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Supervisor:

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Sveučilište u Zagrebu

Prehrambeno-biotehnološki fakultet

Marinela Nutrizio

UTJECAJ PRIMJENE ZELENIH OTAPALA I VISOKONAPONSKOGA ELEKTRIČNOGA PRAŽNJENJA NA EKSTRAKCIJU BIOAKTIVNIH SPOJEVA IZ ODABRANOGA SREDOZEMNOGA BILJA

DOKTORSKI RAD

Mentor:

prof. dr. sc. Anet Režek Jambrak

Zagreb, 2024.

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Impact of high voltage electrical discharges and green solvents in extraction of bioactive compounds from selected Mediterranean herbs

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Anet Režek Jambrak, Ph.D., Full professor (the University of Zagreb, Faculty of Food Technology and Biotechnology, Laboratory for Sustainable Development)

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IMPACT OF HIGH VOLTAGE ELECTRICAL DISCHARGES AND GREEN SOLVENTS IN EXTRACTION OF BIOACTIVE COMPOUNDS FROM SELECTED MEDITERRANEAN HERBS

Marinela Nutrizio, *MSc*

Thesis performed at the Faculty of Food Technology and Biotechnology.

Supervisors: Anet Režek Jambrak, Ph.D., Full professor

Abstract: The aim of the study was to investigate a sustainable food extraction method high voltage electrical discharge (HVED) with green solvents for the extraction of bioactive compounds from Mediterranean herbs rosemary and oregano. The theoretical predictive tools were used to assess the solubility of certain bioactive compounds in green solvents, to reduce solvent consumption during experimentation. Results showed that HVED is a highly efficient green extraction method that can be a sustainable alternative to conventional extraction methods. The obtained extracts were stabilized using microencapsulation which was presented as a valuable tool for preserving polyphenol stability in aqueous extracts, contributing to the development of functional foods with customizable properties.

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Marinela Nutrizio, mag. nutr.

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Sažetak: Cilj istraživanja bio je istražiti održivu metodu ekstrakcije hrane visokonaponskim električnim pražnjenjem (HVED) sa zelenim otapalima za ekstrakciju bioaktivnih spojeva iz mediteranskog bilja ružmarina i origana. Teorijski alati za predviđanje korišteni su za procjenu topljivosti određenih bioaktivnih spojeva u zelenim otapalima, kako bi se smanjila potrošnja otapala tijekom eksperimentiranja. Rezultati su pokazali da je HVED vrlo učinkovita metoda zelene ekstrakcije koja može biti održiva alternativa konvencionalnim metodama ekstrakcije. Dobiveni ekstrakti stabilizirani su pomoću mikrokapsulacije koja se pokazala kao vrijedan alat za očuvanje stabilnosti polifenola u vodenim ekstraktima, pridonoseći razvoju funkcionalne hrane s prilagodljivim svojstvima.

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First and foremost, I would like to express my deepest appreciation to my supervisor prof. Anet Režek Jambrak for the opportunity to be part of her project. I want to thank her for all the effort, shared knowledge, support, guidance, and encouragement throughout this scientific adventure. I am very grateful for her immeasurable contribution she made to my development. I would also like to thank her for the opportunity for numerous collaborations and conferences abroad, these experiences have not only enriched my academic knowledge but have also led to unforgettable adventures and new friendships in my life.

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Thanks to my family and friends for being by my side from the day I chose what I wanted to study until the completion of my Ph.D. I want to express my deepest gratitude to all my loved ones who had support and believed in my abilities. Your encouragement played an integral role in my accomplishments. To my mom, dad, and Karlo: Thank you for raising me to be a good person, to enjoy life, for having faith in me, and thank you for teaching me to believe in myself!

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I want to raise a toast to everyone who has been a part of this incredible journey with me.

Extended Abstract

The growing global population presents a substantial challenge to the world's food production systems, prompting the need for sustainable food processing methods. Furthermore, the global market has a significant demand for natural and functional foods, incorporating bioactive compounds (BACs) sourced from natural origins. These phytochemicals found in various foods play a crucial role in regulating metabolic processes, contributing to overall health improvement. BACs from the Mediterranean and aromatic plants like rosemary and oregano exhibit antioxidant properties crucial for preventing various health conditions. The initial and crucial step in obtaining BACs involves extraction, where plant material is combined with a suitable solvent. The conventional thermal extraction methods rely on high temperatures, and face challenges in terms of energy efficiency and waste management. For that reason, research in food technology is oriented towards innovative nonthermal extraction methods as efficient alternatives to conventional thermal methods. This study aimed to determine the efficiency of high voltage electrical discharge (HVED) as a nonthermal extraction technology for the extraction of BACs from rosemary and oregano, in combination with green solvents. Theoretical predictive tools, including Hansen solubility parameters and COSMO–RS, were utilized to evaluate the solubility of BACs from rosemary and oregano in various green solvents. The results demonstrated the potential of these tools in predicting solvent suitability, aiding in the selection of green solvents for extraction processes. To conduct additional experiments, water, and ethanol at concentrations of 25 % and 50 % (v:v) were chosen. The study provides insights into the physicochemical changes during the extraction process, emphasizing the nonthermal nature of HVED. Furthermore, HVED proved to be a highly effective method for extracting BACs from rosemary and oregano, demonstrating efficiency in terms of total phenolic content, antioxidant capacity, and individual concentrations of volatile and non-volatile BACs. The obtained extracts, rich in BACs, were found to meet safety standards for human consumption concerning pesticide and metal levels, supporting their use as dietary supplements. Furthermore, the life cycle assessment of HVED provided a comprehensive understanding of its environmental, economic, and social aspects, positioning it as a sustainable alternative to conventional extraction methods. In conclusion, HVED stands out as a green extraction technology with high extraction yields for BACs from rosemary and oregano. Its effectiveness, coupled with its sustainability, positions it as a promising technique for various industries, including food and pharmaceuticals. However, the obtained extracts demand stabilization to be used for functional food. Therefore, microencapsulation was

employed as an innovative technology that addresses the stability issues associated with polyphenols extracted from plants. The study demonstrates that microencapsulation, performed at room temperature using the ionic gelation method, effectively preserves the stability of polyphenols in aqueous rosemary extracts obtained by HVED. Various encapsulation coatings, including sodium alginate, zein, and hydroxypropyl methylcellulose, influence the physicochemical properties of microparticles, offering controlled release under different conditions. The study highlights microencapsulation as a valuable tool for preserving polyphenol stability in aqueous extracts, contributing to the development of functional foods with customizable properties.

Keywords: *high voltage electrical discharge, rosemary, oregano, bioactive compounds, green solvents, sustainability, microencapsulation*

Prošireni sažetak

Rastuće svjetsko stanovništvo predstavlja značajan izazov za svjetske sustave proizvodnje hrane, potičući potrebu za održivim metodama obrade hrane. Globalno tržište također ima značajnu potražnju za prirodnom i funkcionalnom hranom koja sadrži bioaktivne spojeve prirodnog porijekla. Ove fitokemikalije koje su prisutne u raznim namirnicama igraju ključnu ulogu u regulaciji metaboličkih procesa, pridonoseći ukupnom poboljšanju zdravlja. Bioaktivni spojevi iz mediteranskih i aromatičnih biljaka poput ružmarina i origana, pokazuju antioksidativna svojstva ključna za prevenciju raznih zdravstvenih stanja. Početni i ključni korak u dobivanju bioaktivnih spojeva uključuje ekstrakciju, gdje se biljni materijal miješa s odgovarajućim otapalom. Konvencionalne metode toplinske ekstrakcije oslanjaju se na visoke temperature i suočavaju se s izazovima poput energetske neučinkovitosti i gospodarenja otpadom. Zbog toga su istraživanja u prehrambenoj tehnologiji usmjerena na inovativne netoplinske metode ekstrakcije kao učinkovite alternative konvencionalnim toplinskim metodama. Cilj ovog istraživanja bio je utvrditi učinkovitost visokonaponskog električnog pražnjenja (HVED) kao netoplinske ekstrakcijske tehnologije za ekstrakciju bioaktivnih spojeva iz ružmarina i origana, u kombinaciji sa zelenim otapalima. Teorijski prediktivni alati, uključujući Hansenove parametre topljivosti i COSMO–RS, korišteni su za procjenu topljivosti bioaktivnih spojeva iz ružmarina i origana u različitim zelenim otapalima. Rezultati su pokazali potencijal ovih programa u predviđanju prikladnosti otapala, pomažući u odabiru zelenih otapala za procese ekstrakcije. Za provođenje daljnjih eksperimenata odabrani su voda i etanol u koncentracijama od 25 % i 50 % (v:v). Rad pruža uvid u fizikalno-kemijske promjene tijekom procesa ekstrakcije, naglašavajući netoplinsku prirodu HVED-a. Nadalje, HVED se pokazao kao vrlo učinkovita metoda ekstrakcije bioaktivnih spojeva iz ružmarina i origana, pokazujući učinkovitost s obzirom na sadržaj fenola, antioksidativni kapacitet i pojedinačne koncentracije hlapljivih i nehlapljivih bioaktivnih spojeva. Utvrđeno je da dobiveni ekstrakti, s visokim udjelom bioaktivnih spojeva, zadovoljavaju sigurnosne standarde za ljudsku prehranu u pogledu razine pesticida i metala, što podržava njihovu upotrebu za dodatke prehrani. Nadalje, procjena životnog ciklusa HVED-a pružila je sveobuhvatno razumijevanje njegovih ekoloških, ekonomskih i društvenih aspekata, predstavljajući ga kao održivu alternativu konvencionalnim metodama ekstrakcije. Nadalje, HVED se ističe kao tehnologija zelene ekstrakcije s visokim prinosima ekstrakcije za bioaktivne spojeve iz ružmarina i origana. Učinkovitost uz održivost, čini HVED obećavajućom tehnologijom za razne industrije, uključujući prehrambenu i farmaceutsku. Međutim, dobiveni ekstrakti zahtijevaju stabilizaciju kako bi se mogli koristiti

kao dodaci u funkcionalnoj hrani. Stoga je korištena metoda mikrokapsulacije kao inovativna tehnologija koja rješava probleme stabilnosti povezane s polifenolima ekstrahiranim iz biljaka. Mikroinkapsulacija provedena na sobnoj temperaturi metodom ionskog geliranja, učinkovito čuva stabilnost polifenola u vodenim ekstraktima ružmarina dobivenim HVED-om. Različiti omotači za inkapsuliranje, uključujući natrijev alginat, zein i hidroksipropil metilcelulozu, utječu na fizikalno-kemijska svojstva mikročestica te imaju mehanizme kontroliranog otpuštanja u različitim uvjetima. U radu je prikazana mikroinkapsulacija kao vrijedan alat za očuvanje stabilnosti polifenola u vodenim ekstraktima, pridonoseći razvoju funkcionalne hrane s prilagodljivim svojstvima.

Ključne riječi: *visokonaponsko električno pražnjenje, ružmarin, origano, bioaktivni spojevi, zelena otapala, održivost, mikroinkapsulacija*

Information about the supervisor – Anet Režek Jambrak, Ph.D., Full Professor

ANET REŽEK JAMBRAK is a tenured full professor at the Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia. She received her Ph.D. in 2008 titled: *Ultrasound effect on the physical and functional properties of whey proteins.* She underwent international training at Coventry University in the UK and the University of Avignon in France. Additionally, she completed training in 2021 at the EIT *Food Executive Academy* and the 2021 Algebra *Mini MBA Digital training*. Since 2019, she has been the head of the Laboratory for Sustainable Development. Her areas of research are nonthermal processing, advanced thermal processing, sustainability, food processing, industry 4.0, digitalization, food chemistry, food physics, etc. In the period since 2007, Anet Režek Jambrak has published over 130 significant scientific papers with more than 6350 citations (h-index 42) and is the author of numerous chapters in books by recognized scientific publishers such as Wiley, Elsevier, Springer, etc. She is the editor of the book *Nonthermal Processing in Agri -Food-Bio Sciences: Sustainability and Future Goals;* Springer Nature Switzerland AG 2022. Anet has been invited to more than 40 conferences as an invited lecturer or keynote speaker. She is the winner of many awards and recognitions: in 2021 she received *the Emerging Sustainability Leader Award* from *MDPI Sustainability Foundation*; In 2019, she was included in the *Highly Cited Researcher list*, among 0.1 % of world scientists (*Web of Science).* In 2009 and 2019, she received state awards for science *from the Parliament of the Republic of Croatia, Ministry of Science and Education, Government of the Republic of Croatia*; In 2016, she was awarded the *Young Scientist Award - International Union of Food Science and Technology* (IUFOST).

Professor Anet Režek Jambrak is the mentor of 3 doctorates; more than 45 diploma theses, more than 30 final theses, and 3 student research theses that were awarded the Rector's Award of the University of Zagreb. She is currently mentoring the preparation of 6 doctorates. She is a member of the *Global Young Academy* (GYA), the *Young Academy of Europe* (YAE); the *European Union for Food Science and Technology* (EFFoST), *the International Academy of Food Sciences and Technology* (IAFoST), and the Executive Committee *of the European Union for Food Science and Technology* (EFFoST). She is currently leading the project of the Croatian Science Foundation: ***Digitization of nonthermal protein extractions from plant by-products and electroforming as an output product,*** and as a partner work package on the project: **PRIMA/HORIZON 2021-2025 – *Functionalized tomato products – FunTomP*.** She was the principal investigator of the project *Extraction of bioactive compounds from Mediterranean plants with "green solvents" using high-voltage electrical discharge – GREENVOLTEX*.

Author's publications included in the doctoral dissertation:

Paper I:

Nutrizio, M., Gajdoš Kljusurić, J., Marijanović, Z., Dubrović, I., Viskić, M., Mikolaj, E., Chemat, F., Režek Jambrak, A. (2020) The potential of high voltage discharges for green solvent extraction of bioactive compounds and aromas from rosemary (*Rosmarinus officinalis* L.) - computational simulation and experimental methods. *Molecules* **25**(16), 3711. doi: 10.3390/molecules25163711

Paper II:

Nutrizio, M., Maltar-Strmečki, N., Chemat, F., Duić, B., Režek Jambrak, A. (2020) High-Voltage Electrical Discharges in Green Extractions of Bioactives from Oregano Leaves (*Origanum vulgare* L.) Using Water and Ethanol as Green Solvents Assessed by Theoretical and Experimental Procedures. *Food. Eng. Rev.* **13**(1), 161-174.

Paper III:

Nutrizio, M., Režek Jambrak, A., Rezić, T., Djekic, I. (2022) Extraction of phenolic compounds from oregano using high voltage electrical discharges - sustainable perspective. *Int. J. Food Sci. Tech.* **57**, 1104-1113.

Paper IV:

Nutrizio, M., Jurić, S., Kucljak, D., Švaljek, S. L., Vlahoviček-Kahlina, K., Režek Jambrak, A., Vinceković, M. (2023) Encapsulation of Rosemary Extracts using High Voltage Electrical Discharge in Calcium Alginate/Zein/Hydroxypropyl Methylcellulose Microparticles. *Foods* **12**(8), 1570. doi: 10.3390/foods12081570

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GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

The exponential growth of the global population has become a significant concern for the world's food production systems. The current food processing industry has a high impact on the environment, such as carbon dioxide (CO_2) emission (Arshad et al., 2022; Jurgilevich et al., 2016). To address this challenge, it is crucial to focus on sustainable food processing methods that minimize waste, reduce energy consumption, and promote efficient resource utilization. Embracing innovative approaches and technological advancements in food processing can help meet the growing demand for food while ensuring long-term sustainability and preserving the planet's resources (Otles et al., 2015).

There is a great demand in the global market for natural and functional foods. Such food contains bioactive compounds (BACs) from natural sources (Granato et al., 2017). BACs are phytochemicals present in various foods, and have the ability to regulate metabolic processes, contributing to improved health. BACs have a wide range of beneficial effects on human health, where antioxidant properties are the most important because they are essential for the prevention of cardiovascular diseases, diabetes, obesity, hypertension, and stimulation of immune responses (Godos et al., 2017; Cuevas et al., 2013). Mediterranean and aromatic plants, such as rosemary and oregano, are an important source of various BACs and essential oils whose medicinal properties have already been medically proven (Roohinejad et al., 2017; Giacometti et al., 2018).

Extraction is the initial and crucial step in obtaining and purifying, wherein the plant material is combined with a suitable solvent. Conventional thermal extraction techniques, such as Soxhlet, heat reflux, and maceration, require high temperatures for management. Despite considerable attempts to enhance heat recovery and reduce water consumption, conventional methods are not considered efficient in terms of energy requirements and waste management. Consequently, in recent decades, much research has been dedicated to exploring alternative nonthermal technologies as more sustainable alternatives (Picart-Palmade et al., 2019). Unlike conventional extractions which rely on heat transfer to achieve desired outcomes, nonthermal extraction methods utilize alternative technologies to preserve food quality, extend shelf life, and ensure safety without the application of high temperatures. Nonthermal food extraction methods offer several advantages over thermal processing. They enable the preservation of nutritional quality, flavor, and texture while achieving microbial safety (Safwa et al., 2023). Additionally, nonthermal techniques can reduce processing time and energy consumption,

resulting in improved operational efficiency and environmental sustainability. These technologies also offer opportunities for the development of minimally processed, fresh-like foods with extended shelf life, meeting the demands of modern consumers for convenient, healthy, and minimally processed food options (Allai et al., 2023; Jadhav et al., 2021). These technologies include various innovative approaches, such as high pressure processing, ultrasonication, high voltage electrical discharge (HVED), supercritical technology, pulsed electric fields (PEF), pulsed light (PL), etc. (Watts, 2022; Chemat et al., 2020).

HVED has emerged as a novel and promising technology for food extractions, offering unique advantages in terms of preserving the quality and BACs of extracted products. The application of HVED in food extractions involves the use of low-temperature and short-duration treatment, minimizing the thermal degradation of sensitive BACs (Coutinho et al., 2021). HVED parameters such as voltage, frequency, treatment time, and gas composition play a crucial role in influencing the efficiency of extractions by affecting the type and concentration of reactive plasma species. This method proves effective in enhancing the extraction of BACs, including antioxidants and essential oils, while simultaneously minimizing the loss of nutritional value and sensory attributes in the extracted food products. Despite its potential benefits, safety considerations, sensory impacts, and regulatory evaluations need careful attention to ensure the successful integration of HVED in food extraction processes (Li et al., 2019; Nutrizio et al., 2020).

Over the past decade, there has been a growing interest in the production of novel food, cosmetic, and pharmaceutical products with natural compounds that preferably contain antioxidants and other properties that are beneficial for human health (Hcini et al., 2021). The most desirable compounds are polyphenols that are generally hydrophilic and are therefore extracted with liquid organic solvents. The obtained aqueous extracts enriched in polyphenols are unstable medium for further processing due to their specific structure. The unsaturated bonds in their molecular structure heighten sensitivity to external environmental factors, including oxidants, light, pH, temperature changes, and enzymatic activities. Consequently, it becomes essential to enhance phenolic compounds' stability and shelf life by protecting them from chemical and physical damage before application (Choudhury et al., 2021).

Microencapsulation is an emerging technology that addresses this challenge by encapsulating sensitive compounds within a protective coating. This innovative approach protects against various processing conditions, ensuring a controlled release under specific conditions.

Furthermore, the use of microencapsulation holds promise for improving the stability of polyphenols and enhancing the final product's food safety and sensory quality (Choudhury et al., 2021; Suganya and Anuradha, 2017).

THEORETICAL BACKGROUND

2. THEORETICAL BACKGROUND

2.1. Sustainability in food technology

Sustainability in food technology has gained special emphasis within the global efforts towards achieving the ambitious targets set forth by Agenda 2030 (UN, 2015c). The Agenda 2030 for sustainable development was adopted at the United Nations Sustainable Development Summit on 25 September 2015, presenting a plan of action for people, plants, and prosperity. Sustainable development is defined as *"development that meets the needs of the present without compromising the ability of future generations to meet their own needs"* (WCED, 1987). The ability of the science and technology communities to jointly address present and future requirements must be improved as part of sustainable development, which connects social, economic, technological, scientific, and environmental challenges through Sustainable Development Goals (SDGs). There are 17 SDGs with 169 targets in total. The SDGs are presented in Figure 1. Each goal represents a critical aspect of fostering a more sustainable and equitable world (UN, 2015b).

Figure 1. UN Sustainable Development Goals (SDGs) (Adopted from UN, 2015a).

Within this Agenda, food technology plays an important role in reshaping the way we produce, consume, and distribute food. Consequently, there is an increased interest in advancing

sustainable development goals due to the close connection between food production and numerous sustainable development goals (Djekic et al., 2021).

Food production and consumption are related to all SDGs. The most important SDG for the food industry is SDG 2 (Zero hunger) to end hunger, achieve food security and improved nutrition, and promote sustainable agriculture. The direct impact is also notable with other SDGs, namely SDG 1 (No poverty), SDG 3 (Good health and wellbeing), SDG 10 (Responsible production), SDG 12 (Responsible consumption and production), SDG 13 (Climate action), SDG 14 (Life below water), and SDG 15 (Life on land). In addition to these direct links, food production also shows broader links with the remaining goals, thus positioning the food industry as a contributor to all the SDGs (Djekic et al., 2021; Grosso et al., 2020; Chandan et al., 2023).

A standard that describes the issue of sustainability and methods for its assessment is ISO 14040 – Life cycle assessment (LCA) (ISO, 2006). LCA plays a crucial role in the sustainability of the food industry. It enables comprehensive analysis of the environmental impact of food products throughout the entire life cycle, from production and processing to distribution, consumption, and disposal. By considering factors such as CO_2 emissions, energy consumption, and resource use, LCA helps identify areas for improvement in the food supply chain (Roy et al., 2009).

In essence, sustainability in food technology, aligned with the LCA and the principles of Agenda 2030, is a transformative force that empowers the global community to build a more sustainable, resilient, and equitable food system. By embracing innovation, collaboration, and a commitment to the SDGs, the food technology sector plays a crucial role in shaping a future where nourishing the planet goes hand in hand with preserving its natural resources and supporting the well-being of all its inhabitants. With this in mind, the food industry is searching for modified and improved processing methods that are more sustainable and within the principles of Agenda 2030 and SDGs (Arshad et al., 2022).

In that context, scientists are searching for innovative green processing methods that overcome the unfavorable effects of conventional processing with the aim of waste reduction, the (re)use of abandoned by-products, and a decrease in the processing-related carbon footprint. Finally, the goal is to find green processing methods that are in line with sustainable development (Chemat et al., 2017).

2.2. Innovative green extraction processes

Since food is a complex mixture of various proteins, carbohydrates, fats, water, and micronutrients such as vitamins, mineral compounds, antioxidants, aromas, etc., the extraction of individual compounds plays one of the most important steps for the food industry. Especially in the creation of new products, such as functional food containing added natural compounds. The extraction processes have a significant negative impact on the environment, need considerable energy inputs, and use a lot of solvents (Giacometti et al., 2018). Mancini et al. (2019) presented a great overview of the impact of extractions on SDGs. It was presented that extractions have a positive and negative impact on SDGs, a positive direct impact on SDGs 6, 8, 9, 13, and 15, and an indirect to SDGs 1, 2, 3, 4, 7, and 17. On the other side, there is a huge adverse impact, including direct to SDG 3, 5, 6, 8, 11, 13, 14, and 15, and indirect to SDG 1, 2, 7, 10, and 16. For that reason, there is a need to find novel or improved extraction methods to overcome the high environmental impact (Mancini et al., 2019; Nutrizio et al., 2022). Innovative methods ought to be financially feasible, ecologically sustainable, and effectively executed. An alternative to the traditional method of extracting compounds from food and/or natural products needs to require less energy, solvent use, and shorter extraction (processing) time to be considered "green" (Giacometti et al., 2018).

Following this concept, Chemat et al. (2012) defined green extraction as *"Extraction based on the discovery and design of extraction processes which will reduce energy consumption, allows the use of alternative solvents and renewable natural products, and ensure a safe and high quality extract/product".* Furthermore, they have developed six principles of green extraction of natural compounds as innovative examples to follow by scientists and to apply by the industry:

- Principle 1: Innovation by selection of varieties and use of renewable plant resources.
- Principle 2: Use of alternative solvents and principally water or agro-solvents.
- Principle 3: Reduce energy consumption by energy recovery and using innovative technologies.
- Principle 4: Production of co-products instead of waste to include the bio-and agro-refining industry.
- Principle 5: Reduce unit operations and favor safe, robust, and controlled processes.
- Principle 6: Aim for a nondenatured and biodegradable extract without contaminants (Chemat et al., 2012).

Various green innovative extraction methods have been investigated with this green extraction approach, especially following Principle 3. Such technologies are also of interest for the industry to control energy usage, cost, process and product safety, quality, and usability. Green extraction methods include advanced thermal and nonthermal technologies (Zia et al., 2022).

Advanced thermal technologies have been developed to increase the efficiency of heat processing while ensuring food safety and avoiding unfavorable effects on foods' nutritional and organoleptic properties. In conventional thermal methods, heat is delivered to the material by convention, conduction, and radiation from the surface to one or more cold points, all of which take a long time. In advanced thermal processing, heat is generated within the product (Kubo et al., 2023). These technologies include ohmic, infrared, and dielectric heating (such as microwave and radio frequency), and all of them except ohmic heating have a basis in the electromagnetic spectrum. These processes have a shorter time of processing compared to conventional heating (Leong and Oey, 2022; Moreno-Vilet et al., 2018). Each technology covers a certain frequency (and thus wavelength) range: the infrared frequency ranges from 60,000 to 150,000 MHz (Aboud et al., 2019), the radio frequency range is from 300 kHz to 300 MHz, and the microwave range is from 300 MHz to 300 GHz (Orsat and Raghavan, 2014).

There are some other advanced thermal technologies, such as subcritical water extraction. Subcritical water extraction involves using high-temperature and high-pressure liquid water existing at temperatures and pressures below its critical point (T_c = 374.15 °C, p_c = 22.1 MPa) to extract target compounds from various solid or semi-solid samples. The unique properties of subcritical water, such as altered dielectric constant, viscosity, and surface tension at elevated temperatures, enhance its ability to solubilize and extract analytes effectively, making it a valuable method for extractions (Cheng et al., 2021).

Nonthermal technologies have been developed to overcome the disadvantages of high temperatures (for extraction) to foods and meet the demand for convenient, high-quality, and minimally processed food products. These technologies include ultrasound-assisted extractions (UAE), high-pressure extraction (HPE), supercritical fluid extraction (SFE), HVED, PEF, accelerated solvent extraction (ASE), enzyme-assisted extractions (EAE), electron-beam extraction, and PL. Each of these techniques offers distinct benefits and applications in the food industry. Nonthermal technologies are generally considered greener than conventional extraction techniques and have higher extraction yields (Zia et al., 2022; Ijod et al., 2022). A detailed overview of nonthermal technologies including their working principle, advantages,

disadvantages, and sustainability is given in Table 1. To fully utilize their benefits for effective, environmentally friendly, and sustainable extraction, advanced thermal and nonthermal extraction technique combinations could be used.

Some nonthermal technologies such as HPE, PEF, SFE, and UAE have already been commercialized at industrial scales, offering advantages such as energy and water savings, minimal environmental impact, and improved product quality. Despite their promise, challenges such as equipment development, parameter control, and regulatory approval interfere with the widespread industrial implementation of these nonthermal technologies, emphasizing the need for further research and optimization for large-scale production (Ali et al., 2021; Zia et al., 2022).

Table 1. An overview of nonthermal technologies for extractions

Nonthermal technology	Working principle	Advantages	Disadvantages	Sustainability	References
UAE	Mechanical energy generated by ultrasound waves is applied to the sample. The sonication results in acoustic cavitation that causes the disintegration of the sample matrix cell wall and release of target compounds to the solvent.	Fast method, low solvent consumption, low power consumption, low cost, simplicity, high extraction yield, and high purity.	Free radical formation outside optimal processing parameters, possible degradation and inactivation of the extracted compound made by ultrasonic waves, without control temperatures may rise, possible erosion of probe tips.	Reduced energy consumption, short processing time, and reduced solvent usage compared to traditional extraction methods.	(Tiwari, 2015; Martins Strieder et al., 2019; Wen et al., 2018)
PEF	Application of short, high-voltage electric pulses to cause membrane permeabilization by electroporation, facilitating the release of	Reduced heat generation (nonthermal process), improved extraction yield, short processing time, low solvent usage, and energy-efficiency.	The initial investment in PEF equipment can be relatively high, structural changes and oxidation of lipid compounds due to electrochemical reactions, PEF does not work on	Reduced energy consumption, short processing time, minimal solvent use, preserved product quality, and extended product shelf life,	(Ricci et al., 2018; Yan et al., 2017; Pateiro et al., 2021)

	intracellular compounds into the surrounding solvent.		conductive or fully insulated media, possible electrode erosion.	reduced waste generation, potential for using renewable energy sources.	
HPE	Physically applied high pressure results in the disruption of the plant tissue, facilitating efficient and rapid extraction of desired compounds from the material.	Short processing time, increased extraction yield, mild extraction conditions (nonthermal), preservation of nutritional qualities of food, the extended shelf life of the product, reduced solvent usage, possible in-package processing.	High cost for investment and maintenance, limited industrial-scale application due to materials limitations, non-uniformity in processing (reproducibility problems), food enzymes and bacterial spores are very resistant to pressure and require very high pressure for inactivation.	The process requires only electric energy, short processing time, reduced waste products, reduced solvent usage.	(Balasubramaniam et al., 2015; Naveena and Nagaraju, 2020)
HVED	Applying electrical pulses to generate plasma leads to electroporation, facilitating the extraction of compounds from the	Rapid extraction, reduced solvent usage, efficiency for various matrices, high extraction yield.	Equipment complexity, the potential for sample degradation, safety concerns due to high-voltage, limited industrial-	Reduced solvent usage, short extraction time, reduced energy consumption, and	(Li et al., 2019; Nutrizio et al., 2022)

	sample through physical and chemical processes.		scale application, free radicals production, and possible electrode erosion.	reduced waste products.	
SFE	Utilizing fluids, usually CO_2, in its supercritical state, as an extraction solvent to selectively dissolve and extract target compounds from a material, based on their relative solubility.	Selectivity, mild operating conditions, minimal residue, low solvent usage, versatility, high extraction yield, low temperatures, and easy recovery.	High investment and operational costs, the complexity of the operation, limited solubility for polar compounds, the moisture content in the supercritical fluid may interfere with extraction.	Minimal waste generation, reduced chemical usage, and low temperatures.	(Ramsey et al., 2009; Uwineza and Waśkiewicz, 2020)
ASE	The application of high pressure maintains the solvent in a liquid state below its critical point and promotes the extraction process.	Short processing time, automated process, high extraction yield, reproducibility, and versatility.	High investment and operational costs, the complexity of the operation, higher extraction yields at high temperatures.	Automation, some ASE systems are designed for the recovery and recycling of solvents.	(Alvarez-Rivera et al., 2019; Zia et al., 2022)
EAE	Catalytic hydrolysis reaction by breaking the plant cell wall under	Low energy consumption, improved extraction yield, easy scale-up, mild	High cost, high time-consuming, specificity of enzymes, enzyme	Low energy consumption, reduced usage of chemicals	(Phong et al., 2018; Majik

	optimal experimental conditions, followed by the release of intracellular components into the extraction medium.	operating conditions, reduced chemical usage and by-product formation, simplicity, safe working conditions, selectivity, and recyclability.	sensitivity to reaction conditions (such as temperature and pH), enzyme stability, immoderate use of solvents.	and toxic substances, recyclability, and waste reduction.	and Gawas, 2023)
Electron-beam extraction	The use of high-energy electron beams to break down and release target compounds from a sample, utilizing the energy transferred to the sample by the electrons.	Rapid extraction, reduced solvent usage, selectivity, precision, and accuracy.	Equipment complexity, limited penetration depth, and potential for sample degradation.	Reduced solvent usage, energy efficiency, and reduced waste.	(Shen et al., 2022; Oks and Brown, 1998)
PL	Utilizing short time pulses of intense light, rich in ultraviolet (UV) light selectively disrupts cell structures and facilitates the extraction of bioactive compounds.	Rapid extraction, minimal heat generation, and potential pathogen inactivation.	High cost investment, limited penetration depth, and potential degradation of light-sensitive compounds.	Reduced solvent usage, energy efficiency, reduced waste, and low temperatures.	(Hossain et al., 2015; Unni and Chauhan, 2019)

2.2.1. High voltage electrical discharge (HVED)

In food research, the application of HVED was mostly focused on food decontamination, food quality enhancement, degradation of toxins, and surface modification of packaging materials (Pankaj and Keener, 2017). In recent years, HVED has become an increasingly popular innovative method for extractions. This method is recognized for its effectiveness as a nonthermal, environmentally friendly extraction technique, falling within the technology of discharge in the liquid phase (Li et al., 2019). It allows for an accelerated extraction rate, and reduced diffusion time and temperature, all achieved with minimal energy input. Water is commonly used as a solvent, and the extraction process is conducted from plant material. HVED in liquid initiates chemical reactions along with physical processes whereby energy is transferred through the plasma channel between two opposite electrodes (Boussetta and Vorobiev, 2014).

Extraction by HVED is an extraction method based on an electrical discharge between two electrodes and the formation of a cold plasma. By using a sufficient potential difference between the electrodes, the gas breaks down into positive ions and electrons, an electric field is created, and a potential difference is established. Cells are introduced into an electric field, which opens the pores of the cell membranes and, consequently, biomolecules are released from the cells, which is called the phenomenon of electroporation (Rajha et al., 2015).

2.2.1.1. Cold plasma

Plasma can be defined as a quasi-neutral, partially or fully ionizing gas consisting of electrons, ions, free radicals, atoms and molecules in the ground or excited state, and UV radiation photons. Along with the solid, liquid, and gas states, plasma is the fourth state of matter existing in the universe (Misra et al., 2011). Creating cold plasma involves supplying particles with external energy through the electrical discharge (ionization of gas) between electrodes connected to an energy source. The resulting electric field attracts electrons to the positively charged electrode, causing collisions between electrons, atoms, and molecules (Fridman, 2009; Kaushik et al., 2019).

Electrons receive energy from the electric field, resulting in numerous collisions with atoms and molecules in the gas. The distinct masses of electrons and particles prevent energy exchange during collisions. Energy exchange between particles of different masses, such as ions with a mass similar to neutral molecules, leads to a complete distribution of energy. Given

the significantly smaller mass of electrons compared to neutral molecules, collisions with neutral molecules involve no energy exchange. Consequently, electrons heat up more than ions, causing the electron temperature in the presence of an electric field to surpass the gas temperature rapidly (Turner, 2016).

In a cold plasma, electron temperatures can range from 10,000 to 100,000 K (1 – 10 eV), while the gas temperature may remain as low as room temperature (Petitpas et al., 2007). This imbalance creates a thermodynamic state known as a non-equilibrium nonthermal plasma or cold plasma. The plasma's color results from electrons returning from the excited state to the ground state, emitting energy in the visible spectrum. The color depends on the type of gas used, with argon, nitrogen, helium, and carbon dioxide being the most commonly used gases in laboratory conditions (Turner, 2016).

The efficacy of cold plasma depends on various factors, which is mostly due to the distinct characteristics of different plasmas and the methods employed for their generation. For instance, the choice of gas utilized in the process plays a key role in determining the type and quantity of reactive species produced through electrical discharge, thereby influencing the efficiency of the treatment. Similarly, the characteristics of the active species generated are contingent upon the frequency and input voltage, with higher values corresponding to increased energy density (Guo et al., 2015). Another process variable impacting the effectiveness of cold plasma treatment is the exposure method, with direct exposure being the preferred approach for process enhancement, as opposed to indirect or remote exposure. The latter reduces the heat transferred to the material due to the self-igniting nature of charged particles and their tendency to recombine before interacting with the sample (Patil et al., 2014).

Another parameter influencing the production of plasma is the electrode configuration. There are different electrode configurations used to generate electrical discharge in liquids: point-to-point, point-to-plate, wire-cylinder, Diaphragm discharge, etc. Figure 2 shows the main proposed electrode configurations. The choice of electrode configuration depends on factors such as the type of plasma desired, the properties of the liquid, and the application requirements. Each configuration has its advantages and limitations, and researchers may select a specific setup based on the characteristics of their intended plasma-liquid system. A point-to-point configuration with a needle is used to inject gas bubbles into the reactor. Furthermore, needle-point electrodes can concentrate the electric field to a very high value (Locke et al., 2012; Magureanu et al., 2018; Palma et al., 2022).

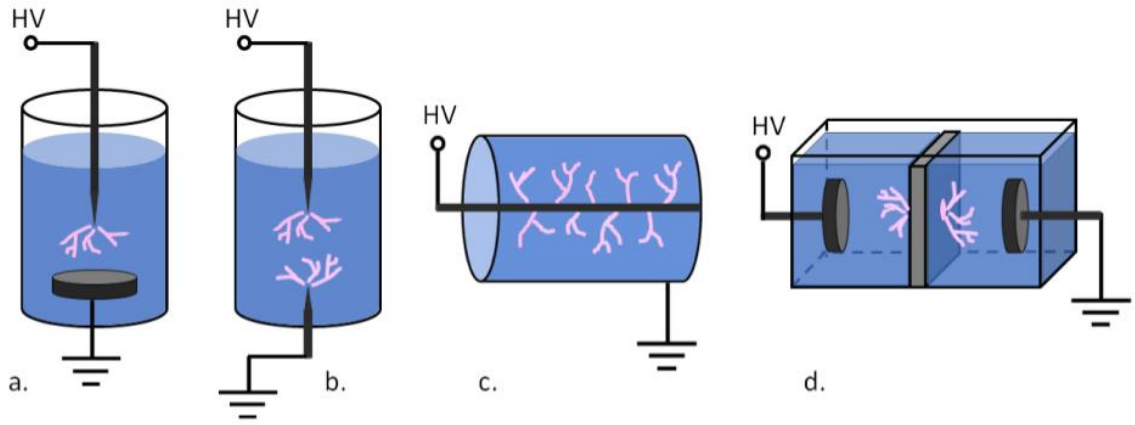

Figure 2. Basic electrode configurations for the generation of electrical discharges in liquids: (a) point-to-plate; (b) point-to-point; (c) wire-cylinder; (d) Diaphragm discharge (Adopted from Magureanu et al. (2018))

2.2.1.2. Electroporation

When a cell is exposed to an electric field, electroporation occurs, wherein the cell membrane pores open, releasing biomolecules. The formation of an electrical discharge occurs in two phases. In the first "streamer" phase, an electrically conductive channel of ionized gas is formed. During the transition from the first to the second phase, the strength of the current increases sharply and an electrical discharge occurs, i.e. a drop in the strength of the current and the release of energy. During the second or "arc" phase, plasma formation and the formation of high-intensity shock waves occur, which can lead to cell damage and the formation of free radicals (Boussetta and Vorobiev, 2014; Li et al., 2019; Žuntar et al., 2019). The relationship of voltage, current, and energy of the system is shown in Figure 3.

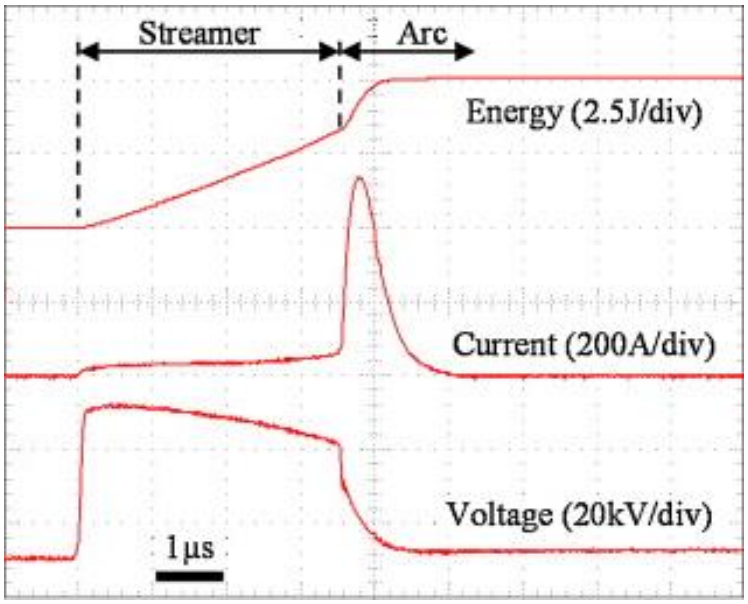

Figure 3. Relationship between voltage, current, and energy during electrical discharge (Adopted from Boussetta and Vorobiev (2014)).

During the "arc" phase, the generation of high-pressure shock waves (90-100 bar) occurs, posing a potential threat to the integrity of biological cells. Simultaneously, this process produces hydroxyl radicals through photodissociation. Following the shock wave formation, refraction waves emerge, initiating the creation of cavitation bubbles containing gas. The collapse of these cavitation bubbles generates secondary shocks, which may result in the weakening and rupture of cellular structures. These phenomena contribute to the macroscopic fragmentation of the treated material and induce turbulence in the liquid, expediting the release of biomolecules from the cells of the biological material and enhancing mass transfer processes. Monitoring the electrical conductivity of the resulting suspension allows for the assessment of damage to the biological material, as the release of bioactive components alters the ionic composition (Boussetta and Vorobiev, 2014).

The electrical discharge in liquid induces damage to cell structures and particle fragmentation, facilitating more efficient and rapid extraction of intracellular components known as electroporation. Consequently, the occurrence of an electrical discharge induced by high voltage electricity will trigger liquid turbulence, the emission of high intensity UV light, the generation of hydrogen peroxide (H_2O_2), and the formation of shock waves and cavitation bubbles (Figure 4) (Boussetta and Vorobiev, 2014).

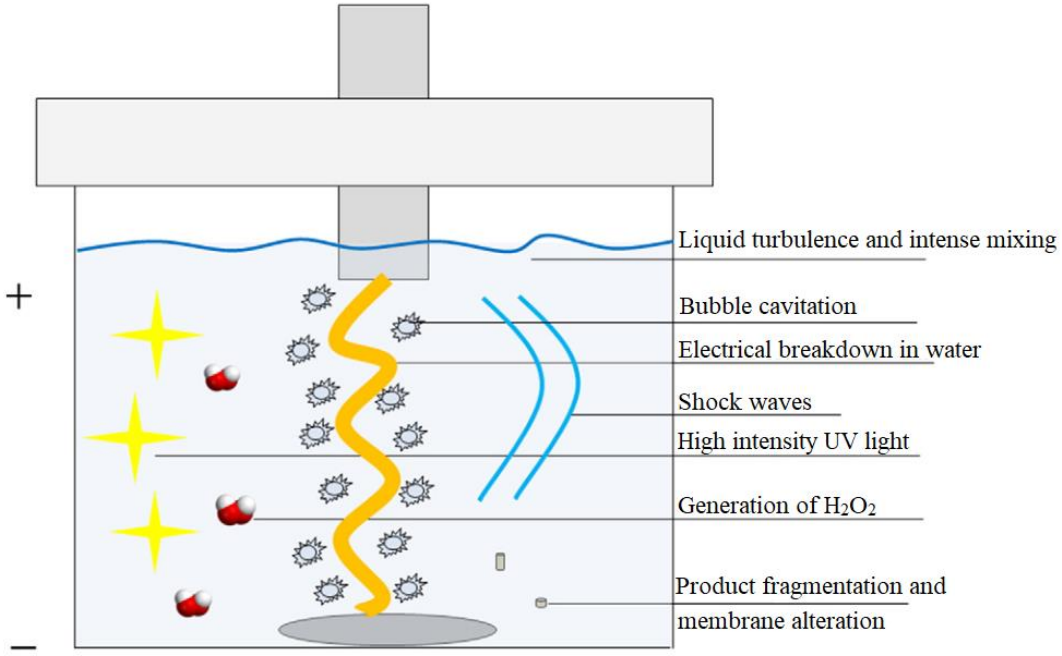

Figure 4. HVED between two electrodes in solution (Adopted from Rajha et al. (2015)).

The application of HVED presents an opportunity to accelerate the extraction of bioactive components from plant material with minimal energy consumption (Lebovka et al., 2012). This nonthermal method has gained attraction in the food industry. Similar to traditional extraction processes, the successful use of HVED for extracting bioactive components relies on carefully chosen extraction conditions (such as voltage, temperature, and time), tailored to the specific material and target component. The energy of sample treatment stands out as a pivotal parameter for optimizing extraction through HVED. The addition of other solvents to water also significantly influences the efficiency of extraction by HVED, as demonstrated by Boussetta et al. (2013) who found a synergistic effect on the yield of total phenolics from flax cake with the addition of ethanol to water.

The HVED was shown to be a suitable approach for extracting phenolic compounds from different plant materials. Adjusting voltage, frequency, and treatment time allows for control over the extraction efficiency. HVED extraction preserves total phenols in the extract, and the antioxidant activity remains relatively stable. As a relatively new technology, further exploration of the antioxidant effects of extracts obtained through HVED is necessary. However, the method holds great potential as it aligns with the principles of green chemistry (Nutrizio et al., 2020).

2.2.1.3. Types of HVED processes

Extraction by HVED can be divided into three categories. These are batch, continuous, and circular extraction types. The working principle is the same, and in all of them, a discharge occurs due to a strong electric field, which causes accelerated mass transfer and damage to cellular material. In their work, Li et al. (2019) described three types of HVED extraction.

The batch system, conducted in mutually independent batch treatment chambers employs a needle-plane electrode with positive voltage. When the pulsating voltage is high enough, a discharge occurs in the aqueous solution (Boussetta and Vorobiev, 2014). The discharge occurs due to the high intensity of the electric field concentrated in the electrode, which causes shock waves, turbulence, emission of UV radiation, and the formation of radicals, which causes cell damage and diffusion of intracellular material into the solvent. In the batch HVED process, extraction procedures are divided into three steps: sample preparation, HVED treatment and diffusion, and the collection process. Sample preparation includes cleaning, drying, and sieving raw material, while HVED treatment mixes stored dry material with solvent at a specified ratio, treated in the chamber. Additional solvent may be added to the suspension for diffusion, and the supernatant is separated from residues through centrifugation for analysis (Li et al., 2019).

The continuous type takes less time, and instead of needle-shaped electrodes, electrodes with parallel disk grids made of stainless steel are used. The distance between the electrodes is 20 mm, and the voltage is applied to one electrode, while the other is grounded. Due to the very construction of the electrodes, the possibility of discharge is much smaller, and it is necessary to take measures that will concentrate the electric field. An insulating plate with a smaller hole with a diameter of 1 mm in the middle is placed. Such a structure enables a stronger electric field, that is, the electric field in the hole itself is higher than in other parts of the reactor. Given that the voltage is not concentrated on the electrodes, the possibility of material corrosion is avoided (Boussetta et al., 2012; Li et al., 2019).

The third type of extraction, i.e. the circulating type, consists of a generator, a reactor, a transport unit, and an extraction tank. The walls of the reactor are made of insulating polycarbonate, and there is a small hole at the top that enables pressure regulation inside and outside the reactor. The electrode is built in the shape of a needle and there is a stainless steel ring under it. The electrode is placed so that the tip of the needle is in the middle of the ring. Then a high voltage is applied to the needle, while the ring is grounded. This results in a very strong electric field at the ring's center, followed by discharge. This type of extraction enables

higher capacities with a small treatment area, which increases the efficiency of the process and reduces energy consumption (Li et al., 2019).

2.2.1.4. Physical and chemical changes during extraction with HVED

Electrical discharge causes various physical and chemical changes in liquids. The most significant physical changes include the appearance of shock waves, cavitation, and UV radiation, while chemical changes include the formation of reactive oxygen (ROS) (such as hydroxyl, superoxide, and peroxyl radicals) and nitrogen species (NOS) (such as nitric oxide and nitrogen dioxide radicals), and various reactive molecules such as hydrogen peroxide (H_2O_2) and ozone (O_3) (Jiang et al., 2016).

Free radicals are molecules characterized by an unpaired electron, making them highly reactive. In the biological realm, they assume a crucial role as they are naturally generated during regular physiological processes (Nimse and Pal, 2015). Due to their unpaired electrons, radicals exhibit extreme instability, continuously seeking equilibrium by promptly reacting with other molecules in their surroundings upon formation, thereby disrupting the stability of these molecules. During free radical reactions, a continuous generation of new free radicals occurs, initiating chain reactions that have the potential to spiral out of control. Frequently, these uncontrolled chain reactions are implicated in various diseases and premature aging. Free radicals, including reactive oxygen species, can originate within our body or be introduced from external sources, such as UV radiation, air pollution, or smoking. These reactive forms subsequently break down in our system, giving rise to short-lived hydroxyl radicals (HO·), which further react with the body's proteins and DNA molecules, causing damage. The accumulation of such damage is associated with the development of cancer, diverse heart diseases, and premature aging (Lobo et al., 2010).

2.3. Computational programs for green solvents selectivity

The crucial step in any extraction process is the careful selection of an appropriate solvent. While experimental laboratory procedures for solvent selection are accurate and dependable, they often demand substantial quantities of solvent and time, resulting in high costs and unsustainable energy consumption (Zhou et al., 2020). To reduce this energy consumption for solvent selection, Principle 2 (use of alternative solvents) and Principle 3 (innovative methods to reduce energy consumption) of green chemistry should be followed (Chemat et al., 2012).

To address the need for reduced solvent usage, cost-effectiveness, and a shift towards environmentally friendly solvents, theoretical and computational methods and models have been developed. These methods aim to predict the solubility parameters of new solvents and solutes, offering an alternative to traditional, resource-intensive approaches. Given the expanding array of solvents and the inherent limitations in user knowledge and experience, computational methods have emerged as crucial tools in guiding the selection and design of solvents for diverse applications (Nutrizio et al., 2022).

The influence of solvents on a system is widely acknowledged and is associated with specific sets of solvent properties. This underscores the significance of identifying trustworthy models for predicting these properties. Many prediction models rely on the physical properties of solvents and are based on simple scale-based methods, such as the Kauri-butanol index and Kamlet–Taft scale. More advanced models, like Hansen solubility parameters (HSPs) and Conductor like screening model for realistic solvents (COSMO-RS), go beyond by enabling the prediction of not only electrostatic interactions between a solute and a solvent but also encompassing thermodynamic properties (Sicaire et al., 2018).

2.3.1. Hansen solubility parameters (HSPs)

Hansen solubility parameters (HSPs) were developed by Charles M. Hansen in his Ph.D. thesis in 1967 as a way of predicting if one material will dissolve in another and form a solution. It is based on the "like dissolves like" principle, meaning that a liquid is a good solvent for a solute if its solubility parameters closely align (Sánchez-Camargo et al., 2019).

The premise of the HSP is that the cohesive energy (E) is the sum of three individual energies: nonpolar (dispersion) interactions (E_d), polar (dipole-dipole and dipole-induced-dipole) interactions (E_p), and hydrogen-bonding or other specific association interactions (E_h) (Eq. 1)

$$E = E_d + E_p + E_h \qquad /1/$$

To scale them so that molecules in a series are more comparable, we need cohesive energy density which is obtained by dividing by molar volume V (Eq. 2):

$$\frac{E}{V} = \frac{E_d}{V} + \frac{E_p}{V} + \frac{E_h}{V} \qquad /2/$$

This cohesive energy density is more convenient in terms of the solubility parameter δ, where $\delta^2 = E/V$. This brings the classic formula (Eq. 3) for HSPs where the total parameter is composed of three components: a dispersion force (δ_d), a polar force (δ_p), and a hydrogen bonding (δ_h).

$$\delta^2 = \delta_d^2 + \delta_p^2 + \delta_h^2 \quad /3/$$

A solvent solubility can be presented easily through HSP programming with a three-dimensional space, where axes are δ_d, δ_p, and δ_h. The program additionally suggests a sphere including potentially good solvents inside and poor solvents outside the parameters of a solute under consideration (Hansen, 2007). The Hansen solubility sphere, represented by the radius of the sphere (R_o), serves as a crucial metric for identifying the optimal solvent that closely aligns with the center of the sphere, considering the probable maximum difference in solute and solvent parameters. The R_a parameter represents the distance of a solvent from the center of the Hansen solubility sphere, i.e. the distance between solute (*i*) and a solvent (*j*) determined by the components of each of their partial solubility parameters (Eq. 4).

$$R_a = \sqrt{4(\delta_{d,i} - \delta_{d,j})^2 + (\delta_{p,i} - \delta_{p,j})^2 + (\delta_{h,i} - \delta_{h,j})^2} \quad /4/$$

To enhance the outcomes of Hansen solubility parameters, a simplified composite affinity parameter, known as the relative energy difference (RED) number, has been introduced (Eq. 5).

$$RED = \frac{R_a}{R_0} \quad /5/$$

Accordingly, a RED value of 0 indicates a "perfect solvent" since no energy difference was found, RED < 1 represents high affinity, 1 to 3 denotes medium solubility, and RED > 3 indicates low affinity or poor solvent performance (Sánchez-Camargo et al., 2019; Nutrizio et al., 2022; Hansen, 2007).

In recent years, there has been a significant increase in the application of HSP for assessing the solubility of various analytes, particularly those derived from natural sources. This approach offers notable advantages in terms of simplicity and efficient utilization of time and resources. Green solvents like ethanol, D-limonene, vegetable oils, CO_2, and ionic liquids are incorporated into the Hansen software database, facilitating the prediction of solubility for high-value compounds such as polyphenols (Pagano et al., 2018), carotenoids (Yara-Varón et al., 2016; Aissou et al., 2017), triacylglycerides (Angles et al., 2017), and volatile aroma compounds (Li et al., 2014). The HSP strategy, guided by principles of green chemistry and sustainability, facilitates the selection of bio-solvents based on their HSPs, contributing to the development of efficient and selective extraction methods (Sánchez-Camargo et al., 2019; Nutrizio et al., 2022).

2.3.2. Conductor like screening model for realistic solvents (COSMO-RS)

COSMO-RS is a more advanced method for solvent screening. It is a quantum chemistry-based statistical thermodynamics model for the prediction of thermodynamic properties of solvents and liquid mixtures. COSMO-RS uses a dual-step calculation process at different scales: a microscopic scale and a macroscopic scale, to capture the transition from solutes' molecular states to real solvents. In the microscopic step, the COSMO model is employed to envelop the solute within a virtual conductor, inducing a polarization charge density on its surface known as the σ-surface. The produced σ-surface represents the redistribution of charge density across the molecular surface, where colors ranging from green to yellow indicate weakly polar surfaces, blue highlights electron-deficient regions, and red signifies electron-rich regions (Figure 5b) (Sicaire et al., 2018; Nutrizio et al., 2022).

In the macroscopic step, statistical thermodynamic calculations are used. The polarization charge density serves as a quantification metric for assessing the interaction energy between pairwise interacting surface segments, particularly focusing on essential molecular interaction modes like electrostatics and hydrogen bonding. Converting the 3D distribution of polarization charges (σ) on the surface of each molecule into a surface composition function results in a histogram called σ-profile (COSMOlogic, 2011). These profiles provide insights into the molecular polarity distribution. The chemical potential of the surface segment referred to as the σ-potential, is then calculated from the thermodynamics of molecular interactions based on the obtained σ-profile. The solvent's chemical potential (σ –potential), is computed through the thermodynamics of molecular interactions using the acquired σ-profile. By examining the σ-profile of the solvent, its chemical potential, can be understood as an expression of affinity of the solvent S for a surface characterized by polarity σ. COSMO-RS integrates the σ-profiles and σ-potentials, alongside other thermodynamic parameters, to enable effective prescreening and classification of various solvents. All calculating steps are presented in Figure 5. Through multivariate statistical analyses, COSMO-RS facilitates the selection of optimal solvents for diverse industrial applications (Filly et al., 2015; Ying et al., 2019; Nutrizio et al., 2022).

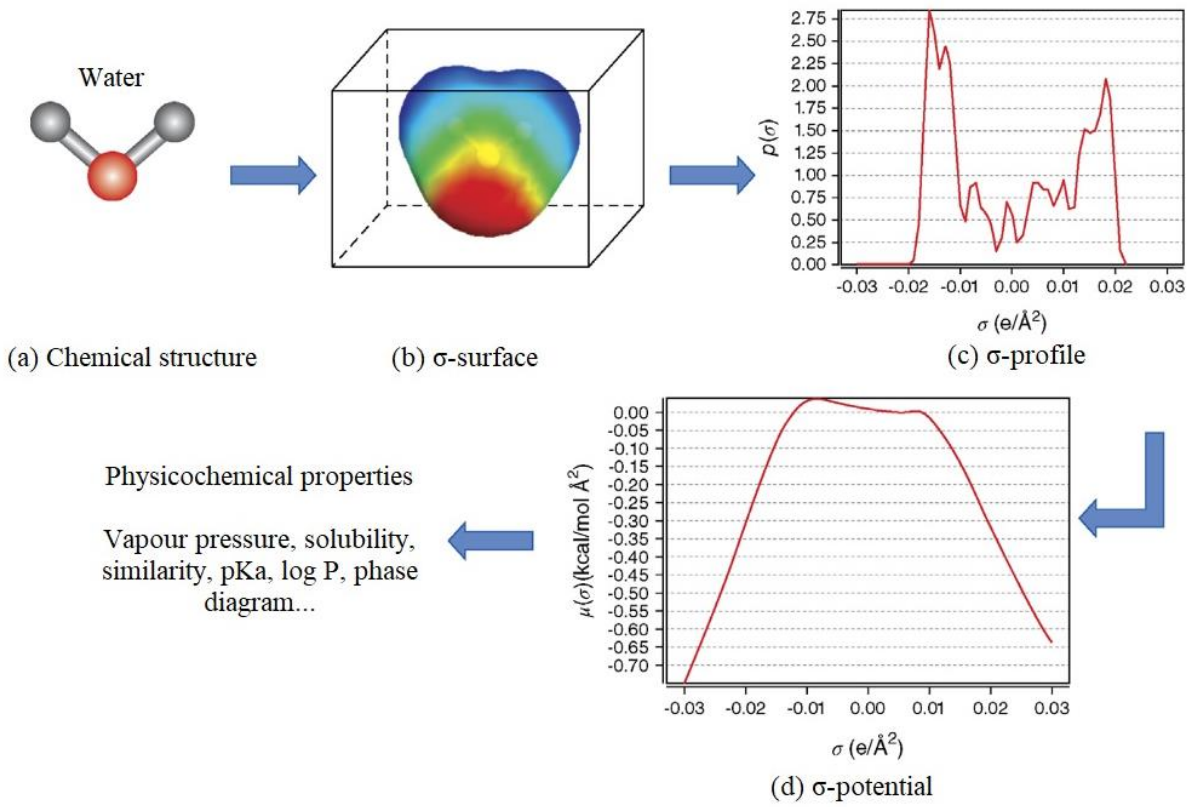

Figure 5. Schematic diagram of COSMO-RS method calculation steps: starting with (a) the 2D molecular structure, followed by (b) the generation of σ-surfaces, (c) the conversion into a σ-profile, and finally (d) the derivation of σ-potentials of water as a representative solvent (Adopted from Sicaire et al. (2018)).

The number of published research and literature on the utilization of COSMO-RS is expanding every day. To date, COSMO-RS stands out as the most precise method for screening the solubility of solvents. This method has found diverse applications in the pharmaceutical and cosmetic industry, material science, chemical industry, petrochemicals, and biotechnology. Numerous studies highlighted the efficacy of COSMO-RS in the extraction of natural compounds from food, paving the way for the substitution of traditional solvents with environmentally friendly alternatives (Sicaire et al., 2018).

2.3.3. Green solvents

Solvents are widely used in many different applications, which underscores their substantial influence on health, safety, and the environment. Traditionally, n-hexane is commonly used for extracting BACs from natural sources due to its low polarity, optimal boiling point, evaporation ease, and stability. However, its petrochemical origin faces strict regulations. In the face of increasing consumer demands and expanding production, both society and industries have

recognized the importance of sustainable practices and high-quality products. This recognition has led to the implementation of new regulations such as The European Union Directive 1999/13/EC (EC, 1999) which aims to prevent or reduce the direct and indirect effects of emissions of volatile organic compounds on the environment and human health, and The Regulation on the registration, evaluation, authorization, and restriction of chemicals (REACH) (EC, 2006), as main European Union's law to protect human health and the environment from the risks that can be posed by chemicals. This led to the creation of solvents' Environmental, health, and safety (EHS) characteristics. Some pharmaceutical companies have released their green solvent selection recommendations, which are based on the companies' policy compliance and EHS ranking (Clarke et al., 2018; Capello et al., 2007).

The request for sustainable alternatives, i.e. green solvents, initially emerged from Principle 2 of green extractions, intending to replace conventional solvents with environmentally friendly options (Chemat et al., 2012). These solvents, essential for green extractions, possess characteristics such as being non-volatile, non-flammable, inexpensive, low toxicity, easy biodegradability, having low environmental impact, and sufficient dissolving power and selectivity. While no single green solvent is universally perfect for all situations, researchers must balance the various demands of green chemistry by choosing the best alternative for each experiment (Capello et al., 2007; Winterton, 2021).

The most environmentally friendly solvent to consider for green extractions is water. Furthermore, water has been recognized for its safety, cheapness, and wide availability, it is renewable and can dissolve a wide range of substances. Water, existing in a liquid state at 0-100 °C under atmospheric pressure, is a crucial solvent with excellent solvation properties, facilitating nutrient transport and supporting biological processes. Water's remarkable solvent capabilities, characterized by its dipole moment and relative permittivity, make it effective for dissolving a wide range of compounds, particularly ionic and polar substances. However, its high evaporative nature leads to increased energy consumption and emissions. Innovative methods, such as pressurized hot water extraction or subcritical water, operate efficiently in the subcritical region, offering an economical approach to resource recovery without compromising product quality. Additionally, co-solvents like hydrotropes or surfactants can enhance water's properties, influencing solubility and extractive potential (Castro-Puyana et al., 2017; Hartonen and Riekkola, 2017).

Ethanol, derived from natural sources through the fermentation of sugar-rich materials, enzymatic hydrolysis of starch, or processing lignocellulosic raw materials, serves as another commonly employed green solvent - bioethanol. It is readily accessible in high purity, cost-effective, and entirely biodegradable. The production of bioethanol contributes significantly to reducing greenhouse gas (GHG) emissions associated with traditional fuel combustion. Furthermore, ethanol is a well-established solvent in plant extraction processes, allowing bioethanol to be promptly employed in such procedures (Chemat et al., 2019; Nutrizio et al., 2020). Some other alcohols like methanol, isopropanol, and 1-butanol are also considered green solvents (Sicaire et al., 2018).

Terpenes constitute a diverse group of naturally occurring organic compounds present in essential oils, oleoresins, fruits, and herbs. Extraction from plant-based raw materials can be achieved through physical methods like hydrodistillation or cold pressing, particularly evident in the extraction of citrus peel oils. Terpenes are prevalent in various products, including perfumes, pharmaceuticals, dyes, and food, where they primarily contribute to their aromatic properties. Despite their aromatic role, terpenes, due to their abundance, also serve as effective solvents. One of the most commonly employed terpenes in this capacity is likely D-limonene, a significant byproduct of the citrus industry. There is also α-pinene, a natural terpene hydrocarbon obtained from pine trees or some plants like mint, lavender, sage, and ginger, cymene, an aromatic hydrocarbon that occurs widely in tree leaf oils and β-myrcene that exists as a key component of numerous plant species, such as cannabis and hops. These solvents, characterized by biodegradability, low flammability, low polarity, and powerful solvent capabilities, are particularly effective for extracting natural compounds like bioactive molecules, fats, and oils (Aissou et al., 2017; Tanzi et al., 2012; Calvo-Flores et al., 2018).

Various esters are also considered as green solvents. Organic acid esters are produced by esterifying organic acids like acetic, citric, gluconic, or lactic acid. These organic acid esters include ethyl acetate, methyl acetate, and ethyl lactate. Ethyl acetate is the most used organic acid ester as a green solvent. Traditionally, ethyl acetate was produced through the esterification of ethanol and acetic acid. In novel methods, ethyl acetate can be produced through the dehydrogenation of bioethanol, offering the advantage of relying on a single raw material obtained primarily through fermentation. It is a biodegradable solvent but is classified as highly flammable (Gadewar, 2012). Furthermore, some fatty acid esters (i.e. ethyl oleate) are also considered as green. Derived from vegetable oils like rapeseed or sunflower, fatty acid esters

undergo transesterification with alcohols such as methanol, ethanol, or 2-ethoxyhexanol (Sicaire et al., 2018).

In the field of biorefinery, leftover plant materials from cereal production, known as lignocellulosic residues, can be used to make furfural. Furfural serves as a precursor for various molecules, including 2-methyltetrahydrofuran (MeTHF). MeTHF is synthesized through the hydrogenation of products derived from the carbohydrate fractions of hemicellulose, sourced from diverse feedstock such as corn cobs or sugar cane bagasse (Zheng et al., 2006). MeTHF, commonly used as a solvent in organic synthesis, showed promise as an eco-friendly alternative solvent in extraction processes due to its biodegradability, environmentally friendly footprint, easy recyclability, and effective solvating properties (Sicaire et al., 2014).

Green solvents can be sourced not only from plants but also from by-products of the petrochemical industry or through environmentally friendly chemical synthesis such as dimethyl carbonate and cyclopentyl methyl ether (CPME). DMC is nowadays produced through transesterification of propylene carbonate or a reaction involving carbon monoxide, methanol, and oxygen. Recognized for its non-toxicity and biodegradability, dimethyl carbonate is considered a green solvent, with studies exploring its use in biodiesel production. CPME, also synthetically derived, serves as an alternative to traditional ether solvents like tetrahydrofuran or dioxane. While not derived from renewable sources, CPME's advantageous properties, such as its boiling point (106 °C) that reduces peroxide formation, position it as a strong candidate for directly replacing ethers. CPME surpasses other ether solvents in terms of environmental impact, health, and safety, making it increasingly appealing for plant extractions.

Notably, supercritical CO_2, ionic liquids, and deep eutectic solvents have emerged as highly investigated green solvents in recent studies. Ionic liquids are a combination of organic heterocyclic cations and organic or inorganic anions, while deep eutectic solvents are a combination of various hydrogen bond acceptors (Wu and Han, 2019; Płotka-Wasylka et al., 2020).

2.4. Mediterranean herbs

Mediterranean herbs have played a significant role since ancient times, serving both medicinal and culinary purposes. Over 30 % of all plant species have been utilized for medicinal benefits. Mediterranean regions boast a variety of herbs, including oregano, basil, rosemary, bay leaf, lavender, mint, parsley, thyme, sage, chives, dill, fennel, marjoram, saffron, etc. (Giacometti et al., 2018; Bower et al., 2016; Kumar et al., 2012).

Mediterranean herbs are rich sources of beneficial BACs and essential oils. Studies indicate that Mediterranean herbs and their BACs impact the quality of foods in terms of nutrition, chemistry, microbiology, and taste. Above that, recent clinical studies have explored the potential of plant extracts containing BACs in treating conditions like cardiovascular disease, diabetes, and obesity. Recognizing the potential risks of synthetic antioxidants, consumers and industries are increasingly turning to Mediterranean herbal extracts as safer alternatives in the production of food, pharmaceuticals, and cosmetics (Vinceković et al., 2017).

A significant emphasis in the Mediterranean region is given to the Lamiaceae family, encompassing around 236 genera and 7,200 species. Notably, oregano, sage, rosemary, and thyme stand out as the most commercially important members of this family (Ramasubramania, 2012; Giacometti et al., 2018).

2.4.1. Rosemary (*Salvia rosmarinus* Spenn.)

Rosemary (*Salvia rosmarinus* Spenn.) is an aromatic plant native to the Mediterranean region and belongs to the Lamiaceae family. The plant was commonly known as *Rosmarinus officinalis* L., but recently undergone taxonomic changes. Recent phylogenetic analysis merged the genus *Rosmarinus* into *Salvia*. Consequently, in 2019, the Royal Horticultural Society formally approved the incorporation of Rosmarinus into the Salvia genus (RHS, 2019). This branched, evergreen plant with short, leathery, dark green leaves can reach a height of up to 3 meters and blooms twice a year, in April and September. Archaeological evidence indicates the historical use of rosemary for medicinal, culinary, and cosmetic purposes in ancient Egypt, Mesopotamia, China, and India (Sasikumar, 2012; Ribeiro-Santos et al., 2015).

Rosemary is versatile and can be utilized in various forms, such as fresh, dried, or essential oil. This aromatic plant exhibits various biological activities attributed to its BACs, including volatile fractions and phenolic constituents. According to the European Medicines Agency (2022), officially recognized medicinal components of rosemary include its leaves (Rosmarini folium) and the essential oil (Rosmarini aetheroleum) obtained through distillation. It was reported that rosemary leaves have a beneficial impact when used orally for the symptomatic relief of dyspepsia and mild spasmodic disorders of the gastrointestinal tract. As a bath additive, it has been used for the relief of minor muscular and articular pain and in minor peripheral circulatory disorders. The essential oil showed successful applications for the relief of minor muscular and articular pain, and in minor peripheral circulatory disorders (EMA, 2022). Additionally, studies have reported diverse health benefits of rosemary BACs, including

antioxidative, antifungal, antibacterial, anti-diabetic, antiulcerogenic, anti-inflammatory, antithrombotic, and antidepressant effects (Ribeiro-Santos et al., 2015; Sueishi et al., 2018).

Nowadays, its applications are widespread globally. In the food industry, rosemary serves as a spice, enhancing the flavor and organoleptic properties of fatty products, meats, and soups. Rosemary extracts and essential oils have found commercial use as natural food biopreservatives. The Food and Drug Administration (FDA) has recognized rosemary as generally recognized as safe (GRAS) for its intended use. This recognition follows Commission Directive 2010/67/EU and Commission Directive 2010/69/EU, which recognized rosemary extracts as a new food additive under label E392. As a result, rosemary may be a helpful functional ingredient for creating novel functional foods (EC, 2010; FDA, 2023; FSA, 2023).

2.4.1.1. Chemical composition of rosemary

Rosemary has a highly complex chemical composition. 208 variations in species, varieties, growth circumstances, harvesting times, soil qualities, climate, origin, and geographic features contribute significantly to the variety in the concentration of macro and micronutrients in rosemary (Ribeiro-Santos et al., 2015).

Regarding the macronutrients, dried rosemary leaves consist of 4.88 % of proteins, 15.20 % of lipids, 64.10 % of carbohydrates, and 9.31 % of water. The most abundant minerals in rosemary leaf are calcium (1280 mg/100 g), potassium (955 mg/100 g), and magnesium (220 mg/100 g), while the most abundant vitamins include vitamin C (61.2 mg/100 g), vitamin B6 (1.74 mg/100 g), and niacin (1 mg/100 g) (USDA, 2019a).

In a study by Mena et al (2016) identification of 57 polyphenolics was made possible by the ultra-high-performance liquid chromatography (UHPLC)-based characterization of the phenolic fraction of the rosemary extract. In the rosemary extract, 24 flavonoids (mostly flavones, though there were also flavonols and flavanones found in the extract), 5 phenolic acids, 24 diterpenoids (carnosic acid, carnosol, and rosmanol derivatives), 1 triterpenoid (betulinic acid), and 3 lignans (medioresinol derivatives) were found. It was reported that diterpenes constitute the majority of phenolic compounds (97.2 %). Among the essential BACs, carnosic acid, rosmarinic acid, and carnosol are highlighted as crucial components (Mena et al., 2016).

Regarding the volatile fraction, the composition of rosemary essential oil includes monoterpenes, monoterpenes derivatives (95-98 %), and sesquiterpenes (2-5 %), with α-pinene,

verbenone, camphor, 1,8-cineole, borneol, and camphene identified as the main volatiles (Szumny et al., 2010). However, the content of BACs can vary based on factors like climate, plant parts, drying, extraction methods, and analytical approaches (Achour et al., 2018; Giacometti et al., 2018).

2.4.2. Oregano (*Origanum vulgare* L.)

Oregano (*Origanum vulgare* L.) is a perennial aromatic plant belonging to the Lamiaceae family. It grows wild in the Mediterranean region, especially in high-altitude areas between 1500 to 3600 meters. The plant's structure is characterized by a branched woody stem 20-80 cm in height and stalked hairy leaves. The glandular hairs on the leaves contain volatile and essential oils responsible for the distinctive aroma and fragrance of oregano. The flowering period, occurring from June to August, produces small reddish-brown seeds (Bhatt et al., 2020; Kintzios, 2012).

In traditional medicine, oregano was used for treating respiratory disorders, dyspepsia, painful menstruation, rheumatoid arthritis, and urinary system disorders (Teixeira et al., 2013). The Greeks considered it a symbol of happiness, believing the deceased would be eternally happy if it grew on a grave (Roby et al., 2013). Due to its aroma and fragrance, oregano stands out as one of the most well-known and economically significant culinary herbs worldwide. Besides its traditional culinary use, special attention is given to the chemical compounds and their benefits for human health (Yan et al., 2016; Pezzani et al., 2017). Numerous in vitro and in vivo preclinical studies have demonstrated the potential of oregano as an effective agent against chronic degenerative and infectious diseases due to the anticancer, anti-inflammatory, antioxidant, antifungal, and antimicrobial effects of its bioactive components. According to Pezzani et al. (2017), more effectiveness is shown by complex systems such as extracts and essential oils compared to pure compounds, attributed to the synergistic and/or additive effects of different components in the system (Bhatt et al., 2020).

2.4.2.1. Chemical composition of oregano

Under the name oregano, at least 61 species from 17 genera and six families are listed. The genus *Origanum*, which is the source of the popular Greek and Turkish oregano spices, is thought to belong to the most significant family, Lamiaceae (Kintzios, 2012; Singha et al., 2017).

In terms of macronutrients, the contents of oregano leaves are as follows: 9.00 % proteins, 4.28 % lipids, 68.90 % carbohydrates, and 9.93 % water. The most prevalent vitamins in oregano leaf are niacin (4.64 mg/100 g), vitamin C (2.3 mg/100 g), and vitamin B6 (1.04 mg/100 g). The most prevalent minerals in rosemary leaf are calcium (1600 mg/100 g), potassium (1260 mg/100 g), and magnesium (270 mg/100 g) (USDA, 2019b).

Phytochemicals in oregano, based on their hydrophilic and hydrophobic properties, can be divided into two categories - essential oils and phenolic compounds. The content of bioactive components varies depending on the chemotype of the same species, the geographical region of growth, the harvest time, and the applied extraction method (Gutiérrez-Grijalva et al., 2018). Essential oils are predominantly composed of terpenoids, with carvacrol and thymol as two primary phenolic monoterpenoids (78–82 %). They are in charge of the distinct aroma in addition to antioxidant and antibacterial properties. Other chemical groups found in oregano include monoterpene hydrocarbons (p-cymene and γ-terpinene), oxygenated monoterpenes (thymol, 4-terpineol, carvacrol and trans-sabinene hydrate), sesquiterpene hydrocarbons (β-caryophyllene, β-bisabolene, β-bourbonene, α-humulene), oxygenated sesquiterpenes (β-caryophyllene oxide), acyclic monoterpenoids (geraniol, geranyl acetate, linalool, linalyl acetate, and β-myrcene), bornyl- compounds (camphene, camphor, borneol, and bornyl and isobornyl acetate) (Pezzani et al., 2017; Kintzios, 2012).

In oregano, phenolic acids are most prevalent and categorized into two primary groups - hydroxycinnamic acid derivatives and hydroxybenzoic acid derivatives. Hydroxycinnamic acid is more prominently recorded in oregano. The most abundant phenolic acids found in oregano are rosmarinic and caffeic acid. Additionally, it contains significant quantities of phenolic compounds, with catechin, epicatechin, cinnamaldehyde, and carvacrol being the main components of soluble extracts, while gallic acid and p-coumaric acid dominate in bound extracts. Other BACs including phenolic glycosides, flavonoids, tannins, sterols, and terpenoids were also detected (De Martino et al., 2009).

2.5. Microencapsulation for the improved delivery of bioactive compounds into foods

One of the most fascinating topics in the area of active agent delivery systems is encapsulation. Encapsulation is a process of packaging solids, liquids, or gaseous components as active material (i.e. encapsulated material, core material, filler, internal phase) with a continuous film as a coating (i.e. immiscible material, matrix, shell, external phase). In microencapsulation,

those capsules are called microparticles (i.e. microcapsules, microspheres) and have a diameter of between 1 μm and 1 mm (Ozkan et al., 2019; Champagne and Fustier, 2007).

This interdisciplinary field of microencapsulation requires expertise in chemistry, material science, and a profound understanding of active agents' stabilization. In the food industry, the main goal of encapsulation is protecting the core from degradation by reducing its reactivity with the environment. Simultaneously, it aims to extend shelf life, reduce evaporation and mass transfer from the capsule to the environment, change the physical characteristics of the encapsulated material to facilitate its handling, control the release time of the core from the capsule, ensure uniform dispersion of the core from the material, improving the quality of the final product and separating components that would react without encapsulation. Additionally, encapsulation works to mask undesirable flavors (Aceval Arriola et al., 2016; Najafi-Soulari et al., 2016; Fang and Bhandari, 2010). Due to the slow and controlled release of encapsulated compounds, greater efficiency and safety for the environment are ensured (Kumari et al., 2010; Jurić et al., 2019).

Microencapsulation is of significant interest to the food industry, particularly in functional foods. Adding bioactive ingredients to functional foods poses challenges in maintaining stability during processing and storage (Donsì et al., 2015). Microencapsulation helps address these challenges by protecting BACs from environmental stresses and enabling controlled release in the gastrointestinal system. Microencapsulation facilitates the preservation of nutritional value, bioavailability, solubility, and functionality of BACs, contributing to health-beneficial and disease-prevention functionalities in food products (Vinceković et al., 2017).

Microencapsulation methods can be categorized into three main groups: (i) physical methods like spray drying, lyophilization, supercritical fluid precipitation, and solvent evaporation; (ii) physicochemical methods, such as coacervation, liposomes, and ionic gelation; and (iii) chemical methods, including interfacial polymerization and molecular inclusion complexation. Each method has its own set of advantages and disadvantages across various aspects. However, the choice of the microencapsulation process is primarily influenced by the thermosensitivity and solubility of the active compounds. Optimizing parameters associated with the physicochemical properties of the encapsulated material is essential for each encapsulation method, as well as for the core and wall materials. This optimization facilitates the achievement of narrower size distributions, minimizing product loss, and enhancing nutritional value (Ozkan et al., 2019).

2.5.1. Ionic gelation

In recent decades, ionic gelation (i.e. ionotropic gelation) has emerged as a highly effective technology for encapsulating liquid samples within biopolymer matrices. This method is advantageous due to its low encapsulation process temperatures, minimizing the negative impact on the quality properties of the encapsulated solution. The ionic gelation process involves the interaction between polymers with opposite charges or a polymer with a polycation or polyanion, leading to the formation of gel particles that encapsulate the desired substance (Benavides et al., 2016; Guerra-Valle et al., 2022).

In the ionic gelation technique, there is an increasing interest in using natural biopolymers as active ingredient carriers. Biopolymers are diverse and versatile class compounds, that originate from biological systems or are synthesized from biological sources. Similar to other polymers, biopolymers consist of recurring units (monomers) that are connected. The distinctive attributes of biopolymers, such as biodegradability, accessibility, and the potential for manipulating physicochemical characteristics, make them integral in innovative formulations. As the focus shifts toward green sustainable processing, biopolymers provide a foundation aligning to create an eco-friendly environment (Fazal et al., 2023).

The choice of biopolymers plays a pivotal role in achieving the desired encapsulation properties. Some of the most used biopolymers in the ionic gelation method include polysaccharides- and proteins-based systems. Polysaccharides are monosaccharide units connected through glycosidic linkages. They are promising biomaterials for the development of microparticles within complex biological settings. Various cationic (such as chitosan, chitin, and curdlan), anionic (such as alginate, hyaluronic acid, xanthan gum, dextran, dextrin, pectin, and carrageenan), and neutral (such as cellulose, starch, agarose, and pallulan) polysaccharides, as well as their composites and derivatives, are being used as biopolymers (Fazal et al., 2023).

Proteins suitable for consumption, notably whey protein, caseinate, zein, gelatin, albumin, and soy protein, are highly valued in the food industry due to their associated health benefits. Their functional attributes, such as gelation, emulsification, and binding capacity, make them appealing choices as substitutes for the creation of microparticles with BACs. Moreover, the hydrophobic region of proteins can interact with the benzene ring of BACs, while the carbonyl and amine groups of proteins form hydrogen bonds with the hydrophilic regions (Song et al., 2022).

Among all biopolymers, sodium alginate stands out due to its nontoxic, biodegradable, low-cost, and biocompatible properties, making it the most commonly employed biopolymer with diverse applications (Ozkan et al., 2019). The process involves the addition of sodium alginate drop by drop to a bath containing polyvalent cations. The affinity of sodium alginate for divalent cations depends on its composition, with guluronic acid-based alginate exhibiting higher ion binding capability (Ozkan et al., 2019; Jurić et al., 2021). The process results in the formation of polymeric microparticles known also as hydrogel beads. The widely preferred gelling cation is Ca^{2+} (from calcium chloride) due to its chemical versatility and safety. The gel formation follows the "egg-box" model, where Ca^{2+} ions bridge adjacent guluronic acid blocks, creating stable bonds that hold the alginate chains together. The kinetics of gelling are fast and adaptable, depending on the type and concentration of the polymer and cation used. The method's versatility allows for the preparation of microparticles using a microdroplet generator (Figure 6) (Sikorski et al., 2007; Montanucci et al., 2015). Overall, ionic gelation is favored for its mild conditions, economical production costs, and suitability for various applications, including controlled active substance release (Ozkan et al., 2019).

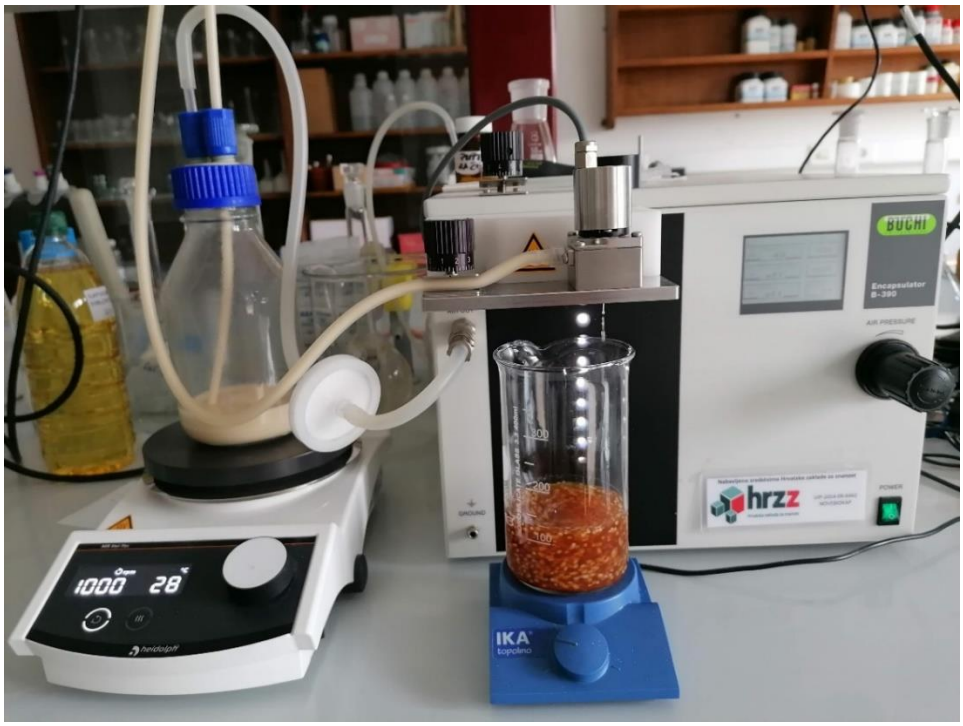

Figure 6. Microencapsulation process using microdroplet generator Encapsulator Büchi-B390, BÜCHI Labortechnik AG, Switzerland (Author's photo).

In general, sodium alginate is known for creating firm hydrogels (Eiselt et al., 2000); however, it often exhibits a porous structure and lacks robust physical and mechanical properties, which are crucial for delivering active agents. By blending different biopolymers (especially

combining carbohydrate- and protein-based biopolymers), it is possible to modify their properties, enhancing chemical stability and producing microparticles with improved controlled release of encapsulated materials (Belščak-Cvitanović et al., 2017; Jurić et al., 2021).

2.6. Hypothesis, objectives, and expected scientific contribution of the research

The hypothesis of the research is that HVED in combination with green solvents, contributes to a higher yield of extracted BACs and lower environmental impact compared to conventional extraction.

The general aim of the work is to determine the efficiency of extraction of BACs from selected Mediterranean herbs using HVED and green solvents. Furthermore, the following specific research objectives include:

- Evaluation of the potential use of model predictive tools as a preliminary method to reduce solvent consumption during experimentation and comparison with experimental data (*Paper I, Paper II*).
- Determination of the efficiency of extraction of BACs from rosemary and oregano using HVED and green solvents, in comparison with conventional extraction (*Paper I, Paper II*).
- Process optimization of HVED extraction (*Paper I, Paper II*).
- Evaluation of sustainability of HVED in comparison with conventional extractions (*Paper III*).
- Stabilization of obtained optimal extract by microencapsulation in biopolymer-based microparticles for the potential use in functional food preparation (*Paper IV*).

The scientific contribution of the proposed research is in the development and optimization of a new and sustainable method for the extraction of BACs from Mediterranean herbs, which, in combination with green solvents, contributes to a higher yield of the extraction process compared to conventional methods. Additionally, a contribution is the use of model predictive tools as a preliminary method based on predictions of solvent-solute properties and interactions to reduce solvent consumption during experimentation.

SCIENTIFIC PAPERS

3. SCIENTIFIC PAPERS

This Ph.D. thesis is based on four scientific papers presenting the work performed on the extraction of BACs from rosemary and oregano using HVED as nonthermal technology and green solvents.

List of scientific papers:

Paper I: **Nutrizio, M.**, Gajdoš Kljusurić, J., Marijanović, Z., Dubrović, I., Viskić, M., Mikolaj, E., Chemat, F., Režek Jambrak, A. (2020) The potential of high voltage discharges for green solvent extraction of bioactive compounds and aromas from rosemary (*Rosmarinus officinalis* L.) - computational simulation and experimental methods. *Molecules* **25**(16), 3711. doi: 10.3390/molecules25163711

Paper II: **Nutrizio, M.**, Maltar-Strmečki, N., Chemat, F., Duić, B., Režek Jambrak, A. (2020) High-Voltage Electrical Discharges in Green Extractions of Bioactives from Oregano Leaves (*Origanum vulgare* L.) Using Water and Ethanol as Green Solvents Assessed by Theoretical and Experimental Procedures. *Food. Eng. Rev.* **13**(1), 161-174.

Paper III: **Nutrizio, M.**, Režek Jambrak, A., Rezić, T., Djekic, I. (2022) Extraction of phenolic compounds from oregano using high voltage electrical discharges - sustainable perspective. *Int. J. Food Sci. Tech.* **57**, 1104-1113.

Paper IV: **Nutrizio, M.**, Jurić, S., Kucljak, D., Švaljek, S. L., Vlahoviček-Kahlina, K., Režek Jambrak, A., Vinceković, M. (2023) Encapsulation of Rosemary Extracts using High Voltage Electrical Discharge in Calcium Alginate/Zein/Hydroxypropyl Methylcellulose Microparticles. *Foods* **12**(8), 1570. doi: 10.3390/foods12081570

Paper I

Nutrizio, M., Gajdoš Kljusurić, J., Marijanović, Z., Dubrović, I., Viskić, M., Mikolaj, E., Chemat, F., Režek Jambrak, A. (2020) The potential of high voltage discharges for green solvent extraction of bioactive compounds and aromas from rosemary (*Rosmarinus officinalis* L.) - computational simulation and experimental methods. *Molecules* **25**(16), 3711. doi: 10.3390/molecules25163711

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Article

The Potential of High Voltage Discharges for Green Solvent Extraction of Bioactive Compounds and Aromas from Rosemary (*Rosmarinus officinalis* L.)—Computational Simulation and Experimental Methods

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Abstract: Rosemary (*Rosmarinus officinalis* L.) is a Mediterranean medicinal and aromatic plant widely used due to valuable bioactive compounds (BACs) and aromas. The aim of the study was to evaluate the extraction of intracellular compounds from rosemary combining experimental procedure by means of high voltage electrical discharge (HVED), with a theoretical approach using two computational simulation methods: conductor-like screening model for real solvents and Hansen solubility parameters. The optimal HVED parameters were as follows: frequency 100 Hz, pulse width 400 ns, gap between electrodes 15 mm, liquid to solid ratio 50 mL/g, voltage 15 and 20 kV for argon, and 20 and 25 kV for nitrogen gas. Green solvents were used, water and ethanol (25% and 50%). The comparison was done with modified conventional extraction (CE) extracted by magnetic stirring and physicochemical analyses of obtained extracts were done. Results showed that HVED extracts in average 2.13-times higher total phenol content compared to CE. Furthermore, nitrogen, longer treatment time and higher voltage enhanced higher yields in HVED extraction. HVED was confirmed to have a high potential for extraction of BACs from rosemary. The computational stimulation methods were confirmed by experimental study, ethanol had higher potential of solubility of BACs and aromas from rosemary compared to water.

Keywords: high voltage electrical discharge; rosemary; COSMO–RS; Hansen solubility parameters; bioactive compounds; food aromas; extractions

1. Introduction

Rosemary (*Rosmarinus officinalis* L.) is an autochthonous Mediterranean herb from *Lamiaceae* family. From ancient times, rosemary has been used as flavoring agent and for medicinal purposes due to its intense aromatic odor and health benefits [1]. The biological activity of rosemary is mostly related to the phenolic compounds, such as carnosol, carnosic acid, and rosmarinic acid [2], and volatile compounds from essential oil like α-pinene, camphor, eucalyptol, or 1,8-cineole [3]. Due to valuable bioactive

compounds (BACs), rosemary possess antioxidant [2], anticancer [4], diuretic [5], antimicrobial [6], antiproliferative [7], anti-inflammatory [8], and anti-hyperglycemic properties [9]. Rosemary leaves, extracts, and essential oil have received recognition as generally recognized as safe (GRAS) for their intended use, from Food and Drug Administration [10] and according to Commission Directive 2010/67/EU and Commission Directive 2010/69/EU. Therefore, rosemary products can be a useful functional ingredient for the production of new functional foods [11].

Mostly used conventional extraction (CE) techniques are often associated with long extraction time, use of organic solvents in huge amounts and possible thermal degradation of thermosensitive compounds such as antioxidants [12]. For that reason, various innovative methods have been developed that are within the principles of green extraction processes. Apart from solvent extraction and steam distillation techniques, rosemary extracts have already been prepared by several green extraction methods: ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE) [13,14], supercritical fluid extraction (SFE) [15–17], and pressurized liquid extraction (PLE) [18–20]. For example, UAE was used for extraction of bioactive compounds from dried rosemary. Increased recovery of carnosic acid was obtained in ethanol, while rosmarinic acid was better extracted using methanol as a solvent. [21,22]. In a similar report, UAE of rosemary produced a three-fold increase in concentration of rosmarinic and carnosic acid when compared to the solid–liquid extraction [23]. In a work by Jacotet-Navaro et al., (2015), several extraction techniques were compared. Carnosic and ursolic acid extraction from rosemary was enhanced by ultrasound, while microwave extraction was more suitable for rosmarinic acid extraction. Both procedures were performed with reduced energy consumption and carbon emission when compared to heat reflux extraction. UAE was performed at 40 °C, while MAE was performed at 70–150 °C. The extraction yield increased with temperature but might have caused increased degradation and loss of volatile components, which makes thermal methods less suitable for extraction of aromatic compounds.

A green nonthermal extraction method that has not been investigated for extractions from rosemary is high voltage electrical discharge (HVED)—cold plasma treatment [24]. HVED extraction is a novel, eco-friendly extraction technique, that has been efficiently used in the extraction of BACs from various plant sources. Furthermore, as a nonthermal technology, extraction with HVED is performed in mild temperatures (usually at room temperature) and the temperature elevation after the extraction is low, so the thermal degradation of BACs is prevented [25,26]. The extraction of intracellular compounds by HVED is enhanced by the phenomenon of electrical breakdown in liquid that provokes cell structure damage and formation of pores (electroporation). The electrical breakdown is managed by the liquid ionization that is presented from the application of a high voltage between two electrodes with a gas flow. This leads to the liquid turbulence and intense mixing, emission of high-intensity ultraviolet light, generation of active radicals, production of shock waves and also a bubble cavitation [27].

Most conventional organic solvents are volatile, flammable, explosive, toxic, and have a negative environmental impact. For that reason, another principle of green extraction is directed towards use of alternative green solvents. In order to define green solvents, Gu and Jérôme (2013) proposed 12 criteria for green solvents that should be fulfilled related to availability, price, recyclability, grade, synthesis, toxicity, biodegradability, performance, stability, flammability, storage, and renewability. The solvent is considered greener when compared to conventional solvents that should be replaced with alternative solvent that fulfils at least some of the mentioned criteria [28]. Among green solvents, the water and agro- or bio-solvents play an important role for the replacement of organic solvents. Such solvents are derived from a renewable resource produced from biomass such as wood, starch, vegetable oils, or fruits. They have a high solvent power, are biodegradable, non-toxic, and non-flammable [29]. In a work by Barbieri et al., (2020), UAE of polyphenols from rosemary was performed in ethanol and natural deep eutectic solvents (NADES). High viscosity of NADES was reduced by the addition of 10% of water. The extraction efficiency was comparable to the results of ethanol extraction, while the antioxidant capacity of choline-based extracts was significantly improved [30].

It is important to choose an optimal solvent for the desired extraction, and to reduce solvent usage during experimentation. Traditional experimental procedures for solvent selection use high amount of solvents to choose which one is suitable for the extraction of compounds. For that reason, various computational simulations for solvent selection have been developed, such as Hansen solubility parameters (HSPs) and conductor-like screening model for real solvents (COSMO–RS). These models use theoretical predictions for assessing the solubility of targeted compounds in each solvent [31].

Since HVED treatment involves many processes, different reactive compounds are also formed. Beside generation of free radical species, there is also a possibility of electrodes abrasion and release of metals to the sample. For that reason, it is important to monitor preferred substances like phenols, antioxidants and volatile compounds, but also metals and other undesired compounds. Near infrared (NIR) spectroscopy has been utilized widely in the food and agribusiness ventures in the course of the last 20–30 years to decide significant parts in numerous agrarian items and plant materials [32–34]. In regard to determination of qualitative and quantitative characteristics of agricultural and food products, NIR offers a number of advantages over traditional analytical methods: it is a fast, physical, non-destructive, and non-invasive method, requires minimal or no sample preparation, no reagents are required, and no hazardous wastes are produced [35]. The wavelength range of the NIR spectroscopy from 750 to 2500 nm [36] is related to the vibration of molecules, especially the bands that are due to hydrogen (C-H, O-H, and N-H) vibrations [37,38]. But although the spectra recording is simple, user friendly (no additional sample preparation and use of chemicals) the interpretation of NIR spectra is very complex and chemometric methods are required to extract relevant information and reduce those that are less informative. The most common used tool is principal component analysis (PCA), quantitative analysis using multivariate calibration methods and qualitative analysis using multivariate classification techniques [34,38].

The aim of this work was to understand the green extraction of BACs and volatile compounds from rosemary using computational programs (HSPs and COSMO–RS) and experimental extractions. The experimental solvent extraction was carried out using HVED as a nonthermal technology. The obtained extracts were analyzed for physical and chemical (analytical) parameters. Furthermore, analyses of pesticides and metals in dry rosemary leaves and in HVED extracts were also performed.

2. Results

The extraction of BACs and volatile compounds from dried rosemary leaves were assessed by computational simulation methods and experimental analysis using HVED. Computational simulation methods were performed by HSPs and COSMO–RS where various green solvents were assessed in comparison with conventional solvent n-hexane in order to theoretically predict the probability of solution of BACs from rosemary. Experimental extraction method from rosemary was done using water and ethanol solutions (25 and 50%) as green solvents by means of HVED, and it was compared with modified CE under the same extraction conditions. The obtained extracts by CE and HVED were analyzed for physical parameters (pH, conductivity, temperature, and power), non-volatile compounds (total phenolic content (TPC), antioxidant capacity by 2-diphenyl-2-picrylhydrazyl (DPPH) free radical assay and ferric reducing antioxidant power (FRAP) method, NIR and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC–MS/MS)), volatile compounds by headspace solid-phase microextraction/gas chromatography-mass spectrometry (HS–SPME/GC–MS), and metal content. Additionally, analyzes of pesticides and metals in dry rosemary leaves were performed to assess the safety of raw material for further processing and human consumption. The flowchart of all performed analysis from rosemary is presented in Figure 1.

2.1. Computational Simulation Methods for Assessing Solubility of Rosemary Compounds

The solubility parameters of green solvents for extraction of BACs and aromas from rosemary leaves have been studied by means of the HSP and COSMO–RS theoretical predictions. For both models, solubility results have been presented for various green solvents (ethyl acetate, methylacetate,

ethyl oleate, ethanol, 1-butanol, isopropanol, methanol, limonene, α-pinene, cymene, β-myrcene, cyclopentyl methyl ether (CPME), dimethyl carbonate, methyltetrahydrofuran (MeTHF), and water) compared to conventional *n*-hexane (first column). The HSPs for rosemary compounds were assessed at room temperature (20 °C) for different solvents and relative energy difference (RED) values are summarized in Table 1. RED results have been used to quantify the solutes–solvents interaction.

COSMO–RS combines quantum chemical considerations (COSMO) and statistical thermodynamics (RS) to determine and predict thermodynamic properties without experimental data. The computational simulation results derived by COSMO–RS for rosemary compounds at room temperature is presented in Table 2.

2.2. Experimental Method for Extraction of Bioactive Compounds and Aromas from Rosemary by High Voltage Electrical Discharges

Experimental method for assessing the solubility of BACs from rosemary was performed by green extraction method using HVED and green solvents water and ethanol (25 and 50%). For comparison of results, a modified CE by magnetic stirring was performed at same conditions as HVED: at room temperature, extraction times 3 and 9 min, ratio plant:solvent 1 g:50 mL. For all results in experimental procedure, "R" stands for rosemary, "N" for nitrogen, and "A" for argon. For HVED, numbers 1–12 show the order of conducted treatment. For CE treatments, 3 and 9 are referred to extraction time while 0, 25, and 50 stands for concentration of an ethanol solvent (%).

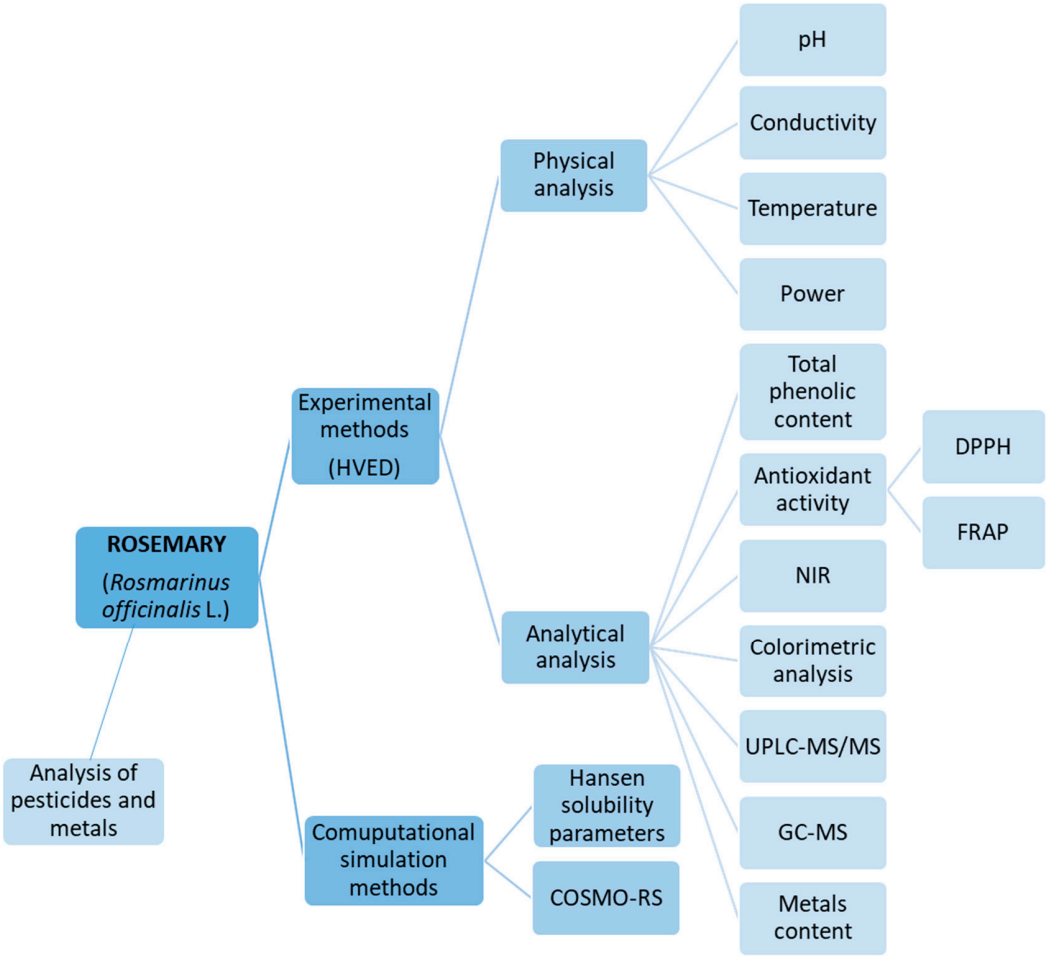

Figure 1. Flowchart of performed analysis in the paper.

Table 1. Hansen Solubility Parameter (HSP) values of relative energy difference (RED) of bioactive compounds from rosemary for different solvents.

Solvents / Compounds	n-Hexane	Ethyl Acetate	Methyl Acetate	Ethyl Oleate	Ethanol	1-Butanol	Isopro-panol	Methanol	Limonene	α-Pinene	Cymene	β-Myrcene	CPME	Dimethyl Carbonate	MeTHF	Water
Monoterpenes																
β-myrcene	1.04	1.45	2.77	0.1	4.5	3.39	3.56	5.57	0.67	0.32	0.67	0	0.5	2.36	0.91	10.49
p-cymen-7-ol	3.01	1.37	1.68	1.93	2.97	1.96	2.15	4.13	1.48	2.17	1.82	1.99	1.59	1.66	1.32	8.84
α-pinene	0.94	1.75	3.05	0.4	4.77	3.64	3.82	5.86	0.72	0	0.59	0.32	0.77	2.65	1.18	10.75
β-pinene	0.83	1.79	3.09	0.43	4.82	3.69	3.87	5.9	0.83	0.11	0.7	0.34	0.82	2.69	1.23	10.8
camphene	0.83	1.79	3.09	0.43	4.82	3.69	3.87	5.9	0.83	0.11	0.7	0.34	0.82	2.69	1.23	10.8
sabinene	1.03	1.74	3.08	0.42	4.78	3.67	3.85	5.87	0.7	0.15	0.47	0.37	0.74	2.6	1.14	10.77
α-phellandrene	1.19	1.48	2.75	0.25	4.46	3.34	3.52	5.56	0.46	0.32	0.51	0.22	0.5	2.38	0.9	10.44
α-terpinene	1.35	1.46	2.66	0.4	4.34	3.21	3.4	5.46	0.3	0.47	0.51	0.38	0.52	2.35	0.87	10.32
δ-terpinene	1.33	1.42	2.63	0.35	4.33	3.2	3.1	5.44	0.34	0.46	0.54	0.34	0.48	2.32	0.84	15.5
Oxygenated monoterpenes																
camphor	1.88	1.42	2.93	0.99	4.49	3.5	3.67	5.52	1.02	1.14	0.73	1.06	0.83	1.99	0.87	10.45
borneol	2.28	0.91	1.72	1.22	3.33	2.23	2.42	4.46	0.9	1.5	1.3	1.28	0.9	1.63	0.71	9.3
α-terpineol	2.37	0.98	1.68	1.32	3.26	2.15	2.34	4.4	0.97	1.58	1.38	1.37	1.01	1.67	0.82	9.22
piperitone	1.82	1.18	2.62	0.8	4.22	3.19	3.37	5.28	0.74	1.02	0.63	0.88	0.55	1.86	0.57	10.19
Sesquiterpenes																
β-caryophyllene	1.04	1.73	2.97	0.42	4.68	3.54	3.73	5.78	0.61	0.14	0.57	0.33	0.74	2.63	1.15	10.65
Diterpenes																
carnosol	2.49	1.55	2.37	1.45	3.78	2.73	2.93	4.95	0.87	1.57	1.12	1.49	1.21	2.1	1.12	9.65
carnosic acid	2.99	1.69	2.05	1.94	3.29	2.26	2.46	4.48	1.38	2.1	1.72	1.98	1.66	2.09	1.48	9.09
rosmanol	2.95	1.42	1.78	1.88	3.07	2.05	2.24	4.24	1.4	2.09	1.74	1.93	1.56	1.78	1.32	8.93
epirosmanol	2.95	1.42	1.78	1.88	3.07	2.05	2.24	4.24	1.4	2.09	1.74	1.93	1.56	1.78	1.32	8.93
rosmadial	2.6	1.25	2.42	1.53	3.8	2.88	3.05	4.85	1.25	1.75	1.28	1.61	1.19	1.56	0.94	9.69
Triterpenes																
betulinic acid	2.05	1.51	2.63	1.04	4.15	3.08	3.27	5.3	0.49	1.11	0.62	1.07	0.88	2.2	0.94	10.08
ursolic acid	2.12	1.54	2.6	1.11	4.11	3.03	3.23	5.26	0.53	1.18	0.71	1.14	0.94	2.22	0.99	10.02
rosmarinic acid	4.56	2.95	2.76	3.49	3.11	2.55	2.69	4.2	2.93	3.65	3.18	3.53	3.17	2.85	2.91	8.29
Flavonoids																
apigenin	4.37	2.78	2.61	3.30	3.07	2.43	2.59	4.19	2.74	3.46	3.01	3.35	2.99	2.74	2.74	8.35
hispidulin	4.44	2.88	2.71	3.37	3.14	2.52	2.67	4.26	2.8	3.52	3.07	3.42	3.07	2.85	2.83	8.38
diosmetin	4.13	2.63	2.6	3.07	3.21	2.5	2.66	4.35	2.5	3.21	2.75	3.11	2.77	2.65	2.53	8.59
hesperidin	4.92	2.87	2.43	3.82	2.38	2.19	2.26	3.27	3.42	4.09	3.67	3.9	3.44	2.4	3.08	7.53
cirsimaritin	4.13	2.64	2.6	3.07	3.21	2.49	2.65	4.36	2.5	3.22	2.76	3.12	2.78	2.68	2.55	8.59
genkwanin	4.04	2.49	2.45	2.97	3.11	2.36	2.53	4.26	2.41	3.12	2.67	3.01	2.66	2.52	2.42	8.55

HSP: Relative energy difference (RED) very good solubility 0–1 (green color); medium solubility 1–3 (yellow color); poor solubility >3 (red color). CPME—cyclopentyl methyl ether, MeTHF—methyltetrahydrofuran.

Table 2. Conductor-like screening model for real solvents (COSMO–RS) probability of solubility (%) of bioactive compounds from rosemary for different solvents.

Compounds \ Solvents	n-Hexane	Ethyl Acetate	Methyl Acetate	Ethyl Oleate	Ethanol	1-Butanol	Isopro-panol	Methanol	Limonene	α-Pinene	Cymene	β-myrcene	CPME	Dimethyl-Carbonate	MeTHF	Water
Monoterpenes																
β-myrcene	69.18	81.28	60.26	100.00	11.22	20.89	16.98	4.17	95.50	81.28	100.00	100.00	100.00	39.81	100.00	0.00
α-pinene	99.08	42.66	26.92	100.00	10.47	22.91	17.38	3.63	95.50	100.00	83.18	87.10	89.13	16.60	85.11	0.00
β-pinene	95.50	57.54	34.67	100.00	12.59	25.70	19.95	4.57	97.95	99.08	89.13	89.13	97.72	22.39	95.50	0.00
camphene	97.72	51.29	34.67	100.00	12.59	25.70	19.95	4.68	97.95	99.31	89.13	91.20	97.72	22.39	93.33	0.00
sabinene	87.10	66.07	46.77	100.00	13.49	25.70	20.42	5.13	99.98	93.97	95.50	95.50	100.00	30.90	100.00	0.00
α-phellandrene	87.10	63.10	44.67	100.00	12.30	24.55	19.50	4.57	100.00	95.50	95.50	95.50	100.00	28.84	100.00	0.00
β-phellandrene	83.56	69.18	48.98	100.00	12.88	25.12	19.95	4.79	100.00	93.33	95.50	97.72	100.00	32.36	100.00	0.00
Oxygenated monoterpenes																
camphor	48.54	86.98	70.07	99.25	44.06	70.94	58.92	21.45	85.15	63.09	91.76	89.39	95.89	53.75	100.00	0.10
borneol	11.22	100.00	89.13	81.28	85.11	100.00	100.00	41.69	17.78	13.49	16.98	16.60	100.00	38.02	100.00	0.02
α-terpineol	11.22	75.86	57.54	50.12	60.26	87.10	77.62	30.90	20.42	14.45	20.89	20.42	97.72	31.62	100.00	0.02
piperitone	32.36	100.00	89.13	85.11	91.20	100.00	100.00	51.29	72.44	46.77	87.10	83.18	87.10	70.79	100.00	0.07
Sesquiterpenes																
β-caryophyllene	99.95	53.70	33.11	100.00	7.94	18.62	14.13	2.34	100.00	100.00	89.13	89.54	100.00	18.20	100.00	0.00
Diterpenes																
carnosol	2.86	100.00	100.00	60.81	100.00	100.00	100.00	58.48	10.59	4.67	13.46	11.99	100.00	100.00	100.00	0.00
carnosic acid	0.86	100.00	100.00	100.00	100.00	100.00	100.00	100.00	3.37	1.41	3.89	3.49	100.00	100.00	100.00	0.00
rosmanol	0.69	100.00	100.00	41.70	100.00	100.00	100.00	100.00	3.22	1.23	4.33	3.76	100.00	100.00	100.00	0.00
epirosmanol	0.69	100.00	100.00	41.70	100.00	100.00	100.00	100.00	3.22	1.23	4.33	3.76	100.00	100.00	100.00	0.00
rosmadial	0.58	100.00	100.00	38.89	100.00	100.00	100.00	96.82	4.06	1.22	6.35	5.58	100.00	100.00	100.00	0.00
Triterpenes																
betulinic acid	6.64	100.00	100.00	48.56	68.99	93.73	90.54	22.83	17.90	9.08	19.53	17.24	100.00	45.69	100.00	0.00
ursolic acid	1.07	100.00	100.00	83.73	100.00	100.00	100.00	100.00	2.78	1.39	2.59	2.32	100.00	45.15	100.00	0.00
rosmarinic acid	0.00	100.00	100.00	34.92	100.00	100.00	100.00	100.00	0.07	0.01	0.13	0.11	100.00	100.00	100.00	0.02
Flavonoids																
apigenin	0.00	100.00	100.00	25.70	100.00	100.00	100.00	100.00	0.02	0.00	0.03	0.03	100.00	100.00	100.00	0.18
hispidulin	0.02	100.00	100.00	25.13	100.00	100.00	100.00	100.00	0.26	0.05	0.47	0.41	100.00	100.00	100.00	0.00
diosmetin	0.00	100.00	100.00	23.44	100.00	100.00	100.00	100.00	0.02	0.00	0.03	0.03	100.00	100.00	100.00	0.07
hesperidin	0.00	100.00	100.00	0.72	100.00	100.00	100.00	100.00	0.00	0.00	0.00	0.00	100.00	100.00	100.00	0.00
cirsimaritin	0.03	100.00	100.00	13.12	100.00	100.00	100.00	100.00	0.38	0.09	0.71	0.63	100.00	100.00	100.00	0.00
genkwanin	0.03	100.00	100.00	13.48	100.00	100.00	100.00	100.00	0.35	0.08	0.65	0.57	100.00	100.00	100.00	0.00

COSMO–RS: Low probability of solubility 0–20% (red color); medium probability of solubility 20–60 % (yellow color); high probability of solubility 60–100% (green color). CPME—cyclopentyl methyl ether, MeTHF—methyltetrahydrofur.

Since the aim of the extraction with HVED was to achieve electrical discharge that is responsible for plant cell disruption and consequently the extraction of BACs from intracellular area. During the experiments, it was difficult to achieve discharge using nitrogen under 20 kV, therefore, for nitrogen treatments voltage of 20 and 25 kV were chosen, while lower voltages of 15 and 20 kV were obtained with argon treatments. All results were measured in duplicates and are presented as average ± standard deviation (SD).

The experiment design performed in STATGRAPHICS Centurion software is presented in Table 3.

Table 3. Denotation of samples, experimental design, and process parameters.

Sample	Treatment Time (min)	Voltage (kV)	Ethanol Content (%)	Stirring Time (min)	Extraction Type
3 R0	0	0	0	3	CE
9 R0	0	0	0	9	
3 R25	0	0	25	3	
9 R25	0	0	25	9	
3 R50	0	0	50	3	
9 R50	0	0	50	9	
RN1	3	20	50	/	HVED
RN2	9	20	0	/	
RN3	3	20	0	/	
RN4	3	25	0	/	
RN5	9	25	25	/	
RN6	9	20	25	/	
RN7	9	20	50	/	
RN8	9	25	50	/	
RN9	3	25	25	/	
RN10	9	25	0	/	
RN11	3	25	50	/	
RN12	3	20	25	/	
RA1	3	15	50	/	
RA2	9	15	0	/	
RA3	3	15	0	/	
RA4	3	20	0	/	
RA5	9	20	25	/	
RA6	9	15	25	/	
RA7	9	15	50	/	
RA8	9	20	50	/	
RA9	3	20	25	/	
RA10	9	20	0	/	
RA11	3	20	50	/	
RA12	3	15	25	/	

2.2.1. Determination of Physical Parameters of Rosemary Extracts

Results of physical parameters of CE and HVED treatment are given Figure 2, including pH, conductivity, power and temperature before and after the HVED treatment.

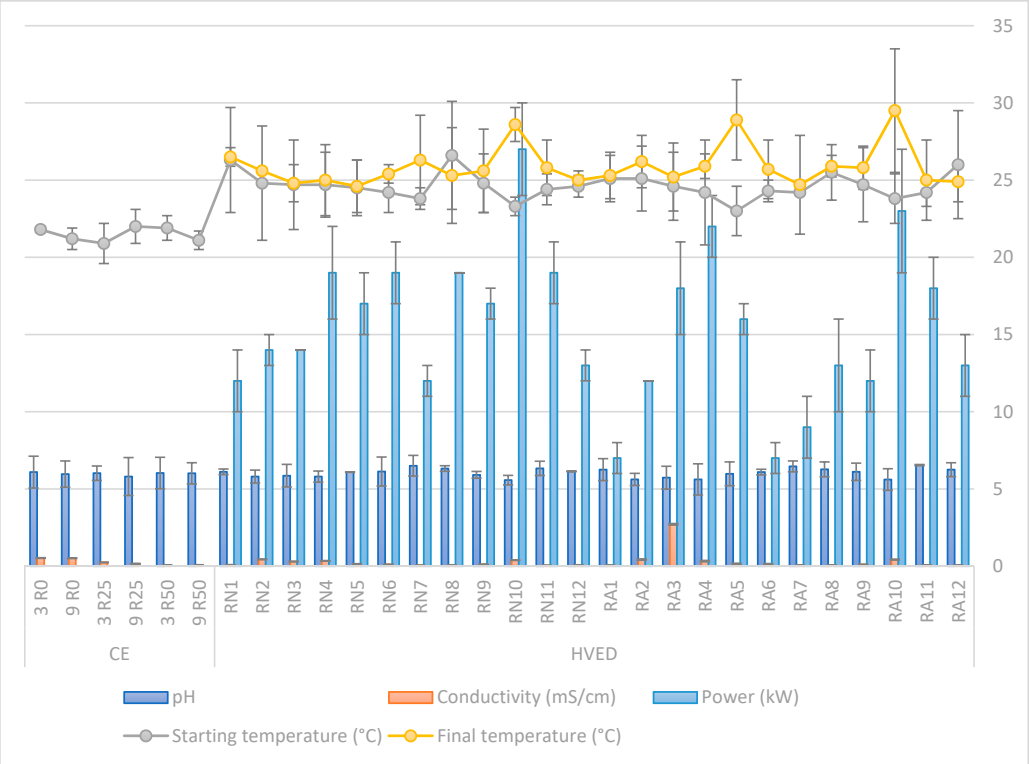

Figure 2. Values of pH, conductivity (mS/cm) for CE and HVED treated samples, starting temperature (°C), final temperature (°C) and power (kW) after HVED treatments.

2.2.2. Determination of Phenols and Antioxidant Activity of Rosemary Extracts

The difference between CE and HVED extraction according to results of TPC, antioxidant parameters and extraction yields are presented in Figure 3. TPC results ranged from 7.21 to 31.64 mg GAE/g of sample. Antioxidant capacity was measured with two different methods—DPPH and FRAP. DPPH ranged from 25.85 to 32.92 µmol TE/g of sample, while FRAP ranged from 44.07 to 562.64 µmol FE/g of sample. Yield of extraction was calculated as g GAE/g of sample × 100 (%).

2.2.3. Near Infrared Spectroscopy and Qualitative Modeling

The recorded NIR spectra in wavelength range from 904 to 1699 nm were used in the qualitative modelling using principal component analysis (PCA) to identify potential grouping (Figure 4) where the sample 9 R50 seemed to be an outlier. Additionally, the Grubbs test was conducted and this sample was confirmed as an outlier.

Furthermore, modeling with different wavelength ranges was performed (Table S1) using the partial least squares regression (PLSR). Four different wavelength ranges were used for four models: Model 1: λ = 904–1699 nm; Model 2: λ = 1349–1699 nm; Model 3: λ = 904–932 and 1349–1699 nm; and Model 4: λ = 904–932 nm. Model efficacy was evaluated using the coefficient of determination (R^2) and the regression point displacement that is the ratio of the standard error of performance (RPD) and the ratio of the range of reference chemistry values to standard error of prediction (RER). RPD is the ratio of standard deviation of the validation data set (SDv) and the standard error of prediction (SEP). How efficient this quantitative prediction of TPC, FRAP, and DPPH is on the rest of 40 % of the samples is presented in Figure 5.

2.2.4. Determination of Color of Rosemary Extracts

The colorimetric analyses of rosemary extracts were measured under International Commission on Illumination (CIE)—L*a*b* color system. Results are shown in Table 4. Results of ΔC, ΔE, and ΔH are

presented for HVED extracts as a result of total color difference, difference in tone color and difference in saturation, respectively, compared to same conditions with CE.

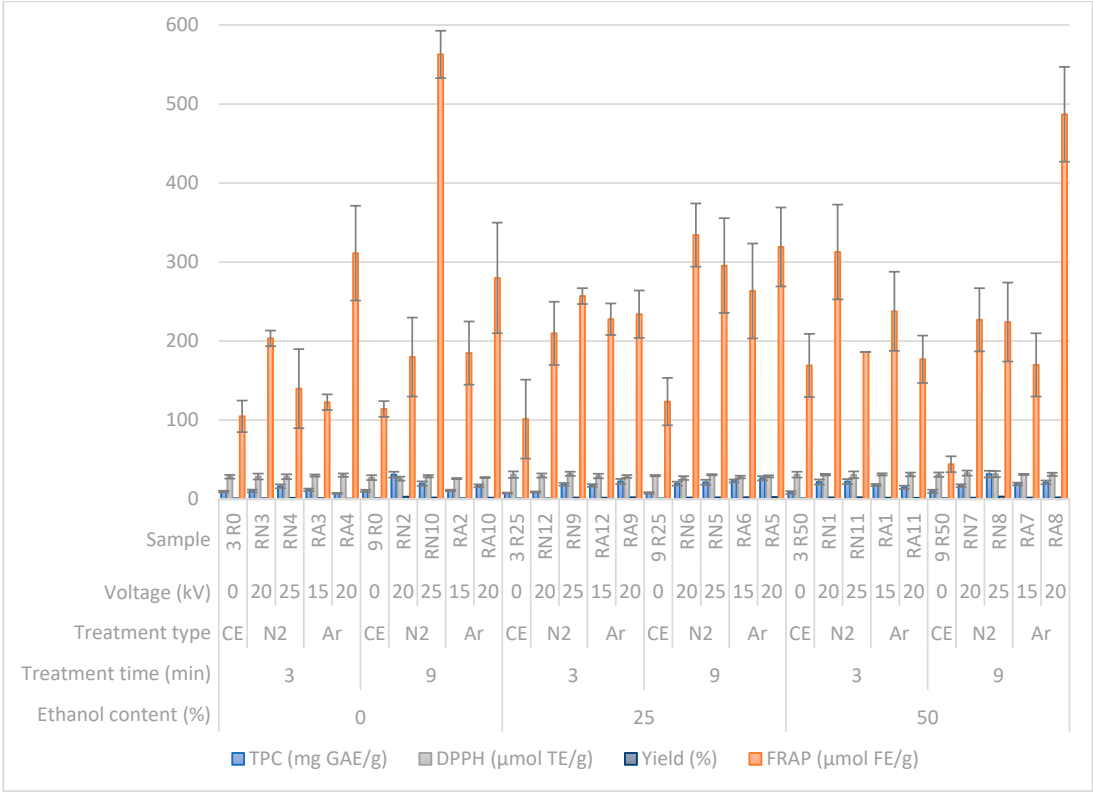

Figure 3. Determination of bioactive compounds—total phenolic compounds (TPC) values, antioxidant activity (2-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing ability of plasma (FRAP) and yield of extraction—measurements for CE and HVED treated samples. TPC = total phenolic content, DPPH = 2,2-diphenyl-2-picrylhydrazyl free radical assay, FRAP = ferric reducing ability of plasma; Treatment type: CE—conventional extraction, N_2—HVED treatment with nitrogen, Ar—HVED treatment with argon.

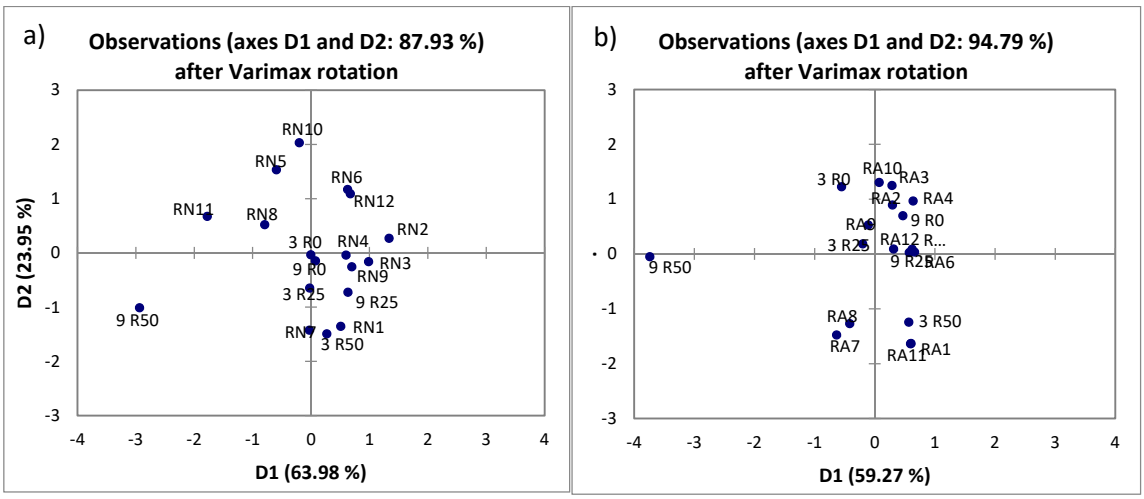

Figure 4. The principal component analysis (PCA) of rosemary extracts: (**a**) The data is denoting untreated rosemary samples, and rosemary samples treated with HVED using nitrogen; (**b**) The data is denoting untreated rosemary samples, and rosemary samples treated with HVED using argon.

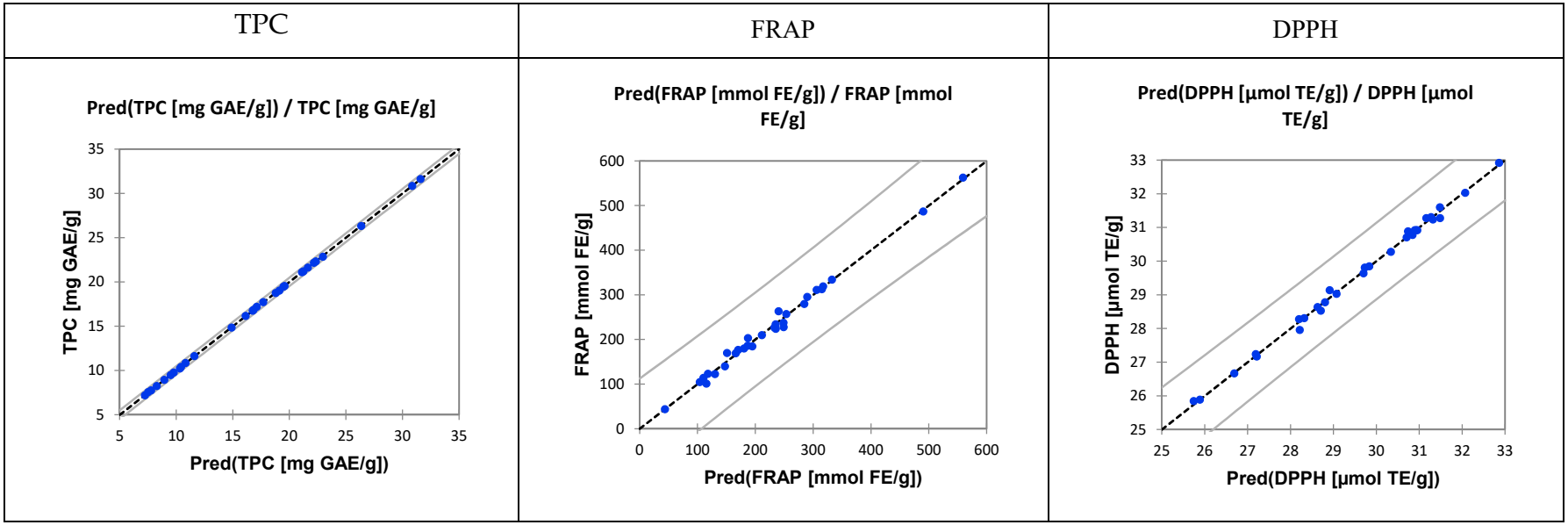

Figure 5. Control chart for predicted and experimental data of analyzed samples, using principal component regression (PCR) models.

Table 4. International Commission on Illumination (CIE)—L*a*b* color parameters of CE and HVED treated rosemary extracts.

Sample	L*	a*	b*	C	h	ΔC	ΔE	ΔH	Extraction Type
3 R0	86.01 ± 2.01	3.87 ± 0.52	35.77 ± 1.79	35.98 ± 1.03	1.46 ± 0.02	/	/	/	CE
9 R0	82.09 ± 3.64	7.09 ± 1.07	44.15 ± 0.61	44.72 ± 1.56	1.41 ± 0.43	/	/	/	
3 R25	95.13 ± 1.97	−0.77 ± 0.03	15.26 ± 3.72	15.28 ± 0.82	−1.52 ± 0.06	/	/	/	
9 R25	93.55 ± 2.27	−0.41 ± 0.04	20.56 ± 4.08	20.56 ± 1.35	−1.55 ± 0.15	/	/	/	
3 R50	95.32 ± 4.31	−1.16 ± 0.37	13.79 ± 1.06	13.84 ± 1.10	−1.49 ± 0.09	/	/	/	
9 R50	94.11 ± 2.55	−0.77 ± 0.15	15.79 ± 2.35	15.81 ± 2.74	−1.52 ± 0.07	/	/	/	

Table 4. *Cont.*

Sample	L*	a*	b*	C	h	ΔC	ΔE	ΔH	Extraction Type
RN1	88.56 ± 1.96	−1.81 ± 1.06	43.33 ± 1.71	43.37 ± 2.67	−1.53 ± 0.16	29.53	30.31	1.03	HVED
RN2	80.89 ± 1.74	6.58 ± 1.21	49.65 ± 2.09	50.08 ± 1.65	1.44 ± 0.27	5.37	5.65	1.30	
RN3	83.86 ±0.72	4.45 ± 0.47	43.24 ± 2.52	43.47 ± 1.79	1.47 ± 0.20	7.49	7.79	0.21	
RN4	84.41 ± 1.82	4.38 ± 0.69	43.40 ± 1.79	43.62 ± 3.40	1.47 ± 0.13	7.64	7.81	0.28	
RN5	92.19 ± 4.18	−1.70 ± 0.82	28.03 ± 0.67	28.08 ± 1.06	−1.51 ± 0.06	7.52	7.70	0.98	
RN6	92.22 ± 2.33	−2.38 ± 0.04	28.18 ± 1.38	28.28 ± 1.75	−1.49 ± 0.00	7.72	7.98	1.55	
RN7	92.42 ± 1.79	−5.22 ± 0.57	36.22 ± 1.64	36.59 ± 0.89	−1.43 ± 0.14	20.79	20.98	2.27	
RN8	92.52 ± 0.64	−4.07 ± 0.03	32.48 ± 0.82	32.73 ± 2.07	−1.45 ± 0.01	16.93	17.09	1.73	
RN9	92.40 ± 2.87	−5.28 ± 0.50	37.19 ± 2.07	37.56 ± 1.46	−1.43 ± 0.03	22.28	22.55	2.17	
RN10	81.96 ± 2.91	6.35 ± 0.73	47.41 ± 4.23	47.83 ± 2.37	1.44 ± 0.12	3.12	3.35	1.21	
RN11	91.92 ± 5.01	−4.42 ± 0.06	36.02 ± 3.16	36.29 ± 1.68	−1.45 ± 0.07	22.45	22.72	0.86	
RN12	95.30 ± 2.69	−1.86 ± 0.07	18.18 ± 1.06	18.27 ± 0.69	−1.47 ± 0.06	3.00	3.12	0.86	
RA1	92.23 ± 3.15	−2.04 ± 0.00	28.69 ± 2.74	28.76 ± 1.41	−1.50 ± 0.04	14.92	15.24	0.26	
RA2	82.44 ± 1.82	5.79 ± 1.14	43.08 ± 1.95	43.47 ± 2.38	1.44 ± 0.19	−1.25	1.72	1.13	
RA3	86.00 ± 2.47	3.43 ± 0.97	36.88 ± 0.56	37.04 ± 1.06	1.48 ± 0.02	1.06	1.19	0.55	
RA4	84.95 ± 0.53	3.72 ± 0.38	38.07 ± 2.03	38.25 ± 0.76	1.47 ± 0.14	2.27	2.54	0.38	
RA5	90.25 ± 2.74	−0.73 ± 0.05	30.17 ± 1.73	30.18 ± 0.19	−1.55 ± 0.12	9.61	10.17	0.11	
RA6	90.89 ± 3.17	−0.76 ± 0.14	28.81 ± 2.49	28.82 ± 1.03	−1.54 ± 0.10	8.26	8.68	0.16	
RA7	92.36 ±1.56	−2.91 ± 0.09	29.96 ± 0.86	30.10 ± 2.07	−1.47 ± 0.06	14.29	14.44	1.05	
RA8	91.24 ± 2.40	−2.84 ± 0.16	33.36 ± 1.78	33.48 ± 1.56	−1.49 ± 0.15	17.67	17.92	0.83	
RA9	92.84 ± 3.77	−1.43 ± 0.62	24.87 ± 0.93	24.91 ± 0.59	−1.51 ± 0.08	9.63	9.90	0.14	
RA10	81.04 ± 1.82	5.72 ± 0.11	43.50 ± 2.00	43.87 ± 2.24	1.44 ± 0.20	−0.84	1.84	1.26	
RA11	94.56 ± 3.09	−2.81 ± 0.23	23.50 ± 1.37	23.67 ± 1.38	−1.45 ± 0.04	9.83	9.88	0.63	
RA12	93.93 ± 2.66	−1.57 ± 0.07	21.44 ± 1.08	21.50 ± 1.59	−1.50 ± 0.13	6.22	6.35	0.41	

L*—lightness from black to white; a* from green to red, and b* from blue to yellow; C—chroma; h—hue angle; ΔE—total color difference, ΔC—difference in chroma; ΔH— difference in hue.

2.2.5. Principal Component Analysis (PCA) of Rosemary Extracts

Data matrix used to identify similarities and/or differences in the data set presenting the samples as well as the physical and chemical properties. The PCAs ability to reduce the dimensionality and increasing the interpretability with minimal information lost was used on the data matrix of experimental data. The matrix included as active variables the physical parameters, total phenolic content and antioxidant capacity, as well as the parameters of color, while the supplementary data set were the experimental conditions (extraction type, HVED treatment time, stirring time, treatment time, voltage, and the ethanol content). The Extraction type (CE or HVED) were included in the analysis as qualitative variables (Figure 6). To present the differences in the samples which are extracted by CE or HVED, in the Supplementary Information are added boxplots for the TPC and AOX by use of DPPH and FRAP method and the yield, as well as the PCA biplot for different extractions and the physical-chemical properties Figures S3 and S4).

Modeling that has followed included the steps of calibration, validation and prediction. In the modelling were included the NIR spectra what is in detail explained in the Section 4.5. Calibration model was developed by principal component regression (PCR). From the data matrix 2/4 of it was used for the calibration and 1/4 was used for the validation while the remaining $\frac{1}{4}$ was used for the prediction. Validation was done by K-fold cross-validation.

2.2.6. Ultra-Performance Liquid Chromatography–Tandem Mass Spectrometry (UPLC–MS/MS) Analysis of Phenolic Compounds from Rosemary Extracts

UPLC–MS/MS analysis was performed to quantify individual phenolic compounds (apigenin, carnosol, diosmetin, hydroxytyrosol, luteolin, oleanolic acid, quercetin, rosmarinic acid, p-cymene, camphor, thymol, and carvacrol) from rosemary extracts (Table 5).

2.2.7. Determination of Volatile Compounds from Rosemary Extracts

Determination of main volatile compounds from rosemary extracts was performed by HS–SPME/GC–MS and analysis included eucalyptol, camphor, borneol and linalool. Results of performed analysis are presented in Table 6.

2.2.8. Determination of Pesticides and Metals

Results of pesticides and metals measured from dried rosemary leaves are shown in Table 7. Also, table includes results of heavy metals measured in selected rosemary extracts. Maximum residue levels (MRLs) are given according to European Commission (EC) Regulations EC No. 396/2005 for pesticides for a rosemary plant and No. 1881/200 for metals for food supplements with rosemary, since rosemary is not listed in this Regulations as a plant.

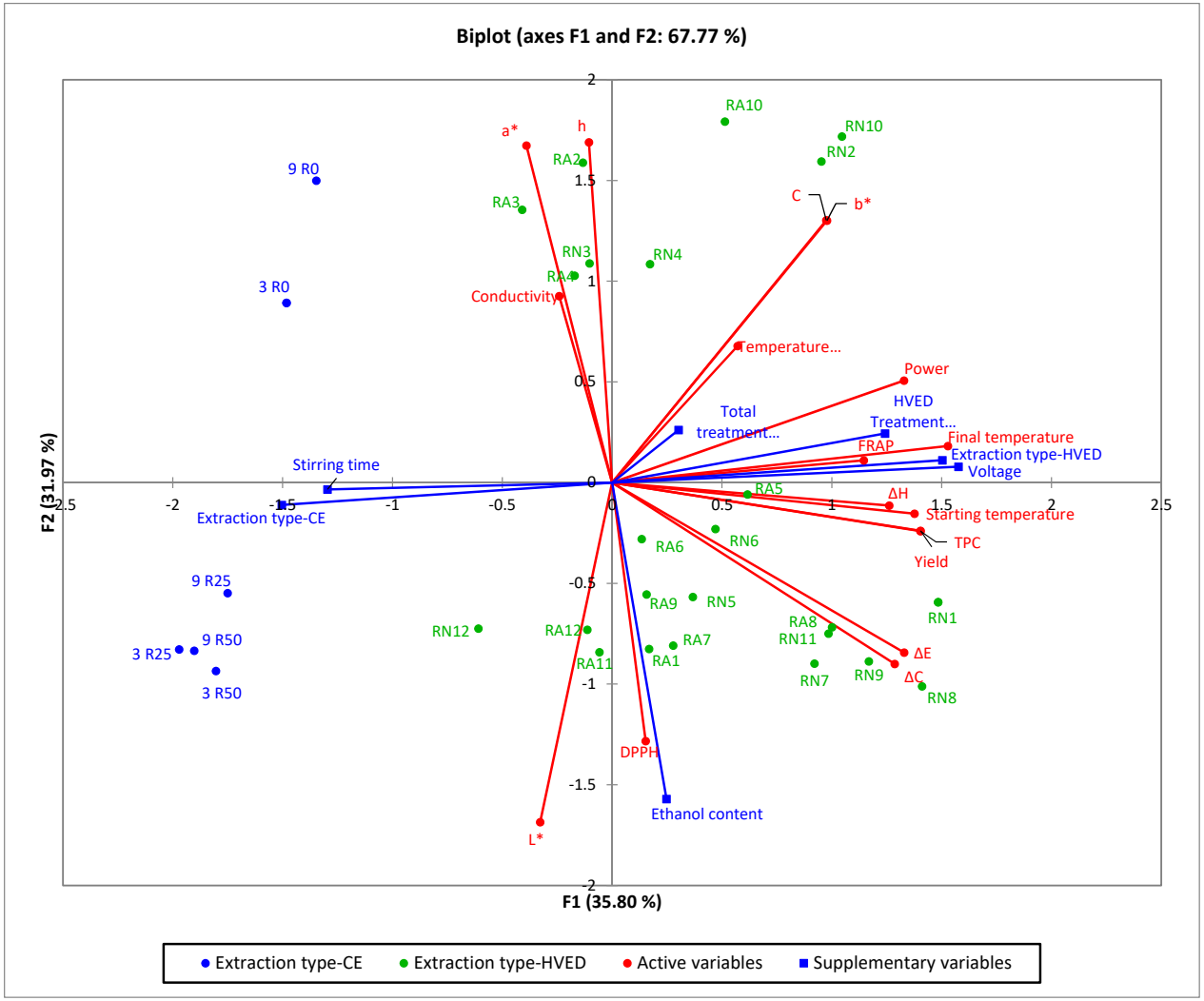

Figure 6. Biplot of the principal component analysis applied on all samples including the active variables (physical parameters, phenols and antioxidants, and parameters of color) and parameters of the experiment design as supplementary variables.

Table 5. Ultra-Performance Liquid Chromatography–Tandem Mass Spectrometry (UPLC–MS/MS) analysis of extractive compounds from rosemary (measurements for CE and HVED treated samples) (ng/mL).

Sample	Apigenin	Carnosol	Diosmetin	Hydroxytyrosol	Luteolin	Oleanolic Acid	Quercetin	Rosmarinic Acid	p-Cymene	Camphor	Thymol	Carvacrol	Extraction Type
3 R0	44.460	0.940	115.897	0.394	180.406	/	/	13.030	0.009	0.602	0.002	0.013	CE
9 R0	32.818	0.869	111.415	0.104	152.254	/	/	0.408	0.059	0.038	0.002	0.001	
3 R25	29.996	1.849	80.044	3.241	154.296	/	0.035	0.767	/	0.007	0.032	0.002	
9 R25	27.342	2.410	82.583	2.983	147.022	/	/	0.761	0.070	0.003	0.002	0.002	
3 R50	66.946	69.323	140.454	15.749	107.979	307.057	0.481	4756.226	0.033	0.949	0.031	0.029	
9 R50	90.244	34.363	179.350	59.951	236.985	390.762	0.711	5100.455	0.729	0.251	0.020	0.300	
RN1	159.160	207.346	310.578	68.141	305.866	288.807	11.271	5797.821	0.001	0.066	0.001	0.035	HVED
RN2	80.659	2.548	255.637	0.362	291.207	/	/	23.421	/	0.003	1.576	/	
RN3	50.146	1.143	166.246	0.583	239.840	/	/	5.173	0.012	0.214	0.062	0.224	
RN4	60.764	1.317	218.043	0.469	399.846	/	/	2.544	0.001	0.069	0.001	0.000	
RN5	137.663	27.816	376.440	96.537	415.194	/	1.608	4228.058	0.010	0.238	/	/	
RN6	107.933	8.988	314.863	61.995	326.021	/	0.269	3591.086	0.025	0.005	0.094	0.004	
RN7	119.723	349.797	177.469	39.265	126.156	2091.128	1.510	5950.966	0.001	0.003	0.002	0.001	
RN8	164.683	117.627	335.963	72.962	304.784	325.866	7.824	5745.552	0.002	0.004	0.003	0.005	
RN9	123.606	303.095	191.720	37.747	122.569	2053.066	1.380	6002.350	/	0.006	0.205	0.000	
RN10	95.125	3.532	362.800	3.845	600.262	4.907	/	29.489	0.001	0.153	0.001	0.000	
RN11	116.828	195.651	194.323	38.968	124.223	1464.630	1.115	5700.140	0.001	0.015	0.001	0.000	
RN12	50.827	6.240	125.214	11.977	167.060	14.068	/	68.983	0.000	0.007	0.000	0.000	
RA1	112.850	286.709	206.963	37.852	167.721	920.212	3.707	5648.074	0.002	0.054	0.005	0.069	
RA2	71.536	2.555	179.178	0.377	246.834	/	/	32.988	0.032	1.776	7.304	0.011	
RA3	42.925	1.156	141.806	0.298	151.249	/	/	16.758	0.009	0.239	0.000	0.000	
RA4	42.412	0.521	140.039	0.268	181.444	/	/	1.106	0.002	0.003	0.000	0.687	
RA5	95.701	11.270	266.810	58.260	285.150	/	0.124	236.826	0.001	0.030	0.013	0.059	

Table 5. *Cont.*

Sample	Apigenin	Carnosol	Diosmetin	Hydroxytyrosol	Luteolin	Oleanolic Acid	Quercetin	Rosmarinic Acid	p-Cymene	Camphor	Thymol	Carvacrol	Extraction Type
RA6	73.333	7.219	207.054	18.538	197.301	/	/	9.584	0.001	0.007	0.000	0.000	HVED
RA7	90.353	236.740	164.949	40.098	127.597	954.465	2.300	5829.363	0.001	/	0.000	0.000	
RA8	111.501	291.279	193.902	39.888	159.881	1001.253	4.224	5872.906	0.018	0.019	0.000	0.000	
RA9	56.649	10.003	173.879	11.916	203.449	/	/	30.652	0.019	0.137	0.000	0.000	
RA10	34.136	1.326	111.703	0.276	239.407	/	/	5.206	0.000	0.006	0.000	0.001	
RA11	76.580	157.254	131.357	26.408	98.402	757.572	1.479	5531.217	0.002	/	0.002	0.001	
RA12	38.301	7.088	129.120	5.393	148.453	/	0.022	21.810	0.000	0.015	0.001	/	

/—not detected.

Table 6. Headspace solid-phase microextraction/gas chromatography-mass spectrometry (HS–SPME/GC–MS) analysis of volatile compounds from rosemary (measurements for CE and HVED treated samples) (%).

Sample	Area (%)				Extraction Type
	Eucalyptol (RI = 1038)	Camphor (RI = 1150)	Borneol (RI = 1172)	Linalool (RI = 1103)	
3 R0	40.33	26.70	13.46	/	CE
9 R0	34.89	24.81	15.78	/	
3 R25	/	/	/	/	
9 R25	/	/	/	/	
3 R50	/	/	/	/	
9 R50	/	/	/	/	
RN1	/	/	/	/	HVED
RN2	31.04	22.8	14.73	3.46	
RN3	39.44	24.63	15.58	2.96	
RN4	32.56	22.19	13.42	2.79	
RN5	2.52	1.12	0.24	/	

Table 6. *Cont.*

Sample	Area (%)				Extraction Type
	Eucalyptol (RI = 1038)	Camphor (RI = 1150)	Borneol (RI = 1172)	Linalool (RI = 1103)	
RN6	2.28	0.99	0.40	/	
RN7	/	/	/	/	
RN8	/	/	/	/	
RN9	/	/	/	/	
RN10	25.66	20.88	15.99	/	
RN11	/	/	/	/	
RN12	/	/	/	/	
RA1	/	/	/	/	
RA2	28.36	25.01	15.91	1.56	
RA3	34.58	24.14	11.60	/	HVED
RA4	30.27	24.64	9.51	/	
RA5	3.92	2.07	0.46	/	
RA6	3.20	1.68	0.61	/	
RA7	/	/	/	/	
RA8	/	/	/	/	
RA9	/	/	/	/	
RA10	30.65	37.83	6.22	1.19	
RA11	/	/	/	/	
RA12	/	/	/	/	

/—not detected.

Table 7. Residue levels and maximum residue levels (MRL) of pesticides (mg/kg) and metals (mg/kg) in rosemary samples.

	Component	MRL (mg/kg)	Content (mg/kg)	HVED Extracts			
				RA8	RN7	RN9	RN11
Pesticides	Alachlor	0.02	<0.005	/	/	/	/
	Aldrin and Dieldrin (Aldrin and dieldrin combined expressed as dieldrin)	0.01	<0.002	/	/	/	/
	Captan (Sum of captan and THPI, expressed as captan)	0.06	<0.020	/	/	/	/
	DDT (sum of p,p′-DDT, o,p′-DDT, p-p′-DDE and p,p′-TDE (DDD) expressed as DDT)	0.05	<0.004	/	/	/	/
	Endosulfan (sum of alpha- and beta-isomers and endosulfan-sulphate expresses as endosulfan)	0.05	<0.002	/	/	/	/
	Endrin	0.01	<0.004	/	/	/	/
	Heptachlor (sum of heptachlor and heptachlor epoxide expressed as heptachlor)	0.01	<0.002	/	/	/	/
	Hexachlorobenzene	0.01	<0.002	/	/	/	/
	Hexachlorocyclohexane (HCH), alpha-isomer	0.01	<0.002	/	/	/	/
	Hexachlorocyclohexane (HCH), beta-isomer	0.01	<0.002	/	/	/	/
	Iprodione	0.02	<0.010	/	/	/	/
	Lindane (Gamma-isomer of hexachlorocyclohexane (HCH))	0.01	<0.002	/	/	/	/
	Methoxychlor	0.01	<0.010	/	/	/	/
	Tolylfluanid (Sum of tolylfluanid and dimethylaminosulfotoluidide expressed as tolylfluanid)	0.05	<0.020	/	/	/	/
	Vinclozolin	0.02	<0.004	/	/	/	/
Metals	Lead (Pb)	3.00	<0.050	/	/	/	/
	Cadmium (Cd)	1.00	<0.006	/	/	/	/
	Mercury (Hg)	0.10	0.026	/	/	/	/
	Chromium (Cr)	/	0.240	55.3	66.1	71.0	60.5
	Nickel (Ni)	/	0.322	2.10	1.10	1.20	0.950
	Manganese (Mn)	/	21.00	7.10	5.10	5.45	6.20
	Iron (Fe)	/	163	23.6	17.6	17.0	19.8
	Copper (Cu)	/	6.40	3.00	3.75	3.95	6.90
	Zinc (Zn)	/	26.0	6.65	9.10	10.7	20.5

/—no data available.

3. Discussion

The aim of the present study was to provide the potential of green solvents to extract BACs from rosemary leaves comparing theoretical and experimental methods.

3.1. Computational Simulation Methods for Assessing Solubility of Rosemary Compounds

For extractions of BACs, *n*-hexane is one of the most used solvents due to its low polarity, optimal boiling point, easy removal from the product by evaporation and stability. On the other side, n-hexane is a solvent of petrochemical origin, which are nowadays strictly regulated by European Directives. For that reason, industries are forced to replace such solvents with more sustainable alternative solvents [39]. Therefore, various green solvents have been chosen for assessing the potential for extraction of BACs from rosemary (Tables 1 and 2). Every solvent showed different theoretical solubility for rosemary BACs, and that can be explained by the differences in the solvent polarities. Generally, the optimum HSPs for very good solubility of solutes in solvents are presented with green color (Table 1). It can be concluded that many alternative (green) solvents are capable for extraction of BACs from rosemary, some even with higher affinity for extraction, compared to conventionally used *n*-hexane. According to RED results, by evaluating compounds with most green color (very good solubility), followed by yellow (medium solubility) and red color (poor solubility), the potential for extractions of BACs from rosemary was in the following order: CPME > limonene > cymene > MeTHF > ethyl oleate > β-myrcene > α-pinene > ethyl acetate > *n*-hexane > methylacetate > dimethyl carbonate > isopropanol > 1-butanol > methanol > ethanol > water. Results showed that α-pinene, cymene, β-myrcene, CPME, and ethyl oleate had high potential for extraction of volatile compounds such as monoterpenes and sesquiterpenes. For that reason, these solvents should be used for extraction of volatile compounds of essential oil from rosemary. Moreover, water and ethanol showed low potential for extraction of most evaluated compounds from rosemary.

Results of COSMO–RS solubility assessment (Table 2) showed similar trend for solvents like HSPs, although some differences have been noticed. COSMO–RS results showed following order according to most results with high probability of solubility (green color): MeTHF > CPME > ethyl acetate > methylacetate > 1-butanol > isopropanol > ethanol > ethyl-oleate > dimethylcarbonate > methanol > limonene > cymene > β-myrcene > α-pinene > *n*-hexane > water. It is clear that all green solvents have higher potential for extraction of rosemary compounds compared to conventional n-hexane, except water. According to results, ethyl acetate, methylacetate, ethanol, 1-butanol, isopropanol, methanol, CPME, dimethylcarbonate and MeTHF, showed high probability of solubility for diterpenes, triterpenes and flavonoids, which was not showed with HSPs assessment.

3.2. Experimental Analysis of Extraction of BACs and Aromas from Rosemary Using HVED

3.2.1. Physical Parameters of Rosemary Extracts

Results of pH, conductivity, power and temperature during extraction of rosemary BACs using HVED and CE are given in Figure 2. Temperature was measured before and after the HVED treatment and the maximum elevation of 5.9 °C was noted with sample RA5. In average, extracts treated for 9 min had 1 °C higher final temperature compared to extracts treated for 3 min. Also, maximum temperature was 29.5 °C after HVED treatment and it can be concluded that no significant elevation in temperature was noted during HVED treatment. Since all temperatures were under 30 °C, HVED was confirmed as a non-thermal extraction method. Results of pH for all extracts variated between 5.57 and 6.54 and no significant changes in pH were observed during the HVED treatment. pH and conductivity significantly depended ($p \leq 0.05$) only on ethanol content. With higher content of ethanol in the solution, pH increased and conductivity decreased for both extraction types that is expected according to literature data [40].

The power used for the treatment with HVED changed from 7.0 to 27.0 kW. The highest power was noted for sample RN10 (27.0 ± 3.0 kW) which is expected since it was the sample treated for longer

time 9 min) and the highest voltage (25 kV). Accordingly, power significantly increased with higher voltage used for the treatment and decreased with higher ethanol content in the sample.

3.2.2. Total Phenolic Content and Antioxidant Activity of Rosemary Extracts

The goal of the extraction with HVED was to extract highest yield of phenolic compounds and antioxidants and compare results with same conditions by modified CE (at room temperature) (Figure 3). HVED treated samples showed 0.76–3.39-times higher yield of phenolic content for same extraction parameters than CE. On average, HVED had 2.13-times better yield of extraction of phenols than CE. The highest TPC value was noted for sample RN8 that was treated with HVED for 9 min at 25 kV with 50% of ethanol as a solvent. Nitrogen, longer treatment time and higher voltage yielded higher results of phenolic compounds, but only the treatment time had a statistically significant influence to TPC score ($p \leq 0.05$). Ethanol content showed different trend for each treatment, with CE, the highest scores were obtained with water, HVED treatment with nitrogen was highest with 50% of ethanol, while treatment with argon had highest yield of TPC with 25% of ethanol. Bellumori et al., (2016) performed extractions from dried rosemary leaves by ultrasound-assisted extraction and microwave-assisted extraction for 10 min. Their results also showed higher amounts of TPC with ethanol as a solvent, compared to water, but their maximum obtained results were slightly higher compared to HVED, 35.0 mg/g for ultrasound and 36.6 mg/g DL for microwave-assisted extraction with ethanol (ACS grade, ≥99%), but different method for calculation was used [23].

Results of DPPH did not vary notably between extracts. However, the significant correlation was observed for DPPH with treatment time and ethanol content, it was higher with shorter treatment time and higher percentage of ethanol in the solution. Some differences in DPPH results could be due to generation of free radicals that is characteristic for HVED treatment that could influence to the antioxidant activity of extracts and have a possibility to interact with DPPH radical. FRAP results showed similarities with TPC values for HVED extraction, it was higher with longer treatment time, higher voltage used and with treatment using nitrogen, compared to argon. In average, HVED extraction showed 2.39-times higher antioxidant capacity, according to FRAP results, when compared with CE.

In total, higher results for phenolic content and antioxidants was noted with ethanol than with water. These results are in line with theoretical predictions with HSPs and COSMO–RS, although pure ethanol was used for calculations, while in experimental procedure 25 and 50% ethanol was used. Additionally, other green solvents were used for experimental assessment for extraction of BACs using HVED including limonene, α-pinene, glycerol, ethyl acetate and dimethyl carbonate. However, no electrical discharge was achieved during the extraction with HVED when mentioned solvents were used. This could be explained by high viscosity and density of these solvents [41,42]. Also, terpenes, such as limonene and pinene are oil solvents that are not able to mix with water and are therefore suitable for extraction of volatile compounds and not water-soluble compounds [41].

3.2.3. Near Infrared Spectroscopy of Rosemary Extracts and Modeling

The recorded NIR spectra showed grouping in the range of λ = 904–1699 nm which is the result of different vibrations of molecules as C–H, O–H, and N–H bonds. NIR spectra was specific in two regions: 904–925 nm and 1350–1699 nm. Overlapping of NIR scans is visible with specific differences in the range of 904–925 nm, indicating absorption detecting differences of the third overtone region and detecting different vibration of C–H bonds as well as HOH region at 1400 nm (water λ = 1400–1460 nm) and continues to the end of the recorded spectra indicating differences in the vibrations of the first overtone of C–H3, Ar–CH, C–H, and C–H2 bonds and second overtone of O–H, N–H, Ar–CH, and R–OH [43,44].

From those findings it was clear that the content of TPC and the antioxidative activity could be predicted. A part of the input spectral data was used for the model training (60%). After the training followed the PLSR model evaluation and its testing on unknown samples. Our aim was to predict those parameters not only qualitative but also quantitatively. In order to gain more accurate models,

four different wavelength ranges were used (Model 1: λ = 904–1699 nm; Model 2: λ = 1349–1699 nm; Model 3: λ = 904–932 and 1349–1699 nm; and Model 4: λ = 904–932 nm). The first model (Table S1) which included the total range of NIR spectra resulted with the best model efficiency parameters (RPDs > 3 and RER > 10) what is an indication of very good quantitative model prediction [45]. The efficacy of model for prediction of TPC, DPPH, and FRAP using NIR is presented in Figure 5.

3.2.4. Colorimetric Analysis of Rosemary Extracts

The color of the product is an important aspect that specifies the commercial quality of the product and has an effect to the consumer's final purchase decision [46]. The color measurements of the different extracts prepared by conventional and HVED extraction are given in terms of L*, a*, b*, C, and h values under CIE—L*a*b* color system. According to the results, HVED treated samples had lower values of L* (darker), a* (more green), and higher values of parameter b* (more yellow) when compared to CE in same conditions (Table 4). The HVED extraction also caused increasing in parameter C resulting with increase and discernible difference in color intensity and slight increase in hue of extracts. Differences with untreated extracts are expressed as ΔE—total color difference, ΔC—difference in tone color; ΔH—saturation. Based on these data with more intense coloring of HVED extracts (mass and the solvent used were equally as with CE), it can be concluded that HVED treatment had damaged the cell structure and that the pigments contained within the plant cells exited the cell surface causing changes in extract color [47]. Since the cavitation is caused, the process of extraction of BACs is also facilitated [48]. In our study, ethanol content had significant effect on color parameters (L*, a* and b*) in rosemary HVED treated samples with both argon and nitrogen. All colorimetric parameters, except ΔH, had statistically significant dependence ($p \leq 0.05$) on ethanol content—with higher ethanol content, parameters L, ΔC, and ΔE increased, while a*, b*, C, and h decreased. Saturation significantly depended on treatment time and gas used, more saturation was noted with longer treatment time and use of nitrogen during the treatment.

In general, results indicated that HVED extracts had dark greenish-brown color with darker color when compared to CE which is associated also with higher phenolic compounds and antioxidant content. Plasma reactive species induce release of some BACs that are covalently bonded to the plant matrix which accordingly results with increase in TPC and greater antioxidant capacity and consequently with changes in extract colors. The reason is that phenolic compounds result with different color in free and bound forms [49,50].

3.2.5. Principal Component Analysis (PCA) of Rosemary Extracts

Based on the PCA biplot showed in Figure 6, the extraction type divided samples on the left and right part of chart quadrants. Extraction with CE positioned the samples in the second and third quadrant while the HVED extraction has spread the samples in all four quadrants with the main sample concentration in the fourth quadrant. The physical composition, content of TPC and antioxidant capacity as well the color parameters of the extracts. The experiment conditions are dominantly positioned near to the first principal component, PC1 (time (of HVED treatment, stirring and total treatment), voltage) with the exception—ethanol content.

The subjected table to the PCA chart-squared cosines of the variables asserts the HVED treatment time, stirring time, total treatment time and voltage used as experiment condition as values for which the squared cosine is the largest.

Biplot in the form where the active variables (physical properties, TPC, antioxidant capacity (DPPH and FRAP), and color parameters) are related to the supplementary which are the experimental design parameters, show the correlations between them and for which samples are they dominant. So accordingly, in the fourth quadrant the almost overlap of parameters as the DPPH content and the ethanol content indicating that the antioxidant capacity by DPPH method was higher in those samples where the ethanol content was 25% or 50% (Samples RA1; RA7; RA8; and all other positioned in the fourth quadrant, with the higher DPPH values of 31.28% (RA1 and RA7) and 31.31% (RA8),

respectively. Applying the same rule of data relation analysis in the third quadrant, samples RN12; RA11; and RA12 as well as 3 R25; 3 R50; 9 R25; and 9 R50 will have the highest values of the parameter L*, what confirmed the results presented in Table 4.

3.2.6. Analysis of Individual Bioactive Compounds from Rosemary Extracts

Data of UPLC–MS/MS analysis for individual phenolic compounds (Table 5) showed that main phenolic compounds in rosemary are: apigenin, diosmetin and rosmarinic acid. Comparison of phenolic compound in different type of extraction (CE and HVED) and conditions in extraction type showed that: apigenin, carnosol, diosmetin, hydroxytyrosol, luteolin, oleanolic acid, oleuropein, quercetin, and rosmarinic acid were higher in HVED extracts compared with CE extracts. The example of chromatograms for selected extracts is presented in Supplementary Materials (Figure S1). For this purpose, the extract with highest content of phenolic compounds detected with UPLC–MS/MS was chosen (RN9, Figure S1b) and compared with extract extracted with CE (3 R25, Figure S1a) under same conditions (3 min, 25 % of ethanol).

Statistical analysis showed that most of the measured compounds significantly depended only on ethanol content (apigenin, carnosol, diosmetin, hydroxytyrosol, luteolin, oleanolic acid, oleuropein, quercetin, and rosmarinic acid) and additionally, apigenin, carnosol, diosmetin, hydroxytyrosol, and luteolin depended on treatment time.

HVED is considered to be energy- and cost- saving method for successful extraction of phenolic compounds from rosemary. However, further analysis of energy and environmental impact should be performed. Hirondart et al., (2020) have obtained rosmarinic acid, carnosic acid, and carnosol by PLE in hydroalcoholic solution and conventional Soxhlet extraction. Extract yields of bioactive compounds were similar with both methods, energy consumption was lower for PLE extraction because less solvent had to be heated, and the cost was reduced with a smaller amount of waste generated [51].

When compared with theoretical results, it is clear that a similar trend for solution in water and ethanol was noticed. With higher ethanol content, solubility of most of extracted compounds increased, as well as a sum of all phenolic compounds. According to HSP results, all compounds that were analyzed with UPLC–MS/MS showed poor solubility (red color in Table 1) in both water end ethanol, but better results (higher solubility) was presented with ethanol as a solvent. However, COSMO–RS results gave better solubility results for extraction with ethanol. Results showed that carnosol, rosmarinic acid, apigenin and diosmetin have 100% of solubility in ethanol (green color in Table 2), while camphor has 44.06% solubility (yellow color). Experimental results confirmed these results since apigenin and carnosol showed the highest results with 50% of ethanol as a solvent, 164.68 and 349.80 ng/mL respectively, and diosmetin and rosmarinic acid showed highest measured results 25% of ethanol as an extraction solvent, 376.44 and 6002.35 ng/mL, respectively. Camphor was found in small amounts in all extracts (<1 ng/mL) except in water extract RA2 (1.78 ng/mL). Results are in line with one previously reported for oregano [52].

3.2.7. Analysis of Volatile Compounds from Rosemary Extracts

HS–SPME is a rapid, simple, inexpensive, solvent-free and highly sensitive technique [53]. Volatile organic compounds composition is strongly dependent on the extraction method. Results of the chemical composition of HS is presented in Table 6. The predominant HS compound was the cyclic monoterpene ether eucalyptol (40.33–2.28%). A second compound was cyclic monoterpene ketone camphor (26.70–0.99%), followed by bicyclic monoterpene borneol (15.99–0.24%). Terpene alcohol linalool (3.46–1.19%) was found in a smaller percentage. The concentration of these terpenes depends on the treatment of the plant with ethanol content or gas used (nitrogen or argon). From Table 6, it is notable that when water is used as an extraction solvent, the percentage of all three monoterpenes is high except for linalool. It was difficult to characterize volatile compounds in extracts with ethanol since overlapping profile of peaks happened with ethanol peak. Therefore, no results for most samples with ethanol were presented. The traceability is similar in the RN2-RN6 sample as in the RA2-RA6

sample, except that the concentration of linalool monoterpene alcohol in these samples deviated. Linalool was not found in the RN10 sample, while it was found in RA10. Chromatograms for two extracts with highest concentrations obtained by CE (3 R0, Figure S2a) and HVED (RA10, Figure S2b) are given in Supplementary Materials.

It was not possible to compare data with theoretical predictions for ethanol and water solubility of volatile compounds, since incomplete results are provided for ethanol extracts. However, the comparison between camphor and borneol can be provided to compare theoretical and experimental data. HSPs showed poor solubility in both ethanol and water for both compounds, but borneol had slightly higher chances for solubility (RED = 3.33 in ethanol and 9.3 in water), compared to camphor (RED = 4.49 for ethanol and 10.45 for water). From COSMO–RS results, it was also predicted that camphor and borneol have low chances for solubility in water, 0.1% and 0.02%, respectively, but different results were given for ethanol: camphor has medium probability of solubility (44.06%), while borneol has high probability (85.11%). Experimental results were opposite and higher concentrations of camphor were found in all extracts, compared to borneol. Moreover, results were closer to COSMO–RS results than HSPs since both compounds were extracted in higher concentrations: 37.83% of camphor was extracted in sample RA10 and 15.99% of borneol in sample RN10. Mena et al., (2016) showed similar results in conventionally performed acetone-based extraction from rosemary, they have extracted more camphor (41.52 ± 6.00 μg/g) than borneol (11.92 ± 2.01 μg/g) in their extracts [54].

SFE is a procedure recently used for the extraction of bioactive compounds and purification of rosemary essential oil. Mouahid et al., (2017) have compared the efficiency of SFE and hydrodistillation of rosemary leaves. The essential oil obtained by SFE had an increase in yield of monoterpenoids for 37%, while yields of individual monoterpenes varied [16]. Pereira et al., (2007) have performed a cost analysis of the extraction of rosemary essential oil by SFE and steam distillation. The manufacturing cost with SFE was lowered, while the lower profitability of steam distillation was a consequence of higher energy consumption and lower content of essential oil in the extracts [55]. SFE was used to extract the volatiles from rosemary and combined with SWE for the recovery of polyphenols from the produced extract. A combination of these processes resulted in a 28% reduction of operating cost when compared to the separate use of these techniques [56]. Since HVED was presented as a method that is more efficient for extraction of non-volatile than volatile compounds, it could be considered to be used in a combination with some other techniques for better extraction efficiency as well.

3.2.8. Analysis of Pesticides and Metals

Although rosemary could be considered as a nutritional supplement, there are still no categories in European Commission (EC) Regulation, herbs or plant tea, therefore the high levels of pesticides and heavy metals that can be found in its dried leaves could possess serious toxicological effects on human health. For that reason, the analysis of pesticides and heavy metals in the rosemary samples were measured and are presented in Table 7. This analysis is important for preparing healthy extracts from dried rosemary leaves that could be further used for new functional food. Trace analysis of pesticides residues were analyzed in dried leaves and for heavy metals residues were analyzed in dried leaves and HVED extracts. Residues levels of all pesticides were lower than limit of quantitation of method which is quite below maximum residue level (MRL) according to EC Regulations No. 396/2005 for rosemary as a plant. In EC regulation MRL of some pesticides with similar structures (such as DDT, endosulfan, aldrin, heptachlor, etc.) are grouped.

Residue levels of Lead (Pb) and Cadmium (Cd) in dried rosemary leaves were also below the limit of quantitation of the method, and only the level of Mercury (Hg) was slightly higher than limit of quantitation. However, all these values were quite below MRL according to EC No. 1881/2006. Furthermore, other metals (Chromium (Cr), Nickel (Ni), Manganese (Mn), Iron (Fe), Copper (Cu), and Zinc (Zn)) were measured in selected HVED extracts with high phenolic and antioxidant content (RA8, RN7, RN9, and RN11) as well and results are presented in Table 7. These metals are not included in EC Regulations so no MRL data were provided. Although the data should not be compared since

data before the HVED extraction are given per g of dried herb and data after HVED extraction are given per gram of extract, it is clear that content of Cr and Ni increased in extracts, while level of other metals decreased after HVED extraction. From this data it is notable that during the HVED treatment, levels of some toxic metals are increasing that could be the result of abrasion of electrodes during the treatment.

Rosemary plant was compliant regarding content of contaminants, pesticide residues and toxic metals. With respect to this, obtained results showed that rosemary samples are safe for use in human dietary. On the other hand, some changes in levels of metals could happen during the treatment and further detailed analyses should be done to assess this issue.

4. Materials and Methods

The concept of this work is presented in Figure 1, where all analysis performed for rosemary are presented as a flowchart.

4.1. Plant Materials

Dried rosemary leaves (*Rosmarinus officinalis* L.) were provided by local drugstore (Suban d.o.o., Samobor). Herbs were stored in polyethylene bags in a dark and dry place until extractions. Dried rosemary leaves were grinded to plant particle size distribution of d(0.1) ≤ 3 9.683 μm; d(0.5) ≤ 224.816 μm; d(0.9) ≤ 425.819 μm measured by the laser particle size analyzer Mastersizer 2000 (Malvern Instruments GmbH, Herrenberg, Germany). For the extraction, 1 g of herb material was weighted into the beaker of 100 mL and mixed with 50 mL of extracting solvent at room temperature (22 °C). Extraction was carried out using distilled water, 25% and 50% aqueous ethanol (*v*/*v*) as extraction solvents.

4.2. Computational Simulation Methods

4.2.1. Hansen Solubility Parameters (HSPs)

HSP provide a convenient and efficient way for characterization of solute-solvent interactions according to the classical "like dissolves like" rule. A detailed concept of HSPs is described in Aissou et al., (2017) [57]. For solvent optimization, a simple composite affinity parameter, the RED number, has been calculated to determine the solubility between solvents and solutes.

$$\mathrm{RED} = \frac{\mathrm{Ra}}{\mathrm{Ro}} \quad (1)$$

where R_o is the radius of a Hansen solubility sphere and R_a is the distance of a solvent from the center of the Hansen solubility sphere.

A potentially good solvent has RED number smaller than 1 (the compound has similar properties and will dissolve), while medium and poor solvents have RED values of from one to three and more than 3, respectively. The chemical structures of the solvents and solutes discussed in this article could be mutually transformed by JChemPaint version 3.3 (GitHub Pages, San Francisco, CA, USA) to their simplified molecular input line entry syntax (SMILES) notations, which were subsequently used to calculate the solubility parameters of the solvents and compounds (HSPiP Version 4.0, Hansen Solubility, Hørsholm, Denmark).

4.2.2. COSMO–RS Software

The COSMO–RS was developed by Klamt and co-workers as a statistical thermodynamic method for molecular description and solvent screening based on a quantum-chemical approach [58]. COSMO–RS prediction is a two-step procedure—microscopic and macroscopic. The procedure was explained in details by Aissou et al., (2017) [57]. The COSMOthermX program (version C30 release 13.01, COSMOlogic, Leverkusen, Germany) was used to calculate the relative solubility between the

solid compound and the liquid solvent in terms of the logarithm of the solubility in mole fractions ($\log_{10}(x_{solub})$). The logarithm of the best solubility was set to 0 and all other solvents were given relative to the best solvent. Also, the logarithm was transformed into probability of solubility (%). The calculation was performed at room temperature (20 °C) and at boiling temperature for each solvent.

4.3. High Voltage Electrical Discharge (HVED) and Conventional Extraction (CE)

HVED was performed with "IMP-SSPG-1200" generator (Impel group d.o.o., Zagreb, Croatia) that generated rectangular pulses using direct current and achieving high voltage. Maximum adjustable current was 30 mA and voltage up to 25 kV. Based on conducted preliminary experiments with different HVED parameters (frequency, voltage, pulse length, distance between electrodes, and ratio mass to solvent), fixed HVED parameters were chosen as follows: frequency of 100 Hz, pulse width 0.4 microseconds, voltage 15 and 20 kV for argon gas, and 20 and 25 kV for nitrogen gas, the gap between electrodes of 15 mm, treatment duration 3 and 9 min, and ratio mass to solvent 1 g:50 mL (according to pharmacopoeia). Mixture of herb material and solvent was transferred to beaker shaped reactor of 100 mL. This reactor that is opened on both sides was fitted with silicone tops with 1 cm in diameter. Silicone tops were used due to easier mounting of the electrode from the top and needle form the bottom. Gases (argon or nitrogen) were flowed in through the needle with the flow 0.5–1 L/min. Set-up of generator and reactor are shown in Figure 7. For measuring the output voltage (data not shown), oscilloscope Hantek DS05202BM (Tektronix, Inc., Beaverton, OR, USA) connected to the high voltage probe Tektronix P6015A (Hantek Electronic Co., Ltd., Qingdao, China) was used.

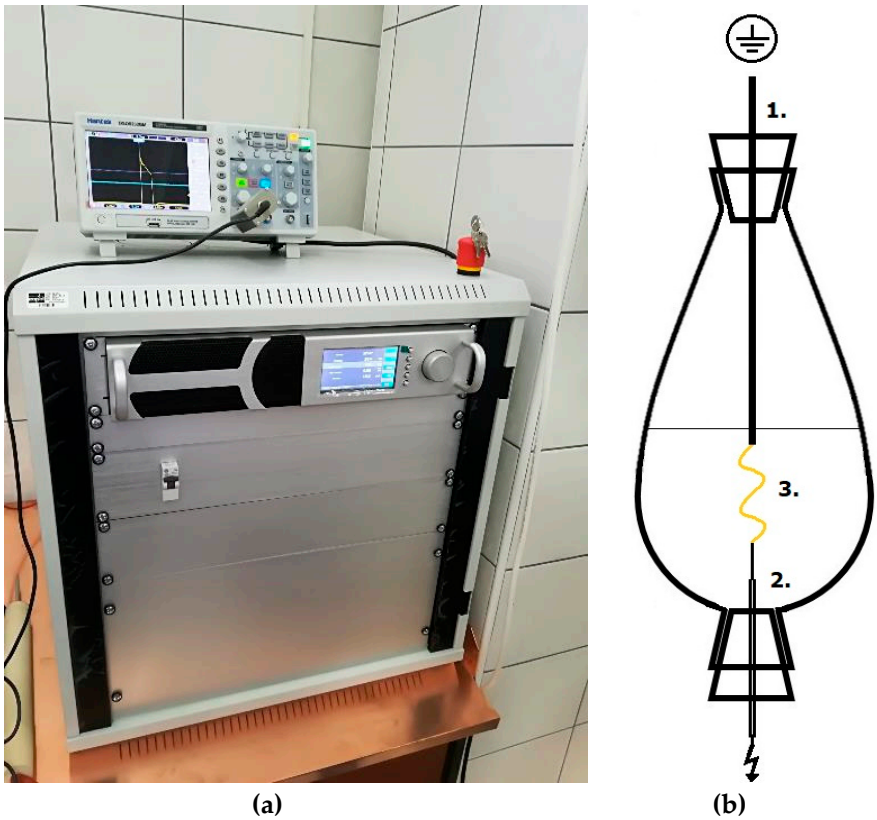

Figure 7. Set-up of generator and reactor for HVED treatments: (**a**) HVED and plasma generator „IMP-SSPG-1200" (Impel group d.o.o., Zagreb, Croatia); (**b**) Beaker shaped reactor: (1)—ground electrode; (2)—high voltage electrode (needle with empty interior for argon and nitrogen flow) during treatments; and (3)—discharge (plasma).

For comparison, modified CE (untreated samples) was performed at room temperature as well, by dissolving the dried rosemary material in the solvent with light magnetic stirring during 3 or 9 min. Both extractions, HVED and CE, were performed in duplicates.

4.4. Analytical Methods

4.4.1. Determination of Total Phenolic Content (TPC)

For determination of TPC of rosemary extracts, a Folin–Ciocalteu method was used [59] with slight modifications. A volume of 0.1 mL of extract (appropriately diluted) was mixed with 0.2 mL of Folin–Ciocalteu reagent. After 3 min 1 mL of 20% Na_2CO_3 (*m*/*v*) was added. After thorough mixing by vortex, the reaction mixtures were incubated at 50 °C for 25 min, followed by absorbance reading at 765 nm against blank (instead of an extract, extraction solvent was used). The calibration curve was prepared using 50 to 500 mg/L of gallic acid in ethanol as a standard. The concentration of TPC was expressed in mg of gallic acid equivalents per gram of sample (mg GAE/g of sample).

4.4.2. 2-Diphenyl-2-Picrylhydrazyl (DPPH) Free Radical Assay

The antioxidant activity of rosemary extracts determined by DPPH method was determined as reported by Shortle et al., (2014) [59] with slight modifications. An aliquot (0.75 mL) of rosemary extracts or methanol solution of Trolox (25–200 mM) was mixed with 1.5 mL of 0.5 mM DPPH methanolic solution. After mixing, the solutions were stored in the dark for 20min at room temperature and the absorbance was measured at 517 nm against 100% methanol as a blank. The results were calculated using calibration curve for Trolox and expressed as µmol of Trolox equivalents per gram of sample (µmol TE/g of sample).

4.4.3. Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was conducted according to literature [59] with modifications. The FRAP reagent was prepared by mixing 0.3 M acetate buffer (pH 3.6) with 10 mM TPTZ solution and 20 mM $FeCl_3$ solution in ratio 10:1:1. An aliquot (80 µL) of rosemary extract was mixed with 240 µL of water and 2080 µL of FRAP reagent. Following incubation at 37 °C for 5 min, the absorbance was measured at 595 nm. FRAP values were calculated according to the calibration curve for $FeSO_4{\cdot}7H_2O$ and expressed as µmol of Fe^{2+} equivalents (FE) per g of sample (µmol FE/g of sample).

4.4.4. Near Infrared Spectroscopy (NIR)

NIR spectroscopy was conducted using the NIR-128-1.7-USB/6.25/50µm (Control Development Inc., South Bend, IN, USA) to record sample spectra using the SPEC 32 Control Development software. NIR spectra was recorded in the wavelength range from 904 to 1699 nm. Each sample was recorded in triplicate and the average spectrum was calculated which was used for further processing.

4.4.5. Colorimetric Evaluation of Rosemary Extracts

Color parameters for all trials was measured by Konica Minolta colorimeter CM 3500d (Konica Minolta, Tokyo, Japan) at CIE Standard Illuminant D65 by 8 mm thick plate. All measurements were conducted in the Specular Component Included (SCI) mode as previously reported [60]. The color measurements of the different extracts prepared by CE and HVED extraction are given in terms of L*, a*, b*, C, and h values under CIE—L*a*b* color system (L*—lightness from black to white; a*—from green to red, and b*—from blue to yellow; C—chroma; and h—hue angle). Differences compared to CE were expressed as ΔE—total color difference, ΔC—difference in tone color; and ΔH—saturation.

4.4.6. Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry Characterization of Phenolic Compounds (UPLC–MS/MS)

UPLC–MS/MS Eksigent Expert Ultra LC 110, SCIEX 4500 QTRAP (SCIEX, USA) method for reference conditions [61] was conducted using Luna Omega 3 µm Polar C18 100 Å, 100 × 4.6 mm (column), thermostat column temperature 40 °C, automatic sampling temperature 4 °C, and injection volume of 10 µL. Mobile phases consisted of: A 100% H_2O with 0.1% HCOOH (*v*/*v*) and B 100% acetonitrile with 0.1 % HCOOH (*v*/*v*) with mobile phase flow 0.40 mL/min. Gradient was set as follows: 1 min 10% B, 2 min 10% B, 15 min 90% B, 25 min 90% B, 27 min 10% B, 30 min 10% B. Determination conditions for MS/MS detector were: ionization -negative ionization mode atmospheric pressure (API)—negative ionization at atmospheric pressure; ionization temperature: 500 °C, i.e., gas temperature combining the mobile phase at the exit from the capillary before ionization. Voltage on the electrode after capillary and next to ionization (Ion Spray Voltage) was −4500 V.

4.4.7. Headspace Solid-Phase Microextraction (HS–SPME) Followed by Gas Chromatography and Mass Spectrometry Analysis (GC–MS)

HS–SPME was performed with a manual SPME holder using three fiber covered with DVB/CAR/PDMS obtained from Supelco Co. (Bellefonte, PA, USA). For HS–SPME, the finely samples 2 mL were placed separately in 10 mL glass vials and hermetically sealed. The vials were maintained at 60 °C during equilibration (15 min) and extraction (45 min). Thereafter, the SPME fiber was withdrawn and inserted into GC–MS injector (250 °C) for 6 min for thermal desorption. The procedure was similar as in previous paper [62].

Gas chromatography and mass spectrometry (GC–MS) analyses were done on an Agilent Technologies (Palo Alto, CA, USA) gas chromatograph model 7890A equipped with a mass spectrometer (MSD) model 5977E (Palo Alto, CA, USA) and HP-5MS capillary column (5% phenyl-methylpolysiloxane, Agilent J & W). The GC conditions were the same as reported previously [62]. In brief, the oven temperature was set at 70 °C for 2 min, then increased from 70 to 200 °C (3 °C /min) and held at 200 °C for 18 min; the carrier gas was helium (1.0 mL/min). The compounds identification was based on the comparison of their retention indices (RI), determined relatively to the retention times of *n*-alkanes (C_9–C_{25}), with those reported in the literature [63] and those from Wiley 9 (Wiley, New York, NY, USA) and NIST 14 (National Institute of Standards and Technology; Gaithersburg, MD, USA) mass spectral database. The percentage composition of the samples was computed from the GC peak areas using the normalization method (without correction factors).

4.4.8. Determination of Pesticides and Metals in Rosemary Samples

The contents of the pesticides were performed by modified procedures with following national regulations HRN EN ISO 12393-1, 12393-2, and 12393-3: 2013, i.e., extraction with petroleum ether/dichloromethane and determination using the GC-ECD Varian CP-3800 instrument (Varian, Inc., Walnut Creek, CA, USA). Metal trace content was determined according to the HRN EN ISO 14084: 2005 procedure, or by wet sample digestion by HNO_3 (microwave digestion) with microwave reaction system Multiwave 3000 (Anton Paar GmbH, Graz, Austria). Determination of metals were conducted on the Perkin Elmer AAS Analyst 800 and ICP-MS Perkin Elmer NexION 300× (PerkinElmer, Inc., Waltham, MA, USA), while Hg traces were determined by the Leco AMA254 Hg analyzer (LECO Inc., St. Joseph, MI, USA).

4.5. Experimental Design and Statistical Analysis

The experiment was designed in STATGRAPHICS Centurion (StatPoint Technologies Inc., Warrenton, VA, USA) software. Multifactorial design consisting of 12 experimental trials using per gas (argon and nitrogen). The three chosen independent variables for HVED assisted extraction were: treatment time (3 and 9 min), voltage applied and gas type (15 or 20 kV for argon, and 20 or 25 kV for nitrogen) and concentration of ethanol (0%, 25%, or 50%). For CE, the independent

variables included: concentration of ethanol (0%, 25%, or 50%) and treatment time (3 and 9 min). The experimental design is presented in Table 3. A total of 30 extracts were prepared in duplicates.

In order to provide information about experimental results, a descriptive statistic was used. Analysis of covariance (ANCOVA) was used for assessment of correlation and all parameters according to dependent variables (gas, treatment time, voltage, ethanol content). The *p*-values present the statistical significance of each of the factor and it was significant at $p \leq 0.05$. Statistics was performed using XLStat (MS Excel 2010) (data not shown).

Information investigation of NIR spectra includes preprocessing and calibration modeling [64] where preprocessing will minimize commotions and undesirable components in spectra, which are subjected to construction of calibration models. Data matrix which included NIR spectra and physical-chemical properties of the samples consisted of 128 rows and 797 columns. This matrix was used for the identification of qualitative differences, by use of principal component analysis (PCA) as well as in the modelling that followed. The NIR spectra were pre-treated to enhance the prediction accuracy. Several spectra pre-treatment methods were arranged to the original absorbance spectra such as multiplicative scatter correction (MSC), standard normal variate (SNV), Smoothing (Moving Average, Gausian and Median filter, and Savitzky–Golay), first and second derivative absorbance (d1a and d2a), Savitzk–Golay first and second derivative absorbance (S-G d1a and S-G d2a), and the combination of the MSC and SNV + d1a or d2a, but as the most effective was the Savitzky–Golay first derivation (S-G d1a). Calibration model was developed by principal component regression (PCR). From the data matrix 2/4 of it was used for the calibration and 1/4 was used for the validation while the remaining $\frac{1}{4}$ was used for the prediction. Validation was done by K-fold cross-validation. [65]. Then followed the application of the multivariate tool mostly used in model prediction, the PLSR and PCR [45]. As in the case of pretreatments, one or more multivariate tools can be used in the calibration, which implies quantitative or qualitative analysis. Model efficacy is evaluated using R^2 and the regression point displacement that is the ratio of the standard RPD and the ratio of the range of reference chemistry values to RER. RPD is the ratio of SDv and SEP, while RER range, min and max, and minimum and maximum values of the validation set. All data analyses were conducted in MS Excel and its additional statistical tool pack: XLStat.

5. Conclusions

In this study, the potential of high voltage discharges for green solvent extraction of BACs and aromas from rosemary leaves was assessed by computational simulation and experimental method by means of HVED. The experimental results were compared with untreated samples (modified CE) and HVED was presented to yield 2.13-times higher TPC and 2.39-times higher antioxidant capacity. Nitrogen, longer treatment time, and higher voltage yielded higher results of phenolic compounds and antioxidants. Also, NIR spectra and modelling with analytical data were shown as an extremely useful tools that can help in assessing whether there is a "cost-effectiveness" of extracting phenols or antioxidants from specific samples, in a quick and easy way. The results presented that NIR spectroscopy combined with chemometrics approach gave accurate TPC, FRAP, and DPPH content prediction, showing that indicates the potential of the method in estimating the quantitative expected antioxidant potential as well as the content of total phenols. Generally, HVED extracts had a dark greenish-brown color with darker color when compared to CE which is associated also with higher phenolic compounds and antioxidant content. An UPLC–MS/MS showed that main phenolic compounds in rosemary were apigenin, diosmetin, and rosmarinic acid, while the predominant volatile compounds in rosemary extracts was eucalyptol. Altogether, results showed that HVED confirmed high potential for extraction of BACs and food aromas from rosemary with increased yield of individual compounds and total phenolic and antioxidant properties, compared to untreated samples. Furthermore, rosemary was presented as safe raw material for further processing in human nutrition in terms of pesticides and metals. The computational stimulation methods were confirmed by experimental study, ethanol had higher potential of solubility of BACs and aromas from rosemary compared to water. Therefore,

these theoretical prediction methods present a new approach for assessment of solubility of individual compounds in selected solvents that could impact to lower solvent usage during experimentation and lower environmental impact.

Supplementary Materials: The following are available online. Table S1: Model statistics for prediction of compositional parameters of rosemary extracts based on the NIR spectra, Figure S1: UPLC-MS/MS chromatograms of representative extracts: (a) CE (sample 3 R25), and (b) HVED (sample RN9), Figure S2: UPLC-MS/MS chromatograms of representative extracts: (a) CE (sample 3 R0), and (b) HVED (sample RA10), and (c) chemical structure of main detected compounds, Figure S3: Box plots for the (A) content of total phenols; antioxidant activity of the samples conducted by the (B) DPPH and (C) FRAP method and the (D) yield for samples treated by CE (R) and HVED (RN & RA), Figure S4: PCA biplot for different extraction types (CE: R; HVED; RN & RA).

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Sample Availability: Samples of the compounds for UPLC-MS/MS and GC-MS are available from the authors.

Paper II

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High-Voltage Electrical Discharges in Green Extractions of Bioactives from Oregano Leaves (*Origanum vulgare* L.) Using Water and Ethanol as Green Solvents Assessed by Theoretical and Experimental Procedures

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Abstract
Development of green extractions of natural compounds is an ongoing challenge for researches. The aim of the study was to evaluate the extraction of bioactive compounds (BACs) from oregano combining an experimental procedure with a theoretical approach using two computational simulation methods, Hansen solubility parameters (HSP) and conductor-like screening model for real solvents (COSMO-RS) software. In this study, the high-voltage electrical discharge-plasma (HVED) was used as one of new promising green extraction techniques. Optimization of processing extraction parameters (argon and nitrogen for generation of plasma, voltage—15 kV, 20 kV, and/or 25 kV), frequency (100 Hz), pulse duration (400 ns), and treatment time (3 and 9 min) was done using software STATGRAPHICS. Oregano extracts were prepared by pharmacopoeia, 1 g per 50 mL of solvents (water or aqueous ethanol (25 and 50% v/v) treated by HVED. Extraction process was controlled by analytical methods (determination of total phenolic compounds (TPC); antioxidant properties by electronic paramagnetic resonance (EPR), 2,2-diphenyl-2-picrylhydrazyl (DPPH) free radical assay; near-infrared (NIR) spectroscopy; and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) characterization of phenolic compounds, and changes during and after extraction process were investigated. Process follows six principles of green extractions. Theoretical results were in line with experimental for solvent selection; ethanol had higher potential of solubility of BACs than water. Results showed that HVED, as a green extraction method, confirmed high potential for extraction of BACs from oregano with increased yield of individual BACs and TPC in obtained extracts and increased antioxidant activity, compared with untreated samples.

Keywords Oregano · High-voltage electrical discharge · COSMO-RS · Hansen solubility parameters · Bioactive compounds

Introduction

For centuries, medicinal plants have been used in medicine for improving human health, mostly due to its naturally presented bioactive compounds (BACs). This has led to increased demand for extraction of BACs from medicinal herbs and their application in various industries such as food, pharmaceutical, and cosmetic industry [1]. Oregano extracts have shown strong antioxidant and antimicrobial properties related to its BACs, mostly phenolic compounds. Main compounds found in oregano are phenols (gentisic, chlorogenic, p-coumaric, rosmarinic, cinnamic, caffeic, p-hydroxybenzoic, syringic, protocatecholic and vanillic acids, carvacrol, and thymol) and flavonoids (hyperoside, isoquercitrin, rutin, quercitrin, and luteolin). These compounds have shown anticarcinogenic, antimicrobial, antiviral, hypolipidemic, antimutagenic, anti-inflammatory, and anticardiovascular disease properties [2–4].

There is a growing interest in industry to replace conventional extraction (CE) methods with more sustainable, green extractions. Green extractions are based on the discovery of extraction processes that will reduce energy consumption, allow the use of alternative solvents and renewable natural products, and ensure a safe and high-quality extract/product [5]. This definition led to the development of six principles of

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green extraction established to guide researchers in their action toward green innovation. According to these principles, medicinal plants, such as oregano, that are favored with sustainable rather than wild harvesting, allow the preservation of biodiversity and meet the demand of a growing market. Also, the second principle of green extraction promotes the use of alternative solvents and principally water or agro-solvents. Ethanol is the most common green solvent, obtained by the fermentation of sugar-rich materials like sugar beet and cereals. Even though ethanol is flammable and potentially explosive, it is used on a large scale because it is easily available in high purity, it has a low price, and it is completely biodegradable. Water and ethanol have been showed as cheap and environmentally acceptable green solvents and a great option for replacement of toxic organic solvents, such as methanol and hexane [6].

In order to reduce solvent consumption during experiments, computational methods that use theoretical prediction of solubility parameters for selected solvents can be used. Such softwares are conductor-like screening model for real solvents (COSMO-RS) and Hansen solubility parameter (HSP) theoretical prediction. These model predictive tools are used to predict the properties and behavior of the interaction of solvent-solute and to predict the most favorable performance of solvents for targeted ingredients and application [7].

Furthermore, principles of green extraction promote reducing energy consumption using innovative technologies. Innovative technologies have been developed in order to improve extraction yield in a shorter time, compared with CE, with minimal consumption of solvents and energy [5]. Among them, the use of electrotechnologies like pulsed electric field (PEF) and high-voltage electrical discharge (HVED) has raised the interest of both industry and academia as they showed great potential to improve the recovery of biocompounds from plant sources quickly, economically, and more sustainably [8].

HVED is a non-thermal technology that has impact based on electroporation, a damage of cell structure with membrane pore formation presented when electrical discharges are in a contact with liquid [9]. This process is the result of liquid ionization when high voltage is applied with high intensity and short pulses duration between two electrodes. The mechanism consists of three phases: generation of electric pulses, current discharge, and formation of an electric arc–electrical breakdown [8]. Additionally, several associated phenomena can occur during the process: shock waves, cavitation, turbulence in the liquid, and production of reactive species [10].

The aim of the study was to evaluate the extraction of BACs from oregano leaves combining an experimental procedure with a theoretical approach carried out by means of two computational simulation methods, HSP and COSMO-RS software. The experimental procedure was performed by HVED extraction following principles of green chemistry. Furthermore, the obtained extracts were compared with CE by means of physical (pH, conductivity, power, and temperature) and chemical characteristics (total phenolic content (TPC), antioxidant capacity, near-infrared spectroscopy (NIR), and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) characterization of phenolic compounds).

Materials and Methods

Plant Materials

Dried oregano (*Origanum vulgare* L.) herb was provided by local drugstore (Suban d.o.o., Samobor). Herbs were stored in polyethylene bags in a dark and dry place until extractions. Plant particle size distribution was as following: 90% of particles were up to diameter of $d\ (0.9) \leq 164.315$ μm; 50% of particles was up to median diameter $d\ (0.5) \leq 297.345$ μm; and 10% was $d\ (0.1) \leq 461.460$ μm measured by the laser particle size analyzer (Malvern, Mastersizer 2000, Germany). Before extraction, herb material was weighted (1 g) into the 100 mL beaker and mixed in 50 mL of extracting solvent at room temperature (22 °C). Extraction was carried out using distilled water and 25% and 50% aqueous ethanol (v/v) as extraction solvents.

Computational Methods: Theoretical Prediction

The solubility parameters of water and ethanol used to dissolve BACs from oregano leaves have been studied by means of the COSMO-RS and HSP theoretical prediction.

COSMO-RS Software

The COSMO-RS was developed by Klamt and co-workers as a statistical thermodynamic method for molecular description and solvent screening based on a quantum chemical approach [11]. COSMO-RS combines quantum chemical considerations (COSMO) and statistical thermodynamics (RS) to determine and predict thermodynamic properties without experimental data. COSMO-RS prediction is a two-step procedure—microscopic and macroscopic. The procedure was explained in details by Aissou et al. (2017) [12]. The COSMOthermX program (version C30 release 13.01) was used to calculate the relative solubility between the solid compound and the liquid solvent in terms of the logarithm of the solubility in mole fractions ($\log_{10}(x_{solub})$). The logarithm of the best solubility was set to 0, and all other solvents were given relative to the best solvent. Also, the logarithm was transformed into probability of solubility (%). The calculation was performed at room temperature (20 °C) and at boiling temperature for each solvent.

Hansen Solubility Parameters (HSPs)

Solubility parameters for predicting the solubility of a solute were proposed by Hansen [13]. HSP provide a convenient and efficient way for characterization of solute-solvent interactions according to the classical "like dissolves like" rule. The concept of HSPs is described in details in Aissou et al. (2017) [12]. For HSP solvent optimization, a simple composite affinity parameter, the relative energy difference (RED) number, has been calculated to determine the solubility between solvents and solutes:

$$\mathrm{RED} = \frac{Ra}{Ro} \quad (1)$$

where R_o is the radius of a Hansen solubility sphere and R_a is the distance of a solvent from the center of the Hansen solubility sphere. The smaller the value of R_a is, the greater the affinity between the solutes and solvents. It means that a potentially good solvent has a RED number smaller than 1 (the compound has similar properties and will dissolve), while medium and poor solvents have RED values of from one to three and more than 3, respectively. The chemical structures of the solvents and solutes discussed in this article could be mutually transformed by JChemPaint version 3.3 (GitHub Pages, San Francisco, CA, USA) to their simplified molecular input line entry syntax (SMILES) notations, which were subsequently used to calculate the solubility parameters of the solvents and compounds (HSPiP Version 4.0, Hansen Solubility, Hørsholm, Denmark).

High-Voltage Electrical Discharge (HVED) and Conventional Extraction (CE)

HVED was conducted by a generator "IMP-SSPG-1200" (Impel group, Zagreb, Croatia) that generated rectangular pulses using direct current (DC) and achieving high voltage. Maximum adjustable current was 30 mA and voltage up to 25 kV. Based on conducted preliminary experiments with different HVED parameters (frequency, voltage, pulse length, distance between electrodes, as well as ratio mass to solvent), fixed HVED parameters were chosen as follows: frequency was 100 Hz, pulse width was 400 ns, voltage was 15 and 20 kV for argon gas and 20 and 25 kV for nitrogen gas for HVED treatments, the gap between electrodes was 15 mm, and ratio mass to solvent 1 g:50 mL (according to pharmacopeia). Mixture of herb material and solvent was transferred to beaker-shaped reactor of 100 mL. This reactor was opened on both sides; therefore, it was fitted with silicone tops with diameter of 1 cm. Silicone tops were used due to easier mounting of the electrode from the top and needle form the bottom. Gases (argon or nitrogen) were flowed in through the needle with the flow 5 L min^{-1}. Set-up of generator and reactor is shown in Fig. 1. For measuring the output voltage (data not shown), oscilloscope (Hantek DS05202BM) connected to the high-voltage probe (Tektronix P6015A) was used.

For comparison, modified CE (untreated samples) was performed at the same (room) temperature as the HVED extraction by dissolving the dried oregano material in the solvent with light magnetic stirring during 3 or 9 min. Both extractions, HVED and CE, were performed in duplicates.

Analytical Methods

Determination of Total Phenolic Content (TPC)

TPC of oregano extracts was determined using Folin-Ciocalteu method previously described [14] with slight modifications. A volume of 0.1 mL of extract (appropriately diluted) was mixed with 0.2 mL of Folin-Ciocalteu reagent. After 3 min, 1 mL of 20% Na_2CO_3 (m/v) was added. After thorough mixing by vortex, the reaction mixtures were incubated at 50 °C for 25 min, followed by absorbance reading at 765 nm against blank (instead of an extract, extraction solvent was used). The calibration curve was prepared using 50 to 500 mg/L of gallic acid in ethanol. The concentration of TPC was expressed in *mg* of *gallic acid equivalents* per *g* of sample (mg GAE/g of sample).

2,2-Diphenyl-2-Picrylhydrazyl (DPPH) Free Radical Assay

DPPH assay of oregano extracts was determined according to previously reported procedure [14] with modifications. An aliquot (0.75 mL) of oregano extracts or methanol solution of trolox (25–200 mM) was mixed with 1.5 mL of 0.5 mM DPPH methanolic solution. After mixing, the solutions were stored in the dark for 20 min at room temperature, and then, the absorbance was measured at 517 nm against 100% methanol as a blank. The results were calculated using calibration curve for trolox and expressed as μmol of trolox equivalents per gram of samples (μmol TE/g of sample).

Electron Paramagnetic Resonance (EPR) Spectroscopy

EPR spectra were recorded at room temperature using Varian E-9 spectrometer equipped with Bruker ER 041 XG microwave bridge working at microwave frequency 9.5 GHz (i.e., X-band). The spectrometry settings were magnetic field modulation frequency (100 kHz), central field (331 mT), sweep range (10 mT), sweep time (20 s), microwave power (10 mW), and modulation amplitude (0.1 mT).

DPPH stable free radical was used to screen scavenging capability in EPR measurements. The stability of its freshly prepared ethanol solution was monitored, and no significant loss of signal was detected within 24 h. The 3968 μL

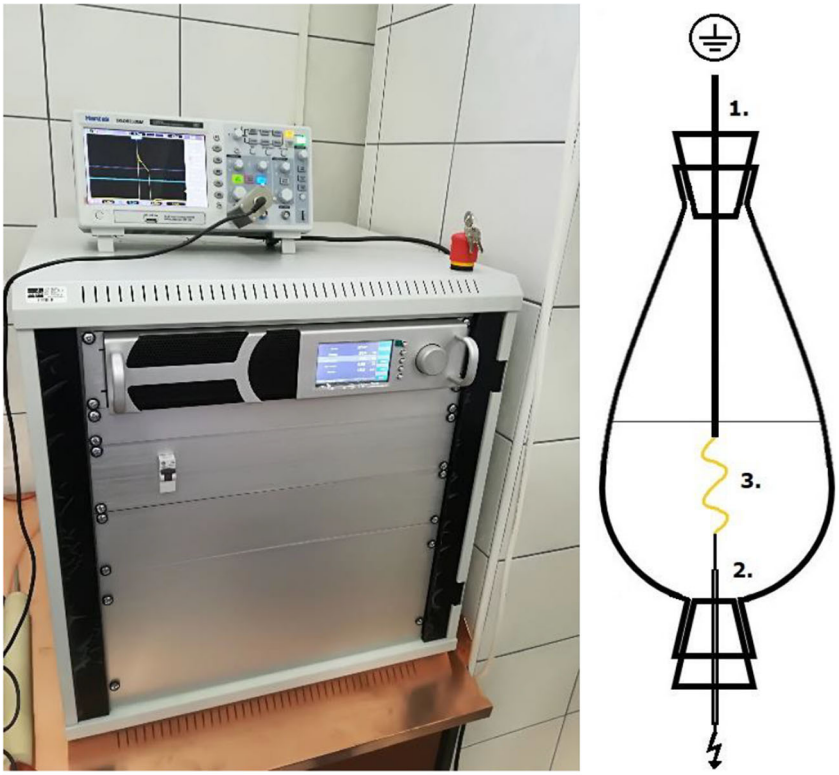


Fig. 1 Set-up of generator and reactor for HVED treatments: (a) HVED and plasma generator "IMP-SSPG-1200″ (Impel group, Zagreb, Croatia). (b) Beaker-shaped reactor. (1) Ground electrode; (2) high-voltage electrode (needle with empty interior for argon and nitrogen flow) during treatments; and (3) discharge (plasma)

volume of a DPPH stock ethanol solution (0.5 mmol/L) was added to 32 μL of the solution of the oregano extract and mixed in the case of argon plasma treatment, and 3984 μL volume of a DPPH stock ethanol solution (0.5 mmol/L) was added to 16 μL of the solution of the oregano extract and mixed in the case of nitrogen plasma treatment. The mixture was immediately put into the capillary which was then placed in a standard EPR tube. EPR spectra were recorded as a function of time starting from the oregano extract solution and radical solution contact. The scavenging effect of oregano extracts on the DPPH radicals was obtained from the EPR signal intensities of samples calculated by the double integration of EPR spectra and expressed in arbitrary units. The signal intensity of the pure 0.5 mmol/L DPPH solution, measured just before starting the sample measurement, was taken as the reference signal intensity (I_o) for the reaction time $t = 0$ min. EPR signal intensity of DPPH radicals was decreased upon extract addition. The remaining DPPH radicals, expressed as a percentage, (I_R) after the reaction time t, were calculated from following expression:

$$I_R = \frac{I}{Io} \times 100 \quad (2)$$

where I is the signal intensity of DPPH in extract solution measured at time t.

Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry Characterization of Phenolic Compounds (UPLC-MS/MS)

Method for UPLC-MS/MS (Eskigent Expert Ultra LC 110, SCIEX 4500 QTRAP) reference conditions [[15]] was conducted using Luna Omega 3 μm Polar C18 100 Å, 100 × 4.6 mm (column), thermostat column temperature 40 °C, automatic sampling temperature 4 °C, and injection volume 10 μL. Mobile phases consisted of A 100% H_2O with 0.1% HCOOH (v/v) and B 100% ACN with 0.1% HCOOH (v/v) with mobile phase flow 0.40 mL/min. Gradient was set as follows: 1 min 10% B, 2 min 10% B, 15 min 90% B, 25 min 90% B, 27 min 10% B, and 30 min 10% B. Determination conditions for MS/MS detector were ionization -negative API (ionization mode atmospheric pressure) - negative ionization at atmospheric pressure; ionization temperature: 500 °C, i.e., gas temperature combining the mobile phase at the exit from the capillary before ionization. Voltage on the electrode after capillary and next to ionization (ion spray voltage) was − 4500 V.

Near-Infrared Spectroscopy (NIR)

NIR spectroscopy was conducted using the Control Development Inc., NIR-128-1.7-USB/6.25/50 μm to record sample spectra using the SPEC 32 Control Development

software. NIR spectra were recorded in the wavelength range from 904 to 1699 nm. Each sample was recorded in triplicate and afterward was calculated in the average spectrum which was used for further processing.

Experimental Design and Statistical Analysis

The experiment was designed in STATGRAPHICS Centurion (StatPoint Technologies, Inc., Warrenton, VA, USA) software. Multifactorial design consisting of 12 experimental trials using per gas. For argon, 12 experimental trials and 12 experimental trials for nitrogen. The three chosen independent variables for HVED-assisted extraction were: (A) treatment time (3 and 9 min), (B) voltage applied and gas type (15 kV or 20 kV for argon and 20 kV or 25 kV for nitrogen), and (C) concentration of ethanol (0%, 25%, or 50%). For CE, the independent variables included concentration of ethanol (0%, 25%, or 50%) and treatment time (3 and 9 min). The experiment design is shown in Table 1. A total of 30 extracts were prepared in duplicates. Each model and factor were significant at $p \leq 0.05$.

In order to provide information about experimental results, a descriptive statistic was used. Kolmogorov–Smirnov and Levene's test were used for normality and homoscedasticity analysis. Differences in extractions were tested using multivariate analysis of variances (MANOVA; three-way ANOVA with interactions; data not shown). The significance levels for rejection of a null hypothesis in all tests were $\alpha \leq 0.05$. Statistics was performed using STATGRAPHICS Centurion software (StatPoint Technologies, Inc., Warrenton, VA, USA).

Table 1 Denotation of samples, experimental design, and process parameters

Sample	A: Treatment time (min)	B: Voltage (kV)	C: Ethanol content (%)	Stirring (min)	Extraction type
3 O0	0	0	0	3	CE
9 O0	0	0	0	9	
3 O25	0	0	25	3	
9 O25	0	0	25	9	
3 O50	0	0	50	3	
9 O50	0	0	50	9	
ON1	3	20	50	/	HVED
ON2	9	20	0	/	
ON3	3	20	0	/	
ON4	3	25	0	/	
ON5	9	25	25	/	
ON6	9	20	25	/	
ON7	9	20	50	/	
ON8	9	25	50	/	
ON9	3	25	25	/	
ON10	9	25	0	/	
ON11	3	25	50	/	
ON12	3	20	25	/	
OA1	3	15	50	/	
OA2	9	15	0	/	
OA3	3	15	0	/	
OA4	3	20	0	/	
OA5	9	20	25	/	
OA6	9	15	25	/	
OA7	9	15	50	/	
OA8	9	20	50	/	
OA9	3	20	25	/	
OA10	9	20	0	/	
OA11	3	20	50	/	
OA12	3	15	25	/	

O oregano; *N* nitrogen; *A* argon. For HVED, numbers 1–12 are the order of conducted treatment. For CE treatments, 3 and 9 are referred to treatment time, while 0, 25, and 50 stands for concentration of an ethanol solvent (%)

Also, analysis of covariance (ANCOVA) was used for assessment of individual phenolic compounds analyzed by UPLC with TPC, DPPH, and EPR results (data not shown). The p values present the statistical significance of each of the factor, and it was significant at $p \leq 0.05$. The PCA analysis was performed in XLStat (MS Excel 2010).

The principal component analysis (PCA) was used as a multivariate statistical analysis tool in the processing of the NIR spectrum to reduce the dimensionality of the data and detect qualitative similarities or differences [16]. The PCA analysis was performed in XLStat (MS Excel 2010).

Results and Discussion

The study of the extraction of BACs using water and ethanol as green solvents was performed by a theoretical procedure, using two computational predictive methods (COSMO-RS and HSP) and via experimentation.

Analysis Using Computational Methods

COSMO-RS Calculations

The COSMO-RS simulation was conducted in order to determine the potential of water and ethanol for the extraction of BACs from oregano. The software integrates a quantum chemistry approach that permits the calculation of various properties such as the relative solubility of a compound in several solvents [12]. Table 2 shows results of solubility of various BACs from oregano in water and ethanol. The results are expressed in $\log_{10}(x_{solub})$ with best solubility set to 0 and probability of solubility (%) for better understanding of results. Results of probability of solubility are presented at room temperature and at boiling temperature of each solvent for better comparison of temperature influence to solubility. Ethanol showed higher probability of solubility for all compounds, compared with water. The probability of solubility was low for all compounds using water as a solvent (0–20%). Although probability was higher for water at boiling temperature (100 °C), it was still low (0–20%), and no significant influence of temperature was noted.

According to the rule ″like dissolves like,″ the substances with similar chemical characteristics will dissolve in each other. More specifically, polar solvents have a tendency to dissolve polar solutes, while non-polar solvents tend to dissolve non-polar solutes. In line with this rule, ethanol, as a poplar solvent, showed high probability of solubility (60–100%) at room temperature for carvacrol, thymol, borneol, α-terpineol, piperitone, and palmitic acid and medium probability of solubility (20–60%) for menthone, pulegone, eucalyptol, α-cadinol, linalool, and isospathulenol. These are all compounds with polar groups in their molecules, and most of them are oxygenated monoterpenes with hydroxyl group. The solubility of compounds at boiling temperature of ethanol was not significantly different. The only difference in solubility categories was that p-cymen-7-ol showed medium probability of solubility (20.89%) and linalool showed high probability of solubility (60.25%). Since no significant differences were shown with probability of solubility of solvents at room and boiling temperature, it is suitable to perform a non-thermal extraction with HVED (at room temperature) using water and ethanol as solvents.

The low probability of solubility of ethanol and water for phenolic compounds could be due to the hydroxyl groups of phenolics that give a high polarity to molecules. Moreover, most of the oxygenated compounds (oxygenated monoterpenes, oxygenated sesquiterpenes, and palmitic acid) are more polar than the abovementioned and showed a higher probability of solubility in ethanol. Also, it is expected that experimental analyses of oregano extracts should show higher content of BACs when extracted with higher yield of ethanol.

Hansen Solubility Parameters (HSP)

The solubility of BACs in water and ethanol is assessed by HSP and presented in Table 2. The software allowed the assessment of the RED, the estimation of capacity of a solvent to dissolve solutes. RED values < 1 represent good solubility, meaning that water and ethanol are not the best solvents, from a theoretical perspective, for the extraction of the evaluated phenolics from oregano. From the HSP results, it is clear that water has low solubility (RED > 3) of all selected BACs from oregano. Ethanol showed higher results of solubility for all compounds compared with water and medium solubility (RED 1–3) for dissolving p-cymen-7-ol and α-cadinol. The poor theoretical solubility of presented polar solvents can be explained by the difference in the polarity of these solvents regarding to the solutes.

Experimental Analyses of Oregano Extracts

High-Voltage Electrical Discharge (HVED) and Physical Properties of Obtained Extracts

In Fig. 2, results of pH, conductivity, power, and temperatures before and after HVED treatment are given. MANOVA statistical analysis, for oregano extracts, showed significant influence ($p \leq 0.05$) of ethanol concentration on pH, conductivity, and temperature difference values of obtained oregano extract using HVED and nitrogen. For HVED extraction obtained from oregano using argon, there is significant influence on conductivity influencing ethanol content and treatment time. Higher ethanol concentration increased pH and decreased conductivity for all extractions (CE, HVED, nitrogen, and argon).

Table 2 COSMO-RS relative solubility ($log_{10}(x_{solub})$) and probability of solubility (%) and HSP values of relative energy difference (RED) of bioactive compounds from oregano using water and ethanol as solvents

Compounds	*COSMO-RS*						*HSP*	
	$log_{10}(x_{solub})$		*Probability (%)*				**RED**	
	Ethanol (room temperature)	*Water (room temperature)*	*Ethanol (room temperature)*	*Water (room temperature)*	*Ethanol (73°C)*	*Water (100°C)*	*Ethanol (room temperature)*	*Water (room temperature)*
Monoterpenes								
carvacrol	0.00	-3.56	**100.00**	**0.02**	**100.00**	**0.04**	3.43	9.34
thymol	0.00	-4.14	**100.00**	**0.007**	**93.32**	**0.01**	3.43	9.34
β-myrcene	-0.95	-6.02	**11.22**	**9.55E-05**	**17.37**	**0.0005**	4.5	10.49
p-cymen-7-ol	-0.86	-5.42	**13.8**	**0.0003**	**20.89**	**0.001**	2.97	8.84
α-pinene	-0.98	-5.59	**10.47**	**0.0002**	**15.48**	**0.001**	4.77	10.75
β-pinene	-0.9	-5.32	**12.58**	**0.0004**	**18.19**	**0.002**	4.82	10.8
camphene	-0.9	-5.28	**12.58**	**0.0005**	**18.19**	**0.002**	4.82	10.8
sabinene	-0.87	-5.36	**13.48**	**0.0004**	**19.95**	**0.001**	4.78	10.77
α-phellandrene	-0.91	-5.58	**12.3**	**0.0002**	**18.62**	**0.001**	4.46	10.44
α-terpinene	-0.92	-5.57	**12.02**	**0.0002**	**18.19**	**0.001**	4.34	10.32
δ-terpinene	-0.94	-5.69	**11.48**	**0.0002**	**17.37**	**0.001**	4.33	15.5
α-terpinolene	-0.92	-5.62	**12.02**	**0.0002**	**17.78**	**0.001**	4.25	10.22
β-phellandrene	-0.89	-5.5	**12.88**	**0.0003**	**19.49**	**0.001**	4.53	10.52
cis-β-ocimene	-0.94	-5.94	**11.48**	**0.0001**	**17.78**	**0.0006**	4.41	10.39
trans-β-ocimene	-0.94	-5.94	**11.48**	**0.0001**	**17.78**	**0.0006**	4.45	10.43
menthone	-0.44	-3.65	**36.30**	**0.02**	**45.7**	**0.01**	4.39	10.37
pulegone	-0.27	-3.16	**53.70**	**0.06**	**60.25**	**0.04**	4.08	10.03
Oxygenated monoterpenes								
eucalyptol	-0.35	-3.74	**44.66**	**0.018**	**43.65**	**0.01**	4.51	10.5
borneol	-0.07	-3.77	**85.11**	**0.01**	**81.28**	**0.03**	3.33	9.3
α-terpineol	-0.22	-3.67	**60.25**	**0.02**	**79.43**	**0.04**	3.26	9.22
α-cadinol	-0.38	-5.80	**41.68**	**0.0001**	**45.7**	**0.005**	3.95	9.91
linalool	-0.28	-4.29	**52.48**	**0.005**	**60.25**	**0.009**	3.49	9.46
piperitone	-0.04	-3.16	**91.2**	**0.06**	**87.09**	**0.14**	4.22	10.19
Sesquiterpenes								
β-caryophyllene	-1.1	-6.95	**794**	**1.12E-05**	**14.12**	**8.90E-05**	4.68	10.65
copaene	-1.22	-7.25	**6.02**	**5.62E-06**	**10.47**	**4.70E-05**	4.99	10.97
α-cubebene	-1.20	-7.31	**6.3**	**4.90E-06**	**10.96**	**4.10E-05**	4.67	10.64
β-elemene	-1.00	-6.79	**10**	**1.62E-05**	**16.59**	**0.0001**	5.92	10.84
β-cadinene	-1.15	-7.22	**7.07**	**6.03E-06**	**12.02**	**5.00E-05**	4.64	10.62
δ-cadinene	-1.11	-6.93	**7.76**	**1.17E-05**	**13.18**	**9.10E-05**	4.86	10.84
Oxygenated sesquiterpenes								
isospathulenol	-0.32	-5.1	**47.86**	**0.0007**	**58.88**	**0.002**	3.84	9.79
Other Oxygenated								
palmitic acid	-0.06	-6.33	**87.09**	4.68E-05	**93.32**	0.0001	3.67	9.67

COSMO-RS: Low probability of solubility 0–20% (red color); medium probability of solubility 20–60% (yellow color); high probability of solubility 60–100% (green color)

HSP: Relative energy difference (RED) very good solubility 0–1 (green color); medium solubility 1–3 (yellow color); poor solubility >3 (red color)

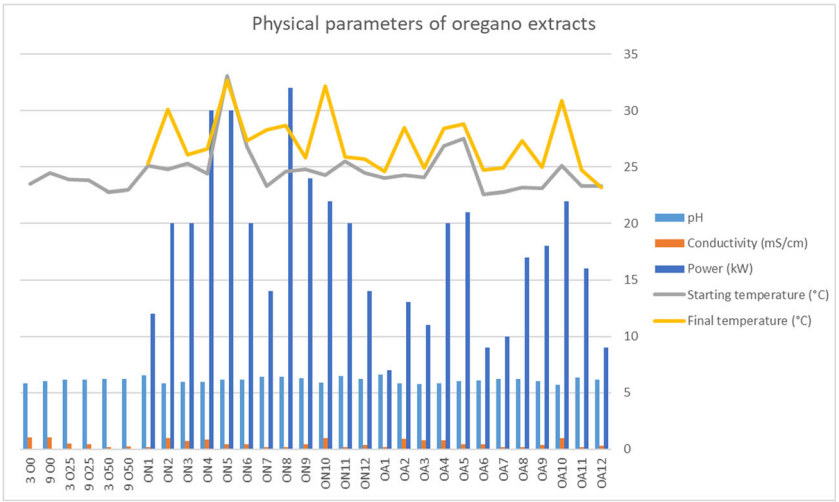


Fig. 2 Values of pH, conductivity (μS/cm) for CE- and HVED-treated samples, starting temperature (°C), final temperature (°C), and power (kW) after HVED treatments. O oregano; N nitrogen; and A argon. For HVED, numbers 1–12 are the order of conducted treatment. For CE treatments, 3 and 9 are referred to treatment time, while 0, 25, and 50 stands for concentration of an ethanol solvent (%)

The final temperature for untreated samples was not noted since no heating during stirring in CE occurred. The maximum temperature during extractions was 33.1 °C, and maximum difference between final and starting temperature for HVED treatment was 7.9 °C (ON10). Since there was no significant heating during the treatment with HVED, it confirms HVED as a non-thermal technology. There is also a significant influence of AC variable (combination of variables A-treatment time and C-ethanol content) to temperature difference (the difference between final and starting temperature during the treatment). The highest power (32.0 ± 2.0 kW) was noted for sample ON8 (nitrogen, 9 min, 25 kV, 50% of ethanol). Higher power was used accordingly with increased voltage and treatment time. Also, more power was used for treatment with nitrogen than with argon because the applied voltage was higher for nitrogen (20 and 25 kV) than argon (15 and 20 kV). The reason is that it was difficult to achieve discharge when nitrogen was used during the extraction, and therefore, higher voltage and more power were needed for electrical discharge. The extraction was performed in order to extract BACs from intracellular area through membrane pores to solvent due to electroporation, under optimized conditions to maintain their activity [17]. During the liquid phase discharge, more secondary phenomena may occur to enhance extraction: UV light, shock waves, and strong liquid turbulence during the treatment may lead to plant fragmentation and cell damage; also, a high density of radicals can also cause a cell damage by cell oxidation [18]. Process conditions were optimized according to results depending of extraction parameters.

Extraction of Bioactive Compounds from Oregano Extracts and its Antioxidant Potential

Different novel techniques, including HVED, have been recognized to recover polyphenols from plant materials with non-thermal technology and in principles of sustainable development [19–24]. This HVED technology with pulsed rapid discharge voltages (from 20 to 80 kV/cm electric field intensity) is based on the phenomenon of electrical breakdown in liquids which induces physical and chemical processes that affect both the cell walls and the membranes while freeing intracellular components [25]. In order to observe impact of electroporation using HVED, TPC as well as antioxidant properties (DPPH and EPR) of obtained extracts were selected to test efficiency of HVED. In this study, CE was done in order to perform a control for comparison with HVED extraction.

The difference between CE and HVED extraction according to results of TPC, antioxidant parameters, and yields is presented in Table 3. For CE, the highest values of TPC (93.02 ± 6.18 mg GAE/g) for oregano extracts were determined after 9 min extraction using water as a solvent. It is notable that HVED had higher yield (regarding TPC) compared with CE. Regarding TPC and yield, from oregano samples, there is 0.9–6.5 times higher efficiency using HVED in the same conditions. It is obvious that there is an increase in content of phenolics with increase of treatment time for both CE and HVED and with increase of voltage. For oregano and HVED using nitrogen, the highest TPC and yield is for ON5 (25 kV; 9 min; 25% ethanol), whereas using argon highest values are for OA6 (15 kV; 9 min; 25% ethanol) (Table 3). Since cold plasma is an ionized gas that can be produced with many different gases, the gas type used for plasma generation has an influence to the results. The gas composition significantly affects the quality of the final extract. Different gases ionize at different voltages, and also, different radical species are being produced during the treatment. Therefore, the efficiency of cold plasma is affected by the type of gas used [26]. The highest yield of phenolics extracted with both argon and nitrogen was obtained in extracts with 25% ethanol, likely due to increased solubility of phenolic compounds [7]. HVED or other electrically assisted extractions are less thermally

Table 3 Determination of bioactive compounds—total phenolic compounds (TPC) values, antioxidant activity (DPPH and EPR), and yield of extraction—measurements for CE and HVED treated samples

Sample	TPC (mg GAE/g)	DPPH (μmol TAE/g)	I_R (%)	Yield (%)	Extraction type
3 O0	19.17 ± 1.19	29.10 ± 1.71	78.04 ± 3.98	1.92 ± 0.12	CE
9 O0	93.02 ± 6.18	22.35 ± 1.74	70.94 ± 2.79	9.30 ± 0.62	
3 O25	36.52 ± 4.15	27.96 ± 1.53	57.76 ± 3.01	3.65 ± 0.41	
9 O25	59.03 ± 5.45	27.17 ± 1.45	55.00 ± 2.16	5.90 ± 0.55	
3 O50	39.95 ± 3.34	31.17 ± 1.38	42.75 ± 1.88	4.00 ± 0.33	
9 O50	57.03 ± 4.17	30.60 ± 1.57	38.86 ± 1.74	5.70 ± 0.42	
ON1	86.50 ± 6.16	29.21 ± 1.22	28.23 ± 1.32	8.65 ± 0.62	HVED
ON2	112.80 ± 8.13	23.60 ± 1.40	17.95 ± 0.80	11.28 ± 0.81	
ON3	73.02 ± 7.33	16.96 ± 1.11	27.07 ± 2.01	7.30 ± 0.73	
ON4	124.98 ± 8.17	22.56 ± 1.64	29.28 ± 0.94	12.50 ± 0.82	
ON5	191.28 ± 7.12	22.10 ± 1.12	14.95 ± 0.87	19.13 ± 0.71	
ON6	137.80 ± 8.30	23.89 ± 1.53	4.99 ± 0.42	13.78 ± 0.83	
ON7	74.54 ± 4.11	28.85 ± 1.44	18.29 ± 1.12	7.45 ± 0.41	
ON8	81.28 ± 5.54	27.67 ± 1.12	3.59 ± 0.33	8.13 ± 0.55	
ON9	125.20 ± 5.32	25.89 ± 1.26	22.07 ± 0.96	12.52 ± 0.53	
ON10	92.37 ± 7.11	25.71 ± 1.25	28.23 ± 1.04	9.24 ± 0.71	
ON11	62.15 ± 6.11	26.67 ± 1.18	17.95 ± 0.83	6.22 ± 0.61	
ON12	128.89 ± 9.33	26.78 ± 1.42	27.07 ± 1.27	12.89 ± 0.93	
OA1	73.46 ± 5.14	30.31 ± 2.10	4.79 ± 0.41	7.35 ± 0.51	
OA2	82.59 ± 6.16	27.99 ± 2.11	4.46 ± 0.37	8.26 ± 0.62	
OA3	36.93 ± 3.38	29.78 ± 2.14	5.93 ± 0.53	3.69 ± 0.34	
OA4	77.37 ± 5.15	28.53 ± 1.72	8.24 ± 0.61	7.74 ± 0.52	
OA5	122.80 ± 7.12	24.71 ± 2.12	27.35 ± 1.07	12.28 ± 0.71	
OA6	129.54 ± 8.43	27.42 ± 3.11	22.38 ± 0.99	12.95 ± 0.84	
OA7	86.28 ± 3.19	30.06 ± 2.26	29.44 ± 1.35	8.63 ± 0.32	
OA8	75.63 ± 4.66	29.28 ± 2.19	9.12 ± 0.77	7.56 ± 0.47	
OA9	114.76 ± 6.14	25.85 ± 3.16	21.59 ± 0.89	11.48 ± 0.61	
OA10	80.20 ± 5.65	25.14 ± 2.13	4.79 ± 0.12	8.02 ± 0.57	
OA11	74.76 ± 5.58	29.46 ± 1.44	4.46 ± 0.25	7.48 ± 0.56	
OA12	116.07 ± 8.42	24.10 ± 1.67	5.93 ± 0.68	11.61 ± 0.84	

O oregano; *N* nitrogen; *A* argon. For HVED, numbers 1–12 are the order of conducted treatment. For CE treatments, 3 and 9 are referred to treatment time while 0, 25, and 50 stands for concentration of an ethanol solvent (%)

TPC total phenolic content, *DPPH* 2,2-diphenyl-2-picrylhydrazyl free radical assay, *EPR* electron paramagnetic resonance, I_R the intensity of remaining DPPH radicals in EPR measurements

destructive than standard CE, and they are useful for extraction of specific thermolabile BACs. With increased effectiveness, such extracts are obtained at lower temperatures in a shorter period. Another study found HVED as and efficient pre-treatment technique without impact in chemical composition of papaya peels [27].

Moreover, HVED generates hot and localized plasma during photonic dissociation of water, with emission of the UV light and OH* radicals. At the same time, HVED will create shockwaves and pyrolysis caused by electrohydraulic cavitation [28]. With generating electric fields and electrical discharges of up to 25 kV, there is additional formation of free radical species (ROS and RNS) [28]. Since antioxidants bind with free radicals by giving up their own electrons, the antioxidant activity would be lowered by destabilization. This can be negative and destabilizing effect for sensitive BACs, especially in a long non-controlled treatment, where they can deteriorate. For that reason, DPPH and EPR measurements were performed. DPPH values for HVED-treated samples were similar or lower than for CE. For HVED extraction using nitrogen, there is significant influence of ethanol content on DPPH values of oregano extracts. For HVED extraction using argon, there is significant influence of ethanol content on yield values of oregano extracts ($p \leq 0.05$). The I_R values of obtained extracts were obtained using an EPR spectroscopy, and lower values indicate the higher antioxidant activity of

Table 4 UPLC-MS/MS analysis of extractive compounds from oregano (measurements for CE- and HVED-treated samples) (ng/mL)

Sample	Apigenin	Carnosol	Diosmetin	Hydroxytyrosol	Luteolin	Oleanolic acid	Oleuropein	Quercentin	Rosmarinic acid	p-cymene	Carvacrol	Camphor	Thymol	**Extraction type**
3 O0	26.7190	0.1317	49.5192	1220.0221	191.0806	N/A	N/A	0.1343	64.8690	0.2319	0.0125	0.1790	0.0771	CE
9 O0	0.0988	0.0596	0.1146	0.2550	1.8565	N/A	N/A	N/A	0.3698	0.0242	0.0008	0.0424	0.0015	
3 O25	67.8901	0.6560	73.1606	139.7604	139.7604	N/A	1.3525	1.2726	4721.5901	0.4230	0.0047	6.7108	0.3370	
9 O25	66.2952	0.0554	61.7534	175.6552	295.7906	N/A	1.2147	2.3875	6279.3696	2.7216	16.254	0.3366	0.3932	
3 O50	21.4445	0.0910	10.2109	80.1791	67.5741	292.7768	1.9606	2.5666	7120.3091	0.0002	0.0344	0.0134	0.0111	
9 O50	29.4085	0.2435	14.3638	99.5673	78.6565	607.8882	0.9001	2.0585	7015.5906	0.0001	0.1772	0.0010	0.0066	
ON1	29.5422	6.4244	44.4430	163.0726	128.8512	571.6491	0.4215	1.4564	6778.1671	0.0002	0.0009	0.0009	0.0002	HVED
ON2	161.9087	0.1860	161.0401	201.3003	545.8774	N/A	0.8027	0.9466	307.3124	0.0004	0.0005	0.0022	0.0002	
ON3	130.2587	0.0648	157.4162	2.5138	213.5677	N/A	0.0901	N/A	9.5986	0.0001	0.0001	0.0005	0.0002	
ON4	241.6326	0.0852	230.1913	5.1477	505.7684	N/A	N/A	N/A	6.3204	0.0004	0.0003	0.0008	0.0010	
ON5	37.9383	0.1652	46.2547	226.4722	193.5792	N/A	0.2247	6.4996	5845.0470	0.0002	0.0002	0.0016	0.0004	
ON6	38.0113	0.1151	45.4213	176.9062	186.3631	N/A	0.2948	5.2647	5092.9196	0.0010	0.0003	0.0013	0.0001	
ON7	28.8378	0.2585	38.9683	123.5226	129.6773	137.8594	0.2764	0.9395	6148.9590	0.0002	0.0003	0.0018	0.0005	
ON8	32.1221	0.1153	39.6107	126.1000	138.5636	114.4831	0.1040	0.9403	6304.5726	0.0019	0.0004	0.0056	N/A	
ON9	30.4893	0.0826	36.0572	143.8500	131.8861	N/A	0.1596	3.6732	4673.8530	0.0000	0.0001	0.0007	0.0000	
ON10	150.6299	0.3325	157.2593	3.9222	191.0310	N/A	N/A	N/A	24.7603	0.0002	0.0003	0.0008	0.0001	
ON11	41.3586	0.2474	59.5840	127.2977	224.9529	91.4115	2.4120	2.0493	6073.8332	0.0003	0.0000	0.0024	0.0002	
ON12	36.5870	0.1053	44.7293	147.2922	175.7205	N/A	0.2169	2.9289	4608.0827	0.0006	0.0001	0.0006	0.0001	
OA1	33.0985	1.5446	53.9525	136.8496	168.9821	384.1643	0.1981	1.2420	6824.2333	0.0002	0.0002	0.0014	0.0003	
OA2	102.9958	0.4277	121.3095	14.5805	286.8336	N/A	0.1118	N/A	36.2145	0.0003	0.0004	0.0056	0.0003	
OA3	140.8111	0.2452	157.5651	72.7284	371.9303	N/A	0.1643	N/A	6.4155	0.0006	0.0004	0.0017	0.0001	
OA4	115.0587	0.2637	210.3784	355.8469	556.8231	N/A	0.2214	1.6395	N/A	0.0011	0.0003	0.0004	0.0003	
OA5	26.5438	0.2216	32.7508	166.4899	141.8778	N/A	0.1624	5.4779	5301.6813	N/A	N/A	0.0011	N/A	
OA6	25.4582	0.1476	36.2213	156.9322	135.6786	N/A	0.1093	6.3645	5429.7199	0.0002	0.0002	0.0047	0.0007	
OA7	31.7985	0.2892	47.8602	119.9150	135.2109	118.4562	0.1478	1.5840	6292.1307	0.0004	0.0001	0.0012	0.0005	
OA8	38.2096	0.4530	56.9014	160.6655	172.5675	409.6722	0.2196	1.3671	6951.8650	0.0001	0.0002	0.0023	0.0001	
OA9	43.1587	0.1104	51.9164	157.4797	233.6387	N/A	0.2057	4.0596	5051.4470	0.0012	0.0007	0.0007	0.0001	
OA10	156.9071	0.1528	146.9811	1.4572	203.7296	N/A	N/A	N/A	20.3466	0.0005	0.0016	0.0034	0.0002	
OA11	29.7964	0.2417	36.4388	123.4133	124.0078	261.4641	3.4429	1.0654	6526.4812	0.0049	0.0003	0.0015	0.0002	
OA12	25.2101	0.1159	31.7368	137.4879	136.2554	N/A	0.3335	4.0693	5026.0186	0.0003	0.0001	0.0016	0.0002	

O oregano; *N* nitrogen; *A* argon. For HVED, numbers 1–12 are the order of conducted treatment. For CE treatments, 3 and 9 are referred to treatment time while 0, 25, and 50 stands for concentration of an ethanol solvent (%)

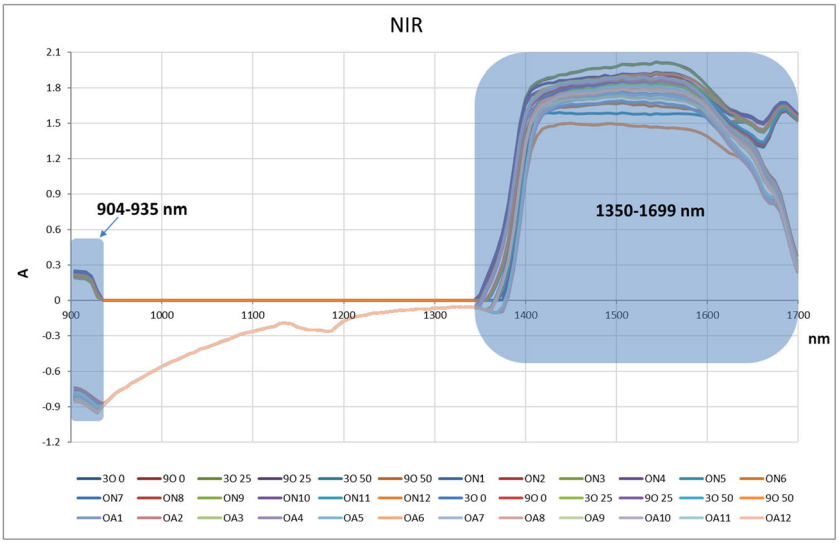


Fig. 3 Near-infrared (NIR) spectra of oregano extracts with significant regions from 904 to 935 nm and 1350–1699 nm. O oregano; N nitrogen; and A argon. For HVED, numbers 1–12 are the order of conducted treatment. For CE treatments, 3 and 9 are referred to treatment time, while 0, 25, and 50 stands for concentration of an ethanol solvent (%)

extracts. All HVED extracts had lower I_R values than CE, meaning that HVED extracts had higher antioxidant activity. In average, HVED extracts had 3.54× lower I_R than extracts obtained by CE indicating that there are still more antioxidants in extracts than free radicals.

UPLC-MS/MS Analysis of Obtained Oregano Extracts

An UPLC-MS/MS analysis of individual phenolic compounds has shown that main constituents of phenolics in oregano extracts were in following order according to the average concentration in all extracts: rosmarinic acid > luteolin > hydroxytyrosol > oleanolic acid > diosmetin > apigenin > quercetin > carvacrol > oleuropein > carnosol > camphor > p-cymene > thymol but in different concentrations depending of the extract (Table 4). Rosmarinic acid is the main constituent detected by UPLC-MS/MS in oregano extracts, having the highest values with increase in ethanol content up to 50% of ethanol. Hydroxytyrosol is also one of the main constituents [29], and there is higher amount in samples extracted in water (0% ethanol). Hydroxytyrosol is a precursor of oleuropein, scavenger of superoxide anions, and inhibitor of neutrophils and hypochlorous acid-derived radicals. On the other side, oleuropein was detected in very low amounts from 0.00 to 3.44 ng/mL.

The ANCOVA analysis was performed to see correlation between each phenolic compound and TPC and antioxidant capacity. Results showed significant correlation ($p \leq 0.05$) for thymol with all three variables (TPC, DPPH, EPR), for oleanolic acid with DPPH and for quercetin with TPC. Additionally, carvacrol and thymol were compared with theoretical results obtained by COSMO-RS and Hansen (Table 2). Both compounds showed high probability of solubility (60–100%) in ethanol and low probability (0–20%) in water. The highest content of these compounds was found experimentally in extracts with 25% of ethanol.

Near-Infrared (NIR) Spectra and Principal Component (PCA) Analysis of Oregano Extracts

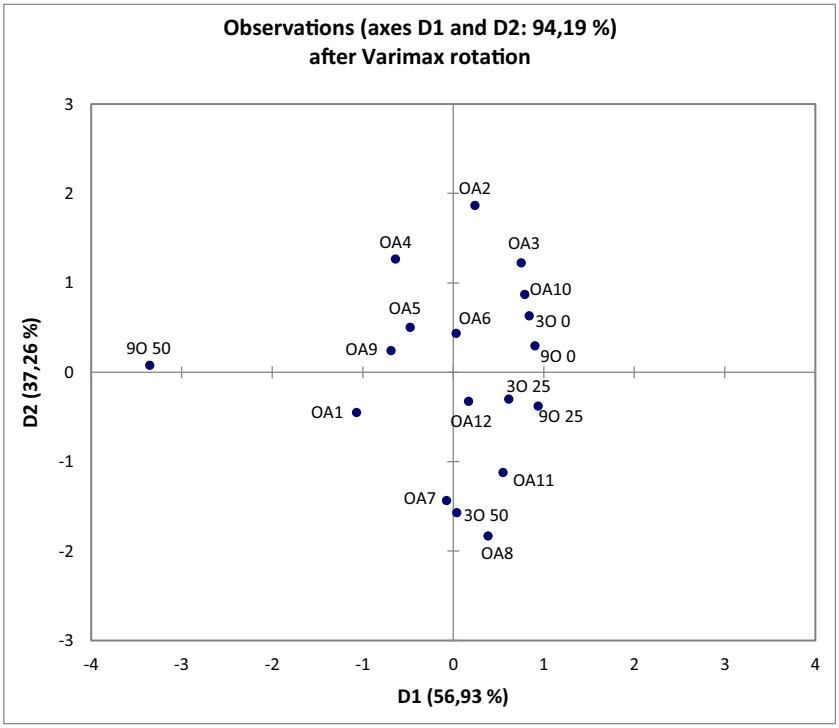
The NIR spectra were recorded for all oregano extracts over a wavelength range of 899–1699 nm (Fig. 3). Two significant ranges were identified, the first from 904 to 935 nm and second from 1350 to 1699 nm. The second significant spectral region (1350–1699 nm) is a characteristic of the first overtone of the R–OH and O–H stretching vibrational bands (alkyl alcohols or water) and the intermolecular H-bonds of water absorption [30]. When analyzing the spectra, it is clear that most of the recorded spectra are very similar.

The most significant difference between argon- and nitrogen-treated samples is that nitrogen samples start from the positive results of absorbance (around 0.2), while argon and untreated samples start from negative part of the spectrum (around − 0.8) and have lower values of absorbance (negative) until the second significant range (1350–1699 nm). Also, all nitrogen-treated samples have an increase in the absorbance in the last part of the spectra (with peak around 1687 nm), while untreated and argon-treated samples have decreased trend of absorbance starting from 1553 nm. On the other hand, a complex chemical composition of oregano results in a large set of overtones and vibrations that can be observed on the NIR spectrum. It can be also observed that samples prepared in a solvent with a higher percentage of ethanol are more pronounced in the NIR spectrum.

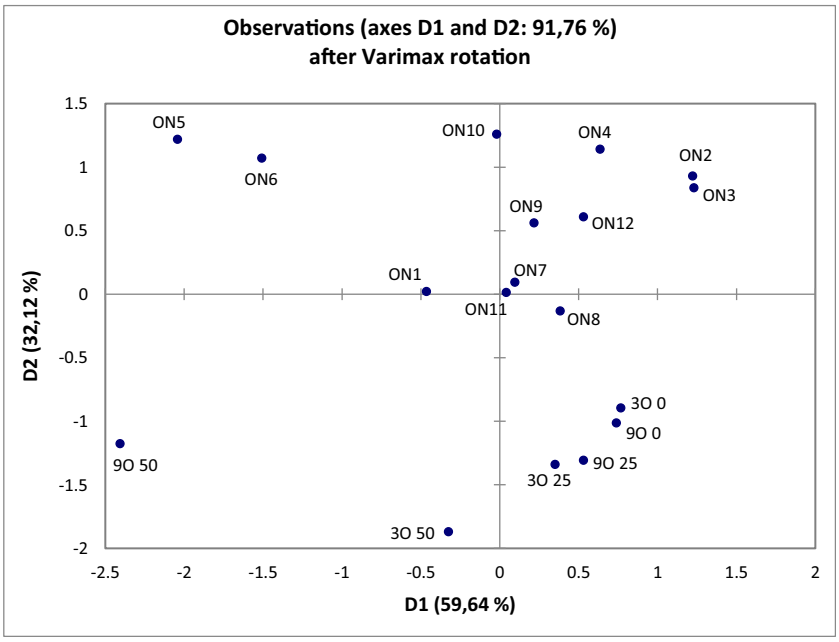
Since recorded spectra of oregano extracts are very similar, and some overlapping occurred, the further chemometric analyses were observed. For this purpose, only the most significant range of recorder spectra with the least overlapping (1350–1699) was taken for further analyses for better

explanation of variables. The PCA analysis was used for extraction of information about the quality of data collected with NIR spectrum. The analysis of major components enables grouping of data without a set physical model.

Using the PCA analysis, we have determined which of the observed parameters had a significant effect to the distribution of samples. For argon-treated samples (Fig. 4a), the first major component encompasses 56.93% of variance in the observed data set, whereas the other major component explains 37.26% of the variance in the observed data set. A total of 94.19% of variance can be clarified in the observed data set. Aqueous extracts were



a)



b)

Fig. 4 The principal component analysis (PCA) of oregano extracts: (a) The data is denoting untreated oregano samples and oregano samples treated with HVED using argon. (b) The data is denoting untreated oregano samples, and oregano samples treated with HVED using nitrogen.O oregano; N nitrogen; and A argon. For HVED, numbers 1–12 are the order of conducted treatment. For CE treatments, 3 and 9 are referred to treatment time, while 0, 25, and 50 stands for concentration of an ethanol solvent (%)

placed in the upper part (higher values of D2) in the first and second quadrant. Extracts with 25% ethanol had lower D2 values and were all placed around origin, while extracts with 50% ethanol content were all placed in third and fourth quadrant, except untreated sample 9O 50. The second PCA analysis of nitrogen-treated samples (Fig. 4b) had a total of 91.76% of the variance with 59.64% major component encompasses of variance in the first and 32.12% in the other major component. In this graph, grouping by ethanol content was also notable. Furthermore, clear separation by untreated and HVED-treated samples was noted; all untreated extracts were placed in the third and fourth quadrant.

Provided results indicated that NIR spectroscopy used with chemometrics such as PCA presented as a fast, useful, and non-invasive method for determination of phenolics from oregano extracts. Although overlapping of untreated and HVED-treated extracts happened, the PCA analysis showed differences in each extract, and separation results were in line with results of TPC. Similar results were found in cold plasma treatment of pomegranate juice where NIR spectra recorded two significant ranges from 968 to 1115 nm and 1269 to 1457 nm and PCA analysis was also successfully performed [31].

Conclusions

The potential of water and ethanol as green solvents in the extraction of BACs from oregano leaves was evaluated experimentally and by theoretical models using two softwares (COSMO-RS and HSP). Experimental study was performed with HVED extraction that was compared with modified CE. Results confirmed the high potential of HVED for extraction of BACs from oregano, 0.9–6.5 times higher efficiency using HVED in the same conditions compared with CE. The extraction yield presented as value of TPC per g of sample (%) was increased with longer treatment, the use of nitrogen, higher voltage, and ethanol concentrations of 25%. Similar results were noted for antioxidant capacity and content of each individual BAC. NIR spectroscopy was presented as a fast and efficient method for evaluation of BACs from oregano extracts when additional statistical analysis like PCA is used. Due to electroporation of oregano performed with HVED treatment, more BACs were extracted, and significant changes in physical properties of extracts were noted accordingly. Theoretical results were in line with experimental for solvent selection; ethanol had higher potential of solubility of BACs than water. However, theoretical results showed that some BACs from oregano are poorly soluble in both water and ethanol. Therefore, other green solvents should also be considered in extraction of BACs from oregano.

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Compliance with Ethical Standards

Conflict of Interest All authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human or animal subjects.

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Paper III

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Original article

Extraction of phenolic compounds from oregano using high voltage electrical discharges–sustainable perspective

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Summary The aim of the study was to observe the sustainability parameters of high voltage electrical discharge (HVED) as a novel technology for extraction of phenolic compounds from oregano. The life cycle assessment (LCA) of HVED was evaluated and compared with conventional extraction methods–infusion and maceration. The results showed that HVED extraction with nitrogen gave 2.19%–34.04% higher yield of phenolic compounds compared to infusion and 6.13%–90.95% higher yield compared to maceration, depending on the voltage and treatment time used for HVED. The environmental pillar of LCA, assessed by global warming potential, ranged from 1.130 to 4.635 kg CO_2e for HVED samples treated with nitrogen and from 1.207 to 4.487 kg CO_2e for samples treated with argon, which was mainly related to the duration of treatment. Other pillars of sustainability, namely society, economy, food safety and quality, were discussed. HVED was found to be the more sustainable technology (based on the results from LCA), with prospects for future scale-up for the food industry.

Keywords high voltage electrical discharge, life cycle assessment, nonthermal technology, oregano, polyphenols, sustainability.

Introduction

In recent years, there has been a growing interest by industries to replace conventional extraction methods with more sustainable, green extraction techniques. These extraction methods are based on the search for processes that reduce energy consumption while allowing the use of alternative solvents and renewable natural products, as well as ensuring a safe and high quality extractor product (Chemat *et al*., 2012). Several innovative technologies based on green extraction principles have been developed to improve extraction yields in less time and with minimal solvent and energy consumption compared to conventional methods (Chemat *et al*., 2020). Among them, the use of electrotechnologies such as HVED has attracted the interest of industry and academia as they show great potential to improve the recovery of bioactive compounds from plant sources in a fast, economical and more sustainable manner (Li *et al*., 2019).

Bioactive compounds are molecules that are gaining popularity among scientists, primarily because of their many beneficial nutritional and medicinal properties. They are also increasingly used in a variety of industries (Srivastava *et al*., 2021). Oregano is a traditional Mediterranean plant belonging to the mint family Lamiaceae with health properties due to its various bioactive compounds, mainly polyphenols (Kintzios, 2012). These polyphenols have high potential for extraction using novel technologies such as HVED.

At this stage, no assessment of the environmental impact of HVED treatment can be found in the literature. Emerging (non-thermal) technologies are presented as more sustainable compared to conventional extraction methods, but no concrete calculations can be found in the literature. Life cycle assessment (LCA) of emerging technologies could help industry and academia in further research optimisation and cost assessment. Sustainability has gained much attention with the 2015 United Nations 2030 Agenda (FAO, 2015). This agenda aims to achieve sustainable development and the coexistence of people, technology and the environment through 17 sustainable development goals. Within these goals, the use of new technologies for food processing is supported as they are considered sustainable, environmentally friendly, fast, clean and green techniques with low CO_2 emissions and lower environmental impact (Picart-Palmade *et al*., 2019; Režek Jambrak *et al*., 2021). However, there are no clear or concise recommendations that the extraction

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of natural products is a green sustainable process. For this reason, LCA should be conducted for all emerging technologies and compared with conventional technologies to obtain real data on environmental impacts.

This work is a beginning of such an approach to present HVED as a sustainable extraction technology. HVED is a non-thermal technology that is effective at room temperature or slightly elevated temperature, reduces the negative effects of heat on nutrient composition and food quality, and produces less CO2 emissions because there is no heating (Stoica *et al*., 2013). HVED has the potential for industrial scale-up where larger quantities could be processed in a similar time frame. Although scale-up leads to process adaptation compared to laboratory scale, the LCA evaluation of HVED at laboratory scale could also be of great interest for the food industry (Boussetta *et al*., 2012; Carmelia Bălănică Dragomir *et al*., 2020).

The aim of this study was to compare HVED with conventional extraction methods–infusion and maceration–in terms of sustainability. The comparison was based on analyses of phenolic compound yields, environmental impacts–particularly global warming and ozone depletion potential–and economics of the extractions. Water and ethanol were used as green solvents for the extraction, which have been shown to be cheap, environmentally safe and have the potential to replace toxic organic solvents such as methanol and hexane (Chemat *et al*., 2019). Nitrogen and argon have been used as inert gases that are environmentally neutral and safe in HVED treatment (Greig *et al*., 2016).

Materials and methods

Plant materials

The dried herb of the oregano plant (*Origanum vulgare* L.) was provided by a local specialty drugstore (Suban d.o.o., Samobor, Croatia). The plant material was collected during the flowering season in 2017 in northwestern Croatia, naturally dried, ground and stored in polyethylene bags in a dark and dry place at room temperature until extraction. The particle size distribution of the plants measured by laser particle size analyzer (Malvern, Mastersizer 2000, Germany) was d (0.1) ≤164.315 μm; d(0.5) ≤297.345 μm; d(0.9) ≤461.460 μm. Before extraction, the herbal material was weighed and mixed with 1:50 (w/v) solvent. Extraction was carried out using distilled water, 25% and 50% aqueous ethanol (v/v) as extraction solvent.

High Voltage Electrical Discharge (HVED)

HVED extraction was performed using the generator 'IMP-SSPG-1200' (Impel Group, Zagreb, Croatia) (Fig. 1), which generates rectangular pulses of direct current and high voltage. HVED parameters were as follows: Frequency (100 Hz), pulse duration (400 ns), high voltage current (30 mA), distance between electrodes (15 mm), voltage (15 and 20 kV for argon and 20 and 25 kV for nitrogen) and treatment time (3 and 9 min). The mixture of herbal material and solvent was transferred to beaker-shaped reactor of 100 mL. This reactor was open on both sides and was therefore fitted with silicone lids of 1 cm diameter. The silicone tops were used because it is easier to attach the electrode from the top and the needle from the bottom. Gases (argon or nitrogen) were introduced through the needle at a flow rate of 0.75 NL min^{-1}. Extractions were performed using 1 g of dry plant material and 50 mL of solvent. The HVED parameters and setup have been used previously and described in more detail by Nutrizio *et al*. (2020a). After HVED extractions, all samples were filtered and quantitatively transferred to a 50 mL volumetric flask. A total of 24 extracts were prepared in duplicates.

Infusion

Infusion extraction was performed by heating the sample-solvent mixture in a Sonorex Digitec DT 100H water-ultrasonic bath (Bandelin electronic, Berlin, Germany) at 80 °C for 30 min (ultrasound was not turned on) according to the method of Vuong *et al*. (2011). Extractions were performed using 2 g of dry plant material and 100 mL of solvent. After extraction, the sample was filtered and quantitatively transferred to a 50-mL volumetric flask. The infusion was prepared in duplicate.

Maceration

Maceration was performed according to Trusheva *et al*. (2007) with slight modifications. Extraction involved mixing 2 g of dry plant material with 100 mL of solvent for 48 h at room temperature. Throughout the maceration period, the mixture was stirred several times. After completion of maceration, the extract was separated by filtration and quantitatively transferred to a 50-mL volumetric flask. The maceration was carried out in duplicate.

Determination of Total Phenolic Content (TPC)

The TPC of oregano extracts was determined using the Folin-Ciocalteu method as previously described (Shortle *et al*., 2014) with slight modifications. A volume of 0.1 mL of the extract (appropriately diluted) was mixed with 0.2 mL of Folin-Ciocalteu reagent. After 3 min, 1 mL of 20% Na_2CO_3 (m/v) was added. After thorough vortex mixing, the reaction mixtures were incubated at 50 °C for 25 min, followed by

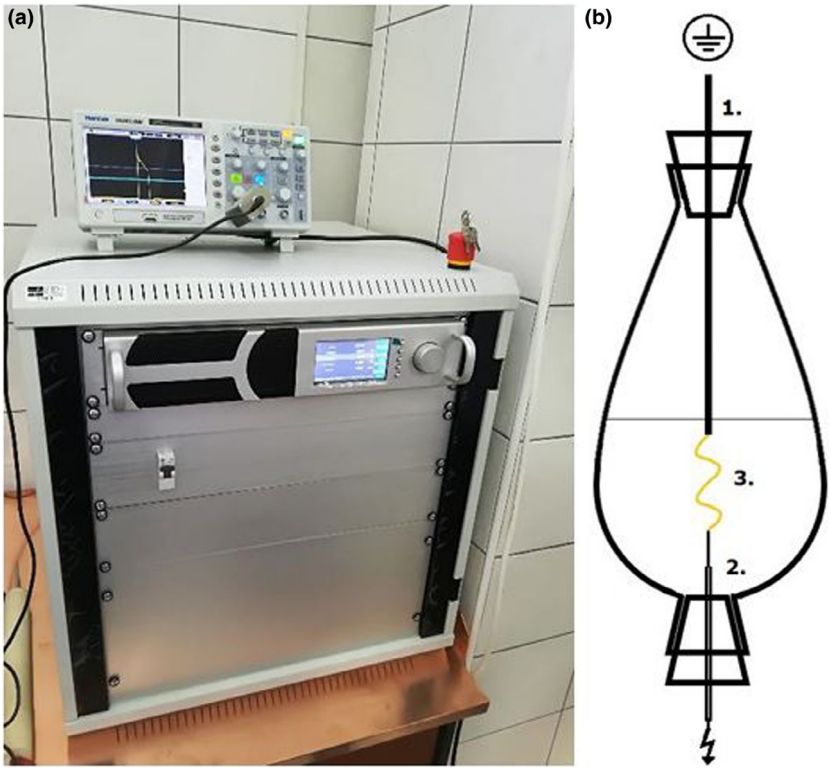

Figure 1 Set-up of generator and reactor for HVED: (a) HVED and plasma generator 'IMP-SSPG-1200' (Impel group d.o.o., Zagreb, Croatia); (b) Beaker-shaped reactor-(1)–ground electrode; (2) high voltage electrode (needle with empty interior for argon and nitrogen flow) during treatments and (3) discharge (plasma).

absorbance measurement at 765 nm against blank (extraction solvent was used instead of extract). The calibration curve was prepared using 50 to 500 mg L^{-1} gallic acid in ethanol. TPC concentration was expressed in mg gallic acid equivalents per gram dry weight of sample (mg GAE g^{-1} of sample). All TPC measurements were performed in duplicates.

Life cycle approach

The assessment of the environmental impact of oregano treatment, carried out using three extraction methods (HVED, infusion and maceration), was carried out using a partial LCA approach. It involved mapping the process of all treatments, defining the scope and limits at laboratory scale, collecting and calculating data and evaluating the results (ISO, 2006). A treatment of oregano was set as the functional unit (FU) output reference. The boundaries of the system in this study are shown in Fig. 2.

The inventory analysis included the use of natural resources (water, energy), the use of chemicals, namely ethanol (as a solvent) and nitrogen and argon gas for HVED treatments. The flow of gases (nitrogen and argon) was estimated to be 0.75 NL min^{-1}. The calculation of environmental impacts was performed using data from ©CCaLC and Ecoinvent databases (CCaLC, 2018).

The environmental footprints calculated in this study included global warming potential (GWP) and ozone depletion potential (ODP). GWP represents the damage level in kg of CO2 equivalent (CO_2e), which is the weighted impact of greenhouse gases (GHGs) for a 100-year period (IPCC, 2013). ODP calculates the destructive impact of halogenated hydrocarbons on the stratospheric ozone layer over a 100-year time horizon. The impacts of the chemical compounds are weighted relative to the impacts of trichlorofluoromethane (R-11 or CFC-11) (Hischier *et al.*, 2010).

Economic assessment

The price of each FU was estimated based on the ethanol content and the average electricity consumption for each treatment (kWh). The power (W) consumed during HVED treatment was measured directly at the HVED device, while the power consumed for heating during infusion was taken from the device's manual. The electricity price was calculated according to the current prices in Croatia based on information from the national electricity company (HEP Elektra d.o.o, Croatia), while the ethanol price was taken as the purchase price from a local supplier. The price is in Euro, based on the exchange rate on 7th of June 2021.

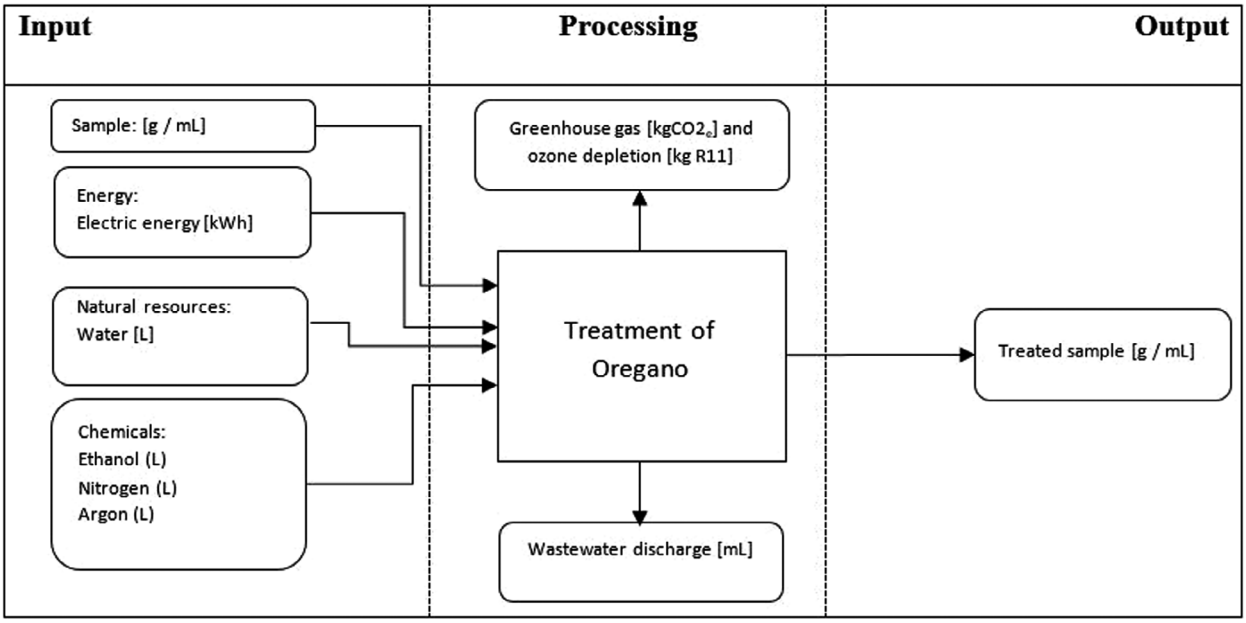

Figure 2 Generic model of oregano treatments employed.

Statistical analysis

Statistical analysis was performed using Microsoft Excel v2016. To evaluate the statistical difference between the different extractions, data were analysed using the one-way ANOVA test. In addition, a paired Student's *t*-test was used for the HVED results of TPC and for economy assessment. The significance level for rejecting a null hypothesis was *P*-value ≤0.05 for all tests.

Results and discussion

Total Phenolic Content (TPC) of oregano extracts

Detailed results of physical and chemical analysis after HVED extractions of oregano were given in a previous paper (Nutrizio *et al*., 2020b). For the purpose of this study, only the TPC results are presented (Table 1).

In general, a higher yield of phenolic compounds was found in HVED extracts when nitrogen was used compared to argon, a higher voltage, a longer treatment time and ethanol concentrations of 25%. The highest content of TPC was 191.28 ± 7.12 in an extract obtained with nitrogen at 25 kV, treatment time of 9 min and 25% ethanol as solvent. Nitrogen is an inert gas that could replace atmospheric oxygen in the extraction system and interrupt oxygen-induced oxidation to protect plant polyphenols. From the sustainability point of view, it is also worth mentioning

Table 1 Total phenolic content (TPC) of HVED extracts

Gas	Solvent	Treatment time (min)	Voltage (kV)	TPC (mg GAE g^{-1})
Nitrogen	H_2O	3	20	73.02 ± 7.33[b]
			25	124.98 ± 8.17[a]
		9	20	112.80 ± 8.13[a]
			25	92.37 ± 7.11[a]
	25% EtOH	3	20	128.89 ± 9.33[a]
			25	125.20 ± 5.32[a]
		9	20	137.80 ± 8.30[b]
			25	191.28 ± 7.12[a]
	50% EtOH	3	20	86.50 ± 6.16[a]
			25	62.15 ± 6.11[a]
		9	20	74.54 ± 4.11[a]
			25	81.28 ± 5.54[a]
Argon	H_2O	3	15	36.93 ± 3.38[b]
			20	77.37 ± 5.15[a]
		9	15	82.59 ± 6.16[a]
			20	80.20 ± 5.65[a]
	25% EtOH	3	15	116.07 ± 8.42[a]
			20	114.76 ± 6.14[a]
		9	15	129.54 ± 8.43[a]
			20	122.80 ± 7.12[a]
	50% EtOH	3	15	73.46 ± 5.14[a]
			20	74.76 ± 5.58[a]
		9	15	86.28 ± 3.19[a]
			20	75.63 ± 4.66[a]

*Results are given as mean ± standard deviation; Values represented with different letters are statistically different at $P \leq 0.05$.

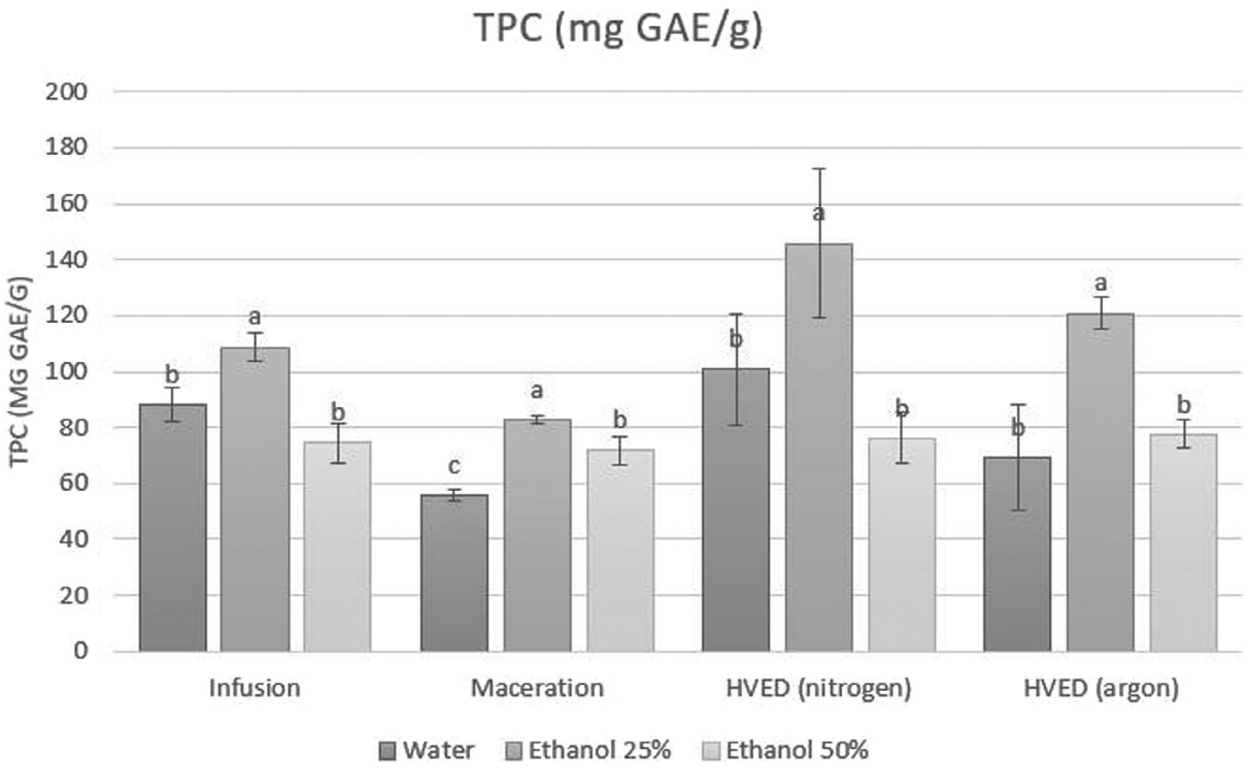

Figure 3 Mean values of total phenolic content (TPC) of extracts obtained by HVED and conventional extractions (infusion and maceration) per solvent.

that the nitrogen could be recycled during the extraction process and reused for further extractions (Ding *et al.*, 2019). Similar results in the extraction of polyphenols were also observed in other Mediterranean plants such as rosemary and wild thyme (Nutrizio *et al.*, 2020a,c). The detailed phenolic profile obtained by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) of the extracts obtained by HVED can be found in the supplementary material Table S1 (Nutrizio *et al.*, 2020b). The results obtained were in agreement with the TPC and confirmed the improved nutritional profile of the HVED extracts.

The reported results of TPC for HVED were compared with the phenolic content of two conventional extractions–infusion and maceration. The results of all extraction methods are shown in Fig. 3.

The highest yield of phenolic compounds extracted by all extraction methods (HVED, infusion and maceration) was obtained from extracts containing 25% ethanol. The observed difference was statistically significant with a P-value ≤ 0.05. This trend was also observed with different extraction methods of tiger nut by-products (Roselló-Soto *et al.*, 2019), grape pomace (Huaman-Castilla *et al.*, 2019) and rosemary (Nutrizio *et al.*, 2020a). The solubility of phenolic compounds depends mainly on the composition of the compounds themselves, including the presence and position of hydroxyl groups, which have a direct influence on the polarity of the phenolic compound. For this reason, phenolic compounds are often extracted in higher yields with more polar solvents such as aqueous ethanol, compared to pure water (Iloki-Assanga *et al.*, 2015; Bundeesomchok *et al.*, 2016). The polarity of each phenolic compound from oregano and their solubility in ethanol and water was also observed by computational prediction methods, the Conductor-like screening model for real solvents (COSMO-RS) and the theoretical prediction of Hansen solubility parameters (HSPs), which confirmed a higher solubility of phenolic compounds from oregano in ethanol than in water (Nutrizio *et al.*, 2020b).

Analysis of the yield of phenolic compounds showed that higher results were obtained when extracted with HVED than with the two conventional extraction methods–infusion and maceration. Higher results were observed when nitrogen was used than argon in HVED. On average, 2.19%–34.04% higher yields were obtained with nitrogen HVED compared to infusion and 6.13%–90.95% higher yields were obtained compared to maceration when the same processing conditions (ethanol content) were considered. The difference

Table 2 Estimated environmental impacts (GWP and ODP) of HVED treatment [functional unit–1 treatment]

Gas	Solvent	Treatment time (min)	Voltage (kV)	Extraction yield (mg GAE g^{-1} min^{-1})	GWP (kg CO_{2e})	ODP (mg $R11_e$)
Nitrogen	H_2O	3	20	24.34	1.255	0.074
			25	41.66	1.489	0.092
		9	20	12.53	3.766	0.223
			25	10.26	3.906	0.233
	25% EtOH	3	20	42.96	1.130	0.064
			25	41.73	1.364	0.082
		9	20	15.31	3.781	0.223
			25	21.25	4.481	0.276
	50% EtOH	3	20	28.83	1.098	0.061
			25	20.72	1.284	0.075
		9	20	8.28	3.374	0.192
			25	9.03	4.635	0.287
Argon	H_2O	3	15	12.31	1.239	0.067
			20	25.79	1.449	0.083
		9	15	9.18	3.857	0.211
			20	8.91	4.487	0.259
	25% EtOH	3	15	38.69	1.207	0.064
			20	38.25	1.418	0.080
		9	15	14.39	3.592	0.190
			20	13.64	4.432	0.254
	50% EtOH	3	15	24.49	1.175	0.061
			20	24.92	1.385	0.077
		9	15	9.59	3.676	0.196
			20	8.40	4.166	0.233

between the results depended on the duration of HVED treatment and the voltage used. Although HVED extraction was performed in a significantly shorter time compared to conventional extraction, the extraction yields were higher. HVED is an extraction method performed at room temperature and is therefore a less thermally destructive method than conventional extractions that use high temperatures. This is of great importance for thermolabile compounds such as plant phenols (Parniakov *et al*., 2014; Li *et al*., 2019).

Environmental impact of oregano treatments

Two environmental potentials associated with non-thermal (HVED) treatment were calculated from the experimental design and inventory analysis (Table 2). The range of CO_2 emissions from the HVED treatments was between 1.130 and 4.635 kg CO_2e for the nitrogen-treated samples and between 1.207 and 4.487 kg CO_2e for the argon-treated samples, mainly related to the duration of the treatments (gas and energy consumption). Although nitrogen and argon are not counted as greenhouse gases, their impact is high due to the production facilities where these gases are produced. On the other hand, the ODP is not high and ranges between 0.064 mg R11 and 0.287 mg R11 for samples treated with nitrogen and 0.064 mg R11 and 0.259 mg R11 for samples treated with argon.

At longer treatment times and higher voltage, more energy was used for extraction and, accordingly, higher results for GWP and ODP were obtained at these settings. Statistical analysis showed that ethanol content had no significant effect on GWP and ODP, but treatment time and voltage did. Based on these results, the treatment parameters can be optimised both in terms of extraction yield and environmental impact.

As for the other two conventional treatments (infusion and maceration) as completely different treatments, infusion performed at 80 °C for 30 min in baths with an operating volume of 2 L using solvents and maceration using only solvents showed a large difference in environmental impact (Table 3).

In terms of energy consumption, it was evident that HVED had promising potential compared to infusion (shorter treatment time and lower equipment power consumption). Maceration had the lowest environmental impact as no heating or electricity is used for this treatment. However, maceration required a long treatment time (48 h) compared to the other extraction methods and significantly lower extraction yields were observed (Fig. 3). Considering the extraction yield in the same time (expressed as TPC per minute of a treatment) (Tables 2 and 3), it is clear that the yield was highest for HVED treatment (8.28–42.96 mg GAE g^{-1} min^{-1}), followed by infusion (2.48–3.63 mg GAE g^{-1} min^{-1}) and maceration (0.02–0.03 mg GAE g^{-1} min^{-1}), with significantly lower yield values. Considering the environmental impact per treatment yield, it can be concluded that for 1 min of extraction, HVED had the lowest environmental impact, while extraction by infusion had the highest impact.

Other sustainability aspects of oregano treatments

In terms of sustainability, there are three main pillars: environment, society and economy (Brundtland, 1987). Gast *et al*. (2017) emphasises that none of the three

Table 3 Estimated environmental impacts (GWP and ODP) of conventional treatments [functional unit–1 treatment]

Sample	Extraction yield (mg GAE g^{-1} min^{-1})	GWP (kg CO_{2e})	ODP (mg $R11_e$)
I - H_2O	2.94	53.696	4.076
I - 25%EtOH	3.63	53.745	4.078
I - 50% EtOH	2.48	53.794	4.079
M - H_2O	0.02	$0.032 * 10^{-3}$	$1.610 * 10^{-6}$
M - 25%EtOH	0.03	$48.999 * 10^{-3}$	$1.530 * 10^{-3}$
M - 50% EtOH	0.02	$97.968 * 10^{-3}$	$3.060 * 10^{-3}$

I, infusion; M, maceration.

Table 4 Estimated economic impacts of HVED and conventional treatments [functional unit–1 treatment]

Extraction type	Gas	Solvent	Treatment time (min)	Voltage (kV)	Power (W)	Price (€)*10^{-3}
HVED	Nitrogen	H_2O	3	20	20.0 ± 1.0	0.06[a]
				25	30.0 ± 3.0	0.09[a]
			9	20	20.0 ± 2.0	0.18[a]
				25	22.0 ± 2.0	0.20[a]
		25% EtOH	3	20	14.0 ± 1.0	28.17[a]
				25	24.0 ± 2.0	28.20[b]
			9	20	20.0 ± 1.0	28.31[a]
				25	30.0 ± 4.0	28.40[a]
		50% EtOH	3	20	12.0 ± 2.0	53.47[a]
				25	20.0 ± 2.0	53.50[a]
			9	20	14.0 ± 0.0	53.57[a]
				25	32.0 ± 2.0	53.73[b]
	Argon	H_2O	3	15	11.0 ± 1.0	0.03[a]
				20	14.0 ± 1.0	0.04[a]
			9	15	13.0 ± 2.0	0.12[a]
				20	22.0 ± 3.0	0.20[a]
		25% EtOH	3	15	9.0 ± 0.0	28.15[a]
				20	18.0 ± 1.0	28.18[b]
			9	15	9.0 ± 1.0	28.21[a]
				20	21.0 ± 3.0	28.32[a]
		50% EtOH	3	15	7.0 ± 1.0	53.46[a]
				20	16.0 ± 2.0	53.49[a]
			9	15	10.0 ± 2.0	53.53[a]
				20	17.0 ± 1.0	53.59[a]
Infusion	/	H_2O	30	/	140	4.29[a]
		25% EtOH			140	32.41[b]
		50% EtOH			140	57.73[c]
Maceration	/	H_2O	2880	/	0	0[a]
		25% EtOH			0	28.12[b]
		50% EtOH			0	53.44[c]

*The price was estimated based on ethanol content and power used for each treatment (kWh). The price is shown in euros, based on exchange rate on 7th of June 2021. Values represented with different letters are statistically different at $P \leq 0.05$.

pillars should be given priority, but that a balance of sustainable production should be sought. Finally, from a food perspective, an additional pillar related to food safety (and quality) should be considered (Režek Jambrak *et al*., 2018).

The economic pillar is an important value to consider when defining FU in food LCA (Mouron *et al*., 2006; Djekic *et al*., 2017). The economic value of extractions depends on all steps during the extraction process–plant material, solvent, equipment and electricity consumption (energy). Since the equipment used during pretreatment is the same for all extraction methods and the solvent to solid ratio was also the same, we can assume that the main economic impacts are electricity consumption during treatment and solvent (ethanol) content. The economic impacts considering these two aspects are shown in Table 4. The power consumed for HVED treatment increased with higher voltage and longer treatment time and ranged from 7.0 ± 1.0 to 32.0 ± 2.0 W. However, the power consumed for heating during infusion extraction was significantly higher (140.0 W). Therefore, the final cost (€) for each treatment extracted under the same conditions with HVED and infusion was higher for infusion extraction. Maceration extraction was evaluated as less economical because no electricity is consumed during extraction. However, maceration is a long time extraction (48 h) with low efficiency, so it is not suitable for industry to extract polyphenols quickly and effectively.

From an economic point of view, energy consumption has the greatest impact on product price, since lower energy consumption leads to a lower final price. Especially when it is known that the prices of energy sources are constantly increasing (Sorrell, 2015). On the other hand, lower consumption of other resources has a high impact on the economic value. For this reason, it is better to use non-thermal technologies such as HVED, where low temperatures are used, treatment time is short and higher extraction yield is obtained compared to conventional extraction techniques.

Therefore, when the laboratory scale technology is transferred to the industrial level, if the process is further optimised, lower material and solvent consumption per treatment could be used, including electricity and other sources that affect the economic value (Piccinno *et al*., 2016; Zuin & Ramin, 2018).

The societal pillar of sustainability represents the good social well-being of a society, which can be assessed through different life spectra such as poverty, inequality, peace and others (Griggs *et al*., 2013). The adoption of HVED as a technology with lower environmental impact and by reducing costs, along with higher productivity and higher quality of a product, has a direct impact on a society (Rüßmann *et al*., 2015; Režek Jambrak *et al*., 2021). Moreover, HVED is a new technology that has a potential for further development and application in various industries. On the other hand, it is important to raise consumer awareness of non-thermal food processing technologies and their impact on the environment and economy. Awareness of natural, organic, sustainable and less processed foods is increasing worldwide, so further education of society could have a positive impact on awareness of novel technologies, including HVED (Song *et al*., 2020).

Finally, the additional pillar deals with food safety and the quality of the product obtained by HVED. Many literature data show that HVED technology improves food safety and quality, including microbial inactivation (Han *et al*., 2016; Yannam *et al*., 2018), enzyme inactivation (Pankaj *et al*., 2013; Gu *et al*., 2021) and nutritional value improvement (Hebert *et al*., 2020; Nutrizio *et al*., 2020b). Our study also showed improved nutritional quality of the extracts in terms of higher total phenolic content compared to conventional extractions (Fig. 3). However, further studies are needed to analyse all the positive and negative effects of HVED on the final extract. This should include microbial analysis, sensory analysis and determination of possible free radical formation.

Considering all the benefits of HVED in terms of LCA, including environmental, social, economic, food safety and quality, it can be considered as a 'green' extraction technology (Režek Jambrak *et al*., 2018; Chemat *et al*., 2020). With further improvements and optimisations, HVED has a high potential for industrial scale-up.

High voltage electrical discharges from a perspective of United Nations SDGs

The main results of this study show two main dimensions of environmental improvements–reduction of energy consumption and lower CO2 emissions. From the perspective of the United Nations Sustainable Development Goals (SDGs), these results confirm the assumption of some authors that the application of non-thermal processing technologies (HVED) not only ensures food safety and quality, but also strives to become a green, clean and energy efficient technology (Režek Jambrak *et al*., 2021), with a direct correlation with SDG7–Affordable and Clean Energy. At the same time, the low emission of greenhouse gases from these technologies has a positive impact on climate change, in line with SDG 13–Climate change mitigation (Djekic *et al*., 2021). Based on their positive outcomes (adequate food safety and quality), Augustin *et al*. (2016) identified their crucial role in food and nutrition security (SDG 2–Zero Hunger) and the pursuit of sustainable diets (SDG 12–Responsible Consumption and Production).

Conclusion

This study demonstrated the high potential of HVED for extraction of phenolic compounds from oregano compared to conventional extraction methods–infusion and maceration. HVED is capable of extracting phenolic compounds at room temperature and in shorter time, thus preventing thermal degradation of thermolabile phenolic compounds. Since HVED uses lower temperatures and shorter time for the extraction of phenolic compounds, better extract quality and lower CO2 emission are obtained than conventional extraction methods. Moreover, HVED has the potential to be used on an industrial scale with the aim of reducing energy consumption and energy costs, thus helping society by reducing the use of energy sources and CO2 emissions. Based on the data obtained in this study, the food industry could consider upscaling HVED to industrial scale. It is expected that they should have a short return of investment.

A limitation of this study is that all calculations were based on the three treatments only and did not consider the potential impact of the following factors: (i) sample preparation; (ii) equipment changeover (cleaning/disinfection) and (iii) use of consumables such as gloves, pipettes, etc.

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Social Fund. The data that support the findings of this study are available on request from the corresponding author. All authors declare that they have no conflict of interest.

Author contributions

Marinela Nutrizio: Conceptualization (equal); Data curation (equal); Formal analysis (lead); Investigation (equal); Methodology (equal); Visualization (lead); Writing–original draft (lead); Writing–review & editing (equal). **Anet Režek Jambrak:** Funding acquisition (lead); Project administration (lead); Resources (equal); Supervision (equal); Writing–review & editing (equal). **Tonči Rezić:** Formal analysis (supporting); Supervision (equal); Writing–review & editing (equal). **Ilija V. Djekic:** Conceptualization (equal); Data curation (equal); Investigation (equal); Methodology (equal); Software (lead); Supervision (equal); Validation (equal); Visualization (equal); Writing–original draft (supporting); Writing–review & editing (equal).

Ethical approval

This article does not contain any studies with human or animal subjects. Therefore, ethics approval was not required for this research.

Peer review

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Data available on request from the authors.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. UPLC-MS/MS analysis of extractive compounds from oregano (measurements for HVED treated samples) (ng/mL) (Nutrizio et al., 2020).

Paper IV

Nutrizio, M., Jurić, S., Kucljak, D., Švaljek, S. L., Vlahoviček-Kahlina, K., Režek Jambrak, A., Vinceković, M. (2023) Encapsulation of Rosemary Extracts using High Voltage Electrical Discharge in Calcium Alginate/Zein/Hydroxypropyl Methylcellulose Microparticles. *Foods* **12**(8), 1570. doi: 10.3390/foods12081570

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Article

Encapsulation of Rosemary Extracts using High Voltage Electrical Discharge in Calcium Alginate/Zein/Hydroxypropyl Methylcellulose Microparticles

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Abstract: The increased demand for functional food with added health benefits is directing industrial procedures toward more sustainable production of naturally added bioactive compounds. The objective of this research was to investigate the potential of bioactive compounds from rosemary extract obtained using high-voltage electrical discharge as a green extraction method, for microencapsulation as a protective method for future application in functional food. Four types of microparticles were made via the ionic gelation method using alginate (Alg), zein (Z), and hydroxypropyl methylcellulose (HPMC) biopolymers and were analyzed considering the physicochemical properties. The diameter of dry microparticles ranged from 651.29 to 1087.37 µm. The shape and morphology analysis of microparticles showed that the obtained microparticles were quite spherical with a granular surface. The high encapsulation efficiency was obtained with a loading capacity of polyphenols up to 11.31 ± 1.47 mg GAE/g (Alg/Z microparticles). The microencapsulation method showed protective effects for rosemary polyphenols against pH changes during digestion. Specifically, the addition of both zein and HPMC to calcium-alginate resulted in microparticles with a prolonged release for better availability of polyphenols in the intestine. This research background indicates that the release of rosemary extract is highly dependent on the initial biopolymer composition with high potential for further functional food applications.

Keywords: microencapsulation; rosemary extract; high-voltage electrical discharge; bioactive compounds; alginate; zein; hydroxypropyl methylcellulose

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1. Introduction

Consumer interest in the beneficial health effects of natural bioactive compounds with antioxidant activity is continuously growing. This is especially noticeable in the food, pharmaceutical, and cosmetic industries, with an emphasis on bioactive compound bioavailability, metabolism, and biological effects [1,2]. Nevertheless, antioxidants, particularly polyphenols, are not stable and they easily interact with other compounds from matrices that surround them. For that reason, it is important to find the best methods for their management from the extraction process to the final incorporation into a new product [3,4].

Firstly, a suitable extraction method should be considered. Due to the disadvantages of conventional extraction techniques that include long processing times and high working temperatures, innovative non-thermal extraction techniques present a favorable alternative for the extraction of bioactive compounds [5,6]. In 2012, Chemat et al. introduced the

concept of green extraction of natural products that are within the principles of green chemistry and engineering [7]. Such an extraction method is a high-voltage electrical discharge (HVED) that is a novel, efficient, eco-friendly extraction method which, in comparison with conventional methods, works with reduced solvent consumption, low operating temperatures, and temperature rise during the extraction, higher extraction yield, and less processing time [8,9]. The HVED principle of operation is based on electroporation [9,10], a phenomenon caused by the electrical field that increases the permeability of the cell membrane, allowing intracellular bioactive compounds to easily diffuse into the extraction solvent [11].

Due to its structure and nature, the aqueous extract reached in polyphenols is not a stable medium for further processing. Their unsaturated bonds in the molecular structure make them sensitive to external environmental conditions such as oxidants, light, pH and temperature changes, enzymatic activities, etc. [1,4]. Therefore, the polyphenolic stability should be increased via protection from external conditions for safe delivery. Microencapsulation is an emerging technology that ensures the protection of sensitive compounds against various processing conditions by encapsulating them inside a coating material and providing a controlled release under specific conditions. Additionally, the food safety and sensory quality of the product can also be improved [12]. Depending on their rheological and functional properties and intended use, various coatings can be used for microencapsulation, either polymeric or nonpolymeric materials. Some coating materials include carbohydrates (such as starch, dextran, cellulose, alginate, pectin, carrageenan), proteins (such as gluten, casein, gelatin, zein), and others (such as glycol, polyethylene, cellulose derivatives) [13,14]. Zein is a class of prolamin proteins obtained from corn. It is composed of approximately equal amounts of hydrophilic and hydrophobic amino acid residues, and these amphiphilic properties ensure a high potential for zein to form microstructures such as spheres and films. Therefore, this edible coating material presents a good option for the microencapsulation of functional ingredients such as polyphenols and essential oils due to its biocompatibility, low water uptake value, thermal resistance, and excellent mechanical properties [15–17]. However, zein has been continuously reported as an encapsulating material for hydrophobic compounds, while studies are lacking regarding its usage for hydrophilic substances [18]. Hydroxypropyl methylcellulose (HPMC) is a partly O-methylated and O-(2-hydroxypropylated) cellulose ether derivative that is odorless, tasteless, and non-toxic. It is widely used in oral controlled delivery systems and can be used as a matrix for both hydrophilic and hydrophobic constituents [19,20]. The use of biodegradable polysaccharides, combining sodium alginate with HPMC, has been presented as a suitable combination for controlled drug delivery systems including bioactive compounds [21].

Rosemary (*Rosmarinus officinalis* L.) is a long-lasting evergreen aromatic herb from the *Lamiaceae* family, typical of the Mediterranean region [22]. Rosemary leaves have traditionally been used in Mediterranean cuisine for improving the flavor of food, for food preservation, and as a medicinal herb for its astringent, anti-inflammatory, antioxidant, antimicrobial, antiaging, antirheumatic, analgesic, and hypotensive properties [23,24]. The beneficial properties of rosemary are mostly related to its bioactive compounds, specifically phenolic compounds and flavonoids rosmarinic acid, carnosic acid, carnosol, and rosmanol [25]. Due to its therapeutic effects, rosemary extract, rich in bioactive compounds, presents a desirable natural substrate for encapsulation to preserve its properties for further production. Until today, rosemary essential oil was mostly used for encapsulation [26,27]. Only a few research papers reported the encapsulation of aqueous rosemary extracts and showed high potential for several applications in food technology or nanomedicine [28,29].

This study aimed to encapsulate rosemary extract obtained using HVED in biopolymer-based microparticles prepared from different combinations of calcium alginate, zein, and HPMC. The obtained microparticles were evaluated considering the physical properties including diameter, swelling degree, and morphology. Chemical analysis of the composition and its functional properties including encapsulation efficiency, loading capacity,

and in vitro release profiles of polyphenols in simulated gastrointestinal tract conditions were also investigated. Additionally, a detailed characterization of molecular interactions between bioactive ingredients from the rosemary extract and encapsulating materials was performed. The results of this research will increase the possibility to use stabilized aqueous rosemary extracts obtained using green technologies in functional food preparation and the pharmaceutic/cosmetic industry.

2. Materials and Methods

2.1. Materials

Dried rosemary leaves (*Rosmarinus officinalis* L.) were provided by a local specialized drugstore (Suban d.o.o., Samobor). Plant material was collected in 2017, in the northwestern part of Croatia. Leaves were air-dried and stored in polyethylene bags in a dark and dry place, at ambient temperature before extractions were performed. Dried rosemary leaves were ground to a plant particle size distribution of d(0.1) $\leq$ 39.683 µm; d(0.5) $\leq$ 224.816 µm; d(0.9) $\leq$ 425.819 µm measured using the laser particle size analyzer Mastersizer 2000 (Malvern Instruments GmbH, Herrenberg, Germany).

Alginic acid sodium salt (CAS Number: 9005-38-3, M/G ratio of ~1.56, molecular weight 280,000) g/mol was purchased from Sigma Aldrich (USA). A commercially available product $CaCl_2$ was purchased from Gram-Mol (Croatia), zein from Acros Organics BVBA (Belgium), and hydroxypropyl methylcellulose (HPMC) from Alfa Aesar (Germany). All chemicals were of analytical grade and used as received without further purification.

2.2. Methods

2.2.1. Extraction Using HVED

HVED extraction was conducted using the "IMP-SSPG-1200" generator (Impel group d.o.o., Zagreb, Croatia) that generated rectangular pulses using direct current and achieving high voltage. Based on the previous study by Nutrizio et al. [10], optimized HVED parameters were chosen for the extraction from rosemary: frequency of 100 Hz, high-voltage current of 30 mA, pulse width 0.4 µs, voltage 25 kV using nitrogen as a reaction gas, with the gap between electrodes of 15 mm, treatment duration 9 min, and ratio mass to solvent 1:50 (*w/v*) (according to pharmacopeia). The mixture of herb material and the solvent was transferred to a beaker shaped reactor of 100 mL. The reactor, which is opened on both sides, was fitted with silicone tops that were 1 cm in diameter. Silicone tops were used due to easier mounting of the electrode from the top and needle from the bottom. The setup of the generator and reactor, as well as a detailed extraction description, have been described previously [10].

2.2.2. Microparticle Preparation

Microparticles were prepared via ionic gelation at room temperature in a sterile environment as described by Jurić et al. [30]. Ionic gelation involved the preparation of microparticles by dripping a solution of sodium alginate without or with zein and/or HPMC using Encapsulator Büchi-B390 (BÜCHI Labortechnik AG, Flawil, Switzerland) under constant magnetic stirring. The total concentration of coating materials was constant (1.5% *w/v*), and four types of microparticles were made: 1.5% sodium alginate (Alg), 1.3% sodium alginate + 0.2% zein (Alg/Z), 1.2% sodium alginate + 0.3% HPMC (Alg/HPMC), and 1.0% sodium alginate + 0.2% zein + 0.3% HPMC (Alg/Z/HPMC). Before mixing with other biopolymers, zein was dissolved in distilled water (100 g/L (*w/v*)) and 1 mL of 1 mol/L NaOH was added. The solution was stirred for 30 min at 50 °C. The mixtures were dripped into 2% $CaCl_2$ (*w/v*) solution through the encapsulator nozzle size of 1000 µm. Both coating materials and $CaCl_2$ were dissolved in the rosemary extract obtained using HVED. Encapsulation parameters are shown in Table 1.

Table 1. Encapsulation parameters.

Microparticles	Vibration Frequency (Hz)	Amplitude	Pressure (mbar)
Alg	50	3	30
Alg/Z	60	3	20
Alg/HPMC	40	3	80
Alg/Z/HPMC	40	3	60

To promote gel strengthening, formed microparticles were kept at room temperature for an additional 30 min under constant magnetic stirring. Afterward, microparticles were filtered, washed with deionized water, air-dried to constant weight, stored in plastic Falcon tubes at room temperature, and protected from light, until further studies.

For comparison, blank (without extract) microparticles were made with the same coating materials and the same encapsulation parameters, just using distilled water instead of the extract as loading material. All microparticle formulations were prepared in triplicates.

2.2.3. Determination of Total Polyphenolic Content (TPC)

The Folin–Ciocalteu method was used to estimate the total polyphenol content (TPC), as reported by Jatoi et al. [31]. The data for the TPC of extracts were expressed as mg of gallic acid equivalent weight (GAE) per L of the aqueous supernatant or rosemary extract.

2.2.4. Determination of Antioxidant Capacity of Extracts Using ABTS and DPPH Assays

2,20-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assays were used to determine the antioxidant capacity of rosemary extract. The Trolox equivalent antioxidant capacity was estimated using both the DPPH radical scavenging assay and the ABTS assay, as described previously by Vinceković et al. [32]. The results, obtained from triplicate analyses, were expressed as Trolox equivalents (TE) and derived from a calibration curve determined for Trolox (100–1000 µmol/L).

2.2.5. Determination of Total Flavonoids of Extracts (TF)

The total flavonoid content (TF) was determined with a spectrophotometric method as previously described [33]. A total of 1 mL of extract was added to a 10 mL volumetric flask containing 4 mL of distilled water. A volume of 300 µL of $NaNO_2$ (0.5 g/L (*w/v*)) solution was added to the suspension, and after 5 min, 300 µL of $AlCl_3$ (1 g/L (*w/v*)) was added. After 6 min, 2 mL of NaOH (1 mol/L) was added to the mixture. The final volume was set to 10 mL with the addition of distilled water. Absorbance was measured at 360 nm and calculated as mg quercetin equivalents (QE) per L of extract (mg QE/L).

2.2.6. Determination of Total Protein Content in Extracts (TP)

The total protein content (TP) was determined with the Lowry method using bovine serum albumin (BSA) as the standard protein [34]. The results were expressed as mg equivalent of BSA per volume (mL) of the aqueous supernatant or rosemary extract.

2.2.7. Encapsulation Efficiency, Loading Capacity, and Swelling Degree of Microparticles

Detailed procedures for the determination of encapsulation efficiency (EE), loading capacity (LC), and swelling degree (S_w) of microparticles were previously described [35,36].

EE expressed as a percentage was determined from the total TPC content (TPC_{tot}) from polymer solution (TPC_p), TPC content from $CaCl_2$ solution (TPC_c), and content in the filtrate (TPC_f) and calculated using the equation:

$$EE(\%) = \frac{TPC_{load}}{TPC_{tot}} \times 100 \quad (1)$$

where $TPC_{tot} = TPC_p + TPC_c$, and $TPC_{load} = TPC_{tot} - TPC_f$.

LC expressed as a TPC content (mg GAE/g) was calculated with the equation:

$$LC = \frac{c \times V}{W_c} \times 100 \quad (2)$$

where c is the concentration of TPC in the sample, V is the volume of the sample, and W_c is the weight of dry microparticles.

For S_w determination, dry microparticles were swelled in distilled water. Microparticle S_w was calculated using the equation:

$$S_w = \frac{w_t - w_0}{w_0} \times 100 \quad (3)$$

where w_t is the weight of the swollen microparticles and w_0 is their initial weight. All measurements were replicated three times, and the results are presented as the mean values.

2.2.8. In Vitro Phenolic Release from Microparticles

In vitro release studies of TPC from microparticles were carried out at 37 °C in distilled water, and simulated gastric (HCl, pH 1.64) and intestinal solutions (pH 7.40, phosphate buffer −0.2 mol/L Na_2HPO_4, 0.2 mol/L $NaH_2PO_4 \times 2H_2O$). The release of polyphenolic compounds from the microparticles was performed by measuring the released TPC cumulative concentration using the Folin–Ciocalteu assay as described in Section 2.2.3. The results are presented as the percent of cumulatively released TPC (f) using the equation:

$$\text{Cumulative polyphenolic release}(\%) = \frac{TPC_t}{LC} \times 100 \quad (4)$$

where TPC_t presents the cumulative concentration of TPC released in time t, and LC is the total amount of TPC loaded in microparticles.

2.2.9. Fourier-Transform Infrared Spectroscopy Analysis

The Fourier-transform infrared spectroscopy (FTIR) spectra of individual constituents and microparticles were recorded with the FTIR Instrument—Cary 660 FTIR (MIR system) spectrometer (Agilent Technologies, Santa Clara, CA, USA). To make pellets, samples were combined with potassium bromide. The spectral scanning ranged from 500 to 4000 cm^{-1}.

2.2.10. Microscopic Observations

Microparticles were observed using three different microscopic techniques:

1. Optical microscopy (Leica MZ16a stereomicroscope, Leica Microsystems Ltd., Saint Gallen, Switzerland) was used to examine the size and shape of the microparticles. An average diameter of prepared dry microparticles was determined using Olympus Soft Imaging Solutions GmbH, version E_LCmicro_09Okt2009. Diameters of about 100 microparticles, randomly selected from batches produced in triplicate, were measured.
2. Scanning electron microscopy (SEM) (FE-SEM, model JSM-7000 F, Jeol Ltd., Akishima City, Japan) was used to determine the microparticle morphology properties. Microparticles were put on the high-conductive graphite tape. Energy-dispersive X-ray spectroscopy (EDS) was used to determine the elemental composition of the surface. FE-SEM was linked to an EDS/INCA 350 (energy dispersive X-ray analyzer) manufactured by Oxford Instruments Ltd. (Abingdon, UK). Various compounds and elements were analyzed and marked as: C ($CaCO_3$), O (SiO_2), Na (Albite), Cl (KCl), Ca ($CaCO_3$), Ca ($CaCO_3$), Ca ($CaCO_3$), Ca ($CaCO_3$), Ca ($CaCO_3$), Ca (C (Wollastonite)).
3. The atomic force microscopy (AFM) using the MultiMode Scanning Probe Microscope with Nanoscope IIIa controller (Bruker Corporation, Billerica, MA, USA) was used to determine the surface morphology and obtain the topography of microparticles. The samples for AFM imaging were prepared by deposition of a microparticle suspension

on the mica substrate. The microparticles were flushed three times with 50 µL MiliQ water to remove all residual impurities. The microparticle surface, cross-section, and grain size distribution within each sample were analyzed using MultiMode Scanning Probe Microscope with Nanoscope IIIa controller (Bruker Corporation, Billerica, MA, USA) with SJV-JV-130 V ("J" scanner with vertical engagement); Vertical engagement (JV) 125 µm scanner (Bruker Corporation, Billerica, MA, USA); Tapping mode silicon tips (R-TESPA, Bruker, Nom. Freq. 300 kHz, Nom. spring constant of 40 N/m). Accordingly, three-dimensional information about the surface topology was obtained and the roughness was quantified. All AFM imaging was performed at three different regions of each microparticle to ensure the consistency of obtained results.

2.2.11. Statistical Analysis

All experiments were carried out in triplicates at room temperature. Results are presented as mean values ± standard deviation. Microsoft Excel 2016 and the XLSTAT statistical software add-in were used to examine the data.

3. Results and Discussion

The results and discussion are presented in three interconnected sections. In the first section, the chemical properties of obtained rosemary extracts are analyzed; the second section evaluates the physical properties of microparticles; and the final section discusses the functional properties of microparticles loaded with rosemary extracts.

3.1. Chemical Properties of Rosemary Extract for Encapsulation

The rosemary extract for encapsulation was obtained using the HVED method under optimal conditions determined in a previous paper by Nutrizio et al. [10]. For extraction purposes, distilled water was chosen as the cheapest and the most available green solvent. The rosemary extract was subjected to chemical methods to assess the composition of obtained extract for further encapsulation. Following the characterization of rosemary extract, the study of TPC, TF, TP, as well as antioxidant activity (ABTS and DPPH methods) was carried out. As shown in Table 2, the TPC of rosemary extract was 333.07 mg GAE/L which corresponds to 16.65 mg GAE/g of the dry weight of rosemary. This result is in line with previous results of the extraction of polyphenolic compounds from rosemary obtained using HVED [10]. In contrast, the TPC level of rosemary extract obtained with conventional extraction methods was significantly lower, as Alfonso et al. reported a TPC level in aqueous rosemary extract of 166.7 µg/g dry weight [37], and Pereira et al. measured a level of 409.1 µg/g dry weight in 80% ethanol extract [38].

Table 2. Chemical properties of rosemary extract (the total polyphenolic content (TPC), total flavonoid content (TF), antioxidant capacity (ABTS and DPPH methods), and total protein content (TP)).

Method	Determination Value
TPC (mg GAE/L)	333.07 ± 12.32
TF (mg QE/L)	333.08 ± 24.22
ABTS (mmol TE/L)	1.94 ± 0.15
DPPH (mmol TE/L)	1.98 ± 0.16
TP (mg BSA/mL)	8.48 ± 0.07

Results are expressed as mean ± standard deviation.

Both antioxidant capacity methods, ABTS and DPPH, showed similar results: 1.94 mmol TE/L and 1.98 mmol TE/L, respectively. Antioxidant capacity in this work was also relatively higher when compared to that obtained via conventional extraction methods [1,28] and in line with extracts obtained with supercritical fluid extraction by Justo et al. [39]. The number of polyphenolic compounds and antioxidant capacity presented in this study were higher compared to other extraction methods, especially conventional ones. That may be related to the rosemary specifications such as environmental, harvesting,

and genetic conditions, as well as different measurement methods, but mostly due to differences in the extraction procedure. The extraction using HVED induced electroporation of rosemary plant cells, which increases extraction potential and provides a higher extraction yield.

The TF in rosemary extract was 333.08 mg QE/L, which corresponds to 16.65 mg QE/L. The obtained result is in line with the research from Munekata et al. who found TF in rosemary extract obtained using various extraction methods, ranging from 16.29 (for ultrasound-assisted extraction) to 24.99 mg of catechin equivalents/g DW (for conventional extraction) [40].

3.2. Physical Properties of Microparticles Composed of Variable Coating Materials

In the second section, the physical properties of obtained microparticles are described to compare the morphological properties of microparticles prepared with rosemary extract to microparticles prepared with distilled water instead of the extract in the encapsulated core material. Furthermore, edible and readily available biopolymers were chosen as coating materials. Therefore, prepared microparticles were further analyzed to assess their properties and functionality. Physical properties included analysis of microparticle swelling behavior and diameter, as well as microscopic observations.

3.2.1. Swelling Degree and Diameter of Microparticles

In Table 3, the swelling degree (%) and microparticle diameter (µm) are presented. The swelling behavior of prepared microparticles was analyzed in distilled water, as described in Section 2.2.7, while the diameter of microparticles was measured under the optical microscope (Figure 1).

Table 3. Physical properties of microparticles with different coating materials loaded with rosemary extract and distilled water as loading materials (swelling degree and diameter of microparticles).

Microparticles	Loaded Material	Swelling Degree (%)	Diameter (µm)
Alg	water	55.88 ± 1.79^{bA}	651.29 ± 79.22^{aA}
	extract	52.63 ± 6.98^{aA}	698.79 ± 90.39^{aB}
Alg/Z	water	48.95 ± 1.82^{aA}	691.32 ± 60.17^{bA}
	extract	50.57 ± 5.86^{aA}	711.57 ± 69.12^{bB}
Alg/HPMC	water	126.75 ± 11.64^{cA}	874.51 ± 166.64^{cA}
	extract	114.72 ± 22.97^{bA}	1202.90 ± 311.42^{cB}
Alg/Z/HPMC	water	138.95 ± 12.34^{cA}	1034.12 ± 163.56^{dA}
	extract	119.83 ± 11.94^{bA}	1087.37 ± 236.51^{dB}

Results are expressed as mean ± standard deviation; values presented with the different lower case are statistically different at $p = 0.05$ for different microparticles (coating material) but the same loading material; values presented with the different upper case are statistically different at $p = 0.05$ for different loading material but same coating material.

When microparticles are dispersed in water, they swell. The swelling of microparticles depends on the hydrogel structure, temperature, properties, and composition of the core material [41]. To avoid the influence of electrolytes on swelling properties, the swelling behavior of prepared microparticles was observed in distilled water, and the results are shown in Table 3. Swelling degree (%) showed that there was a statistically significant difference for microparticles loaded with rosemary extract when HPMC was introduced in the structure. The swelling degree was 2.18–2.84 times higher with HPMC compared to microparticles prepared under the same conditions without HPMC. This is consistent with the results of Nochos et al., who found that swelling degree increases with higher HPMC content in microparticles [42]. The addition of zein did not significantly influence the swelling degree ($p < 0.05$). When compared to microparticles prepared with the same coating materials, but with distilled water instead of the rosemary extract, no significant difference was noted. Therefore, the addition of rosemary extract did not have a significant

influence on the swelling behavior of microparticles. These results can be explained due to the high hydrophilicity and swellability of HPMC, as HPMC is the dominant hydrophilic polymer that swells significantly upon contact with water [42,43].

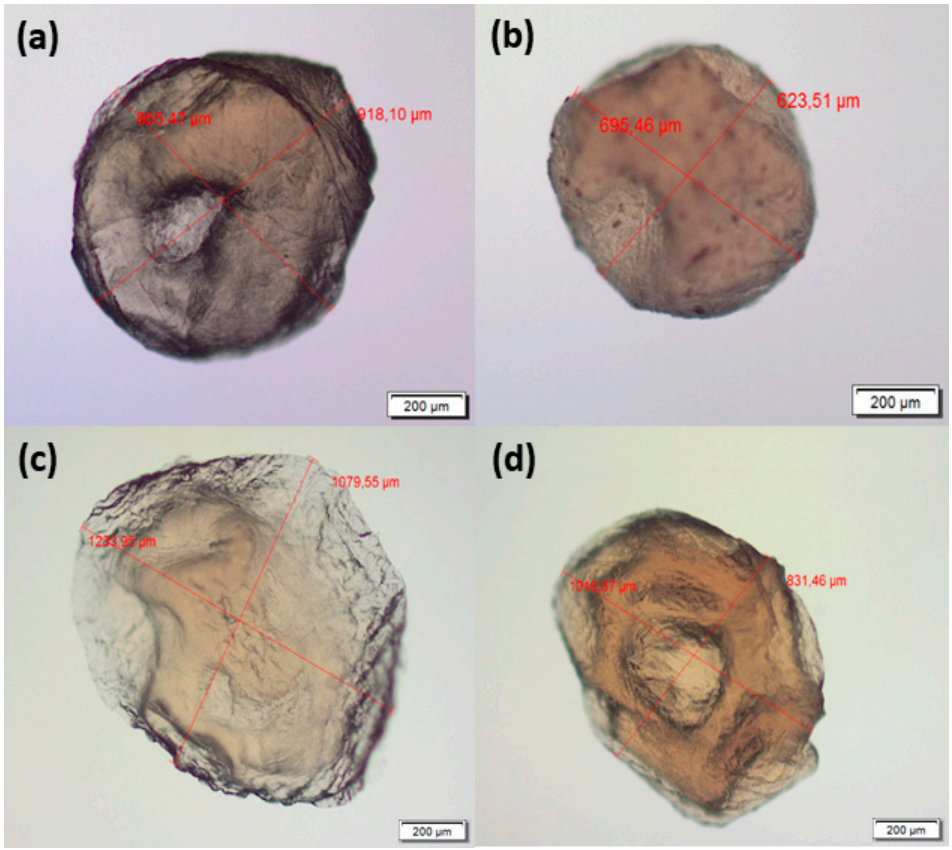


Figure 1. Images of dry microparticles obtained under an optical microscope (Leica MZ16a stereomicroscope, Leica Microsystems Ltd., Switzerland): (**a**) Alg (**b**) Alg/Z (**c**) Alg/HPMC, and (**d**) Alg/Z/HPMC. Bars are indicated.

The prepared fresh microparticles were observed with the optical microscope and showed that microparticles were consistent in size (around 2 times larger than nozzle size) and had a spherical shape. However, after drying to constant mass, the regular spherical shape was lost and dents on the surface were noted (Figure 1). These results are consistent with the results of Jurić et al. where the surfaces of dry microparticles and wet microparticles were compared [44]. The wet microparticles kept their oval shape, unlike the dry microparticles, which had visible indentations on the surface. The mean diameter of microparticles is shown in Table 3. The results showed that the addition of rosemary extract, compared to distilled water as a loading material, significantly increased the microparticle diameter for all investigated types. Moreover, the variation in coating material (zein and HPMC) significantly increased the diameter of the microparticles. Consequently, the largest diameter noted in Alg/Z/HPMC microparticles was 1087.37 µm and 1034.12 µm for microparticles with rosemary extract and water, respectively. This is supported by the research from Hosseini et al. who reported an increase in the diameter of zein-electrospun fibers with higher concentrations of encapsulated rosemary essential oil [27].

3.2.2. Morphological Characterization of Microparticles Using SEM

Dried samples were placed on high-conductive graphite tape for SEM analysis. The surface morphology of prepared microparticles was observed using SEM and is presented in Figure 2 at the magnifications of 100×, 5000×, and 20,000×, respectively. The surface of microparticles changed with the composition. The surface of all microparticles was

wrinkled and very porous with pores of different sizes. The surface of Alg microparticles was the smoothest with quite a regular structure. The addition of other biopolymers (zein and HPMC) induced significant changes in the surface structure and increased the roughness while reducing the porosity of microparticles. However, the surface of Alg/Z/HPMC microparticles was smoother and with reduced roughness compared to Alg/Z and Alg/HPMC ones. This could be supported by the explanation that the presence of HPMC reduced the agglomeration of zein [18].

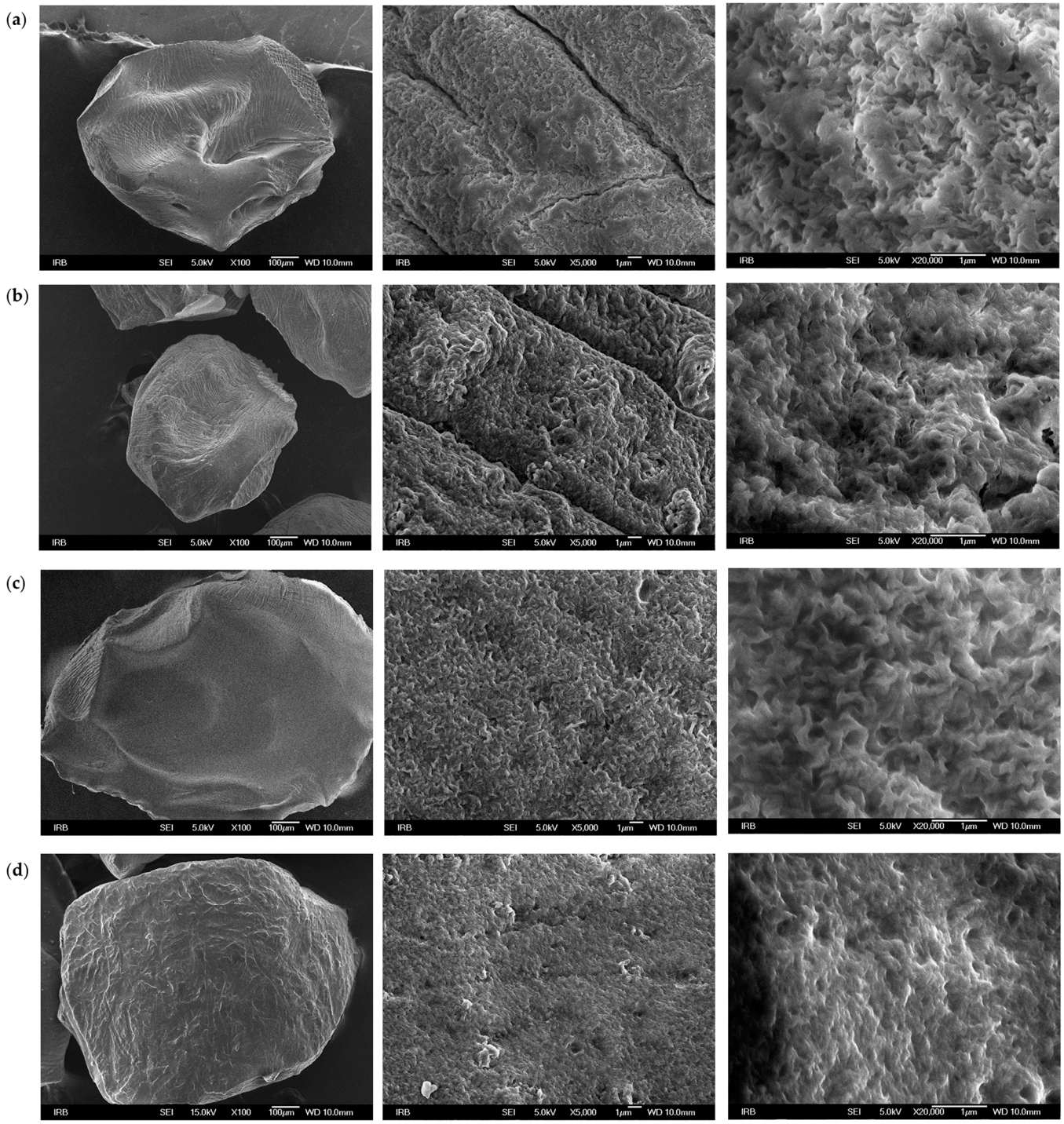


Figure 2. SEM microphotographs of (**a**) Alg (**b**) Alg/Z (**c**) Alg/HPMC, and (**d**) Alg/Z/HPMC microparticles loaded with rosemary extract. Bars are indicated.

The surface imaging results of SEM microscopy were compared to that of microparticles prepared with distilled water instead of the rosemary extract, as presented in Figure 3. Compared to microparticles loaded with distilled water, those with rosemary extract had significant changes in surface morphology. The addition of rosemary extract caused a rougher structure with sharper edges of wrinkles that resemble the furrows of the human intestine. In comparison, a study by Sheng et al. reported that SEM analysis showed a more compact surface of microparticles composed of calcium alginate and HPMC when compared to the blank calcium alginate ones. A compact surface without pores hindered the diffusion during passage through the gastrointestinal tract and additionally slowed the extract release in simulated gastric conditions [21].

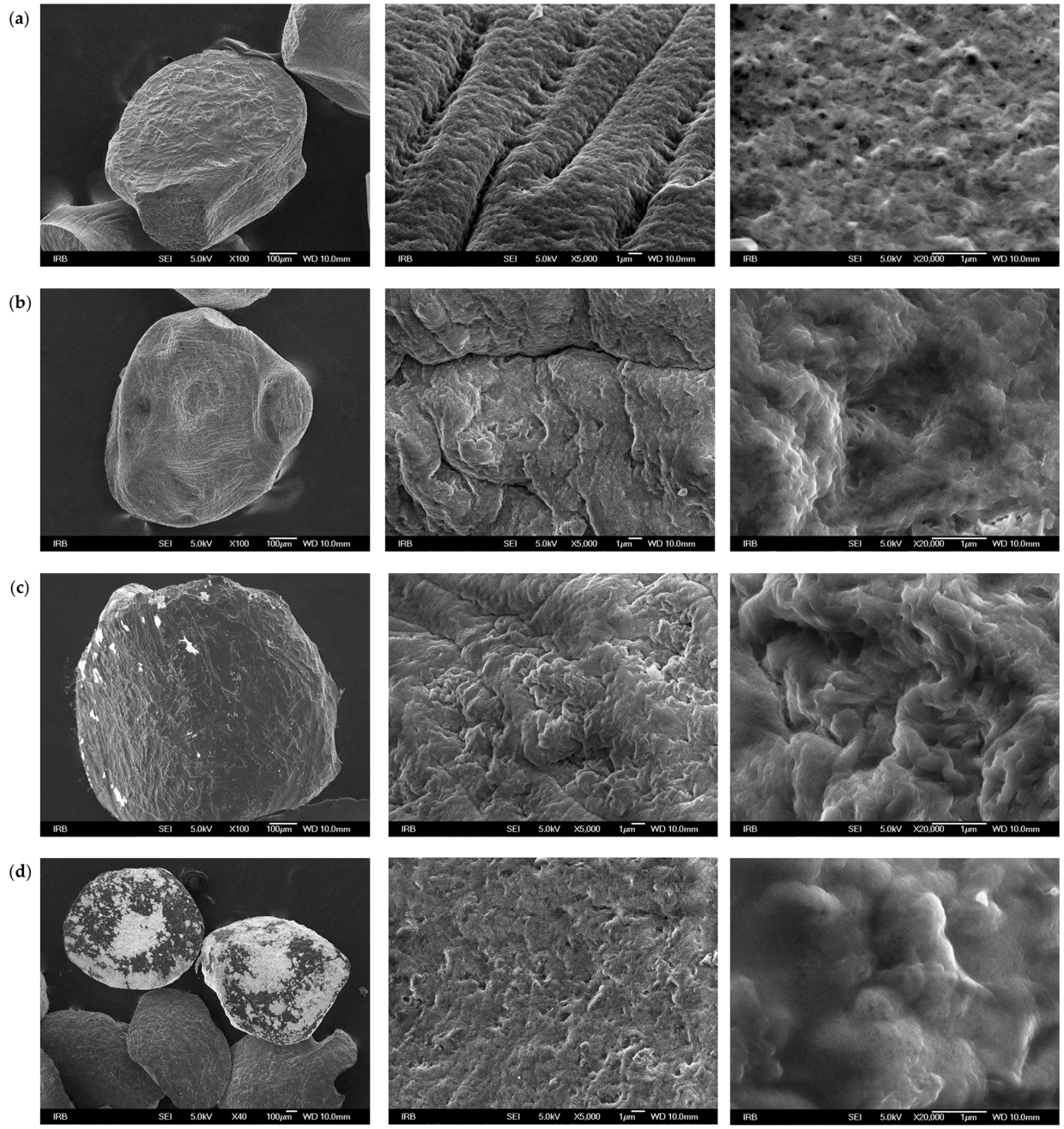


Figure 3. SEM microphotographs of (**a**) Alg (**b**) Alg/Z (**c**) Alg/HPMC, and (**d**) Alg/Z/HPMC microparticles loaded with distilled water. Bars are indicated.

SEM microscopy allowed the determination of the surface elemental composition via EDS spectra analysis. The example of a spectrum of Alg/Z/HPMC microparticles is presented in Figure 4. The analysis using EDS, applied to the nearest surface of microparticles, revealed that the major percentage corresponded to carbon and oxygen. The same trend with the dominant peaks was noted in all formulations. Low amounts of sodium and chloride were noted, which are probably residues during microparticle preparation. Similar results were found in the paper by Kolar et al. [45]. The SEM-EDS analysis is a non-destructive analytical technique for the sample, and it gives information on the chemical composition of the surface. The obtained results suggested that rosemary extract is completely encapsulated inside the biopolymer particles, and it is not present on the surface.

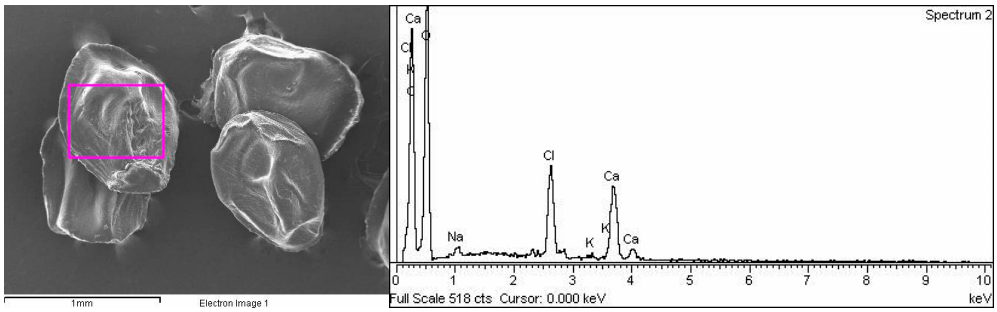


Figure 4. Morphology and surface elemental analysis using EDS (expressed in the atomic weight percent) of Alg/Z/HPMC microparticles loaded with rosemary extract. Bar is indicated.

3.2.3. Morphology of Microparticles Determined Using AFM

The AFM analysis (Figure 5) of obtained microparticles loaded with rosemary extract was performed to complement SEM surface morphology data. The scanned sample area is presented using topographic images of height data presented as the "top view" and 3D surface view, with appropriate color scale characterizing the microparticle height. All AFM imaging was performed at different regions of each microparticle to ensure the consistency of the obtained results.

The AFM analysis was presented as 3D-topographic height images and amplitude images data in Figure 5. The surfaces of microparticles showed substructures consisting of abundant smaller grains, but in comparison with Alg/Z/HPMC microparticles with sharp boundaries, grains are oriented in different ways in the remaining samples with increased alginate percentage. The average size and mean diameter of grains on the surface are presented in Table 4. The individual grains on the surface area are visible with a height of around 8 nm and a diameter that ranged from 25 ± 64 (for Alg microparticles) to 77 ± 59 nm (for Alg/HPMC microparticles). The 3D topographic images of microparticles showed the finer grain morphological characteristics of microparticles prepared only with calcium alginate (Alg). This is followed by the results of grain diameter. Similar results were found in the work by Jurić et al. who investigated the morphology of alginate microparticles [46].

Table 4. Roughness parameters of the microparticle surfaces (grain mean height, grain mean diameter, average roughness—R_a, root mean square of roughness—R_q and Z range for microparticles loaded with rosemary extract).

Microparticles	Grain Height (nm)	Grain Diameter (nm)	R_a (nm)	R_q (nm)	Z (nm)
Alg	7.9 ± 5.5 [a]	25 ± 64 [a]	31 ± 2 [a]	39 ± 3 [a]	327 ± 22 [a]
Alg/Z	8.5 ± 4.2 [a]	76 ± 164 [a]	29 ± 1 [a]	38 ± 2 [a]	342 ± 16 [a]
Alg/HPMC	11.3 ± 2.8 [a]	77 ± 59 [a]	46 ± 3 [b]	53 ± 4 [b]	340 ± 13 [a]
Alg/Z/HPMC	6.3 ± 5.5 [a]	58 ± 54 [a]	28 ± 2 [a]	38 ± 1 [a]	432 ± 21 [b]

Results are expressed as mean ± standard deviation; values presented with different letters are statistically different at $p = 0.05$.

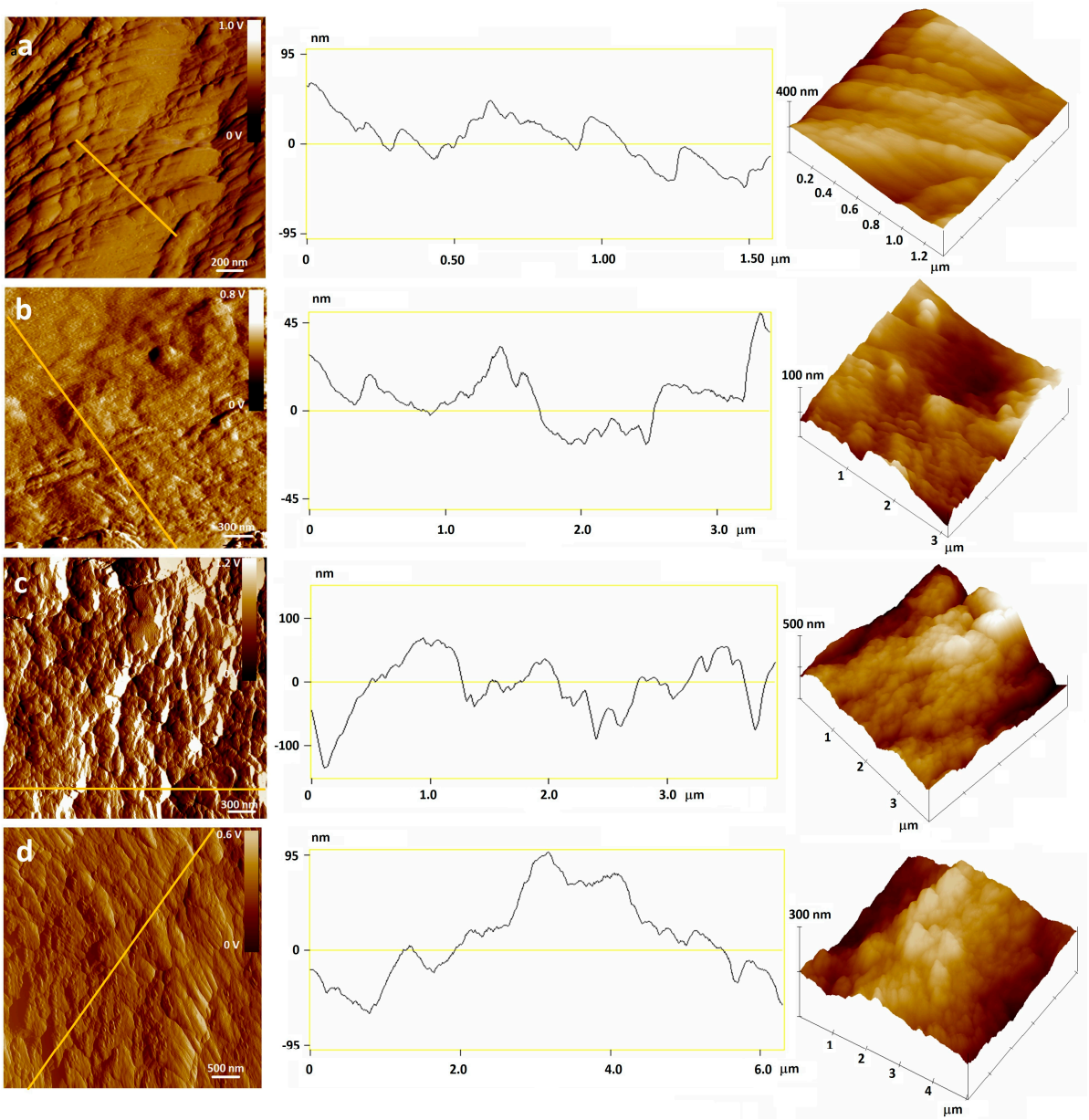


Figure 5. AFM amplitude image (**left**), section analysis profile along the labeled line (**middle**) and 3D-topographic images of height data—top view (**right**) of microparticles loaded with rosemary extract: (**a**) Alg, (**b**) Alg/Z, (**c**) Alg/HPMC, and (**d**) Alg/Z/HPMC. Bars are indicated.

The roughness analysis of a single microparticle was performed using AFM, and the results are presented in Table 4. The surfaces of the investigated microparticles were highly rough (from Ra = 28 ± 2 to Ra = 46 ± 3 nm for Alg/Z/HPMC and Alg/HPMC microparticles, respectively) and changed with the composition of microparticles. The surface of the microparticles is very rough and heterogeneous, with a lot of small grains and some bigger grains. For that reason, great deviations between grains are notable. The limitation of the instrument is that it provides insight into only a small part of the microparticle surface. In the analyzed surface roughness, the results showed that there was no statistical difference in grain height and grain diameter of microparticles due to variations in grains. Average roughness (R_a) and root mean square of roughness (R_q) showed that there was no statistical difference among Alg, Alg/Z, and Alg/Z/HPMC microparticles; however, Alg/HPMC microparticles were showed to have statistically higher roughness. The Z range was the highest for Alg/Z/HPMC microparticles (432 ± 21 nm).

3.3. Functional Properties of Microparticles Composed of Variable Coating Materials

In the third section, the functional properties of obtained microparticles with rosemary extract are described. The analysis included the determination of encapsulation efficiency (EE), loading capacity (LC), and TP (Table 5). A cumulative release of polyphenols from microparticles was also observed. Additionally, molecular interactions in microparticles were investigated via FTIR analysis.

Table 5. Functional properties of microparticles with different coating materials (encapsulation efficiency (EE), loading capacity (LC), and total protein content (TP)).

Microparticles	EE (%)	LC (mg GAE/g)	TP (mg BSA/mL)
Alg	110.94 ± 2.14 [a]	5.55 ± 0.42 [a]	3.73 ± 0.68 [a]
Alg/Z	117.75 ± 7.28 [a]	10.42 ± 0.72 [d]	11.31 ± 1.47 [d]
Alg/HPMC	113.31 ± 3.26 [a]	6.74 ± 0.74 [b]	4.30 ± 0.81 [b]
Alg/Z/HPMC	120.59 ± 2.37 [b]	9.33 ± 0.62 [c]	9.77 ± 1.29 [c]

Results are expressed as mean ± standard deviation; values presented with different letters are statistically different at $p = 0.05$.

3.3.1. Encapsulation Efficiency (EE), Loading Capacity (LC), and Total Protein Content (TP)

To determine the content of polyphenols in obtained microparticles and to assess the efficiency of the encapsulation process, EE and LC were determined. Results are shown in Table 5. High results of EE were noted for all microparticles (113.31 to 120.59%) with approximately similar effectiveness. The highest encapsulation efficiency was measured for Alg/Z/HPMC microparticles, which were the only ones with statistically significantly higher EE compared to all other microparticles. Efficiencies greater than 100% are attributed to the fact that the rosemary extract was added both to the encapsulation solution (biopolymers) and the $CaCl_2$ solution. Due to this encapsulation step, no losses of polyphenols via diffusion from microparticles to $CaCl_2$ solution occurred [45,47].

The results of loading capacity showed that the lowest LC was noted in microparticles prepared with only calcium alginate (Alg) with the value of 5.55 mg GAE/g, while the introduction of co-biopolymers significantly increased the LC. The LC ranged with increasing results as follows: Alg < Alg/HPMC < Alg/Z/HPMC < Alg/Z. Furthermore, the LC increased in accordance with TP (Table 5). This could be the result of possible interactions between proteins and phenolic compounds [48]. The highest TP was noted in microparticles composed partly of zein, which is expected since zein is a protein itself. These results are following the work of Papoutsis et al. who compared the LC of capsules with and without protein (soy protein). Their results also showed that particles containing the protein had significantly higher LC compared to non-protein freeze-dried particles [49]. The LC and TP are also highly correlated with the results of AFM and SEM. The microparticles with lower LC and TP had rougher surfaces.

3.3.2. Molecular Interactions in Microparticles Formulations

Designing and preparing microparticle formulations for a specific purpose requires a good knowledge of the molecular interactions between biopolymers and the encapsulated material. In Figure 6, the FTIR spectra of individual biopolymers (zein and HPMC) as well as rosemary extract are shown. The characteristic spectra of pure sodium alginate and $CaCl_2$ have been reported earlier [36,45]. Characteristic FTIR bands of both constituents are in accordance with literature data [50,51]. Characteristic FTIR bands of zein and HPMC with assignments are listed in Table 6 and are following literature data [52–54]. For zein spectra, the presence of a higher amount of α-helices secondary structure is confirmed by the symmetrical spectral peak at 1643 cm^{-1} [54–56]. The FTIR spectra of rosemary extract exhibited two characteristic peaks. One broad absorption in the wavelength range (3376–3241 cm^{-1})—corresponding to OH stretching bands of alcohols and/or carboxylic acids vibrations and one at 1627 cm^{-1}, which corresponds to bending out of plane C-H and

stretching vibrations of conjugated C-C of a benzenoid ring, respectively, in the rosemary extract [57].

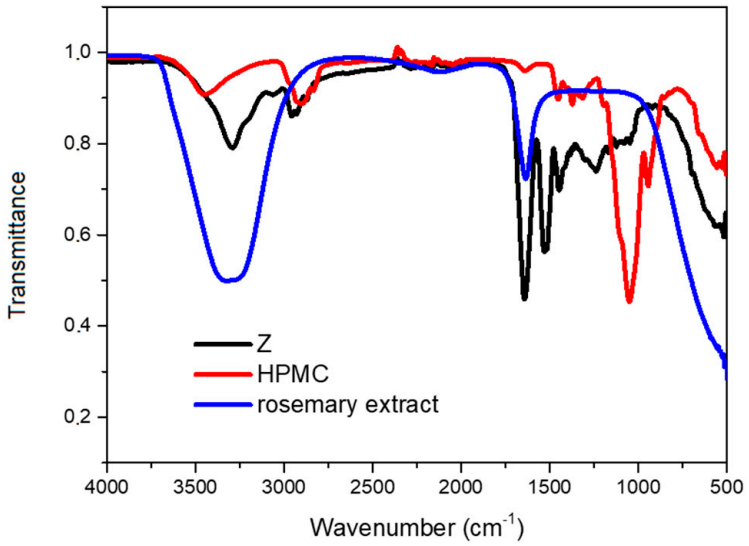


Figure 6. FTIR spectrum of zein (Z)-black line; HPMC—red line; and rosemary extract—blue line.

Table 6. FTIR bands of zein and HPMC with assignments.

Coating Material	Vibration (cm^{-1})	Assignment
Zein	1643	Amide I (C=O stretching)
	1531	Amide II (N-H bending)
	1240	Amide III (C-N stretching)
	3292	N-H stretching
	3304	O-H stretching
	2958	reflected the stretching vibration of the intermolecular bonded hydroxyl group
HPMC	3444	stretching frequency of the hydroxyl (-OH) group
	1373	bending vibration of -OH
	2929, 1055	stretching vibration bands related to C-H and C-O
	1300 to 900	C-O-C stretching
	3000–2800	C-H stretching

On the comparison of FTIR spectra of pure constituents (Figure 6) and dry formulations of Alg microparticles (Figure 7a), changes in the position and intensity of individual bands are noticeable. This is especially noticeable with a decrease in the intensity and position of the band corresponding to the elongation of the carboxyl group (1556 cm^{-1}), but also with the band corresponding to the elongation vibration region; the O-H bond in calcium alginate is narrower than the absorption region in sodium alginate. The difference occurs due to the participation of hydroxyl and carboxylate groups of sodium alginate with calcium ions in the process of forming a chelate structure, which leads to the process of reducing hydrogen bonds between hydroxyl functional groups. The vibrations of the asymmetric elongation of the carboxylate ion are shifted towards lower values of the wavenumbers because the calcium ion replaces the sodium ion in the sodium alginate, and there is a change in the charge density, radius, and atomic weight of the cation. These carboxylate group-linked bands are extremely useful for monitoring changes in alginate structure. The obtained results are following the literature data [58]. Compared to microparticles prepared with distilled water, the presence of rosemary extract in the Alg formulation causes slight changes in the intensity of individual bands (carboxyl group 1556 cm^{-1}) and with the band corresponding to the elongation vibration region of the O-H bond

(wavelength range (3376–3241 cm^{-1}). These observations represent evidence of an effective extract encapsulation [59].

The FTIR spectroscopy studies reveal that molecular interaction of zein and alginate occurs in the microparticles (Figure 7b). The spectrum of alginate shows bands at 3337, 1609, 1414, and 1034 cm^{-1} that can be attributed to (H_2O and OH), (-COO), and (C-O-C) vibrations, respectively. The cross-linking process with Ca^{2+} causes an obvious shift of the 1609 and 1414 cm^{-1} bands to higher wavenumber values, while the band at 1034 cm^{-1} appears as in the polysaccharide, indicating the formation of an ionic bond between calcium ions and deprotonated carboxylate groups of alginate. Furthermore, the FTIR spectrum of the zein is characterized by the presence of a band at 3308 cm^{-1}, which is assigned to the NH stretching vibration mode of the so-called amide A groups from the protein. Bands at 1658 and 1538 cm^{-1} are attributed to vibrations of C=O of amide I bond C-N-H of amide II of peptide groups, respectively. The amide I band is essentially associated with the stretching vibration mode of the carbonyl group (C=O), although it also receives a contribution of the C-N stretching and the C-C-N deformation vibrations. However, the amide II band is mainly due to the NH bending vibration mode. It is a mixed contribution of the N-H in-plane bending, the C-N stretching, and the C-C stretching vibrations. In the FTIR spectrum of the Alg/Z microparticles, the band at 1658 cm^{-1} characteristic of amide I vibrations of zein appears to be overlapped by the alginate characteristic band at 1609 cm^{-1}, being now observed only as a single band at 1614 cm^{-1}. This band is asymmetric, suggesting that there is a band at a low frequency related to the amide I component and a high-frequency band associated with the amide II component, which is an indication that a hydrogen-bonded aggregate is formed. This effect can be a consequence of possible interactions between the carboxylate groups in alginate with protonated amino groups from the protein. The band corresponding to vibrations of the amide II groups of zein appears as a low-intensity band, being displaced at 1545 cm^{-1}. These shifts suggest the existence of interactions between the protein and the polysaccharide. In the FTIR spectrum of the Alg/Z microparticles after cross-linking with Ca^{2+} ions, a shift of the (COO) band to 1632 cm^{-1} is observed, in a similar way to that observed in cross-linked alginate. However, the band ascribed to OH vibration modes of alginate appears at a higher wavenumber (3462 cm^{-1}) than in cross-linked alginate. This phenomenon suggests the existence of hydrogen bonding interactions between the biopolymers, similar to that reported for xanthan–alginate blends [60]. In comparison to microparticles prepared with distilled water, the presence of aqueous rosemary extract in the Alg/Z microparticles causes intensive changes in the intensity and position of individual bands. The spectrum of the Alg/Z microparticles prepared with rosemary extracts incorporated in the matrix shows a displacement of the typical COO bands appearing at 1609 cm^{-1} in the pure formulations and 1596 cm^{-1} formulations with extracts. Moreover, the change of bend in the range of 3359–3207 cm^{-1} indicates the change in hydrogen bonding because of the presence of rosemary extracts. The characteristic bands of rosemary extracts are overlapped with the bands of biopolymers [60]. Similar results were found in a paper from Hosseini et al. who performed encapsulation of rosemary essential oil in zein via the electrospinning technique [27]. A higher wavenumber was observed as a result of adding rosemary essential oil to zein, indicating a lower α-helix length. The rosemary essential oil affected the secondary structure of zein protein and confirmed that the rosemary essential oil was successfully encapsulated.

The FTIR spectra of Alg/HPMC formulations (Figure 7c) with and without the rosemary extract are very similar with changes in peak intensity when rosehip extracts are encapsulated. In both cases, a broad peak at the intensity of 3341 cm^{-1} was observed, which indicates the presence of stretching of the hydroxyl groups of HPMC, but also the presence of hydrogen bonds, which are strengthened by the addition of rosemary extract. There is also an increase in the intensity of the peak at the value of 1021 cm^{-1}, which corresponds to the process of cross-linking of calcium ions with sodium alginate, i.e., electrostatic interaction between calcium ions and the hydroxide group of sodium alginate [61,62].

The addition of both zein and HPMC into calcium alginate-based formulations caused some changes in the spectra of formulations samples (Figure 7d). The peaks at 2926, 1696, and 1461 cm^{-1} were shifted to higher wavenumbers. These spectral changes can be attributed to the possible interaction (hydrogen bonds) between HPMC, calcium alginate, and zein. It can be observed that hydrogen bonding in COO groups was checked in Alg/Z/HPMC formulations by shifting the peak at 1647 cm^{-1} for COO- asymmetric stretching and shifting the peak at 1455 cm^{-1} for COO- symmetric stretching [63]. The presence of rosemary extract in the Alg/Z/HPMC formulation causes intensive changes in the intensity and position of individual bands.

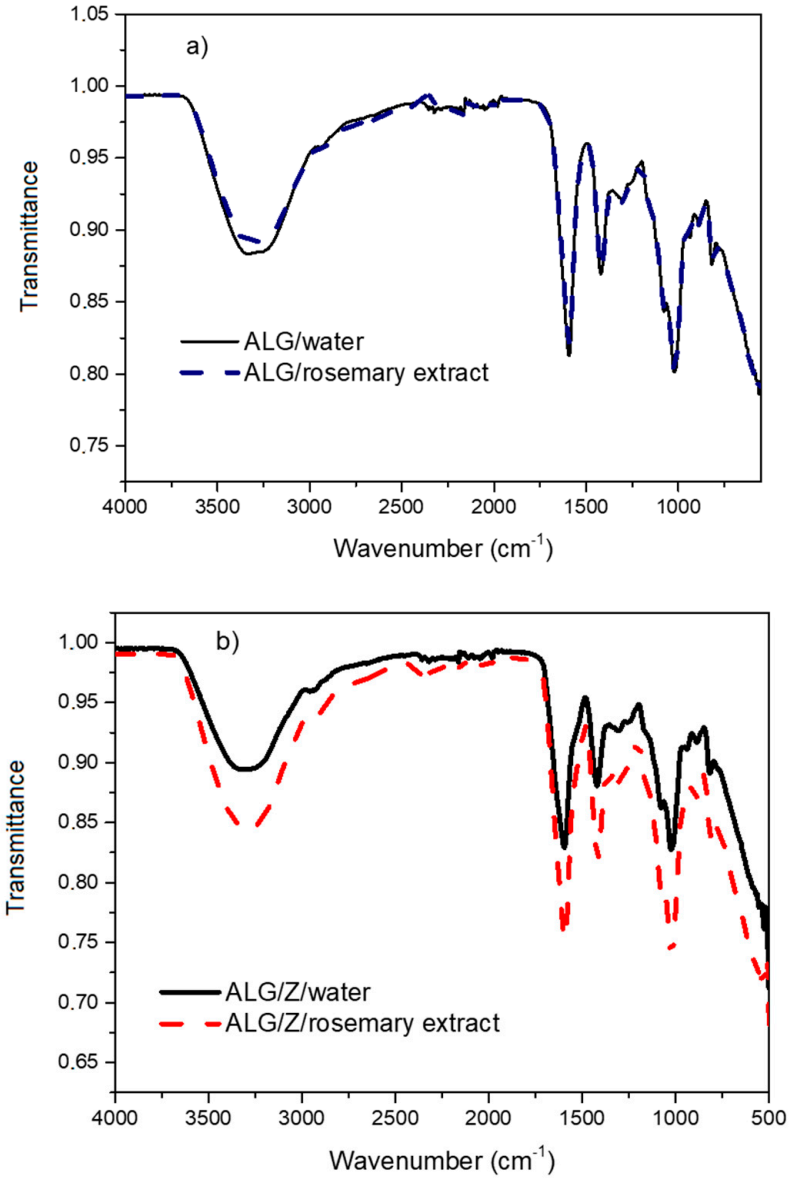


Figure 7. *Cont.*

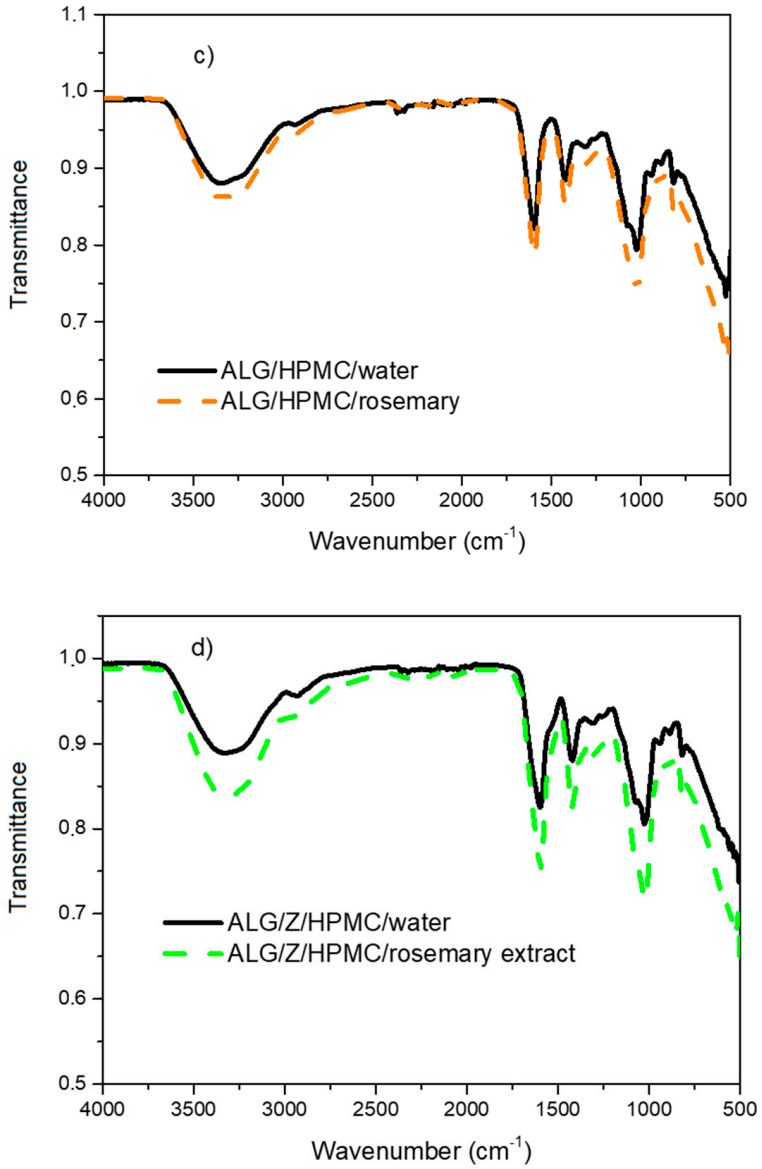
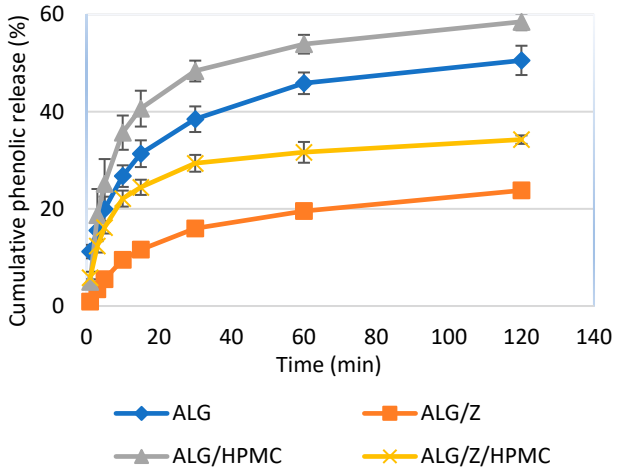


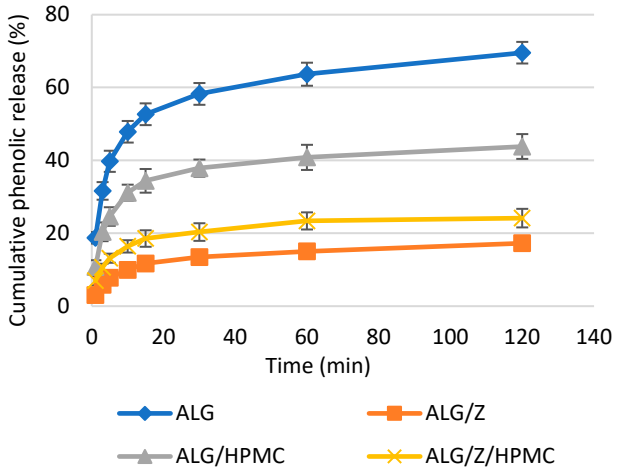
Figure 7. FTIR spectra of microparticles loaded with distilled water and rosemary extracts: (**a**) ALG/water—black line, ALG/rosemary extract—blue line; (**b**) ALG/Z/water—black line, ALG/Z/rosemary extract—red line; (**c**) ALG/HPMC/water—black line, ALG/HPMC/rosemary extract; (**d**) ALG/ZHPMC/rosemary extract—green line.

3.3.3. In Vitro Release Profiles of Total Polyphenols from Microparticle Formulations

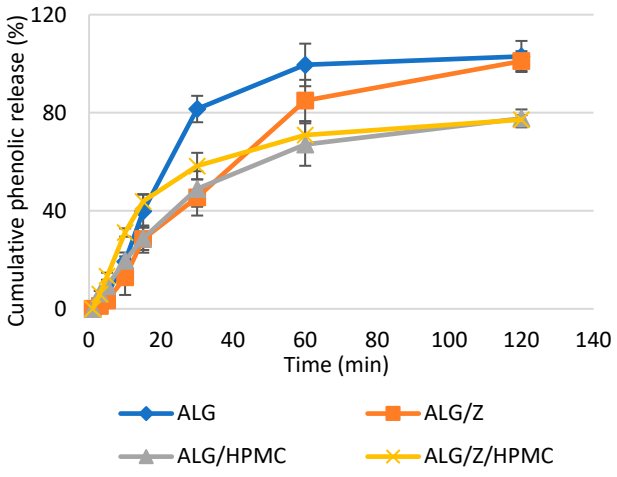
The antioxidant potential of polyphenols, i.e., their potential bioactivity in vivo depends on their metabolism, absorption, distribution, and excretion from the body after their intake and the reducing properties of the resulting metabolites [64]. As TF, DPPH, and ABTS have high correlations with TPC, only TPC was chosen as the most propriate method for the evaluation of bioactivity of valuable phenolic compounds from rosemary [10,65]. For that reason, the in vitro TPC release from microparticles was observed in neutral (distilled water), acidic HCl solution (gastric simulation), and alkaline buffer solution (intestinal simulation) at 37 °C. The results are shown in Figure 8 as cumulative release presented as a percentage (%) of the total LC of each prepared microparticle type over time.



(**a**)



(**b**)



(**c**)

Figure 8. Cumulative release of total polyphenolic content (TPC) from microparticles in different dissolution media: (**a**) distilled water; (**b**) HCl solution (pH 1.64); and (**c**) buffer solution (pH 7.40). The error bars indicate the standard deviation of the means.

The TPC release in pure water showed quick initial release (11.6–40.6% in the first 15 min) that was followed by slower gradual release (Figure 8). The addition of HPMC in the initial formulation (Alg/HPMC microparticles) increased cumulative release compared to Alg microparticles by 19% on average. On the other side, zein significantly decreased the cumulative release of TPC (Alg/Z microparticles), by an average of 67%. Therefore, zein causes a slowdown in the polyphenols release in water. The isoelectric point of zein protein is at pH 6.2, and for that reason, it is poorly soluble in neutral pH. Microparticles with both HPMC and zein (Alg/Z/HPMC) had on average 26.7% lower release in time compared to Alg microparticles. The cumulative release trend was in between the trend of Alg/Z and Alg microparticles. The results of cumulative release in water are correlated with microparticles morphology analyzed using SEM and AFM. The microparticles with rougher surface and higher grains on the surface had less pores and slower release of TPC.

In vitro simulated gastric TPC release analyzed in HCl solution (pH 1.64) showed changes in release trend compared to release in water. In an acidic medium, Alg microparticles had increased cumulative release in time compared to a neutral medium (distilled water). With this faster release, after 120 min, 27.3% more polyphenols were released in the tested medium, and the highest proportion of released polyphenols from all four formulations, during 120 min. The release rate of polyphenols from microparticles in an acidic medium was in the following order: Alg > Alg/HPMC > Alg/Z/HPMC > Alg/Z. The addition of both HPMC and zein into the microparticle structure decreased the TPC release in HCl. Consequently, Alg/Z microparticles had the lowest TPC release during time, which was 1.38 × lower after 120 min compared to release in water. According to these results, it is evident that compared to the microparticles without zein in their structure, the microparticles with zein release polyphenols in HCl more slowly. These results concur with the results of the work of Karthikeyan et al. where they compared the release from microparticles coated with zein and microparticles without zein. The addition of zein to the microparticle structure slowed down the release in the acidic medium [66].

Furthermore, the cumulative release was analyzed in a buffer solution (pH 7.40) for simulated intestinal conditions. When compared to water and HCl, the release drastically changed. The greatest increase in the concentration of released polyphenols takes place in the first 15 min, that is when the release is the fastest. In that period, the highest proportion of released polyphenols was measured in microparticles containing calcium alginate, HPMC, and zein (Alg/Z/HPMC—43.8%). After this period, the rate of release of polyphenols from this type of microparticles decreases, and after two hours, the measured proportion of released polyphenols is 77.2%—the smallest proportion of polyphenols released from microparticles in phosphate buffer. No polyphenols were released in the first minute from any microparticle formulations. After that, the rapid growth of release rate occurs, and after two hours, the complete release of polyphenols from the microparticles (100%) was measured in both Alg and Alg/Z microparticles. From the measurement results, it is evident that in phosphate buffer, microparticles containing HPMC release polyphenols more slowly than microparticles that do not contain HPMC. The results are consistent with the results obtained in the work of Patole and Pandit (2018) where the release of mesalamine from capsules coated with HPMC slows down the release under small intestine simulation conditions [67]. The total release of TPC in phosphate buffer happened due to the rapid degradation of microparticles. The degradation is a consequence of ion exchange between sodium and calcium cations that causes a gel network to relax, diffusion of calcium ions from the gel to the medium, and finally microparticle erosion [45,68].

Comparing the results of measuring the release of polyphenolic compounds in water, HCl, and phosphate buffer, it is evident that each type of microparticle reacts differently relative to the medium type. All types of microparticles release polyphenols the fastest in phosphate buffer, while it was the slowest in acidic conditions (except for Alg microparticles that had the lowest release rate in water). Therefore, it is evident that the addition of co-biopolymers (HPMC and zein) to the microparticle composition slowed down the release of polyphenols from the particles (except for Alg/HPMC in water). The TPC release

rate increased with high pH for microparticles composed of alginate, zein, and/or HPMC (Alg/Z, ALG/HPMC, and Alg/Z/HPMC). The pH significantly affected the release of rosemary polyphenols from microparticles as a result of zein's high level of solubility in acidic media. This was also confirmed by Hosseini et al. who analyzed the release of rosemary essential oil from zein-electrospun fibers [27] and by Colín-Orozco et al. who reported higher rates of release with a pH increase of rosemary extract from polyethylene oxide/whey protein isolate fibers [69]. The release level of grape seed proanthocyanidin extract from microparticles prepared with calcium alginate and HPMC also showed an acceleration of release and microparticles disintegration with higher pH in work from Sheng et al. [21]. The solubility is also correlated with possible protein-phenols interactions that showed higher solubility in alkaline pH, and therefore the release of phenolic compounds [48].

Thus, most of the polyphenols will remain in the microparticles after passing through the stomach and will be released from the microparticles in the small intestine. This is important since most polyphenols are metabolized in the intestine by the microflora (mostly in the jejunum and ileum) [64]. With the right combinations of biopolymers, the desired level of phenolic release and stability can be achieved, depending on the desired purpose. The investigated formulations of microparticles show great potential in the production of functional foods, where different polymer combinations can be used depending on the desired food product properties.

The potential limitations of our study include more precise simulation of gastrointestinal tract conditions that can include enzymatic digestion, and application in real food systems. Microparticle composition is important to consider when implementing the latter into food production. Future studies should focus on the investigation of microparticle behavior in real, complex matrices, such as food systems where interactions of many components might interfere with the active ingredient release.

4. Conclusions

In this research, rosemary extract obtained using HVED has proven to be an effective potential source of bioactive compounds, especially polyphenols. The microencapsulation of aqueous rosemary extract was successfully carried out by the ionic gelation method using calcium alginate, zein, and HPMC as coating materials. Each formulation resulted in specific microparticles different in physicochemical characteristics, and functional properties in terms of bioactive content and release properties.

The swelling behavior significantly changed ($p < 0.05$) with the addition of HPMC to calcium alginate, while zein and rosemary extract did not make significant changes to the swelling degree of microparticles. The mean diameter of dry microparticles increased with the addition of rosemary extract and with both zein and HPMC to calcium alginate. The morphological surface of microparticles analyzed using SEM and AFM was shown to be granular with visible individual grains of 6.3–11.3 nm in height. The addition of the second biopolymers to calcium alginate increased the roughness and reduced the porosity of microparticles. Moreover, the addition of rosemary extract caused a rougher structure with sharper edges of grains on the surface of the microparticles.

The encapsulation efficiency for all formulations was high (>100%), indicating an efficient encapsulation process where rosemary extract was added to both encapsulation solution (biopolymers) and $CaCl_2$ solutions before encapsulation. The loading capacity of polyphenols from rosemary extract was the highest in microparticles prepared with calcium alginate and zein (10.42 ± 0.72 mg GAE/g). Furthermore, the FTIR analysis with and without rosemary polyphenols presented specific molecular interactions for each formulation. Blending alginate with other polymers influenced molecular interactions, mainly hydrogen bonds and electrostatic interactions. A better understanding of molecular interactions between microparticle constituents ensured its potential for controlling their release behavior and the further development of new microparticles for specific use. Microencapsulation provided a protective effect for rosemary polyphenols against pH changes

during digestion. The addition of both HPMC and zein decreased the polyphenols release in gastric conditions, which resulted in an increase of polyphenol bioavailability in the gut. The results indicated a high potential of microparticles loaded with rosemary extract for further use, especially in functional food development.

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GENERAL DISCUSSION

4. GENERAL DISCUSSION

The overarching objective of the thesis is to assess the efficacy of extracting BACs from selected Mediterranean herbs (rosemary and oregano) using HVED and green solvents. The green solvent selectivity was performed by theoretical and experimental procedures. Furthermore, the extraction from rosemary and oregano was performed using HVED and selected green solvents in comparison to conventional extraction, optimizing the HVED extraction process. Additionally, the sustainability of HVED was assessed and the obtained optimal extracts were stabilized by microencapsulation for potential use in functional food preparation.

4.1. The potential of model predictive tools as a preliminary method to reduce solvent consumption during experimentation

The significance of selecting an appropriate solvent in extraction processes cannot be overstated. The choice of solvent plays a key role in determining the efficiency and success of the extraction, impacting factors such as yield, purity, and selectivity of the target compounds. The selection of the extraction solvent relies on the chemical characteristics of both solvents and desired natural substances undergoing extraction. An optimal solvent should possess the ability to dissolve the desired compounds while minimizing the extraction of unwanted impurities (Popova and Bankova, 2023).

Additionally, conventional methods for solvent selection often involve using large quantities of solvents to determine their suitability for extracting compounds. Considering environmental and economic factors, the judicious selection of a solvent contributes to the sustainability and cost-effectiveness of the overall extraction process (Diorazio et al., 2016). To address this issue, two computational simulation methods for solvent selection have been used in this thesis, including the HSPs and COSMO–RS. These models rely on theoretical predictions to evaluate the solubility of specific compounds in each evaluated solvent.

The solubility of rosemary BACs was evaluated at room temperature (20 °C) to create conditions for nonthermal extractions. The results for HSPs are given for different solvents, and the RED values are presented in Table 1 (*Paper I*). The RED results were utilized to quantify the interaction between solutes and solvents. The assessment results for green solvents including ethyl acetate, methyl acetate, ethyl oleate, ethanol, 1-butanol, isopropanol, methanol, limonene, α-pinene, cymene, β-myrcene, CPME, dimethyl carbonate, MeTHF, and water were compared to conventionally used solvent n-hexane. Depending on solvent polarities, the

theoretical solubility of each solvent for BACs from rosemary exhibited different results. Some of the assessed green solvents are capable of extracting BACs from rosemary even with a higher extraction affinity than conventional hexane. According to produced RED results of HSPs, the order of potential for BAC extractions from rosemary was as follows: CPME > limonene > cymene > MeTHF > ethyl oleate > β-myrcene > α-pinene > ethyl acetate > n-hexane > methyl acetate > dimethyl carbonate > isopropanol > 1-butanol > methanol > ethanol > water. Conversely, water and ethanol exhibited a low potential for extracting BACs from rosemary since most of the evaluated compounds are non-polar.

The results of HSPs align with the trend observed in COSMO–RS assessments, with certain variations. The results of the COSMO-RS assessment confirmed the higher potential of green solvents over n-hexane for rosemary compound extraction, except for water. The probability of solubility was presented according to a number of compounds with high probability (green color): MeTHF > CPME > ethyl acetate > methyl acetate > 1-butanol > isopropanol > ethanol > ethyl oleate > dimethyl carbonate > methanol > limonene > cymene > β-myrcene > α-pinene > n-hexane > water. To summarize all results, from both HSPs and COSMO-RS assessment it can be concluded that ethyl oleate, limonene, α-pinene, cymene, β-myrcene, CPME, and MeTHF demonstrated a high potential for extracting volatile compounds like monoterpenes and sesquiterpenes, making them suitable for extracting essential oil from rosemary. COSMO-RS results confirmed the high potential of ethyl acetate, methyl acetate, ethanol, 1-butanol, isopropanol, methanol, CPME, dimethyl carbonate, and MeTHF, for solubility of diterpenes, triterpenes, and flavonoids, which is not fully confirmed by the HSP results. Therefore, these solvents are considered suitable for the extraction of BACs from rosemary.

However, it’s not always simple to verify theoretical predictions through experimentation. In experimental study, not only the properties of solvents and salutes are taken into consideration, but there are also extraction conditions that influence the solubility. All evaluated green solvents were analyzed in laboratory conditions using HVED for extractions. During experimentation, no electrical discharge was achieved using HVED when employing all of the mentioned green solvents, except for water and ethanol. This can be attributed to the solvent’s high viscosity (ethyl oleate, 1-butanol, isopropanol, α-pinene, cymene) and high density (DMC) (Tanzi et al., 2012; Mohammad and Inamuddin, 2012; Winterton, 2021). Furthermore, hydrophobic solvents like methyl acetate, ethyl oleate, 1-butanol, limonene, α-pinene, cymene, β-myrcene, CPME, dimethylcarbonate, and MeTHF are not suitable for hydrophilic BACs. Moreover, all green solvents are highly flammable and therefore pose a danger during experimentation with HVED.

For that reason, water and ethanol (mixtures) were chosen as appropriate solvents for further experimentation.

In *Paper II*, the theoretical predictions were shown only for water and ethanol as chosen green solvents for further analysis and are presented in Table 2. Both HSPs and COSMO-RS confirmed the low potential of solubility of BACs from oregano. These results are in line with the results for rosemary. Ethanol exhibited a higher solubility probability for all compounds compared to water evaluated with both theoretical methods. Water showed low probability of solubility (0–20 %) for all compounds, even at boiling temperature (100 °C), with no significant temperature influence noted. According to the "like dissolves like" rule, ethanol demonstrated high solubility probability (60–100 %) at room temperature, evaluated by COSMO-RS, for carvacrol, thymol, borneol, α-terpineol, piperitone, and palmitic acid. As there were no significant differences in solubility probability between room and boiling temperature, nonthermal extraction with HVED (at room temperature) using water and ethanol as solvents was confirmed as suitable. According to theoretical results, a higher content of ethanol in solvent mixtures results in a higher yield of BACs extracted from both rosemary and oregano. Theoretical findings concurred with experimental outcomes in solvent selection, revealing that ethanol exhibited a greater capacity for solubilizing BACs compared to water (Figure 3 – *Paper I*, Table 3 – *Paper II*).

The results showed that theoretical prediction is accurate and confirmed with experimental data in terms of solubility. The comparison with experimental data for individual BACs in extracts (Table 5, *Paper I*; Table 4, *Paper II*) revealed a similar trend for solutions in both water and ethanol. Higher ethanol content led to increased solubility of most extracted compounds, along with a rise in the total phenolic compound concentration, and some individual BACs like apigenin, carnosol, diosmetin, rosmarinic acid, carvacrol, and thymol. However, experimental data showed higher extraction yield than expected theoretically. The utilization of the predictive COSMO-RS model proved more suitable than the HSPs for choosing alternative solvents in the extraction of BACs from Mediterranean plants. This pattern was also observed in the data presented in the literature for the extraction of α-mangostin from *Garcinia mangostana* L. (Bundeesomchok et al., 2016).

However, although the theoretical prediction of the presented green solvents showed a high potential of various green solvents for extraction of BACs from rosemary, the experimental data cannot confirm all theoretical results concerning the extraction conditions and difficulties

during experimentation. Nevertheless, theoretical data indicated that certain BACs found in oregano exhibited low solubility in both water and ethanol, emphasizing the need to explore alternative green solvents for the extraction of BACs from oregano. For that reason, a broader approach for green solvents such as vegetable oils (Li et al., 2019), deep eutectic solvents, or solvent mixtures (Wojeicchowski et al., 2020; Winterton, 2021) should be assessed to find an optimal solvent, depending not only on solvent properties, but also on extraction method and conditions, and also properties of the final product. HSPs and COMO-RS showed great potential for the prediction of the solubility of desired compounds in a solvent, however, experimentation is still needed to confirm the theoretical data. For now, computational theoretical predictions could be used to find an optimal solvent mixtures (for example ethanol: water ratio) or to choose the most suitable solvent for further investigations.

4.2. Determination of the efficiency of extraction of bioactive compounds from rosemary and oregano using HVED and green solvents

The experimental extraction techniques for rosemary and oregano involved the use of selected green solvents, specifically water and ethanol solutions at concentrations of 25 % and 50 % (v:v). The efficiency of extraction of BACs from rosemary and oregano using HVED and selected green solvents was evaluated experimentally through numerous physicochemical analyses and compared to conventional extraction.

The HVED extraction was conducted with the "IMP-SSPG-1200" generator (Impel group, Zagreb, Croatia) that generated rectangular pulses using direct current to achieve high voltage. Fixed HVED parameters, determined through preliminary experiments, included a frequency of 100 Hz, pulse width of 400 ns, an electrode gap was 15 mm, and a mass-to-solvent ratio was 1 g:50 mL as per pharmacopeia standards. During the HVED process, a gas flows through the needle (electrode) and undergoes ionization, resulting in the formation of a cold plasma. The choice of gas in the treatment is crucial, as various gases ionize at distinct voltages, leading to the generation of different radical species during discharge. Consequently, using different gases may yield different results (Mir et al., 2019). Due to the challenge of achieving electrical discharges with nitrogen below 20 kV, nitrogen treatments were conducted at 20 and 25 kV, while lower voltages of 15 and 20 kV were selected for argon treatments.

The herb-solvent mixture was transferred to a 100 mL beaker-shaped reactor. Gases (argon or nitrogen) were introduced through the needle at a flow rate of 5 L/min. Treatment time was chosen for 3 and 9 minutes. The generator and reactor setup is depicted in Figure 7 (*Paper I*).

For comparison, untreated samples (modified conventional extraction) were processed at room temperature by dissolving dried plant material in a solvent with light magnetic under the same extraction conditions regarding extraction time, plant mass, and solvent volume.

4.2.1. Physical properties of extracts

Obtained extracts were analyzed for changes in physical parameters in terms of pH, conductivity, power, and temperature measurements conducted during the extraction of rosemary and oregano extracts using HVED and conventional extraction methods. Results for rosemary are given in Figure 2 (*Paper I*) and for oregano in Figure 2 (*Paper II*).

Temperature readings were taken both before and after HVED treatment. The highest temperature recorded during the extraction process reached 33.1 °C (oregano sample with 25 % ethanol, treated for 9 minutes, 25 kV, and nitrogen), and the maximum elevation during HVED treatment was 7.9 °C (observed in the sample with oregano treated for 9 min with water and nitrogen at 25 kV). Although temperatures increased with longer treatment time and higher voltage during HVED treatment, no significant heating during the treatment with HVED was noted, which confirmed the classification of HVED as a nonthermal technology (< 35 °C).

The pH values were similar for all extracts not depending on the plant used. The variations in pH were found to be significantly influenced ($p \leq 0.05$) only by the ethanol content. Higher ethanol content increased pH for all extractions (conventional extraction, HVED using nitrogen and argon).

The power of extracts increased with higher applied voltage and treatment duration of the HVED process. Furthermore, the use of nitrogen required more power compared to argon, as the applied voltage for nitrogen (20 and 25 kV) was higher than that for argon (15 and 20 kV). The differences were made because of the difficulty in achieving discharge with nitrogen during the extraction, necessitating higher voltage and increased power for electrical discharge.

The results of conductivity were found to be significantly influenced ($p \leq 0.05$) by the ethanol content for all extractions and by treatment time (for extractions with argon). Higher ethanol content decreased conductivity which is consistent with existing literature (Maeda et al., 2013). Furthermore, the measurements of conductivity in extractions by HVED may have an importance in assessing the level of electroporation. The procedure of electroporation, also known as electropermeabilization, involves applying an electric field of sufficient strength to an external surface to produce transmembrane potential, which increases the permeability and

conductivity of a cell's plasma membrane (Mahnič-Kalamiza et al., 2014; Pavselj et al., 2013). The extraction process in this thesis aimed to extract BACs from the intracellular area by inducing electroporation, allowing the substances to pass through membrane pores into the solvent. This is supposed to be carried out under optimized conditions to preserve the activity of the extracted compounds (Donsì et al., 2010). Results of the Pearson correlation indicated that there is a significant medium positive relationship between conductivity and TPC results for oregano ($r = 0.475$, $p < 0.001$). These results are in line with the literature data for PEF treatments (Pavselj et al., 2013; Wiktor et al., 2015; Mahnič-Kalamiza et al., 2014). However, this correlation was not confirmed with rosemary. Therefore, further analysis of the possible relationship between physical parameters and extracted BACs with HVED should be performed in further research.

4.2.2. Determination of pesticides and metals in plant samples

The determination of pesticides and metals in oregano and rosemary plants was analyzed to evaluate the safety of usage of extracts obtained from these plants for human use.

While oregano and rosemary may be viewed as nutritional supplements, there is currently no specific categorization for them in the European Commission (EC) Regulation, as herbs or plant tea. Consequently, the elevated levels of pesticides and heavy metals present in the dried plant could potentially lead to significant toxicological consequences for human health. The existing regulation of trace metal and pesticide content in foods is governed by EC Regulations No 1881/2006 and No 396/2005, respectively. Therefore, in the absence of a specific classification, the regulations for dietary supplements were chosen as the closest category for evaluating the health safety requirements. The Regulations include databases with all active substances and their maximum residue levels (MRLs). The analyses for pesticides and heavy metals in rosemary are given in Table 7 (*Paper I*), while results for oregano samples are presented in Table 2 below.

Table 2. Residue levels and maximum residue levels (MRL) of pesticides (mg/kg) and metals (mg/kg) in oregano samples

	Component	MRL (mg/kg)	Residue level (mg/kg)
Pesticides	Alachlor	0.02	<0.005
	Aldrin and Dieldrin (Aldrin and dieldrin combined expressed as dieldrin)	0.01	<0.002
	Captan (Sum of captan and THPI, expressed as captan)	0.06	<0.020
	DDT (sum of p,p´-DDT, o,p´-DDT, p-p´-DDE and p,p´-TDE (DDD) expressed as DDT)	0.05	<0.004
	Endosulfan (sum of alpha- and beta-isomers and endosulfan-sulphate expresses as endosulfan)	0.05	<0.002
	Endrin	0.01	<0.004
	Heptachlor (sum of heptachlor and heptachlor epoxide expressed as heptachlor)	0.01	<0.002
	Hexachlorobenzene	0.01	<0.002
	Hexachlorocyclohexane (HCH), alpha-isomer	0.01	<0.002
	Hexachlorocyclohexane (HCH), beta-isomer	0.01	<0.002
	Iprodione	0.02	<0.010
	Lindane (Gamma-isomer of hexachlorocyclohexane (HCH))	0.01	<0.002
	Methoxychlor	0.01	<0.010
	Tolylfluanid (Sum of tolylfluanid and dimethylaminosulfotoluidide expressed as tolylfluanid)	0.05	<0.020
	Vinclozolin	0.02	<0.002
Metals	Lead (Pb)	3.00	<0.050
	Cadmium (Cd)	1.00	0.014
	Mercury (Hg)	0.10	0.013
	Chromium (Cr)	/	0.180
	Nickel (Ni)	/	0.564
	Manganese (Mn)	/	29.00
	Iron (Fe)	/	79.00
	Copper (Cu)	/	8.00
	Zinc (Zn)	/	42.00

Data showed that all residue levels of pesticides and heavy metals found in both rosemary and oregano are below MRLs for each compound. Rosemary and oregano plants demonstrated conformity in terms of the presence of contaminants, pesticide residues, and toxic metals. The results obtained indicate that both samples are safe for human consumption.

However, it is important to note that certain alterations in metal levels may occur during the treatment, necessitating further in-depth analyses to thoroughly evaluate this matter. The

ground electrode in the HVED system is made from galvanized steel, while on the other side, the high voltage electrode is a medical needle made from stainless steel. Therefore, the main metals in these materials are Zn and Fe with impurities such as Mn, Cr, Ni, etc. (Marder, 2000). For that reason, additional metals (Cr, Ni, Mn, Fe, Cu, and Zn) were assessed in selected HVED extracts characterized by high phenolic and antioxidant content, and the findings are outlined in Table 7 (*Paper I*). The Cr and Ni content increased in the extracts. In contrast, the levels of other metals decreased after HVED extraction. This data suggests that, during the HVED treatment, certain metal levels increase, possibly as a result of electrodes erosion during the process (Cogollo de Cádiz et al., 2021; Potocký et al., 2009). However, there are no MRL levels provided for these metals, so these results do not show a health concern and indicate that the obtained extracts are safe for human dietary use.

4.2.3. Effect of HVED extraction on total phenolic content and antioxidant activity of extracts

The main goal of the thesis was to employ HVED for the extraction of BACs from Mediterranean herbs rosemary and oregano. The results were compared with modified conventional extraction (conducted at room temperature). The impact of process parameters (ethanol concentration, applied voltage, and treatment duration) was assessed for statistical significance ($p \leq 0.05$). The yield of extracted BACs was evaluated in terms of total phenolic content (TPC), antioxidants (2-diphenyl-2-picrylhydrazyl (DPPH), electron paramagnetic resonance (EPR), ferric reducing antioxidant power (FRAP)) and individual BACs.

The results of TPC and antioxidant activity for rosemary are presented in Figure 3 (*Paper I*) and for oregano in Table 3 (*Paper II*). On average, HVED yielded 2.13 times higher content of phenols than CE for rosemary, and 1.94 times for oregano. For both plants, an increase in TPC content was noted with the usage of nitrogen, longer treatment time, and higher voltage. The highest score of TPC was 31.64 and 191.28 mg GAE/g of rosemary and oregano, respectively. Ethanol content showed different trends for each treatment. For rosemary, with conventional extraction, the highest scores were obtained with water as an extraction solvent, while HVED treatment with nitrogen was highest with 50 % ethanol, and treatment with argon had the highest yield of TPC with 25 % ethanol. For oregano, the highest yield of phenolic compounds for conventional extraction was also noted with water, while for HVED extraction with both argon and nitrogen were obtained in extracts with 25 % ethanol, likely due to increased solubility of

phenolic compounds. Furthermore, oregano yielded significantly higher phenolic content compared to rosemary, on average 41.97 % higher.

The antioxidant activity of obtained extracts was measured with DPPH. The results of the DPPH assessment exhibited minimal variation among the extracts and type of extraction. However, a significant correlation was identified between DPPH and treatment time as well as ethanol content for rosemary. The DPPH results were higher with a shorter treatment time and a higher percentage of ethanol in the solution. For oregano extracts, only ethanol content significantly impacted the DPPH values. Discrepancies in DPPH results could potentially arise from the generation of free radicals inherent in HVED treatment, impacting the antioxidant activity of the extracts and possibly interacting with the DPPH radical (Baliyan et al., 2022).

Additionally, a FRAP assessment was done for rosemary extracts. In the case of FRAP results, similarities were observed with TPC values for HVED extraction. Antioxidant capacity was higher with longer treatment times, increased voltage, and nitrogen treatment compared to argon. On average, HVED extraction demonstrated a 2.39-fold increased antioxidant capacity, when compared to conventional extraction. For oregano, the EPR method was performed to confirm the antioxidant activity of extracts. Results of EPR are expressed as intensity of radical (I_R) where lower values signify greater antioxidant activity in the extracts. All extracts obtained through HVED exhibited lower I_R values compared to those obtained through CE, indicating that HVED extracts possessed higher antioxidant activity. On average, HVED extracts displayed an I_R value 3.54 times lower than extracts obtained through conventional extraction, suggesting that there are still more antioxidants present in the extracts than free radicals.

Furthermore, near infrared spectroscopy (NIR) was employed for all samples, recorded within the wavelength range of 904 to 1699 nm (Figure 3, *Paper II*). The two most significant ranges were recognized for both plant samples: the initial one spanning from 904 to 935 nm, and the second one from 1350 to 1699 nm. The latter spectral region (1350–1699 nm) is distinctive for the first overtone of the R–OH and O–H stretching vibrational bands (related to alkyl alcohols or water) and the intermolecular H-bonds of water absorption (Rébufa et al., 2018). Upon examination of the spectra, it becomes apparent that the majority of the recorded spectra exhibit significant similarity. For that reason, differences among the samples could not be determined, necessitating the application of some chemometric techniques. To better explain the large number of variables using a minimal number of components, the technique of principal component analysis (PCA) was employed (Figure 4, *Paper I*; Figure 4, *Paper II*). The obtained

results demonstrated that the application of NIR spectroscopy in combination with chemometrics such as PCA represents a fast, valuable, and non-invasive approach for determining BACs in obtained extracts. Despite the occurrence of overlap between conventional extraction and HVED extracts, the PCA analysis revealed distinctions in each extract. It can be concluded NIR in combination with PCA can explain results qualitatively.

Based on these findings, it became evident that the prediction of both the TPC and antioxidant activity could be predicted using NIR. For rosemary, a part of the input spectral data was utilized for modeling and evaluation with partial least squares regression (PLSR) and principal component regression (PCR). The objective was to predict these parameters not only qualitatively but also quantitatively. The model with the entire range of NIR spectra, yielded the most favorable model efficiency parameters), indicating a highly accurate quantitative model prediction (Kujundžić et al., 2017). The effectiveness of the model in predicting TPC, DPPH, and FRAP using NIR is illustrated in Figure 5 (*Paper I*). Graphical results revealed the best modeling predictions for TPC, FRAP, and DPPH. It was demonstrated that utilizing the entire spectrum of recorded wavelengths resulted in high-quality models, especially for TPC and FRAP results, and that this method could be successfully used for quantitative prediction of TPC and antioxidant activity using NIR.

4.2.4. The analysis of bioactive compounds in the extracts

The ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was utilized for the assessment of the concentrations of non-volatile BACs (including apigenin, carnosol, diosmetin, hydroxytyrosol, luteolin, oleanolic acid, oleuropein, quercetin, rosmarinic acid, p-cymene, camphor, thymol, and carvacrol) in rosemary and oregano extracts.

Results from the UPLC–MS/MS analysis of individual BACs in rosemary extracts (presented in Table 5, *Paper I*) revealed that the key bioactive compounds include apigenin, diosmetin, and rosmarinic acid. A comparison of phenolic compounds in different extraction types indicated that HVED extracts exhibited higher levels of apigenin, carnosol, diosmetin, hydroxytyrosol, luteolin, oleanolic acid, oleuropein, quercetin, and rosmarinic acid compared to extracts obtained by conventional extraction. Statistical analysis demonstrated that the majority of the measured compounds were significantly influenced only by ethanol content (apigenin, carnosol, diosmetin, hydroxytyrosol, luteolin, oleanolic acid, oleuropein, quercetin, and rosmarinic acid). Additionally, apigenin, carnosol, diosmetin, hydroxytyrosol, and luteolin exhibited dependence on treatment time.

The UPLC-MS/MS analysis of individual BACs in oregano extracts showed that the predominant compounds were rosmarinic acid, luteolin, and hydroxytyrosol, (Table 4, *Paper II*). Notably, rosmarinic acid exhibited the highest values, particularly with an increase in ethanol content. Hydroxytyrosol, identified as a major constituent, showed higher amounts in samples extracted in water. Additionally, ANCOVA analysis showed significant correlations ($p \leq 0.05$) for thymol with all three variables (TPC, DPPH, EPR), oleanolic acid with DPPH, and quercetin with TPC.

Furthermore, the composition of volatile compounds in rosemary extracts was performed by headspace solid-phase microextraction/ gas chromatography-mass spectrometry (HS–SPME/GC–MS) (Table 6, *Paper I*). The primary compound was the cyclic monoterpene ether eucalyptol, ranging from 40.33 % to 2.28 %, followed by camphor, borneol, and linalool. The concentrations of these terpenes were influenced by the solvent and the gas used (nitrogen or argon). Notably, when water was used as the extraction solvent, the percentage of all three monoterpenes was high, except for linalool. Characterizing volatile compounds in ethanol extracts proved challenging due to peak overlap with ethanol, and therefore, results for most ethanol samples were not presented. The results of the analysis of volatile compounds in oregano are given in Table 3.

Table 3. HS-SPME/GC-MS analysis of volatile compounds from oregano extracts (measurements for conventional extraction and HVED extraction).

Sample	Area (%)			Extraction type
	Linalool (*RI=1103*)	Terpinen-4-ol (*RI=1182*)	Thymol (*RI=1302*)	
3 O0	n.d.	n.d.	16.16	Conventional extraction
9 O0	n.d.	n.d.	15.69	
3 O25	n.d.	n.d.	n.d.	
9 O25	n.d.	n.d.	n.d.	
3 O50	n.d.	n.d.	n.d.	
9 O50	n.d.	n.d.	n.d.	
ON1	n.d.	n.d.	n.d.	HVED
ON2	1.18	3.17	9.33	
ON3	3.89	7.23	16.23	
ON4	3.54	6.88	21.68	
ON5	n.d.	n.d.	n.d.	
ON6	n.d.	n.d.	n.d.	
ON7	n.d.	n.d.	n.d.	
ON8	n.d.	n.d.	n.d.	
ON9	n.d.	n.d.	n.d.	
ON10	3.04	7.11	14.26	
ON11	n.d.	n.d.	n.d.	
ON12	n.d.	n.d.	n.d.	
OA1	n.d.	n.d.	n.d.	
OA2	5.20	13.18	37.70	
OA3	n.d.	13.18	36.38	
OA4	n.d.	10.63	60.84	
OA5	n.d.	n.d.	n.d.	
OA6	n.d.	n.d.	n.d.	
OA7	n.d.	n.d.	n.d.	
OA8	n.d.	n.d.	n.d.	
OA9	n.d.	n.d.	n.d.	
OA10	n.d.	11.77	24.32	
OA11	n.d.	n.d.	n.d.	
OA12	n.d.	n.d.	n.d.	

*Sample denotation and process parameters for each sample are provided in Table 1 (*Paper II*), n.d. – not detected

The results of HS–SPME/GC–MS analysis of volatile compounds in oregano extracts showed that the predominant compound was thymol, followed by terpinen-4-ol and linalool. As well as for rosemary extracts, it was not easy to measure concentrations in ethanol extracts. For that reason, purified samples without ethanol should be provided for GC analysis in the future.

4.2.5. Process optimization of HVED extraction

Through the efficient extraction of BACs from rosemary and oregano, it is possible to minimize waste while generating extracts suitable for diverse industries such as food and pharmaceuticals. This not only supports the sustainable utilization of resources but also facilitates the creation of innovative products with potential health benefits. Consequently, it is essential to investigate methods for enhancing the extraction of BACs from Mediterranean herbs and encourage their valuable applications.

The influence of each extraction parameter on extraction yield has been in detail studied in *Paper I* for rosemary and in *Paper II* for oregano. For both plants, results revealed that nitrogen, longer treatment time, and higher voltage yielded higher results of phenolic compounds and antioxidants. The highest TPC results were achieved with 25 % ethanol as an extraction solvent for both analyzed plants.

Furthermore, the optimization was performed for each plant sample. The optimization was performed in STATGRAPHICS Centurion (StatPoint Technologies, Inc., Warrenton, VA, USA) software from the previously conducted multifactorial design consisting of 12 experimental trials per gas (nitrogen and argon). The optimization was performed using the response surface methodology (RSM). RSM is a summary of mathematical and statistical methods used to model and analyze the effects of several factors (independent variables) on the observed response (dependent variable). The basic idea of the methodology is to establish the relationship between the influences of independent variables on the dependent variable through a response function (Dean et al., 2017). The three chosen independent variables for HVED-assisted extraction were: (A) treatment time (3 and 9 min), (B) voltage applied and gas type (15 kV or 20 kV for argon and 20 kV or 25 kV for nitrogen), and (C) concentration of ethanol (0 %, 25 %, or 50 %).

For rosemary, observed output/dependent variables were set as results of TPC, DPPH, and FRAP, with the aim of maximizing all three parameters during the optimization process. Two separate optimizations were performed, one for each gas utilized. This procedure helps determine the combination of experimental factors which simultaneously optimize several

responses. In the case of argon, the optimal conditions were a treatment time of 9 minutes, a voltage of 20 kV, and an ethanol content of 25.5892 %, which closely approximates the target of 25 % from the specified extraction parameters. For nitrogen, the optimum values were a treatment time of 9 minutes, voltage of 25 kV, and ethanol content of 50 %. The estimated response surfaces for rosemary are presented in Figure 7.

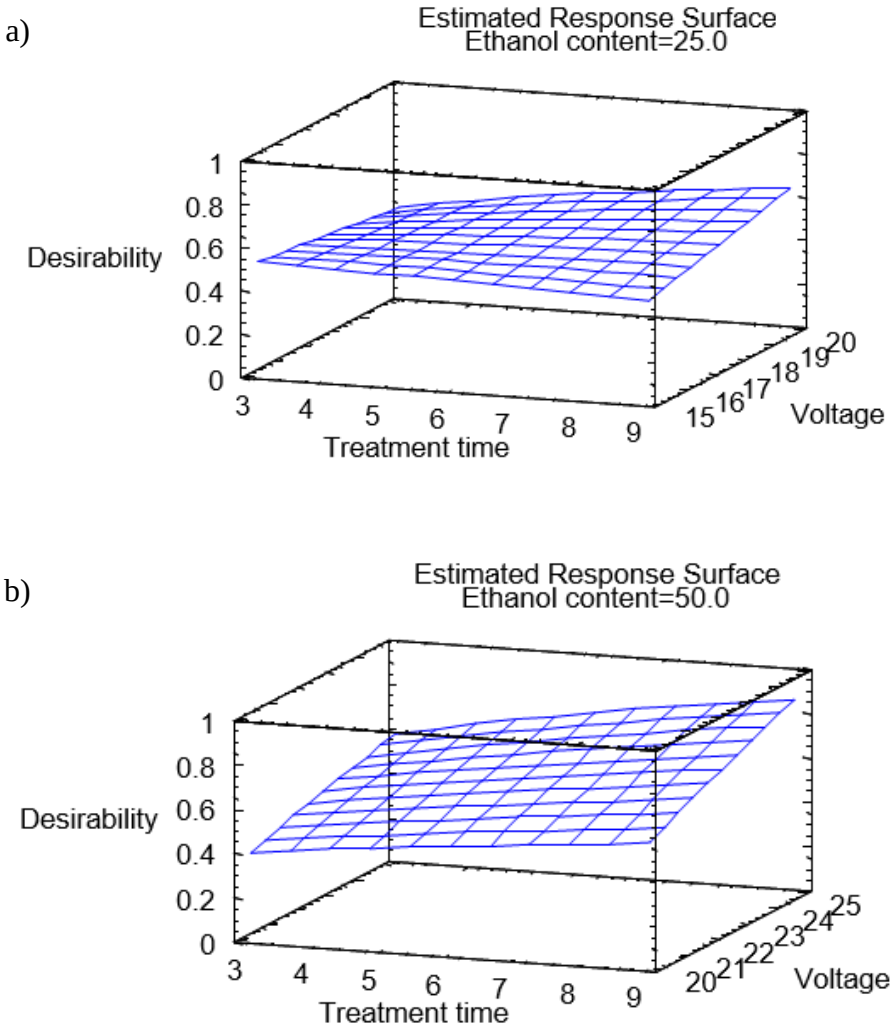
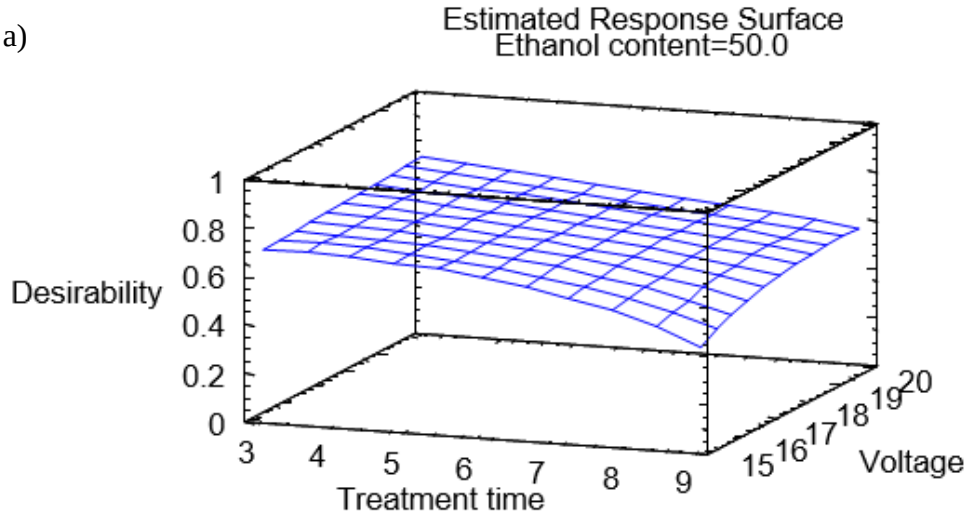


Figure 7. Estimated response surface of multiple response optimization for rosemary treated with HVED using a) argon, and b) nitrogen.

For oregano, observed output variables were set as results of TPC, DPPH, and EPR. Two optimizations were performed, one for each gas used. The optimization goal was set as the maximization of TPC and DPPH and the minimization of EPR results. For argon, optimum values were a treatment time of 3 minutes, a voltage of 20 kV, and an ethanol content of 49.8183 % which is the closest to 50 %. For nitrogen, optimum values were 9 minutes, 24,9996 kV, and 31,4087 % of ethanol. From the used extraction conditions, these results are the closest to 9

min, 25 kV, and 25 % ethanol. The estimated response surfaces for oregano are presented in Figure 8.

a)



b)

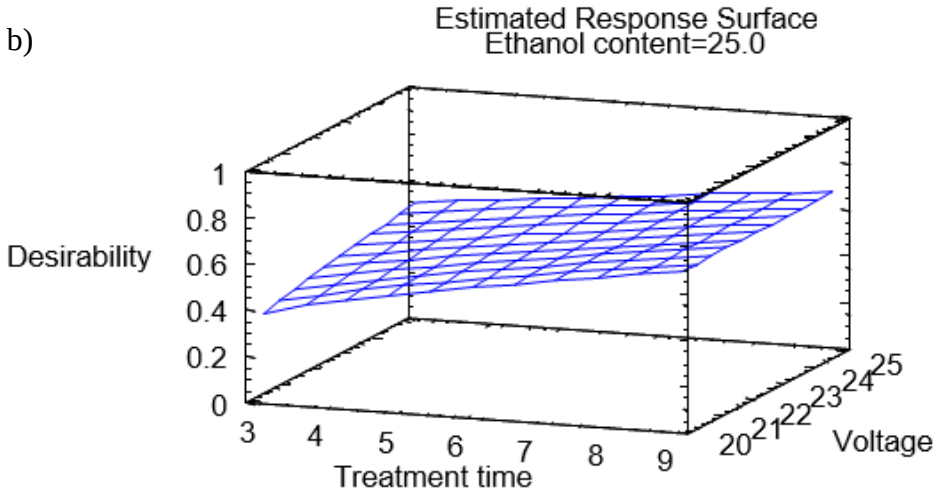


Figure 8. Estimated response surface of multiple response optimization for oregano treated with HVED using a) argon, and b) nitrogen.

The experiment was adequately designed utilizing the multilevel factorial design, and the results were examined and the process optimized using RSM. Similar findings were found for both rosemary and oregano. RSM has proven to be a valuable mathematical and statistical tool for modeling and optimizing the extraction process of BACs from rosemary and oregano. This method facilitates the identification of optimal conditions for the extraction process, yielding extracts with both high BACs content and antioxidant activity, while significantly reducing the

number of experiments required. Given these findings, rosemary and oregano appeared as cost-effective and relatively stable plant materials containing BACs with notable antioxidant activity, offering opportunities for its valorization. These results are important for further experimentation with HVED and for potential scale-up to industrial level to minimize further experimentation.

4.2.6. Comparison of HVED with other extraction methods

In *Paper I* and *Paper II*, HVED was compared with modified conventional extraction at room temperature. The results revealed that HVED showed significantly higher yields of extracted BACs in terms of TPC, antioxidant capacity, and individual compounds measured with UPLC and GC methods, compared to modified conventional extraction. However, since the conventional extraction was performed at room temperature and short treatment time (like HVED), it was expected that results could be quite low, even compared to traditional thermal extraction techniques. Conventional thermal extraction techniques use higher temperatures to extract desired compounds and/or long extraction time. Higher temperatures increase phenolic yield up to 60–80 °C, above that, thermal degradation of phenolic compounds occurs (Antony and Farid, 2022). For that reason, it is expected that some phenolic compounds and other thermolabile BACs would be thermally degraded when extracted at high temperatures.

An additional comparison of the efficiency of HVED was made with conventional thermal extractions – infusion and maceration (Figure 3, *Paper III*). The infusion was made in a water bath at 80 °C for 30 minutes, while maceration was made at room temperature for 48 h. Infusion showed a higher potential for extraction compared to maceration. Despite HVED extraction being conducted in a considerably shorter duration than conventional extractions, the resulting yields of TPC were higher (depending on extraction conditions). HVED can extract phenolic compounds at ambient temperature and for a shorter duration, thereby avoiding the thermal degradation of heat-sensitive phenolic compounds.

Furthermore, HVED extraction was compared with ASE as another nonthermal innovative extraction method. The extraction of phenolic compounds was conducted using an ASE extractor (ThermoScientific™ Dionex™ ASE™ 350) and oregano as a plant with a higher content of BACs as evaluated in this thesis. A 25 % aqueous ethanol solution was used as the extraction solvent (as it was presented as the most appropriate solvent), with variable parameters of temperature for comparison of nonthermal and extraction with elevated temperature (22 and 50 °C), extraction cycles (1, 2, 3), and extraction time (3 and 9 min). The

results of ASE extraction in terms of TPC and DPPH are given in Table 4. The obtained results were statistically processed using the XLStat program (MS Excel 2010). An analysis of variance (ANCOVA test) was conducted to determine the influence of extraction parameters (temperature, static time, and the number of extraction cycles) on the results of TPC and DPPH.

Table 4. Analysis of ASE extracts in terms of total phenolic content (TPC) and antioxidant capacity (DPPH)

Temperature (°C)	Static extraction time (min)	Number of extraction cycles	TPC (mg GAE/g)	DPPH (μmol TE/g)
22	3	1	8.51 ± 0.41	35.32 ± 0.03
	3	2	13.08 ± 1.73	36.59 ± 0.04
	3	3	16.59 ± 1.16	36.34 ± 0.03
	9	1	13.09 ± 0.37	36.40 ± 0.13
50	3	1	17.88 ± 0.48	83.87 ± 0.01
	3	2	26.91 ± 1.70	84.08 ± 0.25
	3	3	29.53 ± 0.77	84.00 ± 0.03
	9	1	24.07 ± 0.65	83.81 ± 0.58

The results of ASE extraction of BACs from oregano were conducted in different extraction parameters. For comparison, extraction was performed at room temperature (22 °C) and elevated temperature (50 °C). Furthermore, different combinations of extraction cycles and extraction times were performed to analyze the differences between extractions with ASE, however, total extraction time variated from 3 to 9 minutes, like in HVED. The results showed that the highest amount of TPC was exhibited at 50 °C, 3 cycles by 3 minutes (29.53 mg GAE/g). The lowest content of phenolic compounds was 8.51 mg GAE/g, extracted under conditions of 22 °C, a static time of 3 min, and 1 extraction cycle. Increasing the temperature, time, and extraction cycles led to a higher efficiency in isolating phenolic compounds, a trend observed in most extracts. Higher efficiency was demonstrated in samples extracted with shorter extraction times but during a greater number of extraction cycles.

In the oregano extract samples, the antioxidant activity determined by the DPPH method ranged from 35.32 to 84.01 μmol TE/g sample. Additionally, it can be observed that samples extracted at higher temperatures exhibit greater antioxidant activity. The sample with the highest antioxidant activity (84.01 μmol TE/g sample) was extracted at 50 °C, 3 min for 2 extraction cycles, while the lowest antioxidant activity was demonstrated by the sample extracted at 22 °C, 3 min for 1 extraction cycle (35.32 μmol TE/g sample).

According to the statistical analysis, all extraction parameters (temperature, extraction time, and the number of extraction cycles) have shown a significant impact on the proportion of phenolic compounds and antioxidant activity. The highest yield, indicating the best efficiency, was achieved with the extraction method conducted at a temperature of 50 °C, and extraction static time of 3 min during 3 extraction cycles, as it yielded the highest content of phenolic compounds from samples of all three plants. It was also demonstrated that higher efficiency is attained with higher temperatures and a greater number of cycles compared to longer static extraction times.

When compared with HVED, it is evident that HVED yielded a significantly higher yield of TPC than ASE. The highest TPC result with HVED extraction was 191.28 mg GAE/g which is 6.48 times higher compared to the ASE extract with the highest yield of TPC. Moreover, a substantial quantity of solvents is employed in ASE treatment. Since solvents are one of the main focuses of this thesis, ASE is considered a less sustainable method compared to HVED.

It can be concluded that HVED yields a high content of BACs from Mediterranean plants rosemary and oregano, compared to conventional extraction methods and nonthermal extraction such as ASE.

4.3. Evaluation of sustainability of HVED in comparison with conventional extractions

As mentioned before, green extraction involves the exploration and development of extraction processes aimed at minimizing energy consumption, and employing alternative solvents and renewable natural resources, all while ensuring the production of a secure and high-quality extract or product. From the definition of green extraction, 6 principles were developed for scientists and industry to follow towards more sustainable extraction methods. Many research papers have been based on guessing that some technology is more sustainable compared to conventional extractions, because of the shorter extraction time, higher extraction yield, or some other advantages that are considered sustainable. However, the literature is lacking in critical analyses of the extraction conditions, optimized conditions, advantages, and disadvantages of nonthermal extraction techniques. Furthermore, no experimental analysis of the sustainability of HVED was found in the literature.

For that reason, a sustainability assessment of HVED was performed in this thesis and is presented in *Paper III*. As the plant sample does not influence sustainability significantly, the chosen plant was oregano as it yielded higher yields of BACs. The sustainability of oregano treatment employed a partial LCA approach and was compared with conventional extraction

methods (infusion and maceration). This involved mapping out the entire treatment processes, establishing scope and limits at the laboratory scale, gathering and computing data, and evaluating the outcomes according to ISO standard 14040 (ISO, 2006). The functional unit (FU) output reference was set as the treatment of oregano.

The input data encompassed the utilization of plant material, natural resources such as water and energy, as well as chemicals like ethanol (as a solvent) and nitrogen and argon gases for HVED treatments. The computation of environmental impacts utilized data from the CCaLC (CCaLC, 2018) and Ecoinvent databases (Ecoinvent, 2021). The environmental footprints assessed in this research comprised two metrics: global warming potential (GWP) and ozone depletion potential (ODP). GWP quantifies the level of damage in kilograms of CO_2 equivalent (CO_{2e}), representing the weighted impact of GHGs over 100 years (IPCC, 2013). ODP measures the detrimental effect of halogenated hydrocarbons on the stratospheric ozone layer within a 100-year time frame. The impacts of the chemical compounds are assessed relative to trichlorofluoromethane (R-11 or CFC-11) (Hischier et al., 2010).

The results revealed varying CO_2 emissions for nitrogen and argon treatments, mainly influenced by treatment duration (gas and energy consumption). While ODP remained relatively low, its values were impacted by treatment parameters. Statistical analysis highlighted the significance of longer treatment time and higher voltage on environmental impact, with ethanol content showing no significant effect on both GWP and ODP. Despite nitrogen and argon not being classified as GHGs, their notable impact is attributed to the production facilities.

Comparing nonthermal HVED with conventional infusion and maceration treatments, significant differences in environmental impact were observed (Table 3, *Paper III*). HVED demonstrated promising potential in terms of energy consumption compared to infusion, owing to shorter treatment times and lower equipment power consumption. Maceration, although having the lowest environmental impact due to no heating or electricity usage, required an extended treatment duration (48 h) with considerably lower extraction yields.

Considering extraction yield per unit time (expressed as TPC per minute of treatment), HVED consistently outperformed infusion and maceration. The environmental impact per unit of extraction yield indicated that, for a 1-minute extraction, HVED had the lowest environmental impact, while infusion had the highest impact. The findings suggest the possibility of optimizing treatment parameters for both extraction yield and environmental impact.

As mentioned in *Paper III*, there are three main pillars of sustainability: environment, society, and economy. It is important not to prioritize any of the pillars but rather to seek a balanced approach to all of them for sustainable production. Additionally, from a food standpoint, an extra dimension linked to food safety (and quality) needs to be taken into account.

The economic value of extractions was determined by various elements in the extraction process, including plant material, solvent, equipment, and electricity consumption. When comparing extraction methods such as HVED and infusion, the economic analysis considers electricity consumption during treatment and solvent content (Table 4, *Paper III*). HVED treatment exhibited lower power consumption than infusion, resulting in a more economical final cost. Maceration extraction, despite not consuming electricity, was deemed less economical due to its lengthy duration and lower efficiency. From an economic standpoint, energy consumption plays a significant role in product pricing, and adopting nonthermal technologies like HVED, with lower energy requirements, is advantageous. The transition from laboratory-scale to industrial-level implementation holds potential for further optimization, reducing material and solvent consumption per treatment and contributing to economic value. This emphasizes the importance of considering economic factors alongside environmental and societal aspects in achieving sustainable and cost-effective extraction processes.

The social pillar of sustainability, focuses on the overall well-being of society, considering factors such as poverty, inequality, and peace. HVED offers lower environmental impact, reduced costs, increased productivity, and improved product quality, contributing to societal progress. This novel technology, with the potential for further development across industries, plays a role in enhancing societal well-being. Simultaneously, there is a need to raise consumer awareness about nonthermal food processing technologies, such as HVED, and their environmental and economic benefits. With a growing global interest in natural, organic, sustainable, and minimally processed foods, increased education can positively influence awareness of innovative technologies like HVED.

The additional pillar focuses on food safety and product quality in HVED. Numerous studies indicate that HVED enhances food safety and quality by addressing microbial and enzyme inactivation, as well as improving nutritional value. This thesis demonstrated increased TPC compared to conventional extractions. However, further investigations are required to assess both positive and negative effects, including microbial and sensory analyses, and detailed detection of potential free radical formation.

In *Paper III*, it was presented that HVED had a direct correlation with SDG7 (Affordable and Clean Energy), SDG 13 (Climate change mitigation), SDG 2 (Zero Hunger), and SDG 12 (Responsible consumption and production). Considering the overall benefits of HVED, encompassing environmental, social, economic, food safety, and quality aspects, it can be recognized as a green extraction technology. With ongoing improvements, HVED holds significant potential for industrial scale-up.

4.4. Stabilization of obtained optimal extract by microencapsulation in biopolymer-based microparticles for the potential use in functional food preparation

Because of its composition and characteristics, the aqueous extract containing polyphenols is not a stable medium for additional processing. The presence of unsaturated bonds in the molecular structure makes the polyphenols sensitive to various external environmental factors, including oxidants, light, pH variations, temperature changes, and enzymatic activities. Among these factors, water is particularly significant due to its essential role in numerous chemical reactions. Hence, it is essential to enhance the stability of polyphenols by protecting them from external conditions to ensure safe delivery (Hcini et al., 2021; Saénz et al., 2009).

Microencapsulation stands as an innovative food processing method wherein any given compound can be enclosed within a specific material, forming small microparticles. The primary purpose of microencapsulation is to protect sensitive compounds, ensuring their secure delivery (Choudhury et al., 2021). Usually, in the food industry, thermal processes are predominantly employed to produce edible and microbiologically safe foods, enhance digestibility, and modify textures, flavors, and colors. However, these processes can induce structural alterations that often result in the degradation of polyphenols (Buljeta et al., 2022). For that reason, microencapsulation in this thesis was performed at room temperature to preserve BACs stability. The microencapsulation was performed by ionic gelation method on aqueous rosemary extracts obtained by HVED with the highest TPC values (nitrogen, voltage of 25 kV, treatment duration of 9 min). Even though extraction with 50 % ethanol yielded higher TPC values compared to water, ethanol is not an adequate solvent for microencapsulation due to fast gelation with sodium alginate (Hermansson et al., 2016). The process of microencapsulation and results of analysis of obtained microparticles are given in *Paper IV*.

For this study, sodium alginate, zein, and hydroxypropyl methylcellulose (HPMC), and their combinations were chosen as encapsulation coatings. Sodium alginate is the most often utilized

biodegradable polymer in the process of the production of microparticles (Jurić et al., 2021). Its role in the process of creation of microparticles and advantages is described in section 2.5.1. During the encapsulation with $CaCl_2$ solution, it forms calcium alginate as a coating. Furthermore, zein, extracted from corn, is a prolamin protein with balanced hydrophilic and hydrophobic amino acid components, enabling the formation of microstructures. This makes it an excellent choice for edible coatings, particularly in microencapsulating functional ingredients like polyphenols and essential oils (Chen et al., 2019; Bhawani et al., 2019). Zein has consistently been acknowledged as a substance for encapsulating hydrophobic compounds, but there is a lack of research on its application for hydrophilic substances (Wong et al., 2020). HPMC is a cellulose ether derivative that possesses characteristics such as being odorless, tasteless, and non-toxic. Widely employed in oral controlled delivery systems, HPMC serves as a matrix for both hydrophilic and hydrophobic components (Deshmukh et al., 2017; Brady et al., 2017). The utilization of biodegradable polysaccharides, specifically the combination of sodium alginate with HPMC, has been proposed as an effective strategy for controlled drug delivery systems, including those with BACs (Sheng et al., 2021).

In *Paper IV*, four types of microparticles were made: 1. calcium alginate (Alg), 2. calcium alginate + zein (Alg/Z), 3. calcium alginate + HPMC (Alg/HPMC), and 4. calcium alginate + zein + HPMC (Alg/Z/HPMC). Various physicochemical analyses were performed to characterize microparticles. The obtained microparticles were consistent in size (around 2 times larger than nozzle size) and had a spherical shape with a diameter ranging from 698.79 ± 90.39 μm (Alg) to 1202.90 ± 311.42 μm (Alg/HPMC) (Table 3, *Paper IV*). However, upon drying to a constant mass, the original spherical shape was no longer evident, and surface indentations were observed (Figure 1, *Paper IV*). The results indicate that the inclusion of rosemary extract significantly increased the microparticle diameter compared to using distilled water as a loading material. Additionally, the choice of coating material (zein and HPMC) had a notable impact on microparticle diameter.

The scanning electron microscopy and atomic force microscopy analyses (Figures 2-5, and Table 4, *Paper IV*) revealed that the surface morphology of the obtained microparticles was granular, featuring individual grains with heights ranging from 6.3 to 11.3 nm. The incorporation of additional biopolymers with calcium alginate led to increased roughness and decreased porosity in the microparticles. Furthermore, the introduction of rosemary extract resulted in a rougher structure, characterized by sharper edges of grains on the microparticle surface.

The functional properties of microparticles were further analyzed and the results are summarized in Table 5 (*Paper IV*). The encapsulation efficiency was consistently high (>100 %) across all formulations, indicating an effective encapsulation process where rosemary extract was introduced into both the encapsulation solution (biopolymers) and $CaCl_2$ solutions before encapsulation. Regarding loading capacity, Alg microparticles had the lowest value, while adding co-biopolymers significantly increased the loading capacity. The microparticles with the highest loading capacity for polyphenols from rosemary extract were Alg/Z microparticles (10.42 ± 0.72 mg GAE/g). Additionally, the Fourier-transform infrared spectroscopy conducted with and without rosemary polyphenols revealed distinct molecular interactions for each formulation (Figure 7, *Paper IV*). The incorporation of alginate with other polymers influenced molecular interactions, particularly involving hydrogen bonds and electrostatic interactions. A deeper understanding of these molecular interactions among microparticle constituents enhances the potential for controlling their release behavior and the development of novel microparticles for specific applications.

Finally, the in vitro release of polyphenols from microparticles was investigated in three different media: neutral (distilled water), acidic HCl solution (gastric simulation), and alkaline buffer (intestinal simulation) conditions at 37 °C (Figure 8, *Paper IV*). In distilled water, microparticles exhibited an initial quick release (11.6–40.6 % in the first 15 min), with Alg/HPMC microparticles increasing release by 19 % compared to Alg microparticles, and Alg/Z microparticles decreasing release by 67 %. The cumulative release results in water align with the analysis of microparticle morphology using obtained microscopically. Microparticles with a rougher surface and higher grain density exhibited fewer pores and a slower release of TPC.

In simulated gastric conditions, Alg microparticles had a higher cumulative release compared to neutral conditions, releasing 27.3 % more polyphenols after 120 minutes, with the release rate following the order: Alg > Alg/HPMC > Alg/Z/HPMC > Alg/Z; the addition of both HPMC and zein decreased TPC release in HCl, resulting in Alg/Z microparticles having 1.38x lower release after 120 minutes compared to water. Simulated intestinal conditions resulted in a rapid initial release, with Alg/Z/HPMC microparticles showing the highest proportion (43.8 %) in the first 15 min, followed by a slower release. Overall, the addition of co-biopolymers (HPMC and zein) influenced polyphenol release, demonstrating the potential for tailored applications in functional foods based on desired properties.

The release of polyphenolic compounds from the microparticles varied in different media, with the fastest release observed in phosphate buffer and the slowest in acidic conditions. The incorporation of both HPMC and zein led to a reduction in polyphenol release under gastric conditions, consequently enhancing polyphenol bioavailability in the gut. Microencapsulation demonstrated a protective effect on rosemary polyphenols. The findings suggest that, with suitable biopolymer combinations, tailored phenolic release and stability can be achieved, indicating the potential of these microparticle formulations in the development of functional foods with customizable properties.

CONCLUSIONS

5. CONCLUSIONS

In this thesis, the extraction of BACs from Mediterranean herbs rosemary and oregano was performed using HVED in combination with green solvents. The following specific conclusions were drawn from the thesis.

Theoretical predictive tools, HSPs and COSMO–RS were employed to assess the solubility of BACs from rosemary and oregano in different green solvents. The results demonstrated the potential of these tools in predicting solvent suitability, aiding in the selection of green solvents for extraction processes. The theoretical predictions aligned with experimental data, particularly highlighting the higher solubility of BACs in ethanol compared to water. While theoretical predictions showed promising results for certain green solvents, experimental challenges emphasized the need for further exploration of alternative solvents, considering extraction conditions and difficulties.

The study provided valuable insights into the physical changes occurring during the extraction process, emphasizing the nonthermal nature of HVED and highlighting the influence of ethanol content on pH and conductivity. The observed correlation between conductivity and TPC in oregano extracts suggested a potential link to assess the level of electroporation that warrants further investigation in future research.

The analysis of pesticides and metals in oregano and rosemary plants, along with their extracts, demonstrated that all residue levels were below the established MRLs for each compound. This conformity with regulatory standards indicated that both plant samples are safe for human consumption, supporting their use as dietary supplements. However, the study acknowledges the potential for alterations in metal levels during HVED extraction. Additional assessments of metals in selected HVED extracts revealed an increase in Cr and Ni content, potentially resulting from electrode erosion during the process. While no MRL levels have been provided for these metals, the results indicate that the obtained extracts remain safe for human dietary use.

The research highlighted the efficacy of HVED in enhancing the extraction of bioactive compounds from rosemary and oregano, as evidenced by increased phenolic content and antioxidant activity. The results indicated that HVED extraction yielded significantly higher phenolic content and antioxidant capacity compared to conventional extraction for both rosemary and oregano. Additionally, oregano yielded higher phenolic content and antioxidant capacity compared to rosemary. For both plants, an increase in TPC content was noted with the

usage of nitrogen, longer treatment time, and higher voltage. The study also underscored the potential of NIR in combination with chemometric methods for both qualitative and quantitative prediction of phenolic content and antioxidant activity in herbal extracts.

The research provided a detailed characterization of non-volatile and volatile bioactive compounds in rosemary and oregano extracts, shedding light on the influence of extraction methods, solvents, and treatment conditions on the composition and concentrations of these compounds. The predominant bioactive compounds in rosemary were apigenin, diosmetin, and rosmarinic acid, and in oregano rosmarinic acid, luteolin, and hydroxytyrosol. The individual BACs content was higher in HVED extracts compared to conventional extraction, and significant correlations were found with TPC and antioxidant activity of extracts.

The study demonstrated that RSM is a valuable mathematical and statistical tool for modeling and optimizing the extraction process of BACs from rosemary and oregano. This approach allowed for the identification of optimal conditions, resulting in extracts with high BAC content and antioxidant activity, while minimizing the number of required experiments. The cost-effectiveness and relative stability of rosemary and oregano as plant materials containing BACs with notable antioxidant activity make them promising candidates for further experimentation with HVED and potential scale-up to industrial levels. These findings contribute to the advancement of sustainable and efficient extraction processes for bioactive compounds from Mediterranean herbs.

The findings indicated that HVED is a highly effective method for extracting BACs from rosemary and oregano when compared to both conventional and nonthermal extraction method - ASE. The superior performance of HVED, coupled with its ability to operate at ambient temperatures, positions it as a promising and sustainable technique for obtaining BACs from these plant sources, with potential applications in various industries, including food and pharmaceuticals.

The LCA of HVED provided a comprehensive understanding of the environmental, economic, and social aspects of this nonthermal extraction technology. The study aimed to address the existing gap in the literature by critically analyzing the sustainability of HVED compared to conventional extraction methods. The study aligned HVED with SDG2, SDG7, SDG12, and SDG13, suggesting that HVED offers a sustainable and promising alternative to conventional extraction methods, contributing positively to environmental, economic, and social pillars of sustainability.

Overall, HVED has been recognized as a green extraction technology with a high extraction yield for BACs from rosemary and oregano, with significant potential for industrial-scale implementation.

Microencapsulation, performed at room temperature using the ionic gelation method, effectively preserved the stability of polyphenols in aqueous rosemary extracts obtained by HVED. The choice of encapsulation coatings, including sodium alginate, zein, and HPMC, influenced the physicochemical properties of microparticles. These microparticles exhibited high encapsulation efficiency, and tailored release profiles under different conditions. The study provided valuable insights into the development of functional foods with improved bioavailability and customizable properties, showcasing the importance of microencapsulation as a tool for preserving the stability of polyphenols in aqueous extracts.

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6. REFERENCES

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AUTOBIOGRAPHY

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Marinela Nutrizio, MSc, is a research assistant with a Master's degree in Nutrition science received from the Faculty of Food Technology and Biotechnology, University of Zagreb in 2015. During the Master's study, she was awarded the University Rector's Award in 2014 for the research project "Potential of Secondary Plant Raw Materials as a Source of Functional Ingredients in Food Products." She further enhanced her expertise through an Erasmus+ internship at the Institute of Food and Nutrition (Rome, Italy), gaining international experience and knowledge about food sustainability.

Since October 2018, Marinela has been a research assistant at the project "Extraction of Bioactive Compounds from Mediterranean Herbs with 'Green Solvents' Using High-Voltage Discharge". She also collaborates on the PRIMA project "Functionalized Tomato Products (FunTomP)" and the Erasmus+ project "European Qualifications & Competences for the Vegan Food Industry – EQVEGAN," contributing to the development of subjects related to the vegan industry. Marinela Nutrizio is involved in teaching activities for the mandatory undergraduate course "Physical Properties of Food," and elective courses such as "Industry 4.0 in Biotechnical Sciences" and "Sustainability of Advanced Food Processing Techniques." She has assisted in numerous final and master's theses and three student research papers awarded the Rector's Award.

During her doctoral studies, Marinela was awarded CEEPUS grant for mobility in Novi Sad (Serbia), focusing on supercritical CO_2 extractions, and in Osnabrück (Germany), excelling in a summer school for innovative food processing techniques. Furthermore, she received Grant for the 13th European Ph.D. Workshop on Food Engineering and Technology EFFoST/EFCE. Her achievements include receiving the Giract European Flavor Research Award in 2018 and securing 2nd place at the 21st IUFoST World Congress of Food Science & Technology for Food Sustainability Idea/Concept Development in 2022. Committed to continuous learning, Marinela actively participates in congresses, workshops, and seminars, where she presents her research results. Until now, she has co-authored 15 scientific papers indexed in Web of Science/Current Contents Connect and three book sections (519 citations; h-index 9).

List of author's publications:

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- Dukić, J., Košpić, K., Kelava, V., Mavrić, R., **Nutrizio, M.,** Balen, B., Butorac, A., Halil Öztop, M., Režek Jambrak, A. (2023) Alternative methods for RuBisCO extraction from sugar beet waste: A comparative approach of ultrasound and high voltage electrical discharge. *Ultrasonics Sonochemistry*, **99**, 106535.
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