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Napredne tehnike ekstrakcije i inkapsulacija fenolnih spojeva iz lista lovora (*Laurus nobilis* L.)

DOKTORSKI RAD

Mentor:

izv. prof. dr. sc. Ivona Elez Garofulić

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Short abstract:

Laurel leaf (*Laurus nobilis* L.) is a rich source of phenols, which have numerous beneficial effects on health. To maximize the yield of phenols and increase their stability and bioavailability for use in functional products, three advanced extraction techniques and two encapsulation methods were tested and optimized. Pressurized liquid extraction proved to be the most desirable method for achieving maximum yield and antioxidant activity of extracts with low energy, time, and solvent consumption. In comparison to electrostatic extrusion, spray drying resulted in higher retention of phenols and antioxidant activity. Both encapsulation methods increased the bioaccessibility of the phenols.

Keywords: *Laurus nobilis L., polyphenols, extraction, encapsulation, bioavailability, antioxidant activity*
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Napredne tehnike ekstrakcije i inkapsulacija fenolnih spojeva iz lista lovora (*Laurus nobilis* L.)

Erika Dobroslavić

Sažetak: Lovorov list (*Laurus nobilis* L.) bogat je izvor fenolnih spojeva (fenola) koji pokazuju brojne pozitivne učinke na zdravlje. Kako bi se omogućili maksimalni prinosi fenola i povećanje njihove stabilnosti i biodostupnosti za primjenu u funkcionalnim proizvodima, ispitane su i optimirane tri napredne metode ekstrakcije te dvije metode inkapsulacije. Ubrzana ekstrakcija otapalima pri povišenom tlaku pokazala se najpoželjnijom za postizanje maksimalnog prinosa i antioksidacijske aktivnosti uz nižu potrošnju energije, vremena i otapala. U usporedbi s elektrostatskom ekstruzijom, sušenje raspršivanjem rezultiralo je većim zadržavanjem fenola i antioksidacijske aktivnosti. Obje metode inkapsulacije povećale su biodostupnost fenola.

Ključne riječi: *Laurus nobilis L., polifenoli, ekstrakcija, inkapsulacija, bioraspoloživost, antioksidacijska aktivnost*
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Extended abstract

Laurel leaf (*Laurus nobilis* L.) is a rich source of phenolic compounds (phenols) that show numerous positive biological effects. In order to enable the maximum use of the mentioned effects and the application of the obtained extracts in the industry, it is necessary to define the optimal parameters of phenol extraction, whereby conventional techniques that consume large amounts of energy, time and solvents are being replaced by advanced green techniques. Due to the tendency of phenols to degrade during handling, storage and consumption in the human gastrointestinal system, it is crucial to convert them into a more stable form by various encapsulation methods. The aim of this work was to optimize three advanced extraction techniques (microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE) and pressurized liquid extraction (PLE)) for achievement of maximum phenol yield and to compare them with the conventional reflux technique (CRE). As a second step, the aim was to optimize the encapsulation of bay leaf phenols using spray drying (SD) and electrostatic extrusion (EE) with the aim of achieving maximum retention of phenols and antioxidant activity with desirable physicochemical characteristics, and to examine their effect on the bioaccessibility of laurel leaf phenols. The optimal parameters of advanced extraction techniques were defined, and it was shown that under optimal conditions, MAE (50% EtOH, 80 °C, 10 min, 400W) and UAE (70% EtOH, 10 min, 50% amplitude) resulted in lower phenol yields and antioxidant activity than CRE (50% EtOH, 30 min), while PLE (50% EtOH, 150 °C, 1 extraction cycle, static time 5 min) resulted in the same yield and antioxidant activity in a significantly shorter time than CRE, which made this technique the most successful. The antioxidant capacity of the extracts was in correlation with the content of total phenols, especially flavan-3-ols and flavonols. The optimal encapsulation parameters of SD (β-CD + MD 50:50, sample:carrier ratio 1:2, 180 °C) and EE (1% alginate, 1.5% $CaCl_2$ + 0.5% chitosan) were defined. SD resulted in higher retention of phenols and antioxidant activity. Physicochemical properties of microcapsules depended on the applied carriers, and combinations of biopolymers in both techniques resulted in more desirable properties and higher encapsulation efficiency. Both techniques resulted in an increase of phenols' bioaccessibility compared to the initial extract. EE resulted in better preservation of phenols during the gastric phase of digestion and higher absorption, while SR resulted in greater preservation of phenols available for the gut microbiota in the colon. The above results represent a contribution to the knowledge of the extraction and encapsulation of phenols from

laurel leaves and as such form the basis for future research and their application in the segment of functional food and nutraceuticals.

Proširenisažetak

Lovorov (*Laurus nobilis* L.) list bogat je izvor fenolnih spojeva (fenola) koji pokazuju brojne pozitivne biološke učinke. Kako bi se omogućilo maksimalno iskorištenje navedenih učinaka te primjena dobivenih ekstrakata u industriji, nužno je definirati optimalne parametre ekstrakcije fenola pri čemu se neučinkovite konvencionalne tehnike koje troše velike količine vremena i otapala zamjenjuju naprednim zelenim tehnikama. Zbog sklonosti fenola degradaciji u različitim uvjetima skladištenja te u ljudskom gastrointestinalnom sustavu, nakon ekstrakcije ih je ključno prevesti u stabilniji oblik primjenom različitih metoda inkapsulacije. Cilj ovoga rada bio je optimirati tri napredne tehnike ekstrakcije (ekstrakcija potpmognuta mikrovalovima (MAE), ekstrakcija potpomognuta ultrazvukom (UAE), ubrzana ekstrakcija otapalima pri povišenom tlaku (PLE)) s ciljem postizanja maksimalnih prinosa fenola i usporediti ih s konvencionalnom tehnikom refluksa (CRE). Kao drugi korak, cilj je bio optimirati inkapsulaciju fenola lista lovora primjenom sušenja raspršivanjem (SR) i elektrostatske ekstruzije (EE) s ciljem postizanja maksimalnog zadržavanja fenola i antioksidacijske aktivnosti uz poželjne fizikalno-kemijske karakteristike te ispitati njihov učinak na biodostupnost fenola lovora. Definirani su optimalni parametri naprednih tehnika ekstrakcije, te se pokazalo kako su pri optimalnim uvjetima MAE (50% EtOH, 80 °C, 10 min , 400W) i UAE (70% EtOH, 10 min, 50% amplituda) rezultirale nižim prinosima fenola i antioksidacijskom aktivnosti od CRE (50% EtOH, 30 min), dok je PLE (50% EtOH, 150 °C, 1 ciklus ekstrakcije, statičko vrijeme 5 min) rezultirao jednakim prinosom i antioksidacijskom aktivnosti u značajno kraćem vremenu od CRE, čime se ova tehnika pokazala najuspješnijom. Antioksidacijski kapacitet ekstrakata bio je u korelaciji sa sadržajem ukupnih fenola, osobito flavan-3-ola i flavonola. Definirani su optimalni parametri inkapsulacije primjenom SR (β-CD + MD 50:50, omjer uzorak:nosač 1:2, 180 °C) i EE (1% alginata, 1.5% $CaCl_2$ + 0.5% kitozan). SR je rezultiralo većim zadržavanjem fenola i antioksidacijske aktivnosti. Fizikalno-kemijska svojstva mikrokapsula ovisila su o primijenjenim nosačima te su kombinacije biopolimera primjenom obaju tehnika rezultirale poželjnijim svojstvima te većom učinkovitosti inkapsulacije. Obje tehnike rezultirale su povećanjem biodostupnosti fenola u odnosu na početni ekstrakt. EE je rezultirala boljim očuvanjem fenola tijekom želučane faze probave te višom apsorpcijom, dok je SR rezultiralo većim očuvanjem spojeva dostupnih za djelovanje mikrobiote u debelom crijevu. Navedeni rezultati predstavljaju doprinos znanju o ekstrakciji i inkapsulaciji fenola iz lista lovora te kao takvi tvore temelj za buduće istraživanje i primjenu u segmentu funkcionalne hrane i nutraceutika.

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Information about the supervisor

Associate Professor Ivona Elez Garofulić, PhD was born on June 27, 1985 in Šibenik where she completed primary and secondary education. She graduated from the Faculty of Food Technology and Biotechnology, University of Zagreb in 2009. In the same year, she was employed as a research assistant in the Laboratory for the processes of preservation and processing of fruits and vegetables at the Faculty of Food Technology and Biotechnology in Zagreb. In July 2015 she defended her doctoral thesis entitled " Optimization of spray drying process of sour cherry Marasca (*Prunus cerasus* var. Marasca) juice " which was a part of the international scientific project "Sour cherry Marasca as an ingredient for functional food" financed by the European Union. During her doctoral studies, she trained for 3 months at the Faculty of Biotechnical Sciences of the University of Ljubljana, Slovenia, in the field of application of chromatographic techniques for the characterization of bioactive compounds. She was elected associate professor on December 14, 2022. She is an associate in several courses at undergraduate and graduate studies: Chemistry and technology of fruits and vegetables, Spices and Aromatic Plants, Biologically active components in food and mechanisms of action, Food technology of plant origin foods, Refreshing soft drinks, Instrumental analysis and Modern methods in food quality control. Also, as an external associate, she participates in teaching Food technology at the University North within the course Technology of Fruit and Vegetable Products and Production and Processing of Medicinal and Aromatic Plants. During her employment, she was also a member of the Committee on International Cooperation and the Committee on Publishing. She was also a member of the organizing committee of the 9th International Congress of Food Technologists, Biotechnologists and Nutritionists in 2018. As an associate, she participated in the realization of several national and international projects, financed by the Ministry of Science, Education and Sports (1 project), HRZZ (3 projects), and EU funds (3 projects). She also participated in the implementation of the BICRO professional technology project, and in several projects related to the transfer of knowledge and technology in the industry. She is also an associate on the EU-funded project Centre of Excellence for Marine Bioprospecting. She is the member of Food and Soft Material Section Editorial board listed in the journals Frontiers in Food Science and Technology and Frontiers in Soft Matter, Processes Topical Advisory Board and has served as a guest editor in special editions of Processes and Applied Sciences journals. Her scientific research is related to isolation, stabilization and application of bioactive compounds from fruits, vegetables, spices and aromatic herbs. It is particularly focused on the application of

new extraction techniques and various encapsulation methods for the preservation of biologically active components. During her work so far, she has published 50 scientific papers (41 indexed A1, 5 indexed A2) and 4 papers in conference proceedings (A3). Published scientific publications have been cited 980 times according to WoS (h-index 16). She has participated in more than 60 national and international scientific conferences. Under her mentorship, 1 doctoral dissertation, 9 graduate and 8 final theses were made. She is the winner of the AgroArca Award for the successfully implemented project "Marasca Cherry as an Ingredient of Functional Food" in 2015.

Author's publications included in the doctoral dissertation:

Publication No.1:

Dobroslavić, E., Repajić, M., Dragović-Uzelac, V., & Elez Garofulić, I. (2022). Isolation of *Laurus nobilis* Leaf Polyphenols: A Review on Current Techniques and Future Perspectives. *Foods*, *11*(2), 235. https://doi.org/10.3390/foods11020235

Publication No.2:

Dobroslavić, E., Elez Garofulić, I., Zorić, Z., Pedisić, S., & Dragović-Uzelac, V. (2021). Polyphenolic Characterization and Antioxidant Capacity of *Laurus nobilis* L. Leaf Extracts Obtained by Green and Conventional Extraction Techniques. *Processes, 9*(10), 1840. https://doi.org/10.3390/pr9101840

Publication No.3:

Dobroslavić, E., Elez Garofulić, I., Šeparović, J., Zorić, Z., Pedisić, S., & Dragović-Uzelac, V. (2022). Pressurized Liquid Extraction as a Novel Technique for the Isolation of *Laurus nobilis* L. Leaf Polyphenols. *Molecules, 27*(16), 5099. https://doi.org/10.3390/molecules27165099

Publication No.4:

Dobroslavić, E., Elez Garofulić, I., Zorić, Z., Pedisić, S., Roje, M., & Dragović-Uzelac, V. (2023). Physicochemical Properties, Antioxidant Capacity, and Bioavailability of *Laurus nobilis* L. Leaf Polyphenolic Extracts Microencapsulated by Spray Drying. *Foods*, 12(9), 1923. https://doi.org/10.3390/foods12091923

Publication No.5:

Dobroslavić, E., Zorić, Z., Dragović-Uzelac, V., & Elez Garofulić, I. (2023). Microencapsulation of Laurus nobilis L. Leaf Extract in Alginate-Based System via Electrostatic Extrusion. Foods, 12(17), 3242. https://doi.org/10.3390/foods12173242

CONTENTS

GENERAL INTRODUCTION

Recently, the demand for alternative approaches in the food, pharmaceutical and cosmetic industries has been steadily increasing. In response to the growing consumer concern for health, the food and pharmaceutical industries are recording significant growth in the segment of functional products and nutraceuticals based on natural plant sources. Various species of medicinal and aromatic plants possess a wide range of bioactive molecules that can be used as natural antioxidants and preservatives (Ameer et al., 2017). Among the many plant species, a plant that has stood out in folk medicine for centuries is laurel (*Laurus nobilis* L.), a Mediterranean shrub whose leaves are known to contain bioactive molecules with a wide range of biological effects, such as antioxidant, antimicrobial, antifungal and anti-inflammatory (Alejo-Armijo et al., 2017). Many of these activities can be attributed to different groups of phenols, including flavonoids, phenolic acids, tannins, and lignans (Konovalov & Alieva, 2019). The chemical structure of these compounds varies from simple to complex, often in combination with other components such as proteins and carbohydrates, making their isolation a challenging process (Routray & Orsat, 2013).

In order to achieve successful utilization of the phenols' beneficial properties, it is crucial to establish an optimal extraction and isolation methodology. Conventional extraction techniques, such as reflux, are easily applicable, but also have certain disadvantages, such as a long extraction time with large consumption of organic solvents and energy. Also, due to prolonged exposure to higher temperatures, thermal decomposition of thermosensitive phenolic compounds may occur (Antony & Farid, 2022). Therefore, many authors focus their research on advanced green extraction techniques of phenols from various plant materials which use different mechanisms (e.g. microwaves, ultrasound or elevated pressure) to disrupt the structure of plant cell walls and enable the release of phenols through a more solvent-, energy- and time-efficient process (Ameer et al., 2017).

Since phenols are unstable and prone to losing their active properties during storage, it is of great importance to improve their stability. This can be achieved by various encapsulation techniques, such as spray drying and electrostatic extrusion. Spray drying is the most commonly used method for encapsulation of bioactive molecules in which the liquid extract with the dissolved carrier is passed through a stream of hot air during which the solvent evaporates and a powder with bioactive molecules encapsulated in the protective coating of the carrier is formed (Shishir et al., 2018). Another method suitable for the encapsulation of phenolic compounds is electrostatic extrusion in which the mixture of carrier (usually sodium alginate) and extract is passed through a nozzle by electrostatic force and uniform gel beads

with encapsulated bioactive compounds are formed in contact with the gelling solution containing polyvalent ions (Bamidele & Emmambux, 2021). Since the properties of the encapsulated systems produced by the aforementioned techniques largely depend on the applied parameters, it is of great importance to carry out research focused on optimization with the aim to attain high encapsulation efficiency and stability of the encapsulated systems with the required release characteristics, biocompatibility, bioaccessibility and bioavailability of active compounds (Aguiar et al., 2016). The research on bioaccessibility for use in functional foods and dietary supplements is extremely important since the abundance of phenols does not necessarily imply the best bioaccessibility and bioavailability (D'Archivio et al., 2010). *In-vitro* bioaccessibility assessment methods cannot reproduce the complex environment of the human digestive system that *in-vivo* methods can, however, are relatively fast, simple, inexpensive, and repeatable, thus allowing a more efficient product formulation (Dima et al., 2020).

The aim of this research was to determine the optimal conditions under which advanced extraction techniques, namely pressurized liquid extraction (solvent, number of extraction cycles, static time and temperature), microwave-assisted extraction (solvent, temperature, irradiation power and time) and ultrasound-assisted extraction (solvent, time, amplitude) lead to laurel leaf extracts with the highest phenolic yields, to compare them with conventional heat reflux extraction and to determine their individual phenolic composition, antioxidant activity and bioaccessibility. The second phase of research was focused on optimizing the encapsulation by spray drying (temperature, type of carrier, sample:carrier ratio) and electrostatic extrusion (percentage of alginate, type of gelling solution) with the objective of achieving optimal physicochemical characteristics and retention of phenols in obtained microcapsules, as well as to determine their antioxidant activity and bioaccessibility during *in-vitro* digestion.

Chapter 1

Theoretical background

- *L. nobilis* L. – general information
- *Publication No.1*
- Encapsulation techniques
- Bioavailability of polyphenols
- Biological activity of *L. nobilis* L. leaf polyphenols' metabolites
- Hypotheses, objectives and expected scientific contribution

1. LAURUS NOBILIS L.

Laurel (*Laurus nobilis* L.) (Figure 1.), also known as bay leaf, daphne or sweet bay is an aromatic and medicinal plant which belongs to the large Lauraceae family that comprises around 3500 species (Batool et al., 2020).

Figure 1. *Laurus nobilis* L. (own photo)

This slow-growing evergreen perennial shrub is native to the Mediterranean region (Figure 2), but can also be found in other areas with mild climate characterized by average annual temperatures between 17–25 °C and a mean annual rainfall of 600–1000 mm (tolerates the range 300–2200 mm since overabundance of water leads to root rotting) (Paparella et al., 2022). The shrub can survive at temperatures around -5 °C, however young growth may become severely damaged in such conditions. Optimal growth conditions include sunny and well-drained, moisture-retentive soils with moderate fertility and pH 5–6.5 without extreme maritime exposure and cold dry winds (Paparella et al., 2022).

Laurus nobilis, Laurus azorica and *Laurus novocanariensis* are three traditionally recognized species of the Laurus genus (Khodja et al., 2023), while many plants outside the genus Laurus such as *Pimenta racemosa* (Myrtaceae) (Batool et al., 2020), *Cinnamomum tamala* (Laureaceae, genus Cinnamomum) (V. Sharma & Rao, 2014) or *Litsea glaucescens* (Lauraceae, genus Litsea) (López-Caamal & Reyes-Chilpa, 2021) share the common name "bay leaf", but are botanically different plants with different phytochemical composition.

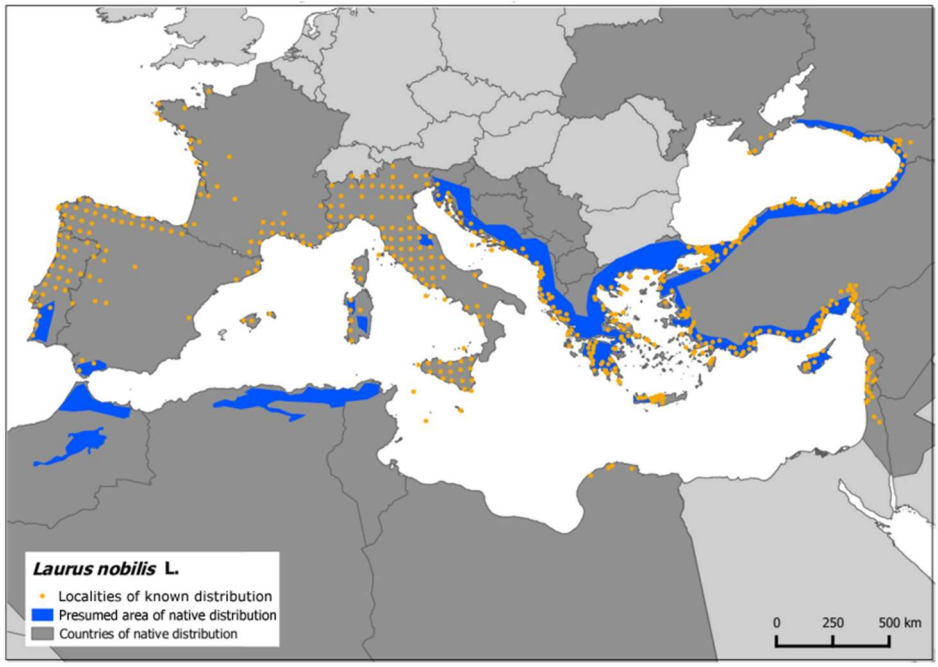

Figure 2. Native distribution of *Laurus nobilis* L. (FAO, 2015)

Botanically, laurel is an evergreen shrub which can grow up to 20 m in natural environment, while in cultivation it is usually pruned to 2–4 m. The species has several trunks, and the bark of the stem and branches is dark brown to almost black (Khodja et al., 2023). The foliage of laurel is characterized by a dark green color on the upper surface and a lighter green on the lower one. The leaves are elongated, lanceolate or lanceolate-acuminate with pointed tips and short petiole with a width of 3-5 cm and a length around 10 cm (Paparella et al., 2022). Laurel is a dioecious plant, with star-shaped male and female flowers on different plants. Flowering occurs in the spring period from March to May, and the inflorescence consists of small umbels with four or five axillary flowers of creamy-white to greenish-white color. The fruit is a 10-15 mm long, ovoid shaped, fleshy aromatic drupe bright green at first and purplish black when ripe in autumn. It consists of pericarp, mesocarp, and endocarp and contains a single seed, formed by two cotyledons rich in fat. The berries usually remain on the plant all winter and may last until next flowering season, where they coexist with the new flowering (Batool et al., 2020; Paparella et al., 2022).

Laurel leaves have a sharp, bitter taste due to the presence of essential oils with over 150 constituents in the leaves. Fresh leaves have a water content of about 50%, whereas dry leaves contain 5-10% water, 65% carbohydrates, 8-11% protein, 5-9% fat, and 4% ash. Four sugars (glucose, fructose, sucrose, trehalose), three organic acids (malic, oxalic, and ascorbic) and three polysaccharides (alginate, fucoidan and laminarin) have been detected in laurel leaves

(Khodja et al., 2023) . They also contain several fatty acids, palmitic acid being the most important, followed by linoleic acid (Alejo-Armijo et al., 2017). Four tocopherols have been detected in bay leaves, among which α- and γ-tocopherols are the most abundant (Chahal et al., 2017). The nutrient composition of *L. nobilis* leaves is shown in Table 1.

Table 1. Nutrient composition of *L. nobilis* leaf (Ambrose et al., 2016)

Constituent	Value per 100 g dw[a]
Water (g)	5.44
Energy (kcal)	313
Protein (g)	7.61
Carbohydrates (g)	74.96
Ash (g)	3.62
Fat (g)	8.36
Saturated fatty acids (g)	2.28
MUFA[b]	1.64
PUFA[b]	2.29
Calcium (mg)	834
Iron (mg)	43
Magnesium (mg)	120
Phosphate (mg)	113
Potassium (mg)	529
Sodium (mg)	23
Zinc (mg)	3.70
Folate (µg)	180
Niacin (mg)	2.005
Riboflavin (mg)	0.421
Thiamine (mg)	0.009
Vitamin A (IU)	6185
Vitamin A (µg)	309
Vitamin C (mg)	46.5
Vitamin E (mg)	139

[a]leaf dry weight; [b]monounsaturated fatty acids; [c]polyunsaturated fatty acids

Laurel leaves have been historically used in the folk medicine of the Mediterranean area for alleviating various health problems including gastrointestinal issues such as bloating and flatulence due to their capacity to stimulate the secretion of gastric fluids (Awada et al., 2023). The plant's leaves have also been known to possess antioxidant, anticonvulsant, neuroprotective, antiepileptic, anti-hemorrhoidal, hepatoprotective, antihyperlipidemic, antiproliferative, antirheumatic, anti-inflammatory, antidiabetic, antifungal, antibacterial and dermoprotective properties widely described in literature reviews (Awada et al., 2023; Batool et al., 2020; Chahal et al., 2017; Khodja et al., 2023; Paparella et al., 2022). These properties are today attributed to the rich content of bioactive compounds which include phenolic compounds (phenols), monocyclic and aliphatic monoterpenes, sesquiterpenes, sesquiterpene

lactones, alkaloids and norisoprenoids (Batool et al., 2020; Khodja et al., 2023). Among these, phenols represent the most numerous group of compounds in the laurel leaves which are known to exhibit many biological activities and were therefore selected as a focus for further research. The phenols in laurel leaves, as well as the importance of extraction procedures and the overview of current state of knowledge are given in the *Publication No.1*.

2. REVIEW OF THE PHENOLIC CONTENT OF LAUREL LEAF AND THE CURRENT KNOWLEDGE OF EXTRACTION PROCEDURES

PUBLICATION NO.1

Dobroslavić, E., Repajić, M., Dragović-Uzelac, V., & Elez Garofulić, I. (2022). Isolation of *Laurus nobilis* Leaf Polyphenols: A Review on Current Techniques and Future Perspectives. *Foods*, *11*(2), 235.

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foods

MDPI

Review

Isolation of *Laurus nobilis* Leaf Polyphenols: A Review on Current Techniques and Future Perspectives

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Abstract: In recent years, the market demand for products enhanced with ingredients derived from natural products, such as polyphenols, is rapidly increasing. *Laurus nobilis* L., known as bay, sweet bay, bay laurel, Roman laurel or daphne is an evergreen Mediterranean shrub whose leaves have traditionally been used in cuisines and folk medicine due to their beneficial health effects, which can nowadays be scientifically explained by various biological activities of the leaf extracts. Many of these activities can be attributed to phenolic compounds present in *L. nobilis* leaves which include flavonoids, phenolic acids, tannins (proanthocyanidins) and lignans. In order to enable efficient industrial utilization of these valuable compounds, it is crucial to establish optimal extraction procedures resulting in the highest yields and quality of the extracts. This paper offers the first systematic review of current literature on the influence of conventional and advanced extraction techniques, including microwave-assisted, ultrasound-assisted, enzyme-assisted, supercritical-CO_2 and mechanochemical-assisted extraction on the phenolic content of *L. nobilis* leaf extracts, allowing more efficient planning of further research and simplifying the steps towards industrial utilization of this plant.

Keywords: *Laurus nobilis* L.; green extraction; conventional extraction; plant extracts; polyphenols

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1. Introduction

Laurus nobilis L., known as bay, sweet bay, bay laurel, Roman laurel or daphne is an evergreen shrub (2–20 m of height) of the Lauraceae family which includes 2500–3500 plant species that grow in the subtropics and tropics of East Asia, and South and North America [1]. The natural habitats of this plant are located in the Mediterranean area characterized by high annual precipitation [2]. Therefore, *L. nobilis* leaves have traditionally been used in Mediterranean cuisine [3] for seasoning, as well as in folk medicine along with *L. nobilis* fruits for treating viral infections, cough, rheumatism, impaired digestion, diarrhea and other health conditions [4]. Numerous scientific studies highlight the antimicrobial [5,6], antifungal [7,8], anticonvulsant [9], antioxidant [10–12], anti-inflammatory [13,14], antidiabetic [15–17], anticancer [12,18], neuroprotective [19] and anticholinergic [20] activities of *L. nobilis* leaf extracts and essential oils. These properties offer various application possibilities of *L. nobilis* extracts in the food, pharmaceutical and cosmetic industries. Due to the traditional use and commercial value of *L. nobilis* leaves, their chemical composition has been studied to a larger extent than other parts of this plant. Some of the constituents found in *L. nobilis* leaves are polyphenolic compounds, alkaloids, norisoprenoids, sugars, polysaccharides, organic acids and tocopherols [1]. The leaves also contain volatile oils which accumulate in the palisade and mesophyll cells and are present in a percentage of 1–3% on a fresh weight basis [21]. The main constituent out of around 150 identified by GC-MS in the essential oil is usually 1,8-cineol with a content ranging up to 50%, or even 70% [1,22,23]. The leaves also contain a small portion of fixed oils with 25 identified fatty acids with levels of polyunsaturated (PUFA) fatty acids higher than saturated fatty

acids (SFA) and the levels of omega-3 fatty acids higher than omega-6 fatty acids, which is considered desirable for the human diet [12]. Sesquiterpene lactones also represent a characteristic group of phytochemicals present in *L. nobilis* leaves. These compounds have been reported to inhibit nitric oxide (NO) production [14] and ethanol absorption [24], as well as to increase the activity of hepatic glutathione S-transferase [25]. This group of phytochemicals is also considered as a possible cause of allergic contact dermatitis that may occur in contact with laurel leaves [26]. One of the most significant groups of bioactive compounds in *L. nobilis* leaves are polyphenolic compounds that will be more thoroughly discussed later. The total content of phenolic compounds (TPC) in laurel leaves has been reported to range from 53 to 9200 mg of gallic acid equivalent (GAE) 100 g^{-1} of extract, depending on the extraction method used [1]. Considering that the extraction of bioactive compounds from plant material is the first and crucial step in their industrial utilization, and the connection between biological activities and phenolic content of plant extracts is well-explored [27], it is of great importance to summarize the knowledge on the effects of different extraction techniques and the applied parameters on the TPC of the extracts in order to allow more directed research planning. Since, to our knowledge, no review discussing the aforementioned effects for the *L. nobilis* L. leaf polyphenols has been published, the aim of this paper was to summarize the current knowledge on the influence of different extraction techniques on the polyphenolic content of *L. nobilis* leaf extracts through a detailed search of the available literature and to propose future research possibilities.

2. Phenolic Compounds in *L. nobilis* Leaves

L. nobilis leaves are a source of numerous different phenolic compounds that include flavonoids, phenolic acids, tannins (proanthocyanidins) and lignans [1]. Figure 1 shows an average composition of phenolic compounds that have been detected in *L. nobilis* leaves to date [1,22,28].

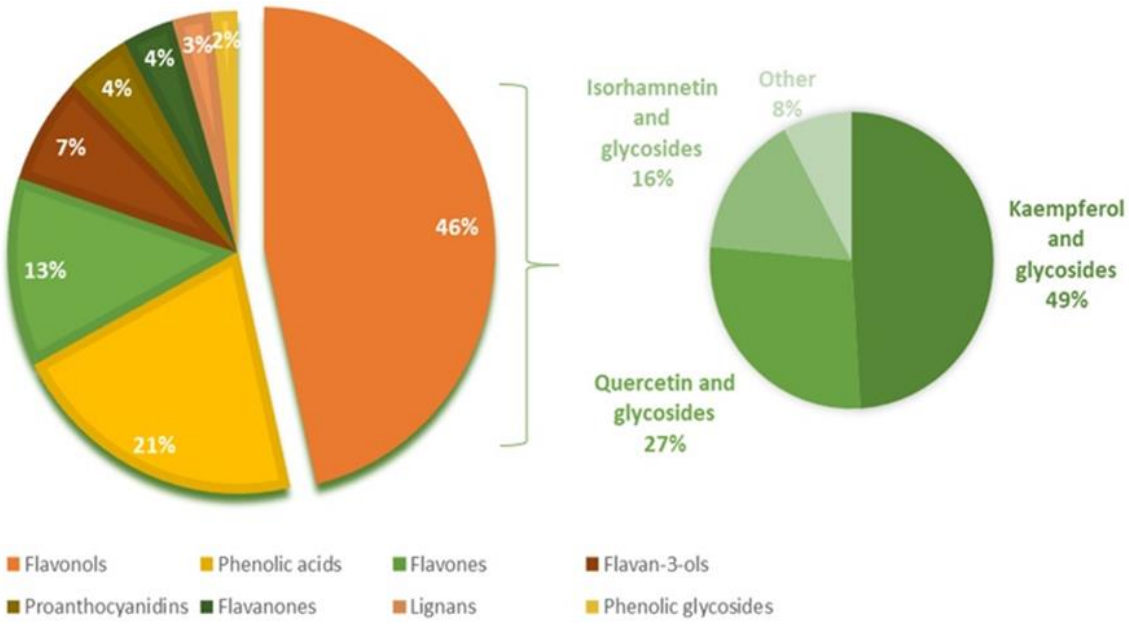
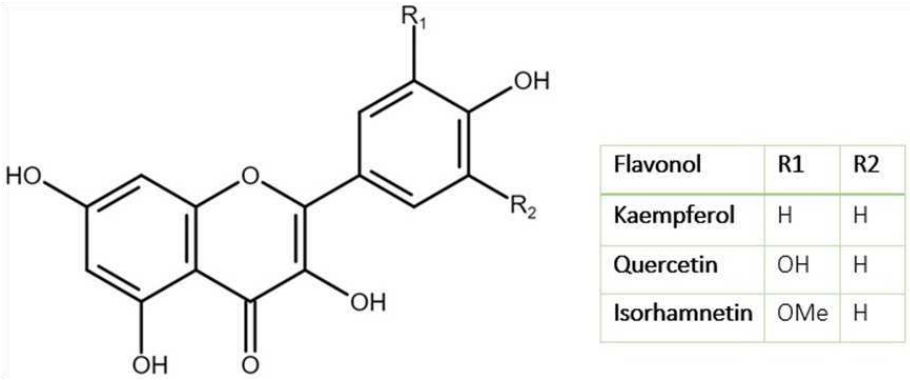


Figure 1. Phenolic compounds found in *L. nobilis* leaves (according to Alejo-Armijo et al. [1]; Diaz-Maroto et al. [22] and Zhilyakova et al. [28]).

As can be seen, flavonoids present the main constituents of alcoholic leaf extracts with a variety of detected compounds (Figure 1). Flavonols are present in the highest amount, with kaempferol and its glycosides being the main representatives (almost 50%), followed by quercetin and isorhamnetin and their glycosides, which are also present in significant amounts. The basic structure of the main *L. nobilis* flavonols is shown in Figure 2.



Flavonol	R1	R2
Kaempferol	H	H
Quercetin	OH	H
Isorhamnetin	OMe	H

Figure 2. Chemical structure of the main flavonols found in *L. nobilis* L. leaves (adapted from Li et al. [29]).

Kaempferol glycosides from *L. nobilis* have shown a variety of biological activities, such as an inhibition of NO production in lipopolysaccharide (LPS)-activated murine macrophages (J774) [30], inhibition of sodium-potassium adenosine triphosphatase [31], antioxidant activity [32], antibacterial activity against *Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, Salmonella typhimurium* and *Proteus vulgaris* [31], as well as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci [33]. Followed by these findings, kaempferol glycosides from *L. nobilis* leaves are especially interesting for further research focused on extraction methods that would result in their highest yield and quality. Phenolic acids from *L. nobilis* leaves have also shown antioxidant activity [34,35], and more than 20 of them have been detected [1]. Levels of *p*-coumaric and ferulic acid detected in hydroalcoholic laurel leaf extracts seem to be higher than in other herbs with similar biological potential [27]. Most of the flavones present in *L. nobilis* leaves are apigenin and its glycosides [1,23]. In a study by Al-Samarrai et al. [36] who investigated the effect of flavonoids and glycosides isolated from *L. nobilis* leaves on the lipid profile of female rabbits, apigenin-7-glucoside and luteolin-7-*O*-glucoside reduced the levels of total cholesterol and triglycerides. Tannins (proanthocyanidins) of the *L. nobilis* plant are mostly present in wooden parts [1]; however, a few, mostly lacking in structure elucidation, have also been detected in the leaves [12,37]. Cinnamtannin B-1 detected in the leaves was reported to show antioxidant activity [32].

3. Extraction of Phenolic Compounds from *L. nobilis* Leaves

3.1. Preextraction Sample Preparation

The first step in any plant extraction process is the preparation of plant samples and protection of the target compounds from deterioration. Phenolic compounds can be extracted from fresh, dried or frozen plant material. Flavonoids, particularly glycosides, which are abundant in *L. nobilis* leaves, can be degraded by intact enzymes when the plant material is fresh and undried [38]. It has been reported that the time between harvest and experimental work should be limited to 3 h in order to maintain the freshness of samples [39]. For this reason, dried and frozen plant material is usually preferred for the extraction of bioactive compounds. Plant material can be dried using several methods that include air-, oven-, microwave-, and freeze-drying (lyophilization). Air-drying at ambient temperature for a period ranging from 36 h [40] up to a few months or even a year [41], depending on the plant material, is the most preferred method since no special equipment is needed, followed by lyophilization, which is often chosen despite its complexity due to the fact that it often results in higher TPC of the final extracts [42,43]. In contrary, Papageorgiou et al. [35] have reported higher TPC and total flavonoid content (TFC) in air-dried as opposed to freeze-dried *L. nobilis* leaf extracts. Microwave- and oven-drying can cause degradation of thermolabile compounds depending on the used parameters, which could influence the final extract quality and composition. However, oven-drying at 60 °C for 48 h resulted in similar TPC as air-drying prior to heat-reflux extraction of *L. nobilis* leaves

performed using the same extraction parameters [11,44]. Generally, air-drying has been the most frequently used drying method of *L. nobilis* leaves for phenolic compounds extraction, regardless of the implemented extraction method (See Sections 3.2.1 and 3.2.2).

Drying is usually followed by milling, grinding and homogenization of the plant samples which are carried out in order to lower particle size and to increase surface contact between the sample and extraction solvent [42]. A particle size less than 500 µm is considered as the most suitable for efficient extraction [45]. Scientific data show that *L. nobilis* leaf samples were mostly ground into fine powder prior to extractions of phenolic compounds, and the size of the particles, if reported, ranged between 250–800 µm [12,45–47]. The presence of non-phenolic substances, such as lipids and proteins in plant material, can affect the composition and activities of phenolic compounds in the final extracts [48], thus, different purification and fractionation procedures can be applied on the crude extracts when the research is focused on composition analysis and quantification of the constituents [49]. *L. nobilis* leaves contain only 1–1.2 g of proteins and fat in traces [50], so these procedures are most often left out. Simić et al. [51] carried out a defatting process of *L. nobilis* leaves using petroleum ether and observed that defatted methanolic extracts showed a higher inhibition of lipid peroxidase. However, the research contained no data on the phenolic content, therefore the result could have been influenced by different factors.

3.2. Extraction Techniques

Extraction is the crucial step in isolation, analysis and utilization of phenolic compounds. Unsuitable extraction conditions may result in a lower yield of phenolic compounds or cause structural changes that would lead to undesirable effects on their biological activity [49]. The choice of the extraction procedure depends on various factors including the goal of conducted research, and nature of the plant material and target compounds. Currently, extraction processes of phenolic compounds can be divided into two groups: conventional and advanced extraction techniques [52]. Subsequent sections give a review on both groups of extraction techniques and discuss their efficacy in obtaining high TPC and TFC from *L. nobilis* leaves.

3.2.1. Conventional Techniques

Conventional extraction techniques, such as infusion, decoction, digestion, maceration, and percolation, as well as Soxhlet and reflux, include the use of solvent. They are, due to their wide applicability and no special equipment requirements, the most commonly used procedures for obtaining extracts from plant material. Plant material usually contains various phenolic compounds in different quantities, ranging from simple to highly polymerized substances that may also be conjoined with other components, such as proteins and carbohydrates [53]. Therefore, an individual and systematic approach is needed to select suitable extraction parameters for every plant sample. The yield of chemical extraction depends on several parameters, including the type of solvent, solid–liquid ratio, the number of repeated extractions, stirring, extraction time, and temperature, as well as the chemical composition and physical characteristics of the plant material [54]. Parameters of conventional phenolic compounds extraction from *L. nobilis* leaves available in the literature are shown in Table 1.

Table 1. Parameters used in conventional extraction techniques of phenolic compounds from *L. nobilis* leaves.

Drying Method	Extraction Parameters	Solid–liquid Ratio (g mL^{-1})	TPC [a]	TFC [b]	Ref.	Publication Year
			Maceration			
Electric dryer at 30 °C	99.5% acetone; 72 h; 30 °C	2:1	71.2 ± 2.5 mg GAE [c] g^{-1} extract	39.2 ± 7.4 mg CAE [d] g^{-1} extract	[55]	2020
Oven dried at 60 °C for a week	distilled water; 45′; 80 °C	1:10	137.0 mg PE [e] g^{-1} sample	604.12 mg CAE g^{-1} sample	[56]	2020
Air-drying	80% ethanol; 5 days; room temperature	1:5	110.43 mg GAE g^{-1} extract	-	[57]	2019
Air-drying	absolute methanol; 30′; room temperature	1:10	-	149.2 ± 8.3 mg ECE [f] g^{-1} extract	[58]	2018
Air-drying	hexane/ethyl acetate/ethanol/water 5× in 24 h; room temp.	-	11.04–54.42 mg PE [f] g^{-1} sample	1.01–8.60 mg QE [g] g^{-1} extract	[59]	2017
Unspecified	80% ethanol; 48 h; room temperature; Successive 24 h; evaporation at 40 °C Defatting: petroleum ether 2× Lyophilization Ethyl acetate; 20% ammonium sulphate; 2% ortho-phosporic acid	1:1001:50	25.70 mg GAE g^{-1} extract	12.11 mg QE g^{-1} extract	[60]	2016
Air-drying	90% methanol + acetic acid at 24 °C for 24 h	1:10	288.15 ± 1.34 mg GAE g^{-1} extract	-	[61]	2016
Air-drying	99% ethanol/deionized water; 3 days; room temperature deionized boiling water; 3 h	1:10	53–132 mg GAE g^{-1} extract	-	[62]	2012
Air-drying	70% methanol 3× in 24 h Ether/chloroform/ethyl acetate/*n*-butanol until colorless	1:20	-	0.68–1.56 mg g^{-1} extract	[63]	2010
Unspecified	70% ethanol, 3× 48 h	-	201 mg g^{-1} leaves	-	[64]	2006

Table 1. *Cont.*

Drying Method	Extraction Parameters	Solid–liquid Ratio (g mL^{-1})	TPC [a]	TFC [b]	Ref.	Publication Year
			Infusion			
Unspecified	DMSO	-	44.07 mg GAE g^{-1}	60.56 mg NAE [h] g^{-1}	[65]	2021
Air-drying	methanol; 2 × 1 h; 25 °C at 150 rpm/ boiling distilled water (100 °C), 5′; room temperature	1:301:200	76.16 ± 0.34 mg g^{-1} extract/ 64.77 ± 2.14 mg g^{-1} extract	-	[12]	2014
Unspecified	water; 15 min; 90 °C centrifuge 6000 rpm	1:40	17.66 mg GAE g^{-1} extract	-	[66]	2010
Air-drying	boiling water (100 °C); 15′	1:8	1.03 ± 0.04 mg GAE L^{-1} infusion	-	[32]	2009
Air-drying	boiling distilled water; 15′/ ethanol; reextracted until colorless	1:20	81.7 mg GAE g^{-1} extract/ 84.5 mg GAE g^{-1} extract	-	[67]	2006
			Heat-reflux extraction			
Unspecified	50–70% ethanol	1:50	42.21−42.35 mg GAE g^{-1} leaves	-	[68]	2021
Oven dried at 60 °C for 48 h	35% ethanol; 2 h; 60 °C	1:4	2.34 ± 0.93 mg GAE g^{-1} dry leaves	-	[44]	2018
Unspecified	ethanol water	1:7.5	94.07 mg GAE g^{-1} extract 66.70 mg GAE g^{-1} extract	-	[69]	2015
Air-drying	ethanol (0, 35, 70%); 0–8 h; 60 °C	1:4	1.5–10.23 mg GAE g^{-1} leaves	-	[11]	2014
			Soxhlet extraction			
Oven dried at 55 °C until moisture level < 10%	water/methanol/ethanol 5 h	1:40	30.73–83.41 mg GAE g^{-1} extract 10.42–12.59 mg GAE g^{-1} dry leaves	-	[70]	2019
Air-drying	chloroform/ methanol	-	0.36 ± 0.01 mg L^{-1} extract/ 0.90 ± 0.06 mg L^{-1} extract	-	[71]	2011

Table 1. *Cont.*

Drying Method	Extraction Parameters	Solid–liquid Ratio (g mL^{-1})	TPC [a]	TFC [b]	Ref.	Publication Year
			Water bath shaker			
Oven dried hydrodistilled residues (temperature unspecified)	water/methanol/80% methanol/ethyl acetate/dichloromethane 48 h; 150 rpm shaker; 2× (water 1×)	1:20	0.50–5.87 mg GAE g^{-1} extract	0.15–5.18 mg QE g^{-1} extract	[72]	2015
Air-drying	60% ethanol; 24 h; 35 °C	1:20	46.79 ± 3.22 mg GAE g^{-1} dry leaves	-	[73]	2011
			Centrifuge			
Oven dried at 25 ± 2 °C for 3 weeks	water/50% ethanol/ethanol 1 h, 40 °C at 600 rpm	1:10	14.37–43.03 mg GAE g^{-1} extract	14.12–30.15 mg ECE g^{-1} extract	[37]	2015
Freezed fresh leaves	phosphate buffer (75 mM, pH 7.0) 20 min; 20,000 rpm	1:7.5	4.02 mg GAE g^{-1} leaves	-	[74]	2001
			Solid–liquid extraction			
Unspecified	80% ethanol; 60 min; 60 °C	1:50	148.3 mg GAE g^{-1} leaves	110.5 mg GAE g^{-1} leaves	[75]	2019
Unspecified	water; 50 °C	-	59.85 mg GAE g^{-1} leaves	-	[76]	2009
			Orbital shaker			
Unspecified	80% acetone with 0.2% formic acid; 1 h; room temperature (2× successive) centrifuge 6000 rpm	1:40	70.81 mg GAE g^{-1} extract	-	[66]	2010

[a] Total phenolic content; [b] Total flavonoid content; [c] Gallic acid equivalents; [d] Catechin equivalents; [e] Pyrocatechol equivalents; [f] Epicatechin equivalents; [g] Quercetin equivalents; [h] Naringin equivalents.

Influence of Different Conventional Extraction Parameters on the Extraction Yield

The solvent type can affect the extraction yield of phenolic compounds due to the fact that their polarity varies between groups. For example, lower molecular flavanols and phenolic acids can be efficiently extracted using water or alcohol, such as methanol and ethanol, while polymerized procyanidins are more efficiently extracted when an aqueous solution of acetone is used [77]. Methanol, ethanol, ethyl acetate, acetone, or their combinations, often with different proportions of water, have most often been used to extract phenolic compounds from different plant material [77]. Water and hydroalcoholic mixtures of ethanol and methanol have most often been used in the extraction of *L. nobilis* leaves, as well. However, acetone was used in two studies. In the first study by Kratchanova et al. [66], the extract obtained using 80% acetone with 0.2% of formic acid after successive extraction (total time: 2 h) contained a significantly higher quantity of phenolic compounds when compared to the water extract obtained after 15 min of infusion at 90 °C. In another study [67], 15 min of infusion in boiling water resulted in higher TPC in comparison with mentioned acetone extract. This could be a result of the difference in solid–liquid ratio, which will be discussed later. The phenolic content obtained by Kratchanova et al. [66] using 80% acetone with 0.2% formic acid was similar to the one obtained by Batiha et al. [55] who used 99.5% acetone during 3 days of maceration. Since different techniques such as maceration in 80% ethanol for 5 days [57], as well as extraction in ethanol and water in a much shorter time [67] resulted in significantly higher TPC, it can be proposed that acetone is less efficient than water and hydroethanolic mixtures. However, a further comparison using the same plant material and extraction conditions would be useful in order to make valid conclusions. Other, less polar solvents, such as ethyl acetate, hexane, dichloromethane and chloroform were used in a few studies. Ethyl acetate and dichloromethane were shown to be more efficient than water, but less efficient than methanol for obtaining higher TPC [72]. Ethyl acetate was also shown to be a more efficient solvent than ethanol [59], with a more than two-fold higher TPC obtained. In the same study, use of hexane was shown to result in a slightly higher TPC than using water. As for TFC, ethyl acetate was shown to be more efficient than other non-polar solvents and water [63], but less efficient than absolute methanol [72] and ethanol [59]. According to studies which compared the efficacy of different solvents on TPC, water is a less efficient solvent than hydroalcoholic mixtures during maceration [12,37,72], as well as Soxhlet extraction [70].

Elevated temperature seems to significantly improve the efficacy of water as a solvent for extraction of *L. nobilis* phenolic compounds. Ramos et al. [62] obtained higher TPC in boiling water after 3 h, than at room temperature after 3 days. Moreover, the TPC of water extraction at 80 °C for 45 min [56] was 10-fold higher when compared to TPC obtained at room temperature during 24 h [62]. Extraction temperature and time are two significantly linked parameters, where extraction at lower temperatures requires a longer extraction time, while shorter extraction time is achieved when using moderate or high temperatures of extraction [52]. Elevated values of temperature can increase solubility of analyte and mass transfer rate, as well as decrease the viscosity and the surface tension of the solvents, which helps the solvent reach the sample matrix, resulting in an improved extraction rate. However, long extraction combined with high temperatures can increase the chance for undesirable reactions, such as hydrolysis and enzymatic oxidation of the phenolic compounds [78,79], which consequently decrease their yield in the extracts. The effect of temperature was less obvious during a comparison of TPC from different studies when other solvents were used. The highest TPC out of all conventional extraction parameters expressed as mg GAE g^{-1} extract was obtained when using 90% methanol with the addition of acetic acid (1% of volume) during 24 h maceration at room temperature [61], and it was two-fold higher than TPC obtained in 80% ethanol after 5 days of maceration at room temperature [57]. Addition of acid into organic solvent was shown to have an effect when preparing anthocyanins-rich extract because the mixture denatures the cell membranes and dissolves the anthocyanins while stabilizing them at the same time [49], so it is possible that a similar effect on other phenolic compounds of *L. nobilis* leaves enhanced the extraction

yield in the mentioned study. Methanol was also reported as a more efficient solvent in comparison with ethanol and chloroform for Soxhlet extraction [70,71]. This data implies that methanol is the most efficient solvent for extraction of phenolic compounds from *L. nobilis* leaves. However, the concentration of methanol seems to have a significant effect on TPC. The TPC obtained when 50% methanol was used [34] was lower than the yields obtained by most other conventional extraction parameters. Boulila et al. [72] also reported a difference in the TPC connected to the methanol concentration. The TPC obtained in their research was higher in absolute methanol than in 80% methanol. Methanol also seems to be the most efficient solvent for obtaining higher TFC. Dhifi et al. [58] obtained more than five-fold higher TFC when using absolute methanol during a two-fold shorter extraction time than Vinha et al. [37] when using water, 50% ethanol and absolute ethanol. Due to its known toxicity, however, methanol is not suitable for research that includes organisms and animal models which often take place after the extraction processes. Since ethanol is much less toxic, it is also a more suitable extraction solvent. Therefore, it is not surprising that ethanol has been most often used for extraction of phenolic compounds from *L. nobilis* leaves.

The efficiency of ethanol as a solvent depends on the water content, and ethanol–water mixtures were shown to be more efficient than absolute ethanol [37]. Muniz-Marquez et al. [11] reported that there was no significant difference in the TPC between using 35% and 70% ethanol for heat-reflux extraction, which implies that the concentration of 35% is sufficient to obtain maximum TPC. In addition, Dobroslavić et al. [68] reported that no significant difference was observed in the TPC when 50% and 70% ethanol were used during heat-reflux extraction. In contrast to their conclusions, the highest TPC out of all included studies (expressed as mg GAE g^{-1} leaves) was the one obtained using 80% ethanol during 60 min of extraction [75]. Muniz-Marquez et al. [11] also reported that 2 h was sufficient for obtaining maximum TPC, and further extension of extraction for 8 h had no positive effect. However, the TPC obtained in a water bath shaker after 24 h in 60% ethanol [73] was four-fold higher than in the study by Muniz-Marquez et al. [11]. Moreover, the highest TPC obtained using aqueous ethanol (expressed as mg GAE g^{-1} extract) was achieved after maceration in 80% ethanol after 5 days [57]. These results might be caused by a combination of extraction parameters; however, they suggest that the extraction time cannot be excluded as an important factor for using ethanol as a solvent.

Generally, an increase of the solvent amount enhances phenolic extraction. However, it is advisable to determine an optimum ratio of the sample to solvent in order to minimize solvent input and saturation effects. Different ratios have been used in studies where bioactive compounds were extracted from plant material, and 1:12 [plant material (g): solvent (mL)] seems to be the most commonly used [52]. A ratio of 1:60 is considered sufficient for the extraction of most phenolic compounds from plant tissues [80]. In conventional extractions of phenolic compounds from *L. nobilis* leaves, a solid–liquid ratio of 1:10 and 1:20 were the most often applied. In a study where acetone was used as a solvent [55] with a resulting ratio of 2:1 after 72 h in TPC, similar to the TPC obtained when 80% acetone with 0.2% formic acid was used at a ratio of 1:40 for 2 h [66]. The difference in the extraction time indicates that the increased amount of solvent significantly influenced the extraction efficiency. At the same ratio of 1:40, water infusion at 90 °C during 15 min resulted in significantly lower TPC when compared to the acetone extract [66]. However, the extract obtained by boiling water infusion during 15 min at a ratio of 1:20 [67] resulted in significantly higher TPC than both acetone and water extract at 1:40, indicating the importance of establishing an optimum ratio for each solvent.

3.2.2. Advanced Extraction Techniques

Conventional extraction techniques require a longer extraction time and large amounts of organic solvents which can cause environmental pollution. Furthermore, they have low extraction selectivity and are difficult to be automated [81]. Because of these limitations, a number of new techniques have been developed aiming to reduce organic solvent con-

sumption and sample degradation, eliminate additional steps after the extraction, and improve overall extraction efficiency and selectivity [82]. Microwave- (MAE), ultrasound- (UAE), and enzyme-assisted (EAE) techniques, supercritical fluid extraction (SFE), and an emerging new technology called mechanochemistry have been used for the extraction of phenolic compounds from *L. nobilis* leaves, and will be further discussed. Basic principles of these techniques are shown in Figure 3. Table 2 summarizes the advantages and disadvantages of the advanced extraction techniques. Parameters from available studies on advanced extraction of phenolic compounds from *L. nobilis* leaves are shown in Table 3.

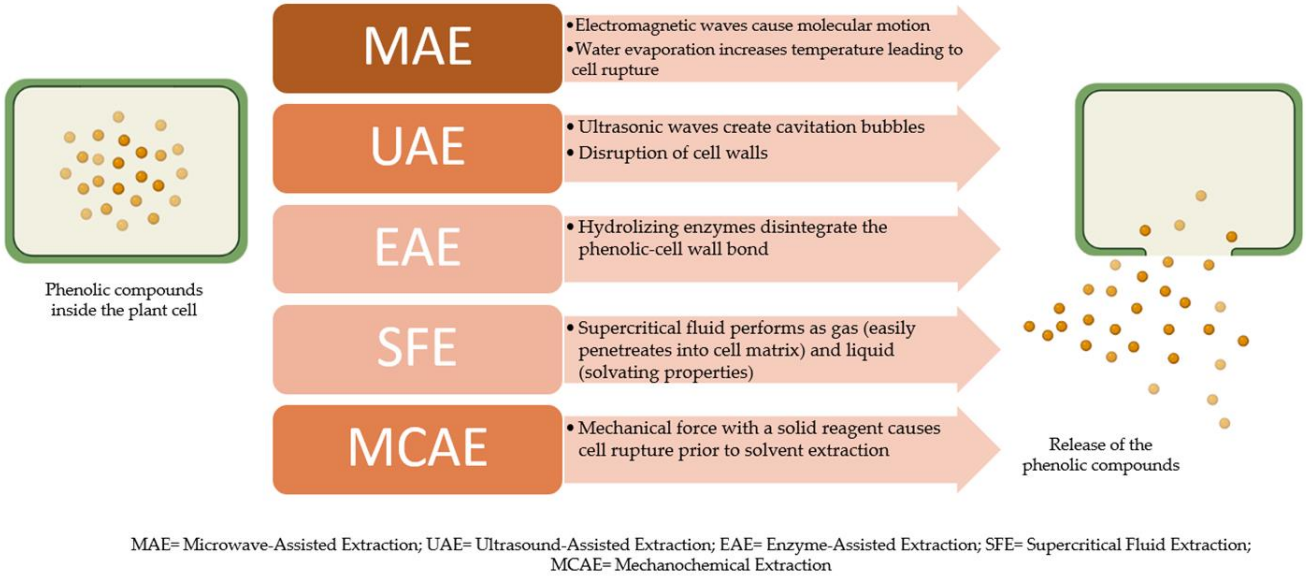


Figure 3. Basic principle of the advanced extraction techniques applied in *L. nobilis* leaf polyphenols isolation.

Table 2. Summary of the advanced extraction techniques applied for the extraction of *L. nobilis* L. leaf polyphenols.

Extraction Technique	Advantages Over Conventional Techniques	Disadvantages	Precautions	Number of Studies on *Laurus nobilis* L. Leaf Polyphenols Isolation	Ref.
MAE	• Reduced solvent consumption • Reduced extraction time • Increased selectivity under right choice of solvent	• Limited to small-molecule phenolic compounds	• Solvents with high dielectric constant should be chosen	3	[41,44,68,70,83]
UAE	• Reduced solvent consumption • Reduced extraction time • Low-cost technology • Suitable for thermolabile compounds	• Ultrasound waves over 20 kHz may cause free radical formation and undesirable changes of target compounds	• The exposure time to high frequencies should be limited	7	[35,45,47,68,70,83–85]
EAE	• Possible enhancment of the solvent permeability	• High costs of required enzymes • Difficulty of applying laboratory scale conditions in industrial scale	• The composition of plant material might limit the access of enzymes	1	[72,86,87]
SFE	• Lower possibility of sample contamination by solvent impurities • Air- and light-free (avoidance of degradation and oxidation of extracted compounds)	• High initial cost of the SFE equipment • High cost of industrial scale application	• Addition of polar modifiers recommended for phenolic compounds	1	[46,88,89]
MCAE	• Water can be used as solvent (increased solubility) • Reduced cost • Simplified purification processes	• Inconsistent data due to novelty of the technique	• Solid reagents should be chosen depending on their alkaline strength and the chemical properties of the target compounds	1	[68,90,91]

MAE = microwave-assisted extraction; UAE = ultrasound-assisted extraction; EAE = enzyme-assisted extraction; SFE = supercritical fluid extraction; MCAE = mechanochemical extraction.

Table 3. Parameters used in advanced extraction techniques of phenolic compounds from *L. nobilis* leaves.

Drying Method	Extraction Parameters	Solid–liquid Ratio (g mL^{-1})	TPC [a]	TFC [b]	Ref.	Publication Year
Microwave-assisted extraction						
Unspecified	50–70% ethanol; 40–80 °C; 400/800 W; 5–15 min	1:50	30.88–53.57 mg GAE [c] g^{-1}	-	[68]	2021
Oven dried at 55 °C until moisture level <10%	ethanol, 500 W; stirring power 50% 15–75′; 90 °C	-	25.03–135.47 mg GAE g^{-1} extract 2.74–21.56 mg GAE g^{-1} dry leaves	-	[70]	2019
Oven dried at 60 °C for 48 h	60 ± 2 °C; three-stage irradiation power (800 W, 15 s; 400 W, 15 s; 200 W, 30 s). ethanol 25–50% 3,6,9′	-	1.91–10.63 mg GAE g^{-1} plant	-	[44]	2018
Ultrasound-assisted extraction						
Unspecified	50–70% ethanol; 5–15 min; 50–100% amplitude; 20 kHz	1:50	24.43–36.74 mg GAE g^{-1} leaves	-	[68]	2021
Air-drying + 45 min oven at 50 °C	ethanol/water/50% ethanol; 20′; 45 °C; 20 kHz	1:10	476.94–796.94 µg GAE g^{-1} extract	192.82–398.71 µg CAE [d] g^{-1} extract	[85]	2020
Oven dried at 55 °C until moisture level <10%	ethanol; 30–150′; 360 W; 50/60 kHz	1:40	44.35–164.04 mg GAE g^{-1} extract 3.33–24.77 mg GAE g^{-1} dry leaves	-	[70]	2019
Air-drying	50% ethanol + 0.1% formic acid, 5′ sonication; centrifuge: 3000× *g*; 10′; 4 °C 2×	1:5	1.12 ± 0.08 mg GAE g^{-1} extract	-	[45]	2014
Air-drying	ethanol (0,35,70%); 20–60′; room temperature; 40 kHz	1:4; 1:8; 1:12	3.52–17.32 mg GAE g^{-1} plant	-	[47]	2013
Air drying (a) Freeze drying (f): 6 h at −60 °C	70% methanol; 6 M HCl 15′ sonication + water bath reflux: 90 °C; 2 h	1:100	a: 22.90–80.30 f: 21.50–41.20 mg GAE g^{-1} extract	a: 2.90 ± 0.18 mg ECE [e] g^{-1} extract f: traces	[35]	2008
Unspecified	methanol; 2 h; 40 °C ultrasonic bath	1:100	99.7 mg GAE g^{-1} extract	80.1 mg kg^{-1} extract	[84]	2005

Table 3. *Cont.*

Drying Method	Extraction Parameters	Solid–liquid Ratio (g mL^{-1})	TPC [a]	TFC [b]	Ref.	Publication Year
			Enzyme-assisted extraction			
Oven dried (no defined temperature) hydrodistilled residues	Pretreatment: distilled water + cellulase/hemicellulase/xylanase/ternary mixture; 1 h; 40 °C methanol; 48 h; 150 rpm shaker; 2×	1:5 1:20	5.85–7.12 mg GAE g^{-1} extract	5.18–6.33 mg QE [f] g^{-1} extract	[72]	2015
			Supercritical fluid extraction			
Air-drying	250 bar; 60 °C; 4% ethanol; 75′ 1. separator: 100 bar, 60 °C 2. separator: 20 bar, 20 °C	-	1. 51.6 ± 0.98 mg GAE g^{-1} extract 2. 87.38 ± 1.32 mg GAE g^{-1} extract	-	[46]	2006
			Mechanochemical extraction			
Oven dried at 55 °C until moisture level <10%	Na_2CO_3, $BaCO_3$, Li_2CO_3, $CoCO_3$, K_2CO_3, $CaCO_3$ (excess of 25 or 50%) ball mill: 400 rpm; 10′ ethanol; 20′; magnetic stirring. centrifuge: 2683.2× *g*, 10′	-	33.01–75.54 mg GAE g^{-1} extract 1.91–9.52 mg GAE g^{-1} dry leaves	-	[70]	2019

[a] Total phenolic content; [b] Total flavonoid content; [c] Gallic acid equivalents; [d] Catechin equivalents; [e] Epicatechin equivalents; [f] Quercetin equivalents.

3.2.3. Microwave-Assisted Extraction (MAE)

MAE is an extraction technique that uses non-ionizing radiation of electromagnetic waves with a frequency between 300 MHz to 300 GHz in order to induce molecular motion in polar or polarizable materials or solvents by working with dipoles [92]. The molecular motions result in heating of the sample, which leads to evaporation of moisture from plant cells that creates pressure, causing rupture of the cell wall and release of target compounds [93]. During radiation, the solvent molecules are induced to align themselves in a normal phase with an electric field. Under the rapid change of the electric field which occurs in MAE, solvent molecules fail to realign and start vibrating, which causes heating of the solvent due to frictional forces [53]. This allows the solvent to penetrate the plant matrix easily and promotes the extraction of the target compounds. Solvents should be chosen based on their boiling points, and dissipation and dielectric properties. Based on those properties, aqueous acetone, ethanol, or their mixtures have often been used to extract phenolic compounds using MAE [53]. Since the microwave energy is transferred by dielectric absorption only [83], non-polar solvents with lower dielectric constants can absorb much less energy, which may result in poor heating and lower extraction yields. Therefore, MAE is considered to be a selective method in the case of polar molecules and solvents with a high dielectric constant [83]. MAE has many advantages similar to UAE, including the use of less solvents, reduced extraction time and processing costs, as well as increased extraction yields. However, this technique is limited to small-molecule phenolic compounds, such as phenolic acids, quercetin, isoflavone, and trans-resveratrol, which were shown to be stable under microwave heating conditions up to 100 °C for 20 min [94]. Phenolic compounds with a higher number of hydroxyl-type substituents, such as tannins, or thermosensitive compounds, such as anthocyanins, may not be suitable for MAE. *L. nobilis* leaves, as described earlier in the text, are abundant in small-molecule flavonoids and phenolic acids, which makes MAE a suitable technique for their extraction.

This technique was previously used in three studies [44,68,70], where phenolic compounds were extracted from *L. nobilis* leaves. In all of them, aqueous solutions of ethanol in different concentrations were used as the solvent. Muniz-Marquez et al. [44] reported that ethanol concentration was the most significant influencing factor for TPC, contrary to the results reported by Dobroslavić et al. [68] where ethanol concentration had no significant influence on the TPC. At lower ethanol concentrations, Muniz-Marquez et al. [44] reported that irradiation time had very little effect on yield, while at a concentration of 50%, the TPC increased proportionally with prolonged irradiation time. The highest TPC was achieved after 9 min, and it was two-fold lower than the TPC obtained by Rincon et al. [70] after 60 min when using pure ethanol as solvent. However, in their study, the TPC after 15–30 min was lower than the yield that Muniz-Marquez et al. [44] achieved after 6 min with 50% ethanol. This indicates that use of 50% ethanol under MAE conditions of Muniz-Marquez et al. [44] is more time-efficient, which can be substantiated by results recently reported by Dobroslavić et al. [68] where 10 min was optimal during the extraction or *L. nobilis* leaf polyphenols with 50% ethanol. The presence of water in ethanol increases the dielectric constant of the system, which could result in an increased extraction yield by improving the swelling of the plant material and therefore increasing the surface contact of the matrix and solvent [95,96]. Moreover, a high ethanol concentration might interrupt the extraction of some phenolic compounds due to lower solubility and lower penetration of ethanol into the plant matrix [97]. The influence of the irradiation power and temperature must not be excluded, since Rincon et al. [70] performed the extraction at 90 °C and 500 W, which might have caused degradation of thermosensitive phenolic compounds over a prolonged time. In accordance, Dobroslavić et al. [68] reported that the increase of temperature from 40 to 80 °C resulted in higher TPC; however, with an irradiation time prolonged from 10 to 15 min, a stagnation of the TPC was observed, which was brought by the authors into connection with possible thermal degradation. The authors have also observed a decline in the TPC when an irradiation power higher than 400 W was applied. The results of these studies were most likely influenced by a combination of extraction parameters, so further

research would be needed for better conclusions. Moreover, it would be interesting to see how other solvents would influence TPC.

3.2.4. Ultrasound-Assisted Extraction (UAE)

UAE, often referred to as sonication, is a technique that uses ultrasonic waves ranging from 20 to 2000 kHz [83] in order to create cavitation bubbles near the sample tissue, which break down and disrupt cell walls. Consequently, surface contact between the sample and solvent increases, thereby improving mass transfer, which helps the target compounds to be extracted more efficiently [98]. Extract recovery is influenced by several factors, including sonication time, extraction temperature, solvent selection, solid–liquid ratio, wave frequency, and ultrasonic wave distribution [99]. Ultrasonic wave distribution is usually not uniform and the wave power decreases with an increased distance from the radiating surface, which is why agitation or shaking can be useful. The main benefits of UAE are reduction in extraction time and solvent consumption, which makes it a simple and relatively low-cost technology. In addition, a reduced processing time makes this technique suitable for the extraction of thermolabile compounds. However, ultrasound waves over 20 kHz may cause free radical formation and undesirable changes of target compounds [83].

Water, ethanol and methanol with different proportions of water have been used as solvents for the UAE of phenolic compounds from *L. nobilis* leaves. Hydroethanolic mixtures were shown to be more efficient than water [47,85] and absolute ethanol [85] for obtaining higher TPC and TFC. According to Muniz-Marquez et al. [47], 35% ethanol is sufficient for obtaining maximum TPC, while further increase of ethanol proportion results in lower yields. On the other hand, Dobroslavić et al. [68] reported a higher TPC when 70% ethanol was used. Since the shortest irradiation time in the study by Muniz-Marquez et al. [47], who applied the frequency of 40 kHz, was two-fold longer than 10 min, which was reported as optimal by Dobroslavić et al. [68] where 20 kHz ultrasonic probe was used, it is possible that the yield was influenced by the duration of exposure to high frequency (over 20 kHz), which might have caused undesirable changes to the phenolic compounds [90]. On the other hand, Rincon et al. [70] reported that the highest TPC was obtained after 2 h with frequency of 50/60 kHz. However, the TPC obtained in their study after 45 min of sonication at 50/60 kHz was two-fold lower than the one obtained after 40 min in 35% ethanol at 40 kHz [47], which could have been a result of the effect of the solvent mentioned in Section 3.2.3, as well as the frequency of ultrasonic waves. Another factor which, according to Muniz-Marquez et al. [47], significantly influences the TPC, was a solid–liquid ratio, which when decreased from 1:4 to 1:12 g of sample per mL of solvent, lead to an increased TPC. In accordance, Dobroslavić et al. [68] achieved a two-fold higher TPC by applying a solid–liquid ratio of 1:50 g of sample per mL. As for other solvents, methanol appears to be a less efficient solvent for UAE when compared to ethanol, since 2 h of extraction in absolute ethanol with a solid–liquid ratio of 1:40 [70] resulted in significantly higher TPC in comparison with 2 h of extraction in absolute methanol with a solid–liquid ratio of 1:100 [84]. However, it is possible that the difference in the solid–liquid ratio might have also influenced the results. Further research on the same plant samples would be necessary to make more valuable conclusions.

3.2.5. Enzyme-Assisted Extraction (EAE)

EAE is considered as a novel and efficient technique for the extraction of numerous secondary plant metabolites with antioxidant properties [81]. It is based on the fact that these metabolites in plant matrices, including phenolic compounds, often interact with a polysaccharide-lignin complex in the cell wall by ester, hydrogen or hydrophobic bonding [86], which can sometimes make them unreachable for solvent during extraction. The addition of specific hydrolyzing enzymes, such as cellulase, α-amylase, pectinase and hemicellulase might enhance extraction of phenolic compounds by promoting disintegration of the phenolic-cell wall matrix bonds, thus allowing the entrance of solvent [87,100]. The

most important factor for extraction efficiency of phenolic compounds, along with the pH of the system, extraction temperature and time, and enzyme concentration, was found to be the particle size of the samples [101]. With an increased contact surface caused by a smaller particle size, the enzyme action is increased. EAE has important shortcomings, which include high costs of required enzymes and the difficulty of applying laboratory scale conditions in industrial scale [102]. Boulila et al. [72] used enzyme pre-treatment in extraction of phenolic compounds from *L. nobilis* leaves and observed no significant difference in TPC and TFC between pre-treated methanolic extracts and control. The authors explained this with the presence of lignin in the cell walls (27.61% in *L. nobilis* leaves), which might limit the accessibility of cellulase and hemicellulase to their substrate.

3.2.6. Supercritical Fluid Extraction (SFE)

SFE is a method where supercritical fluid, a substance that shares physical properties of both gas and liquid above its critical point [103], is used. These properties allow the performance of gas in terms of penetration power into the cell matrix, as well as the solvating properties of liquid [104,105]. CO_2, with a critical point above 31.1 °C and 7380 kPa, is the most frequently utilized supercritical fluid in SFE. It is inflammable, relatively non-toxic, chemically stable, inexpensive, and produces zero surface tension [89]. Its mild critical temperature is suitable for extraction of thermolabile compounds [106]. However, since it is non-polar, the addition of polar modifiers, such as ethanol, methanol, ethyl acetate, or acetone is recommended for the extraction of polar phenolic compounds [107]. A pressure between 50–600 bar, temperature of 20–35 °C and time of 5–180 min are considered as the parameters that result in the highest yields of phenolic compounds extracted by SFE from various plant materials [88]. SFE has many advantages over conventional extraction techniques that include lower organic solvent consumption, increased selectivity and separation of the extract, as well as reduced extraction time [108]. The main advantage of this method is its lower possibility of sample contamination by solvent impurities and avoidance of degradation and oxidation of extracted compounds, since it is performed in the absence of air and light [103]. However, the initial cost of the SFE equipment is very high [109] and the cost of applying it in an industrial scale often outweighs the technical benefits [49].

SFE has been used to extract essential oil from *L. nobilis* leaves [2,110,111], however only Santoyo et al. [46] determined the TPC in extracts obtained using this technique. Extraction parameters which are shown in Table 3 were chosen by the authors based on their previous research on rosemary (*Rosmarinus officinalis*) and oregano (*Origanum vulgare*) leaves. It was shown that the temperature and pressure of the separators had a significant effect on TPC, as well as on antioxidant activity, of which values were higher at 20 bar and 20 °C when compared to the conditions of 100 bar and 60 °C. TPC obtained from both separators is comparable to the content obtained by other extraction techniques, including advanced and conventional ones (Tables 1 and 3). However, more data are needed to make a valid comparison of SFE with other techniques. This can be achieved by varying different extraction parameters in order to find optimal conditions for SFE of phenolic compounds from *L. nobilis* leaves, since they can differ significantly for different plant materials [49]. SFE resulted in higher antioxidant capacity of myrtle (*Myrtus communis*) extracts when compared to conventional extraction [112]. Authors put this into correlation with a higher concentration of the myricetin-*O*-glycosides (flavonol glycosides). Since, as previously mentioned, *L. nobilis* leaves are rich in flavonol glycosides, SFE could potentially result in their higher yield and antioxidant capacity as well. A study on *Ziziphus jujuba* Mill. leaves is in agreement with this hypothesis, since it showed that the SFE technique was superior to UAE for the recovery of kaempferol and quercetin glycosides, which are abundant in *L. nobilis* leaves [113].

3.2.7. Mechanochemical-Assisted Extraction (MCAE)

In order to overcome the purification difficulties due to low selectivity and solvent residues after other advanced extraction techniques, an innovative technology, MCAE, has recently emerged. This technology is based on the research of physicochemical and chemical transformation of compounds caused by mechanical force, such as grinding in a ball mill [90,114]. It consists of mechanochemical processing of plant material under highly insensitive mechanical pressure in the ball mill, with a solid reagent (usually carbonated salts) prior to solvent extraction [115]. Cell walls rupture due to this process, allowing the extraction of target compounds whose water solubility is also improved [91]. This allows the use of water instead of other conventional solvents, reducing the cost of extraction and simplifying the purification process. The most commonly used reagents have been solid alkali reagents, such as NaCO, NaHCO and NaOH, depending on their alkaline strength and the chemical properties of the target compounds [116]. Some studies [91,115] have shown that MCAE results in higher flavonoid yields while being more time-efficient at lower extraction temperatures and without use of organic solvents. However, since the technique is quite novel, the influence of different extraction parameters is still inconsistent and there is a lack of complete understanding, which is essential for the scale-up process and further application [114].

Rincon et al. [70] used Na_2CO_3, $BaCO_3$, Li_2CO_3, $CoCO_3$, K_2CO_3 and $CaCO_3$ in excess of 25 or 50% as solid reagents prior to *L. nobilis* leaf extraction with ethanol. The excess of 25% was shown to result in higher TPC than when 50% excess was used. Adding 25% of Li_2CO_3 resulted in the highest TPC; however, the value was slightly lower than the one obtained by Soxhlet extraction with ethanol in the same study. Additionally, the highest yields obtained by MAE and UAE in the same study were significantly higher than the one obtained by MCAE. It is important to note that the highest yields in MAE and UAE were obtained after 60 and 120 min, respectively, while the total extraction time in MCAE was 40 min. Since TPC from *L. nobilis* obtained by MCAE is comparable, and even higher than the TPC obtained at certain parameters of other extraction techniques, there is definitely potential for further research and optimization of the MCAE for the extraction of phenolic compounds from *L. nobilis* leaves, which could lead to higher yields and/or lower extraction costs than other techniques.

4. Future Perspectives

Laurel leaves, due to a wide range of structurally diverse bioactive molecules and their antioxidant, antimicrobial, anti-inflammatory, and other health beneficial properties, are an excellent base for the production of high-quality extracts with potential applications in the food, pharmaceutical, and cosmetic industries. Insights into the biopotential of laurel require new approaches in the production of plant extracts, and consequently, the use of advanced green techniques that allow the development of formulations and high value-added products with improved biological properties and actions. This paper presents a systematic review of conventional and advanced extraction techniques for the isolation of phenolic compounds from *L. nobilis* leaves, emphasizing the importance of optimization and achieving high yields of polyphenols under optimal conditions, regardless of the applied technique. It has been shown that similar total phenolic yields can be achieved by adjusting the extraction parameters of both conventional and advanced extraction techniques. Therefore, further research should be focusing on including more extraction parameters in optimization with the aim of achieving higher yields of total polyphenols and on overall extract quality, with an emphasis on isolation of target bioactive compounds, such as kaempferol glycosides which have shown diverse biological activities. To the best of the authors' knowledge, pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE), has not yet been applied for the extraction of phenolic compounds from *L. nobilis* leaves. Nevertheless, comparison of PLE with conventional methods [117–119] has shown that PLE resulted in comparable or higher yields of phenolic compounds while

being time-efficient and economic, which sets a promising perspective for application of this technique on *L. nobilis* leaves.

Since phenolic compounds are prone to losing their active properties during storage, it is of great importance to preserve their bioactivity and improve their stability to make them applicable in the industry. Therefore, future research should also be focused on various encapsulation techniques that would result in more stable forms of beads or powders with required release characteristics, biocompatibility and bioavailability of the active compounds [120,121]. Investigating bioavailability for the purposes of application in functional food and supplements is extremely important, since the abundance of polyphenols does not necessarily mean the best bioavailability profile [122]. In vitro methods for the evaluation of bioavailability cannot reproduce the complex environment of human digestion that in vivo methods can; however, they are relatively fast, simple, cheap, and reproducible, allowing more efficient formulation of the products [123]. All of the mentioned steps present future perspectives and open new areas for the multidisciplinary research and development (R&D) of sustainable, efficient and economic procedures that would result in the maximum use of the great potential which *L. nobilis* leaves and their bioactive molecules hold.

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3. ENCAPSULATION TECHNOLOGY

The major concern in utilizing the valuable properties of phenols is their sensitivity to environmental effects such as temperature, light, oxygen, pH and moisture, which leads to poor stability during storage as well as in the human gastrointestinal tract, resulting in their low bioaccessibility (Bamidele & Emmambux, 2021). For this reason, numerous encapsulation techniques have emerged over the years aiming to preserve the beneficial properties of bioactive molecules, prolong their shelf life and achieve controlled release and targeting precision. In addition, encapsulation contributes to the masking of undesirable flavors such as bitter and acrid taste which polyphenols tend to have (Shishir et al., 2018).

In general, encapsulation is a process of coating the bioactive molecules by a carrier material (also known as shell, matrix, external phase or wall material) in order to form micro- or nanocapsules (Shishir et al., 2018). There are two approaches which are followed in the production of encapsulates, the top-down and bottom-down approach. The top-down approach is based on the size reduction and shaping of large structured materials by mechanical disruption forces and it includes techniques such as emulsification, evaporation and extrusion techniques (Joye & McClements, 2014). These techniques are suitable for the encapsulation of both lipophilic and hydrophilic compounds, but they also allow less control over particle size and often require sophisticated equipment. On the other hand, bottom-down approach is based on the development of larger particles through self-assembly of small molecules and it includes techniques such as spray drying, coacervation, electrospinning and inclusion complexation (Jia et al., 2016). These techniques provide better control over particle size and morphology and usually require less energy, but can also be more sensitive to environmental effects during formation (Shishir et al., 2018). There is no standard encapsulation technique applicable to all phenols, since the choice of technique depends on various elements, including the intended purpose of the encapsulate, chemical structure of the individual compounds and type of carriers applied (Aguiar et al., 2016).

To date, encapsulation of laurel leaf phenols has only been researched in two studies. In the first study, microencapsulation by spray drying was applied and the influence of carriers (chitosan, sodium alginate and arabic gum) on the yield, particle size and release properties was examined (Chaumun et al., 2020). In the second study, nanoencapsulation of hydroalcoholic laurel leaf extract by nanoliposomes was applied and it was shown that the encapsulated laurel leaf extract has the potential to delay microbial spoilage and oxidation of minced meat (Tometri et al., 2020).

In this dissertation, two techniques were chosen for the encapsulation of laurel leaf phenols. The first was spray drying as the most researched and the most widely used technique for the encapsulation of phenols from various plant materials (Shishir & Chen, 2017). In addition, phenolic extracts from different plant materials encapsulated by spray drying were successfully utilized for the enrichment of different food products such as biscuits (Hidalgo et al., 2018; Kaderides et al., 2020; Papillo et al., 2019), chocolate bars (Grassia et al., 2021), gummy candies (Sarabandi et al., 2019), yogurt (Yadav et al., 2018), ice cream (Çam et al., 2014), apple puree (Lavelli et al., 2016), hazelnut paste (Kaderides et al., 2015) and fish burgers (Spinelli et al., 2016), showing potential for a wide use of spray dried laurel leaf phenols in food fortification. The other encapsulation technique which was chosen for the encapsulation of laurel leaf phenols was electrostatic extrusion as one of the most recent techniques used for the encapsulation of bioactive compounds (Bamidele & Emmambux, 2021). This technique was shown to be efficient for encapsulation of thyme (*Thymus serpyllum* L.) phenols (Stojanovic et al., 2012), as well as six other medicinal plant extracts including nettle (*Urtica dioica* L.), hawthorn (*Crategus laevigata*), raspberry leaf (*Rubus idaeus* L.), olive leaf (*Olea europea* L.), yarrow (*Achillea millefolium* L.) and ground ivy (*Glechoma hederacea* L.) (Belščak-Cvitanović et al., 2011). Moreover, bioactive compounds from carrot waste encapsulated by this technique were successfully applied in a fortified yogurt formulation (Šeregelj et al., 2021), indicating potential for use of this technique in functional food production.

a. SPRAY DRYING

Spray drying is a widely implemented physical technique for the microencapsulation of bioactive compounds which is based on the principle of atomization of a liquid feed through a stream of hot air, thereby resulting in formation of microcapsules in which the bioactive compounds are coated by a layer of wall material (Figure 3) (Shishir et al., 2018). The main reasons for the wide use of spray drying are relatively low operational cost, high stability of the obtained powders due to low moisture content and water activity and short drying contact time (5–100 s) which allows preservation of targeted molecules, as well as flavors and colors (Shishir & Chen, 2017).

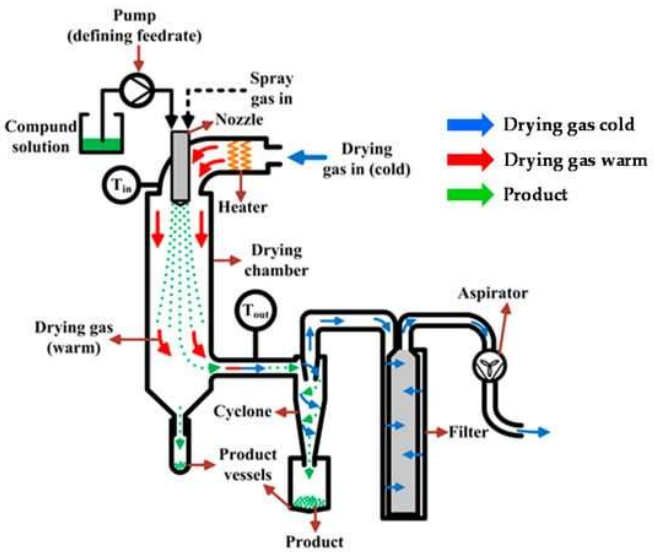

Figure 3. Schematic representation of the spray drying process (Piñón-Balderrama et al., 2020)

The efficiency of spray drying depends on several parameters including type and speed of the atomizer, the atomization pressure, drying temperature, feed and drying flow rate, as well as type and concentration of carrier agent (Shishir & Chen, 2017). For this reason, optimization of the process is crucial for obtaining powders with the most desirable properties.

i. PROCESS PARAMETERS

Since atomization is the main feature of a spray dryer, choosing the right atomizer is the key to a successful spray drying process. The main function of the atomizer is to disperse the liquid feed into small droplets in order to maximize the surface volume and control the rate at which the feed enters the drying chamber (Patel et al., 2009). The most common types of atomizers are rotary atomizers, hydraulic nozzles, pneumatic nozzles and ultrasonic nozzles. The atomization pressure and speed have a great influence on the physical properties of the spray-dried product. It has been shown that up to a certain point, higher atomizer pressure and speed result in smaller particles, enhance the drying process and increase the total solids content and bulk density of the obtained powders (Shishir & Chen, 2017). It has also been reported that the use of excessive atomizer pressure wastes energy without further reduction in the droplet size (Shishir et al., 2018), while the consequent shorter exposure of droplets to drying air leads to insufficient drying which is why finding the optimal combination is crucial.

Drying (inlet) temperature is defined as the temperature of the drying medium at the moment of initial contact with feed which directly influences the amount of solvent that can be

removed per unit of time (Cal & Sollohub, 2010). As such, it is the most important factor affecting the physicochemical characteristics of the obtained powders since it allows evaporation of the solvent and directly influences the moisture content, particle size and consequently solubility and bulk density of the final product (Shishir & Chen, 2017). The main objective in choosing the drying temperature is to achieve the lowest possible moisture content without causing thermal degradation of the targeted compounds, and the temperature range of 140–200 °C is the most commonly applied during spray drying of various plant extracts (Piñón-Balderrama et al., 2020). In order to obtain powders of the highest quality while avoiding particle agglomeration, adhesion to the drying chamber walls, stickiness and collapse of the microstructure, inlet temperature should not be higher than 20 °C above the glass transition temperature (T_g) of the dry solid (temperature at which interchanging glassy and rubbery state occurs) (Fazaeli et al., 2012). In addition to the inlet temperature, the outlet temperature in the cyclone where separation of powder occurs is also an important parameter representative of droplet drying rate which affects the moisture content, process yield and morphology of the obtained particles (Shishir & Chen, 2017). This temperature cannot be directly influenced, but is the result of other factors such as inlet temperature, gas flow rate, characteristics of the feed and enthalpy of evaporation (Piñón-Balderrama et al., 2020).

Feed flow rate relies on the speed of the atomizer where a higher pump speed results in a higher feed flow rate. The high flow rate does not allow sufficient interaction time between the feed droplets and the drying air resulting in less effective heat and mass transfer leading to higher moisture content in the final product (Shishir & Chen, 2017). The drying gas flow rate is defined as the volume of drying air (usually atmospheric air, followed by nitrogen) supplied to the system per unit of time which determines the rate of particle movements through the system (Cal & Sollohub, 2010). According to some literature reports, it is advisable to operate the drying at the maximum flow rate in order to maximize the cyclone operation efficiency, since higher gas flow rates result in better atomization of the liquid feed and smaller product particles (Maury et al., 2005). However, since a high flow rate leads to shorter contact time between feed and drying air, the moisture content of the final product may increase due to insufficient drying (Santos et al., 2018), highlighting the need for optimization.

ii. SPRAY DRYING CARRIERS

In general, plant extracts have low glass transition temperatures due to the presence of organic acids and polysaccharides, which is why different carriers are added during the process of spray drying (Hussain et al., 2018). The selection of carriers and their appropriate

concentration depends on the proposed purpose of the final product, and optimization procedures are necessary to find the most suitable combinations. All carriers must fulfill basic requirements including "generally recognized as safe" (GRAS) status and biodegradability (Azhar et al., 2021). In addition, they should be highly soluble, have low viscosity at high concentrations, film forming ability, high molecular weight and T_g, as well as be able to protect the targeted compounds from environmental effects and increase their adherence at the target sites of the gastrointestinal tract (Shishir et al., 2018). The most commonly used carriers in spray drying are carbohydrate polymers including starch derivatives (maltodextrins, cyclodextrins) and gums (gum arabic, gum karava) which were chosen as carriers in this dissertation, followed by pectin, inulin and cellulose derivatives (cellulose, carboxymethylcellulose). Materials such as proteins (soy protein, pea protein, wheat flour) and lipids (lecithin, oils) are less often used due to less desirable physicochemical characteristics (Azhar et al., 2021).

Polysaccharides are naturally occurring carbohydrate polymers comprised of monosaccharide units linked by glycosidic bonds whose varying chemical structure results in different molecular characteristics important for the spray drying process such as solubility, emulsification capability, digestibility and water retention capacity (Fathi et al., 2012). Starch is one of the most abundant polysaccharides in plants consisting mainly of linear amylose and branched amylopectin polymer units (Nedovic et al., 2011). Natural starch is mostly hydrophilic and is not emulsifiable, which limits its application for the encapsulation of hydrophobic bioactive compounds (Fathi et al., 2012). For this reason, different chemical, physical and enzymatic methods are applied in order to produce modified starch derivatives with higher functionality. Dextrins are hydrolyzed, water-soluble modified starches with varying dextrose equivalent (DE) values. Dextrins with DE values lower than 20 are referred to as maltodextrins, while those DE higher than 20 are denoted to as glucose solids, glucose syrups or corn syrup solids. The functionality of dextrins with the same DE can vary depending on the type of starch (e.g. potato, corn) (Shishir et al., 2018). Maltodextrin (DE value of < 20) is a hydrolyzed starch widely used in the food and drug industry due to its relatively high water solubility (70%), low viscosity and contribution in bulking, coating and protection of bioactive compounds from thermal degradation and oxidative loss (Alvani et al., 2011). Maltodextrins with higher DE have higher solubility and lower molecular weight, but also result in higher moisture content of the powders due to more hydrophilic groups so the most commonly used maltodextrin is the one with DE 10 (Shishir & Chen, 2017).

Cyclodextrins are cyclic oligosaccharides mostly composed of 6 (α-cyclodextrin), 7 (β-cyclodextrin) or 8 (γ-cyclodextrin) glucopyranose units produced by the enzymatic modification of starch. They possess hydrophobic cavity and a hydrophilic external surface which enable the formation of molecular inclusion complexes with poorly soluble molecules such as phenols (Đorđević et al., 2015). Among cyclodextrins, β-cyclodextrin is the most commonly applied for encapsulation purposes due to its low cost. However, due to its limited aqueous solubility, derivatives with improved characteristics such as 2-hydroxypropylted-β-cyclodextrin and methylated-β-cyclodextrin are produced and widely applied for the encapsulation of bioactive compounds (Shishir et al., 2018).

Gums are non-starch, water-soluble polysaccharides widely applied in the food and pharmaceutical industry due to their non-toxicity, biocompatibility and biodegradability. Naturally occurring plant-based gums can be classified into four categories based on their origin, including plant exudates gums (e.g. arabic gum), seed gums (e.g. guar gum), sea weed gums (e.g. carrageenans) and microbial exudates gums (e.g. xanthan gum) (Shishir et al., 2018). Gum Arabic, deriving from the *Acacia senegal* tree, is the most widely applied exudate gum consisting of galactose, arabinose, rhamnose, glucuronic and 4-*O*-methyglucuronic acid units. It has low viscosity and high solubility, stable emulsion forming ability and high retention of volatile compounds as well as some disadvantages such as low production yield and a higher cost which is why it is often combined with other carriers (Buljeta et al., 2022). In fact, none of the carriers has all the desirable characteristics for spray drying. For example, the widely used starch and its derivatives possess high molecular weight and T_g, low viscosity and high solubility in cold water, but have low film forming ability which is crucial for the preservation of bioactive molecules (Shishir et al., 2018). On the other hand, gums have high film forming ability and low T_g, which is why combination of starch derivatives and gums in a mass ratio higher than 1 usually results in the most desirable physicochemical properties of the powders (Azhar et al., 2021).

b. Electrostatic extrusion

Extrusion technology includes processes in which a material is forced to flow through an orifice of different diameters at predefined rates under a variety of conditions, depending on the desired product specifications (Alam et al., 2016). Electrostatic extrusion is a dispersion technique based on the formation of microspheres by dropping polymers (most often sodium alginate) into hardening solutions by applying electric field between the charged needle and the hardening solution (Figure 4) (Bamidele & Emmambux, 2021). This technique enables

production of varying sized beads for multiple purposes by alternating needle sizes and adjusting process parameters including the applied voltage, encapsulant and carrier concentration in order to obtain maximum encapsulation efficiency, stability and optimal release kinetics (Low & Lim, 2014).

Sodium alginate, a linear anionic polysaccharide derived from marine algae polysaccharide composed of β-D-mannuronate (M) and α-L-guluronate (G) residues linked by 1-4 glycosidic bonds, is the most commonly used carrier in electrostatic extrusion due to its ability to form gels in the presence of polyvalent ions (Shishir et al., 2018). Ca^{2+} is the most suitable and widely applied ion in the hardening solutions since it results in non-toxic and biocompatible complexes through a relatively cheap and simple process which was also shown not to interact with polyphenols (Silva et al., 2020). Other biopolymers can be combined with alginate in order to enhance encapsulation efficiency and preserve biological activity of polyphenols. For example, it was shown that the addition of chitosan, a non-toxic and biocompatible cationic polysaccharide built by the β-(1–4)-linked D-glucosamine and N-acetyl-D-glucose-amine residues, results in forming stable complexes with other anionic crosslinking agents and leads to more controlled release of polyphenols (Yeh et al., 2022) and increase in their bioavailability (Liang et al., 2017).

i. Process parameters

The main components of a device designed for the electrostatic extrusion (Figure 4) include a pump that allows the polymer solution to be forced out of the reservoir at different flow rates through a stainless steel capillary or needle that can have different diameters. Electrostatic potential, which can be constant or pulsed, is applied by a high voltage generator. It occurs in the space of varying distance between the capillaries (needle) and the hardening solution where the particles are collected (Kostić et al., 2012).

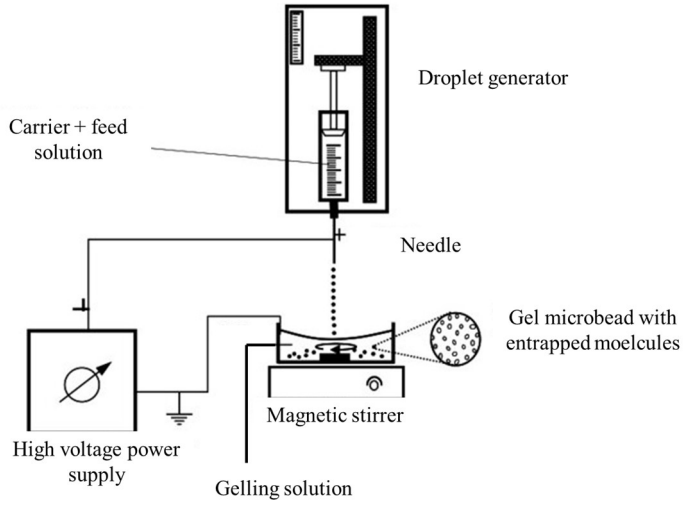

Figure 4. Schematic representation of electrostatic extrusion (adapted from Manojlovic *et al.*, 2008)

The main feature of this technique is the gradual decrease of the droplet diameter with increasing electrostatic voltage, up to a certain value of the critical electrostatic voltage. When the critical electrostatic potential is reached, an unstable jet of the polymer solution is formed, which breaks up into a large number of charged droplets that collect in the hardening solution, where the formation of particles occurs (Poncelet et al., 1999). By measuring the size of the obtained droplets, it was observed that the distribution of the droplet size is not always unimodal and that it is often bimodal or multimodal size distribution, which is a consequence of the droplet formation process itself from the liquid meniscus (Lewińska et al., 2004). This clearly indicates the importance of adjusting the process parameters for each individual carrier-material system for encapsulation, using the electrostatic extrusion procedure.

It is now well known that the properties of beads are a complex function of several process parameters as well as the properties of the extrusion equipment and the polymer solutions themselves. Apart from the electrostatic potential, which is the main feature of the electrostatic extrusion process, as already explained, three other parameters can be singled out that significantly affect the size of the beads obtained, namely the flow of the polymer solution used for encapsulation, the needle diameter (capillaries) and the geometry of the extrusion device. Increasing the flow leads to an increase in the size of the alginate beads obtained by increasing the amount of surface active molecules moving to the surface droplets, and thus the surface tension (Poncelet et al., 1999). Reduction of the diameter of the needle (capillaries) leads to the formation of smaller beads and the reduction of the average diameter of the final

beads in this way also depends on the properties of the encapsulated material (e.g., chemical structure of phenols, protein molar mass, etc.) as well as the pressure drop through the needle (Amsden & Goosen, 1997). By changing the concentration of the alginate solution, the viscosity of the solution is altered resulting in a modified formation of solution droplets and the final diameter of the obtained beads.

The process of extracting beads from the formed droplets includes their curing in the hardening solution, which further increases the number of factors that can affect the final properties and size of the resulting beads (Kostić et al., 2012). Part of the research on obtaining alginate beads by electrostatic extrusion was therefore aimed from the beginning at investigating the influence of the concentration and composition of the alginate solution, as well as the concentration of divalent cations necessary for gelation, on the physical and chemical properties of the beads. The situation is further complicated by the presence of encapsulated compounds, which can influence both the properties of the alginate solution and the extrusion process itself, for example by changing the microhydrodynamics within the capillary due to electrochemical and physical interactions (Amsden & Goosen, 1997). One of the most important data is the minimum amount of divalent cations such as Ca^{2+} required for polymer crosslinking. Therefore, extensive research has been conducted on alginate gelation in the presence of limited amounts of divalent cations. It was found that the presence of other compounds slows down the crosslinking and affects the hardness of calcium alginate beads obtained by the electrostatic extrusion process (Kostić et al., 2012). Therefore, in the process of preparation of beads, their retention in hardening solution is practiced from 30 min to several hours.

Electrostatic extrusion is a technique suitable for the encapsulation of thermolabile compounds since it is performed at ambient temperature and it allows encapsulation of high concentrations of bioactive molecules. The main limitation of this technique is the difficulty of scale-up which restricts the industrial exploitation of this technique that is generally considered to be low energy, cost saving and able to produce microspheres with unique characteristics. For this reason, several solutions have been suggested including modification of structural aspects by developing continuous operation and multi-needle systems that would allow wider use of the technique and utilization of its benefits (Bamidele & Emmambux, 2021).

4. BIOAVAILABILITY OF PHENOLS

Bioavailability represents the portion of bioactive compounds which the human body can absorb and utilize and as such is the key factor influencing the bioactivity of phenols (Carbonell-Capella et al., 2014). The bioactivity of bioactive compounds, including phenols, determined by *in-vitro* methods on the parent compounds may differ significantly from those detected *in-vivo* due to the low stability and challenging absorption of phenols during digestion (Quirós-Sauceda et al., 2014). Since the determination of bioavailability is complicated and raises ethical issues, most research is focused on *in-vitro* simulation of digestion and determination of bioaccessibility which represents the content of bioactive compounds released from food matrix into the gastrointestinal tract which can be absorbed or bioavailable (Carbonell-Capella et al., 2014). The bioaccessibility of phenols depends on the chemical structure of the compound, the degree of polymerization, glycosylation, acylation and conjugation with other compounds. Phenols with high molecular weight (e.g. hydrolysable and condensed tannins), complex flavonoids conjugated with sugars and acetylated hydroxycinnamic acids have been shown to have lower bioaccessibility compared to aglycones and phenols with lower molecular weight (Quirós-Sauceda et al., 2014). Additionally, the gut microbiota, enzyme modifications, and bloodstream transport affect the bioavailability of phenols after absorption (Gao & Hu, 2010).

When phenols are consumed from food and not in the form of supplements that enter the stomach directly, their digestion begins in the oral cavity, where phenols come in contact with saliva (pH 6-7). Phenols are not significantly affected by amylase (the most dominant enzyme in saliva) (Bohn, 2014), however they might bind to acidic proteins through hydrophobic interactions or hydrogen bonds, as in case of tannins (Soares et al., 2011). Also, the phenols bound to a sugar moiety may go through deglycosylation by residual bacteria in the oral cavity (Bohn, 2014).

Most phenols are released from the food matrix during gastric digestion due to the occurrence of hydrolysis and deconjugation, while some (e.g. phenolic acids) may be absorbed in the stomach in their free form (Bohn, 2014). After gastric digestion, the unabsorbed phenols enter the small intestine, where the pH changes from acidic (pH 2–4) to neutral (pH 7). The acidic conditions prevailing at the beginning of small intestinal digestion may favor the presence of the phenols (Singh et al., 2009). It is estimated that only 5–10% of dietary phenols are absorbed in the small intestine, while the rest reach the colon (Selma et al., 2009). Since phenols are mostly present in the polar form as glycosides, their absorption in the small

intestine is unlikely, but those that are hydrolyzed and become aglycones may enter the liver via the portal vein (Rein et al., 2013). When pH increases, pancreatic enzymes and bile are activated and contribute to the absorption of phenols. There are two suggested pathways leading to the absorption of phenols. The first involves the hydrolysis in the intestinal lumen to form aglycones that are absorbed into enterocytes by passive diffusion, while the other involves a sodium-dependent glucose transporter (SGLT) that assists in the active transport of phenols across the brush membrane (Crozier et al., 2010). Two enzymes present in the small intestine, cytosolic β-glucosidase (CBG) and lactase-phlorizin hydrolase (LPH), hydrolyze glycosylated flavonoids, with LPH showing selectivity for flavonoid *O*-β-D-glucosides (Rein et al., 2013). The resulting aglycones enter epithelial cells by passive diffusion due to their increased lipophilicity and are adjacent to the cell membrane. Passive diffusion transfer is characteristic of low molecular weight phenols such as phenolic acids, epicatechin and catechin (B. Hu et al., 2017). After absorption of aglycones, they undergo further metabolism at the enterocyte level. Phenols reach the bloodstream either in free form or bound to other compounds such as proteins (Selma et al., 2009). The degree of polymerization has been shown to have a significant effect on cellular uptake. For example, the absorption of procyanidin dimers is much lower than monomeric units of catechin, while polymeric procyanidins are considered inaccessible for absorption (Actis-Goretta et al., 2013).

Further metabolism of phenols includes conjugating glucuronidation, sulfation, and methylation reactions (Lewandowska et al., 2016). Conjugation is a detoxification process characterized by the presence of conjugation enzymes such as sulfotransferases (SULT), UDP-glucuronosyltransferases (UGT), catechol-*O*-methyltransferases (COMT), and glutathione transferases (GT), which aim to prevent the potential toxic effects of xenobiotics, in this case polyphenols, by increasing the hydrophilicity of the ingested compounds and consequently promoting their excretion. Following these processes, polyphenols may be further metabolized in the liver by the action of phase I cytochrome P450 (CYP450) and enter target tissues via the bloodstream or be excreted via bile, thus returning to the intestinal tract (Kawabata et al., 2019). Hydrophilic conjugates and phenols that are not absorbed by the colon become a substrate of action in the intestinal microflora in the colon, where bacteria hydrolyze the glycosidic bonds between polyphenols and secrete extracellular glycosidases (Liu & Hu, 2007). In addition, the bacteria have a catabolic effect that enables the cleavage of C-C bonds of heterocyclic and aromatic rings, dehydroxylation, demethylation, decarboxylation, and hydrogenation of alkene residues (Kawabata et al., 2019). They also express high levels of glucuronidases and

sulfatases, which enable the release of aglycones (Liu & Hu, 2007). Different bacterial species and genera have different metabolic properties, and therefore the fate of phenols is largely dependent on the composition of the gut microflora (Hoyles & Swann, 2019). In addition, polyphenols reaching the colon can also improve the composition of gut microflora by acting as prebiotics and exhibiting selective bactericidal activity towards pathogenic bacteria, resulting in improved health status of the host and reduced risk of several chronic diseases related to gut dysbiosis (Lippolis et al., 2023).

The metabolites formed by the liver (e.g. quercetin-3-*O*-glucuronide and kaempferol-3-*O*-glucuronide), as well as those formed by gut microflora (e.g. 4-hydroxyphenylocytic acid as a common metabolite of kaempferol) reach the target tissues and cells through bloodstream where they exert various biological activities beneficial to human health including antioxidant (Baeza et al., 2016; Dueñas et al., 2010; González-Manzano et al., 2011; Stepanić et al., 2019), anti-inflammatory (Derlindati et al., 2012; di Gesso et al., 2015; Ha et al., 2017; W. Hu et al., 2016; Kamalakararao et al., 2018; Kure et al., 2016), antitumor (Delgado et al., 2014; Stanisławska et al., 2019; Z. Zhang et al., 2014), cardioprotective (Baeza et al., 2017; Terao, 2023; Van Rymenant et al., 2017; Warner et al., 2016) and neuroprotective effects (Ho et al., 2019; Mukai et al., 2012), showing that the biological activity of the parent compounds determined in the plant material is preserved in their metabolites.

All of the mentioned findings highlight the importance of preserving the stability of phenols throughout all of the stages of digestion in order to utilize their maximum potential. Encapsulation by different techniques has been shown to be an efficient tool for increasing the stability and bioaccessibility of plant derived phenols. For example, spray drying of phenolic olive leaf extract using sodium alginate resulted in 58% higher bioaccessibility of phenols during the small intestinal phase and 20% higher bioavailability compared to the initial extract (González et al., 2019). In another research, spray dried chitosan- liposome powders containing cocoa hull waste phenols had sixfold higher bioaccessibility of phenols in the small intestinal phase of digestion compared to the initial extract (Altin et al., 2018a). Similar results were observed in a study where electrospinning of sour cherry anthocyanins using gelatin increased their bioaccessibility by eightfold (Isik et al., 2018). Moreover, encapsulation using β-cyclodextrin and a mixture of gelatin and pectin increased the stability of flavonoid taxifolin by 1.2 and 1.8 fold throughout the digestive system compared to a water solution, respectively (Fatkullin et al., 2021). Similar results were observed in a study where resveratrol was encapsulated by electrospraying with zein as carrier and 1.5 fold higher stability of this

compound was observed during in-vitro digestion compared to the non-encapsulated compound (Jayan et al., 2019).

In addition, the effectiveness of encapsulation in increasing bioaccessibility of phenols has been confirmed in several studies where the encapsulated phenols were applied in functional food. In one study, roselle encapsulated within gelatin (confectionary gum) was compared to grounded roselle during in-vitro digestion and it was found that gelatin enabled 30% higher controlled release of phenols compared to the grounded roselle (Villanueva-Carvajal et al., 2013). Another study showed that lyophilized colloidal cinnamon nanoparticles incorporated in white chocolate resulted in an increased physicochemical stability and controlled release compared to chocolate enriched with non-encapsulated cinnamon extract during in-vitro digestion (Muhammad et al., 2018). In a research where drinking yogurt was enriched with cocoa hull waste phenols encapsulated by different techniques (freeze drying, spray drying, chitosan-coated liposomes, spray dried chitosan coated liposomes), it was shown that spray dried chitosan-coated liposomes provided the highest stability of cocoa hull waste phenols as they protected the phenols from interacting with the proteins from yogurt (Altin et al., 2018b). Green tea phenols encapsulated in soy lecithin liposomes were incorporated into a hard low-fat cheese and the total phenolic content and antioxidant capacity were increased after in-vitro digestion showing that encapsulation efficiently preserved the beneficial effects of phenols during digestion (Rashidinejad et al., 2016). In another study, a gellified fish product was enriched with curcumin-gelatin microparticles and compared to a gel containing non-encapsulated commercial curcumin during in-vitro digestion (Gómez-Estaca et al., 2015). The results showed that bioaccessibility of curcumin was similar in both gels, however the gel fortified with microparticles allowed higher antioxidant activity due to the protection of curcumin from complexation with water soluble proteins. These results show that encapsulation not only ensures the stabilization of phenols and allows them to reach the consumers at appropriate levels, but also allows preservation of their biological activity during digestion and increases their bioaccessibility and bioavailability from various functional food products.

5. AIMS, HYPOTHESES AND EXPECTED CONTRIBUTION

This research hypothesized that:

(i) extracts obtained by advanced extraction techniques including ASE/PLE (accelerated solvent extraction/pressurized liquid extraction), MAE (microwave-assisted extraction) and UAE (ultrasound assisted extraction) will have higher phenolic content

(ii) stability and quality of encapsulates will depend on encapsulation conditions, especially on the carrier used

(iii) extracts with higher phenolic content will have higher antioxidant capacity, while the bioaccessibility of the powders will depend on the carrier applied

In order to confirm or decline established hypotheses, the following objectives were defined:

(i) to determine the optimal conditions at which ASE, MAE and UAE result in laurel leaf extracts with the highest phenolic yields and compare them with CRE (conventional heat reflux extraction)

(ii) to optimize encapsulation by spray drying and electrostatic extrusion

(iii) to determine the antioxidant capacity of the extracts and physicochemical properties and bioaccessibility of encapsulated phenols.

Due to its complexity and scope, this research was divided into 2 parts.

In the first phase of research, the influence of advanced extraction techniques' parameters on the phenolic yield of the obtained extracts was examined and optimal conditions were defined for each technique (*Publication No.2* and *Publication No.3*).

In the second phase of research, the influence of two encapsulation techniques' parameters on the physicochemical properties and bioccessibility of the obtained microcapsules was examined and the optimal parameters were defined.

Throughout this dissertation, the following questions were examined:

1. Which percentage of ethanol in an aqueous solution is the most efficient for the conventional heat reflux extraction of laurel leaf phenols? (*Publication No.2*)
2. What are the most efficient extraction conditions of MAE (ethanol percentage, temperature, microwave power, extraction time), UAE (ethanol percentage, amplitude, extraction time) and PLE (ethanol percentage,

temperature, number of extraction cycles and static extraction time) that result in the highest phenolic yield? (*Publication No.2* and *Publication No.3*)

3. Do the advanced extraction techniques improve the extraction efficiency of laurel leaf phenols and how do they affect individual phenolic content and antioxidant activity of the obtained extracts? (*Publication No.2* and *Publication No.3*)
4. How do encapsulation conditions of spray drying (carrier type, sample:carrier ratio and inlet temperature) affect the physicochemical properties, phenolic profile, antioxidant activity and bioaccessibility of the encapsulated laurel leaf phenols? (*Publication No.4*)
5. How do the encapsulation conditions of electrostatic extrusion (percentage of sodium alginate, content of $CaCl_2$ and presence of chitosan in the cross-linking (gelling) solutions) affect individual phenolic content, antioxidant activity, release kinetics and bioaccessibility of encapsulated laurel leaf phenols? (*Publication No.5*)

Throughout this dissertation, the following was achieved:

1. The understanding of the influence of MAE, PLE and UAE and their parameters on the phenolic content and antioxidant activity of laurel leaf extracts was improved
2. The most efficient extraction parameters for MAE, PLE and UAE were defined
3. The knowledge of the influence of spray drying and electrostatic extrusion and their parameters on the physicochemical characteristics, phenolic profile and bioaccessibility of laurel leaf phenols was improved
4. The most efficient encapsulation parameters of spray drying and electrostatic extrusion were defined
5. A basis for future development of functional products based on liquid or encapsulated herbal laurel extracts was established

CHAPTER 2

Publication No.2: Polyphenolic Characterization and Antioxidant Capacity of Laurus nobilis L. Leaf Extracts Obtained by Green and Conventional Extraction Techniques.

Processes

Publication No.2

Dobroslavić, E., Elez Garofulić, I., Zorić, Z., Pedisić, S., & Dragović-Uzelac, V. (2021). Polyphenolic Characterization and Antioxidant Capacity of *Laurus nobilis* L. Leaf Extracts Obtained by Green and Conventional Extraction Techniques. *Processes, 9*(10), 1840.

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Erika Dobroslavić: conceptualization, methodology, formal analysis, data curation, writing-original draft preparation

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Article

Polyphenolic Characterization and Antioxidant Capacity of *Laurus nobilis* L. Leaf Extracts Obtained by Green and Conventional Extraction Techniques

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Abstract: *Laurus nobilis* L. is an evergreen Mediterranean shrub whose leaves have been known for various health-promoting effects mainly attributed to polyphenols. Microwave- (MAE) and ultrasound-assisted extraction (UAE) are green extraction techniques that enable effective isolation of polyphenols from plant material. Therefore, the aim of this research was to optimize the extraction conditions of MAE (ethanol percentage, temperature, extraction time, microwave power) and UAE (ethanol percentage, extraction time, amplitude) of polyphenols from *Laurus nobilis* L. leaves and to assess their polyphenolic profile by ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) and antioxidant capacity by oxygen radical absorbance capacity (ORAC) assay. Optimal MAE conditions were 50% ethanol, 80 °C, 10 min and 400 W. Optimal UAE conditions were 70% ethanol, 10 min and 50% amplitude. Spectrophotometric analysis showed the highest total phenolic content in the extracts was obtained by MAE, compared to conventional heat-reflux extraction (CRE) and UAE. The polyphenolic profile of all obtained extracts included 29 compounds, with kaempferol and quercetin glycosides being the most abundant. UPLC-MS/MS showed the highest total phenolic content in the extracts obtained by CRE. ORAC assay showed the highest antioxidant capacity in extracts obtained by CRE, which is in agreement with the polyphenolic profile determined by UPLC-MS/MS.

Keywords: *Laurus nobilis* L.; plant extracts; polyphenols; microwave-assisted extraction; ultrasound-assisted extraction; UPLC-MS/MS; ORAC

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1. Introduction

Laurus nobilis L., a representative of the family Lauraceae, is an evergreen shrub native to the Mediterranean area. The leaves of this plant have traditionally been used in folk medicine to treat various health conditions, mainly respiratory and gastrointestinal disorders [1]. Due to their beneficial effects, which nowadays can be attributed to various biological activities of leaf extracts and essential oils including antioxidant [2,3], anti-inflammatory [4,5], antimicrobial and antifungal [6,7], the chemical composition of *Laurus nobilis* L. leaves has been studied to a greater extent than that of other plant parts. *Laurus nobilis* L. leaves comprise the aforementioned essential oils, alkaloids, norisoprenoids, sugars, polysaccharides, organic acids, tocopherols and a wide range of polyphenols including different flavonoids, phenolic acids, tannins and lignans [8]. Polyphenols are a group of compounds that are of particular interest due to their redox properties, as they can act as antioxidant agents [9] and, thus, are largely responsible for the antioxidant activity of *Laurus nobilis* L. [3,10,11]. Plant material usually contains a wide range of polyphenols, including simple to highly polymerized compounds which can also be conjoined with various other components, making their recovery a challenging process [12]. Establishing an optimal methodology for the isolation of polyphenols is a crucial step

for the utilization of their beneficial properties, and various techniques can be applied in order to achieve their effective recovery. Conventional extraction techniques, such as heat-reflux, although easily applicable, are often time-, energy- and solvent-consuming with difficulties when it comes to scale-up processes [13]. These techniques also carry the risk of thermal degradation of heat-sensitive polyphenolic compounds [14]. In recent years, advanced green extraction techniques, such as microwave-assisted (MAE) and ultrasound-assisted extraction (UAE), have been in focus when it comes to the extraction of polyphenols from different plant materials [15–19]. The main advantage of both MAE and UAE over conventional heat-reflux extraction (CRE) is the reduction in extraction time, resulting in lower solvent consumption and higher extraction efficiency along with less thermal degradation of sensitive compounds [20,21]. In MAE, the dipole rotation induced by the electromagnetic wave radiation leads to homogeneous heating of the sample, which leads to disruption of the plant cell and release of the targeted compounds from the plant matrix [22]. In UAE, the cell disruption is caused by ultrasonic waves, which generate cavitation bubbles that burst near the sample tissue. The distribution of ultrasonic waves is not homogenous and the wave power decreases with the increase in distance between the sample and radiating surface, which is why shaking and agitation are useful during UAE [23]. The efficiency of both MAE and UAE depends on their parameters (e.g., extraction time, temperature, solvent type, microwave power (MAE), frequency and amplitude of ultrasonic waves (UAE)), which should be chosen with respect to the properties of the plant material and the targeted compounds. In addition to the isolation of polyphenols, chemical characterization of the obtained extracts, including the identification and quantification of individual compounds and evaluation of their antioxidant capacity, is also of great interest. Combined chromatographic and spectral techniques, such as UPLC-MS/MS, have been shown to be the most effective for chemical characterization of even the most complex of polyphenolic structures such as flavonoid glycosides and proanthocyanidins [24]. Antioxidant capacity can be determined using several assays divided into two categories: single electron transfer (SET) assays (DPPH, FRAP, ABTS) and hydrogen atom transfer (HAT) assays (ORAC, TRAP, TOSC, CL) [25]. ORAC (oxygen radical absorbance capacity) is a method that uses the most biologically prevalent peroxyl radical as a source of free radicals [26] and can measure both hydrophilic and lipophilic antioxidants [27], making it one of the most significant assays in terms of its biological relevance. Therefore, ORAC has been established as an assay of choice for determining the antioxidant capacity of plant material and food.

The aim of this research, therefore, was to investigate the influence of different extraction parameters in MAE (solvent, temperature, extraction time and microwave power) and UAE (solvent, extraction time and amplitude) on the total phenolic content of *Laurus nobilis* L. leaf extracts and to establish optimal extraction conditions for both extraction techniques. Moreover, data on the polyphenolic profile of *Laurus nobilis* L. leaves obtained by MAE and UAE are scarce [28–30], and, to our knowledge, no comparison of the polyphenolic profiles obtained between the two techniques has been reported so far. Hence, the aim of this research was to determine and compare the UPLC-MS/MS polyphenolic profile of the extracts obtained with MAE, UAE and CRE and to determine their antioxidant capacity using the ORAC assay.

2. Materials and Methods

2.1. Chemicals and Reagents

Ethanol (96%) was purchased from Lach-ner (Neratovice, Czech Republic), HPLC grade acetonitrile from J.T. Baker Chemicals (Deventer, Netherlands) and formic acid (98–100%) from T.T.T. d.o.o. (Sveta Nedjelja, Croatia). Distilled water was purified by Milli-Q water purification system (Millipore, Bedford, MA, USA). Folin–Ciocalteu reagent was obtained from Merck (Darmstadt, Germany), anhydrous sodium carbonate (≥99.5%) and sodium phosphate (96%) from Kemika (Zagreb, Croatia), 6-hydroxy-2,5,7,8-

tetramethylchroman-2-carboxylic acid (Trolox) from Acros Organics (Thermo Fisher Scientific, Geel, Belgium), 2,20-Azobis (2-amidinopropane) hydrochloride from Sigma-Aldrich (Steinheim, Germany) and fluorescein sodium salt from Honeywell Riedel-de-Haën (Bucharest, Romania). Authentic standards of quercetin-3-glucoside, myricetin, caffeic acid, gallic acid, ferulic acid, protocatechuic acid, syringic acid, rosmarinic acid, chlorogenic acid and *p*-coumaric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Catechin, epigallocatechin gallate, epicatechin gallate, kaempferol-3-glucoside, rutin, apigenin, procyanidin B2 and luteolin were procured from Extrasynthese (Genay, France). All standards were prepared as methanol stock solution except apigenin, which was dissolved in ethanol with 0.5% (v/v) dimethyl sulfoxide. Working standard solutions were prepared by dilution of the stock solutions to produce five concentrations.

2.2. Plant Material

A sample of dry leaves of *Laurus nobilis* L., collected in November 2020 in the Rijeka region, Croatia, was purchased from Šafram d.o.o (Zagreb, Croatia). The dry leaves were stored at room temperature and ground into fine powder using an electric grinder (GT11, Tefal, Rumilly, France) before extraction. The obtained powder was analyzed for total solids by drying to constant mass at 103 ± 2 °C [31]. Content of dry matter in the sample was >95%.

2.3. Conventional Heat-Reflux Extraction (CRE)

The polyphenols of *Laurus nobilis* L. leaves were extracted from 1 g of ground sample with 40 mL of aqueous ethanol solution (50% and 70% *v*/*v*) in a flat bottom Erlenmeyer flask. The solvents for all extractions were selected based on previous literature reports showing that 50% and 70% aqueous ethanol were suitable for isolation of polyphenols from *Laurus nobilis* leaves and similar plant material [29,32]. The mixture was extracted with reflux for 30 min, filtered through Whatman No. 40 filter paper (Whatman International Ltd., Kent, UK) and made up to 50 mL in volumetric flasks with the extraction solvent. The extracts were transferred into plastic Falcon tubes and stored at −18 °C in nitrogen gas atmosphere. All extracts were prepared in duplicate.

2.4. Microwave-Assisted Extraction (MAE)

The MAE of polyphenols from *Laurus nobilis* L. leaves was performed using Ethos Easy (Milestone, Italy) microwave reactor. General extraction parameters: the time required to achieve extraction temperature, stirring and ventilation after extraction were kept constant at 2 min, 50% and 1 min, respectively. The varied extraction parameters were temperature (40, 60 and 80 °C), microwave power (400 and 800 W) and time (5, 10 and 15 min). For each extraction, 1 g of ground sample was mixed with 40 mL of ethanol solution in the extraction vessel with a magnetic stirrer and placed into the microwave reactor. After cooling at room temperature, the obtained extracts were filtered through Whatman No. 40 filter paper into 50 mL volumetric flasks, made up to volume with solvent, transferred into plastic Falcon tubes and stored at −18 °C in nitrogen gas atmosphere. All extracts were prepared in duplicate.

2.5. Ultrasound-Assisted Extraction

For the UAE of polyphenols from *Laurus nobilis* L. leaves, 1 g of ground sample was mixed with 40 mL of the extraction solvent in a glass beaker. UAE was performed using an ultrasonic processor (UP) 400 S (Dr. Hielscher GmbH, Teltow, Germany) that has maximal nominal output power 400 W and the ultrasonic frequency 24 kHz. The UP is equipped with an ultrasonic probe (surface 3.8 cm^2), which was immersed 1 cm into the beaker with sample mixture. The varied parameters were extraction time (5, 10 and 15 min) and amplitude (50, 75 and 100%). The temperature was monitored using an infrared thermometer and it did not exceed 30 °C, which was achieved by placing the beaker in a

cooling bath with ice during the extraction. The extracts were filtered through Whatman No. 40 filter paper, made up to 50 mL in volumetric flasks, transferred to plastic Falcon tubes and stored at −18 °C in nitrogen gas atmosphere. All extracts were prepared in duplicate.

2.6. Determination of Total Phenolic Content

Total phenolic content of *Laurus nobilis* L. leaves was determined by the spectrophotometric Folin–Ciocalteu method previously described by Shortle et al. (2014) [33] with some modifications. A 100 μL aliquot of sample extract (solvent extraction for blank) was mixed with 200 μL Folin–Ciocalteu reagent and 2 mL distilled water. After 3 min, 1 mL of 20% w/v sodium carbonate solution was added into the mixture. After tempering for 25 min at 50 °C in a water bath, the absorbance was read at 765 nm. All measurements were performed in duplicate. A gallic acid standard calibration curve (y = 0.0035x, R2 = 0.9995) was prepared from working standard solutions in concentration range from 50 to 500 mg L^{-1}. Total phenolic content (TPC) of the samples was calculated and expressed as mean value in mg gallic acid equivalents (GAE) per g of sample ± standard deviation.

2.7. Identification and Quantification of Polyphenols

Identification and quantification of polyphenols in extracts obtained at optimized conditions were performed on UPLC-MS/MS in positive and negative ionization mode on Agilent 6430 Triple Quad LC/MS mass spectrometer (Agilent, Santa Clara, CA, USA) connected to UPLC system (Agilent series 1290 RRLC instrument) consisting of binary pump, autosampler and a column compartment thermostat. Ionization of the analytes was performed by ESI ion source and nitrogen was used as desolvation and collision gas with following parameters: drying gas temperature 300 °C, flow rate 11 L h^{-1}, capillary voltage 4000/−3500 V and the nebulizer pressure 40 psi. Agilent's Zorbax Eclipse Plus C18 column (100 × 2.1 mm; particle size 1.8 μm) was used for separations with following conditions: column temperature 35 °C, injection volume 2.5 μm. The composition of solvents as well as gradient conditions that were used were previously described by Elez Garofulić et al. (2018) [34]. Instrument control and data processing was performed using Agilent MassHunter Workstation Software (ver. B.04.01). The identification and quantitative determination was carried out on the basis of the calibration curves of the standards: myricetin, caffeic acid, gallic acid, ferulic acid, protocatechuic acid, syringic acid, rosmarinic acid, chlorogenic and *p*-coumaric acid, quercetin-3-glucoside, quercetin-3-rutinoside, kaempferol-3-glucoside, catechin, epigallocatechin gallate, epicatechin gallate, apigenin, procyanidin B2 and luteolin. For compounds lacking reference standards, identification was based on mass spectral data and literature reports of mass fragmentation patterns, while quantification was performed as follows: kaempferol-3-rutinoside, kaempferol-3-O-hexoside, kaempferol-3-O-deoxyhexoside and kaempferol-3-O-pentoside were calculated according to kaempferol-3-glucoside, apigenin-6-C-(O-deoxyhexosyl)-hexoside according to apigenin, luteolin-6-C-glucoside according to luteolin, isorhamnetin-3-hexoside, quercetin-3-rhamnoside and quercetin-3-pentoside according to quercetin-3-glucoside, epicatechin according to catechin, 3,4-dihydroxybenzoic acid hexoside according to protocatehuic acid while *p*-hydroxybenzoic acid was calculated as gallic acid equivalent. Quality parameters for the analytical method, including calibration curves, instrumental detection (LOD) and quantification (LOQ) limits, were reported previously [34]. Concentrations of analyzed compounds were expressed as mg per 100 g of sample as mean value ± standard deviation. All analyses were performed in duplicate.

2.8. Oxygen Radical Absorbance Capacity (ORAC) Assay

The oxygen radical absorbance capacity (ORAC) assay was carried out on an automated plate reader (BMG LABTECH, Offenburg, Germany) following a previously reported method [35] and the data analysis was performed using MARS 2.0 software. In total, 75 μM phosphate buffer (pH 7.4) was used for preparation of 240 mM 2,20-Azobisradical (2-amidinopropane) dihydrochloride (AAPH) solution, 70.3 nM fluorescein solution and different dilutions (3.12–103.99 μM) of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). Briefly, Trolox standard or appropriately diluted sample were added into a 96-well microplate containing 150 μL of fluorescein and the plate was incubated at 37 °C for 30 min. After the first three cycles (baseline signal), AAPH solution was injected to generate the peroxyl radical. During the total measurement period (120 min), the fluorescence intensity (excitation at 485 nm and emission at 528 nm) was monitored every 90 s. Determinations were performed in duplicate (n = 4) and the results were expressed as μmol Trolox equivalent (TE) per g of sample as mean value ± standard deviation.

2.9. Statistical Analysis

Statistica ver. 10.0 software (StatSoft Inc., Tulsa, OK, USA) was used for statistical analysis. Total phenolic content was the dependent variable, while the independent variables were: (a) solvent (50% and 70% ethanol) for all extraction techniques, (b) time (MAE and UAE, 5, 10 and 15 min), (c) temperature (40, 60 and 80 °C) and microwave power (400 and 800 W) for MAE and (d) amplitude (50, 75 and 100%) for UAE. Multifactorial analysis of variance (MANOVA) was used for the continuous variable analysis while marginal means were compared using Tukey's HSD multiple comparison test. One-way analysis of variance (ANOVA) and post-hoc Tukey's HSD multiple comparison test was carried out for comparison of the ORAC values, and individual and total phenolic contents obtained by CRE, MAE and UAE. All of the tests were significant at $p \leq 0.05$.

3. Results and Discussion

This study examined the influence of different extraction parameters of MAE and UAE on the content of polyphenols in *Laurus nobilis* L. leaf extracts compared to conventional heat-reflux extraction. The total phenolic content of the obtained extracts was measured by the Folin–Ciocalteu spectrometric method (Table 1) and the optimal extraction conditions were determined by statistical analysis. The identification and quantification of polyphenols in extracts obtained at optimized extraction conditions was assessed by UPLC-MS/MS and their antioxidant capacity was characterized by ORAC assay.

Table 1. Total phenolic content of L. nobilis L. leaf extracts obtained by different extraction parameters and techniques.

Extraction Technique	Extraction Parameters				TPC (mg GAE g^{-1})
CRE	%EtOH				
	50%				42.35 ± 0.86
	70%				42.21 ± 0.65
MAE	%EtOH	Time (min)	Temperature (°C)	Microwave power (W)	
	50	5	40	400	38.75 ± 1.01
	50	5	40	800	32.91 ± 1.21
	50	10	40	400	33.88 ± 0.35
	50	10	40	800	39.99 ± 1.25
	50	15	40	400	39.63 ± 2.06
	50	15	40	800	33.30 ± 1.36

	50	5	60	400	36.91 ± 0.70
	50	5	60	800	35.57 ± 0.70
	50	10	60	400	36.15 ± 0.60
	50	10	60	800	36.60 ± 0.80
	50	15	60	400	36.86 ± 2.47
	50	15	60	800	36.91 ± 0.95
	50	5	80	400	40.90 ± 0.40
	50	5	80	800	39.74 ± 1.36
	50	10	80	400	53.57 ± 1.01
	50	10	80	800	43.75 ± 0.25
	50	15	80	400	49.44 ± 2.11
	50	15	80	800	44.51 ± 1.81
	70	5	40	400	33.60 ± 0.80
	70	5	40	800	40.17 ± 0.80
	70	10	40	400	33.31 ± 0.50
	70	10	40	800	31.87 ± 1.15
	70	15	40	400	30.88 ± 1.61
	70	15	40	800	34.29 ± 0.30
	70	5	60	400	41.08 ± 1.31
	70	5	60	800	36.64 ± 1.01
	70	10	60	400	38.40 ± 0.75
	70	10	60	800	42.68 ± 0.76
	70	15	60	400	42.13 ± 0.65
	70	15	60	800	39.13 ± 0.40
	70	5	80	400	42.79 ± 0.95
	70	5	80	800	43.36 ± 1.05
	70	10	80	400	46.17 ± 0.55
	70	10	80	800	44.21 ± 0.15
	70	15	80	400	46.51 ± 1.91
	70	15	80	800	46.53 ± 1.71
	%EtOH	Time (min)	Amplitude (%)		
	50	5	50		24.43 ± 1.31
	50	5	75		31.18 ± 1.41
	50	5	100		27.46 ± 0.50
	50	10	50		29.78 ± 1.16
	50	10	75		31.70 ± 1.71
	50	10	100		29.12 ± 1.11
	50	15	50		36.74 ± 2.12
	50	15	75		33.96 ± 1.01
UAE	50	15	100		28.89 ± 0.30
	70	5	50		30.16 ± 1.16
	70	5	75		25.23 ± 1.31
	70	5	100		27.77 ± 0.25
	70	10	50		32.85 ± 1.16
	70	10	75		32.52 ± 0.61
	70	10	100		35.04 ± 0.10
	70	15	50		31.98 ± 0.20
	70	15	75		31.88 ± 0.55
	70	15	100		33.36 ± 0.96

TPC = total phenolic content, CRE = conventional heat-reflux extraction, MAE = microwave-assisted extraction, UAE = ultrasound-assisted extraction. Results are expressed as mean ± SD

The total phenolic content of the *Laurus nobilis* L. leaf extract obtained by CRE was 42.21−42.35 mg GAE g^{-1}, which is higher than the 10.23 mg GAE g^{-1} reported by Muniz-Marquez et al. (2014) [11] and similar to the 46.79 mg GAE g^{-1} reported by Lu et al. (2011) [36]. The total phenolic content obtained by MAE ranged from 30.88 to 53.57 mg GAE g^{-1}, which is higher than the 10.63 mg GAE g^{-1} reported by Muniz-Marquez et al. (2018) [29] and the 21.56 mg GAE g^{-1} reported by Rincon et al. (2019) [37]. The values of total phenolic content obtained by UAE ranged from 24.43 to 36.74 mg GAE g^{-1} which is higher than the 17.32 mg GAE g^{-1} reported by Muniz-Marquez et al. (2013) [28] and similar to the 24.77 mg GAE g^{-1} reported by Rincon et al. (2019) [37].

3.1. Conventional Heat-Reflux Extraction (CRE)

The influence of ethanol concentration used for MAE and UAE (50% and 70%) on the yield of polyphenols was also examined in extracts obtained by CRE (Table 2). It was shown that ethanol concentration had no statistically significant influence on the yield of polyphenols, which was also observed in the conventional extraction of polyphenols from Olea europaea L. leaves [38] with 50% and 70% aqueous ethanol, as well as in the conventional extraction of polyphenols from Limnophila aromatica [39] when 50% and 75% aqueous ethanol were used. Therefore, 50% aqueous ethanol solution was chosen as optimal to obtain maximum total phenolic content in the *Laurus nobilis* L. leaf extracts obtained by CRE.

Table 2. Influence of extraction parameters on total phenolic content of L. nobilis leaf extracts.

Extraction Technique	Source of Variation	Total Phenolic Content (mg GAE g^{-1})
CRE	% EtOH	p = 0.86 ‡
	50% *w*/*w*	42.35 ± 0.54 [a]
	70% *w*/*w*	42.21 ± 0.55 [a]
MAE	% EtOH	p = 0.38 ‡
	50% *w*/*w*	39.41 ± 0.19 [a]
	70% *w*/*w*	39.65 ± 0.19 [a]
	Temperature (°C)	p ≤ 0.01 †
	40 °C	35.22 ± 0.24 [a]
	60 °C	38.25 ± 0.24 [b]
	80 °C	45.12 ± 0.24 [c]
	Time(min)	p ≤ 0.01 †
	5 min	38.53 ± 0.24 [a]
	10 min	40.05 ± 0.24 [b]
	15 min	40.01 ± 0.24 [b]
	Microwave power (W)	p ≤ 0.01 †
	400 W	40.05 ± 0.19 [b]
	800 W	39.01 ± 0.19 [a]
UAE	% EtOH	p ≤ 0.05 †
	50% *w*/*w*	30.36 ± 0.26 [a]
	70% *w*/*w*	31.20 ± 0.26 [b]
	Time (min)	p ≤ 0.01 †
	5 min	27.70 ± 0.31 [a]
	10 min	31.84 ± 0.31 [b]
	15 min	32.80 ± 0.31 [b]
	Amplitude (%)	p =0.17 ‡
	50%	30.99 ± 0.31 [a]
	70%	31.10 ± 0.31 [a]
	100%	30.27 ± 0.31 [a]

CRE = conventional heat-reflux extraction, MAE = microwave-assisted extraction, UAE = ultrasound-assisted extraction. Results are expressed as mean ± SE. Values with different letters are statistically different at $p \leq 0.05$. † Statistically significant variable at $p \leq 0.05$. ‡ Statistically insignificant variable at $p \leq 0.05$.

3.2. Microwave-Assisted Extraction (MAE) Optimization

Ethanol concentration (50 and 70%), temperature (40, 60 and 80 °C), time (5, 10 and 15 min) and microwave power (400 and 800 W) were varied during MAE of polyphenols from *Laurus nobilis* L. leaves. The obtained results were statistically analyzed and the results are shown in Table 2. There was no statistically significant difference in the total phenolic content of the extracts obtained with 50 and 70% aqueous ethanol. Lovrić et al. (2017) reported the same observation during MAE of polyphenols from Prunus spinosa L. flowers [40], while Shang et al. (2020) [41] reported a higher total phenolic content of the Lithocarpus polystachyus Rehd. extracts obtained with 60% ethanol in comparison to 50% ethanol. In addition, Ismail-Suhaimy et al. (2021) [42] reported an increase in total phenolic content of Barleria lupulina L. extracts with the increase in ethanol concentration from 40% to 80%. On the other hand, Dahmoune et al. (2015) [43] observed a decline in total phenolic content in Myrtus communis L. leaf extracts with the increase in ethanol concentration from 40% to 60%. The differences in the results obtained by these authors might be attributed to different content and polarity of polyphenols of the investigated plants considering the ''like dissolves like'' principle and the fact that the polarity of the hydroethanolic solvent mixtures depends on the ethanol–water ratio [44].

Temperature plays a key role in MAE by influencing the desorption rate, solubility and degradation of targeted compounds. Most often, elevated temperatures result in higher extraction yields due to increased diffusion of the solvent into the plant matrix and enhanced solubility and desorption of the targeted compounds from the matrix [45]. However, degradation of heat-sensitive compounds may occur when higher temperatures are applied [46]. The influence of temperature on the total phenolic content of *Laurus nobilis* L. leaf extracts was significant ($p \leq 0.01$). Increasing the temperature from 40 to 80 °C resulted in higher total phenolic content of the obtained extracts. This is in accordance with the aforementioned effects of elevated temperature, with the absence of degradation effects since different plant extracts and standard solutions of phenolic compounds were shown to be relatively stable during exposure to temperatures in the range of 60–100 °C [47]. Other authors have also reported similar results. Dobrinčić et al. (2020) [48] reported a higher content of total phenolic compounds extracted from Olea europaea L. leaves with the increase temperature from 45 to 80 °C, while Putnik et al. (2016) [49] observed an increase in total phenolic content of Salvia officinalis L. extracts with the increase in temperature from 30 to 80 °C.

Generally, increased extraction time results in higher yields of targeted compounds until the optimal level of efficiency is achieved, after which the extraction yields may decrease due to degradation of thermolabile compounds [45]. In our study, extraction time significantly ($p \leq 0.01$) influenced the total phenolic content of the extracts. Maximum total phenolic content was obtained after 10 min, which is in agreement with results reported by Muniz-Marquez et al. (2018) [29] where a maximum total phenolic content in *Laurus nobilis* L. leaf extract was achieved after 9 min of extraction. Saraktsianos et al. (2020) [50] reported that 10 min of MAE resulted in the highest total phenolic content of Sideritis raeseri, Sideritis scardica and Origanum vulgare L. extracts. Putnik et al. (2016) [49] also reported a maximum total phenolic yield of Salvia officinalis L. extracts after 10 min of MAE.

Microwave power is another important factor that enhances the extraction efficiency by increasing molecular interactions between the sample and the electromagnetic field [51]. However, degradation of some phenolic compounds may occur during prolonged exposure of the sample to a higher microwave power [52]. Microwave power was also a significant parameter ($p \leq 0.01$) in the MAE of polyphenols from *Laurus nobilis* L. leaves. The total phenolic content of the extracts was lower when 800 W was applied compared

to 400 W. Other authors also reported a decrease in total phenolic content in extracts of different plant material when microwave power higher than 600 W was applied [16,41–43].

Considering the results of statistical analysis, optimal MAE parameters for obtaining the highest content of polyphenols from *Laurus nobilis* L. leaves were: 50% ethanol, temperature 80°C, time 10 min and microwave power 400 W.

3.3. Ultrasound-Assisted Extraction (UAE) Optimization

Ethanol concentration (50 and 70%), time (5, 10 and 15 min) and amplitude (50, 75 and 100%) were varied during the UAE of polyphenols from *Laurus nobilis* L. leaves and the statistically analyzed results are shown in Table 2. Ethanol concentration significantly ($p \leq 0.05$) influenced the yield of the obtained polyphenols. A higher total phenolic content of the extracts was achieved in 70% ethanol, which is different than results reported by Muniz-Marquez et al. (2013) [28] where a maximum total phenolic content of 17.32 mg GAE g^{-1} from dry leaves in *Laurus nobilis* L. extracts obtained by UAE was achieved in 35% ethanol. The achieved total phenolic content in the mentioned study was significantly lower than those achieved under various conditions in our study. This difference might be attributed to a variation in the content of polyphenols in the plant material, possibly due to different phenological phases and environmental growth conditions such as soil quality and climate [53], as well as other extraction parameters including sample-to-solvent ratio, ultrasonic power and extraction time, which can affect the quality and quantity of targeted compounds [54]. Cao et al. (2021) [55], Bouadia-Madi et al. (2019) [56] and Ghitescu et al. (2015) [57] achieved maximum total phenolic content using 70% ethanol during UAE from Triarrhena lutarioriparia, Myrtus communis L. pericarp and Picea abies L. wood bark, respectively. These results are in accordance with our observations.

Extraction time is an important factor in UAE. Prolonged exposure of the sample to the solvent promotes the diffusion of targeted compounds, thus enhancing the extraction yield [58], but may also cause oxidation of phenolic compounds [59], so it is crucial to establish the optimal extraction time for the plant material of interest. Extraction time had a significant ($p \leq 0.01$) effect on the total phenolic content of *Laurus nobilis* L. extracts obtained by UAE. The highest concentration of polyphenols was achieved after 10 min of sonication and prolongation of time to 15 min had no significant effect. This can be explained by the application of Fick's second law of diffusion, which states that final equilibrium is established after a certain time between the solid and the bulk solution [60]. Muniz-Marquez et al. (2013) [28] observed the same trend of achieving maximum concentration at medium time value with no effect of further prolongation of time during the UAE of polyphenols from *Laurus nobilis* L. In accordance with our results, Falleh et al. (2012) [61] reported that 10 min of UAE was optimal for achieving the highest concentration of polyphenols from Mesembryanthemum edule L. Aizoaceae, while Bouadia-Madi (2019) [56] reported that 7.5 min was optimal for the UAE of polyphenols from Myrtus communis L. pericarp.

Amplitude is a parameter that indicates the height of the ultrasonic waves and represents the intensity of sonication that is transmitted to the plant material [62]. The cavitation effect of the ultrasonic waves enhances the extraction rate by increasing local temperature and pressure, which results in breakage of the plant material's cell walls and improved mass transfer rate [63]. This effect is caused by the compression and rarefaction cycle of the waves that depends on their amplitude and, generally, a higher amplitude results in higher extraction efficiency [64]. In our study, the amplitude within the selected range had no significant effect on the total phenolic content of the obtained extracts. Borras-Enriquez et al. (2021) [19] reported the same results when a range of 30–90% amplitude was applied for the UAE of polyphenols from Mangifera indica L. var. Manililla residues. On the other hand, several authors reported a positive influence of higher amplitude on the yield of polyphenols from different plant material [48,56,65]. The

different observations might be attributed to variations in the polyphenolic contents of different plant material. Moreover, in our study, the temperature was constantly kept under 30 °C, which might have reduced the effect of temperature provoked by higher amplitude on the mass transfer rate, thus resulting in the absence of amplitude influence on the concentration of the obtained polyphenols.

Based on the results of statistical analysis, the optimal parameters for the UAE of polyphenols from *Laurus nobilis* L. leaves were selected as follows: 70% aqueous ethanol, 10 min and 50% amplitude.

3.4. Polyphenolic Characterization

In order to investigate the polyphenolic profile of the *Laurus nobilis* L. leaf extracts obtained at defined optimal extraction parameters, UPLC/MS-MS analysis was carried out (Table 3). A total of 29 phenolic compounds, consisting of phenolic acids, flavonols, flavan-3-ols, flavones and proanthocyanidins, were identified in extracts obtained by all three extraction techniques (Figure 1).

Table 3. Mass spectrometric data and identification of phenolic compounds in *Laurus nobilis* L. leaves obtained by optimized extraction conditions.

Compound	RT Min	Precursor Ion (m/z)	Fragment Ions (m/z)	Tentative Identification	Concentration mg 10^{-2} g^{-1}		
					CRE	MAE	UAE
				Phenolic acids			
1	0.874	359.1	161	rosmarinic acid *	0.53 ± 0.03 ^{a}	1.25 ± 0.07 ^{b}	1.44 ± 0.07 ^{b}
2	1.145	197	182	syringic acid *	0.03 ± 0.00 ^{a}	0.04 ± 0.01 ^{a,b}	0.06 ± 0.00 ^{b}
3	2.052	317	155	3,4-dihidrobenzoic acid hexoside	1.75 ± 0.14 ^{a}	2.89 ± 0.14 ^{b}	2.49 ± 0.09 ^{b}
4	3.508	153	109	protocatehuic acid *	2.80 ± 0.18 ^{b}	3.54 ± 0.16 ^{c}	2.04 ± 0.02 ^{a}
5	4.913	353	191	chlorogenic acid *	0.38 ± 0.01 ^{a}	0.38 ± 0.01 ^{a}	0.39 ± 0.03 ^{a}
6	5.074	137	93	*p*-hydroxybenzoic acid	0.72 ± 0.02 ^{a}	1.02 ± 0.01 ^{b}	1.30 ± 0.07 ^{c}
7	5.711	179	135	caffeic acid *	2.55 ± 0.00 ^{a}	34.31 ± 0.52 ^{c}	20.73 ± 0.49 ^{b}
13	7.28	163	119	*p*-coumaric acid *	1.40 ± 0.07 ^{b}	0.83 ± 0.04 ^{a}	0.82 ± 0.01 ^{a}
17	7.917	193	134	ferulic acid *	9.44 ± 0.24 ^{b}	0.78 ± 0.00 ^{a}	1.10 ± 0.03 ^{a}
25	11.443	169	125	gallic acid *	0.45 ± 0.02 ^{a}	1.05 ± 0.03 ^{b}	0.48 ± 0.00 ^{a}
				Flavonols			
12	6.831	433	286	kaempferol-3-O-deoxyhexoside	0.14 ± 0.01 ^{c}	0.06 ± 0.01 ^{a}	0.10 ± 0.00 ^{b}
14	7.301	611	303	Rutin *	28.07 ± 0.57 ^{a}	98.21 ± 2.04 ^{b}	23.14 ± 0.46 ^{a}
16	7.839	465	303.1	quercetin-3-glucoside	51.34 ± 0.64 ^{a}	102.74 ± 2.18 ^{c}	91.83 ± 0.70 ^{b}
19	8.219	595	287	kaempferol-3-rutinoside	24.17 ± 0.21 ^{c}	5.78 ± 0.35 ^{a}	7.52 ± 0.21 ^{b}
20	8.39	435	303	quercetin-3-pentoside	28.32 ± 0.57 ^{c}	8.62 ± 0.28 ^{b}	5.43 ± 0.14 ^{a}
21	8.51	449	287	kaempferol-3-O-hexoside	111.63 ± 1.13 ^{c}	18.73 ± 0.28 ^{b}	14.25 ± 0.35 ^{a}
22	8.616	479	317	isorhamnetin-3-hexoside	40.56 ± 0.35 ^{c}	25.10 ± 0.35 ^{b}	21.62 ± 0.35 ^{a}
23	8.767	449	303	quercetin-3-rhamnoside	12.74 ± 0.21 ^{a}	14.34 ± 0.28 ^{a}	39.96 ± 1.33 ^{b}
24	9.048	419	287	kaempferol-3-O-pentoside	43.90 ± 0.35 ^{b}	8.37 ± 0.21 ^{a}	7.92 ± 0.14 ^{a}
28	12.045	319	273	Myricetin *	0.65 ± 0.05 ^{a}	0.73 ± 0.05 ^{a}	0.78 ± 0.05 ^{a}

				Flavan-3-ols			
8	5.93	291	139	epicatechin	71.17 ± 0.42 [b]	13.65 ± 0.35 [a]	13.90 ± 0.28 [a]
9	5.937	291	139	catechin *	72.37 ± 0.42 [c]	12.62 ± 0.18 [a]	19.88 ± 0.70 [b]
27	12.028	442.9	139	epicatechin gallate *	0.10 ± 0.02 [a]	0.45 ± 0.02 [c]	0.26 ± 0.02 [b]
29	12.268	459	289	epigallocatechin gallate *	0.49 ± 0.05 [b]	0.22 ± 0.02 [a]	0.08 ± 0.04 [a]
				Flavones			
11	6.677	449	329	luteolin-6-C-glucoside	2.10 ± 0.07 [a]	5.23 ± 0.28 [c]	4.04 ± 0.28 [b]
15	7.77	271	153	apigenin *	0.65 ± 0.07 [a]	3.74 ± 0.07 [b]	8.52 ± 0.21 [c]
18	8.157	287	153	luteolin*	3.80 ± 0.21 [a]	7.17 ± 0.21 [b]	11.36 ± 0.35 [c]
26	11.998	579	459	apigenin-6-C-(O-deoxyhexosyl)-hexoside	0.09 ± 0.01 [a]	0.13 ± 0.04 [a]	0.10 ± 0.01 [a]
				Proanthocyanidins			
10	6.249	865	713	procyanidin trimer	20.33 ± 0.28 [c]	7.72 ± 0.21 [a]	15.20 ± 0.42 [b]
Total phenols UPLC-MS/MS (mg 10^{-2} g^{-1})	-	-	-	-	531.35 ± 1.84 [c]	375.74 ± 5.55 [b]	311.47 ± 7.47 [a]
Total phenols A = 765 nm (mg GAE g^{-1})	-	-	-	-	42.35 ± 0.86 [b]	53.57 ± 1.01 [c]	32.85 ± 1.16 [a]
ORAC (μmol TE g-1)	-	-	-	-	100.09 ± 0.21 [b]	86.04 ± 1.26 [a]	90.27 ± 1.11 [a]

CRE = conventional heat-reflux extraction, MAE = microwave-assisted extraction, UAE = ultrasound-assisted extraction. Results are expressed as mean ± SD. * identification confirmed using authentic standards. Values with different letters are statistically different at $p \leq 0.05$

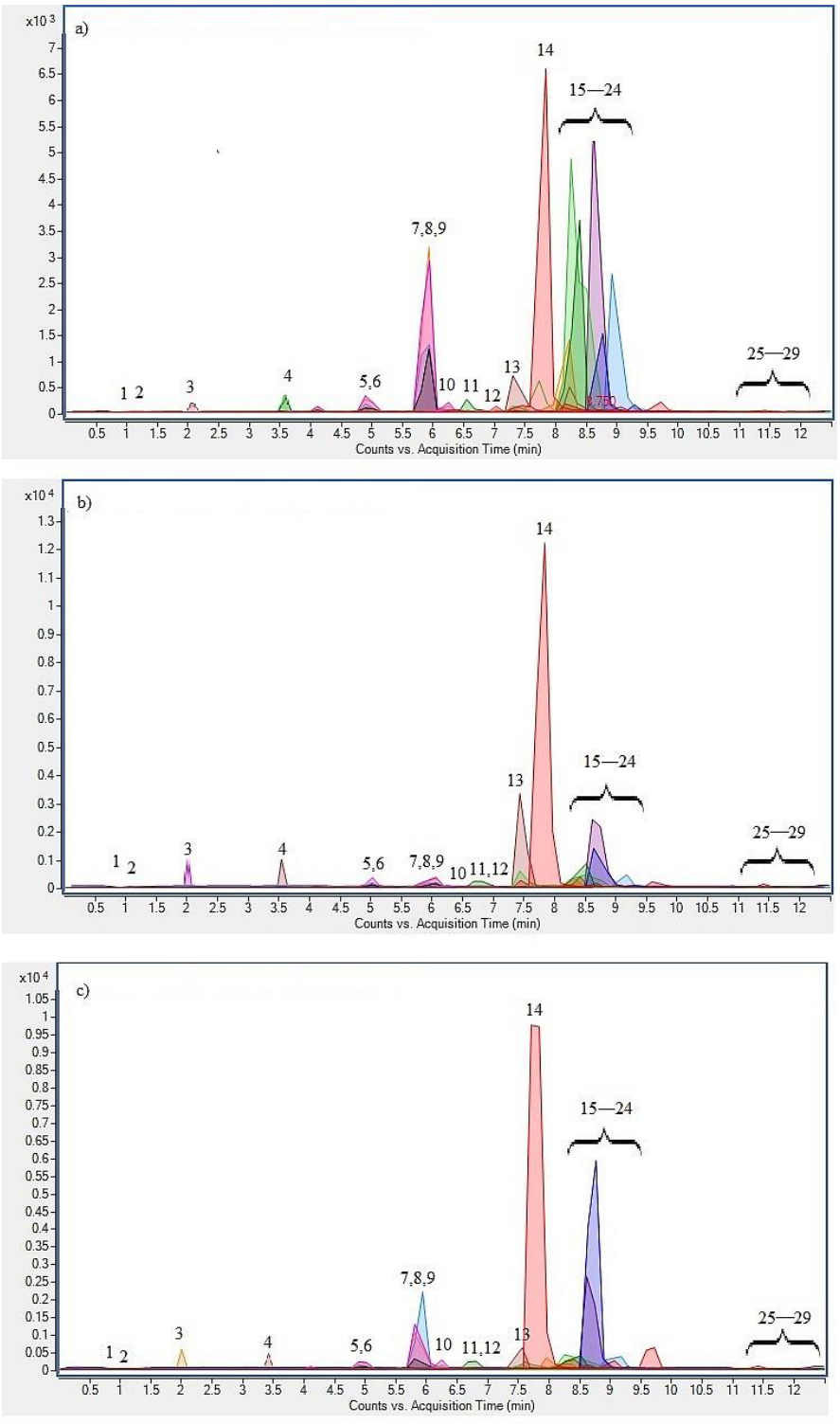

Figure 1. UPLC-MS/MS chromatogram in MRM acquisition mode of *Laurus nobilis* L. leaf extracts obtained by optimized extraction conditions of CRE (**a**), MAE (**b**) and UAE (**c**): (1) rosmarinic acid, (2) syringic acid, (3) 3,4-dihidrobenzoic acid hexoside, (4) protocatechuic acid, (5) chlorogenic acid, (6) *p*-hydroxybenzoic acid, (7) caffeic acid, (8) epicatechin, (9) catechin, (10) procyanidin trimer, (11) luteolin-6-C-glucoside, (12) kaempferol-3-O-deoxyhexoside, (13) *p*-coumaric acid, (14) rutin, (15) apigenin, (16) quercetin-3-glucoside, (17) ferulic acid, (18) luteolin, (19) kaempferol-3-rutinoside, (20) quercetin-3-pentoside, (21) kaempferol-3-O-hexoside, (22) isorhamnetin-3-hexoside, (23) quercetin-3-rhamnoside, (24) kaempferol-3-O-pentoside, (25) gallic acid, (26) apigenin-6-C-(O-deoxyhexosyl)-hexoside, (27) epicatechin gallate, (28) myricetin, (29) epigallocatechin gallate.

Among the phenolic acids, compounds 1, 2, 4, 5, 7, 13, 17 and 25 were identified through comparison with authentic standards as rosmarinic, syringic, protocatechuic, chlorogenic, caffeic, p-coumaric, ferulic and gallic acid, respectively. Compound 3 was tentatively assigned as 3,4-dihidrobenz-A hexoside based on a fragment ion at 153 m/z and fragmentation loss of -162 amu, characteristic of hexose residue [66]. Compound 6 was assigned as p-hydroxybenzoic acid due to the previously described fragmentation pattern [67]. All of the detected phenolic acids were previously found in *Laurus nobilis* L. leaves in varying amounts [30,68–70]. Among the flavonols, compounds 14 and 28 were identified through comparison with authentic standards as rutin and myricetin. Compounds 12, 19, 21 and 24 were distinguished by a specific fragment ion at m/z 287 consistent with kaempferol. They were tentatively assigned, due to the specific loss of sugar moieties, as kaempferol-3-O-deoxyhexoside (deoxyhexose -146 amu), kaempferol-3-rutinoside (rhamnose −146 amu; glucose −162 amu), kaempferol-3-glucoside (glucose −162 amu) and kaempferol-3-pentoside (pentose −132 amu) [35], respectively. Compounds 16, 20 and 23 were tentatively assigned as quercetin-3-glucoside, quercetin-3-pentoside and quercetin-3-rhamnoside due to a characteristic fragment ion at m/z 303 and specific loss of sugar moieties: glucose (−162 amu), pentose (−132 amu) and rhamnose (−146 amu), respectively. Compound 17 was identified by a precursor ion at m/z 479 and fragment ion at m/z 317 corresponding to the loss of hexose (−162 amu) as isorhamnetin-3-hexoside.

Flavonols, mainly kaempferol and quercetin glycosides, were the most abundant compounds detected in our study. This is in accordance with previous reports that have shown the presence of various flavonol glycosides in *Laurus nobilis* L. leaves, kaempferol glycosides being the most diverse [8,71]. To our knowledge, the presence of myricetin in *Laurus nobilis* L. leaves was only reported by Stefanova et al. (2020) [69] in leaves grown in Greece and Georgia. In their study, the amount of myricetin was comparable to that of quercetin, while in our study it was significantly lower. As for flavan-3-ols, compounds 9, 27 and 29 were identified through comparison with authentic standards as catechin, epicatechin gallate and epigallocatechin gallate, respectively. Compound 8 was tentatively assigned as epicatechin due to a precursor ion at m/z 291 and fragment ion at m/z 139. All detected flavan-3-ols have previously been found in *Laurus nobilis* L. leaves [3,70,72,73]. Catechin and epicatechin were the most abundant with similar concentrations, which is in agreement with results reported by Vallverdu-Queralt et al. (2014) [70].

Among flavones, compounds 15 and 18 were identified through comparison with authentic standards as apigenin and luteolin. Compound 11 was tentatively assigned as luteolin-6-C-glucoside due to a precursor ion at m/z 449 and fragment ion at m/z 329 corresponding with the loss of −120 amu, characteristic for hexose residue in C-glycosylation [74]. Different authors have reported the presence of these flavones in *Laurus nobilis* L. leaves [3,30,69]. Compound 26 was tentatively identified as apigenin-6-C-(O-deoxyhexosyl)-hexoside due to a precursor ion at m/z 579 and fragment ion at m/z 459 consistent with the fragmentation pattern previously described by Pacifico et al. (2014) [68] during the identification of phenolic compounds in *Laurus nobilis* L. leaves. Among proanthocyanidins, only compound 10 was detected and tentatively assigned as procyanidin trimer due to a precursor ion at m/z 865 and fragment ion at m/z 713 produced by previously described retro Diels–Alder (RDA) fission of the heterocyclic ring system subunits [75]. Vinha et al. (2015) [10] reported the presence of various proanthocyanidins in *Laurus nobilis* L. leaves, with dimeric proanthocyanidins being the most abundant, followed by trimers. Dias et al. (2014) [3] also confirmed the presence of different proanthocyanidins, including procyanidin trimer with the same fragmentation pattern as in our study.

The highest total concentration of phenolic compounds, according to UPLC-MS/MS results, was achieved in the extract obtained by CRE. Even though the MAE and UAE are generally considered to increase the phenolic content of plant extracts, it is possible that

the application of microwaves and ultrasonic waves resulted in the degradation of certain constituents in *Laurus nobilis* L. leaves. The concentration of flavonols and flavan-3-ols was significantly higher in CRE extracts than in MAE and UAE extracts (Figure 2). The presence of hydroxyl-substituents in these compounds [76] was shown to increase the degradation of polyphenols caused by microwaves [20], which could explain the lower concentration obtained by MAE. As for UAE, it was shown that a frequency over 20 kHz might cause the degradation of phenolic compounds [21]. This can occur due to the increased generation of hydrogen atoms (H) and hydroxyl radicals (OH*), which promote the decomposition and polymerization of polyphenolic compounds [54]. The mentioned generation of free radicals can also influence biological activity of the extracts obtained by UAE [77]. On the other hand, another possible explanation for CRE suitability is related to plant material properties. *Laurus nobilis* L. leaves are stiff and leathery, so their firm structure allows the application of more intense extraction conditions, such as in CRE, providing better extraction yield in terms of polyphenols.

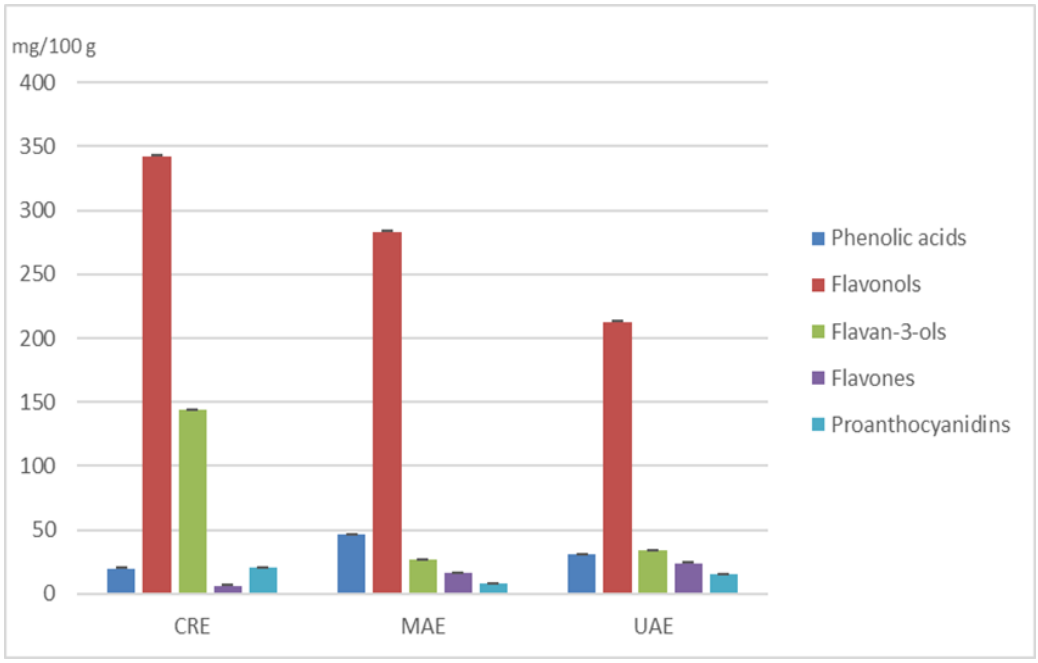

Figure 2. The content of different groups of polyphenolic compounds determined by UPLC-MS/MS in extracts obtained by CRE, MAE and UAE.

The total phenolic content in the extracts determined by the Folin–Ciocalteu (FC) spectrometry method was significantly higher than that revealed by the UPLC-MS/ MS analysis, which can be explained by the fact that some non-phenolic compounds, such as various polysaccharides, sugars and organic acids, present in the leaves of *Laurus nobilis* L. [8], are known to be detectable by spectrophotometer, resulting in a higher reported polyphenolic concentration [25,78]. In addition, El-Hamidi et al. (2016) [79] reported the interaction of chlorophyll with the Folin–Ciocalteu reagent, leading to an apparent increase in the total polyphenol content of chlorophyll-rich plants, which provides an additional explanation for the observed differences between polyphenol content determined by the spectrophotometric and chromatographic techniques.

3.5. Antioxidant Capacity

The oxygen radical absorbance capacity (ORAC) assay was performed on the extracts obtained at defined optimal extraction conditions in order to determine their antioxidant capacity. As shown in Table 3, the antioxidant capacity of the extracts obtained by CRE, MAE and UAE ranged between 86.04 and 100.09 μmol TE g^{-1}, showing that a similar

antioxidant capacity can be achieved with less time and energy expenditure since the extraction time at optimal MAE and UAE conditions was three times shorter than in CRE, which is important for potential scale-up processes. The antioxidant capacity of *Laurus nobilis* L. leaves determined by ORAC assay reported in the literature has varied significantly. For example, Zheng et al. (2001) [80] reported the value of 37.7 μmol TE g^{-1} for phosphate buffer (75 mM, pH 7) *Laurus nobilis* L. leaf extract where the total phenolic content was also significantly lower than in our study (4.04 mg GAE g^{-1}). On the other hand, Kratchanova et al. (2010) [81] reported a higher ORAC value of 170 μmol TE g^{-1} for a water extract with the total phenolic content of 17.66 mg GAE g^{-1}, which is lower than in our study. Moreover, Kim and Kim (2021) [82] and Dudonne et al. (2009) [83] reported significantly higher ORAC values of 2600 μmol TE g^{-1} in DMSO extract and 2963 μmol TE g^{-1} in a water extract, with total phenolic contents similar to those in our study, 44.07 and 59.85 mg GAE g^{-1}, respectively. There are several explanations for the discrepancies between the total phenolic contents, which were shown to correlate with the antioxidant capacity in *Laurus nobilis* L. leaf extracts [30,81], and the reported ORAC values. First, different environmental growth factors, harvesting season and the choice of the extraction method could have influenced the presence of other non-phenolic antioxidants such as tocopherols organic acids and volatile compounds in the extracts [8]. Moreover, the possible synergistic or antagonistic mechanisms between the constituents in the extracts cannot be represented solely by the amount of total polyphenols present, so further qualitative research in this regard is needed [84,85]. Apart from influencing the presence of non-phenolic compounds, the previously mentioned factors could have also influenced the content of individual polyphenolic compounds whose antioxidant capacity may differ significantly depending on their structural features [86]. This effect can be observed in the results of our study since the extracts obtained by CRE showed slightly higher antioxidant capacity than those obtained by both MAE and UAE that can be brought into connection with the concentration of flavonols and flavan-3-ols determined by the UPLC/MS-MS, which were shown to influence the antioxidant activity [87], as well as procyanidin trimer content, which was the highest of the CRE extracts. It was shown that procyanidin dimers and trimers were more effective against different radical species than monomeric flavonoids due to the higher polymerization degree [88]. Muniz-Marquez et al. (2018) [29] observed that *Laurus nobilis* L. leaf extracts obtained by CRE (76.86%) were slightly more efficient in lipid peroxidation inhibition than those obtained by MAE (70.71%), which, as the authors explained, was in agreement with the phenolic content of the extracts. In another study by Muniz-Marquez et al. (2014) [28], the lipid peroxidation inhibition of the *Laurus nobilis* L. leaf extracts obtained by UAE was 73.55%, which is also lower than the inhibition percentage previously reported for CRE. These results are in accordance with the trend observed in our study.

4. Conclusions

MAE and UAE, as green extraction techniques, were optimized for the rapid and effective isolation of the polyphenols of *Laurus nobilis* L. leaves and were compared with CRE. The determined optimal MAE conditions were 50% ethanol, temperature 80 °C, time 10 min and microwave power 400 W, while for UAE they were 70% ethanol, 10 min and 50% amplitude. The polyphenolic profile of *Laurus nobilis* L. leaves, regardless of the extraction technique used, included 29 compounds belonging to the classes of phenolic acids, flavonols, flavan-3-ols, flavones and proanthocyanidins. Flavonols were the most abundant phenolic group consisting mainly of kaempferol and quercetin glycosides. Although according to the spectrophotometric determination of the total phenolic content MAE was shown to be the most effective technique, the individual polyphenolic profile revealed that the highest polyphenolic yield and, consequently, the highest antioxidant capacity was obtained by CRE. Although green extraction techniques have not overcome the CRE yield, they produced polyphenol rich extracts with similar antioxidant capacity

in a significantly shorter time, demonstrating their advantages in reducing time and energy consumption.

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CHAPTER 3

Publication No.3: Pressurized Liquid Extraction as a Novel Technique for the Isolation of *Laurus nobilis* L. Leaf Polyphenols.

Molecules

Publication No.3

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molecules

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Article

Pressurized Liquid Extraction as a Novel Technique for the Isolation of *Laurus nobilis* L. Leaf Polyphenols

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Abstract: *Laurus nobilis* L., known as laurel or bay leaf, is a Mediterranean plant which has been long known for exhibiting various health-beneficial effects that can largely be attributed to the polyphenolic content of the leaves. Pressurized liquid extraction (PLE) is a green extraction technique that enables the efficient isolation of polyphenols from different plant materials. Hence, the aim of this research was to determine optimal conditions for PLE (solvent, temperature, number of extraction cycles and static extraction time) of laurel leaf polyphenols and to assess the polyphenolic profile of the optimal extract by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) as well as to evaluate the antioxidant activity determined by FRAP, DPPH and ORAC assays. The optimal PLE conditions were 50% ethanol, 150 °C, one extraction cycle and 5 min static time. The polyphenolic extract obtained at optimal PLE conditions comprised 29 identified compounds, among which flavonols (rutin and quercetin-3-glucoside) were the most abundant. The results of antioxidant activity assays demonstrated that PLE is an efficient green technique for obtaining polyphenol-rich laurel leaf extracts with relatively high antioxidant activity.

Keywords: *Laurus nobilis* L.; polyphenols; pressurized liquid extraction (PLE); UPLC-MS/MS; antioxidant activity

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1. Introduction

Laurus nobilis L., also known as laurel or bay leaf, is an evergreen plant characteristic of the Mediterranean area with high annual rainfall, whose leaves have been widely used as a spice in traditional cuisine, as well as in folk medicine for treating various health conditions. For that reason, their chemical composition and biological activities have been more well researched than other plant parts [1]. Laurel leaves, which have shown antioxidant [2], anti-inflammatory [3] and antimicrobial activity [4], comprise essential oils, alkaloids, polysaccharides, sugars, norisoprenoids, tocopherols, organic acids and a variety of polyphenols comprising flavonoids and non-flavonoids (phenolic acids and lignans) whose structure varies in complexity [5]. Polyphenols can be considered largely responsible for the laurel leaf extracts' antioxidant activity [2,6] since they possess redox properties which allow them to act as antioxidant agents [7]. Recovery of these antioxidants is a challenging process since the plant material comprises a variety of polyphenolic structures including simple to complex and highly polymerized polyphenols that often interact with other constituents such as polysaccharides and lipids [8]. Therefore, a key step in the utilization of polyphenols' beneficial properties is establishing an optimal isolation methodology that would result in their effective recovery, and various techniques may be applied for this purpose. Conventional extraction techniques, such as maceration, heat-reflux and infusion, are generally easily applicable, but also often solvent-, time- and energy-consuming. These techniques may also result in the degradation of thermosensitive polyphenolic compounds and are often difficult to automate, making them inapplicable on larger scales. For this reason, many advanced extraction techniques have emerged in

recent years. Pressurized liquid extraction (PLE), known as accelerated solvent extraction (ASE), is an automated green extraction technique which was shown to be an economic and time-efficient alternative to conventional techniques since it resulted in comparable or higher contents of polyphenols in various plant extracts [9]. During PLE, the use of elevated pressures allows the liquid solvents to be used at temperatures above their atmospheric boiling point, resulting in enhanced solubility and diffusion rate of the targeted compounds, while the surface tension and solvent viscosity decrease, which results in a drained matrix after the extraction [10]. PLE can be performed in a dynamic or static setup. During the dynamic mode of operation, the solvent is delivered at a constant flow rate, while in the static mode, the extraction process includes one or several cycles in a predetermined time (most often 5–15 min), with solvent replacement between the cycles [10]. Various parameters, such as solvent, temperature, pressure and time of extraction, can be varied in order to improve the extraction performance. After the optimal isolation methodology has been established, the next step is to identify and quantify individual polyphenols and to evaluate the extracts' antioxidant activity. Combined chromatographic and spectral techniques such as ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) are the most useful tools which allow chemical characterization of both simple and complex polyphenolic structures [11]. Several assays divided into two groups (single electron transfer (SET) and hydrogen atom transfer (HAT)) can be used for determination of antioxidant capacity [12]. The ferric reducing antioxidant power (FRAP) and 2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) assays are both SET colorimetric assays widely used in estimation of plant extracts' antioxidant capacities, usually applied together or in a combination with other techniques [13]. Oxygen radical absorbance capacity (ORAC) is a HAT method often chosen for antioxidant capacity determination in plant material since it can measure both polar and nonpolar antioxidants, and it is also the most biologically relevant due to the use of the most biologically prevalent peroxyl radical [14].

Therefore, the purpose of this research was to examine how different PLE parameters (solvent, temperature, number of extraction cycles, static extraction time) affect the phenolic content of laurel leaf extracts and to determine the optimal extraction conditions. To the best of our knowledge, PLE has not been applied for the extraction of laurel leaf polyphenols to date; therefore, there are no data on polyphenolic profiles obtained by this technique. Hence, the goal of this research was to identify and quantify individual polyphenols of the laurel leaf extract obtained by PLE at optimal conditions using the UPLC-MS/MS and to evaluate the antioxidant activity of the extract by using the ORAC, DPPH and FRAP assays.

2. Results and Discussion

This study examined the effect of several PLE extraction parameters on the polyphenolic content of laurel leaf extracts. The TPC of the extracts, as determined by the spectrophotometric Folin–Ciocalteu method, is shown in Table 1. The results of the statistical analysis, which was used to determine optimal extraction conditions, are shown in Table 2. Individual compounds in the extract obtained at optimal conditions were identified and quantified by UPLC-MS/MS, while the antioxidant capacity of the optimal PLE extract was characterized by ORAC, DPPH and FRAP assays.

The TPC of the laurel leaf extracts obtained by PLE ranged from 31.87 to 49.30 mg GAE g^{-1}, which is similar to the previously reported value of 46.79 mg GAE g^{-1} [15], lower than 59.85 mg GAE g^{-1} [16] and higher than 10.23 mg GAE g^{-1} [17], all obtained by conventional extraction techniques. The TPC of the extracts is also in the range of phenolic contents obtained by microwave-assisted extraction (MAE) (30.88 to 53.57 mg GAE g^{-1}) and ultrasound-assisted extraction (UAE) (24.43 to 36.74 mg GAE g^{-1}) in our previous research [18].

Table 1. Total phenolic content of laurel leaf extracts obtained by PLE.

Extraction Parameters				TPC mg GAE g^{-1}
% EtOH	Temperature (°C)	Extraction Cycles	Static Extraction Time (min)	
50	90	1	5	34.10 ± 2.02
50	90	1	10	33.82 ± 1.87
50	90	2	5	35.38 ± 1.77
50	90	2	10	36.46 ± 0.81
50	90	3	5	35.39 ± 0.71
50	90	3	10	36.46 ± 0.81
50	120	1	5	36.63 ± 1.31
50	120	1	10	44.03 ± 2.02
50	120	2	5	38.93 ± 1.46
50	120	2	10	40.78 ± 1.56
50	120	3	5	39.85 ± 0.50
50	120	3	10	44.60 ± 1.11
50	150	1	5	46.34 ± 1.21
50	150	1	10	44.46 ± 1.82
50	150	2	5	46.09 ± 1.82
50	150	2	10	47.99 ± 1.11
50	150	3	5	45.82 ± 1.67
50	150	3	10	49.30 ± 1.01
70	90	1	5	31.87 ± 1.51
70	90	1	10	32.41 ± 1.87
70	90	2	5	33.25 ± 0.61
70	90	2	10	34.76 ± 1.56
70	90	3	5	35.39 ± 1.51
70	90	3	10	36.24 ± 2.72
70	120	1	5	35.33 ± 2.27
70	120	1	10	36.42 ± 2.57
70	120	2	5	38.19 ± 0.55
70	120	2	10	40.44 ± 1.51
70	120	3	5	37.49 ± 1.97
70	120	3	10	40.12 ± 2.02
70	150	1	5	43.39 ± 0.86
70	150	1	10	42.98 ± 1.92
70	150	2	5	42.46 ± 1.56
70	150	2	10	47.06 ± 1.46
70	150	3	5	40.56 ± 0.55
70	150	3	10	39.73 ± 1.36

TPC = total phenolic content. Results are expressed as mean ± SD.

Table 2. Influence of different PLE parameters on total phenolic content of laurel leaf extracts.

N	Source of Variation	TPC (mg GAE g^{-1})
	% EtOH	$p < 0.05$ †
36	50% w/w	40.91 ± 0.87 [b]
36	70% w/w	38.23 ± 0.72 [a]
	T	$p < 0.01$ †
24	90 °C	34.63 ± 0.39 [a]
24	120 °C	39.40 ± 0.62 [b]
24	150 °C	44.68 ± 0.62 [c]
	Extraction cycles	$p = 0.37$ ‡
24	1	38.48 ± 1.10 [a]
24	2	40.15 ± 1.01 [a]
24	3	40.08 ± 0.91 [a]
	Static extraction time	$p = 0.14$ ‡
36	5 min	38.69 ± 0.76 [a]
36	10 min	40.45 ± 0.86 [a]

TPC = total phenolic content. N = number of trials. Results are expressed as mean ± standard error. Values marked with different letters are statistically different at $p \leq 0.05$. † Statistically significant variable at $p \leq 0.05$. ‡ Statistically insignificant variable at $p \leq 0.05$.

2.1. Pressurized Liquid Extraction (PLE) Optimization

Ethanol percentage in an aqueous solution (50 and 70%), temperature (90, 120 and 150 °C), number of extraction cycles (one, two and three) and static extraction time (5 and 10 min) were varied during the PLE of laurel leaf polyphenols. As shown in Table 2, the ethanol percentage significantly ($p \leq 0.05$) influenced the TPC of the extracts, which was higher when 50% ethanol was used as a solvent. Similarly, Leyva-Jimenez et al. [19] reported that, in the tested range of 15–85% ethanol, 46% ethanol was optimal for the PLE of polyphenols from *Lippia citriodora* leaves. The use of 50% ethanol also resulted in the highest TPC apple pomace [20] and grape skin extracts [21] obtained by PLE. On the other hand, 71% ethanol was found optimal during PLE of polyphenols from *Myrtus communis* L. leaves [22]. The different observations might be a result of different polyphenolic contents of the samples and the polarity of present compounds which consequently influences the extraction yield when the "like dissolves like" principle is taken into account [23].

Temperature is an important parameter in PLE extraction since it influences the molecular diffusivity and viscosity of the solvent [24]. Generally, applying higher temperatures increases solubility and recovery of compounds from the plant matrix; however, thermosensitive compounds may degrade at higher temperatures, which is why it is crucial to determine optimal temperatures for each plant material [25]. In the present study, the temperature significantly ($p < 0.01$) influenced the TPC of the extracts, which increased proportionally with the increase in temperature. Zhao et al. (2012) [26] have observed a similar effect during the extraction of lignans from *Fructus Schisandrae* where the yield increased proportionally with temperature rise from 80 to 160 °C. A further increase to 180 °C resulted in a lower yield of lignans, possibly due to the mentioned thermal degradation. Repajić et al. have also observed an increase in the TPC of *Urtica dioica* L. leaf extracts [27], as well as *Foeniculum vulgare* Mill. seed extracts [28], with the increase in temperature during PLE. In contrast, temperature had no effect during the PLE of phenolic compounds from *Olea europaea* L. fruit [29].

The number of extraction cycles and the static time are also important since a longer exposure of the analytes to the extraction solvent at elevated temperatures increases the diffusion rate, while multiple extraction cycles may result in the complete extraction of targeted compounds [10]. In the present study, the number of extraction cycles and static extraction time did not significantly influence the TPC of the extracts, contrary to the results reported by Repajić et al. (2020) [27], Li et al. (2019) [21] and Wibisono et al. (2009) [30] where PLE was optimized for the extraction of polyphenols from *Urtica dioica* L. leaves, grape skin and different plant food materials, respectively. For the PLE of phenolic compounds from *Foeniculum vulgare* Mill. seeds, Repajić et al. (2021) [28] reported that static time had no significant influence on the phenolic yield, so 5 min was chosen as optimal, while the number of extraction cycles was a significant parameter. Static time of 5 min was also chosen as optimal for the PLE of *Rosmarinus officinalis* phenolic compounds [31]. Sandei and Vadala (2013) [32] applied one extraction cycle and a static time of 5 min for the optimization of other PLE parameters during the extraction of tomato polyphenols, which are the same as the lowest values applied in our study.

Based on the results of statistical analysis, 50% ethanol, 150 °C, one extraction cycle and a static time of 5 min were chosen as optimal for obtaining the maximum content of polyphenols from laurel leaves.

2.2. Polyphenolic Characterization

In order to provide insight into the polyphenolic composition of the laurel leaf extract produced under optimal extraction conditions, UPLC-MS/MS analysis was performed (Table 3). Twenty-nine phenolic compounds, including flavonoids (flavones, flavonols, flavan-3-ols and proanthocyanidins) and phenolic acids, were identified in the extract (Figure 1). The identification of compounds was carried out as described in our previous research [18].

Table 3. Mass spectrometric data on laurel leaf extract obtained at optimal PLE conditions.

Compound	Retention Time	Tentative Identification	Concentration mg/100 g
		Phenolic acids	
1	1.008	chlorogenic acid *	0.46 ± 0.01
2	3.638	protocatechuic acid *	58.63 ± 1.66
3	4.259	rosmarinic acid *	0.99 ± 0.03
4	4.937	p-coumaric acid *	4.25 ± 0.12
5	5.961	syringic acid *	0.07 ± 0.00
8	7.917	ferulic acid *	1.01 ± 0.03
19	10.788	caffeic acid *	74.44 ± 2.11
20	10.802	p-hydroxybenzoic acid	2.83 ± 0.08
23	11.573	gallic acid *	0.28 ± 0.01
22	11.426	3.4-dihidrobenz-A-hexoside	4.57 ± 0.13
		Flavones	
6	6.938	luteolin-6-C-glucoside	3.91 ± 0.11
13	8.678	luteolin *	7.15 ± 0.20
21	11.415	apigenin *	9.40 ± 0.27
27	11.998	apigenin-6-C-(O-deoxyhexosyl)-hexoside	0.27 ± 0.01
		Flavonols	
7	7.561	rutin *	97.31 ± 2.75
9	7.969	quercetin-3-glucoside	94.41 ± 2.67
10	8.349	kaempferol-3-rutinoside	6.00 ± 0.17
11	8.39	quercetin-3-pentoside	7.92 ± 0.22
12	8.64	kaempferol-3-*O*-hexoside	18.02 ± 0.51
14	8.747	isorhamnetin-3-hexoside	24.93 ± 0.71
15	8.791	myricetin *	2.25 ± 0.06
16	8.897	quercetin-3-rhamnoside	9.57 ± 0.27
18	9.178	kaempferol-3-*O*-pentoside	8.04 ± 0.23
29	12.299	kaempferol-3-*O*-deoxyhexoside	0.14 ± 0.00
		Flavan-3-ols	
17	9.014	epigallocatechin gallate *	0.15 ± 0.00
24	11.658	catechin *	31.35 ± 0.89
25	11.898	epicatechin gallate *	0.34 ± 0.01
28	12.055	epicatechin	29.20 ± 0.83
		Proanthocyanidins	
26	11.977	procyanidin trimer	3.89 ± 0.11
Total phenols (mg 10^{-2} g^{-1})	-	-	501.84 ± 2.27

Results are expressed as mean ± standard deviation. * identification was confirmed with authentic standards.

According to UPLC-MS/MS results, flavonols were the most abundant group of phenolic compounds (Figure 2), with rutin and quercetin-3-glucoside being the main representatives. Rutin and quercetin were shown to be stable during exposure to elevated temperatures [33], and data on different thermal degradation rates of various quercetin glycosides showed that quercetin-3-*O*-glucoside was among the more stable glycosides [34]. The content of rutin and quercetin-3-glucoside was also the highest in laurel leaf extracts obtained by MAE in our previous research [18]. In the same research, the content of phenolic acids of the extracts obtained by conventional heat-reflux extraction (CRE), MAE and UAE was more than 100 mg g^{-1} lower than that in the extract obtained by PLE in the present study, where caffeic acid was the main representative. Since phenolic acids, especially hydroxycinnamic acids, were shown to be thermally stable [33], the exposure to higher temperatures during PLE may have increased their recovery.

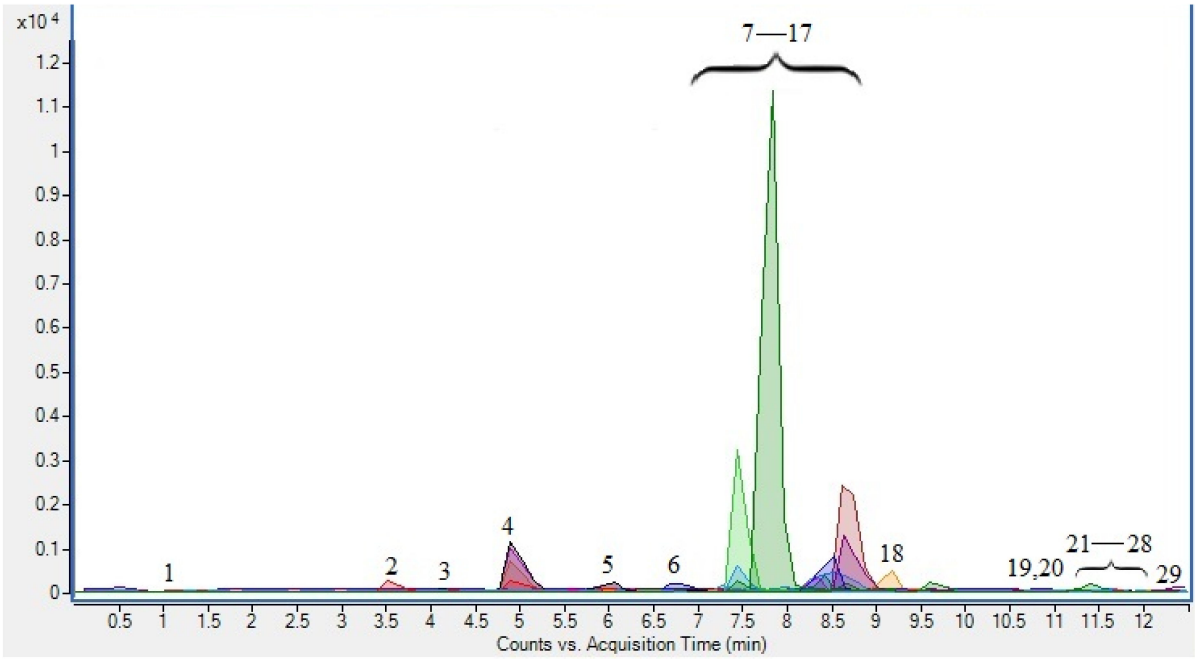

Figure 1. UPLC-MS/MS chromatogram of laurel leaf extracts obtained at optimal PLE conditions in MRM acquisition mode: (1) chlorogenic acid, (2) protocatechuic acid, (3) rosmarinic acid, (4) *p*-coumaric acid, (5) syringic acid, (6) luteolin-6-C-glucoside, (7) rutin, (8) ferulic acid, (9) quercetin-3-glucoside, (10) kaempferol-3-rutinoside, (11) quercetin-3-pentoside, (12) kaempferol-3-*O*-hexoside, (13) luteolin, (14) isorhamnetin-3-hexoside, (15) myricetin, (16) quercetin-3-rhamnoside, (17) epigallocatechin gallate, (18) kaempferol-3-*O*-pentoside, (19) caffeic acid, (20) *p*-hydroxybenzoic acid, (21) apigenin, (22) 3,4-dihidrobenzoic acid hexoside, (23) gallic acid, (24) catechin, (25) epicatechin gallate, (26) procyanidin trimer, (27) apigenin-6-C-(*O*-deoxyhexosyl)-hexoside, (28) epicatechin, (29) kaempferol-3-*O*-deoxyhexoside.

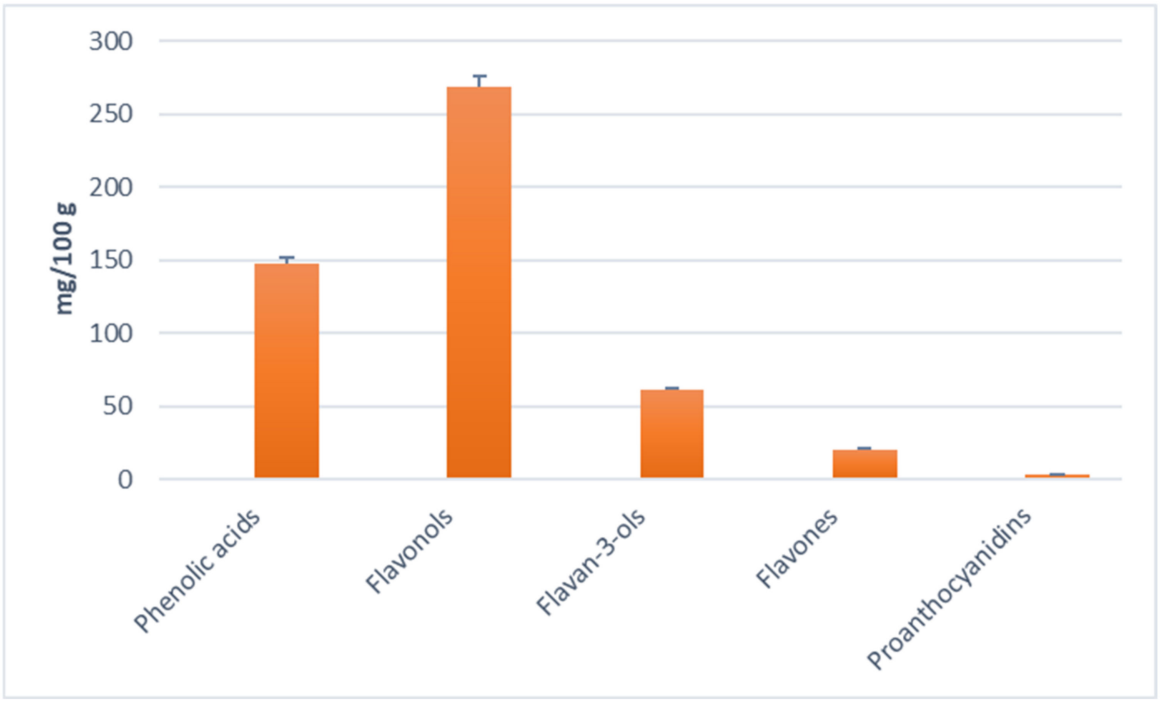

Figure 2. Concentration of different groups of polyphenols determined in the laurel leaf extract obtained at optimal PLE conditions.

The content of the main flavan-3-ol representatives, catechin and epicatechin, was more than 2-fold lower than their content obtained previously by CRE and more than 3-fold higher than their content obtained previously by UAE and MAE [18]. Even though the high temperature applied during PLE increases the extraction rate, explaining the higher content than in UAE and MAE, thermal degradation of catechin and epicatechin may occur during prolonged exposure to elevated temperatures, leading to their lower content than in the CRE extracts [33,35]. Apigenin was the most abundant flavone in the PLE extract, with a concentration higher than that in extracts obtained by all three techniques applied in the previous research where luteolin was the most abundant flavone [18]. This is in agreement with the findings that apigenin is resistant to prolonged exposure to temperatures around 100 °C, resulting in its higher recovery [36]. Luteolin and its glycosides were previously shown to be less thermally stable than apigenin [33], which is consistent with their recovered amount. The content of procyanidin trimer in the PLE extract was lower than the content in MAE, CRE and UAE extracts obtained previously [18], which is in agreement with previous findings where proanthocyanidins from blueberry and grape pomace were shown to be thermosensitive [37]. In addition, procyanidin oligomers, especially B-type procyanidins, such as the procyanidin trimer detected in the present study, were shown to be the most thermosensitive phenolic compounds in cloudy apple juice [34].

The results of Folin–Ciocalteu spectrophotometry showed significantly higher TPC than that revealed by UPLC-MS/MS, which might be a result of interference caused by non-phenolic constituents present in laurel leaves, such as organic acids, various polysaccharides and sugars [5] which were shown to be detectable by the spectrophotometer [12]. Moreover, it was reported that chlorophyll may interact with the Folin–Ciocalteu reagent, resulting in a seeming increase in the TPC of chlorophyll-rich plant material [38], which could serve as another explanation for the observed discrepancies between the results obtained by chromatographic and spectrophotometric techniques.

2.3. Antioxidant Activity

Antioxidant activity of the extract obtained at the defined optimal extraction conditions was determined by the ORAC, DPPH and FRAP assays, and the results are shown in Table 4.

Table 4. Antioxidant activity of laurel extracts obtained by PLE determined by various assays.

Assay	μmol TE g^{-1}
ORAC	97.27 ± 2.01
DPPH	73.51 ± 0.22
FRAP	311.10 ± 5.67

Results are expressed as mean ± SD.

The ORAC value of the laurel leaf extract obtained by PLE was in the range of those reported in the literature, which have varied from 37.7 μmol TE g^{-1} [39] to 170 μmol TE g^{-1} [40] in laurel leaf extracts with lower TPCs (4.04 and 17.66 mg GAE g^{-1}, respectively) than in the present study, to even 2600 μmol TE g^{-1} [41] for an extract with a TPC similar to that of the present study (44.07 mg GAE g^{-1}). The ORAC value was also in the range of those reported in our previous research carried out on the same laurel leaf sample where CRE resulted in a higher ORAC value (100.09 μmol TE g^{-1}) and MAE and UAE resulted in lower ORAC values (86.04 and 90.27 μmol TE g^{-1}, respectively), which might have been influenced by the previously discussed differences in the individual phenolic compounds' contents since the antioxidant activity is dependent on the structural features of phenolic compounds [42]. The DPPH value was lower than the 300 μmol TE g^{-1} reported for a laurel leaf extract [43] where the TPC was 1.01 mg GAE g^{-1}. The FRAP value was in the range of 278 μmol TE g^{-1} determined in a hydromethanolic laurel leaf extract where the phenolic content was not analyzed [44], and also lower than 504.25 μmol TE g^{-1} [15] where the TPC of laurel leaf extract was 46.79 mg GAE g^{-1}. The differences between the antioxidant activity and the phenolic contents of laurel leaf extracts indicate that the antioxidant

activity is not influenced only by the TPC. Several other factors, including the plant growth environment, harvest season, storage conditions and different extraction techniques, may have affected the extracts' contents of nonphenolic antioxidants such as organic acids, tocopherols and terpenoids [5]. In addition, it is possible that antagonistic or synergistic mechanisms occur between certain components of the extracts which cannot be clarified only by the TPC and could possibly be explained by further qualitative research [45]. Moreover, the content of individual phenolic compounds may significantly influence the antioxidant activity of the extracts since their antioxidant activity depends on the structural features [46]. This is supported by the results of the present study since the same ORAC and DPPH values were determined in the extracts with different TPCs, as well as the contents of individual compounds.

3. Materials and Methods

3.1. Chemicals and Reagents

Ethanol (96%) and methanol (99.8%) were procured from Lach-ner d.o.o. (Neratovice, Czech Republic), and acetonitrile (HPLC grade) was obtained from J.T. Baker Chemicals (Deventer, the Netherlands). Purified distilled water was produced in a Milli-Q water purification system (Millipore, Bedford, MA, USA). Anhydrous sodium carbonate (≥99.5%), anhydrous sodium acetate (≥99%), formic acid (98–100%), $FeCl_3 \times 6H_2O$, sodium phosphate (96%) and Folin–Ciocalteu reagent were from Kemika (Zagreb, Croatia); 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were from Acros Organics (Thermo Fisher Scientific, Geel, Belgium); fluorescein sodium salt was from Honeywell Research Chemicals (Bucharest, Romania); and hydrochloric acid (37%), glacial acetic acid, 2,20-azobis (2-amidinopropane) hydrochloride (AAPH) and 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH) were from Sigma-Aldrich (Steinheim, Germany). Authentic standards of myricetin, quercetin-3-glucoside, caffeic, protocatechuic, gallic, ferulic, rosmarinic, syringic, p-coumaric and chlorogenic acids were procured from Sigma-Aldrich (St. Louis, MO, USA). Kaempferol-3-glucoside, catechin, epicatechin gallate, epigallocatechin gallate, apigenin, rutin, luteolin and procyanidin B2 were purchased from Extrasynthese (Genay, France). Apigenin standard was prepared as an ethanol–0.5% *v/v* dimethyl sulfoxide stock solution, while all other standards were dissolved in methanol. Stock solutions were diluted in order to produce working standard solutions at five concentrations.

3.2. Plant Material

Dried laurel leaves collected in the region of Rijeka, Croatia, in November 2020 were purchased from Šafram d.o.o (Zagreb, Croatia) and stored at room temperature. Prior to extraction, the leaves were ground in an electric grinder (OmniBlend, Vervita, Croatia) until a coarse powder was obtained. The total solids of the obtained powder (>95%) were analyzed by drying to constant mass at 103 ± 2 °C [47].

3.3. Pressurized Liquid Extraction (PLE)

The extractions were performed on a Dionex ASE 350 Accelerated Solvent Extractor (Thermo Fisher Scientific Inc., Sunnyvale, CA, USA) in static mode. One gram of the ground leaves was combined with 2 g of diatomaceous earth and transferred to 34 mL stainless steel cells previously fitted with three cellulose filters. The extraction conditions were varied following the full factorial design (shown in Table 1): ethanol percentage in aqueous solution (50 and 70%), extraction temperature (90, 120 and 150 °C), extraction cycles (1, 2 and 3) and static extraction time (5 and 10 min). The pressure, purge with nitrogen and volume flush were kept constant at 10.34 MPa, 30 s and 50%, respectively. The 250 mL glass vials with Teflon septa used for the collection of the extracts were filtered into volumetric flasks (50 mL) through Whatman No. 40 filter paper (Whatman International Ltd., Kent, UK) and made up to volume with the extraction solvent. The extracts were transferred and

stored in plastic Falcon tubes at −18 °C in a nitrogen gas atmosphere until further analysis. All extracts were prepared in duplicate.

3.4. Total Phenolic Content (TPC)

The TPC of laurel leaves was determined following a modified methodology previously established by Shortle et al. [48]. First, 100 µL of the extract (extraction solvent for blank), 200 µL Folin–Ciocalteu reagent and 2 mL of distilled water were put into the reaction tube; 1 mL of sodium carbonate solution (20% *w*/*v*) was added into the reaction after 3 min, and the mixture was shaken using Vortex MS2 Minishaker IKA (IKA, Staufen, Germany) at 1800 rpm and incubated at 50 °C in a water bath. The absorbance was read after 25 min on a VWR UV-1600PC Spectrophotometer (VWR, Wayne, PA, USA) at 765 nm. All measurements were carried out in duplicate. Working standard solutions of gallic acid (50–500 mg L^{-1}) were used to prepare a standard calibration curve (y = 0.0035x, R^2 = 0.9995). The calculated TPC was expressed as a mean value of mg gallic acid equivalents (GAE) per g sample ± standard deviation.

3.5. UPLC-MS/MS Conditions

Individual polyphenols in the extracts obtained at optimal conditions were identified and quantified using a UPLC-MS/MS system (Agilent series 1290 RRLC instrument) coupled with an Agilent 6430 Triple Quadrupole LC/MS mass spectrometer (Agilent, Santa Clara, CA, USA). The ionization was performed in positive and negative ionization mode by ESI ion source with nitrogen as a desolvation and collision gas. Drying gas temperature was set at 300 °C, flow rate at 11 L h^{-1}, nebulizer pressure at 40 psi and capillary voltage at 4/−3.5 kV. The Zorbax Eclipse Plus C18 column from Agilent (100 × 2.1 mm; 1.8 µm particle size) was used for separations under the following conditions: injection volume 2.5 µm and column temperature 35 °C. Other parameters including gradient conditions, solvent composition and instrumental limits of detection (LOD) and quantification (LOQ) were previously reported by Elez Garofulić et al. (2018) [49]. Agilent MassHunter Workstation Software (ver. B.04.01) (Agilent, Santa Clara, CA, USA) was used for data processing and instrument control. Identification and quantitative determination were performed as described by Dobroslavić et al. (2021) [18]. The concentrations of the analyzed polyphenols were expressed as mg per 10^2 g sample (mean value ± standard deviation). The analyses were carried out in duplicate.

3.6. Antioxidant Activity

In order to determine the antioxidant activity of the laurel leaf extract obtained at optimal extraction conditions, three assays were applied.

3.6.1. Oxygen Radical Absorbance Capacity (ORAC) Assay

An automated plate reader (BMG LABTECH, Offenburg, Germany) was used to perform the oxygen radical absorbance capacity (ORAC) assay, while the MARS 2.0 software (BMG LABTECH, Offenburg, Germany) was used for data analysis. The assay was performed following a previously reported method [50]. A 240 mM solution of AAPH, a range of Trolox dilutions (3.12–103.99 µM) and 70.3 nM fluorescein solution were prepared with phosphate buffer (pH 7.4). Afterward, a properly diluted sample or Trolox standard was added to 150 µL of fluorescein in a 96-well microplate which was then incubated at 37 °C for 30 min. The first three cycles set the baseline signal, after which the AAPH solution was injected in order to produce the peroxyl radical. The fluorescence intensity (excitation and emission at 485 and 528 nm, respectively) was monitored for 120 min every 90 s. Determinations were carried out in duplicate. The results were expressed as mean value ± standard deviation of µmol Trolox equivalent (TE) per g sample.

3.6.2. DPPH Radical Scavenging Assay

The DPPH radical scavenging assay was performed following the methodology previously described by Brand-Williams et al. (1995) [51], with some modifications. Briefly, 0.75 mL of the extract and 1.5 mL of 0.2 mM methanolic DPPH solution were mixed in a test tube and shaken at 1800 rpm using Vortex MS2 Minishaker IKA (IKA, Staufen, Germany). As a blank, 2.25 mL of methanol was used. The samples were placed in absence of light at room temperature, and the absorbance was measured after 20 min at 517 nm on a VWR UV-1600PC Spectrophotometer (VWR, Wayne, PA, USA). A standard calibration curve ($y = -0.008x + 1.3476$, $R^2 = 0.9948$) was prepared using standard Trolox solutions in a concentration range of 10–150 μM. The results were expressed as mean value ± standard deviation of μmol TE per g sample.

3.6.3. Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was performed following a methodology previously described by Shortle et al. (2014) [48], with some modifications. Sodium acetate buffer (0.3 M, pH 3.6), 0.01 M TPTZ solution in 0.04 M hydrochloric acid and 20 mM $FeCl_3 \times 6H_2O$ aqueous solution in a ratio 10:1:1, respectively, were used to prepare the FRAP reagent. The reagent was incubated at 37 °C for 10 min prior to analysis. Afterward, 80 μL of the extract (extraction solvent for blank), 240 μL of distilled water and 2080 μL of FRAP reagent were added into test tubes, shaken at 1800 rpm using Vortex MS2 Minishaker IKA (IKA, Staufen, Germany) and incubated at 37 °C for 5 min. The absorbance was read on a VWR UV-1600PC Spectrophotometer (VWR, Wayne, PA, USA) at 593 nm. A standard calibration curve ($y = 0.0013$, $R^2 = 0.9995$) was prepared using standard Trolox solutions (25–1000 μM). The results were expressed as mean value ± standard deviation of μmol TE per g sample.

3.7. Statistical Analysis

Statistica ver. 12.0 (Statsoft Inc., Tulsa, OK, USA) software was used for the statistical analysis of the results. For the determination of optimal extraction conditions, TPC was the dependent variable, while a full factorial design (mixed two- and three-level) comprising 72 trials was applied in order to evaluate the influence of the following independent variables: (a) solvent (50% and 70% ethanol), (b) temperature (90, 120 and 150 °C), (c) extraction cycles (1, 2 and 3) and (d) static extraction time (5 and 10 min). The normality and homoscedasticity of the data were analyzed using the Shapiro–Wilk W test and Levene's test, respectively. Normally distributed data were analyzed using one-way and multifactorial analysis of variance (ANOVA), while Tukey's HSD multiple comparison test was used to compare marginal means. Nonparametric tests including Kruskal–Wallis one-way ANOVA and multiple comparison of mean ranks were applied for analysis of the data which were not normally distributed and/or not homoscedastic. All of the tests were significant at $p \leq 0.05$.

4. Conclusions

PLE, as a novel advanced green extraction technique, was optimized for the efficient isolation of polyphenols from laurel leaves. The optimal extraction conditions determined were 50% ethanol, a temperature of 150 °C, one extraction cycle and a static time of 5 min. The polyphenolic profile of the laurel leaf extract obtained by PLE comprised 29 compounds, including flavonoids (flavones, flavonols, flavan-3-ols and proanthocyanidins) and phenolic acids, and quantitative analysis has shown that flavonols (rutin and quercetin glucoside as the main representatives) were the most abundant group. The antioxidant activity assays have demonstrated that PLE yields extracts with relatively high antioxidant activity through a time-, energy- and solvent-efficient automated process, demonstrating its advantages over conventional extraction techniques in terms of scaling-up processes and reducing solvent, time and energy consumption. Therefore, PLE was proven to be an efficient green extraction technique suitable for the isolation of polyphenols from laurel leaves.

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CHAPTER 4

Publication No.4: Physicochemical Properties, Antioxidant Capacity, and Bioavailability of *Laurus nobilis* L. Leaf Polyphenolic Extracts Microencapsulated by Spray Drying.

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foods

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Article

Physicochemical Properties, Antioxidant Capacity, and Bioavailability of *Laurus nobilis* L. Leaf Polyphenolic Extracts Microencapsulated by Spray Drying

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Abstract: Laurel (*Laurus nobilis* L.) leaves are a rich source of polyphenols with the potential for use in functional foods, where the main obstacle is their low stability and bioavailability, which can be improved by spray drying (SD). This research examined the influence of SD parameters, including inlet temperature (120, 150, and 180 °C), carrier type (β-cyclodextrin (β-CD); β-CD + maltodextrin (MD) 50:50; β-CD + gum arabic (GA) 50:50), and sample:carrier ratio (1:1, 1:2 and 1:3) on the physicochemical properties, encapsulation efficiency, polyphenolic profile, antioxidant capacity and bioaccessibility of laurel leaf polyphenols. The highest encapsulation efficiency was achieved at a sample:carrier ratio 1:2 and the temperature of 180 °C by using either of the applied carriers. However, the application of β-CD + MD 50:50 ensured optimal solubility (55.10%), hygroscopicity (15.32%), and antioxidant capacity (ORAC 157.92 μmol Trolox equivalents per g of powder), while optimal moisture content (3.22%) was determined only by temperature, demanding conditions above 150 °C. A total of 29 polyphenols (dominantly flavonols) were identified in the obtained powders. SD encapsulation increased the bioaccessibility of laurel flavonols in comparison to the non-encapsulated extract by ~50% in the gastric and ~10% in the intestinal phase, especially for those powders produced with carrier mixtures.

Keywords: laurel; polyphenols; microencapsulation; spray drying; bioaccessibility; antioxidant activity

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1. Introduction

Laurel (*Laurus nobilis* L.) is a Mediterranean shrub well known in folk medicine due to the many health-beneficial properties attributed mainly to the leaves extracts, which contain significant amounts of various polyphenols belonging to the groups of flavonols, flavan-3-ols, flavones, proanthocyanidins, and phenolic acids [1]. Laurel leaf polyphenols are known for their numerous biological effects, which hold potential for their utilization in the food industry as natural preservatives, antioxidants, or as functional food ingredients [2]. However, polyphenols are prone to degradation under different storage conditions such as temperatures, humidity, light, and pH [3] and also have low bioavailability derived from their low solubility, instability during digestion, and difficult cell membrane diffusion [4]. For this reason, it is of great importance to improve their stability which can be achieved by various microencapsulation techniques.

Spray drying is a widely used method for microencapsulation of bioactive molecules in which the liquid extract with the dissolved carrier is passed through a stream of hot air in which the solvent, evaporates and a powder is formed with bioactive molecules encapsulated in the protective coating of the carrier [5]. The physicochemical properties of powders

depend on the applied process parameters, properties of the feed, and the adequate choice of carrier type and the proportion in the mixture [6]. Some of the carriers which are often used in spray drying technology are starch and its derivatives (e.g., maltodextrins and cyclodextrins) and gums (e.g., gum arabic). Maltodextrins (MD) are highly soluble linear polymers obtained by partial hydrolysis of starch consisting of 3–20 β-d-glucose units [7], often mixed with other carriers due to their low emulsifiability [8]. Cyclodextrins (CD), including α-, β- and γ-cyclodextrin, are enzymatically hydrolyzed starch derivatives consisting of a hydrophobic cavity that allows accommodation of various guest molecules and of a hydrophilic external surface providing aqueous solubility [8]. Gum arabic (GA) is a variable complex natural derivative of the acacia plant consisting of a mixture of arabinogalactan, monosaccharides (galactose, rhamnose, arabinose) and glycoprotein [9]. These carriers are widely used since they meet the necessary requirements, including the "generally recognized as safe" (GRAS) status, relatively high water solubility, high molecular weight, and high glass transition temperature, which allow the protection of the final product from external factors such as heat, oxygen, humidity, and light [5]. To our knowledge, there is only one work related to the spray drying of *Laurus nobilis* L. leaf extracts [10] and another on the Mexican laurel leaf (*Litsea glaucescens*) [11], which has similar chemical composition as *Laurus nobilis* L. [12]. The physicochemical characterization of the powders obtained in the mentioned studies did not cover some of the parameters important for the stability and applicability of powders, such as hygroscopicity, moisture content, or solubility. In addition, the individual polyphenolic content or bioaccessibility during different stages of digestion was not investigated, showing the need for further research in order to find optimal process parameters that would result in the highest quality of the powders.

The aim of this research was to examine the influence of inlet temperature (120–180 °C), carrier type (β-CD, β-CD + MD 50:50, and β-CD + GA 50:50), and sample:carrier ratio (1:1, 1:2, and 1:3) on the process yield, physicochemical parameters (moisture content, solubility, hygroscopicity) and encapsulation efficiency of spray-dried laurel leaf extract, with the hypothesis that all of the applied process parameters would have significant influence. In addition, microcapsule morphology, antioxidant capacity, polyphenolic profile, and bioaccessibility will be examined in the powders where the highest encapsulation efficiency is obtained.

2. Materials and Methods

2.1. Chemicals and Reagents

Mili-Q system (Millipore, Bedford, MA, USA) was applied for the purification of distilled water. Kemika d.d. (Zagreb, Croatia) supplied ethanol (96%), methanol, sodium chloride, Fe (III) chloride hexahydrate, sodium acetate (99%), sodium bicarbonate, and HPLC grade formic acid (99%). Sigma Aldrich (St. Louis, MO, USA) supplied maltodextrin (DE 4–7), bile salts, porcine pancreatin, porcine gastric mucosa pepsin, and 2,2-diphenyl-1-(2,4,6- trinitrophenyl) hydrazyl (DPPH), as well as standards of quercetin-3-glucoside, myricetin, gallic, syringic, ferulic, protocatechuic, caffeic, chlorogenic, p-coumaric, and rosmarinic acid. Gum arabic, β-cyclodextrin, Trolox, and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) were obtained from Acros Organics (Geel, Belgium). Honeywell Riedel-de-Haën (Charlotte, NC, USA) supplied fluorescein sodium salt, while J.T.Baker (Deventer, The Netherlands) supplied glacial acetic acid, hydrochloric acid (37%), and HPLC grade acetonitrile. Extrasynthese (Genay, France) supplied standards of procyanidin B2, rutin, kaempferol-3-glucoside, luteolin, apigenin, catechin, epigallocatechin gallate, and epicatechin gallate. Methanol (ethanol with 0.5% v/v DMSO for apigenin) stock solutions of the standards were used to produce five working standard dilutions.

2.2. Plant Material

Dry laurel leaves collected in November 2021 in Lovran, Croatia (45°17′48.9408″ N/14°16′20.532″ E) were procured from Šafram d.o.o. (Zagreb, Croatia). Prior to extraction,

the leaves were ground in an electric grinder (GT11, Tefal, Rumilly, France) in order to produce a coarse powder. The powdered leaves' total solids (>95%) were determined by drying at 103 ± 2 °C until a constant mass was achieved [13].

2.3. Microwave-Assisted Extraction (MAE)

The MAE of laurel leaf polyphenols was carried out at the sample:solvent ratio 1:6.25 and the previously defined optimal parameters [14]: 50% ethanol as a solvent, temperature 80 °C, microwave power 400 W, and irradiation time 10 min. The extract dry matter (4.74%) was determined by drying to constant mass at 103 ± 2 °C [13], while the total phenolic content of the extract (8300 mg gallic acid equivalents (GAE)/L) was determined spectrophotometrically as previously described [14]. Briefly, 100 µL sample, 200 µL Folin-Ciocalteu reagent, and 2 mL of water were mixed in test tubes, and 1 mL of 20% Na_2CO_3 aqueous solution was added after 3 min. The samples were placed in a water bath at 50 °C for 25 min, and the absorbance was read at 765 nm on the UV-1600PC spectrophotometer (VWR, Wayne, PA, USA).

2.4. Microencapsulation by Spray Drying

Microencapsulation by spray drying of the laurel leaf extract was carried out as shown in the experimental design (Table 1) on the Büchi Mini Spray Dryer B-290 laboratory device in a closed mode paired with the B295 inert loop (Büchi, Switzerland) working with nitrogen as drying gas. β-CD alone or with the addition of MD or GA (1:1 w/w) was used as a carrier at varying extract dry matter:carrier ratios (1:1, 1:2, and 1:3, w/w). An appropriate mass of carrier (4.74 g, 9.48 g, and 14.22 g for 1:1, 1:2, and 1:3 extract dry matter:carrier ratio, respectively) was dissolved in 100 mL of water at 50 °C for 30 min on a magnetic stirrer (IKA, Staufen, Germany) and mixed with 100 mL of the laurel leaf extract. During the process of spray drying, aspirator capacity, pump capacity, and nozzle cleaner were kept at 80%, 15%, and level 4, respectively. Three different inlet temperatures were applied: 120 °C, 150 °C and 180 °C with the corresponding outlet temperatures of 70 °C, 80 °C and 90 °C, respectively. The laurel leaf powders were produced in duplicate and hermetically stored at room temperature in plastic containers until further analysis.

2.5. Microcapsules' Characterization

2.5.1. Process Yield

The process yield was calculated using the equation [15]:

$$\text{Process yield } (\%) = \frac{m_p}{m_d + m_c} \times 100 \quad (1)$$

where m_p is the mass (g) of powder, m_d is the extract dry matter (g) in the volume used for drying, and m_c is the mass of the added carrier (g).

2.5.2. Moisture Content

The powders' moisture content was analyzed by drying to constant mass at 103 ± 2 °C [13].

2.5.3. Solubility

The powders' solubility was determined using a previously described method [15] and calculated using the following equation:

$$\text{Solubility } (\%) = \frac{m_s}{m_p} \times 100 \quad (2)$$

where m_s is the mass (g) of the dried supernatant, and m_p represents the mass (g) of laurel leaf powder used for analysis.

Table 1. Physicochemical properties, encapsulation efficiency, and capacity of laurel leaf powders obtained at different inlet temperatures using different carriers at varying ratios.

Sample	Inlet Temperature	Carrier	Sample:Carrier Ratio	Moisture Content %	Process Yield %	Solubility %	Hygroscopicity mg/100 g	Encapsulation Efficiency %	Encapsulation Capacity %	OEE
1	120	β-CD	1:1	2.69 ± 0.13	77.77 ± 0.55	28.02 ± 0.20	17.23 ± 0.12	84.71 ± 0.28	42.55 ± 2.06	0.36 ± 0.02
2		β-CD + MD 50:50		3.54 ± 0.08	75.69 ± 0.53	57.83 ± 0.41	17.82 ± 0.13	69.07 ± 1.13	42.20 ± 0.63	0.29 ± 0.00
3		β-CD + GA 50:50		3.53 ± 0.35	75.50 ± 0.53	53.86 ± 0.38	21.80 ± 0.15	71.26 ± 0.92	53.22 ± 0.00	0.38 ± 0.00
4		β-CD	1:2	4.58 ± 0.14	78.91 ± 0.56	44.85 ± 0.32	12.70 ± 0.09	82.20 ± 1.19	55.46 ± 0.99	0.46± 0.01
5		β-CD + MD 50:50		4.54 ± 0.24	75.99 ± 0.53	56.20 ± 0.40	15.16 ± 0.11	78.50 ± 1.72	69.46 ± 0.67	0.55 ± 0.01
6		β-CD + GA 50:50		4.72 ± 0.08	71.69 ± 0.50	53.01 ± 0.37	18.30 ± 0.13	81.98 ± 2.61	76.67 ± 1.70	0.63 ± 0.03
7		β-CD	1:3	5.71 ± 0.02	73.70 ± 0.52	45.67 ± 0.32	9.94 ± 0.07	72.40 ± 1.59	38.55 ± 3.49	0.28 ± 0.03
8		β-CD + MD 50:50		5.44 ± 0.21	70.36 ± 0.50	46.04 ± 0.32	11.83 ± 0.08	53.09 ± 0.86	56.79 ± 1.07	0.30 ± 0.01
9		β-CD + GA 50:50		4.39 ± 0.35	84.46 ± 0.59	47.33 ± 0.33	17.59 ± 0.12	50.20 ± 0.71	62.70 ± 1.39	0.31 ± 0.00
10	150	β-CD	1:1	3.70 ± 0.22	72.09 ± 0.51	40.47 ± 0.28	18.02 ± 0.13	63.48 ± 0.29	34.26 ± 0.19	0.22± 0.00
11		β-CD + MD 50:50		3.95 ± 0.15	77.28 ± 0.54	58.90 ± 0.41	16.35 ± 0.12	61.91 ± 0.79	35.91 ± 2.09	0.22 ± 0.01
12		β-CD + GA 50:50		2.70 ± 0.16	73.71 ± 0.52	54.77 ± 0.39	19.46 ± 0.14	60.34 ± 0.84	47.22 ± 3.02	0.29 ± 0.02
13		β-CD	1:2	3.66 ± 0.16	73.73 ± 0.52	45.00 ± 0.32	12.08 ± 0.08	88.67 ± 1.60	62.33 ± 1.47	0.55 ± 0.00
14		β-CD + MD 50:50		2.80 ± 0.17	69.87 ± 0.49	46.03 ± 0.32	15.31 ± 0.11	69.87 ± 5.61	61.65 ± 1.50	0.43 ± 0.05
15		β-CD + GA 50:50		3.84 ± 0.10	67.91 ± 0.48	33.03 ± 0.23	19.80 ± 0.14	83.01 ± 3.39	67.29 ± 0.57	0.56 ± 0.02
16		β-CD	1:3	3.37 ± 0.16	66.20 ± 0.47	47.35 ± 0.33	10.70 ± 0.08	81.84 ± 0.42	41.99 ± 3.18	0.34 ± 0.02
17		β-CD + MD 50:50		2.26 ± 0.17	81.58 ± 0.57	52.36 ± 0.37	13.55 ± 0.10	78.76 ± 1.12	69.67 ± 2.60	0.55 ± 0.03
18		β-CD + GA 50:50		2.72 ± 0.18	80.28 ± 0.56	52.66 ± 0.37	18.08 ± 0.13	74.95 ± 2.93	59.67 ± 3.22	0.45 ± 0.04

Table 1. *Cont.*

Sample	Inlet Temperature	Carrier	Sample:Carrier Ratio	Moisture Content %	Process Yield %	Solubility %	Hygroscopicity mg/100 g	Encapsulation Efficiency %	Encapsulation Capacity %	OEE
19	180	β-CD	1:1	3.65 ± 0.12	68.96 ± 0.49	39.81 ± 0.28	20.15 ± 0.14	81.44 ± 1.72	62.46 ± 1.92	0.51 ± 0.03
20		β-CD + MD 50:50		3.73 ± 0.24	75.14 ± 0.53	58.91 ± 0.41	18.46 ± 0.13	71.18 ± 2.04	59.82 ± 0.92	0.43 ± 0.02
21		β-CD + GA 50:50		3.83 ± 0.22	73.10 ± 0.51	52.63 ± 0.37	21.27 ± 0.15	67.26 ± 0.29	55.88 ± 1.59	0.38± 0.01
22		β-CD	1:2	3.42 ± 0.16	74.69 ± 0.53	47.45 ± 0.33	14.28 ± 0.10	89.83 ± 1.03	67.96 ± 1.46	0.61 ± 0.02
23		β-CD + MD 50:50		2.51 ± 0.26	78.96 ± 0.56	59.47 ± 0.42	14.13 ± 0.10	74.41 ± 3.39	80.23 ± 1.70	0.60 ± 0.04
24		β-CD + GA 50:50		3.49 ± 0.27	75.67 ± 0.53	52.88 ± 0.37	17.99 ± 0.13	75.38 ± 0.62	69.44 ± 0.94	0.52 ± 0.01
25		β-CD	1:3	3.50 ± 0.04	74.38 ± 0.52	45.88 ± 0.32	12.00 ± 0.08	92.07 ± 0.57	58.58 ± 0.86	0.54 ± 0.00
26		β-CD + MD 50:50		3.66 ± 0.17	76.39 ± 0.54	60.15 ± 0.42	15.24 ± 0.11	45.30 ± 2.15	59.17 ± 1.31	0.27 ± 0.01
27		β-CD + GA 50:50		4.39 ± 0.19	73.35 ± 0.52	46.12 ± 0.32	16.41 ± 0.12	82.63 ± 0.85	75.89 ± 1.71	0.63 ± 0.02
Average				3.72	74.72	49.14	16.14	73.54	58.04	0.43

β-CD = β-cyclodextrin; MD = maltodextrin; GA = gum arabic. OEE = overall encapsulation efficiency factor. Results are expressed as mean ± SD.

2.5.4. Hygroscopicity

The hygroscopicity of the obtained powders was determined as previously described [15] and expressed as g of absorbed moisture per 100 g of powder according to the equation:

$$\text{Hygroscopicity (g/100 g)} = \frac{m_7 - m_0}{m_0} \times 100 \quad (3)$$

where m_7 is the mass (g) of the powder after 7 days, and m_0 is the initial mass (g) of the powder.

2.5.5. Encapsulation Efficiency and Capacity

For the determination of encapsulation efficiency (EE) and capacity (EC), the total and surface polyphenols of the obtained laurel leaf powders were determined spectrophotometrically at 765 nm following a previously described method [14]. The total polyphenols were extracted by mixing 0.2 g of powder with 2 mL of methanol:water:acetic acid (50:42:8) solvent mixture in a test tube stirred briefly on a vortex mixer (IKA, Staufen, Germany). The test tube was then put in an ultrasonic bath for 20 min without heating, centrifuged for 10 min at 3000 rpm, and filtered through Whatman no.40 filter paper. The surface polyphenols were extracted following a similar procedure using methanol:ethanol (50:50) solvent mixture without placing the samples in an ultrasonic bath.

The EE was calculated according to the following equation [16]:

$$\text{EE} = \frac{TPC_p}{TPC_i} \times 100 \quad (4)$$

where TPC_p is the total phenolic content in the obtained powder (mg GAE g^{-1} extract dry matter (DM)), and the TPC_i is the total phenolic content in the initial extract (mg GAE g^{-1} extract DM).

The EC was calculated using the following equation [15]:

$$\text{EC (\%)} = \left(\frac{TP - SP}{TP}\right) \times 100 \quad (5)$$

where TP is the concentration (mg GAE g^{-1} powder) of total polyphenols, and SP is the concentration (mg GAE g^{-1} powder) of surface polyphenols.

In order to evaluate the overall encapsulation efficiency of the spray drying process, an overall encapsulation efficiency factor (OEE) was calculated using following equation:

$$\text{OEE} = \frac{EC \times EE}{10,000} \quad (6)$$

where EC is the encapsulation capacity (%), and EE is the encapsulation efficiency (%). By multiplying the TPC of the initial extract with the OEE, the amount of the successfully encapsulated polyphenols can be predicted.

2.5.6. SEM Analysis

The study of particle size and morphology of the obtained microcapsules was carried out at the Ruđer Bošković Institute, Zagreb, Croatia, on the JSM-7000F high-resolution field emission SEM (scanning electron microscope) (Jeol, Tokyo, Japan). Laurel leaf powders were deposited on carbon tape in a thin layer on a sample holder to fix them in place and enable electrical contact with the instrument. An accelerating voltage of 5000 V at the standard objective-sample distance (10 mm) was applied, and photomicrographs of each sample were taken at 2000× and 5000× magnification using a secondary electron detector.

2.5.7. UPLC-MS^2 Analysis

For the UPLC-MS^2 analysis, 1 g of the powders was mixed in a test tube with 10 mL of 80% (v/v) methanol and placed in an ultrasonic bath for 20 min. The samples were filtered into a 10 mL volumetric flask using the Whatman no.40 filter paper and made up to volume with the solvent. An aliquot of 1.5 mL was filtered into glass vials using 0.45 µm syringe filters and stored at −18 °C until further analysis. The polyphenolic profile of the powders was determined on the Agilent 1290 RRLC UPLC-MS^2 system paired with 6430 Series LC-MS Triple Quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA) at the conditions described previously, along with identification and quantification procedure [14]. The concentrations of the analyzed polyphenols were expressed as mg/100 g of the powder (mean value ± standard deviation (SD)). All analyses were carried out in duplicate.

2.5.8. Antioxidant Capacity

The antioxidant capacity of the laurel leaf powders dissolved in 80% methanol as described in Section 2.5.7. was determined by DPPH radical scavenging assay, Ferric Reducing Antioxidant Power (FRAP) assay, and Oxygen Radical Absorbance Capacity (ORAC) assay following previously described methodologies [17]. For the DPPH and FRAP, the absorbances were read on a UV-1600PC spectrophotometer (VWR, Wayne, PA, USA) at 517 nm and 593 nm, respectively. For the ORAC (CLARIOstar Microplate Reader, BMG LABTECH, Germany), the fluorescence intensity with the excitation and emission wavelengths of 485 nm and 528 nm, respectively, were monitored during 120 min in intervals of 90 s. The collected data were analyzed using the MARS 2.0 software (BMG LABTECH, Offenburg, Germany). Trolox was used as a standard for all three methods. All measurements were performed in duplicate, and the results were expressed in µmol Trolox equivalents (TE) g^{-1} powder as mean value ± SD.

2.5.9. Bioaccessibility of Polyphenols

The bioaccessibility of polyphenols encapsulated in the powders was examined in a simulated three-step in vitro digestion following a recently described methodology [15]. Briefly, 250 mg of powders (750 µL of laurel leaf extract) was mixed with 800 µL of pepsin solution (40 mg mL^{-1}) and 10 mL of 0.9% NaCl solution in 50 mL Falcon tubes. The pH was adjusted to 2 by adding an adequate volume of 0.1M HCl, if necessary. The samples were placed in a water bath shaker (IKA, Staufen, Germany) for 1 h at 100 rpm and a temperature of 37 °C. In order to stop the reaction, the samples were then put on ice for 5 min. Pur-A-Lyzer 6–8 kDa dialysis membranes (Sigma-Aldrich, Steinheim, Germany) containing the mixture of 1 mL of 0.5 M $NaHCO_3$ and 1 mL of 0.9% NaCl were added in the Falcon tubes, which were again placed in water bath shaker at the same conditions for 45 min. Afterward, the pH was adjusted to 6.5 by adding 1 M $NaHCO_3$ in the necessary volume, 2.5 mL of pancreatin (2 mg mL^{-1})-bile salts (12 mg mL^{-1}) solution was added to the reaction, and the samples were incubated for another 2 h in a water bath shaker; 2 mL aliquots of each phase were taken for the UPLC-MS^2 analysis of the phenolic content performed as described in Section 2.5.7. The process and analysis were performed in duplicate, and the results were expressed in mg g^{-1} extract DM as mean value ± SD.

2.6. Statistical Analysis

Statistical analysis of the data was performed using the Statistica ver. 10.0 (Statsoft Inc., Tulsa, OK, USA) software. A full factorial design (Table 1) comprising 27 experimental trials performed in duplicate was applied in order to evaluate the influence of spray drying parameters on the physicochemical properties of the obtained powders. The inlet temperature, type of carrier, and sample:carrier ratio were the independent variables (X) observed at three levels, while moisture content, process yield, solubility, hygroscopicity, encapsulation efficiency, and encapsulation capacity were the dependent variables (Y).

The normality of the data set and homogeneity of the residuals were analyzed by Shapiro–Wilk's and Levene's tests, respectively, followed by one-way analysis of variance (ANOVA) paired with Tukey's HSD multiple comparison test on normally distributed and homogenous data. Nonparametric Kruskal–Wallis one-way ANOVA followed by multiple comparison of mean ranks were applied to the data that were not normally distributed and/or homogenous. All of the tests were considered significant at $p \leq 0.05$.

3. Results and Discussion

This study evaluated the influence of drying temperature, applied carrier, and sample:carrier ratio on different properties of the obtained powders relevant to their stability during storage and biological activity. The experimental design and obtained values of observed parameters are shown in Table 1, while the results of the statistical analysis are shown in Table 2.

3.1. Process Yield

When the process yield is higher than 50%, the process of spray drying can be considered successful [18]. As shown in Table 1, the process yield in this study ranged from 66.20 to 84.46% showing that the process was successful at all of the applied drying conditions. Statistical analysis (Table 2) showed that none of the applied conditions had a significant influence on the process yield. This is in agreement with made observations that all produced powders were in a free-flowing form, and stickiness or adherence to chamber walls did not occur at any of the drying conditions applied. Obtained variations in yield may be a result of manual collecting of the particles adhered to the cyclone wall, as well as loss of the fine particles through the outlet air filter [19].

3.2. Moisture Content

Moisture content is a highly relevant requirement for the stability of the obtained powders during packaging and storage. A moisture content lower than 5% is desirable since there is a lower chance of microbial growth, the solubility of powders is higher, and overall stability is greater, making the powders applicable in the industry [5]. As shown in Table 1, the moisture content in this study ranged from 2.26 to 4.72% showing that all of the obtained powders have the moisture content required for their stability. However, statistical analysis (Table 2) showed that the powders obtained at inlet temperatures above 150 °C had a significantly ($p < 0.01$) lower moisture content. This is expected since the temperature gradient between the drying air and the atomized particles is greater at higher inlet temperatures resulting in increased water evaporation [5]. The type of carrier and the sample:carrier ratio had no statistically significant influence on the moisture content.

3.3. Solubility

Solubility is an important quality factor influencing the reconstitution behavior of the powders, and low solubility can cause difficulties during production of the enriched products [5]. The solubility of the powders obtained in the present study ranged widely from 28.20 to 60.15% (Table 1). The statistical analysis showed that only the type of the applied carrier had a statistically significant influence ($p < 0.01$) on the solubility and that the powders obtained using a combination of β-CD with either MD or GA resulted in higher solubility than when β-CD was used alone. This can be explained by the low water solubility of β-CD, which was enhanced by the presence of more water-soluble MD and GA [8].

Table 2. Influence of spray drying parameters on the physicochemical characteristics of the obtained laurel leaf powders.

Source of Variation	N	Process Yield %	Moisture Content %	Solubility %	Hygroscopicity g/100 g	EE %	EC %	OEE
Inlet temperature		$p = 0.27$ ‡	$p < 0.01$ †	$p = 0.31$ ‡	$p = 0.70$ ‡	$p = 0.59$ ‡	$p < 0.05$ †	$p < 0.05$ †
120 °C	18	76.01 ± 0.95 [a]	4.35 ± 0.22 [b]	48.09 ± 2.04 [a]	15.82 ± 0.77 [a]	71.49 ± 2.86 [a]	55.29 ± 2.97 [a]	0.40 ± 0.03 [a]
150 °C	18	73.63 ± 1.22 [a]	3.22 ± 0.14 [a]	47.84 ± 1.81 [a]	15.93 ± 0.77 [a]	73.65 ± 2.38 [a]	53.33 ± 3.14 [a]	0.40 ± 0.03 [a]
180 °C	18	74.52 ± 0.63 [a]	3.58 ± 0.12 [a]	51.48 ± 1.64 [a]	16.66 ± 0.77 [a]	75.50 ± 3.21 [a]	65.49 ± 1.94 [b]	0.50 ± 0.03 [b]
Carrier		$p = 0.22$ ‡	$p = 0.76$	$p < 0.01$ †	$p < 0.01$ †	$p < 0.01$†	$p < 0.05$ †	$p = 0.38$ ‡
β-CD	18	73.38 ± 0.96 [a]	3.81 ± 0.02 [a]	42.72 ± 1.41 [a]	14.12 ± 0.81 [a]	81.84 ± 2.07 [b]	51.57 ± 2.83 [a]	0.43 ± 0.03 [a]
β-CD + MD 50:50	18	75.70 ± 0.96 [a]	3.60 ± 0.02 [a]	55.10 ± 1.29 [b]	15.32 ± 0.47 [a]	66.90 ± 2.65 [a]	59.43 ± 3.14 [a]	0.40 ± 0.03 [a]
β-CD + GA 50:50	18	75.08 ± 0.96 [a]	3.73 ± 0.02 [a]	49.59 ± 1.57 [b]	18.97 ± 0.40 [b]	71.89 ± 2.56 [a]	63.10 ± 2.33 [b]	0.46 ± 0.03 [a]
Sample:Carrier Ratio		$p = 0.79$ ‡	$p = 0.61$ ‡	$p = 0.67$ ‡	$p < 0.01$ †	$p < 0.01$ †	$p < 0.01$ †	$p < 0.01$ †
1:1	18	74.36 ± 0.63 [a]	3.50 ± 0.11 [a]	49.47 ± 2.48 [a]	18.95 ± 0.42 [b]	70.07 ± 1.92 [a]	48.17 ± 2.35 [a]	0.34 ± 0.02 [a]
1:2	18	74.16 ± 0.87 [a]	3.73 ± 0.18 [a]	48.66 ± 1.79 [a]	15.53 ± 0.60 [a]	80.43 ± 1.57 [b]	67.83 ± 1.75 [b]	0.54 ± 0.02 [b]
1:3	18	75.63 ± 1.31 [a]	3.94 ± 0.27 [a]	49.28 ± 1.12 [a]	13.93 ± 0.69 [a]	70.14 ± 3.78 [a]	58.11 ± 2.73 [a]	0.41 ± 0.03 [a]
Total	54							

N = number of trials. β-CD = β-cyclodextrin. MD = maltodextrin. GA = gum arabic. EE = encapsulation efficiency; EC = encapsulation capacity; OEE = overall encapsulation efficiency factor Results are expressed as mean ± SE. Values with different letters within a parameter are statistically different at $p \leq 0.05$. † The variable is statistically significant at $p \leq 0.05$. ‡ The variable is statistically insignificant at $p \leq 0.05$.

3.4. Hygroscopicity

Hygroscopicity is a parameter that shows how much moisture the powder absorbs from a relatively humid environment over a certain time period and, as such, can be a valuable predictor of the powder's stability during storage. In the present study, the hygroscopicity of the powders during 7 days ranged from 9.94 to 21.8% (Table 1). Type of carrier and sample:carrier ratio significantly ($p < 0.01$) influenced the hygroscopicity, while the influence of temperature was insignificant (Table 2). Powders obtained using β-CD alone or in combination with MD had a significantly lower hygroscopicity than the powders obtained using the combination of β-CD + GA 50:50. This can be explained by the branched structure of GA, which allows water molecules to bind to the hydroxyl groups in the chains [20], while MD (DE 4–7) is less polymerized and the β-CD has a specific cyclic structure with hydrophobic cavity and a hydrophilic outer part and are therefore less susceptible for the binding of water molecules [8]. The sample:carrier ratio of 1:1 resulted in a higher hygroscopicity than both 1:2 and 1:3 ratios which can be explained by the increased dry matter content with the addition of carrier and consequently lower water content and lower hygroscopicity [21].

3.5. Encapsulation Efficiency and Capacity

Encapsulation efficiency of the spray drying process wascalculated from the TPC of the initial extract (Supplementary Materials; Table S1), and the TPC of the obtained powders ranged from 45.3 to 92.07% with the mean value of 73.54% (Table 1), which is comparable to the range of 72.9–99.3% [10], as well as the value of 70% [11] achieved during spray drying of laurel leaf (*L. nobilis* L.) and Mexican laurel leaf (*Litsea glaucescens*) extract, respectively. Statistical analysis (Table 2) showed that inlet temperature had no statistically significant influence on the encapsulation efficiency, while the applied carrier and sample:carrier ratio had a significant ($p < 0.01$) influence. The highest encapsulation efficiency was achieved when β-CD was applied as a carrier, possibly due to the structure of β-CD whose hydrophobic central cavity diameter was shown to be suitable for stable binding with flavonoids [22] whose β-ring showed high affinity for binding with β-CD [23]. Encapsulation capacity calculated using the concentration of total and surface polyphenols (Supplementary Materials; Table S1) ranged from 34.26 to 80.23%. Statistical analysis showed that inlet temperature and the sample:carrier ratio had a significant influence ($p < 0.01$) on the encapsulation capacity, while the applied carrier was not a significant parameter. The highest encapsulation capacity was obtained at the temperature of 180 °C, possibly due to faster drying rates which allowed the early structural formation of the complexes and therefore resulted in more efficient entrapment of the polyphenols [24]. The sample:carrier ratio of 1:2 resulted in both the highest encapsulation efficiency and capacity. The ratio of 1:2 was likely more efficient than the 1:1 ratio due to the higher concentration of the carrier, which was allowed to precipitate faster on the surface of the dispersed phase and therefore prevented the diffusion of polyphenols across the phase boundary [25]. Further addition of the carrier at the ratio of 1:3 possibly shortened the time of contact of the feed with drying air which slowed heat and mass transfer and delayed structural formation of the complexes resulting in less efficient entrapment.

The overall efficiency factor calculated from the encapsulation efficiency and capacity values showed that the highest overall encapsulation efficiency of the spray drying process could be achieved at the inlet temperature of 180 °C by using any of the three applied carriers at the ratio of 1:2. The use of β-CD + MD 50:50, however, would result in the most desirable physicochemical properties of the powder since it had a higher solubility than when only β-CD was applied and a lower hygroscopicity compared to β-CD + GA 50:50. Nevertheless, in order to observe the influence of carrier on other properties of the powders, the powders obtained using all three carriers at the temperature of 180 °C and the sample:carrier ratio 1:2 (powder samples 22, 23 and 24) were chosen for further analysis of the particle size by SEM, individual polyphenolic content by UPLC-MS^2, antioxidant capacity, and the in vitro bioavailability.

3.6. Morphology of the Microcapsules

In order to observe the morphology of the microcapsules as influenced by the type of carrier, the powders obtained at 180 °C and a carrier ratio 1:2 were analyzed by SEM at 2000× (Figure 1a–c) and 5000× (Figure 1d–f) magnification. None of the carriers resulted in uniform microcapsules whose size ranged from 1 to 8µm in all powders.

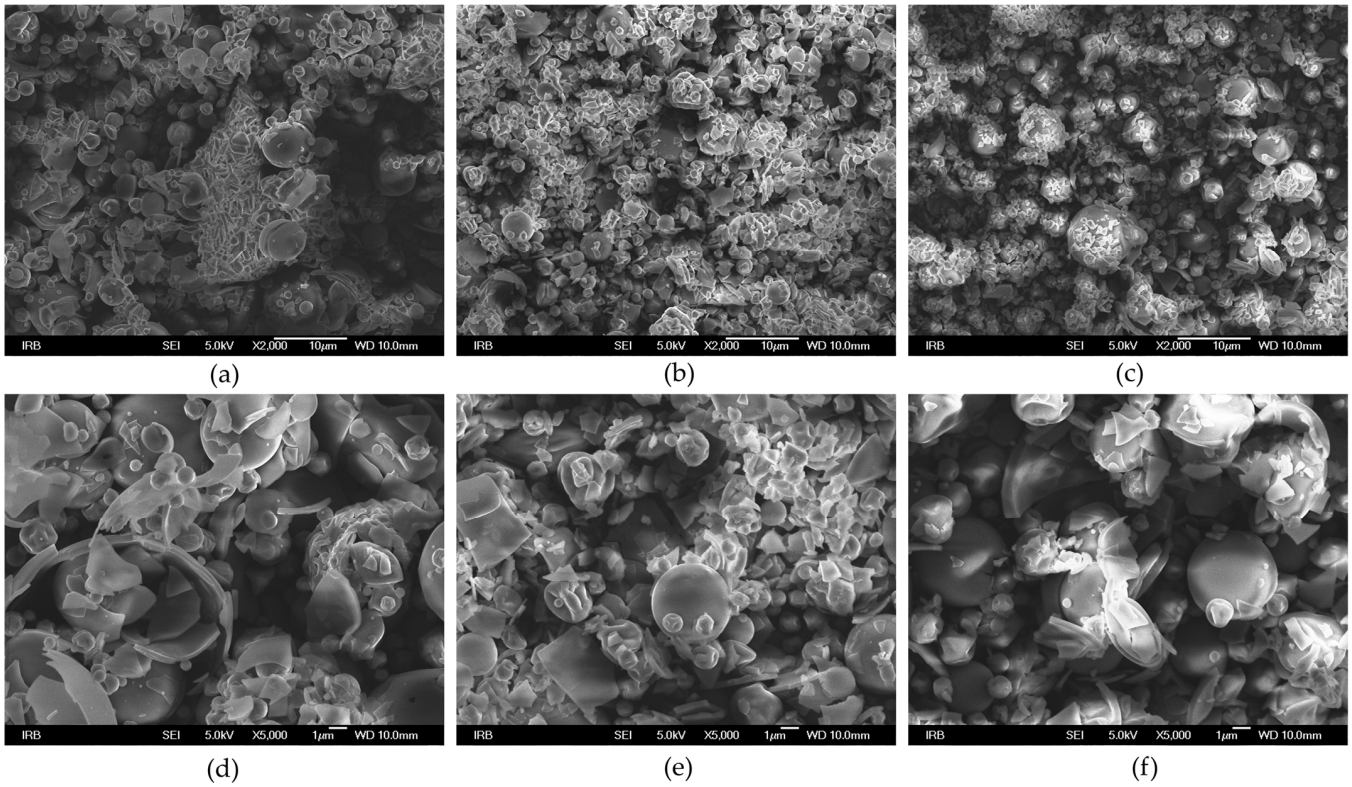

Figure 1. SEM images of the laurel leaf powders obtained at 180 °C and 1:2 sample:carrier ratio using different carriers: (**a**,**d**) β-CD; (**b**,**e**) β-CD + MD 50:50; (**c**,**f**) β-CD + GA 50:50.

Following a classification established by Walton (2000) [26], the type of microcapsules obtained by all the carriers can be classified as skin-forming with a visible mixture of non-broken microcapsules, fractured spheres, and broken shells characteristic for drying at high inlet temperatures that lead to rapid evaporation of the solvent [27].

3.7. Individual Polyphenolic Content

UPLC-MS^2 analysis was performed on the laurel leaf powders obtained at the temperature of 180 °C and sample:carrier ratio 1:2 in order to investigate the influence of the carrier type on the powders' polyphenolic profile (Table 3). The compounds were identified and quantified as described previously [14], and the chromatograms are shown in Supplementary Materials (Figure S1). In total, twenty-nine polyphenols were identified in all powders, among which ten were phenolic acids represented largely by syringic acid, four flavones (mainly luteolin), four flavan-3-ols (mostly catechin and epicatechin in equal amounts), one proanthocyanidin (B-type procyanidin trimer) and ten flavonols (dominantly quercetin glycosides) which were the most abundant group representing around 74% of all polyphenols in each powder which is in accordance with the polyphenolic content of laurel leaf extracts [1].

Table 3. Individual polyphenolic content of laurel leaf powders obtained with different carriers at 180 °C and sample:carrier ratio 1:2 as determined by UPLC-MS^2.

Compound Number	Retention Time	Tentative Identification	Concentration (mg 100 g^{-1} Powder)		
			β-CD	β-CD + MD 50:50	β-CD + GA 50:50
		Phenolic acids			
1	1.679	Gallic acid *	0.26 ± 0.01 ^{b}	0.18 ± 0.01 ^{a}	0.34 ± 0.01 ^{c}
2	2.313	3,4-dihydrobenzoic acid hexoside	0.17 ± 0.00 ^{b}	0.17 ± 0.00 ^{b}	0.13 ± 0.00 ^{a}
3	3.488	Syringic acid *	5.42 ± 0.15 ^{b}	5.39 ± 0.15 ^{b}	4.35 ± 0.12 ^{a}
4	3.508	Protocatechuic acid *	0.69 ± 0.02 ^{b}	0.60 ± 0.02 ^{a}	0.54 ± 0.02 ^{a}
5	4.259	Rosmarinic acid *	0.55 ± 0.02 ^{b}	0.78 ± 0.02 ^{c}	0.44 ± 0.01 ^{a}
6	4.813	*p*-hydroxybenzoic acid	0.45 ± 0.01 ^{a}	0.49 ± 0.01 ^{a}	0.44 ± 0.01 ^{a}
7	5.043	Chlorogenic acid *	0.38 ± 0.01 ^{b}	0.35 ± 0.01 ^{b}	0.27 ± 0.01 ^{a}
8	5.711	Caffeic acid *	0.52 ± 0.01 ^{b}	0.19 ± 0.01 ^{a}	0.80 ± 0.02 ^{c}
9	7.28	*p*-coumaric acid *	0.62 ± 0.02 ^{b}	0.55 ± 0.02 ^{a}	0.50 ± 0.01 ^{a}
10	8.587	Ferulic acid *	0.79 ± 0.02 ^{b}	0.66 ± 0.02 ^{a}	0.61 ± 0.02 ^{a}
		∑ Phenolic acids	9.84 ± 0.28 ^{b}	9.36 ± 0.26 ^{b}	8.44 ± 0.24 ^{a}
		Flavones			
11	2.755	Apigenin-6-C-(*O*-deoxyhexosyl)-hexoside	0.00 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}
12	6.938	Luteolin-6-C-glucoside	0.54 ± 0.02 ^{b}	0.49 ± 0.01 ^{b}	0.38 ± 0.01 ^{a}
13	8.29	Apigenin *	0.07 ± 0.00 ^{b}	0.06 ± 0.00 ^{a}	0.05 ± 0.00 ^{a}
14	9.849	Luteolin *	23.22 ± 0.66 ^{b}	22.94 ± 0.65 ^{b}	16.35 ± 0.46 ^{a}
		∑ Flavones	23.84 ± 0.67 ^{b}	23.50 ± 0.66 ^{b}	16.79 ± 0.47 ^{a}
		Flavan-3-ols			
15	5.93	Catechin *	124.96 ± 3.53 ^{b}	118.19 ± 3.34 ^{b}	103.76 ± 2.93 ^{a}
16	5.937	Epicatechin	123.25 ± 3.49 ^{b}	116.71 ± 3.30 ^{b}	102.97 ± 2.91 ^{a}
17	6.02	Epigallocatechin gallate *	0.04 ± 0.00 ^{a}	0.08 ± 0.00 ^{b}	0.04 ± 0.00 ^{a}
18	7.905	Epicatechin gallate *	0.22 ± 0.01 ^{c}	0.10 ± 0.00 ^{b}	0.07 ± 0.00 ^{a}
		∑ Flavan-3-ols	248.48 ± 7.03 ^{b}	235.09 ± 6.65 ^{b}	206.85 ± 5.85 ^{a}
		Proanthocyanidins			
19	6.249	Procyandinin trimer	78.67 ± 2.23 ^{a,b}	80.41 ± 2.27 ^{b}	71.30 ± 2.02 ^{a}
		∑ Proanthocyanidins	78.67 ± 2.23 ^{b}	80.41 ± 2.27 ^{b}	71.30 ± 2.02 ^{a}
		Flavonols			
20	7.692	Rutin *	136.91 ± 3.87 ^{a}	125.99 ± 3.56 ^{a}	123.21 ± 3.48 ^{a}
21	7.969	Quercetin-3-glucoside	362.32 ± 10.25 ^{b}	358.52 ± 10.14 ^{b}	268.95 ± 7.61 ^{a}
22	8.48	Kaempferol-3-rutinoside	46.60 ± 1.32 ^{b}	47.44 ± 1.34 ^{b}	33.24 ± 0.94 ^{a}
23	8.51	Kaempferol-3-hexoside	85.64 ± 2.42 ^{a}	82.03 ± 2.32 ^{a}	85.67 ± 2.42 ^{a}
24	8.52	Quercetin-3-pentoside	84.33 ± 2.39 ^{a}	82.73 ± 2.34 ^{a}	81.42 ± 2.30 ^{a}
25	8.877	Isorhamnetin-3-hexoside	125.78 ± 3.56 ^{b}	122.43 ± 3.46 ^{b}	86.69 ± 2.45 ^{a}
26	8.897	Quercetin-3-rhamnoside	162.36 ± 4.59 ^{b}	160.58 ± 4.54 ^{b}	133.26 ± 3.77 ^{a}
27	9.178	Kaempferol-3-*O*-pentoside	38.03 ± 1.08 ^{a}	35.19 ± 1.00 ^{a}	35.07 ± 0.99 ^{a}
28	9.825	Kaempferol-3-*O*-deoxyhexoside	0.09 ± 0.00 ^{a}	0.09 ± 0.00 ^{a}	0.08 ± 0.00 ^{a}
29	12.137	Myricetin *	0.19 ± 0.01 ^{a}	0.19 ± 0.01 ^{a}	0.22 ± 0.01 ^{b}
		∑ Flavonols	1042.25 ± 29.48 ^{b}	1015.19 ± 28.71 ^{b}	847.81 ± 23.98 ^{a}
		Total	1403.07 ± 39.68 ^{b}	1363.54 ± 38.57 ^{b}	1151.19 ± 32.56 ^{a}

β-CD = β-cyclodextrin; MD = maltodextrin; GA = gum arabic. Results are expressed as mean ± SD. Values within rows marked with different letters were statistically different at $p < 0.05$. * identification confirmed using authentic standards.

Statistical analysis showed that carrier type significantly ($p < 0.05$) influenced the individual polyphenolic content of the powders. All polyphenolic groups and total polyphenols were more abundant in powders obtained using β-CD or the combination of β-CD + MD 50:50 compared to powders obtained using β-CD + GA 50:50. The largest differences were observed in the concentration of flavonols, namely quercetin-3-glucoside, and isorhamnetin-3-hexoside. Even though the branched structure of GA may facilitate the binding of polyphenols due to more binding sites available for the interaction, the presence of neutral sugars in the structure of GA [28] may result in the steric hindrance of the adsorption as it was previously shown in the case of pectins and procyanidins [29]. In addition, the degree of glycosylation and type of hydroxylation of the glycosides might have affected the solubility of flavonol glycosides and therefore hindered the binding with the complex structure [30] of GA, resulting in lower content than with the other two carriers.

3.8. Antioxidant Capacity

In order to examine the influence of carrier on the antioxidant capacity of the powders, powders obtained at 180 °C and sample:carrier ratio 1:2 were analyzed by DPPH, FRAP, and ORAC assay, and the results are shown in Table 4.

Table 4. Antioxidant capacity of the laurel leaf powders obtained at 180 °C and a sample:carrier ratio 1:2.

Carrier	DPPH μmol TE g^{-1} Powder	FRAP μmol TE g^{-1} Powder	ORAC μmol TE g^{-1} Powder
β-CD	162.18 ± 4.83 ^{a}	210.00 ± 9.06 ^{a}	88.59 ± 1.84 ^{a}
β-CD + MD 50:50	201.43 ± 3.85 ^{b}	267.18 ± 1.93 ^{b}	157.92 ± 3.28 ^{c}
β-CD + GA 50:50	159.30 ± 1.80 ^{a}	196.15 ± 16.77 ^{a}	99.43± 2.06 ^{b}

The results are expressed as mean ± SD. Values marked with different letters within a column are statistically different at $p \leq 0.05$.

As can be observed, all three assays showed that the powder obtained using β-CD + MD 50:50 showed the highest antioxidant capacity. These results indicate that not only the content of polyphenols was responsible for the antioxidant capacity since the powders obtained by using β-CD and β-CD + MD 50:50 had the same concentration of polyphenols as determined by UPLC-MS^2. It is likely that other antioxidant compounds, such as fatty acids or chlorophyll present in the laurel leaves [1], were encapsulated more efficiently by the combination of β-CD and MD due to their different chemical structures, which allow diverse binding mechanisms. In support, it was shown that combinations of carriers often result in higher antioxidant capacity of the encapsulated plant extracts, and maltodextrin was shown to be more efficient than GA in the encapsulation of chlorophyll [31].

3.9. Bioaccessibility of Polyphenols

The bioavailability of polyphenols is defined as the rate and degree of their absorption through the epithelial cells in the gastrointestinal tract, and it includes bioaccessibility (release) of compounds from the food matrix and bioactivity (digestion, absorption, metabolism, distribution, and the physiological response). Since the determination of bioavailability is complex due to ethical issues and impracticality, most of the research is focused on the bioaccessibility of bioactive molecules as the first step and key factor in predicting the bioavailable fraction of the compounds [32]. In order to observe the influence of carriers applied during spray drying on the bioavailability of laurel leaf polyphenols, the initial extract and the powders obtained at the temperature of 180 °C and sample:carrier ratio 1:2 with all three carriers (β-CD (S22); β-CD + MD 50:50 (S23); β-CD + GA 50:50 (S24) were analyzed by UPLC-MS^2. The content of flavonols as the most abundant polyphenolic group was monitored through three stages of in vitro digestion and expressed as a percentage of the concentration in the initial extract/powders (Figure 2a–c). The individual content of flavonols is shown in Supplementary Materials (Table S2).

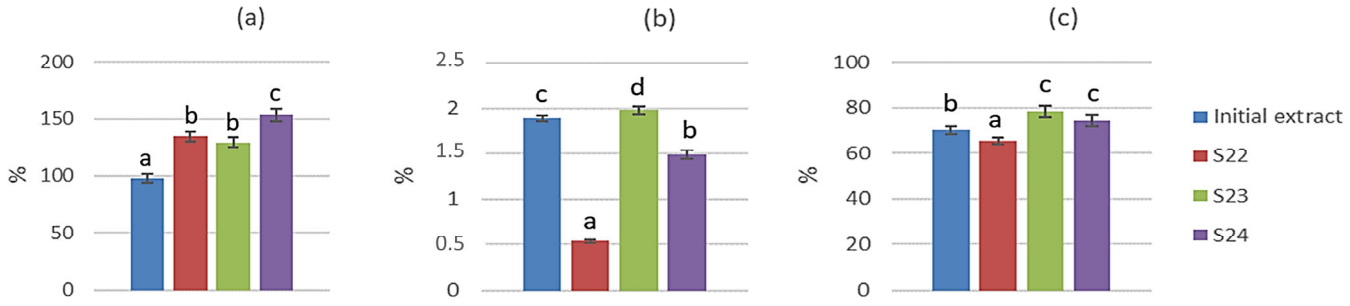

Figure 2. Bioaccessibility of laurel leaf flavonols in the initial extract and powders obtained using β-CD (S22), β-CD + MD 50:50 (S23), and β-CD + GA 50:50 (S24) during (**a**) gastric phase of in vitro digestion; (**b**) absorbed phase of in vitro digestion; (**c**) intestinal phase of in vitro digestion. Columns marked with different letters within picture are statistically different at $p < 0.05$.

As it can be observed, during the gastric phase of in vitro digestion (Figure 2a), the concentration of the flavonols ranged between ~100 and 150% compared to the content in the initial extract and powders. The bioaccessible portion from the initial extract was the lowest, but the polyphenols did not degrade, which is consistent with the data on their stability in the acidic medium [33]. On the other hand, the percentages higher than 100% for the bioaccessible flavonols in powders might be a result of the breaking of the bonds between the carriers and flavonols due to the acidic medium, which enhances the release of polyphenols from the food matrix in general [34]. There was no significant difference between the polyphenolic content in powders obtained using β-CD or β-CD + MD 50:50, while the highest percentage was released from the powder using β-CD + GA 50:50. The absorbed portion of flavonols (Figure 2b) from the intestinal phase ranged between ~0.5 and 2% which is consistent with the literature data which states that less than 10% of polyphenols are absorbed during the intestinal phase of digestion [32], while the majority is a substrate to colon microbiota which produces various metabolites which, when absorbed, potentially possess higher biological potential than their parent compounds [35]. The β-CD + MD 50:50 carrier resulted in the highest absorbed percentage, whereas the use of β-CD resulted in the lowest absorbed percentage of flavonols. In the intestinal phase, the percentage of bioaccessible flavonols (Figure 2c) was significantly lower than in the gastric phase, ranging from ~62 to 80%, which is consistent with previous findings where the largest portion of polyphenols was released during the gastric phase of digestion, and a significant degradation occurred in the intestinal phase [36]. This might be a result of a change of pH from acidic to mildly alkaline in the duodenum, as alkaline pH often causes oxidation and degradation of polyphenols [33]. Encapsulation using β-CD + MD 50:50 or β-CD + GA 50:50 preserved a higher percentage of flavonols compared to the initial extract, while the β-CD alone preserved a lower percentage than the initial extract. These results indicate that applying a combination of carriers is the most efficient way to achieve the stability of laurel leaf flavonols during digestion which is likely due to the difference in their structure allowing more interaction and binding sites for the polyphenols [32]. Overall, spray drying increased the bioaccessibility of laurel leaf flavonols during three different stages of in vitro digestion and showed potential for increasing their bioavailability. Further research, including the colon stage of digestion, as well as investigation of the metabolites' fate in plasma, would provide a detailed insight into the bioavailability of laurel leaf flavonols and their fate in the human body.

4. Conclusions

This study emphasized the importance of optimization for the microencapsulation of laurel leaf polyphenols by spray drying since the physicochemical characteristics depended on the applied process parameters. It was shown that the highest encapsulation efficiency could be obtained by using either of the three applied carrier mixtures at a ratio of 1:2 and the temperature of 180 °C, while the most desirable solubility and hygroscopicity were

achieved using β-CD + MD 50:50. None of the parameters influenced the process yield, while the moisture content depended only on the inlet temperature and was optimal at temperatures above 150 °C. A total of 29 polyphenols were identified in the powders, with flavonols being the dominant group. The individual polyphenolic content was higher when β-CD alone or in combination with MD was applied as a carrier, while β-CD + MD 50:50 carrier combination provided the highest antioxidant capacity. The in vitro digestion showed that microencapsulation by spray drying increased the bioaccessibility of laurel flavonols, demonstrating the potential to enhance their bioavailability in vivo. The use of a carrier mixture (β-CD + MD/GA 50:50) was more efficient in preserving the laurel leaf flavonols during digestion than using only β-CD. Based on the findings of this study, it can be concluded that microencapsulation by spray drying is a promising technique for the stabilization of the laurel leaf polyphenols during storage and digestion, thus enabling efficient utilization of their potential as functional food ingredients.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods12091923/s1, Figure S1: UPLC-MS/MS chromatogram in MRM acquisition mode of encapsulated laurel leaf extracts obtained at 180 °C and 1:2 sample:carrier ratio and (**a**) β-CD, (**b**) β-CD + MD (50:50) and (**c**) β-CD + GA (50:50).; Table S1: Concentrations of total and surface polyphenols in laurel leaf powders; Table S2: Concentration of laurel leaf flavonols in selected powders and the initial extract during in vitro digestion.

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CHAPTER 5

Publication No.5: Microencapsulation of *Laurus nobilis* L. Leaf Extract in Alginate-Based System via Electrostatic Extrusion.

Foods

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Zoran Zorić: formal analysis, methodology

Verica Dragović-Uzelac: funding acquisition, writing-review and editing

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Article

Microencapsulation of *Laurus nobilis* L. Leaf Extract in Alginate-Based System via Electrostatic Extrusion

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Abstract: Bay leaves (*L. nobilis* L.) are a rich source of polyphenols that hold great potential for application in functional food products in which where the main challenges are the polyphenols' low stability and bioaccessibility, which can be overcome through different microencapsulation techniques, such as electrostatic extrusion, which hasn't been applied for the encapsulation of bay leaf polyphenols (BLP) to date. Therefore, the main goal of this research was to evaluate the potential of this technique through monitoring the polyphenolic content, antioxidant activity, release kinetics, and bioaccessibility of the encapsulated BLP. The results showed that electrostatic extrusion was suitable for the encapsulation of BLP, where 1% alginate and 1.5% $CaCl_2$ with 0.5% chitosan resulted in the highest encapsulation efficiency (92.76%) and antioxidant activity in vitro. The use of 1.5% or 2% alginate with 5% $CaCl_2$ + 0.5% chitosan showed the most controlled release of polyphenols, while encapsulation generally increased the bioaccessibility of BLP. The results showed that electrostatic extrusion can be considered an efficient technique for the microencapsulation of BLP.

Keywords: laurel; polyphenols; stability; antioxidant activity; bioavailability; release kinetics

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1. Introduction

Bay leaf (*L. nobilis* L.) is a shrub widely distributed in the Mediterranean area, where its leaves have been traditionally used in cuisine and folk medicine for treating various gastrointestinal and respiratory health problems due to the beneficial effects of the bioactive molecules present in this plant part. Polyphenols, including flavonoids (mainly quercetin and kaempferol glycosides), phenolic acids, and proanthocyanidins, are largely responsible for these beneficial effects due to their antioxidative, antimicrobial, cardioprotective, neuroprotective, antiproliferative, and anti-inflammatory properties [1]. The main obstacle in an efficient utilization of these valuable properties is the tendency of polyphenols to degrade during storage under different temperatures, humidity, light, and pH [2], as well as their low bioavailability (degree of absorption in the gastrointestinal tract) related to their low bioaccessibility (release from food matrix), instability during digestion in the gastrointestinal tract, and difficult cell membrane diffusion [3]. Microencapsulation via different techniques has emerged as a concept which can be applied in order to overcome the mentioned shortcomings and allow the application of polyphenols in different functional products. To the best of the authors' knowledge, encapsulation of BLP was investigated only in two studies [4,5], where it was suggested that BLP can be effectively encapsulated by spray-drying and nano-liposome encapsulation. Further research on these and other encapsulation techniques focusing on the optimization of their parameters with the goal of achieving maximum yields, stability, and bioavailability is a key step in the development of new or enhanced functional products utilizing the maximum potential that BLP holds. Electrostatic extrusion is a microencapsulation technique based on passing a biopolymer (most often sodium alginate) through a nozzle into a cross-linking (gelling) solution with

the use of an electric field, resulting in uniform beads [6]. This technique is suitable for the microencapsulation of both hydrophilic and lipophilic compounds and has been applied for the encapsulation of polyphenols from different herbs [7,8]. It therefore holds the potential to be an efficient tool for preserving the quality and enhancing the bioavailability of BLP. Alginate is a water-soluble linear anionic marine polysaccharide composed of β-D-mannuronate and α-L-guluronate residues linked by 1–4 glycosidic bonds which forms gel in the presence of polyvalent ions, among which Ca^{2+} is the most suitable since it results in non-toxic and biocompatible complexes through a relatively cheap and simple process [6]. The encapsulation efficiency (EE) and preservation of biological activity of polyphenols encapsulated in a calcium–alginate complex may be enhanced by adding other biopolymers, such as chitosan, which is a non-toxic and biocompatible cationic polysaccharide built by N-acetyl-D-glucose-amine and D-glucosamine residues linked by 1–4 glycosidic bonds with the ability to form stable complexes with other anionic crosslinking agents [6]. Moreover, the addition of chitosan may result in a more controlled release of polyphenols [9] from the polymeric complex as well as increase their bioavailability [10]. Research on bioavailability for use in functional foods and dietary supplements is highly relevant since the concentration of polyphenols is not necessarily proportional to bioavailability [11]. However, it is often challenging due to the complexity of human physiology and ethical issues. Therefore, in vitro bioaccessibility assessment methods are often applied since they are relatively fast, simple, inexpensive, repeatable, and representative of data from in vivo studies, thus allowing a more efficient product formulation [12].

Since electrostatic extrusion has not been applied for the microencapsulation of BLP to date, the objective of this study was to evaluate the potential of this technique by varying the encapsulation parameters (percentage of sodium alginate, content of $CaCl_2$, and presence of chitosan in the cross-linking (gelling) solutions) and monitoring the total and individual polyphenolic contents, antioxidant activity, release kinetics, and bioaccessibilities of selected polyphenols in the obtained beads. The results of this work will widen the scarce knowledge on the microencapsulation of BLP, making a step toward industrial utilization of this valuable plant material. In addition, insight into the influence of different electrostatic extrusion parameters on individual polyphenolic compounds will be provided which will offer valuable information for application on polyphenolic extracts from other plant materials as well.

2. Materials and Methods

2.1. Chemicals and Reagents

Milli-Q system (Millipore, Bedford, MA, USA) was used for distilled water purification. Folin–Ciocalteu reagent, sodium carbonate, sodium bicarbonate, calcium chloride, sodium acetate, formic acid (98–100%), and $FeCl_3 \times 6H_2O$ were procured from Kemika d.o.o. (Zagreb, Croatia). Methanol (99.8%), ethanol (96%), and anhydrous sodium citrate were purchased from from Lach-ner d.o.o. (Neratovice, Czech Republic). HPLC grade acetonitrile was procured from J.T. Baker Chemicals (Deventer, the Netherlands). Acros Organics B.V.B.A. (Thermo Fisher Scientific, Geel, Belgium) supplied TPTZ and Trolox. Glacial acetic acid, hydrochloric acid (37%), DMSO, DPPH, low viscosity sodium alginate, chitosan from shrimp shells (≥75% deacetylated), pepsin (≥500 U/mg, from porcine gastric mucosa), bile salts, and pancreatin (4 × USP, from porcine pancreas) were procured from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Sigma-Aldrich Corporation (St. Louis, MO, USA) provided myricetin, quercetin-3-glucoside, gallic, caffeic, ferulic, syringic, protocatechuic, rosmarinic, chlorogenic, and *p*-coumaric acid authentic standards. Extrasynthese (Genay, France) provided authentic standards of rutin, epicatechin gallate, catechin, epigallocatechin gallate, luteolin, apigenin, procyanidin B2, and kaempferol-3-glucoside. The stock solutions of standards were prepared in methanol (ethanol–0.5% v/v DMSO for apigenin) and diluted to five concentrations of working standard solutions.

2.2. Plant Material

A dry commercial sample of bay leaves collected in November 2021 in Lovran, Croatia was procured from Šafram d.o.o. (Zagreb, Croatia) and stored in a paper box at room temperature. The leaves were ground into coarse powder prior to the extraction using an electric grinder (OmniBlend V Blender 1200 W, VerVita, Croatia). Drying to constant mass at 105 °C [13] was used to determine leaf dry matter (>95%).

2.3. Extract Preparation

The extraction was performed on YC-010 5 L multi-functional extracting tank (Pilotech, Shanghai, China). Briefly, the ground bay leaf sample was mixed with distilled water in the extracting tank at a sample:solvent ratio of 1:7.5 and subjected to extraction for 10 min at the pressure of 0.07 MPa and temperature of 70 °C. The extract was collected and filtered through Whatman No. 40 filter paper (Whatman International Ltd., Kent, UK) into 1 L volumetric flask, made up to volume with distilled water, and transferred to a glass bottle which was stored in the refrigerator at 4 °C. The extract's dry matter (2.13%) was analyzed by drying to constant mass at 105 °C [13].

2.4. Total Phenolic Content (TPC)

The total phenolic content (TPC) of the bay leaf extract was determined according to a previously described spectrophotometric method [14]. Briefly, 100 µL of extract (solvent for blank), 200 µL of Folin–Ciocalteu reagent, 2 mL of distilled water, and 1 mL of Na_2CO_3 were mixed in a reaction tube and incubated at 50 °C for 25 min, and the absorbance was read at 765 nm on a VWR UV-1600PC Spectrophotometer (VWR, Wayne, PA, USA) with gallic acid as standard. All samples were analyzed in duplicate and the TPC was expressed as mean value of gallic acid equivalents (GAE) in mg per g of leaf (bead) ± standard deviation.

2.5. Electrostatic Extrusion

For the electrostatic extrusion, alginate solutions (1.0, 1.5 and 2% w/v) were prepared by dissolving the adequate mass of low viscosity alginate in 100 mL of the bay leaf water extract (distilled water for the blank solutions) and stirring overnight at room temperature. Six different gelling solutions were prepared. The 1.5, 3, and 5% w/v calcium chloride solutions (pH 7) were produced by dissolving the adequate mass of calcium chloride in 1000 mL of distilled water. The calcium chloride solutions containing 0.5% w/v chitosan (pH 4) were prepared as previously described [15], with modifications. Briefly, an appropriate amount of chitosan was dissolved in 1% v/v acetic acid. Afterwards, adequate amounts of calcium chloride (1.5, 3 and 5% w/v) were added, and the solutions were made up to volume with 1% v/v acetic acid in a 1000 mL volumetric flask. The encapsulation was performed on Büchi Encapsulator B-390 (Büchi, Switzerland) with a 1 mm nozzle at following fixed parameters: pressure 0.1 bar, frequency 120 Hz, temperature of 37 °C, and voltage of 500 V. A magnetic stirrer (IKA, Staufen, Germany) was placed in front of the encapsulator for the constant stirring of the gelling solution. The obtained beads were left in the gelling solution for 20 min after formation, rinsed with distilled water and filtered through Whatman No. 40 filter paper (Whatman International Ltd., Kent, UK), after which they were frozen at −80 °C for 1 h. The beads were freeze-dried in a laboratory freeze-dryer (Christ, Osterode am Harz, Germany) with isothermal plate temperatures of 20 °C for 24 h under high vacuum (13–55 Pa), vacuumed sealed using a FoodSaver® vacuum sealer (Sunbeam Products, Inc., Boca Raton, FL, USA), and stored at −18 °C in nitrogen gas atmosphere until further analysis. The process was carried out in duplicate.

2.6. Encapsulation Efficiency (EE)

For the determination of EE, the beads were dissolved in a 5% sodium citrate solution at a ratio of 1:100 after 4 h on a magnetic stirrer (IKA, Staufen, Germany) at room temperature. The TPC of the dissolved beads was determined according to the procedure

described in Section 2.4 and expressed as mg GAE L^{-1} of the dissolved beads solution. The EE percentage was calculated using the following equation:

$$EE\ \% = (TPC_B/TPC_0) \times 100 \quad (1)$$

where TPC_B is the TPC in the sodium citrate dissolved beads solution, and TPC_0 is the TPC in the initial bay leaf extract (theoretical load) calculated through a mass balance method. The beads were dissolved and analyzed in a duplicate (n = 4).

2.7. Antioxidant Activity Assays

The antioxidant activity of the beads dissolved in 5% sodium citrate as described in Section 2.6 was analyzed via DPPH radical scavenging assay and Ferric Reducing Antioxidant Power (FRAP) assay as previously described [14]. For DPPH, 0.75 mL of extract was mixed with 1.5 mL of DPPH methanol solution (0.2 mM) and incubated for 20 min in the dark at 23 °C. 2.25 mL methanol was used as blank. For FRAP, 80 µL of extract, 240 µL of distilled water, and 2080 µL of FRAP regent (acetate buffer (pH 3.6):TPTZ (10 mM in 40 mM HCl):$FeCl_3$ (20 mM in distilled water) in a ratio of 10:1:1) were mixed, vortexed and incubated for 5 min at 37 °C. The absorbances were read on a VWR UV-1600PC Spectrophotometer (VWR, Wayne, PA, USA) at 517 nm and 593 nm for DPPH and FRAP, respectively. The standard curves were produced using Trolox. All measurements were performed in duplicate, and the results were expressed as mean value ± standard deviation of µmol Trolox-equivalent (TE) g^{-1} beads.

2.8. UPLC-MS^2

The polyphenolic content of the extracts and dissolved beads was determined using an Agilent 1290 RRLC UPLC-MS^2 system coupled with 6430 Triple Quadrupole LC/MS mass spectrometer (Agilent, Santa Clara, CA, USA). The ionization with ESI source was performed in +/− ionization mode with nitrogen as a desolvation and collision gas. A 100 × 2.1 mm Zorbax Eclipse Plus C18 (Agilent, Santa Clara, CA, USA) with 1.8 µm particle size was used for separations at 35 °C with the injection volume 2.5 µL. Flow rate was set at 11 L h^{-1}, nebulizer pressure, drying gas temperature, and capillary voltage at 40 psi, 300 °C, and 4000/−3500 V, respectively. The limits of detection and quantification as well as solvent composition and gradient were previously described [16]. The data analysis and instrument control were carried out using MassHunter Workstation (ver. B.04.01) software (Agilent, Santa Clara, CA, USA). Identification and quantitative determination of individual polyphenols were described in detail in our previous research [17]. The polyphenols concentrations were expressed as mg L^{-1} of the extract or solution (mean value ± standard deviation). The analyses were carried out in duplicate.

2.9. Release Kinetics of Polyphenols

For the determination of BLP's release kinetics in water, 300 mg of the freeze-dried alginate beads were suspended in 10 mL of distilled water and continuously agitated at 100 rpm on a shaker (IKA, Staufen, Germany) at room temperature. Every 10 min, an aliquot was taken from the supernatant and replaced by the same volume of distilled water. The TPC of the aliquots was determined as described in Section 2.4, and the results were expressed as mg GAE g^{-1} beads. The experiments were performed in a duplicate.

The experimental data from the release kinetics study were fitted to a Korsmeyers–Peppas model using Microsoft Office Excel ver. 2019 according to the equation:

$$f_t = \frac{M_t}{M_\infty} = K \times t^n \quad (2)$$

where M_t and M_∞ stand for the content of released polyphenols at time t and infinity, respectively. The M_∞ can be considered the content of polyphenols in the initial beads. K represents the release velocity coefficient, while n is the release exponent indicating

the release mechanism, including Fickian diffusion ($n < 0.43$), non-Fickian (anomalous) transport ($0.43 < n < 0.85$), and the super case II transport mechanism ($n > 0.85$) [18].

2.10. Bioaccessibility of Polyphenols

The bioaccessibility of the encapsulated BLP was examined according to a recently described three-step in vitro model [19], with modifications. Briefly, 200 mg of freeze-dried beads (750 µL of bay leaf extract) was placed in 50 mL reaction tubes and mixed with 800 µL of 0.1 M HCl pepsin solution (40 mg mL^{-1}) and 10 mL of 0.9% sodium chloride solution. The pH was adjusted to 2 with 0.1 M HCl in required volume if necessary. For the gastric phase of digestion, the samples were shaken at 100 rpm in a water bath for 1 h at 37 °C. The reaction was stopped by placing the reaction tubes on ice for 5 min, and 1 mL of 0.9% NaCl and 1 mL of 0.5 M $NaHCO_3$ were added into Pur-A-Lyzer 6–8 kDa dialysis membranes (Sigma-Aldrich, Steinheim, Germany), which were then placed in the reaction tubes, and the incubation was continued for 45 min to simulate the transition from stomach to the small intestine. Afterwards, the pH was adjusted to 6.5 by adding 1 M $NaHCO_3$ in the required volume, and reaction was continued after adding 2.5 mL of pancreatin–bile salts solution (2 mg mL^{-1}/12 mg mL^{-1}). The samples were returned to the water bath at 37 °C at 100 rpm for 2 h to simulate the intestinal phase, after which they were put on ice to stop the reaction. An aliquot of 2 mL was taken from each phase and filtered through 0.45 µm syringe filters into glass vials for the UPLC-MS^2 analysis of the phenolic content. The samples were stored at −18 °C in in nitrogen gas atmosphere. The process was carried out in duplicate.

2.11. Statistical Analysis

Statistical analysis was performed in the Statistica ver. 12.0 (Statsoft Inc., Tulsa, OK, USA) software. For the determination of optimal encapsulation conditions, TPC of the beads, DPPH, and FRAP values were the variables dependent on the alginate percentage and type of gelling solution, whose influence was evaluated through a full factorial design comprising 36 trials. Shapiro–Wilk and Levene's tests were applied to analyze the normality of the data sets and the homoscedasticity of the data sets' variance. One-way and multifactorial analysis of variance (ANOVA) and Tukey's HSD post hoc multiple comparison test were applied to normally distributed data, while the data which were not normally distributed and/or homogenic were analyzed using nonparametric Kruskal–Wallis one-way ANOVA and multiple comparison of mean ranks. All of the tests were considered significant at $p \leq 0.05$.

3. Results and Discussion

3.1. Influence of the Encapsulation Parameters on the Phenolic Content and Antioxidant Activity of BLP

This study examined the influence of alginate percentage and type of gelling solution on the phenolic content of the encapsulated bay leaf extracts as well as antioxidant activity determined by DPPH and FRAP according to the full factorial design shown in Table 1. In order to exclude the influence of alginate and gelling solution on the results of spectrophotometric analysis, blank beads were produced and analyzed. Phenolic compounds or antioxidant activity were not detected in the blank beads.

Table 1. Total phenolic content, encapsulation efficiency, and antioxidant activity of bay leaf polyphenols encapsulated under different conditions.

Sample	% Alginate	Gelling Solution	Total Phenols mg GAE/g Bead	Encapsulation Efficiency %	DPPH µmol TE/g Bead	FRAP µmol TE/g Bead
1	1	1.5% $CaCl_2$	10.94 ± 0.28	52.44 ± 1.36	10.63 ± 0.94	9.62 ± 0.11
2	1	3% $CaCl_2$	12.31 ± 0.46	59.35 ± 2.23	11.88 ± 0.72	11.12 ± 0.59
3	1	5% $CaCl_2$	10.46 ± 0.16	50.25 ± 0.77	11.66 ± 0.71	8.33 ± 0.38
4	1	1.5% $CaCl_2$ + 0.5% chitosan	19.22 ± 0.78	92.76 ± 3.78	19.47 ± 0.23	16.96 ± 1.3
5	1	3% $CaCl_2$ + 0.5% chitosan	12.68 ± 0.64	60.92 ± 3.1	16.01 ± 0.16	12.12 ± 0.43
6	1	5% $CaCl_2$ + 0.5% chitosan	10.68 ± 0.40	51.2 ± 1.94	14.18 ± 0.21	9.95 ± 0.27
7	1.5	1.5% $CaCl_2$	12.11 ± 1.03	58.12 ± 4.94	11.78 ± 0.37	10.68 ± 0.11
8	1.5	3% $CaCl_2$	9.82 ± 0.04	47.37 ± 0.19	10.49 ± 0.77	7.14 ± 0.38
9	1.5	5% $CaCl_2$	10.33 ± 0.58	50.04 ± 2.81	12.27 ± 0.05	8.15 ± 0.75
10	1.5	1.5% $CaCl_2$ + 0.5% chitosan	19.85 ± 0.93	95.43 ± 4.45	20.18 ± 0.63	17.03 ± 1.19
11	1.5	3% $CaCl_2$ + 0.5% chitosan	13.82 ± 0.69	66.26 ± 3.29	17.42 ± 1.11	14.03 ± 0.92
12	1.5	5% $CaCl_2$ + 0.5% chitosan	12.33 ± 0.40	59.14 ± 1.94	16.01 ± 0.58	10.14 ± 0.33
13	2	1.5% $CaCl_2$	10.22 ± 0.83	49.08 ± 3.97	11.74 ± 0.85	11.59 ± 0.43
14	2	3% $CaCl_2$	9.21 ± 0.54	44.56 ± 2.61	11.76 ± 0.44	11.27 ± 0.65
15	2	5% $CaCl_2$	10.33 ± 0.60	49.97 ± 2.9	12.12 ± 0.35	12.19 ± 0.97
16	2	1.5% $CaCl_2$ + 0.5% chitosan	20.38 ± 0.68	98.3 ± 3.29	19.14 ± 0.32	19.18 ± 1.08
17	2	3% $CaCl_2$ + 0.5% chitosan	15.15 ± 1.11	72.49 ± 5.32	16.87 ± 0.04	16.29 ± 0.38
18	2	5% $CaCl_2$ + 0.5% chitosan	9.22 ± 0.36	44.77 ± 1.74	14.4 ± 0.38	11.46 ± 0.11

Results are expressed as mean ± standard deviation.

As shown in Table 1, the EE was in the range of 44.56–98.30%, while the antioxidant activity determined by DPPH and FRAP ranged from 10.63–20.18 µmol TE g^{-1} and 7.14–19.18 µmol TE g^{-1} bead, respectively. These results show that different conditions significantly influence the examined parameters, showing the importance of optimization processes. The raw data were statistically analyzed, and the results are shown in Table 2.

Table 2. Influence of encapsulation parameters on total phenolic content, encapsulation efficiency, and antioxidant activity of bay leaf polyphenols.

	N	Total Phenols (mg GAE g^{-1} Beads)	EE (%)	DPPH (µmol TE g^{-1} Beads)	FRAP (µmol TE g^{-1} Beads)
% alginate		p = 0.41 ‡	p = 0.39 ‡	p = 0.87 ‡	p = 0.06 ‡
1	12	12.71 ± 0.92 ^{a}	61.15 ± 4.46 ^{a}	13.97 ± 0.92 ^{a}	11.35 ± 0.85 ^{a}
1.5	12	13.04 ± 1.01 ^{a}	62.73 ± 4.84 ^{a}	14.69 ± 1.05 ^{a}	11.19 ± 1.03 ^{a}
2	12	12.42 ± 1.24 ^{a}	59.86 ± 5.97 ^{a}	14.34 ± 0.86 ^{a}	13.66 ± 0.92 ^{a}
Gelling solution		$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †
1.5% $CaCl_2$	6	11.09 ± 0.42 ^{a}	53.21 ± 2.04 ^{a}	11.38 ± 0.34 ^{a}	10.63 ± 0.37 ^{a}
3% $CaCl_2$	6	10.44 ± 0.61 ^{a}	50.43 ± 2.94 ^{a}	11.38 ± 0.35 ^{a}	9.84 ± 0.87 ^{a}
5% $CaCl_2$	6	10.37 ± 0.16 ^{a}	50.09 ± 0.75 ^{a}	12.02 ± 0.18 ^{a}	9.55 ± 0.87 ^{a}
1.5% $CaCl_2$ + 0.5% w/v chitosan	6	19.82 ± 0.33 ^{c}	95.49 ± 1.59 ^{c}	19.60 ± 0.24 ^{c}	17.72 ± 0.59 ^{c}
3% $CaCl_2$ + 0.5% w/v chitosan	6	13.89 ± 0.52 ^{b}	66.56 ± 2.47 ^{b}	16.77 ± 0.33 ^{b}	14.15 ± 0.79 ^{b}
5% $CaCl_2$ + 0.5% w/v chitosan	6	10.74 ± 0.58 ^{a}	51.71 ± 2.70 ^{a}	14.86 ± 0.39 ^{b}	10.52 ± 0.31 ^{a}
Average	36	12.72 ± 0.60	61.25 ± 2.88	14.33 ± 0.53	12.07 ± 0.56

Results are expressed as mean ± SE. † Statistically significant at $p \leq 0.05$. ‡ Statistically insignificant at $p > 0.05$. Values with different letters within the same column are statistically different at $p \leq 0.05$. EE = encapsulation efficiency.

As can be observed in Table 2, the percentage of alginate had no statistically significant influence on any of the dependent variables indicating that 1% is enough to achieve efficient entrapment of BLP. The gelling solution significantly influenced ($p < 0.01$) all the dependent

variables. Generally, higher EE and antioxidant activity were achieved when the gelling solutions containing chitosan were applied. This can be explained by the improvement of alginate porous structure in the presence of other polysaccharides, such as chitosan, which enables higher EE, namely of lower molecular polyphenols [20]. In addition, the lower pH of the gelling solution containing chitosan might have influenced the interaction between the mannuronic and glucuronic acid (pK 3.38 and 3.65, respectively) in alginate with different groups of polyphenols, ensuring the chemical entrapment of polyphenols in the bead's matrix since it was suggested that the protonated form of alginate shows greater ability to bind phenolic compounds than the deprotonated form [21]. The highest EE, as well as antioxidant activity determined using both FRAP and DPPH, were achieved when 1.5% $CaCl_2$ with 0.5% chitosan was used as a gelling solution. Further increase in $CaCl_2$ percentage resulted in lower values in the presence of chitosan, while there was no statistically significant influence of gelling solution when $CaCl_2$ solutions without chitosan were used, indicating that 1.5% $CaCl_2$ is adequate to form calcium alginate beads with maximum EE and antioxidant activity of BLP. The decrease in the values in the presence of chitosan might be due to the interaction of calcium ions from 3% and 5% $CaCl_2$ solutions with the amino groups of chitosan molecule, leading to less amino groups available for the binding of polyphenols [22,23].

Based on the results of statistical analysis, 1% alginate and 1.5% $CaCl_2$ + 0.5% w/v chitosan gelling solution (S4) were chosen as optimal for obtaining maximum EE and antioxidant activity of the encapsulated extracts. Under these conditions, the achieved EE was 92.76%, which is higher than the 73.76% achieved via encapsulation in nanoliposome [5] and in the range of 72.9–99.3% achieved through spray-drying [4], indicating that electrostatic extrusion is an efficient technique for the encapsulation of BLP. The optimal sample was further analyzed for individual phenolic compounds using UPLC-MS^2 and compared to the sample obtained by using 1% alginate and 1.5% $CaCl_2$ (S1) in order to observe the influence of chitosan presence and the difference in the pH of the gelling solution. The content of individual phenolic compounds was analyzed in the initial extract as well, and theoretical load was calculated for each compound through mass balance in order to estimate the EE for each of the detected compounds. The results are shown in Table 3.

Flavonols were the most abundant group of polyphenols in the initial extract as well as in S1 and S4, where quercetin glycosides (mainly quercetin-3-glucoside) were the main representatives. Phenolic acids (dominantly caffeic acid) were also present in large portions, while flavan-3-ols, proanthocyanidins, and flavones were present in significantly lower quantities. Differences were observed in the TPC determined by spectrophotometric analysis and the results of UPLC-MS^2 that can be explained by the presence of other compounds such as organic acids, sugars, and chlorophyll present in the bay leaf extract, which interact with the Folin–Ciocalteu reagent, leading to seemingly higher values of TPC [24,25]. The results of UPLC-MS^2 confirmed the results of spectrophotometric analysis, which showed that gelling solution containing chitosan results in a higher EE of total polyphenols. However, differences were observed in the EE of individual polyphenols and polyphenolic groups. While the EE for phenolic acids was not affected by the gelling solution, higher percentages of flavones and flavonols were encapsulated with the use of gelling solution containing chitosan (S4). On the other hand, higher percentages of flavan-3-ols and proanthocyanidins were encapsulated in absence of chitosan (S1). These differences imply that the entrapment of polyphenols may be affected by the specific structures of individual compounds and by their various moieties (i.e., carboxylic and hydroxylic groups) and molecular masses, resulting in different binding affinities for the alginate and chitosan functional groups. In addition, some polyphenols could interact with the uronate and glucosamine residues of alginate chains, explaining the selective bonding of certain polyphenols [8].

Table 3. Identification and encapsulation efficiency of individual BLP as determined by UPLC-MS^2.

Compound Number	Retention Time	Tentative Identification	Concentration (mg L^{-1})					
			Extract	Bead Extract Theoretical	Bead Extract Experimental		EE (%)	
					S1	S4	S1	S4
				Phenolic acids				
2	3.745	3,4-dihidrobenzoic acid hexoside	0.19 ± 0.01	0.02 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	23.49 ± 1.33 ^{b}	5.82 ± 0.33 ^{a}
3	4.55	Protocatehuic acid	5.41 ± 0.15	0.54 ± 0.02	0.25 ± 0.01	0.29± 0.01	45.69 ± 2.59 ^{a}	53.75 ± 3.04 ^{b}
4	4.79	Syringic Acid	8.62 ± 0.24	0.86 ± 0.02	0.64 ± 0.02	0.66 ± 0.02	74.11 ± 4.19 ^{a}	76.97 ± 4.36 ^{a}
5	4.913	Chlorogenic acid	0.97 ± 0.03	0.10 ± 0.00	0.06 ± 0.00	0.07 ± 0.00	60.61 ± 3.43 ^{a}	72.20 ± 4.09 ^{b}
6	5.43	Rosmarinic acid	1.65 ± 0.05	0.17 ± 0.00	0.16 ± 0.00	0.10 ± 0.00	98.58 ± 5.58 ^{b}	61.20 ± 3.46 ^{a}
7	6.492	Caffeic acid	119.74 ± 3.39	11.97 ± 0.34	5.03 ± 0.14	3.87 ± 0.11	42.02 ± 2.38 ^{b}	32.30 ± 1.83 ^{a}
12	7.931	*p*-coumaric acid	4.13 ± 0.12	0.41 ± 0.01	0.25 ± 0.01	0.36 ± 0.01	59.85 ± 3.39 ^{a}	87.32 ± 4.94 ^{b}
17	8.568	Ferulic acid	2.76 ± 0.08	0.28 ± 0.01	0.24 ± 0.01	0.27 ± 0.01	85.21 ± 4.82 ^{a}	98.03 ± 5.55 ^{b}
24	9.76	*p*-hydroxybenzoic acid	4.10 ± 0.12	0.41 ± 0.01	0.34 ± 0.00	0.41 ± 0.01	83.03 ± 3.48 ^{a}	99.19 ± 4.15 ^{b}
28	11.443	Gallic acid	5.73 ± 0.16	0.57 ± 0.02	0.55 ± 0.02	0.26 ± 0.01	95.65 ± 5.41 ^{b}	46.15 ± 2.61 ^{a}
-	-	∑Phenolic acids	153.30 ± 0.43	15.33 ± 0.04	7.51 ± 0.02	6.30 ± 0.02	49.02 ± 3.66 ^{a}	41.07 ± 3.44 ^{a}
				Flavonols				
1	3.604	Kaempferol-3-*O*-rutinoside	24.44 ± 0.69	2.44 ± 0.07	2.36 ± 0.07	2.32± 0.07	96.57 ± 5.47 ^{a}	94.86 ± 5.37 ^{a}
15	8.343	Rutin	78.43 ± 2.22	7.84 ± 0.22	5.76 ± 0.16	4.44± 0.13	73.45 ± 4.16 ^{b}	56.58 ± 3.20 ^{a}
18	8.62	Quercetin-3-*O*-glucoside	108.39 ± 3.07	10.84 ± 0.31	7.56 ± 0.21	10.70± 0.30	69.78 ± 3.95 ^{a}	98.74 ± 5.59 ^{b}
19	9.161	Kaempferol-3-*O*-hexoside	27.27± 0.77	2.73 ± 0.08	1.43 ± 0.04	1.50 ± 0.04	52.44 ± 2.97 ^{a}	54.84 ± 3.10 ^{a}
20	9.171	Quercetin-3-*O*-pentoside	19.35 ± 0.55	1.93 ± 0.05	1.06 ± 0.03	1.77 ± 0.05	54.83 ± 3.10 ^{a}	91.71 ± 5.19 ^{b}
22	9.528	Isorhamnetin3-*O*-hexoside	40.85 ± 1.16	4.08 ± 0.12	2.86 ± 0.08	4.95 ± 0.07	69.97 ± 3.96 ^{a}	121.21 ± 1.65 ^{b}
23	9.548	Quercetin-3-*O*-rhamnoside	60.23± 1.70	6.02 ± 0.17	4.98 ± 0.14	6.00 ± 0.17	82.73 ± 4.68 ^{a}	99.64 ± 5.64 ^{b}
25	9.829	Kaempferol-3-*O*-pentoside	6.02± 0.17	0.60 ±0.02	0.51 ± 0.01	0.58 ± 0.02	84.64 ± 4.79 ^{a}	96.98 ± 5.49 ^{b}
27	10.346	Kaempferol-3-*O*-deoxyhexoside	0.14± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	40.02 ± 2.26 ^{a}	63.15 ± 3.57 ^{b}
29	12.176	Myricetin	0.85 ± 0.02	0.09 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	98.18 ± 5.56 ^{a}	99.69 ± 5.64 ^{a}
-	-	∑Flavonols	365.96 ± 1.04	36.60 ± 0.1	26.61 ± 0.08	32.36 ± 0.08	72.73 ± 4.09 ^{a}	88.42 ± 4.44 ^{b}

Table 3. *Cont.*

Compound Number	Retention Time	Tentative Identification	Concentration (mg L^{-1})					
			Extract	Bead Extract Theoretical	Bead Extract Experimental		EE (%)	
					S1	S4	S1	S4
				Flavones				
11	7.589	Luteolin-6-C-glucoside	0.31± 0.01	0.03 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	46.97 ± 2.66 [a]	86.95 ± 4.92 [b]
14	8.223	Apigenin-6-C-(*O*-deoxyhexosyl)-hexoside	0.14 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	15.28 ± 0.86 [a]	30.78 ± 0.42 [b]
21	9.261	Luteolin	8.62 ± 0.24	0.86 ± 0.02	0.54 ± 0.02	0.86 ± 0.02	62.80 ± 3.55 [a]	99.61 ± 5.64 [b]
26	10.24	Apigenin	1.07 ± 0.03	0.11 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	21.02 ± 1.19 [a]	28.98 ± 1.64 [b]
		∑Flavones	10.14 ± 0.07	1.04 ± 0.00	0.58 ± 0.02	0.92 ± 0.03	57.25 ± 2.07 [a]	90.82 ± 3.15 [b]
				Flavan-3-ols				
8	6.581	Catechin	14.50 ± 0.41	1.45 ± 0.04	0.34 ± 0.01	0.14 ± 0.00	23.14 ± 1.31 [b]	9.73 ± 0.55 [a]
9	6.588	Epicatechin	14.90 ± 0.42	1.49 ± 0.04	0.33 ± 0.01	0.16 ± 0.00	22.27 ± 1.26 [b]	10.77 ± 0.61 [a]
13	7.993	Epicatechin gallate	1.01 ± 0.03	0.10 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	31.14 ± 1.76 [a]	36.19 ± 2.05 [b]
16	8.363	Epigallocatechin gallate	2.24 ± 0.06	0.22 ± 0.01	0.03 ± 0.00	0.07 ± 0.00	12.11 ± 0.69 [a]	31.26 ± 1.77 [b]
		∑Flavan-3-ols	32.65 ± 0.23	3.27 ± 0.02	0.73 ± 0.02	0.41 ± 0.02	22.24 ± 1.25 [b]	12.50 ± 1.24 [a]
				Proanthocyanidins				
10	6.9	Procyanidin trimer	24.22 ± 0.68	2.42 ± 0.07	0.31 ± 0.01	0.15 ± 0.00	12.65 ± 0.72 [b]	6.38 ± 0.36 [a]
-	-	Total phenols	586.26 ± 2.46	58.63 ± 0.25	35.74 ± 0.31	40.41 ±0.45	60.97 ± 1.15 [a]	68.46 ± 1.37 [b]

Results are expressed as mean ± SD. Values with different letters in the same row are statistically different at $p \leq 0.05$. S1 = 1% alginate and 1.5% $CaCl_2$ gelling solution; S4 = 1% alginate and 1.5% $CaCl_2$ + 0.5% *w*/*v* chitosan gelling solution. EE = encapsulation efficiency.

3.2. Release Kinetics of BLP

The release profile of BLP from the beads produced using 1% alginate (Figure 1a), 1.5% alginate (Figure 1b), and 2% alginate (Figure 1c) and all combinations of gelling solutions were investigated after placing the beads in water and measuring the TPC of the surrounding medium in selected intervals during 60 min. The obtained data on TPC were fitted according to the Korsmeyer–Peppas model equation (Equation (2)). For all data sets, the correlation coefficient R^2 was higher than 0.95 indicating a good correlation between the model and experimental data. Release velocity coefficient K and release exponent *n* were determined from the data fitting (Table 4).

Table 4. The kinetic parameters for the release kinetics of encapsulated bay leaf polyphenols correlated using the Korsmeyers–Peppas model.

Sample	Alginate %	Gelling Solution	K	*n*	R^2
1	1% alginate	1.5% $CaCl_2$	0.0846	0.1499	0.9604
2		3% $CaCl_2$	0.0714	0.4242	0.9606
3		5% $CaCl_2$	0.0962	0.1469	0.9831
4		1.5% $CaCl_2$ + 0.5% chitosan	0.0638	0.5357	0.9680
5		3% $CaCl_2$ + 0.5% chitosan	0.0204	0.7260	0.9605
6		5% $CaCl_2$ + 0.5% chitosan	0.0146	0.7851	0.9582
7	1.5% alginate	1.5% $CaCl_2$	0.0657	0.4396	0.9752
8		3% $CaCl_2$	0.1811	0.0717	0.9578
9		5% $CaCl_2$	0.1156	0.2328	0.9530
10		1.5% $CaCl_2$ + 0.5% chitosan	0.0562	0.6016	0.9611
11		3% $CaCl_2$ + 0.5% chitosan	0.0434	0.5866	0.9747
12		5% $CaCl_2$ + 0.5% chitosan	0.0787	0.1367	0.9768
13	2% alginate	1.5% $CaCl_2$	0.0345	0.5091	0.9747
14		3% $CaCl_2$	0.1545	0.0296	0.9531
15		5% $CaCl_2$	0.1426	0.1382	0.9746
16		1.5% $CaCl_2$ + 0.5% chitosan	0.0158	0.5704	0.9618
17		3% $CaCl_2$ + 0.5% chitosan	0.0246	0.5646	0.9564
18		5% $CaCl_2$ + 0.5% chitosan	0.0871	0.0813	0.9674

K = release velocity coefficient; *n* = release exponent; R^2 = correlation coefficient.

Over 60 min, the highest level of release of polyphenols from 1% and 1.5% alginate beads was obtained using 1.5% $CaCl_2$ + 0.5% chitosan, and the lowest in the sample was obtained using 1.5% $CaCl_2$ and 5% $CaCl_2$ + 0.5% chitosan, respectively, as gelling solutions. The latter gelling solution resulted in the lowest release level in 2% alginate beads as well, while the highest level in the case was obtained by 1.5% $CaCl_2$. These results indicate that chitosan combined with the highest percentage of $CaCl_2$ results in the lowest release level of BLP at alginate percentage 1.5% or higher, most likely due to enhanced alginate–chitosan complexation and alginate cross-linkage, which resulted in the increase in the beads' mechanical strength and consequently lower water penetration [26]. Even though the level of release was higher in some beads obtained by gelling solutions with chitosan, the values of release velocity coefficient *K* showed that these beads generally released BLP at a lower rate than those without chitosan, meaning that they reached equilibrium later than samples obtained without chitosan in gelling solution. This is consistent with previous data on slower release of polyphenols in water from alginate beads reinforced with chitosan [8,9].

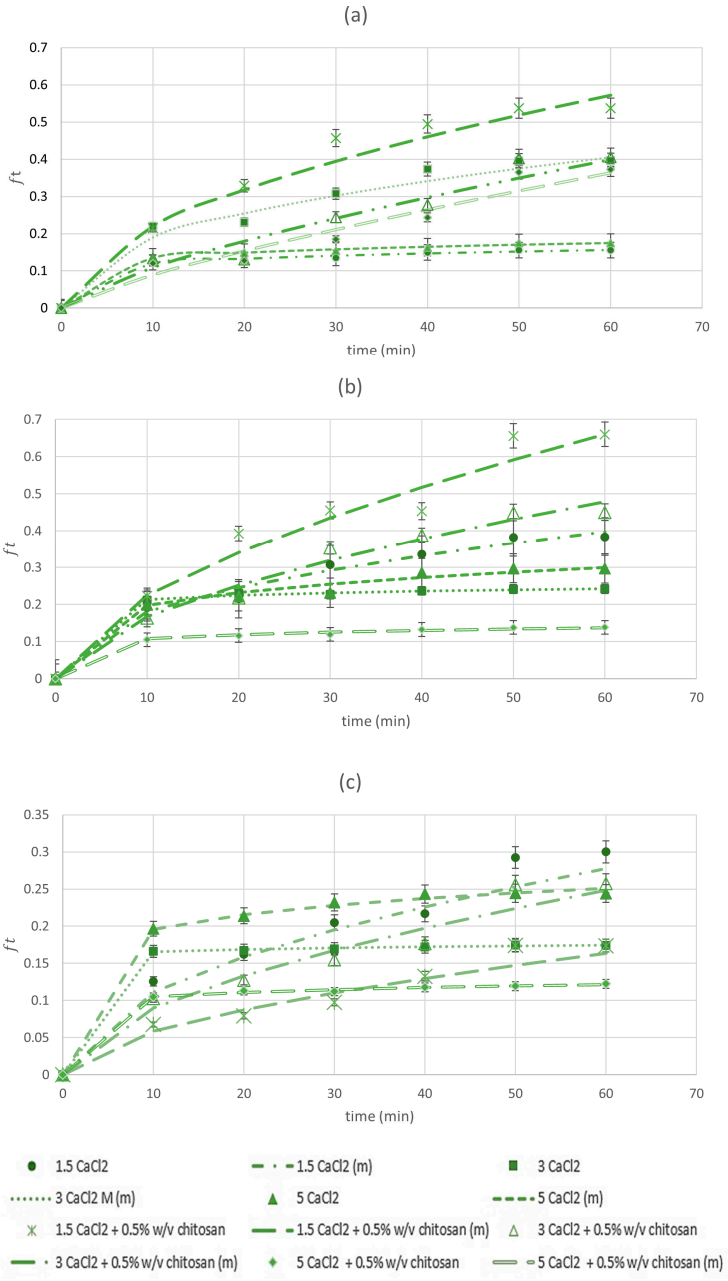

Figure 1. Plots of fraction of bay leaf polyphenols released in water versus time and modelling for the correlation of kinetic parameters: (**a**) 1.0% alginate; (**b**) 1.5% alginate, (**c**) 2% alginate. Data are expressed as mean ± standard deviation. Symbols: experimental data; lines: modelling results. (m) = model.

The values of release exponent n were in the range of 0.07–0.79, indicating that different mechanisms were involved in the release of BLP depending on the conditions under which the beads were produced. The release mechanism of the most beads obtained using gelling solutions without chitosan was consistent with Fickian diffusion ($n < 0.43$), while the release of polyphenols from most beads obtained using gelling solutions with chitosan was controlled by multiple mechanisms ($0.43 < n < 0.85$), including diffusion, swelling, and erosion of the polymeric matrix [18]. According to the kinetic parameters K and n, the beads which showed the lowest levels of release (1.5% or 2% alginate with 5% $CaCl_2$ + 0.5% chitosan) also had relatively low release rates and followed Fickian diffusion, which indicates that beads obtained at these parameters behave in the most predictable manner, which makes them the most suitable for application in the food industry.

3.3. Bioaccessibility of BLP

The bioavailability of polyphenols depends on their release during different stages of digestion (bioaccessibility), which is highly influenced by the food matrix or—in case of microencapsulation—the applied carrier [27]. In order to observe the influence of encapsulation on the bioaccessibility of BLP, the most abundant compounds (quercetin-3-glucoside and caffeic acid) from the initial extract, S1 (1% alginate + 1.5% $CaCl_2$), and S4 (1% alginate + 1.5%$CaCl_2$ + 0.5% chitosan) were analyzed using UPLC-MS^2 and monitored through three stages of in vitro digestion. As expected, encapsulation generally increased the bioaccessibility of both quercetin-3-glucoside (Figure 2a) and caffeic acid (Figure 2b). During the gastric phase of digestion, the bioaccessible percentage of quercetin-3-glucoside was twice as high in S1 and S4 as in the initial extract, showing that encapsulation protected this compound from the hydrolysis in the acidic environment, which occurs in different ratios depending on the food matrix [28]. On the other hand, caffeic acid had the highest bioaccessible percentage from the initial extract, possibly due to its relative stability in the acidic gastric environment [29]. The release of caffeic acid from the polymeric matrices was lower than in the intestinal phase, in which the structural change of alginate and chitosan caused by the change in pH from acidic to neutral possibly resulted in higher release of both caffeic acid and quercetin-3-glucoside [30,31]. Encapsulation using the gelling solution containing chitosan allowed the highest absorption of both compounds, which mirrors the findings in vivo, where—in addition to the sustained stability of polyphenols [10]—chitosan can also enhance the absorption due to its mucoadhesive properties, which allow longer presence in the small intestine and consequently higher absorption [32]. The absorbed percentage of caffeic acid (13.01%) was higher than that of quercetin-3-glucoside (7.07%), which is in agreement with the literature data, which reported the absorption of quercetin and its glycosides to be lower than 10% [33], while the absorption of caffeic acid can reach the percentage of 19.1% [34].

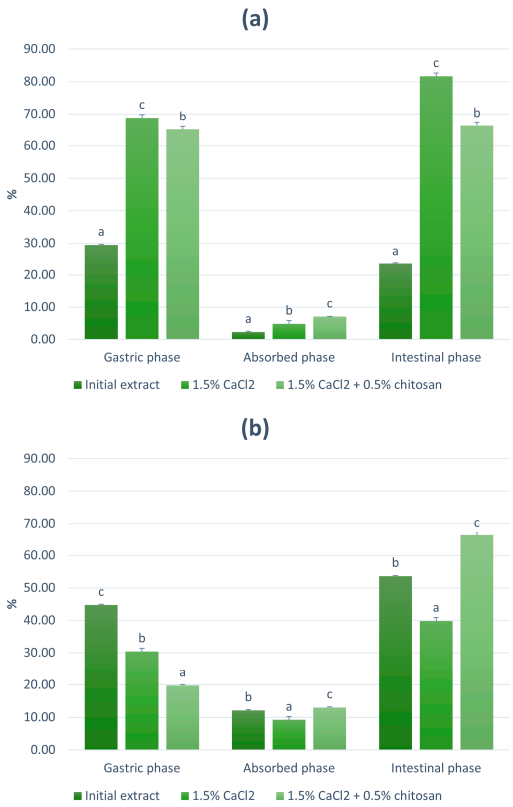

Figure 2. Bioaccessibility profile of (**a**) quercetin-3-glucoside and (**b**) caffeic acid from the initial extract and encapsulated extracts using 1% alginate. Values with different letters are statistically different at $p \leq 0.05$.

These results provide an insight into the bioccessibility of the main BLP, which can be useful in formulating food products with the encapsulated bay leaf extract. Further research of the colon phase of digestion and the interaction with the gut microbiota, which plays a major role in the polyphenols' metabolism [27], as well as following the fate of metabolites in plasma would give further insight into the bioavailability of BLP and their fate and biological activity in the human body.

4. Conclusions

This study demonstrated for the first time that electrostatic extrusion in an alginate-based system can be considered as an efficient technique for the encapsulation of BLP. The encapsulation efficiency, antioxidant activity, and release kinetics largely depended on the applied encapsulation parameters. The combination of 1% alginate and 1.5% $CaCl_2$ with 0.5% chitosan as a gelling solution resulted in the highest encapsulation efficiency and antioxidant activity in vitro. The use of 1.5% or 2% alginate with 5% $CaCl_2$+ 0.5% chitosan as a gelling solution resulted in the most controlled release of BLP. Generally, encapsulation increased the bioaccessibility of BLP and the presence of chitosan in the gelling solution showed potential for higher absorption of the main BLP representatives, quercetin-3-glucoside, and caffeic acid. These results indicate that the combination of calcium alginate with chitosan generally results in more desirable properties of the obtained beads, showing the highest potential for use in functional food products. In order to achieve maximum usage of the potential of BLP, further research should be focused on varying the alginate–chitosan ratio along with other encapsulation conditions, taking into account the characteristics and proposed application purpose of the encapsulated BLP.

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CHAPTER 6

General discussion

- Influence of the applied extraction techniques on the phenolic content of laurel leaf extracts
- Influence of the applied extraction techniques on the antioxidant capacity of laurel leaf extracts
- Influence of the encapsulation techniques on retention of phenols, antioxidant activity and bioaccessibility

1. INFLUENCE OF THE APPLIED EXTRACTION TECHNIQUES ON THE PHENOLIC CONTENT OF LAUREL LEAF EXTRACTS

As mentioned in Chapter 1, extraction is one of the crucial steps in the efficient utilization of plant extracts and the phenolic content may largely vary depending on the applied technique and different parameters applied within a technique. For this reason, one of the main objectives of this dissertation was to optimize three advanced extraction techniques for the extraction of laurel leaf phenols and compare them to a conventional extraction technique with the aim of achieving maximum phenolic yield. MAE and UAE were optimized in *Publication No. 2*, while PLE was optimized in *Publication No.3*.

As discussed in *Publications No. 2* and *3*, flavonols were the dominant phenolic groups in the extracts obtained by all of the applied techniques' optimal parameters (Table 2), while the ratio of other phenolic groups differed depending on the applied extraction technique. When comparing all 4 of the applied techniques, the largest differences can be observed in the content of phenolic acids and flavan-3-ols, leading to differences in the total phenolic content as determined by UPLC-MS^2. CRE and PLE resulted in the same total phenolic content of the laurel leaf extracts, while the content obtained by MAE and UAE was significantly lower. This is contrary to the hypothesis that the advanced extraction techniques would result in higher phenolic yields than CRE.

PLE resulted in almost tenfold and up to fivefold higher content of hydroxybenzoic and hydroxycinnamic acids, respectively, compared to other extraction techniques. This is likely due to the breakage of lignin bonds at high temperatures (150 °C) applied during PLE, leading to the release of bound phenolic acids whose quantity was shown to be twice that of free phenolic acids (Antony & Farid, 2022). CRE extract had a significantly higher content of flavan-3-ols compared to other applied techniques, which might have been enhanced by the extraction time of 30 min which was shown in previous study to be optimal time within the range from 5–120 min for the extraction of catechin at the temperature of 80 °C (Vuong et al., 2011).

Table 2. Content of phenolic groups determined by UPLC-MS^2 in the extracts obtained at optimal extraction conditions for each applied technique

Extraction technique	**Phenolic acids (mg/100 g)**			**Flavonols (mg/100 g)**	**Flavan-3-ols (mg/100 g)**	**Flavones (mg/100 g)**	**Proanthocyanidins (mg/100 g)**	**Total phenols (mg/100 g)**
	HBA	HCA	∑					
	p<0.01	p<0.01	p<0.01	p<0.01	p<0.01	p<0.01	p<0.01	p<0.01
CRE	5.75 ± 0.11[a]	14.29 ± 0.29[a]	20.04 ±0.40[a]	341.51 ± 6.83[c]	144.13 ± 2.88[c]	6.64 ± 0.13[a]	20.33 ± 0.41[d]	532.65 ± 10.65[c]
MAE	8.54 ± 0.17[a]	37.55 ± 0.75[c]	46.09 ±0.92[c]	282.66 ± 5.65[b]	26.93 ± 0.54[a]	16.27 ± 0.33[b]	7.72 ± 0.15[b]	379.67 ± 7.59[b]
UAE	6.37 ± 0.13[a]	24.49 ± 0.49[b]	30.86 ±0.62[b]	212.56 ± 4.25[a]	34.12 ± 0.68[a]	24.02 ± 0.48[d]	15.20 ± 0.30[c]	316.75 ± 6.34[a]
PLE	60.31 ± 1.21[b]	87.22 ± 0.31[d]	147.55 ±2.95[d]	268.61 ± 5.37[b]	61.05 ± 1.22[b]	20.74 ± 0.41[c]	3.90 ± 0.08[a]	501.84 ± 10.04[c]
Mean	20.24 ± 8.76	40.89 ±3.52	61.13 ±19.19	276.34 ±17.46	66.57 ± 17.61	16.92 ± 2.48	11.79 ± 2.42	432.73 ± 33.45

CRE= conventional heat reflux extraction; MAE= microwave-assisted extraction; UAE= ultrasound-assisted extraction; PLE= pressurized liquid extraction. HBA= hydroxybenzoic acids; HCA= hydroxycinnamic acids. Values within column marked with different letter are statistically different at p<0.05.

Even though the extraction time in PLE was much shorter, this technique resulted in twofold higher content of flavan-3-ols compared to MAE and UAE, possibly due to the effect of high temperature, since the yield of catechins was shown to increase proportionally with the increase of temperature up to 150 °C, after which thermal degradation occurs (Antony & Farid, 2022).

The highest concentration of flavonols was found in the extract obtained by CRE, followed by MAE, PLE and UAE. This is possibly a result of several factors including the properties of laurel leaf whose firm structure allows efficient extraction during prolonged time in CRE, as well as chemical properties of flavonols whose hydroxyl-substituents may increase degradation caused by microwaves (Liazid et al., 2007) and ultrasound frequencies over 20 kHz (Handa et al., 2008). In the case of PLE, the high temperatures which increase the extraction rate might have caused degradation of flavonols (Antony & Farid, 2022), resulting in a lower concentration compared to CRE.

The highest concentration of proanthocyanidins was found in the extract obtained by CRE, possibly due to the longer extraction time which was shown to enhance the extraction of proanthocyanidins (Wissam et al., 2012), followed by UAE where the mechanochemical breakage of the polymer units and plant material bonds likely occurred due to cavitation (Lv et al., 2021). The lowest concentration of proanthocyanidins was obtained by PLE, likely due to thermal degradation since it was shown that B-type procyanidins trimers, such as the one detected in the laurel leaf, are susceptible to thermal degradation at temperatures above 70 °C (Maldonado & Figueroa, 2023). The same explanation can be applied to MAE, since the optimal conditions included the application of 80 °C.

As opposed to other phenolic groups, CRE resulted in the lowest content of flavones compared to MAE, UAE and PLE which yielded similar contents. Since the most of flavones detected in the laurel leaf were present in the form of aglycones without the stabilization by sugar moieties, it is possible that they degraded more due to the longer extraction time in CRE (Biesaga, 2011).

These findings suggest that PLE is the most suitable technique for the extraction of laurel leaf phenols since it yielded the same phenolic content as CRE in a significantly shorter time through a more solvent- and energy-efficient process.

2. Influence of the applied extraction techniques on the antioxidant capacity of laurel leaf extracts

Another objective of this dissertation was to determine the antioxidant capacity of the extracts obtained by different extraction techniques, which was carried out in *Publications No. 2* and *3*. As summarized in Table 3, all of the extracts had relatively high antioxidant capacity.

Table 3. Antioxidant activity of laurel leaf extracts obtained at optimal extraction conditions for each applied technique determined by ORAC

Extraction technique	ORAC (μmol TE g^{-1})
	$p<0.05$
CRE	100.09 ± 2.00^{b}
MAE	86.04 ± 1.72^{a}
UAE	90.27 ± 1.81^{a}
PLE	97.27 ± 1.95^{b}
Mean	93.42 ± 2.22

CRE= conventional heat reflux extraction; MAE= microwave-assisted extraction; UAE= ultrasound-assisted extraction; PLE= pressurized liquid extraction. ORAC= oxygen radical absorbance capacity. Values within column marked with different letters are statistically different at $p<0.05$.

However, the extracts obtained by CRE and PLE showed statistically higher antioxidant capacity which was shown to highly correlate (Table 4) with the content of total phenols, especially flavan-3-ols and flavonols, as determined by UPLC-MS^2. These results confirmed the hypothesis that the extracts with higher phenolic content have higher antioxidant capacity, which is in accordance with previous data reporting that phenols are major contributors to antioxidant activity of medicinal plant extracts (Muflihah et al., 2021; Osman et al., 2020; Piluzza & Bullitta, 2011; Turumtay et al., 2014). Flavonols and flavan-3-ols likely contributed to the antioxidant capacity the most, due to their structure which is completely (in case of e.g. quercetin) or partially (flavan-3-ols) consistent with the three criteria postulated to predict the best radical scavenging activity for flavonoids, namely including the presence of two hydroxy-groups on the 3',4' position on the B ring, a double bond in the 2,3 position and 3- and 5-hydroxyl groups with 4-oxo function (Bors et al., 1997).

Table 4. Pearson's correlations between the content of phenolic groups and antioxidant activity determined by ORAC

Group of compounds	Pearson for ORAC	Description of correlation
Phenolic acids	0.230177*	low positive
HBA	0.350912*	low positive
HCA	-0.89529*	very high negative
Flavonols	0.553904*	moderate positive
Flavan-3-ols	0.824174*	very high positive
Flavones	-0.44815*	moderate negative
Proanthocyanidins	0.317411*	low positive
Total phenols UPLC-MS^2	0.84472*	very high positive

HBA= hydroxybenzoic acids; HCA= hydroxycinnamic acids.*$p<0.05$

3. Influence of the encapsulation technique on retention of phenols, antioxidant activity and bioaccessibility

After selecting and optimizing the most suitable extraction technique, encapsulation represents the next crucial step to ensure stability and highest quality of the active ingredients which allows their efficient utilization in the industry. In this dissertation, spray drying (*Publication No.4*) and electrostatic extrusion (*Publication No.5*) were optimized for the encapsulation of laurel leaf extracts and the hypothesis that stability and quality of encapsulates depend on the encapsulation conditions was confirmed. The main characteristics of the obtained encapsulates including the encapsulation efficiency, antioxidant activity and bioaccessiblity as the main interests for the application in functional food as a function of the applied techniques are further discussed.

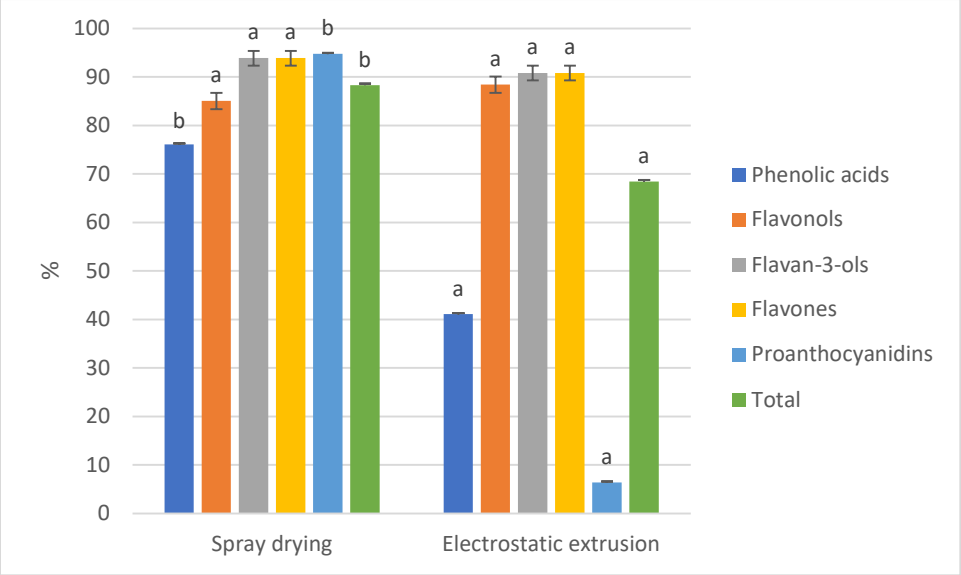

Figure 6. Encapsulation efficiency of different phenolic groups in laurel leaf extract obtained by spray drying and electrostatic extrusion. Columns of same color marked with different letters are statistically different at $p<0.05$.

As seen in the Figure 6, both of the applied extraction techniques resulted in relatively high encapsulation efficiency comparable to those reported in literature for laurel leaf phenols, where spray drying using chitosan, sodium alginate and arabic gum resulted in the range of encapsulation efficiency between 72.9%–99.3% (Chaumun et al., 2020) and encapsulation by nanoliposomes in the encapsulation efficiency of 73.76% (Tometri et al., 2020). Spray drying was shown to be more efficient for the encapsulation of total phenols (88.32%), phenolic acids (76.10%) and proanthocyanidins (94.76%) than electrostatic extrusion where the encapsulation of these compounds was 68.46%, 41.10% and 6.38%, respectively. The lower encapsulation efficiency of phenolic acids during electrostatic extrusion can be explained by their low molecular weight since it has been shown that compounds with lower molecular weight are not as efficiently encapsulated in biopolymer beads (G. Gómez-Mascaraque et al., 2019) due to diffusion through the hydrogel network during formation (Li et al., 2021). Even though polymers such as proanthocyanidins have more hydroxy-groups theoretically allowing them a stronger affinity for the alginate molecules (Li et al., 2021), it is likely that these complex and large compounds weren't efficiently entrapped due to the reaction between the carboxyl groups of sodium alginate and the amine groups of chitosan leading to fewer functional groups available for reaction with phenols where smaller compounds might have accessed more easily (Kulig et al., 2016). Low encapsulation efficiency of proanthocyanidins was also reported in

literature for alginate-chitosan nanocomplexes (15.62%) (Ding et al., 2021), as well as alginate-pectin biopolymer (9.62–15.91%) (Li et al., 2021).

The antioxidant activity of the encapsulates (Table 5) was reduced compared to the initial extract, which was expected due to the loss of phenols. However, the reduction in antioxidant activity was not proportional to the amount of lost phenols and was likely affected by other parameters, which can be supported by previous findings where the loss of phenols of 10% was accompanied by loss of antioxidant activity higher than 20%, possibly due to structural changes of the present compounds and the subsequent change in their interaction (Ou et al., 2002; Réblová, 2012). In the case of spray drying, it is possible that the high temperature was the main factor resulting in the reduction of antioxidant activity. The literature data on the effect of temperature are conflicting and various findings were reported for different plant materials. For example, heat treatments of green leafy vegetables (Turkmen et al., 2005) and powdered onion (K. Sharma et al., 2015) resulted in significant increase of antioxidant activity, due to structural alterations of the present compounds or the formation of novel compounds by Maillard reactions. On the other hand, the spray drying process is more intensive and even though the heat exposure is relatively short, several authors reported deterioration of various encapsulates' antioxidant activity (Ballesteros et al., 2017; Kha et al., 2010; Sarabandi & Jafari, 2020; Suhag & Nanda, 2016; Tonon et al., 2010), indicating that even though the high temperatures resulted in high encapsulation efficiency and physicochemical characteristics of the powders, lower temperatures might result in higher antioxidant capacity of the obtained powders. The encapsulation efficiency of electrostatic extrusion was lower than that of spray drying, so the low retention of antioxidant activity can be partially assigned to the lower content of total phenols, as well as the content of specific groups of compounds, namely proanthocyanidins whose retention was under 10% (Figure 6). It was shown that, due to a higher polymerization degree, procyanidin dimers and trimers tend to be more effective against different radical species than monomeric flavonoids (Heim et al., 2002), and could therefore be major contributors to antioxidant activity.

The bioaccessibility of laurel leaf flavonols was investigated in *Publications No.4* and *5* for spray drying and electrostatic extrusion, respectively. The results confirmed the hypothesis that the bioaccessiblity of the phenols from encapsulates depends on the applied carriers. In both techniques, the combination of polymers (β-cyclodextrin with either maltodextrin or gum arabic in spray drying, alginate with chitosan in electrostatic extrusion) resulted in the most desirable behaviour of flavonols during simulated *in-vitro* digestion, most

likely due to the different chemical structures of the carriers which in combination allowed more binding sites for potential interaction with polyphenols which consequently increased their stability (Grgić et al., 2020). As shown in Table 5, encapsulates obtained by spray drying released twofold higher percentage of flavonols during the gastric phase of digestion compared to encapsulate obtained by electrostatic extrusion, likely due to the breakage of bonds between carriers and phenols in the acidic medium (Q. Zhang et al., 2020). This effect was lower in the case of electrostatic extrusion which can be explained by the fact that protonated form of alginate at lower pH shows greater affinity for the phenols, resulting in a stronger binding (Plazinski & Plazinska, 2011).

Table 5. Comparison of encapsulation efficiency, antioxidant activity retention and bioaccessibility of laurel leaf polyphenols in optimal encapsulates obtained by two different techniques

Microencapsulation technique	**EE%**	**Phenols (mg/g encapsulate)**	**Retention of AA (% of initial extract's AA)**		**Bioaccessibility of flavonols (% of initial encapsulate content)**		
			DPPH	FRAP	Gastric	Absorbed	Intestinal
Spray drying	88.32 ± 0.13^{b}	42.42 ± 1.94^{b}	48.13 ± 0.42^{b}	52.55 ± 0.71^{b}	129.22 ± 2.23^{b}	1.97 ± 0.04^{a}	78.26 ± 1.61^{b}
Electrostatic extrusion	68.46 ± 0.07^{a}	19.22 ± 0.78^{a}	26.71±0.31^{a}	14.23 ± 0.25^{a}	65.05 ± 0.95^{a}	7.07 ± 0.19^{b}	66.25 ± 1.21^{a}

EE= encapsulation efficiency. AA= antioxidant activity. Values within column marked with different letters are statistically different at $p<0.05$.

The absorption of flavonols in the encapsulates obtained by both techniques was in the range reported in the literature (<10%) (Grgić et al., 2020), however higher absorption was achieved from the encapsulates obtained by electrostatic extrusion. This would also be expected *in-vivo* since chitosan was shown to possess mucoadhesive properties that enhance the absorption of phenols by allowing their longer presence in the small intestine (Niu et al., 2022). In the case of both encapsulation techniques, the bioaccessible portion of phenols was significantly lower than in the gastric phase indicating that degradation of flavonols occured due to the alkaline pH (Xiao, 2022). Nevertheless, the encapsulates obtained by spray drying allowed higher preservance of laurel leaf phenols which would further be a substrate for the gut microbiota which plays major role in the polyphenols' metabolism (Grgić et al., 2020).

Overall, both encapsulation techniques increased bioaccessibility of laurel leaf flavonols compared to the initial extracts and showed the potential to increase their bioavailibility. Applying electrostatic extrusion would likely result in higher absorption of phenols in the small intestine, while spray drying allows greater concentration of phenols available for the production of valuable metabolites by the gut microbiota. These findings can be useful in the formulation of functional products depending on their intended use, while further research including the colon phase of digestion and the health beneficial effects of metabolites produced during small intestinal and colon phase would provide better insight into the targeted sites of action and consequently the choice of encapsulation technique.

CHAPTER 7
Conclusions and prospects

- This study confirmed that laurel leaf is a valuable plant material rich in phenolic compounds with potential for contribution to human health.
- A total of 29 compounds belonging to the classes of flavonols, phenolic acids, flavan-3-ols, flavones and proanthocyanidins were detected in laurel leaf extracts. Flavonols were the most abundant phenolic group consisting mainly of kaempferol and quercetin glycosides, regardless of the applied extraction technique.
- Advanced extraction techniques including MAE, UAE and PLE were successfully optimized and compared with CRE (50% EtOH, 30 min). At the optimal extraction conditions MAE (50% EtOH, 80 °C, 10 min, 400W) and UAE (70% EtOH, 10 min, 50% amplitude) resulted in lower phenolic contents and antioxidant capacity, while PLE (50% EtOH, 150 °C, 1 extraction cycle, static time 5 min) resulted in the same phenolic content and antioxidant capacity as CRE in a significantly shorter time. These findings suggest that PLE should be taken as the method of choice for the extraction of laurel leaf phenols as it is energy-, time- and solvent- efficient, as well as suitable for scale-up processes.
- Antioxidant capacity was in correlation with the content of total phenols, especially flavonols and flavan-3-ols which can therefore be considered major contributors to biological activity of laurel leaf extracts.
- Spray drying and electrostatic extrusion were successfully applied and optimized for the encapsulation of laurel leaf phenols. At optimal conditions, spray drying (β-CD + MD 50:50, sample:carrier ratio 1:2, 180 °C) resulted in higher encapsulation efficiency and antioxidant activity of the extracts compared to electrostatic extrusion (1% alginate, 1.5% $CaCl_2$ + 0.5% chitosan). The physicochemical characteristics of the encapsulates depended on the applied carriers in both techniques, and the combinations of carriers resulted in more desirable properties and better entrapment of laurel leaf phenols.
- Encapsulation by both applied techniques resulted in higher bioaccessibility of laurel leaf phenols compared to initial extracts. Electrostatic extrusion resulted in better preservation of phenols during the gastric phase of digestion and higher absorption, while spray drying preserved greater concentration of phenols available for the production of valuable metabolites by the gut microbiota.
- The results of this dissertation represent a significant contribution to the knowledge on the extraction and encapsulation of laurel leaf phenols, thus providing a fundamental

platform for future research and industrial utilization of this valuable plant material in the field of functional food and nutraceuticals.

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CURRICULUM VITAE

Erika Dobroslavić was born in 1997 in Dubrovnik. After graduating from high school, during which she studied diatoms as a research project, she enrolled Nutrition at the Faculty of Food and Biotechnology in Zagreb. During her studies and while preparing her master thesis in the Laboratory of Drying Processes and Stability Monitoring of Biologically Active Compounds, she developed an exceptional interest in the isolation and application of biologically active molecules from plant sources. Since 2020, she has been working as an assistant on the research project "Bioactive molecules of medicinal plants as natural antioxidants, microbiocides and preservatives", within the framework of which she is preparing her PhD thesis. In 2022, she completed the postgraduate specialised studies "Dermatopharmacy and Cosmetology" at the Faculty of Pharmacy and Biochemistry in Zagreb, in the framework of which she worked on the application of bioactive molecules from by-products of the food industry in cosmetics. She is the co-author of 7 scientific papers indexed in the Web of Science/Current Contents Connect database, and she has presented her research results at numerous domestic and international congresses. She participated in supervising 3 graduation and 4 final theses.

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