatiti kao rešenje kojim se prevazilaze nedostaci homogene kisele i bazne katalize [96]. Chen i sar. [97] su izveli dvostepeni proces koji se sastojao iz esterifikacije ulja iz slatkovodnih (*Scenedesmus*), morskih (*Nannochloropsis*) i heterotrofnih (*Dinoflagellate*) vrsta algi metanolom u prisustvu sumporne kiseline, kako bi se smanjio sadržaj SMK, nakon čega je u drugom koraku izvršena bazno katalizovana transesterifikacija ulja. Dvostepenim procesom za vreme od 30 min postignuta je 100% konverzija TAG na 65 °C pri molskom odnosu metanol:ulje 12:1 i 2% KOH. Međutim, metanolizom suncokretovog ulja u jednostepenom postupku na 20 °C, pri molskom odnosu 6:1, 1% KOH kao katalizatora i intenzitetu mešanja 120 rpm, ostvaren je prinos u opsegu 80–90% nakon 15–20 min reakcije [85].

Heterogeno katalizovane reakcije

Primena heterogenih katalizatora u proizvodnji biodizela ima niz prednosti: proces odvajanja i prečišćavanja proizvoda se pojednostavljuje, smanjuje se zagađenje životne sredine, mogućnost ponovne upotrebe regenerisanog katalizatora, što kao posledicu daje pozitivan ekonomski efekat [98]. Najveći broj istraživanja heterogeno katalizovane transesterifikacije različitih biljnih ulja odnosi se na primenu oksida zemnoalkalnih metala kao katalizatora reakcije. Katalitička aktivnost

baznih heterogenih katalizatora raste sa povećanjem njihove baznosti, odnosno katalizatori najveće baznosti ostvaruju najveću konverziju [99]. Najčešće korišćeni heterogeni katalizator u reakciji transesterifikacije biljnih ulja je CaO, čijom upotrebom se mogu postići visoki prinosi MEMK i preko 98% [100].

Ne postoji mnogo objavljenih rezultata o primeni heterogenih katalizatora za dobijanje biodizela iz mikroalgi, uglavnom zbog činjenice da su mikroalge relativno nova sirovina i nisu komercijalno dostupne na tržištu [78]. Čisti CaO i MgO nisu pokazali aktivnost u transesterifikaciji ulja mikroalge *N.oculata*, dok je CaO na Al_2O_3, u koncentraciji od 80% omogućio prinos biodizela od 97,5% pri molskom odnosu metanol:ulje 30:1 na temperaturi od 50 °C [101]. Sadržaj MEMK u biodizelu dobijenom metanolizom lipida mikroalge *Nannochloropsis gaditana* u prisustvu hijerarhijskih beta zeolita bio je manji od 30% [102]. Transesterifikacijom ulja iz mikroalgi *Nannochloropsis* spp. pri temperaturi od 65 °C u prisustvu Mg–Zr kao čvrstog katalizatora u toku 4 h trajanja reakcije postignut je zanemarljivo mali prinos MEMK od 22,2%, pri koncentraciji katalizatora 10% i masenom odnosu metanol:ulje 10:1. Dalje povećanje masenog odnosa metanol:ulje na 20:1 dovodi do opadanja prinosa MEMK [103].

Enzimski katalizovane reakcije

Enzimski katalizovana sinteza biodizela ima određene prednosti: visoku selektivnost, manju potrošnju energije, manje sporednih proizvoda i otpada [104], nižu reakcionu temperaturu [105], eliminisanje stvaranja sapuna [106], laku regeneraciju glicerola i katalizatora [106], a moguća je i potpuna konverzija sirovina sa visokim sadržajem SMK u estre [107]. Ovaj način dobijanja biodizela iz ulja mikroalgi je već proučavan [108–110].

Li i sar. [109] su enzimski katalizovanom transesterifikacijom ostvarili najveću konverziju ulja iz mikroalge *C. protothecoides* od 98,15% za 12 h pri sledećim optimalnim uslovima: 75% imobilisane lipaze, sadržaj vode 10% (računato na količinu lipida), molski odnos metanol: ulje 3:1 (metanol je dodavan postepeno, u tri dela, kako bi se izbegla inhibicija enzima), temperatura 38,8 °C i pH 7.0. Isto tako, Lai i sar. [108] su proučavali uticaj molskog odnosa metanol:ulje, reakcione temperature, zapremine rastvarača i sadržaja vode na prinos biodizela pri enzimski katalizovanoj transesterifikaciji ulja iz mikroalge *Chlorella pyrenoidosa.* Korišćene su dve vrste enzima, i to lipaze plesni *Penicillium expansum* (PEL) i kvasca *Candida antarctica* (Novozym 435), u prisustvu dva sistema rastvarača (jonski rastvarač 1-butil-3-metilimidazol-heksafluorofosfat, [BMIm][PF6], i organski rastvarač *terc*-butanol). Pod optimalnim uslovima, u prisustvu PEL i Novozym 435 viši prinosi MEMK postignuti su u jonskom rastvaraču (90,7 i 86,2%, redom) u odnosu na reakciju u *terc*-butanolu (48,6 i 44,4%, redom).

Tran i sar. [110] primenili su dva načina proizvodnje biodizela iz mikroalge *C. vulgaris* ESP-31 u prisustvu imobilisane lipaze vrste *Burkholderia* sp. C20 kao katalizatora. Prvi je podrazumevao transesterifikaciju prethodno ekstrahovanog ulja, a drugi direktnu transesterifikaciju razorene mikroalgalne biomase. Postupkom transesterifikacije prethodno ekstrahovanog ulja za 48 h reakcije postignuta je konverzija ulja od 72,12% (43,27% biomase) pri sledećim optimalnim uslovima: količina enzima 1203,11 U/g, temperatura 40 °C, sadržaj vode 65,52 mas.%, molski odnos metanol:ulje 12,35:1, sadržaj heksana 67,46 mas.%.

***IN SITU* TRANSESTERIFIKACIJA**

Konvencionalni postupak dobijanja biodizela iz mikroalgi podrazumeva prethodnu ekstrakciju ulja iz mikroalgi pomoću organskih rastvarača, nakon čega se primenjuje reakcija transesterifikacije. Međutim, ovaj međukorak može se izbeći ukoliko se direktno transesterifikuje ulje sadržano u algama. Ovaj postupak je poznat kao *in situ* transesterifikacija [111]. *In situ* transesterifikacija je energetski efikasniji postupak u odnosu na postupak sinteze biodizela iz ekstrahovanog ulja algi [112]. Dalje unapređenje *in situ* transesterifikacije se može postići primenom mikrotalasnog zračenja, jer se ovim postupkom dobija manje sporednih proizvoda i manje otpada u odnosu na klasični postupak ekstrakcije i transesterifikacije [113].

In situ transesterifikacija mikroalgi je izvođena u odsustvu katalizatora, tj. u superkritičnim uslovima, i u prisustvu homogenih i heterogenih katalizatora i enzima.

***In situ* transesterifikacija u superkritičnim uslovima**

Metanol u superkritičnim uslovima rastvara nepolarne TAG, stvarajući jednofazni sistem ulje:metanol i ubrzavajući sintezu MEMK [114]. Tako, na primer, Levine i sar. [115] predlažu proces koji se sastoji iz dve faze: intraćelijske hidrolize lipida vlažne algalne biomase (80% vlage) superkritičnom vodom, praćene superkritičnom *in situ* etanolizom vlažnog čvrstog materijala sa visokim sadržajem masnih kiselina, bez prisustva katalizatora. Na ovaj način eliminiše se potreba za sušenjem biomase i ekstrakcijom ulja iz algi.

Voda prisutna u algalnoj biomasi se ponaša kao kosolvent u procesu sa superkritičnim metanolom, što ubrzava konverziju ulja iz algi u MEMK, a takođe povećava rastvorljivost ulja u metanolu. *In situ* transesterifikacija alge *Nannochloropsis* sp. CCMP1776 sa 90% sadržaja vode pod superkritičnim uslovima metanola u velikoj meri skraćuje reakciono vreme (25 min), pojednostavljuje proces prečišćavanja biodizela i maksimizira konverziju TAG do odgovarajućih alkil estara [114].

Soh i Zimmerman [116] predlažu primenu superkritičnog CO_2, uz metanol kao kosolvent u smeši je efikasna u ekstrakciji, transesterifikaciji i separaciji proiz-

voda pri nižim reakcionim temperaturama i čija se prednost ogleda u povećanoj selektivnosti i rastvorljivosti, kao i manjem utrošku energije. Za razliku od metanola, glicerol je skoro potpuno nerastvoran u superkritičnom CO_2 i po nastajanju će se izdvojiti iz smeše.

In situ transesterifikacijom karbonizovane algalne biomase pri superkritičnim uslovima etanola (270–325 °C) postignut je prinos etil estara masnih kiselina (EEMK) > 90% [117]. Primenom superkritičnih uslova temperature i pritiska etanola u *in situ* transesterifikaciji mikroalgi, moguće je postići visoke prinose EEMK primenom malih količina etanola, čime se doprinosi smanjenju troškova i manjem ulaganju u rekuperaciju alkohola. Naime, pri molskom odnosu etanol:masne kiseline od 5/1, postignut je prinos EEMK od 79% nakon 150 min, dok je pri molskom odnosu 20/1 pronos EEMK bio 89% nakon 180 min, pri temperaturi od 275 °C [118]. Poređenja radi, prinos EEMK pri superkritičnim uslovima etanola nakon 120 min pri molskom odnosu etanol ulje 3/1 i 6/1 bio je u oba slučaja 60%, dok su pri istim molskim odnosima, kiselo katalizovanom (5% H_2SO_4) *in situ* transesterifikacijom na 60 °C ostvareni prinosi između 5 i 10% [118].

Homogeno katalizovana *in situ* transesterifikacija

Johnson i Wen [95] su poredili dva postupka kiselo katalizovanog (H_2SO_4) dobijanja biodizela iz heterotrofne mikroalge *Schizochytrium limacinum*: *in situ* transesterifikaciju algi i transesterifikaciju prethodno ekstrahovanog ulja. Nezavisno od korišćenog rastvarača (hloroform, heksan ili petroletar), *in situ* postupkom su dobijeni veći prinosi MEMK. Pri tome, jedino je transesterifikacija uz hloroform dala visok sadržaj MEMK u sirovom biodizelu. Međutim, u slučaju bazno katalizovane transesterifikacije biomase i ulja esktrahovanog iz mikroalge *C. vulgaris*, pri količini KOH od 4%, molskom odnosu metanol:biomasa, odnosno metanol:ulje 16:1, prinos MEMK nakon 4 h reakcije bio je znatno veći u postupku koji uključuje prethodnu ekstrakciju ulja i transesterifikaciju, zbog veće debljine zida mikroalge [94].

Visoki prinosi i dobar kvalitet biodizela mogu postići *in situ* kiselo-katalizovanom transesterifikacijom mikroalge *C. pyrenoidosa* u prisustvu *n*-heksana [103]. Optimalni uslovi obuhvatili su: 1 g algi u prahu, 6 ml *n*-heksana i 4 ml metanola sa 0,5 M sumporne kiseline, pri temeraturi od 90 °C. Tokom 2 h reakcije pri optimalnim uslovima postignut je prinos od 95% biodizela sa sadržajem MEMK od 99%.

Pri istraživanju *in situ* transesterifikacije mikroalgi vrste *Chaetoceros gracilis*, Wahlen i sar. [119] su definisali sledeće parametre: vrstu alkohola, količinu alkohola po jedinici biomase, temperaturu reakcije i koncentraciju katalizatora kao ključne za uspešnu konverziju masti iz algi u biodizel. Ispitujući ekstrakciju ulja iz mikroalgi *C. gracilis* metanolom, utvrđeno je da se dobija znatno manja količina TAG iz algalne biomase, u odnosu na ekstrakciju etanolom, 1-butanolom, 2-metil-1-propanolom ili 3-metil-1-butanolom. Međutim, kada je postupak izvođen u prisustvu 1,8 zapr.% H_2SO_4 kao katalizatora, vrsta korišćenog alkohola nije imala uticaj na prinos dobijenih MEMK. Pri ovoj metanolizi, prinos MEMK posle 125 min je bio 14,8% (računato na biomasu). Povećavajući zapreminu metanola na 2,5 ml po 100 mg biomase (maseni odnos 20:1), prinos MEMK se povećao 22,6% nakon 150 min. Dalje povećanje masenog odnosa metanol:biomasa do 40:1 nije dovelo porastu prinosa MEMK. Primenjeni maseni odnos metanol:biomasa je daleko veći od uobičajenog masenog odnosa koji se koristi u metanolizi suncokretovog ulja (1:4,5, koji odgovara molskom odnosu 6:1).

Optimalan odnos metanol:ulje je proučavan od strane mnogih istraživača. Tako, povećanje masenog odnosa metanol:suve alge od 0,4:1 na 3.2:1 u kiselo katalizovanoj *in situ* transesterifikaciji mikroalgi *C. pyrenoidosa* dovelo je do povećanja prinosa biodizela sa 69,4 na 94,3%, dok je sadržaj MEMK ostao iznad 98% [103]. Dalje povećanje masenog odnosa metanol:suve alge do 7,9:1 nije doprinelo povećanju prinosa biodizela i sadržaja MEMK, kao posledica otežane separacije usled viška metanola. Međutim, povećanje masenog odnosa metanol:suve alge do 9,5:1 ima pozitivan uticaj na *in situ* metanolizu biomase pod dejstvom mikrotalasnog zračenja, dok dalje povećanje masenog odnosa metanol:suve alge ne doprinosi povećanju prinosa [120].

Generalno, na višim temperaturama esterifikacije postižu se veći prinosi biodizela, zbog veće brzine reakcije, kao i zbog poboljšanog razaranja ćelija, pri čemu ulje lakše dolazi u kontakt sa reaktantima. Međutim, veoma visoke temperature reakcije mogu dovesti do smanjenja prinosa pri dužem trajanju reakcije [33]. Miao i Wu [33] su razvili integrisani metod dobijanja biodizela kiselo katalizovanom metanolizom ulja iz heterotrofne mikroalge *C. protothecoides*. Pri molskom odnosu metanol:ulje 30:1 i koncentraciji katalizatora 100%, računato na masu ulja, nema značajne razlike u prinosu biodizela na 30 (56%) i 50 °C (58%) [33]. Rezultati ispitivanja uticaja temperature (50–110 °C) i vremena trajanja (0,5–4 h) kiselo katalizovane *in situ* proizvodnje biodizela iz mikroalge *C. pyrenoidosa* pokazali su da se viši prinosi biodizela postižu pri višoj temperaturi, naročito u prvom času reakcije, dok nije bilo značajne promene u sadržaju MEMK u uzorcima uzetim u opsegu 0,25 do 4 h, što ukazuje da je efikasna ekstrakcija ulja iz biomase ključni korak *in situ* transesterifikacije. Najveće količine biodizela dobijene su na 90 i 110 °C (95% prinosa za 2 h), dok su prinosi na 20, 50 i 70 °C rasli nakon četvrtog sata reakcije [103]. Povećanje temperature sa 60 na 90 °C, u toku 10 min reakcije, pri direktnoj transesterifikaciji algalne vrste *C. gracilis,* dovelo je do porasta prinosa MEMK sa 23,7 na 33,7%. S

druge strane, porastom temperature sa 60 na 80 °C, tokom 20 min trajanja reakcije, postignuto je značajno povećanje prinosa, uz maksimalni prinos MEMK od 34,1%. Promenom koncentracije H_2SO_4 od 1,2 do 2,4% (v/v), u trajanju 10 min na 80 °C, uočen je neznatan porast prinosa MEMK sa 28,2 na 31,7% [119].

Heterogeno katalizovana *in situ* transesterifikacija

Jedino su Li i sar. [121] istraživali heterogenu *in situ* transesterifikaciju suvih mikroalgi *Nannochloropsis* sp., korišćenjem 10% Mg–Zr kao katalizatora. Pri zapreminskom odnosu metanol:metilen dihlorid 3:1, u toku 4h trajanja reakcije na temperaturi od 65 °C postignut je prinos MEMK od 28%.

Enzimski katalizovana *in situ* transesterifikacija

Primenom postupka direktne transesterifikacije razorene mikroalge *C. vulgaris* ESP-31 u prisustvu imobilisane lipaze iz vrste *Burkholderia* sp. C20, pri optimalnim uslovima (količina katalizatora 1233,1 U/g, temperatura 40 °C, molski odnos metanol:ulje 67,93:1, sadržaj heksana 80,57% i vreme trajanja reakcije 48 h), konvertovano je 97,3% ulja (odnosno 58,3% biomase) u biodizel. Imobilisana lipaza u postupku direktne transesterifikacije razorene biomase bila je otporna na visoke količine metanola (molski odnos metanol:ulje veći od 67,93), aktivna i pri visokom sadržaju vode (>71,39%) i mogla je biti korišćena u 6 ciklusa bez značajnog gubitka aktivnosti [110].

Unapređenje procesa *in situ* transesterifikacije

Jones i sar. [122] su iskoristili mogućnost vezivanja ćelija za jonoizmenjivačku smolu (Amberlit), radi koncentrisanja razblažene suspenzije želija. Nakon toga, smola sa vezanom biomasom tretirana je 5% rastvorom sumporne kiseline u metanolu, radi eluiranja biomase, *in situ* transesterifikacije i regeneracije smole. Na ovaj način, sakupljanje i transesterifikacija algi se odigravaju istovremeno, čime se gubi potreba za međukoracima, kao što su liziranje ćelija, sušenje i ekstrakcija rastvaračem.

U svrhu daljeg unapređenja *in situ* proizvodnje biodizela iz algi ispitivana je primena ultrazvuka [123,124], mikotalasnog zračenja [120,124], kao i primena kosolvenata [125]. Takođe, sprovedena je optimizacija procesnih faktora primenom metodologije površine odziva [112,113,120,126–128].

Primena ultrazvuka i mikrotalasnog zračenja

Ultrazvuk ima sve veću ulogu u hemijskim procesima, naročito u slučajevima kada klasične metode zahtevaju drastične uslove ili veoma dugo trajanje reakcije i predstavlja važno sredstvo zelene hemije u smislu minimizacije otpada i uštede energije [123]. Mikrotalasno zračenje se, takođe, koristi poslednjih godina kao efikasan način skraćivanja vremena reakcije transesterifikacije. Njene prednosti su značajno smanjenje količine sporednih proizvoda i kratko vreme separacije [129].

Koberg i sar. [124] su proučavali značaj ultrazvuka i mikrotalasa u unapređenju procesa proizvodnje biodizela iz algi. Oni su izveli reakciju transesterifikacije prethodno ekstrahovanog ulja i *in situ* transesterifikaciju mikroalgi *Nannochloropsis algae* u prisustvu ultrazvuka (na 50 °C) ili mikrotalasa (na 60 °C), uz SrO kao katalizator, u trajanju od 5 min. U prvom slučaju (ekstrakcija i transesterifikacija) prinos biodizela bio je 18,9, odnosno 32,8% u prisustvu ultrazvuka, odnosno mikrotalasa, redom. Još veći prinosi biodizela postignuti su *in situ* transesterifikacijom, i to 20,9 i 37,1% primenom ultrazvuka, odnosno mikrotalasa, respektivno, što potvrđuje ne samo da je *in situ* transesterifikacija jednostavniji i brži proces, već i da se na ovaj način postižu viši prinosi biodizela. Pored toga, u prisustvu mikrotalasa postižu se veći prinosi biodizela u odnosu na ultrazvuk (i do 99,9%), što se pripisuje većoj izloženosti ćelija mikrotalasima usled njihovog razlaganja na manje klastere, koji su dostupniji za transesterifikaciju. Ubrzanje reakcije transesterifikacije vezuje se za jonsku prirodu prelaznog stanja reakcije transesterifikacije pod dejstvom mikrotalasa. Takođe, SrO se, kao čvrsti bazni katalizator može izdvojiti iz reakcione smeše i ponovo koristiti [124].

Direktna transesterifikacija suve biomase (sa sadržajem lipida 26%) mikroalge *C. vulgaris*, pod dejstvom ultrazvuka u trajanju od 30 min na sobnoj temperaturi, uz sumpornu kiselinu kao katalizator rezultovala je 60% prinosom MEMK [123]. *In situ* transesterifikacijom suve biomase pod dejstvom mikrotalasa postiže se visok procenat ekstrakcije ulja i njegova efikasna konverzija u biodizel, a takođe se smanjuje vreme trajanja reakcije i zapremina rastvarača u odnosu na postupak koji uključuje transesterifikaciju ekstrahovanog ulja [120].

Primena kosolvenata

U pokušaju daljeg pojednostavljenja procesa dobijanja biodizela iz mikroalgi, kao i smanjenja energetskih troškova samog postupka, Xu i Mi [125] su ispitali direktnu bazno katalizovanu (KOH) *in situ* transesterifikaciju mikroalgi, u prisustvu kosolvenata, ali bez ikakvog mešanja i zagrevanja. Od svih analiziranih kosolvenata (toluena, dihlormetana i dietiletra), kao i njihovih kombinacija (etar/toluen, toluen/metanol i dihlormetan/metanol), sistem toluen/metanol (u zapreminskom odnosu 2:1) pokazao se najefikasnijim, uz postignuti prinos biodizela 76% u prvom i 10% u drugom ciklusu transesterifikacije.

Optimizacija procesa primenom metodologije površine odziva

Primenom statističkih metoda moguće je postići bolje razumevanje i poznavanje procesa i odrediti opti-

malne procesne uslove koji obezbeđuju maksimalni prinos biodizela iz lipida mikroalgi.

Centralni kompozitni dizajn 2^3 (6 aksijalnih i tri centralne tačke) primenjen je u planiranju eksperimenata metanolize sintetskog ulja algi (sastav masnih kiselina *C.vulgaris*) [126]. Tri procesne promenljive (molski odnos metanol:ulje, temperatura reakcije i količina katalizatora) ispitivane su na dva nivoa u cilju optimizacije procesa primenom metodologije površine odziva. Model drugog reda predvideo je da se najviši prinos ME postiže pri sledećim optimalnim uslovima: molski odnos metanol:ulje 14:1, 0,42% NaOH na temperaturi od 43 °C.

Haas i Wagner [127] su primenili metodu površine odziva u optimizaciji *in situ* transesterifikacije algi u odsustvu kosolventa. Ispitivan je uticaj pripreme sirovine (netretirana, sušena u pećnici i ispirana vodom/sušena) na prinos proizvoda, kao i uticaj promene reakcione temperature (23 do 65 °C), količine alkohola (maseni odnos metanol:biomasa od 2,5:1 do 6,3:1) i kiselog katalizatora (maseni odnos H_2SO_4:biomasa od 0,588 do 1,254) u toku 2 h reakcije. Rezultati su pokazali da odnos zapremine metanola i biomase (*V*/*w*) i temperatura imaju najznačajniji uticaj na prinos proizvoda, pri čemu najveći odnos metanol:biomasa (*V*/*w*) i najviša primenjena temperatura (65 °C) daju najveće prinose, dok promena koncentracije katalizatora nije imala značajan uticaj u ispitivanom opsegu uslova. Smanjenje sadržaja vode u biomasi smanjuje potrebnu količinu metanola za postizanje visokih prinosa. Sušenje u pećnici smanjuje zapreminu metanola na 4 ml/g supstrata, uz prinos od 83% u odnosu maksimalni teorijski (90%), dok ispiranje biomase vodom praćeno naknadnim sušenjem nije doprinelo ni povećanju prinosa ni smanjenju potrebne zapremine metanola.

Centralni kompozitni dizajn 2^2 primenjen pri istraživanju kisele *in situ* metanolize mikroalge *N. oculata* pokazao je da je zapreminski odnos metanol: HCl najznačajnija promenjiva koja utiče na prinos MEMK, dok vreme trajanja reakcije nije značajno uticalo na prinos [96]. Na osnovu eksperimentalnih rezultata i metodologije površine odziva, optimalni uslovi *in situ* metanolize suvih algi *Nannochloropsis* spp. pod dejstvom mikrotalasnog zračenja definisani su kao: maseni odnos metanol:suva biomasa 9,5:1, koncentracija KOH 2% i reakciono vreme od 4 min [120].

Korišćenjem metode površine odziva, Patil i sar. [128] ispitivali su uticaje tri faktora: maseni odnos metanol:vlažna biomasa (3,2:1 do 9,5:1), temperatura (240–260 °C) i vreme reakcije (10–30 min), vreme reakcije (3–9 min) i koncentracija katalizatora (1–3% u odnosu na suvu biomasu) na proces proizvodnje biodizela iz biomase pri natkritičnim uslovima i mikrotalasnom zračenju, redom. Pri optimalnim uslovima maseni odnos metanol:vlažna biomasa 7,1:1, temperatura 225 °C i vreme 25 min ostvaren je prinos MEMK od oko 84% (računato na ukupni sadržaj lipida). S druge strane, pod dejstvom mikrotalasnog zračenja pri optimalnim uslovima (maseni odnos metanol:vlažna biomasa 9,5:1, koncentracija katalizatora 2% KOH i vreme reakcije 4 do 5 min na temperaturi od 60 do 64 °C) postignut je prinos MEMK oko 80%.

Primenivši metodologiju površine odziva, Patil i sar. [113] su optimizovali postupak *in situ* transesterifikacije suve biomase *Nannochloropsis salina* pod dejstvom mikrotalasnog zračenja. Na osnovu analize eksperimenata i metodologije površine odziva, došlo se do sledećih optimalnih uslova za dati postupak: maseni odnos metanol:suva biomasa 10:1, koncentracija KOH 2,5% (u odnosu na suvu biomasu), reakciono vreme 8–10 min pri utrosku energije od 1400 W tokom procesa.

POVEĆANJE RAZMERE PROCESA

Bioinženjeringom se može postići heterotrofni rast nekih mikroalgi, čime je moguće dobiti veće količine biomase i ulja iz mikroalgi [33,34], a samim tim i veće količine biodizela. Dosadašnja ispitivanja dala su dobre rezultate na laboratorijskom nivou. Postavlja se pitanje: može li se biodizel proizvoditi iz ulja heterotrofnih mikroalgi i na industrijskom nivou? U cilju utvrđivanja primene iste tehnologije i u velikim bioreaktorima, Li i sar. [109] su proučavali enzimski katalizovanu transesterifikaciju ulja iz mikroalge *C. protothecoides* u laboratorijskom (5 dm^3), poluindustrijskom (750 dm^3) i industrijskom (11,000 dm^3) bioreaktoru. Rezultati su pokazali da se sadržaj lipida neznatno smanjuje povećanjem razmere kultivacije, i iznosi 46,1, 48,7 i 43,0% u uzorcima iz laboratorijskog, poluindustrijskog i industrijskog bioreaktora, redom. Utvrđeno je da se kultivacija heterotrofne mikroalge *C. protothecoides*i proizvodnja biodizela iz nje može proširiti i na industrijski nivo.

ZAKLJUČAK

Značaj mikroalgi u prirodi ogleda se u tome što obavljajući fotosintezu učestvuju u obnavljanju i održavanju količine kiseonika u atmosferi. Međutim, alge mogu predstavljati i značajan izvor različitih industrijski značajnih komponenti. Jedna od mogućnosti primene mikroalgi jeste i sinteza ulja koje se može koristiti za proizvodnju biodizela. Za razliku od ostalih sirovina koje se koriste za dobijanje biodizela, upotreba mikroalgi pruža niz prednosti kao što su: veći prinos po jedinici površine, povećana efikasnost, smanjeni troškovi izdvajanja i transporta biomase, kao i smanjeni troškovi proizvodnje biodizela. S obzirom da sadržaj i sastav ulja veoma zavisi od uslova rasta mikroalgi, izmenom sastava podloge ili načina gajenja može se značajno povećati prinos ulja. Pored toga, veoma značajan korak u

povećanju sadržaja ulja predstavlja primena genetičkog inženjerstva. Fototrofno gajenje mikroalgi je energetski povoljnije, međutim veći prinos biomase može se postići heterotrofnim gajenjem. Ipak, održivost proizvodnog sistema najviše zavisi od svojstava vrste mikroalge koja se koristi, a što nameće potrebu za stalnim izolovanjem novih sojeva, proučavanjem uslova rasta i produkcije ulja mikroalgi. Tokom procesa proizvodnje ulja energetski je vrlo zahtevan postupak izdvajanja biomase. Uglavnom se za izdvajanje biomase primenjuju metode koje su već prisutne u industriji (najčešće centrifugiranjem). Kako je podložna kvarenju, za povećanje održivosti biomase nakon izdvajanja koriste se različite metode sušenja.

Proizvodnja biodizela iz mikroalgi može se obaviti na dva načina. Prvi podrazumeva ekstrakciju ulja iz biomase i transesterifikaciju i/ili esterifikaciju ulja odgovarajućim alkoholom u prisustvu katalizatora ili u odsustvu katalizatora pod natkritičnim uslovima metanola. Metode koje se koriste za ekstrakciju ulja iz uljarica nisu pogodne za mikroalge, pa se ekstrakcija odvija nakon razaranja ćelija i uz primenu odgovarajućih organskih rastvarača ili superkritičnih fluida. Dobijanje biodizela iz izdvojenog ulja može se vršiti primenom homogene katalize u prisustvu kiselih katalizatora, kao i heterogenom katalizom. Veoma dobri rezultati prinosa biodizela postignuti su primenom enzimski katalizovane reakcije transesterifikacije. Drugi način predstavlja direktnu transesterifikaciju algalne biomase (*in situ* postupak) kojim se unapređuje proces proizvodnje smanjenjem troškova. Primenom ovog postupka izbegava se proces ekstrakcije ulja, a pokazalo se da *in situ* kiselo katalizovana transesterifikacija može dati veći prinos biodizela u poređenju sa procesom transesterifikacije ektrahovanom ulja.

Usled povećanih potreba za biogorivima, upotreba mikroalgi za proizvodnju biodizela predstavlja značajan korak u zameni fosilnih goriva. U tu svrhu neophodni su stalni razvoj tehnologija i optimizacija procesa gajenja, izdvajanja biomase i ulja mikroalgi, kao i dobijanja biodizela.

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SUMMARY

PRODUCTION OF BIODIESEL FROM MICROALGAE

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(Review paper)

In recent years, more attention has been paid to the use of third generation feedstocks for the production of biodiesel. Microalgae have emerged as one of the most promising sources for biodiesel production. They are unicellular or colonial photosynthetic organisms, with permanently increasing role in industrial application in the production of not only chemicals and nutritional supplements, but also the biodiesel. The biodiesel productivity per hectare of cultivation area can be up to 100 times higher for microalgae than for oil crops. Also, microalgae can grow in a variety of environments that are often unsuitable for agricultural purposes. Microalgae oil content varies in different species and can reach up to 77% of dry biomass, while the oil productivity by the phototrophic cultivation of microalgae is up to 122 mg/l/d. Variations of the growth conditions and the implementation of the genetic engineering can induce the changes in the composition and productivity of microalgae oil. Biodiesel from microalgae can be produced in two ways: by transesterification of oil extracted from biomass or by direct transesterification of algal biomass (so called *in situ* transesterification). This paper reviews the curent status of microalgae used for the production of biodiesel including their isolation, cultivation, harvesting and conversion to biodiesel. Because of high oil productivity, microalgae will play a significant role in future biodiesel production. The advantages of using microalgae as a source for biofuel production are increased efficiency and reduced cost of production. Also, microalgae do not require a lot of space for growing and do not have a negative impact on the global food and water supplies. Disadvantages of using microalgae are more difficult separation of biomass and the need for further research to develop standardized methods for microalgae cultivation and biodiesel production. Currently, microalgae are not yet sustainable option for the commercial production of biodiesel. First of all, the price of biodiesel from microalgae is still higher than the price of diesel, due to high production costs.

Keywords: Biodiesel • Microalgae • Cultivation • Extraction • *In situ* transesterification

In vitro studies of temperature and pH influence on chlorophyll degradation by horseradish peroxidase: Spectroscopic and HPLC studies

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Abstract

In vitro chlorophyll *a* degradation by horseradish peroxidase in the presence of the resorcinol was investigated in this paper, and the influence of pH and temperature was particularly studied. Chlorophyll *a* degradation was followed by UV–Vis and HPLC. Chlorophyll *a* was degraded when hydrogen peroxide was added into reaction mixture containing chlorophyll fraction, horseradish peroxidase, resorcinol and phosphate buffer. HPLC analysis has identified the main degradation product of chlorophyll *a* as 13^2-hydroxychlorophyll *a*. The degradation was traced at different temperatures and pH values. The increasing temperatures led to increase of chlorophyll *a* degradation, with a maximum at 37 °C. The degradation also increased with increasing pH values, reaching maximum at pH 6.

Keywords: chlorophyll, horseradish peroxidase, resorcinol, temperature, pH, HPLC.

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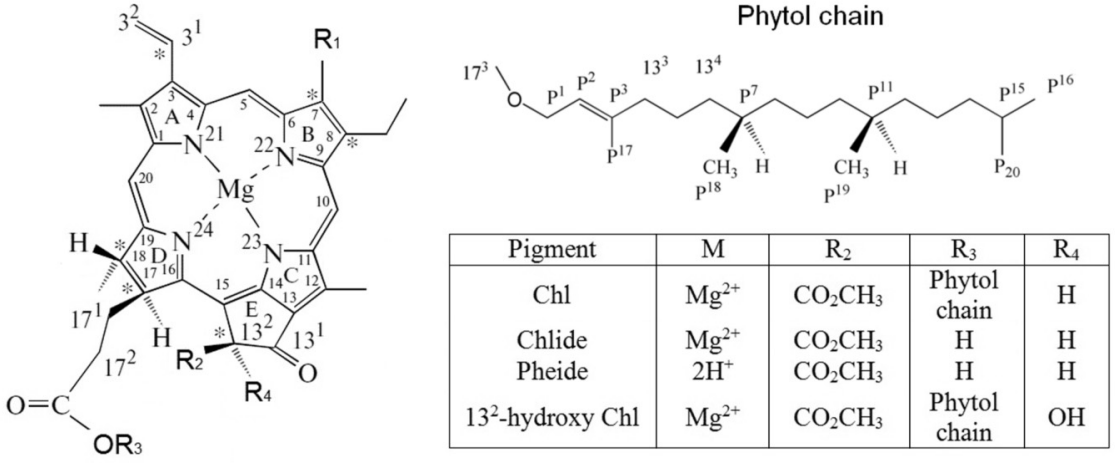
Chlorophylls (Chls) are the most ubiquitous pigments of all natural pigments, which are responsible for the color of all green plants. Biosynthetically, they are derived from protoporphyrin IX [1]. Structurally, chlorophylls are cyclic tetrapyrroles with isocyclic cyclopentanone ring, fused at the edge of the right-bottom pyrrole ring [2], as shown in Figure 1.



Pigment	M	R_2	R_3	R_4
Chl	Mg^{2+}	CO_2CH_3	Phytol chain	H
Chlide	Mg^{2+}	CO_2CH_3	H	H
Pheide	$2H^+$	CO_2CH_3	H	H
13^2-hydroxy Chl	Mg^{2+}	CO_2CH_3	Phytol chain	OH

Figure 1. Chlorophyll structure. The C-atoms are numerated according to IUPAC nomenclature rules. In position C-7, $–R_1$ corresponds to: $–CH_3$ and –CHO in the cases of Chl a and Chl b, respectively.

Chls are susceptible to many chemical or enzymatic degradation reactions. The pathway of chlorophyll (Chl) breakdown can be provided by simultaneous actions of enzymes, weak acids, oxygen, light and heat, which can lead to the formation of a large number of degradation products [3]. The previous studies have shown that the enzymatic Chl degradation may include several enzymes, such as chlorophyllase, Mg-dechelatase, Chl oxidase and peroxidase [4,5]. The role of chlorophyllase is in removal of phytol tail from chlorophyll and the formation of chlorophyllide (Chlide). On the other hand, Mg-dechelatase removes not only phytol tail, but the central Mg atom as well. Chl oxidase and peroxidase are indirectly included in the chlorophyll *a* (Chl *a*) degradation, because their action requires presence of phenolic compound. Both of them, peroxidase and Chl oxidase, as degradation products yield 13^2-hydroxychlorophyll *a* (Chl *a*-1) [6,7]. The role of peroxidase in the mechanism of Chl degradation *in vivo* is not completely understood. Still, it is known that during storage of plants the activity of chlorophyllase is slight decreased while, on the other hand, the activity of Chl

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degrading peroxidase is increased, implying a great role of peroxidase in Chl degradation [8].

Previous studies have shown that phenol, 2,4--dichlorophenol and resorcinol were effective in Chl degradation. In the Chl degradation mechanism, peroxidase oxidizes the phenolic compound, with the hydroxyl group at the *p*-position and forms the phenoxy radical or/and superoxide anion. After, the obtained radical or superoxide anion attack on Chl *a* to form Chl *a*-1 [9], shown in Figure 2. Yamauchi and collaborators (2004) demonstrated that the other phenolic compounds, like *p*-coumaric acid, apigenin, apigetrin, naringenin are highly effective in the peroxidase – hydrogen peroxide system, having electron attracting groups at the *p*-position [8–10].

Funamoto and collaborators [6] have shown that Chl content in stored broccoli decreased significantly after 4 days storage at 15 °C, while the content in broccoli treated at 50 °C for 2 h has remained almost intact during storage [6]. Similary, Martínez *et al.* [11] have examined the influence of temperature and pH on degradation of chlorophyll with peroxidase from strawberry fruits in the presence of *p*-coumaric acid and the obtained results have indicated the biggest degradation at 35 °C and pH 5.2 [11].

The subject of this work is to study the influence of temperature and pH on *in vitro* degradation of Chl *a* from spinach in the presence of horseradish peroxidase (HRP) and resorcinol, as phenolic compound, including the identification of the major degradation product by UV–Vis spectroscopy and HPLC. As opposed to previous studies, this research is the first report about influence of pH and temperature on *in vitro* degradation of Chl *a* from spinach by HRP in the presence of resorcinol as phenolic compound.

EXPERIMENTAL

All experiments, beginning with extraction, were performed under dim light as far as possible, and inside vessels and equipment covered with aluminum foil or black cloth, preventing pigments exposure to light [12].

Extraction of plant pigments

Extraction of plant pigments from spinach leaves, *Spinacia oleracea* L. (found in the local market), was performed by using already published method [13]. The final extract was a mixture of pigments containing large amounts of various Chl forms, as well as accessory pigments, carotenoids (carotenes and xanthophylls).

Chlorophyll fraction

The Chl fraction – the purified mixture, *e.g.*, Chl *a* and chlorophyll *b* (Chl *b*), was isolated from the pigment extract by using column chromatography with silica gel as the adsorbent (silica gel 60, Merck, 0.063––0.200 mm) and *n*-hexane/acetone eluent mixture [13].

Enzyme

HRP (298 U/mg) was purchased from Sigma (Germany). A 2 μM stock solution of HRP was prepared by dissolving the 0.34 mg of the solid HRP in 10 ml of cold 50 mM phosphate buffer pH 6. The enzyme concentration was calculated using a ε_{403} = 102.0 mM^{-1} cm^{-1} [14].

Chlorophyll degradation reaction

Chl degradation was determined as described by Yamauchi and Minamide with slight modifications [4]. The reaction mixture contained 0.2 ml Chl fraction in ethanol solution, 50 μl ethanol solution of resorcinol, 0.1 ml 1% Triton X-100, 0.1 ml 0.3% hydrogen peroxide, 40 μl HRP and 2.0 ml 100 mM phosphate buffer (pH

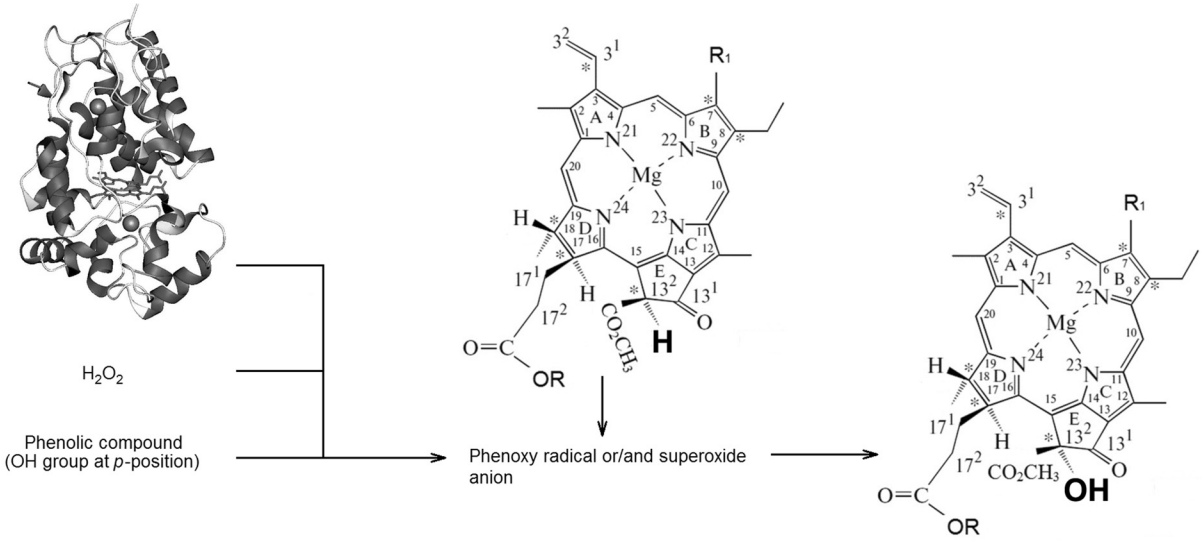

Figure 2. The mechanism of chlorophyll degradation by HRP in the presence of phenolic compound. (R corresponds to phytol tail; R_1 corresponds to: –CH_3 and –CHO in the cases of Chl a and Chl b, respectively).

6.0) in a total volume of 2.5 ml. The reaction was allowed to proceed for 10 min at defined temperatures and pH values, then stopped by the addition of 2.5 ml 96% ethanol. After, all presented Chls from the reaction mixture, were extracted from the reaction mixture by addition of 5.0 ml *n*-hexane.

UV–Vis spectroscopy

The spectrophotometric measurements were made on a Varian Cary-100 spectrophotometer equipped with 1.0 cm quartz cells. All spectra were recorded from 350 to 800 nm with 1.0 bandwidth. Spectra of all compounds are recorded in *n*-hexane solution. Chl concentration in the mixture was set in the range between 10^{-5} and 10^{-6} mol/dm^3 [15]. Chl *a* content in relation to Chl *b*, in Chl fraction, was 5:1.

HPLC Analysis

HPLC Analysis of Chl-containing reaction mixture was performed under isocratic conditions on Agilent 1100 Series set-up (Waldborn, Germany), on Zorbax Eclipse XDB-C18 column, by using diode array detector set at detection wavelength ($\lambda_{det.}$): 660 nm; the isocratic conditions were: mobile phase – acetonitrile/ /methanol/ethyl acetate, 60:20:20, flow rate – 1 ml/min, temperature 25 °C.

RESULTS AND DISCUSSION

HPLC Chromatograms of the degraded Chls, extracted from the reaction mixture, at the different temperatures are shown in Figure 3. The absorption spectra of the main compounds in the eluent mixture observed on the HPLC chromatograms at t_{ret} = 13 min (assigned as Chl *a*), and t_{ret} = 12 min (assigned as Chl *a*-1), are shown in the increments of the Figure 3, respectively; the spectra shown in the increments correspond to the two peaks.

HPLC chromatograms of the extracted Chls from the reaction mixture at different pH values are shown in Figure 4. The absorption spectra of all main compounds in the eluent mixture observed in the HPLC chromatograms at t_{ret} = 13 min (Chl *a*), and t_{ret} = 12 min (Chl *a*-1), are shown in the increments of the Figure 4, respectively; the spectra shown in the increments were taken correspond to the two peaks.

There are many studies about the effects of various phenolic compounds such are 2,4-dichlorophenol, phenol, *p*-coumaric acid, *p*-hydroxyphenylacetic acid, *p*-hydroxybenzoic acid, *p*-hydroxyacetophenone, resorcinol and umbelliferone on peroxidase-mediated Chl oxidation [4,5]. On the other hand, the obtained results with *o*-diphenols and derivatives such as catechol,

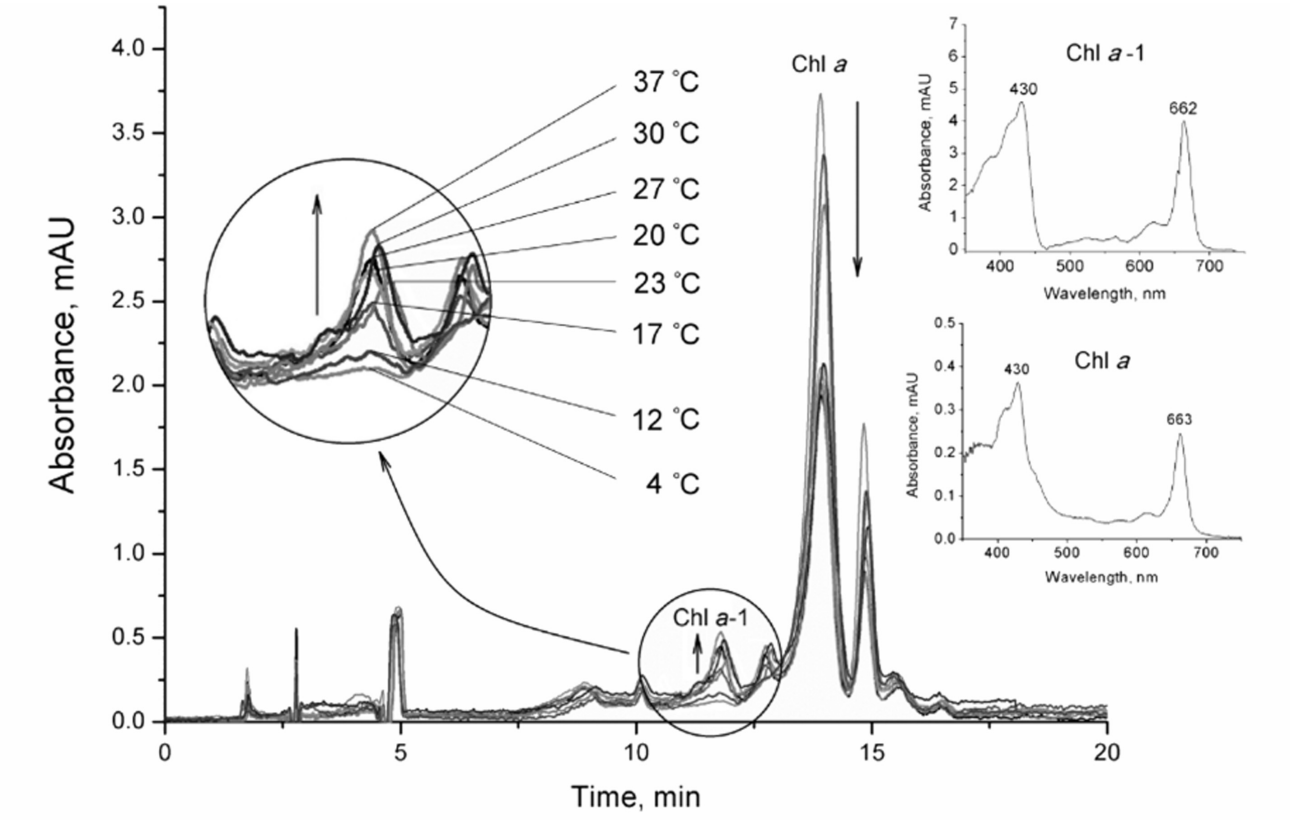

Figure 3. HPLC profile of the extracted chlorophylls from the reaction mixture at the different temperatures. The reaction medium contained 1 μM of Chl a, 16 nM HRP, 0.1 mM resorcinol, 50 mM sodium phosphate buffer, pH 6.0, and 0.1 mM H_2O_2. Detection was carried out at 660 nm.

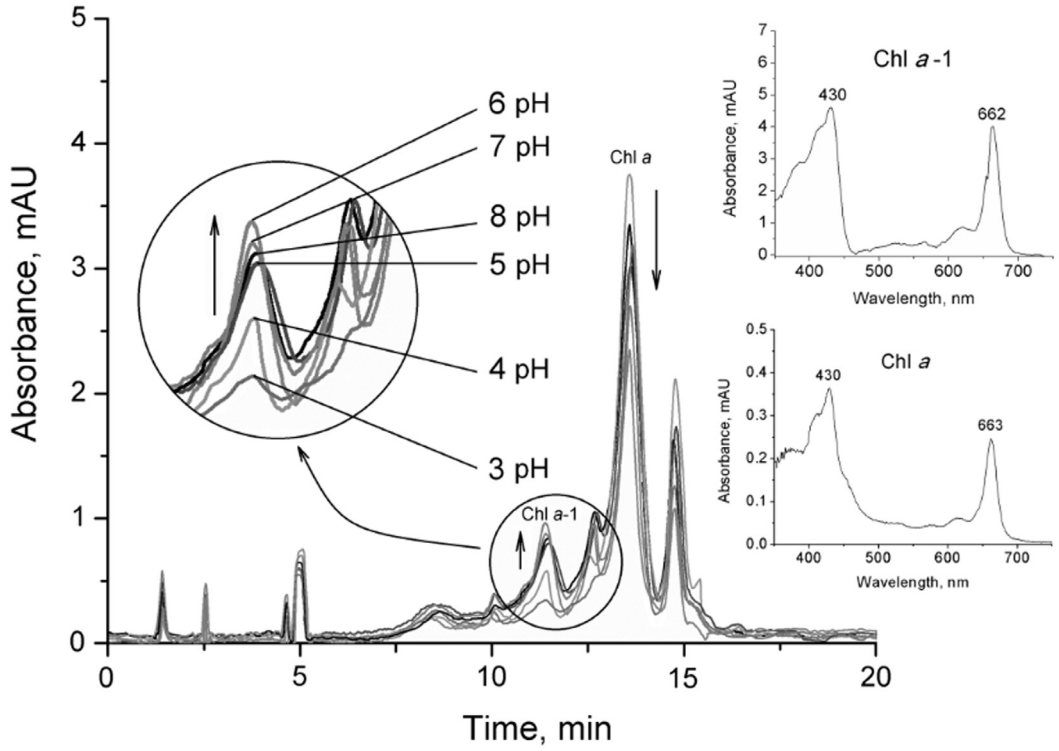

Figure 4. HPLC Profile of the extracted chlorophylls from the reaction mixture at different pH. The reaction was performed at 25 °C in medium contained of 1 μM of Chl a, 16 nM HRP, 0,1 mM resorcinol, 50 mM sodium phosphate buffer, pH 6.0, and 0.1 mM H_2O_2. Detection was carried out at 660 nm.

guaiacol, ferulic acid, caffeic acid and chlorogenic acid on degradation of Chl by peroxidase were negative, *e.g.*, the degradation was not detected [16]. Based on these findings, it can be concluded that the phenolic compounds involved in Chl degradation could be monophenols whith a hydroxyl group at *p*-position. The flavonoids, with hydroxyl group in *p*-position in the B-ring, such as apigenin and its 7-glucoside derivative, as well as naringenin were also effective in peroxidase-mediated degradation of Chl [8]. Based on the presented data, we selected resorcinol to mediate Chl *a* degradation by HRP.

Bacause the peroxidase is capable of abstracting a labile hydrogen atoms from phenolic substrate [17], the interpretation of these results is that the enzyme is involved in production of Chl *a*-1 radicals (at t_{ret} =12 min – Figures 3 and 4) [18]. The absorption spectra of the Chl *a* and Chl *a*-1, given in the increments of Figures 3 and 4, showed good agreement with literature data [9,18–20]. The absorption spectra of Chl *a* and Chl *a*-1 are very similar, which is expected since they are both porphyrine type of components with only difference in C-13^2 position, so identification of Chl *a*-1 is based on the retention time on HPLC chromatogram, obtained under similar conditions [21]. As it is well known, Chls as the porphyrin derivatives have two major absorption bands in the visible range, due to extended π-delocalization at the edge of cyclic tetrapyrrole (porphyrin) skeleton (Figure 1): ''red'' (Q-) band and ''blue'' (Soret or B-) band [22–24]. The "red" and the "blue" bands of Chl *a* (assigned as Q_y- and Soret band) are located at 662 and 430 nm in acetonitrile, respectively [12,22], similar to the ones shown in the increments of Figures 3 and 4. The ratios of absorbance intensities for Soret- and the Q-band is ~1.3 for Chl *a* [22]. Of course, the bands intensities and their maximum absorption positions (λ_Q and λ_{Soret}) in the other solvents are different, and they are also influenced by many other factors like substitution, ligands, H-bonding, the surroundings [24].

Since the analysis of this type of compounds (such as Chls) predominantly uses C-18 column, retention time of the analyzed components always decreases in the same order: chlorophyll *a* > C13^2-hydroxychlorophyll *a* > chlorophyll *b* and predominantly depends on the polarity of the mobile phase [15,25]. The HPLC chromatograms obtained at the different temperatures (Figure 3), show decrease of Chl *a* with increase of temperatures from 4 to 47 °C. On the other hand, HPLC chromatograms obtained at the different pHs at constant temperature (Figure 4) also show decrease of Chl *a* with increase of pH. The amount of Chl *a*-1 is accumulated on the account of the degraded Chl *a*.

Peroxidase-mediated Chl degradation activities at different temperatures and pH are shown in Figures 5

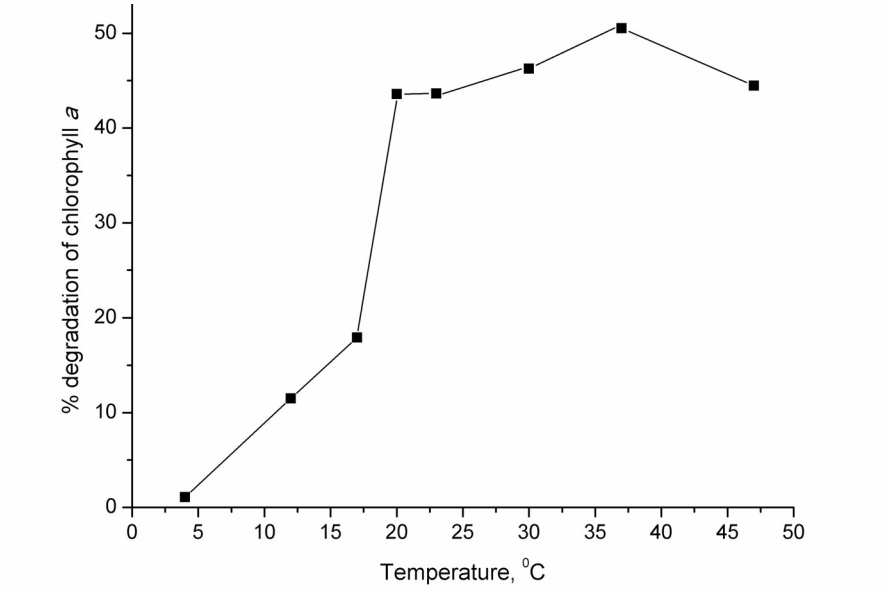

Figure 5. Temperature effects on Chl a degradation by HRP, in the presence of resorcinol at pH 6.

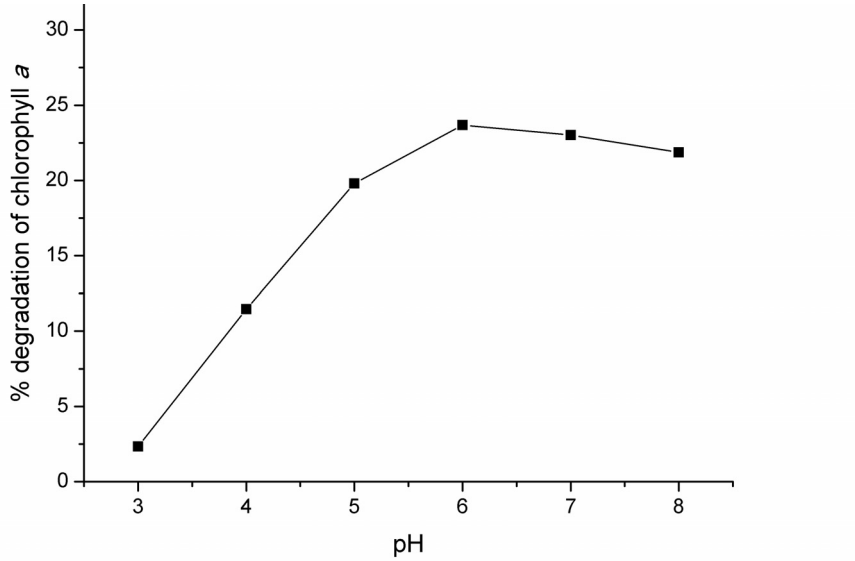

Figure 6. Effect of pH on degradation of Chl a *by HRP in the presence of resorcinol at 25 °C.*

and 6. The degradation rate of Chl a was determined using the absorption values from UV–Vis spectra at 662 nm.

The degradation of Chl *a* by HRP in the presence of resorcinol increases with increasing temperatures, but not in the linear manner in the whole range. An increase of Chl degradation by HRP in the presence of resorcinol was observed in 4–17 °C range, following by a strong rise in 17–20 °C range, a slight increase in 20––37 °C, and a slight decrease afterwards. The obtained results are in line with the results of degradation of Chl by HRP in the presence of *p*-coumaric acid, with optimal degradation temperature at 35 °C [11].

The results reflecting pH effects on degradation reaction of Chl *a* by HRP in the presence of resorcinol are shown on Figure 6. Two remarks might be drawn from it: a slight increase in pH range from 3 to 6, followed by a second region from pH 6–8 in which a slight decrease has been recorded. The achieved results are in line with the results of degradation of Chl *a* by HRP in the presence of *p*-coumaric acid with optimal pH value at 5.2 [11].

Having in mind the optimal temperature and pH ranges for HRP activity (30–40 °C and pH 6–8, depending on the medium), our results (of Chl degradation by HRP in the presence of resorcinol) look expected [26]. On the other side, the comparison with *Martinez et al*., (2001) results leads to similar conclusions [11]. It means that *in vitro* degradation of Chl *a* by HRP needs similar conditions irrespective of the selected phenolic com-

pound, because activity of HRP depends more on pH values and temperature and not on structure of these two phenolic compounds (resocinol and *p*-coumaric acid).

CONCLUSION

In conclusion, the main product of Chl *a* degradation by HRP, in the presence of resorcinol as phenolic compound, is Chl *a*-1 (t_{ret} = 12 min). Obtained results of temperature and pH influence on *in vitro* degradation of Chl *a*, have shown that the degradation increases with increasing temperature and has a maximum at 37 °C. On the other hand, Chl *a* degradation also increases with increasing of pH value and has a maximum at 6. It seems that *in vitro* degradation of Chl *a* by HRP needs similar conditions irrespective of the selected phenolic compound, because activity of HRP depends more on pH values and temperature and not on structure of these two phenolic compounds (resocinol and *p*-coumaric acid).

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IZVOD

IN VITRO ISPITIVANJE UTICAJA TEMPERATURE I pH VREDNOSTI NA DEGRADACIJU HLOROFILA POD DEJSTVOM PEROKSIDAZE IZ RENA: SPEKTROSKOPSKA I HPLC ISPITIVANJA

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(Naučni rad)

Hlorofili su najzastupljeniji biljni pigmenti i pripadaju grupi porfirina. Njihova stabilnost je uslovljena brojnim faktorima. Prisustvo svetlosti, toplote, kiseonika, slabih kiselina i enzima, dovodi do formiranja velikog broja degradacionih proizvoda. U ovom radu je ispitivana *in vitro* degradacija hlorofila *a* iz spanaća pomoću peroksidaze iz rena u prisustvu rezorcinola (kao fenolnog jedinjenja), kao i uticaj pH vrednosti i temperature. Za degradaciju hlorofila pomoću peroksidaze neophodno je da fenolno jedinjenje koje u svojoj strukturi ima hidroksilnu grupu u *p*-položaju. Degradacija hlorofila *a* je praćena pomoću UV-VIS spektrofotometra i HPLC sistema. HPLC analizom kao glavni degradacioni produkt identifikovan je 13^2 -hydroxychlorophyll *a*. Tokom reakcije koncentracija formiranog produkta degradacije se proporcionalno povećava na račun smanjenja koncentracije hlorofila *a* u smeši. Dobijeni rezultati ispitivanja uticaja temperature na degradaciju hlorofila pomoću peroksidaze iz rena, pokazuju da se sa povećanjem temperature povećava degradacija hlorofila *a*, i dostiže svoj maksimum na 37 °C. Sa druge strane, povećanje pH vrednosti takođe povećava degradaciju hlorofila *a,* sa maksimalnim stepenom degradacije pri pH 6. Dobijeni rezultati ispitivanja degradacije hlorofila *a* pokazuju da degradacija hlorofila u najvećoj meri zavisi od vrednosti pH i temperature, što ukazuje na činjenicu da je za degradaciju hlorofila najviše odgovorna peroksidaza, jer se sa povećanjem temperature i pH vrednosti istovremeno približavamo optimalnim uslovima koji su neophodni za dejstvo peroksidaze iz rena.

Ključne reči: Hlorofil • Peroksidaza iz rena • Rezorcinol • Temperatura • pH • HPLC

QUANTITATION OF ELLAGIC ACID IN BLACKBERRIES

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Abstract

The objective of this study was to evaluate the content of ellagic acid in blackberries, as well as in the juice of different blackberry cultivars obtained by using a specific technology. The analysis of the ellagic acid content in the test samples was performed using the high-pressure liquid chromatography with diode-array detection (HPLC-DAD). The results have shown considerable variations in ellagic acid content in the test blackberry fruit samples, the highest being determined in the fruit of the blackberry- raspberry hybrid cv. "Tayberry" (54.794 mg/100 g fresh weight), and the lowest in blackberry cv. "Čačanska Bestrna" (1.852 mg/100 g fresh weight). The ellagic acid content in the "Tayberry" juice produced using the specific technology was very high and almost identical to that in fruits.

Keywords: ellagic acid, bioavailability, human health.

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Over the last years, there has been an increasing awareness among nutritionists, doctors and most importantly consumers, related to the role of nutrition in improving human health and the quality of life [1]. Dark-coloured small fruits belonging to the families Ericaceae (blueberries) and Rosaceae (blackberries, raspberries and strawberries) are particularly known for their polyphenol content [2], with polyphenols making a significant contribution to the total antioxidant capacity of fruits [3,4]. A growing number of research papers and articles are being published in scientific journals dealing with this subject that show positive effects of plant-based foods on the prevention of different diseases [5–8].

Small fruits are singled out from among these foods due to their beneficial effect on human organism, with their high levels of ellagic acid playing a preventive role against malignant diseases [9–11]. Namely, ellagic acid has been proven to slow down or even prevent the division of malignant cells [12–14]. Moreover, ellagic acid exhibits a potent antioxidant activity, thereby preventing and controlling the spread of cancer [15–17]. The antioxidant properties of ellagic acid allow it to neutralize free radicals, chelate toxic metals and activate antioxidant enzymes, thus contributing to strengthening the body's antioxidant defence systems [18]. In addition, several other beneficial properties have been reported for ellagic acid in particular and phenolics in general, including anti-inflammatory, anti-microbial and anti-allergenic properties, as confirmed by many researchers [19–22].

Given the above facts, modern tendencies in fruit and vegetable production are oriented towards the production of functional foods that have special health effects, involving attempts to increase and preserve the ellagic acid content in fruits or edible parts of plants. Although the high content of ellagic acid in strawberry and raspberry cultivars is a long known fact [23,24], its content in different blackberry species and cultivars has not been sufficiently studied. Therefore, the objective of the present study was to evaluate the ellagic acid content in fruits of different blackberry (*Rubus caesius* L.) cultivars largely grown in the south-western part of Serbia. Another objective was to determine the ellagic acid content in the blackberry juice obtained by a specific technology, in order to determine the potential effect of the technology on the preservation of ellagic acid content in the produced juice. Namely, the ellagic acid as an extremely valuable substance is found mostly in the seed, and hardly a third of its juice content, on average, can be retained using conventional fruit processing methods [25]. The preservation of ellagic acid content in fruit juices is of utmost importance for human health, considering the highest consumer acceptance rate of juice as a processed fruit product that is available for consumption throughout the year.

MATERIAL AND METHODS

Chemicals

Methanol (HPLC, gradient grade), acetonitrile and formic acid (HPLC) were supplied by Merck KGaA (Darmstadt, Germany). The standard substance, includ-

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ing ellagic acid, was purchased from Sigma-Aldrich GmbH (Sternheim, Germany). Water used throughout the experiments was purified using a Millipore, Elix UV and Simplicity Water Purification System (Milford, MA, USA). Tert-buthylhydroquinone, HCl and ethanol were purchased from Centrohem (Centrohem, Stara Pazova, Serbia). All other chemicals and reagents were of analytical reagent grade.

Plant species

This study evaluates the content of ellagic acid in the following samples: fruit of the blackberry-raspberry hybrid cv. "Tayberry" (*Rubus fruticosus*×*Rubus idaeus*), fruit of blackberry cv. "Čačanska Bestrna" (*Rubus caesius* L., cv. Čačak Thornless) and fruit of wild grey–blue blackberries (*Rubus caesius* L.).

Preparation of materials

The mature fruits were harvested for analysis of ellagic acid content in the wider region of the Mt. Golija Nature Park (43° 20' N, 20° 17' E) in the south-western part of Serbia at the beginning of August, 2011. Immediately after harvest, the fruits were carefully placed into a portable refrigerator and transported to the laboratory, where they were stored at –20 °C until analysis.

The analysis of ellagic acid content in the blackberry samples was performed at the Laboratory of Chemistry, Fruit Research Institute, Čačak, in March 2012, using the high-pressure liquid chromatography with diode-array detection (HPLC-DAD). Apart from the fruit, ellagic acid content was also determined in the juice produced from hybrid cv. "Tayberry" fruits. The objective of this part of the study was to identify the effect of juice production technology on the preservation of ellagic acid in the juice. The used technology has involved cold pressing of blackberry fruits using a wooden press and juice straining through a 0.8 mm sieve. This process ensures retention of a large number of seeds in the juice, with seeds containing the highest percentage of ellagic acid. Furthermore, the juice is a completely natural product, as no preservatives or additives are added.

Sample preparation was performed as follows*:* blackberry samples were prepared for analysis of ellagic acid content according to the method of Hertog M.G.L., Hollman P.C.H., Katan M.B. [26]. The berry fruits were frozen in liquid nitrogen and homogenised by using a stainless steel blender. An aliquot of 15 g of ground fruit or juice was weighed into a 100-mL Erlenmeyer flask and diluted in 20 mL of 62.5% aqueous methanol containing 2 g/L of tert-buthylhydroquinone (TBHQ). Then, the mixture was ultrasonicated for 5 min, and 5 mL of 6 M HCl was added to the extract. Hydrolysis was carried out in a shaking water bath at 85 °C for 2 h. After hydrolysis, the sample was allowed to cool. Then, it was filtered, made up to 50 mL with methanol and ultrasonicated for 5 min. Before quantification by HPLC, the sample was filtered through a 0.45 μm membrane filter.

HPLC-DAD Analysis

Samples were analysed using an Agilent 1260 series HPLC (Agilent Technologies, Santa Clara, CA, USA) linked to a ChemStation data handling system, using a ZORBAX Eclipse Plus C18 column (4.6 mm×150 mm, 3.5 μm particles). Injection volume was 5 μl and the temperature was at 30 °C. Solvent A was 1% formic acid and solvent B was acetonitrile. The used gradient was as follows: 0–10 min, 10% of B in A; 10–25 min, 15–50% of B in A; 25–30 min, 50–80% of B in A; 30–32 min, 10% of B in A. The good purity and separation were achieved in raspberry samples using this gradient (flow rate 0.5 mL/min). The HPLC equipment was used with a diode array detector (DAD). Ultraviolet–visible spectra (ranging from 190 to 540 nm) were recorded for all peaks. Triplicate analyses were performed for each sample. Ellagic acid was detected at 260 nm, and identified according to peak retention time and UV/Vis spectra, which were compared with those of the standard. The quantities of ellagic acid were based on peak areas, and expressed as mg/100 g fw (or mg/100 g juice).

RESULTS AND DISCUSSION

Chromatograms for the test samples subjected to identification and quantification of ellagic acid are presented in Figure 1.

The ellagic acid content of the test samples is given in Table 1.

The results have shown considerable variations in ellagic acid content depending on the type of the sample. The highest content of ellagic acid was determined in hybrid cv. "Tayberry" fruit (54.794 mg/100 g fresh weight, on average), which was considerably higher than that in the fruit of other blackberry species and cultivars. As compared to the data on ellagic acid content in small fruits, the value determined in hybrid cv. "Tayberry" was extremely high. Hertog *et al*. [27] reported average values of ellagic acid content in strawberries and raspberries of only 5.52 mg/100 g and 40.06 mg/100 g fresh weight, respectively. Same authors also assessed the ellagic acid content in blackberry fruit (*Rubus caesius* L.) and found values of 37.60 mg /100 g fresh weight, as confirmed by the present results. The similar results on ellagic content in blackberries were reported by other authors 0.75 do 6.65 mg/100 g [28,29]. The lowest content of ellagic acid in this study was found in blackberry cv. "Čačanska Bestrna" – only 1.852 mg of ellagic acid per 100 g fresh weight. This was a rather unexpected result, parti-

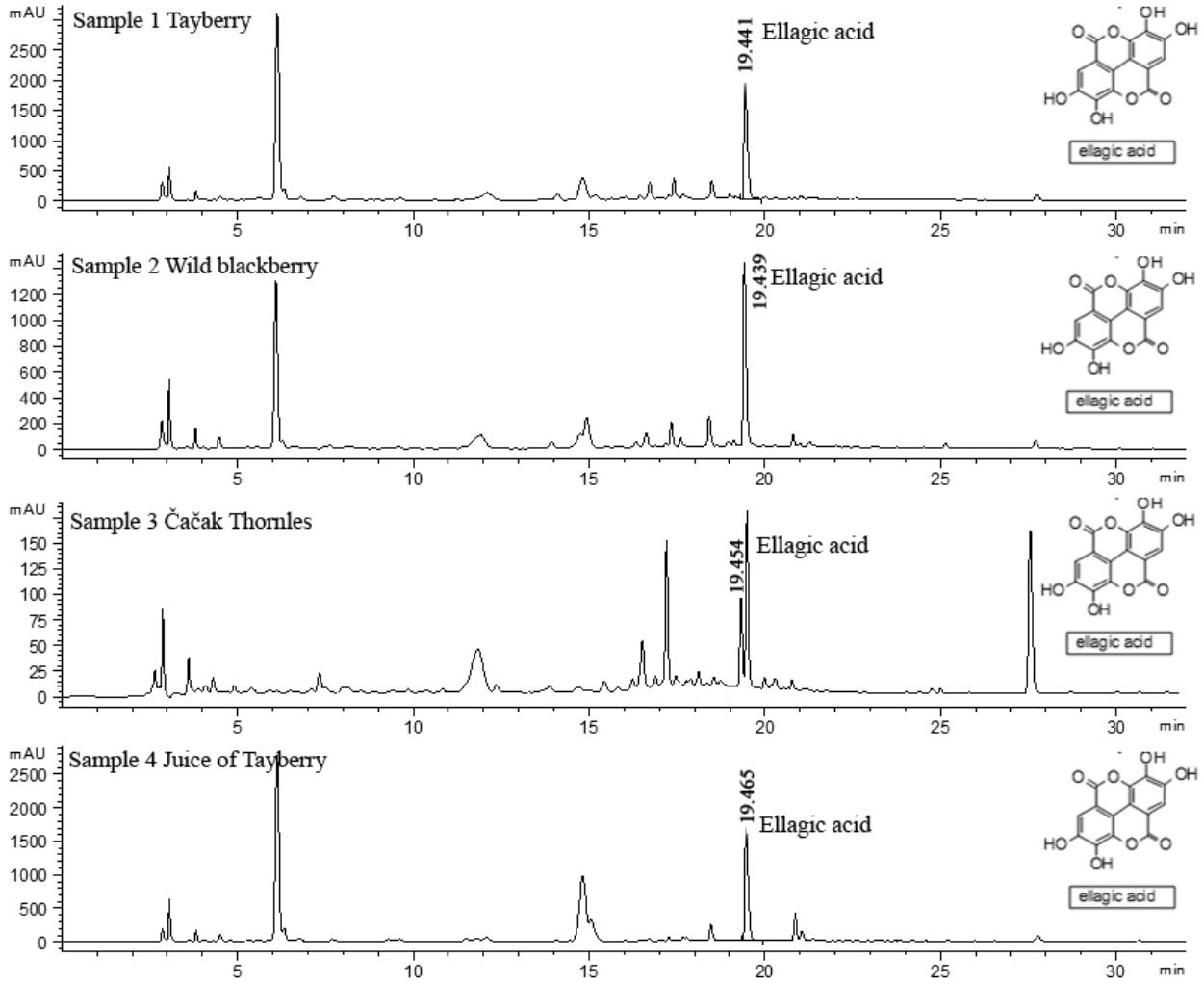

Figure 1. Chromatograms for the test samples using the high-pressure liquid chromatography with diode-array detection (HPLC-DAD).

Table 1. Ellagic acid content of the test samples

Sample	Ellagic acid content (mg/100 g fw)
Hybrid berry cultivar "Tayberry"	54.794±0.536
Rubus caesius L. (wild blackberry)	35.701±0.986
Blackberry cultivar "Čačanska Bestrna"	1.852±0.063
"Tayberry" juice	53.999±1.985 (mg/100 g juice)

cularly in view of the fact that the content of ellagic acid in grey-blue blackberries and hybrid cv. 'Tayberry' fruits was 20-fold and almost 30-fold higher, respectively. Such high differences in ellagic acid content between the samples speak in favour of the proposition that ellagic acid content shows substantial variation among different blackberry cultivars and small fruits in general, as confirmed by a number of studies [30,31]. In the research of authors Hakkinen *et al*. [30] determined values for the content of ellagic acid in fruits of different berries was in the range from 23.8 to 68.6 mg/100 g, and in the research of Vrhovsek *et al*. [31] the value for the same parameter ranged from 1.2 to 39.3 mg/100 g.

An important part of this research involved determination of ellagic acid content in the juice obtained from the fruit of the blackberry-raspberry hybrid cv. "Tayberry". The value identified in the juice slightly deviated from that of the fruit of this cultivar. This result is in disagreement with the literature data reporting a considerably lower content of ellagic acid in the juice than in the fruit used for juice production [25]. The successful preservation of the ellagic acid content in the juice obtained from the fruit of the blackberry–

–raspberry hybrid cv. "Tayberry" can be primarily attributed to the technology used for juice production, as an important factor in preserving valuable substances in fruits. The present findings also indicate an important role of blackberry juice production technology in preserving the valuable substances present in blackberries, particularly in view of the fact that storage of red raspberries and blueberries over a period of 9 months at –20 °C may result in a 30–40% reduction in ellagic acid content [30]. Therefore, the cold pressing procedure used in juice production is of extremely high importance since it significantly contributes to preserving the content of ellagic acid.

CONCLUSIONS

The results obtained in this study suggest considerable variations in ellagic acid content in blackberry fruit, depending on blackberry species and cultivars. A high fruit content of ellagic acid was found in blackberry–raspberry hybrid cv. "Tayberry", which should be taken into account when selecting, purchasing and processing small fruits. Both the juice and the juice production technology used in the research suggest a significant content of ellagic acid. The research has shown that ellagic acid content was highest in the seeds. The ellagic acid content of the juice produced from hybrid cv. "Tayberry" was high, almost identical to that of the fruits, suggesting the need for juice processing technology (involving homogenization of blackberry seeds) to take a targeted approach to the preservation of the nutritional quality of both natural ingredients and fruit flavour. In terms of human health improvement and commercially speaking, this finding is of high importance, considering the highest consumer acceptance rate of juice as a processed fruit product that is available for consumption throughout the year. Regular intake of blackberries and their juice has a preventative effect on human health, thereby considerably reducing the risk of different diseases.

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IZVOD

KVANTIFIKACIJA ELAGINSKE KISELINE U KUPINI

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(Naučni rad)

Rad je koncipiran sa ciljem da se ispita sadržaj elaginske kiseline u plodovima različitih sorti kupine, kao i u soku dobijenom iz plodova kupine primenom posebnog tehnološkog postupka, koji se ogledao u homogenizaciji semenki u plodu kupine. Istraživanja pokazuju da je sadržaj elaginske kiseline najveći u semenu. Sadržaj elaginske kiseline u ispitivanim uzorcima određen je kvantitativno primenom tečne hromatografije uz korišćenje DAD detektora (HPLC-DAD). Dobijeni rezultati su pokazali da najveću vrednost elaginske kiseline ima plod hibridnog kultivara kupine i maline „*Tayberry*“ i iznosi je 54,794 mg/100 g, a najmanju sorta kupine „Čačanska bestrna“, svega 1,852 mg/100 g suve materije. Sadržaj elaginske kiseline u soku dobijenom iz plodova hibridnog kultivara „*Tayberry*“ je bio visok, gotovo isti kao i u plodovima, što upućuje na zaključak da se kod tehnološkg postupka proizvodnje soka mora obratiti posebna pažnja u cilju očuvanja nutritivne vrednosti prirodnih sastojaka, kao i arome voća. Plodovi jagodastog voća, posebno maline i kupine, bogati su polifenolnim jedinjenjima koja bitno doprinose ukusu, aromi i obojenosti plodova. Istraživanja sve više ukazuju da elagina kiselina ima izraženo antimikrobno, antioksidativno, antikancerogeno dejstvo i konzumiranjem ovog voća bitno se doprinosi očuvanju zdravlja a samim tim i smanjuje se rizik od nastanka bolesti.

Ključne reči: Elagina kiselina • Biodostupnost • Zdravlje ljudi

Multielement determination using inductively coupled plasma optical emission spectrometry for metal characterization of water from artesian wells in Semberija region: Multivariate analysis of data

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Abstract

The concentrations of fifteen metals (Mg, Na, Ca, K, Se, Zn, Mn, Fe, Pb, Cr, Cu, Cd, Sb, Ni and Co) were determined in water taken from ten artesian wells (AW) in Semberija in order to obtain a general metal profile of water in this region. The principal components analysis (PCA) was used in this classification. Two factors controlling the metal variability were obtained by using principal component analysis, which accounted for nearly 71.5% of the total variance. Natural (lithogenic) factor is represented by PC1, while anthropogenic factor is represented by PC2. PC1 with high contribution of Mn, Mg, Na, K, Ca, Zn and Se accounting for 41.84% of the total variance, while PC2 exhibits high loading for Cd, Ni, Sb, Cr and Pb (29.66%). Three general areas (clusters) with different metal characteristics were detected. Water from artesian wells in first cluster (AW1–AW6) had much higher metal concentration compared with those in the second (AW7–AW9) and third cluster (AW10). That is as a result of anthropogenic inputs. Also, the analysis of water demonstrated slightly elevated values for Mn (concentrations up to 0.176 mg/L), while concentrations of the other investigated elements are below the values recommended by the World Health Organization (WHO) and the United States Environmental Protection Agency (US EPA).

Keywords: metals, artesian wells, ICP-OES, Semberija, principal component analysis.

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Several metal ions such as sodium, potassium, magnesium and calcium are essential to sustain biological life. At least six additional metals, chiefly transition metals, are also essential for optimal growth, development and reproduction, *i.e.*, manganese, iron, cobalt, copper, zinc and molybdenum. Heavy metals in the environment originate from two anthropogenic sources, one is connected with human activity and the other is in charge for the natural circulation of the metals throughout nature. The occurrence of heavy metal ions in water, soils and sediments can result in serious environmental and human health problems [1]. Heavy metals are dangerous because they tend to bioaccumulate. Several metal ions are very toxic even in low concentrations [2], while others are essential in low concentration, but hazardous in higher concentrations. Determination of metals in environment is important in the context of environmental pollution monitoring, intoxication, clinical diagnosis, etc.

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Well water is an important resource for the northern part of Bosnia and Herzegovina (B&H), the Semberija region. The whole region is abundant in ground water, stored in an alluvial aquifer. The aquifer has very good hydraulic characteristics, but is therefore quite sensitive with respect to water quality. There are several potential groundwater contaminants, both anthropogenic and natural. Some contamination sources include agriculture, livestock waste and faulty septic system. During the development of the sewerage system, the care was not taken to protect groundwater [3]. As a result, there are numerous sources of potential groundwater pollution. Mobile pollutants leaching to the groundwater are quickly transported by the considerable groundwater flow [4]. Domestic well water (100 to 300 m deep) supplies residents of this area, especially those who living in villages. But most of these privately owned domestic wells are not controlled by authorized institutions, which would confirm its chemical and microbiological quality. During decades before the war in B&H started (1992), systematic water quality testing and analysis were done. During the war, the monitoring system was destroyed and establishment of a new one needs appropriate human and financial resources. Therefore, the quality of potable water is still unsatisfactory in some parts of the country, especially in the

rural areas where much of the potable water is supplied from domestic wells [5].

The main approaches to ground water quality monitoring were identified as determination of physical, chemical and biological properties of ground water [6]. However, as far we know, there is no data on metal concentration in water from artesian wells. The study will attempt to identify any potential drinking water hazards. Well water quality is important because of possible health hazards that people may face when using well water for domestic purpose. Also, the second purpose of this work is to assess the pollution distribution in the study area, using pattern recognition technique of principal component analysis.

EXPERIMENTAL

Study area

Semberija is located between two rivers: Drina on the east and Sava on the north and Majevica mountain. Semberija lies on the part of the Pannonian basin. Many lakes and swamps were left after the regression of the Pannonian Sea at the end of Miocene. Alluvial deposits of gravel and sand (60–160 m), layers of clay and sands with intercalations of gravel were found in Semberija [3]. It has variable thickness and even disappearing in some zones. Thus, there are some sensitive areas where the groundwater is not protected naturally. The general slope of the aquifer is from west to east and from south to the north. The principal direction of groundwater flow is from the south to the north, towards the Sava River and parallel with the Drina. The Semberija alluvium aquifer is mainly recharged by the Drina River.

Ten sampling stations were selected along the Sava River in the north Semberija, near the mouth of the Drina River. The working area and sampling points are shown in Figure 1.

Water sampling and analysis

The series of investigation were conducted from February 2011 to January 2012. Artesian well waters were analyzed at depths from 100 to 200 m. Sampling periods occurred once a month, every 18th of the

Figure 1. A map of study area and location of artesian wells.

month. Water samples (3×2 L) were collected in clean PVC sample bottles filtered in a Whatman 0.45 μm membrane filter and acidified by the addition of 3 mL of conc. HNO_3 per liter of water. All samples were stored at 4 °C until analysis. The metal concentration in artesian well water samples were analyzed by iCAP 6000 inductively coupled plasma optical emission spectrometer (ICP-OES) (Thermo Scientific, Cambridge, UK). For ICP-OES measurements emission line was chosen based upon tables of known interferences and baseline shifts. The analytical lines used for each element, as well as the instrumental conditions, are given in Table 1.

Table 1. Operational parameters for ICP-OES measurements

Parameter	Value
Flush pump rate	100 rpm
Analysis pump rate	50 rpm
Rf power	1150 W
Nebulizer gas flow	0.7 L/min
Coolant gas flow	12 L/min
Auxiliary gas flow	0.5 L/min
Plasma view	Axial/radial
Metal	Detection wavelength, nm
Ca	422.673
Cd	228.802
Co	238.892
Cr	283.563
Cu	324.754
Fe	259.94
K	766.49
Mg	285.213
Mn	257.61
Na	589.592
Ni	221.647
Pb	220.353
Sb	252.85
Se	196.09
Zn	213.856

Reagent and standards

Ultra scientific (USA) ICP multi-element standard solution of about 20.00±0.10 mg/L was used. Sample bottles were treated with 5% nitric acid and washed with ultra-pure water 0.05 μS/cm (MicroMed high purity water system, TKA Wasseraufbereitungs system GmbH).

Statistical analysis

Data were reported as mean ± standard deviation (SD) for triplicate determinations. Principal component analysis (PCA) was performed using a statistical package running on a computer (Statistica 8.0, StatSoft, Inc., Tulsa, OK, USA). A probability of $p < 0.05$ was considered to be statistically significant [7].

RESULTS AND DISCUSSION

Fifteen elements were analyzed in water from ten artesian wells during one year. The results, expressed in milligram per liter (mg/L) or microgram per liter (μg/L) (Table 2), were obtained from triplicate measurements and rounded according to their standard deviation (*SD*).

According to World Health Organization [8], Ca, Na, K, Mg, Fe, Zn, Cu, Cr, Co and Se among tested elements are essential for human health. A second group of elements that have some beneficial health effects include Mn and Ni. The third group is composed of the potentially toxic elements Pb, Cd, Hg, As, Al, Li and Sn.

Based on mean values (Table 2), the metals follow the decreasing concentration order: Na > Ca > Mg > K > > Fe > Mn > Zn > Cu > Cr > Co > Ni > Sb > Pb > Cd > Se. Na, Ca, Mg and K, those are the elements with major content in all samples with average concentration of 169; 17.29; 5.44; 2.059 (AW1), 162; 16.67; 4.746; 1.112 (AW2), 164; 20.44; 5.38; 1.607 (AW3), 133; 14.05; 4.44; 0.985 (AW4), 153; 19.25; 4.84; 1.160 (AW5), 82.3; 24.48; 15.81; 1.31 (AW6), 103.6; 14.23; 10.81; 1.209 (AW7), 107.5; 19.33; 14.23; 1.467 (AW8), 52.6; 45.6; 21; 1.462 (AW9) and 63.1 mg/L; 48.5; 20; 1.597 mg/L (AW10), respectively. Sodium is widely distributed in drinking water. It has been detected in almost all evaluated surface and groundwater systems. The taste threshold concentration of Na in water depends on the associated anion and the temperature of the solution. At room temperature, the average taste threshold for Na is about 200 mg/L [9]. World Health Organization (WHO) [9] does not provide health-based guideline for the Na content in drinking water and the limit that has been set by the United States Environmental Protection Agency (US EPA) [10] is 20 mg/L. Taking into account the guidelines, concentrations of Na in all tested samples are below the recommended value. K occurs widely in the environment and is an essential element in humans [8]. Although K may cause some health effects in susceptible individuals, K intake from drinking-water is well below the level at which adverse health effects may occur. The origin of Ca and Mg in the water is related to their high prevalence in nature. WHO [9] and US EPA [10] guideline do not make recommendations regarding minimum concentrations of essential elements, including Ca and Mg, because of the uncertainties surrounding mineral nutrition from drinking-water. The increased presence of soluble salts of these two elements in water (100–300 mg/L) [9] can influence on taste threshold.

Table 2. Range and mean±SD of metal ions concentration in water from artesian wells

Sampling station	Code	Ca, mg/L	Cd, μg/L	Co, μg/L	Cr, μg/L	Cu, μg/L	Fe, mg/L	K, mg/L
Velino selo	AW1	15.73–19.21	0.026–0.768	0.47–3.59	0.513–0.568	0.23–3.36	0.021–0.094	1.637–2.555
		17.29±0.05	0.089±0.001	0.82±0.04	0.709±0.003	1.37±0.04	0.041±0.007	2.059±0.006
Donji Brodac	AW2	15.18–18.07	0.025–0.068	0.46–4.65	0.512–1.972	0.22–1.27	0.018–0.067	0.873–1.714
		16.67±0.07	0.082±0.002	0.96±0.03	0.684±0.004	0.65±0.03	0.029±0.003	1.112±0.004
Gornji Brodac	AW3	17.35–22.61	0.024–0.338	0.469–0.56	0.514–0.539	0.32–3.39	0.017–0.080	1.389–2.052
		20.44±0.08	0.053±0.001	0.57±0.01	0.619±0.002	1.91±0.04	0.036±0.002	1.607±0.007
Begov put	AW4	11.07–15.87	0.023–0.028	0.459–0.492	0.509–0.544	0.29–4.07	0.021–0.125	0.651–1.320
		14.05±0.04	0.043±0.002	0.509±0.008	0.527±0.003	1.05±0.04	0.047±0.003	0.985±0.002
Ostojićevo	AW5	17.34–23.18	0.022–0.198	0.459–1.122	0.512–0.542	0.29–2.47	0.01–0.001	1.828–1.643
		19.25±0.05	0.114±0.002	0.507±0.004	0.528±0.003	1.05±0.04	0.026±0.003	1.160±0.003
Gornje Crnjelovo	AW6	20.53–28.07	0.022–0.159	0.462–0.831	0.522–6.831	0.18–3.17	0.014–0.029	1.196–1.654
		24.48±0.08	0.037±0.002	0.520±0.008	1.056±0.003	2.06±0.03	0.024±0.002	1.31±0.01
Subotište	AW7	10.95–17.62	0.019–0.028	0.459–2.171	0.514–1.966	0.18–2.69	0.022–0.042	0.807–1.592
		14.23±0.04	0.025±0.003	0.747±0.008	0.724±0.004	1.25±0.07	0.028±0.003	1.209±0.007
Burum	AW8	15.74–26.13	0.021–0.028	0.466–0.711	0.516–1.600	0.18–4.06	0.023–0.099	0.117–1.883
		19.33±0.07	0.025±0.002	0.508±0.006	0.664±0.005	1.68±0.09	0.047±0.002	1.467±0.003
Vršani	AW9	38.3–53.5	0.022–0.027	0.456–1.941	0.521–1.724	0.19–2.66	0.022–0.122	0.978–0.843
		45.6±0.2	0.024±0.003	0.508±0.006	0.799±0.006	1.03±0.03	0.057±0.004	1.462±0.004
Čađavica	AW10	41.24–52.79	0.023–0.027	0.48–1.94	0.509–2.54	0.23–4.99	0.026–0.162	1.038–2.059
		48.5±0.3	0.025±0.003	0.71±0.02	0.687±0.006	1.62±0.05	0.051±0.002	1.597±0.006

Sampling station	Code	Mg, mg/L	Mn, mg/L	Na, mg/L	Ni, μg/L	Pb, μg/L	Sb, μg/L	Se, μg/L	Zn, mg/L
Velino selo	AW1	5.15–5.71	0.007–0.009	144.9–200.5	0.07–7.35	0.025–0.113	0.18–0.39	0.041–0.064	0.007–0.021
		5.44±0.03	0.008±0.001	169±1	1.76±0.06	0.043±0.002	0.29±0.05	0.051±0.002	0.013±0.001
Donji Brodac	AW2	4.194–5.120	0.006–0.0098	129.8–181.6	0.05–0.801	0.018–0.052	0.09–0.25	0.023–0.033	0.019–0.03
		4.746±0.002	0.008±0.001	162±1	0.178±0.009	0.040 ± 0.001	0.18±0.01	0.028±0.001	0.023±0.001
Gornji Brodac	AW3	4.86–5.98	0.007–0.023	142.3–179.4	0.07–0.97	0.015–0.037	0.23–0.48	0.035–0.053	0.016–0.04
		5.38±0.02	0.014±0.001	164±1	0.24±0.01	0.025±0.001	0.37±0.01	0.041±0.002	0.031±0.001
Begov put	AW4	4.21–4.73	0.0155–0.0246	103.8–179.4	0.08–3.38	0.032–0.128	0.21–0.36	0.016–0.033	0.008–0.022
		4.44±0.03	0.0207±0.0006	133±1	0.81±0.03	0.049±0.001	0.031±0.02	0.022±0.001	0.013±0.001
Ostojićevo	AW5	4.51–5.32	0.007–0.014	126.3–195.6	0.09–1.87	0.011–0.043	0.35–0.43	0.019–0.041	0.02–0.029
		4.84±0.02	0.0098±0.0001	153±1	0.25±0.01	0.022±0.001	0.39±0.04	0.029±0.001	0.023±0.001
Gornje Crnjelovo	AW6	14.92–17.21	0.064–0.102	68.3–115.4	0.06–1.7	0.039–0.259	0.31–0.52	0.028–0.045	0.006–0.012
		15.81±0.03	0.073±0.001	82.3±0.6	0.49±0.03	0.081±0.006	0.043±0.01	0.033±0.002	0.008±0.001
Subotište	AW7	10.29–11.08	0.02–0.04	89.5–124.9	0.09–0.68	0.023–0.216	0.26–0.42	0.028–0.041	0.008–0.013
		10.81±0.03	0.027±0.001	103.6±0.7	0.21±0.02	0.053±0.001	0.31±0.01	0.031±0.001	0.009±0.001
Burum	AW8	12.93–19.92	0.067–0.111	72.7–182.2	0.08–1.29	0.035–0.101	0.15–0.47	0.028–0.044	0.042–0.056
		14.23±0.07	0.083±0.001	107.5±0.9	0.39±0.01	0.05±0.01	0.36±0.02	0.037±0.001	0.048±0.001
Vršani	AW9	18.8–23.8	0.093–0.164	36.5–81.2	0.06–0.55	0.044–0.231	0.11+0.29	0.029–0.046	0.007–0.015
		21±1	0.116±0.002	52.6±0.5	0.26±0.01	0.062±0.001	0.19±0.02	0.036±0.001	0.011±0.001
Čađavica	AW10	18.5–20.9	0.129–0.221	43.6–99.8	0.08–4.33	0.041–1.737	0.38–0.45	0.026–0.051	0.211–0.535
		20±1	0.176±0.001	63.1±0.5	0.72±0.03	0.21±0.03	0.41±0.01	0.040±0.002	0.276±0.002

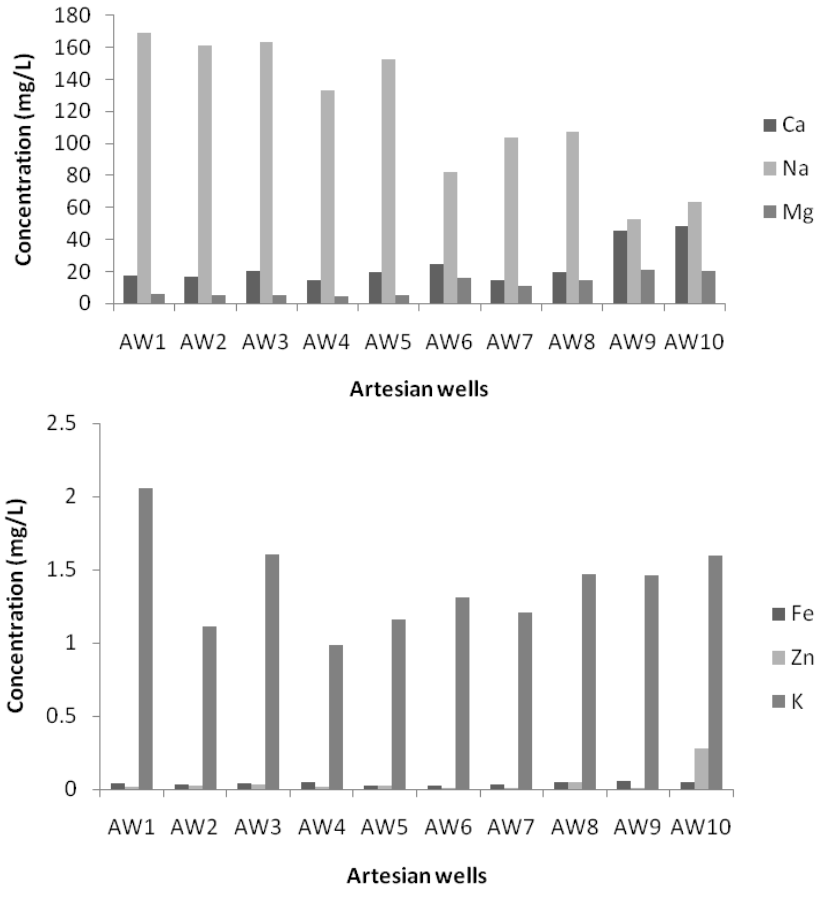
Trace elements, essential for human life, such as Fe, Mn Zn, Cu and Co in higher concentrations can have a negative impact on human health [11]. Based on the results given in Table 2, it can be concluded that these elements are present in the water samples at concentrations lower than the values recommended by the WHO [9] and US EPA [10]: Cu 1.0 and 2.0 mg/L, Fe 0.3 mg/L [10], Zn 5.0 and <3.0 mg/L, respectively. Only concentrations of Mn in water from artesian wells: AW6 (0.073 mg/L), AW8 (0.083 mg/L), AW9 (0.116 mg/L) and AW10 (0.176 mg/L) are near or above of the recommended standard of 0.05 mg/L according to US

EPA [10] and <0.1 mg/L according to WHO [9]. Mn is naturally occurring in many surface water and groundwater sources. Also, several studies suggest a mainly non-anthropogenic source of Mn, which further supports the conclusion that it has mainly natural source [12,13]. There have been several epidemiological studies that report adverse neurological effects following extended exposure to very high levels in drinking-water [14–16]. Cobalt has both beneficial and harmful effects on human health. Cobalt is beneficial for humans because it is part of vitamin B12, which is essential to maintain human health. Small amounts of cobalt are naturally found in most rocks, soil, water, plants and animals, typically in small amounts. This element does not exist in its native form and is most often found in the form of arsenides and sulphides. Generally, cobalt compounds that dissolve easily in water are more harmful than those that are hard to dissolve in water [17]. Because of the low concentration of cobalt in water in relation to potential toxicity, there is no maximum permissible concentration in drinking-water given by WHO and US EPA.

Selenium is well known as an essential element for biological systems, both as a nutrient and as a potential toxicant, the difference between the necessary daily intake and the toxic value is small [18]. Also, selenium can be present as impurities in gravel, sands, and other water contact materials. The data on exposure of the population to selenium in drinking water indicate that selenium in drinking water does not make a significant contribution to total selenium intake for most of the population [8]. WHO [9] and US EPA [10] have established recommended standard for drinking water for selenium of 0.04 and 0.05 mg/L, respectively. The water samples are found to have a lower level for Se than the recommended values.

Toxic metals like Pb, Cr, Sb, Ni and Cd have been associated with various forms of cancer, nephrotoxicity, central nervous system effects and cardiovascular disease in humans. Human activities have substantially altered the natural distribution of these metals in the environment, leading to potentially elevated concentrations of these metals in many environmental media [19]. WHO [9] and US EPA [10] guideline values are: for Pb 0.01 and 0.015 mg/L, for Cr 0.05 and 0.01 mg/L, for Sb 0.02 and 0.006 mg/L, for Ni 0.07 (WHO) and for Cd 0.003 and 0.005 mg/L, respectively. Obtained results (Table 2) showed that the concentrations of Pb, Cr, Ni, Sb and Cd in water samples are lower than those recommended by the WHO and US EPA.

Compared metal concentrations in water from different artesian wells, metal levels in water samples from AW1– AW6 are higher than those in water samples from AW7–AW10 (Figure 2).



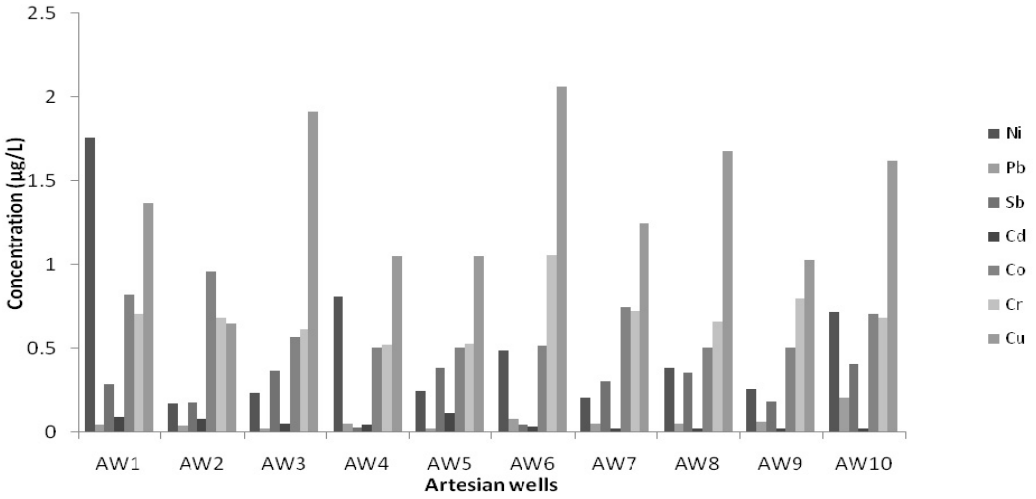


Figure 2. Comparison of metal levels in water from different artesian wells.

Principal component analysis (PCA) and cluster analysis (CA) are the most common multivariate statistical methods used in environmental studies [20,21]. PCA was applied to assist in the identification of source of pollutants. The PCA results are graphically displayed using loading plot (Figure 3).

It shows the correlations between variables (metals). Each point, whose coordinates are the loadings on the principal components, corresponds to a variable. High level of a variable affects most the principal component on which this variable has a high loading. The variables that have small factor loadings in any principal component have little influence. Table 3 displays the factor loadings as well as eigenvalues.

Factor loadings >0.71 are typically regarded as excellent and <0.32 very poor [22]. In this study, all principal factors extracted from the variables were retained with eigenvalues >1.0, as suggested by the Kaiser criterion [23]. Two factors were obtained. The first factor accounts for 41.84% of the total variability

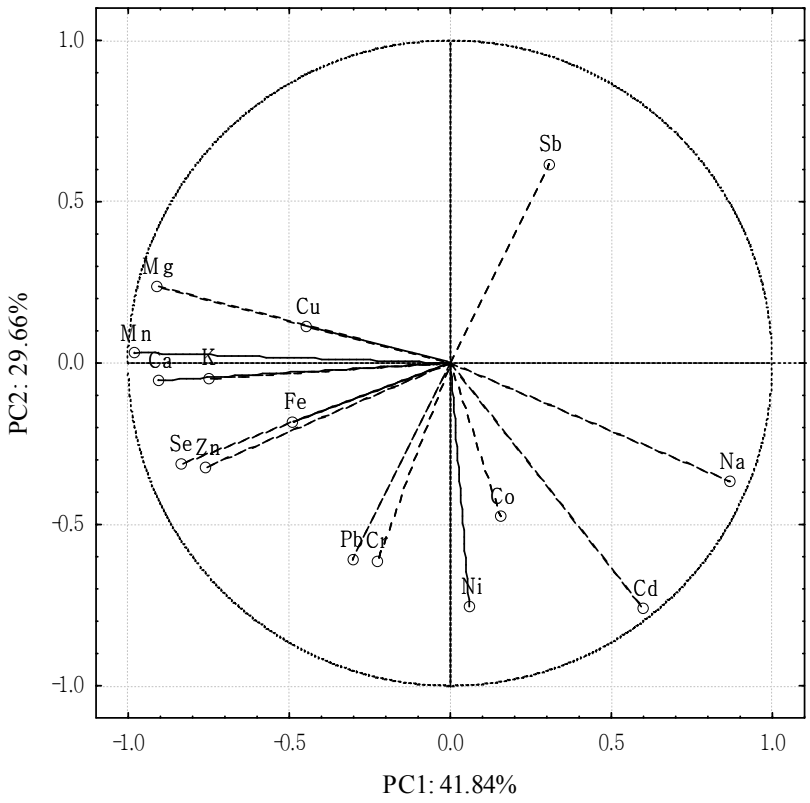


Figure 3. Principal component score plot (PC1 and PC2) of the studied metals' contents based on ICP-OES measurement.

and the second one represents 29.66% of the total variance. PC1 and PC2 explain the largest possible variation in the data and therefore account for most of the information (71.5%). Factor 1 is dominated by Mn, Mg, Na, Ca, K, Zn, Se, Fe and Cu, while Na is negatively correlated with other elements. In this case, factor loadings of Cu (0.447) and Fe (0.448) are not as high as the loadings of the other elements of the group which may suggest a different origin from the other elements. According to International Sava River Basin Commission [13] occurrence of Fe and Mn in alluvium is natural. Moreover, Mn, Mg and Ca are correlated to each other because they have similar factor loadings, as well as Zn and Se. PC1 with high contribution of Mn, Mg, Na, Ca, K, Zn and Se could be considered to be dominated by the natural factor of the lithogenic process. Factor 2 is dominated by Cd, Ni, Sb, Cr, Pb and Co. Co (0.475) is with the smallest factor loading referring to the other elements suggesting another source. A strong correlation between Cd and Ni could be an indicator of the common source or sources of the two elements. Cr and Pb are also correlated, suggesting another common source or sources. PC2 loaded by Cd, Ni, Sb, Cr and Pb indicate the likely influence from anthropogenic factors.

When the location relates to the concentrations of metals found in water from artesian wells, three clearly defined groups can be observed (Figure 4).

Table 3. Principal component analysis (PCA loadings > 0.4 are shown in bold)

Element	Component	
	1	2
Mn	**0.982**	–0.03
Mg	**0.913**	–0.236
Ca	**0.908**	0.053
Na	**–0.869**	0.367
K	**0.836**	0.312
Zn	**0.758**	0.325
Se	**0.748**	0.046
Fe	**0.488**	0.181
Cu	**0.447**	–0.114
Cd	–0.601	**0.761**
Ni	–0.059	**0.755**
Sb	–0.306	**–0.617**
Cr	0.228	**0.612**
Pb	0.303	**0.611**
Co	–0.157	**0.475**
Percent of variance	41.843	29.656
Cumulative percent	41.843	71.499

The first group including artesian wells AW1, AW2, AW3, AW4, AW5 and AW6 are located in the positive quadrant. The mean values of the metals in water from

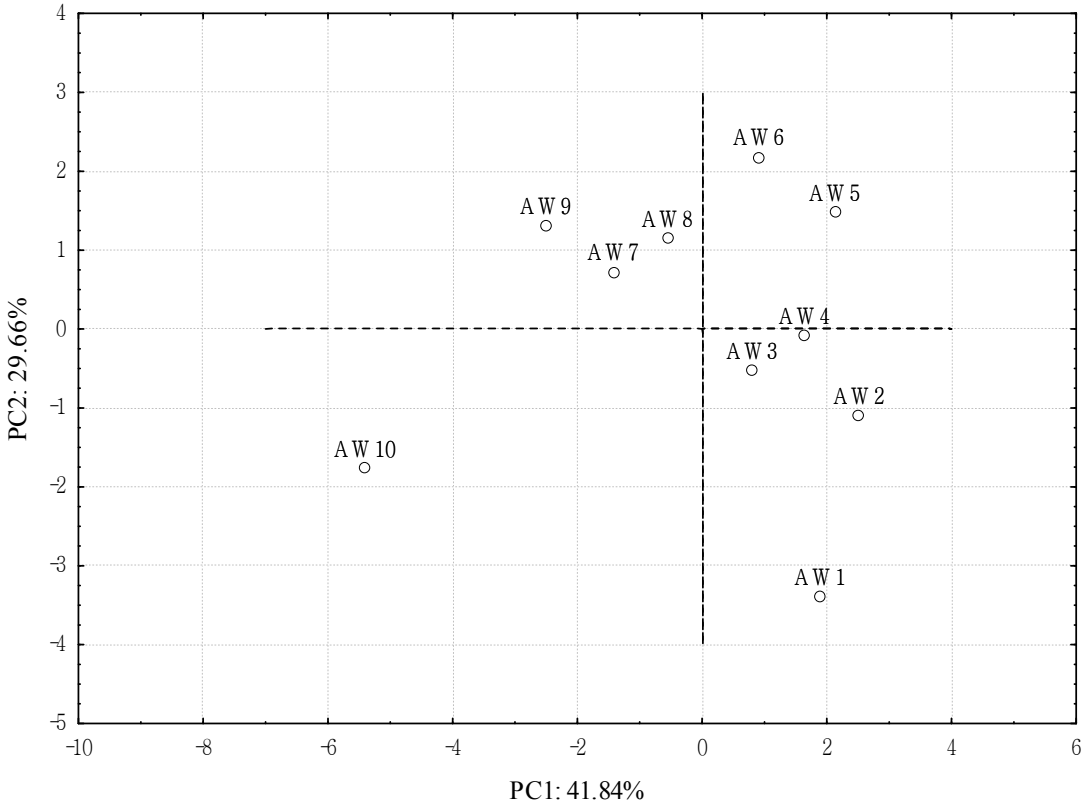


Figure 4. Principal component score plot (PC1 and PC2) of the studied water samples from different artesian wells based on ICP-OES data for the metals contents.

these artesian wells were greater than those in the second and third group. Artesian wells, AW7, AW8 and AW9, in the second group are located in the second quadrant with negative contribution to PC1 and positive contribution to PC2. Artesian well, AW10, from third group is located in the third quadrant with negative contribution to PC1 and PC2.

It is important to note that artesian wells AW1––AW6 are located in region with a high density of traffic and human activities. High mean values of Pb, Cd and Cr in water are related to heavy traffic on the border between Bosnia and Herzegovina and Serbia. Atmospheric deposition of heavy metals is considered to be a significant factor in soil and water pollution [24,25]. This region is also a part of Drina River Basin that there are certain environmental "hot spots" which are potential threat to the environment (chemical factory in Goražde, aluminium factory in Zvornik and Glinica, viscose factory in Loznica, etc.). The Drina River Basin is well known for its significant coal, bauxite and iron resources, the exploitation which has multiple negative impacts on soil and water quality (through contamination of groundwater and as a result of flooding). Other potential sources of pollution include agricultural activities and solid waste disposal [26].

On the other hand, artesian wells, AW7–AW9 are located in the region of the Sava River basin. The main causes of groundwater pollution in the Sava River Basin are intensive agriculture, insufficient wastewater collection and treatment on municipal level, inappropriate waste disposal sites, urban land use and mining activities [13]. Also, the chemical status of surface and underground water can be affected by sediment quality. The quality of sediments in the Sava River Basin has been estimated at the national and international level [27]. The findings of the study based on an analysis of sediments sampled at 20 locations along the Sava River indicated a moderate elevation of Hg levels in sediments (up to 0.6 mg/kg) and Cr and Ni (up to 400 and 210 mg/kg, respectively) in industrially impacted sites. Contamination of Sava sediments by Pb, Zn, Cu, Cd and As was not significant. Obtained data also indicate that the concentrations of elements in sediments of the Sava River gradually increase from the Sava River spring to its outflow to the Danube River. These results are in accordance with Cr, Ni, Pb and Cd concentrations in water from artesian wells located near the Sava River. The water from artesian wells, regarding Cr, Ni, Pb and Cd concentrations, follows the increasing order: AW8 < AW7 < AW4. Higher concentrations of Cr, Cd and Ni at these sampling sites indicate heavy industry and chemical industry activities along the Sava River. Higher concentrations of Pb in water are related to heavy border traffic [27].

In general, influences between air, soil and water pollution are mutual. Just as the atmosphere can transfer a large amount of heavy metals into soil and water [28,29], soil dust can also contribute to the concentrations of heavy metals in the water and air [30].

CONCUSION

The investigation of water from artesian wells in ten sample sites in Semberija region during one year showed that the concentrations of the investigated metals are lower than those recommended by the WHO and US EPA. Mn concentrations were exceeded WHO and US EPA quality guideline values. Higher Mn concentrations are attributed to a main origin in soil. Obtained results also indicate that metal levels in water samples from artesian wells: AW1–AW6 are higher than those in water samples from AW7–AW10. Furthermore, data indicate that, in general, the concentrations of Cr, Ni, Pb and Cd in water from artesian wells along the Sava River (AW8, AW7 and AW4) gradually increase from the Sava River spring to its outflow to the Danube River. This is in accordance with data obtained from the analysis of sediments sampled at 20 locations along the Sava River. PCA was used to identify the sources of metals and classified sampling sites according to metal concentrations. According to the results of PCA, the original variables could be reduced to two factors, which accounted for 71.5% of the total variance. Natural factor controls PC1 of water samples while anthropogenic factor is represented by PC2. Mn, Fe and Co are attributed to natural origin in soil. Cr, Ni, Pb and Cd originate mainly from industrial sources as well as from traffic sources. Beside metal concentrations all artesian wells were classified into three groups: the first group including artesian wells AW1–AW6, second including AW7–AW9 and the third group including AW10. Metal concentration is higher in the first group, followed by the second and the third group. These findings indicate that more attention should be paid to the application of systematic measures for improving the quality of groundwater in this area that is susceptible to pollution.

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ИЗВОД

МУЛТИЕЛЕМЕНТНО ОДРЕЂИВАЊЕ МЕТАЛА КОРИШЋЕЊЕМ ОПТИЧКЕ ЕМИСИОНЕ СПЕКТРОМЕТРИЈЕ СА ИНДУКОВАНО КУПЛОВАНОМ ПЛАЗМОМ У ЦИЉУ КАРАКТЕРИЗАЦИЈЕ ВОДЕ ИЗ АРТЕШКИХ БУНАРА СЕМБЕРИЈЕ: МУЛТИВАРИЈАНТНА АНАЛИЗА ПОДАТАКА

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(Научни рад)

У раду је одређен садржај петнаест метала (Mg, Na, Ca, K, Se, Zn, Mn, Fe, Pb, Cr, Cu, Cd, Sb, Ni и Co) у води из артешких бунара (АБ) на територији Семберије у циљу праћења квалитета подземних вода. Метода главних компоненти (PCA) је коришћена циљу класификације испитиваних узорака у погледу садржаја метала. Методом главних компоненти екстрахована су два фактора који заједно објашњавају 71,5% укупне дисперзије посматраних мерења. Природни (литогени) фактор представља PC1, док антропогени фактор представља PC2. Први фактор објашњавају Mn, Mg, Na, K, Ca, Zn и Se, што представља 41,84% дисперзије посматраних мерења, док други фактор карактерише висока засићеност за Cd, Ni, Sb, Cr и Pb (29,66%). Појава ових метала у води је резултат индустријског загађења и повећаног обима саобраћаја у близини испитиваних локалитета. На основу кластер анализе добијена су три јасно одвојена кластера. Први кластер чине артешки бунари (АВ1––АВ6) који се одликују већом концентрацијом метала у поређењу са онима у другом (АВ7–АВ9) и трећем кластеру (АВ10). Повећане концентрације су резултат антропогеног фактора. Такође, анализа воде је показала благо повишене концентрације за Mn (концентрација до 0,176 mg/L), док су концентрације других испитиваних елемената испод вредности које препоручују Светска здравствена организација (WHO) и Агенција за заштиту околине Сједињених Америчких Држава (US EPA). Добијени резултати указују на то да треба више пажње посветити примени системских мера у циљу побољшања квалитета подземних вода у овој области која је подложна загађењу.

Кључне речи: Метали • ICP-OES • Артешки бунари • Семберија • Анализа главних компоненти

Discrimination of mineral waters using near-infrared spectroscopy and aquaphotomics

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Abstract

Despite that water is one of the most studied materials today its dynamic properties are still not well understood. Water state in human organism is of high importance for normal healthy functioning of human body. Different kinds of water are usually classified according to their present solutes and concentrations of these solutes, but though it is known that water molecules can form clusters around present solutes, the classification of waters based on types of water molecular organization and present clusters is not present in current literature. In this study the multivariate analysis is used for classification of commercial mineral waters based on their near-infrared spectra (NIR). Further, the aquaphotomics has been applied, a new approach for interpretation of near-infrared spectra of water, that gives insight into organization of water molecules in each of these waters.

Keywords: water, discrimination, near infrared spectroscopy, aquaphotomics, multivariate analysis.

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Water is maybe one of the most studied materials today; it has been studied with different tools and methods [1–7], but its behaviour is still the subject of intensive scientific research. On nano- and microlevels, like in biological cellular and extracellular spaces, water is not a homogeneous structure, but rather dynamic equilibrium among changing percentages of assemblages of different oligomers and polymer species [8]. The structure and these assemblages or units themselves are dependent on its chemical contents, temperature and pressure [8,9].

Spring water is consumed as a drink beneficial for human health and is used for therapeutic purpose. Despite mineral water being associated with safer and healthier drink than tap water, the quality and composition of mineral water vary with its origin and require careful monitoring. When it comes to quality of drinking waters, it is usually considered in the terms of the concentrations of different solutes, cations and anions, the ratio of certain ions, etc., with regard to human organism functioning. But little is said about the structure of the water itself, even though with approximately 65–70%, water is the most abundant chemical in the human body and it plays a central role in the regulation of cell volume, nutrient transport, waste removal and thermal regulation [10].

Aquaphotomics [11] is a term, recently proposed to describe the concept in which water as multi-element system could be well described by its multi-dimensional spectra. Aquaphotomics is based on visible-near infrared spectroscopy (Vis-NIRS) and multivariate analysis. It discovers new properties of water hydrogen bonds in different aqueous systems under various perturbations.

Based on years of experience in vis-NIR spectroscopy research of water and different aqueous systems under various perturbations, 12 characteristic wavelength ranges (6–20 nm width each) have been identified in the area of the first overtone of the water NIR spectra, where despite the type of perturbation the observed systems showed predictable spectral variations. To present these changes of water absorbance pattern a star chart named “Aquagram” is used [6]. Aquagram displays normalized absorbance values at several water bands on the axis originating from the center of the graph. Absorbance values at specific water absorbance bands were placed on the respective radial axes. Aquagrams were used in studying aqueous and biological systems to obtain more information on the matter (ions, molecules, etc.) present in the water. In this approach, water is used as a “mirror”, which can reflect functionality of the present structures [12].

Aquaphotomics has been successfully applied in various fields from water characterization, food quality control to early diagnosis of disease [11–17], but very low number of publications exists on the subject of using NIR spectra of waters for its discrimination [1].

A quite number of papers are published on the subject of different chemical composition of water and the respective bio-functionality from the aspect of the concentration of the present ions, but there is virtually none about the bio-functionality of the water struc-

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tured around the present solutes. Thus, the aim of this study was to determine the structural organization of the various mineral and tap waters regularly used in human diet. In this study, several commercial mineral waters and tap waters were investigated by the means of NIR spectroscopy with novel aquaphotomics approach [11]. Potential of multivariate analysis applied on the NIR spectra of waters and Aquaphotomics in discrimination of different types of mineral waters which are commercially available was also assessed. The purpose of applying aquaphotomics was to look for unique aquaphotomic fingerprint for each of these waters which can subsequently be used as a distinctive criterion for their identification.

MATERIAL AND METHODS

Materials

The pure water samples were investigated (Aqua purificata sterilisata, Pharma product, Serbia) and seven commercial mineral waters available on the market, which were purchased in the local shop and stored in dark ambient before analysis. Concentration of some typical cations and anions for analysed waters are presented in Table 1. The sources of information on physicochemical properties presented in this table were labels on the bottles, as well as the information from the manufacturers' websites [18–21]. For waters Aqua viva, Aqua Una and Belgrade tap water, the analysis of physicochemical properties was performed by the Knjaz Miloš laboratory and published in [22]. All commercial mineral waters were non-carbonated.

Methods

NIR Spectra of waters were acquired in transmittance mode, using mini-spectrometer Hamamatsu (TG – Cooled NIR-IC9913GC, Hamamatsu, Japan) in the range from 900 to 1700 nm. A quartz liquid sample cell was used as a container. Order of recorded spectra for water samples was random, and for each water sample 10 consecutive spectra were recorded. For each water sample, at least 10 spectra were acquired, with a total of 80 spectra. Only the region of the first overtone of water (1300–1600nm) was used in further analysis. The temperature in laboratory was 24.4 °C, and humidity was 61%.

Multivariate analysis

All multivariate analysis was carried out by Pirouette ver. 4.0 (Infometrics, USA) software. Multivariate data analysis in the form of Hierarchical Cluster Analysis (HCA) and Soft Modelling of Class Analogies (SIMCA) was applied.

Hierarchical Cluster Analysis (HCA) is unsupervised pattern recognition method. It calculates the distances (or correlation) between all samples using a defined metric such as Euclidean distance. The most similar objects are first grouped [23]. Euclidean distance was here used as a criterion. The results are presented in a form of dendogram.

Soft Independent Modelling of Class Analogies (SIMCA) employs principal components analysis of spectra for the construction of mathematical models for each class to be analysed. This analysis is a supervised method for sample classification which consists of assigning training sets to classes and then a principal component model is formed for each class with different confidence regions. Interclass distances are calculated using between class residuals and variable importance is determined by comparing average residual variance of each class to all classes and residual variance of all classes to themselves. Variable importance, known as discriminating power can be used to define variables with predominant effect on the samples classification [24]. The models were validated using cross-validation (leave-ten-out).

Aquaphotomics

Aquaphotomics is based on visible-near infrared spectroscopy (Vis-NIR) and it uses a part of the water NIR region from 1300 to 1600 nm. The WAMACS coordinates [11] are the 12 wavelength ranges found in this part of the water spectrum, for which specific water vibrations were assigned [11]. The acquired spectra of all waters were normalized, the pure water spectrum was subtracted and the values of the normalized absorbance in 12 WAMACS coordinates are extracted and presented in aquagram.

Table 1. Concentration of some typical cations and anions in investigated waters (mg/L)

Water	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	Reference
Prolom	43,9	0.5	1	1	5	16	[18]
Gala	18.15	1.65	64	29	0.8	14	[19]
Rosa	2.7	1	10.4	0.9	2	6.4	[20]
Zlatibor	4.51	0.9	62.7	30.5	1.4	17.6	[21]
Beograd tap	6.5	1.1	61.1	11.1	11.3	37.5	[22]
Aqua viva	13.7	2.1	90.1	13.3	20.6	28.1	[22]
Aqua una	8.8	1	88.2	19.5	2.8	9.9	[22]

Aquagram displays normalized absorbance values at several water bands on the axis originating from the center of the graph. Absorbance values at specific water absorbance bands were placed on the respective radial axes.

RESULTS AND DISCUSSION

Figure 1 presents results of hierarchical cluster analysis applied on the NIR spectra of analysed waters. The results show grouping of waters in three categories of similar waters. First category is comprised of Aqua Una, Rosa and Zlatibor, the second is comprised of Aqua Viva water samples taken from different bottles (volume 0.5 and 0.33 L) and Prolom, and the third is comprised of Aqua Purificata, Belgrade tap water and Gala.

Figure 2 presents results of the hierarchical cluster analysis applied on the chemical contents data (from Table 1). The clusters of similar waters presented in dendogram in Figure 2, are clearly different from the clusters identified and presented in dendogram in Figure 1.

This clearly illustrates that it is not only the content of different elements what makes one water unique and distinctive comparing to other waters (or on the other way similar), but is the organization of water molecules around this molecules and ions, which is considered when the analysis is applied on the NIR spectra. The spectrum which shows the information on

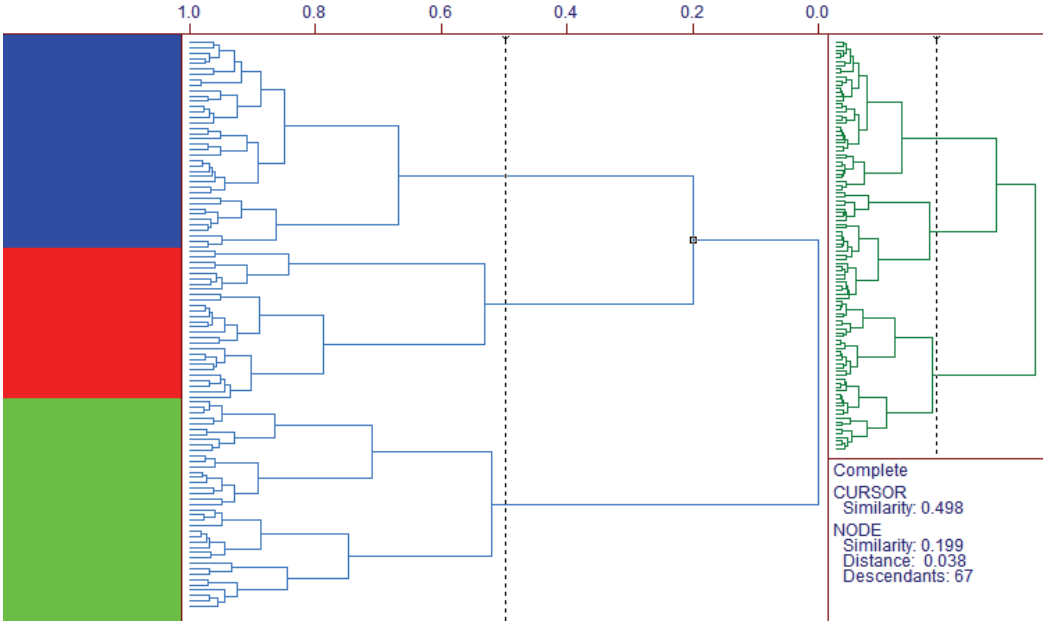

Figure 1. Dendogram showing clustering of NIR spectra of analyzed waters shows three distinctive clusters: blue – Aqua una, Rosa and Zlatibor; red – Aqua viva and Prolom, green – Aqua purificata, Gala and Belgrade tap water.

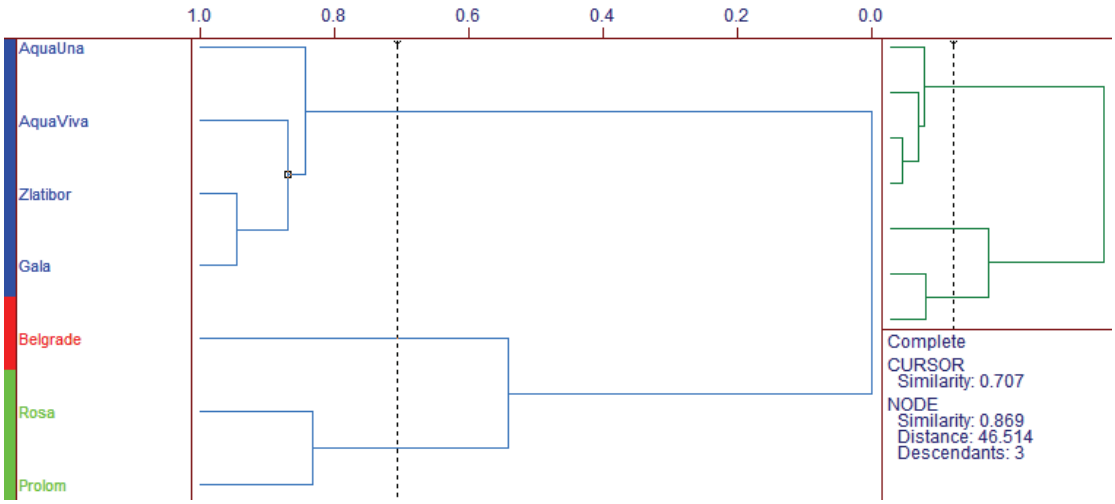

Figure 2. Dendogram of chemical contents of analyzed waters shows three distinctive categories: blue – Aqua una, Aqua viva, Zlatibor and Gala; red – Belgrade tap water; green – Rosa and Prolom.

vibration of water molecular bonds, thus takes into account a parameter which the analysis based on the mineral content of waters are clearly missing.

Figures 3–5 present aquagrams of analysed waters. Similar aquagrams are presented together on one star chart. From these aquagrams, it is obvious that the groups of similar looking aquagrams are in agreement with the clusters identified in dendogram from Figure 1. Thus, the aquagrams of Belgrade tap water and Gala water is presented in Figure 3, Aqua viva and Prolom water in Figure 4, and Aqua una, Zlatibor and Rosa in Figure 5.

The aquagrams are showing how the water molecules are arranged in these waters around present solutes. Thus, aquagrams give insight into water organization. This organization is what makes each and every one of these waters distinctive and different comparing to others, thus this can be viewed as a unique fingerprint for each of these waters under specified conditions.

This fingerprint property of aquagrams is evident on the example of water Aqua viva aquagram (Figure 4). The samples of Aqua viva water are taken from randomly chosen two bottles with different volumes (0.33 and 0.5 L). As it can be seen from the aquagrams of these waters, the aquagram lines are almost identical. However, it should be noted, that the aquagram of any analysed water will be different if the experiment conditions were different. Therefore, any hereby presented aquagram of water should be considered as a fingerprint of water under exactly these conditions (same temperature, atmosphere pressure, humidity etc.).

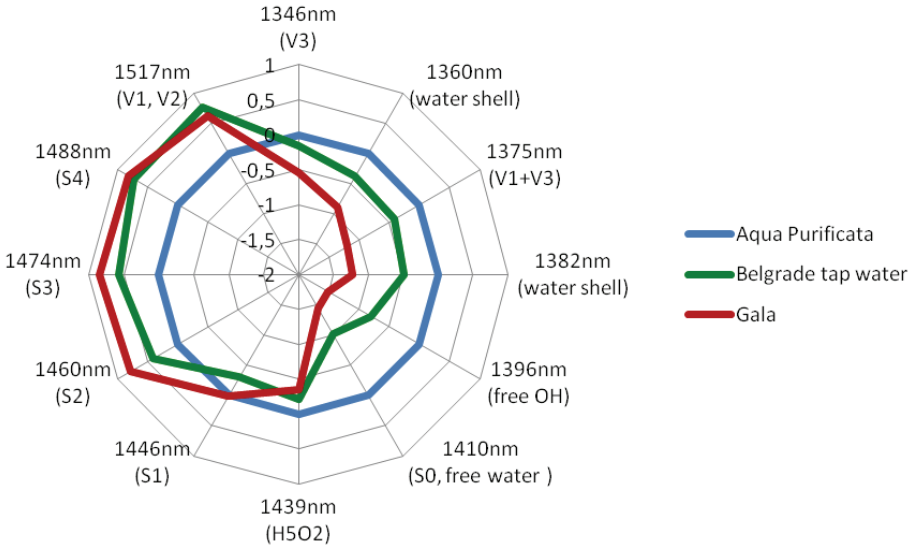

Figure 3. Aquagrams for waters Aqua purificata (AP), Belgrade tap water and Gala water.

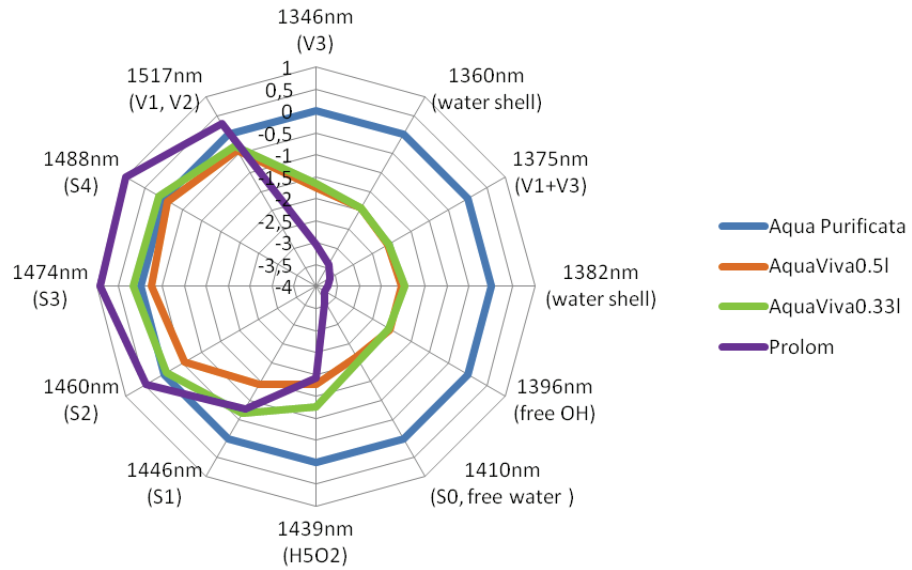

Figure 4. Aquagrams for waters Aqua purificata (AP), Aqua viva water from two bottles (vol. 0.33 and 0.5 L) and Prolom water.

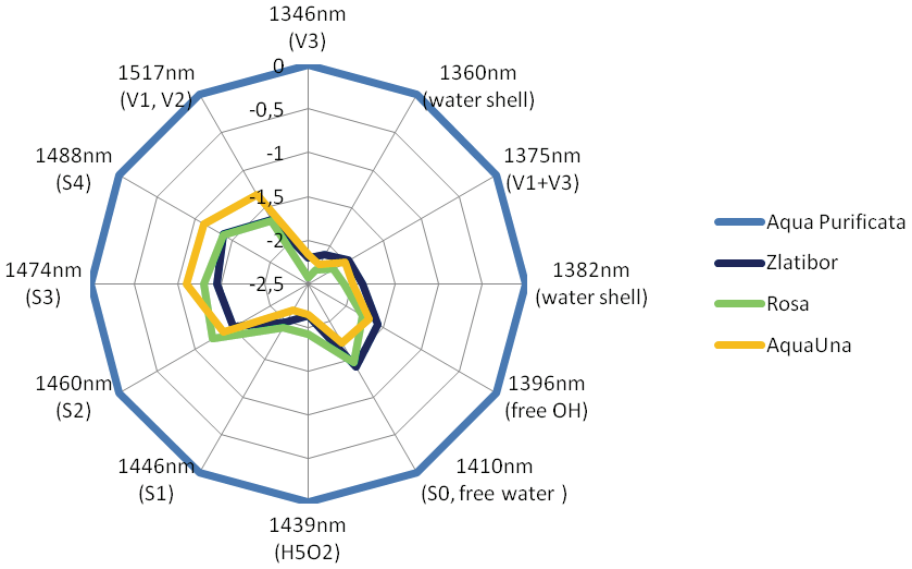

Figure 5. Aquagrams for waters Aqua purificata (AP), Aqua una, Rosa and Zlatibor water.

From aquagrams on Figure 3, it can be seen that common features of Belgrade tap water and Gala water are absorbances at 1517nm (v1,v2) which is a strongly bound water, 1488nm (S4) – water molecules making 4 hydrogen bonds (water pentamers), 1474nm (S3) – water molecules making 3 hydrogen bonds (water tetramers), 1460nm (S2) – water molecules making 2 hydrogen bonds (water trimers). The absorbances at these water matrix coordinates (WAMACS [11]) suggest that water molecules in these two waters are strongly bound to each other – the water molecules are connecting with one another. The absorbance at these water matrix coordinates is higher comparing to pure water which means higher number of these water species comparing to pure water.

Figure 4 presents aquagrams for Aqua viva water and Prolom water, which have similar water organization to previously described waters – water molecules are making hydrogen bonds between themselves creating dimers, trimers, tetramers, pentamers and strongly bound water. However, the intensity of absorbance at these WAMACS is much lower in Aqua viva water, and higher in Prolom water. Prolom water has highest absorbance in these WAMACS, which means that this water has highest number of water molecules bound in dimers, trimers, tetramers pentamers and strongly bound water, with highest number of tetramers and pentamers. For comparison, the number of these larger water formations is highest in Prolom water, then little bit lower in Belgrade tap water and Gala water, and very small in Aqua viva, where it is almost the same as in pure water.

The aquagrams for last three waters – Aqua una, Zlatibor and Rosa are similar and are presented in Figure 4. Their aquagrams are different comparing to other waters. The absorbances are lower in all WAMAC comparing to pure water, and apart from bonded water (1460 nm – S1, 1474 nm – S2, 1488 nm – S3 and 1517 nm (v1,v2)), there is also absorbance at 1410 nm – free water (free water molecules) and at 1396 nm (free OH, water molecules with free OH^-).

Further, we applied method SIMCA for discrimination of analysed waters based on its NIR spectra. A method of internal validation is applied using cross validation (leave-ten-out).

The waters are well separated and the interclass distance is for all waters well over 3, as it can be seen from Table 2, which is a criterion for good separation of classes [25].

All waters were properly classified, based on the developed SIMCA model into previously assigned classes, with no misclassified samples and results are presented in Table 3.

Table 2. Interclass distance between 8 different waters based on SIMCA class projections of the NIR spectra in 1300–1600 nm region (CS1 – Aqua purificata, CS2 – Aqua una, CS3 – Belgrade tap water, CS4 – Gala, CS5 – Prolom, CS6 – Rosa, CS7 – Zlatibor)

	CS1(4)	CS2(4)	CS3 (5)	CS4(4)	CS5(4)	CS6(4)	CS7(3)	CS8(4)
CS1	0							
CS2	43.02	0						
CS3	37.38	12.53	0					
CS4	15.48	46.88	33.32	0				

Table 2. Continued

	CS1(4)	CS2(4)	CS3 (5)	CS4(4)	CS5(4)	CS6(4)	CS7(3)	CS8(4)
CS5	13.64	31.20	22.63	13.80	0			
CS6	53.45	21.57	21.86	56.01	36.40	0		
CS7	46.61	**6.76**	18.68	52.87	36.08	20.15	0	
CS8	42.59	**5.27**	15.63	47.68	31.69	18.64	**4.63**	0

Table 3. Number of misclassified samples based on SIMCA model

Assigned category	Predicted category based on the NIR spectra of water								
	Aqua purificata	Aqua una	Aqua viva	Belgrade tap water	Gala	Prolom	Rosa	Zlatibor	No match
Aqua purificata	10	0	0	0	0	0	0	0	0
Aqua una	0	10	0	0	0	0	0	0	0
Aqua viva	0	0	20	0	0	0	0	0	0
Belgrade tap water	0	0	0	10	0	0	0	0	0
Gala	0	0	0	0	10	0	0	0	0
Prolom	0	0	0	0	0	10	0	0	0
Rosa	0	0	0	0	0	0	10	0	0
Zlatibor	0	0	0	0	0	0	0	10	0

The highest discriminating power was attributed to 1346 nm wavelength in spectra (Figure 6). This wavelength is one of the WAMACS coordinates with assigned vibration mode of v3.

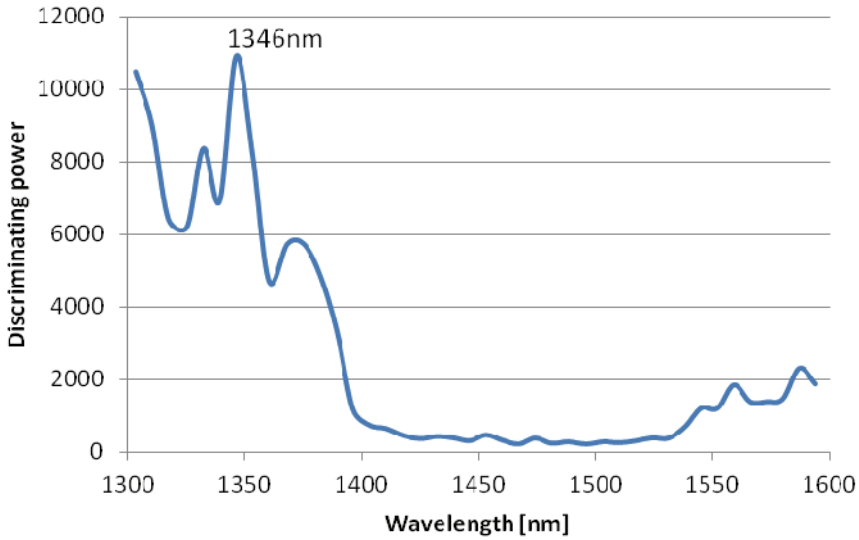

Figure 6. Discriminative power of individual wavelengths of the spectra in SIMCA model. Highest discriminating power has the 1346 nm wavelength.

CONCLUSION

The present study confirms the potential of NIR spectroscopy in discrimination and classification of commercial mineral waters. This shows that apart from the present solutes and their concentration, these waters can be classified according to their NIR spectra, by the means of the multivariate analysis and Aquaphotomics, which are actually giving information about organization of water molecules. This organization of water may have influence on the biomolecular information processing, so in our future study we would investigate if the organization of water molecules has also some health effects.

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IZVOD

DISKRIMINACIJA MINERALNIH VODA UZ POMOĆ BLISKE INFRACRVENE SPEKTROSKOPIJE I AKVAFOTOMIKE

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(Stručni rad)

Voda je jedan od najčešće proučavanih materijala danas, ali uprkos tome mnoga njena svojstva i dalje ostaju nerazjašnjena i neiskorišćena. Voda je neophodna za normalno funkcionisanje ljudskog organizma, između ostalog zbog toga, poremećaji homeostaze vode u ljudskom telu leže u osnovi mnogih bolesti. Analiza vode i njene ispravnosti za upotrebu u ljudskoj ishrani uglavnom se bavi onim što je prisutno u vodi – koncentracijama prisutnih anjona i katjona, prisustvu mikroorganizama i tome slično. Različite vrste voda se uglavnom i klasifikuju upravo prema vrsti elemenata koje sadrže, koncentraciji prisutnih elemenata, ili pak odnosu između koncentracije pojedinih jona i njihov efekat na ljudski organizam razmatra se isključivo sa stanovišta elemenata koji su prisutni u njoj. Međutim, iako je poznato da voda formira različite tipove klastera i može da se organizuje oko prisutnih elemenata na različite načine, klasifikacija voda na osnovu organizacije vodenih molekula, kao i efekti različito klasterizovanih voda na ljudski organizam, za sada ne postoje u literauri. Predmet ovog rada je diskriminacija različitih tipova voda na osnovu njihovog spektra u bliskoj infracrvenoj oblasti, primenom multivarijacione analize i novog pristupa za tumačenje spektara vode u ovoj oblasti, poznatog pod nazivom Akvafotomika. Akvafotomika interpretira spektar vode u bliskoj infracrvenoj oblasti preko posebno definisanih koordinata vodene mreže (*water matrix coordinates* – WAMACS) kojima su pripisani tačno određeni vibracioni modovi molekula vode preko kojih se može zaključiti kako se molekuli vode organizuju. Na taj način, primenom saznanja akvafotomike, voda se može opisati i sa aspekta njene organizacije u klastere, i time se omogućiti i diskriminacija voda na osnovu prisutnih tipova klastera što je prikazano u ovom radu.

Ključne reči: voda • diskriminacija • bliska infracrvena spektroskopija • Akvafotomika • multivarijaciona analiza

КОРОЗИЈА И ЗАШТИТА МАТЕРИЈАЛА

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Књига ***Корозија и заштита материјала,*** обима 405 страна подељених у девет поглавља, објављена је крајем 2012. год. од аутора проф. др Миомира Павловића, проф. др Душана Станојевића и проф. др Сретена Младеновића (постхумно), у издању Технолошког факултета Зворник. Рецензенти ове књиге су били проф. др Недељко Крстајић и проф. др Јово Мандић. Текст садржи 233 слике и фотографије и 46 табела које презентирану материју добро илуструју. На крају сваког поглавља књиге дати су литературни извори којих укупно има 246. Кроз читав текст је доследно примењен Међународни систем мерних јединица (SI) и препоруке о симболима Међународне Уније за чисту и примењену хемију (IUPAC). Књигу сачињавају следећа поглавља:

I. ГРАЂА, СТРУКТУРА И ЕЛЕКТРОХЕМИЈСКО ПОНАШАЊЕ МЕТАЛА

II. ТЕРМОДИНАМИКА ЕЛЕКТРОХЕМИЈСКЕ КОРОЗИЈЕ МЕТАЛА

III. КИНЕТИКА ЕЛЕКТРОХЕМИЈСКЕ КОРОЗИЈЕ МЕТАЛА

IV. КАРАКТЕРИСТИКЕ ЕЛЕКТРОХЕМИЈСКЕ КОРОЗИЈЕ МЕТАЛА

V. ПАСИВНОСТ МЕТАЛА

VI. КЛАСИФИКАЦИЈА КОРОЗИОНИХ ПРОЦЕСА И ВРСТЕ КОРОЗИЈЕ МАТЕРИЈАЛА

VII. МЕТОДЕ КОРОЗИОНИХ ИСПИТИВАЊА

VIII. ХЕМИЈСКА И ГАСНА КОРОЗИЈА МЕТАЛА

IX. ПРИНЦИПИ ЗАШТИТЕ МАТЕРИЈАЛА ОД КОРОЗИЈЕ

Књига је садржајно усмерена према електрохемијској корозији и заштити метала, што је по важности свакако доминатан сегмент корозионе науке, али, такође је обрађена и корозија неметалних материјала (керамика, стакло, емајл, графит, итд.). Код приказивања корозије и заштите неметалних материјала аутори су обрадили полимерне материјале и дрво, али не и бетон јер су 2008. год. објавили посебну књигу под називом „Корозија и заштита бетона и армираног бетона“ у којој је област грађевинских материјала веома детаљно разматрана.

У првом поглављу, као врста увода и усмеравања читаоца ка релевантним појмовима и појавама, даје се кратак преглед грађе и структуре метала, и с тим у вези утицај границе зрна и дефеката кристалне решетке на електрохемијско понашање метала што је од суштинског значаја за корозионо понашање метала и разумевање појаве електрохемијске корозије метала.

У другом поглављу приказана је веза између термодинамичких карактеристика метала и појаве корозије. Објашњени су и повезани принципи електрохемијске термодинамике са смером и током електрохемијских реакција у реверзибилном и корозионом спрегу. Приказана је анализа термодинамичке стабилности метала на бази Пурбеових (*Pourbaix*) дијаграма, као и механизми растварања метала у електролитима. Детаљно су анализирани електродни процеси (анодне и катодне реакције), као услов електрохемијске корозије метала и дискутована улога секундарних корозионих реакција.

Кинетика електрохемијске корозије метала обрађена је у трећем поглављу књиге где су приказани основи кинетике корозионих процеса. У оквиру овог поглавља дискутовани су неки типични модели анодних процеса

растварања метала, а од катодних процеса су детаљно обрађене реакције редукције водоничног јона и редукције кисеоника, као најважнији катодни процеси електрохемијске корозије метала.

Четврто поглавље обухвата карактеристике електрохемијске корозије метала у коме је посебно детаљно анализирана појава поларизације на електродама кроз узроке и ефекте деловања на корозиони процес, као и начини представљања корозионог процеса преко поларизационих дијаграма.

Пето поглавље књиге бави се посивношћу метала. Презентирано је и објашњено пасивно стање метала кроз најважније теорије пасивности, утицај пасивирања метала на кинетику корозије, методе постизања пасивног стања и разлоге и последице пробоја пасивне опне на површини пасивираног метала.

Врло детаљна и прегледна класификација корозионих процеса заузима шесто поглавље у коме су посебно приказане све најважније врсте електрохемијске корозије метала, корозија неметала и корозија (деструкција) полимерних материјала и дрвета.

Методе корозионих испитивања дате су у седмом поглављу књиге. Поред начина избора узорака и метода припреме узорака за корозиона испитивања, приказане су најважије класичне и савремене методе испитивања корозије метала у лабораторијским и реалним условима.

У осмом поглављу представљена је хемијска и гасна корозија метала. Ова врста корозије, због својих специфичности, детаљно је анализирана и са термодинамичког, и са кинетичког становишта. Приказане су све најважније врсте хемијске корозије метала (водонична корозија, карбонилна корозија, корозија у неелектролитима, корозија у растопима метала и растопима соли метала), а представљене су и најзначајније методе заштите метала од гасне корозије.

Девето поглавље третира веома широку област заштите метала од електрохемијске корозије у којој су, сагласно савременим погледима корозионе науке, методе заштите устројене по принципима деловања. Тако су посебно анализиране методе електрохемијске заштите, заштите модификовањем корозионе средине, заштите превлакама, итд., а на посебно инструктиван начин је дискутован најважнији и потенцијално најјефтинији вид заштите метала, заснован на рационалном конструисању.

Сва поглавља рукописа књиге ***Корозија и заштита материјала*** прикладно су опремљена и додатно појашњена сликама, дијаграмима и табелама. Језик и стил рукописа је разумљив, јасан, научно прихваћен, инжењерски концизан и прилагођен читаоцу који има потребна предзнања. Страни изрази којих је све више у нашем техничком језику су коришћени у неопходној мери, тј. тамо где не постоје наши термини или где су се страни термини одавно одомаћили.

Обимна литература коју су аутори користили за припрему рукописа припада научно признатим и познатим литературним изворима, међу којим има класика корозионе науке, савремених аутора, али и референци самих аутора.

Корозија и заштита материјала, према начину на који је писана, одабраној материји, и начину приказивања и анализирања сложених корозионих проблема представља значајан допринос домаћих аутора релативно скромној библиографији везаној за корозију објављеној на нашем језику. Ова књига може бити веома корисна студентима као шира литература за поједине теме из корозије и заштите материјала, инжењерима технологије и машинства код решавања конкретних корозионих проблема, а нарочито инжењерима који се баве заштитом материјала у индустрији.

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