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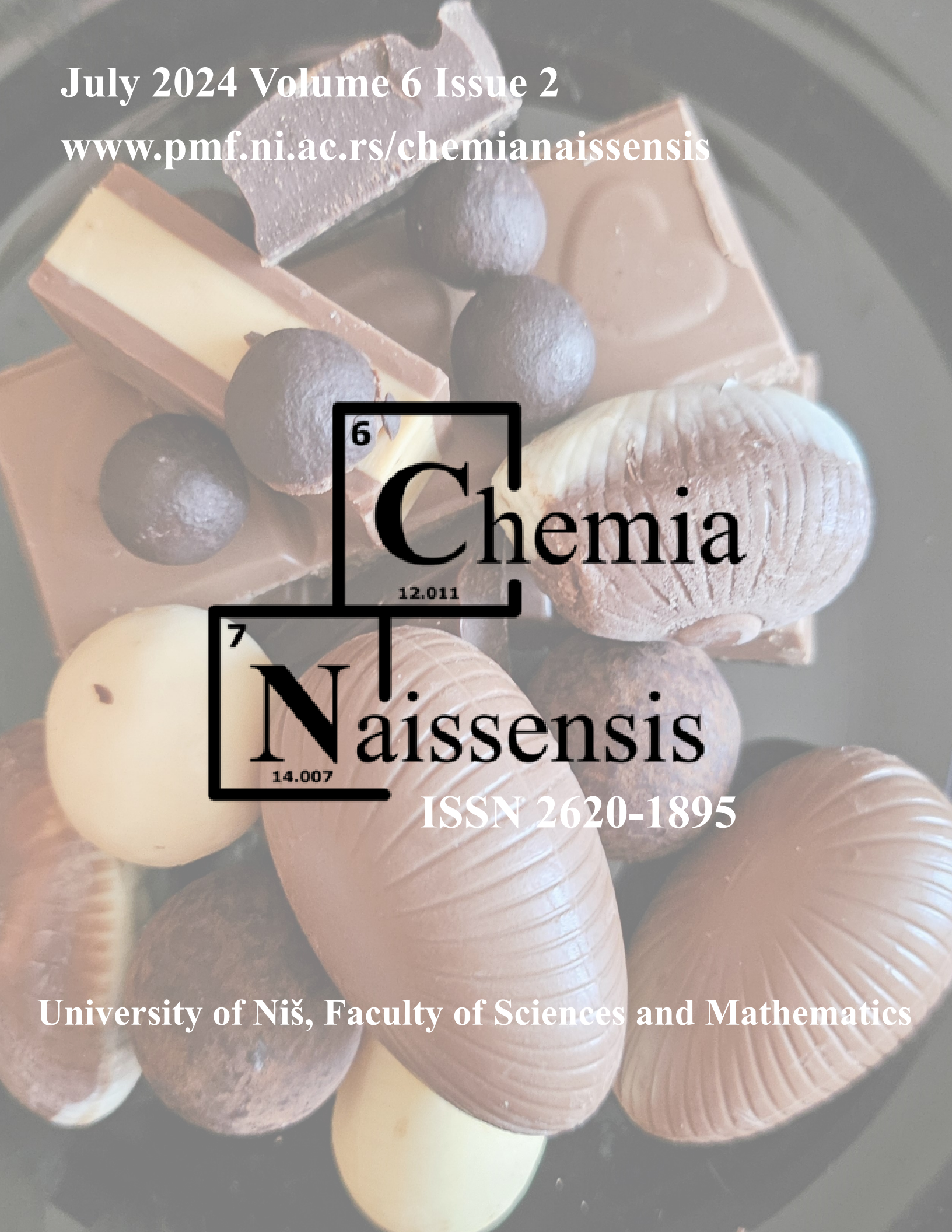

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CONTENT

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Effect of sulfate-reducing bacteria on stainless steel: a review

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ABSTRACT

Corrosion-resistant alloys such as stainless steel provide an ideal substrate for microbial colonization due to the absence of corrosion products, similar to inert non-metallic surfaces. Stainless steels are sensitive to pitting and other types of localized corrosion in chloride-containing media such as seawater. Sulfate-reducing bacteria play an essential role in the corrosion of stainless steel in marine and soil environments. Sulfate is utilized by microbes as a terminal electron acceptor as their respiration drives sulfate reduction leading to the formation of H_2S, which can lead to a significant increase in anodic and cathodic processes and corrosion of materials. In reviewing the literature, it was found that most studies on microbially induced corrosion in stainless steels indicate that it is caused by the influence of chlorides and sulfides in the soil resulting from the secretion of sulfate-reducing bacteria. The influence of sulfate-reducing bacteria on stainless steel is described in detail in this review, which can be seen from the following points: general properties of sulfate-reducing bacteria, morphology and chemical composition of biofilm and corrosion products, mechanisms of microbiological corrosion by sulfate-reducing bacteria and electrochemical studies of corrosion rates of stainless steel by sulfate-reducing bacteria under different experimental conditions.

Keywords: *stainless steel, sulfate-reducing bacteria, corrosion*

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Introduction

All definitions of microbiological corrosion (Javaherdashti, 2011; Liu et al., 2018; Pal end Lavanya, 2022) are based on the following statements: microbiological corrosion is an electrochemical process; microorganisms can influence the duration of corrosion, the strength and the direction in which corrosion takes place; and in addition to the presence of microbes, nutrients and water must be present for microbial corrosion to take place. Microbial corrosion accounts for 20% of all corrosion damage (Flemming, 1996). Damage caused by microbial corrosion has always been a significant concern of corrosion researchers. The authors Maxwell et al., (2004) pointed out that corrosion causes losses to the oil industry of around 100 million US dollars annually. It is estimated that 10% of all corrosion damage in the UK is due to microbiological corrosion (De Romero et al., 2000).

Microbes are one of the leading causes of corrosion in underground pipelines (Li et al., 2003), which is related to the influence of different microbes and the physicochemical properties of the soil. It is known that the chloride content in soil and other environments plays a vital role in the corrosion of steel structures (Sun et al., 2011), so the influence of microbiological corrosion on stainless steel is recognized as an essential corrosion phenomenon (Nguyen et al., 2008). Due to the wide application of stainless steel, numerous studies have focused on the microbiological corrosion of these materials (Sheng et al., 2007; Maruthamuthu et al., 2008; Sun et al., 2011; Tang et al., 2021). Stainless steels are alloys with a wide technical-technological application due to their high corrosion resistance; however, the presence and influence of microbes is unavoidable even with these types of steel (Geesey et al., 1996). Due to the wide application of stainless steel in the industry, both in salt and freshwater environments, researchers have focused on studying the influence of sulfate bacteria on these steels. Various analytical techniques have been used to study microbial corrosion, including electrochemical and surface analysis techniques: electrochemical impedance spectroscopy (EIS), atomic force microscopy (MAC) and X-ray photoelectron spectroscopy (XPS) (Sheng et al., 2007; Nguyen et al., 2008; Ramdane et al., 2023).

Work also presents the main aspects of sulfate-reducing bacteria, the morphology and structure of biofilms and corrosion products on stainless steel, and the mechanisms and corrosion processes of microbiological corrosion of sulfate-reducing bacteria on stainless steel. In addition, the role of sulfate-reducing bacteria in the corrosion and fracture of steel was also explained.

General properties of sulfate-reducing bacteria

Sulfate-reducing bacteria is a general term for different types of bacteria that reduce sulfates to hydrogen sulfide or FeS_x. Sulfate-reducing bacteria represent a group of heterotrophic, mixotrophic, mesophilic and halophilic bacteria (Lane, 2005). Sulfate-reducing bacteria have a spindle-shaped, granular or oval form with sessile cell densities of about 107 cells per cm^2 (Duan et al., 2006). The maximum concentrations of sulfides formed by the reduction of sulfates by sulfate-reducing bacteria do not exceed 600 ppm, while sulfide concentrations in sediments hardly exceed 500 ppm. Sulfate-reducing bacteria can tolerate a pressure of up to 506.6 bar (Barton and Tomei, 1995). The optimum temperature for the growth of sulfate-reducing bacteria is 20-30 °C, but they can also survive at 50-60 °C. Sulfate-reducing bacteria survive under anaerobic conditions in soils, seawater, sludge, underground pipelines and oil wells at pH values of 6 to 9 (Yuan and Pehkonen, 2009). Sulfate bacteria can also be found in wastewater (Chen et al., 1998) and the human body, e.g. in the mouth (Maruthamuthu et al., 2008). These organisms exist in a naturally occurring film and form a complex with which they bind to the surface of the metal (Baker et al., 2003).

In their natural habitat, sulfate-reducing bacteria are essential in the biogeochemical sulfur cycle (Yuan and Pehkonen, 2009; Tran et al., 2021). They are a group of classified anaerobic microbes that dissimilate sulfur compounds such as sulfates, sulfites, thiosulfates and elemental sulfur to sulfides (Gibson, 1990). Sulfate-reducing bacteria do not require oxygen but can be found in aerobic environments. They tolerate significant changes in temperature, pH, chloride concentration and pressure fluctuations (Javaherdashti, 2011). Sulfate-reducing bacteria are involved in microbiological corrosion processes affecting various engineering systems and alloys. For example, the sulfate bacteria *Desulfovibrio* can produce insoluble sulfates of Zn, Pb, Ni, Cr, Cd, Cu, Fe, Hg and other elements (Lane, 2005).

The classification of corrosivity, which occurs depending on the number of cells of the sulfate-reducing bacteria, is expressed in units: cell/ml. According to this classification, the environment is considered low corrosive if the number of sulfate-reducing bacteria is 1000 cells/ml or less, while the content of sulfate-reducing bacteria in the solution between 10^3 and 10^5 cells/ml is a medium corrosive environment. If the sulfate-reducing bacteria content is above 10^5 cells/ml, the environment is classified as highly corrosive (Javaherdashti, 2011).

The authors Cheng and Enhou, (2005) showed that the corrosion of stainless steel increases with the decrease in sulfate-reducing bacteria. Wei et al., (2010) demonstrated a positive correlation between sulfate-reducing bacteria and the corrosion process of stainless steel. However, the authors Ilhan-Sungur et al., (2007) showed that there is no evidence of a correlation between the corrosion rate and the number of bacterial cells in the tested environment. Kuang et al., (2007) even showed that instead of the number of sulfate-reducing bacteria, the metabolic products of the bacteria may be of greater importance when testing metal corrosion.

Formation and structure of the biofilm of sulfate-reducing bacteria and their corrosion behavior

According to the general definition, a biofilm's gradual formation can change the metal surface's chemical composition. The physical meaning of the biofilm indicates a passivity that restricts oxygen diffusion to the metal surface. Therefore, the active metabolism of the microorganisms consumes oxygen and produces metabolites. The result of biofilm formation is reflected in the creation of a concentration gradient of chemical species along the entire length of the biofilm, usually between 10 and 400 μm (Xu et al., 1998).

In order to understand the role of sulfate-reducing bacteria in the corrosion of stainless steel, it is necessary to analyse and define the effects of the biofilm on the metal. The heterogeneity of the biofilm is vital for triggering localized corrosion by increasing the corrosion rate, as it can contribute to significant differences in metabolites, pH or soluble oxygen (differential aeration cell), leading to the formation of an active electrochemical corrosion cell (Yuan and Pekhonen, 2007). The resulting biofilm behaves like the growth of a colony of sulfate-reducing bacteria on the metal surface, leading to localized corrosion (Javaherdashti, 2011; Anusha and Mulky, 2023). Microorganisms easily attach to the steel surface. When the metal is immersed in seawater, bacteria excrete organic polymers and adhere to the steel surface, forming a thin film that changes the properties of the metal surface, significantly static charge and wettability. This leads to the adhesion of bacteria to the steel surface and the growth of a bacterial colony (Zhang et al., 2011). In biofilm formation, the initial attachment of bacteria to the steel surface is the most crucial step, which is achieved by electrostatic attraction and other forces, e.g. van der Waals forces and hydrophobic interactions (Ong et al., 1999; Narayana and Srihari, 2019).

Ong et al., (1999) showed that the adhesion force in cell-cell interaction is greater than the force with which *D. disulfuricans* cells adhere to the steel surface. Most researchers (Beech, 2002 and Xu, 2002) indicated stronger adhesion at the cell-cell surface related to the accumulation of exopolymeric substances at the cell periphery, accelerating the mutual binding of the bacteria to the substrate and the steel surface. These results suggest that the sticky exopolymeric substance accumulating on the cell surface increases cell aggregation, leading to the expansion of the biofilm over the surface of the tested steel (Yuan and Pehkonen, 2009). The exopolymeric substance is essential for cell attachment to the biofilm structure. A class of N-acyl-homoserine lactone signalling molecules released by cells into the local environment has also been shown to interact with neighbouring cells.

Studies (İlhan Sungur et al., 2010; Javaherdashti, 2011; Sun et al., 2011; Elmouaden et al., 2016) have shown that on metal surfaces such as stainless steel, galvanized steel and copper, the number of sulfate-reducing bacteria on the examined surface increases over time, indicating that a biofilm has formed. The heterogeneity of the steel structure and the uneven oxygen concentration at the interface between the biofilm and steel can also contribute to forming an electrochemical cell, such as a differential aeration cell, which accelerates corrosion (Vujičić, 2002). The metal at the edge of the biofilm is in contact with the substrate solution, which is relatively richer in oxygen. Due to the decrease in oxygen in the biofilm's centre, differential aeration occurs, manifesting in the anodic dissolution of metals in the form of Fe^{2+} ions. The transport of these ions by diffusion, convection and ion migration towards the cathodic periphery of the biofilm leads to the deposition of hydrated Fe(III) hydroxide as the end product of corrosion (Vujičić, 2002). Due to the different cation concentrations, it is also more likely that a particular site on the steel will become a local cathode, while the opposite site with lower cation concentrations will form a local anode and thus contribute to the occurrence of pitting (Javaherdashti, 2011). Therefore, local anodes become the sites near which the electrolyte contains a lower concentration of passivating substances or a higher concentration of activating substances. Corrosion activators include chloride ions, hydroxyl and ammonia ions, which make the potential of the metal more negative and make it impossible to reach the passive state of the steel (Vujičić, 2002).

However, the biofilm of anaerobic sulfate-reducing bacteria has other properties: a thick biofilm layer impedes oxygen diffusion. The consequences of such processes are anaerobic conditions that lead to better conditions for the growth and reproduction of bacteria (Javaherdashti, 2011). By

reducing the oxygen concentration on the surfaces where the corrosion product is deposited, these surfaces change their polarity, which means that the anode surface area increases at the expense of the cathode surface area (Vujičić, 2002). As a result of chemical reactions promoted by sulfate-reducing bacteria, a film of iron sulfide (FeS_x) was formed. The film forms a corrosion product on the steel, which is very brittle and leads to pits and cracks. A differentiated aeration cell forms above the cracks. Such processes further accelerate corrosion. As the biofilm develops, it begins to change the electrochemical and physical properties of the metal directly on the surface of the metal (Pal and Lavanya, 2022).

Another consequence of biofilm formation can be the saturation of the biofilm, which increases the pitting potential and thus contributes to the occurrence of localized corrosion and premature failure of the steel (Dexter and Chandrasekaran, 2000). The chemical composition of the biofilm boundary layer may also differ from the bulk of the solution, causing the aerated solution to form oxygen pockets in the biofilm. This biofilm structure can be a barrier to chemicals such as biocides or toxic copper ions (Javaherdashti, 2011).

Looking at the chemical composition of biofilms and corrosion products, the results of Duan et al., (2006) show that the inorganic sulfide species include sulfides of Fe, Cr and Mo, as well as organic sulfide species such as sulfur-containing proteins, amino acids and organic molybdenum sulfide. The resulting biofilm and corrosion products can appear on the surface of stainless steel exposed to sulfate-reducing bacteria for seven days. The reaction of the formed biofilm with the passive film and the substrate Fe produces metal oxide Fe_2O_3, which can be reductively dissolved by biogenic hydrogen sulfide, resulting in iron sulfide. Cytochrome and hydrogenase can participate in the reductive dissolution of iron oxide (Amirbachman et al., 1997). Independent of the local corrosion of the thin passive layer, bacterial cells can act directly on elemental Fe by reduction on the cell surface, e.g. by hydrogenase electron transfer, and thus play an essential role in corrosion processes and biomineralization (Da Silva et al., 2004).

Antony et al., (2007) found that the site of attack occurs mainly at locations with low Cr and Mo content in the steel, as biogenic sulfides trigger the attack on duplex steel. The initial systolic state of the bacteria tends towards metal corrosion, as it depends on the chemical elements of the steel alloy under investigation. Thus, the high content of Ni and N in the structure of stainless steel increases the binding of bacteria to the metal surface (Flint et al., 2000; Lopes et al., 2005). Direct dissolution of Cr_2O_3 with biogenic H_2S forms chromium sulfide, which indicates a possible

sulfidation of chromium oxide in the passive layer through contact with the substrate of sulfate-reducing bacteria. Duan et al., (2006) demonstrated the described phenomenon of passivity reduction on stainless steel 317L exposed to sulfate bacteria of the species Desulfovibrio desulfuricans.

On the other hand, mackinawite ($Fe_{1+x}S$) is generally regarded as the first FeS_x layer on the surface of stainless steel. It is formed by the activity of sulfate-reducing bacteria in water. It has also been hypothesized that the microstructure of pyrrhotite on the surface of stainless steel is due to the action of sulfate-reducing bacteria. Mackinawite is less stable than pyrrhotite under slightly acidic conditions, as pyrrhotite (Fe_7S_8) is stable in slightly acidic, neutral and alkaline environments ($5.3 < pH < 13.3$) (Jeffrey and Melchers, 2003). In contrast, pyrite is not a typical corrosion product for such experimental conditions. Although sulfate-reducing bacteria can produce pyrite from mechinavite in contact with sulfur or polysulfides, no pyrite is formed under the given experimental conditions (120 °C, pH=7.2-8.0, sulfate-reducing bacterial substrate) (Ilkin and Barner, 1996).

Morphology and chemical composition of biofilm and corrosion products

The surface morphology of stainless steel samples buried in soil for 136 days was studied with and without sulfate-reducing bacteria and chloride ion substrate (0.5%). The scanning electron microscopy (SEM) investigations show rough and fine-grained corrosion products on the surface of the tested steel. In some places, pitting was observed to tend to spread over the entire surface. It was also shown that the sulfur content as a corrosion product was much higher in the soil with sulfate-reducing bacteria than in the soil without sulfate-reducing bacteria. This indicates that FeS is present in the corrosion product, which increases the tendency to pit the steel. A high Mn and Si content and a low S and Fe content were also found in the corrosion products, indicating the onset of pitting near the site containing these elements. Stainless steel samples were placed in a soil substrate containing 3.0% Cl^- and sulfate-reducing bacteria, and after 136 days the concentration of sulfate-reducing bacteria was no longer detectable (Sun et al., 2011).

On the other hand, Antony et al., (2008) showed by microscopic examination that the entire surface was covered with a biofilm on the base metal after 40 days of exposure to sulfate-reducing bacteria. Loosely associated extracellular polymeric substances, cracks and pits were seen in biofilm and

stainless steel imaging. Microholes are determined in the ferrite phase, in the heat-affected zone. Microscopy shows a welded spot on steel with a fusion zone, i.e., the base metal zone, under the influence of heat. The microstructure consists of a solidified ferrite zone and transformed austenite, as dendrites, in the fusion zone. In the area affected by heat, the ferrite fraction increased significantly. It was also observed that the welding wire, with a higher Ni content, helped to achieve a balanced microstructure on the duplex surface in the fusion zone. At higher saturations of the solution with sulfate-reducing bacteria, the tested austenite and intragranular austenite growth was observed within the heat-affected zone. The ferrite phase is characterized by the appearance of chromium nitride (Cr_2N) (Antony et al., 2008).

Even though duplex steels are known for their corrosion resistance in a chloride environment, the influence mentioned above of the presence and activity of sulfate-reducing bacteria contributes to the wear of the passive layer (Song et al., 2018). Therefore, this work showed that the welded spot and sulfate-reducing bacteria could contribute to the depassivization of steel via the formation of the inevitable microstructure on the steel surface by controlling these properties. Microstructural studies revealed that the weld spot can cause an imbalance in the heating zone with higher fractions of ferrite than the base metal. Also, the metal welding wire has a high Ni content, which increases appearance of austenite fraction in the fusion zone. Due to the solidification of the welded area in the ferritic form, the cooling rate is higher because there is little time for the transformation to the austenite phase. This leads to the trapping of interstitial nitrogen in the ferrite phase and the formation of Cr_2N (Muthupandi et al., 2003; Chen and Yang, 2002). On the other hand, the transformation process from ferrite to austenite begins in connection with the saturation of Ni in the melting zone at high temperatures. It helps to achieve phase equilibrium and a microstructure free from precipitates in the fusion zone.

The initial concentration of bacteria attached to steel depends on the properties of the metal microstructure. The population of bacteria is higher in the fusion zone than the number of bacteria on the base (parent) metal. The formation of clusters of bacterial cells in the heating zone indicates that the bacteria tend to attach in certain places, as in this case, at the heat-affected zone/fusion zone phase boundary (Antony et al., 2008).

The austenitic phase of the base metal only corrodes in a medium with sulfate-reducing bacteria (Antony et al., 2007). The reformed austenite in the ferritic matrix remained intact at high Cr and Mo content compared to the austenite of the base metal. This can be explained by the low diffusion

of substitutional elements of the alloy during the transformation to the solid state (Antony et al., 2008). The authors pointed out that duplex steel with a higher Cr and Mo content and a defective microstructure may have better passivity in the environment with sulfate-reducing bacteria (Wan et al., 2023). Also, the appropriate choice of molten weld and welding parameters affects the distribution of alloys and the more difficult formation of Cr_2N, which may contribute to the passivity of the welded area (Antony et al., 2008). Chen and Yang, (2002) showed through experiments that the addition of nitrogen from the shielding gas can contribute to the reformed austenite of the heating zone by diffusion of nitrogen at the surface of the fusion zone/heat-affected zone interface, thereby avoiding the formation of Cr_2N in the ferrite phase.

According to Sreekumara et al., (2005), the combination of physical and chemical changes caused by stainless steel welding favors the accumulation of organic matter on the surface and the gradual binding of microorganisms to the metal. The morphological appearance of the corrosion products is masked by a biofilm, which, according to energy dispersive X-ray spectroscopy (EDS) analysis, consists of C, Ca, O, Si and P (Beech and Sunner, 2004). A cross-section of the sample shows further damage where the stainless steel is skeletonized.

A black-colored substance visible on the surface of a stainless steel sample (AISI 304) formed after an incubation period of 7 weeks in seawater containing the substrate *Desulfovibrio desulfuricans*. XRD analysis was used to determine the binding energies and composition of the biofilm on the surface of the investigated steel. The chemical composition of the *Desulfovibrio desulfuricans* biofilm was as follows: monosulfide, iron sulfide, chromium sulfide and molybdenum sulfide, then disulfide, polysulfide and organosulfide-like proteins, which can be formed by bacterial excretion during the experiment with a binding energy of 163 to 168 eV. Organic molybdenum sulfide (Mo(V)-S (cysteine)) was also detected in the passive layer during the described experiment (Nguyen et al., 2008).

The surface saturated with Mo was also considered when the stainless steel was exposed to an abiotic and biotic corrosion environment with a certain chlorine content (Bastidas et al., 2002). Molybdenum is usually found in the +4 and +6 oxidation states in the form of molybdenum chloride salts and MoO_4^{2-}, which can improve the pitting resistance of steel. The study indicated the presence of inorganic MoS in the passive film, which is more insoluble than the molybdenum chloride salt and can, therefore, be converted to molybdenum sulfide by reaction with biotic H_2S. Thus, organic sulfides, including organic molybdenum sulfide, also occur in the passive film. The

extracellular polymeric substance and the bacterial cells adhering to the stainless steel surface are the source of the organic sulfides. The Mo(V) complex can be formed in interaction with a hydrogenase (FeS_x protein) or a cysteine residue formed in the biofilm (Duan et al., 2006).

Based on the experimental data, the XPS results of Nguyen et al., (2008) on the influence of sisal bacteria on corroding steel are presented. The change in the chemical composition of the typical elements on the surface of the SS 304 steel under consideration (Fe and Ni) was determined. The biofilm on stainless steel samples formed under the action of sulfate-reducing bacteria shows the presence of elements such as Na, K, Cl and S. In this way, it was revealed that the biofilm can retain soluble and insoluble particles and metabolic products of sulfate-reducing bacteria.

By analyzing and recording C 1s spectra, three types of carbon bonds were identified: C-C, C-O or C-N, and C=O or C=N. The presence of the analyzed layer is also explained by the CHn hydrocarbon layer of 0.5 to 1 nm on the surface of the metal (Vinnichenko et al., 2002). The presence of the analyzed layer was attributed to production processes, preparation of the metal surface or contact with the atmosphere. The presence of C-O and C=O bonds is explained by polishing paste on the analyzed surface. C-N and O=C-N bonds were explained by proteins from the solution or bacterial cells (Nguyen et al., 2008). Also, in the presence of sulfate-reducing bacteria adsorbed on the metal surface, the presence of the O=C-N bond is explained by the presence of peptide chains of the protein components of the bacteria (Johansson and Saastamoinen, 1999), while the C-C bond indicates the accumulation of long aliphatic chains from bacterial cells on the stainless steel surface. The C=O bond indicates intracellular lipids, while the C-O bond may originate from extracellular polymers (Kusy et al., 2002).

The primary spectrum of O 1s includes the following compounds: metal oxides and hydroxides. A decrease in the oxide layer was observed with increasing concentration of sulfate-reducing bacteria. In addition, O-C=O and N-O-S bonds were detected on the stainless steel surface of the analyzed samples exposed to sulfate bacteria.

In the continuation of the experiment, analyzes of S 2p spectra were performed, and based on the results, a conclusion was drawn about the low sulfur content on polished and bacteria-free steel surfaces, in contrast to surfaces exposed to a solution of sulfate-reducing bacteria (Nguyen et al., 2008). Sulfates are formed from dissolved sulfate salts adsorbed on the metal surface, while sulfites are formed as intermediates during sulfate reduction. Precipitated FeS and FeS_2 are

corrosion products of iron substrates formed in artificially saturated salt water (Keresztes et al., 2001).

Organic sulfides are also present in biofilm as an integral part of the exopolymer. In sulfate-reducing bacteria, FeS_x proteins, such as the hydrogenases of *D. desulfuricans*, play an essential role in biological electron transfer processes and many enzymatic reactions (Keresztes et al., 2001). For this reason, the presence of organic sulfides in the biofilm of sulfate-reducing bacteria suggests that these bacteria play an essential role in biocorrosion by accelerating the cathodic reaction. There are specific changes in the chemical composition of the biolayer in the spectra of C 1s, O 1s and S 2p for the different samples.

The XPS spectra showed changes in stainless steel under the influence of sulfate-reducing bacteria for three elements: Fe, Cr and Ni. The outer oxide layer on the surface of SS 304 steel contains lower amounts of Ni, and Ni is obtained by dissolving the lower oxide layers, which are then exposed to the outer layer of the biofilm. On the other hand, the Fe-2p spectrum indicates higher concentrations of iron on the corroded surface under the influence of sulfate bacteria, suggesting that the passive film becomes thinner due to the dissolution of metal oxides in the studied electrolyte (Nguyen et al., 2008).

The curve corresponding to the S 2p spectrum shows the following compounds: monosulfide S^{2-}, disulfide S_2^{2-} and polysulfide (S_n^{2-}) as well as S^0, SO_3^{2-}, organic sulfide / SO_4^{2-}, and $S_2O_3^{2-}$. A passive film of FeO, Fe_2O_3, Cr_2O_3, $Cr(OH)_3$ and FeOOH formed on the steel surface exposed to sterile seawater and aerobic conditions (Yuan and Pehkonen, 2007). However, sulfidation of the passive layer by biogenic sulfide ions leads to a gradual loss of passivity of the steel.

Therefore, the studies above show that the corrosivity of steel samples with sulfate bacteria is higher than that of samples exposed to *Pseudomonas* bacteria, mainly related to biogenic sulfide anions (Yuan and Pehkonen, 2009).

Mechanisms of microbiological corrosion by sulfate-reducing bacteria

Due to the significant role of sulfate-reducing bacteria in microbial corrosion, many researchers are focusing on determining the corrosion mechanisms of steel by sulfate-reducing bacteria (Abdullah et al., 2014; Liu et al., 2019; Lv et al., 2019). To date, different versions of the

corrosion mechanisms of stainless steel by sulfate-reducing bacteria have been developed, specifically cathodic and anodic depolarization (Nguyen et al., 2008).

The mechanism that explains the metal corrosion caused by sulfate-reducing bacteria is the consumption of hydrogen produced at the cathode by the enzyme hydrogenase. Stott, (1988) pointed out that the main effect of sulfate-reducing bacteria is the elimination of hydrogen from the corroding metal, which refers to the hydrogenase reversibly catalyzing the activation of hydrogen. The classical theory of cathodic depolarization is represented by reactions occurring in three domains: metal, solution and microorganisms, which is given by equations 1, 2, and 3.

In the absence of oxygen, the cathode surface of the metal is rapidly polarized by atomic hydrogen. Under anaerobic conditions, an alternative cathodic reaction, such as oxidation by gaseous or free oxygen atoms, is not possible. Under such conditions, water dissociation occurs, so this reaction, together with the cathodic reaction of the adsorbed hydrogen ions produced, is the most crucial cathodic reaction. The products of the cathodic reactions are then adsorbed on the metal surface (polarization) and consumed by the hydrogenase. The classical theory of microbiological corrosion of metals by sulfate-reducing bacteria is shown schematically in Fig. 1. The bacterial cells are shown separately; in reality, they live directly on the surface of the iron. The reduced hydrogen is transported from the iron to the bacteria at the cathode and used to reduce sulfate to sulfide. Only a quarter of the dissolved iron(II) ions react stoichiometrically with H2S to form FeS at the anode. In the presence of CO_2 and bicarbonate, the Fe^{2+} residue transforms into $FeCO_3$; without bicarbonate, $Fe(OH)_2$ precipitates (Song et al., 2018; Victoria et al., 2021).

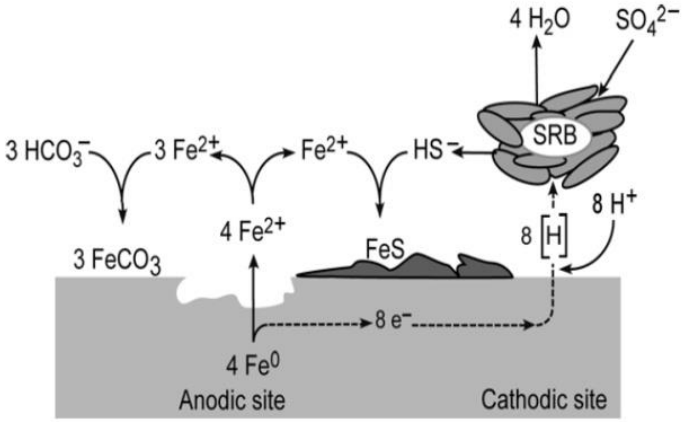

Figure 1. Schematic representation of the classical theory of cathodic depolarization by SRB activity (Hang, 2003).

The most critical corrosion mechanisms of sulfate-reducing bacteria are:

1. Cathodic depolarization mechanism: sulfate-reducing bacteria produce sulfates that oxidize adsorbed H^+ under anaerobic conditions to accelerate corrosion and release hydrogen. The reactions proceed as follows (Javaherdashti, 2011):

$$Anode: 4Fe^0 \rightarrow 4Fe^{2+} + 8e^- \quad (1)$$

$$Cathode: 8H^+ + 8e^- \rightarrow 8H_{ad} \quad (2)$$

$$Cathodic\ depolarization: SO_4^{2-} + 8\,H_{ad} \rightarrow S^{2-} + 4H_2O \quad (3)$$

$$Ionization\ of\ water: 8H_2O \rightarrow 8H^+ + 8OH^- \quad (4)$$

$$Corrosion\ products: Fe^{2+} + S^{2-} \rightarrow FeS \qquad 3Fe^{2+} + 6OH^- \rightarrow 3Fe(OH)_2 \quad (5)$$

$$Overall\ reaction: 4Fe^0 + SO_4^{2-} + 4H_2O \rightarrow FeS + 3Fe(OH)_2 + 2OH^- \quad (6)$$

According to Kuhr's theory, hydrogenase and hydration of the bacterial cell can promote depolarization and accelerate corrosion.

2. The concentration cell mechanism: This mechanism shows that a concentration cell forms when corrosion products, such as iron hydrate, form on the metal surface. In most cases, this type of corrosion would form an area of hypoxia near the metal surface under anaerobic conditions and create suitable conditions for the growth of sulfate-reducing bacteria that accelerate corrosion.

3. Mechanism of metabolites: Anaerobic corrosion of sulfate-reducing bacteria results from phosphide, which is highly active and volatile and is produced by the metabolism of sulfate-reducing bacteria. Phosphides can react with iron compounds or H_2S to form iron phosphide, which enhances corrosion.

4. Other mechanisms (Zhang et al., 2011): (a) extracellular polymers of sulfate-reducing bacteria react with the metal to form Fe^{2+}, which oxidizes to Fe^{3+}, accelerating corrosion; (b) under anaerobic conditions, the accumulation and release of H_2O_2 in the biofilm participate in the electrode reaction of steel, causing an increase in electrode potential and leading to localized corrosion; (c) sulfate-reducing bacteria attack the metal by attacking the grain boundary and selectively releasing austenite; d) the oxygen present reacts with sulfur and leads to the formation of intermediates, accelerating the corrosion process; e) sulfide ions (S^{2-}) react with iron to form FeS, which is the cathode, while Fe is the anode; the cathodic depolarization reaction, which releases hydrogen, can corrode the metal surface.

Based on the proposed mechanisms, there are no unified views, so further investigation of possible mechanisms of microbiological corrosion of sulfate-reducing bacteria on stainless steel should be continued, mainly focusing on the most promising mechanism of cathodic depolarization.

The authors Videla and Herrera, (2005) summarized a new picture of the microbiological mechanisms of corrosion by sulfate-reducing bacteria, which includes the following phases:

- In a saline environment, steel dissolves at high Fe^{2+} concentrations and forms a film of iron hydroxide whose thickness and protective properties depend on the solution's pH.
- Anion adsorption processes, which take place in the boundary layer between the metal and the solution, have a further influence on the acceleration or retardation of corrosion.
- The physicochemical properties of the iron sulfide film can control the effect of sulfide on steel dissolution, which depends on the iron/sulfide anion ratio, the presence of sulfate-reducing bacteria and the coverage of the metal surface with biofilm.

Research by Hang, (2003) has also shown that different types of bacteria play a significant role in corrosion processes. A solution of sulfate-reducing bacteria was tested, enriched directly with iron and sulfate and a CO_2/bicarbonate buffer. The bacterial strains used iron, lactate and pyruvate for sulfate reduction. In the presence of iron, the strains of *Desulfobacterium corrodes* reduce sulfate much faster than *Desulfovibrio*. In contrast, sulfate reduction slows down in the presence of hydrogen and lactate compared to *Desulfovibrio* species. This work indicated a new species of *Desulfovibrio ferrophilus*, which can reduce sulfates faster than other Desulfovibrio species in the presence of iron but slower than *D. corrodens*.

The complete genome of *Desulfovibrio vulgaris* encodes oxidase, oxidoreductase, plasmid-encoded catalase and superoxide dismutase genes. Analysis of the incomplete genome sequences of gram-positive *Desulfitobacterium hafniense* reveals genes encoding three catalyzes, one of which is extracellular. These catalyzes are homologs of hydroperoxidase I and II. The genome of *Desulfovibrio vulgaris* encodes several proteins that are similar to the chemerythrin domain and contain Fe or O. Overall, the genome sequence of *Desulfovibrio vulgaris* indicates the presence of 27 methyl-accepting chemotaxis proteins, which include the oxygen- or redox-potential-responsive proteins DcrA and DcrH (Beech and Sunner, 2004). These proteins may be necessary for sulfate-reducing bacteria on oxygenated or non-oxygenated surfaces.

The development of biofilms is promoted by producing an extracellular polymeric substance containing macromolecules such as proteins, polysaccharides, nucleic acids and lipids. The ability of the exopolymeric substances to bind metal ions depends on the type of bacteria and metal ions (Rohwerder et al., 2003). The binding between the exopolymeric substance and the metal occurs via the bond between metal ions and anionic functional groups (carboxyl, phosphate, sulfate, glycerol, pyruvate and succinate groups) common to the proteins and carbohydrate compounds of the exopolymer. The affinity of anionic ligands can be vital for multivalent ions such as Ca^{2+}, Cu^{2+}, Mg^{2+} and Fe^{3+}. The presence and affinity for metal ions with different oxidation numbers in the biofilm can constantly change the standard reduction potential. For example, the electrode potential of Fe (III/II) changes with the change of ligands (from +1.2 V to -0.4 V). Extracellular polymeric substances bound to metal ions can act as electron acceptors and thus contribute to redox reactions in the biofilm matrix, e.g. by direct electron transfer from metals (e.g. Fe) or biominerals (e.g. FeS). In the presence of a specific acceptor (e.g. O_2 under aerobic conditions or nitrate under anaerobic conditions), the redox pathway would lead to a depolarization of the cathode and thus intensify the corrosion. A schematic model of the corrosion reactions of ferrous metals, which includes the binding of exopolymeric substances to metal ions in an oxidized biofilm, can be found in the work of Beech and Sunner, (2004).

Studies of the Fe-hydroxide biofilm layer have shown that bacterial exopolymers and acidic polysaccharides can occur in acagenite (b-FeOOH) (Chan et al., 2003). It has been demonstrated that polymer production aims to localize the precipitation of iron oxyhydroxide immediately outside the cell to increase the energy of cell metabolism by accelerating proton movement. In the presence of iron, the Fe-oxyhydroxide surface is associated with biofilm polymers to adsorb Fe(II) ions, leading to their oxidation and contributing to the cathodic reaction (Beech and Sunner, 2004). It is well known that catalysis of the cathodic proton/water reaction occurs mainly on an iron alloy under anaerobic conditions (Stott, 1993):

$$2H^+ + 2e^- \rightarrow H_2 \text{ or } 2H_2O + 2e^- \rightarrow H_2 + 2OH^- \quad (7)$$

The production of metabolites of sulfide ions activates sulfate-reducing bacteria:

$$SO_4^{2-} + 4H_2O + 8e^- \rightarrow S^{2-} + 8OH^- \quad (8)$$

This forms a FeS deposit that catalyzes the metal surface's cathodic reaction between protons and water. However, the mechanism of anaerobic biocorrosion is more complex than can be seen in the work of Beech and Sunner, (2004). Hydrogen consumption by sulfate-reducing bacteria cannot

have a direct effect on the corrosion rate because reaction 8 can be decomposed into a Vollmer reaction (Lee et al., 1995):

$M + H_2O + e^- \leftrightarrow M\text{-}H_{ads} + OH^-$ (9)

which is followed by the Tafel reaction:

$2M\text{-}H_{ads} \leftrightarrow 2M + H_2$ (10)

or Hierovski's reaction:

$M\text{-}H_{ads} + H_2O + e^- \leftrightarrow M + H_2 + OH^-$ (11)

Tafel and Hierowski reactions are limiting reactions on the surface of the iron alloy. The consumption of generated hydrogen cannot accelerate them. Sulfate-reducing bacteria have an advantage in hydrogen production during corrosion processes (reaction 7) and use it as an electron donor that supports sulfide production (Mehanna et al., 2009). Hydrogenase enzymes produced by sulfate-reducing bacteria can be adsorbed on the steel surface, catalyzing the reduction of protons (Da Silva et al., 2002), and the presence of phosphate buffers can cause an additional cathodic reaction.

On the other hand, EDS spectra analysis shows the entry of active chloride ions (Cl^-) under the biofilm and the lowering of the pH value in the anode area when $FeCl_3$ is formed. This favored initiating and propagating pitting corrosion (Yuan and Pehkonen, 2007). The anodic reaction describes the effect of chloride ions on the passive layer of stainless steel (Yuan and Pehkonen, 2009):

$Fe^0 \rightarrow Fe^{2+} + 2e^-$ (12)

$Fe^{2+} + 2H_2O + Cl^- \rightarrow Fe(OH)_2 + 2HCl$ (13)

$Fe(OH)_2 + 3Cl^- \rightarrow FeCl_3 + 2OH^- + e^-$ (14)

$FeCl_3 + 3H_2O \rightarrow Fe(OH)_3 + 3HCl$ (15)

Chloride ions react with the oxyhydroxide layer and displace OH^- ions in the oxide layer until the soluble product $FeCl_3$ is formed. The intermediate product $FeCl_3$ is hydrolyzed to the porous precipitate $Fe(OH)_3$ under decreasing pH at the original corrosion site. Such reactions result in a self-reinforcing or autocatalytic mechanism of hole growth.

On the other hand, the EDS spectrum indicates a synergistic interaction of active biogenic sulfide and chloride anions that are responsible for the initiation of grey spot corrosion on the steel surface exposed to sulfate bacteria. It is generally concluded that chloride ions can catalyze sulfide dissolution, which causes metastable pitting corrosion in stainless steel. The anodic dissolution of

sulfide ions (S^{2-}) in the marine environment is represented by the following reactions (Yuan and Pehkonen, 2009):

$$Fe^0 + H_2S \rightarrow FeSH^-_{ads} + H^+ \quad (16)$$

$$FeSH^-_{ads} \rightarrow FeSH^+_{ads} + 2e^- \quad (17)$$

$$FeSH^+_{ads} \rightarrow FeS_{1-x} + xHS^- + (1-x)H^+ \quad (18)$$

$$Fe^0 + 2Cl^- + H_2O \rightarrow [Fe(OH)^+ + Cl^-] + HCl + 2e^- \quad (19)$$

$$Fe(OH)^+ + HS^- \rightarrow FeS + 2H_2O \quad (20)$$

Electrochemical investigations of the corrosion rates of stainless steel by sulfate-reducing bacteria under different experimental conditions

Javed et al., (2022) investigated the corrosion effect of sulfate-reducing bacteria on stainless steel samples, UNS30400, exposed to sulfate-reducing bacteria and conditions where no sulfate-reducing bacteria existed. The samples that exhibited pitting corrosion were exposed to an environment containing iron ions and an environment containing chloride ions for 32 days under anaerobic conditions and at a temperature of 30 °C. The electrochemical tests show that the difference between the passivity breakdown potential, E_b and E_{corr} for the 304 stainless steel was smallest for the samples exposed to sulfate-reducing bacteria and a solution containing chloride ions. Based on the criteria of Pardo et al., (2000), the authors concluded that pitting corrosion occurs on UNS30400 stainless steel specimens due to the interaction of sulfate-reducing bacteria and a relatively high salt concentration in the tested medium.

The influence of sulfate-reducing bacteria on the corrosion behavior of the X80 steel pipeline in acidic soil was investigated using electrochemical impedance spectroscopy (EIS). The formed biofilm of sulfate-reducing bacteria developed over the entire surface of the tested steel in a thin layer of 20μm, and after 14 days, the number of living sessile cells was more significant than the number of dead cells of the sulfate-reducing bacteria. Under the abiotic conditions of the formed biofilm, an EIS analysis was performed, according to which the size of the semicircle of the Nyquist plot decreases with time, indicating an acceleration of the corrosion processes over time. However, under the conditions of the inoculated soil solutions, the activity of the sulfate-reducing bacteria and their metabolic products inhibit the corrosion of the steel during the first four days of the experiment and then accelerate the corrosion until the end of the experiment (Chen et al., 2021).

The authors Sun et al., (2011) investigated the corrosion effect of sulfate-reducing bacteria (23000-35000 cells/g soil) on stainless steel samples, 1Cr18Ni9Ti, buried for 136 days and the addition of a particular concentration of chloride ions to the soil. The work showed that the electrode potential (Eh) decreases with increasing chloride ion concentration, which is explained by the acceleration of the activity of sulfate-reducing bacteria in the soil. These results also indicate that the dissolved oxygen in the soil decreases with increasing Cl^- concentration, which is suitable for the respiration of the sulfate-reducing bacteria.

In addition, the authors presented the relationship between the corrosion potential (mixing potential) and the concentration of chloride ions in the soil with and without sulfate-reducing bacteria injection. In both cases, the corrosion potential of stainless steel decreases with increasing chloride ion concentration. However, the potential of steel in soils with sulfate-reducing bacteria is more negative than the potential of steel samples in soils without sulfate-reducing bacteria, suggesting that the presence of sulfate-reducing bacteria increases the tendency of steel corrosion. The same authors showed changes in corrosion rates and maximum pitting depth of stainless steel as a function of Cl^- concentration, with and without injection of sulfate-reducing bacteria into the soil. It was found that higher corrosion rates occur at lower Cl^- concentrations (1.0%) in the presence of sulfate-reducing bacteria when hydrogenase is also observed. Hydrogenase from sulfate-reducing bacteria consumes hydrogen atoms on the steel surface and thus accelerates the cathodic depolarization reaction (Guo et al., 1992). However, when the Cl^- concentration increases to 2.0%, a constant corrosion rate of the steel in the soil occurs.

Sun et al.'s, (2011) experimental results show that the maximum depth of pits formed on steel with sulfate-reducing bacteria is more remarkable than without sulfate-reducing bacteria. This indicates that sulfate-reducing bacteria increase the pitting tendency. It has also been shown that stainless steel corrosion does not occur when the Cl^- concentration is 0.05% in soils with sulfate-reducing bacteria.

Considering that we are dealing with soil that contains pores, it is clear that the contact area between the steel and the soil particles has a low oxygen content, while the area of the boundary layer between the steel and the pores is in an area with a high oxygen concentration. The metal corrosion that occurs under the influence of unequal oxygen concentrations is called corrosion by differential aeration (Vujičić, 2002). The sites mentioned above may be available for the growth of sulfate-reducing bacteria. As is known, sulfate-reducing bacteria reduce sulfates to sulfide

(FeS), sulfates being electron acceptors under anaerobic conditions. Sites on the surface of stainless steel where FeS accumulates act as cathodes, forming a new electrochemical cell (Iv et al., 1984). Therefore, the passive layer is no longer stable due to increased chloride ion concentration.

EIS results showed that the corrosion processes of stainless steel in the presence of sulfate-reducing bacteria are controlled by concentration polarization (Sheng et al., 2007). Based on the impedance values of the film resistance (R_f), they concluded that the number was higher in soil samples with sulfate-reducing bacteria than in other samples without sulfate-reducing bacteria. On the steel surface occured complex film of a corrosion products and bacterial biofilm, and the compactness of the film thus formed is better than the compactness of the single-layer film.

Effective prevention of surface reactions by the film formation was also found. Feng et al., (2008) showed that the film formed on steel in soil with sulfate-reducing bacteria is less stable than the film formed on steel without sulfate-reducing bacteria. This indicates that the complex film gradually degrades when the Cl^- concentration exceeds 1.0%. Thus, sulfate-reducing bacteria increase the pitting tendency of stainless steel, 1Cr18Ni9Ti. The author concludes that pitting corrosion is due to the influence of chloride and sulfide ions in the soil, which are metabolic products excreted by sulfate-reducing bacteria. In other words, sulfate-reducing bacteria increase the susceptibility of steel to pitting corrosion in Cl^- containing soils.

On the other hand, a thermomechanical process such as welding can form undesirable phases on the steel if not carried out under suitable conditions (Tavares et al., 2007). For example, Antony et al., (2008) investigated the influence of microstructure formation of the weld in tungsten gas on duplex steel (2205) under anaerobic conditions with Cl^- and sulfate-reducing bacteria as substrate. The chemical composition of the welded area consists of Ni and Mo, with a content of more than 2%. The duplex steel samples were exposed to an aqueous medium with a pH of 7.5 and a temperature of 30 °C for 40 days. The shift in corrosion potential, E_{corr}, relative to the active value of the potential, which remained constant until the end of exposure of the steel samples, was indicated. Similar E_{corr} variations were determined for passivated steels in an environment with sulfate-reducing bacteria (Xu et al., 2008). In the work of Antony et al. (2008), it was also shown that the change in sulfide concentration in the given environment does not affect the E_{corr} value. It was revealed that the E_{corr} value reaches a particular active value even at low sulfide concentrations in the reactive environment. In contrast, the E_{corr} value in sterile samples indicates an improvement

in metal passivity compared to samples exposed to an environment with sulfate-reducing bacteria during the 40 days of the experiment (Antony et al., 2008).

The electrochemical data show no significant difference between the base metal and the spot weld. However, the spot has higher anodic currents and a lower E_{corr} value than the base metal. Nevertheless, the destruction of the passive layer of the base metal and the welded spot increased when exposed to sulfate-reducing bacteria compared to a sterile environment.

The current value increases with the potential differences for all samples for the three distinct phases formed on the base metal. The current density of samples previously exposed to sulfate-reducing bacteria for 40 days is twice as high as the current density of samples exposed to a sterile medium during the same period. This indicates a strong influence of sulfate-reducing bacteria on the increase of corrosion of steel 2205. The current density of the samples before exposure was an order of magnitude lower than that of the samples in a sterile environment for the same period. In each of the three different environments (before and after exposure in a sterile environment and in an environment with sulfate-reducing bacteria), the weld exhibited a higher current density at all potentials than the base metal's current density. In the two steel samples previously exposed to an environment with sulfate-reducing bacteria, the current density of the welded area increases faster at higher potentials than the current density of the base metal (Antony et al., 2008).

Continuous exposure of SS 316L samples to sulfate-reducing bacteria and a combination of sulfate-reducing bacteria and ferric bacteria results in a significant decrease in E_{corr} and the steel's polarization resistance, R_p, and corrosion acceleration compared to the observed samples exposed only to ferric bacteria or sterile medium for the same time interval. During the additional exposure to the solution with sulfate-reducing bacteria and iron bacteria, E_{corr} was strongly reduced, and a value of -0.54 V was reached for 24 days. The results thus indicate the metabolic activity of bacteria and its influence on corrosion processes. The presence of sulfate-reducing bacteria led to higher corrosion rates than iron-containing bacteria. Still, it was also shown that the combination of sulfate-reducing and iron-containing bacteria led to higher corrosion rates than the single effect of the same bacteria (Zhang et al., 2007).

Similarly, based on graphical EIS methods, the authors Nguyen et al., (2008) found that the polarization resistance of SS 304 steel is reduced in the presence of sulfate-reducing bacteria. This indicates that the metabolic products of sulfate-reducing bacteria cause changes in the electrochemical properties of steel (Sheng et al., 2007).

In addition, diffusion processes occur within the biofilm, involving bacterial cells and their insoluble products, such as metal sulfides in solution. Impedance parameters indicate the difference between abiotic and biotic corrosion. The first difference between abiotic and biotic corrosion is the charge transfer resistance (R_{ct}), whose value is high at 805.6 Ω in the presence of sulfate-reducing bacteria compared to 600 Ω in the environment without sulfate-reducing bacteria. In addition, the roughness coefficient n for stainless steel in a sterile environment is around 1, indicating a relatively homogeneous passive film. A lower coefficient value of 0.60 for the passive film in the presence of sulfate-reducing bacteria indicates the roughness of the passive film. Also, forming an anaerobic film of stainless steel in a saturated artificial seawater solution with a substrate of sulfate-reducing bacteria leads to a reduction in the resistivity of the passive film, R_{pf}. The decrease in the resistivity and roughness parameters of both types of coatings, the steel and the passive film indicate two critical points. First, sulfate-reducing bacteria and their products can be adsorbed on the metal surface or become part of the passive film, changing the charge of the Me/solution interface and the passive film, which more easily leads to metal surface corrosion. Secondly, the EIS results show that the biofilm plays a vital role in metal corrosion, and this has been confirmed by other authors (Wang and Liang, 2008; Xu et al., 2008).

Conclusion

Based on various tests of stainless steel with sulfate-reducing bacteria, corrosion of the tested metal was demonstrated during a very short period of exposure to these bacteria under anaerobic conditions.

It was shown that sulfate-reducing bacteria lead to more severe pitting corrosion of SS 304 passive steel than *Pseudomonas* bacteria. When investigating metal corrosion, it is not the number of sulfate-reducing bacteria but the metabolic products of the bacteria that may be of greater importance. The exopolymeric substance is also important for binding the cells in the biofilm structure.

The weld on the steel allows access to and binding microorganisms, i.e., grease and oil deposits located on the pipe wall, which could be the primary source of the carbon needed for the bacteria's development and growth. In sulfate-reducing bacteria, FeSx proteins, such as the hydrogenases of

D. desulfuricans, play an essential role in biological electron transfer processes and many enzymatic reactions.

Pitting corrosion is caused by the influence of Cl^- and sulfides in the soil, which are produced by the secretion of sulfate-reducing bacteria. In other words, sulfate-reducing bacteria increase steel's susceptibility to pitting in Cl^- containing soils. Regardless, it was found that the destruction of the passive layer of the base metal and the welded area was greater when exposed to sulfate-reducing bacteria than in a sterile environment.

The sulfate-reducing bacteria resulted in a higher corrosion rate than iron-containing bacteria. Still, it was also shown that the combination of sulfate-reducing and iron-containing bacteria resulted in higher corrosion rates than the single effect of the same bacteria.

Despite the significant research in the field of microbiological corrosion, many questions remain unanswered, such as the influence of biofilms on the electrochemical properties of metals. For this reason, many authors believe that the study of sulfate bacteria on stainless steel should be continued.

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Conflict-of-Interest Statement

Declarations of interest: none

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Efekat sulfat-redukujućih bakterija na nerđajući čelik: revijalni rad

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SAŽETAK

Legure otporne na koroziju, kao što je nerđajući čelik, slično inertnim nemetalnim površinama, pružaju idealnu podlogu za kolonizaciju mikroorganizama zbog odsustva produkata korozije. Nerđajući čelici su osetljivi na tačkastu i druge tipove lokalizovane korozije u medijumima koji sadrže hloride, kao što je morska voda. Bakterije koje redukuju sulfate igraju važnu ulogu u koroziji nerđajućeg čelika u morskim i zemljišnim sredinama. Mikrobi koji koriste sulfat kao terminalni akceptor elektrona za svoje disanje učestvuju u redukciji sulfata, što dovodi do stvaranja H_2S, što može dovesti do značajnog povećanja brzine anodnih i katodnih procesa. Pregledom literature ustanovljeno je da većina studija o mikrobno indukovanoj koroziji nerđajućih čelika ukazuje da je ona uzrokovana uticajem hlorida i sulfida u zemljištu koji nastaju kao rezultat lučenja različitih vrsta mikroba. Uticaj sulfat-redukujućih bakterija na nerđajući čelik detaljno je opisan u ovom preglednom radu kroz nekoliko celina: opšta svojstva sulfat-redukujućih bakterija, morfologija i hemijski sastav biofilma i produkata korozije, mehanizmi mikrobiološke korozije nerđajućih čelika i elektrohemijska ispitivanja brzine korozije nerđajućeg čelika pod uticajem sulfat-redukujućih bakterija pri različitim eksperimentalnim uslovima.

Ključne reči: nerđajući čelik, sulfat-redukujuće bakterije, korozija

Effet des bactéries sulfato-réductrices sur l'acier inoxydable : une revue

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RÉSUMÉ

Les alliages résistants à la corrosion tels que l'acier inoxydable constituent un substrat idéal pour la colonisation microbienne en raison de l'absence de produits de corrosion, similaires aux surfaces inertes non métalliques. Les aciers inoxydables sont sensibles aux piqûres et à d'autres types de corrosion localisée dans les milieux contenant des chlorures tels que l'eau de mer. Les bactéries sulfato-réductrices jouent un rôle essentiel dans la corrosion de l'acier inoxydable dans les environnements marins et du sol. Le sulfate est utilisé par les microbes comme accepteur d'électrons terminal car leur respiration entraîne la réduction du sulfate conduisant à la formation de H_2S, ce qui peut entraîner une augmentation significative des processus anodiques et cathodiques et de la corrosion des matériaux. En examinant la littérature, il a été constaté que la plupart des études sur la corrosion microbienne dans les aciers inoxydables indiquent qu'elle est causée par l'influence des chlorures et des sulfures dans le sol résultant de la sécrétion de bactéries sulfato-réductrices. L'influence des bactéries sulfato-réductrices sur l'acier inoxydable est décrite en détail dans cette revue, qui peut être vue à partir des points suivants : propriétés générales des bactéries sulfato-réductrices, morphologie et composition chimique du biofilm et des produits de corrosion, mécanismes de corrosion microbiologique par les bactéries sulfato-réductrices et études électrochimiques des taux de corrosion de l'acier inoxydable par les bactéries sulfato-réductrices dans différentes conditions expérimentales.

Mots-clés : acier inoxydable, bactéries sulfato-réductrices, corrosion

Влияние сульфатредуцирующих бактерий на нержавеющую сталь: обзор

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Резюме

Коррозионно-стойкие сплавы, такие как нержавеющая сталь, обеспечивают идеальную субстрат для микробной колонизации благодаря отсутствию продуктов коррозии, подобно инертным неметаллическим поверхностям. Нержавеющая сталь чувствительна к точечной коррозии и другим видам локальной коррозии в хлоридсодержащих средах, таких как морская вода. Сульфатредуцирующие бактерии играют важную роль в коррозии нержавеющей стали в морской и почвенной среде. Сульфат используется микробами в качестве терминального акцептора электронов, поскольку их дыхание приводит к восстановлению сульфатов, что приводит к образованию H_2S, что может привести к значительному увеличению анодных и катодных процессов и коррозии материалов. При обзоре литературы было установлено, что большинство исследований микробно-индуцированной коррозии в нержавеющих сталях указывают на то, что она вызвана влиянием хлоридов и сульфидов в почве, образующихся в результате секреции сульфатредуцирующих бактерий. В данном обзоре подробно описано влияние сульфатредуцирующих бактерий на нержавеющую сталь, которое видно из следующих моментов: общие свойства сульфатредуцирующих бактерий, морфология и химический состав биопленки и продуктов коррозии, механизмы микробиологической коррозии сульфатредуцирующих бактерий и электрохимические исследования скоростей коррозии нержавеющей стали сульфатредуцирующими бактериями в различных экспериментальных условиях.

Ключевые слова: нержавеющая сталь, сульфатредуцирующие бактерии, коррозия

Auswirkung von sulfatreduzierenden Bakterien auf Edelstahl: ein Überblick

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ABSTRAKT

Korrosionsbeständige Legierungen wie rostfreier Stahl bieten ein ideales Substrat für die mikrobielle Besiedlung, da sie ähnlich wie inerte nichtmetallische Oberflächen keine Korrosionsprodukte bilden. Nichtrostende Stähle sind empfindlich gegenüber Lochfraß und anderen Arten von örtlicher Korrosion in chloridhaltigen Medien wie Meerwasser. Sulfatreduzierende Bakterien spielen eine wesentliche Rolle bei der Korrosion von nichtrostendem Stahl in Meeres- und Bodenumgebungen. Sulfat wird von Mikroben als terminaler Elektronenakzeptor verwendet, da ihre Atmung die Sulfatreduktion antreibt, was zur Bildung von H2S führt, was wiederum zu einer signifikanten Zunahme der anodischen und kathodischen Prozesse und der Korrosion von Materialien führen kann. Bei der Durchsicht der Literatur wurde festgestellt, dass die meisten Studien über mikrobiell induzierte Korrosion bei rostfreien Stählen darauf hindeuten, dass sie durch den Einfluss von Chloriden und Sulfiden im Boden verursacht wird, die durch die Absonderung sulfatreduzierender Bakterien resultieren. Der Einfluss sulfatreduzierender Bakterien auf nichtrostenden Stahl wird in dieser Übersichtsarbeit ausführlich beschrieben, was aus den folgenden Punkten ersichtlich ist: allgemeine Eigenschaften sulfatreduzierender Bakterien, Aufbau und chemische Zusammensetzung von Biofilm und Korrosionsprodukten, Mechanismen der mikrobiologischen Korrosion durch sulfatreduzierende Bakterien und elektrochemische Untersuchungen der Korrosionsraten von nichtrostendem Stahl durch sulfatreduzierende Bakterien unter verschiedenen experimentellen Bedingungen.

Schlüsselwörter: edelstahl, sulfatreduzierende bakterien, korrosion

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The potential of *Hedera helix* L. stems and leaves for atmospheric pyrene phytomonitoring

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ABSTRACT

The aim of this work was to investigate the phytomonitoring potentials of *Hedera helix* L. from the Bor′s municipality concerning (atmospheric) Pyrene as a well-known hazardous substance. The results of gas chromatographic-mass spectrometric analysis of the unwashed stems and leaves showed that Pyrene concentrations obviously varied at all selected rural and urban/industrial zones (RZ and UIZ). These first signs of various sources of Pyrene in the investigated area were later supported by the results of the performed Pearson's correlation analysis, which showed that the detected Pyrene concentrations came not only from the vicinal heating and smelting plants, as the main sources of pollution in the whole region but also from the domestic heating in RZ, or forest fires, or controlled fires in the cultivated fields, and finally from the traffic in all zones. The correlation analysis also signalized that at some locations, the detected concentrations (especially in stems) came not only from the atmosphere but also from the soil. The calculated factor R confirmed the sites with the greatest atmospheric impact. Very high R values were calculated for 3 sites (two in UIZ and one in RZ) with the highest value of 1.61. Based on the obtained results, it can be concluded that the investigated stems and leaves, with the applied chemical and statistical analyses, and the calculation of R factor, can serve as a useful tool in the atmospheric Pyrene phytomonitoring.

Keywords: phytomonitoring, pyrene, *Hedera helix* L.

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Introduction

Pyrene (Pyr) is a complex organic compound (Fig. 1) belonging to the class of polycyclic aromatic hydrocarbons (PAHs). It comprises 4 fused benzene rings (Fig. 1) with a molecular weight of 202.30 g/mol. The melting point of this compound is 150.6ˆC, whereas the boiling point amounts to 404°C; Henry's Law Constant, which indicates the chemical's potential to volatilize, is 1.2 (Pa/m^3) (Verbruggen, 2012).

Figure 1. Pyrene structural formula (NIST, Chemistry, WebBook, SRD 69)

As with other PAHs, Pyr has a pretty low solubility in water – 77 μL, resulting in better accumulation in the aquatic organisms and sediments. Its vapor pressure – $2.5 \cdot 10^{-6}$ mmHg is also low. For this compound, Log Kow (that provides an indication of the potential of the organic compound to partition between water and lipids) is 4.96, while log Koc (indicates a compound's potential to bind to organic carbon in soils) is 4.75 (Alagić et al., 2015; Papludis et al., 2022). Toxicological characteristics of Pyr are also noticed. Its toxicity was observed not only in humans but in all animals, too (especially in aquatic species). Its toxicity to plants is not defined yet but it can be supposed that, in some extent, it may by have same toxic effects in these organisms. According to USEPA (United States Environmental Protection Agency) IRIS program (Integrated Risk Information System) for humans, Reference Concentration for Inhalation Exposure (RfC) is not assessed, whereas the amount of its Reference Dose (RfD) for Oral Exposure is $3 \cdot 10^{-2}$ mg/kg-day. In the case of Pyr, the kidneys and the complete urinary tract are the most endangered. Fortunately, there is no evidence of human carcinogenicity (https://iris.epa.gov/ChemicalLanding/&substance_nmbr=445).

Similarly to other PAH compounds, Pyr originates from different anthropogenic and natural (eruptions of volcanoes and fires in forests) sources. In general, PAHs are released from the pyrolysis of hydrocarbons present in waste, fossil fuels (tar, wood, and oil), and biomass. They

are present in grilled food and tobacco smoke, too (Alagić et al., 2015; Papludis et al., 2022, 2023a). Its concentrations in the environment are constantly increasing, and Pyr is included in 16 priority PAHs, known as dangerous pollutants, even in micro-concentrations (Alagić et al., 2016, 2017; Morillo et al., 2007).

The mentioned facts raised human need to track the concentrations of these compounds, including Pyr, in the environment, trying to protect the endangered local populations. One of the most appropriate methods for PAH/Pyr monitoring is a biological method known as bio-/phyto-monitoring, which is mostly based on the monitoring of PAH concentrations in the aboveground plant parts (Reinholds et al., 2015; Sari et al., 2020). It is also known that most plants can absorb this substance from the soil but cannot transport it to the aboveground parts (Alagić et al., 2016, 2017). In this work, the concentrations of Pyr were monitored using unwashed, dried, stems and leaves of *Hedera helix* L. (poison ivy, PI) from the Bor region. The concentrations were detected applying gas chromatographic-mass spectrometric, GC/MS method.

Experimental

The locations in the Bor region that were chosen for the conducted phytomonitoring experiment were as follows:

- Flotacijsko jalovište (FJ), Bolničko naselje (BN), Slatinsko naselje (SN) and Naselje Sunce (NS) in the urban-industrial zone (UIZ), and
- Oštrelj (O), Borsko jezero (BJ) Krivelj (K), Zlot (Z) and Gornjane (G) in the rural zone (RZ).

The distances of the chosen locations from the city heating plant and the copper smelter as the main sources of pollution in the whole region, are as follows: for the site FJ (an abandoned flotation tailings pond) - 0.7 km, for the site BN (near the hospital in Bor) - 1.3 km, and for the two suburb sites SN and NS - 3.2 km, and 3.6 km, respectively. The sites from RZ are located regarding to the Bor town as follows: site O - 4.5 km, site BJ - 7 km, site K - 8 km, site Z - 13 km, and site G - 19 km.

It should be mentioned here that the town of Bor (UIZ) is a relatively novel settlement, with the developed central heating based on coal burning, whereas in RZ, the domestic heating based on different kind of fuels is dominant.

Plant material of PI was collected from the selected sites in a way that was described in Papludis et al. (2023a), together with the way of its preparation and GC/MS analysis. Used apparatus was 7890/7000B GC-MS-MS triple quadrupole device (Agilent Technologies, USA), equipped with a Combi PAL auto sampler and an HP-5MS capillary column. The only distinction was that the leaves and stems were analyzed as unwashed.

The processing of the results of PI samples, i.e., in terms of evaluating the level of atmospheric Pyr in the Bor region, the ratio (factor R) of Pyr concentrations in leaves and stems, was calculated as follows: $R_{leaf/stem} = C_{leaf}/C_{stem}$ (C_{leaf}, and C_{stem} are the concentrations of Pyr in the corresponding plant organs at each location). The amounts of factor R bigger than 1 point to the sites with the greatest atmospheric impact (Oliva and Mingorance, 2006; Papludis et al., 2023b).

The relation between the content of Pyr in plant organs and the distance from the main sources of contamination in the entire region (the vicinal heating and smelting plants sited in the mining-metallurgical complex in the town of Bor), the statistical method of Pearson's correlation analysis (Miller, and Miller, 2005; Reinholdset al., 2015; Sari et al., 2020) was performed in IBM SPSS program, version 20.

Results and Discussion

Measured concentrations of Pyr (in μg/kg dry weight) in the stems and leaves of PI are given in Table 1. It shows that almost all investigated locations had very different concentrations of Pyr, which ranged from nd (not detected) in stems from site K to 167.42 μg/kg in the same plant parts from site SN. The lowest leaf concentration was found at site S: 2.41 μg/kg, whereas the highest was in leaves from site O: 18.96 μg/kg. The only sites with similar stem and leaf concentrations were FJ and BN from UIZ. These facts were the first signals that the content of Pyr in the investigated parts from UIZ and RZ was not of the same origin (Alagić et al., 2016, 2017), which was later supported by the results obtained from the performed Pearson's correlation analysis.

Table 1. The concentrations of Pyr (μg/kg, dw) in the leaf and stem samples of PI

Location	Pyr stems	Pyr leaves
FJ	8.73	8.24
BN	8.01	8.45
SN	167.42	9.48
NS	10.26	13.09

O	89.43	18.96
S	68.52	2.41
BJ	8.44	7.19
K	nd	17.01
Z	7.90	16.13
G	4.70	7.56

nd – not detected

More precisely, Pearson′s correlation analysis showed that the concentrations of Pyr in plant stems were in a weak negative correlation with the distance from the main sources of pollution (p = –0.29), which means that with distance increasing, the concentrations of Pyr decreased. However, this low value of the Pearson′s correlation coefficient (generally, less than 0.39; Evans, 1996) has a practical meaning that the main sources of pollution had a very low influence on stem concentrations in UIZ, and RZ. In the other hand, leaf Pyr concentrations were in a positive correlation with the distance from the same sources of pollution but also, with a very low value (p = 0.039); this means that with distance increasing, the concentrations of Pyr also increased but in a very low extent. This fact finally points that the main sources of pollution cannot be treated as the only sources of Pyr in the investigated area - they definitively point on additional sources of Pyr (Alagić et al., 2016, 2017), which may include: the domestic heating in rural areas, forest fires, controled firest in the filds with the cultivated plants, and finally, the developing trafic in all zones. In addition, Pearson′s correlation coefficients also showed that the contents of Pyr in plant stems were in a statistically significant positive correlation (p = 0.740, at the 0.05 level) with the corresponding soil concentrations (given in Papludis et al., 2023a), while leaf and soil contents were in a low positive correlation (p = 0.395). Although the correlation between the pollutant concentration in soil and in the unwashed aboveground part cannot be taken as a confident (only the pollutant concentration in the washed aboveground part can reflect an actual bioaccumulation), these calculated correlation trends may represent an evidence that, in general, stems had a better ability for Pyr assimilation from the soil than leaves, which further explains some cases of locations with higher stem than leaf concentrations (the locations SN, O, S, and BJ), i.e., this may explain that stem Pyr concentrations at mentioned locations, came probably, predominantly from the soil, and not from the atmosphere.

The sites with the greatest atmospheric impact were confirmed by the calculation of factor R, presented in Fig. 2.

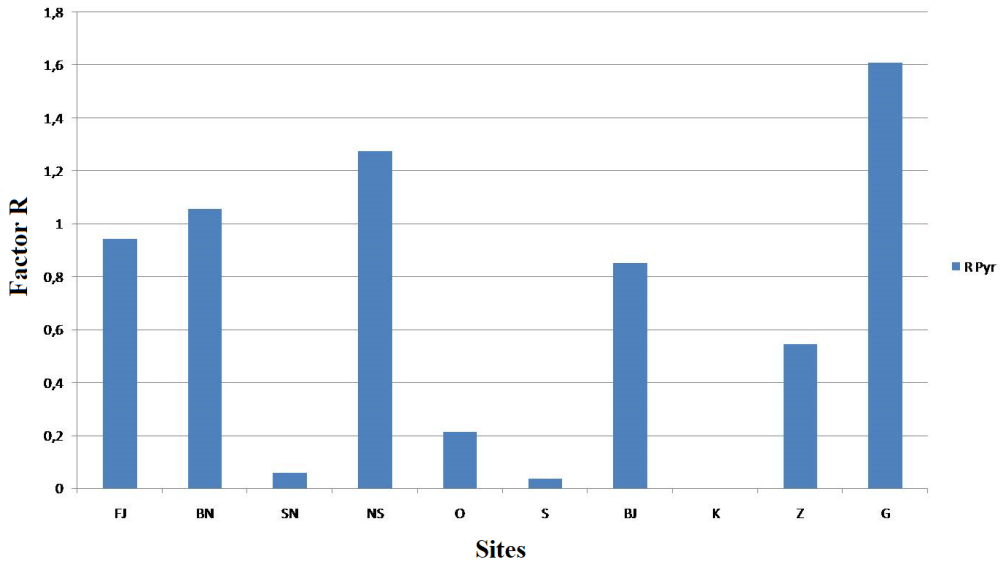

Figure 2. The values of factor R at the investigated sites

R values higher than 1, were calculated for the sites BN, NS, and G (where the highest value were found, 1.61). Also, amounts very close to 1 were found in the case of the sites from UIZ: FJ, and BJ. The lowest R value was found at the site S (0.04), which points that the contribution of atmospheric pollution was the lowest at this place. R factor was not posible to calculate for the site K.

Conclusion

The results for Pyr concentrations in the stems and leaves of PI showed that these concentrations varied at the selected locations from nd in stems from RZ (site K) to 167.42 μg/kg in the same plant parts from the site SN in UIZ. The lowest and the highest leaf concentrations were found at the sites from RZ: S - 2.41 μg/kg, and O - 18.96 μg/kg. The highest R-value was at site G = 1.61, whereas the lowest R-value was found at site S = 0.04.

With the assumption (resulting from the performed Pearson′s correlation analysis) that the pyrene content in the stem is mainly the consequence of accumulation from the soil, whereas the leaf content mainly originates from the atmospheric deposition, the ratio of pyrene concentration in unwashed leaves and stems (R) can serve as an indicator of atmospheric pyrene pollution.

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Potencijal stabljika i lišća biljke *Hedera helix* L. za fitomonitoring atmosferskog pirena

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SAŽETAK

Cilj ovog rada bio je ispitivanje fitomonitoring potencijala *Hedera helix* L. iz Borske opštine u odnosu na (atmosferski) piren, kao dobro poznatu opasnu supstancu. Rezultati gasno hromatografske-maseno spektrometrijske analize neopranih stabljika i lišća pokazali su das u koncentracije pirena uočljivo varirale na svim odabranim ruralnim i urbano/industrijskim zonama (RZ i UIZ). Ovi prvi signali o različitim izvorima pirena u ispitivanoj oblasti bili su kasnije podržani rezultatima izvedene Pearson-ove korelacione analize koji su pokazali da su detektovane koncentracije pirena došle ne samo od susednih postrojenja toplane i metalurške fabrike, kao glavnih izvora zagađenja u celom regionu, već takođe od individualnih ložišta in RZ, ili šumskih požara, ili kontrolisanih požara u kultivisanim poljima i konačno, od saobraćaja u svim zonama. Korelaciona analiza je takođe pokazala da su na nekim lokacijama, detektovane koncentracije (posebno u stabljikama), najverovatnije došle ne samo iz atmosfere, već i iz zemljišta. Obračunati faktor R je potvrdio mesta gde je atmosfersko zagađenje imalo najveći uticaj. Veoma visoke R vrednosti obračunate su za 3 mesta (dva u UIZ i jedno u RZ), sa najvišom vrednošću od 1.61. Na osnovu dobijenih rezultata, može se zaključiti da ispitivane stabljike i lišće, sa primenjenim hemijskih i statističkih analiza, kao i izračunavanjem R faktora, mogu poslužiti kao koristan alat u fitomonitoringu atmosferskog pirena.

Ključne reči: fitomonitoring, piren, *Hedera helix* L.

Le potentiel des tiges et des feuilles d'*Hedera helix* L. pour la phytosurveillance du pyrène atmosphérique

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RÉSUMÉ

L'objectif de ce travail était d'étudier le potentiel phytotechnique d'*Hedera helix* L. de la municipalité de Bor en ce qui concerne le pyrène (atmosphérique) en tant que substance dangereuse bien connue. Les résultats de l'analyse par chromatographie en phase gazeuse et spectrométrie de masse des tiges et des feuilles non lavées ont montré que les concentrations de pyrène variaient manifestement dans toutes les zones rurales et urbaines/industrielles sélectionnées (RZ et UIZ). Ces premiers signes de diverses sources de pyrène dans la zone étudiée ont ensuite été confirmés par les résultats de l'analyse de corrélation de Pearson, qui a montré que les concentrations de pyrène détectées provenaient non seulement du chauffage vicinal et des fonderies, qui étaient les principales sources de pollution dans toute la région, mais aussi du chauffage domestique dans la RZ ou des feux de forêt ou des feux contrôlés dans les champs cultivés et enfin de la circulation dans toutes les zones. L'analyse de corrélation a également signalé qu'à certains endroits, les concentrations détectées (en particulier dans les tiges) provenaient non seulement de l'atmosphère mais aussi du sol. Le facteur R calculé a confirmé les sites ayant le plus grand impact atmosphérique. Des valeurs R très élevées ont été calculées pour 3 sites (deux dans UIZ et un dans RZ) avec la valeur la plus élevée de 1,61. Sur la base des résultats obtenus, on peut conclure que les tiges et les feuilles étudiées, avec les analyses chimiques et statistiques appliquées, et le calcul du facteur R, peuvent servir d'outil utile dans la phytosurveillance atmosphérique du pyrène.

Mots-clés : phytomonitoring, pyrène, *Hedera helix* L.

Потенциал стеблей и листьев *Hedera helix* L. для атмосферного фитомониторинга пирена

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Резюме

Целью данной работы было исследование возможностей фитомониторинга Hedera helix L. из муниципалитета Бор в отношении (атмосферного) пирена как хорошо известного опасного вещества. Результаты газохромато-масс-спектрометрического анализа немытых стеблей и листьев показали, что концентрации пирена явно варьировали во всех выделенных сельских и урбанистических/промышленных зонах (РЗ и УИЗ). Эти первые признаки наличия различных источников пирена в исследуемом районе были впоследствии подтверждены результатами проведенного корреляционного анализа Пирсона, который показал, что обнаруженные концентрации пирена исходят не только от местного отопления и плавильных заводов, как основных источников загрязнения во всем регионе, но и от бытового отопления в RZ. или лесные пожары, или контролируемые пожары на обрабатываемых полях, и, наконец, от транспорта во всех зонах. Корреляционный анализ также показал, что в некоторых местах обнаруженные концентрации (особенно в стеблях) поступали не только из атмосферы, но и из почвы. Расчетный коэффициент R подтвердил участки с наибольшим атмосферным воздействием. Очень высокие значения R были рассчитаны для 3 сайтов (два в UIZ и один в RZ) с наибольшим значением 1,61. На основании полученных результатов можно сделать вывод, что исследованные стебли и листья, с применением химического и статистического анализа, а также расчетом R-фактора, могут служить полезным инструментом в атмосферном фитомониторинге пирена.

Ключевые слова: фитомониторинг, пирен, *Hedera helix* L.

Das Potenzial von Hedera helix L. Stängeln und Blättern für atmosphärisches Pyren-Phytomonitoring

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ABSTRAKT

Ziel dieser Arbeit war es, Phytomonitoring-Potenziale von *Hedera helix* L. aus der Gemeinde Borās im Hinblick auf (atmosphärische) Pyrene als bekannten Gefahrstoff zu untersuchen. Die Ergebnisse der gaschromatographisch-massenspektrometrischen Analyse der ungewaschenen Stängel und Blätter zeigten, dass die Pyrenkonzentrationen offensichtlich in allen ausgewählten ländlichen und städtischen/industriellen Zonen (engl. RZ und UIZ) variierten. Diese ersten Anzeichen verschiedener Pyrensäurequellen im Untersuchungsgebiet wurden später durch die Ergebnisse der durchgeführten Pearson-Korrelationsanalyse bestätigt, die zeigte, dass die nachgewiesenen Pyrenkonzentrationen nicht nur von den benachbarten Heiz- und Schmelzwerken als Hauptverschmutzungsquellen in der gesamten Region stammten, sondern auch von der Hausheizung aus den ländlichen Zonen, oder von Waldbränden oder kontrollierten Bränden auf den bestellten Feldern und schließlich durch den Verkehr in allen Zonen. Die Korrelationsanalyse signalisierte auch, dass an einigen Stellen die nachgewiesenen Konzentrationen (insbesondere in Stängeln) nicht nur aus der Atmosphäre, sondern auch aus dem Boden stammten. Der berechnete Faktor R bestätigte die Standorte mit den größten atmosphärischen Auswirkungen. Für 3 Standorte (zwei städtische/industrielle und eine ländliche Zone) wurden sehr hohe R-Werte mit dem höchsten Wert von 1,61 berechnet. Basierend auf den erhaltenen Ergebnissen kann der Schluss gezogen werden, dass die untersuchten Stängel und Blätter mit den angewandten chemischen und statistischen Analysen sowie der Berechnung des R-Faktors als nützliches Werkzeug für das atmosphärische Pyrenen-Phytomonitoring dienen können.

Schlüsselwörter: phytomonitoring, pyrene, *Hedera helix* L.

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Unveiling the Dynamics of "Vranac" Wine Anthocyanins Oxidation: Insights from Accelerated Chemical Testing

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ABSTRACT

To gain insights into the oxidative behavior of red wines, a comprehensive study was conducted. The rates of anthocyanins decrease were measured for "Vranac" red wine subjected to two different accelerated aging tests: chemical (with hydrogen peroxide) and thermal. The kinetics of malvidin-3-O-glucoside (M3G) and malvidin-3-O-acetylglucoside (M3AG) degradation in this red wine by hydrogen peroxide in aqueous solution at various temperatures were investigated. The trace amount of Cu(II) ions was used to catalyze the reaction, and it was monitored using an HPLC-DAD method through the application of the initial-rate method. The HPLC-DAD method was validated for determining M3G and its derivatives in red wines. The kinetic parameters of the reactions are reported, and rate equations are suggested. The activation energy values for the degradation of M3G and M3AG were calculated to be 57.70 and 57.74 kJ/mol, respectively. The thermodynamic functions of activation (ΔG^*, ΔH^*, *and* ΔS^*) have also been calculated.

Keywords: red wine, oxidation, HPLC-DAD, kinetic parameters, thermodynamic functions

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Introduction

Chemical reactions hinge significantly on the presence of oxygen. Oxidation occurs in red wines, spanning from the winemaking process, aging in barrels and finally in the bottle. These oxidation reactions significantly influence both the chemical and sensory characteristics of the wine, impacting factors such as color (Ugliano, 2013; Ferreira et al., 2014; Deshaies et al., 2020) and organoleptic characteristics (De Beer et al., 2016). At every stage of the winemaking process, the wine is exposed to specific oxygen levels. Given the close relationship between wine composition and its reactivity with oxygen, predicting the outcomes for a specific wine with a designated oxygen amount proves challenging. However, our research provides practical insights that can help in this prediction. Existing literature acknowledges three accelerated aging tests: the heat test, enzyme test, and chemical test (hydrogen peroxide test).

From a chemical perspective, polyphenols stand out as one of the wine constituents most susceptible to oxidation (Oliveira et al., 2011). Red wines undergo chemical oxidation reactions involving polyphenols, such as anthocyanins, proanthocyanidins, and flavan-3-ols (Waterhouse and Laurie, 2006; Kilmartin, 2009).

Reactive oxygen intermediates, including hydrogen peroxide (H_2O_2), hydroxyl radical (HO•), and superoxide anion, play a crucial role in the degradation of plant pigments (Waterhouse and Laurie, 2006; Oliveira et al., 2011). De et al. (1999) observed that the HO• radical serves as the primary reactive species, cleaving the benzene ring and facilitating substrate degradation into CO_2 and H_2O. Sondheimer and Kertesz (1952) and Ozkan (2002) also proposed that the HO• radical is responsible for the oxidation and subsequent degradation of anthocyanins.

Anthocyanins, originating from the Greek words "anthos" (flower) and "kianos" (blue), emerge as essential pigments within vascular plants. Renowned for their safety and effortless integration into aqueous solutions, these pigments are esteemed for their potential as natural water-soluble dyes (Smyk et al., 2008). Exhibiting vivid shades of orange, pink, red, violet, and blue, anthocyanins enrich the vibrant spectrum of colors found in flowers, fruits, and select vegetation. In the domain of red wine, they play a pivotal role in determining its hue, a key characteristic that significantly influences wine quality.

Tannins in wine, alongside anthocyanins, play a significant role in color development and stability. While anthocyanins contribute primarily to the red, purple, and blue hues in wine,

tannins enhance the wine's overall color intensity and longevity.

Various factors influence the stability of anthocyanins, encompassing pH, light, oxygen, enzymes, ascorbic acid, sugars, sulfur dioxide or sulfite salt, metal ions, and co-pigments. Temperature, a well-established factor affecting anthocyanin stability, has been extensively studied (Kechinski et al., 2010; Hillmann et al., 2011; Turturica et al., 2018; Yajing and Yuanping, 2019). Additionally, the impact of hydrogen peroxide on anthocyanin degradation in fruits is documented (Kechinski et al., 2010; Zorić et al., 2014; Gerard et al., 2019).

Notably, the combination of high temperature and oxygen emerges as particularly detrimental to the stability of these compounds (Cavalcanti et al., 2011). While existing literature lacks data on the effects of H_2O_2, Cu(II), and temperature on red wine anthocyanins, this study aims to fill this gap by investigating their degradation in Serbian red wine “Vranac”. The kinetics of anthocyanin degradation will be evaluated from a thermodynamic perspective, considering activation functions such as free energy (ΔG^*), enthalpy (ΔH^*), entropy (ΔS^*), and activation energy (Ea). These thermodynamic parameters offer valuable insights into the thermal degradation kinetics of these compounds in food systems.

Experimental

Materials

For this study, samples of the red wine “Vranac”, produced in Serbia, were purchased in a local supermarket.

Chemicals

The reagents used, namely Cu(II) (chloride salt), hydrogen peroxide, acetonitrile, formic acid, and M3G, were all analytical grade (Merck, Darmstadt, Germany, and Sigma Chemical Co. St Louis, MO, USA). The solutions were prepared using deionized water from MicroMed's high-purity water system, TKA Wasseraufbereitungssysteme GmbH.

Preparation of standards

Preparing a stock solution of M3G (300 mg/L) involved dissolving the specified quantity in deionized water. The solution of Cu(II) ($1 \cdot 10^{-3}$ mol/L) was prepared by dissolving $CuCl_2 \cdot 2H_2O$

in deionized water. The hydrogen peroxide solution (0.979 mol/L) was prepared from a 30% commercial reagent just before it was used.

HPLC-DAD equipment and analysis

The oxidation kinetics for the anthocyanins was followed by measuring the concentration of anthocyanins at 520 nm on the Agilent 1200 chromatographic system equipped with a quaternary pump, an Agilent 1200 photodiode array detector with radiofrequency identification tracking technology for flow cells, a UV lamp, an automatic injection, and Chem-Station software. The column was thermostated at different temperatures (25 °C, 30 °C, 35 °C and 40 °C). After injection of 5 μL of the reaction mixture, the separation was performed on the Agilent-Eclipse XDBC-18 4.6x150 mm column. Two solvents were used for the gradient elution: A-(H_2O+5% HCOOH) and B-(80% ACN+5% HCOOH+H_2O). The elution program used was as follows: from 0 to 28 min, 0.0% B; from 28 to 35 min, 25% B; from 35 to 40 min, 50% B; from 40 to 45 min, 80% B, and finally for the last 10 min again 0% B (Mitić et al., 2012). The identification of the compounds was achieved using their retention times and UV-VIS spectra, analyzed with a Diode Array Detector (DAD). Quantification of M3G and M3AG was performed using calibration curves derived from standard M3G solutions.

The proposed method underwent validation in accordance with the guidelines established by the International Conference on Harmonization (ICH, 1996/2005) and the Commission Decision (2002/657/EC) guidelines. The method was validated by estimating linearity, precision, accuracy and sensibility. Linearity was evaluated by using the standard solution in the range of 2-150 mg/L M3G. Each concentration was analyzed in triplicate. The calibration curve was generated using peak areas of the reference M3G versus their concentration. The correlation coefficient of the calibration curve exceeded 0.999. The precision of the method was determined as repeatability and reproducibility in terms of per cent relative standard deviation (%RSD). The %RSD value for evaluated concentration (10, 50 and 100 mg/L) was lower than 2.20%. The limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated at 0.528 mg/L and 1.584 mg/L, indicating the high sensibility of the method.

Accelerated aging test

The red wine "Vranac" was submitted to accelerated ageing tests experiments (Magalhaes et al., 2010; Deshaies et al., 2021), chemical (H_2O_2 and Cu (II) added) and heat treatment (at 25, 30, 35 and 40 ºC). The reaction was carried out in the following way. In a special four-compartment vessel, the solution of Cu(II) was placed in the first, the solution of H_2O_2 in the second, the red wine "Vranac" in the third, and the deionized water (total volume 2 mL) in the four compartments (adjusted to pH 3.5 with 1mol/L tartaric acid). The vessel was thermostated at 25±0.02 ºC (or 30±0.02 ºC, 35±0.02 ºC and 40±0.02 ºC). The content was mixed well and immediately injected into an Agilent 1200 chromatographic system. The change in concentration of anthocyanins was recorded at 520 nm as a function of time every 35 min (for M3G) and 40 min (for M3AG) for the first 175 min of the reaction. The kinetic analysis utilized the initial rates method. The zero-time concentration value was determined by preparing the red wine with deionized water (total volume 2 mL).

Results and Discussion

Kinetics of M3G and M3AG degradation

The catalytic effect of Cu(II) on the oxidation reaction of M3G and M3AG, by hydrogen peroxide was observed. The HPLC chromatograms for M3G (peak 1) and M3AG (peak 2), in the red wine "Vranac" from Serbia, recorded at 520 nm, are shown in Figure 1. The chromatogram in an aqueous solution (wine diluted with deionized water in a ratio of 1:1) is present in curve 1. The chromatogram of the mixture of wine-H_2O_2-water is shown in curve 2. After the wine was mixed with Cu(II)-H_2O_2, the concentration of anthocyanins strongly decreased. The reaction between anthocyanins and hydrogen peroxide occurs slightly at 25 ºC (curve 2), but the addition of Cu (II) ions particularly accelerates this reaction (curve 3).

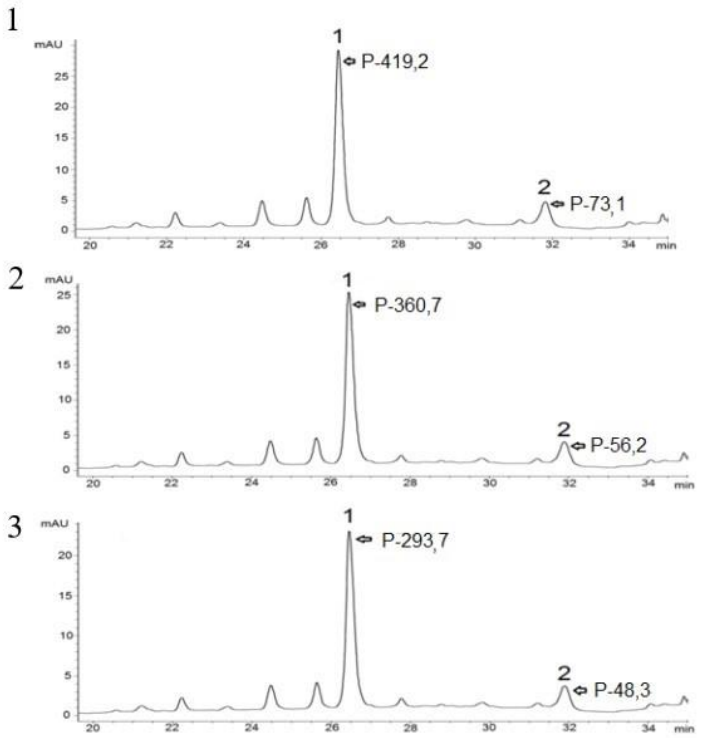
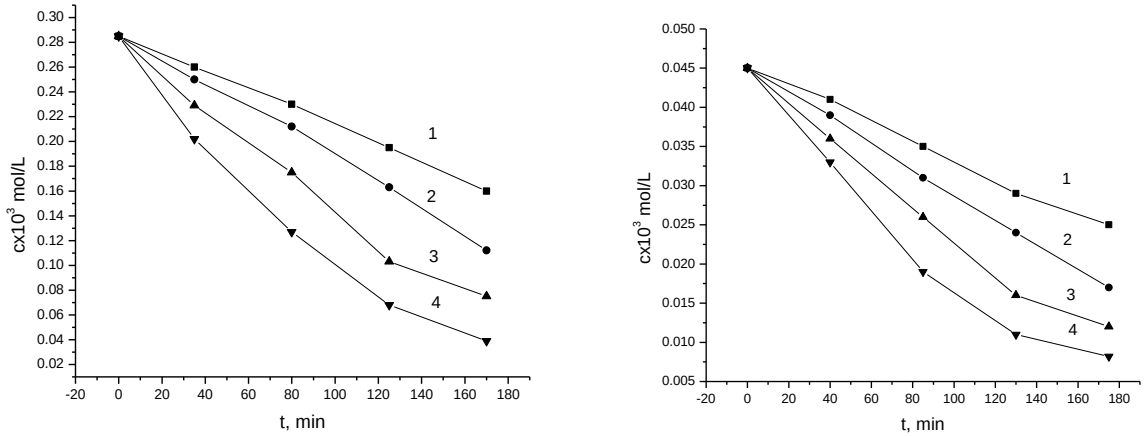


Figure 1. HPLC chromatogram of red wine anthocyanin after 35 min of incubation. Peak 1- M3G (retention time- 26.55 min), peak 2- M3AG (retention time- 31.90 min); (1) wine + water; (2) wine + H_2O_2 + water; (3) wine + H_2O_2 + Cu(II) + water.

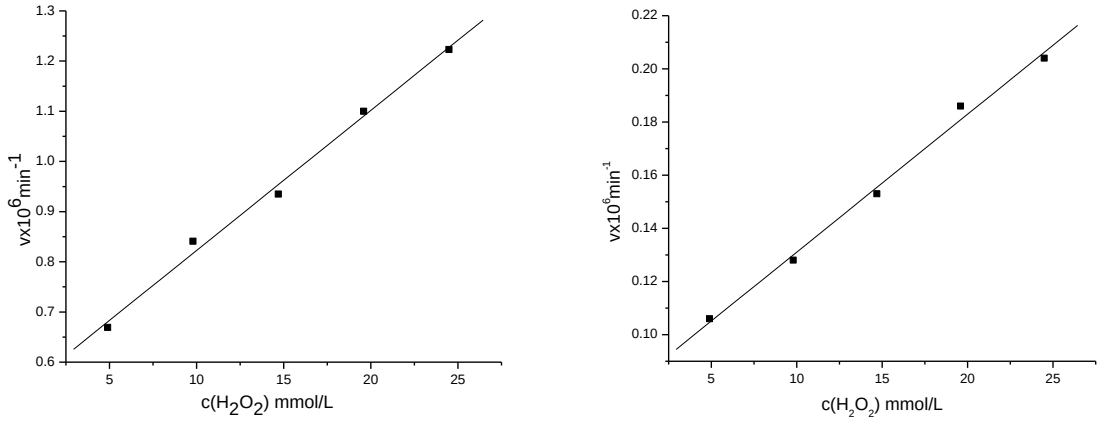


a) b)

Figure 2. Concentration as a function of reaction times for different temperatures: 1 - 25 ^oC; 2 - 30 ^oC; 3 - 35 ^oC; 4 - 40 ^oC; for M3G (a) and M3AG (b). c(H_2O_2) =$14.69\cdot10^{-3}$ mol/L; c(Cu(II))=$0.185\cdot10^{-4}$ mol/L; pH=3.5

Figure 2 shows the relationships between the concentration of M3G and M3AG and time at different temperatures. Anthocyanins' concentration decreases with time. It is possible to follow the reaction rate by the HPLC-DAD method as the change of the pick areas values with time because of the linear dependence of pick area on time during the first 130 min (of reaction at 25º C and 30 ºC) or first 180 min (of reaction at 35 ºC and 40 ºC). The initial rate method was used to determine partial orders (Mottola and Perez-Bendito, 1996). The initial reaction rates were established by measuring the slopes of the initial tangents on the anthocyanin concentration-time curves, represented by dc/dt.

The reaction rate dependence on the H_2O_2 concentration was studied in the range of 4.90-24.49 mmol/L (Figure 3). As the concentration of H_2O_2 increased, the reaction rate accelerated. This figure shows that the degradation of M3G and M3AG in red wine "Vranac", follows the first-order reaction with respect to H_2O_2 concentrations because the curve is linear. For further work, a concentration of H_2O_2 of 14.69 mmol/L was selected as the optimal value.

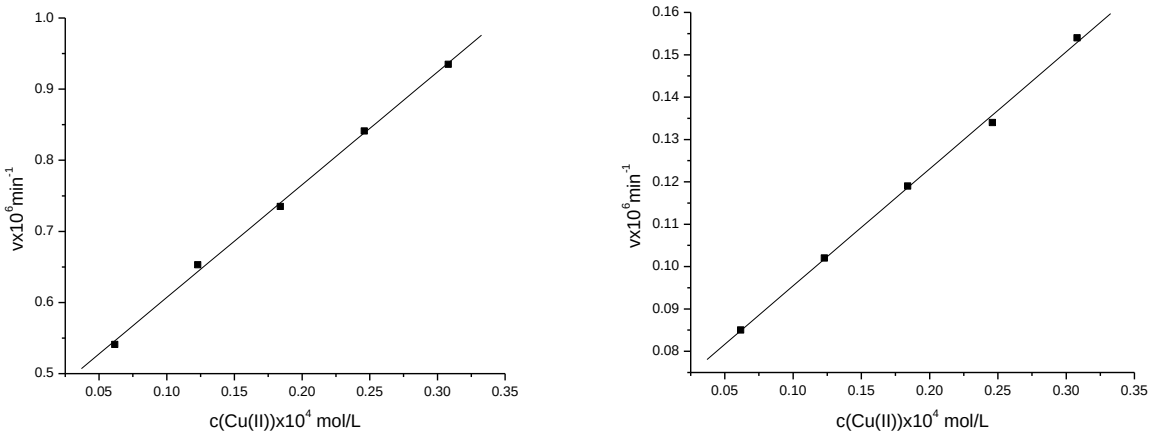


a) b)

Figure 3. The reaction rate's dependence on the concentration of H_2O_2 for M3G (a) and M3AG (b). c(M3G)=2.85·10^{-4} mol/L; c(M3AG)=4.49·10^{-5} mol/L; c(Cu(II))=3.08·10^{-5} mol/L, pH=3.5, t =25 ºC.

Keeping the H_2O_2 and anthocyanins concentration constants, the Cu(II) dependence on the system was studied in the range of 0.62-3.10·10^{-5} mol/L (Figure 4). It was observed that Cu(II) ions had catalytic activity in this reaction. The reaction rate increased with increasing the concentration of Cu(II). The linear relationship indicated that the degradation of anthocyanins in

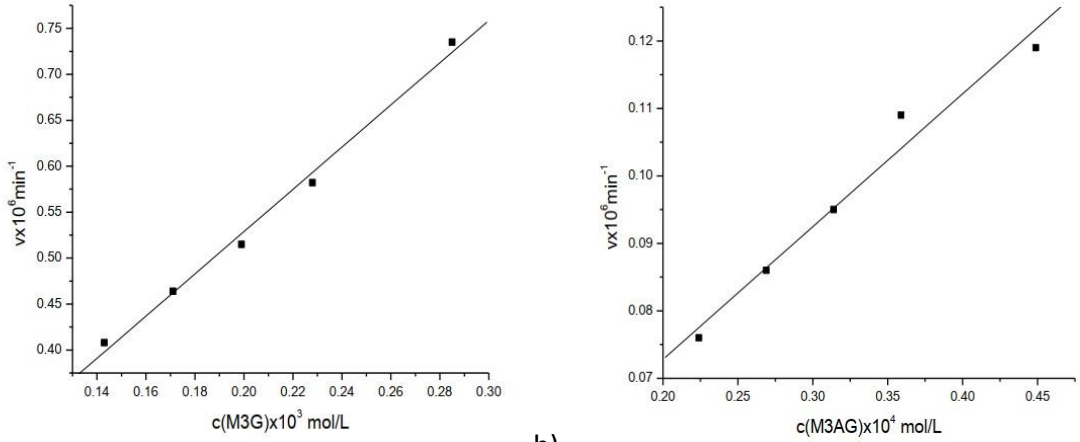
the red wine followed first-order reaction kinetics with respect to Cu(II) concentrations. For further work, a concentration of Cu(II) of $1.85 \cdot 10^{-5}$ mol/L was selected as the optimal value.



a) b)

Figure 4. The reaction rate's dependence on the concentration of Cu (II) for M3G (a) and M3AG (b). c(M3G)=$2.85 \cdot 10^{-4}$ mol/L; c(M3AG)=$4.49 \cdot 10^{-5}$ mol/L; c(H_2O_2) =$14.69 \cdot 10^{-3}$ mol/L, pH =3.5, t =25 ^oC.

The influence of the concentration of M3G and M3AG on the reaction rate was studied in the range 0.142-0.285 mmol/L M3G and 0.224-0.449 mmol/L M3AG, respectively under the following working conditions: c(H_2O_2)= 14.69 mmol/L, c(Cu(II))= $1.85 \cdot 10^{-5}$ mol/L, pH=3.5 and t=25 ^oC. Linear dependence confirmed that the degradation of M3G and M3AG in red wine "Vranac" followed the first-order reaction (Figure 5).



a) b)

Figure 5. Dependence of the reaction rate on the concentration M3G (a) and M3AG (b). c(H_2O_2)=14.69 mmol/L, (Cu(II))=1.85·10^{-5} mol/L, pH =3.5, t =25 ºC.

Based on the present kinetic investigation, the kinetic equations for degradation of M3G and M3AG in the red wine "Vranac" by H_2O_2 in the presence of Cu (II) as a catalyst were formulated:

$$-\frac{dc}{dt} = k_1 \cdot c_{H_2O_2} \cdot c_{Cu(II)} \cdot c_{M3G}$$

$$-\frac{dc}{dt} = k_2 \cdot c_{H_2O_2} \cdot c_{Cu(II)} \cdot c_{M3AG}$$

where k_1 and k_2 are rate constants. Based on these equations, the rate constants for the reactions were calculated and presented in Table 1. As expected, the k values increased with temperature, indicating that greater degradation occurs at higher processing temperatures.

Table 1. Rate constant k of the degradation of anthocyanins *vs* temperature.

t, ºC	k_1 (M3G)·10^{-4}/mol^{2}dm^{-6}min^{-1}	k_1 (M3AG)·10^{-4}/mol^{2}dm^{-6}min^{-1}
25	0.951	0.976·10^4
30	1.315	1.369·10^4
35	1.884	1.895·10^4
40	2.547	2.649·10^4

Mechanism of anthocyanins degradation by H_2O_2

As red wine oxidation induces color changes, measurements were done to compare natural and forced oxidation. The primary contributors to the red color are anthocyanins (Tanaka et al., 2008), and their degradation due to oxidation could explain the differences in absorbance measurements. Absorbance at 520 nm, as shown in Figure 7 (curve 1), corresponds to the flavylium ring of anthocyanin. The malvidin-3-glucoside, the main anthocyanin present in wine, is not readily oxidized (Waterhouse and Laurie, 2006). In the presence of H_2O_2 or Cu(II)/H_2O_2 reagent, it is possible that brown oxidized polyphenols were formed, which could increase the absorbance to 420 nm. As shown in Figure 7 (curve 3), an accelerated degradation of the wine anthocyanin was observed in the presence of Cu(II)/H_2O_2. Therefore, it can be concluded that Cu(II) acts as a catalyst in the anthocyanin's hydrogen peroxide oxidation reaction.

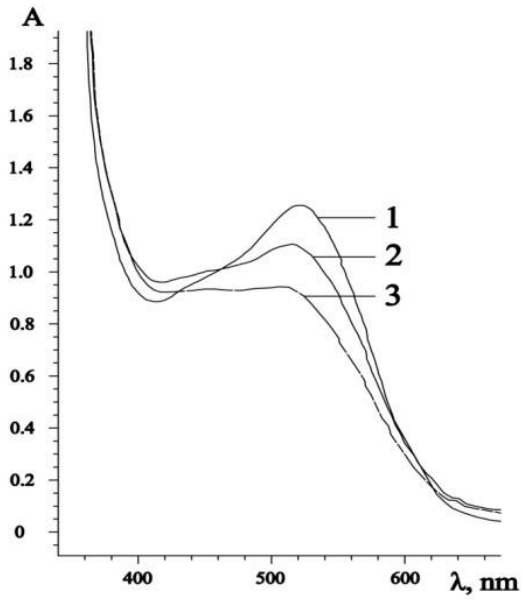


Figure 6. Absorbance measurements (350–650 nm) for three different wine mixtures: 1) aqueous solution of wine; 2) aqueous solution of wine + H_2O_2; 3) aqueous solution of wine + H_2O_2 + Cu(II) -20 min after preparing the mixture.

Hydrogen peroxide is an oxygen species and the simplest peroxide, and this relatively reactive species may influence the biogeochemistry of various transition metals and their complex can act effectively as a reductant or as an oxidant. However, in the presence of certain metals, the presence of H_2O_2 could potentially result in the generation of the highly reactive and

harmful hydroxyl radical (HO•). Many kinetic studies on the reaction of both Cu(I) and Cu(II) with H_2O_2 have been reported (Perez-Bendito, 2004; Pham et al., 2012; Pham et al., 2013).

Gray (1996) suggested that the reaction between Cu(II) and H_2O_2 produced Cu(I) and $O_2^{\bullet-}$ (Eguations I-IV). Thus, free radical pathway has been promoted by several investigations both in the absence and presence of organic ligands with production of Cu(I) subsequently resulting in formation of $HO^{\bullet}$ via a "Fenton-like" reaction between Cu(I) and H_2O_2.

$$H_2O_2 \leftrightarrow H^+ + HOO^- \quad \text{I}$$

$$Cu(II) + HOO^- \rightarrow Cu(I) + HO_2^{\bullet} \quad \text{II}$$

$$HO_2^{\bullet} \leftrightarrow H^+ + O_2^{\bullet-} \quad \text{III}$$

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + HO^{\bullet} + OH^- \quad \text{IV}$$

Anthocyanins, as thermolabile compounds in wine, are the primary targets of chemical changes caused by the presence of hydroxyl radicals. The de-glycosylation and cleavage of anthocyanins will lead to the release of the A and B rings of anthocyanins (Redus et al., 1999). It can be explained by the oxidation of malvidin 3-O-diglucoside in the presence of hydrogen peroxide under acidic conditions, which leads to the formation of ortho-benzoyloxyphenylacetic acid esters through Baeyer–Villiger oxidation type (Harazdina, 1970; Harazdina and Franzese, 1974). New compounds produced from malvidin 3-O-glucoside according to Baeyer–Villiger oxidation are 2,4,6-trihydroxybenzaldehyde (Piffaut et al., 1994), syringic acid or anthocyanone A (8-β-D-glucopyranosyl-2,4-dihydroxy-6-oxo-cyclohexa-2,4-dienyl acetic acid) (Lopes et al., 2007). Hypothetical scheme for the formation of new compounds from malvidin O-glucoside through a Baeyer−Villiger-type oxidation is presented in Figure 7 (Lopes et al., 2007).

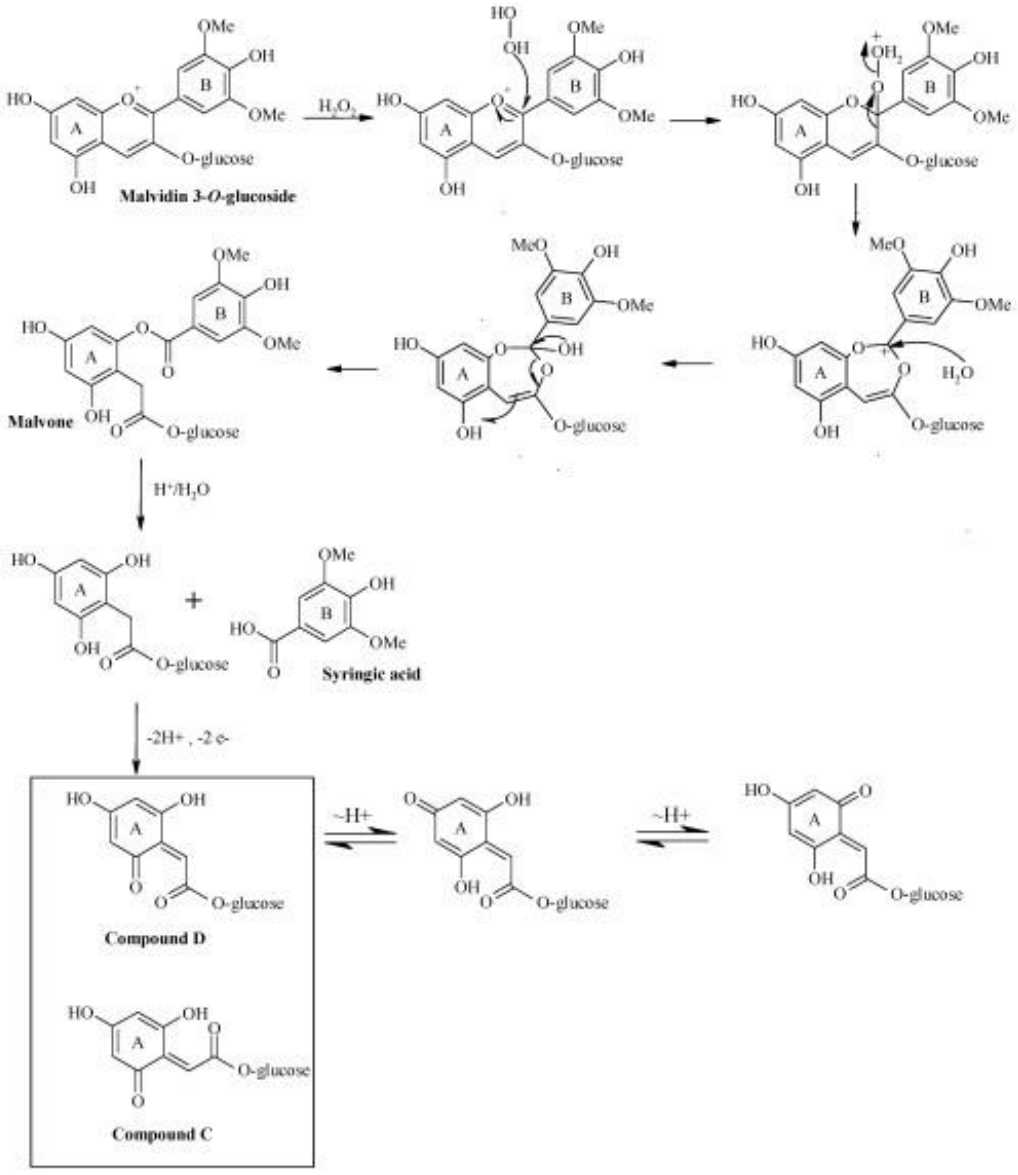


Figure 7. Hypothetical scheme for the formation of compounds C and D (anthocyanone A) from malvidin-O-glucoside through a Baeyer-Villiger-type oxidation (Lopes et al., 2007).

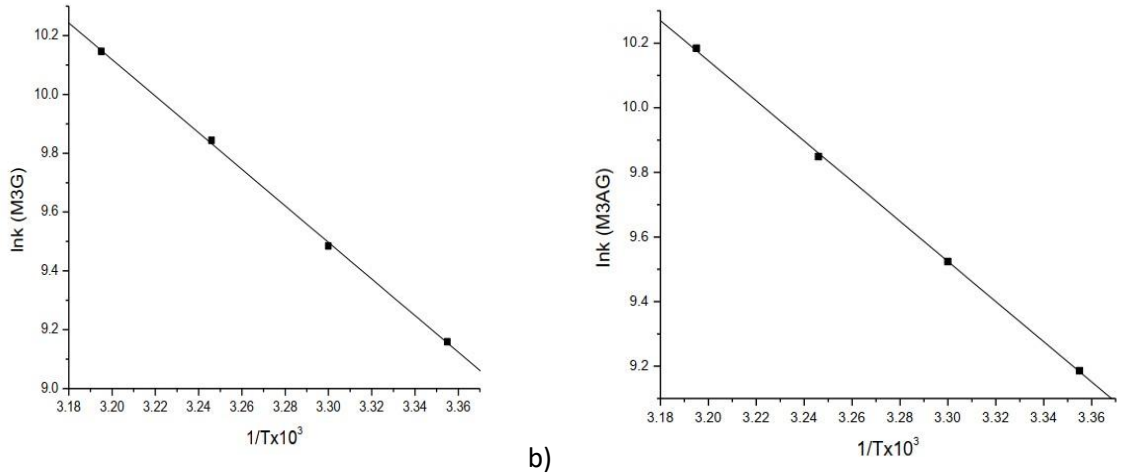
Thermodynamic analysis

Estimating thermodynamic parameters could provide additional and useful information for degradation kinetics. Table 2 presents the activation energy (Ea), activation enthalpy (ˆH*), activation entropy (ˆS*), and free energy of activation (ˆG*).

Table 2. Kinetic parameters for degradation of anthocyanins of red wine "Vranac"

	M3G	M3AG
Ea, kJ/mol	57.70	57.74
ΔH^*, kJ/mol	55.22	55.26
ΔS^*, J/Kmol	16.60	16.94
ΔG^*, kJ/mol	60.17	60.31

The activation energy (*Ea*) describes the energy required to reach the transition state of a reaction. The activation energy is usually evaluated from experimental data using the Arrhenius model. Figure 8 presents the Arrhenius plot developed from the kinetic constant rates of anthocyanins obtained in this study.



a) b)

Figure 8. The Arrhenius plots for degradation of M3G (a) and M3AG (b) in red wine "Vranac" c(H_2O_2) =14.69 mmol/L, c(Cu(II)) = $1.85 \cdot 10^{-5}$ mol/L, pH= 3.5.

The calculated value of *Ea* was 57.78 and 57.74 kJ/mol for M3G and M3AG. This means that there are no statistical differences ($p<0.05$) between the *Ea* values obtained for the two monomeric anthocyanins. Earlier research has demonstrated similar activation energy (*Ea*) values for the degradation of anthocyanins in food items, with studies on black carrots revealing *Ea* values spanning from 68.8 to 95.1 kJ/mol (Kirca et al., 2007); thermal treatment (40–80 ºC) of blueberry juice presented anthocyanin degradation with activation energy value of 80.4 kJ/mol (Kechinski et al., 2010); anthocyanins thermal degradation at 60-90 ºC from wild strawberry showed *Ea* value 21.6 kJ/mol (Özşen and Erge, 2012), 68 kJ/mol for acerola pulp (Silva *et al.*,

2016). The activation energy of the degradation of anthocyanins in grape juice was determined as 64.89 kJ/mol at 70–90 °C (Danisman et al., 2015). Muche et al. (2018) reported that each anthocyanin compound had a different degradation rate and temperature sensitivity. These authors determined activation energy of 49.63 kJ/mol and 29.75 kJ/mol for M3G and peonidin-3-glucoside (Pn3G) in Rudy grape juice in a temperature range of 5-35 ^{o}C. Also, Oliveira et al. (2015) reported an activation energy value of 60.7 kJ/mol for M3G in red Port wine after oxygen addition.

The activation enthalpy (ΔH^*) signifies the minimum energy necessary for the reactant to initiate the reaction, correlating with the strength of the chemical bonds involved in its formation and breakage. The positive value of ΔH^* indicated that the reaction of anthocyanin degradation is an endothermic reaction, proving our previous results that the degradation rate increased with temperatures. ΔH^* for M3G and M3AG degradation with Fenton-like reagent (Cu (II)/H_2O_2) in this study was 55.22 and 55.26 kJ/mol.

The Gibbs free energy (ΔG^*) is defined as the difference between the energies of reactants and activated state and usually serves as a measure of process spontaneity. Both ΔH^* and ΔG^*, values obtained in the current study are like the values represented by Kechinski et al. (2010) for blueberry juice (77.8 and 91.3 kJ/mol for ΔH^* and ΔG^*, respectively); Moldovan et al. (2019) for wild blackthorn fruit extracts (51.51 and 55.82 kJ/mol) and Yajing and Yuanping (2019) for rose anthocyanin extracts (141.53 and 100.93 kJ/mol).

Entropy (ΔS^*) values imply the change of disorder of molecules in the reaction system and it is usually related to the number of molecules with appropriate energy that can react. ΔS^* values determined in this study were positive, 16.60 and 16.94 J/mol K. The positive ΔS^* values of wine anthocyanins indicated that the entropy increased when reaching the transition state (Celli et al., 2016). The relatively low value of ΔS^* implies the low significance of this function (Al-Zubaidy and Khalil 2007). Additionally, the ΔS^* values were relatively smaller than those reported by Yajing and Yuanping (2019) at 118.31 J/molK. In contrary to our results, the negative ΔS^* values were reported for anthocyanins thermal degradation in blueberry juice (Kechinski et al., 2010) and for wild blackthorn fruit extracts (Moldovan et al., 2019).

Conclusion

In this study, the degradation kinetics of M3G and M3AG in "Vranac" red wine were investigated using the Cu(II)/H_2O_2 reagent over a temperature range of 25 to 40 °C. Temperature, H_2O_2 concentration, Cu(II) concentration, and anthocyanin levels were found to notably influence the reaction rate. The degradation rate constants varied with temperature according to the Arrhenius relationship. The positive activation enthalpies for the degradation processes suggested an endothermic nature, while the Gibbs free energy of activation indicated a nonspontaneous character. These findings imply that controlled oxygen levels can stabilize the wine and promote the development of unique aromas during aging. Winemakers should carefully manage their exposure to ensure optimal outcomes without compromising the wine's quality.

Acknowledgement

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Conflict-of-Interest Statement

The author did not declare any conflict of interest.

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Otkrivanje dinamike oksidacije vina „Vranac“: uvidi iz ubrzanog hemijskog testiranja

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SAŽETAK

Da bi se stekao uvid u proces oksidacije vina, praćena je degradacija antocijana u crvenom vinu Vranac. Vino je ispitivano dvama različitim testovima ubrzanog starenja: hemijskim (sa vodonik-peroksidom) i temperaturnim. Ispitivana je kinetika razgradnje malvidin-3-*O*-glukozida i malvidin-3-*O*-acetilglukozida vodonik-peroksidom u vodenom rastvoru (na različitim temperaturama). Reakcija je katalizovana određenim količinama Cu (II) jona u tragovima, a praćena je HPLC-DAD metodom primenom metode početne brzine. Odrađena je validacija HPLC-DAD metode za određivanje malvidin-3-O-glukozida i njegovih derivata. Prikazani su kinetički parametri reakcija i predložene su jednačine brzine. Vrednosti energije aktivacije za razgradnju malvidin-3-glukozida i malvidin-3-acetilglukozida su 57,70 i 57,74 kJ/mol, respektivno. Termodinamičke funkcije aktivacije ΔG^*, ΔH^* i ΔS^* su takođe izračunate.

Ključne reči: crveno vino, oksidacija, HPLC-DAD, kinetički parametri, termodinamičke funkcije

Dévoilement de la dynamique de l'oxydation des anthocyanes du vin Vranac : aperçu des tests chimiques accélérés

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RÉSUMÉ

Pour mieux comprendre le comportement oxydatif des vins rouges, les taux de diminution des anthocyanes ont été mesurés pour le vin rouge Vranac soumis à deux tests de vieillissement accéléré différents : chimique (avec du peroxyde d'hydrogène) et thermique. La cinétique de dégradation de la malvidine-3-*O*-glucoside (M3G) et de la malvidine-3-*O*-acétylglucoside (M3AG) dans ce vin rouge par le peroxyde d'hydrogène en solution aqueuse à différentes températures a été étudiée. Des traces d'ions Cu(II) ont été utilisées pour catalyser la réaction, et elle a été surveillée à l'aide d'une méthode HPLC-DAD par l'application de la méthode du taux initial. La méthode HPLC-DAD a été validée pour la détermination du M3G et de ses dérivés dans les vins rouges. Les paramètres cinétiques des réactions sont rapportés et des équations de vitesse sont suggérées. Les valeurs d'énergie d'activation pour la dégradation de M3G et M3AG ont été calculées à 57,70 et 57,74 kJ/mol, respectivement. Les fonctions thermodynamiques d'activation (ΔG*, ΔH* et ΔS*) ont également été calculées.

Mots-clés : vin rouge, oxydation, HPLC-DAD, paramètres cinétiques, fonctions thermodynamiques

Раскрытие динамики окисления антоцианов вина Вранац: выводы из ускоренного химического тестирования

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Резюме

Чтобы получить представление об окислительных превращениях красных вин, скорость снижения антоцианов была измерена для красного вина Вранац, подвергнутого двум различным испытаниям на ускоренное старение: химическому (с перекисью водорода) и термическому. Исследована кинетика деградации мальвидин-3-О-глюкозида (M3G) и мальвидин-3-О-ацетилглюкозида (M3AG) в этом красном вине перекисью водорода в водном растворе при различных температурах. Для катализа реакции использовали следовое количество ионов Cu(II) и контролировали с помощью метода ВЭЖХ-ДАД с применением метода начальной скорости. Валидирован метод ВЭЖХ-ДАД для определения M3G и его производных в красных винах. Приведены кинетические параметры реакций и предложены уравнения скорости. Значения энергии активации для деградации M3G и M3AG составили 57,70 и 57,74 кДж/моль соответственно. Рассчитаны термодинамические функции активации (ΔG*, ΔH* и ΔS*).

***Ключевые слова*:** красное вино, окисление, ВЭЖХ-ДАД, кинетические параметры, термодинамические функции

Enthüllung der Dynamik der Oxidation von Anthocyanen in „Vranac„ Weinen: Erkenntnisse aus beschleunigten chemischen Tests

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ABSTRAKT

Um Einblicke in das oxidative Verhalten von Rotweinen zu gewinnen, wurde die Geschwindigkeit des Anthocyanabbaus bei Vranac Rotweinen gemessen, der zwei verschiedenen beschleunigten Alterungstests unterzogen wurde: chemisch (mit Wasserstoffperoxid) und thermisch. Die Kinetik des Abbaus von Malvidin-3-O-Glucosid (M3G) und Malvidin-3-O-Acetylglucosid (M3AG) wurde in diesem Rotwein durch Wasserstoffperoxid in wässriger Lösung bei verschiedenen Temperaturen untersucht. Die Reaktion wurde durch Spuren von Cu(II)-Ionen katalysiert und mit Hilfe einer HPLC-DAD-Methode durch Anwendung der Anfangsratenmethode überwacht. Die HPLC-DAD-Methode wurde für die Bestimmung von M3G und seinen Derivaten in Rotweinen validiert. Es wird über die kinetischen Parameter der Reaktionen berichtet, und es werden Geschwindigkeitsgleichungen vorgeschlagen. Die Aktivierungsenergiewerte für den Abbau von M3G und M3AG wurden mit 57,70 bzw. 57,74 kJ/mol berechnet. Die thermodynamischen Aktivierungsfunktionen (ΔG*, ΔH* und ΔS*) wurden ebenfalls berechnet.

Schlüsselwörter: rotwein, oxidation, HPLC-DAD, kinetische parameter, thermodynamische funktionen

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What kind of magic do they put in chocolate, anyway?

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ABSTRACT

Chocolate is the most popular candy in the world. The cocoa tree's seeds are fermented, roasted, and ground, making chocolate the final finished product. The main components of chocolate are carbohydrates, fat, and protein, and it also contains essential minerals like calcium, magnesium, iron, and zinc. The resulting sensory perception of chocolate is linked to many diverse components, making its flavour highly complex. There are different types of chocolate in terms of composition and shape. The cocoa mixture, cocoa bean roasting method, and the ratio of cocoa butter, fat-free cocoa powder, and sugar all play a role in determining the type of chocolate. Chocolate can take different forms, such as bars or sticks, and is often flavored with vanilla, cinnamon, cloves, hazelnuts or almonds. Milk chocolate is the most popular chocolate in the world. Creamy and tender, it melts on the tongue and leaves you wanting more. The second most popular chocolate was dark chocolate, followed by white chocolate. The most popular combinations are soft nougat and crunchy walnuts. Hazelnuts and almonds are also indispensable in the chocolate industry.

Keywords: *chocolate, cocoa butter, anandamide, theobromine, caffeine, types of chocolate*

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Introduction

Chocolate has a fascinating history. The first known cacao plantations were established by the Maya in the lowlands of southern Yucatán around 600 AD. The Maya revered chocolate as divine food, associating the cacao tree with gods and viewing its fruits as a gift to humanity. They held the cacao tree as sacred, often burying dignitaries with bowls of chocolate, symbolizing its importance in the afterlife. The identification of the (Olmec-originated) word ka-ka-w ("cacao") inscribed on those containers was vital to deciphering the Maya's phonetic manner of writing. They believed that the tree belonged to the gods and that the fruits of the cacao tree were God's gift to humans 300. In the new era, the Mayans built castles and temples at the time of the most incredible rise of their empire. They carved images of the cocoa fruit on the "sacred" walls as a symbol of life and fertility. A drink made from cocoa beans was first created in the Mayan Empire. The chocolate drink was so bitter that it was used in drinks known as chocolate or "bitter water". It was the drink of kings and lords to enhance festivities and rituals. Sometimes, chili pepper was added to the bitter drink.

After the mysterious disappearance of the Mayan civilization, this area was inhabited by the Toltecs and later by the Aztecs. The king of the Toltecs, Quetzalcoatl, politically forced to leave the kingdom, sailed away in a small boat, promising to return in a specific year to recover his empire. The legend of his empire became part of mythology. Astrologers of the Aztecs predicted that the year of his return would be 1519.

Before the Europeans reached Central America, the Incas (Peru) and the Aztecs (Mexico) were cultivating cocoa trees (Scholey and Owen, 2013). Precisely, in 1519, the Spanish conquistador Cortez arrived by ship on the soil of the Aztec Empire. The Aztec king Montezuma believed that Cortez was the reincarnation of Quetzalcoatl and welcomed him with the greatest honours. He organized a celebration where a cocoa drink was a tremendous honour. Cortez immediately realized the great potential of cocoa beans. He firmly believed he had found the "gold" he sought. For Cortez, it was also in the literal sense. Namely, according to historical notes, Montezuma drank fifty jugs of cocoa daily. He always drank from a golden cup and threw the cup into the lake. That's how Cortez literally found the "golden lake".

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In the Aztec Empire, a healthy slave could be bought for 100 beans of cocoa, a rabbit for four beans, and a young and beautiful party girl charged for her services for ten beans. Cocoa beans were a "stable currency" for several centuries, and in parts of Central America until the 19th century, cocoa beans remained used as a means of payment.

Cortez, throwing Montezuma in prison, soon organized the establishment of cocoa plantations. The Spanish built plantations in Mexico, Ecuador, and Peru.

Therefore, it is considered that Hernán Cortés, the Spanish conqueror, discovered the secret of making a chocolate drink. However, it should be mentioned that Columbus's crew, returning from a trip to the New World in 1502, brought the first quantities of cocoa beans to Europe, above all out of curiosity and as a souvenir, not recognizing its actual value.

In 1580, the first facility for processing cocoa beans was established in Spain. The Spanish made their version of the chocolate drink by mixing sugar, cinnamon and vanilla with cocoa beans. Soon after, the "chocolate" drink achieved enormous popularity, enjoyed only by the wealthy. Preparing the chocolate drink was a well-kept secret, so it took about 100 years for the preparation process to spread throughout Europe. "Chocolaterias" chocolate houses began to spring up all over Spain. From there, they spread throughout Europe via the Netherlands and Italy as a new fashion among the upper classes of society. Various spices are added, such as chili, cloves, vanilla, and black pepper.

The first chocolate drinking was established in London in 1657. It was mentioned in Pepys' Diary of 1664, where he wrote that "jocolatte" was "very good". In 1727, milk was added to the drink. This invention is generally attributed to Nicholas Sanders. Due to the high costs of cacao and sugar, early consumption of this drink was limited to royalty and the aristocracy. However, with the cultivation of *Theobroma cacao* reducing production costs, chocolate beverage consumption became widespread across all social classes.

The making of chocolates is the result of long discovery and innovation. In making the drink, enormous problems were caused by some kind of butter because over half of the cocoa bean consists of cocoa butter. The cocoa butter tended to melt in hot water, causing the cocoa particles to clump together and giving the appearance of fat rising to the surface, which was visually unappealing. Van Houten, a Dutch chemist, invented a hydraulic press in 1828, with which he managed to extract cocoa butter from cocoa beans on one side and a dark brown cake

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of cocoa powder. To enhance the dispersion of the powder in hot water or milk and reduce its fat content, the Dutch treated cocoa beans with an alkaline solution during the roasting process, a technique later termed the Dutching process. This method also allowed for the adjustment of cocoa powder colour by varying the alkalizing agent used.

Ten years later, Van Houten sold the patent, and the machine entered general use. Mixing extracted cocoa butter with cocoa beans produces a paste that absorbs sugar, which leads to the production of edible chocolate. It has yet to be discovered precisely who first dissolved cocoa butter and re-combined it with cocoa powder, with the addition of sugar. The result was a smooth, moldable mixture. 1847. Company J. S. Fry & Sons first exhibited this product at the Birmingham Fair under the name "Chocolat Delicieuk a Manger". Daniel Peter in Switzerland. Thus, after the wave of chocolate as a beverage, the wave of "eating" chocolate swept across Europe.

Chocolate must have low moisture content to prevent it from turning into a paste when melted due to water reacting with sugar. Thus, Daniel Peter needed to devise a method to dry the abundant liquid milk in his country. The recent development of a condensed milk formula by Henri Nestle' helped him with this. This meant he had much less water to evaporate and could remove the remaining amount using relatively cheap water-powered machines.

Non-fat particles must be smaller than 30 microns to ensure the chocolate melts smoothly in the mouth. Despite grinding chocolates with granite rollers, those made by Fry and Peter still had a gritty texture due to large particles and agglomerates formed by particle groups. Moreover, inadequate fat coating contributed to this texture, while the presence of acidic chemicals led to bitterness. In 1880, Rodolphe Lindt developed a machine in Berne, Switzerland, producing smoother, better-tasting chocolate. This machine was known as a conche, because its shape was similar to the shell with that name. Employing such a machine enabled the breaking up of agglomerates and some larger particles, coating them all with fat. Simultaneously, moisture and certain acidic chemicals were evaporated, resulting in smoother, less astringent chocolate. Chocolate can be considered a dense suspension of solid particles (sugar, cocoa, and milk mixture, depending on the type) dispersed in a continuous fat phase, chiefly composed of cocoa butter (Khampuis, 2010; Nickless, 1996).

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Swedish botanist Carolus Linnaeus renamed the cocoa tree, giving it the Greek name Theobroma Cacao in the 18thcenturyThe name "theobroma" itself comes from the ancient Greek word θεός (theos), meaning "god, deity", and βρῶμα (broma), meaning "food. The cocoa or cacao tree (*T. cacao* L.) originally came from South and Central America but is now commercially cultivated in suitable climates between 20 degrees north and 20 degrees south. These areas have a high average temperature throughout the year and constant high humidity from plentiful rainfall. In commercial plantations, they are often sheltered by intercropping trees such as coconut and banana. Its leaves are evergreen and are up to about 300 mm in length. Trees begin producing pods within 2–3 years, but reaching total yield takes 6 or 7 years. The cacao tree is notable for its cauliflory, where cacao flowers grow directly from the trunk rather than branches, as is typical. One fruit contains about 20-22 cocoa beans.

There are four types of cocoa (https://barandcocoa.com/pages/varieties-of-cocoa-beans). Criollo has beans with white cotyledons and a mild flavour. The trees are, however, relatively low-yielding. Most cocoa is Forastero, which is more vigorous and often grown on smallholdings (a family's cultivated land smaller than a farm) in West Africa. The third form, Trinitario, is usually considered a hybrid of the other two types. The fourth type is Nacional, which is grown only in Ecuador and probably originates from the Amazonian area of Ecuador. Nacional Cocoa produces beans with a full cocoa flavour and additional floral and spicy flavours.

The cacao tree seeds possess a strong bitter taste and require fermentation to develop their flavour. Fermentation consists of a natural, seven-day microbial fermentation of the pulp at temperatures up to 50 °C (Guzmán-Alvarez and Márquez-Ramos, 2021). Some compounds that give flavour to cocoa are formed during this fermentation. Traditional fermentation is driven by a series of reactions catalyzed by a succession of microorganisms-yeasts, lactic acid bacteria, and acetic acid bacteria-that naturally inoculate cocoa pulp. The end products of fermentation, including ethanol, lactic acid, and acetic acid, kill the beans and generate flavour precursors (De Vuyst 2020).

Free amino acids and peptides also form, and sucrose is inverted to produce reducing sugars (Munoz et al., 2020). After fermentation, the beans undergo drying, cleaning, and roasting. The shells are removed, producing cacao nibs, ground into cocoa mass, the purest form

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of chocolate. When the cocoa mass is heated and liquefied, it becomes chocolate liquor, which can be further processed into cocoa solids and butter. (De Vuyst and Weckx, 2016).

Cocoa butter

The cocoa components used in chocolate are cocoa butter (the fat phase), cocoa powder (the fat-free phase), or cocoa mass (also known as cocoa liquor), which is a combination of cocoa powder and the cocoa butter naturally found in the cocoa bean (Talbot, 2012). Cocoa powder is not used as a component in white chocolate. Cocoa butter, the primary structural material in chocolate, consists of monounsaturated, polyunsaturated, and primarily saturated fats.

Cocoa butter is considered the most important by-product of cocoa due to its physical (rheology and texture), chemical, and organoleptic qualities (Lipp and Anklam, 1998), which produce widely sought-after functional properties in the food industry.

Cocoa butter makes up about half the mass of the cocoa bean. It is obtained using a hydraulic press with the help of high pressures. Cocoa beans contain a large amount of fat. It contains 50-54% fat, 10-15% protein, 4-5% moisture, 1% theobromine and 0.44% caffeine (Afoakwa et al. 2013). These unique characteristics, which cannot be compared with other edible vegetable fats, are very useful in producing many products in the chocolate, cosmetic and pharmaceutical industries. In terms of fat, cocoa butter is the only constant because it is present in the fat phase, regardless of the type of chocolate. Cocoa butter is usually the only fat in dark or plain chocolate. Exceptions to this are when a small amount of milk fat is added to increase the chocolate's resistance to blooming or when low levels of non-cocoa vegetable fat are added in countries that allow these fats in chocolate. In milk chocolate, the fatty phase is then enhanced by milk fat. Some countries allow vegetable fats in chocolate, usually at a defined maximum level. As for the low-fat components in chocolate, the most important for taste is the cocoa powder, i.e., the low-fat part of the cocoa.

Cocoa butter itself has many excellent and exciting properties, as well as significant limitations. The aroma composition of cocoa products is closely related to the unique post-harvest processing conditions and the variety and origin of the cocoa itself (Kongor et al., 2016). Pure-pressed cocoa butter has a flavour that will become part of the chocolate. For some

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products, especially white chocolate, this taste is considered unpleasant. In this case, deodorized cocoa butter is used. This is often produced by steam distillation of cocoa butter under a vacuum (Meursing and Zijderveld,1999).

The growing conditions of the cocoa beans influence the cocoa butter content and fatty acid profiles in chocolate products. Identifying and quantifying fatty acids in cocoa butter is crucial for research, development, processing, and quality control during manufacturing. In cocoa butter, fatty acids form triacylglycerols (TAGs), which are mainly composed of 2-oleyl glycerides (O) combined with palmitic (P) and stearic (S) acids (POP, POS, SOS) (Segall et al., 2005). The TAG composition significantly influences chocolate's production performance and final attributes, including texture, viscosity, melting properties, flavour, and taste (Afoakwa, 2010). The simple composition of glycerides allows the chocolate to melt at a temperature range of 23 to 37°C. The crystalline form of V (β2) lipids is preferred in chocolate production and dominant in well-tempered chocolates.

The cocoa composition and FA profile vary depending on geographical origin. At the same time, only carbohydrates and fat content varied significantly in chocolates due to the effect of origin, with no significant effect observed for processing conditions. Quantitatively, the most essential fatty acids were C16:0, C18:0, C18:1, and C18:2 in all samples (Torres-Moreno et al., 2015). The structure of acids directly influences how chocolate behaves during production and affects the final product's texture, viscosity, melting properties, and flavour (Afoakwa, 2010). High-quality chocolates exhibit slower melting rates due to their higher content of saturated fatty acids, resulting in a desirable mouthfeel as they melt smoothly.

Certain vegetable fats have a triglyceride composition similar to cocoa butter, allowing them to be added to chocolate without altering its texture. Legally, such vegetable fats are allowed up to 5% in the EU for a product sold as chocolate (Regulation on Cocoa and Chocolate Products, 2003). The quantity of cocoa butter and the fatty acid composition in chocolate products are determined by the growing conditions of the cocoa beans. Identifying and quantifying fatty acids in cocoa butter is vital for research, development, processing, and quality control during manufacturing. In cocoa butter, fatty acids form triacylglycerols (TAGs), primarily consisting of 2-oleyl glycerides (O) combined with palmitic (P) and stearic (S) acids (POP, POS, SOS). Cocoa butter alternatives like Caprenin, which have unique fatty acids and

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low absorbability in the intestines, can be used as low-calorie fat substitutes. Cocoa butter can be used with non-lauric fats and tempered like usual.

Sugar and sugar substitutes

Conventionally, chocolate was commonly produced with approximately 50% sugar, predominantly sucrose, and a small amount of lactose in milk chocolate. Sugar adds flavour characteristics to a product, helps keep it moist, and inhibits bacterial growth. Monosaccharides like glucose and fructose are rarely used in chocolate because they are difficult to dry. This added moisture in the chocolate would increase the interaction between sugar particles, leading to higher viscosity. Dextrose and lactose are adequate substitutes for sucrose in milk chocolate. Lactose promotes browning through the Maillard reaction. Recently, sucrose-free chocolates have gained popularity among consumers and manufacturers due to their lower caloric content.

Sugar alcohols (polyols) replace sucrose in chocolate when it is required to make a lower-calorie or sugar-free product. Other common sugar alcohols include sorbitol, mannitol, isomalt and lactitol. Some must be processed into chocolate at relatively low temperatures to prevent them from forming gritty lumps. There is also a big difference in sweetness between the different sugar alcohols. Sugar alcohols, including xylitol, sorbitol, mannitol, erythritol, maltitol, maltitol syrup, isomalt, and lactitol, are used to produce low-calorie and sugar-free products (Grembecka, M. (2015). However, the replacement of sucrose with sugar alcohol affects the rheological properties, as well as the processing conditions and the quality of the chocolate. Maltitol is a suitable replacement for sucrose in chocolate because it shares similar rheological properties. The EU limits the consumption of sugar alcohols to 20 g per day due to the laxative effect (Grembecka, M. (2015).

It is sometimes found in unique chocolates for people with diabetes, as, unlike saccharose, it does not raise blood sugar when eaten. However, it does need special processing conditions, especially regarding temperature and humidity.

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Milk

Around 13.5% of the liquid milk is composed of anhydrous components. Lactose makes up the most considerable portion, accounting for nearly 5%. Minerals account for about 0.7 % of the total (Haylock and Dodds, 1999). Milk fat, however, has a limited shelf life as it can be oxidized or attacked by enzymes (lipolysis). The enzymes speed up the decomposition of the acids into shorter chain free acids, resulting in a rancid off-flavour and rendering the chocolate unsuitable. However, when this reaction occurs with cocoa butter, the acids formed are largely tasteless, so the chocolate remains acceptable. When milk is dried, it can produce a wide range of different powders (Fowler, 1999).

Skim milk and full cream milk powder are the most commonly used powders in chocolate making. Both powders can make chocolates with the same overall milk component content. Milk proteins add to the chocolate's nutritional content. They also determine its flavour, texture, and liquid flow properties. If the proteins are subjected to water and heat, they can participate in the Maillard (browning) reaction. Milk powder added as spray-dried skimmed milk powder or whole milk powder contributes to flavour, texture, and flow properties, depending on heat treatment and drying conditions.

There are two very different types of protein, namely caseins and whey proteins. There are four to five times as many caseins as whey proteins. Caseins act as emulsifiers. As water binds the sugar particles, adding powdered milk to liquid milk to chocolate in about 12-25% is better. Milk contains about 5% lactose, 5% milk fat, 3.5% protein and 0.7% minerals. Triglycerides of milk fat, dominated by saturated fatty acids, show different crystal structures. However, adequate amounts of palmitic, stearic, and oleic acids are the primary fatty acids in cocoa butter. Milk fat is mostly liquid (15-20% solid) at room temperature and softens the texture of the chocolate, slows down settling, and uses up to 30% of the total fat, preventing the ripening of the fat (greying). Milk fat is prone to oxidation and affects shelf life with 80% casein and 20% whey protein. The casein fraction acts as a surfactant and reduces the viscosity of chocolate, while whey proteins, on the contrary, increase the viscosity (Sadiq, Gill and Chandrapala, 2021).

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Emulsifiers

Emulsifiers have been used in chocolate for a long time for several crucial reasons: texture enhancement, stabilization, flow properties, reduced viscosity, and cost efficiency. Emulsifiers are critical in guaranteeing a desirable texture, stability, and workability of chocolate, enhancing the general quality of the product (McClements, 2004). Emulsifiers contribute to chocolate's smooth texture, stability, and overall consistency, ensuring it tastes delightful and has a visually appealing appearance. Several common emulsifiers used in chocolate production include:

Polyglycerol Polyricinoleate (PGPR, E476) reduces the viscosity of chocolate, facilitating smoother processing during various production stages like moulding and enrobing. PGPR contributes to chocolate's overall quality and appeal by promoting easy handling, texture refinement, and stability maintenance. It substitutes cocoa butter, ensuring economic efficiency without compromising the delectable texture and stability of the final chocolate product.

Sorbitan Esters are indispensable emulsifiers in chocolate production because they prevent ingredient separation, creating a stable emulsion. They also ensure the homogeneous blending of cocoa solids and fats, inhibit fat crystallization, and preserve the desired texture over the product's shelf life.

Distilled Monoglycerides (DMG) are vital contributors to the art of chocolate making. These emulsifiers are essential for creating a stable emulsion, guaranteeing the seamless incorporation of cocoa solids and fats. DMG inhibits fat crystallization, preserving the chocolate's desired consistency over time and enhancing the overall texture for a creamy and delightful mouthfeel.

Highly complex chocolate

The resulting sensory perception of cocoa is linked to many diverse components, making its flavour highly complex. Both non-volatile and volatile compounds contribute to the overall flavour profile of cocoa and cocoa-derived products. Over 600 odour compounds have been reported to be found in cocoa and chocolate (Aprotosoaie et al., 2015). The aroma composition

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of cocoa products is tightly related to the unique post-harvest processing conditions and the variety and origin of the cocoa itself.

Among the non-volatile compounds in cocoa, alkaloids and polyphenols arguably have the highest impact on flavour perception, as they are linked to bitterness. The polyphenols in chocolate, derived from cacao beans, are believed to contribute to cardiometabolic health benefits by modulating blood pressure and lipid profiles. (Tan et al., 2021). Additionally, polyphenols are associated with astringent sensations and contribute to green and fruity flavours (Noor-Soffalina, 2009). Moreover, proteins and carbohydrates are non-volatile compounds essential in forming volatile aroma compounds during the drying, roasting, and conching processes through Strecker degradation and a Maillard reaction (Guzmán Penella et al., 2023).

On the other hand, volatile aroma compounds found in cocoa products include esters, alcohols, acids, and phenols, mainly derived from the fermentation and drying processes. These compounds tend to be linked to sweet, sour, fruity, and floral notes, except for phenols, which may convey smoky and other undesirable hints (Jinap et al., 1998).

Chocolate also contains the following compounds

- Aldehydes (2-methyl butanal, 2-methyl propanal, 2-phenylacetaldehyde, 3-methyl butanal acetaldehyde, benzaldehyde)
- Esters (1,3-diacetoxypropane, diethyl butanoate, ethyl acetate, isoamyl acetate, propylene glycol diacetate) alcohols and phenols (3-methyl butane-1-ol pungent, 2,3-butanediol (isomere A), 2,3-butanediol (isomere B), 2-methyldopa-1-ol, 2-phenyl ethanol aetoin buttery, ethanol - furfuryl alcohol, pentan-2-ol
- Ketones (acetoin acetate, acetol, acetophenone, butane-2,3-dione)
- Pyrazines (2,3,5,6-tetramethylpyrazine, 2,3,5-trimethyl pyrazine, 2,3-dimethyl pyrazine)
- Other (2,2,4,6,6-pentamethylheptane, 2-acetyl pyrrole, acetic acid, butyrolactone, decane, dimethyl sulfide, toluene (Guzmán Penella et al., 2023).

Pyrazines, aldehydes, and ketones products resulting from Maillard reactions are other volatile compounds of interest.

Pyrazines are aromatic compounds with the formula C_4H_4-n$R_n$$N_2$ (where R represents an alkyl group). Pyrazines bearing methyl substituents, such as 2,3-dimethylpyrazine, exhibit a

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nutty aroma. At the same time, trimethylpyrazines and tetramethylpyrazines are associated with cocoa and coffee scents, respectively. Additionally, particular ketones and aldehydes significantly influence chocolate's flavour profile; for instance, 2- and 3-methyl-butanal contribute to a malt or chocolate aroma, and phenylethanal imparts a honey-like scent. Still, pyrazines are usually associated with the expression of nutty, earthy, roasted, and green notes (Rodriguez-Campos et al., 2012).

Chocolate includes chemicals like – phenylethylamine (PEA), anandamide, theobromine, caffeine, serotonin, phenolics, xanthenes, histamine, thyphylline etc.

Phenylethylamine (PEA) is an organic compound with the chemical formula $C_8H_{11}N$. The phenylethylamine structure is also integrated into more complex ring systems, such as the ergoline system in LSD and the morphinan system in morphine.

It acts as a neurotransmitter in the human central nervous system. Phenylethylamine has pharmacological properties similar to those of amphetamine. It is often called a Love drug because the brain releases this natural alkaloid when people fall in love. PEA is a kind of amphetamine with a very short half-life. It helps the production of serotonin, the neurotransmitter dopamine and norepinephrine, a neurotransmitter and a hormone. However, it acts mainly as a neurotransmitter), and generates optimistic and pleasurable feelings (DeLisi et al., 1984). Chocolate contains PEA, but chocolate cannot make someone fall in love because blood levels of phenylethylamine do not increase after eating chocolate, as most of this enchanting compound is metabolized during digestion. PEA is likely to be broken down by monoamine oxidase during digestion, and the trace amounts that survive are too small to affect the brain. Phenylethylamine is present in relatively high concentrations in chocolate (0.4-6.6 micrograms per gram).

Caffeine (theine, methyl theobromine) is a bicyclic molecule derived from the purine ring system. The chemical composition is 1,3,7-trimethyl-1H-purine-2,6-dione. It is an alkaloid drug found in chocolate at low levels (0.1%). Under the guidelines issued by the Food and Drug Administration (FDA), daily intake of up to 400 milligrams of caffeine is safe. Caffeine offers a plethora of health advantages. It stimulates the central nervous system, improves blood circulation in the brain, facilitates serotonin release, reduces fatigue, enhances mood and alertness, and fortifies respiratory and cardiovascular functions. However, owing to different

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metabolisms, caffeine may lead to various side effects, including headaches, jitteriness, restlessness, increased heart rate, or other reactions (Reyes and Cornelis, 2018).

The amount of caffeine in chocolate can vary depending on the type, the cocoa beans' growing conditions, and the source of cocoa. Darker chocolates (bittersweet and semi-sweet) typically contain the most caffeine. Milk chocolate contains less caffeine because it contains fewer cocoa solids, while white chocolate has no caffeine. The interaction between caffeine and theobromine in chocolate affects the psychoactive effects by relieving unpleasant side effects such as jitters, midday crashes, and sleep disturbance. The effect described is commonly referred to as the "entourage effect."

Theobromine's earlier name is Xantheose, a bitter alkaloid of a cacao plant (Jain et al., 2020.) whose chemical formula is $C_7H_8N_4O_2$, which comprises 1.5-2.7% chocolate. Interestingly, theobromine does not contain bromine in its composition. The name is derived from the cacao tree, Theobroma, which translates to 'god drink' in Greek ('theo' meaning 'god' and 'broma' meaning 'drink'). The Mayan people believed chocolate was their gods' preferred beverage. The main methylxanthines in cocoa, chocolate, and certain plant foods are bitter alkaloids, theophylline, and caffeine.

Theobromine and caffeine differ by only one methyl group but have comparable stimulant effects on the human brain. It is highly fat soluble, with levels peaking in the plasma 1–2 h after ingestion. Recognized (Scholey and Owen, 2013) as an antagonist of the adenosine receptor and inhibitor of the phosphodiesterase, this white crystalline powder has multiple uses, including bronchodilation, antitussive, neurostimulation, cardiac stimulant, vasodilation, muscle relaxation, diuretic properties, anti-inflammatory effects, antitumoral activity, and cardiovascular protection (Carbajal-Valenzuela et al., 2020). The content of theobromine is around 200 mg per 100 g of milk chocolate, and the darker the chocolate, the higher the concentration.

Anadamide is a lipid. The origin of the name anandamide might be a clue to its effects - Ananda is Sanskrit for "bliss". Anandamide is an N-acylethanolamine 20:4 resulting from the formal condensation of the arachidonic acid's carboxy group with the ethanolamine's amino group. This endocannabinoid has a role as a neurotransmitter, a vasodilator agent and a human blood serum metabolite. Anandamide is an endogenous cannabinoid receptor agonist. Anandamide works on two cannabinoid receptors, provoking euphoria, increased appetite, and

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short-term memory deficits. The two most abundant endogenous cannabinoids, N-acyl ethanolamines (NAEs) found in chocolate (N-oleylethanolamine and N-linoleylethanolamine), inhibit the degradation of anandamide without activating brain cannabinoid receptors (Beltramo and Piomelli, 1998.) This observation suggested that these compounds might enhance the pleasurable properties of chocolate by causing non-metabolized anandamide to accumulate at its action sites. Di Marzo and co-workers (Di Marzo et al., 1994) have tested the role of endogenous cannabinoids in chocolate. Based on their findings, they deduce that the levels of NAEs and other cannabinoid-related compounds in cocoa are significantly lower than what would be needed if ingested orally to reach the bloodstream and induce noticeable cannabis-like effects. Therefore, tiny amounts of anandamide in cocoa could be explained as processing artefacts.

Types of chocolate

There are many types of chocolate, which can be classified into various groups depending on their color, composition, uses, and flavor potential.

Milk chocolate

Milk chocolate is sweet chocolate that contains milk powder or condensed milk. Milk chocolate is light brown in colour, has a creamy texture, and has a sweet flavour. It is widely regarded as the most popular type of chocolate (Minifie, 1989). Renowned for its smooth and velvety texture, milk chocolate is known for its mild, sweet, and creamy flavour. It is crafted by blending chocolate liquor (cocoa solids and cocoa butter) with sugar and milk. An emulsifier like soy lecithin is occasionally included to augment its smooth consistency. FDA defines milk chocolate composition as at least 10% chocolate liquor, 3.39% milk fat, and at least 12% milk solids. Milk chocolate is milder and sweeter. The cacao % (amount of actual cocoa bean used) is between 35% to 55% cocoa mass, 20% milk powder, and 20% to 25% sugar. Milk chocolate typically offers a flavour profile characterized by sweetness and chocolate richness, with hints of cooked milk and caramelized sugar, followed by a subtle vanilla undertone. It is notably sweeter and possesses a softer texture compared to dark chocolate. However, it falls slightly short in sweetness and softness compared to white chocolate (The Chemistry of Chocolate).

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When stored properly, milk chocolate maintains its quality for approximately 16 months. It is an excellent choice in baking when a milder chocolate flavour is desired, as demonstrated in recipes such as chocolate waffles. Due to its higher sugar and milk solid content, milk chocolate should not be substituted for semi-sweet chocolate in recipes. Milk chocolate is optimal for crafting dipping and drizzling sauces, pastry creams, and confectionery delights.

White Chocolate

White chocolate comprises a minimum of 20% cocoa butter and 14% milk, with a sugar content not exceeding 55%, along with vanilla and lecithin. These components contribute to the sweet vanilla fragrance associated with white chocolate. Its flavour profile is typically characterized by pronounced sweetness, underscored by rich, sweetened condensed milk and vanilla notes. High-quality white chocolate exhibits a luxurious, velvety texture thanks to its cocoa butter base and elevated sugar and milk concentrations. Notably, white chocolate stands out for its absence of cocoa solids, which are integral to the composition of traditional chocolate varieties. It's dark brown and has a chocolatey taste that we all know and love (Glicerina et al., 2016). White chocolate qualifies as chocolate because the cocoa bean ingredients are derived from the cacao bean. It should not be confused with white-flavored or vanilla-flavored coatings commonly found in lower-quality products. Cocoa butter is an essential constituent of white chocolate. It is relatively expensive due to the high demand for products such as lotions in the cosmetics industry. Consequently, some companies substitute cocoa butter with other vegetable fats to reduce costs. However, these substitutes often fail to meet the FDA's requirement of at least 20% cocoa butter content, preventing them from officially being labelled as white chocolate.

When stored correctly, white chocolate maintains its quality for approximately four months. Besides being delicious, white chocolate is versatile in cooking and baking and a decorative element for drizzling or coating. It is well-suited for creating dipping and drizzling sauces, mousses, pastry creams, and various confectionery delights.

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Blonde chocolate

Blonde chocolate was, for all intents and purposes, a happy accident! It is produced by slowly heating white chocolate, which gives it a golden colour and triggers Maillard reactions. These reactions create a range of flavour compounds, contributing to its caramel-like flavour (Liu et al., 2022). It was discovered by chance in 2004 in Valrhona by a pastry chef, Frédéric Bau when he accidentally left some white chocolate in a bain-marie for a few hours. The chocolate went through a Maillard reaction. Both the Maillard reaction and caramelization involve a chemical reaction that causes browning. However, while caramelization only impacts sugars, the Maillard reaction involves amino acids. Bau found that his white chocolate took on a pale brown colour with irresistible butterscotch, toffee, and shortbread-tasting notes, unlike any chocolate he had eaten before. Because Bau’s blonde chocolate had been created by mistake, it took chocolatiers eight years to reproduce the treat and master its nuanced flavour profile. Although blonde chocolate undergoes the Maillard reaction, it is often mistakenly called 'caramelized chocolate' or even 'toasted chocolate'. And it's not hard to see why. With its golden caramel colour and depth of flavour, brimming with toasted notes, it has a fuller taste than traditional white chocolate, which tends to be subtle and delicate.

Dark chocolate

Dark chocolate is crafted by adding fat and sugar to the cacao mixture. Dark chocolate encompasses a variety of cocoa contents, ranging from 35% to over 85%. With higher cocoa content, chocolate has less sugar and a more pungent, bitter taste. This kind of chocolate, characterized by its distinctive dark brown colour, is in second place in popularity among consumers. Frequently, it is called black or semi-sweet chocolate because it is significantly less sweet than milk chocolate (Glicerina et al., 2016). Recently, dark chocolate has gained popularity due to numerous articles highlighting its health benefits. Its composition is relatively simple, typically comprising two primary ingredients: chocolate liquor and sugar. Occasionally, small amounts of vanilla and soy lecithin, an emulsifier, are added. Most high-quality dark chocolate is free from added dairy, making it an excellent choice for vegans. The absence of

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dairy and reduced sugar content contributes to its firmer texture than milk and white chocolate, resulting in a satisfying snap when adequately tempered.

The flavour profile of dark chocolate varies widely depending on its cocoa content. It often boasts a slightly sweet and chocolatey taste, accompanied by hints of baked brownie, red fruit, and brown spice like cinnamon or allspice. Its robust chocolate-forward flavour makes it perfect for baking, particularly when a rich, chocolatey flavour is desired, as showcased in recipes like classic brownies or decadent chocolate bourbon maple pecan pie.

Dark chocolate has been rediscovered because of its health benefits, making it a preferred snack choice among health-conscious consumers. Dark chocolate is recognized as a functional food due to its anti-diabetic, anti-inflammatory, and anti-microbial properties. Additionally, it provides protection against cardiovascular diseases, certain cancers, and brain-related disorders such as Alzheimer's and Parkinson's (Samanta et al., 2022). When stored correctly, it maintains its quality for approximately 20 months. In contrast to other chocolate varieties, dark chocolate exhibits a more pronounced and bittersweet flavour profile. It is typically classified into two primary types: bittersweet chocolate and semi-sweet chocolate. The FDA stipulates that dark chocolate must contain a minimum of 35% cacao and less than 12% milk solids, leaving manufacturers accountable for categorizing their products as either bittersweet or semi-sweet. Bittersweet chocolate typically contains a higher cocoa percentage than semi-sweet and tends to be less sweet.

Dark chocolate is especially well-suited for recipes where chocolate takes centre stage, such as ganache, mousses, truffles, and puddings.

Bittersweet chocolate

Bittersweet chocolate is mainly composed of chocolate liquor (about 33% of the final mass), cocoa butter, vanilla, and sometimes lecithin. The sugar content is less, and the liquor content is more than semi-sweet chocolate. People are increasingly interested in bittersweet chocolate as they learn about cacao and cocoa percentages (https://www.lakechamplainchocolates.com/types-of-chocolate/). The rise in popularity of this type of chocolate, also known as extra-dark chocolate, occurred when people started advocating for dark chocolate with a cocoa content of over 70% for maximum health benefits. The high

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cacao content gives the chocolate a rich flavor profile, reducing sweetness and imparting a slightly dry or crumbly texture. Bittersweet chocolate is darker than milk and semi-sweet chocolate on the chocolate spectrum. Still, it is less dark than authentic dark chocolate. Bittersweet baking chocolate is excellent for balancing sharp and sweet chocolate notes.

Semi-sweet chocolate

Semi-sweet chocolate typically contains approximately 50-60% cocoa solids, with the remaining portion comprising sugar and potentially small amounts of lecithin, vanilla, or other flavourings. (https://www.lakechamplainchocolates.com/types-of-chocolate/)

Although semi-sweet chocolate contains less sugar than milk chocolate, its sugar content is higher than most dark chocolates. Cocoa butter is mainly responsible for semi-sweet chocolate's smooth texture and glossy appearance. Lecithin is an emulsifier that smoothly blends cocoa and cocoa butter. Vanilla is often added to enhance the chocolate's flavour, offering a moderate level of sweetness that strikes a balance between the inherent bitterness of cocoa and the added sugar. Semi-sweet chocolate has a more robust, less sweet taste than milk chocolate, making it well-suited for desserts that do not require an overpowering chocolate flavour.

When baking, bittersweet and semi-sweet chocolate can be swapped depending on the recipe and personal taste preferences.

Raw chocolate

Unprocessed chocolate, commonly known as raw cacao, is consistently dark and contains at least 75% cacao content. Raw chocolate has not undergone processing, heating, or blending with other ingredients. In its production process, the "raw" chocolate is not exposed to high temperatures, cocoa beans are not subjected to roasting, and the chocolate conching temperature does not exceed 45 °C, which enables preserving its valuable traits. It is available in countries where cocoa is cultivated and, to a lesser extent, in other regions. Raw chocolate is often promoted as a healthier alternative. Raw chocolate includes numerous essential antioxidants, minerals, and vitamins- proteins, iron, and fibers (Urbańska et al., 2019). Improperly tempered chocolate may exhibit whitish spots on its dark surface, referred to as chocolate bloom. This

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indicates that sugar and/or fat have separated due to inadequate storage. While unappealing in appearance, chocolate bloom is not harmful and can be consumed safely.

Ruby Chocolate

Belgian chocolate maker Barry Callebaut discovered Ruby chocolate. This variety developed in 2004 and was introduced to the public in 2017. The exact production process of ruby chocolate has yet to be discovered. However, in 2012, Callebaut obtained a European patent for a specific "process for producing cocoa-derived material". Unlike traditional white chocolate, it obtains its colour from a particular type of cocoa bean known as the ruby cocoa bean, typically cultivated in regions such as Ecuador, Brazil, and the Ivory Coast. As opposed to regular chocolate production, either under fermented cocoa beans, not fermented for more than three days, or preferably, unfermented cocoa beans, the so-called “Lavados” beans, meaning “washed” beans, are used (Tuenter et al., 2021). This chocolate stands out among its counterparts, featuring a distinctive red-pink hue. With a 47.5% cacao content composition and 26.3% milk, ruby cacao boasts intense fruity flavours and refreshing sour notes. Ruby chocolate's flavour profile is sweet and fruity with fresh, tart notes and a red-pink colour despite having no added colours or fruit flavourings. This trendy new type of cacao is great for creating bold, fruit-forward chocolate treats and colourful chocolate confections. Rubbery cacao can have a shelf-life of about 12 months (https://www.lakechamplainchocolates.com/types-of-chocolate/).

Gianduja

Gianduja refers to European-style chocolate crafted from a chocolate and nut paste blend. While hazelnut paste is the most prevalent, almond paste can also be used. It is available in both milk and dark chocolate variations. The history of Gianduja starts during a Napoleonic lockdown. During the Napoleonic wars, Napoleon and Nelson's battle led to a European-wide lockdown. Nelson and the British Navy enforced a blockade on European ports in response to Napoleon's trade war called the "Continental System". This lockdown put an enormous strain on cocoa supplies. Michele Prochet, a chocolatier from Turin, blended his limited chocolate with hazelnuts from Piedmont's hills to make it go further. Due to limited resources, he had to rely on easily accessible, regional ingredients to extend his chocolate production. The birth of Gianduja

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during the Napoleonic lockdown was a response to the high demand for chocolate from customers (https://meltchocolates.com/gianduja-carnival-what-is-gianduja-and-its-connection-to-carnival/).

Gianduja chocolate is a versatile ingredient, suitable for flavouring or as a replacement for milk or dark chocolate. Like conventional chocolate, it is produced in plain and milk versions. It may include other nuts, such as almonds.[18] In bar form, Gianduja resembles conventional chocolate, albeit noticeably softer because of the hazelnut oil content. It maintains a smooth consistency at room temperature, allowing it to be rolled or cut. Yet, it is too soft for moulding chocolates (Medrich, 2015).

Couverture Chocolate

Couverture chocolate is a quality chocolate favoured by professional bakers and confectioners; it is renowned for its superior quality. It boasts a high cocoa butter content comprising at least 30 percent and a significant proportion of chocolate liquor. The abundance of cocoa butter imparts a glossy appearance to the chocolate and ensures a firm snap when expertly tempered. Couverture chocolate melts smoothly, making it ideal for coating, dipping, and enrobing. It is the preferred chocolate for tempering and enrobing candies. It is not recommended for baking. Couverture chocolate is offered in dark, milk, and white chocolate options. It is commonly found in drop form but can also be purchased as a bar or slab.

Couverture chocolate's composition must meet specific criteria. Both couverture and milk chocolate must contain identical amounts of cocoa butter and cocoa solids. Still, couverture chocolate requires at least 31% cocoa butter and 2.5% fat-free cocoa solids. (https://www.valrhona.com/en/l-ecole-valrhona/discover-l-ecole-valrhona/chocolate-terminology/couverture-chocolate). The importance lies in cocoa butter, which provides couverture chocolate with its glossy appearance and smooth consistency.

Chocolate bloom

Chocolate bloom is a white or greyish coating that can form on chocolate. Although it may appear and feel unappealing, chocolate that has bloomed is typically safe for consumption. There are two forms of chocolate bloom: fat bloom and sugar bloom.

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Fat bloom occurs when chocolate undergoes fluctuations in temperature. The process of fat bloom involves the separation of cocoa butter from cocoa solids when it melts. As cocoa butter solidifies, it migrates to the chocolate's surface, producing grey streaks or white blotches, known as fat bloom. Poor storage conditions, particularly temperature fluctuations, often contribute to fat bloom. Chocolate is sensitive to temperature changes and should be stored in a cool, dry environment (Indiarto et al., 2021). While fat bloom can alter the chocolate's colour and sometimes its texture, typically making it soft and crumbly, it remains safe to consume and usually does not affect the taste.

When fat bloom occurs, the chocolate can be safely remelted and tempered. Conversely, sugar blooms arise when chocolate encounters moisture, resulting in water, either in dampness or condensation, impacting the chocolate. This causes the sugar to segregate and form a sugar bloom, giving the chocolate a white and grainy appearance. Preventing sugar bloom necessitates keeping chocolate shielded from moisture and storing it in a cool, dry location, refraining from refrigeration or freezing.

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Koju vrstu magije zapravo stavljaju u čokoladu?

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SAŽETAK

Čokolada je najpopularniji slatkiš na svetu. Fermentisano, prženo i mleveno seme kakao- drveta rezultira čokoladom, krajnjim gotovim proizvodom. Glavne komponente čokolade su ugljeni hidrati, masti i proteini, ali čokolada sadrži i važne minerale kao što su kalcijum, magnezijum, gvožđe i cink. Postoje različite vrste čokolade po sastavu i obliku. Mešavina kakaoa, način pečenja kakao-zrna i odnos kakao-putera, kakao-praha, masti i šećera igraju ulogu u određivanju vrste čokolade. Rezultirajuća senzorna percepcija čokolade povezana je sa mnogim različitim komponentama, čineći njen ukus veoma složenim. Čokolada može imati različite oblike, poput štapića, i često je začinjena vanilom, cimetom, karanfilićem, lešnicima ili bademima. Mlečna čokolada je najpopularnija čokolada širom sveta. Kremasta i nežna, topi se na jeziku i ostavlja osećaj potrebe da je želite još. Druga najpopularnija čokolada je crna, a zatim bela čokolada. Najpopularnije kombinacije su meki nugat i hrskavi orasi. Lešnici i bademi su takođe nezamenljivi u industriji čokolade.

Ključne reči: čokolada, kakao puter, anandamid, teobromin, kafein, tipovi čokolade

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Quel genre de magie mettent-ils dans le chocolat, de toute façon ?

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RÉSUMÉ

Le chocolat est la douceur la ~~p~~lus populaire au monde. Les graines du cacaoyer sont fermentées, torréfiées et broyées, ce qui fait du chocolat le produit fini final. Les principaux composants du chocolat sont les glucides, les lipides et les protéines et il contient également des minéraux essentiels comme le calcium, le magnésium, le fer et le zinc. La perception sensorielle du chocolat qui en résulte est liée à de nombreux composants divers, ce qui rend sa saveur très complexe. Il existe différents types de chocolat en termes de composition et de forme. Le mélange de cacao, la méthode de torréfaction des fèves de cacao et le rapport entre le beurre de cacao, la poudre de cacao sans gras et le sucre jouent tous un rôle dans la détermination du type de chocolat. Le chocolat peut prendre différentes formes, comme des barres ou des bâtonnets et est souvent aromatisé à la vanille, à la cannelle, aux clous de girofle, aux noisettes ou aux amandes. Le chocolat au lait est le chocolat le plus populaire au monde. Crémeux et tendre, il fond sur la langue et vous donne envie de plus. Le deuxième chocolat le plus populaire est e chocolat noir, suivi du chocolat blanc. Les combinaisons les plus populaires sont le nougat moelleux et les noix croquantes. Les noisettes et les amandes sont également indispensables dans l'industrie du chocolat.

Mots-clés : chocolat, beurre de cacao, anadamide, théobromine, caféine, types de chocolat

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Что за волшебство они вкладывают в шоколад?

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Резюме

Шоколад – самая популярная конфета в мире. Семена какао-дерева ферментируются, обжариваются и измельчаются, что делает шоколад конечным готовым продуктом. Основными компонентами шоколада являются углеводы, жиры и белки, а также он содержит необходимые минералы, такие как кальций, магний, железо и цинк. Полученное в результате сенсорное восприятие шоколада связано со многими разнообразными компонентами, что делает его вкус очень сложным. Существуют разные виды шоколада по составу и форме. Смесь какао, метод обжарки какао-бобов и соотношение какао-масла, обезжиренного какао-порошка и сахара играют роль в определении типа шоколада. Шоколад может принимать различные формы, такие как батончики или палочки, и часто ароматизируется ванилью, корицей, гвоздикой, фундуком или миндалем. Молочный шоколад – самый популярный шоколад в мире. Сливочный и нежный, он тает на языке и заставляет вас хотеть большего. Вторым по популярности шоколадом стал темный шоколад, за ним следует белый шоколад. Самые популярные сочетания – мягкая нуга и хрустящие грецкие орехи. Фундук и миндаль также незаменимы в шоколадной промышленности.

Ключевые слова: шоколад, какао-масло, анадамид, теобромин, кофеин, виды шоколада

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Was für ein Zauber steckt überhaupt in Schokolade?

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ABSTRAKT

Schokolade ist die beliebteste Süßigkeit der Welt. Die Samen des Kakaobaums werden fermentiert, geröstet und gemahlen, so dass Schokolade das Endprodukt ist. Die Hauptbestandteile von Schokolade sind Kohlenhydrate, Fett und Eiweiß, und sie enthält auch essentielle Mineralien wie Kalzium, Magnesium, Eisen und Zink. Die daraus resultierende sensorische Wahrnehmung von Schokolade ist mit vielen verschiedenen Komponenten verbunden, was ihren Geschmack sehr komplex macht. Es gibt verschiedene Schokoladensorten, die sich in ihrer Zusammensetzung und Form unterscheiden. Die Kakaomischung, die Röstmethode der Kakaobohnen und das Verhältnis von Kakaobutter, fettfreiem Kakaopulver und Zucker spielen eine wichtige Rolle bei der Bestimmung der Schokoladensorte. Schokolade kann in verschiedenen Formen, wie Riegeln oder Stangen, angeboten werden und ist oft mit Vanille, Zimt, Nelken, Haselnüssen oder Mandeln aromatisiert. Milchschokolade ist die beliebteste Schokolade der Welt. Cremig und zart zergeht sie auf der Zunge und macht Lust auf mehr. Die zweitbeliebteste Schokolade ist Zartbitterschokolade, gefolgt von weißer Schokolade. Die beliebtesten Kombinationen sind weicher Nougat und knackige Walnüsse. Auch Haselnüsse und Mandeln sind aus der Schokoladenindustrie nicht wegzudenken.

Schlagworte: schokolade, kakaobutter, anadamid, theobromin, koffein, schokoladensorten

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Honey: food and a therapeutic agent

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ABSTRACT

Honeybees produce honey. Humans have used honey for more than 9,000 years as food and medicine. Honey has a complex chemical composition (sugars, organic acids, amino acids, peptides, enzymes, micro and macroelements, water-soluble vitamins and natural phenolic compounds), varying depending on the botanical source. Besides its significant role in traditional medicine, honey also has a place in modern medicine. Research has shown that honey has an inhibitory effect on about 60 types of bacteria, some types of fungi and viruses. The antioxidant capacity of honey is significant for many diseases and is primarily due to many natural phenolic compounds in honey. Therefore, honey is also used to treat inflammatory, dermal, diabetic, gastrointestinal, cardiovascular, and neoplastic conditions. This article briefly overviews the composition, physico-chemical properties and application of natural honey as a nutraceutical agent.

Keywords: honey, composition, organoleptic characteristic, food, therapeutic agent

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Introduction

Bee honey is considered the most consumed bee product. It is defined by the Codex Alimentarius as a sweet substance produced by Apis mellifera bees from the flower's nectar or secretions of plants, which bees collect, transform by combining with their specific enzymes and store in honeycombs (CODEX ALIMENTARIUS, International Food Standards, STANDARD FOR HONEY CXS 12-1981). Unlike white sugar and other sweeteners, honey is a treasure trove of substances necessary for the healthy functioning of the body.

History of honey use

Evidence from Stone Age paintings shows that treatment of disease with bee products such as honey originated 9000 years ago (Figure 1).

Figure 1. A honey hunter (a cave painting from Spain) (~8000 BCE) (Qamar & Rehman, 2020)

Ancient scrolls, tablets and books-Sumerian clay tablets (6200 BC), Egyptian documents (1900–1250 BC), Veda (a body of Hindu religious texts) (5000 years old), Holy Koran, Bible, and Hippocrates (460–357 BC) illustrated that honey had been widely used as a medicine. Quran vividly indicated the activity of therapeutic value of honey. According to this holy book of Muslims, the Lord inspired the bees to build their hives in hills, on trees, and in man's habitations; from within their bodies comes a drink of varying colours, representing a healing for humankind. In Hinduism, honey contains specific religious attributes as one of the "five nectars" (Qamar & Rehman, 2020). Jewish traditions link honey to the New Year, and use of honey-dipped apples as a traditional meal on the day (Qamar & Rehman, 2020). Honey was used for a variety of disease conditions, including eye diseases, throat infections, asthma, tuberculosis, hiccups, fatigue, hepatitis, dizziness, constipation, worm infestation, piles, eczema, healing of ulcers, and wounds (The History of Honey and Beekeeping: https://localhoneyfinder.org/HistoryOfHoney.php).

Organoleptic characteristics of honey

The organoleptic analysis includes honey's taste, colour, aroma, and viscosity. The taste is something that the sense of taste can feel, and the colour is visible and can be observed in honey. Aroma is the result of sensing through smell. The smell is also an indicator of damage to the product (Lawless, 1991). For example, a foul odour indicates the product has been damaged. In honey products, the quality standard for the aroma and colour of honey depends on the origin and type of flower (Puscion-Jakubik et al., 2020). In addition, the colour varies from white, yellow, brown, red to black. The colour variation depends on the nectar of the flower (Puscion-Jakubik et al., 2020). The colour of honey can divide types into white, light, and dark honey. Red honey is usually dark honey, which was previously yellow, then brownish yellow, and then becomes slightly reddish. White honey is not much different from red honey. The only difference is the flower nectar that is sucked by the worker bees. White honey is mainly obtained from citrus, kapok, and durian trees, while dark honey is from daisies and insect fluids (Anupama et al., 2003).

Chemical composition of honey

About 300 substances in honey have been reported, and research on this topic is continued.

Sugars

Honey is composed primarily of fructose (32.56 to 38.2%) and glucose (28.54 to 31.3 %), disaccharides such as maltose, sucrose, isomaltose turanose, nigerose, melibiose, panose, maltotriose, melezitose (Fuente et al., 2011). Honey contains 4 to 5% fructooligosaccharides, known as probiotic agents (Ahmad et al., 2017).

Water

Water is the second most crucial component of honey. The maximum allowed water content in honey is up to 20%.

Organic acids

Organic acids are responsible for 0.57% of honey and include gluconic acid (a byproduct of enzymatic digestion of glucose). Acetic, citric, formic, glutaric, fumaric, succinic, D-gluconic, oxalic, D-glucuronic, L-malic, propionic, D-quinic, L-tartaric and many others are present in honey. The organic acids give a contribution to the acidity of honey and are mainly responsible for its characteristic taste (Suto et al., 2020).

Mineral composition

The concentration of mineral compounds goes from 0.1% to 1.0 %. Potassium is the major element, followed by Cl, S, Ca, Na, P, Mg, Si, Fe, Mn and Cu. Trace elements contents of honey depend mainly on the botanical and geographical origins of honey. Although mineral compounds in honey do not make a considerable proportion, they significantly raise the value of honey for human consumption (Tafere, 2021).

Vitamins

The vitamins of honey are mainly represented by B-group vitamins (thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid), and vitamin C. The content of vitamins in honey depends on its botanical origin (León-Ruiz et al., 2011; Popkova et al., 2021).

Amino acids

Honey contains most amino acids, such as proline, tyrosine, methionine, lysine, phenylalanine, histidine, glycine, *etc.* Proline is the major free amino acid in honey, and its concentration (greater than 180 mg/kg) is an indicator of the authenticity of the honey (da Silva et al., 2016). Since the nectar plant is the major provider of amino acids in honey, and geographic and floral sources can lead to subtle differences in amino acid types and content, some studies have indicated that differences in amino acids can be used as a tool for the identification of the floral source of the honey (Kowalski et al., 2017).

Enzymes

Honey contains numerous enzymes. The honey enzymes originate from bees, plant nectars, secretions or excretions of plant-sucking insects, or microorganisms such as yeasts (Alaerjani et al., 2022). Enzyme-catalyzed and non-enzymatic reactions can characterize honey. Notable examples of enzyme-catalyzed reactions are hydrogen peroxide production through glucose oxidase activity and the involvement of catalase enzymes in the conversion of hydrogen peroxide to water and oxygen (Alaerjani et al., 2022). The production of hydroxymethylfurfural (HMF) from glucose or fructose is an example of a non-enzymatic reaction in honey. The most spread enzymes in honey are glucose oxidase, protease, invertase, diastase, acid phosphatase, dihydroxyacetone phosphatase, catalase, reduced glutathione and superoxide dismutase (Alaerjani et al., 2022). Diastase enzymes are included as honey quality parameters and indicators for assessing honey storage conditions in all honey standards. Invertase is not adopted as a honey quality parameter; however, it is suggested to be a helpful quality parameter in the European standards for honey. Although diastase and invertase originate from honeybees, they can be used as indicators for honey floral origins because the concentration of the substrates affects the enzyme activity (Alaerjani et al., 2022).

The phenolic compound and flavonoid profiles

The phenolic compounds and flavonoid profiles of honey allow the evaluation of its quality, since they identify emerging risks, facilitate the differentiation of the varietals, botanical origin, and detect adulteration and bioactive compounds with health-promoting properties. The profiles of phenolic compounds and flavonoids in honey are analysed mainly by High-

Performance Liquid Chromatography (HPLC) and Fourier-Transform Infrared Spectroscopy (FTIR). The predominant phenolic compounds found in samples have been *p*-coumaric, caffeic, chlorogenic, ferulic, ellagic, protocatechuic, and vanillic acids, rutin, myricetin, apigenin and quercetin, pinocembrin, kaempferol, galangin, chrysin and hesperetin and their glucosides (Becerril-Sánchez et al., 2021).

Honey as a food

One hundred grams of honey has an energy value of 1229 kJ, and contains no fat, very little protein (about 0.4%), carbohydrates 80%, of which fructose and glucose are the most abundant. The percentage of water ranges from 15 to 20 (Bogdanov et al., 2008).

There is no recommended daily intake of honey. However, honey should be consumed in moderate quantities due to its high sugar content. The World Health Organization (WHO) recommends that free sugars should not represent more than 10% of a daily energy intake. For a person requiring 2000 kcal per day, ~ 60 grams of honey (if honey is used as the unique external source of sugar) is recommended. Due to the reason that honey may contain the bacteria *Clostridium botulinum* (which can cause severe infection in infants), it is recommended to avoid its consumption in children below 12 months of age (WHO, 2015).

Honey as a therapeutic agent

For ages, honey has been traditionally used to treat human diseases (Figure 2). Recently, it has become acceptable as a therapeutic agent that is relatively cheap and lacks side effects. The therapeutic and beneficial properties of honey are present due to:

- Its antibacterial, antiviral, antifungal, and antiparasitic activities against various organisms.
- Its anti-inflammatory and immunomodulatory activities because of substantial amounts of phenolic contents.
- Its antioxidant capacity to scavenge free radicals.
- Its natural immune-boosting capability.

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Honey could also be used to treat other medical conditions, such as:

- *Escherichia coli*, *Salmonella* and *Shigella* caused diarrhea.
- Various gastrointestinal conditions such as gastric and duodenal ulcers, and gastritis.
- Canine recurrent dermatitis, seborrheic dermatitis and diaper dermatitis.
- Diabetics, based on results from animal model studies, preclinical and clinical studies, and human studies.
- Cancer and tumour, may be due to its antiapoptotic, antitumor, antiproliferative, antimutagenic, and estrogenic modulatory activities.
- Wounds, especially in diabetic patients.

The most known factors that give honey its above-mentioned properties include its acidity, hydrogen peroxide, high sugar, and other non-peroxide properties (Bucekova et al., 2019). The therapeutic properties of honey can be diminished by its exposure to heat or higher temperature, and also under the influence of light, sunlight, or UV light.

In general, honey has profound potential as a therapeutic agent which might complement/replace conventional drugs. Therefore, further research is still needed to establish the scientific basis for classifying honey as a medical agent (Nweze et al., 2020).

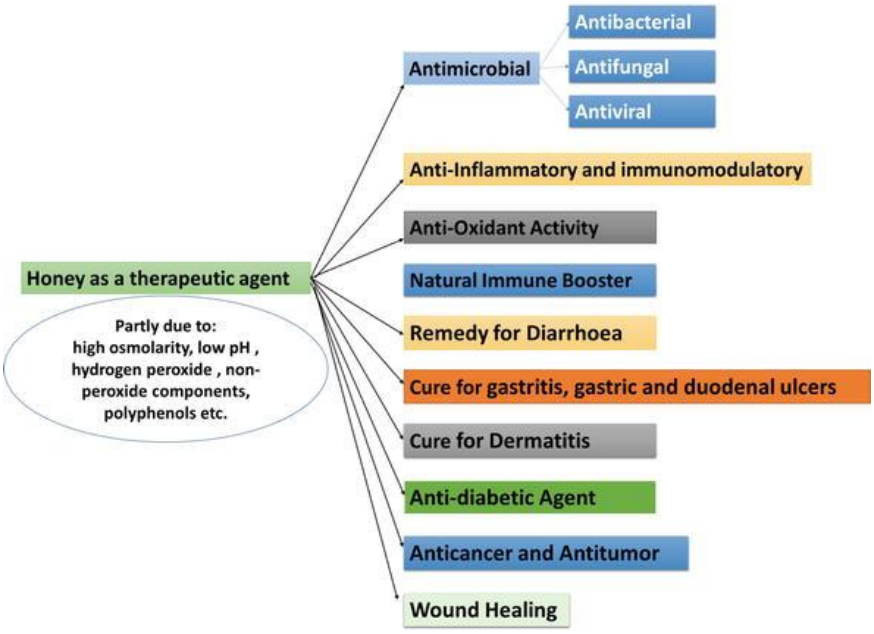

Figure 2. Bee honey as a therapeutic agent (Nweze et al., 2020)

Conclusion

Besides macroconstituents, honey contains microconstituents responsible for its nutraceutical properties. Therefore, it is not surprising that its use and significance originate from the distant past of humankind. Properties of honey depend on botanical origin. Further studies will enable the use of honey as a medicinal agent.

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Conflict-of-Interest Statement

None.

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Med: hrana i terapeutski agens

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SAŽETAK

Pčele prave med. Ljudi koriste med više od 9.000 godina kao hranu i lek. Med ima složen hemijski sastav (šećeri, organske kiseline, aminokiseline, peptidi, enzimi, mikro i makroelementi, vitamini rastvorljivi u vodi i prirodna fenolna jedinjenja), koji varira u zavisnosti od botaničkog porekla. Pored značajne uloge u tradicionalnoj medicini, med ima i mesto u savremenoj medicini. Istraživanja su pokazala da med ima inhibitorno dejstvo na oko 60 vrsta bakterija, neke vrste gljivica i virusa. Antioksidativni kapacitet meda je značajan za mnoge bolesti i prvenstveno je posledica mnogih prirodnih fenolnih jedinjenja u medu. Zato se med koristi i za lečenje upalnih, dermalnih, dijabetičarskih, gastrointestinalnih, kardiovaskularnih i neoplastičnih stanja. Ovaj članak daje kratak pregled sastava, fizičko-hemijskih svojstava i primene prirodnog meda kao hrane i leka.

Ključne reči: med, sastav, organoleptičke karakteristike, hrana, terapaeutski agens

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Le miel : aliment et agent thérapeutique

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RÉSUMÉ

Les abeilles produisent du miel. Les gens utilisent le miel depuis plus de 9 000 ans comme aliment et médicament. Le miel a une composition chimique complexe (sucres, acides organiques, acides aminés, peptides, enzymes, micro et macroéléments, vitamines hydrosolubles et composés phénoliques naturels), variant selon la source botanique. Outre son rôle important dans la médecine traditionnelle, le miel a également sa place dans la médecine moderne. La recherche a montré que le miel a un effet inhibiteur sur environ 60 types de bactéries, certains types de champignons et de virus. La capacité antioxydante du miel est importante pour de nombreuses maladies et est principalement due à de nombreux composés phénoliques naturels du miel. Par conséquent, le miel est également utilisé pour traiter les affections inflammatoires, cutanées, diabétiques, gastro-intestinales, cardiovasculaires et néoplasiques. Cet article donne un bref aperçu de la composition, des propriétés physico-chimiques et de l'application du miel naturel en tant qu'agent nutraceutique.

Mots-clés : miel, composition, caractéristique organoleptique, aliment, agent thérapeutique

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Мед: пища и лечебное средство

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Резюме

Медоносные пчелы производят мед. Люди использовали мед более 9000 лет в качестве пищи и лекарства. Мед имеет сложный химический состав (сахара, органические кислоты, аминокислоты, пептиды, ферменты, микро- и макроэлементы, водорастворимые витамины и природные фенольные соединения), изменяющийся в зависимости от растительного источника. Помимо своей значительной роли в народной медицине, мед имеет место и в современной медицине. Исследования показали, что мед оказывает угнетающее действие примерно на 60 видов бактерий, некоторые виды грибков и вирусов. Антиоксидантная способность меда имеет большое значение при многих заболеваниях и обусловлена, прежде всего, многими природными фенольными соединениями в меде. Поэтому мед также используется для лечения воспалительных, кожных, диабетических, желудочно-кишечных, сердечно-сосудистых и опухолевых заболеваний. В данной статье кратко рассмотрены состав, физико-химические свойства и применение натурального меда в качестве нутрицевтического средства.

Ключевые слова: мед, состав, органолептические характеристики, пища, лечебное средство

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Honig: Lebensmittel und ein Therapeutikum

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ABSTRAKT

Honigbienen produzieren Honig. Der Mensch verwendet Honig seit mehr als 9.000 Jahren als Nahrungsmittel und Medizin. Honig hat eine komplexe chemische Zusammensetzung (Zucker, organische Säuren, Aminosäuren, Peptide, Enzyme, Mikro- und Makroelemente, wasserlösliche Vitamine und natürliche phenolische Verbindungen), die je nach botanischer Quelle variiert. Neben seiner bedeutenden Rolle in der traditionellen Medizin hat Honig auch einen Platz in der modernen Medizin. Untersuchungen haben gezeigt, dass Honig eine hemmende Wirkung auf etwa 60 Arten von Bakterien sowie auf einige Arten von Pilzen und Viren hat. Die antioxidative Kapazität von Honig ist für viele Krankheiten von Bedeutung und beruht in erster Linie auf den vielen natürlichen phenolischen Verbindungen im Honig. Daher wird Honig auch zur Behandlung von entzündlichen, dermalen, diabetischen, gastrointestinalen, kardiovaskulären und neoplastischen Erkrankungen verwendet. Dieser Artikel gibt einen kurzen Überblick über die Zusammensetzung, die physikochemischen Eigenschaften und die Anwendung von natürlichem Honig als nutrazeutisches Mittel.

Schlüsselwörter: honig, zusammensetzung, organoleptische eigenschaft, lebensmittel, therapeutikum

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