Optimization of extraction and encapsulation techniques of bioactive molecules from nettle (Urtica dioica L.)

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DOCTORAL DISSERTATION

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Supervisor:

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Zagreb, 2023.

Sveučilište u Zagrebu

Prehrambeno-biotehnološki fakultet

Ena Cegledi

Optimiranje postupaka ekstrakcije i inkapsulacije bioaktivnih molekula koprive (*Urtica dioica* L.)

DOKTORSKI RAD

Mentor:

prof.dr.sc. Verica Dragović-Uzelac

Zagreb, 2023.

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This is an article-based doctoral thesis, known as Scandinavian model, which consists of already published scientific papers accompanied by a chapter with the critical review, which was written in accordance with Article 14 of the Doctoral Studies Regulations at the University of Zagreb (2016).

BASIC DOCUMENTATION CARD

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Optimization of extraction and encapsulation techniques of bioactive molecules from nettle (*Urtica dioica* L.)

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Short Abstract: Nettle (*Urtica dioica* L.) is a plant rich in bioactive active molecules such as phenols, pigments and phytosterols, which have beneficial effects on human health and were the subject of this research. In order to isolate higher yields of mentioned bioactives from nettle, the parameters of accelerated solvent extraction were studied and optimized. In addition, the influence of phenological stage and habitat on the accumulation of bioactive molecules during the growing season was investigated. Furthermore, to protect and stabilize phenolics from nettle leaves, the parameters of encapsulation technique of spray drying were optimized, and it was determined that encapsulation had a positive effect on the bioavailability of phenolic compounds.

Key words: nettle, bioactive molecules, accelerated solvent extraction, spray drying

The thesis contains: 139 pages, 4 figures, 4 tables, 174 references

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Optimiranje postupaka ekstrakcije i inkapsulacije bioaktivnih molekula koprive (*Urtica dioica* L.)

Ena Cegledi 420/PT

Sažetak: Kopriva (*Urtica dioica* L.) je biljka bogata biološki aktivnim molekulama poput fenola, pigmenata i fitosterola koji pozitivno utječu na zdravlje te su predmet ovog istraživanja. Za učinkovitu izolaciju spomenutih komponenata s maksimalnim prinosom iz ekstrakta koprive, ispitani su i optimirani parametri ubrzane ekstrakcije otapalima. Nadalje, ispitan je utjecaj fenotipske faze i staništa na akumulaciju biološki aktivnih molekula tijekom vegetacije. Nadalje, u svrhu zaštite i stabilizacije fenola iz lista koprive optimirani su parametri inkapsulacijske metode sušenja raspršivanjem te je utvrđeno kako inkapsulacija pozitivno utječe na bioraspoloživost fenolnih spojeva.

Ključne riječi: kopriva, bioaktivne molekule, ubrzana ekstrakcija otapalima, sušenje raspršivanjem
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Extended abstract

Nettle (*Urtica dioica* L.) is a perennial plant rich in bioactive molecules such as phenols, pigments and phytosterols, which are found in all parts of the plant. Since the mentioned molecules have a positive effect on a human health (mainly as powerful antioxidants), it is necessary to extract them efficiently from the plant material. Nowadays, advanced extraction techniques are increasingly used, because they reduce extraction time, consume less solvent, and isolate the target molecules more effectively. Among the various advanced extraction techniques, accelerated solvent extraction (ASE) is highlighted as very successful and efficient. The aim of this study was to optimize the ASE conditions for the efficient isolation of phenols and pigments from nettle leaves and stems, and for the isolation of phytosterols and pentacyclic triterpenoids from nettle roots. Furthermore, the study also aimed to compare the efficiency of this technique (yield and antioxidant capacity) with the efficiency of other extraction techniques. In general, higher temperature, relatively short extraction time, and multiple extraction cycles were sufficient for successful extraction of bioactive molecules, making ASE an excellent technique for isolation of high-quality antioxidants compared to other techniques. Moreover, another aim was to investigate how the plant part, phenological stage, and habitat affect the accumulation of bioactive molecules. According to the results obtained, the highest levels of bioactive molecules were present in the leaves, and nettle should be harvested before and during flowering, when the accumulation of phenols and pigments is the highest. In addition, the content of phenols and pigments in nettle varied depending on the habitat of the plant. Samples from seaside areas had higher pigment content, while samples from continental and mountainous habitats contained greater amounts of phenols. Since bioactive molecules in the liquid extract become unstable and lose their properties over time, it is necessary to protect and stabilize them. The next objective of this dissertation was to apply and optimize spray drying as a technique for encapsulation of phenolic compounds from nettle leaf liquid extract in order to obtain powders with desirable physicochemical properties and high encapsulation capacity. Moreover, the encapsulation of phenols increased their bioavailability. In summary, the research results provide new insights into the improvement of extraction techniques of bioactive molecules from nettle (phenols, pigments, sterols) and the preparation of powders using the spray drying microencapsulation technique. This is a scientific

contribution in the field of knowledge of the biological potential of nettle and the potential application of extracts and/or encapsulations in the production of functional foods.

Keywords: nettle (*Urtica dioica* L.), bioactive molecules, accelerated solvent extraction, spray drying

Proširenisažetak

Kopriva (*Urtica dioica* L.) je višegodišnja biljka bogata biološki aktivnim molekulama poput fenola, pigmenata i fitosterola smještenim u svim dijelovima biljke. Budući da spomenute molekule pozitivno utječu na zdravlje (ponajviše kao jaki antioksidansi) potrebno ih je učinkovito ekstrahirati iz materijala. Danas se sve više koriste napredne tehnike ekstrakcije, jer skraćuju vrijeme ekstrakcije, troše manje otapala te efikasnije izoliraju ciljane komponente. Među raznim naprednim metodama ekstrakcije, ubrzana ekstrakcija otapalom (ASE) ističe se kao vrlo uspješna i učinkovita. Cilj ove disertacije bio je optimirati uvjete ASE za učinkovitu izolaciju fenola i pigmenata iz lišća i stabljike koprive te za izolaciju fitosterola i pentacikličkih triterpenoida iz korijena koprive. Nadalje, disertacija je također imala za cilj usporediti učinkovitost ove metode (prinos i antioksidativni kapacitet) s učinkovitošću drugih tehnika ekstrakcije. Generalno, viša temperatura, relativno kratko vrijeme ekstrakcije i nekoliko ekstrakcijskih ciklusa bili su dovoljni za uspješnu ekstrakciju bioaktivnih molekula, što ASE čini izvrsnom metodom za izolaciju visoko vrijednih antioksidansa u usporedbi s drugim tehnikama. Također, jedan od ciljeva je bio uvidjeti kako dio biljke, fenotipska faza i stanište utječu na akumulaciji bioaktivnih molekula. Prema dobivenim rezultatima, najviše razine bioaktivnih molekula su prisutne u lišću, a koprivu bi trebalo brati prije i tijekom cvjetanja, kada je najbogatija fenolima i pigmentima. Nadalje, količine fenola i pigmenata u koprivi varirale su ovisno o staništu biljke. Uzorci sakupljeni s primorskog područja pokazali su veći sadržaj pigmenata, dok su veće količine fenola bile prisutne u uzorcima s kontinentalnog i planinskog staništa. Budući da bioaktivne molekule u tekućem ekstraktu tijekom vremena postaju nestabilne i gube svoja svojstva, potrebno ih je zaštititi i stabilizirati. Idući cilj ove disertacije bio je primijeniti i optimirati sušenje raspršivanjem kao metodu inkapsulacije fenolnih spojeva iz tekućeg ekstrakta lista koprive, kako bi se dobili prahovi poželjnih fizikalno-kemijskih svojstava, s visokim inkapsulacijskim kapacitetom. Također, inkapsulacija fenola povećala je njihovu bioraspoloživost. Zaključno, rezultati istraživanja ukazuju na nove spoznaje o unaprijeđenju postupaka ekstrakcije bioaktivnih molekula iz koprive (polifenola, pigmenata, sterola) te proizvodnje prahova primjenom mikroinkapsulacijske tehnike sušenja raspršivanjem. Time se ostvaruje znanstveni doprinos u segmentu poznavanja biološkog potencijala koprive i potencijalnoj primjeni ekstrakata i/ili inkapsulata u proizvodnji funkcionalnih proizvoda.

Ključne riječi: kopriva (*Urtica dioica* L.), bioaktivne molekule, ubrzana ekstrakcija otapalima, sušenje raspršivanjem

Prije svega, želim se zahvaliti svojoj mentorici prof.dr.sc. Verici Dragović-Uzelac na pruženoj prilici i povjerenju kroz cijeli period mog školovanja, a posebno tijekom protekle 3 godine. Hvala Vam na svakoj pomoći, prenesenom znanju i meni osobno najvažnije – izuzetno ugodnoj atmosferi i velikodušnosti s Vaše strane tijekom ovog mog znanstvenog putovanja. Budući da ste me mentorirali kroz završni, diplomski i doktorski rad, slobodno mogu reći da sam se uz Vas oblikovala i razvila poslovno i privatno. Ovo je bio jedan od najljepših, ali i najizazovnijih perioda u mome životu, a iskreno se nadam da Zvrko najstavlja put i dalje!

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Information about the supervisor - Verica Dragović-Uzelac, Ph.D. Full Professor

VERICA DRAGOVIĆ-UZELAC has been working at the Faculty of Food Technology and Biotechnology at the University of Zagreb since 1993 and was appointed full professor in 2013. She is actively involved in scientific research and has published over 128 scientific papers, 80 of which are high-ranking articles (a1) and have been cited 4810 times. She has also published a number of papers in journals indexed in secondary databases (a2), in congress books with international peer review (a3), and she has participated in a large number of national and international congresses. Since 2013 she has been the head of the Laboratory for drying technologies and monitoring of biologically active compounds. She actively participates in teaching at undergraduate, graduate, and doctoral studies, and has successfully supervised 8 doctoral dissertations, 3 professional theses, and over 150 undergraduate and graduate theses. She has particularly excelled in leading national and EU-funded projects in the field of food technology and food chemistry: “Sour cherry Marasca (*Prunus cerasus* var. Marasca) as an ingredient for functional food“, “The application of innovative technologies in bioactive compounds isolation from organic waste in the wine production”, “Application of innovative technologies for the production of plant extracts as ingredients for functional food”, “Isolation and encapsulation of bioactive molecules of wild and cultivated nettle and fennel and effects on organism physiology” and “Medicinal plants' bioactive molecules as natural antioxidants, microbicides and preservatives”. She participated as a collaborator in the project “Processing raw materials into excellent and sustainable end products while remaining fresh”, “Equipping the semi-industrial practicum for the development of new food technologies”, "Center of Excellence for Marine Bioprospecting BioProCro" and the project "Bioprospecting of the Adriatic Sea". She has received numerous awards including: annual state award for significant scientific achievement (2015), RegioStars recognition for the most successfully implemented project from the Operational Program: Regional Competitiveness 2007-2013 (Brussels, 2016), award from the Faculty of Food Technology and Biotechnology for published papers in recognized international journals and exceptional achievements in scientific research and teaching (2014), annual state award for popularization and promotion of science (2012) and certificate for the most successful local EU project in the Croatia for the period from 2010 to 2013 (Cherry Marasca (*Prunus cerasus* var. Marasca) as an ingredient in functional food), ARCA Gold Plaque 2015 - for the same IPA project.

Authors publications included in the doctoral dissertation:

Publication No.1

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Publication No.2

Repajić, M., **Cegledi, E.,** Zorić, Z., Pedisić, S., Elez Garofulić, I., Radman, S., Palčić, I., Dragović-Uzelac, V. (2021) Bioactive Compounds in Wild Nettle (*Urtica dioica* L.) Leaves and Stalks: Polyphenols and Pigments upon Seasonal and Habitat Variations. *Foods,* **10(1)**, 190. https://doi.org/10.3390/foods10010190

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Cegledi, E., Repajić, M., Balbino, S., Peričić, M., Dragović-Uzelac, V. (2022) Sterols and pentacyclic triterpenoids from nettle root: content and composition as affected by pressurized liquid extraction. *Journal of the Science of Food and Agriculture,* **103(8)**, 4058-4067. https://doi.org/10.1002/jsfa.12373

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Cegledi, E., Garofulić Elez, I., Zorić, Z., Roje, M., Dragović-Uzelac, V. (2022) Effect of Spray Drying Encapsulation on Nettle Leaf Extract Powder Properties, Polyphenols and Their Bioavailability. *Foods*, **11(18)**, 2852. https://doi.org/10.3390/foods11182852

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General introduction

Food quality and health have become extremely important to consumers, so the market is increasingly offering foods with high content of different bioactive molecules which possess positive effects on the body and mind. Manufacturers should follow nutritional trends and offer their consumers what they expect from them - products that improve and maintain health, so-called functional foods. The global functional foods market was estimated at $305.4 billion in 2022 and is expected to reach $597.1 billion by 2032 (Precedence Research, 2023). The trends of the modern market increasingly include ingredients of natural origin, rich in bioactive molecules that not only have nutritional value, but also protect against diseases. Nettle (*Urtica dioica* L.) as one of the medicinal plant species, due to its chemical composition and content of bioactive molecules, represents an excellent basis for obtaining products with high biological potential. It is a perennial plant distributed worldwide and most often grows as a weed, which makes it extremely accessible and cheap material for consumers. Besides being a nutritionally rich plant, nettle is a rich source of bioactive molecules like phenols, pigments and phytosterols that possess numerous anti-inflammatory and antioxidant properties (Otles and Yalcin, 2012; Đurović et al., 2017; Grauso et al., 2020).

To isolate these highly valuable molecules from plant material, a suitable extraction technique must be used. Nowadays, advanced extraction techniques are becoming more common compared to conventional techniques because they not only save time, solvent and energy, but also increase efficiency and extraction yield. Among the advanced techniques, accelerated solvent extraction (ASE) is highly appreciated because of its efficiency, ease of use and the possibility to work in multiple cycles (Sun et al., 2012). It is carried out at temperatures above the boiling point of the solvent and under increased pressure, which allows better diffusion of the solvent into the matrix, accelerating mass transfer and consequently leading to higher yields (Mottaleb and Sarker, 2012). However, in order to extract as much as possible from the plant material, optimization of the process parameters is necessary and required.

Apart from the extraction technique, the structure and composition of bioactive molecules may also be influenced by the phenological stage and habitat. Different amounts of bioactive molecules are produced as the plant matures in response to various forms of abiotic stress (Ncube and Van Staden, 2015). Although nettle is a highly adaptable plant, the accumulation of bioactive molecules depends on climate and habitat. Plants growing in colder climates often have higher levels of

antioxidants as a result of defense against oxidative stress (Tolić et al., 2017), while plants adapted to warmer climates contain more pigments due to more sunlight and higher temperatures (González-Talice et al., 2013; Saini et al., 2015). In addition, different parts of the nettle contain different proportions of bioactive molecules. According to the literature, the leaf is the richest part of the nettle and is therefore the most commonly analyzed (Pinelli et al., 2008; Otles and Yalcin, 2012).

Since bioactive molecules in liquid plant extracts become unstable over a long period of time due to external influences and sensitivity to processing conditions and have low bioavailability, they need to be stabilized and protected. Encapsulation has been shown to be an effective technique for protecting sensitive compounds and can be defined as a process in which a barrier is created around the active component that inhibits chemical interactions, protects against environmental factors such as temperature, pH, enzymes, and oxygen, and allows the controlled release of the active component under certain conditions (Dias et al., 2017). One of the most frequently used techniques in the food industry is spray drying, in which the raw material in liquid state is dispersed in hot air with the addition of a carrier and dried, resulting in a powder form (Caliskan and Dirim, 2013; Santos et al., 2018). Also, in order to obtain a powder with desirable physicochemical properties, with high encapsulation capacity and improved bioavailability the process itself needs to be optimized.

This dissertation, in the form of published papers and a final comprehensive review, aims to evaluate and optimize the influence of ASE parameters (temperature, static extraction time, and number of cycles) to efficiently extract bioactive molecules from nettle and see how the parameters affect its structure and antioxidant capacity. Phenols and pigments were extracted from the aerial part of nettle, and phytosterols and pentacyclic triterpenoid derivatives were extracted from the underground part. The influence of plant part, habitat and phenological stage on the accumulation of bioactive molecules from nettle was also examined. Furthermore, in order to protect and stabilize phenols from nettle leaves, the aim was also to examine and optimize spray drying parameters (temperature, type of carrier, and carrier:sample ratio) on the physicochemical properties (yield, moisture content, solubility, and hygroscopicity), encapsulation and loading capacity, and bioavailability of phenols.

Chapter 1

Theoretical background

- nettle (*Urtica dioica* L.) – general information
- bioactive molecules from nettle and their importance in the food industry
- accelerated solvent extraction for the isolation of nettle bioactive molecules
- significance of spray drying encapsulation
- hypothesis, research objectives, and expected scientific contributions

1. Nettle (*Urtica dioica* L.)

Stinging nettle or nettle (*Urtica dioica* L.) is the most popular nettle species in the genus Urtica, which consists of 46 species belonging to the Urticaceae family (Kregiel et al., 2018). It is a perennial wild plant that is widespread throughout the world, especially in Europe, Asia, Africa and America. Due to its rapid growth, wide distribution and adaptability in nature, it is considered a weed. It consists of roots, stems and leaves and survives the winter in the form of underground rhizomes (di Virgilio et al., 2015). It is also adapted to various climatic conditions and is best suited to a moderate and warm climate. Nettle prefers moist, nitrogen-rich soils and therefore grows in meadows, forests and abandoned fields in partially shady places up to an altitude of 1,800 m (Kregiel et al., 2018; Devkota et al., 2022). The genus name comes from the Latin word *urere*, meaning "to burn", while the species name *dioica* means dioecious, indicating that the plants usually contain either male or female flowers (Upton, 2013).

The plant can grow up to 2 m high. The stem of stinging nettle is upright and unbranched, while the dark green, heart-shaped leaves are arranged opposite each other on the stem. Leaves and stems are covered with hairs - trichomes, the liquid content of which causes local irritation with redness and burning when in contact with the skin. The main components of the irritant liquid are: acetylcholine, formic acid, serotonin and histamine (Kregiel i sur.,2018). Nettle blooms from May to September with small green flowers that are in dense, drooping inflorescences (Paulauskienė et al., 2021; Bhusal et al., 2022). The fruit of the nettle is a green nut with a flat shape and contains a brown seed. Nettle has a widely branched root system with many long rhizomes, which allows it to overwinter at very low temperatures, so that a new plant can grow from it in the spring. Propagation of nettle is carried out by sowing, rhizomes or cuttings (di Virgilio et al., 2015; Viotii et al., 2022).

Figure 1. Stinging nettle (*Urtica dioica* L.) (www.shutterstock.com accessed 23rd June, 2023)

Although it grows as a weed, nettle has been known in folk medicine since ancient times. For example, Hippocrates mentions it as a strong medicinal and nutritional agent (Kavalali, 2003). Today, due to its nutritional and health potential, it is mainly used in the food, cosmetic and pharmaceutical industries, as all parts of the nettle (leaves, stems and roots) have a rich composition of bioactive molecules with high antioxidant capacity (Kukrić et al., 2012; Otles and Yalcin, 2012). It is consumed in the form of stew, mixed like spinach with milk, in pasta, etc. Nettle leaves and roots are also consumed as tea (Upton, 2013; Marchetti et al., 2018). Due to its richness in chlorophyll, nettle is also used as a natural dye. In the pharmaceutical industry, various tinctures, capsules and other supplements based on nettle are used. In the textile industry, the stems of nettle, rich in fibers, are used for the production of various fabrics and fishing accessories (sails, ropes, and fishing nets), because of their strength and fineness of the material (Samanta et al., 2021). In the cosmetic industry, many creams containing nettle are known, where nettle can be used alone or in combination with other plants (e.g., nettle root and burdock root as a remedy for acne) (Kumar et al., 2005; Joshi et al., 2014). It also appears as an ingredient in shampoos and

tinctures. In addition, the nettle is also used as a natural fertilizer and insecticide in ecological agriculture to reduce an excessive use of mineral fertilizers and pesticides in agriculture. When plants are treated with nettle extract, they are provided with nutrients (especially nitrogen), which promotes growth and development and protects the plant from diseases when it is used as an insecticide (for example, against aphids) (Popescu et al., 2014; Garmendia et al., 2018).

Table 1 shows the nutritional value of nettle leaves, as they are considered the most valuable source of nutrients (Otles and Yalcin, 2012). As it can be seen, nettle is a low-calorie plant, but a rich source of proteins, carbohydrates, fats, etc. The proteins contain numerous amino acids necessary for humans.

Table 1. Nutritional composition of nettle leaves (Said et al., 2015)

Nutrient	Amount (%)
water	65 - 90
proteins	4.3 - 8.9
ashes	3.4 - 18.9
carbohydrates	7.1 - 16.5
lipids	0.7 - 2
fibers	3.6 - 5.3
calories (kcal/100 g)	57 - 99.7

It also contains a great amount of vitamins, especially vitamins A, B complex (B_1, B_2, B_3, B_6), C, K and E (Rutto et al., 2013; Mahlangeni et al., 2016; Bhusal et al., 2022), as well as minerals such as calcium, iron, phosphorus and potassium (Viotti et al., 2022). Shonte et al. (2020) and Dimitrijević et al. (2016) stated that nettle contains more vitamins A and C than spinach and it covers more than half of the daily requirement of these vitamins.

2. Bioactive molecules from nettle and their importance in the food industry

Plants are capable of producing and synthesizing different groups of organic compounds, which can be divided into primary and secondary metabolites. The primary metabolites are involved in the growth, development and reproduction of the plant, they have no pharmacological effect, but are very nutritious components (Erb and Kliebenstein, 2020). The secondary plant metabolites, on

the other hand, are produced in response to stress and are not directly involved in the plant's metabolism and are not necessary for its survival, but are important components of the plant's defense mechanisms and its interaction with the environment. They also have a positive influence on human health (Eshalfie et al., 2023).

Nettle is not only a nutritionally valuable plant, but also contains a large number of bioactive molecules. Although bioactive molecules also include nutritionally valuable ingredients, secondary plant constituents are natural, non-nutritive, physiologically active food components that have certain functional properties in the body and act as aids in the prevention and treatment of diseases and improvement of body condition (Astley and Finglas, 2016).

According to the available literature, nettle has beneficial effects on human health achieved through the action of bioactive molecules. In particular, it is a powerful antioxidant (Flórez et al., 2022), it is used to relieve joint pain (Randall et al., 2013), respiratory diseases and allergies (Roschek et al., 2009), as well as digestive disorders (Joshi et al., 2014; Upton, 2013). It also has a diuretic effect, relieves urinary tract infections and has beneficial effects on the kidneys and liver (Eldamaty, 2018; Fatima et al., 2018). Sometimes it is not strong as an independent medicine, but it is a very useful additional therapy. Nettle root is also used in prostate diseases, in the treatment of benign prostatic hyperplasia (Safarainejad, 2005). Detailed pharmacological actions of nettle can be found in the review papers by Bhusal et al. (2022), Grauso et al. (2020) and Jan et al. (2017).

Bioactive molecules can be found in the aerial and underground parts of the nettle. Table 2 gives an overview of the main bioactive molecules in all parts (leaves, stems, and roots) of the nettle. For example, the leaves and stems are a rich source of phenols, like flavonoids and phenolic acids, as well as pigments, especially chlorophylls and carotenoids, while the root is a rich source of phytosterols, pentacyclic triterpenoids, lignans and coumarins.

Table 2. Bioactive molecules found in different parts of nettle (*Urtica dioica* L.)

PART OF THE NETTLE	CHEMICAL GROUP	CHEMICAL COMPOUND	REFERENCE
LEAVES	flavonoids	kaempferol, quercetin, rutin, naringenin, isorhamnetin, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glucoside, isorhamnetin-3-*O*-glucoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, catechin, epicatechin	Jeszka-Skowron et al., (2022); Otles and Yalcin, (2012); Kregiel et al., (2018)
	phenolic acids	syringic acid, fumaric acid, protocatechuic acid, ellagic acid, *p*-coumaric acid, ferulic acid, gentisic acid, quinic acid, caffeic acid, cinnamic acid	Otles and Yalcin, (2012); Kregiel et al., (2018); Orčić et al., (2014)
	carotenoids	β-carotene, lutein, 13-*cis*-lutein, 9-*cis*-lutein, neoxhantin, violaxanthin, zeaxanthin	Guil-Guerro et al., (2003); Rafajlovska et al., (2002)
	chlorophylls	chlorophyll *a*, chlorophyll *b*	Rafajlovska et al., (2002); Hojnik et al., (2007)
	organic acids	oxalic acid, citric acid, malic acid	Jeszka-Skowron et al., (2022)
	fatty acids	stearic acid, palmitic acid, lauric acid, archidic acid, beheic acid, linoleic acid, α-linoleic acid, palmitoleic acid, oleic acid	Tarasevičiene et al., (2023); Rutoo et al., (2013)
STEMS	fatty acids	stearic acid, palmitoleic acid, olic acid, gadoleic acid, arucic acid, linoleic acid,	Guil-Guerro et al., (2003)
	flavonoids	myricetin, quercetin, kaempferol, rutin, naringin, isorhamnetin	Otles and Yalcin, (2012)
	phenolic acids	syringic acid, fumaric acid, vanillic acid, ellagic acid, *p*-coumaric acid, ferulic acid, quinic acid, caffeic acid	Otles and Yalcin, (2012); Kregiel et al., (2018)
	chlorophylls	chlorophyll *a*, chlorophyll *b*	Hojnik et al., (2007)
ROOTS	phytosterols	β-sitosterol, stigmasterol, campesterol, campestanol, Δ^5-avenasterol, Δ^5-stigmasterol, cycloartenol, 24-methylene cycloartenol, obtusifoliol, citrostadienol	Obranović et al. (2022); Kovacheva et al. (1990)
	pentacyclic triterpenoids	α-amyrin, β-amyrin, β-amyrin acetate	Obranović et al., (2022)
	phenolic acids	*p*-hydroxybenzoic acid, protocatechuic acid, quinic acid, *p*-coumaric acid, ferulic acid, caffeic acid, 5-*O*-caffeoylquinic acid	Franciśković et al., (2017)
	flavonoids	myricetin, quercetin, kaempferol, rutin	Otles and Yalcin, (2012)
	coumarins	esculetin, scopoletin	Franciśković et al., (2017); Sajfrtova et al., (2004)
	fatty acids	behenic acid, stearic acid	Tarasevičiene et al., (2023)
	lignans	neo-olivil, secoisolariciresinol, dehydrodiconiferyl alcohol, isolariciresinol, pinoresinol, 3,4-divanillyltetrahydrofuran	Schöttner et al., (1997); Chaurasia and Wicthl (1986)

Phenolic compounds are secondary plant metabolites that have one or more aromatic bond rings with one or more hydroxyl groups. Considering their structure and function, slightly more than 8,000 different phenolic compounds are known, so they are divided into two major groups: flavonoids (flavones, isoflavones, flavonols, flavanones, flavanols, and anthocyanins) and non-flavonoids (hydroxybenzoic acid, hydroxycinnamic acid, tannins, coumarins, lignans, and stilbenes) (Tungmunnithum et al., 2018; Dobson et al., 2019). They are widely distributed in the plant kingdom, where they naturally protect plants from UV radiation and pathogen attacks, and contribute to plant color, aroma, and flavor (Dai and Mumper, 2010). Phenolic compounds are mainly known to act as powerful antioxidants, i.e., substances that prevent the oxidation of other substances, and serve to neutralize free radicals in biological systems (Scalbert et al., 2005). By reducing oxidative stress and protecting against oxidative damage, phenols contribute to the prevention of chronic diseases such as cardiovascular disease (Michalska et al., 2010), anti-inflammatory diseases (Serafini et al., 2010), cancer (Khan and Mukhtar, 2013), and neurodegenerative disorders (Braidy et al., 2016).

Pigments are natural substances, carriers of colors and are found in the cells and tissues of plants, fruits, vegetables, fungi, bacteria and algae. The main source of pigments are plants, especially green ones. These pigments play vital roles in plant biology, including photosynthesis, protection against environmental stresses, and attraction of pollinators. The most common types of plant pigments are chlorophyll, carotenoids, and anthocyanins. They are sensitive to physical and chemical influences throughout processing, during which they can be degraded (Nabi et al., 2023). Chlorophyll is the most abundant pigment in plants with the highest concentration in chloroplasts. The structure of the chlorophyll is a porphyrin derivative conjugated with a magnesium ion. It plays a crucial role in the process of photosynthesis and gives plants their characteristic green color (Zhong et. Al, 2021). Although 6 types (a, b, c, d, e, and f) of chlorophyll are known, the most widespread and best studied are chlorophylls *a* and *b* (Nabi et al., 2023). While chlorophyll is not an essential nutrient for humans, it has been studied for its potential health benefits; antioxidant and anti-inflammatory properties (Ferruzzi et al., 2002; Lin et al., 2013), detoxification and liver health (Kumara et al., 2020; Wunderlich et al., 2019) as well as wound healing (Zhiqiang and Hao., 2022). Carotenoids are tetraterpene pigments in yellow, orange, red, and violet colors. They can be divided into two groups, namely carotenes (α-carotene, β-carotene, and lycopene) and xanthophylls (lutein and zeaxanthin) (Langi et al., 2018). Together with chlorophyll, they represent

essential pigments in photosynthetic organs. Carotenoids are involved in photosynthetic reactions by collecting energy from sunlight and transporting it to chlorophyll (Zulfiqar et al., 2021). They also attract pollinators with their color, are precursors of plant hormones and have antioxidant and anti-inflammatory properties (Hozawa et al., 2007; Maoka, 2020), participate in eye protection (Ma et al., 2012) and reduce risk of cardiovascular disease (Sesso et al., 2004).

Pentacyclic triterpenoids are formed by the oxidation of triterpenes and contain a hydrocarbon skeleton with a functional group containing oxygen. They include lupane, ursane and oleanane groups, where α-amyrin and β-amyrin are the main representatives. Plant sterols or phytosterols are derivatives of triterpenoids, which are polycyclic alcohols whose core consists of three cyclohexane rings and one cyclopentane (Kushiro and Ezibuka, 2010; Xu et al., 2018). As structural components of cell membranes, sterols have protective, transport, and signaling functions in plants. One of the primary functions of phytosterols is their ability to interfere with the absorption of cholesterol in the digestive system. Phytosterols compete with cholesterol for absorption, resulting in reduced cholesterol uptake into the bloodstream (Ostlund, 2002). Both pentacyclic triterpenoids and phytosterols have a positive effect on human health. In addition to being strong antioxidants (Yoshida and Niki, 2003), they reduce the risk of cardiovascular diseases (Ras et al., 2014) and the development of cancer (Ludwiczuk et al., 2017; Semalty et al.,2017; Yin et al., 2018).

Chemical composition of bioactive molecules from nettle is influenced by various environmental, genotypic, phenotypic and agronomic factors. Different parts of nettle contain different proportions of bioactive molecules. The accumulation of bioactive molecules also depends on the phenological stage during plant growth, while the harvest time varies depending on the final product. If nettle is used for the preparation of fresh drinks or food, it must be harvested before flowering. For the production of dry preparations, it is harvested before and during flowering. However, it is recommended to harvest before the appearance of seeds, because then the plant loses its vitality and contains a smaller amount of ingredients (Upton, 2013; Paulauskienė et al., 2021). The climate in which the plant grows also plays a major role in the accumulation of compounds. Nettle plants are adaptable and can grow at different altitudes. However, altitude can affect temperature, precipitation patterns, and sunlight intensity, which, in turn, affect nettle physiology and metabolism. Different altitudes may result in variations in the types and amounts

of bioactive molecules produced by nettle plants. Warmer temperature and more sunlight have a positive effect on the synthesis and accumulation of bioactive molecules. Sufficient rainfall or irrigation provides adequate hydration, which supports the metabolic processes involved in the production of bioactive molecules (Paulauskienė et al., 2021). For all these reasons, more efforts are made to cultivate the nettle to obtain a plant with a uniform chemical composition and improved quality (Opačić et al., 2022).

By implementing the mentioned bioactive molecules in food, a new functional product is obtained. It is a product enriched with bioactive ingredients that have a positive effect on health in the aforementioned way, acting mainly as powerful antioxidants. Moreover, according to European legislation, pigments are used as natural colorants in food industry; carotenoids as E160 and E161 and chlorophylls as E140 and E141 (Regulation No. 1333/2008 and its amendments). Color is an important characteristic of food that shapes first impressions and customer expectations. Color additives are important to the food industry to improve sensory quality lost during food processing, to enhance existing colors, to color otherwise uncolored foods, and generally to expand product variety. As consumers become increasingly aware of the impact of nutrition on their health, functional products have a promising future.

3. Accelerated solvent extraction for the isolation of nettle bioactive molecules

In order to isolate bioactive molecules from plant material, various extraction techniques are used. Extraction is a process for selective isolation or separation of desired substance or component from a mixture using a suitable solvent or extraction technique. In this process, the target substance is transferred from its original starting material or matrix into a separate phase, usually a liquid or solid phase.

Since there is no standard extraction technique for the isolation of bioactive molecules, the appropriate technique and extraction conditions must be chosen for each plant material. The choice of extraction technique depends on the nature, chemical structure, and solubility of the bioactive molecules, as well as the type and physicochemical properties of the material from which they are to be extracted (Zhang et al., 2018). Extraction techniques can be divided into conventional and

advanced. Conventional extraction techniques refer to traditional methods that are widely used for the extraction of target compounds from various sample matrices. These techniques are often simple, easily accessible, and have been used for many years. Conventional extraction techniques include: maceration, percolation, infusion, decoction, cold pressing and Soxhlet extraction (Blicharski and Oniszczuk, 2017; Rasul, 2018). Although they have been used for a long time, these techniques have some drawbacks. Conventional extraction techniques are not only time-consuming and use a lot of energy and solvents, resulting in low efficiency (Bursać Kovačević et al., 2018), but also have a negative impact on the yield and quality of the extracts due to the thermal degradation of bioactive molecules and are therefore generally not considered as "green" technologies since they are not sustainable or environmentally friendly (Putnik et al., 2019). Hence, advanced extraction techniques have been developed and are increasingly used in recent years. These are automated techniques that use shorter extraction times, less solvent, and provide improved extraction efficiency. By optimizing extraction parameters such as temperature, pressure, and choice of solvent, these techniques allow selective extraction of desired compounds while minimizing co-extraction of undesired components (Bitwell et al., 2023). Advanced extraction techniques often use more environmentally friendly solvents, such as supercritical fluids or organic solvents with lower toxicity and environmental impact. They may also require reduced sample size, minimizing the amount of waste generated (More et al., 2022). These factors contribute to safer and more environmentally friendly extraction processes. Some of the advanced extraction techniques include: microwave assisted extraction (MAE), ultrasound assisted extraction (UAE), supercritical fluid extraction (SFE), pulsed electric field (PEF), high pressure extraction (HPE), subcritical water extraction (SWE) and accelerated solvent extraction (ASE).

ASE showed to be one of the most effective techniques for extracting plant material. Compared to other techniques, it gives extremely high yields. In the study of Zengin et al. (2019), the highest content of total phenols was obtained from the plant *Erica arborea* L. using ASE compared to MAE, UAE, maceration and Soxhlet extraction. Abdel-Aal et al. (2014) concluded that ASE proved to be the best technique for isolating anthocyanins from different grains compared to MAE and conventional extraction. It also resulted in less changes in anthocyanin composition and anthocyanin content compared to MAE.

ASE, also known as pressurized solvent extraction (PLE) or enhanced solvent extraction (ESE), was developed and introduced in the late 1990s. It was invented by Dionex Corporation, which later became part of Thermo Fisher Scientific. The ASE technique was developed to improve the efficiency and speed of the extraction process compared to conventional extraction techniques. Most research papers on ASE before 2000 were related to environmental analysis, i.e., extraction of environmental pollutants in soil, sludge from wastewater, and sediments (Wang et al., 2008), but since then, the use of ASE for the extraction of bioactive molecules from plant materials has increased significantly (Shams et al., 2015) and has recently been applied in various fields, including biology, pharmaceutical and food industry (Sun et al., 2012). ASE is an automated extraction technique used to obtain extracts from solid or semisolid samples in a short time (less than 20 min) and with a small amount of solvent (less than 50 mL). The procedure is carried out at temperatures above the boiling point of the solvent (50-200 °C), while the pressure inside the extraction cell is high (10-15 MPa) (Sun et al., 2012). As it can be seen in Figure 2, the device mainly consists of a solvent tank, a pump, an oven containing an extraction cell, various valves and chokes, nitrogen tank and collection bottles (Alvarez-Rivera et al., 2020).

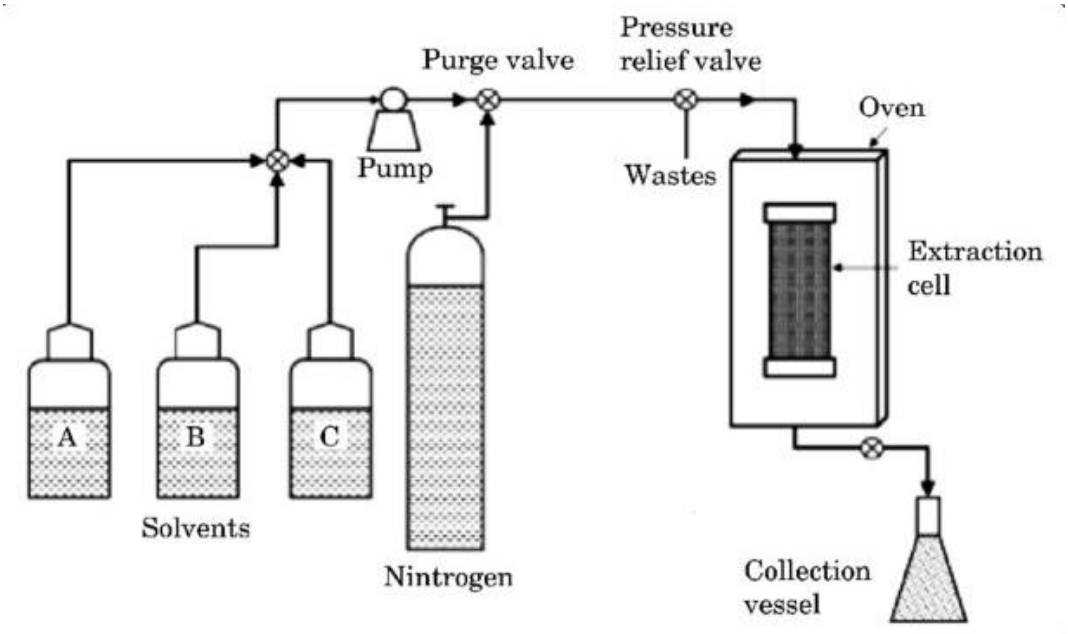

Figure 2. Principle of accelerated solvent extraction (Shahid et al., 2015)

The process itself starts with placing the stainless steel cells filled with crushed plant material on the device and setting the extraction parameters, the rest of the procedure is automated. Since the process is carried out inside the cell, the material is not exposed to light or oxygen. A solvent is pumped into the cells, the sample is heated in an oven and the extraction is carried out at a certain temperature and pressure. At the end of the extraction, the cells are purged with nitrogen and dried, and the extract is filtered and collected in vials, ready for analysis (Mottaleb and Sarker, 2012; Ameer et al., 2017). To isolate as much of the target components as possible, the process itself must be optimized. Parameters that can be optimized include: extraction solvent, temperature, pressure, extraction time, number of extraction cycles, and sample composition.

The selection of a suitable solvent for the extraction of bioactive molecules is the first challenge in the development of the ASE extraction technique. Since "like dissolves like," the polarity of the extraction solvent should match the polarity of the target compounds. There is a wide range of solvents of different polarity that can be used in ASE, from *n*-hexane to methanol. Strong acids and bases as well as self-igniting liquids in the temperature range of 50 to 200 °C cannot be used (Juan et al., 2005). Methanol is a widely used extraction solvent because it allows higher yields of target compounds and offers the possibility of extracting various bioactive molecules from the plant material, although it is a toxic solvent (Truong et al., 2019; Alhallaf et al., 2022). Preferably, “generally recognized as safe” (GRAS) and environmentally safe solvents (ethanol, ethyl acetate, and water) should be used. Goh et al. (2021) concluded that the mitragynine content in kratom leaves was not significantly different when organic solvents of different polarity were used. On the other hand, the highest content of phenols was found when ethyl acetate was used as a solvent, while the highest content of flavonoids was found in ethanol extracts. For the extraction of the lipid fraction from the Apiaceae seeds and nettle root by ASE, Balbino et al. (2021) and Obranović et al. (2023) used *n*-hexane. From a toxicological point of view, ethanol and water are considered safer solvents than acetone, methanol and other organic solvents and are therefore more suitable for use in the food industry (Oktay et al., 2003). However, in some cases, mixtures of polar and nonpolar solvents result in higher yields (He and Balasubramanian, 2009). In conclusion, the solvent must be chosen based on the components to be extracted.

Temperature is considered as the most important factor during ASE. High temperatures accelerate the extraction kinetics, increase the solubility and diffusion rate of the analyte, decrease the

viscosity and surface tension of the solvent, leading to better penetration into the pores of the analyte and improving mass transfer (Ameer et al., 2017; Alvarez-Rivera et al., 2020). Consequently, the increased temperature leads to a higher yield of bioactive molecules. This was noted by Dobroslavić et al. (2022), where increasing the ASE temperature from 90 to 150 °C increased the yield of phenols from bay leaves. However, it should be kept in mind that some of the bioactive molecules extracted with ASE are thermolabile and high temperatures may negatively affect their bioactivity. Piñeiro et al. (2004) claimed that a temperature of 130 °C during ASE is optimal for the isolation of catechins from tea leaves and grape seeds. Above that temperature, their concentration decreased, probably due to thermal degradation. Also, increasing the temperature alone may not be sufficient to increase the extraction efficiency, because many of the organic solvents used for extraction have their boiling point at relatively low temperatures. One way to overcome this problem is to apply sufficient pressure during extraction (Mottaleb and Sarker, 2012). The high pressure allows the solvent to remain in a liquid state at high operating temperatures (above the boiling point) and penetrate into the pores of the sample that it would not have reached under atmospheric conditions (Mottaleb and Sarker, 2012). Cho et al. (2007) studied the effect of pressure (500-2500 atm) on the isolation of volatile metabolites in *Angelica* roots using ASE. The optimal pressure was 1500 atm (10.34 MPa), which is the pressure mainly used in ASE when the pressure is kept constant, since this pressure is above the necessary threshold to maintain the solvent in a liquid state and is not considered a critical parameter of ASE (Mottaleb and Sarker, 2012).

The extraction time required for complete extraction of a certain matrix depends on the matrix, the type of compound, and the extraction method (static or dynamic). In the static mode, temperature and extraction time are the determining factors. In this mode, the cell containing the analyte is heated to a specific temperature, followed by a static extraction process. During this process, the analytes are isolated from the sample under stable static conditions, and the process can be repeated several times to achieve higher yields (Sun et al., 2012). The long exposure to the solvent allows the analytes to swell and the solvent to better penetrate the interstitial spaces of the sample. Extending the static extraction time allows better penetration of the extraction solvent into the pores of the sample, resulting in higher yields of the target components, especially if they are hard to reach. The extraction is finished in max 20 min using max 50 mL of solvent (Moattaleb and Sarker, 2012). Dynamic extraction, on the other hand, improves mass transfer, but is rarely used

due to higher solvent consumption compared to static extraction (Sun et al., 2012). For the extraction of target components to be complete and effective, the combination of static time and number of cycles should be examined. The purpose of static cycle is to constantly introduce fresh solvent during the extraction process, which helps to maintain a favorable extraction balance. If the sample contains a higher concentration of a particular constituent or if these constituents are difficult to obtain, more extraction cycles are performed. Introducing multiple extraction cycles can lead to complete extraction of the target components. Saha et al. (2015) concluded that the optimal conditions for the isolation of carotenoids from carrots are 60 °C/ 15 min/ 3 cycles. Longer time improved the extraction of carotenoids, while their extraction did not improve after 3 extraction cycles.

The influence of the sample matrix on the extraction depends on its chemical composition, matrix interferences and the particle size of the material. The complexity of the analytical procedure increases with the number of compounds present in the sample. Plant materials often contain various compounds such as carbohydrates, lipids, pigments, and cellulose components that can contribute to matrix interference. Matrix interference refers to the presence of compounds that can co-extract with the bioactive molecules and interfere with their detection or analysis. They can affect the selectivity and accuracy of the extraction process. It is important to consider appropriate sample preparation techniques, such as degreasing or pretreatment steps, to minimize matrix interference and improve extraction efficiency (Sun et al., 2012). The chemical composition of the plant material, including the presence of various bioactive moleculess, can affect the extraction process. Some bioactive molecules may be more soluble in certain solvents, while others may require specific extraction conditions. As mentioned above, it is important to select an appropriate solvent that can selectively extract the desired bioactive molecules from the plant material based on their chemical properties. If other bioactives are extracted with the analyte, they should be removed before analysis (Giergielewicz-Możajska et al., 2001). The particle size of the plant material is important because it affects the surface area available for extraction. Smaller particle sizes provide a larger surface area for solvent penetration and contact with bioactive molecules, resulting in higher extraction yields. Therefore, finely ground or powdered plant material generally results in better extraction efficiency in ASE. Thus, solid samples are usually crushed or ground, but cannot be too fine, because they can create a layer at the bottom of the cell through which the

solvent then passes with difficulty and consequently the yield of bioactive molecules is reduced (Yunus et al., 2013).

According to the mentioned, ASE is an effective technique, uses less solvent and energy, shortens the extraction time, includes the possibility of working in multiple cycles, which significantly contributes to a higher extraction yield and more efficient isolation of the target components when parameters of extraction are optimized.

4. Significance of spray drying encapsulation

Although liquid plant extracts are most commonly used by consumers, the stability of bioactive molecules in such extract depends on numerous factors such as light, oxygen, heat and moisture (Shishir and Chen, 2017), which affect the reduction or complete loss of their biological activity over time. The food, pharmaceutical, and cosmetic industries are increasingly emphasizing the use of herbal products that contain natural bioactive ingredients that have a positive impact on human health, while at the same time the shelf life of the product should be prolonged. One of the techniques used to extend the shelf life of such products and protect the ingredients from environmental influences is encapsulation.

Encapsulation in the food industry uses specialized technologies to create a protective barrier (shell, wall, coating or carrier material) around active ingredients (core or active material) such as microorganisms, flavors, colors, nutrients, oils, or bioactive molecules (Timilsena et al., 2020). These ingredients are very sensitive to environmental conditions, processing, storage, and/or conditions in the gastrointestinal tract. Encapsulation provides effective protection of functional food ingredients in a way that increases their stability during processing and storage, prevents undesirable reactions with other food ingredients, slows degradation processes (oxidation, hydrolysis, etc.), or prevents decomposition until the ingredient is delivered to the desired location (Bratovcic and Suljagic, 2019). In the food industry, there are several techniques for encapsulating ingredients: vacuum and freeze drying, fluid bed coating, melt injection, melt extrusion, extrusion, emulsification, coacervation, molecular inclusion, spray chilling, spray cooling, and spray drying (Nedović et al., 2013).

Among the above techniqus, spray drying is the most commonly used microencapsulation technique in the food industry (about 80-90% of encapsulates are spray-dried), having some advantages over other encapsulation techniques. It is a highly efficient process that enables fast and continuous production. It can process large quantities of liquid feed and produce powdered products on a large scale in a short time, making it suitable for industrial applications (Buljeta et al., 2022). It can be used for a wide range of food ingredients and can encapsulate both hydrophilic and lipophilic ingredients (Nedović et al., 2013).

In general, spray drying is a technique in which liquid or semi-liquid feed are dried in a stream of hot air, producing a powder as the final product. It was invented in 1872 by Samuel Percy, whose initial experiments focused on the production of milk powder from liquid milk. Today, it is increasingly used for encapsulating bioactive molecules from various plant materials (Kandasamy and Naveen, 2022). The aim of spray drying is to quickly and efficiently remove the water from the feed, which helps to inhibit the growth of microorganisms and enzymatic reactions that can cause spoilage, and obtain a powder with the desired physical and chemical properties (Tonon et al., 2008). Before the drying process, a certain amount of carrier is added to the extract solution, which not only improves the drying effect, but also forms a protective layer around the bioactive ingredient (Caliskan and Dirim, 2013). The size of the powder particles obtained by spray drying varies from a few μm to a few mm and they are most often spherical shape (Guajardo-Flores et al., 2015). As it can be seen in Figure 3, the process itself consists of four steps: atomization, establishing contact of the sprayed feed with the air used for drying, evaporation of water from the dispersed droplets, and separation of the dried powder from the air (Mohammed et al., 2020).

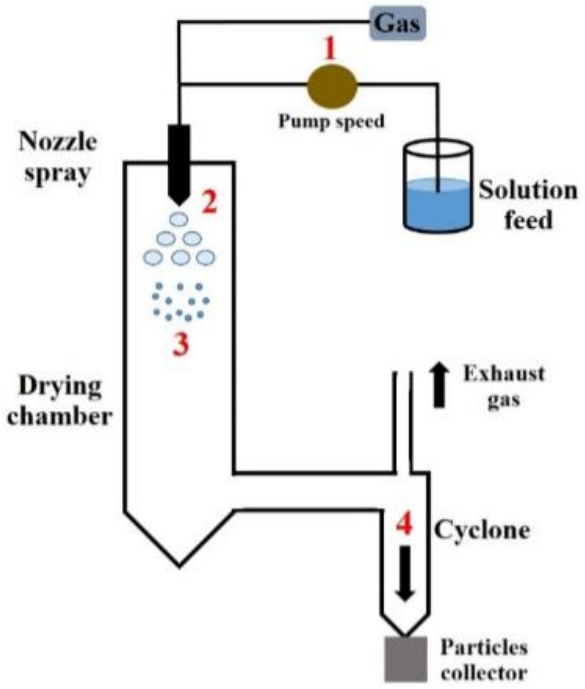

Figure 3. Steps of spray drying process (Lechanteur and Evrard, 2020)

In the first step, the solution feed enters the sprayer and is passed through the nozzles and sprayed onto smaller droplets to create larger area between the dry air and the solution, which increases the heat and mass transfer, and the degree of water evaporation (Phisut, 2012). In the second step, the dispersed droplets come into contact with the hot air in the drying chamber, and thus the actual drying process begins. The air is heated to a certain temperature using of the heat exchanger and introduced into the chamber. The third step is the drying process in which the water evaporates from the droplets. The heat is transferred from the air to the product, creating a temperature difference, while the transfer of the water occurs in the opposite direction due to the difference in vapor pressure (Santos et al., 2018). The final step in the drying process is the separation of the dried powder from the exhaust air. Separation is done either at the bottom drying chambers, where larger particles are separated, or in cyclones, where smaller particles are separated, which reduces product loss, although some of the particles are discharged outside together with the exhaust air.

It is important to optimize the spray drying process in order to obtain a high yield of powder with high encapsulation efficiency of the targeted bioactive molecules without degradation. Also, it is important to obtain a powder with desirable properties, such as low moisture content and

hygroscopicity, and high solubility. The influence of drying temperature, feed flow rate, choice of the carrier and its concentration in the feed are some of the parameters that can affect the efficiency of the encapsulation.

The choice of carrier is a very important step in the encapsulation of food ingredients in order to increase the productivity of the process itself and obtain a stable final product. Their choice depends on type and properties of the substance to be encapsulated (Timislena et al., 2020). In general, the addition of carriers to the material to be dried increases the content of dry matter, resulting in the production of a powder that ultimately contains lower amount of water and has lower hygroscopicity, but higher solubility and product yield. Masking of undesirable tastes and odors, and controlled or sustained release of ingredients after application, leading to an increase in bioavailability of the encapsulated substance, is also achieved in this way (Ghosh et al., 2021). In addition to the fact that the carrier must form a barrier between the active ingredient and the environment, protecting it from high temperatures, enzymatic and chemical changes, it also must be biodegradable, food grade (certified as "GRAS") and highly soluble (Shishir and Chen, 2017; Buljeta et al., 2022). The most commonly used carriers in encapsulation by spray drying are polysaccharides (starch hydrolysates, gums, and cyclodextrins), proteins and other polymers (Akbarbaglu et al., 2021). Maltodextrin (MD), the most commonly used carrier in food encapsulation, is a long-chain, high molecular weight starch hydrolysate consisting of glucose units linked by α-glycoside bonds. It is described by its dextrose equivalent (DE), which is inversely related to its average molecular weight and indicates the degree of starch degradation. It is used because of its mild taste, low price, low viscosity and high solubility (Saavedra-Leos et al., 2015). Another carrier is gum arabic (GA), which is a natural plant excretion of the acacia tree, composed of about 2% glycoproteins and polysaccharides such as L-rhamnose, D-galactose, L-arabinose, and D-glucuronic acid, with a highly branched structure (de Barros Fernandes et al., 2014). It is the only gum used in foods that has high solubility and low viscosity in aqueous solutions, as well as good emulsifying properties that facilitate the drying process. Among cyclodextrins (CD), α-, β- and γ-CD are the most commonly used as carriers. They have a hydrophobic cavity in the center that allows the binding of other molecules inside, while the hydrophilic outer part allows solubility in water (Dobroslavić et al., 2023). Şahin-Nadeem et al. (2013) studied the physicochemical properties of spray-dried sage leaf extract powder where MD, GA and β-CD were used as carriers. They concluded that increasing the proportion of carriers

increased the yield, bulk density, and solubility and decreased the moisture content. Moreover, when β-CD was used as the carrier, most of the 1,8-cineole was trapped in the carrier, due to its structure. When adding the carrier to the spray drying solution, it is necessary to determine the optimal concentration, as it affects the properties of the powder. The concentration of the carrier must not be too low, otherwise the yield will decrease and the active ingredient will be insufficiently protected. Dobroslavić et al. (2023) found that the ratio of 1:2 of the dry matter of the extract to the carrier was optimal for the maximum encapsulation capacity of phenols from laurel extract. Further increase in the ratio led to a decrease in the encapsulation value due to the shorter contact time of the raw material with the drying air, which slows down the heat and mass transfer, resulting in lower entrapment of the phenolic compounds. Since each carrier has its own advantages, their combination is often used in spray drying. Aliakbarian et al. (2015) concluded that the optimal combination of carriers for encapsulation of phenols from olive pomace by spray drying was MD:GA in a 60:40 ratio, as opposed to their individual proportions.

Temperature plays a crucial role in spray drying processes, as it significantly affects drying kinetics, product quality and overall efficiency. Typically, the inlet temperature of the hot air or gas in a spray drying process is between 120 and 200 °C (Ferrari, 2012). The temperature directly affects the rate of water evaporation during spray drying. Higher temperatures accelerate the drying process by increasing the vapor pressure and causing the water to evaporate from the droplets faster (Phisut, 2012; Elez Garofulić et al., 2016). This results in a shorter residence time and faster drying, which can be an advantage for large-scale production. Tran and Nguyen (2018) concluded that increasing the temperature from 110 to 150 °C led to higher solubility and antioxidant activity of the lemongrass extract powder and the powder contained a lower moisture content. On the other hand, total phenol and flavonoid contact decreased with increase of temperature. Accordingly, there are many bioactive molecules that are sensitive to heating. For example, many authors pointed out that drying at temperatures above 160 °C can lead to degradation of color and content of bioactive molecules, leading to the creation of a lower quality product (Fang and Bhandary 2010; Paini et al., 2015; Zorić et al., 2017). Temperature can affect the particle size and morphology of the dried product. Higher temperatures usually lead to the formation of larger particles, as the water evaporates faster and a protective layer form more quickly on the surface of the droplet, while a vapor bubble forms inside, which consequently increases the size of the particles (Tonon et al., 2008). Observing the morphology of the powder,

it is evident that increasing the temperature leads to the formation of a greater number of particles with a smooth surface and larger dimensions, since the degree of drying is greater, which results with higher process yield (Corrêa-Filho et al., 2019).

The flow rate of the feed is another parameter that affects the effective formation of powder during spray drying. In general, the faster the flow rate, the shorter the contact time between the feed and the drying air, making heat transfer less efficient and consequently removing less water from the feed. Also, at higher flow rates, dripping may occur in the chamber, because the feed is not dispersed, resulting in a lower yield during the process (Singh and Dixit, 2014). In the study by Thi Anh Dao et al. (2021), the amount of phenols from the green tea extract powder increased when the flow rate was increased from 6 to 7 rpm, and then decreased when the flow rate was increased further. At low flow rates, the mixture is exposed to heat for too long, resulting in a loss of phenols, while at too high flow rates, not all of the water is removed from the mixture because the contact with the hot air is too short, reducing the drying efficiency and phenolic content. Therefore, aiming for the powder yield as high as possible, it is important to find the optimal flow of the mixture.

In recent years, the use of encapsulated bioactive substances incorporated into foods has increased, so the market for such functional foods will certainly continue to grow. New food products are being developed to ensure the stability, activity, and bioavailability of bioactive functional ingredients after processing and storage (Morais et al., 2018). Numerous studies show that phenols from various plants can be spray-dried and later incorporated into various products such as yogurt (Pimpley et al., 2022), bread (Pasrija et al., 2015), chocolate (Belšak-Cvitanović et al., 2012), and cookies (Caleja et al., 2018) to improve their quality. Spray-dried pigments such as chlorophylls and carotenoids, especially those derived from algae, can also be used as natural colorants in various foods such as chocolate (Polat et al., 2020), ice cream (Durmaz et al., 2020), and chewing gum (Palabiyik et al., 2018). Phytosterols are another ingredient that can be incorporated into a functional product. Incorporating spray-dried phytosterols into dark chocolate increases its stability and bioavailability and also lowers cholesterol levels (Tolve et al., 2018). As for stinging nettle, there is only one paper that investigates the effects of spray-dried nettle extracts as a functional food ingredient. Kalajahi et al. (2022) concluded that nettle powder can enrich cakes with antioxidant compounds and replace synthetic additives in bakery products with natural ones.

Therefore, further research is needed to see how spray dried nettle can enrich different food with its composition.

5. Hypothesis, research objectives, and expected scientific contributions

The research hypothesizes that:

i. bioactive molecules yield will depend on applied extraction conditions;
ii. content of bioactive molecules and antioxidant activity will vary according to the phenological stage and habitat of the nettle;
iii. microencapsulation will affect stability and bioavailability of phenols.

In order to accept or decline established hypotheses, the following objectives are defined:

i. to optimize the extraction process of bioactive molecules (phenols, pigments, and phytosterols) from all parts of nettle by ASE;
ii. to determine the influence of phenological stage and habitat on the accumulation and composition of bioactive molecules and antioxidant activity of nettle;
iii. to optimize microencapsulation of extracted phenols from nettle leaf by spray drying and to investigate the physicochemical properties and bioavailability of phenols.

The research plan was divided into three parts:

The first part of the research included the optimization (temperature, static extraction time, and number of cycles) of ASE of nettle phenols, pigments, sterols and pentacyclic triterpenoids. The optimal conditions of the advanced extraction technique were compared with those of other extraction techniques. The influence of extraction parameters on the chemical composition of bioactive molecules and antioxidant activity was examined (***Publication No.1 and Publication No.3***).

In the second part, the influence of phenological stage and habitat on the presence and chemical profile of phenols and pigments as well as antioxidant activity of wild nettle leaves and stems was investigated (***Publication No.2***).

The third part dealt with the optimization (temperature, type of carrier, and sample to carrier ratio) of microencapsulation of phenols from nettle leaf extract by spray drying. In addition, the effect of the encapsulation parameters on the physicochemical properties, encapsulation and loading capacity of the obtained nettle leaf extract powders, as well as the effect of encapsulation on the antioxidant activity and bioavailability of phenols were investigated (***Publication No.4***).

Throughout this dissertation the following questions were examined:

1) What is the optimal temperature, static extraction time and number of extraction cycles for the extraction of phenols, pigments and sterols and pentacyclic triterpenoids from nettle by ASE? How do the different extraction parameters affect the chemical composition of the bioactive molecules? (***Publication No.1 and Publication No.3***)
2) Can an advanced extraction technique, ASE, improve the extraction of bioactive molecules from nettle compared to other techniques and how does it affect their yield, chemical composition and antioxidant activity? (***Publication No.1 and Publication No.3***)
3) How do the different habitats and phenological stages affect the chemical profile of phenols and pigments of wild nettle leaves and stems and their antioxidant activity? (***Publication No.2***)
4) What is the optimal temperature, type of carrier and sample to carrier ratio for encapsulation of phenols from nettle leaf extract by spray drying? How do the different encapsulation parameters affect the chemical composition, physicochemical properties, encapsulation capacity and antioxidant activity of the obtained powder? (***Publication No.4***)
5) Can encapsulation of nettle leaf extract improve the bioavailability of phenolic compounds from nettle leaf extract? (***Publication No.4***)

Throughout this dissertation next was achieved:

1) knowledge of the optimal parameters for the ASE of nettle phenols, pigments, sterols and pentacyclic triterpenoids
2) a better understanding of the influence of extraction technique on the yield, chemical composition and antioxidant activity of nettle extracts
3) characterization of the chemical structure of phenols, pigments, sterols and pentacyclic triterpenoids of nettle
4) a better understanding of the influence of phenological stage and habitat on the accumulation of phenols and pigments in nettle leaves and stems
5) knowledge of the optimal parameters for the encapsulation of phenols from nettle leaf extract by spray drying
6) better understanding of the properties of encapsulated extracts and their potential application in food industry

Chapter 2

Publication No.1: Accelerated Solvent Extraction as a Green Tool for the Recovery of Polyphenols and Pigments from Wild Nettle Leaves

Processes

Publication No.1

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processes

MDPI

Article

Accelerated Solvent Extraction as a Green Tool for the Recovery of Polyphenols and Pigments from Wild Nettle Leaves

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Abstract: This study aimed to investigate the performance of accelerated solvent extraction (ASE) as a green approach for the recovery of polyphenols and pigments from wild nettle leaves (NL). ASE was operated at different temperatures (20, 50, 80 and 110 °C), static times (5 and 10 min) and cycle numbers (1–4) using ethanol (96%) as an extraction solvent. In order to compare the efficiency of ASE, ultrasound assisted extraction (UAE) at 80 °C for 30 min was performed as a referent. Polyphenol and pigment analyses were carried out by HPLC and antioxidant capacity was assessed by ORAC. Seven polyphenols from subclasses of hydroxycinnamic acids and flavonoids, along with chlorophylls *a* and *b* and their derivatives and six carotenoids and their derivatives were identified and quantified. Chlorogenic acid was the most abundant polyphenol and chlorophyll *a* represented the dominant pigment. ASE conditions at 110 °C/10 min/3 or 4 cycles proved to be the optimal for achieving the highest yields of analyzed compounds. In comparison with UAE, ASE showed better performance in terms of yields and antioxidants recovery, hence delivering extract with 60% higher antioxidant capacity. Finally, the potential of NL as a functional ingredient from natural sources can be successfully accessed by ASE.

Keywords: *Urtica dioica* L.; nettle leaves; accelerated solvent extraction; polyphenols; chlorophylls; carotenoids; HPLC; ORAC

1. Introduction

Nettle (*Urtica dioica* L.) is a herbaceous and perennial wild plant for which numerous studies show that almost every part of it (stem, flowers, leaves, roots and seeds) has a significant content of various bioactive compounds with corresponding antioxidant capacity (polyphenols, carotenoids, chlorophyll, phytosterols, etc.) [1–3]. Therefore, different parts of this plant may have different applications for functional food production due to its valuable nutritional and biological composition [4–7]. In particular, aerial parts of the nettle are good sources of polyphenols [8–10] and pigments [11,12] with different pharmacological and medicinal properties [13–15]. Dried nettle extract has already been used as commercially formulated food supplement that may have positive effects on reducing osteoarthritis symptoms [16]. Consumer preferences are driving rapidly towards the natural products, hence, the interest of the industry in the production and application of natural extracts is constantly growing

as they show multiple benefits and could represent a valuable ingredient of functional foods, foods supplements and nutraceuticals [17,18].

Nettle extracts are the most common form of its application in the industry, where for each individual species, as well as its part, optimal extraction conditions should be determined with an emphasis on maximum process efficiency and selective isolation of target compounds [19]. Conventional extraction techniques, such as maceration and solvent extraction, use large amounts of solvent, are long-lasting and ultimately do not result in extracts of adequate quality and yield. Therefore, currently priority is given to green extraction techniques that enable fast and environmentally friendly efficient extraction with less energy and solvent consumption [20]. Among these techniques, Accelerated Solvent Extraction (ASE) is highly appreciated for its effectiveness, easy use and fully automated process [21]. The ASE is carried out with a liquid solvent in a combination of elevated temperature and elevated pressure. The method is suitable for the extraction of bioactive compounds sensitive to oxygen and heat [22]. The great advantage of this technique is the ability to work with a larger number of extraction cycles, which significantly contributes to a higher extraction yield [23]. Other benefits of ASE include better diffusion of solvent into the sample due to cell-wall disruption upon high pressure, reduced viscosity of the solvent at elevated pressure and temperature resulting in better solubility, advanced mass transfer, and reduced extraction time [24]. However, ASE may be incomplete due to the limited volume of the solvent and also lower extraction yields of thermolabile components can be reached due to elevated temperatures [24]. Nevertheless, as each extraction parameter can have a significant effect on the extraction efficiency of target compounds, ASE should be optimized in order to maximize its potential [25].

Novel green solvent extraction approaches follow the requirements of being free of toxic solvents. Also, to be performed in miniaturized [26] and automated [27] fashion, are other features of greenness of analytical chemistry. Although the ASE system has been successfully used for isolation of bioactive compounds from various plant material, studies investigating the application of ASE for the isolation of bioactive compounds from nettle are very scarce. Only one research was conducted with aim to investigate ASE extracts of nettle roots, stems, leaves and flowers with respect to anti-inflammatory activity [28]. The extraction methodology was taken from the publication of Johnson et al. [29] and included temperature (22–27 °C and 100 °C), static time 5 min, flushing volume 50%, nitrogen purge time 100 s, and number of cycles 3. Nevertheless, the aim of this work was to investigate the anti-inflammatory and cytotoxic effects of obtained nettle extracts, therefore, did not give a conclusion about the efficiency of ASE in terms of the influence of its process parameters on bioactives recovery. In conclusion, authors stated that further chemical investigation of ASE extracts of nettle is required to identify the individual bioactive compounds responsible for their observed therapeutic potential [28].

Therefore, the aim of this study was to investigate the potential of ASE as a green strategy for the recovery of hydrophilic and lipophilic antioxidants such as polyphenols and pigments (chlorophylls and carotenoids) from wild nettle (*Urtica dioica* L.). As a referent extraction technique for the comparison with ASE efficiency, ultrasound assisted extraction (UAE) was also performed. Moreover, ASE parameters such as extraction temperature, static time and cycle number were optimized with respect to the highest recovery of target bioactive compounds and antioxidant capacity.

2. Materials and Methods

2.1. Chemicals

HPLC grade acetonitrile was purchased from J.T. Baker Chemicals (Deventer, Netherlands). Water was purified in a ableMilli-Q water purification system (Millipore, Burlington, MA, USA). Ethanol (96%) was obtained from Gram–mol d.o.o. (Zagreb, Croatia) and formic acid (98–100%) from T.T.T. d.o.o. (Sveta, Nedelja, Croatia). Chlorogenic acid (≥95%), *p*-coumaric acid (≥98%), ferulic acid (≥99%), quercetin-3-glucoside (≥99%), (−)-β-carotene, α-carotene, chlorophyll *a* (from *Anacystis nidulans* algae), chlorophyll *b* (from spinach) and 2,2′-Azobis (2-amidinopropane) dihydrochloride were obtained

from Sigma-Aldrich (St. Louis, MO, USA). Fluorescein sodium salt was purchased from Honeywell Fluka™ (Seelze, Germany) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) from Acros Organics (Geel, Belgium).

2.2. Plant Material

Wild nettle (*Urtica dioica* L.) was collected in April 2019 in Sela Žakanjska, Croatia (altitude 244 m, latitude 45°36′27.8″ N, longitude 15°20′38.2″ E). Immediately after harvesting, nettle leaves (NL) were separated from stalks and freeze-dried (Alpha 1-4 LSCPlus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Afterwards, dry leaves were grinded using a mortar and obtained powder was instantly used for the extraction. Dry matter content of nettle powders was determined by drying at 103 ± 2 °C to constant mass [30].

2.3. Extraction Procedures

2.3.1. Accelerated Solvent Extraction

Polyphenols and pigments of NL were isolated using Accelerated Solvent Extraction (ASE) (Dionex™ ASE™ 350 Accelerated Solvent Extractor, Thermo Fisher Scientific Inc., Sunnyvale, CA, USA). Extractions were performed in 34 mL stainless steel cells fitted with two cellulose filters (Dionex™ 350/150 Extraction Cell Filters, Thermo Fisher Scientific Inc., Sunnyvale, CA, USA) containing 1 g of the sample mixed with 2 g of diatomaceous earth. In order to establish the highest extraction efficiency, extractions were performed under different extraction conditions as follows: extraction temperatures (20, 50, 80 and 110 °C), static extraction times (5 and 10 min) and extraction cycles (1, 2, 3 and 4), while all other parameters remained constant: 10.34 MPa, 30 s of purge with nitrogen and 50% volume flush. Ethanol (96%) was used as the extraction solvent and obtained extracts were collected in 250 mL glass vial with Teflon septa, transferred into 50 mL volume flask and made up to volume with the extraction solvent.

2.3.2. Ultrasound Assisted Extraction

In order to compare ASE efficiency, an ultrasound assisted extraction (UAE) of NL polyphenols and pigments was simultaneously conducted at previously optimized conditions. Briefly, sample (0.5 g) was put into a sealed test tube (50 mL) and 25 mL of ethanol (96%) was added and homogenized on the Vortex ZX3 (Velp Scientifica Srl, Usmate (MB), Italy). The test tube was placed in an ultrasound bath with frequency of 40 kHz (Bandelin electronic GmbH & Co., Berlin, Germany) at 80 °C for 30 min. Afterwards, the suspension was centrifuged (Z 206 A, Hermle Labortechnik GmbH, Wehingen, Germany) at 6000 rpm for 15 min. The supernatant was filtered using Whatman No. 4 filter into 25 mL volumetric flasks, and made up to volume with the extraction solvent.

Experiental setup is shown in Figure 1. All extracts were prepared in duplicate. Extracts were stored at −18 °C in inert gas atmosphere and filtered through a 0.45 µm membrane filter (Macherey-Nagel GmbH, Düren, Germany) prior to HPLC analysis.

2.4. HPLC Analysis

Separation and quantification of polyphenols and pigments were performed using HPLC analysis with Agilent 1260 Infinity quaternary LC system (Agilent Technologies, Santa Clara, CA, USA) equipped with photodiode array detector (PDA), an automatic injector and ChemStation software. The separation of phenolic compounds was performed on a Nucleosil 100-5C18, 5 mm (250 mm × 4.6 mm i.d.) column (Macherey-Nagel, GmbH, Düren, Germany). The composition of solvents and gradient elution conditions were previously described by [31]. For gradient elution, mobile phase A contained 3% of formic acid in water (*v*/*v*), while mobile phase B contained 3% of formic acid in 100% acetonitrile (*v*/*v*). The used elution program commenced with 10% A in B, raising to 40% A after 25 min, then to 70% A after 30 min and then to 10% A after 35 min. Operating conditions were as follows: column

temperature 20 °C, injection volume 20 µL and the flow rate was 0.9 mL min^{-1}. Detection was performed with UV/VIS–PDA detector by scanning from 220 to 360 nm. Identification was assessed by comparing retention times and spectral data with those of authentic standards (phenolic acids were identified at 280 nm and flavonol glycosides at 360 nm) and previous literature reports [1,32–34]. Quantitative determinations were carried out using external standard method.

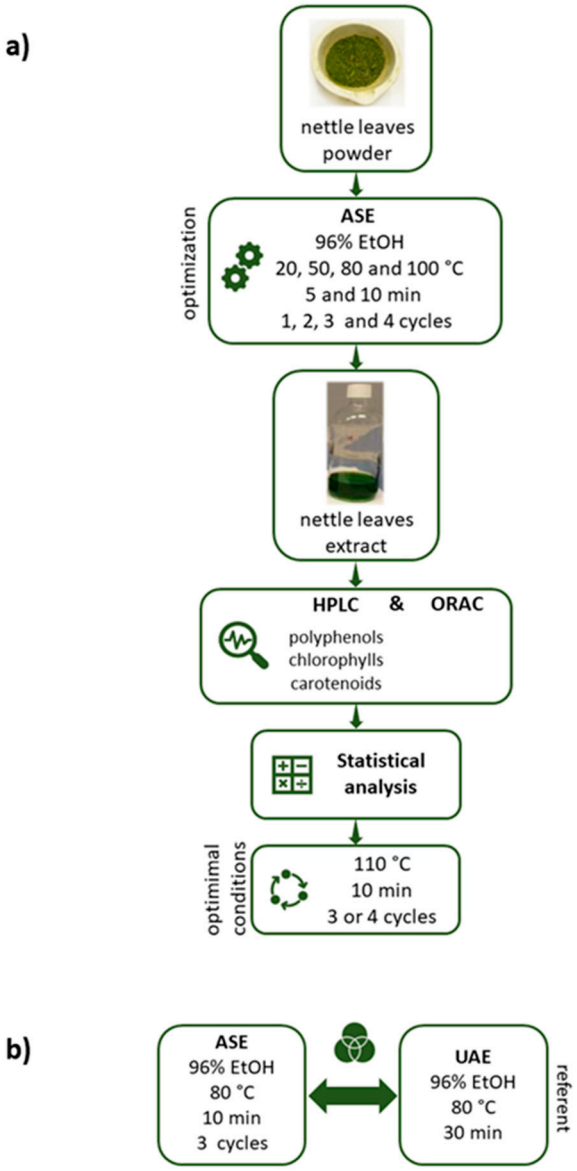

Figure 1. Experimental setup: (**a**) optimization of ASE conditions, (**b**) comparison of ASE and UAE efficiency. (ASE = accelerated solvent extraction, UAE = ultrasound assisted extraction).

For HPLC analysis of carotenoids and chlorophylls, Phenomenex Develosil RP-Aqueus C 30, 3 μm, (250 × 4.6 mm i.d.) (Phenomenex, Torrance, CA, USA) column was used. The solvent composition and the used gradient conditions were described previously by Castro-Puyana et al. [35]. The mobile phase consisted of a mixture of MeOH: Methyl tert-butyl ether (MTBE): water (90:7:3, *v/v/v*) as mobile phase A and MeOH: MTBE (10:90, *v/v*), as mobile phase B. The flow rate was 0.8 mL min^{-1} and the injection volume 10 μL. The chromatogram was monitored by scanning from 240 to 770 nm and the signal intensities detected at 450 nm and 660 nm were used for carotenoid and chlorophyll quantitation. Identification was carried out by comparing retention times and spectral data with those of the authentic standards (α- and β-carotene, chlorophyll *a* and *b*) or in case of unavailability of standards by comparing the absorption spectra reported in the literature [36,37]. Quantifications were made by the external standard calculation, using calibration curves of the standards β-carotene, α-carotene, chlorophyll *a* and chlorophyll *b*. The quantification of individual carotenoid compounds (neoxantine, violaxantine, lutein and its derivatives, derivative of zeaxantine and lycopene) was calculated as β-carotene equivalents and derivatives of chlorophylls as chlorophyll *a* and *b* equivalents using the equation based on the calibration curves, respectively. The concentrations of analyzed compounds were expressed as mg 100 g^{-1} of dry matter, as mean values ± SD (N = 4).

2.5. Antioxidant Capacity

The antioxidant capacity of the extracts was assessed by the oxygen radical absorbance capacity (ORAC) assay according to the study of Prior et al. [38] and Bender et al. [39] with minor modifications. The ORAC procedure used an automated plate reader (BMG LABTECH, Offenburg, Germany) with 96-well plates and data were analyzed by MARS 2.0 software. The 2,2′-Azobis radical (2-amidinopropane) dihydrochloride (AAPH), fluorescein solution, different dilutions of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and samples were prepared in 75 μM phosphate buffer (pH 7.4). Briefly, 25 mL of appropriate diluted samples were added in a 96-well black plate containing 150 μL of fluorescein solution (70.3 nM). The plate was incubated for 30 min at 37 °C and after the first three cycles (representing the baseline signal), AAPH (240 mM) was injected into each well to initiate the peroxyl radical generation. On each plate, different dilutions of Trolox (3.37–107.88 μM) were used as a reference standard. Fluorescence intensity (excitation at 485 nm and emission at 528 nm) was monitored every 90 s over a total measurement period of 120 min. The measurements were performed in duplicates and results are expressed as mmol of Trolox equivalents (TE) 100 g^{-1} of dry matter, as mean values ± SD (N = 4).

2.6. Statistical Analysis

Statistical analysis was carried out using Statistica ver. 10.0 software (Statsoft Inc., Tulsa, OK, USA). Experimental part was designed as full factorial randomized design and descriptive statistic was assessed for the basic evaluation of the data. Multivariate analysis of variance (MANOVA) was used for the analysis of continuous variables (polyphenols, pigments and antioxidant capacity) and marginal mean values were compared with Tukey's HSD test. Obtained results are expressed as mean ± SE. Relationships between antioxidant capacity and determined compounds were tested by calculation of Pearson's correlation coefficient. All tests were carried out at the significance level $p \leq 0.05$.

3. Results and Discussion

3.1. Influence of Accelerated Solvent Extraction on Polyphenols Recovery

ASE of NL was performed using 96% ethanol as solvent. The reason for choosing this solvent is that ethanol has a GRAS status ("Generally Recognized as Safe"), so it meets one of the criteria of green chemistry, i.e., green extraction [40] that coupled with ASE might be an efficient green tool for bioactives recovery. Therefore, in order to achieve the maximum efficiency of the extraction process and to provide a high extraction yield of target compounds, the extraction operating parameters need to be

optimized [41]. Therefore, present study involved the optimization of the ASE operating conditions in terms of temperature (20, 50, 80 and 110 °C), static time (5 and 10 min) and number of extraction cycles (1–4) for NL polyphenols and pigments extraction. In obtained extracts various polyphenols have been identified and quantified by HPLC analysis: chlorogenic acid (ChA), *p*-coumaric acid (*p*-CA), ferulic acid (FA) and chicoric acid (CA) belonging to the group of hydroxycinnamic acids (HCA) and flavonoids (F) quercetin-3-glucoside (Q-3-G), kaempferol-3-rutinoside (K-3-R) and luteolin (LTL) (Table 1). As it can be observed, the most abundant polyphenol was ChA with the highest content of 278.14 mg 100 g^{-1} dm at 110 °C/10 min/2 cycles. CA was the least represented compound, detected only at 110 °C/10 min/2–4 cycles. Similar polyphenols profile in NL was found by other authors [2,9,10,34,42,43]. Carvalho et al. [10] studied the polyphenols composition in leaves of three nettle species (*Urtica dioica* L., *Urtica membranacea* Poir and *Urtica urens* L.), where *Urtica dioica* L. had the highest concentration of polyphenols and HCA were the most dominant, especially derivatives of caffeic and *p*-CA. In accordance, Vajić et al. [42] identified two groups of polyphenols in NL, HCA and F, among which the most dominant were 2-*O*-caffeoyl malic acid, ChA and rutin. Orčić et al. [9] reported that neochlorogenic acid was the most abundant compound in overground parts of nettle, consisting up to 3.6% of the extract by weight. The following most represented components were quercetin-3-*O*-rhamnosylglucoside and Q-3-G. Slight differences in composition of polyphenols could be due to the type and various extraction conditions, as well as the pre-harvest and post-harvest conditions [1,2,44,45]. In study of Pinelli et al. [34] ChA and 2-*O*-caffeoylmalic acids represented 71.5 and 76.5 % of total polyphenols (TP) in cultivated and wild NL, respectively. However, CA was not reported in previous research, while in our study it was identified only at the highest applied conditions (110 °C/10 min/2–4 cycles), pointing ASE as very efficient for the isolation of bioactive polyphenolic compounds.

The influence of ASE parameters (temperature, static time and cycle number) on yield of NL polyphenols are presented in Table 2. The sum of TP includes determined total HCA (THCA) and total F (TF). As it can be seen, the effect of temperature, static time and cycle number had a significant influence ($p < 0.01$) on all polyphenols groups as well as on TP, except THCA were not significantly affected by the cycle number ($p = 0.19$). The increase of temperature from 20 to 110 °C resulted with significantly higher THCA, ranging from 16.87 to 255.51 mg 100 g^{-1} dm, TF (from 3.67 to 80.16 mg 100 g^{-1} dm) and TP (from 20.54 to 335.67 mg 100 g^{-1} dm). These results are in accordance with the findings of Zgórka (2009) [46] who reported that the concentration of isoflavone from clover increased with temperature increase (75–125 °C) during ASE and there was no degradation of the analyzed compounds. Increase of the temperature during ASE increases the solubility of the compounds, diffusion rate and mass transfer, enhances the penetration of the solvents into the matrix and thus affects the extraction efficiency [47]. However, Erdogan and Erdemoglu [48] reported that optimum temperature for ASE of polyphenols from apricots was 60 °C, above which the amount of polyphenols decreased due to the possible degradation, which accents the need for the adjustment of proper temperature depending on the used matrix. Considering static time, all polyphenol groups showed the highest content at static time of 10 min (THCA = 164.31 mg 100 g^{-1} dm, TF = 50.47 mg 100 g^{-1} dm, TP = 214.78 mg 100 g^{-1} dm) (Table 2). It is evident that longer static time (10 vs. 5 min) promoted almost double yields of all compound groups. Regarding the cycle number, results revealed that the highest yields of polyphenols were achieved during the third cycle, with an increase of 42–58% when compared to the initial cycle number (1). The same trend was observed by Gomes et al. [49] where the maximum of studied ASE conditions (80 °C/10 min/5 cycles) resulted in highest polyphenols yields. Moreover, Mottaleb and Sarker [21] confirmed the significance of the combined effects of static time and number of cycles in the recovery of natural products.

Table 1. Nettle leaves polyphenols (mg 100 g^{-1} dm) and ORAC values (mmol TE 100 g^{-1} dm) in extracts obtained at ASE different conditions.

Temperature (°C)	Static Time (min)	Cycle Number	ChA	*p*-CA	FA	CA	Q-3-G	K-3-R	LTL	ORAC
20	5	1	6.69 ± 0.05	1.33 ± 0.02	nd	nd	nd	nd	nd	2.66 ± 0.01
		2	8.63 ± 0.31	1.73 ± 0.04	nd	nd	nd	nd	nd	3.08 ± 0.03
		3	9.03 ± 0.34	1.51 ± 0.20	nd	nd	nd	nd	nd	2.42 ± 0.04
		4	10.53 ± 0.17	1.76 ± 0.08	nd	nd	nd	nd	nd	2.96 ± 0.14
	10	1	10.14 ± 6.63	1.75 ± 1.10	nd	nd	5.41 ± 0.04	nd	nd	5.37 ± 0.07
		2	19.74 ± 0.80	2.82 ± 0.57	nd	nd	7.32 ± 0.52	nd	nd	7.54 ± 0.06
		3	24.09 ± 1.01	3.61 ± 0.33	nd	nd	8.27 ± 0.67	nd	nd	10.22 ± 0.02
		4	27.65 ± 1.00	3.95 ± 0.12	nd	nd	8.32 ± 0.42	nd	nd	9.91 ± 0.05
50	5	1	10.08 ± 0.25	1.82 ± 0.23	nd	nd	6.57 ± 0.27	nd	nd	4.61 ± 0.02
		2	13.00 ± 0.24	1.63 ± 0.22	nd	nd	3.84 ± 0.16	nd	nd	4.77 ± 0.04
		3	13.70 ± 0.72	1.55 ± 0.32	nd	nd	4.69 ± 0.40	nd	nd	3.53 ± 0.04
		4	15.56 ± 0.53	1.55 ± 0.37	nd	nd	4.80 ± 0.20	nd	nd	3.43 ± 0.06
	10	1	23.36 ± 0.94	4.01 ± 0.73	nd	nd	5.63 ± 0.55	nd	nd	7.50 ± 0.04
		2	31.13 ± 1.47	4.89 ± 0.79	nd	nd	7.17 ± 0.98	nd	nd	10.08 ± 0.01
		3	37.35 ± 1.70	6.14 ± 1.01	nd	nd	8.67 ± 1.16	nd	nd	10.74 ± 0.06
		4	51.01 ± 0.54	7.14 ± 0.87	nd	nd	11.71 ± 1.93	2.62 ± 3.70	nd	12.53 ± 0.05
80	5	1	74.73 ± 2.56	45.79 ± 1.78	nd	nd	29.48 ± 4.99	10.13 ± 1.05	1.61 ± 0.36	14.13 ± 0.03
		2	72.28 ± 1.76	26.68 ± 10.49	nd	nd	39.24 ± 10.96	10.66 ± 1.11	2.06 ± 0.72	13.43 ± 0.02
		3	150.77 ± 41.24	30.19 ± 7.08	1.81 ± 0.00	nd	30.93 ± 9.27	9.14 ± 0.80	1.28 ± 0.39	13.24 ± 0.07
		4	109.74 ± 31.04	17.70 ± 2.30	1.55 ± 0.03	nd	34.49 ± 10.31	9.57 ± 0.85	1.37 ± 0.34	13.67 ± 0.03
	10	1	154.35 ± 19.17	54.71 ± 20.50	2.07 ± 0.05	nd	50.73 ± 11.06	13.02 ± 0.21	1.86 ± 0.21	14.26 ± 0.06
		2	188.32 ± 6.66	104.22 ± 3.84	2.60 ± 0.09	nd	73.60 ± 10.53	17.88 ± 1.05	2.75 ± 0.21	21.11 ± 0.03
		3	231.86 ± 37.64	68.26 ± 16.06	2.49 ± 0.04	nd	68.51 ± 8.61	17.12 ± 0.70	2.45 ± 0.22	22.07 ± 0.03
		4	173.65 ± 7.56	47.85 ± 8.91	3.18 ± 0.02	nd	64.14 ± 9.80	15.82 ± 0.87	2.21 ± 0.18	21.71 ± 0.02
110	5	1	83.78 ± 13.87	29.49 ± 6.89	1.31 ± 0.10	nd	31.04 ± 3.17	9.81 ± 0.40	0.89 ± 0.05	14.51 ± 0.05
		2	134.55 ± 23.01	39.40 ± 9.57	1.89 ± 0.06	nd	40.48 ± 10.86	12.76 ± 0.27	1.31 ± 0.06	14.52 ± 0.02
		3	163.61 ± 23.95	52.84 ± 3.21	2.41 ± 0.05	nd	48.66 ± 7.59	14.86 ± 0.19	1.14 ± 0.05	14.05 ± 0.02
		4	143.13 ± 6.73	52.59 ± 1.18	2.35 ± 0.10	nd	51.42 ± 8.06	14.85 ± 0.50	1.84 ± 0.70	14.52 ± 0.02
	10	1	174.46 ± 2.09	57.49 ± 22.23	1.94 ± 0.00	nd	55.63 ± 13.15	16.81 ± 0.75	1.14 ± 0.24	14.34 ± 0.03
		2	278.14 ± 55.96	76.83 ± 19.23	4.26 ± 0.05	4.34 ± 0.03	71.22 ± 16.81	21.87 ± 0.81	3.20 ± 2.30	16.71 ± 0.04
		3	251.58 ± 38.79	104.56 ± 16.76	5.91 ± 0.00	6.07 ± 0.04	94.67 ± 0.87	24.22 ± 0.98	6.76 ± 4.89	17.78 ± 0.04
		4	248.49 ± 38.24	105.96 ± 3.42	8.22 ± 0.03	8.47 ± 0.08	87.14 ± 4.84	23.34 ± 0.90	6.25 ± 4.63	17.50 ± 0.05

ASE = accelerated solvent extraction, ChA = chlorogenic acid, *p*-CA = *p*-coumaric acid, FA = ferulic acid, CA = chicoric acid, Q-3-G = quercetin-3-glucoside, K-3-R = kaempferol-3-rutinoside, LTL = luteolin. nd = not detected. Results are expressed as mean ± SD.

Table 2. The influence of ASE conditions on yield of nettle leaves polyphenols (mg 100 g^{-1} dm), pigments (mg 100 g^{-1} dm) and ORAC values (mmol TE 100 g^{-1} dm).

Source of Variation	THCA	TF	TP	TCH	TCAR	TPG	ORAC
Temperature (°C)	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *
20	16.87 ± 3.72a	3.67 ± 1.70a	20.54 ± 4.23a	464.37 ± 2.25a	31.48 ± 0.01a	495.85 ± 2.25a	5.52 ± 0.01a
50	27.99 ± 3.72a	6.96 ± 1.70a	34.95 ± 4.23a	678.60 ± 2.25b	44.07 ± 0.01b	722.67 ± 2.25b	7.15 ± 0.01b
80	195.60 ± 3.72b	63.76 ± 1.70b	259.35 ± 4.23b	1070.63 ± 2.25c	64.74 ± 0.01c	1135.38 ± 2.25c	16.70 ± 0.01d
110	255.51 ± 3.72c	80.16 ± 1.70c	335.67 ± 4.23c	1075.25 ± 2.25c	65.81 ± 0.01d	1141.06 ± 2.25c	15.49 ± 0.01c
Static time (min)	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *
5	83.67 ± 2.63a	26.81 ± 1.21a	110.48 ± 2.99a	709.20 ± 1.59a	45.52 ± 0.00a	754.71 ± 1.59a	8.72 ± 0.01a
10	164.31 ± 2.63b	50.47 ± 1.21b	214.78 ± 2.99b	935.23 ± 1.59b	57.54 ± 0.00b	992.77 ± 1.59b	13.71 ± 0.01b
Cycle number	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *
1	92.41 ± 3.72a	29.97 ± 1.70a	122.38 ± 4.23a	746.06 ± 2.25a	47.31 ± 0.01a	793.37 ± 2.25a	9.67 ± 0.01a
2	127.13 ± 3.72b	39.42 ± 1.70b	166.55 ± 4.23b	829.68 ± 2.25b	52.60 ± 0.01c	882.28 ± 2.25b	11.41 ± 0.01b
3	146.17 ± 3.72c	42.67 ± 1.70b	188.84 ± 4.23c	838.54 ± 2.25c	51.94 ± 0.01b	890.48 ± 2.25b	11.76 ± 0.01c
4	130.25 ± 3.72b	42.49 ± 1.70b	172.74 ± 4.23b	874.57 ± 2.25d	54.26 ± 0.01d	928.82 ± 2.25c	12.03 ± 0.01d
Grand mean	123.99	38.64	162.63	822.21	51.53	873.74	11.22

ASE = accelerated solvent extraction, THCA = total hydroxycinnamic acids, TF = total flavonoids, TP = total phenols, TCH = total chlorophylls, TCAR = total carotenoids, TPG = total pigments. * Statistically significant variable at $p \leq 0.05$. Results are expressed as mean ± SE. Values with different letters within column are statistically different at $p \leq 0.05$.

The purpose of the extraction cycles is to introduce a fresh solvent during the extraction with the aim to maintain a favorable extraction balance. This could be useful for samples with a high concentration of analytes or for samples where it is difficult for solvent to penetrate in the pores of matrix. However, extraction cycles need to be adequately combined with static extraction time for the most efficient extraction [21].

Currently, UAE performed by using an ultrasound bath operating at a frequency between 37 and 45 kHz represents an easy and low-cost extraction process used to obtain high valuable compounds from natural products [50]. In line with this, ASE (80 °C/10 min/3 cycles) was compared with UAE under previously optimized conditions (80 °C/30 min). The polyphenols concentrations obtained at UAE optimal conditions are shown in Table 3. Comparing the yields of both techniques obtained at similar setups, ASE outputs are generally three-fold higher for most of the analyzed polyphenols. Zengin et al. [51] reported that ASE accomplished the highest yields of TP content (65.05 mg GAE g^{-1}) of *Tanacetum parthenium* extracts in comparison with other four different extraction techniques, among which UAE was also studied.

Table 3. Nettle leaves polyphenols (mg 100 g^{-1} dm), pigments (mg 100 g^{-1} dm) and ORAC values (mmol TE 100 g^{-1} dm) in UAE extracts obtained at optimal extraction conditions (80 °C/30 min).

Compounds		**Concentration**
Polyphenols	ChA	76.84 ± 3.32
	p-CA	53.23 ± 1.07
	Q-3-G	19.02 ± 2.77
	K-3-R	5.18 ± 0.45
Chlorophylls	CHL *b* der 1	8.42 ± 0.06
	CHL *a* der 1	36.65 ± 2.10
	CHL *a* der 2	38.62 ± 2.44
	CHL *b*	230.46 ± 15.17
	CHL *a*	589.04 ± 22.36
	CHL *a* der 5	5.07 ± 0.02
Carotenoids	VIOLAX der	1.17 ± 0.10
	NEOX	2.38 ± 0.15
	VIOLAX	1.10 ± 0.08
	13′-*cis*-LUT	2.38 ± 0.06
	LUT 5,6-ep	1.10 ± 0.09
	NEOX der	0.67 ± 0.02
	LUT	21.25 ± 1.04
	ZEAX der	1.02 ± 0.03
	9′-*cis*-LUT	1.78 ± 0.11
	α-CAR	6.12 ± 0.41
	β-CAR	14.14 ± 1.03
	LYC der 10	0.32 ± 0.01
Antioxidant capacity		
ORAC		13.26 ± 0.05

UAE = ultrasound assisted extraction, ChA = chlorogenic acid, *p*-CA = *p*-coumaric acid, Q-3-G = quercetin-3-glucoside, K-3-R = kaempferol-3-rutinoside, CHL *b* der 1 = chlorophyll *b* derivative 1, CHL *a* der 1 = chlorophyll *a* derivative 1, CHL *a* der 2 = chlorophyll *a* derivative 2, CHL *b* = chlorophyll *b*, CHL *a* = chlorophyll *a*, CHL *a* der 5 = chlorophyll *a* derivative 5, VIOLAX der = violaxanthin derivative, NEOX = neoxanthin, VIOLAX = violaxanthin, 13′-*cis*-LUT = 13′-*cis*-lutein, LUT 5,6-ep = lutein 5,6-epoxide, NEOX der = neoxanthin derivative, LUT = lutein, ZEAX der = zeaxanthin derivative, 9′-*cis*-LUT = 9′-*cis*-lutein, α-CAR = α-carotene, β-CAR = β-carotene, LYC der 10 = lycopene derivative 10. Results are expressed as mean ± SD.

In their study, UAE yielded 30% less of polyphenols in comparison with ASE, showing its lower efficiency, which is similarly to our results. ASE also showed the best performance for polyphenols extraction in other studies [47,51,52]. Summarizing the obtained results, ASE proved to be effective green technique for recovery of NL polyphenols, whereas conditions 110 °C/10 min/3 cycles showed the highest polyphenols yield. It was shown that temperature presents one of the most important factors among the examined conditions, where generally, application of higher temperature results in higher yields of polyphenols.

3.2. Influence of Accelerated Solvent Extraction on Chlorophylls and Carotenoids Recovery

Chlorophylls and carotenoids are pigments responsible for plant color [53], but they also possess antioxidant properties [54,55], therefore promoting health effects [56]. Along with phenols, NL extracts were also analyzed for the pigment content and the obtained results are given in Tables 4 and 5. HPLC analysis revealed eight chlorophylls including chlorophyll *a* (CHL *a*), chlorophyll *b* (CHL *b*) and their derivatives (Table 4). CHL *a* was the most abundant component ranging between 167.41 and 871.33 mg 100 g^{-1} dm, where the highest level was obtained at 80 °C/10 min/2 cycles. The following component was CHL *b* in a ratio of 1:3 when compared to the amount of CHL *a*, which had been previously confirmed in other studies [12,44]. The component with the lowest concentration was CHL *a* derivative 2 in the range of 1.06–3.70 mg 100 g^{-1} dm obtained only at 50 and 80 °C. Concentrations of other identified chlorophylls were much lower. Identified carotenoids were as follows: violaxanthin derivative (VIOLAX der), neoxanthin (NEOX), violaxanthin (VIOLAX), 13′-*cis*-lutein (13′-*cis*-LUT), lutein 5,6-epoxide (LUT 5,6-ep), neoxanthin derivative (NEOX der), lutein (LUT), zeaxanthin (ZEAX), 9′-*cis*-lutein (9′-cis-LUT), α-carotene (α-CAR), β-carotene (β-CAR) and lycopene derivative 10 (LYC der 10) (Table 5). The most dominant carotenoid was LUT ranging from 5.52 to 30.16 mg 100 g^{-1} dm with the highest yield achieved at 110 °C/10 min/2 cycles. β-CAR was the following carotenoid with a maximum value of 19.02 mg 100 g^{-1} dm, while the least present carotenoid was LYC der 10 (0.32–0.85 mg 100 g^{-1} dm) and it was detected only at higher temperatures (80 and 110 °C). With respect to carotenoids composition, our results are in agreement with the findings from the study of Guil-Guerrero et al. [11] where total of nine carotenoids were identified in NL extracts being LUT, β-CAR and their isomers 60% of total carotenoids (TCAR). However, carotenoids in our study were found in higher concentrations, probably due to the application of different extraction technique and conditions. When supercritical and liquid CO_2 extraction were used to characterize NL chlorophylls and carotenoids, it was revealed that chlorophylls content was lower (CHL *a* 73 mg 100 g^{-1} dm, CHL *b* 100 100 g^{-1} dm), while LUT and β-CAR contents were higher (LUT 39 mg 100 g^{-1} dm, β-CAR 24 mg 100 g^{-1} dm) in comparison with our results [57]. Moreover, it can be observed that the chlorophylls levels are much higher compared to the levels of carotenoids (Tables 4 and 5), which is in accordance with previous studies, where NL extracts had four-fold higher concentrations of chlorophylls in comparison with carotenoids [2,44].

Table 4. Nettle leaves chlorophylls (mg100 g^{-1} dm) in extracts obtained at ASE different conditions.

Temperature (°C)	Static Time (min)	Cycle Number	CHL *b* der 1	CHL *a* der 1	CHL *a* der 2	CHL *b*	CHL *a* der 3	CHL *a* der 4	CHL *a*	CHL *a* der 5
20	5	1	nd	nd	nd	50.13 ± 3.21	2.32 ± 0.14	nd	167.41 ± 10.10	nd
		2	nd	nd	nd	52.20 ± 1.96	2.80 ± 0.09	nd	201.34 ± 1.36	nd
		3	nd	nd	nd	64.08 ± 5.47	2.98 ± 0.25	1.81 ± 0.11	218.83 ± 19.03	nd
		4	nd	nd	nd	76.86 ± 4.49	4.06 ± 0.31	2.53 ± 0.14	266.62 ± 22.25	nd
	10	1	nd	nd	nd	149.34 ± 11.54	5.77 ± 0.11	6.27 ± 0.02	486.82 ± 30.11	9.71 ± 0.66
		2	nd	nd	nd	124.02 ± 9.65	4.23 ± 0.32	4.75 ± 0.33	420.08 ± 3.56	5.63 ± 0.41
		3	nd	nd	nd	144.32 ± 3.63	5.42 ± 0.25	5.31 ± 0.25	493.82 ± 14.79	7.12 ± 0.22
		4	nd	nd	nd	164.12 ± 12.09	6.08 ± 0.04	5.97 ± 0.19	546.09 ± 21.45	6.14 ± 0.31
50	5	1	nd	nd	nd	128.14 ± 2.55	5.28 ± 0.06	2.79 ± 0.08	410.82 ± 9.96	2.15 ± 0.06
		2	nd	nd	nd	118.76 ± 4.58	5.96 ± 0.14	3.81 ± 0.20	405.97 ± 37.05	1.84 ± 0.25
		3	nd	nd	nd	118.89 ± 8.85	5.29 ± 0.24	3.79 ± 0.27	399.27 ± 16.16	2.15 ± 1.04
		4	nd	nd	nd	127.24 ± 4.30	7.26 ± 0.57	5.12 ± 0.15	444.73 ± 7.86	1.86 ± 0.77
	10	1	nd	nd	nd	149.14 ± 6.98	6.13 ± 0.48	5.75 ± 0.43	480.65 ± 25.52	4.90 ± 0.13
		2	nd	2.07 ± 0.03	1.86 ± 0.07	186.43 ± 14.74	9.64 ± 0.08	6.35 ± 0.23	576.32 ± 5.93	6.71 ± 0.52
		3	nd	1.90 ± 0.16	2.34 ± 0.11	199.60 ± 8.55	10.89 ± 0.87	7.97 ± 0.50	618.59 ± 50.38	8.20 ± 0.74
		4	nd	2.61 ± 0.23	3.70 ± 0.18	220.30 ± 18.18	11.09 ± 0.66	6.55 ± 0.42	691.27 ± 7.45	6.74 ± 0.30
80	5	1	nd	4.60 ± 0.33	1.06 ± 0.05	223.73 ± 15.02	8.95 ± 0.73	3.06 ± 0.27	672.71 ± 49.12	8.13 ± 0.18
		2	nd	4.97 ± 0.12	3.59 ± 0.09	250.17 ± 21.37	8.70 ± 0.55	2.96 ± 0.02	722.10 ± 60.17	12.55 ± 1.09
		3	nd	4.52 ± 0.06	2.87 ± 0.14	253.59 ± 7.58	9.23 ± 0.32	2.84 ± 0.03	721.07 ± 58.77	21.77 ± 1.11
		4	nd	5.03 ± 0.11	nd	253.75 ± 8.64	11.06 ± 0.86	3.11 ± 0.18	736.72 ± 8.72	21.21 ± 2.04
	10	1	nd	5.03 ± 0.07	nd	253.75 ± 10.54	11.06 ± 0.74	3.11 ± 0.08	736.72 ± 3.66	21.21 ± 1.45
		2	nd	7.47 ± 0.37	nd	301.80 ± 26.44	10.89 ± 0.71	2.94 ± 0.22	871.33 ± 52.30	13.63 ± 0.99
		3	nd	7.60 ± 0.22	nd	280.77 ± 14.73	8.81 ± 0.42	3.20 ± 0.17	850.56 ± 30.47	15.20 ± 0.56
		4	11.91 ± 0.42	8.92 ± 0.44	nd	239.99 ± 20.19	13.05 ± 0.24	4.39 ± 0.36	860.70 ± 13.96	10.98 ± 0.87
110	5	1	nd	4.23 ± 0.06	nd	217.77 ± 5.66	7.33 ± 0.09	2.55 ± 0.14	660.54 ± 28.87	7.48 ± 0.63
		2	nd	4.82 ± 0.01	nd	259.70 ± 17.05	9.86 ± 0.05	3.40 ± 0.11	739.60 ± 15.47	11.11 ± 1.04
		3	nd	4.70 ± 0.10	nd	280.66 ± 23.56	11.64 ± 0.60	4.13 ± 0.35	714.51 ± 41.41	22.89 ± 1.52
		4	7.20 ± 0.15	4.36 ± 0.08	nd	271.95 ± 3.72	12.57 ± 1.03	4.43 ± 0.40	768.41 ± 1.22	22.19 ± 2.07
	10	1	7.45 ± 0.02	4.16 ± 0.28	nd	233.27 ± 5.27	10.29 ± 0.08	3.89 ± 0.23	756.26 ± 31.28	26.66 ± 1.95
		2	nd	2.42 ± 0.02	nd	308.74 ± 19.54	11.05 ± 1.07	4.89 ± 0.13	775.16 ± 26.55	152.85 ± 12.12
		3	nd	5.05 ± 0.09	nd	255.42 ± 7.46	8.36 ± 0.54	3.06 ± 0.21	821.35 ± 3.33	35.94 ± 3.02
		4	nd	4.68 ± 0.15	nd	271.74 ± 22.47	8.31 ± 0.11	2.97 ± 0.05	793.09 ± 54.06	36.89 ± 0.98

ASE = accelerated solvent extraction, CHL *b* der 1 = chlorophyll *b* derivative 1, CHL *a* der 1 = chlorophyll *a* derivative 1, CHL *a* der 2 = chlorophyll *a* derivative 2, CHL *b* = chlorophyll *b*, CHL *a* der 3 = chlorophyll *a* derivative 3, CHL *a* der 4 = chlorophyll *a* derivative 4, CHL *a* = chlorophyll *a*, CHL *a* der 5 = chlorophyll *a* derivative 5. nd = not detected. Results are expressed as mean ± SD.

Table 5. Nettle leaves carotenoids (mg 100 g^{-1} dm) in extracts obtained at ASE different conditions.

Temperature (°C)	Static Time (min)	Cycle Number	VIOLAX der	NEOX	VIOLAX	13′-*cis*-LUT	LUT 5,6-ep	NEOX der	LUT	ZEAX	9′-*cis*-LUT	α-CAR	β-CAR	LYC der 10
20	5	1	0.60 ± 0.02	0.70 ± 0.05	2.14 ± 0.14	nd	nd	0.30 ± 0.01	5.52 ± 0.21	nd	0.54 ± 0.03	1.45 ± 0.07	4.23 ± 0.22	nd
		2	0.63 ± 0.01	0.83 ± 0.01	2.48 ± 0.21	nd	0.33 ± 0.01	0.35 ± 0.00	6.39 ± 0.45	0.28 ± 0.00	0.57 ± 0.00	1.80 ± 0.11	5.11 ± 0.01	nd
		3	0.65 ± 0.02	0.90 ± 0.03	2.65 ± 0.23	0.21 ± 0.01	0.35 ± 0.01	0.35 ± 0.01	6.91 ± 0.44	0.30 ± 0.02	0.58 ± 0.01	2.01 ± 0.16	5.66 ± 0.51	nd
		4	0.81 ± 0.05	1.06 ± 0.05	3.11 ± 0.17	0.30 ± 0.01	0.41 ± 0.02	0.44 ± 0.03	8.28 ± 0.66	0.37 ± 0.01	0.74 ± 0.02	2.28 ± 0.03	6.75 ± 0.55	nd
	10	1	1.01 ± 0.07	1.90 ± 0.12	3.65 ± 0.30	1.55 ± 0.10	0.30 ± 0.00	0.52 ± 0.01	15.71 ± 1.02	0.54 ± 0.03	1.72 ± 0.01	3.71 ± 0.25	12.40 ± 1.02	nd
		2	0.84 ± 0.06	1.76 ± 0.12	3.45 ± 0.32	1.02 ± 0.03	0.10 ± 0.00	1.06 ± 0.01	13.16 ± 1.10	0.45 ± 0.03	1.52 ± 0.05	3.18 ± 0.24	10.82 ± 0.87	nd
		3	1.11 ± 0.10	1.92 ± 0.01	3.24 ± 0.26	1.54 ± 0.06	0.00 ± 0.00	0.66 ± 0.04	15.27 ± 0.96	0.53 ± 0.01	1.98 ± 0.07	3.61 ± 0.16	12.59 ± 0.99	nd
		4	1.16 ± 0.08	2.29 ± 0.17	4.81 ± 0.41	1.30 ± 0.09	0.13 ± 0.00	1.53 ± 0.09	17.51 ± 1.20	0.67 ± 0.04	1.12 ± 0.06	5.29 ± 0.41	13.87 ± 1.05	nd
50	5	1	1.09 ± 0.08	1.89 ± 0.10	4.40 ± 0.39	0.58 ± 0.01	0.62 ± 0.02	0.51 ± 0.01	12.71 ± 1.05	0.59 ± 0.01	0.91 ± 0.02	3.82 ± 0.12	9.22 ± 0.04	nd
		2	0.92 ± 0.03	2.01 ± 0.15	4.66 ± 0.44	0.45 ± 0.01	0.63 ± 0.03	0.42 ± 0.03	12.95 ± 0.96	0.19 ± 0.00	0.79 ± 0.03	3.87 ± 0.12	9.38 ± 0.23	nd
		3	0.95 ± 0.07	1.85 ± 0.12	4.18 ± 0.32	0.60 ± 0.03	0.60 ± 0.02	0.43 ± 0.02	12.49 ± 0.87	0.20 ± 0.01	0.80 ± 0.03	3.88 ± 0.05	9.38 ± 0.00	nd
		4	1.14 ± 0.05	2.17 ± 0.20	5.00 ± 0.47	0.60 ± 0.05	0.70 ± 0.05	0.52 ± 0.02	14.30 ± 0.56	0.65 ± 0.05	1.02 ± 0.08	4.34 ± 0.33	10.44 ± 0.21	nd
	10	1	1.05 ± 0.06	1.89 ± 0.13	3.29 ± 0.15	1.34 ± 0.11	0.65 ± 0.05	0.40 ± 0.01	14.76 ± 1.11	0.26 ± 0.01	1.03 ± 0.07	4.76 ± 0.45	11.83 ± 0.66	nd
		2	1.23 ± 0.01	2.20 ± 0.17	3.73 ± 0-29	1.67 ± 0.10	0.77 ± 0.04	0.46 ± 0.02	17.67 ± 0.85	0.84 ± 0.06	1.24 ± 0.09	5.74 ± 0.55	14.10 ± 0.36	nd
		3	1.36 ± 0.10	2.21 ± 0.07	3.76 ± 0.21	1.93 ± 0.15	0.89 ± 0.01	0.69 ± 0.04	18.49 ± 0.84	0.89 ± 0.07	1.59 ± 0.10	5.88 ± 0.21	14.56 ± 0.74	nd
		4	1.80 ± 0.13	2.67 ± 0.09	5.38 ± 0.11	1.87 ± 0.06	1.13 ± 0.09	0.92 ± 0.08	21.88 ± 1.57	1.05 ± 0.09	1.76 ± 0.09	6.30 ± 0.11	15.84 ± 0.77	nd
80	5	1	2.07 ± 0.14	2.39 ± 0.13	6.10 ± 0.50	1.48 ± 0.03	1.08 ± 0.08	1.20 ± 0.10	20.38 ± 1.66	0.72 ± 0.05	1.99 ± 0.11	5.13 ± 0.26	14.83 ± 0.91	nd
		2	1.99 ± 0.15	2.71 ± 0.22	5.82 ± 0.33	2.00 ± 0.17	1.13 ± 0.09	1.13 ± 0.08	21.93 ± 2.00	0.82 ± 0.04	3.99 ± 0.12	5.62 ± 0.09	15.54 ± 1.21	0.36 ± 0.01
		3	2.01 ± 0.16	2.78 ± 0.26	5.57 ± 0.45	1.93 ± 0.18	0.14 ± 0.01	1.03 ± 0.07	21.25 ± 1.52	0.29 ± 0.01	3.93 ± 0.06	5.42 ± 0.49	15.64 ± 1.20	0.34 ± 0.00
		4	1.70 ± 0.09	3.12 ± 0.24	5.11 ± 0.28	2.25 ± 0.08	1.08 ± 0.08	0.85 ± 0.07	22.29 ± 0.98	0.82 ± 0.05	3.82 ± 0.14	5.90 ± 0.27	15.35 ± 0.59	0.35 ± 0.01
	10	1	1.70 ± 0.09	3.12 ± 0.12	5.11 ± 0.24	2.25 ± 0.10	1.08 ± 0.07	0.85 ± 0.06	22.29 ± 0.58	0.82 ± 0.05	3.82 ± 0.20	5.90 ± 0.35	15.35 ± 0.85	0.35 ± 0.02
		2	2.19 ± 0.011	3.47 ± 0.21	6.67 ± 0.52	2.38 ± 0.07	1.32 ± 0.07	1.17 ± 0.11	25.40 ± 2.21	0.88 ± 0.06	4.50 ± 0.23	6.59 ± 0.24	17.55 ± 0.08	0.41 ± 0.01
		3	2.12 ± 0.18	3.28 ± 0.25	5.86 ± 0.23	2.49 ± 0.22	1.24 ± 0.10	1.10 ± 0.05	24.30 ± 0.33	0.87 ± 0.04	4.57 ± 0.17	6.37 ± 0.24	17.00 ± 1.05	0.44 ± 0.02
		4	2.45 ± 0.21	3.09 ± 0.11	6.03 ± 0.36	2.17 ± 0.14	1.26 ± 0.12	1.13 ± 0.09	24.40 ± 2.08	0.82 ± 0.03	4.48 ± 0.31	6.35 ± 0.52	16.56 ± 0.74	nd
110	5	1	1.69 ± 0.012	2.87 ± 0.06	5.91 ± 0.35	1.79 ± 0.17	1.00 ± 0.05	0.67 ± 0.02	20.89 ± 0.78	0.65 ± 0.03	2.90 ± 0.18	5.51 ± 0.31	14.72 ± 0.47	nd
		2	1.72 ± 0.14	3.50 ± 0.014	6.16 ± 0.41	2.18 ± 0.20	1.18 ± 0.09	0.82 ± 0.02	23.52 ± 0.32	0.92 ± 0.07	3.25 ± 0.22	6.21 ± 0.20	16.00 ± 1.25	0.32 ± 0.00
		3	1.77 ± 0.13	3.62 ± 0.22	5.94 ± 0.35	2.44 ± 0.19	0.98 ± 0.07	0.81 ± 0.05	24.20 ± 1.47	0.87 ± 0.06	3.57 ± 0.09	6.12 ± 0.54	16.41 ± 1.26	0.34 ± 0.01
		4	1.74 ± 0.14	3.43 ± 0.30	5.26 ± 0.47	2.50 ± 0.23	1.09 ± 0.06	0.74 ± 0.03	23.23 ± 0.88	0.76 ± 0.06	3.63 ± 0.16	5.77 ± 0.20	15.71 ± 0.93	0.36 ± 0.01
	10	1	1.93 ± 0.15	3.40 ± 0.24	4.68 ± 0.43	2.65 ± 0.24	1.08 ± 0.06	0.73 ± 0.04	22.98 ± 1.26	0.81 ± 0.02	3.70 ± 0.08	5.83 ± 0.17	15.53 ± 0.88	0.43 ± 0.03
		2	2.04 ± 0.11	4.33 ± 0.36	2.50 ± 0.21	4.35 ± 0.37	0.95 ± 0.07	0.59 ± 0.03	30.16 ± 2.11	1.07 ± 0.01	4.54 ± 0.25	7.03 ± 0.66	19.02 ± 1.52	0.85 ± 0.04
		3	1.97 ± 0.05	3.45 ± 0.15	4.71 ± 0.12	3.00 ± 0.28	1.13 ± 0.03	0.80 ± 0.07	24.18 ± 1.45	0.90 ± 0.07	3.72 ± 0.15	6.19 ± 0.62	16.29 ± 0.04	0.55 ± 0.03
		4	1.77 ± 0.07	3.17 ± 0.27	3.93 ± 0.33	2.72 ± 018	0.97 ± 0.02	0.71 ± 0.06	23.07 ± 0.63	0.74 ± 0.06	3.74 ± 0.10	5.59 ± 0.47	15.80 ± 0.14	0.58 ± 0.04

ASE = accelerated solvent extraction, VIOLAX der = violaxanthin derivative, NEOX = neoxanthin, VIOLAX = violaxanthin, 13′-*cis*-LUT = 13′-*cis*-lutein, LUT 5,6-ep = lutein 5,6-epoxide, NEOX der = neoxanthin derivative, LUT = lutein, ZEAX = zeaxanthin, 9′-*cis*-LUT = 9′-*cis*-lutein, α-CAR = α-carotene, β-CAR = β-carotene, LYC der 10 = lycopene derivative 10. nd = not detected. Results are expressed as mean ± SD.

Table 2 provides the results of ASE conditions' impact on the yield of NL pigments. The sum of total pigments (TPG) includes total chlorophylls (TCH) and TCAR. The results showed that the temperature, static time and number of cycles significantly affected ($p < 0.01$) content of all analyzed pigments. TCH and TCAR increased two-fold with an increase in temperature (TCH 464.37 vs. 1075.25 mg 100 g^{-1} dm, TCAR 31.48 vs. 65.81 mg 100 g^{-1} dm). Nevertheless, it can be observed that several chlorophylls and carotenoids achieved maximum yield at 80 °C, while at 110 °C a decrease in yield was recorded (Tables 4 and 5). Considering static time, all pigments showed higher content at static time of 10 min and as for cycle number, it can be observed that the highest yields of pigments were achieved at the maximum cycle number (Table 2). Finally, the highest TPG was obtained at 110 °C (1141.06 mg 100 g^{-1} dm), at the static time of 10 min (992.77 mg 100 g^{-1} dm) and at the fourth cycle of extraction (928.82 mg 100 g^{-1} dm) with temperature being dominant for influencing the extraction yield. Temperature increase affects the viscosity and solubility of the solvent, but degradation of the components can also occur if the applied temperature is too high [58]. Optimization of ASE parameters for carotenoids extraction (LUT and β-CAR) from carrot was also conducted by Saha et al. [58] by variation of temperature (40, 50 and 60 °C) and static time (5, 10 and 15 min). An increase in extraction yield for 4–8% was recorded by increasing the time for 5 min at 60 °C, but extraction efficiency was not observed when more than three cycles were carried out, giving 60 °C/15 min/3 cycles as optimal conditions. Furthermore, Cha et al. [59] investigated the effect of temperature (50, 105 and 160 °C) and static time (8, 19 and 30 min) on content of chlorophylls and carotenoids from algae *Chlorella vulgaris* and also concluded that temperature had the strongest influence on pigment extraction. They reported maximum yields of CHL *a* and *b* at the highest temperatures (150–160 °C), while β-CAR showed temperature sensitivity since its yield decreased at temperatures between 120 and 160 °C. Kim et al. [60] characterized twelve carotenoids from different varieties of paprika isolated using ASE at optimal conditions of 100 °C/5 min/3 cycles, and Mustafa et al. [56] reported 60 °C/2 min/5 cycles as the highest efficiency ASE conditions for carotenoids extraction from carrot by-products. They recorded a decrease of α- and β-CAR at temperatures above 120 °C, which is explainable by the thermo-sensitivity of carotenoids. They also proposed the use of several cycles, since some carotenoids are beginning to release during longer extraction time. Hojnik et al. [12] believe that for the extraction of chlorophylls it is necessary to conduct at least 2 extraction cycles in order to obtain a high yield, while Rafajlovska et al. [61] showed that multiple cycle extraction is better technique than one long cycle extraction. Although they applied different technique (supercritical CO_2 extraction), the levels of CHL *a* + *b* and β-CAR in NL significantly raised with the application of several steps of extraction. Further, chlorophylls dissolved better at higher pressure and temperature (210 bar, 50 °C) compared to the carotenoids (140 bar, 40 °C).

Comparing the pigment yields between ASE and UAE (Tables 3–5), it is evident that ASE yielded higher amounts of almost all pigments, especially the dominant ones. Research of Plaza et al. [62] also compared ASE and UAE for the extraction of chlorophylls and carotenoids from the algae *Chlorella*. It was concluded that ASE accomplished higher yields of pigments along with being a faster and more controlled technique. Moreover, Koo et al. [63] achieved a seven-fold higher amount of zeaxanthin from *Chlorella* in ASE extract compared to the extracts obtained with UAE. Based on the above results, ASE is a technique that executes higher yields of targeted compounds and protects sensitive compounds from light and oxygen under controlled conditions of temperature, pressure and extraction time [64].

3.3. Influence of Accelerated Solvent Extraction on Antioxidant Capacity

As already mentioned, it is well established that antioxidants prevent the oxidation of other substances and, in biological systems, they neutralize reactive free radicals, thus protect the body from various diseases. Since NL extracts already proved to be a very rich source of natural antioxidants, antioxidant capacity (AC) in obtained extracts was documented by the ORAC method (Table 1). ORAC values ranged from 2.42 to 22.07 mmol TE 100 g^{-1} dm. The highest value was determined in extract obtained at 80 °C/10 min/3 cycles, after which a decline was recorded. Similar ORAC levels

were recorded by Moldovan et al. [8]. High AC of nettle was also confirmed in Skapska et al. [65] and Tian et al. [66] research.

Considering ASE conditions, temperature, static time and cycle number had a significant influence ($p < 0.01$) on the AC. The most suitable combination of ASE parameters for achieving the extract with the highest ORAC value was 80 °C/10 min/4 cycles, as presented in Table 2. Regarding temperature, AC of the extracts was three-fold higher at 80 °C (16.70 mmol TE 100 g^{-1} dm) compared to the value at the initial temperature (5.52 mmol TE 100 g^{-1} dm) and afterwards it slightly decreased. This points that analyzed compounds of NL extracts were stable at 80 °C. This is in accordance with previously discussed results since the increase of AC derives from the abundant presence of bioactive molecules at higher temperatures as a consequence of the cell-wall disruption and increased mass transfer from the sample to the pressurized solvent. Accordingly, Howard et al. [67] reported an increase of spinach extracts AC combined with elevated temperature as well as Benchikh and Louailèche [68] in study of carob pulp polyphenols.

Calculated correlation coefficients showed a very strong correlation (r = 0.86–0.94) between the analyzed compounds and ORAC levels (Table 6), showing that NL THCA and TF as well as pigments significantly contribute to its antioxidant potential. Previous research showed that phenolic acids and flavonoids are significant antioxidants [69–71] as well as chlorophylls [54] and carotenoids [72–75].

Table 6. Pearson's correlations between nettle leaves polyphenols (mg 100 g^{-1} dm), pigments (mg 100 g^{-1} dm) and ORAC values (mmol TE 100 g^{-1} dm) in ASE extracts.

Parameter	ORAC
THCA	0.86 *
TF	0.87 *
TP	0.87 *
TCH	0.94 *
TCAR	0.92 *
TPG	0.92 *

ASE = accelerated solvent extraction, THCA = total hydroxycinnamic acids, TF = total flavonoids, TP = total phenols, TCH = total chlorophylls, TCAR = total carotenoids, TPG = total pigments. * $p \leq 0.05$.

When comparing AC between ASE and UAE extracts, the higher ORAC values were observed in ASE comparted to UAE extracts (22.07 vs. 13.26 mmol TE 100 g^{-1} dm) (Tables 1 and 3), confirming that ASE is more efficient for achieving high valuable extracts. Similarly, Hossain et al. [47] reported 77.52% higher AC in rosemary ASE extract in comparison with conventional extract, as a result of more efficient ASE of antioxidant phenolic compounds. Other authors also reported similar findings [51].

From all of the above, NL certainly represents a great source of various antioxidants (hydrophilic and lipophilic), where generally all analyzed compounds strongly contribute to AC. Moreover, ASE provides higher extraction yields of bioactive antioxidants in comparison with UAE; thus, it could be considered as an efficient green tool for the production of highly valuable nettle extracts for further industrial use.

4. Conclusions

Due to the increased interest in the industry for application of natural extracts in functional food production, the obtained results clearly demonstrate that ASE nettle extracts could be considered as green extracts for potential further use. Wild NL extracts were shown as a valuable natural source of structurally diverse bioactive compounds, polyphenols and pigments. Moreover, high efficiency for obtaining valuable NL extracts has been successfully obtained by ASE at optimized conditions of 110 °C, 10 min of static time and three or four cycles. Among the bioactive components that contribute to the biological value of NL, seven polyphenols belonging to the groups of hydroxycinnamic acids and flavonoids, chlorophylls *a* and *b* along with six of their derivatives and twelve carotenoids were present. Quantitatively, ChA was the most abundant polyphenol and CHL *a* represented the dominant pigment,

followed by CHL *b*, LUT and β-CAR. Furthermore, ASE showed better performance in comparison with UAE, obtaining higher yields of antioxidant compounds and 60% higher antioxidant capacity. Results of this study are fundamental for future research involving spray-drying of NL extracts and further implementation of the obtained NL powder into various food products.

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Chapter 3

Publication No.2: Bioactive Compounds in Wild Nettle (*Urtica dioica* L.) Leaves and Stalks: Polyphenols and Pigments upon Seasonal and Habitat Variations

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Article

Bioactive Compounds in Wild Nettle (*Urtica dioica* L.) Leaves and Stalks: Polyphenols and Pigments upon Seasonal and Habitat Variations

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Abstract: This study evaluated the presence of bioactives in wild nettle leaves and stalks during the phenological stage and in the context of natural habitat diversity. Thus, wild nettle samples collected before flowering, during flowering and after flowering from 14 habitats situated in three different regions (continental, mountain and seaside) were analyzed for low molecular weight polyphenols, carotenoids and chlorophylls using UPLC-MS/MS and HPLC analysis, while the ORAC method was performed for the antioxidant capacity measurement. Statistical analysis showed that, when compared to the stalks, nettle leaves contained significantly higher amounts of analyzed compounds which accumulated in the highest yields before flowering (polyphenols) and at the flowering stage (pigments). Moreover, nettle habitat variations greatly influenced the amounts of analyzed bioactives, where samples from the continental area contained higher levels of polyphenols, while seaside region samples were more abundant with pigments. The levels of ORAC followed the same pattern, being higher in leaves samples collected before and during flowering from the continental habitats. Hence, in order to provide the product's maximum value for consumers' benefit, a multidisciplinary approach is important for the selection of a plant part as well as its phenological stage with the highest accumulation of bioactive compounds.

Keywords: nettle leaves and stalks; phenological stage; location; accelerated solvent extraction; UPLC-MS/MS; polyphenols; chlorophylls; carotenoids; antioxidant capacity; ORAC

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1. Introduction

Nettle (*Urtica dioica* L.) is a perennial wild plant of the Urticaceae family, genus Urtica, which is widespread in Europe, Asia, America and part of Africa, and has been adapted to different climatic conditions [1,2]. Nettle has long been used in the food, cosmetic and pharmaceutical industries due to its nutritional and health potential, as all parts of nettle (leaves, stalks and roots) show a rich composition of bioactive compounds with high antioxidant capacity [2,3] Previous studies have shown that nettle leaves and stalks are a rich source of vitamins A, B and C, minerals (iron, potassium, calcium, magnesium), polyphenols such as phenolic acids and flavonoids as well as pigments, especially chlorophyll and carotenoids [4–11]. In accordance with the above, aerial parts of nettle have anti-inflammatory and therapeutic effects; these nettle parts are used in the treatment of arthritis, anemia, allergies, joint pain and urinary tract infections, have a diuretic effect and are used to strengthen hair [3,12]. Besides aerial parts, nettle root also presents a rich source of various compounds such as protein lectin, sterols, polysaccharides, lignans and phenols [5,7,13,14] and is mostly used in the treatment of benign prostatic hyperplasia [15]. Apart from medicinal use, other applications of nettle include food preparation, where it

is consumed in the form of tea, soup, stew or salad [3], or for commercial extraction of chlorophyll, which is used as a green coloring agent (E140) [16].

For medicinal purpose and medicinal preparations, nettle is mostly often used in the form of liquid or dry extract; thus, it is important to apply extraction method that will give a highly stabile extract with the greatest possible content of bioactive ingredients. Therefore, new extraction methods are increasingly being used and one of them is accelerated solvent extraction (ASE). In addition to being an efficient method, it uses less solvent, shortens the extraction time and more effectively isolates the target components [17].

Aside from extraction method, extract quality and richness in bioactives also depends on used plant material, either wild or cultivated, where its chemical composition and consequently antioxidant capacity are influenced by environmental, genotypic and phenotypic factors.

Different parts of plant may contain different amounts of particular compounds, e.g., nettle leaves accumulate higher amounts of polyphenols and chlorophylls in comparison with stalks [6,7,18]. In general, leaves are the richest part of a nettle in bioactive compounds, therefore they are mostly used in processing. However, changes in chemical composition and compounds' distribution occur with plant's maturity, where bioactive compounds are present in different proportions during different phenological stages. For example, the content of polyphenols decreases with growth and maturity of the plant [19]. Bioactive compounds are produced in response to different forms of (a)biotic stresses, as well as to fulfil important physiological tasks (attracting pollinators, establishing symbiosis, providing structural components to lignified cell walls of vascular tissues, etc.) [20]. These processes are often connected to specific phenological stages. Hence, harvest time depends on the type of final product. Although opinion on nettle optimal harvest time differs among various authors [3], Moore (1993) [21] stated that for juices and other fresh preparations, nettle leaves are best picked in spring or early summer (before flowering), and according to Upton (2013) [3] for dried preparations, it is best to harvest from mid-spring to late summer. If nettle is used for food purposes, the recommended harvest should be at the pre-flowering and flowering phases, certainly before the appearance of the seeds when it contains the least bioactive ingredients [3].

Nevertheless, nettle herb is mostly wild-harvested [3]. Concerning the natural habitat and climate, nettle is a quite adaptable plant. It grows in areas characterized by mild to temperate climates and prefers open or partly shady habitats with plenty of moisture such as forests, by rivers or streams and on roadsides [2]. Still, accumulation of polyphenols and pigments varies upon climate and habitat diversity. Plants grown in cold climates often show greater antioxidant properties, as a result of oxidative stress defense [22], while pigments synthesis is enhanced due to exposure to higher temperatures and more sunlight [23,24].

Although mentioned scientific literature provides data regarding nettle chemical composition, to our best knowledge there are no comprehensive studies on polyphenols and pigments constituents and their accumulation in wild nettle leaves and stalks during different vegetation periods of growing across diverse regions. These cognitions could be beneficial input data in a production of liquid and dry extracts. Therefore, the current study aimed to examine the presence and profile of low molecular weight polyphenols, carotenoids and chlorophylls as well as to determine antioxidant capacity in wild nettle leaves and stalks collected during three phenological stages (before flowering, during flowering and after flowering) from 14 different natural habitats situated in three regions in Croatia.

2. Materials and Methods

2.1. Chemicals

HPLC grade acetonitrile was procured from J.T. Baker Chemicals (Deventer, Netherlands). Purified water was obtained by a Milli-Q water purification system (Millipore, Bedford, MA, USA). Ethanol (96%) was purchased from Gram–mol d.o.o. (Zagreb, Croa-

tia) and formic acid (98–100%) from T.T.T. d.o.o. (Sveta Nedjelja, Croatia). Commercial standards of quercetin-3-glucoside, kaempferol-3-rutinoside, myricetin, caffeic acid, gallic acid, ferulic acid, sinapic acid, quinic acid, chlorogenic acid, *p*-coumaric acid, esculetin, scopoletin, α-carotene, β-carotene, chlorophyll *a* and chlorophyll *b* were purchased from Sigma–Aldrich (St. Louis, MO, USA). Epicatechin, catechin, epigallocatechin gallate, epicatechin gallate, apigenin, luteolin and naringenin were obtained from Extrasynthese (Genay, France), while quercetin-3-rutinoside was procured from Acros Organics (Thermo Fisher Scientific, Geel, Belgium). Apigenin was dissolved in ethanol with 0.5% (*v/v*) dimethyl sulfoxide, standards of carotenoids and chlorophylls in *n*-hexane. Other standards were prepared as a stock solution in methanol, and working standard solutions were prepared by diluting the stock solutions to yield five concentrations.

2.2. Plant Material

Samples of wild nettle (*Urtica dioica* L.) were collected at three phenological stages [(I) before flowering, (II) during flowering and (III) after flowering] during 2019 from different habitats in Croatia belonging to three regions (continental, mountain and seaside) (Table 1). Plant material was identified by using usual keys and iconographies with support of Department of Vegetable Crops, Faculty of Agriculture, University of Zagreb (Croatia). Immediately after harvesting, leaves were separated from stalks and samples were stored at −18 °C, freeze-dried (Alpha 1-4 LSCPlus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and afterwards grinded into fine powder using a commercial grinder (GT11, Tefal, Rumilly, France). Obtained powders were immediately analyzed for total solids by drying at 103 ± 2 °C to constant mass [25] and further used for the extraction. Content of dry matter in samples was >95%.

Table 1. Location and weather characteristics of wild nettle (*Urtica dioica* L.) habitats.

Region	Location	Altitude/ Latitude/Longitude	Weather Parameters	Phenological Stage					
				I Before Flowering		II During Flowering		III After Flowering	
				April	May	June	July	September	October
Continental	Sela Žakanjska	244 m 45°36′27.80″N 15°20′38.21″E	a.d. T (°C)	11.0	13.4	22.6	22.0	16.3	12.7
			T min (°C)	−0.1	0.4	11.4	9.2	2.9	0.7
			T max (°C)	27.7	26.5	34.7	35.6	30.5	26.7
			a.p. (mm)	143.4	170.1	73.8	85.4	101.8	55.6
	Sopčić Vrh	177 m 45°34′14.88″N 15°20′24.98″E	a.d. T (°C)	11.0	13.4	22.6	22.0	16.3	12.7
			T min (°C)	−0.1	0.4	11.4	9.2	2.9	0.7
			T max (°C)	27.7	26.5	34.7	35.6	30.5	26.7
			a.p. (mm)	143.4	170.1	73.8	85.4	101.8	55.6
	Žakanje	178 m 45°36′34.38″N 15°20′14.96″E	a.d. T (°C)	11.0	13.4	22.6	22.0	16.3	12.7
			T min (°C)	−0.1	0.4	11.4	9.2	2.9	0.7
			T max (°C)	27.7	26.5	34.7	35.6	30.5	26.7
			a.p. (mm)	143.4	170.1	73.8	85.4	101.8	55.6
	Zagreb I (Gračani)	119 m 45°51′31.10″N 15°58′19.34″E	a.d. T (°C)	12.4	13.7	23.8	22.9	17.2	13.2
			T min (°C)	1.9	2.1	13.3	10.4	4.6	2.2
			T max (°C)	27.1	26.1	34.6	35.9	33.1	25.9
			a.p. (mm)	81.1	147.7	70.8	76.8	150.1	42.3
	Zagreb II (Vrapče)	119 m 45°49′8.69″N 15°52′49.84″E	a.d. T (°C)	13.6	14.3	24.8	24.1	18.4	14.8
			T min (°C)	5.4	5.6	15.1	14.0	8.5	5.2
			T max (°C)	22.9	27.1	27.3	35.5	32.7	24.6
			a.p. (mm)	85.2	123.1	83.9	65.8	131.6	39.5
	Koretići	410 m 45°48′47.23″N 15°33′36.18″E	a.d. T (°C)	9.0	9.9	20.4	19.6	14.5	13.3
			T min (°C)	−3.0	−1.8	9.8	7.2	1.5	3.5
			T max (°C)	22.4	21.5	31.2	30.0	28.2	22.8
			a.p. (mm)	135.7	283.7	81.4	184.8	120.2	59.5

Table 1. *Cont.*

Region	Location	Altitude/ Latitude/Longitude	Weather Parameters	Phenological Stage					
				I Before Flowering		II During Flowering		III After Flowering	
				April	May	June	July	September	October
Mountain	Ogulin	320 m 45°15′47.84″N 15°13′42.36″E	a.d. T (°C)	10.8	12.4	21.6	21.4	15.6	13.0
			T min (°C)	0.5	0.5	11.8	8.3	2.8	3.0
			T max (°C)	25.5	25.1	33.4	33.0	29.3	25.9
			a.p. (mm)	167.4	319.2	139.5	109.4	143.6	64.2
	Čovići I	456 m 44°49′44.07″N 15°17′57.29″E	a.d. T (°C)	9.4	11.1	20.1	19.7	14.0	10.6
			T min (°C)	−2.1	−1.3	7.6	5.5	−2.0	1.2
			T max (°C)	24.8	25.0	34.1	34.5	29.6	25.1
			a.p. (mm)	138.6	189.3	25.1	106.2	106.9	31.8
	Čovići II	456 m 44°49′50.05″N 15°17′57.18″E	a.d. T (°C)	9.4	11.1	20.1	19.7	14.0	10.6
			T min (°C)	−2.1	−1.3	7.6	5.5	−2.0	1.2
			T max (°C)	24.8	25.0	34.1	34.5	29.6	25.1
			a.p. (mm)	138.6	189.3	25.1	106.2	106.9	31.8
Seaside	Poreč	0.34 m 45°13′37.03″N 13°35′39.64″E	a.d. T (°C)	13.0	14.5	24.3	24.9	19.4	15.7
			T min (°C)	3.9	6.0	13.2	13.4	7.3	6.3
			T max (°C)	23.5	22.7	33.6	33.6	30.9	25.7
			a.p. (mm)	116.1	210.0	7.3	58.7	143.2	38.6
	Limski zaljev	17 m 45°7′56.45″N 13°39′13.78″E	a.d. T (°C)	13.0	14.5	24.3	24.9	19.4	15.7
			T min (°C)	3.9	6.0	13.2	13.4	7.3	6.3
			T max (°C)	23.5	22.7	33.6	33.6	30.9	25.7
			a.p. (mm)	116.1	210.0	7.3	58.7	143.2	38.6
	Bale	129 m 45°2′25.93″N 13°47′8.88″E	a.d. T (°C)	13.4	14.4	23.9	24.5	19.8	15.5
			T min (°C)	4.9	4.5	13.8	13.6	7.5	5.8
			T max (°C)	23.7	24.5	34.0	34.3	33.0	25.5
			a.p. (mm)	129.5	264.7	37.4	71.5	91.1	42.0
	Vodnjan	141 m 44°57′28.79″N 13°51′6.10″E	a.d. T (°C)	13.4	14.4	23.9	24.5	19.8	15.5
			T min (°C)	4.9	4.5	13.8	13.6	7.5	5.8
			T max (°C)	23.7	24.5	34.0	34.3	33.0	25.5
			a.p. (mm)	129.5	264.7	37.4	71.5	91.1	42.0
	Muntrilj	342 m 45°14′30.84″N 13°48′38.44″E	a.d. T (°C)	11.1	12.5	22.2	22.3	16.4	13.1
			T min (°C)	0.5	1.1	11.2	9.5	2.3	2.5
			T max (°C)	23.3	23.5	35.8	36.1	31.7	25.2
			a.p. (mm)	135.1	295.1	26.0	72.6	90.5	26.4

a.d. T = average day temperature, T min = minimal day temperature, T max = maximal day temperature, a.p. = accumulated precipitation.

2.3. Extraction Conditions

Extraction of polyphenols and pigments from dry nettle leaves and stalks was carried out by ASE. Extraction conditions and procedure were adopted from the study of Repajić et al. (2020) [11]: extraction was performed in Dionex™ ASE™ 350 Accelerated Solvent Extractor (Thermo Fisher Scientific Inc., Sunnyvale, CA, USA) using ethanol (96%) as the extraction solvent. Extraction was accomplished in 34 mL stainless steel cells fitted with 2 cellulose filters (Dionex™ 350/150 Extraction Cell Filters, Thermo Fisher Scientific Inc., Sunnyvale, CA, USA), within which 1 g of sample was mixed with 2 g of diatomaceous earth, placed in cell and filled up with diatomaceous earth to the full cell volume. Extraction parameters differed for leaves and stalks: leaves were extracted under 110 °C with 10 min of static extraction time and 4 cycles, while stalk extracts were obtained at 80 °C, 5 min of static extraction time and 4 cycles (parameters previously optimized). Other extraction parameters remained fixed for the extraction of both plant parts, namely pressure 10.34 MPa, 30 s of purge with nitrogen and 50% of flushing. Obtained extracts were collected in 250 mL glass vessel with Teflon septa, transferred into 50 mL volume flask and made up to volume with the extraction solvent. All extracts were filtered through a 0.45 µm membrane filter (Macherey-Nagel GmbH, Düren, Germany) prior to further analysis. All extracts have been prepared in a duplicate (n = 2).

2.4. UPLC-MS/MS Conditions

Identification and quantification of phenolics were performed on UPLC–MS/MS in both ionization modes on a 6430 QQQ mass spectrometer Agilent Technologies (Agilent, Santa Clara, CA, USA). Analytes were ionized using ESI ion source with nitrogen as desolvation and collision gas (temperature 300 °C, flow 11 L h^{-1}), capillary voltage, +4 −3.5 kV^{-1} and the pressure of nebulizer was set at 40 psi. The mass spectrometer was linked to UPLC system (Agilent series 1290 RRLC instrument) consisted of binary pump, autosampler and a column compartment thermostat. Reversed phase separation was performed on a Zorbax Eclipse Plus C18 column 100 × 2.1 mm with 1.8 µm particle size (Agilent). Column temperature was set at 35 °C and the injection volume was 2.5 µL. The solvent compositions and the gradient conditions used were as described previously by Elez Garofulić et al. (2018) [26]. For instrument control and data processing, Agilent MassHunter Workstation Software (ver. B.04.01) was used. Quantitative determination was carried out using the calibration curves of the standards, where protocatechuic acid, gentisic acid, syringic acid and *p*-hydroxybenzoic acid were calculated as gallic acid equivalents and cinnamic acid according to *p*-coumaric acid. Isorhamnetin rutinoside, quercetin rhamnoside, quercetin, isorhamnetin, quercetin pentoside, quercetin acetylhexoside, quercetin acetylrutinoside and quercetin pentosylhexoside were calculated according to quercetin-3-glucoside, kaempferol hexoside, kaempferol pentoside, kaempferol rhamnoside, kaempferol pentosylhexoside and kaempferol according to kaempferol-3-rutinoside, apigenin hexoside and genistein according to apigenin, while umbelliferone was expressed as scopoletin equivalents. All analyses have been performed in a duplicate and concentrations of analyzed compounds are expressed as mg 100 g^{-1} of dry matter (dm) (N = 4).

2.5. HPLC-UV-VIS/PDA Conditions

The carotenoids and chlorophylls identification and quantification were performed using Agilent Infinity 1260 system equipped with Agilent 1260 photodiode array detector (PDA; Agilent, Santa Clara, CA, USA) with an automatic injector and Chemstation software (ver. C.01.03).

The separation of carotenoids and chlorophylls was performed using Develosil RP-Aqueus C 30 column (250 × 4.6 mm i.d. 3 µm, Phenomenex, Torrance, CA, USA). The solvent composition and the used gradient conditions were described previously by Castro–Puyana et al. (2017) [27]. The mixture of MeOH:MTBE:water (90:7:3, *v/v/v*) (A) and MeOH:MTBE (10:90, *v/v*) (B) formed the mobile phase. The injection volume was 10 µL and the flow rate was kept at 0.8 mL min^{-1}. The chromatogram was monitored by scanning from 240 to 770 nm and the signal intensities detected at 450 nm and 660 nm were used for carotenoid and chlorophyll quantitation. Identification was carried out by comparing retention times and spectral data with those of the authentic standards (α- and β-carotene, chlorophyll *a* and *b*) or in case of unavailability of standards by comparing the absorption spectra reported in the literature [28,29]. Quantifications were made by the external standard calculation, using calibration curves of the standards α-carotene, β-carotene, chlorophyll *a* and chlorophyll *b*. The quantification of individual carotenoid compounds (neoxantine, violaxantine, lutein and its derivatives, derivative of zeaxantine and lycopene) was calculated as β-carotene equivalents and derivatives of chlorophylls as chlorophyll *a* and *b* equivalents using the equation based on the calibration curves, respectively. All determinations have been performed in a duplicate and results are expressed as mg 100 g^{-1} dm (N = 4).

2.6. ORAC Determination

The procedure was based on a previously reported method [30,31] with slight modifications. Briefly, a 96 wells black microplate was prepared containing 150 µL of fluorescein solution (70.30 nM) and 25 µL of blank (75 µM phosphate buffer, pH 7.4), Trolox standard (3.24–130.88 µM) or sample (appropriate diluted) were added. The plate was incubated for 30 min at 37 °C. After the first three cycles (representing the baseline signal), AAPH

(240 mM) was injected into each well to initiate the peroxyl radical generation. Fluorescence intensity (excitation at 485 nm and emission at 528 nm) was monitored every 90 sec over a total measurement period of 120 min using an automated plate reader (BMG LABTECH, Offenburg, Germany) and data were analyzed by MARS 2.0 software. The results were expressed as mmol Trolox equivalent (TE) 100 g^{-1} of dm. Determinations were carried out in duplicate (N = 4).

2.7. Statistical Analysis

Statistica ver. 10.0 software (Statsoft Inc., Tulsa, OK, USA) was applied for the statistical analysis. Full factorial randomized design was designated for the experimental part and descriptive statistic was used for the basic data evaluation. Continuous variables (polyphenols, pigments and antioxidant capacity) were analyzed by multifactorial analysis of variance (MANOVA) and marginal mean values were compared with Tukey's HSD test. Relationships between determined compounds and antioxidant capacity were examined by calculated Pearson's correlation coefficients, while possible grouping of the samples according to the examined sources of variations was tested using Principal Component Analysis (PCA). Significance level $p \leq 0.05$ was assigned for all tests.

3. Results and Discussion

This study examined the influence of plant part (leaves and stalks), phenological stage (before flowering, during flowering and after flowering) and habitat (Table 1) on the concentrations of polyphenols and pigments in wild nettle grown in Croatia. A total of 84 nettle samples were analyzed, where target compounds (polyphenols and pigments) were extracted using ASE and their identification/quantification was assessed by UPLC-MS/MS (polyphenols) and HPLC-UV-VIS/PDA (pigments). Moreover, obtained extracts were characterized for their antioxidant capacity by the ORAC method.

3.1. Influence of Phenological Stage and Habitat on Polyphenols in Nettle Leaves and Stalks

Table 2 shows detailed polyphenolic profile and mass spectrometric data obtained by UPLC-MS/MS analysis of nettle leaves and stalks. A total of 41 polyphenolic compounds were identified, belonging to the classes of benzoic, cinnamic and other phenolic acids, flavonols, flavan-3-ols, flavones, isoflavones, flavanones and coumarins (Supplementary files 1 & 2). Among the benzoic acids, compound **35** was identified as gallic acid by comparison of its retention time and mass spectra data with those of an authentic standard. Other benzoic acids were tentatively identified according to their mass fragmentation patterns. Compounds **2** and **14** showed same fragmentation pattern with molecular ion at *m/z* 153 and fragment ion at *m/z* 109, corresponding to the loss of carbon dioxide moiety and implicating the structure of dihydroxybenzoic acids and were therefore according to their polarity tentatively identified as protocatehuic (3,4-dihydroxybenzoic acid) and gentisic acid (1,3-dihydroxybenzoic acid), respectively [32]. Compound **31** showed precursor ion at *m/z* 197 and fragmentation loss of −15 amu corresponding to the loss of methyl radical characteristic for methoxylated phenolic acids and was tentatively identified as syringic acid. Compound **34** showed precursor ion at *m/z* 137 and characteristic fragmentation pattern for deprotonated phenolic acid with loos of −44 amu due to decarboxylation [33] and was assigned as *p*-hydroxybenzoic acid. The composition of benzoic acids in nettle leaves and stalks is in accordance with previous reports [9,14]. Among the cinnamic acids, compounds **12**, **15**, **19**, **25** and **32** were identified using authentic standards as caffeic, chlorogenic, *p*-coumaric, ferulic and sinapic acid, respectively. Compound **21** was presented with precursor ion at *m/z* 147, and fragment ion at *m/z* 103 as a result of decarboxylation and was due to its mass spectra data assigned as cinnamic acid [32]. Compound **16** was identified as quinic acid comparing its spectral data and retention time with those of an authentic standard. The composition of cinnamic acids is in accordance with previous reports by Orčić et al. (2014) [14] and Franciškovič et al. (2017) [34] with the exception of cinnamic acid which was not detected in their research, but was reported previously

in composition of nettle leaves by Zeković et al. (2017) [35]. The most numerous class of flavonoid polyphenols identified in nettle were flavonols and their glycosides. Compounds **4**, **8**, **17** and **18** were identified by the authentic standard comparison as kaempferol-3-rutinoside, myricetin, quercetin-3-glucoside and quercetin-3-rutinoside, respectively. Other compounds were tentatively identified according to their mass spectra and characteristic fragmentation patterns reported previously. Among the aglycones, compounds **10**, **24** and **41** were assigned as quercetin, isorhamnetin and kaempferol due to characteristic molecular ion at *m/z* 301, *m/z* 315 and *m/z* 285 [36]. The presence of this aglycones in nettle aerial parts was confirmed previously by Bucar et al. (2006) [37]. Flavonol glycosides lacking authentic standards were tentatively identified according to the characteristic loss of sugar moiety and formation of aglycon fragment ion. Therefore, because of fragment ion at *m/z* 317, compound **3** was assigned as isorhamnetin glycoside. Precursor ion at *m/z* 625 implicated glycosylation with rhamnose (+146 amu) and glucose (+162 amu), so it was assigned as isorhametin rutinoside.

Table 2. Mass spectrometric data and identification of polyphenols.

Compound	Rt (min)	Cone Voltage (V)	Collision Energy (V)	Ionization Mode	Precursor Ion (*m/z*)	Fragment Ions (*m/z*)	Tentative Identification
				Benzoic acids			
2	0.828	105	9	-	153	109	Protocatechuic acid (3,4-dihydoxybenzoic acid)
14	0.992	100	9	-	153	109	Gentisic acid (2,5-dihydroxybenzoic acid)
31	8.837	90	7	-	197	182	Syringic acid
34	11.358	80	10	-	137	93	*p*-hydroxybenzoic acid
35	11.375	100	10	-	169	125	Gallic acid *
				Cinnamic acids			
12	0.975	80	10	-	179	135	Caffeic acid *
15	1.254	80	10	-	353	191	Chlorogenic acid *
19	3.332	80	10	-	163	119	*p*-coumaric acid *
21	4.490	100	5	-	147	103	Cinnamic acid
25	6.158	80	5	-	193	178	Ferulic acid *
32	11.012	100	17	-	223	193	Sinapic acid *
				Other phenolic acids			
16	1.620	150	20	-	191	85	Quinic acid *
				Flavonols			
3	0.842	120	15	+	625	317	Isorhamnetin rutinoside
4	0.856	120	15	+	595	287	Kaempferol-3-rutinoside *
5	0.880	100	5	+	449	303	Quercetin rhamnoside
6	0.880	30	5	+	449	287	Kaempferol hexoside
8	0.907	140	25	+	319	273	Myricetin *
10	0.938	130	15	-	301	151	Quercetin
17	1.855	100	5	+	465	303	Quercetin-3-glucoside *
18	2.461	120	5	+	611	303	Quercetin-3-rutinoside *
24	5.963	160	21	-	315	300	Isorhamnetin
27	7.106	100	5	+	419	287	Kaempferol pentoside
28	7.256	100	5	+	435	303	Quercetin pentoside
29	7.930	100	5	+	433	287	Kaempferol rhamnoside
30	8.242	100	10	+	507	303	Quercetin acetylhexoside
33	11.232	100	15	+	653	303	Quercetin acetylrutinoside
36	11.391	100	15	+	597	303	Quercetin pentosylhexoside
39	11.758	120	15	+	581	287	Kaempferol pentosylhexoside
41	11.822	130	0	-	285	285	Kaempferol
				Flavan-3-ols			
23	4.728	100	5, 15	+	459	289, 139	Epigallocatechin gallate *
37	11.615	100	10	+	291	139	Epicatechin *
38	11.621	100	5	+	291	165	Catechin *
40	11.792	100	5	+	443	291	Epicatechin gallate *

Table 2. *Cont.*

Compound	Rt (min)	Cone Voltage (V)	Collision Energy (V)	Ionization Mode	Precursor Ion (*m*/*z*)	Fragment Ions (*m*/*z*)	Tentative Identification
				Flavones			
7	0.890	135	5	+	433	271	Apigenin hexoside
9	0.924	140	35	+	287	153	Luteolin *
22	4.615	80	30	+	271	153	Apigenin *
				Isoflavones			
20	4.468	145	32	-	269	133	Genistein
				Flavanones			
11	0.945	130	16	-	271	151	Naringenin *
				Coumarins			
1	0.821	120	19	-	161	133	Umbelliferone (7-hydroxycoumarin)
13	0.979	105	15	-	177	133	Esculetin *
26	6.333	80	8	-	191	176	Scopoletin *

* Identification confirmed using authentic standards.

Its presence in nettle leaves and stalks was reported previously by Pinelli et al. (2008) [6]. Compounds **5**, **28**, **30**, **33** and **36** were identified as quercetin glycosides due to MS/MS ion at *m*/*z* 303 and were assigned as quercetin rhamnoside, quercetin pentoside, quercetin acetylhexoside, quercetin acetylrutinoside and quercetin pentosylhexoside due to fragmentation losses corresponding to rhamnose (−146 amu), pentose (−132 amu), hexose with acetyl residue (−162 and −42 amu), rutinose with acetyl residue (−308 and −42 amu) and pentose with hexose moiety (−132 and −162 amu) [38]. Previous reports on quercetin glycosides composition in nettle mostly included quercetin glucoside [6,14,34] and quercetin rutinoside [8,14,34], while not reporting the presence of acylated glycosides and diglycosides identified in this study. The latter provides the valuable contribution to detailed insight into nettle polyphenolic profile. Because of the characteristic fragment ion at *m*/*z* 287 corresponding to the kaempferol aglycon, compounds **6**, **27**, **29** and **39** were assigned as kaempferol hexoside, pentoside, rhamnoside and pentosylhexoside, respectively, due to fragment losses of corresponding sugar moieties. Similar to the previous literature reports on quercetin glycosides, the ones on kaempferol glycosides mostly only include kaempferol rutinoside [6,8] or glucoside [14,34,39], while not reporting the presence of kaempferol pentoside, rhamnoside and pentosylhexoside which are therefore being confirmed here for the first time. All compounds belonging to the class of flavan-3-ols (**23**, **37**, **38** and **40**), namely epigallocatechingallate, epicatechin, catechin and epicatechingallate were identified and confirmed according to the authentic standard. Orčić et al. (2014) [14] identified catechin in nettle stalks, epicatechin was reported by Proestos et al. (2006) [40] in leaves, while there are no available reports on previous identification of epicatechingallate and epigallocatechingallate. Compounds **9** and **22** were assigned as luteolin and apigenin due to molecular ions at *m*/*z* 287 and *m*/*z* 271 and confirmed by comparison with standards, while compound **7** was tentatively identified as apigenin hexoside based on fragment ion at *m*/*z* 271 and fragmentation loss of -162 amu specific for hexose residue. Nencu et al. (2012) [41] reported the polyphenolic composition of nettle leaves including aglycones luteolin and apigenin, which is in accordance with our findings, while literature reports on flavone aglycones are scarce. Compound **20** showed precursor ion at *m*/*z* 269 and fragment ion at *m*/*z* 133, corresponding to the previously reported fragmentation mechanism of genistein anion [42], confirmed in nettle leaves extract by Zeković et al. (2017) [35]. Compounds **11**, **13** and **26** were identified by its corresponding authentic standards as naringenin, esculetin and scopoletin, while compound **1** was tentatively assigned as umbelliferone due to molecular ion at *m*/*z* 161 and fragment ion at *m*/*z* 133 formed after the loss of one carbon

monoxide molecule [43]. The composition of flavanones and coumarines reported in our study is in accordance with previous literature data [14,34,35].

To examine the influence of phenological stage and habitat on the content of polyphenols in nettle leaves and stalks, identified polyphenols were arranged in corresponding classes, following which their individual concentrations accordingly summarized and subjected to statistical analysis, as shown in Table 3. Total polyphenols grand mean (GM) was 380.90 mg 100 g^{-1} dm, among which cinnamic acids were the most abundant group (GM 179.22 mg 100 g^{-1} dm), followed by flavonols (GM 134.60 mg 100 g^{-1} dm), flavones (GM 24.56 mg 100 g^{-1} dm), flavan-3-ols (GM 20.70 mg 100 g^{-1} dm) and benzoic acids (GM 10.20 mg 100 g^{-1} dm). Coumarins, isoflavones and other acids were present in lower concentrations: GM values 5.31, 3.09 and 2.88 mg 100 g^{-1} dm, respectively, while the least represented group of polyphenols were flavanones (GM 0.34 mg 100 g^{-1} dm). Moreover, obtained results are in accordance with the results of other authors [6,8,11,14], who reported quite similar phenolic profile in nettle extracts where cinnamic acids accounted for the most of presented total polyphenols.

As can be observed, the plant part, phenological stage and habitat had a significant influence ($p < 0.01$) on amounts of all polyphenols' groups. When comparing amounts of polyphenols between nettle leaves and stalks, it can be seen that leaves accumulated significantly higher concentrations of all polyphenols' groups (Table 3). Otles and Yalcin (2012) [7] also documented higher polyphenols content in wild nettle leaves extracts when compared to stalks extracts, as well as Pinelli et al. (2008) [6] who studied the content of polyphenols in cultivated and wild nettle and reported higher total polyphenols in leaves of both types of nettle (cultivated 7.364 mg g^{-1} fw, wild 2.58 mg g^{-1} fw) as opposed to nettle stalks (cultivated 3.670 mg g^{-1} fw, wild 0.750 mg g^{-1} fw).

Same authors documented the abundance of nettle stalks with fibers, consisting of several components of the lignin. However, in study of Orčić et al. (2014) [14], who examined nettle samples picked at three different locations, several identified polyphenols were recorded in higher levels in stalks, but the cinnamic acids presented in their study with chlorogenic acid were also more abundant in leaves.

Considering the phenological stage, it can be noticed that the 1st phenological stage (before flowering) resulted with higher concentrations of all polyphenols, except flavan-3-ols which were significantly higher during the 2nd phenological stage (flowering) (Table 3). Overall, total polyphenols decreased for almost 50% by the 3rd phenological stage. Similar to our results, in two studies of Nencu et al. (2012, 2013) [41,44], it was concluded that the optimal time for nettle leaves harvest was March, since the polyphenols content greatly decreased (over 80%) by June and September, respectively. Authors reported that the total polyphenols decrease is due to the decrease of non-tannin phenols (phenolcarboxylic acids and flavonoids), which are the most important compounds from nettle leaves. This was also confirmed by Roslon et al. (2003) [45] who reported a sudden drop of phenolcarboxylic acids in leaves harvested at the plant flowering stage. Furthermore, the results of Biesiada et al. (2009, 2010) [46,47] and Kőszegi et al. (2020) [19] also indicated that the beginning of the nettle vegetation period was optimal for harvesting, giving the highest yield of polyphenols, which then decreased by autumn for over 50%. Therefore, in order to obtain extracts with the highest polyphenols content, the optimal time to harvest the aerial parts of the nettle is spring (before the flowering of the plant). It can be assumed that the total polyphenols decrease starting at the flowering stage is a result of the physiological switch from the vegetative to the generative phase and the formation of flowers [48].

Table 3. The differences in polyphenols content (mg 100 g^{-1} dm) in wild nettle (*Urtica dioica* L.) due to the plant part, phenological stage and habitat.

Source of Variation		Benzoic Acids	Cinnamic Acids	Other Acids	Flavonols	Flavan-3-ols	Flavones	Isoflavones	Flavanones	Coumarins	Total Polyphenols
Plant part		$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *
leaves		12.55 ± 0.04b	209.46 ± 0.26b	4.30 ± 0.03b	160.26 ± 0.14b	25.99 ± 0.04b	29.28 ± 0.05b	3.37 ± 0.02b	0.40 ± 0.01b	6.53 ± 0.01b	452.14 ± 0.39b
stalks		7.86 ± 0.04a	148.98 ± 0.26a	1.45 ± 0.03a	108.94 ± 0.14a	15.42 ± 0.04a	19.84 ± 0.05a	2.81 ± 0.02a	0.29 ± 0.01a	4.09 ± 0.01a	309.67 ± 0.39a
Phenological stage		$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *
1st		12.65 ± 0.05c	223.32 ± 0.32c	3.66 ± 0.03c	169.53 ± 0.17c	22.23 ± 0.05b	31.89 ± 0.06c	3.70 ± 0.02c	0.48 ± 0.01c	7.28 ± 0.02c	474.75 ± 0.48c
2nd		11.55 ± 0.05b	202.70 ± 0.32b	3.18 ± 0.03b	141.72 ± 0.17b	23.88 ± 0.05c	22.28 ± 0.06b	2.99 ± 0.02b	0.33 ± 0.01b	5.11 ± 0.02b	413.75 ± 0.48b
3rd		6.42 ± 0.05a	111.63 ± 0.32a	1.78 ± 0.03a	92.54 ± 0.17a	16.00 ± 0.05a	19.50 ± 0.06a	2.58 ± 0.02a	0.22 ± 0.01a	3.53 ± 0.02a	254.21 ± 0.48a
Region/Habitat		$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *
C	Sela Žakanjska	12.06 ± 0.10g	200.25 ± 0.70h	2.10 ± 0.07b	134.87 ± 0.38g	16.68 ± 0.11b	24.18 ± 0.12g	2.92 ± 0.05d	0.31 ± 0.02bcd	4.41 ± 0.04a	397.78 ± 1.03g
	Sopčić Vrh	9.11 ± 0.10cd	215.63 ± 0.70i	4.12 ± 0.07f	150.83 ± 0.38i	26.97 ± 0.11i	26.69 ± 0.12h	3.25 ± 0.05e	0.49 ± 0.02fg	5.20 ± 0.04d	442.29 ± 1.03j
	Žakanje	19.39 ± 0.10i	227.10 ± 0.70j	2.56 ± 0.07c	177.87 ± 0.38j	32.13 ± 0.11j	42.44 ± 0.12j	5.29 ± 0.05i	0.41 ± 0.02def	5.92 ± 0.04f	513.12 ± 1.03l
	Zagreb I	10.21 ± 0.10e	172.62 ± 0.70e	4.22 ± 0.07fg	130.21 ± 0.38f	15.63 ± 0.11a	23.33 ± 0.12f	1.95 ± 0.05a	0.33 ± 0.02cde	6.76 ± 0.04h	365.26 ± 1.03e
	Zagreb II	11.06 ± 0.10f	185.09 ± 0.70f	4.21 ± 0.07fg	125.81 ± 0.38d	18.80 ± 0.11cd	21.49 ± 0.12d	3.47 ± 0.05ef	0.31 ± 0.02bcd	6.51 ± 0.04g	376.74 ± 1.03f
	Koretići	10.87 ± 0.10f	195.03 ± 0.70g	4.53 ± 0.07gh	144.36 ± 0.38h	22.09 ± 0.11g	18.87 ± 0.12b	3.77 ± 0.05g	0.45 ± 0.02efg	5.06 ± 0.04d	405.02 ± 1.03h
M	Ogulin	13.18 ± 0.10h	212.80 ± 0.70i	4.62 ± 0.07h	182.65 ± 0.38k	20.88 ± 0.11f	36.09 ± 0.12i	3.51 ± 0.05f	0.56 ± 0.02g	6.77 ± 0.04h	481.06 ± 1.03k
	Čovići I	9.44 ± 0.10cd	203.51 ± 0.70h	3.73 ± 0.07e	152.62 ± 0.38i	19.69 ± 0.11e	23.92 ± 0.12fg	4.47 ± 0.05h	0.44 ± 0.02efg	5.63 ± 0.04e	423.46 ± 1.03i
	Čovići II	8.96 ± 0.10c	194.18 ± 0.70g	3.08 ± 0.07d	127.91 ± 0.38e	21.62 ± 0.11g	17.14 ± 0.12a	2.91 ± 0.05d	0.53 ± 0.02g	4.70 ± 0.04bc	381.01 ± 1.03f
S	Poreč	9.37 ± 0.10cd	130.43 ± 0.70a	1.31 ± 0.07a	107.51 ± 0.38a	16.43 ± 0.11b	20.64 ± 0.12c	1.87 ± 0.05a	0.16 ± 0.02a	4.40 ± 0.04a	292.12 ± 1.03a
	Limski zaljev	9.53 ± 0.10d	141.67 ± 0.70c	1.07 ± 0.07a	111.21 ± 0.38b	18.32 ± 0.11c	23.43 ± 0.12f	2.36 ± 0.05b	0.23 ± 0.02abc	4.80 ± 0.04c	312.61 ± 1.03c
	Bale	7.14 ± 0.10b	143.55 ± 0.70c	1.82 ± 0.07b	114.39 ± 0.38c	19.17 ± 0.11de	22.41 ± 0.12e	2.68 ± 0.05cd	0.16 ± 0.02a	4.49 ± 0.04a	315.81 ± 1.03cd
	Vodnjan	6.14 ± 0.10a	134.44 ± 0.70b	1.03 ± 0.07a	112.89 ± 0.38bc	22.74 ± 0.11h	22.13 ± 0.12e	2.35 ± 0.05b	0.19 ± 0.02ab	5.15 ± 0.04d	307.05 ± 1.03b
	Muntrilj	6.40 ± 0.10a	152.75 ± 0.70d	1.87 ± 0.07b	111.27 ± 0.38b	18.72 ± 0.11cd	21.06 ± 0.12cd	2.46 ± 0.05bc	0.22 ± 0.02abc	4.55 ± 0.04ab	319.29 ± 1.03d
Grand mean		**10.20**	**179.22**	**2.88**	**134.60**	**20.70**	**24.56**	**3.09**	**0.34**	**5.31**	**380.90**

C = continental, M = mountain, S = seaside. * Statistically significant variable at $p \leq 0.05$. Results are expressed as mean ± SE (N = 4). Values with different letters within column are statistically different at $p \leq 0.05$.

Habitats of wild nettle samples differed according to the climate conditions and could be grouped into three different regions: continental, mountain and seaside (Table 1). As presented in Table 3, habitats significantly ($p < 0.01$) differed regarding polyphenols content, with no uniform pattern regarding individual polyphenolic groups. Thus, Žakanje, belonging to the continental region, was characterized with the highest concentrations of total polyphenols (513.12 ± 1.03 mg 100 g^{-1} dm), benzoic (19.39 ± 0.10 mg 100 g^{-1} dm) and cinnamic acids (227.10 ± 0.70 mg 100 g^{-1} dm), flavan-3-ols (32.13 ± 0.11 mg 100 g^{-1} dm), flavones (42.44 ± 0.12 mg 100 g^{-1} dm) and isoflavones (5.29 ± 0.05 mg 100 g^{-1} dm). Contrarily, Ogulin, situated in mountain areas, was characterized with the highest amounts of other acids (4.62 ± 0.07 mg 100 g^{-1} dm), flavonols (182.65 ± 0.38 mg 100 g^{-1} dm), flavanones (0.56 ± 0.02 mg 100 g^{-1} dm) and coumarins (6.77 ± 0.04 mg 100 g^{-1} dm). Moreover, seaside habitats generally showed the lowest presence of all polyphenols. Still, based on total polyphenols content, a difference between seaside samples and ones from other two regions can be observed, where continental and mountain samples showed significantly higher levels of total polyphenols when compared to the samples from seaside zone. This could be explained as a plant's self-defense against oxidative stress caused by lower temperatures. According to Di Virgillo et al. (2015) [1] habitat greatly affects the accumulation of polyphenolic compounds in nettle. Just as in the current study, other authors also confirmed a diversity in nettle polyphenols content in growing areas [7,14].

3.2. Influence of Phenological Stage and Habitat on Pigments in Nettle Leaves and Stalks

The presence of nettle natural color carriers, carotenoids and chlorophylls was monitored by HPLC analysis, which has detected a total of 13 carotenoids and 9 chlorophylls in wild nettle leaves and stalks, namely neoxanthin and its two derivatives, violaxanthin and its two derivatives, 13′-*cis*-lutein, lutein 5,6-epoxide, lutein, zeaxanthin, 9′-*cis*-lutein, α-carotene, β-carotene, chlorophyll *a* and its six derivatives and chlorophyll *b* and its derivative (Figure 1, Supplementary file 1). A similar chlorophylls and carotenoids composition was previously reported [4,11]. For statistical purposes, identified pigments were grouped and analyzed as total carotenoids and total chlorophylls, as well as their sum (total pigments) (Table 4). Total pigments GM was 644.22 mg 100 g^{-1} dm, most of which were chlorophylls (GM 611.19 mg 100 g^{-1} dm), while carotenoids were less present (GM 33.03 mg 100 g^{-1} dm). Other authors also reported higher chlorophylls content in nettle leaves extracts in comparison with the content of carotenoids [9,11,47,49].

As presented in Table 4, all sources of variation significantly ($p < 0.01$) affected both groups of pigments as well as their sum. When comparing the pigments distribution in examined plant parts, abundance in pigments was expectedly higher in leaves since they are major photosynthesis organs [50]. Accordingly, Hojnik et al. (2007) [18] also reported a much higher concentration of chlorophylls in nettle leaves in comparison with stalks (147.1 vs. 16 mg g^{-1} extract). Furthermore, determined values for total chlorophylls in leaves were similar to previously reported results by Biesiada et al. (2010) [46], Zeipiņa et al. (2014) [49] and Repajić et al. (2020) [11], but were higher than in Đurović et al.'s (2017) [9] study. Also, the obtained total carotenoids content was in accordance with the values documented in Repajić et al.'s (2020) [11] study, but it showed dissimilarity in comparison with the data of other authors [4,9,46,49], probably due to environmental differences.

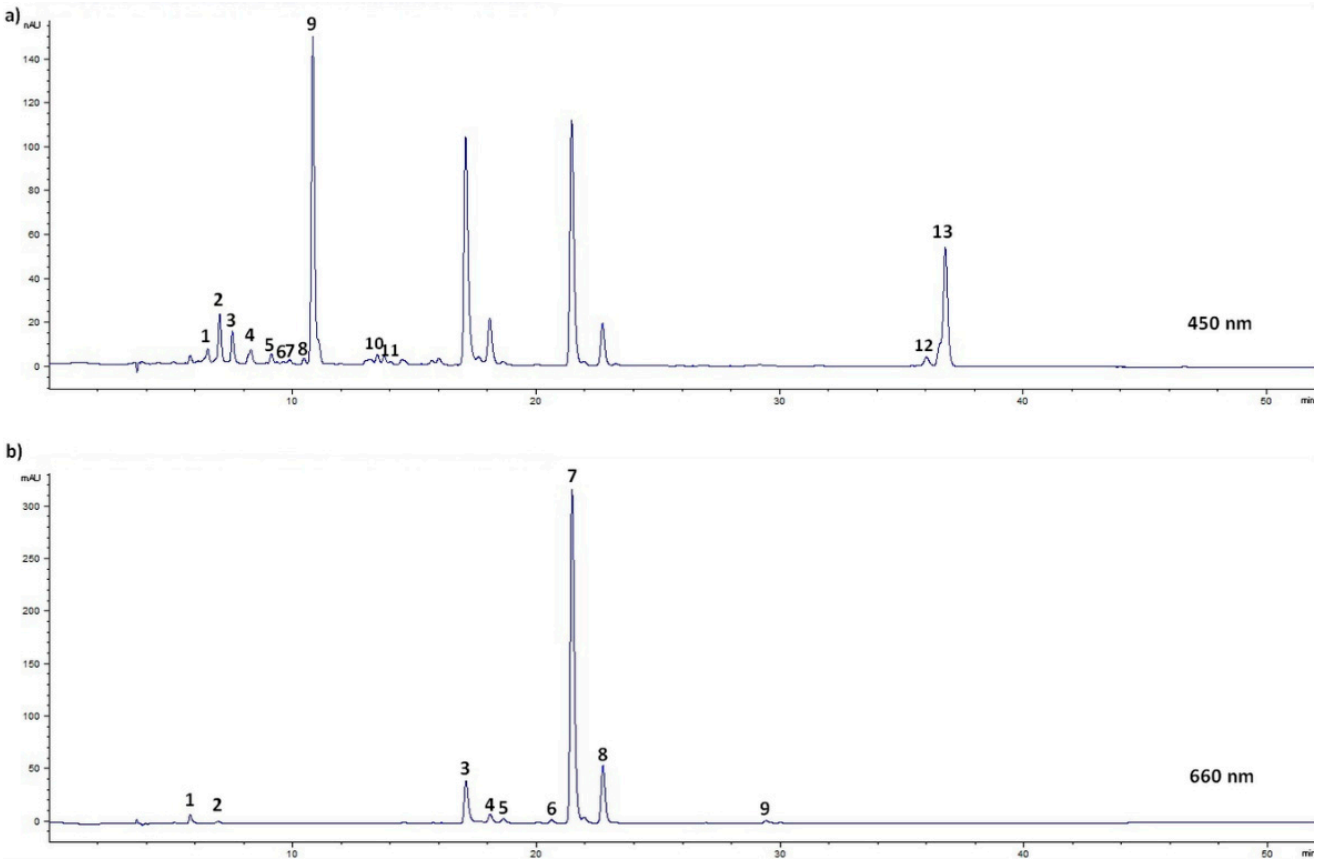

Figure 1. HPLC UV-VIS/PDA detection of pigments in wild nettle leaves (*Urtica dioica* L.) collected from Poreč before flowering: (**a**) at 450 nm (1 = violaxanthin derivative 1, 2 = neoxanthin derivative 1, 3 = neoxanthin, 4 = violaxanthin, 5 = violaxanthin derivative 2, 6 = 13′-*cis*-lutein, 7 = neoxanthin derivative 2, 8 = lutein 5,6-epoxide, 9 = lutein, 10 = zeaxanthin, 11 = 9′-*cis*-lutein, 12 = α-carotene, 13 = β-carotene); (**b**) at 660 nm (1 = chlorophyll *a* derivative 1, 2 = chlorophyll *a* derivative 2, 3 = chlorophyll *b*, 4 = chlorophyll *b* derivative 1, 5 = chlorophyll *a* derivative 3, 6 = chlorophyll *a* derivative 4, 7 = chlorophyll *a*, 8 = chlorophyll *a* derivative 5, 9 = chlorophyll *a* derivative 6).

Regarding the phenological stage, the highest amounts of all analyzed pigments were observed during the 2nd stage (flowering), where chlorophylls were the dominant pigments present in almost a 19-fold higher concentration (691.46 mg 100 g^{-1} dm) when compared to the amount of carotenoids (36.97 mg 100 g^{-1} dm). Similarly, Biesiada et al. (2009) [47] reported increased content of chlorophylls and carotenoids in nettle leaves when harvested in July in comparison with the harvest in May. Additionally, Marchetti et al. (2018) [10] observed that the highest lutein and β-carotene concentrations in nettle leaves occurred during the flowering stage (184 and 6.7 μg g^{-1} dm, respectively). Pajević et al. (1999) [51] also determined the maximum levels of chlorophylls and carotenoids in leaves of five alfalfa (*Medicago sativa* L.) genotypes just before and during the flowering stage. These similar patterns can be explained by enhanced production of secondary metabolites, such as plant pigments, during the flowering stage as a plant mechanism for fulfilling important physiological tasks like attracting pollinators [20].

When observing the differences in nettle pigments among the examined habitats, generally samples grown in seaside regions (particularly in the Limski zaljev and Bale habitats) had the highest pigments content. As this area was generally characterized by higher temperatures and lower accumulated precipitation (Table 1), these results are expected since the level of pigments in nettle is influenced by environmental factors, primarily the climate and growing location, where exposure to higher temperatures and more solar energy will result in a higher pigments content [24]. The results of Candido et al.'s (2015) [52] study, in which they examined carotenoid content in buriti palms pulp grown in two different regions (Amazon and Cerrado, Brazil), supported the aforementioned

results. They concluded that a higher content of carotenoids was measured in samples from the Amazon area, characterized by higher temperatures and humidity which prevent photodegradation of fruit pigments.

Table 4. The differences in pigments content (mg 100 g^{-1} dm) and ORAC values (mmol TE 100 g^{-1} dm) in wild nettle (*Urtica dioica* L.) upon plant part, phenological stage and habitat.

Source of Variation		Carotenoids	Chlorophylls	Total Pigments	ORAC
Plant Part		$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *
leaves		61.46 ± 0.08b	1126.94 ± 0.66b	1188.40 ± 0.71b	11.96 ± 0.02b
stalks		4.60 ± 0.08a	95.45 ± 0.66a	100.05 ± 0.71a	7.37 ± 0.02a
Phenological Stage		$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *
1st		32.49 ± 0.10b	589.07 ± 0.81b	621.56 ± 0.86b	11.26 ± 0.04b
2nd		36.97 ± 0.10c	691.46 ± 0.81c	728.44 ± 0.86c	12.10 ± 0.04c
3rd		29.64 ± 0.10a	553.04 ± 0.81a	582.67 ± 0.86a	5.63 ± 0.04a
Region/Habitat		$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *
C	Sela Žakanjska	37.29 ± 0.21h	701.89 ± 1.76g	739.18 ± 1.87h	11.76 ± 0.06i
	Sopčić Vrh	31.95 ± 0.21d	558.11 ± 1.76c	590.06 ± 1.87d	11.78 ± 0.06j
	Žakanje	27.94 ± 0.21c	466.84 ± 1.76a	494.78 ± 1.87a	12.25 ± 0.06m
	Zagreb I	26.70 ± 0.21b	480.27 ± 1.76b	506.97 ± 1.87b	11.89 ± 0.06k
	Zagreb II	32.14 ± 0.21d	600.73 ± 1.76d	632.87 ± 1.87e	9.46 ± 0.06f
	Koretići	31.81 ± 0.21d	596.83 ± 1.76d	628.64 ± 1.87e	11.22 ± 0.06h
M	Ogulin	33.23 ± 0.21e	598.67 ± 1.76d	631.91 ± 1.87e	12.20 ± 0.06l
	Čovići I	25.65 ± 0.21a	472.09 ± 1.76ab	497.74 ± 1.87a	10.59 ± 0.06g
	Čovići II	34.29 ± 0.21f	650.39 ± 1.76e	684.68 ± 1.87f	9.46 ± 0.06f
S	Poreč	27.40 ± 0.21bc	552.88 ± 1.76c	580.28 ± 1.87c	6.28 ± 0.06b
	Limski zaljev	40.21 ± 0.21j	719.69 ± 1.76h	759.90 ± 1.87i	6.26 ± 0.06a
	Bale	38.35 ± 0.21i	760.95 ± 1.76i	799.30 ± 1.87j	6.58 ± 0.06c
	Vodnjan	35.93 ± 0.21g	678.67 ± 1.76f	714.60 ± 1.87g	8.08 ± 0.06e
	Muntrilj	39.55 ± 0.21j	718.67 ± 1.76h	758.23 ± 1.87i	7.52 ± 0.06d
Grand mean		**33.03**	**611.19**	**644.22**	**9.67**

C = continental, M = mountain, S = seaside. * Statistically significant variable at $p \leq 0.05$. Results are expressed as mean ± SE (N = 4). Values with different letters within column are statistically different at $p \leq 0.05$.

3.3. Influence of Phenological Stage and Habitat on Antioxidant Capacity in Nettle Leaves and Stalks

The results of nettle antioxidant capacity measured by the ORAC method are given in Table 4 and Supplementary file 1. ORAC GM was 9.67 mmol TE 100 g^{-1} dm. Moreover, the nettle antioxidant capacity was significantly influenced ($p < 0.01$) by all examined sources of variation. Nettle leaves showed higher antioxidant capacity in comparison with stalks (11.96 mmol TE 100 g^{-1} dm vs. 7.37 mmol TE 100 g^{-1} dm). Similar ORAC values in nettle leaves were recorded in study of Repajić et al. (2020) [11], while Češlova et al. (2016) [53] obtained the same results by measuring the antioxidant capacity of different nettle parts infusions, where nettle leaves gained higher DPPH levels when compared to stalks. In support, Kırca and Arslan (2008) [54] concluded that leaves and flowers of different examined plants had a higher antioxidant capacity when compared to stalks and seeds.

When observing the influence of phenological stage, the highest ORAC value was observed during flowering, after which it significantly decreased and was the lowest after flowering. Similar to the results of the current study, other authors [19,46] documented that the antioxidant capacity of nettle leaves was higher in the earliest periods (April/May and June/July), after which it decreased (September/October).

Nettle samples showed diversity in antioxidant capacity upon habitat variations. As can be observed, samples from the continental and mountain part were described with the highest ORAC levels as opposed to nettles grown in seaside areas, which were

characterized with the lowest antioxidant capacity levels. These results are in accordance with previously discussed contents of polyphenols and pigments, where a certain grouping of the samples according to the presence of polyphenols and pigments by the growing area is evident. Moreover, calculated correlation coefficients supported this observation, since they showed a strong correlation between ORAC values and cinnamic acids, flavonols and total phenols (Table 5).

Table 5. Pearson's correlations between analyzed compounds (mg 100 g^{-1} dm) and ORAC values (mmol TE 100 g^{-1} dm).

Group of Compounds	ORAC Value
Benzoic acids	0.53 *
Cinnamic acids	0.71 *
Other acids	0.59 *
Flavonols	0.68 *
Flavan-3-ols	0.47 *
Flavones	0.36 *
Isoflavones	0.36 *
Flavanones	0.39 *
Coumarins	0.60 *
Total phenols	0.71 *
Carotenoids	0.46 *
Chlorophylls	0.44 *
Total pigments	0.44 *

* $p \leq 0.05$.

Obtained results clearly demonstrated the importance of the appropriate plant part selection as well as its phenological stage with the presence of the highest bioactive compounds accumulation in order to obtain the maximally enriched product, which will be beneficial for consumers.

3.4. PCA Analysis

Additionally, in order to examine a possible grouping of the nettle samples according to the applied sources of variations, PCA was carried out and obtained results are presented in Figure 2.

According to the preliminary PCA, a communality value of $\geq$0.5 described all 14 variables, thus they were all included in the test. The first two components (PC1 and PC2) explained 71.31% of total variance, where PC1 accounted for 53.47% of total variance, while PC2 attributed to 17.84% of total variance. Since PC1 strongly/very strongly negatively correlated ($-0.77 \leq r \leq -0.96$) with benzoic and cinnamic acids, flavonols, flavan-3-ols, flavones, ORAC values and total polyphenols, while PC2 had a strong/very strong correlation with carotenoids, chlorophylls and total pigments ($-0.79 \leq r \leq -0.81$), these variables could be considered as the most discriminating variables.

As can be seen in Figure 2a, separation of the samples clearly occurs based on the plant part. Most of the leaf samples were distributed at negative PC2 values, while all samples of stalks were situated at positive PC2 values. Regarding the phenological stage, a certain grouping appeared between samples from the 1st and 3rd phenological stage, where samples collected before flowering were mainly situated at negative PC1 values and almost all of the post-flowering samples were located at the positive PC1 values (Figure 2b). a partial grouping of nettle samples is visible in Figure 2c based on the growing region, where the most of separation can be seen to be present between continental and seaside samples, although this did not completely occur.

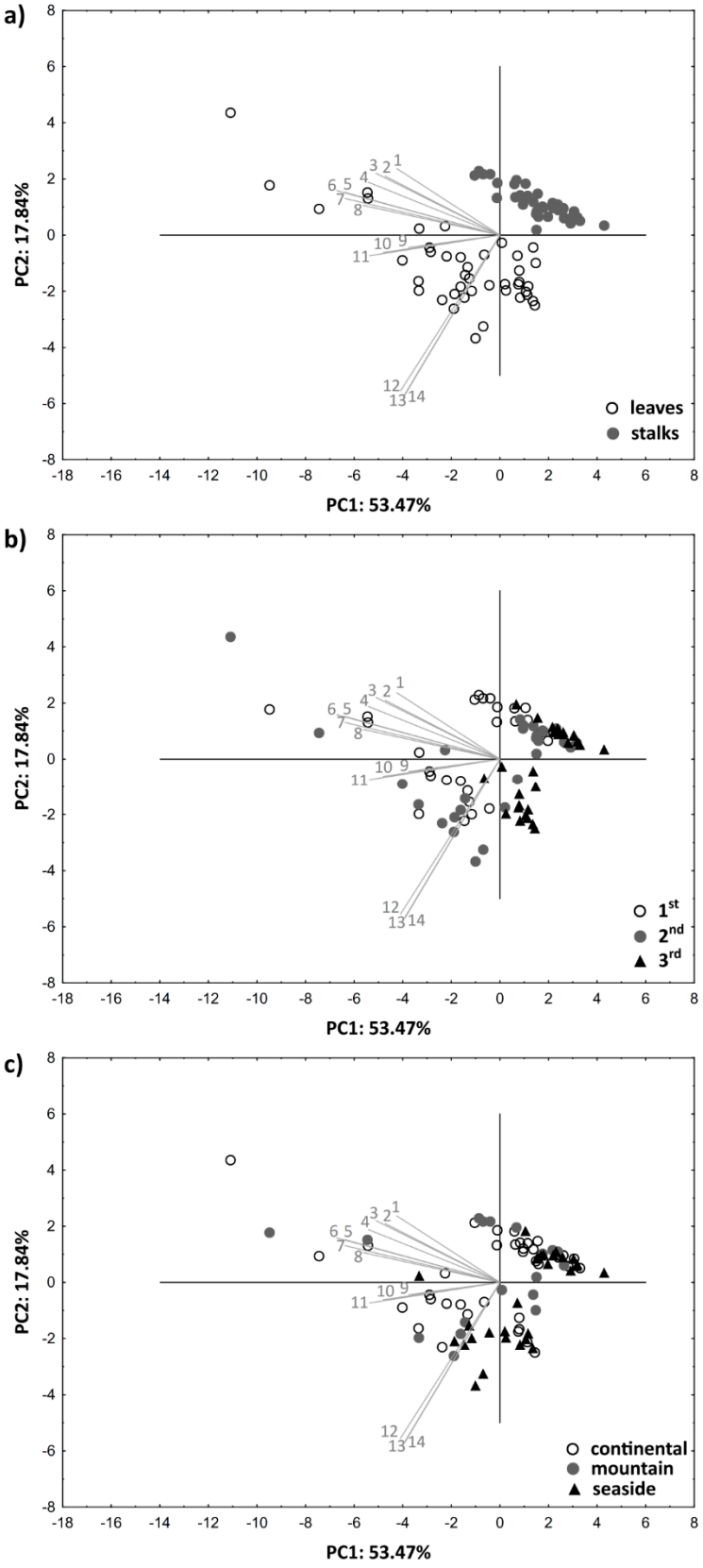

Figure 2. Distribution of wild nettle samples in two-dimensional coordinate system defined by the first two principal components (PC1 and PC2) according to the (**a**) plant part; (**b**) phenological stage; (**c**) growing region (1 = isoflavones, 2 = flavanones, 3 = flavones, 4 = benzoic acids, 5 = cinnamic acids, 6 = total polyphenols, 7 = flavonols, 8 = flavan-3-ols, 9 = other acids, 10 = coumarins, 11 = ORAC, 12 = carotenoids, 13 = chlorophylls, 14 = total pigments).

4. Conclusions

The current study confirmed the abundance of wild nettle with diverse bioactive molecules such as low molecular weight polyphenols and pigments, where 41 phenolic compounds, 13 carotenoids and 9 chlorophylls were documented. By using applied extraction conditions, cinnamic acids and flavonols were found to be the dominant classes of identified polyphenols (33.10–519.81 mg 100 g^{-1} dm and 57.44–383.25 mg 100 g^{-1} dm, respectively), while chlorophylls were the most abundant natural pigments (4.26–1934.38 mg 100 g^{-1} dm). Moreover, the ORAC values of obtained nettle extracts ranged from 3.05 to 19.83 mmol TE 100 g^{-1} dm. However, in order to obtain high valuable wild nettle extracts that are abundant in natural antioxidants, it is of the utmost importance to select appropriate plant parts as well as an appropriate harvest time. Obtained results evidenced that the highest levels of nettle bioactives accompanied by high antioxidant capacity were present in leaves, which should be collected during the early phenological period (before and at the flowering stage). Moreover, the amounts of wild nettle polyphenols and pigments greatly differed based on the natural habitat, as samples from the seaside region were characterized with elevated accumulation of pigments, while higher polyphenols amounts were present in habitats located in continental and mountain areas. This research will surely contribute to the selection of plant part and phenological stage for nettle optimal harvest, as well as to designate nettle natural habitats that have been shown to be a source of valuable plant material. These findings present the basis for the production of nettle seedlings with high bioactives content, which could further be used in the production of liquid and dry extracts. Furthermore, they showed the importance of a multidisciplinary approach for the selection of a plant part as well as its phenological stage in order to provide highly enriched products intended for the benefit of consumers.

In addition, besides low molecular weight polyphenols and pigments covered by this research, future studies could also include other beneficial compounds present in nettle such as oligomers and polymers as well as sterols, to provide a full insight into the nettle's bioactive potential.

Supplementary Materials: The following figures and tables are available online at https://www.mdpi.com/2304-8158/10/1/190/s1, (file 1) Figure S1: LC-MS/MS chromatogram in dMRM acquisition from the extract of wild nettle leaves (*Urtica dioica* L.) collected from Poreč before flowering, (file 2) Tables S1–S3: Concentrations of individual compounds and ORAC values in nettle (*Urtica dioica* L.) samples.

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Chapter 4

Publication No.3: Sterols and Pentacyclic Triterpenoids from Nettle Root: Content and Composition as affected by Pressurized Liquid Extraction

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Sterols and pentacyclic triterpenoids from nettle root: content and composition as affected by pressurized liquid extraction

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Abstract

BACKGROUND: Nettle is a medicinal plant rich in bioactive molecules. The composition of nettle leaves and stems has been extensively studied, whereas the root has been insufficiently investigated. Therefore, the present study aimed to optimize the parameters of advanced extraction technique, pressurized liquid extraction (PLE), for the lipid fraction of nettle root rich in triterpenoid derivatives and to compare the efficiency of isolation under optimal conditions with conventional Soxhlet extraction (SE).

RESULTS: The PLE yields ranged from 0.39–1.63%, whereas the total content of triterpenoid derivatives ranged from 43.50–78.26 mg 100 g^{-1}, with nine sterols and three pentacyclic triterpenoids identified and quantified within a total range of 42.81–76.57 mg 100 g^{-1} and 0.69–1.68 mg 100 g^{-1} dried root, respectively. The most abundant sterol and pentacyclic triterpenoid were β-sitosterol and β-amyrin acetate, with mean values of 50.21 mg 100 g^{-1} and 0.56 mg 100 g^{-1} dried root.

CONCLUSION: The optimal PLE conditions were 150 °C/5 min/four cycles and showed significantly better performance compared to SE (68 °C, 8 h), establishing an excellent technique for the isolation of the nettle root lipid fraction. Also, triterpenoid derivatives from nettle could be used as functional ingredients for the development of new foods and dietary supplements.

Keywords: *Urtica dioica* L.; nettle root; lipid fraction; phytosterols; pentacyclic triterpenoids; pressurized liquid extraction

INTRODUCTION

Nettle (*Urtica dioca* L.) is a medicinal plant from the *Urticaceae* family, distributed worldwide from temperate to tropical areas.[1,2] Because of the presence of various bioactive molecules, nettle possesses anti-inflammatory, anti-cancer, antioxidant, cardiovascular and diuretic effect.[3-5] Previous studies have shown that nettle leaves are rich in vitamins, minerals, phenolic compounds and pigments.[6] Considering their nutritional properties, they are used in soups, sauces, teas and juices.[7-9] On the other hand, nettle root is rich in protein lectin, sterols, polysaccharides, lignans, coumarins and phenols.[10-12] Although the composition of these compounds has not been extensively explored, nettle root extracts are well-known for their positive effects in treatment of benign prostatic hyperplasia (BPH),[13] antioxidant and anti-inflammatory effects.[12,14] In addition to the numerous *in vitro* studies attesting to various beneficial effects of nettle root, numerous studies have also been conducted *in vivo*. For example, in the review by Chrubasik *et al.*,[13] oral nettle root extract was found to have anti-inflammatory potential in the carrageenan-induced rat paw oedema test. Also, in the mentioned review paper there is one research conducted on dogs where treatment with nettle root extract over 100 days decreased the size of the prostate and serum testosterone levels. There is also clinical study by Schneider and Rübben[15] on 246 patients that can be considered as a safe therapeutic option for BPH, especially for reducing irritation symptoms and BPH-related complications. There is also *in vivo* research by Lichius and Muth[16] in which they created a BPH model by direct implantation of a urogenital sinus into the ventral prostate gland of an adult mouse and concluded that the 20% methanolic nettle root extract was most effective with a 51.4% inhibition of induced growth.

Triterpenes are a class of terpenes composed of six isoprene units and are usually classified by the number of rings in the structure, with pentacyclic triterpenes being the most common.[17,18] Oxidation of triterpenes produces triterpenoids, which, besides a hydrocarbon skeleton, contain a functional group with oxygen. One of the most common derivatives of triterpenoids are plant sterols or phytosterols, which are classified into sterols and stanols. The most abundant sterols are β-sitosterol, campesterol and stigmasterol.[19,20] As structural components of cell membranes, sterols possess protective, structural, transport and signal function in plants.[21] Because they have a similar chemical and

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structural function as cholesterol, they compete with it for a receptor site on the membrane, thus reducing the intestinal absorption of cholesterol. Moreover, sterols (especially fungal ergosterol) can modulate the expression of genes related to cholesterol metabolism.[22] Furthermore, sterols act as antioxidants, reduce the risk of cardiovascular diseases and cancer, and are used in anti-aging preparations,[23,24] and they also play a significant role in the treatment of BPH attributed to nettle root.[25] Major dietary sources of sterols include cereals, vegetable oils, nuts and seeds; however, because they are present in relatively small amounts, sterol enriched functional foods such as milk, yogurt, orange juice and snack bars are increasingly consumed.[24,26,27] Pentacyclic triterpenoids are found mainly in aromatic herbs, fruits and vegetables, especially in their leaves, stem bark and fruit peel. Widespread pentacyclic triterpenoids include oleanane, ursane and lupane groups, with the main representatives being α- and β-amyrin. Their biological effects include anti-inflammatory, antimicrobial, anticancer, antidiabetic and antioxidant properties; thus, they also are used as dietary supplements.[17,28-31]

To utilize the great advantage of natural bioactive compounds, various conventional and advanced extraction techniques are applied. Conventional solvent extraction such as Soxhlet extraction (SE), despite its simplicity, has many disadvantages, such as a long extraction time, high hazardous solvents consumption and decomposition of target components.[32,33] To overcome these serious drawbacks and to increase extraction yield, modern extraction techniques are being developed; for example, microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE). PLE is an automated extraction method that maintains solvent liquid under elevated pressure (10–15 MPa) and temperature (50–200 °C).[34,35] PLE uses the principle of high pressure, which allows the solvent to be heated to temperatures above its boiling point, increasing its diffusion rate and decreasing its viscosity, in turn allowing better penetration into the sample matrix and enhancing extraction yield.[36,37] Furthermore, PLE has a number of other advantages over conventional methods: shorter extraction time, lower solvent consumption, controlled temperature and pressure, and automation.[38-40]

Although nettle root is a valuable source of bioactive molecules, to the best of our knowledge, there are no detailed studies on the composition of its sterols and pentacyclic triterpenoids. Hence, the present study aimed to qualitatively and quantitatively examine the profile of nettle root sterols and pentacyclic triterpenoids. This is the first study dealing with the application of PLE as one of the most promising advanced extraction techniques for the isolation of nettle root lipid fraction. Therefore, the present study also aimed to optimize the PLE conditions for the isolation of lipid fraction of nettle root. To test the efficiency of PLE, conventional SE was also performed and compared to PLE. In addition to the numerous *in vitro* and *in vivo* studies demonstrating beneficial effects on human health, triterpenoid derivatives from nettle root could be used as functional ingredients, as well as to produce new functional products.

MATERIALS AND METHODS

Chemicals

Ethanol (96%) was purchased from Gram-mol d.o.o. (Zagreb, Croatia), KOH was obtained from Kemika (Zagreb, Croatia), Al_2O_3 was obtained from Alfa Aesar Chemical (Thermo Fisher Scientific, Geel, Belgium), *n*-hexane was obtained from Fisher Chemical (Thermo Fisher Scientific), diethyl ether was obtained from Lach-Ner d.o.o. (Brno, Czech Republic) and α-cholestanol, α-cholesterol, pyridine, hexamethyldisilazane, chlorotrimethylsilane were purchased from Honeywell Fluka (Seelze, Germany).

Plant material

Commercially available dried nettle root (*Urtica dioica* L.) harvested in 2020 (Suban Ltd, Strmec, Croatia) was used for the experiment. Prior to extraction, sample was ground using an electric grinder (Waring WSG30; Sprzęt Laboratoryjny i Medyczny Labpartner KBS, Warszawa, Poland) and sieved through a 2-mm sieve. The obtained root powder was analyzed for total solids by drying to constant mass at 105 °C[41] and the content of dry matter was 95.13%.

Lipid fraction extraction

Pressurized liquid extraction

To optimize PLE conditions for the isolation of lipid fraction from dried nettle root, the extraction temperature (70–150 °C), static extraction time (5–15 min) and number of extraction cycles[2-4] were varied according to the experimental design (Box–Behnken) and a total of 17 experimental conditions including five central points were obtained (Table 1). PLE was performed using ASE Dionex 350® instrument (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the method previously described by Repajić *et al.*[2] and Balbino *et al.*,[42] with some modifications. Briefly, 5.0000 ± 0.0001 g of ground sample was mixed with 0.5 g of diatomaceous earth and transferred to the stainless steel extraction cell (34 mL) fitted with three cellulose filters (Dionex™ 350/150 Extraction Cell Filters; Thermo Fisher Scientific Inc.) at the bottom of the cell and one more on the top. Extraction was carried out with *n*-hexane as a solvent, at a pressure of 10.34 MPa, rinse volume of 50% and nitrogen purge time of 30 s. After extraction, extract was transferred into round bottom flask and the solvent was evaporated on a rotary vacuum evaporator (Heidolph, Schwabach, Germany) at 40 °C. The resulting oil residue was transferred into weighed glass vial (20 mL) and purged through a stream of nitrogen to remove residual solvent. After weighing, the sample was stored at −18 °C until it was analyzed.

Soxhlet extraction

An amount of 10.0000 ± 0.0001 g of ground sample was weighed into the extraction capsule, which was closed with cotton wool and placed on Soxhlet apparatus. Lipid extraction was performed using *n*-hexane in a multiple continuous extraction procedure at 68 °C for 8 h, after which the solvent was evaporated on a rotary vacuum evaporator. Further preparation of the sample for the analysis was as described above for pressurized liquid extraction.

Determination of sterols and pentacyclic triterpenoids

Isolation of sterols and pentacyclic triterpenoids

Sterols and pentacyclic triterpenoids were isolated using modified ISO method 12228.[43] Nettle root lipid fraction (0.25 g) was refluxed for 15 min with 5 mL of ethanolic KOH solution (0.5 mol L^{-1}) after adding 1 mL of α-cholestanol (1 mg mL^{-1}) as an internal standard. Afterwards, 5 mL of ethanol was added to the mixture. The unsaponifiable fraction was eluted by solid phase extraction on a column containing 10 g of aluminium oxide with 5 mL of ethanol and 30 mL of diethyl ether. Solvent was removed from eluate by evaporation using rotary vacuum evaporator at 40 °C. The residue was dissolved in 2 mL of diethyl ether and transferred to silica gel chromatography plate. The plate was placed in a developing bath

Table 1. Yield of nettle root lipid fraction (%) and content of total triterpenoid derivatives (sterols and pentacyclic triterpenoids), total sterols and total pentacyclic triterpenoids (mg 100 g^{-1} root; mean ± SD, $n = 2$) obtained under different PLE conditions according to a Box–Behnken design

Sample	Temperature (°C)	Static time (min)	Number of cycles	Yield	Total triterpenoid derivatives	Total sterols	Total pentacyclic triterpenoids
1	70	5	3	0.39	43.50 ± 0.05	42.81 ± 0.04	0.69 ± 0.02
2	110	5	4	0.83	66.36 ± 0.14	65.14 ± 0.14	1.22 ± 0.00
3	110	15	2	0.93	72.12 ± 0.01	70.59 ± 0.11	1.53 ± 0.10
4	110	10	3	1.00	65.55 ± 0.19	64.43 ± 0.18	1.12 ± 0.01
5	150	10	4	1.35	78.26 ± 0.35	76.57 ± 0.07	1.68 ± 0.42
6	150	10	2	1.63	72.98 ± 0.33	71.53 ± 0.33	1.45 ± 0.00
7	110	10	3	0.87	71.25 ± 0.14	69.87 ± 0.13	1.38 ± 0.01
8	70	15	3	0.53	61.12 ± 0.02	59.99 ± 0.07	1.13 ± 0.05
9	110	10	3	0.92	65.36 ± 0.10	64.06 ± 0.09	1.30 ± 0.00
10	70	10	4	0.55	62.61 ± 0.19	61.37 ± 0.02	1.24 ± 0.17
11	110	15	4	0.88	64.42 ± 0.18	63.04 ± 0.04	1.38 ± 0.14
12	150	5	3	1.57	70.11 ± 0.17	68.65 ± 0.16	1.46 ± 0.01
13	110	5	2	0.68	53.43 ± 0.11	52.39 ± 0.00	1.03 ± 0.11
14	110	10	3	0.79	62.92 ± 0.06	61.57 ± 0.05	1.35 ± 0.01
15	110	10	3	1.02	66.31 ± 0.13	64.80 ± 0.11	1.51 ± 0.02
16	150	15	3	0.87	49.50 ± 0.21	48.38 ± 0.12	1.11 ± 0.10
17	70	10	2	0.53	57.59 ± 0.04	56.15 ± 0.03	1.44 ± 0.01
Mean				0.90	63.73	62.43	1.30

containing 100 mL of hexane:diethyl ether solution (1:1, *v/v*). After development, the plate was sprayed with methanol and sterol zone was scraped with a spatula, and filtered by 15 mL of diethyl ether. The solvent was then evaporated by rotary vacuum evaporator at 40 °C to a volume of 1 mL, transferred to a 10-mL test tube and purged to dryness through a stream of nitrogen. Silylation reagent (pyridine:hexamethyldisilazane:chlorotrimethylsilane 5:2:1, *v/v/v*) was added to the tube containing the sterols fraction at the ratio of 50 µL per 1 mg of unsaponifiable matter. The tube was closed and heated at 105 °C for 15 min, cooled and centrifuged at 4711 × *g* for 7 min. The clear supernatant, ready for injection, was transferred into 2-mL vial.

Gas chromatography

Content and composition of sterols and pentacyclic triterpenoids were determined using an Agilent 6890N Network gas chromatograph (GC) System (Agilent Technologies, Santa Clara, CA, USA) gas chromatograph equipped with flame ionization detector and 5973 *inert* Mass Selective Detector (MSD). The capillary column DB-17MS (Agilent), 30 m × 0.32 mm, 0.25 µm (50%-phenyl)-methylpolysiloxane, was used for the separation. The initial oven temperature was 180 °C and was heated at a rate of 6 °C min^{-1} to 270 °C at which it was maintained for additional 30 min. The injector temperature was 290 °C and the detector was set at 280 °C. The carrier gas was helium at a flow rate of 1.5 mL min^{-1} and the split ratio was 13.3:1. The injected sample volume was 1.0 µL. For identification of sterol peaks, MSD was used. The transfer line was maintained at 280 °C, the MS source at 230 °C and the quadrupole at 150 °C. The range of the mass spectral scan was set to *m/z* 30–550. The NIST 17 data library (https://chemdata.nist.gov/dokuwiki/doku.php?id=chemdata:nist17) was used for sterol identification. During the identification, three pentacyclic triterpenoids, *α*-amyrin, *β*-amyrin and *β*-amyrin acetate were also identified. The content and composition of sterols and pentacyclic triterpenoids were expressed in mg 100 g^{-1} dried nettle root as the mean ± SD of two replicates ($n = 2$).

Experimental design and statistical analysis

Design-Expert 10.0 (Stat-Ease Inc., Minneapolis, MN, USA) and XLSTAT 2021.23.2.1141.0 (Addinsoft, Paris, France) software systems were used for experimental design and statistical data processing. A Box–Behnken design with three factors was used for the experimental design with upper and lower limits of the tested parameters as follows: (i) extraction temperature 70 to 150 °C; (ii) static extraction time 5–15 min; and (iii) number of extraction cycles 2–4. Each tested parameter was observed at three levels (−1, 0, 1), resulting in a total of 17 experiments including the central point with five replicates (Table 1).

The dependent variables were the yield of the lipid fraction of nettle root (%), the content of total triterpenoid derivatives (sterols and pentacyclic terpenoids) (mg 100 g^{-1} dried root) and the *β*-sitosterol content (mg 100 g^{-1} dried root). To optimize the PLE conditions, response surface methodology (RSM) was used. The normality of the residuals was checked by the Shapiro–Wilks test and homoscedasticity by the Levene test. Regression models for the observed dependent variables were calculated according to:[44]

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=j}^{3} \beta_{ij} X_i X_j$$

where Y is predicted response; β_0 is fixed response at central point; β_i, β_{ii} and β_{ij} are the linear, quadratic and interaction coefficients; and X_i and X_j are the values of an independent, controlled variable. The models were also evaluated by the lack of fit and coefficient of determination (R^2).

To determine the significance of the influence of individual factors (temperature, static time and number of cycles), an anaylsis of variance (ANOVA) was performed at a 95% probability level.

The same independent variables were included in the optimization of PLE conditions performed by the desirability method. All selected dependent variables were marked by importance factor of 5.

To compare PLE *versus* SE, one-way ANOVA was performed, followed by Tukey's post-hoc test. Results are presented as the mean ± SD ($n = 2$ for extraction yield and $n = 4$ for content of total triterpenoid derivatives and β-sitosterol).

RESULTS AND DISCUSSION

Extraction yield and content of total sterols, pentacyclic triterpenoids and triterpenoid derivatives

The results for extraction yield of the lipid fraction from nettle root and the content of total triterpenoid derivatives, as well as total sterols and pentacyclic triterpenoids, obtained under different PLE conditions, are shown in Table 1. The yield ranged from 0.39% to 1.63% with a mean of 0.90%. It is important to note that results obtained are difficult to compare with literature data because, to the best of authors knowledge, there are no data on the extraction of nettle root lipid fraction by PLE. In the research by Guil-Guerrero *et al.*,[8] the content of saponifiable matter in nettle root was 0.1 g 100 g^{-1} sample, which is lower than the results of this study. Sajfrtová *et al.*[11] extracted the lipid fraction from nettle root using supercritical and liquid CO_2 and the highest yield of lipid extract was 9 mg g^{-1} dry mass, which is in agreement with the results of the present study. When comparing the content of triterpenoid derivatives (43.50–78.26 mg 100 g^{-1} dried root), it can be observed that sterols predominated with 97.96% compared to pentacyclic triterpenoids. The content of total sterols was determined in a range of 42.81–76.57 mg 100 g^{-1} dried root with a mean of 62.43 mg 100 g^{-1} and total pentacyclic triterpenoids were present in a range from 0.69 to 1.68 mg 100 g^{-1} dried root with mean of 1.30 mg 100 g^{-1} (Table 1). Kovacheva *et al.*[45] indicated that the content of phytosterols in nettle concentrates was between 3.23% and 6.05%, which is approximately two-fold lower than that obtained in gthe present study in which content of sterols, converted to the lipid extract obtained, was in the range 4.39–11.25%. Jafari *et al.*,[46] who analyzed nettle seeds, determined a total plant sterols content of 93.1 mg 100 g^{-1} oil, which is even lower compared to the results of the present study. In addition, Balbino *et al.*[42] examined sterols in various Apiaceae seeds and total content of sterols were in the range 468.8–903.9 mg 100 g^{-1} oil, whereas Ham *et al.*[47] determined the average sterols content in oily extract from saw palmetto berries to be 186.4 mg 100 g^{-1} oil. Because edible vegetable oils are the main sources of phytosterols, they can be used to evaluate the sterol composition of the lipid fraction of nettle root. For example, in the study by da Silva *et al.*,[48] the content of sterols (expressed as oil content) in different vegetable oils was investigated, and the content ranged from 27.23 mg 100 g^{-1} in coconut oil to 616.97 mg 100 g^{-1} in evening primrose oil. Also, in other edible oils, the mean value of the different varieties was 511.82 mg 100 g^{-1} for sesame seed oil,[49] and variation of sterols was in the range 170–190 mg 100 g^{-1} for tomato seed oil[50]. When the data from the above studies are compared with the results of the present study, it is evident that a remarkably higher amount of sterols was determined in our study which, when converted, ranges from 4463.58 to 11 058.15 mg 100 g^{-1} lipid extract, which is a great advantage of nettle root, for which the extract contains exceedingly large amounts of sterols.

Individual sterols and pentacyclic triterpenoids

There are only few studies on identification and quantification of sterols in the available scientific literature and there are no studies on identification and quantification of pentacyclic triterpenoids from nettle root. Accordingly, the present study aimed to provide a more detailed data on the composition of sterols and pentacyclic triterpenoids found in nettle root. The results of GC analysis are presented in Tables 2 and 3. In all analyzed samples of nettle root lipid fraction, a total of nine sterols and three pentacyclic triterpenoids have been determined. According to the chemical structure and sterols biosynthetic pathway analyzed compounds can be divided into Δ^5-sterols, Δ^7-sterols, stanols, sterol precursors and

Table 2. Content of sterols (mg 100 g^{-1} root; mean ± SD, $n = 2$) in nettle root lipid fraction obtained under different PLE conditions

Sample	Campesterol	Campestanol	Stigmasterol	β-sitosterol	Δ^5-avenasterol	Cycloartenol	Obtusifoliol	24-methylene-cycloartanol	Citrostadienol
1	4.77 ± 0.03	0.75 ± 0.00	0.99 ± 0.00	35.00 ± 0.01	0.44 ± 0.02	0.25 ± 0.01	0.07 ± 0.01	0.18 ± 0.01	0.36 ± 0.00
2	6.90 ± 0.00	0.88 ± 0.02	1.54 ± 0.01	53.14 ± 0.00	0.45 ± 0.00	0.32 ± 0.12	0.12 ± 0.00	0.36 ± 0.00	1.43 ± 0.01
3	7.38 ± 0.01	0.96 ± 0.01	1.79 ± 0.00	56.94 ± 0.05	0.38 ± 0.00	0.87 ± 0.03	0.18 ± 0.01	0.52 ± 0.01	1.56 ± 0.00
4	6.89 ± 0.04	1.02 ± 0.01	1.50 ± 0.00	52.24 ± 0.06	0.43 ± 0.09	0.46 ± 0.01	0.15 ± 0.00	0.34 ± 0.00	1.38 ± 0.01
5	8.08 ± 0.01	1.19 ± 0.01	1.77 ± 0.04	62.88 ± 0.02	0.29 ± 0.02	0.51 ± 0.12	0.16 ± 0.02	0.37 ± 0.05	1.33 ± 0.01
6	7.21 ± 0.01	0.69 ± 0.01	1.68 ± 0.01	56.91 ± 0.26	0.37 ± 0.01	2.78 ± 0.01	0.18 ± 0.01	0.46 ± 0.00	1.25 ± 0.03
7	7.30 ± 0.02	0.98 ± 0.03	1.60 ± 0.03	55.67 ± 0.01	0.31 ± 0.01	2.05 ± 0.01	0.18 ± 0.01	0.46 ± 0.02	1.32 ± 0.03
8	6.50 ± 0.02	0.99 ± 0.00	1.41 ± 0.00	49.52 ± 0.10	0.28 ± 0.01	0.50 ± 0.03	0.15 ± 0.01	0.33 ± 0.01	0.32 ± 0.00
9	6.67 ± 0.02	0.92 ± 0.01	1.47 ± 0.00	49.81 ± 0.05	0.33 ± 0.00	2.75 ± 0.02	0.21 ± 0.00	0.45 ± 0.00	1.45 ± 0.00
10	6.32 ± 0.08	1.03 ± 0.01	1.38 ± 0.00	48.31 ± 0.08	0.32 ± 0.01	2.33 ± 0.01	0.14 ± 0.01	0.39 ± 0.00	1.15 ± 0.01
11	6.72 ± 0.01	0.77 ± 0.01	1.54 ± 0.00	50.83 ± 0.02	0.37 ± 0.01	0.90 ± 0.05	0.19 ± 0.00	0.45 ± 0.00	1.28 ± 0.01
12	7.06 ± 0.01	0.41 ± 0.01	1.69 ± 0.00	55.73 ± 0.10	0.33 ± 0.01	0.95 ± 0.01	0.22 ± 0.01	0.52 ± 0.01	1.74 ± 0.05
13	5.52 ± 0.02	0.90 ± 0.00	1.26 ± 0.00	41.55 ± 0.03	0.29 ± 0.00	1.30 ± 0.00	0.13 ± 0.00	0.31 ± 0.00	1.13 ± 0.00
14	6.49 ± 0.05	0.81 ± 0.02	1.42 ± 0.01	49.35 ± 0.01	0.49 ± 0.00	0.69 ± 0.00	0.20 ± 0.00	0.46 ± 0.00	1.66 ± 0.01
15	6.87 ± 0.01	0.87 ± 0.00	1.49 ± 0.00	52.12 ± 0.05	0.31 ± 0.01	0.71 ± 0.01	0.23 ± 0.01	0.55 ± 0.01	1.64 ± 0.03
16	5.18 ± 0.07	0.68 ± 0.02	1.10 ± 0.02	39.15 ± 0.02	0.18 ± 0.00	0.35 ± 0.01	0.11 ± 0.00	0.41 ± 0.02	1.22 ± 0.01
17	5.86 ± 0.03	0.78 ± 0.00	1.28 ± 0.00	44.39 ± 0.00	0.27 ± 0.00	1.76 ± 0.00	0.16 ± 0.00	0.36 ± 0.00	1.30 ± 0.00
Mean	6.57	0.86	1.47	50.21	0.34	1.15	0.16	0.41	1.27

Table 3. Content of pentacyclic triterpenoids (mg 100 g^{-1} root; mean ± SD, $n = 2$) in nettle root lipid fraction obtained under different PLE conditions

Sample	α-amyrin	β-amyrin	β-amyrin acetate
1	0.10 ± 0.01	0.38 ± 0.00	0.21 ± 0.01
2	0.13 ± 0.01	0.69 ± 0.01	0.40 ± 0.00
3	0.28 ± 0.09	0.54 ± 0.02	0.71 ± 0.01
4	0.20 ± 0.00	0.48 ± 0.01	0.44 ± 0.00
5	0.26 ± 0.02	0.75 ± 0.33	0.67 ± 0.10
6	0.37 ± 0.01	0.50 ± 0.01	0.59 ± 0.00
7	0.18 ± 0.00	0.50 ± 0.01	0.70 ± 0.00
8	0.22 ± 0.02	0.43 ± 0.01	0.48 ± 0.01
9	0.21 ± 0.01	0.48 ± 0.00	0.62 ± 0.01
10	0.23 ± 0.16	0.49 ± 0.00	0.52 ± 0.01
11	0.28 ± 0.12	0.49 ± 0.00	0.61 ± 0.02
12	0.21 ± 0.03	0.51 ± 0.03	0.74 ± 0.00
13	0.20 ± 0.07	0.40 ± 0.01	0.43 ± 0.03
14	0.27 ± 0.00	0.47 ± 0.01	0.60 ± 0.00
15	0.32 ± 0.01	0.51 ± 0.00	0.68 ± 0.01
16	0.21 ± 0.12	0.44 ± 0.01	0.47 ± 0.01
17	0.38 ± 0.00	0.44 ± 0.01	0.63 ± 0.00
Mean	0.24	0.50	0.56

pentacyclic triterpenoids.[51,52] In the present study, the following sterols were determined: the group of Δ^5-sterols consisted of campesterol, stigmasterol, β-sitosterol and Δ^5-avenasterol, whereas citrostadienol belongs to Δ^7-sterols and campestanol is a stanol. Cycloartenol, 24-methylene cycloartanol and obtusifoliol are the precursors in sterol synthesis, and the determined pentacylic triterpenoids were α-amyrin, β-amyrin and β-amyrin acetate. The most dominant group was Δ^5-sterols, which accounted for 91.97% of the sterols and triterpenoids fraction, followed by precursors (2.67%), pentacyclic triterpenoids (2.03%), Δ^7-sterols (1.97%) and stanols (1.37%).

When observing individual sterols, the most dominant sterol in all samples was β-sitosterol, with a mean of 50.21 mg 100 g^{-1} dried root (i.e. 80.42% of total sterols). It was followed by campesterol (mean 6.57 mg 100 g^{-1} dried root), stigmasterol (mean 1.47 mg 100 g^{-1} dried root), citrostadienol (mean 1.27 mg 100 g^{-1} dried root) and cycloartenol (mean 1.15 mg 100 g^{-1} dried root). The results obtained are in accordance with Ostlund *et al.*,[53] who reported that β-sitosterol, campesterol and stigmasterol are the most abundant sterols in plant species in general. Means of other determined sterols were < 1 mg 100 g^{-1} dried root, where the least represented was obtusifoliol (mean 0.16 mg 100 g^{-1} dried root). The results obtained are reasonably consistent with the data in the literature. Horník *et al.*[54] documented that the predominant sterol in the nettle root extract was β-sitosterol with a content of 81%, whereas Sajfrtová *et al.*[11] extracted β-sitosterol and scopoletin from nettle root and the maximum yield of β-sitosterol was 0.63 mg g^{-1} dry matter. Moreover, Petkova *et al.*[55] reported that the most dominant sterol in nettle seed oil was β-sitosterol (90.1%), followed by stigmasterol (8.5%), whereas campesterol was present in much lower content (0.7%). On the other hand, Federovska *et al.*[56] examined the optimal parameters for obtaining oil extract enriched with phytosterols from nettle root. Corn oil was chosen as the base, in which the composition and content of sterols was determined. After the addition of nettle root extract, an increase in phytosterols in the oil extract was observed, where the content of the most abundant sterol (β-sitosterol) increased by 35.72%. Yang *et al.*[57] studied the composition of sterols from different vegetable oils. The most significant sterol in all examined oils was β-sitosterol, accounting for approximately 50% of total sterols. Besides the main representatives of sterols (β-sitosterol, campesterol and stigmasterol), other sterols were also recorded: the content of Δ^5-avenasterol ranged from 3.64 mg 100 g^{-1} in peony oil to 157.41 mg 100 g^{-1} in rice bran oil, cycloartenol ranged from 4.71 mg 100 g^{-1} in soybean oil to 156.25 mg 100 g^{-1} in rice bran oil, whereas the content of 24-methylene-cycloartanol varied from 12.33 mg 100 g^{-1} in camellia oil to 222.88 mg 100 g^{-1} in rice bran oil. The sterol content determined in the present study was similar to that of the mentioned vegetable oils, except for β-sitosterol, the concentration of which was much higher in nettle root lipid fraction.

Beside sterols, MS identification also confirmed the presence of three pentacyclic triterpenoids in nettle root lipid extract, namely α-amyrin, β-amyrin and β-amyrin acetate. Their individual contents are given in Table 3, where it can be seen that the most abundant triterpenoid compound was β-amyrin acetate, with mean of of 0.56 mg 100 g^{-1} dried root. It was followed by β-amyrin (mean 0.50 mg 100 g^{-1} dried root), whereas α-amyrin was determined in the lowest content (mean 0.24 mg 100 g^{-1} dried root). When combining the GC-MS with nuclear magnetic resonance spectroscopy, Grauso *et al.*[58] identified α- and β-amyrin in nettle leaves extract, in the content of 10.9 and 9.7%, respectively. It is the only study that identified pentacyclic triterpenoids in nettle leaves, whereas pentacyclic triterpenoids in nettle root have not been previously reported. Balbino *et al.*[42] identified two pentacyclic triterpenoids, α- and β-amyrin, in Apiaceae seed lipid extracts obtained by PLE. β-amyrin was present in higher content than α-amyrin, which is consistent with the results of current study. Klein *et al.*[59] compared α- and β-amyrin concentrations in extracts from uvaia (*Eugenia pyriformis* Cambess.) leaves obtained by SFE and UAE under different conditions. In both extracts, the concentration of β-amyrin was also higher compared to α-amyrin. According to Zhang *et al.*,[60] who studied the composition of various phytosterols in 31 vegetable oils, α- and β-amyrin were also detected. α-amyrin was detected only in 10 vegetable oils, whereas β-amyrin was detected in 15 vegetable oils. The content of α-amyrin ranged from 2.53 mg 100 g^{-1} oil in samara oil to 1721 mg 100 g^{-1} oil in shea butter oil, and the content of β-amyrin ranged from 3.47 mg 100 g^{-1} oil in poppy seed oil to 271 mg 100 g^{-1} oil in shea butter oil. Compared with the results of the present study, the content of α- and β-amyrins was higher in nettle lipid fraction, except for shea nut butter, which had higher levels of the mentioned amyrins. On the other hand, Czaplicki *et al.*[61] studied the composition of nine unconventional vegetable oils and no amyrins were detected in all tested samples.

In brief, when comparing the results of the present study with other previous studies on the isolation of sterols from nettle, it should be noted that the present study is the first to identify and quantify nine sterols and three pentacyclic triterpenoids in nettle root.

Effect of extraction parameters

Lipid fraction yield, content of total triterpenoid derivatives and β-sitosterol, as dominant bioactive compounds showing a positive effect on health,[62–64] were included in further data analyses (i.e. RSM) as the most important variables for testing the influence

of examined PLE parameters and optimizing the extraction. The results of the statistical analysis are presented in Figs 1 and 2 and Table 4.

As presented in Table 1, the lowest yield was obtained at 70 °C during 5 min and three cycles and the highest yield was obtained at 150 °C, during 10 min and two extraction cycles. The results of ANOVA show that temperature had a statistically significant effect ($P \leq 0.05$) on the yield of the nettle root lipid fraction, whereas static time and the number of extraction cycles showed no significant effect ($P > 0.05$) (Figs 1a and 2a and Table 4). This is also evident in Fig. 1(a), which indicates that increasing of the temperature increased the yield, regardless of the static time. Elevated temperature affects the properties of the solvent and it is one of the most important parameters of PLE. An increase in temperature increases the diffusion rate and solubility of the substance in the solvent, whereas the viscosity decreases and the solvent penetrates better into the pores of the analyte giving more efficient extraction.[34,36] Lohani *et al.*[65] investigated the effect of PLE parameters and different solvents on the yield of different oilseeds. Comparison of the results for the yield obtained with PLE at 80, 100 and 120 °C showed that higher yields were achieved at 120 °C than at lower temperatures. Although different plant species were involved, such research confirms the trend of yield increase depending on the applied temperature. The same conclusion was also reached by Balbino *et al.*[42] where PLE was used for the lipid extraction of several seed spices from the Apiaceae family, and the highest yields were obtained at the highest temperature applied.

When observing the content of total triterpenoid derivatives, the lowest content of total triterpenoid derivatives was obtained under the 70 °C, 5 min and three extraction cycles, whereas their highest content was obtained under the 150 °C, 10 min and four extraction cycles (Table 1). Statistically, all examined PLE parameters significantly ($P \leq 0.05$) affected the content of total triterpenoid derivatives. Moreover, interactions temperature *versus* static time and static time *versus* cycle number also showed a significant effect on their content (Figs 1b and 2b and Table 4 and). The content of total triterpenoid derivatives increased when the temperature increased from 70 to 150 °C during a static time of 5 min; however, with the application of a longer static time (15 min), increasing of the temperature caused a decrease of the content of total triterpenoid derivatives (Fig. 1b). Furthermore, extraction cycles and static time need to be appropriately combined for the most efficient extraction of target compounds.[34] The purpose of the extraction cycle is to introduce fresh solvent during extraction and maintain a favorable extraction balance.[66] It is useful for samples with high analyte concentrations and those where it is difficult to penetrate the matrix with the solvent.[34] As can be observed from Fig. 2(b), two extraction cycles with a simultaneous

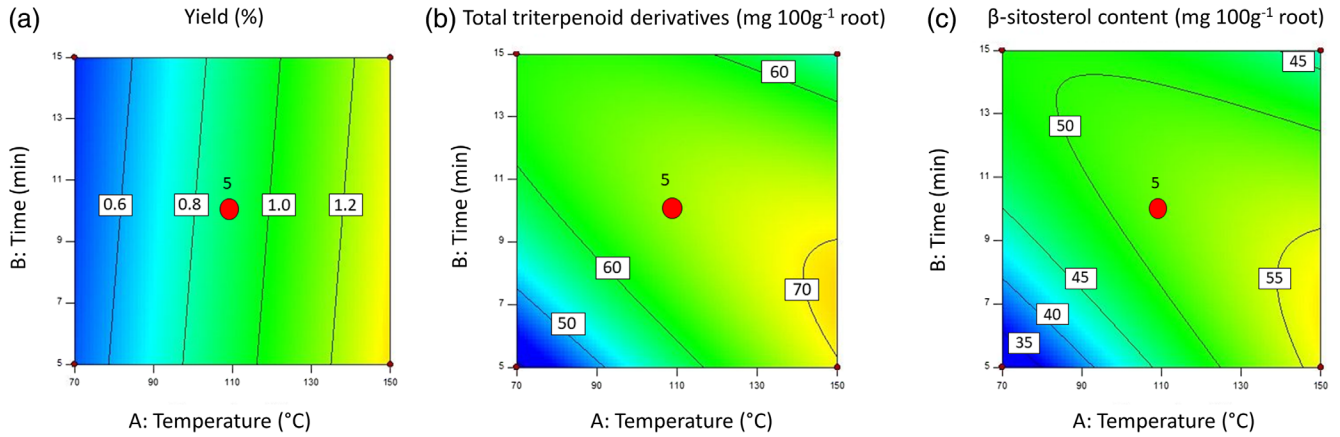

Figure 1. Influence of temperature (°C) and static time (min) at three extraction cycles on (a) extraction yield (%), (b) content of total triterpenoid derivatives (mg 100 g^{-1} root) and (c) content of β-sitosterol (mg 100 g^{-1} root).

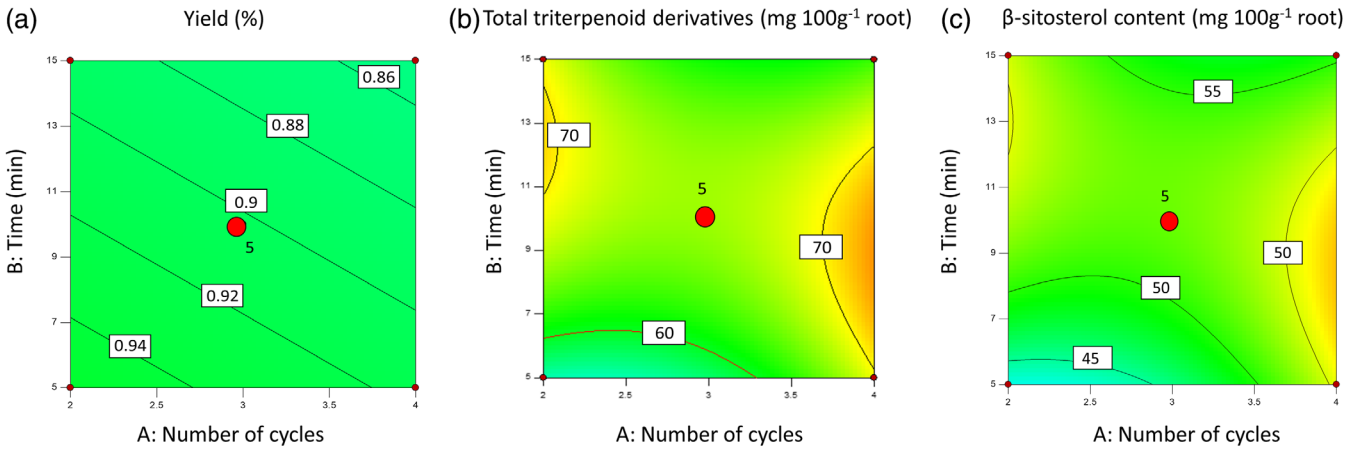

Figure 2. Influence of cycle number and static time (min) at 110 °C on (a) extraction yield (%), (b) content of total triterpenoid derivatives (mg 100 g^{-1} root) and (c) content of β-sitosterol (mg 100 g^{-1} root).

Table 4. Influence of PLE parameters on the yield of nettle root lipid fraction (%), content of total triterpenoid derivatives (sterols and pentacyclic triterpenoids) (mg 100 g^{-1} root) and β-sitosterol (mg 100 g^{-1} root)

Extraction yield (%)

Source of variation	*F*-value	*P*-value
X_1	51.65	<0.001*
X_2	0.01	0.934
X_3	0.08	0.980
Lack of fit	4.08	0.095
R^2	0.799	
Model	$Y = -1.2491 + 0.0108X_1 + 0.0010X_2 - 0.0016X_3$	

Total triterpenoid derivatives (mg 100 g^{-1} root)

Source of variation	*F*-value	*P*-value
X_1	15.25	0.006*
X_1^2	2.51	0.157
X_2	1.36	0.281
X_2^2	11.88	0.011*
X_3	1.73	0.230
X_3^2	5.59	0.050*
X_1X_2	21.05	0.003*
X_1X_3	9.43×10^{-4}	0.976
X_2X_3	6.13	0.043*
Lack of fit	3.00	0.158
R^2	0.778	
Model	$Y = -50.9182 + 1.0597X_1 + 14.2962X_2 - 16.7376X_3 - 0.0478X_1X_2 + 0.0016X_1X_3 - 1.0317X_2X_3 - 0.0020X_1^2 - 0.2800X_2^2 + 4.8029X_3^2$	

β-sitosterol (mg 100 g^{-1} root)

Source of variation	*F*-value	*P*-value
X_1	13.44	0.008*
X_1^2	1.62	0.244
X_2	1.16	0.316
X_2^2	7.28	0.031*
X_3	2.26	0.177
X_3^2	4.01	0.085
X_1X_2	18.53	0.004*
X_1X_3	0.08	0.784
X_2X_3	6.00	0.044*
Lack of fit	3.50	0.129
R^2	0.778	
Model	$Y = -38.8107 + 0.7754X_1 + 11.0044X_2 - 11.8074X_3 - 0.0389X_1X_2 + 0.0129X_1X_3 - 0.8848X_2X_3 - 0.0014X_1^2 - 0.1899X_2^2 + 3.5264X_3^2$	

Note: X_1, temperature; X_2, static extraction time; X_3, number of extraction cycles.
*Statistically significant at $P \leq 0.05$.

increase in static time from 5 to 15 min resulted in a higher content of total triterpenoid derivatives. By contrast, the content of total triterpenoid derivatives decreased with a longer extraction time in combination with four extraction cycles. Gil-Ramírez *et al.*[67] examined the influence of PLE parameters on fungal sterols detected in *Agaricus bisporus* mushrooms. They investigated the influence of temperature in a range from 25 °C up to 200 °C. The optimal extraction temperature was 100 °C because, at that temperature, almost all extractable sterols present in the sample were obtained. They also concluded that with a shorter extraction time and a lower number of cycles, almost all sterols were extracted because, with a longer static time, degradation and conversion between sterols could occur. In a study by de Souza *et al.*,[68] higher concentrations of amyrins were obtained in PLE extracts of *Arctium lappa* leaves when higher temperatures were applied. Because there is a lack of literature on the extraction of triterpenoid compounds by PLE, other groups of compounds were used for the comparison. Accordingly, Repajić *et al.*[69] reported that temperature increase from 20 to 110 °C resulted in a significantly higher content of phenolic compounds and pigments in PLE extracts of nettle leaves and the highest yield of polyphenols and pigments was achieved during 10 min of extraction and three or four extraction cycles.

The lowest and highest content of β-sitosterol was achieved at the same parameters as total triterpenoid derivatives (Table 2).

Comprising the most abundant analyzed compound in nettle root samples, the influence of extraction conditions on the content of β-sitosterol was also examined by statistical analysis, and the results obtained are shown in Table 4. Temperature and static time had a significant effect ($P \leq 0.05$) on the β-sitosterol content, whereas the influence of cycle number was not significant ($P = 0.177$). Similar to total triterpenoid derivative content, the interactions of temperature *versus* static time and static time *versus* cycle number had a significant effect ($P \leq 0.05$) on β-sitosterol content, with their combination showing the same effect on β-sitosterol content as for total triterpenoid derivatives (Figs 1c and 2c). Shen & Shao[70] investigated the effect of PLE on the isolation of sterols and terpenoids in tobacco samples by extraction at different temperatures (100, 125 and 150 °C) and reported the increase of β-sitosterol content accompanied with temperature increase. de Oliveira *et al.*[71] studied the effect of the PLE parameters (solvent and static time) on the quantification of β-sitosterol from Babassu almond oil and concluded that a lower extraction time (about 3 min) was sufficient to obtain the maximum β-sitosterol content.

To predict values of lipid fraction yield, contents of total triterpenoid derivatives and β-sitosterol were calculated for each value of temperature, static time and number of cycles in linear, quadratic and interaction coefficients combined. The equations of the regression model are presented in Table 4. In general, the higher the value next to the variable in the regression model equation, the more significant the influence of the variable in the model.[72] In addition, the coefficient of determination (R^2) and the lack of fit were also calculated because they measure the accuracy of the model and the ability of the model to reproduce the data. Calculated R^2 was above 0.7 for all three models and the values for lack of fit were not significant ($P > 0.05$) (Table 4), indicating very good reliability of the model based on the experimental results.[73]

Because all PLE parameters are interrelated and to maximize the yield of nettle root lipid fraction and content of total triterpenoid derivatives, as well as β-sitosterol, examined PLE conditions were optimized using desirability function method. The optimal conditions, with a desirability of 0.941, were extraction temperature of 150 °C, static extraction time of 5 min and four extraction cycles, ensuring a predicted yield of 1.34% of the lipid fraction, 88.61 mg 100 g^{-1} dried root of total triterpenoid derivatives and 66.31 mg 100 g^{-1} dried root of β-sitosterol. The predicted values were confirmed experimentally under calculated optimal PLE conditions: 1.28% for the yield of lipid fraction, 88.40 mg 100 g^{-1} dried root for total triterpenoid derivatives and 71.20 mg 100 g^{-1} dried root for β-sitosterol. Because the experimental values of all variables studied differed from the predicted values by approximately 7% or less, it can be concluded that the regression models are sufficiently accurate to predict the efficiency of PLE for the isolation of the nettle root lipid fraction, as well as the content of triterpenoid derivatives and β-sitosterol.

Comparison of PLE and SE

With SE being a standard method for the extraction of lipid components in food products, the efficiency of PLE and SE in isolation of nettle root lipid fraction, with the main focus on total triterpenoid derivatives and β-sitosterol, was compared and results obtained are given in Fig. 3.

When comparing the results obtained with both methods, PLE was found to to be a better choice for the extraction of examined target compounds. PLE achieved significantly ($P \leq 0.05$) higher yield of lipid fraction compared to the yield obtained with SE (1.28% *versus* 0.49%) (Fig. 3a). Moreover, the content of total

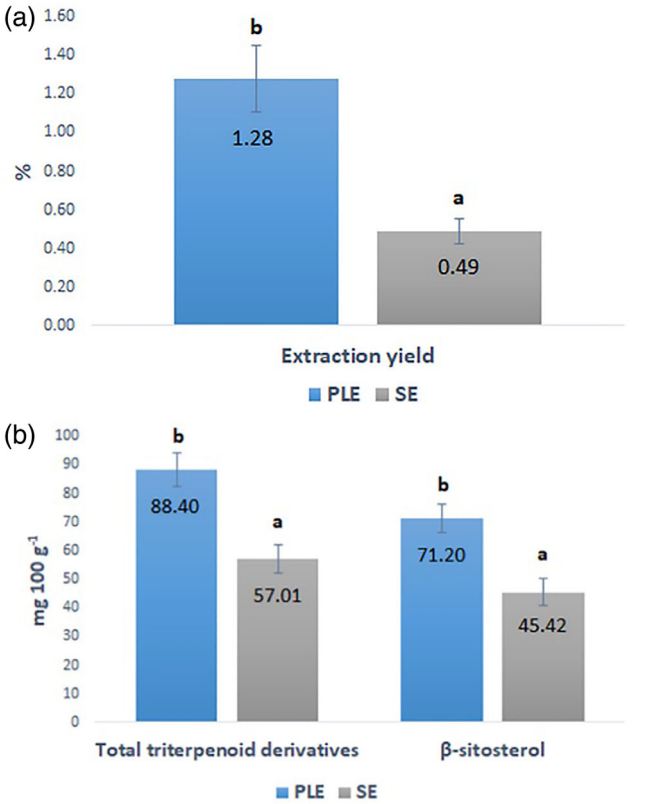

Figure 3. Comparison of PLE at optimal conditions (150 °C/5 min/4 cycles) and SE in (a) extraction yield and (b) content of β-sitosterol and total triterpenoid derivatives. Results are presented as the mean ± SD ($n = 2$ for extraction yield and $n = 4$ for content of total triterpenoid derivatives and β-sitosterol). Different lowercase letters indicate a statistically significant difference at $P \leq 0.05$.

triterpenoid derivatives as well as β-sitosterol was significantly ($P \leq 0.05$) higher in the sample obtained by PLE compared to that obtained using SE (total triterpenoid derivatives 88.4 *versus* 57.01 mg 100 g^{-1}, β-sitosterol 71.2 *versus* 45.42 mg 100 g^{-1}) (Fig. 3b). In the study by Péres *et al.*,[74] the extraction yield and chemical composition of *Piper gaudichaudianum* Kunth leaves were compared when using SE, PLE and UAE. The highest yield values were obtained with PLE and it also gave the highest β-sitosterol content. Additionally, Hirondart *et al.*[75] studied PLE as a green alternative to SE for the extraction of antioxidants from rosemary leaves and PLE was found to be more efficient for achieving higher extraction yields. A similar conclusion was also reached by Hu *et al.*[76] In their research, the total yield of ginger extract obtained by PLE was considerably higher than that obtained with SE (364.8 *versus* 96.3 mg g^{-1} dried ginger) with accompanying higher concentrations of the phenol compound gingerol. Considering that the extraction yield and composition of plant extracts mostly depend upon extraction technique used and based on the obtained results, PLE was shown to be an excellent technique for isolation of nettle root lipid fraction containing a remarkably higher content of lipophilic bioactive components.

CONCLUSIONS

The results of this research significantly contribute to the current knowledge on the comprehensive analysis of nettle root components consisting of sterols and, for the first time, isolated

pentacyclic triterpenoids, in terms of their isolation by an advanced extraction technique. In total, nine sterols and three pentacyclic triterpenoids were identified and quantified in the samples of nettle root lipid fraction. The most abundant sterol in all samples was β-sitosterol, whereas β-amyrin acetate was the dominant pentacyclic triterpenoid. When examining the influence of PLE parameters, temperature had a significant effect on the yield of the lipid fraction, whereas the contents of β-sitosterol and triterpenoid derivatives were significantly affected by all three parameters examined. Moreover, the contents of β-sitosterol and triterpenoid derivatives were significantly affected by the interactions of temperature *versus* static time and static time *versus* cycle number. The optimal PLE conditions for achieving the highest yields and efficient extraction of target compounds were 150 °C/5 min/four cycles. In addition, when optimal PLE conditions were compared to SE (68 °C, 8 h), PLE showed better performance and proved to be effective for isolating the lipid fraction from the nettle root giving remarkably higher yields of lipid fraction, total triterpenoid derivatives and β-sitosterol in a short time using less solvent. Because triterpenoid derivatives from nettle root can be used as functional ingredients for the development of new functional food and dietary supplements as a result of their beneficial effects on human health, future perspectives should include further studies on the stability of the mentioned compounds under processing and storage conditions, as well as *in vivo* studies of their biological effects and metabolism.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Chapter 5

Publication No.4: Effect of Spray Drying Encapsulation on Nettle Leaf Extract Powder Properties, Polyphenols and Their Bioavailability

Foods

Publication No.4

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Article

Effect of Spray Drying Encapsulation on Nettle Leaf Extract Powder Properties, Polyphenols and Their Bioavailability

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Abstract: Nettle (*Urtica dioica* L.) is a plant rich in a health-promoting compounds such as polyphenols, which are sensitive and unstable compounds with low bioavailability, that need to be stabilized and protected from external influences. Therefore, the aim of this study was to examine how the temperature, type of carrier and sample to carrier ratio influence the physicochemical properties and encapsulation and loading capacity of the nettle leaf extract powder and examine the effect of encapsulation on the antioxidant capacity and bioavailability of polyphenols. The process yield ranged from 64.63–87.23%, moisture content from 1.4–7.29%, solubility from 94.76–98.53% and hygroscopicity from 13.35–32.92 g 100 g^{-1}. The highest encapsulation (98.67%) and loading (20.28%) capacities were achieved at 160 °C, β-CD:GA (3:1) and sample:carrier ratio of 1:3. Extracts encapsulated at selected conditions showed high antioxidant capacity and distinct polyphenolic profile comprised of 40 different compounds among which cinnamic acids were the most abundant. Moreover, the encapsulation increased the bioavailability of nettle leaf polyphenols, with the highest amount released in the intestinal phase. Thus, the obtained encapsulated extract represents a valuable source of polyphenols and may therefore be an excellent material for application in value-added and health-promoting products.

Keywords: spray drying; nettle leaves; maltodextrin; gum arabic; β-cyclodextrin; antioxidant capacity

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1. Introduction

It is known that in nature there is a large number of plant species with undervalued biological potential that can be processed into various types of products, among which plant extracts certainly occupy an important place [1]. The reason for the popularity and growth of plant extracts is the increasing awareness among consumers about the quality of the food they consume, the presence of bioactive ingredients, and their potential beneficial effects on health [2]. For standardization and stabilization purposes, liquid plant extracts are often processed into powders that can be used as semi-finished or finished products.

Nettle (*Urtica dioica* L.) is one of the medicinal plant species that, due to its chemical composition and content of bioactive components, is an excellent basis for obtaining products with high biological potential. It is a perennial wild plant, known in folk medicine since ancient times, widely distributed and adapted to different climatic zones [3]. It is used both as food and medicine, as all parts of the nettle (leaf, stem, root) are a rich source of antioxidant phenolic compounds, vitamins and minerals. Accordingly, nettle is consumed in the form of tea, stews, soups, juices, etc. [4,5]. Although all parts of nettle contain significant amounts of biologically active molecules and possess medicinal properties, the leaves are the most valuable source [6,7]. The bioactive constituents of nettle leaves, among which phenolic compounds occupy an important place, act as radical scavengers and play an important role in the prevention of cancer, neurodegenerative and cardiovascular diseases, which is one of the reasons for the potential use of nettle leaves in the production of plant extracts, powders, etc. [8,9].

The emphasis on the use of plant products rich in bioactive ingredients that have a positive effect on human health, also increases the need to apply techniques that would lead to the production of foods with high stability and long shelf life. For these reasons, the obtained liquid plant extracts are often transformed into powder form. One of the ways to achieve this form is encapsulation by spray drying, in which liquid or semi-liquid foods are dried in a stream of hot air to produce a powder as the final product. The goal of this process is to quickly and efficiently remove the water from the food and obtain a powder with the desired physicochemical properties [10]. In addition, a physical barrier is created by protecting the unstable active ingredient from external influences (light, moisture, oxygen) [11,12]. Spray drying is an alternative to improve the preservation of the final product. The result is a product with higher stability, better quality, controlled release of biologically active molecules, longer shelf life and lower volume and weight, which facilitates storage, handling and transportation of the product [13,14]. The production of powders with desirable physicochemical properties is highly influenced by the properties of the solution to be dried, the characteristics and parameters of the spray drying equipment, and the appropriate choice of the carrier and its proportion in the mixture [15]. The most commonly used carriers in spray drying are polysaccharides (starch, maltodextrin, gum), proteins (gelatin, casein, soy proteins) and lipids (waxes, glycerides). In the food industry, these are maltodextrin (MD), gum arabic (GA) and β-cyclodextrin (β-CD). They are used due to their wide commercial availability, low cost, high solubility, low viscosity and ability to stabilize the product [16]. Since encapsulants have the potential to be used in a functional product, it is important to monitor their bioavailability as well. The bioavailability of polyphenolic compounds depends significantly on the structure and form in which they are taken into the body, and encapsulation has shown a protective effect on changes in pH and enzymatic activity during the digestive process. In this way, the polyphenols are delivered to a specific part of the digestion and released in a controlled manner [17,18]. From the above, it is clear that in order to obtain products with the best sensory and nutritional properties and higher yields, it is important to first optimize the encapsulation process itself.

According to the authors, there is only one work dealing with spray drying of nettle extract [19]. In that work, the extract was prepared by conventional techniques and the effects of temperature, flow rate, and different concentrations of maltodextrin on the process yield, total polyphenols, encapsulation efficiency, antioxidant activity and morphology of the powder were studied. That work differs from the methodology used in mentioned study. The aim of this study was to encapsulate a nettle leaf extract rich in phenolic compounds and to investigate the influence of temperature (120, 160 and 200 °C), type of carrier (maltodextrin, β-cyclodextrin and their combinations with gum arabic in the ratio 1:1 and 3:1, *w*/*w*) and the sample:carrier ratio (1:1, 1:2, 1:3, *w*/*w*) on the physicochemical properties of the obtained powders (process yield, moisture content, hygroscopicity, solubility, powder morphology) and on the encapsulation and loading capacity, the antioxidant capacity and bioavailability of polyphenols from the powder.

2. Materials and Methods

2.1. Chemicals

Distilled water was obtained using the Milli-Q water purification system (Millipore, Bedford, MA, USA). Ethanol (96%), sodium acetate (99%) and iron (III) chloride hexahydrate were purchased from Kemika d.d. (Zagreb, Croatia), methanol and sodium bicarbonate from Gram-mol d.o.o. (Zagreb, Croatia), and sodium chloride from Lachner (Neratovice, Czech Republic). Maltodextrin (DE 4–7) was procured from Sigma-Aldrich (St. Louis, MO, USA), and β-cyclodextrin, gum arabic, 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 (Geel, Belgium). Pepsin, pancreatin, bile salts and 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and hydrochloric acid (37%) from Carlo Erba Reagents (Val-de-Reuil, France), while acetic

acid was purchased from J.T.Baker (Deventer, The Netherlands). Standards for gallic acid, chlorogenic acid, protocatechuic acid, synaptic acid, ferulic acid, quinic acid, caffeic acid, *p*-coumaric acid, esculetin, quercetin-3-glucoside, kaempferol-3-glucoside, scopoletin and myricetin were obtained from Sigma-Aldrich (St. Louis, MO, USA) and catechin, epigallocatechin gallate, epicatechin gallate, luteolin, naringenin and apigenin were obtained from Extrasynthese (Genay, France).

2.2. Material

Commercially available dried nettle leaves (*Urtica dioica* L.) harvested in 2020 (Suban Ltd., Strmec, Croatia) were used for the experiment.

2.3. Microwave-Assisted Extraction (MAE)

Prior to extraction, nettle leaves were ground using an electric grinder (Waring WSG30, Sprzęt Laboratoryjny i Medyczny Labpartner KBS, Warszawa, Poland). The extract for encapsulation process was obtained by microwave-assisted extraction in Ethos Easy reactor (Milestone, Sorisole, Italy) which was carried out according to the optimal extraction parameters obtained on nettle leaves (temperature 60 °C, time 5 min, power 300 W) previously determined by Elez Garofulić et al. (2021) [20] with 30% ethanol as solvent, since the powders produced have the potential for application in in value-added and health-promoting functional products. In each extraction cell 10 g of sample, 60 mL of 30% aqueous ethanol solution (v/v) and magnetic stirrer were added. The cells were placed on the rotor of microwave reactor, the extraction parameters were set and an automatic extraction process was started. Subsequently, extract was filtered through Büchner funnel, collected and stored at −18 °C until spray-dried.

2.4. Spray Drying

Spray drying of the nettle extract was carried out using a laboratory device Büchi Mini Spray Dryer B-290 operating in closed mode with an inert loop B295 (Büchi, Switzerland). Nitrogen was used as a drying gas. The dry matter content of the liquid extract was 3.58%. During the process, the following parameters were kept constant: aspirator capacity at 80%, pump capacity at 15% and nozzle cleaner at level 4. Spray drying process was carried out according to the experimental design shown in Table 1. Three different carriers were used to perform the experiment: MD, GA and β-CD, where MD and β-CD were used as single carriers or in combination with GA in the ratio of 1:1 and 3:1 (w/w), respectively. Also, three different dry matter sample:carrier ratios (1:1, 1:2, 1:3, w/w) were used. A certain amount of carrier was added to the 100 mL of the water and stirred for 30 min at 50 °C on a magnetic stirrer RT 5 (IKA-Werke, Staufen im Breisgau, Germany) after which a homogeneous solution was mixed with 100 mL of extract. Spray drying was performed at three inlet temperatures: 120, 160 and 200 °C, while corresponding outlet temperatures were around 70, 85 and 100 °C. Powders were produced in duplicate and stored in hermetically sealed plastic containers in desiccator at room temperature until analyzed.

Table 1. Physicochemical properties and encapsulation capacity of nettle leaves extract powders obtained with different carrier agents added in different ratios under different temperatures.

Sample	Carrier	Sample: Carrier Ratio	Temperature (°C)	Process Yield (%)	Moisture Content (%)	Solubility (%)	Hygroscopicity (g 100 g^{-1})	Encapsulation Capacity (%)	Loading Capacity (%)
1	**MD**	**1:1**	**120**	74.92 ± 0.29	3.8 ± 0.62	82.21 ± 0.46	23.03 ± 0.11	97.61 ± 0.04	15.32 ± 0.09
2	**MD:GA (1:1)**			73.52 ± 0.03	7.29 ± 0.67	82.01 ± 0.79	25.93 ± 0.49	97.07 ± 0.16	14.29 ± 0.32
3	**MD:GA (3:1)**			73.12 ± 0.35	5.18 ± 0.49	87.46 ± 0.14	29.96 ± 0.25	97.37 ± 0.09	16.27 ± 0.16
4	**β-CD**			72.96 ± 0.82	4.55 ± 0.75	76.91 ± 1.34	24.26 ± 0.10	97.37 ± 0.26	17.33 ± 1.30
5	**β-CD:GA (1:1)**			70.87 ± 0.36	5.8 ± 0.37	90.63 ± 0.60	30.21 ± 1.21	97.59 ± 0.04	16.35 ± 0.22
6	**β-CD:GA (3:1)**			70.35 ± 0.99	5.49 ± 0.85	75.18 ± 0.72	27.66 ± 0.00	97.64 ± 0.09	17.67 ± 0.15
7	**MD**	**1:2**		75.22 ± 0.68	5.36 ± 0.01	83.75 ± 0.54	13.35 ± 0.65	98.51 ± 0.04	9.81 ± 0.25
8	**MD:GA (1:1)**			73.83 ± 1.23	6.88 ± 0.35	85.18 ± 0.51	15.04 ± 0.27	98.66 ± 0.06	9.32 ± 0.12
9	**MD:GA (3:1)**			77.61 ± 0.09	3.27 ± 0.64	80.95 ± 0.12	21.77 ± 0.42	97.76 ± 0.04	10.59 ± 0.04
10	**β-CD**			74.76 ± 0.45	4.87 ± 0.21	88.86 ± 0.24	14.11 ± 0.32	97.46 ± 0.23	10.56 ± 0.14
11	**β-CD:GA (1:1)**			75.93 ± 0.42	4.12 ± 0.28	88.35 ± 0.68	19.49 ± 0.74	97.76 ± 0.11	11.37 ± 0.07
12	**β-CD:GA (3:1)**			73.49 ± 0.13	4.81 ± 0.53	78.98 ± 0.29	20.29 ± 0.52	97.51 ± 0.11	10.98 ± 0.48
13	**MD**	**1:3**		77.17 ± 0.53	4.11 ± 0.46	90.41 ± 0.24	14.57 ± 0.75	98.40 ± 0.04	6.69 ± 0.07
14	**MD:GA (1:1)**			76.25 ± 0.59	3.74 ± 0.06	89.27 ± 0.57	18.08 ± 0.21	98.65 ± 0.09	7.24 ± 0.04
15	**MD:GA (3:1)**			82.42 ± 0.86	4.9 ± 0.6	91.32 ± 0.06	22.71 ± 0.84	97.72 ± 0.20	7.55 ± 0.08
16	**β-CD**			74.90 ± 0.44	4.49 ± 0.89	86.10 ± 0.75	14.60 ± 0.65	95.42 ± 0.13	11.02 ± 0.09
17	**β-CD:GA (1:1)**			81.47 ± 0.76	3.97 ± 0.52	91.43 ± 0.44	19.27 ± 0.54	97.84 ± 0.03	9.47 ± 0.04
18	**β-CD:GA (3:1)**			77.00 ± 0.45	3.48 ± 0.75	89.25 ± 0.79	18.19 ± 0.64	96.82 ± 0.05	9.60 ± 0.08
19	**MD**	**1:1**	**160**	80.35 ± 0.61	3.55 ± 0.83	85.01 ± 0.75	27.59 ± 0.36	98.14 ± 0.04	19.07 ± 0.26
20	**MD:GA (1:1)**			73.61 ± 0.10	4.9 ± 0.33	90.19 ± 0.03	32.92 ± 0.47	97.03 ± 0.07	14.31 ± 0.25
21	**MD:GA (3:1)**			75.06 ± 0.66	4.88 ± 0.64	87.28 ± 0.84	32.11 ± 0.62	97.26 ± 0.07	17.10 ± 0.06
22	**β-CD**			68.98 ± 0.42	4.9 ± 0.89	57.09 ± 0.15	25.55 ± 0.62	96.61 ± 0.05	12.88 ± 0.08
23	**β-CD:GA (1:1)**			64.63 ± 0.71	6.92 ± 0.6	88.24 ± 0.78	31.41 ± 0.23	97.11 ± 0.02	15.29 ± 0.19
24	**β-CD:GA (3:1)**			70.38 ± 0.23	4 ± 0.62	78.20 ± 0.92	29.36 ± 0.84	96.55 ± 0.03	14.50 ± 0.20
25	**MD**	**1:2**		77.56 ± 0.40	2.31 ± 0.49	89.59 ± 0.09	19.98 ± 0.41	97.51 ± 0.03	9.30 ± 0.04
26	**MD:GA (1:1)**			75.92 ± 0.12	2.66 ± 0.64	87.88 ± 0.86	24.21 ± 0.91	97.68 ± 0.09	9.93 ± 0.13
27	**MD:GA (3:1)**			80.16 ± 0.12	6.43 ± 0.3	87.63 ± 0.17	21.98 ± 0.40	97.31 ± 0.04	10.07 ± 0.07
28	**β-CD**			74.41 ± 0.08	3.52 ± 0.84	62.13 ± 0.96	15.88 ± 0.35	96.92 ± 0.13	11.56 ± 0.05
29	**β-CD:GA (1:1)**			78.52 ± 0.84	2.48 ± 0.77	88.64 ± 0.22	21.85 ± 0.28	97.72 ± 0.05	12.61 ± 0.02
30	**β-CD:GA (3:1)**			73.85 ± 0.32	4.45 ± 0.11	80.82 ± 0.41	21.79 ± 0.87	97.23 ± 0.02	12.96 ± 0.03
31	**MD**	**1:3**		87.23 ± 0.41	2.98 ± 0.76	87.77 ± 0.85	13.93 ± 0.13	97.91 ± 0.09	8.69 ± 0.20
32	**MD:GA (1:1)**			84.54 ± 0.93	3.61 ± 0.09	91.11 ± 0.80	15.92 ± 0.49	98.16 ± 0.15	7.61 ± 0.08
33	**MD:GA (3:1)**			78.59 ± 0.86	4.08 ± 0.55	83.63 ± 0.72	20.13 ± 0.65	97.90 ± 0.12	9.66 ± 0.18
34	**β-CD**			76.01 ± 0.80	2.65 ± 0.71	86.53 ± 0.04	16.37 ± 0.43	95.68 ± 0.07	13.20 ± 0.02
35	**β-CD:GA (1:1)**			81.86 ± 0.71	3.79 ± 0.66	89.51 ± 0.47	22.57 ± 0.37	97.47 ± 0.11	10.42 ± 0.08
36	**β-CD:GA (3:1)**			80.28 ± 0.70	3.6 ± 0.1	90.69 ± 0.73	18.25 ± 0.44	98.67 ± 0.07	20.28 ± 0.17

Table 1. *Cont.*

Sample	Carrier	Sample: Carrier Ratio	Temperature (°C)	Process Yield (%)	Moisture Content (%)	Solubility (%)	Hygroscopicity (g 100 g^{-1})	Encapsulation Capacity (%)	Loading Capacity (%)
37	**MD**	**1:1**	**200**	76.32 ± 1.05	4.05 ± 0.61	90.24 ± 0.62	20.82 ± 0.56	96.68 ± 0.10	13.11 ± 0.05
38	**MD:GA (1:1)**			74.51 ± 0.80	4.82 ± 0.45	91.37 ± 0.14	22.75 ± 0.55	95.52 ± 0.20	11.68 ± 0.07
39	**MD:GA (3:1)**			72.41 ± 0.04	3.87 ± 0.19	88.96 ± 0.55	28.11 ± 0.47	96.56 ± 0.00	14.59 ± 0.05
40	**β-CD**			69.50 ± 0.55	3.27 ± 0.03	60.24 ± 0.53	24.23 ± 0.72	96.81 ± 0.14	16.10 ± 0.33
41	**β-CD:GA (1:1)**			72.57 ± 0.18	2.69 ± 0.35	90.36 ± 0.17	29.76 ± 0.85	96.96 ± 0.14	17.13 ± 0.53
42	**β-CD:GA (3:1)**			68.96 ± 1.03	1.64 ± 0.55	75.80 ± 1.01	26.01 ± 0.61	96.54 ± 0.18	15.41 ± 0.37
43	**MD**	**1:2**		78.25 ± 0.68	3.92 ± 0.6	86.89 ± 0.06	15.77 ± 0.67	96.68 ± 0.00	9.20 ± 0.12
44	**MD:GA (1:1)**			76.60 ± 0.00	3.43 ± 0.27	89.37 ± 0.73	17.47 ± 0.53	97.32 ± 0.03	11.20 ± 0.10
45	**MD:GA (3:1)**			79.42 ± 0.71	4.13 ± 0.4	90.72 ± 0.59	21.31 ± 0.61	97.48 ± 0.03	10.19 ± 0.03
46	**β-CD**			67.96 ± 1.41	1.4 ± 0.04	64.80 ± 0.77	19.08 ± 0.16	96.03 ± 0.08	11.31 ± 0.08
47	**β-CD:GA (1:1)**			76.18 ± 0.94	3.87 ± 0.53	90.48 ± 0.85	26.40 ± 0.26	96.31 ± 0.06	11.49 ± 0.08
48	**β-CD:GA (3:1)**			74.78 ± 0.41	3.5 ± 0.76	81.52 ± 0.92	24.01 ± 1.18	96.00 ± 0.01	11.20 ± 0.07
49	**MD**	**1:3**		83.42 ± 0.07	3.11 ± 0.3	83.07 ± 0.30	13.64 ± 0.55	96.13 ± 0.03	7.16 ± 0.09
50	**MD:GA (1:1)**			83.29 ± 0.48	3.38 ± 0.11	91.52 ± 0.75	17.10 ± 0.25	97.47 ± 0.09	7.75 ± 0.03
51	**MD:GA (3:1)**			81.62 ± 0.56	2.63 ± 0.72	92.83 ± 0.67	19.90 ± 1.30	97.45 ± 0.16	7.95 ± 0.03
52	**β-CD**			78.54 ± 0.81	2.66 ± 0.22	87.24 ± 0.84	15.63 ± 0.38	95.57 ± 0.10	13.70 ± 0.02
53	**β-CD:GA (1:1)**			75.44 ± 0.31	4.32 ± 0.15	90.05 ± 1.15	23.17 ± 0.90	97.37 ± 0.03	9.14 ± 0.24
54	**β-CD:GA (3:1)**			70.07 ± 0.28	2.51 ± 0.78	91.22 ± 0.92	18.01 ± 0.45	96.16 ± 0.09	9.24 ± 0.01
	MEAN			75.88	4.02	89.94	21.62	97.20	12.01

Results are expressed as mean ± SD (N = 4).

2.5. Characterization of the Microcapsules

2.5.1. Process Yield

The yield of the spray drying process was calculated according to the following equation [21]:

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

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

2.5.2. Moisture Content

The moisture content of encapsulated extracts was determined by drying in an oven Heratherm OMH100 (Thermo Scientific, Dreieich, Germany) at 105 °C to constant weight (AOAC, 1984).

2.5.3. Solubility

The solubility of the encapsulated extracts was determined according to the modified method described by Anderson et al. (1969) [22]. 1 g of encapsulated extract was dissolved in 10 mL of distilled water in a test tube, stirred at vortex mixer for 1 min, thermostated at 37 °C in a B-490 water bath (Büchi, Flawil, Switzerland) for 30 min and then centrifuged (Rotofix 32, Hettich, Kirchlengern, Germany) for 20 min at 5500 rpm. The resulting supernatant was dried in an oven at 105 °C to a constant mass.

Solubility was calculated according to the following equation:

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

where m_s is the mass (g) of powdered extract obtained by drying the supernatant to constant weight and m_p is the mass (g) of powdered extract taken for analysis.

2.5.4. Hygroscopicity

The hygroscopicity of the encapsulated nettle extracts was determined by the method described by Tonon et al. (2008) [10]. A mass of 1 g of microcapsules was placed in an open Petri dish in a desiccator containing saturated NaCl solution (75.29% humidity) for 7 days at 25 °C. After 7 days, the sample was weighed and hygroscopicity was expressed as grams of adsorbed moisture per 100 g of microcapsules (g 100 g^{-1}) according to the following equation:

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

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

2.5.5. Encapsulation and Loading Capacity

Encapsulation capacity is determined through the ratio of surface and total phenolic compounds in microcapsules, according to the method of Robert et al. (2010) [23] and loading capacity is determined through amount of total phenolic compounds in microcapsules and weight of microcapsules after spray drying [24].

For extraction of total polyphenols, 0.2 g of powder was mixed with 2 mL of methanol: acetic acid:water solvent (50:8:42, $v/v/v$) in a test tube. The mixture was stirred on a vortex mixer for 1 min and extracted in an ultrasonic bath at room temperature for 20 min. After extraction, the mixture was centrifuged at 3000 rpm for 10 min. The content of total polyphenols was determined using the Folin–Ciocalteu method reagent [25].

To extract the surface polyphenols 0.2 g of powder was mixed with 2 mL of ethanol: methanol solvent (50:50, v/v) and stirred on a vortex mixer for 1 min and centrifuged at 3000 rpm for 10 min. The supernatant was filtered through a filter paper and the surface polyphenol content was determined in the same way as total polyphenols.

The encapsulation capacity is calculated through the ratio of surface and total polyphenols according to the following formula:

$$EC(\%) = \left(\frac{TP - SP}{TP}\right) \times 100 \quad (4)$$

where TP is concentration of total polyphenols (mg gallic acid g^{-1}) and SP is concentration of surface polyphenols (mg gallic acid g^{-1}).

The loading capacity is calculated through the ratio of total polyphenols and weight of microcapsules after spray drying according to the following formula:

$$LC\ (\%) = \frac{TP}{MC} \times 100 \quad (5)$$

where TP is amount of polyphenols in microcapsules (g) and MC is weight of microcapsules (g) after spray drying.

2.5.6. Bioavailability

Bioavailability was determined according to in vitro method described by McDougall et al. (2007) [26] and Gunathilake et al. (2018) [27] with some modifications. In the first phase, gastric conditions were simulated by mixing 250 mg of the powder with 10 mL of 0.9% NaCl solution and 800 µL of 40 mg/mL pepsin dissolved in 0.1 M HCl in Falcon tubes. Samples were adjusted to pH 2 with 0.1 M HCl and incubated at 37 °C for one hour with shaking at 100 rpm. Then, 2 mL aliquot was taken from the tube to determine the polyphenol content. The intermediate phase simulated the transition from the stomach to the small intestine, where 1 mL of 0.9% NaCl and 1 mL of 0.5 M $NaHCO_3$ are added to the dialysis membranes (6–8 kDa) and returned to the gastric solution. Samples were incubated at 37 °C at 100 rpm for 45 min, and then adjusted to pH 6.5 by addition of 1M $NaHCO_3$. In the final phase, conditions in the small intestine were simulated by adding 2.5 mL of pancreatin (2 mg/mL)-bile salt (12 mg/mL) solution to the samples at adjusted pH and incubating the samples for 2 h at 37 °C with shaking at 100 rpm. Subsequntly, 2 mL aliquots were taken from the membrane and tube to determine the polyphenol content by Folin–Ciocalteu method.

2.5.7. Antioxidant Capacity

Antioxidant capacity of nettle encapsulated extracts was determined by two types of assays. Both assays were determined according to the method described by Dobroslavić et al. (2022) [28].

FRAP (Ferric Reducing Antioxidant Power) Assay

The FRAP reagent was prepared by mixing 0.3 M sodium acetate buffer, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution dissolved in 40 mM hydrochloride acid and an aqueous solution of 20 mM iron (III) chloride hexahydrate in a 10:1:1 ratio. In addition, 240 µL of distilled water, 80 µL of sample and 2080 µL of FRAP reagent were added to the glass test tubes, stirred in vortex mixer and thermostatted at 37 °C for 5 min. Then, the absorbance was measured at 593 nm using a spectrophotometer. A calibration curve (y = 0.0013) was prepared using Trolox standard solutions (25–1000 µM).

DPPH Radical Scavenging Assay

Prior to analysis, a 0.2 mM DPPH (2,2-diphenyl-1-picrylhydrazyl radical) solution in methanol was prepared. Then, 0.75 mL of the sample and 1.5 mL of the 0.2 mM DPPH

solution were added to the glass test tubes. The tubes were placed in the dark at room temperature for 20 min after which the absorbance was measured at 517 nm using a spectrophotometer. A calibration curve (y = −0.008x + 1.3476) was prepared using Trolox standard solutions (10–150 μM).

2.5.8. Scanning Electron Microscopy (SEM)

The morphology of the microcapsules was studied using the high-resolution field emission scanning electron microscope (SEM) JSM-7000F (Jeol, Tokyo, Japan) at the Ruđer Bošković Institute, Division of Materials Chemistry, Zagreb, Croatia. Nettle powder samples were deposited in a thin layer on a carbon tape on the sample holder of the electron microscope to fix them and ensure electrical contact with the rest of the instrument. Images were acquired with an accelerating voltage of 5.0 kV at a standard distance of the objective from the sample (WD = 10 mm), and photomicrographs were taken of each sample at 2000× magnification. A secondary electron detector was used to produce the micrograph or image. The morphological characteristics were studied on the microcapsules with the highest polyphenol encapsulation capacity prepared using different carriers at 160 °C and a ratio of dry matter of extract and carriers 1:3.

2.5.9. UPLC-MS/MS Analysis of Polyphenols

Prior to UPLC analysis, 1 g of powder was dissolved in 10 mL of 80% methanol solution and extracted for 20 min at 50 °C in an ultrasonic bath. The obtained extract was filtered through 0.45 μm PTFE membrane filter. Identification and quantification of polyphenols of nettle leaf extract powder with the highest encapsulation capacity was performed by ultra-high performance liquid chromatography with mass spectrometry (UPLC/MS-MS) (Agilent 6430 Triple Quad LC/MS, Agilent Technologies, Santa Clara, CA, USA). Analytes were ionized using an ESI ion source with nitrogen as an inert gas (temperature 300 °C, flow rate 11 L h^{-1}), capillary voltage +4 −3.5 kV^{-1} and nebulizer pressure set at 40 psi. The mass spectrometer was connected to a UPLC system (Agilent series 1290 RRLC instrument) which consisted of a binary pump, an autosampler and a column thermostat. Reverse phase separation was performed on Zorbax Eclipse Plus C18 columns 100 × 2.1 mm with a particle size of 1.8 μm (Agilent, Santa Clara, CA, USA). The column temperature was set at 35 °C, and the injection volume was 2.5 μL. Solvent composition and gradient parameters were as previously described by Elez Garofulić et al. (2018) [29]. Software was used for instrument control and data processing Agilent MassHunter Workstation (ver. B.04.01). The identification of phenolic compounds was carried out by comparing the retention time of separated compounds (Rt) with the retention times of standards, polarity and comparing the characteristic values of precursor ions (m/z) and fragment ions (m/z) that are specific for each individual compound.

Quantitative determination was carried out using the calibration curves of the standards, where *p*-hydroxybenzoic acid was calculated as gallic acid equivalent and genistic acid according to protocatechuic acid. Quercetin, isorhamnetin, quercetin pentoside, quercetin 3-*O*-rhamnoside, quercetin acetyl-hexoside, quercetin pentosyl-hexoside, quercetin-acetyl-rutinoside, isorhamnetin 3-*O*-rutinoside and quercetin 3-*O*-rutinoside were calculated according to quercetin-3-glucoside, kaempferol 3-*O*-rutinoside, kaempferol pentoside, kaempferol rhamnoside, kaempferol pentosylhexoside and kaempferol according to kaempferol-3-glucoside, epicatehin according to catechin, apigenin 7-*O*-glucoside and genistein according to apigenin, while umbelliferone was expressed as scopoletin equivalent. All analyses have been performed in a duplicate and concentrations of analyzed compounds are expressed as mg 100 g^{-1} of dry matter (dm) (N = 4).

2.6. Experimental Design and Statistical Analysis

Statistica 12.0 (StatSoft, Inc., Tulsa, OK, USA) was used for experimental design and statistical data processing. The experiments were designed as mixed full factorial design with 2 factors on three and 1 factor on six levels. The influence of temperature (120, 160

and 200 °C), carrier type (MD, MD:GA (1:1), MD:GA (3:1), β-CD, β-CD:GA (1:1) and β-CD:GA (3:1)) and sample:carrier ratio (1:1, 1:2, 1:3) were observed as independent variables, giving in total 54 experimental runs. The dependent variables (process yield, dry matter, solubility, hygroscopicity, encapsulation and loading capacity) were analyzed by analysis of variance (ANOVA). The normality of the residuals was checked by Shapiro-Wilks test and homoscedasticity by the Levene test. A statistically significant difference was considered at the level of $p \leq 0.05$ (95% confidence interval), and marginal means were compared using Tukey's HSD test.

3. Results and Discussion

In order to obtain an encapsulated nettle extract with the best physical and chemical properties and with the highest retention and stability of polyphenols, the spray drying encapsulation process needs to be optimized. In addition to the inlet temperature, the type and proportion of the carrier also plays an important role. The experimental design for the production of powders from nettle leaf extract is shown in Table 1 as well as the results of physicochemical properties (process yield, moisture content, solubility, hygroscopicity) and encapsulation and loading capacity of obtained powders. The influence of spray drying parameters on the analyzed properties was tested by ANOVA and is presented in Table 2. Also, the morphology of the selected powders is shown in Figure 1, and antioxidant capacity in Table 3 In addition, the bioavailability of polyphenols in selected powders was studied (Figure 2) and the difference in bioavailability of polyphenols in non-encapsulated and encapsulated extract was demonstrated (Figure 3). Also, UPLC-MS/MS identification and quantification of polyphenols was carried out (Table 4).

Table 2. Influence of spray drying parameters on the process yield, moisture content, solubility, hygroscopicity, encapsulation and loading capacity of nettle leaves extract powders.

	N	Process Yield (%)	Moisture Content (%)	Solubility (%)	Hygroscopicity (g 100 g^{-1})	Encapsulation Capacity (%)	Loading Capacity (%)
Temperature (°C)		$p = 0.23$	$p < 0.01$	$p = 0.12$	$p = 0.26$	$p < 0.01$	$p = 0.36$
120	36	75.32 ± 0.51 ^{a}	4.78 ± 0.19 ^{c}	85.46 ± 0.84 ^{a}	20.70 ± 0.89 ^{a}	97.62 ± 0.13 ^{c}	11.75 ± 0.58 ^{a}
160	36	76.77 ± 0.91 ^{a}	3.98 ± 0.22 ^{b}	84.00 ± 1.57 ^{a}	22.88 ± 0.97 ^{a}	97.38 ± 0.11 ^{b}	12.74 ± 0.58 ^{a}
200	36	75.55 ± 0.78 ^{a}	3.29 ± 0.15 ^{a}	85.37 ± 1.54 ^{a}	21.29 ± 0.76 ^{a}	96.61 ± 0.11 ^{a}	11.53 ± 0.49 ^{a}
Carrier		$p < 0.01$	$p = 0.28$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$
MD	18	78.94 ± 0.94 ^{b}	3.69 ± 0.21 ^{a}	86.55 ± 0.73 ^{ab}	18.08 ± 1.16 ^{a}	97.51 ± 0.19 ^{b}	10.93 ± 0.93 ^{ab}
MD:GA (1:1)	18	76.90 ± 0.96 ^{ab}	4.52 ± 0.37 ^{a}	88.66 ± 0.74 ^{c}	21.05 ± 1.35 ^{ab}	97.51 ± 0.23 ^{b}	10.37 ± 0.62 ^{a}
MD:GA (3:1)	18	77.82 ± 0.83 ^{b}	4.38 ± 0.27 ^{a}	87.87 ± 0.86 ^{b}	24.22 ± 1.05 ^{b}	97.41 ± 0.09 ^{b}	11.55 ± 0.80 ^{ab}
β-CD	18	73.11 ± 0.83 ^{a}	3.59 ± 0.30 ^{a}	74.43 ± 3.04 ^{a}	18.86 ± 1.05 ^{a}	96.43 ± 0.21 ^{a}	13.07 ± 0.53 ^{ab}
β-CD:GA (1:1)	18	75.27 ± 1.24 ^{ab}	4.22 ± 0.33 ^{a}	89.74 ± 0.28 ^{c}	24.90 ± 1.07 ^{b}	97.35 ± 0.11 ^{b}	12.08 ± 0.68 ^{ab}
β-CD:GA (3:1)	18	73.24 ± 0.86 ^{a}	3.72 ± 0.29 ^{a}	82.41 ± 1.45 ^{ab}	22.62 ± 1.00 ^{ab}	97.01 ± 0.13 $_{ab}$	13.54 ± 0.86 ^{b}
Ratio sample:carrier		$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p = 0.16$	$p < 0.01$
1:1	36	72.39 ± 0.57 ^{a}	4.53 ± 0.13 ^{b}	82.08 ± 1.66 ^{a}	27.32 ± 0.57 ^{b}	97.02 ± 0.10 ^{a}	15.47 ± 0.31 ^{c}
1:2	36	75.80 ± 0.46 ^{b}	3.97 ± 0.24 ^{ab}	83.70 ± 1.35 ^{a}	19.65 ± 0.61 ^{a}	97.32 ± 0.12 ^{a}	10.76 ± 0.18 ^{b}
1:3	36	79.45 ± 0.70 ^{c}	3.56 ± 0.24 ^{a}	89.05 ± 0.46 ^{b}	17.89 ± 0.50 ^{a}	97.27 ± 0.17 ^{a}	9.80 ± 0.54 ^{a}

Results are expressed as mean ± SD (N = 4). Values with different letters within column are statistically different at $p < 0.05$.

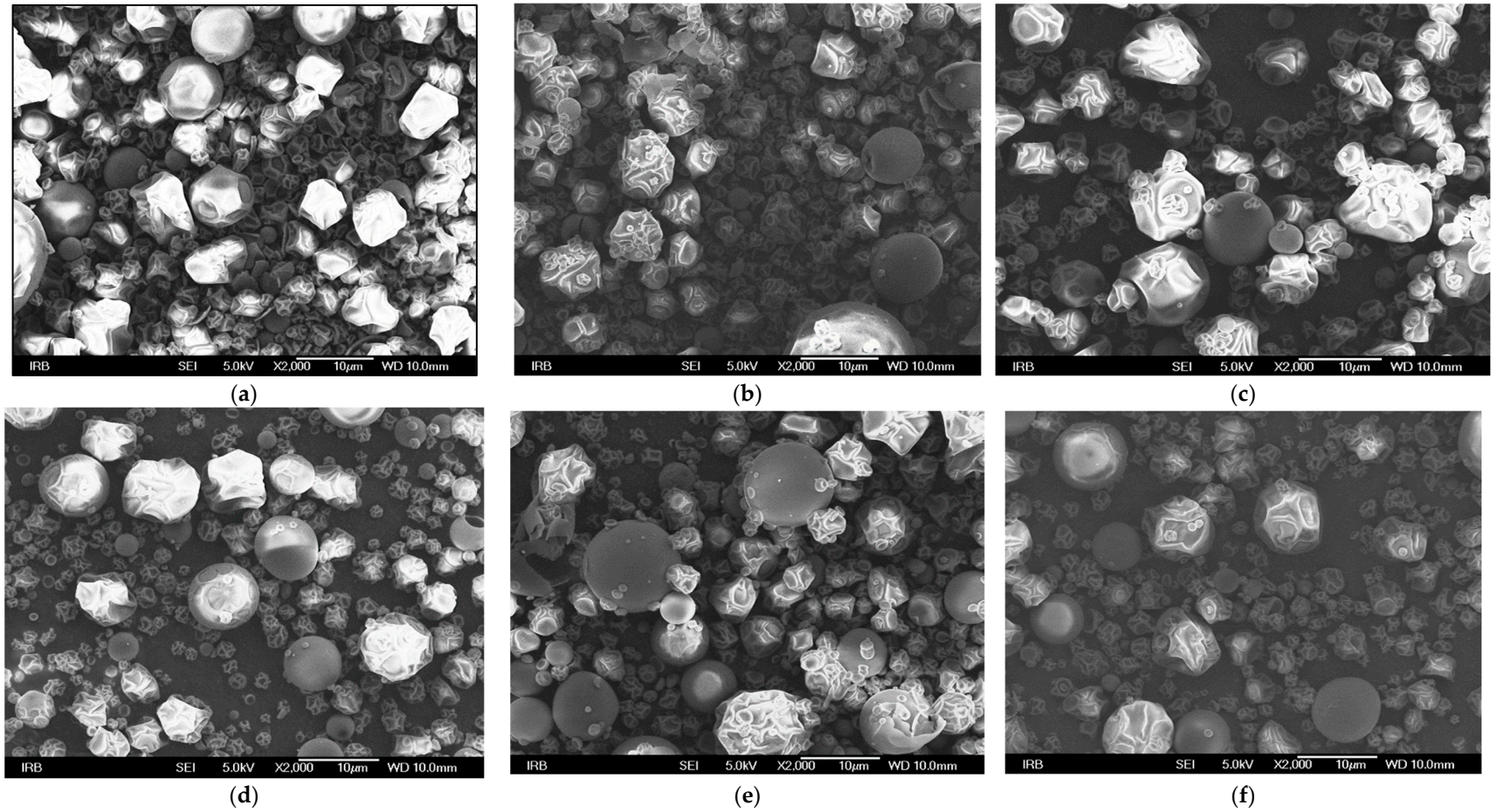

Figure 1. SEM images of the microcapsules of encapsulated nettle leaves extract with different carriers: (**a**) MD (**b**) MD:GA (1:1) (**c**) MD:GA (3:1) (**d**) β-CD (**e**) β-CD:GA (1:1) (**f**) β-CD:GA (3:1) at 160 °C and sample:carrier ratio 1:3.

Table 3. Antioxidant capacity of nettle leaf powder obtained at same conditions (160 °C and sample:carrier ratio 1:3) with different carriers.

Sample	Carrier	Sample: Carrier Ratio	Temperature (°C)	FRAP (mmol TE $100g^{-1}$ dm)	DPPH (mmol TE $100g^{-1}$ dm)
31	**MD**	**1:3**	**160**	6.13 ± 0.18^{a}	9.10 ± 0.08^{a}
32	**MD:GA (1:1)**			8.57 ± 0.40^{b}	12.42 ± 0.16^{b}
33	**MD:GA (3:1)**			8.30 ± 0.62^{b}	12.11 ± 0.03^{b}
34	**β-CD**			9.59 ± 0.16^{bc}	12.74 ± 0.12^{b}
35	**β-CD:GA (1:1)**			10.55 ± 0.20^{c}	12.13 ± 0.34^{b}
36	**β-CD:GA (3:1)**			11.04 ± 0.27^{cd}	15.57 ± 0.01^{c}

Results are expressed as mean ± SD (N = 4). Values with different letters within column are statistically different at $p < 0.05$.

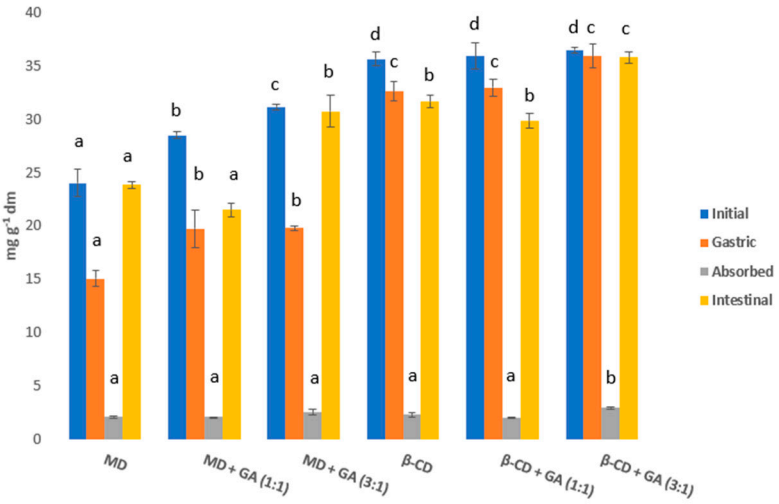

Figure 2. In vitro bioavailability of polyphenols from encapsulated nettle extracts obtained at same conditions (160 °C and sample:carrier ratio 1:3) with different carriers. Values with different letters within phases are statistically different at $p < 0.05$.

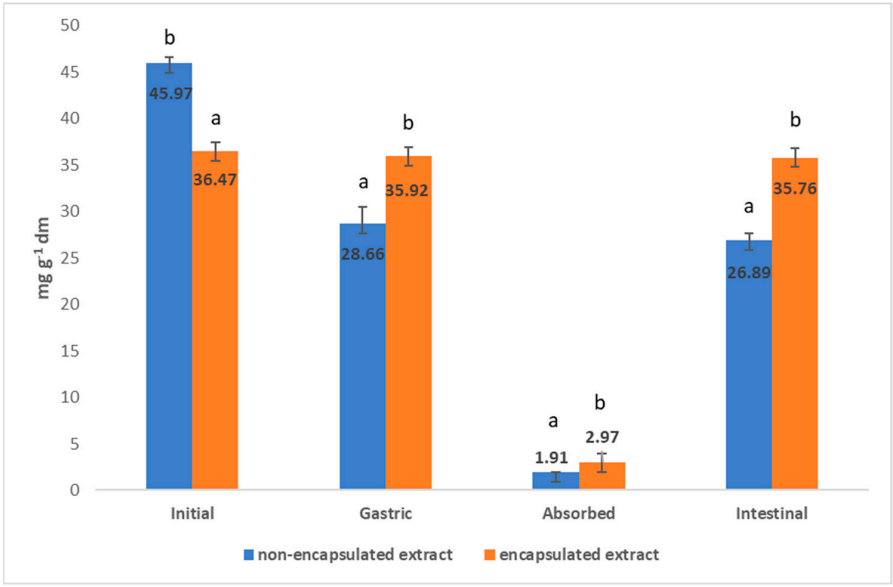

Figure 3. Comparison of the in vitro bioavailability of non-encapsulated extract and encapsulated extract with best properties. Values with different letters within phases are statistically different at $p < 0.05$.

Table 4. Polyphenolic profile including mass spectrometric data and concentration of identified individual compounds of nettle leaves extract powder with the highest encapsulation capacity.

		Mass Spectrometric Data		Concentration (mg 100 g^{-1})
Compound	**Rt (min)**	**Precursor Ion (*m*/*z*)**	**Fragment Ion (*m*/*z*)**	**Encapsulated Sample 36**
FLAVONOLS				
Quercetin-acetyl-rutinoside	11.317	653	303	1.45 ± 0.08
Isorhamnetin 3-*O*-rutinoside	1.384	625	317	0.36 ± 0.05
Quercetin 3-*O*-rutinoside	11.16	611	303	1.00 ± 0.05
Quercetin-pentosyl-hexoside	11.498	597	303	0.11 ± 0.03
Kaempferol 3-*O*-rutinoside	2.173	595	287	0.29 ± 0.03
Kaempferol-pentosyl-hexoside	11.344	581	287	0.28 ± 0.02
Quercetin-acetyl-hexoside	11.511	507	303	0.21 ± 0.04
Kaempferol 3-*O*-glucoside *	2.193	449	287	0.09 ± 0.03
Quercetin 3-*O*-rhamnoside	12.013	449	303	0.65 ± 0.10
Quercetin-pentoside	9.236	435	303	0.26 ± 0.07
Kaempferol-rhamnoside	10.551	433	287	0.63 ± 0.04
Kaempferol-pentoside	8.054	419	287	0.12 ± 0.07
Quercetin	7.732	301	151	0.01 ± 0.00
Isorhamentin	6.265	315	300	0.01 ± 0.00
Myricetin *	1.201	319	273	5.79 ± 0.12
Kaempferol	11.58	285	285	9.44 ± 0.04
FLAVAN-3-OLS				
Epigallocatechin gallate *	9.711	459	289, 139	0.31 ± 0.05
Epicatechin gallate *	10.872	443	291	0.09 ± 0.03
Epicatechin	12.067	291	139	57.66 ± 0.04
Catechin *	11.127	291	165	0.29 ± 0.07
FLAVONES				
Apigenin 7-*O*-glucoside	1.863	433	271	2.00 ± 0.11
Apigenin *	7.025	271	153	3.25 ± 0.05
Luteolin *	1.266	287	153	0.83 ± 0.07
ISOFLAVONES				
Genistein	7.65	269	133	2.76 ± 0.11
FLAVANONES				
Naringenin *	1.091	271	151	0.11 ± 0.05
COUMARINS				
Umbelliferone	0.803	161	133	0.99 ± 0.09
Esculetin *	1.417	177	133	13.59 ± 0.11
Scopoletin *	0.947	191	176	0.80 ± 0.05
BENZOIC ACIDS				
Protocatechuic acid *	0.807	153	109	14.41 ± 0.43
Gallic acid *	11.292	169	125	2.86 ± 0.12
Syringic acid *	10.037	197	182	0.25 ± 0.04
Gentisic acid	1.148	153	109	14.78 ± 0.63

Table 4. *Cont.*

***p*-hydroxybenzoic acid**	11.313	137	93	6.70 ± 0.21
CINNAMIC ACIDS				
Chlorogenic acid *	0.909	353	191	1.73 ± 0.05
Sinapic acid *	4.213	223	193	0.25 ± 0.04
Ferulic acid *	6.544	193	178	8.82 ± 0.15
Caffeic acid *	1.414	179	135	194.82 ± 2.92
***p*-coumaric acid ***	3.624	163	119	2.30 ± 0.10
Cinnamic acid *	4.465	147	103	828.47 ± 1.09
OTHER ACIDS				
Quinic acid *	0.786	191	85	109.61 ± 0.24
TOTAL POLYPHENOLS				1288.39

* Identification confirmed using authentic standards. Results are expressed as mean ± standard deviation.

3.1. Process Yield

As shown in Table 1, the process yield of obtained encapsulated extracts ranged between 64.63–87.23%, with a mean of 75.88%. According to Bhandari et al. (1997) [30], spray drying process can be considered successful when the achieved process yield is above 50%, which was obtained on all encapsulated extracts produced in this study. Losses of powder particles which consequently lead to lower yields, can occur due to particles sticking to the wall of the drying chamber, being pumped off through the outlet air filter, or due to manual operations when collecting powder [31]. The results of spray drying process in this study are relatively high compared to the process yields of other similar plant species [19,32–34]. This could be due to the use of different feed compositions, drying conditions, carriers in different proportions and manual operations.

Table 2 shows that both type and proportion of carrier had a statistically significant influence ($p < 0.01$) on process yield, while temperature had no effect ($p = 0.23$). The highest process yield was obtained when MD or its combination with GA (3:1) was used as carrier while the lowest process yield was obtained with β-CD and its combination with GA (3:1). Navarro-Flores et al. (2020) [34] investigated the influence of different carriers on the physicochemical properties of the powder obtained from the methanolic extract of *Crotalaria longirostrata* leaves. The highest process yield in spray drying was obtained when a combination of MD and GA was used as carrier and MD as single carrier. Also, Nadeem et al. (2011) [35] who spray dried the water extract of mountain tea concluded that higher process yield was obtained with MD compared to β-CD and GA. Moreover, the process yield increased when the amount of carrier in the feed solution was increased. This is due to an increase in the total solids in the drying solution, and the addition of a carrier reduces the stickiness so that the particles do not stick to the chamber, which in turn increases the process yield. The same conclusion was reached by Daza et al. (2015) [36] who spray dried Cagaita fruit extracts with GA and inulin, and as the proportion of carriers increased from 10% to 30%, the process yield also increased.

3.2. Moisture Content

Moisture content is an important factor affecting the stability of encapsulated extracts. If the moisture content of the powdered extract is relatively low (<5%), this prolongs its shelf life because there is less microbiological contamination, better solubility, and in general greater stability of its properties and thus the possibility of application of the powder for technological purposes [32]. The moisture content of the nettle leaf extract powder was determined in a range of 1.4–7.29% with a mean of 4.02% (Table 1), where most of the powders satisfied the stated thesis. Sablania and Bosco (2018) [37] optimized the spray drying process for *Murraya koenigii* leaves extract and determined moisture content

in a range of 3–5.2% which is in accordance with this study. On the other hand, Tran and Nguyen (2018) [38] indicated that a moisture content in lemongrass powders were ranging from 8.49 to 13.11%, which is higher than in this study.

Moisture content was significantly ($p < 0.01$) affected by temperature and carrier content, whereas carrier type did not play a statistically significant role ($p = 0.28$) (Table 2). As the drying temperature increased from 120 to 200°C, the moisture content decreased. Nadeem et al. (2013) [33] stated that the moisture content in sage powders ranged from 3–5%, and decreased with increasing temperature from 145–165 °C, which is consistent with the results of this study. The same conclusion was also reached by the results of other authors [36,39,40]. As the inlet temperature increases, the moisture content decreases, which is due to a faster heat transfer between material to be dried and the heated air. At higher inlet temperatures, there is a greater temperature gradient between the atomized particles and the drying air, resulting in greater driving forces for water evaporation [10,41]. The lowest proportion of moisture content in powder was found at a dry matter ratio of 1:3 of the sample and the carrier. In general, the addition of a carrier decreases the moisture content in the material and thus reduces the proportion of water available for evaporation [42,43].

3.3. Solubility

Solubility is also one of the most important parameters of encapsulated extract' stability. Poor solubility can lead to difficulties in further processing [36]. Consequently, moisture content and particle size affect solubility. Thus, solubility increases with decreasing moisture content and larger particles sink and dissolve faster. As shown in Table 1, the solubility of nettle leaf extract powder ranged from 57.09 to 92.83%, with mean of 89.94%. In the research by Susantikarn and Donlao (2015) [44], the solubility ranged from 94.76–98.53% in green tea powders which is a bit higher than the results of this study.

Moreover, the solubility was significantly ($p < 0.01$) affected by the type of carrier and its proportion, while temperature had no effect ($p = 0.12$). Combinations of the carriers MD and β-CD with GA in the ratio 1:1 proved to be the best combinations and showed the highest solubility of the nettle extract powder, while the lowest solubility was found when β-CD was used as single carrier. Fazaeli et al. (2012) [39] concluded that a mixture of MD and GA is better than using each carrier separately. The increase in solubility by combined carriers is probably due to the chemical structure of the carrier itself. Maltodextrin contains numerous hydroxyl groups that facilitate the dissolution process, while GA has good emulsifying properties and highly branched structure in addition to good solubility [45,46]. The solubility was the lowest when a β-CD was used as a carrier. This is because of β-CD is the least soluble in water of all the carriers observed due to intramolecular hydrogen bonds between the hydroxyl groups of adjacent glucose units. A similar conclusion was reached by Pudziuvelyte et al. (2019) [47] who studied the influence of different carriers on the physicochemical properties of powder from *Elsholtzia ciliate* herb. The highest solubility of the powder was determined with resistant-maltodextrin, and the lowest with β-CD. In addition, increasing the amount of carrier in the drying solution, the solubility also increased. Thus, the highest solubility of the powder was found at a ratio of 1:3 between the dry matter of the extract and the carrier. Daza et al. (2015) [36] investigated the effect of carrier content on the solubility of Cagaita fruit powder. They also concluded that the solubility of the powder increased by increasing the carrier content from 10% to 30%.

3.4. Hygroscopicity

Hygroscopicity is a parameter that can be used to predict the behavior of an encapsulated extract during storage and indicates its stability. The results of the research show that the hygroscopicity of the encapsulated nettle leaf extract ranged between 13.35–32.92 g 100 g^{-1} (Table 1) with mean of 21.62 g 100 g^{-1}. Zokti et al. (2016) [48] and Susantikarn and Donlao (2015) [44] encapsulated green tea extracts by spray drying

and concluded that hygroscopicity values of obtained powders were lower than in this research (3.22–4.71% and 8.61–13.72%).

Carrier type and sample:carrier ratio had a statistically significant effect ($p < 0.01$) on hygroscopicity, while temperature did not ($p = 0.26$). The lowest hygroscopicity was observed in powders when MD and β-CD were used as single carriers, and the value was slightly lower when MD was used as a carrier, while it was higher when they were used in combination with GA. This could be due to the chemical structure of the carriers. Maltodextrin itself has low hygroscopicity and is therefore a very effective carrier for spray drying, and the lower the degree of polymerization of the carrier, the lower the degree of water adsorption, while GA has a branched structure, so water molecules bind more easily to hydroxyl groups in the GA structure [49]. Also, as the proportion of carrier in the solution increased, the hygroscopicity decreased. In general, the addition of a carrier to the material to be dried increases the dry matter content of the material, resulting in the production of a powder that ultimately contains less water and consequently has a lower hygroscopicity [50,51]. Mishra et al. (2013) [52] studied the effect of concentrations of MD on the hygroscopicity of powder obtained from amla juice and Vidović et al. (2014) [32] on *Satureja montana* powder and concluded that the higher the carrier concentration, the lower the hygroscopicity, which is consistent with the results of this study.

3.5. Encapsulation and Loading Capacity

The encapsulation capacity, calculated from the ratio of surface and total polyphenols (Table S1), ranged from 95.42–98.67% (Table 1) with mean of 97.20% and the loading capacity ranged between 6.69–20.28%, with mean of 12.01%, showing a very high degree of polyphenol encapsulation under all spray drying conditions applied. Compared to other study conducted on nettle powders [19] it is evident that they got lower values for polyphenol encapsulation capacity (63.23–87.21%), while loading capacity of nettle powder was not recorded in literature data

According to the statistics, the temperature and type of carriers had a statistically significant effect ($p < 0.01$) on the encapsulation capacity, while the proportion of carriers had no effect ($p = 0.16$). On the other side, the type of carrier and their proportion had significant effect ($p < 0.01$) on the loading capacity and temperature did not ($p = 0.36$). The highest encapsulation capacity was observed at the lowest drying temperature of 120 °C and decreased with increasing temperature. Polyphenols are bioactive compounds that are sensitive to external conditions and thus to high temperature where thermal decomposition, polymerization and transformation reactions, can occur. Also, drying at high temperatures directly affects the formation of lower quality products due to color decrease and loss of nutrients [41,53]. Regarding the influence of the carrier on encapsulation capacity, MD and its combinations with GA and β-CD with GA (1:1) proved to be most effective in achieving the best encapsulation capacity, while the lowest capacity was achieved when β-CD was used as single carrier. Navarro-Flores et al. (2020) [34] investigated the microencapsulation efficiency of powders obtained from chipilin leaf extract using different carriers. The lowest encapsulation efficiency of polyphenols was obtained when MD was used as single carrier, while it was significantly higher when combined with other carriers. Watson et al. (2017) [54] concluded that MD and GA exhibit better solubility and are more heat stable than β-CD so these carriers capture the active substance better when the mixture passes through a spray dryer. Moreover, the carrier combination is a better choice than using a single carrier because each carrier contributes to the encapsulation thanks to its structure. In a study by Zokti et al. (2016) [48] on the encapsulation of green tea leaf extract, the combination of MD and GA resulted in a higher encapsulation capacity than the use of a single MD.

3.6. SEM Analysis

Figure 1a–f show SEM microstructural analyses of nettle leaf powders with the highest encapsulation capacity prepared under the same drying conditions but with different

carriers. The particle size was not uniform, ranging from 2 to 12 µm. In all the images, it can be seen that the some microparticles had a regular round spherical shape and some had depressions on the surface without cracks, which means that the encapsulation was well performed.

Comparing the particle sizes obtained with MD and β-CD, it can be seen that the larger particles were obtained with MD which is in agreement with the research by Chong et al. (2014) [55] who encapsulated betacyanins from *Amaranthus gangeticus* with MD and β-CD. An expansion in the particles size occurs due to the addition of carriers and the inability of water to evaporate rapidly because the carrier retains them [35] and rapid drying at high temperatures leads to the formation of wrinkles and dents on the surface of the microparticles. Kalajahi and Ghandiha (2022) [19] who encapsulated nettle extract using MD as a carrier, concluded that MD resulted in the formation of particles with irregular surface but without cracks and holes. The same conclusion was obtained by Pudziuvelyte et al. (2019) [47] who studied the morphology of *Elsholtzia ciliata* herb powder particles and demonstrated that MD resulted in smoother particles compared to GA and β-CD.

3.7. Bioavailability of Polyphenols

Bioavailability is the amount of a nutrient or bioactive ingredient that the human body can store or use in various metabolic processes, and it is necessary to determine it because the beneficial effects of the bioactive ingredient depend on its bioavailability in the body.

To study the bioavailability of polyphenols, the group of powders with the highest encapsulation capacity was selected, prepared under the same spray drying conditions (160 °C and sample:carrier ratio 1:3), using different types of carriers. The concentrations of phenolic compounds in the initial samples of the encapsulated nettle leaf extracts and in all phases of the simulated digestion are shown in Figure 2. The concentrations of polyphenols in the initial powders ranged from 24.01 to 36.47 mg g^{-1} dm of extract. The polyphenol concentrations in the gastric phase were slightly lower (15.09–35.92 mg g^{-1} dm of extract) than in the initial sample in all samples. The results show that in the gastric phase 62.85–98.49% of polyphenols were available for bioavailability, based on the amount in the initial powder, and the values of absorbed polyphenols ranged from 2.03–2.97 mg g^{-1} dm of the extract, and an average 6.16–13.95% were absorbed into the bloodstream through the small intestine. This is consistent with the literature data stating that a very small amount of polyphenols (5–10%) is absorbed during the digestive phase in the small intestine, while most of it is absorbed in the colon due to chemical modification carried out by the microorganisms present there [17]. The largest amount of polyphenols (75.42–99.37%) was released during the intestinal phase and was available for degradation by the microflora in the colon. Shahidi and Peng (2018) [56] concluded that in a three-phase in vitro digestion test, the greatest release of phenolic compounds occurs in the intestinal phase. Zokti et al. (2016) [48] considered that most phenolic compounds are released in the intestinal phase because the interactions between water molecules and amorphous powder microparticles are strong, thereby increasing the solubility of the phenolic compounds. Similarly, dissociation of powder microparticles, which occurs due to the change in pH as the contents pass from the gastric to the intestinal phase, contributes to the release of phenolic compounds. Ydjedd et al. (2017) [57] also demonstrated that the concentration of phenolic compounds of the encapsulated carob extract gradually increased during digestion, with the highest concentrations recorded in the intestinal phase. As can be seen, powders produced with β-CD and its mixture with GA had higher concentration of polyphenols during the bioavailability process than those produced with MD and GA. Grgić et al. (2020) [17] stated that the use of cyclodextrin as a carrier in the encapsulation process increased the solubility of active ingredients and permeability through the intestinal membrane and contributed to higher bioavailability of the encapsulated compound, while GA formed a dry layer and prevented contact between the core and air.

The sample obtained at 160 °C with carrier β-CD:GA (3:1) and sample:carrier ratio 1:3 showed the highest concentrations of polyphenols at all stages of the in vitro diges-

tion test (Figure 2). Also, the mentioned encapsulated extract showed a high retention of polyphenols (79.33%) compared to the initial extract. Tuan et al. (2016) [58] encapsulated guava leaves extracts with mixture of MD and GA. Only 48% of polyphenols was encapsulated by spray drying when compared with polyphenols before encapsulation. On the other hand, Jovanović et al. (2021) [59] concluded that polyphenols retention of willowherb leaves encapsulated extract was 75.80% when 20% MD was used as carrier, which is still lower than in this research. In accordance, in this study the mentioned encapsulated extract was compared with the non-encapsulated extract for the concentrations of phenolic compounds during the in vitro bioavailability phases (Figure 3). As it can be seen, the bioavailability of the phenolic compounds of the encapsulated powder is higher than that of the non-encapsulated extract, indicating that encapsulation effectively protects the phenolic compounds from the adverse conditions in the gastrointestinal tract. For the non-encapsulated extract, it is observed that the polyphenol concentration decreased at each digestive stage due to the influence of enzymes and digestive fluids on polyphenol degradation. On the other hand, the encapsulated extract retained a high level of polyphenolic compounds at each stage, precisely because the carrier protects them from adverse conditions. Comparing the concentration of phenolic compounds of the initial extract and powder with the concentration of the absorbed compounds, a significantly higher absorption of phenolic compounds of the encapsulated powder was observed compared to the non-encapsulated extract. The concentration of phenolic compounds of the initial non-encapsulated extract was 45.97 mg g^{-1} dm of extract, and in an in vitro digestion test, 1.91 mg g^{-1} dm of extract was absorbed, corresponding to 4.16%. On the other hand, the concentration of initial encapsulated extract was 36.47 mg g^{-1} dm of extract and was absorbed by 2.97 mg g^{-1} dm of extract, corresponding to 8.13% and was two-fold higher than non-encapsulated extract. In order to study the full bioavailability of phenolic compounds, it would be necessary to simulate digestion in the colon, since in the work of Grgić et al. (2020) [17] and Bonetti et al. (2016) [60] it was found that the highest concentration of phenolic compounds was absorbed in the colon.

3.8. Antioxidant Capacity

The antioxidant capacity of the group of powders with the highest encapsulation capacity prepared under the same spray drying conditions (160 °C and sample: carrier ratio 1:3), and using different carriers was investigated using FRAP and DPPH tests and is shown in Table 4. The FRAP values of the nettle leaf powder were in the range of 6.13–11.04 mmol TE $100g^{-1}$ dm of the extract and for DPPH ranged from 9.10 to 15.57 mmol TE $100g^{-1}$ dm of th extract. In both tests, the lowest value was obtained when MD was used as the carrier and the highest value when β-CD:GA in the ratio of 3:1 was used as the carrier. Bhusari and Kumar (2014) [61] spray dried tamarind pulp and investigated how the carrier type affects the antioxidant activity. They concluded that powders with GA had higher antioxidant activity than those obtained with MD. This is due the fact that GA has a little of protein content which could contribute to the increase in antioxidant capacity [62]. Sharayei et al. (2020) [63] encapsulated pomegranate peel extract with MD and β-CD and concluded that the powder obtained with β-CD had higher total polyphenol concentration and antioxidant capacity. Other authors also concluded that cyclodextrins as carriers improved antioxidant capacity of phenolic compounds [64]. This can be attributed to the different structure of studied carriers and the influence of drying parameters [15]. The values of antioxidant capacity follow the values of total phenols, but not completely, which means that the antioxidant capacity is influenced by other components besides phenols, such as chlorophylls and carotenoids [6].

3.9. UPLC-MS/MS Identification and Quantification of Polyphenols

For the encapsulated extract with the highest encapsulation efficiency, obtained at 160 °C with β-CD:GA (3:1) and a sample:carrier ratio of 1:3, a complete characterization of the polyphenolic compounds was performed by UPLC-MS/MS (Table 3). A detailed

description of identification pathways of phenolic compounds lacking standards from nettle leaves was described by Repajić et al. [6]. A total of 40 compounds belonging to the classes of flavonols, flavan-3-ols, flavones, isoflavones, flavanones, coumarins, benzoic acids, cinnamic acids and other acids were identified (Figure S1). The most abundant group were cinnamic acids (80% of total polyphenols) with cinnamic acid being the major compound (828.47 mg 100 g^{-1}), followed by caffeic acid (194.82 mg 100 g^{-1}). Other authors also reported that cinnamic acids were predominant group in nettle leaves [6,20,65,66]. The second most dominant group of encapsulated nettle leaves were other phenolic acids (quinic acid), followed by flavan-3-ols, benzoic acids and flavonols and the least respresented group were flavanones. Flavonols were the most numerous groups with 16 phenolic compounds, where kaempferol was the most abundant compound (9.44 mg 100 g^{-1}). Elez Garofulić et al. (2021) [20] also concluded that kaempferol was the most dominant polyphenol in flavonol group in nettle leaves. Comparing the identification and quantification of phenolic compounds of nettle leaves with other studies, differences may occur due to different growing conditions and harvesting times [6], different preparation of the extract [20], and in this case, due to the effects of encapsulation on the polyphenols.

4. Conclusions

The results of this study emphasized the necessity of careful selection of encapsulation parameters in order to obtain encapsulated nettle leaf extract with desirable physicochemical properties and preserved polyphenolic content. Therefore, the highest process yield was obtained at 160 °C, when MD was used as carrier, in sample:carrier ratio 1:3. The lowest moisture was achieved at 200 °C with β-CD and sample:carrier ratio 1:2, the highest solubility at 120 °C with MD and sample:carrier ratio 1:2, while the lowest hygroscopicity was achieved at 200 °C with MD:GA (3:1) and sample:carrier ratio 1:3. For the encapsulation and loading capacity, 160 °C, β-CD:GA (3:1) and sample:carrier ratio 1:3 were conditions for obtaining highest values. Since the positive effects of polyphenols significantly depend on their bioavailability in the human organism, it has been proven that encapsulation of nettle leaf extracts enabled a two-fold increase in polyphenol bioavailability. The present study showed that nettle leaf extract powders are a rich source of polyphenols and have high antioxidant capacity. In UPLC-MS /MS profiling, 40 phenolic compounds were identified, with cinnamic acids being the most abundant. Therefore, spray drying encapsulation of nettle leaf extract showed to be a promising tool for preservation and stabilization of valuable antioxidants with increased bioavailability, thus enabling their application in functional food products.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods11182852/s1, Table S1: Concentrations of total and surface polyphenols in nettle leaves extract powders; Figure S1: UPLC-MS/MS chromatogram of nettle leaves extract powder.

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Chapter 6

General discussion

- optimal parameters of ASE and their influence on the yield and antioxidant capacity of bioactive molecules
- comparison with other extraction techniques
- chemical composition and influence of ASE parameters on their concentration
- influence of phenological stage and habitat on the accumulation of phenols and pigments in nettle
- optimal spray drying parameters for the encapsulation of phenols from nettle extract and their bioavailability

1. Optimal parameters of ASE and their influence on the yield and antioxidant capacity of bioactive molecules

Since optimization of extraction parameters is an important step to obtain the maximum yield of the targeted components from the plant material, one of the objectives of this dissertation was to optimize ASE for the isolation of bioactive molecules from all parts of nettle and to see how the extraction parameters (temperature, static extraction time, and number of extraction cycles) affect the yield, chemical composition, and antioxidant capacity of nettle extracts. ASE was chosen as an advanced extraction technique where the combination of heat and pressure helps to dissolve bioactive molecules more efficiently, requiring less solvent and shorter extraction time (Luthria et al., 2019). Moreover, it can be applied to a wide range of plant matrices and extract different classes of bioactive molecules (Sun et al., 2012; Manousi et al., 2019; Jha and Sit, 2022).

In *Publication No. 1*, ASE of phenols and pigments from nettle leaves was optimized using 96% ethanol as an extraction solvent. Ethanol was used because it is a polar solvent, meaning that it can dissolve a wide range of compounds and is classified as a food grade solvent (GRAS). As it is shown in Table 1 in the publication, the optimization process included temperature parameters from 20 to 110 °C, static extraction times of 5 and 10 min, and the number of extraction cycles from 1 to 4. Table 2 shows that the content of total phenols and pigments increased with increasing temperature and static extraction time. Depending on the number of cycles, 3 cycles were optimal for phenol extraction, while 4 extraction cycles were required for maximum yield of pigments. This could be due to the higher concentration of pigments, especially chlorophylls, requiring more extraction cycles to extract as much as possible (Sun et al., 2012). Since nettle leaf extract is the most valuable source of mentioned antioxidant molecules, their antioxidant capacity was evaluated (Hudec et al., 2007; Otles and Yalcin, 2012). It was tested using the Oxygen Radical Absorbance Capacity (ORAC) method, which is based on the transfer of hydrogen atoms and is suitable for hydrophilic and lipophilic antioxidants (Huang et al., 2005). All the extraction parameters studied affected the antioxidant activity of nettle leaf extracts. As the temperature increased, the antioxidant capacity also increased, but only up to 80 °C, after which a slight decrease was observed, indicating that the bioactive molecules contributing as antioxidants are most stable at this temperature. As for static extraction time and number of extraction cycles, the highest values were measured at 10 min and 4 extraction cycles. From Table 6 it can be seen that all analyzed

compounds strongly contributed to antioxidant capacity (r=0.86-0.94), which was also confirmed by other authors (Shonte et al., 2020; Elez Garofulić et al., 2021; Paulauskienė et al., 2021).

A very similar optimization process for the ASE of phenols and pigments was performed with the nettle stem, with the difference that the temperature range was from 50 to 110 °C, since very low concentrations of ingredients were found or could not be detected at all when phenols and pigments were extracted from the leaf at 20 °C. The statistical analysis of the results for the bioactive nettle stem molecules is presented in Table 3., as it is not included in the publications of this dissertation (unpublished data). As it can be seen, all the parameters analyzed affected the concentration of bioactive components, with the exception of static extraction time on phenolic content. Thus, a temperature of 80 °C is optimal for the isolation of all bioactive molecules, after which their content decreased, probably due to degradation. The optimal static extraction time was 5 min and 4 extraction cycles.

Table 3. The influence of ASE conditions on the yield of nettle stem phenols and pigments

Source of variation	Total phenols (mg/100 g^{-1} dm)	Total chlorophylls (mg/100 g^{-1} dm)	Total carotenoids (mg/100 g^{-1} dm)	Total pigments (mg/100 g^{-1} dm)
Temperature (°C)	p<0.01*	p<0.01*	p<0.01*	p<0.01*
50	142.93±3.97^{a}	83.56±0.57^{a}	14.52±0.11^{a}	98.07±0.60^{a}
80	309.13±3.97^{c}	101.87±0.57^{c}	17.69±0.11^{c}	119.57±0.59^{c}
110	219.89±3.97^{b}	99.66±0.57^{b}	15.96±0.11^{b}	115.62±0.59^{b}
Static extraction time (min)	p=0.47	p<0.01*	p<0.01*	p<0.01*
5	222.31±3.24^{a}	96.66±0.47^{b}	16.64±0.09^{b}	113.30±0.49^{b}
10	225.66±3.24^{a}	93.40±0.47^{a}	15.47±0.09^{a}	108.87±0.49^{a}
Extraction cycle number	p<0.01*	p<0.01*	p<0.01*	p<0.01*
1	177.02±4.59^{a}	88.60±0.66^{a}	15.26±0.13^{a}	103.86±0.69^{a}
2	212.43±4.59^{b}	93.03±0.66^{b}	16.75±0.13^{b}	108.91±0.69^{b}
3	235.62±4.59^{c}	99.69±0.66^{c}	17.88±0.13^{bc}	116.41±0.69^{c}
4	270.87±4.59^{d}	98.81±0.66^{c}	19.35±0.13^{c}	118.17±0.69^{d}
Grand mean	**223.99**	**95.03**	**16.06**	**111.09**

* Statistically significant variable at p≤0.05. Results are expressed as mean±standard error (SE). Values with different letters within column are statistically different at p≤0.05.

On the other hand, a study of optimization of ASE of lipid fraction from nettle root from which phytosterols and pentacyclic triterpenoids were isolated was published in *Publication No. 3*. For

the isolation of the lipid fraction, *n*-hexane was used as the solvent because it is the most common solvent for lipid extraction due to its high selectivity for lipids and low cost (Shin et al., 2018). The temperature of 70 to 150 °C, static time of 5 to 15 min, and 2 to 4 extraction cycles were used for optimization, as shown in Table 1. Phytosterols and pentacyclic triterpenoids were isolated according to the ISO method 12228. Increase of the temperature resulted in increased concentrations of triterpenoid derivatives, while 5 min and 4 cycles were sufficient for their most effective extraction.

To summarize the results obtained in *Publication No.1*, *Publication No.3* and unpublished data, the optimal conditions for the isolation of bioactive molecules from all parts of nettle (leaves, stems, and roots) obtained by ASE are listed in Table 4. It can be seen that higher temperatures, a relatively short time, and multiple extraction cycles are optimal for the isolation of phenols, pigments, phytosterols, and pentacyclic triterpenoids from the nettle. It is also clear that the optimization must be carried out for each part of the plant, since each part has a specific chemical composition and certain compounds are bound differently in plant tissue, making some molecules easier to access than others.

Table 4. Optimal conditions for obtaining maximum content of bioactive molecules from nettle (*Urtica dioica* L.)

Part of plant	Compound	Temperature/static extraction time/number of extraction cycles
leaves	phenols	110 °C/10 min/3
	chlorophylls and carotenoids	110 °C/10 min/4
stems	phenols	80 °C/5 min/4
	chlorophylls and carotenoids	80 °C/5 min/4
root	phytosterols and pentacyclic triterpenoids	150 °C/5 min/4

Olech et al. (2020) also concluded that a higher temperature contributed to a higher amount of phenols extracted from sweet leaves of *Rhododendron luteum* using ASE. High temperature leads to a significant improvement in the efficiency of ASE, as it helps to break the interactions between the analyte and the matrix, increase the solubility of the components, and promote the diffusion of

the analyte into the solvent. Similar to the results of this dissertation, Repajić et al. (2020) concluded that the optimal conditions for the isolation of carotenoids and chlorophylls from fennel seeds using ASE were 110 °C/5 min/3 cycles and 110 °C/10 min/4 cycles, respectively. Longer extraction may lead to the degradation of carotenoids as they are more heat sensitive than other pigments, possibly due to their linear and rigid structure. Efthymiopoulos et al. (2018) concluded that the maximum oil fraction obtained from spent coffee grounds with *n*-hexane as solvent using ASE was obtained at a temperature of 145 °C, which was the optimal temperature for the fast lipid diffusion rate. They also stated that the high oil yields could be due to the extraction of bound lipids and impurities from the matrix. After 145 °C, the oil concentration decreased slightly, which could be due to lower lipid stability and degradation.

2. Comparison with other extraction techniques

To determine the efficiency of ASE, in *Publication No. 1* the yield of phenols and pigments from nettle leaves obtained with ASE were compared with the yield obtained with UAE. UAE was performed using an ultrasonic bath, as it is a simple, easy to handle and inexpensive technique for isolating highly valuable compounds from plants (Kumar et al., 2021). It was previously optimized, and the optimal conditions (80 °C/30 min) were compared with the similar conditions at ASE (80 °C/10 min/3 cycles). It was found that ASE gave 3-fold higher values of phenol concentrations as well as higher values of pigment concentrations. Also, antioxidant capacity was much higher when ASE was used as extraction technique, indicating that ASE produces extracts containing more bioactive molecules that contribute to the antioxidant activity. Elez Garofulić et al. (2021) also examined the influence of MAE, ASE and the conventional reflux extraction on the isolation of phenols and antioxidant capacity of nettle leaves. ASE proved to be the most efficient technique, yielding the extracts with the highest phenol concentration and antioxidant capacity compared to the other two techniques.

In the *Publication No. 3*, the optimal ASE conditions for the efficient isolation of the oil fraction from nettle root were compared with Soxhlet extraction, the conventional, most commonly used technique for extracting the oil fraction. The main focus was on the comparison of extraction yield, total triterpenoid derivatives and β-sitosterol as the dominant phytosterol. The extraction yield

obtained with ASE (1.28%) was more than 2-fold higher than one obtained with Soxhlet extraction (0.49%), as shown in Figure 3a. The amount of triterpenoid derivatives and β-sitosterol was also significantly higher in the extracts obtained using ASE (88.40 mg 100 g^{-1} dm and 71.20 mg 100 g^{-1} dm) in comparison with the extracts obtained by Soxhlet (57.01 mg 100 g^{-1} dm and 45.42 mg 100 g^{-1} dm) (Figure 3b).

Considering that the yield and composition of the plant depend primarily on the appropriate selection of the extraction technique and its parameters, it can be concluded that ASE is an excellent technique for the extraction of bioactive molecules from nettle. Compared to other extraction techniques, the extracts obtained by ASE contained a significantly higher content of the targeted components, characterizing them as a very valuable source of antioxidants for further processing and implementation in food. To enable the use of foods containing high-value nettle molecules such as triterpenoid derivatives, future research should focus on the use of other "GRAS" solvents and other advanced techniques such as SFE to avoid the use of hexane in their isolation.

3. Chemical composition and influence of ASE parameters on their concentration

Although the chemical composition of bioactive molecules in the plant depends mainly on environmental, phenotypic and genotypic factors, the choice of a suitable extraction technique also plays an important role in their isolation (Srivastava et al., 2021). In summary, the average composition of phenols and pigments in nettle leaves and stems and the average composition of triterpenoid derivatives (both phytosterols and pentacyclic triterpenoids) in nettle roots, all determined under optimal ASE conditions, are shown in Figure 4. The mean value of the total content of phenols and pigments was calculated based on data from *Publication No. 2*, regardless of phenological stage and habitat, as the detailed composition of individual compounds determined by UPLC-MS/MS is given in the publication. The mean value for the concentration of triterpenoid derivatives was taken from *Publication No. 3*, which confirms the predicted value under optimal ASE conditions.

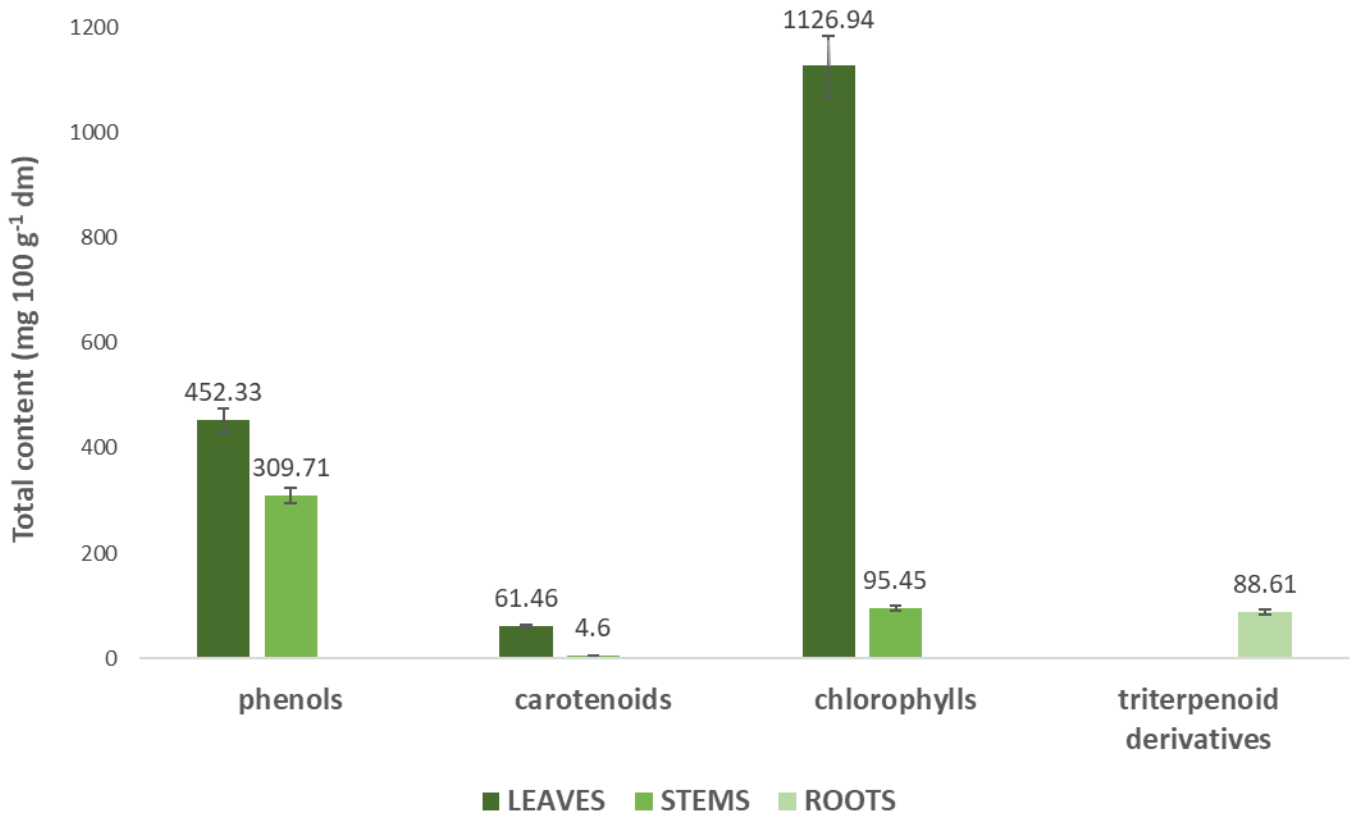

Figure 4. Approximate content of phenols and pigments in nettle leaves and stems and triterpenoid derivatives in nettle root

The average amount of phenols in the nettle leaves was 452.33 mg 100 g^{-1} dm, while in the stem it was 309.71 mg 100 g^{-1} dm. From these data, it can be concluded that the phenolic content in nettle leaves was higher than in stems, which was also found by other authors (Pinelli et al., 2008; Otles and Yalcin, 2012). There is only one study by Elez Garofulić et al. (2021), who extracted phenols from stinging nettle leaves using ASE, where a total content of 425.13 mg 100 g^{-1} dm was determined, which is consistent with the present data. Depending on the chemical composition, phenols were identified and quantified using UPLC-MS/MS. As it is shown in Table 2 in *Publication No. 2*, a total of 41 phenolic compounds were determined in both nettle leaves and stems extracts. The compounds were classified into the groups of benzoic acids, cinnamic acids, other phenolic acids, flavonols, flavan-3-ols, flavones, isoflavones, flavanones, and coumarins. The most dominant group was cinnamic acids (grand mean 179.22 mg 100 g^{-1} dm), followed by

flavonols (grand mean 134.60 mg 100 g^{-1} dm). Generally, it has been demonstrated that the content of bound phenolic acids is twice the amount of free phenolic compounds, and some authors stated that phenolic acids bound to cell wall structural compounds such as lignin, protein, and polysaccharides can be released when high temperatures are used during ASE (Mitrović et al., 2021; Antony and Farid, 2022). In this way, their concentration in the extract increases as the bonds between the mentioned compounds and the acids are broken and the compound breaks down. Flavonoids are more sensitive to an increase in extraction temperature than phenolic acids, so their content decreases at very high temperatures, usually above 150 °C (Palma et al., 2001; Ross et al., 2011).

The amount of carotenoids was very low in both leaves (61.46 mg 100 g^{-1} dm) and stems (4.6 mg 100 g^{-1} dm). Among the analyzed groups of pigments, chlorophylls are the most represented. Their approximate amount was 1126.94 mg 100 g^{-1} dm in the nettle leaves, while it was much lower in the stems (95.45 mg 100 g^{-1} dm), which was also confirmed by Hojnik et al. (2007). It can be concluded that nettle leaves are an extremely valuable source of chlorophylls. HPLC-UV-VIS/PDA was used to identify and quantify the pigments. Pigments were divided into carotenoids and chlorophylls, of which 13 carotenoids and 9 chlorophylls were identified and quantified. The most dominant carotenoid was lutein (18.39 mg 100 g^{-1} dm), and among chlorophylls it was chlorophyll *a* (594.67 mg 100 g^{-1} dm). In general, an increase in temperature resulted in an increase in the content of all pigments in the nettle extract. Similarly, higher temperatures, longer exposure of the solvent to the matrix, and more extraction cycles extracted some chlorophyll and carotenoid derivatives that were not extracted at lower conditions. Although these are sensitive components, they are protected from external influences such as light and oxygen in the ASE system inside the extraction cell, which prevents oxidative degradation of the compounds (Fikselová et al., 2008; Antony and Farid, 2022).

On the other hand, the average amount of triterpenoid derivatives in nettle root was 88.61 mg 100 g^{-1} dm, which is slightly higher than the amounts reported by Obranović et al. (2023). Phytosterols and penatcyclic triterpenoids were identified and quantified by GC-FID and GC-MS. Their composition is shown in Tables 2 and 3 of ***Publication No. 3***, where a total of 9 phytosterols and 3 pentacyclic triterpenoids were identified. Among the plant sterols, β-sitosterol dominated with a mean value of 50.21 mg 100 g^{-1} dm (80.42% of the total phytosterols), while among the pentacyclic

triterpenoids, β-amyrin acetate dominated with a mean value of 0.56 mg 100 g^{-1} dm. This is the first publication using ASE for the isolation of triterpenoid derivatives from nettle roots. Almost the same composition of plant sterols and pentacyclic triterpenoids was obtained in the work of Obranović et al. (2023).

4. Influence of phenological stage and habitat on the accumulation of phenols and pigments in nettle

Since ASE proved to be an excellent technique for the extraction of bioactive molecules from nettle, the next objective of this dissertation was to determine how the phenological stage and habitat affect the accumulation of phenols and pigments (chlorophylls and carotenoids) in nettle leaves and stems. In the *Publication No.2*, wild nettle samples were collected from 14 natural habitats situated in continental, mountain and seaside area in Croatia during 3 phenological stages (before, during and after flowering). The collected samples were subjected to ASE at the optimal parameters obtained in *Publication No.1*, and the composition and amounts were determined by UPLC-MS/MS and HPLC-UV-VIS/PDA methods.

Plant part, phenological stage and habitat had a statistically significant influence on the accumulation of phenols in both nettle leaves and stems. Among plant part nettle leaves had higher amounts of all types of phenols. Observing the influence of the phenological stage, it was evident that the highest concentration of phenols was recorded before flowering of the nettle, while after flowering content of phenols was 2-fold lower. In general, the samples from continental region in Croatia had the highest phenol accumulation, followed by the samples from mountain region. Some groups of phenols were the highest in the samples from continental region, while the other were the highest in the samples collected in mountain region. Among the analyzed natural habitats, the place Žakanje stood out with the highest content of phenols (513.12 mg 100 g^{-1} dm).

Pigments, especially chlorophylls, are responsible for the process of photosynthesis in plants. Since light is one of the key parameters in photosynthesis, it is evident from Table 4 in the *Publication No.2* that the highest amount of carotenoids and chlorophylls was recorded in the seaside region of Croatia, where samples from Bale had the highest content of pigments (799.30 mg 100 g^{-1} dm). Between samples from other two regions there was no significant difference in

the accumulation of pigments. The highest accumulation of carotenoids, chlorophylls and total pigments was recorded in nettle samples collected during flowering, while the lowest content of pigments was determined after flowering, the same as for phenols. Moreover, nettle leaves had a significantly higher values of all analyzed pigments compared to stems.

Influence of phenological stage and habitat on antioxidant capacity of nettle extracts was also examined using ORAC method. The highest values were determined in extracts from leaves collected during flowering, while the diversity upon habitat variations was observed. Extracts obtained from samples collected in continental and mountain region had higher antioxidant capacity, compared to extracts of samples from seaside region. Also, cinnamic acids, flavonols and total phenols had a strong correlation (r=0.68-0.71) with antioxidant capacity.

It is evident that accumulation of bioactive molecules depends on habitat and phenological stages during nettle vegetation. Also, different parts of plant accumulate different amounts of compounds like phenols and pigments. Based on the results obtained, nettle should definitely be collected before and during flowering, since the accumulation of all analyzed compounds reduced after flowering. Depending on the components to be isolated, nettle should be harvested from the areas that contain more of the targeted components.

5. Optimal spray drying parameters for the encapsulation of phenols from nettle extract and their bioavailability

According to literature, among the bioactive molecules studied in this dissertation, phenolic compounds have a wide range of biological activities, which has been confirmed by both *in vivo* and *in vitro* studies (Fernandez-Panchon et al., 2008; Martins et al., 2016; González-Burgos and Gómez-Serranillos, 2021). Therefore, the further focus of this research was on the protection of these sensitive compounds and their conversion to a more stable form, since extracted phenolic compounds in liquid form have low bioavailability and are very sensitive to environmental, processing, and storage conditions (Lu et al., 2021). One of the objectives of this dissertation was to optimize the microencapsulation of extracted phenols from nettle leaves by spray drying and to study the physicochemical properties of the powders obtained and bioavailability of phenols. The spray drying was method chosen, which is widely used in the food industry because it is efficient, fast and can be used for a wide range of materials.

As it was stated in the *Publication No. 4*, the parameters that were optimized were temperature (120-200 °C), type of carrier (MD, β-CD, and their combinations with GA in 1:1 and 3:1 ratio, *w/w*), and sample:carrier ratio (1:1, 1:2, and 1:3, *w/w*). The different parameters have different effects on the physicochemical properties of the powders obtained. Depending on the powder use, the encapsulation conditions should be optimized. In general, produced powders had high yield and solubility, low hygroscopicity and moisture content. With the focus on the encapsulation of phenols, the highest encapsulation (98.67 %) and loading capacity (20.28%) were obtained at 160 °C, with combination of β-CD and GA as carriers in 3:1 ratio and sample:carrier ratio of 1:3. This powder also had a high antioxidant capacity. In general, the encapsulation of phenols was carried out very successfully, during which all powders were produced with an encapsulation capacity greater than 95%. Also, UPLC-MS/MS analysis showed that all 40 identified compounds were also presented in non-encapsulated and encapsulated nettle extract.

Another goal of encapsulation is to increase the bioavailability of phenols, since their bioavailability depends largely on the structure and form in which they enter the body, interactions with other macromolecules in food and modifications during digestion. *Publication No. 4* also investigated the bioavailability of phenolic compounds from non-encapsulated and encapsulated extracts of nettle leaves using simulated digestion. The *in vitro* test consisted of three phases - gastric, absorption and intestinal. The gastric phase takes place in the stomach at a pH of 2 and contributes to the release of phenolic compounds from the food, especially those with weaker bonds. The acidic conditions in the stomach cause hydrolysis and deconjugation of the phenolic compounds (Bohn, 2014; Corona et al., 2014) and when comparing non-encapsulated and encapsulated phenols, it was clear that these gastric conditions have a negative effect on the non-encapsulated phenols, while the encapsulated phenols were not degraded, as it is shown in Figure 3 in the publication. Since nettle contains a large amount of phenolic acids, free phenolic acids with low molecular weight can be absorbed in this phase. The absorbed levels of phenolic compounds for non-encapsulated and encapsulated nettle extracts were about 2 to 3% (with a slightly higher value of the encapsulated extract), which is consistent with literature data where it was reported that less than 10% of phenolic compounds are absorbed by the epithelium of the small intestine and can exert an effect on a target cell type or tissue (Grgić et al., 2020). During transport from the stomach to the duodenum, the pH changes from acidic to alkaline due to secretion of bile salts and pancreatic juice. Depending on their chemical structure, some phenolic

compounds are unstable in an alkaline medium and are lost, resulting in lower bioavailability (Velderrain-Rodríguez et al., 2014). This statement is particularly true for anthocyanins, flavonols, and flavan-3-ols. The remaining content of phenolic compounds is passed into the colon, where most of them are further metabolized and absorbed. Since nettle contains many glycosides that are more resistant to digestion than free compounds, during the digestive process they are broken down into aglycones, which are further available for absorption into the bloodstream. Comparing the bioavailability of the encapsulated extract, it appears that encapsulation improved the bioavailability of phenols and protected them from the effects of the digestive process. Future research could include monitoring the bioavailability of specified phenolic compounds and the further digestion process in the colon.

Chapter 7

Conclusions and prospects

- The results of this study showed that all parts (leaves, stems and roots) of the nettle (*Urtica dioica* L.) are a great source of bioactive molecules, with emphasis on phenols and pigments from leaves and stems, and phytosterols and pentacyclic triterpenoids from the root.
- ASE was successfully applied and optimized to extract phenols and pigments from leaves and stems, as well as phytosterols and pentacyclic triterpenoids from roots of nettle. The optimal ASE parameters for sufficient phenol extraction from nettle leaves were 110 °C/10 min/3 cycles, for pigment extraction 110 °C/10 min/4 cycles, while for both phenol and pigment extraction from stems they were 80 °C/5 min/4 cycles. The maximum values of the triterpenoid derivatives were extracted at 150 °C/5 min/4 cycles.
- ASE showed better performance compared to UAE in isolating phenols and pigments and compared to Soxhlet in isolating triterpenoid derivatives, achieving higher yields and protecting sensitive components from environmental conditions under controlled temperature, pressure and time.
- The chemical structure and antioxidant capacity of the extracted bioactive molecules were influenced by the applied ASE parameters. In general, higher temperature with short static extraction time and few extraction cycles provided a higher concentration of analyzed compounds and higher antioxidant capacity. Both phenols and pigments contributed to the antioxidant capacity of nettle extracts measured by the *in vitro* ORAC method.
- Plant part, phenological stage, and habitat had a significant effect on the accumulation of bioactive molecules in nettle. The obtained results show that the highest levels of bioactive nettle molecules associated with high antioxidant capacity were present in leaves that should be collected in the early phenological stage (before and during flowering). Moreover, the amounts of phenols and pigments in nettle varied greatly depending on the natural habitat: samples from the seaside region showed increased accumulation of pigments, while higher amounts of phenolics were present in samples from continental and mountain habitats.
- A total of 41 phenolic compounds, 9 chlorophylls, and 13 carotenoids were identified and quantified in nettle leaves and stems obtained using ASE. Among phenolics, cinnamic acids dominated, followed by flavonols, chlorophyll *a* was the major chlorophyll, while lutein was the major carotenoid. In addition, a total of 9 phytosterols and 3 pentacyclic

triterpenoids were identified and quantified in the oil fraction of nettle root isolated using ASE. The predominant phytosterol was β-sitosterol and β-amyrin acetate was the predominant pentacyclic triterpenoid.

- Encapsulation by spray drying was applied and optimized to protect and stabilize phenols from nettle extracts. It proved to be a promising technique for encapsulating phenols from nettle extract and obtaining powders with desirable physicochemical properties. The highest encapsulation and loading capacities were obtained at 160 °C, β-CD:GA (3:1) and a sample:carrier ratio of 1:3.
- Encapsulation of the phenols of nettle leaves increased their bioavailability 2-fold compared to the non-encapsulated extract, thus enhancing their beneficial effects on health.
- The results of this dissertation represent a significant contribution to a better understanding of the extraction and encapsulation of bioactive molecules of nettle and their potential use as functional ingredients for the development of new food supplements.

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Autobiography

Autobiography

Ena Cegledi is a research assistant working in the research and higher education since the 2020. Previously, she worked as a health associate at the Andrija Štampar Teaching Institute of Public Health, Department of Environmental Protection and Health Ecology (2018-2019). She received a Bachelor's degree in Food Technology and a Master's degree in Food Engineering from the University of Zagreb Faculty of Food Technology and Biotechnology. During her graduate studies, she received the Rector's Award for her scientific work entitled "Microwave-assisted extraction of polyphenolic compounds from grape skin pomace of Cabernet Sauvignon, Merlot and Teran". She is currently finishing her Ph.D. study at the University of Zagreb Faculty of Food Technology and Biotechnology in the field of Food Technology. She has been employed as a research assistant in the project "Isolation and encapsulation of bioactive molecules of wild and cultivated nettle and fennel and effects on organism physiology", funded by the Croatian Science Foundation. Her research is based on extraction and encapsulation techniques, as well as analysis of bioactive molecules from nettle extracts. Until now, she has co-authored 6 scientific papers in journals indexed in Web of Science/Current Contents Connect (71 citations; h-index 3). She participated in many international and national congresses where she presented her research results in the form of oral or poster presentations. She has participated in the supervision of two final and nine diploma theses.

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