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Supercritical CO_2 Extraction: Fundamentals, Industrial Relevance, and Future Perspectives

Running title: Supercritical CO_2 extraction

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ABSTRACT

Supercritical carbon dioxide extraction is an advanced separation technique that is increasingly applied in various industries due to its environmental friendliness, selectivity and efficiency. This method enables the extraction of bioactive compounds without the use of toxic chemicals and at lower temperatures compared to classical extraction techniques. This review paper outlines the fundamental principles of supercritical extraction, including the physicochemical properties of supercritical CO2, its advantages over conventional methods, and key process parameters such as pressure, temperature, flow rate, extraction time, and modifiers. Special attention is paid to the industrial applications of this technique, particularly in the pharmaceutical, food, and cosmetic industries, where it is utilised to extract essential oils, antioxidants, polyphenols, lipids, and other valuable components. Moreover, environmental aspects of supercritical extraction were discussed, with an emphasis on sustainability and the potential for minimizing the environmental impact. Through the analysis of current research and technological innovations, this paper provides insight into the perspectives of further development and optimization of supercritical extraction using CO_2, as well as its possible application in new industrial areas.

Keywords: *supercritical extraction, carbon dioxide, sustainable technology, separation, bioactive components, industrial application*

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Introduction

The application of supercritical extraction for obtaining natural products has advanced significantly in recent years. Many studies focus on developing such processes (Reverchon et al., 2006). Supercritical fluids (SCF) are of great interest owing to their unique properties that blend characteristics of gases and liquids, making them ideal for extraction. Supercritical fluid extraction (SFE) involves a mass transfer process conducted under pressure and temperature conditions above the solvent's critical point. The concept of a biorefinery emerged from the need to replace non-renewable energy sources in the production of fuels and chemicals. In a biorefinery, various chemical products are derived from renewable resources using separation, isolation, and chemical or biochemical transformation methods (Attard et al., 2016). The ability to implement supercritical extraction at different stages of a biorefinery is particularly valuable due to the advantageous properties of this method (Temelli et al., 2009). From a green chemistry perspective, extraction processes should ideally use natural solvents such as water, ethanol, and carbon dioxide. Supercritical carbon dioxide is a popular extraction solvent, generally recognized as safe (GRAS), which means it is a non-polluting chemical capable of producing highly pure products, aligning with green chemistry principles (Yıldırım et al., 2024). However, one of the key challenges in process optimization is removing the solvent from the solute to achieve the highest purity of the final product, and in many cases, supercritical extraction is the best option.

Biorefineries contribute to sustainability by reducing harmful gas emissions and preserving natural resources through the use of renewable feedstocks and waste recycling. Economically, they can create new industries, jobs, and markets, reducing dependence on fossil fuel imports and promoting local production. By integrating supercritical extraction, biorefineries can enhance efficiency and environmental benefits, reinforcing their role as a sustainable alternative in fuel and chemical production (Mantell et al., 2013).

The primary raw materials used in biorefineries and the potential applications of SFE technology are outlined below:

- **Sugar/Starch-rich Crops**: These are the most common raw materials in current biorefineries. They contain large amounts of sucrose, which can be easily extracted and fermented to produce ethanol or bio-based chemicals. Sugarcane is the most preferred feedstock due to its economic and environmental benefits, as it's easier to produce. In this case, water is typically used as the solvent to extract the solutes, and supercritical fluid extraction is not necessary at this stage. However, there are several studies that explore the use of supercritical fluid extraction with a counter current column to separate ethanol from aqueous solutions (Di Giacomo et al., 1991; Pereyra et al., 1995; Ruiz-Rodriguez et al., 2010)
- **Vegetable Oil**: This raw material is mainly used to produce biodiesel through the transesterification process. The oil can come from natural sources such as palm, soybean, rapeseed, and sunflower seeds, or as a waste product like used cooking oil or animal fat.

Producing biodiesel from vegetable oils faces challenges related to land use, sustainability, and the high cost of refining impure waste oils (Anagha et al.,2025; Chabni et al., 2025; Mantell et al., 2013; Mouahid et al., 2024; Sahena et al., 2009).

- **Lignocellulosic Biomass**: This type of biomass comes from non-edible plant material, mainly composed of cellulose, hemicellulose, and lignin. It is considered a promising second-generation feedstock for the future production of biofuels and bio-based chemicals using various conversion technologies. Many studies focus on using supercritical fluid extraction to separate secondary metabolites from agricultural waste and lignocellulosic materials (Barbini et al., 2021; Da Costa Lopes, 2016; Mantell et al., 2013).
- **Microalgae**: This group consists of a wide variety of single-celled organisms that can be photoautotrophic or heterotrophic. Microalgae can produce a range of valuable compounds. These include carotenoids, polyunsaturated fatty acids, and antioxidants, which are useful in the food industry, as well as various lipids that can be used to produce biodiesel through transesterification. Additionally, the remaining carbohydrates in microalgae can be fermented to produce bioethanol. To maximize the efficiency of bioactive compound recovery, supercritical fluid extraction is being explored as an advanced method for their separation (Grierson et al., 2012; López-Limón et al., 2025; Mantell et al., 2013; Singh et al., 2021).

One of the most important applications of supercritical extraction is the extraction of bioactive substances from plant materials (Ivakhnov et al., 2024; Mohammadi et al., 2024). Supercritical CO_2, as a solvent, enables the efficient extraction of lipophilic compounds, which makes it particularly suitable for the following substances:

1. **Essential oils**: Supercritical CO_2 is commonly employed for extracting essential oils from plants such as lavender, rosemary, eucalyptus, lemon, and many others. These oils are often sought after for their aromatic, fragrant, and therapeutic properties (Kessler et al., 2024).
2. **Flavonoids and phenolic compounds**: These bioactive compounds are present in many plants and are known for their antioxidant, anti-inflammatory, and other health benefits (Díaz-Reinoso et al., 2006). Flavonoids from plants such as onions, citrus fruits, and tea can be effectively extracted with supercritical CO_2 (Christaki et al., 2024; Talmaciu et al., 2015).
3. **Alkaloids**: Alkaloids, such as caffeine from coffee, theobromine from cocoa, and nicotine from tobacco, can also be extracted using supercritical CO_2. These compounds are of interest due to their stimulating effects and pharmacological properties (Saldaña et al., 1999).
4. **Carboxylic acids**: Supercritical CO_2 is used for the extraction of fatty acids, such as omega-3 fatty acids from plant sources (e.g., flaxseed and hemp) and essential fatty acids from plant oils (e.g., olive oil) (Kumar et al., 2024; Min et al., 2010).

5. **Carotenoids**: These plant pigments, such as beta-carotene, lutein, and zeaxanthin, play a key role in defending against oxidative stress and being vital for human health (Sabio et al., 2003). Supercritical CO_2 is an efficient solvent for carotenoids from plant materials such as carrots, papayas, and spinach.
6. **Terpenes**: Terpenes, which are often responsible for the scent and aroma of many plants, such as limonene from lemon peel or pinene from pine, can also be extracted with supercritical CO_2. Terpenes are widely used in the perfume, food, and beverage industries (Bañares et al., 2024).
7. **Steroidal saponins**: Plants containing saponins (such as ginseng or licorice root) can be used for the extraction of these bioactive compounds, which have potential health benefits.
8. **Vitamin E (tocopherol)**: Vitamin E, which is found in plant oils like wheat germ oil, can also be extracted using supercritical CO_2 (Shi et al., 2024; Shi et al., 2025).

Market and Industrial Applications

Supercritical fluid extraction can be applied on large industrial scales (tons of raw materials). It has several important industrial applications, particularly in the extraction, purification, and fractionation of edible oils, fats, and waxes. The goal of this process is to separate specific compounds from natural solid materials, such as seeds, fruits, and citrus peels. "Refining" refers to the removal of certain substances, such as carotenoids, phospholipids, and free fatty acids, that can promote oxidation and cause rancidity in oils. Fractionation, on the other hand, allows the selective separation of short-chain triglycerides, unsaturated vegetable and animal oils, and valuable compounds from natural sources, such as vitamins, flavors, and polyunsaturated fatty acids.

One of the earliest applications of supercritical fluid extraction was the extraction of alkaloids from plant materials, including processes like decaffeinating coffee and tea. To preserve aroma and flavor, caffeine extraction is typically performed on green coffee beans before roasting and grinding. Today, supercritical CO_2 decaffeination accounts for 20% of the global coffee production.

The growing trend in SFE process is propelled by the growing consumer demand for natural and health-promoting food additives. Consumers are increasingly seeking products derived from natural sources, as the market shifts toward eco-friendly extraction methods. Supercritical fluid extraction has made significant industrial progress in this area, particularly for obtaining food additives such as colorants, flavors, and antioxidants from natural products (Fersi et al., 2024). The use of agricultural by-products for these extractions adds value and reduces waste. Additionally, numerous studies have investigated the health benefits of natural compounds, including anticancer, antimutagenic, and anticonvulsant properties, which further enhance the appeal of these products.

The growing preference for natural products with clean labels in the market has significantly increased the adoption of SFE in the food and cosmetics industries. As consumers become increasingly aware of the health and environmental risks associated with synthetic chemicals, natural alternatives derived from SFE are becoming the preferred choice. For example, in the cosmetics industry, the demand for organic skin care products has created opportunities for SFE to extract pure active ingredients, such as essential oils, antioxidants, and vitamins, that meet the growing consumer preference for non-toxic and environmentally friendly formulas. Additionally, SFE is expected to play a crucial role in the biofuel production sector as industries seek more sustainable alternatives to fossil fuels (Sato et al., 2022). As the focus intensifies on lowering greenhouse gas emissions, supercritical CO_2 offers a promising solution for extracting valuable biofuels and bio-based chemicals with minimal environmental impact.

CO_2 as a Supercritical Fluid

Supercritical fluid is any substance that exists in a state above its critical temperature (T_c) and critical pressure (P_c). A typical temperature-pressure diagram for a pure substance can be used to visualize this, which divides the diagram into three distinct regions corresponding to the solid, liquid, and gas phases. Two other key points are visible in the diagram shown in Figure 1: the triple point, where all three phases (solid, liquid, and gas) coexist, and the critical point, which marks the end of the liquid-gas transition. The critical point is defined by critical temperature and critical pressure, beyond which distinct liquid and gas phases no longer exist, and a supercritical fluid phase emerges.

When either pressure or temperature (or both) changes, it also alters the density of the supercritical fluid. This is significant because the solvent power of a substance depends largely on its density. Solvation occurs when intermolecular forces in the solvent cause molecules to surround and interact with the solute molecules. These forces are influenced by the solvent's density, meaning the solvating ability of supercritical fluids can vary widely, ranging from gas-like to liquid-like densities, depending on the conditions.

The density of an SCF can be controlled to modify its solubility power, allowing for selective extraction or fractionation of multiple solutes. The most efficient conditions for these processes are found near the critical point, where even slight changes in pressure or temperature can result in significant variations in density and, therefore, solvent power.

In addition to these density and solvent power characteristics, SCF exhibit other beneficial properties, particularly in terms of mass transfer. While the solvent power of an SCF is similar to that of a liquid, its viscosity and diffusivity resemble that of gases. This means SCF combine the dissolving ability of liquids with the rapid movement and transfer properties of gases, enhancing transfer characteristics compared to traditional liquid solvents.

Given these unique properties, SCF provide significant advantages over conventional solvents. Separations using SCF tend to be much more efficient, and the process is simple by adjusting the pressure, the supercritical fluid can be easily transformed into a gas, allowing for easy separation of the products.

Carbon dioxide is the most widely used supercritical solvent due to its low critical temperature (31.1 °C) and pressure (73.8 bar), which are easy to achieve (Mantell et al., 2013). Carbon dioxide is also fully miscible with low-molecular-weight hydrocarbons and oxygenated organic compounds, making it an effective solvent for many organic materials. Additionally, it has low mutual solubility with water, which makes it a good choice for selective extractions where water needs to be excluded.

Carbon dioxide offers several benefits as a supercritical solvent. It is non-toxic, non-flammable, non-corrosive, and environmentally friendly. Additionally, it is inexpensive, abundant, and can be obtained in various purity levels. Carbon dioxide also has advantageous transport properties, such as low viscosity, high diffusion coefficients, and suitable heat conductivity and heat of vaporization, particularly near the critical point. These properties make it energy-efficient for many processes. Moreover, after the extraction process is complete and the pressure is reduced, carbon dioxide transitions to a gas under normal environmental conditions. It can then be compressed and recycled for repeated use, ensuring no residue remains in the extracted product and minimizing environmental impact.

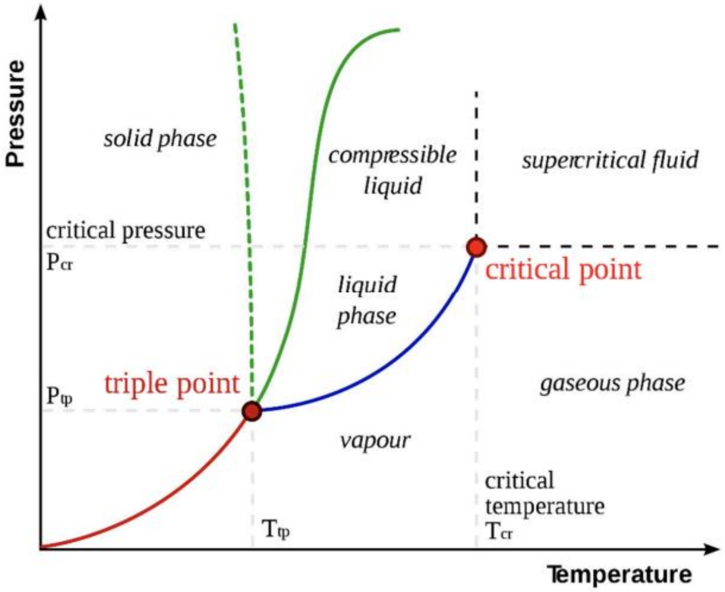

Figure 1. Phase diagram (Image taken from the Yıldırım et al., 2024, according to CC BY licence).

The technological scheme of the plant for extraction using supercritical CO_2 (SC-CO_2) is shown in Figure 2., illustrating all parts of the facility and the complete extraction process.

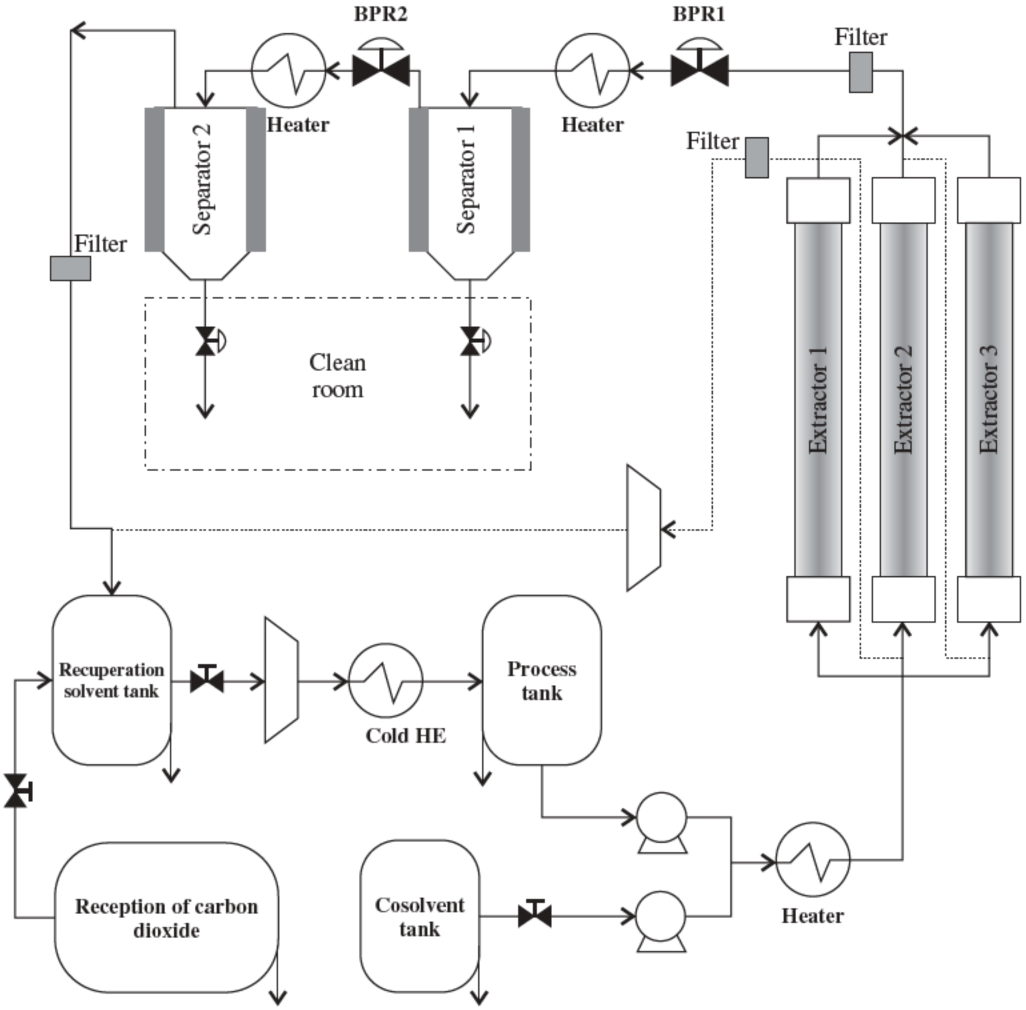

Figure 2. Schematic diagram of SC-CO_2 system (Image taken from the Mantell et al., 2013 with the permission of Copyright Clearance Center).

Supercritical CO_2 extraction systems typically operate in a discontinuous mode for the solid phase and a continuous mode for the supercritical fluid. To enhance process efficiency, three extractors are often used in parallel: one undergoes extraction, the second is in the loading and pressurization stage, while the third is in depressurization and unloading. Fractionation of extracts is achieved through progressive depressurization across a series of cascade separators. By adjusting pressure and temperature conditions, the solvent density and solubility are gradually reduced, leading to the sequential precipitation of compounds according to their solubility: less soluble substances precipitate first, while more soluble components remain in solution until reaching subsequent separators. A critical economic aspect of the process is solvent recovery and recycling, typically performed using two tanks. One operates at low pressure to collect the separated solvent, while the other maintains higher pressure conditions to replenish and prepare the CO_2 for reintegration into the extraction cycle.

Comparing the Advantages and Disadvantages of Supercritical Fluid Extraction with Other Methods

Supercritical fluid extraction and hydrodistillation are two different techniques for extracting bioactive compounds from plants, but they differ in their application, equipment, and the types of substances that can be effectively extracted. During the hydrodistillation, plants are exposed to steam, which helps release essential oils or other volatile substances. The steam passing through the plant material carries with it the volatile components, which are then condensed and collected. Hydrodistillation is simple and inexpensive technology, which does not require high temperatures or complex equipment. It is good choice for easily evaporating components, such as essential oils and can be classified as natural process, as it only uses steam and minimal handling of plant material. It is most applied in the production of essential oils, perfumes, and for obtaining floral waters (hydrosols). It is also used for extracting flavors and fragrances in the food industry.

SFE is especially effective for extracting lipophilic (fat and oil) and thermolabile components, such as essential oils, flavonoids, terpenes, and sterols. It can also be used to extract other bioactive molecules such as alkaloids. Supercritical extraction is used in industries that require high efficiency and selectivity, such as the production of essential oils, dietary supplements, cosmetics, pharmaceuticals, and bioenergy materials (Cassel et al., 2000).

Microwave-assisted extraction (MAE) and SFE while share the common goal of efficient extraction of compounds, they rely on different principles and technologies. Microwave-assisted extraction uses microwave radiation to plant material causing them to vibrate and rapidly generate heat, which increases the rate of extraction. Microwaves are sometimes used in combination with solvents (such as water, ethanol, or acetone) to enhance the extraction efficiency. Microwave-assisted extraction can be used for a wide range of bioactive substances, including phenols, flavonoids, alkaloids, terpenoids, proteins, enzymes, and other water-soluble and organic-soluble compounds. Microwave-assisted extraction is fast and energy-efficient method, as microwaves enable rapid heating and temperature rise of plant material as well as lower solvent consumption compared to traditional extraction methods, reducing environmental impact. Additional benefits are: quicker heating for bioactive compounds extraction, reduced use of solvents, low risks and short extraction time (3-30 min). Microwave-assisted extraction is commonly used in laboratory research, as well as in industries for extracting bioactive substances from plants, such as polyphenols, alkaloids, and flavonoids. It is also used in the production of food, beverages, and pharmaceuticals.

Advantages of SFE

SFE enables high efficiency in extraction, especially for thermolabile substances, as it does not use high temperatures. It is more environmentally friendly compared to traditional solvents, as it uses CO_2, which is non-toxic and easily removed after extraction. Moreover, it provides greater selectivity for certain components, as parameters (temperature and pressure) can be adjusted to optimize extraction.

Supercritical extraction is known for its high selectivity for certain components because parameters (temperature and pressure) can be fine-tuned to enhance extraction efficiency. Moreover, it provides high efficiency in extracting thermolabile substances without the need for high temperatures. It is more environmentally friendly method, as it uses CO_2, which is non-toxic and easily removed from the extract. Additional benefits are: moderate extraction time (10-60 min), possibility to on-line coupling with chromatographic process, reduced use of organic solvents, small amount of sample (1-5 g) and better separation of solute from solvent.

Disadvantages of SFE

SFE usually requires high initial capital and equipment complexity, as it requires specialized systems to achieve supercritical conditions (high pressure and temperature). For this reason, it can be more expensive than other methods, such as hydrodistillation.

Supercritical extraction demands high initial capital and equipment complexity, as it requires specialized systems to achieve supercritical conditions. This process may be economically challenging for small- and medium-scale applications and has limited ability to dissolve polar compounds.

Disadvantages of hydrodistillation and MAE

Hydrodistillation has limited efficiency in extracting components that are not easily volatile and may lead to loss of some thermolabile or non-volatile components due to high temperatures. Additionally, large water consumption can be impractical in large-scale industrial processes.

Microwave-assisted extraction has limited application for certain substances that do not respond well to microwaves. Due to rapid and uneven heating, potential degradation of some thermolabile components can be occurred. Additionally, careful monitoring of parameters (microwave power, heating time, solvent type) is necessary because the system is more sensitive to process conditions. It requires additional steps such as filtration and has poor efficiency when either the target compounds or solvents are non-polar or volatile. It should be kept in mind that the solvent must absorb microwave energy.

Table 1. summarizes and compares the investment costs for MAE and SFE procedures.

Table 1. Comparative investment costs of MAE and SFE procedures (Data adapted from Talmaciu et al., 2015).

Extraction technique	MAE[1]	SFE[2]
Capital cost	medium	high
Operating cost	medium	low
Total value ($/kg extract)	120-150	150-200

[1] microwave-assisted extraction; [2] supercritical fluid extraction

Supercritical Fluid Extraction Efficiency Using Cosolvent

A cosolvent, or entrainer, is an organic compound that has a volatility between that of the SCF solvent and the solute being extracted. It is typically added in very small amounts (1 to 5 mol%) to the SCF solvent to alter its properties, such as polarity and specific interactions, without substantially altering the density and compressibility of the original SCF solvent.

The SCF solvent mixed with a cosolvent becomes supercritical when its pressure exceeds the mixture's critical pressure and its temperature is higher than the mixture's critical temperature, which are usually close to the critical values of the pure SCF solvent. In Figure 3.a, the mixture's critical pressure is shown as the highest pressure on an isothermal P–x diagram of the binary mixture, beyond which there is no two-phase region for a given temperature. The mixed SCF solvent remains supercritical at all pressures above its mixture's critical pressure, indicated by the shaded area. If the pressure is below the critical pressure, such as at point A, the system is in a gaseous state, since it lies outside the two-phase region. However, as seen in Figure 3.b, the two-phase vapor-liquid region can extend past the mixture's critical temperature for a specific pressure. This means that a mixture is not necessarily supercritical at temperatures above its mixture's critical temperature. The critical point (CP) marks the distinction between the gas phase and the liquid phase.

When a binary mixture of an SCF solvent and a cosolvent is used above the critical pressure of the binary mixture to dissolve a liquid solute, the system is represented by a ternary diagram (Figure 3.c). In these situations, all three components are typically distributed between both the liquid and SCF phases. The extent to which each component is dissolved in the two phases is described by the distribution coefficient, as shown by the two endpoints of a tie line.

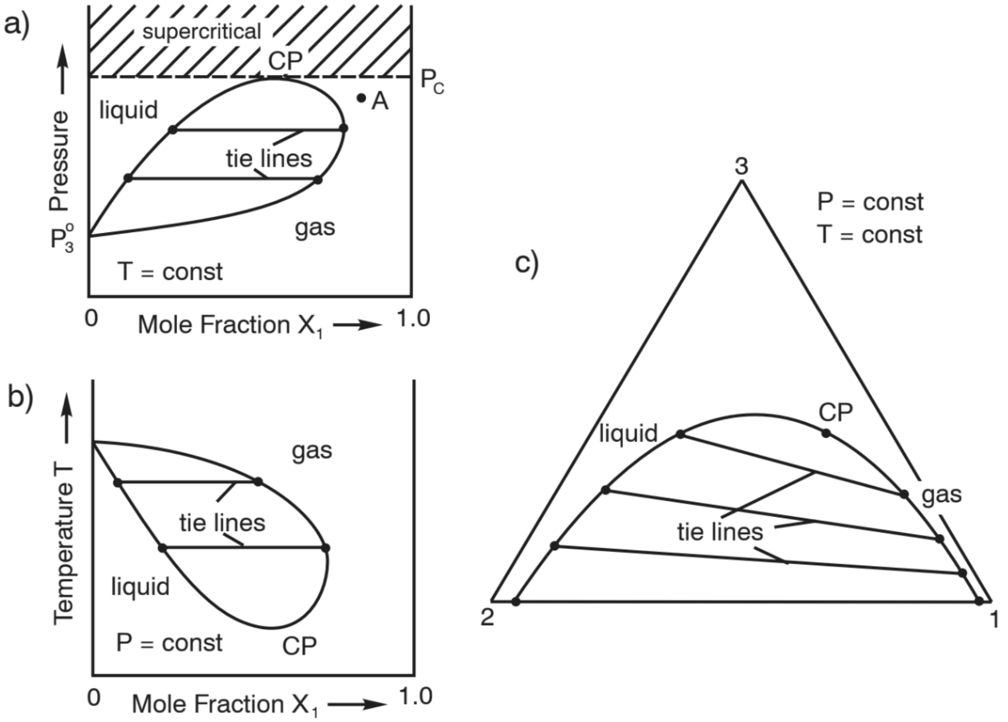

Figure 3. Critical points in a), b) binary and c) ternary mixtures (Image taken from the Mukhopadhyay, 2000).

A comparison of the physical properties of gases, liquids, and supercritical fluids is shown in Table 2. Supercritical fluids (SF) have densities similar to those of liquids, while their viscosity and diffusivity resemble those of gases. As the density increases, their ability to dissolve substances also improves, allowing them to dissolve more compounds than gases. Thanks to higher diffusivity and lower viscosity, SFs can easily penetrate the pores of solid structures, further enhancing their dissolving power. Their physical properties can vary significantly depending on temperature and pressure, as shown in Table 2., which presents the physical constants of the gas, liquid, and supercritical fluid phases under different conditions (P and T).

Table 2. A list of physical constants for gases, liquids, and supercritical fluids (Data adapted from Yildirim et al., 2024).

Solvent	Density g/cm^3	Viscosity g/(cm·s)	Diffusion cm^2/s	Surface tension N/m
Gas[1]	$6\cdot10^{-4}$-$2x10^{-3}$	$1\cdot10^{-5}$-$3\cdot10^{-5}$	0.1-0.4	0
Liquid[1]	0.6-1.6	$2\cdot10^{-4}$-$3\cdot10^{-3}$	$2\cdot10^{-6}$-$2\cdot10^{-5}$	3-$6\cdot10^{-2}$
SF[2]	0.2-0.5	$1\cdot10^{-4}$-$3\cdot10^{-4}$	$7\cdot10^{-4}$	0
SF[3]	0.4-0.9	$3\cdot10^{-5}$-$9\cdot10^{-5}$	$2\cdot10^{-3}$	0

[1]P=1 bar, T=298 K; [2]P=Pc, T=Tc; [3]P=4Pc, T=Tc

The addition of cosolvents, typically polar solvents like ethanol, methanol, acetone, or water, can:

1. **Increase the solubility of polar substances**: CO_2 is a nonpolar gas, so it struggles to dissolve polar substances. Adding cosolvents enhances the solubility of polar molecules, allowing their extraction under supercritical conditions.
2. **Improve extraction selectivity**: Cosolvents can modify the solubility of specific components, enabling selective extraction of certain substances. For example, a combination of CO_2 and ethanol can effectively extract bioactive compounds from plant sources, such as flavonoids, alkaloids, and essential oils.
3. **Lower extraction temperature**: The addition of cosolvents can reduce the temperature and pressure required for extraction, which is beneficial for protecting heat-sensitive substances like certain vitamins and antioxidants.
4. **Expand application range**: Using cosolvents broadens the scope of supercritical CO_2 extraction, allowing the extraction of bioactive compounds from food, medicinal plants, polymers, and other materials that are otherwise difficult to dissolve in pure CO_2.

In conclusion, cosolvents can enhance the capabilities of supercritical CO_2 extraction by enabling more efficient and selective removal of a wide range of substances, including those that are traditionally hard to dissolve in pure CO_2.

Parameters of CO_2 Supercritical Fluid Extraction

Several factors influence the efficiency of SFE, including temperature, pressure, flow rate, and the use of modifiers. Additionally, aspects such as sample grinding, size, and drying agents also play a role. While other factors may be important, they are less commonly discussed in research. Here's a breakdown of these key parameters:

Temperature

Temperature impacts the extraction efficiency by altering the solubility of the solute. As the temperature increases, the vapor pressure of the solute rises, enhancing solubility, but the solvent concentration decreases, which can reduce the overall extraction efficiency. The effect of temperature on yield can vary depending on the plant's characteristics. For example, a study on rice bran oil found the highest yield at 40 °C, while a different study on lycopene extraction from tomatoes showed increased yields with higher temperatures (Yıldırım, et al., 2024).

Pressure

Pressure is one of the most crucial factors in SFE. When pressure increases, so does the solvent density and its solvation power, improving extraction efficiency. High pressure enhances the density of supercritical CO_2, reducing resistance to mass transfer, which helps isolate both polar and non-polar compounds. However, high pressure can affect the stability of some compounds and raise operational costs, so it must be carefully controlled.

Flow Rate

Flow rate, which refers to the amount of CO_2 passing through the plant material over a set period, greatly influences extraction efficiency. A higher flow rate speeds up the process but may lead to incomplete dissolution or transport of components. Lower flow rates allow for longer interaction times between the supercritical fluid and plant material, enhancing extraction, but also extending the process and increasing costs. The ideal flow rate strikes a balance between efficiency, energy consumption, and equipment performance.

Modifiers

Carbon dioxide is not very effective at extracting polar compounds, so modifiers like water, ethanol, or methanol are often added to enhance the extraction of these compounds. These modifiers change the behavior of supercritical CO_2, but they also come with challenges. They can affect critical temperature and pressure of carbon dioxide, which may compromise its beneficial properties, like low viscosity and high dispersibility. Additionally, separating modifiers from CO_2 after extraction can be difficult and costly, often requiring more energy and additional processes, which can reduce the environmental sustainability of the extraction.

Grinding Time and Particle Size

The size and texture of the plant material can significantly influence extraction results. Smaller particles provide more surface area for the supercritical fluid to interact with, reducing mass transfer resistance and increasing extraction efficiency. However, excessive grinding can raise the temperature of the plant material, potentially causing the loss of volatile compounds, which negatively affects extraction efficiency.

Preparation of Plant Material for SC-CO_2

The timing of plant collection plays a crucial role in determining the composition of the extract that will be obtained. Factors such as the season, time of day, specific location, and whether it’s a rainy or dry day can all influence the chemical makeup of the plant. Plants for extraction can either be harvested from the wild or bought from suppliers and herbalists. Once collected, the specific part of the plant to be extracted (such as flowers, fruits, stems, roots, bark, seeds, etc.) is carefully separated from the rest of the plant material. The plant is then dried, either naturally or by using heat in drying systems resembling ovens. Procedures like grinding and drying can greatly impact the phytochemical content of the plant. To prevent enzymes from misfunctioning and to stop the plant’s metabolism, it’s essential to remove the water from the plant material. The drying temperature must be carefully regulated to prevent promoting microorganism growth while maintaining the plant's chemical integrity. Drying can be done under either natural or controlled (artificial) conditions. Natural drying typically involves leaving the plant material in an open or semi-open environment, avoiding direct sunlight. In artificial conditions, drying can be managed by adjusting temperature and pressure. It’s crucial to carefully choose the drying temperature to maintain volatile components and avoid degradation of important compounds. Methods such as freeze-drying, vacuum chambers, ovens, tunnel dryers, and shaft dryers are commonly used in laboratories for drying purposes.

Before performing supercritical fluid extraction, the moisture content of the plant should be under 10%, and the material should be ground into very fine particles. Using techniques that preserve the plant’s volatile components and phytochemical content while reducing moisture and particle size is key. As moisture increases, the extraction yield tends to decrease. Furthermore, smaller particle sizes allow the supercritical fluid to interact more effectively with the plant material, leading to a higher yield of extraction. Once the grinding process is completed, the plant material is passed through sieves with specific pore sizes, and if necessary, the particle size of the remaining material can be calculated. After completing all these steps, the plant material should be stored in dark bottles to prevent exposure to light and placed in a deep freezer (between −4 and −20 °C) until the extraction process begins.

Extraction examples utilizing SC-CO_2

Supercritical fluid extraction is an extremely efficient technique for isolating essential oils and other bioactive compounds from plants. Yields and parameters for supercritical extraction can vary depending on the plant, but the main parameters to consider include temperature, pressure, solvent type (most commonly CO_2), as well as extraction time.

Here is an overview of possible yields and parameters for supercritical extraction of essential oils from plants such as lemon, lavender, orange, chamomile, and rosemary:

Citrus peels (*Citrus limon, Citrus reticulata* and *Citrus sinensis*)

Rich in bioactive compounds, citrus peels represent a valuable economic resource with potential applications in the food, cosmetic, and pharmaceutical industries. In the study by Romano et al., the peels of orange (*Citrus sinensis*), tangerine (*Citrus reticulata*), and lemon (*Citrus limon*) were subjected to CO_2 extraction to obtain bioactive compounds (Romano et al., 2022). Carbon dioxide was applied under both liquid and supercritical (SC) conditions at various temperatures and pressures, and the results were compared with ethanol extraction, which served as a control. The study also investigated the use of ethanol as a co-solvent at low concentrations (up to 20%) during the CO_2 extraction process.

Supercritical extraction at 20 MPa with 20% ethanol as a co-solvent (SC-20-20) produced lower yields (14.56%, 13.01%, and 24.32% for orange, tangerine, and lemon peels, respectively) compared to liquid CO_2 extraction (L-20), despite both being conducted at the same pressure (20

MPa) and with the same ethanol concentration (20%). However, SC-20-20 was performed at a higher temperature (60 °C) than L-20. At 20 MPa, the higher temperature reduces the density of the solvent, which decreases the solubility of the solutes and consequently lowers the extraction yield.

Temperature has two opposing effects on solute solubility: on one hand, it increases the vapor pressure of the solutes, enhancing their solubility in the fluid phase and improving the extraction yield. On the other hand, it decreases the solvent density, reducing its solvating power. At pressures near the critical point, the effect of temperature on solvent density tends to dominate over its effect on solute vapor pressure, often leading to lower yields.

The yields obtained from SC-20-20 were also lower than those from SC-30-20, as the higher pressure of 30 MPa at 60 °C increased the density and solvent power of CO_2, enhancing ethanol penetration into the matrix and improving the extraction yield. However, it is important to note that excessive pressure can reduce extraction efficiency. A similar trend was observed with SC-20-10, which resulted in a lower yield (7.51%) than both L-10 and SC-30-10 in the case of lemon peels.

Lavender (*Lavandula angustifolia*)

Lavender essential oil is extensively utilized in the cosmetic, pharmaceutical, and food industries for its well-established aromatic, antimicrobial, and anti-inflammatory properties. In the study by Cruz-Sánchez, the authors optimized the extraction process based on yield by evaluating the effects of pressure, temperature, and the addition of a co-solvent (Cruz-Sanchez et al., 2024). The performance of lavender essential oil extraction was performed at pressures ranging from 180 to 300 bar and temperatures of 40 °C and 60 °C, with and without co-solvent. The results show that the extraction yield increases with higher temperatures at constant pressure. Specifically, the process is more efficient at 60 °C compared to 40 °C. As discussed in previous sections, this improvement is attributed to the higher vapor pressure of solutes at elevated temperatures. The maximum oil solubility and extraction yield were achieved at 250 bar and 60 °C. Moreover, the addition of ethanol as a co-solvent (0.2% v/v) resulted in a significant increase in extraction yield.

Chamomile (*Matricaria chamomilla*)

Supercritical CO_2 extracts of chamomile are extensively applied in the cosmetic, pharmaceutical, and food industries because of their high content of bioactive compounds like matricine, bisabolol, and flavonoids. Kotnik et al. conducted SFE experiments using CO_2 in a semi-continuous flow system (Kotnik et al., 2007). The system was specifically designed to operate at pressures reaching up to 500 bar and temperatures up to 100 °C. Approximately 15 grams of finely ground material were placed into the extractor (volume: 60 cm^3). The water bath temperature was carefully controlled and maintained within ±0.5 °C. The system was first purged with nitrogen, followed by the introduction of the extraction gas. CO_2 was then continuously pumped at high pressure (450 bar) through a preheating coil and across the sample bed in the extractor. The extracted product was collected in a glass trap, where separation took place at 1 bar and 0° C. The collected extract was weighed (±0.1 mg), and the extraction yield was calculated. In a two-step separation process, the first separator was a high-pressure vessel capable of operating at 120 bar and 100 °C, while the second separator was a glass trap at atmospheric pressure and 0 °C. The extracts were immediately analyzed after collection.

The chamomile flower heads were extracted with CO_2 at pressures 100, 150 and 250 bar and temperatures 30 and 40 °C followed by one step separation. According to extraction yield the best extraction conditions were 250 bar and 40 °C where the yield was 3.81%. Extraction yield increased with increasing solvent density and at constant solvent density the extraction yield was increased with higher temperature. According to the content of active compounds in extracts, the best extraction conditions using one step separation were determined to be 250 bar, 30 ºC, where the content of matricine reached 9.81 mg/g.

Rosemary (*Rosmarinus officinalis*)

Supercritical carbon dioxide extraction is an efficient method for isolating natural antioxidants from rosemary, offering several advantages over traditional extraction techniques. By adjusting process parameters such as pressure and temperature, the selectivity of SC-CO_2 for specific components can be enhanced, enabling phase separation to obtain solvent-free extracts.

Some studies have incorporated modifiers like ethanol to increase antioxidant yields from rosemary, although higher concentrations of modifiers may reduce CO_2 selectivity. It is generally not recommended to use modifiers at high pressures (e.g., 50 MPa and above) as they can decrease antioxidant activity in the extracts.

Research of Ivanović et al. has demonstrated that antioxidant fractions can be effectively extracted from Lamiaceae herbs, including rosemary, sage, thyme, and oregano, using SC-CO_2 without modifiers at pressures ranging from 50–100 MPa and temperatures between 90–110 °C (Ivanović et al., 2009). Other studies have employed lower pressures (15–35 MPa) and temperatures (40–60 °C), either with or without small amounts of ethanol, to isolate antioxidants. Additionally, some researchers have combined traditional methods, such as distillation, with SC-CO_2 extraction to concentrate antioxidant fractions from rosemary. SC-CO_2 extraction of rosemary yields antioxidants with equal or stronger activity compared to synthetic alternatives. The resulting extracts are semi-solid at room temperature and can be further processed by grinding at low temperatures and dissolved in oils.

Table 3. collects yields of rosemary antioxidant fractions at different P and T from the study of Ivanović et al. (2009).

Table 3. Yields of rosemary and sage antioxidant fractions in the performed experiments (Data adapted from the Ivanović et al., 2009).

Herbaceous material	P MPa	t °C	W wt.%
Rosemary	30	40	1.10
		100	1.57

Conclusion

Supercritical extraction is an effective technique for extracting essential oils and other bioactive compounds from plants. Yields depend on the plant species, as well as specific extraction parameters, such as temperature, pressure, flow rate, and extraction time. Using CO_2 as a solvent enables the production of high-quality products without toxins or solvent residues, which is an advantage in the essential oil and bioactive compound industries.

SC-CO_2 is a selective and environmentally friendly separation technique that is increasingly applied in various industrial sectors. Its advantages, such as low toxicity, preservation of thermolabile compounds and the possibility of fine control of extraction conditions, make it superior to conventional extraction methods. It is particularly important in the food, pharmaceutical, and cosmetic industries, where it enables the obtaining of high-quality bioactive compounds without organic solvent residues.

Although supercritical extraction has already been well-examined and applied in many fields, further research is necessary to improve the process's efficiency, reduce costs, and expand its use in new industries. The development of new modifiers, optimization of operating parameters, and integration with other technologies could further expand its application.

Given the increasing demand for sustainable and environmentally friendly technological solutions, supercritical CO_2 extraction has the potential to become the dominant extraction method in the future. Further research and innovation in this area can enhance its economic profitability and contribute to the development of new green technologies in industry.

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Declarations of interest:

The authors declare that they have no conflict of interest.

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Ekstrakcija superkritičnim CO_2: Osnovni principi, industrijski značaj i buduće perspektive

Tekući naslov: Ekstrakcija superkritičnim CO_2

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Sažetak

Ekstrakcija superkritičnim ugljen-dioksidom je napredna separaciona tehnika koja se sve više primenjuje u različitim industrijama zbog svoje ekološke prihvatljivosti, selektivnosti i efikasnosti. U poređenju sa klasičnim tehnikama ekstrakcije, ova metoda omogućava ekstrakciju bioaktivnih jedinjenja bez upotrebe toksičnih hemikalija i pri nižim temperaturama.

Ovaj pregledni članak sagledava osnovne principe superkritične ekstrakcije, uključujući fizičko-hemijska svojstva superkritičnog CO_2, prednosti u odnosu na konvencionalne metode, kao i ključne parametre procesa poput pritiska, temperature, protoka, vremena ekstrakcije i upotrebe modifikatora. Posebna pažnja posvećena je industrijskim primenama ove tehnike, uključujući farmaceutsku, prehrambenu i kozmetičku industriju, gde se koristi za ekstrakciju etarskih ulja, antioksidanata, polifenola, lipida i drugih značajnih komponenti.

Takođe, razmatarani su ekološki aspekti superkritične ekstrakcije, sa naglaskom na održivost i potencijal za smanjenje negativnog uticaja na životnu sredinu. Kroz analizu trenutnih istraživanja i tehnoloških inovacija, ovaj rad pruža uvid u perspektive daljeg razvoja i optimizacije superkritične ekstrakcije uz upotrebu CO_2, kao i njene moguće primene u novim industrijskim oblastima.

Ključne reči: *superkritična ekstrakcija, ugljen-dioksid, održiva tehnologija, separacija, bioaktivni spojevi, industrijska primena*

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Density Functional Theory (DFT) Investigation on the Radical-Scavenging Activity of Galantamine and Norgalantamine

Running title: Computational studies on galantamine and norgalantamine

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ABSTRACT

Galantamine, a natural alkaloid with inherent antioxidant properties, effectively crosses the blood-brain barrier, making it a promising therapeutic agent for treating certain brain-related disorders in humans. This density functional theory (DFT) study presents the results of quantum chemical calculations on the dissociation enthalpies of galantamine's O-H and C-H bonds, elucidating its radical-scavenging activities. The findings highlight galantamine's propensity to interact with radicals in biological systems, emphasizing the bond strength and acidity of its O-H and C-H groups. Additionally, the study explores the implications of hydride ion abstraction, shedding light on its potential reactivity and antioxidant mechanisms.

Keywords: *galantamine, norgalantamine, radical-scavenging activity, DFT calculations*

Introduction

Oxidative damage is a critical factor in the development of many diseases. The brain is especially vulnerable due to its high content of easily oxidizable fatty acids, significant oxygen consumption, and limited endogenous antioxidant defences (Behl & Moosman, 2002). Within this context, oxidative stress emerges as a pivotal player in the neuronal degeneration observed in the brains of Alzheimer's disease patients (Ansari & Scheff, 2010; Marlatt et al., 2008; Petersen et al., 2007). Reactive oxygen species (ROS) instigate cell membrane dysfunction (Axelsen et al., 2011), subsequently triggering the initiation of apoptosis in nerve cells (Behl & Moosman, 2002).

Plant antioxidants with radical-scavenging activity have the potential to protect against brain diseases or, at the very least, slow their progression. However, their efficacy largely depends on their ability to cross the blood-brain barrier. Galantamine, renowned for its radical-scavenging properties (Traykova et al., 2003), efficiently crosses the blood-brain barrier while exhibiting relatively low toxicity. This makes it a reliable therapeutic option for treating various brain disorders in humans (Tsvetkova et al., 2013).

The antioxidant properties of galantamine hydrobromide have been substantiated through *in vitro* experiments (Traykova et al., 2003). The capacity of galantamine and galantamine hydrobromide to neutralize ROS such as $O_2^{\bullet -}$, $^{\bullet}OH$, and HOCl is attributed to the hydroxyl group at the sixth position of the galantamine cyclohexene ring (Figure 1). This conclusion is based on the observation that any modification of the hydroxyl group affects the efficacy of radical-scavenging activity (RSA) (Karadjova et al., 2021). Its effectiveness disappears entirely upon the alkylation of the OH group. Moreover, research indicates an increase in galantamine's radical-scavenging potency when it is converted into its hydrobromide salt form.

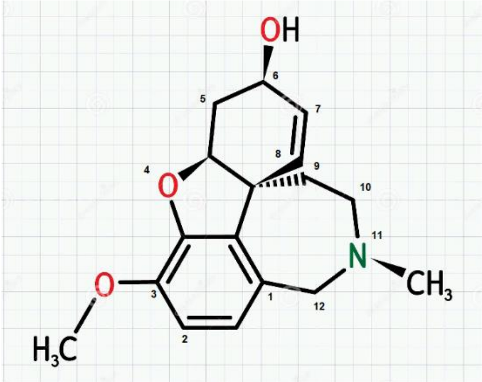


Figure 1. Structure of galantamine.

Galantamine has a significant impact on a variety of human brain pathologies (Harvey, 1995; Marco & do Carmo Carreiras, 2006; Melo et al., 2009). Its primary application is as a competitive, reversible, and selective inhibitor of acetylcholinesterase, which enhances cognitive function in individuals with Alzheimer's disease. Additionally, it has been observed to play a role in nicotine addiction (Harvey, 1995).

The quantum chemical studies available in literature primarily focus on the electron density distribution and the resulting electrostatic potential of galantamine (Parameswari et al., 2013). Alternatively, some studies delve into the correlation between the energy of the highest occupied orbitals and their role as inhibitors of acetylcholinesterase within a group of galantamine analogues (Ece & Pejin, 2015). Yet, none of these studies have explored galantamine's propensity for engaging in chemical reactions linked to its most readily dissociable O-H and C-H bonds.

In this paper we will discuss the results of quantum chemical calculations of the enthalpies of dissociation of O-H and C-H bonds in galantamine (Figure 1 and the Schemes 1-5) and norgalantamine - an analogue of galantamine without the methyl group at N11. Norgalantamine is widely used in synthetic chemistry (Vezenkov et al., 2009, 2020). The results provide valuable insights into their overall reactivity, the mechanism of radical scavenging activity, and the most likely metabolic pathways.

Experimental

The calculations were performed using the density functional theory and the hybrid Becke-3-parameter-Lee-Yang-Parrfunctional (DFT/B3LYP) coded in the Gaussian09 software package (Frisch et al., 2016) and orbital basis 6-311++g(d,p) (Parr, 1980). The aforementioned procedure was previously employed on a different set of radical-scavengers, yielding entirely satisfactory outcomes (Velkov et al., 2019). No constraints on geometry were imposed during the optimization. The energy minima for all structures were confirmed by frequency analysis. Solvent effects were calculated with the self-consistent reaction field (SCRF) approach of the polarized continuum model (PCM) (Tomasi et al., 2005).

The species (X) enthalpy is calculated using the following equation:

$$H(X) = E_0 + ZPE + \Delta H_{trans} + \Delta H_{rot} + \Delta H_{vib} + RT$$

where E_0 is the total electronic energy, ZPE is the unscaled zero-point energy, ΔH_{trans}, ΔH_{rot}, and ΔH_{vib} are respectively the translational, rotational, and vibrational shares in the enthalpy, RT represents the work term converting the internal energy into enthalpy (T = 298 K).

The enthalpies of the hydrogen atom, proton and electron in vacuum and in water are taken from the literature (Markovic et al., 2016; Rimarcik et al., 2010). The used proton enthalpy ($\mathbf{H}_{(H^+)}$) in vacuum is 6.197 kJ/mol, and in water is –1083.803 kJ/mol; the used enthalpy of an electron ($\mathbf{H}_{(e^-)}$) in vacuum is 3.145.kJ/mol, and in water is –232.676 kJ/mol, the used enthalpy of a hydrogen atom ($\mathbf{H}_{(H^\cdot)}$) in vacuum is –1312.479 kJ/mol and in water is –1316.479 kJ/mol.

Results and Discussion

Table 1 provides the obtained enthalpies of O-H and C-H bond dissociations.

Table 1. Enthalpies of O-H and C-H bond dissociations (in kJ/mol, in water).

	BDE	IP	PDE	PA	ETE
C-H galantamine	321.81	288.16	24.46	316.00	-3.38
C-H norgalantamine	321.80	308.33	4.28	316.52	-3.61
O-H galantamine	428.52	288.16	131.16	218.26	201.07
O-H norgalantamine	428.44	308.33	110.92	218.18	201.07

X-H bond breaking. HAT mechanism of O-H bond dissociation

$$\text{Gal-X-H} \longrightarrow \text{Gal-X}^{\bullet} + \text{H}^{\bullet}$$

Scheme 1. HAT mechanism

The most prevalent mechanism for X–H bond dissociation in a nonpolar environment is the Hydrogen Atom Transfer (HAT). The characteristic descriptor of this mechanism is the bond dissociation enthalpy (BDE), which is calculated as the enthalpy difference between the resultant radical and hydrogen atom and the corresponding galantamine molecule: $BDE = H(\text{Gal-O}^{\bullet}) + H(\text{H}^{\bullet}) - H(\text{Gal-OH})$. The spin density within the radical, which emerges subsequent to the homolytic dissociation of the hydroxyl group, is notably centred at the oxygen atom. The Mulliken spin density at this oxygen is substantial – 0.85. The calculated O-H BDE confirmed that a dissociation of this bond by the HAT mechanism is quite difficult - BDE is 428.52 kJ/mol.

In tandem with galantamine, we also computed the O-H bond BDE for norgalantamine. The only structural difference between the two compounds is the presence of a methyl group linked to the nitrogen at position 11 (Figure 1). However, this distinction does not exert any influence on the BDE of the O-H bond at position 6. In norgalantamine it is 428.44 kJ/mol. For the sake of comparison, it's worth noting that the calculated BDEs for the most reactive hydroxyl groups in flavones and coumarins fall within the range of 320 to 350 kJ/mol (Karadjova et al., 2021).

The results in Table 1 are calculated taking into account the polarizing effect of water. Since the HAT mechanism is expected to take place in non-polar environments, such as those in the human brain, we calculated the BDE of the O-H bond in a vacuum (Table 2). It is not surprising that, within a vacuum, these bonds exhibit a greater propensity for dissociation compared to their behavior in a water environment (Table 1).

Table 2. BDE in a vacuum (in kJ/mol)

	Galantamine	Norgalantamine
BDE(O-H)	425.53	425.27
BDE(C-H)	319.78	319.65

Therefore, the radical-scavenging reaction involving the hydroxyl group through the HAT mechanism appears to be unattainable due to thermodynamic constraints. However, it gains greater feasibility within nonpolar environments.

SET-PT mechanism of O-H bond dissociation

Two-steps mechanisms for O-H bond dissociation exhibit higher likelihood within polar environments. Within this mechanism, an initial step involves the detachment of an electron from galantamine (or norgalantamine), followed by the subsequent removal of a proton from the resulting cation-radical (Scheme 2).

$$\text{Gal-X-H} \xrightarrow{-e^{-}} \text{Gal-X-H}^{\cdot +} \xrightarrow{-H^{+}} \text{Gal-X}^{\cdot}$$

Scheme 2. SET-PT mechanism

The feasibility of the SET-PT mechanism can be approximated through the assessment of the ionization potential (IP = $H_{(Gal\text{-}O\text{-}H\bullet+)} + H_{(e-)} - H_{(Gal\text{-}O\text{-}H)}$) and the proton dissociation enthalpy (PDE = $H_{(Gal\text{-}X\bullet)} + H_{(H+)} - H_{(Gal\text{-}X\text{-}O\bullet+)}$).

The process of electron detachment from galantamine (in water) is more likely to occur compared to homolytic dissociation (in vacuum). IP of galantamine (288.16 kJ/mol) (norgalantamine 308.33 kJ/mol) is less than BDE, even in vacuum (319.78 kJ/mol).

Proton separation from the O-H group in the cation-radical of galantamine (and norgalantamine) in the second step of the SET-PT mechanism is even easier: the change in the enthalpy of this process (PDE) is 131.16 kJ/mol (110.92 kJ/mol for norgalantamine).

Therefore, the dissociation of the O-H bond in galantamine and the norgalantamine by the SET-PT mechanism is more probable than the dissociation by the HAT mechanism. The ionization potential of the phenolic compounds we have worked with before is between 369 and up to 393 kJ/mol (Markovic et al., 2016), and the PDE is a small positive or even negative number.

SPLET mechanism of O-H bond dissociation

$$\text{Gal-X-H} \xrightarrow{-H^{+}} \text{Gal-X}^{-} \xrightarrow{-e^{-}} \text{Gal-X}^{\bullet}$$

Scheme 3. SPLET mechanism

For the description of the SPLET (Sequential Proton Loss Electron Transfer) mechanism (Scheme 3) the proton affinity {PA = H(Gal-O^-) + H(H^+) - H(Gal-OH)} and the electron transfer enthalpy ETE = H(Gal-$O^\bullet$) + H(e^-) - H(Gal-O^-) are needed.

The enthalpy of heterolytic dissociation of the O-H bond (PA) in galantamine is 218.26 kJ/mol (in norgalantamine, 218.18 kJ/mol). Simultaneously, the enthalpy of electron transfer (ETE) for both compounds is 201.07 kJ/mol. In this case, the first stage of the SPLET mechanism – the heterolytic dissociation of the O-H bond presents greater difficulty. However, the results indicate that SPLET is the most likely mechanism for O-H bond dissociation.

These results strongly suggest that O-H bond within galantamine plays a pivotal role in defining its chemical and physiological properties.

Protonated galantamine and norgalantamine

To verify the experimentally found results (Traykova et al., 2003), which indicate that the RSA of galantamine increases when it is converted to the hydrobromide salt, we calculated the PA and ETE of the O-H bonds in protonated galantamine and norgalantamine. It turns out that the PA of the O-H bond in the protonated galantamine is lower: 210.68 kJ/mol (respectively, 210.39 kJ/mol for the norgalantamine), and the ETE for the detachment of an electron from the obtained anion from the hydroxyl group is 209.34 kJ/mol (209.62 kJ/mol for norgalantamine) (Table 3).

Table 3. Enthalpies of C-H и O-H bond dissociation (kJ/mol) in the protonated galantamine and norgalantamine in the SPLET mechanism.

	$PA_{O\text{-}H}$	$ETE_{O\text{-}H}$	$PA_{C\text{-}H}$	$ETE_{C\text{-}anion}$
Galantamine cation	210.68	209.34	295.83	-14.67
Norgalantamine cation	210.39	209.62	295.87	-14.63

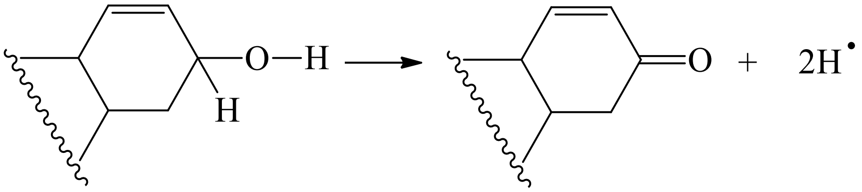
PA in galantamine (and norgalantamine) with the protonated amino is about 8 kJ/mol less than that in the non-protonated, which agrees with the experimental results, according to which in an acidic environment galantamine is a more active radical-scavenger.

The O-H bond in protonated galantamine (and norgalantamine) dissociates more easily *via* the SPLET mechanism compared to the unprotonated form, which accounts for the higher RSA observed in its bromide salt.

On the other hand, this supports the conclusion that the SPLET mechanism occurs during galantamine's reaction with active radicals *in vitro*.

Dissociation of the C(6)-H bond

The C-H bond located at the C(6) position (Scheme 1) is anticipated to undergo relatively easier dissociation across all mechanisms. This is attributed to the proximity of the double bond and the oxygen atom. The spatial arrangement facilitates the sharing of spin density and charge with neighboring carbon atoms (C7 and C8) as well as the oxygen, if a positive charge is localized at the C(6) position. Certainly, the galantamine radical, acquired after the homolytic dissociation (Scheme 4) of the C-H bond, manifests a maximum spin density of 0.676. This is significantly less than in the radical obtained after the homolytic dissociation of the O-H bond. BDE of this C-H bond in galantamine (in water) is 321.81 kJ/mol, (and 321.80 kJ/mol in norgalantamine). This is about 107 kJ/mol lower than the BDE of the O-H bond (Table 1). As mentioned above, it is necessary to inspect the HAT mechanism for C-H bond dissociation in a vacuum (Table 2). It turned out that BDE of this bond in galantamine is 319.78 kJ/mol, and in norgalantamine: 319.65 kJ/mol.



Scheme 4. Conversion to oxygalantamine.

If a HAT mechanism of the C-H bond dissociation is realized, it becomes possible at the next step to break much easier the O-H bond according to Scheme 4, and the enthalpy change of the O-H bond becomes now 148.82 kJ/mol (148.60 kJ/mol for norgalantamine).

Let us consider the changes in the enthalpies in the two-steps mechanisms of C-H bond breaking. The conversion of galantamine to a cation-radical in the SET-PT mechanism has already been considered (Scheme 2). As said above, this is a completely feasible process from a thermodynamic point of view: IP is 288.16 kJ/mol (308.33 kJ/mol for norgalantamine). PDE of the C-H bond dissociation from the cation-radical of galantamine is 24.457 kJ/mol (4.280 kJ/mol in norgalantamine). It turns out that it is much easier to tear off a proton from the C-H bond than from the O-H bond of the cation-radical.

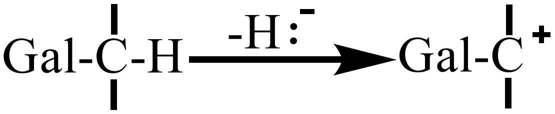
Heterolytic dissociation of the C-H bond to an anion and a proton is less possible than the dissociation of the O-H bond. PA of the C-H bond dissociation is 316.00 kJ/mol in galantamine (316.52 kJ/mol in norgalantamine). If such an anion is formed, it would spontaneously release an electron to form a radical (the ETE of the galantamine anion is -3.38 kJ/mol and of the norgalantamine anion -3.61 kJ/mol).

The protonation of the amino group in galantamine and norgalantamine may benefit the SPLET mechanism (Table 3). The enthalpy of the C-H bond heterolytic dissociation (PA) in the protonated galantamine is 295.83 kJ/mol (in the protonated norgalantamine: 295.87 kJ/mol). ETE of the anions with a protonated amino group are bigger negative numbers: -14.67 kJ/mol (and -14.63 kJ/mol).

In a non-polar environment, galantamine readily undergoes oxidation to oxygalantamine during its interaction with two active radicals.

Hydride ion detachment

The presence of a heteroatom and a conjugate system adjacent to C(6) and C(12) are the reasons to consider another way of C-H bond dissociation - the detachment of the hydride ion according to Scheme 5:



$$\text{Gal-}\overset{|}{\underset{|}{\text{C}}}\text{-H} \xrightarrow{-\text{H}:^{-}} \text{Gal-}\overset{|}{\underset{|}{\text{C}}}{}^{+}$$

Scheme 5. Hydride ion separation

A positive charge in these positions can be readily delocalized due to the nearby double bond and heteroatom.

The change in the enthalpy of this process at C6 is 319.72 kJ/mol (in norgalantamine: 318.48 kJ/mol). The same dissociation at C12 adjacent to the nitrogen atom, is more probable: 228.51 kJ/mol for galantamine and 237.69 kJ/mol for norgalantamine. The probability of this process is therefore fully comparable to the SPLET dissociation mechanism of the O-H bond.

For comparison, we calculated the change in enthalpy during the detachment of the hydride ion from NADH (202.62 kJ/mol), and from morphine, where the change in enthalpy is 187.72 kJ/mol.

Conclusion

Using DFT methods, the dissociation enthalpies of the O-H bond and the two most easily dissociable C-H bonds in galantamine and norgalantamine were calculated. The calculations were performed in a non-polar environment (vacuum) and with consideration of the polarizing effect of water. This approach enables modeling the RSA of these compounds in aqueous and lipid environments within the human body. To explain the experimentally observed higher activity of galantamine hydrobromide, studies were conducted on both the free bases and the protonated forms of the two alkaloids. It was found that the higher activity of the salt is due to the preferred SPLET mechanism and the greater acidity of the O-H bond in the ammonium ion compared to its acidity in the free base. The possibility of two-electron oxidation processes was also evaluated: a two-step oxidation of galantamine *via* the HAT mechanism to oxygalantamine and a one-step process involving hydride ion abstraction from the C12 atom, located adjacent to the aromatic ring A and the nitrogen atom.

Conflict-of-Interest Statement

The authors declare that they have no conflict of interest.

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Istraživanje aktivnosti galantamina i norgalantamina u uklanjanju radikala pomoću teorije funkcionala gustine (DFT)

Tekući naslov: Računarske studije o galantaminu i norgalantaminu

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Sažetak

Galantamin, prirodni alkaloid sa inherentnim antioksidativnim svojstvima, efikasno prelazi krvno-moždanu barijeru, što ga čini obećavajućim terapeutskim sredstvom za lečenje određenih poremećaja povezanih sa mozgom kod ljudi. Ova studija teorije funkcionala gustine (DFT) predstavlja rezultate kvantno-hemijskih proračuna entalpija disocijacije galantaminovih O-H i C-H veza, razjašnjavajući njegove aktivnosti uklanjanja radikala. Rezultati ističu sklonost galantamina ka interakciji sa radikalima u biološkim sistemima, naglašavajući jačinu veze i kiselost njegovih O-H i C-H grupa. Pored toga, studija istražuje implikacije apstrakcije hidridnih jona, bacajući svetlo na njegovu potencijalnu reaktivnost i antioksidativne mehanizme.

Ključne reči: *galantamin, norgalantamin, aktivnost uklanjanja radikala, DFT proračuni*

Chemia Naissensis, Vol 7, Issue 2, 36-53, RESEARCH ARTICLE

The antioxidant capacity determination of *Rosa canina* L. fruit extracts using optimized cyclic voltammetry and spectrophotometric assays, and determination of vitamin C

Running title: Antioxidant capacity and vitamin C in *R. canina* extract

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ABSTRACT

The optimized electrochemical method, cyclic voltammetry, was applied to investigate the antioxidant capacity of water extracts of *Rosa canina* L. fruits. Scan speeds (25, 50, and 75 mV/s) and pH (2, 4.5, and 7) were varied and optimal conditions were found at 75 mV/s and pH 4.5. Cyclic voltammograms, recorded from 0 to 1200 mV, showed anodic peaks for catechin-type flavonoids and quercetin. Spectrophotometric tests (FRAP, CUPRAC, DPPH, ABTS) and determination of total polyphenols content (TPC), total flavonoids content (TFC), and vitamin C content were also conducted. FRAP and CUPRAC values ranged from 0.534 to 0.710 mmol Fe/g dw and from 103 to 174 mg TE/g dw, respectively. ABTS values were in the interval from 566 to 623 mg TE/g dw, while DPPH values ranged from 113 to 188 mg TE/g dw. Total polyphenols content and total flavonoids content ranged from 24.0 to 38.9 mg GAE/g dw, and from 12.7 to 21.5 mg CE/g dw, respectively. The concentrations of vitamin C were in the interval from 2.78 to 3.67 mg/g dw. Pearson's analysis showed good correlations between ABTS and DPPH ($R^2 = 0.87$), FRAP and DPPH ($R^2 = 0.85$), FRAP and ABTS ($R^2 = 0.86$), TPC and *in vitro* tests ($0.5 < R^2 < 0.77$), and TFC and *in vitro* tests ($0.47 < R^2 < 1$). For the purpose of classifying spectrophotometric tests for determining antioxidant activity, hierarchical cluster analysis was performed.

Keywords: *Rosa canina L., antioxidant activity, cyclic voltammetry, spectrophotometric assays, cluster analysis, Pearson correlation analysis*

Introduction

Ensuring a sufficient supply of nutritionally rich food represents one of the key global challenges of modern society. The growing world population, along with adverse environmental factors and changes in agroecosystems, increasingly affects the stability of food resources. Climate change, including frequent droughts, floods, and extreme weather events, significantly reduces agricultural yields and complicates the availability of essential nutrients. Consequently, investigating plant species rich in bioactive components has become crucial for finding sustainable solutions in the fields of nutrition and public health.

The fruits of plants from the genus *Rosa* (family *Rosaceae*), particularly rosehip (*Rosa canina* L.), have been extensively studied in scientific research because of their rich chemical composition, which includes high levels of vitamin C, polyphenols, minerals, fatty acids, and other bioactive compounds.

Rosehip has been traditionally used in folk medicine and nutrition. At the same time, modern research approaches have revealed their potential in the prevention and treatment of various diseases, including inflammatory conditions, cancer, and cardiovascular disorders (Larsen et al., 2003; Rein et al., 2004; Christensen et al., 2008; Fujii et al., 2009; Andersson et al., 2012). Numerous studies indicate that rosehip fruits contain significantly higher amounts of vitamin C compared to citrus fruits, classifying them among the most valuable natural sources of this essential nutrient (Demir and Özcan, 2001; Ercisli and Esitken, 2004). The concentration of ascorbic acid in rosehip varies from 300 to 4000 mg per 100 g of fruit, enabling its widespread application in the food and pharmaceutical industries, particularly in the production of teas, marmalades, and dietary supplements (Taneva et al., 2016). Besides being rich in vitamin C, rosehip fruits are also rich in flavonoids and phenolic acids, which contribute to their pronounced antioxidant, anticancer, and anti-inflammatory effects. These bioactive components play an essential role in nutrition and can contribute to health improvement by protecting cells from oxidative stress (Elmastaş et al., 2017). Furthermore, research has shown that rosehip consumption may positively influence blood sugar regulation, indicating its potential for diabetes prevention (Orhan et al., 2007).

Flavonoids are secondary metabolites of plants that exhibit a range of positive effects on human health, such as antioxidant and anti-inflammatory properties, antimutagenic, and anticancer properties. For this reason, they are widely used in various industries, such as pharmaceuticals and cosmetics (Panche et al., 2016). For example, rutin and quercetin, which are present in *R. canina* L. fruits, have protective effects against cardiovascular diseases, positive impacts on diabetes, and antimicrobial properties against certain infections (Elmastaş et al., 2017; Kumar et al., 2017; Semwal et al., 2021).

The composition of bioactive compounds in rosehip fruits varies depending on numerous factors, including environmental conditions, geographical origin, cultivation methods, as well as extraction and processing techniques (Taneva et al., 2016; Fascella et al., 2019). During the ripening process, significant changes occur in the concentration of flavonoids and phenolic acids, which can influence their antioxidant capacity and nutritional value (Elmastaş et al., 2017).

The aim of this study was to determine the antioxidant activity of *Rosa canina* L., the most prevalent species from the Rosaceae family, collected from two different locations in the Republic of Serbia. The antioxidant activity of the water extracts of *Rosa canina* L. fruits was determined using the electrochemical method**,** cyclic voltammetry (CV), and *in vitro* spectrophotometric tests. Also, a correlations were established between the obtained results.

Experimental

Chemicals

For the preparation of samples and standards, deionized water (0.05 µS/cm) was used (MicroMed purification system, TKA Wasseraufbereitungssysteme GmbH, Niederelbert, Germany). Ethanol and methanol from J.T. Baker (Deventer, Netherlands) were used for the preparation of standards. Chemical reagents, including ABTS (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonate), DPPH (2,2-diphenyl-1-picrylhydrazyl hydrate), TPTZ (2,4,6-tri(2-pyridyl)-S-triazine), as well as flavonoids such as (+)-catechin, kaempferol, rutin, and quercetin (HPLC grade), phenolic compounds such as gallic acid and protocatechuic acid, along with neocuproine and thiourea, were purchased from Sigma-Aldrich (Steinheim, Germany). Cyanidin-3-O-glucoside chloride was obtained from ChromaDex (Irvine, California, USA), while trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Acros Organics (Morris Plains, New Jersey, USA). Folin–Ciocalteu reagent, as well as various salts and acids used in the analyses, including aluminum chloride hexahydrate, ammonium acetate, copper(II) chloride, iron(III) chloride, iron(II) sulfate heptahydrate, sodium acetate, sodium nitrite, sodium carbonate, sodium hydroxide, sodium sulfate, sodium persulfate, as well as acetic acid, ascorbic acid, bromine water, hydrochloric acid, formic acid, metafosforic acid, nitric acid, sulfuric acid, and trichloroacetic acid, were obtained from Merck® (Darmstadt, Germany).

Sample preparation for analysis

Rosa canina L. fruit samples were collected from two locations in the southeastern region of Serbia during October 2023. At each location, sampling was conducted at four different points, with a maximum distance of 500 m between them. Between 300 and 500 g of fruits were collected and stored at -20 °C until sample preparation for analysis. Herbarium specimens were registered and are preserved housed in the collection of the Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš. The voucher numbers, along with details of the sampling locations, are provided in Table 1 (Milenković et al., 2024). Approximately 30 g of frozen samples were taken and completely dried by lyophilization (Freeze-dryer Alpha 1-2 LDplus, Osterode am Harz, Germany). After full moisture removal, the samples were homogenized using a laboratory grinder. For extraction, 20 ml of deionized water were added to 1 g of lyophilized powder, mixed and treated on a laboratory shaker for 60 minutes. All the samples were centrifuged at 4000 rpm (10 minutes). This procedure was repeated three times. All extracts were filtered through a PTFE microfilter (0.45 μm), combined, and evaporated to dryness under reduced pressure at 40–50 °C. The dry extract residues were dissolved in the deionized water and transferred into a volumetric flask with a final volume of 25 mL.

Table 1. Geographical characteristics of *Rosa canina* sampling sites and corresponding voucher numbers

Sample mark	Locality	Latitude (N)*	Longitude (E)*	Altitude (A)	Voucher No.
S1-S4	Senokos, Dimitrovgrad	43° 8'35.73"	22°55'27.27"	938 m	18613
S5-S8	Visočka Ržana, Pirot	43° 9'26.45"	22°48'59.04"	741 m	18614

*the geographical coordinates are given in decimal degrees (°N, °E)

Electrochemical analysis

One of electrochemical techniques, cyclic voltammetry (CV), was used to examine the redox properties of the investigated compounds. In this study, measurements were conducted using a CHI 760B electrochemical instrument (CH Instruments, Austin, Texas, USA). The experimental electrochemical cell consisted of a glassy carbon (GC) electrode as the working electrode, a platinum electrode as the auxiliary electrode (Model CHI221), and a reference electrode made of silver-silver chloride (Ag/AgCl) (Model CHI111). Before measurements, the working electrode was mechanically treated by polishing with aluminum oxide powder of different grain sizes (1.0, 0.3, and 0.05 μm) and subsequently degreased in ethanol to remove impurities and ensure optimal surface conductivity. Rosehip extracts and standard compound solutions were prepared by mixing them with sodium acetate-acetate buffer at pH 4.5, and with phosphate buffer at pH 7 in a 1:1 volume ratio, while pH 2 was adjusted by adding hydrochloric acid. All measurements were performed at room temperature. The potential range for measurements was set from 0 to 1200 mV, while the scan rate varied at 25, 50, and 75 mV/s, with an increment of 2 mV. Additionally, voltammograms were recorded for trolox within a concentration range of 2 to 80 μmol/L, following previous studies (Piljac-Žegarac et al., 2010; Veljković et al., 2013). The area under the dominant anodic peak (Q600), depending on the concentration of the standard compound, was used to obtain the calibration curve. The antioxidant capacity of the analyzed rosehip samples was determined as the trolox equivalent antioxidant capacity (TEAC).

Spectrophotometric analysis

The absorbance of the samples was measured using an Agilent 8453 UV/Vis spectrophotometer (Agilent Technologies, Santa Clara, California, USA), utilizing optical cuvettes with a path length of 1 cm.

Total polyphenolic content (TPC)

The TPC was determined following the method outlined by Prior et al. (2005). Measurements were performed at 760 nm, and the results were reported as gallic acid equivalents (GAE) per gram of dry weight sample (mg GAE/g dw).

Total flavonoid content (TFC)

The TFC was determined using the method developed by Yang et al. (2004). Absorbance was measured at 520 nm, and the total flavonoid content was quantified as milligrams of catechin equivalents (CE) per gram of dry weight sample (mg CE/g dw).

Antioxidant assays

The antioxidant activity of water extracts was determined using four spectrophotometric assays. The ABTS assay is based on 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), an organic compound with strong reducing power over phenolic compounds, acting as an electron donor. The method was conducted according to Re et al. (1999). In this assay, the reduction in absorbance is correlated with the concentration of antioxidants. Absorbance was measured at 734 nm, and the results were given as milligrams of trolox equivalents (TE) per gram of dry weight sample (mg TE/g dw). The DPPH method was used to determine the radical scavenging capacity according to Brand-Williams et al. (1995), employing a prepared DPPH solution with a concentration of 10^{-4} mol/L in methanol. After a 30-minute incubation, absorbance was recorded at 520 nm. Antioxidant activity determined by the DPPH method was quantified as milligrams of trolox equivalents per gram of dry weight sample (mg TE/g dw). The FRAP method relies on the ability of the extract to reduce Fe^{3+} ions to Fe^{2+} ions in the presence of 2,4,6-tripyridyl-s-triazine (TPTZ) under acidic conditions (Benzie et al., 1999). The reduction process was monitored by measuring changes in absorbance at 595 nm. The obtained results were expressed as millimoles of Fe^{2+} equivalents per gram of sample (mmol Fe/g). The CUPRAC assay determines antioxidant activity based on the ability to reduce Cu^{2+} ions to Cu^{+}. This method was first described by Apak et al. (2004). The mixture containing the CUPRAC reagent and the sample was thermostated at 25 °C for 30 minutes in the dark, after which absorbance was measured at 450 nm. The obtained antioxidant activity values were expressed as milligrams of trolox equivalents per gram of dry weight sample (mg TE/g dw).

Vitamin C content

The content of vitamin C was determined using the method outlined by Khan et al. (2006). Absorbance of the reaction mixture was recorded at 521 nm, and the results were expressed as milligrams of vitamin C per gram of dry weight sample (mg/g dw).

Results and Discussion

To achieve optimal conditions for determining the antioxidant activity of rosehip water extracts using cyclic voltammetry, the pH values and scan rates were varied. The samples were prepared in three series, with pH values of the extract set at 2.0, 4.5, and 7.0, while the scan rate was monitored at 25, 50, and 75 mV/s. The effect of the scan rate on the anodic current intensity is presented in Figure 1.

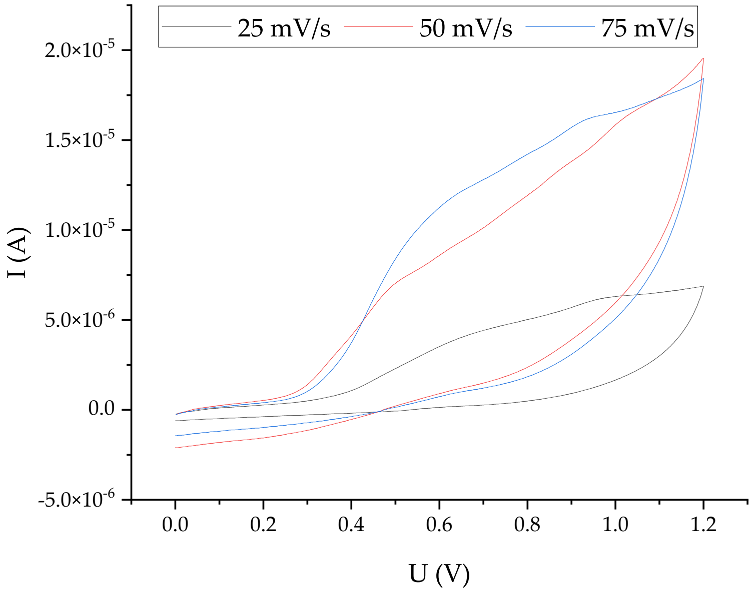

Figure 1. Effect of scan rate on anodic current intensity

As shown in Figure 1, a higher scan rate leads to a more intense anodic current, while a lower scan rate allows sufficient time for the stabilization of the double-layer charge on the glassy electrode. To adjust the pH of the aqueous rosehip extracts, acetate buffer (pH 4.5), phosphate buffer (pH 7), and hydrochloric acid (pH 2) were used. Regarding the effect of pH on the intensity of the anodic current, the most optimal current was observed at a pH value of 4.5, as presented in Figure 2. This finding is consistent with the study by Yakovlev et al. (2007), which reported that an increase in the pH of the electrolyte solution results in the shift of anodic potential (E_{pa}) towards the less positive values of potential, explained by the reduced degree of antioxidant protonation and a shift in the molecular charge toward more negative values.

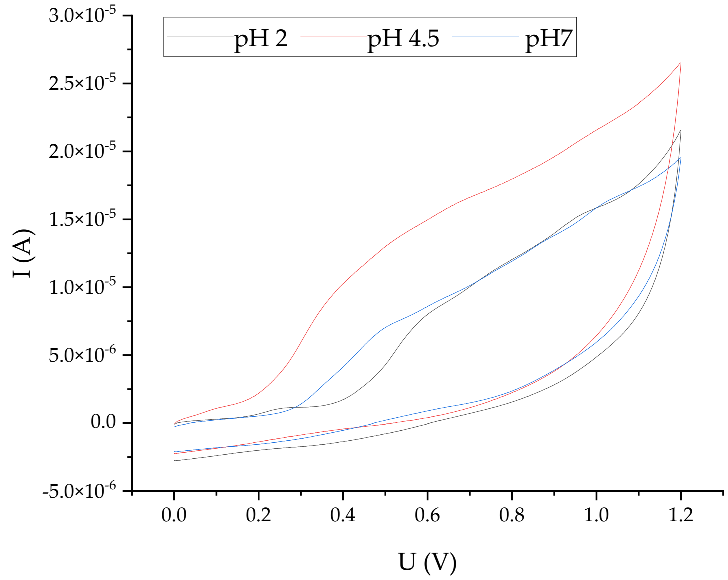

Figure 2. Effect of solution pH on anodic current intensity

Based on the obtained data, the optimal conditions used for cyclic voltammetry were a pH of 4.5 and a scan rate of 75 mV/s. Before recording the sample, standards were recorded under the optimal conditions, and their values of oxidation (E_{pa1}, E_{pa2}, E_{pa3} - potential of first, second, and third anodic pick) and reduction potentials (E_{pc1} and E_{pc2} - potential of first and second catodic peak) are presented in Table 2.

Table 2. Oxidation (E_{pa1}, E_{pa2}, E_{pa3}) and reduction (E_{pc1}, E_{pc2}) potentials of phenolic compounds

Phenolic compound	E_{pa1} (V)	E_{pa2} (V)	E_{pa3} (V)	E_{pc1} (V)	E_{pc2} (V)
Ellagic acid	0.355	0.704	-	0.519	-
(+)-Catechin	0.485	0.779	-	-	-
Quercetin	0.617	1.002	-	0.774	0.069
Rutin	0.526	0.886	-	-	-
(-)-Epicatehin	0.510	0.735	-	-	-
Protocatechuic acid	0.605	-	-	0.155	-
Caffeic acid	0.435	-	-	0.189	-
Galic acid	0.553	0.884	-	-	-

Abou Samra et al. (2011) evaluated the antioxidant potential of the investigated compounds based on the E_{pa} values of the first anodic peak. Lower E_{pa} values indicate a greater ability to donate electrons, meaning the compound exhibits stronger antioxidant activity. Comparing the anodic peaks obtained for the standards to those obtained for the samples (Table 3), it can be concluded that the first anodic peak, with values ranging from 0.485 to 0.513 V, and the second anodic peak, ranging from 0.712 to 0.746 V, correspond to the oxidation of flavonoids-catechins. According to the study by Medvidović-Kosanović et al. (2010), the first oxidation peak for (+)-catechin, which is reversible, corresponds to the oxidation of the 3',4'-dihydroxyl substituent on the B-ring. The second oxidation peak is irreversible and has been attributed to the oxidation of the 5,7-dihydroxy substituent on the A-ring. Catechin and epicatechin exhibit similar electrochemical properties due to their nearly identical chemical structures and the same oxidation mechanism (Jara-Palacios et al., 2024). In the voltammograms for the epicatechin standard, the oxidation peak appeared at a potential of 0.510 V, corresponding to the oxidation of the catechol group. The second anodic peak was detected at 0.735 V, indicating the oxidation of the resorcinol group. However, according to some sources, this anodic peak may result from the irreversible oxidation of the hydroxyl group at the third position of the non-aromatic C-ring (Petrović, 2009; Rebelo et al., 2013). The values obtained for the third anodic peak of the samples ranged from 0.931 to 0.991 V, which may be attributed to the oxidation of quercetin. The oxidation mechanism of quercetin involves the first anodic peak and the corresponding cathodic peak, which corresponds to the reversible oxidation of the catechol group. In contrast, the second anodic peak indicates the oxidation of the resorcinol group (Jara-Palacios et al., 2024).

Table 3. Peak potential (E_p) and current (I_p) values of analyzed rosehip samples

Sample	E_{pa1} (V)	I_{pa1} (A)	E_{pa2} (V)	I_{pa2} (A)	E_{pa3} (V)	I_{pa3} (A)	E_{pc} (V)	I_{pc} (A)
S1	0.492	0.58	0.714	1.19	0.980	1.44	-	-
S2	0.513	1.05	0.746	1.63	0.988	1.68	-	-
S3	0.505	0.51	0.712	0.95	0.957	1.34	-	-
S4	0.502	0.66	0.743	1.36	0.989	1.60	-	-
S5	0.485	0.53	0.717	1.09	-	-	-	-
S6	0.495	0.46	0.713	0.88	0.931	1.14	-	-
S7	0.485	0.61	0.737	1.33	0.991	1.50	-	-
S8	0.485	0.61	0.744	1.30	0.972	1.42	-	-

The lowest value of TPC (24.0 mg GAE/g) was found in the fruits from the Senokos locality, while the highest value (38.9 mg GAE/g) was recorded in the samples from Visočka Ržana (Figure 3). The average TPC at the Senokos site was 30.65 mg GAE/g (24.00–37.50), whereas at Visočka Ržana, it was 35.49 mg GAE/g (30.55–38.90). The difference in TPC may be attributed to the environmental conditions (Ciornea et al., 2018), as well as to differences in altitude between the two sites (Lacramioara and Rosu, 2021). The obtained results for TPC were lower than those reported by Paunović et al. (2019), who analyzed raw and dried samples of this species from Serbia. They were also lower than the TPC of *R. canina* L. fruits from Sicily, extracted with 80% methanol (Fascella *et al.*, 2019), but higher than results obtained for samples from Slovakia, extracted with 96% ethanol (Rovna et al., 2020). This suggests that, in addition to the origin of the sample, the choice of extraction solvent may significantly influence the total polyphenol content.

The TFC in the water extracts ranged from 12.7 to 21.5 mg CE/g. The average TFC in fruits at the Senokos site was 15.9 mg CE/g (12.7–20.5 mg CE/g), while in fruits at Visočka Ržana, it was 19.4 mg CE/g (16.4–21.5 mg CE/g). Similar to total polyphenols, the total flavonoid content was higher in fruits at the Visočka Ržana site, as shown in Figure 3. Paunović et al. (2019) reported a TFC (miligrame of quercetin equivalents, QE, per gram of raw and dried fruit sample) of 38.5 mg QE/g in raw and 26.47 mg QE/g in dried samples using a 70% ethanol extract. In contrast, Tahirović and Bašić (2017) found much lower values for aqueous extracts of samples from Bosnia and Herzegovina, reporting 0.214 mg QE/g, while the ethanol (50%) extract yielded 0.675 mg QE/g. Similarly, Nađpal et al. (2016) reported a total flavonoid content of 1.14 mg QE/g in dried *R. canina* fruits. The differences amoung TFC in the literature may be caused by the geographical origin of the samples, soil conditions, climate, as well as using different compounds as equivalents (quercetin or catechin) for the calculate of flavonoid contents (Taneva et al., 2016; Fascella et al., 2019).

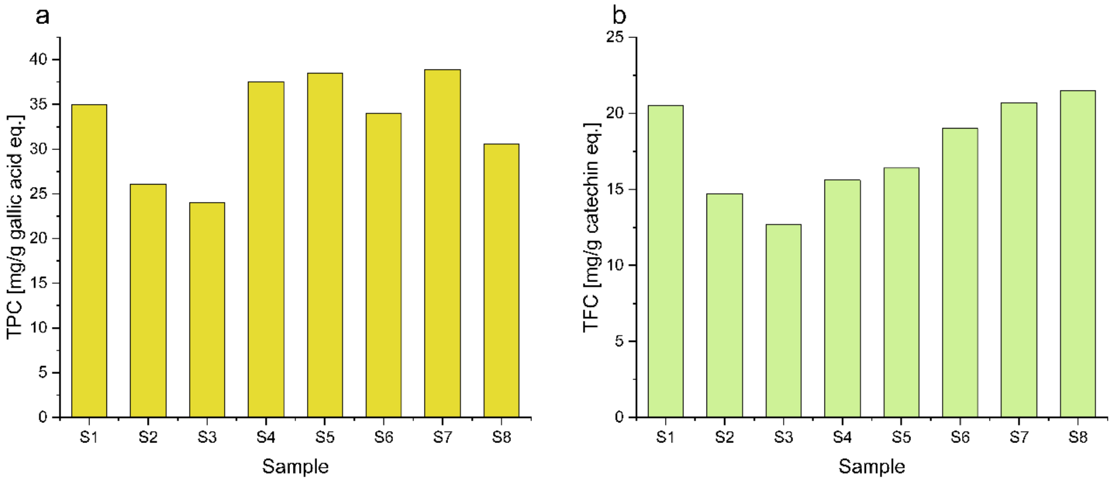

Figure 3. Total polyphenol and flavonoid content in the analyzed water extracts of *R. canina* L. fruits

Rosa canina L. is a good source of antioxidants, which is one of the reasons for its widespread application. The analysis of the antioxidant activity of the aqueous extract of *R. canina* L. fruits using the DPPH assay showed that the average value for samples from the Senokos locality was 127 µmol TE/g (113–144 µmol TE/g), while for the samples from the Visočka Ržana locality, the average value was 141 µmol TE/g (120–188 µmol TE/g). These results are consistent with those found in the literature, for example, 250 µmol TE/g for the ethanol (70%) extracts of dried fruits and 320 µmol TE/g for the fresh samples (Paunović et al., 2019), as well as 382.3 µmol TE/g for the aqueous extracts (Tahirović and Bašić, 2017). The average ABTS values for the water extracts of fruits from Senokos and Visočka Ržana were 589 mmol TE/g and 592 mmol TE/g, respectively, with ranges of 566–609 and 580–623 mmol TE/g. Unlike the values for TPC, TFC, and DPPH, the results obtained for ABTS antioxidant activity of samples from two locations were very similar. Taneva et al. (2016) analyzed the antioxidant activity of *R. canina* L. aqueous extract using the ABTS assay and reported a value of 313.1 mmol TE/g, while for the ethanol (50%) extract, the ABTS value was 368.4 mmol TE/g. The average FRAP test value for the fruit extracts from Senokos was 0.61 mmol Fe/g (0.53–0.69), while for the fruit extracts from Visočka Ržana, it was 0.66 mmol Fe/g (0.61–0.71). The mean CUPRAC test values were 129 mg TE/g (103–166) and 157 mg TE/g (133–174 mg TE/g) for the extracts of fruits from two locations, respectively. The FRAP test results were similar to those obtained for the aqueous extract of *Rosa* species investigated by Tahirović and Bašić (2017). Adamczak et al. (2012) emphasized that vitamin C is highly stable and exhibits significant bioavailability in the human body, as organic acids and flavonoids prevent its oxidation. The vitamin C concentrations in the analyzed samples ranged from 2.78 to 3.67 mg/g of dry sample. As shown in Table 4, the content of vitamin C were slightly higher in the samples from Visočka Ržana. The obtained results are in good agreement with the findings of Barros et al. (2011) and Nađpal et al. (2016), while Demir et al. (2014) reported significantly lower vitamin C concentrations in similar samples. The effect of ripeness on vitamin C content was investigated by Nojavan et al. (2008), who found that *R. canina* rosehips contain the highest concentration of vitamin C in their fully ripe stage-six times higher than in oranges. All previous studies confirm the traditional use of rosehips as a rich source of vitamin C.

For the purpose of classifying spectrophotometric tests for determining antioxidant activity, hierarchical cluster analysis was performed using Euclidean distance and Ward's clustering method on standardized data for these methods. The resulting dendrogram is presented in Figure 4.

Table 4. Antioxidant activity of rosehip extracts and vitamin C content (mean ± standard deviation, number of repetitions -3)

Sample	ABTS (mg TE/g)	DPPH (mg TE/g)	CUPRAC (mg TE/g)	FRAP (mmol Fe/g)	Vitamin C (mg/g)
S1	609±6	132±9	166±1	0.63±0.01	2.78±0.06
S2	566±5	113±3	119±1	0.53±0.01	3.02±0.07
S3	581±4	120±9	103±2	0.59±0.01	3.11±0.06
S4	598±6	144±4	126±1	0.69±0.01	3.27±0.06
S5	582±8	120±9	133±2	0.61±0.01	3.67±0.06
S6	581±6	125±4	154±1	0.63±0.01	3.57±0.07
S7	623±5	188±9	168±2	0.71±0.01	3.33±0.07
S8	580±50	130±1	174±1	0.62±0.01	3.43±0.07

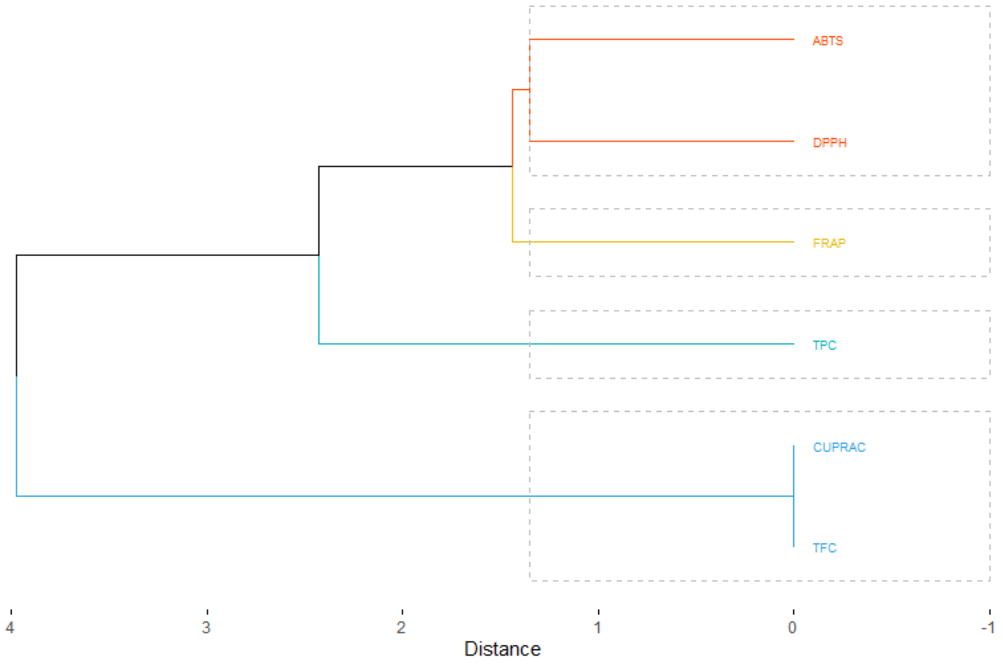

Figure 4. Classification of spectrophotometric tests

In the first cluster, CUPRAC and TFC are grouped together, indicating their interrelation in determining the extract's total antioxidant activity. This can be explained by flavonoids' ability to reduce copper in the CUPRAC assay, as confirmed by Apak et al. (2004). They developed a method known as "cupric reducing antioxidant capacity."

Regarding the second cluster, TPC is grouped into a separate subcluster, suggesting that it is not a decisive factor in determining antioxidant activity; however, it remains closely associated with other antioxidant analysis methods. In another subcluster, FRAP is separated from the DPPH and ABTS methods, which can be explained by its significantly different mechanisms of action. ABTS and DPPH are grouped in a single subcluster, which is expected since both analytical methods measure the ability of compounds to neutralize free radicals through electron or hydrogen transfer.

To explore potential correlations between the spectrophotometric tests for determining antioxidant activity, total phenol and flavonoid content, and vitamin C content, Pearson correlation analysis was performed among these parameters, and the results are presented graphically in Figure 5.

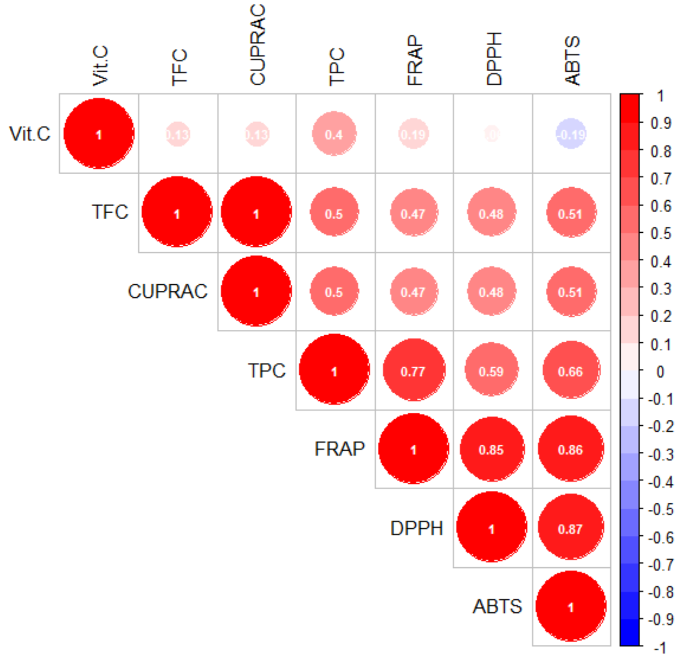

Figure 5. Correlations between the applied methods for determining TPC, TFC, antioxidant activity, and vitamin C content

As shown in Figure 5, there is a strong correlation between the antioxidant tests themselves, as well as between the antioxidant tests and the total phenol and flavonoid content. This can be explained by the fact that phenolic compounds, including flavonoids, exhibit significant antioxidant effects (Rice-Evans et al., 1997).

The correlation between vitamin C content and the antioxidant tests is considerably weaker, which is consistent with the findings of Rice-Evans et al. (1997), who reported that polyphenols are more effective antioxidants *in vitro* than vitamin E and vitamin C. Consequently, polyphenols play a more significant role in protecting the body against oxidative stress than these vitamins.

Conclusion

The results of this study indicate that the antioxidant properties of *R. canina* L. fruit extracts can be determined using an optimized electrochemical method, cyclic voltammetry. Based on the anodic peaks recorded for both standards and samples, it was established that the antioxidant activity of this species is significantly influenced by the presence of catechin, epicatechin, and quercetin. Furthermore, the results obtained from spectrophotometric assays show a significant correlation with the total polyphenol and flavonoid content but not with the results of vitamin C determination, confirming that this vitamin is a weaker antioxidant compared to polyphenols.

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Conflict-of-Interest Statement

The authors declare that they have no conflict of interest.

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Chemia Naissensis, Vol 7, Issue 2, 54, RESEARCH ARTICLE

Određivanje antioksidativnog kapaciteta ekstrakta plodova Rosa canina L. korišćenjem optimizovane ciklične voltametrije i spektrofotometrijskih testova i određivanje vitamina C

Tekući naslov: Antioksidativni kapacitet i vitamin C u ekstraktu *R. canina*

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Sažetak

Za ispitivanje antioksidativnog kapaciteta vodenih ekstrakata plodova vrste *Rosa canina* L. primenjena je optimizovana elektrohemijska metoda, ciklična voltametrija. Ispitivanjem uticaja različitih brzina skeniranja (25, 50 i 75 mV/s) i pH vrednosti (2, 4,5 i 7) određeni su optimalni uslovi (75 mV/s i pH 4,5). Ciklični voltamogrami, snimljeni u opsegu potencijala od 0 do 1200 mV, pokazali su anodne pikove karakteristične za flavonoide, kao sto su katehin i kvercetin. Takođe su urađeni spektrofotometrijski testovi (FRAP, CUPRAC, DPPH i ABTS) i određen ukupni sadržaj polifenola (TPC), ukupni sadržaj flavonoida (TFC) i sadržaj vitamina C. FRAP i CUPRAC vrednosti kretale su se u opsegu od 0,534 do 0,710 mmol Fe/g suve materije (*eng. dry weight*, dw), odnosno od 103 do 174 mg TE/g dw. ABTS vrednosti bile su u opsegu od 566 do 623 mg TE/g dw, dok su se DPPH vrednosti kretale između 113 i 188 mg TE/g dw. Ukupni sadržaj polifenola i flavonoida iznosio je od 24,0 do 38,9 mg GAE/g dw, odnosno od 12,7 do 21,5 mg CE/g dw. Koncentracije vitamina C kretale su se u opsegu od 2,78 do 3,67 mg/g dw. Pearsonova analiza pokazala je dobre korelacije između ABTS i DPPH ($R^2 = 0{,}87$), FRAP i DPPH ($R^2 = 0{,}85$), FRAP i ABTS ($R^2 = 0{,}86$), kao i između TPC i *in vitro* testova ($0{,}50 < R^2 < 0{,}77$) i TFC i *in vitro* testova ($0{,}47 < R^2 < 1{,}00$). U cilju klasifikacije spektrofotometrijskih testova za određivanje antioksidativne aktivnosti, sprovedena je hijerarhijska klaster analiza.

Ključne reči: *Rosa canina L., antioksidativna aktivnost, ciklična voltametrija, spektrofotometrijske metode, klaster analiza, Pirsonova korelaciona analiza*

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Unveiling the Chemistry of E-Cigarettes: What's Inside Your Vape?

Running title: Chemical Composition of E-Cigarettes

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ABSTRACT

Smoking tobacco represents one of the most harmful habits for health, causing addiction, a series of somatic problems ranging from allergic reactions to cardiovascular diseases and lung cancer. Recently, electronic cigarettes (e-cigarettes) are increasingly being promoted as a healthier alternative to smoking. Though e-cigarettes have been marketed as safer alternatives to traditional tobacco products, the composition of e-liquids, the substances inhaled during vaping, require critical evaluation. E-liquids typically contain nicotine, propylene glycol, glycerol, flavoring agents, and other additives. However, studies have identified harmful constituents, including tobacco-specific nitrosamines (TSNAs), a group of carcinogenic compounds found in tobacco and tobacco smoke, volatile organic compounds (VOCs), heavy metals, and carbonyl compounds, which can form during heating.

Nicotine, a primary component of most e-liquids, poses addiction risks and cardiovascular effects. TSNAs and VOCs, even in trace amounts, are known carcinogens. Heavy metals (lead, cadmium, and nickel) released from e-cigarette components raise toxicological concerns. It's important to note that when inhaled, flavoring agents, often considered safe for ingestion, may have adverse respiratory effects, underscoring the need for caution.

Despite their popularity, e-cigarettes' long-term health impacts remain largely unknown, complicating public health assessments (Omaiye et al., 2020). The diverse chemical composition of e-liquids, combined with the alarming lack of strict quality standards, raises additional concerns. Present review emphasizes the urgent need for comprehensive research into the components of e-liquids and their potential health effects. Regulatory frameworks should introduce mandatory declarations of ingredient and product safety to reduce the risks associated with e-cigarette use. Addressing these gaps is essential to inform consumers, guide policymakers, and ensure public health protection amidst the growing popularity of e-cigarettes.

Keywords: *e-cigarettes, nicotine, tobacco-specific nitrosamines, volatile organic compounds, carbonyls, heavy metals*

Introduction

Electronic cigarettes represent battery-powered devices that intend to simulate standard tobacco cigarettes by converting e-liquid into an inhalable aerosol. This e-liquid can be contained in a disposable cartridge or tank, allowing consumers to refill it as needed. Because they do not burn tobacco, it is expected that they do not produce the same number of chemicals that can be found in conventional tobacco products. With that in mind, they can be proposed as potential products for tobacco harm reduction (Bullen et al., 2013; Capponeto et al., 2013a,b; Etter et al., 2011; Farsalinos et al., 2013a,b; Manzoli et al., 2013; O'Connor et al., 2012; Polosa et al., 2013). While their safety and efficacy as a substitute for standard tobacco cigarettes are controversial, many available studies are suggesting the potential of these products in the promotion of smoking reduction. Over the last few years, there have been numerous concerns regarding these products' manufacturing processes and quality since used e-liquids are not manufactured to standards such as those regarding medications. The constituents of cartridges and refill e-liquids could not comply with the label. They could contain impurities or many toxic substances (Etter et al., 2013; O'Connor et al., 2012).

E-liquids, the substances used in e-cigarettes, consist of nicotine (in different concentrations) in a mixture of vegetable glycerine and/or propylene glycol with water, followed by many other constituents (Omaiye et al., 2020; Krüsemann et al., 2021). Nicotine, a highly addictive substance, is the primary component that makes e-cigarettes appealing to smokers. When heated by an atomizer, the alcohols mentioned are used as humectants for aerosol creation. A wide variety of different chemicals are added for the flavor and aroma. As the usage of e-cigarettes requires heating the e-liquid, a variety of new chemical compounds can be produced, and many of them can be considered harmful.

The very first tests on the chemical composition of e-cigarette liquids were performed way back in 2008 by Health New Zealand Ltd. (Laugesen, 2008). After one year, the Food and Drug Administration Agency (FDA) was asked to quantitate the amount of nicotine and impurities in two brands of e-cigarette cartridges, which highlighted the presence of low amounts of nicotine in liquids labeled as 'no nicotine'. The same report showed the presence of toxic compounds as impurities, one of them being diethylene glycol. The presence of tobacco-specific nitrosamines, potent human carcinogens, was also highlighted (IARC Monogr., 2007). Moreover, some studies reported compounds such as formaldehyde, acetaldehyde, and acrolein in small amounts (Etter et al., 2013; Laugesen M., 2008). In contrast, others reported the presence of other volatile organic compounds such as benzene, toluene, xylene, and styrene (Etter et al., 2013; FDA. Laboratory analysis of electronic cigarettes conducted by FDA. 2009; Goniewicz et al., 2014b). Although e-cigarettes contain various metal components, many metals have been found in the e-liquids and aerosol. More recent data has shown that differences between nicotine content and labels are more minor than previously reported, suggesting an improvement in manufacturing (Davis et al., 2014; Etter et al., 2013).

Nicotine and tobacco-specific nitrosamines

Nicotine is a highly addictive central and peripheral nervous system stimulant with a lethal dose estimated to be 0.8-1.0 *mg/kg* of body weight in adult nonsmokers (Etter et al., 2011; Yildiz et al., 2004). Because it is both toxic and addictive, it is essential that its content in refill liquids and cartridges comply with the label (Davis et al., 2014). Nicotine used for e-liquid production is extracted from tobacco. Its extraction, however, can be followed by the production of some tobacco-specific nitrosamines (TSNAs), such as N-nitrosonornicotine, 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone, N-nitrosoanatabine and N-nitrosoanabasine. They are believed to be highly toxic (FDA. Laboratory analysis of electronic cigarettes conducted by FDA. 2009). Other impurities that can be found are minor alkaloids like nornicotine, anatabine, anabasine, myosmine, cotinine, nicotine-N'-oxides (*cis* and *trans* isomers), β-nicotyrine and β-nornicotyrine (Etter et al., 2013). Nicotine and cotinine in tobacco are mainly present as the levorotary *(S)*-isomers (only 0.1%–0.6% of total nicotine content is *(R)*-nicotine). In contrast, anabasine, anatabine, and nornicotine in tobacco exist as a mixture of enantiomers (Omaiye et al., 2020) According to European Pharmacopeia, nicotine of pharmaceutical grade may, as raw material, contain up to 0.3% of each of the specified nicotine impurities. However, since the e-liquid manufacturing process is not strictly controlled, some products can show impurities above these acceptance limits for pharmaceutical products. For both compound types, methods such as HPLC-DAD, GC-MS, and LC-MS/MS have shown reliable results and can be used for regular content assessment.

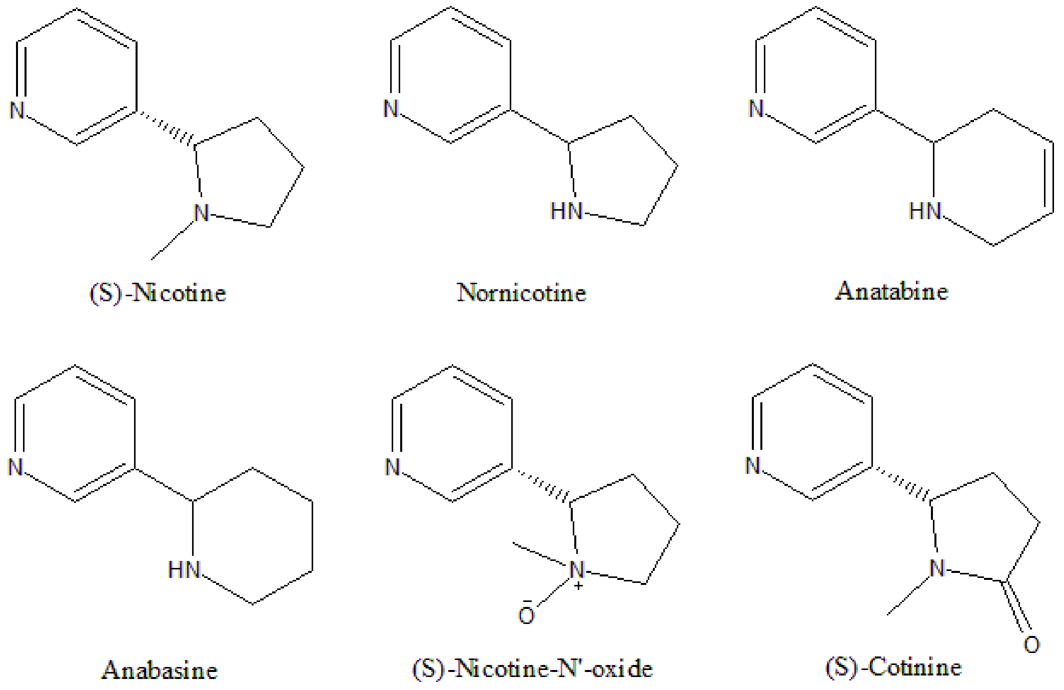

Figure 1. Structures of nicotine and nicotine related compounds that are most abundant in e-liquids.

Glycols used as humectants

E-liquids, containing propylene glycol, vegetable glycol, or a combination of both, are designed to generate aerosol upon heating (Laugesen, 2008). Both glycols have been marked as ’generally safe’ by the FDA and GRAS, making them approved solvents 59oru se in pharmacy. This safety reassurance should leave the audience with a sense of security about the composition of e-liquids. Vegetable glycol is derived from plant oils, while propylene glycol is prepared by hydrolysis of propylene oxide under pressure at high temperatures. Unlike inhalers or nebulizers, which can use the mentioned glycerols, e-cigarettes consist of a heating component, and the heating of glycols can generate various potentially toxic carbonyl compounds. One of the tests for propylene glycol identification in pharmaceutical products requires using a GC/FID method, and the reported chromatographic parameters can also be used to determine propylene glycol content on e-liquids. Chromatographic separation was achieved using a column coated with 14% cyanopropylphenyl-86% methylpolysiloxane stationary phase. Additionally, these components can be easily identified by ^{1}H NMR spectroscopy after purification and confirmed by GC-MS. Rest assured, these components are safe 59oru se in e-liquids.

Volatile organic compounds and carbonyls

Volatile organic compounds are a group of various compounds usually recognized as harmful to human health. They have high vapor pressure, low-to-medium water solubility, and low molecular weight (Moran et al., 2006). Published results (Laugesen, 2008; Lim et al., 2013; Uchiyama et al., 2013) demonstrated the presence of small amounts of formaldehyde and acetaldehyde in some cartridge liquids. Acetaldehyde may occur in some liquids because of its intentional addition as a flavor compound. The International Agency for Research on Cancer [IARC] classified formaldehyde as carcinogenic to humans (group 1) and acetaldehyde as possibly carcinogenic to humans (group 2B). Acrolein can irritate the nasal cavity and damage the lining of the lungs. Benzene (group 1 by IARC) and other solvents (toluene, xylenes, and styrene) could be present in e-liquids because of their use as solvents for nicotine extraction from tobacco leaves (Etter et al., 2013). Other volatile organic compounds in the liquid phase produce aromas and flavor through heating (Bahl et al., 2012). For analysis of these compounds, GC-MS can use the Environmental Protection Agency (EPA) method 826030. The chemical composition of aerosol can be different from liquid: using e-cigarettes requires heating the liquid, and under such conditions, chemical reactions may result in the formation of new compounds. In some models, the temperature measured in the center of the heating coil can be notably high (≥ 350 °C), promoting pyrolysis reactions of e-liquid chemical components (Moran et al., 2006). Many studies report that short-chain aldehydes (formaldehyde, acetaldehyde, and acrolein) are formed under the heating of e-liquids (Etter et al., 2013; Laugesen, 2008; Schober et al., 2013, Uchiyama et al., 2020). Uchiyama et al. (2013) demonstrated that 70% of examined e-cigarette brands generated formaldehyde, acetaldehyde, and acrolein with maximum concentrations of 260, 210, and 73 mg/m^3, respectively. They also detected two toxic carbonyl compounds, glyoxal, and methylglyoxal, which, until now, have not been detected in the smoke from tobacco cigarettes.

Heavy metals

Studies concerning the quantification of metals in refill liquids show that their content doesn't exceed limits that harm human health (Burstyn et al., 2014). In Ruyan's report (Laugesen, 2008) cartridge liquids were tested for heavy metals. Still, none of them was found at a concentration higher than 0.1–0.2 ppm. Since e-cigarettes contain various metal components, metals can migrate to the generated aerosol, constituting a health risk to users and bystanders. Williams et al. (2013) showed that small particles comprised of various elements (Sn, other metals, semimetals, and silicate) passed through cartomizer fibers and were present in the aerosol of e-cigarettes. A total of 22 elements were identified in aerosol, including Pb, Ni, and Cr. Pb and Cr concentrations in aerosol were within the range of conventional cigarettes (Zhao et al., 2023). In contrast, Ni concentration was about 2–100 times higher in e-cigarette aerosol than in Marlboro brand cigarettes. Ni particles likely originated from the nichrome wire. Significant amounts of Sn, other metals, and silicate beads escaped into an aerosol. They would result in human exposure, which, in some cases, is probably more remarkable than those observed in a conventional cigarette. All of the mentioned metals were determined through validated ICP-OES or ICP-MS methods (Laugesen, 2008; Williams et al., 2013.).

Table 1. Published and approved analytical methods for various components of e-liquids analysis

Analytes / classes of analytes	Matrices	Analytical techniques	References
Nicotine	Refill liquid	GC/FID, HPLC/DAD	Schober et al., 2013; Davis et al., 2014.
	Cartridge[a]	GC/FID, HPLC/UV	Cheah et al., 2014; Westenberger et al., 2009.
Nicotine and nicotine related compounds	Cartridge, aerosol	GC-TSD	Goniewicz et al., 2014a,b.
	Cartridge	HSGC-MS	Westenberger et al., 2009
	Cartridge[a], refill liquid, aerosol	HPLC/DAD	Trehy et al., 2011.
Tobacco specific nitrosamines	Cartridge[a]	LC-MS/MS	Laugesen , 2008; Westenberger et al., 2009
	Refill liquid	LC-MS/MS	Schober et al., 2013; Kim et al., 2013.
Propylene glycol	Refill liquid	GC/FID (GC/MS[b])	Etter et al., 2013.
Volatile organic compounds	Refill liquid	GC-MS (^{1}H NMR[b])	Schober et al., 2013
Carbonyl compounds	Refill liquid	HS-SPME GC-MS[c]	Lim et al., 2013.
	Aerosol	HPLC/DAD[c]	Goniewicz et al., 2014a,b; Kosmider et al., 2014.
Heavy metals	Cartidge[a]	ICP-MS	Laugesen , 2008
	Aerosol	ICP-MS, ICP-OES	Goniewicz et al., 2014b; Williams et al., 2013

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[a] It requires extraction with organic solvent; [b] Confirmatory method; [c] Derivatization step previously

Conclusion

Electronic cigarettes (e-cigarettes) are a complex technological alternative to traditional tobacco products, offering potential harm reduction benefits due to the absence of combustion. However, the safety of e-cigarettes remains controversial, with concerns over manufacturing standards, impurities, and possible harmful byproducts. E-liquids, composed primarily of nicotine, glycols, and flavor additives, can contain toxic substances such as tobacco-specific nitrosamines, carbonyl compounds, volatile organic compounds, and heavy metals. While some improvements in product consistency and manufacturing quality have been noted, significant variability still persists. It is crucial to assess the composition and potential risks associated with e-cigarettes using comprehensive chemical analyses and advanced techniques, such as GC-MS, LC-MS/MS, and ICP-MS. This emphasis on vigilance is key to understanding the health implications of e-cigarettes and ensuring the safety of their users

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Otkrivanje hemije e-cigareta: šta je unutar vašeg vejpa?

Tekući naslov: Hemijski sastav e-cigareta

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Sažetak

Pušenje duvana predstavlja jednu od najštetnijih navika po zdravlje, izazivajući, pored zavisnosti, i niz somatskih tegoba u rasponu od alergijskih reakcija do kardiovaskularnih bolesti i raka pluća. U poslednje vreme elektronske cigarete (e-cigarete) se sve više promovišu kao zdravija alternativa pušenju. Iako se e-cigarete plasiraju kao sigurnija alternativa tradicionalnim duvanskim proizvodima, sastav e-tečnosti, supstanci koje se udišu, zahtevaju kritičku procenu. E-tečnosti obično sadrže nikotin, propilen glikol, glicerol, arome i druge aditive. Međutim, studije su identifikovale štetne sastojke, uključujući nitrozamine specifične za duvan (TSNA, grupa kancerogenih jedinjenja koja se nalaze u duvanu i duvanskom dimu), isparljiva organska jedinjenja (VOC), teške metale i karbonilna jedinjenja, koja se mogu formirati tokom zagrevanja.

Nikotin, primarna komponenta većine e-tečnosti, nosi rizik od stvaranja zavisnosti i šteti kardiovaskularnom sistemu. TSNA i VOC, čak i u tragovima, poznati su kancerogeni.Zabrinutost izaziva i prisustvo teških metala (olova, kadmijuma i nikla) koji se oslobađaju iz komponenti e-cigareta.

Važno je napomenuti da, kada se udišu, agensi za ukus, koji se često smatraju bezbednim za gutanje, mogu imati štetne respiratorne efekte, što naglašava potrebu za oprezom.

Uprkos njihovoj popularnosti, dugoročni uticaji e-cigareta na zdravlje ostaju uglavnom nepoznati, što komplikuje procene javnog zdravlja. Raznovrsni hemijski sastav e-tečnosti i alarmantan nedostatak strogih standarda kvaliteta izazivaju dodatnu zabrinutost. Ovaj pregled naglašava hitnu potrebu za sveobuhvatnim istraživanjem komponenti e-tečnosti i njihovih potencijalnih efekata na zdravlje. Zakonski okviri treba da uvedu obavezne deklaracije o sastojcima i bezbednosti proizvoda kako bi se smanjili rizici povezani sa upotrebom e-cigareta. Usled rastuće popularnosti e-cigareta, pored informisanja potrošača, od suštinskog je značaja usmeravanje kreatora politike na otklanjanje ovih nedostataka radi zaštite javnog zdravlja.

Ključne reči: *elektronske cigarete, nikotin, nitrozamini specifični za duvan, isparljiva organska jedinjenja, karbonili, teški metali*

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Presentation of activities in the Erasmus+ project: "Master degree in integrating innovative STEM strategies in higher education"

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ABSTRACT

The integration of STEM (Science, Technology, Engineering, and Mathematics) in higher education is a key priority for preparing future educators to address interdisciplinary challenges and support sustainable development. This article presents the goals, structure, and early-stage achievements of the Erasmus+ project *Master Degree in Integrating Innovative STEM Strategies in Higher Education*. The project aims to design a transnational master's program focused on innovative STEM methodologies by leveraging international collaboration among universities and experts across Europe.

To date, the project has completed its foundational stages, including national reports on the state of STEM education, a transnational comparative analysis, a joint strategic framework, and the launch of a dedicated digital platform. The methodology involves six specialized working groups, addressing areas such as curriculum development, digitalization, sustainability, professional development, and educational policy.

The project emphasizes inquiry-based and project-based learning approaches, supported by emerging digital tools such as virtual and augmented reality and interactive online simulations. Sustainability principles are being integrated into the educational model, with a focus on green technologies and climate awareness. These efforts lay the groundwork for the forthcoming development of the joint curriculum and digital resources. By fostering cross-border knowledge exchange and aligning educational practices with future workforce needs, the project contributes to the modernization and internationalization of STEM education in higher education.

Keywords: *STEM education, Erasmus+, innovative teaching, higher education, digital learning, sustainability*

Introduction

The rapid advancement of science, technology, engineering, and mathematics (STEM) fields has fundamentally reshaped societal structures and economic landscapes, making STEM education an essential driver for innovation, competitiveness, and sustainable development (National Science Board, 2018). In contemporary higher education, the integration of STEM disciplines through interdisciplinary approaches fosters critical thinking, creativity, and problem-solving skills that are crucial for addressing complex global challenges (Honey, Pearson, & Schweingruber, 2014). Despite its recognized importance, STEM education still faces significant challenges, including gender disparities, limited accessibility, insufficient incorporation of digital technologies, and a gap between theoretical knowledge and practical application (Marginson, Tytler, Freeman, & Roberts, 2013; UNESCO, 2017).

Innovative pedagogical strategies are increasingly viewed as critical tools to revitalize STEM education and enhance student engagement. Inquiry-based learning, project-based learning, and problem-solving methodologies have demonstrated positive effects on learners' motivation and academic achievements (Hmelo-Silver, Duncan, & Chinn, 2007; Freeman et al., 2014). These approaches encourage active participation, deeper understanding, and the development of transferable skills. Moreover, the incorporation of digital technologies, such as virtual laboratories, augmented reality (AR), and online simulations, offers flexible and immersive learning environments that bridge physical limitations and democratize access to quality education (Makransky, Terkildsen, & Mayer, 2019).

One of the critical aspects of contemporary STEM education is its alignment with sustainability goals. The integration of sustainability principles within STEM curricula fosters environmental stewardship, social responsibility, and the promotion of green technologies (Wals & Corcoran, 2012). Green STEM education emphasizes renewable energy, climate change mitigation, and eco-friendly practices, thereby preparing future professionals to contribute to the United Nations' Sustainable Development Goals (SDGs) (UNESCO, 2017).

The Erasmus+ project "Master Degree in Integrating Innovative STEM Strategies in Higher Education" emerges as a timely initiative addressing these multifaceted challenges. By developing an advanced academic program that incorporates innovative pedagogical techniques, digital tools, and sustainability principles, the project seeks to create a transformative impact on STEM education at the European and global levels. International collaboration among universities enables the sharing of best practices, harmonization of academic standards, and creation of standardized STEM education models that are adaptable across diverse educational contexts (European Commission, 2020).

Bridging the gap between academia and industry is another pivotal dimension of modern STEM education. Employers increasingly demand graduates who are not only knowledgeable but also capable of applying their skills in real-world contexts (Carnevale, Smith, & Strohl, 2013). Embedding case studies, industry projects, and internships within STEM curricula enhances students' readiness for the workforce and nurtures innovation ecosystems (Bok, 2013). Therefore, the Erasmus+ project's emphasis on integrating real-world applications and problem-solving approaches is critical for producing graduates who are capable of driving societal and technological advancements.

Equally important is the focus on professional development for STEM educators. Teachers play a central role in facilitating meaningful learning experiences, and their ongoing professional development is crucial for the successful implementation of innovative teaching strategies (Desimone & Garet, 2015). Professional development programs that focus on STEM content knowledge, pedagogical content knowledge, and digital literacy empower educators to design and deliver impactful instruction (Darling-Hammond, Hyler, & Gardner, 2017).

The digital transformation in education, accelerated by the COVID-19 pandemic, has further underscored the need for flexible, technology-enhanced learning environments. Blended learning models, combining face-to-face instruction with online components, offer resilience and adaptability in times of disruption (Hodges et al., 2020). However, the crisis also underscored challenges related to assessment and grading in remote contexts. To address this, the Erasmus+ project integrates alternative digital assessment methods, including e-portfolios, formative feedback tools, online project-based evaluation, and rubrics specifically designed for virtual environments. These approaches prioritize continuous, competency-based assessment over traditional exams, aligning with the pedagogical emphasis on real-world skills. The project's dedication to developing virtual learning environments and digital resources ensures that both educators and students are equipped to navigate and thrive in a digitally connected world.

Moreover, educational policy and international collaboration are essential for scaling and sustaining innovations in STEM education. Policymakers, institutional leaders, and industry stakeholders must work collaboratively to create an enabling environment that supports curriculum reforms, resource allocation, and accreditation processes (OECD, 2019). Through its dedicated working groups, the Erasmus+ project addresses these systemic dimensions, thereby enhancing its potential for long-term and widespread impact.

The integration of innovative STEM strategies in higher education is not only desirable but necessary for building a future-ready workforce and fostering sustainable societies. By embracing interdisciplinary learning, digital innovation, sustainability principles, real-world applications, and continuous professional development, the Erasmus+ project "Master Degree in Integrating Innovative STEM Strategies in Higher Education" sets a pioneering example. Its collaborative, holistic approach addresses current shortcomings in STEM education and paves the way for a more inclusive, dynamic, and impactful educational paradigm.

What distinguishes this project is its focus on creating a fully integrated, transnational Master’s program in STEM education, grounded in digital innovation, sustainability, and international cooperation. It is among the first initiatives in Eastern Europe to simultaneously address the curriculum gap in STEM teacher training, the integration of green technologies into STEM content, and the alignment of academic programs with industry needs. The project's emphasis on interdisciplinary collaboration, joint strategy building, and early adoption of immersive digital tools reflects a pioneering model with potential for replication across other regions in Europe and beyond.

OVERVIEW OF WORKING GROUPS (WG) INSIDE THE ERASMUS+ PROJECT

WG1: Curriculum Development and Innovative Teaching Methods

This working group focuses on the design and structuring of the master's program curriculum, emphasizing innovative STEM teaching strategies. The objective is to integrate cutting-edge pedagogical techniques, such as inquiry-based learning, project-based approaches, and problem-solving methodologies, into STEM education. This WG is responsible for developing syllabi, learning outcomes, and assessment methods tailored to STEM disciplines.

WG2: Digital Tools and Virtual Learning Environments

The primary aim of this group is to incorporate digital technologies into STEM education. This includes the development of virtual and augmented reality applications, interactive simulations, and online platforms to support distance learning. The WG collaborates with IT experts to design digital learning resources and train educators in their implementation.

WG3: Sustainable Development and Green STEM Approaches

Sustainability in STEM education is a core focus of this working group. It explores the integration of environmental and sustainability topics into STEM curricula. The team investigates best practices for teaching sustainability, including renewable energy, climate change mitigation, and eco-friendly laboratory experiments.

WG4: Educational Policy and International Collaboration

This working group addresses policy-related aspects of STEM education at the national and European levels. It aims to align the master's program with international educational standards and accreditation frameworks. Additionally, the WG fosters partnerships with other universities, policymakers, and industry leaders to enhance the global impact of the project.

WG5: Professional Development for STEM Educators

Ensuring that educators are well-equipped with the necessary skills and knowledge is critical for the success of the project. This group designs and delivers professional development courses, workshops, and training sessions for teachers, providing them with innovative STEM teaching strategies and digital literacy skills.

WG6: Coordination, Dissemination, and Stakeholder Engagement

The dissemination of project outcomes is essential for maximizing its impact. This working group is responsible for maintaining the project website, publishing newsletters, organizing international conferences, and engaging stakeholders, including policymakers, educators, and industry representatives. The goal is to ensure widespread adoption of the project's findings and best practices.

RISK ASSESSMENT AND MITIGATION

While the Erasmus+ project "Master Degree in Integrating Innovative STEM Strategies in Higher Education" is progressing steadily, several potential risks have been identified that could affect implementation. These risks, however, are being proactively monitored and mitigated by the project management and partner institutions.

One of the primary risks concerns delays in curriculum development due to the complexity of aligning academic standards across four international partners. To address this, the consortium has agreed on a clear work breakdown structure, strict internal deadlines, and regular virtual coordination meetings to ensure timely task completion.

A second risk involves the varying levels of digital readiness among academic staff across institutions, which could limit the uptake of advanced digital tools. This is being mitigated through targeted professional development activities and ongoing IT support provided by digitally advanced partners.

Lastly, limited dissemination or engagement with target groups may affect the project's long-term impact. To counter this, the project team is investing in multilingual dissemination strategies, early stakeholder engagement, and integration with ongoing institutional initiatives.

By identifying and addressing these risks at an early stage, the project aims to ensure successful completion of its objectives and sustainability of its outcomes beyond the project lifecycle.

ACHIEVED RESULTS UP TO NOW IN THE ERASMUS+ PROJECT

The Erasmus+ project "Master Degree in Integrating Innovative STEM Strategies in Higher Education" has successfully completed analysis and strategy development. The main achievements include:

1. Establishment of the official project website, providing open-access updates, background information, and downloadable materials for educators and institutional stakeholders.
    - Website: https://stem-md.swu.bg/
2. Development of national reports on the status of STEM education in each partner country, providing a comparative foundation for curriculum planning.
3. Completion of a transnational synthesis report and the drafting of a joint strategic framework for the master’s degree program.
4. Partnership-building and collaboration with the following institutions:
    - South-West University "Neofit Rilski" (Bulgaria, coordinator)
    - Trakya University (Turkey, partner)

    - University of Niš (Serbia, partner)
    - Tashkent Institute of Chemical Technology (Uzbekistan, partner)

5. Launch of the project's social media channels, used to share updates, events, and dissemination materials:

    - Facebook: https://www.facebook.com/61576844852816

6. Ongoing dissemination efforts, including presentations at academic conferences, newsletters, and internal institutional publications to promote project results.

These outputs provide the groundwork for the development of the joint master's curriculum, digital learning tools, and sustainability-focused teaching resources in the upcoming project phases.

COLLABORATIVE EXPERIENCE AND RECOMMENDATIONS FOR ERASMUS+ PARTICIPATION

The project's success thus far reflects the strong interdisciplinary collaboration among academic experts, institutional leaders, and policy stakeholders dedicated to advancing STEM education. The involvement of higher education institutions, industry representatives, and decision-makers has ensured that the emerging master's program responds to the evolving demands of the education sector. Participation in the Erasmus+ initiative has created valuable opportunities for international networking, mutual learning, and the exchange of innovative pedagogical practices. Active contributions within the project's working groups - particularly in curriculum development, digital learning strategies, and sustainable STEM integration - have fostered the incorporation of modern teaching methods. Regular online meetings, partner workshops, and knowledge-sharing sessions continue to support the co-creation of a forward-looking, interdisciplinary academic program.

Conclusion

The Erasmus+ project *"Master Degree in Integrating Innovative STEM Strategies in Higher Education"* represents a transformative initiative aimed at modernizing STEM education through interdisciplinary collaboration, digital innovation, and sustainability. Although still in its early stages, the project has already achieved significant milestones, including the completion of national and transnational reports, the development of a joint strategic framework, and the establishment of a dedicated online platform for dissemination.

What sets this project apart is its complete and future-oriented approach to designing a master's program that integrates cutting-edge pedagogical strategies, such as inquiry-based and project-based learning, with digital learning environments and green STEM principles. The structure of six interrelated working groups ensures that the curriculum development process is comprehensive, inclusive, and aligned with both academic standards and industry expectations.

The project's long-term vision is to establish a replicable European model for STEM teacher training that bridges the gap between theory and practice. The integration of sustainability, gender inclusivity, and digital readiness into the core of STEM higher education has the potential to create a significant impact at both institutional and policy levels. Through sustained collaboration and knowledge exchange among partner institutions, the project makes a meaningful contribution to the broader goals of the European Education Area.

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Conflict-of-interest statement

The author declares that she has no conflict of interest.

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Prezentacija aktivnosti u okviru Erazmus+ projekta: Master studije integracije inovativnih STEM strategija u visokom obrazovanju

Tekući naslov: Inovativne STEM strategije u visokom obrazovanju

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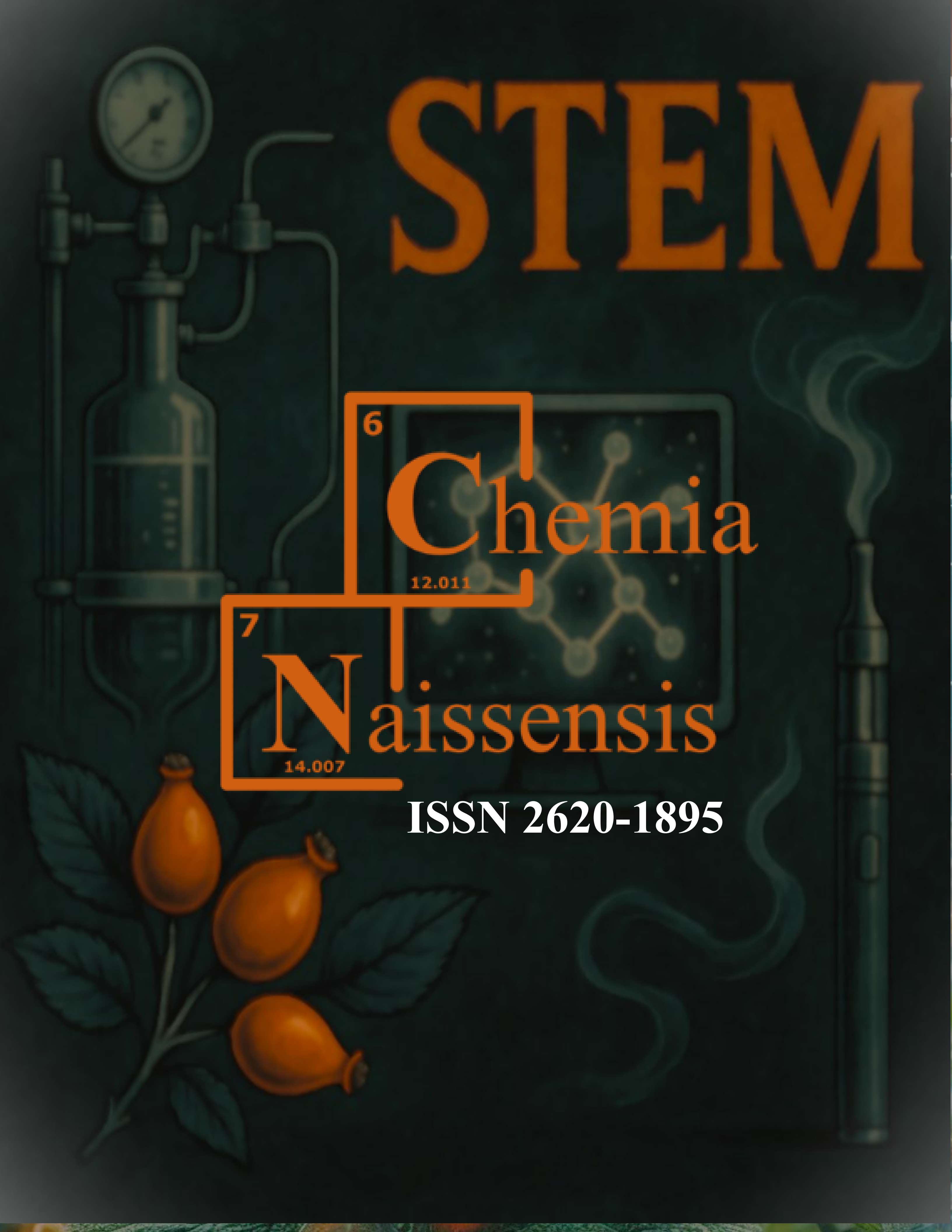
Sažetak

Integracija STEM obrazovanja (nauka, tehnologija, inženjerstvo i matematika) u visoko obrazovanje je ključni prioritet za pripremu budućih edukatora za rešavanje interdisciplinarnih izazova i podršku održivom razvoju. Ovaj članak predstavlja ciljeve, strukturu i dostignuća u ranoj fazi Erazmus+ projekta Master studije integracije inovativnih STEM strategija u visokom obrazovanju. Cilj projekta je da se putam saradnje među univerzitetima i stručnjacima širom Evrope osmisli transnacionalni master program usmeren na inovativne STEM metodologije.

Do danas je projekat završio svoje osnovne faze, koje uključuju nacionalne izveštaje o stanju STEM obrazovanja, transnacionalnu komparativnu analizu, zajednički strateški okvir i pokretanje namenske digitalne platforme. Metodologijom je predviđen rad šest specijalizovanih radnih grupa, koje se bave oblastima kao što su razvoj kurikuluma, digitalizacija, održivost, profesionalni razvoj i obrazovna politika.

Projekat naglašava pristupe učenju zasnovane na istraživanju i projektima, uz podršku novih digitalnih alata, kao što su virtuelna i proširena stvarnost i interaktivne onlajn simulacije. Principi održivosti se uključuju u obrazovni model, fokusirajući se na zelene tehnologije i svest o klimi. Ovi napori postavljaju temelje za predstojeći razvoj zajedničkog kurikuluma i digitalnih resursa. Podsticanjem prekogranične razmene znanja i usklađivanjem obrazovnih praksi sa budućim potrebama radne snage, projekat doprinosi modernizaciji i internacionalizaciji STEM obrazovanja u visokom obrazovanju.

Ključne reči: *STEM obrazovanje, Erazmus+, inovativna nastava, visoko obrazovanje, digitalno učenje, održivost*



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